Directed Evolution of a Subtilisin Carlsberg Variant towards Increased Oxidative Stability

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ABBREVIATIONS

Ap  Ampicillin
AU  Absorbance unit
BSA Bovine serum albumin
*B. subtilis*  *Bacillus subtilis*
Cm  Chloramphenicol
Da  Dalton
DMSO Dimethyl sulfoxide
DNA Deoxyribonucleic acid
*E. coli*  *Escherichia coli*
epPCR Error-prone PCR
HTS High throughput screening
LB Luria-bertani medium
MEGAWHOP Megaprimer PCR of whole plasmid
MTP Microtiter plate
OD Optical density
PAA Peroxyacetic acid
PCR Polymerase chain reaction
pI Isoelectric point
PMSF Phenylmethylsulfonyl fluoride
SDM Site directed mutagenesis
SSM Site saturation mutagenesis
SeSaM Sequence saturation mutagenesis
StEP Staggered elongation process
Suc-AAPF-pNA Succinyl-L-Ala-L-Ala-L-Pro-L-Phe-\(p\)-nitroanilide
TCA Trichloroacetic acid
Tc Tetracycline
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I dedicate this dissertation to my brother and parents for their unconditional love and support in every possible way during the process of making this dissertation and beyond.
ABSTRACT

Serine proteases are very important enzymes employed mainly in detergent industry. A recently described subtilisin Carlsberg variant Thr59Ala/Leu217Trp (SC Perhydrolase) is a potentially important industrial hydrolase. The key feature of this variant is its ability to catalyse perhydrolysis, in addition to hydrolysis, forming peroxycarboxylic acids in the presence of esters and hydrogen peroxide. Employing SC Perhydrolase for in situ generation of peroxycarboxylic acids by hydrolysis of esters or amides in the presence of hydrogen peroxide is an alternative approach for producing peroxycarboxylic acids when required in application. However, the level of perhydrolytic activity of SC Perhydrolase is still not sufficient to be used in industrial scale since its activity and oxidative stability are far from the industry required standards. In order to enhance the perhydrolytic performance and the oxidative stability of this subtilisin variant the directed evolution was performed. An optimized microtiter plate expression system in Bacillus subtilis DB104 along with a transformation protocol for a particular Bacillus strain (DB104) were established yielding $10^5$ transformants per µg DNA. The assays for the detection of perhydrolytic and proteolytic activity were developed and optimized for high throughput screening (HTS). Screening of two site saturation mutagenesis libraries for increased oxidative stability resulted in a set of nine SC Perhydrolase variants. Amino acid substitutions were identified and combined into two variants M7 (Trp217Met) and M4 (Trp217Leu/Met222Cys) with increased oxidative resistance for 4.5-fold (M7) and 2.2-fold (M4). The $k_{cat}$ values for perhydrolysis of methylbutyrate of these two variants moderately decreased for 1.4-fold. The analysis of beneficial substitutions showed that the oxidation-sensitive amino acids close to the active site can be replaced with oxidation-resistant residues resulting in a less active but functional variant. Further analysis revealed that solvent exposure of amino acid residues could determine the level of its oxidation and subsequently inactivation of the enzyme.

The investigation on the catalytic promiscuity in SC Perhydrolase and employing directed evolution for engineering its catalytic efficiency towards diacetate esters as an attractive substrate for industrial application was the second goal of the thesis. The main approach in the directed evolution campaign was screening error prone
PCR (epPCR) and site saturation mutagenesis (SSM) libraries having mutations at positions proposed after docking analysis and visual inspection of the subtilisin Carlsberg model. A microtiter plate screening was performed using a novel fluorescent assay for the detection of in situ generated peroxycarboxylic acids. Although a variant with increased catalytic efficiency towards diacetate ester was not identified, the investigation of molecular interactions between SC Perhydrolase and the ester substrate contributed to gain a significant insight in understanding of molecular reasons for the catalytic promiscuity in SC Perhydrolase. The novel insights can be helpful for designing enzyme variants with improved level of perhydrolysis towards more suitable ester substrates in the future.
MOTIVATION OF THE WORK

The development of protein engineering techniques and their application in tailoring proteases as an important group of enzymes has brought enormous benefit due to the improvement in terms of activity, substrate specificity and stability. Despite all success achieved up to date, there are still many challenges for serine proteases and their adaptation for a more efficient use in industry.

Peroxycarboxylic acids are widely used for mild bleaching and disinfection for instance in agricultural industry, food establishments and medical facilities. Despite their attractive synthesis potential, peroxycarboxylic acids are limited in applications due to hydrolysis in aqueous solutions, and their explosive character in concentrated form. Employing enzymes able to catalyze *in situ* generation of peroxycarboxylic acids by hydrolysis of esters or amides in the presence of hydrogen peroxide is an alternative approach for producing peroxycarboxylic acids when required in application. The subtilisin proteases are group of industrially employed enzymes having promiscuous activity. Enzyme promiscuity describes the ability of biocatalysts to catalyze conversions beyond their “natural” reactions. Subtilisin Carlsberg is the first example of a promiscuous protease which is able to catalyze perhydrolysis besides its natural proteolytic activity. Variant subtilisin Carlsberg T59A/L21W (SC Perhydrolase) was patented by Henkel AG & Co. KGaA (Wieland, 2009) due to increased peroxycarboxylic acid production. Enzymatic generation of chemical oxidants is often limited by enzyme stability. A concentration around 1.5 mM is sufficient for bleaching in liquid detergent formulations. Unfortunately SC Perhydrolase has a relatively low oxidative resistance; therefore reengineering of this enzyme to promote side reaction of perhydrolysis and its oxidative resistance for synthetic and industrial applications was the main motivation of this work.
PART I: GENERAL INTRODUCTION

1. Protein engineering and directed evolution

Enzymes are proteins that catalyse a wide variety of chemical reactions (e.g. oxidation, hydrolysis, transglycosilation, transamination, isomerisation, ligation). Due to their efficiency and specificity, enzymes are exploited to produce compounds that traditionally were made by chemical synthesis (e.g. ester hydrolysis, oxido-reduction of alcohols and ketons, aldol reaction, glycosidic bond formation, isomerization, addition, elimination). Their application is continuously expanding for chemical, pharmaceutical and food industry generating, at the same time, a demand for enzymes exhibiting increased specific activity, stability, specificity, and enantioselectivity. The development of genetic engineering methods enabled an expansion on the use of enzymes in industry. Naturally occurring enzymes (wild type) are optimized for physiological conditions and often need to be optimized for industrial conditions such as extreme temperatures, pH and salt concentration along with non-aqueous solvents. Despite enzymes with suitable properties that have been isolated from extremophile organisms, most of the enzymatic reactions of industrial interests lack a natural enzyme that fits 100 % on the pre-established working conditions.

Most common molecular level approaches in protein engineering to generate and isolate an enzyme having tailored characteristics are directed evolution and rational design.

The concept of directed evolution was introduced in 1967 (Yamane et al 2004). Directed evolution is a method developed to overcome the limitations of natural enzymes by tailoring their properties to the needs of a particular application or working conditions and simultaneously identify and investigate structure–function relationships (Roccatano et al 2006). It basically mimics the process of Darwinian evolution in a test tube with a few differences: directed evolution requires an outside intelligence, it does not create new species (macroevolution) only improvements within a species (microevolution) and it happens rapidly (months or years timescale) (Stemmer and Holland 2003). Besides improving an activity that already exists, directed evolution can be used for combining properties not necessarily found
together in natural enzymes (Matsuura and Yomo 2006). It has been successfully applied in the field of biotransformations, biosensors, bioremediation, vaccines, therapeutic proteins and others (Roccatano et al 2006).

A typical directed evolution experiment composes of three major steps: I) diversity generation, II) screening for improved variants in a condition directly comparable with the final application and III) isolating the gene encoding for the improved protein variant (Fig. 1.1) (Güven et al 2010). This process is repeated in iterative cycles of random mutagenesis and screening resulting in tailored proteins having the desired properties.

![Fig. 1.1 Scheme of a directed evolution experiment (iterative cycles). Step I-mutant generation, Step II-screening for the improved variants, Step III-isolation of gene encoding for the improved protein variant (Güven et al 2010)](image)

The limitation steps in performing a successful directed evolution experiment is the generation of a library with unbiased diversity and developing a screening/selection system that mimics as close as possible the desired working conditions in order to apply a correct selection pressure (Schmidt-Dannert and Arnold 1999). The great advantage of the directed evolution approach is that it requires no prior structural or functional knowledge of the target protein, nor it is necessary to be able to predict an effect of a given mutation.

Diversity generation methods are crucial to the success of a directed evolution campaign. Techniques for introducing random mutations into a DNA sequence
include error prone PCR (Cadwell et al 2007), UV radiation (Cox and Parry 1968), DNA shuffling (Stemmer 1994), Staggered Extension Process (StEP) (Zhao et al 1998), and Sequence Saturation Mutagenesis (SeSaM) (Wong et al 2004). The most common method for introducing random mutations in a gene is epPCR, while DNA shuffling and StEP are used for recombination, which allow the accumulation of beneficial mutations in a separate genes. SeSaM is one of the latest methods developed for the generation of random mutagenesis libraries. It enables introduction of mutations by saturating every amino acid position in a given protein and overcomes significant bias for transition mutations over transversions. A perfect diversity generation method should fulfill seven criteria: I) unbiased mutational spectrum, II) controllable mutation frequency, III) consecutive nucleotide substitutions or codon–based substitutions, IV) enable subset mutagenesis, V) independence of gene length, VI) technically simplicity and reproducibility, and VII) low cost (Wong et al 2006).

While the methods for generation of gene diversity are independent of the synthesized protein, the screening formats for activity need to be developed and optimized for each specific enzyme and reaction. The bottleneck for most directed enzyme evolution experiments is therefore the availability of genuinely high throughput screening (HTS) systems or selection for target activity (Aharoni et al 2005). Various screening methods have been employed in protein evolution. The most common methods used are the colorimetric or fluorometric reaction in microtiter plate format (Cohen et al 2001) and the solid phase screening on agar plates, filter papers or membranes (Lin and Cornish 2002). Microtiter plate based screenings offer medium throughput ($10^3$-$10^5$) whereas solid phase screenings are high throughput ($10^4$-$10^6$) but have less detail level (Olsen et al 2000). Because of their ultra-high throughput screening capabilities (>10$^7$/hour) (Aharoni et al 2005), flow cytometry based screening methods coupled with emulsion technology are becoming more and more attractive. They offer qualitative differentiation between positive or negative activity enabling enrichment of active variants; however, dye diffusion and the limited number of fluorescent assays suitable for flow cytometry screening are some of the challenges of this technology. The comparison of current available high throughput screening formats and technologies are summarized in Table 1.1.
**Table 1.1. The comparison of screening and selection technologies (Leemhuis et al 2009)**

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Library size</th>
<th>Advantage</th>
<th>Disadvantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selection</td>
<td>$\sim 10^9$</td>
<td>Yields desirable variants only</td>
<td>Only possible if activity confers an advantage</td>
</tr>
<tr>
<td>Agar plate screening</td>
<td>$\sim 10^5$</td>
<td>Simple to operate</td>
<td>Limited dynamic range</td>
</tr>
<tr>
<td>Microtiter plate</td>
<td>$\sim 10^4$</td>
<td>Most analytical methods possible. Excellent dynamic range</td>
<td>Relative low screening capacity</td>
</tr>
<tr>
<td>screening</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell–in–droplet</td>
<td>$\sim 10^9$</td>
<td>Large Libraries</td>
<td>Fluorescence detection and DNA modifying enzymes</td>
</tr>
<tr>
<td>screening</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell as microreactor</td>
<td>$\sim 10^9$</td>
<td>Large libraries</td>
<td>Fluorescent detection</td>
</tr>
<tr>
<td>Cell surface display</td>
<td>$\sim 10^9$</td>
<td>Large libraries</td>
<td>Fluorescence detection</td>
</tr>
<tr>
<td><em>In vitro</em> Compartmentalization</td>
<td>$\sim 10^9$</td>
<td>No cloning steps. Large libraries</td>
<td>Fluorescence detection and DNA modifying enzymes</td>
</tr>
</tbody>
</table>

In the last step of directed evolution campaign, identified variant with desired improvement is isolated and mutations introduced in the gene are analysed. The mutated gene is used as a parent for further iterative cycles of directed evolution, or all identified mutations are recombined to reach satisfactory level of improvement. The number of iterative cycles depends on the starting activity of the protein, susceptibility of its gene to mutations and the level of improvement to be achieved.

Rational design, on the other hand, is based on enzyme structural information (crystal structure or results of homology modeling), necessary to identify residues that can be exchanged to yield a desired improvement. It is considered as the most dynamic and rapidly developing area in computational biology. The experiments based on rational design are significantly less time consuming and composes of site directed mutagenesis or site saturation mutagenesis experiments followed with screening of a limited number of variants. The identification of beneficial residues and their combination are helpful for understanding of function–structure relationship of proteins, in general. The rational design approach brings us closer to fully characterize improved catalysts.

Since structure-function relationships are naturally complex, the development of new computational tools became a very important area of protein engineering. For
Part I: General introduction

example, identification of routes from buried active sites to the external solvent in static protein structure a new algorithm, ‘CAVER’, has been developed. The algorithm can be used for static and dynamic analysis of molecular dynamic trajectories (Petrek et al 2006). Quantitative Structural Activity Relationship (QSAR) methods were developed, like COMparative BINding Energy (COMBINE) analysis, whose application has been explained by Wang et al (2002), and Volsurf computational method (Braiuka et al 2007).

The combination of both approaches, random mutagenesis methods of directed evolution with the elements of rational enzyme modification is most likely the best tool to yield remarkable improvements in target protein.

2. Proteases

Proteases are enzymes that hydrolyse peptide bonds. They account for 2 % of the total number of proteins present in living organisms. Proteases can be classified into different groups; based on pH conditions under which they show optimal activity, they can be separated into acid, neutral or alkaline types. The second criterion for their classification are the essential catalytic residues present in their active site, therefore proteases can be classified as serine-, thiol-, acid-, and metallo–proteases (Gupta et al 2002).

Proteases are present in many important physiological processes like maturation of enzymes and hormones, hydrolysis of proteins in cell-free environment enabling the source of nutrients for the cells (Maurer 2004). Besides their importance in the physiology of living cells, they are also attractive for industrial technology. Industrial production of bacterial proteases began in 1960s and since then great interest for their exploitation in technical processes was noted. Proteases represent approximately 40 % of the total enzymes sales in food, detergent, diagnostics and pharmaceutical industries (Gupta et al 2002).

In food industry they are used in the production of meat extract powder, hydrolysates of gelatin, caseine and other proteins. Also they are used in fish, seafood and animal protein processing for enhancing oil recovery, improving digestibility, reducing allergenicity, and improving a flavor. Rennet (mainly chymosin), has been used traditionally in the production of cheese. Proteases are used in leather industry i.e. alkaline proteases that are used to remove hair from hides. In wool industry proteases are used to hydrolyse the overlapping scales that
are covering the wool fibers contributing to silky luster. Proteases are also useful and important components in biopharmaceutical products such as contact lens cleansing agents. Proteases also find use in medicine for their anti-inflammatory effects in addition to their proteolytic effects such as in wound debridement, the removal of dead wound tissue to assist healing, and in the treatment of osteoarthritis with a benefit comparable to that of other anti-inflammatory drugs. All this applications make proteases an important group of enzymes in biology, medicine and biotechnology.

3. Serine proteases and subtilisins

Serine proteases are the most commercially exploited among proteases. Besides the essential serine at the catalytic site, they have two additional residues, histidine and aspartic acid which form the catalytic triad (Fig. 1.2). The pH optimum of this proteases ranges from 7 to 11. Serine proteases are further divided into subclasses depending on their side chain specificity and structural homology to well established proteases. The main subclasses are chymotrypsin-like, subtilisin-like, wheat serine carboxypeptidase II-like, prolyligopeptidase-like, myxobacter $\alpha$-lytic and staphylococcal proteases (Gupta et al 2002).
Subtilisin-like proteases are a family of serine proteases containing an essential serine residue at the active site that initiates the nucleophilic attack on peptide bond. These proteases are secreted to the medium and have a pH optimum around 10, molecular weight range of 15 to 90 kDa, and isoelectric point near 9. They are produced by various Bacillus sp. like B. amyloliquefaciens, B. licheniformis and B. subtilis (Gupta et al 2002). The structure of different subtilisins has been
determined by X-ray crystallography and the enzymes have been widely used as a model system for protein engineering.

All subtilisins have the same reaction mechanism (Fig. 1.3). Their catalytic triad consists of aspartic acid, histidine and serine. Once the substrate is bound, a hydroxyl group of a nucleophile serine attacks the sessile peptide carbonyl group. A covalent bond forms between the serine and the substrate to yield the complex known as tetrahedral intermediate. The tetrahedral intermediate, which resembles reaction’s transition state, is stabilized by two amide hydrogens coordinating the anionic oxygen. This region is known as an oxyanion hole since it is occupied by the intermediate’s oxianion group. The tetrahedral intermediate decomposes back to an acyl-enzyme intermediate by breaking the peptide bond and removing the N-terminal portion of the substrate. The remaining substrate is temporarily covalently linked to the enzyme. The ester bond is broken by a nucleophilic attack of water molecule present in the active site. Histidine is protonated while forming a covalent bond to the carbonyl carbon. The result is another tetrahedral intermediate stabilized by amide groups in the oxianion hole. In the last step the tetrahedral intermediate decomposes by breaking the bond to serine hydroxyl group. The hydrogen held by the histidine is transferred to the serine and the substrate is released with a carboxylic acid terminus and enzyme is restored to its initial state (Polgár 2005).
4. Subtilisin proteases: application in detergent industry and protein engineering

Subtilisins are the most widely used proteases in detergent industry, especially high alkaline subtilisins which entered industry of detergents in the 1980s (Maurer 2004). Apart of removing proteinaceous stains and increasing the level of cleaning, they have contributed to some environmental benefits such as reducing energy consumption by decreasing water use, lowering washing temperature and shortening washing times. Despite proteases can be produced by many organisms, only few are considered as appropriate for industrial exploitation. Strains of Bacillus sp. dominate the industrial sector, especially B. licheniformis, B. subtilis, B. amyloliquefaciens and...
Part I: General introduction

*B. mojavensis* (Gupta *et al* 2002). These strains are an attractive source of proteases due to the fact that they can be cultured in large quantities in a relatively short time and they produce large amounts of extracellular protease making downstream processing easier.

Besides attractiveness and important role in industry, subtilisins are a key model for understanding the genetic engineering approaches. Although subtilisins are very efficient and broadly specific proteases, some properties could be improved for application in detergents. The requirements of industry for improved catalytic efficiency, stability towards temperature and oxidative agents generated an increased interest in protein engineering studies in the 1980s. In a short period of time, mutations in over 50% of 275 amino acids of subtilisin were reported (Bryan 2000). Engineering of subtilisins included altering of catalytic amino acids, substrate binding region and mutations that influence stability (Bryan 2000). The properties that have been altered in subtilisins by means of directed evolution are oxidative stability (Estell *et al* 1985), thermal stability (Heringa *et al* 1995), alkaline pH stability (Cunningham and Wells 1987), stability in organic solvents (Takagi *et al* 2000), substrate specificity (Rheinnecker *et al* 1993), nucleophile specificity (Rheinnecker *et al* 1994) and pH activity profile (Sternberg *et al* 1987).

Among subtilisins, BPN’ from *B. amyloliquefaciens*, subtilisin E from *B. subtilis* and savinase from *B. lentus* have been the most studied (Bryan 2000). The first model for genetic manipulation was subtilisin BPN’ and it was engineered for thermal stability (Pantoliano *et al* 1987), catalytic mechanism (Carter *et al* 1989) and oxidative stability (Estell *et al* 1985). Engineering of subtilisin E to improve thermal stability was achieved by introducing disulfide bridges between residues Gly61Cys and Ser98Cys (Takagi *et al* 1990). Stability of aprA subtilisin from *B. subtilis* was improved by substitution Asn to Ser in position 109 and 218. Thermal stability of the enzyme was additionally increased by an amino acid substitution (Asn76Asp) that increased affinity for calcium in a primary binding site (Narhi *et al* 1991). The calcium binding site was also altered in subtilisin BPN’. Negatively charged amino acids were introduced in proximity to a second calcium binding site (Pro172Asp, Gly131Asp) resulting in a variant with decreased dependence on the calcium concentration (Pantoliano *et al* 1988). The stability of a neutral protease from *B. stearothermophilus* was improved by replacing Gly residues located in a central α-helix by Ala. Thermal stability of this enzyme was improved by increasing hydrophobicity of its core
Part I: General introduction

(Imanaka et al 1986). Stability of subtilisin BPN’ in the presence of oxidative agents was improved by substitution of Met 222 with amino acid that are less susceptible to oxidation such as Ala (Estell et al 1985). All mentioned substitutions are summarized in Table 1.2.

Table 1.2. Engineering of different subtilisin proteases

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Property</th>
<th>Substitutions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subtilisin E</td>
<td>Thermal stability</td>
<td>Gly61Cys and Ser98Cys</td>
<td>(Takagi et al 1990)</td>
</tr>
<tr>
<td>aprA subtilisin</td>
<td>Thermal stability</td>
<td>Asn109Ser, Asn218Ser, Asn76Asp</td>
<td>(Narhi et al 1991)</td>
</tr>
<tr>
<td>Subtilisin BPN’</td>
<td>Calcium dependency</td>
<td>Pro172Asp, Gly131Asp</td>
<td>(Pantoliano et al 1988)</td>
</tr>
<tr>
<td>Neutral protease from B. stearothermophilus</td>
<td>Thermal stability</td>
<td>Gly→Ala</td>
<td>(Imanaka et al 1986)</td>
</tr>
<tr>
<td>Subtilisin BPN’</td>
<td>Oxidative stability</td>
<td>Met222Ala</td>
<td>(Estell et al 1985)</td>
</tr>
</tbody>
</table>

5. Subtilisin Carlsberg and catalytic promiscuity

Subtilisin Carlsberg was the first reported bacterial serine protease (1954); it is naturally produced by B. licheniformis and the complete amino acid sequence of the mature protein is described (Smith et al 1966). Protein sequence analysis revealed that subtilisin Carlsberg differs from BPN’ in 83 amino acids (Neidhart and Petsko 1988). Despite this difference, the structures of these enzymes are similar and the active sites are identical.

Thermal stability is one of the most interesting features of subtilisin Carlsberg and due to this property it became an important industrial enzyme, resulting in the fact that it is produced in greater quantities than any other enzyme (Jacobs et al 1985). Subtilisin Carlsberg is produced as a pre-pro-subtilisin. The N-terminal pre-peptide is a signal peptide consisting of 29 residues, the pro-peptide is 76 residues long and the mature protein comprises 274 residues (Jacobs et al 1985). The molecular weight of the mature protein is 27 kDa. The pro-region guides the folding of the subtilisin into its active conformation acting as a chaperone. The pro-region holds the pro-protease associated with the membrane and the release of the protease takes
place as a result of the autocatalytic removal of the pro-region (Takagi and Takahashi 2003). Point mutation experiments showed that the autocatalytic cleavage and recognition of this junction of the subtilisin Carlsberg pro-region is independent of the amino acid sequence around the cleavage site (Egnell and Flock 1992). On the other hand, a high level of sequence identity at the cleavage site of several subtilisins suggests that the amino acid sequence at the cleavage site is important for its recognition (Jacobs et al 1985).

Besides hydrolysis of peptide bonds, subtilisin Carlsberg is able to catalyse the reversible formation of peroxycarboxylic acids from esters in the presence of hydrogen peroxide. This catalytic promiscuity was for the first time reported for hydrolases in 1985 (Zaks and Klibanov 1985). Promiscuity has been classified in three major classes: “condition” promiscuity, when the enzymatic reaction is happening in the conditions which are different from natural conditions of the enzyme; “substrate” promiscuity, when the enzyme can catalyse a reaction using a broad range of similar substrates; and “catalytic” promiscuity, when the enzyme can catalyse several different reactions (Busto et al 2010). Subtilisin Carlsberg fits in two of the three mentioned classes of promiscuity; the switch from water to hydrogen peroxide as nucleophilic agents makes it a member of the “substrate” promiscuous enzymes. On the other hand, the chemically different products of the catalysed reactions make it an example of the “catalytic” promiscuity group. Catalytic promiscuity of subtilisin Carlsberg is of great importance for biotechnology and industry since it catalyses the production of peroxycarboxylic acids, well known as bleaching agents.

However, the reported level of perhydrolytic reaction is significantly lower compared to the hydrolytic activity in subtilisin Carlsberg; thus, increasing the fraction of perhydrolytic activity in this hydrolase could open a new application field for this enzyme.

