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Improving the substrate selectivity of an oxidase used in biosensors

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Verbessern der Substratselektivität einer in Biosensoren verwendeten Oxidase

Oxidase, Selektivität, Proteinreinigung.

Kurzfassung

Ziel dieser Arbeit war es, die Substratspezifität einer in Biosensoren verwendeten Oxidase zu untersuchen und durch zielgerichtete Mutagenese zu verändern, um das Ansprechen des Sensors auf ein unerwünschtes Substrat zu verhindern. Durch die eingeführte Mutation sollte aber die Aktivität des Enzyms mit dem Hauptsubstrat nicht zu stark beeinflusst werden.
Basierend auf dem Vergleich mit anderen Enzymen der Enzymfamilie wurden vier Mutanten geplant und generiert sowie ein Reinigungsprotokoll für den Labormaßstab etabliert.
Die Charakterisierung der Mutanten zeigte, dass eine von ihnen mit einer verbesserten Selektivität für das Hauptsubstrat aufwarten konnte. Die kinetischen Konstanten der Mutante mit diesem Substrat verschlechterten sich dabei nicht maßgeblich gegenüber dem Wildtyp des Enzyms. Zwei der Mutanten zeigten keinerlei Aktivität. Die Aktivität der hergestellten Doppelmutante war 100-fach geringer als die des Wildtypenzyms.

Improving the substrate selectivity of an oxidase used in biosensors.

Oxidase, Selectivity, Protein purification

Abstract

The aim of the work was to change the substrate specificity of an oxidase through site directed mutagenesis to minimize the activity of the oxidase with an undesirable substrate without impairing its activity with the main substrate. Said oxidase is used in commercially available biosensors.
After extensive literature research and comparison with other enzymes of its enzyme family four mutants were planned and created and a purification protocol was established.
Characterization of the mutants showed that one of the four mutants displayed an improved selectivity for the preferred substrate while retaining an acceptable kcat. Two of the mutants were found to be inactive. The double mutant displayed a 100-fold lower activity than the wild type enzyme.

Contents

1 Introduction

1.1 Motivation

L-Lactate oxidase (LOX) is used in clinical diagnostics to determine blood lactate levels via biosensors. Recently it was reported by Pernet (Pernet et. al. – 2009) that this sensor is not only specific for *L*-lactate but its *L*-lactate oxidase also reacts with glycolate. Comparison of the structures of these two substrates shows that they only differ in an additional α-methylgroup of lactate. (See Figure 1.1)

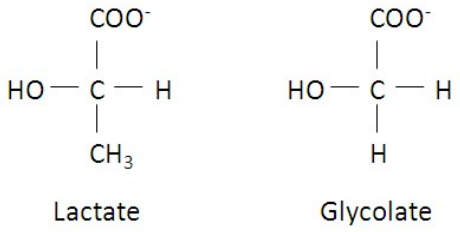

Figure 1.1 Similar chemical structure of lactate and glycolate

The false elevation of blood lactate can lead to the misdiagnosis of lactate acidosis while the patient actually suffers from ethylene glycol poisoning. Ethylene glycol poisoning poses a serious threat to the health of the patient therefore it is of great importance to give the appropriate medical treatment as soon as possible.
Ethylene glycol is the primary ingredient of automobile antifreeze, engine coolants and hydraulic brake fluids. Ethylene glycol poisoning may occur either intentionally or accidental. It is often imbibed deliberately due to the fact that its cheaper than ethanol. By itself ethylene glycol is nontoxic but its metabolites are harmful.
After ingestion ethylene glycol is rapidly absorbed from the gastrointestinal tract and metabolized primarily in the liver (See Figure 1.2 Metabolism of ethylene glycol - bold arrows indicate the major pathway). During the first step of the metabolism the enzyme alcohol dehydrogenase converts ethylene glycol to glycolaldehyde which is then rapidly metabolized to glycolate via aldehyde dehydrogenase. In the third step the conversion of glycolate to glyoxylate occurs relatively slow and therefore leads to the accumulation of glycolate. Due to the accrued glycolate the metabolic acidosis characteristic of poisoning by ethylene glycol takes place. In the fourth and last step glyoxylate is metabolized to produce oxalate, which then rapidly precipitates as calcium oxalate and is deposited in a crystalline form in many areas of the body including the brain, heart, lungs and especially the kidneys where it can lead to kidney failure.
The first neurological symptoms of ethylene glycol poisoning which are similar to the symptoms of ethanol intoxication appear thirty minutes after ingestion. Additionally irritation to the stomach may cause nausea and vomiting. Twelve hours later the symptoms associated with the accumulation of organic acids from the ethylene glycol metabolism as mentioned above start to appear.

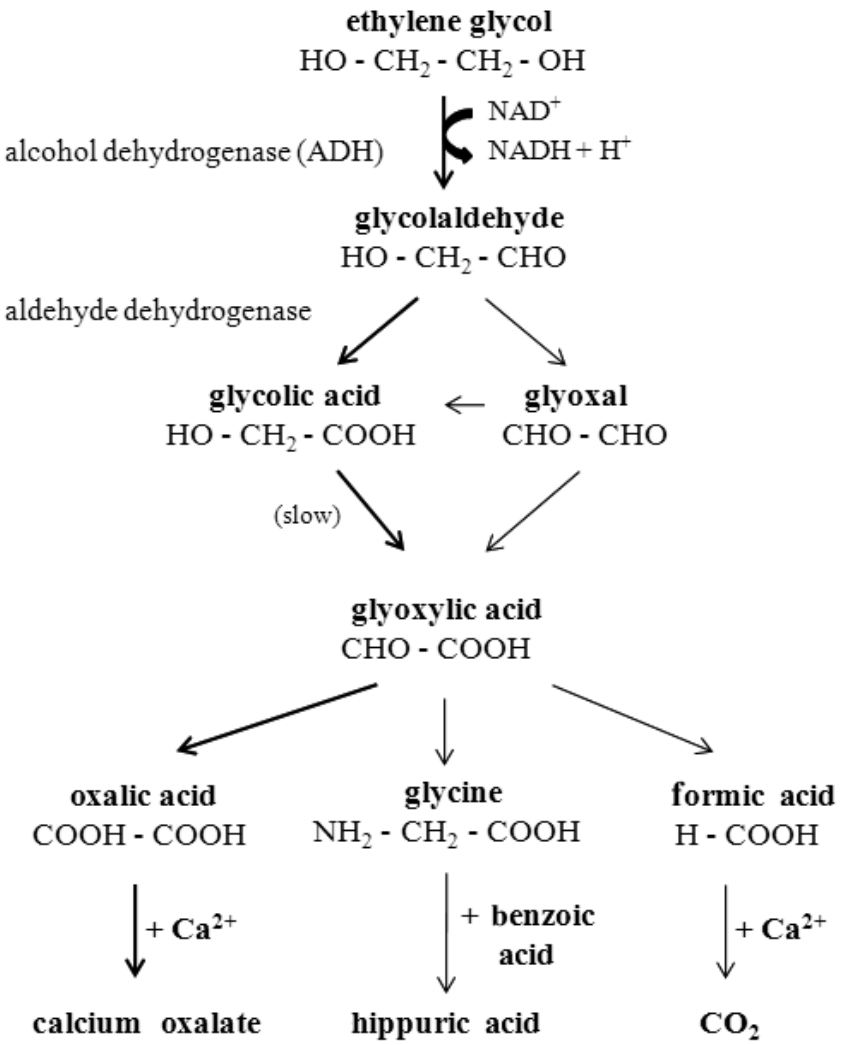

Figure 1.2 Metabolism of ethylene glycol - bold arrows indicate the major pathway

The aim of this work is to change the substrate specificity of lactate oxidase through site directed mutagenesis to minimize the activity of lactate oxidase with glycolate without impairing its activity with lactate.

1.2 α-Hydroxyacid-oxidase family

L-Lactate oxidase from *Aerococcus viridians* is a member of the α-hydroxyacid-oxidase family. This group of flavoproteins catalyzes the flavin mononucleotide (FMN)-dependent oxidation of their respective substrates. The enzymes of this family are found in various organisms that range from bacteria and yeast to mammals. Other members of this family are lactate dehydrogenase (flavocytochrome b2) (B2) from *Saccharomyces cerevisiae*, *L*-lactate monooxygenase (LMO) from *Mycobacterium smegmatis*, glycolate oxidase (GLO) from *Spinacia oleracea*, *L*-mandelate dehydrogenase (MDH) from *Pseudomonas putida* and long-chain α-hydroxyacid oxidase (HAO) from *Rattus norvegicus*.

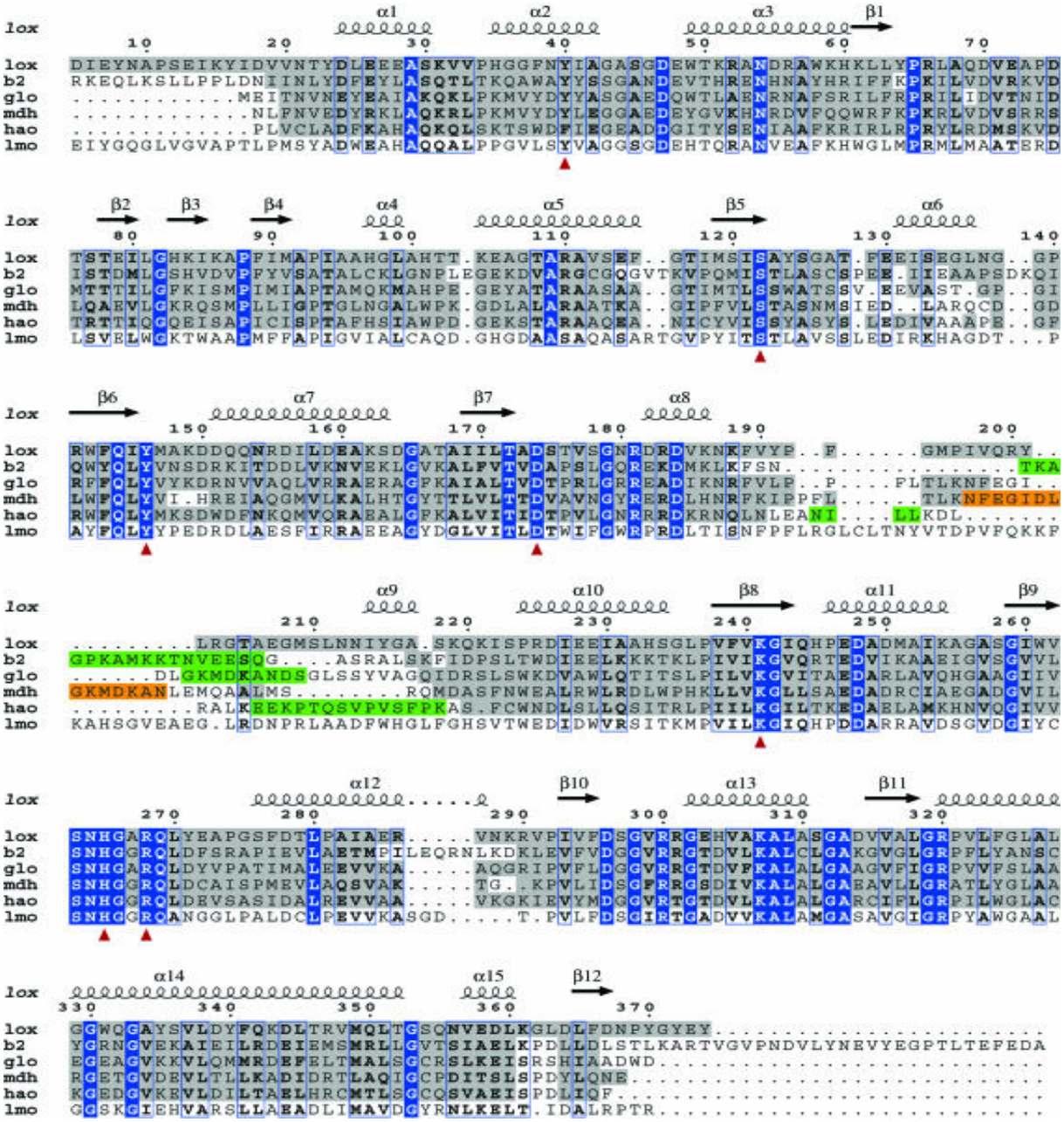

Figure 1.3 Structure based sequence alignment of selected FMN- dependent enzymes (Leiros et al. - 2006)

1.3 Structure of *L*-lactate oxidase

Comparison of the solved crystal structures of some members of the family (Umena et. al. - 2006) reveals similar protein folding patterns with each monomeric unit consisting of eight α-helices and eight β-sheets in an α/β barrel arrangement. The FMN prosthetic group is located at the C-terminal end of the β-strands. The conserved amino acid residues that form the binding pocket for the FMN cofactor are located at the top of the barrel.
The quaternary structure of the enzyme is a tetramer in which each of the monomers adopts a nearly identical structure. Each subunit consists of 374 amino acid residues and has a molecular weight of 41 kDa.

