Combinatorial approaches for introducing additional modifications into lantibiotics
Mu, Dongdong

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Chapter 5

The cinnamycin aspartic acid hydroxylase CinX is able to modify class I lantibiotics

Dongdong Mu, Manuel Montalbán-López, Xin Zhao and Oscar P. Kuipers in preparation
Abstract

Lantibiotics are ribosomally synthesized antimicrobial peptides secreted by various Gram-positive bacteria. They undergo extensive posttranslational modifications that lead to the insertion of dehydrated serine/threonine residues and lanthionine/methyllanthionine rings. Some lantibiotics display additional modifications. CinX was proven to be the modification enzyme responsible for the in vitro hydroxylation of aspartic acid in cinnamycin, a class II lantibiotic from Streptomyces cinnamoneus DSM 40005. Here, we investigate the functionality of CinX in vivo on other substrates by coexpressing it with nisin mutants (class I lantibiotic), harboring engineered aspartic acid and the nisin modification machinery. Both electrospray mass spectrometry and tandem mass spectrometry data demonstrate the ability of CinX to hydroxylate aspartic acid in vivo in designed nisin derivatives, in a leader-independent way.
Introduction

The hydroxylation of aspartic acid residues is widespread in nature. Different stereoisomers of hydroxyaspartate have been found in various organisms as part of a metabolic pathway, in different receptors or in cytotoxic compounds (1-4). It has been reported that human plasma protein C contains hydroxylated aspartic acid in its epidermal growth factor domain although a clear function of this modified residue remains unknown. It has been hypothesized that hydroxylation of aspartic acid might relate to the negative regulation of fucosylation of the epidermal growth factor domain of human plasma protein C (5). In addition, hydroxylated aspartic acid is an inhibitor of the mouse serine racemase that catalyzes the formation of D-serine, an N-methyl-D-aspartate receptor agonist (6). Interestingly, hydroxylated aspartic acid itself can inhibit several fungi and bacteria (7).

The hydroxylation of aspartic acid has been identified in the lanthipeptides duramycin and cinnamycin (Figure 1) (8, 9). Lanthipeptides are a specific kind of ribosomally synthesized peptides produced by Gram-positive bacteria (10, 11). They all undergo extensive posttranslational modifications, such as the dehydration of serine and threonine that results in dehydroalanine (Dha) and dehydrobutyryl (Dhb), respectively. Lanthionine and methyl-lanthionine rings are formed by the addition of a thiol group of a cysteine to Dha and Dhb, respectively (10, 11). Lanthipeptides can be subdivided in 4 different classes based on the enzyme(s) that catalyze(s) the formation of (methyl)lanthionine rings (11). Those lanthipeptides that display antimicrobial activity are referred to as lantibiotics, and belong to classes I and II. In class I, serine and threonine residues are dehydrated by LanB and cyclized by LanC, while in class II both steps are catalyzed by a bifunctional enzyme LanM (11). Moreover, in class II lantibiotics, the Cys residue to form a thioether bond can be located N-terminally from the cognate dehydrated residue, while this never occurs in class I lantibiotics.

Cinnamycin is a class II lantibiotic with potential medical applications (12-14). In addition to lanthionine, the uncommon amino acids hydroxy aspartic acid and lysinoalanine are present in cinnamycin. Recently, Ökesli et al. investigated the maturation of cinnamycin (9). Besides the bifunctional lantibiotic synthetase CinM, Cinorf7 is responsible for the formation of lysinoalanine and CinX catalyzes the hydroxylation of aspartic acid (9). The lysinoalanine bridge linking the ε-amino group of lysine at position 19 and a serine residue at position 6 is essential for the activity of cinnamycin against Bacillus subtilis LH45 (9). CinX is an α-ketoglutarate/iron(II)-dependent hydroxylase that can hydroxylate the aspartic acid at position.
15 in cinnamycin yielding erythro-3-hydroxy-L-aspartic acid (9). The function of hydroxy aspartic acid in cinnamycin is binding the ammonium group of its target phosphatidylethanolamine (PE) (15, 16). CinX has been shown to act in a leader-independent fashion (9), which makes it a promising tool for peptide design.

