Cloning and characterization of alfalfa hydroperoxide lyase

A biocatalyst for the production of green note flavors

Klonering en karakterisering van alfalfa hydroperoxide lyase

Een biokatalysator voor de produktie van ‘groene’ geur- en smaakstoffen

(met een samenvatting in het Nederlands)

Proefschrift

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Education is understanding the rules;
Experience is understanding the exceptions.
-Farmer's Almanac

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Abbreviations

AEA	*N*-arachidonoylethanolamine
AOS	allene oxide synthase
CB	cannabinoid receptor
CD	circular dichroism
CP	chiral phase
EPR	electon paramagnetic resonance
FID	flame ionization detection
DNPH	2,4-dinitrophenylhydrazone
GC-MS	gas chromatography-mass spectrometry
FPLC	fast protein liquid chromatography
HHE	4-hydroxy-(2*E*)-hexenal
HNE	4-hydroxy-(2*E*)-nonenal
15-HPETE	15-hydroperoxy-arachidonic acid
13-HPOD	13(*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid
13-HPOT	13(*S*)-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid
13-H(P)OTNH$_2$	13(*S*)-hydro(pero)xy-*N*-linolenoylamine
13-H(P)OTNHEtOH	13(*S*)-hydro(pero)xy-*N*-linolenoylethanolamine
9-HPOD	9(*S*)-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid
9-HPOT	9(*S*)-hydroperoxy-(10*E*,12*Z*,15*Z*)-octadecatrienoic acid
HPO lyase	hydroperoxide lyase
HRP	horseradish peroxidase
IMAC	immobilized metal affinity chromatography
IPTG	isopropyl-β-D-thiogalactoside
LOX	lipoxygenase
NAE	*N*-acylethanolamine
NAPE	*N*-acylphosphatidylethanolamine
NDGA	nordihydroguaiaretic acid
NTA	nitrilotriacetic acid
OD	(9*Z*,12*Z*)-octadecadienoic acid (linoleic acid)
OT	(9*Z*,12*Z*,15*Z*)-octadecatrienoic acid (linolenic acid)
PEA	*N*-palmitoylethanolamine
POX	peroxygenase
RP-HPLC	reversed phase high performance liquid chromatography
S.D.	standard deviation
SPE	solid phase extraction
SPME	solid phase micro extraction
TMBZ	3,3',5,5'-tetramethylbenzidine
TMS	trimethylsilane
U	unit

1

General introduction

1 Wound responses in plants

Plants are continuously exposed to 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 as the cuticle, toxic compounds, or enzymes able to digest 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 is considered to be the primary systemic response upon wounding [1,2]. Systemin is readily transported from wound sites throughout the whole plant and propagates the signal by inducing the transcription of defense genes (Fig. 1). The products of the upregulated genes can be classified into four categories: (1) defensive proteins, (2) signal pathway components, (3) proteinases and (4) enzymes with unknown function. Defensive proteins are for example proteinase inhibitors and polyphenol oxidase, which protect the plant against digestive serine proteases of herbivorous insects. Proteolysis of the ingested food in the gut of herbivores can also be affected, retarding their growth and development, and making the plant less attractive for animals.

The most important signal pathway induced by wounding is probably the octadecanoid or lipoxygenase pathway. Wounding and systemin induce the release of linolenic (OT) and linoleic acid (OD) [3], which are then converted by enzymes of the lipoxygenase pathway 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 [4-6] and the production of more defense proteins [7-9]. Furthermore, they are involved in developmental processes such as seedling growth, pollen formation and flower development [10,11]. Traumatin and traumatic acid also act as wound hormones and growth-promotors [12]. The epoxy, hydroxy and divinyl ether fatty acids are known to have antifungal activity [13,14]. 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 [6,15-21]. Besides, they might be a signal for other plants in the surroundings to induce a defense response as well [22]. They can also act as attractants or repellents of insects and might have a role in the production of floral scent [23,24]. 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 acetyl salicylic acid [6,25].

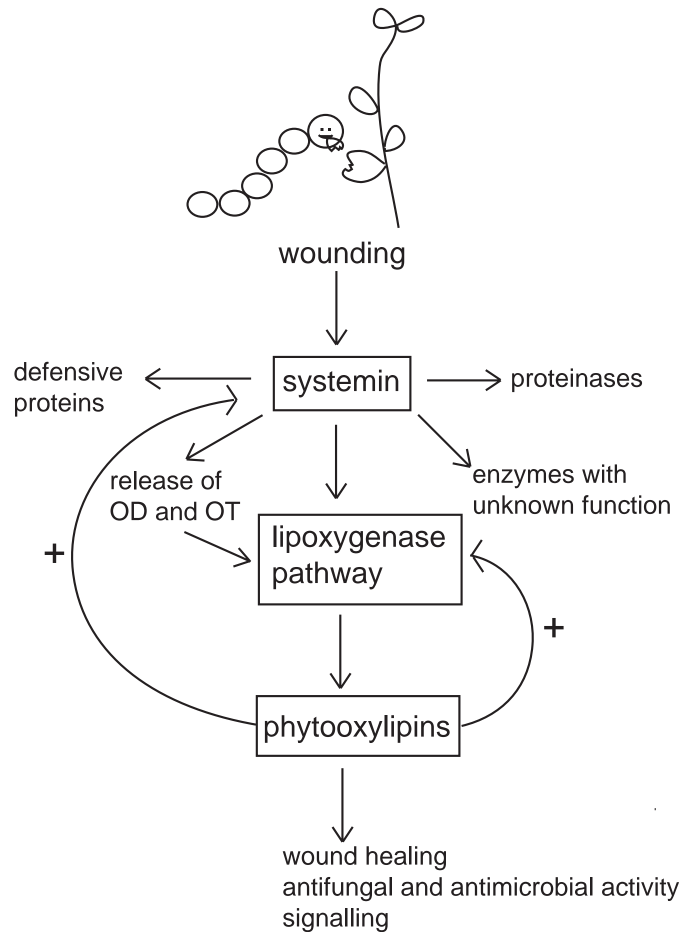


Fig. 1 Induced wound responses in plants

2 Lipoxygenase pathway

2.1 Lipoxygenase

The first step in the lipoxygenase pathway is the dioxygenation of OT or OD 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 [26]. Lipoxygenase in its native form contains Fe(II) and must first be oxidized into Fe(III) by its own hydroperoxide product [27]. Upon binding the substrate, a hydrogen atom is abstracted and a fatty acid radical is formed (Fig. 2). 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 regio-specificities of plant lipoxygenases with OD and OT as substrates are known: some lipoxygenases form 13(*S*)-HPOD or 13(*S*)-HPOT, others the 9(*S*)-isomers or a mixture of both.

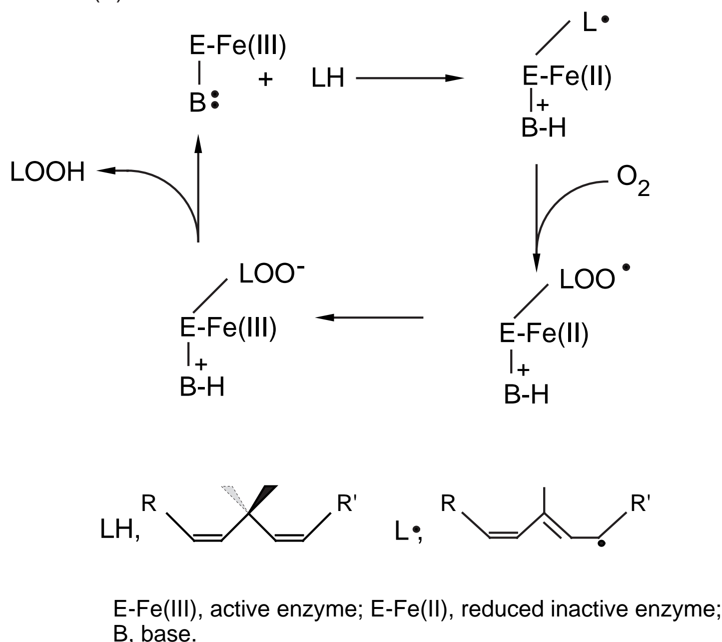


Fig. 2 Reaction mechanism of lipoxygenase

2.2 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 like allene oxide synthase (AOS), peroxygenase (POX), divinyl ether synthase and hydroperoxide lyase (HPO lyase).

Allene oxide synthase (EC 4.2.1.92) dehydrates the hydroperoxy fatty acids to unstable allene oxides, which are readily hydrolyzed into α- and γ-ketols, or cyclized by allene oxide cyclase (Fig. 3). 12-Oxo-(10,15*Z*)-phytodienoic acid, the cyclized product derived from α-linolenic acid can be converted into jasmonic acid by 12-oxo-phytodienoic acid reductase followed by three β-oxidation steps [28]. Several allene oxide synthases have been cloned and the enzyme has been characterized as a cytochrome P450 enzyme (CYP74A) [29-33]. Transcipts of AOS accumulate upon wounding, insect attack or treatment with methyl jasmonate or ethylene [6,24,32,34]. Overexpression of AOS in tobacco does not increase jasmonic acid levels in healthy plants, but wounding leads to a dramatic increase of jasmonic acid in the transgenic plants [35].

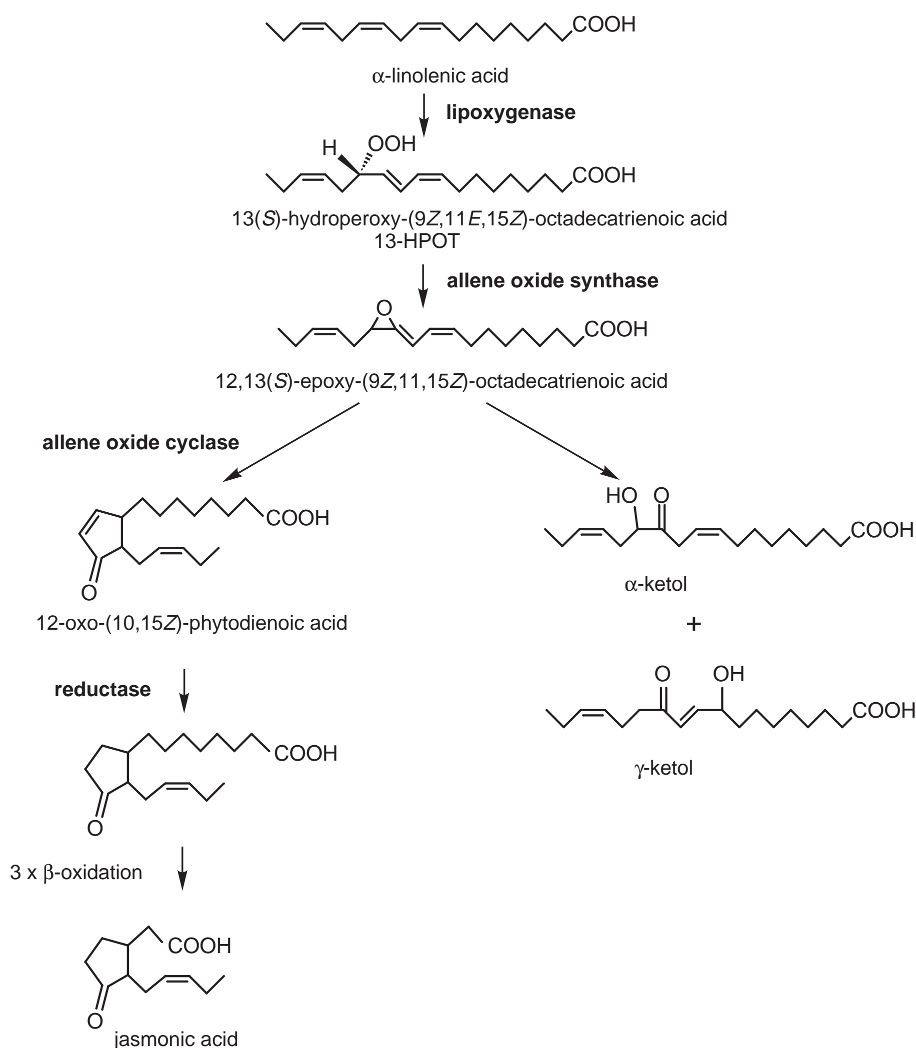


Fig. 3 Lipoxygenase-allene oxide synthase pathway

The action of peroxygenase on hydroperoxy linoleic and α-linolenic acids results in the formation of hydroxy and epoxy-hydroxy fatty acids [36]. In the presence of hydroperoxy fatty acids, peroxygenase can also act directly on OD and OT, forming 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 [37]. Little is yet known about this enzyme, but it acts in a regio- and stereospecific way and it is mainly found in the microsomal fraction [38,39].

Hydroperoxide lyases are cytochrome P450 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 (Fig. 4). The (3*Z*)-aldehydes can be isomerized to their (2*E*)-isomers and reduced by alcohol dehydrogenase to their corresponding alcohols. Furthermore, the (3*Z*)-alkenals can be oxygenated to 4-hydroxy-(2*E*)-alkenals [40,41]. Gardner *et al*. suggested that this oxygenation was catalyzed by a lipoxygenase [42].

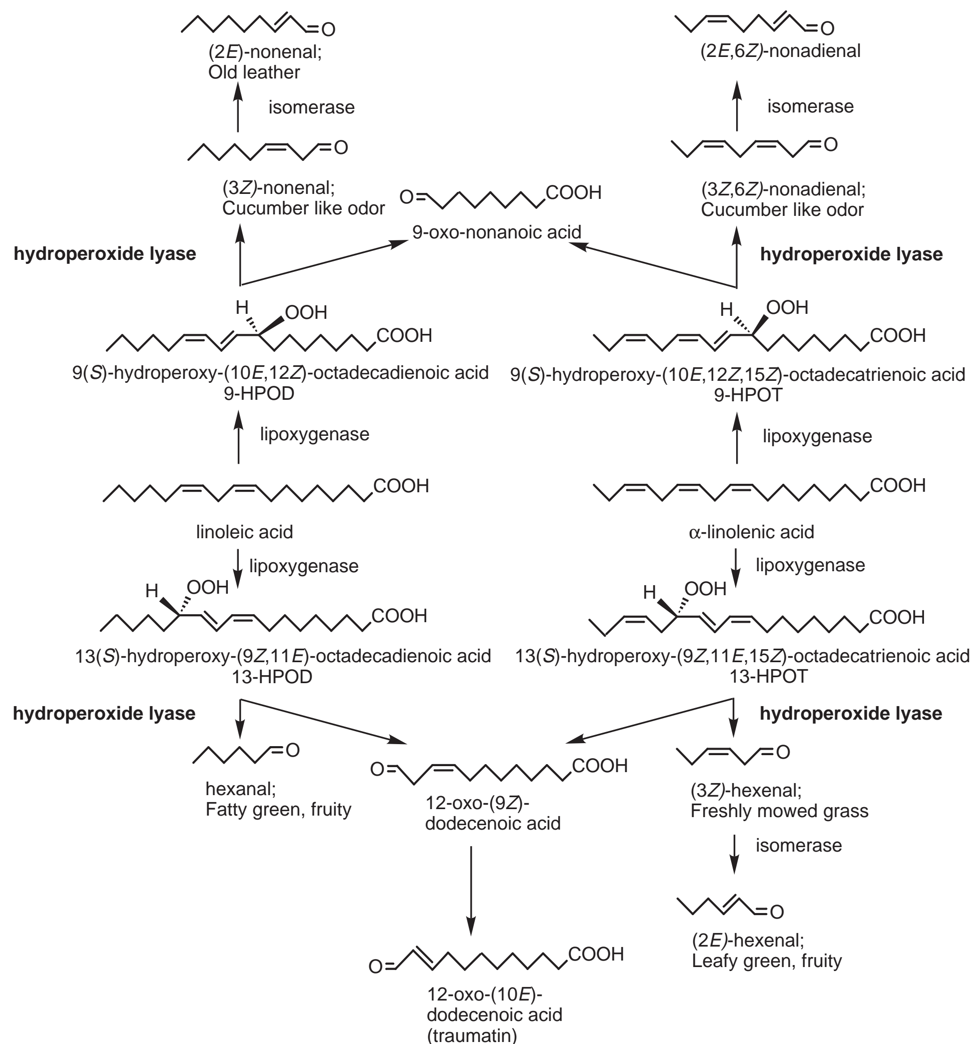


Fig. 4 Lipoxygenase-hydroperoxide lyase pathway

3 Cytochrome P450 enzymes

Allene oxide synthase and hydroperoxide lyase belong to the class of cytochrome P450 enzymes. Cytochrome P450 enzymes are heme-containing mono-oxygenases, 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 (P for pigment) [43]. These proteins are, in fact, not cytochromes because electrons are not transferred to another acceptor (other than O_2).

3.1 Physiological role and general reaction mechanism

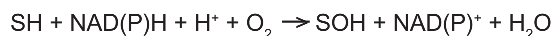
Cytochrome P450 enzymes play an important role in nature and are involved in the biosynthesis and metabolism of a large number of endogenous compounds, as well as in the detoxification of xenobiotics. Plant cytochrome P450 enzymes are for example involved in the biosynthesis of wound-signaling compounds (e.g. jasmonic acid - allene oxide synthase, salicylic acid, traumatic acid - hydroperoxide lyase) and hormones (e.g. gibberellins, brassinosteroids) [44]. Cytochrome P450 enzymes catalyze many different reactions such as hydroxylations, epoxydations, N-demethylations, O-dealkylations, deaminations, sulfoxidations, desulforations 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. Cytochrome P450 enzymes can also utilize peroxides such as cumene hydroperoxide and t-butylhydroperoxide as an oxygen donor. This bypass is usually referred to as the peroxide shunt, and is supposed to occur via homolytic scission of the hydroperoxy group [45,46]. The general reaction mechanism of these enzymes is shown in Fig. 5.

3.2 Structure

Cytochrome P450 enzymes are 45-60 kDa proteins. Six crystal structures of cytochrome P450 enzymes have been solved, five soluble prokaryotic cytochrome P450 enzymes and one eukaryotic microsomal P450 [48-54]. Analysis of the crystal structures revealed that these P450 enzymes have a triangular shape and are rich in secondary structure. They consist of an α-helix domain (40-70%) and a β-sheet rich domain (10-22%) (Fig. 6). The active site heme is bound by a highly conserved cysteine and located within 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, is probably involved in the binding of oxygen and proton delivery [48,55-57]. The structures of the B’ helix and of the F/G loop are rather variable which

might account for the different substrate specificities [53-55]. The question arises if these structures are useful as a general model for all P450 enzymes. The overall three-dimensional structures of the six crystalized P450 enzymes are highly similar, despite their low sequence identity, indicating that all P450 enzymes might have a comparable structure [54,56].

Most eukaryotic cytochrome P450 enzymes are membrane bound, associated to microsomes, mitochondria or chloroplasts. Two general models of the membrane topology have been proposed: (1) the deep immersion model (protein deeply imbedded in the membrane) and (2) the partial immersion model (anchoring of the protein by membrane spanning helices) [58]. The crystal structure of the microsomal cytochrome P450 suggests a monofacial attachment to the membrane via a broad, hydrophobic surface that is adjacent to an anchor provided by a transmembrane helix at the N-terminus [54].



SH = substrate
POOH = organic peroxide

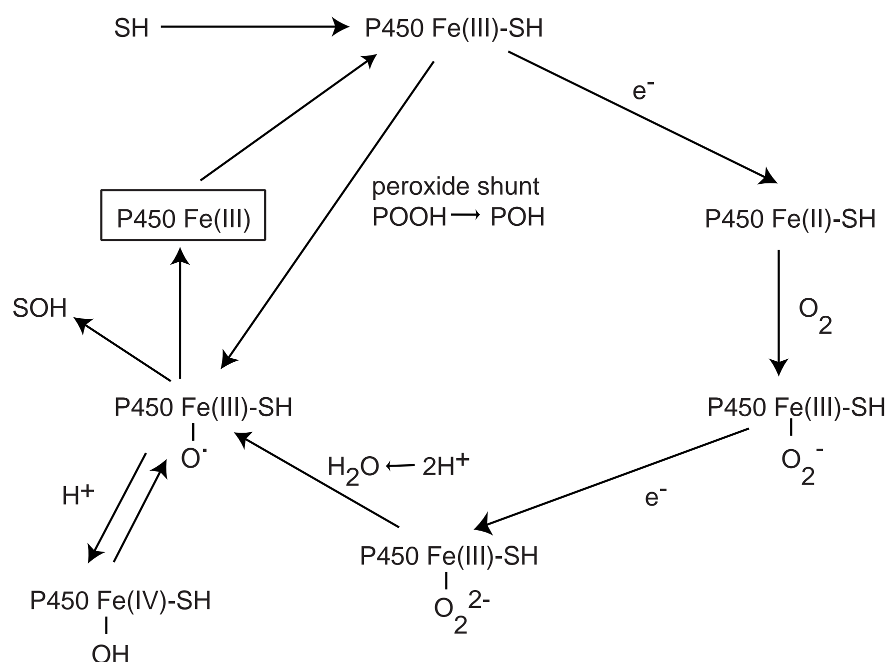


Fig. 5 General reaction mechanism of cytochrome P450 enzymes [47]

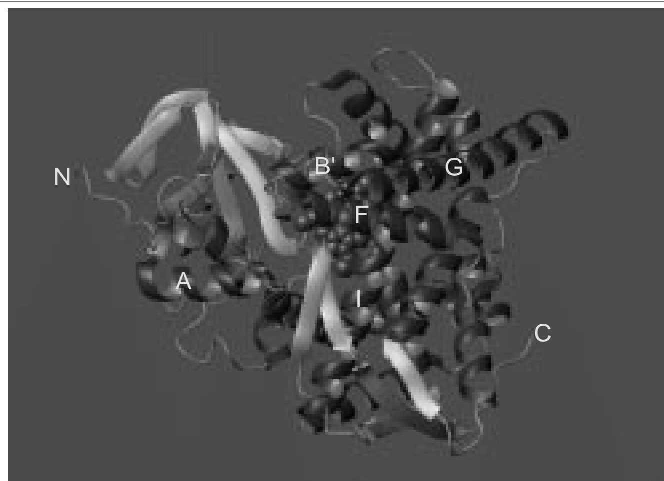


Fig. 6 A ribbon representation of P450BM-3. Reprinted from Julian Peterson's homepage [http://p450terp.swmed.edu/Bills_folder/billhome.htm]. Helices are represented by cylinders and are labeled according to the P450cam nomenclature. β-sheets are indicated by arrows.

3.3 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 center [59]. The iron of the heme is located in the center of the protoporphyrin ring, bound by the four pyrrol nitrogens. The heme-binding domain contains the characteristic sequence FxxGxxxCxxG, wherein the thiolate anion of the conserved cysteine serves as the fifth (axial) ligand to the heme iron [48]. The sixth (axial) ligand is an OH group from a water molecule or hydroxide ion [49,60]. The Fe(III) exists in high spin (5/2) and low spin (1/2) forms, depending on the configuration of the five 3d electrons. These forms are usually in equilibrium and possess different spectral properties [61]. Changes in the spin state of hemoproteins are usually caused by a change in ligand state of the heme iron: the 6-coordinated state is low spin and the 5-coordinated 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 to high spin (type I interaction), but stabilization of the low spin state has been observed too (type II interaction) [61-63]. In a type I interaction, displacement of water by the substrate might lead to the movement of iron out of the plane of the porphyrin ring, which makes the high spin configuration favorable (Fig. 7) [49,64]. 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 [65]. The functional importance of the spin state is still not completely understood. It has been suggested that the high spin form is more rapidly reduced [66], but on the other hand the reduction is mostly not the rate-determining step.

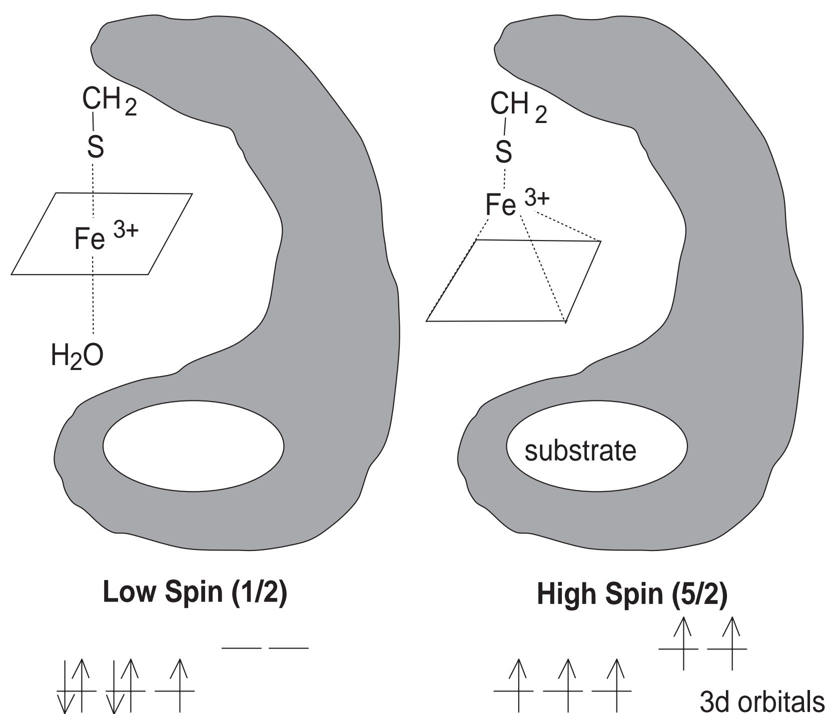


Fig. 7 Spin state change in cytochrome P450 enzymes due to substrate binding (type I interaction)

3.4 Nomenclature

The diversity of P450 enzymes required the development of a systematic nomenclature that assigns each protein to a specific family and subfamily based upon its primary sequence [67]. The systematic name starts with *CYP* (CYP for mRNA and proteins), followed by the family number which is assigned chronologically. Proteins are members of the same family if they share over 40% amino acid identity. Cytochrome P450 enzymes in one family which are less than 55% identical are members of different subfamilies. Subfamilies are given different letters, such as CYP74A (allene oxide synthase) and CYP74B (hydroperoxide lyase). Proteins of the same subfamily are numbered chronologically, such as CYP74B1 and CYP74B2.

