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FUNCTIONAL CHARACTERIZATION OF A NOVEL BACTERIAL PEROXIDOCKERIN WITH HOMOLOGY TO HUMAN HEME PEROXIDASES

Master Thesis

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ABSTRACT

Today five heme peroxidase (super) families are known, including the peroxidase-catalase superfamily and the peroxidase-cyclooxygenase superfamily. The latter contains seven subfamilies. The first subfamily, which includes the mammalian peroxidases lactoperoxidase (LPO), myeloperoxidase (MPO), eosinophil peroxidase (EPO) and thyroid peroxidase (TPO), is very well investigated. For other subfamilies some biochemical data can be found in literature. In this thesis we focused on subfamily 5, the peroxidockerins. We choose a cyanobacterial peroxidockerin from *Lyngbya sp.* PCC8106 (LspPOX) with high homology to mammalian LPO for biochemical and biophysical characterization.

In general the mammalian peroxidases play an important function in innate immunity and hormone biosynthesis. Their primary role is killing of invading microbes, parasites and related organisms, by generating and releasing a battery of substances that promote oxidation and halogenation reactions. In contrast, the native function of LspPOX is fully unknown and speculations include unspecific defense mechanism, xenobiotic detoxification and distinct roles in metabolism.

In this thesis the heterologous expression of the bacterial protein in *E. coli* was established. The protein was characterized by a broad set of biochemical and biophysical methods including mass spectroscopy, differential scanning calorimetry, stopped flow spectroscopy, circular dichroism and UV-Vis spectroscopy.

ZUSAMMENFASSUNG

Häm Peroxidasen sind in zwei Superfamilien unterteilt, zum einen die Peroxidase-Katalase Superfamilie und zum anderen die Peroxidase-Cyclooxygenase Superfamilie. Die zweite Superfamilie, umschließt Oxidoreduktasen aus Vertebraten und Invertebraten, Pflanzen, Pilzen und Bakterien. Sie kann in sieben Unterfamilien geteilt werden. Die erste bereits sehr gut untersuchte Unterfamilie enthält Säugetier-Peroxidasen, wie zum Beispiel Lactoperoxidase (LPO), Myeloperoxidase (MPO) und Eosinophile Peroxidase (EPO). Für die anderen Unterfamilien können meist nur wenig biochemische Daten in der Literatur gefunden werden. In dieser biochemischen Arbeit fokussieren wir uns auf die noch nicht sehr genau erforschte Unterfamilie der Peroxidockerine. Es wurde ein Peroxidockerin aus dem Cyanobakterium *Lyngbya sp.* PCC8106 (LspPOX) mit hoher Homologie zu LPO ausgewählt.

Säugetier-Peroxidasen spielen in der unspezifischen Immunabwehr (LPO, EPO und MPO) und der Hormonbiosynthese (TPO) wichtige Rollen. Durch ihre Fähigkeit Halogenide in starke Oxidationsmittel umzuwandeln, bilden MPO, LPO und EPO die erste Barriere gegen das eindringen von Mikroorganismen in den menschlichen Körper. Die Funktion von LspPOX ist im Moment noch unklar, die Überlegungen dazu reichen von antimikrobieller Aktivität bis zu noch unbekannten Funktionen in Stoffwechselwegen.

In dieser Arbeit haben wir uns die Aufgabe gestellt, einen geeigneten und einfachen Weg zu finden um LspPOX in *Escherichia coli* zu exprimieren und ein schnelles Reinigungsprotokoll zu entwickeln. Weiters wurde das rekombinant hergestellte Protein mit unterschiedlichen Techniken, wie zum Beispiel Massenspektroskopie, Differenzkalorimetrie, Stopped-Flow-Spektroskopie, Circulardichroismus und UV-Vis Spektroskopie untersucht, um einen tieferen Einblick in den Reaktionsmechanismus und die allgemeinen Eigenschaften von LspPOX zu erhalten.

1. INTRODUCTION

Heme peroxidases (EC number 1.11.1.7) are oxidoreductases present in all living cells. Their molar mass (MW) ranges from 35 to 100 kDa. They can occur as monomeric (e.g. horseradish peroxidase) or dimeric (e.g. myeloperoxidase) proteins.

In general, peroxidases use hydrogen peroxide (H_2O_2) or other hydroperoxides (ROOH) as one of the substrates. Their field of activity is manifold, as they participate in cell wall biosynthesis, lignin biosynthesis, hormone biosynthesis, antimicrobial activity or simply H_2O_2 detoxification. They also may contribute to the pathogenesis of many inflammatory diseases [Klebanoff, S.J., 2005].

1.1. PHYLOGENETICS

Beside non-heme peroxidases, the majority of heme peroxidases belongs to two distinct superfamilies. The first superfamily (peroxidase-catalase superfamily) is comprised of bacterial, fungal and plant heme peroxidases [Welinder, 1992]. Members of this first superfamily are catalase-peroxidases, ascorbate peroxidases, cytochrome *c* peroxidases, manganese, lignin peroxidases and secretory plant peroxidases [Zámocký *et al.,* 2010]. Mammalian peroxidases such as myeloperoxidase (MPO), eosinophil peroxidase (EPO), lactoperoxidase (LPO) and thyroid peroxidase (TPO) belong to the second superfamily, the peroxidase-cyclooxygenase superfamily [Zámocký *et al.,* 2008].

Its representatives are found in all kingdoms of life. Today the names of both superfamilies indicate the main enzymatic activities. They evolved independently during evolution and show significant differences in overall fold and heme cavity architecture [Zámocký *et al.,* 2008].

1.1.1. CATALASE-PEROXIDASE SUPERFAMILY

The first superfamily (catalase-peroxidase superfamily) or "superfamily of plant, fungal and bacterial superfamily" includes three classes of structurally related proteins, first described by Karen Welinder in 1992. All of them possess a heme *b* (ferriprotoporphyrin IX) and at the proximal side of the heme a conserved histidine is found. Additionally, there are conserved histidine and arginine residues at the distal side.

Class I contains eukaryotic and prokaryotic non-glycosylated heme peroxidases, without disulfide-bridges and Ca^{2+} binding [Zámocký *et al.,* 2010]. Well-known members of class I peroxidases are cytochrome *c* peroxidase found in the mitochondrial intermembran space, catalase-peroxidase and ascorbate peroxidase found in eukaryotic photosynthetic organisms [Passardi *et al.,* 2007].

Class II peroxidases are glycosylated, Ca^{2+}-binding proteins that are stabilized by disulfide bridges. Class II peroxidases are mainly secreted by fungi. Lignin peroxidase and manganese peroxidase can oxidize molecules with high redox potential [reviewed in Conesa *et al.,* 2002].

Glycosylated class III peroxidases also contain a calcium binding side and disulfide bridges, but they are found only in land plants. They play an important role all along the plant life cycle such as lignin polymerization, cross-linking of cell wall constituents and auxin metabolism [Bakalovic *et al.,* 2006] [Passardi *et al.,* 2005].

1.1.2. PEROXIDASE-CYCLOOXYGENASE SUPERFAMILY

Peroxidase-cyclooxygenase superfamily, which includes LspPOX, is divided into seven subfamilies (Figure 1-1). As mentioned before, the main structural difference to the first superfamily is the nature of the heme *b* group. Heme *b* in the catalase-peroxidase superfamily is non-covalently bound; whereas the prosthetic group of the second superfamily forms two covalent links with the polypeptide chain *via* ester bounds; MPO forms an additional sulfonium ion linkage [Fiedler and Davey, 2000]. The members of this family are able to bind and oxidize small anionic substrates such as halides, thiocyanate and a multitude of organic molecules. The oxidized products (hypohalous acids, hypothiocyanate and organic radicals) participate in the unspecific mammalian immune defense.

The first subfamily of the peroxidase-cyclooxygenase superfamily comprises chordata peroxidases. They can be found in all branches and are represented by MPO, LPO, EPO and TPO.

Peroxidasins found in vertebrates and invertebrates represent the second subfamily. They are multidomain proteins first found in *Drosophila* [Nelson *et al.,* 1994].

Peroxinectines form the third subfamily. They contain a peroxidase domain and an integrin-binding motif. The first peroxinectin was discovered in crayfish blood in 1988 [Johansson and Söderhall, 1988].

The fourth subfamily is represented by cyclooxygenases. This family contains human, bacterial, fungal and plant peroxidases with different physiological roles. Human cyclooxygenases are often target of non-steroidal anti-inflammatory drugs (e.g. aspirin) to relief from symptoms of inflammation [reviewed in Patrignani and Patrono, 2014]

The fifth subfamily is constituted by bacterial perocicins. At the moment there is no member of this subfamily known with experimentally verified physiological function.

The sixth subfamily is formed by peroxidockerins with different origins. Beside the peroxidase domain, peroxidockerins contain a transmembrane domain and two Dockerin type I repeats. LspPOX is arranged in this subfamily according to phylogentic data, even if LspPOX does not contain a transmembrane domain and Dockerin type I repeats.

The seventh subfamily is constituted by eukaryotic dual oxidases. They are multidomain peroxidases, with a peroxidase domain and a flavin domain connected by a linker.

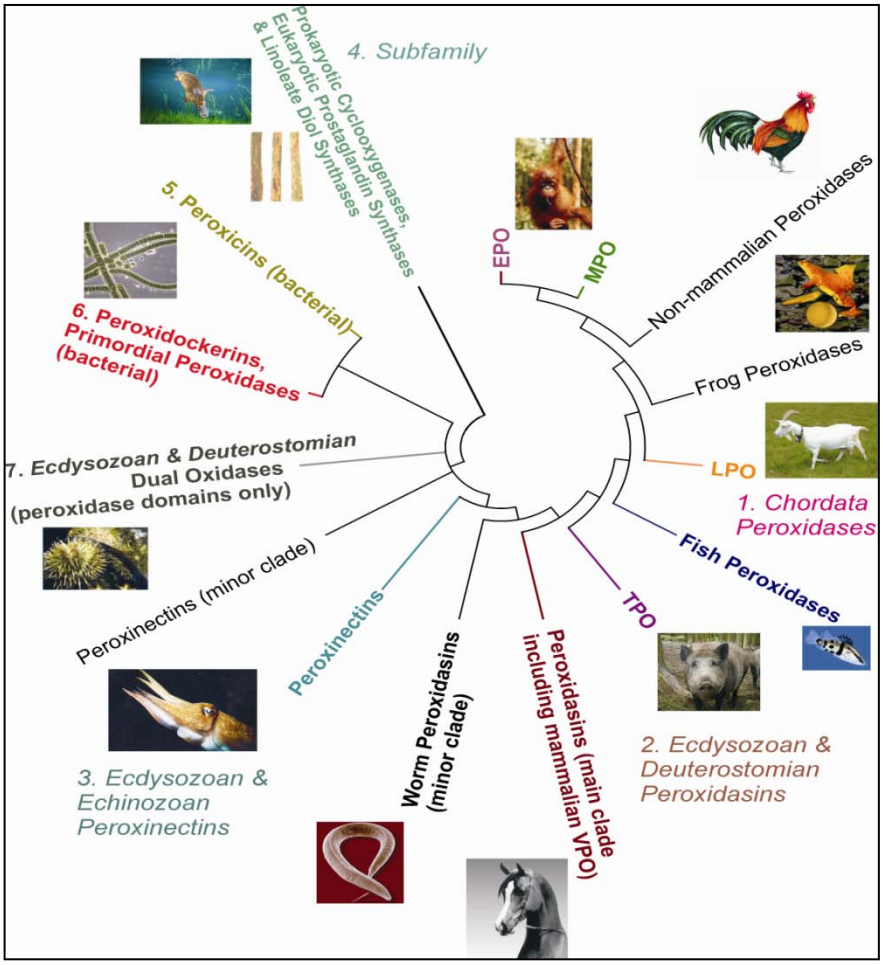


Figure 1-1 – Phylogenetic tree of peroxidase-cyclooxygenase superfamily divided into seven subfamilies (chordata peroxidases, peroxidasins, peroxinectins, cyclooxygenases, peroxicins, peroxidockerins (with LspPOX) and dual oxidases)

1.2. STRUCTURE OF HEME PEROXIDASES

Heme peroxidases are found in all kingdoms of life. They differ in their primary and tertiary structures and heme cavity architectures. Typically they contain heme *b* as prosthetic group. Peroxidases from the peroxidase-cyclooxygenase superfamily are the only enzymes with covalently linked heme [Furtmüller *et al.,* 2006]. The mammalian peroxidases MPO, LPO, EPO and TPO have two covalent ester bonds between the modified heme *b* and the protein [Colas *et al.,* 2002] [DePillis *et al.,* 1997]. MPO has an additional vinyl-sulfonium linkage. These covalent bonds are posttranslational modifications and occur *via* autocatalytic formation [Furtmüller *et al.,* 2006]. The porphyrin ring, usually a planar molecule, distorts because of the different covalent bonds. This variation of the prosthetic group has a crucial influence on biochemical and biophysical properties, e.g. MPO variant Met243 (without vinyl-sulfonium linkage) losses completely the ability to oxidize chloride to hypochlorous acid [Zederbauer *et al*., 2007].

1.2.1. HEME STRUCTURE

Heme is a prosthetic group incorporated in the active site of many peroxidases and it is indispensible for their reactivity. A heme consists of a large porphyrin ring, made up of four pyrrole rings joined together by methine bridges. Ferriprotoporphyrin IX (also called heme *b*) (Figure 1-2) is the most abundant heme in peroxidases [Dunford, 1999]. Embedded in the centre of the porphyrin ring is an iron (III), which is in the ferric native state of e.g. LPO displaced toward the proximal site (below the heme) by 0.1 Å [Singh *et al*., 2009]. In the native resting enzyme iron (III) is five-coordinated, the sixth position is left open for ligands and substrates such as H_2O_2, cyanide or halides.

The nitrogen atoms of the four pyrroles are the coordination positions 1-4. The fifth position is located on the proximal side of the heme and is occupied by the imidazole side chain of a conserved histidine residue. At the distal side of heme a water molecule resides. It is also the region were the reactions take place. Cyanide and H_2O_2 diffuse to the distal cavity and directly change the coordination number of the iron (III) from 5 to 6 [Dunford, 1999].

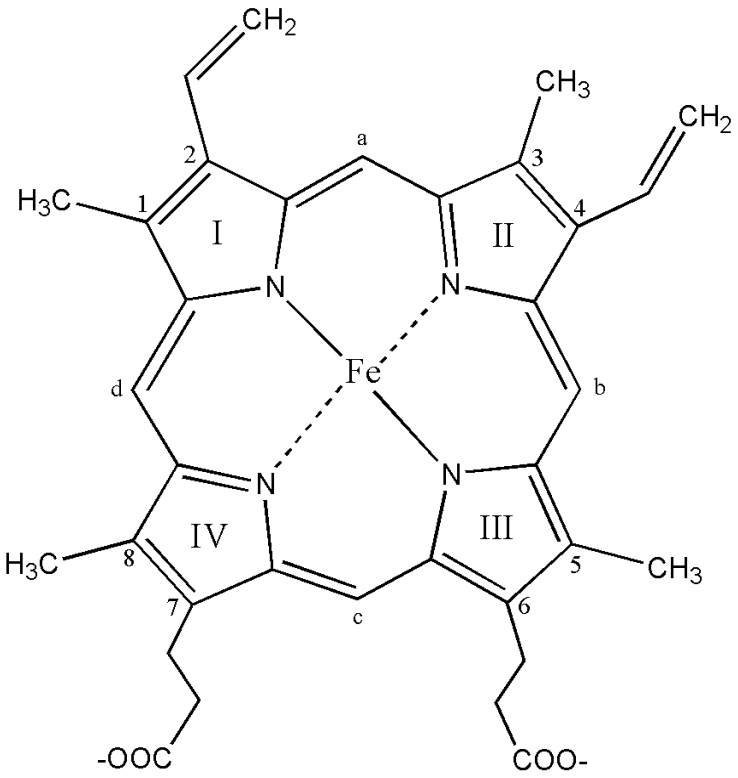


Figure 1-2 - Structure of protoheme IX (heme *b*) with its four pyrrole rings I, II, III and IV. There are eight side chains as follows: four methyl (position 1, 3, 5 and 8), two vinyl groups (2 and 4) and two propionate groups (6 and 7). Methine bridges are labeled a, b, c and d. Figure created with ChemDrawUltra 8.0.

1.2.2. ACTIVE SIDE RESIDUES

Within the active site of heme peroxidases, there are several essential amino acids responsible for heme *b* binding, iron coordination and substrate binding. The active site and its residues of LPO are now explained in more detail, because *Lyngba* PCC8106 peroxidase (LspPOX) has high homology to LPO (see 1.4.3).

The heme group of LPO is a modified protoporphyrin IX. The pyrrole rings I and III are methylated. These methyl groups are further hydroxylated, which allows the formation of ester bonds with the carboxyl groups of Glu258 and Asp109 of LPO [Andersson *et al*., 1996] (Figure 1-3). These bonds are posttranslational modifications and can occur autocatalytically. It has been shown, that incubation with low amounts of hydrogen peroxide increases the extent of covalent heme binding and the catalytic activity [DePillis *et al.,* 1997].

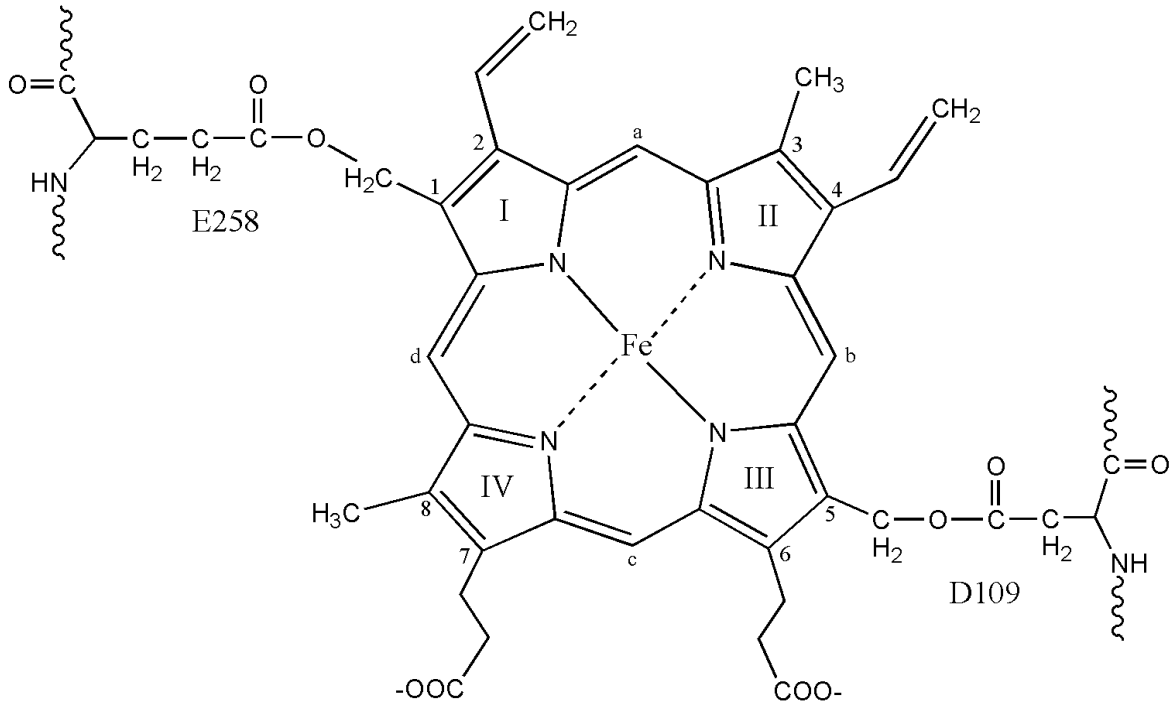


Figure 1-3 – Modified protoporphyrin IX of LPO. The methylated pyrrole rings I and III are covalently linked *via* ester bonds to Asp109 and Glu258. Waved lines symbolize the polypeptide chain of LPO. Figure created with ChemDrawUltra 8.0.

The proximal His and Asn (His351 and Asn437 in LPO) are conserved in all members of the second superfamily. In LPO His351 is the fifth coordination position with a distance of 2.2 Å to the iron (III), whereas Asn437 forms a hydrogen bond with the proximal histidine [Singh *et al*., 2009]. On the distal side the three residues His108 (responsible for heterolytic hydrogen peroxide cleavage), Gln105 (involved in halide binding) and Arg255 are fully conserved (Figure 1-4). These residues form hydrogen bonds with all in all four water molecules in the active cavity of LPO. One water molecule is located mid-way between His108 and iron (III), though according to the distances (His108 – H_2O 2.6 Å and H_2O – Fe (III) 2.7 Å) it is suggested that the water molecule is stronger bound to the distal histidine than to the iron [Zederbauer *et al.,* 2007]. The different hydrogen bonds, between the three residues and the four water molecules, are thought to be a hydrogen bond chain that conducts protons away from the distal histidine [Dunford, 1999].

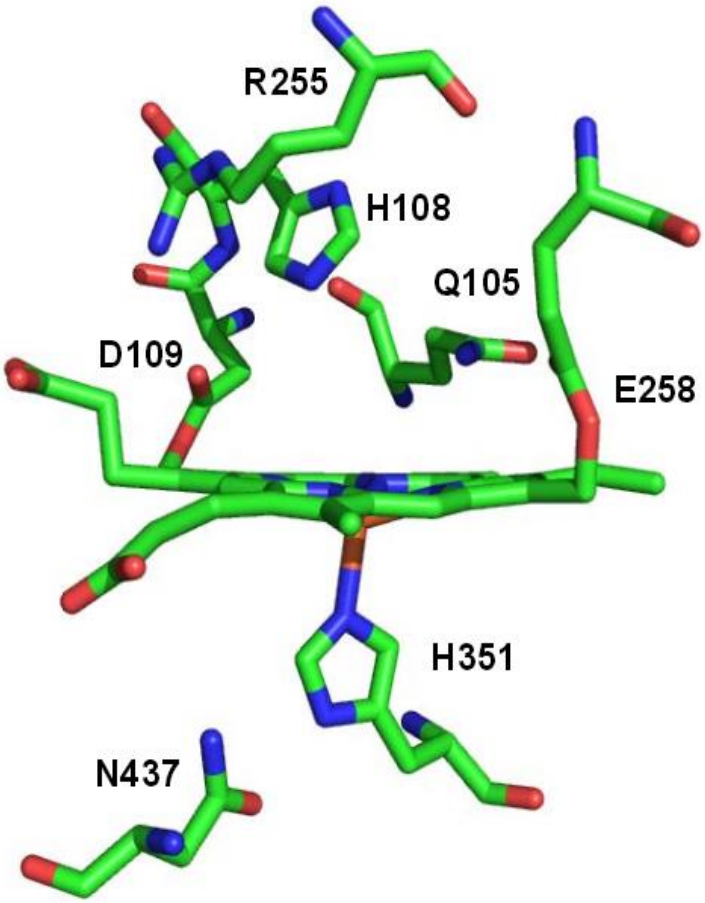


Figure 1-4 - Active side of bovine LPO, with the crucial amino acids at the distal (above the heme) and proximal (below the heme) side. Two covalent bonds are formed between heme *b* and the protein (D109 and E258). H351 at the proximal side is the fifth position that coordinates iron (III) in the centre of heme *b*. R255, H108 and Q105 play an important role in substrate binding and electron transfer.

1.3. Reaction Mechanism of Peroxidases

Peroxidases can heterolytically cleave peroxidic bonds. Many of these enzymes use hydrogen peroxide as substrate, which is reduced to water, but others are more active with organic hydroperoxides. Regarding peroxidase substrates, peroxidases have a great variety of one- and/or two-electron donors, these can be organic (AH_2) and inorganic molecules (e.g. halides, X^-). It is often a great problem to identify the native substrate of peroxidases; in question of LspPOX no appropriate substrate is found yet. The products are on the one hand organic radicals and on the other hypohalous acids according to Reaction 1 and 2.

$$H_2O_2 + X^- + H^+ \rightarrow H_2O + HOX \qquad \text{Reaction 1}$$

$$H_2O_2 + 2\,AH_2 \rightarrow 2\,H_2O + 2\,AH^{\cdot} \qquad \text{Reaction 2}$$

Both overalls reactions can be broken down into several individual steps, resulting in the halogenation cycle (reaction 1) and the peroxidation cycle (reaction 2). Due to the change of oxidation- and spin-status of heme peroxidases during these reactions, both cycles with their intermediate states and interconversions can be observed spectro photometrically.