Figure 1. Structure of cinnamycin, nisin, nisin mutants with designed aspartic acid residues and their modified precursor peptides. The residues involved in the formation of (methyl)lanthionine are yellow shaded. Lysinoalanine bridges are purple shaded. Hydroxylated aspartic acid residues are depicted in blue. Dha and Dhb are colored into green and purple, respectively, while the aspartic acid residues in N20D-M21G and nisin(Δ30-34)-KIHIHVDG appear in red.

In this study, we aim to extend the in vivo lantibiotic production system containing the modification enzymes of nisin (NisBTC), previously established by our group (17, 18), with CinX to probe its substrate tolerance in another class of lantibiotic. Two expression systems were developed, using Lactococcus lactis and Escherichia coli, respectively, for this goal. The data obtained show that CinX is compatible with class I modification enzymes (NisBC), it has a rather broad substrate tolerance and can hydroxylate aspartic acid at distinct positions of nisin, also at the C-terminal region, suggesting that this hydroxylase can be used as a tool for further peptide hypermodifications.

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
CinX is able to modify class I lantibiotics in a minimal expression medium (MEM) for peptide expression and purification assays (17). *E. coli* strains were cultured in LB medium at 37 °C, with vigorous shaking when inoculated in liquid medium. Chloramphenicol and/or erythromycin were used at 5 μg/ml for *L. lactis* when necessary, and ampicillin (100 μg/ml) and/or kanamycin (20 μg/ml) for *E. coli* when required.

**Molecular Cloning**

Molecular cloning techniques were performed according to Sambrook and Russell (19). Preparation of *L. lactis* competent cells and transformation was performed as described previously (20). Fast digest restriction enzymes and ligase were supplied by Thermo Fisher Scientific and used according to the manufacturer.

**Construction of recombinant vectors**

pNZ-derived plasmids: Plasmid isolation was performed with the plasmid DNA extraction kit (Roche). The gene *cinX*, including the ribosome binding site in front of *nisA* and an N-terminal 6-Histidine tag, was codon optimized for *L. lactis* and synthesized by Baseclear (Table S1). HindIII and KpnI were fused to the 5’-terminal end while XhoI and BamHI were fused to the 3’-terminus. pNZ-N20D-M21G was constructed by round PCR with the primers: P-for-N20D-M21G and P-rev-N20D-M21G (Table 2), using pNZ-nisA as template. The *cinX* gene was ligated into pNZ-N20D-M21G as a HindIII-XhoI fragment generating the vector designated as pNZ-N20D-M21G-cinX.

<table>
<thead>
<tr>
<th>Name</th>
<th>Characteristic</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. lactis</em> NZ9000</td>
<td>pepN::nisRK; Expression host strain</td>
<td>(21)</td>
</tr>
<tr>
<td><em>E. coli</em> BL21DE3</td>
<td>lacO, T7 promoter</td>
<td>NEB</td>
</tr>
<tr>
<td>Vectors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pL3EzyBTC</td>
<td>EzyR, nisBTC; modification and transport of lantibiotics</td>
<td>(18)</td>
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<tr>
<td>pNZ-nisA</td>
<td>CmR, nisA; expression of nisin</td>
<td>(18)</td>
</tr>
<tr>
<td>pNZ-N20D-M21G</td>
<td>CmR, ΔnisA, expression of N20D-M21G</td>
<td>This work</td>
</tr>
<tr>
<td>pNZ-N20D-M21G-cinX</td>
<td>CmR, ΔnisA, expression of N20D-M21G and 6His-CinX</td>
<td>This work</td>
</tr>
<tr>
<td>pRSF-Duet-nosCB</td>
<td>KanR, nosBC; modification of lantibiotics</td>
<td>Unpublished; Gift from Andrius Buivydas</td>
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<tr>
<td>pET-nisA</td>
<td>AmpR, nisA; expression of nisin</td>
<td>Lanthio Pharma</td>
</tr>
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<td>pET-N20D-M21G</td>
<td>AmpR, ΔnisA, expression of N20D-M21G</td>
<td>This work</td>
</tr>
<tr>
<td>pET-N20D-M21G-cinX</td>
<td>AmpR, ΔnisA, expression of N20D-M21G and CinX</td>
<td>This work</td>
</tr>
<tr>
<td>pET-S33D-K34G</td>
<td>AmpR, ΔnisA, expression of S33D-K34G</td>
<td>This work</td>
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</table>
Chapter 5  