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 [68]. Vick *et al.* demonstrated such lyase activity in watermelon seedlings and named it hydroperoxide lyase in 1976 [69]. Since then, hydroperoxide lyases from a number of organisms have been purified and characterized. Recently, HPO lyases from bell pepper, *Arabidopsis thaliana*, cucumber, tomato and guava fruit have been cloned and expressed in *Escherichia coli* [24,34,70-73].

4.1 Substrate and product specificities of different HPO lyases

Plant HPO lyases can be classified into two groups, according to their substrate specificity. One group cleaves 13-HPOD into 12-oxo-(9*Z*)-dodecenoic acid and hexanal, and α-13-HPOT 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-oxo-nonanoic acid and (3*Z*)-nonenal or 9-oxo-nonanoic 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-HPO lyase activity is present [69,74-78]. Pears only contain 9-HPO lyase activity [79]. Soybean and pea seeds/seedlings, cucumber fruits and seedlings contain both HPO lyase activities [80-82]. Matsui *et al*. succeeded in separating the 13- and 9-HPO lyase activities from cucumber seedlings, indicating that different enzymes are specific for the different substrates [83]. However, recently a cucumber HPO lyase was cloned which uses both substrates [72]. This HPO lyase is remarkable because it shows a higher sequence similarity to AOS than to 13-HPO lyases.

Conversion of the carboxyl group of the fatty acid hydroperoxide to a methyl ester or alcohol greatly reduces the activity of hydroperoxide lyases from tea chloroplasts and soybeans [77,84], indicating that the carboxyl group of the substrates is important for HPO lyase activity.

The influence of the position and amount of double bonds in the substrate on plant HPO lyase activity was studied as well. HPO lyases from tea leaves, tomatoes and green bell pepper fruits show nearly ten times higher initial activity towards 13-HPOD than towards α-13-HPOT [74,76,85,86], whereas this introduction of a (15*Z*)-double bond in linoleic acid increases the activity of tea chloroplast HPO lyase by 2% only [84]. Introduction of a (6*Z*)-double bond (γ-13-HPOT) strongly decreases the activity of hydroperoxide lyases [74,84,86]. 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 the C_{22} acids, and not the natural substrates (C_{18}), show the highest reactivities towards HPO lyase [87]. 15-hydroperoxy arachidonic acid (C20:4, 15-HPETE) however, does not act as a substrate for tea chloroplast hydroperoxide lyase [88]. From a racemic mixture of 13(*R*)-HPOD and 13(*S*)-HPOD, only the *S*-isomer is converted by HPO lyase [77]. In conclusion, the structural requirements of substrates for plant HPO lyases 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 HPO lyase which is specific for 10-hydroperoxy fatty acids [89-92]. The products of this HPO lyase 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 (*Chlorella*) [93] or pentanol (*Oscillatoria*) [94]. In mammals, only one HPO lyase has been found, in rabbit leukocytes [95]. This HPO lyase is proposed to cleave 15-HPETE into pentanol and 15-oxo-(5*Z*,8*Z*,11*Z*,13*E*)-pentadecatetraenoic acid.

4.2 Localization and regulation

In watermelon seedlings the highest HPO lyase activity is present in the hypocotyl-root junction [69]; in cucumber seedlings, roots are the richest source of HPO lyase activity [96]. Tissue print immunoblot analysis of bell peppers showed that most HPO lyase is located in the outer parenchymal cells of the pericarp [76]. This is in accordance with its primary role in protection against attacks from outside. Furthermore, the localization of HPO lyase seems to be linked to rapid cell growth and development [97-99]. Analogously, the activity of HPO lyase in *Marchantia polymorpha* cells is highest when the cells are growing logarithmically and rapidly decreases when the cells enter the stationary phase [100].

HPO lyases are thought to be membrane proteins and detergents are needed for their solubilization. In some plant sources HPO lyases are localized in the chloroplasts [101-105] or in the microsomes [74,106,107], whereas in others no specific localization in a particular organelle is observed [76,108]. The HPO lyase gene from *Arabidopsis thaliana* contains a chloroplast-directing transit sequence [34], but the gene from tomato does not [24,109]. The subcellular localization thus remains unclear.

Transcription of hydroperoxide lyase increases in response to insect attack and mechanical wounding, but, in contrast to AOS, not by methyl jasmonate treatment [24,34]. The rapid release of HPO lyase products after wounding indicates that HPO lyase is already present in the plant and that *de novo* synthesis is not necessary. The enzyme might be activated upon contact with its substrate. Phospholipase D is activated in response to wounding and promotes the release of fatty acids by supplying phosphatidic acids as a lipolytic substrate, or by activating phospholipase A and nonspecific acyl hydrolases [110]. Furthermore, phospholipase D may induce LOX-2 expression. In this case, the enzyme should be present in the membrane or be transported to the wounding site.

4.3 Structure

The HPO lyase genes described so far code for 55 kDa proteins [24,34,70,71,73]. Most isolated plant HPO lyases consist of subunits of 55-60 kDa [74,76,94,111,112], but based on native-PAGE, it has been suggested that the enzymes exist as trimers or tetramers of about 200-250 kDa [69,73,75,76,78,112].

Based on its sequence homology, HPO lyase was identified as cytochrome P450 enzyme [70]. The masses of the HPO lyases found 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 [113]. Similar to other cytochrome P450 enzymes, HPO lyases contain a highly conserved cysteine, which is supposed to bind the heme group [24,34,70]. HPO lyases however, do not show an absorption maximum at 450 nm after reduction and treatment with CO [109,113]. This behaviour is similar to AOS that shows a low affinity for CO too [114]. Furthermore, HPO lyase, as well as AOS, do not need molecular oxygen nor an NADPH-dependent cytochrome P450 reductase for their activity. These enzymes are, therefore, unique within the cytochrome P450 family. HPO lyases and AOS, show little homology to other cytochrome P450 enzymes in the I-helix region (oxygen-binding pocket) and lack the conserved threonine, which might be explained by the fact that these enzymes do not have to interact with molecular oxygen for their reaction. The absence of the oxygen binding pocket might explain their low affinity for CO.

4.4 Reaction mechanism

It is known that fatty acid hydroperoxides can be cleaved homolytically in an autoxidation reaction [115,116], as well as heterolytically in an acid catalyzed reaction [117]. For a long time, a heterolytic reaction mechanism seemed to be most likely for plant HPO lyase action, because the reaction products of the heterolytic reaction resemble the ones formed by hydroperoxide lyase, whereas the complex volatile profile obtained by homolysis does not [116]. To obtain more information about the reaction mechanism, both oxygen atoms of the hydroperoxy group of 13-hydroperoxy-linoleyl alcohol were labeled with ^{18}O. This hydroperoxide was incubated with tea chloroplasts and it was observed that the ^{18}O of the hydroperoxide was not transferred into hexanal but instead into 12-oxo-(9*Z*)-dodecenol [118]. Based on these results, Hatanaka *et al.* proposed a heterolytic reaction mechanism for 13-hydroperoxide cleavage by HPO lyase (Fig. 8). In the first step, hydroperoxide lyase protonates the hydroperoxide, causing cyclization to a 12,13-epoxycarbocation and loss of a water molecule. An allylic ether cation localized at C_{13} is formed via a 1,2-shift of the bond from C_{13} to the electron-deficient oxygen. Subsequently, addition of water to the oxygen-stabilized carbocation occurs and spontaneous rearrangement of the intermediate would result in formation of a C_{12}-oxoacid and a C_6-aldehyde. An attempt to detect reaction intermediates by using [1-^{14}C]-labeled 13-HPOD was not successful [112]. The stereospecificity of the cleavage was studied by conversion of the reaction products to 2,4-dinitrophenylhydrazone derivatives [74]. The results suggested that HPO lyase from tea leaves retains the *Z*-configuration in the substrates.

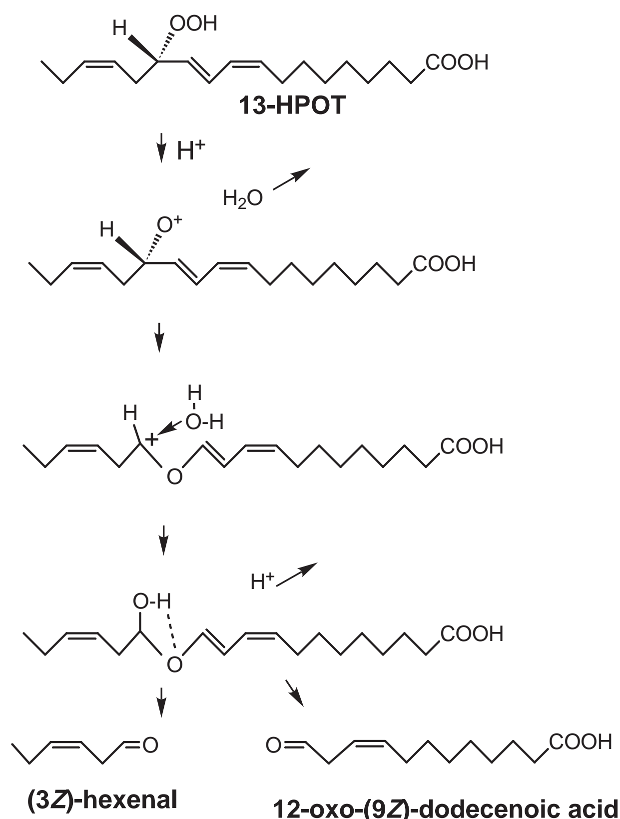


Fig. 8 Proposed mechanism of heterolytic cleavage of 13-hydroperoxy fatty acids to (3*Z*)-hexenal and 12-oxoacid by tea chloroplast hydroperoxide lyase [86]

However, since the characterization of HPO lyase as a cytochrome P450 enzyme, a homolytic mechanism seems to be more likely. In support of a radical process, inhibition of the enzymatic activity by radical scavengers is observed. Furthermore, dithiothreitol and 13-hydroxy-linoleic acid protect against inactivation of the enzyme by 13-HPOD, which suggests that inactivation is caused by destruction of an essential -SH-group near the reaction centre of HPO lyase [119]. The observed protection by organic antioxidants and radical scavengers (α-tocopherol, nordihydroguaiaretic acid, butylated hydroxyanisole and butylated hydroxytoluene) suggests that the -SH-group is destroyed by radical formation from fatty acid hydroperoxides near the reaction centre of HPO lyase.

The cleavage of 13-hydroperoxy fatty acids by green algae is supposed to be homolytic, but since pentane or pentene is formed instead of C6- or C9-aldehydes, this reaction might occur in a different way [93].

In mushrooms, one oxygen of the 10-hydroperoxy fatty acid is transferred to 1-octen-3-ol, the other oxygen might be transferred to 10-oxo-(8*E*)-decenoic acid. It was suggested that the -OH group of the peroxide group is transferred to C_{13}, the Δ^{12}-double bond rearranges and the oxygen remaining at C_{10} forms the C=O group of the 10-oxo-(8*E*)-decenoic acid [90,120]. It is still unclear if this mechanism is homolytic or heterolytic.

5 Aim of this thesis

Volatile C_6- and C_9-aldehydes and alcohols 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. Until now, these green note flavors are mainly obtained by chemical synthesis, but the present preference of consumers for 'natural' instead of 'nature identical' food additives urges the industry to develop biocatalytic production processes. Lipoxygenase and hydroperoxide lyase are suitable biocatalysts for the production of 'natural' food flavors. More information about the properties of HPO lyase could contribute to the development of such a biocatalytic process and extend our knowledge of the biological role of this enzyme. The aim of the studies described in this thesis is to characterize the enzyme HPO lyase and to make it available for use as a biocatalyst.

Alfalfa seedlings are used as a HPO lyase source, since they contain a relatively high HPO lyase activity. A purification method is developed, and the substrate and product specificities of alfalfa HPO lyase are determined. Furthermore, the presence of alcohol dehydrogenase and (3*Z*:2*E*)-enal isomerase in alfalfa are studied. The results are described in chapter 2.

Hydroperoxide lyase isolated from alfalfa extracts is impure and the amounts are too small to perform characterization studies or use it as a biocatalyst. To improve the availability of the enzyme, a cDNA library of alfalfa seedlings is constructed and screened with a HPO lyase gene from bell pepper to obtain HPO lyase genes from alfalfa. These genes are expressed in *E. coli*, with an N-terminal 6xHis-tag to facilitate purification. The cloning, expression and characterization of the expressed enzymes are described in chapter 3. This expression system provided us with sufficient HPO lyase to further characterize its active site, using a combination of spectrophotometry and EPR. The influence of the detergent Triton X-100, commonly used to solubilize the enzyme, on the enzyme conformation is studied as well. Furthermore, cysteine point mutants are constructed to determine if the conserved cysteine is involved in binding the heme group. The results of these characterization studies are described in chapter 4.

N-acylethanolamines (NAEs) constitute a new class of plant lipids and are thought to play a role in plant defense. Because oxylipins generated by the lipoxygenase pathway are

also important actors in plant defense, it is interesting to determine if members of the NAE class can be converted in this pathway as well, to form other possible defense compounds. Furthermore, the ability of HPO lyase to use the LOX products of NAEs as substrates may give an indication of the importance of the carboxy group of the substrates for HPO lyase activity. In chapter 5, four different linolenic acid amide derivatives are tested as substrates for soybean lipoxygenase-1, alfalfa HPO lyase and flax seed allene oxide synthase. The products are characterized, as well as the kinetic parameters of the dioxygenation and subsequent metabolism of these novel lipids.

(3Z)-Alkenals produced by HPO lyase can be oxygenated to 4-hydroxy-(2*E*)-alkenals, interesting compounds because of their high cytotoxicity. These 4-hydroxy-(2*E*)-alkenals appeared to be present in incubations of hydroperoxy fatty acids with pure HPO lyase as well. This is in contrast to the previously proposed oxygenation of (3*Z*)-alkenals by lipoxygenase. In chapter 6, the formation of these products is studied to obtain more information about the role of lipoxygenase and HPO lyase in the oxygenation process, and about the possible physiological significance of 4-hydroxy-(2*E*)-alkenals in plants.

In chapter 7 the production and isolation of active HPO lyase with the expression system described in chapter 3 are optimized. For the development of a biocatalytic process, the optimal reaction conditions for the production of volatile aldehydes from hydroperoxy linole(n)ic acids by HPO lyase are determined as well.

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2

Alfalfa contains substantial 9-hydroperoxide lyase activity and a 3*Z*:2*E*-enal isomerase

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Abstract

Fatty acid hydroperoxides formed by lipoxygenase can be cleaved by hydroperoxide lyase resulting in the formation of short-chain aldehydes and ω-oxo-acids. Plant hydroperoxide lyases use 13- or 9-hydroperoxy linoleic and linolenic acids as substrates. Alfalfa (*Medicago sativa* L.) has been reported to contain a hydroperoxide lyase specific for 13-hydroperoxy linoleic and linolenic acids only. However, in addition to 13-hydroperoxide lyase activity we found substantial 9-hydroperoxide lyase activity in alfalfa seedlings as well. The specific activity for 9-hydroperoxy fatty acids was ~50% of the activity for the 13-isomers. Furthermore, alfalfa seedlings contain a 3*Z*:2*E*-enal isomerase that converts the 3*Z*-enal products to their 2*E*-enal isoforms.

Introduction

Many higher plants have been reported to be able to produce volatile C6- and C9-compounds such as hexanal, (3*Z*)- and (2*E*)-hexenal, (3*Z*)- and (2*E*)-nonenal, (3*Z*,6*Z*)- and (2*E*,6*Z*)-nonadienal and the corresponding alcohols. These compounds are important constituents of the characteristic flavors of fruits, vegetables and green leaves and are widely used as food additives. Besides, they might also be involved in wound healing and pest resistance [1,2]. They are derived from linoleic and linolenic acids in a catalytic route involving three enzymes. Fatty acids are peroxidized by lipoxygenase and subsequently cleaved by hydroperoxide lyase (HPO lyase). The resulting short-chain aldehydes and ω-oxo-acids can be reduced to alcohols by alcohol dehydrogenase. Besides, double bond isomerization can occur. It is still unclear, however, whether this reaction is catalyzed by an isomerase or a nonenzymatic isomerization factor.

Although HPO lyase has been purified from different sources, little is yet known about its structure and reaction mechanism. Hatanaka *et al*. [3] proposed a heterolytic scission mechanism similar to the acid-catalyzed cleavage of linoleate hydroperoxides in an aprotic solvent [4]. However, the recently found homology of HPO lyase with cytochrome P450 enzymes could suggest a homolytic reaction mechanism [5]. Two different types of substrates for plant HPO lyases are known: 13- and 9-hydroperoxy linoleic or linolenic acids. HPO lyases from watermelon seedlings, tea leaves, tomato fruit and leaves, apples, green bell peppers and soybeans are specific for 13-HPO fatty acids [6-11], whereas HPO lyase from pears is specific for 9-HPO fatty acids [12]. Soybean seeds/seedlings and cucumber fruit and seedlings contain both HPO lyase activities [13,14] and Matsui *et al*. [15] succeeded in separating 13- and 9-HPO lyase from cucumber seedlings. In alfalfa, only 13-HPO lyase was found, and no 9-HPO lyase activity was observed [16-18]. The work presented here, however, provides evidence for the presence of both 13- and 9-HPO lyase activity in alfalfa seedlings, which makes alfalfa interesting for application in a biocatalytic process. Furthermore, a heat sensitive 3*Z*:2*E*-enal isomerase was found in alfalfa seedlings, which could be separated from HPO lyase by anion exchange chromatography.

Materials and methods

Materials

All chemicals used were commercially obtained and of analytical grade. 13-HPOD and 13-HPOT were prepared from linoleic and α-linolenic acid, respectively (~99%, Fluka Chemie AG, Buchs, Switzerland), with soybean lipoxygenase-1 [19]. Analogously, 9-HPOD and 9-HPOT were prepared with tomato lipoxygenase [20].

Enzyme activity measurements

HPO lyase activity was determined with the indirect assay described by Vick [21] or by measuring the decrease of the A_{234} due to the cleavage of substrate. One unit of activity corresponds to the amount of enzyme that converts 1 μmol of substrate per min. Protein concentrations were determined with the bicinchoninic acid method after freeze-drying of the samples to remove β-mercaptoethanol [22].

Enzyme purification

Alfalfa seeds (*Medicago sativa* L.) were purchased from a local nursery. Seeds were soaked in tap water for 6 h and germinated in the dark for 3 days at 20 °C. Seedlings were homogenized with 1.7 $ml•g^{-1}$ buffer A (15 mM potassium phosphate, pH 7.5, 0.2% Triton X-100, 10 mM β-mercaptoethanol) in a blender and filtered through four layers of cheesecloth. The filtrate was centrifuged at 40 000 × g for 20 min and the lipid top-layer was discarded. The supernatant (crude extract) was concentrated by ultrafiltration through a 30 kDa membrane (Diaflo PM30, Amicon Inc., Beverly, MA, USA) and applied to a Sephadex G150 (2.5 × 23 cm) and a Sepharose Cl4B column (2.5 × 30 cm) in series (Pharmacia, Uppsala, Sweden). Elution was performed with buffer B (buffer A with 0.1% Triton X-100 instead of 0.2%) at 0.25 $ml•min^{-1}$. The fractions containing HPO lyase activity were pooled and applied to a DEAE Sepharose Cl6B column (1.5 × 10 cm, Pharmacia, Uppsala, Sweden). Elution was performed at 0.5 $ml•min^{-1}$ for 8 h with a linear gradient of 0-0.5 M NaCl in buffer B. All purification steps were carried out at 4 °C.

Product identification

For qualitative product analysis 1 U of HPO lyase (determined with 13-HPOD as substrate) was diluted to 8 ml with buffer C (50 mM potassium phosphate, pH 6). 80 μM of substrate 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 polydimethyl-siloxane coated fiber, Supelco Inc., Bellefonte, USA) 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 temperature program used was 35-200 °C, 10 $°C•min^{-1}$ with a begin and end isotime of 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 temperature program used was 140-280 °C, 6 °C•min^{-1} with a begin and end isotime of 2 min. Electron impact mass spectra were recorded with an ionization energy of 70 eV.

Quantitative product analysis was performed with HPLC after derivatization with DNPH. 0.5 U of enzyme was diluted to 5 ml with buffer C and incubated with 80 μM of substrate in a stirred vessel at 20 °C. After different time periods the reaction was stopped by addition of 5 ml ethanol containing 0.1% DNPH and 0.5 M HAc. 200 μl of 2.5 mM octanal in isopropanol/water (1/1) was added as an internal standard. After 30 min the reaction products were extracted with 2 × 4 ml hexane, the hexane was evaporated and the products were dissolved in 0.5 ml methanol. HPLC was performed with a C18 column (Chromsphere 5, 250 × 4.6 mm, Chrompack) and acetonitril/THF/water (80/1/19, 1 ml•min^{-1}) as eluent. The products were quantified by measuring the absorption at 350 nm.

Results and discussion

The substrate specificity of alfalfa HPO lyase was determined with the assay described by Vick [21], in order to distinguish between HPO lyase and other hydroperoxide converting enzymes. The results are shown in Table 1. A crude extract of alfalfa seedlings was used to prevent possible changes in the ratios of the activities for the different substrates caused by purification steps. Although HPO lyase activity is highest for the 13-isomers, there is substantial 9-HPO lyase activity as well. This is in contrast to previous findings where no 9-HPO lyase activity was observed in alfalfa [16-18]. In the latter studies however, HPO lyase activity was determined by measuring the amount of aldehydes formed by GLC of essential oils. It might be possible that the C9-aldehydes were not observed because of their reduced vapor pressure compared to the C6-aldehydes. In this paper we show that it is possible to analyze C9-aldehydes with SPME-GC, because they are strongly absorbed to the polydimethylsiloxane coated fiber. Furthermore, alfalfa HPO lyase has a slight preference for the hydroperoxytrienes derived from linolenic acid compared to the hydroperoxydienes derived from linoleic acid.

Table 1 Substrate specificity of HPO lyase in a crude extract of alfalfa.
Enzyme activities were determined with the assay described by Vick [21].

Substrate	Specific activity ($U \cdot mg^{-1}$) ± S.D.	Relative activity* ± S.D.
13-HPOD	0.15 ± 0.008	90% ± 5%
13-HPOT	0.17 ± 0.008	100% ± 5%
9-HPOD	0.077 ± 0.008	46% ± 10%
9-HPOT	0.086 ± 0.004	51% ± 5%

The data are mean values of 3 crude extracts
*Activity compared to the activity with 13-HPOT as a substrate

The enzymatic pH optimum was determined and appeared to be equal for 13- and 9-hydroperoxy fatty acids, namely pH 5.5. The pH optimum of alfalfa HPO lyase is in accordance with the optimal pH values described for HPO lyases from other origins, which range from 5.5 in tomatoes and green bell pepper fruit to 8 in cucumber fruits [8,9,15]. In contrast to alfalfa, 13- and 9-HPO lyase from cucumber do not have the same pH optimum. Cucumber 13-HPO lyase has an optimal pH of 6.5, whereas the pH optimum of cucumber 9-HPO lyase is 8 [15]. The specific activities of alfalfa HPO lyase for both types of substrates slightly decrease during the first 8 days of germination and are highest in the 123 000 × g pellet which contains the microsomes. Typical results are shown in Fig. 1.

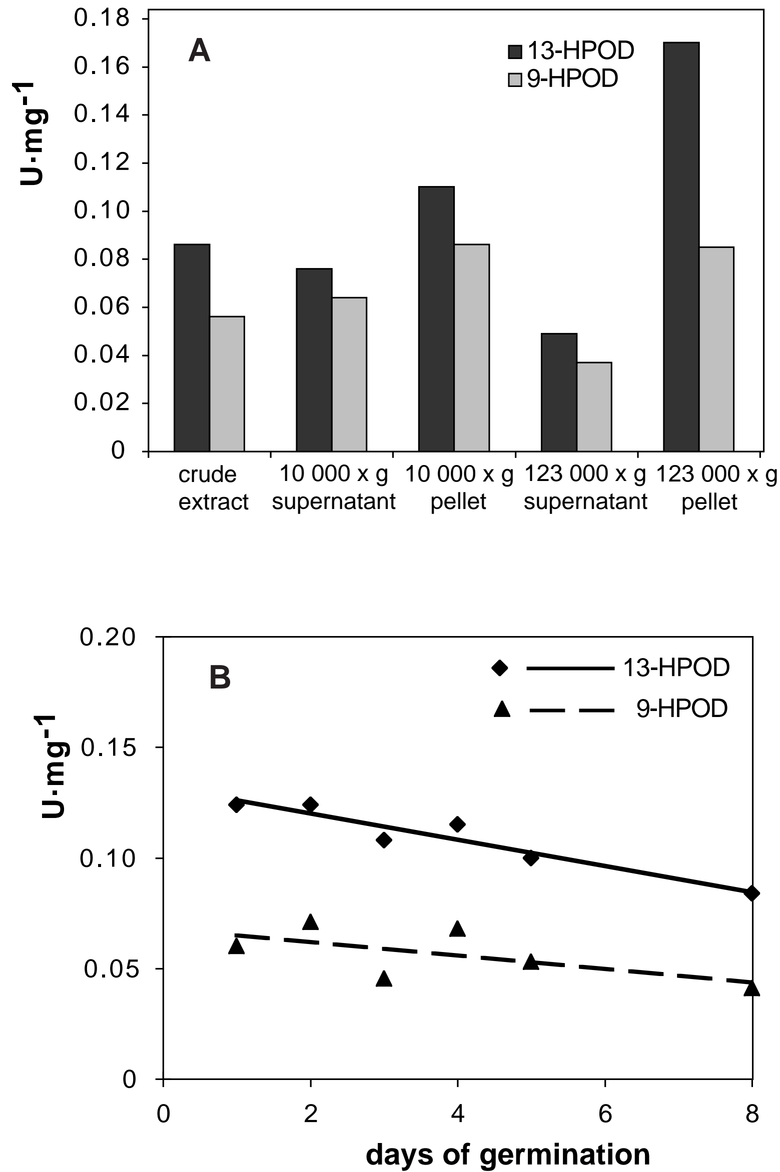

Fig.1 HPO lyase activity in different cell fractions of alfalfa seedlings (A) and during germination (B). HPO lyase activity was determined by measuring ΔA_{234} of a crude extract incubated with 13-HPOD and 9-HPOD. Crude extracts were prepared with 50 mM phosphate buffer pH 7.0, with (B) or without (A) 0.2% Triton X-100.

