

In Vitro Reconstitution and Substrate Specificity of a Lantibiotic Protease[†]

L. A. Furgerson Ihnken, Champak Chatterjee, and Wilfred A. van der Donk*

Department of Chemistry, University of Illinois at Urbana-Champaign, 600 South Mathews Avenue, Urbana, Illinois 61801

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ABSTRACT: Lacticin 481 is a lanthionine-containing bacteriocin (lantibiotic) produced by *Lactococcus lactis* subsp. *lactis*. The final steps of lacticin 481 biosynthesis are proteolytic removal of an N-terminal leader sequence from the prepeptide LctA and export of the mature lantibiotic. Both proteolysis and secretion are performed by the dedicated ATP-binding cassette (ABC) transporter LctT. LctT belongs to the family of AMS (ABC transporter maturation and secretion) proteins whose prepeptide substrates share a conserved double-glycine type cleavage site. The *in vitro* activity of a lantibiotic protease has not yet been characterized. This study reports the purification and *in vitro* activity of the N-terminal protease domain of LctT (LctT150), and its use for the *in vitro* production of lacticin 481. The G(−2)A(−1) cleavage site and several other conserved amino acid residues in the leader peptide were targeted by site-directed mutagenesis to probe the substrate specificity of LctT as well as shed light upon the role of these conserved residues in lantibiotic biosynthesis. His₁₀-LctT150 did not process most variants of the double glycine motif and processed mutants of Glu−8 only very slowly. Furthermore, incorporation of helix-breaking residues in the leader peptide resulted in greatly decreased proteolytic activity by His₁₀-LctT150. On the other hand, His₁₀-LctT150 accepted all peptides containing mutations in the prepeptide or at nonconserved positions of LctA. In addition, the protease domain of LctT was investigated by site-directed mutagenesis of the conserved residues Cys12, His90, and Asp106. The proteolytic activities of the resulting mutant proteins are consistent with a cysteine protease.

Lantibiotics are a class of ribosomally synthesized antimicrobial peptides that undergo extensive post-translational modification (1–3). These modifications include enzyme-catalyzed dehydration of serine and threonine residues followed by cyclization of the resulting dehydro amino acids through reaction with cysteine residues to form thio-ether linkages (Figure 1). Following dehydration and cyclization, a leader peptide that is not modified is removed by a protease to give rise to the biologically active species. The modified peptide is secreted from the cell just prior to or immediately following the cleavage event by a transport protein. This sequence of biosynthetic events is shown in Figure 1 for lacticin 481 (1, 4). Serine and threonine residues of the lacticin 481 precursor peptide, LctA, are first dehydrated and then cyclized by the bifunctional protein LctM (5).¹ The unmodified leader sequence is removed, and mature lacticin

481 is exported by the LanT protein LctT (6). Because LctM and LctT are bifunctional enzymes, lacticin 481 is a member of the class II lantibiotics (7). In class I lantibiotics distinct proteins perform the dehydration (LanB), cyclization (LanC), cleavage (LanP), and export functions (LanT).

The role(s) of the leader peptide in lantibiotic biosynthesis has been the subject of much speculation (8, 9). It may act in a protective role for the producing organism, as its removal is required for bioactivity (10–15). The leader peptide could also serve as a recognition motif for the transport machinery because nonlantibiotic peptides attached to leader peptides are secreted (16, 17), or as a scaffold for the lantibiotic synthetases (11, 18–20). Recently, it was shown that the leader sequence of LctA, while important for processing by LctM, is not absolutely required for activity (21). When just the prepeptide was provided to the enzyme, dehydration still proceeded but with much decreased efficiency. These results demonstrated the importance of the leader sequence for efficient dehydration prompting an investigation of the conserved residues in the leader peptide by site-directed mutagenesis. The accompanying paper describes the results of these studies that indicate that none of the conserved leader residues are absolutely required for dehydration activity, but that the secondary structure of the peptide as well as Leu−7 are important for efficient processing by LctM (22). In this work, we investigated the importance of the conserved residues in the leader peptide for proteolytic processing.

In the biosynthesis of most class I lantibiotics, removal of the leader peptide is performed by a dedicated LanP serine-type protease (23). However, *lanP* genes are absent

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* To whom correspondence should be addressed. Tel: 217 244 5360; 217 244 8533. E-mail: vddonk@uiuc.edu.

¹ Abbreviations: AMS, ABC transporter maturation and secretion; Dha, 2,3-didehydroalanine; Dhb, 2,3-didehydrobutyrine; DTT, dithiothreitol; GST, glutathione S-transferase; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB, Luria–Bertani; LanB, class I lantibiotic dehydratase enzymes; LanC, class I lantibiotic cyclase enzymes; LanP, class I lantibiotic protease; LanM, class II lantibiotic synthetase enzymes; LanT, class I lantibiotic transporter or class II lantibiotic ABC transporter/protease; LctT, lacticin 481 transporter/protease; LctM, lacticin 481 synthetase; MALDI-TOF MS, matrix assisted laser desorption ionization time-of-flight mass spectrometry; RP-HPLC, reverse phase high performance liquid chromatography; TCEP, tris(2-carboxyethyl)phosphine; TFA, trifluoroacetic acid.

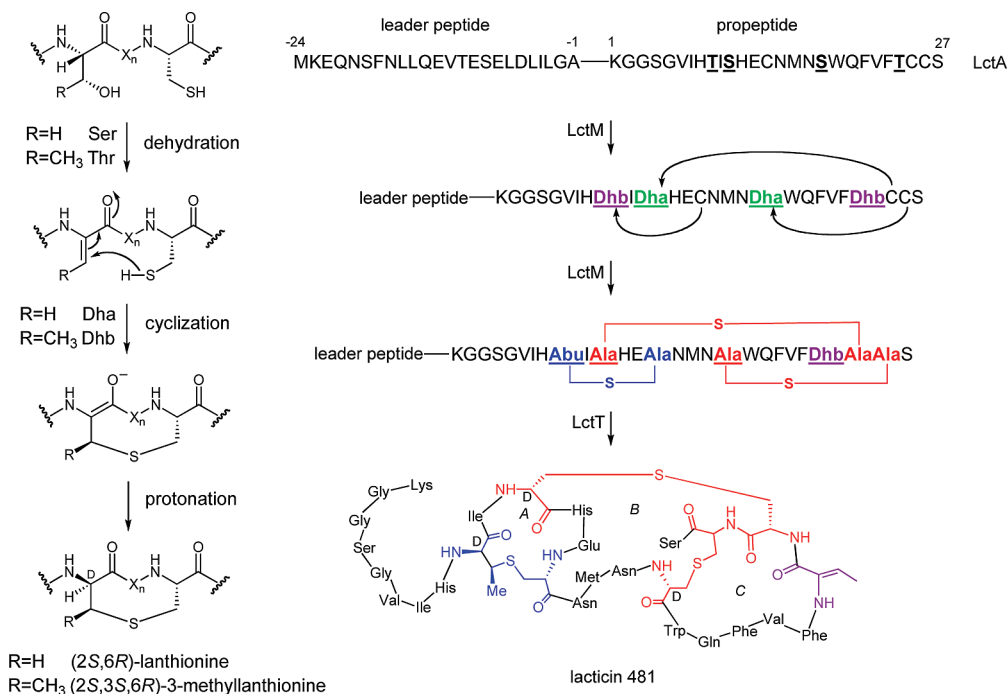


FIGURE 1: Biosynthesis of lacticin 481.

from the biosynthetic gene clusters of class II lantibiotics, and the *lanT* genes instead encode an N-terminal peptidase domain in the transporter proteins that is absent in the class I lantibiotic ABC transporters. This domain is proposed to remove the leader sequence from the modified precursor peptides concomitant with export of the mature species. Håvarstein and co-workers recognized a correlation between bacteriocin transporters that contain this N-terminal proteolytic domain and the presence of a double glycine motif in the leader sequences of their cognate prepeptide substrates (24). They showed that the N-terminal 150 amino acids of the bacteriocin transporter LagD were sufficient for lactococcin G precursor cleavage. This investigation was the first of three studies on the *in vitro* activity of nonlantibiotic bacteriocin protease domains, with more recent studies describing *in vitro* hydrolysis of ComC by the N-terminal domain of ComA (25), and cleavage of the colicin V precursor by the protease domain of CvaB (26). With the exception of a very recent report on ComC (27), these previous studies focused mainly on characterization of the protease with investigation of substrate recognition receiving little attention. The study of lantibiotic proteases has been restricted to limited *in vivo* mutagenesis of the leader peptides (10, 13, 15, 28, 29).