<table>
<thead>
<tr>
<th>Construct</th>
<th>Description</th>
<th>Source</th>
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<tr>
<td>pET-S33D-K34G-cinX</td>
<td>AmpR, ΔnisA, expression of S33D-K34G and CinX</td>
<td>This work</td>
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<tr>
<td>pET-nisA(Δ30-34)-KIHIHVDG</td>
<td>AmpR, ΔnisA, expression of nisin(Δ30-34)-KIHIHVDG</td>
<td>This work</td>
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<tr>
<td>pET-nisA(Δ30-34)-KIHIHVDG-cinX</td>
<td>AmpR, ΔnisA, expression of nisin(Δ30-34)-KIHIHVDG and CinX</td>
<td>This work</td>
</tr>
<tr>
<td>pNZ-nisP</td>
<td>CmR, nisP; expression of NisP</td>
<td>Unpublished; Manuel Montalbán-López (22)</td>
</tr>
</tbody>
</table>

*pET*-derived plasmids: pET-N20D-M21G was constructed by round PCR with the primers P-for-N20D-M21G and P-rev-N20D-M21G (Table 2), and pET-nisA (kind gift from Lanthio Pharma) as template. In order to insert cinX into pET-N20D-M21G, another two PCRs were performed. First, primers P-for-rou-xhoi-pET and P-rev-rou-kpni-pET were used to insert the restriction sites XhoI and KpnI into pET-N20D-M21G. The same sites were fused to the two ends of the cinX gene with pNZ-N20D-M21G-cinX as template and primers P-for-kpni-T7RBS-cinX and P-rev-xhoi-cinX. In this way the ribosomal binding site and His-tag coding sequence at the N-terminus of cinX were replaced. By ligating these two amplicons as an XhoI-KpnI fragment, pET-N20D-M21G-cinX was generated.

The vector pET-S33D-K34G was constructed by round PCR with the primers P-for-S33D-K34G-pET and P-rev-S33D-K34G (Table 2), and pET-nisA as template. Similarly, the construction of pET-N20D-M21G-cinX, pET-S33D-K34G-cinX was done by inserting cinX gene into pET-S33D-K34G. An additional round PCR was performed to construct pET-nisA(Δ30-34)-KIHIHVDG/pET-nisA(Δ30-34)-KIHIHVDG-cinX with primers P-for-S29insKIH/pET-rev-S29insKIH and pET-S33D-K34G/pET-S33D-K34G-cinX as templates.

All the constructs are listed in Table 1 and have been verified by sequencing. Primers used were listed in Table 2.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Characteristics</th>
</tr>
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<tbody>
<tr>
<td>P-for-N20D-M21G</td>
<td>gacggaaaacagcaactgtgctag</td>
<td></td>
</tr>
<tr>
<td>P-rev-N20D-M21G</td>
<td>acaacccatcagagctcctg</td>
<td>5’ phosphorylation</td>
</tr>
<tr>
<td>P-for-S33D-K34G-pET</td>
<td>gacggataaggatccggctgctaac</td>
<td></td>
</tr>
<tr>
<td>P-rev-S33D-K34G</td>
<td>tacgtgaatactacaatgac</td>
<td>5’ phosphorylation</td>
</tr>
<tr>
<td>P-rev-rou-kpni-pET</td>
<td>cggggtaccgcgttggtcagcagccggatc</td>
<td></td>
</tr>
<tr>
<td>P-rev-rou-xhoi-pET</td>
<td>cggcgctgagcaaggaagctgagttggctg</td>
<td></td>
</tr>
<tr>
<td>P-rev-xhoi-cinX</td>
<td>cgcgcggaggaacagcagcagctgctgctag</td>
<td></td>
</tr>
<tr>
<td>P-for-kpni-T7RBS-cinX</td>
<td>cggggtaccagagagctgctgctgctgctag</td>
<td></td>
</tr>
</tbody>
</table>

