Engineering of Epimerases towards Pharmaceutical Applications

Sandro Giger completed his apprenticeship as a chemical laboratory technician at Novartis and then studied chemistry at the Zurich University of Applied Sciences (ZHAW). During his military service after graduation, he realized that he wanted to further deepen his knowledge. Back at the ZHAW, he was able to complete his master's degree under the supervision of Prof. Dr. Rebecca Buller, head of the biocatalysis group at the ZHAW. Currently, Sandro Giger is a PhD student at the ZHAW in collaboration with the Vienna University of Technology. His research focuses on engineering enzymes for synthetic applications.

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The World Health Organization (WHO) states that the current clinical pipeline of antibacterial agents is insufficient to tackle the challenge of increasing emergence and spread of antimicrobial resistance [1]. Many pharmaceutical companies have abandoned research on new antibiotics leading to a shortage of new drugs available [2]. Making matters worse, antibiotics are excessively used as human therapeutics and for livestock farming, thus facilitating and accelerating the development of bacterial resistances against existing drugs.

Antimicrobial peptides (AMPs) are found in all five kingdoms of live. They offer an alternative to classical antibiotic substances by selectively acting upon the negatively charged bacterial membranes, while the uncharged cell membranes of plants and animals remain unaffected [3]. So far, only a few AMPs such as vancomycin or polymyxin B are used in human therapy. AMP's oral bioavailability is typically limited due to low stability and poor penetration of the intestinal mucosa. Furthermore, regulatory agencies struggle with guidelines defining the minimum purity for peptide therapeutics [4]. D-amino acid containing peptides, however, offer a promising approach to overcome some of the drawbacks of AMPs [5]. In various studies, which compared D-amino acid containing AMPs with their all L-counterparts, D-amino acid containing AMPs have shown higher protease stability while maintaining or increasing their antimicrobial activity without exhibiting toxicity against mammalian cells [6, 7].

Radical S-adenosylmethionine (rSAM) enzymes create a radical species by cleaving S-adenosylmethionine (SAM) reductively, which enables them to catalyze a wide range of different reactions, such as epimerisations [8]. The first rSAM enzyme capable of selectively inverting the stereoconfiguration of amino acids in peptides, called PoyD, has been described in 2012 [9]. Later, OspD, AvpD and PlpD [10], as well as YydG [11] have been added to the rSAM epimerase family. The proposed reaction mechanism of rSAM epimerases works as follows: SAM is cleaved reductively by a [4Fe – 4S] iron-sulfur cluster resulting into a radical 5'-deoxyadeonsine species, which then abstracts the C_α-hy-

drogen atom from an amino acid in the peptide substrate. A solvent derived thiol hydrogen atom, provided from a cysteine residue, quenches the radical species stereoselectively resulting in the formation of a D-amino acid [11]. In my thesis, the rSAM epimerase YydG was expressed as a Strep-tag fusion protein in E. coli. In vivo YydG epimerizes one valine and one isoleucine residue in YydF [11]. The activity of YydG was verified by in vitro reactions with a truncated version of its native substrate, called YydFcore. To allow for analysis by LC-MS, the epimerized peptide was digested with trypsin resulting in three short peptides.

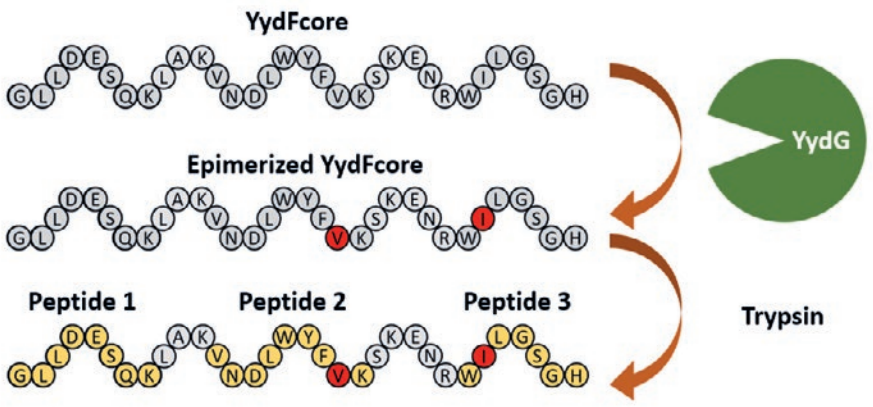

Figure 1: Schematic representation of the epimerization of peptide YydFcore with the epimerase YydG and subsequent digestion with trypsin for LC-MS analysis. The epimerized residues are colored in red, the three resulting peptides after trypsin digest are colored in yellow.

