Lipoxygenase; a game-changing enzyme

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Lipoxygenase; a game-changing enzyme

Ruud Heshof

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“Our greatest weakness lies in giving up. The most certain way to succeed is always to try just one more time.”

Thomas A. Edison
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Introduction
We now have reached an era in which a transition takes place from a petroleum-based economy towards a biobased economy. The foundation of a biobased economy is the conversion of biological waste into valuable chemicals and energy. Biological waste streams, like plant based agricultural waste, contain many sugars that can be used to grow microorganisms. These microorganisms can be developed to cell-factories for the production of all sorts of chemicals and proteins. Plant waste residues also contain polyunsaturated fatty acids (PUFAs), which are very valuable raw materials that can be used by industry for production of biopolymers such as plastics. Since the biobased economy concept is relatively young, new processes have to be developed to make this type of economy viable for industry.

I aim to contribute to the development of novel concepts for a biobased economy by exploring the potential of lipoxygenase (LOX) for the production of valuable chemicals. LOX catalyzes the dioxygenation of PUFAs, thereby rendering them suitable for further degradation reactions into oleochemicals. These oleochemicals can be used as building blocks for the chemical industry. Different oleochemicals can be formed depending on the position of the introduced peroxide group to the PUFA. Large quantities of LOX, however, are needed for this process to become industrially relevant. Therefore we aimed to use Aspergillus niger as an expression host. A. niger is a fungus used by industry to generate organic compound like citric acid, and can be used for production of enzymes. A. niger is also capable to consume the sugar xylose, which is abundant in plant waste material. Therefore the degradation of plant waste material and the LOX enzyme production can in principle be performed in one step, which is an important contribution to the concept of a biobased process.

This thesis is a result of an approach to contribute to a sustainable environment using LOX. To achieve this goal it is important to ascertain the biological role of LOX. This identification assesses the potential that this enzyme can have in industrial processes. These different potentials are described in Chapter 2. Also the current state of heterologous production of LOX using microorganisms is discussed.

Since LOX is abundant in the kingdom of life, a good selection of LOXs is needed for heterologous expression. For this selection of LOXs a bioinformatics script was written as discussed in Chapter 3. We identified a novel class of fungal LOXs that we wanted to exploit for its potential in a biobased economy. Four potential candidates were selected: Pleurotus sapidus LOX, two Aspergillus fumigatus LOXs, and Gaeumannomyces graminis LOX. These LOXs were chosen for their potential in food industry, to investigate the potential pathogenic role in lung tissue, and the potential in plastic production respectively.
To select the desired transformants of A. niger expressing different lox genes high-throughput assays are needed. In Chapter 4 several plate assays are discussed, which could serve as valuable assays in selecting wanted transformants. Using this screening method, hundreds of clones can be screened simultaneously for LOX expression and can be applied to different expression hosts. Since the results of the described potassium iodide assay were negative for A. niger, we decided to switch to Aspergillus nidulans as an expression host. In this thesis we show these plate assays are effective using expression hosts such as Escherichia coli, A. nidulans, P. pastoris, and Trichoderma reesei. 

Chapter 5 describes the results using A. nidulans as an expression host for heterologous production of LOX. Unfortunately, A. nidulans did not give the results we hoped for. Therefore we investigated the cause for the ineffective use of A. nidulans in producing LOX at various levels. We analyzed the LOX production on mRNA level, we used Western blotting techniques to identify small quantities of LOX, and eventually used a proteomics approach to verify the presence of LOX. We concluded A. nidulans produces proteases able to degrade the LOX. Thus making A. nidulans unsuitable for the production of LOX in high quantities.

Finally, in Chapter 6 we reflect on the steps taken during this thesis and analyze what would be a better approach to produce LOX in high quantities. We discuss the use of LOX in a biobased economy in a broader perspective, e.g. the origin of the needed PUFA for LOX. Also it is discussed how enzyme engineering can help in making LOX more specific or even broaden its use in the production of oleochemicals.

This thesis research is combined with an ERA-IB (European Research Area in Industrial Biotechnology) collaboration of six partners in Europe. The project was called “Novel enzyme tools for production of functional oleochemicals from unsaturated lipids” (ERA-NOEL). The project partners worked in two areas: one on the biochemical aspects and one on the oleochemical and downstream processing aspects. AB Enzymes in Germany, VTT in Finland, and Wageningen University in the Netherlands were the partners responsible for heterologous expression of LOX by using different fungal expression hosts. Where AB Enzymes worked with T. reesei and VTT worked with P. pastoris, Wageningen University was responsible to investigate the potential of A. niger in heterologous expression of LOX. Aarhus University in Denmark and the University of Aveiro in Portugal were responsible for the chemical procedures in making oleochemicals suited for industry with use of LOX. Finally, Fraunhofer Institute in Germany was responsible for the chemical upscaling and downstream processing. After three years of collaboration we were able to manufacture chunks of plastic that...
can be used to produce plastic products. This illustrates the basic concept of the project: that using fatty acids from plant waste material plastics can be produced by use of LOX as is shown in Fig. 1.

Figure 1: Plastics produced by from the reaction products of linoleic acid and LOX. The chunks had a size varying from 5 cm to 10 cm and can be used as start material to be moulded into plastic products. a) Product of dodecanedioic acid with a diamine and b) dodecanedioic acid with PEG 1000. The pictures are from the final report of the ERA-NOEL project. Pictures are courtesy of Prof. J. Buchert, final report ERA-NOEL project 053.80.702
Chapter 2

This chapter was adapted from:

Industrial potential of lipoxygenases
Abstract

Lipoxygenases (LOXs) are iron- or manganese-containing oxidative enzymes found in plants, animals, bacteria, and fungi. Lipoxygenases catalyze the oxidation of polyunsaturated fatty acids to the corresponding highly reactive hydroperoxides. Production of hydroperoxides by LOX can be exploited in different applications such as in bleaching of coloured components, in modification of lipids originating from different raw materials, in production of lipid derived chemicals, and in production of aroma compounds. Most application research has been carried out using soybean LOX, but currently also the use of microbial LOXs has been reported. Development of LOX compositions with high activities by e.g. heterologous expression in suitable production hosts would enable full exploitation of the potential of LOX derived reactions in different applications. Here we review the biological role of LOXs, their heterologous production, as well as potential uses in different applications. Lipoxygenases may fulfil an important role in the design of processes that are far more environmental friendly than currently used chemical reactions. Difficulties in screening for the optimal enzymes and in producing LOX enzymes in sufficient amounts prevent large-scale application so far. With this review we summarize the current knowledge of LOX enzymes and the way in which they can be produced and applied.

1. Lipoxygenase and its biological role

Lipoxygenases (LOX) are found in a wide variety of organisms throughout the kingdoms of Plant, Animal, Bacteria, and Fungi (Nemchenko et al. 2006, Gilbert et al. 2011, Oliw 2002, Hansen et al. 2013). LOXs are iron- or manganese-containing dioxygenases that catalyze the conversion of unsaturated fatty acids (UFA) into the corresponding hydroperoxides called oxylipins (Mita et al. 2007). These oxylipins have important signalling functions in many organisms, though the language of oxylipins is not fully understood. Fungal LOXs are involved in interaction between plants and fungi by using the LOX signalling pathway to infect plant tissue (Oliw 2002, Tsitsigiannis et al. 2005). Oxylipins are also reported to have a role in antimicrobial or defence mechanisms in plants and animals, in cell division in animals, and to play a role in seed germination in plants (Prost et al. 2005, Peters-Golden 1998, Feussner et al. 2001, Pidgeon et al. 2007). The phylogenetic relation of LOXs has been investigated using bioinformatics as shown in Fig. 1 (Heshof et al. 2014a). Based on this analysis it was found LOXs can be divided into two groups: plant LOXs vs. other LOXs. LOXs can be subdivided into classes based on the C-atom they oxygenate in the UFA. For example, a 9-LOX generates the hydroperoxide group to C-9 position of
the substrate UFA. The main difference between LOXs of plant and animal origin is their specificity. Plant LOXs consist of 9- and 13-LOXs and mainly use linoleic acid as a substrate, while animal LOXs consist of 5-, 8-, 12-, and 15-LOXs and use arachidonic acid as a substrate (Brash 1999, Tsitsigiannis et al. 2007). Compared to plant and animal LOXs, LOXs from microbial origin have gained less attention. However, some LOXs from microbial origin are identified as 10- and 11-LOXs using the substrates oleic and linoleic acid respectively (Vidal-Mas et al. 2005, Su & Oliw 1998). LOXs can produce enantiomeric specific oxylipins and the substrate can enter the binding-pocket in head-first or tail-first position (Coffa et al. 2005). Therefore thirty-two different oxylipins can be generated (Coffa et al. 2004). A schematic overview of the structures of the oxylipins produced by LOXs is shown in Fig. 2.

Figure 1: A schematic presentation of the phylogenetic relationship of LOXs throughout the kingdom of life. Planta LOXs are coloured in light green and divided into subclasses: Marchantiophyta, Angiosperms, Lycodiophyta, and Bryophyta. In yellow Animalia LOXs, in blue Bacteria LOXs, in dark green a Chromalveolata LOX, and in red Fungi LOXs. This figure is reproduced with permission of the publisher from the original paper (Heshof et al. 2014a).
**Figure 2:** Overview of different oxylipins that are produced by LOXs from various origins. *Planta* use linoleic acid for 9- and 13-LOXs, *Animalia* use arachidonic acid for 5-, 8-, 12-, and 15-LOXs. In addition to these LOXs the *Fungi* kingdom revealed a so far unique 11/13-LOX, and in the *Bacteria* kingdom a 10-LOX was identified using oleic acid as a substrate.
1.1 Lipoxygenase in Plant

In Plant two types of LOXs are found. These LOXs are called type 1 and type 2 LOXs. Type 1 LOXs consist of cytosolic 9- and 13-LOXs and these LOXs share high similarity with each other. Type 2 LOXs only consist of 13-LOXs and have a putative chloroplast targeting sequence (Nemchenko et al. 2006). Plant LOXs have a function in seed maturation by converting storage lipids to acetyl-CoA to be used as carbon feedstock by the seed. LOXs also have a role in defence mechanisms due to the production of jasmonic acid, which in turn functions as a signal molecule in wound healing and programmed cell death (Howe et al. 2008, Mosblech et al. 2009, Reinbothe et al. 2009). Initial steps in jasmonic acid production are caused by LOX as the hydroperoxide fatty acids produced by 13-LOX are further converted to 12-oxo phytodienoic acid (OPDA) and finally reduced via β-oxidation to jasmonic acid (Mosblech et al. 2009). An antimicrobial defence mechanism is induced via hydroperoxides produced by cytosolic 9-LOXs (Blee 2002, Hughes et al. 2009, Vellosillo et al. 2013). For example, in Nicotiana tabacum down-regulation of 9-LOX causes infection by Phytophthora parasitica while over expressing this 9-LOX results in higher resistance against the pathogen (Rance et al. 1998, Mene-Saffrane et al. 2003). The susceptibility of microorganisms towards different oxylipins was investigated by Prost et al. (Prost et al. 2005).

1.2 Lipoxygenase in Animalia

1.3 Lipoxygenase in Bacteria

LOXs are found in Bacteria although they are not very abundant. So far LOXs have been found in Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Cyanothecae* sp., *Anabaena* sp., and *Myxococcus xanthus* (Vidal-Mas et al. 2005, Andreou et al. 2010, Hansen et al. 2013, Zheng et al. 2008, Goldman et al. 2006). The only Gram-positive bacteria known so far to have LOXs are *Streptomyces canosus* and *Streptomyces massasporus* (Hansen et al. 2013). A secreted LOX from *P. aeruginosa* has been successfully expressed in *Escherichia coli* and its structure has been solved (Vidal-Mas et al. 2005, Garetta et al. 2013). This LOX is known to function as a 10-LOX with oleic acid as a substrate, but also as a 13-LOX with linoleic acid, and a 15-LOX with arachidonic acid as a substrate (Vance et al. 2004, Vidal-Mas et al. 2005, Garetta et al. 2013).

1.4 Lipoxygenase in Fungi

In Fungi genes encoding extracellular LOXs have been found in *Gaeumannomyces graminis*, *Aspergillus fumigatus*, *Magnaporthe salvinii*, *Magnaporthe grisea*, and *Botryotinia fuckeliana* (Hörnsten et al. 2002, Nierman et al. 2005, Wennman et al. 2013, Dean et al. 2005, Amselem et al. 2011). Although their function is still speculative, it is hypothesized that fungal LOXs can interfere with plant signalling cascades (Rance et al. 1998, Mene-Saffrane et al. 2003, Gao et al. 2008). For example, the pathogenic fungus *G. graminis* secretes a manganese-containing 11/13-LOX, which is hypothesized to interfere with the host-cell signalling cascade upon infection (Su et al. 1998, Oliw 2002). Also, oxylipins produced in *Aspergillus flavus* are suggested to act as quorum sensing signals (Tsitsigiannis et al. 2007, Horowitz Brown et al. 2008).

2. The structure of lipoxygenases

The 3D structures of LOXs originating from *Glycine max*, *O. cuniculus*, *P. homomalla*, *H. sapiens*, *S. scrofa*, *Gersemia fructicosa*, and *P. aeruginosa* have been determined (Minor et al. 1996, Skrzypczak-Jankun et al. 1997, Gillmor et al. 1997, Oldham et al. 2005, Gilbert et al. 2011, Xu et al. 2012, Eek et al. 2012, Garetta et al. 2013). These LOXs generally consist of two domains: an antiparallel N-terminal β-barrel domain and a helical catalytic domain, shown in Fig. 3 (Kuhn et al. 2005, Ivanov et al. 2010). The N-terminal β-barrel of LOX shows homology with pancreatic lipase bound to thylakoid membranes (Ivanov et al. 2010, Chahinian et al. 2000, Emek et al. 2013). The role of the β-barrel in membrane binding and its role in catalytic properties of the
enzyme has been elucidated by its deletion from LOXs originating from *H. sapiens, Mus musculus, Macaca mulatta*, and *Pongo pygmaeus*. Although the N-terminal β-barrel was not necessary for membrane binding, binding capacity to membranes of mutants was lower. The mutant LOXs showed decreased activity as compared to wild type LOXs, whereas product specificity was not changed (Walther et al. 2011). LOXs devoid of N-terminal β-barrel have also been identified in *e.g.* *P. aeruginosa* and *Anabaena* sp. (Garetta et al. 2013, Zheng et al. 2008). These LOXs are called mini-LOXs due to their relatively low molecular weight (Zheng et al. 2008). Instead of an N-terminal β-barrel the *P. aeruginosa* LOX contains an extension of about 100 amino acid residues that forms a flexible lid covering the entrance to the binding pocket (Garetta et al. 2013). Another example is the *Anabaena* sp. LOX that is fused to a catalase-like hemoprotein (Zheng et al. 2008). Deletion of this catalase-like hemoprotein did not affect the LOX activity when expressed in *E. coli*.

**Figure 3:** 3D-models of the (A) *Glycine max* (PDB 3PWZ) and (B) *Pseudomonas aeruginosa* (PDB 4G33) LOX. The *G. max* LOX has a membrane binding β-barrel, whereas the *P. aeruginosa* LOX has a removable lid covering the entrance over the binding pocket.

The quaternary structure of the catalytic domain of LOXs consists mainly of α-helices in which the catalytic centre and the substrate-binding pocket are located (Ivanov et al. 2010). The proposed reaction mechanism of LOX is schematically drawn in Fig. 4 (Zoia et al. 2011). The cofactor is activated from Fe$^{2+}$ to Fe$^{3+}$ using its product or atmospheric O$_2$ (Gardner et al. 1991). The Fe$^{3+}$ cofactor binds to the PUFA (1), abstracts a hydrogen, and changes to Fe$^{2+}$, which results in a radical formation on the PUFA (2). The radical rearranges with a double bond (3), and oxygen can be inserted (4). *Via* peroxy radical reduction (5) the hydroperoxy fatty acid product is formed. This results in active Fe$^{3+}$, which creates a chain reaction effect. The cofactor can be inactivated to its Fe$^{2+}$ state by free radical decomposition that occurs when oxygen becomes the rate-limiting step, which was investigated by Zoia *et al.* (Zoia et al. 2011). They
determined the amount of radical products by performing the LOX reaction with and without oxygen. Their findings show that a lower oxygen concentration leads to an increase of free radicals formed in the reaction solution.

**Figure 4:** Schematic overview of the proposed reaction mechanism of LOX (Zoia et al. 2011). The reaction consists of active Fe^3+ that binds to the PUFA (1), hydrogen abstraction (2) radical rearrangement (3), insertion of oxygen (4), and peroxy radical reduction (5). The reaction is stopped when oxygen or substrate becomes limiting. The cofactor will then turn to its resting state Fe^{2+} via free radical decomposition.

The cofactor in the catalytic domain is held in place by a water molecule and five amino acids; three histidines, a fourth serine, histidine or asparagine, and a fifth C-terminal amino acid as shown in Fig. 5 (Brash 1999, Skrzypczak-Jankun et al. 1997). The cofactor is usually iron, but the secreted LOXs from the fungi *G. graminis*, *M. salvinii*, and *A. fumigatus* have manganese as a cofactor (Cristea et al. 2005, Wemnan et al. 2012, Heshof et al. 2014a). The substrate-binding pocket is located near the cofactor-binding site (Minor et al. 1996). Site-directed mutagenesis of the binding pocket revealed that conserved amino acids alanine or glycine in the so-called Coffa-Brash site are involved in product specificity. An alanine in Coffa-Brash site will result in an S-enantiomer oxylipin while a glycinecine will result in an R-enantiomer oxylipin (Coffa et al. 2005). Exceptions of this rule, however, are known in e.g. the *Danio rerio* LOX (Jansen et al. 2011). Sloane *et al.* identified amino acids that are involved in the product specificity of human 15-LOX. A M418V mutation resulted in a conversion of 15-LOX to a 12/15-LOX with an equal ratio of oxylipin-products (Sloane et al. 1991). By mutating the neighbouring amino acids Q416K and I417A the 15-LOX was converted to a 12-LOX with a ratio of 15:1. Borngräber *et al.* discovered that a phenylalanine at position 353 in the rabbit 15-LOX forces the substrate to bend in the substrate-binding pocket. By introduction of a F353L mutation the substrate is able to get deeper into the binding-pocket, resulting in a 12-LOX (Börngraber et al. 1996).
Figure 5: 3D-model of the catalytic site of the *Glycine max* LOX (PDB 1IK3) in complex with 13(S)-hydroperoxy-9(Z),11(E)-octadecadienoic acid (13(S)-HPOD). The iron cofactor is held in place by three histidines, an asparagine, an isoleucine, and a water molecule. The substrate linoleic acid substitutes the water molecule and binds to the iron cofactor. When this complex has been formed oxygen will attach to the substrate generating 13(S)-HPOD.

