
# Characterization of the nisin dehydratase NisB

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# Abstract

Nisin, consists of 34 amino acids, is antimicrobial active against Gram-positive bacteria and comprises five (methyl)lanthionine rings. Thus, nisin belongs to the group of lantibiotics. Nisin is ribosomally produced by *Lactococcus lactis* as a 57 amino acids long prepeptide. Posttranslational modifications introduce the dehydrated amino acids dehydrobutyrine and dehydroalanine as well as the cyclic thioether amino acids lanthionine and methyllanthionine. The enzymes involved in the posttranslational modification are NisB, which dehydrates specific serine and threonine residues in prenisin, and the cyclase NisC, which catalyzes (methyl)lanthionine formation. Fully modified prenisin is exported by the ATP-binding cassette (ABC) transporter NisT and the 23 amino acids long leader peptide is cleaved off by the extracellular protease NisP. Although much is known about its antimicrobial activity and mode of action, well-founded knowledge about the modification process is still rather limited.

In this work the *in vitro* interaction of the dehydratase NisB with unmodified, dehydrated and fully modified prenisin was investigated. Therefore, purifications of the enzyme and the different peptides were optimized to gain homogenous enzyme and prepeptides. Furthermore, the enzyme and the prepeptides were characterized by light scattering and HPLC analysis. Light scattering analysis demonstrated that purified NisB is a dimer in solution and HPLC analysis was used to identify unmodified, dehydrated and fully modified prenisin.

Using size exclusion chromatography and surface plasmon resonance, the interaction of NisB and unmodified, dehydrated and fully modified prenisin was studied. Unmodified prenisin binds to NisB with a dissociation constant of 1.05 ± 0.25 μM, whereas the dehydrated and the fully modified derivatives bind with respective dissociation constants of 0.31 ± 0.07 μM and 10.5 ± 1.7 μM. The much lower affinity for the fully modified prenisin related to a >20-fold higher off rate. For all three peptides the stoichiometry of binding was 1:1. Active nisin, which is the equivalent of fully modified prenisin lacking the leader peptide did not bind to NisB, nor did prenisin in which the highly conserved FNLD-box within the leader peptide was mutated to AAAA. Taken together the data indicate that the leader peptide is essential for initial recognition and binding of prenisin to NisB, and that NisB can discriminate between unmodified, dehydrated and fully modified prenisin.

# Zusammenfassung

Nisin ist ein antibakteriell wirksames Peptid, welches vom Milchsäurebakterium *Lactococcus lactis* ribosomal als ein 57 Aminosäuren langes Prepeptid synthetisiert wird. Posttranslationale Modifikationen führen zu den unnatürlichen Aminosäuren Dehydrobutyrin und Dehydroalanin als auch zu den Thioetheraminosäuren Lanthionin und Methyllanthionin, die intramolekulare Ringstrukturen ausbilden. Da Nisin ein Lanthionin-haltiges Peptidantibiotikum ist zählt es zu der Gruppe der Lantibiotika. Für die posttranslationale Modifikationen sind die Enzyme NisB und NisC verantwortlich. Die Dehydratase NisB katalysiert die spezifische Dehydrierung von Serin und Threonin und die Cyclase NisC katalysiert die Bildung der (Methyl)lanthioninringe. Das komplett modifizierte Prenisin wird von dem Transporter NisT exportiert und das 23 Aminosäuren lange Leader Peptide wird von der Protease NisP abgeschnitten.

Diese Dissertation untersuchte die Interaktion der Dehydratase NisB mit unmodifizierten, dehydrierten und komplett modifizierten Prenisin *in vitro*. Um homogenes Enzym und homogene Peptide zu erhalten, wurden die Reinigungen des Enzyms und der Peptide optimiert. Das gereinigte Enzym und die Peptide wurden mittels statischer Lichtstreuung und HPLC analysiert. Die statische Lichtstreuung zeigte das NisB in Lösung einen stabilen Dimer bildet. Unmodifiziertes, dehydriertes und komplett modifiziertes Prenisin wurden durch HPLC Analysen identifiziert.

Mittels Größenausschluss-Chromatographie und Oberflächen-Plasmon-Resonanz Messungen wurde die Interaktion zwischen NisB und den verschiedenen Prenisin analysiert. Unmodifiziertes Prenisin bindet mit einer Dissoziationskonstante von 1.05 ± 0.25 μM an NisB, wohingegen dehydriertes und komplett modifiziertes Prenisin mit Dissoziationskonstanten von jeweils 0.31 ± 0.07 μM und 10.5 ± 1.7 μM an NisB binden. Dabei wies komplett modifiziertes Prenisin eine mehr als 20fach höhere Dissoziationsrate auf. Alle drei Prenisine binden an dimerisiertes NisB mit einer 1:1 Stöchiometrie. Nisin, welches kein Leader Peptid aufweist und ein weiteres Prenisin, bei welchem die im Leader Peptid konservierte FNLD-Box zu AAAA mutiert wurde, konnten ebenfalls nicht an NisB binden. Das intakte Leader Peptid ist somit essentiell für die initiale Erkennung und Bindung der Prenisine an NisB. Dabei ist NisB in der Lage zwischen unmodifizierten, dehydrierten und komplett modifizierten Prenisin zu unterscheiden.

# Index

# 1. Introduction

## 1.1 Antibiotics and Resistance

Antibiotics are originally defined as substances, produced by living organisms, which inhibit the growth of another living organism (1,2). Today, the term antibiotic also includes semi-synthetic or synthetic produced compounds such as amikacin and sulfonamides (3,4). According to their biological effect, antibiotics can be subdivided into two groups. Bactericidals kill bacteria via inhibition of cell wall synthesis and bacteriostatics, which inhibit cell growth by inhibiting protein or DNA synthesis (1). Antibiotics exhibit a selective toxicity, which means that a particular antibiotic is toxic to one organism but not to another (1,2). If an antibiotic is selectively active against a pathogenic organism (bacteria) without harming the human host of this pathogenic organism, it can be used for medical treatment (1,2). Examples of such antibiotics are penicillin, vancomycin and tetracycline, which are often used to treat microbial pathogens within the human or animal body (1,2,5). Antibiotics can be subdivided in four groups according to their mode of actions (5).

1. Interference with cell wall synthesis.

    β-lactams such as penicillin or cephalosporin and glycopeptides such as vancomycin or teicoplanin inhibit bacterial cell wall synthesis of growing cells by interfering with enzymes involved in the synthesis of the peptidoglycan layer, which is a part of the cell wall, and thereby causing death of the cell (6-8). Penicillin as well as cephalosporin inhibits the crosslinking of peptidoglycan polymers by binding covalently to the active center of transpeptidases, which prevents the crosslinking reaction (6,9).

    Teicoplanin as well as vancomycin inhibits cell wall synthesis by binding to a cell wall precursor molecule, which inhibits the elongation of a peptidoglycan polymer (this will be described in more detail below) (8). By both mechanisms, the cell wall synthesis of a growing cell is inhibited, which causes disruption of the cell wall and subsequently cell death. Therefore, β-lactams and glycopeptides are bactericidal antibiotics.

2. Inhibition of protein synthesis.

Antibiotics such as tetracycline or chloramphenicol exhibit their antimicrobial activity by inhibition of bacterial protein synthesis. Chloramphenicol interacts with the peptidyl transferase region of the ribosomal 50S subunit where it blocks the polypeptide exit tunnel (10). By binding to the 30S subunit of bacterial ribosome tetracycline prevents the association of the aminoacyl-tRNA with the 30S subunit (11). By inhibition of protein synthesis, growth and reproduction of the bacteria is inhibited. Therefore, chloramphenicol and tetracycline are bacteriostatic antibiotics.

3. Interference with nucleic acid synthesis

Antimicrobial agents like fluoroquinolones interrupt DNA synthesis. Fluoroquinolones can inhibit topoisomerase IV, which is responsible for separation of interlinked chromosomes or plasmids. Therefore, the daughter chromosome or plasmid cannot be separated from the parental one. Topoisomerase IV is targeted by fluoroquinolones preferential in Gram-positive bacteria (12,13). Furthermore, fluoroquinolones inhibit gyrase, which is involved in relaxing supercoiled DNA prior to DNA replication. Flouroquinolones trap a DNA-gyrase complex, which then blocks movement of the replication fork (12). Gyrases are fluoroquinolone targets preferential in Gram-negative bacteria (13). Both mechanisms inhibit DNA replication during cell division. Therefore, fluoroquinolones are bacteriostatic antibiotics.

4. Disruption of the bacterial membrane

Antibiotics such as gramicidin increase the permeability of bacterial membranes. A gramicidin dimer builds an ion channel in target membranes, which selectively transports monovalent cations across the lipid bilayer, which allows monovalent ions to cross the membrane in an unrestricted manner (14). Therefore, it makes the target membrane more permeable for monovalent cations and destroys the ion gradient between the cytosol and the extracellular environment and causes cell death (15). Thus, gramicidin is a bactericidal antibiotic.

The use of antibiotics since the early 20$^{th}$ century has mitigated a lot of suffering and has saved millions of lifes. There was however also an unwanted side effect of the use of

antibiotics (3). Bacteria, which were treated by antibiotics, developed various forms of resistance (5). Whereas, some bacteria are innately not affected by a certain class of antibiotics, for example Gram-negative bacteria are not harmed by vancomycin, the ability of bacteria to acquire resistance is of greater concern (5). Bacteria, which acquired resistance to an antibiotic survive antibiotic treatment while still susceptible bacteria are killed. Therefore, these bacteria, which under normal condition could be a subpopulation can proliferate and spread under the selective pressure of the antibiotic (5,9). For example *Staphylococcus aureus* (S*. aureus*) expressing penicillin-binding protein 2a (PBP2a), which is a transpeptidase that exhibits low affinity to penicillin proliferates only under the selective pressure of penicillin (9). The development of resistance occurred by mutations, which may cause alteration of the antibiotic binding site (5,16). For example, the T338A/M339F mutations in PBP2x from *S. pneumoniae* reduces the penicillin efficiency more than 1000-fold by modifying the binding site of penicillin (17).

Another mechanism to acquire resistance is the uptake of genetic information, which encodes resistance (16). Genetic transfer could occur by conjugation, transduction or by transformation (16). During conjugation the resistance gene is directly transferred from one cell to another by for example a pilus, which joins the two cells (5). Transduction occurs via bacteriophages that transfer resistance genes from one bacterium to another (16). In contrast, transformation is the uptake of DNA of lysed cells from the environment (16). For example the *vanA* gene, which lead to an alteration of the vancomycin binding site, was transferred from vancomycin resistant *enterococci* (VRE) to vancomycin susceptible S*. aureus*, which resulted in vancomycin resistant *S. aureus* (VRSA) (18).

Bacteria exhibit several mechanisms to protect themselves against antibiotics. These mechanisms can be subdivided into four groups, which will be described below.

1. Enzymatic inactivation of the antibiotic.

   Classical examples of such resistance mechanism are β-lactamase enzymes. β-lactamases cleave the β-lactam ring of penicillin and cephalosporin, which changes the structure of the antibiotics and prevents binding to transpeptidases. Therefore, the crosslinking of the peptidoglycan polymers is not inhibited (7).

2. Reduction of the membrane permeability.

Reduced expression of outer membrane porins like OmpF in *E. coli*, due to a mutated transcription regulator, reduces the drug diffusion through the membrane and mediates drug resistance against fluoroquinolones, tetracycline and β-lactams (19,20). Therefore, accumulation of the antibiotic above inhibitory concentrations in the target cell is abolished (16).

3. Efflux of antibiotics.

Some bacteria protect themselves against antibiotics by the use of efflux pumps. Efflux pumps are found in Gram-positive and Gram-negative bacteria as well as in eukaryotic organisms. Efflux pumps extrude antibiotics from inside the cell to the external environment and therefore the concentration of the intracellular antibiotic is reduced (21).

The AcrAB/TolC complex of *E. coli* is the major antibiotic efflux pump and exports antibiotics entering the cell. AcrB is a proton gradient driven exporter located in the in the inner membrane of *E. coli* (22). It exhibits a broad substrate spectrum and exports many antibiotics including fluoroquinolones, chloramphenicol and tetracyclines (23). For direct export of substrates into the external medium AcrA, a periplasmic membrane fusion protein (MFP), TolC, an outer membrane factor (OMF) and AcrB assemble to a complex, which spans both membranes and the periplasma of *E. coli* (22). As it mediates resistance against multiple antibiotics the AcrAB/TolC complex is termed a multidrug resistance (MDR) efflux pump. A mutation in the repressor, which regulates the *acrAB* gene expression leads to overexpression of AcrAB, which causes a constant drug efflux (24). Therefore, antibiotics cannot accumulate in the target cell and the bacteria becomes resistant, because it tolerates higher concentrations of the antibiotic (3). Similar efflux systems also occur in Gram-positive bacteria and in eukaryotes. For example, in *S. aureus* NorA which also is a proton motive force dependent efflux pump, exports several fluoroquinolones (25). In human cancer cells, overexpression of ATP-binding cassette transporters such as P-glycoprotein (Pgp), ABCG2 or MRP1 is responsible for MDR since they export a wide range of structurally unrelated chemotherapeutics (26). The ABC transporters Pgp, ABCG2 and MRP1 are also

expressed in non-cancer cells, where they are important for detoxification of healthy tissue (21).

4. Alteration of the target site.

Mutations in the penicillin binding site of transpeptidases, which are targeted by penicillin and are termed penicillin-binding proteins (PBPs), significantly reduces the affinity of penicillin to PBPs (as mentioned above). Therefore, a higher penicillin concentration is required to kill the bacteria (3,9). However, such high concentrations of an antibiotic could also be toxic to the patient who is infected by the resistant bacteria (16).

During the last 60 years bacteria have developed resistance due to the extensive use of antibiotics (27,28). Nowadays, the emergence of multidrug resistant pathogens is a public health problem. Therefore, development and discovery of new antibiotics is necessary to overcome such highly resistant microbial pathogens (29-31). As a consequence of the dissemination of MDR bacteria, the first generation of antibiotics like penicillin has become less effective (30) and the number of therapeutic options to treat live-threatening diseases is decreasing (32).

Prominent multidrug resistant bacteria are methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *enterococci* (VRE) (30). MRSA strains are resistant to all β-lactams and other groups of antibiotics like erythromycin and tetracycline (33). The resistance against β-lactam derivatives is mediated by penicillin-binding protein 2a (PBP2a), which has a low affinity to β-lactams (30,31). This leads to tolerance of otherwise inhibitory concentrations of β-lactams (30,31). Therefore, MRSA is also resistant against methicillin, which is not inactivated by β-lactamases (9). VRE strains like vancomycin resistant *Enterococcus faecium* exhibit also ampicillin and fluoroquinolone resistance (34). Resistance to vancomycin and other glycopeptides in *enterococci* is mediated by conversion of the vancomycin-binding site (35,36). Vancomycin exhibits antimicrobial activity by binding to the essential cell wall precursor lipid II and therefore inhibiting cell wall synthesis (8,37).

The cell wall of Gram-positive bacteria consists of a thick peptidoglycan layer (38). In Gram-negative bacteria a thin peptidoglycan layer is located in the periplasma between the inner and outer membrane (39). Vancomycin is only active against Gram-positive bacteria,

since it is unable to penetrate the outer membrane of Gram-negative bacteria (40). Peptidoglycans consists of two amino sugars, N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc), and a pentapeptide which is attached to MurNAc consisting of L-Ala, D-Glu, L-Lys, D-Ala and D-Ala in the case of Gram-positive bacteria (41). Lipid II consists of a membrane anchored carrier, an undecaprenyl phosphate and an attached peptidoglycan monomer (Figure 1A) (42).

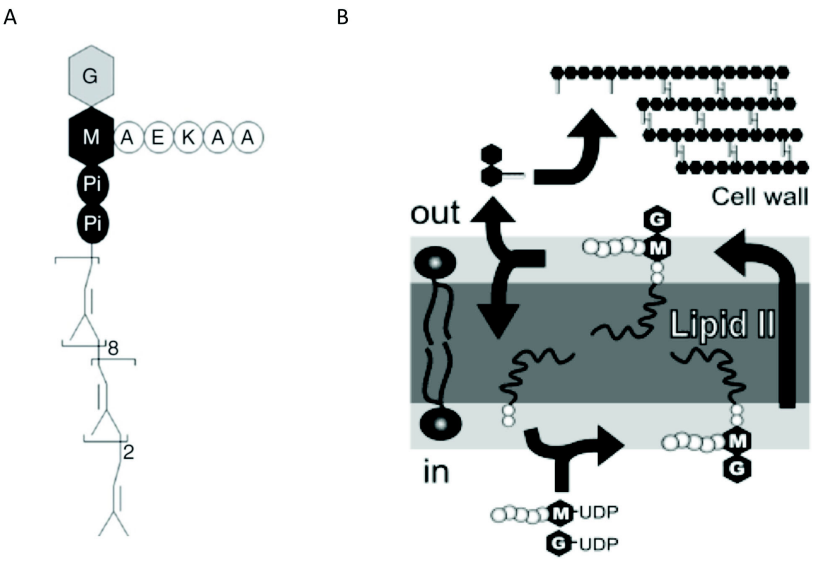


**Figure 1. Structure of lipid II and a model for cell wall synthesis of Gram-positive bacteria. A:** Structure of lipid II. The peptidoglycan monomer consists of N-acetylglucosamine (G) and N-acetylmuramic acid (M). Attached to N-acetylmuramic acid is the pentapeptide L-Ala, D-Glu, L-Lys, D-Ala, D-Ala (AEKAA). The undecaprenyl chain, which is attached to the pyrophosphate (PiPi) consists of 8 isoprene units in the cis-conformation followed by 2 units in the trans-conformation and the terminal isoprene. **B:** Assembly of lipid II in the inner leaflet and subsequent transport of lipid II across the membrane where peptidoglycan is released and elongates the peptidoglycan polymer. After elongation of the peptidoglycan polymer the pentapeptide (white circles) is cross-linked with another peptidoglycan layer to confer rigidity of the cell wall. The remaining undecaprenyl phosphate flips back for the next cycle. Taken from (43,44).

After assembly of the sugar complex in the cytosol lipid II is transported to the outer leaflet by the recently found transporter FtsW (43). Hereupon, the peptidoglycan is released and elongates the peptidoglycan polymer to form the cell wall (Figure 1B) (38,42). The essential role of lipid II in cell wall synthesis makes it a suitable target for many antibiotics. Vancomycin specifically targets the C-terminal D-Ala-D-Ala residues of the pentapeptide of lipid II (Figure 1A), which causes a steric hindrance and prevents the elongation of a peptidoglycan polymer. By changing the vancomycin binding site from D-Ala-D-Ala to D-Ala-D-lactate (D-Ala-D-Lac) vancomycin resistant bacteria prevent binding of vancomycin to lipid II (35).

Although antibiotic resistance of Gram-positive and Gram-negative bacteria is on the rise, the number of newly approved antibiotics has decreased to only six since 2003 (31). Therefore, new antibiotics are necessary to battle the increasing number of resistant pathogens (31). Since antibiotics used in healthcare facilities belong to a few classes of molecules, which have been explored and modified (e.g. β-lactams and glycopeptides), it seems unlikely that new improved agents or variants within those classes will be found (31). Therefore, it is necessary to search for antimicrobial agents that are not related to those in clinical use (31). In recent years, a class of antibiotic peptides which exhibit a high antimicrobial activity, the so-called lantibiotics, have gained special interest (30).

## 1.2 Lantibiotics

Lantibiotics are produced by Gram-positive bacteria mainly for self-defense purposes as for example nisin from *Lactococcus lactis* or lacticin 481 from *Streptococcus lactis* (45). They can also function as quorum sensors to activate their own biosynthesis like nisin (46) or cytolysin from *Enterococcus faecalis* (47). Quorum sensing is used by bacteria to regulate cell-density dependent gene expression (48). Herein, cells sense a specific molecule, which functions as signal molecules to induce gene expression (49). For example, the nisin production, which is induced by sensing extracellular nisin, starts at early- to mid-logarithmic growth phase and reaches a maximum at the early stationary phase (48). Furthermore, some lantibiotics act as morphogens like SapB from *Streptomyces coelicolor*, which functions as a biological surfactant decreasing the surface tension at the air-water interface. This allows its producer strain to escape the aqueous milieu and grow into the air (50).

Lantibiotics are ribosomally synthesized as a prepeptide and are posttranslationally modified by several enzymes to be converted into an active lantibiotic (51). The posttranslational modifications lead to 2,3-dehydroalanine (Dha) and 2,3-dehydrobutyrine (Dhb) by dehydrating serine and threonine residues. Whereas, lanthionines and methyllanthionines result from coupling of Dha/Dhb to cysteines (see below and Figure 2) (45). The terminus "lantibiotic" is derived from "lanthionine containing antibiotic" (52). Lanthionines are thioether bridged amino acids and introduce intramolecular ring formation within the peptide (51). Since it was shown that nisin and Pep5 from

*Staphylococcus epidermidis 5* consisting (methyl)lanthionine rings were not harmed by proteases it is assumed that the (methyl)lanthionine rings protect the lantibiotics against proteolytic degradation by making the potential cleavage site less accessible to proteases (53,54). Whereas, elimination of a (methyl)lanthionine ring makes Pep5 accessible to proteolytic degradation (53).

In general, lantibiotic prepeptides consist of two parts, an N-terminal leader peptide, which directs the prepeptide to the modification enzymes (55-57) and a C-terminal propeptide, where the modifications occur (Figure 2) (58).

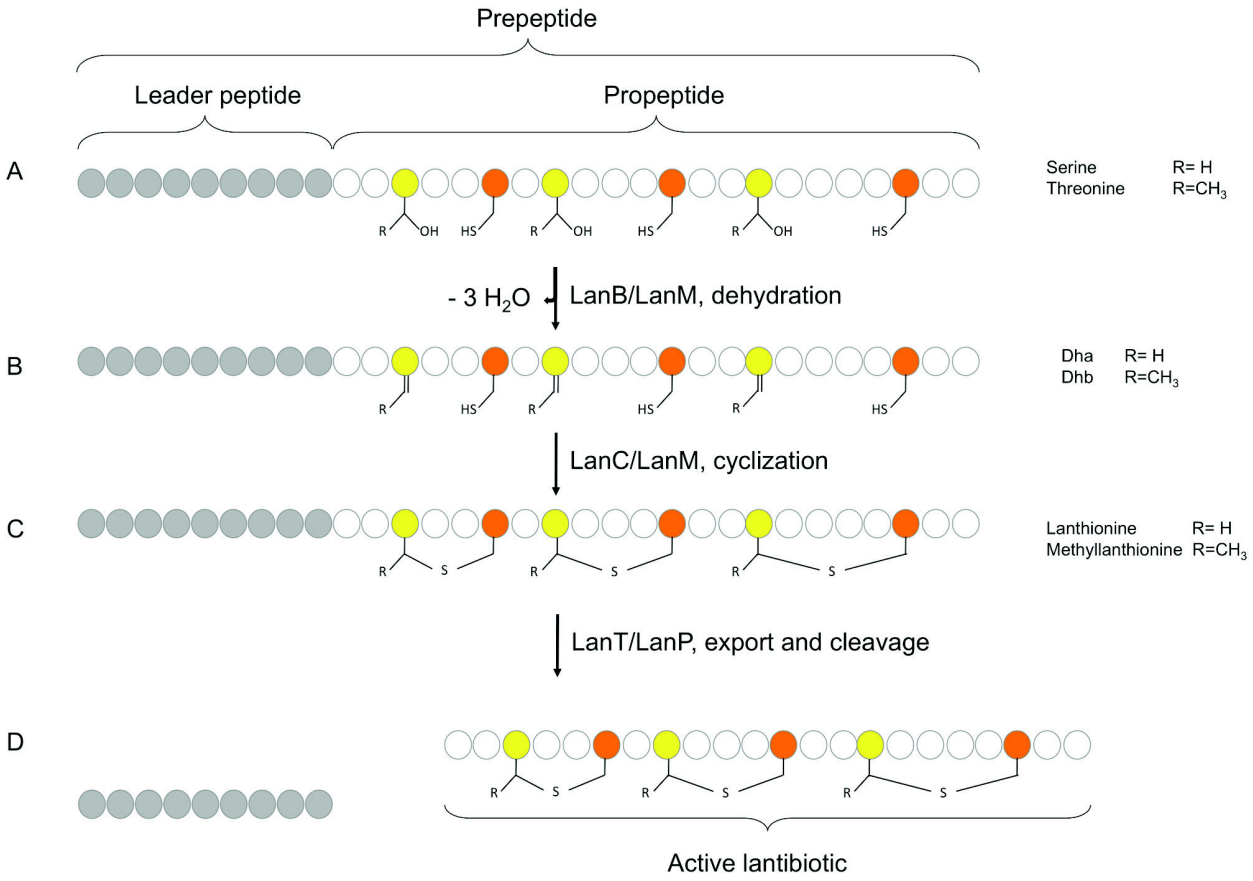


**Figure 2. Schematic illustration of lantibiotic modification and export.** The leader peptide is depicted in grey and the propeptide is represented by white circles. **A:** Unmodified prepeptide with yellow highlighted serine or threonine residues becomes dehydrated by LanB or LanM enzymes. **B:** Dehydrated prepeptide with Dha/Dhb (yellow) becomes regio- and stereospecifically coupled to cysteine residues (orange) by LanC or LanM enzymes. **C:** Fully modified prepeptide with (methyl)lanthionine rings becomes exported by the ABC-transporter LanT. **D:** Active lantibiotic and the leader peptide, which was cleaved off by either LanP (class I) or LanT (class II).

Common to all lantibiotics are the dehydrations of serine and threonine residues in the propeptide yielding 2,3-dehydroalanine and 2,3-dehydrobutyrine, respectively (Figure 2B). Subsequently, these dehydrated residues are coupled to cysteines to form lanthionine or

methyllanthionine rings (Figure 2C) (59). When the prepeptide is fully modified, the peptide is exported and the leader peptide is cleaved off by a protease. Thus, the active lantibiotic is released into the extracellular space (Figure 2D), whereas lantibiotics with the leader peptide still attached exhibit almost no antimicrobial activity (54,56-58).

Besides serine/threonine dehydration and ring formation, 15 other types of posttranslational modifications have been identified in various lantibiotics, which are shown in Figure 3 (59).

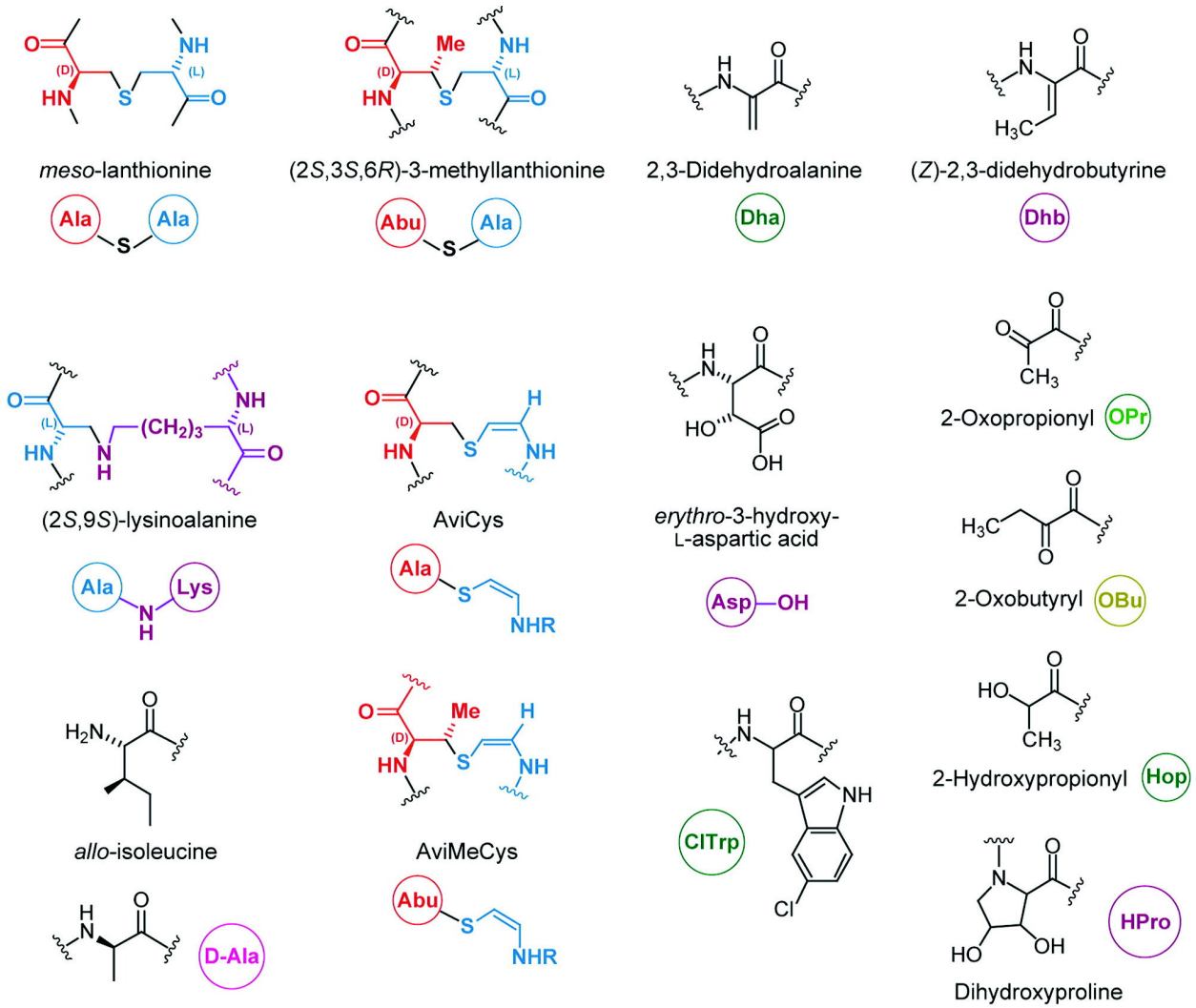


**Figure 3. Posttranslational modifications identified in lantibiotics.** ClTrp: chlorinated tryptophan. Taken from (59).

In 1991, lantibiotics were classified based on their structure and mode of action. Type A lantibiotics like nisin from *L. lactis*, subtilin from *B. subtilis* or epidermin from *S. epidermidis* exhibit an elongated flexible conformation, are positively charged, amphiphilic and disturb bacterial membranes (60). Type A lantibiotics are further subdivided in type AI and type

AII according to the enzymes, which introduce the modifications. In type AI lantibiotics such as nisin and subtilin, the (methyl)lanthionine rings are introduced by two distinct modification enzymes, which belong to either the LanB or the LanC family. LanB enzymes dehydrate serine and threonine residues in the propeptide (Figure 2B) and LanC enzymes couple the dehydrated residues to cysteines (Figure 2C). In contrast, in type AII lantibiotics like lacticin 481 both reactions are catalyzed by a single enzyme belonging to the LanM family (Figure 2) (61). Type B lantibiotics, like mersacidin produced by *Bacillus* sp. strain HIL Y-85,54728 and cinnamycin from *S. cinnamoneus* are of more globular shape, negatively or neutral charged and they act by inhibiting enzymes (45). The modification of type B lantibiotics is exclusively catalyzed by enzymes of the LanM family (60,61). Currently, over 60 lantibiotics are known, with some of them lacking antimicrobial activity (45). Since so many lantibiotics are known the clarity of the old classification diminished and a new classification was proposed, which is based on the biosynthesis machinery used for maturation of the active lantibiotic and on the presence or absence of antimicrobial activity (59). Therefore, lantibiotics were subdivided into three classes (Figure 4). Class I lantibiotics like nisin, subtilin or epidermin are modified by LanB (lantibiotic dehydratase) and LanC (lantibiotic cyclase) enzymes and subsequently the modified prepeptide is exported by a dedicated ABC transporter, which belongs to the LanT family. The maturation of the class I lantibiotics is completed after an extracellular anchored protease (LanP) cleaves off the leader peptide. Class II lantibiotics like lacticin 481, cinnamycin or mersacidin are modified by LanM enzymes, which possess dehydration as well as cyclization activity with the C-terminus of LanM enzymes showing a low sequence identity to LanC enzymes (62). A single, multifunctional protein performs the export and cleavage of class II lantibiotics with the protease domain conserved at the N-terminus of the protein. Note that this protein family is also designated as LanT, like the class I transporter family, although it has a additional function (59). The two-component lantibiotics like lacticin 3147 from *Lactococcus lactis* subsp. *lactis* DPC3147 and haloduracin from *Bacillus halodurans*, which are assembled by two peptides to form the active lantibiotic belong also to the class II lantibiotics (45,60). Lantibiotics of class I and class II exhibit antimicrobial activity whereas class III lantibiotics lack significant antimicrobial activity (59). Examples of the latter are SapB and SapT form *Streptomyces tendae*, which are morphogenetic peptides (50,63). Through out this work the class I, II and III classification is used.