YydG was then tested upon additional substrates, including antimicrobial peptides. Notably, the enzyme was able to epimerize several non-native peptides and their modified analogues. Analysis of the epimerization pattern gave first insights into the mechanism at the basis of the enzymatic epimerization reaction, i.e., which amino acid sequence in the peptide might define the epimerization site.

Since the conformational change of a single or two amino acids in a peptide often only has a low impact on the analyzable characteristics of the molecule, analysis of epimerase products is delicate. To overcome this problem, the in vitro reactions with YydG were performed in buffer that was prepared with deuterated water. As the hydrogen atom which is donated by the enzyme to form the D-configured amino acid is ultimately derived from solvent, these experiments allowed for the incorporation of a deuterium atom into the peptide. Utilizing MALDI-TOF-MS, the isotopic shift of a deuterium incorporation can be observed in this way confirming the epimerization reaction. To pinpoint the exact epimerization site, epimerized peptides were subjected to LC-qTOF analysis, which allowed to determine the epimerization site by investigation of b- and y-ions of the peptides.Overall, only selected peptides were epimerized by YydG possibly indicating that certain

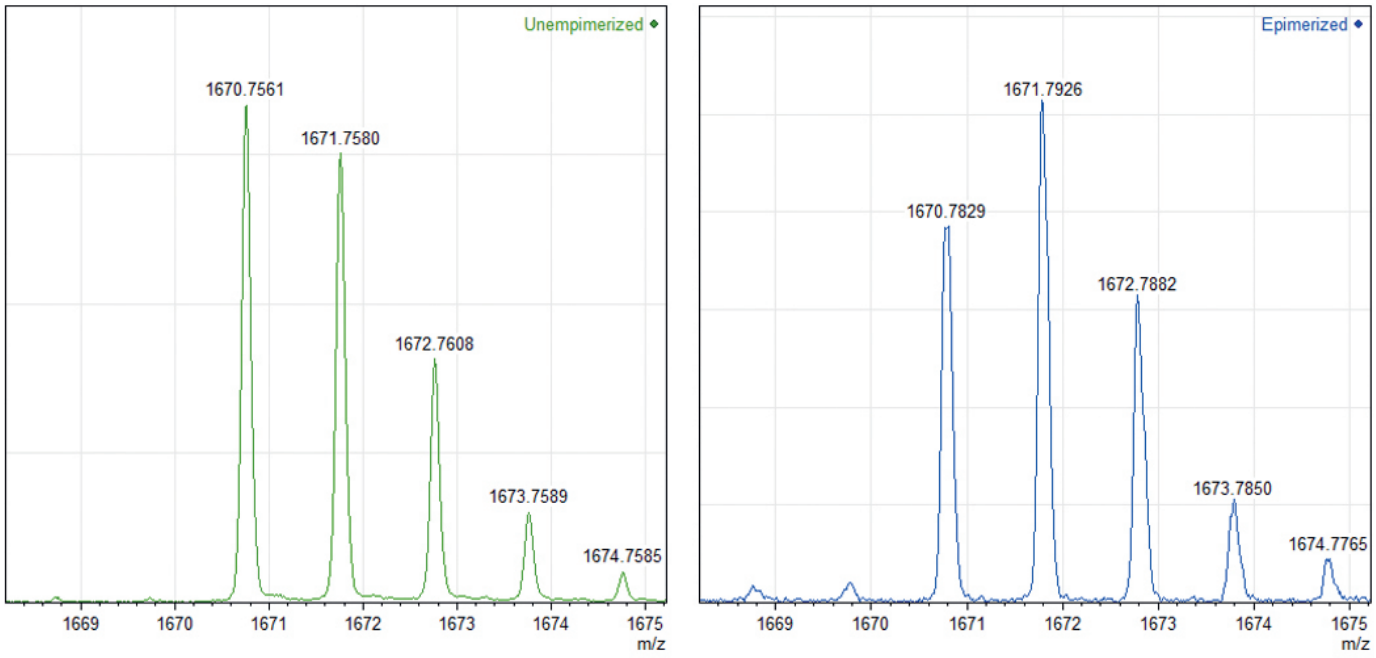

Figure 2: MALDI-TOF-MS spectra of two peptides. Left: Non-epimerized reference peptide. Right: Peptide epimerized in deuterated buffer, resulting in an observable mass shift.

peptide sequence motifs are crucial for epimerization by YydG. Using this knowledge, further studies will aim to investigate if additional non-native peptides can be epimerized by YydG.

References

[1] *World Health Organization, Antibacterial agents in clinical development: an analysis of the antibacterial clinical development pipeline. 2019.*

[2] *Ventola, C. L. The Antibiotic Resistance Crisis. P&T 2015, 40, 278–283.*

[3] *Zasloff, M. Antimicrobial peptides of multicellular organisms. Nature 2002, 415, 389–395.*