Herein, we report the first purification and *in vitro* activity of a lantibiotic protease, the N-terminal domain of LctT responsible for the maturation of lacticin 481. The N-terminal 150 amino acids were overexpressed and purified as a soluble His₁₀-fusion protein designated His₁₀-LctT150. The importance of conserved leader peptide residues and the substrate scope of proteolysis by His₁₀-LctT150 were examined by subjecting various His₆-LctA mutant peptides to the protease followed by mass spectrometric analysis. His₁₀-LctT150 did not process most variants of the double glycine motif and processed mutants of Glu-8 only very slowly. Furthermore, incorporation of helix-breaking residues in the leader peptide resulted in greatly decreased protease activity. On the other

hand, His₁₀-LctT150 accepted all mutant peptides containing mutations in the propeptide or at nonconserved positions of LctA. In the course of this study, the first complete *in vitro* synthesis of lacticin 481 was achieved by treating His₆-LctA consecutively with His₆-LctM and His₁₀-LctT150.

MATERIALS AND METHODS

General Methods. Molecular biology techniques were carried out using standard protocols. Mass spectrometry was performed by the Mass Spectrometry Laboratory at the University of Illinois using a Voyager matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) spectrometer. Unless otherwise noted, all substrates including the chimeric peptides were cloned, overexpressed, and purified according to (22).

Construction of Plasmids pGSTLctT150 and pHis₁₀-LctT150. *Lactococcus lactis* CNRZ 481 was grown in M17 broth supplemented with 0.5% glucose (GM17). Genomic DNA was isolated using a DNAzol kit from Molecular Research Center, Inc., and was used as the template for generating the 450 bp fragment corresponding to the N-terminal 150 amino acids of LctT. This PCR product was obtained using a forward primer containing a *Bam*HI restriction site (primer 1, Table S1) and a reverse primer containing an *Xho*I site (primer 2, Table S1). The PCR product and pGEX-6p-1 (GE Healthcare Life Sciences) were digested with *Bam*HI-*Xho*I and ligated to produce the vector pGSTLctT150 encoding the N-terminal 150 amino acids of LctT fused at its N-terminus to a glutathione S-transferase tag and a PreScissionProtease cleavage site (LEVLFQGP). pGSTLctT150 was used as template for amplification of a 450 bp *lctT150* gene fragment containing a 5'-*Nde*I site and a 3'-*Bam*HI site (primers 3 and 4, Table S1). The PCR product and pET16b (Novagen) were digested with *Nde*I-*Bam*HI and ligated to produce the vector pHis₁₀LctT150 encoding the N-terminal 150 amino acids of LctT fused at

Table 1: His₁₀-LctT150 Cleavage of Wild-Type and G(−2)A(−1) Mutant His₆-LctA Peptides^a

peptide	full length (calcd)	leader (calcd)	propeptide (calcd)	result ^b
LctA wt	7709 (7709)	4754 (4754)	2973 (2973)	Ala−1/Lys1
G−2V	7752 (7751)	4797 (4796)	2973 (2973)	Ala−1/Lys1*
G−2E	7781 (7781)	— (4826)	— (2973)	no cleavage
G−2K	7781 (7780)	— (4825)	— (2973)	no cleavage
A−1I	7752 (7751)	— (4796)	— (2973)	no cleavage
A−1D	7754 (7753)	— (4798)	— (2973)	no cleavage
A−1K	7765 (7766)	— (4811)	— (2973)	no cleavage
A−1G	7695 (7695)	4738 (4740)	2971 (2973)	Gly−1/Lys1

^a Masses for peaks observed by MALDI mass spectrometry are shown, with calculated values in parentheses, (—) indicates that no cleavage products were observed, and (*) indicates reduced enzyme activity based on the intensity of the product ion relative to the substrate ion. ^b Site of observed cleavage, indicating N-terminal amino acid/C-terminal amino acid with respect to cleavage site.

its N-terminus to a His₁₀-tag (30) containing a Factor Xa site (MGHHHHHHHHSSGHIEGRH). The correct sequences of the *lctT* inserts were confirmed by sequencing at the Biotechnology Center at the University of Illinois at Urbana-Champaign.

Construction of Plasmids pGSTLctT150(C12A) and pGSTLctT150(C12S). The *lctT150* gene was amplified from pGSTLctT150 by PCR employing forward primers encoding the C12A or C12S mutation and containing a *Bam*HI restriction site (Table S1, primers 5 and 6, respectively) and the wild-type reverse primer with an *Xho*I site (Table S1, primer 2). The PCR products and vector pGEX-6p-1 (GE Healthcare Life Sciences) were digested with *Bam*HI-*Xho*I, and ligated to produce the plasmids pGSTLctT150(C12A) and pGSTLctT150(C12S).

Construction of Plasmids pGSTLctT150(H90A), pGSTLctT150(H90N), and pGSTLctT150(D106A). The entire pGSTLctT150 plasmid was amplified by QuikChange Site-Directed Mutagenesis (Stratagene) using the appropriate mutant forward primers (Table S1, primers 8, 10, and 12) and reverse primers (Table S1, primers 7, 9, and 11).

Construction of Plasmids for Mutants of His₁₀-LctT150: C12A, C12S, H90A, H90N, and D106A. The *lctT150* mutant genes were amplified by PCR from the corresponding pGSTLctT150 mutant plasmids using the wild-type *Nde*I forward primer (Table S1, primer 3) and wild-type *Bam*HI reverse primer (Table S1, primer 4). The mutant genes and pET16b were digested with *Nde*I-*Bam*HI and ligated producing plasmids encoding the N-terminal 150 amino acids of LctT with the appropriate single mutation fused at its N-terminus to a His₁₀-tag.

Construction of Plasmids for Mutants of His₆-LctA: E−13A, L−7A, A−1G, and K1A. A 5′ gene fragment was amplified by PCR from the pHis₆-LctA plasmid (11) using the T7 forward primer and an internal mutant reverse primer (Table S1, primers 11, 13, 15, and 17). An overlapping 3′ gene fragment was also generated by PCR with the T7 reverse primer and an internal mutant forward primer (Table S1, primers 12, 14, 16, and 18). Finally, a nested PCR with the overlapping gene fragments and the T7 forward and reverse primers was used to generate full-length LctA mutants. After digestion of the corresponding genes and pET15b with *Nde*I and *Bam*HI, ligation produced plasmids encoding LctA point mutants fused at their N-termini to a hexa-histidine tag (MGSSHHHHHHSSGLVPRGSH).

Overexpression of Wild-Type and Mutant His₁₀-LctT150. The *Escherichia coli* strain BL21(DE3) containing pHis₁₀-LctT150 (wild-type or mutant) was grown overnight in 5 mL of Luria–Bertani (LB) medium containing 100 μg/mL

ampicillin. The cells were harvested at 4750g and the supernatant decanted. The cell pellet was resuspended in 1 mL of fresh LB, and the suspension used to inoculate 500 mL of LB medium containing 100 μg/mL ampicillin. The culture was incubated at 37 °C with aeration until OD₆₀₀ = 0.7. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. The culture was then grown at 25 °C for 5 h and the cells harvested by centrifugation at 11900g for 25 min at 4 °C. The cell pellet was frozen in liquid nitrogen and stored at −80 °C.