*pET*: chloramphenicol resistance; EryR: erythromycin resistance; KanR: kanamycin resistance; AmpR: ampicillin resistance.

Unpublished; Manuel Montalbán-López (22)
Expression and purification of 6His-CinX and nisin derivatives in L. lactis

The expression and purification of 6His-CinX and nisin derivatives were performed as described previously (18, 23). A 100 ml culture of L. lactis NZ9000 harboring either pNZ-N20D-M21G-cinX or pNZ-N20D-M21G was induced using 5 ng/ml nisin when the OD (600nm) reached 0.4-0.5 and harvested after 3 h induction. The cell pellet was resuspended in suspension buffer (50 mM Tris-HCl, pH 7.4) containing lysozyme (100 mg/ml) and incubated at 30 °C for 2 h. The treated cells were lysed by sonication (65% amplitude; 10 s pulse, 20 s pause, 5 min) and the cytoplasmic fraction was collected by centrifugation (7000g; 4 °C; 5 min) for Ni-NTA purification. 1.0 ml of 50% superflow Ni-NTA resin (Qiagen) was equilibrated twice with 5 ml lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0) in a 50 ml tube by mixing on a rotor for 30 min. Subsequently, the column material was resuspended in the 5 ml cytoplasmic fraction and transferred into a 15 ml tube. Lysis buffer containing a protease inhibitor (Roche) was added to a final volume of 12 ml and the His-tagged protein was allowed to bind to the column material on a rotor at 4 °C for 2 h. Subsequently, the column was washed twice with 35 ml of wash buffer (50 mM NaH₂PO₄, 300 mM NaCl and 20 mM imidazole, pH 8.0). His-tagged protein was eluted with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl and 250 mM imidazole, pH 8.0). Fractions were analyzed by SDS-PAGE and Western blot using anti-His-tag antibodies.

MEM medium was inoculated at 2% from an overnight culture of L. lactis NZ9000(pIL3EryBTC; pNZ-N20D-M21G-cinX) or L. lactis NZ9000(pIL3EryBTC; pNZ-N20D-M21G) 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 4000g, and the supernatant was kept for the isolation of the peptides. To concentrate protein from supernatant, trichloroacetic acid (TCA) precipitation was performed according to Sambrook and Russell (19).

Expression and purification of nisin variants in E.coli

Vectors containing the structural nisin (variant) gene with/without cinX were transformed into E. coli BL21DE3 (pRSFDuet-nisCB). An overnight culture grown from a single colony transformant was inoculated at 1:100 ratio into fresh LB medium with kanamycin and
ampicillin. When the OD (600nm) reached 0.8, IPTG was added for induction at a final concentration of 0.1 mM. 100 ml of cell culture was harvested by centrifugation (7000g, 4 °C; 5 min) after 16 h induction. The obtained cytoplasmic fractions were analyzed on SDS-PAGE gel. Ni-NTA purification as described before was performed to purify His-tag labeled nisin variants from the cytoplasmic fractions. Eluted samples were desalted by a PD-10 column (GE Healthcare) and assessed on Tricine-SDS-PAGE (24).

The concentration of His-tag purified peptides was estimated 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. The solvents used 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.

**Mass spectrometry techniques**

The PD10-desalted peptide was digested with 1 μl of 1 mg/ml sequencing-grade trypsin in 5mM CaCl₂ and 50mM Tris (pH 6.8) solution at 37 °C for 16 h. After trypsin digestion, the proteolytic mix was analyzed by LC-MS or LC-MS/MS as described in chapter 4.