In general, mechanism of perhydrolysis (Fig. 1.4) is similar to mechanism of hydrolysis (Fig. 1.3). The main difference occurs after formation of the acyl-enzyme complex. The acyl-enzyme complex is attacked by a nucleophilic hydrogen peroxide molecule instead of water. In the following steps of the reaction, histidine protonates the oxygen of the catalytic serine, leading to the release of a peroxycarboxylic group. This mechanism is called ping–pong bi–bi mechanism (Yin de et al 2010).
Part I: General introduction

![Proposed mechanism of perhydrolysis in a subtilisin-like protease (Bernhardt et al 2005). Differences in perhydrolysis compared to hydrolysis mechanism are labeled in red.](image)

Fig. 1.4 Proposed mechanism of perhydrolysis in a subtilisin-like protease (Bernhardt et al 2005). Differences in perhydrolysis compared to hydrolysis mechanism are labeled in red.

Alternatively, another mechanism was reported; called bi-bi nonconventional mechanism, suggesting that the carboxylic acid substrate is stabilized by serine through a hydrogen bond instead of forming an acyl-enzyme intermediate, but this proposed mechanism still needs an experimental confirmation. The fact that some subtilisins do not show perhydrolytic activity suggests that the catalytic triad is not crucial for the reaction (Bernhardt et al 2005). In order to determine the essential residues for perhydrolytic activity, amino acid sequence of six hydrolases and six perhydrolases were aligned. The identified residues were mutated in hydrolases to achieve the same amino acid sequence as in perhydrolases. The study showed that the molecular basis for the increase in level of perhydrolysis is the presence of a carbonyl group in vicinity of the active site. Carbonyl group serves as stabilizer of
hydrogen peroxide attack on a putative acyl-enzyme intermediate. The formation of the second tetrahedral intermediate is stabilized by a key hydrogen bond.

In the case of subtilisin Carlsberg, a second tetrahedral intermediate is formed after hydrogen peroxide attacks the acyl-enzyme complex. According to Bernhard, the stabilization of the second transition state is of key importance for the perhydrolytic reaction.

Subtilisin Carlsberg has been engineered to increase the level of perhydrolysis and simultaneously to decrease the level of hydrolysis. As a result, a double mutant of subtilisin Carlsberg has been found, Thr59Ala/Leu217Trp, (SC Perhydrolase) with increased perhydrolytic activity (Wieland 2009). Molecular dynamic (MD) simulations were used to study the influence of these amino acid substitutions in the second tetrahedral intermediate state. Results showed that during perhydrolysis a hydrogen bond is formed between Trp 217 and the second oxygen of the perhydroxyl group. On the other hand, the equivalent hydrogen bond in reaction of hydrolysis is not formed between the water oxygen atom and Trp 217 due to a larger distance (Fig. 1.5). Besides stabilization of the second tetrahedral intermediate state by hydrogen bond the formations of other hydrogen bonds were monitored during the simulation of SC Perhydrolase and wild type subtilisin Carlsberg. In wild type, the interaction between His 64 and Ser 221, important for completion of hydrolytic reaction, is not interrupted by additional hydrogen bonding to His 64. In SC Perhydrolase, the interaction between the oxygen atom of water and His 64 is stronger than that of His 64 and Ser 221. This result was experimentally proven by measuring the rate of hydrolysis of the double mutant, which is reduced compared to subtilisin Carlsberg wild type while perhydrolysis rate is improved (Lee et al 2010).

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**Fig. 1.5** Hydrogen bond between Trp 217 and oxygen of the second tetrahedral intermediate of the hydrolysis (A) and perhydrolysis (B) (Lee et al 2010)
6. Perhydrolases

Peroxycarboxylic acids (RCO$_3$H) are very powerful oxidizing agents which are used in chemical processing, synthesis, bleaching and disinfection (Rüsch gen. Klaas et al. 2002). They have a broad application in various industry branches such as synthesis of epoxides (Zhao et al. 2011), alcohols oxidation (Jones 1999) and nylon manufacturing (Dicosimo 2009). As antimicrobial agents, they cause cell lysis and microbial death (Alasri et al. 1992). Besides hydrogen peroxide as traditional bleaching species, peroxycarboxylic acids such as peroxyacetic acid (PAA) and medium chain (C7-C12) peroxycarboxylic acids, have been introduced in the detergent industry as a bleaching agents (Akerman et al. 2003).

Chemically, peroxycarboxylic acids can be synthesized by mixing a carboxylic acid and hydrogen peroxide. The reaction is catalysed by a strong inorganic acid. This is an equilibrium reaction and the production of peroxycarboxylic acid is favored by excess of substrate or by its removal from the reaction system. The chemical production of peroxycarboxylic acids is not widely applied due to I) instability of peroxycarboxylic acids in aqueous solution which result in decrease of its concentration over time, and II) the chemical mixture is strongly acidic due to the presence of catalyst (i.e. sulfuric acid). To overcome these disadvantages, an enzymatic production of peroxycarboxylic acids has been proposed. Currently, enzymatic reactions are classified as environmentally friendly especially where conditions and hazardous catalysts are avoided. Enzymes able to produce peroxycarboxylic acids are known as perhydrolases and generally comprise lipases, esterases or proteases. These enzymes are called promiscuous since the perhydrolytic activity they exhibit is a secondary reaction (Khersonsky et al. 2006). Besides promiscuous enzymes, a naturally occurring perhydrolase from Mycobacterium smegmatis capable of using long chain ethyl ester substrates (Wichmann et al. 2010) for perhydrolysis was reported.

Some examples of enzymes with increased perhydrolytic activity are Pseudomonas fluorescens esterase (PFE) (Kazlauskas 2006), Pseudomonas mendocina lipase (Poulouse 1994), lipase from Pseudomonas putida (Poulouse 1992), polypeptide (465 amino acids) from Candida parapsilosis (Dubreucq Eric et al. 2007), CE–7 esterases (cephalosporin C deacetylases and acetyl xylan esterases) from B. subtilis and Thermotoga sp. (Dicosimo 2009) and subtilisin Carlsberg from B.
licheniformis (Wieland 2009). From the literature there are only few publications regarding perhydrolases with reported values for hydrolytic and perhydrolitic activity. The best enzyme is esterase mutant from *Pseudomonas fluorescens* with ratio of hydrolytic versus perhydrolytic activity of only 0.02 (Table 1.3).

**Table 1.3. Hydrolytic vs. perhydrolytic activity for enzymes engineered by directed evolution**

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>H/P*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas fluorescens</em> esterase mutant</td>
<td>0.02</td>
</tr>
<tr>
<td><em>T. maritima</em> CE-7</td>
<td>0.20</td>
</tr>
<tr>
<td>Double mutant of subtilisin Carlsberg (Thr59Ala/Leu217Trp)</td>
<td>0.23</td>
</tr>
<tr>
<td><em>T. lettingae</em> CE-7</td>
<td>0.30</td>
</tr>
<tr>
<td><em>T. neapolitana</em> CE-7</td>
<td>1.00</td>
</tr>
<tr>
<td>Polypeptide from <em>C. parapsilosis</em></td>
<td>1.45</td>
</tr>
<tr>
<td><em>P. mendocina</em> lipase mutant</td>
<td>2.00</td>
</tr>
<tr>
<td><em>P. putida</em> lipase mutant</td>
<td>2.00</td>
</tr>
<tr>
<td><em>B. subtilis</em> 31954 CE-7</td>
<td>4.50</td>
</tr>
</tbody>
</table>

*H/P–ratio of hydrolytic over perhydrolytic activity

It is important to mention that the calculated activity values were obtained using different substrates, thus in order to eliminate the relativity on the activities of these enzymes they should be tested with the same substrate.

By employing enzymes having perhydrolytic activity for production of peroxycarboxylic acids *in situ* the challenge of its instability and storage is overcome. Additionally, the required concentrations of the carboxylic esters or amides as substrates are much lower compared to those used in chemical production.
PART II: AN EFFICIENT TRANSFORMATION METHOD FOR Bacillus subtilis DB104

1. INTRODUCTION

1.1. Aim of the work

The aim of this work was to develop and establish a transformation protocol for B. subtilis DB104 as an expression host of subtilisin Carlsberg and its variant Thr59Ala/Leu217Trp (SC Perhydrolase). B. subtilis DB104 is an attractive host for directed evolution due to its low proteolytic activity and efficient secretion. Prerequisite for successful directed protease evolution are efficient transformation protocols to generate a sufficient number of variants for large mutant libraries.

1.2. Transformation in Bacillus

Directed evolution is a powerful algorithm to improve enzyme properties in iterative cycles of diversity generation and screening. A typical directed evolution experiment comprises three major steps: I) diversity generation, II) screening to identify improved mutants out of a large pool of variants and III) isolating the gene encoding for the improved protein variant (Tee and Schwaneberg 2007). The first step in a directed evolution campaign includes the generation of a DNA mutant library and its subsequent transformation into the host organism. Advances in screening technologies like flow cytometry based screening systems (Aharoni et al 2005; Prodanovic et al 2011; Tu et al 2011) allow a throughput of up to $10^8$ variants so that transformation efficiencies are increasingly becoming the limiting step in directed evolution experiments.

B. subtilis is used as host for production of secretory proteins especially proteases and lipases which have a significant market value (Westers et al 2004). Low transformation efficiencies are a main challenge when using Bacillus strains in directed evolution campaigns. In order to ensure an efficient and secreted expression of the targeted protease or lipase in a Bacillus host it is essential to improve the targeted enzymes directly in the Bacillus production strain. B. subtilis DB104 is one of the most used strains for the production of industrially important extracellular enzymes, especially subtilisin proteases. B. subtilis DB104 is a
Part II: An efficient transformation method for *B. subtilis* DB104

derivative of *B. subtilis* 168 Marburg strain (Kawamura and Doi 1984), generated by lesions in the genes coding for alkaline protease (aprA3) and neutral protease (nprE18). As a consequence, *B. subtilis* DB104 shows less than 4% of the extracellular protease activity of *B. subtilis* 168 Marburg strain, which is a key advantage when proteases are expressed heterologously. *B. subtilis* DB104 requires histidine as an essential amino acid which is usually supplemented to the growth medium (Kawamura and Doi 1984).

Transformation of *Bacillus* strains can be accomplished by natural competence or artificial methods. Natural competence for DNA uptake is in gram positive and gram negative bacteria a physiologically and genetically determined trait in response to environmental stress (Hamoen *et al* 2003). *B. subtilis* develops competence for DNA uptake in dependence of nutritional conditions and growth stage. In addition, development of natural competence is strain specific due to divergent structure of the quorum sensing components which control development of natural competence (Tran *et al* 2000). Transformation based on natural competence allows DNA uptake from various sources, for instance phage DNA, plasmid DNA and chromosomal DNA. DNA uptake occurs by a common pathway through the following stages: binding, fragmentation, uptake, as well as additionally integration and replication/or mismatch repair in the case of chromosomal DNA (Dubnau 1991). Reported transformation efficiency for *B. subtilis* 168 strain reached $10^6$ transformants per µg chromosomal DNA (Anagnostopoulos and Spizizen 1961). Artificial transformation methods employ, in contrast to the above mentioned natural competence methods, physical and chemical treatments. The latter comprise electroporation or addition of chemicals such as polyethylene glycol or mannitol (Brigidi *et al* 1990; Chang and Cohen 1979) which are summarized in Table 2.1. The technically least demanding method, reported as “simple and rapid method” (Table 2.1: No. 1) allows transformation on solid media by overlaying plated *Bacillus* cells with chromosomal or plasmid DNA (2-3 µg). The “agar plate transformation” is described to yield approximately 100–200 transformants per µg DNA (Hauser and Karamata 1994). An advancement of the latter method was achieved by adding DNA in protoplast lysates (Table 2.1: No. 2) yielding transformation efficiency of $2.3 \times 10^3$ per µg chromosomal DNA (Akamatsu and Taguchi 2001); transformation efficiency decreased a 100 times in case when plasmid DNA was transformed. For transformation of plasmid DNA an electroporation method (Table 2.1: No. 3) with transformation efficiency of $10^4$ per µg
DNA was developed (Brigidi et al 1990). Electroporation combined with a previous treatment of the cells with glycine (Table 2.1: No. 4) increased efficiency to $1.7 \times 10^6$ transformants per µg of plasmid DNA. Preparation and transformation of *B. subtilis* protoplasts (Table 2.1: No. 5) in presence of polyethylene glycol yielded up to $1.4 \times 10^7$ transformants per µg plasmid DNA (Chang and Cohen 1979).

<table>
<thead>
<tr>
<th>No.</th>
<th>Transformation method</th>
<th>Bacillus strain</th>
<th>Transformants per µg DNA</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Solid media</td>
<td><em>B. subtilis</em> 168/W23</td>
<td>100–200</td>
<td>2-3 µg of DNA needed</td>
</tr>
<tr>
<td>2.</td>
<td>Solid media in protoplast lysates</td>
<td><em>B. subtilis</em> AYG2</td>
<td>$2.3 \times 10^3$</td>
<td>Optimized for chromosomal DNA</td>
</tr>
<tr>
<td>3.</td>
<td>Electroporation of intact <em>Bacillus</em> cells</td>
<td><em>B. subtilis</em> PB1424</td>
<td>$1 \times 10^4$</td>
<td>Low survival rate due to applied voltage</td>
</tr>
<tr>
<td>4.</td>
<td>Electroporation with glycine treatment</td>
<td><em>B. pseudofirmus</em> OF4</td>
<td>$1.69 \times 10^6$</td>
<td>Survival rate due to applied voltage 2-16 %</td>
</tr>
<tr>
<td>5.</td>
<td>Protoplasts</td>
<td>Derivatives of <em>B. subtilis</em> Marburg 168 strain</td>
<td>$4 \times 10^7$</td>
<td>2-3 days recovery time</td>
</tr>
</tbody>
</table>

2. MATERIAL AND METHODS

2.1. MATERIALS

2.1.1. Chemicals

All chemicals were purchased from AppliChem (Darmstadt, Germany) or Carl Roth GmbH (Karlsruhe, Germany) except casamino acids (ForMedium™, Norfolk, United Kingdom).

2.1.2. Equipment

The cell culture was cultivated in a Certomat® RM shaker (Sartorius Stedim Biotech GmbH, Goettingen, Germany). The amount of DNA in the experiments was quantified by using a NanoDrop photometer (ND-1000, NanoDrop Technologies, Wilmington, DE, USA). Plasmid isolation kit was purchased from Qiagen (Hilden, Germany). Optical density of cell culture at 600 nm ($OD_{600}$) was measured using BioPhotometer plus photometer (Eppendorf AG, Hamburg, Germany). For electroporation of *Bacillus* cells at 2100 V Electroporator 2510 (Eppendorf AG,
Hamburg, Germany) was used. For centrifugation Eppendorf centrifuge 5810 R with Rotor F-34-6-38 max. 12,000 rpm was employed.

2.1.3. Bacterial strain and plasmids

The bacterial strain used in this study was *B. subtilis* DB104 (Kawamura and Doi 1984). Plasmids used for transformation were I) pC194 (2910 bp; Cat. No. 4393, DSMZ, Braunschweig, Germany), II) pHY300PLK *Bacillus-E. coli* shuttle vector (4870 bp; Takara Bio Inc., Shiga, Japan), III) pHY300Car, derivative of pHY300PLK vector containing a subtilisin Carlsberg (6221 bp) and IV) pHCMC04 *Bacillus-E. coli* shuttle vector (8089 bp) (Nguyen et al. 2005). The final concentrations of antibiotics in agar plates were: 15 µg/ml tetracycline for cells containing pHY300PLK/pHY300Car plasmids, and 10 µg/ml or 5 µg/ml chloramphenicol for cells containing the pC194 or the pHCMC04 plasmid.

2.2. METHODS

2.2.1. Transformation of *B. subtilis* DB104 by electroporation (Brigidi et al. 1990)

The competent cells were prepared using buffers supplemented with polyethylene glycol (50%) and mannitol (136 mM). The competent cells were electroporated at 1400 V using Electroporator 2510. The cells were recovered (37°C; 150 rpm for 4h) and plated on agar plate with selective antibiotic.

2.2.2. Transformation of *B. subtilis* DB104 based on glycine treatment (Ito and Nagane 2001)

This protocol is based on the treatment of *Bacillus* cells with glycine in order to reach concentration at which growth and synthesis of the cell wall is inhibited since glycine is considered as a cell weakening agent. Before the treatment with glycine, cells were grown at 37°C, 250 rpm to OD_{600nm} = 0.5. The cell culture was split into aliquots (20 ml) to which different concentrations of glycine (1 %-2.5 %, v/w) were added. The cells were shaking for additional 1 hour; the OD_{600nm} should not change significantly during this time. The cells were transferred to 50 ml Falcon tube and cooled on ice for 15 min. Cells were washed in cold buffer containing 0.5 M sorbitol, 0.5 M mannitol and 10 % glycerol and centrifuged (4°C; 8500 rpm for 10 min). After the washing step cells were ready for transformation by the electroporation at
Part II: An efficient transformation method for *B. subtilis* DB104

2100 V. The cells were recovered (37°C; 250 rpm for 3 h) and plated on LB skim milk plates supplemented with tetracycline (final conc. 15 µg/ml).

### 2.2.3. Transformation of *B. subtilis* DB104 cells in late exponential growth phase using optimized transformation buffer (Martinez *et al* 1999)

In this method cells were diluted in the optimized transformation buffer containing 0.075 % yeast extract, 0.8 % glucose, 0.01 % calcium chloride and 0.09 % magnesium chloride, pH 7.0. A dilution is made to obtain \( \text{OD}_{600 \text{nm}} = 0.5–0.8 \). After addition of the plasmid DNA, the cells were recovered (37°C; 250 rpm for 2 h) and plated on selective media.

### 2.2.4. Transformation of *B. subtilis* DB104 with DNA multimers (Shafikhani *et al* 1997)

DNA multimers were generated with commercial Qiagen REPLI–g Mini Kit. The Kit is design to amplify genomic DNA of ~ 10 ng in an overnight reaction at 37°C. The cells were grown in minimal media and transformed with multimer PCR product.

### 2.2.5. Transformation of *B. subtilis* DB104 with plasmid DNA using protoplast method (Chang and Cohen 1979)

The method is based on preparation of *B. subtilis* DB104 protoplast like described (Chang and Cohen 1979). The adapted protocol of reported method by Henkel AG & Co. KGaA was used for transformation. For transforming the high amounts of plasmid DNA were used (≥ 1 µg). After transformation a 3-day recovery phase is necessary.

### 2.2.6. Transformation protocol based on natural competence of *B. subtilis* DB104

*Media composition*

Starvation Medium 1 (SM1) contains: 0.2 % ammonium sulfate, 1.4 % dipotassium hydrogen phosphate, 0.6 % potassium dihydrogen phosphate, 0.07 % sodium citrate, 0.5 % glucose, 0.02 % magnesium sulfate heptahydrate, 0.2 % yeast extract and 0.025 % casamino acids. All the components were mixed together and autoclaved. Starvation Medium 2 (SM2) is less reach in nutrients and includes: 0.2 % ammonium sulfate, 1.4 % dipotassium hydrogen phosphate, 0.6 % potassium dihydrogen phosphate, 0.07 % sodium citrate, 0.5 % glucose, 0.08 % magnesium sulfate heptahydrate, 0.1 % yeast extract, 0.01 % casamino acids and 0.05 %
Part II: An efficient transformation method for *B. subtilis* DB104

calcium chloride. All components of SM2 medium were mixed and autoclaved together. Histidine solution was sterilized by filtration using 0.2 µm filters (Puradisc™ 25 mm, Cat. No. 6780-2502, GE Healthcare, Munich, Germany).

**Preparation of competent cells**

*B. subtilis* DB104 cells were spread on a LB agar plate without antibiotics (37°C; for 9 h). Antibiotic-free SM1 media was inoculated by transferring a single colony and subsequent cultivation (37°C; 250 rpm for 14–16 h). Overnight culture was diluted in SM1 medium by adjusting optical density at 600 nm (OD$_{600}$) to 0.5 (approximately 2.9 x 10$^7$ cells per ml) in a volume of 10 ml and incubated (37°C; 200 rpm for 3 h). The cell culture volume was doubled by addition of SM2 medium and subsequently supplemented with varied histidine concentrations (final concentration 0, 10, 50, 200, 500, and 1000 µg/ml; total volume 20 ml) and incubated (37°C; 300 rpm for 2 h). After the latter treatment, *B. subtilis* DB104 cells were competent for approximately 1 h. Histidine concentration of 200 µg per ml (final conc.) was used in final protocol.

**Transformation procedure**

The competent cells (500 µl) were mixed with varied amounts of plasmid DNA (pHY300Car: 2, 5, 10, 20, 40, 100, 250, 500, 1000 ng) and incubated (37°C; 200 rpm for 30 min). In order to recover the cells, 300 µl of fresh LB medium was supplemented and competent *B. subtilis* DB104 were additionally incubated (37°C; 200 rpm for 30 min). 200 µl of *B. subtilis* DB104 cell suspension was subsequently spread on LB agar plates with selective antibiotic (tetracycline, final conc. 15 µg/ml).

3. RESULTS

The transformation efficiency represents a bottleneck in the directed evolution of proteases in *B. subtilis* DB104 as an expression host. All above mentioned methods were investigated and yielded in case of *B. subtilis* DB104 up to the 5 x 10$^3$ transformants per µg plasmid DNA using protoplast. Due to this, a highly efficient transformation method based on development of natural competence in *B. subtilis* DB104 was optimized for plasmid DNA. The developed protocol is based on transformation protocol for *B. subtilis* 168 strain employing chromosomal DNA (Anagnostopoulos and Spizizen 1961). Table 2.2 summarized optimization steps during competence development and transformation procedures. Protocol
optimization comprises steps like omitting centrifugation, adapting incubation times, optimization of cell growth as well as transformation times, and histidine concentration. Subsequently, the influence of plasmid DNA amount and size on transformation efficiency were investigated.

Table 2.2 Comparison of the developed plasmid transformation protocol for *B. subtilis* DB104 to the transformation protocol of Spizizen for *B. subtilis* 168 strain (Anagnostopoulos and Spizizen 1961)

<table>
<thead>
<tr>
<th>Spizizen’s protocol</th>
<th>Optimized protocol for <em>B. subtilis</em> DB104</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organism: <em>B. subtilis</em> 168</td>
<td>Organism: <em>B. subtilis</em> DB104</td>
</tr>
<tr>
<td>Overnight culture on an LB agar plate</td>
<td>Overnight culture in SM1 liquid medium</td>
</tr>
<tr>
<td>10^8 cells per ml inoculums cultivated in SM1 medium containing essential tryptophan (50 µg/ml)</td>
<td>Dilution in SM1 medium (no essential histidine amino acids in medium) to reach 10^7 cells per ml (OD₆₀₀ ~ 0.5)</td>
</tr>
<tr>
<td>Time of incubation: 4 h</td>
<td>Time of incubation: 3 h</td>
</tr>
<tr>
<td>Centrifugation of the cells</td>
<td>No centrifugation step</td>
</tr>
<tr>
<td>1:10 dilution in SM2 medium containing tryptophan (5 µg/ml)</td>
<td>1:1 dilution in SM2 medium containing histidine (200 µg/ml)</td>
</tr>
<tr>
<td>No additional incubation step in SM2 medium</td>
<td>Incubation for additional 2 h (37 ºC; 300 rpm without DNA; final volume 20 ml)</td>
</tr>
<tr>
<td>Chromosomal DNA added to the cell culture followed by incubation for 90 min (37ºC; rpm not defined, final volume 1 ml)</td>
<td>Plasmid DNA added to the cell culture followed by incubation for 30 min (37ºC; 200 rpm; final volume 0.8 ml)</td>
</tr>
<tr>
<td>No recovery step in LB medium</td>
<td>Recovery of the cells with 300 µl fresh LB medium for 30 min (37ºC; 200 rpm)</td>
</tr>
<tr>
<td>Plated on agar plates with selective antibiotic</td>
<td>Plated on agar plates with selective antibiotic</td>
</tr>
</tbody>
</table>

3.1. Optimization of time of the growth of *B. subtilis* DB104

*B. subtilis* develops its natural competence at the end of exponential growth phase with expression of the comK gene (Hamoen *et al* 2003). In order to determine the time required for the cells to enter into a stationary phase, the growth of *B. subtilis* DB104 in SM1 and SM2 medium was monitored. Overnight culture (37ºC; 200 rpm for 14-16 h) of *B. subtilis* DB104 in SM1 medium was diluted with SM1 medium until OD₆₀₀ reached a value of 0.5. *B. subtilis* DB104 cells were grown
Part II: An efficient transformation method for *B. subtilis* DB104

(37°C; 200 rpm) and aliquots (1 ml) were taken for OD$_{600}$ measurements every 30 min. Figure 2.1 shows the growth curve of *B. subtilis* DB104 in SM1 medium (closed square) reaching a stationary phase after 3 h. At the end of exponential phase (3 h growth time) the total cell culture volume was diluted 1:1 with SM2 medium and supplemented with varied histidine concentrations (final conc. 0, 10, 50, 200, 500, and 1000 µg/ml). Upon dilution with SM2 medium (SM1/SM2, open square) the end of exponential phase was reached after 2 h of growth. Different histidine concentrations show no detectable effect on growth of *B. subtilis* DB104. After optimization, *B. subtilis* DB104 cells reach a state of natural competence after a cultivation time of 3 h in SM1 and of 2 h in SM1/SM2 medium.