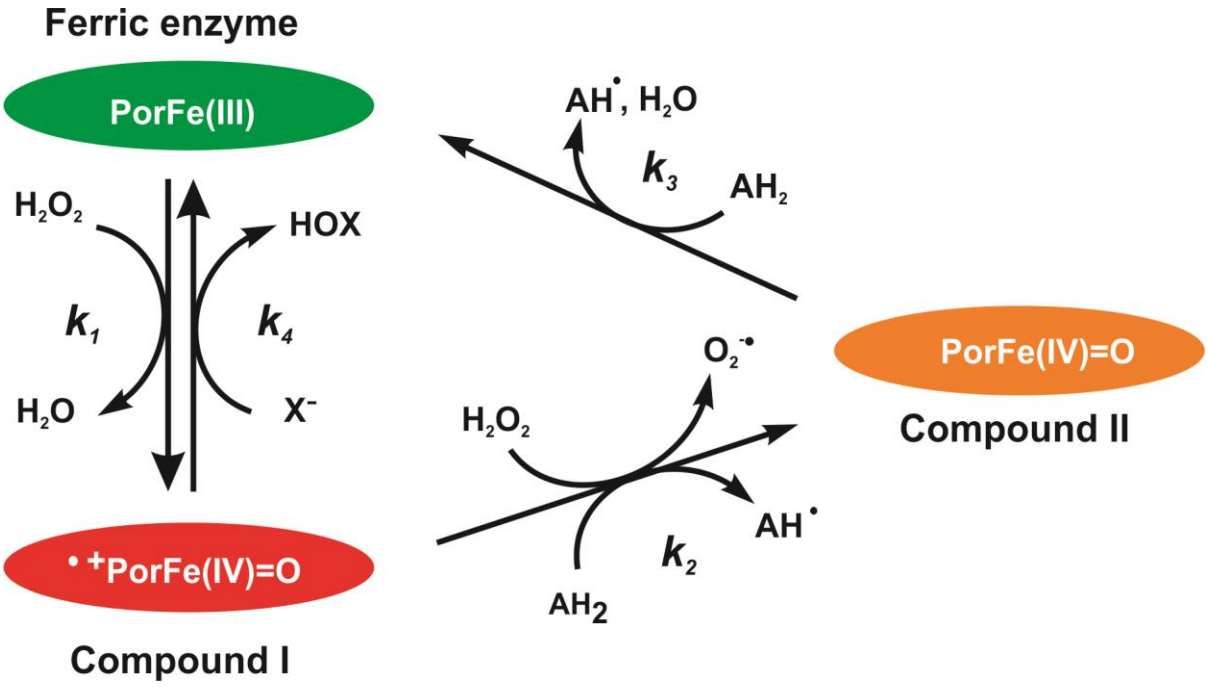


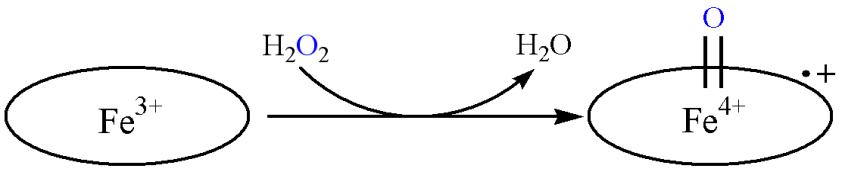
Figure 1-5 – Reaction mechanism of mammalian peroxidases. The native ferric enzyme is located in the left upper corner. Halogenation cycle is described in reaction k_1 and k_4. Peroxidation cycle is described in reaction k_1, k_2 and k_3.

1.3.1. HALOGENATION CYCLE

Mammalian peroxidases (LPO, EPO and MPO) efficiently oxidize two-electron donors like iodide, bromide and thiocyanate; only MPO is able to oxidize even chloride, because of the higher redox potential of the enzyme [Harrison and Schultz, 1976].

The halogenation cycle starts with the oxidation of hydrogen peroxide to water and the formation of Compound I (Reaction 1a). The Compound I formation runs as follows: the distal deprotonated histidine binds hydrogen then the anionic peroxide attaches to the iron followed by the heterolytic cleavage of the peroxide. One of the oxidized equivalents is stored as oxyferryl moiety on the iron. The second equivalent can either form a porphyryl radical cation or it can be transferred to the protein matrix by forming a protein-radical.

Reaction 1a:

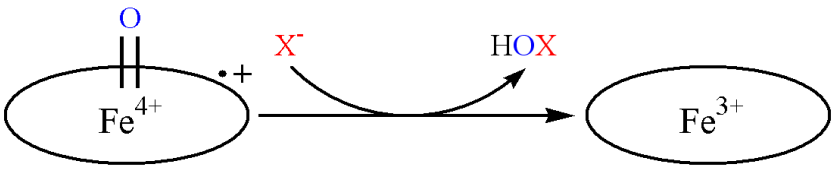


$$[\mathrm{Fe(III)\ (Por)}] + \mathrm{H_2O_2} \rightarrow [\mathrm{Fe(IV) = O(Por)^{\cdot +}}] + \mathrm{H_2O}$$

Figure 1-6 – Formation of Compound I. Ferric enzyme heterolytically cleaves hydrogen peroxide.

In the following step Compound I undergoes a two-electron reaction with halides or pseudohalides (Reaction 1b). Compound I is thereby reduced back to its ferric resting state and the halides are oxidized to their corresponding hypohalous acids.

Reaction 1b:



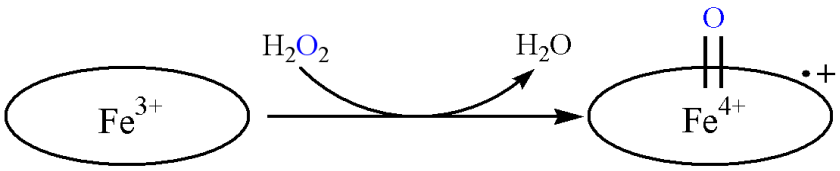
$$[Fe(IV) = O\ (Por)^{\cdot +}] + X^- \rightarrow [Fe(III)\ (Por)] + HOX$$

Figure 1-7 - Back reaction of the halogenation cycle. A halide (X-) is oxidized to a hypohalous acid (HOX).

1.3.2. PEROXIDATION CYCLE

The first step of the peroxidation cycle is identically to the halogenation cycle. Hydrogen peroxide is oxidized to water and Compound I is formed (Reaction 2a). The redox equivalents are stored as oxyferryl moiety and as radical on the heme *b* or the protein matrix.

Reaction 2a:

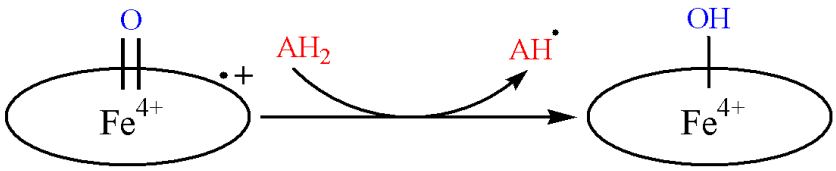


$$[\mathrm{Fe(III)\ (Por)}] + \mathrm{H_2O_2} \rightarrow [\mathrm{Fe(IV) = O\ (Por)^{\cdot +}}] + \mathrm{H_2O}$$

Figure 1-8 – Starting reaction of peroxidase cycle and formation of Compound I. Hydrogen peroxide is oxidized to water.

In the second step Compound I is reduced by an one-electron substrate to Compound II, an oxyferryl species (Reaction 2b). The substrate is oxidized to the corresponding free radical and is released from the active cavity.

Reaction 2b:

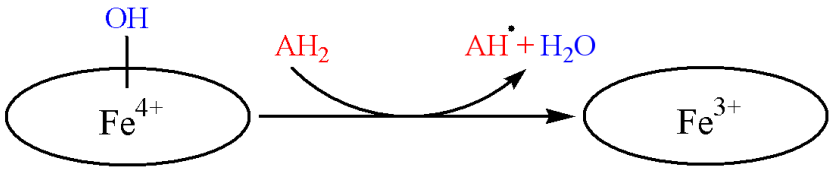


$$[Fe(IV) = O\ (Por)^{\cdot +}] + AH_2 \rightarrow [Fe(IV) - OH\ (Por)] + AH^{\cdot}$$

Figure 1-9 –Formation of Compound II from Compound I. A one-electron substrate is reduced and forms a free radical.

A second one electron reduction transfers Compound II back to the ferric resting state (Reaction 2c). Compound I and Compound II usually react with the same reducing substrate. A second substrate radical is formed, thus the stoichiometry of hydrogen peroxide and substrate is 1:2 over the entire peroxidation cycle.

Reaction 2c:



$$[Fe(IV) - OH\ (Por)] + AH_2 \rightarrow [Fe(III)\ (Por)] + AH^{\cdot}$$

Figure 1-10 –Reduction of the second substrate. The enzyme is back in its native ferric state.

1.4. PEROXIDASE FROM CYANOBACTERIUM *LYNGBYA* PCC8106

1.4.1. CYANOBACTERIA

Cyanobacteria are photosynthetic bacteria and often called blue-green algae, but this terminus might be misleading since algae are eukaryotes. All cyanobacteria contain chlorophyll *a* and phycobilines, which occur in blue. Blue phycobilines in combination with green chlorophyll *a* leads to the blue-greenish (cyan) coloring and consequently to the name of these bacteria. The photosynthetic mechanism of cyanobacteria is very similar to that of algae and plant chloroplast, and according to endosymbiotic theory, chloroplasts found in algae and plants evolved from cyanobacterial ancestors. Cyanobacteria are ubiquitary and exist in almost every terrestrial and aquatic habitat [Herrero and Flores, 2008]. They are usually unicellular (a typical cell varies between 0.5 to 1 µm), though they often grow in colonies as filaments (Figure 1-11) [Madigan *et al.,* 2006]. About 3.5 billion years ago the oldest known fossil of a cyanobacterium was found in Archaean rocks in Western Australia [Stanley, 2004].

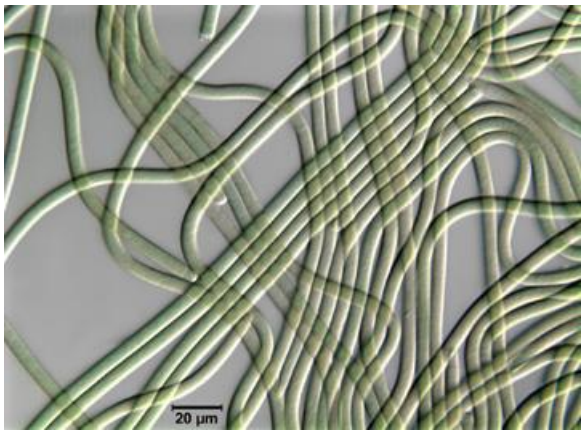


Figure 1-11 – Cyanobacteria forming long filaments.

The atmosphere of the earth changed between approximately 2.4 billion and 0.5 billion years ago from a reducing (anoxic) to an oxidizing (oxic) one [Holland, 2006]. Cyanobacteria might be the most important group of organism that is responsible for this transition. Some are capable to fix atmospheric nitrogen with their nitrogenase enzyme system which might be located in heterocysts; as primary producers they play a crucial role in the carbon cycle; and as by-product of photosynthesis they produce gaseous oxygen [reviewed in Grula, 2006]. These abilities changed the atmosphere in a way that made new physiologies and morphologies possible.

1.4.2. *LYNGBYA* PCC8106

Cyanobacteria are sub-divided into five groups according to their morphology. The genus *Lyngbya* is located in the third group of the Oscillatoriaceae. Oscilatoric cells form unbranching cylindrical filaments (trichomes) up to 10 cm long; these trichomes often become tangled with other filaments and form large mats (Figure 1-12). The reproduction of *Lyngbya* happens asexually. The filamentous cells break apart and each cell forms a new filament.

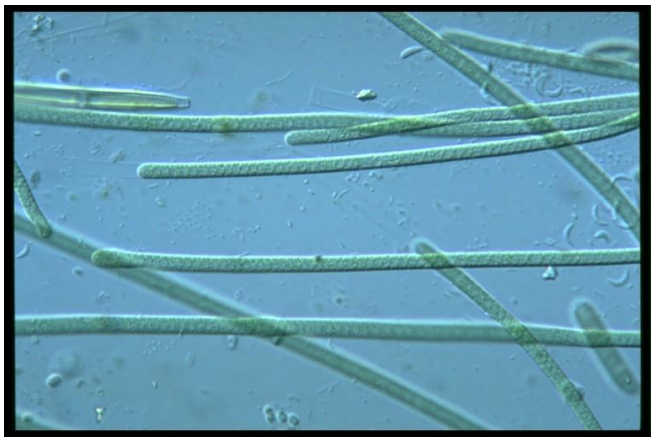


Figure 1-12 – Microscopic view of *Lyngbya*.

1.4.3. *LYNGBYA* PCC8106 PEROXIDASE (LSPPOX)

Lyngbya sp. (PCC 8106) peroxidase (LspPOX) is a cyanobacterial peroxidase with high homology to lactoperoxidase (LPO). Auer *et al.,* 2013, conducted structural alignments of 20 peroxidockerins (subfamily 6) and the known structure of bovine LPO. A high level of conservation was found both on the distal and on the proximal heme side. The distal important residues histidine and glutamine (H_2O_2 cleavage and halide binding) as well as two aspartate (involved in Ca^{2+} binding) residues are embedded in a conserved helix α2 found in all analyzed peroxidockerins. Furthermore on the distal side, the aspartate and glutamate (responsible for the covalent links in LPO) are conserved in a region before a helix α8. On the proximal side the heme coordinating histidine and its neighbor asparagine can be found in all investigated peroxidockerins. Auer *et al.,* 2013, came to the conclusion that the active site structure of halogenating heme peroxidases is strongly conserved. Thus LspPOX with high homology to LPO is an ideal candidate to start recombinant expression for further biochemical and physiological investigations and serves as model protein for mammalian peroxidases.

Furthermore it is a striking fact that a bacterial enzyme like LspPOX might be able to form posttranslational covalent links between the protein and the prosthetic heme *b*, as the mammalian counterparts do.

2. AIMS OF INVESTIGATION

An enduring problem in analyzing mammalian peroxidases like LPO, EPO or MPO is the fact that only small amounts of the enzymes can be produced recombinantly in either insect cells or animal cell factories. Beside poor yield and the time-consuming expression in cell factories, unsatisfying heme occupancy is a further problem when producing mammalian peroxidases.

In this thesis we focus on *Lyngbya sp.* peroxidase (LspPOX), a cyanobacterial peroxidase with high homology to LPO. We take advantage of this homology and want to use LspPOX as a model protein for LPO and other mammalian peroxidases. Establishing an expression system with *Escherichia coli* solves many problems compared to cell factories. Beside economic reasons such as lower price and shorter length of one production batch, expression of LspPOX with *E. coli* promises higher yields, better heme incorporation and easier preparation of LspPOX mutants. Following expression, a preferably fast and basic purification protocol has to be established. Beside essential procedures as concentration and desalting, we try to get along with only one chromatography step.

LspPOX forms autocatalytically covalent links between heme *b* and the polypeptide chain. The amount of these linkages is increased when the enzyme is treated with the 15 molar excess of hydrogen peroxide. First of all we want to identify these covalent links with both a chemiluminescence based method and mass spectroscopy. The new linked form (LspPOX (H_2O_2)) seems to be faster in pre steady-state kinetics and more stable in comparison to the recombinant form LspPOX. Thus, the spectral change and its time traces of LspPOX and LspPOX (H_2O_2) after adding cyanide have to be measured in the conventional stopped-flow mixing mode. Circular dichroism spectroscopy and differential scanning calorimetry are the methods of choice to get more insight into thermal stability of both enzyme species.

Summarizing, a fast and easy expression and purification protocol has to be established for LspPOX. This protocol and the characterization data of recombinant LspPOX and LspPOX (H_2O_2) are the basis for the design of further mutants as well as biochemical and biophysical characterizations.

3. MATERIALS AND METHODS

3.1. CLONING OF RECOMBINANT PROTEIN

The peroxidase of *Lyngbya* PCC8106 (LspPOX) was expressed recombinantly in *E. coli*. The gene of LspPOX was isolated from a preexisting plasmid *via* polymerase chain reaction (PCR). With a second PCR affinity tags on the C- and N-terminal site of the protein were added. These tags can be used to purify the protein from the crude biologically source using affinity chromatography. Furthermore overlapping regions were added for cloning with Gibson Assembly. The insert was cloned into a pET-52b(+) vector. Then the plasmid was transformed into chemically competent *E. coli* cells.

3.1.1. VECTOR FOR TRANSFORMATION INTO *E. COLI*

The vector pET-52b(+) from Novagen (Figure 3-1) was used for cloning and expression in *E. coli* cells. It possesses a gene for beta-lactamase that provides resistance to ampicillin and a lac operon to induce expression of inserted proteins with IPTG or lactose.

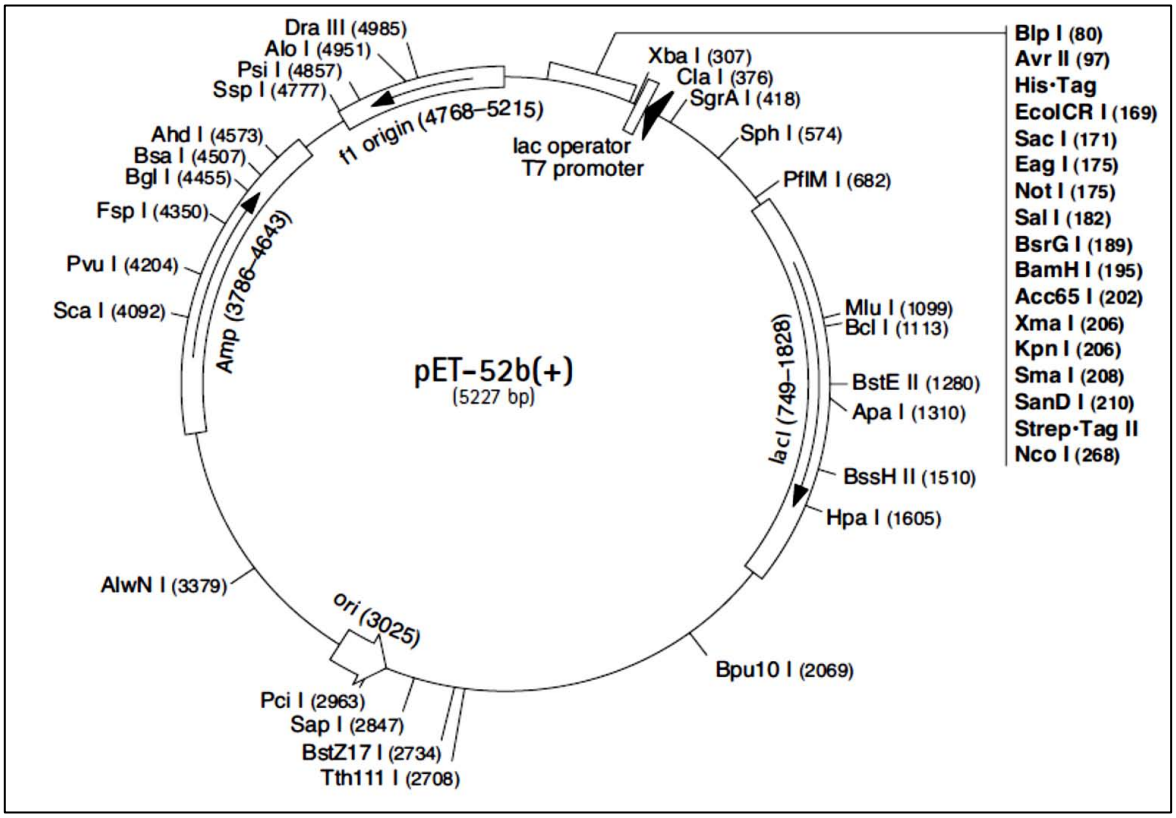


Figure 3-1 - A pET-52b(+) vector map with unique restriction sites and their positions, origin of replication (ori), ampicillin resistance gene (Amp), lac operator and T7 promoter.

The multiple cloning site (Figure 3-2) contains an N-terminal StrepTag II followed by a recognition site for human rhinovirus (HRV) 3C protease for fusion tag removal. At the C-terminal end of the multiple cloning site, an optional thrombin binding site is located followed by a HisTag coding sequence. Genes cloned into pET-52b(+) vectors are under the control of bacteriophage T7 transcription and translation signals. Expression of recombinant proteins is induced by providing a source of T7 RNA polymerase in the *E. coli* host cells.

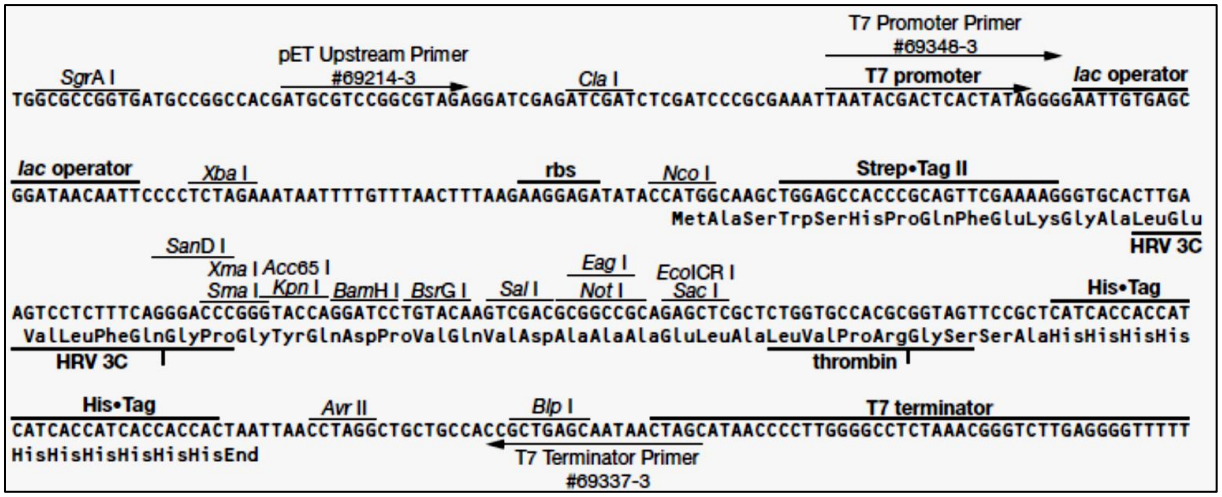


Figure 3-2 - Multiple cloning site of pET-52b(+) with T7 promoter and terminator site, lac operator, ribosome binding site (rbs), StrepTag II and HisTag for protein purification, HRV 3C and thrombin cleavage site and different endonuclease recognition sites in non-bold letters.

Material and Equipment:

pET-52b(+) (Novagen)

3.1.2. Restriction Enzyme Digest

A restriction enzyme is a bacterial or archaeal enzyme that cuts DNA. Each restriction enzyme cuts at a specific nucleotide sequence also known as recognition site. Originally these enzymes are used as defense mechanism against invading viruses, in laboratory they are an important tool for cloning issues in biotechnology.

To perform a restriction enzyme digest the double stranded DNA has to be incubated with the appropriate restriction enzymes in a suitable buffer recommended by the supplier. To save time, it is possible to digest DNA with two restriction enzymes simultaneously (double digestion). A buffer for both enzymes and the reaction temperature with the best enzyme performance are recommended by the supplier. In order to prepare the vector for further cloning issues pET-52b(+) was on the one

hand incubated with AvrII and NcoI and on the other with AvrII and BstBI. Table 3-1 shows all used restriction enzymes.

Table 3-1- List of restriction enzymes used for digestion of pET-52b(+), with their recognition site and optimal conditions.

	Recognition site	Temperature	Buffer	Concentration	Producer
AvrII	5‘ – C\CTAGG – 3‘	37 °C	CutSmart Buffer	5 units/µL	NEB
NcoI	5‘ – C\CATGG – 3‘	37 °C	NEBuffer 3.1	10 units/µL	NEB
BstBI	5‘ – TT\CGAA – 3‘	65 °C	CutSmart Buffer	20 units/µL	NEB

Procedure:

For double digestion with AvrII and NcoI prepare the reaction setup (Table 3-2 left side) on ice. Incubate the sample in a thermocycler at 37 °C for 2 h.

For double digestion with AvrII and BstBI set up all reaction components on ice (Table 3-2 right side) except BstBI. Transfer reaction tube from ice to a thermocycler and incubate the sample at 37 °C for 1.5 h. Afterwards add BstBI and incubate at 65 °C for another 1.5 h.

Table 3-2 – Double digestion of pET-52b(+) with AvrII/NcoI (left) and AvrII/BstBI (right).

