Expression of Manganese Lipoxygenase and Site-Directed Mutagenesis of Catalytically Important Amino Acids

Studies on Fatty Acid Dioxygenases

MIRELA CRISTEA
Abstract


Polyunsaturated fatty acids can be bioactivated by two families of dioxygenases, which either contain non-heme iron (lipoygenases) or heme (cyclooxygenases, linoleate diol synthases and α-dioxygenases).

Lipoygenases and their products play important roles in the pathophysiology of plants and fungi. The only known lipoygenase with catalytic manganese (Mn-lipoygenase) is secreted by a devastating root pathogen of wheat, the Take-all fungus Gaeumannomyces graminis. Its mycelia also contains linoleate diol synthase (LDS), which can oxidize linoleic acid to sporulation hormones.

Mn-lipoygenase belongs to the lipoygenase gene family. Recombinant Mn-lipoygenase was successfully expressed in the yeast Pichia pastoris with an expression level of 30 mg/L in fermentor culture. The tentative metal ligands of Mn-lipoygenase were studied by site-directed mutagenesis. The results show that four residues His-274, His-278, His-462 and the C-terminal Val-602 likely coordinate manganese, as predicted by sequence alignments with Fe lipoygenases.

Mn-lipoygenase (∼100 kDa) contains an Asp-Pro peptide bond in the N-terminal region, which appears to hydrolyze during storage and in the acidic media during Pichia expression to an active enzyme of smaller size, mini-Mn-lipoygenase (∼70 kDa). The active form of Mn-lipoygenase can oxygenate fatty acids of variable chain length, suggesting that the fatty acids enter the catalytic site with the α-end (“tail first”).

Mn-lipoygenase is an R-lipoygenase with a conserved Gly316 residue known as a determinant of stereospecificity in other R/S lipoygenases. The Gly316Ala mutant showed an increased hydroperoxide isomerase activity and transformed 18:3n-3 and 17:3n-3 to epoxyalcohols.

The genome of the rice blast fungus, Magnaporthe grisea, contains putative genes of lipoygenases and LDS. Mycelia of M. grisea were found to express LDS activity. This enzyme was cloned and sequenced and showed 65% amino acid identity with LDS from G.graminis.

Take-all and the rice blast fungi represent a constant threat to staple foods worldwide. Mn-lipoygenase and LDS might provide new means to combat these pathogens.

Keywords: Dioxygenase, Gaeumannomyces graminis, lipoygenase, Magnaporthe grisea, oxylipin, Pichia pastoris, hydroperoxide isomerase, polyunsaturated fatty acids

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List of Original Papers

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II **Mirela Cristea** and Ernst H. Oliw (2006). A single mutation (Gly316Ala) of manganese lipoxygenase augments the hydroperoxide isomerase activity. Mechanism of biosynthesis of epoxyalcohols *(submitted)*.


IV **Mirela Cristea**, Åke Engström and Ernst H. Oliw (2006). Catalytic properties of recombinant manganese(III)-lipoxygenase and acid-catalyzed hydrolysis to mini-manganese-lipoxygenase *(manuscript)*.


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<td>α-dioxygenase</td>
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<tr>
<td>AOX</td>
<td>alcohol oxidase</td>
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<td>BMG</td>
<td>buffered minimal glycerol</td>
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<td>BMM</td>
<td>buffered minimal methanol</td>
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<td>CP-HPLC</td>
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<td>epidermal lipoxygenase 3</td>
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<td>HOTrE</td>
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<td>HPOTrE</td>
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<td>potassium phosphate buffer</td>
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<td>linoleate diol synthase</td>
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<td>lipoxygenase</td>
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<td>MALDI-TOF</td>
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<td>Mn-LO</td>
<td>manganese lipoxygenase</td>
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<td>peroxidase</td>
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<td>sLO</td>
<td>soybean lipoxygenase</td>
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<td>sodium dodecyl sulphate–polyacrylamide gel electrophoresis</td>
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<td>SP-HPLC</td>
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<td>slow reacting substance of anaphylaxis</td>
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<td>RP-HPLC</td>
<td>reverse phase-HPLC</td>
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<td>RT-PCR</td>
<td>reverse transcription-polymerase chain reaction</td>
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<td>YPDS</td>
<td>yeast peptone dextrose sorbitol</td>
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Oxygenated fatty acids, also known as oxylipins [1], are found in both the plant and the animal kingdoms. Their biosynthesis involves at least one step of oxygenation, which can be achieved by two main enzymatic systems: fatty acid dioxygenases with catalytic non-heme iron (the lipoxygenases) and the dioxygenases with heme e.g. cyclooxygenases, linoleate diol synthases and α-dioxygenases. The third important system, which can catalyze the oxygenation of unsaturated fatty acids, is the cytochrome P450 family of monoxygenases (Fig. 1). The presence of various structural differences between the oxylipins formed in plants and animals might be due to the different fatty acid profiles and the fact that there are different sets of enzymes catalyzing their biosynthesis and metabolism.

**Figure 1.** Oxidative metabolism of polyunsaturated fatty acids in mammals, plants and fungi.
Fatty acid dioxygenases form local mediators, which regulate important aspects of the physiology of man and plants. Human lipoxygenases are important in asthma and allergic inflammation, whereas cyclooxygenases are inhibited by aspirin and other non-steroidal anti-inflammatory drugs. In plants, lipoxygenases are involved in defense mechanisms against fungal pathogens. The present thesis focuses on lipoxygenases and heme-containing dioxygenases in pathogenic fungi.

Lipoxygenases

Historical background
A first scientific report on a lipoxygenase was presented in 1932, when Andre and Hou [2] described oxidation of lipids in soybeans by an enzyme named lipoxidase. The importance of the lipoxygenase products in humans generates from the discovery made by Harkavy [3] in 1930 of a substance from the sputum of asthma patients that caused spasm in the smooth muscle preparations. About 10 years later, the substance was found in perfusates of dog and monkey lungs treated with cobra venom [4] and was named “Slow Reacting Substance of Anaphylaxis” (SRS-A) [5, 6]. It took almost 40 years until the structure of SRS-A was determined [7-9]. Samuelsson and co-workers found that the first step in SRS-A biosynthesis was catalyzed by a lipoxygenase similar to the ones previously described in soybean [10, 11].

Theorell [12] crystallized the lipoxygenase from soybeans in 1947, and this enzyme has been one of the most studied lipoxygenases. Lipoxygenases were considered exclusively plant enzymes until the mid seventies, when 12-LOX activity was discovered in human and bovine platelets [10]. Just one year later, Schewe et al [13] identified 15-LOX activity in the rabbit reticulocytes. From their discovery for more than 30 years ago and until now, the lipoxygenases have been under intensive study in order to elucidate their structure, mechanism of action and functions.

Occurrence and function of lipoxygenases
The lipoxygenases belong to the big family of dioxygenases that are widely spread in nature in plants [14] and animals [15]. They have also been detected in bacteria [16-18] and lower marine organisms [19-21]. All lipoxygenases cloned and sequenced so far belong to the same gene family, which is defined by sequence homology of catalytically important amino acids.

Lipoxygenases in plants
There are at least 35 plant lipoxygenases cloned, sequenced and characterized [22]. Some plants contain multiple lipoxygenases with at least 8 identified in soybean [23]. The most common substrates for lipoxygenases in plants are linoleic and α-linolenic acids.
According to the positional specificity of the linoleic acid oxygenation, the plant lipoxygenases are classified into 9- and 13-LOX [24]. The enzymes catalyze oxygenation at C-9 respective C-13 of the hydrocarbon backbone of the fatty acid with the formation of 9S-hydroperoxy and 13S-hydroperoxy derivatives. A more complex classification based on their primary structure divides the plant lipoxygenases into type 1-LOXs, characterized by a high sequence similarity (~75%), and type 2-LOXs with a relative lower sequence similarity (~35%) [24].

Some plant lipoxygenases are constitutive but most of them are often activated by pathogen attack, as a means of pathogen resistance [24-26]. Their products can have diverse important functions in signalling [27], plant defense or wounding [28, 29]. The lipoxygenases in plants can also be used as storage proteins during vegetative growth [30]. Since lipoxygenases are normally present in the seeds of plants they are also involved in a series of developmental processes [31, 32] and in the mobilization of storage lipids during germination [33].

The mammalian lipoxygenases

Mammalian lipoxygenases belong to the lipoxygenase gene family [23, 34, 35]. Their main substrate is arachidonic acid. Depending on the position of the oxygenation, they are divided into 4 classes, designated as follows: 5-LOX, 8-LOX, 12-LOX and 15-LOX. The 8-LOX has only been identified in mouse [36], while 5-, 12- and 15-LOX are present in humans. Both 12- and 15-LOX are characterized by the presence of several isoforms. A more accurate classification based on their phylogenetic relatedness, divides the mammalian lipoxygenases into four groups [15]:

1. 5-LOX (found, for example, in man [37], mouse [38], rat [39] and hamster [40]).
2. platelet-type 12-LOXs (found, for example, in man [41] and mouse [42]).
3. epidermis-type LOXs (see below).
4. 12/15 LOXs (see below).

The third category includes epidermis-type lipoxygenases like 12R-LOX and 15S-LOX from man [43, 44] and 15S-LOX and 8S-LOX from mouse [45, 46]. In the last category are grouped together leukocyte-type 12-LOXs from mouse [47], rat [48], rabbit [49], bovine [50] and porcine [51], together with reticulocyte-type 15S-LOX from man [52] and rabbit [53]. The human reticulocyte 15-LOX is closely related to porcine leukocyte 12-LOX, thus these two isoforms are grouped together in 12/15 LOXs although they have different positional specificity. Usually, isoenzymes that exhibit the same position specificity are named after the prototypical tissue of their occurrence. This is the case of 12-LOXs, which can be platelet-, leukocyte- or epidermis-type.

5-LOX catalyses the oxygenation of arachidonic acid to 5S-HPETE but also the dehydration to leukotriene \( \Lambda_4 \) (LTA\(_4\)). The latter can be further metabolized either to leukotriene \( \Lambda_5 \) by LTA\(_4\) hydrolase or by glutathione conjugation catalyzed by LTC\(_4\).
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Lipoxygenases in fungi

The fungi are organisms characterized by a variable content in fatty acids [71]. The C_{18} fatty acids are predominant in ascomycetes and basidiomycetes, while the oomycetes and the true fungi present higher levels of arachidonic and eicosapentanoic acids. There are some reports of lipoxygenases in fungi like Saprolegnia parasitica [72], Pityrosporum orbiculare [73] and Psallioata bispora [74, 75].

Interesting for study are the fungi that act as pathogens and secrete lipoxygenases that may be involved in their pathogenic mechanism. One of these fungi is
Gaëumannomyces graminis also known as the Take-all fungus – name given by the Australian farmers in the 19th century [71]. Take-all attacks wheat cultures and other grasses by causing the roots to rotten. G. graminis secretes a lipoxygenase with some unique features [76]. First of all, the catalytic metal is not iron as in all the other lipoxygenases, but manganese. Therefore the enzyme has been designated Mn-LO. Secondly, the enzyme transforms linoleic and α-linolenic acid to 13R-hydroperoxy fatty acids and to novel products, 11S-hydroperoxy fatty acids. The function of Mn-LO is unknown but it may cause oxidative damage to the root cells of wheat and grasses [76]. An alignment of partial amino acid sequences from Mn-LO, lipoxygenases with known 3D structures and two putative fungal lipoxygenases from Magnaporthe grisea and Aspergillus fumigatus showed that many important residues are conserved (Table 1).