3. Expression and production of LOX in microbial hosts

So far there are only a few reports of the successful heterologous production of LOXs in microbial hosts. Several attempts have been made to express LOXs from a eukaryotic origin in prokaryotic hosts such as *E. coli*. As extracellular fungal LOXs are glycosylated, the prokaryotic expression system is not the preferred one as only few bacteria can glycosylate proteins (Nothaft et al. 2010). However, plant LOX-2 and LOX-3 from *Pisum sativum*, and human reticulocyte-type 15-LOX have been successfully expressed in *E. coli* (Hughes et al. 1998, Walther et al. 2002). The extensively glycosylated *G. graminis* LOX could not be expressed in active form in *E. coli*, whereas its expression was successful using *Pichia pastoris* as an expression host with yields of 30 mg/l (Cristea et al. 2005). Knust and Wettstein tried the extracellular production of pea-seed LOXs in *Saccharomyces cerevisiae* by using the yeast invertase secretion signal. Despite the addition of a secretion signal, most of the enzyme remained inside the cell in an inactive form (Knut & Wettstein 1992). Several bacterial LOXs
have been successfully expressed in prokaryotes. As claimed by Lu et al. the highest level of expressed LOX so far is 3.89 U/ml and has been achieved by expressing _P. aeruginosa_ LOX in _E. coli_ (Lu et al. 2013). Most heterologous production of LOXs in _E. coli_ is performed at temperatures ranging from 8°C – 20°C. This low temperature can cause additional production costs in up-scaling the production process due to the cooling energy that is required for fermenters during growth of the expression host. An overview of successful heterologous expression of LOXs is given in Table 1. The limited number of examples present in scientific and patent literature is indicative that the expression of LOX of any origin in microbial hosts is not a trivial matter. The success of expression of LOX can probably be increased by using a host that is genetically closely related to the organism that produces the enzyme. Also the biological role of the LOX produced oxylipins as precursors for signal molecules, e.g. inducing cell death or cell division, can be anticipated to interfere with the expression system used (Heshof et al. 2014b). Extracellular production of LOX would thus be beneficial and this can be achieved by selecting a naturally extracellular LOX or, alternatively incorporating a secretion signal to the LOX protein. Recent developments in synthetic biology will further contribute to improved expression levels. The use of BioBricks allows rapid screening of codon-optimized constructs that carry promoter or secretion signal variants (Shetty et al. 2008). Mutagenesis of the LOX of interest can be a useful strategy in production of specific oxylipins. This can be achieved by changing amino acids affecting the substrate specificity (Sloane et al. 1991). Alternatively the catalytic activity of the molecule can be altered by removing the N-terminal β-barrel, either by tryptic digestion or genetic modification. Resulting mini-LOXs are catalytically complete, but seem to be more flexible and less stable (Di Venere et al. 2003, Zheng et al. 2008). Although the N-terminal β-barrel is considered to be non-essential for enzymatic activity, it may have other functions, e.g. in membrane binding or calcium binding (Walther et al. 2002). In all cases, successful production of industrial quantities of this enzyme class will require more fundamental research and also needs improvements in bioprocess engineering.
Table 1: Overview of heterologous expressed LOXs in different expression systems. The LOXs can be classified as 5-, 8-, 9-, 10-, 11-, 12-, 13-, or 15-LOXs. The number indicates the attachment of the peroxide group to the fatty acid. The ValLOX represents a LOX that uses valencene as a substrate.

<table>
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<tr>
<th>LOX-type</th>
<th>LOX host organism</th>
<th>Expression organism</th>
<th>Yield</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-LOX</td>
<td>Homo sapiens</td>
<td>Saccharomyces cerevisiae</td>
<td>50 μg/l</td>
<td>Nakamura et al. 1990</td>
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<tr>
<td>9-LOX</td>
<td>Oryza sativa</td>
<td>Escherichia coli</td>
<td>3% of total soluble protein</td>
<td>Schirano et al. 1990</td>
</tr>
<tr>
<td>13-LOX</td>
<td>Glycine max</td>
<td>Escherichia coli</td>
<td>22-30 mg/l</td>
<td>Steczko et al. 1991</td>
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<tr>
<td>9/13-LOX</td>
<td>Pisum sativum</td>
<td>Saccharomyces cerevisiae</td>
<td>100-200 U/μl</td>
<td>Knust et al. 1992</td>
</tr>
<tr>
<td>13-LOX</td>
<td>Lens culinaris</td>
<td>Escherichia coli</td>
<td>N/A</td>
<td>Hilbers et al. 1996</td>
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<td>Escherichia coli</td>
<td>N/A</td>
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<tr>
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<td>Escherichia coli</td>
<td>N/A</td>
<td>Hughes et al. 1998</td>
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<tr>
<td>9-LOX</td>
<td>Magnaporthe salvinii</td>
<td>Aspergillus oryzae</td>
<td>N/A</td>
<td>Sugio et al. 2002</td>
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<td>15-LOX</td>
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<td>Escherichia coli</td>
<td>0.33 mg/l</td>
<td>Walther et al. 2002</td>
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<tr>
<td>10/13-LOX</td>
<td>Pseudomonas aeruginosa</td>
<td>Escherichia coli</td>
<td>N/A</td>
<td>Vidal-Mas et al. 2005</td>
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<td>Pichia pastoris</td>
<td>30 mg/l</td>
<td>Cristea et al. 2005</td>
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<td>Escherichia coli</td>
<td>N/A</td>
<td>Koeduka et al. 2007</td>
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<td>85 mg/l</td>
<td>Zheng et al. 2008</td>
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<td>Aspergillus oryzae</td>
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<td>Christensen et al. 2008</td>
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<td>9/13-LOX</td>
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<td>Huang &amp; Schwab 2011</td>
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<td>11/13-LOX</td>
<td>Gaeumannomyces graminis</td>
<td>Trichoderma reesei</td>
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<td>9-LOX</td>
<td>Anabaena PCC7120</td>
<td>Bacillus subtilis</td>
<td>171.9 μg/ml</td>
<td>Zhang et al. 2012</td>
</tr>
<tr>
<td>ValLOX</td>
<td>Pleurotus sapidus</td>
<td>Escherichia coli</td>
<td>60 mg/l</td>
<td>Zelena et al. 2012</td>
</tr>
<tr>
<td>10/13-LOX</td>
<td>Pseudomonas aeruginosa</td>
<td>Escherichia coli</td>
<td>3.89 U/ml</td>
<td>Lu et al. 2013</td>
</tr>
<tr>
<td>9-LOX</td>
<td>Magnaporthe salvinii</td>
<td>Pichia pastoris</td>
<td>N/A</td>
<td>Wennman et al. 2013</td>
</tr>
<tr>
<td>13-LOX</td>
<td>Fusarium oxysporum</td>
<td>Escherichia coli</td>
<td>30 mg/l</td>
<td>Brodhun et al. 2013</td>
</tr>
<tr>
<td>13-LOX</td>
<td>Aspergillus fumigatus</td>
<td>Pichia pastoris</td>
<td>1 mg/l</td>
<td>Heshof et al. 2014a</td>
</tr>
<tr>
<td>ValLOX</td>
<td>Pleurotus sapidus</td>
<td>Pichia pastoris</td>
<td>100-150 mg/l</td>
<td>Kelle et al. 2014</td>
</tr>
</tbody>
</table>
4. Potential applications of LOXs

The primary reaction of LOX, e.g. production of hydroperoxides, can be exploited in different industrial applications ranging from food processing to oleochemical production as summarized in Table 2. Fatty acid derived hydroperoxides produced by LOXs can be converted to a wide variety of oleochemicals and material precursors by different chemical reactions. On the other hand, co-oxidation reactions caused by the hydroperoxide can be exploited in different bleaching applications in food, pulping, and textile applications. Formation of hydroperoxides by intrinsic LOXs present in food raw materials, e.g. in cereals or legumes, and its subsequent reactions can, however, be harmful to the final product quality due to off-flavour formation as reviewed by Robinson et al. (Robinson et al. 1995). Although the potential of LOXs has been widely discussed in different scientific papers, the only commercial applications so far is the use of LOX enriched soybean flour in baking for bleaching of wheat flour (de Roos et al. 2006). Up to now most of the application research has also been carried out using soybean LOX, although recently the use of microbial LOXs in different applications have been reported. Development of LOX compositions with high activities, by e.g. heterologous expression in suitable production hosts, would enable full exploitation of the potential of LOX derived reactions in different applications.
<table>
<thead>
<tr>
<th>Application</th>
<th>LOX source</th>
<th>Key reaction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavour production</td>
<td><em>Glycine max</em></td>
<td>Cleavage of LOX produced hydroperoxide to e.g. hexanal by hydroperoxide lyase</td>
<td>Muller et al. 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Furusawa et al. 2005</td>
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<td></td>
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<td>Gigot et al. 2010</td>
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<td></td>
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<td>Noordermeer et al. 2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Márczy et al. 2002</td>
</tr>
<tr>
<td>Flour bleaching in bread or noodle</td>
<td><em>Glycine max</em></td>
<td>Co-oxidation of carotenoids by LOX produced hydroperoxide</td>
<td>Faubion &amp; Hoseney 1981</td>
</tr>
<tr>
<td>manufacture</td>
<td></td>
<td></td>
<td>Cato &amp; Halmos 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Frazier et al. 1977</td>
</tr>
<tr>
<td>Annatto decolourization in waste</td>
<td><em>Glycine max</em></td>
<td>Co-oxidation of annatto colour by LOX produced hydroperoxide</td>
<td>de Roos et al. 2006</td>
</tr>
<tr>
<td>whey</td>
<td></td>
<td></td>
<td>Kang et al. 2010</td>
</tr>
<tr>
<td>Dough structure improvement by</td>
<td><em>Glycine max</em></td>
<td>Oxidation of SH-groups by LOX produced hydroperoxide</td>
<td>Faubion &amp; Hoseney 1981</td>
</tr>
<tr>
<td>protein crosslinking</td>
<td></td>
<td></td>
<td>Frazier et al. 1977</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hoseney et al. 1980</td>
</tr>
<tr>
<td>Production of oleochemicals</td>
<td>*Gaeumannomyces</td>
<td>Hydroperoxide production and its further chemical reactions</td>
<td>Villaverde et al., 2013a</td>
</tr>
<tr>
<td></td>
<td>*graminis, Glycine</td>
<td></td>
<td>Villaverde et al. 2013b</td>
</tr>
<tr>
<td></td>
<td>*max, Pseudomonas</td>
<td></td>
<td>Hayes 2004</td>
</tr>
<tr>
<td></td>
<td><em>aeruginosa</em></td>
<td></td>
<td>Maurer &amp; Schmid 2005</td>
</tr>
<tr>
<td>Removal of wood extractives in</td>
<td>*Gaeumannomyces</td>
<td>Oxidative degradation of extractives</td>
<td>Zhang et al. 2007</td>
</tr>
<tr>
<td>mechanical pulping</td>
<td>*graminis, Glycine</td>
<td></td>
<td>Marques et al. 2011</td>
</tr>
<tr>
<td></td>
<td><em>max</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Textile bleaching during manufacture</td>
<td>*Magnaporthe</td>
<td>Co-oxidation of coloured components</td>
<td>Salmon et al. 2004</td>
</tr>
<tr>
<td>or washing</td>
<td><em>salvinii</em></td>
<td></td>
<td>Adriaanse et al. 2000</td>
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<td></td>
<td></td>
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<td>Sugio &amp; Takagi 2002</td>
</tr>
</tbody>
</table>
4.1. LOX as tool in oleochemistry

Biomass derived lipids are potential renewable raw materials for the chemical industry (Metzger et al. 2006). Lipid derived hydroperoxides produced by LOX can be converted to a wide variety of functional oleochemicals precursors by three main conversion routes: acid catalyzed decomposition, free radical decomposition, and base catalyzed decomposition as schematically illustrated in Fig. 5 (Gardner 1984, Gardner 1989, Frankel 1989, Blanksby et al. 2002, Frankel et al. 2005). LOX-catalyzed production of hydroperoxides can be regarded more sustainable than chemical technologies using e.g. ozone oxidation (Nishikawa et al. 1995, Reynolds et al. 2006). In contrast to enzymatic oxidation, chemical oxidation is non-specific. Therefore specific regio-isomers can be obtained in a low yield. In addition, the chemical reaction is difficult to control. Most reactions yield degradation products like the radicals formed in a Fenton’s reaction (Villaverde et al. 2011, Villaverde et al. 2012). *G. graminis* LOX has been shown to have a high specificity for generation of 13-hydroperoxy-(9Z,11E)-octodecadienoic acid when linoleic was used as substrate (Su & Oliw 1998). This result was recently confirmed by Villaverde *et al.* (Villaverde et al. 2013a). They obtained high yields of hydroperoxides (88.0%) at low linoleic acid concentrations and a selectivity for 13-(Z,E)-isomer of around 74%. Furthermore, cloned *Pseudomonas aeruginosa* LOXs also demonstrated, at laboratory scale, to efficiently convert linoleic acid into hydroperoxides with yields higher than 75% and selectivity for the 13-(Z,E)-isomer up to around 70% (Villaverde et al. 2013b).
Figure 6: Schematic overview of the chemical conversion of unsaturated fatty acids by acid and base and enzymatic conversion by radical degradation. The chain length of the degradation products depends on the position of the peroxide group on the fatty acid. By using specific LOXs and/or fatty acids various sizes of polymers can be generated.

Production of hydroperoxides by LOX has been investigated on laboratory scale using different process conditions. The process efficiency is hampered by low solubility of the lipid and oxygen substrates in water. The use of detergents or organic solvents that enable a higher accessibility of the fatty acid substrate to the enzyme, using pressurized oxygen to improve solubility of O₂ or using immobilized enzymes, have been studied as reviewed by Hayes, and Maurer and Schmid (Hayes 2004, Maurer & Schmid 2005). Studies on the production of hydroperoxides by *G. graminis* LOX using industrially feasible linoleic acid concentrations (100-300 g/l) concluded that normal aeration can be used as oxygen source thus lowering the costs of the process (Villaverde et al. 2013a). Fatty acid hydroperoxides can be used as intermediates for the preparation different oleochemicals, e.g. mid-chain hydroxy fatty acids, epoxides, alcohols, carbonylic compounds, and diacids all being monomers for polymer production (Kharasch et al. 1950, Hamberg & Gotthammar 1973, Gardner 1989, Frankel 2005). Fatty acid hydroperoxides are also involved in the mechanism of oils polymerization in painting formulations (van Gorkum & Bouwman 2005).
4.2. Use of LOX in food applications

The use of LOXs in food applications has been reviewed by Casey and Hughes (Casey & Hughes 2004). Depending on the targeted food product, LOX-catalysed lipid oxidation can be regarded as harmful or beneficial. The intrinsic LOX present in e.g. cereals may cause formation of off-flavours in the final product such as in beer (Robinson et al. 1995, Hirota et al. 2006). Thus, significant amount of research has been carried out to develop methods for LOX inhibition during food processing (Baysal & Demirdöven 2007). Another application is the production of nootkatone from valencene using soybean LOX (Muller et al. 1998). Nootkatone is a grapefruit flavour that can be used in industry for food applications and cosmetics (Furusawa et al. 2005). To date no known commercial application using LOX in the production of nootkatone is known. The main commercial LOX application so far is co-oxidation of carotenoid pigments in cereal flour by LOX enriched soya flour. As a result a bleaching effect is obtained. LOX can also co-oxidize SH-groups in proteins resulting in crosslinking or aggregate formation, which in turn affects the structure of the product (Permyakova & Trufanov 2011). The role of LOXs in bread and pasta quality has been addressed in several studies (Frazier et al. 1977, Hoseney et al. 1980, Faubion & Hoseney 1981, Cato et al. 2006, Junguera et al. 2007). The bleaching effect caused by hydroperoxides can also be exploited in dairy products, e.g. cheese, milk, butter oil, cream, and whey products (de Roos et al. 2006). The use of LOX to bleach annatto, a carotenoid colourant used in the manufacture of certain cheeses, has also been reported as whey decolourisation treatment instead of chemical bleaching agents (Kang et al. 2010). LOX catalysed bleaching in food processing is expected to be more positively perceived by the consumers as compared to chemical bleaching.

LOX and hydroperoxide lyase catalysed fatty acid conversion in vivo is responsible of the aroma of e.g. freshly cut grass, cucumbers, or green apples (Gardner et al. 1991, Gigot et al. 2010). Traditionally these natural green notes have been produced by extraction from plants, but currently more efficient means for their production are investigated. Production of hexanal and hexenal from vegetable oils by LOX in combination with hydroperoxide lyase reaction has been reported by many papers (Cass et al. 2000, Noordermeer et al. 2002, Santano et al. 2002, Omar et al. 2003, Gigot et al. 2010). A two-step process with soybean LOX and spinach leaf hydroperoxide lyase was reported resulting in hexanal production with a yield of 54% (et al. 2002). The LOX catalysed production of the aroma compound β-ionone from β-carotene
has also been reported (Waché et al. 2006). Limitation of the processes using plant homogenates as source of LOX and hydroperoxide lyase is low enzyme activities and low specificity of these crude enzyme preparations (Gigot et al. 2010, Buchhaupt et al. 2012).

4.3. Other LOX applications

Lipophilic extractives from lignocellulosic materials, e.g. alkanes, fatty acids, steroids, and triglycerides, cause pitch deposits in papermaking (Dubé et al. 2008). This can result in product defects or problems in paper runnability (Gutiérrez et al. 2009). Global paper and board production volumes are significant, accounting for 400 million tonnes annually (FAO 2013). Therefore different chemical and biotechnical approaches to minimize the amount of lipids, especially during mechanical pulping, are being pursued. LOXs can be used for pitch control of mechanical pulps (Zhang et al. 2007). The role of *G. graminis* LOX to remove lipophilic extractives from chemical eucalyptus and flax pulps in presence and absence of linoleic acid followed by peroxide bleaching was studied by Marques et al. (Marques et al. 2011). The main lipophilic extractives in eucalyptus pulp were reduced by 40% and 7% in presence and absence of linoleic acid respectively, with simultaneous pulp delignification (Marques et al. 2011). Degradation of xenobiotics, e.g. chlorpromazine, by immobilized LOX has also been reported by Santano et al. (Santano et al. 2002). LOX derived bleaching effect used in food and pulp processing can also be exploited in textile bleaching or in detergents, e.g. triglycerides (Adriaanse et al. 2002, Sugio & Shinobu 2002, Salmon et al. 2004).

5. Conclusions

LOX catalysed hydroperoxide formation and the reactivity of hydroperoxides produced can be exploited in many different applications ranging from production of oleochemical compounds or flavour compounds to bleaching of different materials. Despite the potential, the development of applications is hampered by lack of commercial LOXs with high activity. So far only LOX enriched soya flour is commercially available for application development. LOXs may fulfil an important role in the design of novel environmentally friendly processes especially in oleochemistry and in this way partially replacing chemical reactions. The major problem thus far, however, is to produce LOX enzymes in sufficient amounts for large-scale application. Although there are reports on successful heterologous expression of LOXs in microorganisms, development of efficient production systems for LOXs with different specificities are needed in order to harness its potential as industrial biocatalyst.
6. Declaration of interest

The authors wish to thank the ERA-IB funding for the ERA-NOEL project EIB.08.002. The authors also wish to thank the national funding agencies for funding: TEKES in Finland; FCT-Portugal (Fundação para a Ciência e Tecnologia) and the Associate Laboratory CICECO in Portugal, DATSI (Styrelsen for Forskning og Innovation) in Denmark, Fachagentur Nachwachsende Rohstoffe e.V. (FNR) in Germany and NWO (Netherlands Organization for Scientific Research) in the Netherlands. The authors declare they have no competing interest.
Chapter 3

This chapter is adapted from:

A novel class of fungal lipoxygenases
Abstract

Lipoxygenases (LOXs) are enzymes that have been well-studied enzymes in plants and mammals but less studied in fungi. In this study we compared fungal LOX protein sequences to all known characterized LOXs. To do this, a script was written using Shell commands to extract sequences from the NCBI database. The extracted sequences were then aligned using MUltiple Sequence Comparison by Log-Expectation (MUSCLE). We constructed a phylogenetic tree with the use of Quicktree to visualize the relationship of fungal LOXs with other LOXs. Sequences were analyzed with respect to the signal sequence, C-terminal amino acid, the stereochemistry of the formed oxylipin, and the metal ion cofactor usage. This study shows fungal LOXs are divided into two groups: the Ile-group and the Val-group. The Ile-group has a conserved WRYAK sequence that appears to be characteristic for fungal LOXs and has as a C-terminal amino acid isoleucine. The Val-group has a highly conserved WL-L/F-AK sequence that is also found in LOXs of plant and animal origin. We found that fungal LOXs with this conserved sequence have a valine at the C-terminus in contrast to other LOXs of fungal origin. We also found that these LOXs have signal sequences, implying they will be expressed extracellular. Our results show that in this group, in addition to the Gaeumannomyces graminis and the Magnaporthe salvinii LOXs, the Aspergillus fumigatus LOX uses manganese as a cofactor.
Introduction

Lipoxygenase (LOX) is a non-heme iron-containing or manganese-containing dioxygenase that catalyzes the conversion of polyunsaturated fatty acids (PUFAs) to fatty acid hydroperoxides. Substrates such as linoleic acid (C18:2ω6), linolenic acid (C18:3ω3) and arachidonic acid (C20:4ω6) are converted to hydroperoxy octadecadienoic acid (HPOD), hydroperoxy octadecatrienoic acid (HPOT) and hydroperoxy eicosatetraenoic acid (HPETE), respectively (Andreou et al. 2009). In many LOX enzymes the catalytic center consists of the sequence H-X₄-H; H-X₃-N and the enzyme has an isoleucine as a C-terminal amino acid. The composition of the catalytic center, however, can vary as is found for Gaeumannomyces graminis (H-X₃-H; H-X₃-N), Homo sapiens (H-X₄-H; H-X₃-H) and Mus musculus (H-X₄-H; H-X₃-S) LOXs (Brash 1999; Hornsten et al. 2002; Jisaka et al. 1997; Wennman and Oliw 2012). LOX reaction products are called oxylipins and have different functions in various organisms. In vertebrates, 5-LOXs regulate the biosynthesis of leukotrienes and are involved as signal molecules in the inflammatory response in asthma and allergic rhinitis. In platelets, 12-LOX products contribute to chemotactic and mitogenic responses in the smooth muscle cells of the circulatory system (Schwarz et al. 2001; Lagarde et al. 2010; Duroudier et al. 2009; Izumi et al. 1990). In plants, oxylipins function as signal molecules in the development of seeds but are also involved in the initiation of a defensive system against pathogenic fungi. The hydroperoxides formed are further converted to jasmonic acid, which activates proteinase inhibitor encoding genes in wounded and non-wounded leafs (Jensen et al. 1997; Hamberg et al. 2003; Baysal and Demirdoven 2007).

