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Cloning and characterization of superoxide dismutase from the ectoparasite *Sarcoptes scabiei*

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Degree project in biology, Master of science (1 year), 2008

Examensarbete i biologi 30 hp till magisterexamen, 2008

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Summary

Sarcoptic mange or scabies is a cutaneous disease of veterinary and human significance caused by the microscopic mite *Sarcoptes scabiei* and encountered all over the world. The fertilized female excavates a tunnel in the epidermis of the host where she lays her eggs, and as these hatch, the larvae leave the tunnel to complete their development on the skin surface.

Resistance among *S. scabiei* to various drugs has been reported. To understand this development, the defense mechanisms used by the parasite to metabolize foreign substances needs to be better understood. Since *S. scabiei* cannot be cultivated in vitro, recombinant proteins are used as tools to increase the knowledge about the parasite and how it interacts with its surroundings. This project reports the construction of an expression vector with the *S. scabiei* superoxide dismutase cDNA (*sssod*) inserted. This gene codes for an enzyme that catalyses the dismutation of superoxide free radicals. It is an important defense system in all cells exposed to oxygen. The *sssod* was cloned into the pET-14b expression vector and the final construct was designated pPU118. The vector has a sequence coding for a hexahistidine tag fused downstream of the open reading frame of *sssod*. This expression vector can be used in further studies to evaluate the enzyme activity of *S. scabiei* SOD protein (ssSOD) and to increase the knowledge about this parasite.

In this study, I also report the expression and purification of a recombinant ssSOD from an earlier described expression vector, pPU110. This expression vector carries the open reading frame for *sssod* fused to a hexahistidine tag sequence downstream and a maltose binding protein (MBP) coding sequence upstream. Different types of affinity and ion exchange purification of ssSOD were used, where affinity purification using maltose binding protein (MBP) gave the best purification results. The enzyme activity was also evaluated, under the condition for the assay, one unit of ssSOD was defined as the amount that gives 50 % inhibition of nitroblue tetrazolium (NBT). One unit of enzyme activity was estimated to correspond to 0.61 $\mu\text{g}/\mu\text{l}$ ssSOD.

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1 Introduction

1.1 *Sarcoptes scabiei*

The parasitic mite *Sarcoptes scabiei* is the causative agent of a skin disease referred to as sarcoptic mange or scabies. This disease affects both animals and humans with an estimated prevalence of 300 million infected individuals worldwide (Orion *et al.* 2004). The infection results from the burrowing of the mite in the epidermis of the host, which is done both mechanically and through secretion of cytolytic compounds. The mite and the secreted compound induce an immunopathological response in the host (Pettersson *et al.* 2005, Molin & Mattsson 2007).

The parasite is a small arachnid mite that can be up to 0.4 mm in diameter. It is round with very short legs, but there are some important characters that distinguish it from other mange mite, e.g. numerous transverse ridges and triangular scales on the dorsum (Ljunggren *et al.* 2006) (figure 1a).

The female mates only once in her life time and when the fertilization is completed she excavates a one centimeter long tunnel in the epidermis of the host where she lays 10-25 eggs. Approximately 10 % of the eggs hatch within 3-4 days and the larvae leave the tunnel to mature on the skin surface of the host for additional an 14-17 days (Orion *et al.* 2002) (Figure 1b).

An early sign of infection is intense itching, and later on consistent skin eruptions and lesions can form (Ljunggren *et al.* 2002). There can also be secondary infections resulting from bacteria that colonize the tunnel that is burrowed by the mite (Pasay *et al.* 2006). Examples of secondary infections are septicemia (presence of bacteria in the blood), renal damage and rheumatic fever, which can further lead to rheumatic heart disease (Ljunggren *et al.* 2002). Another more severe form of *S. scabiei* infections is crusted scabies, a condition that is more frequently found in individuals with immune-system problems, including AIDS, diabetes, and lupus (Ljunggren *et al.* 2006). *S. scabiei* infection is a common health problem around the world since it affects people of all social, economic and ethnic categories, but it is a particular problem for individuals in developing countries because of overcrowding and delayed treatments (Orion *et al.* 2002, Dragos *et al.* 2004)

The infection occurs in a wide range of domestic and wild animals where some are more susceptible than others (Pettersson *et al.* 2004). This causes a particular problem with both respect to animal welfare and economic aspects. Between 50 % and 95 % of the herds in the international pig industry are estimated to be infected (Molin & Mattsson 2007) and the cost for this problem in the US alone is estimated to hundreds of millions of dollars (Ljunggren *et al.* 2002). As a consequence, the pig industry has taken preventive measures to eliminate this problem (Ljunggren *et al.* 2006). Domestic and wild dogs are among the animals that also often get infested (Arlian *et al.* 1996). During the 1980's, the red fox population in Sweden was almost extinguished due to mange (Pettersson 2002).

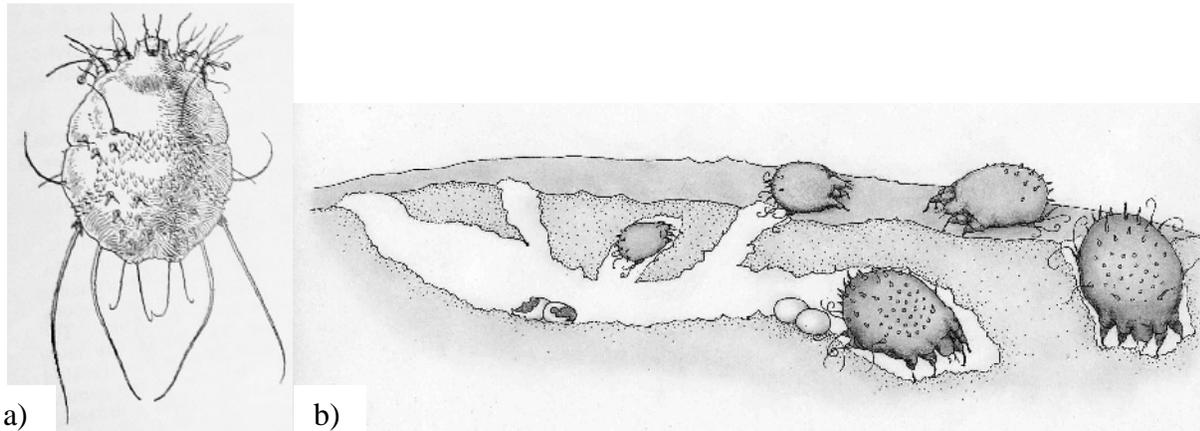


Figure 1: The mite *Sarcoptes scabiei* that causes sarcoptic mange. a) The body is round and can be up to 0.4 mm in length. The legs are short (Image: Kendall, D. 2007). **b)** Life cycle. The mite's lifecycle lasts about two weeks; it begins by excavating a tunnel in the skin where the mite lays the eggs. The larvae leave the tunnel to mature in the skin surface (Image: Näslund, K., National Veterinary Institute (SVA)).

1.2 Background to project

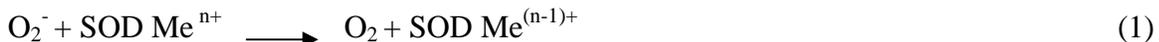
Infestations of the parasitic mite *S. scabiei* are a common health problem around the world and are almost an everyday diagnosis in the dermatologist's office. The parasite is microscopic and as it digs its way through the skin it triggers the immune response of the host (Arlian *et al.* 1995). Despite the immune response *S. scabiei* manages to maintain itself successfully in the skin of some hosts, but how it does that is still unknown (Mattsson *et al.* 2001). Increased resistance has been observed against some of the drugs (acaricides) used to treat *S. scabiei* infections (Arlian *et al.* 1996, Elder *et al.* 2006). Also, it takes a longer time for the parasite to be eliminated and a longer time for the host to recover after infections (Ljunggren *et al.* 2005). Among many things one needs to know more about the defense mechanisms against foreign substances in order to obtain a better understanding of the parasite's success to cope with various drugs. Superoxide dismutase (SOD) is an enzyme acting in the defense against toxic free radicals in many types of cells (Zemlyak *et al.* 2006) and is the enzyme investigated in this study. A strain of the malaria mosquito, *Anopheles gambiae*, resistant to some of the insecticides called pyrethroids has shown high over-expression of SOD (Muller *et al.* 2007). Pyrethroids affect the nervous system in arthropods; this occurs at the sodium ion channels in the nerve cell membrane. These channels are essential for normal transmission of nerve impulses which can be disrupted by pyrethroids. Pyrethroids are also frequently used to treat scabies infections. Thus the reason for investigating SOD.

Because *S. scabiei* is impossible to grow in the laboratory, a recombinant SOD enzyme (ssSOD) needs to be used for the characterization of the enzyme. The pET-system is a powerful approach for the production of recombinant proteins, originally developed by Studier and colleagues (Moffatt & Studier 1986, Rosenberg *et al.* 1987, Studier *et al.* 1990). The system uses the bacteriophage T7 promoter to direct the expression of the protein. The cloned gene therefore is not expressed unless T7 RNA polymerase is added because the *E. coli* RNA polymerase does not recognize the T7 promoter. The DE3-prophage used, encodes the T7 RNA polymerase under *lac* repressor control, the expression is induced by the addition of isopropylthiogalactoside (IPTG).