Purification of Wild-Type and Mutant His₁₀-LctT150. His₁₀-LctT150 was isolated by affinity chromatography using a 5 mL HiTrap chelating column (GE Healthcare Life Sciences) charged with 0.1 M CoCl₂ and equilibrated with phosphate start buffer (50 mM Na₂HPO₄, 500 mM NaCl, pH 7.5 at 4 °C). The cell pellet was thawed and resuspended in 10 mL of phosphate start buffer. The cells were lysed by sonication with a Sonics & Materials Inc. Vibra Cell and the cell lysate was cleared by centrifugation at 17400g for 30 min at 4 °C. The supernatant was filtered through a 0.45 μm syringe-tip filter (Millipore) and applied to the HiTrap column. The column was washed with two column volumes of phosphate start buffer and the protein eluted using a step gradient of imidazole. Three column volumes each of phosphate start buffer containing 0 mM, 50 mM, 100 mM, 250 mM, 500 mM, and 1 M imidazole were used. The fractions containing protein, as visualized by SDS–PAGE analysis, were concentrated by Amicon filtration with a YM-1 ultrafiltration membrane (Millipore). Glycerol was added to the concentrated protein to generate a 10% (v/v) solution, which was frozen in liquid N₂ and stored at −80 °C.

Cleavage of His₆-LctA Substrates by His₁₀-LctT150. Assays were performed with 2 mg/mL stock solutions of each of the substrates shown in Tables 1–3. Wild-type and mutant His₆-LctA peptides and His₁₀-LctT150 were incubated in the presence of 50 mM Na₂HPO₄ pH 7.5, 50 mM Na₂SO₄, and 1 mM DTT (buffer A) at 25 °C for 2–4 h. The presence of reductant was required for His₁₀-LctT150 activity. The final concentration of each peptide was 0.4 mg/mL (~50 μM), and that of His₁₀-LctT150 was 0.2 mg/mL (10 μM). Reactions were quenched by the addition of 1% trifluoroacetic acid (TFA) to a final concentration of 0.1%, and samples for mass analysis were prepared by C18 Zip-tip (Millipore). Reaction progress was monitored by MALDI-TOF mass spectrometry.

Cleavage of His₆-LctA Substrates by His₁₀-LctT150 at Low Substrate Concentrations. His₆-LctA (wild-type, V−12A, L−7E, D−6P, and E−13A) and His₆-MutLctA were incu-

Table 2: His₁₀-LctT150 Cleavage of Wild-Type and Leader Sequence Mutant His₆-LctA Peptides^a

peptide	full length (calcd)	leader (calcd)	propeptide (calcd)	result ^b
LctA wt	7709 (7709)	4754 (4754)	2973 (2973)	Ala-1/Lys1
E-13A	7653 (7651)	4697 (4696)	2972 (2973)	Ala-1/Lys1
V-12A	7677 (7681)	4727 (4726)	2975 (2973)	Ala-1/Lys1
V-12P	7708 (7707)	4753 (4752)	— (2973)	Ala-1/Lys1*
E-8A	7652 (7651)	4696 (4696)	— (2973)	Ala-1/Lys1*
E-8P	7680 (7677)	4724 (4722)	— (2973)	Ala-1/Lys1*
		4653 (4651)	— (3044)	Gly-2/Ala-1*
L-7A	7668 (7667)	4712 (4712)	2972 (2973)	Ala-1/Lys1
L-7E	7727 (7725)	4772 (4770)	2975 (2973)	Ala-1/Lys1
L-7K	7728 (7724)	4773 (4769)	— (2973)	Ala-1/Lys1
D-6P	7695 (7691)	4739 (4736)	2975 (2973)	Ala-1/Lys1
L-5Q	7725 (7724)	4769 (4769)	2973 (2973)	Ala-1/Lys1
L-5P	7695 (7693)	4738 (4738)	— (2973)	Ala-1/Lys1*
I-4P	7695 (7693)	4742 (4738)	— (2973)	Ala-1/Lys1*
MutLctA	8085 (8081)	5127 (5126)	2973 (2973)	Gly-1/Lys1

^a Masses for peaks observed by MALDI mass spectrometry are shown, with calculated values in parentheses, and (*) indicates reduced enzyme activity based on the intensity of the product ion relative to the substrate ion. Based on the relative intensities of the leader peptide and the propeptide in all mass spectra, the ionization efficiency for the leader peptide is likely much higher, potentially because of the His-tag. In some cases, the propeptide was not detected at all (—) in the expected mass range, presumably because of poor ionization efficiency and ion suppression. ^b Site of observed cleavage, indicating N-terminal amino acid/C-terminal amino acid with respect to cleavage site.

Table 3: His₁₀-LctT150 Cleavage of Wild-Type and Propeptide Mutant His₆-LctA Peptides and Chimeric Substrates^a

peptide	full length (calcd)	leader (calcd)	propeptide (calcd)	result ^b
LctA wt	7709 (7709)	4754 (4754)	2973 (2973)	Ala-1/Lys1
K1A	7652 (7652)	4753 (4754)	2914 (2916)	Ala-1/Ala1
G3I/G5H	7850 (7845)	4755 (4754)	3107 (3109)	Ala-1/Lys1
K1-G2insAAA ^c	7925 (7922)	4683 (4683)	— (3257)	Gly-2/Ala-1
		4755 (4754)	3187 (3186)	Ala-1/Lys1
		5153 (5152)	— (2788)	Gly5/Gly6
		5210 (5209)	— (2731)	Gly6/Ser7
LctMutA	8427 (8423)	5127 (5126)	3317 (3317)	Ala-1/Asn1
LctRumA	7858 (7853)	5131 (5126)	2748 (2747)	Ala-1/Gly1
LctNukA	7769 (7767)	4752 (4752)	3029 (3031)	Ala-1/Lys1*
LctSpaS	8573 (8568)	5126 (5126)	3462 (3465)	Ala-1/Trp1*
LctNisA	8605 (8601)	— (5126)	— (3498)	no cleavage

^a Masses for peaks observed by MALDI mass spectrometry are shown, with calculated values in parentheses, and (*) indicates reduced enzyme activity based on the intensity of the product ion relative to the substrate ion. Based on the relative intensities of the leader peptide and the propeptide in all mass spectra, the ionization efficiency for the leader peptide is likely much higher, potentially because of the His-tag. In some cases, the propeptide was not detected at all (—) in the expected mass range, presumably because of poor ionization efficiency and ion suppression. ^b Site of observed cleavage, indicating N-terminal amino acid/C-terminal amino acid with respect to cleavage site. ^c For full linear amino acid sequence, see Chatterjee *et al.* (35).

bated with His₁₀-LctT150 in 1 mL of diluted cleavage buffer (10 mM Na₂HPO₄ pH 7.5, 10 mM Na₂SO₄, and 0.2 mM DTT) at 25 °C for 4 h. The final concentration of each peptide was 0.2 μM, and that of His₁₀-LctT150 was 0.5 μM. The reactions were quenched by addition of 1% TFA and then concentrated 10-fold by a Centrивap concentrator (Labconco). Samples for mass analysis were prepared by C18 Zip-tip (Millipore), and reaction progress was monitored by MALDI-TOF mass spectrometry.

In Vitro Synthesis of Lactacin 481. His₆-LctA and His₆-LctM were incubated in the presence of 50 mM MOPS pH 7.2, 2.5 mM ATP, 2.5 mM TCEP, and 10 mM MgCl₂ at 25 °C for 2–4 h. The dehydrated material was applied to a C18 Zip-tip and eluted in 10 μL of 50% acetonitrile, 0.3% TFA. The solvent was removed by a Centrивap concentrator, and the residue dissolved in 10 μL of buffer A. This product mixture was incubated with 10 μL of 0.5 mg/mL His₁₀-LctT150 at 25 °C for 12 h. Aliquots for MS analysis were quenched with 1% TFA, partially purified by Zip-tip and analyzed by MALDI-TOF mass spectrometry.

Synthesis and Activity of Short Peptide Substrates. The 10-mer peptide DLILGAKGGS corresponding to the P1–P6 and P1'–P4' residues of LctA was synthesized using standard Fmoc-based solid phase peptide synthesis and subsequent

HPLC purification using previously described protocols (31). In addition, the peptide Ac-LILGA-pNP (with pNP = C-terminal *p*-nitrophenyl ester) was prepared by solid phase peptide synthesis of AcLILGA and subsequent esterification. Both peptides were incubated with His₁₀-LctT150 or with GST-LctT150 but no activity was observed by either LC-MS or UV-vis spectroscopy.