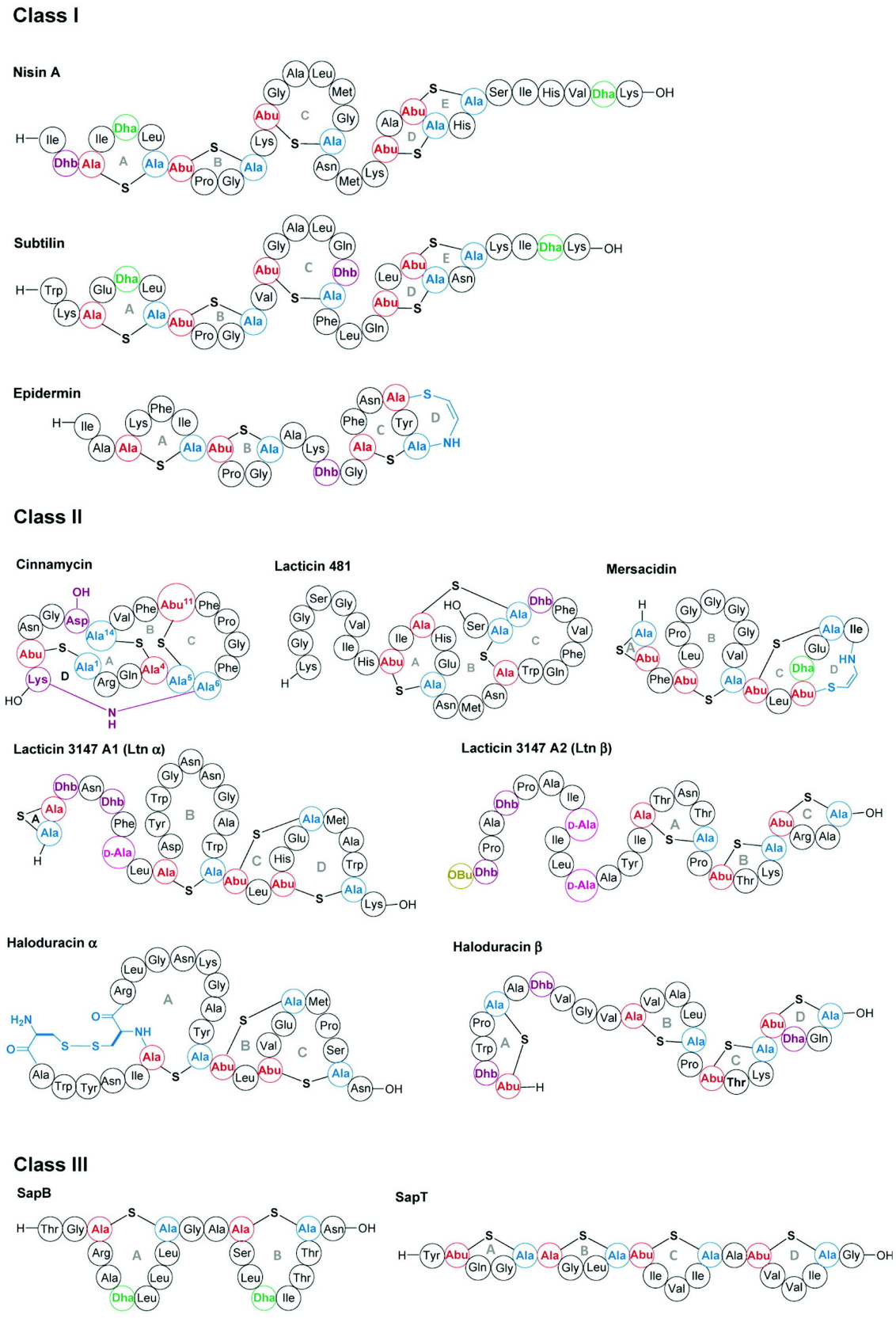


**Figure 4. Examples of the three lantibiotic classes.** In lanthionine and methyllanthionine rings the red Ala/Abu segments are derived from Ser/Thr residues, respectively. Dha/Dhb that are not involved in ring formation are highlighted in green and purple, respectively. Other posttranslational modifications are highlighted as well. Taken from (59).

The genes and proteins involved in lantibiotic biosynthesis have been assigned the generic locus symbol *lan* and Lan, respectively (64). Genes encoding the prepeptide (*lanA*), the proteins involved in lantibiotic modification (*lanB*, *lanC*, *lanM*), transport (*lanT*), processing (*lanP*), immunity (*lanI*, *lanFEG*) and regulation (*lanRK*) are arranged in gene clusters (Figure 5).

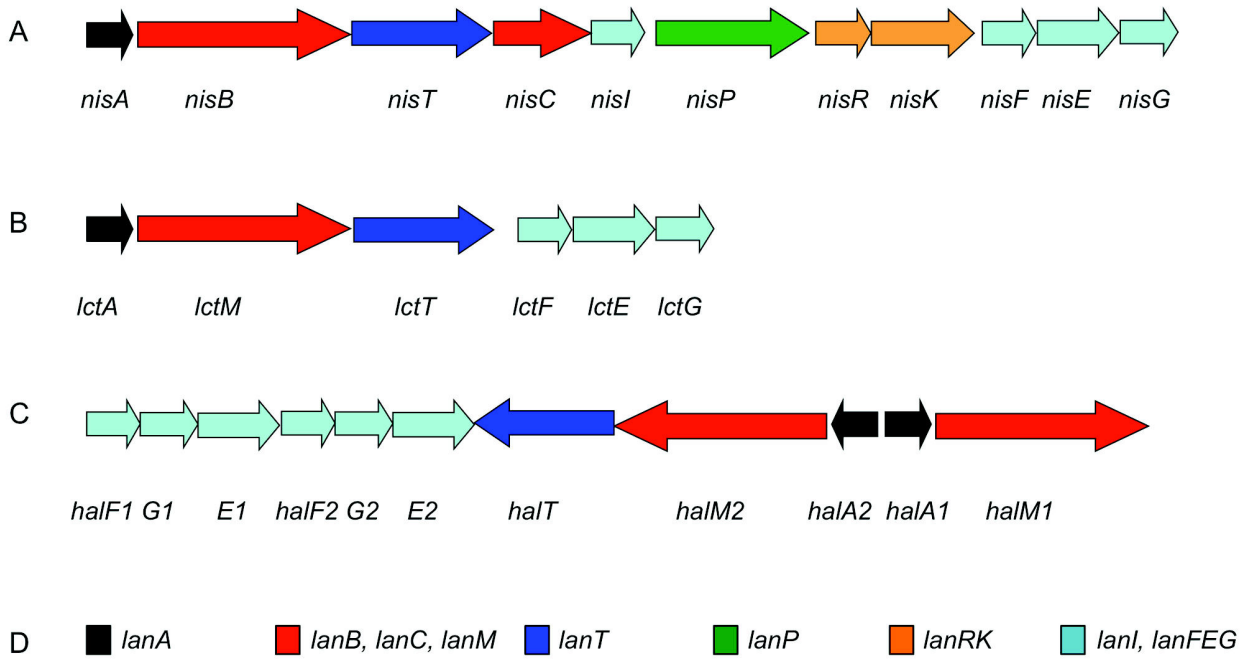


**Figure 5. Representative gene clusters of selected lantibiotics. A:** Nisin gene cluster from *L. lactis*; **B:** Lacticin 481 gene cluster from *S. lactis*; **C:** Haloduracin gene cluster from *B. halodurans*. **D:** Genes, which encode prepeptides (black), modification enzymes (red), transporters (blue), protease (green), the two component regulatory system (orange) and autoimmunity proteins (cyan) are highlighted in identical colors.

These clusters can be encoded chromosomally (subtilin) or on transposons (nisin), which are gene sequences that can move themselves to a new position within a genome of a cell (39), or on plasmids (nukacin ISK-1) (59,64). The biosynthesis of many lantibiotics like nisin or subtilin is triggered by a two-component regulatory system. This two-component system consists of a receptor histidine kinase (LanK) and a transcriptional response regulator (LanR) (60,65). In case of nisin or subtilin the extracellular lantibiotic act as a signal peptide, which is recognized by the corresponding LanK and induce autophosphorylation of a histidine residue in LanK (45). The high energy phosphoryl group is subsequently transferred to an aspartate residue within the response regulator protein (LanR) (65). This initiates the binding of LanR to distinct promoters, which activates the

transcription of the lantibiotic gene as well as the genes of the modification and immunity proteins (Figure 6) (60).

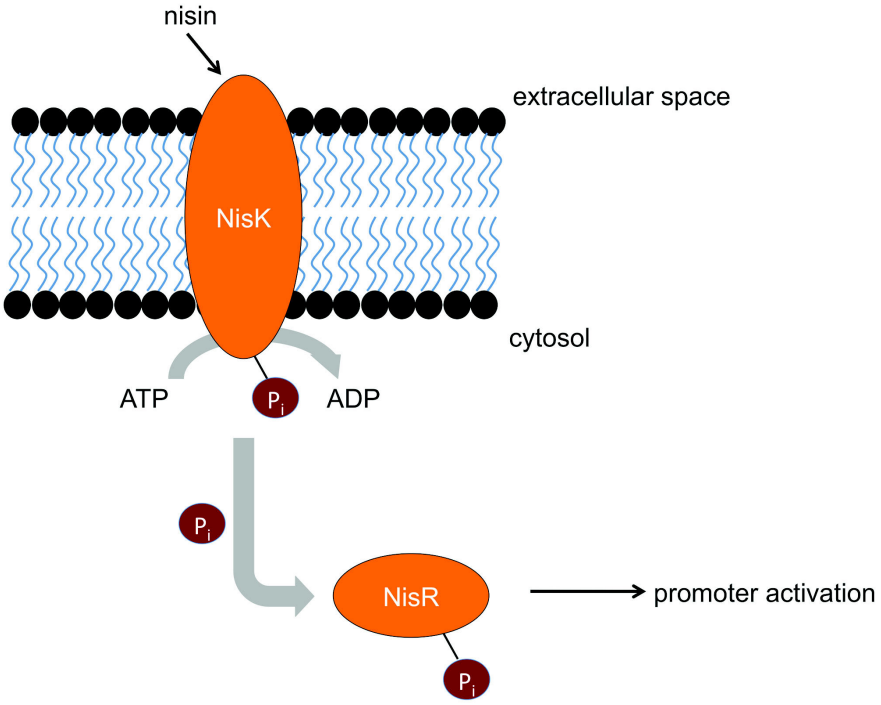


**Figure 6. Two-component regulatory system of nisin.** Upon recognition of an extracellular signal autophosphorylation of LanK occurs. Subsequently, the phosphoryl group is transferred to LanR, which activates the promoters for lantibiotic biosyntheses and autoimmunity.

Several lantibiotics like nisin and subtilin in non-lethal concentrations serve as a signal peptide, which induce their own biosynthesis (45,59,60). Biosynthesis of other lantibiotics is regulated by systems unrelated to a two-component regulatory system like NisRK. For example, a change in pH of the cell environment induces lacticin 481 production via the transcription regulator RcfB (66). Furthermore, the production of the two-component lantibiotic cytolysin from *E. faecalis* is stimulated upon the presence of a potential target cell (47). Cytolysin consists of $CylL_S$'' and $CylL_L$'', which form a stable complex that has neither toxic nor regulatory functions. In the presence of a target cell $CylL_L$'' preferentially binds to the target membrane. Whereas, dissociated $CylL_S$'' functions as a signal peptide to induce cytolysin production, which is repressed by CylR2 in the absence of target cells (47). Because lantibiotics are produced by and are active against Gram-positive bacteria the producer strains have to protect themselves against their own antimicrobials. The autoimmunity is commonly mediated by one or both of the two methods. First, a specific immunity protein, which is extracellular anchored to the cell wall is supposed to intercept

extracellular lantibiotics and therefore protects the cell wall for being targeted (LanI) (67,68). Secondly, ABC-transporters (LanFEG) reduce the amount of nisin that is associated to the cell suggesting an export function (68). LanF is the nucleotide-binding domain (NBD), which hydrolyzes ATP and provides the energy for the transport. LanE and LanG are the transmembrane domains (TMD) of the transporter (68,69). Since ABC transporters consists of four domains, two NBDs and two TMDs, it is assumed that a $LanF_2EG$ complex is formed (61). The autoimmunity proteins of *L. lactis*, NisI and NisFEG, were demonstrated to work synergistically, as deletion of either one of NisI or NisFEG resulted in 5-20 % of immunity compared to strains having both present (70,71).

Lantibiotics act mainly against Gram-positive bacteria, including pathogens as MRSA, *enterococci* or *Clostridium difficile* (72-74). Some class I lantibiotics as nisin and subtilin and probably many more are characterized by a dual mode of action. One mode of action is the inhibition of cell wall synthesis and the other one is the permeabilization of the cytosolic membrane by forming pores. Both have in common that the lantibiotic binds to lipid II, which is an essential cell wall precursor molecule of Gram-positive bacteria (see below) (30,45). Other class I lantibiotics like epidermin, gallidermin from *S. gallinarum* and mutacin 1140 from *S. mutans* also bind to lipid II but their ability to form pores is strain specific as the length of the lantibiotic and the thickness of the target membrane determines the ability to permeabilize the membrane of sensitive strains (75-77). However, they are able to delocalize lipid II from the septum, the division site of two cells, and thus cell wall synthesis is abolished (78). Mersacidin, a class II lantibiotic, was also shown to interact with lipid II but does not form pores. It inhibits cell wall synthesis by blocking the transfer of a peptidoglycan monomer to the peptidoglycan polymer (79).

Alignment of class II lantibiotics revealed that there is a TxS/TxE/DC motif conserved within one ring structure of these lantibiotics (80), whereas in class I lantibiotics rings A and B share conserved motifs (Figure 7) (30,45). Therefore, it is likely that class I and class II lantibiotics bind to different sites within lipid II. These binding sites differ from the vancomycin binding site (the pentapeptide) as these lantibiotics are active against vancomycin resistant bacteria (45).

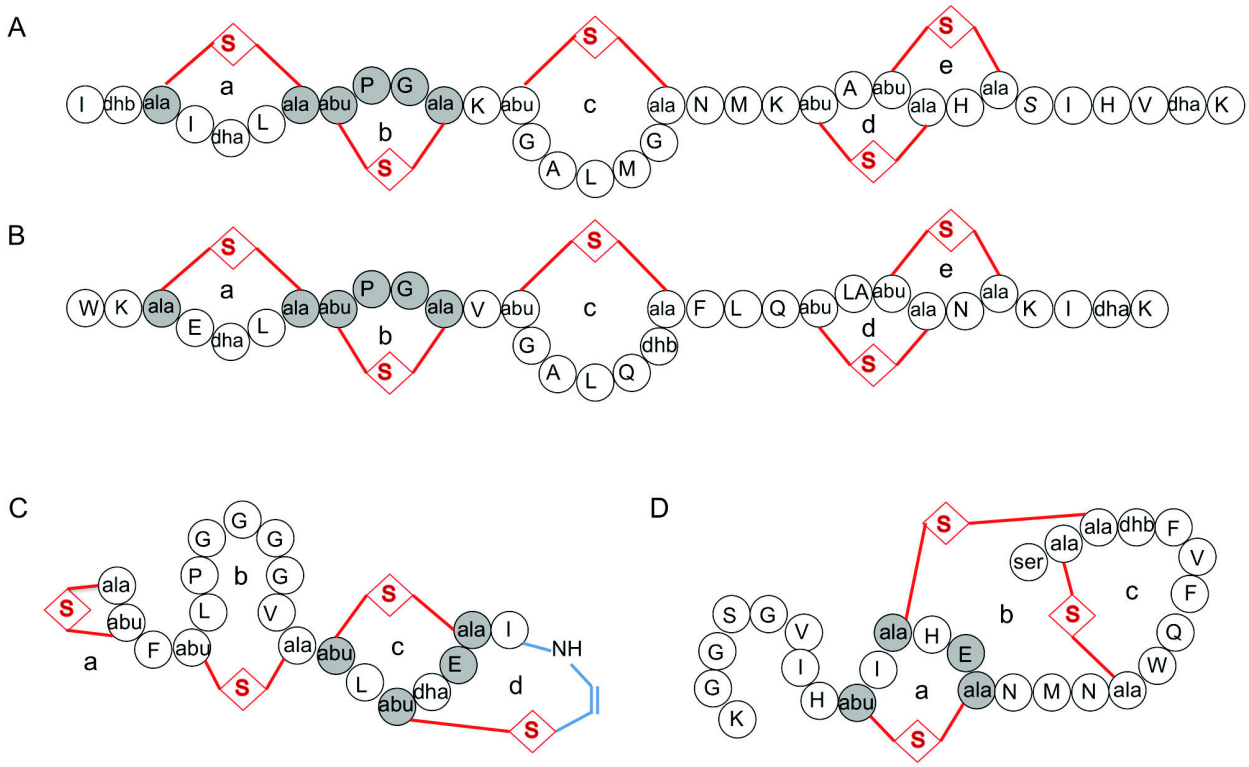


**Figure 7. Conserved motifs within class I and class II lantibiotics. A:** Nisin (class I); **B:** Subtilin (class I); **C:** Mersacidin (class II); **D:** Lacticin 481 (class II). Conserved motifs are represented in grey.

## 1.3 Nisin production by *L. lactis*

Nisin is produced by *L. lactis* as a prepeptide consisting of 57 amino acids and is the best-studied lantibiotic (45,61). After maturation, active nisin consists of 34 amino acids (54). Its antimicrobial activity against Gram-positive bacteria was first described in 1928 by Rogers and Whittier who showed that a substance secreted by *L. lactis* cells inhibited growth of other species as well as the growth of the producer strain after the culture reached a certain cell density (81). Nisin, is used as a food preservative since 40 years for example in milk products, canned vegetables, meat and fish (82) and is marked with the E number E234. E numbers mark food additives that have been assessed in the European union (83). Nisin is highly active against Gram-positive bacteria such as *Bacillus cereus*, *Listeria monocytogenes*, e*nterococci*, *staphylococci* and *streptococci* (61). It is classified as a class I lantibiotic, because it is modified by two distinct enzymes (NisB and NisC) and exported by NisT. The maturation of nisin is finished after the leader peptide is cleaved off by NisP. The genes responsible for nisin production, modification, export, regulation, processing and auto-immunity are encoded on a gene cluster (61,69,71).

The gene *nisA* encodes the prepeptide of nisin whereas the modification enzymes are encoded by *nisBC*. The gene *nisP* encodes the protease, which cleaves off the leader peptide and the nisin exporter is encoded by *nisT*. The auto-immunity factors are encoded by the genes *nisIFEG*, whereas *nisRK* encodes the two-component regulatory system (61,69,71) (Figure 8).

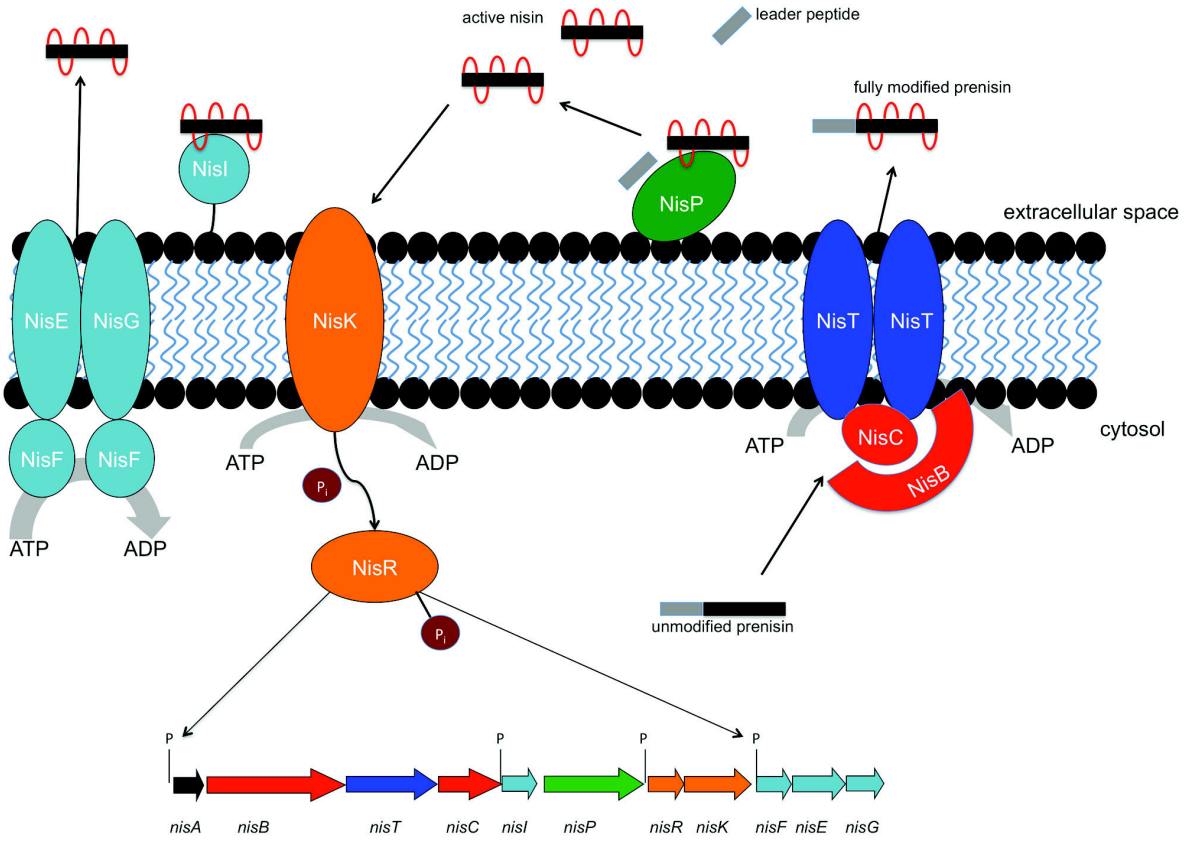


**Figure 8. Nisin biosynthesis and auto-immunity.** Genes and proteins, which are involved in similar events like modification (red), transport (blue), processing (green), regulation of biosynthesis (orange) and autoimmunity (cyan) are highlighted in identical colors. The nisin prepeptide is shown in grey/black whereas active nisin is black with indicated (methyl)lanthionine rings (red). P*nisI* and P*nisRK* are constitutive promoters.

### 1.3.1 Nisin biosynthesis

Nisin is ribosomally synthesized as a 57 amino acids long prepeptide. The prepeptide is subdivided in a N-terminal leader peptide comprising 23 amino acids (numbered -23 to -1 in Figure 9) and a 34 amino acid long propeptide (numbered 1 to 34 in Figure 9). Prenisin is modified posttranslationally by a specific modification machinery, which is responsible

for dehydration (NisB), cyclization (NisC), export (NisT) and cleavage of the leader peptide (NisP) (61).

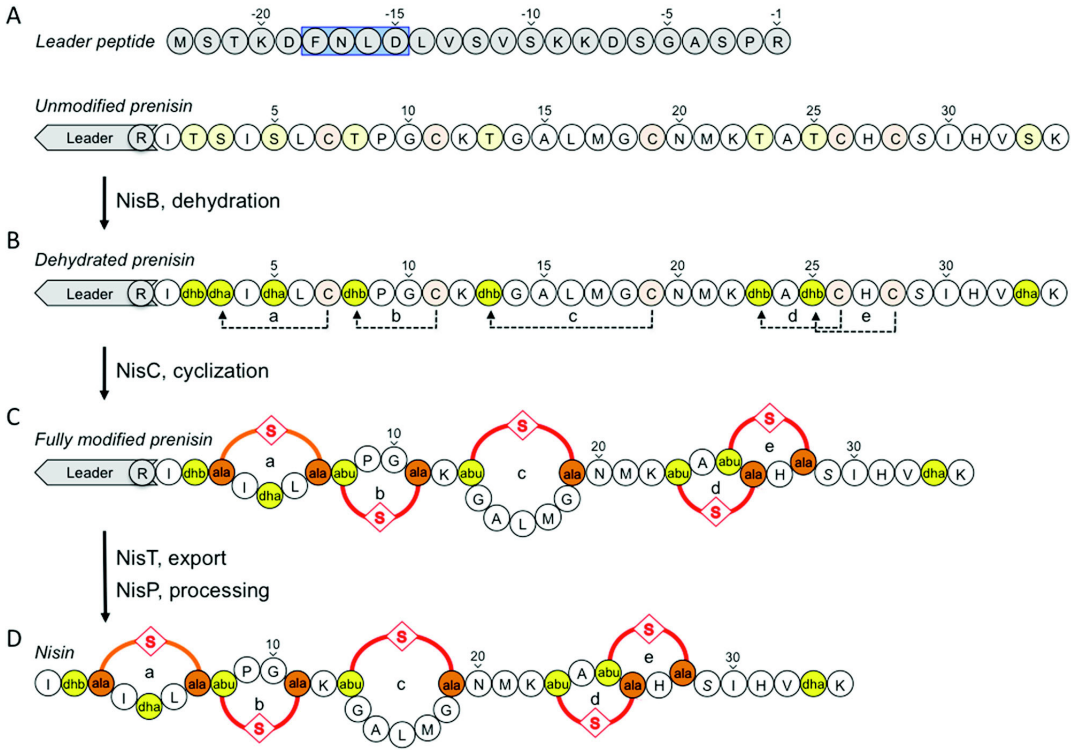


**Figure 9. Posttranslational modification of nisin.** For nisin maturation, the ribosomally synthesized precursor peptide undergoes a series of modifications. **A:** The leader peptide (in grey) directs prenisin to the nisin modification and transport machinery. The conserved FNLD-box is highlighted in blue. **B:** Specific serine and threonine residues (highlighted in yellow) are converted by the dehydratase NisB into dehydroalanines (Dha) and dehydrobutyrines (Dhb) respectively. **C:** The dehydrated residues are specifically coupled to cysteine residues (highlighted in orange) by the cyclase NisC yielding five thioether rings comprising one lanthionine (a) and four methyllanthionines (b-e). **D:** Fully modified prenisin is subsequently exported by the ABC transporter NisT and processed by the extracellular protease NisP that cleaves off the leader peptide to liberate active nisin. Note that Ser29 is never dehydrated in nisin. Taken from (84).

### 1.3.1.1 The dehydratase NisB

The dehydratase NisB consists of 993 amino acids and has a molecular mass of 117.5 kDa. NisB exhibits only 30 % sequence identity with other LanB enzymes and almost no homology to any other proteins (61). NisB catalyzes the dehydrations of serine and threonine residues exclusively in the propeptide of unmodified prenisin, yielding dehydrated prenisin (Figure 9B) (85). The dehydration of prenisin catalyzed by NisB presents the first step in nisin maturation and is thus critical for nisin biosynthesis (61,86). Herein, serine at position 29 is never dehydrated whereas serine at position 33 could escape dehydration in some cases (85,87). Overexpression of NisB was shown to efficiently

dehydrate Ser33 in all nisin molecules (85). However, Ser29 was still not dehydrated, which indicated that Ser29 was not accessible for NisB (85). A mutational study revealed that when ring formation of rings D and E is abolished, due to mutations of Thr23 or Thr25, an additional dehydration occurred, which is likely a result of the dehydration of Ser29 (87). Therefore, it is assumed that rings D and E prevent dehydration of Ser29 due to steric hindrance (87). *In vivo* studies have demonstrated that NisB is promiscuous as it is able to dehydrate a multitude of nisin derivatives and even therapeutic peptides not related to nisin if they are fused to the nisin leader peptide (88,89). Further studies have shown that dehydration of serine and threonine residues is favored when they are flanked by hydrophobic residues, whereas a hydrophilic environment disfavored dehydration (90,91). Unfortunately, detailed information about the actual dehydration reaction is lacking.

#### 1.3.1.2 The cyclase NisC

Following NisB-mediated dehydration of serine and threonine residues, NisC catalyzes the coupling of dehydrated residues to C-terminal located cysteine residues, which form (methyl)lanthionines rings (Figure 9C) (92). However, Dhb2, Dha5 and Dha33 remained uncoupled. NisC, consisting of 418 amino acids has a molecular weight of 47.9 kDa and shows a sequence homology to other LanC enzymes of about 30 % (61). In 2006 the structure of NisC was solved and the cyclization activity was reconstituted *in vitro* (57). The structure revealed that a $Zn^{2+}$ ion is coordinated by Cys284, Cys330, and His331. These residues as well as His212 and Asp141 are essential for NisC activity and are conserved among the LanC enzymes (93). A mechanism for the cyclization reaction was proposed (57). After binding of dehydrated prenisin to NisC a thiol from a cysteine residue of the substrate replaces a water molecule, which is coordinated by His212 and the $Zn^{2+}$ ion. Subsequently, the thiol is deprotonated by a base or polarized water and the thiolate attacks the β-carbon of Dha/Dhb generating an enolate. The enolate is stereospecifically hydrated to provide a D-conformation of the α-carbon (Figure 10) (57).

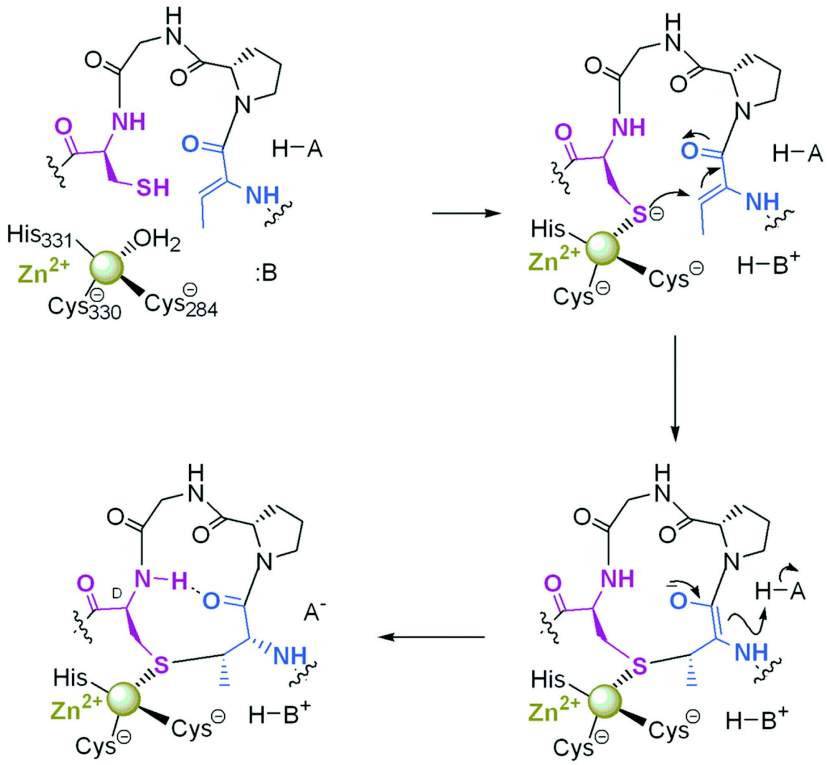


**Figure 10. Proposed mechanism for the cyclization reaction catalyzed by NisC.** Shown is the formation of ring B of nisin. Taken from (57).