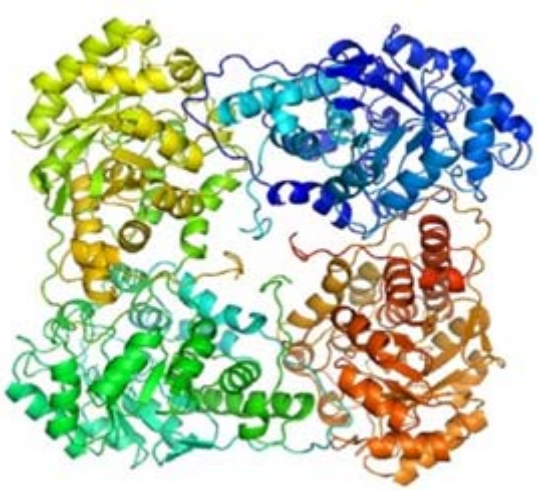

Figure 1.4 Tetramer form of the LOX (Umena et al. - 2006)

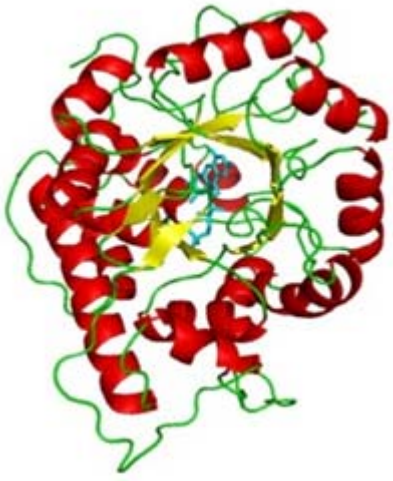

Figure 1.5 One subunit of the LOX tetramer (Umena et al. - 2006)
red: α-helices, yellow: β-strands, blue: FMN group

1.4 Reaction mechanism

L-Lactate oxidase catalyzes the oxygen-dependent conversion of *L*-lactate to pyruvate and hydrogen peroxide in a ping-pong mechanism (See Figure 1.6)
In such a ping-pong reaction the enzyme can exist in two states, such as E and a chemically modified form E*. When substrate A binds, it changes the enzyme from E to E* (for instance through transferring a chemical group to the active site) and is subsequently released. Only after the first product is released substrate B can bind and react with the modified enzyme, regenerating the unmodified E form.

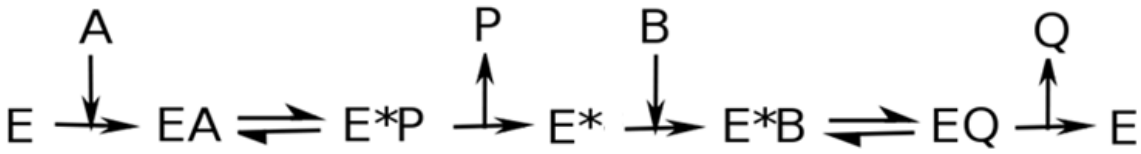

Figure 1.6 Ping-pong reaction mechanism

In the first part of the lactate oxidase reaction (reductive half-reaction) the α-hydroxyacid lactate is oxidized to form the keto acid pyruvate as the product, and the flavin is reduced to the hydroquinone. In the oxidative half reaction the reduced flavin is re-oxidized by an electron acceptor.

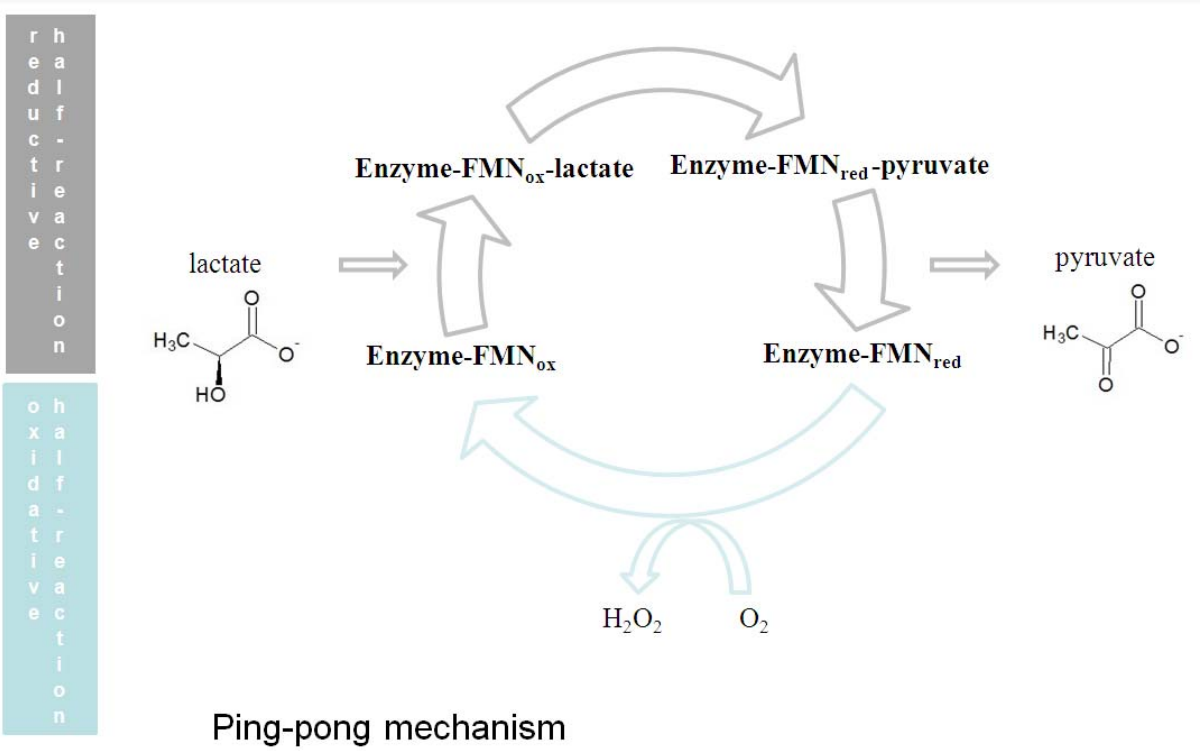

Figure 1.7 Reaction mechanism of lactate oxidase

There are two proposed mechanistic alternatives for the reductive half-reaction. (See Figure 1.8). In the first model a proton of the α-carbon of the substrate is abstracted by a catalytic histidine (His 265) leading to the formation of a carbanion intermediate and the subsequent transfer of two electrons to the flavin to yield reduced FMN and pyruvate. In the hydride transfer mechanism the substrate's α-hydroxyl is deprotonated by a catalytic histidine and the α-hydrogen is transferred to the N5 atom of the FMN cofactor as a hydride. The more recent publications (e.g. Furuichi et al. - 2008) support the latter mechanism.

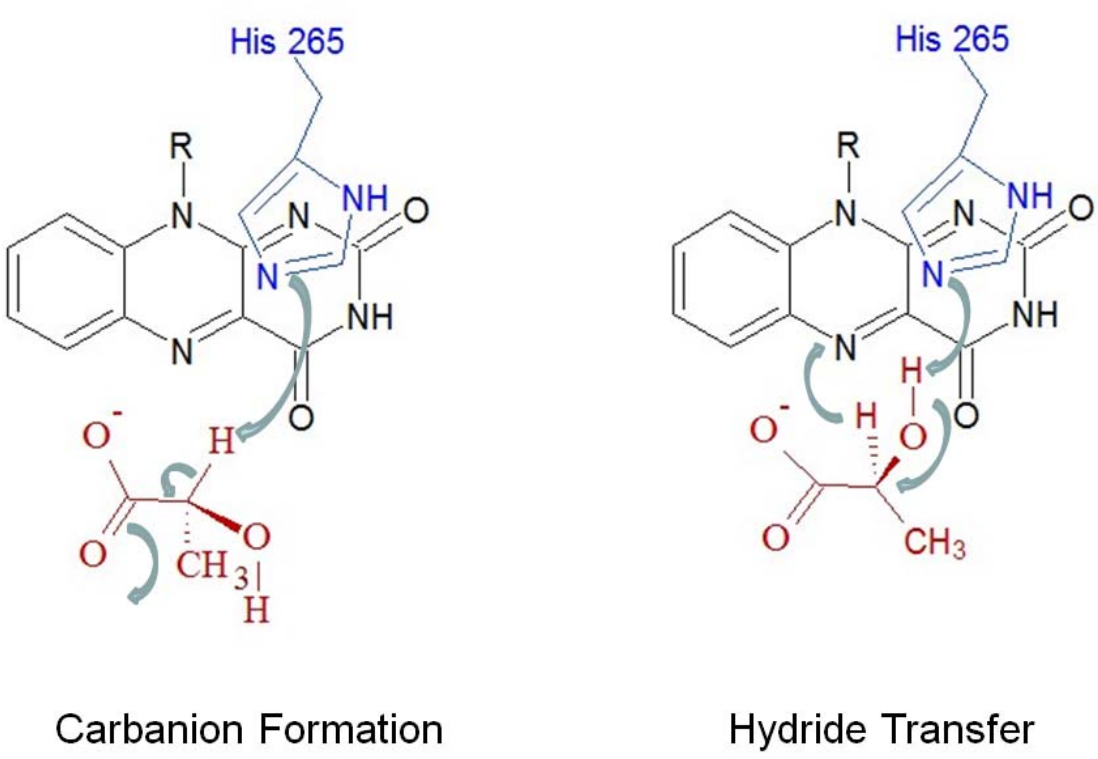

Figure 1.8 Proposed mechanisms for the reductive half-reaction of lactate oxidase

1.5 The active site of *L*-lactate oxidase

There are six amino acid residues near the active site which are conserved in all enzymes of the α-hydroxyacid-oxidase family. The only exception is the long-chain α-hydroxyacid-oxidase, where a phenylalanine residue is found instead of an otherwise conserved tyrosine (tyrosine 40). The function of this tyrosine is to bind to the substrate carbonyl group (Maeda-Yorita et al. - 1995). According to other sources (Leiros et al. – 2006) it binds to FMN and stabilizes the reactive isoalloxazine ring which forms as a transition state.
Older publications state that tyrosine 146 interacts with the hydroxyl group of the substrate (Maeda-Yorita et al. - 1995). In contrast to this opinion Murray et al. – 2008 propose that this tyrosine is not important for substrate binding but instead stabilizes the reactive isoalloxazine ring and in addition binds FMN (Leiros et al. – 2006).
Asparagine 174 is supposed to stabilize a protonated histidine 265 (Maeda-Yorita et al. - 1995).
The positively charged side chain of the lysine 241 residue stabilizes the anionic forms of the flavin group (Maeda-Yorita et al. - 1995). Leiros et al. – 2006 suggest that it also develops a hydrogen bond to FMN O2. Murray et al. – 2008 propose that it lowers the pk_A of the N5 of the isoalloxazine ring to facilitate an easier hydride transfer.
Histidine 265 functions as catalytic base abstracting a proton from the substrate.
And lastly the amino acid residue arginine 268 binds to the carbonyl group of the substrate.

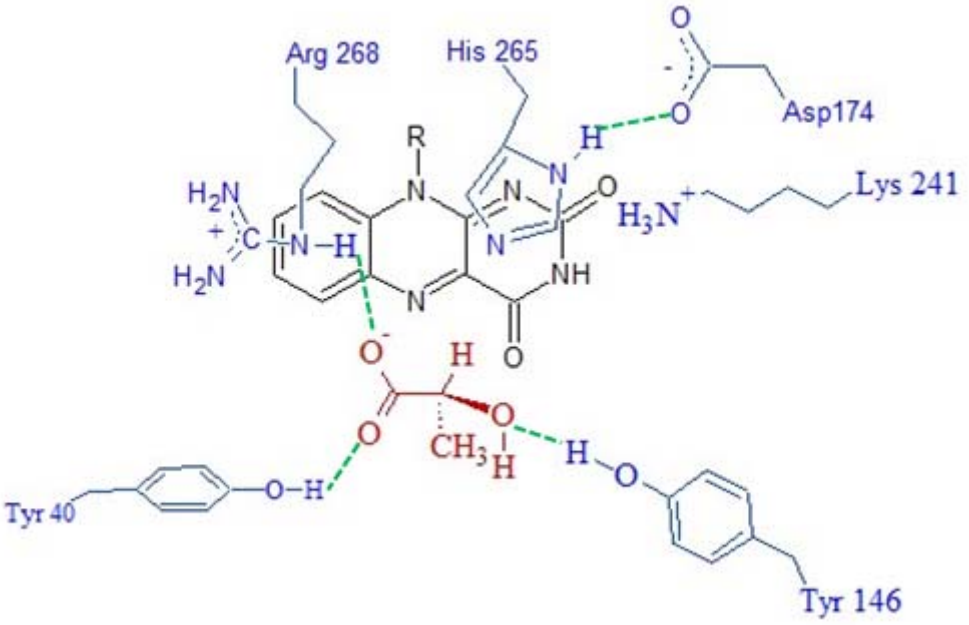

Figure 1.9 Conserved amino acid residues in LOX

Recently Furuichi et. al. - 2008 solved the X-ray structure of lactate oxidase at pH 4.5 and made some interesting discoveries about its oxidation mechanism. They discovered that the His265 flips away from the active site after it is protonated. This structure seems to mimic an intermediate after His265 abstracts a proton of the substrate. The flip results in a large structural rearrangement in which a new hydrogen bond network is fashioned between His265-Asp174-Lys221 and additionally a space for molecular oxygen is created in between the substrate and His265. In this way the structure of the mobile loop 4 (between the strand β_4 and the helix α_4) is altered. Furuichi proposes that in the following oxidative half-reaction His265 flips back and pushes the oxygen into the substrate binding site as the second substrate to produce hydrogen peroxide.