So far, two fungal LOXs are characterized that use manganese as a cofactor instead of iron (Su and Oliw 1998; Wennman and Oliw 2012). These Mn-LOXs are found in the “take-all” fungus G. graminis and the fungus Magnaporthe salvinii and the biological function of Mn-LOXs is unknown. It has been suggested that these Mn-LOXs play a role in programing cell death and damaging the root cells of wheat as part of the invasion process (Wennman et al. 2012; Andreou et al. 2009).

Several classification systems for LOXs have been developed and are based on information regarding substrate, phylogenetic relatedness, and function. However, no unifying nomenclature of LOXs has been developed (Ivanov et al. 2010). Much is known of Planta and Animalia LOXs but LOXs have only been partially described for other organisms such as bacteria, algae, and fungi (Hansen et al. 2013; Kuo et al. 1996; Oliw 2002). To gain more insight in the characteristics of LOXs throughout the kingdom of life, we analyzed these enzymes based on their amino acid sequence and ordered them in a phylogenetic tree.
Materials and methods

Databases and sequences used for the analysis

NCBI’s protein database was searched for LOXs containing the tag “lipoxygenase OR lox”. The identified LOXs were filtered according to five criteria: a) the sequences should start with methionine to be a complete sequence b) contain the highly conserved sequence W-X\textsubscript{2}-AK where X is any amino acid (Brash 1999; Hornsten et al. 2002), c) sequence titles do not contain the tags putative, hypothetical, patent, predicted, unnamed, unknown, and related in the title, d) the C-terminal amino acid ends with valine or isoleucine, and e) the sequences have the catalytic center H-X\textsubscript{3/4}-H; H-X\textsubscript{3}-N/H/S (Tomchick et al. 2001). All these criteria were written in a script using Shell commands (see Supplementary material 1). We chose the X\textsubscript{2} combination because not all LOXs contain the WLLAK sequence but have variants. The tags in the titles were eliminated from selection, however, these settings were set as a filter and could be turned on or off. The C-terminal amino acid is an important part of the catalytic center as it is involved in coordinating the catalytic metal. In most cases this is isoleucine but also sometimes valine is found.

MUltiple Sequence Comparison by Log-Expectation (MUSCLE) was used to align the isolated sequences and this alignment was then analyzed with ClustalX to manually delete improperly aligned sequences (Edgar 2004; Larkin et al. 2007). This concerns sequences the script selected relating to H-X\textsubscript{3/4}-H; H-X\textsubscript{3}-N/H/S fragments existing in another position in the sequence. Sequences were then re-aligned with the use of MUSCLE. A phylogenetic tree was constructed with the use of Quicktree and this was then visualized using the program FigTree (v1.2.3) (Howe et al. 2002; Page 1996).

Production and purification of LOXs

Purification of Glycine max LOX

G. max 15-LOX was obtained from Cayman Chemical (Estland, no. 60700). We purified the protein by loading 250 µl onto a Superdex 200 10/300 GL (Pharmacia Biotech) using 0.1 M Tris-HCl 0.15 M NaCl pH 7.0 as a running buffer with a flow rate of 0.75 ml/min. Fractions of 1 ml were collected and stored. LOX activity containing fractions were separated again on the column with the same settings to further purify the enzyme.
Production and purification of G. graminis LOX

G. graminis LOX has been produced by AB enzymes, Darmstadt, Germany as described earlier (Nyyssölä et al. 2012). The G. graminis lipoxygenase-encoding gene AAK81882.1 was codon-optimized for Trichoderma reesei [GI:500229533] and synthesized by GeneArt (Regensburg, Germany). To facilitate cloning in Trichoderma, SacII and BamHI restriction sites were added at the 5’ and 3’ ends of the fragment, respectively. The DNA fragment of the synthesized gene was ligated into the SacII and BamHI sites of the expression vector pAB100 containing the strong cbhl-promoter of T. reesei. The native signal sequence of the recombinant LOX was used for secretion. T. reesei strain RH32578 was transformed as described and transformants were screened for growth on plates with acetamide as sole nitrogen source (Penttilä et al. 1987). Resulting transformants were analyzed by PCR for integration of the LOX gene by isolating genomic DNA (Cenis 1992). A PCR reaction was performed using the forward 5’-ATGCAGGCCTTCGTCGACTC-3’ and reverse 5’-GGATCCTTAGACGCTCAGGAAGAAGG-3’ primers resulting in a band of 630 bp. Crude G. graminis LOX extract was filtered using 0.45 µm filters. The 50 ml was loaded manually onto an equilibrated G75 column (GE Healthcare, Brøndby, Denmark) (phosphate buffer, 678 cm bed). Fractions of 14 ml were eluted with a flow rate of 0.5 ml/min. Dialysis was performed of the pooled fraction in 8 L of 20 mM NaH2PO4 H2O pH 6.6 (Buffer A) at 4°C overnight. The dialyzed pool was diluted 1:1 in Buffer A and the 28 ml sample was loaded onto the Resource S column (GE Healthcare, Brøndby, Denmark) with a flow rate of 2 ml/min. Samples of 1 ml were eluted using a linear gradient (Buffer A + 1 M NaCl; 10 column volumes).

Production and purification of A. fumigatus LOX

A. fumigatus LOX was codon-optimized for Pichia pastoris [GI:500229553] and has been produced by VTT, Espoo, Finland as previously described (Nyyssölä et al. 2012). A P. pastoris transformant expressing LOX of A. fumigatus was selected for growth in a 1 L Sartorius Stedim Biotech Biostat Qplus fermenter containing 0.4 L basal salt medium (Sartorius, Goettingen, Germany) (Stratton et al. 1998). One colony was pre-grown in 50 ml YNB medium containing 2% glycerol at 30 °C for two days (Madzak et al. 2005). This culture was added to the fermenter to an OD600 of 2.5. The transformant was grown at 30 °C until the glycerol in the medium was completely consumed. A glycerol fed-batch was initiated for 8 h to increase biomass in the fermenter before methanol induction at 25 °C was started. The medium was controlled with 35% dissolved oxygen at pH 5.0 by addition of NH3 and H3PO4. After 4 days the culture reached an OD600 of ~800 and the fermentation process was ceased.
The supernatant was isolated by centrifugation and the pH was adjusted to pH 8.0. Precipitation was removed by centrifugation and the supernatant was filtered using a 0.22 µm filter and loaded onto a HisPrep FF 16/10 column (GE Healthcare, Diegem, Belgium) using 300 mM NaCl, 50 mM Tris-HCl pH 8.0 as a running buffer at a flow rate of 2 ml/min. Active fractions of 1 ml were isolated using a gradient at 1 ml/min for 60 min. using 300 mM NaCl, 50 mM Tris-HCl, 500 mM imidazole pH 8.0 as elution buffer. Finally, the protein was stored at 4 °C.

**Lipid hydroperoxide diene isomers**

Three independent samples of 10 µl of reaction mixture were injected at room temperature onto a reverse phase Kinetex column (2.6 µm C18 100 Å column 150 x 2.1 mm, Phenomenex, Værløse, Denmark) that was equilibrated with an aqueous 0.1% formic acid 10% acetonitrile solution with a flow of 1.0 ml/min. Elution was achieved with 0.1% formic acid in water (eluent A) and 0.1% formic acid in 95% acetonitrile (eluent B). Elution started with 10% B for 2 min. and was increased to 55% B in 9 min., to 70% B in 15 min. and to 95% B in 5 min where it was then held for 10 min. Analyzes were performed using an Agilent Technologies (Waldbronn, Germany) HPLC series 1100 comprising a model G1312A binary pump, a model G1379A microvacuum degasser, a model G1327A thermostated auto sampler, a model G1316A thermostated column compartment, a model G1315B diode-array detector, and a model G2707DA LC/MSD SL detector fitted with a model G1948A electrospray source. The station was controlled and the results were analyzed with Agilent’s ChemStation software (Rev. A.10.02). UV spectra were recorded from 190 to 400 nm using a reference of 700 nm and the secondary derivative was used to differentiate between cis and trans forms of the different hydroperoxides isomers (Banni et al. 1996). The MS-scan spectrum was recorded in negative mode using a fragmenter voltage of 200 V and a capillary voltage of 3500 V. The ESI ion-source was operating at 350°C with nebulizer pressure of 55 psig and drying gas flow of 12 l/min. The identification of the hydroperoxide isomers with respect to the position of the hydroperoxide was performed according to the fragmentation pattern (Haefliger et al. 2007).

**ICP-AES**

To determine the metal ion cofactor in LOXs, Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) was used for three different LOXs: Purified *G. max* 15-LOX and *G. graminis* LOX were used as references to determine the iron and
manganese cofactors, respectively and purified *A. fumigatus* LOX was used to confirm the hypothesis. Measurements were carried out using a radial ICP-AES of Varian Vista Pro (Varian, Mulgrave, Australia). RvA certified (Dutch Accreditation Council) solutions of 0, 10, and 20 mg/l iron in dH$_2$O were used as standards. For manganese measurements, RVA certified solutions of 0, 1, and 2 mg/l manganese in dH$_2$O were used. Samples containing purified 0.96 mg *G. max* LOX, 2.53 mg *G. graminis* LOX, and 1.00 mg *A. fumigatus* LOX were used to determine the cofactor.
Results

Bioinformatics scripts

The script-identified LOX protein sequences from the NCBI database all contained the highly conserved W-X$_2$-AK sequence, the catalytic H-X$_3$-H; H-X$_3$-N amino acid sequence, and the C-terminal isoleucine or valine. Variants of the catalytic site were described and different script settings were used to select these variants (Table 1). These settings selected for four different catalytic variants (A, B, C and D) divided in four subsections (filter on/off, C-terminal Ile/Val) as presented in Table 1.

Script A1 identified 314 LOX amino acid sequences containing the H-X$_3$-H; H-X$_3$-N catalytic variant. Most of these sequences are found in plant sequences but are also present in mammalian, bacterial, algae, and fungal LOXs. Script B1I identified 26 sequences that are all 12/15-LOXs Animalia LOXs and script B1V identified an arachidonic 5-LOX originating from the bacterium Nitrosomonas europaea [GI:30180541].

Another variant of the LOX catalytic site is H-X$_3$-H; H-X$_3$-S with a C-terminal isoleucine. The number of sequences extracted by script C1I was 17 that code for 15-LOXs with exception of a M. musculus 8-LOX [GI:56800274]. The only non-Animalia LOX in this group is a 9-LOX originating from Brassica napus [GI:27372773] (Terp et al. 2006). Script C2I (filter off) identified 45 sequences. The addition of these sequences resulted in the identification of another LOX sequence that does not code for a 15-LOX: a predicted 12S-LOX originating from Monodelphis domestica [GI:126309204], which makes a total of three non-15-LOX serine catalyzed LOXs. The variant with a C-terminal valine identified by script C1V finds its origin in two different organisms: Zea mays LOX6 [GI:162463394] and Sorghum bicolor LOX6 [GI:258618873]. Script C2V identified seven different LOXs originating from Hordeum vulgare [326494396], Z. mays, and S. bicolor, which are all cereal-like organisms.

Two different LOXs were found using script D1: G. graminis Mn-LOX [GI:17861374] and Arabidopsis thaliana LOX2 [GI:6911852]. The G. graminis LOX has a C-terminal valine whereas the A. thaliana LOX has a C-terminal isoleucine.
Table 1: Criteria used in the scripts for selection of LOXs and the number of identified sequences.

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Phylogenetic tree

A total of 369 LOX amino acid sequences were identified from the NCBI database using the scripts A1, B1, C1 and D1. These sequences were used to generate a phylogenetic tree (Fig. 1). The different colours in the figure identify the various groups of the kingdom of life where all LOXs from plant origin are coloured green. The plant group is a large group and is further categorized into four sub-classes: Machantiophyta, Angiosperms, Lycopodiophyta, and Bryophyta. The plant group in the phylogenetic tree differs from the other LOXs. This is probably a reflection of the substrate difference, since plants commonly use linoleic acid, while mammalians use arachidonic acid (Tsitsigiannis and Keller 2007).

The group of LOXs clustered in yellow is found in Animalia, which is an isolated group. The blue group represents Bacteria, which has three branches. The smallest branch splits up via the route of Animalia LOXs according to Fig. 1 and is a single LOX with a C-terminal valine belonging to the Gram-negative bacteria Myxococcus xanthus [GI:108463641]. The group in dark green represents Chromalveolata LOXs. This group currently exists of Ectocarpus siliculosus LOXs. The Fungi group is coloured red and is sub-divided into two groups: the Iso-group LOXs and the Val-group LOXs.
The sequences used to prepare Fig. 1 are listed from top to bottom in Supplementary material 2.

**Figure 1:** Alignment of 369 LOX amino acid sequences extracted from the NCBI database. The upper branches (with green background) are all classified Plant LOXs and the lower branches (no background) are classified as Other LOXs. The colours represent different organism groups. Plants (light green) are divided into four subclasses: *Machantiophyta*, *Angiosperms*, *Lycopodiophyta* and *Bryophyta*. The other colored groups represent *Animalia* (yellow), *Bacteria* (blue), *Chromovelolata* (dark green) and *Fungi* (red). The number of sequences that were identified determines branch width and the evolutionary length of the LOX determines branch length.

**Fungal LOXs**

**Phylogenetic tree**

The phylogenetic tree displayed in Fig. 1 consists of 14 different fungal LOXs. Switching off the filter, implicating also hypothetical sequences are selected, results in 40 different fungal sequences. In this study we use these sequences since they have the conserved W-X$_2$-AK sequence, the H-X$_4$-H; H-X$_3$-N or the H-X$_5$-H; H-X$_3$-N catalytic center, and an isoleucine or valine as a C-terminal amino acid. The sequences were aligned using MUSCLE and the phylogenetic tree is shown in Fig. 2. This phylogenetic tree splits into two different groups. In this study we refer to these groups as the “Ile-group” and the “Val-group”. The Ile-group contains sequences with a C-terminal
isoleucine and the conserved WRYAK sequence. This conserved sequence is only found in fungal LOXs. The Val-group consists of the WL-L/F-AK sequence that has a C-terminal valine with exception of the *M. salvinii* LOX.

**Figure 2:** Phylogenetic tree derived from 40 LOX amino acid sequences of fungal origin. Two groups can be distinguished: the Ile-group and the Val-group. The Ile-group has a conserved WRYAK sequence and a C-terminal isoleucine. With exception of the *Magnaporthe salvinii* LOX, the Val-group has a conserved WL-L/F-AK sequence and a C-terminal valine residue.

**Stereochemistry**

An alanine or a glycine at a specific location in the sequence, called the Coffa-Brash site, is important with respect to the stereochemistry of the formed oxylipin called the A/G stereo specificity rule (Coffa and Brash 2004; Cristea and Oliw 2006). We analyzed the application of this rule for the fungal LOX sequences. Box 3 in Fig. 3 highlights the part of the alignment where the A/G stereo specificity rule applied to LOXs. All variations of the A/G stereo specificity rule regarding the sequences shown in Fig. 1 are listed in Table 2.
Figure 3: Alignment of fungal LOXs with the conserved W-X$_2$-AK (box 1; 905-909), catalytic H-X$_4$-H (box 2; 925-930) and the Coffa-Brash site (box 3; 968). Box 3 indicates most of the LOXs in the Ile-group 1 will form $S$-hydroperoxides due to the alanine at the specific location, while the Val-group will form $R$-hydroperoxides due to the glycine.
**Table 2:** The variance of amino acids seen in the 369 LOX sequences regarding the Coffa-Brash site.

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Cofactor

With the exception of Neosartorya fischeri [GI:119487626] and Arthrobotrys oligospora [GI:345562408] LOXs (Emanuelsson et al. 2007), LOXs that belong to the Val-group originate from plant pathogenic fungi and have a secretion signal according to SignalP 4.0 server. The N. fischeri LOX is identical to the A. fumigatus [GI:70981869] LOX but it lacks the secretion signal and the A. oligospora LOX has a SignalP prediction of 0.449 where the cutoff is 0.450 in value.

Given that G. graminis is classified into the Val-group, we investigated whether more LOXs in this group have a manganese cofactor. Based on the amino acid sequence of the G. graminis LOX and the similarities found in this group, we hypothesized that the Val-group LOXs use manganese as a cofactor. The 14 fungal sequences displayed in Fig. 1 all split into either the Ile-group or Val-group, where the Val-group consists of the G. graminis and A. fumigatus LOX sequence. Therefore, we elected to analyze the A. fumigatus LOX for cofactor usage.

ICP-AES analysis showed purified A. fumigatus LOX uses manganese and not iron as a cofactor. No iron could be detected in the purified A. fumigatus LOX sample, whereas 0.058 mg/l manganese was measured. This resulted in a manganese usage of > 0.50 manganese molecules per enzyme molecule and indicates the A. fumigatus LOX uses manganese instead of iron as a cofactor.

Figure 4: ICP-AES results of the A. fumigatus LOX. a) The blank measurement indicates no manganese. b) The purified A. fumigatus LOX contains manganese in a concentration of 0.058 mg/l. c) The blank measurement contains no iron and d) the purified A. fumigatus LOX also shows no iron content.
Fungal LOXs lipid hydroperoxide diene isomers

The specificity of the *A. fumigatus* LOX was measured by identifying the presence of hydroperoxide isomers that are generated after reaction with linoleic acid. Reverse phase separation coupled with absorbance detection at 234 nm and MS was used to detect hydroperoxide conjugated isomer dienes. As illustrated in Fig. 5, four isomeric forms of conjugated dienes were identified in each chromatogram according to the UV-spectra and the MS-ions fragmentation patterns (Banni et al. 1996; Haefliger et al. 2007). The interpretation of the UV-absorbance spectra was performed using two derivatives showing local minimum according to the cis/trans conformation. The position of hydroperoxide was identified by EIS-MS; the ions 293, 249, 223, 195, and 113 m/z being specific for 13-HPOD and 293, 249, 185, 183, and 169 m/z being specific for 9-HPOD, respectively. The identification was performed by LC-UV/MS and the LOX was highly specific towards the 13-Z,E-HPOD yielding 89% of the total HPOD. The other HPOD isomers 9-Z,E-HPOD, 13-E,E-HPOD, and 9-E,E-HPOD were 1, 4, and 5% respectively.