Similar to NisB, NisC has a broad substrate spectrum, as NisC can cyclize a wide variety of nonlantibiotic peptides, which are fused to the nisin leader peptide (94). Furthermore, it was shown that cysteine residues, which are N-terminal flanked by glutamate and C-terminal flanked by lysine, are favorable for cyclization, whereas a adjacent N-terminal tryptophan abolishes cyclization in model peptides (94).

### 1.3.1.3 The nisin secretion machinery

After dehydration and cyclization, the fully modified prenisin is exported via the ABC-transporter NisT. The extracellular localized fully modified prenisin remains inactive until the protease NisP, which is anchored to the cell membrane, cleaves off the leader peptide and releases active nisin (Figure 9D).

By yeast-two hybrid and co-immunoprecipitation studies it was shown that NisBCT likely form a nisin synthetase complex, as NisT was shown to interact with NisC but not with NisB. On the other hand NisC was shown to interact with both, NisT and NisB (95). In analogy a synthetase complex was also proposed for the subtilin synthetase proteins SpaB, SpaC and SpaT (96). Both proposed models differ in the stoichiometry of the proposed

synthetase complex. The nisin synthetase complex is believed to comprise a NisT homodimer, a NisB monomer and two NisC molecules (Figure 11A) (95). Whereas, the subtilin synthetase complex is assumed to comprise two molecules of each protein (SpaB, SpaC and SpaT) (Figure 11B) (96). However, isolation and characterization of such a synthetase complex has remained unsuccessful (85,87,95,96).

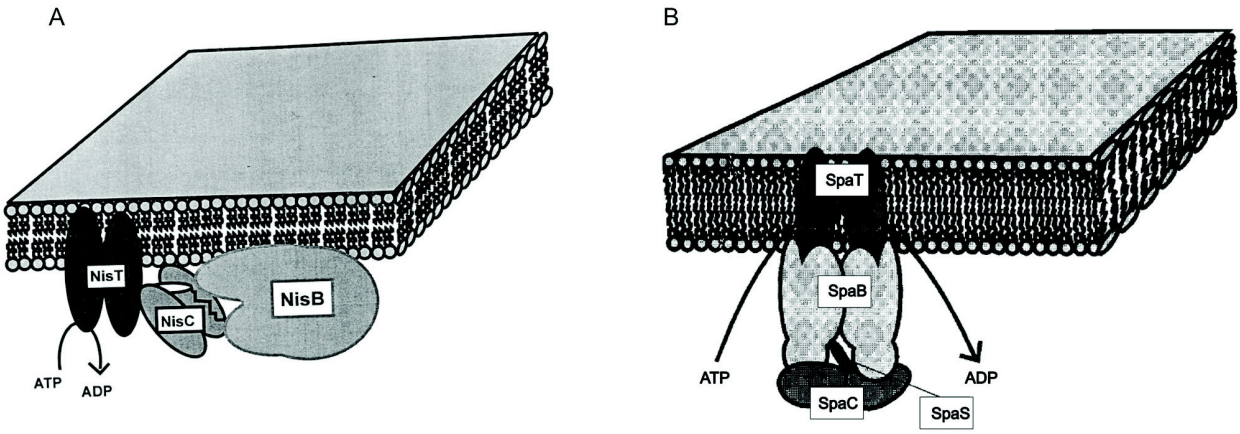


**Figure 11. Models of lantibiotic synthetase complexes. A:** The nisin synthetase complex model consists of a NisT homodimer a NisC dimer and a NisB monomer. **B:** The subtilin synthetase complex model consists of a SpaT homodimer a SpaB dimer and a SpaC dimer. Neither the nisin synthetase nor the subtilin synthetase complex has been isolated to date. Taken from (95,96).

*In vivo* studies have shown that NisB and NisC function in a cooperative manner. It was shown that in the absence of NisC, NisB mediated dehydration of a nisin unrelated peptide fused to the nisin leader peptide is more efficiently (97). Thus, formation of a preceding ring impairs dehydration of threonine residues which are involved in a (methyl)lanthionine ring (97). This finding was further supported by a mutational study in which serine and threonine residues were cloned directly after cysteines involved in ring formation. Herein, it was shown that NisB did not dehydrate these residues when formation of a preceding ring had occurred previously (87).

Another *in vivo* study showed that nisin production was decreased to 30 % when NisC was lacking and when NisB was lacking nisin production was 2 % compared to control cells where all enzymes (NisBCT) were present. The low production level in the absence of NisB was independent of the presence or absence of NisC (86). Moreover, it was found that when either NisB or NisC was missing, prenisin accumulated in the cells. This indicated that comparable amounts of peptide were produced in the cell, whereas the transport of those intermediates (unmodified and dehydrated prenisin, respectively) was suppressed

(86). These data indicate that the modification reactions catalyzed by NisB and NisC and the transport are coupled events.

In absence of both NisC and NisB or just NisC unmodified or dehydrated prenisin could be isolated. Therefore, the *L. lactis* strain NZ9000 was used, which does not contain the protease NisP (49). This strain does not produce nisin and lacks the proteins involved in nisin modification, export and processing. However, this strain harbors the two-component regulatory system NisRK to activate a P*nisA* promoter encoded on a plasmid (49). NZ9000 cells harboring the *nisA* and *nisT* genes on plasmids produce and secrete unmodified prenisin, whereas cells encoding *nisA*, *nisB* and *nisT* produce dehydrated prenisin (see materials and methods) (86). Fully modified prenisin, which is biologically inactive due to the leader peptide that is still attached (57) could be achieved by using NZ900 cells harboring *nisA*, *nisB*, *nisC* and *nisT* (91).

## 1.4 Nisin: Mode of action

Active nisin consists of 34 amino acids including 1 lanthionine, 4 methyllanthionines, 2 dehydroalanines and 1 dehydrobutyrine, which are introduced posttranslationally. The (methyl)lanthionines introduce 5 intramolecular rings into the peptide which protect nisin against proteolytic degradation (98). Nisin possesses two mechanisms to kill target cells. First it was shown that nisin was able to form pores in model membranes, but up to three orders of magnitudes higher concentration were needed compared to permeabilization of intact cells (75,99). This indicated that nisin specifically interacts with a component of the target membrane. The significant difference in activity against model membranes and intact cells was explained by the finding that nisin interacts with lipid II in a highly specific manner, since model membranes supplemented with lipid II were more efficiently permeabilized than model membranes lacking lipid II (75,99). Furthermore, it was shown that addition of vancomycin, which also targets lipid II (see section 1.1), prior to nisin treatment blocks nisin activity. This also indicated that nisin interacts with lipid II (99). Since nisin is active against vancomycin resistant *enterococci*, nisin apparently binds to a different site than vancomycin, which binds to the C-terminus of the pentapeptide attached to lipid II (Figure 12A) (99).

To date it is known that nisin targets the pyrophosphate moiety of lipid II. This interaction occurs between the backbone amine groups of residues Dhb2, Ala3, Ile4, Dha5 and Abu8 of nisin and the oxygen atoms of the pyrophosphate via hydrogen bonds (Figure 12B) (42). After binding to lipid II the C-terminus of nisin can insert into the membrane of the target cell and form pores, which are composed of 4 lipid II and 8 nisin molecules (Figure 12C) (44).

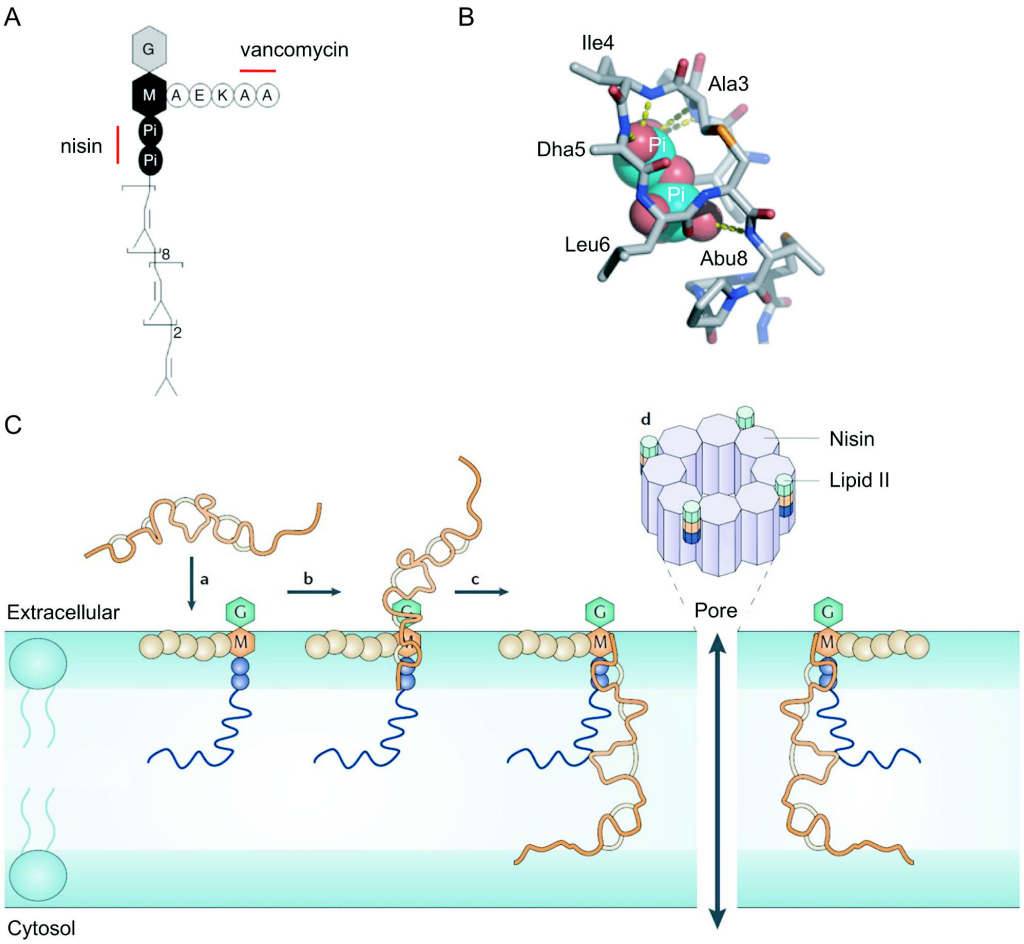


**Figure 12. Mode of action of nisin. A:** Nisin and vancomycin binding sites in lipid II. **B:** Nisin lipid II interaction **C:** Nisin induced pore formation upon nisin binding to lipid II. Taken from (38)**.**

A prerequisite for pore formation is the flexible hinge region (amino acids 20-22) of nisin, as mutations of the hinge region impaired pore formation, due to reduced flexibility. Rigid nisin was unable to form pores but still had antimicrobial activity, which was 10-fold reduced compared to wild type nisin (100). On the other hand, a truncated nisin mutant, which lacks the rings D and E and therefore cannot span the target membrane showed a 100-fold decrease in activity (101). Since pore formation is not essential for the antimicrobial activity of nisin a second mode of action was assumed.

The second mode of action was revealed, by the findings that nisin was shown to delocalize lipid II from the septum, where cell wall synthesis occur (78). After addition of nisin or of the nisin hinge-region mutants lipid II was sequestered in large patches in the membrane apart from the septum (78). Thus, nisin inhibits cell wall synthesis and cell division by removing lipid II from the septum and permeabilize the target membrane by forming pores, which leads to cell death. This dual mode of action explains the high antimicrobial activity of nisin, which is indicated by the low amount of nisin that is sufficient to kill the target organism. For example, to inhibit the growth of the nisin sensitive *L. lactis* strain NZ9000 by 50 % a nisin concentration of 2.6±0.1 nM is sufficient (102). The ability to delocalize lipid II is currently unique for lantibiotics and perhaps the reason why until now no significant resistance against lantibiotics such as nisin has occurred (78).

## 1.5 Mechanisms of lantibiotic modification

Since the modification enzymes, which catalyze lantibiotic maturation, are supposed to enhance the stability and/or activity of lantibiotic unrelated peptides, it becomes more important to understand the catalytic mechanism of such enzymes. Several *in vivo* and some *in vitro* studies were performed to reveal the mechanisms by which lantibiotic modifications are catalyzed. To date, *in vitro* reconstitution of some active LanM enzymes like LctM, NukM and HalM1/HalM2 (56,103,104) and of the nisin cyclase NisC (57) were reported, whereas *in vitro* reconstitution of any LanB enzyme, such as NisB, EpiB and SpaB, was unsuccessful (45,85,105,106).

### 1.5.1 Modification of class II lantibiotics

Several studies have provided mechanistic insights for LanM enzymes, which catalyze both, the dehydration and the cyclization of lantibiotic prepeptides. The C-termini of LanM enzymes possess low sequence homology to LanC enzymes including two cysteine residues and one histidine residues that are thought to be involved in $Zn^{2+}$ coordination. Therefore, it was assumed that the C-terminus represents the cyclase domain in LanM enzymes (107). Whereas, the N-terminus is designated as the dehydratase domain, which does however not exhibit any sequence homology to LanB enzymes (56). Successful *in vitro*

reconstitution of LctM and HalM1/HalM2 activity revealed that the enzymes process the prepeptides completely, but only upon addition of ATP and $Mg^{2+}$ ions (56,103). The use of C-terminally truncated LctA as substrate strongly reduced the dehydration efficiency of LctM. Therefore, it was possible to observe that serine and threonine residues in the propeptide were phosphorylated prior to dehydration. Moreover, the elimination of the phosphate group only occurred in the presence of ADP/$Mg^{2+}$. Strikingly, no ATP binding motif was found in LctM (108). By site directed mutagenesis of the N-terminus of LctM the involvement of certain residues (Asp242 and Asp259) in dehydration of the substrate was demonstrated (109). Furthermore, it was shown that LctM lacking cyclization activity, due to a point mutation of a zinc coordinating amino acid, was still able to catalyze the dehydration of serine and threonine residues *in vitro* (107). LctM mediated dehydration was shown to be processive *in vitro*, meaning that the substrate LctA after binding to LctM is fully dehydrated prior to dissociation from the enzyme, as no intermediates could be isolated (110). Moreover, the production of fully dehydrated LctA lacking the three possible cyclizations demonstrated that dehydration and cyclization catalyzed by LctM are not strictly coupled (110). In addition, it was shown that LctM is able to dehydrate and cyclize non-lantibiotic peptides as well as peptides containing non-proteinogenic amino acids *in vitro*, when they are C-terminally fused to the leader peptide of lacticin 481. Thus, LctM has a broad substrate spectrum (111-113). LctM activity was also obtained when the propeptide was applied to LctM without the leader peptide, but here only a single dehydration could be observed (55). In contrast, when the leader peptide and the propeptide were applied *in trans* as two distinct peptides increased dehydration and cyclase activity was observed as three dehydrations out of four were catalyzed in 150 minutes (55). This activity is however still strongly reduced when compared to the wild type substrate (leader peptide attached to propeptide) where four dehydrations occurred within 15 minutes (55). In addition, the processivity of LctM was lost when leader peptide and propeptide were supplied *in trans* to LctM as intermediates were obtained with N-terminal residues being favored for dehydration indicating some directionality of LctM (55). These findings support the necessity of the leader peptide for the processive activity of LctM when the substrate is attached to the leader. Therefore, the leader peptide seems to provide important binding affinity that allows the lantibiotic prepeptide to remain associated to the modification enzyme for dehydration and cyclization (55).

Another *in vivo* study showed that heterologous co-expression of NukM, the modification enzyme of nukacin ISK-1, and NukA, the prepeptide, in *E. coli* results in fully processed peptide (114). Furthermore, NukM seems to be able to distinguish between serine and threonine residues as replacement of serine with threonine and vice versa results in a decrease or loss of dehydration activity (104). This indicates a difference in substrate recognition between LctM and NukM, as LctM dehydrates serine and threonine residues with the same efficiency (104,113). For NukM as well as for LctM it was shown that applying a C-terminal truncated form of the substrate leads to less efficient processing of the prepeptide (104,108). *In vitro* reconstitution of NukM also revealed that its activity is ATP/$Mg^{2+}$ dependent like LctM (104). Furthermore, an *in vitro* interaction of NukM with its natural substrate pre-nukacin ISK-1 was shown by SPR analysis (115).

### 1.5.2 Modification of the class I lantibiotic nisin

The activity of NisC, the cyclase of the prenisin modification machinery, was reconstituted *in vitro* and its structure was solved (57,116). It was shown that a $Zn^{2+}$ ion is involved in the cyclization reaction. The $Zn^{2+}$ ion is coordinated by two cysteines and by one histidine residue that are also conserved in the C-termini of LanM enzymes like LctM and in other LanC enzymes like SpaC (107,116). This suggests that LanM and LanC enzymes share a similar mechanism of cyclization (45). Mutations of the $Zn^{2+}$ ion coordinating residues in NisC and LctM resulted in impaired cyclization activity, which further indicates that LanC and LanM have a similar cyclization mechanism (87,93,107). *In vivo* studies demonstrated that NisC introduces (methyl)lanthionine rings into nonlantibiotic peptides, when they are fused to the prenisin leader peptide (94). Thus, NisC also has a broad substrate spectrum similar to LctM. In addition, a directionality of NisC activity was revealed, because NisC could couple Dha/Dhb only to more C-terminal located cysteine residues (94).
Most studies regarding class I modification enzymes (LanB, LanC, LanT and LanP) were performed *in vivo*, as *in vitro* reconstitution of especially LanB activity has failed (61,105,106). NisB was shown to dehydrate nonlantibiotic peptides, which are fused to the prenisin leader peptide. Therefore, NisB has a broad substrate spectrum similar to NisC or LctM (88,117). Furthermore, it was demonstrated that serine and threonine residues flanked by hydrophobic amino acids are preferential dehydrated, whereas hydrophilic

flanking residues disfavor dehydration by NisB (90,91). This is in contrast to LctM that apparently does not show preferences for either hydrophobic or hydrophilic residues flanking the serine or threonine residues (113).

Remarkably, NisB does not show homology to LanM enzymes, the sequence homology even to other LanB enzymes is rather low (61). Therefore, it remains unknown whether LanB and LanM enzymes catalyze the dehydration reaction in a similar manner. Moreover, it was demonstrated that NisB dehydrates stretches of threonines, and even residues at positions further away than in the natural prenisin (90). Those findings indicate that NisB has a broad substrate spectrum, which include substrates that are longer or shorter than prenisin, and nisin unrelated peptides. However, the dehydration mechanism of LanB enzymes still remains unclear.

Several studies have demonstrated that the lantibiotic modification enzymes can function independent of each other. NisC and LctM were shown to be active *in vitro* in the absence of NisB/NisT and LctT, respectively (56,57). NisB and NisT activity in the absence of the other modification enzymes were demonstrated *in vivo* (89,117). NisB was able to dehydrate peptides which were fused to the leader peptide of prenisin in the absence of NisC and NisT, where the export of the peptides was mediated by N-terminal fused SEC or TAT secretion signals (89). Moreover, NisT was able to export unmodified prenisin in the absence of NisB and NisC (117). NisT also showed relaxed substrate specificity, as it could export nonlantibiotic peptides fused to the prenisin leader peptide (117). However, NisT also showed substrate specificity, because fully modified prenisin is more efficiently secreted than unmodified and dehydrated prenisin (86).

#### 1.5.2.1 The leader peptide of prenisin

A prerequisite for modification by NisB or NisC and for the transport by NisT is the leader peptide of prenisin, which is never modified (57,88,117). Since NisB, NisC and NisT are shown to be active independently, it is likely that each enzyme recognizes and binds the leader peptide individually. A recent study showed that the nisin modification enzymes tolerate single as well as multiple mutations within the leader peptide. However, the conserved FNLD motif (Figure 9A) within the leader is essential for prenisin modification but not for export of the mutant, since the FNLD/AAAA mutant showed no dehydrations

but was secreted into the medium (118). Therefore it is possible that the individual enzymes recognize different sites of the leader peptide.

# 2. Aims and objectives

The function of leader peptides in lantibiotic maturation is not well investigated. It is suggested that the leader peptide directs the substrate to the lantibiotic modification machinery (88,91,94,119). The finding that LctM, which catalyze both, the dehydration as well as the cyclization of the class II lantibiotic lacticin 481, dehydrates the propeptide of pre-lacticin without the leader peptide attached, showed that the leader peptide is not crucial for dehydration by LctM (55). However, the leader peptide attached to the propeptide strongly increases the efficiency of LctM (55). Whether the increased efficiency is due to high affinity of the leader peptide to the enzyme or due to a conformational change of the enzyme induced by the leader peptide, could not be revealed, as the substrate was poorly soluble (119).

The leader peptide of the class I lantibiotic nisin was shown to direct nonlantibiotic peptides, which were fused to the leader peptide, to the nisin modification machinery *in vivo* (88,94,117). Whereas, dehydrated prenisin, lacking the leader peptide of nisin, was not cyclized by NisC *in vitro* (57). This indicates that the leader peptide of nisin is crucial for the substrate to become modified and exported by the nisin modification enzymes (NisB and NisC) and the nisin exporter NisT (88,94,117).

Since *in vivo* studies are not suitable to characterize a direct interaction of prenisin with either one of the modification enzymes *in vitro* investigation could give further insight. As the dehydration of unmodified prenisin is the first step in nisin maturation, this work focus on the substrate recognition and specificity of NisB, *in vitro*. Therefore, all components have to be purified and characterized. Furthermore, a suitable binding assay has to be developed to demonstrate and characterize the interaction of NisB and different prenisin forms. This can reveal how NisB may recognize its substrate and whether NisB can discriminate between the different prenisin forms. Herein, the function of the leader peptide for the NisB-prenisin interaction can be investigated in detail. The results may provide further knowledge of how prenisin is modified.

# 3. Materials and Methods

## 3.1 Materials

### 3.1.1 Chemicals

| | |
|---|---|
| 1,4-Dithiotreitol (DTT) | AppliChem |
| 2-(N-morpholino)ethanesulfonic acid (MES) | Carl Roth GmbH & Co. |
| 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) | Carl Roth GmbH & Co. |
| 3-(Cyclohexylamine)-1-propanesulfonic acid (CAPS) | Carl Roth GmbH & Co. |
| 3-(N-morpholino)propane-1-sulfonic acid (MOPS) | Carl Roth GmbH & Co. |
| Acetic acid (98 %) | Carl Roth GmbH & Co. |
| Acryl amid | Carl Roth GmbH & Co. |
| Agar-Agar | Serva |
| Ammoniumperoxodisulphate | Carl Roth GmbH & Co. |
| Asparagine | Sigma-Aldrich Chemie GmbH |
| Bromphenol blue | Sigma-Aldrich Chemie GmbH |
| Calcium chloride | Carl Roth GmbH & Co. |
| Casein hydrolysate | Carl Roth GmbH & Co. |
| Chloramphenicol | Carl Roth GmbH & Co. |
| Coomassie Brilliant Blue R-250 | Carl Roth GmbH & Co. |
| $ddH_2O$ Milli-$Q^{50}$ Plus | Millipore |
| Deoxyribonuclease I | Sigma-Aldrich Chemie GmbH |
| Disodium hydrogen phosphate | VWR |
| Erythromycin | Merck KGaA |
| Ethanol | Carl Roth GmbH & Co. |
| Ethylendiamine-tetra-acetate (EDTA) | Carl Roth GmbH & Co. |
| Folic acid | Carl Roth GmbH & Co. |

| | |
|---|---|
| Formaldehyde 37% | Carl Roth GmbH & Co. |
| Glucose | Carl Roth GmbH & Co. |
| Glycerol | Carl Roth GmbH & Co. |
| Glycerol phosphate | Carl Roth GmbH & Co. |
| Glycine | Carl Roth GmbH & Co. |
| Hydrochloric acid, 37 % | Carl Roth GmbH & Co. |
| Imidazole | Fluka Chemie GmbH |
| Iron (III) chloride | J.T. Baker |
| Isopropanol | Carl Roth GmbH & Co. |
| Lysozyme | Sigma-Aldrich Chemie GmbH |
| Magnesium chloride | Carl Roth GmbH & Co. |
| Magnesium sulfate | Merck KGaA |
| Methanol | Carl Roth GmbH & Co. |
| Nickel (II) chloride | Grüssing |
| Nickel (II) sulfate | Sigma-Aldrich GmbH |
| Nicotinic acid | Carl Roth GmbH & Co. |
| Nisin, from *Streptococcus lactis* 2.5% | Sigma-Aldrich Chemie GmbH |
| PageRuler™ Prestained Protein Ladder | Fermentas |
| PageRuler™ Unstained Protein Ladder | Fermentas |
| Peptone from casein/soy/beef | Carl Roth GmbH & Co. |
| Potassium chloride | Fluka Chemie GmbH |
| Potassium dihydrogen phosphate | Grüssing |
| Protease inhibitor cocktail tablets | Roche |
| Riboflavin | Carl Roth GmbH & Co. |
| Silver nitrate | Fluka |
| Sodium acetate | Sigma-Aldrich Chemie GmbH |
| Sodium azide | Fluka Chemie GmbH |
| Sodium carbonate | Carl Roth GmbH & Co |
| Sodium chloride | Carl Roth GmbH & Co. |
| Sodium dodecyl sulfate (SDS) | Carl Roth GmbH & Co. |
| Sodium hydroxide | Carl Roth GmbH & Co. |
| Tetramethylethylendiamine (TEMED) | Carl Roth GmbH & Co. |

| | |
|---|---|
| Trichloracetic acid | Fluka Chemie GmbH |
| TRIS(hydroxymethyl)-aminomethan (TRIS) | VWR |
| Trypsin | Sigma-Aldrich Chemie GmbH |
| Tween-20 | Carl Roth GmbH & Co. |
| Yeast extract | Carl Roth GmbH & Co. |
| Zink sulfate | Sigma-Aldrich Chemie GmbH |

### 3.1.2 Antibodies

| | |
|---|---|
| anti-His | Qiagen |
| anti-Leader | (117) |
| anti-rabbit antibody coupled to horseradish peroxidase (HRP) from goat | Sigma-Aldrich Chemie GmbH |
| anti-mouse antibody coupled to HRP from goat | Dianova |

### 3.1.3 Consumables

| | |
|---|---|
| Amicon Ultra 15 (MWCO 100 kDa) | Millipore |
| Amicon Ultra 4 (MWCO 30 kDa) | Millipore |
| Amicon Ultra 4 (MWCO 3 kDa) | Millipore |
| PVDF blotting membrane | Pall corporation |
| Reaction tubes 1.5 ml and 2 ml | Eppendorf |
| Reaction tubes 15ml and 50 ml | Sarstedt |
| Pipette tips | Sarstedt |
| Non-sterile 4 mm Millex syringe filter units | Millipore |
| Tube-O-DIALYZER, micro (MWCO 50 kDa) | G-Bioscience |
| Sensor Chip NTA | GE Healthcare |
| Supor 450 membrane 47mm | Pall corporation |
| Supor 100 membrane 47mm | Pall corporation |

### 3.1.4 Strains

| | |
|---|---|
| *Lactococcus lactis* strain NZ9000 | MG1363 derivative with *pepN*::*nisRnisK* (120) |

### 3.1.5 Plasmids

| | |
|---|---|
| pNGnisBhis | *L. lactis* expression vector; $cm^R$; provided by BiOMaDe |
| pNZnisA-E3 | *L. lactis* expression vector; $ery^R$ (117) |
| pIL3BTC | *L. lactis* expression vector; $cm^R$ (91) |
| pIL3hpBT | *L. lactis* expression vector; $cm^R$ (86) |
| pIL3hpT | *L. lactis* expression vector; $cm^R$ (86) |

### 3.1.6 Media

Media components were dissolved in distilled or double distilled water and sterilized by autoclaving.

M17-medium

- 5 g/l Peptone from soy
- 5 g/l Peptone from casein
- 5 g/l Peptone from beef
- 2.5 g/l yeast extract
- 0.5 g/l Ascorbate
- 1 ml/l Magnesium sulfate (1M)
- 19 g/l beta-Glycerol phosphate

Minimal medium

- Solution A (20 % (v/v))
    - 10 g/l Ammonium sulfate
    - 30 g/l Disodium hydrogen phosphate
    - 15 g/l Potassium dihydrogen phosphate
    - 5 g/l Sodium chloride

- Solution B (80 % (v/v))
    - 12.5 g/l Casamino acid
    - 2.5 g/l Sodium acetate
    - 0.1 g/l Asparagine
    - 0.25 g/l Magnesium chloride
    - 0.0125 g/l Calcium chloride
    - 0.00075 Iron(III) chloride

- 20 % Glucose (2.5 % (v/v))

- Vitamin Mix (0.1 % (v/v))
    - 0.1 g/l Biotin
    - 1 g/l Folic acid
    - 1 g/l Riboflavin
    - 1 g/l Nicotinic acid
    - 1 g/l Pantothenic acid
    - 2 g/l Pyridoxal

GM 17 Agar

- M17 medium supplemented with 0.5 % (w/v) glucose and 1.2 % (w/v) Agar-Agar

### 3.1.7 Buffers

Resuspensionbuffer

- 50 mM HEPES-NaOH pH 8
- 150 mM NaCl

SDS-PAGE running buffer

- TRIS-HCl (50 mM, pH 8.3)
- SDS (1 g/l)
- Glycine (190 mM)

SDS-PAGE stacking gel buffer

- TRIS-HCl (1.5 M, pH 8.85)
- SDS 4 g/l

SDS-PAGE separating gel buffer

- TRIS-HCl (0.5 M, pH 6.8)
- SDS 4 g/l

5 x SDS sample buffer

- Stacking gel buffer (20 % (v/v))
- SDS (2.5 % (w/v))
- Glycerin (40 % (v/v))
- Bromophenol blue (0.02 % (w/v))
- DTT (10 mM)

Transfer buffer for western blotting

- SDS-PAGE running buffer
- Methanol (20 % (v/v))

TBS buffer

- TRIS-HCl (20 mM, pH 8.0)
- NaCl (250 mM)

TBS-T buffer

- TRIS-HCl (20 mM, pH 8.0)
- NaCl (250 mM)
- Tween 20 (0.1 % (v/v))

Coomassie staining solution

- Coomassie Brilliant Blue R-250 (0.25 % (w/v)
- Methanol (40 % (v/v))
- Acetic acid, 98 % (10 % (v/v))

Coomassie destaining solution

- Methanol (40 % (v/v))
- Acetic acid, 98 % (10 % (v/v))

Fixing solution for silver staining

- Ethanol (30 % (v/v))
- Acetic acid, 98 % (10 % (v/v))

Thiosulfate buffer

- Sodium acetate (100 mM)
- Ethanol (30 %)
- pH 6.0 adjust with acetic acid

Silver nitrate solution

- Silver nitrate (0.1 % (w/v))

Developer solution (prepare fresh before usage)

- Sodium carbonate (2.5 % (w/v))
- Formaldehyde 37 % (100 µl/100ml)

### 3.1.8 Instruments

Purification systems

| | |
|---|---|
| • Äkta Basic | GE Healthcare |
| • Äkta Purifier | GE Healthcare |
| • Äkta Explorer | GE Healthcare |
| • Äkta Prime | GE Healthcare |
| • Äkta micro | GE Healthcare |
| • HPLC LaChrom | Merck-Hitachi |

Chromatography columns

| | |
|---|---|
| • HiTrap Chelating HP Columns 5 ml | GE Healthcare |
| • HiTrap SP HP 5 ml | GE Healthcare |
| • Superdex 200 10/300 GL | GE Healthcare |
| • Superdex 200 PC 3.2/30 | GE Healthcare |
| • HiLoad 16/600 Superdex 200 pg | GE Healthcare |
| • LiChrospher WP 300 RP-18 end capped column | Merck |

Optical instruments

| | |
|---|---|
| • Nanodrop ND-1000 | Peqlab |
| • FLUOstar OPTIMA | BMG Lab technology |
| • CO8000 cell density meter | WPA Bioware |
| • Biacore X | GE Healthcare |

Centrifuges

| | |
|---|---|
| • Centrifuge 5417R | Eppendorf |
| • Centrifuge 5424 | Eppendorf |
| • MEGAFUGE 1.0R | Thermo scientific |
| • Avanti J-26 XP | Beckman coulter |
| • Sorvall Evolution RC | Thermo scientific |
| • Optima L-90K Ultracentrifuge | Beckman coulter |

Electrophoretic instruments

| | |
|---|---|
| • Power supply PowerPac HC | Bio-Rad |
| • Trans-Blot Semi-Dry system | Bio-Rad |
| • SDS vertical electrophoresis unit | Chem. Werkstätten HHU |
| • Mini-PROTEAN Tetra Electrophoresis System | Bio-Rad |

Incubators

| | |
|---|---|
| • Function Line | Heraeus Instruments |
| • Multitron Standard | Infors HT |
| • Ecotron | Infors HT |

Others

| | |
|---|---|
| • Basic Z cell disruptor | Constant systems |
| • SDS-PAGE casting unit | Chem. Werkstätten HHU |
| • Milli-$Q^{50}$ Plus | Millipore |
| • Gilson pipettes | Gilson |
| • Macro™ Pipette Controller | Brandtech scientific, Inc |
| • Thermomixer comfort | Eppendorf |
| • Vortex VF 2 | Janke & Kunkel IKA Labortechnik |
| • Rocking platform | VWR |
| • neoMag Magnetic Stirrer | neoLab |
| • pH Meter pH 526 | WTW |

## 3.2 Methods

### 3.2.1 Cultivation of *L. lactis*

M17 medium containing 0.5 % (w/v) glucose (GM17) and the appropriate antibiotics was inoculated with *L. lactis* NZ9000 cells stored as cryostocks. Cultures were grown at 30°C. Overnight grown pre-cultures were transferred to fresh GM17 medium or minimal medium containing the appropriate antibiotics. The cell density was determined by measuring the optical density of the culture at 600 nm with a CO8000 cell density meter. After induction cells were grown either for an additional 3 h or overnight at 30°C. For long-term storage cells from a 5 ml stationary pre-culture were harvested at 6000 xg for 10 min. The supernatant was discarded and the pellet was suspended in 500 µl medium and diluted 1:1 with 100 % glycerol. This suspension was quickly frozen in liquid nitrogen and stored as cryostock at -80°C.