RESULTS

Cloning and Isolation of LctT150. Part of the *lctT* gene corresponding to the N-terminal 150 amino acids was amplified by PCR from genomic DNA of *L. lactis* CNRZ 481. This specific truncation of the LctT protein was chosen for study as sequence alignment with known bacteriocin AMS proteins followed by application of a previously reported algorithm (32) identified a possible interdomain linker region between residues 125–175. The gene fragment was inserted into pGEX-6p-1 to generate an N-terminal glutathione S-transferase (GST) fusion of the LctT protease domain (LctT150). This fusion protein was heterologously overexpressed in *E. coli* and purified by glutathione affinity chromatography. Incubation of the protein with His₆-LctA resulted in very low protease activity and surprisingly

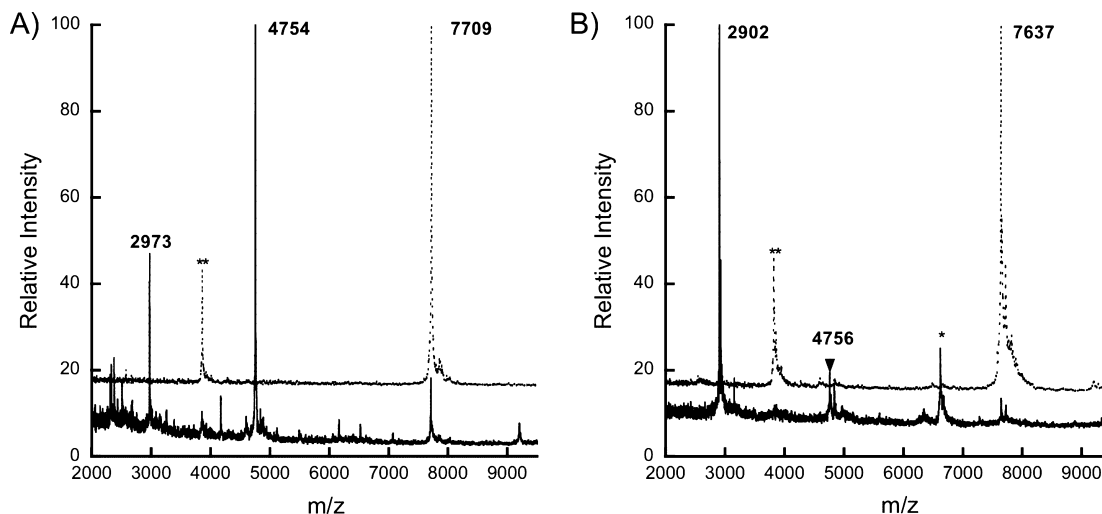


FIGURE 2: *In vitro* reconstitution of LctT proteolytic activity. MALDI mass spectra for (A) linear His₆-LctA and (B) His₆-LctM-processed His₆-LctA after incubation with (solid lines) or without (dotted lines) His₁₀-LctT150. The double asterisk (**) indicates a peak corresponding to doubly charged starting material, and the single asterisk (*) to a triply charged His₁₀-LctT150 ion.

cleavage between Gly-2 and Ala-1 (Figure S1). Given the low activity and the incorrect position of proteolysis, this fusion protein was not further investigated. The 450 bp *lctT150* gene fragment was instead inserted into pET16b to produce an N-terminal ten histidine (His₁₀) fusion of LctT150. His₁₀-LctT150 was heterologously expressed in *E. coli* as a soluble fusion protein and purified by Co²⁺-NTA affinity chromatography, resulting in 2 mg of fusion protein per liter of cell culture. It should be noted that attempts to purify this fusion protein by Ni²⁺ affinity chromatography were unsuccessful.

His₁₀-LctT150 Activity with Wild-Type His₆-LctA. Small-scale assays were performed with wild-type His₆-LctA in order to assess enzyme activity. All assays were performed at 25 °C for 2–4 h at pH 7.5, and reaction progress was monitored by MALDI-TOF mass spectrometry. Figure 2A shows the mass spectrum for His₁₀-LctT150-catalyzed cleavage of His₆-LctA. A small peak for starting material was observed at 7709 Da, along with two proteolysis products. The peak at 4754 Da corresponds to residues -24 to -1 of His₆-LctA, which is the N-terminal fragment resulting from amide bond hydrolysis at the predicted site between Ala-1 and Lys1 (Figure 1). The corresponding C-terminal product, LctA1-27, was observed at 2973 Da. The amide hydrolysis reaction did not require any metals or cofactors and was not inhibited by EDTA. The presence of a thiol reducing agent (DTT or TCEP) was required for activity during assay but not during purification or for storage of the enzyme. Quantitative analysis to determine kinetic parameters was hampered by the previously documented poor solubility of the substrate peptide (33, 34). Attempts to use a synthetic truncated substrate DLILGA-KGGS corresponding to the P1-P6 and P1'-P4' residues of LctA were unsuccessful, as this peptide was not processed by the enzyme. Similarly, a *p*-nitrophenyl ester of a truncated substrate was not hydrolyzed by His₁₀-LctT150.

The same cleavage pattern was observed when His₆-LctA was dehydrated and cyclized using His₆-LctM (Figure 1) and then subjected to cleavage by His₁₀-LctT150 (Figure 2B). The His₆-LctM-processed His₆-LctA was detected at 7637 Da after the loss of four water molecules. After His₁₀-LctT150 treatment, the leader sequence was observed at 4756

Da and lactacin 481 was detected at 2902 Da, indicating that the loss of four water molecules occurred in the propeptide. Comparing Figures 2A and 2B, it appears that the ionization efficiency of the C-terminal fragment is enhanced upon dehydration and cyclization, but this observation was not further investigated. These results are the first *in vitro* biosynthesis of authentic lactacin 481 as previous *in vitro* studies before the availability of LctT150 utilized the commercial protease Lys-C, which produces Δ1-lactacin 481 (11, 34, 35). As no difference was observed in LctT activity between the linear and cyclized substrates, further studies were continued with linear peptides for simplicity.

Proteolytic Activity of His₁₀-LctT150 Mutant Proteins. Three conserved residues of LctT proposed to be involved in peptide bond cleavage (Cys12, His90, and Asp106) (24) were mutated to investigate their importance in amide bond hydrolysis. The involvement of these residues in catalysis has been suggested, but never experimentally tested for lantibiotic proteases. Mutation of Cys12 to either Ser or Ala resulted in loss of all proteolytic activity (Figure 3A and 3B, respectively) consistent with the LctT protease domain being a cysteine protease. Surprisingly, mutation of His90 to Ala resulted in incorrect cleavage between Gly-2 and Ala-1 (Figure 3C). Mutation of Asp106 to Ala had no effect on peptidase activity (Figure 3D), indicating that this residue is not essential for catalysis.

His₁₀-LctT150 Activity with His₆-LctA Double Glycine Mutant Peptides. Enzymatic assays were performed with a number of His₆-LctA mutant peptides. Because LctT was predicted to recognize the double glycine motif at the cleavage site, seven peptides with mutations at residues -2 and -1 were generated. The results of the assays with these mutants are summarized in Table 1. Mutation of Gly-2 or Ala-1 to a charged residue (Glu, Asp, or Lys) completely abolished cleavage by His₁₀-LctT150 (Figure 4A and Figure S2a–c). Exchange of Ala-1 for Ile also resulted in complete inhibition of proteolysis (Figure S2d). Out of seven double glycine motif mutant peptides, only His₆-LctA(G-2V) (Figure S2e) and A-1G (Figure 4B) were accepted as substrates by His₁₀-LctT150. However, the G-2V peptide was not a good substrate and was processed to a much lesser