To find out if alfalfa seedlings contain one enzyme that accepts both types of substrates, or two enzymes each being specific for one positional isomer like cucumber [15], alfalfa HPO lyase was partially purified by ultrafiltration, gelfiltration and anion exchange chromatography. A clear, colorless solution was obtained and the purification factors obtained with this method were typically around 10. With anion exchange chromatography HPO lyase was separated from lipoxygenase, but the 13- and 9-HPO lyase activities showed the same elution profile and could not be separated.

To obtain more information about the products formed by alfalfa HPO lyase, the following substrates were incubated with alfalfa HPO lyase obtained from the gelfiltration pool: 13-HPOD, 9-HPOD, 13-HPOT and 9-HPOT. Headspace analysis of the incubation mixtures showed production of hexanal, (2*E*)-hexenal, (2*E*)-nonenal and (2*E*,6*Z*)-nonadienal, respectively (Fig. 2A). The absence of alcohols is probably caused by the loss of alcohol dehydrogenase during gelfiltration, as headspace analysis of incubations of 13-HPOD and 13-HPOT with a crude extract of alfalfa did show production of hexanol and (2*E*)-hexenol.

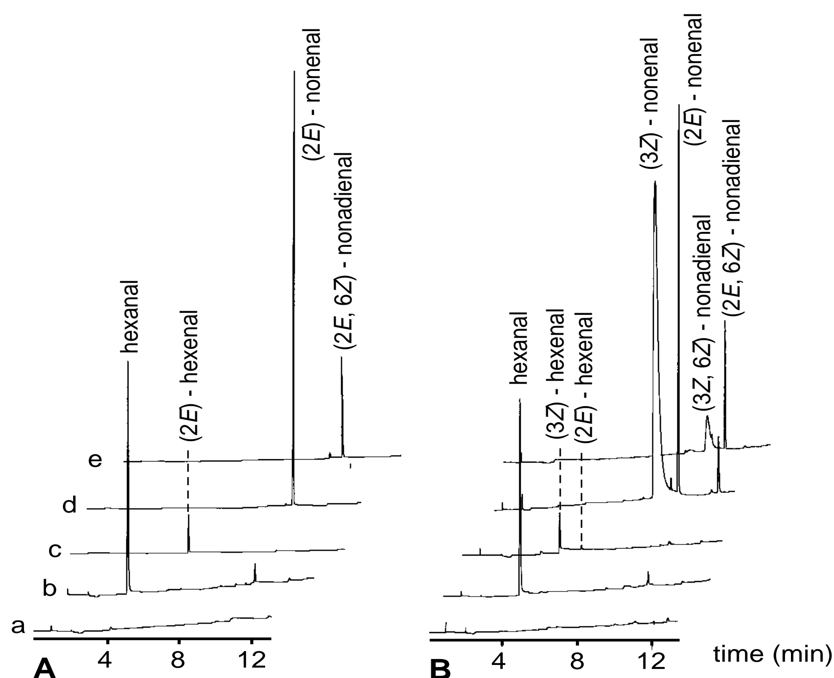

Fig. 2 Volatile reaction products formed by alfalfa HPO lyase obtained from the gelfiltration pool (A) or from the anion exchange pool (B) incubated with (a) no substrate, (b) 13-HPOD, (c) 13-HPOT, (d) 9-HPOD and (e) 9-HPOT. Headspace compounds were trapped by SPME and analyzed by GC-FID. Identification of the compounds occurred by MS.

GC analysis of the nonvolatile compounds in the incubation mixtures with 13-HPOD and 13-HPOT showed a large peak with a retention time of 12.04 min (Fig. 3A). Peaks of the following characteristic ions were present in the electron impact mass spectrum: *m*/*z* [ion attribution; relative intensity], 300 [M^+; 1.6%], 285 [M^+-CH_3; 8.7%], 253 [M^+-CH_3O_2; 51.9%], 129 [$C_3H_4OTMS^+$; 100%] and 73 [TMS^+; 81.1%], which corresponds to the mass spectrum of 12-oxo-(10*E*)-dodecenoic acid [6].

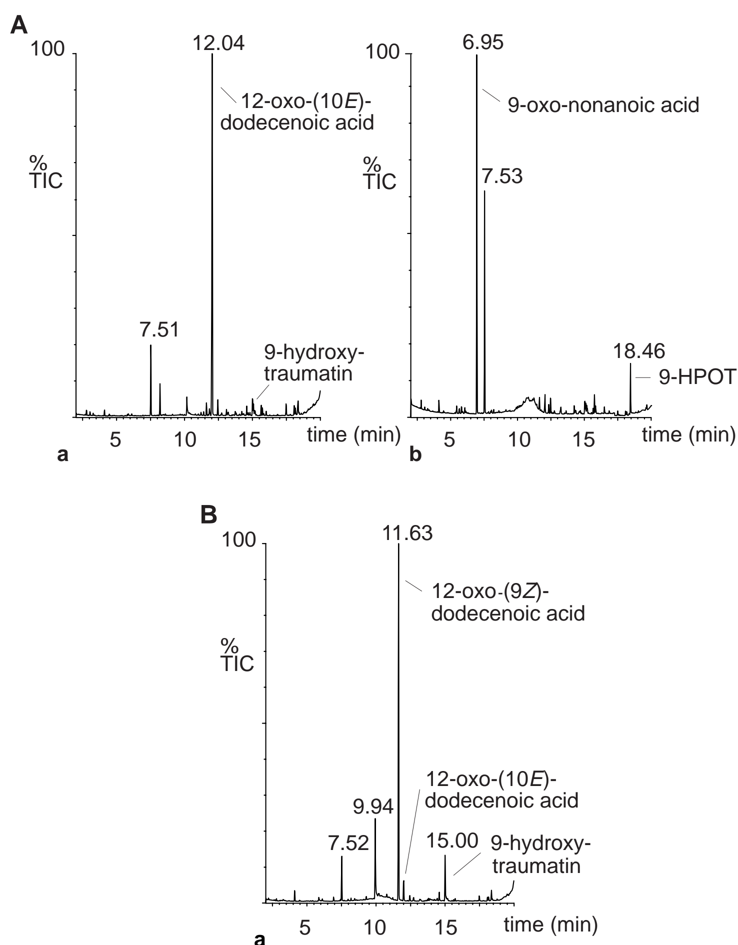

Fig. 3 Nonvolatile reaction products formed by alfalfa HPO lyase obtained from the gelfiltration pool (A) or from the anion exchange pool (B) incubated with (a)13-HPOT and (b)9-HPOT. GC-MS analysis of the methyl esters, trimethylsilyl ethers of the $NaBH_4$ -reduced reaction products. The chromatograms of the incubations with 13-HPOD and 9-HPOD were similar to the chromatograms of the incubations with 13-HPOT and 9-HPOT, respectively.

The mass spectrum of the peak at 15.00 min corresponds to 9-hydroxy-traumatin, another product of the lipoxygenase pathway recently described by Gardner [23]. GC-analysis of the nonvolatile compounds in the incubation mixtures with 9-HPOD and 9-HPOT showed a large peak with a retention time of 6.95 min. The electron impact mass spectrum showed peaks of the following characteristic ions: *m*/*z* [ion attribution; relative intensity], 245 [M^+-CH_3; 27.7%], 213 [M^+-CH_3O_2; 100%], 103 [CH_2OTMS^+; 34.2%], 89 [$OTMS^+$; 43.9%] and 73 [TMS^+; 71.6%]. This product was identified as 9-oxo-nonanoic acid. 13-HPOD and 13-HPOT were almost completely converted, whereas in the cases of 9-HPOD and 9-HPOT there was still substrate left. This might be due to the lower specific activity of alfalfa HPO lyase for the 9-isomers. The peak at 7.5 min also appeared in the sample without substrate and is therefore due to a component in the extract.

Remarkably, only the *E*-isomers were formed, which suggests the presence of an isomerase or an isomerization factor in alfalfa seedlings. When the same experiments were performed with further purified HPO lyase from the anion exchange pool, the *Z*-isoforms were the main products (Fig. 2B,3B). The mass spectrum of the peak with retention time 11.63 min showed peaks of the following characteristic ions: *m*/*z* [ion attribution; relative intensity], 300 [M^+; 0.06%], 285 [M^+-CH_3; 3.2%], 253 [M^+-CH_3O_2; 11.6%], 103 [CH_2OTMS^+; 100%], 73 [TMS^+; 91.4%] and was identified as 12-oxo-(9*Z*)-dodecenoic acid. Thus, the isomerase or isomerization factor is lost during anion exchange chromatography. The loss of isomerase activity was also observed during the purification of cucumber HPO lyase [14]. To obtain more information about the 3*Z*:2*E*-enal isomerization, 13-HPOT was incubated for different periods of time with a crude extract or with HPO lyase obtained from the anion exchange pool. The products were derivatized with DNPH and analyzed with HPLC. As expected, the (3*Z*)-hexenal formed by a crude extract isomerized to (2*E*)-hexenal, whereas with the partially purified enzyme isomerization did not occur (Fig. 4). Remarkably, the isomerization appears to be a much slower process than the lyase reaction which was completed within 1 min. After boiling the crude extract for 5 min, no isomerization activity could be observed anymore, which suggests involvement of an enzyme in the isomerization.

These results clearly show that both 13- and 9-HPO lyase activities and a 3*Z*:2*E*-enal isomerase are present in alfalfa seedlings. This makes an end to the former discrepancy between the product specificity of alfalfa lipoxygenase, which forms almost equal amounts of 13- and 9-hydroperoxy fatty acids [17,24], and the substrate specificity of HPO lyase, which was thought to use only the 13-isomers. The fact that alfalfa HPO lyase shows a high activity for both 13- and 9-substrates and an almost equal activity for the hydroperoxides derived from linoleic or linolenic acid, makes it also very interesting as a biocatalyst for the production of short-chain aldehydes. The characterization of the enzymes and their application as biocatalysts are the subjects of future investigations.

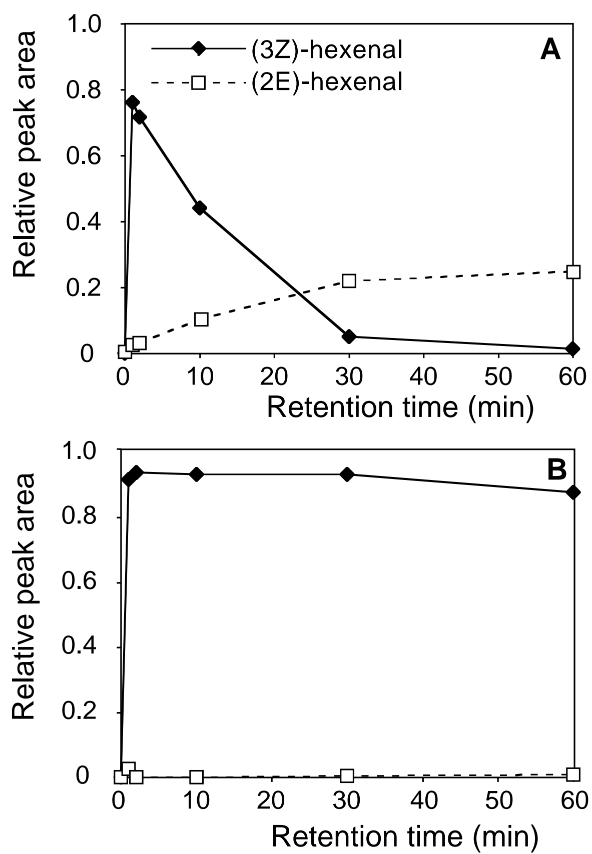

Fig. 4 Volatile products formed by a crude extract of alfalfa (A) and by partially purified HPO lyase (B) incubated with 13-HPOT. HPLC analysis after derivatization of the products with DNPH. The relative peak area is the ratio of product and internal standard peak areas.

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3

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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Abstract

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 substrate. 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 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 extension 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.

Note: The DNA sequences of the *CYP74B4v1, CYP74B4v2* and *CYP74B4v3* genes have been deposited in the EMBL Database under the accession numbers AJ249245, AJ249246 and AJ249247, respectively.

Introduction

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].

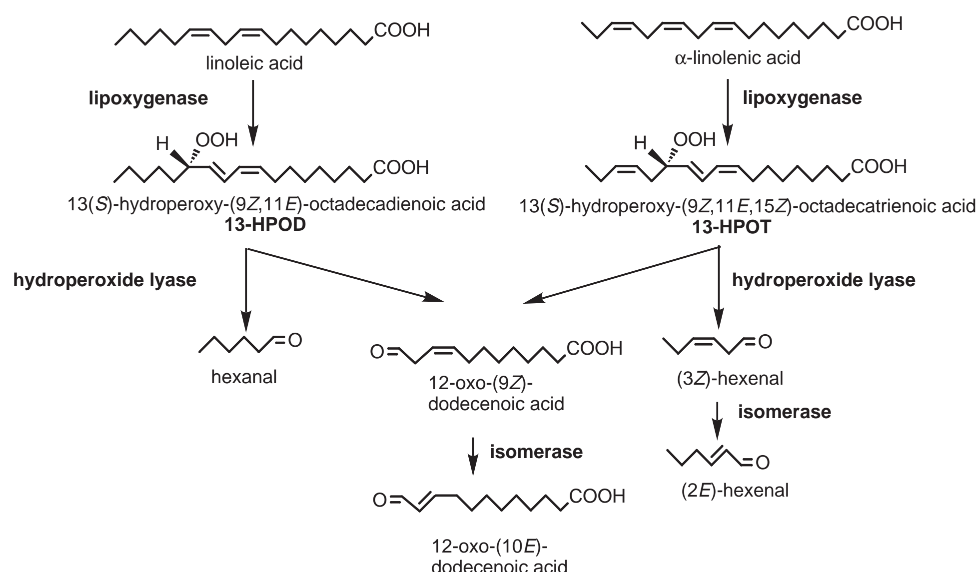

Fig. 1 **Lipoxygenase pathway.** HPO lyase has been purified from different sources and two genes coding for HPO lyases have been published [4,5]. Currently, little is known about its structure and reaction mechanism, due to its instability and difficult purification. Hatanaka *et al*. [6] proposed a 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 linoleic or linolenic 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.

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 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 5 × Denhardts (1 × Denhardts 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 × SSC, 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'-GGGGATCC-TACCGATCCGGCAGATTC-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 1 × LB medium, large cultures (up to 10 liter) in 1.5 × LB 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-NTA 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-NTA 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

1 U of HPO lyase (determined with 13-HPOD as substrate) was diluted to 8 ml with 50 mM potassium phosphate buffer pH 6.0. 80 μM of substrate 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]. 3 Volumes of a 6.3 mM TMBZ-solution (3,3',5,5'-tetramethylbenzidine) in methanol were mixed with 7 volumes 0.25 M sodium acetate buffer pH 5.0. One ml 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. 19 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 found 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 (Cys442) (Fig. 2). The amino acid sequence of CYP74B4v1 showed 61% identity and 69% homology to bell pepper and *Arabidopsis thaliana* HPO lyases. As the similarity starts at leucine-23, the first 22 amino acids probably are 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.

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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
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 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
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 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
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 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
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 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
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 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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 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
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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.

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•mg^{-1}, by measuring the decrease in A_{234} due to the decomposition of 13-HPOT.

	CYP74B4v1	CYP74B4v1	CYP74B4v2	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

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 non-volatile 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.

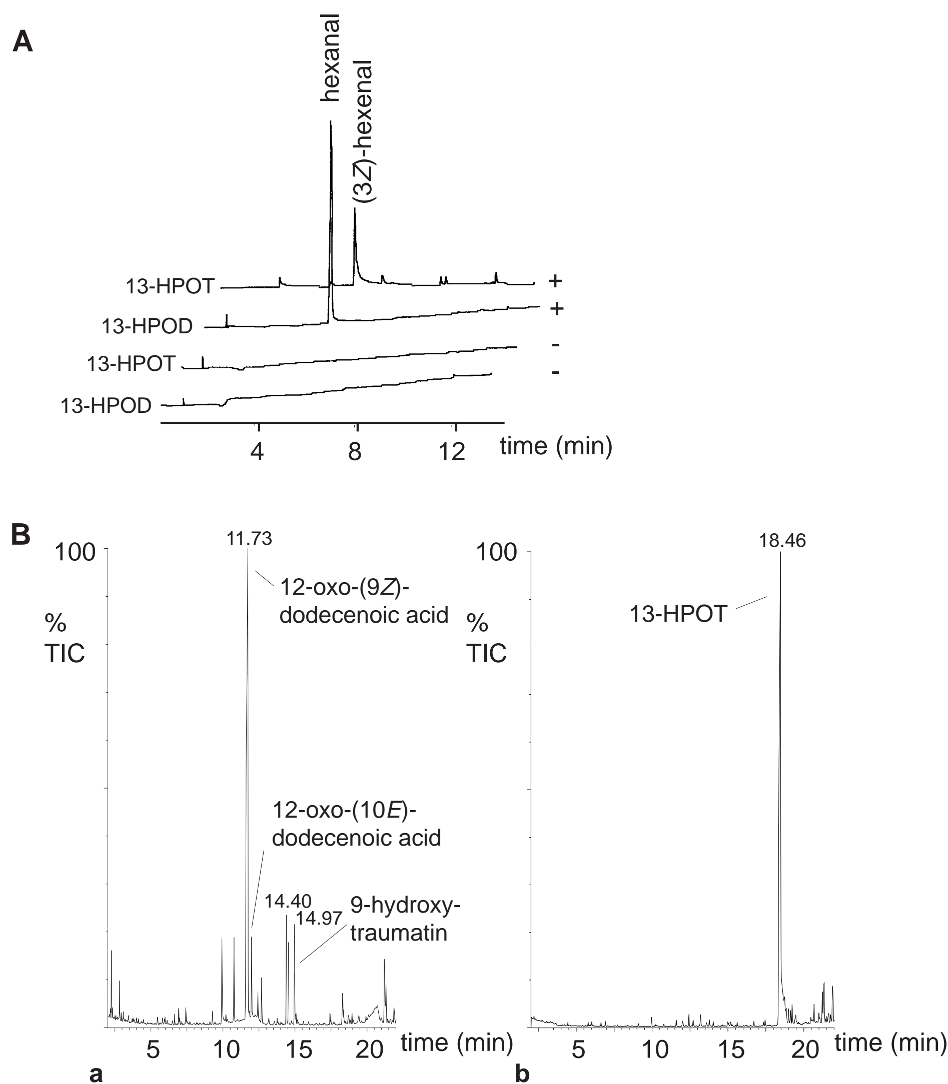

Fig. 3 **A: Volatile products of incubations of 13-HPOD and 13-HPOT with the solubilized membrane fractions of *E. coli* containing the pQE32 plasmid with (+) or without (-) alfalfa HPO lyase gene.** Volatile products were trapped by SPME and analyzed with GC-FID.

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 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 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 liter 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/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-NTA 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.

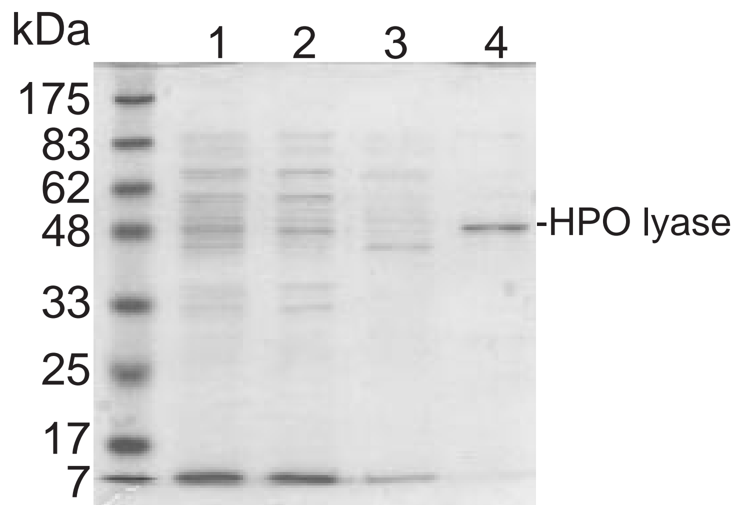

Fig. 4 Purification of expressed HPO lyase by IMAC.
10% polyacrylamide SDS/PAGE gel of the purification fractions as detected by Coomassie Blue staining. Lane 1, solubilized membrane proteins; Lane 2, flow through; Lane 3, wash; Lane 4, elution fraction.

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.

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	0.0073	1.63

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 ± S.D.)

	CYP74B4v1	CYP74B4v1	CYP74B4v2	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

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].

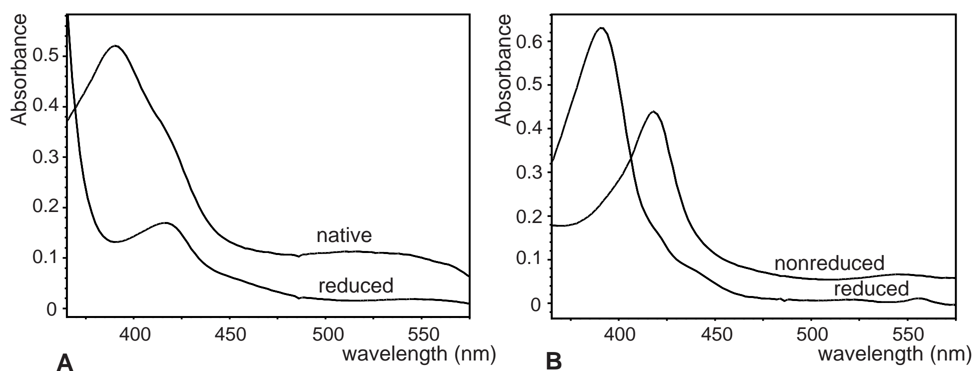

Fig. 5 Absorption spectra of purified alfalfa HPO lyase

A: Absorption spectra of native and sodium dithionite reduced HPO lyase.

B: Absorption spectra of reduced and nonreduced pyridine hemoferrochrome of HPO lyase.

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].

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 *Arabidopsis 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 *Arabidopsis thaliana* gene from the EST database [5]. The N-terminal sequences of the alfalfa HPO lyases are unusual, and different from the *Arabidopsis 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.

	Domain A	Domain B
Alfalfa HPO lyase	LLFVLGFNSYGGFS**I**FL	ELELINSVVYETLRMNPPV
A. thaliana HPO lyase	LLFVLGFNAYGGFS**V**FL	EMELVKSVVYETLRFNPPV
Bell pepper HPO lyase	LLFILGFNAFGGFT**I**FL	EMELVQSFVYESLRLSPPV
A. thaliana AOS	LLFATSFNTWGGMK**I**LF	KMELTKSVVYECLRFEPPV

	Domain C	Domain D
Alfalfa HPO lyase	RDPVVFDEPEQFKPERFTKEKGA	PTVSNKQ**C**AGKDIVTFTAALIV
A. thaliana HPO lyase	RDANVFDEPEEFKPDRYVGETGS	PSASNKQ**C**AAKDIVTLTASLLV
Bell pepper HPO lyase	KDPKVFDEPEKFMLERFTKEKGK	PTESNKQ**C**AAKDAVTLTASLIV
A. thaliana AOS	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. Alfalfa hydroperoxide lyases; *Arabidopsis thaliana* hydroperoxide lyase [5]; Bell pepper hydroperoxide lyase [4]; *Arabidopsis thaliana* allene oxide synthase [32].

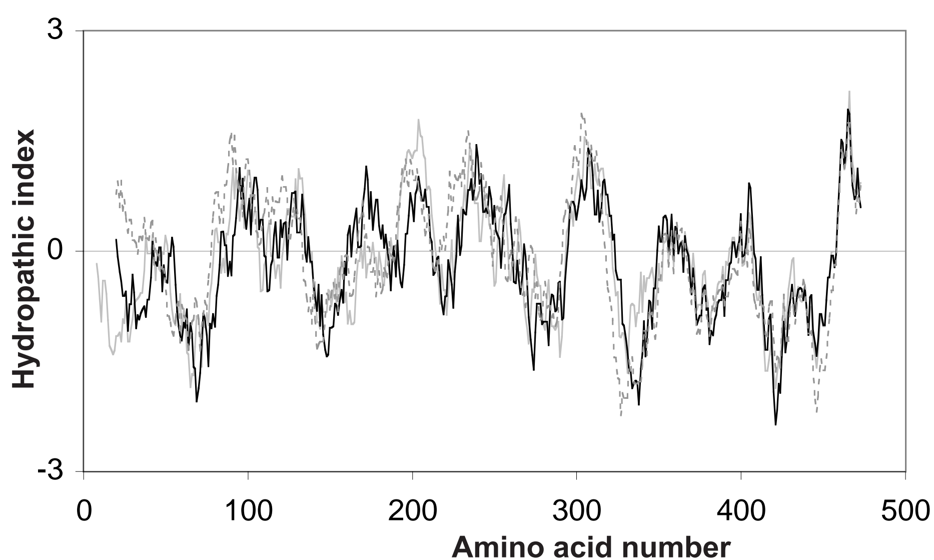

Fig. 7 Hydropathy plots of alfalfa, *Arabidopsis thaliana* and bell pepper HPO lyases indicate that these enzymes have a similar structure.
The hydrophobicity scale of Kyte *et al.* [43] and a 15-amino acid window were used. — Alfalfa HPO lyase (CYP74B4v1), - - - Bell pepper HPO lyase [4], — *Arabidopsis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

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 (TMPred-Prediction of Transmembrane Regions and Orientation; 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 *Arabidopsis 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 Ser-377 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 hydroxylgroup. Bell pepper HPO lyase contains Ser at position 377 [4], whereas *Arabidopsis 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 *Arabidopsis 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.

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 2 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.

Fraction	Relative activity
Crude homogenate	100%
10 000 x g supernatant	72%
10 000 x g pellet	18%
123 000 x g supernatant	49%
123 000 x g pellet	17%

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.

