
# The Nisin ABC

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# Investigating the maturation process of the lanthipeptide nisin

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Tag der mündlichen Prüfung:

*no pain - no game*
(Peter Kurowski, Dormagen 2000)

# Abstract

The antimicrobial lanthipeptide nisin is produced ribosomally as a precursor peptide by the lactic acid bacteria *Lactococcus lactis*. The nisin precursor consists of a N-terminal leader peptide and a C-terminal core peptide. Nisin is active against several Gram-positive bacteria including human pathogens. The biological activity of nisin derives from the characteristic post-translational modifications within the core peptide, which are installed via four essential steps. First, the dehydratase NisB dehydrates serine and threonine residues. Second, the cyclase NisC forms the (methyl)lanthionine rings by linking the dehydrated amino acids to C-terminal located cysteine residues via thioether bridges. In the third maturation step the fully modified precursor peptide is secreted across the cytoplasmic membrane via the ABC transporter NisT. The fourth and last step involves the subtilisin-like protease NisP. The N-terminal located leader peptide is proteolytically cleaved off by NisP to release the activated mature nisin.
In this thesis, the maturation steps of nisin were investigated on a molecular level. The modification enzymes NisB, NisC and NisP as well as the nisin precursor peptide and the mature nisin were isolated in order to analyze their interactions *in vitro*. The basic principles of gene expression and protein purification are summarized in chapter one. The activity of nisin is as well directed against its producing host and therefore *L. lactis* developed a self-immunity system, which is described in chapter two.
Chapter three, four and five deal with the cytosolic interactions between the modification enzymes NisB, NisC and the nisin precursor peptide. The interaction of NisB with the nisin precursor peptides was investigated by surface plasmon resonance. NisB is specifically interacting with the nisin leader peptide, especially recognizing the highly conserved -FNLD- box. NisB is able to distinguish between the three different maturation states of the core peptide (unmodified, dehydrated and modified). The interactions between the nisin precursor peptide and NisC were determined using isothermal titration calorimetry. The obtained binding affinities revealed that in contrast to NisB, NisC is specifically interacting with the leader peptide and cannot distinguish between the three different maturation states *in vitro*. Additionally, a recognition motif -FxLx- within the leader peptide was identified.
Chapter six is dedicated to the serine protease NisP. The *in vitro* activity of NisP could be demonstrated by reversed phase high-performance liquid chromatography. This analysis revealed that NisP cleaves the nisin precursor peptide independently of its maturation state.
Finally, in chapter seven a complex of NisB, NisC and the nisin precursor peptide was obtained and characterized. The unmodified precursor peptide initiates the assembly of the maturation complex with a stoichiometry of 2:1:1 *in vitro*.

# Zusammenfassung

Das ribosomal synthetisierte, antimikrobiell wirksame Lanthipeptid Nisin wird vom Milchsäure Bakterium *Lactococcus lactis* in Form einer Vorstufe produziert. Diese Vorstufe besteht aus einer N-terminalen Erkennungssequenz und einer C-terminalen Kernsequenz. Nisin wirkt antibakteriell gegen verschiedene Gram-positive Bakterien, unter ihnen einige humanpathogene Stämme. Die antibakterielle Wirkung beruht auf den charakteristischen und namensgebenden Lanthioninringen. Die Bildung der post-translationalen Modifikationen kann in vier Phasen eingeteilt werden. In der ersten Phase werden die Aminosäurereste Serin und Threonin durch das Enzym NisB dehydratisiert. Diese werden in der zweiten Phase über Thioetherbrücken mit C-terminal positionierten Cysteinresten verbunden. Dies hat eine Ringbildung zur Folge, welche durch die Zyklase NisC katalysiert wird. Anschließend wird in der dritten Phase das modifizierte Peptid in den extrazellulären Raum transportiert. Diese Sekretion wird durch den ABC-Transporter NisT katalysiert. Nach erfolgreicher Sekretion wird die N-terminale Erkennungssequenz durch die Protease NisP abgespalten. Diese Spaltung überführt die Vorstufe in das antimikrobiell aktive Peptid Nisin.

In dieser Dissertation wurden die Interaktionen zwischen den Nisin-Vorstufen und den Modifikationsenzymen NisB, NisC und NisP auf molekularer Ebene untersucht. Hierzu wurde die Isolation der Enzyme, des Peptids Nisin und der Nisin-Vorstufen erfolgreich etabliert und optimiert. Die Grundlagen der Genexpression und Proteinisolation sind in Kapitel 1 beschrieben.

Das gereifte, aktive Nisin ist ebenfalls gegen den Nisin produzierenden Stamm antimikrobiell wirksam. *L. lactis* hat daher einen Mechanismus entwickelt, um sich gegen sein eigenes Peptid zu schützen. Dieser Mechanismus ist in Kapitel zwei genauer beschrieben.

Die Kapitel drei, vier und fünf widmen sich der Untersuchung der zytosolischen Interaktion der Modifikationsenzyme NisB und NisC mit den Nisin-Vorstufen. Die Interaktion zwischen der Dehydratase NisB und den Nisin-Vorstufen wurde durch Oberflächen-Plasmon-Resonanz untersucht. NisB interagiert spezifisch mit der Erkennungssequenz, in welcher die hoch konservierte -FNLD- Box als Erkennungsmotiv identifiziert wurde. Zusätzlich kann NisB zwischen den verschiedenen Modifikationszuständen der Nisin-Vorstufen unterscheiden.

Die zweite Stufe der Modifizierung wird durch NisC katalysiert. Die Interaktionen zwischen NisC und den Nisin-Vorstufen wurden mit Hilfe der isothermen Titrationskalorimetrie bestimmt. NisC bindet *in vitro* ausschließlich die Erkennungssequenz und kann demnach nicht zwischen den Modifikationsstufen

der Kernsequenz unterscheiden. NisC bindet spezifisch die beiden Aminosäurereste Phenylalanin und Leucin, daraus ergibt sich ein Erkennungsmotiv -FxLx-.
Die Aktivität der Protease NisP konnte erfolgreich *in vitro* rekonstruiert werden (Kapitel 6). Durch analytische Hochleistungsflüssigkeitschromatographie wurde die Spaltungsreaktion kinetisch untersucht. NisP proteolysiert die Nisin-Vorstufe unabhängig des Modifikationszustand der Kernsequenz.
Aufbauend auf diesen Erkenntnissen konnte in Kapitel sieben ein *in vitro* Komplex aus den Enzymen NisB, NisC und der Nisin-Vorstufe isoliert und charakterisiert werden. Die unmodifizierte Vorstufe des Nisins initiiert hierbei die Bildung des Modifikationskomplexes in einer 2:1:1 Stöchiometrie *in vitro*.

# Contents

# List of Figures

# Abbreviations

| | |
|---|---|
| **ABC** | ATP binding cassette |
| **ADP** | adenosine-5-diphosphate |
| **ATP** | adenosine-5-triphosphate |
| **cIEX** | cation exchange chromatography |
| **Da** | Dalton |
| **DNA** | deoxyribonucleic acid |
| **E. coli** | *Escherichia coli* |
| **EDTA** | ethylenediaminetetraacetic acid |
| **e.g.** | exempli gratia |
| **et al.** | et alii |
| **GTP** | guanosine-5-triphosphate |
| **$His_6$-tag** | hexahistidine-tag |
| **$His_8$-tag** | octahistidine-tag |
| **$IC_{50}$** | concentration which inhibits the growth by 50 % |
| **IMAC** | immobilized metal ion chromatography |
| **ITC** | isothermal titration calorimetry |
| **$K_a$** | acid dissociation constant |
| **$K_D$** | dissociation constant |
| **kDa** | kilo Dalton |
| **$K_M$** | Michaelis-Menten constant |
| **L. lactis** | *Lactococcus lactis* |
| **m** | meter |

| | |
|---|---|
| **M** | molar |
| **MALS** | multi-angle light scattering |
| **mg** | milligram |
| **min** | minute |
| **mL** | milliliter |
| **NBD** | nucleotide binding domain |
| **nm** | nanometer |
| **nM** | nanomolar |
| **NRP** | nonribosomal peptide |
| **NRPS** | nonribosomal peptide synthetase |
| **p$K_a$** | -$\log_{10}$ $K_a$ |
| **PTM** | post-translational modification |
| **RiPP** | ribosomally-synthesized and post-translationally modified peptide |
| **RP** | ribosomal peptide |
| **RP-HPLC** | reversed phase high-performance chromatography |
| **sec** | second |
| **SEC** | size exclusion chromatography |
| **SPR** | surface plasmon resonance |
| **TFE** | trifluorethanole |
| **TMD** | transmembrane domain |

| **Amino acid** | **three letter code** | **one letter code** |
|---|---|---|
| Alanine | Ala | A |
| Arginine | Arg | R |
| Asparagine | Asn | N |
| Aspartic acid | Asp | D |
| Cysteine | Cys | C |
| Glutamic acid | Glu | E |
| Glutamine | Gln | Q |
| Glycine | Gly | G |
| Histidine | His | H |
| Isoleucine | Ile | I |
| Leucine | Leu | L |
| Lysine | Lys | K |
| Methionine | Met | M |
| Phenylalanine | Phe | F |
| Proline | Pro | P |
| Serine | Ser | S |
| Threonine | Thr | T |
| Tryptophan | Trp | W |
| Tyrosine | Tyr | Y |
| Valine | Val | V |

# 1 Introduction

## 1.1 Peptides

The information of life is encoded in the genetic code, DNA. DNA is the abbreviation for deoxyribonucleic acid, a polymer build up by four different nucleotides[1,2]. It encodes the blueprint for ribosomal protein synthesis and therefore DNA dictates the entire protein sequences. Proteins comprise essential functions in almost every biological process. Proteins are polymers composed of a set of 20 different proteiniogenic amino acids. Amino acids are organic molecules characterized by a central $\alpha$-carbon atom substituted with a carboxyl group, an amino group, a hydrogen atom and a specific side chain. Amino acids are connected by peptide bonds, the product of a condensation reaction which connects the $\alpha$-carboxyl group of amino acid n with the $\alpha$-amino group of amino acid n+1 (shown in Figure 1)[1,2,3].

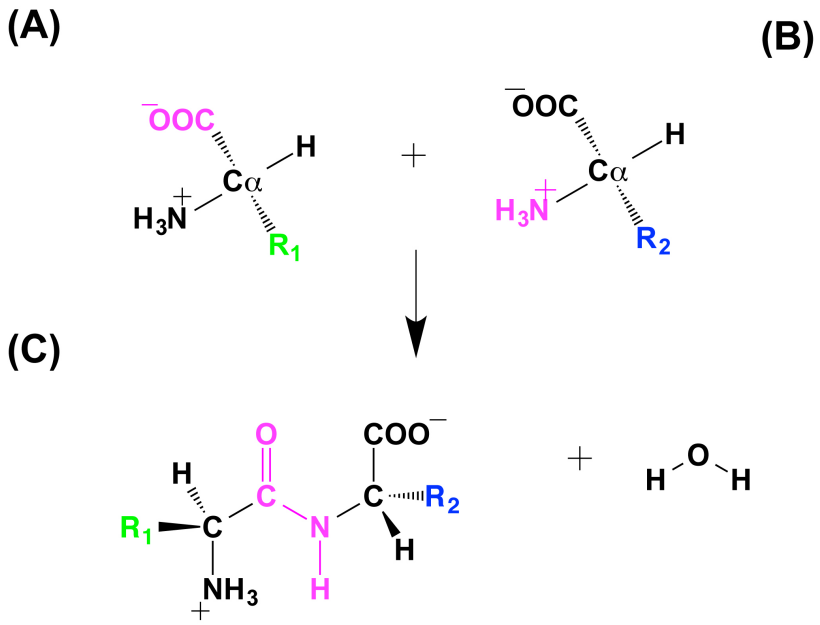


Figure 1: **Formation of a peptide bond**. The carboxyl group of amino acid n (A) and the amino group of amino acid n+1 (B) are highlighted in pink. The condensation reaction results in the formation of the peptide bond (shown in pink) between both amino acids (C) plus a water molecule as by-product. Created with CS BioDraw Ultra 12.0.

Per definition, proteins consist of more than 100 amino acids, whereas polypeptides below this threshold are termed peptides[1,2]. Proteins and peptides can be described by four structural characteristics.

The primary structure defines the amino acid composition in respect to the polypeptide length and precise order of connected amino acids[1,2]. The secondary

structure describes abundant structural motifs of polypeptides, namely for example $\alpha$-helices, $\beta$-sheets and hairpins. The formation of hydrogen bonds is the major determinant for the formation of secondary structural elements. The tertiary structure characterizes the specific, three-dimensional conformation of polypeptides. A subset of various interactions between the amino acids, like disulfide bridges, van der Waals interactions, hydrophobic interactions or salt bridges are responsible for forming the tertiary structure. A set of at least two polypeptide chains can build more global structural elements. These oligomeric states are characterized by the quaternary structure[1,2]. Peptides are frequently composed of primary and secondary structural elements, like $\alpha$-helices or $\beta$-sheets. In contrast, proteins typically contain a tertiary structure and are often described by quaternary structural elements[1,2].

Peptides are ubiquitous in all three domains of life and have several functions[4]. For examples they are involved in the immune system of eukaryotes[5,6] or antimicrobial agents of bacteria[7,8]. Remarkably, peptides are not only synthesized by the classical, ribosomal synthesis (ribosomal peptides - RPs[3]) but also by a second pathway called the non-ribosomal peptide synthetase (NRPS)[9], yielding in the class of non-ribosomal peptides (NRPs)[10,11]. The NRPS enzymes or enzyme complexes contain four domains[9,12]. The so-called adenylation domain (A) activates a dedicated amino acid. Afterwards, the peptidyl carrier protein domain (PCP) transports the activated amino acid to a second amino acid. The condensation domain (C) catalyzes the formation of the peptide bond. Finally, the peptide is released by the thioesterase domain (TE) that catalyzes a hydrolysis or cyclization reaction[9,12].

Various NRPs exist and the antibiotic vancomycin[13] and $\delta$-(L-$\alpha$-aminoadipyl-L-cysteinyl-D-valine) (ACV)-tripeptide, a penicillin precursor molecule, are prominent members[14]. Others are responsible for cytostastic activities (epothilone)[15] or iron uptake in bacteria (enterobactin)[16].

The main difference of NRPs compared to RPs is the possibility of NRPs to contain non-natural amino acids like ornithine[17]. They are often macrocyclic and differ in the post-translational modifications (PTMs). Furthermore NRPs can contain fatty acids[17].

RPs are subdivided in distinct classes, for example conus peptides describe spider venom peptides[18] or defensins are involved in the human immune system[19,20]. The bacteriocin and the lanthipeptide class possess antimicrobial activity and are described in more detail below[7,8,21,22,23].

Ribosomally-synthesized and post-translationally modified peptides (RiPPs) are a fascinating group of RPs[4]. These peptides are characterized by modifications immediately introduced after ribosomal synthesis[4]. These modifications confer unique properties to these peptides such as high stability against proteases and highly increased activity[4].

## 1.2 Ribosomally-synthesized and post-translationally modified peptides

Ribosomally-synthesized and post-translationally modified peptides (RiPPs) are produced by bacteria[24], archaea[25], fungi[26], insects[27] and also humans[28]. Therefore RiPPs exist in all three kingdoms of life with a vast structural and functional diversity[4,29]. Human defensins are produced by phagocytic cells and mucosal tissues and are effector molecules in the innate immune system[30]. Another example is the magainin produced by the skin of frogs to protect them against bacterial infections after injury[6].The first discovered prokaryotic RiPP was nisin produced by *Lactococcus lactis* (*L. lactis*), which is used as a food preservative in the food industry for over 50 years[24].
A common feature of all RiPPS are the PTMs that increase stability, life-time and function, which causes a maximum efficiency[7]. Due to the large effort in genome sequencing approaches, the number of identified gene clusters encoding for RiPPs increased drastically during the last years[31].
RiPPs are produced as precursor molecules, which are modified and processed during maturation to the mature peptides[4]. Figure 2 summarizes the maturation process of a RiPP.

Every RiPP consists of a leader peptide and a core peptide[4]. The N-terminal leader peptide serves as a recognition site for the modification and transport machinery[32,33]. In eukaryotes, an additional signal might be present N-terminal to the leader peptide to direct the peptide to the endoplasmatic reticulum[34].
The core peptide represents the part of the precursor peptide, which finally forms the active peptide[33]. The PTMs occur exclusively in the core peptide and not in the leader peptide[33]. In some peptides a C-terminal recognition sequence is responsible for cyclization and excision of the precursor peptide[34].
The directionality of the process is mostly directed from the N- to the C-terminus, although a few examples are known with reverse direction[35]. The common cleavage of the leader peptide from the modified core peptide by a protease represents the final step and activates the RiPP[33].
The function of the leader peptide for recognition and coordination of the modification process is described in more detail in chapter 1.3.3.

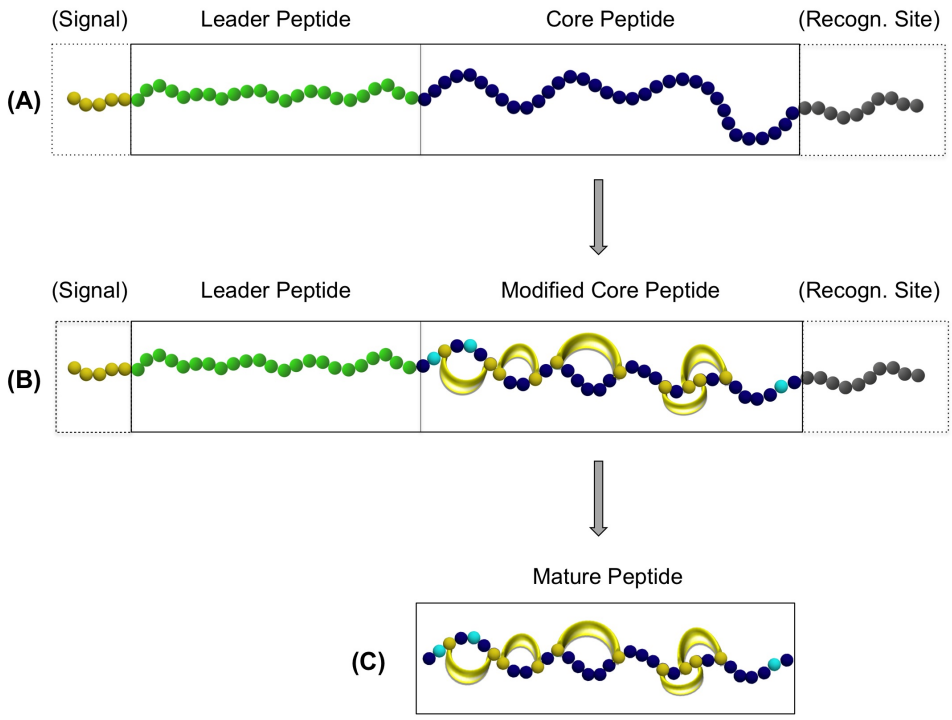


Figure 2: **Maturation process of RiPPs**. The ribosomally synthesized precursor peptide is shown in (A). After modification the core peptide is called modified core (B). After cleaving off the leader, the final RiPP is released, which is called mature peptide (C). The yellow colored signal is often found in eukaryotic RiPPs. This N-terminal elongation directs the peptide e.g. to the endoplasmatic reticulum. The grey colored recognition site is responsible for cyclization and excision of the precursor peptide. The signal as well as the recognition site are motifs which are not essentially found in every RiPP. In green the leader peptide is shown which ensures the recognition by the modification enzymes and the transport machinery. The dark blue chain represents the core peptide within the light blue balls indicate PTMs as well as the yellow rings which symbolize cyclizations such as (methyl)lanthionine rings.

## 1.3 Lanthipeptides

Sulfur atoms of cysteine residues are the predominant modification sites of RiPPs[4,36]. Intramolecular thioether or disulfide bonds are frequently observed[4,36]. In nisin, the best characterized RiPP since its discovery in 1928[37], five thioether bridges link cysteine residues with dehydrated serine and threonine residues. In 1971, the structure of nisin revealed the unique conformation of the lanthionine rings[38]. These intramolecular cyclizations (3-methyllanthionine or *meso*-lanthionine rings) represent the eponymous motif of the so-called lanthipeptides (lanthionine-containing peptides)[21].

Together with nisin, 50 other lanthipeptides display antimicrobial activity and build the class of lantibiotics (lanthionine-containing antibiotic)[39].

### 1.3.1 Biological Activity and Application of Lanthipeptides

Nisin is used in the food industry as a preservative for example in milk products, cheese and packed foods[24]. In Europe it has been included in the food additive list (E number 234)[40]. Nisin inhibits the growth of Gram-positive organisms and clostridial spores, furthermore it prevents microbial spoilage[24].
Since lantibiotics are antimicrobial, they are interesting candidates as potential therapeutics[5]. Nisin and mersacidin for example are effectively applied against the methicillin-resistant *Staphylococcus aureus* (MRSA) as well as against *Clostridium difficile*, *Streptococcus pneumoniae* and *enterococci* species[41,42,43]. Since the antibiotic resistance is rising a new generation of antibiotics is needed[5]. Also several other diseases can be treated by lantibiotics. Lacticin 3147 shows spermicidal activity and is investigated as an agent against the transmission of sexual diseases[44,45,46]. *Propionibacterium acnes* are killed by gallidermin and epidermin, making them interesting candidates as acne drugs[47]. Nisin is already included in therapeutics against bovine mastitis[48], as well as lacticin 3147, which is a promising candidate for incorporating in agents against teat seals[49].
RiPPs themselves are potent therapeutical peptides in the treatment of human and animal diseases. Furthermore the modification machinery can be used to modify conventional pharmaceutical peptides to enhance their activity or stability. The modification machinery of nisin has already been used several times to introduce PTMs like dehydrated amino acids and lanthionine rings[35,50,51]. Angiotensin for example is a cardiovascular peptide and displays increased stability and activity[52] after modification by the nisin modification machinery[35,50,51].

### 1.3.2 Classification of RiPPs

All members of the lanthipeptide family are encoded on a coherent gene cluster[4,8,21]. These operons contain several genes for proteins that are involved in the regulation of expression, the modification process or self-immunity against the self produced active RiPP. Up to now more than 90 members of this family were identified[25]. Based on the precise operon organization they were subdivided into four classes[4,8,21]. These classes are described in the next section and a schematic overview is shown in Figure 3. The nisin operon is described in more detail in chapter 1.4.

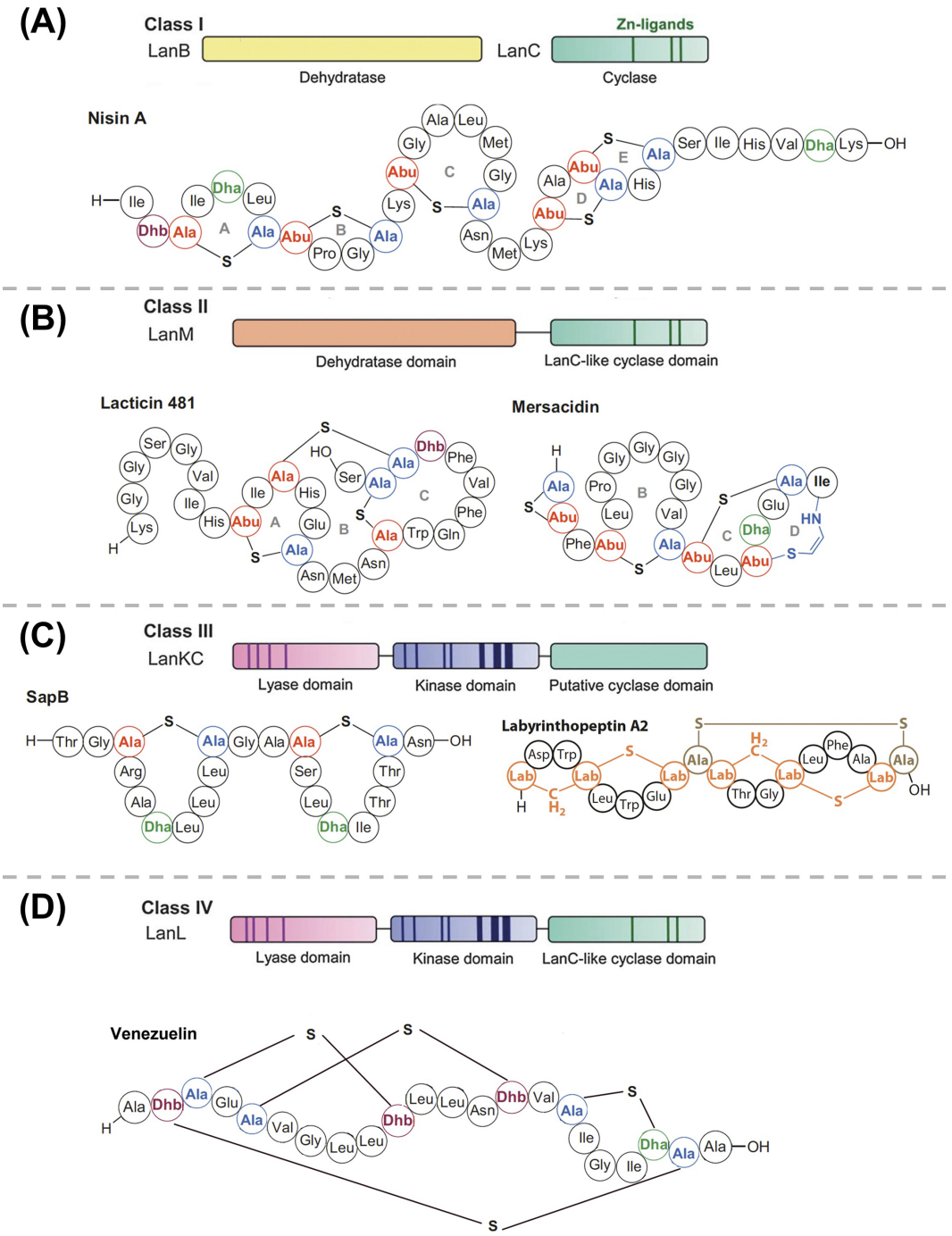


Figure 3: **The classification of lanthipeptide operons**. (A) Class I is characterized by two modification enzymes LanB (dehydratase) and LanC (cyclase). (B) Class II lanthipeptide operons contain one modification enzyme LanM, that includes a dehydration and a cyclization domain. (C) Class III comprises a modification enzyme with three distinct catalytic domains. A N-terminal lyase domain is followed by a kinase domain. At the C-terminus a cyclase domain is located, which lacks the characteristic LanC zinc finger-like motif. (D) The order of domains in class IV is homolog to class III although the cyclase domain possesses the zinc finger-like motif. The green vertical lines within the cyclase domain represent the zinc-ligands. For each class, representative lanthipeptides are shown. Class I: nisin A. Class II: lacticin 481 and mersacidin. Class III: SapB and labyrinthopeptin A2. Class IV: venezuelin. Based on Willey *et al.* 2007[8] and Knerr *et al.* 2012[21].

#### 1.3.2.1 Class I

Every operon of class I lanthipeptides includes two families of modification enzymes, LanB and LanC[53,54,55]. In Figure 3 A the overall composition of the class I modification enzymes and the prominent member nisin are shown.
The LanB protein family represents a dehydratase, which catalyses the dehydration of serine and threonine residues to didehydroalanine (Dha) and didehydrobutyrine (Dhb), respectively[54,55,56,57]. The family of LanB proteins comprise a low sequence identity of approximately 30 %[7,58,59]. However, all members have a molecular weight of approximately 110 kDa[58,59]. No high-resolution structure of a lanthipeptide dehydratase has been determined and only recently, the *in vitro* activity of such a dehydratase was demonstrated (see chapter 1.4.2)[57,60].
The cyclase LanC catalyses the formation of thioether bridges between dehydrated amino acid residues and thiol groups of dedicated cysteine residues[61]. Hereby, a (methyl)lanthionine ring is build. All members of the LanC family possess a sequence identity of approximately 20 - 30 % and a molecular weight of approximately 44 kDa[7,58,59]. A zinc finger consisting of a central zinc ion, two cysteines and one or two histidine residues build up the active site[61,62]. While LanB and LanC were shown to catalyze their specific reaction independently of the other modification enzyme[57,61,63], the presence of the other modification enzyme increases the efficiency drastically[55].
The transport of class I lanthipeptides across the Gram-positive cell wall is energized by an ABC transporter named LanT[64,65,66]. After transport, the serine protease LanP proteolytically cleaves the leader peptide from the active lanthipeptide[7,67]. LanP proteases are either anchored to the outer leaflet of the cytoplasmic membrane or located cytoplasmically[7]. The nisin protease NisP, for example, is membrane anchored via lipidation of a threonine residue[67,68]. In 2011, the *in vitro* activity of a class I cytoplasmic protease was demonstrated[69].

#### 1.3.2.2 Class II

In class II lanthipeptides, a single enzyme called LanM introduces the PTMs (Figure 3 B)[7,21]. This multifunctional protein combines the dehydration and the cyclization reaction[21,60]. The dehydration reaction is typically catalyzed by a N-terminal domain that possesses no homology to LanB. The C-terminal located cyclase domain comprises sequence identity to LanC proteins of approximately 25 %[21]. The molecular weight of the LanM proteins varies between 99 and 130 kDa[7]. The paradigm of class II lanthipeptides is lacticin 481 (Figure 3 B)[60]. The dedicated LanM is named LctM and previous studies demonstrated its *in vitro* activity and revealed important details

regarding the dehydration and cyclization mechanisms[60]. In a first step, specific serine and threonine residues are phosphorylated in an ATP and $Mg^{2+}$ dependent manner. In a second step, the phosphorylated serine and threonine residues are dehydrated[70]. The mechanism of class I and class II dehydratases is different, which is reflected by a low degree of sequence identity[60].
Like LanC cyclases, the cyclase domain of LctM acts zinc dependent. The substrate specificity is inverse to LanC cyclases and Dha and not Dhb are the preferred targets for the cyclization reaction[71,72]. For lacticin 481 and haloduracin II a directionality of the cyclization reaction from the N- to the C-terminus of the peptides was proven[60,73,74]. In contrast to that, the cyclization occurs bidirectionally for cinnamycin[75]. In general the dehydration and cyclization reactions are not strictly coupled to each other, both reactions were demonstrated to be independent from each other[21,36,70].
Beyond the modification machinery, the transport and processing machinery is also assembled as a multi-enzyme complex[7,21]. Compared to class I LanT ABC transporters, members of class II are approximately 100 amino acids longer[7]. These N-terminal elongation encodes C39 peptidase domains which represent papain-like protease domains[76]. Transport and proteolysis are not strictly coupled[77,78,79]. In case of lacticin 481, the proteolytic activity of the isolated protease domain was demonstrated[77]. For nukacin, however, proteolytic activity is coupled to the ABC transporter NukT[78,79]. Cells with hydrolysis-deficient NukT accumulated uncleaved nukacin inside the cytoplasm[78,79].
As exception, class II lanthipeptides contain hetero-dimeric lanthipeptides like haloduracin[74,80]. Two peptides assemble, after modification and transport, and only in combination they display the antimicrobial mode of action[74,80].

#### 1.3.2.3 Class III and IV

In 2004, the first class III lanthipeptide was discovered in *Streptococcus coelicolor* and is called SapB (Figure 3 C)[81]. The corresponding modification enzyme family is called LanKC[21,81]. The SapB corresponding modification enzyme SapC contains three distinct catalytical domains, a lyase, a kinase and a cyclase domain[81]. In contrast to LanC or LanM, the cyclase domain of class III modification enzymes does not contain the zinc finger motif[8].
Studies with the LanKC of the labyrinthopeptin modification enzyme (LabKC) from *Actinomadura namibiensis* were performed and the *in vitro* activity was demonstrated[82]. The dehydration reaction is catalyzed in a stepwise mode of action. The kinase domain phosphorylates the serine and threonine residues, the lyase domain preforms an elimination reaction and the dehydrated amino acid residues are released[82]. The lyase domain shares characteristic motifs with phosphothreonine

lyases of the OspF family[83]. The cyclization reaction is catalyzed by the C-terminal located cyclization domain[21,70]. In case of labyrinthopeptin the Michael addition, which is responsible for forming the thioether bridge, is performed twice resulting in a thioether bridge and a carbocyclic ring, introducing the PTM labionin (Figure 3 C)[82] . The mode of action of the cyclization reaction is described in detail in chapter 1.4.3.1. Until now, no protease domain was identified to be involved in the maturation of class III lanthipeptides[82,84,85] but a LanT-like ABC transporter is present in the operon suggested to catalyze the transport out of the cell[82,84,85].
In 2010, the lanthipeptide class IV was discovered[21,33]. The peptide venezuelin and the corresponding modification enzyme, member of the LanL family were identified in *Streptomyces venezuelae* (Figure 3 D)[86]. The dedicated venezuelin modification enzyme VenL contains three distinct domains, similar to class III. A OspF-like lyase domain, a kinase domain followed by a cyclization domain[83]. The difference to class III is due to the cyclization domain, which contains the class I and class II-like zinc finger motif[86].

### 1.3.3 Function of the leader peptide

The leader peptides of lanthipeptides are crucial for efficient modification[87,88,89], transport[64,90] and proteolytical maturation[60,91]. For two lanthipeptide classes it was demonstrated that the leader peptide is crucial for any interaction with the dedicated modification enzymes (e.g. class IV - labyrinthopeptin[85], class II - cinnamycin and haloduracin[73,75]). In class II (e.g. lacticin 481[60]) the modification machinery is existent in an equilibrium between an inactive and an active state[89]. Upon binding of the leader peptide the equilibrium is shifted towards the active state, starting to install the PTMs[89].
In each class, the leader peptide contains defined regions that direct the precursor peptides to the modification machineries[33]. These regions are highly conserved and mutations affect the modification efficiency negatively[33,92]. In non-conserved regions, mutations show little or no impact[33,67,92]. The composition of such regions is arbitrary and all amino acids, beside cysteine residues, are found in leader peptides[33]. Serine and threonine residues are frequently found in leader peptides, however, these residues are never post-translational modified[33].

In class I lanthipeptides, the leader peptide is specifically recognized by LanB and LanC via four highly conserved amino acid residues (called -FNLD- box, Figure 4)[87,88,92]. For nisin, it was shown that the exchange of the complete -FNLD- box abolished modification, which demonstrates the importance of the -FNLD- box for the modification machinery[92].
For class II lanthipeptides the conserved leader peptide binding motif of LanM


Figure 4: **Leader peptide alignments of class I - IV**. The alignments shown are created with ClustalW2[93] based on the amino acid sequence taken from the UniProtKB database. The UniProtKB entry number is shown in parenthesis. The asterisk (*) represents single fully conserved amino acids. Conserved residues are highlighted by an (:) similarity by a (.). Based on Plat *et al.* 2013[33].

comprises the amino acid residues -(L/V/I)(E/Q)EV- (Figure 4)[92]. Previous studies with haloduracin and cinnamycin demonstrated a strict correlation between the presence of the leader peptide and the modification process[92,94,95]. For lacticin 481, however, the leader peptide is not essential for partially formation of PTMs[92,94,95]. The three domain harboring class III LanKC enzyme recognizes the -(I/L)L(E/D)LQ- box (Figure 4) within the leader peptide of class III lanthipeptides[33]. Regarding class IV lanthipeptides, the binding motif of LanL is currently unknown[33].

An additional parameter which influences modification efficiency is the distance between the leader peptide and the target residues of the core peptide, more precisely the number of amino acids[85,92,96]. For class I lanthipeptides the leader peptide consists of 20 - 30 amino acid residues[33,92]. Truncations of the leader peptide caused a shift of the PTMs to the C-terminus of the core peptide and modification of the N-terminal located targets were skipped because they were too closely positioned to the leader peptide[92]. Apparently, the leader peptide represents a spacer for the modification

machinery that directs the core peptide to the active center, as shown for nisin (class I)[92], lacticin 481 (class II)[96] and labyrinthopeptin (class III)[85].
Remarkably, non-lanthipeptides are modified by the nisin modification machinery if they are fused C-terminal to the nisin leader peptide[35,50,51,97]. Moreover, they are transported to the extracellular space via the ABC transporter NisT[35,50,51,97]. This highlights the importance of the leader peptide within the modification as well as the secretion process.
The C-terminus of the leader peptide comprises a consensus sequence which represents the recognition site for the dedicated protease[92]. In class I, the sequence PR/Q[92,94,95] is recognized by LanP and in class II the sequence GG or GA is responsible for cleavage by the multi-enzyme complex LanTP[76,77]. In class II leader peptides a secondary structure is often found, which forms an $\alpha$-helix and supports the interaction between the leader peptide and the protease[85,96,98].

### 1.3.4 Post-translational modifications (PTMs)

PTMs are important for the unique properties of RiPPs[36]. Until now, 15 PTMs were identified including the frequently used dehydrated amino acids didehydroalanine, didehydrobutyrine and the (methyl)lanthionine rings (Figure 5)[8,21,36]. The dehydration and cyclization reaction represents the most frequently used PTM and is described in more detail in chapter 1.4.2.1 and 1.4.3.1, respectively.

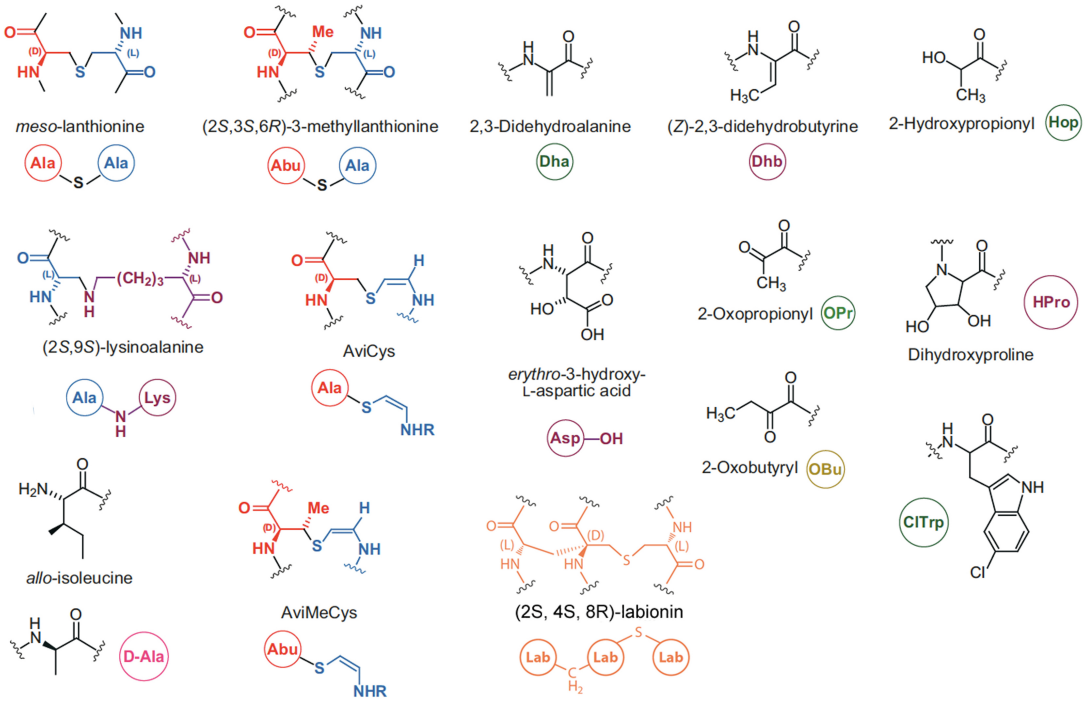


Figure 5: **Overview of PTMs found in lanthipeptides of different classes**, the corresponding examples are shown in Figure 3 and in Figure 6. Based on Willey *et al.* 2007[8].

In Figure 5[8,21] an overview of identified PTMs is shown. Until now, not every function for all discovered PTMs is known, but they are mainly responsible for stability, especially against protease digestion[8,21,36]. Among these, the C- and N-termini are mainly protected against carboxypeptidase and aminopeptidase, respectively[99]. In mersacidin[100] (Figure 6) the AviCys and AviMeCys (Figure 5) are installed via oxidative decarboxylases (LanL). They oxidize the cysteine at the C-terminus to a thioaldehyd, which afterwards is decarboxylated[99,101]. Another two-step mechanism converts the L-Ser in D-Ala (Figure 5) via dehydration to Dha and hydrogenation by a dehydrogenase to D-Ala, e.g in lacticin 3147 and lacticin S[102].

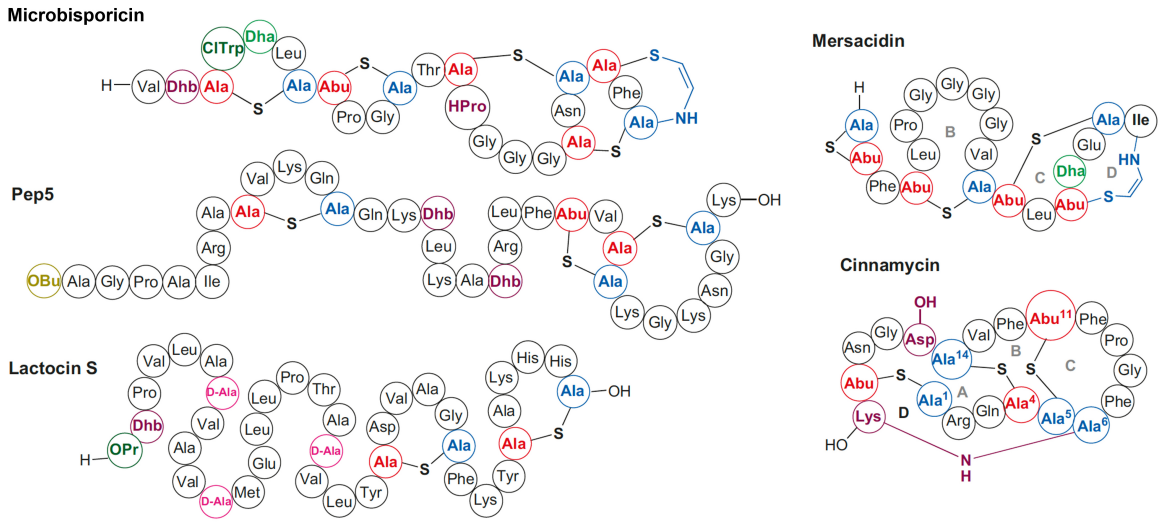


Figure 6: **Selected examples of lanthipeptides with uncommon PTMs**. Microbisporicin is produced by *Microbispora sp.* and harbors the recently obtained chlorinated tryptophane residues (ClTrp) and the hydroxylated proline residue (HPro) as PTMs. *Staphylococcus epidermidis 5* produces the lanthipeptide Pep5[103] and modifies its N-terminus with a ketoamide (OBu). This PTM is also found in lacticin S of *Lactobacillus sake* that also incorporates D-Ala into the lanthipeptide[104]. A hydroxylated aspartate (Asp-OH) is found in cinnamycin from *Streptomyces cinnamoneus cinnamoneus* DSM 40005[105]. Mersacidin produced by *Bacillus subtilis* HIL Y-85,54728 harbors the MeAviCys[100]. Based on Willey *et al.* 2007[8].

The N-terminus is capped with $\alpha$-ketoamides to install the 2-oxobutyryl (OBu, Figure 5) in Pep5[106] and $\alpha$-hydroxyamides to install the 2-oxopropionyl (OPr, Figure 5) in lacticin S[102] (Figure 6). Furthermore, in cinnamycin nine PTMs are installed (Figure 6), within the lysinoalanine and $\beta$-hydroxylated aspartate (Asp-OH, Figure 5)[75]. Very recently two other PTMs were identified in microbisporicin (Figure 6), which imply a tryptophan chlorination (ClTrp, Figure 5) and proline hydroxylation (HPro, Figure 5)[43,107].

## 1.4 The Nisin System

The class I lanthipeptide nisin is produced by *L. lactis*[108]. All eleven genes for synthesis, modification and transport as well as for self-immunity and regulation are located on the nisin operon in the order *nisABTCIPRKFEG* (Figure 7)[109].

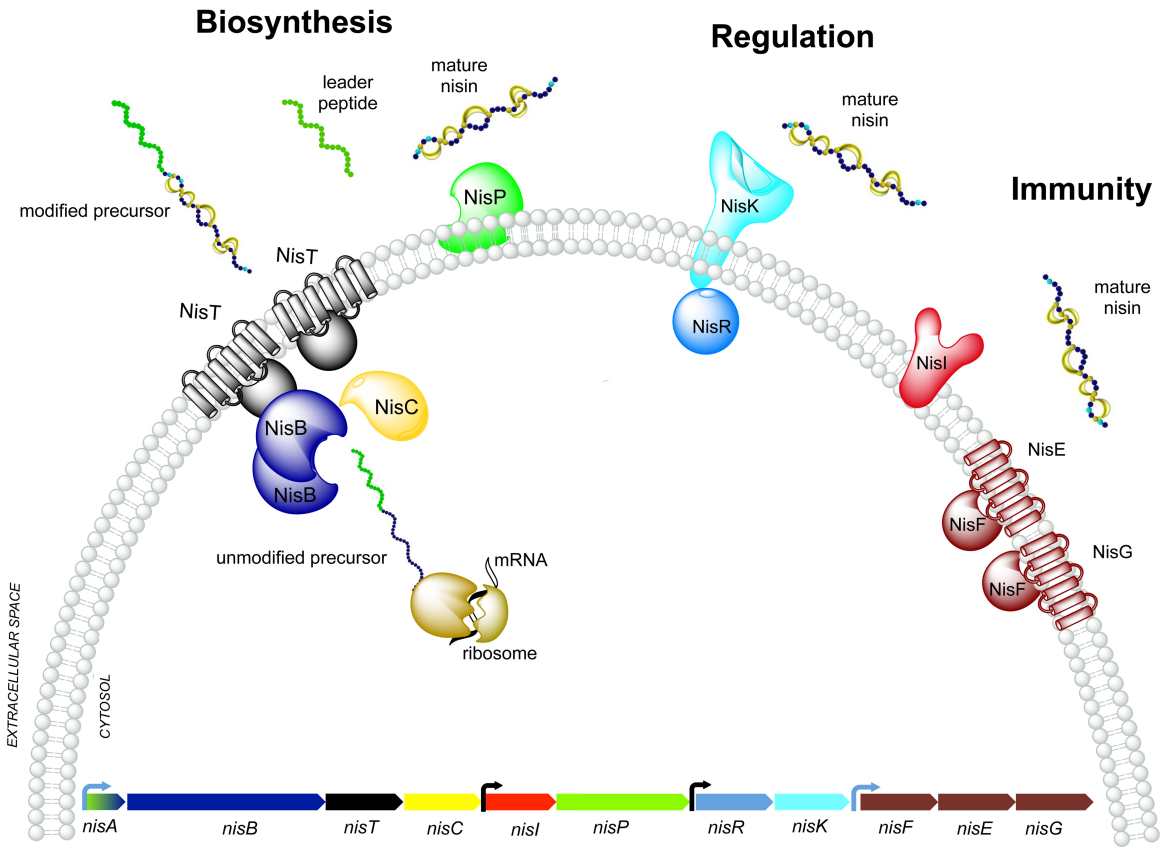


Figure 7: **Overview of the nisin operon consisting of biosynthesis, immunity and regulation**. On the left side the proteins taking part at the biosynthesis of nisin are shown comprising NisB (blue), NisC (yellow), NisT (black) and NisP (green). The center describes the regulatory proteins NisR (light blue) and NisK (turquoise). In the right part the immunity proteins NisI (red) NisFEG (dark red) are shown. The promotor *nisA* and *nisF* is shown with blue arrows and the constitutive promotors *nisI* and *nisR* are shown in black. Created with CS BioDrawUltra 12.0.

Nisin is ribosomally synthesized as a precursor peptide consisting of 57 amino acids[110]. This precursor peptide consists of the N-terminal leader peptide (23 amino acid residues) and the C-terminal core peptide (34 amino acid residues) (Figure 8)[111]. All PTMs are introduced exclusively in the core peptide[55]. The modification machinery consists of two enzymes, the dehydratase NisB and the cyclase NisC[55]. The dehydratase NisB is responsible for the dehydration of serine and threonine residues[55,109,112]. The cyclization reaction, catalyzed by NisC, generates one lanthionine and four methyllanthionine rings, which are responsible for the

antimicrobial activities of nisin[7]. In addition to these PTMs, the transport[65] out of the producer cell and the proteolytic cleavage[67] of the leader peptide is essential for the antimicrobial activity. For secretion, the ABC transporter NisT specifically recognizes the leader peptide and translocates the peptide across the cytoplasmic membrane[64,65]. Here, the protease NisP cleaves the precursor peptide C-terminal to the conserved amino acids PR of the leader peptide and mature nisin is released to the surrounding[67,109]. An overview of the steps during maturation of nisin is given in Figure 8.

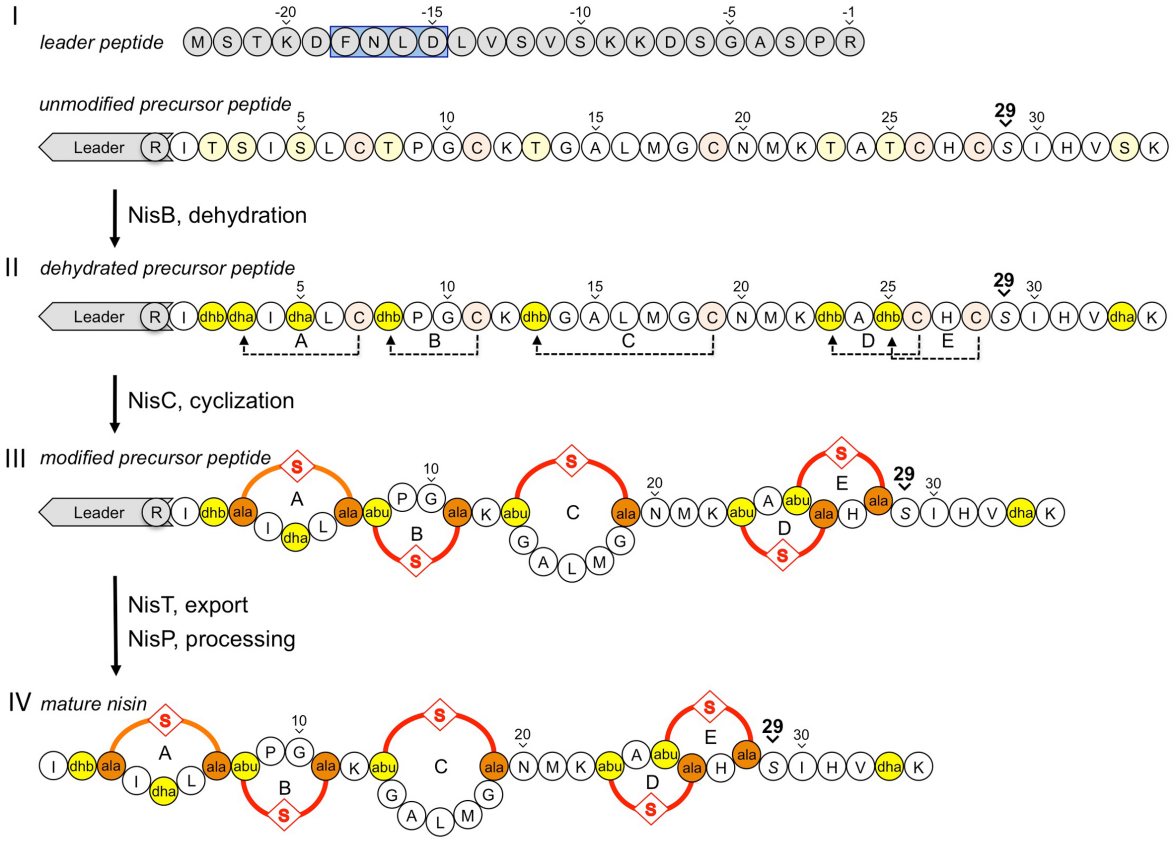


Figure 8: **PTMs of nisin**. (I) The leader peptide (in grey) directs the precursor peptide to the nisin modification and transport machinery. The conserved -FNLD- box of the leader peptide is highlighted in blue. (II) Serine and threonine residues (highlighted in yellow) are converted by the dehydratase NisB into didehydroalanines (dha) and didehydrobutyrines (dhb), respectively. (III) Dehydrated residues are coupled by NisC to cysteine residues (highlighted in orange) forming one lanthionine (A, orange) and four methyllanthionine rings (B-E, red). (IV) The modified precursor peptide is exported by the ABC-type transporter NisT and processed by the extracellular protease NisP that cleaves off the leader peptide to liberate mature nisin. The serine at position 29 does not undergo any dehydration reaction. Taken from chapter 3.4.

The antimicrobial effect of nisin is directed against Gram-positive bacteria and here specifically against a component of the cytoplasmic membrane, lipid II[113,114,115,116].

Lipid II is a precursor molecule for cell wall synthesis[113]. Since lipid II is also a member of the cytoplasmic membrane of the producer cells, a self-immunity mechanism has evolved to protect the producer cell against nisin[117]. Previous studies demonstrated two different immunity mechanisms that are encoded by the nisin operon[53]. The lipoprotein NisI[118,119] and a complex of the proteins NisFEG perform the immunity reactions[117,120]. Both immunity mechanisms are described in more detail in chapter 3.2.
The genes *nisI* and *nisRK* are constitutively expressed[121,122]. NisI ensures a basal immunity and NisRK are responsible for quorum sensing regulation response against nisin[121,122]. The initial expression of the nisin gene *nisA* is not identified but previous studies revealed a nisin expression depending on the carbon sources galactose and lactose[123]. When nisin is present in the extracellular medium of *L. lactis* an auto-regulation mechanism is induced[121]. Here, nisin activates the two-component system consisting of NisK and NisR[121]. NisK is a histidine kinase anchored to the cytoplasmic membrane, which binds mature nisin and activates a signal transduction cascade[67,110]. Following the binding event NisK is auto-phosphorylated at a histidine residue[67,110]. The high-energy potential of the phosphor group of NisK is transferred to an aspartate residue of NisR, the transcriptional activator[122]. NisR binds specifically to the *nisA* promotor sequence on the nisin operon. This binding event stimulates expression of the nisin operon[122]. The first two rings A and B of nisin were shown to be essential for this regulation mechanism, the third ring C is important for an efficient induction of expression while rings D and E do not influence the expression at all[121].

### 1.4.1 NisA - the nisin mature peptide

The three-dimensional solution structure of nisin was solved via NMR spectroscopy in 1991[124] (Figure 9). Based on this structure, nisin is subdivided into three regions: First: The N-terminal part consists of the rings A-C. Second: A flexible part called ‘hinge region‘. Third: The C-terminal region comprises the intertwined rings D and E followed by six amino acids. Nisin is an amphipathic molecule with a hydrophobic N-terminal and a hydrophilic C-terminal part[124].
The target molecule of nisin, lipid II, is a precursor molecule of cell wall synthesis, which is anchored to the cytoplasmic membrane by an undecaprenyl residue (Figure 10)[116]. The anchor is connected via an ester bridge to a diphosphate moiety that carries a N-acetylmuramic acid as well as a N-acteylglucosamine molecule[8,113,125]. The attachment of a pentapeptide (for *S. aureus* L-Ala-D-Glu-L-Lys-D-Ala-D-Ala[125]) completes the lipid II molecule[8,113,125]. Catalyzed by a transglycosylation reaction, more N-acetylmuramic acids molecules are fused to lipid II. These linear polymers are cross-linked by a transpeptidation reaction to form the mature peptide glycane of

Gram-positive bacteria[8,113,125].

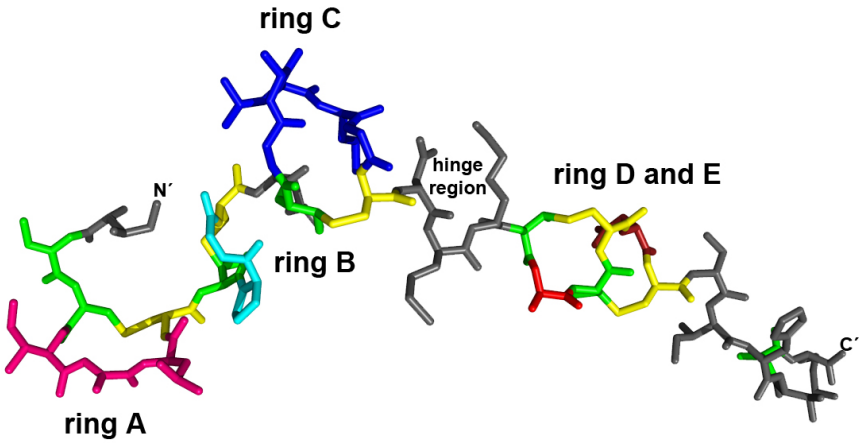


Figure 9: **The NMR structure of nisin**. Green: dehydrated amino acid residues. Yellow: cysteine residues. Pink: lanthionine ring A. Turquoise: methyllanthionine ring B. Blue: methyllanthionine ring C. Red: methyllanthionine rings D and E. Figure is based on pdb entry 1WCO and is created with PyMol 1.5.0.4.

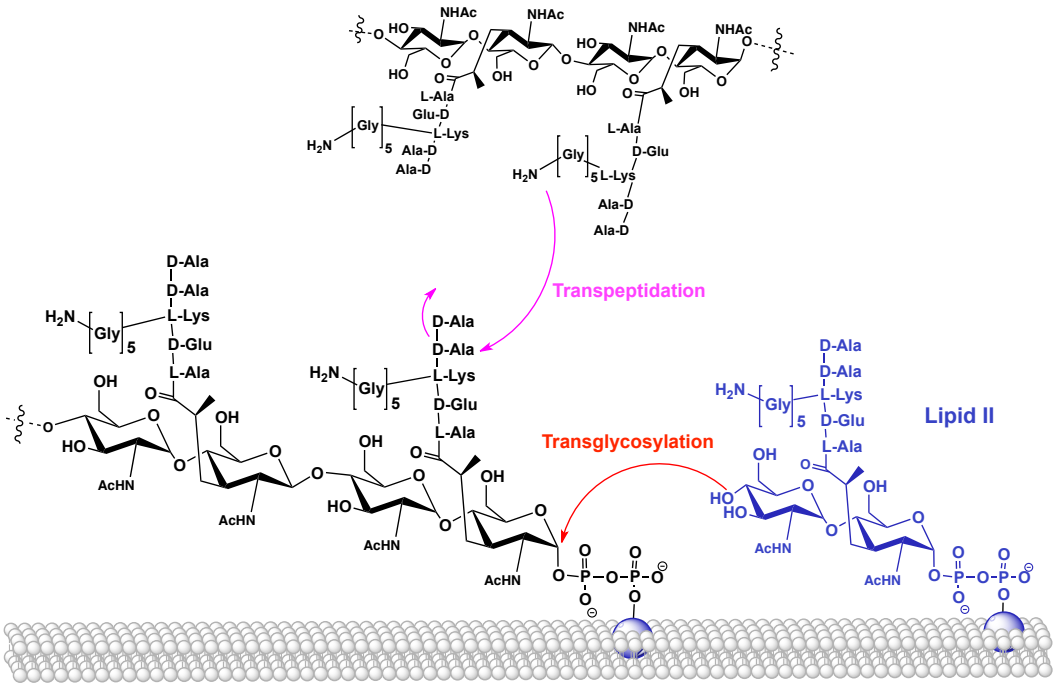


Figure 10: **Lipid II dependent cell wall synthesis**. A lipid II monomer (blue) is fused to other peptidoglycan monomers via $\beta$ 1,4 glycosidic bonds (transglycosylation). The cross-connection between the linear, polymeric peptidoglycan is obtained by the transpeptidase, which catalyzes the connection of D-Ala to L-Lys of a second polymer (transpeptidation). Created with CS Biodraw Ultra and based on Willey *et al.* 2007[8] and Schneider *et al.* 2010[125].

Nisin binds with the first two (methyl)lanthionine rings (A and B) to the diphosphate moiety of lipid II (Figure 11 A)[114,115,126] and prevents the transglycosylation reaction thereby the cell wall synthesis is inhibited (Figure 11 A)[116]. When the concentration of nisin-lipid II complexes increases beyond a certain threshold an intermediate complex is formed (Figure 11 B). Thereupon, the insertion of the C-terminus of nisin into the cytoplasmic membrane is induced and a polymerization reaction occurs resulting in a supramolecular complex consisting of eight nisin and four lipid II molecules[114]. This complex assembly induces a pore with a diameter of 2 - 2.5 nm (Figure 11 C). This causes a collapse of the membrane potential and leads to cell death[114]. The threshold concentration for this reaction is in the low nM range[127].

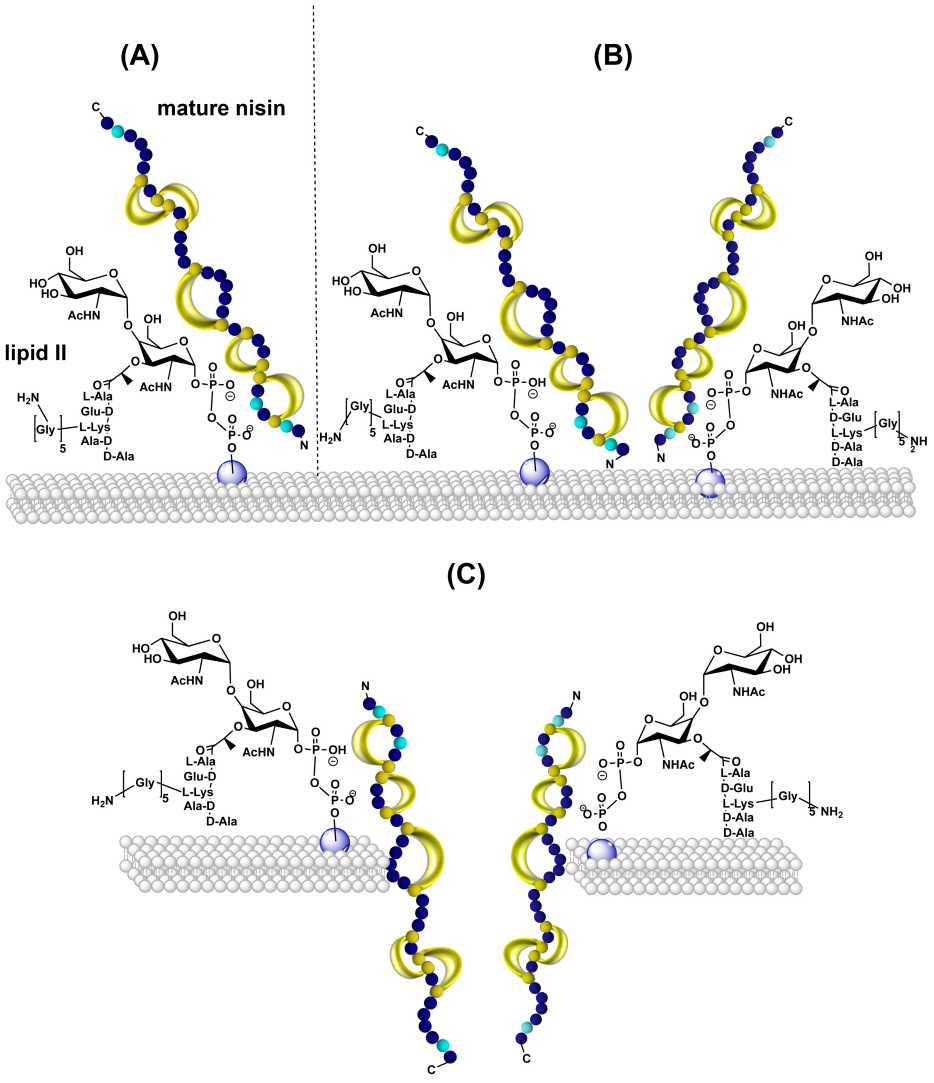


Figure 11: **Mode of action of nisin**. (A) Nisin binds with ring A and B to the diphosphate moiety of lipid II. (B) The increasing concentration of nisin - lipid II complexes induces an intermediate complex. (C) The C-terminus inserts into the cytoplasmic membrane and nisin and lipid II build a supramolecular complex with a stoichiometry of eight nisin molecules to four lipid II molecules. This complex forms a pore that leads to cell death. Created with CS Biodraw Ultra 12.0.