### 3.2.2 Electrophoresis and protein detection

#### 3.2.2.1 Protein determination

The concentration of soluble protein was determined by measuring the absorbance at 280 nm with a NanoDrop ND-1000 spectrophotometer. The theoretical extinction coefficient and the theoretical molecular weight for each protein was calculated using ProtParam (www.expasy.org/tools/protparam.html).

For peptides lacking aromatic amino acids the concentration could not be determined by the absorption at 280 nm. Therefore, the Pierce BCA Protein Assay Kit was used. The BCA assay detects peptide bonds as well as cysteines, tyrosines and tryptophans by reducing $Cu^{2+}$ ions to $Cu^{+}$ in an alkaline environment. Herein, the peptide bonds are the major contributor to the reduction of copper. Subsequently, the $Cu^{+}$ ions form a colorimetric complex with bicinchoninic acid (BCA) which can be detected by measuring the absorption at 584 nm with a FLUOstar OPTIMA plate reader (121). A calibration curve with bovine serum albumin (BSA) with known concentrations was used to calculate the peptide concentration.

### 3.2.2.2 SDS polyacrylamide gel electrophoresis

Protein solutions were analyzed using discontinuous SDS polyacrylamide gel electrophoresis (SDS-PAGE). A discontinuous SDS-PAGE consists of a stacking gel, which has a fixed acrylamide content of 4.5 %, and a separation gel, in which the acrylamide content is adjusted for the proteins of interest. For large proteins (30-150 kDa) 10 % separation gels were used, whereas small proteins (3-100 kDa) were analyzed with 15 % separation gels. The composition of the used SDS-gels is shown in Table 1.

**Table 1. Recipe for 9 SDS polyacrylamide gels**

| | **Stacking gel** | **Separation gel** | |
|---|---|---|---|
| | 4.5 % | 10 % | 15 % |
| (Bis-)Acrylamide solution 30 % | 5.25 ml | 25 ml | 37.5 ml |
| Stacking gel buffer | 5 ml | | |
| Separation gel buffer | | 18.75 ml | 18.75 ml |
| $ddH_2O$ | 21 ml | 31.25 ml | 18.75 ml |
| APS | 210 µl | 300 µl | 300 µl |
| TEMED | 70 µl | 70 µl | 70 µl |

SDS-PAGE separates proteins according to their size as SDS unfolds and homogenously charges the proteins. Therefore, smaller proteins migrate faster through the gel matrix than bigger ones. The pore size of the gel matrix is determined by the content of acrylamide and decreases with increasing amount of acrylamide.

SDS gels were prepared by applying the separation gel mixture to a gel-casting chamber and subsequently the gels were covered with isopropanol. After 1 h of polymerization the isopropanol was decanted and the stacking gel mixture was poured over the separation gel. Teflon combs were used to generate pockets into the stacking gel. Protein samples were supplemented with $1/5^{th}$ (v/v) of 5 x sample buffer prior to electrophoresis. SDS-PAGE was performed at 150 V until the bromophenol blue running front migrated through the separation gel. For smaller proteins the electrophoresis was stopped when the dye front had migrated to about 75 % of the total separation gel distance.

### 3.2.2.3 Coomassie staining and silver staining

To visualize the proteins analyzed by SDS-PAGE, the SDS gels were stained using either Coomassie staining or silver staining, depending on the amount of protein that was applied to SDS-PAGE. Coomassie staining has a sensitivity of approximately 100 ng whereas silver staining is more sensitive allowing detection of protein amounts as low as 5-10 ng (122).
Coomassie staining was performed by incubating the gel in Coomassie staining solution on a shaking platform for 30 min. Excess dye was removed by incubating the gel in destain solution, which had to be exchanged several times.
For a more sensitive detection silver staining was used. First the proteins needed to be fixed in the gel by incubating the gel 15 min in fixing solution. Then the gel is washed in thiosulfate buffer (containing 1mg/ml thiosulfate) for 15 min. Afterwards, the gel was washed 2 times in $ddH_2O$ for 10 min. Subsequently, the gel was incubated for 25 min in silver nitrate solution. For detection the gel was shortly washed with $ddH_2O$ and developer solution. The gel was then covered with developer solution to allow silver staining of the protein bands. To stop the silver staining reaction, acetic acid was added.

### 3.2.2.4 Immunodetection

Immunodetection by western blotting is a common technique to specifically detect a certain protein in a mixture of several proteins. Therefore proteins are first separated by SDS-PAGE and subsequently transferred to a PVDF membrane. The SDS gel was applied on a PVDF membrane, which was on top of 3 wet Whatman filter papers. On top of the gel, 3 additional wet Whatman papers were applied gently to avoid any air bubbles. Transfer was performed with a current of 100 mA per gel for 1 h, or alternatively for small peptides the transfer lasted 25 min. To block any free binding sites the PVDF membrane was incubated with milk powder solution for 30 min. After washing with TBS-T and TBS to remove unbound compounds, the first antibody was applied to the membrane for 1 h. Subsequently the washing steps were repeated and the secondary HRP conjugated antibody was added to the membrane for 1 h. Unbound antibody was removed by washing with TBS-T and TBS. For detection the ECL advanced kit was used.

## 3.2.3 Protein expression and purification

### 3.2.3.1 Expression of His-tagged NisB

*L. lactis* NZ9000 containing the plasmid pNGnisBhis was grown overnight in 100 ml M17 medium containing 0.5 % (w/v) glucose (GM17) and 5 μg/ml chloramphenicol at 30°C. Cells were transferred to two liters of fresh GM17 medium and growth was continued to an $OD_{600}$ of 0.8 whereupon NisB expression was induced by the addition of nisin to a final concentration of 25 ng/ml. Three hours after induction, the cells were harvested by centrifugation at 8000 xg for 20 min. at 4°C. The cell pellet was suspended in buffer composed of 50 mM HEPES-NaOH pH 8.0 and 150 mM NaCl and stored at -20°C until use.

### 3.2.3.2 Purification of His-tagged NisB

For purification, cells were thawed at 4°C, supplemented with protease inhibitor cocktail and DNase I, and lysed with a cell disruptor, generally 4-5 cycles at a pressure of 2.5 kbar. Remaining cells were removed at 18000 xg for 30 min. at 4°C, and the resulting supernatant was cleared from membranes by centrifugation at 130000 xg for 75 min. at 4°C. The supernatant containing NisB was supplemented with imidazole pH 8.0 to a final concentration of 5 mM and incubated at 4°C for 10 min.

Prior to immobilized metal ion affinity chromatography (IMAC) a 5 ml HiTrap chelating column was saturated with $Ni^{2+}$ ions and equilibrated with 50 mM HEPES-NaOH pH 8.0, 500 mM NaCl, 10 mM imidazole and 10 % (v/v) glycerol. Subsequently, the NisB containing supernatant was applied to the 5 ml HiTrap chelating column saturated with $Ni^{2+}$ ions, using a flow rate of 2 ml/min at 4°C. The column was washed with buffer containing 50 mM HEPES-NaOH pH 8.0, 500 mM NaCl, 10 mM imidazole and 10 % (v/v) glycerol followed by a wash step in which the imidazole concentration was increased to 50 mM imidazole to remove unspecifically bound proteins. Finally, NisB was step-eluted by increasing the imidazole concentration to 250 mM using a flow rate of 1 ml/min. Eluted protein was monitored by measuring the absorbance at 280 nm and analyzed by SDS-PAGE. NisB containing fractions were pooled and 5 ml of the NisB containing solution were applied to a Superdex 200 16/600 size exclusion column using 50 mM HEPES-NaOH pH 8.0, 500 mM

NaCl and 10% (v/v) glycerol as elution buffer. NisB containing fractions were pooled and concentrated with an Amicon Ultra centrifugal filter (100 kDa cut-off). The protein concentration was determined by measuring the absorbance at 280 nm with a NanoDrop ND-1000 spectrophotometer using the theoretical extinction coefficient of NisB of 128400 $L \cdot mol^{-1} \cdot cm^{-1}$ and the theoretical molecular weight of NisB of 118.3 kDa, as calculated for the his-tagged NisB using ProtParam web server (http://www.expasy.ch).

#### 3.2.3.3 Optimization of buffer solutions for protein purification

Different buffer conditions were tested to enhance the yield of purified NisB after size exclusion chromatography. Different buffers covering a pH range from pH 4.0 to pH 10.0, in combination with different salt concentrations of 50 mM, 150 mM and 500 mM sodium chloride were tested for each pH. The used buffer systems are listed in Table 2.

**Table 2. Used buffer systems**

| | |
|---|---|
| pH 4 | 50 mM Acetate |
| pH 5 | 50 mM Acetate |
| pH 6 | 50 mM MES |
| pH 7 | 50 mM HEPES |
| pH 8 | 50 mM TRIS or 50 mM HEPES |
| pH 9 | 50 mM TRIS |
| pH 10 | 50 mM CAPS |

NisB purified by immobilized metal ion affinity chromatography was divided in 150 µl aliquots and dialyzed overnight at 4°C against 200 ml of the indicated buffer (Table 2), using the tube-o-lyzer dialysis units with a 50 kDa MW cut-off. After dialysis NisB was transferred to a 1.5 ml reaction tube and centrifuged at 14000 rpm at 4°C for 10 min, to sediment aggregates. The supernatants, which still contained protein, were filtered through a 0.45 µm syringe filter prior to applying the samples to an analytical Superdex 200 pc 3.2/30 column using an Äkta micro system. SEC analysis was performed at 4°C using filtered (0.1 µm) and degassed corresponding dialysis buffer as elution buffer. Protein elution was monitored as the absorbance at 280 nm and protein-containing fractions were analyzed by SDS-PAGE.

### 3.2.3.4 Expression of prenisin and its derivatives

*L. lactis* NZ9000 containing the plasmid pNZnisA-E3 together with either pIL3BTC (for fully modified prenisin), pIL3hpBT (for dehydrated prenisin) or pIL3hpT (for unmodified prenisin) were grown overnight in 50 ml M17 medium containing 0.5 % (w/v) glucose (GM17), 5 μg/ml chloramphenicol and 2 μg/ml erythromycin at 30°C. Cells were transferred to half a liter of minimal medium and growth was continued to an $OD_{600}$ of 0.3 whereupon prenisin expression was induced by the addition of nisin to a final concentration of 25 ng/ml. After overnight incubation at 30°C, cells were sedimented by centrifugation at 8000 xg for 30 min. at 4°C. Purification was subsequently performed with the prenisin containing medium supernatant.

### 3.2.3.5 Purification of prenisin and its derivatives

Cell-free medium containing the peptide was diluted 1:1 with 50 mM lactic acid pH 3.0, filtered through a 0.45 μm membrane filter and subjected to SP-Sepharose chromatography. After peptide binding, the lactic acid buffer pH 3.0 was gradually changed to 50 mM HEPES-NaOH pH 7.0 by applying a gradient (0 - 100% 50 mM HEPES-NaOH pH 7.0) for 8 column volumes at a flow rate of 2 ml/min, followed by a wash step for 8 column volumes with 50 mM HEPES-NaOH pH 7.0. At this stage, the eluent showed a pH of 7.0. Finally, bound prenisin was eluted with 50 mM HEPES-NaOH pH 7.0, 1 M NaCl and 10 % (v/v) glycerol. Peptide elution was monitored at 215 nm and fractions were analyzed by SDS-PAGE. Prenisin containing fractions were pooled and filtered through an Amicon Ultra centrifugal filter (30 kDa cut-off) to remove high molecular weight contaminants. The flow through containing the prenisin was then concentrated with an Amicon Ultra centrifugal filter (3 kDa cut-off). Peptide concentrations were determined with a Pierce BCA Protein Assay Kit at 584 nm.

### 3.2.3.6 Purification of active nisin

Nisin was obtained as a lyophilized powder from commercial source, which contains ~2.5 % (w/w) nisin. For purification of active nisin about 1.3 g of powder (corresponding to ~32 mg nisin) was diluted in 100 ml 50 mM lactic acid pH 3.0 and filtered through a 0.45

μm membrane filter. The nisin solution was then applied to a 5 ml HiTrap SP HP ion exchange column using a flow of 2 ml/min. After binding, the column was washed with 50 mM lactic acid pH 3.0 until a stable base line was reached. The elution was performed by block elution in which the NaCl concentration was increased from 0 to 1 M in 200 mM steps. Protein elution was monitored at 215 nm and fractions were analyzed by SDS-PAGE. Nisin containing fractions were pooled and protein was precipitated with 25 % (v/v) trichloroacetic acid overnight at 4°C. Precipitated protein was washed two times with ice-cold acetone to remove residual NaCl and TCA and then suspended in 50 mM lactic acid pH 3.0 and stored at 4°C. Peptide concentrations were determined with a Pierce BCA Protein Assay Kit at 584 nm.

## 3.2.4 Protein analysis

### 3.2.4.1 Molar mass determination

Molar masses were determined by multi-angle light scattering (MALS) experiments. MALS is an analytical technique for determining the absolute molar masses of macromolecules such as proteins. Herein, a polarized light beam illuminates a solution containing the macromolecules of interest. The macromolecules scatter the irradiated light in dependency of their molar mass.

For mass determination of NisB the dialyzed protein was applied to a Superdex 200 10/300 column on an Äkta purifier using dialysis buffer as running buffer. Eluted protein was analyzed using a triple-angle light scattering detector and a differential refractive index (RI) detector. Data were analyzed with the ASTRA software package according to the equation (1).

$$\Delta LS = \left(\frac{I_\theta}{I_0}\right)_{solution} - \left(\frac{I_\theta}{I_0}\right)_{buffer} = K \cdot \left(\frac{dn}{dc}\right)^2 \cdot M_w \cdot c \qquad (1)$$

Where $I_\theta/I_0$ is the ratio of scattered light at angle θ and the incident light. ΔLS is the difference between the excess of light scattered by a solution containing a macromolecule at angle θ and the light scattered by the buffer. $M_w$ is the molecular mass of the scattering

macromolecule. C is the concentration of the macromolecule in solution, which can be determined by the absorbance at 280 nm if the extinction coefficient (ε) of the macromolecule is known.

$$c = \frac{Abs_{280\,solution} - Abs_{280\,buffer}}{\varepsilon} = \frac{\Delta Abs_{280}}{\varepsilon} \text{ with path length 1cm} \quad (2)$$

The dn/dc is the change of refractive index in dependency of the concentration of the macromolecule. For most soluble proteins the dn/dc has a value of 0.187 ml/g. The dn/dc can be determined experimentally by measuring the differences of the refractive index in dependency of known macromolecule concentrations.
Therefore, the concentration can be described as:

$$c = \frac{\Delta RI}{(dn/dc)} \quad (3)$$

K is a constant, which depends on the refractive index of the buffer without the macromolecule (n), the wavelength of the irradiating light (λ), the angle of the scattered light (Θ) and the distance between the scattering molecule and the detector (r). With $N_A$ is the Avogadro number.

$$K = \frac{2 \cdot \pi^2 \cdot n^2}{\lambda_0^4 \cdot N_A} \cdot \left( \frac{1 + \cos^2 \theta}{r^2} \right) \quad (4)$$

The molecular mass can be determined in dependency of the UV (absorption) or RI (refractive index) signal. By using equations (5) or (6).

$$M_w = \frac{\Delta LS}{K \cdot \left( \frac{dn}{dc} \right)^2 \cdot \frac{\Delta Abs_{280}}{\varepsilon}} \quad (5)$$

$$M_w = \frac{\Delta LS}{K \cdot \left( \frac{dn}{dc} \right)^2 \cdot \frac{\Delta RI}{(dn/dc)}} \quad (6)$$

### 3.2.4.2 HPLC analysis of prenisin and its derivatives

Analytical RP-HPLC was performed with a LiChrospher WP 300 RP-18 end capped column (Merck) at room temperature. Purified prenisin or nisin was injected at a concentration of 50 μM and eluted by mixing the aqueous buffer A (10% acetonitrile, 0.1% (v/v) trifluoroacetic acid) with the organic solvent buffer B (90% acetonitrile, 0.1% (v/v) trifluoroacetic acid). Elution was performed by applying a gradient of 0 - 100% of buffer B over the course of 60 min. at a flow rate of 1 ml/min. The eluent was monitored by measuring the absorbance at 220 nm.

### 3.2.4.3 Interaction studies using size exclusion chromatography (SEC)

For complex formation, purified prenisin or nisin diluted in 50 mM HEPES-NaOH pH 7.0, 1 M NaCl and 10% (v/v) glycerol was mixed with purified NisB, which was stored in 50 mM HEPES-NaOH pH 8.0, 500 mM NaCl and 10% (v/v) glycerol, giving a molar ratio of 2:1. Complex formation was allowed to proceed for 1 h at room temperature. Next, the samples were applied to a Superdex 200 pc 3.2/30 size exclusion column on an ÄKTA micro system at 4°C using 50 mM HEPES-NaOH pH 7.4 and 500 mM NaCl as elution buffer. Protein elution was monitored at 215 nm and 280 nm and the co-elution of NisB and prenisin was analyzed by SDS-PAGE and western blotting. Protein was visualized by silver staining and immunodetection.

### 3.2.4.4 Surface plasmon resonance (SPR) measurements

SPR measurements are used to study biomolecular interactions. The binding events are detected using the phenomenon of surface plasmon resonance (SPR). SPR occurs when polarized light under conditions of total internal reflection strikes an interface of two transparent media with different refractive indexes (for example glass and buffer) from the side of higher refractive index (glass). Although the incident light is totally reflected, an electromagnetic field component called the evanescent wave penetrates the medium of lower refractive index (buffer). If the interface between the media is coated with a thin gold layer the evanescent wave causes a reduction of the intensity of the reflected light (Figure 13). The angle at which this phenomenon is obtained is called the SPR angle.

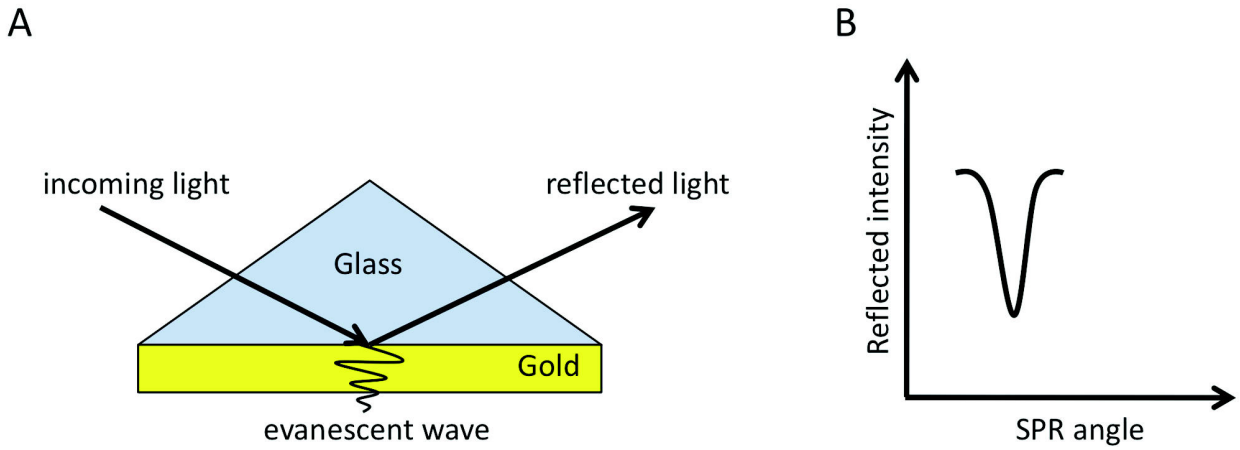


**Figure 13. Surface Plasmon resonance. A:** Under the conditions of total internal reflection at a gold-coated interface, an evanescent wave propagates into the medium of lower reflective index. **B:** The intensity of the reflected light decreased at the SPR angle.

The SPR angle depends on the refractive index of the medium into which the evanescent wave propagates (the thin gold layer). A sensor chip for SPR measurements consists of a glass layer and a thin gold layer. The gold layer is covered with a surface matrix on which biomolecules can be immobilized. Interaction between an analyte and an immobilized ligand causes a change of the refractive index of the gold layer and therefore the SPR angle also changes (Figure 14A and 14B). The change of the SPR angle due to mass changes on the chip surface is the measured signal. Therefore, the association and dissociation of an analyte to an immobilized ligand can be monitored without the need to label any interaction partner. Association of the analyte to the ligand results in a signal increase whereas dissociation leads to a signal decrease (Figure 14C) (123).

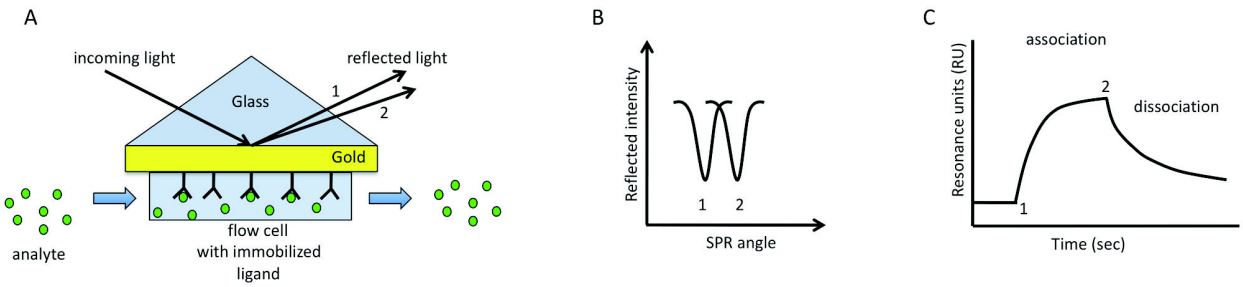


**Figure 14. SPR measurement of biomolecule interaction. A:** An interaction between an analyte and immobilized ligand leads to a change of mass on the sensor chip surface. **B:** The change of the SPR angle due to the mass difference on the sensor chip surface (1 and 2). **C:** Typical sensorgram for the analyte-ligand interaction. During association the signal increases (from 1 to 2) and during dissociation the signal decreases.

All measurements were performed with a Biacore X (GE Healthcare) at 25°C and at a constant flow rate of 30 µl/min using a NTA sensor chip (GE Healthcare). The SPR buffer

was composed of 50 mM HEPES-NaOH pH 7.4, 250 mM NaCl and 50 μM EDTA. Prior to immobilization of his-tagged NisB both flow cells were saturated with $Ni^{2+}$ ions by injecting 5 μl of a 10 mM $Ni^{2+}$ solution using a flow rate of 10 μl/min. For immobilization, purified NisB was diluted in SPR buffer to a final concentration of 170 nM, whereupon 5 μl was injected on flow cell 2 with a flow rate of 10 μl/min. The first flow cell was left with just $Ni^{2+}$ as reference. Prenisin and its derivatives (including active nisin and the FNLD/AAAA mutant) were diluted from stock solutions in SPR buffer to final concentrations ranging from 46 nM to 2.9 μM. Due to the low binding responses of the fully modified prenisin higher concentrations were tested in addition, with no apparent effect on the kinetic constants and the affinity (data not shown). For each peptide concentration, 50 μl of the prenisin samples were injected at a flow rate of 30 μl/min. Sensorgrams were recorded for 250 seconds. After every analyte injection, the chip surface was regenerated by injecting successively 10 μl of 350 mM EDTA, 10 μl of 100 mM NaOH and 10 μl of 0.5 % (w/v) SDS at a flow rate of 10 μl/min. Subsequently NisB was immobilized again as described above. All sensorgrams presented are corrected for background and bulk flow effects. Each measurement was repeated at least three times. Data were analyzed using the BIAevaluation 4.1 software according to equation 7, which describes the association, equation 8, which describes the dissociation and equation 9, which describes the dissociation constant, $K_D$, as reported in (124). Where dR/dt is the binding rate, $k_{on}$ is the association rate, C is the concentration of injected analyte, $R_{max}$ is proportional to the ligand concentration, R is proportional to the formed ligand-analyte complex and $k_{off}$ is the dissociation rate.

$$\frac{dR}{dt} = k_{on} \bullet C \bullet \left(R_{\max} - R\right) - k_{off} \bullet R \tag{7}$$

$$\frac{dR}{dt} = -k_{off} \bullet R \tag{8}$$

$$K_D = \frac{k_{off}}{k_{on}} \tag{9}$$

As the binding responses from SPR measurements correlates to the mass of molecules bond to the surface, the stoichiometry of the interaction can be evaluated by equation 10 (125,126).

$$stoichiometry = \frac{R_{\max} \bullet MW_l}{MW_a \bullet R_l} \tag{10}$$

Herein, the analyte binding capacity $R_{max}$ can be extrapolated from experimental data and the immobilized ligand response $R_l$ is obtained directly from a sensorgram recorded during ligand immobilization.

# 4 Results

## 4.1 Purification of NisB

The nisin biosynthesis machinery consists of several enzymes, namely, the dehydratase NisB, the cyclase NisC, the ABC transporter NisT and the protease NisP. This work focused on the dehydratase NisB that dehydrates specifically serine and threonine residues in the propeptide part of prenisin. To investigate the interaction of NisB *in vitro* with unmodified prenisin as well as with prenisin derivatives, such as dehydrated prenisin, fully modified prenisin and the FNLD/AAAA mutant, NisB had to be purified to homogeneity in sufficient quantity.

For this, NisB with a carboxy-terminal $His_6$-tag was expressed in *L. lactis* NZ9000 and purified from the cytosol using IMAC with 50 mM HEPES-NaOH pH 8.0, 150 mM NaCl, 10 % glycerol as wash buffer and with 50 mM HEPES-NaOH pH 8.0, 150 mM NaCl, 10 % glycerol and 500 mM imidazole as elution buffer (Figure 15A). SDS-PAGE analysis showed that NisB and two major contaminants (~ 80 kDa and ~ 35 kDa) were present in the elution fractions (Figure 15B, lanes 5-10 marked by asterisk).

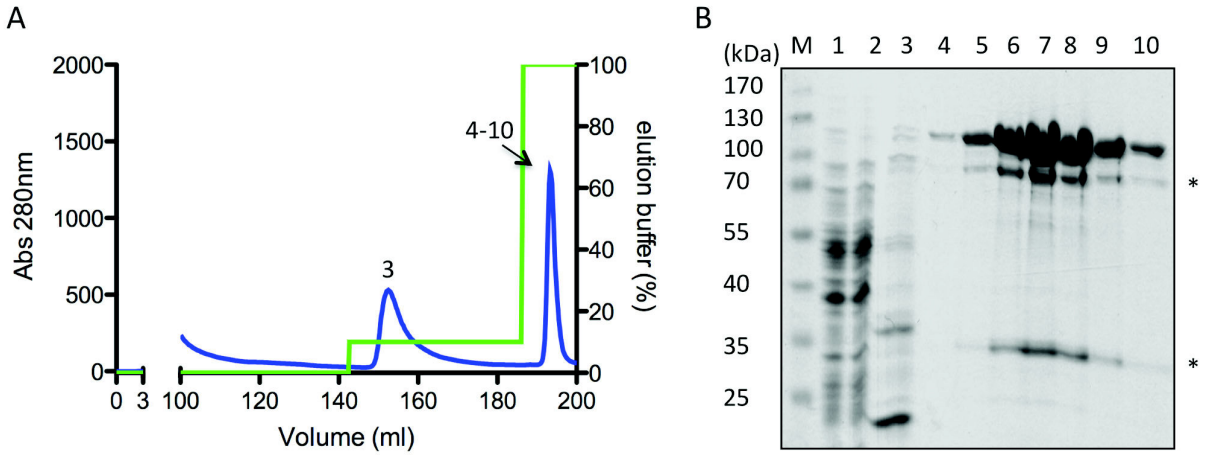


**Figure 15. Purification and SDS-PAGE analysis of NisB. A:** Immobilized metal ion chromatography (IMAC) of NisB. The elution was monitored by measuring the absorbance at 280 nm (blue line). Concentration of the elution buffer (50 mM HEPES-NaOH pH 8.0, 150 mM NaCl, 500 mM imidazole, 10 % glycerol) is shown in % (green line). **B:** SDS-PAGE analysis of NisB IMAC elution fractions. M: molecular weight marker proteins (kDa); lane 1: supernatant after high-speed centrifugation of *L. lactis* cell lysate; lane 2: flow through of IMAC; lane 3: 50 mM imidazole IMAC wash; lanes 4-10: 500 mM imidazole IMAC step elution.

The contaminants turned out to be NisB degradation products as determined by immunoblotting and mass spectrometry (Figure 16). The 80 kDa protein fragment was isolated from a polyacrylamide gel and digested with trypsin. The trypsin fragments were subsequently analyzed by mass spectrometry. Identified peptides were all attributed to NisB. From the data it can be deduced that the 80 kDa contaminant comprises at a minimum a NisB fragment ranging from Ser25 to Arg707. The theoretical molecular weight of this fragment is 80.7 kDa and is in line with the molecular weight deduced from the SDS-PAGE analysis (Figure 16A). In addition, the 35 kDa fragment was identified as a NisB degradation product by immunoblotting. By using an anti-His antibody a 35 kDa fragment carrying a His-tag, which is fused to the C-terminus of NisB, was identified (Figure 16B, lane 1).