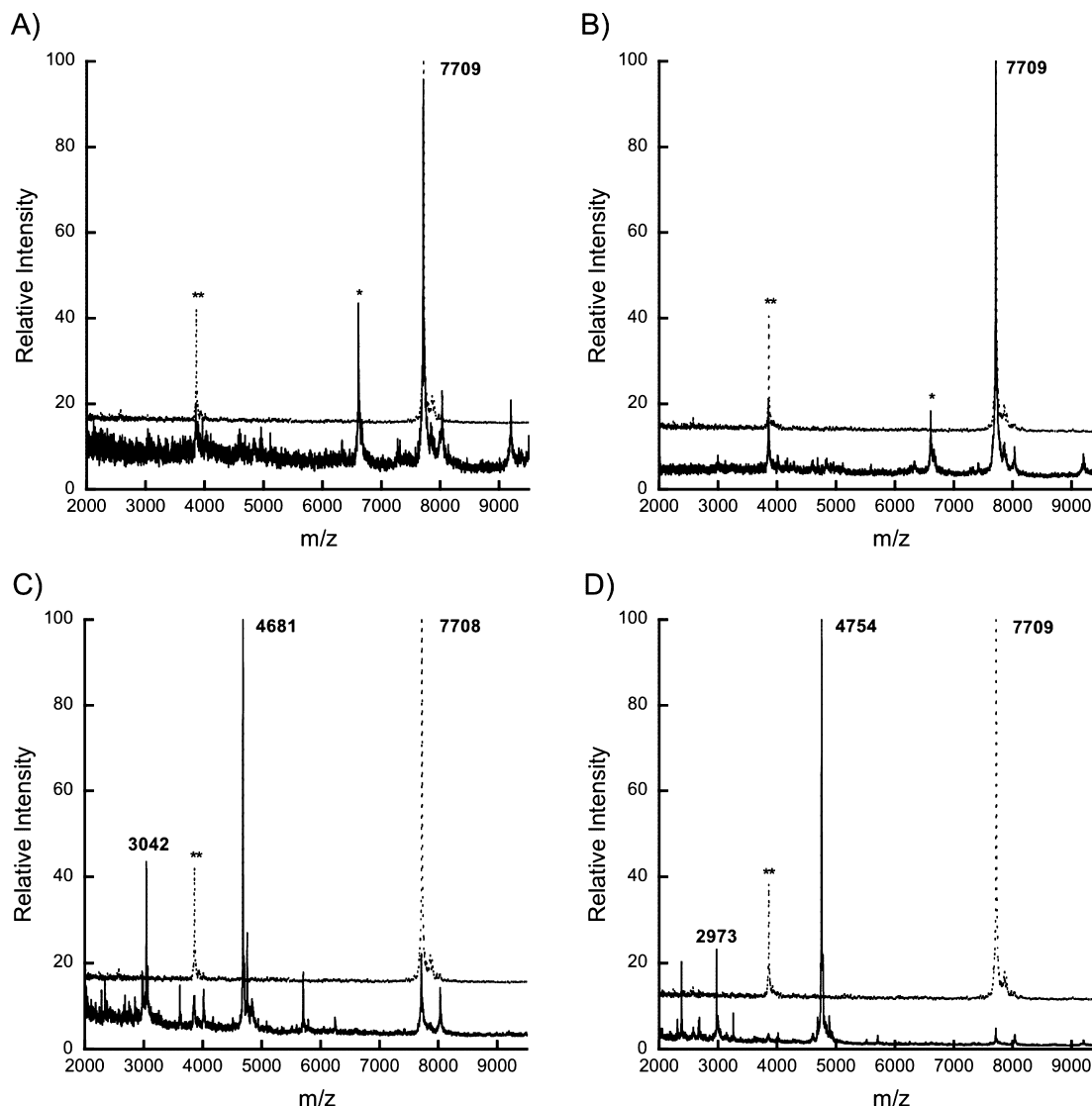


FIGURE 3: MALDI mass spectra for His₆-LctA wild-type after incubation with (solid lines) or without (dotted lines) (A) His₁₀-LctT150 C12S, (B) His₁₀-LctT150 C12A, (C) His₁₀-LctT150 H90A, or (D) His₁₀-LctT150 D106A. The double asterisk (**) indicates a peak corresponding to doubly charged starting material, and the single asterisk (*) to a triply charged His₁₀-LctT150 ion.

extent than the wild-type sequence under identical conditions (Figure S2e).

His₁₀-LctT150 Catalyzed Proteolysis of His₆-LctA Leader Sequence Mutant Peptides. In order to evaluate the importance of other conserved residues in the leader sequence besides Gly-2 and Ala-1, the mutants E-13A, V-12A, E-8A, L-7A, L-7E, L-7K, and L-5Q were investigated. Assay results for these peptides are summarized in Table 2 and show that these mutations did not prohibit hydrolysis by His₁₀-LctT150 (Figure S3), except in the case of E-8A, for which the cleavage efficiency was reduced (Figure S3d). As discussed above, we have not been able to obtain quantitative kinetic parameters and efforts to use competition assays to determine relative catalytic efficiencies with respect to the wild-type sequence provided unreliable results. Qualitatively, however, with the exception of E-8A all mutant substrates were processed with very similar efficiencies as wild-type LctA based on relative intensities of the substrate and product ions observed by MALDI-TOF MS under identical assay conditions.

Five additional mutant peptides (V-12P, E-8P, D-6P, L-5P, and I-4P) were generated to disrupt potential

secondary structure in the leader peptide that could influence the activity of LctT150, as structure prediction programs suggest that the leader sequence may be helical (22). Interestingly, all of these mutants except D-6P resulted in reduced efficiency of cleavage by His₁₀-LctT150 (Figure S3). As a representative example, Figure 4C shows the level of cleavage of His₆-LctA E-8P by His₁₀-LctT150 under conditions where His₆-LctA would be completely processed. The effects of these mutations are not entirely due to disruption of secondary structure, however. For example, for Glu-8, mutation to Ala had a similar effect on proteolysis as mutation to Pro (Figure S3d), suggesting that the glutamate side chain provides an important recognition point. On the other hand, mutation of Val-12 to Ala and Leu-5 to Gln resulted in substrates that were processed with similar efficiencies as the wild-type LctA peptide (Figures S3b and S3i, respectively), suggesting that the observed decreased proteolysis with V-12P and L-5P (Figure S3j) is related to disruption of the structure of the peptide.

In addition to these point mutants, the possible importance of the nonconserved residues in the leader sequence was investigated via a chimera (MutLctA) joining the leader

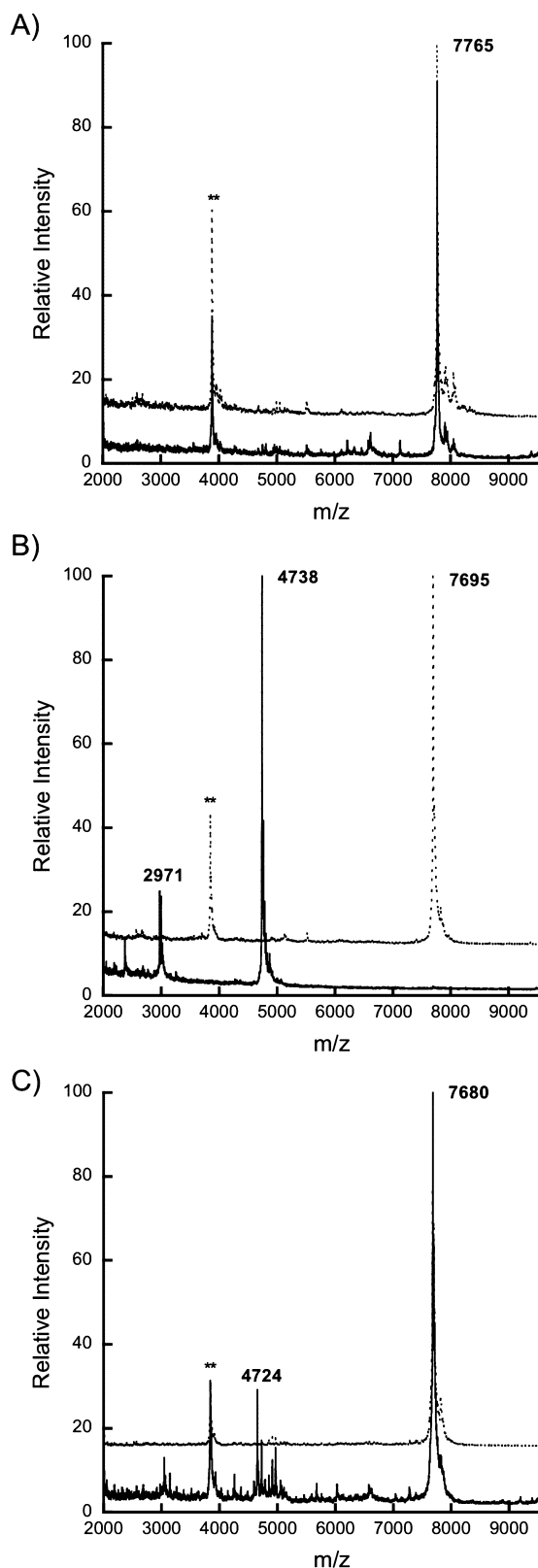


FIGURE 4: MALDI mass spectra for (A) His₆-LctA A-1K, (B) His₆-LctA A-1G, and (C) His₆-LctA E-8P after incubation with (solid lines) or without (dotted lines) His₁₀-LctT150. The double asterisk (**) indicates a peak corresponding to doubly charged starting material ion.

sequence of the mutacin II prepeptide, MutA, to the propeptide of LctA (22). The leader peptides from MutA and LctA share significant sequence homology, with 11 out of 24 residues identical in the two peptides (Figure 5).