### 1.4.2 NisB - the nisin dehydratase

The enzyme NisB consists of 993 amino acids and comprises a theoretical molecular weight of 117.5 kDa[7,109,128]. In the primary sequence of NisB a region was identified, which is putatively attached to the cytoplasmic membrane[128]. Previous experimental studies to elucidate the localization of NisB demonstrated that NisB is attached to the cytosolic membrane in the nisin producer strain *L. lactis* 6F3[128], which points towards a membrane associated modification complex[58]. The activity of NisB was demonstrated by deletion of *nisB*, thereby the production of functional, antimicrobial nisin was abolished[109], caused by the absence of the dehydrated amino acid residues within the core peptide[55]. Therefore the function of NisB was shown to dehydrate all serine and threonine residues in the core peptide of the unmodified precursor peptide with one exception, the serine residue at position 29[129]. The reason for the missing dehydration of this specific serine residue was traced back to lanthionine rings D and E, which cause a steric hindrance, therefore the serine residue at position 29 is not accessible for NisB[129]. Additionally it was observed that NisB dehydrates the serine residue at position 33 to a percentage of 90 % in a nisin producer strain[112]. When *nisB* is expressed via a plasmid the NisB concentration is increased and thereby the dehydrated efficiency is enhanced, resulting in 100% dehydration efficiency for the serine residue at position 33[112].

In 2011, *in vitro* data of NisB showed that NisB assembles as a homodimer (see chapter 3.4)[88]. This dimer specifically recognizes the -FNLD- box within the nisin leader peptide *in vitro*[88]. Furthermore, the interaction between NisB and the nisin precursor peptide depends on the maturation state of the core peptide[88]. As shown by surface plasmon resonance (SPR), the affinities of NisB for the precursor peptide increases in the order modified precursor peptide ($K_D = 10.5 \pm 1.7\ \mu M$) << unmodified precursor peptide ($K_D = 1.05 \pm 0.25\ \mu M$) < dehydrated precursor peptide ($K_D = 0.31 \pm 0.07\ \mu M$)[88]. These results suggest that NisB is able to distinguish between the different maturation states of the core peptide by interacting with the core peptide as well as with the leader peptide (chapter 3.4)[88]. The binding of the -FNLD- box of the leader peptide alone is sufficient for the dehydration reaction[35,50,97]. This was demonstrated by chimeric peptides of the nisin leader peptide fused N-terminal to therapeutic peptides as angiotensin. Here, angiotensin was dehydrated by NisB *in vivo*[35,50,97].

Despite the absence of a high-resolution structure of NisB, *in vivo* analysis in combination with sequence alignments allowed the identification of distinct domains within the protein[57,130]. In the C-terminal part of NisB, the membrane-associated region is located between the residues 838 and 851[57,128,130]. Truncational studies showed that this domain is involved in binding to the nisin precursor peptide[130]. This

truncation caused a loss of the dehydration reaction suggesting an important function of the C-terminus for activity [57,130].

The N-terminal part of NisB is highly conserved among LanB proteins [56,57,128,130]. Deletion of the residues at position 77 - 79 (Ile-Tyr-Lys) or the mutation of the tyrosine at position 80 to phenylalanine abolished the activity of NisB [130].

Recently, *in vitro* studies proposed a mechanism for the dehydration that involves a glutamylation reaction of serine or threonine residues prior to elimination of the glutamate resulting in dehydrated amino acid residues (chapter 1.4.2.1) [57]. In this study, the C-terminal part, mentioned above, was not responsible for the glutamylation of the target residues, but the elimination reaction was impaired [57]. Moreover, essential amino acid residues were identified (His961, Arg786 and Arg826) [57]. This suggests an impact of this domain on the elimination reaction. Due to these results, it was hypothesized that NisB might contain a C-terminal lyase domain as shown for class III and IV dehydratase domains. Whether the N-terminal part of NisB catalyzes the glutamylation reaction needs to be clarified [57].

### 1.4.2.1 Dehydration

Recently, the dehydration reaction for the class I dehydratase NisB was demonstrated *in vitro* [57]. According to this study, it is proposed that NisB harbors two domains, a glutamylation and an elimination domain [57].

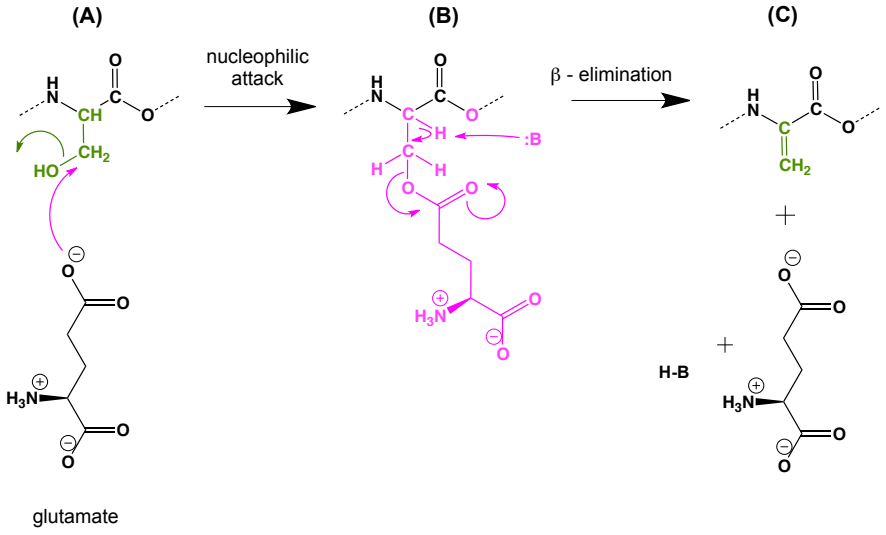


Figure 12: **Proposed dehydration reaction for nisin.** (A) The carboxylic acid group of glutamate attacks the $\beta$-carbon atom of the serine residues nucleophilically and a glutamate ester is formed (B). A base B: abstracts the $\alpha$-proton as first step of the elimination reaction. (C) After release of the glutamate, a dehydrated serine is produced. Based on Ke *et al.* 2011 [131] and Garg *et al.* 2013 [57] and created with CS BioDrawUltra 12.0.

Mechanistically, the reaction starts with an activation of the glutamate by ATP or by a direct attack of the carboxylic acid group of the glutamate. This carboxylic acid group attacks (Figure 12 A) the side chain of the corresponding serine or threonine, resulting in a glutamate ester (Figure 12 B). Now, the $\alpha$-hydrogen of the target residue is deprotonated. A $\beta$-elimination releases the glutamate and an $\alpha$, $\beta$-unsaturated amino acid Dha or DhB is formed (Figure 12 C and Figure 5). The base for the deprotonation of the $\alpha$-hydrogen still needs to be identified. For class III and class IV lyases, a conserved lysine was shown to abstract the proton [131]. For NisB two arginine residues (Arg796 and Arg826) were identified, which are essential for the elimination [57]. Glutamylation reactions are common, for example during the formation of microtubule [132,133]. In contrast to the proposed NisB glutamylation mechanism, the glutamates are linked between the amino group and the C$\gamma$ of the next residue [132,133]. Threonine residues are more preferred targets for dehydration than serine residues [35,97]. Besides this preference, neighboring residues frequently define the target sites and hydrophobic, non-aromatic acids are often in close proximity to dehydrated residues [35]. The basic principle for class II to class IV dehydration reactions is proposed to be performed by a two-step mechanism. A serine/threonine kinase domain phosphorylates the target residues and a lyase domain performs an E1cB-like $\beta$-elimination reaction (Figure 13) [81,82,83,86].

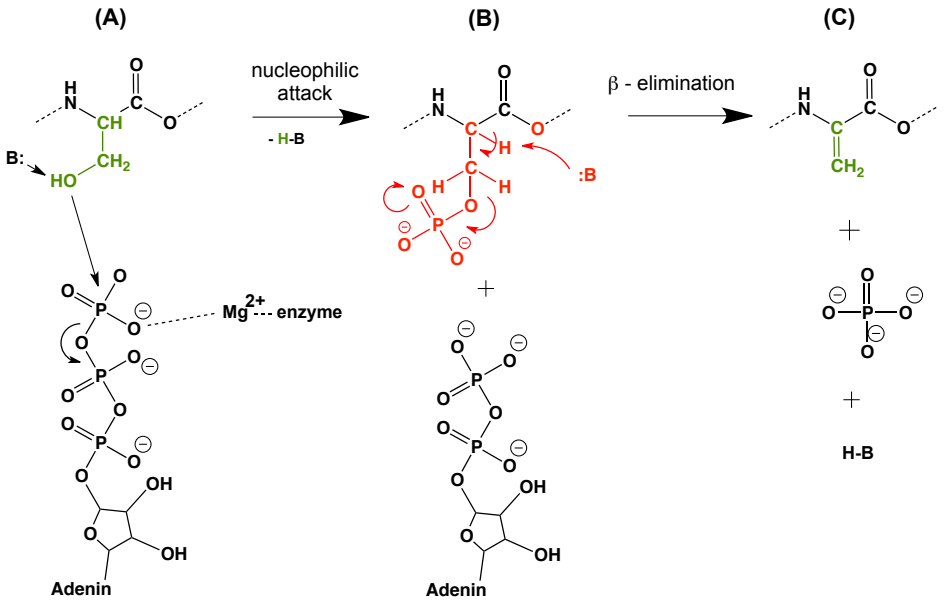


Figure 13: **Proposed dehydration reaction catalyzed in class II-IV lanthipeptides based on serine/threonine kinase and phosphoserine/threonine lyase domains**. (A) The first step is the ATP and $Mg^{2+}$ dependent phosphorylation of the serine residue. (B) Afterwards the so called eliminylation reaction is performed starting with deprotonating the $\alpha$-proton by lysine 136, here represented by B:. After releasing the phosphate the dehydrated serine is produced (C). Based on Ke *et al.* 2011 [131] and created with CS BioDrawUltra 12.0.

Currently, only preliminary data are available for kinase or lyase domains of lanthipeptides to elucidate the molecular mechanisms similar enzymes classes were investigated[60,70,83,86,131,134]. The mode of action of homolog serine/threonine kinases is well understood, for example for the protein kinase A (PKA)[135,136,137]. Additionally, the mechanism of phosphothreonine lyases belonging to the OspF family[83,86,134], which is homolog to class III and IV modification enzymes (SpvC[131]), was demonstrated[83,86,134]. Based on the general mode of action of serine/threonine kinases and homolog lyase domains, a dehydration mechanism for class II - IV lanthipeptides was proposed (Figure 13).
Target residues are activated by a serine/threonine kinase and not by a glutamylation[21]. LctM hereby transfers the $\gamma$-phosphate of ATP to a serine or threonine residue in a $Mg^{2+}$ dependent manner[60,70] similar to phosphoserine phosphatase (Figure 13 A and B)[138,139]. The phosphorylation decreases the activation energy for the following elimination reaction[60,70].
The $\beta$-elimination follows an E1cB-like mechanism initiated by the abstraction of the $\alpha$-proton by a lysine residue, demonstrated for SpvC[131,140]. The intermediate state with a negative charge at the C$\alpha$ atom subsequently forms an enolate, which is stabilized by an oxyanion hole of the residues Tyr185 and Lys104. The final rate-limiting step is the cleavage of the C$\beta$ - $OPO_3{}^{2-}$ bond (Figure 13 C). This PTM is called eliminylation[131,140].

### 1.4.3 NisC - the nisin cyclase

NisC (418 amino acids with a theoretical molecular weight of 48 kDa) catalyzes the second maturation step of nisin (Figure 8 II) and deletion of *nisC* results in the production of a dehydrated, non-active nisin molecule[55]. For the antimicrobial activity of nisin the presence of at least three of the five (methyl)lanthionine rings is essential. Therefore, the missing biological activity is derived from the inactivity of NisC[141]. NisC was shown to be at least partially associated at the cytoplasmic membrane of nisin producing cells[58].
In 2006, the X-ray structure of NisC was reported and the *in vitro* activity was demonstrated[61]. The crystal structure is shown in Figure 14 A. NisC consists of two domains, the $\alpha$-toroid core and a SH2-like domain. The $\alpha$-toroid core comprises 14 $\alpha$-helices and is characterized by a two-layer bowl structure. The three highly conserved residues Cys284, Cys330 and His331 coordinate a zinc ion in the central cavity. In combination with a water molecule, this structural feature represents a zinc finger with a tetrahedral coordination of the $Zn^{2+}$ ion[61] (Figure 14 B). SH2-like domains typically interact with partner proteins or peptides, but the function within

NisC is currently unknown. The mechanism of the cyclization reaction is described in the following chapter 1.4.3.1. *In vivo* studies highlighted the importance of the -FNLD- box within the nisin leader peptide for interacting with NisC[92,142]. Here, the exchange by four alanines abolished any post-translational modification[92]. Moreover, four additional amino acids in the leader peptide (-LVSV- at position -14 to -11) were shown to be involved in the binding event with NisC[142]. However, no *in vitro* data describing the interaction of NisC with the precursor peptide are available.

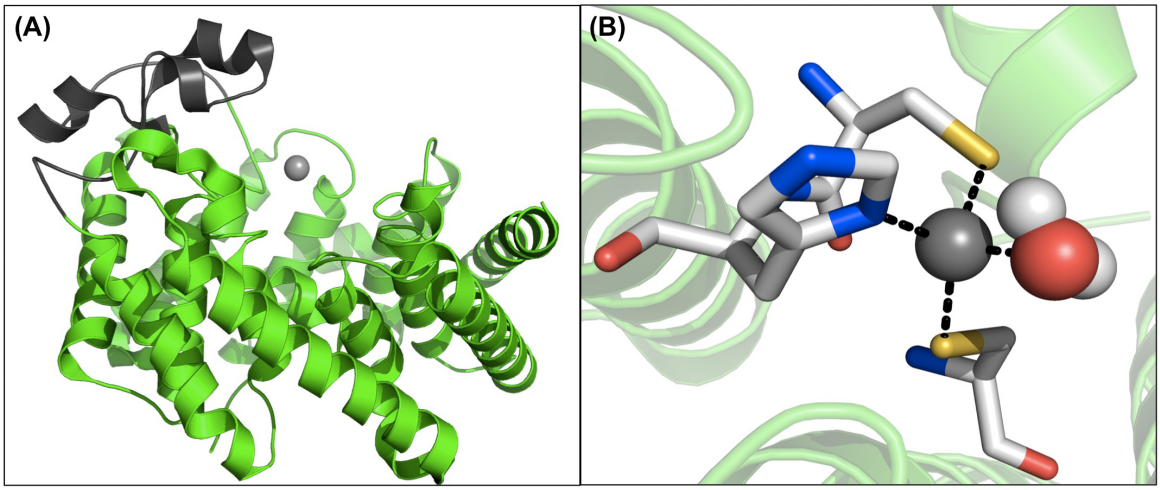


Figure 14: **X-ray structure of NisC**. (A) Overall X-ray structure of NisC. The $\alpha$-toroid core is shown in green and the SH2-like extension is colored black. (B) Zoom in the active site. The $Zn^{2+}$ ion is colored in dark grey, surrounded by the conserved residues Cys284, Cys330 and His331 colored in light grey. The coordinated water molecule is shown in red (oxygen) and white (hydrogen). Figure is based on the pdb entry 2G02 and created with PyMol 1.5.0.4.

### 1.4.3.1 Cyclization

The determination of the X-ray structure of the nisin cyclase NisC in combination with *in vitro* activity studies allowed the identification of essential residues for the cyclization reaction[61,63]. For class I lanthipeptides the involvement of a zinc ion in cyclization was demonstrated[62]. In NisC a zinc finger-like motif is also present and the zinc ion is coordinated by two cysteine and one histidine residue and a water molecule (Figure 14 B). This zinc ion coordinates the thiol groups of cysteine residues, which lowers their $pK_a$ value and shifts the equilibrium towards the thiolate (Figure 15). This thiolate represents the active species in the reaction and comprises a much higher rate constant for the addition to the $\beta$-carbon atom of the $\alpha$, $\beta$-unsaturated serine or threonine residue. An enolate intermediate abstracts a proton at the $\alpha$-carbon atom of the dehydrated residues. This reaction is highly stereospecific and introduces a D-configuration at the newly formed stereogenic center[61]. Whether a water molecule

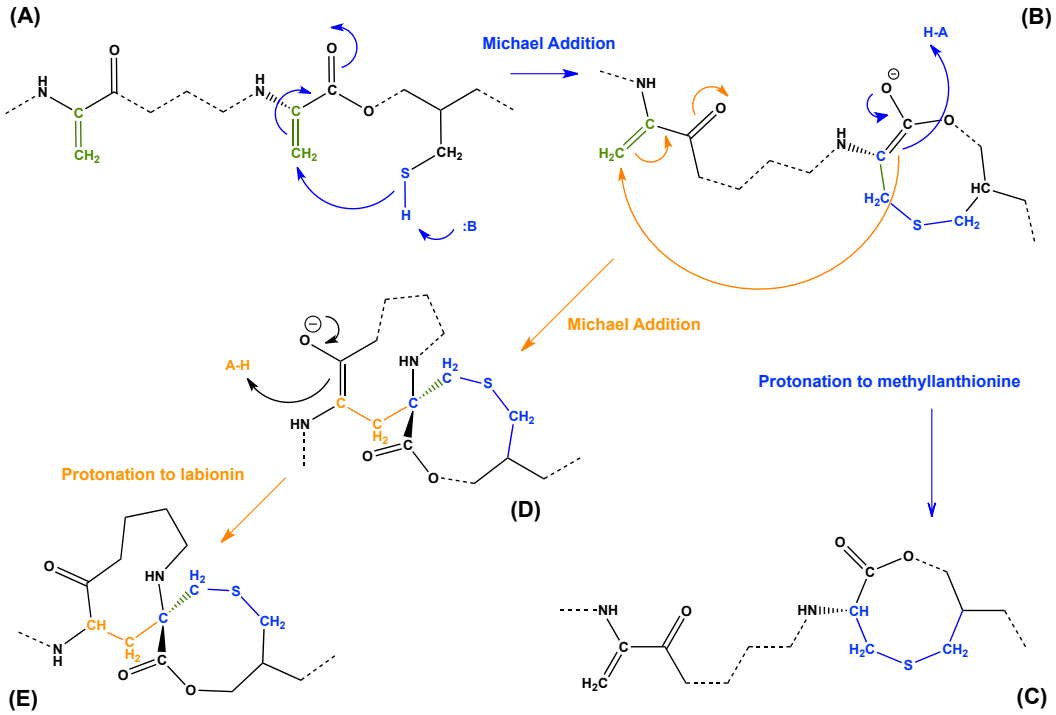


Figure 15: **Proposed cyclization reactions in class I (shown in blue) and class III (shown in orange) lanthipeptides**. (A) A thiolate is formed by deprotonating a thiol group of a cysteine residue. Either via the base B: or for NisC, the deprotonation is suggested to be catalyzed by the $Zn^{2+}$ coordinated in the zinc finger. This thiolate subsequently performs a Michael addition to the C$\beta$ of the Dha. For lanthionine rings the intermediate (B) is protonated by an acid. For NisC the His212 might act as a proton donor (C). For labionin, the intermediate state (B) is not protonated. Instead the enolate attacks the C$\beta$ of another Dha via a second Michael addition (D). Now a protonation in analogy to (C) occurs and the carbocycle of labionin is formed. Based on Mueller *et al.* 2010[82] and Li *et al.* 2006[61]. Created with CS BioDrawUltra 12.0.

or the histidine residue at position 212 of NisC acts as base is currently unknown (Figure 15) and no additional cofactors are known to be involved in the cyclization reaction of class I LanC proteins[61,63].

The class II LanM family consists of two domains: a dehydration and a cyclase domain[60]. The cyclase domain comprise a low degree of sequence identity to LanC enzymes from class I[7,8,21]. Sequence alignments and mutational studies revealed the presence of two cysteine and one histidine residue that might form the zinc finger to coordinate the catalytical zinc ion, comparable to NisC[56]. Here, the mutational exchange of these residues abolished the *in vitro* activity of LctM. Comparable results were achieved by the addition of EDTA during the cyclization reaction of LctM demonstrating the crucial catalytic function of $Zn^{2+}$ ions[60]. Based on theses data, the reaction mechanism of class I and class II cyclases was suggested to be homolog to each other (Figure 15)[60].

Maybe this holds true for the very recently discovered class IV lanthipeptides as well, which contain a C-terminal located LanC-like cyclase domain[21,33]. The characteristic

zinc containing cyclase domain of class I and II lanthipeptides is not existing in class III lanthipeptide processing proteins[82]. Nevertheless the proposed mechanism for the Michael addition is believed to be analog to class I lanthipeptides (Figure 15)[82]. The thiolate undergoes a Michael addition at the $\beta$-carbon atom of a dehydrated serine or threonine residue. The resulting enolate is not protonated, as in class I reactions, furthermore it undergoes a second Michael addition with a nucleophilic attack on another $\alpha$, $\beta$-unsaturated amino acid. This second Michael addition does not result in a sulfur atom containing bridge, it creates a carbocyclic structure instead, the whole PTM is named labionin[82].

### 1.4.4 NisT - the nisin translocase

The ABC transporter NisT catalyzes the secretion of the modified precursor peptide across the cytoplasmic membrane to the exterior[65]. In absence of NisT the nisin precursor peptide accumulates in the cytoplasm[143]. ABC transporters typically consist of four domains: two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs)[144]. As a so called half-size ABC transporter, *nisT* encodes one TMD and one NBD and the functional unit is suggested to be homodimeric[58]. In general, TMDs are thought to build a pore across the membrane and create substrate specificity. NBDs bind and hydrolyze ATP, which energizes the whole transport[145]. As shown for NisB and NisC, NisT interacts with the leader peptide of the nisin precursor peptide[91]. NisT is able to secret all three different maturation states of nisin[64] and furthermore also non-lantibiotic peptides which are fused to the nisin leader peptide[97].

### 1.4.5 NisP - the nisin protease

The proteolytic cleavage of the leader peptide by NisP represents the final maturation step of nisin (Figure 8 IV)[67]. NisP belongs to the subtilisin-like serine protease (Pfam entry: Peptidase S8)[67,146] that are characterized by a catalytic triad comprising an aspartate or glutamate, a histidine and a serine residue[147]. For NisP, the identified residues are Asp64, His111 and Ser317[67,94]. A fourth residue, Asn212 builds the so-called oxyanion hole that stabilizes a transition state during cleavage[67,94]. NisP (682 amino acids) is produced as a proprotein with a N-terminal signal sequence (amino acids 1-195). This signal directs NisP to the Sec-dependent secretion system and NisP is transported across the cytoplasmic membrane[67]. Afterwards, the signal sequence is cleaved off C-terminal to the motif -VSLR- (amino acid residues 192 - 195), which was found to be auto-catalytically performed. Thereby, the proprotein is

converted to the functional protease (amino acids 196 - 682) that results in theoretical molecular weight of 54 kDa[67,94]. The C-terminal part of NisP contains a -LPxTG-motif, which is recognized by a sortase that catalyzes the lipidation of the threonine residue at position 655 and this modification anchors NisP to the outer leaflet of the cytoplasmic membrane[68]. The substrate specificity of NisP expressing cells was investigated with the three different maturation states of nisin[64]. This previous study demonstrated a substrate specificity to the modified precursor peptide and the contained (methyl)lanthionine rings, the other precursor peptides were not cleaved. After chemical introduction of the (methyl)lanthionine rings NisP was able to digest the dedicated precursor peptide[64]. Additionally this proofs, that the cleavage reaction is not depending on the export function of NisT[64].

### 1.4.6 The Nisin multimeric lanthionine synthetase complex

Whether the modification and transport machinery of nisin assembles as a multi-enzyme complex or not is currently unknown. Nevertheless, yeast-two-hybrid studies and immuno-precipitation studies suggested the presence of such a complex[58]. In addition, NisB and NisC are associated at the membrane fraction of nisin producing cells[58,128], indicating a membrane associated multi-enzyme complex as well. Furthermore, the interaction of NisT and NisC, NisB and NisC, and the interaction of NisA with all three proteins was demonstrated[58]. Based on these data and the above-mentioned studies, the presence of a multi-enzyme complex is supported including two NisT, one NisB, two NisC and one nisin precursor peptide molecule[58]. Previous studies demonstrated that each modification enzyme (NisT, NisB and NisC) could function independently of each other *in vivo* and *in vitro*[55,57,61,64]. Lubelski *et al.* investigated the dependency of NisB with NisC and *vice versa*[129]. Here, both proteins were functional *in vivo* without the corresponding pendant; however a cooperativity was observed for both proteins. The simultaneous presence of NisB and NisC increased the efficiency of the modification reaction significantly[129]. For example, in the absence of NisC the nisin precursor production is reduced by 70%, in the absence of NisB by 98%[66]. The dehydration reaction catalyzed by NisB is more efficient in the presence of NisC - even in the presence of a catalytical inactive NisC variant. These data indicate that the nisin maturation process is a concerted mechanism of the three modification enzymes[66].

The observation that the serine residue at position 29 of nisin is not dehydrated in the presence of NisB and NisC was a milestone for understanding the modification process[129]. The serine residue at position 29 is located directly C-terminal to the rings D and E (Figure 8). In contrast, the serine residue at position 29 is dehydrated in the absence of NisC and in the presence of a catalytically inactivated NisC variant.

Apparently, only the presence of the (methyl)lanthionine rings prevents dehydration of the serine residue at position 29, presumably by a sterical hindrance for the dehydratase NisB[129]. Since the serine residue at position 29 is dehydrated in the presence of inactivated NisC and not in the presence of active NisC, the cyclization reaction of ring E prevents the dehydration of this specific serine residue at position 29. Based on these results, the modification process of the core peptide happens stepwise and NisC catalyzes the cyclization reaction prior to the dehydration of the next target residue by NisB[129]. This hypothesis was further validated by studies using mutants of the core peptide. Several serine residues were inserted directly C-terminally to the lanthionine or methyllanthionine rings, respectively. As expected, these mutations inhibited the dehydration reaction. One exception is the dehydration reaction for target residues of ring A and B. Here, NisB dehydrates the target residues at position 2, 3, 5 and 8 prior to the cyclization by NisC. This exception is necessary, otherwise the threonine residue at position 8 could not be dehydrated because of ring A. Additionally the reaction needs to be performed in an alternating fashion, demonstrating that the second ring is formed prior to NisB reaches position 12. When a serine residue is introduced at position 12 it is never dehydrated, proving an alternating process between NisB and NisC[129].

The first trials to isolate an *in vitro* modification complex were investigated in 2011[130]. Khusainov *et al.* isolated the assembled modification complex[130]. A C-terminal his-tagged precursor peptide was extracted out of a cell lysate sample by immobilized affinity chromatography (IMAC) and NisB as well as NisC were bound to the precursor peptide. NisC was found in relatively low, non-stoichiometric amounts in the IMAC elution fractions. Mutation of the histidine residue of NisC at position 331 to an alanine residue, however, increased the relative amount of NisC. This suggests that the inactivation of NisC tightens the modification complex and that the complex is trapped in a transient state. Moreover, the influence of the -FNLD- box on this assembly was investigated and the exchange of the -FNLD- box by four alanine residues reduced the amounts of co-eluted NisB and no NisC co-eluted[130]. In addition to the -FNLD- box, further binding motifs for NisB and NisC were identified within the leader peptide[142]. The sequence -STKD- (amino acid residues -2 to -5) are important for binding of NisB, however, all dehydrations were present in the core peptide. The sequence -LVSV- (amino acid residues -14 to -11) represents an additional binding motif for NisC and the alanine substituted leader peptide did not force the co-elution of NisC. Contrary to these data, the exchange of the amino acid residues at position -13 to -8 by histidine residues did not impair the cyclization reaction[92,142].

# 2 Aims

The elucidation of the nisin maturation process on a molecular level and *in vitro* was the overall topic of this thesis. The modification processes can be separated into cytosolic and extracellular reactions. In the cytosol, PTMs are introduced via NisB and NisC. On the cell surface the protease NisP cleaves off the leader peptide and mature nisin is released.

An essential requirement to introduce PTMs into the core peptide of nisin is the N-terminal located leader peptide[7,33,55,59,61,64,92]. When the leader peptide is fused to non lanthipeptide encoding amino acids sequences, this recombinant core peptide gets modified to a certain extend by NisB and NisC and secreted via NisT[35,50,51,97]. This rises the question how at least three enzymes are interacting with this leader peptide. The relationship between the modification enzymes NisB and NisC within this system remains elusive. In 1996 using yeast-two-hybrid systems an interaction between the modification enzymes was observed[58]. So far, no direct evidences for an interaction between the modification enzymes NisB and NisC have evolved in the absence of the nisin precursor peptide. In 2006, the X-ray structure and the *in vitro* activity of NisC was observed. Neither a binding site within the enzyme NisC nor in the substrate could be identified. Accordingly, a ping - pong mechanism for NisB and NisC was proposed in 2009[129] and in 2011 a precursor peptide was identified which was bound to NisB and NisC[130]. Subsequent *in vivo* studies revealed a class I conserved -FNLD- box, which is involved in the interaction between the nisin precursor peptide and the modification enzymes[92].

These results give only limited insights into the molecular events happening during the maturation process. Furthermore the recognition sites of the modification enzymes are unknown, as well as a biochemical characterization of the binding event and the enzymatic catalysis is missing.

In order to elucidate the binding motifs between the modification enzymes NisB, NisC, NisP and the precursor peptide, the first aim of this thesis was the establishment of a reliable purification strategy for the substrate nisin, the nisin precursor peptides and for all three mentioned modification enzymes.

After achieving the isolation of enzymes and substrates, the second aim was to analyze the binding characteristics and affinities in order to clarify the molecular processes involved in the modification reactions.

Finally, the third aim was to obtain and characterize an *in vitro* complex of the modifications enzymes, NisB, NisC which insert the PTMs in the nisin precursor peptide.

# 3 Publications

| | |
|---|---|
| Chapter I | **Rational and Irrational Approaches to Convince a Protein to Crystallize**<br>Book Chapter - InTech |
| Chapter II | **Lantibiotics: How do producers become self-protected?**<br>Review - Journal of Biotechnology |
| Chapter III | **Easy and Rapid Purification of Highly Active Nisin**<br>Research Article - International Journal of Peptide |
| Chapter IV | **Substrate Recognition and Specificity of the NisB Protein, the Lantibiotic Dehydratase Involved in Nisin Biosynthesis**<br>Research Article - The Journal of Biological Chemistry |
| Chapter V | **NisC binds the FxLx motif of the nisin leader peptide**<br>Research Article - Biochemistry |
| Chapter VI | **NisP cleaves the nisin precursor peptide *in vitro***<br>Research Article - in preparation |
| Chapter VII | **Assembly of the nisin maturation complex *in vitro***<br>Research Article - in preparation |

## 3.1 Chapter I - Gene Expression & Protein Purification

| | |
|---|---|
| Published in: | InTech |
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# Rational and Irrational Approaches to Convince a Protein to Crystallize

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# Rational and Irrational Approaches to Convince a Protein to Crystallize

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## 1. Introduction

The importance of structural biology has been highlighted in the past few years not only as part of drug discovery programs in the pharmaceutical industry but also by structural genomics programs. Mutations of human proteins have been long recognized as the source of severe diseases and a structural knowledge of the consequences of a mutation might open up new approaches of drugs and cure. Although the function of a protein can be studied by several biochemical and/or biophysical techniques, a detailed molecular understanding of the protein of interest can only be obtained by combining functional data with the knowledge of the three-dimensional structure. In principle three techniques exist to determine a protein structure, namely X-ray crystallography, nuclear magnetic resonance spectroscopy (NMR) and electron microscopy (EM). According to the protein data bank (pdb; http://www.rcsb.org) that provides a general and open-access platform for structures of biomolecules, X-ray crystallography contributes more than 90% of all structures in the pdb, a clear emphasis of the importance of this technique.
To perform X-ray crystallography it is essential to have large amounts of pure and homogenous protein to perform an even today still "trail and error"-based screening matrix to obtain well diffracting protein crystals. Therefore, successful protein crystallization requires three major and crucial steps, all of them associate with specific problems and challenges that need to be overcome and solved. These steps are (I) protein expression, (II) protein purification and (III) the empirical search for crystallization conditions. As summarized in Figure 1, every single step needs to be optimized along the long and stoney road to obtain protein crystals suitable for structure determination of your "most-beloved" protein via X-ray crystallography. This chapter will focus on these three steps and suggests strategies how to perform and optimize each of these three steps on the road of protein structure determination.

## 2. Protein expression (I)

To crystallize a protein, the first requirement is the expression of your protein in high amounts and most importantly on a regular basis. This implies that it is possible to obtain a freshly purified protein at least weekly. In general, it is possible to express a protein either homologously or heterologously (see Figure 1 – (I) expression). Especially for large proteins,

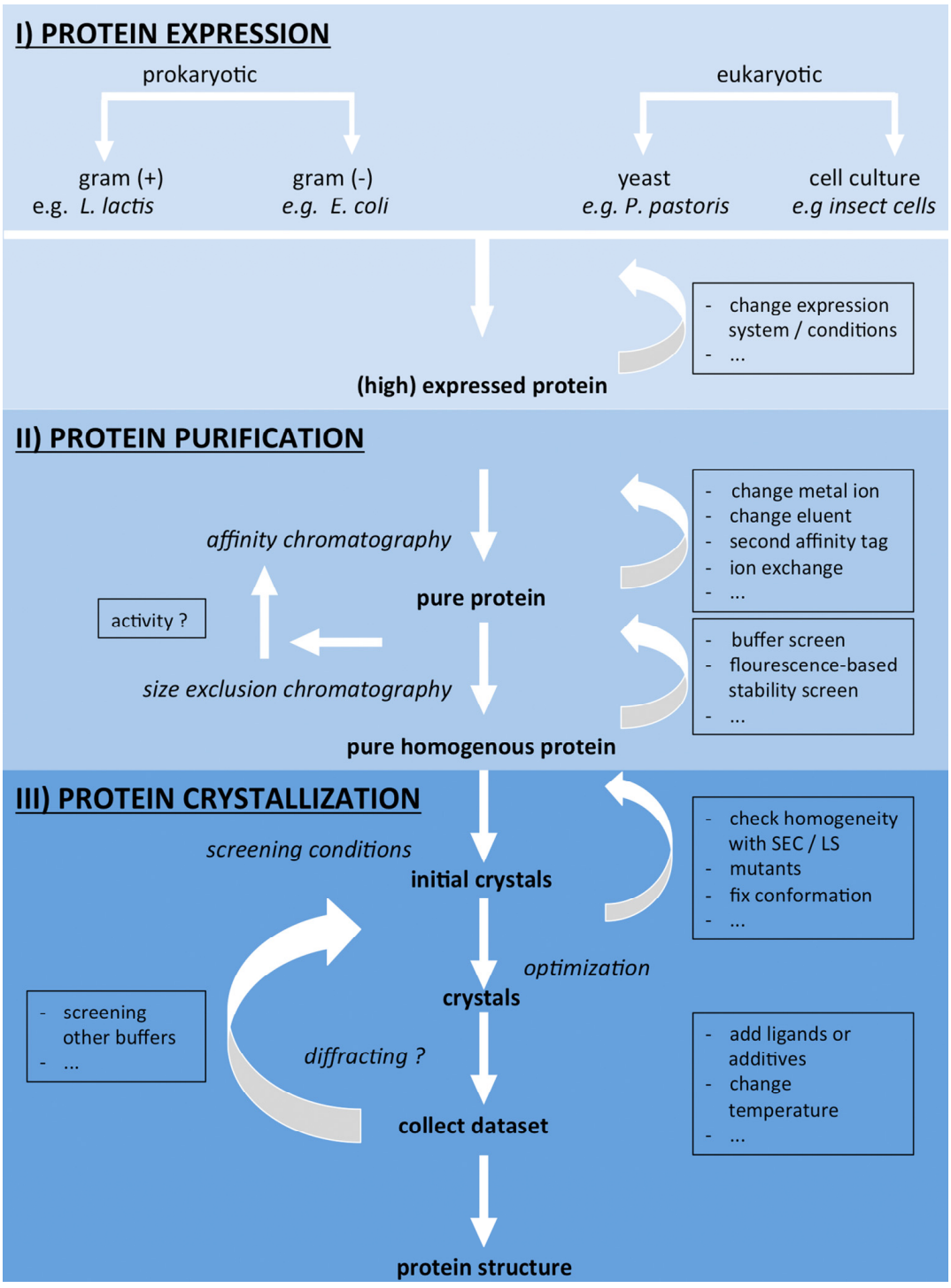


Fig. 1. Schema highlighting the three steps towards a protein crystal. (I) Expression (II) Purification (III) Protein crystallization.

proteins containing a co-factor or a ligand, the natural habitat is likely the best choice to express the protein. However, often the natural host, for example humans, produce only low amounts of protein and suitable overexpression protocolls are not available. To circumvent this problem, several expression strains, cell lines as well as a large number of expression vectors have been developed to allow expression of any protein in a different host (heterologous expression). In general, the used organisms for protein expression can be divided into two different groups: prokaryotic and eukaryotic expression hosts. The natural organism of the protein of interest mainly dictates the choice, which expression system to use. If working with a bacterial protein, it is very likely that also a prokaryotic host is able to express the protein in high amounts. The same holds true for proteins originating from a eukaryotic host, which is likely best overexpressed in a eukaryotic host. These proteins often require posttranslational modification such as glycosylation or disulfide bond formation, which are possible in eukaryotic expression hosts. The most common used heterologous expression host is the gram negative bacteria *Escherichia coli* since it is commercially available and a large number of expression cassettes have been developed. Thus, it is the most widely used expression system with expression rates of several mg/L of culture. The best characterised and understood expression hosts are described in more detail below and the commecially available systems are listed in table 1.

### 2.1 Expression hosts – Prokaryotic

#### 2.1.1 Gram negative - *E. coli*

As mentioned above, *E. coli* is the most common used expression system (Figure 1 – (I) expression, left side). This is further highlighted by the fact that 80% of all protein structures deposited in the protein data bank were overexpressed in *E. coli* (Sorensen and Mortensen 2005). There are several advantages promoting *E. coli* as expression host: (A) Cultivation of *E. coli* is simple and a doubling time of 30 minutes is rather quick allowing the fast generation of biomass, (B) genetics are well understood and any genetical manipulation is well established, (C) expression levels of up to 60 % of the total protein mass within the cell make the next step, protein purification rather straight forward and finally (D) the cultivation does require only standard equipment normally present in every biochemical laboratory and therefore expression using *E.coli* is relatively cheap. In the last decades, many different plasmid based expression systems have been developed such as the pET vector systems, which contain several different expression plasmids with a choice for the affinity tag on either termini of the protein as well as the possiblity to use a dual cassette when expressing two or more proteins at once. The selection pressure derived from different antibiotics, and the resistance genes encoded on these plasmids further simplify laboratory practice. Only cells harbouring the right plasmids are able to grow and therefore expenditure on sterility is low.

The typical *E. coli* expression system is plasmid-based, which can be transferred to different *E. coli* strains ((Sorensen and Mortensen 2005), Novagen pET vector table). An *E. coli* expression vector consists mainly of five important parts: the replicon, a resistance marker, a promotor and a so-called multiple cloning site (MCS) (Baneyx 1999; Jonasson, Liljeqvist et al. 2002). The replicon is the crucial part of a plasmid to maintain it inside a cell. It is recognized and duplicated by the replication machinery (Baneyx 1999). The selection marker allows the identification of cells carrying a plasmid as it encodes for a resistance, e.g. against antibiotics (see above)(Sorensen and Mortensen 2005). The promotor sequence is the recognition site for the RNA polymerase, however it is inactive under initial cultivation conditions. The addition of an inducer (sometimes also a temperature change) switches the

promotor from 'off' to 'on' whereby the expression is initiated (Jana and Deb 2005). Common inducers are isopropyl-β-D-thiogalactopyranosid (IPTG) in the pET system or arabinose for pBAD vectors (Invitrogen™). The multiple cloning site (MCS) is a short DNA segment combining many (up to 20) restriction sites. This feature simplified the insertion of genes into the plasmid enormously and made cloning procedures very convenient. However, new cloning strategies, which are independent of restriction enzymes and ligases, are emerging and will replace the standard approaches some day (see for example Li and Elledge 2007).

### 2.1.2 Gram positive - *L. lactis*

Within the prokaryotic expression system also some gram (+) bacteria are used for protein overexpression (Figure 1 – (I) expression left side). Here, lactic acid bacteria play a privotal role. They are used in food industry and are known since 1873 when Joseph Lister isolated the first strain (Teuber 1995; Mierau and Kleerebezem 2005). For the overexpression of recombinant proteins, there are many lactic acid bacteria around, *lactococci, lactobacilli, streptococci* and *leuconostocs*. (Gasson 1983; van de Guchte, Kok et al. 1992; de Vos and Vaughan 1994). The best characterized and most widely used host is *Lactococcus lactis*, which is famous for its usage within food fermentation and like for *E. coli* the genome, metabolisms and molecular modifications are well known and established (Bolotin, Wincker et al. 2001; Guillot, Gitton et al. 2003). Thus, it has been called the 'bug of the next millennium'(Konings, Kok et al. 2000). All established gram (+) bacteria expression hosts are able to overexpress proteins homologously or heterologously. Since only one, namely the cyctoplasmic membrane is present (Kunji, Slotboom et al. 2003), this host is in comparison to the two-membrane system of gram (-) bacteria, a good choice to express eukaryotic as well as prokaryotic membrane proteins or proteins with membrane anchors (Kunji, Slotboom et al. 2003). The promoter used for expression in *L. lactis* is induced by the external addition of nisin. Nisin is an antimicrobial active peptide, which interacts with lipid II in the cytoplasmic membrane of gram (+) bacteria and causes cell lysis. Interestingly, nisin is produced by *L. lactis* itself. The expression strain is deleted of the nisin producing genes and therefore external nisin can be used as inductor. Nisin binds to NisK, which as part of a two-component system, phosphorylates NisR, which in turn binds to the promotor $P_{nisA}$ thereby allowing synthesis of the protein located downstream on the plasmid. Since nisin is also active against *L. lactis* itself, the concentration range of nisin used in such expression studies is relatively narrow to circumvent killing of the *L. lactis* expression strain. This is clearly a draw back of this expression system since expression can basically only turned on with a certain nisin concentration. Between the inducer concentration and the expressed protein a linear behaviour is observed. Unfortunately the nisin concentration range between the minimal and maximum nisin concentration is very small, nisin concentration higher than 25 ng per liter of cells, cause cell death.

However, *L. lactis* has been proven to be a very efficient expression system. Kuipers et al. created many expression hosts and plasmids to produce any protein of interest by cloning it downstream of the $P_{nisA}$ promotor. With this nisin inducible (NICE) expression system, it is now possible to induce the protein production with minimal concentration (0.1 – 5 ng) of nisin (de Ruyter, Kuipers et al. 1996; Kuipers, de Ruyter et al. 1998). The amount of produced recombinant protein can reach up to 50 % of the total intracellular proteins (Kuipers, Beerthuyzen et al. 1995; de Ruyter, Kuipers et al. 1996). Following a few examples for expressed proteins in *L. lactis* whose structures have been solved: an ECF-type ABC transporter (PDB:3RLB)(Erkens, Berntsson et al. 2011), a peptide binding protein OppA

(PDB:3RYA)(Berntsson, Doeven et al. 2009) and the multidrug binding transcriptional regulator LmrR (PDB:3F8B)(Madoori, Agustiandari et al. 2009).

### 2.2 Eukaryotic expression hosts

The great benefit of choosing a eukaryotic host for overexpression of a protein of interest are the availability of a posttranslational modification system as well as the frequently enhanced protein folding (Midgett and Madden 2007). Eukaryotic proteins tend to misfold or lack biological activity when expressed in prokaryotic expression systems such as *E. coli* (Cregg, Cereghino et al. 2000; Midgett and Madden 2007). To overexpress these proteins, different yeast strains, insect cells or even mammalian cell lines have been developed as expression hosts (Figure 1 - (I) expression, right side). Eukaryotic expression systems are often more expensive, provide low expression levels and are sometimes hard to handle, when compared to bacterial systems. However, the genetic and cellular contexts are more similar to the original protein-expressing organism (Midgett and Madden 2007). In the following sections, some of the commonly employed eukaryotic expression systems will be described.

#### 2.2.1 Yeast expression systems - *Saccharomyces cerevisiae* and *Pichia pastoris*

The most widely used yeast strains to express protein are *Saccharomyces cerevisiae* and *Pichia pastoris*, which offer the major advantage of a posttranslational modification system for glycosylation, proteolytic processing as well as disulfide bond formation, which for some proteins are essential for the function and/or correct folding (Cregg, Cereghino et al. 2000; Midgett and Madden 2007). The handling of yeast expression systems is similar to prokaryotic systems with respect to the genetic background and cultivation. Similar to the bacterial vector systems, expression in yeast starts with a plasmid-based cloning part which can be performed in *E. coli* (Cregg 2007). Afterwards the expression cassette gets integrated into the genome by simple homologous recombination in the yeast. One major advantage in *P. pastoris* is the insertion of multiple copies of the protein DNA-sequence into genomic DNA, which increases expression yield.

The biggest advantage of yeast as expression system is that well established protocols for fermentation are available. Optimal fermentation of *P. pastoris* can end up with more than 130 gram of cells per liter of culture. Even if expression levels in the cell are not that high the mass of cells easily compensates for this disadvantage (Wegner 1990; Cregg, Cereghino et al. 2000; Hunt 2005; Cregg 2007; Midgett and Madden 2007). Examples of crystal structures from proteins expressed in *P. pastoris* are a human monoamine oxidase B (PDB:3PO7) (Binda, Aldeco et al. 2010) and a protein involved in cell adhesion NCAM2 IG3-4 (PDB:2XY1)(Kulahin, Kristensen et al. 2011).

#### 2.2.2 Insect cells

The expression system in insect cells is beside yeast a well-characterised alternative to express eukaryotic proteins (Midgett and Madden 2007). As insect cells are higher eukaryotic systems their posttranslational modification machinery can carry out more complex alterations than yeast strains. They also have a machinery for the folding of mammalian proteins. The most commonly used vector system for recombinant protein expression in insect cells is baculovirus, which can also be used for gene transfer and expression in mammalian cells (Smith, Summers et al. 1983; D., L.K. et al. 1992; Altmann, Staudacher et al. 1999). A few examples of proteins expressed in insect cells that resulted in

crystal structures are the transferase Ack1 (PDB:3EQP)(Kopecky, Hao et al. 2008), a human hydrolase (PDB: 2PMS)(Senkovich, Cook et al. 2007) and myosin VI (PDB:2BKI)(Menetrey, Bahloul et al. 2005).

### 2.2.3 Mammalian cell lines

The expression of proteins in mammalian cell lines is the most expensive and complex alternative. Especially for human membrane proteins this expression system has been proven to express the most active protein (Tate, Haase et al. 2003; Lundstrom 2006; Lundstrom, Wagner et al. 2006; Eifler, Duckely et al. 2007). The resulting protein amount, however, obtained from mammalian cell lines is mostly only sufficient for functional studies. Using mammalian cells lines is the most challenging variant of protein overexpression and therefore only choosen if any of the other expression system described failed. Some examples of protein structures expressed in mammalian cell lines are the hydrolase PCSK9 (PDB:2QTW)(Hampton, Knuth et al. 2007) and the acetylcholine receptor AChBP (PDB:2BYQ)(Hansen, Sulzenbacher et al. 2006).
Table 1 sums up advantages and disadvantages of the above mentioned overexpression systems used for protein crystallography.

| Expression system | Pros | Cons |
|---|---|---|
| **Prokaryotic** | | |
| Gram negative *E. coli* | - costs<br>- simplicity (genetic/culture)<br>- yield | - inclusion bodies<br>- protein folding<br>- posttranslational modifications<br>- protein secretion |
| Gram positive *L. lactis* | - costs<br>- protein secretion<br>- one membrane | - posttranslational modifications |
| **Eukaryotic** | | |
| Yeast | - high cell densities<br>- costs<br>- simple cultivation<br>- posttranslational modifications | - hyper glycosylation, non-native<br>- lipid composition is different to mammalian cells |
| Insect cells | - more native lipid environment<br>- good track record of functional proteins | - costs<br>- non-native lipid environment<br>- glycosylation pattern different to mammalian cells<br>- protein amount |
| Mammalian cells | - native conditions for human membrane proteins to investigate diseases<br>- posttranslational modifications<br>- lipid environment<br>- good track record of functional protein | - costs<br>- difficult to establish<br>- protein amount |

Table 1. Overview of expression systems. Summarized are the advantages and disadvantages.

## 3. Purification

After having expressed your protein of interest, the race for crystals is by no means finished. The next step on the long road to structure determination is to isolate the protein or - phrasing it differently - to remove all other proteins present in the cell (Figure 1 – (II) purification). An elegant method to do so is the genetic attachment of an affinity tag on either site of the protein or in some cases on both sides (Waugh 2005). This affinity tag has the possibility to bind high affine to a immobilized ligand on a matrix, while all other proteins have a much more reduced binding affinity and therefore flow through the matrix (Figure 1 – (II) purification 1st step). This allows a one-step purification, which in almost all cases is relatively harmless for the protein and likely does not interfere with folding and/or overall structure of the protein. There are a lot of affinity tags available as well as matrix materials (Terpe 2003). The well known and most often used affinity tag is the poly-histidine tag (Porath, Carlsson et al. 1975; Gaberc-Porekar and Menart 2001), which can vary in length as well as in position but the overall purification strategy is the same. From all the structures solved nowadays, almost 60 % of the proteins are purified via a histidine tag; mainly due to the great purification efficiency, which can be as large as 90% after a single purification step (Gaberc-Porekar and Menart 2001; Arnau, Lauritzen et al. 2006). Therefore, most commercially available expression systems and methods contain a his-tag encoded on the plasmid. Besides the his-tag, there are other tags avaible and used for protein purification, of which the Strep-, CBP-, GST-, MBP-tag are described below.

### 3.1 Choice of the right tag

#### 3.1.1 Polyhistidine-tag (his-tag)

As mentioned above the polyhistidine-tag is the most common affinity tag and the required affinity resins and chemicals are relatively inexpensive. The purification step is a so-called immobilized metal ion affinity chromatography (IMAC) (Porath, Carlsson et al. 1975). Here, a matrix is able to bind bivalent metal ions. For example nitrilotriactetic acid (NTA), which is a chelator and binds metal ions like $Ni^{2+}$, $Zn^{2+}$, $Co^{2+}$ or $Cu^{2+}$ (Hochuli, Dobeli et al. 1987). These metal ions have a high affinity to the imidazole group of the amino acid histidine. A stretch of histindines in a row with for example an *E. coli* protein is very unusual. Thus, **t**he genetical introduction of several, in most cases 6- 10 histidines in a row selects for specific binding of this protein. As eluant very elegantly imidazole can be used, which competes with the histidine tag and elutes the protein of interest. When used in low concentrations, imidazole can also be used to remove unspecifically bound proteins, which bind with low affinity to the matrix (Hefti, Van Vugt-Van der Toorn et al. 2001). Normally, a protein with a 6-10 histidine tag should be bound to the matrix relative strongly and 100-250 mM imidazole in the buffer is required to elute the protein from the resin. In contrast, proteins with a low affinity to the matrix can already be eluted with 10-50 mM imidazole (the "impurities" of *E. coli*). Therefore, a linear imidazol gradient, for example, separates the protein of interest and impurities (Hochuli, Dobeli et al. 1987; Gaberc-Porekar and Menart 2001). Although the polyhistidine-tag is the most common and mostly an efficient variant, there are a few applications where the his-tag can cause problems. Metalloproteins can interact either directly with the his-tag or with the ions immobilized on the matrix. In comparison to some other affinity-tags, the specificity of the his-tag is not that high and in some cases this results in the co-purification of other proteins (Waugh 2005).

### 3.1.2 Strep-tag

In comparison to the his-tag, which binds to immobilized metal ions, the strep-tag II constists of a small octapeptide (WSHPQFEK), which binds to the protein streptavidin (Schmidt, Koepke et al. 1996). The commercial available matrix is a streptavidin variant and is called Strep-Tactin. This variant is able to bind the Strep-tag II octapeptide under mild buffer conditions and can be gently eluted with biotin derivates such as desthiobiotin (Schmidt, Koepke et al. 1996; Voss and Skerra 1997). Especially for metal-ion containing enzymes it is a promising alternative to the his-tag (Groß, Pisa et al. 2002). However, as chemicals are more expensive and the matrix has a lower binding capacity, compared to NTA resins, it is often not the first option choosen. Moreover, it cannot be used under denaturating conditions since Strep-Tactin denatures and will not bind the tag anymore (Terpe 2003; Waugh 2005). Examples of proteins crystallized after a Strep-tag purification are OpuBC (PDB:3R6U)(Pittelkow, Tschapek et al. 2011) and *Af*ProX (PDB:3MAM)(Tschapek, Pittelkow et al. 2011) as well as the sodium dependent glycine betain transporter BetP from *Corynebacterium glutamicum* (PDB:2WIT)(Ressl, Terwisscha van Scheltinga et al. 2009).

### 3.1.3 CBP-tag

Another peptide tag, is the calmodulin binding peptide, first described in 1992 (Stofko-Hahn, Carr et al. 1992). This peptide is prolonged compared to the Strep-tag II, consisting of 26 amino acids and binds with nanomolar affinity to calmodulin in the presence of $Ca^{2+}$ (Blumenthal, Takio et al. 1985). It is derived from the C-terminus of the skeletal-muscle myosin light-chain kinase, which makes the system an excellent choice for proteins expressed using a prokaryotic expression system, since in prokaryotic systems nearly no protein interacts with calmodulin. This allows extensive washing to remove impurities and elution with EGTA, which complexes specifically $Ca^{2+}$, and a protein recovery around 90 % can be achieved (Terpe 2003). A drawback of this tag however is that the CBP tag can only be fused to the C-terminus of the protein since it has been shown that CBP on the N-terminus negatively influences the translation and thereby the expression rate (Zheng, Simcox et al. 1997).

### 3.1.4 GST-tag

With respect to the length of the tags, the his-tag contains only a few amino acids, the Strep-tag II and the CBP-tag already contain 8 – 26 amino acids, but it is possible to fuse whole proteins with 26 – 40 kDa to a recombinant protein. Here, the high affinity binding of the protein to their substrate is used to purify the protein of interest (Smith and Johnson 1988). In the case of the glutathione S-transferase (GST, 26 kDa) the protein specifically binds to immobilized glutathione. To elute the fusion protein from the resin, non-denaturating buffer conditions employing reduced glutathione are used (Terpe 2003). The tag can help to protect the recombinant protein from degradation by cellular proteases. It is recommended to cleave off the GST-tag after purification with a specific protease like thrombin or TEV (Tobacco Etch Virus) protease (Terpe 2003).

### 3.1.5 MBP-tag

Another affnitiy tag, which can be fused to the protein of interest, is the maltose binding protein (MBP) from *E. coli*. This protein has a molecular weight of 40 kDa and has the ability to bind to a cross-linked amylose matrix. The binding affinity is in the micro molar range

and the tag can be used in a pH range from 7.0 – 8.0, however, denaturating buffer conditions are not possible (di Guan, Li et al. 1988). The elution of the recombinant protein is recommended with 10 mM maltose. A great opportunity of the MBP-tag is the increasing solubility effect of the recombinant protein in prokaryotic expression systems and even more pronounced in eukaryotic systems (Sachdev and Chirgwin 1999). Like the CBP-tag, a fusion at the N-terminal side might influence translation and expression rates (Sachdev and Chirgwin 1999).

### 3.1.6 Tag position and double tags

As described above, the position of the tag either at the N- or C-terminus has a considerable influence on translation and expression rate as well as on the biological function (Arnau, Lauritzen et al. 2006). If information regarding activity of the protein is already available especially about the location of interaction sites, this should be included in the protein design, meaning tag position etc. In general, the tag should be placed at the position of the protein, which is less important for interactions and/or expression. To minimize the influence of the tag on folding and/or activity in some cases it helps to create a linker region of a few amino acids between the tag and the protein (Gingras, Aebersold et al. 2005). A very efficient and sophisticated solution is, the addition of amino acids between tag and protein of interest, which functions not only as an accessibility increasing factor, but, also encodes for a recognition site for proteases like thrombin or TEV. Due to this arrangement the tag – protein interaction is minimized and the tag can be cleaved off if necessary (Arnau, Lauritzen et al. 2006). In some special cases a combination of two affinity tags results in enhanced solubility and more efficient purification. To enhance the purity of a protein, often a construct of two different short affinity tags like his-tag and Strep-tag or CBP-tag can be engineered (Rubio, Shen et al. 2005). Also a combination of two his-tag or two strep-tag kept apart by a linker region enhances the binding affinity extremely. This allows more stringent washing steps prior to elution of the protein (Fischer, Leech et al. 2011).

## 3.2 Size exclusion chromatography and ion exchange chromatography

Despite the usage of affinity tags a second purification step is sometimes required (Figure 1 – (II) purification). Which kind of purification procedure is required depends on the nature of impurities. If these impurities differ in molar mass compared to the protein of interest, a method based on size separation can be applied. Size exclusion chromatography (SEC) also separates different oligomeric species of the protein from each other, which otherwise would strongly inhibit crystallization and also allows analysis of stability and monodispersity of the protein (Regnier 1983a; Regnier 1983b).

However, in many cases, SEC is not sufficient to remove all impurities. Then separation by overall charge of the protein might be an option. Depending on the isoelectric point of the protein either anion or cation exchange chromatography can be performed. The protein binds to a matrix under very low ionic strength and is eluted afterwards either by increasing the ionic strength or by pH variation. Similar results can be achieved by hydrophobic interaction chromatography. Here, proteins with different surface properties show differences in their binding strength and binding of the protein is done inversely as during ion exchange chromatography. High ionic strength favors protein binding to a hydrophobic matrix and elution takes place when reducing the ionic strength. Although there are many other possibilities to increase the purity of a protein, the above mentioned techniques are without any doubt the most widely used and general applicable methods.

### 3.3 How to get a homogenous protein solution

In some cases isolated proteins are stable and homogenous at high concentrations after the purification and can be directly used for crystallization experiments. Often, however, the protein does not behave ideal and precipitates at high concentrations or forms aggregates or inhomogenous, oligomeric species; all of them prohibit crystal growth. SEC is a very elegant method to visualize the stability and oligomeric state of a protein. If the stability or the homogeneity of a protein sample is critical, you need to adapt your purification protocol and search for an optimized procedure. Different approaches are summarized below, for example a buffer screen to enhance protein solubility, multi-angle light scattering experiments to determine the absolute mass and the oligomeric state of the protein sample or fluorescence-based experiments to investigate the stability of the protein of interest.

#### 3.3.1 Purified proteins – An *in vitro* system

After a protein is expressed in a soluble form, the subsequent purification procedure changes the environment of the protein dramatically. The cytoplasm of the cells, where the overexpression takes place, is packed with macromolecules. In *E. coli*, for example, the concentrations of proteins, RNAs and DNAs are about 320 mg/mL, 120 mg/mL, and 18 mg/mL, respectively (Cayley, Lewis et al. 1991; Zimmerman and Trach 1991; Elowitz, Surette et al. 1999) resulting in an overall concentration of macromolecules of above 450 mg/mL. During cell lysis and the first purification step, likely an IMAC (see above), the protein is separated from almost all other cell components. This rigorous procedure is accompanied with a severe change of the environment into an *in vitro* system. As a result proteins often tend to aggregate, precipitate or form inhomogeneous oligomeric states that prevent the formation of crystals in further experiments. Therefore one of the biggest challenges in structural studies is the preparation of protein solutions with high concentrations (as a rule of thumb 10-20 mg/mL) in a homogenous state. To fulfill these requirements, the *in vitro* system needs to be optimized with respect to different parameters as highlighted in *Figure 1 – (II) purification*. If a sufficient protein sample cannot be obtained, different strategies are available to increase the important characteristics of the protein: purity and homogeneity. As mentioned above, the usage of different metal ions during IMAC, ion exchange, a second affinity chromatography etc. can be sufficient to enhance purity. This might also lead to an increased stability. However, if the stability and/or homogeneity of a protein is still a problem, screening for a new buffer composition is essential to succeed during crystallization trials.

#### 3.3.2 Buffer composition

Many examples illustrate the importance of an adequate buffer composition for protein stability, homogeneity, conformation, and activity (Urh, York et al. 1995; Holm and Hansen 2001; Jancarik, Pufan et al. 2004; Collins, Stevens et al. 2005). Some buffers are very frequently used and recommended by manufactures (see for example Qiagen, Roche, New England BioLabs, Fermentas, etc.). All of them contain a buffer reagent that keeps the pH constant in a well-defined range. Well-known examples are phosphate, tris (hydroxymethyl) aminomethane (Tris), or HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) that buffer at the physiological relevant pH range of 6- 9 (Durst and Staples 1972; Chagnon and Corbeil 1973; Tornquist, Paallysaho et al. 1995). In recent years, the development of other buffer systems has been quite successful (Taha 2005) (for a list of buffers and corresponding

pH ranges, see for example: http://delloyd.50megs.com/moreinfo/buffers2.html). Next to the well-defined pH, the stability and homogeneity of proteins depend on many other parameters, for example ionic strength, the presence of ligands and/or co-factors, divalent ions, glycerol, etc. The appropriate buffer composition cannot be predicted so far and needs to be identified by trial-and-error approaches.

### 3.4 Protein purification – How to overcome problems

In this part we would like to present some pitfalls that might occur during protein purification and provide some ‚rationales' to overcome these problems. As usual, the crucial step of solving a problem is its identification. Here, we are trying to sensitize the reader to indications, which might point towards problems related to instability and/or inhomogeneity of the protein sample. Moreover, such problems cannot always be recognized without the adequate technique(s). Therefore, we are introducing techniques that are capable to visualize the state of proteins.

#### 3.4.1 Visible protein precipitations during IMAC

A very obvious stability problem is the formation of precipitations in the elution fractions of a chromatography step (see Figure 2). In this example, the his-tagged protein was eluted with a linear imidazole gradient from 10 to 500 mM imidazole and eluted at about 250 mM imidazole. Protein precipitation occured immediately after elution (Figure 2A and B) and continued (Figure 2C) resulting in a low amount of soluble protein. This aggregation can be reduced by dilution with a IMAC buffer (typically lacking imidazole) immediatly after the elution. Thereby, dilution hinders the concentration-dependent aggregation. In many cases, this rational is not sufficient to prevent precipitation. After applying, for example, a *buffer screen* (see Figure 1 – (II) purification) the new defined buffer is used for the chromatography or the eluting protein is diluted into the new buffer (see Figure 2D).