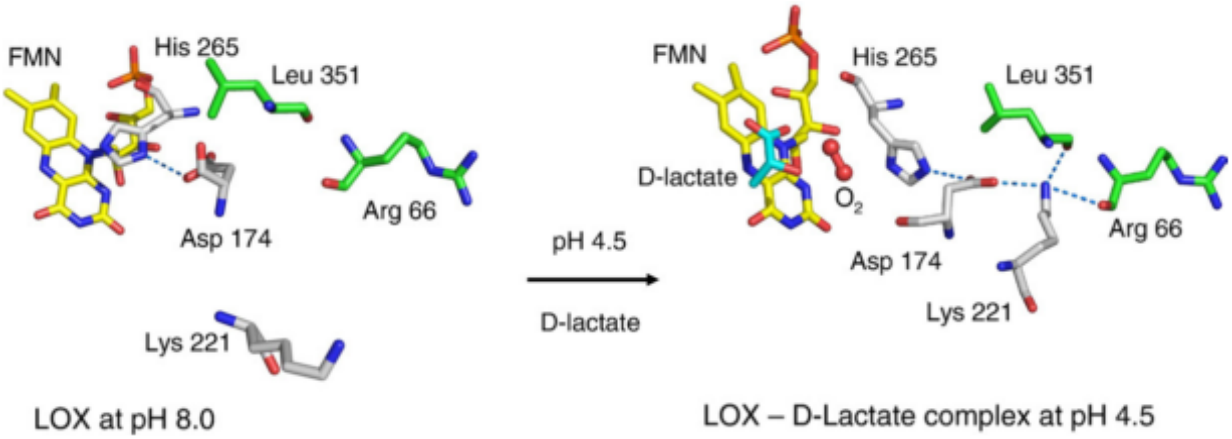

Figure 1.10 Mode of catalysis (Furuichi et. al. – 2008)

1.6 Mechanistic variations of *L*-lactate oxidase and *L*-lactate monooxygenase

While *L*-lactate oxidase and *L*-lactate monooxygenase share many characteristics, they vary in their reaction mechanism. The reason for this is that an intermediate of these enzymes – the reduced enzyme and pyruvate charge transfer complex – is not equally stable in both of them. The complex is very stable in the *L*-lactate monooxygenase where it reacts with O_2 to form a complex in which the oxidative decarboxylation takes place. This mechanism yields the products acetate, CO_2 and H_2O. Contrary to this in *L*-lactate oxidase the complex releases pyruvate rapidly. As a consequence the free reduced flavin form of the enzyme reacts with O_2 leading to the formation of pyruvate and H_2O_2 (See Figure 1.11).

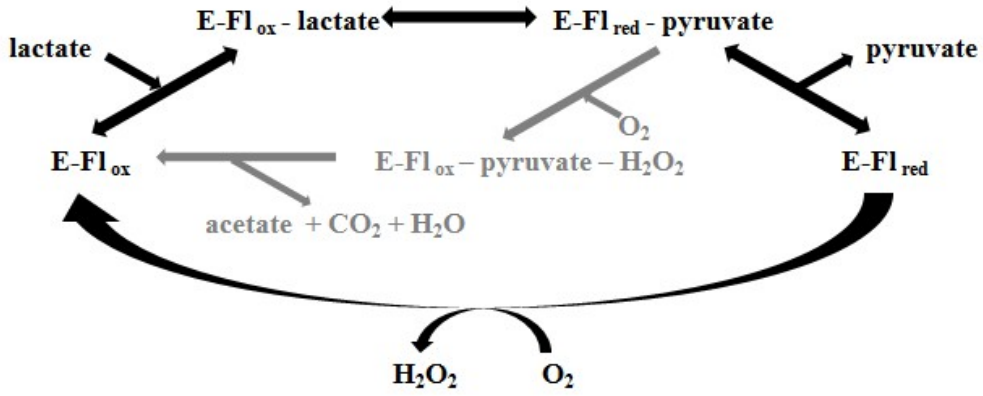

Figure 1.11 *L*-Lactate monooxygenase catalysis follows the inner pathway, *L*-lactate oxidase the outer pathway

1.7 Mutant design

Daff et. al. – 1994 tried to alter the substrate specificity of the α-hydroxyacid-oxidase flavocytochrome b_2 from *Saccharomyces cerevisiae*. The X-ray crystal structure of flavocytochrome b_2 shows that alanine 198 and leucine 230 are two not very well conserved residues which show van der Waals interactions with the substrate methyl group. Daff et al. attempted to change the specificity of flavocytochrome b_2 to favor larger substrates and created the following mutants: A198G, L230A and the double mutant A198G/L230A. An overview of the resulting kinetic data for the substrates of interest is given in Table 1.1. To summarize the results as far as they concern this work: In A198G the k_{cat} decreased for both substrates to about half of the values of the wild type enzyme while the K_M values increased about 10-fold. L230A shows a more than 10-fold decrease of k_{cat} for lactate while the k_{cat} for glycolate is the same as in the A198G mutant. The K_M values for both lactate and glycolate are slightly higher than in the A198G mutant. The double mutant provided the most interesting results - there was no activity with glycolate anymore while the k_{cat} for lactate was 10-fold lower than in the wild type and the K_M value increased more than 70-fold. (See Mowat et. al. – 2004 for insights to the mechanism of *L*-lactate dehydrogenation these mutations provide.)

Table 1.1 Change of substrate specificity in flavocytochrome b_2 (Daff et. al. – 1994)

	FCB2 Wild type		**FCB2 A198G**		**FCB2 L230A**		**FCB2 A198G / L230A**	
substrate	k_{cat} [s^{-1}]	K_M [mM]	k_{cat} [s^{-1}]	K_M [mM]	k_{cat} [s^{-1}]	K_M [mM]	k_{cat} [s^{-1}]	K_M [mM]
L-lactate	400	0.49	185	4.1	30	6.1	41	38
glycolate	7	0.34	3	2	3	3	-	-

An alignment of the structures of lactate oxidase and flavocytochrome b_2 showed that alanine 198 is conserved (alanine 95) while leucine 230 is a tyrosine 124 in lactate oxidase. (See Figure 1.12)

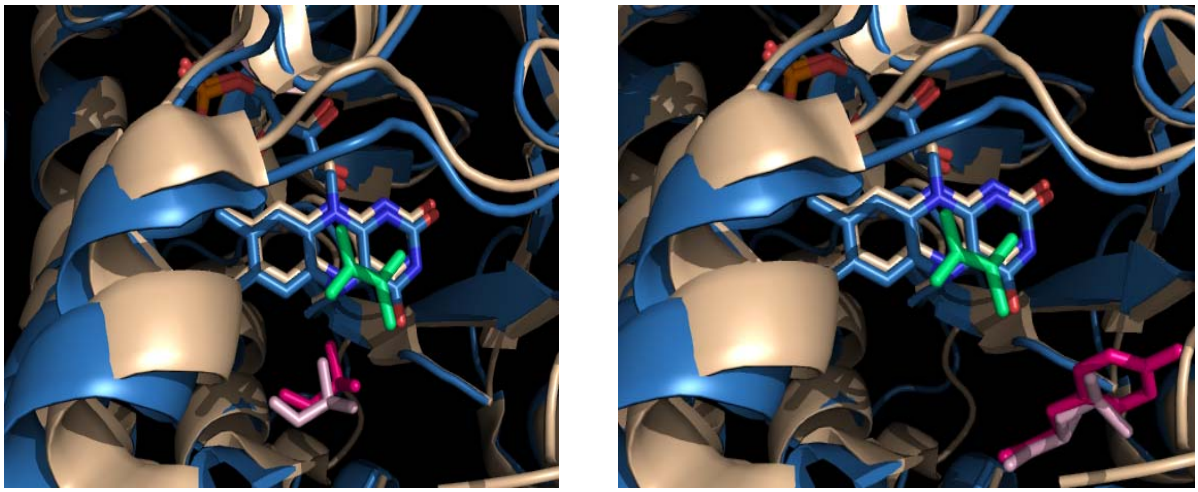

Figure 1.12 Structure alignment of the active site: light brown: LOX; blue: flavocytochrome b_2, green: pyruvate
Left: structure alignment highlighting LOX A95 (hot pink) and flavocytochrome b_2 A198 (light pink)
Right: structure alignment highlighting LOX Y124 (hot pink) and flavocytochrome b_2 L230 (light pink)

According to these alignments the following mutations were generated: A95G, Y124L, Y124A and the double mutant A95G/ Y124A.

Yorita et. al. – 1996 already reported the creation of an A95G lactate oxidase mutant. This mutant was constructed in an attempt to mimic the *L*-lactate monooxygenase (LMO) from *Mycobacterium smegmatis.* In this endeavor the group was not successful but instead they improved the LOX activity with long chain *L*-α-hydroxyacids and larger α-hydroxyacids such as the aromatic *L*-mandelate. The k_{cat} value of the mutant was only slightly lower than in the wild type enzyme while the K_M value increased nearly 8-fold. (See Table 1.2) The activity with glycolate was not investigated.

Table 1.2: Change of substrate specificity in the LOX A95G mutant (Yorita et. al. – 1996)

	LOX Wild type		**LOX A95G**	
substrate	k_{cat} **[s^{-1}]**	K_M **[mM]**	k_{cat} **[s^{-1}]**	K_M **[mM]**
L-lactate	283	0.87	250	6.75

2 Materials and methods

2.1 Spectrophotometric stop rate determination of *L*-lactate oxidase

Reagents were supplied by Roth and Sigma. The measurements were carried out with a Varian Cary 50 Bio UV-Visible Spectrophotometer.
For the kinetic measurements an adaption of the assay developed by Massey et. al. - 1972 was used. The assay is based on the fact that LOX oxidizes *L*-lactate and oxygen to pyruvate and hydrogen peroxide. Hydrogen peroxide is together with aminoantipyrine and N,N-dimethylaniline converted to a quinonediimine dye and water by the horseradish peroxidase (POD).

LOX: *L*-lactate + O_2 → pyruvate + H_2O_2
POD: 2 H_2O_2 + 4-aminoantipyrine + N,N-dimethylaniline → quinonediimine dye + H_2O

In a 0.51 ml reaction mix, the final concentrations are 39 mM 3,3 dimethylglutaric acid, 2.5 units peroxidase, 1.5 mM 4-aminoantipyrine, 49 mM L(+)lactic acid, 0.04% (v/v) N,N-dimethylaniline, 0.20 mM potassium phosphate and 0.001 - 0.002 unit lactate oxidase.
After 10 minutes 1 ml 0.25% (w/v) dodecylbenzenesulfonic acid solution is used to stop the reaction. Measuring conditions: T = 37°C, pH = 6.5, A_{565nm}, Light path = 1 cm

2.2 Measurement of the oxygen depletion

An oxygen electrode was used to check if the reaction mechanisms of the mutants changed to the mechanism of *L*-lactate monooxygenase which does not produce hydrogen peroxide. Using the oxygen electrode the consumption of oxygen was monitored. The O_2 sensor used was the Microx TX3 sensor supplied by PreSens.
For the measurement 50 mM potassium phosphate buffer pH 7 was preheated to 37°C in a water bath. Then 1450 µl of the buffer were transferred into a cuvette and 50 µl of substrate solution in an appropriate dilution (e.g. to a 0.17 – 17 mM *L*-lactate final concentration in the reaction mix for the wild type enzyme) were added. The mixture was stirred by a magnetic stirrer at 300 rpm. At last 10 µl enzyme were added (e.g. the LOX wild type was added to a final concentration of 6.6 nM in the mix for measuring the K_M value of *L*-lactate) and the cuvette was blanked off using a rubber plug. Finally the sensor was inserted into the solution and the measurement was conducted. Only the values measured during the first 3 minutes after adding the enzyme were considered in the determination of the kinetic constants because for this time frame the temperature was assumed to be constant at 37°C.

