Combinatorial approaches for introducing additional modifications into lantibiotics
Mu, Dongdong

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Chapter 4

Lantibiotic reductase LtnJ substrate selectivity assessed with a collection of nisin derivatives as substrates

Abstract

Lantibiotics are potent antimicrobial peptides characterized by the presence of dehydrated amino acids, dehydroalanine and dehydrobutyrine, and (methyl)lanthionine rings. In addition to these posttranslational modifications, some lantibiotics exhibit additional modifications that usually confer increased biological activity or stability to the peptide. LtnJ is a reductase responsible for the introduction of D-alanine in the lantibiotic lacticin 3147. The conversion of L-serine into D-alanine requires dehydroalanine as substrate, which is produced \textit{in vivo} by the dehydration of serine by a lantibiotic dehydratase, \textit{i.e.} LanB or LanM. In this work we probe the substrate specificity of LtnJ using a system that combines the nisin modification machinery (dehydratase, cyclase and transporter) and the stereospecific reductase, LtnJ, in \textit{Lactococcus lactis}. We also describe an improvement in the production yield of this system by inserting a putative attenuator from the nisin biosynthesis gene cluster in front of the \textit{ltnJ} gene. In order to clarify the sequence selectivity of LtnJ, peptides composed of truncated nisin and different mutated C-terminal tails were designed and co-expressed with LtnJ and the nisin biosynthetic machinery. In these tails, serine was flanked by diverse amino acids to determine the influence of the surrounding residues in the reaction. LtnJ successfully hydrogenated peptides when hydrophobic residues (Leu, Ile, Phe and Ala) were flanking the intermediate dehydroalanine, while those in which dehydroalanine was flanked by one or two polar residues (Ser, Thr, Glu, Lys, Asn) or Gly were either less prone to be modified by LtnJ or not modified at all. Moreover, our results showed that dehydrobutyrine cannot serve as a substrate for LtnJ.
Introduction

Since the discovery of penicillin by Alexander Fleming in 1928, antibiotics have saved the lives of countless people. Regrettably, due to abuse and overuse, increasing resistance to antibiotics has been found among pathogenic bacteria, which has led to an urgent need for new antimicrobial compounds (1-4). Lanthipeptides, defined as posttranslationally modified peptides containing lanthionine and/or methyllanthionine ring(s), are a type of ribosomal peptides produced by many Gram-positive bacteria (5-6). They can be subdivided into 4 different classes based on the enzyme(s) that catalyze(s) the formation of lanthionine residues (6). Some of them (i.e. classes I and II) show antimicrobial activity and are referred to as lantibiotics (7).

The capability of lantibiotics to inhibit the growth of obstinate pathogens, including multidrug-resistant bacteria such as methicillin-resistant Staphylococcus aureus, vancomycin-resistant enterococci, and oxacillin-resistant Gram-positive organisms, makes them very promising candidates for future antimicrobial development (7-9). So far, only few lantibiotics have been commercially applied or are under development for medical use in spite of their promising properties (7,10). Nisin, the model Class I lantibiotic produced by the Gram-positive bacterium Lactococcus lactis, has been applied in industry as a food preservative for decades without triggering effective resistance in pathogens (11-12); Duramycin, a class II lantibiotic, is in phase II clinical trial and was proven to be safe and effective for the symptomatic treatment of cystic fibrosis by inhalation (13). Another class II lantibiotic, deoxyactagardine B (NVB302, Novacta Biosystems Limited), is undergoing a phase I clinical trial as a drug candidate for the treatment of Clostridium difficile infections (14). Furthermore, recent research has shown that some Class III lanthipeptides have unexpected bioactivity to relieve neuropathic pain (15) and as antiviral compounds (16).