**Figure 5:** Conjugated dienes produced by the *Aspergillus fumigatus* LOX yield in 89% specificity for 13-Z,E-HPOD. The other HPOD isomers 9-Z,E-HPOD, 13-E,E-HPOD, and 9-E,E-HPOD were 1, 4, 5% respectively. Percentages are relative to the absorption (Abs.).
Discussion

Bioinformatic scripts

Several bioinformatics scripts were used to identify and cluster LOXs from the kingdom of life. Script C2V identified LOXs with a C-terminal valine and a serine in the catalytic site and these LOXs belong to cereal-like organisms originating from *H. vulgare*, *Z. mays*, and *S. bicolor*. This result raises the question whether plant pathogenic fungal LOXs with a C-terminal valine such as *G. graminis* have relatedness towards these specific plant LOXs regarding infection and intracellular communication.

Script D1 identified two LOXs originating from *G. graminis* and *A. thaliana*. The sequence of the *A. thaliana* LOX [GI:6911852] is almost identical to the *A. thaliana* LOX2 protein [GI:17065070]. It only differs in an insertion of the amino acid sequence RTHACTEPYIIANRQLSAMHPIYRL at position 557-582, which is the region of the alignment where the catalytic site is found. Therefore, it is unlikely that sequence [GI:6911852] is a splice variant from *A. thaliana* LOX2 [GI:17065070]. Based on our alignment sequence, [GI:17065070] belongs to the (A) H-X$_4$-H; H-X$_3$-N family whereas sequence [GI:6911852] belongs to the (D) H-X$_4$-H; H-X$_3$-N family. Sequence [GI:17065070] fits the alignment better because more LOXs have the RTHACTEPYIIANRQLSAMHPIYRL-like sequence. It could be that sequence [GI:6911852] is incorrectly sequenced.

Phylogenetic tree

The phylogenetic tree given in Fig. 1 contains a bacterial LOX sequence originating from *M. xanthus*. According to MUSCLE, this LOX is clustered in the *Animalia* group. *M. xanthus* is a spore-forming predatory proteobacterium that interacts with conspecifics as a social group and is typically found in marine topsoil (Konovalova et al. 2010). *M. xanthus* is known for its large genome in which gene families have likely been inserted via horizontal gene transfer (Goldman et al. 2006). This might explain the presence of a bacterial LOX in a group that has more characteristics of LOXs of marine origin. Analysis of the *M. xanthus* genome shows two different LOX encoding genes incorporated in the genome: one encoding a C-terminal valine LOX [GI:108463641] and the other encoding a hypothetical C-terminal isoleucine LOX [GI:108461295]. Both LOXs meet the criteria set in the methods section to be classified as a LOX. One may speculate these LOXs have functions in the processes of spore formation and predatory behavior.
Cofactor

Fig. 2 shows the Ile-group and the Val-group of fungal LOXs. We hypothesized the fungal Val-group LOXs use manganese as cofactor. This opens the way to a discussion on why this group would use manganese as a cofactor. *G. graminis* can infect the root cells of wheat and other grasses and releases Mn-LOX to oxidize fatty acids to generate hydroperoxides and reactive oxygen species (Cristea and Oliw 2007). As stated earlier, fungal LOXs of the Val-group are plant pathogenic and their LOXs show a secretion signal and a high similarity in primary protein structure. Mn-LOX is heavily glycosylated, has a broad pH spectrum, and is heat stable. Mn$^{2+}$ is chemically more stable than Fe$^{2+}$ suggesting these features are useful in an extracellular expressed enzyme (Oliw 2002).

Another interesting feature of the *G. graminis* Mn-LOX is it produces 11S-oxylipins and it is able to isomerize this product to 13R-oxylipins because Mn-LOX has the capacity for suprafacial hydrogen extraction from its substrate rather than antarafacial extraction such as occurs with Fe-LOX (Cristea and Oliw 2007). These Mn-LOX features suggest that manganese is a better choice of cofactor than iron because this LOX has the capacity to be a more stable enzyme and have a broader use in product formation.

Stereochemistry

An alanine or a glycine at the Coffa-Brash site is important with respect to the stereochemistry of the formed oxylipin (Coffa and Brash 2004; Cristea and Oliw 2006). Glycine is a small amino acid and can penetrate the substrate into the LOX binding pocket and results in *R*-enantiomer oxylipins, where the methyl group of alanine diminishes the space and resulting in the production of *S*-enantiomer oxylipins. In contrast, this rule is not applicable for *Danio rerio* LOX1, because this LOX produces *S*-enantiomer oxylipins although a glycine is present at the specific position. Another variation of the A/G stereo specificity rule is found in the *G. graminis* LOX, because it has a glycine at the specific location. This LOX can produce both *S*- and *R*-enantiomer oxylipins (Hamberg et al. 1998). Also the secretable 9S-LOX from *M. salvinii* has a glycine at the Coffa-Brash site, which contradicts the A/G stereo specificity rule (Wennman et al. 2012). These results suggest the Coffa-Brash site does not determine the stereochemistry of the produced oxylipin for fungal LOXs.

Comparing the two fungal groups (Ile and Val groups) indicates Ile-group LOXs tend to have alanine at the specific location. There are three exceptions to this: LOXs originating from *Chaetomium globosum* [GI:88178689], *Thielavia terrestris* [GI:346996741] and *Myceliophthora thermophila* [GI:347009196]. With exception of
A. oligospora, which has an alanine at the specific location, the Val-group tends to use glycine.

**Fungal LOXs**

On the basis of primary structures we have shown that fungal LOXs can be divided into two groups: the Ile-group and the Val-group. Unlike other known LOXs, the Ile-group has a unique conserved WRYAK sequence and all these sequences have a C-terminal isoleucine. With the exception of the *M. salvinii* LOX, the Val-group has the conserved WL-L/F-AK sequence seen mostly in plants and end with a C-terminal valine. The Ile-group is composed of plant pathogenic fungal LOXs, which are expressed extracellular. Therefore, it is likely they use manganese as a cofactor instead of iron because Mn$^{2+}$ is more stable than Fe$^{2+}$.

The *G. graminis* and *M. salvinii* LOXs use manganese as a cofactor and we have found that the *A. fumigatus* LOX in the Val-group also contains manganese. Our results show that with use of a bioinformatics script, amino acid sequences can be compared and patterns can be revealed. Pattern identification then leads to new hypotheses and new research questions. In this way we have predicted and acknowledged the use of a cofactor in a novel class of fungal lipoxygenases.

**Competing interests**

The authors declare that they have no competing interests.

**Acknowledgements**

We would like to thank dr. ir. P. J. Schaap for his help writing the scripts used in this study, ing. P. R. Nobels for his help with the ICP-AES, and C. Nebel for her help with the HPLC experiment. The authors gratefully acknowledge the financial support provided by the European Research Project (Novel enzyme tools for production of functional oleochemicals from unsaturated lipids (ERA-NOEL), ERA-IB/BIO/0001/2008).
Supplementary materials

Supplementary material 1 – Bioinformatics script used in isolating 369 LOXs

```bash
cat all_lox.fasta|sed ‘/>/>s/$/@/’ |tr -d "\n" |tr "\n" "\n" |grep @M |grep W..AK |grep H...H |grep H...N |rev |grep -f endlist |rev |grep -f filter -i -v |awk -F@ ‘(print $2)’ |sort -u >lox1

cat -n lox1 |awk ‘{print “>@”$1”@””\n”$2}’ >lox2

cat all_lox.fasta |grep -f filter -i -v >database_blast/.../bit_prog/blast-2.2.22/bin/formatdb -i database_blast/.../bit_prog/blast-2.2.22/bin/blastall -pblastp -i lox2 -d database_blast -FF -m9 -b10 -v10 |tee lox3

cat lox3 |grep gi |awk ‘{$3 == 100.00 && $7==$9 && $8==$10 {print “s/”$1”/”$2}’ >sedin

cat lox2 |sed -f sedin >./../lox_final.fasta/.../bit_prog/muscle -in lox_final.fasta -out lox_final_align.fasta
```
A NOVEL CLASS OF FUNGAL LIPOXYGENASES

Supplementary material 2 – GI-numbers belonging to Fig. 1

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A NOVEL CLASS OF FUNGAL LIPOXYGENASES

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A NOVEL CLASS OF FUNGAL LIPOXYGENASES
Chapter 4

This chapter is adapted from:


* Contributed equally
Methods for identifying lipoxygenase producing microorganisms on agar plates
METHODS FOR IDENTIFYING LIPOXYGENASE PRODUCING MICROORGANISMS ON AGAR PLATES
Abstract

We developed plate assays for the screening of lipoxygenase-producing microorganisms on agar plates. Both potassium iodide-starch and indamine dye formation methods were effective for detecting soybean lipoxygenase activity on agar plates. A positive result was also achieved using the β-carotene bleaching method, but the sensitivity of this method was lower than the two other methods. The potassium iodide-starch and indamine dye formation methods were also applied for detecting lipoxygenase production by \textit{Trichoderma reesei} and \textit{Pichia pastoris} transformants expressing the lipoxygenase gene of the fungus \textit{Gaeumannomyces graminis}. In both cases, lipoxygenase production in the transformants could be identified. The potassium iodide-starch method was successful for detection of the \textit{G. graminis} lipoxygenase produced by \textit{Aspergillus nidulans}. When \textit{Escherichia coli} was grown on agar and soybean lipoxygenase was applied to the culture, lipoxygenase activity could clearly be detected by the indamine dye formation method. This suggests that the indamine dye method has potential for screening of metagenomic libraries in \textit{E. coli} for lipoxygenase activity.

Introduction

Lipoxygenases (EC 1.13.11.12, EC 1.13.11.34 and EC 1.13.11.33) are non-heme, iron or manganese containing enzymes, that catalyze the oxidation of unsaturated fatty acids containing a 1-\textit{cis}, 4-\textit{cis}-pentadiene structure into fatty acid hydroperoxides. Linoleic acid, linolenic acid and arachidonic acid are the natural substrates of lipoxygenases (Su and Oliw 1998; Goldsmith et al. 2002).

Lipoxygenase-catalyzed oxidative coupling reactions are utilized extensively in bread making. The addition of lipoxygenase-rich soybean flour results in the production of fatty acid hydroperoxides, which bleach the pigments of wheat flours. Lipoxygenase activity also improves the mixing tolerance and rheological properties of the dough. (Frazier et al. 1973; Faubion & Hoseney 1981; Boussard et al. 2012). Furthermore, lipoxygenases have been used for bleaching dairy products, such as cheese, milk, butter oil, cream, and whey (de Roos et al. 2006). Alcohols and aldehydes have been produced industrially from the fatty acid hydroperoxides that are formed in the lipoxygenase-catalyzed reactions. These compounds are used for flavouring foods (Whitehead et al. 1995).
Lipoxygenases have been detected in plants, mammals, and in eukaryotic and prokaryotic microorganisms (Feussner & Wasternack 2002; Baysal & Demirdöven 2007; Hammarberg et al. 1993; Su & Oliw 1998; Koeduka et al. 2007; Vance et al. 2004). The recombinant production of secreted, stable lipoxygenases at high levels, however, has only been achieved by the expression of the lipoxygenase gene of the fungus *Gaeumannomyces graminis* in *Pichia pastoris* (Cristea et al. 2005).

Various assays to detect lipoxygenase activity have been developed (Axelrod et al. 1981; Williams et al. 1986; Suda et al. 1995; Villafuerte Romero & Barrett 1997; Anthon & Barrett 2001). The majority of these assays are based on identifying the formation of fatty acid hydroperoxides via indirect detection using colourimetry or direct detection using UV-spectroscopy. In most cases, however, plate assays for the screening of microorganisms for industrially interesting enzymatic activities are the method of choice because it allows high-throughput screening. We are unaware of any agar plate assays to screen microorganisms for the production of secreted lipoxygenases.

In this paper we present methods for detecting lipoxygenase activity in microorganisms. The tested organisms include *Trichoderma reesei*, *P. pastoris* and *Aspergillus nidulans* expressing the *G. graminis* lipoxygenase gene, as well as the native lipoxygenase producer *G. graminis* var. *triciti*.

**Materials and methods**

**Cloning the *G. graminis* lipoxygenase gene**

The gene encoding the *G. graminis* lipoxygenase AAK81882.1 was codon-optimized and synthesized by GenScript, a gene synthesis service (GenScript Corporation, Piscataway, USA), for expression in *P. pastoris*. To facilitate cloning, *Eco*RI and *Kpn*I restriction sites were added at the 5' and 3' ends of the fragment, respectively. The DNA fragment of the synthesized gene was ligated into the *Eco*RI and *Kpn*I sites of the vector pPICZαA (Invitrogen) bringing the expression under the control of the methanol inducible AOX1 promoter. The native signal sequences were replaced with the prepro sequence of the *Saccharomyces cerevisiae* α-factor secretion signal for secretion of the recombinant protein. *P. pastoris* wild type strain X-33 (Invitrogen) was transformed by using electroporation with the lipoxygenase expression vector that had been linearised with *Pme*I to target the integration into the AOX1 locus. The transformants were plated on YPDS plates containing 100 μl ml⁻¹ Zeocin (Invitrogen).
and cultivated at 30 °C. Single-crossover recombination at the AOX1 locus was verified by PCR using 5’ and 3’ AOX1 primers.

For expression in *Trichoderma reesei* the *G. graminis* lipoxygenase-encoding gene AAK81882.1 was codon-optimized and synthesized by GeneArt (Regensburg, Germany). To facilitate cloning, *Sac*II and *Bam*HI restriction sites were added at the 5’ and 3’ ends of the fragment, respectively. The DNA fragment of the synthesized gene was ligated into the *Sac*II and *Bam*HI sites of the expression vector pAB100 containing the strong *cbhl*-promoter of *T. reesei*. The native signal sequence of the recombinant lipoxygenase was used for secretion. *T. reesei* strain RH32578 was transformed as described and transformants were screened for growth on plates with acetamide as sole nitrogen source (Penttilä et al. 1987). Resulting transformants were analyzed by PCR for integration of the lipoxygenase gene into the genome.

The gene encoding the *G. graminis* lipoxygenase AAK81882.1 was codon-optimized for expression in *Aspergillus niger* and synthesized by DNA 2.0 (Menlo Park, USA). For expression the promoter and secretion signal of the *xlnD* gene, from *Aspergillus niger* [GI: 74626559], replaced the native secretion signal of *G. graminis* (Van Peij et al. 1997). The *Xba*I and *Bam*HI restriction sites ensured the synthesized gene was incorporated into a pUC19 vector and was used to transform the *pyrA* strain *A. nidulans* WG505. The transformants were plated on MMS plates for four days at 37 °C (Kusters et al. 1991). Positive transformants were analyzed using PCR for verification of integration of the lipoxygenase gene into the genome.

**Lipoxygenase plate assay methods**

A 25 mM linoleic acid solution containing 14 mg ml⁻¹ Tween 20 was used in the β-carotene bleaching assay. The indamine dye formation assay was prepared as previously described (Anthon & Barrett 2001). All incubations were carried out in the dark at room temperature.

**β-carotene bleaching**

The half-saturated solution of β-carotene in acetone used in the assay was prepared as follows. Five mg of β-carotene was dissolved in 5 ml of acetone. The suspension was mixed thoroughly and centrifuged for 10 min at 25 000 g. The supernatant was diluted with the same volume of acetone to give 50% saturation with respect to β-carotene.
The linoleic acid agarose solution contained 2.3 mM linoleic acid, 1% (w v⁻¹) low melting point agarose (Seaplaque) and 50 mM potassium phosphate buffer (pH 7.0). The linoleic acid agarose solution was mixed at a ratio of 39:5 (w v⁻¹) with half-saturated solution of β-carotene in acetone and applied as an overlay. This method is based on a previously described lipoxygenase assay of liquid samples (Villafuerte Romero & Barrett 1997).

**Indamine dye formation**

The indamine dye formation assay is based on the chemical reaction described by Ngo and Lenhoff (Ngo & Lenhoff 1980). A 10 mM DMAB [3-(dimethylamine) benzoic acid] stock solution buffered with 200 mM disodium phosphate pH 6.0; the 10 mM MBTH (3-Methyl-2-benzothiazolinone) stock solution and the 0.1 mg ml⁻¹ hematin stock solution buffered with 5 mM NaOH were prepared as previously described (Anthon & Barrett, 2001). An overlay of agarose with linoleic acid was used and the colouring reagents contained 2.3 mM linoleic acid, 1% (w v⁻¹) agarose, 50 mM K-phosphate buffer (pH 7.0), 4.6 mM DMAB, 0.1 mM MBTH, and 1 μg l⁻¹ hematin.

**Potassium iodide (KI) - starch method**

The potassium iodide assay described by Williams *et al.* was modified for detecting lipoxygenase activity of agar cultures (Williams *et al.* 1986). A linoleic acid solution was prepared by mixing linoleic acid (700 μl) and Tween 20 (700 μl) with 5 ml water. The resulting emulsion was clarified by adding 1 ml 1 M NaOH and diluted to a final volume of 25 ml. For detecting lipoxygenase activity, 100 μl of the linoleic acid solution was added into wells on agar containing 40 g l⁻¹ starch. After incubation, 100 μl of saturated potassium iodide solution and 75 μl of 75% (v v⁻¹) acetic acid were added into the wells and mixed.

**Comparison of assay sensitivity**

An ammonium sulfate suspension of type V soybean lipoxygenase (sLOX-1) (Sigma) was diluted with a 50 mM potassium phosphate buffer at pH 7.0. A lipoxygenase solution of 25 μl was applied to wells in LB-agar in amounts ranging from 8 ng to 1 μg lipoxygenase per well. For the KI-starch method, starch was added to the LB-agar as described above. For comparison of the sensitivities, the plates were incubated at room temperature for 2 h and then were visually examined.
Cultivation of the microorganisms on agar plates for detection of lipoxygenase activity

*P. pastoris* transformants were grown for three days at 30°C on BMMY (Buffered Methanol Complex Medium, Invitrogen) agar. Inducer-methanol was added daily to the lid of the inverted plate to compensate for loss due to evaporation. *T. reesei* transformants were grown on potato dextrose agar at 28–30°C. For analysis using the KI-starch method, plates were supplemented with starch as described above. *A. nidulans* transformants were grown on 50 mM D-xylose minimal medium plates containing 1.2% agar and potato dextrose was omitted to prevent carbon catabolite repression (Pontecorvo et al. 1953). *G. graminis* var. *tritici* CBS 905.73 was grown on potato dextrose agar at 25°C. For the KI-starch analysis method, the medium was supplemented with starch as described above. Colouring reagents were applied as an agarose overlay for the indamine dye formation assay.

Effect of the linoleic acid oxidation products on the growth of *E. coli*

To study inhibitory effects of the reaction-products of lipoxygenase catalyzed oxidation, linoleic acid was dispersed by mechanical homogenization into LB-agar at a concentration of 0.4% (w v⁻¹). A 4 μl solution containing 0.5, 1.8 and 7.2 μg of soybean lipoxygenase (Sigma L-7395) in 50% (w v⁻¹) glycerol, 100 mM Na-phosphate buffer, pH 7.0 was applied to wells present in the agar. Plates were incubated for 30 min at room temperature. *E. coli* (XL1-blue) suspensions were spread on the plates after the incubation and cells were grown overnight.

Detection of lipoxygenase activity by the indamine dye formation method in the presence of *E. coli* cells

Wells were made in the LB-agar plates and each well was inoculated with 400 μl of *E. coli* (XL1-blue) suspension in LB medium. Cells were cultivated overnight at 37°C. A lawn on the plates sLOX-1 (Sigma) was diluted with a 50 mM potassium phosphate buffer, pH 7.0, and 25 μl of the lipoxygenase solution was applied into the agar wells at final amounts ranging from 8 ng to 1 μg. After the enzyme solutions had been absorbed, the indamine dye formation agarose was applied either as a single layer or sequentially as two layers of equal volume. In the latter case, the first layer contained 4.6 mM linoleic acid and 100 mM K-phosphate buffer pH 7.0 and the second layer contained 9.2 mM DMAB, 0.2 mM MBTH and 2 μg hematin. After the application of the first layer the plates were incubated overnight at room temperature before the
second layer was applied. Experiments both with and without 0.5 mM EDTA in the overlays were carried out.

Results

Comparison of assays for detection of soybean lipoxygenase

The formation of lipoxygenase-catalyzed hydroperoxides resulted in a violet-blue colour with the indamine dye formation method, a brown colour with the potassium iodide-starch method and bleached halos in the yellow-orange agarose background with the β-carotene bleaching method.