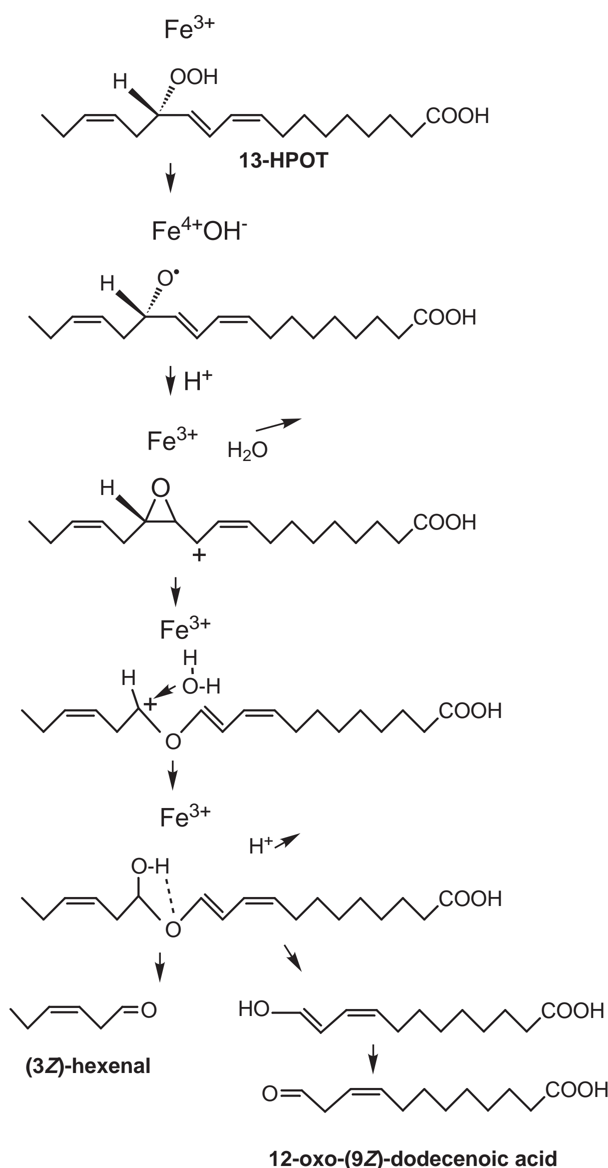

Fig. 8 Proposed reaction mechanism for HPO lyase.

Acknowledgements

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Spectroscopic studies on the active site of hydroperoxide lyase; the influence of detergents on its conformation

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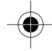

Abstract

Expression of high quantities of alfalfa hydroperoxide lyase in *E. coli* made it possible to study its active site and structure in more detail. CD spectra showed that hydroperoxide lyase consists for about 75% of α-helices. EPR spectra confirmed its classification as a cytochrome P450 enzyme. The positive influence of detergents on the enzyme activity is paralleled by a spin state transition of the heme Fe(III) from low to high spin. EPR and CD spectra showed that detergents induce a subtle conformational change, which might result in improved substrate binding. Because hydroperoxide lyase is thought to be a membrane bound protein and detergents mimic a membrane environment, the more active, high spin form likely represents the *in vivo* conformation. Furthermore, the spin state appeared to be temperature-dependent, with the low spin state favored at low temperature. Point mutants of the highly conserved cysteine in Domain D indicated that this residue might be involved in heme binding.

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Introduction

Plants continuously fight against life-threatening events such as drought, mechanical damage, temperature stress, and potential pathogens. The most important signal pathway induced by wounding is the octadecanoid or lipoxygenase pathway. The first step in the lipoxygenase pathway is the dioxygenation of linolenic or linoleic acid by lipoxygenase (LOX) [1]. The formed hydroperoxy fatty acids are further metabolized by enzymes like allene oxide synthase (AOS, CYP74A), peroxygenase, divinyl ether synthase or hydroperoxide lyase (HPO lyase, CYP74B). HPO lyase cleaves the C-C bond adjacent to the hydroperoxy group, resulting in the formation of ω-oxo acids and volatile aldehydes. The (3*Z*)-aldehydes can be isomerized to their (2*E*)-isomers and both can be reduced by alcohol dehydrogenase to their corresponding alcohols. The volatile aldehydes and alcohols, which have a characteristic 'green, fruity' smell, are involved in wound healing and pest resistance [2-5], whereas 12-oxo-(10*E*)-dodecenoic acid (traumatin) is considered to be a wound hormone [6].

HPO lyases have been purified from a number of organisms, and recently HPO lyases from bell pepper, *Arabidopsis thaliana*, tomato, alfalfa and guava fruit were cloned and expressed in *E. coli* [7-11]. Based on its sequence homology, HPO lyase is supposed to belong to the class of cytochrome P450 enzymes [7]. Similar to other cytochrome P450 enzymes, the heme group in the active site of HPO lyase was identified as heme *b* (protoheme IX) [10,12], and is probably bound by a highly conserved cysteine [7-10]. But in contrast to other cytochrome P450 enzymes, hydroperoxide lyases do not show the characteristic absorption maximum at 450 nm after reduction and treatment with CO [12,13]. This behavior is similar to allene oxide synthases that also have a low affinity for CO [14]. Furthermore, HPO lyases and AOS show little homology to other cytochrome P450 enzymes in the I-helix region (oxygen binding pocket) and lack the conserved threonine. This can be explained by the fact that HPO lyases and AOS do not require molecular oxygen nor an NADPH-dependent cytochrome P450 reductase for their activity. These enzymes are, therefore, unique within the cytochrome P450 family.

The structure and reaction mechanism of HPO lyase are still unclear, because the amounts of HPO lyase available so far were too low for characterization studies. Unraveling the structure of the active site is a primary step towards the elucidation of the reaction mechanism of HPO lyase. Our previously described expression system [10] made it for the first time possible to obtain enough enzyme to further characterize its active site by EPR, and determine if HPO lyase indeed belongs to the class of cytochrome P450 enzymes. EPR is superior to UV/Vis spectrophotometry in providing information about heme symmetry, identifying heme ligands, and understanding the electronic structure of the heme iron. Point mutants of the conserved cysteine (C_{442} of alfalfa HPO lyase) were constructed and studied to determine if this residue is involved in binding of the active site heme. Furthermore, the influence of detergents like Triton X-100, commonly used to solubilize cytochrome P450 enzymes, on HPO lyase was studied.

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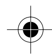

Materials and methods

Enzyme preparations

Alfalfa HPO lyase was expressed in *E. coli* M15 cells containing a pQE32 vector (Qiagen) with the *CYP74B4v1* gene (EMBL Database, accession number AJ249245) without N-terminal sequence encoding the first 22 amino acids, and solubilized from the membrane fraction by 0.2% Triton X-100 [10]. The enzyme was purified to homogeneity by immobilized metal affinity chromatography, as described previously [10]. Triton X-100 was removed from the sample by performing the second wash step and the elution with buffer without Triton X-100.

Construction of HPO lyase point mutants

$C_{442}A$ and $C_{442}S$ point mutants were constructed by site-directed mutagenesis of the *CYP74B4v1* gene without the N-terminal extension of 22 amino acids. The following primers were used for PCR: N-terminal fragment: 5' GTTCAAGCATATGGGTACCAG 3' and 5' TACGGCCGCCTGTTTATTGGACACAG 3' ($C_{442}A$) or 5' TACGGCCGACTGTTTATTGGAC-ACAG 3' ($C_{442}S$), C-terminal fragment: 5' TACGGCCGGTAAGGACATCGTG 3' and Primer-Reverse Sequencing (Qiagen). The N- and C-terminal fragments were restricted with *Eag* I, *Pst* I and *Eag* I, *Hind* III, respectively, and cloned into pBluescript KS+ and pBluescript SK- vectors, respectively. The N-terminal fragments were then cloned into the pBluescript SK- vectors containing the C-terminal fragment, by restrictions with *Afl* III and *Eag* I. The combined fragments were isolated by restriction with *Pst* I. These were cloned into *Pst* I restricted and dephosphorylated pQE32 vectors (Qiagen) containing the first part of the *CYP74B4v1* gene. The sequences were checked by sequence analyses and the enzymes were expressed and purified as described [10].

Enzyme activity measurements

HPO lyase activity was determined in 50 mM potassium phosphate buffer pH 6.0 containing 100 μM of substrate by measuring the decrease of the A_{234} due to the cleavage of substrate. Protein concentrations were determined with the bicinchoninic acid method [15]. The substrates, 13-HPOD and 13-HPOT were prepared from linoleic and α-linolenic acid, respectively (~99%, Fluka), with soybean LOX-1 [16].

Heme analyses

The heme staining procedure was adapted from Thomas *et al*. [17]. Heme staining of the HPO lyase point mutants was performed as described previously, with wild-type HPO lyase as a positive control [10]. Spectrophotometric analyses of purified HPO lyase were carried out with native and reduced enzyme. Reduction occurred 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% (v/v) and NaOH to 0.2 M.

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CD and EPR analyses

CD spectra were recorded on a Jasco J-600 Spectropolarimeter. Prior to the CD measurements, the HPO lyase samples were dialyzed against 50 mM potassium phosphate buffer, pH 7.5, with or without 0.2% Triton X-100, to remove NaCl. The CD spectrum of this buffer was subtracted from the HPO lyase CD spectra. EPR spectra were recorded on a Bruker ECS-106 EPR spectrometer equipped with a 5350 B Hewlett Packard microwave frequency counter and a cryostat from Oxford Instruments. Liquid He was used to regulate the temperature of the sample. Prior to the EPR measurements, the HPO lyase samples were concentrated to 0.5 mg•ml^{-1} with a 30 kDa microcon filter (Amicon/Millipore, Bedford, USA).

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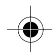

Results and discussion

The rhombic EPR spectrum of purified alfalfa HPO lyase in the presence of Triton X-100 at 45 K (Fig. 1) is typical for a heme Fe(III) in the low spin state, bound to an axial thiolate anion (from cysteine) and an OH-group [18-20]. In the EPR spectrum of HPO lyase recorded at 7.3 K, no high spin Fe(III) signal could be detected in the g = 8 region. The g values, g_z = 2.40, g_y = 2.24 and g_x =1.92 are characteristic for cytochrome P450 enzymes and are clearly distinguishable from the g values of other heme proteins [18,19]. The EPR spectrum confirms that HPO lyase belongs to the cytochrome P450 class. UV/Vis spectra of HPO lyases and AOS recorded at room temperature indicated that these enzymes contain Fe(III) in the high spin state [10,12,13,21]. This points to temperature dependency of the spin state. The high spin state seems to be favored at room temperature, whereas at the low temperature of the EPR measurements the low spin form is favored. The high spin state was not detectable with EPR at higher temperatures due to fast electron-spin relaxation. Temperature dependency of the spin state of cytochrome P450 enzymes has been observed before and is due to the fact that transitions between the spin states are accompanied by slight energy alterations [19,22].

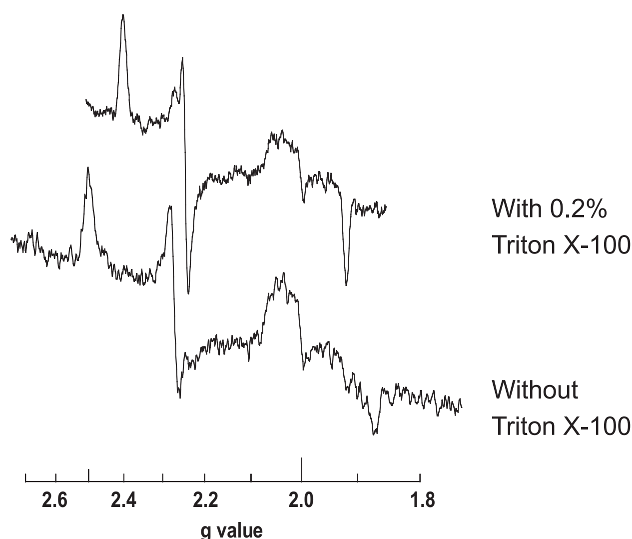

Fig. 1 **EPR spectra of alfalfa HPO lyase in the presence or absence of 0.2% Triton X-100.** The spectra were recorded at 45 K, 20 dB, 9424.2 MHz and 9421.1 MHz, respectively, with a modulation amplitude of 1.27 mT. g Values of HPO lyase with Triton X-100: g_z = 2.40, g_y = 2.24, g_x =1.92, and g values of HPO lyase in the absence of Triton X-100: g_z = 2.50, g_y = 2.27, g_x = 1.87.

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All HPO lyases and AOS described were solubilized by the use of detergents, mostly Triton X-100. Removal of Triton X-100 leads to a remarkable change in the spectral properties of HPO lyase (Table 1). The Soret maximum shifted from 390 to 418 nm, which indicates that the spin equilibrium moves from high towards low spin. By readdition of Triton X-100, the equilibrium shifted back towards the high spin state (Fig. 2). This change in the spin state could also be induced by addition of the detergent *n*-octyl-β-D-glucopyranoside (1%), and was thus not specific for Triton X-100. Addition of a mixture of triglycerides to low spin HPO lyase slightly moved the spin equilibrium towards the high spin state as well. The EPR spectrum of Triton-free HPO lyase showed g values different from the ones of HPO lyase in the presence of Triton X-100 (g_z = 2.50, g_y = 2.27 and g_x = 1.87) (Fig. 1). This indicates that Triton X-100 affects the conformation of the heme site in the enzyme. Previously, we observed that the His-tag of recombinant HPO lyase was only exposed in the presence of Triton X-100, which also indicates that Triton X-100 causes a change in the structure of the enzyme [10].

Table 1 Absorption maxima of HPO lyase with or without 0.2% Triton X-100. Reduction occurred by addition of sodium dithionite to a final concentration of 0.2% (w/v). Pyridine hemoferrochrome was prepared by addition of pyridine to a concentration of 20% (v/v) and NaOH to 0.2 M.

HPO lyase	+ 0.2 % Triton X-100	- Triton X-100
native	390 nm	418 nm
reduced	416 nm	420 nm
pyridine hemoferrochrome	418 nm	390 nm
pyridine hemoferrochrome reduced	390 nm	390 nm

CD spectra of HPO lyase in the presence or absence of 0.2% Triton X-100 showed no obvious structural differences between the two enzyme states (Fig. 3). This indicates that the structure perturbation caused by Triton X-100 is apparently subtle. The CD spectra also indicate that HPO lyase consists for about 75% of α-helices, which is similar to previously described structures of prokaryotic cytochrome P450 enzymes [23-25].

The effect of detergents on the spin state of HPO lyase is similar to a type I interaction. Most cytochrome P450 enzymes are in the low spin state and can be shifted towards high spin by addition of a hydrophobic substrate or a ligand (type I interaction) [26-29]. A substrate molecule probably displaces the water molecule at the sixth, axial, ligand position of the heme iron. This might lead to the movement of iron out of the plane of the porphyrin ring, which makes the high spin configuration favorable [30,31]. Because Triton X-100 is too large to fit into the distal ligand pocket, it can not directly interact with the iron. The different EPR spectra

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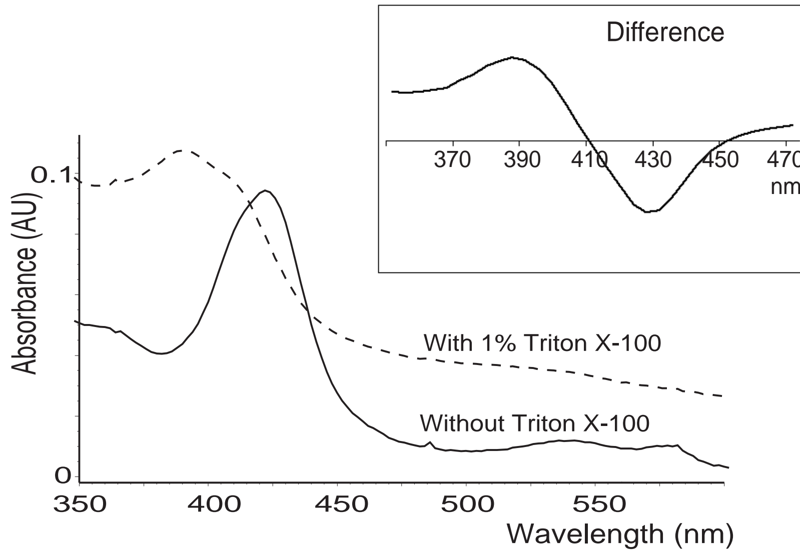

Fig. 2 UV/Vis spectra of alfalfa HPO lyase before and after addition of 1% Triton X-100. The difference spectrum was obtained by subtraction of the spectrum without Triton X-100 from the spectrum of HPO lyase with Triton X-100.

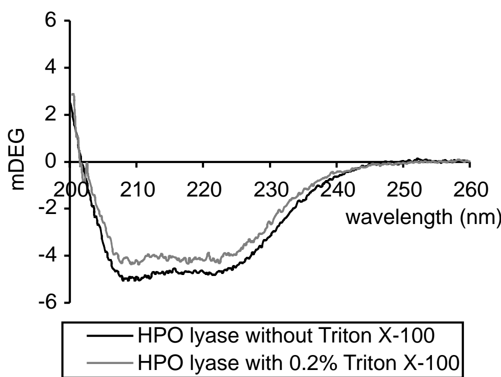

Fig. 3 CD spectra of alfalfa HPO lyase in the presence or absence of 0.2% Triton X-100.

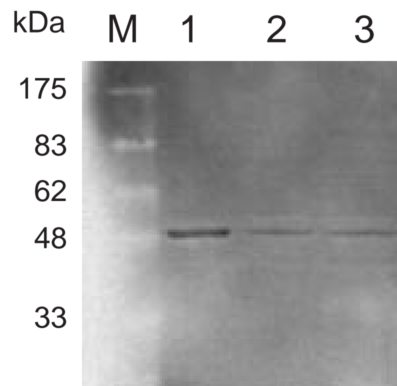

Fig. 4 Western blot of a 10% polyacrylamide SDS/PAGE gel. Lane 1: purified wild-type HPO lyase, lane 2: C_{442}A mutant, and lane 3: C_{442}S point mutant, M: molecular weight marker. Detection was performed with Ni-NTA HRP conjugate (Qiagen), as described by the manufacturer.

of HPO lyase in the presence or absence of Triton X-100 however, indicate that Triton X-100 does change the conformation of the active site. Detergents probably induce the formation of a high spin complex by indirectly perturbing the heme structure and excluding the original axial heme ligand (H_2O), or change the bond length between the heme iron and the sulfur atom. A similar effect was observed when the cytochrome P450 nitric oxide synthase was incubated with a number of large ligands [29,32]. The influence of detergents on the spin state might be typical for enzymes of the CYP74 family, because the cytochrome P450 obtusifoliol 14α-demethylase, which was also solubilized by Triton X-100, was still in the low spin state [33].

Interestingly, the enzyme activity of HPO lyase decreased by about 50% due to the removal of Triton X-100 and subsequent shift in the spin state. Previously, it was also observed that the activity of HPO lyase and AOS in crude extracts increased about twofold by addition of Triton X-100 or other detergents such as polyvinylpyrrolidone or the nonionic Emulgen 911 [21,34-36]. This suggests that the spin state of HPO lyase is important for the enzymatic activity of HPO lyase. The functional importance of the spin state of cytochrome P450 enzymes is not yet understood. It has been suggested that the high spin form is more rapidly reduced [37], 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, showed no correlation with the enzyme activity [29]. In the case of HPO lyase, the increased activity upon transition from low to high spin state might be caused by a lower binding enthalpy and facilitated substrate binding. Because the sixth ligand position in the high spin state is vacant, the heme group might be better accessible for the substrate.

Detergents might mimic a membrane environment. Because HPO lyase is thought to be a membrane protein, it is likely that the more active, high spin state is the *in vivo* conformation of the enzyme. The ability of triglycerides to induce a shift in the spin state as well confirms the essence of a membrane-like environment. It is also possible that HPO lyase activity is partly regulated by the organization of the membrane around the enzyme, as has been suggested before [35].

$C_{442}A$ and $C_{442}S$ point mutants of alfalfa HPO lyase were constructed, expressed in *E. coli* and purified by immobilized metal affinity chromatography (Fig. 4). The cysteine point mutants appeared to be inactive and no coloring was observed upon heme staining, indicating that these mutants do not contain heme. These results suggest that the highly conserved cysteine (C_{442} of alfalfa HPO lyase) is involved in binding of the active site heme.

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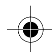

Acknowledgements

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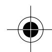

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Formation of a new class of oxylipins from *N*-acyl(ethanol)amines by the lipoxygenase pathway

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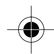

Abstract

N-acylethanolamines (NAEs) constitute a new class of plant lipids and are thought to play a role in plant defense strategies against pathogens. In plant defense systems, oxylipins generated by the lipoxygenase pathway are important actors. To date, it is not known whether plants also use endogeneous oxylipins derived from NAEs in their defense reactions. We tested whether members of the NAE class can be converted by enzymes constituting this pathway, such as (soybean) lipoxygenase-1, (alfalfa) hydroperoxide lyase and (flax seed) allene oxide synthase. We found that both α- and γ-*N*-linolenoylethanolamine (18:3), as well as α- and γ-*N*-linolenoylamine were converted into their 13(*S*)-hydroperoxide derivatives by lipoxygenase. Interestingly, only the hydroperoxides of α-*N*-linolenoyl(ethanol)amines and their linoleic acid analogs (18:2) were suitable substrates for hydroperoxide lyase. Hexanal and (3*Z*)-hexenal were identified as volatile products of the 18:2 and 18:3 fatty acid (ethanol)amides, respectively. 12-Oxo-*N*-(9*Z*)-dodecenoyl(ethanol)amine was the nonvolatile hydrolysis product. Kinetic studies with lipoxygenase and hydroperoxide lyase revealed that the fatty acid ethanolamides were converted as readily or even better than the corresponding free fatty acids. Allene oxide synthase utilized all substrates, but was most active on 13(*S*)-hydroperoxy-α-*N*-linolenoylethanolamine and the 13(*S*)-hydroperoxide of linoleic acid and its ethanolamine derivative. α- and γ-ketols were characterized as products. In addition, cyclized products, i.e. 12-oxo-*N*-phytodienoylamines, derived of 13(*S*)-hydroperoxy-α-*N*-linolenoylamines were found. The results presented here show that, in principle, hydroperoxide NAEs can be formed in plants and subsequently converted into novel phytooxylipins.

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Introduction

Fatty acid amides are emerging as important signal transduction molecules in mammals and plants. In mammals various compounds of this new lipid family, i.e. *N*-acylethanolamines (NAEs), exert several neurological and immunological functions. It is speculated that these NAEs may function as stress-recovery factors [1]. Little attention has been paid to the family of fatty acid amides in plants. The discovery of the important function of NAE in mammals, however, has renewed the interest in plant NAEs. *N*-Acylphosphatidylethanolamines (NAPEs) were first detected in plants more than 30 years ago and represent 1-5% of the lipid content [2]. The hydrolysis products of NAPEs, NAEs, may play a role in germination and in the defense system of plants [3,4]. During early germination of soybeans the amount of NAPE decreased from 3.5 to 1.5% of the total lipid content [3]. In tobacco-cell cultures the [^{14}C]-NAPE content decreased 5-fold after stimulation with a fungal elicitor, whereas the [^{14}C]-NAE content in the culture-medium increased approximately 6-fold [4]. *N*-Laureoylethanolamine (12:0) and *N*-myristoylethanolamine (14:0) were identified as endogeneously released compounds. Two hours after elicitor stimulation NAPE-synthase activity was increased, thereby replenishing NAPE [5,6].

The plant defense system comprises a complex array of constitutive and inducible responses. Oxygenated fatty acids, termed oxylipins, were shown to be important metabolites in plant resistance [7]. These (phyto)oxylipins are generated in the lipoxygenase pathway. The first step in this cascade is catalyzed by lipoxygenases, which convert fatty acids containing one or more (1*Z*,4*Z*)-pentadiene systems into 1-hydroperoxy-(2*E*,4*Z*)-pentadiene derivatives in a regio- and stereospecific manner. The hydroperoxy fatty acids derived from linoleic (18:2) and linolenic (18:3) acids can be metabolized further by hydroperoxide lyase (HPO lyase), allene oxide synthase (AOS) or peroxygenase (POX). HPO lyases cleave the C-C bond next to the hydroperoxy group, resulting in the formation of short-chain aldehydes and ω-oxo acids. The short-chain aldehydes are important constituents of the characteristic flavors of fruits, vegetables and green leaves and might be involved in wound healing and pest resistance [8,9]. The ω-oxo acid 12-oxo-(9*Z*)-dodecenoic acid can be converted to the wound hormones traumatin (12-oxo-(10*E*)-dodecenoic acid) and traumatic acid [10]. AOS dehydrates the hydroperoxy fatty acids to unstable allene oxides, which hydrolyze spontaneously to α- and γ-ketols or are enzymatically cyclized to 12-oxo-phytodienoic acid, the precursor of jasmonic acid. Jasmonic acid is a wound hormone which is generally considered to be a key mediator in signal transduction of defense reponses. POX converts the hydroperoxy fatty acids into phytoalexins, a class of natural pesticides [7].

Anandamide and *N*-linoleoylethanolamine (ODNHEtOH) (18:2) have been shown to be suitable substrates for plant lipoxygenases [11,12]. Because 13(*S*)-hydroperoxylinolenic acid (18:3) is an important precursor for a broad range of phytooxylipins, it is interesting to know whether its corresponding *N*-linolenoyl(ethanol)amines (18:3, OTNHEtOH and $OTNH_2$) can also serve as substrates for lipoxygenases. Furthermore, it is not yet known whether

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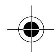

hydroperoxy *N*-linoleoyl(ethano)amines and *N*-linolenoyl(ethanol)amines can be further metabolized by HPO lyases and AOS to form possible defense compounds. Therefore, we tested four different linolenic acid amide derivatives as substrate for soybean lipoxygenase-1. Hydroperoxy *N*-linoleoyl(ethanol)amine and *N*-linolenoyl(ethanol)amines were tested as substrates for alfalfa HPO lyase and flax seed AOS. Here, we present the characterization of the products and the regio- and stereospecificities, as well as the kinetic parameters, of the dioxygenation and subsequent metabolism of these novel lipids.