Reagent		Final
DNA pET-52b(+)	30 µL	1.53 µg
CutSmartBuffer 10x	5 µL	1 x
BSA 10x	5 µL	1 x
AvrII	2.5 µL	10 Units
NcoI	2.5 µL	25 Units
HQ-H_2O	5 µL	
Total	50 µL	

Reagent		Final
DNA pET-52b(+)	30 µL	1.50 µg
CutSmartBuffer 10x	5 µL	1 x
BSA 10x	5 µL	1 x
AvrII	5 µL	20 Units
BstBI	1 µL	20 Units
HQ-H_2O	4 µL	
Total	50 µL	

Do not treat the sample with alkaline phosphatase after digestion since the cut DNA has two different sticky ends, prevention of the DNA from ligation is not necessary. Furthermore the gene of LspPOX has no recognition site of the used restriction enzymes, thus do not heat deactivation the enzymes.

To purify the digested DNA, perform a TAE agarose gel electrophoresis (see 3.1.5). Afterwards cut out the specific DNA gel bands and extract the DNA from the gel slices (see 3.1.6).

Material and Equipment:

Microcentrifuge Nr. 86272	(Sigma)	BstBI	(NewEngland Biolabs)
Arktik™ Thermal Cycler	(Thermo Scientific)	pET-52b(+)	(Novagen)
AvrII	(NewEngland Biolabs)	BSA	(Sigma)
NcoI	(NewEngland Biolabs)	CutSmartBuffer 10x	(NewEngland Biolabs)

3.1.3. Preparation of Insert DNA

A plasmid vector pET-21b(+) with the wild type (wt) gene of LspPOX derived as starting point. Originally, the strain of *Lyngbya* sp. PCC8106 was ordered from the Pasteur Culture Collection and the peroxidase gene was amplified from the genomic DNA.

To achieve the insert DNA ready to clone into the vector two following PCR steps were performed (Figure 3-3). Aim of the first PCR step was isolating the coding region of wt LspPOX and adding the first nucleotides of the N-terminal StrepTag II and C-terminal HisTag. The product of the first PCR step served as template for the second PCR step. With the second PCR step the remaining nucleotides of the different fusion tags on the C- and N-terminal site were added, as well as the nucleotides for the overlapping region needed for Gibson Assembly (see 3.1.7). After each PCR the reaction mix was purified and analyzed (see 3.1.5).

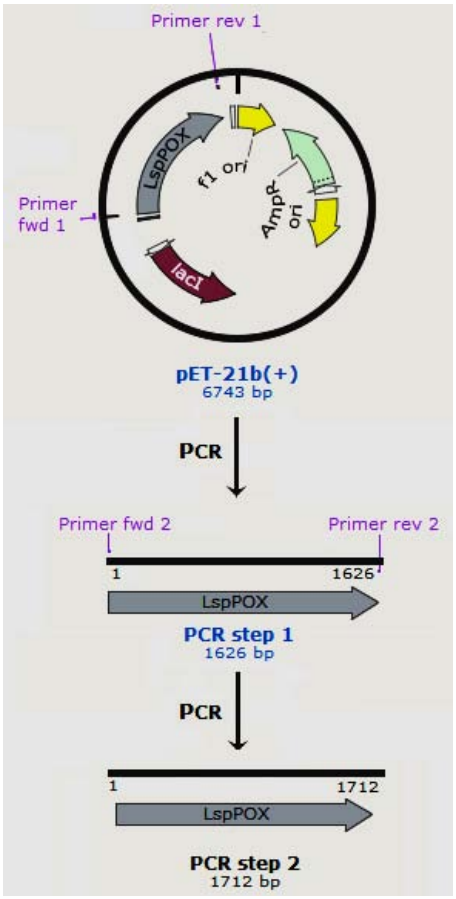


Figure 3-3 - Scheme of cloning LspPOX. The first PCR step was performed to isolate the gene of interest and the second PCR step to add affinity tags and overlapping regions for Gibson Assembly at the C- and N- terminal end.

Procedure:

The detailed procedure of polymerase chain reaction can be seen at 3.1.4.

Material and Equipment:

See Table 3-3.

Table 3-3 - All primers were designed and controlled with SnapGene and/or Gene Runner and synthesized by Sigma-Aldrich. "Primer fwd 1" and "Primer rev 1" were used for the first PCR and "Primer fwd 2" and "Primer rev 2" were used for the second PCR. Annealing bases of the primers are underlined. Overlapping regions for Gibson Assembly are bluish. HisTag is orange. StrepTag is red. Linker between the two affinity tags and the gene of interest are green. Stop and start codon are in capitalized LETTERS.

Primer Name	Sequence 5' - 3'
Primer fwd 1	gaattcatgattggcaaaccgggcaacagctatg
Primer rev 1	ggaactacccaccagaaatgcgttgc
Primer fwd 2	actttaagaaggagatatacATGcatcatcaccatcaccacgcttctattggcaaaccgggc
Primer rev 2	gctcagcggtggcagcagcctaggTTActttttcgaactgcgggtggctccaaccattgaccagaaatgcgttgctttg

3.1.4. Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a biochemical technology to amplify DNA *in vitro*, generating a high amount of a particular DNA sequence. It is used for a variety of applications, such as diagnosis of diseases, DNA profiling, analysis of genes, DNA cloning for sequencing and many more.

The method relies on thermal cycling; the optimal temperatures for DNA melting, primer DNA annealing and enzymatic replication are repeated up to 40 times. A typically PCR cycle is as followed: at first a double-stranded DNA sample (the so called template) denatures at high temperature. Subsequently two DNA primers anneal to the single-stranded DNA template at lower temperature. The primers are complementary to the 3' ends of the sense and anti-sense strand of the DNA target. In the last step of the cycle a DNA polymerase (such as Taq polymerase from *Thermus aquaticus*, which generates sticky ends, or Pfu polymerase from *Pyrococcus furiosus*, which generates blunt ends) binds to the primer-template formation and starts synthesizing a new DNA strand complementary to the DNA template. Since the newly formed double-stranded DNA serves as template in the next cycle, a high amount of a particular DNA sequences can be generated.

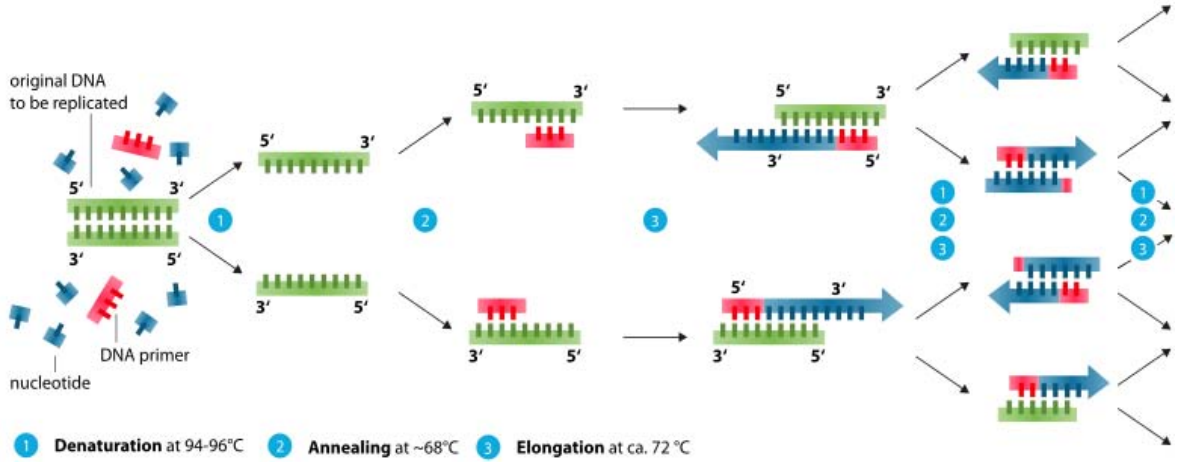


Figure 3-4 - A Schematic drawing of the PCR cycles. At first a DNA template denatures 1, then primers anneal 2 and a polymerase synthesizes a two new DNA strands 3, which serve as new templates in the next cycle.

Procedure:

Prepare the reaction setup (Table 3-4) on ice. Add Phusion polymerase last in order to prevent primer degradation caused by the exonuclease activity. Quickly transfer the reaction mix to a thermocycler preheated to the denaturation temperature. To purify the newly synthesized DNA perform a TAE agarose gel electrophoresis (see 3.1.5). Afterwards cut out the specific DNA gel bands and extract the DNA from the gel slices (see 3.1.6).

Table 3-4 - A 50 µL PCR setup on the left site and the thermocycling conditions for the PCR on the right site (annealing and elongation step at the same temperature).

Reagent		Final
HQ-H_2O	32.5 µL	
5x Phusion HF Buffer	10 µL	1 x
dNTPs (10 mM)	1 µL	200 µM
Primer fwd. (10 µM)	2.5 µL	0.5 µM
Primer rev. (10 µM)	2.5 µL	0.5 µM
DNA template	1 µL	50 ng/50µL
Phusion poymerase	0.5 µL	1 Unit/50 µL
Total	50 µL	

	Temperature	Duration
Inital	98 °C	30 s
35x cycles	98 °C	10 s
	72 °C	60 s
Final	72 °C	10 min
Hold	4 °C	5 min

Material and Equipment:

Arktik™ Thermal Cycler	(Thermo Scientific)	dNTPS	(NewEngland Biolabs)
Phusion HF DNA Polymerase	(NewEngland Biolabs)	synthesized Primers	(from Microsynth)
5x Phusion HF Buffer	(NewEngland Biolabs)	DNA template	

3.1.5. TAE-AGAROSE GEL ELECTROPHORESIS

Gel electrophoresis is a method used in biochemistry and molecular biology to separate molecules. TEA (tris based acetic acid EDTA) agarose gel electrophoresis is especially used for separation and purification of nucleic acids such as DNA or RNA. By applying an electric charge, nucleic acids move through an agarose matrix field. Smaller molecules move faster than larger molecules, because they migrate more easily through the matrix. Different staining methods are available to make DNA or RNA bands visible. Most common staining dyes are SYBR-Green I or ethidium bromide which intercalate into the planar bases of DNA or RNA. They fluoresce when exposed to ultraviolet light.

Procedure:

For each DNA purification prepare a fresh 1% TAE-gel (Table 3-5 left site). Add SYBR-green after melting agarose in a microwave. Pour the molten agarose into the flat mold for hardening. A comb forms pockets for the samples. Let the agarose gel harden for at least 30 min then carefully remove the comb.

Table 3-5 - Ingredients of a 1% TAE-agarose gel on the left site and the sample setup on the right site.

Reagent	
Agarose	1.2 g
TAE 50x	2.4 mL
HQ-H_2O	to 120 mL
SYBR-Green (1:10000)	12 µL

Reagent	
DNA Sample	50.0 µL
Mass Ruler Loading Dye	10.0 µL

Transfer the hard agarose gel into an electrophoresis chamber filled with TAE-buffer. Pipette the sample mixes (Table 3-5 right site) and a ready to use ladder mix into the pockets and apply a constant electric potential of 120 V for 50 min. The DNA bands are visible under UV-light. Cut out and purify the DNA bands (see 3.1.6).

Material and Equipment

LE Agarose			(Biozym)
TAE 50x :	242 g/L	Tris base	(Sigma)
	57.1 mL/L	acetic acid	(Sigma)
	18.61 g/L	EDTA	(Sigma)
	adjust pH to 8.3		
SYBR-Green Safe DNA gel stain			(Invitrogen)

Mass Ruler Ladder Mix	(Fermentas)
Mass Ruler DNA Loading Dye	(Fermentas)
Microdrive 1 Power Supply	(LKB)
PerfectBlue Gel System Mini L	(PEQLAB)
Gene Flash Bio Imaging	(Syngene)

3.1.6. DNA-Purification from Agarose Gel Electrophoresis

After an enzymatic reaction, an agarose gel electrophoresis is often needed. On the one hand for purification reasons and on the other hand for qualitative analysis. To purify the DNA from the gels, a gel band purification kit was used. In the first step of this kit, proteins denature and agarose dissolves, then DNA binds to a silica membrane, salts and other contaminations are removed and finally the DNA can be eluted in TE-buffer (for further cloning applications) or sterile nuclease free water (for sequencing only) (Figure 3-5).

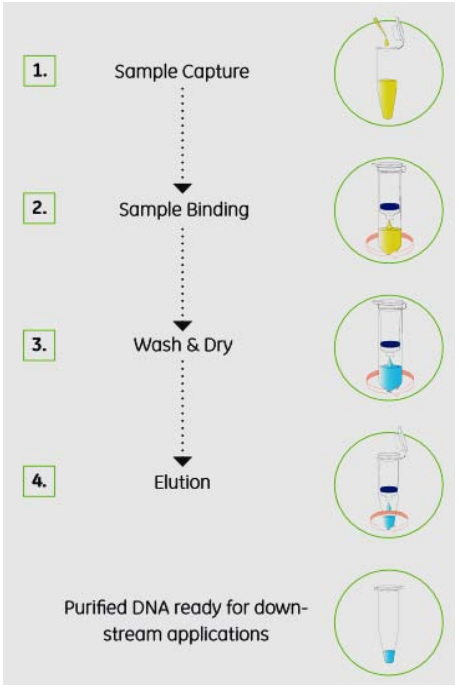


Figure 3-5 - Scheme of Gel Band Purification Kit, Figure from illustra GFX PCR DNA and Gel Band Purification Kit.

Procedure:

Excise the DNA band of interest from the agarose gel with a scalpel under UV light. Transfer the slice into a pre-weighted Eppendorf tube. For 100 µg of gel slice add 100 µL binding buffer. Incubate the mix at 60 °C until the agarose slice is completely melted. Pipette the sample-buffer mixture to a silica membrane column and incubate the column at room temperature for 1 minute. Spin down the DNA to bind it to the membrane. Discard the flow through. Afterwards add 500 µL washing buffer to the column. Spin down the washing buffer and remove the flow through with impurities. Finally, elute DNA with 50 µL (or less for higher concentration) TE-buffer or water.

Material and Equipment

Illustra PlasmidPrep Mini Spin Kit	(GE Healtcare)	Microcentrifuge	(Sigma)

3.1.7. GIBSON ASSEMBLY

Gibson Assembly developed by Dr. Daniel Gibson is a fast and easy tool to assemble multiple DNA fragments within a single tube. This is achieved with three enzymatic activities. First an exonuclease cuts back the 5' ends of the double stranded DNA fragments to create single-stranded 3' overhangs. These overhangs facilitate the annealing of complementary fragments (overlap region). Secondly a DNA polymerase extends the 3' ends to fill the gaps. And last but not least a DNA ligase seals the nicks in the assembled DNA (Figure 3-6).

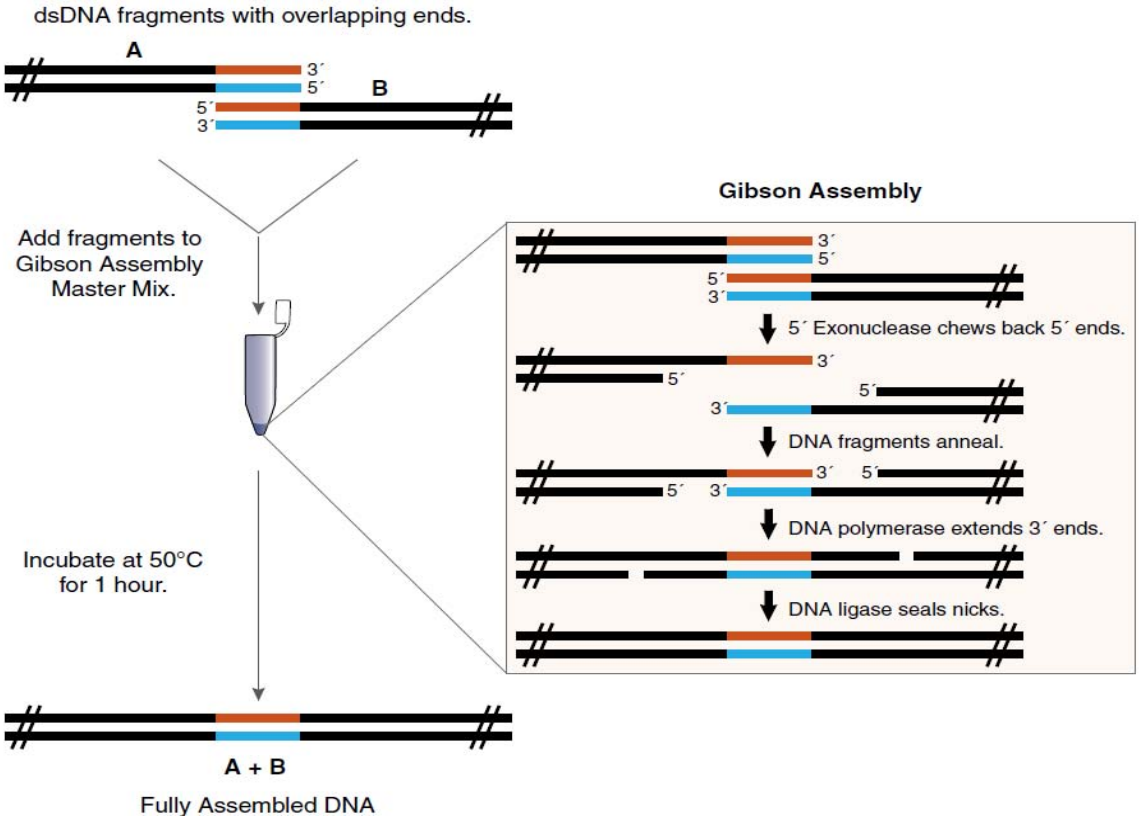


Figure 3-6 - Overview of the Gibson Assembly method. The overlapping nucleotides (in red and blue) are cut back at 5' end, the single stranded DNA fragments anneal and the missing nucleotides are filled up by a DNA polymerase and a DNA ligase. Figure from Gibson Assembly Master Mix Instruction Manuel.

Procedure:

Prepare the reaction mix shown in Table 3-6 on ice. The optimal amount of vector DNA (pET-52b(+)) should be about 100 ng per reaction and at least 2-fold excess of insert DNA (LspPOX) gene. After adding DNA and Gibson Assembly Master Mix fill up to a total volumn of 20 µL. Incubate the sample

in a thermocycler at 50 °C for 60 min. Afterwards store the samples at -20 °C or transform 5-α competent *E. coli* cells with 2 µL of the reaction mix (see 3.1.9).

Table 3-6 - Reaction setup for Gibson Assembly.

Reagent	Volume
DNA fragments	xx µL
Gibson Assembly Master Mix (2x)	10 µL
HQ-H_2O	xx - 10 µL
Total	20 µL

Material and Equipment:

Gibson Assembly® Cloning Kit	(New England BioLabs)	Arktik™ Thermal Cycler	(Thermo Scientific)
5-α competent *E. coli* cells	(New England BioLabs)		

3.1.8. Preparation of Chemically Competent Cells

For transformation, competent cells are needed which take up extracellular DNA from their environment. Thereby cells are treated to make them transiently permeable to DNA. Chemically competent cells are treated with high divalent cation concentration (often calcium chloride) and then exposed to a heat shock. Electrically competent cells are prepared in HEPES buffer and shocked with an electrically field. In either way, the cell membrane becomes permeable for plasmid DNA. In this work chemically competent *E. coli* cells were used because transformation protocol and preparation of the competent cells is easier and faster than for electrically competent cells.

Procedure:

Inoculate 10 mL of LB-medium with a single colony of *E. coli* TOP 10 cells from an LB agar plate. Since this strain has no plasmid or vector with antibiotic resistance, no antibiotics have to be added to the medium. Incubate the culture over night at 37 °C. 100 µL of the overnight culture serve as inoculum for 50 mL LB medium. Incubate at 37 °C until OD_{600} reaches 0.6. All following steps are done at 4 °C. Spin down the cells and discard the supernatant and dissolve the pellet in 15 mL cold 0.1 M $CaCl_2$. Spin down again, discard supernatant and dissolve the pellet in 2 mL 0.1 M $CaCl_2$ containing 15% glycerol. Prepare cell suspension to 50 µL aliquots and freeze them with liquid nitrogen, before storing at -80 °C.

Test chemically competent cells on agar plates with different antibiotics such as ampicillin or kanamycin. If colonies are visible, either competent cells are contaminated or an unwanted plasmid was taken up. Additionally test competent cells with pUC19 vector for transformation efficiency.

Material and Equipment:

E. coli Top 10	(Invitrogen)	0.1 M $CaCl_2$	
LB-agar plates		Microcentrifuge	(Sigma)
LB media			

3.1.9. TRANSFORMATION

During transformation a bacterial cell takes up exogenous genetic material from the surroundings through the cell membrane. *In vivo* transformation can occur naturally, but it can also be affected by artificial means, nevertheless bacteria must be in the state of competence. Competent cells in biotechnology are usually exposed to conditions that do not occur in nature, such as high cation concentrations connected with a heat shock, or cells are briefly shocked with an electric field, which makes the cell membrane permeable for all types of DNA. After transformation the bacterial cells have to be incubated in an optimal medium for recovery, because the state of competence and the transformation cause massive cell stress.

Procedure:

Carefully thaw the chemically competent cells (*E. coli* DH5α after Gibson Assembly, *E. coli* BL21 for expression or *E. coli* TOP 10 for plasmid production) on ice. Add plasmid DNA either from Gibson Assembly or from purification to the cells and incubate the mix on ice for 30 min. Heat-shock the cell DNA mix in a water bath at 42 °C for 30 s without shaking and immediately cool the transformation mix on ice for 2 min. Add preheated SOC-medium to the cells and incubate at 37 °C for 1 h. Plate different amounts of the cell solution on LB_{Amp} agar plates (e.g. 10 µL, 100 µL and 300 µL) and incubate them over night at 37 °C. Since pET-52b(+) contains an ampicillin resistance only transformed cells will grow on LB_{Amp} agar plates. If the amount of used DNA in the reaction is known the transformation efficiency can be calculated.

Table 3-7 - Transformation mix for chemically competent cells.

Reagent	Amount
competent cells	50 µL
Plasmid DNA	2 µL
SOC medium	400 µL

Material and Equipment:

E. coli strainBL21 DE3 Gold	(Invitrogen)	LB_{Amp} agar plates	
E. coli DH5α	(NewEngland Biolabs)	SOC-media	
Water bath		Plasmid DNA (from purification or Gibson Assembly)	

3.1.10. CRYO-CULTURES

When a specific cell line is used over a long time a double-staged cell banking system has to be established. This system consists of a master cell bank (MCB) and a working cell bank (WCB). Cells from MCB are expanded to form the WCB, which is used in all day processes. Both MCB and WCB are cryocultures, which are stored in ideal case separately from each other in -80 °C.

During this thesis, from each produced cell line a MCB was made-up and in a row a WCB. This WCB served as inoculum for all expressions.

Procedure:

Prepare a 20 LB_{Amp} medium overnight culture from a single colony after transformation. Pipette 1050 µL of the overnight culture and 450 µL 30% glycerol in a Cryo vial and mix gently. Store cryo-culture at -80 °C.

Material and Equipment:

LB_{Amp} medium		E. coli strain (single colony)
30% glycerol	(Sigma)	

3.1.11. DNA PURIFICATION FROM CULTURES

Before sequencing or further use of plasmid DNA, purification of the plasmid DNA is needed from the transformed cells. Therefore the illustra plasmidPrep Mini Spin Kit was used. Starting with an overnight culture of the *E. coli* cells containing the plasmid of interest, the cells are lysed and pelleted. The supernatant containing plasmid DNA is loaded onto a column, washed several times and eluted to achieve purified plasmid DNA.

Procedure:

According to illustra plasmidPrep Mini Spin Kit, spin down cells of 3 mL LB_{Amp} overnight culture (37 °C, 180 rpm) and discard the supernatant. Lyse cells to release plasmid DNA from cytosol. Spin down cell lysate and load supernatant onto a column. After a washing step, elute plasmid DNA. Measure the DNA concentration with a nanodrop photometer and store DNA at -20 °C.

Material and Equipment:

Illustra plasmidPrep Mini Spin Kit	(GE Healthcare)	LB_{Amp} media	
Microcentrifuge	(Sigma)	NanoDrop 1000 Spectrometer	(Peqlab)

3.1.12. SEQUENCING

Before using a plasmid DNA or PCR product, the precise order of nucleotides of the DNA molecule has to be determined. Due to an enormous development of sequencing methods in the last several years, DNA sequencing has become easier, cheaper and in order of magnitude faster.