Besides the common (methyl)lanthionine, more than fifteen extra structures have been unveiled in lanthipeptides playing a significant role on the antimicrobial activity, resistance against proteases and/or physico-chemical resistance (12, 17-20). For instance, D-alanine was found within two lantibiotics: lactocin S and the two-component lantibiotic lacticin 3147 (Figure 1) (21-23). According to the work of Cotter and his co-workers, the replacements of D-alanine by other residues (L-alanine, L-threonine, glycine, and L-valine) in lacticin 3147 caused a dramatic decrease in activity against L. lactis HP (24). Additionally, a gene designated as ltnJ was predicted to encode a protein with significant similarity to zinc-
dependent alcohol dehydrogenases and NAD(P)H-dependent quinone oxidoreductases of the zinc containing alcohol dehydrogenase superfamily (24). LtnJ was shown to be responsible for the formation of D-alanines in lacticin 3147 with dehydroalanine (Dha) as intermediate (24). This dehydroalanine reductase activity to generate D-alanine has been observed among some homologues of LtnJ, like SacJ from *S. aureus* C55 and PenN from *Pediococcus pentosaceus* FBB61 (25). In fact, D-alanine in lacticin 3147 can be formed by some homologues of LtnJ, although low efficiency was observed when compared to LtnJ (25).

Figure 1. Structure of lactocin S, lacticin 3147 (peptides LtnA1 and LtnA2), nisin, nisin with designed C-terminal tail and nisin(insS29KIH; K34L). The residues involved in the formation of (methyl)lanthionine are in yellow, D-alanines are depicted shaded into blue, Dha and Dhb are colored into green and purple, respectively. The pathway for D-alanine conversion from serine is depicted.

It has been shown that the nisin biosynthetic machinery possesses high substrate tolerance, and can modify diverse peptides fused to the nisin leader peptide, not only related to lanthipeptides but also to unrelated peptide sequences (26-30). Previously, we successfully introduced D-alanine into nisin by expressing LtnJ together with the nisin biosynthetic machinery in a dual-plasmid system in *L. lactis* (18). The Dha at position 5 of nisin has been demonstrated to be modified by LtnJ, while the other Dha at position 33 is most likely not (18).
In this study nisin was used as a model peptide to investigate if the surrounding residues of Dha affect the conversion of Dha into D-alanine. Therefore a mutant oligopeptide tail (AAIS^{26}LALTIK) was fused at the C-terminus of truncated nisin (i.e. NisA(Δ23-34)) generating a peptide designated as NisAtail (Figure 1). Another 18 variants were designed to probe the influence of flanking residues in the conversion of Dha into D-alanine by LtnJ. We show that the polar or hydrophobic nature of flanking amino acids in the substrate plays a vital role on this reaction.

**Materials and Methods**

**Bacterial strains, plasmids, and growth conditions**

Strains and vectors used in this work are listed in Table 1. *L. lactis* strains were cultured in M17 medium supplemented with 0.5% glucose (GM17) at 30 °C for genetic manipulation or in a minimal expression medium (MEM) for protein expression and purification assays (29). Chloramphenicol and/or erythromycin were used at 5 μg/ml when necessary.

**Table 1. Strains and vectors used in this work**

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<tr>
<th>Name</th>
<th>Characteristic</th>
<th>Reference</th>
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<td><strong>Strains</strong></td>
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<td>pepN::nisRK; Expression host strain</td>
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<td><em>L. lactis</em> NZ9700</td>
<td>Nisin-producing transconjugant containing Tn5276; Used for cloning of the attenuator</td>
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<td><strong>Vectors</strong></td>
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<td>CmR, nisA; expression of nisin</td>
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pNZ-S26T  Derivative of pNZ-nisA; ΔnisA; S26T  This work
pNZ-nisin(Δ30-34)-KIHIHVSL  Derivative of pNZ-nisA; ΔnisA; nisin(Δ30-34)-KIHIHVSL  This work
pNZ-nisA-T-lnJ  Derivative of pNZ-nisA-T-lnJ; ΔnisA; S26T  This work
pNZ-S26T-lnJ  Derivative of pNZ-nisA-T-lnJ; ΔnisA; S26T  This work
pNZ-S26T-lnJ  Derivative of pNZ-nisA-T-lnJ; ΔnisA; S26T  This work
pNZ-L27K-T-lnJ  Derivative of pNZ-nisA-T-lnJ; ΔnisA; L27K  This work
pNZ-I25K-T-lnJ  Derivative of pNZ-nisA-T-lnJ; ΔnisA; I25K  This work
pNZ-L27E-T-lnJ  Derivative of pNZ-nisA-T-lnJ; ΔnisA; L27E  This work
pNZ-I25E-T-lnJ  Derivative of pNZ-nisA-T-lnJ; ΔnisA; I25E  This work
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pNZ-I25F-T-lnJ  Derivative of pNZ-nisA-T-lnJ; ΔnisA; I25F  This work
pNZ-L27N-T-lnJ  Derivative of pNZ-nisA-T-lnJ; ΔnisA; L27N  This work
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pNZ-I25G-T-lnJ  Derivative of pNZ-nisA-T-lnJ; ΔnisA; I25G  This work
pNZ-L27A-T-lnJ  Derivative of pNZ-nisA-T-lnJ; ΔnisA; L27A  This work
pNZ-I25A-T-lnJ  Derivative of pNZ-nisA-T-lnJ; ΔnisA; I25A  This work
pNZ-S26T-T-lnJ  Derivative of pNZ-nisA-T-lnJ; ΔnisA; S26T  This work
pNZ-nisin(Δ30-34)-KIHIHVSL-T-lnJ  Derivative of pNZ-nisA-T-lnJ; ΔnisA; nisin(Δ30-34)-KIHIHVSL  This work