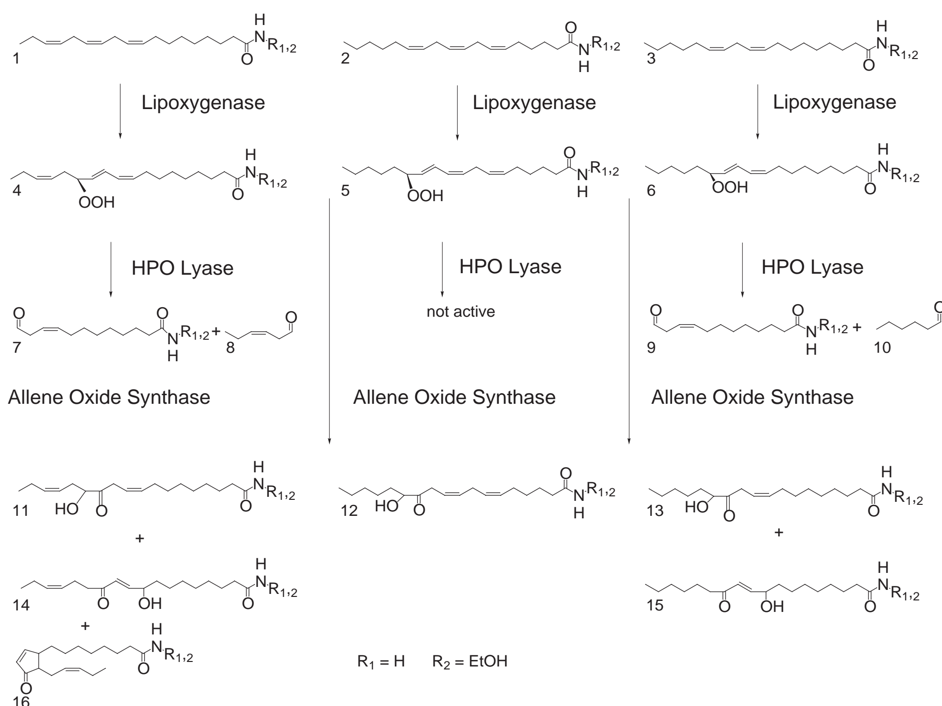

Fig. 1 Structures of products in the lipoxygenase-pathway of α- and γ-*N*-linolenoyl-(ethanol) amine (1,2) and their linoleic analogs (3).

Lipoxygenase products: 13(*S*)-hydroperoxy-*N*-(9*Z*,11*E*,15*Z*)-octadecatrienoyl(ethanol)amine (4); 13(*S*)-hydroperoxy-*N*-(6*Z*,9*Z*,11*E*)-octadecatrienoyl(ethanol)amide (5) and 13(*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoyl(ethanol)amine (6). HPO lyase products: 12-oxo-*N*-(9*Z*)-dodecenoyl(ethanol)amine (7,9); 3(*Z*)-hexenal (8) and hexanal (10). Allene oxide synthase products, α-ketols: 12-oxo-13-hydroxy-*N*-(9*Z*,15*Z*)-octadecadienoyl(ethanol)amine (11), 12-oxo-13-hydroxy-*N*-(6*Z*,9*Z*)-octadecadienoyl(ethanol)amine (12), 12-oxo-13-hydroxy-*N*-(9*Z*)-octadecenoyl(ethanol)amine (13) and γ-ketols: 12-oxo-9-hydroxy-*N*-(10*E*,15Z)-octadecadienoyl(ethanol)amine (14), 12-oxo-9-hydroxy-*N*-(10*E*)-octadecenoyl(ethanol)amine (15) and 12-oxo-*N*-phytodienoyl(ethanol)amine (16).

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Materials and methods

Materials and enzymes

Linoleic acid, α- and γ-linolenic acid (99% pure) were obtained from Sigma. *N*-linole(n)oyl-ethanolamine (OTNHEtOH) and *N*-linole(n)oylamine ($OTNH_2$) were synthesized using standard protocols [12]. All reagents used were of the purest grade available. Lipoxygenase-1 was purified from soybean (Maple Glen) as described previously [13]. The specific activity of the lipoxygenase preparation was 40 U (μmol linoleic acid•min^{-1})•mg^{-1}. AOS was extracted from an acetone powder of flaxseed as described previously [14]. The clear extract was purified further using solid phase extraction (SPE) on BakerBond (solid state C18, 500 mg, J.T. Baker) and OASIS (30 mg, Waters). The specific activity of the AOS preparation was 0.81 U (μmol 13-hydroperoxy linoleic acid•min^{-1})•mg^{-1}.

Alfalfa HPO lyase was expressed in *E. coli* cells containing the pQE32 vector (Qiagen) with the *CYP74B4v1* gene without N-terminal sequence coding for the first 22 amino acids (EMBL Database, accession number AJ249245). A 10 l culture of these *E. coli* cells was grown at 37 °C until an A_{600} of 0.7, in 1.5 x LB medium supplemented with 0.5% glucose, 1 x minimal medium buffer, 25 μg•ml^{-1} kanamycine and 100 μg•ml^{-1} ampicilline, with maximal stirring and O_2 flow. Expression of HPO lyase was induced by addition of 1 mM IPTG and the cells were grown overnight at 30 °C to prevent sequestering of the HPO lyase in inclusion bodies. Cells were harvested by centrifugation and the supernatant was discarded. The cell pellet was resuspended in 350 ml 50 mM potassium phosphate buffer pH 7.5 and sonicated on ice 15 x 1 min with 1-min intervals, in batches of 35 ml. The suspension was recentrifuged and the membrane pellet was resuspended in 400 ml 50 mM potassium phosphate buffer pH 7.5 containing 0.2% (m/v) Triton X-100 (membrane solubilization buffer). After centrifugation, HPO lyase was present in the supernatant. The enzyme was purified to homogenity by immobilized metal affinity chromatography (IMAC) using FPLC with a flowrate of 1 ml•min^{-1}. 100 ml of the supernatant was applied to a column containing 10 ml Ni-NTA superflow (Qiagen), equilibrated previously with membrane solubilization buffer. The column was washed with 20 ml membrane solubilization buffer and 50 ml 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. The active fractions were pooled and the specific activity of the HPO lyase preparation was 249 U (μmol α-13-hydroperoxy linolenic acid•min^{-1})•mg^{-1}. All steps were carried out at 4 °C.

Lipoxygenase assay: extraction and purification of reaction products

Typically 40 μM of substrate in 30 ml rigorously stirred, air-saturated 0.1 M sodium borate buffer pH 9.0 was incubated with 1 U of lipoxygenase-1 for approximately 60 min at 20 °C. The reaction was stopped by acidifying the reaction mixtures to pH 3 with 3 M HCl. The fatty acid amides were extracted with a BakerBond solid state C18-column (500 mg) according to

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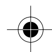

van Aarle *et al.* [15]. The eluate was concentrated under a N_2-flow and residual water was evaporated azeotropically with methanol. The products were dissolved in 1 ml methanol and stored at –25 °C until use. For characterization, the products were reduced with $NaBH_4$ in methanol and purified by using HPLC; the main products were analyzed by ^{1}H-NMR, UV-spectroscopy, CD-spectroscopy, CP-HPLC and GC-MS as described previously [12].

Spectrophotometric assays of enzyme kinetics

The enzyme kinetics were determined on a Hewlett Packard 8452A diode array spectrophotometer by following the change in absorbance at 234 (linoleic acid derivatives) or 236 nm (linolenic acid derivatives; ε = 27 500 $l \cdot mol^{-1} \cdot cm^{-1}$) due to the formation or cleavage of conjugated hydroperoxydienes. The kinetic experiments with lipoxygenase were performed as described previously [13]. HPO lyase activity was determined in 50 mM potassium phosphate buffer pH 6.0 containing 100 μM substrate in a total of 0.5 ml. The AOS assay was performed with 30 μM substrate in 0.1 M potassium phosphate buffer pH 7.0 with 10-40 μl enzyme solution in a total of 1 ml. The changes in absorbance were corrected for the decrease in absorbance caused by the assay. Apparent K_m and k_{cat} were determined from the means of six determinations. The data were fitted to the standard Michaelis-Menten kinetic equation (Graphpad Prism). Protein concentrations were determined by using the bicinchoninic acid method [16].

Characterization of HPO lyase and AOS products

Characterization of HPO lyase products was carried out as described previously [17]. Volatile products were trapped by SPME (solid phase micro extraction) and analyzed using GC. Non-volatile products were extracted from the acidified reaction mixture by SPE (BakerBond solid state C18-column, 500 mg), reduced, methylated, trimethylsilylated and analyzed by using GC-MS. AOS reaction products were concentrated and purified with SPE (OASIS, 30 mg, Waters) without acidification, and analyzed by HPLC and GC-MS as described previously [11], except that the HPLC solvent was methanol/water/acetic acid (80/20/0.1). For GC-MS analyses the samples were reduced with sodium borodeuteride and analyzed without hydrogenation.

Results

Lipoxygenase products

In each reaction with sLOX one major hydroperoxide regio-isomer with a maximal absorbance (λ_{max}) at 236 nm was formed according to RP-HPLC analysis. Interestingly, for both α- and γ-$OTNH_2$ the formation of some minor regio-isomeric and double-dioxygenated (λ_{max} = 269 nm) products was observed. All reactions could be inhibited with NDGA, a widely used LOX-inhibitor. ^{1}H-NMR-spectra were recorded after reduction with sodium borohydride and additional purification by RP-HPLC, to establish the geometry of the 1-hydroperoxy-2,4-diene moiety in the main products (Table 1). The geometric configurations of the conjugated diene at the positions 9-12 were determined as 9*Z*,11*E* on the basis of the coupling constants ($J_{10,9}$= 11 Hz; $J_{10,11}$= 11 Hz; $J_{11,12}$= 15 Hz). The secondary hydroxyl group was located at position 13 in all main products, as determined on the basis of the mass spectra of the fully reduced fatty acid amides. The prominent fragment at *m/z* 173 in all spectra was produced by cleavage at C_{13}-C_{12}. Minor side products were identified by GC-MS analysis as 6,13-dihydroxy-γ-*N*-linolenoylamine (6,13-di$HOTNH_2$), 9-hydroxy-α-*N*-linolenoylamine (9-$HOTNH_2$) and 9,16-dihydroxy-α-*N*-linolenoylamine (9,16-di$HOTNH_2$) (Fig. 2). In order to determine the absolute configuration of the chiral center, the pure main components were analyzed by CD-spectroscopy. Since the CD-spectra of the products showed a positive Cotton effect, like 13(*S*)-HOD (data not shown) [18,19] and CP-HPLC analysis did not reveal the presence of two different enantiomers, it is concluded that the dioxygenation of the substrates yielded predominantly the *S*-enantiomer. This is in accordance with our previous observations, where C18:2 and C20:4 ethanolamides converted by soybean LOX also yielded mainly the *S*-enantiomer (>92%) [11,12].

Thus, the main reaction products formed from α- and γ-OTNHEtOH and $OTNH_2$ with soybean LOX were identified as 13(*S*)-hydroperoxy-*N*-(9*Z*,11*E*,15*Z*)-octadecatrienoyl-(ethanol)amine and as 13(*S*)-hydroperoxy-*N*-(6*Z*,9*Z*,11*E*)-octadecatrienoyl(ethanol) amine, respectively (Fig.1).

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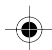

Table 1 **NMR-data of sodium borohydride reduced sLOX-products.** ^{1}H-NMR spectra were recorded in $CDCl_3$ with a Bruker AC 300 (300 MHz) spectrometer at 27 °C. TMS was used as internal standard. (ppm,Hz)

Proton	γ-13-HOTNH$_2$	γ-13-HOTNHEtOH	α-13-HOTNH$_2$	α-13-HOTNHEtOH
1'	-	3.42;q;$J_{2',1'}$=4.5	-	3.43;q; $J_{2',1'}$=4.5
2'	-	3.72;t;$J_{1',2'}$=4.5	-	3.72;t; $J_{1',2'}$=4.5
2	2.23;t;$J_{2,3}$=7.5	2.21;t;$J_{2,3}$=7.5	2.21;t;$J_{2,3}$=7.4	2.20;t; $J_{2,3}$=7.6
3	1.70;m	1.70;m	1.64;m	1.61;m
4	1.47;m	1.52;m	1.33-1.54;m	1.31-1.61;m
5	2.09;q	2.10;m	1.33-1.54;m	1.31-1.61;m
6	5.40;m	5.41;m	1.33-1.54;m	1.31-1.61;m
7	5.40;m	5.41;m	1.33-1.54;m	1.31-1.61;m
8	2.94;m	2.96;m	2.08;m	2.07;m
9	5.40;m	5.41;m	5.40;m	5.40;m
10	6.00;t; $J_{10,9}$=11.0	5.99;t; $J_{10,9}$=10.7	5.98;t; $J_{10,9}$=10.8	5.98;t;$J_{10,9}$=10.8
	$J_{10,11}$=10.8	$J_{10,11}$=11.0	$J_{10,11}$=10.7	$J_{10,11}$=11.0
11	6.55;db[a].d	6.55;db.d	6.53;db.d	6.53;db.d
	$J_{11,10}$= 11.0	$J_{11,10}$=11.1	$J_{11,10}$=11.0	$J_{11,10}$= 11.3
	$J_{11,12}$=15,1	$J_{11,12}$=15,1	$J_{11,12}$=15,3	$J_{11,12}$=15,3
12	5.71;db.d.	5.71;db.d.	5.70;db.d.	5.70;db.d.
	$J_{12,13}$=6.3	$J_{12,13}$=6.5	$J_{12,13}$=6.4	$J_{12,13}$=6.4
	$J_{12,11}$=15.2	$J_{12,11}$=15.2	$J_{12,11}$=15.2	$J_{12,11}$=15.3
13	4.18;q;$J_{12,13}$=6.1	4.19;q;$J_{12,13}$=6.3	4.22;q;$J_{12,13}$=5.3	4.21;q; $J_{12,13}$=6.7
14	1.47;m	1.52;m	2.34;db.d.	2.35;db.d;$J_{14,13}$=6.4
15	1.26-1.32;m	1.32-1.37;m	5.57;m	5.57;m
16	1.26-1.32;m	1.32-1.37;m	5.40;m	5.40;m
17	1.26-1.32;m	1.32-1.37;m	2.08;m;	2.08;m
18	0.89;t;$J_{18,17}$=6.2	0.89;t;$J_{18,17}$=6.2	0.97;t;$J_{18,17}$=7.5	0.97;t; $J_{18,17}$=7.5

a) db.d.= double doublet; m=multiplet; q= quintet; t=triplet

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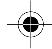


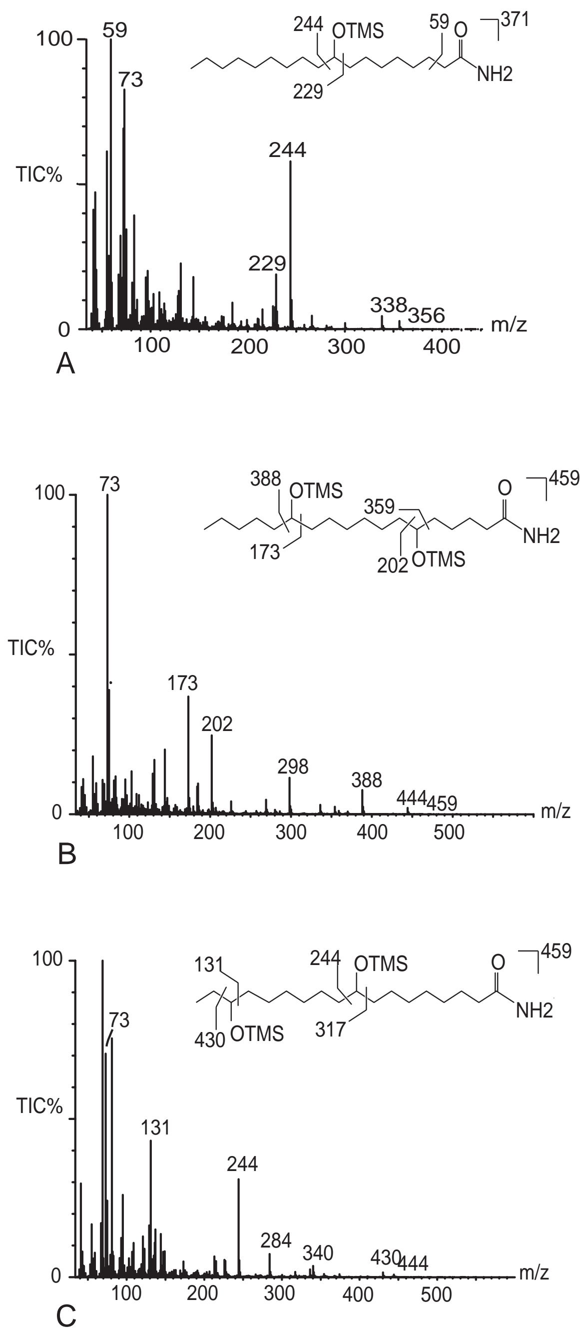
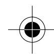


Fig. 2 Mass spectra of fully reduced TMS-ethers of a) 9-hydroxy-*N*-linolenoylamine, b) 6,13-dihydroxy-*N*-linolenoylamine; c) 9,16-dihydroxy-*N*-linolenoylamine. GC-MS analyses were performed with a Carlo Erba GC 8060 with a Fisons MD800 mass detector equipped with a CP-Sil 5 CB-MS column (25m x 0.25 mm x 0.25 µm, Chrompack). The column temperature was held at 140 °C for 2 min, increased from 140 to 300 with 6.0 °C•min^{-1} and held at 300 °C for 2 min. Mass spectra were recorded under electron impact with an ionization energy of 70 eV.



Kinetics of the lipoxygenase reaction

Apparent K_m and k_{cat} of the four substrates with sLOX are given in Table 2, as well as the kinetic parameters of the corresponding fatty acids. K_m values are in the low micromolar range (14-39 µM) and the k_{cat} values are of the same order of magnitude, demonstrating a similar rate profile for both the α- and γ- fatty acid series: OTNHEtOH > OT > $OTNH_2$.

Table 2 Kinetic parameters of sLOX with different substrates. Initial linear reaction rates were determined spectrophotometrically at 236 nm with various concentrations of substrate (4-80 µM) in 1 ml 0.1 M sodium borate buffer pH 9. K_m and k_{cat} were determined from the means of six measurements. Data were fitted to the standard Michaelis Menten kinetic equation (Graphpad Prism).

Substrate	K_m (µM) ± S.D.	k_{cat} (s^{-1}) ± S.D.	k_{cat}/K_m
α-OT	39 ± 4	335 ± 70	8.6
α-$OTNH_2$	24 ± 4	113 ± 20	4.7
α-OTNHEtOH	16 ± 2	443 ± 66	28
γ-OT	23 ± 4	290 ± 62	13
γ-$OTNH_2$	14 ± 3	265 ± 69	19
γ-OTNHEtOH	17 ± 3	355 ± 81	21

HPO lyase and AOS activity with hydroperoxy N-acyl(ethanol)amines

To determine whether hydroperoxy fatty acid (ethanol)amides can be metabolized further into possible plant defense compounds, 13-$HPODNH_2$ and 13-HPODNHEtOH, α- and γ-13-$HPOTNH_2$ and 13-HPOTNHEtOH were tested as substrates for HPO lyase and AOS (Table 3). The HPO lyase activity with 13-HPODNHEtOH and α-13-HPOTNHEtOH as substrates was almost equal to the activity with the corresponding hydroperoxy fatty acids. The HPO lyase activity on the hydroperoxy fatty acid amides however, was ~50% lower. HPO lyase was barely active on hydroperoxy γ-linolenic acid and its derivatives. Apparent K_m and V_{max} of the 13-hydroperoxy-*N*-linoleoyl- and 13-hydroperoxy-α-*N*-linolenoyl(ethanol)amines with HPO lyase, as well as the kinetic parameters of the corresponding hydroperoxy fatty acids, are shown in Table 4. The presence of the ethanolamide group in the substrate greatly increased the K_m and V_{max}, whereas the amide group decreased K_m and V_{max}. AOS was able to use all hydroperoxy fatty acid amides as substrates. It was most active with 13-HPOD, 13-HPODNHEtOH and α-13-HPOTNHEtOH (Table 3).

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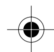
Table 3 **HPO lyase and AOS activity with different substrates.** Initial linear rates were determined spectrophotometrically at 234 or 236 nm with a substrate concentration of 100 μM (30 μM for AOS) in 0.5 ml 50 mM (1 ml 100 mM for AOS) phosphate buffer, pH 6 (7 for AOS). Data reported are the means of six independent measurements.

Substrate	**HPO lyase activity ($U \cdot mg^{-1}$) ± S.D**	**AOS activity* ($U \cdot mg^{-1}$) ± S.D**
13-HPOD	**249 ± 10 (100%)**	**0.81 ± 0.04 (100%)**
13-HPODNH$_2$	107 ± 20 (43%)	0.13 ± 0.01 (16%)
13-HPODNHEtOH	293 ± 18 (118%)	0.44 ± 0.01 (54%)
α-13-HPOT	**428 ± 30 (100%)**	**0.12 ± 0.01 (100%)**
α-13-HPOTNH$_2$	264 ± 20 (61%)	0.13 ± 0.001 (107%)
α-13-HPOTNHEtOH	394 ± 31 (92%)	0.32 ± 0.01 (268%)
γ-13-HPOT	**24.6 ± 6.3 (100%)**	**0.09 ± 0.01 (100%)**
γ-13-HPOTNH$_2$	34.9 ± 7.1 (141%)	0.04 ± 0.003 (48%)
γ-13-HPOTNHEtOH	42.5 ± 12 (173%)	0.08 ± 0.004 (86%)

*Because of the formation of a conjugated system in γ-ketols and 12-oxo PDA analogs (λ_{max} = 227 and 225 nm, respectively) the reaction rate of AOS with the substrates from which they are produced is actually higher than calculated from the decrease in absorbance at λ = 234 (or 236) nm.

Table 4 **Kinetic parameters of HPO lyase with different substrates.** Initial rates were determined as described in Table 3. Apparent K_m and V_{max} were determined from the means of six determinations with concentrations of substrate ranging from 5 to 150 μM. The data were fitted to the standard Michaelis-Menten kinetic equation (Graphpad Prism).

Substrate	**K_m (μM) ± S.D.**	**V_{max} ($U \cdot mg^{-1}$) ± S.D.**	**V_{max}/K_m**
13-HPOD	152 ± 14	449 ± 25	3.0
13-HPODNH$_2$	85.3 ± 10	158 ± 9.0	1.9
13-HPODNHEtOH	199 ± 26	678 ± 60	3.4
α-13-HPOT	140 ± 15	676 ± 44	4.8
α-13-HPOTNH$_2$	213 ± 49	245 ± 40	1.2
α-13-HPOTNHEtOH	221 ± 21	987 ± 66	4.5



Products formed by HPO lyase and AOS

The volatile products formed in the HPO lyase reactions with 13-hydroperoxy-*N*-linoleoyl (ethanol)amine or 13-hydroperoxy-*N*-linolenoyl(ethanol)amine were absorbed by SPME and identified by using GC as hexanal or (3*Z*)-hexenal, respectively. The nonvolatile products were reduced, methylated, trimethylsilylated and analyzed using GC-MS. The main product of the incubations with 13-HPODNH$_2$ and 13-HPOTNH$_2$ was identified as 12-oxo-*N*-(9*Z*)-dodecenoylamine (Fig. 3A). A small amount of the isomer 12-oxo-*N*-(10*E*)-dodecenoylamine was also present. This product showed a mass spectrum similar to the 9*Z*-isomer, except that the *m/z* 103 peak was replaced by a *m/z* 129 peak [$C_3H_4OTMS^+$]. Furthermore, 9-hydroxy-12-oxo-*N*-(10*E*)-dodecenoylamine was present, as judged by its mass spectrum which shows the same characteristic features as the mass spectrum of 9-hydroxy-traumatin [20] (Fig. 3A). The main product of the incubations with 13-HPODNHEtOH and 13-HPOTNHEtOH was identified as 12-oxo-*N*-(9*Z*)-dodecenoylethanolamine (Fig. 3B). 12-oxo-*N*-(10*E*)-dodecenoylethanolamine and 9-hydroxy-12-oxo-*N*-(10*E*)-dodecenoylethanolamine were also present. The mass spectrum of 12-oxo-*N*-(10*E*)-dodecenoylethanolamine was similar to the 9*Z*-isomer with the *m/z* 103 peak replaced by a *m/z* 129 peak [$C_3H_4OTMS^+$]. When a crude extract of alfalfa seedlings was incubated with α-OTNHEtOH, (2*E*)-hexenal, (2*E*,6*Z*)-nonadienal, 12-oxo-*N*-(10*E*)-dodecenoylethanolamine, 9-hydroxy-12-oxo-*N*-(10*E*)-dodecenoylethanolamine and 9-oxo-*N*-nonanoylethanolamine were formed, indicating that both lipoxygenase and HPO lyase in alfalfa were active on this new substrate.

RP-HPLC analysis revealed that three products were formed out of 13(*S*)-hydroperoxy-α-*N*-linolenoyl(ethanol)amines by AOS, i.e. α- and γ-ketols and the 12-oxo-*N*-phytodienoyl(ethanol)amines, as judged by GC-MS. The 13(*S*)-hydroperoxy-γ-*N*-linolenoyl-(ethanol)-amines yielded mainly the α-ketol, whereas the linoleoyl analogs gave rise to both α- and γ-ketols. Characteristic mass fragments of the AOS products of α-13-HPOTNHEtOH and α-13-HPOTNH$_2$ are listed in Table 5 and Fig. 4. The mass fragments of the AOS products of 13-HPODNHEtOH, 13-HPODNH$_2$, γ-13-HPOTNHEtOH and γ-13-HPOTNH$_2$ can be found in the Tables 6 and 7. The mass spectrum of the γ-ketol from 13-HPOTNH$_2$ is not given because of a low response and similar retention times of the α- and γ-ketols. The acetone extract of flax seed does not contain an allene oxide cyclase [21], therefore, the 12-oxo-PDA analogs are generated by a spontaneous nonenzymatic reaction, yielding a racemic mixture.