The pET expression vector can be designed to give a histidine-tagged protein, which means that the protein has six extra amino acids of histidine (His₆-tag) in one end. The His₆-tag is used for the detection and purification of the protein (Hainfeld *et al.* 1999).

1.3 The defense mechanism-superoxide dismutase

All aerobic organisms use oxygen to produce energy. A certain amount of the oxygen that is taken in by the body is always converted to reactive oxygen species (ROS) that are highly reactive and toxic to the cells; this has been shown for instance in earlier studies with SOD null mutants (Touati 1991, Touati 2002). ROS is formed by the addition of electrons one at the time to molecular oxygen, which thereby obtains a negative charge. Superoxide anions (O₂⁻) are one of the most toxic ROS formed. It is very unstable and will attack molecules in the cells. In order to protect the body against these toxic free radicals, organisms have acquired an enzyme that provides the first line of defense in the form of superoxide dismutase (SOD) (Zemlyak *et al.* 2006). SOD was first discovered by Dr. McCord and Dr. Fridovich in 1969 and since then, interest has arisen to further study this enzyme (McCord & Fridovich 1988, Bannister & Bannister 1988). There are three different classes of SOD that are characterized by their different metal content. The first enzyme isolated was a Cu, Zn SOD and after that a Mn SOD and finally a Fe SOD were discovered (Zelko *et al.* 2002, Josephy & Mannervik 2006). SODs are antioxidant metalloenzymes, where the metal ion in the active site first is reduced by superoxide (O₂⁻), thereby releasing oxygen (Reaction 1). The metal ion is then oxidized by a second superoxide releasing hydrogen peroxide (Reaction 2) (Choudhury *et al.* 1999, Hart *et al.* 1999).



1.4 Chromatography methods

1.4.1 Affinity chromatography using a hexahistidine tag

The HiTrap HP column is a fast and economical tool to purify recombinant proteins fused to a hexahistidine tag (His₆-tag) (Hainfeld *et al.* 1999, GE Healthcare 2008). This column can be loaded with different metal ions, where Ni²⁺ is preferred because it has six coordination sites for bonding (Hainfeld *et al.* 1999, Polosukhina *et al.* 2006). The column is equilibrated with starting buffer, the protein mixture is loaded on the column and the target protein will bind to Ni²⁺ by its His₆-tag. The column is washed to remove all unwanted protein that did not bind. The elution step can be performed in different ways but in this study, imidazole was used. Imidazole has a ring structure similar to histidine and will therefore compete with the His₆-tag for Ni²⁺ binding. (Polosukhina *et al.* 2006).

1.4.2 Ion exchange chromatography

Ion exchange chromatography separates proteins based on charge differences. Proteins with a negative charge will bind to positively charged columns and vice versa. First an appropriate buffer with the appropriate pH is chosen based on the isoelectric point (pI, the pH value at which a protein has no net charge) of the desired protein (Figure 2). At pH above its pI the protein will carry a negative net charge and will bind to a positively charged column and vice versa. Proteins can be eluted by adding sodium chloride (NaCl). The salt will compete for binding to the ions of the column, thereby releasing the desired proteins from its bound state (Millard 2006)

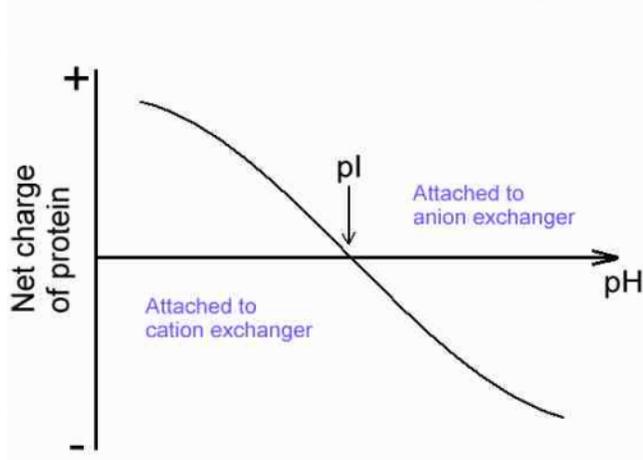


Figure 2: Ion exchange chromatography. At the isoelectric point (pI) the protein has no net electric charge. At pH above pI the protein will carry a negative charge and can be further separated by attachment to the anion exchanger. At pH lower than pI, the protein will carry a positive charge and can be further separated by attachment to the cation exchanger.

1.4.3 Affinity chromatography using the maltose binding protein

This method is an affinity purification method for recombinant proteins fused to a maltose binding protein (MBP). It contains a cross linked affinity matrix of the polysaccharide amylose. The MBP binds to the amylose matrix while other proteins flow through. The protein of interest is eluted by adding maltose, the maltose will bind to the amylose column and thereby releasing the desired protein (Sabrock & Russell 2001).

1.5 Enzyme activity assay

There are more than 20 methods to determine the activity of SOD. One of these assays involves a xanthine-xanthine oxidase reaction, a reaction that involves the xanthine oxidase (XOD) conversion of xanthine to uric acid and hydrogen peroxide (Figure 3). This reaction produces O_2^- which is a substrate for SOD (Kirby & Fridovich 1982) (Reactions 1 and 2). There are both direct and indirect methods to determine the SOD activity. When using the direct method, the O_2^- concentration is monitored directly by its absorbance in the UV region. In one indirect method developed by Bauchamp and Fridovich (Goldstein et al. 1988) O_2^- is allowed to react with nitroblue tetrazolium (NBT). NBT is yellow but when reduced by O_2^- , it yields a purple/blue pigment called formazan (Picker & Fridovich 1984) that can be detected spectrophotometrically at 560 nm. A decrease in absorption is measured when SOD is added since less O_2^- is available in

the sample and hence less NBT is reduced. In other words, the activity of SOD is measured by the reduction of NBT.

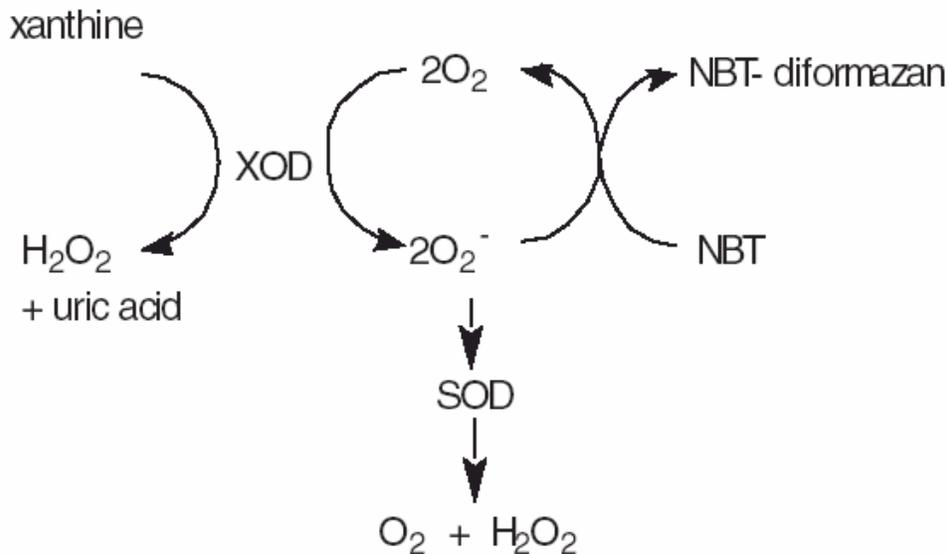


Figure 3: The SOD activity assay reaction. Superoxide anions are generated by the xanthine oxidase (OXD) conversion of xanthine to uric acid and hydrogen peroxide. The O_2^- is a substrate for the superoxide dismutase. Nitroblue tetrazolium (NBT) is reduced to formazan, which can be detected spectrophotometrically at 560 nm. (Image: Kamiya Biochemical Company 2008).

1.6 Aim

The aim of this study was to clone one Cu, Zn superoxide dismutase DNA previously identified in an EST-project (Ljunggren *et al.* 2003) from *S. scabiei* (*sssod*) into the expression vector pET-14b, express the recombinant *S. scabiei* SOD protein (ssSOD) from both this construct and from another expression plasmid called pPU110. Additionally, the recombinant proteins should be purified by chromatography and enzyme activity evaluated.

2 Results

2.1 Construction of an expression plasmid

In order to clone the superoxide dismutase gene from *Sarcoptes scabiei* (*sssod*), it was first amplified using an earlier constructed expression vector called pPU110 as template. The construct contains the open reading frame (ORF) for *sssod* with maltose binding protein (MBP) sequence upstream and a hexahistidine (His₆)-tag sequence downstream (Lecaros 2006). The gene was amplified in duplicates through PCR with primers corresponding to the 5'-end and 3'-end of the ORF. The amplified products were analyzed on 1 % agarose gel (Figure 4). The results confirmed an amplicon of ~ 700 nucleotides, which corresponds well with the ORF of 696 nucleotides.