*CmR: chloramphenicol resistance. EryR: erythromycin resistance

Molecular Cloning

Molecular cloning techniques were performed according to Sambrook and Russell (31). Preparation of competent cells and transformation was performed as described previously (32). Fast digest restriction enzymes and ligase were supplied by Fermentas and used according to the manufacturer.

Construction of recombinant vectors

Plasmid isolation was performed with the plasmid DNA extraction kit (Roche). The transcriptional attenuator region between nisA and nisB was amplified from genomic DNA of L. lactis NZ9700 using primers P-for-nisA-T and P-rev-nisA-T-2nd. After digestion using BglIII and KpnI, it was ligated into pNZ-nisA-lnJ, amplified using the primers P-for-kpnI-lnJ and P-rev-bglIII-Cmr to insert the KpnI site resulting in the plasmid pNZ-nisA-T-lnJ. Primers
LtnJ substrate selectivity assessed with nisin derivatives as substrates

P-for-ALTIK and P-rev(01-19) (Table 2) were designed for the construction of the 19 nisin analogues (Table 3; Figure 1). Round PCR was performed with pNZ-nisA as the template, primer P-for-ALTIK as forward primer and P-rev(01-19) as reverse primers to create 19 different derivatives of pNZ-nisA (Table 1). These resulting pNZ-nisA derivatives were digested by HindIII and XhoI, and ligated with a fragment containing the transcriptional attenuator and ltnJ to generate 19 different derivatives of pNZ-nisA-T-ltnJ (Table 1). This fragment was amplified with the primers P-for-HindIII-T-ltnJ and P-ltnJ-rev-XhoI (Table 2) using pNZ-nisA-T-ltnJ (18). By applying two set of round PCR on pNZ-nisA with two pairs of primers, P-for-K34L/P-rev-K34L and P-for-S29insKIH/P-rev-S29insKIH (Table 2), pNZ-nisin(Δ30-34)-KIHIHVSL was created (Table 1). Similarly, pNZ-nisin(Δ30-34)-KIHIHVSL-T-ltnJ was obtained with pNZ-nisA-T-ltnJ as template (Table 1).

All the constructs were verified by sequencing.

Table 2. Primers used in this study

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<th>Primer</th>
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<td>Construction of nisin(Δ30-34)-KIHIHVSL</td>
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Protein expression and purification

The pNZ-derivative vectors containing the mutant structural gene with or without \( ltnJ \) were transformed into NZ9000 (pIL3EryBTC). The expression assays were performed as described previously (29). Briefly, MEM medium was inoculated at 2% from an overnight culture of the producer strain grown in GM17. When the fresh culture reached an OD (600 nm) of 0.4–0.6, nisin was added at a final concentration of 5 ng/ml. Cells were harvested after 2 h of induction by centrifugation at 4 °C for 10 min at 5000 rpm, and the supernatant was kept for the isolation of the peptides. The cell-free supernatant was mixed at a ratio 1:1 with a 100 mM lactic acid solution and applied to a 5 ml HiTrap SP-Sepharose (GE Healthcare) column for cationic exchange chromatography. Bound peptides were washed with 50 mM lactic acid pH 4.0 and eluted with 50 mM lactic acid, 1 M NaCl pH 4.0. Subsequently, a PD-10 desalting column (GE Healthcare) was used to desalt the sample following the provider instructions. The production was evaluated by tricine-SDS-PAGE as described before (33).