As it has been mentioned above, there are plant pathogen interactions that may induce secretion of lipoxygenases. It is the case of another pathogenic fungus - Magnaporthe grisea or the rice blast fungus. M. grisea can induce a linoleate 13-LOX (rice LOX-1) as an early response of the host to the pathogen [77].

Table 1. Partial amino acid sequence of lipoxygenases with known 3D structures, Mn-LOX, and two putative fungal lipoxygenases

<table>
<thead>
<tr>
<th>Metal binding regions</th>
<th>sLOX-1</th>
<th>sLOX-3</th>
<th>Rabbit 15S-LOX</th>
<th>Coral 8R-LOX</th>
<th>Mn-LOX</th>
<th>LOX_Mg</th>
<th>LOX_Af</th>
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LOX_Mg, putative LOX of Magnaporthe grisea, LOX_Af, putative LOX of Aspergillus fumigatus. Conserved metal ligands are in bold.

Structural aspects of lipoxygenases

Lipoxygenases are long, single chain proteins of molecular masses of ~75-80 kDa in animals and ~94-104 kDa in plants [23]. There are over 50 sequences of lipoxygenases reported and many expressed as active proteins [78]. The enzymes range in length
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from 923 residues (rice LOX-2) to 661 residues (rabbit erythrocyte 15-LOX). The plant sequences are longer than mammalian sequences by 150 to 200 residues in range. The sequence identity between plant and mammalian lipoxygenases is highest in the regions of the catalytic domain, which are near to the iron.

The crystal structures of two soybean lipoxygenases (LOX-1 and LOX-3) [79, 80], rabbit reticulocyte 15-LOX [81] and a coral 8-LOX [82] have been elucidated. The protein structure is composed of two major domains: a small N-terminal β-barrel domain and a large catalytic C-terminal domain, which is predominantly α-helical and contains the active site iron. The function of the β-barrel might be related to lipid binding [83] and to membrane translocation [84]. In sLO-1 the iron is apparently six coordinated [85]. It binds to three His nitrogens, one oxygen from the carboxy-terminus of Ile 839, a water molecule and a distant Asn 694 residue. The three His and the Ile are residues conserved in all the sequenced iron lipoxygenases. The Asn ligand in sLO-1 is at least 2.9 Å away from the catalytic iron, indicating a weak interaction. In the area around the iron atom there are two large cavities: one is thought to be involved in substrate binding and the second is actually a funnel-shaped channel that is thought to be the means of entry for the dioxygen substrate [86]. The bound water points toward the mouth of the substrate-binding channel.

Lipoxygenases are enzymes known to contain non-heme iron in their catalytic center. There is only one lipoxygenase that does not follow this rule and this is Mn-LO of G. graminis. Atomic absorption spectroscopy studies showed that it contained 0.94 Mn per molecule [76]. Upon alignment with the primary structure of sLO-1 over the α-helices, the three His ligands and the distal metal ligand Asn seemed to be conserved. Electron paramagnetic resonance (EPR) studies showed that it is likely that Mn-LO has three N-ligands to the metal center and O-ligands in the six remaining positions [87]. The C-terminal amino acid of Mn-LO is valine [88]. Site-directed mutagenesis of murine platelet and leukocyte 12-LOX showed that the C-terminal isoleucine could be substituted with valine with retention of enzymatic activity [89].

Reaction mechanism of lipoxygenases

Lipoxygenases are enzymes that catalyze the oxygenation of polyunsaturated fatty acids with one or several (1Z,4Z)-pentadiene units to hydroperoxy fatty acids with cis-trans conjugated dienes as main products.

The lipoxygenation starts with the oxidation of Fe²⁺ to Fe³⁺ during a short time lag. During catalysis the iron redox cycles between Fe²⁺-OH₂ in the inactive form and the base Fe³⁺-OH⁻ in the active form of sLO-1. The catalytic base has been identified by studies upon the mutated amino-acids from the second coordination sphere of sLO-1 and the catalytic activity of these mutants in H₂O and ²H₂O at different temperatures [90].

The lipoxygenase reaction consists of three consecutive steps of which the first one is stereospecific and rate limiting [91]:

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1. The hydrogen abstraction from the allylic methylene group with the formation of a carbon-centered radical.
2. Rearrangement of the radical formed with Z,E-diene conjugation
3. Insertion of molecular oxygen at C-1 or C-4 of the pentadienyl structure with the formation of an oxygen-centered hydroperoxide fatty acid radical. The radical intermediate is reduced to the corresponding anion and the enzyme is oxidized back to the ferric form.

The main products of the lipoxygenase reaction are the hydroperoxy fatty acids. They can still contain double allylic methylenes so they can be further metabolized by lipoxygenase via double or triple oxygenation [92, 93]. Under reduced oxygen tension or anaerobiosis, the hydroperoxy fatty acids can be substrates for a hydroperoxide isomerase reaction. The reaction requires the presence of a reductant and it consists in a homolytic cleavage of the hydroperoxy bond with the formation of keto dienes and epoxyalcohols as main products.

A comparison between the reaction mechanism of Mn-LO and sLO-1 can reveal similarities but also important differences. Mn-LO presents the characteristic lag phase of iron lipoxygenases. If linoleic acid is used as substrate, the first step of the lipoxygenation is the stereospecific abstraction of the bis-allylic pro-S hydrogen from C-11 of the (9Z,12Z)-pentadiene unit of linoleic acid and the likely formation of a delocalized alkyl radical over C9 to C13. This is done in the same way by sLO-1 and Mn-LO. In regard to the dioxygenation, the differences appear in the way the molecular oxygen reacts with the alkyl radical and they might be due to steric factors at the active site [94]. SLO-1 allows antarafacial oxygenation and forms 13S-HPODE, while Mn-LO allows a suprafacial attack of the oxygen at either C13 or C11 in a ~3:1 ratio [95] with the formation of 13R-HPODE and 11S-HPODE, respectively. Mn-LO can also isomerize 11S-HPODE to 13R-HPODE [95]. The presumptive mechanism for the isomerization would be the formation of the peroxy radicals in equilibrium with the pentadienyl or the Δ9-[11,12,13]-allyl radicals and molecular oxygen [71]. The EPR could detect a peroxy radical during sLO-1 catalysis and an alkoxyl radical has also been identified [96]. The radicals are likely formed also by Mn-LO but they have not been detected by EPR. The biosynthesis of 13R-HPODE takes place with a higher rate (k_{cat}=19) than the isomerization of 11S-HPODE to 13R-HPODE (k_{cat}=7 and 9 respectively) and this might be due to the steric factors that favor the oxygen insertion at C-13. The turnover of linoleic acid by Mn-LO is ~26 s^{-1}, while for sLO-1 it is 10 times faster [97].

If α-linolenic acid is used as substrate (Fig. 2) the products formed are ~27% 11S-HPOTrE and ~73% 13R-HPOTrE at steady state with a K_{m} of 2.2 μM. The ω3 fatty acid proved to be a better substrate for Mn-LO with a turnover of ~47 s^{-1} [98].
Substrate alignment at the active site

Although direct structural data on LOX/fatty acids complexes is not currently available, attempts have been made to describe the alignment of the fatty acid substrate at the catalytic site of lipoxygenases [99]. There are two current theories that describe how the substrate enters the active site. The first theory is based on the hypothesis that the active site is a hydrophobic pocket in which the fatty acid enters with the methyl-end first (“tail first”). This allows the positioning of the hydrogen atom, which is abstracted from the bisallylic methylene, in close proximity to the non-heme iron, acting as electron acceptor [99, 100]. In favour of this hypothesis comes the three point enzyme/substrate interaction suggested by Kuhn and co-workers [101]. This interaction is based on the structural model of 15-LOX/arachidonic acid complex and includes first a hydrophobic interaction between the methyl-end of the substrate and the amino acids located at the bottom of the active site. Secondly, an ionic interaction of the fatty acid carboxylate with the positively charged side chain might occur and, last but not least, the π-electron interaction between the double bonds of the fatty acid and aromatic amino acid residues.

The “tail first” theory is also supported by studies on human reticulocyte-LOX-1 [102] and site-directed experiments made by Gan and co-workers on human 15-LOX-1 [103]. The latter showed evidence for the presence of a positive charged residue, located close to the surface of the protein, which might interact with the negatively charged carboxyl group of the substrate. These observations are consistent with the methyl-end binding of the substrate in the active site of mammalian 15-LOX [104, 105].

The second hypothesis regarding the alignment of the substrate in the active site involves a carboxylate-end binding. Amzel and co-workers [106] proposed a carboxylate-end first binding for sLO-1, which is supported by the crystal structure of the ferric form of sLO-3 (purple LOX) [107]. A recent report [108] indicated that the substrate might bind carboxyl-end first in the active site of sLO-1, orientation directed by the presence of two residues Trp500 and Arg707 located on the side, respectively the bottom of the substrate pocket.
Dioxygenases with heme

Cyclooxygenases

An important pathway in the oxygenation of fatty acids is represented by the cyclooxygenases or prostaglandin H synthases, as they are also referred to. These are heme-dependent enzymes, which have arachidonic acid and dihomo-\(\gamma\)-linolenic acid as preferential substrates and catalyze prostanoid biosynthesis [109, 110]. The reaction comprises two sequential steps that illustrate two types of activity: first the bis-oxygenation of arachidonic acid by the cyclooxygenase (COX) activity yielding the hydroperoxy endoperoxide PGG\(_2\), and second, the peroxidase activity (POX), which leads to PGH\(_2\). Although the POX reaction is considered the second step in the formation of PGH\(_2\), the COX reaction is dependent on POX activity for its activation with formation of a tyrosyl radical [111, 112].

Regarding the reaction mechanism, the initial step is the abstraction of the 13-pro-S hydrogen from arachidonic acid and antarafacial insertion of oxygen at C-11 to generate an 11-R peroxyl radical [113]. The stereospecific abstraction of the hydrogen is catalyzed by a tyrosyl radical, which has been detected by EPR [110, 114, 115], and it is likely formed after oxidation of the heme iron to a ferryl intermediate by PGG\(_2\) [115, 116]. A recent review [117] showed how the COX reaction might actually be divided into four consecutive steps as follows:

1. The radical at Tyr385 abstracts the 13-pro-S hydrogen with the formation of an 11-arachidonyl radical.
2. The molecular oxygen attacks on the side antarafacial to hydrogen abstraction and generates the 11-R-peroxyl radical.
3. The 11-R-peroxyl radical likely swings over C8, by rotation of the C10-C11 bond, in order to attack at C9 and to form an endoperoxide. The rotation of the C10-C11 bond brings C12 closer to C8 and makes possible the closure of a cyclopentane ring. As a result of this transition, C15 is repositioned for the addition of a second oxygen molecule.
4. The 15-S-peroxy radical is aligned below Tyr385 for donation of the radical and formation of PGG\(_2\).