To compare the three methods, soybean lipoxygenase was applied on the agar plate and analyzed. As shown in Fig. 1, soybean lipoxygenase activity on linoleic acid was detectable with all three colourimetric methods. The sensitivities of the indamine dye formation and KI – starch methods were of the same order with the detection limits ranging from 63 and 130 ng soybean lipoxygenase. β-carotene bleaching was not used for the assays for lipoxygenase activity in further experiments; this method appeared to be the least sensitive of the three methods tested with the detection limit ranging from 0.5 μg to 1.0 μg of soybean lipoxygenase.

Figure 1: Comparison of sensitivities of (A) indamine dye formation, (B) KI-starch, and (C) β-carotene bleaching methods. Different concentrations of sLOX-1 were applied on the agar plates. Plates were incubated for two hours at room temperature.
Detection of lipoxygenase production by microbial transformants

*P. pastoris*, *T. reesei* and *A. nidulans* transformants with and without the lipoxygenase gene of *G. graminis* were grown on agar plates and were induced as described in Materials and Methods. Transformants were analyzed for lipoxygenase activity using the KI-starch and indamine colour formation methods. As shown in Fig. 2, both *P. pastoris* transformants with the lipoxygenase gene gave clear positive signals with both methods. For the assay using the KI-starch method it proved to be necessary to grow the *Pichia* transformants on plates composed of a layer without starch on top and a layer with it on the bottom. Wells were punched in the upper layer in the vicinity of the colonies and the reagents added. Possibly the starch used contained traces of free sugars that caused carbon catabolite repression of expression.

![Figure 2: Lipoxygenase detection of *Pichia pastoris* transformants expressing the lipoxygenase of *Gaeumannomyces graminis* (A) analyzed by indamine dye formation method and (B) analyzed by KI-starch method.](image)
As displayed in Fig. 3, there was a clear brown colour around the mycelium of *T. reesei*. This was not found for the wild type and indicates the plate assay can be used for this organism. For the plate assays using *A. nidulans*, potato dextrose was replaced by D-xylose to prevent inhibition of lipoxygenase by carbon catabolite repression. In these transformants the D-xylose inducible *xlnD* promoter (Van Peij et al. 1997) was used for the expression of the lipoxygenase. Results of the assay are displayed in Fig. 4. Positive transformants for lipoxygenase activity are visible as a brown colony. No positive transformant occurred with the *A. nidulans* wild type. The indamine colour assay was not successful for *A. nidulans* and is likely the result of the low enzyme expression level in this organism.

**Figure 3:** Detection of *Gaeumannomyces graminis* lipoxygenase activity in *Trichoderma reesei* transformants grown on plate using the KI staining method. Positive transformants show a brown colour around the mycelium.

**Figure 4:** Detection of *Gaeumannomyces graminis* lipoxygenase activity in *Aspergillus nidulans* transformants using the KI-assay. Positive transformants are visualized as brown dots in the plate.
Effects of lipoxygenase reaction products on growth of *E. coli* and effects of *E. coli* cells on lipoxygenase activity

To study the effect of the oxidation products of linoleic acid on the growth of *E. coli*, soybean lipoxygenase was applied to linoleic acid LB-agar. After incubation, *E. coli* cells were grown overnight as a lawn on the plates. As shown in Fig. 5, clear growth-inhibiting zones could be detected on the plates around the soybean lipoxygenase (between 0.11 μg and 7.2 μg of the enzyme sample) application points.

![Figure 5: Overnight growth *Escherichia coli* on linoleic acid agar supplemented with sLOX-1. Plates were incubated for 30 min at room temperature in the presence of different amounts of the lipoxygenase and inoculated with the cell suspension.](image)

The possibility that *E. coli* cells interfere with the lipoxygenase activity was investigated by applying soybean lipoxygenase on a lawn of growing cells. When all indamine dye formation reagents were added as a single layer, only 0.5 μg to 1 μg soybean lipoxygenase could be detected after five hours of incubation. When plates were incubated overnight, the blue indamine colour had disappeared. When linoleic acid agarose was added first, however, followed by an overnight incubation and addition of the colouring reagent agarose, the violet-blue colour was clearly visible between 63 ng and 130 ng of soybean lipoxygenase (Fig. 6). In both cases the addition of 0.5 M EDTA intensified the signal (data not shown).
METHODS FOR IDENTIFYING LIPOXYGENASE PRODUCING MICROORGANISMS ON AGAR PLATES

Discussion

The methods used in the current work for identifying lipoxygenase-producing transformants on agar plates are based on detecting the presence of linoleic acid hydroperoxides formed in the lipoxygenase-catalyzed reaction. During the indamine dye formation method, 3-methyl-2-benzothiazolinone (MBTH) is coupled oxidatively with 3-(dimethylamino) benzoic acid (DMAB) in a hematin-catalyzed reaction. This results in the generation of the indamine dye (Anthon & Barrett 2001). In the potassium iodide starch method, the formed fatty acid hydroperoxides oxidize iodide to iodine, which then react with starch (Williams et al. 1986). β-Carotene can then be used for lipoxygenase detection because the fatty acid hydroperoxides cause it to bleach (Villafuerte Romero & Barrett 1997).

The indamine dye formation and KI-starch methods appeared to be more sensitive than the β-carotene bleaching method for detecting lipoxygenase activity. The poor sensitivity of the β-carotene bleaching method has been established in a study where several liquid assays for detecting lipoxygenase activity from vegetable extracts were compared (Villafuerte Romero & Barrett 1997).

The reaction products of lipoxygenase-catalyzed oxidation of linoleic acid appeared to be toxic for E. coli cells. It has been shown that fatty acid hydroperoxides and their degradation products (oxylipins) impair the growth of many microbial plant pathogens (Prost et al. 2005). If linoleic acid is present in the growth medium from the onset of the cultivation, lipoxygenase producers may not be able to survive.
Linoleic acid can also be spontaneously degraded via auto-oxidation during longer incubations (Seo et al. 1999). For these reasons, we chose to omit linoleic acid from the agar medium. Lipoxygenase positive *T. reesei* and *P. pastoris* transformants expressing the *G. graminis* lipoxygenase gene were clearly identified using either KI-starch or indamine dye formation methods. For *A. nidulans* transformants, only the KI-starch assay was successful. The indamine dye formation method did not show clear results for detecting lipoxygenase (data not shown).

A potential problem in screening *E. coli*-hosted metagenomic libraries is that the cells are able to use the lipoxygenase substrate, linoleic acid, for growth. This problem can, however, theoretically be overcome by disrupting genes encoding fatty acid transport and/or β-oxidation present in the *fad* regulon (DiRusso & Nyström 1998). As stated above, adding linoleic acid to the medium may not be feasible because positive transformants can produce toxic substances via the lipoxygenase catalyzed oxidation of the substrate.

Preferably tens of thousands of metagenomic library colonies would have to be screened to find lipoxygenase activity. Furthermore, it is typical for metagenomic expression libraries that the activities are low and may be detectable only after prolonged cultivations (Uchiyama & Miyazaki 2009). The use of the KI-starch method is problematic for the detection of lipoxygenase producing transformants of metagenomic libraries because the colour reaction takes place under very acidic conditions. Iodine is also a well-known microbicide (Vasudevan & Tandon 2010). We, therefore, selected the indamine dye formation method to investigate whether lipoxygenase activity can be detected in the presence of *E. coli* cells. If linoleic acid and the colouring reagents were applied as one layer on the *E. coli* culture supplemented with soybean lipoxygenase, the violet-blue colour was for unknown reasons only transiently visible. When the substrate agarose was applied first, however, followed by incubation and application of the colouring agarose, the soybean lipoxygenase catalyzed reaction was clearly detectable. EDTA intensified the signal, possibly because it chelates metal ions, which can catalyze hydroperoxide degradation.

In conclusion, both the KI-starch and the indamine dye formation methods can be used to detect lipoxygenase producing transformants of *P. pastoris* and *T. reesei*. These methods may also be suitable for other microbial hosts. An advantage of the indamine dye formation method is its possible use in identifying lipoxygenase positive transformants of *E. coli*-hosted metagenomic libraries. At present, however, detecting lipoxygenase production using natural isolates grown on agar remains a challenge.
Chapter 5

This chapter is adapted from:

Heterologous expression of *Gaeumannomyces graminis* lipoxygenase in *Aspergillus nidulans*
HETEROLOGOUS EXPRESSION OF GAEUANNOMYCES GRAMINIS LIPOXYGENASE IN ASPERGILLuS NIDuLANS
Abstract

Aspergillus sp. express Ppo enzymes that produce oxylipins from polyunsaturated fatty acids. These oxylipins function as signal molecules in sporulation and influence the asexual-to-sexual ratio of Aspergillus sp. Another enzyme capable of synthesizing oxylipins is lipoxygenase. Lipoxygenase is hypothesized to be involved in quorum-sensing abilities and in the invasion of plant tissue by the fungus. Aspergillus nidulans and Aspergillus niger only contain ppo genes, whereas the human pathogenic Aspergillus flavus and Aspergillus fumigatus contain ppo in addition to lipoxygenase genes. In this study we introduced the Gaeumannomyces graminis lipoxygenase gene in A. nidulans WG505 to heterologously express the lipoxygenase protein.

The production of the G. graminis lipoxygenase induced phenotypic changes in A. nidulans transformants. The G. graminis LOX, however, could not be detected using SDS-PAGE or Western Blot analysis due to unspecific binding of polyclonal antibodies. Instead, the antibodies showed high homology for a ~125 kDa protein proposed to be PpoC. A proteomic analysis of an A. nidulans strain producing the G. graminis lipoxygenase revealed the presence of G. graminis lipoxygenase, PpoC, thioredoxin reductase, and aminopeptidase Y. These proteins were not detected in the A. nidulans wild type.

It is suggested that thioredoxin reductase protects A. nidulans against oxidative stress, which is induced by active G. graminis lipoxygenase. As a result the G. graminis lipoxygenase is degraded in the vacuole by aminopeptidase Y. Therefore, A. nidulans WG505 is not a suitable production host for heterologous production of G. graminis lipoxygenase.

Introduction

Aspergillus sp. contains ppo genes coding for dioxygenases that belong to the linoleate diol synthase (LDS) protein family. These dioxygenases produce a subset of oxylipins, called precocious sexual inducer (psi) factors. Psi-factor is a collective term for C18:1, C18:2 and C18:3 derived oxylipins (Gao et al. 2007). Oxylipins are signal molecules that are used by fungi to control sporulation (Brodhun & Feussner 2011). These oxylipins can be categorized into three groups depending on the position of hydroxyl group on the polyunsaturated fatty acid (PUFA): psiB (8'-hydroxy-PUFA), psiC (5',8'-dihydroxy-PUFA), or psiA, which has a δ-lactone ring at the 5’ position of the psiC oxylipin (Tsitigiannis et al. 2004). Depending on the PUFA, the oxylipin can be further categorized as α (18:2, linoleic acid), β (18:1, oleic acid), or γ (18:3, linolenic acid) (Tsitigiannis & Keller 2007).
Amongst the *Aspergillus* sp., there are differences in the spectrum of *ppo* genes that are present in the genome. The *Aspergillus nidulans* genome contains the *ppo* genes *ppoA*, *ppoB*, and *ppoC*, which code for enzymes synthesizing the oxylipins psiBα, psiBβ, and psiBβ, respectively (Brodhun et al. 2011). Although *Aspergillus niger* also contains three *ppo* genes, it lacks *ppoB* but contains *ppoD* (Wadman et al. 2009). In *A. nidulans*, PpoA and PpoC have antagonistic roles. PpoB upregulates the *ppoA* gene and, at the same time, represses *ppoC* (Tsitsigiannis & Keller 2005; Brodhun & Feussner 2011). Removing the *ppoA* and *ppoB* genes increases the ratio of asexual-to-sexual sporulation, and removing the *ppoC* gene decreases the ratio of asexual-to-sexual sporulation (Tsitsigiannis & Keller 2005). Developing conidia is preferred in asexual sporulation, whereas scleroatia is preferred in sexual reproduction. It has been speculated, however, that these sporulation phenotypes cannot be explained by the psiB-oxylipin-levels alone. Hence it is suggested that other oxylipins are also involved (Tsitsigiannis & Keller 2005).

Another enzyme capable of producing oxylipins is lipoxygenase (LOX). LOX is a non-heme, iron-containing or managese-containing dioxygenase that can be found in a wide variety of organisms, including fungi (Heshof et al. 2014a). Lipoxygenase is absent in *A. nidulans* and *A. niger*, but the human pathogens *Aspergillus flavus* and *Aspergillus fumigatus* contain both *ppo* genes and *lox* genes (Brown et al. 2008; Wadman et al. 2009; Affeldt et al. 2012). The distribution of *ppo* and *lox* genes in these *Aspergilli* is schematically shown in Fig. 1.
Studies have shown that LOX in A. flavus is involved in quorum-sensing and phenotypic differences are found when the gene is disrupted (Brown et al. 2008; Affeldt et al. 2012). The Gaeumannomyces graminis LOX is a secreted enzyme capable of producing 11S-HPODE and 13R-HPODE oxylipins and is hypothesized to be involved when invading plant tissue (Oliw 2002). Plant oxylipins 9S-HPODE and 13S-HPODE induce sporogenic effects in A. nidulans similar to those produced by the psi factor (Calvo et al. 1999). Both oxylipins cause decreased mycelial growth where 13S-HPODE in a 10-100 µM concentration also reduces mycotoxin production of aflatoxin and sterigmatocystin (Burow et al. 1997). In the present study we introduce the G. graminis lox gene in the A. nidulans WG505 expression host to verify whether A. nidulans is a suitable host for the heterologous expression of G. graminis LOX.

**Materials and methods**

**Expression of G. graminis LOX in A. nidulans**

Heterologous expression of G. graminis LOX in A. nidulans was performed according to the method published by Nyyssölä et al. (2012). The gene encoding the G. graminis LOX AAK81882.1 was codon-optimized for expression in A. niger and synthesized by DNA 2.0 (Menlo Park, USA) [GI: 676899629]. The G. graminis LOX was expressed under control of the promoter of the A. niger xlnD gene and using the secretion signal encoded by this gene [GI: 74626559] that replaced the native secretion-signal of G. graminis (Van Peij et al. 1997, Van der Straat et al. 2014). With help of the XbaI and BamHI restriction sites, the synthesized gene was incorporated into a pUC19 vector and was used to transform A. nidulans WG505, a pyrA derivative of A. nidulans WG096 (ATTC 48756) (Nyyssölä et al. 2012). The resulting A. nidulans GG-LOX transformants were plated on MMS plates and incubated for four days at 37°C (Kusters-van Someren et al. 1991).

We selected 10 positive A. nidulans GG-LOX transformants using the KI-assay according to the procedure published in Nyyssölä et al. (2012) and we used PCR to verify the integration of the lox gene into the genome. A. nidulans GG-LOX transformants were grown in 500 ml Erlenmeyer flasks for 48 hours at 37°C at 250 rpm in 100 ml MM + 50 mM D-xylose. The culture broth was separated from the mycelium using funnel filtration and both culture broth and mycelium were submerged into liquid nitrogen to freeze and preserve the materials. The culture broth and the mycelium were stored at -80°C for use in future research.
Growth and induction of *A. nidulans* wild type and *A. nidulans* GG-LOX

*A. nidulans* WG505 and an isogenic transformant expressing the *G. graminis* LOX (*A. nidulans* GG-LOX) were cultivated on agar plates containing complete medium (6.0 g/l NaNO₃, 1.5 g/l KH₂PO₄, 0.5 g/l KCl, 0.5 MgSO₄ · 7 H₂O, 2 g/l peptone, 1 g/l yeast extract, 1 g/l casamino acids, 0.3 g/l yeast ribonucleic acids, 2 ml/l vitamin solution, 1 ml/l Vishniac solution, 50 mM D- (+)xylose, 12% agar) (Pontecorvo et al. 1953). Liquid cultures of *A. nidulans* were grown at 37°C in 500 ml Erlenmeyers containing 100 ml complete medium without agar and inoculated with 10⁶ spores/ml. Both media contained 50 mM D-xylose to induce the xlnD promotor and stimulate production of *G. graminis* LOX.

**Mutagenesis of the *G. graminis* LOX**

To test whether *G. graminis* LOX interferes with *A. nidulans* as an expression host, a double site-directed H306Q-H310E mutagenesis was performed resulting in inactive *A. nidulans* GG-LOX mutants. These mutations result in a catalytically inactive enzyme but do not affect protein expression levels (Cristea et al. 2005). For mutagenesis the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Amstelveen, The Netherlands) was used. The forward 5’-GTCTACTCCCCAAATGTACCAGGTGCTGTTCAGAGCATCCCAGGAG-3’ primer and the reverse 5’-CTCCGGGATGGTCTCGAACAGCACCTGGTACATTTGGGAGTAGAAC-3’ primer were designed according to the protocol set out in the mutagenesis kit. Sequencing was used to identify positive transformants of this mutant protein (Baseclear, Leiden, The Netherlands).

**Western blot analysis**

Western blot analysis was performed on *A. nidulans* GG-LOX transformants 5, 7, and 9 to identify the presence of *G. graminis* LOX. Disrupted mycelium and 20 µl of culture broth was loaded and run on a 10% SDS-PAGE using a voltage of 100 V for 1 h and Tris-HEPES-SDS running buffer (Thermo Scientific PI28398). The gel was then rinsed with dH₂O and pre-soaked with CAPS-blot buffer. A nitrocellulose membrane was used to absorb the proteins from the SDS-PAGE and was blotted overnight at 70 mA. The membrane was washed for 30 min in TBST and afterwards it was blocked using TBST + 1% BSA for 30 min. Rabbit polyclonal antibody of *G. graminis* LOX (Eurogentec, Seraing, Belgium) was used in a 1/1000 dilution to detect LOX on the membrane. After 3x washes with TBST for 10 min, the membrane was incubated for 30 min using a 1/1000 dilution of secondary anti-rabbit peroxidase antibody
(Sigma-Aldrich Lot. A0545-1ML). After the final 3x 10 min wash step with TBST, the membrane was submitted to AP-detection. This was done by mixing two solvents: 60 mg of 4-chloro-1-naphtol to 20 ml methanol (A) and by adding 60 µl of 30% ice-cold H₂O₂ to 100 ml TBS (B). Prior to use solvents A and B were mixed and the membrane was added to this mixture. The reaction was stopped with dH₂O after 30 minutes of incubation.

mRNA isolation and identification of the G. graminis LOX

Mycelium from A. nidulans WG505 and A. nidulans GG-LOX transformant 5 was submerged in peqGOLD TriFast (peqLAB, De Meern, The Netherlands) and disrupted using glass beads and a MP FastPrep-24 beadbeater (MP Biomedicals, Eindhoven, The Netherlands). The RNA isolated was treated with DNase I and transcribed to cDNA using Omniscript RT enzyme (Qiagen, Venlo, The Netherlands). The resulting cDNA was submitted to PCR using the forward primer 5’-TGAGTTGCAGAACTGGATCG-3’ and reverse primer 5’-GCAGAACGCCAGAAAACTTC-3’ for detection of the G. graminis lox mRNA. cDNA from positive reactions were sequenced (Baseclear, Leiden, The Netherlands).

As a positive control the pyruvate kinase (pkiA) gene was amplified using the forward 5’-GCCAGTCTTGAACTGAACGC-3’ primer and the reverse 5’-GCCAGATCTTGACGTTGAAGTC-3’ primer (de Graaff et al. 1992). Amplified gDNA results in a 304 bp fragment whereas amplified cDNA results in a 204 bp fragment because of an intron in the fragment.

Immunoprecipitation using G. graminis LOX antibody and proteomics analysis

A. nidulans wild type and A. nidulans GG-LOX were grown with 50 mM D-xylose for 48 h and the culture broth and mycelium were separated by funnel filtration. Mycelium was disrupted using French press with a pressure of 1,000 psi (three times) and a cell-free extract was obtained using centrifugation. Samples of 5 ml from both the culture broth and cell-free extract were taken and incubated with 5 ml of polyclonal G. graminis LOX antibody (Eurogentec, Seraing, Belgium). The samples were incubated overnight at 4°C while being continuously stired (200 rpm).