Therefore, the chimeric peptide amounted to a mutant in which about 55% of the leader peptide residues were mutated simultaneously, with the mutations concentrated in nonconserved positions. We do note that the leader peptides of these two peptides differ in the double glycine motif residues with the MutA leader ending in G(-2)G(-1) as opposed to G(-2)A(-1) for LctA. However, because the LctA A-1G mutant was a good substrate for His₁₀-LctT150 as shown above, this difference was not considered a problem. As shown in Figure 6A, the hybrid peptide was a substrate for His₁₀-LctT150, displaying about the same proteolytic efficiency as the His₆-LctA and His₆-LctA A-1G substrates.

As has been reported previously (34), the LctA peptide has very poor solubility ($\sim 1 \mu\text{M}$) in aqueous buffer at pH 7, which has precluded quantitative assessment of the concentration dependence of enzyme activity. All data discussed so far were obtained at the solubility limit. One possible explanation for the apparent lack of effect of several of the mutations in LctA discussed above is that at the concentrations used the observed activities report on k_{cat} and that this parameter was unaffected by the mutations. If so, the possibility still existed that these mutations could affect K_m . To test potential binding effects, several of the His₆-LctA substrates (wild-type, E-13A, V-12A, L-7E, L-7A, L-5Q, D-6P, A-1G, and His₆-MutLctA) were incubated with His₁₀-LctT150 at lower substrate and enzyme concentrations ($0.2 \mu\text{M}$) and in a larger reaction volume to obtain sufficient material for MS analysis. Comparison of the data so obtained showed that V-12A, L-7A, L-5Q, A-1G, and MutLctA were processed to a similar extent as wild-type His₆-LctA, but E-13A, L-7E, and D-6P were poorer substrates under these conditions (Figure S4).

His₁₀-LctT150 Catalyzed Proteolysis of His₆-LctA Propeptide Mutant Peptides. Three His₆-LctA peptides containing mutations in the P1'-P5' sites were generated to determine if the propeptide residues C-terminal to the cleavage site are important for proteolysis by His₁₀-LctT150. The results for these peptides are summarized in Table 3. Mutation of Lys1 in the P1' position to Ala did not abolish proteolysis (Figure S5a). Similarly, simultaneous mutation of Gly3 in the P3' position to Ile and Gly5 in the P5' position to His resulted in a peptide that was accepted by His₁₀-LctT150 (Figure S5b). Analysis of a previously generated mutant in which three Ala residues were inserted between Lys1 and Gly2 (35) showed that this peptide was hydrolyzed in the correct location after Ala-1 but also after Gly-2, Gly5 and Gly6 (Figure S5c).

His₁₀-LctT150 Cleavage of His₆-LctA Chimeras. In addition to the MutLctA chimeric substrate discussed above, five other chimeric peptides were generated in order to investigate the role of the propeptide as a whole in cleavage by LctT. These peptides contain the LctA leader sequence fused at its C-terminus to the propeptide of other lantibiotics including MutA (mutacin II), RumA (ruminococcin), SpaS (subtilin), NukA (nukacin ISK-1), and NisA (nisin) (for sequences, see Figure 5). Both the LctRumA and LctMutA peptides were cleaved efficiently by His₁₀-LctT150 (Figures 6B and S6a, respectively). The LctNukA chimera was cleaved correctly (Figure S6b), albeit to a lesser degree than the other class II chimeras, in agreement with a previous report on the *in vivo* processing of preNukA by LctT (36). Interestingly, fusion of the LctA leader to the class I lantibiotic subtilin propeptide

Class I lantibiotics	leader peptide	↓	propeptide
Nisin A	MSTKD F N L D L VSVSKK-- D SGAS P R	-1 ↓ 1	ITSISLCTPGCK-TGALMGCNMKTATCHCSIHVSK
Subtilin	MSKFDD F D L D V VKVSQ-- D SKIT P Q		WKSESLCTPGCV-TGALQTCFLQTLTCNCKISK
Gallidermin	MEAVKEKNEL F D L D V KVNAKES N D S GA E P R		IASKFLCTPGCAKTGSFNSYCC
Epidermin	MEAVKEKNDL F N L D V KVNAKES N D S GA E P R		IASKFICTPGCAKTGSFNSYCC
Pep5	MKNKNL F D L E I KKETSQNT D -ELE P Q		TAGPAIRASVKQCQKTLKATRLFTVSCKGKNGCK
Class II lantibiotics			
Lactacin 481	MKEQNSFNLLQ E V T E S E L D L I L G A		KGGSGVIHTISHECNMNSWQFCFTCCS
Mutacin II	MNKLNSNAVSLN E V S D S E L D T I L G G		NRWWQGVVPTVSYECRMNSWQHVTCC
Ruminococcin A	MRNDVLTLTN P M E E K E L E Q I L G G		GNGVLKTIHECNMNTWQFLFTCC
Nukacin ISK-1	MENSKVMKDIEVANLLE E V Q E D E L N E V L G A		KKKSGVIPTVSHDCHMNSFQFVFTCCS
Variacin	MTNAFQALDE V T D A E L D A I L G G		GSGVIPTISHECHMNSFQFVFTCCS
Streptococcin A-FF22	MEKNNEVINSIQ E V S L E E L D Q I G A		GKNGVFKTISHECHLNTWAFATCCS
Butyriovibriocin OR79	MNKELNALTNP I D E K E L E Q I L G G		GNGVIKTISHECHMNTWQFIPTCCS
Salivaricin A	MKNSKDILNNAIE E V S E K E L M E V A G G		KRGSGWIATITDDCPNSVFVCC
Sublancin 168	MEKLFKE V K L E E L E N Q K G S		GLGKAQCAALWLQCASGGTIGCGGGAACQNYRQFCR
Non-lantibiotic bacteriocins			
PedA	-----MKKIE K L T E K E M A N I I G G		
ComC	--MKNTVKLEQ F V A L K E K D L Q K I K G G		
CvaC	-----M R T L T L N E L D S V S G G		
ChnB2	-----M N S V K E L N V K E M Q L H G G		
LcnGα	-----M K E L S E K E L R E C V G G		
LcnGβ	MKNNNN-FFKG M E I I E D Q E L V S I T G G		
LcnA	MKNQLN-FN----I V S D E L S E A N G G		

FIGURE 5: Sequence alignment of lantibiotic and nonlantibiotic bacteriocin prepeptides. The residues in red indicate the positions that are fully conserved within that class, and those in blue are highly conserved. For the nonlantibiotic bacteriocins, only the leader sequences are shown. The site of proteolysis is indicated by the arrow.

(LctSpaS) resulted in a peptide that was a substrate for His₁₀-LctT150 although, under identical assay conditions, it was not cleaved to the same degree as the class II chimeric substrates (Figure 6C). LctNisA was not a substrate for His₁₀-LctT150 (Figure S6c).