The hypothetical transition from step 3 likely occurs through a movement of the \(\omega\)-end of arachidonic acid towards the carboxyl half. Previous studies have shown that the COX products might be formed from different competent conformers of arachidonic acid [118]. Cyclooxygenases can also catalyze the oxygenation of other polyunsaturated fatty acids into active compounds [119-123].

Cyclooxygenases present two isoforms, COX-1 and COX-2. COX-1 is constitutively expressed and involved in the physiological homeostasis, while COX2 is the inducible isoform, whose expression is triggered by specific cellular events. Both isoforms are membrane bound and localized on the luminal surfaces of the endoplasmic reticulum [124, 125]. COX-1 and -2 present 60-65% sequence identity.
but differences occur in what regards the signal peptides and the membrane binding domains. Specific for COX-2 is an insert of 18 amino acids that is located six residues in from the C terminus. Crystallization studies showed that the structures of the two isoforms are homologous and quite superimposable. They undergo significant conformational changes following the binding of fatty acid substrates and NSAIDs [126, 127].

The COX inhibition reduces inflammation, pain and fever. Aspirin treatment has long-term effects on platelet aggregation by inhibition of COX-1 [128]. The inhibition achieved by aspirin presents a different mechanism, as the drug binds in the active site and acetylates Ser530 thus blocking the access of the substrate [111, 129]. NSAIDs inhibit both COX-1 and COX-2. The selective inhibition of COX-2, achieved by the coxibs, proved to be efficient in the treatment of inflammation, pain and arthritis [130]. The coxibs might be connected to severe adverse effects regarding the cardiovascular system and rofecoxib has been retrieved from the market for these reasons [131].

The two COX isoforms have manifested important roles in many human pathologies which include thrombosis [132, 133], inflammation, pain and fever [134], various cancers [135-137], Alzheimer’s [138] and Parkinson’s [139] diseases.

Linoleate diol synthases

Linoleate diol synthase (LDS) is a hemeprotein isolated from the fungus *Gaeumannomyces graminis* [140, 141]. The enzyme belongs to the fatty acid heme oxygenase family, having a similar oxygenation mechanism with cyclooxygenases, although the products formed are different.

The reaction catalyzed by LDS begins with the abstraction of the 8-pro-\(\text{S}\) hydrog en from linoleic acid followed by the oxygen insertion with formation of 8-hydroperoxyoctadecadienoic acid (8-HPODE) [142]. A tyrosyl radical likely catalyzes the hydrogen abstraction [142], in analogy with COX. The second activity of the linoleate diol synthase is the hydroperoxide isomerase activity, which converts 8R-HPODE into 75,8S-dihydroxylinoleic acid (7,8-DiHODE). This isomerization is achieved by elimination of the 7-pro-\(\text{S}\)-hydrogen and intramolecular insertion of oxygen [140, 143]. The enzyme oxygenates linoleic, \(\alpha\)-linolenic, oleic and ricinoleic acids, while arachidonic, \(\gamma\)-linolenic and stearic acids were not substrates [144]. In conclusion, the formation of a tyrosyl radical and the presence of ferryl intermediates during catalysis are similar for LDS and COX but the peroxidase activities differ.

The results of the cloning of the LDS gene supported the functional similarities between the two classes of dioxygenases with a sequence identity up to 23-24% and 36-37% positive homology with COX-2 over the catalytic domain [145]. The sequence identity increases up to 36% over the core \(\alpha\)-helices including the proximal and distal heme ligands and the critical Tyr residue of cyclooxygenases [145].

LDS homologs are present among expressed sequence tags (EST) from, for example, *Neurospora crassa*, *Mycosphaerella graminicola* and *Glomus intraradices* (see www.ncbi.nlm.gov). The presence of 8R-HPODE biosynthesis but not the
corresponding LDS-like gene, has been reported in *Leptomitus lacteus* [146] and *Laetisaria arvalis* [147].

The function of LDS is largely unknown but 8R-hydroxylinoleate (8-HODE) has been identified as a sporulation hormone and anti-fungal agent [148-150]. A mutant of *Aspergillus nidulans* has been systematically studied by Champe, Keller and their colleagues. In addition to 8-HODE (PsiB) they have identified 5,8-dihydroxylinoleic acid (5,8-DiHODE) (PsiC) as sporulation hormones [149]. This was recently confirmed by excellent gene knockout studies [151]. A summary of products likely formed from 8R-HPODE is shown in Fig. 3. 8,11-DiHODE was identified as a fungal metabolite only recently [152]. Whether 5,8-DiHODE and 8,11-dihydroxylinoleic acid (8,11-DiHODE) are formed from 8R-HPODE has not been determined. These results underline the importance of LDS in fungal reproduction and growth.

**Figure 3.** Structures of the fungal metabolites formed from 8-HPODE.

**Fatty acid α-dioxygenases**

Besides the lipoxygenase pathway, the fatty acids from the plant leaves can undergo transformations by α-oxidation. The process involving the oxidation at the α-carbon of the fatty acid chain was discovered for the first time in 1956, when Stumpf [153] found that palmitic acid can be converted into pentadecanal by a preparation from peanut cotyledons. In 1999, the enzyme catalyzing oxygenation of fatty acids into 2-hydroperoxides was discovered in tobacco as a pathogen-induced oxygenase (PIOX) [154]. The enzyme was named α-dioxygenase-1 (α-DOX1). Together
with the homologous oxygenase from *Arabidopsis* [155, 156], it belongs to the new class of enzymes called \( \alpha \)-dioxygenases. Other \( \alpha \)-dioxygenases have been found in cucumber [154], rice [157], pea [158] and the green alga *Ulva pertusa* [159, 160]. The discovery of a second \( \alpha \)-DOX like-sequence in *Arabidopsis* [156] and the FEEBLY sequence from tomato [161] represented evidence for the existence of an isoform designated \( \alpha \)-DOX2. A strong \( \alpha \)-DOX activity has been recently reported in the moss *Physcomitrella patens* [162]. The fact that this new \( \alpha \)-dioxygenase prefers as substrate palmitic acid while oleic acid proved to be a very poor substrate marks a difference in comparison with \( \alpha \)-DOX1 from tobacco and *Arabidopsis*. The amino acid identity between \( \alpha \)-dioxygenases from higher plants is quite high (59-95%). The alignment with the *P. patens* \( \alpha \)-DOX places the latter as a distinct phylogenetic group.

The \( \alpha \)-DOX1 from tobacco and *Arabidopsis* possessed heme-binding motifs and showed homology to mammalian COX-1 and -2 [155, 156]. The peroxidase activity of \( \alpha \)-seem to be insignificant in \( \alpha \)-DOX1 from tobacco and *Arabidopsis* [154, 155], or even absent for the related enzymes from cucumber [154] and rice [157].

The reaction mechanism consisted in stereospecific abstraction of the pro-\( R \) hydrogen from C-2 followed by insertion of molecular oxygen with retention of absolute configuration. The 2-hydroperoxy fatty acids are chemically unstable and can easily be transformed to aldehydes [162].

In regard to the biological roles of \( \alpha \)-dioxygenases, several studies on \( \alpha \)-DOX1 from tobacco and *Arabidopsis* seem to indicate the participation in plant defence against microbial infection and in the defense mechanism induced to protect cells from oxidative stress. Interestingly, the isoform \( \alpha \)-DOX2 seems not to be induced in response to microbial infection but involved in the plant development. The high levels of \( \alpha \)-DOX2 transcripts, noticed in leaves subjected to artificial senescence by leaf detachment, might speak about an important role in the protecting mechanisms that control cell disruption [162].

### Expression systems for lipoxygenases

Structural and functional analyses of proteins, especially those that have potential therapeutic or industrial use, require usually large amounts of proteins. The supply of many valuable proteins is often limited by their low natural availability. The use of recombinant DNA technology and different expression systems has overcome this limitation and opened the way for the production of recombinant proteins. Among the most used expression systems are the bacteria, the yeasts and the insect cells. Since the latter represents an area that lies outside the scope of this thesis, only the first two systems will be described.

#### Bacterial systems - *E. coli*

The development of the bacterial expression systems, particularly *E. coli* has been a major advance in the production of large amounts of proteins from cloned genes.
The E. coli expression has facilitated the efficient production of therapeutic-grade proteins such as insulin [164], growth hormone [165] and interferon [166]. Many plant and mammalian lipoxygenases have been expressed successfully in E. coli [89, 90, 167-169].

The fact that it is a well-characterized organism and the high availability of the techniques used for transformation and expression places E. coli as one of the first choices among expression systems. However, the high-level expression of recombinant proteins in E. coli often results in the formation of inclusion bodies [170]. These aggregates are both insoluble and inactive and might represent an important hinder in obtaining biologically active recombinant proteins. Although procedures can be developed for renaturation of the proteins [171, 172], they can be difficult to achieve and time-consuming.

Proteins derived from eukaryotic genomes require post-translational modifications that cannot be achieved by the use of a prokaryotic expression system. For example, E. coli has no capacity to glycosylate recombinant proteins and this might alter the function of certain proteins [173, 174]. The inability to fold correctly the foreign protein and perform post-translational modifications limits the types of proteins that can be expressed in E. coli. Many of the proteins that cannot be expressed in E. coli due to the inconveniences presented above have been produced successfully in eukaryotic systems like the yeast P. pastoris [175].

Expression in yeast - Pichia pastoris

P. pastoris was discovered for more than 30 years ago [176] as one of the yeasts species capable of metabolizing methanol [177]. Since its development as a heterologous expression system in 1980 [178], P. pastoris has proven to be a powerful tool for large-scale production of proteins of interest. This methylotrophic yeast is using methanol as sole source of carbon and energy. The first step in the metabolic pathway is the oxidation of methanol to formaldehyde by the enzyme alcohol oxidase (AOX). The reaction generates hydrogen peroxide and, in order to avoid the toxicity, the process is located in a peroxisome capable of sequestering the hydrogen peroxide away from the rest of the cell. There are two genes in P. pastoris that code for AOX - AOX1 and AOX2. The first one is responsible for the majority of AOX activity [179]. Expression of AOX1 is controlled at the level of the transcription [179-181] and is induced by methanol to high levels.

The strains used for expression are either the wild type X-33 or derivated strains such as GS115, KM71 and SMD1168, which have a mutation in the histidinol dehydrogenase gene (HIS4) and allow for selection of expression vectors containing HIS4 upon transformation [182].

Although the vectors used for transformation into P. pastoris are designed in accordance with the expression into the different P. pastoris strains, they present also several common features [178]. One of them is represented by the foreign gene expression cassette composed of DNA sequences containing the P. pastoris AOX1 promoter, followed by one or more unique restriction sites for insertion of the foreign
gene, followed by the transcriptional termination sequence from the *P. pastoris* AOX1 gene that directs efficient 3' processing and polyadenylation of the mRNAs. Certain vectors contain dominant drug-resistance markers allowing introduction of multiple copies of the foreign gene. One of these set of vectors is pPICZ, which contains the *Sh ble* gene from *Streptoalloteichus hindustanus*. The *Sh ble* gene confers resistance to the drug Zeocin and acts as selectable marker for both *E. coli* and *P. pastoris*. The pPICZ vectors present two important advantages: their size (~3 kb), which makes them easier to manipulate and the presence of a multiple cloning site for the integration of multiple copies of the foreign gene. All expression vectors contain at least one *P. pastoris* DNA segment such as the AOX1 promoter fragment with unique restriction sites that can be used for cleaving and integration of the vector into the host genome. Thus stable *P. pastoris* transformants are generated via homologous recombination [182].