The peptides were purified to homogeneity by reverse phase high performance liquid chromatography (RP-HPLC). A Jupiter 4 μm Proteo 90 Å column, 250 x 4.6 mm (Phenomenex) was used. Solvents for RP-HPLC were solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile). Following a 10 min washing step with 20% solvent B, a gradient of 27.5-47.5% of solvent B over 35 min was executed at a flow rate of 1 ml/min. Peptides were detected measuring the absorbance at 205 nm. The production level of the different mutants was assessed determining the area of the peaks.

LC-MS/MS and LC-MS

The PD10-desalted peptide was digested with 1 μl of 1 mg/ml sequencing-grade trypsin in 5mM CaCl\(_2\) and 50mM Tris (pH 6.8) solution at 37 °C for 16 hours.

After trypsin digestion, the proteolytic mix was injected into an LC-MS/MS system Ultimate 3000 nano (Dionex, Amsterdam, The Netherlands) in-line connected to an LTQ Orbitrap XL (Thermo Fisher Scientific, Bremen, Germany). The sample mixture was loaded
LtnJ substrate selectivity assessed with nisin derivatives as substrates

on a trapping column (Acclaim PepMap C18 5 mm length x 300 μm I.D., 5 μM particle size, 100 Å porosity, Dionex) and washed. After 3 minutes the mixture was separated using a 42 minutes linear gradient from 95% solvent C (0.1% formic acid) to 90% solvent D (0.1% formic acid in acetonitrile) at a flow rate of 250 nl/min. The mass spectrometer was operated in data dependent mode, automatically switching between MS and MS/MS acquisition for the five most abundant doubly and triply charged ions with a minimal signal of 2500 in a given MS spectrum. Full scan MS spectra were acquired from m/z 300-1300 in the Orbitrap at a target value of 1E6 with a resolution of 60,000. The five most intense ions which met the set criteria were then isolated for fragmentation in the linear ion trap, with a dynamic exclusion of 10 seconds. Peptides were fragmented after filling the ion trap at a target value of 1E4 ion counts. Data were analyzed using Peak6 software (www.bioinfor.com).

The intact peptides were analyzed using the same setup with the following modifications: the column temperature was 50 ºC and after a 5 minutes wash, a 44 minutes gradient from 10% to 40% of solvent D at a flow rate of 300 nl/min was performed. The mass range was set to 400-1900 Da. Data was processed using Xtract software (Thermo). Arbitrary abundance of peptide fragments of interest was calculated on the basis of proportional relationship between the area of the chromatographic peak and the corresponding peptide. The conversion rate of LtnJ was obtained by dividing the arbitrary abundance of tail peptide containing D-alanine26 by the summed arbitrary abundance of tail peptides containing D-alanine26 and Dha26 respectively.