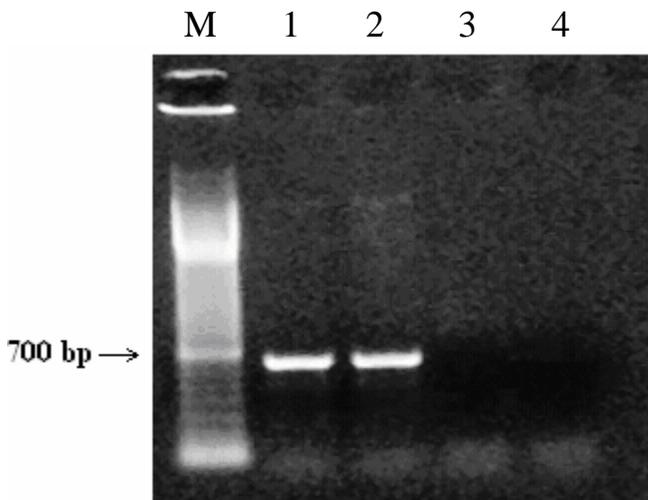


Figure 4: PCR amplification of the superoxide dismutase gene from *Sarcoptes scabiei* (*sssod*). M: DNA size marker (100bp, GE Healthcare). Lane 1 and 2 shows the *sssod* gene amplified with the primer pairs OP553 and OP554. Lane 3 and 4 is negative controls.

The *sssod* DNA identity was confirmed by sequencing. The DNA sequence showed 100 % homology with *sssod* when aligning it against the correct sequence using The Basic Local Alignment Tool (BLAST, www.ncbi.nlm.nih.gov/blast/Blast.cgi).

The amplified *sssod* DNA was subcloned into a vector permitting blue-white screening. Both white (positive clones of cells carrying the plasmid with the insert cloned into it) and blue colonies (negatives) could be spotted on the plates. A total of 18 clones (white) were analyzed using colony PCR with primers complementary to the vector. The PCR products were run on a 1 % agarose gel to verify the result (Figure 5). Here the expected amplicon size was 866 nucleotides. One negative colony (blue) was also analyzed to compare the difference. The plasmid was also control sequenced and the result verified the correct *sssod* insert

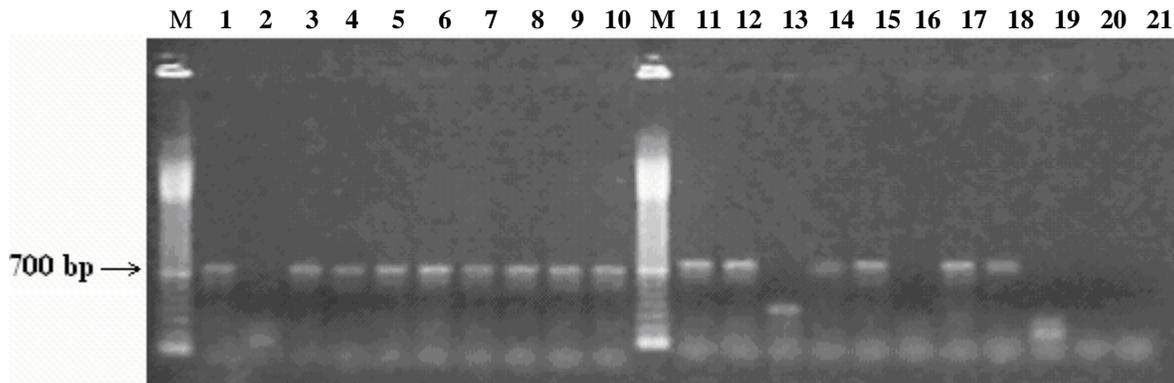


Figure 5: Colony PCR results from the pCR-Script Amp Sk (+) blue-white cloning of *sssod* analyzed on 1 % agarose gel. Lane M shows the DNA size marker (100 bp ladder, GE Healthcare). The DNA was amplified with the primer pair T7 and T3 yielding a 866 bp amplicon if the plasmid contained the correct insert. Lane 19 was from a blue colony. Lanes 20 and 21 shows negative controls.

One colony of the transformed *E. coli* cells corresponding to lane 11 (Figure 5) was inoculated in 100 ml LBamp and grown overnight. Thereafter the plasmid DNA was purified, and its concentration was determined spectrophotometrically to 215.2 ng/μl.

This DNA and the DNA from pET-14b were then digested by *Bam*HI and *Nde*I, gel purified on 0.8 % agarose gel. Undigested and partially digested plasmids were used as controls. Both digested products gave sufficient amounts for the ligation.

The ligation mix (pET-14b plasmid with the *sssod* insert) was transformed into electro competent *E. coli* cells (XL1-blue-MRF[']), spread on LBamp plates and incubated overnight at 37 °C. Colony PCR was performed on 24 colonies and analyzed on a 1 % agarose gel (Figure 6). A total 18 out of the 24 analyzed clones carried an insert of the expected size (896 nucleotides).

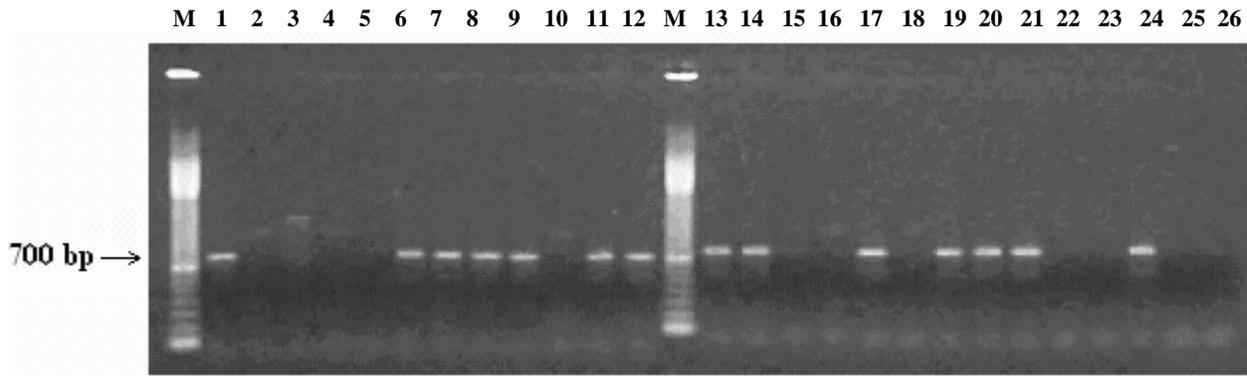


Figure 6: Colony PCR results from the pET-14b cloning analyzed on 1 % agarose gel. Lane M shows the DNA size marker (100 bp ladder, GE Healthcare). The DNA was amplified with the primer pair T7pro and T7ter yielding an 896 bp amplicon if the plasmid contained the correct insert. Lanes 25 and 26 are negative controls.

A single colony of transformed *E. coli* cells amplified in lane 7 in figure 6 was chosen for the purification of pET-14b-*sssod* plasmid. The plasmid was purified and sequenced to check if the *sssod* was inserted into pET-14b in the correct reading frame for correct protein expression of *Sarcoptes scabiei* SOD protein (ssSOD). The DNA sequence showed 100 % homology with *sssod* when aligning it to the correct sequence using BLAST, and was also in the correct reading frame for further expression of ssSOD. The new construct (pET-14b-*sssod*) was designated pPU118 (Figure 7). Because of persistent contamination problems during the construction steps, there was not enough time to carry out the expression studies with this construct.

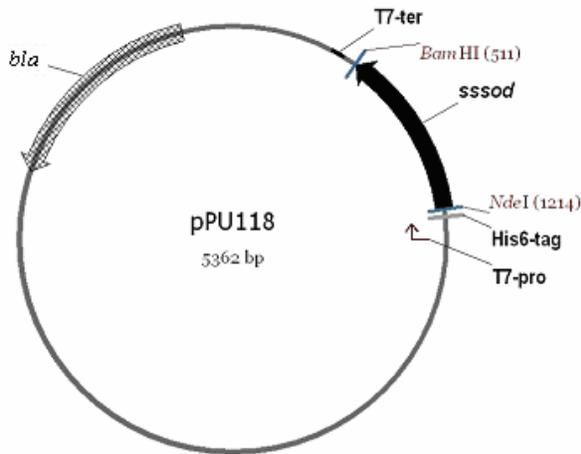


Figure 7: The expression vector pPU118: pPU118 carries the *sssod* open reading frame sequence with a hexahistidine tag downstream. The expression vector is T7 promoter driven and includes the ampicillin resistant (*bla*) gene for screening.

2.2 Expression of ssSOD from pPU110

The recombinant ssSOD (MBP-SOD-His₆-tag) from pPU110 expressed in *E. coli* BL21 (DE3) cells upon induction with isopropylthiogalactoside (IPTG). Proteins from uninduced (without IPTG) and induced cultures (with IPTG) were analyzed on 12 % SDS-PAGE (Figure 8). The result showed a clear induction of expression and a high yield of ssSOD with a molecular weight of ~70 kDa from induced cells.