Using the *P. pastoris* system, the heterologous proteins can be expressed intracellularly or secreted into the medium. The secreted protein represents the majority of the total protein in the expression medium, since *P. pastoris* secretes only low levels of endogenous proteins and there are no other added proteins to the culture medium to promote growth [183].

Due to its respiratory mode of growth, *P. pastoris* can be very well adapted for expression at high-cell densities in fermentors [178]. The secreted proteins can be produced in larger amounts in a fermentor than in the shake-flask cultures since their concentration is proportional to the concentration of *P. pastoris* cells in the medium. Another important advantage of the fermentor culture is the fact that methanol can be added in growth-limiting rates which increases the level of transcription of the AOX1 promoter. The controlled environment in the fermentor ensures also a constant and quite high oxygen level needed for the methanol metabolism of the yeast.

*P. pastoris* is an eukaryote capable of achieving many post-translational modifications such as folding, disulfide bridge formation and O- and N-linked glycosylation [178]. The pattern of glycosylation differs from the one achieved by higher eukaryotes posing problems for the proteins used in the pharmaceutical industry. The differences between the mammalian and the yeasts N-linked glycosylation might limit the use of this, otherwise, extremely efficient expression system.

There are only two reports describing lipoxygenases expressed in *P. pastoris*. They are porcine leukocyte 12-LOX [184] and rat leukocyte 12-LOX [185].

More than 20 mammalian LOX-isoforms have their primary structures elucidated but only one three-dimensional structure has been reported until now [83]. In order to facilitate the study of the structure and the oxygenation mechanism, the need for detailed crystal trials is imposing. *P. pastoris* represents the recombinant expression system that might offer the necessary amount of lipoxygenases for these studies.
Aims

Mn-LO is a fungal enzyme secreted by the devastating root pathogen of wheat, *Gaeumannomyces graminis*. This lipoxygenase contains manganese in its catalytic center, which makes it unique. All other lipoxygenases contain iron. *G. graminis* also expresses a heme-containing dioxygenase, linoleate diol synthase (LDS), with structural and catalytical similarities to cyclooxygenases and fatty acid α-dioxygenases.

The aims of the present studies were to

- Develop a robust and efficient system for heterologous expression of Mn-LO (*paper I*).
- Determine the effects of site-directed mutagenesis of the putative metal ligands of Mn-LO on enzyme activity and manganese content (*paper I*).
- Characterize the effects of three mutations (Ala, Thr and Val) of the conserved Mn-LO Gly316 residue on product formation, as the homologous Gly of *R* lipoxygenases is known as a determinant of R/S stereospecificity (*paper II*).
- Develop an LC-MS/MS method for analysis of epoxyalcohols (*paper III*).
- Study the catalytical properties of Mn-LO (~100 kDa) and its smaller form of ~70 kDa designated mini-Mn-LO (*papers I and IV*).
- Study the thermostability of Mn-LO and the mechanism of formation of mini-Mn-LO (*paper IV*).
- Determine the biosynthesis and the transformations of *R* and *S* stereoisomers of 11-HPODE by Mn and Fe containing lipoxygenases (*paper V*).
- Determine whether mycelia of the rice blast fungus, *Magnaporthe grisea*, can metabolize linoleic acid by the LDS or lipoxygenase pathways (*paper VI*).
- Determine whether *Magnaporthe grisea* expresses homologous genes to LDS and Mn-LO (*paper VI*).
Expression in *Pichia pastoris*

Expression construct

The vector chosen for expression was pPICZαA, which offers the advantage of the high-level, methanol inducible expression of the gene of interest in *P. pastoris* [178]. This vector contains the AOX-1 promoter and can be used in any *P. pastoris* strain including X-33. pPICZαA is conveniently small (3.6 kb) and contains the Zeocin resistance gene for selection in both *E. coli* and *P. pastoris*. In the expression construct pPICZαA-Mn-LO-602, the native secretion signal had been replaced with the yeast secretion α-signal for secreted expression of the recombinant protein.

PCR technology was used in order to delete the native secretion signal of the Mn-LO precursor (Fig. 4). The sequence of Mn-LO in the vector pGEM-7Zf(+) was used as a template with a sense primer containing restriction sites for *Xba*I and *Eco*RI, whereas the antisense primer was located downstream of an *Nco*I restriction site. *Xba*I and *Nco*I were used for restriction of the 681 bp fragment, which was ligated into pGEM-Zf(+) yielding pGEM7-Mn-LO-602. The latter was restricted with *Eco*RI, and the Mn-LO-602 fragment (1.8-kb) was cloned into the *Eco*RI-restricted pPICZαA. Sequencing confirmed that the α-factor signal sequence of pPICZαA was in frame with the coding sequence of Mn-LO-602.

Transformation into *P. pastoris* – Electroporation

The electroporation method uses a pulsed electrical field to introduce DNA into the cells [186]. The strength of the applied field, the length of the electric pulse and the temperature can influence the efficiency of transfection along with the conformation, the concentration of the DNA and the ionic composition of the medium.

The procedures for transformation described by Invitrogen were followed [187]. pPICZαA-Mn-LO-602 and the mutants were grown in low salt Luria-Bertani medium using Zeocin resistance for screening. The purified and linearized DNA was first mixed with freshly prepared electro competent *P. pastoris* X-33 cells in 0.2 cm cuvettes and pulsed (1.5 kV, 25 mF, 200 Ω; Gene Pulser, Bio-Rad). After recovery for 1.5-3 h at 28.5°C aliquots were spread on YPDS-agar plates containing 100 mg/L Zeocin and incubated at 28.5°C for 3-4 days. PCR screening was routinely done in order to recheck the presence of Mn-LO DNA in the colonies.
**Secretion signal**

1 MRSRLAIVF AARHVA

**Recombinant Mn-LOX**

<table>
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<th>15</th>
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<tr>
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</tr>
<tr>
<td>601</td>
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</table>

Figure 4. Amino acid sequences of the native secretion signal and of the recombinant Mn-LO. The peptides in red are identified by MALDI-TOF analysis of tryptic peptides of mini-Mn-LO. The Ser and Thr residues of the first 50 amino acids and the putative N-glycosylation sites are underlined. Mini-Mn-LO is likely formed by cleavage between Asp and Pro (shown in bold and underlined).

**Expression in baffled flasks**

The first goal of the expression process is to generate biomass by growing the Zeocin resistant colonies in buffered minimal glycerol (BMG). In order to induce expression of the protein, the cells were transferred to buffered minimal methanol (BMM) and grown at 21°C or 28.5°C in 3 or 5 L baffled flasks (250 rpm). Methanol was added daily (0.5%) until the OD600 of the culture reached ~15 after 5-6 days. The cells were then precipitated by centrifugation and the supernatant stored at +4°C. The pH of the medium at the end of the expression experiments reaches pH 4.5 or even lower, which is normal for a healthy P. pastoris culture. An adjustment of the pH to 7 was performed in order to run the purification experiments.

**Expression in fermentor**

Many proteins can be well expressed in P. pastoris in shake-flask cultures but the expression can reach ultra-high cell densities in a fermentor [188, 189]. This is possible due to the controlled environment from the fermentor, which includes the control of the pH, the oxygen and the carbon source feed rate. The fermentor expression can be used for P. pastoris cultures, since this organism prefers a respiratory rather than fermentative mode of growth, thus the toxic fermentation products do not accumulate in the culture. This procedure is advantageous for secreted proteins since their concentration increases with the cell density.
Mirela Cristea

For large-scale biosynthesis of recombinant Mn-LO, an FE2 bioreactor (10 L, 20°C) was chosen for growing one *P. pastoris* transformant in basal salt medium with 20% dissolved oxygen and pH 5.0, controlled by addition of NH₃ and H₃PO₄. Protein biosynthesis was induced by 0.5% methanol. The BMM medium was assayed for lipoxygenase activity daily. The culture reached an OD₆₀₀ of 440 and a biomass of 340 g/L on day 5 with little gain in lipoxygenase activity from the previous day. The supernatant was recovered by centrifugation, processed directly or stored at -80°C.

Site-directed mutagenesis

Site-directed mutagenesis based on PCR technology was used in order to study the putative ligands of Mn-LO and other catalytically important amino acids. This rapid four-step procedure [190] requires only a small amount of DNA template and ensures high mutation efficiency together with a decreased potential for random mutations.

Point mutations of different amino acids from the expression construct pPICZαA-Mn-LO-602 were performed with the QuikChange kit (Stratagene), using Pfu DNA polymerase and oligonucleotides (31–43 nt). The two-stage PCR method was used with the following conditions 95°C for 5 min followed by 12–16 cycles (95°C for 30 s, 55°C for 1 min, and 68°C for 12 min). After the first 3 cycles, the PCR containing sense and antisense primer pairs were combined [191]. The PCR reaction was followed by treatment with *Dpn*I (3 h) in order to digest the parental DNA template, which was methylated. The plasmids obtained were transformed into *E. coli.* Mutated plasmids were screened by restriction analysis in some cases and all were sequenced.

Protein analysis and purification

Chromatography

The two first steps in the purification of recombinant Mn-LO and its mutants were hydrophobic interaction and ion-exchange chromatography. The protocol described by Su et al [76] was followed. The ionic strength of the cell free *P. pastoris* BMM medium from the fermentor culture was adjusted by addition of ammonium sulphate to 0.6 M concentration. The pH was adjusted to 7.0 (10 M KOH) and precipitates were removed by centrifugation. The supernatant was then loaded on a phenyl-Sepharose CL-4B column in 5 mM KPB (pH 7.2)/0.6 M ammonium sulfate. The column was washed with the same buffer and captured proteins were eluted with 2.5 mM KPB (pH 7.2). After dialysis, the material was further purified by cation exchange chromatography (CM Sepharose CL-4B) in 0.01 mM KPB (pH 6.8). The column was then eluted in one step with 0.01 mM KPB (pH 6.8)/0.2 M NaCl. The eluted protein was stored in the elution buffer at +4°C (with 1 mM NaN₃) and concentrated by diafiltration, as required for further analysis.
The HPLC system for gel filtration consisted of pump and diode array detector from Waters (Waters 626 and Waters 996 PDA) and the column (Biosep SEC-S3000; 300 x 8.2 mm, Phenomenex) was eluted at 0.5 ml/min with 0.1 M KFB (pH 6.8)/0.15 M NaCl.

The purification of mini-Mn-LO was done by gel filtration and anion exchange chromatography with a Q-Sepharose FF column. The sample was applied to the column in 20 mM TrisHCl pH 8. The elution buffer contained 20 mM Tris HCl pH 8 with 0.5 M NaCl.