Results

Improvement of the production of nisin and its derivatives

The production level of nisin decreased 3-fold when co-expressed with LtnJ using L. lactis NZ9000 (pIL3EryBTC; pNZ-nisA-ltnJ) as a host strain compared to the strain L. lactis NZ9000 (pIL3EryBTC; pNZ-nisA) (Figure 2). An attenuator structure located downstream of nisA gene in the nisin biosynthesis cluster (11) was cloned into pNZ-nisA-ltnJ between the genes nisA and ltnJ resulting in pNZ-nisA-T-ltnJ. This regulatory element ensures high transcription of the structural gene but a reduced transcription level of ltnJ from the nisA promoter. The peptides produced in the culture supernatant of NZ9000 (pIL3EryBTC; pNZ-nisA), NZ9000 (pIL3EryBTC; pNZ-nisA-ltnJ) and NZ9000 (pIL3EryBTC; pNZ-nisA-T-ltnJ)
were purified by cationic exchange chromatography. Tricine-SDS-PAGE was used to visualize the semipurified peptide. The peptides from the three strains migrated as a band of approximately 6 KDa, which is in line with the theoretic mass of the nisin precursor (Figure 2A). Peptides from NZ9000 (pIL3EryBTC; pNZ-nisA), NZ9000 (pIL3EryBTC; pNZ-nisA-ltnJ) and NZ9000 (pIL3EryBTC; pNZ-nisA-T-ltnJ) were further purified via RP-HPLC. An intense peak with a retention time of 24.6 min was observed in all the strains (Figure 2B). The purified peptide from NZ9000 (pIL3EryBTC; pNZ-nisA-T-ltnJ) was collected for a more accurate characterization by LC-MS. We could observe a peak corresponding to a peptide with a positive mass shift of 2 Da compared to the peptide from NZ9000 (pIL3EryBTC; pNZ-nisA) indicating that one Dha was converted into D-alanine by LtnJ (Supplementary Figure S1). These results are consistent with the previous results when the attenuator was not present in the construction (18). Comparing the area under the curve of the different peaks from nisin and its derivatives on the HPLC profile, we observed that the production level of nisin produced by NZ9000 (pIL3EryBTC; pNZ-nisA-T-ltnJ) was 2-fold higher compared to NZ9000 (pIL3EryBTC; pNZ-nisA-ltnJ), although it was still lower than nisin without the addition of LtnJ to the system (Figure 2B).

Figure 2. A: Tricine-SDS-PAGE analysis after cationic exchange chromatographic purification of nisin with/without D-Alanine. M, Molecular weight marker; I, Protein purified from NZ9000(pIL3EryBTC; pNZ-nisA); II, Protein purified from NZ9000(pIL3EryBTC; pNZ-nisA-ltnJ); III, Protein purified from NZ9000(pIL3EryBTC; pNZ-nisA-T-ltnJ). B: HPLC chromatographic profile of nisin with/without D-alanine. Blue, Protein purified from NZ9000(pIL3EryBTC; pNZ-nisA); Red, Protein purified from NZ9000(pIL3EryBTC; pNZ-nisA-ltnJ); Green, Protein purified from NZ9000(pIL3EryBTC; pNZ-nisA-T-ltnJ).
LtnJ can modify the C-terminus of peptides

D-alanine has been observed at the N-terminal part of the two peptides, LtnA1 and LtnA2, that compose lacticin 3147 (22). Recently van Heel et al. showed that when LtnJ was expressed with the nisin machinery, the Dha at position 5 in nisin could be modified into D-alanine while Dha at position 33 was most likely unmodified (18). To investigate if the function of LtnJ is position-dependent, plasmids pNZ-NisAtail and pNZ-NisAtail-T-ltnJ producing mutants of nisin in the absence or presence of LtnJ, respectively, were constructed (Table 1). In these mutants, the C-terminal region of nisin (residues 23-34) was replaced by a linear peptide sequence (AAIS<sup>26</sup>LALTIK) (Table 3) containing a serine that could be dehydrated more effectively than Ser33 in nisin (34). Additionally, the lack of cysteine residues in this fragment (and therefore the absence of lanthionine rings) facilitates MS/MS fragmentation and characterization. Peptides purified by cationic exchange chromatography from both NZ9000 (pIL3EryBTC; pNZ-NisAtail) and NZ9000 (pIL3EryBTC; pNZ-NisAtail-T-ltnJ) were digested by trypsin and analyzed by LC-MS/MS. The conversion of Dha to D-alanine was only detected in the case of NZ9000 (pIL3EryBTC; pNZ-NisAtail-T-ltnJ) compared to the control strain NZ9000 (pIL3EryBTC; pNZ-NisAtail), as expected (Figure 3). De novo sequencing data of the C-terminus of NisAtail obtained following trypsin digestion showed that when LtnJ was expressed 70% of Dha26 was hydrogenated into D-alanine in the fully dehydrated peptide. This percentage is 57% for peptides where Thr30 was not dehydrated (Figure 4). Our results prove that LtnJ can modify Dha when it is located at the C-terminal part of the precursor peptide.