Other elution strategies of his-tagged proteins from an IMAC column are available. As described before, competing the poly-histidine from the IMAC column by imidazole is the most common elution strategy, however, for some proteins other strategies are superior, for example, replacing imidazole by histidine. Imidazole is only a mimic for histidine. If one uses histidine instead of imidazole aggregation can be avoided as concentration of the eluent can be reduced by a factor of ten. An example for a protein sensitive to imidazole concentration is shown in Figure 3B. Here a comparative SEC chromatogram is shown. After elution from the IMAC column with imidazole only a very small amount of the protein elutes at the volume corresponding to the size of a monomer or the dimer, respectively (Figure 3B, continuous line). Most of the protein passes the column very fast and elutes at the void volume indicating large radii meaning aggregated protein. Yields of dimeric (at about 150 mL) and monomeric (at about 180 mL) proteins are strongly increased after an elution with histidine (dashed line) compared to an elution with imidazole (continous line) and only the monomeric species could be crystallized (data not shown). The choice of the eluent in IMAC might therefore be an important step in a purification protocol. Another elution strategy of his-tagged proteins is a pH change from 8 to 4. In an acidic environment, histidines become positively charged and are therefore released from the column matrix. This strategy results in a sharp elution from the matrix and the protein is eluted highly concentrated. Although this strategy is recommended by the manufacturers (see GE Healthcare, Qiagen, etc.) the desired protein needs to retain activity at acidic pHs. The

bivalent metal ions ($Ni^{2+}$, $Co^{2+}$, $Zn^{2+}$,…, see above) that complex the his-tag can be removed from the matrix by chelating reagents as ethylenediaminetetraacetic acid (EDTA) as another elution strategy (Muller, Arndt et al. 1998)

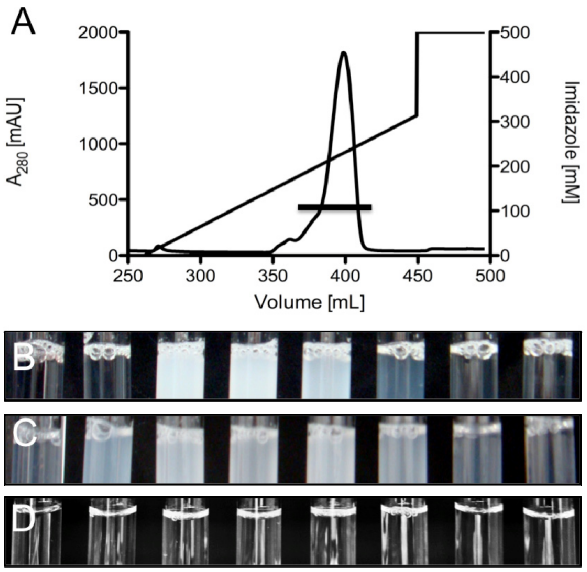


Fig. 2. Elution fractions of an IMAC. The protein was eluted via a linear imidazole gradient from 10 to 500 mM and the absorption at 280 nm was recorded. The elution fractions were collected and photographed. A: IMAC chromatogram of the his-tagged protein. Elution fractions containing the desired protein (indicated by a bar) are collected and shown in B – D. B and C: Elution fractions of the protein in 50 mM Tris-HCl, 150 mM NaCl, pH 8.0 immediately and 10 min after the elution, respectively. D: The elution fractions were immediately mixed in a 1 to 1 ratio with a buffer that enhances the protein stability (50 mM citrate, 50 mM LiCl, pH 6.00) evaluated during a solubility screen.

### 3.4.2 Invisible aggregations

Sometimes aggregation of proteins in solution can not be detected directly by eye. This inhomogeneity of protein samples can be visualized SEC, a method that separates proteins by their hydrodynamical radius (see above). Protein aggregates are eluting at the void volume, since they are clumbed together resulting in a big hydrodynamical radius (see Figure 3A and B). If invisible aggregation is detected the buffer composition needs to be adjusted. In one case we applied this technique to visualize the state of a protein after an IMAC, and the resulting elution profile is shown in Figure 3A (continous line). Comparable to the imidazole-induced precipitation described above, the protein aggregated and elutes within the void volume of the column (about 40 mL). Moreover, several other protein species elute from 55 to 80 mL indicating a highly inhomogeneous protein sample. The running buffer of the SEC was 50 mM Tris-HCl, pH 8.0 and 150 mM NaCl. Remarkably, a simple change to a new buffer (20 mM HEPES, 150 mM NaCl, pH 7.0) Resulted in a stable and homogenous protein sample (Figure 3A, dotted line), which was suitable for

crystallization trials. Next to the rigorous change in the homogeneity of the protein, the biological activity of the protein could only be determined in the new buffer system. The influence of the buffer composition for the protein activity is a well-known phenomenon (Urh, York et al. 1995; Holm and Hansen 2001; Zaitseva, Jenewein et al. 2005) and in many cases the activity goes hand in hand with an optimal buffer for the purification. Mentionable, the new buffer was not found by trial-and-error approaches. We searched for literature dealing with homologous proteins, especially for established purification protocols. This literature search revealed the new buffer, illustrating that not every step towards a protein structure determination must be a trial-and-error process.

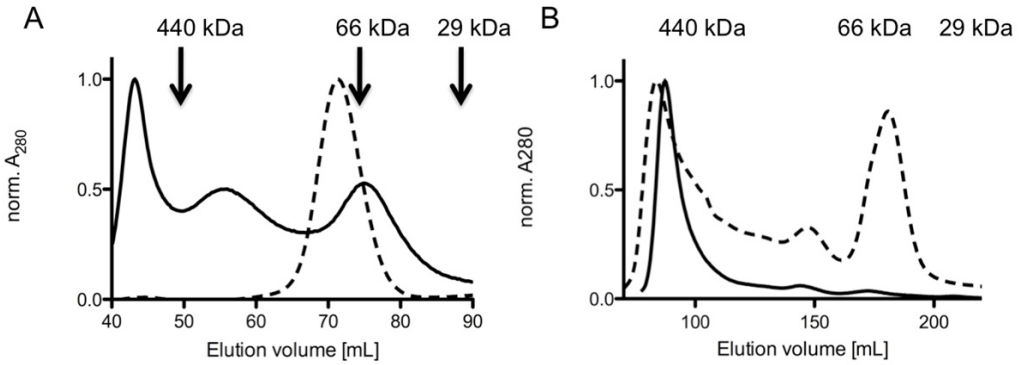


Fig. 3. Size exclusion chromatograms (UV 280nm) of proteins in different buffers. A: The homogeneity of a protein was analyzed in two different buffers; continuous line: 50 mM Tris-HCl, 150 mM NaCl, pH 8.00; dotted line: 20 mM Hepes, 150 mM NaCl, pH 7.00. B: The protein was eluted of the IMAC column either with imidazole (continous line) or with histidine (dotted line), concentrated and applied to the SEC.

Another example for the influence of the buffer composition was published bei Mavaro et al. (Mavaro, Abts et al. 2011). Instead of the buffer agent, the ionic strength of the buffer was the crucial determinant. Purification of the protein in low-salt buffer resulted in an inhomogenous protein sample containing a mixture of aggregates, dimers and monomers without biological activity. However, a simple change to high-salt buffer allowed the purification of homogenous dimeric protein, that was able to bind its substrate.

### 3.4.3 Overcoming protein instability

In the previous sections different strategies were mentioned to enhance the stability and the homogeneity of purified proteins and in all cases the buffer composition was the solution. Still, the essential question how to determine the optimal buffer to make a protein feel happy *in solution* is not answered? Some rationales and experiences are listed above: different elution strategies for IMAC purifications, the usage of frequently used buffer agents and a literature research for established purification protocols of related proteins. However, in many cases these approaches do not solve the problems occuring during the purification. But, is there a general methodology to overcome the problems? Unfortunately, the answer is as frustrating as challenging - there is not a general panacea around for the right buffer composition of a protein. If a new buffer needs to be found, trial-and-error

approaches have to be applied. A lot of different parameters are influencing the state of a protein, i. e. the buffer agent, the salt concentration, presence of metal ions with different valences, the hydrophobicity, and even the temperature of the buffer. The analysis of the protein in different buffers can be done by SEC and/or light scattering experiments. However, screening of all the different variables is very labor- and cost-intensive, and time-consuming, moreover only combinations of two or more additives might be sufficient to enhance the solubility and homogeneity of the protein. Therefore high-throughput methods are needed that handle a lot of different conditions simultaneously using as few as possible protein sample.

### 3.4.4 Buffer screen – Enhancing the solubility

Many publications are available suggesting methods for a solubility screening to allow the crystallization of initially inhomogeneous, aggregating protein samples (Jancarik, Pufan et al. 2004; Zaitseva, Holland et al. 2004; Collins, Stevens et al. 2005; Sala and de Marco 2010; Schwarz, Tschapek et al. 2011). In all of these methods aggregating protein samples are mixed with commercially available crystallization screens incubated for a period of time, and analyzed for precipitation visually using a light microscope. Screening conditions resulting in no precipitations are analyzed upon their composition, and protein samples are further examined with respect to their solubility and homogeneity under these conditions by SEC or light scattering experiments. This technique allows high-throughput screening in a 96-well format, where an automated pipetting system mixes only 50-200 nL of protein solution with 50-200 nL of buffer solutions to minimize the needed protein sample and increase the screening efficiency. Several buffer screens are commercially available that cover many different buffer agents, salt concentrations and other buffer parameters (i. e. from Hampton Research, Molecular Dimensions, Sigma, Jena Bioscience, Qiagen). After a solubility screening was applied, we were able to stabilize a previously unstable protein at concentrations above 3 mg/mL (see above "Protein precipitations during IMAC" and Figure 2D) at concentrations of up to 100 mg/mL for weeks (Schwarz, Tschapek et al. 2011). Typically, the new buffer (50 mM citrate, 50 mM $LiCl_2$, pH 6,00) should be used during the entire purification procedure starting with cell lysis. In the described case, the new buffer contains citrate, which is incompatible with an IMAC purification. Therefore the protein was immediately mixed with the new buffer after the elution of the IMAC column.

### 3.4.5 Size-exclusion chromatography versus light scattering experiments

Size-exclusion chromatography (SEC) and light scattering experiments (LS) are very helpful tools to analyze the homogeneity (Collins, Stevens et al. 2005) and the molecular mass of proteins; however both of them have advantages and disadvantages compared to each other. In SEC experiments proteins are separated based on their hydrodynamic radius by partitioning between a mobile phase and a stationary liquid within the pores of a matrix. All SEC columns are characterized by the volumes $V_0$, the liquid volume in the interstitial space between particles, $V_i$, the volume contained in the matrix pores and $V_T$, the total diffusion volume ($V_0 + V_i$) (Regnier 1983a; Regnier 1983b). In dependency of the hydrodynamic radius molecules are eluting at specific retention volumes in between $V_0$ and $V_T$ with big molecules eluting first. After a calibration of a SEC column with proteins of known

molecular weight (i. e. Sigma-Aldrich, "Kit for Molecular Weights") the molecular mass of the protein of interest can be roughly estimated; the elution volume is correlated to the $\log_{10}$ of the molecular weight (therefore, the hydrodynamic radius is considered to be proportional to the molecular weight). However, many extraneous mechanisms such as adsorptive, hydrophobic and ionic effects are further limiting the correlation between the retention volume and the molecular mass giving sometimes rise to wrong estimations.
Light scattering (LS) experiments can be applied to overcome these disadvantages and investigate the exact molecular weight of the protein sample. The rayleigh scattering of particles of monochromatic light depends directly on the molar mass of the particle. If you know the exact number of particles you can calculate the average molar mass of these particles. This technique is very powerful when used online after separation of the protein depending on their hydrodynamic radius, meaning SEC. This technique is always superior to normal SEC but requires special equipment and especially more time. However, if the protein fold is not really globular or other effects occur (see above: ionic, hydrophobic, etc.) assumption on size and oligomeric state based on SEC is not possible at all. For protein crystallization information about monodispersity, which can be provided by such an experiment, is an additional benefit.

### 3.4.6 Analysis of the homogeneity – High-throughput methods

Despite the development of various sophisticated methods, a bottleneck of homogeneity screening is high-throughput analysis. As mentioned above, proteins need to be analyzed by SEC and/or LS experiments after visual read-out of the protein-buffer droplets. Therefore, fluorescence-based solubility screens were developed that allow the high-throughput analyzes of many samples in a 96-well format (Ericsson, Hallberg et al. 2006; Alexandrov, Mileni et al. 2008; Kean, Cleverley et al. 2008). All these assays use fluorophores as reporters of the protein state. A suitable fluorophore is, for example, Sypro Orange, which exhibits different fluorescence properties as a function of its environment. This dye is almost dark in hydrophilic environment, however, after binding to hydrophobic molecules, it emits light at 570 nm. In inhomogenous and unfolded protein samples hydrophobic amino acids are exposed to the surface of proteins (Murphy, Privalov et al. 1990). An increase in the fluorescence signal of Sypro Orange correlates therefore with unfolding events of proteins. The homogeneity screening can be performed in basically two ways: temperature- or time-dependent. For the first setup the protein sample is heated gradually in distinct steps (i. e. 1 °C) and the emission is monitored at 570 nm. Hereby, a "melting" temperature is determined, which is characterized by 50% fluorescence of the maximal fluorescence at the highest temperature; the higher the melting temperature, the higher the stability of the protein (Ericsson, Hallberg et al. 2006). Secondly, the protein sample is incubated at a specific temperature (i. e. 40°C) and the fluorescence is measured for a period of time. The "half-life" time, at which 50 % fluorescence of the maximum fluorescence in one sample is detected, can be compared to all buffer conditions. In Figure 4 an example of the time-dependent approach is shown. Here, the protein is incubated in different buffers with various salt concentrations. The emission of Sypro Orange is recorded each minute at 570 nm. An analysis of all time-dependent fluorescence plots indicates that the protein is most stable in buffers containing 125 mM NaCl but unfolds fast in 1 M ammonium sulfate. These assays result in qualitative indications about a favourable environment of proteins that enhance the stability. Ericsson et al. proved the concept of this method by applying it to

different proteins (Ericsson, Hallberg et al. 2006). The stability optimization yielded a twofold increase in initial crystallization leads. Moreover these assays enable the search for putative ligands of the protein. Upon binding of a substrate in the binding pocket or an inhibitor, the stability of the protein increases, which can be detected experimentally.

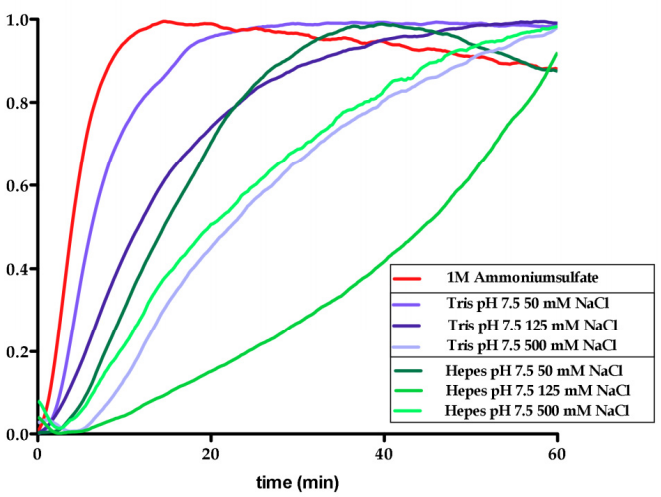


Fig. 4. Time-dependent stability optimization screen using Sypro Orange as reporter. The protein is diluted 1:50 into each test buffer containing Sypro Orange, excited with 490 nm and the fluorescence at 570 nm is measured for 60 minutes automatically with a PLATE READER (Fluorostar, BMG Labtech). Normalized fluorescence is plotted against the time.

## 4. Protein crystallization: Introduction

Protein crystals suitable for X-ray diffraction experiments and usable for subsequent structure determination are normally relatively large with a size of at least 10 to 100 µm. In contrast to crystals of mineral compounds, protein crystals are rather soft and sensitive to mechanical stress and temperature fluctuations. These properties are due to weak interactions between single proteins within the crystal, their high flexibility as well as the size of the macromolecules. The periodic network of building blocks is held together by dipole-dipole interactions, hydrogen bonds, salt bridges, van der Waals contacts or hydrophobic interactions. All of them have binding energies in the low kcal/mol range.
Especially the limited number of crystal contacts and their directionality are the largest difference to the high interactions generally observed in salt crystals. An example of the interactions within a protein crystal is shown in Figure 5. This picture highlights the main pitfalls in protein crystallization. A protein is a highly irregular shaped and flexible macromolecule which allows weak and stinted interactions at very specific locations of its surface. All vacuity is filled with buffer, in general not contributing to any kind of interactions between the protein molecules. Figure 5A shows a protein of around 30 kDa, which crystallizes in a rather small unit cell (shown in black). Only one protein monomer is located in the asymmetric unit of the unit cell, the other shown monomers represent symmetry related proteins. Figure 5B highlights the three-dimensional packing of protein molecules within a crystal.

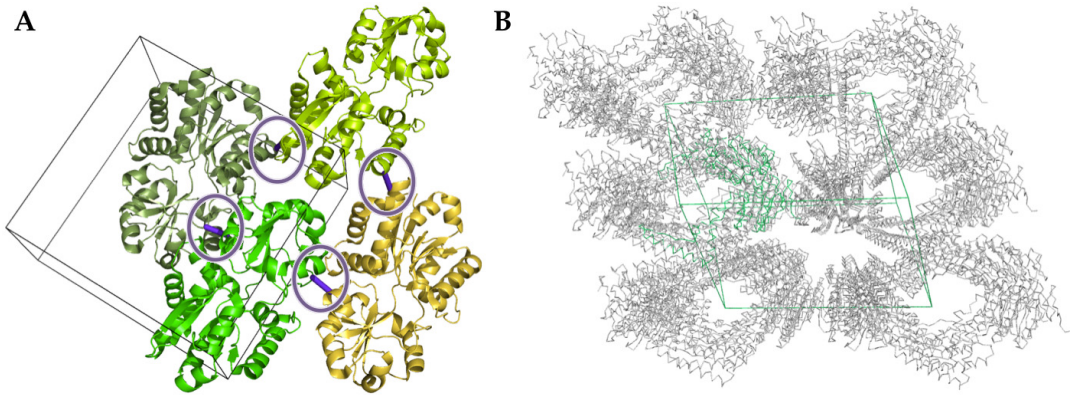


Fig. 5. Example of the packing within a crystal. A: The unit cell is shown in black, crystal contacts are highlighted with purple circles and lines. B: Three-dimensional crystal packing of a different protein. The unit cell as well as one protein monomer are depicted in green.

The flexibility as well as the other mentioned characteristics of proteins are responsible for the problems occuring during crystallization trials and despite extensive efforts not every protein is suitable for crystallization. If one cannot generate crystals one has to move back several steps and change the properties of the protein, e. g. surface properties by mutation of single amino acids, truncation of the protein or sometimes only changing buffer compositions that result in a more suitable protein for crystallization (see Figure 1 and also below). There are several prediction servers available that help choosing the 'right' protein and modification (Linding, Jensen et al. 2003; Goldschmidt, Cooper et al. 2007). However, protein crystallization still remains an empirical approach, sometimes called voodoo, while crystallography is science.

### 4.1 Phase diagram

The conditions or protocols for obtaining good crystals are still poorly understood and despite all progress and efforts protein crystallization is a trail-and-error approach. However, a step towards a better understanding of crystal growth can be achieved by analyzing the phase diagram of a protein-water mixture. The phase diagram is a simple illustration to help understanding how protein crystals are formed. Mostly, it is shown as a function of two ambient conditions that can be manipulated, i. e. the temperature and the concentration. Three-dimensional diagrams (two dependent parameters) have also been reported (Sauter, Lorber et al. 1999) and even a few more complex ones have been determined as well (Ewing, Forsythe et al. 1994). Figure 6 shows a schematic phase diagram for a protein solution as a function of protein concentration and precipitant concentration. The phase diagram is broken down into four distinct zones (Rosenbaum and Zukoski 1996; Haas and Drenth 1999; Asherie 2004):

1. Undersaturated zone: Under these condition the protein will stay in solution as neither the concentration of the protein nor of the precipitant is high enough to reach supersaturation.
2. Precipitation zone: Is the protein concentration or the precipitant concentration too high, the protein precipitates out of solution; this kind of solid material is not useful for crystallographic studies.

3. Labil zone: This is the most important configuration of the two parameters, as nucleation and initial crystal growth take place under these conditions.
4. Metastable zone: After initial crystals are formed and start growing in the labil zone, protein concentration decreases in the drop and the metastable zone will be reached. Here the crystal can grow further to its final maximum size.

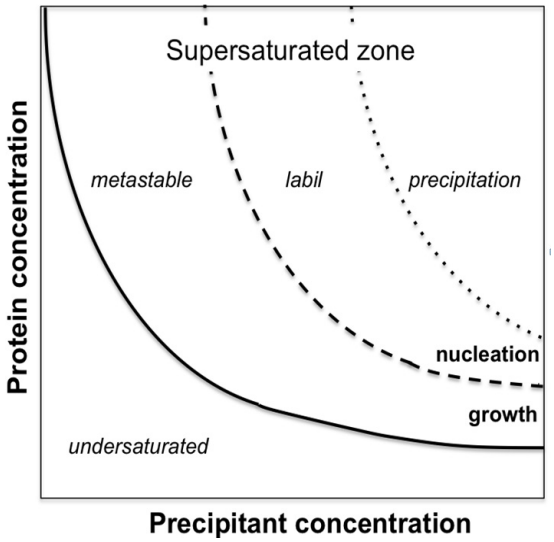


Fig. 6. A basic solubility phase diagram for a given temperature (adapted from (Rupp 2007).

The curve separating the undersaturated zone from the supersaturated one is called *solubility curve*. If conditions are chosen below the solubility curve, the protein will stay in solution and never crystallize. This means when a protein crystal is placed in a solvent, which is free of protein, it will start to dissolve. If the volume of the droplet is small enough it will not dissolve completely: it will stop dissolving when the concentration of the protein in the droplet reaches a certain level. At this concentration the crystal loses protein molecules at the same rate at which protein associate to the crystal – the system is at equilibrium. Determination of the solubility of the protein of interest might be a helpful information at the beginning of crystallization experiments. This can be done in a two-dimensional screen varying for example ammonium sulfate concentrations as well as the protein concentration.

### 4.2 Crystallization techniques

Crystallization is a phase transition phenomenon. Protein crystals grow from a supersaturated aqueous protein solution. Varying the concentration of precipitant, protein and additives, pH, temperature and other parameters induce the supersaturation. However, as mentioned before, prediction of this kind of phase diagrams is *a priori* impossible.
Protein crystallization can be divided into two main steps:

1. Generating initial crystals: 'Searching the needle in a haystack'
2. Empirical optimization of these crystallization condition

The first step is mostly based on experiences from other crystallization trials with different proteins. Nowadays several supplier offer crystallization screens that contain solutions for

initial experiments that were used successfully in the past for crystallization trials (Jancarik and Kim 1991), so-called "sparse matrix screens". There are also some trials around to use more systematic approaches (Brzozowski and Walton 2000) to get more information about solubility prior and simultaneous to crystallization (incomplete factorials, solubility assays). Both kinds of screens can be applied to different crystallization techniques.

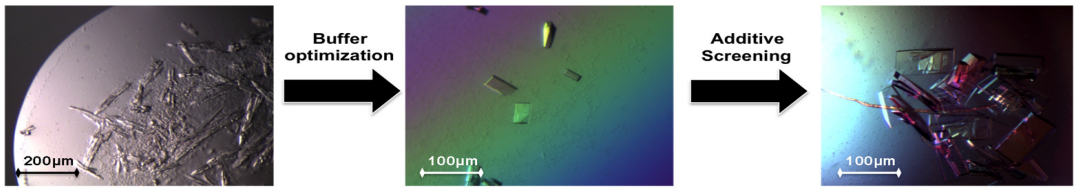


Fig. 7. Crystal optimization. First steps in crystal optimization are shown. Initial protein crystals look weak and fragile, after screening around this initial buffer composition crystal evaluation by eye results in less fragile, homogeneous looking crystals. However, diffraction quality was poor. Therefore an additive screening was performed that resulted in a different crystal form. These crystals finally were able to diffract X-rays to a reasonable resolution.

A lead/hit in that initial step might not be a 'real' crystal rather than a crystalline precipitate or just phase separation. In the next step, fine-tuning the buffer composition further optimizes this hit. Varying pH, salt concentration, type and concentration of precipitant and protein concentration are expected to yield larger and hopefully also better-diffracting crystals. In this step, the chemicals used are much more defined and therefore it is a more systematic than empirical screening (see Figure 7).

### 4.2.1 Vapor diffusion

The most popular and simplest technique to obtain protein crystals is the vapor diffusion method either in the sitting or hanging drop variant (see Figure 8). For both a defined volume (mostly < 1µl) of protein solution is mixed with an equivalent volume of screening solution and then equilibrated against the original precipitant/screening concentration. During this equilibration, the vapor pressure of the solution rises as the protein crystallizes (protein in solution lowers water activity) while the water evaporates to maintain equilibrium, which causes the precipitant concentration to rise. Therefore, if the crystal growth is sensitive to the precipitant concentration, vapor diffusion can rapidly force the mixture to unstable conditions where growth and nucleation are too rapid. This is the main disadvantage of vapor diffusion: Growing large crystals might be problematic!

### 4.2.2 Micro batch method

In this set-up the protein solution is mixed with screening solution at concentrations required for supersaturation right at the beginning of the experiment. Typical drop sizes of micro batch experiments ranges from 1-2 µl. The drop is then covered with oil, which acts as an inert sealing to protect the drops during incubation from evaporation (see Figure 8).

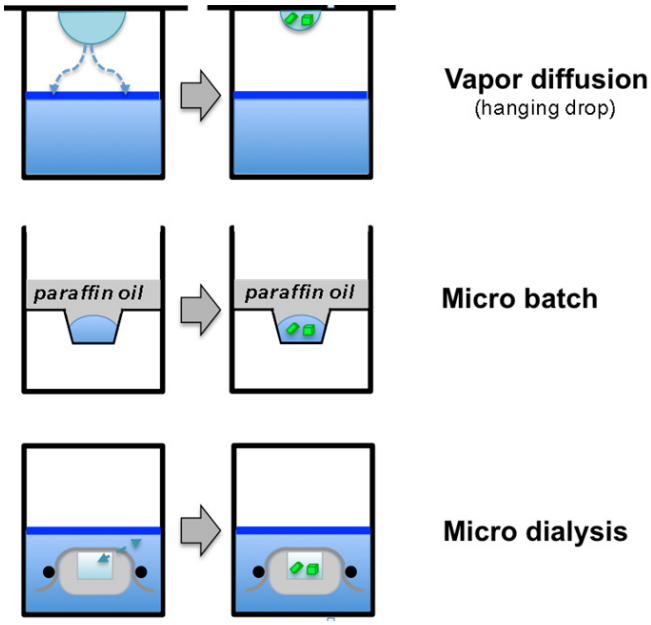


Fig. 8. Protein crystallization techniques. Schematic representation of a) vapor diffusion, b) micro batch and c) micro dialysis crystallization techniques widely used for crystal growth (adapted from (Drenth 2006)).

#### 4.2.3 Micro dialysis

Dialysis is another way to change the buffer composition and increase its concentration in the crystallization experiment gradually (see Figure 8). Micro-dialysis buttons are exposed to different screening buffers. This method requires rather high amounts of protein but might yield large crystals.

After obtaining initial crystal hits in a commercial screen the tough part of crystal optimization starts. By varying pH, salt concentration, temperature, precipitant concentration or protein concentration these initial crystals should be reproduced and become larger, more regular shaped or are simply growing faster. A further improvement of crystal quality might be achieved by the addition of small amounts of so called 'additives'. At this point basically each chemical compound might be sufficient to improve the crystal quality. Luckily, there are some preferable working additives, which have been proven to produce better crystal in more than one case. Especially compounds that are known to reduce undirected interactions in proteins like organic solvents, i. e. DMSO or phenol, or detergents and reducing agents are very often used at this stage and helpful to force more homogeneous well diffracting crystals.

### 4.3 Crystal nucleation

There are two fundamental steps during protein crystallization: Nucleation and crystal growth. If one cannot obtain single crystals of adequate quality for analysis, this is generally a consequence of problems associated with the growth phase (see above). But failure to obtain any crystals at all or failure to obtain single, supportable nuclei reflects difficulties in the nucleation step. Therefore control of nucleation is a powerful tool to optimize protein crystals

or sometimes it is the only way to get crystals at all. Nucleation can take place either homogeneous meaning in the bulk of the solution, when the supersaturation is high enough for the free-energy barrier to nucleus formation to be overcome or heterogeneous mostly by solid material in the crystallization solution. This can also occur even when the supersaturation is not achieved. Therefore in order to control nucleation one has to work with highly clean solutions to avoid nucleation by the second mentioned possibility. The nucleation zone can be bypassed by insertion of crystals, crystal seeds or other nucleants to the protein/precipitant mixture. Addition of crystals or tiny fragments of crystals is called seeding. This method is then subdivided into macro- and micro-seeding dependent on the size of the nucleant added. In macro-seeding experiments one single, already well-formed but small crystal is placed into a new crystallization solution at lower saturation. Microseeding in contrast requires small fragments of a crystal or almost invisible microcrystalline precipitate. These 'seeds' are then transferred into a fresh crystallization solution either by a seeding wand which is dipped into the microseed mixture to pick up seeds and then touched across the surface of the new drop or by a animal whisker or hair that is stroked over the surface of the parent crystal to trap the nuclei and then is drawn through the new drop. As this method also enhances the speed of crystal growth it can be used with sensitive substrate that undergo decomposition over time. Oswald et al. proved this in 2008 by solving the structure of ChoX from *Sinorhizobium meliloti* in complex with a highly hydrolyzing substrate, acetylcholine (Oswald, Smits et al. 2008). In classical vapor diffusion experiments crystals appear after four weeks but data showed only little electron density in the ligand-binding site and turned out to result from a choline bound instead of acetylcholine. Hydroxylation was favored due to the relatively long time for crystal growth but also because of an acetic pH in the crystallization set-up. To circumvent these problems accelerated crystal growth was required. In this case micro-seeding results in crystals suitable for data collection in less than 24 hours.

Recent years more effort in nucleation control yielded in fancy materials that can be used as nuclei for crystals. These methods use the second way of nuclei formation, as a solid material is introduce into the crystallization solution as an 'universal' nucleant (Chayen, Saridakis et al. 2006). There have been several substances that have been tried more or less successful. Some have been useful for individual proteins, but mostly they were not applicable in general (McPherson and Shlichta 1988; Chayen, Radcliffe et al. 1993; Blow, Chayen et al. 1994). In 2001, Chayen et al. proposed the idea of using porous silicon whose pore size is comparable with the size of a protein molecule. In theory such pores may confine and concentrate the protein molecules at the surface of the silicone and thereby encourage them to form crystal nuclei (Chayen, Saridakis et al. 2001). These nucleants have made it to commercial availability (www.moleculardimensions.com) and have proven to be suitable for different kinds of proteins and even membrane proteins that have not been possible to crystallize before formed nice crystals in the presence of these nucleants.

### 4.4 Cryoprotection

Exposure of a protein crystal at room temperature results in dramatic radiation damage due to radicals formed by the ionizing X-ray photons. To reduce that harmful disintegration of the protein crystal the crystal is cooled to ≈100K with the help of liquid nitrogen (Low, Chen et al. 1966; Hope 1988; Rodgers 1994; Garman 1999). However, it is common for the cooling process to disrupt the crystal order and decrease diffraction quality. Thus, the crystal must be cooled fast so that the water in the solvent channels is in the vitreous rather than in the

crystalline state at the end of this procedure. As for pure water this cooling has to take place very quick ($10^{-5}$s, (Johari, Hallbrucker et al. 1987), some water molecules can be replaced by a cryoprotective solution prior to cooling (Juers and Matthews 2004). This exceeds the time window for freezing up to 1-2s (Garman and Owen 2006) however, finding a good 'cryoprotectant' for a special protein crystal again involves substantial screening. Once flash frozen in liquid nitrogen, the crystal must be kept below the glass transition temperature of the cryobuffer at or below 155K at all times (Weik, Kryger et al. 2001).

### 4.5 What can you do when all efforts did not succeed in crystals?

#### 4.5.1 Buffer composition – Again!

The choice of the right buffer used for crystallization experiments is very crucial. As shown above, every protein needs its own buffer composition to feel kind of happy in this aqueous artificial environment. Especially as high protein concentrations (>10mg/ml) are required for crystallization, one might has to test several buffer compositions again (see also Figure 1). As a rule of thumb you should obtain around 50% of clear drops immediately after mixing protein and buffer solution. If you detect drastically more precipitation in your drops you should think first about lower protein concentration but of course secondly about changing your buffer system again.

#### 4.5.2 How to obtain a rigid protein suitable for crystallization?

To overcome the problem of flexibility of some regions in the protein addition of ligands is often a very powerful tool to fix the protein in a single conformation that is more favorable for crystallization. A good example for this strategy is the crystallization of so-called substrate binding proteins (for a recent review see (Berntsson, Smits et al. 2010)). These proteins catch their substrate in the periplasm of bacteria or on the outer membrane of archaea and then deliver it to their cognate transport system located in the membrane. The mechanism of substrate binding is quite well understood. These binding proteins all consist of two domains, which rotate towards each other during the binding event. In solution without substrate they are quite flexible and NMR-studies proved a equilibrium between open and closed conformation (Tang, Schwieters et al. 2007). Analysis of all available structures for this class of proteins showed that more than 95% were crystallized with a ligand bound (Berntsson, Smits et al. 2010). Thus, a stabilization of the two domains seems to simplify crystal contact formation dramatically. Although people always want to obtain a functional conformation of the protein in their crystal structure, it is sometimes helpful to think about how to stop the protein from doing its job. A non-functional protein is in general less flexible and fixed in one conformation. One example for successful implementation of this strategy is the crystal structure of NhaA from *Escherichia coli* solved in 2005 (Hunte, Screpanti et al. 2005). Here, Hunte et al., downregulated the protein activity by working at an acidic pH of 4. Although the protein shows almost no activity at this pH the structure reveals the basis for mechanism of Na+/H+ exchange and also its regulation by pH could be understood.

#### 4.5.3 Rational protein design for crystallization: Surface engineering

The first example of rational protein design that yielded a good diffracting protein crystal is given by Lawson et al. in 1991 (Lawson, Artymiuk et al. 1991). They compared amino acids

involved in crystal contact formation of the rat ferritin protein L. (which is highly homologous to human ferritin H, the target protein) with the amino acids present at that position in human ferritin H. A replacement of Lys86, found in the human sequence, with Glu, which occurs in rat, recreated a $Ca^{2+}$ binding bridge that mediates crystal contacts in the rat ortholog. As this method was successful for several other proteins (McElroy, Sissom et al. 1992; Braig, Otwinowski et al. 1994; Horwich 2000), a general protocol was required. The concept Derewenda et al. proposed in 2004 is based on the general equation for the free energy that drives protein crystallization:

$$\Delta G = \Delta H - T(\Delta S_{protein} + \Delta S_{solvent})$$

As the enthalpy values of intermolecular interactions in a crystal lattice are rather small (see above), crystallization is very sensitive to entropy changes of both protein and solvent. The formation of ordered protein aggregates carries a negative entropy term. This can only be overcome by positive entropy from the release of water bound to the protein. However, large hydrophilic residues (e.g. lysines, arginies, glutamates, glutamines) exposed on the protein surface need to be ordered. Since they are rather flexible this can cause problems. This can be overcome by mutating large amino acids into smaller ones, for example alanines. Among these large amino acids lysines and glutamates play a particular role, as they are always (with only very few exeptions) located on the protein surface (Baud and Karlin 1999). Both lysines and glutamates are typically disfavored at interfaces in protein protein complexes (Lo Conte, Chothia et al. 1999), therefore it is rather straight forward to assume that lysine and glutamate to alanine mutants are good targets for protein crystallization if wildtype protein hardly forms crystals. However this also means that you have to go several steps backwards on road to a protein structure determination (see Figure 1).

#### 4.5.4 Affinity tag removal: Philosophic question???

Another variant in protein crystallization nowadays is the affinity tag used for purification of the desired protein. The decision about position and choice of the affinity tag are mostly made at the beginning of the long way to a crystal structure (see Figure 1). However, it becomes crucial again at the crystallization step. In general most people like to remove the tag before crystallization to prove a physiological conformation. But, there are examples where the tag played a pivotal role in crystallization (Smits, Mueller et al. 2008a). The crystal structure of the octopine dehydrogenase from *Pecten maximus* is shown in Figure 9 (Smits, Mueller et al. 2008b), with the interactions sides/crystal contacts highlighted in green.
In Figure 9A contacts look quite similar to that presented in Figure 5. However when having a closer look on the his-tag, you recognize that it is located in a cavity formed by another monomer of that protein. In that cavity it can perform several hydrogen bonds with amino acids from the other monomer resulting in a very strong interaction which yields good quality crystals.

#### 4.5.5 Crystallization using antibody fragments

A number of ways to stabilize proteins for crystallography have been developed, for example genetic engineering, co-crystallization with natural ligands and reducing surface

entropy (see above). Recently, crystallization mediated by antibody fragments has moved into the focus of crystallographers especially to obtain crystals of membrane proteins (Ostermeier, Iwata et al. 1995; Hunte and Michel 2002). Membrane protein crystallization is even tougher compared to soluble proteins, because of the amphipathic surface of the molecules. As they are located in the lipid bilayer most of their surface is hydrophobic and must be covered to keep them in solution. This is maintained by detergents. The detergent micelles cover the hydrophobic surface and therefore this area is no longer available to form crystal contacts. Crystal contacts can only be formed by the polar surfaces of these proteins. As many membrane proteins contain only relatively small hydrophilic domains, a strategy to increase the probability of getting well-ordered crystals is required. Antibody fragments can play this role. They can be designed for binding at specific regions in the protein and then function as additional polar domain in the membrane protein complex (for example see (Ostermeier, Iwata et al. 1995; Huber, Steiner et al. 2007).

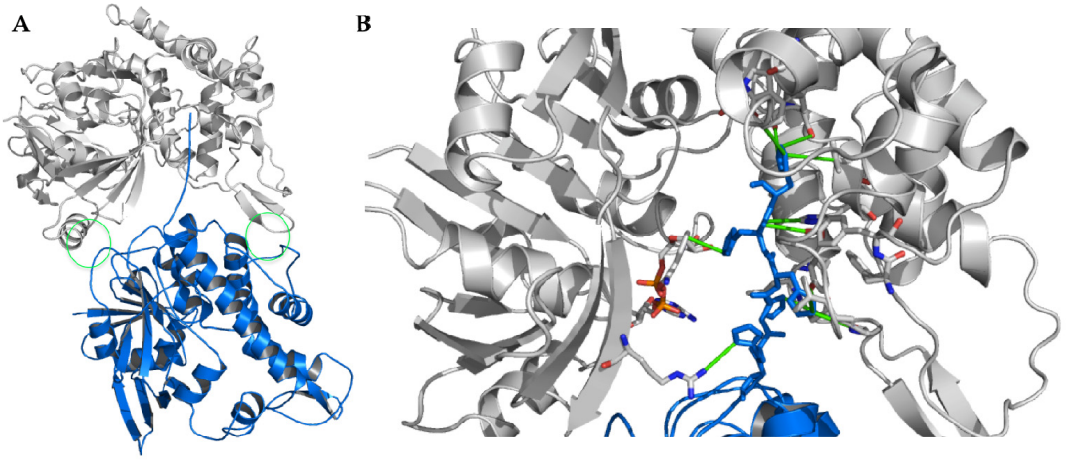


Fig. 9. Crystal contacts in OcDH protein. A: Overall view on two monomers. Surface Crystal contacts are highlighted in the green circles. B: Zoom in on the His-tag of one monomer. The his-tag of one monomer in the crystal structure is located near the binding site in a deep cavity formed by the other monomer. Therefore it is able to form several hydrogen bonds (highlighted in green) with side and main chains of the other protein but also with the ligand bound in this binding site (orange).

## 5. Conclusion

*For what reason do we effort so much work on good quality crystals?*

Single good quality crystals constitute an essential prerequisite for structural investigations of biological macromolecules using X-ray diffraction. The harder one works on crystal quality the easier the determination of a reasonable atomic model of the molecule of interest becomes. The vast majority of problems encountered in crystal structure determination can typically be traced back to data-quality issues caused by crystal imperfections. Consequently, although primary focus of structural biology is on the macromolecule that makes up a crystal, there is also considerable interest in the physical properties, nucleation

and growth of the crystals themselves. Statistics of various Structural Genomics Centers proved that protein crystallization is still despite all the progress in the technology of crystallization robotics is still a rather tough field in biological science. Success rate ranges for small prokaryotic proteins from 10-30% and decreases dramatically to a few percent for human proteins. The struggle obtaining crystals for protein structure determination is justified. After all efforst looking at electron density and subsequent the protein structure is still one of the most intriguing as well auspicious parts in structural biology

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Top of page

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## 3.2 Chapter II - Nisin Gene Cluster and Immunity

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# Lantibiotics: How do producers become self-protected?

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Zainab Alkhatib, **André Abts**, Antonino Mavaro, Lutz Schmitt and Sander H. J. Smits

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# Lantibiotics: How do producers become self-protected?

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ABSTRACT

Lantibiotics are small peptides produced by Gram-positive bacteria, which are ribosomally synthesized as a prepeptide. Their genes are highly organized in operons containing all the genes required for maturation, transport, immunity and synthesis. The best-characterized lantibiotic is nisin from *Lactococcus lactis*. Nisin is active against other Gram-positive bacteria via various modes of actions. To prevent activity against its producer strain, an autoimmunity system has developed consisting of different proteins, the ABC transporter NisFEG and a membrane anchored protein NisI. Together, they circumvent the ability of nisin to fulfill its action and cause cell death of *L. lactis*. Within this review, the mechanism of regulation, biosynthesis and activity of the immunity machinery will be discussed. Furthermore a short description about the application of these immunity proteins in both medical and industrial fields is highlighted.



## 1. Introduction

Lantibiotics are produced by Gram-positive bacteria. Well-known examples are nisin from *Lactococcus lactis* and lacticin 481 from *Streptococcus lactis* (Bierbaum and Sahl, 2009). They can function as quorum sensors to activate their own biosynthesis like nisin (Kuipers et al., 1995) or cytolysin from *Enterococcus faecalis* (Coburn et al., 2004). Quorum sensing is used by bacteria to regulate cell-density dependent gene expression (Kleerebezem et al., 1997). Herein, cells sense a specific molecule, which functions as a signal and induces gene expression (Kuipers et al., 1998). For example, nisin production and secretion starts at the early growth stage of *L. lactis* and the produced nisin is absorbed on the cell surface and induces its own biosynthesis (Hilmi et al., 2006). Due to a drop in pH during growth, absorbed nisin is released from the cell surface into the media during the mid-logarithmic growth phase at a pH below 5.5. Only then, nisin activity can be detected in the medium and nisin production reaches a maximum at the early stationary phase (Kleerebezem et al., 1997).

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 in air (Kodani et al., 2004).

Lantibiotics are ribosomally synthesized as a prepeptide, post-translationally modified by several enzymes and finally converted into the active form (Hansen, 1993). The terminus "lantibiotic" is derived from "*lan*thionine containing an*tibiotic*" (Schnell et al., 1988). Lanthionines are thioether bridged amino acids and introduce intramolecular ring formation within the peptide (Hansen, 1993). The fact that nisin and Pep5 from *Staphylococcus epidermidis 5*, which contain (methyl)lanthionine rings, were not degraded by proteases, led to the conclusion that (methyl) lanthionine rings protect lantibiotics against proteolytic degradation (Bierbaum et al., 1996; van der Meer et al., 1993).

Three classes of lantibiotics are known according to their biosynthesis and function (Willey and van der Donk, 2007). Class I lantibiotics like nisin, subtilin and epidermin are posttranslationally modified by two distinct enzymes, LanB and LanC, and subsequently this modified prepeptide is exported by a dedicated ABC transporter, LanT. The maturation of class I lantibiotics is terminated after the leader peptide is cleaved off by a protease. Class II lantibiotics like lacticin 481 or mersacidin are posttranslationally modified by a single enzyme, LanM, which catalyzes both modification reactions (Siezen et al., 1996). A single, multifunctional protein performs the export of the lantibiotic and the cleavage of the leader peptide. This membrane protein is also designated LanT. Both classes have significant biological antimicrobial activity, while class III of lantibiotics is lacking this activity, which is the definition of the latter class (Willey and van der Donk, 2007).

Both, class I and class II lantibiotics are active against Gram-positive bacteria and exert various mechanisms of activity. Some of these mechanisms are described below. Nisin inhibits cell-wall

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**Table 1**
Lantibiotics are listed together with the producers strains, its classification, as well as the immunity proteins present within its strain.

| Lantibiotics | Strain | Class I or II | Immunity protein |
|---|---|---|---|
| Nisin | *Lactococcus lactis* | Class I | NisFEG, NisI |
| Subtilin | *Bacillus subtilis ATCC6633* | Class I | SpaFEG, SpaI |
| Ericin | *Bacillus subtilis A1/3* | Class I | EriFEG, EriI |
| Epidermin | *Staphylococcus epidermidis* | Class I | EpiFEG, EpiH |
| Gallidermin | *Staphylococcus gallinarium* | Class I | GdmFEG, GdmH |
| Pep5 | *Staphylococcus epidermidis* | Class I | PepI |
| Epicidin | *Staphylococcus epidermidis BN280* | Class I | EciI |
| Epilancin | *Staphylococcus epidermidis K7* | Class I | ElxI |
| Lacticin 481 | *Lactococcus lactis CNRZ 481* | Class II | LctFEG |
| Mutacin II | *Streptococcus mutans T8* | Class II | MutFEG |
| Nukacin ISK-1 | *Staphylococcus warneri ISK-1* | Class II | NukFEG, NukH |
| Lactocin S | *Lactobacillus sakei L45* | Class II | LasJ |
| Lacticin 3147 | *Lactococcus lactis DPC3147* | Class II | Ltn FEG, LtnI |
| Mersacidin | *Bacillus sp. strain HIL* | Class II | MrsEFG |

synthesis by binding to lipid II (Hasper et al., 2006), an essential membrane-anchored cell-wall precursor. Another mechanism is the binding of nisin to lipid II, which induces membrane integration of nisin resulting in the formation of pores composed of nisin and lipid II molecules (Hasper et al., 2004).

There is a highly specific interaction between nisin and lipid II and this is reflected by low micromolar concentrations of nisin, which are sufficient to permeabilize the membrane of the targeted bacteria (van Heusden et al., 2002). The bactericidal activity of lantibiotics is generally measured by quantifying growth inhibition of the target bacteria, either on agar plates or in liquid culture. On agar plates, the zones of growth inhibition of the indicator organism can be visualized and allow a precisely determination of the minimum inhibitory concentration (MIC) (Wiedemann et al., 2001). Alternatively, bacterial growth can be monitored in liquid culture in dependence of the lantibiotic concentration by measuring the optical density. This method allows a simple and quantitative determination of the $IC_{50}$, the concentration of the antibiotic that inhibits cell growth by 50% (Oman and van der Donk, 2009). However, due to the differences in the purification protocols, a quantitative comparison between different studies is difficult as slight difference in the preparation can result in up to 10-fold difference in the determined $IC_{50}$ (Abts et al., 2011).

Since low amounts of a lantibiotic are already sufficient to harm or even kill the producer strain, it has to prevent such a suicidal mode of action. Thus the producer strain regulates the expression of an immunity system induced via measurement of the extracellular concentration of the lantibiotic. The proteins participating in immunity are generically called LanI and LanFEG. Table 1 summarizes some of the known immunity genes.

We focus on the self-immunity proteins of the strains producing antimicrobial peptides with a special emphasis on the nisin immunity proteins located in the nisin operon of *L. lactis*. In this review, the genetic structure, expression, and mode of action of these immunity proteins will be summarized.

## 2. Biosynthesis and gene organization

All lantibiotics biosynthesis pathways are arranged in a single "gene cluster" or "operon" within the genome of the organism. These operons are responsible for the synthesis of these lantibiotics and are, in general, induced by their own products or by environmental changes. Although the genes have been given the generic locus symbol "*lan*", each member of this family has its own designated name based on the produced lantibiotic (e.g., *nis* for nisin, *epi* for epidermin). The operon may be found on a transposon (e.g. nisin), on the chromosome of the host (e.g. subtilin), or on a plasmid (e.g. epidermin) (Champak Chatterjee, 2005). Several sequences of the genes involved in lantibiotic maturation, regulation, transport as well as immunity have been revealed. The ability of organisms to produce and secrete novel lantibiotics can be detected based on the sequence homology of candidate proteins found in newly sequenced genomes.

Genes with similar function have been found in almost all lantibiotic gene clusters, sometimes however arranged differently (see Fig. 1). One report indicates that no uniform orientation or order in the position of the genes in different operons can be derived (Siegers and Entian, 1995), while some exceptions exist, for example the *nis* (*L. lactis*) and *spa* (*Bacillus subtilis*) operons, which have a high similarity, or the *epi* and *gdm* operons, which are identically organized.

Operons of the class I as well as class II lantibiotics are depicted in Fig. 1. To highlight their similarity, genes are color-coded according to the function of the resulting protein. Thereby, the similarity in the operon structure as well as their encoded proteins are visualized according to their involvement in biosynthesis and maturation (green), transport (orange), processing (purple), expression and regulation (dark blue), as well as immunity (yellow).

The nisin operon consists of 11 genes expressing the following proteins (Fig. 1): NisA, which is composed of a leader sequence corresponding to amino acids 1–23 at the N-terminus and 34 amino acids that encode the active peptide, NisB, the dehydratase, catalyzes the dehydration of serine and threonine residues in the prepeptide (Karakas Sen et al., 1999) and specifically recognizes parts of the leader sequence (Mavaro et al., 2011). In the next step of maturation, dehydrated prenisin is modified by the cyclase NisC, which catalyzes the condensation of dehydrated residues to C-terminal positioned cysteine residues to form (methyl)lanthionine rings (Koponen et al., 2002). After that, the ABC transporter NisT exports fully modified prenisin (Qiao and Saris, 1996), which is still inactive until the signal sequence is cleaved off by the cell-membrane anchored protease NisP (van der Meer et al., 1993).

For class II lantibiotics, i.e. lacticin 481, (Fig. 1), the functions of the dehydratase and the cyclase are fused to a single gene coding for a protein called LctM (Paul et al., 2007). Furthermore, the LctT transporter has two functions, it exports the modified peptide, similar to class I lantibiotics, and it cleaves the leader sequence (Champak Chatterjee, 2005).

Compared to nisin, other class I lantibiotics gene operons have similar operon structures. The subtilin operon, shown in Fig. 1, shares a high degree of similarity with the nisin operon with a single exception. Here, the protease LanP is missing in the subtilin operon. However, it has been shown that pre-subtilin is cleaved by several extracellular serine proteases, which are not encoded on the subtilin operon (Corvey et al., 2003). Another example is epidermin. This operon contains additional genes like the *epiD* gene encoding an enzyme responsible for specific post-translational modifications on the N-terminus of epidermin during biosynthesis (Majer et al., 2002).

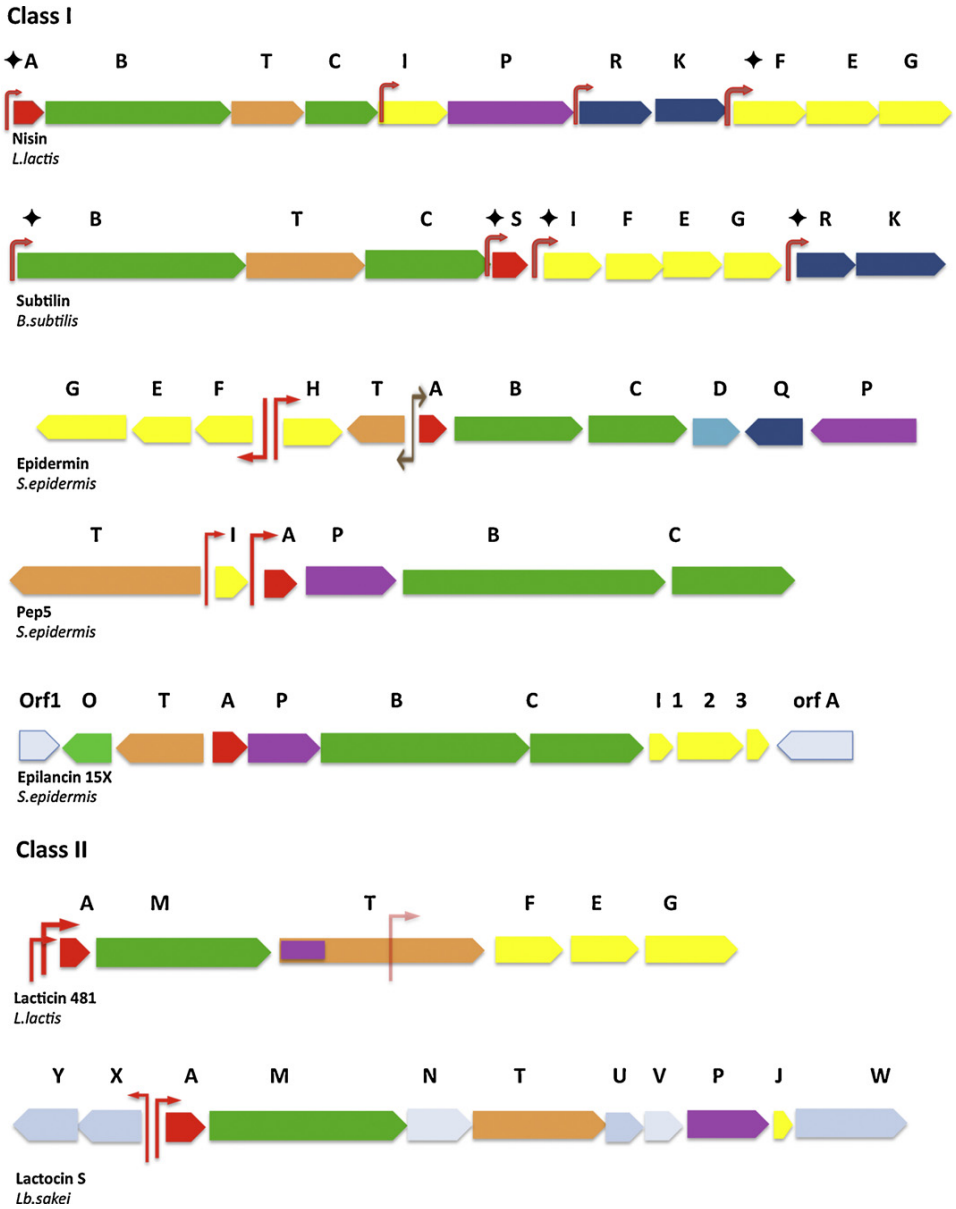


**Fig. 1.** Representative gene clusters of selected class I and class II lantibiotics. The selected examples are nisin, subtilin, epidermin, Pep5, epilancin, lacticin 481 and lactocin S. Genes with the same function are presented in identical colors. Operons associated with a transcriptional promoter (constitutive promoter) are highlighted by a red arrow, while arrows with * refers to a regulated promoter.

Nisin biosynthesis is auto-regulated by a specific two-component regulatory system, NisR and NisK, which are also found in other lantibiotic systems like the subtilin operon of *B. subtilis* (Cheigh and Pyun, 2005) (described in more details in paragraph 3 of this review).

The nisin operon contains four other genes (shown in yellow in Fig. 1): *nisF*, *nisE* and *nisG*, which, when expressed, assemble an ABC transporter and *nisI* encoding a lipoprotein. These proteins together form an immunity system within *L. lactis* against nisin (Siegers and Entian, 1995). The exact regulation and functions of these proteins will be discussed in more details below.

A comparison of the immunity systems of all lantibiotics reveals that nisin, subtilin, epidermin, lacticin 3147, streptococcin AFF-22, mutacin II and lacticin 481 all harbor a member of the LanFEG family as part of the immunity system. In contrast, Pep5, epicidin and lactocin S only require a LanI protein – PepI, EciI and LacJ – to gain resistance against their own lantibiotic.

Interestingly, lantibiotics that are produced by the strains that contain both proteins, LanFEG and LanI, form pores and bind lipid II (Guder et al., 2002). Since, mersacidin which is produced by a strain that only contains LanFEG, binds to lipid II, but does not form pores, it is assumed that both, LanFEG and LanI, are required to inhibit pore formation (Champak Chatterjee, 2005) within the membrane of the producer strain.

## 3. Regulation of lantibiotic biosynthesis and its immunity system

The expression of many lantibiotics, like nisin or subtilin, is regulated by a two-component regulatory system, consisting of

a receptor histidine kinase (LanK) and a transcriptional response regulator (LanR) (Chatterjee et al., 2005; Stock et al., 2000). In case of nisin or subtilin, the extracellular lantibiotic acts as a signal, which is recognized by the corresponding LanK, and induces autophosphorylation of a histidine residue within LanK (Bierbaum and Sahl, 2009). The high energetic potential of the phosphoryl group is subsequently transferred to an aspartate residue within the response regulator protein (LanR) (Stock et al., 2000). 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 (Chatterjee et al., 2005). In non-lethal concentrations, these lantibiotics serve as a signal and induce their own biosynthesis (Bierbaum and Sahl, 2009; Chatterjee et al., 2005; Willey and van der Donk, 2007). The biosynthesis of some other lantibiotics is regulated by systems unrelated to a two-component regulatory system, for example, a change in pH of the environment induces lacticin 481 production via the transcription regulator RcfB (Madsen et al., 2005). Furthermore, the production of the two-component lantibiotic cytolysin from *E. faecalis* is stimulated by the presence of a potential target cell (Coburn et al., 2004). 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, the $CylL_L$ preferentially binds to the target membrane. On the other hand, monomeric $CylL_S$ functions as a signal peptide to induce cytolysin production, which is repressed by CylR2 in the absence of target cells (Coburn et al., 2004).

Similar regulation loops of lantibiotic production and immunity were observed for subtilin from *B. subtilis* (Stein et al., 2002) (Fig. 1). Here, three subtilin inducible promoters regulate the production of the subtilin prepeptide, the modification enzymes as well as the immunity proteins SpaIFEG. In contrast to the nisin system, the two-component system SpaRK of the subtilin-producer strain *B. subtilis* ATCC 6633 is positively controlled by the sigma factor H, SigH. The transcription of *sigH* is negatively controlled by the suppressor AbrB when the *B. subtilis* culture is at the late growth phase (Stein et al., 2002). Nisin, the activator of the *nisA* promoter, results in the transcription of the *nisABTCIP* genes (Kuipers et al., 1995) (see Fig. 1). Another three promoters have been identified within the nisin operon. Two of them depend on the concentration of external nisin, the *nisA* and *nisF* promoters (Qiao and Saris, 1996). Whereas, the *nisR* promoter ensures a constitutive expression of the sensor, NisK and regulator, NisR (de Ruyter et al., 1996). Additionally, the activation of the *nisA/Z* promoter also leads to transcription of *nisRK* (Ra et al., 1996). This guarantees that the signal transduction cascade is always present at sufficient basal levels. The fourth promoter is the *nisI* promoter, which is also a constitutively active promoter ensuring a basal level of immunity against nisin. Without these feed-back loops, the first expression of nisin would be a severe problem for any host. However, upon the activation of the *nisA* promoter, the expression of NisI is also elevated. Thus, an elevated level of resistance is provided. Full immunity, however, is only obtained via the activation of the *nisF* promoter, which initiates the expression of the *nisFEG* genes. The *nisF* promoter is also induced via the NisR/NisK system as disruption of the *nisR/nisK* genes leads to higher susceptibility of *L. lactis* strains against nisin, due to the lack of the full or elevated transcription of the *nisFEG* as well as *nisI* genes (Saris et al., 1996).

However, the *nisF* promoter has a weaker transcription efficiency compared with the *nisA* promoter (de Ruyter et al., 1996). Higher nisin concentrations are required to reach the same transcription levels of *nisF* promoter dependent genes as compared with the *nisA* promoter controlled genes (de Ruyter et al., 1996). As a consequence, genes directed by the *nisF* promoter became transcript at high external nisin concentrations.

## 4. Immunity against nisin

Lantibiotics, produced by Gram-positive bacteria, possess an antimicrobial activity and are produced to ensure survival of the organism in times of, for example, nutrition shortage. Furthermore it has been shown that small peptides are used for cell–cell communication (Sturme et al., 2002). The producer strain, however, should not be affected by the lantibiotic. Therefore, they developed an immunity mechanism containing a distinct set of genes, which when expressed, lead to a specific resistance against their own produced lantibiotics. These self-immunity proteins are called LanFEG and LanI (Draper et al., 2008).

LanFEG is an ABC transporter, which is energized by binding and/or hydrolysis of ATP and allows transport (export or import) of substances across the membrane of a bacterial cell. The second protein, LanI, is an immunity protein expressed in the cells. Some part is covalently attached to a palmotyl moiety at the N-terminus, and thereby localized to the outer leaflet of the cell membrane, while the remaining part is secreted into the medium.

### 4.1. The ABC transporter NisFEG

ATP binding cassette (ABC) transporters comprise one of the largest families of membrane proteins present in all kingdoms of life. They transport a large variety of substrates ranging from small ions to large proteins of up to 800 kDa of size (Hinsa et al., 2003). They can be subdivided in two major classes, the export and import ABC transporters (Davidson et al., 2008). Generally ABC transporters consist of four domains, two hydrophobic membrane domains (TMDs) and two hydrophilic cytosolic nucleotide binding domains (NBDs). The NBDs show a high sequence similarity between all ABC transporters despite their widely different transport substrates, while the TMDs display very little sequence homology. In the genome of an organism, NBDs are recognized by certain sequence motifs that are important for ATP binding and hydrolysis. Strikingly, all of the characteristic sequence motifs of an ABC transporter reside within the NBD. These are the Walker A, the Walker B, the H-loop and the two hallmarks of ABC transporters, the C-loop or ABC signature motif (LSGGQ) (Schmitt and Tampe, 2002), and the equally distinctive feature, the D-loop (Higgins and Linton, 2004; van der Does and Tampe, 2004; Zaitseva et al., 2006). Generally, within an operon, the membrane component(s) are located next to the NBD. Although, in some cases both proteins are fused to one polypeptide.

Several high-resolution structures of full length ABC transporters and several NBDs have now been solved (Dawson and Locher, 2006; Hollenstein et al., 2007; Hvorup et al., 2007; Locher et al., 2002; Schmitt et al., 2003; Smith et al., 2002; Zaitseva et al., 2005a). Based on these structures as well as biochemical experiments, we know that ABC transporters function as dimers (Fetsch and Davidson, 2002; Zaitseva et al., 2005a,b). They need an ATPase domain that is involved in the binding and hydrolysis of ATP that, in turn, provides energy for the transport of the substrate via the transmembrane domains. ABC importers contain an extra domain (Berntsson et al., 2010), either fused to the transporter or as an extra lipophilic membrane associated protein which binds substrate with high affinity and releases it into the designated transport for subsequent import into the cell. Since the function of these proteins is to bind substrate, they are called “substrate binding proteins (SBPs)”. These SBPs undergo a substantial domain movement upon binding of the substrate as well as during release of the substrate to the ABC importer, which has been called the “Venus Fly trap” mechanism and has been well studied over the last decades (for a recent review see Berntsson et al., 2010). Generally, there are at least two ABC transporters found in the lantibiotic operon, like LanT, which transport the prepeptide and LanFEG, which is involved

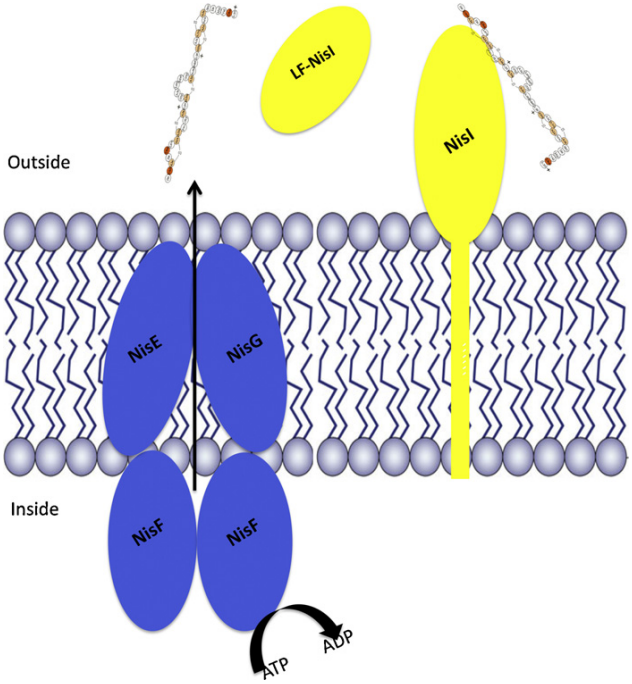


**Fig. 2.** The nisin immunity system. The nisin immunity system consist of NisFEG (color coded in blue) as well as NisI (color coded in yellow) which can be anchored at the membrane or in a soluble form (LF-NisI). For more details see text.

in the immunity. A schematic view of the immunity transporter is shown in Fig. 2. Here, NisF represents the NBD, which binds and subsequently hydrolyzes ATP. The membrane component consists of two membrane proteins, called NisE and NisG, forming a heterodimeric membrane complex. NisG is a 24 kDa integral membrane protein consisting of 214 amino acids with six predicted transmembrane spanning helices. NisE is a 28-kDa integral membrane protein containing a number of six predicted transmembrane helices as well. Assembled as a complex, these three proteins form a functional ABC transporter. Deletion of one of the subunits abolishes the resistance to the lantibiotic completely (Siegers and Entian, 1995).