SDS-PAGE

SDS-PAGE is used to assess the homogeneity and the molecular weight of purified proteins. Deglycosylation analyses can also be run effectively on a SDS-PAGE gel.

After purification and ultracentrifugation the protein samples were applied on 7.5% separation gels and SDS–PAGE was performed as described [76]. The analysis was used routinely in order to confirm the expression of the mutated proteins. Protein bands were analyzed by MALDI-TOF after trypsin digestion (see below).

For deglycosylation, Mn-LO was denaturated by heating and then treated with O-glycosidase or with O-glycosidase plus N-glycosidase F overnight at 37°C. Mini-Mn-LO was deglycosylated by the same procedure.

Lipoxygenase and hydroperoxide isomerase activity assay

UV analysis

Light absorption was measured with a dual beam spectrophotometer (Shimadzu UV-2101 PC). The same instrument was used for stopped-flow with a spectrophotometer accessory (RX 1000 Rapid Kinetics, Applied Photophysics). The cis-trans conjugated hydro(pero)xy fatty acids were assumed to have a molar extinction coefficient of 25,000 cm⁻¹ M⁻¹ at 235 nm (linoleic acid) and at 237 nm (α-linolenic acid) [192, 193]. The lipoxygenase activity was determined by UV spectroscopy from the maximal rate of biosynthesis of cis-trans conjugated hydroperoxy fatty acids during the linear part of the reaction in 0.1 M NaBO₃ buffer pH 9.0.

To assess C-11 and C-13 dioxygenation of α-linolenic acid, recombinant Mn-LO and the mutants were usually incubated in triplicate with 50-100 μM linoleic acid in 0.1 M NaBO₃ buffer (pH 9.0) and the UV absorbance was followed until 50% of the substrate appeared to be consumed (at the middle of the linear UV reaction curve).

The hydroperoxide isomerase activity was monitored by following the conversion of 13R-HPOTrE (1-100 μM) to 13-KOTrE at 280-282 nm in duplicate or triplicate, and the rate was determined from the linear part. Apparent K_m was estimated by Michaelis-Menten kinetics. Hydroperoxide isomerase activity was also monitored by the decline in UV absorbance at 237 nm.
Experiments under oxygen-18 atmosphere were performed essentially as described [95].

**HPLC analysis**

HPLC is a convenient technique for separation and analysis of lipoxygenase metabolites.

RP-HPLC (octadecasilane silica, 5-μm; 200 x 8 mm) was used to purify 9-, 11- and 13-HPODE using methanol/water/acetic acid, 80/20/0.01, and detected by UV absorbance at 235 and 210 nm [194]. Fractions with 11-HPODE were diluted with water and extracted on a cartridge of octadecasilane silica (SepPak/C₁₈). For analytical HPLC analysis of HODE and HOTrE, the RP-HPLC column was Kromasil 5 C₁₈ (250 x 2 mm; 5 μm, 100 Å, Phenomenex) and it was eluted with methanol/water/acetic acid, 80/20/0.01, at 0.3-0.4 ml/min (P2000, SpectroSystem), whereas 70/30/0.01 was used for partial separation of epoxyalcohols.

Separation of methyl 13- and 9-hydroxylinoleates were performed by straight phase-HPLC (SP-HPLC; Nucleosil 50-5, 250x4.6 mm; eluted with 2 ml/min) or by chiral phase-HPLC (CP-HPLC; R(-)-N-3,5-dinitrobenzoyl-α-phenylglycine, 250 x 4.1 mm; eluted with 0.8 ml/min) with 0.5% isopropanol in hexane (v/v) as eluent [93].

SP-HPLC with MS/MS analysis of epoxyalcohols was performed on a Kromasil-RP column (250 x 2 mm, 5 μm), which was eluted at 0.3 ml/min with 1 or 3% isopropanol in hexane (with 0.1-0.03 ml acetic acid L⁻¹) for hydroxy fatty acids and for epoxyalcohols, respectively. To reduce variations in retention times on SP-HPLC, the flow rate was usually adjusted so that *threo* 11-hydroxy-12S,13S-epoxy-9Z-octadecenoate had a retention time of ~12 min.

**Mass spectrometry**

LC-MS/MS analysis

LC-MS/MS has been proven to be a very useful tool for analysing polar, non-volatile or thermolabile molecules, without the need for prepurification or derivatization.

For LC-MS/MS analysis, the column mostly used contained octadecasilane silica (5-μm, 250 x 2 mm) and it was eluted with methanol/water/acetic acid, 80/20/0.01, at 0.4 ml/min. The effluent was subject to electrospray ionization (ESI) in an ion trap mass spectrometer (LCQ, ThermoFinnigan) with monitoring of the negative ions as described [98].

**GC-MS analysis**

GC-MS analysis is useful for determining the position of the hydroperoxy group of lipoxygenase metabolites after reduction to alcohols and derivatization (methylation and silylation).
A capillary GC (Varian 3100) with a non-polar column (30 m; DB-5, film, 0.25-μm; diameter, 0.25-mm) and an ion trap mass spectrometer (ITS40, Finnigan MAT) were used [144]. After splitless injections of samples in heptane, the GC was programmed from 120 to 200°C with 40°C/min, to 260°C with 28°C/min and then to 285°C with 3°C per min. C-values were determined by the retention times of fatty acid methyl esters. Trimethylsilyl ether and methyl ester derivatives were prepared as described [144].

MALDI-TOF analysis

Matrix Assisted Laser Desorption Ionization (MALDI) utilizes the energy from a laser to desorb and ionise the analyte molecules in the presence of a light-absorbing matrix [195]. The smallest ions travel fastest through the flight tube and arrive at the detector first. Thus, the time of flight (TOF) in the electrical field is a measure of the mass (or, more precisely, the mass/charge ratio). This method allows the analysis of small amounts of biomolecules ranging from a few picomoles to femtomoles.

Mn-LO was analyzed after gel filtration and desalting by MALDI-TOF (Bruker Ultraflex TOF/TOF) and the same instrument was used for analysis of tryptic peptides, which were obtained by digestion of the band in the polyacrylamide gel as described [196].

Bioinformatic resources and sequence analysis

DNA sequencing analysis was performed at the department of Animal Breeding and Genetics, SLU, and at the Uppsala Genome Center, Rudbeck Laboratory, Uppsala, Sweden. Sequencing was performed using a Big Dye Terminator sequencing kit and ABI 377 automatic sequencer (Perkin Elmer, Applied Biosystems). The Lasergene program (DNASTAR, Madison, WI) and the BLAST algorithm (www.ncbi.nlm.nih.gov) were used for sequence analysis [197].

The genome of *M. grisea* was analysed by the TBLASTN algorithm at the Whitehead Institute Centre for Genome Research (www-genome.wi.mit.edu) with the protein sequence of LDS. (www.ncbi.nlm.nih.gov).
RESULTS

Expression of Mn-LO in the yeast *P. pastoris* (*paper I*)

The *P. pastoris* colonies were grown overnight in BMG in order to generate biomass. The OD_{600} was ~1 when the induction was started by transferring the cells to BMM at room temperature. After 4-5 days of methanol induction, the cultures in baffled flasks reached an OD_{600}~15. At this point the cells were centrifuged and the supernatant was collected by centrifugation.

Enzyme activity measurements by UV spectroscopy and LC-MS analysis showed that the cultures secreted active enzyme, which formed 11S-HOTrE and 13R-HPOTrE in the same ratio as Mn-LO [76]. The shake-flask cultures yielded a relatively low amount of enzyme ~0.5-1 mg/L. Thus, a scale-up of the expression was performed in a fermentor. The fermentor culture reached an OD_{600} of 440 and the lipoxygenase activity of the culture medium suggested secretion of ~30 mg/L.

Recombinant Mn-LO (*papers I and IV*)

Protein analysis

From the bioreactor culture, 10 mg of Mn-LO was purified to apparent homogeneity by hydrophobic interaction and ion exchange chromatography. The recombinant Mn-LO had an apparent size of ~90 kDa (range 80-100 kDa) on SDS-PAGE; thus, it appeared to be slightly less glycosylated than the native Mn-LO from *G. graminis var. avenae* [76].

The manganese content of the recombinant protein was 0.98 mol Mn/mol protein and the UV absorbance at 280 nm of 1 mg protein/ml was 1.14 absorbance units.

Catalytical properties

The *K_{m}* and *V_{max}* of recombinant Mn-LO with α-linolenic acid as a substrate in 0.1 M NaBO_3 (pH 9.0; 21°C) was 7.1 μM and 18 nmol/min/μg. The maximal linear increase in UV absorbance in 0.61 ml of 0.1 M sodium borate buffer with 0.05 mM α-linolenic acid and 2.74 μg enzyme was ~0.490 absorbance units/μg at 237 nm (biosynthesis of 12 nmol 13R-HPOTrE min^{-1} μg^{-1}). Corresponding numbers for 0.05 mM linoleic acid was ~0.255 absorbance units/μg, or 6.2 nmol 13R-HPODE min^{-1}.
The maximal turnover number of oxygen, as measured by an O$_2$ electrode [76], suggested formation of 18% 11S-HPODE while the corresponding figure for native Mn-LO was 21% [76]. The recombinant enzyme seemed to have similar catalytical properties as the native enzyme.

A marked kinetic lag phase of 2-3 min was observed upon incubation of recombinant Mn-LO with 50 μM α-linolenic acid. The lag phase decreased with the substrate concentration and it was reduced in the presence of 13R-HPODE. After the lag phase the reaction continued at a linear rate and slowed down with consumption of substrate. These experiments suggested that recombinant Mn-LO was mainly in inactive form (Mn$^{III}$-LO) and could be converted to active form (Mn$^{III}$-LO) by the products formed.

A series of C$_{16}$-C$_{22}$ fatty acids were incubated with Mn-LO. 17:3n-3 and 18:3n-3 proved to be the preferred substrates while other C$_{16}$-C$_{22}$ fatty acids were oxygenated with low efficiency.

Mn-LO could be activated to Mn$^{III}$-LO by preincubation with 13R-HPOTrE (1-10 μM). Interestingly, the active form Mn$^{III}$-LO catalyzed the formation of n-6 hydroperoxides as main products of all the fatty acids tested, except 22:6n-3. The MS/MS analysis could also detect n-8 hydroperoxides in variable amounts from traces (17:3n-3) to 40-50% (22:5n-3 and 22:5n-6). These results suggested that the carbon chain length of the fatty acid and the double bond configuration could play an important role in the oxidation of Mn$^{II}$ to the active form, Mn$^{III}$, by n-6 hydroperoxides.

The thermostability studies performed in paper IV in TrisHCl buffer showed that Mn-LO was almost totally inactivated (>95%) after 5 min at 67°C, 30 min at 65°C, and 90 min at 62°C. Upon incubation of Mn-LO (1 mg) with Chelex-100 resin in sodium bicarbonate/NaCl buffer at 50°C, more than 97% of the manganese could be extracted from Mn-LO, as confirmed by atomic absorption spectroscopy.