The method of choice is the so called Sanger sequencing. This method relies on the incorporation of chain-terminating dideoxynucleotides (ddNTPs) by DNA polymerase during *in vitro* replication. Chain-terminating ddNTPs lack a 3'-OH group, which is required for prolonging a nucleotide sequence. Nowadays all four ddNTPs are labeled on the 5' end with four different fluorescent dyes. During DNA replication chain-terminating ddNTPs are randomly inserted to a newly synthesized DNA sequence and end replication. That leads to DNA strands with different lengths each with a labeled ddNTP at the 3' end. Following rounds of template DNA extension, the reaction mix is introduced into a gel capillary electrophoresis to separate the DNA fragments. The nucleotides appear as peaks with different retention times detected by a laser.

Procedure:

Pipette a small amount of purified DNA and a primer into a sterile tube. Send the reaction mix to Microsynth and wait for 1 day. Download sequencing results and align sequences with e.g. SnapGene or DNAStar Lasergene 7.

3.2. *E. COLI* STRAINS

Escherichia coli is a gram-negative, facultative anaerobic bacterium. The cells are rod-shaped, about 2 µm long and 0.25 to 1 µm in diameter. Their optimal growth temperature occurs at 37 °C. Most *E. coli* strains are harmless (they are part of the mammalian flora of the gut), but some strains are origin of serious food poisonings. In biotechnology, *E. coli* plays an important role because of its long history in laboratory and ease of manipulation. There are four strains of *E. coli* (K-12, B, C and W), which are frequently used in biotechnology and serve as model organism in microbiological studies.

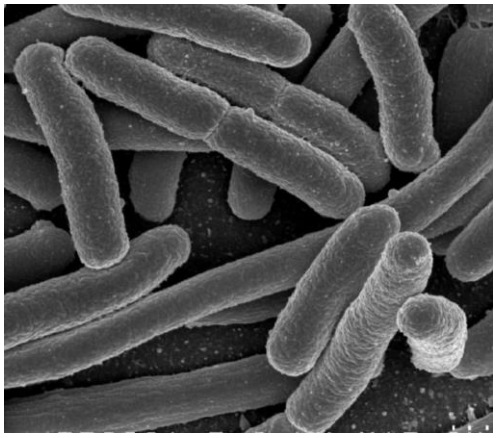


Figure 3-7 - Scanning electron micrograph of *Escherichia coli.*

3.2.1. *E. COLI* BL21 STAR (DE3)

For all expressions *E. coli* strain BL21 Star (DE3) from Invitrogen was used. This strain with DE3, a λ prophage, carries the T7 RNA polymerase gene and the $lacI^q$. Expression of T7 promoter containing plasmids is repressed until induction of IPTG or Lactose.

E. coli BL21 (DE3) genotype:

F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ rne131 λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])

3.2.2. *E. COLI* TOP 10

E. coli TOP 10 from Invitrogen Life Technologies was used for transformation and to prepare high amounts of the empty pET-52b(+) vector. Since *E. coli* TOP 10 is hsdR negative, unmethylated DNA from PCRs is more efficiently transformed. Furthermore, recA1 and endA1 carry a loss of function mutation for better plasmid propagation.

E. coli TOP 10 genotype:

F^- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ^-

3.2.3. *E. COLI* DH5ALPHA

E. coli DH5α from New England BioLabs was used for transformation of Gibson Assembly products. This *E. coli* strain was included in the Gibson Assembly Master Mix.

E. coli DH5α genotype:

F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17 (r_K^- m_K^+), λ^-

3.3. RECOMBINANT PROTEIN PRODUCTION

After cloning the gene of LspPOX into pET-52b(+) and transformation, protein production can be started. The recombinant protein was expressed in *E. coli* BL21 (DE3) Gold. An overnight culture in LB-medium served as inoculum for an expression culture with M9ZB media (both cultures at 37 °C). To the overnight culture and the expression medium ampicillin was added to avoid bacterial contaminations. Hemin was added to the culture additionally the temperature was reduced to 20 °C. Adding IPTG induced expression. After 4 hours cells were pelleted by centrifugation, frozen to -80 °C and after thawing lysed before further purification.

3.3.1. STERILE TECHNIQUE

When operating with cell material sterile techniques are required for two main reasons. At first, it is important to avoid contaminations of the cell material and the solutions or equipment in contact with the cells. Secondly, it is necessary to protect the operator from potential harmful material.

All operations with cells and solutions in contact with cells were either performed in the laminar flow hood or near the Bunsen burner flame. Furthermore, it is highly recommended to wear gloves and a laboratory coat during sterile working.

For operating with the laminar flow hood, fan and UV-lamp were turned on 15 min before working and the working place was cleaned with 70% ethanol. Additionally, every flask, vessel and other materials inserted into the laminar were cleaned with 70% ethanol. After working the bench has to be cleaned again with 70% ethanol and the fan and the UV-lamp were turned on for at least 30 min.

Material and Equipment:

Laminar Flow hood	(Heraeus Instruments)	UV lamp	(Osram HNS 15 watt)
70% Ethanol	(Merck)	Bunsen burner	

3.3.2. PREPARATION OF STOCK SOLUTIONS

All solid substances were weighted and dissolved in HQ-H_2O if not mentioned differently. Hemin is an iron-containing porphyrin, which has to be prepared freshly for each expression. It was dissolved in 0.5 M sodium hydroxide and filled up with HQ-H_2O. It is needed for improvement of heme incorporation during expression of heme containing proteins. The protease inhibitor,

phenylmethanesulfonflouride (PMSF) was prepared in 96% ethanol. All stock solutions were filtered through a 0.2 µm sterile capsule filter unit and stored at -20 °C or 4 °C.

Table 3-8 - Stock solutions, their concentrations and recommended storage conditions.

Reagent	Concentration	Storage
Hemin-chloride	100 ng/mL	4 °C
Ampicillin	5 mg/mL	-20 °C
IPTG	238 mg/mL	-20 °C
PMSF	35 mg/mL	-20 °C

Material and Equipment:

Isopropyl β-D-1-thiogalactopyranoside – IPTG	(Sigma)	Ampicillin sodium-salt	(Roth)
Ampicillin sodium-salt	(Roth)	NaOH	(Roth)
Phenylmethanesulfonfluoride – PMSF	(Sigma)	Sterile filter syringe 0.22 µm, P666.1	(Rotilabor)
Hemin	(Sigma)		

3.3.3. Preparation of Media and Buffers

A growth medium used for microbiological cultures contains all elements that the cells need for growth. A common non selective medium contains a carbon source such as glucose for cellular growth, water, different cations and anions (e.g. Na^+, Mg^{2+}, Cl^-, etc.) and a source of amino acids and nitrogen such as yeast extract. Selective media are often prepared for the growth of only selected microorganism and/or to avoid contaminations. For example, if an antibiotic (e.g. ampicillin) is added to the medium, only microorganisms with a resistance to this antibiotic are able to grow.

All solid substances were weighted and dissolved in HQ-H_2O if not mentioned differently. All buffers and media were sterilized in an autoclave with high pressure saturated steam at 121 °C for at least 20 min. The sterile media and buffers were stored at 4 °C.

Material and Equipment:

Mettler Toledo PB 3002-S/FACT		$MgSO_4*7H_2O$	(Merck)
pH-meter Radiometer PHM 92		Peptone	(Applichem)
Mettler Toledo AE 240		LB-Agar	(Sigma)
Magnetic stirrer IKAMAG RCT		Yeast extract	(Applichem)
Autoclave Fritz Grössner GLA 30		NaH_2PO_4	(Sigma)
NH_4Cl	(Sigma)	Glucose	(Sigma)
KH_2PO_4	(Roth)	NaCl	(Sigma)
$Na_2HPO_4*7H_2O$	(Sigma)		

3.3.3.1. LB MEDIUM AND LB AGAR

Lysogenic broth medium is widely used for cultivation of *Escherichia coli* since first published in 1951. It can be used as liquid medium (LB medium) as well as in the solid form (LB agar). Peptides are provided by yeast extract and peptone, whereas yeast extract is also a potent source for organic compounds helpful for bacterial growth such as vitamins and trace elements. Sodium chloride is added for osmotic balance. Before usage 1 mL ampicillin stock solution is added to 1 L LB medium (5 mg/L final concentration).

Table 3-9 - Composition of LB medium and LB agar.

LB medium/agar			
Peptone	1%	10	g/L
Yeast	0.5%	5	g/L
NaCl	1%	10	g/L
Agar	3.5%	35	g/L

3.3.3.2. M9ZB MEDIUM

For cultivation of recombinant strains of *Escherichia coli* M9ZB growth medium is often used. As buffering agents sodium and potassium phosphate are added; as nitrogen source M9ZB contains ammonium chloride, whereas sodium chloride provides osmotic support. Glucose is added as carbon source to supply carbohydrates. Magnesium increases the growth of recombinant *E. coli*. M9ZB consists of four solutions prepared and sterilized separately to avoid Maillard reaction. They are mixed together just before application. Before usage 1 mL ampicillin stock solution is added to 1 L M9ZB medium (5 mg/L final concentration).

Table 3-10 - Composition of M9ZB medium and the four solutions.

M9ZB-Medium	
Solution A	800 mL
Solution B	100 mL
Solution C	100 mL
Solution D	1 mL

Solution A	
Pepton	24 g/L
Hefexetrakt	12 g/L
NaCl	6 g/L
NH_4Cl	1.2 g/L

Solution B	
KH_2PO_4	30 g/L
$Na_2HPO_4 * 7\ H_2O$	60 g/L

Solution C	
Glucose	50 g/L

Solution D	
$MgSO_4 * 7\ H_2O$	24.6 g/L

3.3.3.3. PHOSPHATE BUFFER

A buffer (e.g. phosphate buffer - PPi) is used to prevent changes in the pH of a solution. It is a mixture of a weak acid and the conjugated base or vice versa. For good buffering both concentration of the buffer must be sufficient and the pK_a must be close to the pH of the solution.

Procedure:

Prepare two separate solutions one of the acidic and one of the basic form of the phosphate buffer, both with the same desired concentration. Pour one of the solutions to the other while monitoring the pH with a pH electrode.

Table 3-11 - Weight samples of acidic and basic solution for phosphate buffer with different concentrations.

Buffer Concentration	**$Na_2HPO_4 \cdot 7H_2O$ solution**	**NaH_2PO_4 solution**
10 mM	2.68 g/L	1.20 g/L
20 mM	5.36 g/L	2.40 g/L
100 mM	26.81 g/L	12.00 g/L

3.3.4. CULTIVATION OF CELLS

Procedure:

Inoculate two Erlenmeyer flasks with 20 mL LB_{Amp} medium with transformed *E. coli* from a cryo-culture under sterile conditions. Incubate over night or at least 16 h at 37 °C and 180 rpm.

Prepare four 2 L Erlenmeyer flasks with 500 mL $M9ZB_{Amp}$ each. Inoculate each of the 2 L Erlenmeyer flasks with 6 mL of the overnight culture and incubate at 37 °C and 180 rpm until the absorbance at 600 nm (OD_{600}) reaches a value of 0.6 - 0.8. Add 5 mL of hemin stock solution to each 2 L flasks and lower the temperature to 20 °C. Incubate for 1 h at 20 °C with shaking at 180 rpm until the cultures reach the temperature. Then start expression by adding 0.5 mL IPTG stock solution (0.5 mM final concentration). After 4 h of expression at 20 °C and 180 rpm harvest cells.

Material and Equipment:

Electronic shaker	(Infors HT Ecotron 349635)	IPTG	(Sigma)
Automatic pipette	(Peqlab 103820050)	LB_{Amp} medium	
Erlenmeyer flasks		$M9ZB_{Amp}$ medium	
Lactose	(Sigma)		

3.3.5. HARVESTING OF CELLS

Procedure:

After protein expression harvest cells by centrifugation (3500 rpm, 20 min, 4 °C). Discard the supernatant, transfer the cell pellet into a 50 mL tube and centrifuge again (3500 rpm, 20 min, 4 °C). Discard the supernatant and store the cell pellet at -80 °C until starting with protein purification. Sterilize all supernatants before discarding.

Material and Equipment:

50 mL tubes	(Greiner Bio-One)	Autoclave	(Grössner GLA 30)
Centrifuge	(Sorvall RC 6 Plus A1548)	Freezer	(Thermo Scientific)

3.4. PURIFICATION OF RECOMBINANT PROTEIN

Since LspPOX has no signal peptide, the protein is expressed in the cytoplasm. Therefore cell lysis has to be performed before further purification. The purification starts with an affinity chromatography and is followed by freezing the pooled fractions at -30 °C. After thawing, the sample is centrifugated and then the supernatant is concentrated and desalted.

3.4.1. CELL LYSIS

Since LspPOX is not transported out of the cell into the culture supernatant, it is necessary to perform cell lysis. There are several methods of breaking down a cell, such as mechanically (e.g. French pressure cell press, ball mill or sonication), chemically and enzymatically (e.g. lysozyme). Usually combinations of the different methods are performed. Due to the disruption of the cell wall, cytoplasm and the protein of interest are released into the supernatant. To avoid proteolysis protease inhibitors are added before lysis (e.g. PMSF, pepstatin A, leupeptin heminsulfate salt, etc.).

Procedure:

Thaw the cell pellet and in the meantime prepare the lysis buffer (Table 3-12). Resuspend the pellet in 40 mL lysis buffer and keep the homogenized cell suspension on ice. Disrupt the cell wall and release the cellular contents by sonication with an ultrasonicator (5 times for 40 s with 50% pulses). Cool the cell suspension during the whole process and wait if necessary between the pulses.

After cell disruption, centrifuge the cell solution (17 000 rpm, 25 min, 4 °C). Among other proteins, LspPOX is located in the supernatant. Autoclave the cell pellet, which contains the cell debris, and discard it.

Dilute the supernatant (approximately 50 mL) with the same volume of Buffer A (Table 3-13). Filter the solution with a filter funnel (pore diameter: 0.45 µm), to remove remaining cell debris and then degas the solution by applying a vacuum for 15 min before starting metal ion affinity chromatography.

Table 3-12 - Buffer setup for lysis buffer.

Reagent	Amount
Tris/HCl 1 M, pH 8.0	2 mL
EDTA 0.5 M, pH 8.0	160 µL
Triton-X-100	40 µL
HQ-H_2O	40 mL
PMSF	200 µL
Protease Inhibtor cocktail	1 tablet

Material and Equipment:

Sonicator	(VibraCell CV 17 350W)	EDTA 0.5 M pH 8.0	146.12 g/L EDTA
Centrifuge	(Sorvall RC 6 Plus A 1548)		adjust to pH 8
Tris/HCl 1 M pH 8.0	121.14 g/L Tris base	Triton-X-100	(Merck)
	adjust to pH 8	Protease Inhibitor cocktail	(Sigma)

3.4.2. IMMOBILIZED METAL ION AFFINITY CHROMATOGRAPHY

Chromatography is used for the separation of mixtures. The mixture, dissolved in a mobile phase (liquid or gas), is carried through a stationary phase (solid or liquid). Separation of the various constituents of the mixture occurs due to different retention times in the stationary phase.

Metal chelating affinity chromatography (MCAC) uses the affinity of amino acids, in particular histidine, to various metals such as nickel, cobalt, zinc or copper. Sepharose (Separation Pharmacia-Agarose), the stationary phase consists of iminodiacetic acid groups, which act as a strong tridentate ligand for metal ions. At least six consecutive histidines usually at the C- or N-terminal end of the protein (His-tag) form a coordinate covalent bound to the immobilized metal ions. This interaction will selectively retain a protein with an exposed HisTag. All unbound or only slightly bound molecules can be washed away. Elution of the proteins is done by changing the pH and/or by adding a competitive ion, such as imidazole.

Procedure:

Pack the column as recommended by the supplier. Degas all buffers before usage in an ultrasonic bath or by applying a vacuum for 15 min (the second method is especially recommended for solutions containing the protein of interest). Purge the column with at least 2 column volumes (CV) of HQ-H_2O, then apply $NiCl_2$ to the column until the column gains a homogenous bluish color. Wash the column with at least 4 CV of HQ-H_2O to remove excess of metal ions. Equilibrate the column with 2 CV of buffer B. Then apply the supernatant solution (see 3.4.1) to the column and wash with buffer B until flow through is completely colorless. For better purification results, check the flow through

with an UV-Vis spectrometer and wash the column with buffer B until protein band at 280 nm vanishes.

After washing, elute the bound protein with a linear gradient of 45 mL (1.5 CV) Buffer B and 45 mL (1.5 CV) Buffer C. The protein will be eluted by the linear increase in the concentration of a competing ion, such as imidazole. Collect the eluate with a fraction collector, the fraction volume should not exceed 2 mL. To reveal which fraction contains the protein of interest, record an UV-Vis spectrum from each fraction. Store all fractions of choice at -20 °C overnight.

Strip of the immobilized metal ion by purging the column with at least 0.5 CV of 50 mM ETDA pH 8.0 or until the bluish color completely disappears. Wash column with 3 CV of HQ-H_2O to remove residual EDTA. To remove precipitated and hydrophobically bound proteins, purge column with 1 M NaOH, the contact time should be at least 1-2 h. Wash column with HQ-H_2O until pH of the flow through is neutral. Store the column in 20% ethanol.

Table 3-13 - Composition of buffers used for protein purification and immobilized metal ion affinity chromatography.

Buffer A				**Buffer B**				**Buffer C**			
Imidazol	40 mM	2.72	g/L	Imidazol	20 mM	1.36	g/L	Imidazol	250 mM	17.02	g/L
NaCl	1 M	58.44	g/L	NaCl	0.5 M	29.22	g/L	NaCl	0.5 m	29.22	g/L
Na_2HPO_4	134 mM	24.56	g/L	Na_2HPO_4	67 mM	12.28	g/L	Na_2HPO_4	67 mM	12.28	g/L
NaH_2PO_4		5.08	g/L	NaH_2PO_4		2.54	g/L	NaH_2PO_4		2.54	g/L

Material and Equipment:

Chelating Sepharose Fast Flow	(GE Healthcare)	$NiCl_2$	5 g/L	(Sigma)
30 mL Fast flow column	(GE Healthcare)	EDTA 50 mM	14.61 g/L	(Sigma)
Gradient former	(Biorad, Model 395)	NaOH 1 M	18 g/L	(Roth)
Filter Funnel reuseable	(Nalgene)	NaCl		(Sigma)
Membrane Type Hvlp 45 µm	(Milipore)	NaH_2PO_4		(Sigma)
Vacuumpump CVC2	(Vacuubrand)	Na_2HPO_4 *$7H_2O$		(Sigma)
Peristaltic pump P1	(GE Healhcare)	Imidazole		(Sigma)

3.4.3. FREEZING, CONCENTRATING AND DESALTING

After metal ion affinity chromatography the eluate contains a high salt and imidazole concentration. These molecules and ions might interfere with further protein characterizations. Therefore the frozen fractions have to be thawed and centrifugated. In this freezing step misfolded proteins participate and remain in the pellet after centrifugation. The supernatant with soluble LspPOX is concentrated and desalted. For concentration, the amount of solvent is reduced *via* CentriPreps by centrifugation. The increased hydrolytic pressure in the tubes forces particles, smaller than the pore

size of the membrane, into the filtrate collector. Molecules larger than the pore size remain in the sample container.

Desalting of LspPOX was done with PD-10 desalting columns, which allows a separation of high molecular weight substances form low molecular weight substances. The technique is based on gel filtration. Larger particles are excluded from the gel matrix and elute first, whereas smaller particles penetrate the pores and retain longer in the gel matrix.

Procedure:

Collect all fractions containing the protein of interest according to the UV-Vis spectra. Store the fractions at -30 °C overnight. Gently thaw the fractions and centrifuge (12000 rpm, 25 °C for 10 min). Discard the pellet and add up to 15 mL of the supernatant to the filter device of the CentriPreps. Centrifuge the filter device at 3000 rpm, 4 °C for 20 min. If the volume of the supernatant exceeds 15 mL, repeat the first step several times. The desired end volume is 2.5 mL.

To desalt the concentrate, equilibrate the PD-10 desalting column with 25 mL equilibration buffer (5 mM phosphate buffer, pH 7.0). Add 2.5 mL of the concentrate to the column and allow the sample to enter the column completely. Elute with 3.5 mL buffer (5 mM phosphate buffer, pH 7.0) and collect the eluate.

The desalted protein solution was concentrated once more with CentriPrep and stored at -80 °C.

Material and Equipment:

CentriPrep 30 K	(Millipore)	Phosphate buffer 5 mM	(Sigma)
PD-10 Desalting Columns	(GE Healthcare)	Centrifuge	(Sorvall RC 6 Plus)

3.5. PROTEIN CHARACTERIZATION

Several assays were performed for biochemical and kinetic characterization of LspPOX. Steady state kinetics were measured with different substrates over a wide pH range. Stopped flow spectroscopy showed the pre-steady state kinetics of LspPOX with cyanide. The existence of the covalent link between heme *b* and the protein was demonstrated with chemiluminescence, whereas the increase of the linkages after incubation with H_2O_2 was tested with mass spectroscopy. Thermal stability was examined with differential scanning calorimetry and circular dichroism, whereat CD revealed additional data about the secondary protein structure and the heme cavity. Along the way many SDS-PAGEs, Western Blots and UV-Vis spectroscopy measurements were performed to determine to quality and efficacy of the expressions and purifications.

3.5.1. SDS-PAGE

SDS-PAGE (Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis) is a widely used analytic method in biochemistry to separate protein-mixtures according to their electrophoretic properties. SDS, an anionic detergent, is added to the protein solution to linearize the protein, to cover the intrinsic charge of the different proteins and to apply a constant negative loading per unit mass. Additionally all samples are heated to 70 °C for 15 min for better linearization and denaturing of the proteins. The velocity of the proteins depends on ionic strength, viscosity of the gel and temperature. A molecular weight marker (ladder) has to be added to determine the molecular mass of the proteins.

Since proteins are mostly colorless, there are different staining methods for proteins such as Coomassie Brilliant Blue, Silver Staining or Western Blotting. During this work three SDS-PAGES were performed for each protein sample; one was stained with Coomassie Brilliant Blue and with the remaining gels two Western Blots (see 3.5.2) were performed.

Procedure:

Prepare sample setup as shown in Table 3-14 (left side) and heat samples at 70 °C for 10 min. Fill the buffer chambers with running buffer (Table 3-14 right side). Load the sample solution of the protein and proper molecular weight marker on the gel.

Table 3-14 - Sample setup for SDS-PAGE and running buffer.

Reagent	Amount
Sample 5 µM	3 µL
NuPAGE Smaple Buffer 4x	2.5 µL
HQ-H_2O	4.5 µL

Reagent	Amount
MOPS 50x running buffer	40 mL
HQ-H_2O	760 mL

If a constant electric field is applied, the proteins will travel towards the positive electrode. Stop electrophoresis when the running front reaches the end of the gel.

After electrophoresis open the gel chamber and wash the gel with water. First incubate the gel in Coomassie Blue staining solution (Table 3-15 left side) for 30 min at room temperature on a shaker and then thrice in Coomassie Blue destaining solution (Table 3-15 right side) for 15-20 min under the same conditions. When the gel is colorless except bluish protein bands, scan the gel and discard it.

Table 3-15 - Setup for Coomassie Blue Staining- and destaining-solution.

Coomassie Blue Staining Solution	
Reagent	**Amount**
Methanol	40%
Acetic Acid	10%
HQ-H_2O	50%
Coomassie Blue R-250	0.1%

Coomassie Blue Destaining Solution	
Reagent	**Amount**
Methanol	40%
Acetic Acid	10%
HQ-H_2O	50%

Material and Equipment:

NuPAGE® LDS Sample Buffer (4x)	(Novex)	Acetic Acid	(Merck)
NuPAGE® 4-12% Bis Tris Mini Gels	(Novex)	Coomassie Blue R-250	(Thermo Scientific)
NuPAGE Running Buffer (20x)	(MOPS, Invitrogen)	Power supply Model 1000/500	(Bio-Rad)
SeeBlue Plus2 Prestained Standard	(Novex)	Shaker	(Janke & Kunkel)
Mark12 Unstained Standard	(Novex)	Electrophoresis Cell Xcell SureLock	(Inivitrogen)
Methanol	(Merck)		

3.5.2. WESTERN BLOT

Blotting is a method to transfer proteins or nucleotides (DNA or RNA) onto a carrier such as nitrocellulose or nylon. Afterwards proteins or nucleotides are visualized by colorant staining (e.g. silver staining of proteins, labelling with chromophoric antibodies or radioactive molecules). The most common blotting methods are Western Blotting to transfer and detect proteins, Nothern Blotting for RNA and Southern Blotting for DNA.