![Growth curve of *B. subtilis* DB104 in SM1 medium (■) and SM1/SM2 medium (□). Optical density (OD$_{600}$) monitored at 600 nm in function of time. The cells were grown (37°C, 200 rpm) and 1 ml aliquots were taken for OD$_{600}$ measurements every 30 min. In order to ensure that OD$_{600}$ reading is within the linear range (0.1-0.5) of accuracy of photometer the cell suspension was diluted prior to the measurement.](image)

**3.2. Influence of histidine concentration on transformation efficiency**

To investigate the dependence of different histidine concentrations on transformation efficiency of *B. subtilis* DB104, the concentration range of histidine as supplement to SM1/SM2 medium was varied (final conc. 0, 10, 50, 200, 500, and 1000 µg/ml). Figure 2.2 shows that concentrations of histidine up to 500 µg/ml transformation efficiency of *B. subtilis* DB104 varied in the range from $8.0 \times 10^4$ to $1.5 \times 10^5$. At higher concentrations of histidine (1000 µg/ml) the competence of *B.
subtilis DB104 decreased to $4.5 \times 10^4$. In the final protocol 200 µg/ml histidine was used.

![Transformation efficiency graph](image)

**Fig. 2.2** Transformation efficiency (transformants per µg plasmid DNA = cfu/µg) at varied concentrations of histidine supplemented (0, 10, 50, 200, 500 and 1000 µg/ml) to SM1/S2 medium. Error bars represent standard deviation between three triplicate experiments.

### 3.3. Dependence of different shuttle vectors on the transformation efficiency of *B. subtilis* DB104 cells

Three different shuttle expression vectors for *B. subtilis* (pC194, pHY300PLK, pHCMC04) were used for transformation of natural competent *B. subtilis* DB104 in order to investigate the dependence of vector size and antibiotic resistance on the transformation efficiency. The used plasmids were ranged from 2.9 to 8.1 kb [pC194 (2.9 kb), pHY300PLK (4.9 kb), pHY300Car (6.2 kb), pHCMC04 (8.1 kb)]. The highest number of transformants was obtained using pHY300Car ($1.5 \times 10^5$ transformants per µg DNA) (Table 2.3). The latter indicates that the employed vectors sizes do not determine the transformation efficiency. In case of the antibiotics (chloramphenicol and tetracycline) five to ten fold higher transformation efficiency could be obtained for the plasmids bearing tetracycline resistance cassette.

**Table 2.3** Influence of plasmid resistance (Cm<sup>R</sup>/Tc<sup>R</sup>) and size on the transformation efficiency of *B. subtilis* DB104

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Size (kb)</th>
<th>Phenotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Transformants per µg plasmid DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>pC194</td>
<td>2.9</td>
<td>Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>$1.3 \times 10^4$</td>
</tr>
<tr>
<td>pHY300PLK</td>
<td>4.9</td>
<td>Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>$1.2 \times 10^5$</td>
</tr>
<tr>
<td>pHY300Car</td>
<td>6.2</td>
<td>Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>$1.5 \times 10^5$</td>
</tr>
<tr>
<td>pHCMC04</td>
<td>8.1</td>
<td>Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>$2.2 \times 10^4$</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cm<sup>R</sup>—chloramphenicol resistance, Tc<sup>R</sup>—tetracycline resistance
3.4. Dependence of amount of plasmid DNA on the transformation efficiency of \textit{B. subtilis} DB104 cells

Transformation efficiencies of \textit{B. subtilis} DB104 cells were determined as a function of DNA amount (pHY300Car: 2, 5, 10, 20, 40, 100, 250, 500 and 1000 ng). Results presented in Figure 2.3 indicate a correlation between transformation efficiency and DNA amount (range of 2-1000 ng). An optimum was found in range between 5-10 ng of plasmid DNA. At amounts higher than 40 ng the transformation efficiency declines continuously from $7 \times 10^4$ to $4 \times 10^3$ transformants per µg plasmid DNA.

![Fig. 2.3](image)

**Fig. 2.3** Transformation efficiency (transformants per µg DNA = cfu/µg) in function of amount of DNA (ng). \textit{B. subtilis} DB104 cells were transformed with different amounts of plasmid DNA (pHY300Car: 2, 5, 10, 20, 40, 100, 250, 500 and 1000 ng). Error bars represent standard deviation between three triplicate experiments.

4. DISCUSSION

Advances in screening technologies with throughputs up to $10^8$ variants (Prodanovic \textit{et al} 2011; Tu \textit{et al} 2011) enable novel directed evolution strategies for instance with high mutational loads. Prerequisite are efficient transformation protocols to generate a sufficient number of variants. The latter is especially important for industrially used hydrolases (proteases and/or lipases) which have to be secreted into media for high level production. \textit{B. subtilis} DB104 represents an attractive directed evolution host due to its low proteolytic activity and efficient secretion.
Transformation protocols for *Bacillus* strains shown in Table 2.1 were investigated for *B. subtilis* DB104 and yielded a transformation efficiency of up to 5 x 10^3 variants per µg plasmid DNA (Table 2.1: No. 5). The latter protoplast method required after transformation a 3-day recovery phase and resulted for *B. subtilis* DB104 in a growth of high number of clones without plasmid DNA despite of antibiotic use. The obtained transformation efficiency and requirement of high DNA concentrations (µg range: Table 2.1: No.1, 2 and 5) made the physical and chemical transformation methods not suitable for directed evolution in *B. subtilis* DB104.

Based on Spizizen’s protocol for transformation of chromosomal DNA by natural competence into the *Bacillus* 168 strain a novel transformation protocol for *B. subtilis* DB104 was developed to transform plasmid DNA. After various optimizations [growth phase, histidine/DNA concentration, shuttle vectors (size, resistance/four constructs)] the highest transformation efficiency of *B. subtilis* DB104 was obtained by addition of plasmid DNA at the end of the exponential growth phase with efficiencies up to 1.5 x 10^5 transformants per µg DNA. The latter are two orders of magnitude higher compared to efficiency obtained after transformation of *B. subtilis* DB104 with any of the methods summarized in Table 2.1. The obtained result is in a concordance with a reported study of Dubnau (1991) that natural competence is developed at the end of exponential phase.

The robustness of the transformation method was determined by investigating whether supplementing essential histidine affects the growth rate and transformation efficiency of *B. subtilis* DB104. In addition, the dependence of employed plasmid system and the amount of transformed plasmid DNA on transformation efficiency was determined. Varied histidine concentrations showed no effect on growth curve of *B. subtilis* DB104 but influenced transformation efficiency (Fig. 2.2). A histidine concentration of 200 µg/ml was finally selected for *B. subtilis* DB104 transformation. Table 2.3 shows that the transformation efficiency of *B. subtilis* DB104 does not depend on the plasmid size. For instance the empty vector pHY300PLK (4.9 kb) and harboring a Carlsberg protease gene (pHY300Car; 6.2 kb) have a similar transformation efficiency (Table 3: 1.2 vs. 1.5 x 10^5 variants per µg plasmid DNA). These results can be explained by differences in DNA uptake between natural competent and physically treated (voltage) *Bacillus* cells (Brigidi et al 1990). In case of natural competence uptake the double stranded DNA is first digested to single stranded DNA by NucA nuclease (Hamoen et al 2003), taken up through pilin-like
structures at the *Bacillus* surface and transported as single stranded DNA across the membrane, complementary strand synthesis and nick repair (Dubnau 1999; Kidane et al. 2009). Interestingly the transformation efficiency for the tetracycline resistant shuttle vectors (pHY300PLK, pHY300Car) is 5-10-fold higher compared to the pC194 and pHCMC04 vectors which use chloramphenicol for selection.

The natural competence transformation protocols allows in contrast to many other artificial transformation protocols (Table 2.1: No 1, 2, and 5) transforming efficiently low amounts of plasmid DNA. An optimum was found in range between 5-10 ng of plasmid DNA (Fig. 2.3) and can likely be attributed to the number of specific receptors (~ 50) (Dubnau 1999) on the *Bacillus* surface which can be regarded as limiting factor for the uptake of high amounts of plasmid DNA.

In summary, a transformation protocol was developed for *B. subtilis* DB104 as an expression host in directed protease evolution experiments. The natural competence protocol allows for the first time to generate large libraries (~ 10^5 transformants per µg plasmid DNA) in *B. subtilis* DB104 and is furthermore robust, fast and simple in handling. The current protocol could be extended to other *Bacillus* strains and might be used to encourage researchers to perform directed evolution experiments employing *Bacillus* species as expression hosts.
Part III: Directed evolution of SC Perhydrolase for oxidative stability

Part III: DIRECTED EVOLUTION OF A DOUBLE MUTANT SUBTILISIN T59A/L217W (SC PERHYDROLASE) TOWARDS INCREASED OXIDATIVE STABILITY

1. INTRODUCTION

1.1. Aim of this work

The aim of this work was to improve an industrially important double mutant of subtilisin Carlsberg Thr59Ala/Leu217Trp (SC Perhydrolase) by means of directed evolution towards higher oxidative stability in the presence of peroxycarboxylic acids while keeping its perhydrolytic activity unaffected. A second objective was to study the amino acid substitutions identified in the improved variants in order to investigate the possible structure-function relationships involved in this adaptation. The chosen approach was the construction of site saturation mutagenesis libraries targeted at positions susceptible to oxidation. A microtiter plate expression and proteolytic assay systems were adapted for high throughput screening for increased residual activity in the presence of peroxycarboxylic acids.

1.2. Oxidative stability of peroxycarboxylic acid producing enzymes

Due to their abundance in nature, proteins are a major target for oxidants (peroxides, hypochlorous acid, and singlet oxygen). Susceptibility of proteins to oxidation is a primary source of their inactivation and denaturation (Weng et al 2009). The studies on oxidation of amino acids residues are reported by Dakin (1906). All 20 natural amino acids have potential reaction sites for oxidation. Excluding Met and Cys, aliphatic amino acids are susceptible to oxidation only by the most reactive radicals such as HO\(^*\) (Davies 2005). Amino acids susceptible to oxidation are Met, Cys and Trp directly affecting enzyme activity when located in or around the active site (Estell et al 1985). Oxidations of the surface-exposed Met residues result in a mixture of R- and S-isomers of methionine sulfoxide (Stadtman et al 2003). Additionally, methionine sulfoxide can be, in a lesser extent, further oxidized to form methionine sulfonate. Besides hydrogen peroxide, a number of other agents can oxidize Met, some examples are periodate, azide,
Part III: Directed evolution of SC Perhydrolase for oxidative stability

tetranitromethane N–bromosuccinimide, and 2,4,5-tribromocyclohexadienone, (Shechter et al 1975).

The phenomenon of enzyme inactivation by oxidants has been investigated by a number of research groups. One of the first enzyme models was chymotrypsin, whose modification was performed photochemically and chemically. The oxidation of a Met residue in vicinity of the active site resulted in an increased $K_M$ value for the studied ester substrate (Stauffer and Etson 1969). The oxidation of Met residues in lysozyme and ribonuclease A reported by Jori et al (1968) drastically reduced their enzymatic activity. Met residue was replaced using site directed mutagenesis with non-oxidizable amino acids such as Ala, Ser or Gln to improve their resistance to the oxidative agents in detergent formulation. This substitution resulted in variants retaining their activity in the presence of 1 M hydrogen peroxide (Estell et al 1985).

Oxidation of Trp potentially yields 16 possible compounds that are classified in pyrole-, phenyl-, or alanyl-moiety (Simat and Steinhart 1998). It was experimentally proven by Simat et al that during Trp oxidation, the pyrole ring is first attacked and subsequently a second reaction step takes place in the phenyl moiety. To understand this mechanism, the oxidation of free and protein bound Trp by hydrogen peroxide was examined. It is reported that the rate of oxidation in protein bound Trp is slower compared to oxidation of the free amino acid. Possible explanations for this phenomenon include I) steric hindrance, (protected Trp residues since they are buried among other amino acids), II) no chain reaction between oxidized products, or III) the lack of availability of carboxyl and amino groups of protein-bound Trp (De Weck et al 1987). The oxidation of Trp residue and its consequences were investigated in lysozyme. One out of the 6 available Trp residues was oxidized, resulting in a loss of enzymatic activity (Kuroda et al 1975). The oxidation of Trp by N–bromosuccinimide was performed in a nuclease isolated from Staphylococcus sp. (Omenn et al 1970). The oxidized enzyme has a reduced activity of around 80 %. Besides all these reports on oxidation of Trp residues the mechanism and influence of the different product of oxidation on enzyme activity is still not completely understood.

Subtilisins constitute the most important group of enzymes used in detergents (Maurer 2004). Their effective use faces several challenges such as high temperature, alkalinity, the presence of ionic and non–ionic detergents surfactants, and the oxidative agents. In order to use subtilisins in an industrial scale, it is
necessary to tailor them to withstand these conditions. The effect of hydrogen peroxide oxidation in subtilisin Carlsberg was investigated (Stauffer and Etson 1969). Out of 5 Met residues present in the protein only one, at the position 222, was oxidized resulting in a dramatically decreased activity. The Met residue at position 222 is conserved along subtilisins and it is the main target for engineering towards increased residual activity in the presence of oxidative agents. Despite the fact that oxidation of this conserved Met residue close to the active site in most subtilisins leads to its inactivation, it has been described that a member of the subtilisin-like alkaline serine proteases, named KP-43, isolated from Bacillus KSM, exhibits an increased resistance towards hydrogen peroxide (Nonaka et al 2004). The crystal structure of the protease was published in 2004, showing 120 additional residues in C–terminus. Due to its increased oxidative stability KP–43 has been incorporated into laundry detergents (Saeki et al 2007). In order to investigate the reasons behind this oxidative resistance, the oxidized and non-oxidized forms of KP-43 were crystallized. The oxidized structure showed methionine sulfoxide formation as in subtilisin proteases, still retaining proteolytic activity.

A recently described subtilisin Carlsberg double mutant Thr59Ala/Leu217Trp (SC Perhyrdolase) showed an increased level of perhydrolysis using ester substrates and hydrogen peroxide (Wieland 2009) to generate peroxycarboxylic acids. Since peroxycarboxylic acids are used as bleaching/oxidative agents in cleaning compositions, engineering of SC Perhydrolase towards oxidative stability would extend current and open a new application field for this enzyme in detergent industry. Previous reports on engineering of subtilisin proteases to increase their oxidative stability, the characterization of a naturally oxidative resistant subtilisin-like protease (KP-43), combined with reviewing the chemistry behind the common pathway of amino acid oxidation provided a good starting point for studying and ultimately increasing the oxidative stability of SC Perhydrolase by means of directed evolution.
Part III: Directed evolution of SC Perhydrolase for oxidative stability

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.2. Plasmids

The plasmid used in this work was pHY300PLK *E.coli-Bacillus subtilis* shuttle vector (Takara Bio Inc., Shiga, Japan) with a size of 4870 bp. This vector has ampicillin resistant gene and tetracycline resistant gene (Ap<sup>R</sup>, Tc<sup>R</sup>) as selective marker. Both genes can be expressed in *E. coli*, but only tetracycline resistant gene can be expressed in *B. subtilis*.

2.1.1. Chemicals

All chemicals were of analytical reagent grade or higher quality and purchased from Sigma-Aldrich (Taufkirchen, Germany), Applichem (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany) or Invitrogen (Darmstadt, Germany). All enzymes were purchased from New England Biolabs (Frankfurt, Germany), Fermentas GmbH (St. Leon-Rot, Germany) or Sigma-Aldrich. Protease inhibitor PMSF was purchased from Sigma-Aldrich.

2.1.3. Bacterial strains

The bacterial strains used in this work are summarized in Table 3.1.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>F'/endA1 hsdR17(rK-mK+) supE44 thi-1 recA1 gyrA (Nalr) relA1 D(lacZYA-argF)U169 deoR(F80dlacD(lacZ)M15)</td>
<td>Stratagene (Waldbronn, Germany)</td>
</tr>
<tr>
<td><em>B. subtilis</em> DB104</td>
<td>nprE aprE</td>
<td>(Kawamura and Doi 1984)</td>
</tr>
</tbody>
</table>

2.1.4. Oligonucleotides

The oligonucleotides used in this work are summarized in Table 3.2. All variants mentioned in this work were numbered according to Protein Data Bank entry 1yu6.
Table 3.2. The oligonucleotides used in this work

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’–3’)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC Per_seq_FW</td>
<td>GAACGTAGTGGCGCGAGCAAG</td>
<td>Sequencing primer for T59A/L217</td>
</tr>
<tr>
<td>SC Per_seq_RP</td>
<td>GTGAAGCTGAAGGATCGGATG</td>
<td>Sequencing primer for T59A/L217W</td>
</tr>
<tr>
<td>SSM_Met 222_FW</td>
<td>CATGGAACGGAACGTCAANNKGGCTCTTCTTCATGAGC</td>
<td>SSM of T59A/L217W</td>
</tr>
<tr>
<td>SSM_Met 222_RV</td>
<td>GCTACATGAGGAGAAGCMNNTGAGTCCGTTCCATG</td>
<td>SDM of T59A/L217W</td>
</tr>
<tr>
<td>SSM_Trp217_FW</td>
<td>GCCATTGACGTTCCGTTNNTGGTGCATAATGTTTC</td>
<td>SDM of T59A/L217W</td>
</tr>
<tr>
<td>SSM_Trp217_RV</td>
<td>CGAACACTATGCAACANNAACGGAACGTCAATGG</td>
<td>SDM of T59A/L217W</td>
</tr>
<tr>
<td>SDM_Met222Ser_FW</td>
<td>CATGGAACGGAACGTCAANNKGGCTCTTCTTCATGAGC</td>
<td>SDM for generation of T59A/W217M/M222S</td>
</tr>
<tr>
<td>SDM_Met222Ser_RV</td>
<td>GCTACATGAGGAGAAGCTGATGACGTTCCGTTCCATG</td>
<td>SDM for generation of T59A/W217M/M222S</td>
</tr>
<tr>
<td>SDM_Met222Cys_FW</td>
<td>CATGGAACGGAACGTCAANNKGGCTCTTCTTCATGAGC</td>
<td>SDM for generation of T59A/W217M/M222C</td>
</tr>
<tr>
<td>SDM_Met222Cys_RV</td>
<td>GCTACATGAGGAGAAGCAGCATGACGTTCCGTTCCATG</td>
<td>SDM for generation of T59A/W217M/M222C</td>
</tr>
<tr>
<td>SDM_Trp217Leu_FW</td>
<td>CGAACACTTATGCAACATGACGTTCCGTTCCATG</td>
<td>SDM for generation of T59A/W217L/M222S and T59A/W217L/M222C</td>
</tr>
<tr>
<td>SDM_Trp_217Leu_RV</td>
<td>GCCATTGACGTTCCGTTCAAGTGTCATAAGTGTTC</td>
<td>SDM for generation of T59A/W217L/M222S and T59A/W217L/M222C</td>
</tr>
</tbody>
</table>

2.1.5. Cell culture media and cultivation

*E. coli* and *B. subtilis* cells were cultivated in 1.5 x Luria Broth (LB) medium supplemented with appropriate antibiotics using a shaking incubator (Multitron II, Infors GmbH, Einsbach, Germany) at 37°C, 250 rpm for 24 h. For cultivation on agar plates, LB skim milk agar was used. Antibiotics used for selection in liquid and solid media are listed in Table 3.3.

**1.5 x LB medium:** 1.5 % (w/v) peptone from casein, 0.75 % (w/v) yeast extract, 1 % (w/v) NaCl, 90 mM potassium phosphate buffer pH 7.6.
Part III: Directed evolution of SC Perhydrolase for oxidative stability

**Skim milk medium:** 1 \% (w/v) peptone from casein, 0.5 \% (w/v) yeast extract, 1 \% (w/v) NaCl, 1 \% (w/v) skim milk, 1.5 \% (w/v) agar.

| Table 3.3. Antibiotics used for cell culture in this work |
|-----------------|-----------------|-----------------|-----------------|
| Antibiotic      | Stock (mg/ml)   | Solvent         | Working conc.(µg/ml) |
| Ampicillin      | 100             | Water           | 100             |
| Tetracycline    | 15              | 50 \% Ethanol   | 15              |

2.2. METHODS

2.2.1. PCR and colony PCR

All PCRs were performed using a thermal cycler (Mastercycler gradient, Eppendorf AG, Hamburg, Germany). The amount of DNA in the experiments was quantified by using a NanoDrop photometer (ND-1000, NanoDrop Technologies, Wilmington, DE, USA). Colony PCR was performed with a slight modification of standard PCR using colony from agar plate as template. An additional 10 min at 95°C of denaturing step at beginning was applied for colony PCR.

2.2.2. Cloning

All gene cloning and manipulation steps were carried out according to the molecular cloning methods (Sambrook 2001). Preparation of *E. coli* competent cells and transformation were carried out as standard heat shock transformation (Inoue et al 1990). Double mutant subtilisin Carlsberg Thr59Ala/Leu217Trp (SC Perhydrolase) gene along with its pre–pro sequence including the *Bacillus* promoter was cloned into pHY300PLK shuttle vector (Takara Bio Inc, Shiga, Japan) by using XbaI and BamHI restriction sites. The generated construct was named pHY300SCPer (6221 bp). Plasmids were verified by PCR, restriction enzyme digestion and sequence analysis. Sequence results were analysed by Vector NTI 9 (Invitrogen). Plasmid isolation, gel purification and PCR purification Mini prep kit were purchased from Qiagen (Hilden, Germany).

The cloned gene was subsequently transformed into chemical competent *E. coli* DHα cells and plated on LB agar plates containing ampicillin (final conc. 100 µg/ml).
Part III: Directed evolution of SC Perhydrolase for oxidative stability

A single colony was used for isolation of the plasmid using Qiagen Mini prep kit. The resulting plasmid was transferred into electrocompetent *Bacillus subtilis* DB104 cells and plated on LB skim milk agar plates with tetracycline as selective antibiotic (final conc. 15 µg/ml).

### 2.2.4. Protein expression-SDS and Experion analysis

After the transformation, colonies forming a clearance halo on skim milk agar plate were picked and inoculated in 4 ml 1.5 x LB media supplemented with tetracycline (final conc. 15 µg/ml). The cell culture was incubated overnight (37°C, 250 rpm for 24 h). After centrifugation, the cell culture supernatant was used for analysis of an extracellular expressed protein. Trichloroacetic acid (TCA) precipitation and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) analysis protein were carried out according to the standard protocol described (Sambrook 2001).

The Experion system (Experion Pro260, Bio-Rad, München, Germany) employs LabChip® microfluidic technology to automate electrophoresis for protein and RNA analysis. 80 µl of the supernatant from the cell culture was precipitated with TCA, the pellet was dissolved in 4 µl of 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer pH 8.0 and the samples were prepared for the electrophoresis.

### 2.2.5. Protein expression in microtiterplate

Single colonies of *B. subtilis* DB104 from LB skim milk agar plate were cultivated in 96-well flat bottom microtiter plates (Greiner, Frickenhausen, Germany) containing 150 µl of 1.5 x LB medium supplemented with 15 µg/ml tetracycline pH 7.6 and incubated (37°C, 900 rpm, 24 h, 70 % humidity; master plate). After 24 h, 12 % (final conc.) glycerol was added to the master plate and the plate was stored at -80°C. The expression was carried out by replicating the master plates into flat-bottom microtiter plates (expression plates) containing 200 µl of 1.5 x LB medium pH 7.6 and 15 µg/ml tetracycline and incubating (37°C, 900 rpm, 22 h, 70% humidity). After expression, the microtiter plates were centrifuged at 3220 g during 30 min at 4°C and the supernatant was transferred to a new microtiter plate for screening.
Part III: Directed evolution of SC Perhydrolase for oxidative stability

2.2.6. Proteolytic activity: Suc-AAPF-pNA

Proteolytic activity was determined using the synthetic peptide substrate succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide (suc-AAPF-pNA) by quantification of the release of free p-nitroaniline (pNA) at 410 nm (DelMar et al 1979). The concentration of the peptide substrate was determined using an extinction coefficient of $\varepsilon_{315} = 14000/M \times cm$ (DelMar et al 1979). For the screening in microtiter plate, the assay conditions were: 5 µl of 10 times diluted cell supernatant, 0.2 µl suc-AAPF-pNA substrate (110 mM) in sodium phosphate buffer (100 mM, pH 7.5; in final volume 100 µl). Absorbance was monitored at 410 nm for 11 cycles with the cycle interval of 30 seconds.