The composition of an ABC transporter by three single polypeptide chains is not unusual for ABC transporter, however it is mainly found for ABC importers. Two of the best characterized systems are the maltose uptake system of *Escherichia coli*, $MalFGK_2$ where MalF and MalG are the transmembrane components (Davidson et al., 2008), and the histidine uptake system consisting of an ABC transporter $HisQMP_2$ and a soluble substrate-binding receptor HisJ (Ames et al., 2001). In both cases, the ABC transporter consists of three proteins. Only one example of a bacterial ABC exporter is known where the ABC transporter consists of three individual polypeptides. The $LolD_2CE$, which is involved in sorting of lipoproteins and subsequent detachment from the membrane leading to the water soluble lipoprotein LolA in the periplasmic space of *E. coli* (Narita, 2011).

Next to the above mentioned sequence motifs that are characteristic for ABC transporters, the NBD harbor a highly conserved glutamine in the so-called "Q-loop". This glutamine is involved in rigid-body motions within the NBD upon ATP binding and seems to act as a sensor for ATP. Recently, Okuda et al. suggested that this glutamine of the Q-loop is replaced by a glutamate residue in the family of lantibiotic immunity ABC transporters. In addition to the sensor function, the Q-loop has been shown to be involved in communication between the NBD and transmembrane segments of the TMD (Dawson et al., 2007; Oldham et al., 2008). Mutational studies of the NukFEG transporter, which gives resistance to nukacin ISK-1 revealed that resistance has been lowered or completely abolished upon mutation of this glutamate residue to either Gln or Ala (Okuda et al., 2010). This highlights the important role of the unusual sequence of the Q-loop in the immunity activity of lantibiotic ABC transporters.

Sequence comparison revealed that NisFEG is conserved in all species producing nisin and also shares a significant amount of similarity with strains producing other lantibiotics like for example *B. subtilis*, which produces subtilin.

Hydrophobicity analysis of NisE and NisG demonstrated that both proteins contain six transmembrane helices. However, the conservation of tryptophan residues within these sequences is more important (see Fig. 3). An alignment of the NisE and NisG with homologues from *Streptococcus uberis* (NusE and NusG), *Bacillus cereus G9241* (BsaE and BsaG), *B. subtilis* (SpaE and SpaG) and *Finegoldia magna ATCC 53516* (MutE and MutG) is shown in Fig. 3A and B. The appearance of tryptophan residues is generally rare in proteins. In NisE and NisG 9 (out of a total of 242; 4%) and 5 (out of a total of 214; 2.5%) tryptophan residues are present, respectively. More striking is the fact that these Trp residues are almost exclusively located within the predicted transmembrane helices. In helix II of NisE, two Trp residues are located in the middle, while in helix IV, one Trp is located slightly more towards the cytoplasm. At the end of helix V, a conserved Trp is followed by a – PYTY – sequence motif. This motif is conserved among the immunity genes and suggests that these aromatic or even Trp residues fulfill a specific role during substrate transport. Also, sequence alignment of NisG revealed a conserved Trp residue at the end of helix V. In the middle of helix V as well as after helix VI two other Trp residues are observed within BsaG, SpaG, and MutG. These Trp are not conserved in NusG and NisG. The latter two, however, have two other Trp residues located in the middle of helix III and IV; maybe these residues counterbalance missing Trp residues (see Fig. 3). The conservation of these residues distributed at the cytosolic, middle and exterior site of the transmembrane helices suggest a distinct transport mechanism conserved among all these proteins.

The NisFEG complex is expelling nisin molecules, before or during pore formation into the surrounding media. It is confirmed that NisFEG exports nisin molecules from the membrane, not modifying or degrading it, as most of the nisin could be recovered when applied to *B. subtilis* cells expressing NisFEG (Stein et al., 2003).

The relative contribution of NisFEG to immunity, first studied in 1995 (Siegers and Entian, 1995), was determined by a knockout approach. Disruption of NisF and NisE made cells more sensitive as in the wild type background. However, mutation of NisG did not have a significant effect on immunity (Siegers and Entian, 1995). Nevertheless, maximal immunity of *L. lactis* cells can only be achieved, if the immunity proteins NisI and NisFEG and the modification and processing machinery NisBTCP are expressed. The independent deletion of *nisABTCI* genes and the effect on the immunity and nisin production is well described (Ra et al., 1999). The NisFEG proteins maintain the crucial part of the nisin immunity in *L. lactis*. This is confirmed by gene deletions (e.g. Δ*nisA*, Δ*nisB*, Δ*nisC*, and/or Δ*nisT*), which result in a nisin non-producing strain. If nisin is not produced, the histidine kinase NisK does not phosphorylate NisR and consequently the *nisA* and *nisF* promoters are not activated. Thus only minimal levels of the immunity proteins are present. If these knockout strains are supplemented with minimal concentrations of nisin, the immunity increases due to the expression of NisI and NisFEG (Ra et al., 1999).

### 4.2. NisI, a second line of defense?

A second protein family involved in lantibiotic immunity is the LanI family. In the case of the nisin operon, NisI is a 245 amino acid

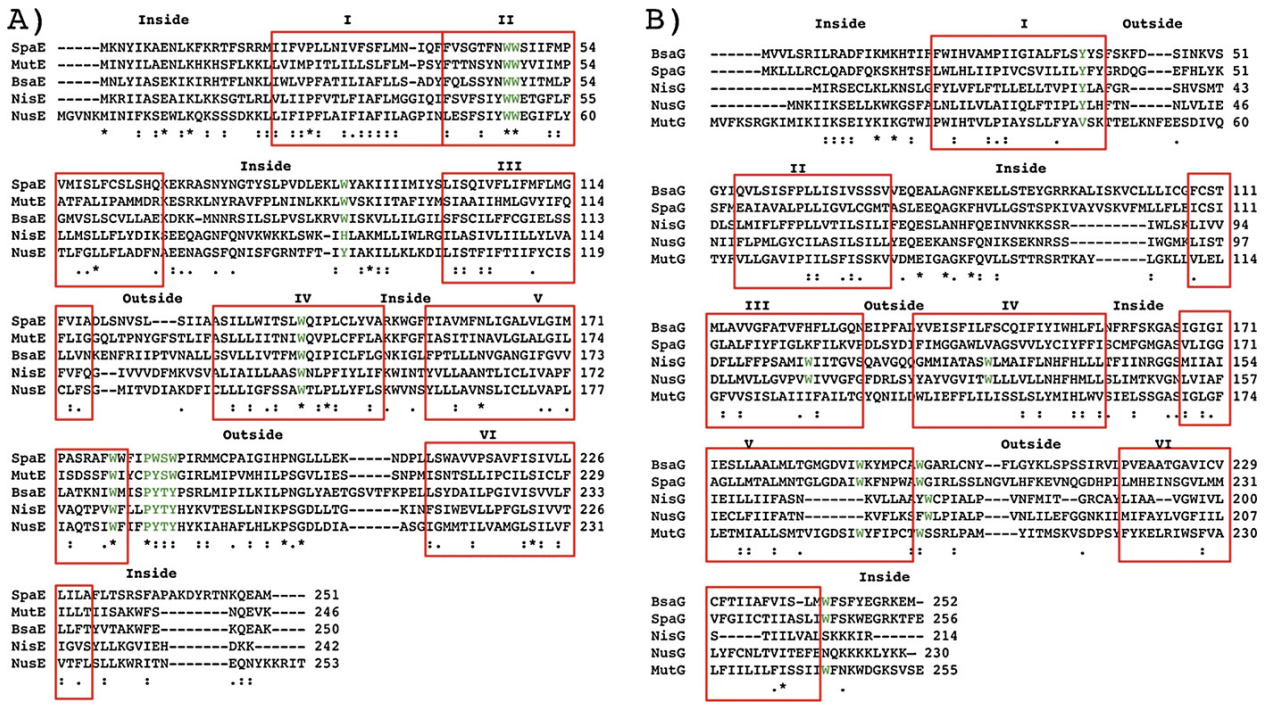


**Fig. 3.** Sequence alignment of NisE and NisG. (A) Sequence alignment of NisEG homologs, SpaEG from *B. subtilis*, MutEG from *Finegoldia magna ATCC 53516*, BsaEG from *Bacillus cereus G9241* and NusEG from *Streptococcus uberis*. Transmembrane helices predicted by a hydrophobicity plot are highlighted by a red square. Tryptophan residues are highlighted in green. Loops, which are predicted to be cytosolic are named "inside", while extracellular loops are labeled "outside".

lipoprotein, with an N-terminal signal sequence, removed during posttranslational modification resulting in the anchoring of NisI to the extracellular side of the cell wall (Qiao et al., 1995). Some of the expressed NisI escape this lipid modifications. The group of Koponen showed that approximately half of the produced NisI is secreted into the medium (Koponen et al., 2004; Takala et al., 2004).

NisI contributes to an immunity against nisin in *L. lactis*, along with NisFEG and displays a high specificity towards nisin. A knock out of NisI resulted in cells more sensitive than the corresponding NisFEG knock out (Siegers and Entian, 1995). This observation resulted in the hypothesis that NisI plays a more important role in the immunity against nisin (Draper et al., 2008). NisFEG conferred around 20% of immunity when expressed alone comparing to the wild type immunity (Ra et al., 1999). There is not much known about the LanI protein family and the proteins within this family exhibit a rather low sequence similarity, presumably due to the specific lantibiotic characteristics that is bound. NisI contains a hydrophobic N-terminal region containing a lipid-protein sequence found also in other LanI proteins like for example PepI the immunity protein of Pep5.

When expressed in *B. subtilis*, NisI provides significant immunity against nisin. However, simultaneous expression of NisFEG and NisI in *B. subtilis*, resulted in higher levels of immunity. This confirmed the initial hypothesis that the specificity of the nisin immunity system against nisin can be transferred into different bacterial species (Stein et al., 2003).

The exact mechanism of NisI to function as an immunity protein is still unknown, although Takala and his colleagues mentioned the importance of the C-terminus of NisI to interact with nisin. Their study identified a 21aa deletion at the C-terminus of NisI, reduced the immunity to 14% compared to the native level. However, this C-terminal region of NisI is not involved in the co-operation with NisFEG as the truncated NisI showed a cooperative effect of nisin resistance when co-expressed with NisFEG (Takala and Saris, 2006). Moreover, they showed that the replacement of the 21 C-terminal amino acids of the subtilin-specific SpaI with the C-terminal 21 amino acid of NisI (SpaI′-NisI′) confers immunity against nisin (Takala and Saris, 2006).

Similar results were obtained for PepI. Hoffmann et al. showed that the C-terminal part mainly provides immunity, while the N-terminal part is more important for exporting PepI out of the cells (Hoffmann et al., 2004). PepI is the simplest immunity system among lantibiotics, which protects *Staphylococcus epidermidis 5* from its own product Pep5. Similar to NisI, it is located and functions at the membrane-cell wall interface, as shown by fusions of PepI and green fluorescent proteins (Hoffmann et al., 2004).

### 4.3. Immunity by non-producer strains: NSR – the nisin resistance protein

Nisin producing strains have developed a resistance machinery to oppose their own lantibiotic consisting of the protein NisI and the ABC transporter NisFEG (Stein et al., 2003). Nevertheless not all species belonging to the *L. lactis* subfamily produce and secrete nisin molecules. Remarkably, the non-producing *L. lactis* strains have developed different mechanisms to prevent growth inhibition when encountering a high level of nisin in the media. In non-producing strains, an enzyme is found which protects the cell against nisin. This enzyme is called nisin resistance protein (NSR) (Froseth et al., 1988; Froseth and McKay, 1991; Tang et al., 2001). Froseth et al. first discovered an open reading frame of 957 nucleotides encoding a 319 amino acid protein on a plasmid in *L. lactis* subsp. *lactis biovar diacetylactis* DRC3. The amino acid composition predicted a N-terminal hydrophobic region from amino acid 7–28, which likely is membrane associated. By sequence homology studies several other NSR like proteins where found in three other Gram-positive bacteria families (Froseth and McKay, 1991). Fig. 4 shows an alignment of these proteins.


**Fig. 4.** Sequence alignment of the NSRs. Shown are sequences from *L. lactis* subsp. *lactis* (lane 1) and *Enterococcus casseliflavus* EC10 (lane 2), *Leuconostoc mesenteroides* subsp. *cremoris* ATCC 19254 (lane 3) and *Streptococcus sanguinis* SK1087 (lane 4). The percentage of amino acid identity are given in different blue colors, where light blue represent low and dark blue high identity. The putative catalytically active serine is highlighted in green.

In 2009, Sun et al. expressed the *nsr* gene in *E. coli* and *L. lactis*. The first construct (NSR) corresponds to the full-length protein including the hydrophobic sequence at the N-terminus (Sun et al., 2009). The second construct lacks the first 27 amino acids and was called NSR-SD. Localization studies showed that NSR is mainly located at the cell membrane whereas NSR-SD is mainly expressed as a soluble cytosolic protein. In vitro studies of NSR-SD revealed a proteolytic digestion of nisin between position MeLan28 and Ser29. The obtained nisin fragment, which still contains the five characteristic lanthionine rings, is 100-fold less active against the nisin sensitive strain *L. lactis* MG1363. Cleavage of the last 6 aa shortens nisin, therefore, it is no longer able to span the target membrane and pore formation is thereby inhibited. The remaining antimicrobial activity is due to the binding of nisin to lipid II, thereby inhibiting cell wall synthesis (Hasper et al., 2006).

In vitro activity of the membrane associated NSR could not be observed (Sun et al., 2009). This indicates that although no full resistance can be observed, non-nisin producing strains developed their own immunity system to deal with the presence of nisin in the medium.

## 5. Immunity system of other lantibiotics

Homologous proteins of the immunity proteins NisI and NisFEG are found in *B. subtilis*, which produces subtilin. SpaI is similar to NisI. It contains an N-terminal hydrophobic region with a lipoprotein signal and it functions as assistance in immunity. It is proposed that SpaI sequesters subtilin at the cytoplasmic membrane, thus preventing subtilin from forming pores and attacking its own cells (Stein et al., 2005). Meanwhile, SpaF showed significant homology to other LanF proteins and the SpaEG proteins have similar sizes and hydrophobicities comparable to other LanEG proteins (Stein et al., 2002). Besides, the expression of the *spaIEFG* genes in the subtilin sensitive *B. subtilis* strain MO1099 confirmed their functions and resulted in even higher levels of immunity than the normal subtilin producer *B. subtilis* ATCC 6633 (Stein et al., 2005). Even in the case of nisin, 90% of nisin was recovered in the media. Both results supported the hypothesis that NisI-FEG and SpaI-FEG are exporting nisin and subtilin, respectively, without modifications or degradations of the lantibiotic (Stein et al., 2003).

In general, all *lanI* genes showed no homology with each other or with any other immunity protein (Twomey et al., 2002). This resulted in sme difficulties to study them. PepI, which showed 74.2% homology to EciI, the immunity gene of epicidin 280, is the huge exception (Heidrich et al., 1998). Although PepI provided immunity to epicidin 280, this is not the case for lantibiotics in general, which are known to be specific to their respective lantibiotic. This cross-immunity could be a result to the fact that the immunity system in both Pep5 and epicidin 280 are simple and no ABC transporter is involved in immunity (Draper et al., 2008). In contrast, nisin and subtilin showed no cross-immunity although they are closely related with 63% of sequence homology (Stein et al., 2005).

## 6. Application of nisin and its immunity pathway

Lantibiotics are of increasing interest in the biotechnological as well as the pharmaceutical industry, since the specific inhibition of bacterial cell growth can be exploited in many ways. Active nisin for example, has now been used for over 50 years in the food industry, for example within cheese- and milk production, canned vegetables, diverse pasteurized dairy and salad dressing productions (de Vuyst and Vandamme, 1993). Although nisin has been added to the European food additive list in 1983 (number E234), it has been identified as a safe product to be used as an additive in food since 1969 by the "Joint Food and Agriculture Organization/World Health Organization Expert Committee of Food Additive" (Cotter et al., 2005).

Besides the lantibiotic itself, the nisin operon has been exploited for industrial and/or basic research purposes. For example, the two-component system (NisR and NisK) has been successfully used as an expression system in *L. lactis* for over more than a decade. This nisin-controlled gene expression system (NICE) is of great interest due to its properties of being tightly regulated and its high degree of induction (Mierau and Kleerebezem, 2005). With NICE system, many proteins can be expressed in order to study their function and/or to produce them for industrial purposes, like for example metabolic proteins, bacteriocins as well as antigens (Zhou et al., 2006). *L. lactis* is a non-pathogenic and a non-toxic bacteria, and therefore it is considered an excellent host for the production of heterologous proteins for experimental or commercial applications (Nouaille et al., 2003). The expression of a protein of interest is induced by external addition of nisin and the "dose" of gene expression can be controlled in some way by the amount of added nisin (Mierau and Kleerebezem, 2005). Thus, both *L. lactis* as well as the

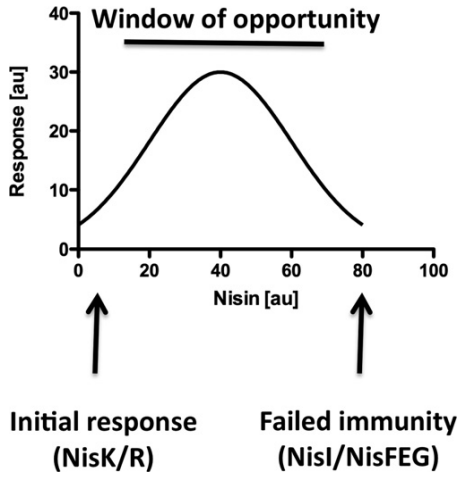


**Fig. 5.** Schematic summary of "working principle" of the NICE system in biotechnological applications. At the first arrow ("initial response") the concentration of nisin in the media is high enough to induce response of the two-component system and expression of the gene under the control of the corresponding *nis* promoter. Increasing concentrations of nisin (plotted in arbitrary units [au]) increases the response (also plotted in arbitrary units [au]). However, the response reaches a maximum at a certain concentration of nisin and starts to decline, because of the bacteriocidal effect of nisin on the producer strain. This effect is counterbalanced by the immunity gene, but at a certain concentration of nisin (indicated by the right arrow). This scheme represents the basic boundaries of the nisin system highlighting the "window of opportunity" by either increasing the affinity of the two component system or by increasing the tolerance towards nisin would extend the possibilities of biotechnological applications.

nisin derived NICE-expression system result in a versatile device to be used for protein/enzyme production.

There is however, one drawback. Since nisin is active against *L. lactis*, the usage of increasing nisin concentrations, which, in principle, should enhance expression levels, is not possible due to the subsequent "killing" activity of the inducer. In Fig. 5, a theoretical dose–response curve is shown. Here, the expression of any gene of interest under the control of a nisin promoter is shown in arbitrary units. At initial concentrations of nisin, where the NisK/NisR response is low, only low amounts of the protein of interest is produced. At a certain concentration of nisin, a critical level is reached and *L. lactis* cells are attacked and eventually lysed by the added inducer (nisin). The usage of the nisin immunity genes will shift this dose–response curve and will create a maximum at higher concentrations of external nisin. Accordingly, an implementation of the immunity genes in the NICE expression system, will allow usage of higher amounts of inducer (nisin) and consequently increase the amount of the expressed protein.

A recent study mentioned that the *nisI* gene could act as an extra factor for the NICE system, as the expression of a recombinant protein (GFP) was roughly 2-fold higher when the *nisI* gene was inserted into the NICE expression vector (Oddone et al., 2009). Before this report, the *nisI* gene was used as a food-grade selectable marker by Takala and Saris in 2002 when they constructed a vector, pLEB590, for lactic acid bacteria (LAB) showing a high and easy selection of the transformants on nisin plates (Takala and Saris, 2002). This *nisI*-mediated vector enabled a selection for many LAB bacteria other than *L. lactis*, like *lactocacilli* bacteria. Lately, in 2011, both *nisI* and *nsr* (nisin-resistance gene) genes were used as a selection markers with 5 μg nisin/ml for *nsr* gene (Li et al., 2011). Here, as noticed, the knowledge about the immunity system can help, after understanding its mechanism, to gain a better controlled expression system eventually leading to higher levels of the interested protein.

Some of lantibiotic producing bacteria are human pathogens. For example, *Streptococcus agalactiae* causes pneumonia and meningitis in neonates and the elderly. *Streptococcus suis*, which is an important pathogen of pigs and causes severe infections including meningitis, septicaemia, endocarditis, or deafness when transferred to human. Another example is *Streptococcus pyogenes*, which is the cause of many human diseases, ranging from mild superficial skin infections to life-threatening diseases. Infections typically begin in the throat or skin, and, in general, a lot of medical treatments are using antibiotics (one or even a cocktail of several different ones) and due to this high usage of antibiotics, the occurrence of resistant strains is a severe problem in the treatment of the patients.

As the genome sequences of these organisms revealed the presence of only lantibiotics immunity genes, a detailed understanding of the LanFEG and LanI protein families could gain insight into their resistance mechanism. Since a specific inhibitor against one of the immunity proteins would inhibit the self-protection of these strains specifically and thereby these human pathogenic strains would become susceptible to their own lantibiotic. This would mean that the pathogenic bacteria would kill itself by the expression of lantibiotics.

The same holds true for pathogenic strains containing a nisin resistance protein, which inactivates lantibiotics by C-terminal cleavage. If a specific inhibitor would exist, these strains could, in principle, be treated by a cocktail of LanI inhibitors and for example nisin.

The major advantage of a medical usage of lantibiotics is that lantibiotics do not harm human cells and therefore will not cause any side effects. Thus, they seem to be an excellent candidates for medical purposes. Although, this is currently only a vision, the potential is truly present and might be used by further studies of the immunity genes encoded in the operons of lantibiotic producing Gram-positive bacteria.

## 7. Concluding remarks

The nisin operon contains eleven proteins, which all have different functions. Interestingly, all eleven proteins act as a symphonic orchestra and only together an efficient and effective production of nisin is possible. The auto-immunity of producer strains against their own lantibiotics, highlights the enormous power of the immunity system, since in comparison the target cells lacking an immunity response are already killed by nM amounts.

Next to the biosynthesis and modification apparatus, the two-component system as well as the immunity system attracts more and more interest of both, basic and industrial researchers.

The immunity against lantibiotics has been observed in producer strains but up to date not in target cells. The LanFEG and LanI superfamilies of proteins have been studied in the past, but due to their potential role in possible medical and industrial applications, they will be move more and more in the research focus of many disciplines within the next years.

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## 3.3 Chapter III - NisA

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# Easy and Rapid Purification of Highly Active Nisin

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*Research Article*

# Easy and Rapid Purification of Highly Active Nisin

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Nisin is an antimicrobial peptide produced and secreted by several *L. lactis* strains and is specifically active against Gram-positive bacteria. In previous studies, nisin was purified via cation exchange chromatography at low pH employing a single-step elution using 1 M NaCl. Here, we describe an optimized purification protocol using a five-step NaCl elution to remove contaminants. The obtained nisin is devoid of impurities and shows high bactericidal activity against the nisin-sensitive *L. lactis* strain NZ9000. Purified nisin exhibits an $IC_{50}$ of ~3 nM, which is a tenfold improvement as compared to nisin obtained via the one-step elution procedure.

## 1. Introduction

The capacity to produce antimicrobial peptides (AMPs) is widespread among Gram-positive bacteria. These AMPs are directed against competitive microorganisms in order to generate a selective advantage for the producer organism [1]. AMPs can be divided in three major classes. Class I consists of the so-called lantibiotics, which are posttranslationally modified peptides containing (methyl-)lanthionines, and have a typical size of <5 kDa. Class II comprises heat-stable, nonmodified peptides of 37–58 amino acids (typical size of <10 kDa) with the leader peptide, as for Class I AMPs, being removed during maturation. Class III contains AMPs that are heat labile and that are generally larger in size (roughly 30 kDa). Especially heat-stable peptides secreted by lactic acid bacteria have been studied extensively because of their potential use as natural preservatives in the food industry.

One of the best characterized AMPs is nisin, which is secreted by *Lactococcus lactis* and is a member of the Class I AMPs (for comprehensive reviews, see [2, 3]). Although nisin has been used as a food preservative for more than fifty years, no significant bacterial resistance against nisin has been reported [4]. Nisin, encoded by the *nisA* gene, is a (methyl-)lanthionine ring containing peptide that is ribosomally synthesized as a prepeptide consisting of 57 amino acids. The NisA prepeptide is modified posttranslationally by the dehydratase NisB, which selectively dehydrates Serine and Threonine residues, and by the cyclase NisC, which catalyzes lanthionine ring formation. NisT finally transports the modified prenisin across the cell membrane, and mature, biologically active nisin is produced upon cleavage of the leader peptide by the extracellular, membrane-anchored protease NisP. Mature nisin harbors three dehydrated amino acids (one dehydrobutyrine and two dehydroalanines), one lanthionine ring, and four methyl-lanthionine rings. In particular, these intramolecular rings are important for the biological activity of nisin [5]. It is worth mentioning that nisin induces its own synthesis via interaction with the two-component regulatory system NisRK.

Nisin is active against Gram-positive bacteria and exerts two killing mechanisms. Firstly, nisin inhibits cell-wall synthesis by binding to lipid II, an essential membrane-anchored cell-wall precursor, and secondly, nisin permeabilizes the target membrane. The binding of nisin to lipid II induces membrane integration of nisin resulting in the formation of a pore, likely composed of eight nisin and four lipid II

molecules [6]. This highly specific interaction with lipid II is reflected by the low nano- to micromolar concentrations of nisin, sufficient to permeabilize the membrane of the target cells [7].

The bactericidal activity of AMPs is generally measured by quantifying growth inhibition of an AMP-sensitive target organism, grown either on agar plates or in liquid culture. On agar plates, zones of growth inhibition of the indicator organism can be easily visualized, and these so-called halo assays allow determination of the minimum inhibitory concentration (MIC) of the tested AMP [8]. Alternatively, bacterial growth can be monitored in liquid culture in dependence of the AMP concentration, by measuring the optical density. This method allows the simple determination of both the MIC and the $IC_{50}$, that is, the concentration of AMP that inhibits cell growth by 50% [9].

Nisin from *L. lactis*, like almost all naturally produced AMPs, can be purified directly from the culture medium [10, 11]. It is a cationic peptide, and therefore, commonly purified using cation exchange chromatography (cIEX) at acidic pH, using high salt concentration for elution, typically a single-step elution with 1 M NaCl [12–16]. By using nisin purified via such a method, the $IC_{50}$ and MIC values were determined for a variety of bacteria, such as various *L. lactis* strains, *Enterococcus faecium*, *Bacillus anthracis*, *Bacillus subtilis*, *Staphylococcus aureus,* and *Streptococcus mutans* [9, 17]. Whereas *E. faecium* and *S. mutans* were equally sensitive to nisin exhibiting an $IC_{50}$ of 5 $\mu$M and MIC of 12.5 $\mu$M, *L. lactis* HP was much more sensitive to nisin, with an $IC_{50}$ of 14 nM and MIC of 32 nM [9]. Nisin shows promising activity towards clinical isolates of the Methicillin-resistant *Staphylococcus aureus* (MRSA) bacterium, *Streptococcus pyogenenes,* and several of the most severe human pathogens, including the multiresistant *Streptococcus pneumoniae* and vancomycin-resistant *E. faecium* or *E. faecalis*, against which new effective antibiotics are most urgently needed [18–20]. In all these, studies purified nisin was used and the bactericidal activity of nisin was measured by determining the MIC or $IC_{50}$.

Here, we describe a rapid and easy nisin purification protocol, optimized to yield active, monomeric nisin. For this purpose, nisin was either produced from *L. lactis* strain NZ9700 or purchased in the form of a lyophilized powder that contains 2.5% (w/w) nisin. Nisin from both sources was purified and tested for bactericidal activity using the nisin-sensitive *L. lactis* NZ9000 strain. During the purification low molecular weight contaminants are removed, which results in purified nisin with high specific activity. This allows a more accurate and reproducible determination of the biological activity of nisin.

## 2. Material and Methods

*2.1. Production and Purification of Nisin*

*2.1.1. Nisin Produced by L. Lactis.* A culture of the *L. lactis* strain NZ9700 was grown overnight in M17 medium containing 0.5% (w/v) glucose (GM17) at 30°C. Next, 100 mL of the overnight culture was used to inoculate 2 l of GM17, and cells were grown at 30°C. At an $OD_{600}$ of 0.8, the culture was supplemented with nisin (Sigma) to a final concentration of 1 ng/ml cell culture to ensure nisin production and growth was continued over night. Next, cells were removed by centrifugation (30 min., 6000xg at 4°C). The supernatant was used and diluted with 1 l of 50 mM lactic acid pH 3 resulting in a 3 l solution with a pH of 5. This solution was loaded on a 5 mL HiTrap SP HP cation exchange (cIEX) column (GE Healthcare) using a flow rate of 4 mL/min. Due to the large volume, this step is optimally performed overnight. Protein elution was monitored by measuring the absorbance at 215 nm. Unfortunately, it is not possible to detect nisin at 280 nm, because it does not contain any aromatic amino acids; therefore, the 215 nm was chosen as wavelength. The column was subsequently washed with 50 mM lactic acid pH 3 until a stable baseline was reached to remove nonspecifically bound material. Peptides were eluted by increasing the NaCl concentration stepwise using a flow rate of 1 mL/min, resulting in elution fractions with 200 mM (Step I), 400 mM (Step II), 600 mM (Step III), 800 mM (Step IV) and 1 M (Step V) NaCl. To remove NaCl, protein in the elution fractions was precipitated with 20% (v/v) trichloroacetic acid (TCA) overnight at 4°C. Precipitated protein was washed two times with ice-cold acetone to remove residual TCA. Finally, the protein pellet was suspended in 50 mM lactic acid pH 3. Nisin concentrations were determined by a colorimetric assay (Pierce BCA Protein Assay Kit, Thermo scientific) by measuring the absorbance at 584 nm according to the protocol of the manufacturer.

*2.1.2. Purchased Nisin.* Commercial nisin (Sigma) is available as a lyophilized powder containing ~2.5% (w/w) nisin. 1.3 g of nisin powder (corresponding to 32.5 mg nisin) was dissolved in 100 mL 50 mM lactic acid pH 3 and filtered through a 0.45 $\mu$m membrane filter (Pall Corporation). The nisin solution was applied to a 5 mL HiTrap SP HP cation exchange column (GE Healthcare) at a flow rate of 2 mL/min, whereas elution was performed at a flow rate of 1 mL/min. Nisin purification, precipitation, and concentration determination were carried out as described above.

*2.2. Tricine-SDS-PAGE.* Tricine-SDS-PAGE was essentially carried out as described in [21]. For analysis, 16 $\mu$L sample was supplemented with 4 $\mu$L 5x SDS sample buffer (0.2 M Tris-HCl, pH 6.8, 10% (w/v) SDS, 40% (v/v) glycerol, 0.02% (w/v) bromophenol blue, and 10 mM DTT) and loaded on a tricine gel consisting of a stacking gel containing 5% acrylamide and a separation gel containing 16% acrylamide. The gel was run at 100 V for 2 hours, and proteins were detected via silver staining. For all purification fractions, 3.2 $\mu$g of total protein was analyzed.

*2.3. MALDI-TOF Mass Spectrometry.* Samples obtained from cIEX chromatography were precipitated with TCA and washed with acetone as described above. The protein pellets

were then dried for 15 min. at 30°C in a vacuum concentrator (Eppendorf concentrator plus). Dried pellets were stored at −20°C until analysis. For mass spectrometric analysis, the samples were dissolved in water with 1% (v/v) formic acid. Measurements were performed using a MALDI-TOF instrument (Voyager-DE STR, Applied Biosystems) with a nitrogen laser ($\lambda = 337$ nm) operating in reflector mode with 25 kV acceleration voltage. The samples were prepared by the standard dried-droplet procedure, by applying 0.5 $\mu$L of 2,5-dihydroxybenzoic acid (DHB) matrix solution (10 mg in 1 mL water) plus 0.5 $\mu$L of sample solution. The droplet was dried by a gentle flow of air. An external calibration with the monomer ion of des-Pro-Bradykinin, Sub P, Bombesin, and Melittin was used. The spectrum was obtained by averaging 200 laser shots.

*2.4. $IC_{50}$ Determination of Nisin.* To investigate the antimicrobial activity of nisin, the nisin sensitive *L. lactis* strain NZ9000 [22] was grown in a 96-well plate in GM17 medium. The total volume in each well was 200 $\mu$L, consisting of 50 $\mu$L sample and 150 $\mu$L GM17 containing *L. lactis* NZ9000 cells (starting $OD_{600} = 0.1$). Samples were prepared by diluting nisin obtained from various cIEX elution fractions in 50 mM lactic acid pH 3 to yield final protein concentrations ranging from 0.15 nM to 300 nM in the wells. 50 mM lactic acid pH 3 without protein served as positive growth control. Cells were grown at 30°C, and the optical density was monitored at 620 nm every 20 min for a period of 8 hours (96 plate reader BMG). To determine the $IC_{50}$ values, the optical density was normalized and plotted against the log of the nisin concentration. Data were evaluated according to

$$Y = \frac{\text{OD min} + (\text{OD max} - \text{OD min})}{1 + 10^{((\log(\text{IC50}) - X) * \text{slope})}}. \quad (1)$$

The $OD_{max}$ value describes the normalized $OD_{600}$ value in the starting plateau. The $OD_{min}$ value corresponds to the normalized $OD_{600}$ of the end plateau value. $Y$ stands for the normalized optical density value, and $X$ represents the logarithmic concentration of the peptide. The $IC_{50}$ value is calculated as the value of the peptide concentration used where the growth inhibition (or $OD_{600}$) is 50%. This corresponds to the inflection point of the resulting curves.

*2.5. Growth Inhibition Visualized by a Halo Assay.* The antimicrobial activity of the different nisin preparations was assessed by means of a halo assay. Purified nisin obtained from the different cIEX elution fractions were supplemented (16 $\mu$L) with 4 $\mu$L of 5x SDS sample buffer and tricine-SDS-PAGE was carried out as described above. After electrophoresis the gel was incubated for 30 min in an aqueous solution containing 20% (v/v) isopropanol and 10% (v/v) acetic acid. Subsequently, the gel was washed two times for 30 min in $ddH_2O$. At this stage, the gel was kept in $ddH_2O$ at 8°C until usage. Finally, the gel was overlaid with GM17-agar (0.5% w/v agar) containing *L. lactis* NZ9000 cells at an $OD_{600}$ of 0.1. After solidification, the overlaid gel was incubated overnight at 30°C to allow for bacterial growth. The bactericidal activity of nisin is readily visualized by the presence of clear zones (halos) resulting from growth inhibition.

## 3. Results

### 3.1. Purification of Nisin

*3.1.1. Lyophilized Commercial Nisin.* To determine the antimicrobial activity of commercially available nisin, we used a lyophilized powder, which contains ~2.5% (w/w) nisin. To further purify nisin, we initially performed SP Sepharose cation exchange chromatography using 1 M NaCl to elute the bound nisin. Subsequent SDS-PAGE analysis of the eluate revealed a major protein band corresponding to a peptide with a molecular mass of about 3.5 kDa (data not shown) in line with the calculated mass of 3354 Da for mature nisin. However, several higher molecular weight components were also present. It is of note that these contaminants are not readily visualized by Coomassie Brilliant Blue staining, whereas these impurities are clearly detected by silver staining. Nisin purified via this method showed antimicrobial activity against *L. lactis* NZ9000 exhibiting an $IC_{50}$ of 30 ± 12 nM.

The presence of contaminants prompted us to optimize the purification of nisin. We first tested elution with a linear gradient (50 times the column volume) ranging from 0–1 M NaCl. This approach, however, resulted in a broad peak eluting throughout the NaCl gradient and further analysis revealed no improvement when compared to the single-step 1 M NaCl elution (data not shown). In contrast, a substantial improvement was achieved when a five-step NaCl step gradient was used to elute nisin from the cIEX column (Figure 1(a)). Bound protein eluted at every step as evidenced by the elution profile and subsequent tricine-SDS-PAGE analysis (Figure 2(a)). The Step II elution fraction contained the bulk of nisin as evident by the major protein band with a corresponding molecular mass of ~3.5 kDa, whereas substantially lower amounts of nisin were detected in elution fractions I, III, and IV. In the Step V fraction no nisin was visible. The Step I and II elution fractions contained exclusively nisin, while elution fractions III–V contained predominantly higher molecular weight compounds (ranging from 6 kDa–70 kDa). In the latter fractions compounds with molecular weights of ~8 kDa and ~10 kDa were most prominent. The total protein concentration of the elution fractions was determined to be: 2.1 mg/mL for Step I (200 mM NaCl), 7.5 mg/mL for Step II (400 mM NaCl), 1.9 mg/mL for Step III (600 mM NaCl), 0.5 mg/mL for Step IV (800 mM NaCl) and 0.4 mg/mL for Step V (1 M NaCl) (Table 1). In Step II, ~60% of the total purified nisin eluted. Thus, it appears that 400 mM NaCl is sufficient to elute the vast majority of the nisin molecules. More importantly, the nisin eluting under these conditions is essentially devoid of contaminants.

*3.1.2. Nisin Produced by L. Lactis.* An alternative to purchasing nisin is to produce it in the laboratory, since *L. lactis* strains that secrete nisin in large amounts are readily

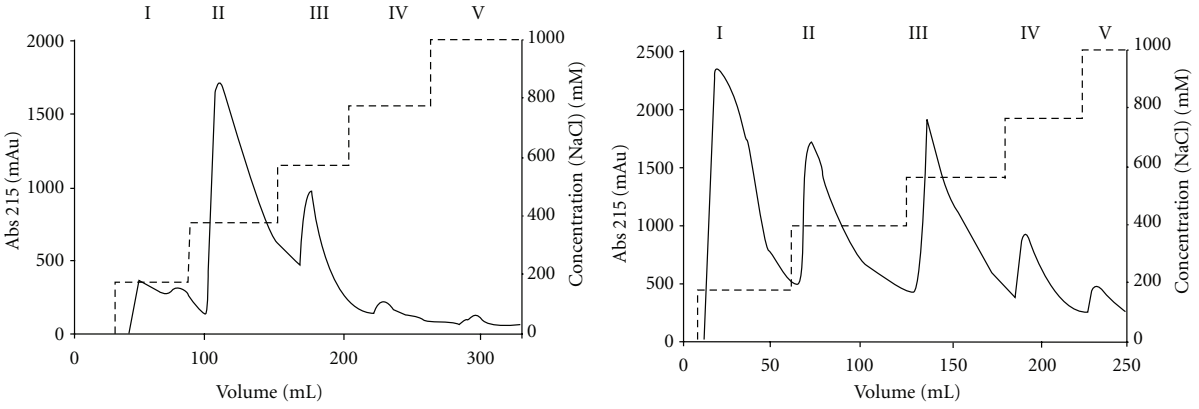


Figure 1: Purification of nisin via cation exchange chromatography. The elution profiles of the purification of commercial nisin (a) and nisin produced by *L. lactis* NZ9700 (b) are shown. In both cases, nisin is eluted from the column using a five-step gradient with 200 mM (Step I), 400 mM (Step II), 600 mM (Step III), 800 mM (Step IV) and 1 M NaCl (Step V). The different elution steps and corresponding NaCl concentrations are indicated by the dashed line and the right *y*-axis, respectively. Protein was detected by measuring the absorbance at 215 nm.

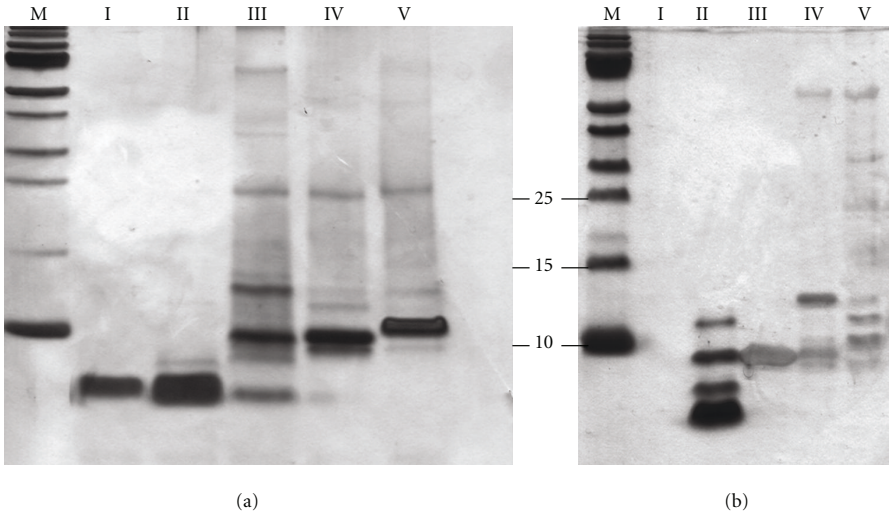


Figure 2: Tricine-SDS-PAGE analysis of the cIEX purification of nisin. Purification of commercial nisin (a) and nisin secreted by the *L. lactis* NZ9700 strain (b). M, marker proteins; I, elution with 200 mM NaCl; II, elution with 400 mM NaCl; III, elution with 600 mM NaCl; IV, elution with 800 mM NaCl; V, elution with 1 M NaCl. Protein was visualized by silver staining. The three lowest marker proteins are indicated with molecular weights (kDa).

Table 1: $IC_{50}$ values of each nisin containing fraction eluted from the cIEX column. Values are combined data from at least three independent nisin purifications and subsequent inhibition experiments.

| | Purchased nisin | | NZ9700 secreted nisin | |
|---|---|---|---|---|
| Elution step | $IC_{50}$ (nM) | Yield (mg) | $IC_{50}$ (nM) | Yield (mg) |
| Step I (200 mM) | 35.1 ± 0.1 | 4.2 | n.i. | 0.06 |
| Step II (400 mM) | 2.6 ± 0.1 | 15.1 | 11.2 ± 0.3 | 5.90 |
| Step III (600 mM) | 6.9 ± 0.2 | 3.8 | n.i. | 3.29 |
| Step IV (800 mM) | 27.0 ± 0.2 | 1.1 | n.i. | 0.14 |
| Step V (1 M) | n.i. | 0.8 | n.i. | 0.45 |

n.i = no inhibition observed under the experimental setup.

available. We used *L. lactis* NZ9700 grown in GM17 medium to produce nisin (see Section 2). Culture supernatant containing nisin was subjected to cIEX chromatography using the same five-step NaCl elution gradient as described above. Here, a high absorbance at 215 nm occurred at the Step I elution (Figure 1(b)), but this did not correspond to nisin or other proteins as evidenced by silver-staining following SDS-PAGE analysis (Figure 2(b), lane 2). Likely, this high absorbance is due to ingredients from the growth medium, which contains large amounts of peptone, tryptone, and yeast extract. This was confirmed by a run with only GM17 media (data not shown). The remainder of the elution profile is similar to that of the lyophilized nisin purification, with two major absorbance peaks observed for the Step II and III fractions (Figure 1(b)). Analysis of the protein content of the different elution fractions revealed that nisin is exclusively found in the 400 mM NaCl elution fraction (Figure 2(b)). The total protein concentration of the 400 mM elution fraction was 2.9 mg/mL. However, whereas nisin is most prominent in this fraction, components with a MW ~ 6 kDa, ~10 kDa, and ~12 kDa are also present. The 0.6–1 M elution fractions on the other hand contained compounds with molecular weights ranging from 8 kDa–70 kDa, similar to those observed for the purification of lyophilized nisin (Figures 2(a) and 2(b)). When compared to the purification of lyophilized nisin, nisin purified from GM17 medium still contained contaminants. This difference in purity may relate to differences in the loaded material. The lyophilized nisin powder (also containing denatured milk solids) was dissolved in 50 mM lactic acid, whereas nisin produced by *L. lactis* NZ9700 was applied to the cationic exchange column as a 1 : 2 mixture of GM17 culture medium and 50 mM lactic acid, respectively.

We considered the possibility that the compounds with molecular weights of 6–8 kDa may represent the unprocessed form of nisin. Immature nisin, that is, nisin still containing the leader sequence, has a molecular weight of 5.9 kDa and may arise if inefficient leader cleavage by the protease NisP occurs. We therefore performed Western-blot analysis using a polyclonal antibody raised against the nisin leader sequence. Purified prenisin secreted by a *L. lactis* strain lacking NisP was used as a positive control for Western-blot analysis [23]. For all elution fractions, no signals were observed suggesting that the observed compounds are not derived from prenisin (data not shown).

*3.2. Biological Activity of Nisin.* To test the biological activity of the purified nisin, we used *L. lactis* NZ9000 as indicator organism. *L. lactis* NZ9000 is a derivative of the plasmid-cured *L. lactis* MG1363 and contains the *nisRK* genes inserted in the chromosomal *pepN* locus [24]. This strain is commonly used as the host for nisin-induced expression system (NICE) purposes [25]. However, since this strain lacks the nisin immunity genes *nisIFEG,* it is sensitive to nisin [22]. The antimicrobial activities associated with the different elution fractions obtained as described above were tested in a so-called halo assay. For this, the various fractions containing nisin were analyzed by tricine-SDS-PAGE and the tricine gel was overlaid with GM17-agar containing nisin sensitive bacteria (see Section 2). The biological activity of nisin is visualized by the growth inhibition zones (halos) at the position where nisin is present. The results for the lyophilized and the laboratory produced nisin are shown in Figures 3(a) and 3(b), respectively. After overnight incubation at 30°C halos were observed for elution Step I–IV for the lyophilized nisin, while for the produced nisin, a halo was only observed for elution fraction II. For both purifications, the highest level of growth inhibition was observed for fraction II. Importantly, the zones of inhibition are located only at the position of the 3.5 kDa nisin peptide (Figure 3). Thus, purified nisin was biologically active and no growth inhibitory activity is associated with the higher molecular weight compounds.

*3.3. Mass Spectrometry.* To assess and confirm the presence of nisin in the individual cIEX elution fractions (Step II–V) of the lyophilized nisin purification, we applied MALDI-TOF mass spectrometry. The 400 mM NaCl elution fraction contained only one peptide with a molecular mass of 3355.09 Da (Figure 4), which is in agreement with the calculated mass of 3354.07 Da for nisin. Peak integration of the total mass spectrum revealed that the 400 mM elution fraction contains >98% of nisin, indicating that this fraction is essentially devoid of contaminants. Nisin was also found in the 600 mM and 800 mM elution fractions, whereas nisin was not detected in the 1 M NaCl fraction. These results are in agreement with tricine-SDS-PAGE analysis (Figure 2(a)).

However, the 0.6–1 M NaCl elution fraction contained several peptides with higher molecular masses. Subsequent tandem MS analysis of these peptides yielded in-sequence tags of eight amino acids and six amino acids, which unfortunately could not be assigned to specific proteins. This was due to the fact that the obtained sequence tags were too short and when blasted gave multiple different protein hits (data not shown). Nevertheless, the obtained sequence tags did not match with the primary sequence of (pre)nisin. Therefore, it can be excluded that the peptide contaminants with molecular masses of ~6–8 kDa are derived from prenisin.

*3.4. Antimicrobial Activity of Purified Nisin.* To quantitatively assess the growth inhibitory activity of nisin obtained from the different purification fractions, a liquid culture assay was performed using *L. lactis* NZ9000 as reporter organism. The optical density of the *L. lactis* NZ9000 cultures after 5 hours of growth was plotted against the total protein concentration of the nisin purification fractions. Results are shown for both, the lyophilized (Figure 5(a)) and the produced nisin (Figure 5(b)), respectively. The resulting growth curves and the calculated $IC_{50}$ values are shown in Figure 5 and Table 1, respectively.

For lyophilized nisin, all cIEX elution fractions exhibited growth inhibitory activity, however, with very distinct $IC_{50}$ values (Figure 5(a) and Table 1). The Step II elution fraction displayed the highest bactericidal activity with an $IC_{50}$ of 2.6 ± 0.1 nM. The other elution fractions showed substantially higher $IC_{50}$ values. Whereas the Step V elution

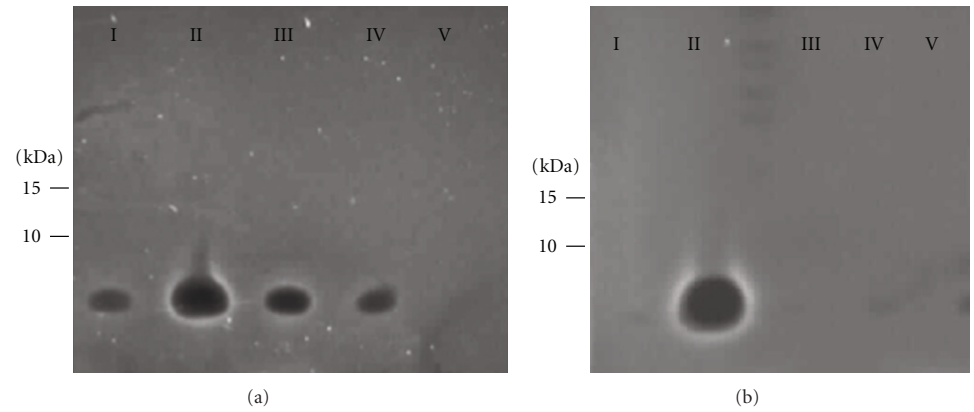


Figure 3: Bactericidal activity of the various nisin purification fractions. Equal amounts of protein of the different elution fractions (Step I–V) from the purification of commercial nisin (a) and from nisin secreted by the *L. lactis* NZ9700 strain (b) were run on a tricine-SDS-PA gel and overlaid with nisin-sensitive *L. lactis* NZ9000 cells (see Section 2). The position of marker proteins with known molecular weight (kDa) are indicated on the left. The growth inhibition zones are visible as dark areas. Lanes I–V represent the five different elution fractions of the cation exchange chromatography. For both purifications, maximum growth inhibition is observed for the Step II elution fraction (400 mM NaCl). Notably, the growth inhibition zone is only visible at a position of ~3.5 kDa.

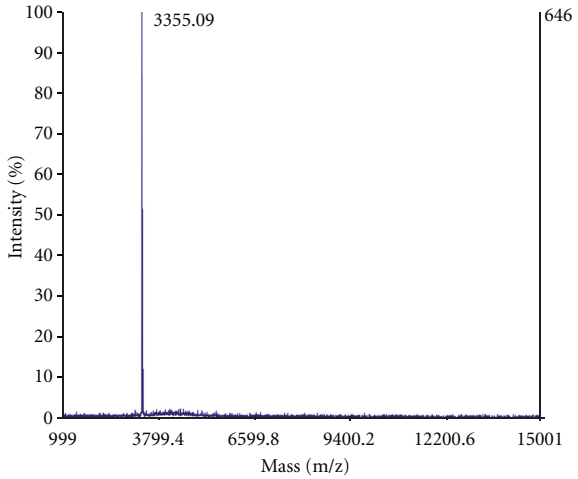


Figure 4: MALDI-TOF mass spectrometry analysis of purified nisin. Mass spectrum of the Step II elution fraction (400 mM NaCl) from the lyophilized nisin purification (for corresponding tricine-SDS-PAGE analysis, see Figure 2(a), lane II).

fraction had only an inhibitory effect at the highest tested concentrations, the Step I, III, and IV elution fractions exhibited an $IC_{50}$ value of 35.1 ± 0.1 nM, 6.9 ± 0.2 nM, and 27.0 ± 0.2 nM, respectively. Thus, the 400 mM NaCl elution fraction contains not only the bulk of nisin, it also contains nisin that displayed the highest specific activity.

A similar observation can be made for nisin purified from the medium (Figure 5(b)). However, here only the 400 mM elution fraction shows bactericidal activity, which is in agreement with tricine-SDS-PAGE analysis (Figure 2(b)) and the halo assay (Figure 3(b)). The calculated $IC_{50}$ of 11.2 ± 0.3 nM is, however, ~4-fold higher than that of the corresponding fraction obtained from the purification of lyophilized nisin. We attribute this difference to the contaminants that are still present (Figure 2(b), lane II). Nevertheless, for both purifications the highest bactericidal activity is associated with the fraction that contains the highest amount of nisin (Figures 2-3 and 5). Taken together the data indicate that nisin obtained from the Step II elution fraction has the highest specific activity.

To determine whether NaCl used for elution has an effect on nisin activity, we repeated the experiment and adjusted the concentration of NaCl after elution in every fraction to 500 mM either by dilution with buffer without salt or by adding buffer and salt. Precipitated protein from these fractions was subsequently used for growth experiments as described above. In all cases, the $IC_{50}$ values of the "salt experiment" were slightly higher than when measured directly after elution, indicating that more nisin is needed to inhibit cell growth by 50% (data not shown). This indicates that residual salt does not have a major influence on the activity of nisin. It can, therefore, be excluded that the differences in $IC_{50}$ values of the various nisin containing elution fractions are induced by the amounts of NaCl used to elute nisin from the column.

## 4. Discussion

AMPs produced by Gram-positive bacteria form a unique group secreted peptides [1]. Their uniqueness of especially the lantibiotic group of AMPs, resides in the posttranslational modifications, such as dehydration of amino acids and intramolecular thioether bridges. One of the best-characterized AMP is nisin, a compound used for more than 40 years in up to 80 countries as an effective agent to combat food-borne pathogens. Nisin has been purified and its antimicrobial activity verified in numerous of studies [9].

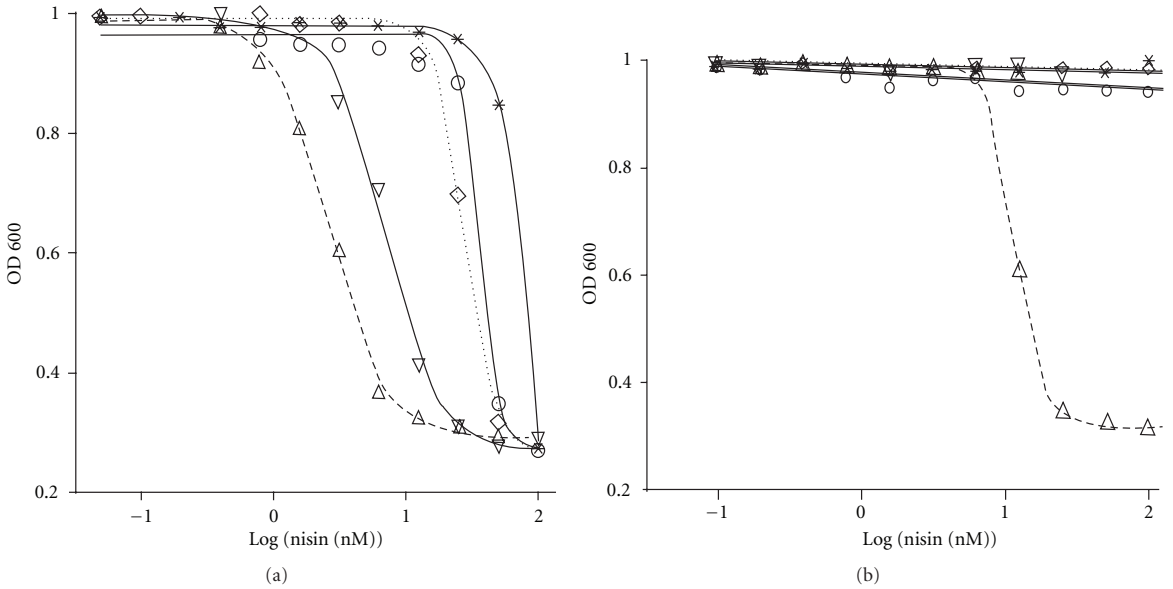


FIGURE 5: $IC_{50}$ determination of the nisin purification fractions. Growth inhibition experiments were performed with nisin obtained from the different elution fractions of the purifications of commercial nisin (a) and of nisin secreted by the *L. lactis* NZ9700 strain (b). The log of the used nisin concentration of each elution fraction is plotted against the normalized optical density of *L. lactis* NZ9000 after five hours of growth. Shown are the inhibition curves for the NaCl elution fractions of 200 mM (o), 400 mM (△), 600 mM (▽), 800 mM (◇), and 1 M (*). Data was fitted and evaluated according to (1).

Commonly, nisin and other cationic AMPs are purified using a single 1 M NaCl elution step, from a cIEX column at an acidic pH [12–14, 16, 26].

The activity of AMPs is strictly dependent on the target organism. For example, the $IC_{50}$ value of nisin ranges from 14 nM for *L. lactis* HP to 5 $\mu$M for Vancomycin-resistant *E. faecium,* with respective MICs of 32 nM and 12.5 $\mu$M [9]. In all these studies, it is noticeable that there is a large variation in the sensitivity to nisin between isogenic strains of Gram-positive bacteria, whereas some bacteria are inherently resistant to nisin. There are several mechanisms by which bacteria can become resistant to an antibiotic. The most prominent example is the enzymatic destruction or modification of the antibiotic, thereby rendering it ineffective. $\beta$-Lactamases, for example, degrade the $\beta$-lactam ring of penicillins. A second important mechanism of resistance is shielding of the target such that the antibiotic cannot get access to it—for example, by cell-surface alterations (capsules S-layers) or by active extrusion by efflux pumps. Moreover, the resistance of the AMP producer organism towards its secreted AMP (autoimmunity) is typically based on ATP-binding cassette (ABC) transporters which expel the AMP from the membrane.

In *L. lactis* NZ9700, cells autoimmunity is mediated by the scavenger protein NisI and the ABC transporter NisFEG [5]. *L. lactis* cells lacking the NisIFEG defense system (e.g., *L. lactis* NZ9000) are sensitive to nisin and can be used as indicator organism to measure the biological activity of nisin. The *nisI* and *nisFEG* genes are part of the *nis* operon and are expressed in concert with the genes involved in nisin production and secretion. Recently, in nisin-non-producing *L. lactis*, nisin resistance was shown to be conferred by a specific nisin resistance gene (*nsr*), which encodes a 35 kDa nisin resistance protein (NSR). NSR proteolytically removes the last six amino acids of nisin, thereby reducing its bactericidal activity by a factor of 100 [27].

The level of intrinsic resistance and the employed mechanisms of antibiotic resistance may differ greatly among microorganisms. It is, therefore, difficult, if not impossible, to directly compare $IC_{50}$ values for a given AMP when comparing strains. Moreover, the purity of the AMP preparation is of great importance to determine accurately the bactericidal activity of the AMP.

In this study, we optimized the purification of the AMP nisin and determined its $IC_{50}$ values against the nisin-sensitive *L. lactis* strain NZ9000. Nisin typically purified via a 1 M NaCl one-step elution yields high levels of active nisin (see above and [28]) but contains a substantial amount of contaminants. We show that these contaminants, which are mainly proteinaceous in nature, are largely removed by using a five-step NaCl elution. Notably, 400 mM NaCl (Step II) was sufficient to elute the bulk of the nisin molecules, while the majority of contaminants remain bound to the column. In this manner, a nisin preparation was obtained that exhibited a high specific activity. When tested against the nisin-sensitive *L. lactis* NZ9000, this highly active nisin exhibited an $IC_{50}$ of 2.6 ± 0.1 nM, which is a 10-fold improvement as compared to the nisin obtained via the one-step elution. The potent bactericidal activity of nisin against *L. lactis* NZ9000 lacking NisI and NisFEG suggests that these

autoimmunity proteins are of great importance for *L. lactis* cells that produce nisin (e.g., *L. lactis* NZ9700).

AMPs get more and more into the focus of biochemical, biophysical, and medical studies due to their antimicrobial activity against a wide variety of bacteria. Here, we report an easy and rapid protocol for the purification of highly active nisin, purified either directly from the culture medium or from a commercially available lyophilized powder. Our studies demonstrate the importance of obtaining AMP preparations with high specific activity. A pure, homogenous, and biologically active preparation will ensure reliable determination of the efficacy of AMPs towards their microbial target(s). Due to the similar chemical and biophysical properties of lantibiotics, our manner of purification may also apply to AMPs other than nisin.

## Abbreviations

$IC_{50}$: 50% Inhibitory concentration
MIC: Minimal inhibitory concentration
MW: Molecular weight
AMP: Antimicrobial peptide.