For Western Blotting, protein bands from a SDS-PAGE are transferred to a nitrocellulose membrane by using an electric current to pull the proteins from the SDS-PAGE gel into the membrane. Then the

nitrocellulose membrane has to be blocked with bovine serum albumin or non-fat dry milk to reduce noise in the final product of the Western Blot. To stain the transferred proteins the membrane is incubated with an antibody (so called primary antibody) specific to a certain amino acid sequence (e.g. HisTag or StrepTag). Then a secondary antibody, conjugated to a chromophoric protein (such as horseradish peroxidase or alkaline phosphatase) binds to the primary antibody. In the end the conjugated protein and a staining solution colorize the specific bound proteins.

Procedure:

Use an iBlot Dry Blotting System to perform the transfer from a pre-run SDS-PAGE gel to a nitrocellulose membrane. Open the anode stack and place the stack on the iBlot device. Place the gel on the transfer membrane. Then place a pre-soaked (HQ-H_2O) filter paper on the gel and remove all air bubbles. Place the cathode stack on the filter paper and remove all air bubbles. Place the sponge on the lid, close the lid and start the electroblot (20 V constant for 7 min). Remove the filter paper and place it into the blocking buffer.

For staining the blotted filter paper, incubate the membrane in blocking buffer at room temperature for 1 h with shacking at 180 rpm. Then wash the membrane thrice in 10 mL binding buffer for 5 min. Add 10 mL binding buffer and 5 µL Anti-HisTag-Antibody (1:2000) or Anti-StrepTag-Antibody (1:20000) both produced in mouse and incubate the membrane at room temperature for 1 h with shaking at 180 rpm. Wash membrane twice with 10 mL binding buffer for 5 min. Incubate the membrane in 10 mL binding buffer and 0.5 µL anti-mouse-IgG antibody conjugated to alkaline phosphatase (AP) (1:20000) at room temperature for 1 h with shaking at 180 rpm. Wash membrane trice with binding buffer for 5 min. Add 10 mL AP buffer and develop Western Blot with BCIP and NBT (amounts recommended by the supplier) until bands become visible.

Table 3-16 - Set up for all solutions used for Western Blotting.

PBS (phosphate-buffered saline)		
Reagent	**Amount**	
NaCl	8	g/L
KCl	0.2	g/L
$Na_2HPO_4*7H_2O$	2.73	g/L
KH_2PO_4	0.24	g/L
adjust pH to 7.4		g/L

AP (alkaline phosphatase) Buffer		
Reagent	**Amount**	
Tris-Base	12.1	g/L
NaCl	5.85	g/L
$MgCl_2$	1.02	g/L
adjust pH to 9.5		

Blocking Buffer		
Reagent	**Amount**	
BSA (bovine serum albumin)	2	g
PBS	100	mL

Blocking Buffer		
Reagent	**Amount**	
PBS	100	mL
BSA (bovine serum albumin)	2	g
Tween-20	100	µL

Material and Equipment:

iBlot® Dry Blotting System	(Invitrogen)	$Na_2HPO_4*7H_2O$	(Sigma)
BSA	(Sigma)	KH_2PO_4	(Sigma)
KCl	(Sigma)	Tween-20	(Merck)
Tris base	(Sigma)	Penta His mouse AB BSA free	(Qiagen)
NaCl	(Sigma)	StrepMAB classic	(iba)
$MgCl_2$	(Sigma)	BCIP/NBT Color Development Substrate	(Promega)

3.5.3. Treatment with Hydrogen Peroxide

All further characterizations (except peroxidase activity) were done with LspPOX and LspPOX pretreated with the 15 fold stoichiometric excess of hydrogen peroxide for 1 h at room temperature. Hence, the pretreated enzyme is called LspPOX (H_2O_2). During this pretreatment, LspPOX consumes H_2O_2 and cycles between the ferric native state, Compound I and Compound II. This reaction leads to an increase of the covalent links between heme *b* and the protein.

Material and Equipment:

H_2O_2	(Sigma)

3.5.4. Spectral Properties

The absorbance spectrum of LspPOX shows the protein peak at 280 nm and the Soret peak at 412 nm. The Soret peak maximum depends on the heme coordination within the protein. The ratio between Soret peak and protein peak describes the purity number (Reinheitszahl, RZ) of the protein solution. Since the molar extinction coefficient of the ferric high spin LspPOX at the Soret maximum

of 412 nm is known ($\varepsilon_{412\ nm}$ = 94 670 M^{-1} cm^{-1}), the concentration of the protein solution can be calculated with the Lambert-Beer's law:

$E_\lambda = \varepsilon_\lambda * c * d$

E_λ = Absorbance of a sample at a certain wavelength

ε_λ = molar extinction coefficient [M^{-1} cm^{-1}]

c = concentration [mol L^{-1}]

d = length of light pathway [cm]

$RZ = E_{280} / E_{Heme}$

RZ = Reinheitszahl

E_{280} = Absorbance at 280 nm

E_{Heme} = Absorbance at Soret peak

Procedure:

For all spectra dilute the protein in 5 mM phosphate buffer pH 7.0. Pipette a sufficient amount of diluted protein solution into a quartz cuvette and set it into the light path of a UV-Vis spectrometer. Record a wavelength scan in the range of 250 - 750 nm at 25 °C. Observe absorbance at the protein peak and Soret maximum and calculate the concentration and purity number.

Material and Equipment:

Spectrophotometer (Zeiss Specord UV-VIS S10; Hitachi U-3900)	Quartz cuvettes, 10 mm path length (Starna, Type 9/B)
Phosphate buffer 5 mM (Sigma)	

3.5.5. CHEMILUMINESCENCE

Covalently bound heme was detected using a chemiluminescence based method. After performing an SDS-PAGE (see 3.5.1) of the purified LspPOX, the gel was blotted onto a nitrocellulose membrane (see 3.5.2). During SDS-PAGE the prosthetic group (heme) remains attached to the polypeptide chain, if the heme is covalently linked to it; without linkage the heme sweeps out of the gel. In this method luminol is used to detect the prosthetic group. Luminol is activated by hydrogen peroxide in the presence of a catalyst such as an iron present in heme. The activated luminol is glowing bluish and can be detected with an imaging system (ChemiDoc XRS+ BioRad).

Procedure:

Perform detection of the covalently bound heme with Clarity Western SCL Substrate from BioRad. Mix the peroxidase solution and the Luminol/enhancer solution in a 1:1 ratio. Prepare 0.1 mL of solution per cm^2 of membrane and incubate the membrane for at least 5 min in the solution. Take a

white light picture of the membrane and a chemiluminescence picture (exposure time 20 s). Merge both pictures.

Material and Equipment:

Clarity Western ECL Substrate	(BioRad)	ChemiDoc XRS+ Imaging System	(BioRad)

3.5.6. PEROXIDASE ACTIVITY

Peroxidase activity can be measured with the one-electron donors guaiacol (2-methoxyphenol) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) in phosphate buffer at pH 7.0. The peroxidase catalyzes the oxidation of the electron donor and the reduction of hydrogen peroxide to water. These assays are used to test the pH optimum of a peroxidase or to compare the activity of different peroxidases with each other. One unit of peroxidase activity is defined as the amount that oxidizes 1 µmol of substrate per min at defined pH values and 25 °C.

3.5.6.1. GUAIACOL ASSAY

One common method to test and quantify peroxidase activity is an assay using guaiacol as substrate. The peroxidase oxidizes two guaiacol molecules to guaiacol radicals; thereby hydrogen peroxide is reduced to water. Two guaiacol radicals form a 3,3'-dimehtoxy-4,4'-dihydroxydiphenyl which is oxidized to the chromophoric 3,3'-dimethoxy-4,4'-biphenoquinone (Figure 3-8). The increase of absorbance at 470 nm ($\varepsilon_{470\ nm}$ = 26.6. M^{-1} cm^{-1}) was followed at 25 °C. To determine the pH optimum of LspPOX, the activity was measure in the pH range 4.0 – 9.0.

Procedure:

Prepare reaction setup for the guaiacol assay shown in Table 3-17 in a stirred quartz cuvette. To start the reaction, add the enzyme in the end to the stirred solution. Measure the increase in absorbance at 470 nm for at least 90 s and calculate the slope of the increase. Repeat the measurement in the pH range of 4.0 - 9.0 with different buffers and perform three repeat determinations for each data point.

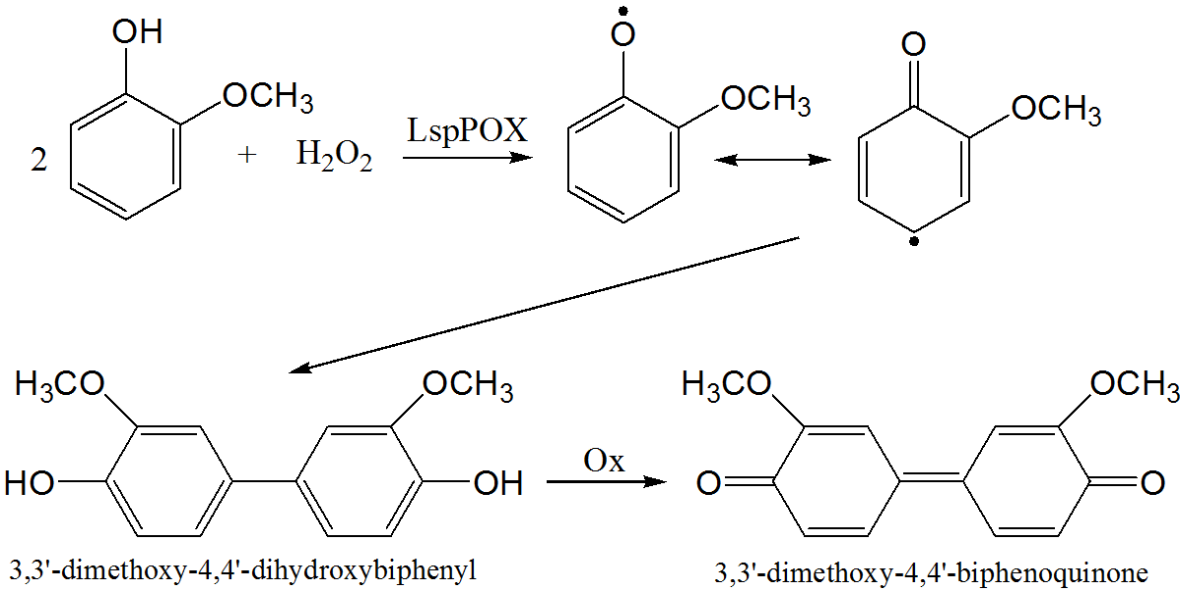


Figure 3-8 - Structure of guaiacol on the right upper corner. Peroxidase-catalyzed the oxidation of two guaiacol molecules to 3,3'-dimethoxy-4,4'-biphenoquinone and the reduction of hydrogen peroxide. Figure created with ChemDrawUltra 8.0.

Table 3-17 - Reaction mix of guaiacol assay.

Volume	Reagent	Final concentration
1 mL	100 mM PPi, varying pH	50 mM
200 µL	10 mM guaiacol	1 mM
200 µL	10 mM H_2O_2	1 mM
500 µL	HQ-H_2O	
100 µL	2 µM enzyme	0.1 µM
2 mL	Total	

Material and Equipment:

Spectrophotometer	(Zeiss Specord UV-VIS S10)	H_2O_2	(Sigma)
Quartz cuvettes 10 mm	(Starna, Type 9/B)	Phosphate Buffer pH 4.0 - 9.0	(Sigma)
Guaiacol	(Sigma)		

3.5.6.2. ABTS ASSAY

In the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay the peroxidase catalyzes the reduction of hydrogen peroxide and oxidation of ABTS to ABTS+● (Figure 3-9). The increase of absorbance at 414 nm ($\varepsilon_{414\ nm}$ = 36.0. M^{-1} cm^{-1}) can be measured at pH 7 at 25 °C. To determine the pH optimum of LspPOX, the activity was measure in the pH range 4.0 – 9.0.

Procedure:

Prepare reaction setup for the ABTS assay shown in Table 3-18 in a stirred quartz cuvette. To start the reaction, add the enzyme to the stirred solution. Measure the increase in absorbance at 414 nm for at least 90 s and calculate the slope of the increase. Repeat the measurement in the pH range of 4.0 – 9.0 with different buffers and perform three repeat determinations for each data point.

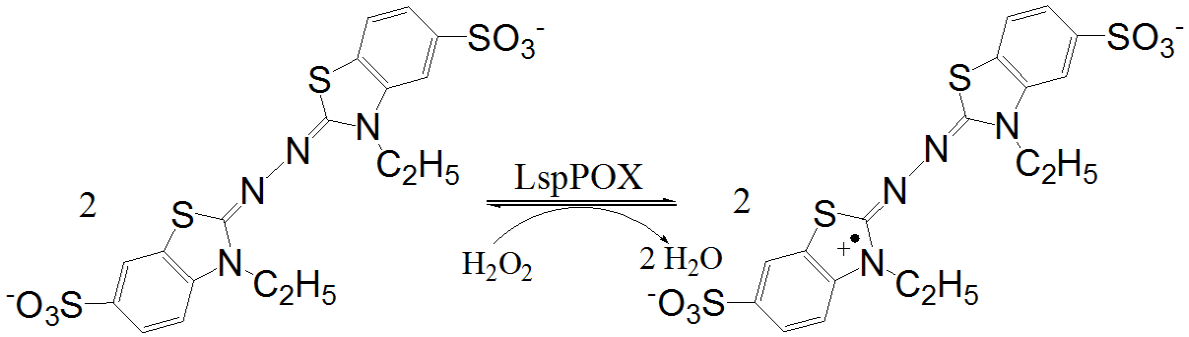


Figure 3-9 - Structure of ABTS on the left side. LspPOX catalyzes the oxidation of ABTS and reduction of hydrogen peroxide. Figure created with ChemDrawUltra 8.0.

Table 3-18 - Reaction mix of ABTS assay.

Volume	Reagent	Final concentration
1 mL	100 mM PPi, varying pH	50 mM
200 µL	10 mM ABTS	1 mM
200 µL	10 mM H_2O_2	1 mM
500 µL	HQ-H_2O	
100 µL	0.2 µM enzyme	10 nM
2 mL	Total	

Material and Equipment

Spectrophotometer	(Zeiss Specord UV-VIS S10)	Phosphate Buffer pH 4.0 - 9.0	(Sigma)
Quartz cuvettes 10 mm	(Starna, Type 9/B)	H_2O_2	(Sigma)
ABTS	(Sigma)		

3.5.7. DIFFERENTIAL SCANNING CALORIMETRY

Differential scanning calorimetry (DSC) is an analytical tool to characterize the stability of proteins and biomolecules. It directly measures the enthalpy and the temperature of thermally induced structural transitions in solution. Thus, DSC provides information on the thermal stability of proteins under different solvent conditions, such as pH, buffer composition or many other factors. For all measurements an identical buffer without protein has to be used as reference.

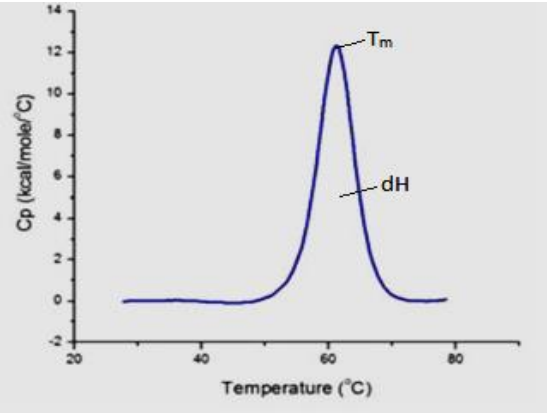


Figure 3-10 - Denaturation of a protein in the DSC. The peak maximum is the T_m and the area under the curve is the enthalpy dH of the process.

In detail, DSC measures the amount of heat, which is absorbed when a protein undergoes a conformation change between a native, biologically active form and an unstructured inactive form. The protein solution is placed in the calorimetric cell and is heated. Unless, melting of a protein is an endothermic event, more heat capacity is required referring to the same solution without protein. The calorimetric enthalpy dH is the area under the melting curve and the midpoint of this melting transition is known as the T_m (Figure 3-10).

Procedure:

Prepare at least 0.5 mL reaction setup shown in Table 3-19. Degas all solution before they are supplied to the sample plate, either in ultrasonic bath or by applying a vacuum. Pipette the air bubble free samples into the sample plate and program the DSC device (temperature gradient 20 – 100 °C, with a heating rate of 60 °C per hour, 4.1. bar cell pressure. and thrice washing of the heating chamber and the syringe between each measurement). Repeat the measurement with buffers in the pH range of 4.5 - 8.0.

Table 3-19 - Reaction setup for differential scanning calorimetry (DSC).

Reagent	Final concentration
LspPOX	5 µM
Phosphate buffer (diff. pH)	10 mM
Guanidine hydrochloride	0.5 mM

Material and Equipment:

DSC	(Microcal VP Capillary DSC)	Phosphate buffer	(Sigma)
Sample plates	(VP-CAP DSC, WEL 190010-010)	Guanidine hydrochloride	(Merck)

3.5.8. CIRCULAR DICHROISM

Circular dichroism (CD) spectroscopy is a technique to analyze optically active chiral molecules. The CD of molecules can be measured over a wide range of wavelengths. Far-UV-region CD (from 180 - 260 nm) delivers insight into the secondary structure of proteins, whereas UV-Vis CD (from 250 - 470 nm) is used to investigate charge transfer transitions.

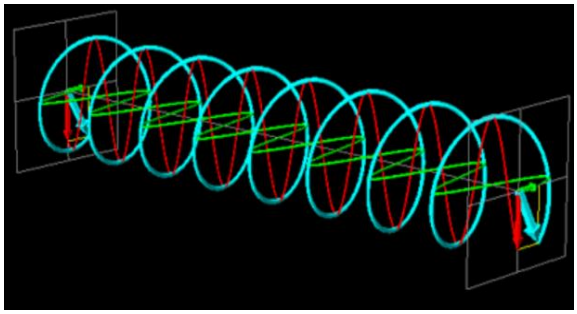


Figure 3-11 - A Left Circularly Polarized (R-CPL) wave (turquoise) is the sum of a horizontally polarized wave (green) and a vertically polarized wave (red) phase-delayed by a quarter wave.

Linearly polarized light oscillates in only one orientation. The sum of two polarized light states at right angle to each other can describe all other polarized light states. For example, if horizontally and vertically polarized light in phase overlap, the resulting light is also polarized light at 45 degrees. If the two light states (horizontal and vertical) are out of phase by a quarter wave, the resulting wave is a helix, also called circular polarized light (Figure 3-11). The helix can either be right handed (R-CPL) or left handed (L-CPL). The superposition of an L-CPL and an R-CPL can result in a linearly polarized wave. A chiral or optically active molecule absorbs R-PCL and L-CPL differently (e.g. 80% of R-CPL and 60% of L-CPL is absorbed), which is the basis for circular dichroism. The difference can be positive or negative, depending on whether R-PCL is absorbed to a higher extent (positive signal) or to a lesser extent (negative signal).

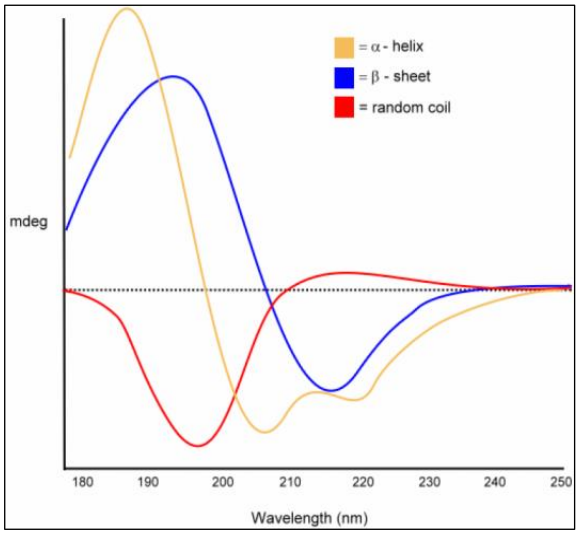


Figure 3-12 - CD spectra of a pure α-helix (yellow), a pure β-sheet (blue) and random coil (red) mixture.

A CD spectrum of a protein is not a sum of the individual amino acids (19 of 20 amino acids are chiral), but is highly influenced by the 3-dimensional structure of the protein itself. The secondary

structural elements of proteins such as α-helices and β-sheets are the most studied circular dichroism signatures (Figure 3-12). The percentage of each secondary structure in a protein can be measured by far-UV CD spectra.

Heating a sample and measuring the CD signal at a specific wavelength provides a thermo-CD. Thereby it is possible to observe the denaturation of the protein and disintegration of the secondary structures. LspPOX and LspPOX (H_2O_2) were heated from 20 °C to 85°C and the decrease of the signal at 208 nm (for the far-UV range) and 408 nm (for the UV-Vis range) was monitored.

The choice of the right specific wavelength is done with a standard CD spectrum. A wavelength with a strong signal is necessary; for LspPOX 208 nm in the far-UV and 408 nm in the UV-Vis range were chosen.

All CD spectra in the far-UV region and the UV-Vis region were done with the Chirascan CD Spectrometer from Applied Photophysics.

Procedure:

Prepare reaction setup shown in Table 3-20 (protein concentration for far-UV CD 2 µM and for UV-Vis CD 4 µM). Pipette the reaction mix into a quartz cuvette and place the cuvette into the CD device. For thermo-CD, heat the sample from 20 °C to 85 °C with a heating rate of 1 °C/min.

Table 3-20 - Reaction setup for circular dichroism spectroscopy.

Reagent	Final concentration
LspPOX	2 or 4 µM
Phosphate buffer pH 6.7	10 mM
Guanidine hydrochloride	0.5 mM

Material and Equipment:

Chirascan CD spectrometer	(Applied Photophysics)	Phosphate Buffer	(Sigma)
Guanidine hydrochloride	(Merck)		

3.5.9. STOPPED-FLOW SPECTROSCOPY

Stopped-flow spectroscopy is a technique to follow chemical reactions in solution in the time range of milliseconds. In general, two reactant solutions are rapidly mixed together through a mixing chamber. The flow is then suddenly arrested in an observation cell. Therein the change of a specific signal is recorded; usually by a coupled UV-VIS/fluorescence spectrometer or a CD spectrometer.

Just after mixing substrate and enzyme, no products or intermediates are formed. The next few milliseconds are called pre-steady-state. This is the time before an equilibrium is established. Every stopped flow spectrometer has a dead time (about 1 millisecond depending on the apparatus); this is the time between the end of mixing the two solutions and the start of observing the reaction kinetics. Analyzing these data provides information to determine reaction rates, reaction mechanisms or information on short-lived reaction intermediates.

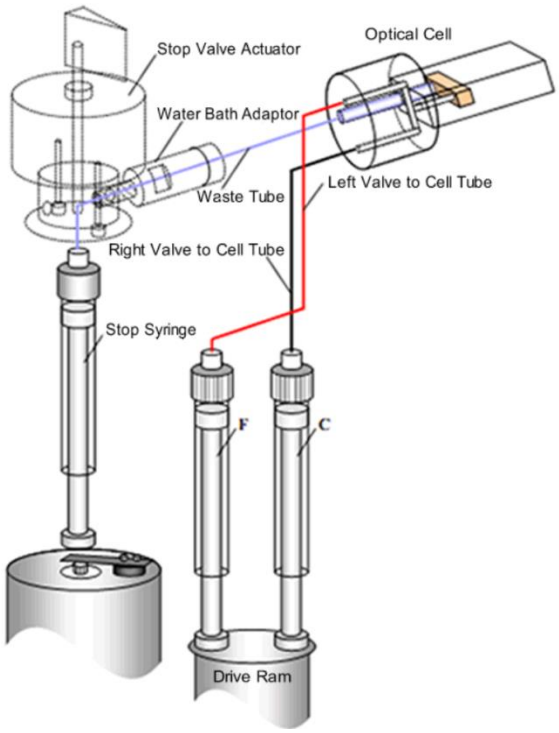


Figure 3-13 - Schematic layout of a mixing module used in stopped flow apparatus. The drive ram pushes two reagents in syringe F and C into the optical cell. This pushes the old reagents out of the optical cell and fills the stop syringe until the plunger hits the trigger. That stops the drive ram and the start of the data acquisition simultaneously.

Procedure:

Wash all parts of the mixing module (syringes, optical cell and tubes) with HQ-H_2O several times before usage. Fill one of the syringes with 2 µM or 4 µM LspPOX in 100 mM phosphate buffer, pH 7.4, and the other syringe with substrates with increasing concentration (be aware that the concentrations in the optical cell decreases by half, when the reactants are mixed in equal parts). Use as a blank 100 mM phosphate buffer and substrate.