Immunoprecipitation was performed using 100 µl Dynabeads Protein G Magnetic Beads (Life Technology, Bleiswijk, The Netherlands) and proteins were separated for 1 cm on a 10% SDS-PAGE at 100 V. Proteins underwent in-gel digestion using 100 ng trypsin/sample in 50 mM ammonium bicarbonate solution before being diluted (1:1 using 2% trifluoroacetic acid to acidify the solution). To purify the samples, the
peptides were bound to a reversed-phase C18 column followed by washing with 0.1% formic acid. Then proteins were eluted using 80% acetonitrile + 0.1% formic acid. Finally, samples were analyzed by LC-MS/MS (Radboud Proteomics Centre, Radboud University, Nijmegen, The Netherlands). MaxQuant software was used for data analysis (Cox & Mann 2008).

**Cultivation of A. nidulans**

A. nidulans wild type and A. nidulans GG-LOX transformant 5 were cultivated in a New Brunswick BioFlo® 310 (Eppendorf, Nijmegen, The Netherlands). The dissolved oxygen level was maintained at 20% via an agitation cascade from 300-1200 rpm using two Rushton impellers at an airflow of 1vvm. The batch-phase was performed using 3 l of medium for 24 h (5 g/kg glucose, 0.5 g/kg KH₂PO₄, 0.5 g/kg MgSO₄·7H₂O, 4.0 g/kg (NH₄)₂SO₄, 1 g/kg yeast extract, 0.1 g/kg Struktol J673). When glucose was completely consumed, fermentation was fed 2 l of D-xylose medium at a speed of 0.35 g/min (75 g/kg D-xylose, 3.1 g/kg KH₂PO₄, 14.85 g/kg (NH₄)₂SO₄, 24.4 g/kg yeast extract). Samples of 5 ml were taken every 2 h to test for the presence of the G. graminis LOX in the culture broth via the ferrous oxidation-xylenol assay (FOX-assay) (Waslidge & Hayes 1995; DeLong et al. 2002).

**Results**

**A. nidulans GG-LOX transformant selection**

As shown in Fig. 2, 10 A. nidulans GG-LOX transformants were identified using the KI-assay procedure as positive for the production of G. graminis LOX. The culture broth of these positive transformants was submitted to a FOX-assay. The cofactor of active LOX binds to xylenol orange and results in a purple. This binding induces an orange to blue colour-shift. We measured light absorbance using spectrophotometry with a wavelength of 560 nm (see Fig. 3 for an overview of the results).

A. nidulans GG-LOX transformant 5 was selected for further research because it had the highest absorbance (0.164). After 48 hours of growth in a liquid culture, A. nidulans GG-LOX showed a different phenotype than both the wild type and the A. nidulans GG-LOX inactive mutant. As shown in Fig. 4, A. nidulans GG-LOX had a darker colour compared to A. nidulans wild type and the mutant. A problem, however, was that SDS-PAGE analysis was unable to detect the G. graminis LOX.
HETEROLOGOUS EXPRESSION OF GAEUMANNOMYCES GRAMINIS LIPOXYGENASE IN ASPERGILLUS NIDULANS

Figure 2: KI-assay performed on Aspergillus nidulans transformed with the Gaeumannomyces graminis lox gene. The ten transformants selected for further research are marked 1 to 10.

Figure 3: Analysis of culture broth from Aspergillus nidulans transformants carrying the Gaeumannomyces graminis lox gene using a FOX-assay (DeLong et al. 2002). Glycine max LOX was used as a positive control and the wild type A. nidulans WG505 was used as a negative control.
HETEROLOGOUS EXPRESSION OF 
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Aspergillus nidulans

Figure 4: a) Aspergillus nidulans wild type WG505, b) GG-LOX transformant, and c) GG-LOX mutant grown in liquid media for 48 hours. All cultures were induced with 50 mM D-xylose. A clear phenotype is found, the transformant expressing Gaeumannomyces graminis LOX is darker than the wild type and the GG-LOX mutant. A dark colour indicates a clear phenotype.

SDS-PAGE and Western blot analysis

Based on the FOX-assay, three A. nidulans GG-LOX transformants giving the highest activities, 5, 7, and 9 (T₁, T₂, T₃), and three A. nidulans GG-LOX mutants transformants (M₁, M₂, M₃) were selected for an SDS-PAGE and Western blot analysis as shown in Fig. 5. The SDS-PAGE analysis revealed A. nidulans has two different protein band fingerprints in samples WT, T₂, T₃, M₂ and T₂, M₁, M₃ (Fig. 5a). This coincides with the single-band and double-band found in the Western blot analysis of the intracellular proteins of A. nidulans (Fig. 5d). The presence of the bands found in the wild type samples showed non-selective binding of polyclonal antibodies. Production of the G. graminis LOX was not detected in the culture broth or in the cell-free extract of the A. nidulans transformants (Fig. 5b and 5c). The mutant version of the G. graminis LOX was also not detected. Therefore a proteomic analysis was performed of the proteins isolated by immunoprecipitation using the polyclonal antibodies against G. graminis LOX.
**Figure 5**: SDS-PAGE and Western blot analysis of *Gaeumannomyces graminis* LOX expression in *Aspergillus nidulans*. Samples T1-T3 are transformants of *A. nidulans* carrying the *G. graminis* lox gene and M1-M3 are transformants of *A. nidulans* carrying the mutated *G. graminis* lox gene. As positive controls, 10 ng, 100 ng, and 1000 ng of the *G. graminis* LOX were applied. The wild type (WT) sample was used as a negative control. a) SDS-PAGE of the proteins in the culture broth of *A. nidulans*; b) SDS-PAGE of intracellular proteins of *A. nidulans*; c) Western blot of the proteins in the culture broth reacting to the antibodies of the *G. graminis* LOX; d) Western blot of intracellular proteins reacting with *G. graminis* LOX antibodies.
mRNA identification of the G. graminis lox gene

We isolated mRNA from *A. nidulans* GG-LOX and the wild type strain WG505 to confirm that the *G. graminis* lox gene was transcribed. This mRNA was then converted to cDNA and amplified using PCR with the aid of specific primers for the *G. graminis* lox gene. As expected, no product was obtained from the wild type strain WG505 but a PCR amplicon of the expected size was readily obtained from *A. nidulans* GG-LOX. Moreover, DNA sequence analysis of this 204 bp amplicon showed it corresponded with the *G. graminis* lox gene [GI: 676899629]. This indicates that the *G. graminis* lox gene was correctly transcribed in *A. nidulans* GG-LOX.

Comparative proteomics analysis of LOX immunoprecipitated fractions from *A. nidulans* WG505 and *A. nidulans* GG-LOX strains

Since phenotypical differences were found between the *A. nidulans* wild type and the *A. nidulans* GG-LOX, we confirmed mRNA was transcribed, but *G. graminis* LOX was not detected by SDS-PAGE and Western Blot analysis. Therefore, we performed a comparative proteomics analysis on *A. nidulans* wild type and *A. nidulans* GG-LOX transformant 5.

Immunoprecipitation was performed on both cell-free extract and culture broth. As displayed in Fig. 6, three different protein bands of ~67 kDa (1), ~54 kDa (2), and ~41 kDa (3) were found after immunoprecipitation in *A. nidulans* GG-LOX transformant 5. Cell-free extract and culture broth protein samples from *A. nidulans* wild type and *A. nidulans* GG-LOX (WT cfe, WT cb, T cfe, and T cb) were subjected to a proteomics analysis. Analysis of the T cfe sample revealed three interesting findings. We identified a peptide with the TNVGVDLTYTPLDDK sequence, which corresponds to a part near the WLLAK-sequence of the *G. graminis* LOX. We identified aminopeptidase Y in the *A. nidulans* GG-LOX transformant by one peptide hit and identified the presence of thioredoxin reductase by four peptide hits in the GG-LOX transformant.

On the basis of the molecular weight, we suspect protein (1) is *G. graminis* LOX, (2) is aminopeptidase Y, and (3) corresponds to thioredoxin reductase (see Fig. 6). A final difference is found in the detection of PpoC with 24 different peptide hits. In the *A. nidulans* wild type this protein is present in the cell-free extract and the culture broth. The cell-free extract of the *A. nidulans* GG-LOX contained an amount of ~30% PpoC compared to the *A. nidulans* wild type and was not detected in the culture broth.
Figure 6: SDS-PAGE analysis of immunoprecipitated proteins in cell free extract (CFE) and culture broth (CB) of *Aspergillus nidulans* wild type (WT) and the transformant (T) using polyclonal antibodies against *Gaeumannomyces graminis* LOX. In sample T_Cfe protein bands are found at (1) ~67 kDa, (2) ~54 kDa, and (3) ~41 kDa. Proteomics analysis and the molecular weight of the proteins suggests these proteins corresponds to *G. graminis* LOX, aminopeptidase Y, and thioredoxin reductase respectively.
Cultivation of *A. nidulans*

To verify whether the uncontrolled pH, substrate consumption, and oxygen consumption in shake flasks were promoting the degradation of the *G. graminis* LOX, a three-to-five liter fed-batch fermentation was run. Culture broth samples were taken every two hours both during the batch phase and the fed-batch phase. Using the FOX assay, these samples were screened for the presence of *G. graminis* LOX. No *G. graminis* LOX, however, was detected. This implies stable culture conditions do not increase heterologous LOX production in *A. nidulans*.

Discussion

**Phenotype differences in *A. nidulans* wild type and *A. nidulans* GG-LOX**

A darker phenotype after 48 hours of growth was found in the *A. nidulans* GG-LOX compared to the *A. nidulans* wild type and the *A. nidulans* GG-LOX mutant. *A. nidulans* is capable of producing yellow, black, red, and green conidia and ascospores (Brown & Salvo 1994). Conidia formation can be induced by intrinsic signals or environmental stress (Adams et al. 1998). The dark pigmentation found in the *A. nidulans* GG-LOX could be a result of its coloured metabolites, however, the pigmentation also suggests the presence of conidia and, therefore, an asexual state of *A. nidulans*. The oxylipins produced by the *G. graminis* LOX might induce this phenotype. This phenotype and the presence of mRNA suggests an active *G. graminis* LOX in the *A. nidulans* GG-LOX transformant.

The *G. graminis* LOX itself, however, could not be detected using SDS-PAGE and Western Blot analysis. Western Blot analysis revealed non-specific binding of *G. graminis* LOX polyclonal antibodies as found in the *A. nidulans* wild type samples shown in Fig. 6d. The non-specific binding of the protein of ~125 kDa is proposed to be PpoC. PpoC was identified using proteomics analysis with 24 peptide hits. This suggests the polyclonal *G. graminis* LOX antibodies have an affinity for PpoC, which might be a reflection of a conserved epitope. One could speculate that *G. graminis* LOX has a repressing effect on the activity of *ppoC* as lower amounts of PpoC were detected in *A. nidulans* GG-LOX. The repressing and up-regulating functions of PpoB on *ppoA* and *ppoC* are in line with this hypothesis (Tsitsigiannis & Keller 2005). The Western Blot analysis, however, did not indicate less activity of the 125 kDa protein thought to be PpoC.

The presence of thioredoxin reductase, a defense-agent against oxidative damage, in *A. nidulans* GG-LOX suggests the *G. graminis* LOX oxylipins induce oxidative stress
to the host (Missall & Lodge 2004). In addition, the presence of aminopeptidase Y suggests the G. graminis LOX is degraded in the vacuole and would explain why the protein was not detected using SDS-PAGE analysis. Thiorexin reductase and aminopeptidase Y, however, were found using G. graminis LOX polyclonal antibodies. It remains unclear how these proteins could be isolated using immunoprecipitation. The non-specific binding of the antibodies, also found with PpoC, offers an explanation. Thiorexin reductase was positive to four peptide hits and aminopeptidase to one peptide hit. Both proteins, however, were only found in the cell-free extract of the A. nidulans GG-LOX transformant.

**Proposed effect of the G. graminis LOX in A. nidulans**

It is unclear why phenotypic changes were observed. Our results suggest active G. graminis LOX is present and that the G. graminis LOX is degraded in the vacuoles. A previous study showed a concentration of 10-100 µM 9S-HPODE and 13S-HPODE had effect on mycelial growth (Burow et al. 1999). In another study, the effect of A. flavus LOX was tested on growth of the mycelium (Horowitz Brown et al. 2008). A. flavus LOX could not be detected but deletion of the A. flavus lox gene resulted in less conidia being produced. This suggests a small quantity of LOX can have an effect on Aspergillus development. This idea is in line with the phenotype found in the A. nidulans GG-LOX.

There are two explanations for the results of the G. graminis LOX in A. nidulans. The first explanation is that G. graminis LOX is active at the endoplasmatic reticulum and is degraded in the vacuole. If this is the case, it suggests the G. graminis LOX is active during transportation from the endoplasmic reticulum towards the Golgi apparatus or from the Golgi apparatus towards the vacuole. Since these transportation vesicles consist of fatty acids, it is possible these fatty acids are used by G. graminis LOX as a substrate to produce oxylipins. The second explanation is that G. graminis LOX is active in an early stage of growth before a defensive system is activated to inactivate G. graminis LOX. This hypothesis could be investigated in a future study by performing a time-lapsed comparative proteomics experiment.

**In conclusion**

Heterologous production of G. graminis LOX was successfully achieved in P. pastoris and Trichoderma reesei (Cristea et al. 2005; Nyysölä et al. 2012). A difference between P. pastoris and T. reesei compared to Aspergillus sp. is the presence of ppo genes. We hypothesize that the introduction of the G. graminis LOX disturbs the oxylipin
balance in *A. nidulans* resulting in a different phenotype. Thioredoxin reductase is then induced to neutralize the oxidative stress that is caused by the *G. graminis* LOX oxylipins. Finally, the *G. graminis* LOX is degraded in the vacuole by protease aminopeptidase Y. A fed-batch fermentation approach did also not induce protein production using the *A. nidulans* GG-LOX transformant. On the basis of these results we conclude that heterologous production of *G. graminis* LOX using *A. nidulans* as an expression system is not effective or efficient for industrial purposes.

**Author’s contribution**

RH carried out the molecular cloning of *A. nidulans* GG-LOX, mRNA analysis, the Western blot experiments, the immunoprecipitation, the proteomics analysis, fermentation experiments, and drafted the manuscript. JPsV was responsible for the mutagenesis work and analysis presented in this paper. JATR conceived and performed the proteomics sample preparation and contributed to the manuscript. RH and LHdG designed the experiments participated to draft the manuscript. All authors read and approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

**Acknowledgements**

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HETEROLOGOUS EXPRESSION OF GAEUMANNOMYCES GRAMINIS LIPOXYGENASE IN ASPERGILUS NIDULANS
Chapter 6
General discussion: what was and was not achieved and what are the implications for the use of lipoxygenase?
This thesis describes results obtained as part of a study on the biobased production of plastics using polyunsaturated fatty acids (PUFAs) and the enzyme lipoxygenase (LOX). By attaching a peroxide group to the PUFA with LOX, smaller building blocks can be produced. These building blocks can function as oleochemicals, which can then be used to produce polymers. In this discussion we summarize the main project results and discuss the industrial use of LOX-based products. For these processes, as written in Chapter 2, sufficient quantities of LOX as well as its substrates are necessary.

In this chapter we also discuss the choice of LOX and possible amino acid adaptations to optimize its biocatalytic properties. By using recently developed methods in bioinformatics, it is possible to define specific targets in the protein to synthetically improve its biocatalytic properties and make the enzyme more suitable for specific applications.

**Evaluation of the approach**

1. **Choice of LOXs used in this thesis**

This thesis was initiated by applying a bioinformatics approach to select LOXs from the kingdom of life as described in Chapter 3. Our main interest was in LOXs from fungal origin because we wanted to exploit *Aspergillus niger* as an expression host. From the dataset obtained, four different fungal LOXs were selected for further investigation: 1) extracellular *Gaeumannomyces graminis* LOX [GI:17861374], 2) extracellular *Aspergillus fumigatus* LOX [GI:70981869], 3) intracellular *A. fumigatus* LOX [GI: 70982632], and 4) intracellular *Pleurotus sapidus* LOX [GI:293330614]. The specific reasons to focus on these four different LOX enzymes are described below.

**G. graminis LOX**

*G. graminis* LOX was chosen to serve as a positive control and as a model enzyme in the production of oleochemicals. The production of this LOX had been achieved by using *Pichia pastoris* as an expression host (Cristea et al. 2005). As *P. pastoris* was used as an expression host by our partner at VTT, Finland, other partners explored the potential of other LOXs using *Trichoderma reesei* and *A. niger* as production hosts.

**A. fumigatus LOX**

Both *A. fumigatus* LOXs were chosen, since their expression in *A. niger* was expected to be relatively easy due to the close relationship between these organisms. Also, we intended to study the differences between an intracellular and extracellular LOX
from the same organism. Heterologous production and purification of these LOXs could be applied to research on its hypothesized pathogenic role in invading lung tissue (Latgé 1999). Due to the close relationship between extracellular *A. fumigatus* LOX and *G. graminis* LOX, we expected that expression levels and the function of *A. fumigatus* LOX would be comparable to *G. graminis* LOX. The effect of LOX in *Aspergillus* had to date not been investigated.

*P. sapidus* LOX

*P. sapidus* LOX is known for the conversion of (+)-valencene to (+)-nootkatone and is, so far, the only known LOX to convert a sesquiterpene. The substrate (+)-valencene is a flavour compound found in the peel of valencene oranges. This substrate can be converted using LOX to (+)-nootkatone, a grapefruit flavour compound. Grapefruit flavour can potentially be used in the food and cosmetics industry as a flavour compound. The (+)-nootkatone production process has been described in a patent application using LOX from soy flour (de Roos et al. 2006). Due to the unique use of the substrate, this LOX could be explored for conversion of other sesquiterpenes. Sesquiterpenes are a class of semiochemicals that function as signal molecules and pheromones. This type of compound can find its use in pest control (Norin 2007). The *P. sapidus* LOX was also chosen because it is a LOX of basidiomycete origin, which could reveal its function in this type of fungus.

2. Expression of LOXs

**Expression of LOX in *A. niger***

All genes as described in the previous section were incorporated into a pUC19-based vector carrying the *xlnD* promoter and the *xlnD* terminator from *A. niger*. Variants of the genes carrying an N-terminal His-tag were also incorporated in this pUC19-based vector. The usefulness of this construct was shown by two colleagues in the expression of an *Aspergillus terreus* cis-aconitate decarboxylase and an *A. niger* galacturonic acid transporter (Van der Straat et al. 2014, Sloothaak et al. 2014). The pUC19-based vector carrying different *lox* genes were used to transform *A. niger* 872.11, a *pyrA* and *argB* deficient strain. Identifying transformant strains that expressed active LOX enzyme was completed by identifying the production of fatty acid hydroperoxides via the KI-plate assay as described in Chapter 4. None of the transformants, however, gave a positive reaction. The usefulness of the KI-assay was shown using *T. reesei* as an expression host. In this assay *T. reesei* transformants are grown on plates and transformants expressing active enzyme are identified by a brown halo around their
colony. The intensity of the halo also gives a first indication of expression levels of the enzyme by the host. The KI-assay can also be used for identification of LOX enzyme in culture broth as shown for *P. pastoris* transformants.

Another assay performed in this thesis is the ferric oxidation-xylenol orange (FOX) assay. This assay is based on the Fe$^{2+}$ to Fe$^{3+}$ conversion that occurs in presence of active LOX enzyme. The Fe$^{3+}$ ion reacts with xylenol orange to form a blue-coloured complex that is measured at a wavelength of 560 nm (Waslidge & Hayes 1995; DeLong et al. 2002). The combination of the KI-assay and the FOX-assay gives a solid confirmation of LOX activity.