DISCUSSION

The biosynthesis of lantibiotics has been extensively studied for several decades, but relatively little is known about the function of the leader peptides. Several roles have been proposed, including acting as a scaffold for the modification enzymes, allowing the prepeptide to be recognized for export, or protecting the producing organism (9). The leader sequences within each lantibiotic class share several conserved residues that could contribute to these functions (Figure 5). Thus far, studies on the importance of these residues have been conducted via *in vivo* mutagenesis in which the leader sequence was changed in a lantibiotic producing bacterial strain. For class I lantibiotics, such studies on nisin biosynthesis demonstrated that the highly conserved leader residues in the -18 to -15 region (Figure 5) are essential for lanthionine incorporation into the precursor peptide, while Ala-4 and Arg-1 are essential for processing by NisP (10). This same region of the Pep5 precursor peptide, however, appears unimportant for correct modification to the mature species, but is important for biosynthetic efficiency (29). Similarly, five mutations of conserved residues in the leader peptide of the class II lantibiotic mutacin II (E-13K, V-12L, E-8K, L-7K, and I-4D) resulted in decreased production of mutacin II as compared to a wild-type *Streptococcus mutans* strain. Two MutA mutant peptides, G-1A and G-2A, prevented the cleavage and/or transport event for production of mutacin II, as dehydrated premutacin accumulation was observed in the cell membranes of these mutant strains (15). The G-2A mutation in the LctA peptide also dramatically reduced *in vivo* cleavage by LctT (13).

Although these studies have provided valuable insights into the importance of certain leader peptide residues for lantibiotic production, to date a comprehensive analysis of their significance for lanthionine formation and proteolytic processing is not available. In this work, we dissected the importance of leader peptide residues for these two processes, reporting on the effects of alteration of the substrate sequence with respect to dehydration and cyclization in the accompanying paper (22), and disclosing in this study the first *in vitro* analysis of the importance of the leader peptide for proteolytic processing.

Because the bifunctional LctT transporter/protease is an integral membrane protein of 691 amino acids (6), we developed a heterologous expression system for only the protease domain. Analogous to similar studies on the protease domain of bifunctional transporters for nonlantibiotic bacteriocins (24-26), expression in *E. coli* of the first 150 residues of LctT resulted in an active protease that correctly hydrolyzed the amide bond between Ala-1 and Lys1 in LctA. These findings indicate that the transmembrane transporter domain of LctT is not required for substrate recognition and proteolysis. Furthermore, His₁₀-LctT150 processed LctM-treated LctA containing the three lanthionine rings about equally well as the linear LctA peptide, demonstrating that the rings are not responsible for substrate recognition. This finding is in contrast to the serine proteases NisP and PepP involved in removal of the leader peptides of the class I lantibiotics nisin and Pep5, for which linear and dehydrated NisA and PepA were not substrates *in vivo* (17, 37). Håvarstein and co-workers proposed that transport and proteolysis of prebacteriocins by ABC transporter maturation and secretion (AMS) proteins may be intimately connected and that substrate recognition for transport may reside in the protease domain (24). Our observation that proteolysis by His₁₀-LctT150 does not depend on the presence of lanthionine rings suggests that

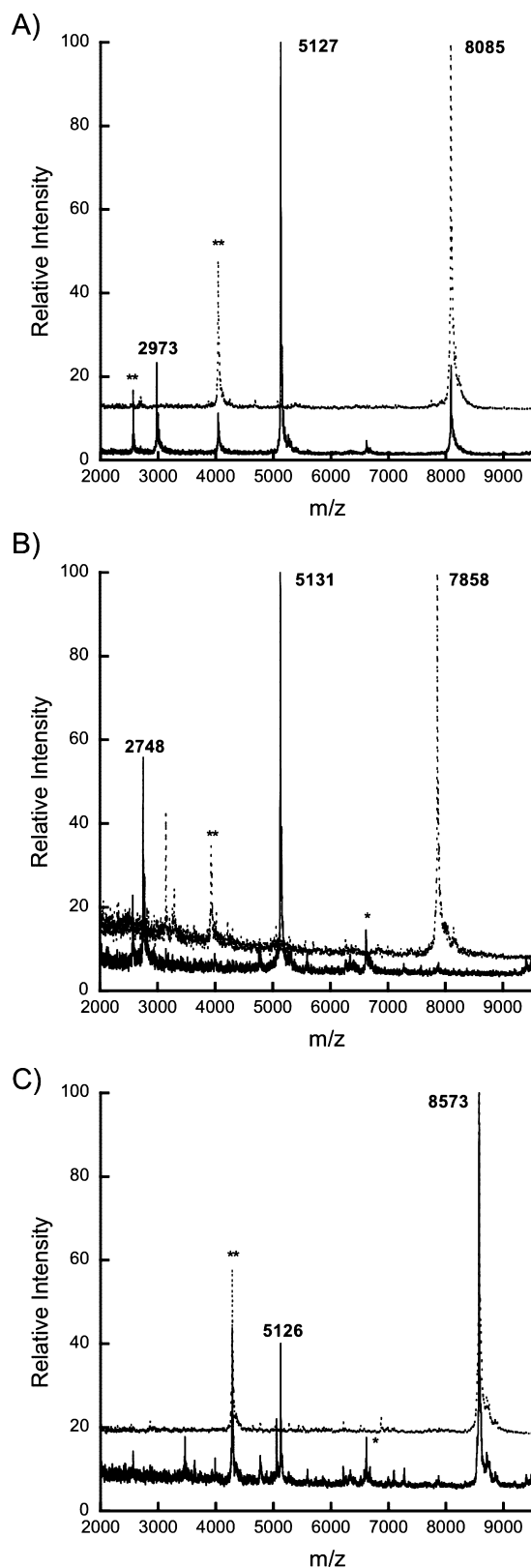


FIGURE 6: MALDI mass spectra for (A) His₆-MutLctA, (B) His₆-LctRumA, and (C) His₆-LctSpaS after incubation with (solid lines) or without (dotted lines) His₁₀-LctT150. The double asterisk (**) indicates a peak corresponding to doubly charged starting material, and the single asterisk (*) to a triply charged His₁₀-LctT150 ion.

AMS proteins can be used to secrete nonlantibiotic peptides attached to the leader sequence of their cognate bacteriocin, similar to previous reports demonstrating such activity for the NisT transporter involved in nisin biosynthesis (17, 19, 38).

Based on sequence homology with papain-like cysteine proteases in the MEROPS database (39), the class II lantibiotic LanT proteins have been proposed to utilize a conserved Cys-His pair in the active site of their protease domains (Figure 7). Although no structural information is currently available on protease domains of bifunctional ABC-type transporters, mutational analysis of the protease domains of the nonlantibiotic transporters LagD, ComA, and CvaB support the importance of these two residues (24, 25). Our results provide additional experimental support for this model for the protease domains of the lantibiotic subgroup of AMS proteins as C12A and C12S mutants of His₁₀-LctT150 were devoid of proteolytic activity. Interestingly, mutation of the putative catalytic histidine to alanine did not prevent cleavage but caused incorrect proteolysis after Gly-2 instead of Ala-1. This surprising result indicates that His90 is not essential for catalysis under the assay conditions employed, and may instead play a role in the regiospecificity of amide bond hydrolysis. This observation is in contrast to previous studies on cleavage of the colicin V precursor by the protease domain of CvaB and hydrolysis of ComC by ComA in which mutation of the corresponding His to Ala abolished protease activity (25, 26). Similar to mutation of His90, replacement of the conserved Asp residue with Ala in His₁₀-LctT150 did not affect the cleavage reaction, suggesting that Asp106 is not essential for catalysis. This residue has not been investigated in LagD, ComA, or CvaB. One possible explanation for the apparent unimportance of His90 and Asp106 involves the protonation state of the protein. The assays in this study were conducted at pH 7.5, but lactacin 481 production has been reported to be induced at pH 5 in the lactic acid bacterium *Lactococcus lactis* (40). If the active site of LctT is in an environment of low pH, Cys may be protonated in the absence of His90 and/or Asp106, resulting in an inactive protease. Unfortunately, we were unable to test this hypothesis, as His₁₀-LctT150 was unstable at pH 5. It has been reported that the activity of the protease domain of CvaB is Ca²⁺ dependent, but like ComA (25) no metal requirement was observed for LctT150.