**Antimicrobial activity assay**

An overnight culture of *L. lactis* NZ9000 (pNZ-nisP; pIL253) (unpublished, Manuel Montalbán-López) was added at 1% (v/v) into melted M17 with 0.75% agar and 0.5% glucose (GM17) at 45 °C and poured in plates. The His-tag purified peptides were prepared at the same starting concentration based on the proportional relationship between the area of the chromatographic peak and the corresponding peptide concentration during HPLC purification. Serial two-fold dilutions were made for each sample and 5 μl of each dilution were dropped on the surface of GM17-agar. The plate was cultured at 30 °C for 16 h before measuring the halo sizes.

**Results and discussion**

*L. lactis* as host strain for co-expression of CinX, NisBC and nisin-leader containing substrates

To mimic the surrounding environment of the hydroxylated aspartic acid in cinnamycin, the residues Asn20 and Met21 in the nisin hinge region were replaced by Asp and Gly,
CinX is able to modify class I lantibiotics respectively, to generate the mutant N20D-M21G. N20D-M21G (Figure 1) was purified from the supernatant of \textit{L. lactis} with and without \textit{cinX}, by cationic exchange chromatography. MS data showed no mass difference between each mutant peptide coexpressed with \textit{cinX} compared to its counterpart without \textit{cinX} (data not shown). To figure out if the lack of modification by CinX was due to the malfunction of this modification enzyme or to the failed expression in \textit{L. lactis}, His-tag purification was performed to purify CinX. The cytoplasmic content of \textit{L. lactis} harboring pNZ-N20D-M21G-cinX after induction was analyzed by SDS-PAGE. No band difference at the expected size was observed when compared to a sample of \textit{L. lactis} with pNZ-N20D-M21G. Western blot using anti-His-tag antibodies did not show any band at the expected size either, thus indicating the failure of CinX expression in \textit{L. lactis} in spite of the codon usage of \textit{cinX} having been optimized for \textit{L. lactis}.

In our constructions, the \textit{cinX} gene with its ribosomal binding site is located downstream the structural gene of nisin variants, thus sharing the same nisin inducible promoter (\textit{P\textsubscript{nisA}}). Although \textit{P\textsubscript{nisA}} has been widely used for the expression of (lantipeptide-related) peptides and posttranslational modification enzymes (17, 18, 25-29), we could not detect the expression of the actinobacterial \textit{cinX} gene in \textit{L. lactis}.

\textit{E. coli} as a producing strain for the expression of CinX and its substrates

Next to the expression in \textit{L. lactis}, we attempted the production in \textit{E. coli}. After being induced by 0.1 mM IPTG, cell extracts of \textit{E. coli} BL21DE3 harboring either pET-N20D-M21G or pET-N20D-M21G-cinX were collected for affinity chromatography purification and SDS-PAGE analysis. An evident extra band between 35 kDa and 40 kDa was present in the sample containing pET-N20D-M21G-cinX, which is in agreement with the expected size of CinX (37 kDa), while the one transformed with pET-N20D-M21G did not show this band (Figure 2). These results indicated a successful expression of \textit{cinX}. This result is in line with the previous report by Ökesli \textit{et al.} in which CinX fused to a His-tag was purified for \textit{in vitro} enzymatic experiments (9).

N20D-M21G was purified from \textit{E. coli} BL21DE3(pET-N20D-M21G, pRSF-Duet-nisCB) and \textit{E. coli} BL21DE3(pET-N20D-M21G-cinX, pRSF-Duet-nisCB) by metal-affinity chromatography. Purified samples were digested by trypsin to cleave the leader peptide followed by LC-MS analysis. The LC-MS data of the peptides purified from \textit{E. coli} BL21DE3(pET-N20D-M21G, pRSF-Duet-nisCB) showed a peak with a mass of 820.6337 Da,
Figure 2. SDS-Polyacrylamide gel electrophoresis of the cytoplasmic content of *E. coli*. M: protein ladder. 1: *E. coli* transformed with pET-N20D-M21G. 2: *E. coli* transformed with pET-N20D-M21G-cinX.