Table 3. Core peptide sequences of NisA-mutant peptides<sup>a</sup>

<table>
<thead>
<tr>
<th>No.</th>
<th>Substrate</th>
<th>Sequence of core peptide</th>
<th>Maximum dehydration on C-terminus detected</th>
<th>Conversion of D-Ala from Dha26</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>NisAtail</td>
<td>ITSISLCTPGCKTGALMGCMKAAI S LALTIK</td>
<td>2 (S26, T30)</td>
<td>++</td>
</tr>
<tr>
<td>02</td>
<td>S26insS</td>
<td>ITSISLCTPGCKTGALMGCMKAAI S S LALTIK</td>
<td>3 (S26, S27, T31)</td>
<td>-</td>
</tr>
<tr>
<td>03</td>
<td>S26insSS</td>
<td>ITSISLCTPGCKTGALMGCMKAAI S S S LALTIK</td>
<td>3 (S26, S27, T32)</td>
<td>-</td>
</tr>
<tr>
<td>04</td>
<td>S26insT</td>
<td>ITSISLCTPGCKTGALMGCMKAAI S LALTIK</td>
<td>3 (S26, T27, T31)</td>
<td>-</td>
</tr>
</tbody>
</table>
Flanking residues of the substrate Dha affect the modification by LtnJ

To explore the role of flanking residues of Dha during biosynthesis 17 nisin mutant peptides (Peptide No. 02-18; Figure 1; Table 3) were designed and constructed similarly to the mutant NisAtail described before. Thus, we replaced the C-terminus of nisin by a peptide sequence (AAIS\textsuperscript{26}LALT\textsuperscript{IK}) in which flanking amino acids of Ser26 (i.e. I25 and L27) were mutated into serine, threonine, lysine, glutamic acid, phenylalanine, asparagine, glycine or alanine (Table 3). The modification extent of this polypeptide tail in nisin was thoroughly studied by LC-MS/MS for all these mutants. According to the LC-MS/MS profile, conversion into D-alanine from Ser26 was observed in L27F, I25F, L27N, L27A, I25A-L27A and I25A (Table 3; Figure S2-S7). The conversion rate of Dha into D-alanine is 71% (L27F), 67%
(I25F), 13% (L27N), 54% (L27A) and 71% (I25A), respectively, when compared to the fully dehydrated peptide (Figure 4 and Figure S3). In the cases where the threonine at position 30 is not dehydrated, the conversion of Dha into D-alanine is not detected for L27F, I25F and hardly detected for L27N and drops to 13% for L27A and 35% for I25A (Figure 4; Figure S8 for L27A). An exception is that I25A-L27A was modified by LtnJ only when threonine was not dehydrated with a conversion rate of 16%.

Figure 3. MS/MS spectra of C-terminal tryptic fragment of NisAtail when LtnJ was present showing the occurrence of D-alanine conversion. Expected masses for y and b ions are listed above and below the peptide sequence, respectively. Ions that were positively identified in the MS/MS spectrum are highlighted in blue (b ions) or red (y ions). D-alanine converted from serine is colored in blue; Threonine and dehydrated threonine are labelled in purple and green respectively. A, Partly dehydrated peptide; B, Fully dehydrated peptide.
In mutant peptides (No. 02-08, 13-15) where Ser26 was neighbored by small polar amino acids (Ser/Thr), charged residues (Lys/Glu) or glycine, hydrogenation was not detected (Table 3), although serine was dehydrated in all cases. A similar result is obtained when asparagine is present at the N-terminus of Ser26. On the other hand, a relatively low conversion rate by LtnJ was detected when asparagine is located C-terminally to Ser26 (Table 3; Figure 4). The presence of charges, either positive or negative, did not allow the conversion into D-alanine in spite of Dha being present. Similarly, the presence of dehydrated residues Dha and Dhb inhibited the hydrogenation of the adjacent Dha (Table 3).

Figure 4. Graphical representation of the conversion percentage of Ser26 flanked by different residues into D-alanine by LtnJ. Fully dehydrated: Both Ser26 and Thr30 were dehydrated. Partly dehydrated: Only Ser26 was dehydrated. A descending tendency of conversion percentage, (XSF>XSL>XSA) shows a preference for larger hydrophobic amino acids at the C-terminus of Ser26.