2.3 Buffer preparation

50 mM potassium phosphate buffer pH 7: A 50 mM monopotassium phosphate solution (solution 1) and a 50 mM dipotassium phosphate solution (solution 2) were prepared by dissolving the solid chemicals (supplied by Roth) in deionized millipore purified water. Solution 1 was adjusted to a pH value of 7 by adding solution 2.

TAE buffer pH 8 for the 1% agarose-gel and gel-electrophoresis: The buffer was prepared as a 50x stock solution. 242 g Tris, 57.1 ml acetic acid and 18.6 mg EDTA were mixed with deionized millipore purified water to a final volume of 1 l.

2.4 Determination of the protein concentration with the BCA protein assay

The BCA protein assay supplied by Thermo scientific was used to determine the LOX protein concentrations. To assemble the working reagent 1 part reagent B was mixed with 50 parts reagent A. 50 µl of the unknown protein sample (diluent for blank) were pipetted into a 1.5 ml tube and additionally samples for a calibration curve with 6 BSA (bovine serum albumin) concentrations ranging from 0.1 to 1 mg/ml were set up. Then 1 ml of the working reagent was added to each sample and the tubes were incubated for 30 minutes at 37°C. After letting the samples cool down for 5 minutes to room temperature their absorbance at 562 nm was measured versus the blank using the Varian Cary 50 Bio UV-Visible Spectrophotometer.

2.5 Overexpression of *L*-lactate oxidase

The plasmid pLO-1 (pLOX) was supplied by Roche. It consists of 4062 bp and contains:

- Ampicillin-resistance
- Tac-Promotor
- LOX
- Several restriction enzyme sites

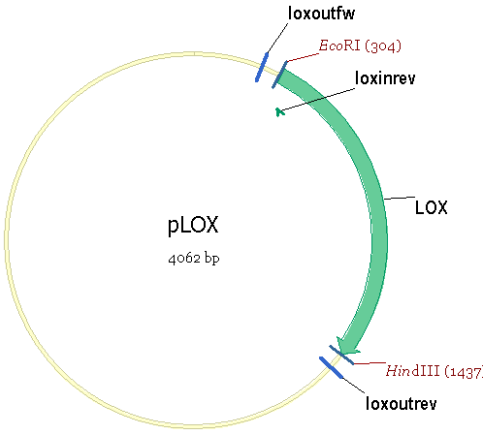

Figure 2.1 pLOX

Roche supplied the strain: *E. coli* RR1 Δ M 15. No further information of this strain is available. The SDS polyacrylamide gel of the crude extract (See results) showed that the amount of produced LOX is not much higher than the amount of some other proteins that accumulate in the *E. coli* cells. To get a higher overexpression of LOX it was decided to transform the vector pLOX into the *E. coli* BL21(DE3) strain which is optimized for protein expression and has a relatively good transformation efficiency. Its chromosomal genotype is *E. coli B dcm ompT hsdS*($r_B^- m_B^-$) *gal*.

2.6 Cell cultivation

Cultivation conditions were 130 rpm and 30°C. The starter culture was grown overnight in 80 ml fermentation medium. For the biomass production the mutants were cultivated in 200 ml fermentation medium in 1 l shake flasks with a starting OD_{600} of 0.1. About 2.5 hours later an OD_{600} of 0.8-1 was reached and induction with sterile filtrated IPTG took place (to a 250 µg/l IPTG concentration in the shake flasks). After 5 hours the cells were harvested by centrifugation at 5000 rpm and 4°C for 15 minutes and then resuspended in 50 mM potassium phosphate buffer pH 7. Lastly the cells were stored in the freezer until purification.
The following medium was used for fermentation: 50 g/l yeast extract, 5 g/l glucose, 2.94 g/l K_2HPO_4 * 7 H_2O, 0.68 g/l magnesium sulfate * 7 H_2O, 0.05 g/l ampicillin

2.7 Protein purification

See appendix for DEAE purification.
The *E. coli* cells were disrupted by a French press. The cell debris was subsequently removed by ultracentrifugation for 45 minutes at 4°C and 30000 rpm. Afterwards 3 M ammonium sulfate stock solution was added to a final concentration of 1.5 M ammonium sulfate and the solution was stirred in the cooling room at 4°C for 30 minutes. Then precipitated proteins were removed by centrifugation for 45 minutes at 4°C and 5000 rpm. Next the cell extract (supernatant of the centrifugation) was purified by hydrophobic interaction chromatography followed by a desalting step. Lastly ion exchange chromatography was used to get pure protein.

Detailed purification protocol after cell harvesting:

1st step: Ammonium sulfate precipitation at 1.5 M ammonium sulfate

2nd step: Hydrophobic interaction chromatography (HIC)
The analytical column was mounted on a fast protein liquid chromatography (FPLC) system (ÄKTA Amersham Biosciences UPC-900 P-920).

Phenyl–Sepharose FF column conditions:

CV: 64 ml
flow: 5 ml/minute
washing buffer: 50 mM potassium phosphate buffer pH 7 + 1.5 M ammonium sulfate
elution buffer: 50 mM potassium phosphate buffer pH 7
maximum pressure: 5 bar
maximum sample load: 30 mg/ml

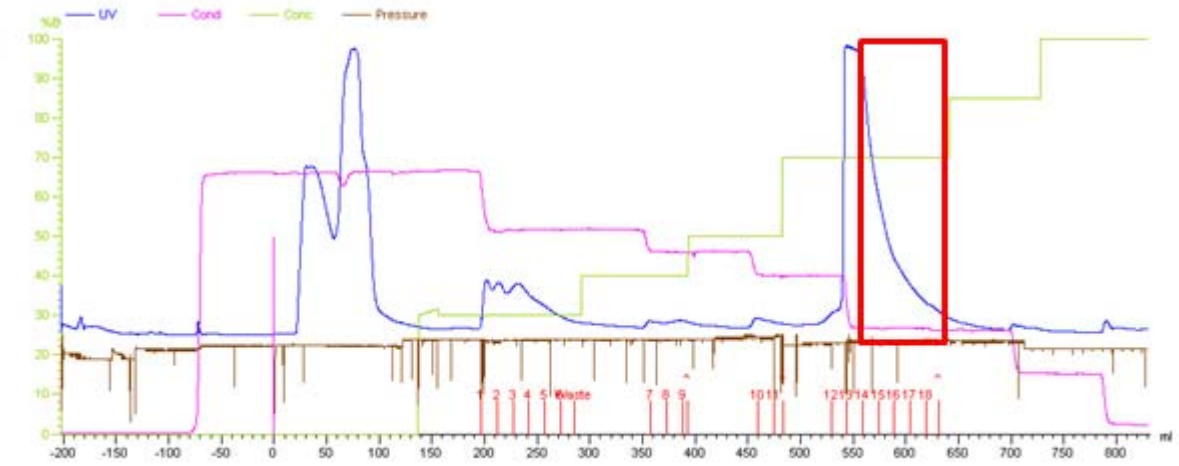

Figure 2.2 Elution profile of the Phenyl–Sepharose FF HIC column

3rd step: Desalting

The wild type LOX elutes in the HIC at an ammonium sulfate concentration of around 1 M. So the fractions with LOX activity had to be desalted because without removal of the salt the enzyme could not bind to the ion exchange column of the next purification step. This was accomplished with vivaspin tubes - using those the buffer was changed to pure elution buffer: 50 mM potassium phosphate buffer pH 7.

4th step: Ion exchange chromatography

The analytical column was mounted on a fast protein liquid chromatography (FPLC) system (ÄKTA Amersham Biosciences UPC-900 P-920).

MonoQ column conditions:

CV: 1 ml
flow: 0.5-3 ml/minute
washing buffer: 50 mM potassium phosphate buffer pH 7
elution buffer: 50 mM potassium phosphate buffer pH 7 + 1 M potassium chloride
maximum pressure: 40 bar
maximum sample load: 45 mg/ml

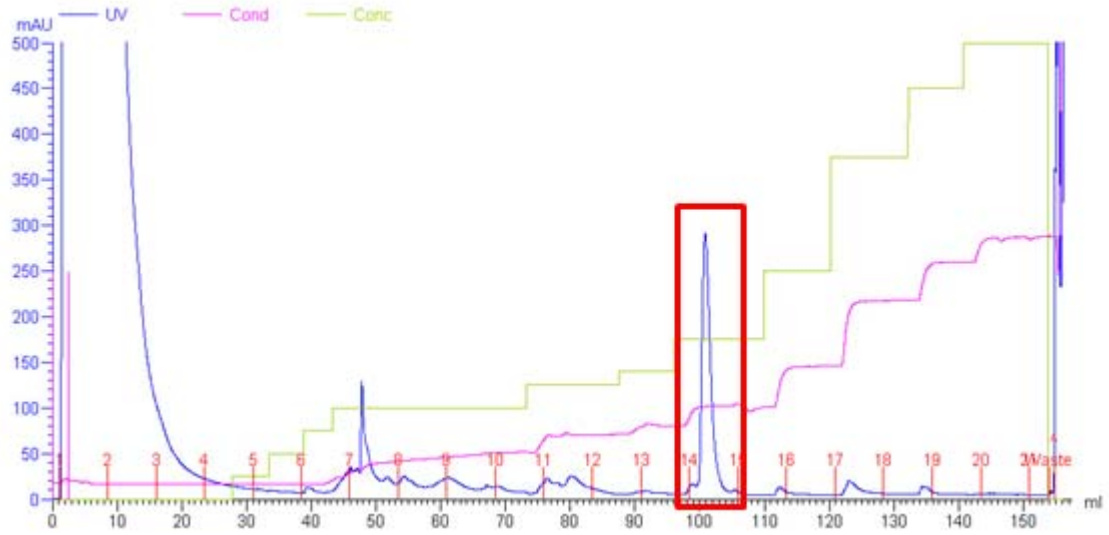

Figure 13 Elution profile of the MonoQ anion exchange column

2.8 DNA purification

For sequencing purposes the DNA was purified employing the Promega Wizard® *Plus* SV Minipreps DNA Purification System. In a deviation of the last step of the protocol the DNA was eluted with 40 µl of deionized millipore purified water instead of 100 µl nuclease-free water supplied by the purification kit.
The PCR reactions were purified by gel-electrophoresis at 90 V for 45 min with a preparative 1% agarose-gel using the Macherey-Nagel NucleoSpin® Extract II Kit. In a deviation of the last step of the protocol the DNA was eluted with deionized millipore purified water instead of elution buffer NE supplied by the purification kit.
1% agarose-gel: A solution of 1% agarose in 70 or 140 ml TEAE buffer was made. This solution was subsequently brought to the boil in a microwave oven to dissolve the agarose. Then the solution was cooled under running water while swirling it. Afterwards ethidium bromide was added (5 µl per 100 ml gel) and dispersed in the solution. Finally the solution was poured into the gel rack and the appropriate comb was inserted into the gel. After the gel became solid it was transferred together with the rack into a tank with TEAE buffer.

2.9 Two stage PCR protocol for site-directed mutagenesis

A polymerase chain reaction (PCR) was employed to introduce the mutation into LOX. The protocol used was published by Wang et. al. – 1999. Important for the primer design was that both primers must overlap (20-30 bases) and carry the mutation. They also should have additional 10-15 bases on the respective 3´-end that correspond to the sequence. Cycling conditions were as follows: 50 seconds at 95°C for denaturation, 50 seconds at 60°C for annealing and 5 minutes at 68°C for extension (4 circles). Two separate primer extension reactions were set up - one reaction for each forward and reverse primer. One reaction (50 µl) consisted of 100-200 ng plasmid template, 0.3 µM of one primer, 10x PfuUltra™-buffer, 200 µM dNTPs and 0.05 U PfuUltra™-polymerase. Then 25 µl from each primer extension

reaction were combined and incubated as above under the same conditions but for 18 cycles. Afterward 25 µl of the reaction were removed and 0.4 U of *Dpn*I restriction endonuclease (Promega) were added. The *Dpn*I digestion of the template DNA took at least 1 hour at 37°C and lastly the enzyme was inactivated through raising the temperature to 75°C for 10 minutes. Subsequently the DNA was transformed into *E. coli* cells.

The primers were designed to include restriction sites which normally do not occur on the plasmid (See Figure 2.4). This gives the opportunity to check on an 1% agarose gel if the mutation was successfully introduced before sequencing.