Material and Equipment:

PiStar CD	(Applied Photophysics)	Phosphate buffer	(Sigma)
SX.18MV-R	(Applied Photophysics)	30% w/w H_2O_2 solution	(Sigma)
2 mL disposable syringes	(Braun)	Cyanide solution	(Sigma)

3.5.10. MASS SPECTROMETRY

To measure the mass-to-charge ratio and the abundance of ions, a mass spectrometry converts molecules to ions so that they can be moved about and manipulated by electric and magnetic fields. Usually the sample is vaporized into a gas and then ionized, mainly to cations by loss of electrons. The ionized gas is accelerated and subjected to an electric or magnetic field. Lighter and higher charged components will deflect more in the field than heavier components or less charged components. A detector capable of detecting charged particles counts the number of ions at different deflections. The result displayed on a chart a so called spectrum gives information about the relative abundance as a function of the ion-to-charge ratio of the ions.

In biochemical areas tandem mass spectrometry (MS-MS) is often used for peptide sequencing and liquid chromatography-mass spectrometry (LC-MS) is used for protein analysis.

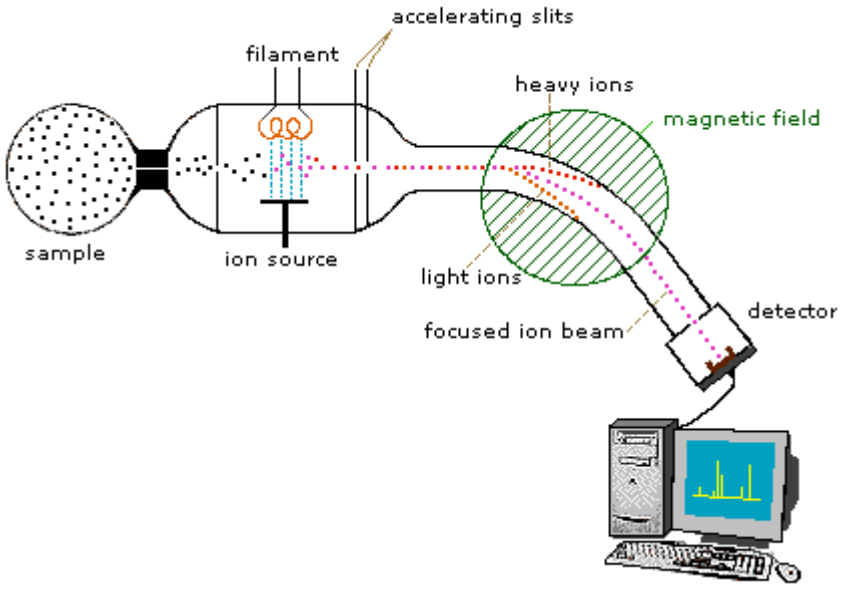


Figure 3-14 - Schematics of a simple mass spectrometer. The sample is vaporized and ionized. The ionized gas is accelerated and subjected to an electric/magnetic field. The detector counts the abundance of deflected ions with different mass-to-charge ratios.

Procedure:

Protein Analysis:

Inject 3 µg of the protein sample (10 mM phosphate buffer, pH 7.0) directly into the LC-MS. Elute the protein by developing a linear gradient from 15% to 70% acetonitrile. Process the data using Data Analysis 4.0 and deconvolute the spectrum with MaxEnt.

Peptide Analysis:

Reduce the protein sample (3 µg of protein in 10 mM phosphate buffer, pH 7.0) with 15 mM dithiothreitol (DTT), carbamidomethylated 55 mM iodoacetamide and acetone precipitated acetone:aqueous phase 4:1. Re-dissolve the pellet in 0.1 M ammonium bicarbonate (ABC) buffer and digest over night with tryspin at 37 °C (enzyme to substrate ration 1:50). Load 3 µg of each digest on a BioBasic C18 column using 65 mM ammon-formate buffer as aqueous solvent. Elute with an acetonitrile gradient from 5% to 65% over 55 min with a flow rate of 6 µL per min. Detect the sample with a Q-TOF mass spectrometer equipped with the standard ESI source in the positive ion, data directed analysis (DDA) mode (= switching to MS-MS mode for eluting peaks). Calibrate the instrument with ESI calibration mixture.

Material and Equipment:

LC: 3000 LC	(Dionex)	Dithiothreitol (DTT)	(Sigma)
MC: Maxi 4G	(Bruker)	Iodoacetamide	(Sigma)
Data Analysis 4.0	(Bruker)	Ammonium bicarbonate	(Sigma)
BioBasic C18 column (150 x 0.32 m, 5 µm)	(ThScience)	Ammon-formate	(Sigma)
Q-TOF MS	(Bruker)	Trypsin	(Life Technology)
Acetonitril	(Sigma)		

4. Results and Discussion

4.1. Cloning of Recombinant Protein

The empty pET-52b(+) vector was cut by the endonucleases AvrII and NcoI. To control the restricted endonuclease reaction and to get rid of the cut out polynucleotide a TAE agarose gel was performed; the estimated length of the linearized vector is 5056 bp and the cut out fragment is 171 bp long. According to the ladder on the TAE electrophoresis gel, the linearized vector has the right length. The cut out DNA fragment is not visible, because on the one hand SYBR-green, which migrates contrary to DNA, is only in the gel and not in the electrophoresis buffer, so the staining qualities are reduced in the lower part of the gel. On the other hand even if the large and the small DNA fragments have the same concentration, the absolute amount of nucleotides and intercalation possibilities are much lower for the small fragment, consequently it is worse visible.

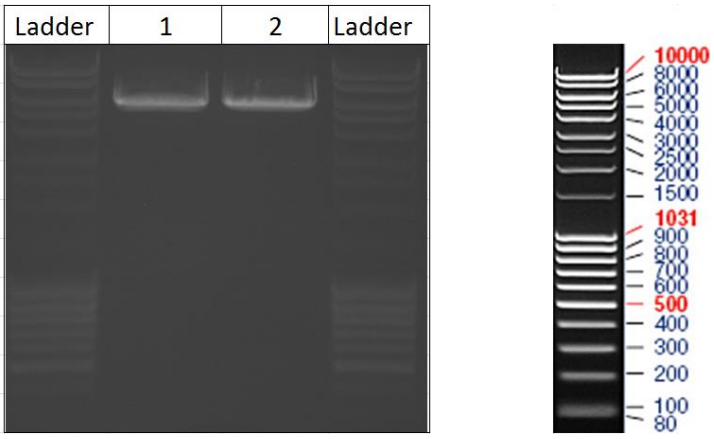


Figure 4-1 - TAE agarose gel electrophoresis of pET-52b(+) vector linearized with AvrII and NcoI in lane 1 and 2. Ladder: MassRuler DNA Ladder Mix.

Purification of the linearized vector yielded 1.14 µg DNA.

Starting from the wild type gene of LspPOX in pET-21b, two PCRs were needed to get the ready to use insert.

The gene of LspPOX was isolated by using two primers at the 5' and the 3' end of the coding region. The estimated length of the first PCR product is 1626 bp long. The height of the band (Figure 4-2) in lane 1 fits with the estimated length of the PCR product.

Purification of the first PCR product yielded 2.17 µg DNA.

The purified DNA of the first PCR served as template for the second PCR. The estimated length of the second PCR product is 1712 bp long. There are two bands visible in lane 1 & 2 (Figure 4-3); the upper

band above the 1500 bp ladder band is the desired PCR product. The lower band is an unwanted PCR product, which might occur due to non-target interactions of the primers with the template.

The upper band was cut out and purified to yield 1.01 µg of DNA.

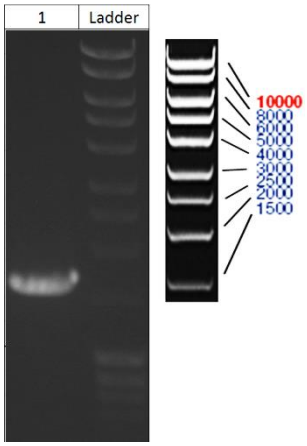


Figure 4-2 - TAE electrophoresis gel of the first PCR (lane 1). Ladder: MassRuler DNA Ladder Mix

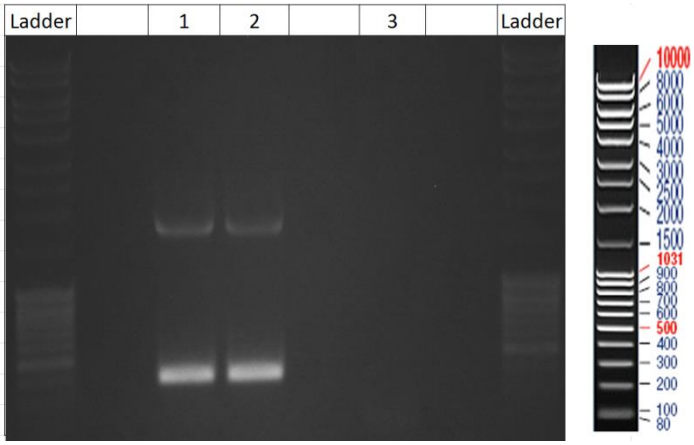


Figure 4-3 - TAE electrophoresis gel of the second PCR (lane 1 & 2); negative control (lane 3). Ladder: MassRuler DNA Ladder Mix

Insert and linearized vector were cloned with Gibson Assembly. After transformation into *E. coli* the cells were plated and incubated overnight at 37 °C. Plasmid DNA from four single colonies was purified and sequenced. The amount of purified DNA can be seen in Table 4-1.

Table 4-1 - Purification of plasmid DNA from four *E. coli* single colonies after transformation.

E. coli	**Plasmid DNA**
Clone 1	5.36 µg
Clone 2	5.93 µg
Clone 3	6.95 µg
Clone 4	5.18 µg

Since the plasmid is 6725 bp long, four primers were used to screen for a proper plasmid without mutations, frame shifts or deletions. All in all two clones (clone 1 and clone 3) had an appropriate plasmid DNA according to the sequencing results. We choose to go on with clone 3 because the concentration of plasmid DNA was higher.

4.2. EXPRESSION OF LSPPOX

For expression of LspPOX, a suitable production system had to be established. At the beginning different problems occurred, such as proteolytic degradation of the protein, low yield and low heme occupancy. Many expression screenings were performed to get rid of the problems. One screening dealt with the induction of the expression; induction with IPTG and lactose at different concentrations was tested. Additionally we varied the starting point of the induction. Other screenings dealt with the duration and temperature of the expression.

As result the expression was performed as follows: we started with two 20 mL LB_{Amp} overnight culture. About 6 mL of the overnight culture served as inoculum for 500 mL $M9ZB_{Amp}$ media; up to four 500 mL batches were performed simultaneously. When OD_{600} reaches a value of 0.6 to 0.8, temperature was decreased to 20 °C and hemin chloride (2.5 µg/L final concentration) was added. After 1 h the expression was induced with IPTG (0.05 mM final concentration). Cells were harvested after 4 h.

Expression overnight at 16 °C had a lower yield because more proteolysis could be observed *via* SDS-PAGEs and Western Blots. Induction of the expression at a lower OD_{600} value of 0.5 just as a higher OD_{600} value of 1.0 had a negative effect on the yield. This may be due to that with a lower cell density less protein is produced and when waiting too long before induction, cells might be already in the stationary growth phase with lower cell activity, thus lower protein production.

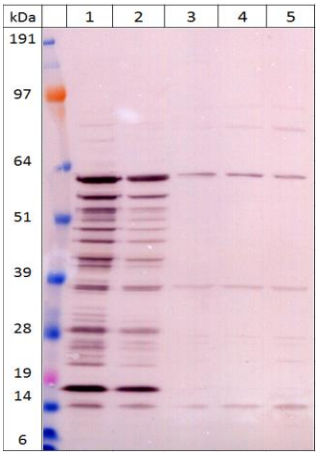


Figure 4-4 – Western blot (stained with anti-His antibody) of supernatant after cell lysis separated by SDS-PAGE. Different IPTG concentrations were tested. Inducing with a final concentration of 100 µM IPTG (lane 3), 10 µM IPTG (4) or no IPTG (5) had any effect on protein production. Starting with 0.1 mM IPTG (2) LspPOX was expressed properly even if there is proteolytic degradation. For 1 mM IPTG (1) yield remains constant according to (2), but proteolysis increases. Ladder: NuPage Novex

The final IPTG and lactose concentration to induce expression had a crucial impact on yield and proteolytic degradation (Figure 4-4). IPTG concentrations higher than 0.1 mM generated too much stress for the cells, proteolytic degradation was the consequence. No IPTG or IPTG concentrations lower than 10 µM caused a lack of protein production. Induction with lactose turned out to be not the right way to induce overexpression; low yield and excessive proteolytic degradation were the consequences.

4.3. PURIFICATION OF LSPPOX

Only a few steps are needed to purify LspPOX. The filtered and degased supernatant of the cell lysis is applied to an affinity chromatography column. The pooled fractions are stored at -30 °C overnight. After thawing the pooled fractions were centrifugated, desalted and concentrated to receive pure LspPOX.

Figure 4-5 shows a progress of metal chelate affinity chromatography (MCAC) purification. On the left side there is an SDS-PAGE stained with Coomassie Blue, in the middle a Western Blot treated with Anti-HisTag antibodies to detect the C-terminal end of LspPOX and on the right side a Western Blot treated with Anti-StrepTag II antibodies to detect the N-terminal end.

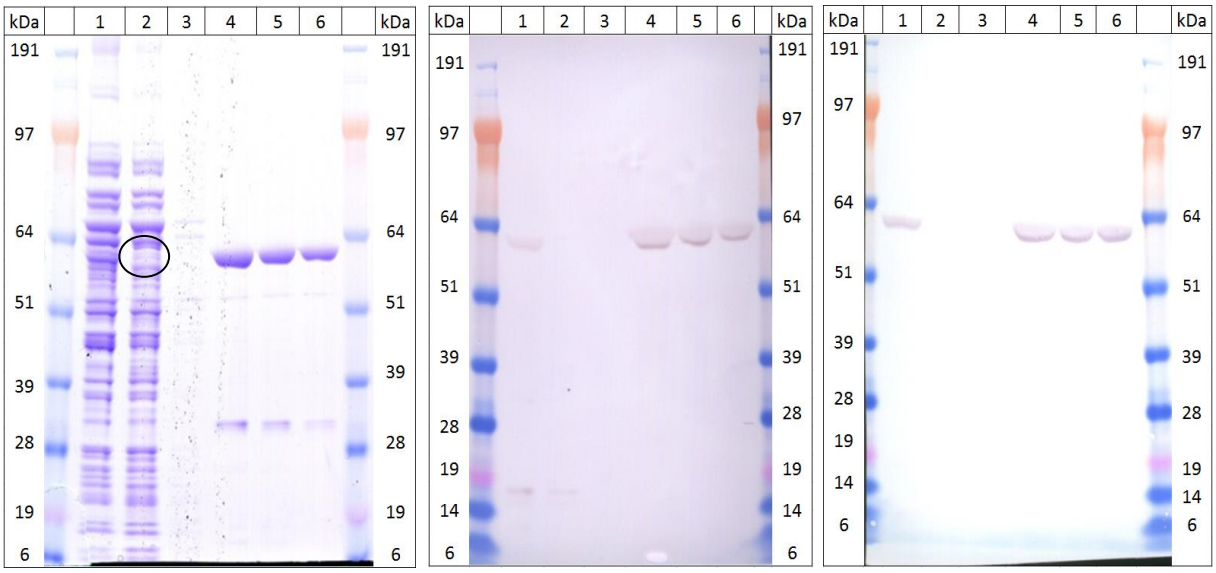


Figure 4-5 – A SDS-PAGE stained with Coomassie Blue (left), a Western Blot incubated with Anti-HisTag antibodies (middle) and a Western Blot incubated with Anti-StrepTag II antibodies (right) show the progress of LspPOX purification. Lane (1) shows the cell lysate, (2) shows the flow through of the MCAC column, (3) shows a washing step, (4) shows the pooled and concentrated fractions, (5) shows the sample after desalting and (6) shows the finished and purified LspPOX. Ladder: NuPAGE Novex

In lane 1 almost all proteins of the cell lysate are visible on the SDS-PAGE. On the Western Blots only the overexpressed LspPOX slightly under the 64 kDa ladder band is visible. Lane 2 shows the flow through of the MCAC column. The 60 kDa band on the SDS-PAGE (black circle) and the Western Blots disappears. Thus LspPOX binds almost completely to the column. Lane 3 shows a washing step of MCAC chromatography, unspecific binding proteins can be seen on the SDS-PAGE. Lane 4 on the SDS-PAGE and the Western Blots shows the pooled and concentrated fractions. There are still impurities on the SDS-PAGE visible (two bands at 30 kDa and 52 kDa).Lane 5 shows LspPOX after desalting. The intensity of the bands at 30 kDa and 52 kDa are decreasing. The impurities are vanishing. Lane 6 shows the purified protein. According to the SDS-PAGE purification of LspPOX works very well, as a

strong band appears at ~60 kDa. The impurities at 30 and 52 kDa are still visible on the SDS-PAGE, but they can be neglected when compared with the main band at 60 kDa. In lane 6 on the Western Blots only one band at 60 kDa is visible. The N-terminal StrepTag II as well as the C-terminal HisTag are intact. Thus there is no proteolytic degradation and the bands are at the expected height. To be sure if the purified protein is LspPOX, peptide sequencing with MS-MS and a protein mass analysis with LC-MS was performed with the purified sample (see 4.4.6).

4.4. CHARACTERIZATION OF LSPPOX

4.4.1. SPECTRAL PROPERTIES

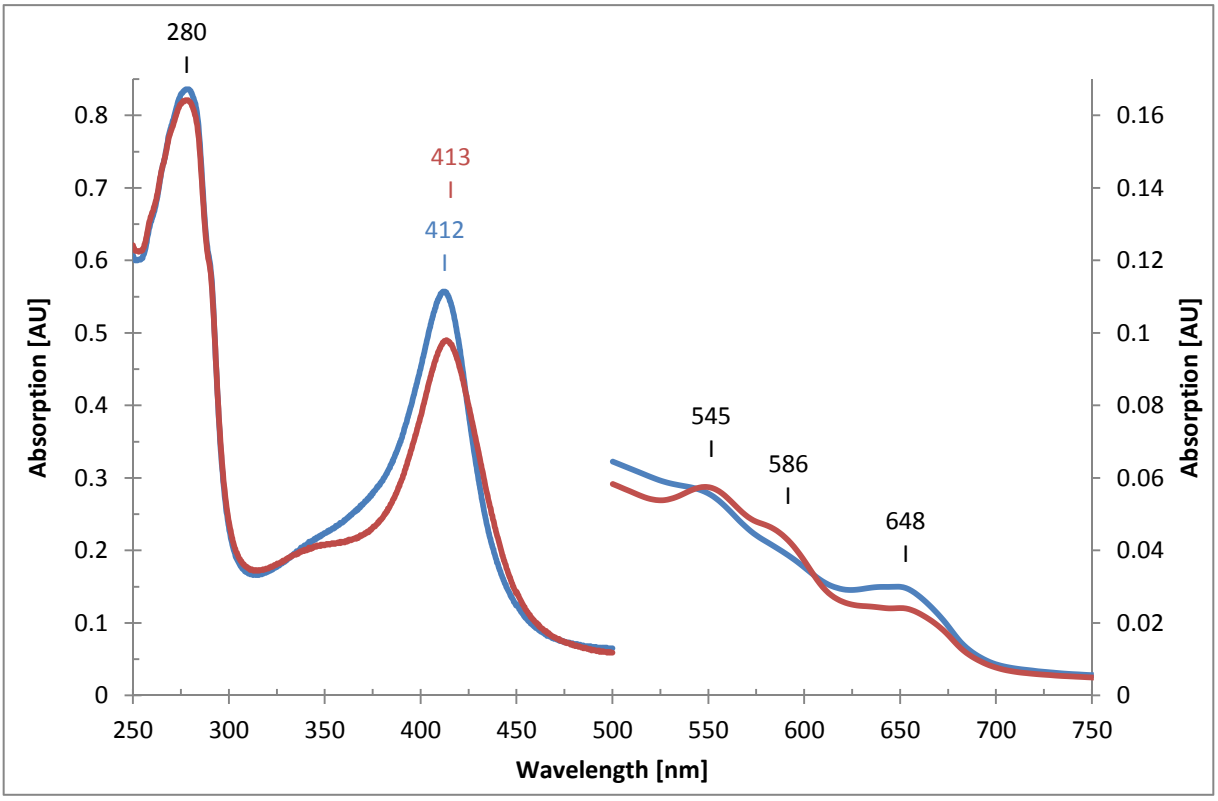


Chart 4-1 - UV-Vis absorbance spectrum of LspPOX (blue) and LspPOX (H_2O_2) (red) in 10 mM phosphate buffer pH 7.0. Values above 500 nm are multiplied by 5 for better visualization. The spectra show the typical protein peak at 280 nm and the Soret band of LspPOX at 412 nm and LspPOX (H_2O_2) at 413 nm. Three charge transfer bands at 545 nm, 586 nm and 648 nm are indicating a high spin state of the protein.

All UV-Vis absorbance spectra of LspPOX were performed in 10 mM phosphate buffer pH 7.0. Chart 4-1 shows a typical UV-Vis absorbance spectrum of LspPOX and LspPOX (H_2O_2). The typical protein peak at 280 nm, which is caused by the absorbance of the two aromatic amino acids tryptophan ($\varepsilon_{280\ nm}$ = 5600 M^{-1} cm^{-1}) and tyrosine ($\varepsilon_{275\ nm}$ = 1400 M^{-1} cm^{-1}); the absorption of phenylalanine is negligible. The peak at 412 nm and 413 nm the so called Soret band is characteristic for proteins containing porphyrin compounds such as heme in LspPOX. The purity number ($A_{412\ nm}$ / $A_{280\ nm}$) of LspPOX is ranging from 0.65 to 0.75 depending on the purification batch. The three bands in the visible region at 545 nm, 586 nm and 648 nm are charge transfer bands, which reflect the electronic configuration of the heme iron. Both spectra show a high spin configuration.

Due to the treatment of LspPOX with hydrogen peroxide the Soret band sharpens and shifts from 412 to 413 nm. Simultaneously the extinction coefficient is decrease by 10%, reflecting heme modification and formation of heme to protein bonds.

Furthermore, the spectral signatures of the bonds in the visible region are sharpened.

4.4.2. CHEMILUMINESCENCE

The covalently bound heme in LspPOX was detected using a chemiluminescence based method. In this method, luminol reacts with the iron of the prosthetic group, if the heme is still covalently linked after denaturation and separation *via* SDS-PAGE. For LspPOX (lane 1) and LspPOX (H_2O_2) (lane 2) in Figure 4-6 the chemiluminescence signal is at the same high as the protein band on the SDS-PAGE, thus LspPOX has a covalent linked heme. However, this analysis is not appropriate to quantify the amount of formed covalent linkages between LspPOX/LspPOX (H_2O_2) and the prosthetic group. Heme in LPO (lane 3) is also covalently bound even if the signal at 64 kDa is very weak. The band at 30 kDa can only be explained by degradation products in the protein solution. Horseradish peroxidase (lane 4) as a negative control has clearly no signal on the blot, thus it has no covalently linked heme.

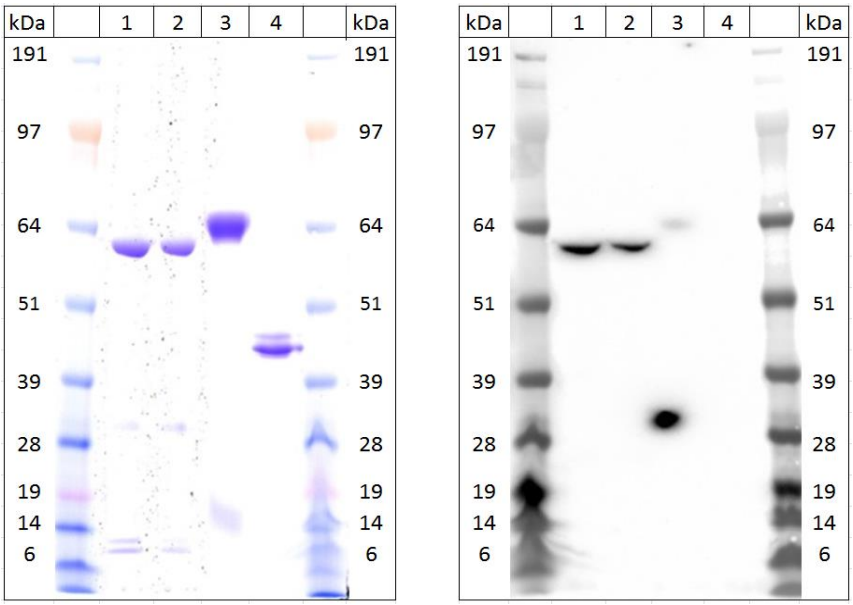


Figure 4-6 - SDS-PAGE on the left side and a blot incubated with luminol on the right side of (lane 1) LspPOX, (2) LspPOX (H_2O_2), (3) lactoperoxidase as positive control with a covalently bound heme and (4) horseradish peroxidase as negative control without a covalent link. Ladder: NuPAGE Novex

4.4.3. Peroxidase Activity

Peroxidase activity was measured with two one-electron donors ABTS and guaiacol at different pH values. Due to the fact that there is a 10 000 fold excess of hydrogen peroxide compared to the used enzyme in the assay, it is not necessary to differentiate between LspPOX and LspPOX (H_2O_2) in this experiment.