2.2.7. Site saturation mutagenesis and site-directed mutagenesis of SC Perhydrolase

Site saturation mutagenesis (SSM) and site directed mutagenesis (SDM) of SC Perhydrolase was performed according to the published method (Wang and Malcolm 2001). The oligonucleotides for SSM and SDM of SC Perhydrolase are listed in Table 3.2. The procedure consists of two steps. In step one, two extension reactions were performed in a separate tubes; one containing the forward primer and the other containing the reverse primer. Subsequently, the two reactions were mixed in the second step. For the mutagenic PCR, conditions were as follows:

**First step:** 98°C for 30 min, one cycle; 98°C for 10 s, 55°C f or 30 sec, 72°C for 3 min, three cycles.

**Second step:** 98°C for 30 sec, one cycle; 98°C for 10 s, 55°C f or 1 min, 72°C for 3 min, 15 cycles; 72°C for 3 min, one cycle, Pfu DNA polymerase (1U), dNTP mix (0.2 mM), each primer (0.4 µM) together with plasmid template (50 ng) were used in 50 µl reaction volume.

Following the PCR, DpnI (20 U) was added, and the mixture was incubated at 37°C overnight. After inactivation of DpnI sample was purified using Qiagen kit and transformed into chemical competent *E. coli* DH5α strain. After cultivation of the cells in the liquid LB medium at 37°C, 250 rpm, overnight plasmid was isolated using Qiagen Miniprep Kit. Isolated plasmid is transferred into naturally competent *B. subtilis* DB104 cells for the expression and analysis (see Part II). Inserted mutations were verified by sequencing (sequencing primers listed in Table 3.2).
2.2.8. Screening for improved oxidative stability using Suc-AAPF-pNA assay

Spectrophotometric suc-AAPF-pNA assay was used to test activity of the protein before and after incubation with peroxyacetic acid (peroxyacetic acid solution 32 wt % in diluted acetic acid, Sigma-Aldrich, Taufkirchen, Germany). For the screening different site saturation mutagenesis libraries assay conditions were: 20 µl supernatant of \( B. \ subtilis \) DB104 was incubated in peroxyacetic acid (final conc. 0.013 % v/v) for 20 min. The assay conditions for the proteolytic activity were: suc-AAPF-pNA (0.22 mM) in sodium phosphate buffer (100 mM, pH 7.5; final volume 100 µl). The proteolytic activity of the treated supernatant was then determined and compared to the activity of untreated supernatant and residual activity was calculated. Residual activity (%) was defined as the activity of the treated sample in Absorbance Unit per min (AU/min) divided by the activity of the untreated sample, multiplied by factor 100.

2.2.9. Protein purification

Shaking flasks (1 l) containing LB medium (250 ml) supplemented with tetracycline (final conc. 15 µg/ml) were inoculated with a 1:250 dilution of overnight culture (\( B. \ subtilis \) DB104) harboring pHY300Car or variants. After 60 h of expression, cell biomass was removed by centrifugation (Sorvall 4°C; 8000 g; 30 min). The resulting supernatant was filtered using a glass fiber filter (#30; Schleicher & Schuell Microscience, Dassel, Germany) and using a Polyethersulfone (PES) membrane filter (0.2 µm; Sartorius, Göttingen, Germany). The supernatant was concentrated to 10 ml using a Millipore ultra-filtration stirred cell with a 10 kDa cut-off (regenerated cellulose ultra-filtration membrane; Millipore, Schwalbach, Germany). The concentrated supernatant was diluted up to 200 ml in HEPES buffer (10 mM, pH 7.0) and concentrated to 10 ml. Subtilisin Carlsberg and variants were purified by anion and subsequent cation exchange chromatography. The concentrated supernatant was loaded onto a Toyopearl Super Q 650c (TOSOH Bioscience GmbH, Stuttgart, Germany) anion-exchange column (ID/Length 15 x 125 mm) pre-equilibrated with running buffer 1 (HEPES, 10 mM, pH 7.0). The flow rate and pressure were managed by an ÄKTA prime system (Amersham, GE Healthcare Europe, Freiburg, Germany) using the Unicorn software package. Subtilisin Carlsberg and variants did not bind to the anion exchange column in contrast to many impurities and were collected in the flow through (buffer 1; flow rate
Part III: Directed evolution of SC Perhydrolase for oxidative stability

of 3 ml/min). Collected subtilisin Carlsberg and variants were loaded on a Toyopearl Super SP-650c (TOSOH Bioscience GmbH, Stuttgart, Germany) cation-exchange column (ID/Length 15 x 125 mm) which was pre-equilibrated with running buffer 2 (HEPES, 20 mM, pH 7.0). Subtilisin Carlsberg and variants were eluted with 4 % NaCl in running buffer 2 (flow rate 1.5 ml/min). Purified samples were concentrated [Amicon ultra-4 centrifugal filter device (Millipore); 10 kDa cut-off membrane], the total protein content determined (BCA assay kit; Pierce, Born, Germany), and the homogeneity analysed by an Experion Pro260 Analysis Kit (Bio-Rad, Munich, Germany).

2.2.10. Determination of kinetic parameters for suc-AAPF-pNA as an artificial proteolytic substrate

After the purification of the selected variants, the kinetic characterization was performed using the synthetic tetrapeptide suc-AAPF-pNA in the range of concentrations from 0.05 to 3 mM in sodium phosphate buffer (100 mM, pH 7.5). The reaction was started by adding 5 µl of the enzyme (final conc. 0.3 nM, 0.5 nM, and 1.4 nM); the increase of absorbance was recorded at 410 nm during 5 min of reaction. Released pNA concentration was calculated using $\varepsilon_{410} = 8800/M \times cm$ as recommended by the manufacturer (Sigma-Aldrich, Taufkirchen, Germany). The initial velocity data was fitted using GraphPad Prism software (GraphPad software, San Diego, CA, USA; hyperbolic fitting). The $k_{cat}$ was calculated from the ratio of $V_{max}$ and protease concentration

2.2.11. Residual activity assay by peroxyacetic acid inactivation: PS$_{50}$ assay

Peroxyacetic acid inactivation was monitored by incubating the enzyme (final conc. 0.5 nM) in different concentrations of peroxyacetic acid in sodium phosphate buffer (100 mM, pH 7.5). The range of concentration of peroxyacetic acid was from 0–40 mM. Residual activity was measured by adding 5 µl of the treated enzyme solution into 95 µl of sucAAPF-pNA (2 mM) solution; the increase of absorbance was recorded at 410 nm during 2.5 min of reaction. Residual activity versus activity without peroxyacetic acid was calculated for each variant.
2.2.12. Perhydrolytic activity: Fluorescent APCC assay determination of kinetic parameters for perhydrolytic activity with methylbutyrate-perhydrolytic activity assay

A highly sensitive fluorescent assay (µM range) which suits needs of high throughput screening systems for identification and quantification of the enzymes with perhydrolytic activity was employed in this work. Figure 3.1 shows the APCC-detection system for quantifying peroxycarboxylic acid concentrations. Firstly, sodium bromide is oxidized to hypobromite by peroxycarboxylic acid which is converted into a carboxylic acid. Subsequently, the fluorogenic substrate 7-(4'-aminophenoxy)-3-carboxy coumarin (APCC) is O-dearlylated by hypobromite, releasing the fluorescent 7-hydroxycoumarin-3-carboxylic acid (HCC) and 4-imino-2,5-cyclohexadien-1-on. The maximum wavelengths for excitation and emission of the generated product are 360 nm and 465 nm, respectively.

![Fig. 3.1 Schematic representation of APCC assay](image)

The $k_{cat}$ and $K_M$ values were determined from initial velocity data measured as a function of methyl-butyrate concentration at 30°C in 96-well microtiter plates (black, flat-bottomed, polystyrene plates; Greiner Bio-One GmbH, Frickenhausen, Germany). After 10 min of pre-incubation at 30°C, the perhydrolytic reaction was initiated by the addition of purified protease or variant (0.93 nM), to the substrate solution containing methylbutyrate (10-200 mM), hydrogen peroxide (100 mM), sodium bromide (100 mM) and APCC (0.5 mM) in sodium phosphate buffer (100 mM, pH 7.5; final volume 100 µl). Fluorescence was measured at 360 nm (ex.)
and 465 nm (em.) (gain 100; 30°C) using an Infinite M1000 (Tecan Group AG, Zürich, Switzerland). The initial velocity data was fitted using GraphPad Prism software (GraphPad software, San Diego, CA, USA; hyperbolic fitting). The $k_{\text{cat}}$ was calculated from the ratio of $V_{\text{max}}$ and protease concentration.

### 2.2.13. Computational analysis

Computational analyses were performed using YASARA version 11.9.18 in combination with Force Filed AMBER03. The initial structure was based on the X-ray crystal structure of subtilisin Carlsberg (Protein Data Bank entry 1yu6). The residue Ser 221 had net charge of -1.00. The partial charges of the atoms were: $\text{OG} = -0.50017$, $\text{O2} = -0.36122$ and $\text{O6} = -0.41595$. The three different conformations resulting in the three different hydrogen bonds ($\text{N}\varepsilon\text{-H of His 64 and side chain OG atom of Ser 221, N}\varepsilon\text{-H of His 64 and O2 from peroxy group, N}\varepsilon\text{-H of His 64 and O6 from peroxy group}$) were simulated for each variant. The energies for involved residues (Asp 32, His 64, Asn 155, Leu/Trp 217, Ser 221, Met 222) in each hydrogen bond conformation were calculated for each variant.

### 3. RESULTS

In this work, directed evolution of a double mutant of subtilisin Carlsberg Thr59Ala/Leu217Trp (SC Perhydrolase) was performed to increase its oxidative stability in the presence of peroxycarboxylic acids. The identified residues from two site saturation mutagenesis libraries were combined by site directed mutagenesis and the selected variants were characterized.

#### 3.1. Directed evolution to improve residual activity in the presence of peroxyacetic acid

##### 3.1.1. Cloning and expression

The SC Perhydrolase gene including the promoter was subcloned from the provided Bacillus expression vector into pHY300PLK shuttle vector, generating the pHY300SCPPer expression plasmid. XbaI and BamHI restrictions enzymes were used (Fig. 3.2). Sequencing results confirmed successful construction of the plasmid. After transformation in electrocompetent *B. subtilis* DB104, the cells were cultivated on LB skim milk agar plates. After overnight growth, clear zones around colonies were formed, indicating expression of an active protease. Additionally, protein expression
was confirmed by measuring proteolytic activity in the supernatant using suc-AAPF-pNA (described in Part III, section 2.2.6).

3.1.2. Optimization of suc-AAPF-pNA based screening system

The proteolytic activity of a neutral and alkaline proteases can be determined and quantified by using an artificial substrate, succinyl-L-Ala-L-Ala-L-Pro-L-Phe-pNA (suc-AAPF-pNA) (DelMar et al 1979). The enzyme activity is quantified via the release of para-nitroaniline (pNA) which is monitored as an increase of the absorbance at 410 nm. The assay advantages are a high conversion rate and a low substrate concentration (final conc. 0.22 mM). The assay was optimized for SC Perhydrolase, having a standard deviation value of 18 % (Fig. 3.3).
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**Fig. 3.3** Validation of the suc-AAPF-pNA screening system. The assay was performed by using 5 µl of 10 times diluted cell supernatant, 0.2 µl suc-AAPF-pNA substrate (110 mM) in sodium phosphate buffer (100 mM, pH 7.5 in 100 µl final volume). The absorbance was monitored at 410 nm for 11 cycles with an interval of 30 seconds (5.5 min). The standard deviation was 18%.

In order to improve the stability of SC Perhydrolase in the presence of peroxycarboxylic acids, the screening conditions were established and optimized. Activity of the protease was measured before and after incubation in buffer containing peroxycetic acid. Different concentrations of peroxycetic acid were tested. The used concentration range of peroxycetic acid was from 0.0016 to 0.26 % (v/v) (0.2 to 32.5 mM). SC Perhydrolase lost activity after 20 min of incubation at high concentrations (> 0.026 %, 3.2 mM) therefore concentrations not higher than 0.013 % (1.7 mM) were used. After 20 min of incubation the remaining proteolytic activity was measured (Table 3.4).

**Table 3.4.** The proteolytic activity of SC Perhydrolase measured after 20 min of incubation of the cell culture supernatant at different concentration of peroxycetic acid (% v/v)

<table>
<thead>
<tr>
<th>Peroxycetic acid (% v/v)</th>
<th>Product formation (mU/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.350</td>
</tr>
<tr>
<td>0.0032</td>
<td>0.267</td>
</tr>
<tr>
<td>0.0064</td>
<td>0.217</td>
</tr>
<tr>
<td>0.013</td>
<td>0.083</td>
</tr>
</tbody>
</table>
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![Graph showing correlation between product increase formation and concentration of peroxyacetic acid.](image)

**Fig. 3.4** Correlation between pNA release and incubation in the presence of different concentrations of peroxyacetic acid (20 min incubation time). Optimized screening conditions were: **Incubation mixture:** 20 µl supernatant of *B. subtilis* DB104 was incubated with peroxyacetic acid (final conc. 0.013 %, 1.7 mM). The assay conditions for **proteolytic activity** —suc-AAPF-pNA (0.22 mM) in sodium phosphate buffer (100 mM, pH 7.5, 100 µl final volume)

After 20 min of incubation with peroxyacetic acid (0.013 %, 1.7 mM) residual activity* of SC Perhydrolase was 23 %. These conditions were used for screening site saturation mutagenesis libraries since they enable the detection and discrimination of residual activity of variants up to 5 times increase compared to SC Perhydrolase (Fig. 3.4).

### 3.1.3. Generation of site saturation mutagenesis libraries

#### 3.1.3.1. Site saturation mutagenesis at position Met 222

Previous studies (Estell *et al* 1985) have implicated Met 222 as a primary site for oxidative inactivation in subtilisins. The treatment of the enzyme with the oxidative agents such as hydrogen peroxide and/or peroxycarboxylic acids leads to inactivation that correlates directly with the production of methionine sulfoxide at the position 222, which is next to the catalytic Ser 221 (Fig. 3.5).

*Residual activity—activity of the enzyme that remains after 20 min of incubation with peroxyacetic acid compared to its activity before the treatment with peroxyacetic acid.
Site saturation mutagenesis at position Met 222 was performed according to the protocol described in Part III, section 2.2.7. The oligonucleotides used to introduce the amino acid substitutions are reported in Table 3.2. The pHY300SCPer was transformed into *B. subtilis* DB104 using a novel natural competence based transformation protocol (described in Part II). The screening of 270 clones was done under the conditions described in Part III, section 2.2.8. Eight different variants were identified and selected for re-screening. Detailed results of re-screening are shown in Table 3.5.

**Table 3.5.** The selected variants from SSM at position Met 222 tested for increased oxidative stability in the presence of 0.013 % peroxyacetic acid compared to SC Perhydrolase

<table>
<thead>
<tr>
<th>Variant</th>
<th>Proteolytic activity* (%)</th>
<th>Stability** (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1B10</td>
<td>30</td>
<td>300</td>
</tr>
<tr>
<td>P2D4</td>
<td>45</td>
<td>300</td>
</tr>
<tr>
<td>P3E10</td>
<td>35</td>
<td>300</td>
</tr>
<tr>
<td>P2F6</td>
<td>120</td>
<td>300</td>
</tr>
<tr>
<td>P2H4</td>
<td>120</td>
<td>270</td>
</tr>
<tr>
<td>P2H6</td>
<td>40</td>
<td>300</td>
</tr>
<tr>
<td>P2H7</td>
<td>20</td>
<td>250</td>
</tr>
<tr>
<td>P3B10</td>
<td>40</td>
<td>400</td>
</tr>
</tbody>
</table>

* Proteolytic (initial) activity is the proteolytic activity of variant before the treatment with 0.013 % PAA
** Stability is defined as residual activity of the variant divided with residual activity of SC Perhydrolase
The selected variants showed increased stability in the presence of peroxyacetic acid (0.013%) compared to SC Perhydrolase from 250 to 400%. Only two variants (P2F6 and P2H4) showed unaffected proteolytic activity, while the other six variants showed a decreased level of the proteolytic activity.

3.1.3.2. Sequencing results

Sequencing results of the selected variants were analysed using Vector NTI 9 (Invitrogen). Each of the variants showed the amino acid substitution at the position 222 (Table 3.6).

Table 3.6. The amino acid substitutions in the selected variants generated in SSM at the position Met 222

<table>
<thead>
<tr>
<th>Variant</th>
<th>Amino acid substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1B10</td>
<td>Met→Ala</td>
</tr>
<tr>
<td>P2D4</td>
<td>Met→Ser</td>
</tr>
<tr>
<td>P3E10</td>
<td>Met→Thr</td>
</tr>
<tr>
<td>P2F6</td>
<td>Met→Cys</td>
</tr>
<tr>
<td>P2H4</td>
<td>Met→Cys</td>
</tr>
<tr>
<td>P2H6</td>
<td>Met→Ser</td>
</tr>
<tr>
<td>P2H7</td>
<td>Met→Val</td>
</tr>
<tr>
<td>P3B10</td>
<td>Met→Ser</td>
</tr>
</tbody>
</table>

3.1.3.3. Protein analysis

The expression level of each SC Perhydrolase variant was analysed using the Experion system according to the protocol that is described in Part III section 2.2.4. Analysis of the chromatograms showed that there was no a significant difference between the expression level of all variants and of the parent (SC Perhydrolase) (Fig. 3.6).
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Fig. 3.6 Expression analysis of different protease variants* using Experion Pro260 BioRad system. SC Perhydrolase variants are highlighted in the red square; L—ladder, supernatant of the cells expressing 1—empty vector (pHY300PLK), 2—SC Perhydrolase (Thr59Ala/Leu217Trp), 3—P1B10 (Met222Ala), 4—P2D4 (Met222Ser), 5—P3E10 (Met222Thr), 6—P2F6 (Met222Cys), 7—P2H7 (Met222Val). The supernatant (80 µl) from the cell culture was precipitated with trichloroacetic acid (TCA) and the pellet was dissolved in 4 µl tris(hydroxymethyl)aminomethane (Tris) buffer (100 mM, pH 8.0). The samples were prepared for electrophoresis according to Experion Pro260 BioRad manual.

3.1.3.4. Determination of the proteolytic activity in the supernatant of selected variants (SSM Met 222) after incubation with higher concentration of peroxycetic acids

Selected variants from SSM Met 222 library were additionally tested by measuring its proteolytic activity after incubation for 20 min with peroxycetic acid in an increased concentration range from 0 to 0.072 % (v/v) (0 to 9.4 mM). The results are summarized in Fig. 3.7.

*All selected variants additionally include amino acid substitutions Thr59Ala/Leu217Trp
**Fig. 3.7** Determination of the proteolytic activity of the selected variants* after incubation with peroxycetic acid ranging from 0 to 0.072 % (v/v). SC Per = SC Perhydrolase (Thr59Ala/Leu217Trp), P1B10 (Met222Ala), P2D4 (Met222Ser), P3E10 (Met222Thr), P2F6 (Met222Cys), P2H7 (Met222Val)

Figure 3.7 shows that the proteolytic activity of SC Perhydrolase dramatically decreases with the increase of peroxycetic acid concentration during the incubation. After the incubation with 0.013 % peroxycetic acid the residual proteolytic activity of SC Perhydrolase is 20 %. The treatment with 0.072 % peroxycetic acid leads to complete inactivation of SC Perhydrolase. All selected variants showed increased resistance to peroxycetic acid compared to SC Perhydrolase. Variants P1B10 and P3E10 showed 50 % residual activity after incubation with 0.072 % peroxycetic acid whereas the remaining four variants showed a lower residual activity (ranging from 20 to 8 %).

*All selected variants additionally include amino acid substitutions Thr59Ala/Leu217Trp*
Variants P1B10, P3E10 and P2H7 showed a drastically decreased initial proteolytic activity of approximately 40% the value of SC Perhydrolase activity. The variant P2F6 showed a similar level of proteolytic activity to SC Perhydrolase while variant P2D4 has an initial activity of 80%. Since variants P2F6 and P2D4 showed increase in the residual activity and moderately decrease in the initial (proteolytic) activities compared to SC Perhydrolase they were selected for the further experimental work.

3.1.4. Site saturation mutagenesis at position Trp 217

Site saturation mutagenesis was performed at position Trp 217 since Trp is also susceptible to oxidation (Fig. 3.8).

![Catalytic triad and position Trp 217](image)

**Fig. 3.8** Catalytic triad (labeled in red) and position Trp 217 in proximity of active center (labeled in orange) in SC Perhydrolase

The protocol for site saturation mutagenesis is described in Part III section 2.2.7. The oligonucleotides for the generation of site saturation mutagenesis library at the position Trp 217 are listed in Table 3.2. In total 270 clones were screened, and two clones were selected for re-screening. The results are summarized in Table 3.7.
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Table 3.7. The rescreening data for the variants generated in SSM at the position Trp217

<table>
<thead>
<tr>
<th>Variant</th>
<th>Proteolytic activity* (%)</th>
<th>Stability** (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P4E10</td>
<td>280</td>
<td>200</td>
</tr>
<tr>
<td>P6C1</td>
<td>350</td>
<td>350</td>
</tr>
</tbody>
</table>

Variants P4E10 and P6C1 show increased initial and residual activities in the presence of 0.013 % peroxyacetic acid (incubation time 20 min) of approximately 3-fold measured in the supernatants when compared to SC Perhydrolase.

3.1.4.1. Sequencing results

Variants P4E10 and P6C1 were sequenced and analysed. In Table 3.8 the substitutions are listed.

Table 3.8. The amino acid substitutions in the selected variants generated in SSM at the position Trp217

<table>
<thead>
<tr>
<th>Variant</th>
<th>Amino acid substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>P4E10</td>
<td>Trp→Leu</td>
</tr>
<tr>
<td>P6C1</td>
<td>Trp→Met</td>
</tr>
</tbody>
</table>

The amino acid substitutions identified in SSM Trp 217 library were combined using site directed mutagenesis together with the identified substitutions from SSM Met 222 library to study the additive effect on increased oxidative stability.

3.1.5. Site directed mutagenesis to generate combined variants at positions 222/217

The identified amino acid substitutions from selected variants (identified in SSM libraries at positions Met 222 and Trp 217) that showed increased residual activity in the presence of peroxyacetic acid were combined. The experiment was performed to investigate possibility of additive effect on increased residual activity and to identify beneficial combinations. SDM was performed in variants P2D4 (Met222Ser)* and P2F6 (Met222Cys)* to introduce the amino acid substitution found in variant P4E10 (Trp217Leu)**. An additional SDM library was constructed for variant P6C1 (Trp217Met)** to introduce the amino acid substitutions found in variants P2F6 (Met222Cys)* and P2D4 (Met222Ser)*. The PCR products of the SDM were analysed and the generated plasmids were transformed into B. subtilis DB104 using

*Proteolytic (initial) activity is the proteolytic activity of variant before the treatment with 0.013 % PAA
**Stability is defined as residual activity of the variant divided with residual activity of SC Perhydrolase
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the transformation protocol as described in Part II. The supernatant of generated variants was used for testing of proteolytic and residual activities in the presence of 0.013%, 1.7 mM peroxyacetic acid employing suc-AAPF-pNA assay. The results are summarized in Table 3.9.

**Table 3.9.** Proteolytic activity and stability of selected variants (SSM Trp 217, SSM Met 222 and SDM Met/Trp 222/217)

<table>
<thead>
<tr>
<th>Variant</th>
<th>Proteolytic activity (%)</th>
<th>Stability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2F6 (Met222Cys)*</td>
<td>120</td>
<td>300</td>
</tr>
<tr>
<td>P2D4 (Met222Ser)*</td>
<td>40</td>
<td>350</td>
</tr>
<tr>
<td>P4E10 (Trp217Leu)**</td>
<td>280</td>
<td>200</td>
</tr>
<tr>
<td>P6C1 (Trp217Met)**</td>
<td>350</td>
<td>350</td>
</tr>
<tr>
<td>P3E10D4 (Trp217Leu/Met222Ser)**</td>
<td>60</td>
<td>240</td>
</tr>
<tr>
<td>P3E10F6 (Trp217Leu/Met222Cys)**</td>
<td>140</td>
<td>280</td>
</tr>
<tr>
<td>P3C1D4 (Trp217Met/Met222Ser)**</td>
<td>60</td>
<td>200</td>
</tr>
<tr>
<td>P3C1F6 (Trp217Met/Met222Cys)**</td>
<td>280</td>
<td>300</td>
</tr>
</tbody>
</table>

The preliminary results showed that the combination of substitutions from SSM Met 222 and SSM Trp 217 libraries did not have an additive effect on the oxidative stability. The variants generated by combining the beneficial mutations from two SSM libraries showed the same stability values as in variants having single substitutions. However, the initial (proteolytic) activity was affected. The introduction of substitutions Trp217Leu and Trp217Met into the variants with substitutions Met222Ser and Met222Cys increased the proteolytic activity compared to the variants with the single substitutions (Table 3.9).