Figure 3. LC-MS analysis of N20D-M21G coexpressed with and without CinX *in vivo*. The first peak of the isotopic distribution is labeled in red.
CinX is able to modify class I lantibiotics

A: *E. coli* transformed with pET-N20D-M21G and pRSF-Duet-nisCB. B: *E. coli* transformed with pET-N20D-M21G-cinX and pRSF-Duet-nisCB.

which is 4 times charged. This matched the theoretical mass of N20D-M21G (820.6348 Da; 4 times charged) (Table 3). Additionally, another 4 times charged peak with a mass of 824.6304 Da was observed in the MS profile as well indicating the oxidation of Met17 (Figure 3A and Table 3). In N20D-M21G, Met21 was mutated to glycine to preserve the original flanking amino acid sequence of the hydroxylated aspartic acid of cinnamycin in nisin. Therefore, this peak was most likely due to the oxidation of Met17, a phenomenon that has been previously reported to appear during purification procedures (30).

For the peptides purified from *E. coli* BL21DE3(pET-N20D-M21G-cinX, pRSF-Duet-nisCB), besides the peak matching N20D-M21G, another two peaks with a mass higher than N20D-M21G (4 times charged mass of 824.6379 and 828.6302 Da, respectively) were detected by MS (Figure 3B and Table 3). Because both oxidation of methionine and hydroxylation of aspartic acid would increase the initial mass of N20D-M21G by 16 Da (i.e. 4 Da in the 4 times charged peak), respectively, the peak with a mass of 824.6379 Da could be caused by either oxidation of Met17 or hydroxylation of Asp20. Only the combination of both oxidation and hydroxylation could generate the 828.6302 Da peak, which was not detected in the mutant that did not express CinX. This indicates that CinX successfully modified aspartic acid in N20D-M21G. The previous study by Ökesli *et al.* showed that CinX was able to modify its native substrate cinnamycin in *E. coli* (9). Our results prove that CinX is able to hydroxylate aspartic acid in nisin variants in *E. coli*, indicating that CinX may have a rather broad substrate tolerance.

Table 3. MS of N20D-M21G\(^a\) expressed with/without CinX

<table>
<thead>
<tr>
<th>Theoretical mass (mono) (M+4H)(^a)</th>
<th>Observed mass (mono)</th>
<th>Oxidation of Met</th>
<th>Hydroxylation of Asp</th>
</tr>
</thead>
<tbody>
<tr>
<td>(M+4H)(^a)</td>
<td>M</td>
<td>Without CinX</td>
<td>With CinX</td>
</tr>
<tr>
<td>820.6348</td>
<td>3278.5175</td>
<td>820.6337</td>
<td>820.6298</td>
</tr>
<tr>
<td>824.6336</td>
<td>3294.5124</td>
<td>824.6304</td>
<td>824.6379</td>
</tr>
<tr>
<td>828.6323</td>
<td>3310.5073</td>
<td>No peak observed</td>
<td>828.6302</td>
</tr>
</tbody>
</table>

\(^a\): All the peptides are 8 times dehydrated.

