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Involvement and Unusual Substrate Specificity of a Prolyl Oligopeptidase in Class III Lanthipeptide Maturation

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Supporting Information

ABSTRACT: Lanthipeptides represent an important group of ribosomally synthesized and post-translationally modified peptides (RiPPs). Commonly, in the last steps of their maturation, a part of the peptide, termed the leader, is removed, providing the active compound. This contribution describes for the first time the identification of a protease involved in the removal of the leader peptide of a class III lanthipeptide. Four putative class III biosynthetic gene clusters were identified in bacterial genomes, each containing a gene encoding a prolyl oligopeptidase (POP). Further in vitro investigations of the gene cluster from *Kribbella flavida*, involving reconstitution of the biosynthesis of the new lanthipeptide flavipeptin, proved that a POP-type FlaP protease is responsible for leader removal. Interestingly, detailed in vitro studies of the substrate specificity revealed that FlaP is specific to the post-translationally modified peptide and can discriminate between N- and C-terminal rings. Therefore, it has been shown for the first time that factors other than size and amino acid sequence might be involved in substrate recognition by POPs.

Over the past decade, substantial progress has been made in the identification and characterization of new ribosomally synthesized and post-translationally modified peptides (RiPPs).[1] Among these, the lanthipeptides comprise one of the most thoroughly investigated groups.[2,3] The biosynthesis of lanthipeptides commences with the ribosomal synthesis of the precursor peptide, which is composed of two segments: an N-terminal leader and a C-terminal core peptide. The latter undergoes post-translational modifications (PTMs) that introduce lanthionine (Lan, class I–IV) or labionin (Lab, class III) bridges (Figure 1). The presence of Lan is a unifying feature of lanthipeptides, and Lab is an unusual structural variation of Lan extended by another amino acid moiety (Figure 1B). Many studies devoted to the role of the leader peptide have shown that it is essential for recognition and processing by the modifying enzymes[4−7] and the export system[8,9] and also provides a self-protection mechanism that keeps the peptide inactive.[10] The proteolytic removal of the leader peptide is usually the final step of lanthipeptide maturation. The corresponding protease can be encoded in the gene cluster as a stand-alone protein (LanP; e.g., extracellular NisP[10] and cytoplasmic PepP[11]) or as an N-terminal domain of an ABC transporter[12] (LanT; e.g., lacticin 481[13]). Alternatively the proteolytic removal of the leader peptide can be accomplished by a protease located elsewhere in the genome

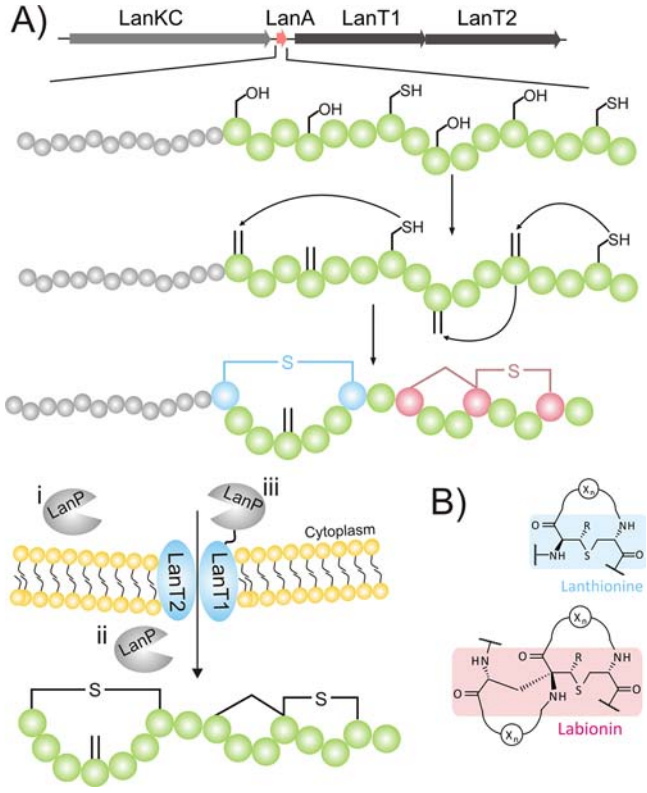

Figure 1. Principles of lanthipeptide biosynthesis. (A) A ribosomally synthesized precursor is composed of the leader (gray) and the core (green) peptides. The latter accommodates post-translational modifications, including dehydrations and subsequent cyclizations to form lanthionine (Lan) and/or labionin (Lab) (B). Commonly, the final step of maturation is proteolytic removal of the leader peptide, which occurs (i) intracellularly, (ii) extracellulary, or (iii) in a transporter-associated fashion.