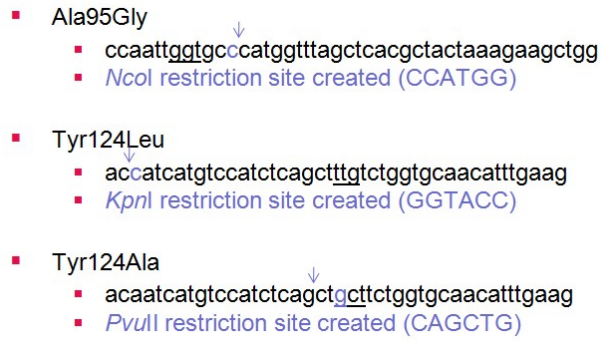

Figure 2.4 PCR primers for site directed mutagenesis

2.10 Competent cell preparation and transformation of *E. coli* cells

The protocol for electro-competent cell preparation written by Barbara Petschacher was used to create competent *E. coli* BL21 and Top 10 cells.
Two Erlenmeyer flasks with 50 ml starter culture were inoculated with *E. coli* cells taken from a glycerol stock and incubated over night at 37°C and 130 rpm. Next day 4 ml of the starter culture were added to each of the six 200 ml main cultures. Then the main cultures were incubated at 37°C and 130 rpm until an OD_{600} of 0.5-0.8 was reached. Subsequently the main culture shake flasks were put on ice and into the cooling room where they were stored for 20 minutes at 4°C. Afterwards the cells were transferred under sterile conditions into centrifuge beakers and centrifuged for 25 minutes at 4°C and 5000 rpm. The pellets were suspended in 10 ml cold sterile H_2O each and two suspended pellets were combined in one centrifuge beaker. In the first washing step cold sterile water was added to the centrifuge beakers until they were 2/3 full. Then followed centrifugation for 25 minutes at 4°C and 5000 rpm. Next the cells were resuspended in 10 ml cold sterile 10% glycerol solution and combined. In the second washing step cold sterile 10% glycerol solution was added to the centrifuge beakers until they were 2/3 full. Then they were centrifuged for 25 minutes at 4°C and 5000 rpm. In the third washing step 30 ml cold sterile 10% glycerol solution were added to the pellet and the cells were suspended and then transferred to a sterile falcon tube. This was followed by centrifugation for 25 minutes at 4°C and 5000 rpm. At last the cells were resuspended in 4 ml cold sterile 10% glycerol solution and portioned to 100 µl amounts in 1.5 ml tubes. Finally the cells were shock frozen with liquid N_2 and stored in the freezer at –70°C.
LB-medium: 10 g/l peptone, 5 g/l yeast extract, 10 g/l sodium chloride.

The following two *E. coli* strains were used for the transformations: TOP10 for genetic work, BL21 Gold for protein expression.

2 µl vector DNA (see 2.9) and 40 µl electro-competent *E. coli* cells were mixed in pre-cooled cuvettes. Then the EC2 pulse was fired and immediately after that 900 µl SOC solution (preheated to 37°C) were added to the cells. Next the cells regenerated for 1 hour at 37°C until finally 100 µl amounts of different cell dilutions were plated on Amp-Agar plates (100 µg/ml ampicillin, preheated to 37°C). The agar plates were incubated over night at 37°C. Subsequently the colonies were transferred to new agar plates. The following day the cells on these plates were used to inoculate over night cultures of cuvettes filled with 10 ml LB-Amp medium (100 µg/ml ampicillin). A day later the DNA was purified (See 2.8) and 5 µl of the DNA were digested for 1-2 hours with 10 U of the respective fast digest enzyme (See Figure 2.4) supplied by Fermentas, the appropriate buffer and H_2O to check if the mutation was successfully introduced. The restriction enzyme was inactivated at 65°C for 10 minutes. This was followed by gel-electrophoresis at 90 V for 45 minutes with a 1% agarose gel. The positive mutants were sent to Agowa for sequencing.

2.11 SDS polyacrylamide gel-electrophoresis

SDS PhastGel™ and buffer strips supplied by GE Healthcare were used. The SDS polyacrylamide gel was run on the Phast system (Amersham Biosciences).
Sample preparation: The sample was diluted to a protein concentration of 2 mg/ml and mixed 1:1 with the dissociation buffer. Afterwards it was held for 2 minutes at 90°C.
Manual Staining: First the gel was put into the staining solution for 1 hour, then it was destained for 15 minutes in the destain solution. After changing the destain solution it was destained for another 45 minutes. Finally it was preserved with the preserve solution over night.
Dissociation buffer: 20 mM Monopotassium phosphate, 6 mM EDTA, 6% SDS, 10% glycerol, 0.05% bromphenol blue (no mercaptoethanol needed because LOX has no disulfide bonds)
Staining: Stock solution: 1 tablet PhastGel™ Blue R, 20 ml H_2O d.d., 180 ml ethanol
Staining Solution: 1 part stock solution + 1 part 20% acetic acid
Destain solution: 3 parts ethanol : 1 part acetic acid : 6 parts H_2O
Preserve solution: 10% acetic acid, 13% glycerol

2.12 Strain maintenance

15% Glycerol stocks of the mutants were produced by shock freezing 1:1 mixtures of 30% glycerol solution and mutant overnight cultures with liquid N_2. They were stored at -70°C.

2.13 Fluorescence spectroscopy

The fluorescence data was collected with the Hitachi F-4500 Fluorescence Spectrophotometer at room temperature. The emission between 300 nm and 380 nm at an excitation wavelength of 280 nm was measured. Enzyme concentrations were for wild type and A95G mutant 1 µM enzyme in 50 mM potassium phosphate buffer pH 7. Other parameters: scan speed 1200

nm/min, excitation and emission slit: 5 nm. The photomultiplier tube was set to a voltage of 700 V and the response time was 0.1 seconds.

2.14 Absorption spectroscopy

The measurements were taken with the Varian Cary 50 Bio UV-Visible Spectrophotometer. The data was collected at room temperature with a 1 µM concentration of the enzyme in 50 mM potassium phosphate buffer pH 7. The absorption between 300 nm and 600 nm was detected.

3 Results

3.1 Overexpression

While the specific activity of the crude extract in the *E. coli* RR strain was only 0.86 U/mg it was more than 10-fold higher in the *E. coli* BL21 cells (9.51 U/mg). The differences in the overexpression of the two strains are clearly visible on the SDS polyacrylamide gel (See Figure 3.1).

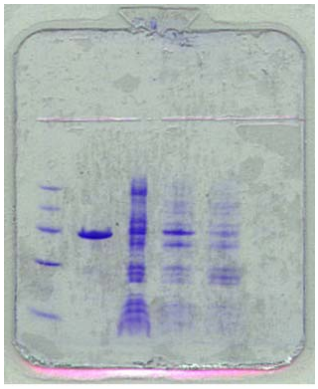

Figure 3.1 Different expression strains: *E. coli* RR1, *E. coli* BL21

3.2 Creation of the mutants

The DNA sequences of the supposed mutants were verified on 1% agarose gels. This was possible because together with each mutation a recognition sequence for a restriction enzyme was added or a previously existing recognition sequence for a restriction enzyme was removed. In the case of A95G a *Nco*I restriction site was added. A 1% agarose gel used to check for the successful insertion of the mutation A95G into the plasmid can be seen below (See Figure 3.2). As pLOX had no *Nco*I recognition sequence before introducing the mutation it should be apparent on the 1% agarose gel that lane 2 which shows pLOX after digestion with *Nco*I exhibits the same bands as lane 10 which shows the undigested pLOX. On the gel below this cannot been seen satisfactorily but the difference of lane 2 or lane 10 to the A95G plasmid cut by *Nco*I on lane 4 which shows 1 sharp band is very pronounced.

After being verified on the 1% agarose gel the mutants were checked additionally by sending them to sequencing at AGOWA.

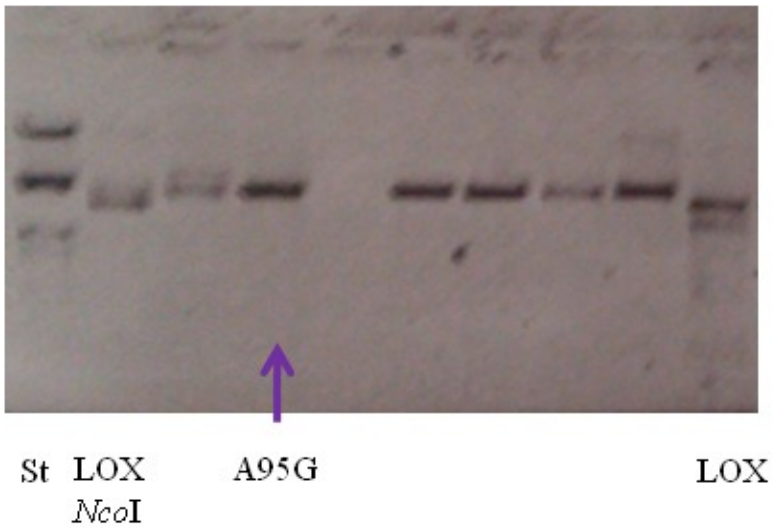

Figure 3.2 1% agarose gel of supposed A95G mutants: lane1: standard λ-*Hin*dIII, lane 2: pLOX digested by NcoI, lane 3-9 supposed A95G mutants, lane 4: A95G mutant verified by sequencing, lane 10: undigested pLOX

After verification of the successful introduction of the mutation to the DNA the expression of the corresponding proteins had to be checked. See Figure for a SDS polyacrylamide gel of the crude extracts of all mutants which also shows that they all were successfully expressed. As LOX has a molecular weight of 41 kDa the corresponding band can be seen just below the 45 kDa band of the standard.

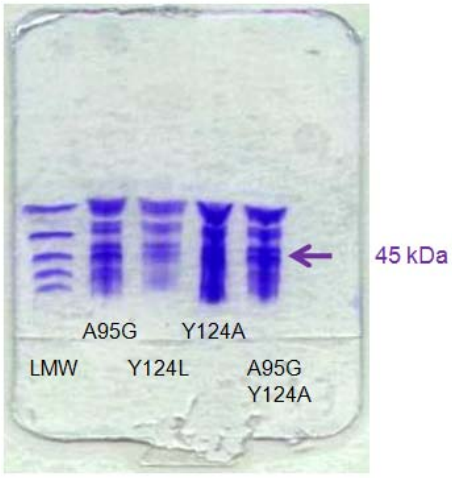

Figure 3.3 SDS polyacrylamide gel with mutant crude extracts. LMW: Low molecular weight standard (GE Healthcare)

3.3 Purification

As is apparent on the SDS polyacrylamide gel in Figure 3.4 this purification protocol yields protein which is pure enough to be used for kinetic measurements.

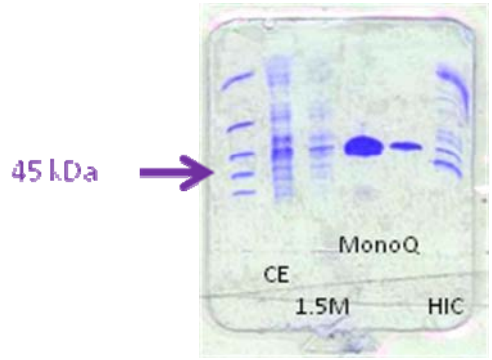

Figure 3.4 SDS polyacrylamide gel of the protein purification: CE: crude extract, 1.5M: ammonium sulfate precipitation supernatant, HIC: fraction after the hydrophobic interaction chromatography, MonoQ: pure protein after the ion exchange chromatography

An overview of the protein and activity yield of this protocol is given in Table 3.1. After the hydrophobic interaction chromatography 64% of the protein activity in the crude extract can still be found but only 14% of the protein activity can be found after the last purification step. There are probably some losses in the desalting step which contribute to the huge loss of activity in the last step. Apparently this protocol is not ideal either but after the last purification step the enzyme is pure enough for my purposes.
Also worth mentioning is that some of the mutants are less stable than the wild type. When they are stored either in the cooling room at 4°C or in the freezer at -20°C a visible amount of precipitate is found which can not be dissolved again.

Table 3.1 Purification overview for the wild type enzyme

Purification step	% protein	% activity
CE	100	100
1.5 M $(NH_4)_2SO_4$	87	64
HIC	11	63
MonoQ	1	14

Table 3.2 shows the purification process of wild type LOX and the A95G mutant. The ammonium sulfate precipitation is not very efficient but indispensable because of the consecutive chromatography step. It is apparent that the hydrophobic interaction chromatography (HIC) with a purification factor of around 7 is the most important step of this purification protocol.

Table 3.2 Purification – specific activities with lactate. CE: crude extract, 1.5 M $(NH_4)_2SO_4$: solution after the first ammonium sulfate precipitation, HIC: solution purified using hydrophobic interaction chromatography, MonoQ: solution purified using ion exchange chromatography

Purification step	Specific activity wild type [U/mg]	Purification factor wild type	Specific activity A95G [U/mg]	Purification factor A95G
CE	8.4		1.7	
1.5 M $(NH_4)_2SO_4$	6.2	0.7	2.0	1.2
HIC	43.7	7	13.4	6.6
MonoQ	177.7	4	23.7	1.8

3.4 Activity measurements

It was checked if another enzyme expressed by the *E. coli* BL21 cells can give false positive results with the employed assays. Neither with the spectrophotometric assay nor with the oxygen electrode a background activity was measurable.