Mini-Mn-LO (papers I and IV)

Recombinant purified Mn-LO (10 mg, ~90 kDa), which was stored in the elution buffer of the CM-Sepharose column (with 1 mM NaN$_3$) at 4°C for 6 months, was enzymatically active, but SDS–PAGE analysis showed that Mn-LO had been quantitatively converted to a protein of only ~70 kDa. This protein was designated mini-Mn-LO (Fig.5).

MALDI-TOF analysis showed that the molecular mass of Mn-LO averaged ~91 kDa (range 80–100 kDa), whereas the molecular mass of mini-Mn-LO averaged ~67.4 kDa (range 64–70 kDa).

The broad mass peak of Mn-LO is consistent with extensive glycosylation. SDS–PAGE analysis of the two proteins after N- and O-linked deglycosylation showed that Mn-LO (~90 kDa) was converted to a less glycosylated protein of ~70 kDa. Mini-Mn-LO was converted only to slightly smaller protein of ~63 kDa.
MALDI-TOF analysis of mini-Mn-LO suggested that mini-Mn-LO could be formed by cleavage in the N-terminal sequence before amino acid 66. The hydrolysis of Mn-LO performed under mild acidic conditions (pH 2.6), according to the method of Fraser et al [198], yielded mini-Mn-LO judged from the SDS-PAGE analysis. The transformation is likely due to the cleavage of an acid-labile Asp-Pro bond present in the N-terminal region. This cleavage might also occur during expression when the \( P. \) \textit{pastoris} culture medium becomes acidic.

The catalytic properties of mini-Mn-LO were investigated and the \( K_m \) and \( V_{\text{max}} \) with \( \alpha \)-linolenic acid as a substrate were 6.4 \( \mu \text{M} \) and 12 nmol/min/\( \mu \text{g} \), respectively. This suggested that the \( V_{\text{max}} \) was reduced by one third in comparison with Mn-LO.

Site-directed mutagenesis of putative manganese ligands  
\textit{(paper I)}

The putative metal ligands of Mn-LO were deduced by analogy with iron lipoxygenases [88]. All mutations were performed by site-directed mutagenesis and the mutants were expressed in \( P. \) \textit{pastoris}. A protein band at 90 kDa on the SDS-PAGE gel indicated that the expression succeeded. The mutated proteins were considered active when a significant increase in UV absorbance at 237 nm with 50 \( \mu \text{M} \) \( \alpha \)-linolenic acid was observed and the two products 11S-HPOTrE and 13R-HPOTrE could be identified by LC-MS.

The mutations of three histidine residues of Mn-LO, His-274 and His-278 of \( \alpha \)-helix 9 (helix numbering refers to sLO-1 [199]) in the partial sequence H^{274}VLFH^{278} and His-462 in the partial sequence H^{462}HVMNQG yielded inactive proteins. The mutants His274Gln, His278Glu, and His462Gln contained less than 0.05 mol Mn/mol Mn-LO protein, according to atomic absorption spectroscopy. His-274, His-278, His-462 appeared to be critical for both enzyme activity and manganese binding and could be manganese ligands.
The deletion of the amino acid residue Val-602 in the C-terminal end yielded protein with undetectable enzyme activity and low manganese content (less than 0.05 mol Mn/mol Mn-LO protein). This suggested that the C-terminal amino acid residue Val-602 likely is a metal ligand in analogy with the C-terminal Ile of iron lipoxigenases [23]. The Val602Ile and Val602Ala mutants were found enzymatically active but the activity appeared to be low.

Other mutations in helix 18, His463Gln, Asn466Glu or Leu, and Gln467Asn in the partial sequence HH463VMN466Q467G retained enzyme activity and neither of them seemed to be important for enzyme activity.

Mutations of the conserved residue Gly316 and the hydroperoxide isomerase activity (paper II)

Alignments of lipoxigenase sequences [200, 201] showed that R-lipoxigenases contain a conserved Gly residue in the central part of their primary structure, which is substituted with an alanine, or serine, in S-lipoxigenases. Previous reports [200, 201] associate this active site residue with the control of stereochemistry of lipoxigenases. In order to investigate if the conserved residue has the same function in Mn-LO, three mutants of Gly316 were prepared and expressed in P. pastoris. These were Gly316Ala, Gly316Val and Gly316Thr.

Gly316Ala and linoleic acid – Mn-LO Gly316Ala metabolized 18:2n-6 to 11S-HPDE and 13R-HPODE as the native enzyme, and 13R-HPODE accumulated as the end product. MS/MS analysis revealed that significant but small amounts of 9-HPODE also were formed (signal at m/z 171 (OOC-(CH2)n-CHO)). Relative to 13R-HODE, the native enzyme formed 2-3% 9S-HPODE and the mutant 7-10% 9S-HPODE, as judged by SP-HPLC and chiral phase HPLC analysis. Hydroperoxide isomerase activity with 13R-HPODE as a substrate was insignificant compared to 13R-HPOTrE.

Gly316Ala and α-linolenic acid – Mn-LO Gly316Ala metabolized 18:3n-3 to 11S-HPOTrE and 13R-HPOTrE with a kinetic time lag similar to the recombinant Mn-LO. After a linear increase at 237 nm due to the formation of 13R-HPOTrE, the kinetic curve declined. The UV analysis showed that the decrease in absorbance at 237 nm was accompanied by an increase in absorbance at ~282 nm with an isosbestic point at 252 nm (Fig. 6). LC-MS/MS analysis confirmed that the increase in absorbance at 282 was due to the formation of 13-KOTrE also identified by RP-HPLC with MS/MS analysis. The Gly316Ala mutant transformed 13R-HPOTrE to 13-KOTrE at a linear rate without apparent time lag and with a K_m=10μM.

Experiments under oxygen-18 atmosphere with Mn-LO Gly316Ala and 18:3n-3 demonstrated complete incorporation of oxygen-18 in the hydroxyl group at C-11 and in the epoxide at C12-C13 of threeo and erythro 11-hydroxy-12,13-epoxy-octadecadienoic acid. In conclusion, the Gly316Ala mutant of Mn-LO possessed hydroperoxide isomerase activity.
Figure 6. Time curve at 237 nm for the biosynthesis of 13R-HPOTrE by Mn-LO Gly316Ala. The decline in UV absorbance is due to the formation of 13-KOTrE with maximum absorbance at 282 (the insert show repeated UV spectra recorded every other min. for 4-18 min after the start of the reaction).

SP-HPLC with MS/MS was used to analyze the polar products formed during the hydroperoxide isomerase reaction. 13-KOTrE eluted as the least polar product while 13-HOTrE, 11-HODE and 9-HODE eluted as major peaks along with small amounts of 12-HOTrE and 16-HOTrE. MS/MS analysis (m/z 309 -> full scan) of the polar products suggested that also epoxyalcohols are formed, as described below.

In comparison with Mn-LO Gly316Ala, the hydroperoxide isomerase activity of the native enzyme seemed to be low but detectable. The studies performed in paper II showed that the mutation of Gly316 to Ala increased the hydroperoxide isomerase activity of Mn-LO about 7-15 times with 18:3n-3 and 17:3n-3 as substrates.

Gly316Ala and (n-3) fatty acids– Mn-LO Gly316Ala oxygenated 17:3n-3 slightly less efficiently than 18:3n-3, but the hydroperoxide isomerase activity was increased. The reduced lipoxygenase activity might be explained by an increase of the distance of the n-8 carbon to the catalytic base caused by the mutation. The oxygenation pattern was interesting, since the mutation seemed to change the oxygenation of 17:3n-3 from exclusively at the n-6 carbon towards the n-8 (7%) and n-10 (11%) carbons. The same phenomenon was noticed upon incubation of Mn-LO Gly316Ala with 18:3n-3 when the oxygenation was shifted with an increased formation of 9S-HPOTrE. Although this is in agreement with the shifts in oxygenation reported before with the Gly to Ala mutations in coral 8R-LOX and 12R-LOX [200, 202] the results presented in paper II showed that the conserved residue not only is a determinant of stereospecificity but it can also confer hydroperoxide isomerase activity to the enzyme. The presence of an n-3 double bond in the structure of the fatty acid substrate seemed to be essential for the hydroperoxide isomerase reaction. LC-MS confirmed that 17:3n-3 also was converted to epoxyalcohols and keto compounds.
Mn-LO Gly316Ala was also incubated with the R- and S-hydroperoxides at n-6 of 17:3n-3, 18:3n-3, and 19:3n-3. The R hydroperoxides were transformed to keto compounds at a rate, which was 5 times higher than for the corresponding S-hydroperoxides. This might be due to the fact that the R-hydroperoxides can be positioned closer to the catalytic metal that the hydroperoxides with S-configuration.

Gly316Val and Gly316Thr – In order to increase the changes produced in the active site by the Gly316Ala mutation, the Gly was mutated to bulkier residues such as Val and Thr. Mn-LO Gly316Val and Gly316Thr did not show any lipoxygenase activity in agreement with the corresponding mutations of 12R-lipoxygenases and S-lipoxygenases, which also yielded inactive enzymes [201](Coffa, 2005 #4, 202).

Epoxyalcohols synthesis and analysis by LC-MS (papers II and III)

Epoxyalcohols synthesis by Mn-LO Gly316Ala

Upon incubation of Mn-LO Gly316Ala with 13R-HPOTrE, the oxygen-oxygen bond is likely cleaved homolytically with the formation of an alkoxyl radical. This radical is transformed by Mn-LO Gly316Ala to erythro and threo 11-hydroxy-12R,13R-epoxy-9Z,15Z-octadecadienoic acid in a ratio of 2:3 and 13-KOTrE as major products. In addition to these two trans epoxides, the corresponding erythro and threo isomers of the corresponding cis epoxide also appeared to be present as minor products. The MS/MS analysis could also confirm the presence of an isomer of 9-hydroxy-12S,13R-epoxy-10E,15Z-octadecadienoic acid.

Epoxyalcohols synthesis by anaerobic incubation of Mn-LO

The epoxyalcohols generated by anaerobic incubation of Mn-LO with 13R-HPOTrE and α-linolenic acid differed from the epoxyalcohols formed from linoleic acid. The main product was identified as erythro 11-hydroxy-12R,13R-epoxy-9Z,15Z-octadecadienoic acid. Small amounts of the threo isomer and 9-hydroxy-12S,13R-epoxy-10E,15Z-octadecadienoic acid were also detected by MS/MS analysis.

Incubation of Mn-LO with linoleic acid under anaerobic conditions generated epoxyalcohols mainly derived from 13R-HPODE. The main epoxyalcohols separated by SP-HPLC are erythro and threo 11-hydroxy-11R,12R-epoxy-9Z-octadecenoic acid and one cis epoxy isomer, 11-hydroxy-12S,13R-epoxy-9Z-octadecenoic acid. Epoxyalcohols from 11S-HPODE and 9S-HPODE were formed as minor products.

Epoxyalcohols synthesis by hematin catalysis

The hematin-catalyzed isomerization of the hydroperoxides to epoxyalcohols was performed in order to generate standards and to compare it with the synthesis
catalyzed by Mn-LO Gly316Ala and the anerobic incubation of Mn-LO (paper II).