(e.g., subtilin).[14] To date, seven proteases have been characterized in the maturation of class I (nisin A,[8] epidermin,[15] epilancin 15X[16]) and class II (nukacin,[17] lacticin 481,[13] cytolysin,[18] lichenicidin[19]) lanthipeptides. In contrast, for the relatively new class III lanthipeptides, removal of the leader peptide remains an unexplored aspect. None of the class III gene clusters described to date contains a gene encoding a protease or a protease domain embedded in the LanT protein. Furthermore, recent investigations of class III lanthipeptide biosyntheses are in

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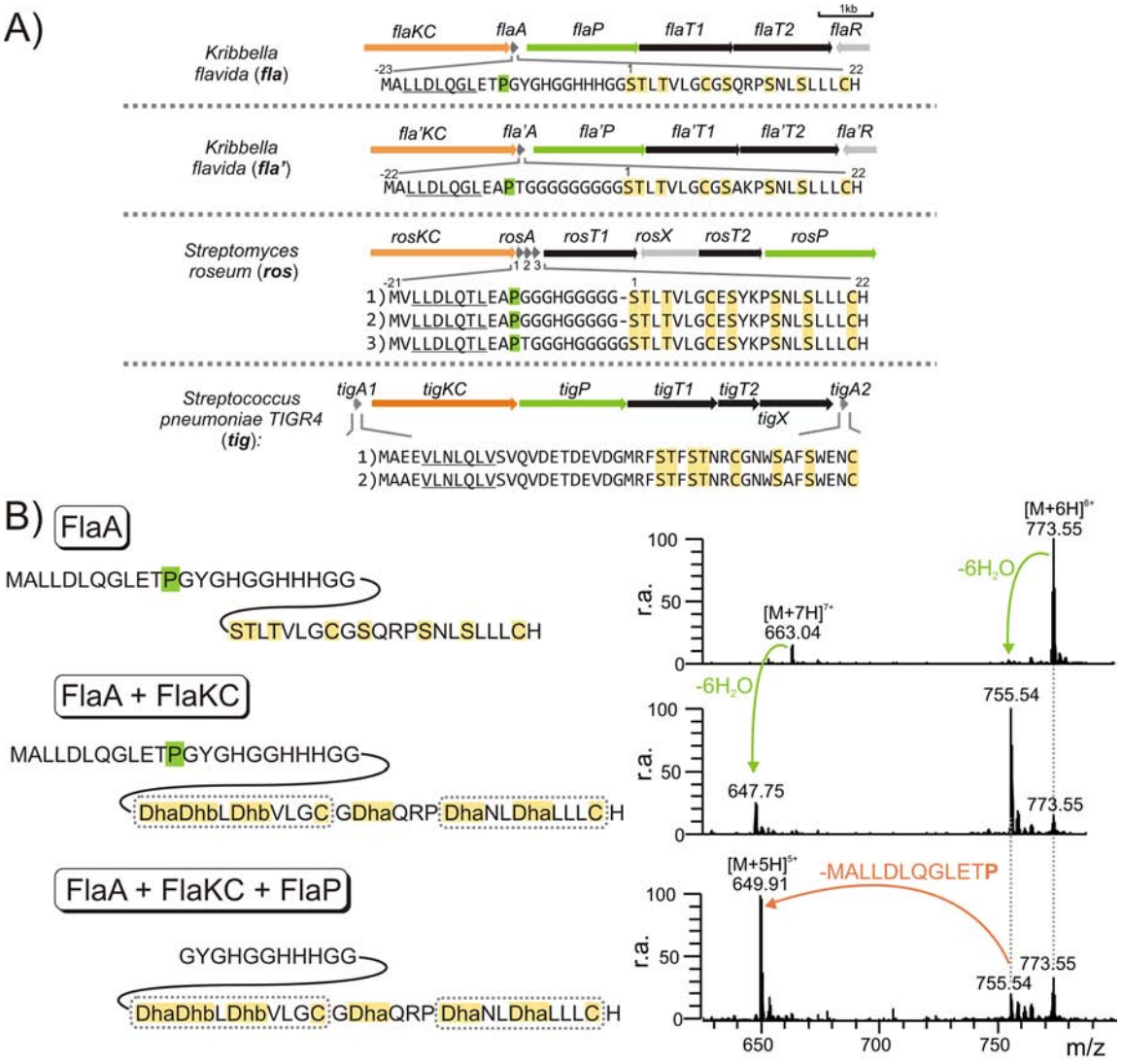