In order to study the modification of Ser33 in nisin, a mutant nisin(Δ30-34)-KIHIHVSL (Table 3; Figure 1) was designed in which the last residue Lys34 was mutated into Leu to favor the dehydratation by NisB and a Lys-Ile-His sequence was inserted downstream Ser29 offering a cleavage site for trypsin digestion. No 2 dalton shift was observed when LtnJ was coexpressed with nisin(Δ30-34)-KIHIHVSL (Table 3).
Dehydrobutyrine is not a substrate for LtnJ

The mutant S26T was designed to mimic the conditions in which Dha is converted into D-alanine and therefore have optimal conditions to investigate the conversion of Dhb into D-aminobutyrate (Table 3). Although Thr at positions 26 and 30 were dehydrate by NisB providing a possible substrate for LtnJ (*i.e.* Dhb), D-aminobutyrate was not detected in any case (Table 3). This result indicated that the additional methyl group in Dhb prevents the hydrogenation catalyzed by LtnJ.

Discussion

The combination of different posttranslational modification enzymes in peptide design and production is a promising approach (35). The success of these efforts depends to a great extent on the correct characterization of the enzymes that can be used (*e.g.* dependence on leader peptide recognition, cofactors, target sequence, substrate tolerance, etc.). In a previous study we demonstrated that the modification machinery of nisin can be expanded with additional posttranslational modification enzymes, in this case LtnJ and GdmD, which do not require their original leader peptide sequence for target recognition (18). However, we observed a reduced production of nisin in the presence of LtnJ (18). Therefore, we attempted to improve the production of the structural peptide. In diverse lantibiotic operons and other bacteriocin systems an inverted repeat sequence is observed between the structural gene and the next ORF within the same operon (36-38). By introducing an attenuator between *nisA* and *ltnJ* in our plug-and-play production system (18), the production level of the structural gene was improved more than 2-fold. These sequences may act as processing sites for the mRNA or as transcriptional attenuators which, in either case, ensure an appropriate ratio of enzymes and structural peptide. Additionally, the inverted repeats can affect the stability of the transcript as in the case of enterocin AS-48 (38) or Pep5 (39).

At present, an impressive number of different posttranslational modifications have been unveiled in lantibiotics (20). For example, aminovinyl-cysteine in gallidermin and epidermin, lysinoalanine in cinnamycin or N-terminal lactate formation in epilancin 15X increase the biological activity and/or constrain the structure of the lantibiotics in which they naturally appear. D-alanine, present in lacticin 3147, is one of the modifications that has been further investigated. Previous studies demonstrated the importance of D-alanine for the activity of
lactocin 3147 and identified LtnJ as the responsible reductase producing D-alanine by deletion-complementation experiments (24). As reported previously, when LtnJ was coexpressed with the model lantibiotic nisin, Dha33 within nisin is likely not modified by LtnJ, whereas Dha5 is partially reduced by LtnJ to form D-alanine (18). In the same article the hypothesis that amino acids flanking Dha may affect the function of LtnJ was raised. In order to clarify the specificity of LtnJ and to extend the applicability of this enzyme as a general tool for the designed modification of peptides, its selectivity was investigated in this study. For this purpose, we designed a set of mutants of nisin where the C-terminus was replaced by a linear polypeptide tail (AAIS26LALTIK) that facilitates de novo sequencing by LC-MS/MS due to the absence of lanthionine rings in this region. In this tail the substrate Dha (i.e. Ser26) is flanked by different residues. In our experiment, the serine in position 26 was dehydrated by NisB in all cases rendering Dha26 (Dha27 for S26insS and S26insSS). However, only in some specific cases, Dha26 was converted into the hydrogenated form D-alanine. The high ratio of hydrogenated peptide compared to the corresponding mutant in NisAtail, L27F, I25F, L27A and I25A emphasizes the importance of hydrophobic residues flanking the intermediate Dha for the function of LtnJ. The conversion observed in L27N (although in low amounts) and not detectable in I25N indicates a clear preference for an N-terminal hydrophobic residue flanking Dha. This phenomenon suggests that the active site in LtnJ is most likely surrounded by nonpolar residues. Additionally, the presence of D-alanine at the C-terminus of the designed mutants demonstrates that the activity of LtnJ is not restricted to the N-terminal part of the peptide, although in its natural substrates D-alanine occurs only in that part of the peptide (Figure 1). A recently published research showed that the reductase CrnJ from *Carnobacterium maltaromaticum* C2 could modify dehydroamino acids into D-amino acids at very different locations in the core peptide of carnolysin (40). Whether LtnJ can do so as well remains to be proved. The failure of modification by LtnJ in the tail of nisin(Δ30-34)-KIHHVSL was probably due to the negative charge of carboxyl group of the C-terminal leucine.