[4] *Mahlapuu, M.; Håkansson, J.; Ringstad, L.; Björn, C. Antimicrobial Peptides: An Emerging Category of Therapeutic Agents. Frontiers in Cellular and Infection Microbiology 2016, 6, 1–12.*

[5] *Gomes, B.; Augusto, M. T.; Felício, M. R.; Hollmann, A.; Franco, O. L.; Gonçalves, S.; Santos, N. C. Designing improved active peptides for therapeutic approaches against infectious diseases. Biotechnology Advances 2018, 36, 415–429.*

[6] *Oren, Z.; Shai, Y. Selective lysis of bacteria but not mammalian cells by diastereomers of melittin: Structure-function study. Biochemistry 1997, 36, 1826–1835.*

[7] *Di Grazia, A.; Cappiello, F.; Cohen, H.; Casciaro, B.; Luca, V.; Pini, A.; Di, Y. P.; Shai, Y.; Mangoni, M. L. D-Amino acids incorporation in the frog skin-derived peptide esculentin-1a(1- 21)NH2 is beneficial for its multiple functions. Amino Acids 2015, 47, 2505–2519.*

[8] *Broderick, J. B.; Duffus, B. R.; Duschene, K. S.; Shepard, E. M. Radical S-adenosylmethionine enzymes. Chemical reviews 2014, 114, 4229–4317.*

[9] *Freeman, M. F.; Gurgui, C.; Helf, M. J.; Morinaka, B. I.; Uria, A. R.; Oldham, N. J.; Sahl, H. G.; Matsunaga, S.; Piel, J. Metagenome mining reveals polytheonamides as post-translationally modified ribosomal peptides. Science 2012, 338, 387–390.*

[10] *Morinaka, B. I.; Vagstad, A. L.; Helf, M. J.; Gugger, M.; Kegler, C.; Freeman, M. F.; Bode, H. B.; Piel, J. Radical S-adenosyl methionine epimerases: Regioselective introduction of diverse D - amino acid patterns into peptide natural products. Angewandte Chemie - International Edition 2014, 53, 8503–8507.*

[11] *Benjdia, A.; Guillot, A.; Ruffié, P.; Leprince, J.; Berteau, O. Post-translational modification of ribosomally synthesized peptides by a radical SAM epimerase in Bacillus subtilis. Nature Chemistry 2017, 9, 698–707.*

[12] *Vagstad, A. L.; Kuranaga, T.; Püntener, S.; Pattabiraman, V. R.; Bode, J. W.; Piel, J. Introduction of D-Amino Acids in Minimalistic Peptide Substrates by an S-Adenosyl-L-Methionine Radical Epimerase. Angewandte Chemie International Edition 2018, 58, 2246–2250.*

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