Combinatorial approaches for introducing additional modifications into lantibiotics
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Chapter 6

General discussion
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The posttranslational modification (PTM) enzymes, LanBC and LanM, involved in the formation of (methyl)lanthionine in class I and II lanthipeptides, respectively, have been studied in some detail (1-3). Various papers proved the functionality and applicability of NisBC on peptide sequences other than the wild type nisin sequence (4). Building thioether bridges in designed peptides fused to the nisin leader peptide using NisBC stressed the important role of the leader peptide in the modification process as a key player in the enzyme-substrate interaction (5). Similar studies have been performed on other enzymes such as the bifunctional dehydratase-cyclase LctM from the lacticin 481 cluster and others (6-8). The extensive characterization and successful application of PTM enzymes (NisBC/LctM) on peptides of medical interest open a new avenue to improve the clinical potential and overcome possible drawbacks of therapeutic peptide drugs. For example, introducing a thioether bridge using NisBC into angiotensin-(1-7) has improved the stability of this therapeutic peptide significantly and enhanced its interaction with its receptor (4). In this thesis we studied the substrate tolerance of other lantibiotic PTM enzymes. We describe the successful applications of a range of PTM enzymes (i.e. GdmD, LtnJ and CinX) on nisin and its derivatives. Our studies prove that GdmD, LtnJ and CinX, similar to NisBC, are promiscuous enzymes able to introduce relevant modifications in designed peptides other than their native substrates. More interestingly, we show that their native leader peptides are not required for substrate recognition and modification.

Previously, a flavoenzyme, EpiD from Staphylococcus epidermidis was found to be responsible for the formation of S-[(Z)-2-aminovinyl]-D-cysteine (AviCys) in the lantibiotic epidermin (9). In the same paper, the EpiD mutant EpiD-G93D reduced the production of modified epidermin showing that native EpiD might play a vital role in stabilizing epidermin against proteolysis. This means that the interaction with EpiD and the subsequent modification stabilizes the end product (9). In Chapter 3 one analogue of EpiD, GdmD, from Staphylococcus gallinarum Tü3928 was applied successfully on nisin mutants in order to create an AviCys-containing nisin-like peptide. Some natural AviCys-containing peptides possess potent bioactivity and can inhibit the growth of sensitive bacteria at nanomolar concentrations (10, 11). Gallidermin has shown low toxicity towards human epithelial cells (12).

The investigation of a number of synthetic peptide libraries containing D-amino acids at certain locations proved the crucial role of D-amino acids in improving stability, altering tertiary structure and affecting the bioactivity of peptides (13). By using complementation
experiments, Cotter et al. reported that LtnJ is the enzyme responsible for the conversion of Dha to D-alanine in the two-component lantibiotic lacticin 3147 (14). The absence of this enzyme resulted in the residues remaining as Dha intermediates, causing a dramatic reduction in the antimicrobial activity and production of lacticin 3147. The decline of production might result from the instability of peptides lacking D-alanine (14). The successful application of LtnJ on nisin variants fused to the nisin leader peptide in chapter 3 proves the versatility of this reductase. A study on conserved residues of LtnJ showed that LtnJ(K359A) modified LtnA1 and LtnA2 of lacticin 3147 in a manner which reduced their antimicrobial activity while not changing their native mass (15). It implied that this mutated LtnJ retained the ability to reduce Dha but lacked the stereospecificity, yielding either L-alanine or a mixture of L-alanine and D-alanine residues rather than exclusively producing D-alanine. In chapter 3 we show that both prenisin (containing Dha at positions 5 and 33) and nisin-(Δ23-34) (containing only Dha5) gave a plus 2 dalton mass shift after being modified by LtnJ indicating that Dha5 is the substrate for LtnJ rather than Dha33. This raises another question, i.e. whether the location and flanking residues of Dha affect the catalytic activity of LtnJ. To answer this question, in chapter 4 a series of nisin analogues with linear polypeptide tails (AAXS\textsuperscript{26}XXALTIK), which facilitate sequencing by LC-MS/MS due to the absence of lanthionine rings in this region, are designed as test-substrates for LtnJ. The results show that hydrophobic flanking residues favor hydrogenation of Dha, while non-hydrophobic ones disfavor this process. Coincidently, hydrophobic residues at both sides of serines are preferred by the dehydratase NisB that catalyzes the dehydration of serine (16), which is mandatory to provide the substrate for LtnJ. This property makes peptide engineering combining NisB and LtnJ even more attractive. Additionally we corroborate the inefficiency of LtnJ to modify Dhb. The other reductase CrnJ with a broader substrate tolerance (either on Dha or Dhb) from Carnobacterium maltaromaticum C2 may be applied to some ribosomally-synthesized and post-translationally-modified peptides (RiPPs) that require both D-alanine and D-aminobutyrate for their activities, although a better characterization of such enzyme is required. Furthermore, the presence of D-alanine at the C-terminus of the designed mutants demonstrates that the activity of LtnJ is not restricted to the N-terminal part of the peptide. Our characterization of LtnJ shows its potential for protein engineering.