In both peptides LtnA1 and LtnA2, the natural substrates of LtnJ, there are Dhb residues that are not converted into a reduced form (24). This is also the case for lactocin S (21). In order to determine whether this effect is caused by the different location and flanking residues or if Dhb cannot serve as a substrate for LtnJ at all, we engineered a mutant where Dha was replaced by Dhb at the same location and surrounded by the same environment. Our data also exclude Dhb as a substrate for LtnJ and indicate that LtnJ is a Dha-specific hydrogenase as
LtnJ substrate selectivity assessed with nisin derivatives as substrates previously suggested (24). Nevertheless, the reductase CrnJ from the carnolysin gene cluster can reduce Dhb flanked by hydrophobic amino acids and render D-aminobutyrate which points at a broader substrate tolerance for CrnJ compared to LtnJ (40).

An interesting observation is that among these modified mutants (NisAtail, L27F, I25F, L27A, I25A-L27A and I25A) where Ser26 was flanked by hydrophobic amino acids, LtnJ preferred to modify Dha with a larger residue (Leu, Phe) on its C-terminal side over a smaller one (Ala).

In this study, we shed light on the specificity of LtnJ in terms of peptide sequence. We show the promiscuity of this leader-independent reductase when hydrophobic residues are neighboring Dha, which is its only substrate as Dhb is never found to be modified by LtnJ. We also show that the reductase action can also take place at the C-terminal region of lantibiotics, although this has not been found in nature. Our data provide a deeper insight into the use of LtnJ as a tool to engineering designed peptides in future research.

References


LtnJ substrate selectivity assessed with nisin derivatives as substrates

Supplementary Information

Figure S1. MS spectrum of peptide purified from NZ9000(pIL3EryBTC; pNZ-nisA-T-LtnJ) showing that part of the peptide was modified by LtnJ. A, mature nisin precursor(MNP); B, mature nisin precursor modified by LtnJ.

Figure S2. MS/MS spectrum and amino acid sequence of the C-terminus of L27F released after tryptic digestion containing D-alanine. Expected masses for y and b ions are listed above and below the peptide sequence respectively.
Figure S3. MS/MS spectrum and amino acid sequence of the C-terminus of I25F released after tryptic digestion containing D-alanine. Expected masses for y and b ions are listed above and below the peptide sequence respectively.

Figure S4. MS/MS spectrum and amino acid sequence of the C-terminus of L27N released after tryptic digestion containing D-alanine. Expected masses for y and b ions are listed above and below the peptide sequence respectively.
LtnJ substrate selectivity assessed with nisin derivatives as substrates

Figure S5. MS/MS spectrum and amino acid sequence of the C-terminus of L27A released after tryptic digestion containing D-alanine. Expected masses for y and b ions are listed above and below the peptide sequence respectively. A, Partly dehydrated peptide; B, Fully dehydrated peptide.

Figure S6. MS/MS spectrum and amino acid sequence of the C-terminus of I25A-L27A released after tryptic digestion containing D-alanine. Expected masses for y and b ions are listed above and below the peptide sequence respectively.
Figure S7. MS/MS spectrum and amino acid sequence of the C-terminus of I25A released after tryptic digestion containing D-alanine. Expected masses for y and b ions are listed above and below the peptide sequence respectively. A, Partly dehydrated peptide; B, Fully dehydrated peptide.
Figure S8. Comparison of the LC-MS profile of L27A tail (AAIS^{26}AALT^{30}IK) expressed with/without LtnJ when threonine30 was not dehydrated. The M/H^2+ data showed 2 mass units difference between fragments of the peaks. By dividing the arbitrary area of peak of modified tail by the summed arbitrary area of two peaks representing either the modified or non-modified, the conversion rate of LtnJ, in this case, was calculated to be 13% (see also figure 4).