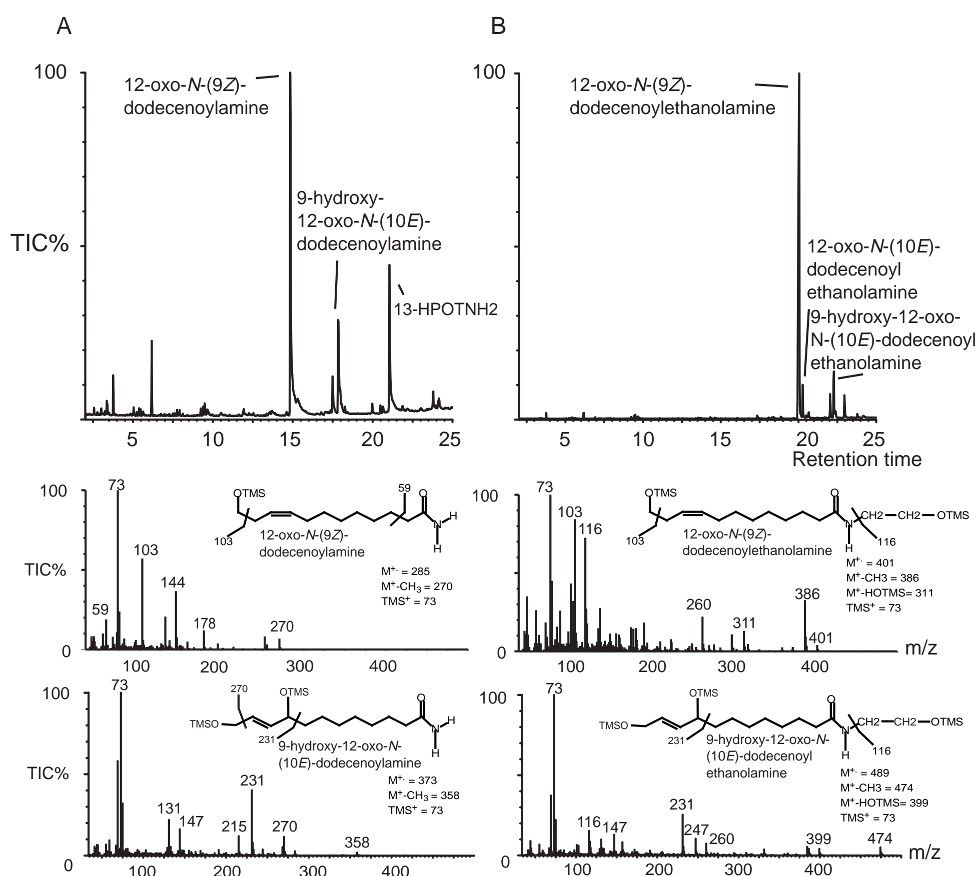


Fig. 3 **Nonvolatile reaction products formed by HPO lyase incubated with a) 13-HPOTNH$_2$ or b) 13-HPOTNHEtOH.** GC-MS analysis of the fully reduced TMS-ethers of the extracted reaction products. The chromatograms of the incubations with 13-HPODNH$_2$ and 13-HPODNHEtOH were similar to a and b, respectively. GC-MS analyses were performed under similar conditions as described in the legend to Fig. 2.

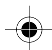


Table 5 Characteristic mass fragments of TMS-ethers of $NaBD_4$-reduced AOS-products of α-13-HPOTNHEtOH and α-13-HPOTNH$_2$: α-ketol, γ-ketol and 12-oxo-PDA derivatives (mass fragment; relative abundance). GC-MS analyses were performed under similar conditions as described in the legend to Fig 2.

mass (*m/z*)	α-ketol		γ-ketol	12-oxo-PDA	
	NHEtOH	NH_2	NHEtOH	NHEtOH	NH_2
$M^{+\cdot}$	572 (<1)	456 (<1)	-	485 (1)	369 (<1)
M^+-15[a]	557 (5)	441 (3)	557 (2)	470 (15)	354 (7)
M^+-90[b]	482 (<1)	-	482 (1)	395 (7)	279 (8)
(M^+-15)-90	467 (1)	-	467 (4)	-	-
M-201/203[c]	371 (13)	255 (36)	-	-	-
a	401 (27)[d]	285 (71)[d]	360 (4)[e]	227 (11)[f]	227 (32)[f]
a-90	-	-	-	137 (5)[f]	137 (28)[f]
b	503 (4)[d]	387 (6)[d]	-	-	-
b-90	413 (14)[d]	297 (43)[d]	-	-	-
c	274 (4)[d]	274 (8)[d]	489 (8)[e]	-	-
c-90	184 (11)[d]	184 (21)[d]	399 (8)[e]	-	-
d	171 (12)[d]	171 (27)[d]	314 (3)[e]	-	-
d-90	-	-	224 (10)[e]	-	-
e	-	-	212 (2)[e]	-	-
TMS	73 (100)	73 (100)	73 (100)	73 (100)	73 (100)

a) loss of $\cdot CH_3$; b loss of TMSOH; c) rearrangement with loss of OHC-CH(OTMS)-C_5H_{11} or OHC-CH(OTMS)-C_5H_9 ; d) See Fig 4a for fragmentation pattern; e) See Fig 4b for fragmentation pattern; f) See Fig 4c for fragmentation pattern

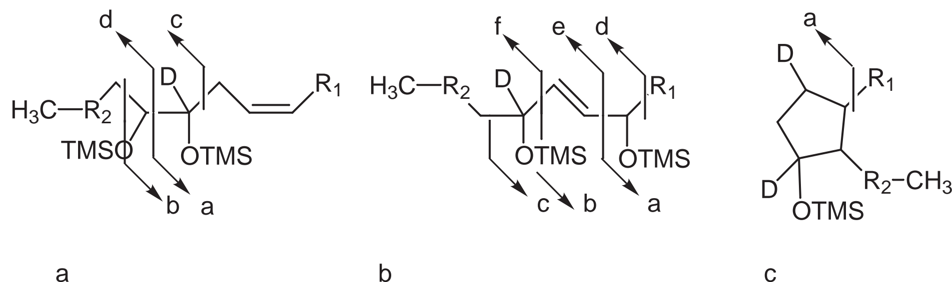


Fig. 4 Fragmentation patterns of TMS-ethers of $NaBD_4$ reduced AOS-products. a) α-ketols b) γ-ketols c) 12-oxo-PDA derivatives. Arrows indicate site of fragmentation and the letters correspond to the letters in Table 5, 6 and 7.

Table 6 **Characteristic mass fragments of TMS-ethers of $NaBD_4$-reduced AOS-products of 13-HPODNHEtOH and 13-HPODNH$_2$: α-ketol and γ-ketol derivatives (mass fragment; relative abundance).** GC-MS analyses were performed under similar conditions as described in the legend to Fig 2.

mass (*m/z*)	α-ketol		γ-ketol	
	NHEtOH	NH_2	NHEtOH	NH_2
$M^{+\cdot}$	574 (<1)	458 (<1)	574 (<1)	-
M^{+}-15[a]	559 (8)	443 (2)	559 (4)	458 (1)
M^{+}-90[b]	484 (2)	-	484 (4)	383 (1)
(M^{+}-15)-90	469 (4)	-	469 (7)	-
M-201/203[c]	371 (25)	255 (31)	-	-
a	401 (65)[d]	285 (70)[d]	360 (4)[e]	259 (4)[e]
a-90	-	-	-	-
b	503 (<1)[d]	-	386 (2)[e]	285 (1)[e]
b-90	413 (1)[d]	-	-	-
c	276 (16)[d]	276 (16)[d]	489 (17)[e]	388 (8)[e]
c-90	186 (14)[d]	186 (5)[d]	399 (11)[e]	298 (9)[e]
d	173 (48)[d]	173 (23)[d]	316 (8)[e]	316 (13)[e]
d-90	-	-	226 (17)[e]	226 (17)[e]
e	-	-	214 (1)	214 (1)
f	-	-	188 (4)	188 (16)
TMS	73 (100)	73 (100)	73 (100)	73 (100)

a) loss of $\cdot CH_3$; b loss of TMSOH; c) rearrangement with loss of OHC-CH(OTMS)-C_5H_{11} or OHC-CH(OTMS)-C_5H_9 ; d) See Fig 4a for fragmentation pattern; e) See Fig 4b for fragmentation pattern

Table 7 Characteristic mass fragments of TMS-ethers of $NaBD_4$-reduced AOS-products of γ-13-HPOTNHEtOH and γ-13-HPOTNH$_2$: α-ketol, γ-ketol and 12-oxo-PDA derivatives (mass fragment; relative abundance). GC-MS analyses were performed under similar conditions as described in the legend to Fig 2.

mass (*m/z*)	α-ketol		γ-ketol		12-oxo-PDA
	NHEtOH	NH_2	HEtOH	NH_2	NH_2
$M^{+\cdot}$	572 (2)	456 (2)	572 (<1)	456 (<1)	369 (<1)
M^{+}-15[a]	557 (9)	441 (3)	557 (2)	441 (3)	354 (1)
M^{+}-90[b]	482 (1)	-	-	-	-
(M^{+}-15)-90	467 (2)	-	467 (2)	-	-
M-201/203[c]	369 (24)	253 (25)			-
a	399 (61)[d]	283 (35)[d]	358 (2)[e]	-	225 (41)[f]
a-90	-	-	-	-	135 (7)[f]
b	501 (1)[d]	385 (<1)[d]	-	-	-
b-90	411 (1)[d]	295 (1)[d]	-	-	-
c	276 (19)[d]	276 (18)[d]	487 (1)[e]	386 (<1)[e]	-
c-90	186 (18)[d]	186 (15)[d]	397 (1)[e]	-	-
d	173 (62)[d]	173 (87)[d]	316 (12)[e]	316 930)[e]	-
d-90	-	-	226 (27)[e]	226 (71)[e]	-
e	-	-	214 (2)[e]	214 (8)[e]	-
f	-	-	188 (3)[e]	-	-
TMS	73 (100)	73 (100)	73 (100)	73 (100)	73 (100)

a) loss of $\cdot CH_3$; b loss of TMSOH; c) rearrangement with loss of OHC-CH(OTMS)-C_5H_{11} or OHC-CH(OTMS)-C_5H_9 ; d) See Fig 4a for fragmentation pattern; e) See Fig 4b for fragmentation pattern; f) See Fig 4c for fragmentation pattern

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Discussion

N-acyl(ethanol)amines as substrates for lipoxygenase

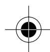
The oxylipins traumatin and jasmonic acid are produced by the lipoxygenase pathway and used by plants as mediators in their defense reaction against pathogens [7]. Recently, the involvement of NAEs in plant resistance strategies was proposed by Chapman *et al*. [4]. To date, it had not been known whether the lipoxygenase pathway could utilize NAEs (18:3) as substrates. We demonstrated that soybean lipoxygenase-1 converted α- and γ-*N*-linolenoyl-(ethanol)amine in a regio- and stereoselective manner into their 13(*S*)-hydroperoxide derivatives. Remarkably, only the *N*-linolenoylamines were double dioxygenated. The extension of the headgroup of the hydroperoxy fatty acid with ethanolamine probably precludes it from being accepted as a substrate. This supports the concept of the inverse substrate orientation for double-dioxygenation of substrates by 15-lipoxygenases [22].

The results presented here extend our previous study in which we found a similar regio- and stereoselectivity and a comparable rate profile of soybean lipoxygenase-1 with 18:2 derivatives [12]. The enzyme kinetics with the fatty acid amides as substrates were quite similar to the reaction with the corresponding fatty acids. The comparable reactivities of the novel substrates and their corresponding fatty acids towards sLOX suggest that the fatty acid amides are possible lipoxygenase substrates *in vivo.* It is noteworthy that the catalytic efficiencies of sLOX with the ethanolamide substrates were even higher than those of the corresponding free fatty acids.

Hydroperoxy N-acyl(ethanol)amines as substrates for HPO lyase and AOS

The formed hydroperoxy fatty acid (ethanol)amides were good substrates for HPO lyase and AOS (Fig. 1). Similar to sLOX, the hydroperoxy fatty acid ethanolamides were as good as or even better substrates for HPO lyase than the corresponding free fatty acids. This was also the case for hydroperoxy α-*N*-linolenoylethanolamine with AOS. In contrast, replacement of the carboxyl group by a methyl ester or alcohol greatly reduced the HPO lyase activity [23,24]. The polarity of the headgroup of the substrates seems to have an important influence on the HPO lyase activity. Because the large ethanolamide group increases the activity, steric hindrance is likely to be of minor interest. Substrates that have a lower K_m, appear to have a lower V_{max} indicating a slower release of the reaction products. Alfalfa HPO lyase was barely active on γ-linolenic acid and its derivatives. Low activity on γ-linolenic acid was also observed for HPO lyase from tea leaves and tea chloroplasts [23,25].

In addition to the known HPO lyase products (i.e. traumatin derivatives and volatile aldehydes), 9-hydroxy-traumatin derivatives were also formed, as judged by GC-MS-analyses. We also observed the formation of 9-hydroxy-traumatin with 13-HPOD or 13-HPOT as substrates. Gardner suggested that the oxygenation of traumatin is catalyzed by lipoxygenase and a peroxygenase [20]. In our purified enzyme preparation, however, there was no



lipoxygenase activity present. In these experiments 9-hydroxy-traumatin might be formed nonenzymatically or by a monooxygenase activity of HPO lyase. It is noteworthy that many cytochrome P450 enzymes are known to have monooxygenase activity [26]. However,z in addition to these experiments further studies are required to get more insight into the mechanism of 9-hydroxy-traumatin formation.

Similarities between plant and animal defense systems and possible functions of NAEs in plants
We have shown that the *N*-acyl(ethanol)amines (18:2) and (18:3) can be converted by LOX, HPO lyase and AOS, yielding a new class of phytooxylipins (Fig. 1). Their physiological roles remain unknown, but they may have a function in plant defense. Several features of the plant signal transduction system for defense against herbivores are strikingly similar to the defense signaling systems of animals against pathogens and parasites, as outlined by Bergey *et al.* [27]. It is thought that the two systems have developed through divergent evolution from an ancestral organism that had fundamental components [27]. Similarities between NAE metabolism in plants and the animal kingdom are also being discovered [4]. As in animals, plant NAEs are released from NAPEs, a minor lipid fraction, by the signal-induced action of phospolipase D [4,28]. They accumulate extracellularly and are degraded by a fatty acid amide hydrolase-like activity [4,29]. We have shown that the oxidative metabolism of NAEs by plant lipoxygenases is also similar to that in animals [12,30,31]. Furthermore, the subsequent metabolism of the lipoxygenase products by AOS is analogous to the action of cycloxygenase-2 on anandamide, which yields the prostaglandin analog PGE_2-ethanolamide [32].

Based on analogy with the animal kingdom it is tempting to speculate that plant NAEs, in particular α-OTNHEtOH and/or their oxylipin derivatives, may function as stress recovery factors in the plant defense system. We demonstrated previously that lipoxygenase products derived from anandamide and *N*-linoleoylethanolamine, i.e. 13(*S*)-hydroxy-*N*-linoleoylethanolamine and 15(*S*)-hydroxyanandamide, may act as competitive inhibitors of mammalian fatty acid amine hydrolase [33]. We suggested that these oxylipins might prolong the pharmacological actions of anandamide [34]. This 'entourage' effect may also be active in plants. Oxylipins of plant NAEs may also have direct physiological effects, as was demonstrated for other structurally related headgroup analogs of jasmonic acid. For example, leucine and isoleucine conjugates with jasmonic acid lead to the up-regulation of specific genes, the down-regulation of house-keeping genes and the production of volatiles [35,36]. Taken together, it would be interesting to screen plants for the occurrence of these novel phytooxylipins and test whether these oxylipins alter the physiological responses of plants to pathogens.

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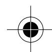

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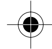

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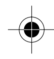

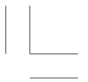
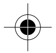
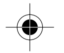
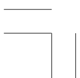
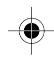
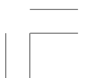

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Oxygenation of (3*Z*)-alkenals to 4-hydroxy-(2*E*)-alkenals in plant extracts: a nonenzymatic process

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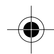

Abstract

There is large interest in 4-hydroxy-(2*E*)-alkenals because of their cytotoxicity in mammals. However, the biosynthetic pathway for these compounds has not been elucidated yet. In plants, 4-hydroxy-(2*E*)-alkenals were supposed to be derived by the subsequent actions of lipoxygenase and a peroxygenase on (3*Z*)-alkenals. The presence of 9-hydroxy-12-oxo-(10*E*)-dodecenoic acid (9-hydroxy-traumatin) in incubations of 12-oxo-(9*Z*)-dodecenoic acid in the absence of lipoxygenase or peroxygenase, has prompted us to reinvestigate its mode of formation. We show here that *in vitro* 9-hydroxy-traumatin, 4-hydroxy-(2*E*)-hexenal and 4-hydroxy-(2*E*)-nonenal, are formed in a nonenzymatic process. Furthermore, a novel product derived from 12-oxo-(9*Z*)-dodecenoic acid was observed and identified as 11-hydroxy-12-oxo-(9*Z*)-dodecenoic acid. The results obtained here strongly suggest that the 4-hydroxy-(2*E*)-alkenals, observed in crude extracts of plants, are mainly due to autoxidation of (3*Z*)-hexenal, (3*Z*)-nonenal and 12-oxo-(9*Z*)-dodecenoic acid. This may have implications for the *in vivo* existence and previously proposed physiological significance of these products in plants.

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Introduction

The formation of 4-hydroxy-(2*E*)-alkenals from polyunsaturated fatty acids was first observed in mammalian systems [1]. Although these compounds have been of great interest because of their cytotoxicity [2], the biosynthetic pathway in mammals has not been unraveled yet. However, it has been suggested that at least 4-hydroxy-(2*E*)-nonenal (HNE) is formed by autoxidation [3].

In plant extracts, 4-hydroxy-(2*E*)-alkenals, such as HNE, 4-hydroxy-(2*E*)-hexenal (HHE) and 9-hydroxy-12-oxo-(10*E*)-dodecenoic acid (9-hydroxy-traumatin), have been found as well [4-7]. These compounds are suggested to be derived from (3*Z*)-hexenal, (3*Z*)-nonenal and 12-oxo-(9*Z*)-dodecenoic acid, which are products of the successive actions of lipoxygenase (LOX) and hydroperoxide lyase (HPO lyase) on linoleic and linolenic acid. Gardner and Hamberg proposed two enzymatic routes for the formation of these 4-hydroxy-(2*E*)-alkenals from (3*Z*)-alkenals in plants [8]. First through an oxygenation reaction of a (3*Z*)-alkenal to a 4-hydroperoxy-(2*E*)-alkenal by a (3*Z*)-alkenal oxygenase activity, followed by reduction of the hydroperoxy group by a hydroperoxide-dependent peroxygenase. By investigating this process in more detail, it was found that the formation of 4-hydroperoxy-(2*E*)-alkenals might be catalyzed by LOX [5,9]. Alternatively, it was suggested that a 4-hydroxy-(2*E*)-alkenal was produced by rearrangement of a 3,4-epoxyalkenal which was also formed from a (3*Z*)-alkenal by a hydroperoxide-dependent peroxygenase.

However, the finding of 9-hydroxy-traumatin in incubations of hydroperoxy fatty acids with pure HPO lyase, in the absence of LOX or a peroxygenase, has prompted us to reinvestigate its mode of formation [10,11]. The data we present here, indicate that the formation of 4-hydroxy-(2*E*)-alkenals from (3*Z*)-alkenals *in vitro* is mainly due to autoxidation.

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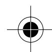

Materials and methods

Enzyme preparations

Lipoxygenase-1 (LOX-1) was isolated from soybeans [12]. Recombinant LOX-100 was isolated as described before [13]. It is a 13-LOX which is induced by methyljasmonate and is located in chloroplasts of barley leaves [14]. Recombinant HPO lyase was isolated as described previously [10]. This HPO lyase from alfalfa is highly specific for 13-hydroperoxy linoleic and linolenic acids. A crude extract of alfalfa was prepared as described previously [7].

Preparation of (3Z)-alkenals

(3*Z*)-hexenal and (3*Z*)-nonenal were prepared from the corresponding alcohols (3*Z*)-hexen-1-ol and (3*Z*)-nonen-1-ol, as described previously [15]. 13(*S*)-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid (13-HPOT) and 13(*S*)-hydroperoxy-(9*Z*,11*E*)- octadecadienoic acid (13-HPOD) were prepared by incubation of α-linolenic or linoleic acid with lipoxygenase-1 [16]. 12-oxo-(9*Z*)-dodecenoic acid was prepared by incubation of HPO lyase with 100 µM 13-HPOT in 50 mM potassium phosphate buffer, pH 6.0. The reaction was followed by measuring the absorbance at 234 nm until all substrate was converted. The reaction mixture was acidified with HCl to pH 5.0 and 12-oxo-(9*Z*)-dodecenoic acid was extracted with an octadecyl solid-phase extraction column (J.T. Baker B.V., Deventer, The Netherlands), previously equilibrated with 100% methanol. The column was washed with 50% methanol, and pure 12-oxo-(9*Z*)-dodecenoic acid was obtained by elution with 75% methanol.

Formation of 4-hydroxy-(2E)-alkenals

For the formation of 9-hydroxy-traumatin, 12-oxo-(9*Z*)-dodecenoic acid was incubated in a stirred vessel for 1.5 h, in 5 ml of 0.1 M sodium borate buffer pH 9.0, 50 mM potassium phosphate buffer pH 6.0 or 50 mM sodium acetate buffer pH 4.0, supplemented with 7 Units of LOX-1, HPO lyase or 1 ml of crude extract (1 U is the amount of enzyme that converts 1 $\mu mol \cdot min^{-1}$). Incubations with heat-denatured enzymes were performed as well. HHE and HNE were formed by incubating 25 µM of (3*Z*)-hexenal or (3*Z*)-nonenal for 1 h, in 2 ml of 100 mM potassium phosphate buffer pH 7.0, supplemented with 100 µM 13-HPOD, 100 µM 13-HPOT, 100 µM H_2O_2, or with 100 µg purified recombinant LOX-100. The reactions were stopped by reducing the hydroperoxy derivatives with an excess of $NaBH_4$.

Analysis of 4-hydroxy-(2E)-alkenals

Hydroxy-traumatin was extracted from the reaction mixture with an octadecyl solid-phase extraction column (J.T. Baker B.V., Deventer, the Netherlands) after acidification with HCl to pH 5.0, or with an Oasis HLB extraction cartridge (Waters, Massachusetts, USA) without acidification, and eluted with 100% methanol. The product was reduced with an excess of $NaBH_4$ at 0 °C, esterified with ethereal diazomethane and silylated with silylating reagent (pyridine/

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1,1,1,3,3,3-hexamethyldisilazane/chlorotrimethylsilane 5:1:1, v/v/v). Identification of the products was performed with GC-MS (Fisons GC 8000 series and Fisons Instruments MD 800 MassLab spectrometer, Alltech AT-1 column, 0.25 µm film thickness, 30 m x 0.25 mm), quantitative analysis was performed with GC-FID (Chrompack CP9002, CP-Sil5 CB column, 0.25 µm film thickness, 25 m x 0.32 mm). 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.

The analysis of HHE and HNE was performed as described before using some modifications [6]. In order to form the 2,4-dinitrophenylhydrazone (DNPH) derivatives of aldehydes, the incubation mixtures were stirred in the presence of 1 ml of DNPH-reagent (0.1% 2,4-dinitrophenylhydrazine in 1 M HCl) at room temperature for 1 h. The reaction mixture was extracted three times with 5 ml hexane each, and the collected organic phases were dried under a stream of N_2. DNPH derivatives were redissolved in 400 µl of acetonitrile. HPLC analysis of the DNPH derivatives was carried out by RP-HPLC on a Jupiter C-18 300A column (250 x 1.0 mm, 5 µm particle size, Phenomenex, Germany) using a binary gradient system (solvent A: acetonitrile/water (60:40, v/v); solvent B: acetonitrile/water (80:20, v/v) with the following gradient program: 100% solvent A for 15 min, followed by a linear increase of solvent B up to 50% within 5 min, a linear increase of solvent B up to 100% within 13.4 min and finally an isocratic postrun at 100% solvent B for 11.6 min. The flow rate was 0.05 ml•min^{-1}. The absorbance at 365 nm was monitored to indicate the presence of DNPH derivatives. All aldehydes were identified by their characteristic UV spectra and by co-injection of authentic standards. Calibration curves (five point measurements) were established.

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Results

To determine if the formation of 9-hydroxy-traumatin was induced by the action of LOX, as proposed, 12-oxo-(9*Z*)-dodecenoic acid was incubated with native or denatured LOX-1. Because formation of 9-hydroxy-traumatin was also observed in incubations of hydroperoxy fatty acids with HPO lyase [10,11], and because many cytochrome P450 enzymes are known to have monooxygenase activity, it was also studied if the formation of 9-hydroxy-traumatin could be catalyzed by HPO lyase. Equal amounts were formed with active and denatured enzymes (Fig. 1A). The formation of hydroxy-traumatin in these incubations is therefore due to autoxidation. With a crude extract of alfalfa, a similar amount of hydroxy-traumatin was formed. Because the amount of hydroxy-traumatin formed in incubations at pH 6.0 was higher than in incubations at pH 9.0, the influence of the pH was studied by incubating 12-oxo-(9*Z*)-dodecenoic acid in buffers with different pH, with or without addition of LOX-1. The oxygenation of 12-oxo-(9*Z*)-dodecenoic acid appeared to be higher at low pH (Fig. 1B) and was independent of the additon of LOX-1. When the sample of pH 9.0 was not acidified prior to solid phase extraction, hardly any hydroxy-traumatin was formed.

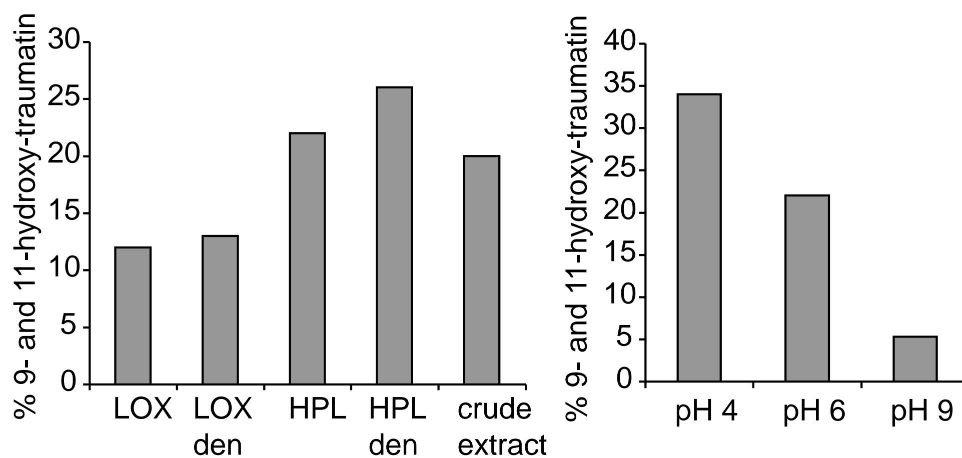

Fig. 1 **Formation of 9- and 11-hydroxy-traumatin from 12-oxo-(9*Z*)-dodecenoic acid as a percentage of the total amount of 12-oxo-dodecenoic acids present.**
A: Formation of 9- and 11-hydroxy traumatin by native or denatured LOX-1 (pH 9.0), HPO lyase (pH 6.0) or a crude extract of alfalfa (pH 6.0). B: Formation of 9- and 11-hydroxy-traumatin in buffers of different pH. Quantification occurred with GC-FID after extraction and derivatization of the products. Representative results are shown.