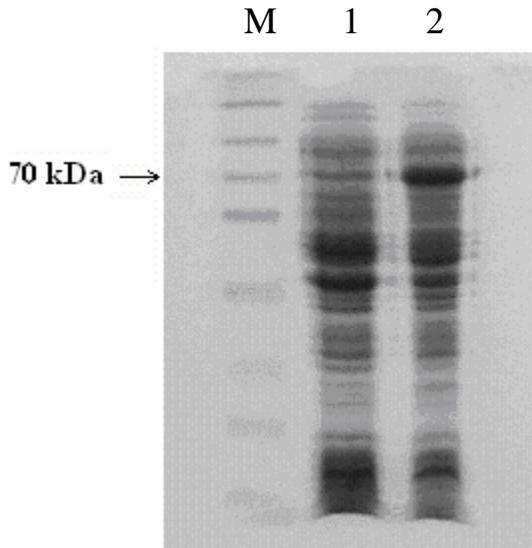


Figure 8: The expression of ssSOD from pPU110 in *E. coli* (BL21 (DE3)) on a 12 % SDS-PAGE: Lane M shows the molecular weight marker (1 kb ladder, Amersham Biosciences). Lane 1 shows the protein expression without IPTG, no expression of the ssSOD from pPU110. Lane 2 shows the protein expression with IPTG. ssSOD has a molecular weight of ~70 kDa.

2.3 Protein purification

2.3.1 ssSOD purification using His₆-tag and ion exchange chromatography

The first attempt to purify ssSOD was by using a HiTrap chelating high performance (HP) column charged with Ni²⁺. The resulting SDS-PAGE (Figure 9) shows a relatively poor purification of ssSOD. Although ssSOD could be observed in the second elution fraction, many unwanted proteins from *E. coli* also were eluted with the desired protein. Also the flow through fraction showed no difference from the total cell lysate, which suggested that the desired protein might not bind properly. An additional step was tested in order to improve the purification of the ssSOD from elution fraction number two (Figure 9; lane 6). The fraction was further purified using ion exchange chromatography by a HiTrap Q HP column (anion exchanger). A negative charge of the ssSOD was obtained by a buffer exchange prior to the separation on the anion exchanger. However, analysis of this purification on SDS-PAGE showed no bands at all. Only the molecular weight marker could be visualized (no data shown).

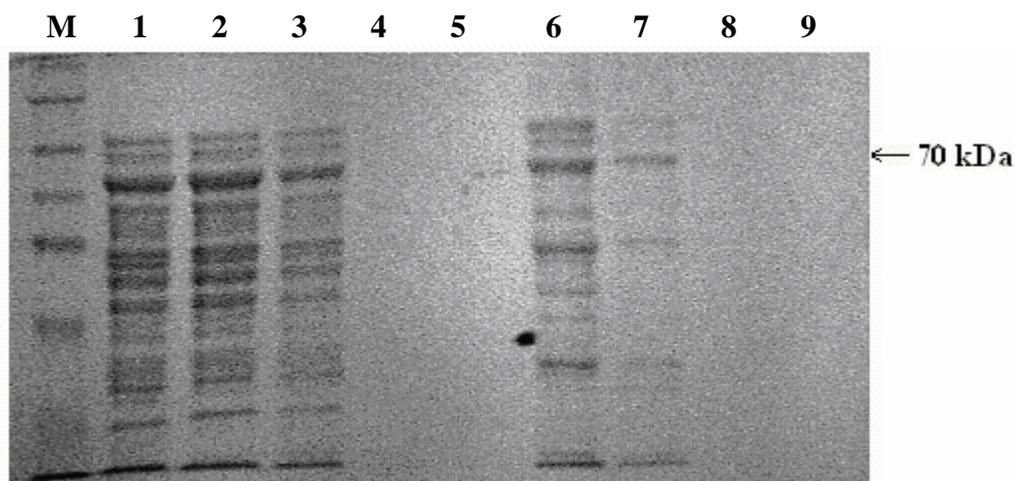


Figure 9: ssSOD purification using the His₆-tag and a HiTrap HP column. The column was first charged with Ni²⁺. Then the protein mixture was loaded and eluted with 0.05 M imidazole, collected in fraction and followed by 12 % SDS-PAGE analysis. Lane M shows the protein size marker (NEB; New England Biolabs). Lane 1 shows total cell lysate fraction, lane 2 shows flow through fraction, Lanes 3 and 4 shows wash fractions, Lanes 5-9 shows final eluted protein fractions.

2.3.2 ssSOD purification using maltose binding protein

The ssSOD then was affinity-purified using an amylose resin column that specifically binds proteins containing maltose binding protein (MBP). The purification was carried out by the binding of MBP, which is fused to ssSOD, to the amylose in the column. I successfully purified ssSOD (Figure 10). The ssSOD protein was observed as a band around 70 kDa in the first three eluted fractions (Figure 10, lane 6, 7 and 8). The first elution fraction (lane 6) contained the least amount of other *E. coli* proteins. This fraction was used in the ssSOD activity assay. The protein concentration of the sample was estimated to 0.34 µg/µl with the Bradford protein assay.

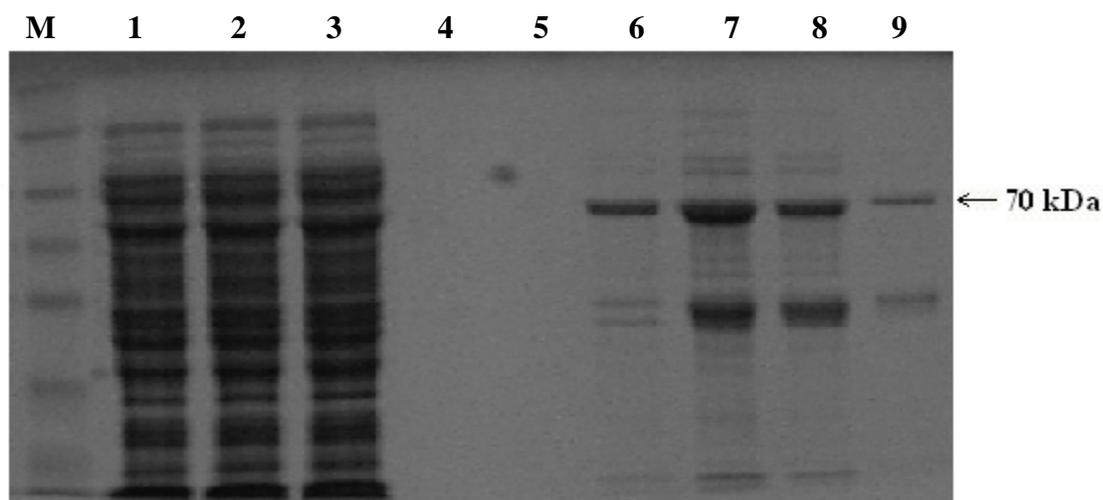


Figure 10: Purification of ssSOD using maltose binding protein (MBP) and an amylose resin column. The protein mixture was loaded, and subsequently eluted with 10 mM maltose, collected in fraction and followed by 12 % SDS-PAGE analysis. Lane M shows the DNA size marker (NEB; New England Biolabs). Lanes 1-3 show flow through fractions. Lanes 4 and 5 shows wash fractions. Lanes 6-9 show final eluted protein fractions.

2.4 SOD activity assay

The activity of ssSOD was determined by its inhibition of nitroblue tetrazolium (NBT) reduction. The ssSOD (Figure 10, lane 6) was diluted up to 20-fold in 50 mM phosphate buffer. The percent inhibition for each dilution per minute was calculated using equations 1 and 2. Thereafter a standard curve with the percentage of inhibition versus the ssSOD concentration was calculated (Figure 11). The protein concentration corresponding to 50 % inhibition was outside the measured range and had to be extrapolated. This estimation gave that one unit of ssSOD activity corresponds to 0.61 $\mu\text{g}/\mu\text{l}$ of ssSOD, where one unit of ssSOD was defined as the amount that gives 50 % inhibition of nitroblue tetrazolium (NBT).