In paper III, a series of hydroperoxides derived from linoleic, [9,10,12,13-2H₄] linoleic and α-linolenic acids were transformed to epoxyalcohols and keto fatty acids by hematin-catalyzed reactions. NP-HPLC with ESI-MS/MS proved to be a useful method for the separation and the analysis of the products formed.

The MS/MS spectra of 1,2-epoxy-3-hydroxy and 1-hydroxy-2,3-epoxy regioisomeric epoxyalcohols showed two characteristic and identical fragments separated by 30 mass units. Identical MS/MS spectra were noticed for 11-hydroxy-9,10-epoxy- and 9-hydroxy-10,11-epoxy-12Z-octadecenoic acids and also for the pair 11-hydroxy-12,13-epoxy and 13-hydroxy-11,12-epoxy-12Z-octadecenoic acids. The identical spectra could be explained by Payne rearrangement. This phenomenon describes an epoxide migration which is based on the deprotonation of the epoxyalcohol to an alkoxide [203] (Fig. 7) and might occur in the gas-phase of the mass spectrometer.

MS/MS spectra of the allylic epoxyalcohols formed from 9-HPODE and 13-HPODE showed characteristic fragments, as these epoxyalcohols contain the 1,2-epoxy-5-hydroxy-3-pentene elements.

**Lipoxygenase inhibitors effect on Mn-LO and Mn-LO Gly316Ala (paper II)**

BW A4C (100 μM) augmented the initial transformation rate of 13R-HPOTrE to 13-KOTrE several fold. BW A4C then slowed down and blocked the reaction after a few min (cf. [204]). This inhibitory effect was less pronounced when smaller amounts of enzyme were used.

The stimulatory effect of BW A4C on the hydroperoxide isomerase activity was concentration-dependent, with a several fold increase in the initial rate of transformation of 13R-HPOTrE to 13-KOTrE at 30-100 μM BW A4C. The stimulatory effect declined at 300 μM BW A4C. The inhibitory effect of BW A4C on lipoxygenation was smaller than its stimulatory effect on the peroxidase activity, as 100 μM BW A4C only inhibited the lipoxygenase reaction by ~40% in these experiments.

Finally, ETYA (100 μM) [205], reduced the rate of conversion of 13R-HPOTrE to 13-KOTrE by Mn-LO Gly316Ala by 75 %.
11-HPODE synthesis and isomerization by iron- and manganese-dependent lipoxygenases (paper V)

Mn-LO transformed 11R,S-HPODE to 13R-HPODE as the main product. The 11R stereoisomer was apparently also transformed to a cis-trans conjugated chromophore. 9R-HPODE was identified by UV, LC-MS, SP-HPLC and CP-HPLC analysis. CP-HPLC showed that the 9R stereoisomer was the main compound formed.

The methyl ester of 11S-HPODE was partly converted by Mn-LO to cis-trans conjugated chromophores and the main metabolite was identified by CP-HPLC analysis as the 13R-HPODE methyl ester. In addition, small amounts of 13S-HPODE methyl ester (~5% of 13R-HPODE methyl ester) and 9S-HPODE methyl ester (~5% of 13(R)-HPODE) were detected. The methyl ester of 11R-HPODE was not isomerized to methyl 9R-HPODE or to other products to any significant extent. The two isomerizations presented above occurred much less efficiently than the isomerization of 11S-HPODE to 13R-HPODE previously reported [95, 97].

Longer time and larger amounts of enzyme were needed in order to isomerize 11S-HPODE by sLO-1. 13R-HPODE was identified as the main product. Methyl 11S-HPODE was metabolized more efficiently than 11S-HPODE by sLO-1. The two main products were methyl 13R-HPODE (62%) and 9S-HPODE (38%), but there was a relatively large formation of 13S-HPODE (about 30% of 13R-HPODE) and 9R-HPODE (about 30% of 9S).

The rice leaf inducible lipoxygenase transformed linoleic acid to 13S-HPODE in accordance with previous studies [206]. The analysis also showed the formation of a second, less abundant compound, which was identified as 11-HPODE (74% 11R) after UV spectroscopy and GC-MS analysis.

Linoleate diol synthase activity from the rice blast fungus Magnaporthe grisea (paper VI)

Mycelia from two strains of M. grisea could rapidly metabolize linoleic acid by the LDS pathway with the formation of 8-HODE and 7,8-DiHODE, as confirmed by GC- and LC-MS analysis. In addition, 17R-hydroxylinoleic acid, 8S,17R-dihydroxylinoleic acid and 8S,16R-dihydroxylinoleic acid could also be detected.

After analysis of the genome of M. grisea from the Whitehead Institute/MIT Center for Genome Research an LDS-like gene was found in contig 2.1121 and a smaller part in contig 2.2091. Expression of mRNA of LDS of M. grisea by the strain Guy 11 could be detected. The three intron borders were confirmed by RT-PCR and sequencing.

Alignment of the deduced protein sequence with LDS of G. graminis yielded 65% identities, 78% positives and 2% gaps. Important residues such as the tyrosine residue (Tyr 384), which likely forms a tyrosyl radical and abstracts a hydrogen atom during catalysis [142], are conserved in the LDS of M. grisea (Fig. 8).
Figure 8. Alignment of the deduced protein sequences of the linoleate diol synthase (LDS) precursor of \textit{M. grisea} with LDS precursor of \textit{G. graminis}. The conserved putative heme-binding histidines and the tyrosine residue are shown in red.

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DISCUSSION

The fungus *G. graminis* – a known pathogen affecting wheat cultures worldwide, secretes Mn-LO. This enzyme presents similar features with the iron lipoxygenases in what regards the protein sequence and some of the catalytical properties [76, 88]. But the presence of manganese in the active center [76] and the reaction mechanism that involves suprafacial oxygen insertion [95] makes it unique. Another important difference between Mn-LO and iron lipoxygenases is marked by the fact that the latter catalyze the conversion of 1Z,4Z-pentadienes to 1-hydroperoxy-2E,4Z-pentadienes, whereas Mn-LO forms 3-hydroperoxy-1Z,4Z-pentadienes and transforms them to 1-hydroperoxy-2E,4Z-pentadienes [95].

In order to study in detail the structural aspects and the metal ligands, an appropriate system for expression had to be chosen. The bacterial cells represented the first choice but in the case of Mn-LO only inactive enzyme could be obtained. Proper protein folding and post-translational modifications, which could only be offered by a eukaryotic expression system, seemed to be required. The *P. pastoris* expression offers numerous advantages like transcriptional regulation of heterologous proteins by AOX1 promoter and suitable methods for molecular genetic manipulations along with technology for the growth of the expression strains in large high-density fermentor cultures.

The yeast *P. pastoris* has proven to be a robust and efficient system for expression of Mn-LO (*paper I*). Different strains and growing conditions have been tested in order to obtain the best level of expression. The wild-type strain was chosen for expression and a lower temperature for induction than the one recommended of 30°C. Sometimes lower temperatures allow a proper folding of the protein of interest especially for the ones with high degree of glycosylation as Mn-LO. With these conditions the recombinant protein reached almost the same glycosylation level (80-100 kDa) as the native Mn-LO (90-130 kDa) with 12 possible O-glycosylation sites and 2 N-glycosylation sites in the N-terminal region. The presence of a smaller, less glycosylated protein on the SDS-PAGE gel could be due to proteolysis at the N-terminal end with loss of sugar moieties. The new protein, designated mini-Mn-LO, retained the enzyme activity. Thus, the cleavage seemed to take place at the N-terminal end, since the C-terminal Val was discovered as essential for the enzyme activity. A “mini-LOX” was obtained by mild tryptic digestion of sLO-1[207]. Interestingly, the presence of a weak and acid-labile Asp-Pro bond in the N-terminal region of Mn-LO seemed to confirm the fact that mini-Mn-LO might be formed by a cleavage in this region. Indeed, Mn-LO was transformed to mini-Mn-LO under acidic conditions. A complex structure with a high level of glycosylation might be an
important hinder in the crystallization process. Mini-Mn-LO could be the perfect candidate for this kind of studies. The X-ray analysis of the crystals would offer a complete image over the structure of this unique LOX.

EPR analysis [87] and extended X-ray absorption fine structure analysis (data not published) suggested that manganese interacts with three nitrogen and three oxygen atoms. The site-directed mutagenesis results from paper I showed that the histidine residues in positions 274, 278 and 462 might provide the three nitrogens. The coordination by two of the oxygen atoms could be done with the carboxyl group of the C-terminal Val and a water molecule. In search of a third oxygen ligand, mutations were performed at Asn466 and Glu467, which yielded active enzymes, and this was in accordance with similar studies performed on mouse 8-LOX [208].

It is well known that enzymes that belong to the same gene family often have conserved 3D structures. Differences occur though between the primary sequences of Mn-LO and iron lipoxygenases and these are the gap between the two putative manganese ligands His-274 and His-278 and the unique C-terminal pentapeptide.

The mechanism of selectivity of manganese and iron lipoxygenases is still unknown and it might be that these differences in the sequence make the enzyme to choose manganese and not iron in the catalytic centre. Studies on mononuclear non-heme iron(II) enzymes have revealed the presence of a conserved structural motif designated as the 2-His-1-carboxylate facial triad motif [209]. As the name suggests, this motif consists of two His residues and one carboxylate (from Asp, Glu, Ile or Val) that bind to the metal and occupy one face of the coordination spheres. The active metal appear to have still three coordination sites available for exogenous ligands such as O₂ or the substrate. The binding of the substrate likely opens the sixth coordination site for O₂ and primes the active metal for dioxygen attack. Thus, the 2-His-1-carboxylate facial triad might act as a platform for dioxygen activation [210]. The conserved triad motif could also be found in enzymes that are not directly involved in oxygen activation like superoxide dismutases [209] and lipoxygenases [211]. Since both iron and manganese dependent superoxide dismutases seemed to contain the 2-His-1-carboxylate facial triad [209], the metal selectivity might be controlled from a region located far from the catalytic centre. This theory was confirmed by site-directed mutagenesis studies on a cambialistic form of superoxide dismutase from Porphyromonas gingivalis, which showed that mutations in the second coordination sphere could change the catalytic properties towards the manganese form [212].

Recent studies have shown that only one mutation of a conserved residue in the active site of lipoxygenases can affect the regio and stereospecificity of the arachidonic acid oxygenation [200, 201]. The residue is conserved as an Ala in S-lipoxygenases and as a Gly in R-lipoxygenases. The mutation of Gly to Ala in human 12R-LOX [200] and coral 8R-LOX [82, 200] changed the position of the oxygenation of the substrate such that an enzyme with R-stereospecificity could make an S-product. The corresponding residue in Mn-LO is Gly316 and site directed mutagenesis was used in paper II in order to investigate if the change in stereospecificity applies to Mn-LO. The mutation of Gly316 to Ala kept the same profile regarding the
lipoygenase reaction but increased considerably the hydroperoxide isomerase activity of the enzyme having as consequence the formation of epoxylalcohols and other less polar products like keto fatty acids. There are reports of a pseudoperoxidase activity observed in sLO-1 [213, 214] and 5-LOX [215] but only in the presence of a reducing agent. Interestingly, the hydroperoxidase activity of the Mn-LO mutant was not dependent of metal reducing agents. Epoxylcohol synthesis under normal oxygen tension is also catalyzed by eLOX3 [67], an enzyme classified as lipoxygenase but lacking lipoygenase activity. The transformation of hydroperoxy fatty acids to epoxylalcohols might be of biological importance since mutations of eLOX3 have been associated with two disorders of skin keratinisation [216]. The fact that a single point mutation in Mn-LO could generate a reaction cycle (Fig. 9) similar to the self-sufficient cycle of eLOX3 might provide new insights into the mechanism of hydroperoxide isomerases and the catalytic differences between iron and manganese lipoygenases.