## Author Contributions

A. Abts and A. Mavaro, contributed equally to this work

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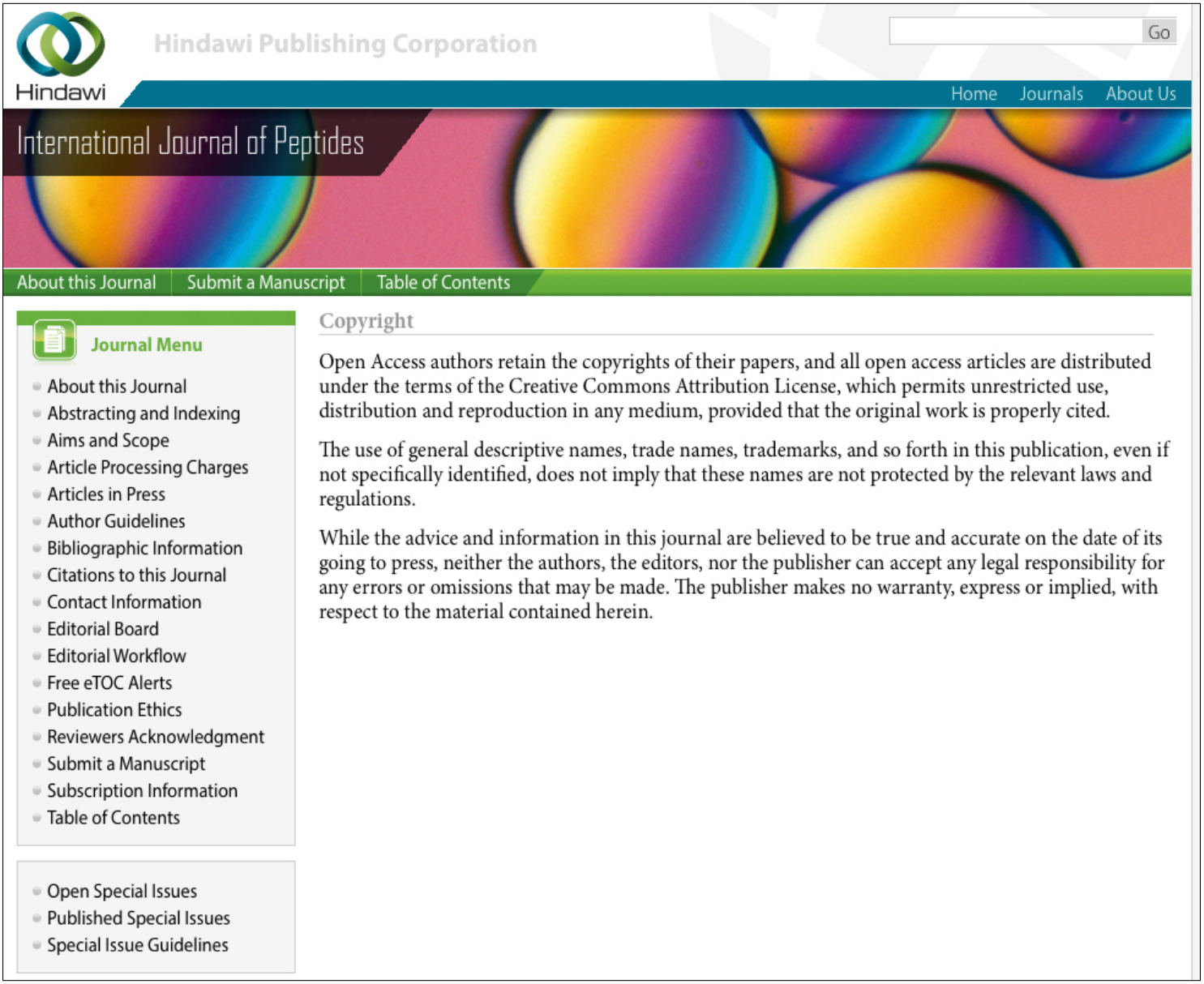

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- Reviewers Acknowledgment
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## 3.4 Chapter IV - NisB

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# Substrate Recognition and Specificity of the NisB Protein, the Lantibiotic Dehydratase Involved in Nisin Biosynthesis

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Antonino Mavaro, **André Abts**, Patrick J. Bakkes, Arnold J. M. Driessen, Sander H. J. Smits, and Lutz Schmitt

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# Substrate Recognition and Specificity of the NisB Protein, the Lantibiotic Dehydratase Involved in Nisin Biosynthesis*

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**Nisin is a posttranslationally modified antimicrobial peptide containing the cyclic thioether amino acids lanthionine and methyllanthionine. Although much is known about its antimicrobial activity and mode of action, knowledge about the nisin modification process is still rather limited. The dehydratase NisB is believed to be the initial interaction partner in modification. NisB dehydrates specific serine and threonine residues in prenisin, whereas the cyclase NisC catalyzes the (methyl)lanthionine formation. The fully modified prenisin is exported and the leader peptide is cleaved off by the extracellular protease NisP. Light scattering analysis demonstrated that purified NisB is a dimer in solution. Using size exclusion chromatography and surface plasmon resonance, the interaction of NisB and prenisin, including several of its modified derivatives, was studied. Unmodified prenisin binds to NisB with an affinity of 1.05 ± 0.25 μM, whereas the dehydrated and the fully modified derivatives bind with respective affinities of 0.31 ± 0.07 and 10.5 ± 1.7 μM. The much lower affinity for the fully modified prenisin was 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 our data indicate that the leader peptide is essential for initial recognition and binding of prenisin to NisB.**

The development of bacterial resistance against clinically relevant antibiotics is on the rise and represents a major scientific challenge (1). There is an ever growing demand for novel and improved antimicrobial agents. As potential antibiotics, bactericidal peptides that are secreted by many bacteria, mainly for self-defense purposes, have gained special interest (2). Among these are the so-called lantibiotics, which are ribosomally synthesized peptides that are posttranslationally modified (3) and produced by a large number of Gram-positive bacteria.

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Nisin, probably the most well known lantibiotic, is produced by the Gram-positive bacterium *Lactococcus lactis* and its antimicrobial activity is directed against microorganisms of similar type. Nisin has been used for more than 40 years as a food preservative, but so far, no significant bacterial resistance has arisen. Recently, *L. lactis* subspecies have been identified that are capable to inactivate nisin via proteolytic cleavage (4).

Nisin exerts its bactericidal mode of action in a dual fashion. First, nisin inhibits bacterial cell-wall synthesis by binding to lipid II, an essential cell-wall precursor molecule (5). Second, binding to lipid II leads to the formation of lipid II-nisin hybrid pores, which depolarizes and permeabilizes the cytoplasmic membrane of the target cell, leading to starvation and cell death (6–8).

The nisin biosynthesis operon comprises the nisin structural gene *nisA* and genes involved in modification (*nisB* and *nisC*), transport (*nisT*), and activation via processing (*nisP*) (9). Nisin biosynthesis is autoregulated via the two-component system *nisRK* (10, 11), whereas the autoimmunity factors of *L. lactis* are encoded by genes *nisFEG* and *nisI* (12). For the maturation of nisin, the 57-amino acid prenisin peptide (NisA) requires modification by several enzymes. The dehydratase NisB catalyzes the dehydration of specific serine and threonine residues to didehydroalanine and didehydrobutyrine residues, respectively (13, 14). These, in turn, are stereospecifically coupled to cysteines by the cyclase NisC (14, 15) yielding five thioether rings (13–15). Finally, the fully modified prenisin is secreted by the ATP-binding cassette transporter NisT (16, 17) and extracellularly processed by the cell-wall anchored protease NisP to liberate active nisin. These modifications are schematically depicted in Fig. 1 (18).

The dehydratase NisB has a central role in nisin biosynthesis. An increase of NisB expression leads to an enhanced dehydration efficiency of prenisin (19). NisB can, however, function in the absence of the cyclase NisC, producing dehydrated prenisin devoid of thioether rings, whereas deletion of NisB results in a substantially reduced production of (unmodified) prenisin (17). Remarkably, NisB can also function in the absence of both the cyclase NisC and the transporter NisT. *L. lactis* cells lacking NisCT secreted dehydrated peptide when the nisin leader peptide is preceded by a Sec signal sequence (22). Yeast two-hybrid and co-immunoprecipitation studies signified interactions between NisB, NisC, and prenisin (9).

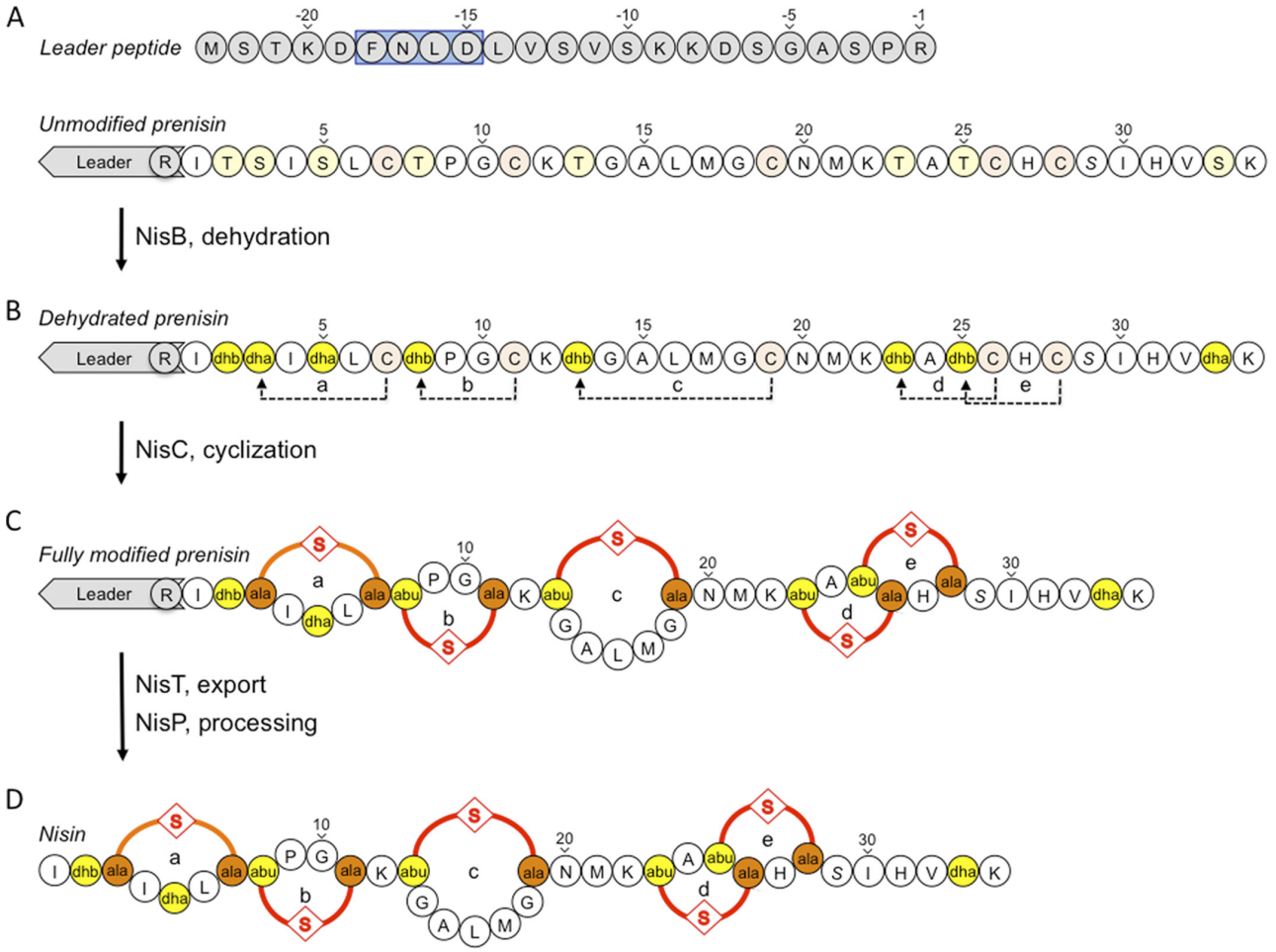


FIGURE 1. **Posttranslational modification of nisin.** For nisin maturation, the ribosomally synthesized precursor peptide undergoes a series of modifications. *A*, the leader peptide (in *gray*) directs the prenisin to the nisin modification and transport machinery. The conserved FNLD box is highlighted 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, in turn, 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-type transporter NisT and processed by the extracellular protease NisP that cleaves off the leader peptide to liberate active nisin. Note that Ser-29 is never dehydrated in nisin.

Recent studies revealed that the modification of prenisin is a progressive and directional process requiring NisB and NisC to function cooperatively in an alternating fashion (20, 21). However, isolation of the proposed nisin modification complex NisBC has so far been unsuccessful (9, 19).

NisB is capable of dehydrating therapeutic peptides (unrelated to nisin) that are fused C-terminal to the nisin leader peptide (13, 22). Combined *in vivo* and *in vitro* data indicate that the leader peptide is essential for NisB and NisC modification (13, 15, 23) as well as for targeting the substrate peptide to the dedicated transporter NisT (17, 24). In addition, it was demonstrated that the leader peptide attached to the fully modified lantibiotic abolishes its antimicrobial activity, suggesting also a role in self-protection (15, 25).

The introduction of thioether rings in therapeutic peptides, either via enzymatic activity (NisBC) or chemical synthesis, has been shown to increase the resistance to proteolytic degradation (26, 27). Thus, a detailed understanding of the molecular mechanisms involved in nisin modification might be very important in the development of novel and improved peptide antibiotics as well as clinically relevant peptides that may be augmented by enzymatic posttranslational modification. However, whereas NisC cyclase activity has been successfully reconstituted *in vitro* and was shown to be independent of NisB and NisT (15), thus far, an *in vitro* activity of NisB has not been reported (2, 28).

Here we show a detailed biochemical and biophysical analysis of NisB and its interaction with prenisin and its modified derivatives, *i.e.* dehydrated and fully modified prenisin. Our results provide strong experimental evidence that the leader peptide and especially the FNLD box herein is a key determinant in substrate recognition and specificity of NisB.

## EXPERIMENTAL PROCEDURES

*Expression and Purification of His-tagged NisB—L. lactis* NZ9000 containing the plasmid pNGnisBhis was grown overnight in 100 ml of M17 medium containing 0.5% (w/v) glucose (GM17) and 5 $\mu$g/ml of chloramphenicol at 30 °C. Cells were

### NisB Interacts with Prenisin in Vitro

transferred to 2 liters of fresh GM17 medium and growth was continued to an $A_{600}$ of 0.8, whereupon NisB expression was induced by the addition of nisin (Sigma) to a final concentration of 25 ng/ml. Three hours after induction cells were harvested by centrifugation at 8000 × *g* for 20 min at 4 °C. The cell pellet was suspended in 14 ml of buffer composed of 50 mM HEPES-NaOH, pH 8.0, and 150 mM NaCl and stored at −20 °C until use.

For purification, cells were thawed at 4 °C, supplemented with protease inhibitor mixture (Roche Applied Science) and DNase I (Sigma), and lysed with a cell disruptor (IUL Instruments), generally 4–5 cycles at a pressure of 2.5 kbar. The remaining cells were removed at 18,000 × *g* for 30 min at 4 °C, and the resulting supernatant was cleared from membranes by centrifugation at 130,000 × *g* 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. The solution was then loaded on a 5-ml HiTrap chelating column (GE Healthcare) 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 applied to a HiLoad 16/60 Superdex 200 (GE Healthcare) 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 Ultracentrifugal filter Ultracel (100 kDa cut-off). Protein concentration was determined by measuring the absorbance at 280 nm with a NanoDrop ND-1000 spectrophotometer (peqlab) using the theoretical extinction coefficient of NisB of 128,400 liters $mol^{-1}$ $cm^{-1}$, as calculated for the His-tagged NisB using ProtParam webserver (EXPASY).

*Expression and Purification of Prenisin and Its Derivatives*—Production of prenisin (NisA) and its derivatives was performed with *L. lactis* strain NZ9000 containing pNZnisA-E3 (17) together with pIL3BTC (for fully modified prenisin) (29), pIL3hpBT (for dehydrated prenisin) (30), or pIL3hpT (for unmodified prenisin) (30) as described in Ref. 21. The FNLD/AAAA mutant was produced as described in Ref. 24. Purification of the various prenisin peptides was performed as described in Ref. 21 with modifications. Cell-free medium containing the peptide was diluted 1:1 with 50 mM lactic acid, pH 3, and subjected to SP-Sepharose chromatography. After peptide binding, the lactic acid buffer, pH 3, was gradually changed to 50 mM HEPES-NaOH, pH 7, by applying a gradient (0–100% 50 mM HEPES-NaOH, pH 7) for 4 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. At this stage, the eluent showed a pH of 7. Finally, bound prenisin was eluted with 50 mM HEPES-NaOH, pH 7, 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 Ultracentrifugal filter (30 kDa cut-off) to remove high molecular mass contaminants. The flow-through containing the prenisin was concentrated with an Amicon Ultracentrifugal filter (3 kDa cut-off). Peptide concentrations were determined with a Pierce BCA Protein Assay Kit (Thermo Scientific) at 584 nm.

*Purification of Nisin*—Nisin was obtained as a lyophilized powder from a commercial source (Sigma), which contains ~2.5% (w/w) nisin. The active nisin was purified as described elsewhere (41). In brief, about 1.3 g of powder (corresponding to ~32 mg of nisin) was diluted in 100 ml of 50 mM lactic acid, pH 3, and filtered through a 0.45-μm membrane filter (Pall Corporation). The nisin solution was then applied to a 5-ml HiTrap SP HP ion exchange column (GE Healthcare) using a flow of 2 ml/min. After binding, the column was washed with 50 mM lactic acid, pH 3, 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. Active nisin eluted at 400 mM NaCl. 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. Peptide concentrations were determined with a Pierce BCA Protein Assay Kit (Thermo Scientific) at 584 nm.

*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 were 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.

*Static Light Scattering*—Size exclusion chromatography (SEC)[2] and multiangle light scattering were performed in line on an Äkta purifier (GE Healthcare) connected to a triple-angle light scattering detector (miniDawn™ TREOS, Wyatt Technology) and a differential refractive index detector (Optilab® rEX, Wyatt Technology). SEC was performed using an analytical Superdex 200 10/300 column (GE Healthcare) equilibrated with 50 mM HEPES-NaOH, pH 7.4, and 250 mM NaCl. The protein concentration of the purified NisB in elution buffer was 2 mg/ml, whereas the sample volume was 100 μl. Data were analyzed with the ASTRA software package (Wyatt Technology).

*Interaction Studies Using SEC*—For complex formation, 10 μl of a 300 μM solution of purified prenisin or nisin (in 50 mM HEPES-NaOH, pH 7, 1 M NaCl, and 10% (v/v) glycerol) was mixed with 100 μl of 15 μM purified NisB (in 50 mM HEPES-NaOH, pH 8, 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 (GE Healthcare) system at 4 °C using 50 mM HEPES-

[2] The abbreviations used are: SEC, size exclusion chromatography; SPR, surface plasmon resonance; IMAC, immobilized metal ion affinity chromatography.

NaOH, pH 7.4, and 500 mM NaCl as elution buffer. Protein elution was monitored at 215 and 280 nm and the co-elution of NisB and prenisin was analyzed by SDS-PAGE. Protein was visualized by silver staining.

*Surface Plasmon Resonance (SPR) Measurements*—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 nitrilotriacetic acid 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 $Ni^{2+}$ by injecting 5 μl of a 10 mM $Ni^{2+}$ solution at 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 s. 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 BIAevaluation 4.1 software (GE Healthcare) according to the Equation 1, which describes the association, Equation 2, which describes the dissociation, and Equation 3, which describes the dissociation constant, $K_D$, as reported in Ref. 32. 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.

$$\frac{dR}{dt} = k_{on} \times C \times (R_{max} - R) - k_{off} \times R \quad \text{(Eq. 1)}$$

$$\frac{dR}{dt} = -k_{off} \times R \quad \text{(Eq. 2)}$$

$$K_D = \frac{k_{off}}{k_{on}} \quad \text{(Eq. 3)}$$

As the binding responses from SPR measurements correlates to the mass of molecules bound to the surface, the stoichiometry of the interaction can be evaluated by Equation 4 (33, 34).

$$\text{Stoichiometry} = \frac{R_{max} \times MW_l}{MW_a \times R_l} \quad \text{(Eq. 4)}$$

Where 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.

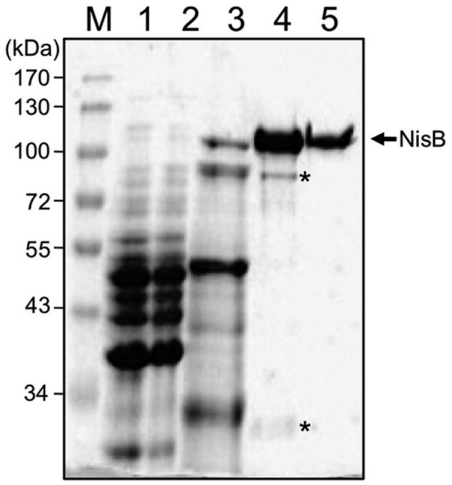


FIGURE 2. **Purification of NisB.** SDS-PAGE analysis of the purification of NisB. *M*, molecular mass marker proteins (kDa); *lane 1*, supernatant after high-speed centrifugation of *L. lactis* cell lysate; *lane 2*, flow-through of IMAC; *lane 3*, 50 mM imidazole IMAC wash; *lane 4*, 250 mM imidazole IMAC step elution; and *lane 5*, NisB after size exclusion chromatography. The *arrow* indicates NisB, whereas the *asterisks* mark the NisB degradation products.

## RESULTS

*Purification of NisB and Prenisin Peptides*—Nisin biosynthesis requires the dehydratase NisB to interact intimately with the nisin precursor peptide. To investigate the interaction of NisB and the nisin precursor peptide *in vitro*, NisB and unmodified prenisin and several modified derivatives thereof, were purified to homogeneity. NisB carrying a carboxyl-terminal $His_6$ tag was expressed in *L. lactis*, and purified from the cytosol using immobilized metal ion affinity chromatography (IMAC) followed by SEC. After IMAC, NisB (~120 kDa) and two contaminants (~90 and ~30 kDa) were present (Fig. 2, *lane 4*). These contaminants turned out to be degradation products of NisB, as determined by immunoblotting and mass spectrometry (data not shown), and could be removed by subsequent SEC (Fig. 2, *lane 5*). Thus, NisB could be purified to homogeneity from the cytosol with a typical yield of 1.5 mg of NisB/liter of cell culture. Notably, in buffer containing 50 mM HEPES-NaOH, pH 8, 500 mM NaCl, and 10% (v/v) glycerol, NisB was stable for at least 1 week at 4 °C as demonstrated by SEC, where no change in the elution profile occurred, and SDS-PAGE analysis (data not shown).

Next, the unmodified prenisin and several of its modified derivatives including the dehydrated and the fully modified prenisin and the FNLD/AAAA mutant (see also Fig. 1) were purified from the culture medium by ion exchange chromatography (see "Experimental Procedures"). Nisin on the other hand was purified from a commercial powder (Sigma). SDS-PAGE analysis of SP-Sepharose fractions and subsequent silver staining revealed that the various peptide preparations were pure (Fig. 3*A*). Subsequent mass spectrometry confirmed the expected masses for the purified peptides (data not shown). Mass spectrometry can, however, not directly distinguish between dehydrated prenisin and the fully modified prenisin as these peptides have identical masses: 5688 Da (peptide without initiating methionine, see also Ref. 30). We therefore sought a

### NisB Interacts with Prenisin in Vitro

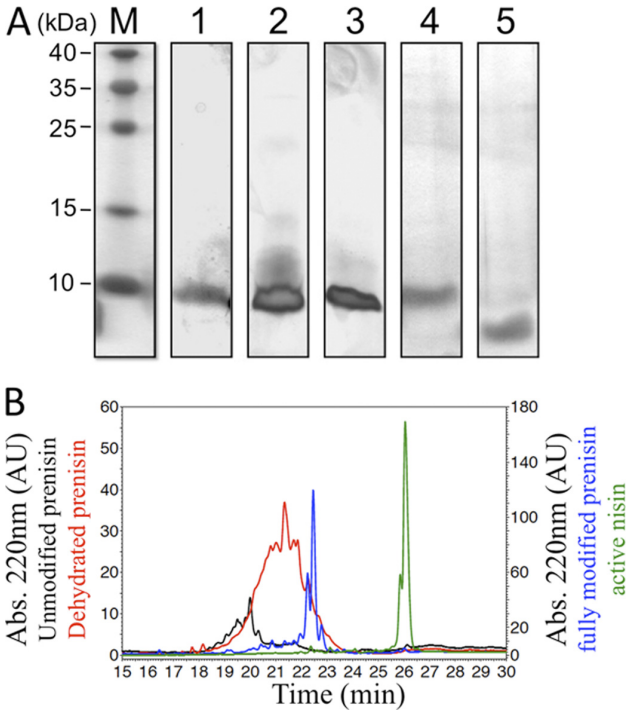


FIGURE 3. **Purification and RP-HPLC analysis of prenisin and prenisin-derived peptides.** *A*, SDS-PAGE analysis of purification of indicated peptides. *M*, molecular mass marker proteins (kDa); *lane 1*, unmodified prenisin; *lane 2*, dehydrated prenisin; *lane 3*, fully modified prenisin; *lane 4*, FNLD/AAAA prenisin mutant; *lane 5*, active nisin. Proteins were visualized by silver staining. *B*, 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.

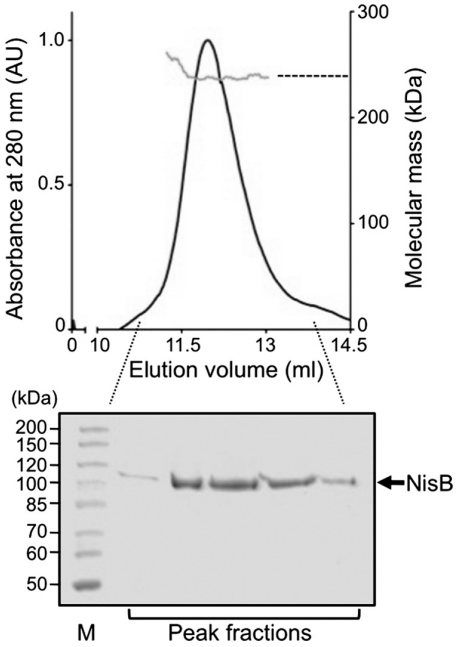


FIGURE 4. **Size exclusion chromatography and static light scattering analysis of NisB.** *Top panel,* NisB elution profile (*black line*) with determined molecular mass (*gray line*); *bottom panel,* SDS-PAGE analysis of the NisB peak fractions.

simple and direct method to discriminate between these peptides. For this, the different prenisin peptides were analyzed by RP-HPLC (Fig. 3*B*). Nisin and the different prenisin peptides were injected separately at a concentration of 50 $\mu$M. Increasing the acetonitrile concentration eluted the peptides. Interestingly, the peptides showed markedly different elution profiles, which relates to significant differences in their hydrophobicity (Fig. 3*B*). The unmodified prenisin eluted between 19.0 and 20.5 min as a broad peak (Fig. 3*B*, *black curve*), whereas the dehydrated prenisin eluted in a similar broad peak but at a substantially later retention time of 19.5–22.5 min (Fig. 3*B, red curve*). Compared with the unmodified prenisin, the 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 the serine and threonine residues, which contribute to the absorbance at 220 nm (35). The fully modified prenisin eluted at 22.5 min and showed a characteristic double peak (Fig. 3*B*, *blue curve*). Nisin also showed a characteristic double peak but eluted much later from the column as compared with the fully modified prenisin, *i.e.* at 26.3 min (Fig. 3*B*, *green curve*). The double peak of nisin has been observed before and has been attributed to a small number of nisin molecules in which Ser-33 has escaped NisB-mediated dehydration (19). 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.

*NisB Is a Dimer in Solution*—The purified components in hand allow the study of the interaction between NisB and prenisin *in vitro*. However, whereas NisB analyzed by SDS-PAGE exhibits the expected molecular mass of ~120 kDa (the calculated mass of His-tagged NisB is 118.3 kDa), we noticed that during preparative SEC NisB eluted as a protein with a molecular mass of ~173 kDa (elution volume 59.6 ml). We therefore determined the molecular mass of NisB in solution using multiangle static light scattering. For this, NisB was loaded on a Superdex 200 10/300 column and the eluted protein was analyzed using a triple-angle light scattering detector. SEC combined with multiangle static light scattering revealed that the purified NisB eluted as a homogenous species with a molecular mass of 238.0 ± 1.2 kDa (Fig. 4, *top panel*). Analysis by SDS-PAGE confirmed that only NisB was present in this peak (Fig. 4, *bottom panel*). Thus, under the tested conditions, NisB is present as a dimer (calculated molecular mass for the NisB-$His_6$ dimer is 236.6 kDa).

*NisB-Prenisin Interaction Studied with SEC*—To investigate the interaction of the various prenisins with NisB, analytical SEC studies were performed. For this, NisB was incubated with the unmodified, dehydrated, or the fully modified prenisin for 1 h 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 active nisin was investigated. Whereas NisB is readily detected at 280

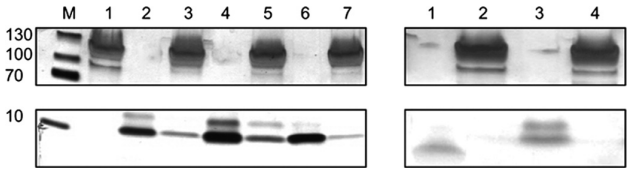


FIGURE 5. **Complex formation between NisB and prenisin.** SDS-PAGE analysis of the NisB peak fractions after size exclusion chromatography showing interaction between NisB and the different prenisins. *Left panels, M*, molecular mass marker proteins (kDa); *lane 1*, purified NisB (2.5 $\mu$M); *lane 2*, purified unmodified prenisin (7 $\mu$M); *lane 3*, co-elution of unmodified prenisin and NisB; *lane 4*, purified dehydrated NisA (7 $\mu$M); *lane 5*, co-elution of dehydrated prenisin and NisB; *lane 6*, purified fully modified prenisin (7 $\mu$M); *lane 7*, co-elution of fully modified prenisin and NisB. Note, due to the presence of free cysteines in unmodified and dehydrated prenisin, oxidative products (*bottom panels,* upper prenisin band) are often observed. *Right panels*; *lane 1*, purified active nisin (7 $\mu$M); *lane 2*, NisB incubated with nisin; *lane 3,* purified FNLD/AAAA prenisin (7 $\mu$M); *lane 4*, NisB incubated with FNLD/AAAA prenisin.

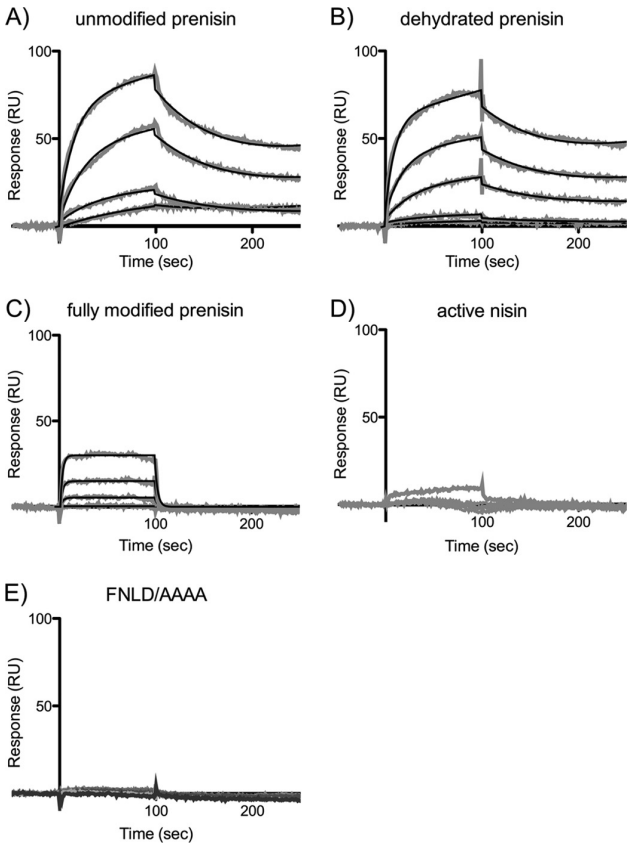


FIGURE 6. **SPR analysis of the interaction of NisB with prenisin.** Sensorgrams showing the interaction of immobilized NisB with unmodified prenisin (*A*), dehydrated prenisin (*B*), fully modified prenisin (*C*), nisin (*D*), and the FNLD/AAAA prenisin mutant (*E*). Injected peptide concentrations, from bottom to top, were 46 nM, 183 nM, 731 nM, 1.5 $\mu$M, and 2.9 $\mu$M.

nm, the prenisin peptides are not, due to a lack of Trp and Tyr 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 (data not shown). However, when NisB was incubated with the dehydrated prenisins, subsequent SEC analysis showed a small decrease in absorbance at 215 nm for the free dehydrated prenisin suggesting that some of the dehydrated prenisin molecules may be bound to NisB. 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 (Fig. 5). SDS-PAGE analysis demonstrated that the unmodified, dehydrated, and the fully modified prenisin co-eluted with NisB (Fig. 5, *left panels*, *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. The dehydrated prenisin was reproducibly present in the 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 (Fig. 5, *right panels*, *lane 2*). 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 also 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 (24). Interestingly, the FNLD/AAAA prenisin did not co-elute with NisB, suggesting that it did not to bind to NisB (Fig. 5, *right panels*, *lane 4*). 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.

*NisB-Prenisin Interaction Studied with SPR*—To characterize the interaction of NisB with the various prenisin peptides in more detail, the binding was quantitatively assessed by SPR. Measurements were performed by immobilizing NisB carrying a C-terminal His tag onto a $Ni^{2+}$-bound nitrilotriacetic acid surface, after which the different peptides were injected at various concentrations. The peptide was injected for 100 s and sensorgrams were recorded for 250 s (Fig. 6). The real-time binding responses showed an exponential association and an exponential dissociation phase for the unmodified, dehydrated, and the fully modified prenisin (Fig. 6, *A–C*, respectively). The data were fitted by 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 1. The unmodified prenisin exhibits an association rate ($k_{on}$) of $1.2 \pm 0.4 \times 10^4\ M^{-1}\ s^{-1}$ 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 $k_{on}$ ($5.1 \pm 1.4 \times 10^4\ M^{-1}\ s^{-1}$), but similar $k_{off}$ ($0.0149 \pm 0.0025\ s^{-1}$). The fully modified prenisin displayed a $k_{on}$ of $3.1 \pm 0.4 \times 10^4\ M^{-1}\ s^{-1}$ and a more than 20-fold higher $k_{off}$ of $0.323 \pm 0.035\ s^{-1}$, when compared with the dehydrated and 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, dehydrated, and the fully modified prenisin peptides were $1.05 \pm 0.25$, $0.31 \pm 0.07$, and $10.5 \pm 1.7$ $\mu$M, respectively. Thus, NisB binds the dehydrated prenisin with highest affinity, whereas 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 (Fig. 5). Consistent with SEC anal-

### NisB Interacts with Prenisin in Vitro

**TABLE 1**
**Kinetic constants for the NisB-prenisin interaction**

| Peptide | $k_{on}$ | $k_{off}$ | $K_D$ |
|---|---|---|---|
| | $_M{}^{-1}\,s^{-1}$ | $s^{-1}$ | $\mu M$ |
| Unmodified prenisin | $1.2 \pm 0.4 \cdot 10^4$ | $0.0117 \pm 0.0014$ | $1.05 \pm 0.25$ |
| Dehydrated prenisin | $5.1 \pm 1.4 \cdot 10^4$ | $0.0149 \pm 0.0025$ | $0.31 \pm 0.07$ |
| Fully modified prenisin | $3.1 \pm 0.3 \cdot 10^4$ | $0.323 \pm 0.035$ | $10.5 \pm 1.7$ |
| Active nisin | | NB[a] | |
| FNLD/AAAA prenisin | | NB | |

[a] NB, no binding observed under tested experimental conditions.

ysis, mature nisin essentially did not bind to NisB. Only at the highest concentration tested was a very weak binding response observed (Fig. 6*D*). Similarly, the unmodified prenisin carrying the FNLD/AAAA mutation did not bind to NisB (Fig. 6*E*). 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, *i.e.* the unmodified prenisin and its modified derivatives, *i.e.* the dehydrated and the fully modified prenisin with different affinity.

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 it can bind. The stoichiometry of the interaction of NisB with the different prenisin peptides was determined using Equation 4 (33, 34). The $R_{max}$ (maximum capacity for analyte binding) is a local fitted parameter and depends on the amount of immobilized NisB. Because 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, dehydrated, and the fully modified prenisin were found to be $0.9 \pm 0.1$, $0.8 \pm 0.1$, and $0.9 \pm 0.1$, respectively.

## DISCUSSION

The antibiotic potency of nisin seems to relate to its structure and dual mode of action. Nisin contains structural elements called (methyl)lanthionine rings that are important for binding to lipid II, an essential precursor molecule of the bacterial cell wall (6–8). Upon binding to lipid II, cell wall synthesis is blocked and nisin pores are formed that permeabilize the cytoplasmic membrane (6–8). The (methyl)lanthionine rings in nisin are installed posttranslationally by the cooperative action of the serine/threonine-specific dehydratase NisB and the cyclase NisC (21). Recently such lantibiotic modification enzymes have gained special interest as they can be successfully exploited to enhance the stability and activity of therapeutic peptides (26, 27). Moreover, a molecular understanding of the reactions catalyzed by these enzymes may further aid the development of novel and improved antibiotics.

From combined *in vivo* and *in vitro* studies it is known that the proteins involved in nisin biosynthesis, *i.e.* the dehydratase NisB, cyclase NisC, transporter NisT, and the leader peptidase NisP can act independent of each other (17–19, 22). The dehydration of the nisin precursor peptide catalyzed by NisB presents an early step in nisin maturation and is thus critical for nisin biosynthesis (Fig. 1). *In vivo* studies have demonstrated that NisB is promiscuous as it is able to dehydrate a multitude of nisin derivatives and even therapeutic peptides non-related to nisin (13, 22). However, for targeting such peptides to NisB the nisin leader peptide is required (13, 22). Unfortunately detailed information about the actual dehydration reaction is lacking. Moreover, no structure of NisB is available and reconstitution of *in vitro* activity of NisB has, thus far, been unsuccessful. In contrast, NisC cyclase activity has been successfully reconstituted *in vitro* and its crystal structure is available (15).

Here, we developed an *in vitro* binding assay to investigate the interaction between the lantibiotic dehydratase NisB and its native substrate the unmodified prenisin, as well as its dehydrated and fully modified derivatives. For this, NisB was expressed in its natural host *L. lactis* and purified to homogeneity (1.5 mg/liter of cell culture), whereas the substrate peptides were purified directly from minimal medium (Fig. 2). Interestingly, NisB purified from the cytosol proved to be dimeric as determined by static light scattering. The dimer was shown to be very stable as even after a week of storage no dissociation or aggregation were observed. To our knowledge, this is the first report that shows a NisB self-interaction. A study that combined a yeast two-hybrid screen with co-immunoprecipitations revealed interactions between members of the putative nisin synthase complex (NisA, NisB, NisC, and NisT) (9). In this study, a NisB self-interaction was, however, not observed. For the yeast two-hybrid screen fragments of NisB were used rather than full-length NisB. It is therefore intriguing to speculate that the self-interaction occurs only when full-length NisB is present. For SpaB, a NisB homolog from *Bacillus subtilis* that catalyzes the dehydration of the lantibiotic subtilin, a self-interaction was demonstrated by yeast two-hybrid analysis and the *in vitro* association of $His_6$-SpaB and Myc-SpaB (36, 37). The proposed subtilin synthase complex consists of two molecules each of SpaB, SpaC, and SpaT, whereas the assumed nisin synthase complex consists of only one NisB molecule and two molecules each of NisC and NisT (9, 36, 37). Our data raises the possibility that in the nisin synthase complex NisB is present as a dimer. Although a body of evidence exists that supports the existence of such multimeric lanthionine synthase complexes, direct isolation of these complexes, and thereby the determination of the stoichiometry of the involved proteins has so far been unsuccessful.

The isolated dimeric NisB exhibited biological activity *in vitro* as it binds its native substrate, the unmodified prenisin, as evidenced by SEC and SPR analysis (Figs. 5 and 6). The interaction between NisB and the unmodified prenisin occurred with an affinity of $1.05 \pm 0.25$ $\mu M$ (Table 1). 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 (9). The modified versions of prenisin, *i.e.* the dehydrated and the fully modified form, were also bound by NisB, although with different affinity (Figs. 5 and 6). Dehydrated prenisin, carrying eight dehydrated residues, showed a 3-fold higher affinity as compared with the unmodified prenisin, which relates to a substantially increased association rate (Fig. 6 and Table 1). This can be explained by an overall increase in hydrophobic interactions due to the presence of the dehydrated residues. In addi-

tion it is possible that NisB interacts in a specific manner with the non-leader part of prenisin. Support for this view comes from binding experiments using the fully modified prenisin (Figs. 5 and 6). Whereas the association rate of the fully modified prenisin was comparable with that of the unmodified and dehydrated prenisin, this peptide dissociated >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 $\mu$M). 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. This indicates a specific interaction of NisB with the propeptide (non-leader part) (29, 38). However, nisin, which is the equivalent of fully modified prenisin minus the leader peptide, did not bind to NisB (Figs. 5 and 6). 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 (13, 22, 23). The FNLD box within the leader peptide (Fig. 1) is highly conserved in the precursor peptides of class I lantibiotics (39). We show that the simultaneous substitution of the FNLD residues in the leader peptide by alanines abolished the interaction of prenisin with NisB *in vitro* (Figs. 5 and 6). These results may explain the *in vivo* observation that this mutant is secreted only in an unmodified form despite the presence of functional NisB and NisC (24). Thus the FNLD box is essential for prenisin interaction with NisB. However, because 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.

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. However, the interaction between NisB and prenisin is not only determined by the nisin leader peptide, but also by the nature of the propeptide (non-leader part). Thus the dehydratase NisB shows substrate specificity *in vitro*, as it is able to discriminate between the unmodified prenisin and its modified derivatives. Similarly, NisB may act as the specificity determinant within the nisin synthase complex *in vivo*. Following modification by NisB and NisC, the fully modified prenisin containing the thioether rings is rapidly released from the modification complex and subsequently exported by NisT.

Recent characterization of a number of prenisin mutants affected in ring formation revealed that NisB-mediated dehydration and NisC cyclase activity are strongly coordinated events. Herein, NisB and NisC alternate in function to install the modifications in a processive and directional manner (20, 21, 30). Our *in vitro* observations that thioether rings reduce the affinity of prenisin for NisB, whereas dehydrated residues appear to increase the affinity, would be compatible with such an alternating mechanism. It remains, however, to be determined whether the NisB dimer is required for biological activity.

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 insight into the mechanistic aspects of lantibiotic modification (31, 40). The dehydratases of the LanB family, *i.e.* the lantibiotic synthases, that require separate enzymes for dehydration and cyclization remain on the other hand 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 (2). Therefore, future work will focus on the aim to reconstitute the dehydratase activity of NisB *in vitro*.

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## 3.5 Chapter V - NisC

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# NisC Binds the FxLx Motif of the Nisin Leader Peptide

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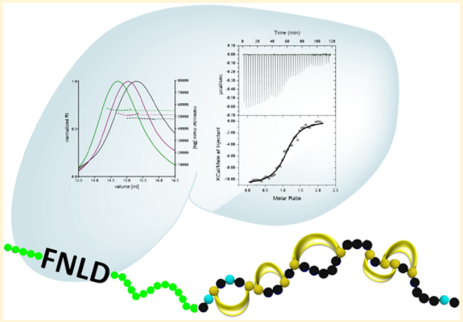
Supporting Information

**ABSTRACT:** Nisin is a model system for lantibiotics, a class of peptides displaying antimicrobial activity against various Gram-positive bacteria. After ribosomal synthesis, the precursor peptide is modified in two steps, of which the last one involves consecutive cyclization reactions mediated by the cyclase NisC. Here, we present a detailed *in vitro* study of the interaction between NisC and the nisin precursor peptide. Our results unravel a specific interaction of NisC with the leader peptide independent of the maturation state. Furthermore, mutagenesis studies identified a specific binding sequence within the leader. Two amino acids ($F_{-18}$ and $L_{-16}$) within the highly conserved -FNLD- box of class I lantibiotics are essential for binding. They represent a potential general binding motif between leader peptides of a group of lantibiotics with their cyclase family. In summary, these *in vitro* data provide a new perception on the complexity of the lantibiotic modification machineries.




The ribosomally synthesized and post-translationally modified antimicrobial peptide nisin is produced by several *Lactococcus lactis* (*L. lactis*) strains.[1−3] Nisin was first discovered in 1928[4] and contains dehydrated amino acids, which become covalently linked to free cysteine thiols to form the characteristic (methyl)lanthionine rings.[3,5] These modifications classify nisin as a member of the lanthipeptide (lanthionine-containing peptides) superfamily, of which lantibiotics are a subclass with antimicrobial activity.[6,7] The N-terminal (methyl)lanthionine rings of nisin are crucial for its mechanisms of antimicrobial activities,[5,8] which are also present in other lantibiotics.[5,9,10] Another example is mersacidin from *Bacillus subtilis*, which displays activity against methilicin-resistant *Staphylococcus aureus* (MRSA).[11]

Nisin is the most intensively used and best-characterized lantibiotic and serves as a model system for these highly effective antibacterial peptides.[3,6,7] Nisin already has antimicrobial activity at nanomolar concentrations.[12]

Within *L. lactis*, a gene operon consisting of *nisABTCIPRKFEG* is responsible for nisin production. These 11 gene products are involved in nisin maturation (*nisBC*), secretion and processing (*nisTP*), regulation (*nisRK*), or immunity (*nisIFEG*).[13−15] Nisin is ribosomally synthesized as a precursor peptide consisting of 57 amino acids. The first 23 amino acids are defined as the leader peptide (residues −23 to −1), while the remaining 34 amino acids are called the core peptide (residues 1−34, Figure 1). The 34 amino acid part corresponds to the unmodified nisin, which by modification is converted in the (methyl)lanthionine-containing nisin.[16] After ribosomal synthesis, several posttranslational modifications occur, affecting the core peptide.[15,17] These reactions result in the formation of the dehydrated amino acids didehydroalanine (from serine) and didehydrobutyrine (from threonine) introduced by the nisin dehydratase NisB.[17−19] After dehydration, the double bond reacts with the thiol group of a cysteine yielding to a (methyl)lanthionine. This reaction is catalyzed by the nisin cyclase NisC in a regio- and stereospecific manner.[17,20]

Subsequent to the modification reactions, fully modified nisin is secreted across the Gram-positive cell membrane. An ABC transporter, NisT, which recognizes the leader peptide catalyzes this step.[21−23] After secretion, the peptide is converted into the active state by catalysis of a serine protease, NisP, which is anchored to the cell membrane and cleaves off the leader peptide C-terminal to the amino acid sequence PR.[24,25] This releases mature nisin into the extracellular space.

The first evidence for the function of the modification machinery was derived from knockout studies in a nisin-producing system *in vivo* indicating that NisB is responsible for dehydration and can function independently of NisC.[17,18] Very recently the *in vitro* activity of NisB was demonstrated, which revealed an additional step before dehydration. The serine and threonine residues become initially glutamylated by NisB before the dehydration reaction can take place.[19] Within an *in vivo* study of NisB, it was also demonstrated that the absence of *nisC* resulted in a peptide that was dehydrated but lacked any antimicrobial activity. This was the first experimental indication for a cyclization function of NisC.[17]

The formation of a potential NisB and NisC complex was investigated via two hybrid studies in yeast.[22] These results

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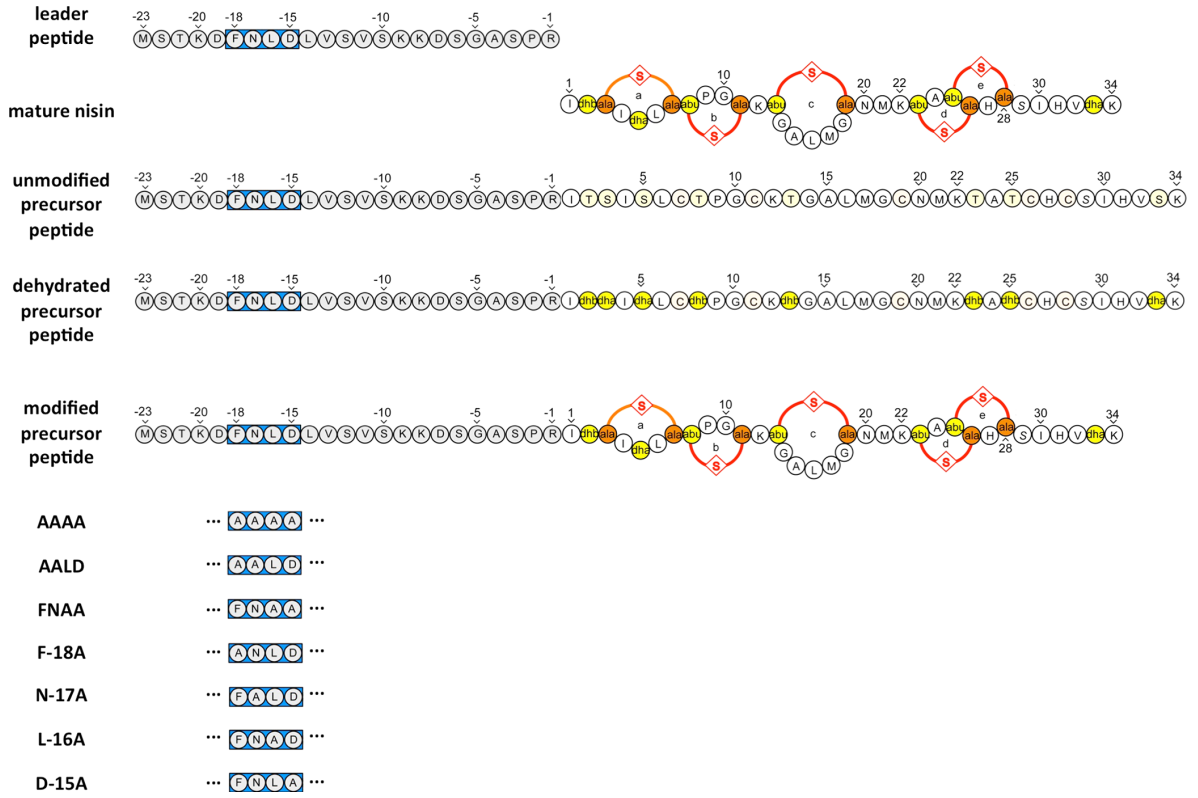


**Figure 1.** Scheme of the nisin precursor peptide variants and mature nisin used in this study. Mature nisin: final product after NisB, NisC, and NisP treatment possessing antimicrobial activity. Unmodified precursor peptide: ribosomally produced precursor peptide without any post-translational modifications. Dehydrated precursor peptide: unmodified precursor peptide after post-translational dehydrations catalyzed by NisB. Modified precursor peptide: fully modified precursor peptide after dehydration by NisB and cyclization by NisC. Leader peptide: the isolated polypeptide chain ranging from amino acid −23 to −1 of the precursor peptide. The following mutations are all based on the modified precursor peptide. -AAAA-: mutation in the leader peptide $F_{-18}A/N_{-17}A/L_{-16}A/D_{-15}A$. -AALD-: mutation in the leader peptide $F_{-18}A/N_{-17}A$. -FNAA-: mutation in the leader peptide $L_{-16}A/D_{-15}A$. The highly conserved -FNLD- box within the leader peptide is highlighted in blue, and specific serine and threonine residues are highlighted in yellow. The five (methyl)lanthionine rings are shown in red and orange, respectively.

together with mutational analysis within the core peptide led to the conclusion that the dehydration and cyclization reactions are related in space and time. It also implied directionality from the N- to the C-terminus and a direct interaction of NisB and NisC with the precursor peptide.[26]

A molecular understanding of the modification processes was given by an *in vivo* identification of a conserved box within the leader peptide of the lantibiotic precursor.[25] Sequence alignments of class I lantibiotic leader peptides identified a conserved -FNLD- box.[27] *In vivo* studies revealed that no posttranslational modifications were introduced into the core peptide in the case of an -FNLD-/-AAAA- mutation.[25] This suggested that at least NisB is interacting with the -FNLD- box.[25] However, a potential influence of mutations within the -FNLD- box on the activity of NisC is unknown.

NisC belongs to the enzyme family LanC, which is present in class I lantibiotic genetic operons. Examples of this family are the cyclases SpaC specific for subtilin,[28] EpiC specific for epidermin,[29] or EciC specific for Epicidin280.[30] On the basis of a phylogenetic study of lanthipeptides synthetases, members of this family can be clustered in a subgroup of the LanC clade.[31] The strict correlation of the presence of an -FNLD- box within the leader sequence and the presence of a LanC cyclase in the lantibiotic operon point towards a general recognition motif.

In this study, we focus on the second and last step of the nisin modification machinery, the cyclization by NisC. We analyzed the molecular mechanisms of interaction of the cyclase NisC with the precursor peptide *in vitro* to obtain a quantitative view on the mode of action. These studies provide the first *in vitro* analysis of the proposed NisC−nisin precursor interaction. Our thermodynamic data demonstrate that NisC is solely interacting with the leader peptide. More precisely, the recognition site of NisC was pinpointed to the -FNLD- box, in which two highly conserved amino acids ($F_{-18}$ and $L_{-16}$) are essential for the interaction with the nisin cyclase.

## MATERIALS AND METHODS

**Cloning of pET-28b-AA-NisC.** NisC was amplified out of *Lactococcus lactis* strain NZ9700[32] with the oligonucleotides NisCpet28for (CAAGAACTTTATTATTCAGGCT-AGCATGAGGATAATGAT) and NisCpet28rev (CACAATA-AGTATTAAATCTCGAGTCATTTCCTCT-TCCCTCCTTTC) containing restriction sites for NheI and XhoI, respectively. Digested PCR product and pET-28b (with NheI and XhoI, Fermentas) were ligated and transformed in



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*Escherichia coli* (*E. coli*) XL-1 Blue cells. The clones were sequenced, and positive hits were transformed in *E. coli* BL21 (DE3) cells for expression. All DNA kits were obtained from Qiagen.

**Overexpression of NisC.** *E. coli* BL21 (DE3) cells containing the pET-28b-AA-NisC plasmid were used for a 200 mL LB preculture containing 30 $\mu$g mL$^{-1}$ kanamycin. Twelve liters of LB medium was supplemented with 100 $\mu$M zinc chloride, inoculated to an $OD_{600}$ of 0.05, and grown at 37 °C with 180 rpm shaking to an $OD_{600}$ of 0.8. At 18 °C, the culture was induced with 100 $\mu$M IPTG and grown overnight. After 20 h, cells were harvested at 6,000*g* and stored at −20 °C.

**Purification of NisC.** Cells were thawed on ice and resuspended in 50 mM HEPES-NaOH, 1 M NaCl, pH 8.0, and 10% glycerol. After adding small amounts of DNase, cells were lysed with a cell disruptor (IUL Instruments) and centrifuged at 4 °C with a low spin step (30 min, 14,5000*g*), followed by a high spin step (45 min, 90,000*g*). The resulting supernatant was supplemented with imidazole (final concentration 10 mM) and applied to an immobilized metal-ion affinity chromatography column (IMAC, Chelating HP column preloaded with $Zn^{2+}$, GE Healtcare). NisC was eluted with 50 mM HEPES-NaOH, pH 8.0, 1 M NaCl, 10% glycerol, and 150 mM imidazole in one step and further purified by size-exclusion chromatography (SEC) (Superdex 200 26/60) (GE Healthcare) pre-equilibrated with SEC buffer (50 mM HEPES-NaOH, pH 7.0, 1 M NaCl, and 10% glycerol). All fractions containing NisC were pooled and concentrated with an Amicon ultracentrifugation unit (30 kDa MWCO). The resulting NisC was digested with the Thrombin clean cleave kit (Sigma) to cleave off the N-terminal $His_6$-tag following the instructions of the manufacturer. After cleavage overnight, noncleaved and cleaved NisC were separated via IMAC following the procedure outlined above. Tag-free NisC was concentrated to 20 mg mL$^{-1}$, aliquoted, frozen in liquid nitrogen, and stored at −80 °C.

**Overexpression and Purification of Mature Nisin, Precursor Peptide, and Its Variants.** Cloning, expression, and purification of mature nisin and the precursor peptide variants were previously described.[12,33] Compared to the published purification protocols, only the elution buffer of the cation exchange chromatography (cIEX) of the different precursor peptide derivatives was changed to 50 mM HEPES-NaOH, pH 7.0, 1 M NaCl, and 10% glycerol.

**RP-HPLC Analysis of Mature Nisin, Precursor Peptide, and Its Variants.** Peptides were analyzed by RP-HPLC with a LiChrospher WP 300 RP-18 end-capped column using an acetonitrile/water solvent system consisting of solvent A (10% acetonitrile/90% water/0.1% TFA) and solvent B (90% acetonitrile/10% water/0.1% TFA). After sample injection, the elution was performed by a linear gradient over 35 min to 60% solvent B at a flow rate of 1 mL min$^{-1}$.

Specific peptide concentrations were determined by the absorption at 205 nm. For calibration, known amounts of nisin (Sigma) and human insulin (Sigma) were injected, and the absorption integrals were determined with the software EZChrom Elite software V.3.3.1. and plotted against the known masses. A linear calibration line is calculated, and this equation was used to determine the specific concentrations from all used peptides.

**Binding Experiments between NisC and the Precursor Peptides by Isothermal Titration Calorimetry (ITC).** The binding parameters between NisC and the precursor peptides were determined by ITC. To circumvent dilution heat or heat resulting from the buffer mixture, the enzyme NisC and the substrate precursor peptide were separately dialyzed against SEC buffer. After dialysis, the concentration of NisC was adjusted to 400 $\mu$M and the concentration of the precursor peptide to 40 $\mu$M. Using an ITC200 (Microcal, GE Healtcare), the enzyme NisC, with a volume of 40 $\mu$L, was titrated to the different precursor peptide variants. Each experiment was performed at least in triplicate. The ITC measurements were performed at 25 °C with 40 injections (1 $\mu$L each). Only the first injection had a volume of 0.5 $\mu$L and was discarded from the isotherm. The other technical parameters were reference power = 5 $\mu$cal s$^{-1}$, stirring speed = 1000 rpm, spacing time = 180 s, and a filter period = 5 s. The control experiment (NisC in buffer) was consistently subtracted from each isotherm, and the resulting isotherm was fitted with a one site binding model using the Origin 7 Microcal software.

**Complex Formation Analysis Using MALS-SEC.** Twenty micromolar NisC was incubated with 200 $\mu$M mature nisin or precursor peptide variants for 1 h at 25 °C. Five hundred microliters were injected on a SEC column (Superdex 200 10/300 (GE Healthcare)) pre-equilibrated with MALS buffer (50 mM HEPES-NaOH, pH 7.0, 500 mM NaCl) with a flow rate of 0.4 mL min$^{-1}$. Absorption signal at 280 nm, light scattering signal (triple-angle light scatter detector miniDAWM TREOS, Wyatt Technology Europe), and differential refractive index (Optilab rEX detector (Wyatt Technology Europe) were monitored. Data were analyzed with the ASTRA software package (Wyatt Technology).

**NisP Purification.** The *L. lactis* strain NZ9000 with plasmid pNG-NisP8His allowed for the expression of the serine protease NisP. A 50 mL GM17 preculture was supplemented with 5 $\mu$g mL$^{-1}$ chloramphenicol and grown overnight at 30 °C. Cells were harvested, and 500 mL of MM-medium was inoculated with a starting $OD_{600}$ of 0.1. Expression was induced with nisin at a final concentration of 0.1 ng mL$^{-1}$. Cells were grown overnight at 30 °C and removed by centrifugation (15,000*g*, 30 min). The pH of the resulting supernatant was adjusted with 3 M Tris-HCl to pH 7.5 and applied to an IMAC HP column (GE Healthcare) preloaded with $Co^{2+}$ at a flow rate of 4 mL min$^{-1}$. The column was intensively washed with buffer (50 mM HEPES-NaOH, pH 8.0, and 150 mM NaCl) until a stable baseline was reached, and NisP was eluted in a single step (50 mM HEPES-NaOH, pH 8.0, 150 mM NaCl, and 300 mM imidazole). Elution fractions were concentrated by ultracentrifugation (10 kDa MWCO) to 2.5 mL, and the buffer was exchanged with a PD10 column (GE Healthcare) to 50 mM HEPES-NaOH, pH 8.0, 150 mM NaCl, and 10% glycerol. The eluting fractions were concentrated to 250 $\mu$L, and NisP was aliquotted and stored at −80 °C.

**Leader Peptide Preparation and Precursor Peptide Digestion.** The nisin leader peptide (amino acids −23 to −1) was produced by digestion of the modified precursor peptide by the protease NisP. To this end, the purified modified precursor peptide was incubated with purified NisP in a molar ratio of 1000:1 at 30 °C overnight. The reaction mixture was applied to a RP-HPLC column, and the eluting leader peptide was fractionated and lyophilized. The corresponding leader peptide was dissolved in SEC buffer and used for ITC and MALS-SEC measurements.

**MALS-SEC Analysis of NisC with NisP Digested Precursor Peptides.** The unmodified, dehydrated, and modified precursor peptides were digested with NisP as described above. Before MALS-SEC analysis, NisP was

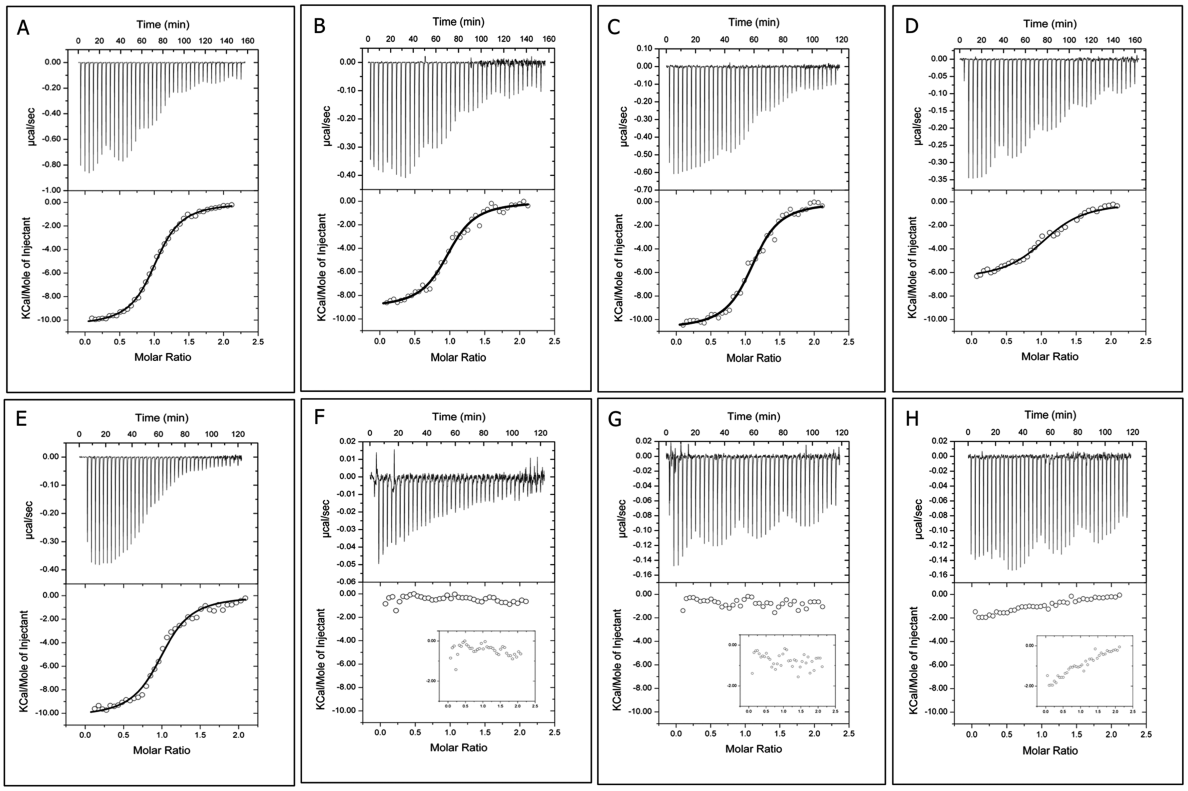


**Figure 2.** ITC experiments of NisC and different precursor peptide variants. A: unmodified precursor peptide. B: dehydrated precursor peptide. C: modified precursor peptide. D: leader peptide. E: $N_{-17}$A variant. F: $F_{-18}$A variant. G: -AAAA- variant. H: NisC is titrated into SEC buffer. All experiments were performed at least in triplicate. For detailed thermodynamic parameters, see Table 1.

extracted from the reaction mixture by binding to $Ni^{2+}$ coupled magnetic beads (Qiagen) for 1 h at room temperature.

## RESULTS

**Purification of the Cyclase NisC.** The enzyme NisC was isolated as described in detail in Materials and Methods. NisC was analyzed by SEC resulting in a single peak of NisC. $His_6$-NisC was purified to more than 95% purity as judged from SDS−PAGE analysis with a yield of roughly 10 mg $L^{-1}$ of cell culture and stored at −80 °C until further use (Figure S1, Supporting Information). Prior to the interaction studies, the $His_6$-tag was removed using thrombin (see Materials and Methods). This was necessary because the $His_6$-tag inhibited the interaction of NisC with the precursor peptide (data not shown). Subsequently, the MALS-SEC analysis demonstrated that tag-less NisC is a monomer in solution (see below and Figure S2, Supporting Information, black line).