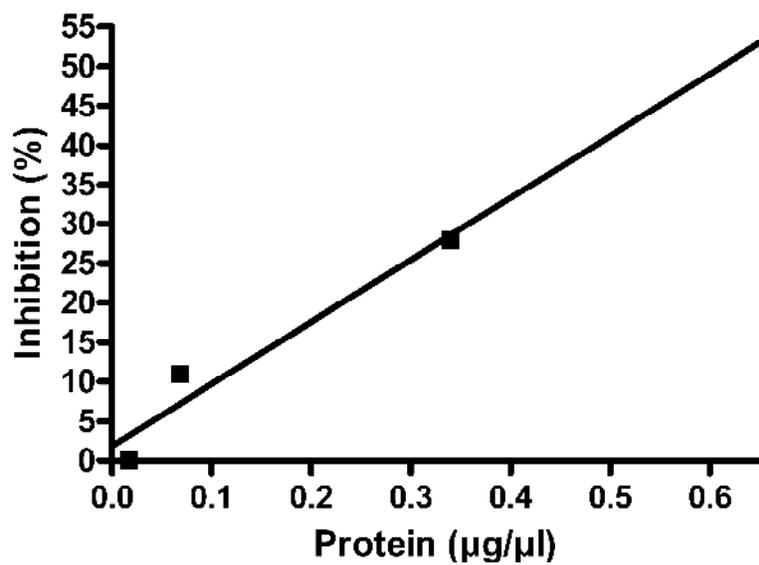


Figure 11: The percentage of inhibition versus ssSOD concentration ($R^2 = 0.94$). The percent inhibition was monitored spectrophotometrically at 560 nm due to the reduction of NBT to formazan by the superoxide radical (O_2^-). The ssSOD concentration corresponding to 50 % inhibition of NBT reduction was estimated to 0.61µg/µl.

3 Discussion

My study was mainly aimed to investigate the superoxide dismutase enzyme from *Sarcoptes scabiei*. To do so, I constructed an expression plasmid for this enzyme, designated pPU118.

3.1 The expression vector pPU118

Although DNA cloning is a straightforward procedure, it can be difficult in some cases. The new recombinant plasmid can be instable and thus difficult to work with. In some cases the plasmid can inhibit the growth of the host bacteria or even kill it. There have also been observed some structural rearrangements of the nascent plasmid in the host cell (Al-Allaf *et al.* 2005). In this case, a subsequent cloning case can be used. In this study, a successful cloning of the *ssod* was accomplished by a subsequent cloning step using the pCR-script Amp SK (+) cloning vector. The pCR-script Amp SK (+) cloning vector is derived from the pBluescript II SK (+) phagemid. The *Srf* I enzyme included in the kit recognizes the rare sequence 5'-GCC CGG GC-3', by using this enzyme one can maintain a high concentration of the digested vector DNA (Lu *et al.* 2008).

The construction of the expression vector pPU118 was a challenging procedure, due to contamination in the laboratory of another expression plasmid with a cloned glutathione transferase gene from *S. scabiei* (*ssgst*). The contamination was verified by sequencing several times. Finally the problem was solved but only at the end of this project and because of the time limit no further work was performed using pPU118. In the mean time the project continued, but with a previously constructed expression vector for *ssod*, designated pPU110.

3.2 Protein purification and enzyme activity assay

The results for ssSOD purification provided evidence that a protein separation of sufficient quality could be achieved by one of the two methods tested in this study. With an affinity purification based on the MBP, I obtained enough purified ssSOD for enzyme measurements. In contrast, purification using the His₆-tag gave a result with little or no differences from the total cell lysate fraction (total cell lysate) of the protein. This suggested that there were other *E. coli* proteins that bound to the Ni²⁺ embedded in the column. Usually in this case a subsequent purification step using ion exchange chromatography works (Pettersson *et al.* 2004), however this step was not successful in this study.

The enzyme activity of SOD was monitored by measuring the percent inhibition of nitroblue tetrazolium (NBT) reduction. The reason for using this indirect method for monitoring the activity instead of using the direct method by monitoring the superoxide or hydrogenperoxid concentration was because of their instability. Although this method is very sensitive, it should be mentioned that most of the reagents used was sensitive to light, especially catalase, which has been reported to sometimes interfere with the results (Chen & Pan 1996).

The results for the enzyme activity was extrapolated to correspond to 0.61 µg/µl of *S. scabiei* Cu, Zn SOD. However, the assumption that there were a linear relationship between protein content and percent inhibition is likely to be false (Mockett *et al.* 2002).

Even though the ssSOD gave a significant inhibition of the reduction of NBT by O_2^- , further work is needed and would be focused on evaluating more samples in a wider concentration range. It would also be good to optimize the purification steps further since there are still contaminating proteins present after the MBP affinity step.

Further work to express the recombinant protein from pPU118 needs to be done in order to compare it with the expression from pPU110. The expression vector pPU118 for *sssod* could provide a molecular tool to increase the knowledge about *S. scabiei* and its defense mechanisms against acaricides and other toxic substances.

4 Material and methods

4.1 Strains and plasmids

4.1.1 *Escherichia coli*

Table 1 summarizes all the strains and genotype of the bacteria used in this study.

Table 1. Strains of bacteria

Strains	Genotype	Source and reference
XL1-Blue MRF ⁺ ¹	<i>(mcrA) 183 (mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F' proAB lacIZ M15 Tn10 (Tet^R)]</i>	Stratagene Donahue & Bebee 1999
BL21 (DE3)	<i>F dcm ompT hsdS(r⁻ m⁻) gal (DE3)</i>	Stratagene Shibata <i>et al.</i> 1999, Donahue & Bebee 1999

¹ All cloning steps were performed with the electro competent *E. coli* strain XL1-Blue MRF⁺, these cells lack a copy for the T7 polymerase, which makes it possible to perform all the cloning steps without inducing any expression.

² Derived from *E. coli* B. The DE3 prophage encodes T7 RNA polymerase under *lac* repressor control. These cells express recombinant proteins encoded in plasmids with the T7 polymerase promoter, which can be induced by isopropylthiogalactoside (IPTG). T7 RNA polymerase is specific, and induce only genes cloned downstream of a T7 promoter.

4.1.2 Plasmid vectors

Table 2 summarizes all the plasmid used in this study.

Table 2. Plasmids

Plasmid	Description	Source or reference
pPU16	Modified pMAL-c2 vector, tac promoter driven and includes the ampicillin resistance for screening.	New England Biolabs, Mattsson <i>et al.</i> 2001
pPU110	The DNA template used for <i>Sarcoptes scabiei</i> superoxide dismutase gene (<i>sssod</i>).	Lecaros 2006
pET-14b	For pET-based cloning. Includes His ₆ -tag, T7 promoter driven and has a multicloning site containing a <i>Bam</i> HI and <i>Nde</i> I.	Novagen, Mattsson 2006
pPCR-Script Amp SK (+)	For subcloning. Derived from the pBluescript II SK(+) phagemid with a multicloning site containing that includes the Srf I restriction endonuclease.	Stratagene

pPU110 (Lecaros 2006) (Figure 12) has the open reading frame (ORF) for a *Sarcoptes scabiei* superoxide dismutase gene (*sssod*), fused to a hexahistidine (His₆)-tag sequence downstream and a maltose binding protein (MBP) sequence upstream in a pPU16 vector. The pPU110 were also used for the expression of *S. scabiei* superoxide dismutase protein (ssSOD).

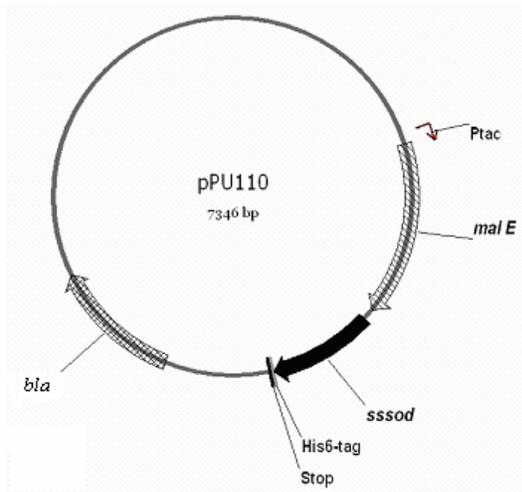


Figure 12: The expression vector pPU110: pPU110 carries the *sssod* open reading frame sequence with a hexahistidine tag sequence downstream and a maltose binding protein coding sequence upstream. The expression vector is tac promoter driven and includes the ampicillin resistant (*bla*) gene for screening.

4.2 polymerase chain reaction - amplification

Two negative controls are prepared for every polymerase chain reaction (PCR) reaction with dH₂O instead of DNA template.

4.2.1 Amplification of *sssod* cDNA

A master mix was prepared using the primer pair OP553 and OP554 (Table 3) with restriction sites for *Nde*I and *Bam*HI (Roche Diagnostics) respectively, for insertion of the amplicon into pET-14b vector (Novagen). The master mix contained: ~ 200 µg *S. scabiei* DNA template (pPU110), 5 µl of 10x cloned *Pfu* buffer (Stratagene), 0.2 mM of each dNTP (GE Healthcare), 20 pmole of each primer, 2.5 units of *Pfu* turbo DNA polymerase (Stratagene) and dH₂O. The following PCR program was used for amplification: 95 °C for 2 minutes followed by 30 cycles of 95 °C for 30 seconds, 57 °C for 30 seconds, 72 °C for 1 minute and a final extension of 72 °C for 10 minutes.