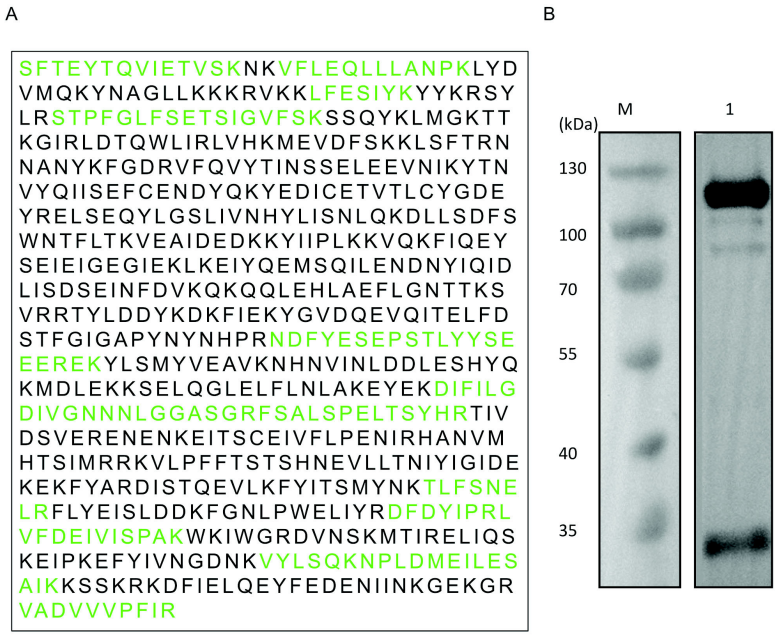


**Figure 16. Analysis of NisB degradation products. A:** NisB primary sequence from amino acids 25 to 707 with identified peptides highlighted in green. **B:** Western blot analysis of NisB after IMAC.

The NisB containing fractions were pooled and concentrated using a 100 kDa cut-off filter device. Subsequent SEC revealed that nearly 50 % of the applied sample eluted as aggregates (Figure 17A and B). Omitting the concentration step prior to SEC significantly reduced the formation of these aggregates (Figure 17C). Elution fractions were analyzed by SDS-PAGE (Figure 17D). The concentrations were determined using a NanoDrop 1000 with the theoretical molecular weight of NisB carrying a His-tag (118.3 kDa) and a calculated extinction coefficient of 128400 $L \cdot mol^{-1} \cdot cm^{-1}$ resulting in a yield of 1 mg/liter of cell culture.

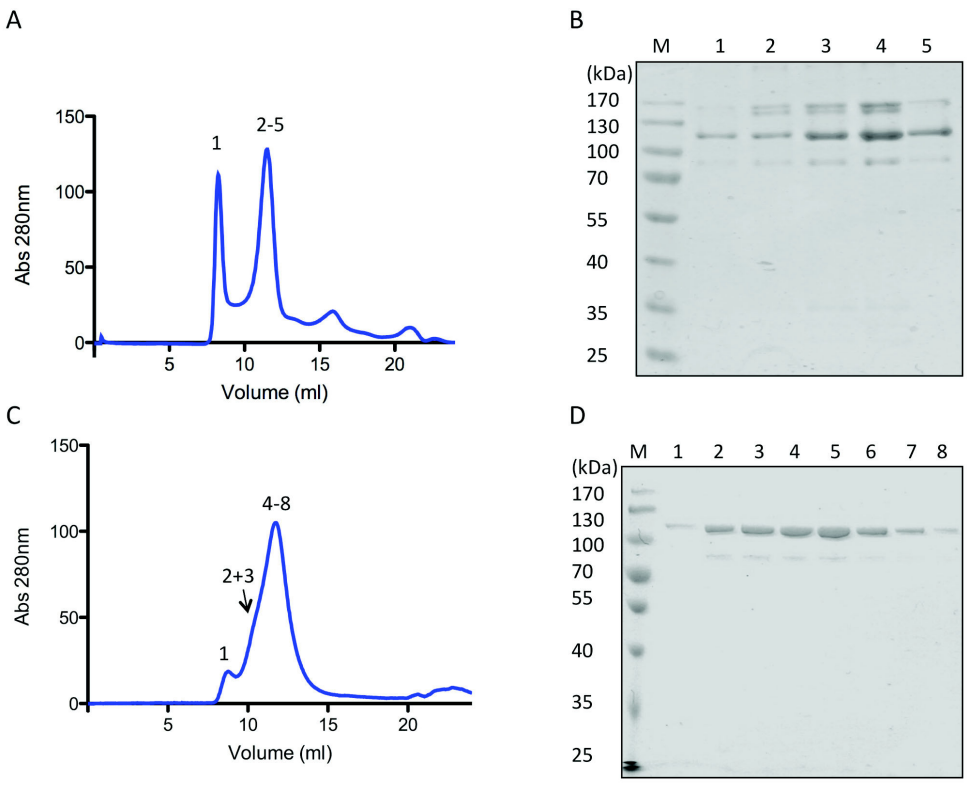


**Figure 17. SEC and SDS-PAGE analysis of NisB. A:** Size exclusion chromatography of concentrated NisB after IMAC using 50 mM HEPES-NaOH pH 7.0, 150 mM NaCl and 10% glycerol. **B:** SDS-PAGE analysis of A: M: molecular weight marker proteins (kDa); lane 1: aggregate peak; lane 2-5: fractions eluting at 11.6 ml **C:** Size exclusion chromatography of NisB after IMAC without previous concentration of NisB using 50 mM HEPES-NaOH pH 7.0, 150 mM NaCl and 10% glycerol. Eluted protein was monitored by measuring the absorbance at 280 nm. **D:** SDS-PAGE analysis of C: M: molecular weight marker proteins (kDa); lane 1: aggregate peak; lane 2-3: fractions eluting at 10.4 ml; lane 4-8: analysis of the peak eluting at 11.9 ml.

SDS-PAGE analysis demonstrated that the peak eluting between 10 and 15 ml contains NisB (Figure 17C and 15D). However, since this NisB peak is rather broad and not symmetric, this suggests that the eluted NisB is not homogenously (Figure 17C, shoulder marked by arrow). Moreover, a small amount of NisB degradation product was also observed in these fractions. Therefore, an optimization of the buffer conditions for the NisB purification was necessary.

## 4.2 Optimization of buffer solutions for NisB purification

The purpose of screening different buffer conditions for NisB purification was to get pure and homogenous NisB after SEC. Therefore, different buffer conditions were tested. The pH of the used buffers ranged from pH 4.0 to pH 10.0 and for each pH NaCl concentrations of 50 mM, 150 mM and 500 mM were tested. NisB after IMAC (as described in 3.1) was separated in aliquots of 150 µl with a protein concentration of 2

mg/ml. The aliquots were dialyzed over night at 4°C against 200 ml of buffer solution. After dialysis the samples were analyzed for precipitated protein. This was done by visual inspection and centrifugation at 14000 rpm and 4°C for 10 min to sediment precipitated protein. All samples with a NaCl concentration of 50 mM and 150 mM showed pellets of precipitated protein, whereas NisB solutions with a pH above 5.0 and a NaCl concentration of 500 mM were all observed to be clear and showed the highest protein concentration after centrifugation and filtration (Table 3). This demonstrated that NisB was soluble under these conditions.

**Table 3. Summary of buffer screen**

| Buffer | Clear after dialysis (+/-) | Clear after spinning (+/-) | Conc. after spinning (mg/ml) | Conc. after filtration (mg/ml) | Retention volume (ml) |
|---|---|---|---|---|---|
| pH 4.0 NaOAc 50 mM NaCl | - | - | 1.2 | 1.0 | 2.14 |
| pH 4.0 NaOAc 150 mM NaCl | - | -- | 0.06 | n.d. | n.d. |
| pH 4.0 NaOAc 500 mM NaCl | -- | -- | 0.04 | n.d. | n.d. |
| | | | | | |
| pH 5.0 NaOAc 50 mM NaCl | -- | -- | 0.02 | n.d. | n.d. |
| pH 5.0 NaOAc 150 mM NaCl | -- | -- | 0.1 | n.d. | n.d. |
| pH 5.0 NaOAc 500 mM NaCl | -- | - | 1.1 | 0.7 | 1.44 |
| | | | | | |
| pH 6.0 MES 50 mM NaCl | -- | -- | 0.08 | n.d. | n.d. |
| pH 6.0 MES 150 mM NaCl | -- | -- | 1.1 | 0.6 | 2.14 |
| pH 6.0 MES 500 mM NaCl | + | + | 1.4 | 1.3 | 1.35 |
| | | | | | |
| pH 7.0 HEPES 50 mM NaCl | -- | -- | 0.6 | 0.6 | 2.1 |
| pH 7.0 HEPES 150 mM NaCl | - | - | 1.2 | 1.0 | 1.48 |
| pH 7.0 HEPES 500 mM NaCl | + | + | 1.5 | 1.25 | 1.35 |

n.d.: not determined

**Table 3. Summary of buffer screen**

| | | | | | |
|---|---|---|---|---|---|
| pH 8.0 HEPES 50 mM NaCl | - | -- | 0.08 | n.d. | n.d. |
| pH 8.0 HEPES 150 mM NaCl | + | - | 1.0 | 0.9 | 1.43 |
| pH 8.0 HEPES 500 mM NaCl | + | + | 1.4 | 1.2 | 1.35 |
| | | | | | |
| pH 8.0 TRIS 50 mM NaCl | - | - | 1.2 | 1.2 | 1.44 |
| pH 8.0 TRIS 150 mM NaCl | + | - | 1.1 | 1.1 | 1.35 |
| pH 8.0 TRIS 500 mM NaCl | + | + | 1.5 | 1.0 | 1.35 |
| | | | | | |
| pH 9.0 TRIS 50 mM NaCl | -- | -- | 0.3 | n.d. | n.d. |
| pH 9.0 TRIS 150 mM NaCl | + | - | 1.2 | 1.0 | 1.44 |
| pH 9.0 TRIS 500 mM NaCl | + | + | 1.2 | 1.2 | 1.35 |
| | | | | | |
| pH 10.0 CAPS 50 mM NaCl | - | - | 0.1 | n.d. | n.d. |
| pH 10.0 CAPS 150 mM NaCl | + | - | 1.3 | 1.2 | 0.96 |
| pH 10.0 CAPS 500 mM NaCl | + | + | 1.5 | 1.3 | 0.96 |

n.d.: not determined

The concentration of the supernatants was measured and samples, which still showed soluble protein, were filtered through a 0.45 µm syringe filtered and analyzed by SEC (Figure 18).

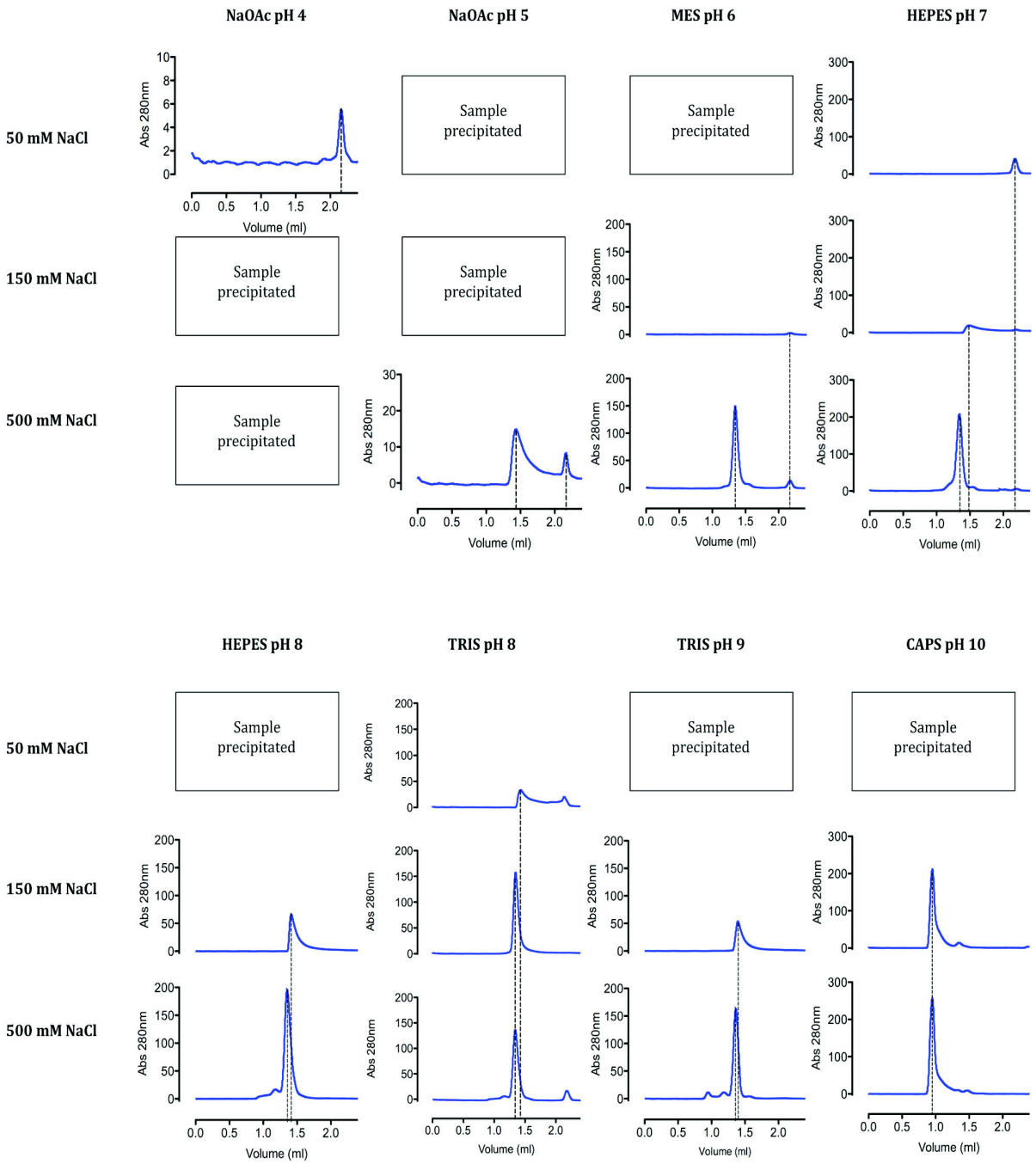


**Figure 18. SEC analysis of NisB buffer screen.** Samples were applied to SEC after dialysis against certain buffers ranging from pH 4.0 to pH 10.0. Each pH was tested with 50 mM NaCl, 150 mM NaCl and 500 mM NaCl as indicated. Samples, which showed precipitation, are indicated as "sample precipitated".

SEC analysis showed that buffer conditions with a NaCl concentration of 500 mM and a pH above 5.0 resulted in symmetric NisB elution peaks, with a high absorbance at 280 nm and a retention volume of 1.35 ml. In addition, NisB in 50 mM TRIS-HCl pH 8.0 and 150 mM NaCl also eluted at 1.35 ml. Whereas, NisB at pH 10.0 eluted at the void volume, which indicated that the protein was aggregated but still soluble. NisB in buffers containing 150 mM NaCl (except for NisB in TRIS-HCl pH 8.0) eluted at 1.44 ml in a

broad asymmetric peak and showed less absorbance at 280 nm as expected for the injected concentrations (Table 3).

This indicated that the protein continued to precipitate during analysis and that eluted NisB is not present in a single conformation, which is in line with the observed pellets for these buffer conditions. Several samples with a NaCl concentration of 50 mM showed a protein concentration suitable for SEC analysis, for example NisB in NaOAc pH 4.0, 50 mM NaCl and NisB in HEPES pH 7.0, 50 mM NaCl. However, SEC analysis of these samples showed only a small peak at 2.1 ml. Moreover, NisB could not be detected by SDS-PAGE in those fractions (Figure 19, lane 1 and lane 5). The fact that the sample loop was clogged after such runs indicated that the majority of the protein had precipitated in the sample loop prior to injection.

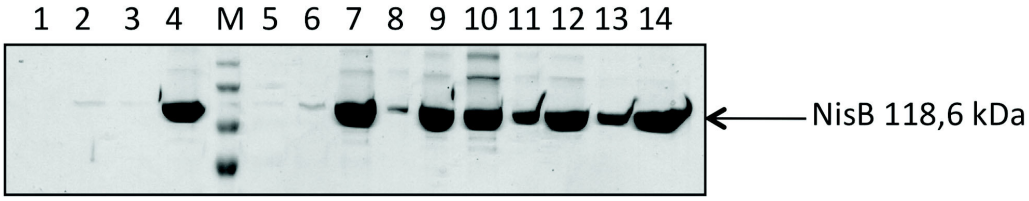


**Figure 19. SDS-PAGE analysis of NisB buffer screen.** Fraction eluting at 1.35 ml were applied on the SDS-gel. lane 1: NisB in NaOAc pH 4.0, 50 mM NaCl; lane 2: NisB in NaOAc pH 5.0, 500 mM NaCl; lane 3: NisB in MES pH 6.0, 150 mM NaCl; lane 4: NisB in MES pH 6.0, 500 mM NaCl; M: molecular weight marker proteins (kDa) lane 5: NisB in HEPES pH 7.0, 50 mM NaCl; lane 6: NisB in HEPES pH 7.0, 150 mM NaCl; lane 7: NisB in HEPES pH 7.0, 500 mM NaCl; lane 8: NisB in TRIS pH 8.0, 50 mM NaCl; lane 9: NisB in TRIS pH 8.0,150 mM NaCl; lane 10: NisB in TRIS pH 8.0, 500 mM NaCl; lane 11: NisB in TRIS pH 9.0, 150 mM NaCl; lane 12: NisB in TRIS pH 9.0, 500 mM NaCl; lane 13: NisB in HEPES pH 8.0, 150 mM NaCl; lane 14: NisB in HEPES pH 8.0, 500 mM NaCl. Samples at pH 10.0 are not included on this gel.

SDS-PAGE analysis of the peak fractions eluting at 1.35 ml confirmed the results of the SEC analysis. NisB in TRIS pH 8.0, 150 mM NaCl and NisB in buffers containing a NaCl concentration of 500 mM showed the highest protein content.

## 4.3 Oligomeric state of purified NisB

The symmetric peak of NisB eluting at 1.35 ml suggests a homogenous species with a molecular weight of 170.2 kDa, as calculated from marker proteins with known molecular weight. This does however not correspond to the molecular weight obtained by SDS-PAGE analysis where NisB migrates between 100 kDa and 130 kDa (Figure 19) indicating a NisB monomer, nor to the molecular weight of a possible NisB dimer, which

is 236.6 kDa. Therefore, the molecular mass of NisB dissolved in MES pH 6.0, 500 mM NaCl; HEPES pH 7.0, 500 mM NaCl; HEPES pH 8.0, 500 mM NaCl; TRIS pH 8.0, 150 mM NaCl; TRIS pH 8.0, 500 mM NaCl and TRIS pH 9.0, 500 mM NaCl was determined by SEC combined with multi angle light scattering (MALS) (Figure 20A-F). Here the blue line represents the normalized absorbance at 280 nm and is depicted on the left y-axis. The determined molecular weight is represented by the green line and is depicted on the right y-axis (Figure 20). For SEC and MALS analysis 200 µl of NisB with a concentration of 4 mg/ml were dialyzed against 250 ml of the above mentioned buffers over night at 4°C. Prior to SEC and MALS analysis samples were centrifuged at 14,000 rpm at 4°C for 10 min and filtered through a 0.45 µm syringe filter.

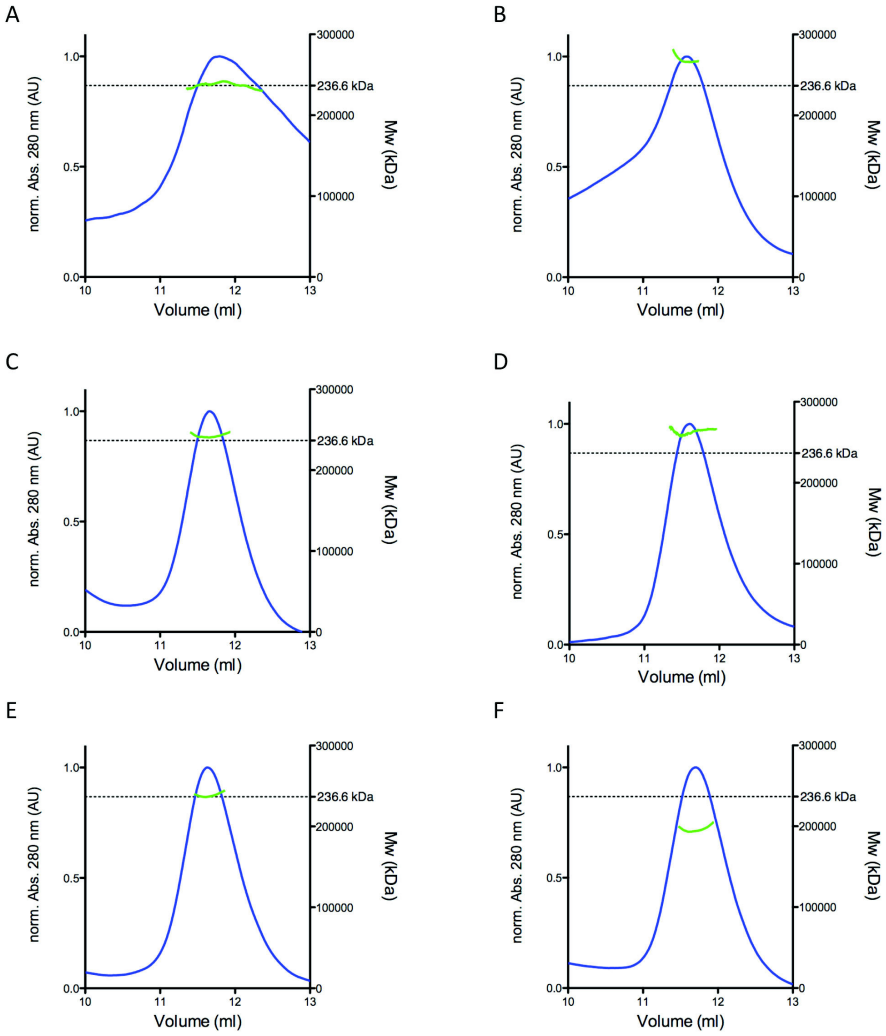


**Figure 20. SEC and MALS-RI analysis of NisB.** NisB in buffers which resulted in a good yield of NisB were applied to SEC. Shown are the elution profiles (blue lines; left y-axis) and the determined masses (green lines; right y-axis). **A:** NisB in MES pH 6.0, 500 NaCl; **B:** NisB in HEPES pH 7.0, 500 NaCl; **C:** NisB in HEPES pH 8.0, 500 NaCl; **D:** NisB in TRIS pH 8.0, 150 NaCl; **E:** NisB in TRIS pH 8.0, 500 NaCl; **F:** NisB in TRIS pH 9.0, 500 NaCl. Dotted black lines indicate the theoretical molecular weight of a NisB dimer (236.6 kDa)

The determined masses were 237.2±2.4 kDa for NisB in MES pH 6.0, 500 mM NaCl (Figure 20A), 268.6±13.4 kDa for NisB in HEPES pH 7.0, 500 mM NaCl (Figure 20B), 242.3±12.1 kDa for NisB in HEPES pH 8.0, 500 mM NaCl (Figure 20C), 263.5±1.6 kDa for NisB in TRIS pH 8.0, 150 mM NaCl (Figure 20D), 239.3±7.1 kDa for NisB in TRIS pH 8.0 500 mM NaCl (Figure 20E) and 196.1±9.8 kDa for NisB in TRIS pH 9.0, 500 mM NaCl (Figure 20F), respectively. The molecular weight determined for NisB in HEPES pH 8.0, 500 mM NaCl or in TRIS pH 8.0, 500 mM NaCl revealed that NisB is present as a dimer, because the calculated molecular weight of the NisB dimer is 236.6 kDa. The determined mass for NisB in MES pH 6.0, 500 mM NaCl was 237.2±2.4 kDa, which also indicated a NisB dimer. However, the elution profile showed a asymmetric broad peak with a leading shoulder, indicating that higher molecular weight compounds were also present (trimers or tetramers), and then the peak signal slowly decreased, which suggests that also smaller molecular weight compounds were eluted (monomers) (Figure 20A). Therefore, it can be reasoned that NisB is not homogenous in MES pH 6.0, 500 mM NaCl. The higher molecular weight observed for NisB in HEPES pH 7.0, 500 mM NaCl can be explained by the shape of the elution peak. Here higher oligomers of NisB likely co-eluted with the dimeric NisB (Figure 20B). A similar high molecular weight of 263±1.6 kDa for NisB was obtained in TRIS pH 8.0, 150 mM NaCl. In this buffer, precipitation of NisB was readily observed after centrifugation of the sample prior to MALS-SEC (Table 4). Therefore, it can be assumed that also some NisB aggregates were co-eluted and due to these aggregates the calculated mass was increased. Compared to the other samples NisB in TRIS pH 9.0, 500 mM NaCl eluted at a later retention volume (Table 4). The determined molecular weight of 196.1±9.8 kDa under these conditions can be explained by the fact that at pH 9.0 a number of the NisB dimers likely dissociate, resulting in a lower overall mass detected.

**Table 4. Summary of mass determination of NisB**

| Buffer | Clear after spinning (+/-) | Conc. after spinning (mg/ml) | Conc. after filtration (mg/ml) | Retention volume (ml) | Determined mass (kDa) |
|---|---|---|---|---|---|
| MES pH 6.0 500 mM NaCl | + | 3.0 | 3.0 | 11.80 | 237.2±2.4 |
| HEPES pH 7.0 500 mM NaCl | + | 3.0 | 2.9 | 11.60 | 268.6±13.4 |
| HEPES pH 8.0 500 mM NaCl | + | 3.2 | 3.0 | 11.65 | 242.3±12.1 |
| TRIS pH 8.0 150 mM NaCl | -- | 2.3 | 2.2 | 11.60 | 263.5±1.6 |
| TRIS pH 8.0 500 mM NaCl | + | 3 | 2.8 | 11.65 | 239.3±7.1 |
| TRIS pH 9.0 500 mM NaCl | - | 2.9 | 2.9 | 11.70 | 196.1±9.8 |

Thus, NisB in HEPES-NaOH pH 8.0, 500 mM NaCl showed a symmetric elution peak corresponding to homogenous NisB dimers as determined by SEC and MALS analysis (Figure 18 and Figure 20C). Furthermore, SDS-PAGE analysis revealed that NisB was pure (Figure 19 lane 14). Therefore, all following NisB purifications were performed with HEPES-NaOH pH 8.0 and 500 mM NaCl as buffer system.

## 4.4 Optimized purification of NisB

NisB carrying a carboxy-terminal $His_6$-tag was expressed in *L. lactis*, and purified from the cytosol using IMAC with 100 mM HEPES-NaOH pH 8.0, 500 mM NaCl, 10 mM imidazole and 10 % (v/v) glycerol as wash buffer and 100 mM HEPES-NaOH pH 8.0, 500 mM NaCl, 500 mM imidazole and 10 % (v/v) glycerol as elution buffer. After washing with 50 mM imidazole to get rid of any unspecifically bond compounds, NisB was eluted with 250 mM imidazole (Figure 21A). To elute protein that was possibly still bound to the column 500 mM imidazole was applied. However, this did not show any protein eluting. SDS-PAGE analysis revealed that the two major degradation products of NisB were still present (~80 kDa and ~35 kDa) (Figure 21). After IMAC, NisB containing fractions were pooled and applied to a Superdex 200 16/600 column for SEC with 50 mM HEPES-NaOH pH 8.0, 500 mM NaCl and 10 % (v/v) glycerol as elution buffer. Under these conditions NisB eluted as a symmetric peak and could be separated from the degradation products as analyzed by SEC and SDS-PAGE (Figure 21C and 19D). The

fractions containing pure NisB were pooled and concentrated and could be stored at 4°C for at least one week, as demonstrated by SEC analysis (data not shown). The typical yield of such purification protocol was 1.5 mg/liter of cell culture.

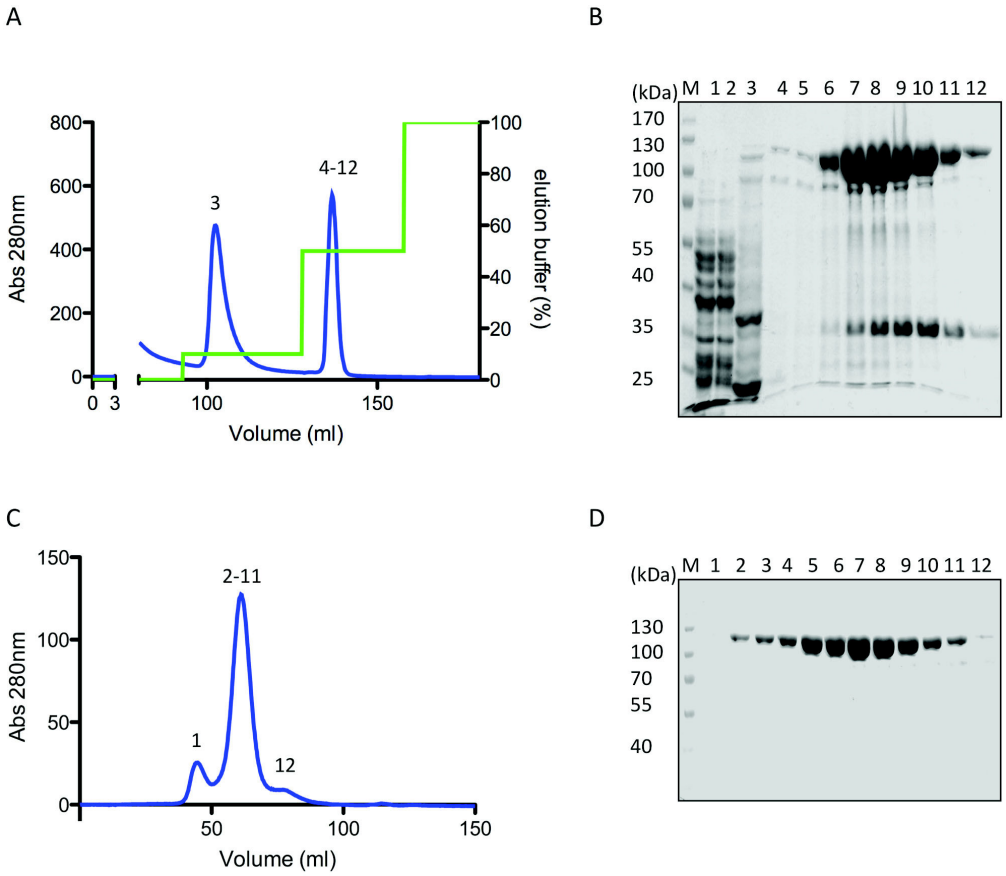


**Figure 21. Purification and SDS-PAGE analysis of NisB. A:** IMAC of NisB. The elution was monitored by measuring the absorbance at 280 nm (blue line). The concentration of imidazole in elution buffer (50 mM HEPES-NaOH pH 8.0, 500 mM NaCl, 500 mM imidazole, 10 % glycerol) is shown in % (green line). **B:** SDS-PAGE analysis of NisB IMAC: M: molecular weight marker proteins (kDa); lane 1: supernatant after high-speed centrifugation of *L. lactis* cell lysate; lane 2: flow through of IMAC; lane 3: 50 mM imidazole IMAC wash; lane 4-12: 250 mM imidazole IMAC step elution. **C:** Size exclusion chromatography of NisB after IMAC using 50 mM HEPES pH8, 500 mM NaCl and 10 % (v/v) glycerol. Eluted protein was monitored by measuring the absorbance at 280 nm. **D:** SDS-PAGE analysis of NisB SEC: M: molecular weight marker proteins (kDa); lane 1: aggregate peak; lane 2-11: analysis of peak eluting at 62.1 ml; lane 12: analysis of peak eluting at 81.3 ml.

Purified NisB was further analyzed by MALS. The determined mass of pure NisB in HEPES pH 8.0, 500 mM NaCl was 238±1.2 kDa, which demonstrated that purified NisB is a dimer under these conditions (Figure 22). Thus, the improved purification protocol leads to pure and dimeric NisB.