3.1.6. Purification and characterization of selected variants

In order to perform a detailed characterization of the selected variants with improved residual activity a protein purification step was performed. The purification is a required step to eliminate the differences in expression levels in order to confirm the data obtained from the cell culture supernatant.

The first purification was performed using an existing protocol for ion-exchange chromatography previously used in our laboratory to purify subtilisin variants. Subtilisin Carlsberg and its variants were expressed in *B. subtilis* DB104 strain at 37°C for 60 h in 250 ml of 1.5 x LB media. Preparation of the samples is described in

* Variants P2D4 and P2F6 additionally have amino acid substitutions Thr59Ala/Leu217Trp
** Variants P4E10 and P6C1 additionally have amino acid substitution Thr59Ala
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detail in Part III section 2.2.9. For removing the impurities from the cell culture supernatant, an anion chromatography column was used. Subtilisin Carlsberg did not bind to this column at pH 7.8 and was collected on the buffer front fractions. One of the reported pI values 6.7 for subtilisin Carlsberg (Vitale and Gamulin 1975) and the theoretically calculated pI value (7.1) indicated that the protein should bind onto a cation exchange chromatography column at pH 7.8. The protein fractions showing protease activity were loaded onto a Toyopearl Super SP-650c (TOSOH) cation exchange chromatography column (20 ml) previously equilibrated with 40 mM HEPES pH 7.8. However, subtilisin Carlsberg did not bind under these conditions, thus an optimization of the purification strategy was required.

Optimization included changes of salt concentration of the buffer from 40 mM HEPES to 20 mM HEPES to reduce the ionic strength of the buffer and changes of pH from 7.8 to 8.6 and 7.0. All the parameter changes were performed to achieve a condition in which the protease would bind to the column matrixes. The decreasing salt concentration of the HEPES buffer at the same pH (7.8) did not result in a successful binding of the protease, where the elution profile was similar to the original chromatography protocol. Shifting the pH from 7.8 to 8.6 required a change in the buffer system from HEPES to Tris/HCl. The first step of purification remained the same, the protease sample was injected into a Super Q 650c (TOSOH) anion exchange column equilibrated with 20 mM HEPES pH 7.8 in order to remove proteins with pI value lower than 7.8. Not bound fractions with proteolytic activity (suc–AAPF-μNA assay) were pooled together and loaded again onto a super Q 650c (TOSOH) anion exchange column that was equilibrated using 20 mM Tris/HCl pH 8.6. The binding of the protease was achieved, however, residual protease activity was observed on the flow-through during the subsequent washing steps (Fig. 3.9), thus further optimization was necessary.
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Fig. 3.9 Super Q 650c (TOSOH) anion exchange chromatography profile. First peak (labeled active fractions) in the figure represents the flow through fractions with proteolytic activity. The second peak represents the protease active fractions eluted with 20 mM Tris pH 8.6

The last optimization step was focused on avoiding leakage of the protease in the first phase of chromatography (anion exchange). Super Q 650c (TOSOH) anion exchange chromatography column (20 ml) was this time equilibrated with 10 mM HEPES pH 7.0 and subtilisin Carlsberg was eluted since it was not bound to the matrix (Fig. 3.10A). The fractions that showed proteolytic activity were pooled together, concentrated to 2 ml and diluted up to 10 ml using distilled water to decrease conductivity (<1.5 mS/cm). This sample was loaded onto the second column [Toyopearl Super SP-650c (TOSOH) cation exchange] previously equilibrated with 20 mM HEPES pH 7.0. The retention of subtilisin Carlsberg was successfully confirmed by analyzing the proteolytic activity of the flowthrough. The elution was performed using a gradient of 20 mM HEPES, 1 M NaCl pH 7.0 with a protease release approximately at 40 mM NaCl (4 %) (Fig. 3.10B). The successful binding conditions indicate that the experimental isoelectric point of subtilisin
Carlsberg and its variants is below pH 7. The collected fractions were analysed on SDS-PAGE (Fig. 3.11).

**Fig. 3.10** Exchange chromatography profiles. A) Elution profile of subtilisin Carlsberg at Tocopearl Super Q 650c anion exchange column, B) Elution profile of subtilisin Carlsberg at Super SP-650c (TOSOH) cation exchange column
Fig. 3.11 SDS-PAGE analysis. A) Toyopearl Super Q 650c anion exchange column fractions 1–starting sample (subtilisin Carlsberg), 2–8–fractions not bound for the column (proteolytically active fractions), 9–10–fractions bound for Super Q column. B) Super SP-650c (TOSOH) cation exchange column fractions 1–sample applied on Super SP column, 2–4–fractions not bound for SP column, 5–9–fractions eluted from the SP column in the first peak (proteolytically active fractions, subtilisin Carlsberg marked in red square), 10–11–fractions eluted in the second peak
The optimized protocol for the purification of subtilisin Carlsberg (HEPES pH 7.0) was used for all variants (Fig. 3.12).

**Fig. 3.12** Scheme of optimized protocol for the purification of subtilisin Carlsberg and its variants

The total protein concentration of the purified samples was determined using BCA assay (Table 3.10). The purity was assessed using Experion electrophoresis to validate the protocol (Fig. 3.13). The purified fractions were stored as described in section 2.2.9.

**Table 3.10.** Calculated specific protein concentration for each variant after the purification

<table>
<thead>
<tr>
<th>Variant</th>
<th>Specific conc. (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subtilisin Carlsberg</td>
<td>1.08</td>
</tr>
<tr>
<td>SC Perhydrolase</td>
<td>0.69</td>
</tr>
<tr>
<td>M1:(Met222Ser)*</td>
<td>1.36</td>
</tr>
<tr>
<td>M2:(Met222Cys)*</td>
<td>1.67</td>
</tr>
<tr>
<td>M3:(Trp217Met/Met222Cys)**</td>
<td>1.28</td>
</tr>
<tr>
<td>M4:(Trp217Leu/Met222Cys)**</td>
<td>0.11</td>
</tr>
<tr>
<td>M5:(Trp217Met/Met222Ser)**</td>
<td>0.27</td>
</tr>
<tr>
<td>M6:(Trp217Leu/Met222Ser)**</td>
<td>3.30</td>
</tr>
<tr>
<td>M7:(Trp217Met)**</td>
<td>1.25</td>
</tr>
<tr>
<td>M8:(Trp217Leu)**</td>
<td>0.65</td>
</tr>
</tbody>
</table>

* Variant additionally has amino acid substitutions Thr59Ala and Leu217Trp
** Variant additionally has amino acid substitution Thr59Ala
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Fig. 3.13 Expression analysis of purified SC Perhydrolase and its variants using Experion Pro260 BioRad system. 1–SC Perhydrolase, 2–M1 (Met222Ser)*, 3–M2 (Met222Cys)*, 4–M3 (Trp217Met/Met222Cys)**, 5–M4 (Trp217Leu/Met222Cys)**, 6–M5 (Trp217Met/Met222Ser)**, 7–M6 (Trp217Leu/Met222Ser)**, 8–M7 (Trp217Met)**, 9–M8 (Trp217Leu)**

3.1.7. Inactivation by peroxyacetic acid: PS$_{50}$ value

The concentration of peroxyacetic acid in which proteolytic activity of the variant is decreased to 50 % after 20 min incubation was defined as PS$_{50}$ value. A detailed protocol is described in section 2.2.11. The range of peroxyacetic acid concentration was from 0 to 40 mM. Figure 3.14 summarizes the PS$_{50}$ values of the variants higher than PS$_{50}$ value of SC Perhydrolase. Four variants showed improved PS$_{50}$ values compared to the parent (SC Perhydrolase), where the variant M6 showed improvement of 4.5 times (3.6 mM compared to 0.8 mM for SC Perhydrolase).

* Variant additionally has amino acid substitutions Thr59Ala and Leu217Trp
** Variant additionally has amino acid substitution Thr59Ala
Fig. 3.14 PS\textsubscript{50} determination and comparison with SC Perhydrolase. Five variants showed increased PS\textsubscript{50} value for 1.8, 2.5, 3.5, 4.2 and 4.5 times compared to SC Perhydrolase. The highest increase (M6) is 4.5 times in respect to the parent.

In addition, the selected variants with increased PS\textsubscript{50} value in respect to parent (SC Perhydrolase) were compared to the wild type (subtilisin Carlsberg) as it is shown (Fig. 3.15). The variants M6, M7, M1 and M5 showed increased PS\textsubscript{50} values for 2.4, 2.3, 1.9 and 1.3 times, respectively. Subtilisin Carlsberg showed 1.8 times increase in PS\textsubscript{50} value compared to SC Perydrolase.

* Variant additionally has amino acid substitutions Thr59Ala and Leu217Trp
** Variant additionally has amino acid substitution Thr59Ala
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Fig. 3.15 PS$_{50}$ determination and comparison with subtilisin Carlsberg. Four variants showed increased PS$_{50}$ for 1.3, 1.9, 2.3, and 2.4 times compared to subtilisin Carlsberg. The highest increase (M6) is 2.4 times in respect to the wild type subtilisin Carlsberg

### 3.1.8. Proteolytic activity characterization using the suc-AAPF-pNA substrate

The kinetic parameters for the proteolytic activity of the selected and purified variants with increased PS$_{50}$ value were determined by using suc-AAPF-pNA assay (DelMar et al 1979). A detailed protocol is described in section 2.2.10. The calculated kinetic parameters for SC Perhydrolase and selected variants with improved PS$_{50}$ are summarized in Fig. 3.16. It has to be considered that characterization using small synthetic substrates (tetrapeptides) does not necessarily represent behavior of the variants towards more complex substrates and full-sized proteins. Despite this, suc-AAPF-pNA is a widely used substrate to characterize proteolytic activity and allows the characterization data to be compared with similar studies. All selected variants have increased $K_M$ values compared to SC Perhydrolase. The $k_{cat}$ value of variant M1 is similar to $k_{cat}$ value measured for SC Perhydrolase, while variants M4**, M5**, M6**, and M7** have an increased $k_{cat}$. M4** showed the highest increase of 5.8 times followed by variants M5**, M6** and M7**

* Variant additionally has amino acid substitutions Thr59Ala and Leu217Trp
** Variant additionally has amino acid substitution Thr59Ala
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with 1.38, 2.34 and 2.20 times improvement, respectively (Fig. 3.16).

**Fig. 3.16** The kinetic parameters for suc–AAPF–pNA substrate. Comparison of $k_{cat}$ and $k_{cat}/K_M$ values (determined for AAPF) of the selected variants with SC Perhydrolase

The kinetic parameters were determined for the wild type subtilisin Carlsberg and compared with the selected variants with increased PS$_{50}$ value (Fig. 3.17).
Part III: Directed evolution of SC Perhydrolase for oxidative stability

![Graph showing comparison of kinetic parameters](image)

**Fig. 3.17** Comparison of $k_{\text{cat}}$ and $k_{\text{cat}}/K_M$ values (determined for AAPF) of SC Perhydrolase and variants with increased $PS_{50}$ with Subtilisin Carlsberg (SC)

As presented in Fig. 3.17 all variant showed drastically decreased $k_{\text{cat}}$ value compared to subtilisin Carlsberg.

### 3.1.9. Determination of kinetic parameters for perhydrolytic activity with methylbutyrate -perhydrolytic activity assay

The kinetic parameters of SC Perhydrolase variants with increased $PS_{50}$ values were determinate also for the perhydrolytic activity using methyl-butyrates as a substrate (concentration of $H_2O_2$ was constant 100 mM) (Fig. 3.18). A detailed protocol and the principle of the assay are described in Part III section 2.2.12. $K_M$ values for all variants compared to the double mutant are increased and $k_{\text{cat}}$ values are significantly decreased except for the variants M4** and M7**. These two variants showed a 4.2- and 2.2-fold increment in $PS_{50}$ values, respectively, whereas $k_{\text{cat}}$ for perhydrolytic activity was decreased 1.5 times.

| Variants | $K_M$ (mM) | $k_{\text{cat}}$ (min$^{-1}$) | $k_{\text{cat}}/K_M$ (min$^{-1}$M$^{-1}$) | M/SC $k_{\text{cat}}/K_M$ | M/SC $k_{\text{cat}}$

| Subtilisin Carlsberg (SC) | 0.59 ± 0.06 | 10353 ± 342 | 17547.45 | 1.00 | 1.00

| SC Perhydrolase (Thr59Ala/Leu217Trp) | 0.15 ± 0.02 | 1983.29 ± 43.28 | 13221.93 | 0.75 | 0.19

| M1 (Met222Ser)* | 0.60 ± 0.08 | 1809.74 ± 70.84 | 3013.72 | 0.17 | 0.17

| M4 (Trp217Leu/Met222Cys)** | 0.98 ± 0.15 | 11551.92 ± 656.90 | 11788.88 | 0.67 | 1.22

| M6 (Trp217Leu/Met222Ser)** | 1.26 ± 0.09 | 4646.24 ± 149.90 | 3702.18 | 0.21 | 0.45

| M7 (Trp217Met)** | 0.43 ± 0.07 | 4374.27 ± 179.55 | 10151.47 | 0.58 | 0.42

* Variant additionally has amino acid substitutions Thr59Ala and Leu217Trp

** Variant additionally has amino acid substitution Thr59Ala
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**Fig. 3.18** The kinetic parameters for perhydrolitic activity of SC Perhydrolase and its variants with increased resistance to peroxycarboxylic acids determined using methyl-butyrate (APCC). Comparison of $k_{\text{cat}}$ and $k_{\text{cat}}/K_M$ values (determined for methylbutyrate in APCC assay) of the selected variants with SC Perhydrolase.

<table>
<thead>
<tr>
<th>Variants</th>
<th>$K_M$ (mM)</th>
<th>$k_{\text{cat}}$ (min$^{-1}$)</th>
<th>$k_{\text{cat}}/K_M$</th>
<th>M/SC Per $k_{\text{cat}}/K_M$</th>
<th>M/SC Per $k_{\text{cat}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC Perhydrolase (Thr59Ala/Leu217Trp)</td>
<td>122.60 ± 36.88</td>
<td>258.35 ± 38.09</td>
<td>2.11</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>M1 (Met222Ser)*</td>
<td>153.70 ± 57.06</td>
<td>37.86 ± 7.48</td>
<td>0.25</td>
<td>0.12</td>
<td>0.15</td>
</tr>
<tr>
<td>M4 (Trp217Leu/Met222Cys)**</td>
<td>160.50 ± 56.19</td>
<td>175.46 ± 33.40</td>
<td>1.09</td>
<td>0.52</td>
<td>0.68</td>
</tr>
<tr>
<td>M5 (Trp217Met/Met222Ser)**</td>
<td>134.30 ± 44.73</td>
<td>44.92 ± 7.00</td>
<td>0.33</td>
<td>0.16</td>
<td>0.17</td>
</tr>
<tr>
<td>M6 (Trp217Leu/Met222Ser)**</td>
<td>164.40 ± 60.17</td>
<td>49.65 ± 9.97</td>
<td>0.30</td>
<td>0.14</td>
<td>0.19</td>
</tr>
<tr>
<td>M7 (Trp217Met)**</td>
<td>139.20 ± 42.33</td>
<td>179.62 ± 28.13</td>
<td>1.29</td>
<td>0.61</td>
<td>0.70</td>
</tr>
</tbody>
</table>

The kinetic characterization of subtilisin Carlsberg for the perhydrolytic activity and its comparison with SC Perhydrolase and variants showing increased PS$_{50}$ value are presented in Fig. 3.19. SC Perhydrolase as already reported (Wieland 2009) showed increased perhydrolytic activity compared to subtilisin Carlsberg (2 times). In addition to this, two variants M4** and M7** showed 1.4 times increased $k_{\text{cat}}$ with methylbutyrate in respect to subtilisin Carlsberg.

* Variant additionally has amino acid substitutions Thr59Ala and Leu217Trp
** Variant additionally has amino acid substitution Thr59Ala
Part III: Directed evolution of SC Perhydrolase for oxidative stability

**Fig. 3.19** Comparison of $k_{\text{cat}}$ and $k_{\text{cat}}/K_M$ values (determined for methylbutyrate in APCC assay) of SC Perhydrolase and the selected variants with increased PS$_{50}$ with Subtilisin Carlsberg (SC)

### 4. DISCUSSION

Due to the importance of subtilisins as industrial enzymes along with existing protocols for cloning, expression, purification, and availability of an atomic resolution structures (Bryan 2000) this subclass of enzymes have become a model system for protein engineering studies. The modification of subtilisins expanded over the years, together with the study of several enzymatic properties, bringing input in the scientific understanding of the rationale behind the changes introduced by engineering. A common feature of subtilisins is their lability to oxidative agents such as hydrogen peroxide and peroxycarboxylic acids, common additives used in washing powders. Therefore, an oxidation resistant subtilisin Carlsberg variant is of interest to the detergent industry. In addition, the generation of such a variant can provide insights

<table>
<thead>
<tr>
<th>Variants</th>
<th>$K_M$ (mM)</th>
<th>$k_{\text{cat}}$ (min$^{-1}$)</th>
<th>$k_{\text{cat}}/K_M$ (min$^{-1}$M$^{-1}$)</th>
<th>M/SC $k_{\text{cat}}/K_M$</th>
<th>M/SC $K_{\text{cat}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subtilisin Carlsberg (SC)</td>
<td>117 ± 26</td>
<td>124.60 ± 13.70</td>
<td>1.06</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>SC Perhydrolase (Thr59Ala/Leu217Trp)</td>
<td>122.60 ± 36.88</td>
<td>258.35 ± 38.09</td>
<td>2.11</td>
<td>1.99</td>
<td>2.07</td>
</tr>
<tr>
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<td>153.70 ± 57.06</td>
<td>37.66 ± 7.48</td>
<td>0.25</td>
<td>0.24</td>
<td>0.30</td>
</tr>
<tr>
<td>M4 (Trp217Leu/Met222Cys)$^{**}$</td>
<td>160.50 ± 56.19</td>
<td>175.46 ± 33.40</td>
<td>1.09</td>
<td>1.03</td>
<td>1.41</td>
</tr>
<tr>
<td>M6 (Trp217Leu/Met222Ser)$^{**}$</td>
<td>164.40 ± 60.17</td>
<td>49.65 ± 9.97</td>
<td>0.30</td>
<td>0.28</td>
<td>0.40</td>
</tr>
<tr>
<td>M7 (Trp217Met)$^{**}$</td>
<td>139.20 ± 42.33</td>
<td>179.62 ± 28.13</td>
<td>1.29</td>
<td>1.22</td>
<td>1.44</td>
</tr>
</tbody>
</table>

* Variant additionally has amino acid substitutions Thr59Ala and Leu217Trp
** Variant additionally has amino acid substitution Thr59Ala
Part III: Directed evolution of SC Perhydrolase for oxidative stability

into the functional consequences of replacing catalytic residues that are susceptible to oxidation since the decrease in enzymatic efficiency and increase in $K_{m}$ value upon oxidation is not completely understood. Three mechanisms that explain the alterations in enzymatic parameters have been previously proposed: I) conformational changes in the protein structure caused by methionine sulfoxide, II) changing the electronic environments of the enzyme by replacing hydrophobic sulfide by hydrophilic sulfoxide and III) steric hindrance due to the presence of an additional oxygen atom that can disturb interactions between substrate and enzyme (Stauffer and Etson 1969). One of the proposed mechanism or combinations of all three could be used to explain the changes regarding the effect of oxidative agents in the particular enzyme, SC Perhydrolase. The main aim of this work was to increase the oxidative stability of a double mutant of subtilisin Carlsberg, Thr59Ala/Leu217Trp (SC Perhydrolase) in the presence of peroxycarboxylic acids while keeping and/or improving the level of its perhydrolytic activity. As previously reported for BPN' subtilisin (Estell et al. 1985), the substitution of the amino acids residues susceptible to oxidation (Met and Trp) by residues that are not prone to oxidation resulted in increased residual activity after incubation with hydrogen peroxide. Highly conserved sequences are present around the active site for almost all subtilisins, therefore the alignment of crystal structures of BPN' and subtilisin Carlsberg protein revealed approximately 70 % homology. As a consequence, it is possible to analyse and explain the amino acid substitutions and their influence on the kinetic properties of the subtilisin Carlsberg identified variants. The generation of site saturation mutagenesis libraries was based on previously described studies on similar subtilisins (i.e. BPN') where the positions susceptible to oxidation have been identified. The latter correspond to positions Trp 217 and Met 222 in SC Perhydrolase situated in proximity to the active site (Fig. 3.20).

The identified variants were compared to wild type subtilisin Carlsberg in order to understand the influence of the amino acid substitutions on oxidative stability and perhydrolytic activity in subtilisins. As reported above, five variants M1 (Met222Ser)*, M4 (Trp217Leu/Met222Cys)**, M5 (Trp217Met/Met222Ser)**, M7 (Trp217Met)** and variant M6 (Trp217Leu/Met222Ser)**, and showed increased $PS_{50}$ value compared to SC Perhydrolase with increase of 4.5 times for variant M6 (Trp217Leu/Met222Ser)**.
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Fig. 3.20 Amino acids susceptible to oxidation located in proximity to the active center of SC Perhydrolase. Catalytic triad-labeled in red, Trp 217-labeled in orange and Met 222-labeled in blue

The amino acid substitutions pattern identified in the variants with increased $PS_{50}$ value was based on the same principle as described for BPN' subtilisin. The amino acids susceptible to oxidation (Met, Trp) were exchanged by amino acids that are less or not prone to oxidation (Cys, Ser, Leu). This type of exchange prevents the conformational changes and steric hindrance in the protein caused by oxidized methionine residues (i.e. methionine sulfoxide). The fact that Cys is more susceptible to oxidation than Ser and/or Leu could explain higher oxidative stability measured in variant M6 (Trp217Leu/Met222Ser)** (4.5 times) than in variant M4 (Trp217Leu/Met222Cys)** (2.5 times) when compared to SC Perhydrolase. Variant M7 (Trp217Met)** having two Met residues around the active site (positions 217 and 222) showed an increased $PS_{50}$ value. This can be explained by the fact that there is a certain level of selective oxidation on residues in the protein. The amino acid residues that are surface exposed are preferentially oxidized, whereas buried residues are protected (Luo and Levine 2009). Visual inspection of ** Variant additionally has amino acid substitution Thr59Ala
the generated three-dimensional SC Perhydrolase model with substitution Trp217Met indicated that residue Met 222 is buried rather than solvent accessible, whereas residue Met 217 is solvent exposed (Fig. 3.21). The oxidation of Met 217 instead of Met 222 constitutes a probable antioxidative defense mechanism for the catalytically more important Met 222 located near the active site. Solvent accessibility of the substituted residue may be the major factor in determining the rate of oxidation and main cause of increased oxidative stability of variant M7 (Trp217Met)**.

** Fig. 3.21 **Structural models of subtilisin Carlsberg variant M7 (Trp217Met) and the catalytic site (Asp32, His64, Ser221). Positions Met217 and Met222 are located in close proximity to the catalytic center. The surface representation of Met217(yellow) and Met222 (blue) show the difference in solvent exposed surfaces

These observations are further supported by a study on an isolated and characterized subtilisin–like alkaline serine protease, KP–43, from *Bacillus* KSM (Nonaka *et al* 2004). KP-43 has a naturally high tolerance for hydrogen peroxide showing activity in up to 0.88 M hydrogen peroxide, a value significantly higher than for most known subtilisins. Nonaka and coworkers proposed that I) distance between sulfoxide atom in methionine residues and oxyanion hole that is longer than

** Variant additionally has amino acid substitution Thr59Ala **
corresponding distance in other subtilisins (3.6 Å in oxidized BPN’ compared to 3.2 Å in oxidized KP-43) (Fig. 3.22) and II) existence of additional methionine residue that gets oxidized readily upon treatment with oxidizing agents than catalytically important Met 256 could be the possible explanations of the oxidatively resistant subtilisin like protease. A structural alignment of the crystal structures of KP-43 and subtilisin Carlsberg revealed that the amino acid positions in KP-43 Met 251 and Met 256 are the structural equivalents of positions 217 and 222 of subtilisin Carlsberg, respectively. This indicates that a similar effect of increased oxidative stability is achieved in two significantly different but structurally similar enzymes. The properties and comparison of these two proteases, KP-43 and subtilisin Carlsberg are summarized in Table 3.11.