**Purification of Mature Nisin and the Precursor Peptide Variants.** All precursor peptide variants used in this study (Figure 1) were expressed in *L. lactis* and secreted using a two-plasmid system as previously described.[23,34,35] The isolation is described in detail in the Materials and Methods section.

All precursor peptide variants, mature nisin, and the leader peptides were analyzed by tricine SDS−PAGE (Figure S3, Supporting Information) and reached at least 90% purity. In contrast, the purity of the -AAAA- mutation of the -FNLD- box within the leader peptide was only around 70%, which is likely due to the low secretion level preventing an efficient purification.[25]

To perform an accurate and reproducible ITC experiment, the exact concentration of each interaction partner had to be known precisely. To determine the specific concentration of the precursor peptides, we employed analytical RP-HPLC. Different nisin and insulin samples of known concentrations were used to calibrate the RP-HPLC column. Here, the absorption of the peptide bond at 205 nm was monitored, which is independent of the exact amino acid composition. The resulting peaks were integrated and plotted against the known nisin and insulin concentrations. Figure S4 (Supporting Information) summarizes the obtained calibration line, which was used in all subsequent experiments to determine the concentration of the different nisin and nisin intermediate samples. For clarity, the RP-HPLC elution profiles are summarized in Figure S5 (Supporting Information).

**Interactions of NisC with the Precursor Peptide Variants.** We applied ITC to characterize potential interactions between NisC and nisin precursor peptides and variants. Because of the high solubility of NisC and the lower solubility of the precursor peptides, especially at high concentrations, the classic ITC setup was inverted. Here, the syringe was filled with a concentrated NisC solution (400 $\mu$M), and the measuring cell was filled with the corresponding precursor peptide (40 $\mu$M). Initially, the unmodified, dehydrated, and modified precursor peptides were measured (see Figure 2A−C). All three precursor peptides resulted in an

**Table 1. ITC Data of NisC and the Precursor Peptide Variants[a]**

| precursor peptide variant | $N$ | $K_D$ [$\mu$M] | $\Delta H°$ [kJ/mol] | $\Delta S°$ [J/mol·K] | $\Delta G°$ [kJ/mol] |
|---|---|---|---|---|---|
| unmodified | 1.1 ± 0.1 | 2.4 ± 0.5 | −44.1 ± 3.3 | −40.1 ± 10.5 | −32.1 ± 0.5 |
| dehydrated | 1.1 ± 0.1 | 2.0 ± 0.2 | −46.0 ± 7.1 | −62.6 ± 8.9 | −27.3 ± 1.5 |
| modified | 1.0 ± 0.1 | 2.0 ± 0.5 | −55.3 ± 2.2 | −75.5 ± 5.6 | −32.9 ± 0.9 |
| leader peptide | 1.0 ± 0.2 | 3.8 ± 0.6 | −34.9 ± 4.6 | −20.3 ± 7.3 | −30.3 ± 1.3 |
| -AAAA- | no binding observed in ITC measurements | | | | |
| -AALD- | no binding observed in ITC measurements | | | | |
| -FNAA- | no binding observed in ITC measurements | | | | |
| -ANLD-($F_{-18}$A) | no binding observed in ITC measurements | | | | |
| -FALD-($N_{-17}$A) | 1.1 ± 0.2 | 1.4 ± 0.2 | −41.2 ± 2.2 | −25.8 ± 8.3 | −33.5 ± 0.3 |
| -FNAD-($L_{-16}$A) | no binding observed in ITC measurements | | | | |
| -FNLA-($D_{-15}$A) | 1.1 ± 0.1 | 7.1 ± 1.6 | −32.9 ± 4.3 | −17.5 ± 3.1 | −29.0 ± 1.1 |
| NisC | control experiment | | | | |

[a]All experiments were performed at least in triplicate, and the error represents the standard deviation of a minimum of three independent experiments. For experimental details, see Materials and Methods.

isotherm indicating specific binding. Furthermore, they displayed a similar equilibrium or dissociation constant ($K_D$) of 2.4 ± 0.5 $\mu$M for the unmodified, 2.0 ± 0.2 $\mu$M for the dehydrated, and 2.0 ± 0.5 $\mu$M for the modified precursor peptide (Figure 2A−C and Table 1). Within experimental error, a 1:1 stoichiometry of the individual complexes was determined (Table 1). This suggests that the maturation state of the core peptide does not influence the binding affinity of NisC to the precursor peptides. In other words, the leader peptide is sufficient for an effective interaction.

To validate this hypothesis, the leader peptide was produced by incubation of the modified precursor peptide with the purified protease NisP (see Materials and Methods and Supporting Information, Figure S6). The two products of the cleavage reaction, the leader peptide and the core peptide, were separated by RP-HPLC (Figure 3A). After purification, the interaction of the isolated leader peptide with NisC was analyzed by ITC (Figure 2D). A binding affinity of $K_D$ = 3.8 ± 0.6 $\mu$M and a 1:1 stoichiometry of the leader peptide and NisC complex were observed (Table 1). A second technique, MALS-SEC, was used to verify this result. MALS-SEC allows for the determination of absolute masses of a protein or a protein complex in solution. Incubation of NisC with the precursor peptides resulted in a shift of the elution profile toward earlier retention times (Figure S2, Supporting Information, and Table 2). The molecular weight determined by MALS was 48550 ± 1085 Da for NisC. For the complex of NisC with unmodified, dehydrated, or modified precursor peptide, the analysis resulted in 55730 ± 1280 Da, 56310 ± 1730 Da, and 55260 ± 1100 Da, respectively (highlighted by the green dotted line in Figure S2 (Supporting Information) for the dehydrated precursor peptide; see Table 2). The difference in molecular mass of the protein−ligand complexes corresponds to the molecular mass of a single precursor peptide within experimental error. The reinjection of the NisC−precursor peptide complex resulted in an identical elution profile and identical difference in molecular mass (data not shown). This highlights that the NisC−precursor peptide complex is stable. Furthermore, a shift in retention time as well as a stable complex with a determined molecular mass of 51250 ± 1420 Da was observed by performing the analysis with the isolated leader peptide (highlighted by the orange line in Figure S7, Supporting Information, and Table 2). Subsequently, MALS-SEC analysis between NisC and mature nisin was performed. The observed mass of 49260 ± 1290 Da corresponded to the molecular mass of monomeric NisC (highlighted by the black lines in Figure 3B and Table 2). This shows that the leader peptide is responsible for binding to NisC.

**Does the Leader Peptide Contain All Required Information for Interaction?** These thermodynamic ITC data demonstrate that the affinity of the complex of NisC and the isolated leader peptide was slightly lower than the affinity of the complex of NisC and the isolated nisin intermediates. This difference was small but statistically reproducible from three independent experiments. One can explain these data by low affinity binding by the whole or by part of the core peptide. This low affinity binding would only become accessible after the leader peptide has formed a stable complex due to, for example, an induced conformational change.

To verify this hypothesis, the modified precursor peptide was digested with NisP resulting in the leader peptide and mature nisin (Figure 3A). This mixture was incubated with NisC and subsequently analyzed by MALS-SEC resulting in only a NisC−leader complex. (Figure S7, Supporting Information, and Table 2). Even a 4-fold access of mature nisin added to a preformed NisC−leader complex provided no indication of binding of the core peptide of mature nisin (Figure 3B and Table 2).

Identical results were obtained for the core peptides of unmodified and dehydrated nisin. Here, the core peptides were obtained by digestion of the corresponding nisin intermediates with NisP. A successful and quantitative cleavage was verified by MS analysis. The mixture was analyzed by MALS-SEC resulting only in a NisC−leader peptide complex (Figure S7, Supporting Information, and Table 2). Altogether, this shows that the leader contains the recognition motif for the cyclase NisC.

**Specific Binding Motif within the Leader Peptide.** To further investigate the binding site within the leader peptide, alanine mutagenesis within the highly conserved -FNLD- box was performed.[25] The seven precursor peptide variants used in this study are summarized in Figure 1 and were expressed and purified as the modified precursor peptides (see Materials and Methods).

The interaction of NisC with the precursor peptide was abolished in the -AAAA- variant of the -FNLD- box (Figure 2G and Table 1). The ITC isotherm resulted in a graph similar to that of the control experiment (Figure 2H and Table 1), where NisC was titrated into buffer only indicating no interaction. This result was verified by MALS-SEC analysis revealing only



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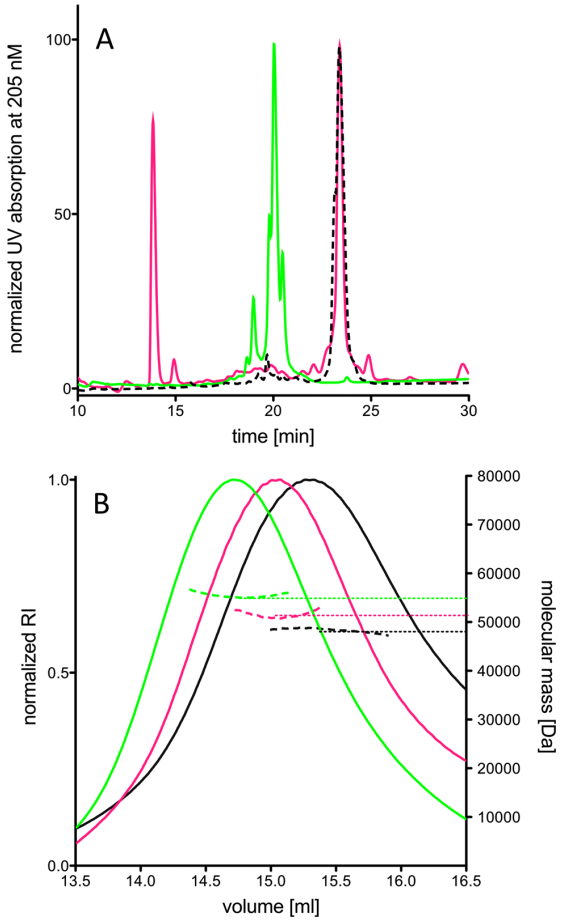


**Figure 3.** (A) NisP digestion of the modified precursor peptide observed by RP-HPLC. The modified precursor peptide (green) was digested with NisP and analyzed by RP-HPLC. The digested sample (pink) showed two peaks. One at an elution time of 14.5 min. This corresponded to the leader peptide, which was confirmed by MS. The second peak eluted at 24 min. The elution time is identical to that of mature nisin shown in black. All experiments were performed at least in triplicate. (B) MALS-SEC analysis of NisC and the modified precursor peptide before and after NisP digestion. Green line: elution profile of NisC incubated with the modified precursor peptide. The observed molecular mass of NisC in complex with the modified precursor peptide is shown in the green dotted line (the horizontal green dotted line indicates the expected molecular mass of a NisC−precursor peptide complex). Black line: elution profile of NisC incubated with mature nisin. The observed mass of NisC incubated with mature nisin shown in the black dotted line (the horizontal black dotted line indicates the expected molecular mass of a NisC monomer). Pink line: elution profile of NisC presaturated with a 2-fold excess of the leader peptide incubated with a 4-fold excess of mature nisin. The pink dotted line shows the observed molecular mass. The horizontal pink line represents the expected molecular mass of a NisC−leader peptide complex. All experiments were performed at least in triplicate. See Table 2 for a detailed summary of the observed molecular masses.

monomeric NisC without any precursor peptide bound (Table 2). Also, the variants -AALD- and -FNAA- were analyzed and did not show any interaction with NisC in the ITC experiment (Table 1).

**Table 2. Overview of the MALS-SEC Analysis between NisC and Mature Nisin and Precursor Peptide Variants[a]**

| (pre)nisin | MALS-SEC analysis [Da] | difference to NisC [Da] |
|---|---|---|
| NisC | 48550 ± 1086 | |
| unmodified | 55730 ± 1280 | 7180 ± 274 |
| dehydrated | 56310 ± 1730 | 7760 ± 911 |
| modified | 55260 ± 1100 | 6710 ± 20 |
| mature nisin | 49260 ± 1290 | 710 ± 288 |
| leader peptide | 51250 ± 1420 | 2700 ± 472 |
| -AAAA- | 48350 ± 1124 | −200 ± 54 |
| leader + modified core peptide (1:1) | 51080 ± 1120 | 2530 ± 48 |
| leader + modified core peptide (1:2) | 50690 ± 1318 | 2140 ± 328 |
| leader + unmodified core peptide (1:1) | 50570 ± 1140 | 2020 ± 76 |
| leader + dehydrated core peptide (1:1) | 51220 ± 1060 | 2670 ± 37 |

[a]The first row summarizes the analyzed samples. The observed molecular masses obtained by MALS-SEC analysis are shown in row two. In the third row, all potential NisC−substrate complexes were subtracted by the mass of isolated NisC. These data indicate that one single precursor peptide, specifically one leader peptide molecule, is interacting with NisC within the inaccuracy of the MALS-SEC analysis. For experimental details, see Materials and Methods.

Within the -FNLD- box sequence of class I lantibiotics, the $F_{-18}$ and the $L_{-16}$ are highly conserved, whereas slight variations are observed at position −17 (N in nisin) and position −15 (D in nisin) (Figure 4). Single amino acid replacements by alanine showed no binding for $F_{-18}$A and $L_{-16}$A to NisC, whereas $N_{-17}$A and $D_{-15}$ both were recognized by the cyclase NisC. $N_{-17}$A had a similar affinity of 1.4 ± 0.2 $\mu$M (Figure 2E) for NisC as the wild type precursor peptide and the $D_{-15}$A mutant showed a slighty lower affinity of 7.1 ± 1.6 $\mu$M (Table 1).

Taken together, these data show that the highly conserved amino acids $F_{-18}$ and $L_{-16}$ within the -FNLD- box are essential for binding to NisC. Both have to be present simultaneously with respect to the ITC result of the -AALD- and -FNAA- variants. The mutation of $N_{-17}$ had no impact on the binding; similarly, $D_{-15}$ played only a minor role in recognition as observed by a slightly increased $K_D$.

## DISCUSSION

Lantibiotics are peptides, which possess antimicrobial activity against Gram-positive bacteria such as *Streptococcus pneumonia*, *Staphylococcus aureus*, *Enterococcus faecium*, and *Enterococcus faecalis*.[11,36] This activity is mediated by at least two modes of action: (i) by binding to the cell wall precursor lipid II, which inhibits cell wall synthesis, and (ii) by forming pores within the membrane.[2,37,38] All lantibiotics share post-translational modifications like dehydration and cyclization.[19] The dehydrated amino acids and more significantly the (methyl)lanthionine rings are essential for antimicrobial activity.[5] Furthermore, the characteristic (methyl)lanthionine rings protect the peptide against proteases and increase the stability of the lantibiotic.[24,39]

Within the nisin biosynthesis system, NisB and NisC, are responsible for the modification and maturation process.[17] The exact stoichiometry of the modification complex consisting of the dehydratase NisB and the cyclase NisC has not been investigated in detail. Recently, it was shown that NisB is a dimer in solution[33] and binds to a single precursor peptide.

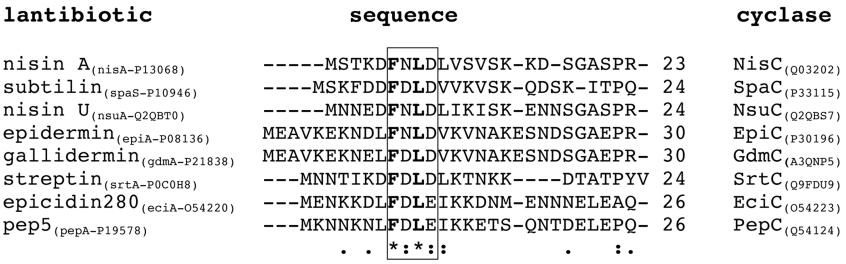


**Figure 4.** Sequence alignment of the -FNLD- box created with ClustalW2 based on the amino acid sequence taken from the UniProtKB database. The UniProtKB entry number is shown in parentheses. The asterisk (*) shows single fully conserved amino acids. Conserved residues are highlighted by (:) and similarity by (.).

Presumably, NisB is also dimeric in the NisBC complex purified directly from the cytosol of *L. lactis*,[40] where it has been suggested to have a stoichiometry of $NisB_2$/NisC/precursor peptide. Our *in vitro* data show that NisC is monomeric in solution and furthermore that NisC is interacting with a single precursor peptide as determined in ITC and MALS-SEC.

The X-ray structure of NisC was solved revealing a monomeric zinc-dependent protein.[20,31] The structure revealed an $\alpha,\alpha$ barrel toroid domain consisting of 14 $\alpha$-helices and an extended domain, which represents a SH2-like fold. The active site contained a zinc finger motif composed of two conserved cysteines and one conserved histidine residue, which are essential for catalysis. A putative reaction mechanism and a binding site for part of the core peptide were proposed by modeling. However, the specific binding sites for the leader as well as for the core peptide could not be identified.[20]

The importance of the leader peptide for both modification enzymes has been shown by fusion with therapeutic peptides in *L. lactis*. The adrenocorticotropic hormone (ACTH), which acts similar to cortisol in the adrenal cortex, the luteinizing hormone-releasing hormone (LHRH), or the cardiovascular peptide angiotensin was dehydrated and cyclized using this methodology, resulting in a higher stability and half-life time.[18,41,42] This shows that the leader peptide contains information, which is sufficient to initiate the modification process.

Here, we show that the leader peptide of the nisin precursor peptide is necessary and more importantly sufficient for an *in vitro* interaction with NisC. The precursor peptides used in this study are composed of two distinct parts, the N-terminal leader and a partially or fully modified C-terminal core peptide. ITC and MALS-SEC showed that only the leader peptide interacts with NisC, while no interaction was observed with the isolated core peptide, independently of the modification state. This is in line with the observation that NisC binds the precursor peptides with similar affinities, but is in contrast to NisB, which displays different affinities for these different forms.[33]

Our observed *in vitro* interaction of NisC with the leader peptide and the -FNLD- box has been suggested by previous *in vivo* data.[16,25,40] This -FNLD- box is conserved in all class I lantibiotics.[27] A sequence alignment of selected examples of class I lantibiotics including the corresponding LanC protein is shown in Figure 4. Here, $F_{-18}$ and $L_{-16}$ are highlighted, which are strictly conserved, while $N_{-17}$ and $D_{-15}$, display variations. This suggests that the side chains of this motif might represent an interaction platform.

We also showed that the -FNLD- box is the major determinant for binding to NisC. Within this box, the highly conserved $F_{-18}$ and $L_{-16}$ are indispensible for this interaction. In light of these results, the variants -AALD- and -FNAA- emphasize that both amino acids, F and L, have to be present to ensure binding to NisC. Mutation of either one abolished binding completely. Thus, the identified recognition motif within the -FNLD- box of the leader peptide is FxLx. Within the crystal structure of NisC, a binding groove lined with hydrophobic and negatively charged residues was identified in close proximity to the $Zn^{2+}$ binding site.[20] This groove was proposed to interact with the positively charged leader peptide of nisin. Our results would be in line with this suggestion. If this groove indeed represents the binding site of the leader peptide, the F and L would interact with the hydrophobic residues of this extended groove, while a repulsion would be generated between the negatively charged residues of the groove and N and D of the leader peptide. This might align the leader peptide in an orientation that would facilitate a $Zn^{2+}$ mediated cyclization of the core peptide.

*In vivo* analysis of the above-mentioned double alanine mutants showed that one to two dehydrations were missing but that the resulting cleaved peptide still displayed antimicrobial active.[25] Also, for the single alanine variants full dehydration pattern and antimicrobial activity were observed. This implies that the *in vivo* situation is different, which is likely due to the presence of both, NisB and NisC, within the cell.

Interestingly, the -FNLD- box also forms the binding site for the dehydratase NisB.[33] This suggests a mechanism in which both modification enzymes compete for the -FNLD- box and that this motif could be the trigger for a handing over of nisin from one modification enzyme to the other, resulting in a sequential reaction mechanism as proposed by Lubelski et al.[26] Pulldown assays performed by Khusainov et al. resulted in predominantly NisB−precursor and only low amounts of the NisB−NisC−precursor complex. Additionally, an alanine mutagenesis study within the leader peptide revealed that that the NisC−precursor complex was not observed if NisB was not present.[43] This suggests that NisB has a tighter interaction with the precursor peptides than NisC[40] suggesting that NisB is responsible for the initial binding to the precursor peptide.[33,40] A ping−pong mechanism was already proposed,[26] and our *in vitro* data support such a scenario. After the modification reactions are completed, the affinities towards the modified precursor peptide become different for the two modification enzymes. Here, we show that NisC possesses a five times higher affinity for the fully modified precursor peptide when compared to NisB ($K_D$ = 10.5 ± 1.7 $\mu$M).[33] This could indicate that NisB initiates the modification steps of the precursor peptide by binding to the unmodified precursor peptide, while the cyclase

reaction of NisC is terminating the modification and a subsequent cycle of modifications can be initiated. After all modifications are terminated, the NisB/NisC/precursor complex disengages. This is supported by a wild type complex consisting of NisB, the precursor peptide, and NisC that was isolated in low amounts.[40] The complex was, however, stabilized, by introducing the H331A mutation inactivating NisC resulting in a higher yield.

In summary, our thermodynamic data demonstrate that NisC is interacting only with the nisin leader peptide independent of the core peptide. In addition and more precisely, NisC interacts with the -FNLD- box of the leader peptide. Especially, the highly conserved FxLx motif might represent a general recognition motif for the LanC protein family.

## ■ ASSOCIATED CONTENT

### Supporting Information

SDS−PAGE of NisC, NisA, and NisP; MALS-SEC analysis of NisC with digested precursor peptides; RP-HPLC calibration and elution profiles of the precursor peptide variants. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS USED

RP-HPLC, reverse phase-high pressure liquid chromatography; ITC, isothermal titration calorimetry; IMAC, immobilized metal-ion affinity chromatography; MALS, multi angle static light scattering; SEC, size exclusion chromatography; Dha, dehydroalanine; Dhb, dehydrobutyrine

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## Supplemental Figure Legends:

### S1:

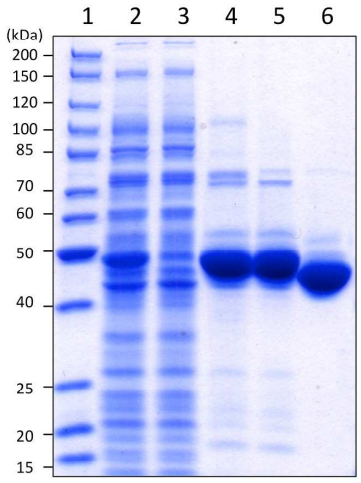


**Figure S1.** SDS-PAGE analysis of the NisC purification stained with Coomassie r-250. ***1:*** protein marker (molecular masses are given in kDa), ***2***: crude extract of *E. coli* cells after the high spin centrifugation step, ***3:*** flow through of IMAC***, 4:*** IMAC elution, ***5:*** $His_6$-NisC after SEC and **6*:*** NisC after thrombin treatment resulting in untagged NisC.

**S2:**

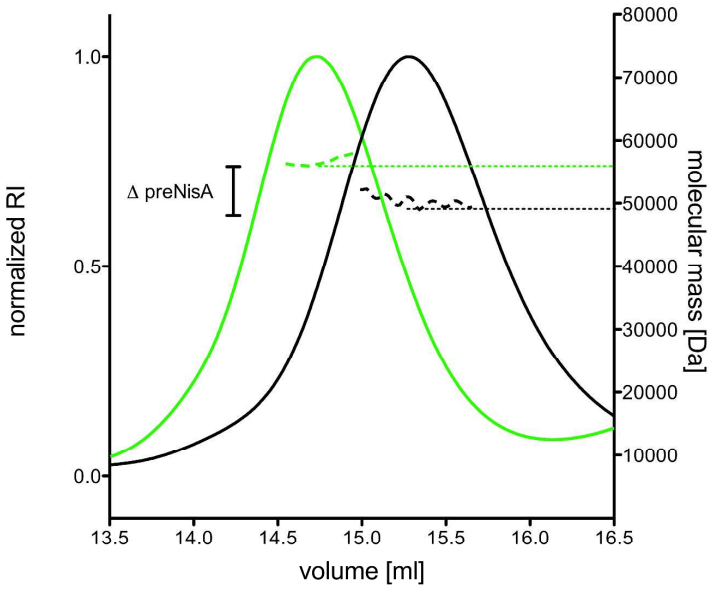


**Figure S2.** MALS – SEC analysis of the interaction of NisC with the dehydrated precursor peptide. ***Green line:*** elution profile of NisC incubated with the dehydrated precursor peptide. ***Green dotted line:*** observed molecular mass of NisC in complex with the dehydrated precursor peptide (the horizontal green dotted line indicates the expected molecular mass of a NisC - precursor peptide complex). ***Black:*** elution profile of NisC***.*** ***Black dotted line:*** observed mass of NisC (the horizontal black dotted line indicates the expected molecular mass of a NisC monomer). The difference between green and black dotted lines fit to the mass of the modified precursor peptide in the range of experimental error (see Table 2). All experiments were performed at least in triplicates. See Table 2 for detailed summary of the molecular masses obtained.

**S3:**

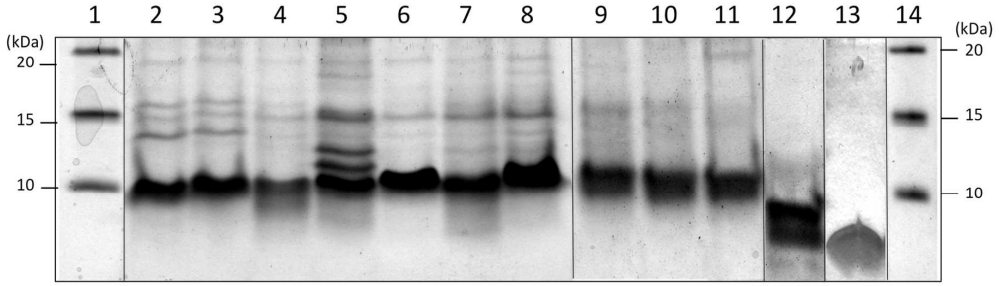


**Figure S3.** Coomassie r-250 stained Tricine SDS-PAGE analysis of mature nisin and precursor peptides variants. Vertical lines indicate separate Tricine SDS-PAGES. ***1:*** protein ladder (molecular masses are given in kDa). ***2:*** unmodified precursor peptide. ***3:*** dehydrated precursor peptide. ***4:*** modified precursor peptide. ***5: -***AAAA- variant. ***6: -*** AALD- variant. ***7: -***FNAA- ***8***: $F_{-18}A$. ***9:*** $N_{-17}A$. ***10:*** $L_{-16}A$. ***11:*** $D_{-15}A$. ***12:*** mature nisin. ***13:*** leader peptide. ***14:*** protein marker (molecular masses are given in kDa).

**S4:**

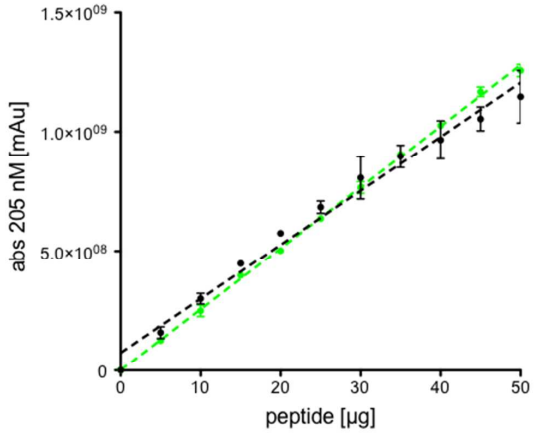


**Figure S4**. Calibration of the RP-HPLC system with insulin (shown in **black**) and mature nisin (shown in **green**). The slope fitted with a linear regression for insulin is : 2260000 ± 820200 mAu $\mu g^{-1}$ and a $R^2$ = 0.9743. The linear regression for nisin has a slope of 25540000 ± 220200 mAu $\mu g^{-1}$ and a $R^2$ = 0.9985.

**S5:**

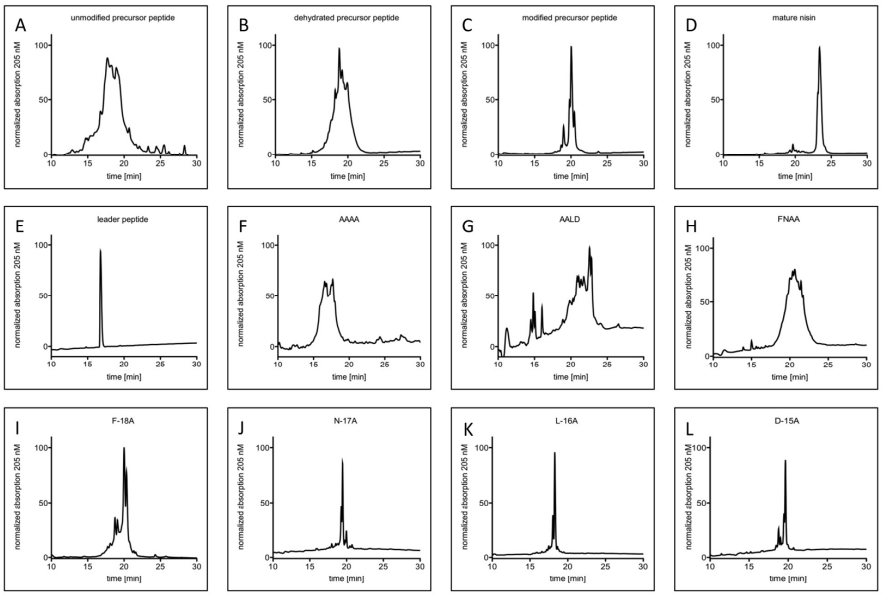


**Figure S5.** RP-HPLC elution profiles of nisin and precursor peptide variants: Unmodified (Figure S5A), dehydrated (Figure S5B), modified precursor peptides (Figure S5C) and mature nisin (Figure S5D) are eluting with a characteristic peak and a characteristic elution time (*1*). The leader peptide eluted at a retention time of around 17 minutes (Figure S5E). The –AAAA- variant (Figure S5F) has a similar elution profile as the unmodified precursor peptide between 16 and 18 min. This reflects the fact that no maturation reaction occurs, which is in line with the mass spectrometry data of Plat *et Al.* who could not determine any dehydration reaction –AAAA- variant(*2*). Both double alanine variants –AALD- (Figure S5G) and –FNAA- (Figure S5H) show a broad peak which maybe reflects the in vivo data, that a mixture is produced in which one to two dehydrations are missing (*2*).

The $F_{-18}A$ variant shows an absorption maximum at around 22 minutes (Figures S5I). The characteristic double peak as well as the retention time could be a hint for a possible cyclisation reaction in the core peptide, which was also identified in Plat *et al.* in the $F_{-18}A$ variant(*2*). Which is analog for the other single alanine variants $N_{-17}A$, $L_{-16}A$ and $D_{-15}A$ (Figure S5J-L).

**S6:**

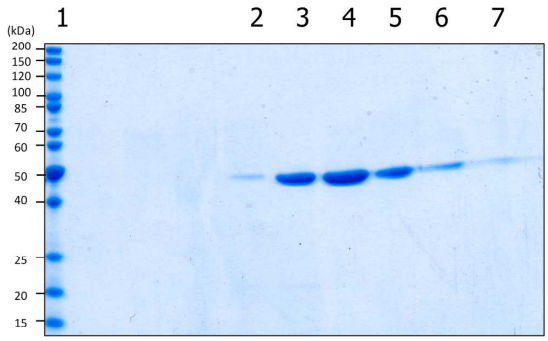


**Figure S6.** SDS-PAGE analysis of the NisP Purification stained with Coomassie r-250. ***1st lane:*** protein marker (molecular masses are given in kDa). ***2nd – 6th lane:*** NisP IMAC elution fractions.

**S7:**

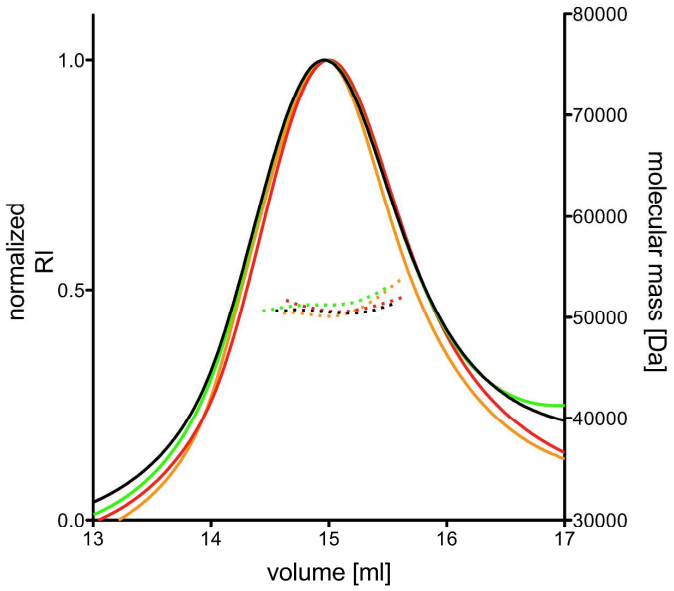


**Figure S7.** MALS-SEC analysis of NisC and digested precursor variants. ***Orange:*** NisC incubated with the leader precursor peptide. ***Red:*** NisC incubated with the digested modified precursor peptide. ***Green:*** NisC incubated with the digested dehydrated precursor peptide. ***Black:*** NisC incubated with the digested unmodified precursor peptide. The corresponding dotted lines represent the observed molecular mass in Da. All experiments were performed at least in triplicates. For detailed molecular masses see Table 2.

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## 3.6 Chapter VI - NisP

in Preparation

Own work: 80%
Establishing and Optimization
of the Purification Strategies
Performing the digestion assay
and RP-HPLC analysis
Writing the manuscript

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# NisP cleaves the nisin precursor peptide *in vitro*

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**André Abts**, Dominik Spiegel, Sander H.J. Smits and Lutz Schmitt

# NisP cleaves the nisin precursor peptide *in vitro*

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**Running Title:** The nisin peptidase NisP - a characterization

**Keywords:** nisin, NisP, precursor peptide, class I lantibiotic protease, *in vitro*

**Abbreviations:**
IMAC - immobilized metal ion affinity chromatography
SEC - size exclusion chromatography
MALS - multi-angle light scattering
*L. lactis* - *Lactococcus lactis*
RP-HPLC - reversed phase high-performance liquid chromatography

## Abstract

The peptide nisin is the best-studied lanthionine ring containing antimicrobial peptide. It is post-translationally modified by the maturation enzymes NisB and NisC during biosynthesis. After translocating across the cell envelope by the ABC transporter NisT, the leader peptide is cleaved off by the subtilisin-like serine protease NisP. This is the final step in the maturation process of antimicrobial active nisin. Here, we present the first *in vitro* characterization of this class I lantibiotic peptidase. Furthermore, the *in vitro* analysis revealed that NisP is cleaving the leader peptide of nisin independently of the post-translational modification state introduced in the nisin core peptide and therefore is purely sequence based. Additionally, this study provides the first kinetic data for a class I lantibiotic protease that elucidates the cleavage process activating the class I lantibiotic nisin.

## Introduction

Nisin is a lantibiotic, which acts against Gram-positive bacteria including some human pathogenic species like methicillin-resistant *Staphylococcus aureus* (MRSA) [1] and is ribosomally synthesized as a precursor peptide consisting of 57 amino acids [2]. It comprises two distinct parts, an N-terminal 'leader peptide' consisting of 23 amino acids and a C-terminal 'core peptide' containing 34 amino acids [3].

After synthesis, the core peptide is post-translationally modified. Here, serine and threonine residues are dehydrated by the dehydratase NisB resulting in formation of the unnatural amino acids didehydroalanine (Dha) and didehydrobutyrine (Dhb), respectively [4, 5]. The next post-translational step forms the eponymous lanthionine rings by the cyclase NisC [5]. These characteristic rings, which are responsible for the antimicrobial activity, arise by linking the dehydrated amino acid with a C-terminally located cysteine [1, 6]. After formation of all post-translational modifications in the core peptide, nisin comprises one didehydrobutyrine, two didehydroalanine, one lanthionine and four methyllanthionine rings. It is called 'modified precursor peptide', which is secreted in the extracellular media by the ABC transporter NisT [7, 8]. The modified precursor peptide is still inactive caused by the presence of the leader peptide. This leader peptide is essential for modification and secretion, since it is the recognition site of NisB and NisC as well as for NisT [9 -11]. To convert the modified precursor peptide in the active state, the leader peptide needs to be cleaved off. This process is catalyzed by the subtilisin-like serine protease NisP [1, 2, 7]. After cleavage of the leader peptide, the N-terminus of the mature peptide is released. The N-terminus of the mature peptide starts to attack the target molecule in the Gram-positive membrane and therefore the cleavage of the leader peptide is the essential step to activate the antimicrobial activity of the mature

peptide [12].
The protease NisP belongs to the family of subtilisin-like serine proteases (Pfam entry: Peptidase S8) [1]. This group of proteases comprise a high sequence identity regarding the active site and the amino acid residues located in close vicinity. The supposed active site consists of the conserved catalytic triad Asp64, His111, Ser317. Equally essential for the enzyme activity is residue Asn212, which is supposed to form the oxyanion hole [1, 13].
NisP is ribosomally synthesized as a preprotein consisting of a polypeptide chain of 682 residues [1]. To anchor the protease to the outer leaflet of the cytoplasmic membrane several maturation processes are needed. A secretion signal is localized within the N-terminal 195 amino acid residues, targeting NisP to a Sec-dependent translocation across the cytoplasmic membrane [1]. The propeptide part contains the amino acid sequence -VSLR-, located at position 191 -195, which encodes a putative serine protease cleavage sequence. It has been shown that this sequence results in an auto-cleavage during maturation [1, 7, 13]. It remains unclear if the protease is cleaved after or during translocation or which function these enlarged 191 amino acid residues harbor next to secretion and cleavage.
After translocation NisP is anchored to the membrane. Therefore a -LPxTG- motif is located at the C-terminus, which is recognized by a sortase [14]. Lipidation of the threonine residue belonging to the -LPxTG- motif at position 655, anchors NisP to the surface of the membrane [14]. After these maturation steps, amino acid residues 196 - 682 encode the active, membrane anchored protease NisP resulting in a molecular mass of approximately 54 kDa [1, 13].

The specific cleavage reaction and substrate specificity of NisP remains elusive. The gene encoding NisP was identified in 1993 [1]. The cleavage reaction of the modified precursor peptide was identified as last step in the nisin maturation process [1]. To investigate the cleavage properties of NisP, the protein was expressed on the cell surface of the *L. lactis* strain NZ9000 [7]. When *nisP* is deleted, the nisin precursor peptide is not cleaved. Thereby it is possible to isolate three different maturation states of nisin precursor peptides in an unmodified, dehydrated and modified state of the precursor peptides [15]. These precursors were incubated with NisP expressing cells and this study revealed that NisP is cleaving the modified precursor peptide by recognition of at least the first (methyl)lanthionine ring [7].
The influence of exchanging the conserved cleavage site within the nisin precursor peptide (-PR/Q-) located at position -2 to -1 in respect of NisP cleavage was investigated, which demonstrated an interaction of NisP with the nisin leader peptide [17]. By exchanging the nisin cleavage site (-ASPR-) against other protease sites the influence on the cleavage efficiency was investigated. First, the -ASPR- site was exchanged against the specific cleavage site -VSLR- that is found within NisP. This exchange resulted in successfully cleaved

precursor peptides. Second, the specific protease cleavage sequences for thrombin and factor Xa were introduced and a cleavage was observed as well [17]. These exchanges demonstrate that NisP is able to cleave the nisin precursor peptide even if the conserved amino acids PR of the nisin precursor peptide cleavage site are substituted.

Here, we present the first isolation of the nisin specific protease NisP *in vitro*. The activity and substrate specificity was monitored by RP-HPLC and mass spectrometry. Furthermore, the cleavage reaction was investigated in respect to pH-, temperature and ion strength dependency. Finally, the kinetic parameters of the cleavage reaction of the modified precursor peptide were determined, which represents the first kinetic characterization of a class I lantibiotic cleavage reaction.

## Material and Methods

**Expression and purification of NisP and the nisin precursor peptides**

The serine protease NisP was homologously expressed in *L. lactis*. Details regarding expression and purification are previously described in Abts *et al.* 2013 [9]. All nisin precursor peptides used in this study are expressed and purified as previously described [9, 10]. The purification protocol of the mature peptide nisin is described in Abts *et al.* 2011 [18].

**Characterizing the oligomeric state of NisP by MALS - SEC analysis**

Purified NisP was loaded on a size exclusion column Superdex 200 10/300 (GE Healthcare) equilibrated with 10 mM HEPES-NaOH pH 7.0, 150 mM NaCl. For mass determination the multi-angle light scattering (MALS) technique in combination with size exclusion chromatography (SEC) was used. The UV absorbance at 280 nm, the multi-angle light scattering signal and the differential refractive index (™Optilab rEX detector - Wyatt Technology Europe) were observed. The Wyatt triple-angle light scatter detector ™miniDAWM TREOS observed the scattering at 0°, 90° and 107°. The resulting peaks were analyzed with the Wyatt software package Astra 5.3.4.20.

**Bioassay of NisP with the nisin precursor peptides**

A ratio between NisP and the nisin precursor peptide of 1 : 1000 was used to analyze the cleavage reaction. 50 $\mu$M of precursor peptide were incubated with 50 nM of NisP in 50 $\mu$L of 10 mM HEPES-NaOH pH 7.0 and 150 mM NaCl. This approach was investigated by changing the parameters regarding temperature (5, 10, 20, 30, 35, 40, 45, 50, 60 °C) pH (3 - lactic acid, 4 -lactic acid, 5 - MES, 6 - MES, 7 - HEPES, 8 - Tris, 9 - Tricine, 10 - CAPS and 11 - $Na_2CO_3$) and NaCl concentration (100 - 900 mM). The mixture was immediately analyzed by RP-HPLC observing the UV absorbance at 205 nm after a reaction time of 20 minutes. Via RP-HPLC analysis the product formation

(leader and mature nisin) and the reactant consumption were detected. The absolute mass of product and reactant was determined by integrating the corresponding peaks.

**Michaelis-Menten kinetic of the NisP cleavage reaction**

To characterize the cleavage reaction, the above-mentioned reaction was performed with different substrate concentrations (5 - 100 $\mu$M). The resulting specific activities were plotted against the substrate concentration and fitted with the Michaelis-Menten equation $Y=V_{max}*X/(K_M+X)$ [19].

**Substrate specificity of NisP**

To test, whether NisP is able to cleave the different maturation states of the precursor peptide. Each different maturation state was purified and incubated with NisP in a molar ratio of substrate to protease of 1000 : 1 at 30 °C over night. After cleavage, NisP was removed with $Ni^{2+}$-NTA-magnetic beads (Qiagen) from the reaction mixture and the resulting peptides were analyzed by mass spectrometry (MS).

## Results

**NisP purification and characterization**

Plasmid pNG-NisP8His was a kind gift of Prof. O. Kuipers, University of Groningen, Netherlands. The plasmid was transformed in *L. lactis* strain NZ9000. This truncated construct consisted of the amino acids 1 to 572 of NisP. The C-terminal truncation allowed secretion of the protease NisP in the extracellular medium. During maturation the N-terminal located amino acids 1 - 195 are cleaved, which resulted in an active protease consisting of the amino acids 196 - 572 followed by a $His_8$-tag. The protein was purified from the supernatant via an immobilized metal ion affinity chromatography (IMAC) and eluted with a step gradient (300 mM imidazole). After IMAC, the imidazole was removed using a PD10 column (GE Healthcare) and concentrated with an Amicon Ultracentrifugation unit. The purity reached over 95 % judged from a SDS-PAGE analysis (Figure 1A). The molecular homogeneity and molecular weight was analyzed using MALS-SEC, which revealed a molecular weight of 41940 ± 1409 Da which fits within experimental error to the theoretic molecular weight of 42458 Da for NisP from amino acid 196 - 572 including the $His_8$-tag (Figure 1B). This shows that NisP is a stable monomer in solution.

***In vitro* activity of NisP**

To test whether NisP is able to cleave the precursor peptide *in vitro*, the isolated modified precursor peptide was incubated with the purified NisP protease overnight and analyzed with RP-HPLC. Using RP-HPLC the substrate, the modified precursor peptide, could be separated from the two products, the leader peptide and mature nisin (Figure 2). The absorbance at 205 nm was monitored because the cor-

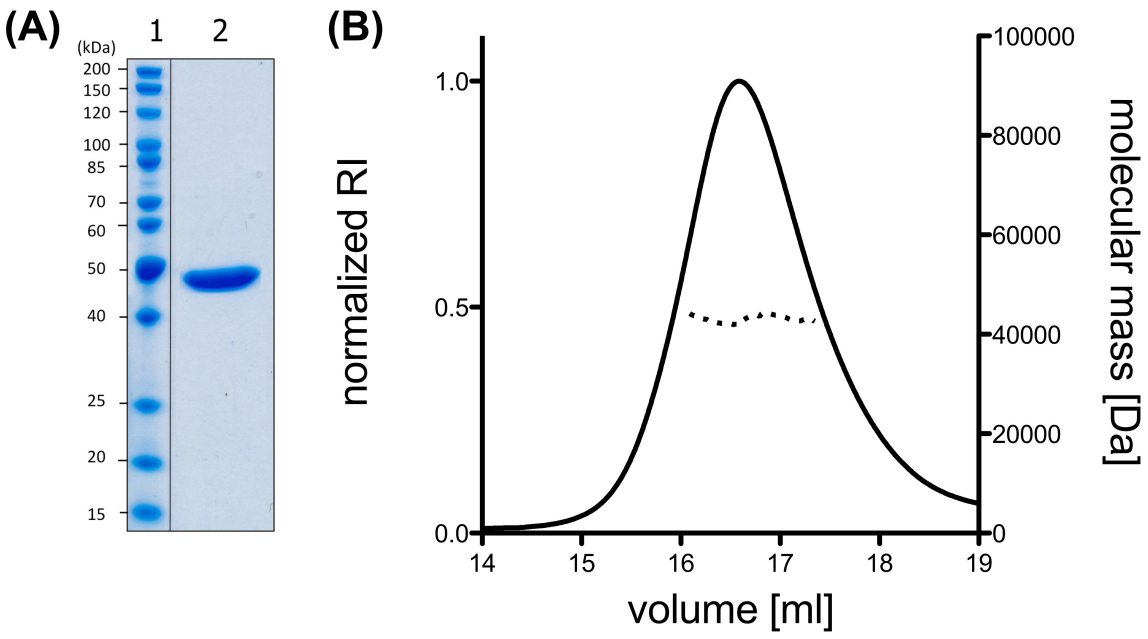


**Figure 1A: SDS-PAGE analysis of the purified nisin serine protease NisP.** Lane 1: protein marker. Lane 2: NisP sample after size exclusion chromatography. Both lanes are obtained from one SDS-PAGE and fused together after staining to simplify the figure. **1B: MALS-SEC analysis of NisP.** Black line: Showing the elution profile of NisP analyzed by a SD200 10/300 (GE Healthcare). Black dotted line: Represents the observed molecular mass of NisP (41940 ± 1409 Da) determined with MALS, the theoretical molecular weight of NisP revealed 42458 Da.

responding peptides did not contain any aromatic amino acids. Furthermore, the absorption at 205 nm was independent from the influence of the amino acids side chains. The modified precursor peptide showed a characteristic elution profile in the RP-HPLC analysis around 20 minutes (green chromatogram in Figure 2). Mature nisin eluted at a retention time of 23.5 minutes (dotted black chromatogram in Figure 2). After incubation of the modified precursor peptide with NisP the blue elution profile was observed in the RP-HPLC analysis. A single peak at a retention time of 14 minutes corresponded to the leader peptide (as shown by MS analysis in the supplemental material Figure S6). A second peak appeared at a retention time of 23.5 minutes, which corresponded to mature nisin. The peak of the modified precursor peptide was no longer detectable, which indicated a 100 % efficient cleavage.

**Activity dependence on ion strength, pH and temperature**

To further investigate the cleavage characteristica, the reaction parameters were defined as follows. The general set up was performed at 30 °C, 150 mM NaCl, pH 7.0, 20 minutes. The molar ratio between protease and substrate was set to 1 : 1000. To investigate the temperature influence on the cleavage reaction different temperatures varying from 5 to 60 °C were an-

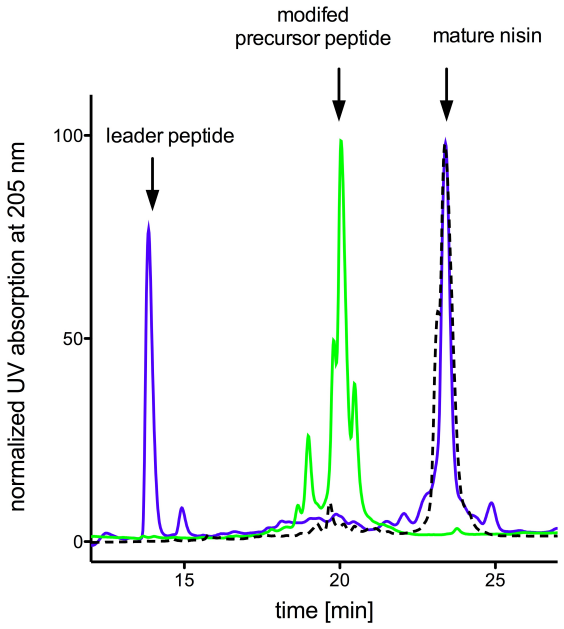


**Figure 2: RP-HPLC profile following the cleavage reaction of NisP with the modified precursor peptide.** The modified precursor peptide (green) was digested with NisP and analyzed by RP-HPLC. The digested sample (blue) showed two peaks. The first peak comprised an elution time of 14 min. This corresponds to the leader peptide, which was confirmed by mass spectrometry. The second peak eluted at 23.5 min. The elution time was identical to mature nisin shown in a black dotted line. In dependency on Abts *et al.* 2013 [9].

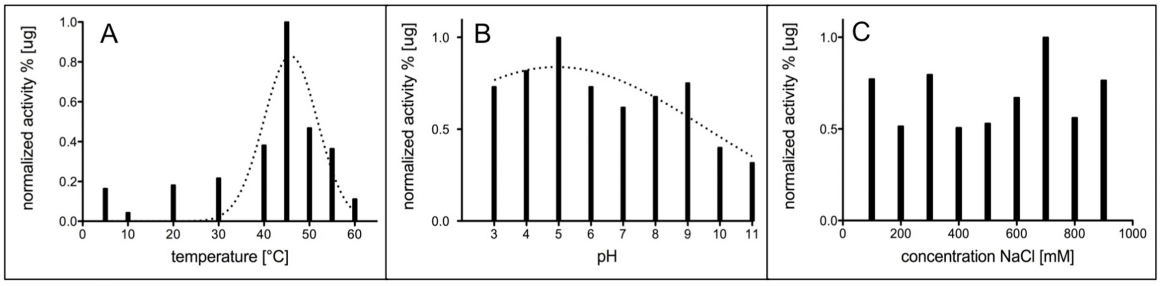


**Figure 3A-C:** Overview of the temperature (3A), pH (3B) and ion strength (3C) dependency of the NisP cleavage reaction. The normalized activity was obtained by integrating the specific peaks via RP-HPLC and plotted on the y-axis. The dotted line represents a Gaussian distribution analysis.

alyzed. The maximum activity could be observed at 45 °C (Figure 3A).

Whether the activity is dependent on different pH values was tested and the result is shown in Figure 3B. A pH range between 3 and 11 was analyzed and revealed that the pH did not influence the activity significantly. The maximum activity was observed at pH 5.0. Nevertheless, the activity was reduced at pH values higher than 9.0 (Figure 3B).

The dependency of the activity on ion strength was analyzed by varying the NaCl concentration from 100 mM to 900 mM. Here, variation of ion strength did not influence the cleavage activity (Figure 3C).

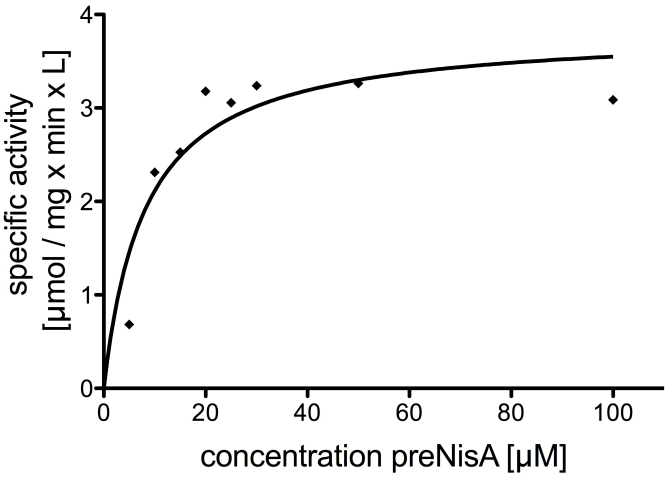


**Figure 4: Determining Michaelis-Menten kinetic parameters.** The specific enzyme activity is plotted against different substrate concentrations (modified precursor peptide) ranging from 5 to 100 $\mu$M. The obtained kinetic parameters were determined to $K_M$ = 8.2 $\mu$mol/L, $V_{max}$ = 3.84 $\mu$mol / min * mg * L and $k_{cat}$ = 4608 $sec^{-1}$ and $k_{cat}/K_M$ = 5.63 $\mu mol^{-1} * sec^{-1} * L$.

**Michaelis-Menten kinetic parameter**

To characterize the cleavage reaction in detail, Michaelis-Menten kinetic parameters were determined. Therefore different substrate concentration were used and the final results are shown in Figure 4 and Table 1.

**Substrate specificity of NisP**

To investigate whether NisP is specifically cleaving in presence of (methyl)lanthionine rings, the three different maturation states of the precursor peptide were digested with NisP overnight. The unmodified precursor peptide, which did not contain any post-translational modification, the dehydrated precursor peptide in which the serine and threonine residues of the core peptide are dehydrated and the modified precursor peptide, which additionally contained the (methyl)lanthionine rings, were analyzed.

By subsequent MS analysis, the leader peptide was detected in every sample with a molecular mass for the reaction mixture of the unmodified-, dehydrated and modified precursor peptide as follows, 2352.14, 2352.37 and 2351.28 kDa, respectively. This indicated that NisP is able to cleave the leader peptide from the nisin precursor peptide independently from the maturation state of the core peptide. As a control the same samples ware analyzed lacking NisP, which revealed no smaller masses of the corresponding peptides.

This highlighted that the ability of NisP to cleave the precursor peptide was not depending on the maturation state of the core peptide. All data are summarized in Table 2 and the corresponding mass spectra are shown in the supplemental material.

**Table 1:** Overview of the Michaelis - Menten parameter obtained for the modified precursor peptide with NisP at 30 °C, pH = 7.0 and 150 mM NaCl.

| **kinetic parameter** | |
|---|---|
| $V_{max}$ | 3.84 $\mu$mol / min * mg * L |
| $K_M$ | 8.18 $\mu$mol/L |
| $k_{cat}$ | 4608 $sec^{-1}$ |
| $k_{cat}$ /$K_M$ | 5.63 $\mu mol^{-1}$ * $sec^{-1}$ * L |

**Table 2: Results of mass spectrometric analysis.** The unmodified-, dehydrated and modified precursor peptides were analyzed in absence and presence of the purified protease NisP.

| | **Sample** | **$M_W$ [Da]** | **- Met [Da]** | **observed $M_W$ [Da]** |
|---|---|---|---|---|
| - NisP | unmodified preNisA | 5963 | 5831 | 5964,1 |
| | dehydrated preNisA (-8 $H_20$) | 5819 | 5687 | 5686,1 |
| | dehydrated preNisA (-7 $H_20$) | 5837 | 5705 | 5702,2 |
| | modified preNisA (-8 $H_20$) | 5819 | 5687 | 5685,1 |
| | modified preNisA (-7 $H_20$) | 5837 | 5705 | 5703,1 |
| + NisP | unmodified preNisA ($M_W$ Leader) | 2483 | 2352 | 2352,3 |
| | dehydrated preNisA ($M_W$ Leader) | 2483 | 2352 | 2352,1 |
| | modified preNisA ($M_W$ Leader) | 2483 | 2352 | 2351,3 |

## Discussion

In 1993, the *nisP* gene was identified as a crucial part of the nisin biosynthesis pathway. The gene is located in the nisin operon *nisABTCIPRKFEG* and expressed simultaneously with the modification and secretion machinery *nisBTC* [20]. Without the corresponding NisP protein no active nisin was revealed [1, 2]. Here, we provide the first *in vitro* analysis of the subtilisin-like serine protease NisP.

The NisP protease was homologously expressed in *L. lactis* and could be purified to homogeneity as presented in Figure 1. Furthermore, the analysis via analytical MALS-SEC demonstrated a homogenous elution profile with a molecular weight of 41940 ± 1409 Da, demonstrating that NisP is a stable monomer in solution.

To analyze, whether the purified protease is active, the natural substrate, the modified precursor peptide, was incubated with NisP. The resulting mixture was analyzed by RP-HPLC and MS, as shown in Figure 2 and Supplemental Material,

which demonstrated that the isolated protein is capable to cleave the modified precursor peptide resulting in the products leader peptide and mature nisin, *in vitro*. It remained unclear which motif of the nisin precursor peptide is recognized by NisP. It was proposed that the first (methyl)lanthionine ring of the nisin core peptide is required for the recognition by NisP [7]. This implies a specific interaction with the core peptide of the nisin precursor peptide.

The leader peptide of the nisin precursor peptide is an essential recognition partner as well, which was concluded from subtilin-nisin chimera [16]. Subtilin comprises the highest sequence identity (approximately 58 %) to nisin. When the nisin core peptide is fused to a subtilin leader peptide, NisP is no longer able to cleave this chimera although the core peptide in this chimera contained at least three (methyl)lanthionine rings [21] as was concluded from the antimicrobial activity [16]. This suggest that the leader sequence plays an essential role, although the (methyl)lanthionine rings are present in the core peptide.

In 2011, a detailed study of the interaction between the nisin leader peptide and the modification enzymes was reported [17]. Here, the conserved cleavage region (-PR/Q-) was exchanged against other amino acids representing cleavage sequences of common proteases. In the nisin leader peptide the cleavage region is composed of the amino acid residues -ASPR-. Within the maturation of NisP the self-cleavage site comprises the amino acids -VSLR-. Therefore the sequence -ASPR- was exchanged against -VSLR- and the modified precursor peptide was successfully cleaved. Furthermore, when the nisin cleavage site was exchanged against the cleavage sites of the protease thrombin or factor Xa, NisP was still able to cleave the substrate [17]. This study highlights that the cleavage reaction is rarely sequence based.

Previous *in vivo* studies revealed that membrane bound NisP cleaves specific the modified precursor peptide [7]. Here, we demonstrate that soluble NisP is able to cleave the nisin precursor peptide independently of the maturation state of the core peptide. The unmodified precursor peptide is cleaved as well from the leader peptide as the dehydrated and the modified precursor peptide. This indicates that NisP recognizes only the nisin leader peptide.

Other lantibiotic proteases are also able to cleave their lantibiotic precursor peptide in an unmodified form, as shown for epidermin and epilancin [22, 23].

These results point to a recognition site within the leader peptide of nisin. The study of the class II lantibiotics, nukacin and lacticin S, revealed that the interaction between the protease and the leader peptide is modulated through an $\alpha$-helix [24, 25]. Equally in class III leader peptides an $\alpha$-helical domain is found [26]. This might also holds true for class I lantibiotics. Although, the affinity of the proteases EpiP and NisP are believed to be dependent on electrostatic interactions between the protease and the arginine at

position -1 [13].

Additionally, the kinetic parameters of the protease were investigated. The pH maximum was revealed at pH 5.0 and the maximum reaction velocity was reached at 45 °C. The ion strength did not influence the cleavage reaction. Furthermore the Michaelis-Menten parameters were determined as summarized in Table 1.

Only a few kinetic parameters of subtilisin-like proteases are described [27-30]. The $K_M$ values range from 3.9 $\mu$mol/L to 17 mmol/L and the $k_{cat}$ from 0.022 - 22 000 $sec^{-1}$ [27-30]. Therefore, a comparison is difficult and especially for class I lanthipeptides protease no kinetic data are available.

In summary, the capability of NisP towards the nisin precursor peptides was investigated and it could be shown that NisP is able to cleave the nisin precursor peptide independently from the maturation state of the core peptide. This points towards a specific interaction between the nisin precursor peptide, directed by the leader peptide.

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## Supplemental Material

Mass spectrometric analysis of the unmodified-, dehydrated and modified precursor peptide in absence and presence of the nisin specific protease NisP (Figure S1 - 6).