In chapter 5 we demonstrate the promiscuity of the newly discovered lantibiotic hydroxylase, CinX, with mutated nisin analogues as substrates and E. coli as producer strain. The hydroxylation of aspartic acid in cinnamycin plays a vital role in the interaction with
phosphatidylethanolamine (PE) in the target cell membrane causing growth inhibition (17). Our in vivo research on CinX creates a perspective to design new artificial peptides that might have different antimicrobial mechanisms and activities.

Besides the enzymes studied in this thesis, some others have been studied on lantibiotics and other posttranslationally modified peptides by other labs. A methyltransferase, CypM, responsible for the methylations of alanine at the N-terminus of cypemycin, which is a peptide antibiotic produced by Streptomyces sp. OH-4156, was exploited in vitro to methylate the α-amino group of the N-terminal residues of nisin and haloduracin (18). For nisin, the ε-amino group of lysine at position 12 was methylated by CypM as well. Surprisingly, the methylated nisin showed higher antimicrobial activity against L. Lactis HP (ATCC11602) and B. subtillis 168 (ATCC6633) (18). The substrate specificity of the peptidase ElxP and the oxidoreductase ElxO required for the maturation of epilancin 15X have been investigated recently (19). It showed that ElxP was able to proteolytically remove the leader peptide when the ElxP recognition motif DLNPQS was introduced into the noncognate lanthipeptide precursor NisA. Moreover, ElxO was shown to exhibit its functionality on peptides resembling the N-terminus not only of epilancin 15X (Pyr-AAIVK) but also of many other lantibiotics, e.g. epilancin K7 (Pyr-AAVLK), epicidin 280 (Pyr-LGPAIK), lactocin S (Pyr-APVLA), and Pep5 (Obu-AGPAIR) demonstrating that the (methyl)lanthionine residues and full length ElxA peptide were not strictly required for substrate recognition by ElxO (19, 20).

The study on the promiscuity of PTMs definitely extends the library of bioengineering tools that can be used to create new compounds with potential desirable traits. Taking into account the high cost and technical challenges that some complex modifications represent in chemical synthesis, especially when the compound is a large molecule, the introduction of modifications by PTM enzymes to ribosomally-synthesized peptides has bright perspectives for peptide design and in the medical field. A feasible way to increase this potential further is the screening of large size libraries for the discovery of novel antibiotics. Hypermodified antibiotics can be synthesized and screened in situ effectively nowadays, e.g. by high-throughput screening techniques (21). A better understanding of the substrate recognition mechanisms of PTM enzymes from lantibiotics and even from other RiPP clusters and their mechanism of action will also allow for a more rational design of peptides.

The low production level of artificially designed peptides is a common drawback needed to be tackled. In chapter 4, introducing a putative attenuator between the structural gene nisA
and the gene encoding LtnJ improved the production of NisA more than 2-fold. This putative attenuator may ensure an appropriate ratio of enzymes and structural peptide. Additionally, it has been reported that the inverted repeats can affect the stability of the transcript as in the case of Pep5 (22) or the enterocin AS-48 (23).

Inducible promoters are used to control protein expression and to obtain large amounts of heterologous proteins. In order to achieve this, starting the expression at the exponential phase is required to avoid the negative effect on the growth of host strain (24-27). Especially when the protein of interest is toxic, the induction at the beginning of growth will lead to the failure of overexpression. Additionally, there are not many available systems where the concomitant overexpression of proteins from two or more different inducible promoters has been achieved.

In chapter 2 we established a zinc-regulated expression system, called Zirex. Zirex is able to tightly control the expression of protein under different concentrations of zinc ions that yielded an amount of protein comparable to the NICE system in *L. lactis* (80% of that obtained with the nisin inducible promoter). Interestingly, the expression controlled by Zirex is not affected when coupled to the NICE system to overexpress two different proteins in the same cell. This makes it possible to regulate the synthesis of products in *L. lactis* that need different production levels of the modification enzymes or a different induction time. Refactoring gene clusters in this manner provides a rational way to increase the production yield of natural products (28). For instance, in chapter 4, D-alanine was observed in the peptide while serine/threonine residues at the C-terminus had not been fully dehydrated by NisB. This might be caused by the co-existence of both NisB and LtnJ acting simultaneously on the peptide. Competition for binding to the substrate peptide might lag the efficiency of NisB, consequently lagging the conversion by LtnJ. By using two different inducible promoters to regulate the time of expression of PTM enzymes could be an ideal way to solve the blockage of one PTM enzyme by another.

All in all, we have made and investigated different tools that can be applied to control the expression of proteins in *L. lactis*, hypermodify diverse peptide substrates with heterologous enzymes, and got an insight into the requirements for these enzymes to work (Figure 1). These data pave the way to the development of optimal production systems for designed substrates using Synthetic Biology.
Figure 1. Overview scheme of this thesis showing the relation between the different chapters. Yin-yang, in Chinese philosophy, describes how apparently opposite forces are actually complementary, interconnected, and interdependent in the natural world. In the battle against pathogens, finding and developing new antimicrobial compounds are not only the effort we should take. Strict administration over the application of antimicrobial compounds may slow down the evolution of those super bugs and contribute to the well-being of all of us.

References

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