Table 3.11. Comparison of enzymatic properties of subtilisin Carlsberg and KP-43

<table>
<thead>
<tr>
<th>Property</th>
<th>Subtilisin Carlsberg</th>
<th>KP–43</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Origin</strong></td>
<td><em>B. licheniformis</em></td>
<td><em>Bacillus sp. strain KSM–KP43</em></td>
</tr>
<tr>
<td><strong>Molecular mass</strong></td>
<td>27 kDa</td>
<td>43 kDa</td>
</tr>
<tr>
<td><strong>Isoelectric point</strong></td>
<td>pH 7.1</td>
<td>pH 8.9–9.1</td>
</tr>
<tr>
<td><strong>N-terminal sequence</strong></td>
<td>AQTVPYGIPLIKADK</td>
<td>NDVARGIVKADVAQ</td>
</tr>
<tr>
<td><strong>Number of amino acids</strong></td>
<td>274</td>
<td>434</td>
</tr>
<tr>
<td><strong>Specific activity (casein)</strong></td>
<td>15.6 U/mg</td>
<td>115 U/mg</td>
</tr>
<tr>
<td><strong>Optimal pH (casein)</strong></td>
<td>pH 10</td>
<td>pH 11-12</td>
</tr>
<tr>
<td><strong>Optimal temperature</strong></td>
<td>55°C</td>
<td>60°C</td>
</tr>
<tr>
<td><strong>pH stability</strong></td>
<td>pH 9</td>
<td>pH 6–11 (40°C for 30 min)</td>
</tr>
<tr>
<td><strong>Effects of oxidants</strong></td>
<td>Sensitive</td>
<td>Stable</td>
</tr>
<tr>
<td><strong>Stability to surfactants</strong></td>
<td>Stable</td>
<td>Highly stable</td>
</tr>
<tr>
<td><strong>Effect of fatty acids</strong></td>
<td>Inhibited</td>
<td>Not inhibited</td>
</tr>
<tr>
<td>(oleic acid)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Inhibitors</strong></td>
<td>PMSF, DFP</td>
<td>PMSF, DFP</td>
</tr>
</tbody>
</table>
Fig. 3.22 Stereo views around the catalytic center of KP-43 and subtilisin BPN’. A) the native form as of KP-43 and subtilisin BPN’ (BPN’ is shown transparently) B) Oxidized forms of subtilisin BPN’ and KP-43, WAT-water (Nonaka et al 2004)

The kinetic parameters for the identified variants with increased oxidative stability showed that subtilisin Carlsberg has the highest proteolytic efficiency. The substitution of Met 222 by amino acids that are less prone to oxidation caused significant decrease in proteolytic activity compared to SC Perhydrolase and subtilisin Carlsberg. The exchange from a hydrophobic amino acid (Met) to a hydrophilic (Ser) may alter the electronic environment around the active site sufficiently to affect the rate of reaction while in the case of substitution Met222Cys, structurally less radical change may reduce the effect on the proteolytic activity as in variant M4 (Trp217Leu/Met222Cys)** (same $k_{cat}$ value as for subtilisin Carlsberg).

** Variant additionally has amino acid substitution Thr59Ala
The analysis of identified amino acid substitutions revealed that position Leu 217 is of crucial importance for the proteolytic activity and exchange with other amino acid leads to its drastic decrease (Lee et al 2010). On the other hand, substitution of Leu 217 by Trp resulted in a variant with increased perhydrolytic activity as described for starting variant in this work, SC Perhydrolase. This increase was explained by Lee et al in a study based on molecular dynamics simulations of the second tetrahedral intermediate (2010). It was proposed that the stabilization of the second tetrahedral intermediate along with maintaining strong hydrogen bond between catalytically important His 64 and Ser 221 are key features necessary for the progression of perhydrolytic reaction in SC Perhydrolase. The stabilization of the second tetrahedral intermediate by weak hydrogen bonding is favored by substitutions Leu217Trp. Since Trp belongs to the group of amino acids prone to oxidation its replacement results in an oxidatively more stable but less active variant in respect to perhydrolysis.

Two of selected variants M4 (Trp217Leu/Met222Cys)** and M7 (Trp217Met)** showed 1.4 increased level of perhydrolysis compared to subtilisin Carlsberg. In order to investigate the mechanism of perhydrolysis a computational analysis was performed. In the catalytic cycle of the perhydrolysis with ester as substrate, hydrogen peroxide is attacking preformed acyl-enzyme complex. The stabilization of the second tetrahedral intermediate is essential for completion of the perhydrolytic reaction (Lee et al 2010). Besides stabilization of the second tetrahedral intermediate, the strength of hydrogen bonds formed between the catalytic residues Ser 221 and His 64 also determines the final product of reaction. In case of hydrogen bond hindrance the second tetrahedral intermediate may reveal starting reactant (i.e. hydrogen peroxide and ester substrate) making the reaction reversible. To confirm the importance of hydrogen bonding between His 64 and Ser 221 for completion of the perhydrolytic reaction some preliminary analysis were performed using YASARA 11.9.18 on the structures of subtilisin Carlsberg, SC Perhydrolase and the identified variants M4 (Trp217Leu/Met222Cys)** and M7 (Trp217Met)**. The initial structure was based on X-ray crystal structure of subtilisin Carlsberg (Protein Data Bank entry 1yu6). The first comparative MD simulations were performed with subtilisin Carlsberg and SC Perhydrolase. The three different conformations resulting in the three possible hydrogen bonds (Nε-H of His 64 and side chain OG atom of Ser 221 (1),

** Variant additionally has amino acid substitution Thr59Ala
Part III: Directed evolution of SC Perhydrolase for oxidative stability

Nε-H of His 64 and O2 from peroxo group (2), Nε-H of His 64 and O6 from peroxo group (3) (Fig. 3.23) were simulated for each variant. The distance between Nε-H of His 64 and side chain OG atom of Ser 221 was used as an indicator of its strength.

Fig. 3.23 The three different conformations resulting in the three possible hydrogen bonds (Nε-H of His 64 and side chain OG atom of Ser 221 (1), Nε-H of His 64 and O2 from peroxo group(2), Nε-H of His 64 and O6 from peroxo group (3). Yellow stick indicates hydrogen bond formation

The preliminary results showed that, in case of subtilisin Carlsberg in the second tetrahedral intermediate, a shorter hydrogen bond was formed between O2 peroxo oxygen and Nε-H of His 64 (approximately 1.9 Å) compared to the bond formed between Nε-H of His 64 and side chain OG atom of Ser 221 (approximately 2.5 Å).
The hydrogen bond formation was also observed between \( N_\varepsilon-H \) of His 64 and O6 peroxy oxygen but was not considered in the analysis since the transfer of hydrogen to this oxygen is not possible. In case of SC Perhydrolase the hydrogen bond between \( N_\varepsilon-H \) of His 64 and side chain OG atom of Ser 221 was shorter (approximately 2 Å) compared to hydrogen bond formed between O2 from peroxy group (2.5 Å). The shorter/stronger bond between \( N_\varepsilon-H \) of His 64 and side chain OG atom of Ser 221 is formed in SC Perhydrolase compared to the same bond in subtilisin Carlsberg.

The stabilization energies for the residues (Asp 32, His 64, Asn 155, Leu/Trp 217, Ser 221, Met 222) involved in hydrogen bond formation were calculated for each variant. The lower energy of the system represents a more stable system and a lower barrier for overcoming the transition state of the reaction. The conformation with the lowest energy in subtilisin Carlsberg was conformation 2 (Fig. 3.22) resulting in formation of a hydrogen bond between \( N_\varepsilon-H \) of His 64 and O2 from perhydroxyl group. In case of SC Perhydrolase conformation 1 (Fig. 3.22) where a hydrogen bond between \( N_\varepsilon-H \) of His 64 and side chain OG atom of Ser 221 is formed had the lowest energy. Based on these observations and considering the fact that catalytically important bond for completion of perhydrolysis is formed between \( N_\varepsilon-H \) of His 64 and side chain OG atom of Ser 221, it can be concluded that completion of perhydrolytic reaction is more favored in case of SC Perhydrolase than in subtilisin Carlsberg. In subtilisin Carlsberg the catalytically important reaction of \( N_\varepsilon-H \) of His 64 and side chain OG atom of Ser 221 was hindered by stronger hydrogen bond between \( N_\varepsilon-H \) of His 64 and O2 from perhydroxyl group. Besides the strength of the hydrogen bonding, the conformations with the lowest energy were additional indicators of higher perhydrolysis level in SC Perhydrolase that was also experimentally measured in SC Perhydrolase and published by Lee et al (2010).

The preliminary computational analysis performed on two variants M4 (Trp217Leu/Met222Cys)** and M7 (Trp217Met)** showed that the hydrogen bond formed between \( N_\varepsilon-H \) of His 64 and side chain OG atom of Ser 221 (1.8 Å) is more favored than the two other possible bonds between \( N_\varepsilon-H \) of His 64 and O2/O6 from peroxy group (2.5 Å). The lower level of interference on hydrogen bonding between \( N_\varepsilon-H \) of His 64 and side chain OG atom of Ser 221 in the selected variants compared to subtilisin Carlsberg gave a hint on the influence of the substitutions on

** Variant additionally has amino acid substitution Thr59Ala
the increased perhydrolytic activity. The conformation with the lowest energy for both variants was one that is resulting in the formation of a hydrogen bond between $N\varepsilon$-$H$ of His 64 and side chain OG atom of Ser 221. Thus, overcoming of the second tetrahedral intermediate in the selected variants is more favored than in subtilisin Carlsberg, while the comparison with SC Perhydrolase showed a higher energy barrier for completion of perhydrolysis.

Additionally, a visual inspection of the protein crystal structure indicated that the voluminous Met residue at position 217 located in proximity of Met 222 may sterically force the second tetrahedral intermediate into a conformation that favors the formation of the hydrogen bond between $N\varepsilon$-$H$ of His 64 and side chain OG atom of Ser 221 in higher extent than in case of less voluminous Leu 217 residue in variant M4 (Trp217Leu/Met222Cys)**. The data obtained in the computational analysis performed on the selected variants supports the experimentally measured data and helped to rationalize the effect of identified amino acid substitutions.

5. CONCLUSIONS

In summary, an expression system for SC Perhydrolase in *B. subtilis* DB104 in a microtiter plate was established whereby the protein expression level were sufficiently high for screening of proteolytic and perhydrolytic activities. The screening and characterization assays were adapted from the classical proteolytic activity quantification methods (suc–AAPF-pNA) and a novel fluorescent assay was developed and optimized for measurement of perhydrolytic activity (APCC assay). The transformation protocol for the low competence strain *B. subtilis* DB104 was adapted and used for transformation of site saturation mutagenesis libraries.

Five variants with increased oxidative stability in the presence of peroxyacetic acid were identified. The highest increase in oxidative stability was achieved by replacing Trp 217 and Met 222 with less oxidizable Leu and Ser as in variant M6 (Trp217Leu/Met222Ser)**.

The substitution of susceptible residues with the residues that are less prone to oxidation additionally resulted in variants M4 (Trp217Leu/Met222Cys)** and M7 (Trp217Met)**. These two variants besides increase in the oxidative stability (approximately 2 times) showed improved perhydrolytic activity when compared to subtilisin Carlsberg (1.4 times).

** Variant additionally has amino acid substitution Thr59Ala
Based on the reported substitutions it can be concluded that two major factors controlling the oxidative stability of subtilisin protease are the susceptibility of amino acid residues to the oxidative agents and its level of exposure to the solvent.

The preliminary computational studies performed on the second tetrahedral intermediate in the aforementioned variants provided a possible explanation of the differences in perhydrolytic activity and can be helpful in further design of new enzyme variants with increased oxidative stability and perhydrolytic activity. The decoration of surface of the protein by Met residues will likely improve its oxidative stability by protecting catalytically important residues for progression of the perhydrolytic activity. Further investigation on the effect of each type of amino acid substitution will provide valuable information for replacement of oxidizable catalytically critical residues of enzyme with beneficial residues for improving oxidative stability while increasing protein catalytic features.

** Variant additionally has amino acid substitution Thr59Ala
PART IV: DIRECTED EVOLUTION OF A DOUBLE MUTANT SUBTILISIN CARLSBERG T59A/L217W (SC PERHYDROLASE) FOR MODIFIED SUBSTRATE SPECIFICITY

1. INTRODUCTION

1.1. Aim of this project

The aim of this project was to generate and identify variants of double mutant subtilisin Carlsberg Thr59Ala/Leu217Trp (SC Perhydrolase) with increased specific activity towards diacetate ester substrates in the presence of hydrogen peroxide. The product of such enzymatic reaction is peroxyacetic acid, known as an efficient bleaching agent used in detergent industry. Diacetate substrates have a number of advantages when used in detergent compositions: I) product effectiveness (one molecule of a diacetate substrate yields two molecules of a peroxyacetic acid), II) the odor (more suitable and pleasant compared to the other ester substrates) and III) the cost effectiveness with respect to the other ester substrates. The main objective of this work was to alter the $k_{cat}/K_M$ value of SC Perhydrolase to achieve a more efficient use of the diacetate substrates. A directed evolution campaign combined with rational design studies, using a SC Perhydrolase as a starting point, was chosen as the strategy to follow. A secondary objective was to achieve, by the study of improved variants, a better understanding of structure-function relationships of the perhydrolysis mechanism in subtilisin Carlsberg variants gaining insight in which residues are important for substrate specificity. If a rational explanation of the substrate specificity can be provided, general means for engineering enzymes for useful and efficient chemical and biochemical reactions \textit{in vivo} and \textit{in vitro} can be proposed.

1.2. Substrate specificity

Chemical forces including hydrogen bonding, electrostatic, hydrophilic and steric interaction for a given substrate determine the substrate specificity as a specific feature of an enzyme (Poulouse 1994). This specificity is determined by the $k_{cat}/K_M$ ratio and commonly differs from substrate to substrate for a given enzyme. When
different substrates are compared for a given enzyme a larger \( \frac{k_{\text{cat}}}{K_M} \) value means that the enzyme takes more efficiently one substrate over the other, making this constant a measure of the catalytic efficiency of an enzyme with a specific/target substrate. The general approach for changing substrate specificity of enzymes having a catalytic triad is based on substitution of amino acid residues within 15 Å of the active site. Exchanging an amino acid residue with a distance greater than 15 Å of the active site is reported to have small or no effect on the substrate specificity value (Poulouse 1994).

A crystal structures of six subtilisin-like serine proteases showed that the catalytic site of the enzyme and its substrate binding pockets (S1, S2…Sn) are positioned in a single domain (Perona and Craik 1995). There are two substrate binding pockets in the enzyme (substrate is described as \( Pn…P2-P1'-P2'…Pn' \), where “P” residue refers to the position proceeding the scissile peptide bond of the substrate), one pocket is formed by two \( \beta \)-strands interacting with P1-P4 substrate residues whereas the second one is a loop composed of residues 156 to 166 (S1 pocket). These two substrate binding sites were the main target for the substrate specificity studies of subtilisin-proteases (Fig. 4.1). The most intensively studied member of subtilisin family is subtilisin BPN’ which was engineered to investigate the substrate specificity, the stability and the catalytic activity (Wells and Estell 1988). This enzyme specifically hydrolyses the peptide bond between the amino acids having hydrophobic side chains. The amino acid substitutions in direct contact with the substrate resulted in alterations of the specificity of the enzyme on hydrophobic residues. The position Gly 166, which lies at the base of the substrate binding pocket, was exchanged by other 12 amino acids. Analysis of these changes showed that substitution of Gly with more voluminous residues decreased the size of the S1 pocket and is followed with reduced activity towards substrates with large amino acids. In the same variants catalytic efficiency towards small amino acids substrates were increased 10 fold. In BPN’, the amino acids composing the substrate binding pocket are Glu 156, Tyr 217, Ala 152 and Ala 169. The residues 156 and 217 have a direct van der Waals contact with the substrate, while residue Ala 169 interacts with the substrate residues through interaction with residue Ala 152. To further investigate the influence of amino acids composing the substrate binding pockets, these positions were exchanged (Glu 156→Ser, Gly 169→Ala, Tyr 217→Leu) (Wells and Estell 1988). The substitution Tyr 217→Leu had the highest influence on \( k_{\text{cat}} \)
value and the substitutions Glu 156→Ser and Gly 169→Ala mostly affected $K_M$. The differences in the substrate specificity were caused by Glu 156→Ser substitution. Since position 156 and 166 showed influence on the substrate specificity sixteen different variants were constructed at these positions. These substitutions altered electrostatic potential of the S1 binding sites by introducing or removing Arg, Lys, Glu, and Asp. The main conclusion of these studies was that hydrophobicity of the S1 binding pocket mainly the substrate specificity, while van der Waals forces and hydration of polar side chains in the substrate binding pocket (S4) have lesser impact.

![Fig. 4.1](image1.png)

**Fig. 4.1** Structure of the S1 and S4 sites of subtilisin BPN’. Catalytic residues are in red, and the inhibitor chain is in green with side chains fitting in substrate binding pockets (S1, S4) (Perona and Craik 1995)

Another experimentally proven strategy for changing substrate specificity in proteases is substrate-assisted catalysis (Wells and Estell 1988). The approach is based on the selection of substrates containing amino acid residues that can participate in the catalytic mechanism. It was shown that imidazol nitrogens from bound substrates containing His (HisP2) can replace imidazole nitrogens of the catalytic His 64. The generated variant with substituted catalytic His 64 to Ala showed approximately 200 fold greater catalytic efficiency for HisP2 substrates than
for related alanine or glutamine containing substrates (AlaP2 and GlnP2) (Carter et al. 1989). This feature makes substrate-assisted catalysis powerful tool for site specific proteolysis of fusion proteins and in general provide a strategy for conversion of a broadly specific to a histidine specific subtilisins.

The modulation of the substrate specificity of the SC Perhydrolase towards methylbutyrate and diacetate was performed based on the studies reported for subtilisin BPN’. Since the secondary structure elements of the subtilisin enzymes are highly conserved, previously reported amino acid positions having an effect in substrate specificity provide a well-founded starting point for this study.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Chemicals

All chemicals were of analytical reagent grade or higher quality and purchased from Sigma-Aldrich (Taufkirchen, Germany), Applichem (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany) or Invitrogen (Darmstadt, Germany). All enzymes were purchased from NewEngland Biolabs (Frankfurt, Germany), Fermentas GmbH (St. Leon-Rot, Germany) or Sigma–Aldrich. Protease inhibitor PMSF was purchased from Sigma-Aldrich.

2.1.2. Bacterial strains

See Part III section 2.1.2.

2.1.3. Plasmids

The plasmids used in this work are summarized in Table 4.1.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHY300PLK</td>
<td>Shuttle vector, Ap’, Tet’</td>
<td>Takara BIO</td>
</tr>
<tr>
<td>pET19b (+)</td>
<td>T7 promoter, Ap’</td>
<td>Novagen</td>
</tr>
<tr>
<td>pNEB</td>
<td>Shuttle vector, Ap’</td>
<td>PURExpress™ In Vitro Protein Synthesis Kit, (NEB)</td>
</tr>
</tbody>
</table>
2.1.4. Oligonucleotides

The oligonucleotides used in this work are listed in Table 4.2. All variants mentioned in this work were numbered according to Protein Data Bank entry 1yu6.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’–3’)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Per_epPCR_FW</td>
<td>CGCTTTGCCCAAGCTTCTAGAATTG</td>
<td>Random mutagenesis of pHY300SCPer</td>
</tr>
<tr>
<td>Per_epPCR_RV</td>
<td>TCTAGAGATCATGTGGCCCATGCGCT</td>
<td>Random mutagenesis of pHY300SCPer</td>
</tr>
<tr>
<td>SSM_Ala152_FW</td>
<td>GGTGCTGCGTTGTAGCTNNKGCAGGGAACACGCGAT</td>
<td>SSM of pHY300SCPer</td>
</tr>
<tr>
<td>SSM_Ala152_RP</td>
<td>ATCCGCTGTTCCCTGMNNAGCTACAACGACGACC</td>
<td>SSM of pHY300SCPer</td>
</tr>
<tr>
<td>SSM_Ala169_FW</td>
<td>TACAATTGGGCTATCTCTNNKAGCTACAACGACGACC</td>
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</tr>
<tr>
<td>SSM_Ala169_RP</td>
<td>TGACAGAATCATTTTNNAAATACGATTCTTCTGTCA</td>
<td>SSM of pHY300SCPer</td>
</tr>
<tr>
<td>SSM_Thr220_FW</td>
<td>TGCAACATGGAACGGANNKTCAATGGCTTCTCTCTC</td>
<td>SSM of pHY300SCPer</td>
</tr>
<tr>
<td>SSM_Thr220_RP</td>
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</tr>
<tr>
<td>SSM_Gly127_FW</td>
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<td>SSM of pHY300SCPer</td>
</tr>
<tr>
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<td>SSM of pHY300SCPer</td>
</tr>
<tr>
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</tr>
<tr>
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<tr>
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<tr>
<td>SSM_Val68_RP</td>
<td>CAGCTACTGTACCCGCMNNATGTGTGCGGCTGTCC</td>
<td>SSM of pHY300SCPer</td>
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Part IV: Directed evolution of SC Perhydrolase for substrate specificity

<table>
<thead>
<tr>
<th>Name</th>
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<th>Description</th>
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<tr>
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<td>CTGATGCTCCCCCAAGMNCAATATTGATAACATCC</td>
<td>SSM of pHY300SCPer</td>
</tr>
<tr>
<td>SSM_Gly166_FW</td>
<td>GAAACACGAATAACAATNNKTATCCTGCGAATACGA</td>
<td>SSM of pHY300SCPer</td>
</tr>
<tr>
<td>SSM_Gly166_RP</td>
<td>TCGTATTTGCAGGATAMNAATTGTATTCGTGTTTC</td>
<td>SSM of pHY300SCPer</td>
</tr>
<tr>
<td>MatPerHnotag_FW</td>
<td>AGAAGGAGATAAACAATGGCGCAACCCGTCCTTCG</td>
<td>In vitro expression</td>
</tr>
<tr>
<td>MatPerHnotag_RP</td>
<td>CTTGGTTAGTTAGTTATTATTGAGCGCAGCTTCGAC</td>
<td>In vitro expression</td>
</tr>
<tr>
<td>ProPerHnotag_FW</td>
<td>AGAAGGAGATAAACAATGGCTCAACCGGCGAATGTCG</td>
<td>In vitro expression</td>
</tr>
<tr>
<td>ProPerHnoag_RP</td>
<td>CTTGGTTAGTTAGTTATTATTGAGCGCAGCTTCGAC</td>
<td>In vitro expression</td>
</tr>
<tr>
<td>PerNdel_FW</td>
<td>ACAGGCAGTCGCAATGCGATGCAAGGGGTGTCGTC</td>
<td>Deletion of Ndel</td>
</tr>
<tr>
<td>PerNdel_RP</td>
<td>GACGACCCTCTTGCAATACGCATTGCGACTGCT</td>
<td>Deletion of Ndel</td>
</tr>
</tbody>
</table>

2.2. METHODS

2.2.1. epPCR

The random mutagenesis library was generated by the standard epPCR (Cadwell and Joyce 1994) and cloned using megaprimer PCR of whole plasmid (MEGAWHOP) (Miyazaki 2003). The mutagenic conditions used in the epPCR were as follows: 95°C for 2 min, 1 cycle; 95°C, 30 s/ 60 °C, 30 s/ 72°C, 60 s, 29 cycles; 72°C for 5 min, 1 cycle), 2.5 U of Taq DNA polymerase, 0.20 mM of dNTP mix, 50 ng of template (pHY300SCPer), 0.05-0.2 mM of MnCl₂ and 10 pmol of each primer. Primer sequence is given in Table 4.2.

The conditions for the generation of the epPCR mutant libraries of the double mutant subtilisin Carlsberg,Thr59Ala/Leu217Trp (SC Perhydrolase) were optimized regarding MnCl₂ concentration by analyzing the percentage of active mutants in the cloned library (Part IV, Results section 3.8). The amplified MEGAWHOP product was digested by DpnI (20 U) at 37°C for 2 h, purified using Qiagen kit and transformed into E. coli DH5α strains. Plasmids were then pooled, isolated and transformed in B.subtilis DB104 following the protocol described in Part II.

Activity of mutants was detected using agar plate skim milk assay as described in Part III section 2.1.5.
2.2.2. Site saturation mutagenesis

Site saturation mutagenesis was performed as described in Part III section 2.2.7. The oligonucleotides used for SSM are listed in Table 4.2.