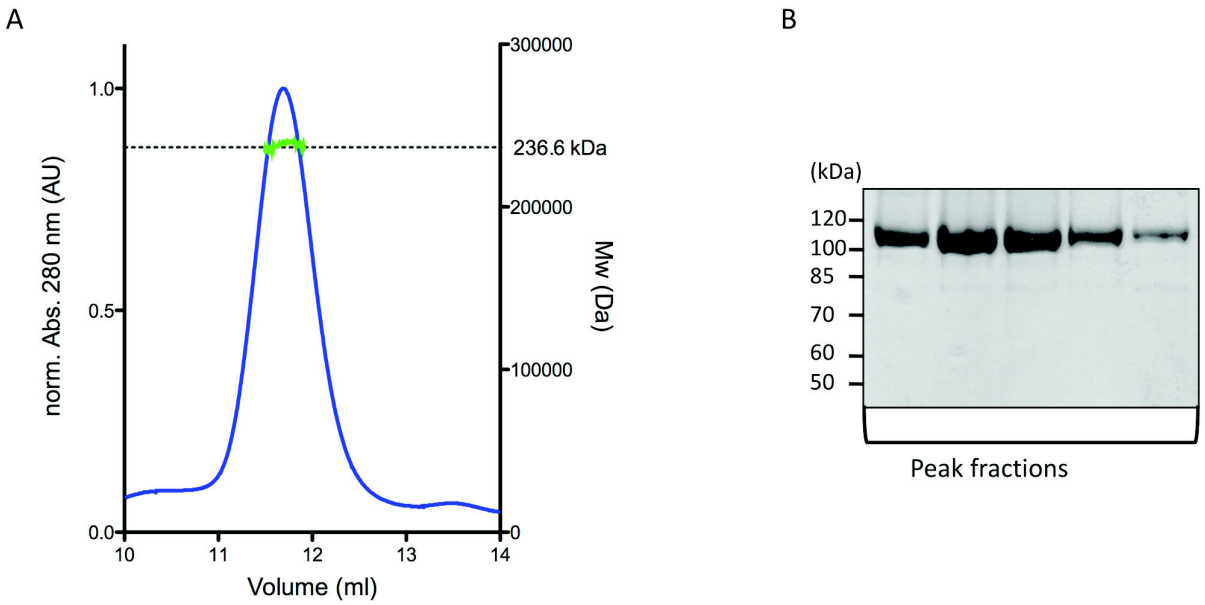


**Figure 22. MALS-SEC analysis of pure NisB. A:** Purified NisB was analyzed by using 50 mM HEPES pH 8.0, 500 mM NaCl as SEC running buffer. Blue curve is the normalized absorbance at 280 nm and is depicted on the left y-axis. Green curve is the molecular weight and is depicted on the right y-axis. The black dotted line marks the molecular weight of dimeric NisB (236.6 kDa). **B:** SDS-PAGE analysis of the elution peak.

## 4.5 Purification of unmodified, dehydrated, fully modified prenisin and the FNLD/AAAA mutant

For investigating potential interaction of the dehydratase NisB with unmodified, dehydrated and fully modified prenisin as well as with the FNLD/AAAA mutant *in vitro* the various prenisins had to be purified. Published protocols for prenisin purification report lactic acid at pH 3.0 as buffer (87). Because of the sensitivity of the analytical technique used for interaction studies prenisin had to be purified in a buffer similar to that of NisB. By changing the lactic acid buffer during prenisin purification it was tried to change the pH from 3.0 to 7.0, such that the eluted prenisin was in buffer pH 7.0. A higher pH is not desirable as it is known that at pH 8.0 and above dehydrated prenisin starts to autocyclize unspecifically (117).

*L. lactis* secreting prenisin was grown in minimal medium. After inducing prenisin expression with active nisin, the cells were grown over night at 30°C. The next day the cells were separated from the medium by centrifugation at 8000 xg. Prenisin was purified from the medium supernatant using cation exchange chromatography. Therefore the supernatant was diluted 1:1 in 50 mM lactic acid pH 3.0 and subsequently filtered through a 0.45 µm membrane filter. After loading prenisin to the cation

exchange column, the column was washed with 50 mM lactic acid pH 3.0 until no further eluting compounds could be detected by the absorbance at 215 nm. The wash buffer was then changed to 50 mM HEPES-NaOH pH 7.0, which resulted in an elution signal, as evidenced by the large absorbance peak at 215 nm (Figure 23A, I). After washing, the eluent showed a stable pH of 7.0. At this stage the NaCl concentration was increased to 1 M to elute bound peptide. This was done by applying 50 mM HEPES-NaOH pH 7.0, 1 M NaCl and 10 % (v/v) glycerol (Figure 23A, II). SDS-PAGE analysis of eluted fractions revealed that due to the drastic change of the buffer a large amount of prenisin eluted that still had an acidic pH (Figure 23A, 1 and 21B, lane 1). Eluted fractions with 50 mM HEPES-NaOH pH 7.0, 1 M NaCl and 10 % (v/v) glycerol (Figure 23A, II) were analyzed by SDS-PAGE and showed pure, unmodified prenisin (Figure 23B, lanes 2-4).

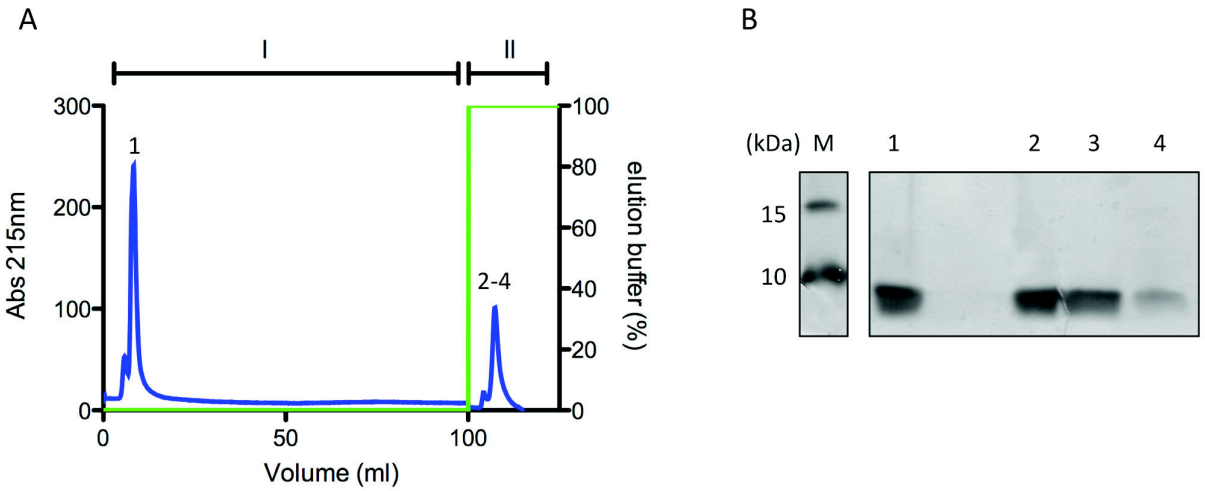


**Figure 23. Purification of unmodified prenisin. A:** Shown is an elution profile of the cation exchange chromatography of unmodified prenisin (blue line) and the concentration of elution buffer (50 mM HEPES-NaOH pH 7.0 and 1 M NaCl) in % (green line) I: after washing with 50 mM lactic acid until a stable baseline was reached, the wash buffer was changed to 50 mM HEPES-NaOH pH 7.0 resulting in a elution peak after one column volume. II: Peptide was eluted using a one step gradient from 0 to 1 M NaCl in 50 mM HEPES pH7.0. **B:** SDS-PAGE analysis of elution fractions. M: molecular weight marker proteins (kDa); lane 1: elution fractions after buffer was changed; lanes 2-4: fractions eluted with 50 mM HEPES-NaOH pH 7.0, 1 M NaCl and 10 % glycerol.

Because not all eluted prenisin could be recovered in 50 mM HEPES-NaOH pH 7.0 likely due to the rapid buffer change, the buffer change and therefore the pH change from 3.0 to 7.0 was performed by using a gradient. For this, after applying prenisin to the column the pH was changed from 3.0 to 7.0 by changing the buffer from lactic acid pH 3.0 to 50 mM HEPES-NaOH pH 7.0 using a gradient over 8 column volumes. Importantly, under these conditions no peptide eluted (Figure 24A, I). Subsequently the column was washed with 8 column volumes of 50 mM HEPES-NaOH pH 7.0. Here, two minor elution

peaks were observed (Figure 24A, II). At this stage the eluent showed a pH of 7.0 and the peptide was eluted by applying 50 mM HEPES pH 7.0, 1 M NaCl and 10 % (v/v) glycerol in a one step gradient (Figure 24A, III). SDS-PAGE analysis revealed that prenisin eluted only in 50 mM HEPES pH 7.0, 1 M NaCl and 10 % glycerol with some higher molecular weight impurities (Figure 24B, lanes 3-10).

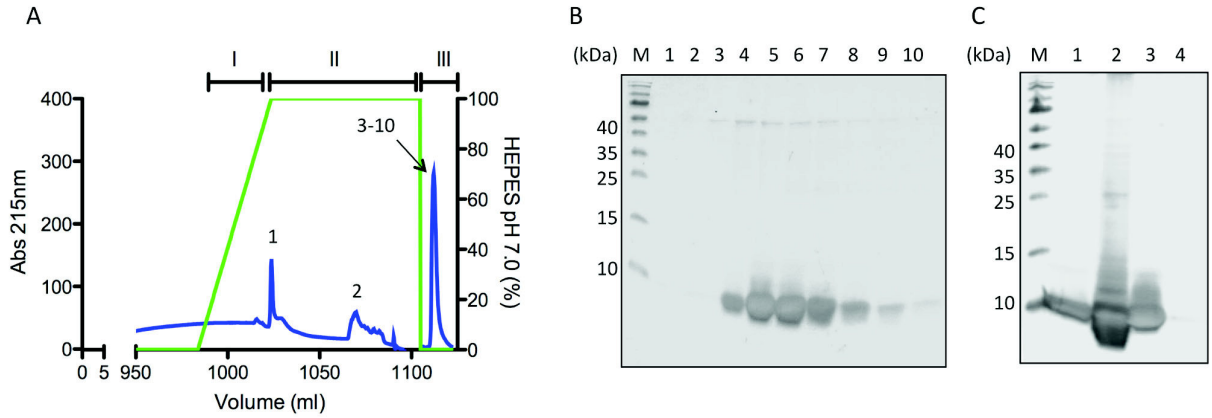


**Figure 24. Purification of unmodified prenisin with gentle pH change. A:** Shown is an elution profile of the cation exchange chromatography of unmodified prenisin (blue line) and the concentration of 50 mM HEPES-NaOH pH 7.0 in % (green line). I: after washing with 50 mM lactic acid until a stable baseline was reached the wash buffer was changed to 50 mM HEPES-NaOH pH 7.0 using a linear gradient over 8 column volumes; II: column was washed additional 8 column volumes with 50 mM HEPES-NaOH pH 7.0 until eluting buffer reached a pH of 7.0; III: Peptide was eluted using a one step gradient from 0 (100 %) to 1 M (0 %) NaCl in 50 mM HEPES pH 7.0 and 10 % (v/v) glycerol. **B:** SDS-PAGE analysis of the elution fractions. M: molecular weight marker proteins (kDa); lane 1: first elution fraction during pH 7.0 wash; lane 2: second elution fraction during pH 7.0 wash; lanes 3-10: fractions eluted with 50 mM HEPES-NaOH pH 7.0 and 1 M NaCl. **C:** SDS-PAGE analysis of unmodified prenisin after filtration and concentration. M: molecular weight marker proteins (kDa); lane 1: flow through 30 kDa cut off filter; lane 2: remaining retantate 30 kDa cut off filter; lane 3: unmodified prenisin concentrated with a 3 kDa cut off filter; lane 4: flow through 3 kDa cut off filter

Fractions containing unmodified prenisin were pooled and filtered through a 30 kDa cut-off filter to remove high molecular weight contaminants (Figure 24C, lane 1). It was observed that during filtration with the 30 kDa cut-off filter device some unmodified prenisin was lost, because it adsorbed to the filter (Figure 24C, lane 2), but pure unmodified prenisin was obtained in the flow through in sufficient amounts. Subsequently the prenisin containing flow through was concentrated by a 3 kDa cut-off filter device (Figure 24C, lane 3). Here, all applied unmodified prenisin was retained in the concentrated, as no peptide was present in the flow through (Figure 24C, lane 4). The yield of pure peptide with pH 7.0 was about 1-2 mg/liter of culture for all purified prenisins. This protocol was used also to purify dehydrated, fully modified prenisin and the FNLD/AAAA mutant as well (Figure 25).

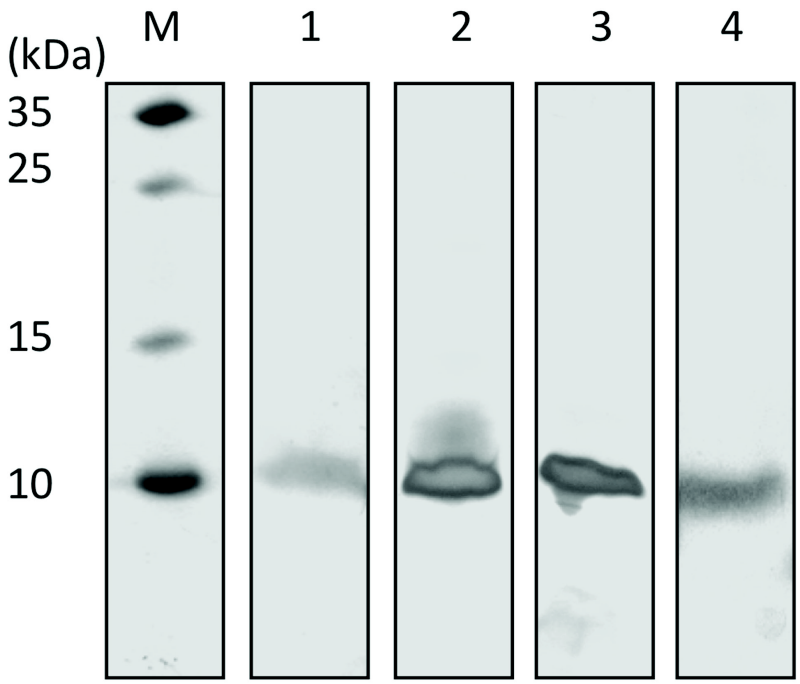


**Figure 25. SDS-PAGE analysis of purified unmodified, dehydrated, fully modified prenisin and the FNLD/AAAA mutant.** M: molecular weight marker proteins (kDa); lane 1: unmodified prenisin; lane 2: dehydrated prenisin; lane 3: fully modified prenisin; lane 4: FNLD/AAAA prenisin mutant

## 4.6 Purification of active nisin

To investigate whether active nisin interacts with the dehydratase NisB, also active nisin was purified. Therefore, commercially available nisin (~2.5 % (w/w) nisin in lyophilized powder) was further purified.

First, nisin was applied to a cation exchange column and eluted using 50 mM lactic acid pH 3.0 and 1 M NaCl. Subsequent SDS-PAGE analysis revealed that nisin was eluted but several higher molecular weight compounds of ~10 kDa were also observed in the analyzed fraction (data not shown). Those contaminants could not be removed by filtration. Hence, it was tried to separate nisin from the contaminants with a five step NaCl step gradient, in which the NaCl concentration was step wise increased from 0 to 1 M using 200 mM steps (Figure 26A) (102).

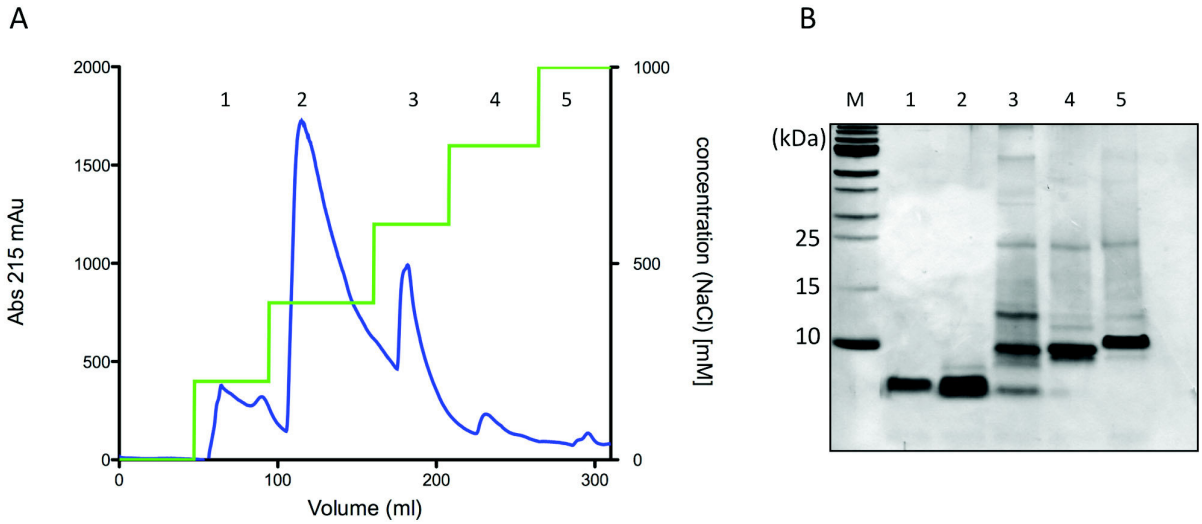


**Figure 26. Purification of active nisin. A:** The elution profile of commercial nisin is shown measured at 215 nm (blue line). Nisin is eluted from the column using a five-step gradient with 200 mM (Step1), 400 mM (Step 2), 600 mM (Step 3), 800 mM (Step 4), and 1 M NaCl (Step 5), respectively. The different steps are indicated by the green line and the right y-axis. **B:** SDS-PAGE analysis of different elution fractions. M: molecular weight marker proteins (kDa); lane 1: elution with 200 mM NaCl; lane 2: elution with 400 mM NaCl; lane 3: elution with 600 mM NaCl; lane 4: elution with 800 mM NaCl; lane 5: elution with 1 M NaCl. Taken from (102).

SDS-Page analysis of eluted fractions revealed that the majority of pure nisin was eluted with 400 mM NaCl (Figure 26B lane 2), whereas at 200 mM NaCl (Figure 26B lane 1) nisin also eluted also but in lower amounts. The elution fractions eluted with 600 mM, 800 mM and 1 M NaCl showed predominantly higher molecular weight contaminants and virtually no nisin (Figure 26B lanes 3-5). Thus, the protocol with a stepwise NaCl gradient led to pure nisin. Note that trials to purify nisin directly in a buffer with pH 7.0 failed, because nisin at pH 7.0 is soluble at low concentrations only.

To test if the purified nisin was biological active the fractions of the stepwise purification were applied to SDS-PAGE. After electrophoresis the gel was overlaid with GM17-agar containing the nisin sensitive *L. lactis* strain NZ9000 and incubated at 30°C over night (halo assay).

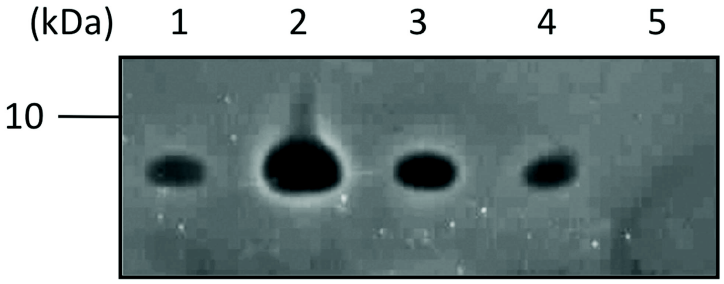


**Figure 27. Biological activity assay for purified nisin.** Various fractions containing nisin were analyzed by SDS-PAGE and the gel was overlaid with GM17-agar containing nisin sensitive bacteria. The biological activity of nisin is visualized by the growth inhibition zones (dark halos) at the position were nisin is present. Lane 1: elution with 200 mM NaCl; lane 2: elution with 400 mM NaCl; lane 3: elution with 600 mM NaCl; lane 4: elution with 800 mM NaCl; lane 5: elution with 1 M NaCl. Taken from (102).

Biological activity of nisin was obtained as zones of growth inhibition (dark halos). Zones of growth inhibition were observed for nisin eluted with 200 mM, 400 mM, 600 mM and 800 mM NaCl, respectively (Figure 27 lanes 1-4). The highest level of growth inhibition was observed for nisin at 400 mM NaCl (Figure 27 lane 2), which also showed the highest content of pure nisin (Figure 26B lane 2).

To investigate the eluted fractions further and confirm the presence of nisin in the individual purification fractions MALDI-TOF mass spectrometry was used. The fraction eluted with 400 mM NaCl contained only one peptide with a molecular mass of 3355.09 Da, which was in agreement with the calculated mass of nisin (3354,07 Da) (Figure 28).

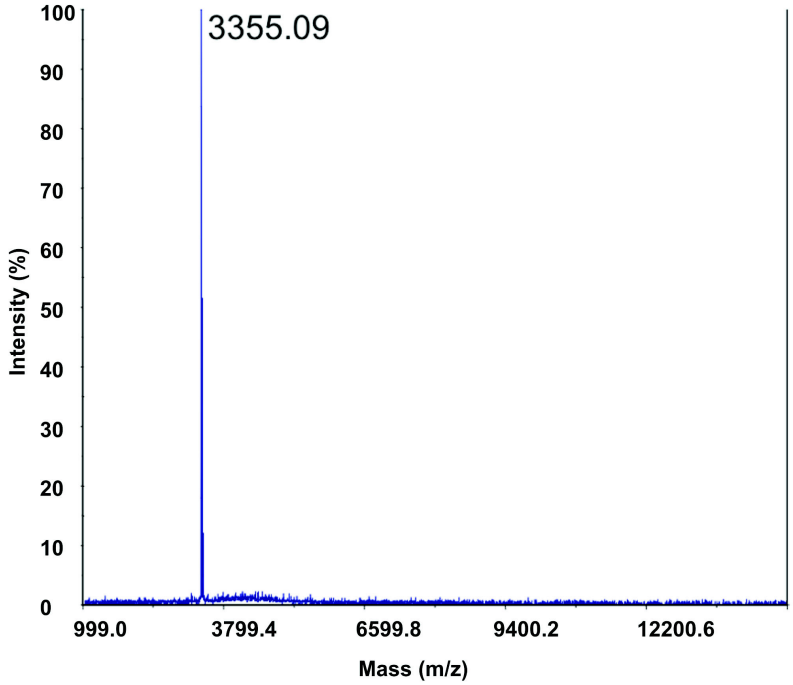


**Figure 28. Mass spectrometry of eluted nisin.** Shown is the analysis of the fraction eluted at 400 mM NaCl. Taken from (102).

Peak integration of the total mass spectrum revealed that the 400 mM elution fraction contains more than 98 % of nisin. Nisin was also found in the 600 mM and 800 mM elution fractions, whereas no nisin was detected in the 1 M NaCl fraction. These results are in agreement with SDS-PAGE analysis (Figure 26B). In addition, it was found that higher molecular mass contaminants were not nisin nor prenisin derived, as the amino acid sequence did not match with the primary sequence of prenisin (102).

## 4.7 Characterization of unmodified, dehydrated and fully modified prenisin

After purification it was important to distinguish between unmodified, dehydrated and fully modified prenisin. It is not possible to distinguish between the different prenisin forms by SDS-PAGE analysis, because the molecular weights of the peptides are in the same range (5.9 kDa – 5.8 kDa). Therefore, the peptides do not migrate differently through a polyacrylamide gel. During maturation of prenisin to active nisin certain serine and threonine residues are dehydrated by NisB.

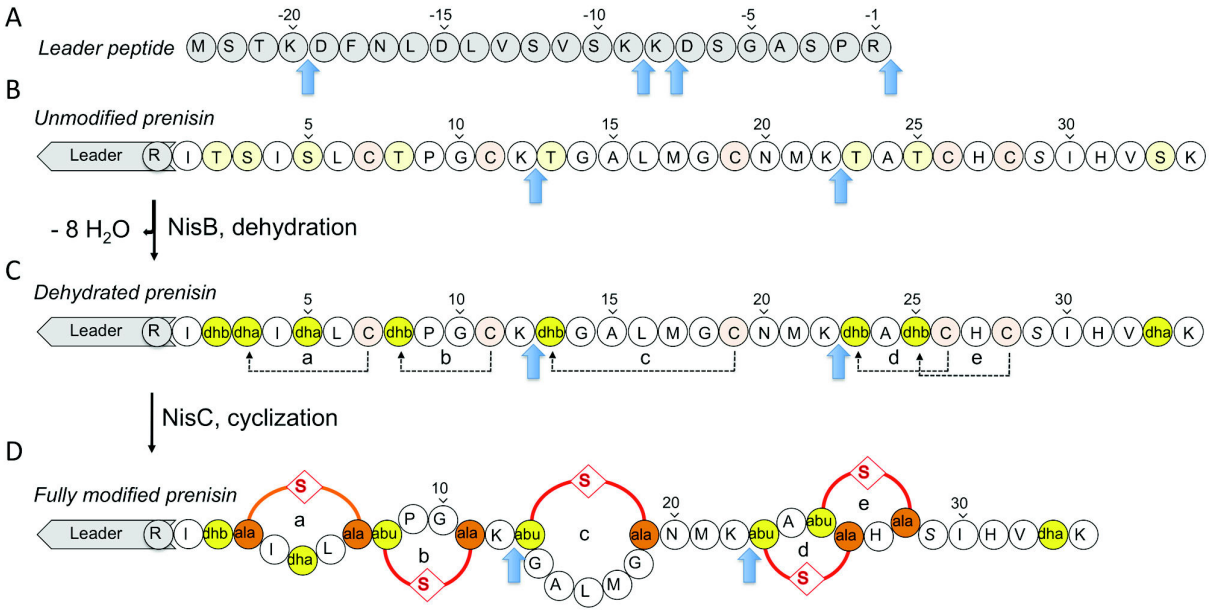


**Figure 29. Posttranslational modifications of prenisin. A:** The leader peptide. The amino acids are number from N- to C-terminus (-23 to -1). **B:** Unmodified prenisin becomes dehydrated by NisB and therefore up to 8 water molecules are eliminated. Dehydrated Ser and Thr residues are highlighted in yellow. **C:** Dehydrated prenisin becomes cyclized by NisC, which results in **D:** fully modified prenisin. Coupled cysteines are highlighted in orange. Blue arrows mark the trypsin cleavage sites at Lys-20, Lys-9, Lys-8 and Arg-1 in the leader peptide and at Lys12 and Lys22 in the propeptide (amino acids 1 to 34).

The masses of unmodified and dehydrated prenisin differ by 126 or 144 Da, which corresponds to seven or eight dehydrations, respectively (Figure 29B and C). Because the introduction of the (methyl)lanthionine rings does not create a difference in molecular weight, the masses of dehydrated and fully modified prenisin are identical (Figure 29C and D). Therefore, it is not possible to distinguish between dehydrated and fully modified prenisin by mass spectrometry. According to literature it is possible to cleave off the leader peptide from the modified propeptide by tryptic digestion (57). Trypsin cleaves peptide chains after lysine and arginine residues, except when these residues are followed by a proline. As the last amino acid of the leader peptide is an arginine, trypsin is suitable for cleaving off the leader (Figure 29, blue arrows). However, there are trypsin cleavage sites also within the propeptide (Figure 29, blue arrows), which are inaccessible in the case of fully modified prenisin possibly due to the adjacent (methyl)lanthionine rings (54). Fully modified prenisin treated with trypsin is expected to result in active nisin, whereas cleavage of unmodified and dehydrated prenisin should not, because the (methyl)lanthionine rings are not present, which are essential for nisin activity and protect the peptides against tryptic digestion. Unmodified, dehydrated and fully modified prenisin with a concentration of 12.5 µM were incubated separately with 0.2 µM trypsin for 1 h at room temperature. Subsequent SDS-PAGE analysis revealed that unmodified prenisin was digested to undetectable small fragments (Figure 30A, lane 5), as six possible cleavage sites are present in prenisin, which are Lys-20, Lys-9, Lys-8, Arg-1 within the leader peptide, and Lys12 and Lys22 within the propeptide (Figure 29, blue arrows). The majority of dehydrated prenisin was fully digested but a small number of molecules were only partial digested as a peptide with a smaller molecular weight than dehydrated prenisin could be observed (Figure 30A, lane 7 marked by asterisk). Perhaps this was due to the double bonds of dehydrobutyrines at positions 13 and 23, which may negatively influence trypsin activity (57). However, fully modified prenisin treated with trypsin showed a band with a molecular weight (Figure 30A, lane 3) smaller than untreated fully modified prenisin (Figure 30A, lane 2). Importantly, the smaller peptide migrated at the same height as nisin (Figure 30A, lane 1, not purified), suggesting that the leader peptide was efficiently removed by trypsin.

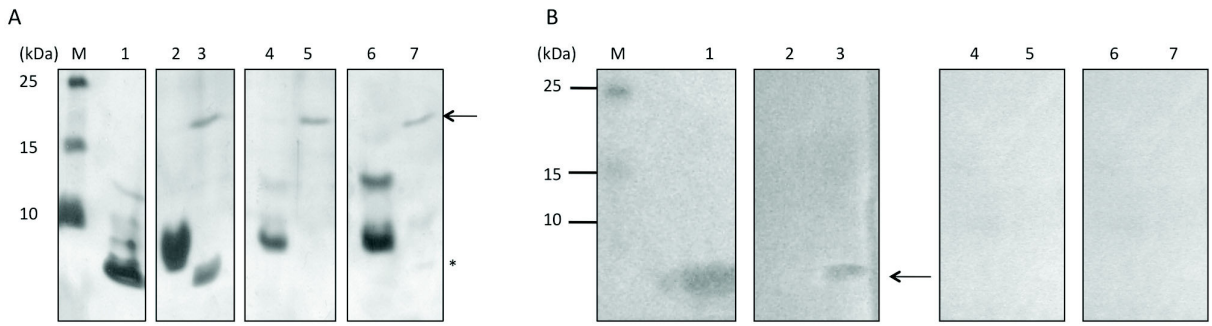


**Figure 30. SDS PAGE and biological activity analysis of prenisins digested with trypsin. A:** SDS-PAGE analysis of prenisin treated with trypsin. M: molecular weight marker proteins (kDa); lane 1: active nisin; lane 2: fully modified prenisin; lane 3: fully modified prenisin treated with 0.2 µM trypsin; lane 4: unmodified prenisin; lane 5: unmodified prenisin treated with 0.2 µM trypsin; lane 6: dehydrated prenisin; lane 7: dehydrated prenisin treated with 0.2 µM trypsin. Arrow indicates trypsin. **B:** Biological activity assay of fully modified prenisin after trypsin digestion. A SDS-PAGE containing trypsin digested fully modified prenisin and active nisin as a positive control was overlaid with M17-agar containing the nisin sensitive strain NZ9000. M: molecular weight marker proteins (kDa); lane 1: active nisin; lane 2: fully modified prenisin; lane 3: fully modified prenisin digested with 0.2 µM trypsin; lane 4: unmodified prenisin; lane 5: unmodified prenisin treated with 0.2 µM trypsin; lane 6: dehydrated prenisin; lane 7: dehydrated prenisin treated with 0.2 µM trypsin. Arrow indicates zones of growth inhibition.

Subsequently, nisin and cleaved fully modified prenisin as well as cleaved unmodified and dehydrated prenisin were tested for biological activity using a halo assay as described in 3.7. Both nisin and cleaved fully modified prenisin led to zones of growth inhibition (Figure 30B, lanes 1 and 3), whereas the cleavage product(s) of unmodified and dehydrated prenisin did not (Figure 30B, lane 5 and 7). Thus, correctly produced fully modified prenisin was identified by the biological activity assay. Whereas, the unequivocally identification of unmodified or dehydrated prenisin was not possible with the biological activity assay.