By using PCR, 15 out of 28 *P. sapidus lox* transformants were identified as positive for integration of the LOX expression construct into the genome of *A. niger* 872.11. On the basis of phenotypical differences between *A. niger* wild type and *A. niger* strains carrying the *P. sapidus lox* gene, transformant 28 was chosen for further investigation in LOX expression. The transformant was grown using D-xylose to induce LOX expression according to the methods described in Chapter 4 and Chapter 5. Phenotypical differences were found and compared to wild type *A. niger* 872.11. The main difference was in regard to sporulation: the wild type strain had big black spores whereas *A. niger* transformants expressing *P. sapidus* LOX showed spores smaller in size but higher in number.

This phenotype can be explained by an increase of formed conidia, which form the basis of asexual sporulation. The role of the *P. sapidus* LOX on this phenotype was not further investigated. Culture broth was analyzed by SDS-PAGE analysis for the presence of LOX. Proteomics analysis was performed on the protein indicated by a red arrow in Fig. 1. This resulted in identification of aspergilliopepsin A-like aspartic endopeptidase [GI:145249508]. We could not validate, however, the presence of active LOX expression under study. In addition, both *A. fumigatus* LOXs were also not detected using the KI-assay and the FOX-assay. Due to negative results of the KI-assay and FOX-assay, the lack of presence of LOX, and the detection of aspergilliopepsin A-like aspartic endopeptidase in the *P. sapidus* LOX transformant, we concluded *A. niger* is not a suitable host for heterologous expression of LOXs. Therefore we abandoned *A. niger* as an expression host for the production of heterologous LOX.
Expression of LOX in Aspergillus nidulans

Due to the failure to express the selected LOXs in \textit{A. niger}, we changed the expression host to \textit{A. nidulans} WG312. \textit{P. sapidus} \textit{lox} was integrated into the genome of \textit{A. nidulans} WG312. Its expression resulted in a phenotype. A white fluffy layer, found in 34 out of 88 transformants, grew over the transformants where spores were expected. Also, the mycelium colour turned pinkish. The KI-assay, however, showed negative results for all transformants. On the basis of these results, we discontinued using \textit{A. nidulans} WG312 as an expression host.

We were able to use \textit{A. nidulans} WG505, kindly provided by Dr. A. Debets, as an expression host in a follow-up study. The same \textit{lox} genes as described in the previous section were used to transform this \textit{A. nidulans} strain. The \textit{G. graminis} \textit{lox} was also used as our partners at VTT and AB Enzymes had successfully produced \textit{G. graminis} LOX in \textit{P. pastoris} and \textit{T. reesei} as shown in Chapter 4. The strains transformed with the \textit{G. graminis} \textit{lox} gene gave the best initial results. KI-plate assays showed positive results and ten strains were selected as described in Chapter 5. From these ten transformants \textit{A. nidulans} GG-LOX transformant 5 was chosen to investigate the outcome of the \textit{G. graminis} LOX in \textit{A. nidulans}. The LOX activity of the \textit{A. nidulans} transformants, however, disappeared over time. This result raised the question about what happened to LOX in the expression host and whether the strain was genetically stable.
The steps undertaken in the analysis to identify the presence of active LOX are described in Chapter 5. mRNA of the LOX transformant was isolated to verify whether the gene was transcribed correctly, which was confirmed by sequencing of the resulting cDNA. We hypothesize that the production level of LOX is too low to be detected in SDS-PAGE analysis. Therefore G. graminis LOX produced in T. reesei was used to raise polyclonal antibodies for detection of LOX in Western blot analysis. The results are presented in Chapter 5 from which it became clear that certain wild type proteins interfered with the G. graminis LOX antibodies. Two distinctive protein fingerprints in A. nidulans were found based on the performed SDS-PAGE analysis. These fingerprints correlated with the presence of one or two interfering protein bands found in Western Blot analysis. By using a comparative proteomics analysis we investigated intracellular proteins of an A. nidulans transformant carrying the G. graminis lox gene. Immunoprecipitation and proteomics analysis enabled the detection of aminopeptidase Y. The detection of proteases is in accordance with the results found using A. niger as an expression host. In addition, identification of thioredoxin reductase suggests G. graminis LOX is actively supressed. The hydroperoxides formed by G. graminis LOX may induce oxidative stress and in response thioredoxin reductase is expressed to minimize these effects.

Finally, the lesser detection of PpoC in the A. nidulans transformant compared to the wild type might be a result of the activity of the G. graminis LOX. The results led to the hypothesis that G. graminis LOX activity interferes with these Ppo enzymes and results in phenotypical differences. This interference then would result in genetical instability and selection of strains with deceased LOX activity. This would provide an explanation for the decrease in activity of G. graminis LOX in time in A. nidulans GG-LOX transformant strains.

To test whether the presence of active G. graminis LOX or the oxylipins generated by this LOX are lethal or induce phenotypical changes, A. nidulans and A. niger were grown on plates as shown in Fig. 2. G. graminis LOX was applied in a concentration of 1 mg/ml extracellularly to the medium with and without linoleic acid (LA). We found that LA has an effect on sporulation of both the A. nidulans transformant and A. niger transformant: the A. nidulans transformant has a darker mycelium colour compared to the wild type and the A. niger transformant shows a yellow halo when LA is added. Both these phenotypical changes are thought to be a result of active LOX produced by the transformants. Extracellular G. graminis LOX has no effect on the growth of A. nidulans and A. niger compared to their wild type. These results can be explained by a LOX inhibiting compound called nigerloxin, produced by A. niger in solid state
fermentation (Sekhar Rao et al. 2005). It could well be *A. nidulans* produces such a compound as well, although this has not been reported in literature or investigated in this work.

Finally, to exclude the effect of the activity of the expressed LOX, a fermenter experiment was performed in which the growth phase and biomass formation was separated from the enzyme expression and production phase. Both *A. niger* transformant 28 and *A. nidulans* transformant 5 were tested for production of LOX. As described in Chapter 5 this separation of biomass formation and production phase had no effect on the heterologous production of LOX.

**Figure 2:** *Aspergillus nidulans* wild type (A) and *A. nidulans* carrying the *Gaeumannomyces graminis* lox gene (B) were grown in presence of 1 mg/ml of *G. graminis* LOX (GGlox) and/or 1 mg/ml linoleic acid (LA). This results shows linoleic acid has effect on growth of *A. nidulans*, whereas *G. graminis* LOX has no effect. The same phenomenon is seen for *Aspergillus niger* (C) and *A. niger* carrying the *P. sapidus* lox gene (D).
Expression of *G. graminis* and *A. fumigatus* LOX in *P. pastoris*

Heterologous expression and production of extracellular *G. graminis* LOX was shown to be successful using *P. pastoris* as an expression host (Cristea et al. 2005). On the basis of expression of this LOX in *P. pastoris*, we investigated the expression of the closely related extracellular *A. fumigatus* LOX in this host. Fermentation experiments of the wild type strain *P. pastoris* X-33, the *P. pastoris* strain carrying the *G. graminis* *lox* gene, and the *P. pastoris* carrying the *A. fumigatus* *lox* gene were performed simultaneously using 1.5 L Sartorius fermenters (Stratton et al. 1998). After the batch phase of ~30 h on glycerol an extra 8 h glycerol fed-batch was performed. This was done to generate extra biomass and simultaneously to perform the dissolved oxygen-spiking experiment. DO-spiking was used to determine the consumption rate of glycerol and to make sure the carbon source is depleted. The DO rose within a minute to 100% oxygen saturated in the culture broth when the carbon source was depleted because there was no need for oxygen consumption to metabolize the carbon source. We then added methanol to induce the AOX promoters in front of the *G. graminis* and the *A. fumigatus* *lox* genes. On the basis of the FOX-assay of the *G. graminis* transformant, we identified the production of *G. graminis* LOX after 12 h. Unlike the *G. graminis* LOX transformant, the *A. fumigatus* LOX transformant revealed no significant production levels. After 3 days of induction the fermentations were stopped because the positive control, *P. pastoris* producing *G. graminis* LOX, did not show increased production of *G. graminis* LOX.

The culture broth of *P. pastoris* carrying the *A. fumigatus* *lox* gene was obtained. Since a His-tag was added to the *A. fumigatus* LOX, purification was done via nickel affinity chromatography. This revealed a production of about 1 mg/l of *A. fumigatus* LOX. Despite the low production it was sufficient protein to analyze using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) which metal cofactor is used by this protein. This technique requires pure protein that is burned in a plasma flame, which excites metals to a higher energy state. The metals decay to a lower energy state, which results in emission of light that is specific for the metal ion which is measured. Different metal ions can be identified due to their distinctive emission patterns. This revealed the presence of manganese and no presence of iron. We concluded, therefore, that *A. fumigatus* LOX uses manganese as a cofactor instead of iron. This is in line with the cofactor used of *G. graminis* LOX. Manganese is a more stable compound compared to iron and is therefore hypothesized to be a more suitable cofactor for extracellular LOX (Oliw 2002).
Aarhus University, Denmark, determined the oxylipins produced by A. fumigatus LOX as described in Chapter 3. This revealed high specificity for production of 13-(Z,E)-HPOD. The differences between the yields of the G. graminis LOX and A. fumigatus LOX using P. pastoris as an expression host suggested the cause is due to a difference in amino acid level. It might be P. pastoris is more effective in degrading A. fumigatus LOX during the fed-batch phase by proteases. This has, however, not been further investigated. Table 1 lists a summary of heterologously expressed LOXs as described in this discussion.
Table 1: overview of heterologous expression of different LOXs in different expression hosts.

<table>
<thead>
<tr>
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<th>Escherichia coli</th>
<th>Pichia pastoris</th>
<th>Aspergillus nidulans</th>
<th>Aspergillus niger</th>
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Expression of LOX in Aspergillus oryzae and Aspergillus flavus

We recently found that the heterologous expression of LOX could be achieved using Aspergillus oryzae as an expression host. The production of G. graminis LOX and M. salvinii LOX using A. oryzae is described in a patent application by Novozymes (Sugio and Takagi 2002; Christensen et al. 2008). A. oryzae is considered to be a less sporulating and domesticated variant of A. flavus due to its use in oriental fermented foods (Kurtzman et al. 1986). A difference between these organisms is the lack of aflatoxins in A. oryzae, making it a non-pathogenic fungus (Chang & Ehrlich 2010). This non-pathogenicity makes the fungus suitable for industry. The function of LOX in sporulation has been shown in earlier research (Brown et al. 2008). Mutation of lox in A. flavus decreased the amount of conidia, which form the basis of asexual sporulation.

A. oryzae contains a hypothetical LOX [GI: 317147480], which is closely related to the hypothetical A. flavus LOX [GI: 220694095]. When we compare the A. oryzae lox gene to the A. flavus lox gene, two differences are found. The main difference is that unlike the A. flavus LOX, A. oryzae LOX contains a secretion signal, as predicted by SignalP 4.1. This is due to the different position of the start codon found in both lox genes. The start codon of the A. flavus lox gene is predicted to be 99 bp earlier in the genome compared to the A. oryzae lox gene (see Fig. 3) and means that an N-terminal secretion sequence is found in the A. flavus lox gene. Therefore, the A. flavus lox sequence is most likely a falsely annotated protein-coding gene. The second difference between the genes is that despite the identical sequence of the two genes, the A. oryzae lox sequence contains an intron that is not identified as an intron in A. flavus lox. As illustrated in Fig. 3, a premature stop codon emerges because of this intron and as a result, A. oryzae LOX lacks a significant part of the C-terminus including the catalytically important C-terminal isoleucine or valine.

The DNA sequences of A. oryzae lox gene and the A. flavus lox gene are, however, almost identical. The question is whether the intron found in A. oryzae is due to the difference in DNA sequence upstream of this intron or is due to the intron being wrongly predicted. According to Novozymes’ patents, however, there is no LOX activity present using their untransformed strain. On the basis of the sporulation phenotype and the DNA sequence of the lox gene in A. oryzae, we hypothesize LOX is catalytically inactive in A. oryzae. This hypothesis can be tested by the heterologous expression of A. flavus LOX in A. oryzae to verify whether conidia formation increases. Such an increase would be in line with the results found in Chapter 5, where phenotype differences were found between the wild type A. nidulans and the A. nidulans carrying the G. graminis lox gene.
Figure 3: Comparison of *Aspergillus oryzae* lox and *Aspergillus flavus* lox. In green are predicted introns, in red predicted exons. The high similarity between the LOXs shows the close relationship between these organisms. The additional N-terminal amino acids found in *A. flavus* disrupt the putative secretion signal present in *A. oryzae* as predicted by SignalP 4.1. The corrected start position of *A. flavus* lox is indicated with a red arrow. In addition, in *A. oryzae* lox an intron is detected, which is not detected as an intron in *A. flavus* lox (red boxes). This leads to a misinterpreted translated protein sequence resulting in a premature stop codon.
How to make lipoxygenase-derived products feasible in a biobased economy

1. Biobased economy

Our society is moving towards a biobased economy in which not only first, but also second, third, and fourth generation of biomasses are used as a resource by industry. The first generation consists of food crops, the second generation includes waste products from food and the third and fourth generations include the conversion of CO₂ to sugars by non-food photosynthetic organisms, such as algae and fast-growing plants (i.e. poplars). These third and fourth generations are considered CO₂-neutral because the production organisms use CO₂ as a carbon source. The difference between the two generations is that the fourth generation produces biofuels using genetically engineered organisms (Vanholme et al. 2013; Lee & Lavoie 2012; Demirbas 2009).

One of the major drawbacks of first-generation biomass is deforestation in using food for fuels. For instance, the demand in Argan oil boomed in 1999 for use as a food source, cosmetics, and for medical purposes and this demand threatens the endemic forest in Morocco (Lybbert et al. 2011). Another example is the deforestation of the Amazon due to pasture. Afterwards this land is used for soybean production known for its high linoleic acid content (Barona et al. 2010; Chowdhury et al. 2007). The second generation of biomass is better suited for industry, since valuable compounds are available in waste biomass (Schieber et al. 2001). A drawback of using plant-based waste material from first-generation and second-generation biomass is the green degradation using enzymes due to the robustness of the plant cell wall, which consists of hard-to-degrade compounds such as hemicellulose, cellulose, pectin, and lignin (Vanholme et al. 2013). Since fungi are specialists in degrading plant cell wall material, the use of fungi in a biobased economy is essential.

2. Potential role of lipoxygenase

LOX may have a valuable potential in a biobased economy in many different applications (Chapter 2). A relatively unexplored use of LOXs is in the production of polymers. These polymers can be used as a building block for paints, plastics, and lubricants. Modern plastics are generally made from petroleum although the amount of bioplastics being produced is rising (Gironi & Piemonte 2011). LOXs have specificity for attaching the peroxide group to its specific substrate: polyunsaturated fatty acids (PUFAs). The PUFAs used as a LOX substrate are mainly linoleic acid (C18:2)
and arachidonic acid (C20:4) (Andreou et al. 2009). In this way LOXs are classified as 5-, 8-, 9-, 10-, 11-, 12-, 13-, and 15-LOXs with the number referring to the PUFA carbon atom the peroxide group is attached to. Via the acid or radical degradation route, and depending on the PUFA and LOX used, many different polymers can be formed. Fig. 4 shows the possible polymers that can be formed by the *G. graminis* 13-LOX using the acid or radical degradation pathway (Chapter 2).

**Figure 4:** Different polymers that can be formed using the *Gaeumannomyces graminis* 13-LOX. The acid degradation pathway delivers two polymers, whereas in the radical degradation pathway β-scission and the acidic or alkaloid environment determine the oxylipins that are formed.
3. Oil production in microorganisms

One of the hurdles in making LOX attractive for industry is the supply of PUFAs. One approach is to produce PUFAs with the use of microorganisms. The filamentous fungus *Mortierella alpina* is capable of producing higher fatty acids such as arachidonic acid (C20:4). *M. alpina* includes many different strains and *M. alpina* strain 1S-4 is an industrialized producer of arachidonic acid with a yield of 13 g/l when produced in a 10.000 l fermenter (Higashiyama et al. 2002). The research in oil-producing microorganisms, however, is mainly focussed on yeasts. Yeasts usually have a duplication time of less than 1 hour and the production is relatively easy to scale up (Ageitos et al. 2011). Yeast is called an oily yeast when 20% of its dry weight is composed of lipids. To date, only 5% of yeasts have been reported to accumulate more than 25% lipids (Beopoulos et al. 2009) and *Cryptococcus curvatus, Lipomyces starkeyi, Rhodosporidium turoloides, Rhodosporidium glutinis, and Yarrowia lipolytica* are all oily yeasts feasible for use in oil production of industrial proportions.

A drawback to using yeasts, however, is extracting oil from the cell. Yeasts have thick cell walls and, to date, there is no feasible method to obtain the oils from the cells (Jacob 1992, Ageitos et al. 2011). Another drawback of yeasts is that oleic acid (C18:1) is the main product of fatty acid synthesis whereas linoleic acid (C18:2) and arachidonic acid (C20:4) are desired as a substrate for LOX (Ageitos et al. 2011). The use of desaturases overcomes this problem.

Desaturases are able to convert (unsaturated fatty acids to (higher) unsaturated fatty acids. Heterologous expression of the *Mucor rouxii* Δ12-desaturase in *Saccharomyces cerevisiae* results in the accumulation of linoleic acid in the cell (Passorn et al. 1999). Another enzyme involved in fatty acid synthesis is elongase. Elongase adds two carbons to fatty acids generating higher fatty acids (Haslam & Kunst 2013). Overexpression of this gene in *M. alpina* pushes the fatty acid synthesis towards production of higher fatty acids (Takeno et al. 2005). A combination of the correct desaturases and elongases makes it theoretically possible to produce higher fatty acids such as arachidonic acid (C20:4) from palmitic acid (C16:0). In this manner, specific PUFAs needed for LOXs are created in a biobased way and therefore do not compete with first-generation biomass.
4. Current lipoxygenase expression systems

There are many different expression hosts, such as bacteria *Escherichia coli* and *Bacillus subtilis*, budding yeasts *P. pastoris* and *S. cerevisiae*, and filamentous fungi *A. oryzae* and *T. reesei*. In Chapter 2, we showed that all these expression hosts are suitable for the heterologous production of LOXs and in Chapter 4 we developed the use of plate assays to screen developed libraries to rapidly detect the highest producing clone. The success of one specific heterologously expressed LOX, however, does not imply the successful expression of another LOXs in that particular host. An example of this is the expression of the *G. graminis* LOX and the *A. fumigatus* LOX in *P. pastoris*. This uni-cellular budding yeast is used as an expression host due to its high cell-density cultures and relatively easy transformation methods. The alcohol oxidase (AOX) promoter used is strong and uses toxic methanol as an inducer as well as a carbon source. The *G. graminis* LOX expressed in *P. pastoris* resulted in the production of 30 mg/l, where the *A. fumigatus* LOX was expressed in an amount of 1 mg/l (Cristea et al. 2005, Chapter 3). These two different LOXs are closely related and there is no explanation for the different expression levels.

Similar problems have been encountered in the heterologous expression of *P. sapidus* LOX in *P. pastoris* where the secretion signal did not function properly. It is suspected the LOX is relocated towards membranes inside the cell due to its predicted N-terminal β-barrel (Zelena et al. 2014). However, the *A. fumigatus* LOX does not contain this putative N-terminal β-barrel but does contain a secretion signal. Also, the biological role of the to be expressed LOX is important in the choice of expression host. The *G. graminis* LOX is hypothesized to be involved in programmed cell death and the production of developmental signal compounds (Oliw 2002). Expression of *G. graminis* LOX in *A. nidulans* resulted in a phenotype and the production of aminopeptidase Y. Thus, *A. nidulans* is not a suitable host for heterologous expression of LOXs (Chapter 5).

The use of a bacterium such as *E. coli* as an expression host has pros and cons. The pros are that *E. coli* is easy to transform and grows rapidly. The cons are the formation of inclusion bodies and the lack of protein glycosylation. Cristea *et al.* discovered the *G. graminis* LOX does not need to be glycosylated to be active. Heterologous expression in *E. coli*, however, results in inclusion bodies, resulting in inactive LOX (Cristea et al. 2005). The highest reported heterologous expression of a LOX, however, was produced in *E. coli* (Lu et al. 2013). In this study, the authors successfully expressed the *Pseudomonas aeruginosa* LOX with its endogenous secretion signal. Due to the extracellular production of the *P. aeruginosa* LOX, the costs for down-
stream processing could be drastically decreased, making it a potential LOX for production in industrial proportions.