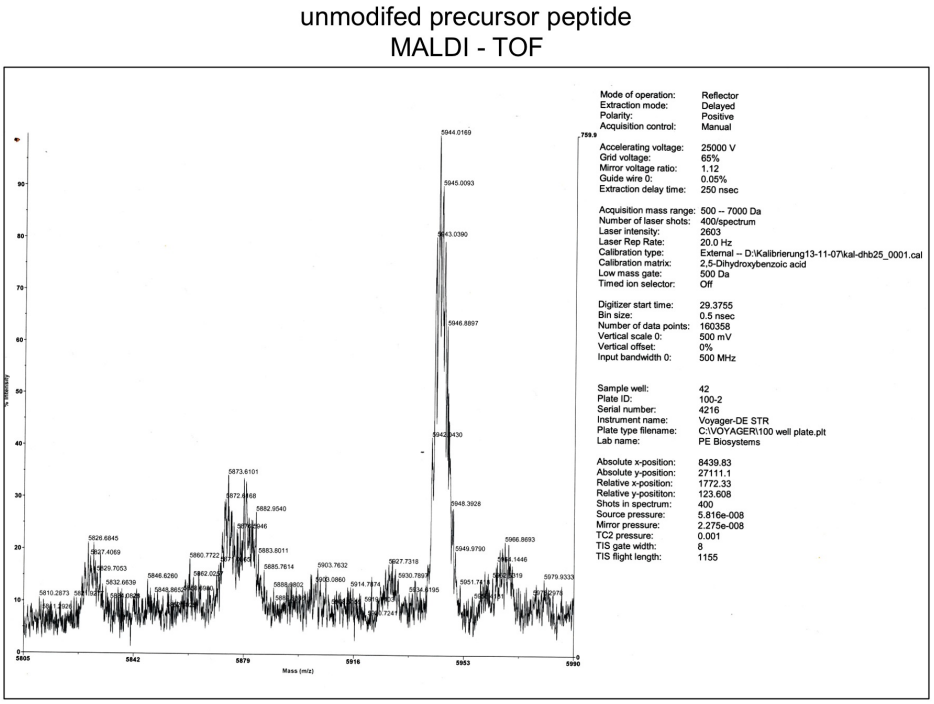


**Figure S1:** MS analysis of the unmodified precursor peptide

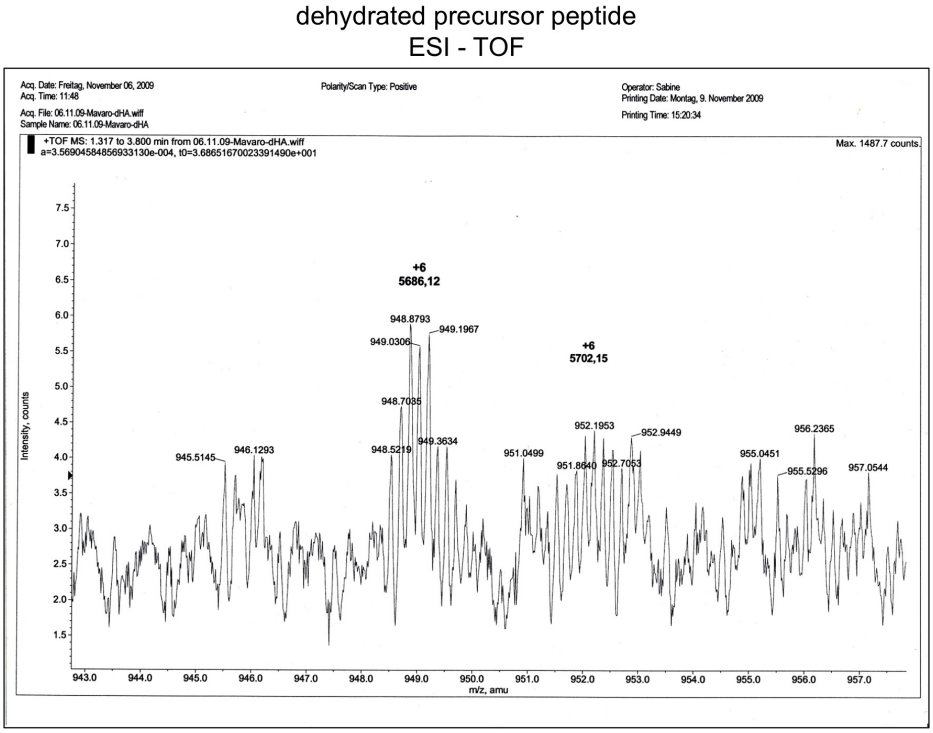


**Figure S2:** MS analysis of the dehydrated precursor peptide

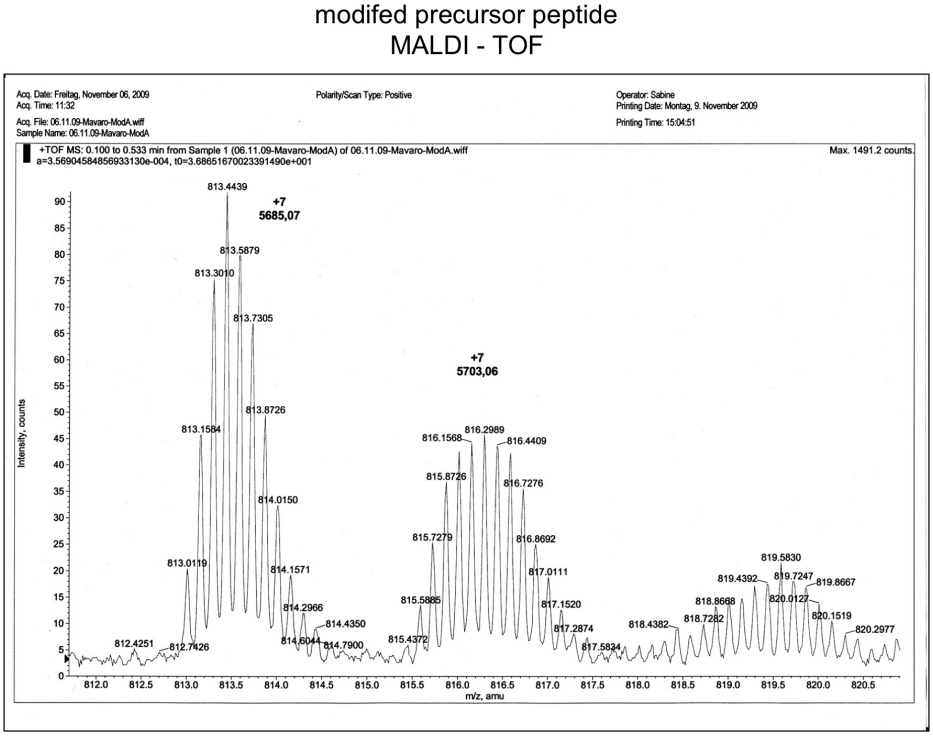


**Figure S3:** MS analysis of the modified precursor peptide

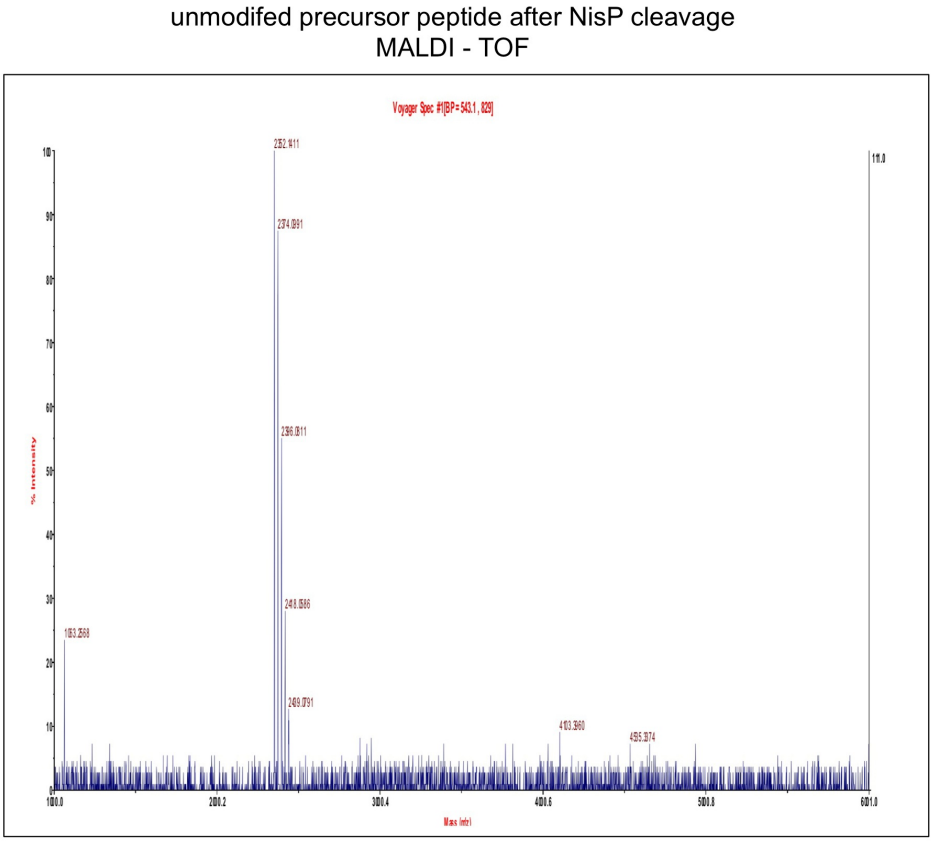


**Figure S4:** MS analysis of the unmodified precursor peptide after NisP treatment

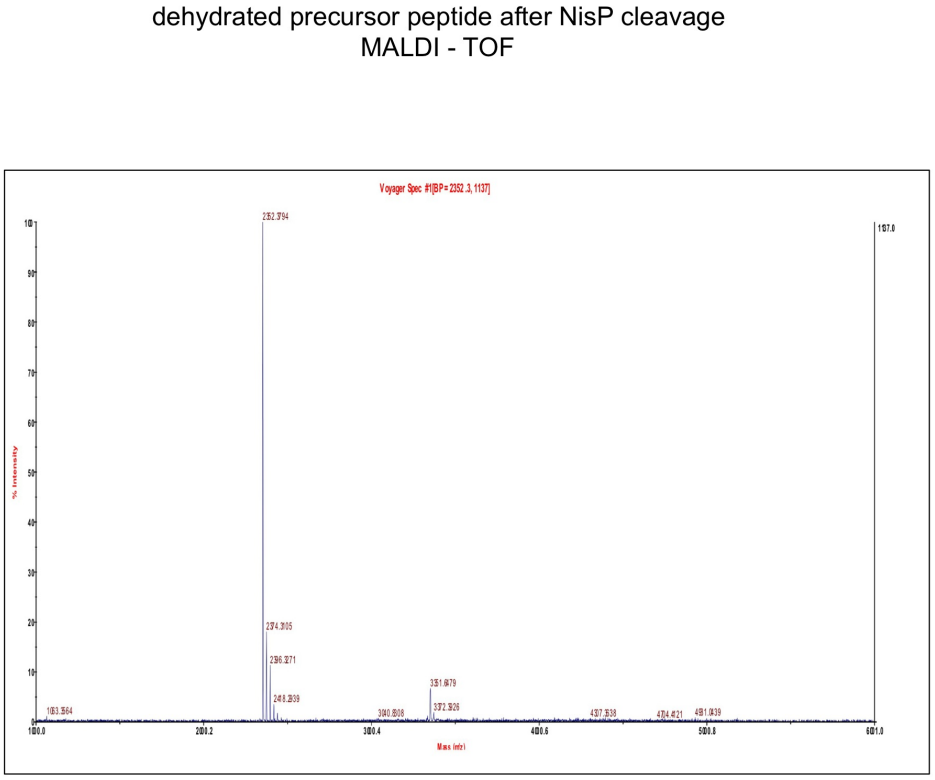


**Figure S5:** MS analysis of the dehydrated precursor peptide after NisP treatment

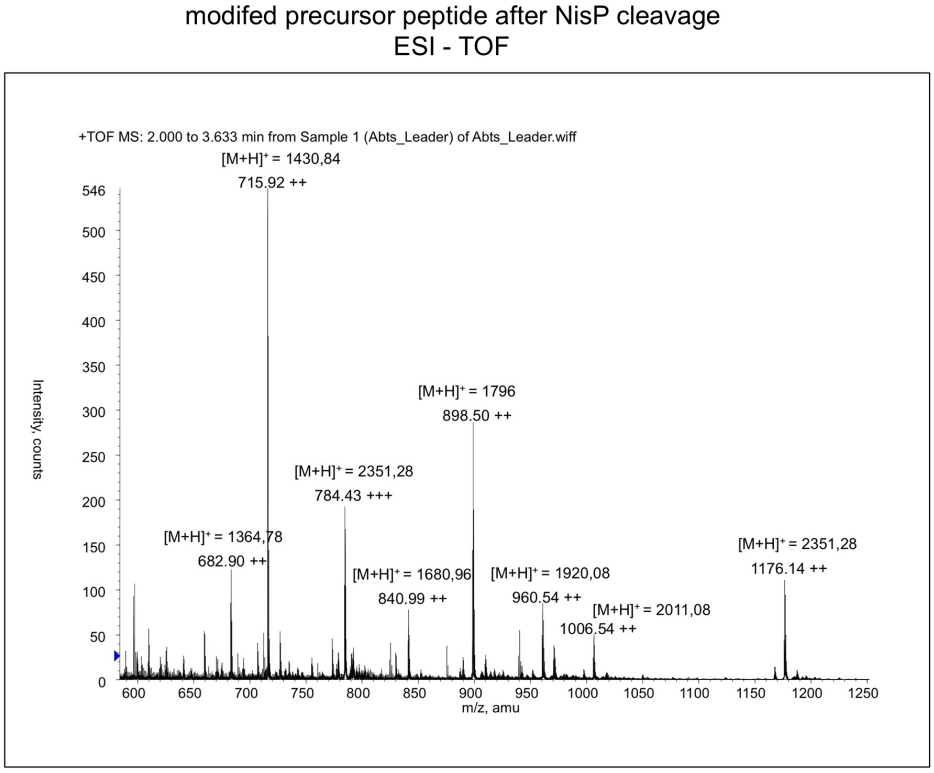


**Figure S6:** MS analysis of the modified precursor peptide after NisP treatment

## 3.7 Chapter VII - The nisin maturation complex NisABC

in Preparation

| | |
|---|---|
| Own work: | 80% |
| | Establishing and Optimization |
| | of the Purification Strategies |
| | Performing the interaction studies |
| | Writing the manuscript |

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# Assembly of the nisin maturation complex *in vitro*

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**André Abts**, Jens Reiners, Diana Kleinschrodt, Iris Fey, Manuel Wagner, Antonino Mavaro, Sander H.J. Smits and Lutz Schmitt

# Assembly of the nisin maturation complex *in vitro*

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**Running Title:** *In vitro* assembly of the nisin modification complex

**Keywords:** nisin, NisB, NisC, maturation complex, lantibiotics, analytical size exclusion chromatography, -FNLD- box

**Abbreviations:** IMAC - immobilized metal ion affinity chromatography
SEC - size exclusion chromatography
MALS - multi-angle light scattering
*L. lactis* - *Lactococcus lactis*
*E. coli* - *Escherichia coli*

## Abstract

Nisin is used as antimicrobial peptide in the food industry for the past five decades and is characterized by unique post-translational modifications. The dehydrated amino acids didehydroalanine and didehydrobutyrine are installed by the nisin dehydratase NisB. The cyclase NisC links free cysteine residues to these dehydrated amino acids and installs eponymous (methyl)lanthionine rings. This reaction is proposed to be catalyzed by a complex consisting of the modification enzymes NisB and NisC in an alternating fashion.

The introduction of post-translational modifications in a regio- and stereospecific manner is an enzyme-based procedure and it was demonstrated for a few examples that it is possible to use the modification enzymes NisB and NisC *in vivo* to introduce post-translational modifications in therapeutic peptides. However, with a better understanding of the *in vitro* process this machinery might be used as a general tool to introduce specific post-translational modifications in any peptides containing serine and threonine residues.

Here, we show the first *in vitro* assembly of the nisin modification complex comprising of a dimer of NisB combined with a monomer of NisC. This complex is only initiated by the unmodified precursor peptide. The complex formation is dependent on the modification state of the core peptide and also on the covalently bound leader peptide. Within the leader peptide, the highly conserved -FNLD- box was identified to be essential for complex formation.

## Introduction

The antimicrobial peptide nisin contains characteristic post-translational modifications, dehydrated amino acids and (methyl)lanthionine rings [1, 2]. These post-translational modifications are responsible for the antimicrobial activity against several Gram-positive bacteria [3-5]. Therefore, nisin belongs to the class of lantibiotics (lanthionine-containing antibiotics)[6].

Nisin was used in the food industry for the past five decades and is the best investigated lantibiotic [6]. It is ribosomally synthesized as a precursor peptide comprising of two parts [7]. The N-terminal part is named ‘leader peptide‘ and is responsible for the recognition of the modification enzymes and essential for transport [8-11]. The C-terminal part is called ‘core peptide‘ and post-translational modifications are only introduced in this part [7].

After ribosomal synthesis of nisin the enzyme NisB dehydrates serine and threonine residues in the core peptide (Figure 1 - I/II) [12-15]. Subsequently, NisC links the dehydrated amino acids via thioether bridges to C-terminal localized cysteine residues, forming the characteristic (methyl)lanthionine rings (Figure 1 - II/III) [13, 16, 17]. After these modifications, the ABC transporter NisT secretes the modified precursor peptide across the cytoplasmic membrane [18-20]. Here, the

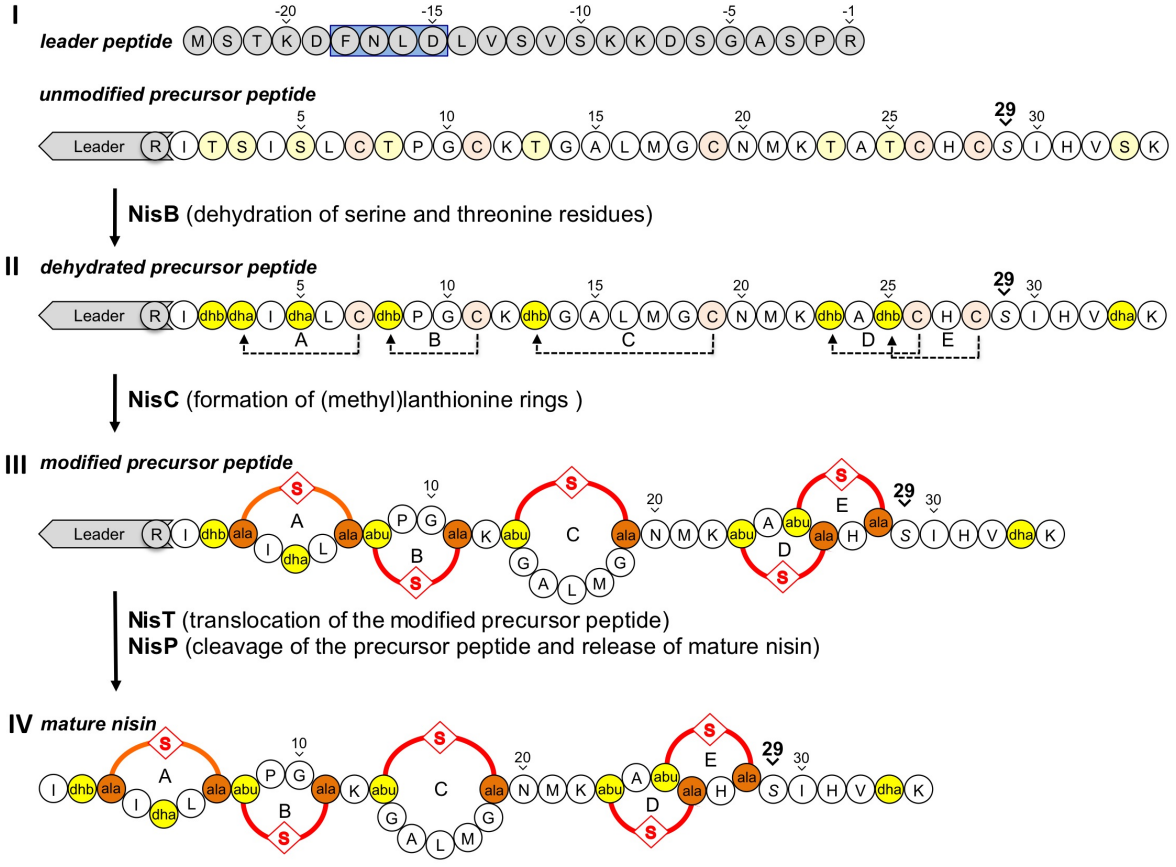


**Figure 1: Schematic overview of the nisin maturation process.** I) The 'leader peptide' is shown on the top of the Figure and the highly conserved -FNLD- box is highlighted by a blue box. The 'unmodified precursor peptide' represents the ribosomally synthesized peptide. II) NisB dehydrates specific serine and threonine residues (yellow) in the core peptide. The resulting peptide is named 'dehydrated precursor peptide', which contains the dehydrated amino acids didehydroalanine (dha) and didehydrobutyrine (dhb). III) NisC links these dehydrated amino acid residues to C-terminal located cysteine residues (light in red) resulting in the 'modified precursor peptide'. In the core peptide one lanthionine ring A (orange) and four methyllanthionine rings B-E (red) are present. IV) After completion of the post-translational modification reactions the modified precursor peptide is secreted by NisT across the cytoplasmic membrane and processed by the serine protease NisP. The modified precursor peptide is cleaved in leader peptide and 'mature nisin' is released from the cell. Adapter from [11].

protease NisP, which is anchored to the outer leaflet of the cytoplasmic membrane, cleaves off the leader peptide (Figure 1 - IV) [19, 21]. The cleavage reaction transforms the modified precursor peptide to mature nisin. The whole maturation process is schematically summarized in Figure 1 for a better understanding [21].

The nisin modification machinery has been used to introduce post-translational modifications in certain, therapeutic relevant peptides *in vivo* [22-24]. For example, the cardiovascular peptide angiotensin was successful dehydrated when fused to the nisin leader peptide. However, the dehydration pattern is very diverse and varies from zero to five dehydrations [22]. These post-translational modifications increase

lifetime by protecting the peptide against proteolytic degradation [22-24]. Although, the system has been successfully employed for a set of peptides, fusion of these peptides to the nisin leader peptide is required.

The essential role of the nisin leader peptide in the maturation process is furthermore supported by *in vitro* investigations of the isolated NisB and NisC enzymes, which recognize specifically the nisin leader peptide [10, 11]. However, a detailed knowledge of the modification complex and modification reaction remains unclear.

A detailed *in vitro* analysis of the complex assembly and function might lead to a better understanding of substrate specificity and recognition. Finally, the post-translational modification machinery may be used *in vitro* to introduce post-translational modifications in user-defined peptides, which might enhance productivity and general usage.

The modification enzymes NisB, NisC, NisP and the ABC transporter NisT are capable to catalyze their specific reaction independently of the presence of the other enzymes [10, 17, 19, 20]. The maturation process starts with the dehydration and cyclization of the unmodified precursor peptide and is finished by release of mature nisin (Figure 1). This process is much more efficient if all enzymes are present simultaneously [20, 25]. For example, the production rate for the dehydrated precursor peptide is decreased by 70 % if NisC is absent [20]. Additionally, the dehydration efficiency as well as the secretion level is increased if NisC is present but inactive [20].

This suggests that a certain level of interaction between these enzymes is required. In 1996 a study revealed an interaction between NisC and NisB as well as between NisC and NisT in presence of the nisin precursor peptide [26]. The enzymes NisB, NisC and NisT were all found to be membrane associated. Consequently, a model describing a membrane associated lantibiotic synthetase complex was proposed. The stoichiometry of NisB, NisC and NisT was suggested to be 1:2:1 [26].

An analog complex was identified for the homolog of nisin, subtilin [27]. The subtilin synthetase complex was proposed to function as a tunnel containing a dimer of SpaB, a dimer of SpaC and a dimer of SpaT [27].

Another study proposed an alternating mechanism between NisB and NisC [25]. Here, a crucial observation pointed to a directionality of the nisin modification process. The maturation state of the specific serine residue at position 29 was analyzed and revealed no dehydration (Figure 1). This serine is located C-terminal to the lanthionine rings D and E (Figure 1) and therefore it was proposed that the (methyl)lanthionine rings D and E cause a steric hindrance for NisB and that the serine residue at position 29 is not accessible for NisB. This explanation required a temporal coordination of the reaction, i.e. that NisC forms the methyl(lanthionine) rings D and E prior to the NisB catalyzed dehydration at position 29. Otherwise the

serine 29 would be dehydrated. Therefore it can be concluded that NisB and NisC are working in an alternating fashion [25]. The first isolation of a NisB, NisC and nisin precursor peptide complex was achieved in 2011 [28]. Khusainov *et al.* fused a $His_6$-tag C-terminally to the nisin precursor peptide, which enabled isolation of the peptide and associated proteins. The isolated peptide was analyzed and the associated proteins were identified as NisB and NisC. However, NisC was detected only in sub-stoichiometric amounts. The -FNLD- box (blue box in Figure 1), which is highly conserved among class I lantibiotics [8], was identified as the crucial factor for the assembly of the modification complex *in vivo* [28].

No detailed information about the complex stoichiometry of the enzymes NisB and NisC are available, also the complex assembling process remains elusive.

Here, we provide the first *in vitro* study addressing the formation of the nisin modification complex composed of NisB, NisC and the nisin precursor peptide. The nisin precursor peptide is the essential trigger initiating complex formation of the enzymes NisB and NisC *in vitro*. More importantly, the -FNLD- box was identified as a crucial part triggering the complex assembly. Furthermore, a stoichiometry of the nisin precursor complex of 2:1:1, consisting of a functional NisB dimer, a monomer of NisC and one molecule of the unmodified precursor peptide was determined.

## Material and Methods

**Purification of NisB, NisC and the nisin precursor peptide variants**

NisB was homologously expressed in *L. lactis* and purified as previously described [11]. The expression and purification of NisC and the nisin precursor peptides was performed as described [10].

**Cloning the nisin precursor ring deficient variants**

For producing ring deficient precursor peptides of nisin, a shuttle vector accessible for *L. lactis* and *E. coli* was created. A kanamycin resistance gene and the *E. coli* origin of replication (Ori) ColE1 were amplified from a pET24 vector with the primer pair pET24aBglIIfor (CTTGCGGTATTCGAGATCTTGCACG) and pET24BamHIrev (CTAAATACATTCAAATATGGATCCGCTC). The *L. lactis* plasmid encoding the nisin precursor peptide was amplified with the following primer pair pNZE3-BglIIfor (GATGCATCGATAGATCTAGTCTTATAAC) and pNZE3-BamHIrev (CTAGATCTATCGATGGATCCCTTAACTTAC). The resulting PCR products were ligated after digestion with the restriction enzymes BgIII and BamHI. Positive results were identified by DNA sequencing.

**Analytical co-elution studies**

The co-elution studies were performed on an Äkta Micro system using a SD200 PC 3.2 column (GE Healthcare) equilibrated with 50 mM HEPES-NaOH, pH 7.0, 500

mM NaCl. The reaction mixtures ratio I (50 $\mu$L consisting of 15 $\mu$M NisB and 20 $\mu$M NisC) and ratio II (50 $\mu$L consisting of 20 $\mu$M NisB and 80 $\mu$M NisC) were applied to the column and the elution was observed at 280 and 215 nm. The corresponding precursor peptide was added at a concentration of 200 $\mu$M. After co-elution, the corresponding fractions were analyzed by a 4 - 20% gradient Tris-Glycin SDS-PAGE (Biorad) stained with Page-Blue (Thermo Fisher).

**MALS-SEC analysis**

To determine the molecular weight and stoichiometry of the NisB/NisC/nisin precursor peptide complex, a combination of multi-angle light scattering and size exclusion (MALS-SEC) was used. The procedure was performed using an Agilent 1260 HPLC system in combination with an triple-angle light scatter detector (miniDAWM TREOS) and an differential refractive index detector (Optilab rEX - both Wyatt Technology Europe). These technique requires higher protein amounts and therefore three different ratios between NisB, NisC and the nisin precursor peptide were used. Ratio I comprised 1:2:10 (20 $\mu$M NisB, 40 $\mu$M NisC and 200 $\mu$M nisin precursor peptide), ratio II comprised 1:4:10 (20 $\mu$M NisB, 80 $\mu$M NisC and 200 $\mu$M nisin precursor peptide) and ratio III comprised 1:8:10 (20 $\mu$M NisB, 160 $\mu$M NisC and 200 $\mu$M nisin precursor peptide) with an applied volume of 250 $\mu$L. The reaction mixture was injected on a Agilent Bio SEC-5 (300 Å; 7.8 x 300 mm) column equilibrated with 50 mM HEPES-NaOH, pH 7.0 and 500 mM NaCl. Data were analyzed using the Astra 5.3420 software package of Wyatt Technology Europe.

**Immunoblotting and SDS-PAGE analysis**

All SDS-PAGE and Western blotting experiments were performed with standard laboratory techniques. The antibodies for NisB [28], NisC [28] and the nisin leader peptide [8] were kindly provided by Dr. Moll, LanthioPharma, Groningen (Netherlands).

## Results

**Characterization of the modification enzymes NisB and NisC**

The enzymes NisB and NisC comprise the nisin modification machinery, which is responsible for the formation of the post-translational modifications of nisin. NisB was homologously expressed in *L. lactis* and could be purified to homogeneity via immobilized metal ion affinity chromatography (IMAC), followed by size exclusion chromatography (SEC). NisC was heterologously expressed in *E. coli* and could be isolated via a three-step purification strategy. The enzyme NisC was first isolated via an IMAC followed by a SEC purification step. The N-terminal $His_6$-tag was removed by thrombin treatment. To remove uncleaved protein, a second IMAC was performed. Both proteins were obtained to 95 % purity as judged from SDS-PAGE analysis (Figure 2A).

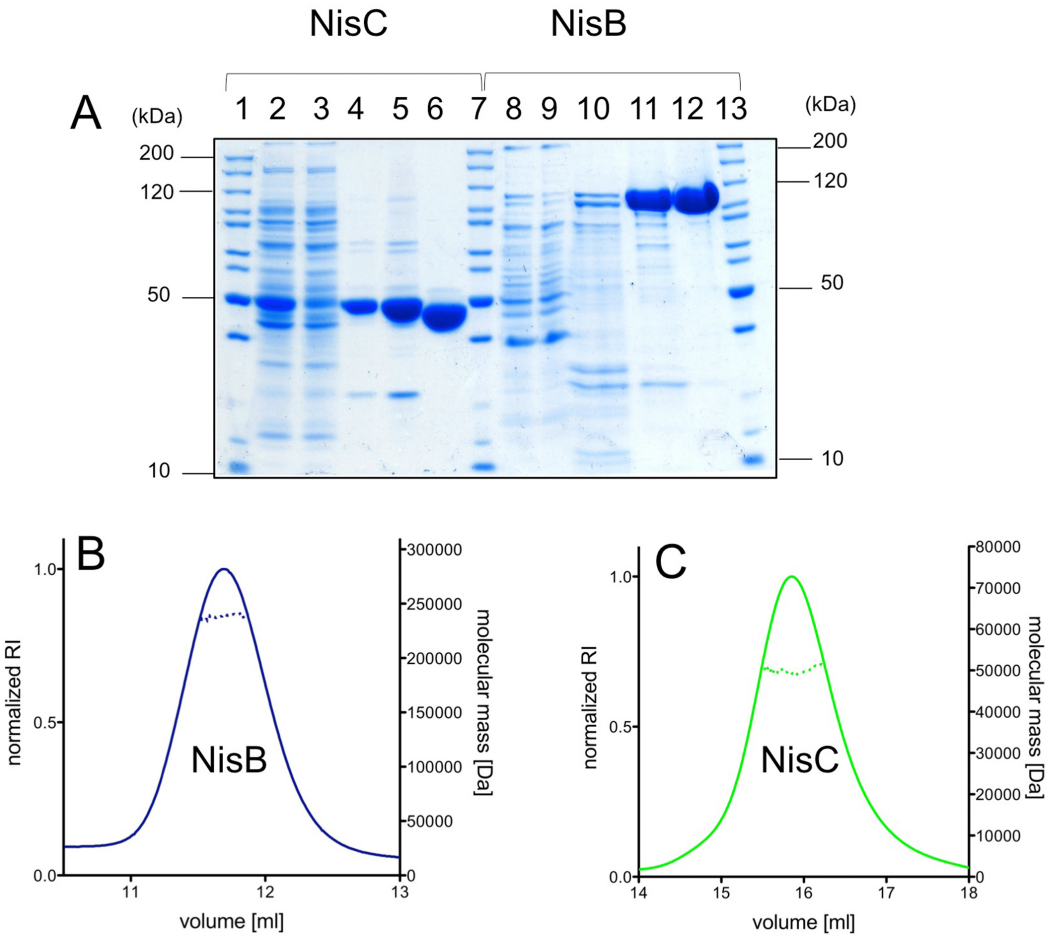


**Figure 2 A: SDS-PAGE analysis presenting the isolation process of NisC and NisB.** Lane 1: Protein marker. Lane 2: Crude extract of $His_6$-NisC expressing *E. coli* cells. Lane 3: IMAC flow through. Lane 4: $His_6$-NisC IMAC elution fraction. Lane 5: $His_6$-NisC elution fraction after SEC. Lane 6: Tag-free NisC after thrombin treatment. Lane 7: Protein marker. Lane 8: Crude extract of *L. lactis* cells, expressing NisB-$His_6$. Lane 9: Flow through after IMAC column. Lane 10: NisB IMAC wash fraction. Lane 11: IMAC elution fraction containing NisB-$His_6$. Lane 12: NisB-$His_6$ elution fraction after passing a SEC column. Lane 13: protein marker. **B: Molecular weight determination of NisB by MALS-SEC.** The elution profile of a purified NisB sample analyzed with MALS-SEC is shown in blue. The observed molecular weight of 239.2 ± 0.3 kDa fits with the theoretical molecular weight of a NisB dimer 236.6 kDa (www.expasy.org). **C: Molecular weight determination of NisC by MALS-SEC.** The green elution profile represents the MALS-SEC analysis of a purified NisC sample. A molecular weight of 49.6 ± 0.8 kDa was determined, which is in-line with the theoretical calculated molecular weight of a NisC monomer of 47.93 kDa (www.expasy.org).

The oligomeric state of the isolated proteins was determined using a combination of multi-angle light scattering and size exclusion chromatography (MALS-SEC). A molecular weight of 239.2 ± 1.2 kDa was determined for NisB, which was in-line with previous studies [11] and indicated that a NisB dimer was present with a theoretical molecular weight of 236.6 kDa (blue elution profile in Figure 2B and Ta-

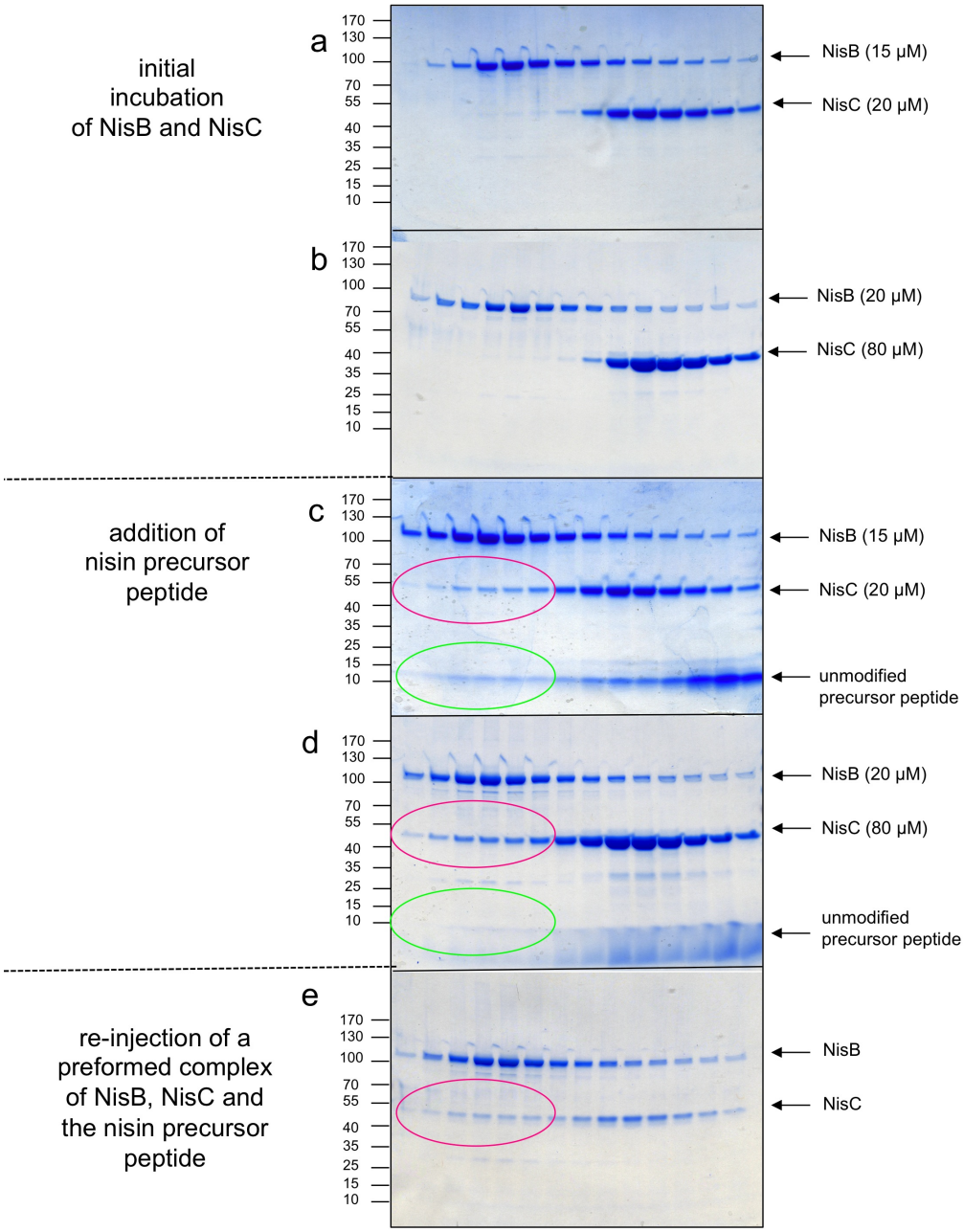


**Figure 3: SDS-PAGE analysis of co-elution experiments.** In the upper panel the results of the initial incubation of NisB and NisC are shown. (a) 15 $\mu$M NisB and 20 $\mu$M NisC (ratio I). (b) 20 $\mu$M NisB and 80 $\mu$M NisC (ratio II). In the middle panel the effect of the addition of the nisin precursor peptide regarding complex formation is shown. (c) 15 $\mu$M NisB, 20 $\mu$M NisC and 200 $\mu$M unmodified precursor peptide (ratio I). (d) 20 $\mu$M NisB, 80 $\mu$M NisC and 200 $\mu$M unmodified precursor peptide (ratio II). In the lower panel the preformed complex of NisB, NisC and the unmodified precursor peptide obtained by the first five fractions of (d) is re-injected and the result is represented in (e). The pink circle indicates the co-eluted NisC and the green circle indicates the co-eluted nisin precursor peptide, respectively.

ble 1). The analysis of NisC revealed a molecular weight of 49.6 ± 0.8 kDa and the theoretical molecular weight of NisC is calculated to 47.93 kDa. The observed molecular weight fitted to previous described data [10] indicating a stable NisC monomer in solution (green elution profile in Figure 2C). The isolation of the nisin precursor peptide using cation exchange chromatography was preformed as previously described [10, 11].

**Modification complex formation upon substrate binding**

The purified enzymes NisB and NisC were incubated at room temperature for 30 minutes and the reaction mixture was analyzed via analytical SEC as well as by MALS-SEC.

The analytical SEC and the corresponding SDS-PAGE analysis (Figure 3a) revealed no interaction between NisB and NisC. Additionally, no interaction between NisB and NisC were observed in the MALS-SEC analysis. The molecular weight of NisB was determined to 237.5 ± 0.3 kDa and the molecular weight of NisC revealed 49.4 ± 1.0 kDa, which is in-line with the molecular weight determined for the isolated enzymes (Figure 4 and Table 1). To investigate if a maturation complex might be formed with an increased amount of NisC the molar ratio between NisB and NisC was set to 1:4 (ratio II: 20 $\mu$M NisB and 80 $\mu$M NisC). The analysis of ratio II also revealed no visible complex formation between NisB and NisC (Figure 3b).

To test whether a potential complex is formed in presence of the precursor peptide, the unmodified precursor peptide was added at a concentration of 200 $\mu$M. The resulted reaction mixture comprised 15 $\mu$M NisB, 20 $\mu$M NisC and 200 $\mu$M of the unmodified precursor peptide (ratio I) and was analyzed by analytical SEC.

In the corresponding SDS-PAGE analysis NisC was detected in the elution fractions of NisB (Figure 3c). In contrast, when the unmodified precursor peptide was absent, NisC was not co-eluting with NisB (Figure 3a). Furthermore, the unmodified precursor peptide was also present in the fractions of NisB and NisC (Figure 3c). The presence of NisC and the nisin precursor peptide in the elution fractions of NisB represents the first visualization of an assembled nisin maturation complex *in vitro*.

To investigate whether the amount of co-eluting NisC could be increased, the NisC concentration was increased to 80 $\mu$M (ratio II), which represented a 4-fold molar access of NisC in respect to NisB (20 $\mu$M ).

Figure 3d represents the result of ratio II, which revealed that the amount of NisC co-eluting with NisB was increased. As a control, no co-elution was visible in the absence of the unmodified precursor peptide (Figure 3b).

The stability of the maturation complex was investigated as follows. The first five fractions of ratio II, containing the nisin maturation complex (NisB, NisC and nisin precursor peptide), were concentrated and re-injected on an analytical SEC column. The resulting SDS-PAGE analysis is presented in Figure 3e. Here, NisC was again

present in the elution fractions of NisB, although dissociation of NisC was visible. This experiment demonstrated, that the complex consisting of the three components was only stable to a certain extent. The nisin precursor peptide was not visible in Figure 3e due to the decreased amount, which was below the detection limit of the coomassie stained SDS-PAGE analysis. The presence of the precursor peptide was verified by immunoblotting of the dedicated fractions (shown in Figure 6 - lane 3).

**MALS-SEC quantification of the nisin maturation complex**

For quantification of the complex a MALS - SEC analysis was performed and the apparent molecular weight of NisB in the absence of the unmodified precursor peptide was compared to the NisB elution fraction in presence of the unmodified precursor peptide. Excess NisC was always present, but excluded from the analysis. The analysis of ratio I resulted in a shift of the NisB elution peak to earlier retention times caused by the increased hydrodynamic radius (data not shown) when NisC and the unmodified precursor peptide were present as compared to the NisB/NisC mixture in absence of the unmodified precursor peptide (shown in pink in Figure 4).

The apparent molecular weight of the NisB elution peak determined for the ratio I was determined to 268.9 ± 0.8 kDa. In the absence of the unmodified precursor peptide the molecular weight for NisB was determined to 237.5 ± 0.3 kDa. The apparent molecular weight of the elution fractions, in which the complex was expected, is increased by 31.4 kDa indicating that the complex was formed only partially. The MALS-SEC analysis determined the apparent molecular weight of the average of all particles, which were eluting simultaneously. Therefore, the determined values correspond to a mixture of complex and isolated NisB. Thus, the apparent molecular weight can be used to calculate the mole fractions of NisB and the complex present in the mixture. Here, the determined apparent molecular weight consisted of isolated NisB and NisB of the complex fractions, which was bound to NisC and the unmodified precursor peptide (shown by SDS-PAGE analysis, Figure 3c). The molecular weight average of this mixture resulted in an apparent molecular weight of 268.9 kDa, which corresponds to a mole fraction of 40 % isolated NisB and 60 % of a complex composed of a NisB dimer, a NisC monomer and one molecule of unmodified precursor peptide.

The presence of isolated NisB might be caused by the decreased concentration of NisC during SEC. The access of NisC was removed during SEC, since dissociation occurs due to the law of mass action responding to a decrease of the apparent concentration of complex. This assumption was supported by the re-injection experiment, which is shown in Figure 3e. Here, a preformed NisB-NisC-nisin precursor peptide complex was re-injected on the analytical SEC column. This experiment represented the situation on the SEC

column when the access of NisC was already separated. The SDS-PAGE analysis revealed that NisC was still bound to NisB to a certain extend but also that a major fraction already dissociated. This is in-line with the lower apparent molecular weight determined for ratio I in the complex formation experiment.

To circumvent the dissociation of NisC from the modification complex during SEC the NisC concentration was increased. Here, the ratio of NisC to NisB, was increased to 1:4 (ratio II), which resulted in an apparent molecular weight of the complex fraction of 291.2 ± 1.2 kDa (shown in green in Figure 4 and Table 1). This difference of ratio II compared to isolated NisB was calculated to 53.7 kDa, which fits within experimental error to a bound NisC monomer (47.93 kDa) and a bound nisin precursor peptide (5.9 kDa), which demonstrates the formation of a nisin maturation complex *in vitro* with a stoichiometry of 2:1:1 (NisB, NisC, nisin precursor peptide) with an overall molecular weight of 290.43 kDa. To verify the specificity of the formed stoichiometry the NisC concentration was further increased to 160 $\mu$M which resulted in ratio III (20 $\mu$M NisB, 160 $\mu$M NisC and 200 $\mu$M unmodified precursor peptide). The MALS-SEC analysis revealed an apparent molecular weight for ratio III of 293.6 ± 1.2. This result verified the specificity and determined stoichiometry of ratio II of 2:1:1 (NisB, NisC, nisin precursor peptide - 290.43 kDa).

**Table 1: MALS - SEC data summarizing the molecular weight ($M_W$) of the isolated enzymes and the complex in different molar ratios.** The theoretical molecular weight of a NisB dimer is calculated to 236.6 kDa, 47.93 kDa for a NisC monomer and 5.9 kDa for the unmodified precursor peptide.

| **Sample** | **apparent $M_W$ [kDa]** | **$M_W$ difference to (B) [kDa]** |
|---|---|---|
| (A) NisB | 239.2 ± 1.2 | |
| (B) NisB:NisC (ratio I) | 237.5 ± 0.3 | 0.0 |
| (C) NisB:NisC (ratio II) | 238.1 ± 0.3 | 0.6 |
| (D) NisB:NisC:nisin precursor (ratio I) | 268.9 ± 0.8 | 31.4 |
| (E) NisB:NisC:nisin precursor (ratio II) | 291.2 ± 1.2 | 53.7 |
| (F) NisB:NisC:nisin precursor (ratio III) | 293.6 ± 1.2 | 56.1 |

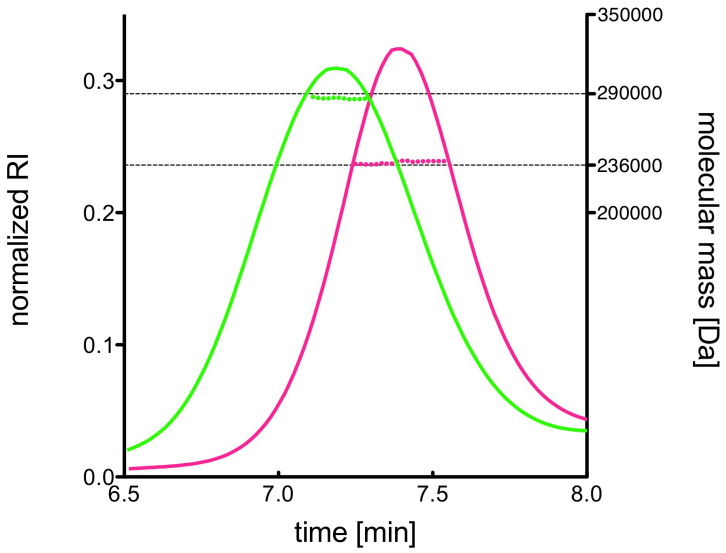


**Figure 4: MALS-SEC analysis of the mixed protein samples consisting of NisB and NisC, in presence or absence of the unmodified precursor peptide.** The elution profile of a mixture of 20 $\mu$M NisB and 80 $\mu$M NisC is shown in pink, resulting in a molecular weight of NisB of 238.1 $\pm$ 0.3 kDa (pink dotted line). The analysis of 20 $\mu$M NisB, 80 $\mu$M NisC and 200 $\mu$M unmodified precursor peptide is shown in green revealing an apparent molecular weight of 291.2 $\pm$ 1.1 kDa (green dotted line) for the formed complex. The two black dotted lines indicate the theoretical molecular weight of isolated NisB (236 kDa) and of a complex consisting of a dimer NisB, a monomer NisC and one molecule of the nisin precursor peptide (290 kDa).

**Identifying specific regions within the nisin precursor peptide, which are responsible for complex formation**

The unmodified precursor peptide initiated a complex formation between NisB and NisC (Figure 3a and Figure 5a). Is the modification state of the core peptide essential for complex formation?

To answer this question, the unmodified, dehydrated and modified precursor peptides were incubated with 15 $\mu$M NisB and 20 $\mu$M NisC. Ratio I was used in the analytical SEC to determine the specific interactions between NisB, NisC and the nisin precursor peptide qualitatively, only for a quantitative MALS-SEC analysis ratio II and III were used due to the different experimental requirements. Further details are provided in Material and Methods. Within the dehydrated precursor peptide all serine and threonine residues of the core peptide are dehydrated. The SDS-PAGE analysis of the analytical SEC is shown in Figure 5b. NisC was only shifted one fraction towards NisB, which might be caused by a weak interaction between NisB/NisC and the dehydrated precursor peptide. The modified precursor peptide, which contains all post-translational modifications in the core peptide, was not able to induce any complex formation between NisB and NisC (Figure 5c).

To verify this result an immunoblot analysis was performed. The fraction containing the peak maximum of the potential NisB-NisC-nisin precursor peptide complex was

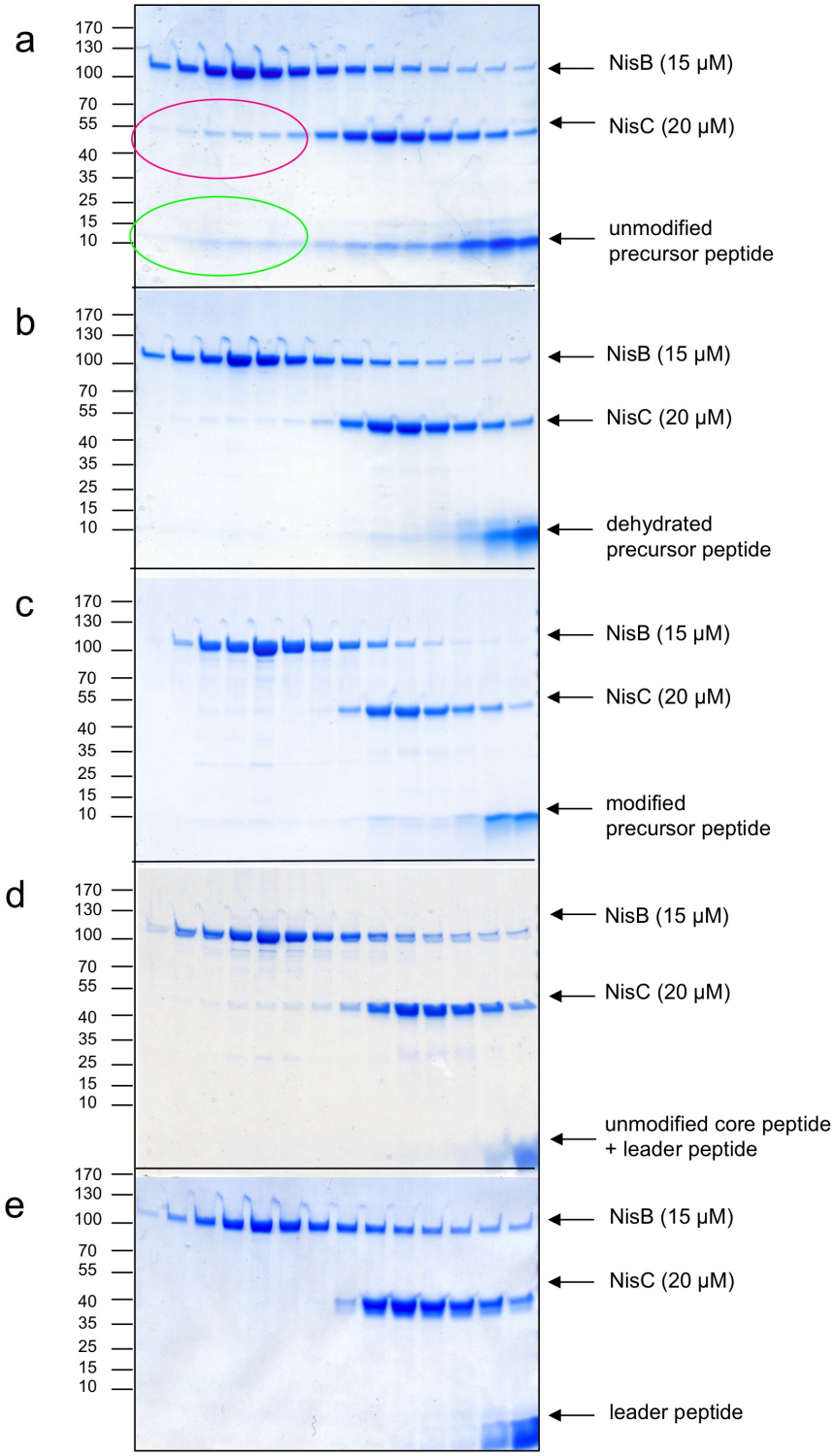


**Figure 5: SDS-PAGE analysis showing the results of determining the core- and leader peptide dependency on complex formation.** (a) 15 $\mu$M NisB, 20 $\mu$M NisC and 200 $\mu$M o the unmodified precursor peptide. (b) 15 $\mu$M NisB, 20 $\mu$M NisC and 200 $\mu$M of the dehydrated precursor peptide. (c) 15 $\mu$M NisB, 20 $\mu$M NisC and 200 $\mu$M of the modified precursor peptide. (d) 15 $\mu$M NisB, 20 $\mu$M NisC and 200 $\mu$M of the digested unmodified precursor peptide. Resulting in an unmodified core peptide and the leader peptide in a molar ratio of 1:1. (e) 15 $\mu$M NisB, 20 $\mu$M NisC and 200 $\mu$M of the leader peptide. The pink circle indicates the co-eluted NisC and the green circle indicates the co-eluted nisin precursor peptide, respectively

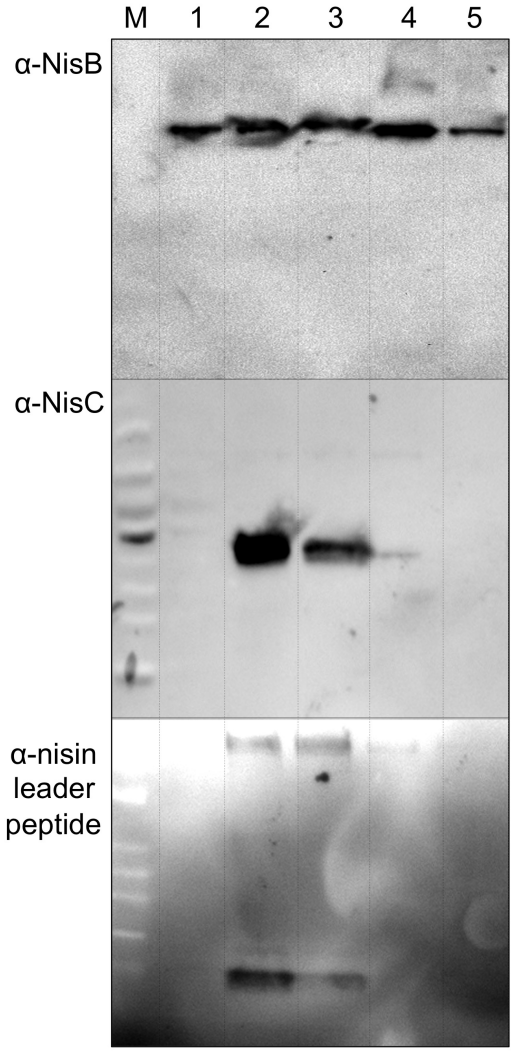


**Figure 6: Western blot analysis of the fractions containing the potential complex.** Top: Western-blot using antibodies recognizing the NisB protein. In the middle a western blot analysis using $\alpha$ - NisC antibodies is presented. At the bottom an immunoblot using antibodies against the nisin leader peptide is shown. M: protein marker. Lane 1: The fraction resulting of the analytical SEC of 15 $\mu$M NisB and 20 $\mu$M NisC. Lane 2: This sample represents the complex formed by 15 $\mu$M NisB, 20 $\mu$M NisC and 200 $\mu$M unmodified precursor peptide. Lane 3: The complex from lane 2 is re-injected. Lane 4: 15 $\mu$M NisB, 20 $\mu$M NisC and 200 $\mu$M dehydrated precursor peptide. Lane 5: 15 $\mu$M NisB, 20 $\mu$M NisC and 200 $\mu$M modified precursor peptide.

analyzed. Antibodies against NisB [28], NisC [28] and the nisin leader peptide [8] were used to analyze each sample. The resulting Western blots are summarized in Figure 6.

In lane 1 the NisB - NisC mixture in the absence of the nisin precursor peptide is shown. No NisC was detected in the NisB elution fraction verifying the results of the analytical SEC experiment and the corresponding SDS-PAGE analysis (Figure 3a/5a) and the MALS-SEC analysis

(Figure 4). In lane 2 the reaction mixture containing NisB, NisC and the unmodified precursor peptide is shown. A signal for NisC and the unmodified precursor peptide was detectable, which proofed complex formation between NisC and NisB, which was mediated by the unmodified precursor peptide. In lane 3 a sample of the preformed and re-injected complex is shown and a signal for NisC and the unmodified precursor peptide is still detectable. Furthermore, the antibody against the nisin leader peptide showed a signal in the same region where NisB is migrating in the SDS-PAGE analysis in lane 2 and 3. This result demonstrated that the unmodified precursor peptide was bound to NisB, which was still detectable in a SDS-PAGE analysis. When NisB and NisC were incubated with the dehydrated precursor peptide (lane 4). NisC was only detected at very low intensity, which might also be caused by an experimental artifact (swap over). The analysis of the modified precursor peptide incubated with NisB and NisC after analytical SEC is shown in lane 5, which demonstrate that neither NisC nor the nisin precursor peptide were detectable, which proofed the analytical SEC experiment presented in Figure 5c. Therefore the modification state of the nisin precursor peptide is the major determinant of complex formation *in vitro*.

**Involvement of the (methyl) lanthionine rings in complex formation**

As presented above, the presence of (methyl)lanthionine rings abolished complex formation. To test whether the presence of all (methyl)lanthionine rings inhibits complex formation or if only one unique ring is responsible for this inhibition, four nisin precursor variants were produced, which differed in the number of (methyl)lanthionine rings in the core peptide. The nisin precursor peptide contains five cysteine residues in the core peptide. Each one is an essential part of one of the five (methyl)lanthionine rings A-E (Figure 1). The cysteine residue at position 7 is part of ring A, the cysteine residue at position 11 is part of ring B, following position 19 which is part of ring C. Positions 26 and 28 comprise cysteine residues involved in the intertwined rings D and E. By changing these cysteine residues against alanines, nisin precursor peptides were derived which vary in the number and position of (methyl)lanthionine rings. The variants CAAAA (comprises only ring A as highlighted by the 'C'), CCAAA (rings A and B), CCCAA (rings A - C) and CCCCA (rings A - D) were incubated with 15 $\mu$M NisB and 20 $\mu$M NisC. After analyzing the different ring deficient precursor peptides by analytical SEC and SDS-PAGEs, no complex formation was detected (Figure S1 a-d). This indicated that the complex formation of NisB, NisC and the nisin precursor peptide was neither possible in the presence of the (methyl)lanthionine rings nor in the presence of the dehydrations. This suggests that only an unmodified core peptide is able to initiate a complex formation between NisB and NisC *in vitro* at sufficient efficiencies.

**Is the leader peptide necessary for initiating a complex?**

The above-described results demonstrated an essential role of an unmodified core peptide in complex formation. Does the leader peptide participate in the complex assembly? The influence of the isolated leader peptide was investigated by incubation of the isolated leader peptide with the modification enzymes 15 $\mu$M NisB and 20 $\mu$M NisC. As shown in Figure 5e the isolated leader peptide did not mediate complex formation.

This suggested that both, the unmodified core peptide and the leader peptide, need to be present to ensure complex formation. Whether the leader peptide needs to be covalently bound to the core peptide, was also investigated. The unmodified precursor peptide was digested by the protease NisP, which resulted in release of the leader peptide and the unmodified core peptide (described in [10]). This peptide mixture was incubated with NisB and NisC (Figure 5d). No interaction between NisB and NisC was detected by SDS-PAGE analysis.

This suggested that the physically presence of the leader peptide in trans did not ensure complex formation. This experiment highlighted the requirement of a covalently bound leader peptide to assemble the nisin maturation complex.

**Is the -FNLD- box of the leader peptide involved in complex formation?**

NisB and NisC are both recognizing the leader peptide, especially the highly conserved -FNLD- box, *in vitro* [10, 11]. To test whether the -FNLD- box is involved in complex formation, the -FNLD- box was mutated to -AAAA-. The SDS-PAGE analysis of the analytical SEC revealed only low amounts of NisC and the -AAAA- precursor peptide variant in the elution fractions of NisB (Figure S2a). In comparison to the unmodified precursor peptide the amount of induced transient complex was reduced by more than 90 % highlighting the importance of the -FNLD- box in complex formation.

Furthermore, each amino acid of the -FNLD- box was exchanged against alanine. All four variants ($F_{-18}A$, $N_{-17}A$, $L_{-16}A$ and $D_{-15}A$) were not able to induce complex formation. A SDS-PAGE analysis summarizes these results in Figure S2 a-e.

The used single point -FNLD- box mutations were purified from the supernatant of a *L. lactis* strain which contains the nisin maturation machinery to secrete the precursor peptide variants. The analysis of the maturation state revealed that they were modified by NisB and NisC, *in vivo* [8]. Therefore they contained already the (methyl)lanthionine rings in the core peptide prior to incubation with NisB and NisC. To circumvent the influence of the modified core peptide on complex formation, the experiments with the single point mutations need to be repeated with unmodified core peptide. The -AAAA- variant did not contain any post-translational modifications in the core peptide [8]. This result further supports the suggestion that only a nisin precursor peptide, which is not post-translational modified by NisB and /

or NisC, contains the essential requirement to induce complex formation of the nisin maturation complex.
Furthermore this suggested that the -FNLD- box played an important role in complex assembly. By manipulating this box, complex formation was reduced at least by 90 %.

## Discussion

### Complex assembly upon substrate binding

The existence of a nisin maturation complex has been proposed but was only rarely investigated due to experimental limitations. Here, we provide the first *in vitro* data on the assembly of the nisin modification complex consisting of a dimeric NisB in combination with a monomer of NisC initiated via the unmodified precursor peptide, which elucidates the detailed process involved in complex assembly.
The enzymes NisB and NisC are proposed to work in an alternating fashion to introduce the post-translational modifications in the nisin precursor peptide. Nevertheless, a direct interaction of NisB and NisC was never observed *in vivo* nor *in vitro* in the absence of the substrate (6, 7, 26, 28, 30). The interaction between NisB and NisC was analyzed in two different ratios (Figure 3). Even in the presence of a 4-fold molar access of NisC no interaction between NisB and NisC, could be demonstrated, which is in-line with other studies (6, 7, 26, 28, 30). Therefore the interaction between NisB and NisC requires an additional factor.
Here, we present the factor required to induce a complex between NisB and NisC. The addition of the unmodified precursor peptide initiated a complex formation between NisB and NisC. This complex was demonstrated by the presence of NisC and nisin precursor peptide in the elution fractions of NisB (Figure 3c and d).
In previous *in vivo* studies, NisB and NisC were already identified to bind simultaneously to the nisin precursor peptide [28]. In this study, the nisin precursor peptide was isolated via an affinity tag from the cytosol of *L. lactis*. NisB was identified in the elution fractions of the nisin precursor peptide and NisC was found in substoichiometric amounts [28]. These data provide a strong evidence for the existence of a complex, but a detailed characterization of the complex remained open. The data presented here, revealed the first visualization of the nisin maturation complex comprising the three components NisB, NisC and the nisin precursor peptide *in vitro*. Furthermore, this is the first evidence that the substrate, the unmodified precursor peptide, initiates the assembly of the modification complex between NisB and NisC.

### Interaction between NisB, NisC and the nisin core peptide

Complex formation between NisB, NisC and the nisin precursor peptide was only observed if the core peptide of the substrate remained unmodified (Figure 5a). The presence of post-translational modifications within the core peptide (dehy-

drations and (methyl)lanthionine rings) abolished complex formation (Figure 5b and c). This might suggest that the complex is able to recognize the maturation state of the substrate and therefore already modified substrates are not accepted as potential substrates and prohibit complex assembly.

Additionally the presence of a defined number of rings were analyzed to investigate their influence in complex assembly (Figure S1). Supporting the above model, the presence of already a single ring prevented complex formation. This is in-line with the data of Khusainov *et al.*. In this study, it was possible to isolate a nisin precursor peptide with bound NisB and NisC in sub-stoichiometric amounts [28]. These isolated precursor peptides were active against a nisin sensitive strain and therefore contained at least three (methyl)lanthionine rings [29]. Furthermore, it was observed that the inactivation of NisC, which results in the absence of the (methyl)lanthionine rings, increased amounts of NisC bound to the nisin precursor peptide were observed [28]. Our data explain these observations. The presence of (methyl)lanthionine rings results in disruption of the interaction of NisB and NisC (Figure 5d) and thereby cause a sub-stoichiometric amount of NisC. In the absence of the (methyl)lanthionine rings, a complex is able to be formed between NisB, NisC and the nisin precursor peptide (Figure 5a).

This highlights that the entire maturation complex is only assembled efficiently if the corresponding substrate contains an unmodified core peptide, which serves as the recognition motif.

**Recognition motif within the nisin leader peptide**

To elucidate the role of the N-terminal located leader peptide in complex formation, the isolated leader peptide as well as an in trans expressed leader peptide were analyzed in respect to complex assembly (Figure 5e). This analyses revealed the leader peptide was essential and was required to be covalently bound to an unmodified core peptide. This result represents a second factor which is required for interaction between NisB, NisC and their substrate. Additionally, this is in-line with *in vivo* data obtained by Khusainov *et al.* [30]. Here, full dehydration activity of NisB was only obtained if the leader peptide was covalently bound to the core peptide [30].