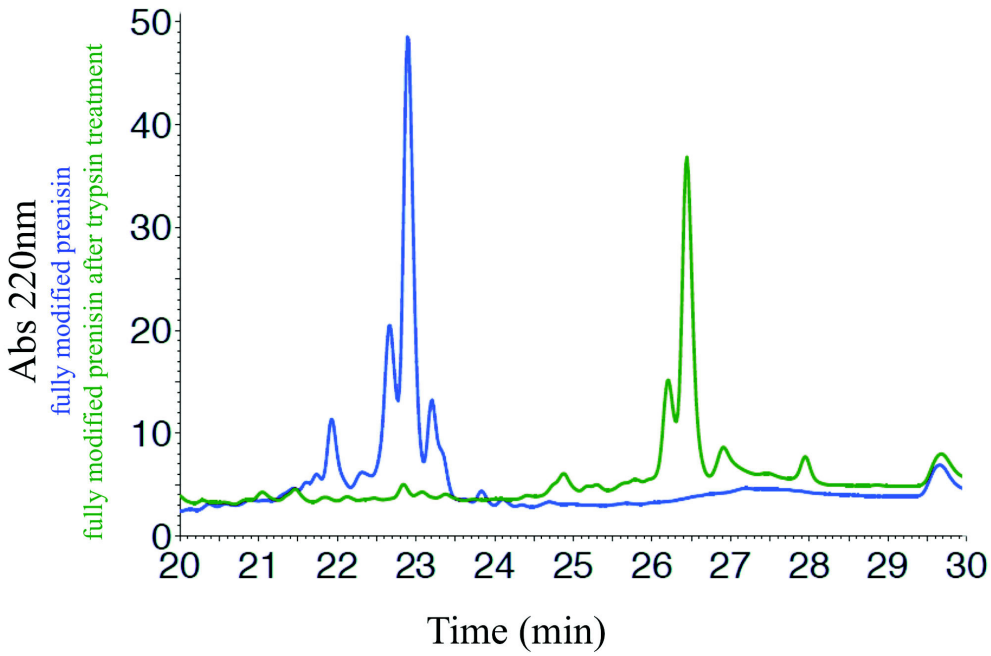


**Figure 31. HPLC analysis of trypsin treated fully modified prenisin.** Blue: fully modified prenisin; Green: fully modified prenisin after trypsin treatment.

To verify those findings another technique was needed to analyze the cleavage of fully modified prenisin. Therefore, reversed phase high-performance liquid chromatography (RP-HPLC) was used. Prior to RP-HPLC analysis, fully modified prenisin was treated with trypsin. Then uncleaved and cleaved fully modified prenisin were applied to RP-HPLC analysis. Fully modified prenisin eluted at 22.75 min. whereas cleaved fully modified prenisin eluted at 26.5 min. (Figure 31). The major product of trypsin cleavage eluted at a position identical to that of active nisin (Figure 32, green curve). These results, in combination with the halo assay, clearly show that fully modified prenisin can be converted into active nisin by cleavage of the leader peptide with trypsin.

To investigate if it is possible to distinguish between different purified prenisins and active nisin, the retention volumes for each of these peptides was analyzed by RP-HPLC. Nisin and the different prenisin peptides were injected separately at a concentration of 50 μM. Interestingly, the peptides showed markedly different elution profiles, which relates to significant differences in their hydrophobicity (Figure 32). The unmodified prenisin eluted between 19.0 and 20.5 min. as a broad peak (Figure 32, black curve), whereas dehydrated NisA eluted in a similar broad peak, but at a substantially later retention time of 19.5-22.5 min (Figure 32, red curve). Compared to unmodified prenisin, dehydrated prenisin showed reproducibly a higher absorbance at 220 nm likely due to the presence of an increased amount of double bonds as a result of the

dehydration of serine and threonine residues. Double bonds are known to contribute to the peptide absorbance at 220 nm (127). Fully modified prenisin eluted at 22.5 min and showed a characteristic double peak (Figure 32, blue curve). Nisin also showed a characteristic double peak but eluted much later from the column as compared to the fully modified prenisin, i.e. at 26.3 min. (Figure 32, green curve). The double peak of nisin has been observed before and has been attributed to a small number of nisin molecules in which Ser33 has escaped NisB-mediated dehydration (85). Taken together, these data demonstrate that RP-HPLC can be used to assess the identity of the different prenisin peptides in a qualitative manner. Importantly, RP-HPLC is able to distinguish directly between the dehydrated prenisin and the fully modified prenisin, which is in clear contrast to mass spectrometry and important for subsequent analysis.

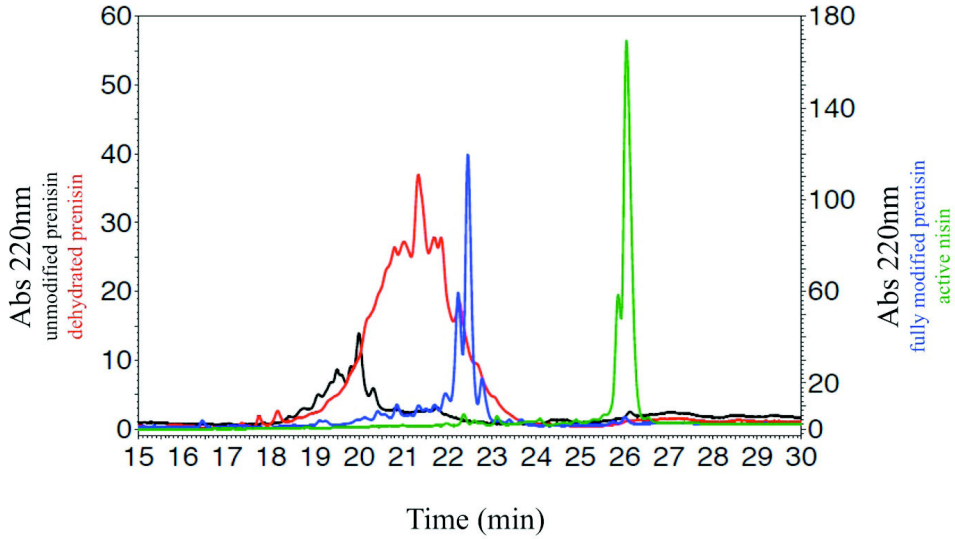


**Figure 32. RP-HPLC elution profiles of the purified peptides.** Elution profiles as a function of time are shown for unmodified prenisin (black), dehydrated prenisin (red), fully modified prenisin (blue) and nisin (green). Left Y-axis shows the absorbance at 220 nm for unmodified and dehydrated prenisin. Right Y-axis shows the absorbance at 220 nm for fully modified prenisin and active nisin.

## 4.8 NisB-prenisin interaction studied with size exclusion chromatography (SEC)

To investigate the interaction of the different prenisin forms with NisB, analytical SEC studies were performed. For this, NisB was incubated with either unmodified, dehydrated or fully modified prenisin for 1 h at room temperature to allow complex formation. The protein mixtures were then subjected to SEC analysis. In addition, the interaction of NisB with the FNLD/AAAA prenisin mutant as well as with active nisin

was investigated. Whereas NisB is readily detected at 280 nm, the prenisin peptides are not, due to a lack of tryptophan or tyrosine residues. The peptides could however be detected at 215 nm. Incubation of NisB with the various peptides and subsequent SEC analysis did not reveal significant changes in the elution behavior of NisB (Figure 33A, blue and black curves). However, when NisB was incubated with dehydrated prenisin a small decrease in absorbance at 215 nm for free dehydrated prenisin was observed suggesting that some of the dehydrated prenisin molecules may be bound to NisB (Figure 33A, red and magenta curves). To verify if NisB is able to bind prenisin, the NisB peak fraction was analyzed by SDS-PAGE for co-elution of the different peptides (Figure 33B). SDS-PAGE analysis demonstrated that unmodified, dehydrated and fully modified prenisin co-eluted with NisB (Figure 33B, lanes 3, 5 and 7, respectively), indicating that these peptides form a complex with NisB. The amount of the various prenisin forms co-eluting with NisB differed substantially. Dehydrated prenisin was reproducibly present in highest amount, whereas a somewhat lower amount of unmodified prenisin was observed. In contrast, only very low amounts of fully modified prenisin were observed to co-elute with NisB. Active nisin, which lacks the leader peptide and is therefore not expected to bind to NisB, indeed did not co-elute with NisB (Figure 33B, lane 9). To further address the importance of the leader peptide in prenisin binding to NisB, a prenisin mutant, in which the conserved FNLD-box within the leader peptide was mutated to AAAA, was analyzed. This FNLD/AAAA prenisin is secreted in low levels into the culture medium by *L. lactis* cells harboring *nisBTC*, but is apparently not modified (118). Interestingly, the FNLD/AAAA prenisin did not co-elute with NisB, suggesting that it did not bind to NisB (Figure 33B, lane 11). Taken together these data indicate that NisB shows functional binding of its native substrate *in vitro* and that this activity strictly depends on the presence of an intact leader peptide.

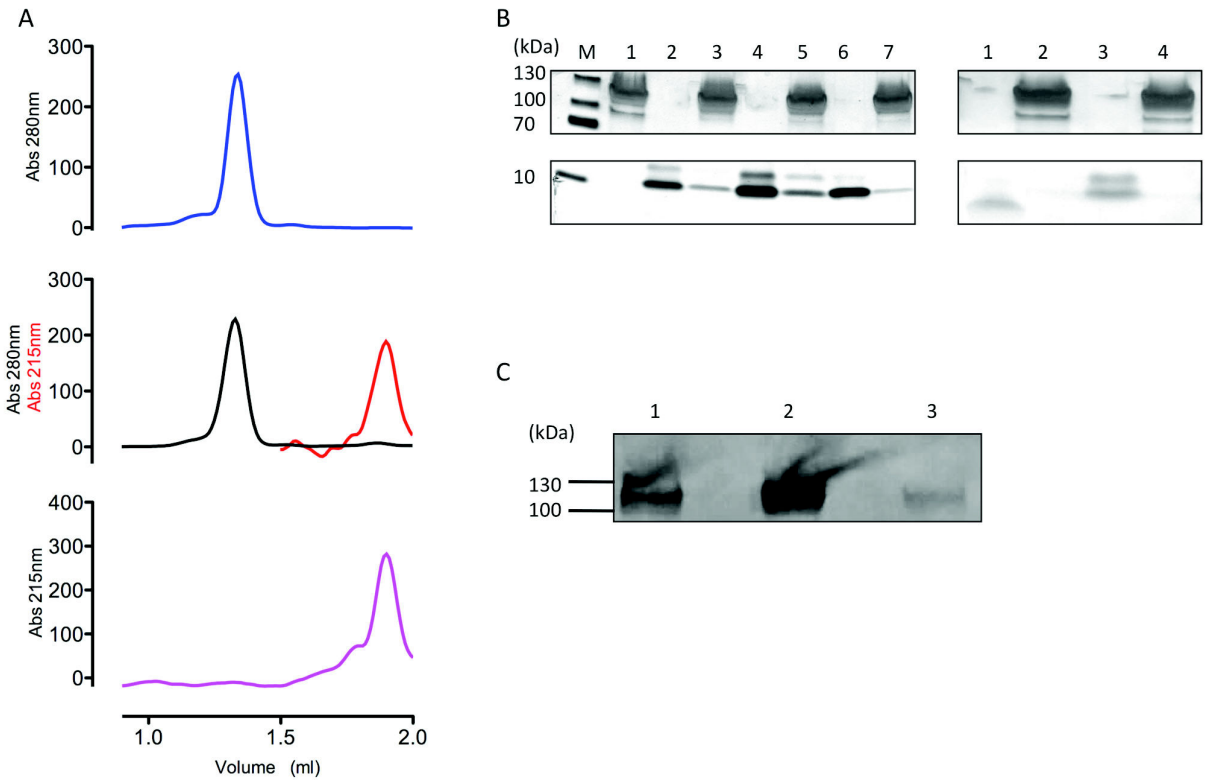


**Figure 33. Analysis of complex formation between NisB and prenisin. A:** NisB and prenisin were incubated together and the reaction mixtures were analyzed by SEC using an Äkta micro system. Shown are the elution profiles of NisB alone at 280 nm (top panel, blue line), NisB incubated with dehydrated prenisin at 280 nm (middle panel, black line) and 215 nm (middle panel, red line) to visualize dehydrated prenisin and the elution profile of dehydrated prenisin alone at 215 nm (bottom panel, magenta line). **B:** SDS-PAGE analysis of the peak fractions after SEC showing interaction between NisB and the different prenisins. M: molecular weight marker proteins (kDa); lane 1: purified NisB (2.5 μM); lane 2: purified unmodified prenisin (7 μM); lane 3: co-elution of unmodified prenisin and NisB; lane 4: purified dehydrated NisA (7 μM); lane 5: co-elution of dehydrated prenisin and NisB; lane 6: purified fully modified prenisin (7 μM); lane 7: co-elution of fully modified prenisin and NisB; lane 8: purified active nisin (7 μM); lane 9: NisB incubated with nisin; lane 10: purified FNLD/AAAA prenisin (7 μM) and lane 11: NisB incubated with FNLD/AAAA prenisin. Note, due to the presence of free cysteines in unmodified and dehydrated prenisin and FNLD/AAAA prenisin, oxidative products (bottom panels, upper prenisin band) are often observed. **C:** Western blot analysis using anti-leader peptide antibodies. Prenisin was detected attached to NisB. Lane 1: unmodified prenisin attached to NisB; lane 2: dehydrated prenisin attached to NisB; lane 3: fully modified prenisin attached to NisB.

The samples were further investigated by western blot analysis. For immunodetection an anti-leader antibody was used. Unmodified, dehydrated and fully modified prenisin could all be detected at the migration height of NisB (Figure 33**C**, lanes 1-3). This indicated that a stable complex of NisB and prenisin is formed *in vitro*.

The FNLD/AAAA mutant and active nisin could not be detected by the used antibody as the epitope it was raised against was either mutated or not present, respectively. Taken together, the different amounts of the various prenisin forms found to co-elute with NisB point to a difference in affinity of these peptides for NisB.

## 4.9 NisB-prenisin interaction studied with surface plasmon resonance (SPR)

### 4.9.1 Immobilization of NisB

To characterize the interaction of NisB with the various prenisin peptides in more detail, the binding was quantitatively assessed by surface plasmon resonance (SPR). Measurements were performed by immobilizing NisB carrying a carboxy-terminal $His_6$-tag onto a $Ni^{2+}$ saturated NTA surface using 50 mM HEPES-NaOH pH 7.4, 500 mM NaCl and 50 µM EDTA as running buffer. NisB was immobilized to 2200 RU at a flow rate of 10 µl/min (Figure 34A). Subsequent the NisB bound surface was equilibrated with the running buffer at a flow rate of 30 µl/min. However, after 2000 sec. a decrease of the signal was obtained, indicating that NisB was dissociating from the surface. The measurement was continued until nearly 1500 RU were washed off the surface (Figure 34B).

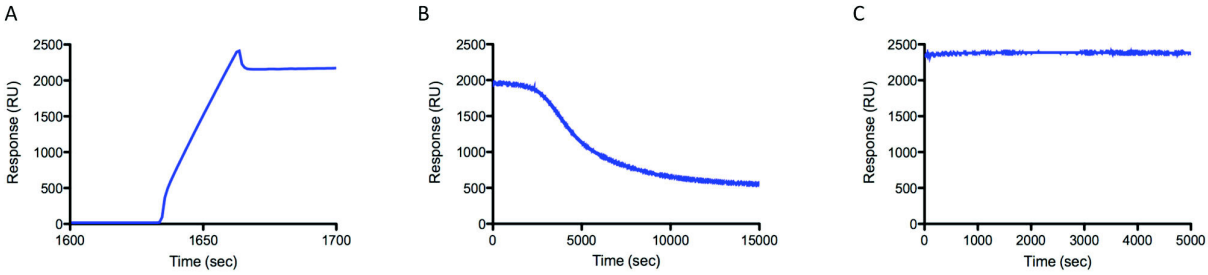


**Figure 34. Immobilization of NisB. A:** NisB was immobilized by injecting 5 µl NisB (170 nM) at 10 µl/min; **B:** Equilibration of immobilized NisB with 500 mM NaCl in the running buffer resulted in a decrease of the signal, indicating that NisB dissociates from the chip surface. **C:** Equilibration of immobilized NisB with 250 mM NaCl in the running buffer resulted in a constant signal.

As NisB was immobilized via electrostatic interaction of the $His_6$-tag to the $Ni^{2+}$ ions the ionic strength of the buffer was reduced to avoid any disturbance of the electrostatic interaction. Therefore the NaCl concentration in the running buffer was reduced to 250 mM. After immobilization of NisB at 10 µl/min the surface was equilibrated at 30 µl/min. During equilibration the signal stayed constant, demonstrating that with 250 mM NaCl in the running buffer immobilized NisB remained bound to the $Ni^{2+}$-NTA surface (Figure 34C).

MALS analysis demonstrated that NisB was still present as a dimer at 250 mM NaCl (Figure 35).

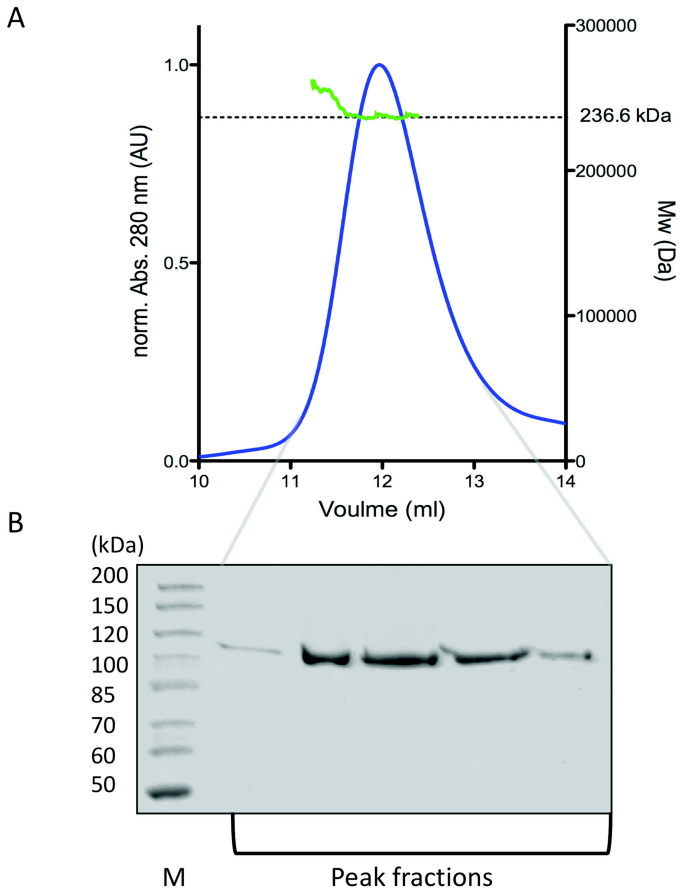


**Figure 35. Size exclusion chromatography and static light scattering analysis of NisB.** SEC and MALS analysis was performed with 50 mM HEPES-NaOH pH 7.4 and 250 mM NaCl. **A:** NisB elution profile (blue line) with determined molecular mass (green line); **B:** SDS-PAGE analysis of the NisB peak fractions. Dotted line indicates the molecular weight of dimeric NisB (236.6 kDa). Taken from (84).

### 4.9.2 NisB-prenisin interaction studies

Subsequent to NisB immobilization the different peptides were injected at various concentrations. Peptide was injected for 100 seconds and sensorgrams were recorded for 250 seconds (Figure 36). The real-time binding responses showed an exponential association and an exponential dissociation phase for the unmodified, the dehydrated and the fully modified prenisin (Figure 36 A, B and C, respectively). The data were fitted using a 1:1 binding model with drifting baseline and local fitted $R_{max}$. The obtained association and dissociation rate constants as well as the calculated equilibrium binding constants are summarized in Table 5. Unmodified prenisin exhibits an association rate ($k_{on}$) of $1.2 \pm 0.4 \cdot 10^4$ $M^{-1}$ $s^{-1}$ and a dissociation rate ($k_{off}$) of $0.0117 \pm 0.0014$ $s^{-1}$. Dehydrated prenisin on the other hand showed a ~ 4-fold higher $k_{on}$ ($5.1 \pm 1.4 \cdot 10^4$ $M^{-1}$ $s^{-1}$), but similar $k_{off}$ ($0.0149 \pm 0.0025$ $s^{-1}$). The fully modified prenisin displayed a $k_{on}$ of $3.1 \pm 0.4 \cdot 10^4$ $M^{-1}$ $s^{-1}$ and a more than 20-fold higher $k_{off}$ of $0.323 \pm 0.035$ $s^{-1}$, when compared to the dehydrated and the unmodified prenisin. The equilibrium constant or binding affinity ($K_D$) for the different peptides were all found to be in the low micromolar range. The $K_D$ for the unmodified, the dehydrated and the fully modified prenisin peptides were $1.05 \pm 0.25$ μM, $0.31 \pm 0.07$ μM and $10.5 \pm 1.7$ μM, respectively. Thus, NisB binds the dehydrated prenisin with the highest affinity, whilst the affinity for fully modified prenisin is ~30-fold lower. These results are in line with the SEC experiments where the amount of the different prenisin peptides that co-eluted with NisB followed the order: dehydrated > unmodified > fully modified (Figure 33). Consistent with SEC analysis, mature nisin essentially did not bind to NisB. Only at the highest concentration tested a very weak binding response was observed (Figure 36D). Similarly, the unmodified prenisin carrying the FNLD/AAAA mutation did not bind to NisB (Figure 36E).

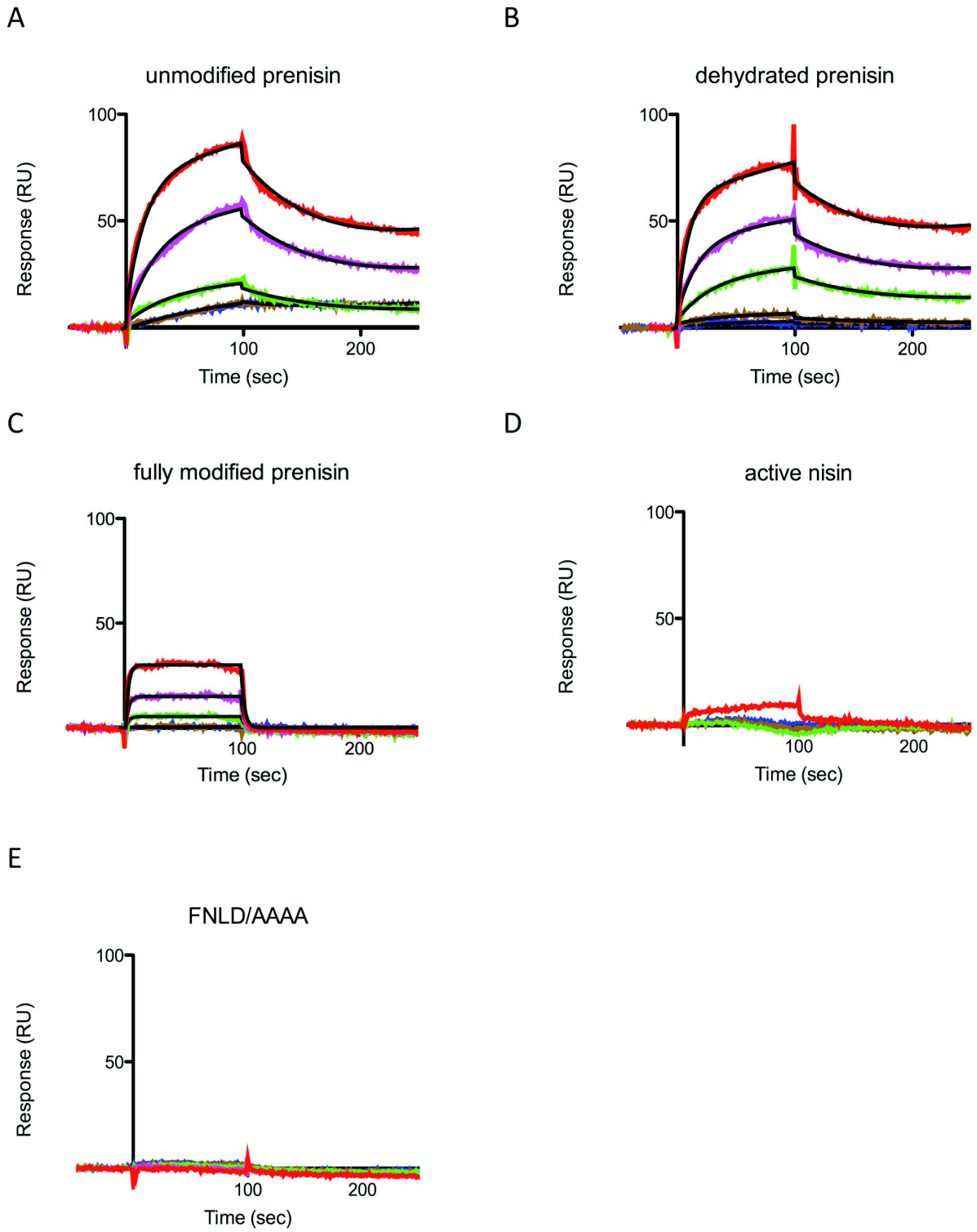


**Figure 36. SPR analysis of the interaction of NisB with prenisin.** Sensorgrams showing the interaction of immobilized NisB with **A:** unmodified prenisin; **B:** dehydrated prenisin; **C:** fully modified prenisin; **D:** nisin and **E:** the FNLD/AAAA prenisin mutant. Injected peptide concentrations, from bottom to top, were 46 nM, 183 nM, 731 nM, 1.5 μM and 2.9 μM. Taken from (84).

Thus, the data indicate that the leader peptide, and especially the FNLD-box herein, is essential for binding of prenisin to NisB. Moreover, NisB shows substrate specificity as it binds its natural substrate unmodified prenisin and its modified derivatives, dehydrated and the fully modified prenisin, with different affinity.

**Table 5. Kinetic constants for the NisB-prenisin interaction.**

| Peptide | $k_{on}$ ($M^{-1}$ $s^{-1}$) | $k_{off}$ ($s^{-1}$) | $K_D$ (μM) |
|---|---|---|---|
| Unmodified prenisin | $1.2 \pm 0.4 \cdot 10^4$ | 0.0117 ± 0.0014 | 1.05 ± 0.25 |
| Dehydrated prenisin | $5.1 \pm 1.4 \cdot 10^4$ | 0.0149 ± 0.0025 | 0.31 ± 0.07 |
| Fully modified prenisin | $3.1 \pm 0.3 \cdot 10^4$ | 0.323 ± 0.035 | 10.5 ± 1.7 |
| Active nisin | n.b. | | |
| FNLD/AAAA prenisin | n.b. | | |

n.b., no binding observed under tested experimental conditions

SPR binding responses are generally proportional to mass and therefore one can assess the stoichiometry of an interaction by comparing the amount of ligand immobilized to the amount of analyte that can be bound. The stoichiometry of the interaction of NisB with the different prenisin peptides was determined using equation 6 (125,126). $R_{max}$ (maximum capacity for analyte binding) is a local fitted parameter and depends on the amount of immobilized NisB. Since NisB was freshly immobilized for each measurement and its immobilized levels differed somewhat for each measurement, the average values of $R_{max}$ and $R_l$ were used. Assuming that the immobilized NisB is dimeric (236.6 kDa), the calculated binding stoichiometry for the unmodified, the dehydrated and the fully modified prenisin were found to be 0.9 ± 0.1, 0.8 ± 0.1 and 0.9 ± 0.1, respectively.

# 5. Discussion

The occurrence and dissemination of drug-resistant pathogens, such as *S. aureus* and *E. faecium*, is a major problem in clinics of developing and developed nations (29). Over recent years a class of antimicrobial peptides, called lantibiotics, have become of great interest since they exhibit a high antimicrobial activity against Gram-positive bacteria. Moreover, due to the dual mode of action of lantibiotics, target organisms so far have not developed significant resistance (30,128). Lantibiotics are produced by certain Gram-positive bacteria and possess antimicrobial activity mainly against Gram-positive bacteria, including important pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) and *Clostridium difficile* (73,74).

Lantibiotics are ribosomally synthesized as prepeptides, which become posttranslationally modified by several enzymes (51). The lantibiotic prepeptides consist of two parts, a N-terminal leader peptide and a C-terminal propeptide (45). The leader peptide directs the prepeptide to the modification enzymes and is in addition crucial for secretion (55-57). All lantibiotics contain (methyl)lanthionine rings, which are introduced during maturation (60). The (methyl)lanthionine rings confer conformationally defined structures, which are important for the biological activity and in addition protect lantibiotics against proteolytic degradation (53,54).

This work focused on the nisin modification machinery of *L. lactis*. Nisin possesses five (methyl)lanthionine rings, which protects nisin against proteolytic degradation and are important for its antimicrobial activity. Nisin possesses a dual mode of action (Figure 12). First, upon binding to lipid II, cell wall synthesis is blocked as nisin bound lipid II is not able to deliver peptidoglycan monomers for cell wall synthesis (78). Second, nisin is able to form pores with lipid II. The complex forming the pore consists of 4 lipid II molecules and 8 nisin molecules (44). Herein, especially rings A and B of nisin are responsible for binding to the pyrophosphate moiety of lipid II (42). Target cell death is then accomplished by permeabilization of the cytoplasmic membrane, which causes uncontrolled efflux of essential cell components (75,99,129).

The (methyl)lanthionine rings in nisin are installed posttranslationally by the cooperative action of the serine/threonine specific dehydratase NisB and the cyclase NisC (87,97). Recently, such lantibiotic modification enzymes have gained special interest as they can be successfully used to introduce (methyl)lanthionine rings into

therapeutic peptides, which enhance the stability and activity of those peptides (98,130). In 2009, Kluskens *et al.* showed that the enzymatic introduction of a thioether ring by NisB and NisC enhances the stability of angiotensin-(1–7) against proteolytic degradation, while maintaining its natural function (131). Angiotensin-(1–7) can be used to treat high blood pressure (131). Thus, a molecular understanding of the reactions catalyzed by the nisin modification machinery may further aid the development of novel and improved antibiotics or therapeutics.

From combined *in vivo* and *in vitro* studies it is known that the proteins NisB, NisC, NisT and NisP from *L. lactis*, can function independent of each other (57,89,117). The dehydration of the nisin precursor peptide catalyzed by NisB presents the first step in nisin maturation and is thus critical for nisin biosynthesis. It was shown that NisB is crucial for nisin production because lack of NisB results in secretion of unmodified prenisin into the culture medium as well as accumulation of unmodified prenisin within *L. lactis* cells (86,92).

Furthermore, when NisB is present and NisC is lacking *L. lactis* cells are able to produce dehydrated prenisin (92,117). *In vivo* studies have demonstrated that NisB is able to dehydrate a multitude of nisin derivatives and even therapeutic peptides non-related to nisin when they are fused to the nisin leader peptide (88,90,132). Furthermore, studies with model peptides have shown that dehydration of serine and threonine residues is favored when they are flanked by hydrophobic residues, whereas a hydrophilic environment disfavored dehydration (90,91). Moreover, the dehydration of threonine seems to be preferred over serine (90). In addition, it was demonstrated that NisB is able to dehydrate residues at C-terminal positions further away than is the case in natural prenisin (90). Taken together, NisB exhibits a broad substrate spectrum and can be used to introduce dehydrated residues into a wide range of nisin unrelated peptides.

However, for targeting such peptides to NisB the nisin leader peptide is required (88,90). Unfortunately, detailed information about the actual dehydration reaction is still lacking and no structure of NisB is available. In this respect, reconstitution of the *in vitro* activity of NisB or any other enzyme belonging to the LanB family has, thus far, been unsuccessful (45,61,105,106). In contrast, NisC cyclase activity has been successfully reconstituted *in vitro* and its crystal structure is available (57). In this work an *in vitro* binding assay was developed to investigate the interaction between the lantibiotic dehydratase NisB and its native substrate unmodified prenisin, as well as its

dehydrated and fully modified derivatives. For this, NisB and the various substrate peptides had to be purified to homogeneity and in sufficient quantity.