It was also tested if ethylene glycol is a substrate for LOX which was not the case (for a LOX concentration of 1 mg/ml). As ethylene glycol is not an α-hydroxyacid but a diol this outcome was not surprising.

The mutants Y124L and Y124A were found to be inactive even after purification by hydrophobic interaction chromatography. This was determined by using the spectrophotometric assay and by measuring the oxygen consumption. According to these results it can be ruled out that either the Y124L or the Y124A changed their mechanism to the *L*-lactate monooxygenase mechanism.

The activity of double mutant A95G Y124A could not be determined with the coupled assay but the oxygen electrode showed it to be more than 100-fold less active than the wild type enzyme. Because the mutant showed activity with the spectrophotometric assay – but not enough to be quantifiable – it still follows the reaction mechanism of LOX.

Table 3.3 shows the kinetic constants and their method of determination. For comparison the values measured by Yorita et. al. – 1996 are included.
Compared to the WT lyophilisate supplied by Roche the k_{cat} value of the self purified enzyme is slightly lower; probably because the self purified WT enzyme lost activity through purification and storage. The K_M value of the Roche wild type enzyme determined by oxygen measurement is similar to the one ascertained via assay.

Table 3.3 Activity measurements. WT*: wild type lyophilisate supplied by Roche

	K_M lactate [mM]	K_M glycolate [mM]	k_{cat} lactate [s^{-1}]	k_{cat} glycolate [s^{-1}]	Method of determination
WT	0.62	1.35	71	1.8	coupled assay
A95 G	1.63	3.92	16	0.3	coupled assay
WT	0.87		283		Yorita et. al. – 1996
A95 G	6.75		250		Yorita et. al. – 1996
WT *	0.23		101		oxygen electrode

Table 3.4 shows the kinetic constants calculated with the values obtained by coupled assay in Table 3.3. The A95G mutant shows a 1.6 higher selectivity for lactate than the wild type enzyme.

Table 3.4 Kinetic constants

	k_{cat}/ K_M lactate	k_{cat}/ K_M glycolate	k_{cat} lactate / k_{cat} glycolate	Selectivity for lactate
WT	113	1	40	86
A95 G	10	0.1	58	139

LOX wild type (WT)

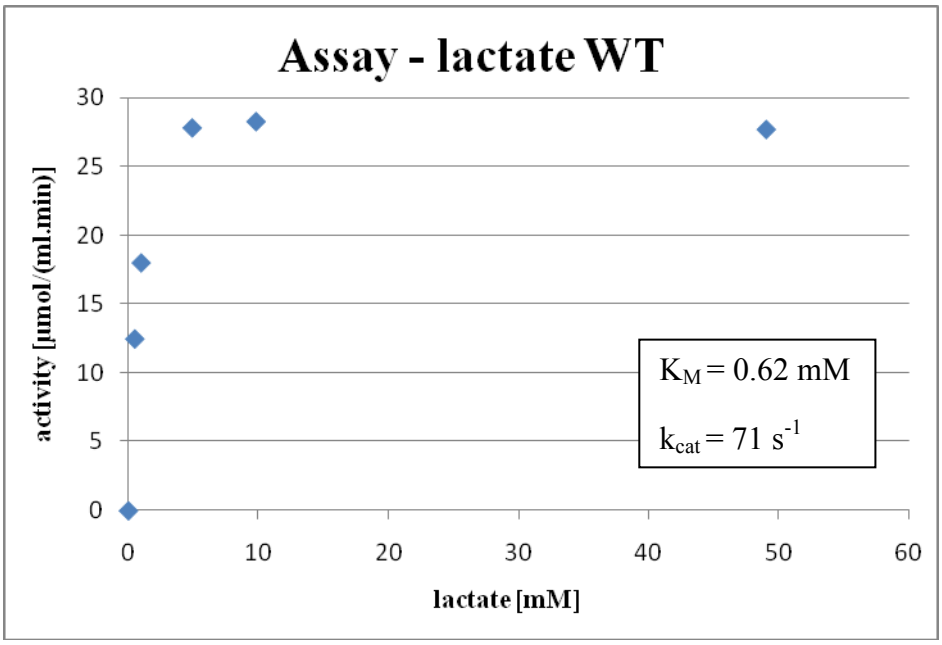

Figure 3.5 Assay lactate WT

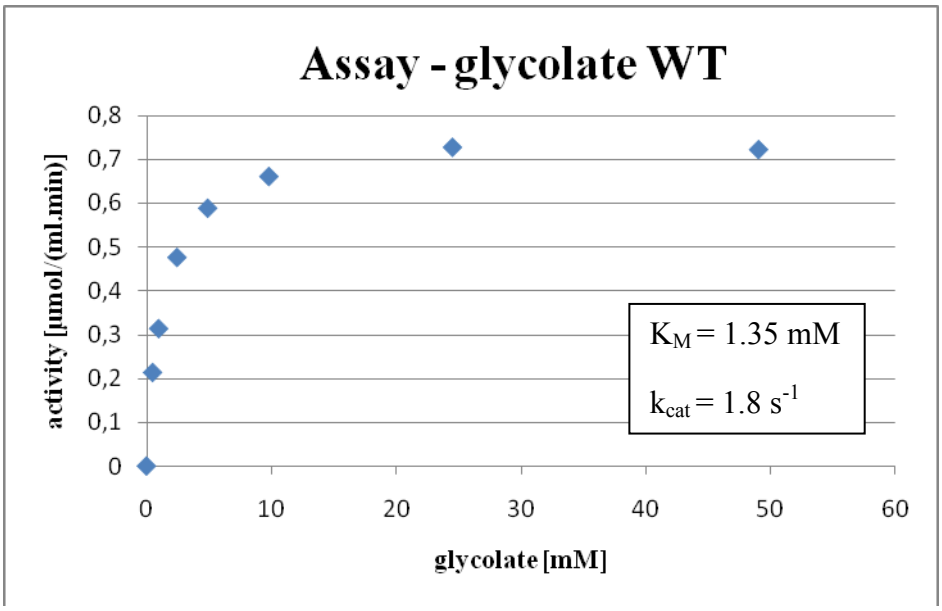

Figure 3.6 Assay glycolate WT

LOX A95G mutant

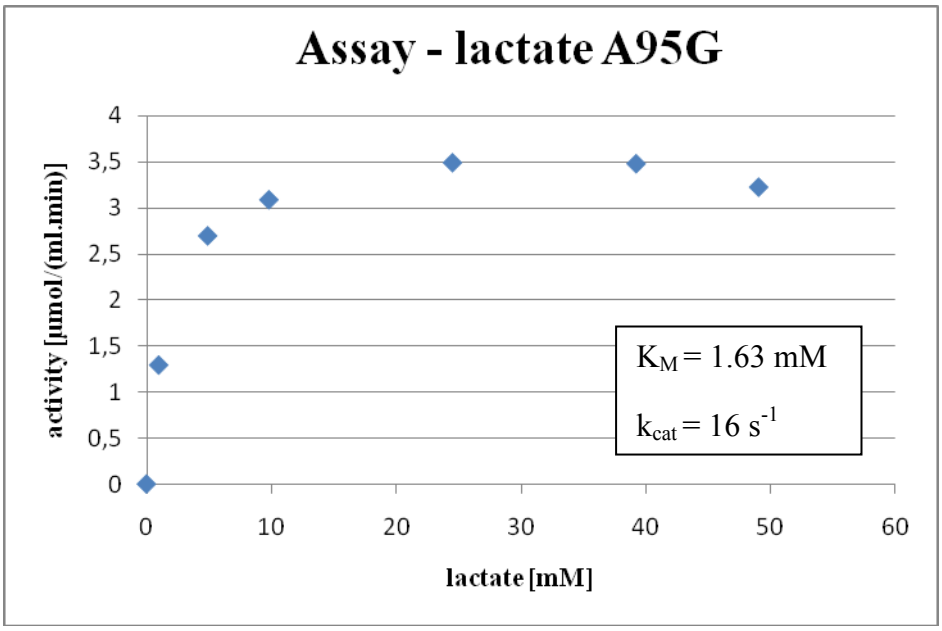

Figure 3.7 Assay lactate A95G

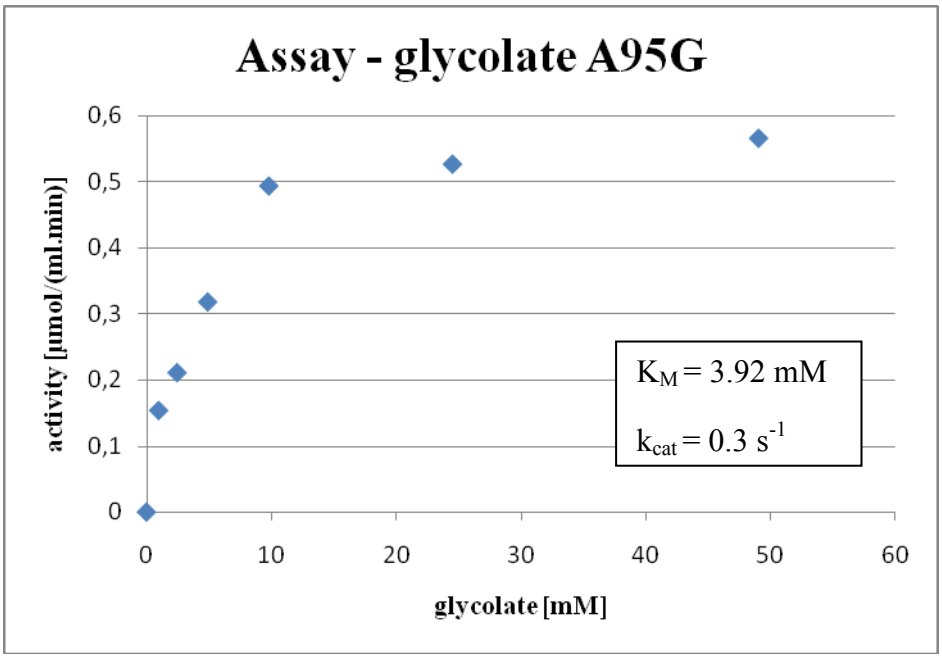

Figure 3.8 Assay glycolate A95G

LOX wild type (WT) lyophilisate supplied by Roche

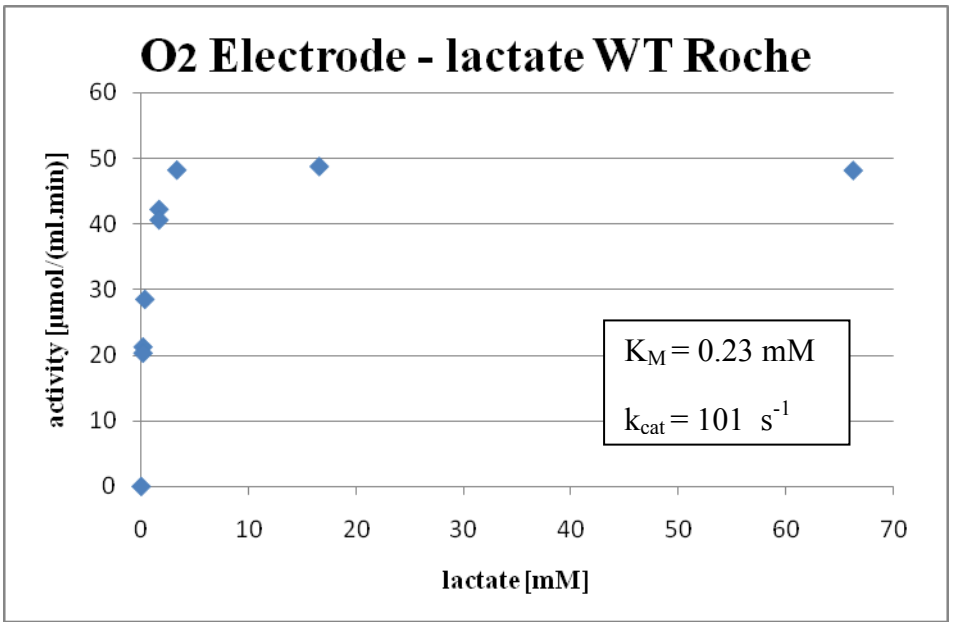

Figure 3.9 Oxygen electrode measurement of lactate with the WT Roche lyophilisate

3.5 Fluorescence spectra

The fluorescence curves of the aromatic amino acids of wild type LOX and the A95G mutant were compared. The peaks of both enzymes look approximately the same so their structure is probably nearly identical.