C)

Mutant	Sequence	Cleavage efficiency [%]	Ring presence N-term	Ring presence C-term
	-23 -10 1 10 22			
FlaA(linear)	MALLDLQGLETPGYGHGGHHHGGSTLTVLGCGSQRPSNLSLLLCH	ND	(-)	(-)
FlaA	MALLDLQGLETPGYGHGGHHHGGSTLTVLGCGSQRPSNLSLLLCH	72 ± 5	(+)	(+)
$FlaA_{C8A,C21A}$	MALLDLQGLETPGYGHGGHHHGGSTLTVLGAGSQRPSNLSLLLAH	8 ± 2	(-)	(-)
$FlaA_{S/T\rightarrow A}$	MALLDLQGLETPGYGHGGHHHGGAALAVLGCGAQRPANLALLLCH	8 ± 2	(-)	(-)
$FlaA_{S10A,S14A,S17A}$	MALLDLQGLETPGYGHGGHHHGGSTLTVLGCGAQRPANLALLLCH	9 ± 4	(+)	(-)
$FlaA_{S1A,T2A,T4A}$	MALLDLQGLETPGYGHGGHHHGGAALAVLGCGSQRPSNLSLLLCH	53 ± 2	(-)	(+)
$FlaA_{\Delta(9-22)}$	MALLDLQGLETPGYGHGGHHHGGSTLTVLGC----------	12 ± 1	(-)	(-)
$FlaA_{\Delta(1-13)}$	MALLDLQGLETPGYGHGGHHHGG----------SNLSLLLCH	51 ± 1	(-)	(+)
$FlaA_{S17A}$	MALLDLQGLETPGYGHGGHHHGGSTLTVLGCGSQRPSNLALLLCH	20 ± 1	(+)	(-)
$FlaA_{S14A}$	MALLDLQGLETPGYGHGGHHHGGSTLTVLGCGSQRPANLSLLLCH	20 ± 1	(+)	(-)
Leader	MALLDLQGLETPGYGHGGHHHGG	ND		

Figure 2. (A) Class III lanthipeptide gene clusters encoding prolyl oligopeptidases (LanP, in green), with sequences of the corresponding precursor peptides. The conserved hydrophobic patches in the leaders are underlined, and the putative PTM sites are marked in yellow. (B) Flavipeptin biosynthesis. Processing of the precursor peptide FlaA by the modifying enzyme FlaKC in the presence of ATP and Mg^{2+} results in a 6-fold dehydrated and cyclized product (localizations of rings are marked with dashed lines). Further incubation with FlaP results in the proteolytic removal of the leader peptide. (C) Cleavage efficiencies of different peptide substrates modified with FlaKC (2 h incubation with FlaP). For all of the mutant peptides, full dehydration was observed. The cyclizations in the N- and C-terminal regions of the core peptide were evaluated by MS/MS analysis and are indicated with (+)/(−).

favor of a sequential removal of the leader in a multistep process.[20−22] The presence of lanthipeptides with different leader peptide overhangs suggests that in the first step a part of the leader is removed by an endopeptidase, with the remaining overhang being successively degraded by aminopeptidases. Such behavior was observed for the labyrinthopeptins erythreapeptin,

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curvopeptin, and avermipeptin,[20−22] but none of the proteases involved in the maturation of class III lanthipeptides has been identified.