Table 3. Primers used in this study

Primer name	Sequence	Sequence complementary
OP553 ¹	5'- GGA ATT <u>CCA TAT GAT</u> GTT CCA TCA AAG GTT T-3'	sssod
OP554 ¹	5'- CGG GAT <u>CCC</u> TAT CCA ATG CTG ACA AA-3'	sssod
T3	5'-ATT TAA CCC TCA CTA AAG GG-3'	pCR-script- <i>sssod</i>
T7	5'-GTA ATA CGA CTC ACT ATA GGG C-3'	pCR-script- <i>sssod</i>
T7 pro	5'-TAA TAC GAC TCA CTA TAG G-3'	pET-14b- <i>sssod</i>
T7 ter	5'-GCT AGT TAT TGC TCA GCG G-3'	pET-14b- <i>sssod</i>

¹The underlined nucleotides correspond to the restriction enzyme recognition sites.

4.2.2 Colony PCR

Single white (positive clones) and one single blue (negative clone) colonies were resuspended in 50 µl dH₂O and spread on LBamp plates (LB agar (Lennox L Agar) with 50µg/ml ampicillin) and incubated overnight at 37 °C. The 50 µl colony resuspension was boiled for five minutes. Master mix was prepared containing: 2.5 µl 10x PCR buffer (Applied Biosystems), 0.2 mM dNTP (GE Healthcare), 20 pmole/µl of each primer (Table 3), 0.1 µl AmpliTaq DNA polymerase (Applied Biosystem) and dH₂O. The following PCR program was used: 30 cycles of 96 °C for 15 seconds, 40 °C for 20 seconds and 72 °C for 2 minutes.

4.2.3 Purification of PCR products

The PCR products were purified with JetQuick PCR purification kit (Qiagen). This purification method uses micro spin cups to get rid of the primers, nucleotides, polymerases and salts from the PCR product required. The principle of this procedure is that the DNA fragment will selectively bind to a specified silica membrane during centrifugation, the purified PCR fragments is eluted with TE-buffer pH 8.0 (10mM Tris-HCl and 1 mM EDTA).

4.3 Construction of expression plasmid

4.3.1 Ligation of *sssod* into pCR-script Amp sk (+) cloning vector

A ligation mix was prepared as follows: 1 µl pCR-script Amp sk(+) cloning vector (10 ng/µl), 1 µl pCR-script 10x reaction buffer, 0.5 µl rATP (10mM), 4 µl *sssod* DNA, 5 units *srf*I restriction enzyme, 4 units T4 DNA ligase and dH₂O. The mixture was incubated at room temperature for one hour and then heated for 10 minutes at 65°C water bath. The 10 µl ligation mix was added to 40 µl electro competent *E.coli* cells (XL1-Blue-MRF') in an electroporation cuvette (2 mm, EquiBio) and electroporated (BTX Electro Cell Manipulator: 2.45 kV, resistance

R5). Directly after the pulse, 960 µl of LB medium (LB-broth (Miller) (Merck)) was added. The mixture was incubated on a slowly shaking platform for one hour at 37°C. Cells were spread on LBamp plates (LB agar (Lennox L Agar) with 50 µg/ml ampicillin) containing 7.1 mg/ml 5'-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Promega) and 70 mM isopropylthiogalactoside (IPTG) for blue/white selection of the positive clones. Many vectors carry the first 146 amino acids of β-galactosidase gene, this gene are integrated in the multicloning site were the insert cDNA is inserted. If the inserted DNA is cloned into that region, cells turn white when plated on plates containing X-gal. If the β-galactosidase gene is expressed, then blue colonies are formed (Ullmann *et al.* 1967). The plates were incubated at 37 °C overnight, before colony PCR.

4.3.2 Endonuclease digestion

pCR-Script-*sssod* and pET-14b genomic DNA was digested with the restriction enzymes *Bam*HI and *Nde*I (Roche Diagnostics). For each digestion, the following ingredients were added: 2x One Phor All buffer (Amersham Biosciences), 1.94 µg pCR-Script-*sssod* or 0.99 µg pET-14b, 1 µl of each restriction enzyme and dH₂O. Both digestion tubes were incubated at 37°C for 2 hours. Partially cleaved plasmid were used as controls, here the plasmids were only cleaved by one of the restriction enzyme respectively and incubated for 30 minutes at 37 °C. Uncleaved plasmids and a molecular weight marker were also used as controls. The DNA fragments were separated on 0.8 % agarose gel stained with ethidium bromide (~8 µg/ml) and run in 1x TAE-buffer (40 mM Tris-acetate and 1mM EDTA) at 95 V for 45 minutes. The cleaved products were excised from the gel with a scalpel and purified with GEL-M Gel Extraction system (Viogene). GEX buffer is added to the gel fragment and melted at 60°C. The mixture is loaded on a Gel-MTM column with silica-based membranes were the DNA will bind. By decreasing the ionic strength with TE-buffer pH 8.0 (10 mM Tris-HCl and 1 mM EDTA), bound DNA can be eluted.

4.3.3 Ligation of *sssod* into pET-14b

The following ligation mix was prepared: 0.1 µg of pET-14b, 1 µl T4 DNA ligase buffer (New England Biolabs), 1 µl T4 DNA ligase (New England Biolabs) and 4 or 6 µl of DNA (*sssod*). To one sample dH₂O were added instead of the DNA, to serve as a negative control. All reactions were incubated at 16 °C for 17 hours. The 10 µl ligation mixes were electroporated into *E. coli* cells as described in section 4.3.1. The electroporated cells were spread on LBamp plates and incubated at 37 °C overnight, before colony PCR.

4.4 Purification of plasmids

Single clones of transformed *E. coli* cells were resuspended in LBamp medium and incubated overnight at 37°C on a shaking platform. The plasmids were purified using the Wizard plus Midipreps DNA purification system or the Wizard Plus Minipreps DNA purification system (both Promega). The midipreps is used to generate larger amounts of plasmids than the minipreps. In both purification methods, cells are first lysed and passed through a column operated on a vacuum pump. TE-buffer pH 8.0 is used for the plasmid elution. The purified plasmid concentration was measured spectrophotometrically at 260 nm using Nanodrop ND-1000 spectrophotometer. The computer belonging to the Nanodrop ND-1000 spectrophotometer was set on the “nucleic acid measurement” program. For the blank test, 2 µl dH₂O were added to the

detector. The DNA concentrations (ng/μl) were measured by adding 2 μl DNA sample, results were read on the computer screen.

4.5 DNA sequencing

One sequence reaction (20 μl) contained: 2 μl Big Dye terminator ready reaction mix v.3.1, 3 μl Big Dye dilution buffer (Applied Biosystem), 5 pmol/μl of each primer (OP553 or OP554 for control sequencing of *sssod*, T7 or T3 for control sequencing of pCR-Script-*sssod* and T7pro or T7ter for control sequencing of pET-14b-*sssod*), DNA template (~200 ng of purified *sssod* PCR-amplicon, ~215 ng purified pCR-Script-*sssod* plasmid or ~324 ng purified pET-14b-*sssod* plasmid) and dH₂O.

The following program was used for the sequencing reactions: 96 °C for 1 minute followed by 25 cycles of 96 °C for 10 seconds, 40 °C for 5 seconds and 60 °C for 4 minute. The reaction products were purified and concentrated with 2 μl EDTA (125 mM), 50 μl 99.5 % ethanol (EtOH) and 2 μl sodium acetate (NaAc) pH 4.6. The resulting pellet was dissolved in 11 μl formamide (HiDi) before analysis, using the ABI PRISM 3100 DNA Analyzer (Applied Biosystems). The sequencing data were processed using Vector NII Advance 10 (Invitrogen).

4.6 Expression of *Sarcoptes scabiei* SOD protein (ssSOD)

A chemical transformation was performed by adding 2 μl of the expression plasmid pPU110 to 40 μl of competent *E. coli* cells BL21 (DE3) (Stratagene). The cells were incubated for 2 minutes on ice, 1 minute in a 42°C water bath and finally 2 minutes on ice. After the incubation 500 μl of LB broth were added to the cells and incubated on a shaking platform in 37 °C for 30 minutes. The cells were spread on LBamp plates and incubated overnight at 37 °C. The following morning, one colony of the transformed cells was inoculated in to 20 ml of LBamp broth and incubated overnight on a shaking platform at 37 °C. Ten ml of the overnight culture was transferred to 1000 ml of minimal medium with casamino acids (MM-CA-medium), this medium contained: 0.125 % glucose, 0.25 μg/ml biotin, 0.5 μg/ml thiamine, 0.25 mg/ml (NH₄)₂SO₄, dH₂O, 0.004 % heavy metal stock (Pryor & Leiting 1997), 0.125 %, Casamino acids, 1x MM-CA phosphate buffer (Pryor & Leiting 1997), 0.0125 μg/ml ampicillin. The cells were incubated at 37°C on a shaking platform until the optical density (OD) reached 0.8-1.0 at 600 nm. The culture was cooled in a water bath to room temperature for five minutes. Thereafter 1 ml of that culture was transferred to a microcentrifuge tube and centrifuged at ~17000g for 2 minutes; the resulting pellet was resuspended in 50 μl of 1x SDS-PAGE buffer (70 mM Tris-HCl pH 6.8, 0.2 % glycerol, 2 % sodium dodecyl sulfate (SDS), 0.005 % bromophenol blue (BFB) and 7.7 mg/ml dithiothreitol (DTT)) and stored at -20°C until further analysis on a 12 % SDS-polyacrylamide gel. To the remaining culture, IPTG was added to the final concentration of 0.5 mM and incubated at ~18°C on a shaking platform overnight. The following morning, 0.5 ml of the overnight culture was transferred to a microcentrifuge tube and centrifuged at ~17000g for 2 minutes. The pellet was resuspended in 100 μl of 1x SDS-PAGE buffer and stored at -20°C until further analysis on 12 % SDS-polyacrylamide gel. The remaining culture was centrifuged at 4000g for 10 minutes at 4°C and the resulting pellet was resuspended in buffer containing: 20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA and complete protease inhibitor (Roche Diagnostic), according to manufacturers instruction. The harvested cells were stored at -20°C.