## 5.1 Purification and characterization of prenisin

Unmodified, dehydrated and fully modified were purified to homogeneity and in sufficient amounts (1-2 mg/L of culture) to allow further *in vitro* investigations. Although it was shown that the amounts of unmodified and dehydrated prenisin found in the medium after 4 h of production were significantly smaller compared to fully modified prenisin (86), nearly identical amounts of unmodified, dehydrated and fully modified prenisin were obtained after overnight expression and purification (Figure 25).

Van den Berg van Saparoea *et al.* (2008) showed that prenisin accumulates to comparable level in cells expressing *nisABCT* (fully modified prenisin), *nisABT* (dehydrated prenisin) or *nisATC* (unmodified prenisin) after 4 h, indicating that the prenisins are produced in similar amount within the cells but not exported with similar efficiency (86). The prenisin exporter NisT may therefore be a limiting factor that causes the export of unmodified and dehydrated prenisin to occur with reduced kinetics (86). However, when using overnight expression, it was possible to obtain similar amounts of unmodified, dehydrated and fully modified prenisin after purification. Nisin expression occurs during the logarithmic growth phase of *L. lactis* and stops when the stationary phase is reached (133,134). Therefore, NisT may export accumulated prenisin during over night expression as no new prenisin is produced after reaching the stationary growth phase, although NisT has reduced affinities for unmodified and dehydrated prenisin (86).

Prior to interaction studies, the various prenisins were identified as correctly produced by HPLC analysis. Importantly, it was demonstrated that HPLC analysis can be used to discriminate between the purified prenisins, because unmodified, dehydrated and fully modified prenisin exhibit significant differences in their retention times and elution profiles (Figure 32). Therefore, HPLC can be used as a direct and fast method to discriminate between these peptides. Especially, the ability to discriminate between dehydrated and fully modified prenisin in a fast and direct manner makes HPLC a

valuable tool for analysis of prenisin derived peptides. This method could likely also be used for other lantibiotics.

To date mass spectrometry is commonly used to distinguish between unmodified, dehydrated and fully modified prenisin. Mass spectrometry allows discriminating directly between unmodified (5963 Da) and dehydrated prenisin (5837 or 5819 Da) because 7 or 8 water molecules are eliminated during dehydration resulting in a mass difference of 126 or 144 Da, respectively (86,92,117). However, it is not possible to distinguish between dehydrated and fully modified prenisin as the cyclization does not result in a change of mass and therefore both peptides have the same molecular mass (86,87). An indirect method can be used to discriminate between these two peptides by mass spectrometry. To reveal if free cysteines are present in dehydrated prenisin, CDAP (1-Cyano-4-dimethylaminopyridinium tetrafluoroborate) can be added to react with free thiol groups leading to a mass increase of 25 Da for each free cysteine (88,97). In a similar fashion, the number of dehydrations within a peptide can be revealed by mass spectrometry. For this, ethanethiol can be added to the peptide, which reacts with dehydrated residues and forms an ethylcysteine. This leads to a mass increase of 62 Da for each dehydrated residue present (88,117).

By using HPLC, it is not necessary to use reagents to modify the peptides to determine whether they are cyclized or not, because their elution profiles differ significantly, apparently due to their different hydrophobicity (Figure 32).

Furthermore, HPLC can be used to reveal NisB dehydration activity since dehydration leads to a change of the elution profile. For investigating NisB activity *in vitro*, prenisin mutants, which feature up to eight dehydratable residues can be produced *in vivo* and used to compare the *in vitro* dehydration efficiency of NisB.

Another method to discriminate between dehydrated and fully modified prenisin is to investigate the antibacterial activity of these peptides after treatment with trypsin, since trypsin can be used to cleave off the leader peptide (54,57,117). The propeptide however contains two trypsin cleavage sites, but the (methyl)lanthionine rings, which are exclusively present in fully modified prenisin protect this peptide against proteolytic degradation (54,57,117). Therefore, trypsin treatment of fully modified prenisin liberates active nisin, whereas unmodified and dehydrated prenisin treated with trypsin does not result in active nisin. These peptides lack the (methyl)lanthionine rings and are efficiently digested (54,57,117).

Since the biological activity assay cannot reveal whether prenisin was completely modified, HPLC can now be used to monitor if all modifications occurred.

## 5.2 NisB is a dimer

NisB expressed in its natural host *L. lactis* was purified to homogeneity from the cytosol using buffers containing 500 mM NaCl since 150 mM NaCl led to heterogeneous and instable protein (Figure 18). As MALS analysis revealed NisB is also stable at a NaCl concentration of 250 mM, which was used for the SPR analysis (Figure 35). This indicates that salt stabilizes NisB by for example increasing the surface tension of the surrounding water, which may lead to increased intra- and intermolecular forces that cause a stabilized protein conformation (135). This indicates that within the cell another factor may stabilize NisB. This factor could be for example another protein (NisC) or the cytoplasmic membrane. Also the class I dehydratases EpiB from *S. epidermidis* and SpaB from *B. subtilis* as well as the class I cyclase NisC from *L. lactis* were purified using buffers containing 500 mM NaCl (57,105,106).

The oligomeric state of purified NisB was determined by MALS analysis. It was found that NisB is a dimer in solution (Figure 22). Although NisB was shown to associate to the cellular membrane (95), a hydrophobicity analysis of LanB proteins revealed that these proteins are rather hydrophilic (60,136). By yeast two-hybrid analysis and co-immunoprecipitation studies it was shown that probably only one NisB molecule is involved in a nisin synthetase complex, which is located at the membrane (95). This putative synthetase complex consists of a NisT dimer, two NisC and one NisB (Figure 11A) (95). Moreover, an interaction between NisB and NisT was not found (95). A possible explanation for the observation that a NisB-NisB interaction could not be detected by the yeast two-hybrid analysis can be that in this study only NisB fragments were used (95). The NisB self-interaction may occur only with full length NisB. Moreover, full length NisB may also be able to interact with NisT.

Also for subtilin from *B. subtilis*, which is a class I lantibiotic closely related to nisin, a membrane associated synthetase complex was proposed. From yeast two-hybrid and co-immunoprecipitation studies it was suggested that the subtilin synthetase complex comprises two molecules each of SpaB, SpaC and SpaT (96). SpaB the subtilin dehydratase was demonstrated to interact with itself and with the transporter SpaT

(96). The self-interaction of SpaB was confirmed by an *in vitro* pulldown assay using soluble SpaB fused to a His-tag or to a Myc-tag (105). In 2007 Helfrich *et al.* reported that SpaB was found exclusively in the cytosol of *B. subtilis*, which led to the assumption that the membrane associated subtilin synthetase complex could be a transition state (137). Moreover, also for the class II modification enzyme, NukM, a self-interaction was reported (115).

In addition Karakas Sen *et al.* (1999) proposed that a second NisB in the nisin synthetase complex could balance the stoichiometry of the putative nisin synthetase complex and make an important contribution to the activity of the complex (85).

Taken together, the data indicate that oligomerization is not uncommon to lantibiotic modification enzymes. Still, much is unclear about the actual organization and the localization of the proteins involved in forming such lantibiotic synthetase complexes. Isolation of an intact lantibiotic synthetase complex may solve these issues in future.

## 5.3 Interaction of NisB and prenisin

Whereas numerous *in vivo* studies have demonstrated that the leader peptides of lantibiotics are responsible for directing the prepeptide to the modification machinery and to the exporter, only limited knowledge is available on the *in vitro* interaction of a lantibiotic prepeptide with its dedicated modification enzyme(s).

For the well studied class I lantibiotic nisin no interaction studies were done to investigate how prenisin interacts with its modification enzymes. Since the modification enzymes of nisin are able to dehydrate and cyclize nonlantibiotic peptides that are fused to the leader peptide of prenisin it is concluded that the leader peptide is crucial for those peptides to become modified by either NisB or NisC and then exported by NisT (88,90,94,117).

NisB was shown to preferentially dehydrate hydrophobic flanked serine and threonine residues (90). Herein, threonines are more effectively dehydrated than serines (90). This indicates that NisB is able to recognize more than the leader peptide of a putative substrate (90,91). To date insights into substrate recognition and specificity of NisB were missing. The work presented here shows for the first time that isolated dimeric NisB exhibits biological activity *in vitro*, as it binds its native substrate, unmodified prenisin, as evidenced by SEC and SPR analysis (Figure 33 and Figure 36). The

interaction between NisB and unmodified prenisin occurred with an affinity of 1.05 ± 0.25 μM (Table 5). Notably, binding to NisB did not require special additives and occurred in the absence of a cellular membrane. These results are consistent with yeast two-hybrid analysis and co-immunoprecipitation studies that showed an interaction between NisB and unmodified prenisin (95). Similarly, the soluble subtilin dehydratase SpaB, was shown to interact *in vitro* with its substrate, unmodified SpaS (137). Moreover, also for the class II modification enzyme, NukM, *in vitro* association with its natural substrate was observed (115). This study revealed via SPR analysis that NukM binds to immobilized pre-nukacin as applied crude extract containing NukM led to a higher binding response than extract, which lacked NukM (115). However, for these interaction studies no purified NukM was used and therefore the dissociation constant could not be determined accurately. Calculation of the dissociation constant requires an accurate determination of the concentration of the analyte (138).

The modified versions of prenisin i.e., the dehydrated and the fully modified form, were also bound by NisB, although with different affinities (Figure 33 and Figure 36). Dehydrated prenisin, carrying eight dehydrated residues, showed a 3-fold higher affinity as compared to unmodified prenisin, which relates to a substantially increased association rate (Figure 36 and Table 5). This can be explained by an overall increase in hydrophobic interactions due to the presence of the dehydrated residues. Indeed, RP-HPLC analysis indicates that dehydrated prenisin is more hydrophobic than unmodified prenisin as it elutes later from the column (Figure 32). In addition, the higher association rate of dehydrated prenisin can be explained by the fact that NisB interacts in a specific manner with the propeptide of prenisin. Support for this view comes from binding experiments using the fully modified prenisin (Figure 33 and Figure 36). Whereas the association rate of the fully modified prenisin was comparable to that of the unmodified and the dehydrated prenisin, this peptide dissociated more than 20-times faster ($k_{off}$ 0.323 ± 0.035 $s^{-1}$). As a consequence NisB exhibits a much lower affinity for this prenisin peptide, which harbors five thioether rings ($K_D$ of 10.5 ± 1.7 μM). This indicates a specific interaction of NisB with the propeptide (90,91). In line with this, dehydration of serine and threonine residues was shown to be favored when they are flanked by hydrophobic residues, whereas a hydrophilic environment disfavored dehydration. Furthermore, the *in vitro* observation that thioether rings reduce the affinity of prenisin for NisB, whereas dehydrated residues appear to increase the affinity

could explain why secretion of dehydrated prenisin is reduced compared to fully modified prenisin (86). Since dehydrated prenisin is more tightly bound to NisB it may be less efficiently transferred to NisT for export, whereas fully modified prenisin is rapidly released from NisB and could therefore be more efficiently exported by NisT.

The importance of the leader peptide for binding to NisB was also investigated. Nisin, which is the equivalent of fully modified prenisin minus the leader peptide, did not bind to NisB (Figure 33 and Figure 36). This demonstrates the requirement of the leader peptide for binding to NisB *in vitro*. The importance of the leader peptide for NisB recognition has been firmly established *in vivo*, as *L. lactis* cells harboring NisB can modify nonlantibiotic therapeutic peptides fused to the nisin leader peptide (88,90).

A recent study showed that the nisin synthetase machinery tolerates single and double mutations within the leader peptide, but simultaneous replacement of the FNLD box, which is conserved among class I lantibiotics (139), with four alanines abolished dehydration and subsequent cyclization (118). By SEC and SPR analysis it was shown that the simultaneous substitution of the FNLD residues in the leader peptide by alanines abolished the interaction of prenisin with NisB *in vitro* (Figure 33 and Figure 36). These results may explain the *in vivo* observation that this mutant is secreted only in an unmodified form in spite of the presence of functional NisB and NisC (118). Thus, the FNLD-box is essential for the interaction of prenisin with NisB. However, since the FNLD/AAAA prenisin is still secreted, this suggests that leader peptide recognition by NisT is less stringent or determined by different part(s) of the leader peptide. This raises the question, whether NisB and NisC may also recognize different motifs within the nisin leader peptide?

These results give first insights into the interaction of a class I lantibiotic prepeptide with one of its modification enzymes. For the well studied class II enzyme LctM, interaction studies with its substrate pre-lacticin were hampered due to the poor solubility of the peptide (119). It is assumed that the leader peptide is responsible for efficient binding of the substrate to LctM, either to keep the prepeptide bound during modification, or to induce a conformational change in LctM to activate the enzyme (55,119). However, the function of the lacticin 481 leader peptide still remains unclear.

Taken together the data indicate that the nisin leader peptide is important for recognition and initial binding to NisB, with an essential role for the FNLD-box. Moreover, the interaction between NisB and prenisin is not only determined by the nisin

leader peptide, but also by the nature of the propeptide. Thus the dehydratase NisB shows substrate specificity *in vitro*, as it is able to discriminate between the unmodified prenisin and its modified derivatives.

## 5.4 NisB dimer binds one prenisin

By SPR analysis it was found that the interaction of an immobilized NisB homodimer with each applied prenisin occurred with a 1:1 stoichiometry. This showed that a NisB dimer binds one prenisin molecule, which indicates that two NisB molecules may be needed to build the binding site for prenisin. A self-interaction to form homodimers is a common phenomenon in biology (140). HIV-1 protease and type II restriction nucleases like EcoRV from *E. coli* also dimerize to build the binding site for their substrates (141,142). Herein, each subunit of a homodimer can coordinate the substrate from another site.

Another explanation for the observation that a NisB dimer binds one prenisin could be that the binding of prenisin to NisB causes negative cooperativity between the NisB subunits. Such a phenomenon was obtained for the metabotropic glutamate receptor type I, a member of the G-protein-coupled receptors, where the binding of the substrate (glutamate) to one subunit prevents binding of a second substrate to the other subunit of the homodimer due to a conformational change (143).

In addition to the findings for the NisB-prenisin interaction, SpaB was shown to interact with itself (96,105). By yeast two-hybrid studies it was revealed that the C-terminal part of SpaB interacts with itself (Figure 37) (96). Furthermore, the subtilin prepeptide (SpaS) was shown to interact with the C-terminus of SpaB only (Figure 37) (96). Therefore it can be hypothesized that SpaS may bind to the SpaB interface, which may be also the case for the NisB prenisin interaction.

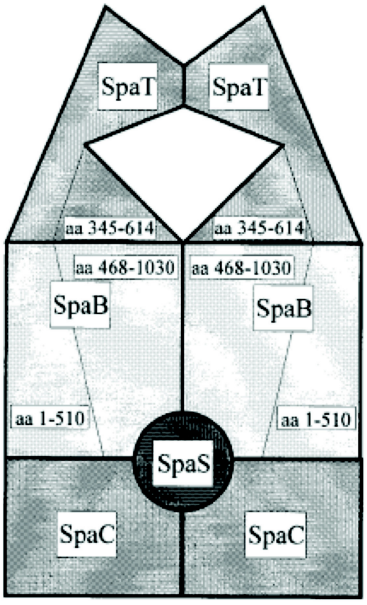


**Figure 37. Composition of the suggested subtilin synthetase complex.** The model based on the data form yeast two-hybrid studies. The thin lines represent protein fragments. The thick lines represent protein interaction interfaces. Taken from (96).

Moreover, Helfrich *et al.* (2007) showed that SpaB was able to bind to SpaS *in vitro*, supporting the finding for the nisin system (105,137). The stoichiometry of this interaction however was not determined.

## 5.5 Mechanism of prenisin modification

Since SPR analysis revealed that NisB binds dehydrated prenisin more tightly than unmodified prenisin it can be assumed that dehydrated prenisin remains bound to NisB to be cyclized by NisC in a putative modification complex. This points to a cooperative function of NisB and NisC. Subsequently, fully modified prenisin is released from NisB and can be transported by NisT. Van den Berg van Saparoea *et al.* (2008) showed that when the proposed nisin synthetase complex is disrupted by deleting one (or several) of its compounds, the optimal production of prenisin is defective (86). This strongly points to the importance of a functional NisBTC complex and a cooperation of the three proteins.

NisB and NisC are assumed to function in concert to introduce the (methyl)lanthionine rings into prenisin, where ring formation can prevent dehydration of residues involved in or adjacent to (methyl)lanthionine rings (87,97).

Kuipers *et al.* (2008) showed that truncated lacticin 3147 fused to the nisin leader peptide was dehydrated by NisB to a higher extent in the absence of NisC (97). Whereas, when NisC was present, dehydration of threonine residues localized in (methyl)lanthionine rings was significantly reduced suggesting that (methyl)lanthionine rings prevent dehydration of residues, which are located in the rings (97). However, Ser5 in active nisin is always dehydrated although Ser5 is located in (methyl)lanthionine ring A (87). Therefore, Lubelski *et al.* (2009) suggested that the first four dehydrations (Ser2, Thr3, Ser5 and Thr8) likely occur before the first cyclization is catalyzed, indicating a high specificity of NisB for the N-terminus of unmodified prenisin (87). Subsequently, NisC catalyzes the first two cyclizations before the next dehydration can occur (87). Evidence for this came from mutating Lys12 into a serine, which becomes strongly dehydrated when ring B formation was impaired (87). In contrast, when ring B precedes the serine introduced at position 12 this residue was mostly not dehydrated. The same was observed for serines that were introduced behind ring C or E (87). They were all dehydrated when no preceding ring was present, whereas a preceding ring impaired dehydration of these residues (87). Since Thr8 is also always dehydrated in nisin this residue must be dehydrated before ring A is made, as otherwise ring B formation would be impaired (87). Therefore, it can be assumed that NisB-mediated dehydration and NisC cyclase activity are coordinated events for building (methyl)lanthionine rings C, D and E. Taken together, NisB and NisC alternate in function to install the modifications in a processive and directional manner (87,97).

Since, NisB was shown to dehydrate residues farther away from the leader peptide than in natural prenisin (90), the question raised how these serines and threonines become dehydrated. Lubelski *et al.* (2009) suggested that prenisin moves through the active sites of NisB and NisC with NisT as the motor, which pulls prenisin through the modification complex (87). However, NisB and NisC were shown to modify prenisin even in the absence of NisT, since in *L. lactis* cells expressing *nisABC* modified prenisin was found, from which antimicrobially active nisin could be liberated upon trypsin treatment (89,144). Unfortunately, the content of modification was not revealed since (methyl)lanthionine rings A, B and C are sufficient for antimicrobial activity of nisin (101). In addition, the growth of *L. lactis* cells expressing *nisABC* was inhibited possibly by the intracellular formation of active nisin, whereas expression of a truncated form of nisin, which comprises only (methyl)lanthionine rings A, B and C did not inhibit culture

growth (86). Therefore, it remains unclear if prenisin found in *L. lactis* cells expressing *nisABC* is fully modified in the absence of NisT (86,89). Furthermore, NisC was able to introduce five (methyl)lanthionine rings into dehydrated prenisin *in vitro* without NisT and NisB present (57). Taken together, this indicates that for modification of prenisin, NisT is not required as a motor.

The data of the SPR analysis suggest that prenisin is tighter bound to NisB after the dehydrations occurred and that the introduction of the (methyl)lanthionine rings led to a weaker affinity of prenisin to NisB. These finding may explain how prenisin is released from the modification enzyme and is available for NisT mediated export. Thus, the different affinities of the propeptide side chains could be the driving force for the modification, which was also suggested by Kuipers *et al.* (2011). These authors suggest that prenisin is bound at a fixed position to NisB and NisC. The finding that a shortened leader peptide causes impaired dehydration of the N-terminal serine and threonine residues supported this suggestion (118). Therefore, the modifiable residues have a distinct distance to the active centers of NisB and NisC. The residues, which are located near the active centers of the enzymes can be modified with a higher efficiency than more distal residues. This might explain why the first four dehydratable residues (Ser2, Thr3, Ser5 and Thr8) apparently are modified before ring A is made by NisC. Whereas, the more distal residues are dehydrated and cyclized in an alternating fashion by NisB and NisC, because they may reach the active centers one after another due to their distances to the active sites. The result could be interpreted as directionality but is indeed a result of different binding affinities of the certain residues due to the distance to the active sites of the modification enzymes and due the adjacent environment of these residues (145).

## 5.6 Model for prenisin modification

NisB was found in the cytosol of *L. lactis* as a dimer, which binds its natural substrate unmodified prenisin with an affinity of 1.05 ± 0.25 μM. Furthermore, NisC was also shown to interact with dehydrated prenisin in solution (57). A similar behavior was reported for SpaB and SpaC, which were both found in the cytosol of *B. subtilis* and were shown to interact with SpaS by *in vitro* pull down assays (137). In addition, the *in vitro* activity of the class II enzymes LctM and HalM was reconstituted in solution (56,103). Although, the modification complexes are assumed to be located at the cytoplasmic membrane of the cell to deliver the fully modified prepeptide to its dedicated transporter for efficient secretion, the modification enzymes apparently do not require the cellular membrane for interaction with their substrate. So far, all attempts to isolate an intact lantibiotic synthetase complex by either blue-native PAGE or co-purification failed (95,96). This indicates that the putative synthetase complex could be rather unstable or a transition state.

Based on the reports in literature and the novel findings, a model for the modification and transport of prenisin is presented (Figure 38). Dimeric NisB, either complexed with NisC or not, first recognizes and binds prenisin (Figure 38A). The leader peptide and especially the FNLD-box herein is a prerequisite for efficient binding to NisB. Upon prenisin binding the NisBA complex might recruit NisC and the modification complex is formed, which comprises a NisB dimer and two molecules of NisC, similar to the subtilin synthetase complex (Figure 11B) (96). Since a direct interaction of NisB and NisT was not demonstrated by the yeast two-hybrid and co-immunoprecipitation studies the whole complex could be located near NisT due to a weak interaction between two NisC molecules and a NisT homodimer (Figure 38B) (95).

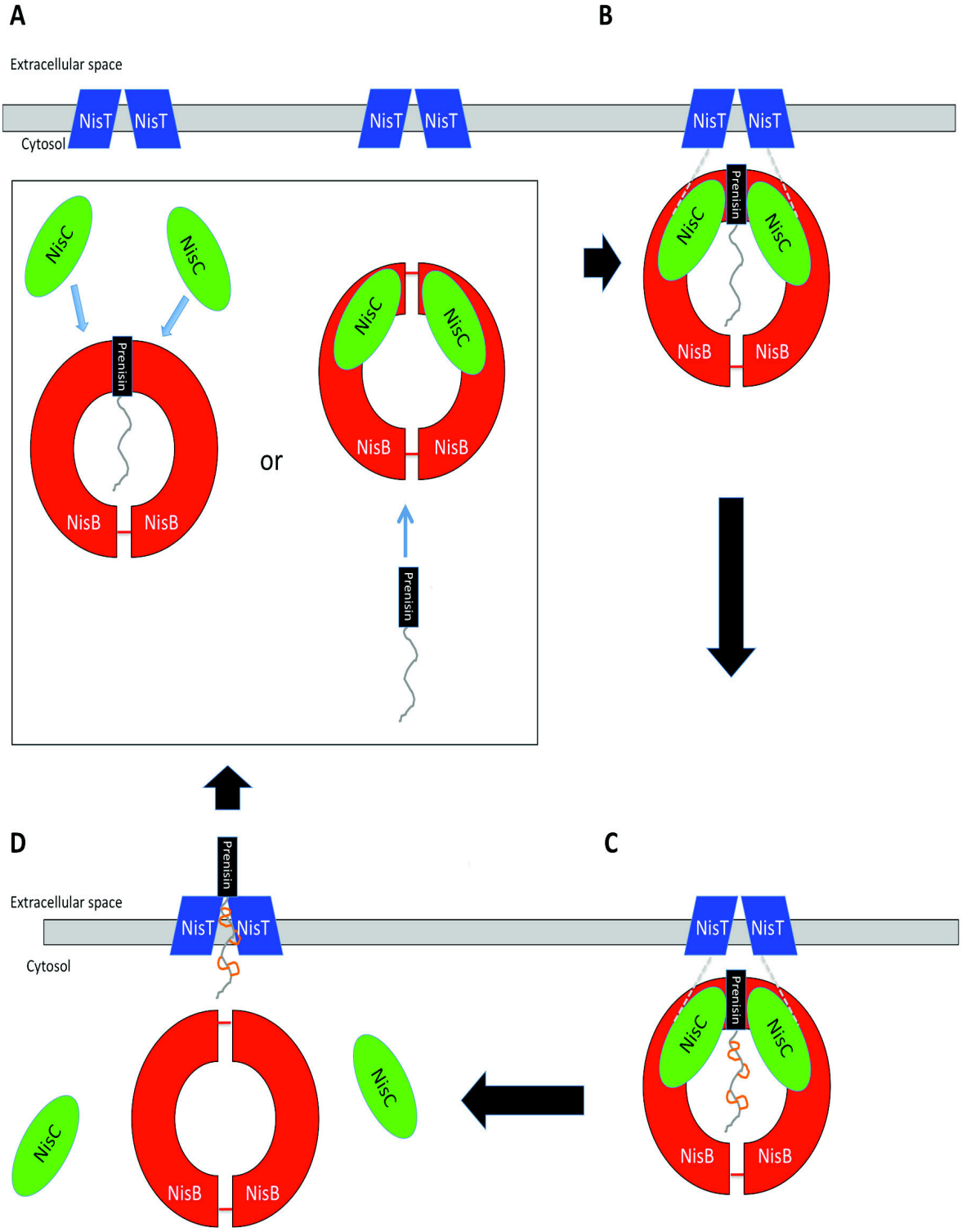


**Figure 38. Model of prenisin modification and transport. A:** Unmodified prenisin may either bind to NisB and then NisC is recruited, or a preformed NisBC complex binds the prepeptide. **B:** An interaction between NisC and NisT coordinates the synthetase complex at the cytoplasmic membrane. **C:** The five (methyl)lanthionine rings are introduced into prenisin by NisB and NisC. **D:** After prenisin is fully modified the synthetase complex dissociates and fully modified prenisin is subsequently exported via NisT. After dissociation of the nisin synthetase complex the enzymes may be available for a new modification cycle.

Then, NisB and NisC catalyze their modification reactions in a alternating and directional fashion (Figure 38C). The high dissociation rate of fully modified prenisin from NisB suggests that when the modification of prenisin is finished, the prepeptide is rapidly released from NisB and subsequently exported via NisT. Since the putative nisin synthetase complex could not be isolated it is possible that the complex dissociates after modification and secretion of prenisin (Figure 38D). Similarly, for the type I secretion system of HlyA from *E. coli*, which comprise an ABC-transporter (HlyB), a membrane fusion protein (HlyD) and an outer membrane factor (TolC), it is known that the translocation complex is a transition state during export (146). Herein, a preformed HlyBD complex binds the substrate and upon binding the outer membrane factor is recruited to assemble the functional translocation complex (147). After the substrate is exported the translocation complex dissociates (146).

# 6. Outlook

The successful *in vitro* reconstitution of the enzymatic activity of several bifunctional LanM enzymes, which harbor both dehydratase and cyclase activity, has provided us with insights into the mechanistic aspects of lantibiotic modification (104,108), on the other hand dehydratases of the LanB family remain enigmatic. Although for many of these LanB enzymes a role in dehydration has been established unequivocally *in vivo*, this still has to be demonstrated in the test tube (61). Since dimeric NisB is available in a pure and homogenous form and was shown to interact specifically with unmodified, dehydrated as well as fully modified prenisin, the next step is to reconstitute the *in vitro* dehydration activity of NisB. For monitoring the dehydration activity of NisB the established protocol for HPLC analysis can be used, since HPLC can distinguish between unmodified and dehydrated prenisin. Moreover, it can be tested if cofactors are required for catalysis, since it was shown that the class II LanM enzymes LctM, HalM and NukM needed ATP/$Mg^{2+}$ for their activity *in vitro*, although they do not contain an ATP binding motif (56,103,115).

Furthermore, HPLC analysis exhibits a convenient and easy method to monitor NisC cyclase activity *in vitro*. Prenisin mutants carrying a different number of *in vivo* introduced (methyl)lanthionine rings could then be analyzed by HPLC and serve as references to determine the number of cyclizations catalyzed by NisC *in vitro*.

It was shown that NisB was able to dehydrate prenisin mutants more efficiently in the presence of an inactive NisC mutant *in vivo* (87). Therefore, it is interesting to investigate whether inactive NisC can positively influence the dehydration by NisB *in vitro*. Such finding would further point to a direct interaction of NisB and NisC. Furthermore, the putative interaction of NisB and NisC can be analyzed by SPR analysis *in vitro*. By SPR analysis, binding kinetics of a putative NisBCA complex can be investigated, which could also answer the question whether NisB or NisC is the first interaction partner of prenisin. With purified NisB, NisC and NisT in hand, it can be investigated whether the putative synthetase complex NisBTC can be formed *in vitro*. Using for example analytical SEC, MALS or blue-native page, the stoichiometry of such complex might be revealed.

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# Abbreviations

| | |
|---|---|
| ABC | ATP binding cassette |
| ADP | Adenosine-5'-diphosphate |
| Ala | Alanine |
| APS | Ammonium persulfate |
| ATP | Adenosine-5'-triphosphate |
| CAPS | 3-(Cyclohexylamine)-1-propanesulfonic acid |
| Dha | Dehydroalanine |
| Dhb | Dehydrobutyrine |
| DNA | Deoxyribonucleic Acid |
| *E. coli* | *Escherichia coli* |
| e.g. | For example |
| EDTA | Ethylendiamine-tetra-acetate |
| GlcNAc | N-acetylglucosamine |
| HEPES | 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid |
| His | Histidine |
| i.e. | Id est (this is) |
| IMAC | Immobilized metal ion affinity chromatography |
| *L. lactis* | *Lactococcus lactis* |
| Lac | Lactate |
| M | Molar |
| MALS | Multi angle light scattering |
| MDR | Multi drug resistance |
| MES | 2-(N-morpholino)ethanesulfonic acid |
| mM | Millimolar |
| MRSA | Methicillin resistant *Staphylococcus aureus* |
| MurNAc | N-acetylmuramic acid |
| NaOAc | Sodium acetate |

| | |
|---|---|
| NTA | Nitrilotriacetic acid |
| PAGE | Polyacrylamide gel electrophoresis |
| PBP | Penicillin binding protein |
| SDS | Sodium dodecyl sulfate |
| SEC | Size exclusion chromatography |
| Ser | Serine |
| SPR | Surface Plasmon resonance |
| TEMED | Tetramethylethylendiamine |
| TRIS | TRIS(hydroxymethyl)-aminomethan |
| VRE | Vancomycin resistant *enterococci* |
| µM | Micromolar |

# Curriculum vitae

## Personal information

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| Name | Antonino Mavaro |
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# List of publications

**Mavaro, A.**, Abts, A., Bakkes, P. J., Moll, G. N., Driessen, A. J., Smits, S. H., and Schmitt, L. (2011) Substrate Recognition and Specificity of the NisB Protein, the Lantibiotic Dehydratase Involved in Nisin Biosynthesis. *J Biol Chem* 286, 30552-30560

Abts, A., **Mavaro, A.**, Stindt, J., Bakkes, P. J., Metzger, S., Driessen, A. J. M., Smits, S. H. J., and Schmitt, L. (2011) Easy and rapid purification of highly active nisin. *International Journal of Peptides*, in press

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# Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Schriften entnommen wurden, sind als solche kenntlich gemacht. Die Arbeit ist in gleicher oder ähnlicher Form oder auszugsweise im Rahmen einer anderen Prüfung noch nicht vorgelegt worden.

Antonino Mavaro Düsseldorf, den 14.09.2011