The His-tag purified nisin mutant produced by *E. coli* BL21DE3(pET-N20D-M21G, pRSF-Duet-nisCB) created a smaller inhibitory halo (19.5 mm diameter) than nisin purified from *E.
coli BL21DE3(pET-nisA, pRSF-Duet-nisCB) (23.5 mm diameter), while the peptides purified from *E. coli* BL21DE3(pET-N20D-M21G-cinX, pRSF-Duet-nisCB) completely lost the antimicrobial activity against *L. lactis* (Figure 4). Relative activity assay showed that the replacement of asparagine (Asn20) by aspartic acid (Asp20) reduced the activity of nisin (Figure 4). It has been reported that the replacement of asparagine (Asn20) by glutamic acid in nisin Z entirely abolished its activity (31). The complete abolishment of activity by Glu20 was assumed to be caused by the decrease of the positive charge of nisin, which hampered the binding of nisin to the bacterial membrane. Based on our results, the abolishment in the activity is determined not only by the net charge of nisin but also by the size and ramification of the side chain of residue 20 in nisin. Another interesting observation was that the His-tag purified sample from *E. coli* BL21DE3(pRSF-Duet-nisCB; pET-N20D-M21G-cinX) consisted of the mixture of both modified and unmodified N20D-M21G by CinX. This mixture did not show any antimicrobial activity against *L. lactis* NZ9000(pNZ-nisP; pIL253) although N20D-M21G produced in the absence of CinX displayed antimicrobial activity against the same indicator strain. This is indicating that the hydroxylated N20D-M21G mutant is inactive and the percentage of N20D-M21G in the mixture is too low to show the activity.

**CinX can modify the C-terminus of nisin**

A previous report showed that switching the order of incubation of the cinnamycin precursor peptide with CinM or CinX *in vitro* resulted in the same fully modified cinnamycin implying that the presence of (methyl)lanthionine and dehydrated residues was not required for the functionality of CinX (9). Therefore, we decided to investigate the tolerance regarding the position of aspartic acid. In order to do so, pET-nisA(Δ30-34)-KIHIHVDG was designed, which encodes a nisin analogue containing a C-terminal tail KIHIHVDG fused behind serine29 (Figure 1). Lysine was intentionally introduced to facilitate the trypsin digestion and further analysis of this fragment. The counterpart vector with *cinX* gene, pET-nisA(Δ30-34)-KIHIHVDG-cinX, was generated as well. Peptides produced by both *E. coli* BL21DE3[pET-nisA(Δ30-34)-KIHIHVDG; pRSF-Duet-nisCB] and *E. coli* BL21DE3[pET-nisA(Δ30-34)-KIHIHVDG-cinX; pRSF-Duet-nisCB] were purified by metal-affinity chromatography and digested by trypsin, respectively. The digestion product was analyzed by LC-MS/MS. The LC-MS/MS data indicated that when CinX was present, hydroxylation of aspartic acid of this nisin analogue was taking place. The modification did not occur in the one without CinX (Figure 5). This finding proves that CinX can recognize and modify aspartic acid located at the C-terminal part of the peptide.
CinX is able to modify class I lantibiotics

<table>
<thead>
<tr>
<th>Peptides purified from E. coli BL21DE3 (pRSH-Duet-miniCB)</th>
<th>Serial two-fold dilutions</th>
<th>Relative activity, %</th>
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<tr>
<td></td>
<td>C</td>
<td>C×2&lt;sup&gt;−1&lt;/sup&gt;</td>
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<tr>
<td>pET-miniA</td>
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<td>pET-N200D-N215</td>
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<td>pET-N200D-N215-cinX</td>
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<tr>
<td>pET-miniA(Δ30-34)-KIHIHVDG</td>
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<tr>
<td>pET-miniA(Δ30-34)-KIHIHVDG-cinX</td>
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Figure 4. Relative activities of nisin and its analogues in agar diffusion assay. Peptides from *E. coli* BL21DE3 containing two plasmids were His-tag purified and applied for serial two-fold dilutions relative activity assay.

<sup>a</sup>: All the five samples were normalized to the same starting concentration (C) and applied in the serial two-fold dilutions.

<sup>-</sup>: no inhibition halo observed

Figure 5. MS/MS analysis of nisin(Δ30-34)-KIHIHVDG with and without CinX after trypsin digestion. Ions that were positively identified in the MS/MS spectrum are highlighted in blue (b ions) or red (y ions)

Fusing the amino acid chain KIHIHVDG to nisin(Δ30-34) kept the activity of nisin(Δ30-34)-KIHIHVDG relatively equal to that of nisin confirming that the C-terminus of nisin has less effect on the activity of nisin, when compared to the N-terminus and hinge region (Figure 4) (32). When CinX was co-expressed, nisin(Δ30-34)-KIHIHVDG only retained 50% activity of nisin indicating that the hydroxylation of aspartic acid at the C-terminal region also reduced nisin activity. The target of nisin is lipid II rather than PE to which hydroxylation of Asp15 of cinnamycin facilitates cinnamycin to bind. We speculate that the possible binding to PE by CinX-modified nisin mutants may negatively affect their binding to lipid II by which nisin exerts its antimicrobial activity (15, 16) and/or hamper translocation of the C-terminal region across the membrane, although this requires further research.

