Pathogenic, versatile and tunable activity of sortase, a transpeptidation machine

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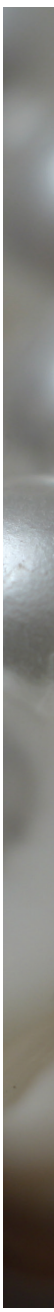
1 INTRODUCTION AND SCOPE OF THE THESIS



INTRODUCTION

Sortase A (SrtA) is a transpeptidase anchored in the membrane of bacteria. As indicated by its name, the role of this enzyme is the "sorting" of proteins with a specific cell wall sorting signal located at the C-terminus; these proteins are secreted from the cytoplasm and covalently attached to the cell wall by the SrtA in a transpeptidation reaction[1,2]. The activity of SrtA enzymes in Gram-positive bacteria was first discovered in *Staphylococcus aureus* bacteria (SaSrtA) by Schneewind and coworkers[3]. Since then, sortase enzymes have been found in many other Gram-positive and even some Gram-negative bacteria[1,4]. Based on their biological function and sequence alignment, sortases were initially divided into four classes, A to D[5]. Later on, two extra classes, E and F, were added to the nomenclature[4]. Class A enzymes are the most ubiquitous and can be found in nearly all Gram-positive bacteria. Therefore, sortases belonging to class A are also known as "housekeeping" sortases. In contrast, class B sortases are only found in a few Gram-positive bacteria and contribute to the regulation of heme uptake in the later phases of infection[6]. Class C sortases are specialized in the assembly of pilus subunits via the isopeptide bond formation. Bacteria use pili for the adhesion to surfaces or other cells as well as for biofilm formation[7]. The remaining three classes of sortases (D - F) are less characterized and currently not much is known about them[1,4,5].

Regardless of the sortase class, the mechanism of "sorting" or transpeptidation is similar for all members of the sortase family: in short, it is comprised of specific peptide cleavage of the substrate followed by the creation of new peptide bonds between two distinct molecules[3,8]. Specifically, in the case of SaSrtA the recognition sequence of the substrate is the pentapeptide LPXTG (X indicating any amino acid), which is followed by a hydrophobic region and a positively charged C-terminal tail[9,10]. Once the target protein is translocated from the cytoplasm to the outer membrane via the general secretory pathway (Sec), sortase recognizes this characteristic motif and cleaves it specifically between the T and G residues[11,12]. The resulting reaction intermediate of sortase and substrate is then attacked by a pentaglycine nucleophile of the bacterial peptidoglycan, resulting in the formation of a new peptide bond between the LPXT motif and the pentaglycine motif[13,14]. In general, the sortase reaction is renowned for its specificity. Nevertheless, some sortases are promiscuous in their substrate recognition and can therefore recognize more than one sorting signal. One example of a sortase with broader substrate specificity profile is the *Streptococcus pyogenes* sortase A (SpSrtA). Apart from the canonical LPXTG sorting motif, SpSrtA is also able to recognize LPXTA and LPKLG sequences[15,16]. In nature the two molecules coupled by sortase A are virulence factors produced in the cytoplasm and lipid II, the building block of the bacterial cell wall. In other words,

sortases are responsible for the attachment of different virulence molecules to the outer envelope of bacteria, thus strongly linking the enzyme to pathogenicity[17]. Not surprisingly, very shortly after its discovery, SaSrtA became a very attractive target for the development of new antivirulence strategies[18,19]. In contrast to other types of virulence factor display, sortases catalyze a covalent attachment of virulence factors to the cell wall. This is a generic strategy, observed in many Gram-positive bacteria[20,21]; most of their virulence factors possess an evolutionary conserved sorting signal[22,23]. Thus, inhibition of the sortase enzyme could lead to a reduced display of a range of essential virulence factors and thus a reduced pathogenicity of the bacteria. The significance of sortase's role in the pathogenicity of bacteria was shown by gene knockout studies conducted in mouse models: *S. aureus* mutant strains lacking the *srtA* gene were incapable of infection due to the failure of surface display of virulence factors[24]. In addition, studies performed on *S. pyogenes* showed that a knockout strain lacking the *srtA* gene became susceptible to phagocytic killing[25]. The search for SrtA inhibitors, including natural products and small molecule screening as well as rational design, has been focused on the interference of those particles with the catalytic cysteine residue. Although several inhibitors of SrtA have been identified, the IC_{50} values leave room for improvement[18,19].

Nature offers a large number of valuable molecules. Recently, even enzymes from pathogenic organisms can be cloned into safe high-production microorganisms and used in a practical way. The transpeptidation reaction performed by sortases has been exploited in and optimized for *in vitro* biochemical applications. Sortases can create new molecules or molecular formats that did not exist before and that cannot be produced by nature or other chemical reactions. Sortase-mediated transpeptidation (sortagging) is not only a site-specific but also a very simple process with a great potential to be used in a variety of biotechnology-based applications. Examples include labeling or modification of recombinantly produced proteins, soluble or coupled to the surface of living cells, and covalent anchoring of proteins to solid supports[26,27]. However, there are some drawbacks related to the catalytic competence of sortases: the catalytic efficiency of sortases is very poor, which lowers the potential of sortagging. To overcome these drawbacks, protein engineering has been successfully applied to improve the catalytic activity of SaSrtA variants[26,28,29]. Directed evolution makes use of the central dogma of molecular biology by introducing modifications into the DNA and selecting proteins with desired properties. Depending on the type of selection, proteins for different purposes can be found and used in a wide range of applications like biocatalysis, diagnostics, therapeutics and biotechnology.

SCOPE OF THE THESIS

My research plan has two directions: the first approach is to use sortase as a target for new antibiotic strategies. The second approach is the biotechnological application of sortases.

For the accomplishment of the first approach described in **Chapter 2**, a library of nitrogen-containing aromatic compounds with different substitution patterns is designed to find a potent inhibitor for SrtA from *S. pyogenes*. Screening of the library is made by using substitution-oriented fragment screening (SOS) method[30]. Compounds with a strong inhibitory potential are found. The engineering part of my research work presented in **Chapter 3** is focused on the improvement of the *S. pyogenes* sortase A[31] enzymes' kinetics and the broadening of substrate specificity by means of semi-rational approach. Selected single mutants are, through iterative saturation mutagenesis, combined into a triple mutant with doubled activity. In line with that, **Chapter 4** further investigates the structure and substrate specificity of the *S. pyogenes* sortase A enzyme by means of loop grafting. This chapter highlights the significance of the less studied β7/β8 loop in substrate recognition by sortase A. Furthermore, **Chapter 5** presents a semi-rational approach towards improvement of thermostability of *S. aureus* SrtA enzyme. By using a consensus analysis, mutants with enhanced activity, improved thermodynamic features and lower dependence on Ca^{2+} ions are found. **Chapter 6** gives an overview of high-throughput techniques employed in protein engineering which can be helpful in screening of big libraries of mutants. One of the high-throughput techniques based on a method named cellular high-throughput encapsulation solubilization and screening (CHESS)[32] is described in more details in **Chapter 7**. This chapter shows the proof-of-concept experiments for the implementation of the CHESS method for the engineering of properties of sortase A.

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