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Interestingly, a novel product derived from 12-oxo-(9*Z*)-dodecenoic acid was observed. This product was identified as 11-hydroxy-12-oxo-(9*Z*)-dodecenoic acid (11-hydroxy-traumatin) (Fig. 2). The amount was about 5 times less than the amount of 9-hydroxy traumatin formed. An incubation in $H_2^{18}O$ (Fluka) was carried out to determine if the oxygen from the hydroxy group is derived from water or from molecular oxygen. The mass spectra of the formed 9- and 11-hydroxy-traumatin only demonstrated exchange of oxygen of the C_{12}-aldehyde group but no incorporation of ^{18}O in the hydroxy group at either C_9 or C_{11}. It is, therefore, likely that 9- and 11-hydroxy-traumatin are formed by a reaction of 12-oxo-(9*Z*)-dodecenoic acid with molecular oxygen.

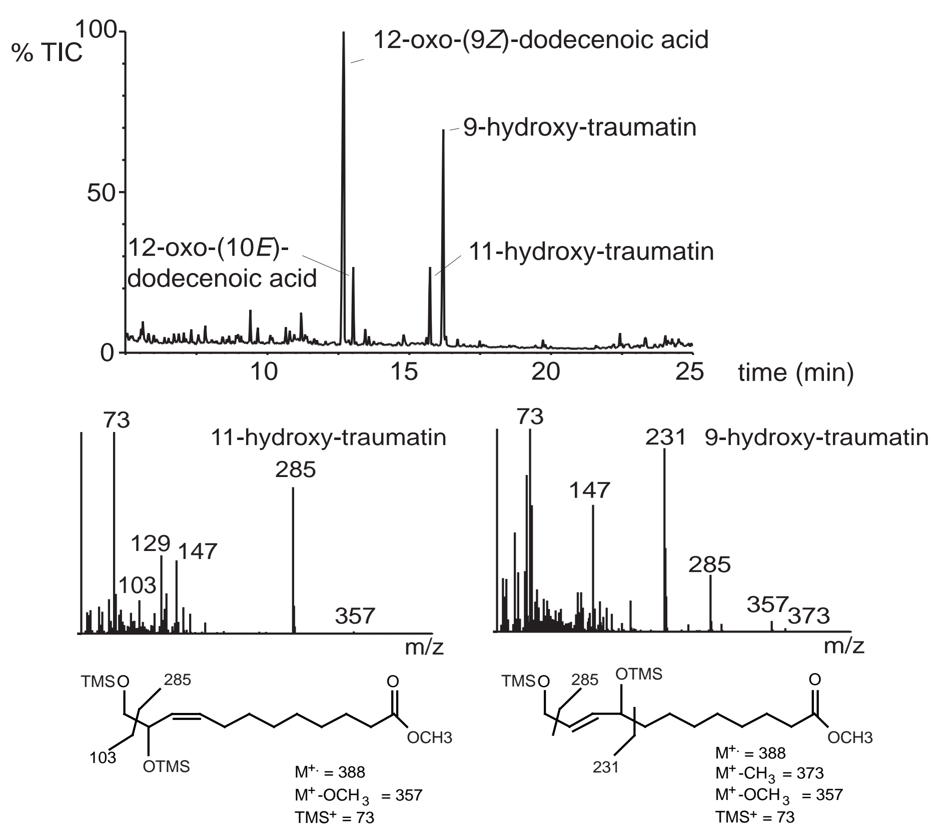

Fig. 2 **Formation of 9- and 11-hydroxy-traumatin upon incubation of 12-oxo-(9*Z*)-dodecenoic acid in 50 mM sodium acetate buffer pH 4.0 for 1.5 h.** The products were extracted, derivatized and identified with GC-MS.

To determine if HHE and HNE were formed in an enzymatic reaction, (3*Z*)-hexenal or (3*Z*)-nonenal were incubated with recombinant LOX-100, which should be the enzyme responsible for HHE formation in case of barley leaves [6]. Furthermore, (3*Z*)-hexenal or (3*Z*)-nonenal were incubated with oxidizing chemicals such as 13-HPOD, 13-HPOT or H_2O_2, or with a combination of 13-HPOD/T or H_2O_2 and LOX-100. For both aldehydes similar results were obtained. As shown for HNE formation (Fig. 3), similar values were found when (3*Z*)-nonenal was incubated with 13-HPOD/T only, or in combination with LOX-100. These results indicate that the formation of HHE and HNE under these conditions is due to autoxidation as well. The formation of 9- and 11-hydroxy-traumatin did not increase in the presence of 100 µM 13-HPOD, which is in contrast to the formation of HHE and HNE. Furthermore, incubation of 12-oxo-(9*Z*)-dodecenoic acid with 100 µM 13-HP^{18}OD did not result in incorporation of ^{18}O.

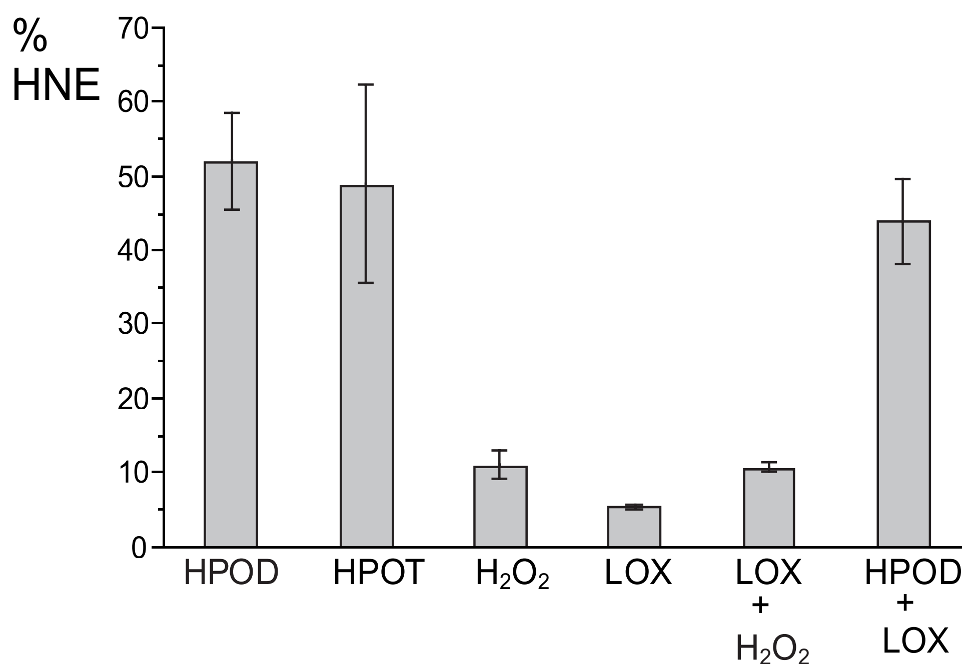

Fig. 3 Formation of HNE upon incubation of (3*Z*)-nonenal with 100 µM 13-HPOD, 100 µM 13-HPOT, 100 µM H_2O_2, or with 100 µg purified recombinant LOX-100, respectively, for 1 h. The remaining aldehydes were derivatized and analyzed by HPLC. The results represent the mean and the standard deviation of 3 experiments.

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Discussion

In contrast to the previously proposed enzymatic routes for the formation of 4-hydroxy-(2*E*)-alkenals in plants, we have shown here that HHE, HNE and 9-hydroxy-traumatin can be formed in a nonenzymatic process. Until now, it had not been studied if 9-hydroxy-traumatin found in crude extracts, could be due to autoxidation. The previously described formation of HNE by LOX-1 spiked with 13-HPOD [9] might be mainly due to the presence of 13-HPOD and not to the action of LOX-1, since we showed that 13-HPOD and 13-HPOT alone induced the formation of much higher amounts of HHE and HNE than LOX. An autoxidative process may also explain the inability of LOX inhibitors to inhibit the oxygenation of (3*Z*)-alkenals [8,15] and the almost racemic mixture of products obtained with crude enzyme preparations [8,9]. The fact that there was no formation of HNE observed with heat-inactivated enzyme preparations in those previous studies, might be due to the reaction conditions used. Since (3*Z*)-nonenal was found to disappear in the incubations with denatured enzyme as well [8,15], it might be possible that the formed HNE was not recovered because of the formation of Schiff bases, as was also proposed for 9-hydroxy-traumatin in undiluted enzyme preparations [5]. In addition, it has been suggested that formation of 4-hydroxy-(2*E*)-alkenals is catalyzed by 9-LOX activity. However, even at neutral pH where LOX-1 has significant 9-LOX activity [17], we could not find enzymatic formation of these oxylipins.

We also studied the reaction itself in more detail. Incubations with $H_2{}^{18}O$ showed that ^{18}O was not incorporated into 9-hydroxy-traumatin, indicating that the hydroxy group is not derived from water. Previously, O_2-uptake during the formation of hydroxy alkenals and the formation of hydroperoxy alkenals was observed [9,15]. This indicates that the oxygenation of (3*Z*)-alkenals to 4-hydroxy-(2*E*)-alkenals is a reaction with molecular oxygen. Furthermore, we observed no incorporation of ^{18}O in 9- and 11-hydroxy traumatin upon incubation of 12-oxo-(9*Z*)-dodecenoic acid with 13-HP^{18}OD. Gardner and Hamberg found some labelled HNE upon incubation of (3*Z*)-nonenal with 13-HP^{18}OD, but only to an extent of 15.7% [8]. This suggests that the majority of 4-hydroxy-(2*E*)-alkenals is not formed by a direct interaction with 13-HPOD/T. The induced formation of HHE and HNE by 13-HPOD/T might therefore be due to initiation of the oxygenation by radicals generated by 13-HPOD/T. The formation of both 9- and 11-hydroxy-traumatin indicates that the reaction might occur via a C_{11}-C_9 delocalized radical intermediate. The lower amount of 11-hydroxy-traumatin compared to the amount of 9-hydroxy-traumatin formed, might be due to the proximity of the oxygen of the aldehyde group, which could make an oxygen attack at C_{11} less favorable than at C_9. The influence of the pH on the oxygenation remains unclear, since formation of 9- and 11-hydroxy-traumatin was optimal at low pH, whereas the formation of HNE and HHE was optimal at pH 9.5 [15].

The results obtained here show that HHE, HNE and 9-hydroxy traumatin, are *in vitro* formed by autoxidation of (3*Z*)-hexenal, (3*Z*)-nonenal and 12-oxo-(9*Z*)-dodecenoic acid, probably similar to the autoxidation of linoleic and linolenic acid. The 400- to 1000-fold lower activ-

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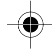

ity of LOX-1 with (3*Z*)-nonenal compared to linoleic acid [9], questions the significance of this enzymatic reaction *in vivo*. It is therefore likely that the previously observed formation of 4-hydroxy-(2*E*)-alkenals in crude extracts of plants is mainly due to autoxidation. This might have implications for the previously proposed physiological significance of these products in plants. However, 4-hydroxy-(2*E*)-alkenals could be excellent markers to distinguish between autoxidative and enzymatic processes.

Acknowledgements

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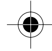

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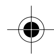

7

Preparatory studies on the application of recombinant alfalfa hydroperoxide lyase as a biocatalyst for the production of food flavors

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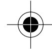

Abstract

Hydroperoxide lyase is a suitable enzyme for the biocatalytic production of volatile aldehydes, which are used as food flavors. To develop such a biocatalytic process, the expression system described in chapter 3, and the reaction conditions were optimized. For optimal expression of HPO lyase in *E. coli,* the cells have to be grown at 25 °C in 1.5 × LB medium supplemented with 1 × minimal medium, 0.5% glucose and the appropriate antibiotics. Induction with 0.1-1 mM IPTG should take place at an $A_{600}<0.8$. After expression for 24 h, the cells have to be resuspended and opened in buffer of pH 8. HPO lyase should be solubilized by addition of Triton X-100 to a final concentration of 0.5% (w/v). The optimal reaction conditions for the conversion of 13-HPOD and 13-HPOT by recombinant alfalfa HPO lyase are pH 8.25, 25 °C. One enzyme molecule can convert $1.6 \cdot 10^5$ molecules of 13-HPOD or $0.9 \cdot 10^5$ molecules of 13-HPOT before it is inactivated. Inactivation is independent of the substrate or product concentration indicating that it is caused by reaction intermediates. The amount of HPO lyase obtained from 1 l of *E. coli* culture can convert up to 24 mmol 13-HPOD or 15 mmol 13-HPOT. The initial turnover rates for these substrates are 330 and 750 s^{-1}, respectively.

Introduction

Volatile C6- and C9-aldehydes and alcohols are main contributors to the characteristic odor of plants. They are widely used as food flavors to reconstitute the 'fresh green' smell of fruit and vegetables lost during processing. Nowadays, consumers have a strong preference for natural food additives. The low abundance of the short-chain aldehydes and alcohols in plants and the high demand have urged the industry to develop biocatalytic processes to produce these compounds on a large scale.

In plants, C6- and C9-aldehydes and alcohols are produced by the lipoxygenase pathway. Linoleic and α-linolenic acids are dioxygenated by lipoxygenase to form 13- or 9-hydroperoxy-linole(n)ic acids. The 13-hydroperoxy fatty acids are subsequently cleaved by hydroperoxide lyase (HPO lyase) into 12-oxo-(9*Z*)-dodecenoic acid and hexanal or (3*Z*)-hexenal, whereas the 9-hydroperoxy fatty acids are cleaved into 9-oxo-nonanoic acid and (3*Z*)-nonenal or (3*Z*,6*E*)-nonadienal. The (3Z)-aldehydes can be isomerized to their (2*E*)-enal isomers and reduced by alcohol dehydrogenase to their corresponding alcohols.

Until now, plant extracts are the most common enzyme source for the biocatalytic production of C6- and C9-aldehydes and alcohols [1,2], [US patents no. 5,464,761, and no. 4,806,379]. However, hydroperoxide lyase in plant extracts is very unstable. Furthermore, crude extracts contain other enzymes that use hydroperoxy fatty acids as substrates, such as allene oxide synthase and peroxygenase, leading to the formation of undesired side-products.

Recombinant expression is an excellent method to increase the availability of hydroperoxide lyase for a biocatalytic process [3], [European patent no. 0 801 133 A2]. The expression system described in chapter 3 [3] makes it possible to obtain large quantities of stable hydroperoxide lyase with a high specificity for 13-hydroperoxy linoleic and α-linolenic acids. This system is optimized to obtain a maximal amount of active enzyme. In addition, the optimal reaction conditions of the recombinant hydroperoxide lyase are determined, as well as the kinetic parameters and the initial and maximal turnover numbers.

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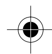

Materials and methods

Expression of HPO lyase

Alfalfa HPO lyase was expressed in *E. coli* cells containing the pQE32 vector (Qiagen) with the *CYP74B4v1* gene without N-terminal sequence coding for the first 22 amino acids (EMBL Database, accession number AJ249245) [3]. For small scale expression 50 ml LB medium containing 25 $\mu g \cdot ml^{-1}$ kanamycine and 100 $\mu g \cdot ml^{-1}$ ampicillin was inoculated with 2.5 ml overnight culture. Expression was induced by addition of IPTG. A 10 l culture of these *E. coli* cells was grown at 37 °C until an A_{600} of 0.7, in 1.5 × LB medium supplemented with 0.5% glucose, 1 × minimal medium (SV buffer), 25 $\mu g \cdot ml^{-1}$ kanamycine and 100 $\mu g \cdot ml^{-1}$ ampicilline, with maximal stirring and O_2 flow. Expression of HPO lyase was induced by addition of 1 mM IPTG and the cells were grown overnight at 25 °C to prevent sequestering of HPO lyase in inclusion bodies.

Isolation of HPO lyase

Cells were harvested by centrifugation and the supernatant was discarded. The cell pellets of the small scale expression cultures were resuspended in 5 ml 50 mM potassium phosphate buffers of different pH, and sonicated on ice 6 × 15 s, with 15 s intervals. Different amounts of Triton X-100 from a 10% solution (w/v) were added after sonication. The suspension was recentrifuged and the supernatant was used for activity measurements.

The cells from the 10 l culture were resuspended in a total volume of 350 ml of 50 mM potassium phosphate buffer, pH 8, and sonicated on ice 15 × 1 min with 1 min intervals in batches of 35 ml. Triton X-100 was added to a final amount of 0.5% and the suspension was recentrifuged. HPO lyase was present in the supernatant. All steps were carried out at 4 °C.

For the determination of the initial turnover number and K_m and V_{max} HPO lyase was purified by immobilized metal affinity chromatography as described [3].

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Enzyme activity measurements

HPO lyase activity was determined in 50 mM potassium phosphate buffer pH 8.25, containing 100 μM substrate, at 25 °C, unless otherwise stated. The decrease of A_{234} due to the cleavage of substrate was followed spectrophotometrically. One unit of activity (U) corresponds to the amount of enzyme that converts 1 μmol of substrate per min. The maximal turnover number was determined by measuring the substrate conversion by various amounts of enzyme, with substrate concentrations of 20 or 80 μM. Apparent K_m and V_{max} were determined from the means of six determinations with concentrations of substrate ranging from 5 to 150 μM. The data were fitted to the standard Michaelis-Menten kinetic equation (GRAPHPAD PRISM). The substrates 13-HPOD and 13-HPOT were prepared with soybean lipoxygenase-1 [4].

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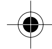

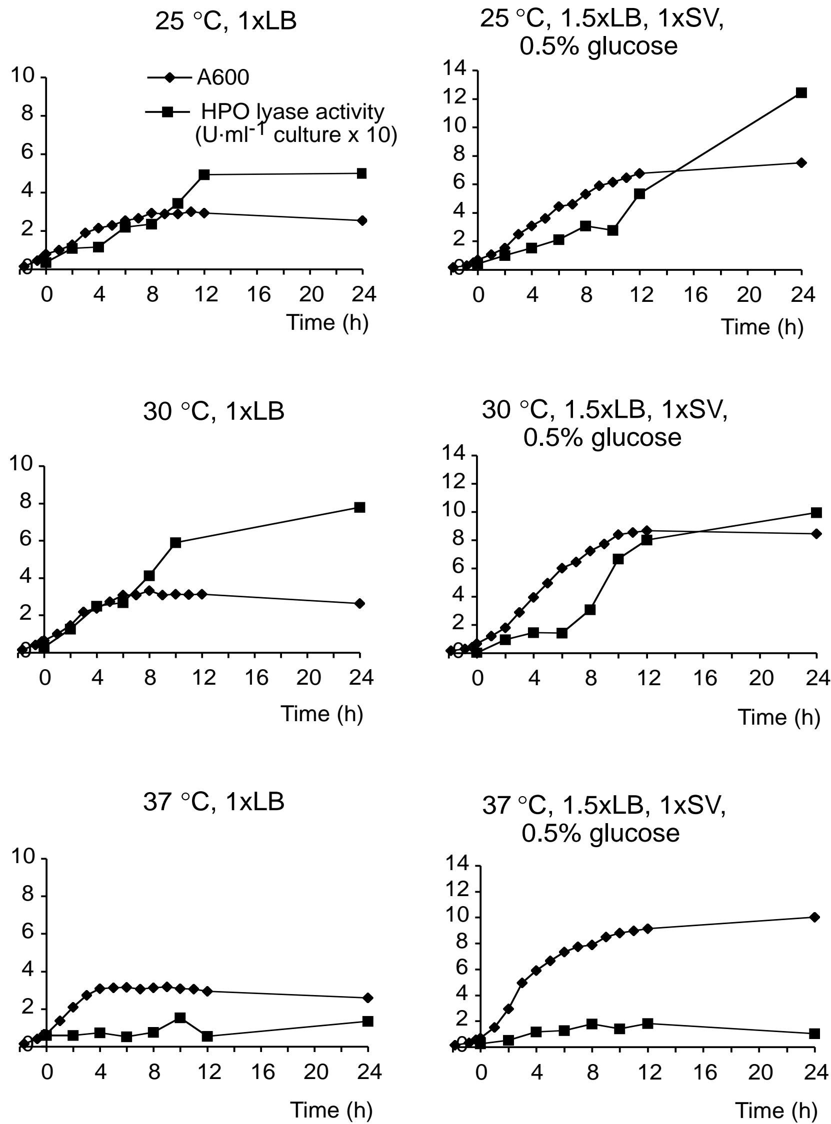

Fig. 1 **Influence of medium and expression temperature on the amount of active HPO lyase produced.** Small scale *E. coli* cultures expressing HPO lyase were grown at different temperatures in 1 × LB medium and in enriched medium (1.5 × LB, 1 × SV buffer, 0.5% glucose). Induction occurred by addition of 1 mM IPTG at A_{600} = 0.7 (t=0). The cells were resuspended in 50 mM potassium phosphate buffer, pH 7.5, and after sonication Triton X-100 was added to a final concentration of 0.2%. HPO lyase activity was determined in the supernatant after centrifugation, with 13-HPOT as substrate.

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Results and discussion

Optimization of expression and isolation conditions

The influences of expression temperature and medium on the production of active HPO lyase in *E. coli* were studied. The results show that the optimal expression temperature is 25 °C (Fig. 1). At higher temperatures most of the expressed HPO lyase is inactive and probably located in inclusion bodies. Enriched medium results in a higher cell density and in an increased amount of HPO lyase produced. HPO lyase expression is maximal in the late log-phase and stationary phase. As cells start to die after 24 h, longer expression times will not lead to a higher expression level.

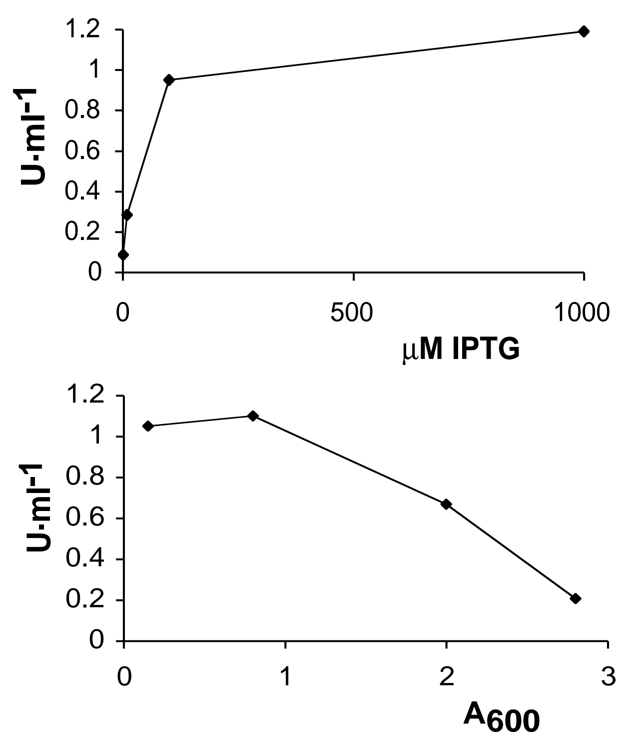

Fig. 2 **Influence of the amount of IPTG and the cell density at the time of addition on the expression of HPO lyase.** Small scale *E. coli* cultures expressing HPO lyase were grown at 25 °C in 1 × LB medium. Induction occurred by addition of different amounts of IPTG at A_{600} = 0.7 (A), or by adding 1 mM IPTG at different cell densities (B). The cells were resuspended in 50 mM potassium phosphate buffer, pH 7.5, and after sonication Triton X-100 was added to a final concentration of 0.2%. The HPO lyase activity was determined in the supernatant after centrifugation, with 13-HPOT as substrate.

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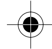


For optimal expression 1 mM IPTG has to be added at A_{600}<0.8 (Fig. 2). However, also with lower concentrations of IPTG (until 100 μM) a reasonably good expression is obtained. Because IPTG is rather expensive, the optimal amount of IPTG for industrial application may, from an economic point of view, be less than 1 mM.

The pH of the extraction buffer and percentage Triton X-100 appeared to have a great influence on the amount of HPO lyase that is solubilized (Fig. 3). The optimal pH for extraction is pH 8 and the optimal amount of Triton X-100 for solubilization is 0.5% (w/v). A second extraction of the cell pellet results in only 15% higher yield of HPO lyase. Higher percentages of Triton X-100 may lead to a slightly higher yield, but will also result in foaming during the enzymatic reaction and are therefore undesirable.

Expression in a 10 l *E. coli* culture under optimal conditions gives a yield of $3 \cdot 10^3$ or $8 \cdot 10^3$ U HPO lyase l^{-1} *E. coli* culture, with 13-HPOD or 13-HPOT as substrates, respectively.

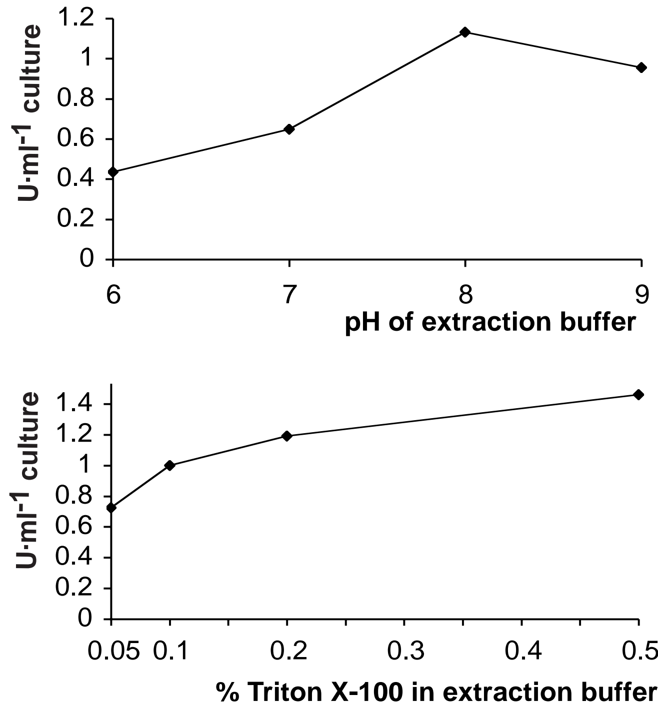
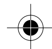


Fig. 3 **Influence of the pH of the extraction buffer and the amount of Triton X-100 on the solubilization of HPO lyase.** *E. coli* cells from small scale cultures (LB medium, 25 °C, 1 mM IPTG added at A_{600} = 0.7) were pelleted and resuspended in A: 50 mM potassium phosphate buffers of pH 6, 7, 8 or 9 with 0.2% Triton X-100, B: 50 mM potassium phosphate buffer, pH 7.5 with different amounts of Triton X-100. The HPO lyase activity was determined in the supernatant after centrifugation, with 13-HPOT as substrate.