5. Expression of lipoxygenase in Aspergillus

*Aspergillus* is a popular expression host used by industry for the production of chemicals, such as citric acid, and for the heterologous expression of enzymes (Meyer et al. 2010; Papagianni 2007; Lubertozzi & Keasling 2009). Furthermore, these organisms are capable of using various sugars found in plant waste material (e.g. C5-sugar xylose) and this makes them suitable for use in a biobased economy (de Vries & Visser 2001).

Expression of LOX in *Aspergillus* grown on plant waste material could result in a one-step procedure in the production of fatty acid hydroperoxides. Chapter 5 describes the use of *A. nidulans* as an expression host for LOX, which did not result in the desired one-step procedure. The same phenomenon is seen using *A. niger* as an expression host. Other *Aspergillus* species, such as *A. fumigatus*, *A oryzae*, and *A. flavus*, naturally produce LOXs according to their genomes. Therefore, it is hypothesized these organisms can be used in the heterologous production of LOXs. The main drawback of using these organisms is their pathogenicity. The most suitable host of these three organisms would be *A. oryzae*.

It is a debated point whether this organism has lost is pathogenicity over time due to its extensive use in biotechnology by mankind (Kurtzman et al. 1986). Its close phylogenetic relationship to *A. flavus*, however, might suggest otherwise. On the basis of genomic data of *A. oryzae lox* compared to the *A. flavus lox*, we hypothesize the *A. oryzae* LOX has lost its functionality by point mutations. These point mutations induce a premature stop codon and result in inactive *A. oryzae* LOX. The use of *A. oryzae* in heterologous LOX expression has been proven (Sugio et al. 2002; Christensen et al. 2008). The question remains whether this positive expression is due to a highly adapted industrial strain.

6. Perspectives

The *G. graminis* fungus, also called the “take-all” fungus due to its devastation on agricultural crops, infects root cells of young wheat plants and reduces the plant’s nitrogen uptake (Macdonald et al. 2012). The fungus secretes a LOX thought to interfere with the plant’s signalling pathways in programmed cell death (Oliw 2002). This LOX is designed by nature to be active extracellular; it is strongly glycosylated, it has a broad pH range, it uses manganese instead of iron as a cofactor, and it is stable.
to heat. This type of enzyme is favoured by industry, because it does not need to be renewed frequently.

It is logical to search for a suitable LOX in the fungal kingdom because fungi are capable of degrading plant waste material and are suitable expression hosts. A novel group of fungal LOXs was identified and resulted in eight different fungal species that naturally secrete LOXs (Chapter 3). Based on this comparison, the secreted LOX from *A. fumigatus* was selected to be heterologously expressed in *P. pastoris*. Although the expression level of the *A. fumigatus* LOX was low, six other fungi house possible LOX candidates for heterologous expression. Also, due to the rapid developments in genome sequencing, more LOXs are being identified which could result in more suitable candidates.

It would be favourable to construct a LOX that can be used to produce multiple oxylipins because LOXs require specific substrates and make specific oxylipins. Through 3D-structures and bioinformatics modelling, this is within our grasp. Many modifications can be made to increase the potential of LOX use. A “perfect” LOX has to be a) small, b) stable, c) active in a broad pH spectrum, d) able to use all PUFAs, and e) enantiomeric specific. An overview of these adaptations is given in Table 2. Many of these specifications are found in secreted fungal LOXs and these may form the basis of a perfect LOX.

**Table 2:** Overview of different adaptations that can be used to create a perfect LOX.

<table>
<thead>
<tr>
<th>Adaptation</th>
<th>Technique</th>
<th>Natural example</th>
<th>Synthetic example</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small size</td>
<td>β-barrel deletion</td>
<td><em>Gaeumannomyces graminis</em> LOX</td>
<td><em>Mus musculus</em> 5-LOX</td>
<td>Walther et al. 2011</td>
</tr>
<tr>
<td>Stability</td>
<td>Glycosylation</td>
<td><em>Gaeumannomyces graminis</em> LOX</td>
<td>N/A</td>
<td>Oliw 2002</td>
</tr>
<tr>
<td>Binding pocket depth</td>
<td>Mutagenesis</td>
<td>All LOXs</td>
<td><em>Orytolagus cuniculus</em></td>
<td>Borngräber et al. 1999</td>
</tr>
<tr>
<td>Use of all PUFAs</td>
<td>Mutagenesis</td>
<td><em>Pseudomonas aeruginosa</em> LOX</td>
<td>N/A</td>
<td>Jisaka et al. 1999</td>
</tr>
<tr>
<td>Enantiomeric specific</td>
<td>Mutagenesis</td>
<td>All LOXs</td>
<td><em>Homo sapiens</em> 15-LOX</td>
<td>Coffa et al. 2005</td>
</tr>
</tbody>
</table>
Regarding LOX size, secreted LOX enzymes are small since they lack the N-terminal β-barrel and this makes so-called “mini-LOXs” (Zheng et al. 2008). As less amino acids are needed, this lowers the cellular burden and might speed up production. For industrial polymer production LOX is not needed to bind to a membrane. Deletion of the N-terminal β-barrel often results in decreased activity, which makes intracellular LOXs less favourable for modification (Walther et al. 2011).

In terms of LOX stability, the secreted fungal G. graminis LOX is glycosylated and this makes the LOX more stable and soluble (Oliw 2002). To enhance protein thermostability, two adaptations can be made: including a larger proportion of salt bridges by a higher frequency of arginine and avoiding proline, cysteine, and histidine in α-helices (Kumar et al. 2000).

The pH level effects how the PUFA enters the substrate-binding pocket because pH is involved in the charge of the carboxyl group of the PUFA. Certain amino acids in the binding pocket of LOX can be altered to broaden the pH range to increase generation of specific oxylipins (Hornung et al. 2008; Walther et al. 2009). The LOX can also be changed to favour the “head” or “tail” entrance of the PUFA into the binding pocket of the LOX, which is important for the oxylipin formation (Jisaka et al. 2000). To adapt a LOX favouring a certain PUFA, the depth of the substrate-binding pocket has to be altered. In this way the LOX will react with a different double bond in the PUFA. An example of such an adaptation is the conversion of a 12-LOX to a 15-LOX in a rabbit LOX, in which the catalytic site is active at the 15th carbon position of the PUFA instead of the 12th carbon (Borngräber et al. 1999). With these changes to the binding pocket, it is possible to create a LOX that uses a variety of PUFAs. For example, the P. aeruginosa LOX is known to use oleic acid, linoleic acid, and arachidonic acid as a substrate (Lu X et al. 2013; Vance et al. 2004).

The final modification that can be made to increase the use of LOX is manipulating the PUFA’s enantiomeric specificity of the peroxide group from S to R. This can be achieved by altering an alanine to a glycine at the Coffa-Brash site. This is due to the larger size of alanine pushing the PUFA in an S-enantiomeric position in the binding pocket (Coffa et al. 2005). This forces the LOX to generate specific oxylipins. These specific oxylipins can be used for medical purposes by signalling cells to follow a certain metabolic pathway (Joo & Oh 2012).

For all these modifications it is recommended to obtain 3D structures of the secreted fungal LOXs. Fig. 5 shows two 3D protein models of the G. graminis LOX using the UCSF Chimera modelling tool (Sali & Blundell 1993). The 3D structures of the Glycine
max LOX and the P. aeruginosa LOX were used as reference models. In these models the native secretion signal of the G. graminis LOX was deleted from the amino acid sequence. The accuracy of the models is based on a zDOPE value calculated by the tool. The lower the score, the better the prediction. The G. graminis LOX based on the G. max LOX scores a zDOPE value of 0.29 whereas the model based on the P. aeruginosa LOX scores a zDOPE value of 0.03. The main differences between the models are found in the N-terminus. The P. aeruginosa LOX and the G. graminis LOX are both naturally secreted enzymes lacking the N-terminal β-barrel. The lower zDOPE value and the lack of the β-barrel suggest the G. graminis LOX looks more like the P. aeruginosa LOX than the G. max LOX. Recently, crystals of the G. graminis LOX have been obtained, but the structure remains unsolved (Wennman et al. 2014). Production and purification of the A. fumigatus LOX can deliver a piece of the puzzle in unravelling the 3D structure of secreted fungal LOXs.

Figure 5: 3D protein models of Gaeumannomyces graminis LOX using Glycine max LOX (A) and Pseudomonas aeruginosa LOX (C) as reference models for the UCSF Chimera modelling tool. Both G. graminis LOX models (B and D) lack the N-terminal β-barrel. The G. graminis LOX has 23.8% sequence homology compared to the G. max LOX and a zDOPE-value of 0.29, whereas compared to the P. aeruginosa LOX is has 24.3% sequence homology and a zDOPE value of 0.03. A lower zDOPE value results in a more accurate model.
7. In conclusion

Although challenges remain in making LOX-derived products viable for industry, LOX-derived products have enormous potential for use in a biobased economy. One of the challenges is the supply of PUFAs for the LOX. This could, for example, be overcome by the production of microbial oil. Another challenge is the relative low yield of heterologous LOX production using various expression hosts. This could be overcome by understanding the biological role of LOX and anticipating what the effect might be on the production host. With the use of bioinformatics techniques, suitable candidates for heterologous expression could be identified and a more suitable LOX could be synthetically engineered. If these challenges could be meet, LOX could become a game-changing enzyme in a biobased economy.
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Summary
Many challenges lie ahead in using LOXs as tools in industrial oleochemistry. One of these challenges is the supply of PUFAs. Although we are moving towards a biobased economy where second and third generation biomass is taking a leading role, it is still faster and cheaper to use first generation biomass. Industrialization of microbial oils is a good alternative to supply the demand of PUFAs. Another challenge is the production of heterologous LOX in sufficient quantities. Since the last decade this problem is being tackled and more research is being done in heterologous expression of LOXs. The LOX with the highest potential so far is the secreted *Pseudomonas aeruginosa* LOX produced in *Escherichia coli*. During this thesis research different *lox* genes were tried for heterologous production of LOX using different *Aspergillus* strains as expression hosts. These LOXs were identified as discussed in Chapter 3 and Chapter 6. Unfortunately, heterologous production in sufficient quantities was unsuccessful using these expression hosts as discussed in Chapter 5 and Chapter 6. Since production of *Gaeumannomyces graminis* LOX was successful in *Trichoderma reesei*, as discussed in Chapter 4, the production of polymers used for bioplastics could be demonstrated in this ERA-NOEL project anyway. Therefore this thesis shifted its focus on resolving the question of the difficulties in the heterologous expression of LOX in different *Aspergillus* species. Chapter 5 is the result of a systematic approach to analyze different aspects of *G. graminis* LOX expression in *A. nidulans*. Chapter 2 shows that heterologous expression of extracellular fungal LOX can be performed using *T. reesei* and *Pichia pastoris* as production hosts, and *E. coli* can be used for the production of intracellular LOXs of plant, mammal, bacterial, and fungal origin. As shown in Chapter 2, *E. coli* is not very efficient in the production of heterologous LOX due to the formation of inclusion bodies and low induction temperature necessary for production. The use of *Aspergillus oryzae* can be exploited further in the heterologous production of LOXs. Due to the choice of using *A. niger* and *A. nidulans* as expression hosts, this expression host was not exploited for its potential. The last challenge is to synthetically engineer LOX to broaden its use in industry. In this way more building blocks for chemicals can be synthetically produced and more products based on LOX origin can be made. Therefore, LOX can be a world-wide game-changing enzyme in a biobased economy as its use can decrease the demand for petroleum-based products.
Appendices
Nederlandse samenvatting

De maatschappij beweegt zich richting een biobased economy; een economie waarin chemicaliën, materialen en energie wordt gemaakt uit biomassa in plaats van fossiele brandstoffen. Voor deze transitie is het noodzakelijk innovatieve oplossingen te bedenken om biomassa zo efficiënt mogelijk te gebruiken. Een voorbeeld van een transitie naar een biobased economy is het produceren van plastic. Plastic is een van de meeste gebruikte stoffen in ons huishouden. Hoewel het meeste nog steeds van petroleum gemaakt wordt, komt er ook meer biologisch (afbreekbaar) plastic op de markt.

Een van de biobased oplossingen in de productie van plastic is het gebruik van enzymen in de industrie. Enzymen voor industriële toepassingen worden geïsoleerd uit bijvoorbeeld planten en dieren. Maar ze kunnen ook geproduceerd worden door genetisch gemodificeerde micro-organismen, zoals bacteriën en schimmels. Deze gastheren zijn vele malen efficiënter in het produceren van enzymen dan via de natuurlijke weg. Schimmels leven van dood organisch materiaal, zoals plantafval, en is bedoeld om deze biomassa om te zetten voor energie en de groei van de schimmel. Deze energie en bouwstoffen kunnen tevens gebruikt worden voor het produceren van enzymen.

Lipoxygenase is een enzym dat allerlei op onverzadigde vetzuur gebaseerde producten kan maken, zoals lijm, coatings en plastics. Een aantal andere toepassingen is het verwijderen van deeltjes tijdens papierproductie, bleekmiddel voor brood en productie van smaakstoffen. Dit doet het enzym door peroxide, een OOH-groep, aan het onverzadigde vetzuur te koppelen. Hierdoor wordt het onverzadigde vetzuur reactief en kan het uiteen vallen in kleinere moleculen door chemische reacties. Deze moleculen kunnen gebruikt worden als bouwstof voor plastics. Door de locatie van de dubbele binding in onverzadigde vetzuren, zoals in de welbekende omega 3 vetzuren uit vis en de omega 6 vetzuren uit plantaardige olie, kunnen er verschillende bouwmoleculen gemaakt worden wat ander soort plastic oplevert. Echter, voor deze toepassingen tot uitvoering gebracht kunnen worden, is het noodzakelijk om het enzym in grote hoeveelheden te kunnen produceren. De biologische functie en toepassingen van lipoxygenase alsmede de productie van plastic met behulp van dit enzym is beschreven in Hoofdstuk 2.

Voordat productie van lipoxygenase in een schimmel kan plaatsvinden, moet er eerst een aantal keuzes gemaakt worden. Er moet een goede gastheer gekozen worden voor de productie van het enzym en er moet bepaald worden welk lipoxygenase
enzym er gebruikt gaat worden. In **Hoofdstuk 3** is onderzocht welke lipoxygenases interessant zijn voor de industrie. Met behulp van bioinformatische technieken is er een nieuwe groep lipoxygenases ontdekt die bestaan in schimmels. Deze groep lipoxygenases worden uitgescheiden door schimmels, wat ze interessant maakt voor de industrie. Dit omdat deze enzymen stabiel zijn, aangezien ze hun functie buiten de cel hebben en opgewassen moeten zijn tegen deze vijandelijke omgeving. In dit proefschrift is de lipoxygenase van de schimmel *Gaeumannomyces graminis* als model gebruikt. Dit enzym komt voor in de nieuw ontdekte groep enzymen.

**Hoofdstuk 4** beschrijft een aantal technieken waarmee we snel en goedkoop de juiste gastheer kunnen kiezen voor productie. Deze technieken zijn toegepast op de schimmels *Trichoderma reesei*, *Pichia pastoris* en *Aspergillus nidulans*; allen zijn bekende schimmels in de industrie voor de productie van enzymen en chemische stoffen. Voor deze schimmels is aangetoond dat de “kalium-iodide” techniek toegepast kan worden.

Omdat het enzym niet goed geproduceerd wordt in *A. nidulans*, is er onderzocht wat de oorzaak hiervan is. Dit onderzoek wordt beschreven in **Hoofdstuk 5**. *A. nidulans* dat lipoxygenase produceert, verandert het fenotype van de schimmel; de schimmel krijgt een andere kleur. Er is aangetoond dat het lipoxygenase-DNA in de schimmel aanwezig is en geschreven wordt naar mRNA. Daarna is gekeken of het enzym vertaald wordt en waar het enzym dan is. Door in de cellen van de schimmel te kijken is er onthkt dat er een kleine hoeveelheid lipoxygenase geproduceerd wordt en in de cel zit. Ook werden er proteases gedetecteerd. Proteases zijn enzymen die andere enzymen afbreken en deze hierdoor onschadelijk maken. Daarom is er geconcludeerd dat *A. nidulans* geen goed productiesysteem is voor lipoxygenase.

Tot slot wordt er in de **Discussie** beschreven hoe we verder kunnen gaan om dit enzym in een biobased economy door te laten dringen. Een aantal knelpunten zal moeten worden opgelost, zoals de hoeveelheid lipoxygenase die geproduceerd kan worden. Deze hoeveelheid zal omhoog moeten om het proces economisch rendabel te maken voor de industrie. De meest succesvolle techniek tot dusver is de productie van de bacteriële lipoxygenase van *Pseudomonas aeruginosa* in de bacterie *Escherichia coli*. Het is bekend dat dit enzym meerdere vetzuren kan bewerken, wat het interessant maakt voor de industrie. Echter, de mogelijkheden van bacteriële lipoxygenases zullen geëxploiteerd moeten worden. Ook zijn de benodigde stoffen, de onverzadigde vetzuren, niet in overmaat aanwezig. Het is niet de bedoeling dat deze techniek moet concurreren met de voedselindustrie. De vetzuren zullen dus niet uit planten, maar op een andere manier verkregen moeten worden. Een oplossing
is gebruik maken van de vetproducerende schimmels als \textit{Yarrowia lipolytica} en \textit{Mortierella alpina}. Deze schimmels kunnen in grote hoeveelheden onverzadigde vetzuren produceren die gebruikt kunnen worden in de lipoxygenase-gebaseerde biobased economy.

Er zal nog een aantal obstakels overwonnen moeten worden, maar dit proefschrift levert een proof-of-principle dat lipoxygenase gebruikt kan worden in een biobased economy. De volgende stap is meer onderzoek doen naar andere lipoxygenases en deze enzymen integreren in een biobased economy.
Dankwoord

En dan nu het gedeelte wat de meeste mensen waarschijnlijk wel zullen lezen. Ik had deze uitdaging voor geen goud willen missen. Als ik denk hoe ik binnenkwam en hoe ik nu wegloop; een wereld van verschil. Dat is niet mogelijk geweest zonder de hulp van vele mensen. Aan iedereen die ik vergeten ben: jullie worden bedankt!

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Ruud Heshof
About the author

Ruud Heshof was born on the 9th of June 1984 in Tholen, The Netherlands. He obtained his Gymnasium VWO diploma at RSG ‘t Rijks in Bergen op Zoom in 2002. Afterwards he started his study in Molecular Life Sciences at Wageningen University. After two master theses in Biochemistry and Microbiology he finished his study by an internship in València, Spain. He then graduated in the specializations Medical Research and Biological Chemistry. During his study he got fascinated by microbiology and enzymology and he wanted to continue in the field in heterologous expression of enzymes. In September 2009 he started his PhD-research focussing on heterologous expression of enzymes for industry using fungal expression hosts under the supervision of Dr. L. H. de Graaff, Prof. W. M. de Vos, and Prof. V. A. P. Martins dos Santos. He is currently working as a project leader in fermentation technology at HAN BioCentre in Nijmegen, The Netherlands.
List of publications

Chapter 2


Chapter 3


Chapter 4


Chapter 5


* Contributed equally
Overview of completed training activities

**Discipline specific activities:**
- Project meetings ERA-IB. 2009-2013. Aarhus University, Denmark; University of Aveiro, Portugal; VTT, Finland; AB Enzymes, Germany; Fraunhofer Institute, Germany. Wageningen University, The Netherlands.

**General activities:**
- PhD competence assessment
- Scientific publishing
- Scientific writing
- Project & Time management

**Optional activities:**
- Advanced Bioinformatics, Wageningen University
- Fungal System Biology group meetings (weekly)
- Systems and Synthetic Biology seminars (monthly)
- Organizing PhD excursion MIB
- Participation PhD trip to China and Japan
- Preparation of PhD Project Proposal
- Laboratory of Microbiology PhD/PostDoc meetings (biweekly)
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