![Figure 9. Proposed mechanism for the hydroperoxide isomerase activity of Mn-LO Gly316Ala.](image)

Hydroperoxy fatty acids (LOOH) are oxidizing Mn$^{2+}$ to Mn$^{3+}$OH with the formation of an alkoxyl radical (LO·). Mn-LO transforms LO· to epoxylalcohols, keto fatty acids and water, and the catalytic metal is reduced to Mn$^{2+}$. BW A4C stimulates the reaction by reducing Mn$^{3+}$ to Mn$^{2+}$, whereas ETYA inhibits it.

Regarding the changes in stereospecifity, the Gly316Ala Mn-LO appeared to change the oxygenation from the n-6 carbons of 17:3n-3 and 18:3n-3 towards the n-8 and n-10 carbons. This seemed to confirm the Coffa and Brash determinant theory on R-lipoygenases, although the change observed in Mn-LO was only modest compared to the previous studied enzymes, but confirmed the studies reported by Meruvu and co-workers on murine 12R-LOX [202].

When the Gly in position 316 is mutated to an Ala the volume of the active site of lipoygenases is diminished [94] and also the flexibility of the protein. In this case the structure of the substrate seemed to play an important role during the hydroperoxide isomerase reaction. The n-3 double bond present in the α-linolenic
acid was apparently essential, since Mn-LO Gly316Ala transformed 13R-HPODE to an insignificant extent in comparison with 13R-HPOTrE. The mutation Gly 316Ala might place the R-hydroperoxide group of 17:3n-3, 18:3n-3 and 19:3n-3 closer to the catalytic metal, allowing a homolytic cleavage of the oxygen-oxygen bond. The positional specificity is strongly influenced by the orientation of the substrate and how deep it can enter the catalytic domain of the protein [201]. By mutating Gly to a bulkier Ala, the active site entrance could be reduced. In the case of R-lipoxygenases the substrate appear to have a more superficial position, closer to the entrance exit and the surface of the active enzyme.

Epoxylcohols and keto fatty acids can also be synthesized by hematin treatment of the 8-, 9-, 11- and 13-hydroperoxyoctadecadienoic acids. NP-HPLC with ESI-MS/MS proved to be an efficient method for analyzing these products (paper III). Reverse-phase LC MS/MS analysis of hydroperoxy fatty acids has been reported before [217-221] but there are only few reports on normal phase LC-MS/MS used for analysis of eicosanoids and oxylipins [222]. NP-HPLC with atmospheric pressure chemical ionization or particle beam interface has been used for analysis of vitamins A, D and E, phospholipids and retinoic acid [223-225]. NP-HPLC with ESI-MS/MS has been used to separate and identify $\omega_2$ and $\omega_3$ hydroxy metabolites of long chain polyunsaturated fatty acids [226]. The results presented in paper III illustrate how NP-HPLC can actually achieve a better separation than RP-HPLC. Interestingly, epoxide migration (Payne rearrangement) [203] might occur in the gas phase of the mass spectrometer, since isomeric epoxylcohols with 1,2-epoxy-3-hydroxy and 1-hydroxy-2,3-epoxy elements showed virtually identical spectra. The Payne rearrangement consists in a deprotonation of the epoxyalcohol to an alkoxyde and occurs usually in aqueous media that contains strong bases [203].

The MS/MS spectra of the allylic epoxylcohols derived form 9- and 13-HPODE seemed to have many ions in common. This might be explained by the transformation of the epoxide and hydroxyl groups to aldehydes during collision-induced fragmentation [217-221]. The differences in ion intensities in the spectra made possible the identification of allylic epoxylcohols. The MS/MS ions in the spectra of unsaturated keto fatty acids could be explained by charge migration and keto-enol tautomerism with breaking of C-C bonds. In conclusion, paper III likely represents one of the first systematic studies of epoxylcohols derived from linoleic and $\alpha$-linolenic acids by LC-MS/MS.

The position of the fatty acid substrate in the active site of lipoxygenases can play an important role in the lipoxygenase reaction and the regio- and stereospecificity of these enzymes. Previous studies have established two theories regarding which part of the structure of the fatty acid enters the active site first: the methyl end or the carboxyl end [101]. Oxygenation experiments with different n-3 and n-6 fatty acids presented in paper IV suggested that the methyl end binding theory (“tail first”) might apply to Mn-LO. In this case the negatively charged carboxyl group of the fatty acid substrate likely interacts with positively charged residues located near the entrance of the active site.
The fatty acid substrates align in a relatively narrow channel in the active site of lipoxygenases [83, 199] thus it seems possible that the site can also accommodate the formed hydroperoxy fatty acids. The investigations performed in paper V looked for evidence of the binding of 11S-HPODE, 11R-HPODE and methyl ester derivatives at the active site of Mn-LO and sLO-1. Difficulties in generating 11-HPODE were overcome by the biosynthesis of 11S-HPODE by Mn-LO [95] and the formation of 11(R,S)-HPODE during vitamin E-controlled autoxidation of linoleic acid [194]. As expected, 11S-HPODE was converted by Mn-LO to 13R-HPODE but 9R-HPODE could be also identified. The results suggested that 11R-HPODE was transformed to 9S- and 13S-HPODE. 11S-HPODE and 11R-HPODE might align in opposite orientations at the active site and further studies are needed in order to confirm this possibility.

Mn-LO belongs to the family of fungal dioxygenases together with LDS from G. graminis [141, 145]. A gene with similar organization with LDS was expressed and sequenced from another pathogenic fungus Magnaporthe grisea (paper VI). PBLAST analysis and alignment with LDS of G. graminis yielded 65% identities, 78% positives, and 2% gaps for the deduced LDS sequence of M. grisea. The 65% identity places the LDS from M. grisea in the same family as LDS from G. graminis. The function of the new enzyme is unknown, but it may be involved in sporulation [148, 149] or even in the pathogenic mechanism of M. grisea.

To summarize, Mn-LO could be efficiently expressed in the yeast P. pastoris and this opened the way for the study of the putative metal ligands by site-directed mutagenesis.

The metal ligands of iron and manganese lipoxygenases appear to be homologous but not entirely conserved. The differences observed in the sequence of Mn-LO compared to iron lipoxygenases may represent the source for the unique biochemical properties of Mn-LO. Although Mn-LO appears to be an R-LOX with the conserved Gly in position 316, the studies presented in this thesis have shown that this residue can be more than a determinant of stereospecificity. Apparently, Gly316 controls both the oxygen insertion and the position of the hydroperoxy group of the n-6 hydroperoxy fatty acids with an n-3 double bond to the catalytic metal thus conferring an augmented hydroperoxide isomerase activity to the enzyme.

The investigations presented in this thesis contribute to the structural and biochemical characterization of Mn-LO, the only lipoxygenase that contains manganese. Its function continues to remain a mystery, although the fact that the enzyme is secreted by G. graminis suggests a presumptive role of Mn-LO in the pathogenic mechanism of this fungus. Its mycelia penetrate and devastate the roots of wheat and other grasses and Mn-LO products might provide the oxidative damage to the root cells [76]. Knowing how to control this mechanism might offer new means in the combat against a pathogen that affects wheat cultures worldwide.
CONCLUSIONS

1. Mn-LO could be efficiently expressed in secreted form in the yeast *Pichia pastoris*. The amount of enzyme was 0.5-1 mg/L in shake flasks and up to 30 mg/L in fermentor.

2. Site-directed mutagenesis suggested that four residues, His-274, His-278, His-462 and the C-terminal amino-acid Val-602, were essential for enzyme activity and for manganese binding.

3. The mutation Gly316Ala of Mn-LO possessed dual enzyme activities – oxygenation of α-linolenic acid and 17:3n-3 to R hydroperoxides at the n-6 carbon, and transformation of these hydroperoxides to epoxyalcohols.

4. The hydroperoxide isomerase activity of the mutant Mn-LO Gly316Ala was increased 7-15 times and a reducing lipoxygenase inhibitor (BW A4C) augmented the activity.

5. Normal phase-HPLC-MS/MS was found to be a suitable method for analysis of epoxyalcohols of linoleic and α-linolenic acids, but epoxide migration complicated the analysis of epoxyalcohols with 1,2-epoxy-3-hydroxy and 1-hydroxy-2,3-epoxy elements.

6. The oxygenation of polyunsaturated n-3 and n-6 fatty acids by Mn-LO and its active form MnIII-LO suggested that the substrates entered the active site with their ω-ends ("tail first").

7. Mn-LO (~ 100 kDa) could be converted to mini-Mn-LO (~ 70 kDa) by mild acidic hydrolysis, likely by cleavage of an Asp-Pro peptide bond in the N-terminal region.

8. The previously established mechanism of isomerization of 11S-HPODE by suprafacial migration of O₂ appeared to be valid also for the isomerizations of 11R-HPODE by Mn-LO and 11S-HPODE by sLO-1.

9. Mycelia of *Magnaporthe grisea* oxygenated linoleic acid to 8-HODE and 7,8-DiHODE in analogy with LDS of *Gaeumannomyces graminis*. A homologous gene was identified in the genome of *M. grisea* and cDNA sequencing revealed that the two LDS enzymes could be aligned with 65% amino acid identity.
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REFERENCES

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74. Würzenberger, M., and Grosch, W., Origin of the oxygen in the products of the enzymatic cleavage reaction of linoleic acid to 1-octen-3-ol and 10-oxo-trans-8-decenoic acid in mushrooms (*Psalliota bispora*), *Biochim Biophys Acta* 794 (1984) 18.

75. Würzenberger, M., and Grosch, W., The formation of 1-octen-3-ol from the 10-hydroperoxide isomer of linoleic acid by a hydroperoxide lyase in mushrooms (*Psalliota bispora*), *Biochim Biophys Acta* 794 (1984) 25.


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144. Brodowsky, I. D., and Oliw, E. H., Metabolism of 18:2(n - 6), 18:3(n - 3), 20:4(n - 6) and 20:5(n - 3) by the fungus Gaeumannomyces graminis: identification of metabolites formed by 8-hydroxylation and by w2 and w3 oxygenation, *Biochim Biophys Acta* 1124 (1992) 59-65.


173. Mendgaard, M., and Svendsen, I., Different effects of N-glycosylation on the thermolability of highly homologous bacterial (1,3-1,4)-beta-glucanases secreted from yeast, *Microbiology* 140 (Pt 1) (1994) 159-166.


198. Fraser, K. J., Pulsen, K., and Haber, E., Specific cleavage between variable and constant domains of rabbit antibody light chains by dilute acid hydrolysis, *Biochemistry* 11 (1972) 4974-4977.


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