**Conclusion**

So far, CinX is the only aspartic acid hydroxylase in lantibiotic biosynthetic clusters, the activity of which has been confirmed experimentally (9). The cinnamycin-like lantibiotic duramycin also possesses hydroxylated aspartic acid but no enzyme has been reported for its formation (33). In addition to hydroxylation of aspartic acid in cinnamycin, the modified amino acid 4,3-dihydroxyproline was detected in microbisporicin, which is produced by the actinomycete *Microbispora corallina* (34). Different ORFs found in the biosynthesis cluster of microbisporicin have been analyzed, and the function of some of them has been predicted. Among them, one ORF, designated as *mibO*, with high similarity to cytochrome P450, could catalyze the hydroxylation of proline in microbisporicin although the characterization of MibO would require a complete novel study. Our results unambiguously prove that CinX can recognize substrates other than cinnamycin. Moreover, it can carry out the hydroxylation of aspartic acid at different positions within the peptide. Being leader-independent, CinX is the only lantibiotic hydroxylase identified and characterized so far that can serve for designing additionally modified lantibiotics. Considering the importance of hydroxyaspartate during binding of cinnamycin to PE, this could be an interesting tool to produce peptides that should be able to interact specifically with PE.
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References


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### Supplementary Information

**Table S1. The nucleotide sequence of codon-optimized cinX**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Nucleotide sequence</th>
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<td>cinX</td>
<td>ATGGGCAAGCAGCCATCATCACATCATATGGGCTCTTTAAAACCTTGTGGAGGAGTTTTTACGTGATGC&lt;br&gt; ATTTGACCCTGCCAGATTTTGGATGGAATTGGATGAAAGCAGTCAGTGACTGAGGTTCCTGAAATCGTCGAACTT&lt;br&gt; GGACGAGGAAGGCAGCTCGGAGCTGGGACCAGAAAGCTGGAAGGCAACAG CCTCAAGACAACAC&lt;br&gt; CACCCAGGGAAAGCCGGCAACTGCGGCTGGTGGACCAGAAGCTGGAGGGCAACAGGCTCCAAGACAAAAAC&lt;br&gt; CACCCAGGGAAAGCCGGCAACTGCGGCTGGTGGACCAGAAGCTGGAGGGCAACAGGCTCCAAGACAAAAAC&lt;br&gt; ATATGTTTAAGATGATTGGGCAAGATTTGTGCGACCTGTGTGCGCAAACACTCGACTGAAAGGTCATGC&lt;br&gt; CTCCATCTGCTAGACTGATGTCGATTGGCCAGAAGATTGGACATCTGGAAGCTGGAAGGCAACAGGCTCCAAGACAAAAAC&lt;br&gt; ATATGTTTAAGATGATTGGGCAAGATTTGTGCGACCTGTGTGCGCAAACACTCGACTGAAAGGTCATGC&lt;br&gt; CTCCATCTGCTAGACTGATGTCGATTGGCCAGAAGATTGGACATCTGGAAGCTGGAAGGCAACAGGCTCCAAGACAAAAAC&lt;br&gt; ATATGTTTAAGATGATTGGGCAAGATTTGTGCGACCTGTGTGCGCAAACACTCGACTGAAAGGTCATGC&lt;br&gt; ATATGTTTAAGATGATTGGGCAAGATTTGTGCGACCTGTGTGCGCAAACACTCGACTGAAAGGTCATGC</td>
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