4.7 Electrophoresis

4.7.1 Sodium Dodecyl Sulfate-Polyacrylamide Gel

Each 12 % sodium dodecyl sulfate-polyacrylamide gel contained a separation gel and a stacking gel. The separation gel contained 0.37 M Tris-HCl pH 8.8, dH₂O, 12 % N, N'-methylene-bis-acrylamide with a crosslinker ratio of 37:5:1 (Bio-Rad Laboratories), 0.10 % sodium dodecyl sulfate (SDS), 0.10 % ammonium persulfate (APS), 10 µl N, N, N', N'-tetramethylethylenediamine (TEMED; Bio-Rad Laboratories). The 4 % stacking gel contained 0.37 M Tris-HCl pH 6.8, dH₂O, 3.9 % N, N'-methylene-bis-acrylamide with a crosslinker ratio of 37:5:1, 0.10 % SDS, 0.10 % APS, 20 µl TEMED. All gels were run in 1x running buffer (25 mM Tris, 250 mM glycine and 0.1 % SDS) at 200 V for ~ 45 minutes

All gels were stained with Coomassie Brilliant Blue solution (0.1 % Coomassie Brilliant Blue, 0.1 % HAc and 0.4 % MeOH). The destaining was carried out by boiling the gel in distilled water in the microwave at full effect for ~20 minutes, changing the water every 5 minutes. The gels were photographed with Kodak Electrophoresis Documentation and Analysis System 120 (Kodak digital science). The protein sizes were calculated using a translate tool on ExpASY web server (www.expasy.org).

4.7.2 Gel electrophoresis

The PCR products were analyzed with 1 % agarose gel stained with ethidium bromide (~8 µg/ml). All gels were run in 1x TAE buffer (40 mM Tris-acetate and 1mM EDTA) at 75V for ~35 minutes. The gels were photographed with a Kodak Electrophoresis Documentation and analysis system 120 (Kodak digital science).

4.8 Purification of ssSOD

4.8.1 General procedure

The harvested cells from the ssSOD expression were thawed in a water bath (room temperature) and lysed by sonication with 6 pulses, each pulse 15 seconds long (amplitude 3, medium power). The lysate were centrifuged at 4 °C for 30 minutes at 9000g and the supernatant was filtered through a 0.45 µm filter (Miller^R-HV).

The protein concentration was determined by using the Bradford method (Bio-Rad) (Bradford 1976) with bovine serum albumin (BSA) as standard. The protein sample was diluted (24, 50, 100 and 200 µg/ml) in dH₂O to wells of an enzyme-linked immunosorbent assay (ELISA) plate and the absorbance was measured at 595 nm in a microplate reader. 1 x Bradford buffer (0.1 mg/ml coomassie brilliant blue, 4.75 % ethanol and 8.5 % H₃PO₄) were added to all measurements. A standard curves were constructed with protein concentrations as a function of absorbance to estimate the final protein concentration.

4.8.2 Affinity purification of ssSOD using the His₆-tag

The ssSOD was purified using a 1 ml HiTrap chelating high performance (HP) (GE Healthcare) operated with a peristaltic pump (Pharmacia Biotech). The column was first loaded with Ni²⁺ ions by 0.1 M metal and sulphate salt solution and then equilibrated by washing with 10 ml of binding buffer (1.25 mM phosphate buffer pH 7.4 and 1 M imidazole pH 7.4, dH₂O). The protein solution

were loaded with a flow rate of 1 ml/minute and finally eluted with 5 ml of elution buffer (0.125 mM phosphate buffer pH 7.4 and 0.05 M imidazole pH 7.4). The elution was collected in fractions of 1 ml each.

4.8.3 Purification of ssSOD using ion exchange chromatography

The ssSOD protein has an estimated isoelectric point (pI) at 6.11 (www.expasy.org), at pH (7.5) above this pI, the protein will carry a negative charge and thus can be purified using an anion exchanger. For the buffer exchange a NAPTM-5 column (GE Healthcare) was first equilibrated with 10 ml of 20 mM Tris-HCl pH 7.5, 2 ml of the protein eluate from the His₆-tag purification was loaded on and washed and collected with 2 ml of 20 mM Tris-HCl pH 7.5. To purify the ssSOD, a HiTrap Q HP column (anion exchanger) (GE Healthcare) was first equilibrated with 20 mM Tris-HCl pH 7.5. The protein fraction was loaded and eluted with 20 mM Tris-HCl pH 7.5 and 0.5M NaCl pH 7.5. The purification step was operated with ÄKTA_{FPLC} (GE Healthcare) with a flow rate of 1 ml/min.

4.8.4 Affinity Purification of ssSOD using the maltose binding protein fusion partner

An amylose resin column (New England Biolabs) was packed according to the manufacturers manual. The column was first equilibrated with start buffer (20 mM Tris-HCl, 200 mM NaCl and 1 mM EDTA). The protein mixture was loaded and passed through the amylose resin and then washed with start buffer. The bound ssSOD was eluted with start buffer supplemented with 10 mM maltose. The eluate was collected in fractions of 2 ml. The chromatography step was preformed using an ÄKTA_{FPLC} instrument (GE Healthcare) with a flow rate of 1 ml/min.

4.9 Enzyme assay

For the ssSOD enzyme assay the following was added to a cuvette: 800 µl working solution (132 ml of potassium phosphate buffer (50 mM, pH 7.8) containing diethylenetriaminepentaacetic acid (DTPA; 1.33 mM) and bovine serum albumin (BSA; 0.20 mg/ml), 5 ml catalase (40 units/ml), 5 ml nitroblue tetrazolium (NBT; 2.24 mM), 17 ml xanthine (1.8 mM) and 1 ml bathocuproine disulfonic acid (BCS; 10mM)) and 100 µl of xanthine oxidase (0.025 units/ml of xanthine oxidase in potassium phosphate buffer (pH 7.8) containing 1.33 mM DTPA and 0.20 mg/ml BSA). The ssSOD purified with the MBP affinity purification (Figure 10, lane 6), was added at different concentrations (1:1,1:10 and 1:20) diluted in 100 µl potassium phosphate buffer (50 mM). The activity was measured spectrophotometrically at 560 nm for 5 minutes for each sample. Each sample was assayed three times. For the blank test, 800 µl working solution, 100 µl xanthine oxidase and 100 µl potassium phosphate buffer (50 mM) was added into a cuvette and measured spectrophotometrically at 560 nm for 6 minutes.

Under the conditions for the assay (Mockett *et al.* 2002), one unit of ssSOD is defined as the amount that gives 50 % inhibition of NBT reduction. Equations 1 and 2 were used to determine the decrease in absorbance per minute (A_{560} nm/ minute) and the present inhibition of the test samples (% inhibition).

$$\frac{(A_{560nm} \text{ at } t_{end}) - (A_{560nm} \text{ at } t_{start})}{t_{end} - t_{start}} = \Delta A_{560nm}/\text{minute} \quad (1)$$

Where t is time in minutes and A is absorbance.

$$\frac{(\Delta A_{560nm}/\text{min})_{\text{negative control}} - (\Delta A_{560nm}/\text{min})_{\text{test sample}}}{(\Delta A_{560nm}/\text{min})_{\text{negative control}}} \cdot 100 = \% \text{ inhibition} \quad (2)$$

An ssSOD standard curve was constructed with the percentage of inhibition as a function of the concentration of ssSOD using GraphPad Prism v.4 (GraphPad Software).

Acknowledgments

I would like to thank my supervisor Jens Mattsson for giving me this opportunity to perform this interesting project and for helping me whenever I needed. I am also very thankful to my other supervisor Eva Molin for everything she taught me and for the time she spent with me. Also thanks for teaching me so much and making every day funny and relaxing. Finally I would like to thank Annie Engström for helping me with the DNA sequencing, Karin Carlson and David Lindell for the critical reading of the report.