Within the leader peptide of class I lantibiotics the -FNLD- box is highly conserved [8] and this box was identified as recognition motif for isolated NisB and isolated NisC [10, 11, 25, 28]. The exchange of -FNLD- against four alanines (-AAAA-) reduced the complex formation drastically by more than 90 %. Additionally the influence of any single amino acid was investigated, however the introduction of single alanines in the -FNLD- box did not result in an visible complex assembly. As shown in Figure 5 and presented above, a modified core peptide abolished the interaction of the modification complex and therefore this explains the missing complex formation for the single point mutations *in vitro*.

**Complex stoichiometry**

The suggested stoichiometry of the modification enzymes in the nisin maturation complex is 1:2:2:1 (1x NisB, 2x NisC, 2x NisT and one nisin precursor peptide) as derived from immuno-precipitation and yeast two-hybrid studies [26].

Here, the apparent molecular weight of the complex comprising of NisB, NisC and the unmodified precursor peptide was determined in three different ratios. The results of ration I have been analyzed in detail in the Results section and will not be discussed here. For ratio II a clear result of the stoichiometry of the nisin maturation complex was obtained.

Here, an apparent molecular weight of 291 kDa was determined for the complex (Figure 4). The difference between the molecular weight of NisB determined in the nisin precursor peptide free sample and ratio II amounts to 53 kDa. The molecular weight of an isolated NisC monomer comprises 48 kDa and the nisin precursor peptide additionally adds 6 kDa which sums up to 54 kDa. Therefore, the observed apparent molecular weight for the complex in ratio II fits within experimental errors to the theoretical calculated molecular weight of 290.43 kDa for a complex consisting of a dimer of NisB (236.6 kDa) a monomer of NisC (47.93 kDa) and one molecule of the nisin precursor peptide (5.9 kDa). To confirm the specificity of this determined stoichiometry the NisB / NisC ratio was further increased to 1:8 (ratio III). The MALS-SEC analysis revealed for ratio III an apparent molecular weight of 293 kDa. This verifies the complex stoichiometry. Furthermore, the determined apparent molecular weight of ratio III verifies that no other stoichiometries between NisB, NisC and the nisin precursor peptide exist *in vitro*. For example, a monomer of NisB, a dimer of NisC and a nisin precursor peptide would result in an apparent molecular weight of a complex of 220 kDa. A summation of the molecular weight of a complex comprised by dimeric NisB and dimeric NisC would lead to approximately 340 kDa.

These data represent the first quantitative view, which demonstrates the apparent molecular weight and thereby elucidates the stoichiometry of the nisin maturation complex. These data suggest a stoichiometry, which contains a NisB dimer, a NisC monomer and one nisin precursor peptide (2:1:1).

In summary, the data obtained in this study identified three factors influencing the complex formation of the nisin modification enzymes NisB and NisC. First, the substrate, the unmodified precursor peptide, mediates an interaction between NisB and NisC and initiates the complex assembly upon binding. Second, the C-terminal located core peptide requires an unmodified state to ensure recognition by the maturation complex. Third, the N-terminal positioned leader peptide plays an important role in complex formation. The leader peptide needs to be covalently bound to the core peptide and the highly conserved -FNLD- box is an essential recognition factor.

Finally, the MALS-SEC analysis revealed

the first quantitative data elucidating the stoichiometry of the nisin maturation complex. This stabilized complex revealed a mass of approximately 291 kDa. This apparent molecular weight demonstrates a stoichiometry of 2:1:1.

## Acknowledgment

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## Supplemental Information

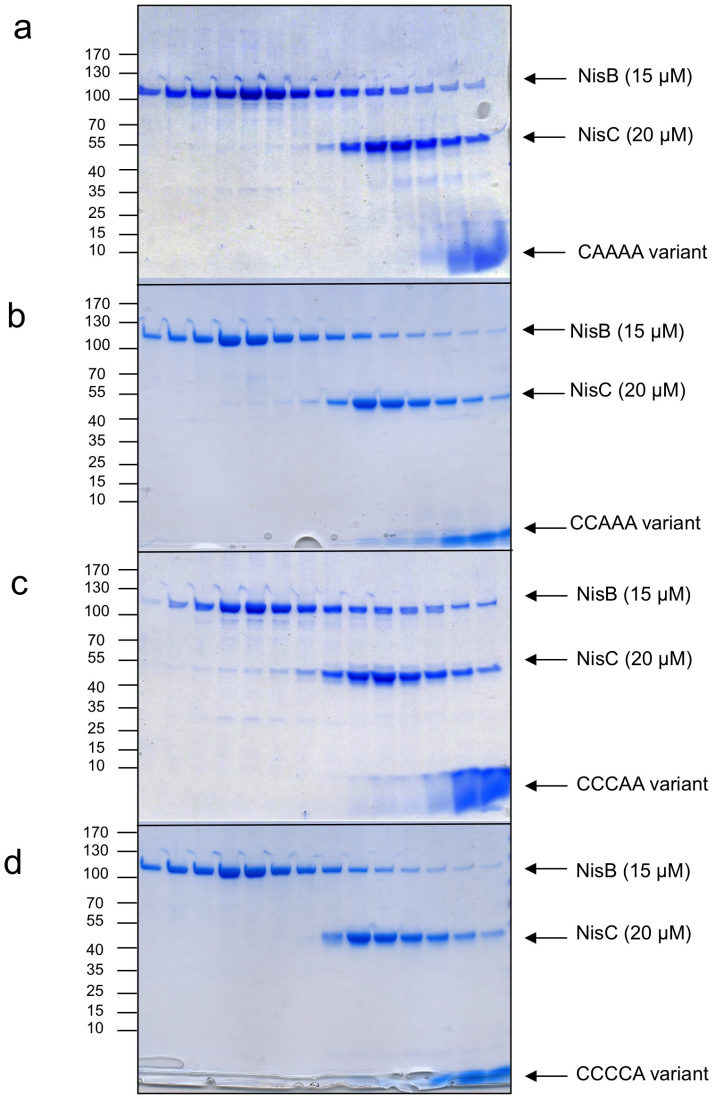


**Figure S1: SDS-PAGE analysis demonstrating the influence in varying the number of formed (methyl)lanthionine rings**. (a) 15 $\mu$M NisB, 20 $\mu$M NisC and 200 $\mu$M of the CAAAA variant. (b) 15 $\mu$M NisB, 20 $\mu$M NisC and 200 $\mu$M of the CCAAA variant. (c) 15 $\mu$M NisB, 20 $\mu$M NisC and 200 $\mu$M of the CCCAA variant. (d) 15 $\mu$M NisB, 20 $\mu$M NisC and 200 $\mu$M of the CCCCA variant.

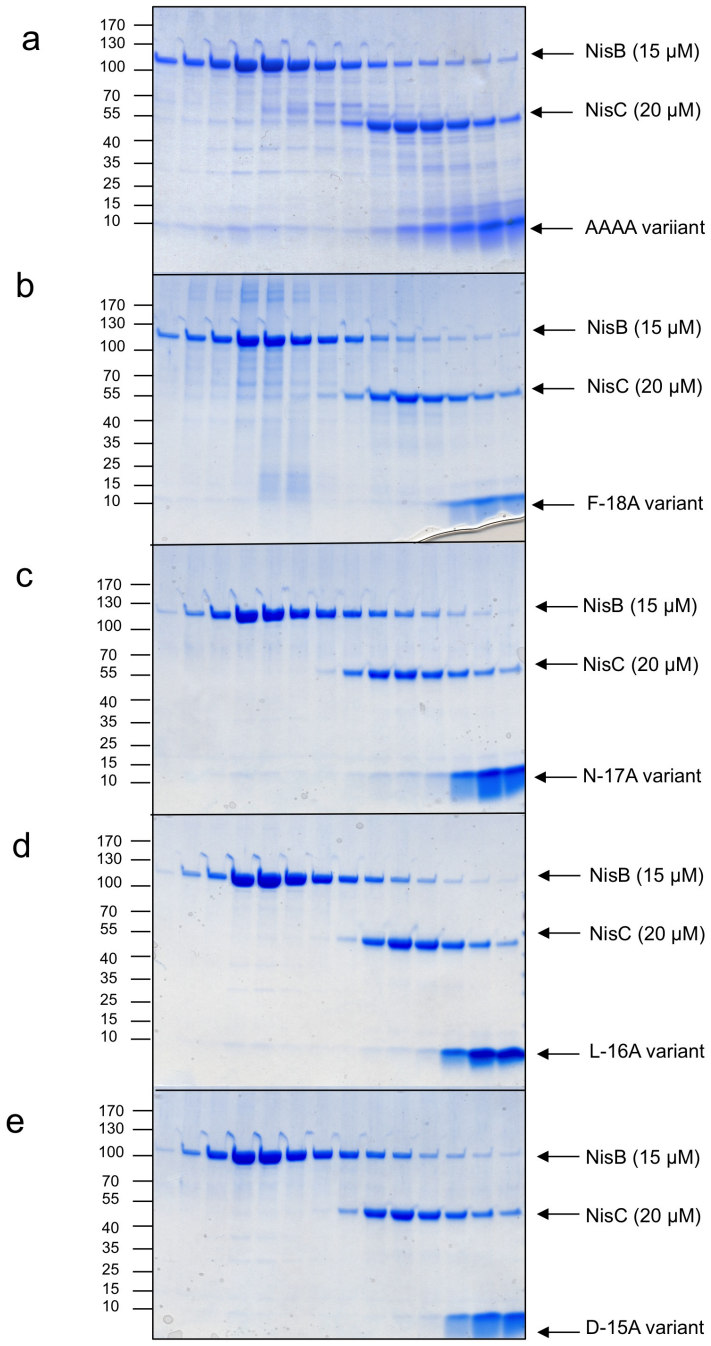


**Figure S2: SDS-PAGE analysis demonstrating the influence on complex formation by mutating the -FNLD-** (a) 15 $\mu$M NisB, 20 $\mu$M NisC and 200 $\mu$M of the AAAA - variant. (b) 15 $\mu$M NisB, 20 $\mu$M NisC and 200 $\mu$M of the $F_{-18}A$ variant. (c) 15 $\mu$M NisB, 20 $\mu$M NisC and 200 $\mu$M of the $N_{-17}A$ variant. (d) 15 $\mu$M NisB, 20 $\mu$M NisC and 200 $\mu$M of the $L_{-16}A$ variant. (e) 15 $\mu$M NisB, 20 $\mu$M NisC and 200 $\mu$M of the $D_{-15}A$ variant.

# 4 Discussion

In this thesis the maturation enzymes of the lanthipeptide nisin were investigated on a molecular level. In addition, a protocol for an efficient production and purification of wildtype and variants of nisin and its precursor peptides was established.

## 4.1 The lanthipeptide nisin

Nisin is synthesized as a precursor peptide in the cytosol of *L. lactis*[58,59]. After several post-translational modification steps, it is secreted into the extracellular space[58,59].

Within this thesis, an optimized purification protocol for nisin from a cell-free culture supernatant was developed, which yielded highly active nisin (chapter 3.3). Compared to known protocols[127,148,149], the step-wise elution increased the purity to $\geq 95$ %, which resulted in a ten-fold higher specific antimicrobial activity of nisin. This purification strategy enabled a reliable and efficient way to obtain nisin (chapter 3.3).

The application of the nisin-controlled gene expression (NICE) system[66,150] for *L. lactis* allowed the expression and secretion of the nisin precursor peptide in three different maturation states (Figure 16). This two-plasmid system is explained in detail in chapter 3.4. One plasmid encodes for the nisin precursor peptide *(nisA)* and the other for the modification enzymes *nisB*, *nisC* and the transporter *nisT*. All genes are regulated by the nisin promotor which is activated by the two-component system NisK and NisR. Thereby, a nisin-dependent induction of all required genes is simultaneously initiated. The presence or absence of genes on the second plasmid dictates the modification state of the secreted nisin precursor peptides (Figure 16 - right hand side).

When all genes are present, the modified nisin precursor peptide is secreted. In absence of NisC, exclusively the dehydrated nisin precursor peptide is produced. When NisB and NisC are absent the nisin precursor peptide is secreted in an unmodified state. The average yield for the nisin precursor peptides variants reached approximately 2 mg / L cell culture. This system formed the basis for isolating nisin precursor peptides in three different maturation states as used in chapter 3.4, 3.5, 3.6 and 3.7.

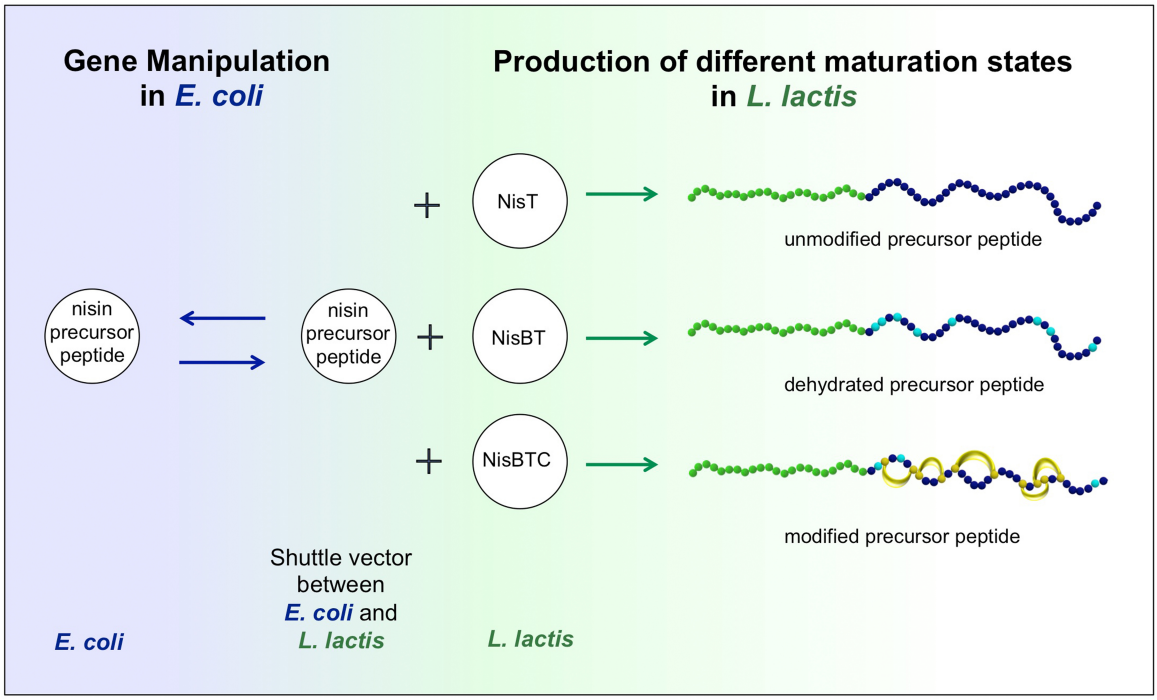


Figure 16: **Manipulation and purification strategy for the nisin precursor peptide.** The nisin precursor peptide is encoded on a shuttle vector for use in *Escherichia coli* (*E. coli*) and *L. lactis*. The left hand side (blue) represents the site-specific mutation of the nisin precursor peptide encoded on the *nisA* shuttle vector in *E. coli*. The right hand side (green) shows the expression and secretion of the nisin precursor peptide in *L. lactis*. The plasmid encoding for the nisin precursor peptide can be combined with one of three different maturation plasmids. The above shown combination (NisT) produces unmodified precursor peptides. The combination shown in the middle (NisBT) results in dehydrated precursor peptides. The addition of the plasmid NisBTC results in modified precursor peptides (chapter 3.4).

Furthermore, a *nisA*-containing shuttle vector was designed and produced for its application in *L. lactis* and *Escherichia coli* (*E. coli*) in cooperation with Diana Kleinschrodt (Protein Production Facility - Heinrich Heine University Düsseldorf) (chapter 3.7). This vector enabled the incorporation of site-specific mutations by standard techniques in *E. coli*. Briefly, an origin of replication (ORI) and an antibiotic resistance gene for both species were included in the plasmid. Several mutations could be inserted in the *nisA* gene in *E. coli* and subsequently transformed, expressed, secreted and purified using *L. lactis* (used in chapter 3.5, 3.6 and 3.7).

## 4.2 The nisin maturation machinery

An essential step for the detailed understanding of the nisin modification process was the *in vitro* characterization of NisB, NisC and NisP. As a first step, NisB was homologously expressed in *L. lactis* and purified to homogeneity (chapter 3.4) with a typical yield of 1.5 mg NisB per liter cell culture. The biochemical characterization of NisB revealed the oligomerization of NisB as a dimer *in vitro*. Moreover, surface plasmon resonance demonstrated that the NisB dimer was able to bind one nisin precursor peptide molecule. The dimeric NisB was specifically interacting with the leader peptide of the nisin precursor peptide and was furthermore able to recognize the modification state of the nisin core peptide (chapter 3.4).

The second step comprised the heterologous expression of the nisin cyclase NisC in *E. coli* (chapter 3.5). NisC was purified to homogeneity yielding approximately 10 mg NisC per liter cell culture. The oligomeric state of NisC was investigated by MALS-SEC and revealed a stable monomer for NisC in aqueous solution. ITC was used to characterize the interaction between NisC and the nisin precursor peptides and demonstrated, that like NisB the nisin leader peptide was also essential for the *in vitro* binding of NisC to the nisin precursor peptide (chapter 3.5).

Third, the protease NisP was characterized *in vitro* and the specificity towards the three different nisin precursor peptides was investigated (chapter 3.6). The protease NisP functions as a monomer and cleaves the nisin precursor peptide C-terminal to the conserved motif -PR-, which yields the leader peptide and the core peptide, independently of the maturation state of the core peptide (chapter 3.6).

As a final step, NisB, NisC and different nisin precursor peptides were incubated and complex formation was examined (chapter 3.7). Here, a complex of all three proteins was assembled *in vitro* and was found to be induced specifically in the presence of the unmodified precursor peptide. Furthermore, a quantitative analysis revealed a stoichiometry of the nisin maturation complex of 2:1:1 comprising of a dimer of NisB, a monomer of NisC and one molecule of the nisin precursor peptide (chapter 3.7).

## 4.3 The nisin maturation complex

The lanthipeptide nisin is a member of the ribosomally synthesized and post-translationally modified peptides (RiPPs)[4]. Furthermore, it contains the unnatural amino acid lanthionine[38,59]. Therefore it belongs to the subclass of lanthipeptides[4,7,21,33]. Despite the fact that, the players involved in maturation and transport of nisin are well described[59,151], the *in vitro* characterization of the involved enzymes remains elusive. Therefore, a main aim of this thesis was the biochemical characterization of the nisin maturation machinery *in vitro*. Major findings of these studies are described within the next chapters and the results for the understanding of the nisin maturation process are discussed.

### 4.3.1 Membrane association and stoichiometry

It was shown by Engelke *et al.*[128] and in more detail by Siegers *et al.*[58] that NisT, NisB and NisC were membrane associated[58,128]. In these studies, membrane fractions of nisin producing *L. lactis* cells were analyzed by immunoblotting with specific antibodies against NisB and NisC[58,128].

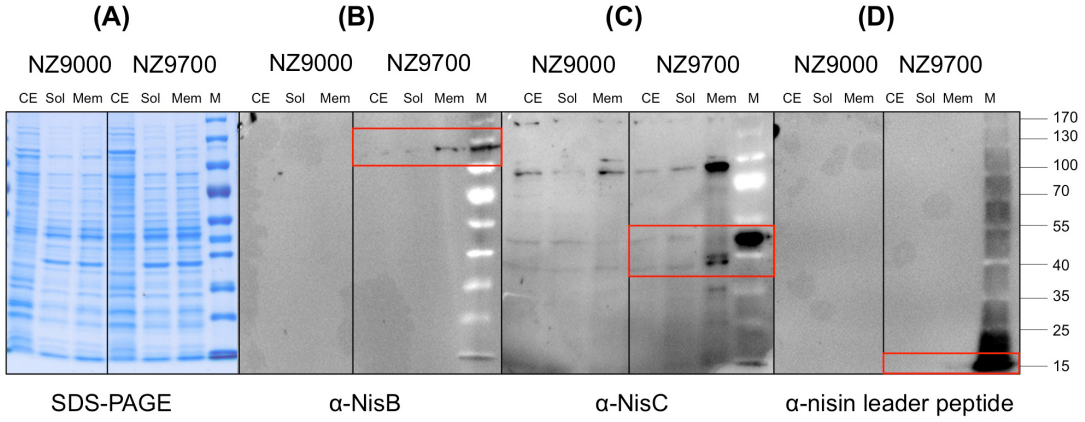


Figure 17: **Localization study of NisB, NisC and the precursor peptide in a chromosomal encoded nisin producing strain**. NZ9000[152] is a non nisin producing strain. The NZ9700[151] contains all genes of the nisin operon including the maturation machinery *nisABTCP*. Both strains were grown in the presence of minimal amounts of nisin (1 ng / mL). As the nisin operon is induced by its own synthesized mature peptide[121]. After harvesting, the cells were lysed and the crude extract (CE) was centrifugated to separate the cytosolic enzymes (Sol) from the membrane fraction. The resulted membrane fraction was washed two times and the gained membrane vesicles are shown in the Mem fraction. All fractions obtained from both species were analyzed via (A) SDS-PAGE[153] and immunoblotting[154] using antibodies against NisB (B), NisC (C) and the nisin leader peptide (D). The red boxes indicate the regions in which the corresponding protein is present / absent. All antibodies used were kindly provided by Dr. Moll, Lanthipep, Groningen and prepared as described in Khusainov *et al.* 2011[130].

The membrane localization of NisB and NisC was also observed in this study (Figure 17). These data support the membrane association of NisB in a nisin producer strain NZ9700 (Figure 17 B) but signals in the fraction for cytosolic proteins were also visible. In the amino acid sequence of NisB, a transmembrane helix is predicted[57,128,130]. Whether the membrane association of NisB depends on the presence of the mentioned putative transmembrane helix is currently not known. The data obtained in this thesis suggest that NisB does not contain a classical transmembrane helix, since NisB was purified in a soluble and homogeneous state in aqueous buffers in the absence of detergents. This isolated NisB was biologically active as it was able to bind the nisin precursor peptide (chapter 3.4 and also shown by[57,130,142,155]). Therefore, NisB might be either associated to the cytoplasmic membrane or localized at the membrane indirectly caused by an interaction with a membrane component, for example with the translocase NisT.

Many studies aimed to determine the stoichiometry of the functional modification complex, so far without any stringent results[58,130]. *In vitro* NisB was shown to assemble as a homodimer (chapter 3.4 and Khusainov *et al.*[130]), NisC was found to be a stable monomer (chapter 3.5 and Li *et al.*[61]). Regarding the ABC transporter NisT, little is known about the oligomeric state. In respect to detailed studies with other ABC transporters[156,157,158,159], the half-size transporter NisT might form a functional dimer. Yeast-two-hybrid studies support this postulate[58]. Based on the available data a stoichiometry between NisB, NisC and NisT might be 2:1:2 (see Figure 18).

In 1996, the first study analyzed the stoichiometry of a potential complex of NisT, NisB, NisC and the nisin precursor peptide and a stoichiometry of 2:1:2:1 was proposed[58]. Studies with the homolog subtilin maturation system revealed a different result[160]. Subtilin comprises the highest sequence identity to nisin (57 %[7]) and the maturation complex was proposed to be a modification tunnel composed of 2:2:2:1 (2x SpaB, 2xSpaC, 2xSpaT and one subtilin molecule)[160]. The difference towards the data obtained in this study might result from the different experimental set-up and additionally only protein domains were analyzed[58].
Our *in vitro* studies (chapter 3.4 and 3.5) regarding NisB and NisC support the 2:1 stoichiometry demonstrated for the nisin modification machinery in chapter 3.7. Here, a functional homodimer of NisB was binding one molecule of the nisin precursor peptide as demonstrated by SPR studies (chapter 3.4). Additionally, a NisC monomer bound specifically one nisin precursor peptide as shown by ITC experiments (chapter 3.5). In chapter 3.7 the assembly of a supramolecular modification machinery consisting of NisB, NisC and the unmodified precursor peptide was presented. Here for the first time, a complex of NisB, NisC and the nisin precursor peptide was obtained and characterized *in vitro*. The complex was visualized by analytical SEC and MALS-SEC

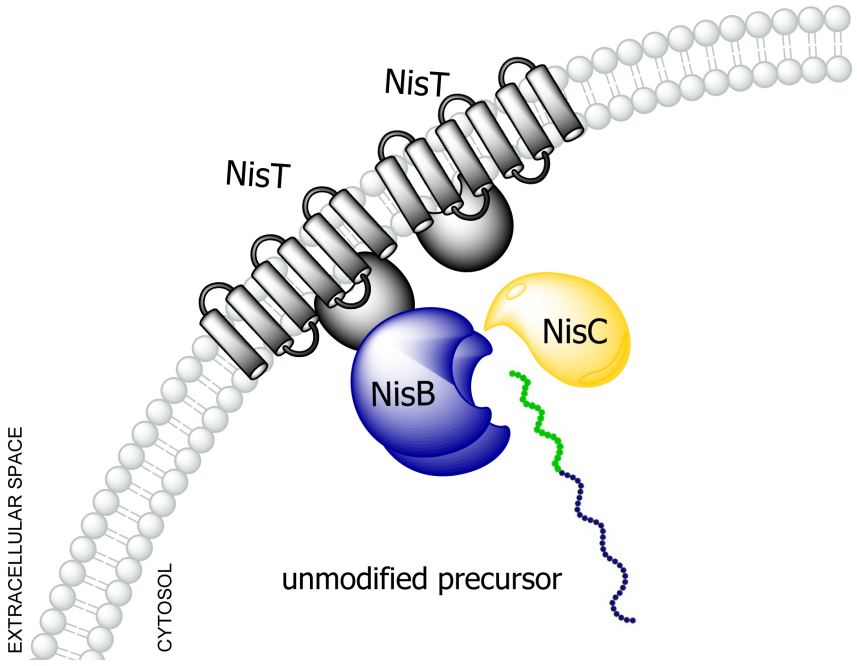


Figure 18: **Proposed stoichiometry of the nisin modification complex members**. Based on this study, NisB (blue) is suggested to be functional as dimer (chapter 3.4 and 3.7). NisB is interacting with a monomer of NisC (yellow - chapter 3.5 and 3.7) and a monomer of the unmodified precursor peptide. In respect to the dimer of NisT (black) the complex comprises a stoichiometry of 2:1:2:1 (2xNisB, 1xNisC, 2xNisT and one molecule of the nisin precursor peptide). Created with CS BioDraw Ultra 12.0.

data, which confirmed a stoichiometry of 2:1:1 between NisB, NisC and the nisin precursor peptide. However these data should be supported by further experiments. Importantly, the assembly of the modification complex is initiated specifically by the unmodified precursor peptide.

### 4.3.2 Activation upon substrate binding

The unmodified precursor peptide is responsible for the assembly of the modification complex *in vitro* (chapter 3.7), especially the leader peptide comprises an important role in the complex formation in lanthipeptides[60,82,89,155,161].

The concept of 'leader activation' was investigated first for the class II lanthipeptide lacticin 481[60]. Here, the corresponding modification enzyme LctM partially dehydrated lacticin 481 in absence of the leader peptide[161]. The dehydration activity could be significantly increased by the presence of an *in trans* expressed leader peptide suggesting a stimulation of LctM activity by the leader peptide[89].

Similar results were shown for NisB and the nisin precursor peptide[155]. The leader peptide was expressed separately from the core peptide. This *in trans* expressed leader peptide stimulated the dehydration activity of NisB. The maximal activity of NisB,

however, was only observed when the leader peptide was covalently bound to the core peptide[155]. This observation might be explained by data obtained in this thesis in respect to the *in vitro* assembly of the modification complex (chapter 3.7). Here, a modification complex of NisB and NisC was only detectable in presence of the unmodified precursor peptide. In contrast, the isolated leader peptide did not induce complex formation. The same holds true for an *in trans* expressed leader peptide in combination with an unmodified core peptide. Apparently, the presence of the unmodified core peptide covalently linked to the leader peptide ensured the maximum modification efficiency by stabilizing a modification complex consisting of NisB, NisC and the nisin precursor peptide. The detailed binding events between the modification enzymes NisB, NisC and the nisin precursor peptide will be discussed in the following two sections.

### 4.3.3 Association of the complex - specific interaction sites between NisB, NisC and the leader peptide

For all lanthipeptide modification enzymes an interaction with the leader peptide of the corresponding lanthipeptide precursor could be demonstrated[33,162]. However, it is currently not known, how specificity towards the dedicated enzymes is realized.
Chapter 3.4 deals with this question and revealed a conserved motif within the nisin leader peptide involved in the interaction with NisB. The highly conserved -FNLD- box (Figure 19) represents the essential recognition motif for NisB. Mutational studies with a fourfold alanine mutation of the -FNLD- box disrupted any interaction with NisB (chapter 3.4). These results are in-line with *in vivo* studies that demonstrated the absence of any dehydrations in the nisin core peptide after alanine mutations in the -FNLD- box[92]. This validates that NisB is not able to introduce any dehydrations to the -AAAA- mutant *in vivo*.
Furthermore, an interaction of NisC with the precursor peptide was described in chapter 3.5. The performed ITC experiments also revealed a specific interaction of NisC and the leader peptide. The determined binding affinity of $\sim 3.8\,\mu M$ for the isolated leader peptide was in the same range as the affinity for the precursor peptide ($K_D$ of $\sim 2\,\mu M$, chapter 3.5). This interaction was also based on the -FNLD- box, which is comparable to NisB. Obviously, NisC and NisB both are binding to the same motif, suggesting a competition between both modification enzymes for the substrate. A similar conclusion was put forward by Khusainov *et al.*. Here, NisC and NisB competed for the nisin precursor peptide in co-elution experiments[130,142].
The influence of any single amino acid residue of the -FNLD- box on the interaction with NisC was investigated in more detail in chapter 3.5 (Figure 19). These studies

demonstrated the importance of the phenylalanine (F) and leucine (L) residues for recognition of the nisin precursor peptide by NisC. Their mutation to alanine residues completely abolished the interaction with NisC. In contrast, the binding affinitiy towards NisC was constant for the mutation regarding asparagine to alanine ($N_{-17}A$) and only slightly decreased by the mutation aspartate to alanine ($D_{-15}A$). Therefore, the binding motif for NisC was defined as -FxLx- (chapter 3.5).

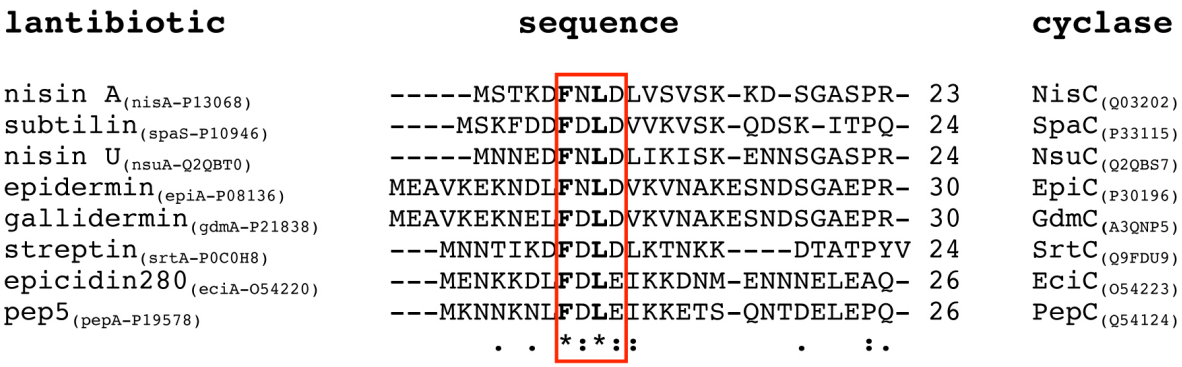


Figure 19: **Sequence alignment of the -FNLD- box.** Created with ClustalW2[93] based on the amino acid sequence taken from the UniProtKB database. The UniProtKB entry numbers are shown in parentheses. The asterisk (*) represents single fully conserved amino acids. Conserved residues are highlighted by (:) and similarity by (.). Taken from chapter 3.5.

The LanC family enzymes were suggested to interact with the leader peptide of class I lanthipeptides (chapter 1.3.2.1)[33,56,92,162]. The results of this thesis (chapter 3.5) support this suggestion. The sequence identity of the lanthipeptides is relatively high, for example 57 % for nisin and subtilin[7] (Figure 19). Additionally, all members of the LanC family cluster in a clade of phylogenetic calculations[56]. Based on these findings, the general recognition site for class I cyclases might be defined in more detail to -FxLx- (chapter 3.5 and Figure 19).

The detailed analysis of the -FNLD- box revealed an alternating hydrophobic / hydrophilic repeat (Figure 19). The residues F and L contain hydrophobic side chains whereas the residues N and D comprise hydrophilic side chains. NisC recognizes the hydrophobic residues, suggesting that NisB interacts with the hydrophilic residues. Furthermore, in class II and class III lanthipeptides the primary sequence of leader peptides often contain secondary structural elements, like $\alpha$-helices[77,85,98,163]. For labyrinthopeptin, a member of class III lanthipeptides[85], an $\alpha$-helical structure was proposed bioinformatically and was shown to be present by CD spectroscopy. The $\alpha$-helical domain was located at the highly conserved -ILELQ- motif (see chapter 1.3.3) which is responsible for the interaction with the modification enzyme LabKC[85]. In class II lanthipeptides lacticin 481[77] and nukacin ISK-1[98] the $\alpha$-helical character of

the leader peptide was already validated and is essential for the interaction between the specific protease[77,98,163] and the precursor peptide. These studies highlight that an $\alpha$-helical character of the leader peptide represents already a general recognition motif for modification enzymes of other lanthipeptide classes[33,162].
In the next step, the structure of the nisin leader peptide was analyzed by nuclear magnetic resonance (NMR) spectroscopy to elucidate the structural character of the -FNLD- box. These experiments are still in the evaluation process, although a first interpretation of the NMR spectra revealed that an $\alpha$-helix might by formed by the amino acids surrounding the -FNLD- box (Figure 20).

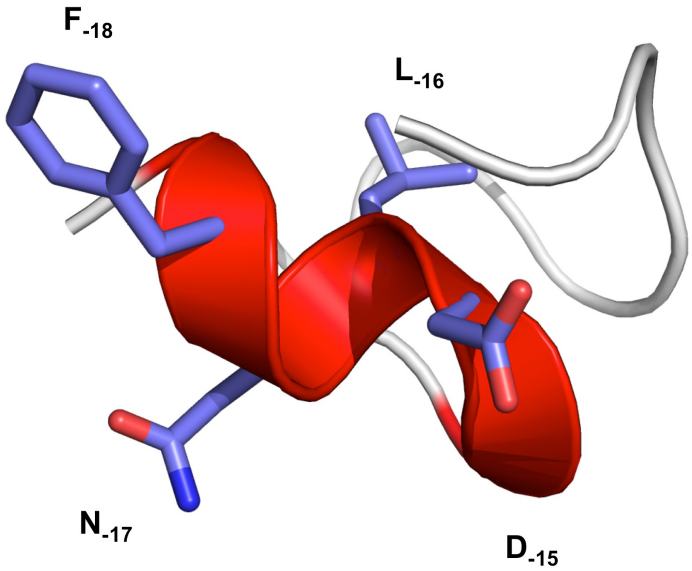


Figure 20: **NMR analysis of the nisin leader peptide**. The predicted helix is shown in red. The highly conserved residues of the -FNLD- box are shown in blue sticks (phenylalanine $F_{-18}$, asparagine $N_{-17}$, leucine $L_{-16}$ and aspartate $D_{-15}$). The structure was analyzed in cooperation with Dr. Hartmann and Prof. Dr. Willbold of the Institute of Complex Systems, Strukturbiochemie (ICS-6) at the Forschungszentrum Jülich.

This result is not in-line with previous studies[111,164]. In these studies, $\alpha$-helices were neither found by CD spectroscopy nor by NMR spectroscopy[111]. But, the addition of trifluorethanol - a reagent that is well known to stimulate the formation of $\alpha$-helices - induced the formation of an $\alpha$-helical structure in the nisin leader peptide[164]. However, the studies in this thesis were performed at pH 6.0 instead of pH 3.5[111], which might explain the structural differences. Further studies will be performed and evaluated to verify the data obtained in this thesis. These current data suggest the presence of a structural motif as binding motif for both, NisB and NisC, comparable to class II and class III leader peptides.

### 4.3.4 Directionality of the post-translational modifications introduced by NisB and NisC

Binding of the leader peptide by NisB and NisC is a prerequisite prior to the formation of PTMs in the core peptide of nisin. In a second or simultaneous step, NisB and NisC modify the core peptide.
Although, the X-ray structure of NisC was solved in 2006[61], the interaction sites for NisB or the nisin precursor peptide were not identified. The active center of NisC was located on the surface of the $\alpha$-barrel (see Figure 14)[61]. It is not covered or obviously closed, therefore it is proposed that NisC might form a 'cover' to the 'base' NisB and by binding to NisB it 'closes' a reaction cavity. One NisC monomer was interacting with one nisin precursor peptide as shown in chapter 3.5. Furthermore, one NisB dimer formed a functional unit, which interacted with one nisin precursor peptide as well, demonstrated in chapter 3.4. So far, neither the structure of the active site in NisB was elucidated nor the essential amino acids were identified. Therefore the molecular properties of the active site are speculative.
Furthermore, the biochemical properties and the directionality of the modification process is only partially understood. All amino acids residues that are target sites for PTMs within the core peptide are presented in Figure 21. Remarkably, the analysis of the serine residue at position 29 revealed that this serine residue was never dehydrated[129]. This observation gave further insights into the directionality and the cooperativity between NisB and NisC during the maturation process. Lubelski *et al.*[129] elucidated that ring D and E, which are localized N-terminal to the serine at position 29, caused a sterical hindrance for NisB. Therefore it is postulated that the presence of the rings cause the missing dehydration of the serine at position 29, consequently these rings must be formed prior to NisB reaches position 29 to dehydrate the serine[129].

When NisC was inactivated by mutating the active center, NisB was able to dehydrate the serine residue at position 29 in absence of the rings D and E[129,130]. This suggests an alternating catalysis of the dehydration and cyclization reaction.
To strengthen this result, several serine residues were introduced at positions C-terminal to cysteines which are involved in lanthionine ring formation (see position 12 next to ring B, highlighted in Figure 21)[129]. Especially the serine and threonine residues located in the N-terminal part of the core peptide demonstrated the directionality of the maturation process. Within the first two (methyl)lanthionine rings A and B, four potential dehydration targets can be found. The threonine residue at position 8, as well as the serine residue at position 29, is C-terminal located to a (methyl)lanthionine ring (ring A). In comparison to serine residue at position 29, which

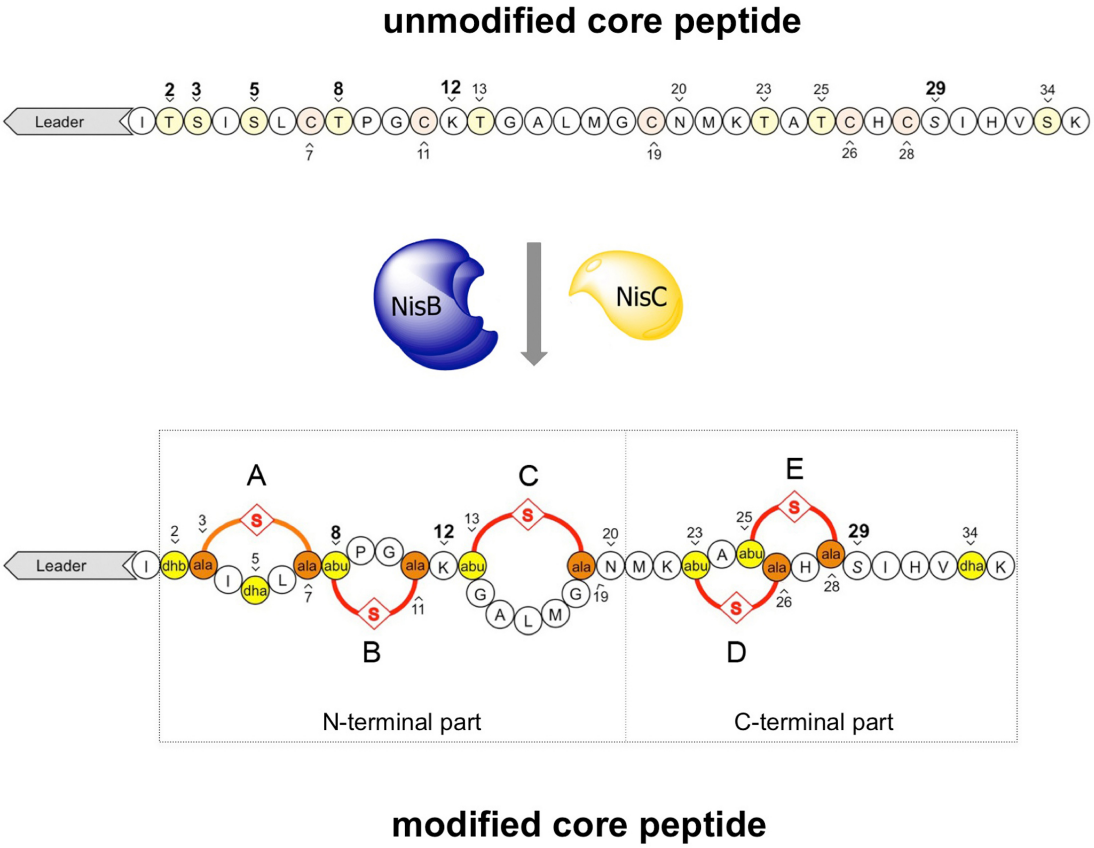


Figure 21: **PTMs in the core peptide of nisin.** At the top of the figure the unmodified core peptide is shown. Serine and threonine residues are highlighted in light yellow and cysteines in light red. Important amino acids are highlighted with bold numbers. After modification by the enzymes NisB (shown in blue) and NisC (shown in yellow) the modified core peptide is presented at the bottom of the figure. A represents the lanthionine ring and B-E the four methyllanthionine rings.

is a potential dehydration target and was never dehydrated, the threonine at position 8 was always dehydrated. The threonine residue at position 8 functions as anchor for ring B and thereby needs to be dehydrated, otherwise ring B is never formed. Therefore it can be concluded, that NisB dehydrates the serine and threonine residues at position 1, 2, 5 and 8 prior to NisC starts installing the first two (methyl)lanthionine rings A and B. Another example supports the alternation between NisB and NisC. The serine at position 12 was never dehydrated by NisB in the presence of NisC, which is a clear evidence that NisC installs ring B prior to NisB reaches position 12[129]. The results regarding position 12 indicate that ring B is formed before NisB is able to dehydrate this residue[129].

Therefore, the formation of ring A and B might cause a movement of the precursor peptide that directs a shift of the molecule by eighth residues. Afterwards, NisB might dehydrate residues 13 - 20, within these residues the next serine residue at position 13 is now accessible for NisB. Thereupon, NisC links the serine residue at position 13

to the cysteine residue at position 19, which represents the largest ring (ring C) that covers five residues in-between. This voluminous ring might cause a conformational change releasing the leader, which moves in direction of NisT. Whether NisT is taking over the precursor peptide and starts pulling or the precursor peptide moves actively through the active site is discussed in more detail in the following section. Thereby, the N-terminal part of the core peptide is released from the active cavity and the C-terminal part is bound[129].

Khusainov *et al.* provided the experimental proof for this hypothesis[155]. A leader-less, unmodified core peptide was expressed in presence of NisB, NisC and NisT. The dehydration pattern of the core peptide were analyzed and revealed 0 - 5 dehydrations[155], which is in-line with the data presented in chapter 3.4 that demonstrate an interaction between NisB and the core peptide. Afterwards the leader peptide was expressed *in trans* to the core peptide in combination with NisB, NisC and NisT[155]. The dehydration pattern revealed only one single dehydration species with 5 dehydrations[155]. Therefore it can be concluded that the *in trans* expressed leader peptide is on the one hand increasing the modification efficiency and on the other hand only the serine and threonine residues in the N-terminal part of the core peptide can be dehydrated. These five dehydratable residues are located at position 1, 2, 5, 8 and 13, highlighted in Figure 21. Therefore, the covalently bound leader directs the C-terminal part of the nisin core peptide in the active cavity after the N-terminal part is modified.
This points towards a three-step formation of the (methyl)lanthionine rings. First, the rings A and B are formed followed by the cyclization of ring C that represents the last modification in the N-terminus of the core peptide. Finally, the rings D and E are installed, after a consequent movement of the precursor peptide.

## Movement of the precursor peptide

Apparently, the leader peptide needs to be released during or at least after the modification process because the modified precursor peptide is translocated to the extracellular space. To elucidate, whether the leader peptide is released during maturation or an unknown release factor causes the release from the active cavity, Rink *et al.*[97] enlarged the core peptide of nisin at the C-terminus[97]. The 14 N-terminal amino acid residues of the core peptide (shown in Figure 21) were fused to the C-terminus of nisin. The nisin molecule was enlarged to 47 amino acids including five more dehydratable residues (positions 36, 37, 39, 42 and 47) and introducing two more putative (methyl)lanthionine rings. The secreted peptides were analyzed by mass spectrometry which identified 11 and 12 dehydrations[97]. This analysis highlights, that

even position 47 was modified, which suggests that the modification enzymes are not regulated by the C-terminal part of the core peptide. NisB and NisC modify extended polypeptide chains proposing that no release factor is included in the substrate.
How the movement of the substrate through the active cavity is realized *in vivo* is still speculative. Whether NisT, which apparently interacts with the leader peptide, takes over and starts pulling or the precursor peptide moves through the modification complex remains unclear[21,33,92,129,155].
Finally, to ensure a complete modification of the core peptide, the alternating maturation process requires a cross-talk between all involved enzymes. NisB and NisC need to interact with NisT ensuring that only complete modified precursor peptides are allowed to be secreted.

## NisB the central player?

The key regulator in the nisin system might be NisB as proposed also by Khusainov *et al.*[130]. NisB was the main component that was attached to the precursor peptide in respect to the amount of NisC. This fact was also described by Siegers *et al.*, who proposed that NisC was only loosely attached to the maturation complex[58]. Furthermore, the deletion of *nisB* had a strong adverse effect on the nisin production[66]. By deleting *nisB* the precursor production was reduced to 2% in respect the *nisC* deletion leaded to a precursor production level of 30 %[66].
This might be explained by a specific interaction of NisB and NisT which controls the nisin maturation process and ensures the complete modification of the nisin precursor peptide. The data presented in chapter 3.4 support this point of view. The affinity of NisB and the unmodified precursor peptide showed a $K_D$ of $\sim 1\,\mu M$, the dehydrated a $K_D$ of $\sim 0.3\,\mu M$ and the final modified precursor peptide a $K_D$ of $\sim 10\,\mu M$. Despite, the dehydrated precursor peptide is the product of the dehydration reactions, here the affinity was maximized. This points towards a product inhibition of the dehydration reaction. Whether this inhibition represents the key regulation step in this process needs to be clarified. However, at the moment it is believed, that when the serine and threonine residues are dehydrated, NisB will not release the substrate because the (methyl)lanthionine rings are still missing. After (methyl)lanthionine ring formation the affinity of NisB drops by a factor of 30. Therefore it can be speculated, that the affinity shift does not arise from the summation of all five (methyl)lanthionine rings, but is caused by single, specific rings. This suggests that each ring located at the end of one part of the core peptide causes a drop in affinity. Therefore, not only the closure of ring E but also the formation of ring C might cause a change in the interaction of NisB and the precursor peptide. These results supports that NisB might represent the key-regulator which fulfills a control mechanism.

### The role of NisC

In chapter 3.5, NisC was demonstrated to interact with the -FxLx- motif of the nisin leader peptide. The *in vitro* interaction of NisC and the precursor peptide was directed by the leader peptide and this affinity was not influenced by the modification state of the core peptide. However, an interaction of NisC with the core peptide is a prerequisite for cyclization. Maybe the regulator NisB presents the core peptide to NisC. This suggestion is supported by *in vivo* studies of Khusainov *et al.*[130,142]. A complex of NisC and the precursor peptide was only found in the presence of NisB[130,142].
Based on the crystal structure of NisC, an external SH2-like domain was identified[61]. Such domains are typically involved in protein-protein interactions, especially for the interaction with protein-tyrosine kinases and phosphatases[165]. The SH2-like domain might also represent an interaction site for the glutamylation (kinase-like) domain of NisB (chapter 1.4.2). However, this assumption needs to be investigated in the future, for example by co-elution experiments of NisB, NisC and the nisin precursor peptide in the absence and presence of the SH2-like domain of NisC and the glutamylation domain of NisB, respectively.
NisC comprises more than the catalytic possibility to install the (methyl)lanthionine rings which is shown by the observation that the presence of NisC increased the catalytical efficiency of NisB[55,129] and NisT[166] *in vivo*, even when a catalytical inactive species of NisC with a mutated zinc-finger motif was used[55,129,130,166].
All these data point towards additional functions of NisC within the maturation process of nisin beside cyclization. NisC directs a correct coordination of the core peptide in the active cavity and increases the efficient interplay of the three modification enzymes NisB, NisC and NisT.

## 4.3.5 Proteolytic cleavage of the leader peptide and release of mature nisin

The final step of the nisin biosynthesis is the proteolytic cleavage of the leader peptide from the modified core peptide[7,67,92]. This cleavage occurs post transport at the outer leaflet of the cytoplasmic membrane. Since little is currently known about NisP, an aim of this thesis was the *in vitro* characterization. For the first time, NisP could be purified in an active state and information regarding the substrate specificity were obtained (chapter 3.6).
This chapter represents the first isolation of a class I lanthipeptide protease. The biochemical characterization of the isolated protease revealed a stable monomer in aqueous solution. Furthermore, the enzyme activity could be demonstrated *in vitro*

by incubating the purified modified precursor peptide with the purified protease NisP. By analyzing the reaction mixture with RP-HPLC, the cleavage reaction was observed and the cleavage products, the leader peptide and mature nisin, were examined by mass spectrometric analysis (chapter 3.6). Furthermore, this chapter represents the first collection of kinetic data describing the proteolytic cleavage process for class I lanthipeptides (chapter 3.6).
In this chapter, NisP was shown to cleave the precursor peptide independently of the maturation state of the core peptide. Therefore, NisP did not seem to contain a binding site within the core peptide of nisin *in vitro*. These results are in clear contrast to previous *in vivo* studies[64]. Here, purified nisin precursor peptides, that differ in the three available maturation states, were incubated with NisP expressing *L. lactis* cells. Only the modified precursor peptide was cleaved. Therefore, the (methyl)lanthionine rings were regarded as recognition site for NisP, most importantly the first ring. However, similar proteases of class II and class III lanthipeptides specifically recognize the corresponding precursor peptide via the leader peptide[77,85,98]. The data obtained in chapter 3.6 support the suggestion that NisP as well as the other modification enzymes is recognizing at least parts of the nisin leader peptide, which highlights the essential role of the nisin leader peptide also involved in the last step of the nisin maturation process.

All available data clearly indicate that the maturation, including modification, transport and processing of nisin, is a complex process that is highly cooperative and the presence of a single enzyme influences the activities of the other enzymes. The involved enzymes, NisB, NisC and NisP could be produced and analyzed *in vitro*. The data of this thesis revealed important insights into the nisin maturation process and the main conclusions of this work are summarized in the next chapter, which proposes a model of the nisin maturation process.

## 4.4 Model for the nisin maturation complex

In the following section the results obtained in the preceding chapters are summarized in a model for the nisin maturation complex represented in Figure 22.

1. Translation of the unmodified precursor peptide by the ribosomes (not shown). A preformed complex consisting of dimeric NisT, dimeric NisB and monomeric NisC is localized in an inactive state at the cytoplasmic membrane, which is only formed in the presence of NisT and a membrane compartment, Figure 22 A.

2. The initial process is directed by binding of the precursor peptide to NisB. In detail, the leader peptide is binding to NisB mediated by the highly conserved -FNLD- box. Upon binding of the leader peptide to NisB the enzyme NisC is binding to the leader peptide as well, precisely to the F and the L of the -FNLD- box. NisB and NisC share a binding site for the leader peptide, furthermore they create a large active cavity to modify the core peptide. This cavity comprises one domain responsible for the dehydration reaction provided by NisB and one cyclization domain facilitated by NisC, Figure 22 B.

3. NisB catalyzes the dehydration of four amino acid residues starting at the N-terminus of the core peptide, Thr1, Ser2, Ser5 and Thr8. This highlights, that NisB dehydrates four amino acid residues consecutively prior to NisC catalyzes the formation of the first two rings, Figure 22 C and D.

4. Formation of ring C is the last PTM in the N-terminal part of the core peptide (blue in Figure 22) and causes a conformational change. Ring C contains the largest number of residues (five) between the Dhb and the Cys and creates a voluminous sterical change in the nisin molecule. Thereby, the affinity of NisB for the precursor peptide is decreased, and the leader peptide is released from NisB, NisC and binds to NisT, Figure 22 D and E.

5. The C-terminal region of the core peptide (shown in red in Figure 22) reaches the active site of NisB. Residues 20 - 28 are dehydrated and cyclized. The formation of the relatively large, intertwined rings D and E cause a conformational change and the affinity of NisB is further decreased, Figure 22 E. Afterwards, the remaining amino acid residues are dehydrated, Figure 22 F.

6. NisT catalyses the transport of the modified precursor peptide after the entire modifications are completed or simultaneously to the PTMs, Figure 22 G.

7. NisP cleaves the transported and modified precursor peptide and produces the final, mature nisin. This cleavage occurs independently of the modification and transport process, Figure 22 H.

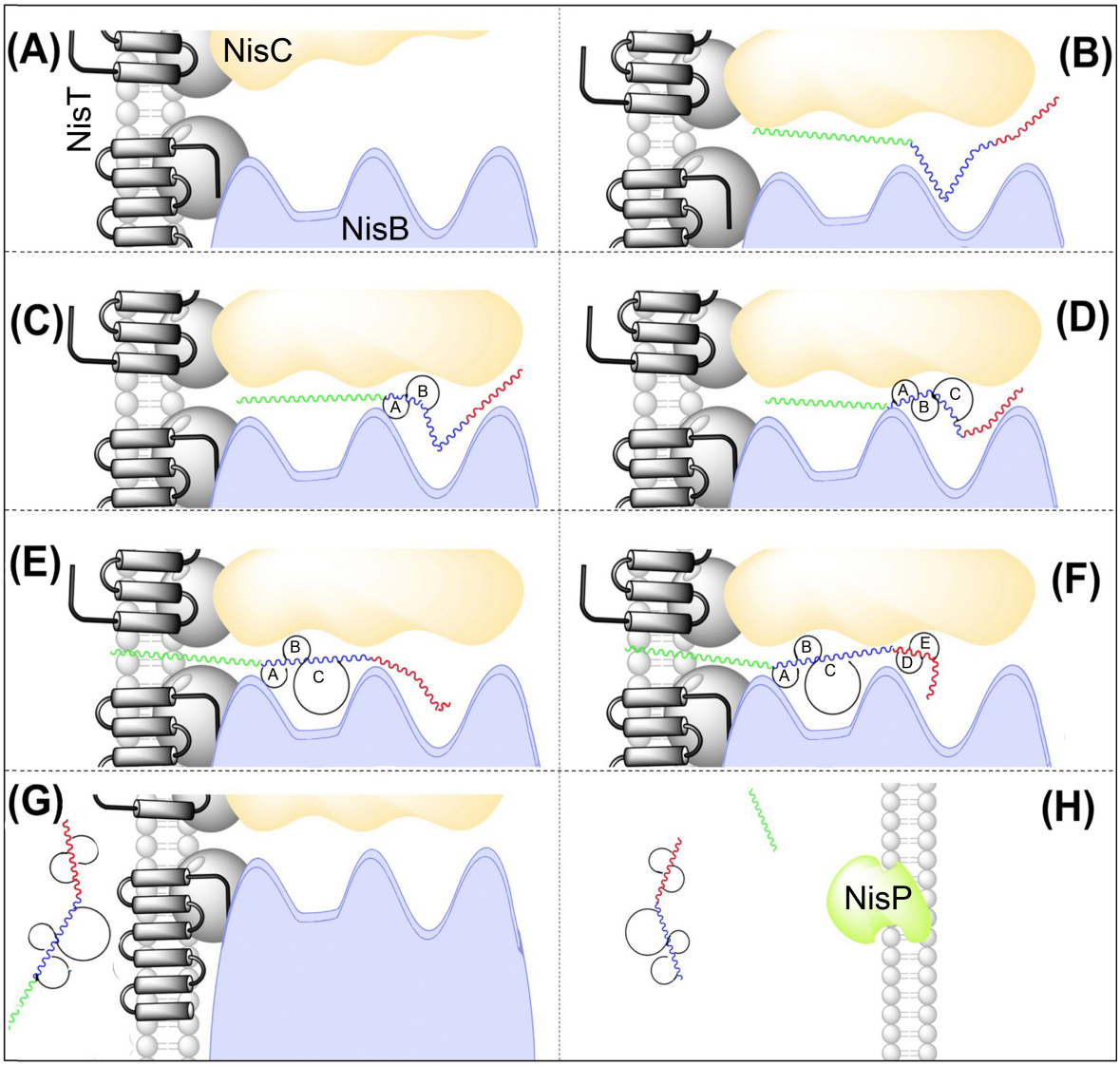


Figure 22: **Proposed model of the nisin maturation machinery of *L. lactis*.** Detailed descriptions are given in chapter 4.4. **(A)** The maturation complex is assembled at the cytoplasmic membrane in an inactive state. **(B)** The nisin precursor peptide binds with the leader peptide (green) and the core peptide (blue and red) to NisB (light blue) and NisC (yellow). This binding event activates the maturation complex. **(C)** After dehydration of all residues up to position 8, NisC installs the rings A and B. **(D)** NisC installs ring C, the last modification in the N-terminal part of the core peptide (blue). **(E)** The formation of ring C causes presumably a conformational change of the core peptide, which results in release of the leader peptide and a movement of the precursor peptide towards NisT (black). Afterwards the C-terminal part of the core peptide (red) reaches the active site of NisB and NisC. **(F)** The formation of the rings within the C-terminal part cause a second conformational change being responsible for the release of the modified core peptide from the modification machinery. **(G)** The modified precursor peptide is secreted by NisT across the membrane to the surrounding. **(H)** Finally, NisP separates proteolytically the leader peptide of the modified core peptide and mature nisin is released.

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# 6 *Curriculum vitae*

## Personal Data

| | |
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| Date of Birth | born in Dormagen, 05.03.1984 |
| Nationality | German |

## Work Experience

| | |
|---|---|
| since 04/2010 | **Ph.D. student** at the Institute of Biochemistry, Heinrich-Heine University Düsseldorf with Prof. Dr. Lutz Schmitt. Member of the Interdisciplinary Graduate and Research Academy Düsseldorf (iGrad). |
| 04/2007 - 10/2008 | **Research Internship** at the Max-Planck Institut für Eisenforschung, Düsseldorf. Member of the Electrochemistry and Corrosion Group with Prof. Dr. Walter Hassel, Department of Interface Chemistry and Surface Engineering |

## Academic Education

| | |
|---|---|
| 10/2003 - 03/2010 | **Diploma in Chemistry**, Heinrich-Heine University Düsseldorf with the Diploma thesis: 'Charakterisierung der Nisin Modifikationsmaschinerie' at the Institute of Biochemistry with Prof. Dr. Lutz Schmitt. |

## School Education

| | |
|---|---|
| 1994 - 2003 | **Abitur**, Norbert Gymnasium Knechtsteden, Dormagen. |

## Workshops and Conferences

02/2013 **Gordon research Seminar and Conference**: Antimicrobial Peptides. *Poster:* Nisin Modification Machinery competes for the FNLD-box, Ventura, California, USA

02/2012 **BAMP 2012: International scientific conference**: Bacteriocins and antimicrobial peptides. *Oral Presentation:* Easy and rapid Purification of highly active Nisin, Kosice, Slovakia

09/2011 **Molecular Life Science 2011**: International Symposium of the German Society for Biochemistry and Molecular Biology (GBM). *Poster:* Substrate Recognition and Specificity of the NisB Protein, Frankfurt, Germany

09/2011 **Isothermal Titration Calorimetry (ITC) Workshop and Conference**: Pushing the Limits of Microcalorimetry, Kaiserslautern, Germany

08/2010 **Wyatt Eclipse Workshop in combination with multi-angle light scattering (MALS) Training**, Dernbach, Germany

## Publications

1 **Abts, A.**, Montalban-Lopez, M., Kuipers, O. P., Smits, S. H. and Schmitt, L. (2013) NisC binds the FxLx motif of the nisin leader peptide. *Biochemistry*, **52 (32)**, 5387-5395, doi:10.1021/bi4008116.

2 Alkhatib, Z., **Abts, A.**, Mavaro, A., Schmitt, L. and Smits, S. H. (2012) Lantibiotics: how do producers become self-protected? *J Biotechnol* **159**, 145-154, doi:10.1016/j.jbiotec.2012.01.032.

3 **Abts, A.**, Schwarz, C., Tschapek, B., Smits, S. H. and Schmitt, L. (2012) Rational and Irrational Approaches to Convince a Protein to Crystallize in Modern Aspects of Bulk Crystal and Thin Film Preparation (eds N. Kolesnikov, E. Borisenko) Ch. 22, 497-528 (*InTech*).

4 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, doi:10.1074/jbc.M111.263210.

5 **Abts, A.**, Mavaro, A., Stindt, J., Bakkes, P. J., Metzger, S., Driessen, A. J., Smits, S. H. and Schmitt, L. (2011) Easy and rapid purification of highly active nisin. *Int J Pept* 2011, 175145, doi:10.1155/2011/175145.

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6 Thakur, H. C., Singh, M., Nagel-Steger, L., Prumbaum, D., Kalawy Fansa, E., Gremer, L., Ezzahoini, H., **Abts, A.**, Schmitt, L., Raunser, S., Ahmadian, M. R. and Piekorz, R. P. (2013) Role of centrosomal adaptor proteins of the TACC family in the regulation of microtubule dynamics during mitotic cell division. *Biol Chem*, **394(11)**, 1411-1423, doi:10.1515/hsz-2013-0184.

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7 Fenster, C., Smith, A. J., **Abts, A.**, Milenkovic, S. and Hassel, A. W. (2008) Single tungsten nanowires as pH sensitive electrodes. *Electrochem Commun* **10**, 1125-1128, doi:Doi 10.1016/J.Elecom.2008.05.008.

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Maschine! Warte bis du erst den Rest richtig kennenlernst! ;) ) Especially I want to thank Sakshi for the last year! I really enjoyed the nice time with you, convincing your protein to be stable. It was a pleasure to help you with your protein purification! Nisin proteins keep their secrets very efficient and you will have to fight for every small step but I am sure that you will finally succeed!
I wish the whole nisin group all the best!

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Mein allergrößter Dank gilt allerdings Maike!

Vielen, vielen Dank mein _ _ _ _ _ _ _ i ;) !

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# 8 Erklärung zur Promotion

Ich versichere an Eides Statt, dass die Dissertation von mir selbstständig und ohne unzulässige fremde Hilfe unter Beachtung der "Grundsätze zur Sicherung guter wissenschaftlicher Praxis" an der Heinrich-Heine-Universität Düsseldorf erstellt worden ist. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf,

(André Abts)

*Hinten kackt die Ente*
(Danke Coach!)