2.2.3. Agar plate assays

Assays on agar plated colonies typically enable the screening of \( >10^4 \) variants in a matter of days, but they are often limited in sensitivity: soluble products diffuse away from the colony and hence only very active variants are detected. In this work, the following agar assays were used:

2.2.3.1. Skim milk assay for proteolytic activity

Protease hydrolyses skim milk to free and soluble peptides. This is visible in clear halos around colonies on skim milk agar plates. A detailed protocol for the preparation of LB skim milk agar is described in Part III section 2.1.5. The cell culture (diluted \( 10^{-6} \)) was plated and grew overnight at 37°C. Proteolytic activity of the colonies was visualized as halos around active colonies.

2.2.3.2. Agar plate assay with azo-dye (2-methylsulfanyl-4-(3-methylsulfanylphenylazo)-aniline for determination of perhydrolytic activity

A semi-quantitative colorimetric method for determination of peroxycarboxylic acids in the presence of hydrogen peroxide in Luria Bertani (LB) agar was tested. For this purpose the redox active azo-dye 2-methylsulfanyl-4-(3-methylsulfanylphenylazo)-aniline for the visual detection of peroxycarboxylic acids was synthesized. The change in color is based on the exclusive oxidation of methylsulfanyl-groups derived from peroxycarboxylic acids (Fig. 4.2) (Minning et al 1999):
Fig. 4.2 Reaction of oxidation of 4-methyltoluene sulfide by peroxycarboxylic acid. A) Formation of peroxycarboxylic acid and B) selective oxidation of 4-methyltoluene sulfide to 4-methyltoluene sulfoxide by peroxycarboxylic acid. \( R_1 \)-alkyl, aryl; \( R_2 \)-alkyl, aryl, H; X–N, O

2.2.3.2.1. Synthesis of 2-methylthio-4-(3-methylsulfanylphenylazo)-aniline

Five ml (41 mM) 3-methylsulfanyl aniline was dissolved in 100 ml hydrochloric acid (half concentrated) and cooled to 58°C under vigorous stirring. 2.85 g (41 mM) sodium nitrite was dissolved in 16 ml water and added drop-wise into the aniline solution. The dark brown emulsion was added carefully to a mixture of 4 ml 3-methylsulfanylaniline (33 mM) dissolved in 40 ml hydrochloric acid (1 M), making sure that the temperature never exceeded 58°C. The color of the solution should change from brown-yellow to red. After the addition of the diazonium salt, the cooling bath was removed and the solution was allowed to reach room temperature. After additional stirring for 36 h the mixture was extracted with 200 ml of chloroform in two portions. Both phases were filtered and the residue was treated with sodium hydroxide (0.1 M) and extracted with chloroform again. The organic layer was dried with anhydrous magnesium sulfate and the solvent was removed; mp: 102°C (Minning et al 1999).

A standard concentration curve of peroxycetic acid (50 µl) was constructed on LB agar plates. Mixture of 1 mg of synthesized azo-dye, 0.3 mg agar and methylbutyrate (0.4 M) dissolved in phosphate buffer (100 mM, pH 9) was boiled shortly and after cooling poured over plates containing peroxycetic acid.
2.2.3.3. Agar plate assay with o-tolidine

The assay is based on oxidation of o-tolidine (3, 3’-dimethyl-(1, 1’-biphenyl)-4, 4’-diamine) by peroxycarboxylic acid (Fig. 4.3). Oxidative-prone groups in the presence of peroxycarboxylic acid were oxidized and dark yellow color was developed. Positive colonies (indicating perhydrolysis) produced a dark yellow halo after 2h.

![Fig. 4.3 Reaction of oxidation of o-tolidine with peroxycarboxylic acid](image_url)

A standard concentration curve of peroxyacetic acid (50 µL) was constructed using overlaying agar (1.5 %) mixture containing o-tolidine (1 mg/mL) dissolved in dimethylsulfoxid (DMSO), hydrogen peroxide (75 mM) and methyl-butyrate (0.4 M) in tris(hydroxymethyl)aminomethane (Tris) buffer (100 mM, pH 8.2).

2.2.4. Purification on small scale based on affinity chromatography

Purification of proteases from supernatant can be carried out in a one-step affinity chromatography with bacitracin as biospecific ligand (Izotova et al 1983). Bacitracin is decapptide with antibiotic properties and is produced by Bacillus species. It is a competitive inhibitor for serine proteases and binds in the active site via a D-Glutamic acid. The protease can be eluted with little or no loss of activity.

The bacitracin purification is a two-step procedure. In the first step, sepharose 4B (CNBr-activated) was suspended and washed in 1 mM hydrochlorid acid (> 200 ml/g matrix). Bacitracin (100 mg/g dry sepharose) was added to the soaked gel with and incubated in sodium hydrogen carbonate (100 mM), sodium chloride (500 mM) while stirring (4°C; 14-15 h). The gel was washed with sodium hydrogen carbonate (100 mM), sodium chloride (500 mM, pH 10) first and then washed with bidestilled water. To block remaining active groups of the sepharose the washed gel was incubated with ethanolamine (1M, pH 8 while rotating at 4°C; 2h). Afterwards the gel was washed with ammonium acetate (100 mM), calcium chloride (10 mM, pH 6.5) in steps using a membrane (regenerated cellulose 0.45 µm) while applying vacuum to remove the washing solution. This gel can be stored at 4°C. The gel was
diluted 1:4 with ammonium acetate (100 mM), calcium chloride (10 mM, pH 6.5) and 800 µl of this suspension is pipetted into each well of a Microlute–Plate for use as Mini Columns (2 ml, Zinsser Analytic, #3239003). For the storage 500 µl sodium acetate (100 mM, pH 4) per well was pipetted onto the sedimented gel. The Microlute-Plate is then firmly closed. In the second step, the supernatant was loaded on the activated matrix as summarized in Table 4.3.

**Table 4.3. Purification steps of serine protease with bacitracin affinity chromatography**

<table>
<thead>
<tr>
<th>Step</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regenerating cycle (3x)</td>
<td>1 ml 100 mM NaAc, 500 mM NaCl pH 4</td>
</tr>
<tr>
<td>Equilibration</td>
<td>3 ml 100 mM NH₄Ac, 10 mM CaCl₂ pH 6.5</td>
</tr>
<tr>
<td>Sample application</td>
<td>300 µl culture supernatant (~ 50 µg protease/ml)</td>
</tr>
<tr>
<td>Washing</td>
<td>3 ml 100 mM NH₄Ac pH 6.5</td>
</tr>
<tr>
<td></td>
<td>2 ml 100 mM NH₄Ac, 50 mM NaCl pH 6.5</td>
</tr>
<tr>
<td>Elution</td>
<td>1.2 ml 100 mM NH₄Ac</td>
</tr>
<tr>
<td></td>
<td>1 M NaCl pH 6.5, 25 % isopropanol</td>
</tr>
<tr>
<td>Regenerating cycle (1x)</td>
<td>1 ml 100 mM NaAc, 500 mM NaCl pH 8</td>
</tr>
<tr>
<td></td>
<td>1 ml 100 mM Tris/HCl, 500 mM NaCl pH 8</td>
</tr>
<tr>
<td>Storage</td>
<td>500 µl 100 mM NaAc pH 4</td>
</tr>
</tbody>
</table>

The solutions were washed through the columns using a vacuum in 1 ml steps through the gel and discarded. The supernatants were applied to the column material using vacuum pump. After the washing steps the protease is eluted from the gel in 200 µl steps. Only 3rd and 4th elutions were collected in a deep well plate.

2.2.5. [2,2’–Azino bis-(3–ethylbenzthiazolin–6-sulfonic acid) diammonium salt] ABTS microtiterplate assay

ABTS assay was established previously by collaborators at Henkel (Pinkernell et al. 1997). The assay can be used for detection of perhydrolytic activity in microtiter plates after purification of SC Perhydrolase by one step chromatography on bacitracin Sepharose as described in Part IV section 2.2.4. Assay enables quantitative determination of enzymatically generated peroxycarboxylic acid via oxidation of ABTS. Oxidation of ABTS was monitored at 405 nm as increase in absorbance. Conditions of the assay were: 20 µl of purified enzyme was mixed with methylbutyrate (50 mM) dissolved in 0.1 % SDS buffer, hydrogen peroxide
(31.3 mM) in sodium phosphate buffer (100 mM, pH 9). The mixture was incubated for 15 min (37°C, 400 rpm). Acetic acid (1.5 M) was added to decrease the pH to 4 prior to the spectrophotometric detection. In aqueous solution there is a dynamic peroxycarboxylic acid-hydrogen peroxide balance. In an acidic environment the balance lies towards peroxycarboxylic acid and ABTS is only oxidized by peroxycarboxylic acid at sufficiently fast rate to be detected. Potassium iodide (0.075 mM) was added to catalyse the quick oxidation of ABTS by peroxycarboxylic acids. The chromogenic reaction was started by addition of the ABTS solution (0.6 mM) and was incubated at room temperature (18–22°C) for 10 to 20 min before measurement using a spectrophotometer at 405 nm. This test system was calibrated with peroxyacetic acid. Therefore a defined concentration of peroxyacetic acid was added to enzyme-free samples before starting the ABTS reaction in order to construct a standard curve for the calculation of enzyme activity.

2.2.6. Inhibition of catalase

In the chromosomal DNA of *Bacillus subtilis* there are at least two major genes encoding for two types of catalases, *katA* and *katB*. *katA* is the gene induced for expression during the exponential growth phase while *katB* is expressed in sporulation phase of the cell cycle. The level of expression of gene *katA* is increased by exposure to hydrogen peroxide and loss of the activity of catalase results in increased sensitivity of the cell to hydrogen peroxide and yields oxidative stress. In its natural environment of *B. subtilis* cells are exposed to limiting concentration of nutrients, high salt concentration, alteration in pH and temperature. To cope with growth restricted conditions *Bacillus* cells are developed a mechanism of defense in form of antioxidative enzymes (stress defense machinery). The expression of catalase is important part of this stress defense mechanism (Loewen and Switala 1987). The decomposition of hydrogen peroxide published by Keilin et al (1939) is based on reduction of the catalase iron by peroxide and its re-oxidation by oxygen:

$$4\text{Fe (III)} + 2\text{H}_2\text{O}_2 = 4\text{H} + 2\text{O}_2$$
$$4\text{Fe (II)} + 4\text{H} + \text{O}_2 = 4\text{Fe (III)} + 2\text{H}_2\text{O}$$

As a final product of the reaction two molecule of hydrogen peroxide are decomposed to two molecule of water and one molecule of oxygen. The mechanism
Part IV: Directed evolution of SC Perhydrolase for substrate specificity

of decomposition of hydrogen peroxide comprises formation of two complexes, complex I and complex II (Fig. 4.4) (Jarnagin and Wang 1958).

![Chemical structure]

**Fig. 4.4** Formation of complex I and complex II during decomposition of hydrogen peroxide

For inhibition, an irreversible inhibitor 3-amino-1, 2, 4-triazole was selected. The irreversible inhibition of catalase by 3-amino-1, 2, 4-triazole occurs in the presence of a continuous supply of hydrogen peroxide. The inhibitor reacts with the catalase-hydrogen peroxide complex I while the reaction of the inhibitor with catalase–hydrogen peroxide complex II leads to uninhibited catalase. (Margoliash and Novogrodsy 1958).

A key requirement for perhydrolytic reaction is the presence of hydrogen peroxide as a substrate. Hydrogen peroxide acts as nucleophile and attacks the putative acyl-enzyme intermediate. Accordingly to the mechanism of perhydrolytic reaction, measurement of perhydrolytic activity of SC Perhydrolase requires inhibition of the existing catalase. The inhibition was performed as follows: supernatant of *Bacillus* cells was mixed with 3-amino-1, 2, 4-triazole (50 mM) and hydrogen peroxide (3.13 mM) in sodium phosphate buffer (100 mM, pH 7.5) and incubated (25°C; 600 rpm for 2.5 h).

**2.2.6.1. Assay for detection of catalase**

The assay was based on the method described by Beers and Sizer in which the decomposition of hydrogen peroxide is monitored spectrophotometrically at 240 nm (1952). The inhibited supernatant was mixed with hydrogen peroxide (31.3 mM) and activity of catalase was determined as decrease of absorbance at 240 nm.
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2.2.7. Determination of perhydrolytic activity using amino–phenoxy–3–carboxy–coumarin assay (APCC)

The conditions of the assay were as follows. After inhibition of catalase (25°C, 600 rpm, 2 h) using 3-amino-1,2,4-triazole (50 mM) and hydrogen peroxide (3.13 mM) activity of SC Perhydrolase was measured by fluorometry (exc./em: 360/465 nm) in sodium phosphate buffer (100 mM, pH 7.5; 25°C) containing hydrogen peroxide (31.3 mM), sodium bromide (100 mM), APCC (0.5 mM) and (a) methylbutyrate (50 mM) or (b) diacetate ester substrate (50 mM).

2.2.8. Determination of esterolytic activity of variants using titration

Esterolytic activity of SC Perhydrolase was determined using a pH stat (TitroLine, alpha, SCHOTT, Mainz, Germany). The mixture of purified subtilisin Carlsberg (0.2 mg/ml) and SC Perhydrolase (0.09 mg/ml), methylbutyrate/ethylbutyrate/diacetate substrates (30 mM) in sodium phosphate buffer (10 mM, pH 7.5) was titrated with 10 mM sodium hydroxide. Sodium hydroxide (10 mM) was used for titration of produced carboxylic acid in order to maintain pH constant. The volume of titrant solution was used for calculation of esterolytic activity of both enzymes.

2.2.9. Docking for methylbutyrate and diacetate as substrates in perhydrolytic reaction

Docking was performed for both substrates (methylbutyrate and diacetate). The software used in this experiment was AutoDock Version 4.2. AutoDock and AutoDockTools, the graphical user interface for AutoDock are available on http://autodock.scripps.edu/. AutoDock is an automated procedure for predicting the interaction of ligands with biomacromolecular targets.

The motivation for this work was to identify important residues in substrates binding pockets (S1 and S2) of subtilisin Carlsberg and relate them to the interaction of the ester substrates with catalytic triad in order to improve perhydrolytic activity of the protein. AutoDock calculations were performed in several steps: I) preparations of coordinate files using AutoDockTools, II) precalculation of atomic affinities using AutoGrid, III) docking of substrates using AutoDock, and IV) analysis of results using AutoDockTools. AutoDock combines an empirical free energy force field with a Lamarckian Genetic Algoritm, providing fast prediction of bound conformations with
predicted free energies of associations (Huey et al 2007; Morris et al 2009). In these experiments, the used parameters for running calculations were:

- Number of individuals in population: 150
- Maximum number of energy evaluations: 250,000,000
- Maximum number of generations: 27,000
- Rate of gene mutation: 0.02

2.2.10. In vitro protein expression

To perform in vitro protein expression of SC Perhydrolase gene two different approaches were followed: I) Starting from a linear DNA expression template and II) Starting from an expression vector.

2.2.10.1. In vitro expression starting from a linear DNA expression template

For in vitro protein expression of SC Perhydrolase commercial EasyXpress Linear Template Kit Plus from Qiagen (Hilden, Germany) was used. The protocol for expression is in detail described in the kit manual. The oligonucleotides used in the first PCR reaction are listed in Table 4.2. The experiment was composed of two parts: I) amplification of SC Perhydrolase gene including pro sequence and II) amplification of mature SC Perhydrolase. Proteolytic activity was measured (suc-AAPF-pNA, Part III section 2.2.6.) in the samples with and without pro-chaperon form after 1 h, 2 h, 3 h, 5 h and overnight incubation.

To validate the efficiency of the in vitro expression kit, positive control (elongation factor protein, EF) was expressed using optimized primers and template for two-step PCR procedure supplied in the kit. The concentration of expressed protein was estimated in SDS electrophoresis with standard concentration of Bovine Serum Albumin (BSA) protein (concentration range from 6–600 µg/ml).

2.2.10.2. In vitro expression starting from an expression vector

To improve the yield of the in vitro expression two different vector systems were used and tested in three different commercial available kits: I) RTS 100 E. coli HY Kit (5 Prime, Hamburg, Germany), II) PURExpress™ In Vitro Protein Synthesis (New England BioLabs, Frankfurt, Germany) and III) EasyXpress® Protein Synthesis (Qiagen, Hilden, Germany). The expression systems were chosen in accordance with the requirements of different kits. SC Perhydrolase gene including pro sequence
was cloned into the expression vectors (Fig. 4.5). Since the SC Perhydrolase gene contains an *NdeI* restriction site in the middle of the gene, prior to cloning, site directed mutagenesis for removing the *NdeI* restriction site was performed successfully. Primers for abolition of *NdeI* restriction site are listed in Table 4.2. The cloning was done using *NdeI* and *XhoI* restriction enzymes (Fermentas, FastDigest®). The proteolytic activity was measured (suc-AAPF-pNa, Part III, section 2.2.6) after 6h and 24h of *in vitro* expression.

**Figure 4.5 In vitro expression systems**—A) pNEB/ProSCPer: the expression vector is provided in the PURExpress™ *In Vitro* Protein Synthesis Kit from New England BioLabs B) pET19b/ProSCPer: commercial available. ProSCPerhydrolase—Pro form of SC Perhydrolase

### 3. RESULTS

In this work, directed evolution of a double mutant of subtilisin Carlsberg Thr59Ala/Leu217Trp (SC Perhydrolase) was performed to increase its substrate specificity towards diacetate ester substrates. Two different approaches were followed: random mutagenesis libraries screening (epPCR) and semi rational approach based on AutoDock analysis and visual inspection of the protein model.
3.1. Directed evolution of double mutant subtilisin Carlsberg Thr59Ala/Leu217Trp (SC Perhydrolase) to change substrate specificity towards diacetate substrates

3.1.1. Optimization of agar plate assay for detection of proteolytic and perhydrolytic activity

3.1.1.1. Skim milk agar plate assay

The skim milk agar plates were prepared as described in Part IV section 2.2.3.1. Proteolytic activity of the cell was visualized as halos around active colonies (Fig. 4.6)

![Fig. 4.6 Proteolytic activity on skim milk agar plate (1 % v/v). Protease hydrolyses skim milk to free and soluble peptides. This is visible in clear halos around colonies on skim milk agar plates](image)

3.1.1.2. Agar plate assay with azo-dye (2-methylsulfanyl-4-(3-methylsulfanylphenylazo)-aniline for determination of perhydrolytic activity

Azo-dye, 2-methylsulfanyl-4-(3-methylsulfanylphenylazo), was synthesized following the protocol described in Part IV section 2.2.3.2. NMR spectrum of the final product confirmed that the desired compound was synthesized (Fig. 4.7).
The lethal effect of hydrogen peroxide and peroxycetic acid on microorganisms and/or degradation of hydrogen peroxide are limiting the addition of the assay reagents directly to the cell culture medium. Thus, the medium was overlaid with a soft agar. Assay was tested by preparing an agar plate spotted with an increasing concentration of peroxyacetic acid ranging from 0.25 to 4 M. (Fig. 4.8). A clear change in color was observed for peroxyacetic acid concentration higher than 1 M, however at lower concentrations there was not an evident color shift implicating that the assay might not be sensitive enough to detect pehydrolytic activity of single colonies.
Part IV: Directed evolution of SC Perhydrolase for substrate specificity

3.1.1.3 Agar plate assay based on oxidation of o-tolidine

To validate the o-tolidine agar plate assay for detection of produced peroxycetic acid a standard agar plate spotted with an increasing concentration of peroxycetic acid was prepared (concentration range from 0.25 to 4 M). Agar plate was overlaid with soft agar containing o-tolidine, the methylbutyrate and hydrogen peroxide. The sensitivity of the o-tolidine assay was higher, compared to the azo-dye assay, reaching sensitivity up to 0.25 M peroxycetic acid (Fig. 4.9).

Fig. 4.8 Agar plate containing azo dye with standard concentrations of peroxycetic acid. Overlaid with mixture of synthesized azo dye (1 mg), agar (0.3 mg) and methylbutyrate (0.4 M) dissolved in phosphate buffer (100 mM, pH 9)

Fig. 4.9 o-tolidine agar plate assay with standard concentrations of peroxycetic acid (0.25-4 M). Standard concentrations of peroxycetic acids (50 µL) were detected using overlaying agar (1.5 %) mixture containing o-tolidine (1 mg/mL), hydrogen peroxide (75 mM) and methyl-butyrate (0.4 M) in tris(hydroxymethyl)aminomethane (Tris) buffer (100 mM, pH 8.2)
3.1.2. ABTS microtiter plate assay

Different concentrations of peroxyacetic acid were measured in ABTS assay as described in Part IV section 2.2.4 to determine the sensitivity of the assay and to generate the calibration curve for calculation of the enzyme activity (Fig. 4.10).

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>Activity (U/mg)</th>
<th>Specific activity (mU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL152</td>
<td>0.51</td>
<td>97.75</td>
</tr>
<tr>
<td>AB35</td>
<td>1.73</td>
<td>123.37</td>
</tr>
</tbody>
</table>
Performing the ABTS assay requires previous purification due to presence of substances which interfere with it such as solvents, media components, proteins (i.e. catalase) and substances which are readily oxidized. Although ABTS assay is optimized for determination of peroxycarboxylic acid content in microtiter plates, it still cannot be used as a continuous assay for measurement of perhydrolytic activity. Reason for this is that ABTS assay requires removal of hydrogen peroxide by catalase before determination of peroxycarboxylic acids, since it interferes with assay, or alternatively lowering pH of the reaction in order to detect peroxycarboxylic acids. Due to these limitations, a fluorescent assay described in Part IV section 2.2.7 was used instead of ABTS for screening.

3.1.3. Inhibition of catalase

Activity of catalase was determined as the decrease of absorbance at 240 nm (hydrogen peroxide absorbs at 240 nm). In Figure 4.11 is shown difference in between samples with and without 3-amino 1, 2, 4-triazole inhibitor. Due to the presence of inhibitor, the absorbance value is higher and was constant over time (no drop in concentration of hydrogen peroxide). On the other hand, sample without 3-amino 1, 2, 4-triazole inhibitor showed dramatically decrease of absorbance over time.

![Graph showing catalase activity with and without inhibitor](image)

**Fig. 4.11** Influence of 3–amino-1, 2, 4-triazole on catalase activity in the supernatant of SC Perhydrolase. A) catalase activity, B) catalase activity in the presence of 3–amino–1, 2, 4-triazole inhibitor
The concentration of hydrogen peroxide did not decrease over time and the level of inhibition caused by the presence of triazole inhibitor was sufficient for further experimental work.


After inhibition of catalase (25°C; 600 rpm for 2 h) using 3-amino-1, 2, 4-triazole (50 mM) and hydrogen peroxide (31.3 mM), the activity of SC Perhydrolase was measured by fluorometry (exc./em.: 360/465 nm) in sodium phosphate buffer (100 mM, pH 7.5; 25°C) containing hydrogen peroxide (31.3 mM), sodium bromide (100 mM), APCC (0.5 mM) and (A) methylbutyrate (50 mM) or (B) diacetate (50 mM). Standard deviation values of developed assay in microtiter plate for methylbutyrate (A) and diacetate substrate (B) were 8.4 % and 14.1 %, respectively (Fig. 4.12).

**Fig. 4.12** Standard deviations for APCC assay in microtiter plate. A) methylbutyrate (8.4 %) B) diacetate (14.1 %)
3.1.5. Determination of esterolytic activity of variants using titration

Esterolytic activity of subtilisin Carlsberg and SC Perhydrolase was determined as described in Part IV section 2.2.7. The ratios of esterolytic vs. proteolytic activity for three different substrates for subtilisin Carlsberg and SC Perhydrolase were calculated (Table 4.5). The calculation was done according to the volume of sodium hydroxide (10 mM) spent to maintain the pH of solution constant and the concentration of used enzyme. Also, perhydrolytic activity was determined using APCC assay previously described.