Optimization of reaction conditions

The pH optimum of the HPO lyase reaction with 100 μM substrate appeared to be pH 8.25. The other HPO lyase isoenzymes (CYP74B4v2 and v3 [3]) have the same optimal pH. This is remarkable because the pH optimum of HPO lyase, isolated from alfalfa seedlings, is pH 5.5 [5]. Allthough the V_{max} of recombinant HPO lyase at pH 6 and pH 8.25 are in the same range (~650 U•mg^{-1} for 13-HPOT), the K_m at pH 8.25 is much lower than at pH 6 (41 and 42 μM for 13-HPOD and 13-HPOT at pH 8.25 instead of 151 and 140 μM for 13-HPOD and 13-HPOT at pH 6). Incubation of recombinant HPO lyase in a crude extract of alfalfa (1:4, v/v) leads to a 20% increase of the activity of recombinant HPO lyase at pH 6, and a twofold decrease of the activity at pH 8.25 with 100 μM substrate, indicating that components of alfalfa change the pH dependency of HPO lyase. In addition, there might exist another HPO lyase isoform in alfalfa, besides the three described, with a lower pH optimum.

An increase of the reaction temperature from 10 to 50 °C leads to an increase in reaction velocity. However, at temperatures above 30 °C HPO lyase is rapidly inactivated, which makes room temperature optimal for the HPO lyase reaction.

The initial turnover numbers with 100 μM 13-HPOD or 13-HPOT are 330 or 750 s^{-1}, respectively. To convert 1 μmol of 13-HPOD or 1 μmol of 13-HPOT, at least 125 mU or 500 mU of HPO lyase are needed, respectively (Fig. 4). These maximal turnover numbers of recombinant HPO lyase are equal to the turnover numbers of HPO lyase in alfalfa [A. van Kooij, unpublished results]. From these numbers it can be derived that an enzyme molecule can convert about 1.6•10^5 molecules of 13-HPOD or 0.9•10^5 molecules of 13-HPOT before it is inactivated. Inactivation of HPO lyase is independent of the substrate concentration (Fig. 4), indicating that inactivation is not caused by the substrate itself. HPO lyase is probably inactivated by reaction intermediates, as it is not inhibited by its reaction products either [A. van Kooij, unpublished results].

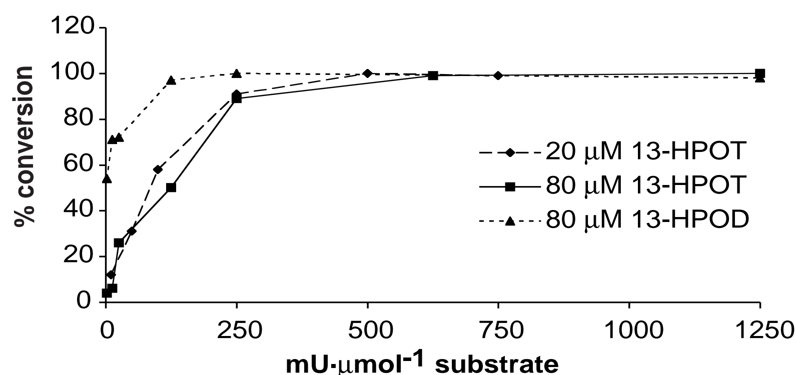


Fig. 4 **Maximal turnover of substrate by HPO lyase.** Different concentrations of HPO lyase were incubated with 20 or 80 μM 13-HPOT or 80 μM 13-HPOD at pH 8.25. The amount of substrate converted was followed by measuring A_{234}.

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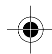

Conclusions

For optimal expression of HPO lyase in *E. coli,* the cells have to be grown at 25 °C in 1.5 × LB medium supplemented with 1 × SV buffer, 0.5% glucose and the appropriate antibiotics. Induction with 0.1-1 mM IPTG should take place at an A_{600}<0.8. After expression for 24 h, the cells have to be resuspended and opened in buffer of pH 8. HPO lyase should be solubilized by addition of Triton X-100 to a final concentration of 0.5% (w/v). The optimal reaction conditions for the conversion of 13-HPOD and 13-HPOT by recombinant alfalfa HPO lyase are pH 8.25, 25 °C. The amount of HPO lyase obtained from 1 l of *E. coli* culture can convert up to 24 mmol 13-HPOD or 15 mmol 13-HPOT. The initial turnover rates for these substrates are 330 and 750 s^{-1}, respectively.

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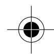

Outlook

HPO lyase 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. Nevertheless, structural characterization of HPO lyase and mechanistic studies could not be performed, because of its instability and difficult purification. HPO lyase is rather different from other cytochrome P450 enzymes, which makes it difficult to derive its structure and mechanism directly from the known structures and general reaction mechanism of cytochrome P450 enzymes. The recent cloning and expression of HPO lyases 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 HPO lyase 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. This is an excellent method to study the conformation of HPO lyase in solution, with special interest for the influence of detergents, as well as the position of the substrate in the active site. EXAFS and AXAFS may provide more information on the conformation of the active site and on the perturbation of the heme group in the high spin complex. In combination with EPR studies on HPO lyase in the presence of substrate, this will lead to a better understanding of the reaction mechanism.

The question if HPO lyase 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 HPO lyase available yet, which might be due to a low immunogenity of the enzyme.

Overexpression or repression of HPO lyase in plants is essential to understand 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. More knowledge about the differences between 13- and 9-HPO lyases can be obtained by cloning specific 9-HPO lyases. Pears could be a good source as they are the species known to contain only 9-HPO lyase.

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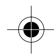
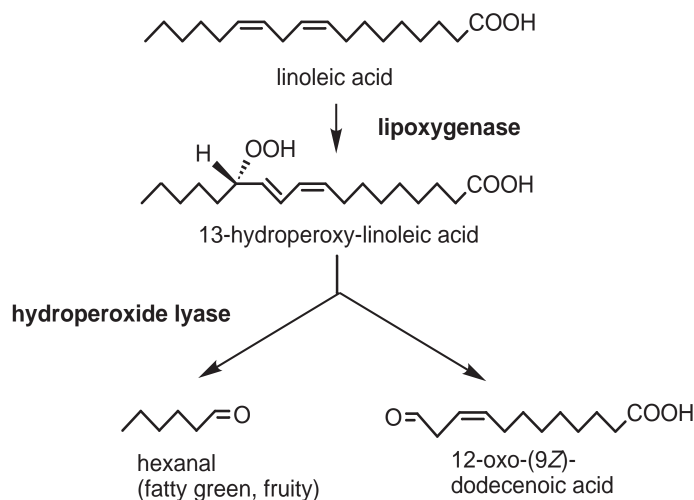


Summary

Plants continuously have to defend themselves against life threatening events such as drought, mechanical damage, temperature stress and potential pathogens. A main component of the plant defense mechanism is the lipoxygenase pathway. Products of this pathway are involved in wound healing, pest resistance, signaling, or 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 formed hydroperoxy fatty acids are highly reactive and dangerous for the plant, and are therefore further metabolized by other enzymes such as allene oxide synthase, hydroperoxide lyase, peroxygenase or divinyl ether synthase.

Hydroperoxide lyases are heme-containing enzymes of the cytochrome P450 class. They cleave the C-C bond adjacent to the hydroperoxy group in the lipoxygenase products, resulting in the formation of ω-oxo acids and volatile C_6- and C_9-aldehydes. The aldehydes and the corresponding alcohols cause the characteristic 'fresh green' odor when fruit and vegetables are wounded, for example by cutting them into pieces. They are widely used as food flavors, for example to restore the fresh odor 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 this study the enzyme hydroperoxide lyase is characterized.



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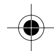

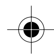

In chapter 2 the presence of hydroperoxide lyase in alfalfa seedlings is studied. Alfalfa has been reported to contain a hydroperoxide lyase specific for 13-hydroperoxy fatty acids. However, in addition to 13-hydroperoxide lyase activity we found substantial 9-hydroperoxide lyase activity in alfalfa seedlings as well. The specific activity for 9-hydroperoxy fatty acids is ~50% of the activity for the 13-isomers. The pH optimum for both activities appeared to be equal, namely pH 5.5, and the activities slightly decrease during germination. Furthermore, alfalfa seedlings contain a heat sensitive 3*Z*:2*E*-enal isomerase that converts the 3*Z*-enal products to their 2*E*-enal isoforms, and an alcohol dehydrogenase that reduces the aldehydes to alcohols.

Hydroperoxide lyase isolated from alfalfa extracts is very unstable and the amounts are low, which makes it difficult to purify. In chapter 3 the cloning and expression of alfalfa hydroperoxide lyase genes in *Escherichia coli* are described. Three full-length cDNAs were found encoding 54 kDa hydroperoxide lyase isoenzymes specific for 13-hydroperoxy fatty acids. Because alfalfa contains both 9- and 13-hydroperoxide lyase activity, this indicates the presence of two different types of hydroperoxide lyases, each specific for one kind of substrate. The cloned genes contain a nonplastidic N-terminal extension of 22 amino acids, which strongly reduces the enzymatic activity. This indicates that the complete enzyme might be a pro-enzyme, activated by cleaving off the N-terminal extension. A serine residue at position 377 may be important for enzymatic activity, as one of the isoenzymes which contains a substitution of this serine by a phenylalanine showed strongly decreased V_{max} and K_m. With this expression system it is possible to obtain large amounts of pure and stable enzyme for characterization studies and to assess its potential for biocatalytic purposes.

In chapter 4 characterization of the recombinant hydroperoxide lyase with EPR, CD spectrometry and spectrophotometry is described. CD spectra showed that hydroperoxide lyase consists for about 75% of α-helices. EPR spectra confirmed its classification as a cytochrome P450 enzyme. The influence of detergents, commonly used to solubilize the enzyme, on the enzyme conformation was studied as well. The positive influence of detergents on the enzyme activity appeared to be paralleled by a spin state transition of the heme Fe(III) from low to high spin. EPR and CD spectra showed that detergents induce a subtle conformational change, which might result in improved substrate binding. Because hydroperoxide lyase is thought to be a membrane-bound protein and detergents mimic a membrane environment, the more active, high spin form likely represents the *in vivo* conformation. Furthermore, the spin state appeared to be temperature dependent, with the low spin state favored at low temperature. Point mutants of the highly conserved cysteine in Domain D indicated that this residue might be involved in heme binding.

In chapter 5 *N*-acylethanolamines were tested as substrates for lipoxygenase, hydroperoxide lyase and allene oxide synthase. These compounds constitute a recently discovered class of plant lipids and are thought to play a role in plant defense as well. We found that both α- and γ-*N*-linolenoylethanolamine (18:3) as well as α- and γ-*N*-linolenoylamine are

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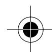

converted into their 13(*S*)-hydroperoxide derivatives by lipoxygenase. Interestingly, only the hydroperoxides of α-*N*-linolenoyl(ethanol)amines and their linoleic acid analogs (18:2) are suitable substrates for hydroperoxide lyase. Kinetic studies with lipoxygenase and hydroperoxide lyase revealed that the fatty acid ethanolamides are at least as readily converted as the corresponding free fatty acids. This indicates that the carboxyl group of the substrate is not essential for hydroperoxide lyase activity. Allene oxide synthase utilizes all substrates, but it is most active on 13(*S*)-hydroperoxy-α-*N*-linolenoylethanolamine and on the 13(*S*)-hydroperoxide of linoleic acid and its ethanolamine derivative. These results show in principle that hydroperoxide *N*-acylethanolamines can be formed in plants and subsequently converted into novel phytooxylipins.

Incubations of substrate with hydroperoxide lyase yield 4-hydroxy-(2*E*)-alkenals, besides the normal lyase products. These compounds are interesting because of their cytotoxicity in mammals. In chapter 6 the origin of these compounds is studied. Their presence in plant extracts appeared to be due to a nonenzymatic reaction of (3*Z*)-alkenals with molecular oxygen, stimulated by low pH. This questions the *in vivo* existence and physiological significance of these compounds in plants.

In chapter 7 the isolation of active hydroperoxide lyase from *Escherichia coli* and the reaction conditions for the production of volatile aldehydes from hydroperoxy fatty acids are optimized for the development of a biocatalytic process. It was determined that one enzyme molecule can convert up to $1.6 \cdot 10^5$ molecules of 13-hydroperoxy linoleic acid or $0.9 \cdot 10^5$ molecules of 13-hydroperoxy linolenic acid before it is inactivated. Inactivation is independent of the substrate or product concentration indicating that it is caused by reaction intermediates. From 1 liter of *Escherichia coli* culture enough hydroperoxide lyase can be obtained to convert 24 mmol or 15 mmol of 13-hydroperoxy linoleic or linolenic acids, respectively. The initial turnover rates for these substrates are 330 and 750 s^{-1}. Remarkably, the pH optimum of recombinant hydroperoxide lyase is pH 8.25, which is different from the optimum of hydroperoxide lyase in a crude extract of alfalfa (pH 5.5). The K_m of recombinant hydroperoxide lyase increases if the pH is lowered, whereas the V_{max} is independent of the pH. Components in the crude extract appeared to influence the pH dependency by increasing the activity of recombinant hydroperoxide lyase at lower pH (pH 6) and decreasing the activity at higher pH (pH 8).

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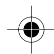
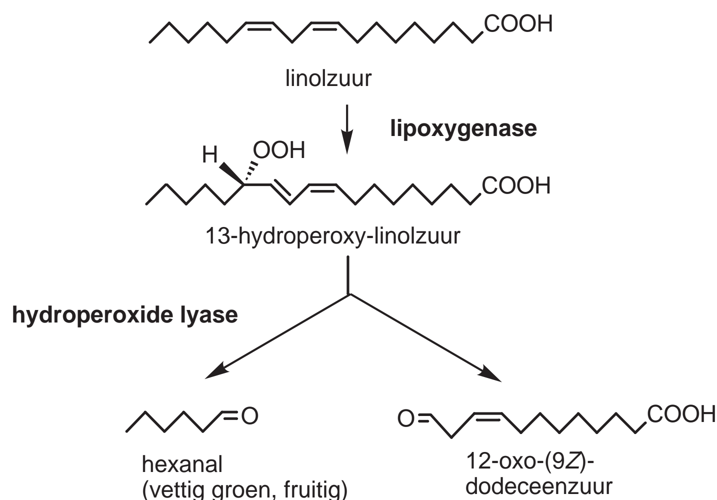


Samenvatting

Planten moeten zich continu verdedigen tegen dreigingen van buitenaf, zoals droogte, mechanische schade, hoge of lage temperaturen en mogelijke pathogenen. Een belangrijke component van het verdedigingsmechanisme van planten is de lipoxygenase route. Produkten van deze route zijn betrokken bij wondheling, resistentie tegen ziekten, signalering of hebben een antimicrobiële en schimmeldodende functie. De eerste stap in de lipoxygenase route is de reactie van linolzuur of linoleenzuur met moleculaire zuurstof, gekatalyseerd door het enzym lipoxygenase. De gevormde hydroperoxy vetzuren zijn erg reactief en gevaarlijk voor de plant. Ze worden daarom verder omgezet door andere enzymen zoals alleen oxide synthase, hydroperoxide lyase, peroxygenase of divinyl ether synthase.

Hydroperoxide lyasen zijn enzymen met een heemgroep die behoren tot de cytochroom P450 klasse. Ze verbreken de C-C binding naast de hydroperoxy groep in de lipoxygenase produkten, resulterend in de vorming van ω-oxozuren en vluchtige C_6- en C_9-aldehyden. De aldehyden en overeenkomstige alcoholen veroorzaken de karakteristieke 'fris groene' geur van groenten en fruit indien deze verwond worden, bijvoorbeeld door ze in stukken te snijden. Ze worden toegepast als geur- en smaakstoffen in voedsel, onder andere om het de frisse, verse geur terug te geven na sterilisatie. De lage hoeveelheden waarin deze stoffen voorkomen in de natuur en de grote vraag ernaar hebben ertoe geleid dat ze op grote schaal moeten worden gesynthetiseerd. Lipoxygenase en hydroperoxide lyase zijn geschikte biokatalysatoren voor de produktie van 'natuurlijke' geur- en smaakstoffen. In dit proefschrift is de karakterisering van het enzym hydroperoxide lyase beschreven.



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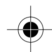

In hoofdstuk 2 is de aanwezigheid van hydroperoxide lyase in alfalfa zaailingen onderzocht. Er was reeds beschreven dat alfalfa een hydroperoxide lyase bevat die specifiek is voor 13-hydroperoxy vetzuren. Wij vonden echter aanzienlijke 9-hydroperoxide lyase activiteit naast de 13-hydroperoxide lyase activiteit. De specifieke activiteit voor 9-hydroperoxy vetzuren is ongeveer 50% van de activiteit voor de 13-isomeren. Het pH optimum bleek voor beide activiteiten gelijk te zijn, namelijk pH 5.5. De activiteiten nemen langzaam af gedurende de ontkieming. Voorts bevatten alfalfa zaailingen een temperatuur gevoelige 3*Z*:2*E*-enal isomerase die de 3*Z*-enal producten omzet in hun 2*E*-enal isovormen, en een alcohol dehydrogenase die de aldehyden reduceert tot alcoholen.

Hydroperoxide lyase geïsoleerd uit een alfalfa extract is erg instabiel en de hoeveelheden zijn laag, wat zuivering ingewikkeld maakt. In hoofdstuk 3 wordt de klonering en expressie van alfalfa hydroperoxide lyase genen in *Escherichia coli* beschreven. Er werden drie volledige cDNA's gevonden die coderen voor 54 kDa hydroperoxide lyase isoenzymen, specifiek voor 13-hydroperoxy vetzuren. Aangezien alfalfa zowel 9- als 13-hydroperoxide lyase activiteit bevat, impliceert dit de aanwezigheid van twee verschillende typen hydroperoxide lyasen die elk specifiek zijn voor een soort substraat. De gekloneerde genen hebben een N-terminale extensie van 22 aminozuren die de enzymactiviteit remt. Bovendien mist deze extensie de kenmerken van een transit-sequentie die het enzym naar de chloroplast dirigeert. Het complete enzym zou een pro-vorm kunnen zijn die wordt geactiveerd door het afsplitsen van de N-terminale extensie. Een serine residu op positie 377 zou belangrijk kunnen zijn voor de enzym activiteit daar één van de isoenzymen met een substitutie van deze serine door een fenylalanine een sterk verlaagde V_{max} en K_m heeft. Met het hier beschreven expressie systeem is het mogelijk om grote hoeveelheden zuiver en stabiel enzym te verkrijgen voor karakterisering en een biokatalytisch proces.

In hoofdstuk 4 is de karakterisering van het recombinant hydroperoxide lyase met EPR, CD en spectrofotometrie beschreven. CD spectra toonden aan dat hydroperoxide lyase voor ongeveer 75% uit α-helices bestaat. EPR spectra bevestigden dat hydroperoxide lyase tot de cytochroom P450 klasse behoort. De invloed van detergentia, die gewoonlijk worden gebruikt om het enzym los te weken uit de membraan, op de conformatie van het enzym is ook onderzocht. De positieve invloed van detergentia op de enzym activiteit bleek samen te gaan met een spin-overgang van het heem Fe(III) van de lage naar de hoge spintoestand. EPR en CD spectra toonden aan dat detergentia een subtiele conformatie verandering induceren die tot een verbeterde substraatbinding zou kunnen leiden. Aangezien er vanuit wordt gegaan dat hydroperoxide lyase een membraangebonden eiwit is en detergentia een membraanachtige omgeving simuleren, is de actievere, hoge spintoestand waarschijnlijk de *in vivo* conformatie. Voorts bleek de spintoestand temperatuur afhankelijk te zijn, waarbij de lage spintoestand de voorkeur heeft bij een lage temperatuur. Puntmutanten van de geconserveerde cysteine in het D-domein gaven een indicatie dat dit residu betrokken is bij het binden van de heemgroep.

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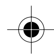

In hoofdstuk 5 zijn *N*-acylethanolamines getest als substraten voor lipoxygenase, hydroperoxide lyase en alleen oxide synthase. Deze stoffen vormen een recentelijk ontdekte klasse van plantenlipiden en worden verondersteld eveneens een rol te spelen bij de afweer van planten. Wij vonden dat zowel α- en γ-*N*-linolenoylethanolamine (18:3) als α- en γ-*N*-linolenoylamine door lipoxygenase omgezet worden tot hun 13(*S*)-hydroperoxide afgeleiden. Het is opmerkelijk dat alleen de hydroperoxiden van α-N-linolenoyl(ethanol)amines en hun linoleenzuur analogen (18:2) geschikte substraten zijn voor hydroperoxide lyase. Kinetische studies met lipoxygenase en hydroperoxide lyase toonden aan dat vetzuur ethanolamines tenminste even goed of zelfs beter worden omgezet dan de overeenkomstige vrije vetzuren. Dit impliceert dat de carboxylgroep van het substraat niet essentieel is voor de hydroperoxide lyase activiteit. Alleen oxide synthase kan alle substraten omzetten, maar heeft een voorkeur voor 13(*S*)-hydroperoxy-α-*N*-linolenoylethanolamine en voor de 13(*S*)-hydroperoxiden van linolzuur en zijn ethanolamine afgeleide. Deze resultaten tonen aan dat hydroperoxide *N*-acylethanolamines in principe in planten kunnen worden gevormd en dat ze vervolgens kunnen worden omgezet in een nieuw soort phytooxylipinen.

Incubaties van substraat met hydroperoxide lyase leveren naast de normale lyase producten ook 4-hydroxy-(2*E*)-alkenalen op. Deze stoffen zijn interessant vanwege hun cytotoxiciteit in zoogdieren. In hoofdstuk 6 is de herkomst van deze stoffen onderzocht. Hun aanwezigheid in plantenextracten bleek voort te komen uit een niet-enzymatische reactie van (3*Z*)-alkenalen met moleculaire zuurstof, gestimuleerd door een lage pH. Dit roept vragen op met betrekking tot het voorkomen *in vivo* en de fysiologische betekenis van deze stoffen in planten.

In hoofdstuk 7 zijn de isolatie van actief hydroperoxide lyase uit *E. coli* en de reactiecondities voor de produktie van vluchtige aldehyden uit hydroperoxy vetzuren geoptimaliseerd ten behoeve van de ontwikkeling van een biokatalytisch proces. Er is bepaald dat één enzym molecuul $1.6 \cdot 10^5$ of $0.9 \cdot 10^5$ moleculen 13-hydroperoxy linol- of linoleenzuur kan omzetten voordat het is geïnactiveerd. Inactivatie is onafhankelijk van de substraat of produkt concentratie waaruit kan worden afgeleid dat het wordt veroorzaakt door reactie-intermediairen. Uit 1 liter *E. coli* cultuur kan voldoende hydroperoxide lyase worden geïsoleerd voor de omzetting van 24 mmol 13-hydroperoxy linolzuur of 15 mmol 13-hydroperoxy linoleenzuur. De initiële activiteiten voor deze substraten zijn 330 s^{-1} en 750 s^{-1}. Het pH optimum van recombinant hydroperoxide lyase is pH 8.25 wat opmerkelijk verschillend is van het optimum van hydroperoxide lyase in een ruw extract van alfalfa (pH 5.5). De K_m van recombinant hydroperoxide lyase neemt af indien de pH toeneemt, terwijl de V_{max} onafhankelijk is van de pH. Componenten in een ruw extract bleken de pH afhankelijkheid ook te beïnvloeden door de activiteit van recombinant hydroperoxide lyase bij lage pH (pH 6) te verhogen en de activiteit bij hogere pH (pH 8) te verlagen.

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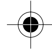

Curriculum Vitae

Minke Arianne Noordermeer werd geboren op 31 december 1972 te Vlaardingen. Na het behalen van het Gymnasium β diploma in 1991 aan het Serviam te Sittard, werd in hetzelfde jaar begonnen met de studie Medische Biologie aan de Universiteit Utrecht. Na het behalen van de propaedeuse (cum laude) werd in 1992 begonnen met de studie Scheikunde aan dezelfde universiteit. Als onderdeel van het doctoraal programma werd onderzoek verricht bij de vakgroep Medische Microbiologie van het Academisch Ziekenhuis Utrecht en bij de groep Pure and Applied Biochemistry aan de universiteit van Lund (Zweden). Voorts werd het vak chemiedidactiek gevolgd. Als hoofdvak werd onderzoek verricht aan het antibacteriële eiwit nisine bij het NIZO (Nederlands instituut voor zuivelonderzoek) te Ede en bij de sectie Biochemie van Membranen van de Universiteit Utrecht. In februari 1997 werd het doctoraal diploma behaald (cum laude). Vanaf 1 maart 1997 was zij als assistent in opleiding aangesteld bij de sectie Bio-organische Chemie van de Universiteit Utrecht, alwaar het in dit proefschrift beschreven onderzoek werd uitgevoerd, onder leiding van Prof. Dr. G.A. Veldink en Prof. Dr. J.F.G. Vliegenthart. De resultaten van dit promotieonderzoek werden onder andere gepresenteerd op het 13th en 14th International Symposium on Plant Lipids te Sevilla (1998) en Cardiff (2000) en op het International symposium on non-mammalian eicosanoids and bioactive lipids te Berlijn (2000). Tijdens het promotieonderzoek werden een cursus massaspectrometrie, een cursus electron paramagnetic resonance en enkele cursussen biokatalyse gevolgd. Vanaf 1 maart 2001 is zij werkzaam als post-doc bij de sectie Bio-organische Chemie waar zij in het kader van het IOP katalyse zal gaan werken aan de toepassing van hydroperoxide lyase als biokatalysator voor de produktie van geur- en smaakstoffen.

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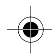

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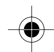

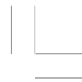
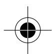
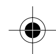

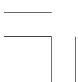
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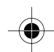
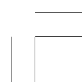

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