The cleavage site for removal of leader peptides of certain members of both lantibiotic and nonlantibiotic bacteriocins is governed by GG or GA, historically called the double Gly motif (41–44). As mentioned above, the importance of this motif for lantibiotic biosynthesis has been investigated through a limited set of mutations. In nonlantibiotic systems, the motif has been probed in one *in vitro* study that showed that mutation of either of the two Gly in the ComC substrate decreased the catalytic efficiency of proteolysis by the protease domain of ComA by 500- to 1000-fold (25). In this investigation, an extensive analysis of the double Gly motif showed that mutation of either Gly-2 or Ala-1 to a charged residue completely abolished His₁₀-LctT150 protease activity. Exchange of Ala-1 for Ile also resulted in loss of peptidase activity. Interestingly, mutation of Gly-2 to Val did not prevent cleavage but did reduce the efficiency, as only small amounts of cleavage products were observed under conditions that led to full conversion of His₆-LctA. Only the LctA A-1G mutation allowed His₁₀-LctT150 to retain good proteolytic activity.

With the exception of mutations of Glu-8, replacement of the other conserved residues did not dramatically alter proteolytic processing by His₁₀-LctT150. Glu-8 is also

CvaB	24	IHQ T ETAE CG -LACLAMICG (60)	FS H FVVLVSVKRNRV LH DPA	123
LctT	4	VL Q NNEQ DCL -LACYSMILG (65)	DN H FVVVTKIYRKNVTL ID PA	108
ComA	9	RP Q VDQMD CG -VASLAMVFG (76)	LL H YYVVTGQDKDSIH IA DPD	114
LcnC	9	TS Q VDEMD CG -CAALSMILK (76)	YP H YYVITGANKNSVFI AD PD	114
papain	17	KN Q GSCGSCWAFSAVVTIEG (120)	VD H AAVAVGYGPNYILIK NS W	177
		* *	* *	

FIGURE 7: Sequence alignment of the protease domain of LctT with papain and the nonlantibiotic bacteriocin peptidase domains of CvaB, ComA, and LcnC. The first and last numbers indicate amino acid positions, and the numbers in parentheses denote the number of excluded residues. The conserved active site residues Cys-His-Asp/Asn and the oxyanion hole Gln are highlighted in blue and marked with asterisks.

implicated as an important residue for proteolysis and/or transport by its conservation in the double Gly type leader peptides of nonlantibiotic bacteriocins (Figure 5). Since these peptides do not undergo any post-translational modifications other than proteolysis (43), the conservation of Glu-8 is likely to be related to proteolysis and secretion. With respect to the observation that mutation of the other conserved residues did not prevent proteolysis, it is important to emphasize that we have been unable to obtain kinetic parameters because of the insolubility of LctA, despite various attempts with truncated synthetic peptides. As a result, we have no information regarding k_{cat} and K_m values and some of the mutants might still be poor substrates under physiological conditions of low substrate concentration as shown for instance for L-7E, E-13A, and D-6P.

As discussed in the accompanying paper, secondary structure predicting programs anticipate α -helical character for the LctA leader peptide between Asn-17 and Leu-3 (22). A helical structure of lantibiotic leader peptides has been suggested previously on the basis of circular dichroism experiments performed with synthetic leader peptides of class I lantibiotics nisin, subtilin, gallidermin, epidermin, and Pep5 (45, 46). These studies were carried out in the presence of trifluoroethanol, which induces helicity, but the biosynthetic proteins may similarly promote or recognize a helical structure, as has recently been demonstrated for binding of the prepeptide ComC to the ComA peptidase domain (27). Circular dichroism and NMR studies also demonstrated α -helical character in the -15 to -5 region of the leader sequence for the nonlantibiotic bacteriocin carnobacteriocin B2 (47). This helix was proposed to be a key recognition element for export and processing and possibly also for keeping the bacteriocin inactive until removal of the leader peptide. In the current work, four of the five Pro mutants in the predicted helical region of His₆-LctA, V-12P, E-8P, L-5P, and I-4P, were poor substrates for His₁₀-LctT150, indicating that putative secondary structure of the leader may indeed be important for substrate recognition. Surprisingly, D-6P was a relatively good substrate being processed about 60% at the highest concentrations allowed by its solubility limit. Under these conditions, wild-type LctA is almost completely proteolyzed. At low substrate concentration, however, D-6P also was a poor substrate, indicating its reduced ability to bind to the enzyme. Hence, all Pro mutants appear to reduce the efficiency of processing by His₁₀-LctT150.

The recognition of a helical secondary structure by LctT may also explain the additional incorrect processing observed when three Ala residues were inserted between K1 and G2 (LctA-K1G2insAAA). Secondary structure predicting programs indicate an extension of the α -helical region of the leader peptide in this mutant to the second inserted Ala

residue. If the active site of LctT is positioned such that it cleaves at a sequence of two consecutive small amino acids (Gly or Ala) immediately C-terminal to a helical region, the observed cleavage between Gly5 and Gly6 and between Gly6 and Ser7 in this mutant can be rationalized. According to this hypothesis, the reason for the lack of cleavage between these residues in wild-type His₆-LctA (in wt LctA these residues are Gly2, Gly3, and Ser4) is that they are not located immediately C-terminal to a helical region. If this hypothesis is correct, LctT150 and other such domains of AMS proteins may have interesting potential for application in biotechnology to remove expression tags similar to the use of Factor Xa, PreScission protease, thrombin, and enterokinase. The demonstrated high promiscuity of His₁₀-LctT150 with respect to the residues C-terminal to the cleavage site as well as the high fidelity with which it cleaved those peptides further highlights its potential, although at low substrate concentrations several of the mutants in which the propeptide was mutated were processed more slowly than wild-type LctA (Figure S4). Because LctT150 appears to recognize only the residues N-terminal to the cleavage site, tag removal is traceless.

A different strategy was employed to probe the importance of nonconserved residues for proteolytic processing to test the possibility that the determinants for substrate specificity for a particular lantibiotic are imparted by the nonconserved residues that differ from compound to compound. The chimeric substrate His₆-MutLctA consisting of the mutacin II leader peptide and lacticin 481 propeptide contained 12 simultaneous mutations of mostly nonconserved residues (Figure 5). Nevertheless, it was processed by His₁₀-LctT150 with about the same efficiency as the LctA propeptide, indicating that these residues are not important for substrate recognition and catalysis. A similar conclusion was reached for the dehydration and cyclization events in the accompanying paper (22).

As previously discussed, formation of the lanthionine rings was also not required for proteolysis by LctT150, suggesting that the propeptide is not important for substrate recognition. However, the N-terminus of lacticin 481 has a linear sequence of eight residues (Figure 1), which might be important for leader sequence removal. Nonconservative mutations of residues in the P1'-P5' positions (K1A, G3I, G5H), however, did not affect proteolysis by His₁₀-LctT150, and several chimeric peptides containing multiple simultaneous mutations in these positions (e.g., P1', Asn, Trp, Gly; P2', Lys, Arg, Asn; P3', Ser, Trp, Gly, Lys (Table 3 and Figure 5)) were likewise processed. Thus, the residues C-terminal to the cleavage site are not important, which may also explain why some two-component lantibiotic biosynthetic gene clusters contain only a single LanT protein despite dramatic differences between the two propeptides of their

substrates (12, 48). A possible exception to the high promiscuity with respect to the residues C-terminal to the cleavage site may be a branched residue at the P1' position because the nisin chimera with an Ile at this position was not accepted. An alternative explanation for the lack of activity with LctNisA is that secondary structure prediction programs anticipate an α -helix for the leader sequence of LctNisA in addition to β -strand character C-terminal to the cleavage site. The presence of this additional secondary structure may be responsible for inhibition of protease activity.

In summary, the double Gly motif is critical for proteolysis with only an Ala \rightarrow Gly mutation in LctA tolerated by the protease. Collectively, the results with mutations of conserved residues to Pro residues support the hypothesis that the region immediately N-terminal to the cleavage site is recognized as a helix by His₁₀-LctT150. Interestingly, Glu-8 appears important for substrate recognition by LctT but mutations other than Pro are tolerated at this position by LctM. Thus, it appears that different residues of the leader peptide are important for specific events in the overall post-translational modification process of lacticin 481 biosynthesis.

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SUPPORTING INFORMATION AVAILABLE

Sequences of oligonucleotide primers used for site-directed mutagenesis of LctT and mass spectrometric data on proteolysis of LctA peptides. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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