Table 4.5. Esterolytic vs. perhydrolytic activities of subtilisin Carlsberg and its variant with different ester substrates

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Ester substrate</th>
<th>Perhydrolytic activity (U/mg)</th>
<th>Esterolytic activity (U/mg)</th>
<th>Esterolytic vs. Perhydrolytic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subtilisin Carlsberg</td>
<td>Methylbutyrate</td>
<td>1.04</td>
<td>1.04</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Ethylbutyrate</td>
<td>0.12</td>
<td>0.66</td>
<td>5.50</td>
</tr>
<tr>
<td></td>
<td>Diacetate</td>
<td>0.17</td>
<td>0.04</td>
<td>0.24</td>
</tr>
<tr>
<td>SC Perhydrolase (Thr59Ala/Leu217Trp)</td>
<td>Methylbutyrate</td>
<td>3.18</td>
<td>5.47</td>
<td>1.72</td>
</tr>
<tr>
<td></td>
<td>Ethylbutyrate</td>
<td>0.45</td>
<td>3.03</td>
<td>6.73</td>
</tr>
<tr>
<td></td>
<td>Diacetate</td>
<td>0.32</td>
<td>2.32</td>
<td>7.25</td>
</tr>
</tbody>
</table>

The perhydrolytic activity of SC Perhydrolase was increased 2 to 3 times for different ester substrates compared to subtilisin Carlsberg. SC Perhydrolase showed highest proteolytic activity with methylbutyrate as substrate while activity with other two ester substrates was decreased for approximately 7 times. The esterolytic activity of SC Perhydrolase showed increased value compared to subtilisin Carlsberg with all three substrates, and the highest value was observed with ethylbutyrate as substrate. The lowest ratio of esterolytic vs. perhydrolytic activity SC Perhydrolase showed with methylbutyrate. Since methylbutyrate is not the preferred ester substrate in detergent industry, the enzyme had to be engineered to decrease the ratio of esterolytic vs. perhydrolytic activity for diacetate substrate.

3.1.6. Rational design for change in substrate specificity and activity of SC Perhydrolase

Based on molecular modeling studies of the second tetrahedral intermediate (Lee et al. 2010) formed during perhydrolytic activity of a SC Perhydrolase and on visual inspection of the model further amino acids substitutions which can lead to increase of perhydrolytic activity were suggested: Thr 33, Ser 125 which are in direct contact
with amino acids of catalytic triad (Fig. 4.13). The overall effect is likely to create a hydrogen bond and steric hindrance based skeleton surrounding the catalytic triad.

![Fig. 4.13 Structure of catalytic site and first shell of SC Perhydrolase. Catalytic triad-red, Thr 33-violet, Val 68-yellow, Ser 125-cyan](image)

In order to change substrate specificity of SP Perhydrolase towards the desired substrate the proposal for amino acids changes was based on the steric hindrance and increasing the polarity of the substrate binding pocket. Less bulky amino acids should improve the accessibility of the substrate molecule to the catalytic triad while an increased polarity of the pocket should improve the “solubility” of the substrate itself. The considered hydrophobic amino acids (Leu 96, Leu 26, Ile 107) present in the substrate binding pocket can be changed into less bulky and more polar aminoacids (Fig. 4.14).
In summary, saturation mutagenesis was performed on the positions: Thr 33, Val 68, Leu 96, Ile 107, Leu 126, Ser 125.

3.1.7. Docking of methylbutyrate and diacetate as substrates in perhydrolytic reaction

The predicted conformation of diacetate as substrate according to AutoDock calculations based on lowest energy of the system is showed in the picture below (Fig. 4.15). Ser from catalytic triad at position 221 is forming a hydrogen bond with the substrate in order to start the reaction. Additionally, Ala at position 169 forms one extra hydrogen bond which is stretching the substrate and making distance of 3.61 Å in between catalytic triad and substrate.
Part IV: Directed evolution of SC Perhydrolase for substrate specificity

In parallel the same computational experiment was performed for methylbutyrate as substrate (Fig. 4.16). The distance between substrate and catalytic triad is around 2.75 Å and there is no formation of additional hydrogen bond with Ala 169. In summary, the distance of diacetate to catalytic triad is larger compare to methylbutyrate and most likely cause of unfavorable enzymatic reaction, so one of the approaches as a result of docking is disfavoring of additional hydrogen bond in case of diacetate. The second approach was based on the fact that position of substrate in catalytic triad and distance can be changed by replacing Ala 169 with more robust amino acid. Amino acids with larger R residue will influence on conformation and position of the substrate in the sense to shorten distance with catalytic triad.
Docking of methylbutyrate showed that the Thr at position 220 is the only amino acid which is surrounding the catalytic triad that can influence on binding and reaction of methylbutyrate with the protein. After positions were selected site saturation mutagenesis libraries for each of them were generated and screened for perhydrolytic activity.

3.1.8. Screening epPCR and SSM libraries

The conditions for the generation of the epPCR mutant libraries of the double mutant subtilisin Carlsberg Thr59Ala/Leu217Trp (SC Perhydrolase) were optimized regarding MnCl$_2$ concentration by analyzing the percentage of active variants in the cloned library (Table 4.6). The differentiation in between active and inactive variants was monitored on skim milk agar plates (described in Part IV, section 2.2.3).
Table 4.6 Percentage of active mutants in the library after epPCR with different concentrations of Mn$^{2+}$

<table>
<thead>
<tr>
<th>Concentration of Mn$^{2+}$ (mM)</th>
<th>Positive colonies (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>0.05</td>
<td>83</td>
</tr>
<tr>
<td>0.08</td>
<td>77</td>
</tr>
<tr>
<td>0.1</td>
<td>43.56</td>
</tr>
<tr>
<td>0.2</td>
<td>20.65</td>
</tr>
</tbody>
</table>

The concentration of Mn$^{2+}$ (0.1 mM) at which ratio of active vs. inactive clones was approximately 50% (43.56%) was selected for generation of epPCR libraries. The primers are listed in Table 4.2 and the library was generated as described in protocol Part IV, section 2.2.1. The ratio of 50% active vs. inactive clones is an internal standard value for generation of epPCR libraries (one or two amino acid substitutions per round of mutagenesis and screening). Screening for modified substrate specificity and increased perhydrolytic activity of 1500 clones from epPCR library was performed by measuring perhydrolytic activities of variants with both substrates (methylbutyrate and diacetate). Unfortunately, none of the clones showed improved ratio of perhydrolytic activity with diacetate substrate.

SSM libraries at positions selected as described (Part IV, section 3.1.6 and 3.1.7) were generated according to the protocol (Part III, section 2.2.7). The primers are listed in Table 4.2. The generated libraries were screened (280 clones per position) for increased perhydrolytic activity towards diacetate and/or methylbutyrate ester substrate. No increase in perhydrolytic activity was observed. SSM library at position Gly 166 was screened for increased ratio perhydrolytic/proteolytic activity and three variants showing changed substrate specificity were selected (Despotovic 2012).

3.1.9. **In vitro expression: testing efficiency of EasyXpress Linear Template Kit Plus (Qiagen)**

Efficiency of the Qiagen kit for *in vitro* expression was validated by expressing the positive control (EF–protein) supplied in the Kit. Linear template (EF$_{\text{G}}$) was generated using control primers and template for two–step PCR procedure supplied in the kit. At the same time, already generated linear template (EF$_{\text{T}}$) supplied in the kit was *in vitro* expressed. *In vitro* expression was performed in a micro tube and detailed protocol is described in Part IV section 2.2.10. Expressed protein was analysed on the SDS-PAGE electrophoresis. The concentration of expressed control
protein is estimated by using the standard BSA protein concentrations (60–600 µg/ml). Expected size on SDS gel for EF–and BSA proteins are 32 kDa and 66.1 kDa, respectively (Fig. 4.17).

**Fig. 4.17** Performing SDS electrophoresis with standard concentration of BSA protein and amount of protein expressed using *in vitro* kit A) 1-Standard BSA (600 µg/ml), 2-Standard BSA (60 µg/ml), 3-Standard BSA (6 µg/ml), 4-EF₉ (overnight), 5-EF₉ (overnight), 6-No-template control (overnight) 7-EF₉ (4h), 8-EF₉ (4h), 9-No-template control (4h); B) 1-no-template control (1h), 2–EF₉, 3-EF₉ (1h), 4-No-template control(2h), 5-EF₉ (2h), 6-EF₉ (2h), 7-Standard BSA (6 µg/ml), 8-Standard BSA (60 µg/ml), 9-Standard BSA (600 µg/ml) (EF₉–elongation factor–already generated template supplied in the kit, EF₉–elongation factor–linear template generated using standard primers and template for two–step PCR procedure supplied in the kit)
According to the SDS-PAGE gel result, incubation time do not influence on amount of expressed protein. By estimation in respect to standard BSA concentration, range of expression of EFₚ was ~ 600 µg/ml while range of expression of EF₅ was ~ 60 µg/ml.

3.1.10. *In vitro* expression: two-step PCR procedure using of linear template SC Perhydrolase

SC Perhydrolase was successfully expressed *in vitro* (Part IV, section 2.2.10.1) using EasyXpress Linear Template Kit Plus from Qiagen. The experiment had two different set-ups in respect to template: I) pro-mature sequence and II) mature sequence of SC Perhydrolase gene. Template containing only mature sequence of gene did not show any proteolytic acitivity. After different times of expression proteolytic activity was checked using suc-AAPF-pNA assay. The highest amount of expression was obtained after overnight expression (Fig. 4.18).

![Graph showing absorbance at 405 nm for different incubation times](image)

**Fig. 4.18** Activity of SC Perhydrolase expressed *in vitro* after different incubation times

For *in vitro* protein expression of functional protease, it is necessary to have Pro-form construct cloned into the desired vector. The overnight expression of the SC Perhydrolase was 0.28 AAPF-Units. The level of expressed protein was not visible on SDS-PAGE electrophoresis.
3.1.11. *In vitro* expression of SC Perhydrolase gene starting from an expression vector

After cloning of Pro- form of SC Perhydrolase gene *in vitro* expression was performed as described in Part IV section 2.2.10.2. The pET19/ProSCPer expression system showed no proteolytic activity in the three tested kits. The pNEB/ProSCPer system showed detectable level of proteolytic activity with the 5 Prime RTS 100 *E. coli* HY Kit. After 6h of expression 0.28 AAPF-Units and after 24h 3.22 AAPF-Units were obtained (Fig. 4.19).

![Graph](image)

**Fig. 4.19** Proteolytic activities of the *in vitro* expressed SC Perhydrolase gene in the pNEB vector system and the RTS 100 *E. coli* HY Kit from 5 Prime after 6 and 24 h of incubation

The level of expression in vector system (pNEB/ProSCPer) was much higher compared to linear template expression. The amount of protein expressed after 6h of expression was the same like amount of protein obtained starting from linear template after overnight incubation. The expressed protein was not visible on SDS-PAGE electrophoresis gel. The pNEB/ProSCPer expression system in combination with 5 Prime RTS 100 *E. coli* HY Kit showed promising preliminary results for further *in vitro* expression experiments.
4. DISCUSSION

4.1. In vitro expression

In vitro protein synthesis offers production of interesting proteins in large quantities avoiding limitations such as aggregation in inclusion bodies, toxicity for expressing cells, and instability due to interaction with the biological system. The use of in vitro expression system as an alternative to cell-based methods within directed evolution experiment could dramatically increase the efficiency in the identification of new or improved enzymes. It enables expression of larger libraries (>10¹⁰) by overcoming problems such as loss of diversity due to low cloning and transformation efficiency, thus offering a wider range of screening techniques, decreasing the experimental time.

The main motivation for establishing an in vitro expression system for SC Perhydrolase was to circumvent the already reported limitations of low cloning and transformation efficiency in B. subtilis DB104 cells. Coupling of high diversity mutant libraries of SC Perhydrolase with the already optimized fluorescent assay for monitoring of perhydolytic activity (APCC) offered the possibility of using flow cytometry screening by applying in vitro compartmentalization technology (IVC) (Tawfik and Griffiths 1998). This powerful screening format would offer time efficiency and increase the probabilities for the identification of SC Perhydrolase variants having increased perhydolytic activity with ester substrates, since it is theoretically possible to screen approximately up to 10⁸ variants per day (Miller et al 2006)

In total, three different template systems (linear template, pET19bSCPer, and pNEBSCPer) and three commercial in vitro expression kits (Qiagen, 5 Prime and NEB) were investigated. The protein expression was performed in a microliter scale as described in Part IV section 3.1.11. The highest activity after protein expression was obtained using pNEBSCPer vector system as a template in combination with 5 Prime in vitro expression kit. The minimal incubation time needed for detection of proteolytic activity using the suc-AAPFpNA assay with this system (pNEBSCPer, 5 Prime in vitro expression kit) was 6 h. The detected activity of the expressed SC Perhydrolase was 0.28 AAPF-Units, however it was not possible to visualize expressed protein using SDS-PAGE electrophoresis (minimal amount of protein
required for visualization approximately 50 ng). The obtained results suggest a slow expression rate and low yield of SC Perhydrolase using an \textit{in vitro} expression system. The required time for expression and the resulting yield of the expressed protease in the \textit{in vitro} expression system were not sufficient for the immediate application in micro compartments for flow cytometry screening. The possible reason for the low expression yield may be due to the fact that the systems are not optimized for protease expression. The complex \textit{in vitro} mixture might contain protease inhibitors and/or dummy substrates (competitive substrates for suc-AAPF-pNA) reducing the measured activity. On the other hand, the proteolytic activity of the expressed SC Perhydrolase might cause disturbance on the components of the \textit{in vitro} machinery, such as degradation of key proteins in the system.

A possible approach to overcome these limitations might be optimization of the already existing systems for protease expression such as replacing inhibitory compounds, pH optimization, or the removal of the expressed protein in order to circumvent the problem of dummy/competitive substrates. On the other hand, the use of serine protease reversible inhibitors such as bacitracin or protecting substrates such as bovine serum albumine can avoid the proteolytic effect of SC Perhydrolase on the key components within the \textit{in vitro} system. Additionally, to increase the yield of expressed protein, it has been reported the development of more advanced \textit{in vitro} expression systems such as a “continuous-flow chamber” in which the supply with amino acids and energy sources is continuous, dramatically increasing the expressed protein yield (Spirin \textit{et al} 1988).

\textbf{4.2. Optimization of agar plate assay for detection of peroxycarboxylic acids}

One of the bottlenecks of a directed evolution campaign is the selection of suitable screening system. Traditional screening formats are agar plate and microtiter plate-based detection systems. Agar plate-based assays are usually applied for prescreening, working as qualitative indicator of positive or negative activity of each colony. Since most of the available liquid phase assays for screening mutant libraries are time consuming (usually 90 different variants can be screened per plate) the existence of an efficient prescreening agar plate format would reduce the amount of clones that will be tested in microtiter plates.

In order to reduce screening efforts in this work, the development and optimization of an agar plate assay for detection of peroxycarboxylic acid was
required. Two possible approaches for agar plate assays were investigated: 2-methylsulfanyl-4-(3-methylsulfanylphenylazo)-aniline, and o-tolidine, both based on oxidation by peroxyacarboxylic acid with subsequent change in color.

The main challenge to be addressed in developing the agar plate screening format was to find an assay with a sensitivity of μM range to correspond the concentration of product formed in reaction catalysed by SC Perhydrolase. The lowest concentration of peroxyacarbocyclic acid that was possible to detect was 0.25 M using the o-tolidine agar plate assay, therefore the perhydrolytic activity of SC Perhydrolase (μM range of product) was not sufficient for detection. Finding and/or optimizing an agar plate assay that enables detection of μM range of peroxyacarboxylic acids would overcome the existing challenge of low SC Perhydrolase activity. There are two possible approaches to circumvent limitation of low product formation. Increase in expression of SC Perhydrolase would be the possible way to increase amount of produced peroxyacarboxylic acid. Use of optimized expression vector systems or the engineering of the vectors to achieve higher expression level of cloned gene are possible alternatives.

The second possible approach might be repeating of iterative cycles of directed evolution of SC Perhydrolase until satisfactory level of improvement is achieved (increase in perydrolytic activity for factor 100).

4.3. Screening for increased level of perhydrolytic activity of SC Perhydrolse with diacetate ester substrate

Understanding of protein engineering principles for perhydrolytic activity as a secondary reaction of a protease is of high academic and economic interest. Investigation of important residues in SC Perhydrolase that influence increase in the perhydrolytic activity as well as specificity for the different ester substrates will provide significant input in engineering enzymes for useful and efficient chemical and biochemical reactions in vivo and in vitro. The first report based on molecular dynamic simulation by Lee et al explained the mechanism of increased perhydrolytic activity towards methylbutyrate ester substrate in double mutant subtilisin Carlsberg Thr59Ala/Leu217Trp (SC Perhydrolase) (2010). From an industry point of view, a protease with commercially sufficient level of the perhydrolytic activity is of great interest since the product of such reaction is a potential efficient bleaching agent for cleaning compositions. In particular, engineering of SC Perhydrolase to effectively
use diacetate ester substrates in production of peroxyacetic acid has several advantages such as substrate/product effectiveness (one molecule of diacetate substrate yields two molecules of peroxyacetic acid), the odor (more suitable and pleasant compared to other ester substrates) and cost effectiveness with respect to other ester substrates.

Our main intention in this work was to provide a validated protocol for directed evolution of SC Perhydrolase and to improve its perhydrolytic activity towards diacetate ester substrate. In the directed evolution campaign, a novel fluorescent microtiter plate screening system for monitoring enzymatically produced peroxycarboxylic acid was developed and optimized. This screening system was employed in screening epPCR and SSM libraries at positions proposed by visual inspection of a subtilisin Carlsberg model and docking analysis using AutoDock Version 4.2 with targeted ester substrates (methylbutyrate and diacetate ester). Screening of different mutant libraries using the diacetate ester did not yield a variant with increased perhydrolytic activity. The studies performed on obtained data helped in understanding the mechanism of perhydrolytic activity and the requirements for its completion.

In a first instance, investigation of chemical properties for diacetate ester substrate was performed. Screening data confirmed that the chemical features of the diacetate ester substrate are not optimal for obtaining variants with increased perhydrolytic activity in the directed evolution campaign. Thus, the project was challenging from the beginning. The understanding and detail explanations of possible negative result were from great scientific input for gaining a useful knowledge about the mechanism of perhydrolysis in proteases/and more general in hydrolases and for selection of more suitable ester substrates.

Detailed analysis of the chemistry for diacetate esters showed several obstacles for reaching optimal level of perhydrolytic reaction when used as a substrate. In aqueous environment diacetate ester shows a tendency for high rate of autohydrolysis of the ester bonds which results in release of two molecule of acetic acid (Rao and Gajanan 2005 ). On the other hand, the feature of diester compounds to form an intramolecular nucleophilic acyl substitution known as Dieckmann condensation to form cyclic compound was taken into account in analyzing the diacetate ester substrate. Since the Dieckmann condensation occurs when a five– or six-membered ring can be formed the completion of reaction with short diacetate
substrate cannot be considered. However, intramolecular interactions in short diacetate esters might cause bending of the substrate and reduce its interactive surface with protein residues in substrate binding pocket. The instability of the substrate in aqueous solution (fast rate of hydrolysis) and its conformation caused by intramolecular interactions are possibly reducing the substrate-protein interaction in the perhydrolysis reaction.

The second challenge to be addressed in this work was the low perhydrolytic activity when diacetate was used as a substrate for SC Perhydrolase. The reaction mechanism of perhydrolysis is similar to that of proteolysis, where the main difference occurs after the formation of the first tetrahedral intermediate. A short computational study on the interaction of ester substrates and substrate binding pocket residues of SC Perhydrolase was performed, based on already known hydrolysis mechanism in subtilisin proteases. As already described for polypeptide substrates, subtilisin Carlsberg binds substrates having bulky aromatic hydrophobic side chain “specificity group” (P1-P4) in the substrate binding pocket. The polypeptide chain of substrate also forms a short β-sheet (hydrogen bond) with subtilisin Carlsberg binding site (S1). The carboxy-terminal end of the substrate binds to the enzyme close to the serine 221 residue in the active site. This position of polypeptide substrate enables formation of tetrahedral intermediate with the serine in addition to alkylation of the catalytic histidine. Thus, the acyl group of the substrate is bound to the enzyme as a tetrahedral intermediate during catalysis in the orientation that is optimal for completion of reaction (Fig. 4.20).
In order to get overview of the different conformational changes in the protein and substrate during the first step of the perhydrolytic reaction, which would result in the formation of the first tetrahedral intermediate, an Autodock analysis was performed with both ester substrates (methylbutyrate and diacetate ester substrates). From experimental results, SC Perhydrolase showed an increased level of perhydrolysis towards methylbutyrate compared to its wild type protein, subtilisin Carlsberg. Considering this, initially, a docking experiment of SC Perhydrolase and methylbutyrate was performed. The position of the methylbutyrate having its carboxyl group orientated towards active serine residue, necessary for formation of first tetrahedral intermediate, was similar to the orientation of polypeptide in the active site of subtilisin Carlsberg. The distance from active serine residue and carbonyl center in the ester substrate were considered as favorable for initiation of the perhydrolytic reaction (<2.5 Å) enabling the formation of the first acyl-enzyme intermediate state (Fig. 4.21).
In case of the diacetate substrate the AutoDock analysis showed that the energy favored conformation state (the lowest energy of the system) was an inhibitory conformation for the perhydrolytic reaction (Fig 4.22A). The orientation of the substrate and the distance between the hydroxyl group of the nucleophile serine and the carbonyl group from the substrate was too large to enable the initial interaction and formation of the first tetrahedral intermediate (distance >6 Å).
To study all possible reasons behind, the low level of perhydrolytic reaction, a putative model of SC Perhydrolase and diacetate substrate was generated using YASARA version 11.9.18 (Fig. 4.22B). The putative conformation of the diacetate substrate in the substrate binding pocket of SC Perydrolase was compared with the predicted conformation obtained by the docking approach. The substrate conformation required for the initiation of the reaction differs considerably to that predicted by docking. The distance between carbonyl group in the substrate as a primary attack center for Serine 221 is approximately 6 Å, which is an unfavorable length for bond formation.
5. CONCLUSIONS

The experimentally obtained evidences in directed evolution of subtilisin variant, comparisons of putative and predicted conformations along with instability of the diacetate substrate may explain the reasons of low perhydrolysis level measured in SC Perhydrolase. The above mentioned obstacles were the limiting factors in directed evolution campaign for identification of SC Perhydrolase variants with increased level of perhydrolysis towards diacetate ester substrate; on the other hand these new insights have implication in understanding the phenomenon of perhydrolysis in SC Perhydrolyse and defining requirements for its completions. Furthermore, the structural-functional knowledge gained in this study can be applied on other hydrolases showing perhydrolytic activity.

The successful application of \textit{in vitro} expression of SC Perhydrolase coupled with high throughput capacity of flow cytometry screening system requires further investigation and optimization. Further optimization will be performed in near future to overcome this bottleneck of high throughput platform.

SUMMARY

In summary, a transformation protocol was developed for \textit{B. subtilis} DB104 as an expression host in directed protease evolution experiment. The natural competence protocol allows for the first time to generate large libraries ($\sim 10^5$ transformants/µg plasmid DNA) in \textit{B. subtilis} DB104 and is furthermore robust, fast and simple in handling. In addition, the screening platform was developed in this work to improve protease and likely other hydrolase (e.g. lipases or esterases) having perhydrolytic activity or find new enzymes with peroxycarboxyllic acid production by screening metagenome libraries. The wide pH range (5-9) and robustness of the APCC detection system offers broad flexibility in terms of assay conditions.

Employing above mentioned developed technology it was possible to identified subtilisin Carlsberg variants in directed evolution experiment. Subtilisin proteases can be tailored for increased perhydrolytic activity and simultaneously increased oxidative resistance in the presence of peroxycarboxylic acids if amino acids close to the active site and which are prone to oxidation are substituted by less sensitive ones. In-depth analysis leads to the hypothesis that a couple of methionine residues
surrounding the catalytic Ser221 residue might represent a general principle to improve oxidative resistance in subtilisin-like proteases. One methionine will thereby sacrifice itself by acting as “suicide” antioxidant and reduce the solvent accessibility of the catalytically important methionine. In terms of performance the variant M7 (T59A/W217M) seems to be “the best” compromise between peroxycarboxylic acid production and oxidative resistance.

FUTURE WORK

Several studies regarding protein stability and substrate specificity, particularly in SC Perhydrolase can be derived from the present work.

The result obtained in the directed evolution study showed protective role of additional methionine residues in the protein exposed to the oxidative agents and opened the new approach within directed evolution campaign to reach a variant with increased oxidative stability. The decoration of protein surface with methionine residues may have positive effect on oxidative stability since the activity important methionine in proximity to the active site would be protected. From the computational studies, preliminary results showed that further investigation of the variants needs to include flexibility studies on the residues surrounding the catalytic triad and more detailed characterization of energies of the interactions. This data would provide additional information regarding the dynamic behavior of identified variants and could suggest modifications to the proposed analysis of the dynamic profiles.

The structure of the binding pocket, the size of the substrate residue and its orientation are the first criteria that determine the rate of perhydrolytic reaction. As already described, site saturation mutagenesis at the positions proposed using visual inspection of a protein model and docking analysis did not result in a variant with increased perhydrolytic activity towards diacetate. Further experiments in which combination of two and/or three site saturation mutagenesis position simultaneously would be introduced using the Omni Change method (Dennig et al 2011) could possible change specificity of the substrate binding pocket and influenced on beneficial conformation of diacetate due to the additive effect of the introduced residues.
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