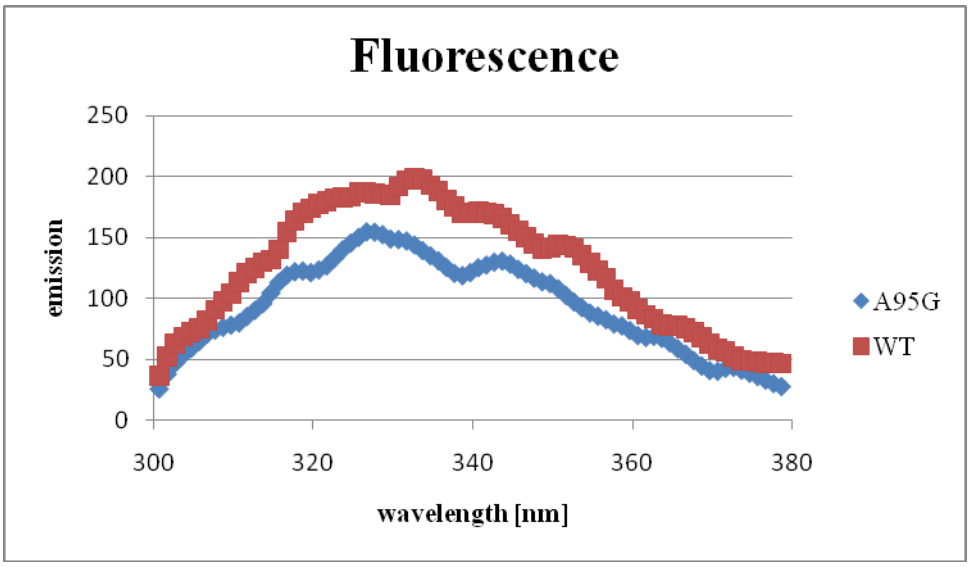

Figure 3.10 Fluorescence spectra

3.6 Absorption spectra

The absorption spectra of the reduced flavin mononucleotide of both enzymes are nearly identical. This means that the A95G mutant is functional and can bind the cofactor like the wild type enzyme. Due to the fact that just one measurement was taken, the release of the cofactor over time was not monitored.

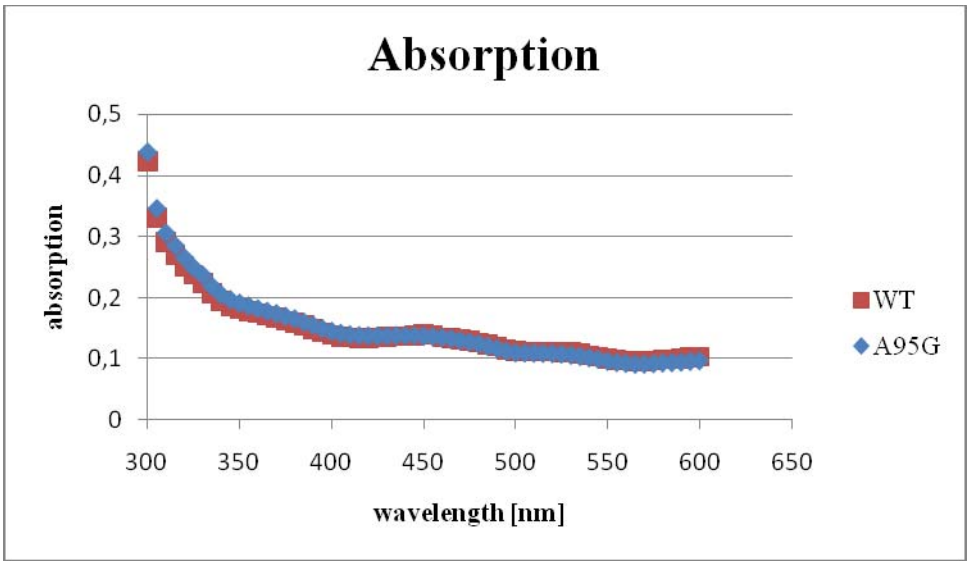

Figure 3.11 Absorption spectra

4 Literature

Daff, S.; Manson, F. D.; Reid, G. A.; Chapman, S. K.: Strategic manipulation of the substrate specificity of *Saccharomyces cerevisiae* flavocytochrome b2. *Biochemical Journal* (1994), 301 (3): 829-834

Furuichi, M.; Suzuki, N.; Dhakshnamoorhty, B.; Minagawa, H.; Yamagishi, R.; Watanabe, Y.; Goto, Y.; Kaneko, H.; Yoshida, Y.; Yagi, H.; Waga, I.; Kumar, P. K.; Mizuno, H.: X-ray structures of *Aerococcus viridans* lactate oxidase and its complex with *D*-lactate at pH 4.5 show an α-hydroxyacid oxidation mechanism. *Journal of Molecular Biology* (2008), 378 (2): 436-446

Leiros, I.; Wang, E.; Rasmussen, T.; Oksanen, E.; Repo, H.; Petersen, S. B.; Heikinheimo, P.; Hough, E.: The 2.1 Å structure of *Aerococcus viridans L*-lactate oxidase (LOX). *Acta Crystallographica Section F – Structural Biology and Crystallization Communications* F (2006), 62 (12): 1185-1190

Lockridge, O.; Massey, V.; and Sullivan, P. A.: Mechanism of action of the flavoenzyme lactate oxidase. *Journal of Biological Chemistry* (1972), 247 (24): 8097-8106

Maeda-Yorita, K.; Aki, K.; Sagai, H.; Misaki, H.; Massey, V.: *L*-Lactate oxidase and *L*-lactate monooxygenase: mechanistic variations on a common structural theme. *Biochimie* (1995), 77 (7-8): 631-642

Mowat, C. G.; Wehenkel, A.; Green, A. J.; Walkinshaw, M. D.; Reid, G. A.; Chapman, S. K.: Altered substrate specificity in flavocytochrome b2: structural insights into the mechanism of *L*-lactate dehydrogenation. *Biochemistry* (2004), 43 (29): 9519-9526

Murray, M. S.; Holmes, R. P.; Lowther, W. T.: Active site and loop 4 movements within human glycolate oxidase: implications for substrate specificity and drug design. *Biochemistry* (2008), 47 (8): 2439-2449

Pernet, P.; Bénéteau-Burnat, B.; Vaubourdolle, M.; Maury, E.; Offenstadt, G.: False elevation of blood lactate reveals ethylene glycol poisoning. *The American Journal of Emergency Medicine* (2009), 27 (1): 132.e1 - 132.e2

Umena, Y.; Yorita, K.; Matsuoka, T.; Kita, A.; Fukui, K.; Morimoto, Y.: The crystal structure of L-lactate oxidase from *Aerococcus viridans* at 2.1 Å resolution reveals the mechanism of strict substrate recognition. *Biochemical and Biophysical Research Communications* (2006), 350 (2): 249-256

Wang, W.; Malcolm, B. A.: Two-stage PCR protocol allowing introduction of multiple mutations, deletions and insertions using quikchange site-directed mutagenesis. *Biotechniques* (1999), 26 (4): 680-682

Yorita, K.; Aki, K.; Ohkuma-Soyejima, T.; Kokubo, T.; Misaki, H.; Massey, V.: Conversion of *L*-lactate oxidase to a long chain α-hydroxyacid oxidase by site-directed mutagenesis of alanine 95 to glycine. *Journal of Biological Chemistry* (1996), 271 (45): 28300-28305

APPENDIX

1. *Adding a tag to LOX*

1.1 Introduction

The purification protocol supplied by Roche involved multiple steps which was deemed inconvenient because a large quantity of mutants was planned and a time consuming purification is unsuitable for a high throughput of mutants.
To solve this problem it was attempted to create a LOX fusion protein with a His tag, an Intein tag or a Strep tag. Both N-terminal and C-terminal taging of LOX was planned. (See Figure 14.1)

His tag:
The polyhistidine tag consists of at least six histidine residues. It was invented by Roche and its vectors are distributed by Quiagen. The purification method of choice is affinity chromatography: A column where e.g. Cu^{2+} is immobilized on the stationary phase and surrounded by water molecules. The histidines of the tag can replace the water molecules and form chelate complexes with the metal ion. Imidazole can be used to elute the protein from the column afterwards because it displaces the histidines in the chelate complexes.

Intein tag:
This method uses the inducible self-cleavage activity of protein splicing elements (inteins) to separate the target protein from the affinity tag. While the His tag is a short polyaminoacid chain, the intein tag is a protein which contains a chitin binding domain for the affinity purification of the fusion protein on a chitin resin. Mercaptoethanol can be used to elute the protein while the intein tag stays bound to the column.

Strep tag:
The strep tag is a synthetic peptide consisting of eight amino acids: WSHPQFEK. It binds with high selectivity to Strep-Tactin (an engineered streptavidin) which can be immobilized on a column. To elute the fusion protein desthiobiotin which is acting as a competitive ligand can be used.

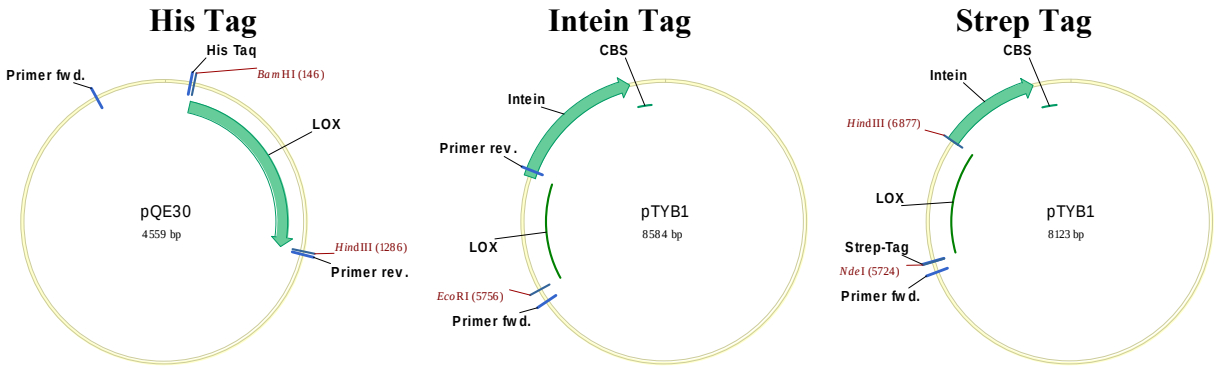

Figure 14.1 Various Tags

1.2 Materials and methods

1.2.1 Colony PCR

Colony PCR was used to screen for correct DNA vector constructs directly by PCR. Colonies were sampled with a sterile toothpick and resuspended in H_2O. To release the DNA from the cells, the solution was held at 95°C for 10 minutes. 2 µl of this solution were used as template for the colony PCR. The 20 µl PCR reaction mix consisted of 2 µl template, 5 pM of each primer, 10x Taq-buffer, 200 µM dNTPs, 1.25 mM magnesium chloride and 0.06 U Taq-polymerase (supplied by Fermentas). Starting temperature was 95°C for 2 minutes. Cycling conditions were as follows: 15 seconds at 95°C for denaturation, 35 seconds at 55°C for annealing and 30 seconds at 72°C for extension (30 circles). After letting the products elongate for 2 minutes at 72°C the PCR mix was cooled down. Then loading dye was added and gel-electrophoresis was performed on an 1% agarose-gel.

Table 4.1 Vectors and their respective primers for the colony PCR

vector	forward primer	reverse primer	vector size
pQE30 His tag	accgctgttgagatccagttcgatg	gtcattactggatctatcaacaggagtccaag	3461 bp
pTYB1 Intein tag	cgatcccgcgaaattaatacgactc	ccatctttacccatgaccttattaccaacctc	7477 bp
pTYB11 Intein tag	ggccatggtggtattcgcaataatc	gctgtaggcataggcttggttatgccggtact	7414 bp

1.2.2 PCR for tag creation

The 20 µl PCR reaction mix consisted of 1 µl 100-200 ng template, 5 pM of each primer, 10x Pfu-buffer, 200 µM dNTPs and 0.04 U Pfu-polymerase (supplied by Promega). Starting temperature was 95°C for 2 minutes. Cycling conditions were as follows: 15 seconds at 95°C for denaturation, 35 seconds at 56°C for annealing and 40 seconds at 72°C for extension (30 circles). After letting the products elongate for 5 minutes at 72°C the PCR mix was cooled down.

The PCR reactions were purified by gel-electrophoresis with a preparative 1% agarose-gel using the Macherey-Nagel NucleoSpin® Extract II Kit.

Table 1.5 Primers for tag creation

		tag/ restriction site	primer
His tag N-terminal	**forward** **reverse**	*Bam*HI: cgaggatcc	atgaataacaatgacattgaatataatgcacc agaccgcttctgcgttctg
Intein tag N-terminal	**forward** **reverse**	*Sap*I: gacgctcttcgaac *Xho*I: cgactcgag	atgaataacaatgacattgaatataatgcacc gtattcataaccgtatgggttatcg
Intein tag C-terminal	**forward** **reverse**	 *Sap*I: cgcgctcttcggca	gtataatgtgtggaattgtgagcctacc gtattcataaccgtatgggttatcg
Strep tag N-terminal	**forward** **reverse**	Strep + *Nde*I: gaccatatgtggagccacccgcag ttcgaaaaa	atgaataacaatgacattgaatataatgcacc agaccgcttctgcgttctg
Strep tag C-terminal	**forward** **reverse**	 Strep: gtattcataaccgtatgggttatcg	gtataatgtgtggaattgtgagcctacc aataaatctagacc

1.2.3 DNA concentration determination

The DNA concentrations were determined with the Beckmann photometer in UVettes at 260 nm wavelength. The absorption value was multiplied with 50 resulting in the concentration value in [ng/µl].