Chart 4-2 shows the activity in percentage at the pH-optimum, with 100% corresponding to 0.7 units/mg (guaiacol) and 208.6 units/mg (ABTS). The activity optimum for both oxidation reactions lies in the acidic region at pH 4.0. Guaiacol is oxidized over a broad pH range (pH 4.0 to pH 9.0), whereas ABTS oxidation only occurred in the acidic region (pH 4.0 to pH 6.0) of reasonable rates.

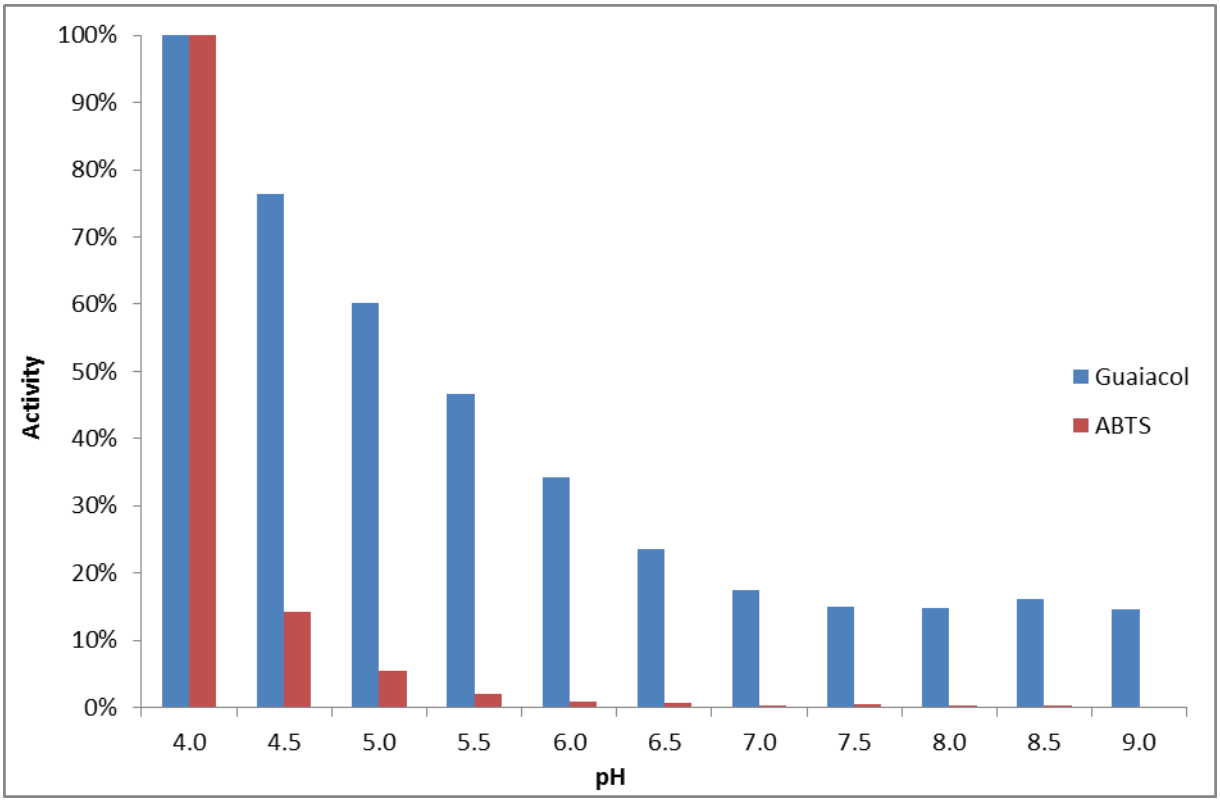


Chart 4-2- Peroxidase activity of LspPOX. Conditions as follows: 50 nM enzyme, 100 µM H_2O_2 with 1 mM guaiacol or ABTS in the pH range of 4.0 to 9.0. Activity is given in percentage at the pH-optimum, 100% are corresponding to 0.7 U/mg (guaiacol) and 208.6 U/mg (ABTS).

4.4.4. DIFFERENTIAL SCANNING CALORIMETRY

The thermal stability of LspPOX and LspPOX (H_2O_2) was measured using the DSC device. The measurements of the enzymes were performed at different pH values (from 4.8 to 7.8) in a temperature range from 20 to 100 °C with a heating rate of 60 °C/h.

Chart 4-3 shows the unfolding pathway of LspPOX. It clearly shows a non-two-state transition (T_{m1} = 70 °C, T_{m2} = 75 °C). Most probably the two events represent unfolding of the apo- and the halo (i.e. heme containing) protein.

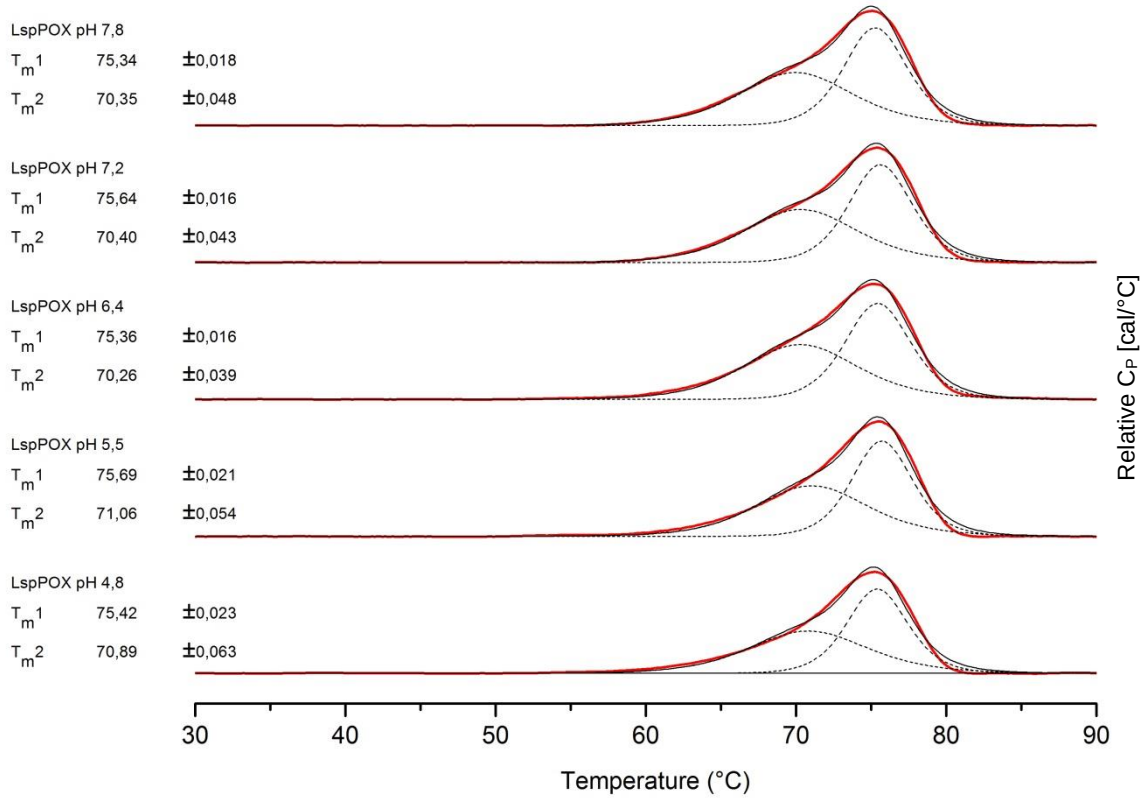


Chart 4-3– Normalized differential scanning calorimetry thermograms of LspPOX. Conditions as follows: 5 µM enzyme, 0.5 M guanidinium chloride, 10 mM phosphate buffer, pH from 4.8 to 7.8. Heating rate is 60 °C/h, temperature range is 20-100 °C and cell pressure is 4.1 bar. DSC thermograms (red) and fitted curves (black lines).

LspPOX (H_2O_2) shows an almost identically thermal unfolding pattern (Chart 4-4). There are no differences of the thermal stability at different pH-values. Just as the native version, LspPOX (H_2O_2) showed two unfolding events, with the only difference that both T_m values for all pH ranges are slightly increased (T_{m1} = 72 °C, T_{m2} = 76 °C).

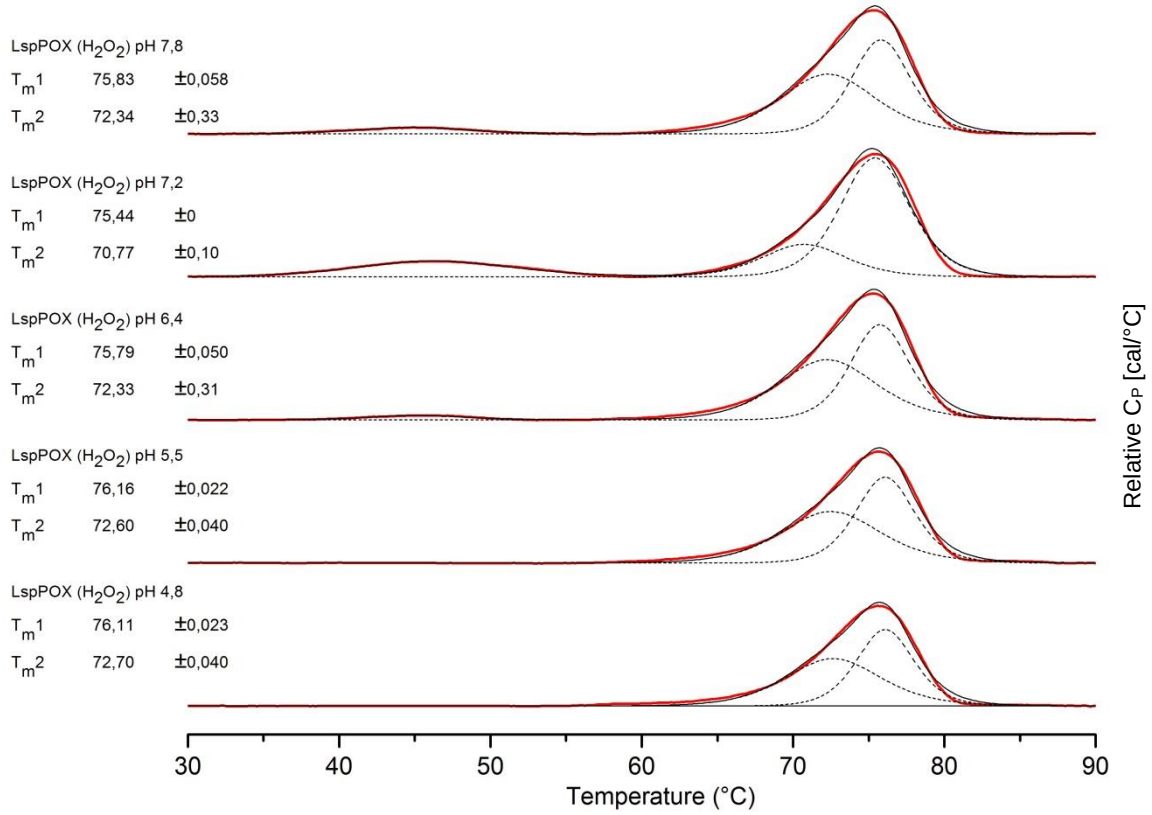


Chart 4-4 – Normalized differential scanning calorimetry thermograms of LspPOX (H_2O_2). Conditions as follows: 5 µM enzyme, 0.5 M guanidinium chloride, 10 mM phosphate buffer, pH from 4.8 to 7.8. Heating rate is 60 °C/h, temperature range is 20-100 °C and cell pressure is 4.1 bar. DSC thermograms (red) and fitted curves (black lines).

4.4.5. CIRCULAR DICHROISM

The electronic circular dichroism spectra of the far-UV range from 180 - 260 nm (Chart 4-5) clearly demonstrate that LspPOX and LspPOX (H_2O_2) have identical secondary structures content. The two spectra are congruent as well they show the almost identical deconvolution results.

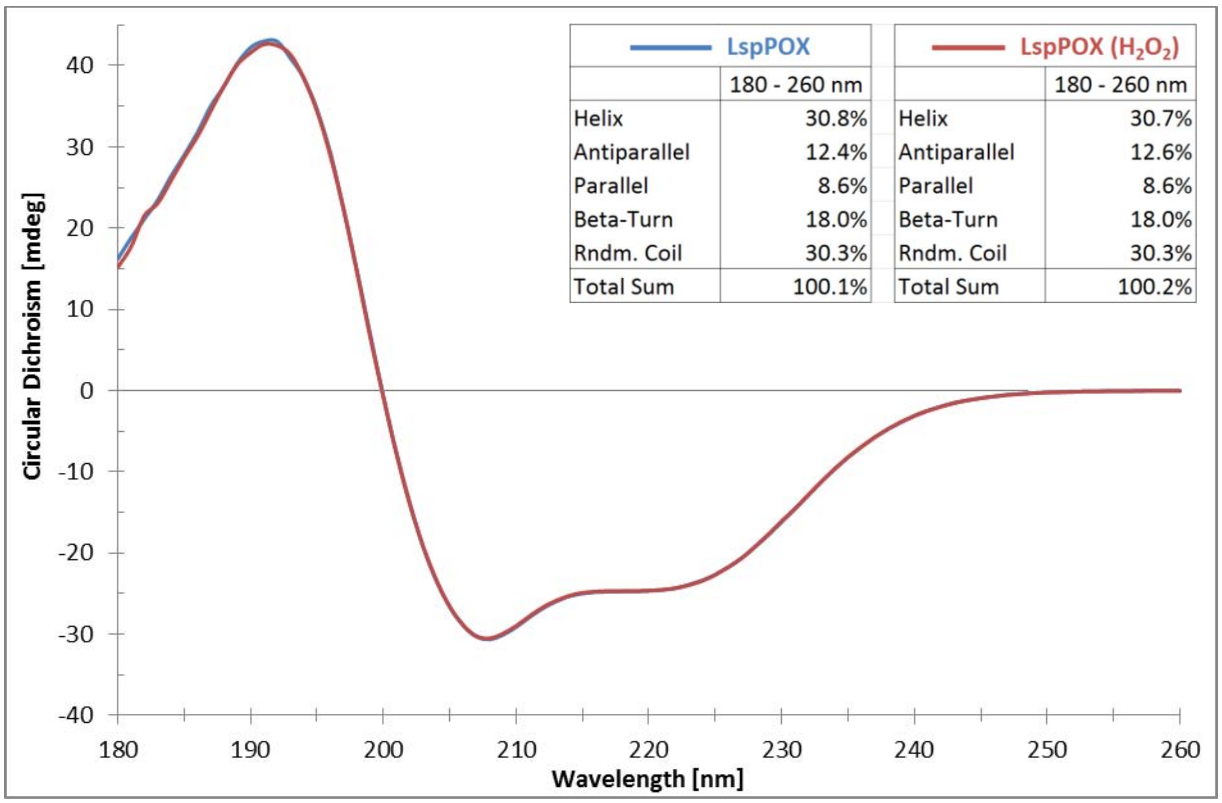


Chart 4-5 - Circular dichroism spectra of LspPOX (blue) and LspPOX (H_2O_2) (red) in the far-UV range. Enzyme concentration of LspPOX and LspPOX (H_2O_2) was identical. In the right upper corner there are the deconvolution results of the spectra (calculated with CDNN). Conditions are as follows: enzyme 4 µM, 10 mM phosphate buffer, pH 7.4.

In the CD spectra in the UV-Vis range of LspPOX and LspPOX (H_2O_2) (Chart 4-6), even small changes of the position of aromatic amino acids cause a deviation in the finger print region (from 270 to 300 nm). Still the patterns of the finger print region for both spectra are quite similar. Treatment with hydrogen peroxide increases the signal at the Soret maximum. Additionally the maxima at the Soret region shift from 408 nm (LspPOX) to 410 nm (LspPOX (H_2O_2)).

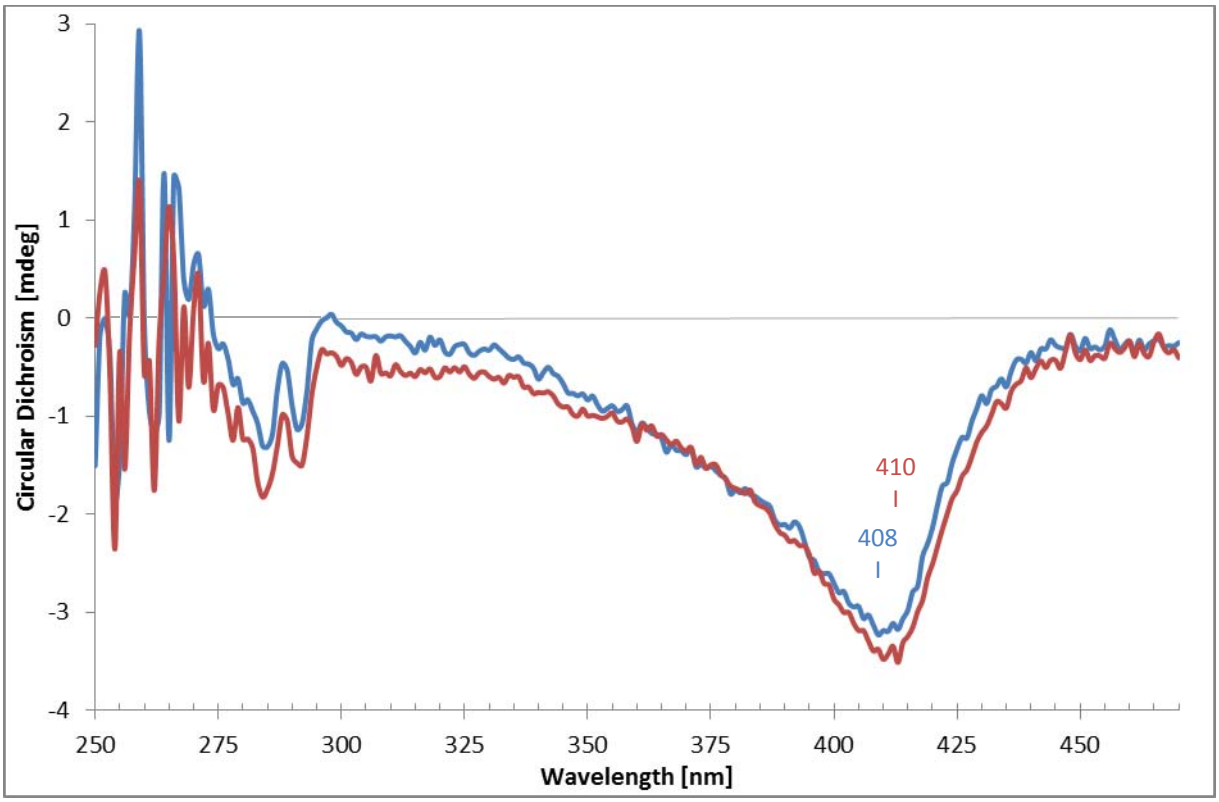


Chart 4-6- Circular dichroism spectra of LspPOX (blue) and LspPOX (H_2O_2) (red) in the UV-Vis range. Conditions are as follows: enzyme 4 µM, 10 mM phosphate buffer, pH 7.4.

Temperature-mediated unfolding was monitored by electronic circular dichroism at 208 nm and 408 nm. At 208 nm in the far-UV range the melting of α-helical structures (Chart 4-7) were followed. At 408 nm in the UV-Vis range the unfolding of the active center and the heme region was monitored (Chart 4-8).

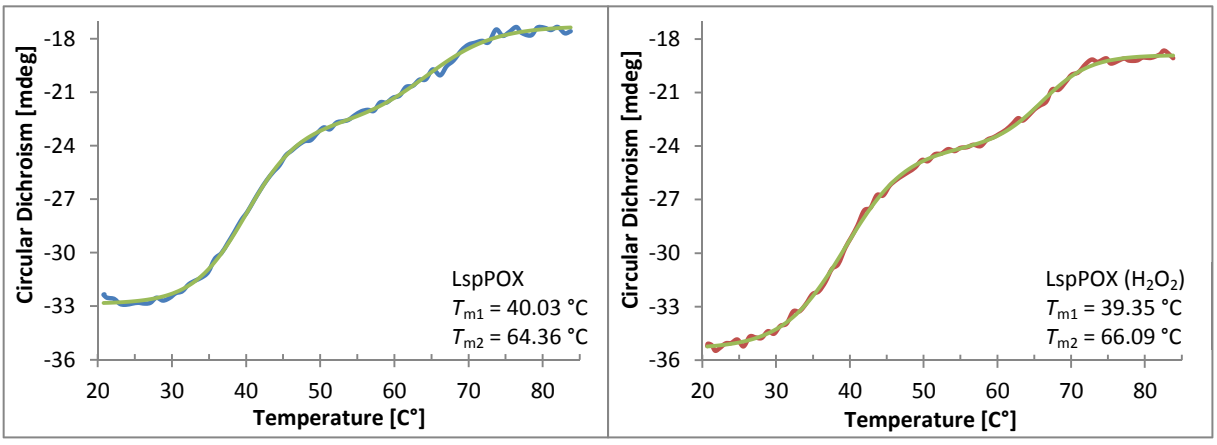


Chart 4-7 – Temperature-mediated unfolding monitored by electronic circular dichroism at 208 nm of LspPOX (blue - left) and LspPOX (H_2O_2) (red – right). Conditions are as follows: 2 µM enzyme, 0.5 M guanidinium chloride, 10 mM phosphate buffer, pH 7.4. Curves are fitted (green) with double sigmoid equations over a range from 20 to 83 °C.

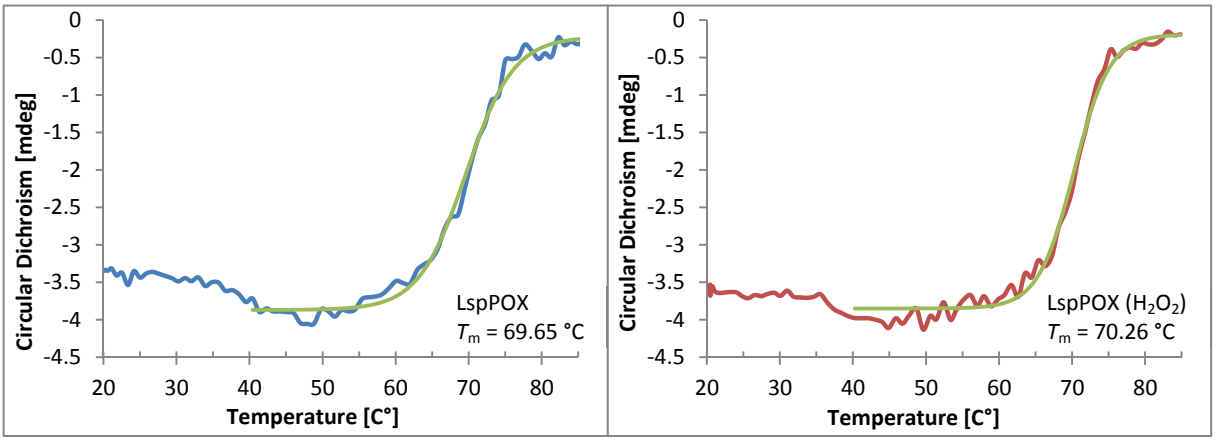


Chart 4-8 – Temperature-mediated unfolding monitored by electronic circular dichroism at 408 nm of LspPOX (blue - left) and LspPOX (H_2O_2) (red – right). Conditions are as follows: 4 µM enzyme, 0.5 M guanidinium chloride, 10 mM phosphate buffer, pH 7.4. Curves are fitted (green) with single sigmoid equations over a range from 40 to 83 °C.