References

- Al-Allaf, F.A., Tolmachov, O., Themis, M. & Coutelle, C. 2005. Coupled analysis of bacterial transformants and ligation mixture by duplex PCR enables detection of fatal instability of a nascent recombinant plasmid. *Journal of biochemical and biophysical methods* 64: 142-146
- Arlan, L.G., Morgan, M.S., Rapp, C.M. & Vyszensky-Moher, D.L. 1996. The development of protective immunity in canine scabies. *Veterinary parasitology* 62: 133-142.
- Bannister, W.H., & Bannister, J.V. 1988. Isolation and characterization of superoxide dismutase: a personal tribute to Joe McCord and Irwin Fridovich. *Free radical biology & medicine* 5:371-376.
- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry* 72: 248-254.
- Chen, C.N. & Pan S.M. 1996. Assay of superoxide dismutase activity by combining electrophoresis and densitometry. *Bot. bull. acad. sin.* 37:107-111
- Choudhury, S.B., Lie, J.W., Davidson, G., Yim, Y.I., Bose, K., Sharma, M.L., Kang, S.O., Cabelly, D.E. & Maroney, M.J. 1999. Examination of the nickel site structure and reaction mechanism in *Streptomyces seoulenis* superoxide dismutase. *Biochemistry* 38: 3744-3752.
- Donahue, R.A. & Bebee, R.L. 1991. B121-SITM competent cells for protein expression in *E.coli*. *Focus* 21: 49-51.
- Dragos, V., Kecelj, N. & Zgavec, B. 2004. Crusted scabies in an 8-year old child. *Acta dermatovenerologica alpina panonica et adriatica* 13: 66-70.
- Elder, B.L, Arlian, L.G. & Morgan, M.S. 2006. *Sarcoptes Scabiei* (Acari: sarcoptidae) mite extract modulates expression of cytokines and adhesion molecules by human dermal microvascular endothelial cells. *Journal of medical entomology* 43: 910-915.
- Fridovich, I. 2001. Reflections of a fortunate biochemist. *Journal of biological chemistry* 276: 28629-28636.
- GE Healthcare. 2008. StrepTrap HP and MBPtrap HP new prepacked columns for tagged protein purification. www.hitrap.com. Downloaded: 2008-01-02.
- Goldstein, S., Michel, C., Bors, W., Saran, M. & Czapski, G.A. 1988. A critical reevaluation of some assay methods for superoxide dismutase activity. *Free radical biology & medicine* 4: 295-303.
- Hainfeld, J.H., Lui, W., Halsey, C.M.R., Freimuth, P., & Powell R.D. 1999. Ni-NTA-gold clusters target his-tagged proteins. *Journal of structural biology* 127: 185-198.

- Hart, P.J., Balbirnie, M.M., Ogihara, N.L., Nersissian, A.M., Weiss, M.S., Valentine, J.S. & Eisenberg, D. 1999. A structure-based mechanism for copper-zinc superoxide dismutase. *Biochemistry* 38: 2167-2178.
- Kamiya Biochemical Company. 2008. SOD assay. <http://www.kamiyabiomedical.com/pdf//KT-019.pdf>. Downloaded: 2008-02-05.
- Kendall, D. 2007. Kendall bioresearch services. <http://www.kendall-bioresearch.co.uk/mite.htm>. Downloaded: 2008-04-05.
- Khalil, A.M., Julius, J.A. & Bachant, J. 2007. One step construction of PCR mutagenized libraries for genetic analysis by recombinant cloning. *Nucleic acids research* 35:104-116.
- Kirby, T.W. & Fridovich, I. 1982. A picomolar spectrophotometric assay for superoxide dismutase. *Analytical biochemistry* 127: 435-440
- Lecaros, L., S. 2006. Cloning and expression of duperoxide dismutase from *Sarcoptes scabiei* in *Eschericha coli*. Degree project in biology, Uppsala University.
- Ljunggren, E.L, Nilsson, D. & Mattsson, J.G. 2003. Expressed sequence tag analysis of *Sarcoptes scabiei*. *Parasitology* 127: 139-145.
- Ljunggren, E.L, Bergström, K., Morrison, D.A. & Mattsson, J.G. 2006. Characterization of an atypical antigen from *Sarcoptes scabiei* containing an MADF domain. *Parasitology* 132: 117-126.
- Lu, Q., Hosfield, T., Buchanan, M. & Padgett, K. 2008. Stratagene. <http://www.biocompare.com/technicalarticle/206/Versatile-Mammalian-Expression-Vectors-A-Review-from-Stratagene.html>. Downloaded: 2008-02-05
- Mattsson, J.G., Ljunggren, E.L. & Bergström, K. 2001. Paromyosine from the parasitic mite *Sarcoptes scabiei*: cDNA cloning and heterologous expression. *Parasitology* 122: 555-562.
- Mattsson, J. 2006. Mite protein. US patent 7105163.
- McCord, J.M. & Fridovich, I. 1988. Superoxide dismutase: the first twenty years. *Free radical biology & medicine* 5: 363-369.
- Millard, J. 2006. The biotechnology project. http://matcmadison.edu/biotech/resources/proteins/labManual/chapter_4/section4_4.htm. Downloaded 2008-02-02.
- Mockett, R., J., Bayne A., V., Sohal, B., H. & Sohal, R., S. 2002. Biochemical Assay of superoxide dismutase activity in *Drosophila*. *Elsevier Science* 28: 287-292.
- Moffatt, B.A & Studier, F.W. 1986. Uses of bacteriophage T7 RNA polymerase to direct high-level expression of cloned genes. *Journal of molecular biology* 189: 113-130.

Molin, E.U. & Mattsson, J.G. 2007. Effect of acaricides on the activity of glutathione transferases from the parasitic mite *Sarcoptes scabiei*. *Parasitology* 135: 115-123.

Muller, P., Donnelly, M.J. & Ramson, H. 2007. Transcription profiling of recently colonised pyrethroid resistant *anopheles gambiae* strain from Ghana. *BMC genomics* 8: 36-48

New England Biolabs. 2008. pMAL protein fusion and purification system. http://www.wooster.edu/biology/wmorgan/BMB303/Resources/Manuals/pMAL_manual_E8000.pdf. Downloaded: 2008-02-24.

Orion, E., Matz, H., Ruocco, V. & Wolf, R. 2002. Parasitic skin infestations II, scabies, pediculosis, spider bites: unapproved treatments. *Clinics in dermatology* 20: 618-625.

Orion, E., Matz, H. & Wolf, R. 2004. Ectoparasitic sexually transmitted diseases: scabies and pediculosis. *Clinics in dermatology* 22: 513-519.

Pasay, C., Walton, S., Fischer, K., Holt, D. & McCarthy, J. 2006. PCR-based assay to survey for knockdown resistance to pyrethroid acaricide in human scabies mites. *American journal of tropical medicine and hygiene* 74: 649-657.

Pryor, K.D. & Leiting, B. 1997. High-level expression of soluble protein in *Escherichia coli* using a his₆-tag and maltose-binding-protein double-affinity fusion system. *Protein expression and purification* 10: 309-319.

Pettersson, E. 2002. Identification and characterization of a novel glutathione S-transferase from the parasite *sarcoptes scabiei*. Degree project in biology, Uppsala University

Pettersson, E.U., Ljunggren, E.L., Morrison, D.A. & Mattsson, J.G. 2005. *International journal for parasitology* 35: 39-48.

Picker, S.D. & Fridovich, I. 1984. On the mechanism of production of superoxide radical by reaction mixtures containing NADH, phenazine methosulfate, and nitroblue tetrazolium. *Archives of biochemistry and biophysics* 228: 155-158.

Polosukhina, D.I., Kanyshkova, T.G., Doronin, B.M., Tyshkevich, O.B., Buneva, V.N., Biko, A.N., Nevinsky, G-A. & Favorova, O.O. 2006. Metal-dependent hydrolysis of myelin basic protein by IgGs from the sera of patients with multiple sclerosis. *Immunology letters* 103: 75-81.

Rosenberg, A.H., Lade, B.N., Chui, D., Lin, S., Dunn, J.J. & Studier, F.W. 1987. Vector for selective expression of cloned DNAs by T7 RNA polymerase. *Gene* 56: 125-135.

Studier, F.W., Rosenberg, A.H., Dunn, J.J. & Dubendorff, J.W. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods in enzymology* 185: 60-89.

Sambrook, J. & Russell, D.W. 2006. Purification of maltose-binding fusion proteins by affinity chromatography on amylose resin. *Cold spring harbor protocols*, doi 10.1101/pdb.prot4087.

Shibata, H., Takahshi, M., Yamaguchi, I. & Kibayashi, S. 1999. Sulfide oxidation by gene expression of sulfide-quinone oxidoreducrae and ubiquione-8 biosynthase in *Escherichia coli*. Journal of bioscience and bioengineering 88: 244-249.

Tauati, D. 1991. The molecular genetics of superoxide dismutase in E-coli. Free radical research communications 12-13: 379-382.

Tauati, D. 2002. Investigating phenotypes resulting from a lack of superoxide dismutase in bacterial null mutants. Methods enzymology 349: 145-154.

Zemlyak, I., Nimon, V., Brooke, S., Moore, T. & Mclaughlin, J. 2004. Gene therapy in the nervous system with superoxide dismutase. Brain research 108: 12-18.