1.2.4 Digestion and ligation

All restriction enzymes were supplied by Fermentas. The plasmids and inserts were digested for 4 hours with their respective restriction enzymes (See below) and buffers. The restriction enzymes were inactivated at 65 °C for 10 minutes. Then the plasmids were dephosphorylated. For ligation of vector and insert 0.5 U/µl T4 ligase (supplied by Fermentas) was used.

Table 1.6 Restriction enzymes used for digestion of vectors/fragments for tag creation

	vector	restriction enzymes	
His tag N-terminal	pQE30	*Bam*HI	*Hin*dIII
Intein tag N-terminal	pTYB11	*Lgu*I	*Xho*I
Intein tag C-terminal	pTYB1	*Eco*RI	*Lgu*I
Strep tag N-terminal	pTYB1	*Nde*I	*Hin*dIII
Strep tag C-terminal	pTYB1	*Eco*RI	*Xho*I

The DNA was purified employing the Promega Wizard® *Plus* SV Minipreps DNA Purification System and transformed into electro-competent cells.

1.2.5 Competent cell preparation and transformation of *E. coli*

The protocol for electro-competent cell preparation written by Barbara Petschacher was used to create competent cells.
The following *E. coli* strains were tested for their transformation efficiency: TOP10, BL21 Gold, XL1 and JMD. TOP 10 and BL21 Gold showed the best performance and were used subsequently.
2 µl vector DNA (see 1.2.4) and 40 µl electro-competent *E. coli* cells were mixed in pre-cooled cuvettes. Then the EC2 pulse was fired and 900 µl SOC solution (preheated to 37°C) were added to the cells immediately. Next the cells regenerated for 1 hour at 37°C until finally 100 µl amounts of different cell dilutions were plated on Amp-Agar plates (preheated to 37°C, 100 µg/ml ampicillin). The agar plates were incubated over night at 37°C. Subsequently the colonies were transferred to new agar plates. The following day the cells on these plates were used to inoculate over night cultures of cuvettes filled with 10 ml LB-Amp medium (100 µg/ml ampicillin). A day later the DNA was purified employing the Promega Wizard® *Plus* SV Minipreps DNA Purification System and 5 µl DNA were digested for 1-2 hours with 10 U of each fast digest enzyme (see 1.2.4) supplied by Fermentas, the appropriate buffer and H_2O to check if the tag was successfully introduced. This was followed by gel-electrophoresis at 90 V for 45 minutes with a 1% agarose gel to check for plasmids with a tagged LOX.
For sequencing purposes the DNA was purified employing the Macherey-Nagel NucleoSpin® Extract II Kit.

1.3 Results

A fusion protein was created containing the N-terminal His tag. But the deletion of one base lead to a frame shift so no functional protein could be expressed (See Figure 1.15). An attempt to correct the frame shift using PCR was not successful.

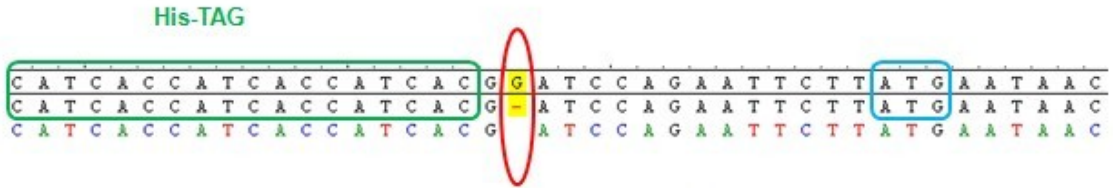

Figure 1.15 His tag with frame shift

2 Protein purification

2.1 Introduction

Because the taging attempts proved to be unsuccessful an alternative way of purifying LOX had to be found. Our first strategy consisted of ammonium sulfate precipitation and anion exchange chromatography but the results were inadequate. So an alternative purification protocol was created which involved hydrophobic interaction chromatography.

2.2 Materials and methods

Ammonium sulfate precipitation and DEAE protocol

1st step: Ammonium sulfate precipitation
Ammonium sulfate was added to reach a concentration of 1.5 M ammonium sulfate which accomplished the removal of other proteins. Then the ammonium sulfate concentration was raised to 2.5 M to precipitate the LOX. After precipitation the LOX was dissolved again in 50 mM potassium phosphate buffer pH 7.

2nd step: Ion exchange chromatography
The analytical column was mounted on a fast protein liquid chromatography (FPLC) system (ÄKTA Amersham Biosciences UPC-900 P-920).

Macro Prep DEAE anion exchange column conditions:

CV: 5 ml
flow: 0.26 ml/minute
washing buffer: 50 mM potassium phosphate buffer pH 7
elution buffer: 50 mM potassium phosphate buffer pH 7 + 1 M potassium chloride
maximum pressure: 5 bar
maximum sample load: 30 mg/ml

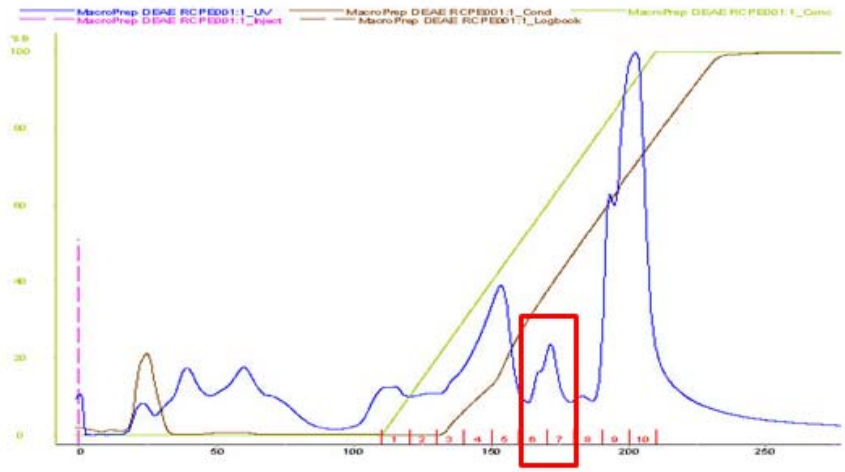

Figure 2.1 Elution profile of the Macro Prep DEAE anion exchange column

2.3 Results

Ammonium sulfate precipitation and DEAE protocol

The appropriate concentrations for the ammonium sulfate precipitation were determined (See Figure). It is apparent that the loss of LOX activity is justifiable until a 1.5 M concentration of ammonium sulfate is reached. It was also ascertained that the entire LOX activity is in the precipitate of the 2.5 M precipitation. The loss of LOX activity in the wild type under these conditions amounts to 36%.

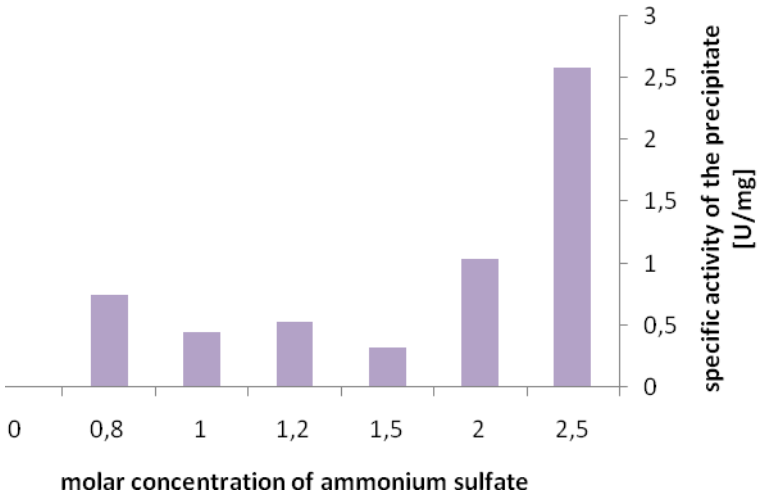

Figure 2.2 Ammonium sulfate precipitation

The ion exchange chromatography (DEAE) in the purification of the wild type enzyme yielded about 22% of the amount of protein that was measured in the crude extract and only 6.6% of the LOX activity. See the elution profile in Figure . On the SDS polyacrylamide gel it was apparent that even after such a great loss of activity the protein was still far from pure (See Figure) and so a different purification protocol had to be established.

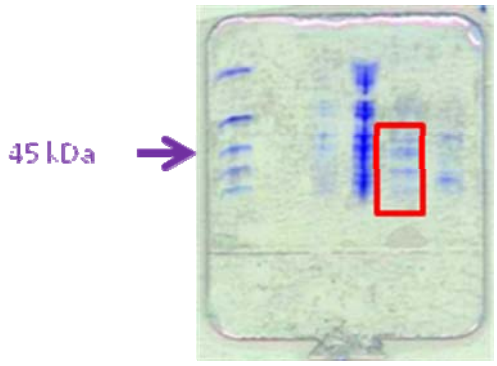

Figure 2.3 SDS polyacrylamide gel of some fractions of the DEAE chromatography

3 Attachments

3.1 DNA sequence of LOX

ATGAATAACAATGACATTGAATATAATGCACCTAGTGAAATTAAGTATATTGATG
TTGTCAATACTTACGACTTAGAAGAAGAAGCAAGTAAAGTGGTACCACATGGTG
GTTTTAACTATATTGCCGGTGCATCTGGTGATGAGTGGACTAAACGTGCTAATGA
CCGTGCTTGGAAACATAAGTTACTATACCCACGTCTAGCGCAAGATGTTGAAGCG
CCTGATACAAGTACTGAAATTTTAGGTCATAAAATTAAAGCCCCATTCATCATGG
CCCCAATTGCTGCACATGGTTTAGCTCACGCTACTAAAGAAGCTGGTACTGCACG
TGCAGTTTCAGAATTTGGTACAATCATGTCCATCTCAGCTTATTCTGGTGCAACAT
TTGAAGAAATTTCTGAAGGCTTGAATGGCGGACCTCGTTGGTTCCAAATTTACAT
GGCTAAAGATGACCAACAAAACCGTGACATCTTAGATGAAGCTAAAGGTGATGG
TGCAACTGCTATTATCCTTACAGCTGACTCAACTGTTTCTGGTAACCGTGACCGTG
ATGTGAAGAATAAATTCGTTTACCCATTTGGTATGCCAATCGTTCAACGTTACTTA
CGTGGTACAGCAGAAGGTATGTCATTAAACAATATCTACGGTGCTTCAAAACAAA
AAATCTCACCAAGAGATATTGAGGAAATTGCTGCTCATTCTGGATTACCAGTATT
TGTTAAAGGTATTCAACACCCTGAAGATGCAGATATGGCAATCAAAGCTGGTGCA
TCAGGTATCTGGGTATCTAACCACGGTGCTCGTCAACTATATGAAGCTCCAGGTT
CATTTGACACCCTTCCAGCTATTGCTGAACGTGTAAACAAACGTGTACCAATCGT
CTTTGATTCAGGTGTACGTCGTGGTGAACACGTTGCCAAAGCGCTAGCTTCAGGT
GCAGACGTTGTTGCTTTAGGTCGCCCAGTCTTATTTGGTTTAGCTTTAGGTGGCTG
GCAAGGTGCTTACTCAGTACTTGACTACTTCCAAAAAGACTTAACACGTGTAATG
CAATTAACAGGCTCTCAAAATGTGGAGGACTTGAAAGGTCTAGATTTATTCGATA
ACCCATACGGTTATGAATACTAAAAGCT

3.2 Protein sequence of LOX

Met N N N D I E Y N A P S E I K Y I D V V N T Y D L E E E A S K V V P H G G F N Y I
A G A S G D E W T K R A N D R A W K H K L L Y P R L A Q D V E A P D T S T E I L G
H K I K A P F I **Met** A P I A A H G L A H A T K E A G T A R A V S E F G T I **Met** S I S A
Y S G A T F E E I S E G L N G G P R W F Q I Y **Met** A K D D Q Q N R D I L D E A K G
D G A T A I I L T A D S T V S G N R D R D V K N K F V Y P F G **Met** P I V Q R Y L R G
T A E G **Met** S L N N I Y G A S K Q K I S P R D I E E I A A H S G L P V F V K G I Q H P
E D A D **Met** A I K A G A S G I W V S N H G A R Q L Y E A P G S F D T L P A I A E R
V N K R V P I V F D S G V R R G E H V A K A L A S G A D V V A L G R P V L F G L A
L G G W Q G A Y S V L D Y F Q K D L T R V **Met** Q L T G S Q N V E D L K G L D L F
D N P Y G Y E Y **Stop** K