Following the loss of ellipticity at 208 nm showed two different unfolding events. For both, LspPOX and LspPOX (H_2O_2) the first event can be found at 40 °C. The second unfolding event can be attributed to the unfolding of the active site. The loss of ellipticity at 408 nm at the Soret minimum suggested the disintegration of the heme cavity. This can be attributed to the second unfolding event monitored at 208 nm. Treatment with hydrogen peroxide slightly increases the value of the second unfolding event at 208 nm (LspPOX: T_{m2} = 64.4 °C, LspPOX (H_2O_2): T_{m2} = 66.1 °C) as well as the unfolding of the active site at 408 nm (LspPOX: T_m = 69.7 °C, LspPOX (H_2O_2): T_m = 70.3 °C).

4.4.6. Mass Spectroscopy

LspPOX was digested by GluC/trypsin overnight at 37 °C to analyze the resulting digests by Q-TOF mass spectrometer. The analyzed peptides and the resulting protein sequence (Figure 4-7) fits exactly with the translated nucleotide sequencing data.

```
  1 MHHHHHHASI GKPGNSYDLV WDDIVPIEFR SIEGLNNNLV NPDWGTPDSQ LIRLSESAYN 60
 61 DEISEPRGGD PSSLVSPREV SNTIFDQSES IPNETGVSDW FWQWGQFIDH DMDLTPGTSG 120
121 ESFNISVPLG DPSFDPFNSG TQEIPLTRSI YDFDTGIDSP REQINEITAY IDGSNVYGSD 180
181 SERAEALRTN DGTGKLKTSV SESGEVLLPF NTDGLDNDNP FGIANDSIFV AGDVRANEQV 240
241 GLTATHTLFV REHNRLADDI ATRLDNGDAE LLDLFAESGL SEGDFIYESA RRIVGAEIQA 300
301 ITYNEFVPLL VGSNALDGYD GYNVTVDSGI SNEFSTAAFR FGHTMLSPTL QNGTNEGLSL 360
361 RDTFFNPDLV VEGGVDSLLL GLASQEAQEV DTQVIDDVRN FLFGAPGSGG LDLVSLNIQR 420
421 GRDHGLPSYT EVREELGLDP ITNFGEITSD PIVQAQLESA YTDVDNIDLW VGGLAEDHVN 480
481 GSLFGETFQV IVVDQFTRLR DGDRFYYEND NLLSVLAPDV AETTLSDVIV ANSAISSIQS 540
541 NAFLVNGWSH PQFEK
```

Figure 4-7 - Amino acid sequence of recombinant LspPOX (molar mass without prosthetic group 60661 kDa). LspPOX in 0.1 M ABC buffer was digested overnight with trypsin and GluC at 37 °C.

To perform protein analysis, 3 µg of LspPOX and LspPOX (H_2O_2) was injected to a LC/MS-system. For LspPOX and LspPOX (H_2O_2) (Char 4-9 A and B) two main peaks can be found.

The enzymes without prosthetic group have a theoretical average mass of 60661 kDa. During sample preparation for mass spectroscopy, the peroxidases undergo denaturation. Thus, all non-covalently linked groups, such as heme, are lost during enzyme unfolding. The second mass peaks at 61275 kDa for LspPOX as well as for LspPOX (H_2O_2) are about 614 Da greater than the theoretical neutral mass peaks. The 614 Da is the exact mass of the heme group. After purification the native untreated LspPOX occurs in a 50% heme-linked to heme-free relation (Chart 4-9 A). Treatment with 15 times molar excess of hydrogen peroxide shifts this relation to almost 70% heme-linked LspPOX (Chart 4-9 B). This indicates that hydrogen peroxide promotes the autocatalytic formation of covalent linkages between heme and enzyme.

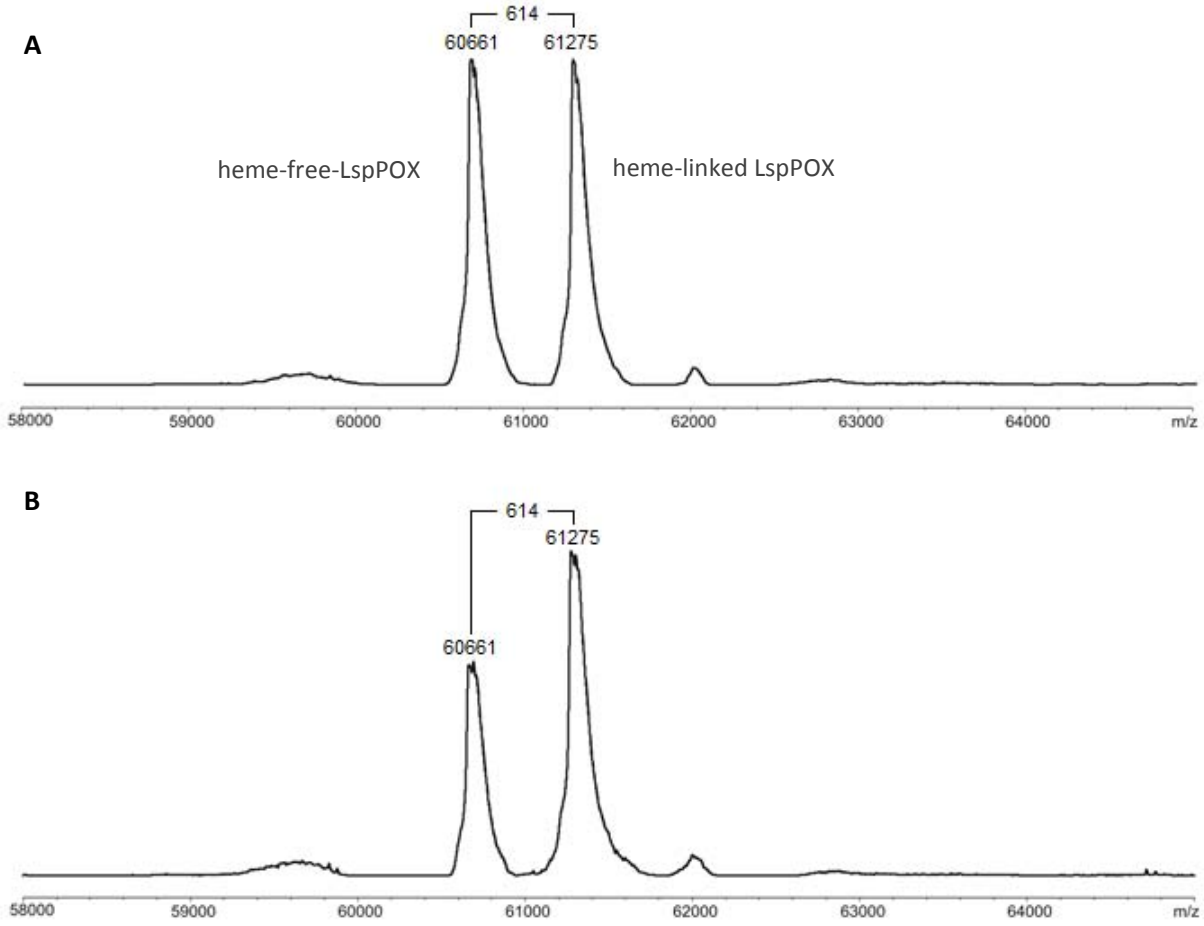


Chart 4-9 - Mass spectroscopy analysis of LspPOX (A) and LspPOX (H_2O_2) (B). The difference of 614 Da is the exact mass of the prosthetic heme group. Treatment with hydrogen peroxide (B) increases the amount of heme-linked LspPOX.

4.4.7. STOPPED-FLOW SPECTROSCOPY

Chart 4-10 shows the spectral change of LspPOX after adding 50 µM cyanide in the conventional stopped-flow mixing mode. Spectra after adding 250 µM cyanide to LspPOX are merged to obtain the maximum cyanide shift. The blue line indicates the ferric recombinant protein with the typical Soret peak at 412 nm in high-spin iron state (S = 5/2). Adding cyanide converts the ferric protein in high-spin state to a low spin-state (S =1/2) (red line). The Soret peak shifts from 412 nm to 429 nm with a distinct isosbestic point at 422 nm. In the visible range above 500 nm a peak at 554 nm arises and two peaks at 642 nm and 662 nm decrease.

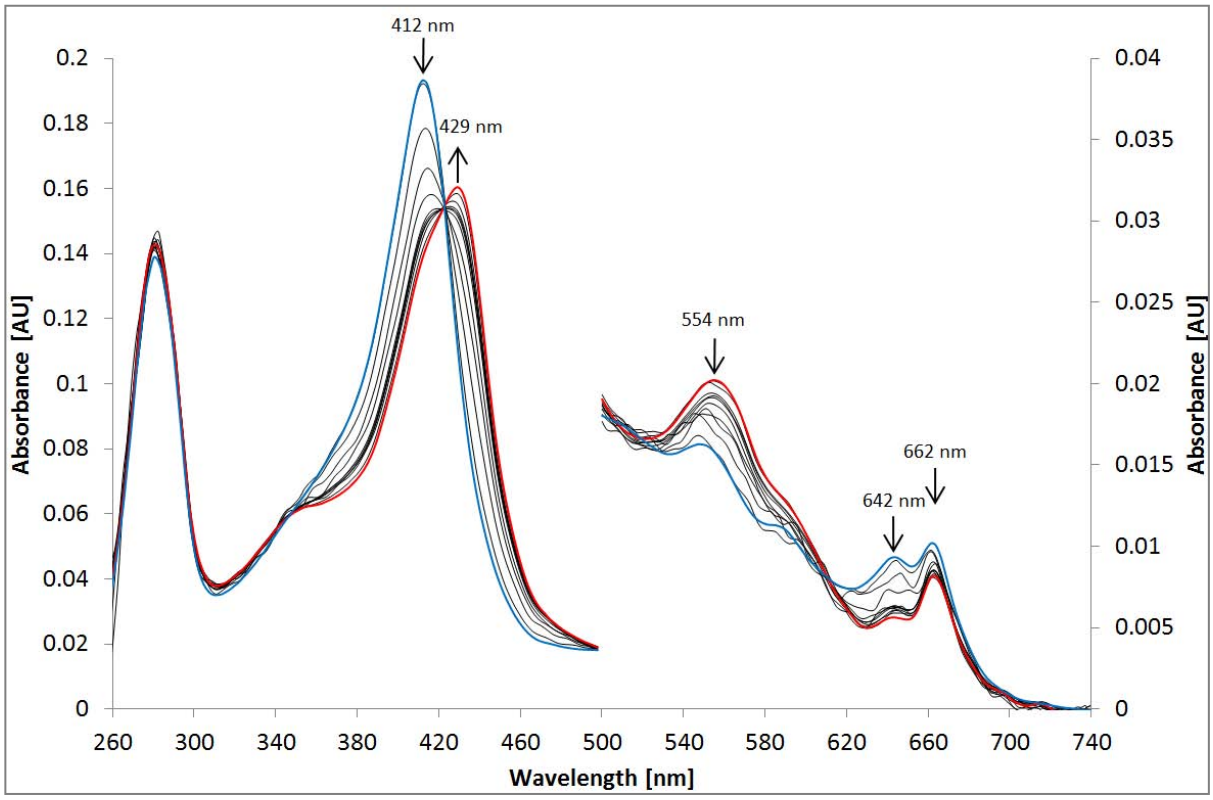


Chart 4-10 - Reaction of ferric LspPOX with cyanide. The first spectrum of the ferric protein in high spin state is shown in blue. The following spectra (black) were recorded after mixing 4 µM LspPOX with 50 µM cyanide at 1 ms, 5 ms, 12 ms, 20 ms, 0.101 s, 0.506 s, 1.079 s and 2.000 s and with 250 µM cyanide at 0.106 s and 0.506 s. The last spectrum (red) shows the formation of the low-spin cyanide complex (250 µM cyanide after 2 s). The arrows indicate the change of absorbance with time at a specific wavelength. Values above 500 nm are multiplied by 5 for better visualization.

With LspPOX (H_2O_2) the spectral changes were very similar, shown in Chart 4-11. The ferric Soret peak of LspPOX (H_2O_2) shifts after addition of 50 µM or 250 µM cyanide from 413 nm to 430 nm with a clear isosbestic point at 423 nm. The changes of the transfer bands of LspPOX (H_2O_2) are similar to LspPOX beside the small shift of the peak at 642 nm to 643 nm.

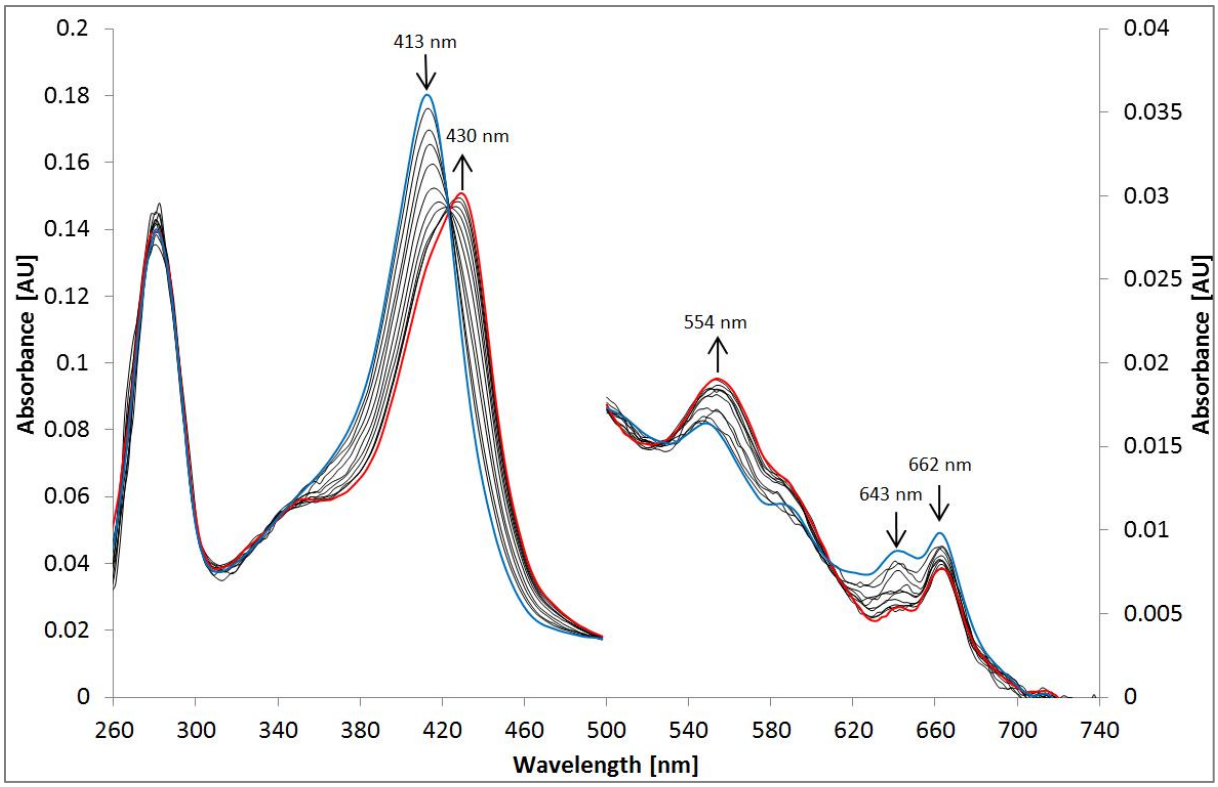


Chart 4-11 – Reaction of ferric LspPOX (H_2O_2) with cyanide. The first spectrum of the ferric protein in high spin state is shown in blue. The following spectra (black) were recorded after mixing LspPOX (H_2O_2) with 50 µM cyanide at 1 ms, 2 ms, 4 ms, 6 ms, 10 ms, 15 ms, 20 ms, 30 ms, 43 ms and 0.101 s. The last spectrum (red) shows the formation of the low-spin cyanide complex (250 µM cyanide after 0.101 s). The arrows indicate the change of absorbance with time at a specific wavelength. Values above 500 nm are multiplied by 5 for better visualization.

The ferric spectrum of LspPOX (H_2O_2) was subtracted from the spectrum of the cyanide complex, resulting in two maxima at 407 nm and 435 nm (not shown). These maxima indicate the most significant changes in absorbance during formation of the cyanide complex. The change in absorbance was detected at 407 nm as a function of time at different cyanide concentrations (Chart 4-12).

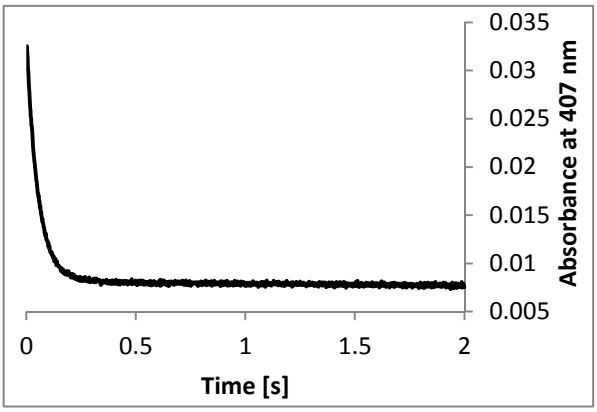


Chart 4-12 – Change of absorbance at 407 nm during the shift from ferric LspPOX (H_2O_2) to cyanide complex. Double exponential fit generates the pseudo-first-order constants $k_{obs\,(1)}$ and $k_{obs\,(2)}$.

The resulting time traces were biphasic with a rapid first phase and a slower second phase. Double-exponential fits gave the associated pseudo-first-order rate constants, $k_{obs\,(1)}$ and $k_{obs\,(2)}$. These constants were plotted (Chart 4-13)) versus the cyanide concentrations ($k_{obs} = k_{on}[CN^-] + k_{off}$). For the rapid first phase a rate constant of $(1.1874 \pm 0.13) \times 10^6\ M^{-1}\ s^{-1}$ ($K_D = k_{off}/k_{on} = 17.6\ \mu M$) and for the slower second phase a rate constant of $(3.536 \pm 0.313) \times 10^5\ M^{-1}\ s^{-1}$ ($K_D = 13.9\ \mu M$) was obtained.

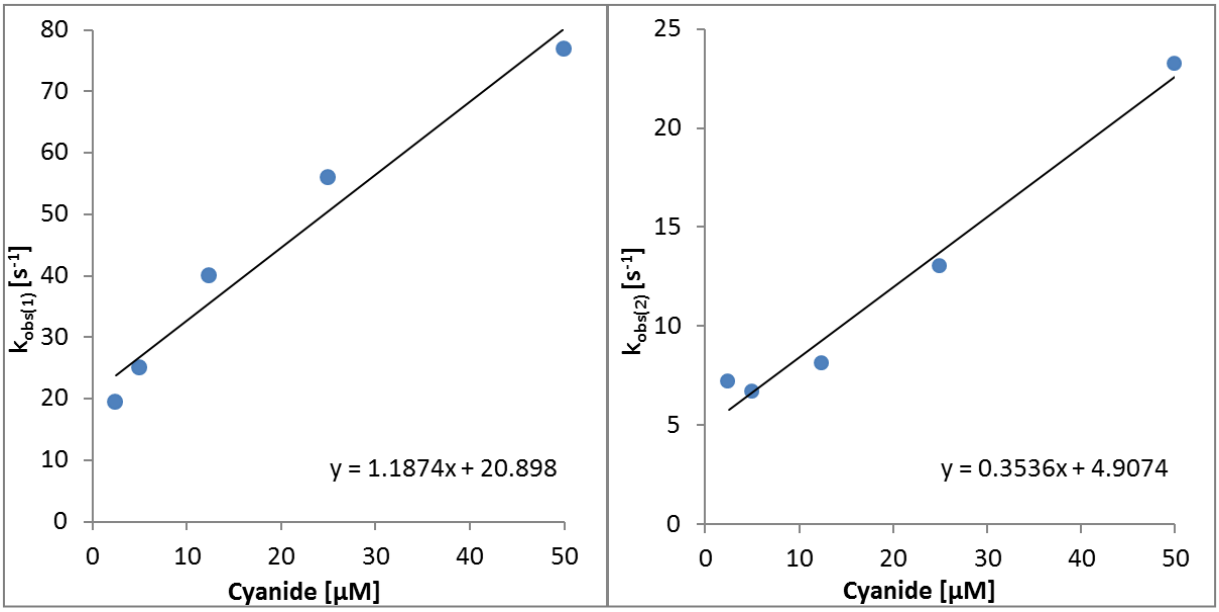


Chart 4-13 - $k_{obs\ (1)}$ (on the left side) and $k_{obs\ (2)}$ (on the right side) in dependence of different cyanide concentrations. Second-order rate constants can be obtained by linear fit ($k_{obs} = k_{on}[CN^-] + k_{off}$).

We assume that the rapid first phase of the reaction reflects the binding of cyanide to the prosthetic group of LspPOX (H_2O_2), whereas the slower second phase represents cyanide binding to LspPOX, when the heme is not covalently bound to the protein. Apparently, the presence of this linkage leads to a higher reaction rate.

5. SUMMARY, CONCLUSION AND OUTLOOK

We were able to express and purify a recombinant peroxidase with high homology to mammalian lactoperoxidase in *E. coli*. The duration and effort from both expression and purification was reduced significantly when compared to the mammalian enzymes. A satisfying amount of recombinant LspPOX can be produced (from overnight culture to purified protein) within a few working days. Purification includes metal chelating affinity chromatography. The usual yield of a purification is 3 mg/L with a heme occupancy of 80% and a purity number ranging from 0.65 to 0.75. It might be possible to increase the yield of LspPOX with further expressions screening, but in my opinion the effort will not cover the benefit.

The main part of the thesis deals with the characterization and comparison of LspPOX and LspPOX (H_2O_2). Mass spectroscopy data shows that treatment of LspPOX with hydrogen peroxide increases the amount of covalent bonds between heme *b* and the enzyme. After purification about 50% of LspPOX has a covalent link, this value raises up to 70% for LspPOX (H_2O_2). Additionally we demonstrated the existence of a covalent link with a chemiluminescence based method (a quantification of the results of this method might be a task for the future). The increase of covalent bonds and the resulting change of the prosthetic group can be seen in the small shift of the Soret band and charge transfer bands in UV/Vis-spectroscopy. CD data shows that the newly formed bonds do not have any influence on the secondary structure of overall protein, but changes occur in the active site around the heme.

We assumed that LspPOX (H_2O_2) is more stable at higher temperature due to additional linkages. Both differential scanning calorimetry as well as temperature mediated unfolding monitored *via* CD show rather identical unfolding pathways for LspPOX and LspPOX (H_2O_2). The melting temperature of LspPOX (H_2O_2) slightly increases, but not significantly. According to protein stability, more measurements beside thermal stability can be done (e.g. long term stability, stability at extreme pH, optimal buffer conditions, precipitation test with ammonium sulfate, etc.)

Peroxidase activity with guaiacol and ABTS shows that the activity optimum for both substrates is in the acidic region and at least guaiacol is oxidized over a broad range of pH. The activity maximum for guaiacol is 0.7 U/mg pH 4.0 and for ABTS it is 208.6 U/mg pH 7.0.

Adding cyanide to LspPOX shows a conversion from high-spin state to low-spin state. The Soret peak of LspPOX and LspPOX (H_2O_2) shifts 17 nm into the higher wavelength region with a clear isosbestic point at 422 nm (LspPOX) and 423 nm (LspPOX (H_2O_2)). The change of the charge transfer bands is for

both enzyme variants rather identical. Stopped-flow measurements with different cyanide concentrations were done only with LspPOX (H_2O_2), thus no comparison is possible. Although the time traces were biphasic, a rapid first phase and a slower second phase could be observed. Given that the reaction solution is still a mixture of linked and unlinked enzyme with a 70% to 30% ratio, we assume that the faster phase (about 70% of overall transition) belongs to the linked LspPOX (H_2O_2) and the slower phase (about 30% of overall transition) to LspPOX. This could be confirmed if the amplitude of the faster phase decreases when measuring the untreated enzyme solution.

This enzyme leaves many questions unanswered, Compound I and Compound II formation needs to be investigated using stopped flow spectroscopy. Different mutations might give a deeper insight into the covalent linkages. Either an aspartic acid (D109) or glutamic acid (E238) at the distal site is responsible for heme binding. Mutation of these amino acids to asparagine and glutamine (or alanine if steric problems with heme *b* occur) might reveal more details about the covalent bonds. An additional methionine at the distal site of the heme cavity (according to the active site structure of MPO) might induce a sulfonium ion linkage. To get more insight into substrate binding the distal amino acids arginine, histidine and glutamine could also be mutated. Additionally, different substrates and inhibitors could be tested to find the native substrate of LspPOX *in vivo*. EPR measurements of LspPOX and its mutants in comparison with HRP and LPO might reveal further information about the distortion of the prosthetic group. And last but not least, a crystal structure would be a substantial contribution to get a better insight into structure and functional relationships of this bacterial peroxidase.

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