In a genome mining survey for novel class III lanthipeptides, we identified four putative gene clusters encoding proteins with homology to prolyl oligopeptidases (POPs) (Figure 2A). POPs belong to a distinct group of serine proteases that were reported to cleave small peptides C-terminally of a Pro residue.[23,24] It was demonstrated that human POPs play a pivotal role in the processing of several important hormone peptides and neuropeptides.[25] Structures of different POPs have been solved, revealing a characteristic architecture. POPs are composed of two domains, an N-terminal serine protease domain and a C-terminal β-propeller domain that covers the active site and therefore serves as a substrate selection filter.[26] To date, no POP involved in the maturation of lanthipeptides has been described. Instead all of the identified lanthipeptide proteases belong to the papain and subtilin-like protease families.[2] The only POP involved in natural product maturation was reported for mushroom toxin biosynthesis (amanitin and phalloidin).[27−29]

Therefore, it was important to provide more details about the identified POP with respect to lanthipeptide processing. Interestingly, the *Kribbella flavida* DSM 17836 genome contains two homologous class III gene clusters (Figure 2A). For the studies described herein, we selected the cluster encoding genes for the synthetase FlaKC (accession number YP_003384295) and the POP-like protein FlaP (designated as the *fla* gene cluster, whose putative product is named flavipeptin). Importantly, the sequence of a precursor peptide (FlaA) contains a Pro residue in the leader peptide, which could serve as the primary cleavage site for FlaP (Figure 2A). In addition, the FlaA sequence displays other characteristic features of class III precursor peptides, namely, a conserved hydrophobic patch (LLDLQ) in the leader and a double Ser/Ser(Thr)/Cys motif. In silico analysis of the FlaP sequence did not indicate the presence of any signal peptide, suggesting cytoplasmic localization of the protein. It was possible to express heterologously and purify soluble His_6-tagged FlaP (FlaP-His_6) from *Escherichia coli* [see the Supporting Information (SI)]. However, subsequent incubation of FlaP with a synthetic linear FlaA peptide did not result in proteolytic cleavage of the precursor peptide as monitored by LC−ESI-MS. This suggested that the protease FlaP may be specific to the modified peptide containing dehydrated residues and/or intramolecular Lan/Lab bridges.

To test this hypothesis, the biosynthesis of flavipeptin was reconstituted in vitro to provide a mature flavipeptin still attached to its leader peptide. We expressed the modifying enzyme FlaKC as a C-terminal fusion protein with a His_6-SUMO domain because of solubility problems encountered using different constructs. Incubation of FlaA with His_6-SUMO-FlaKC in the presence of ATP and Mg^{2+} for 2 h (for details, see the SI) resulted in a mass shift of −108 Da, corresponding to a sixfold dehydration (Figure 2B and Figure S2 in the SI). The presence of rings and their topologies were further evaluated by MS/MS product-ion analysis (see the SI).

In the subsequent step, the ability of FlaP to process the leader peptide still attached to the modified flavipeptin was evaluated. After enzymatic conversion of FlaA with FlaKC, protease FlaP was added, and the mixture was incubated for an additional 2 h. Subsequent LC−ESI-MS analysis revealed the presence of two peptides resulting from the hydrolysis of a peptide bond between Pro(−12) and Gly(−11) (Figures 2B and S4). This result clearly indicates that FlaP is a Pro-specific protease that cleaves the leader peptide C-terminally of Pro(−12). Remarkably, FlaP is specific for the modified peptide only. This finding is in contrast to the observed behavior of EpiP (epidermin, class I)[15] and LctT150 domain (lacticin 481, class II)[13] proteases, which do not discriminate between modified and unmodified peptide. The only reported example of a POP that is involved in the removal of the leader of a RiPP, namely, that for the fungal toxin phalloidin, also cleaves an unmodified linear peptide.[29] On the other hand, in vivo studies on nisin A suggest that extracellular NisP cleaves only dehydrated and cyclized peptide, which is in line with the specificity of FlaP observed herein.[8] Remarkably, flavipeptin attached to the leader peptide is the largest POP substrate described to date.

At this point of the study, the structural requirements of the flavipeptin peptide for recognition and processing by FlaP were still unclear. This includes the presence of didehydroalanine (Dha)/didehydroaminobutyric acid (Dhb) and/or intramolecular rings introduced by the modifying enzyme FlaKC. We therefore exploited the advantage of the in vitro reconstituted flavipeptin biosynthesis to study the substrate specificity of the protease. Its activity was assessed on the basis of the depletion of modified flavipeptin still attached to its leader peptide upon incubation with FlaP (for details, see the SI). In the first step, we tested the mutant peptide $FlaA_{C8A,C21A}$, which is unable to form rings because of the absence of Cys thiol side chains. Incubation of this peptide with the modifying enzyme FlaKC yielded a sixfold-dehydrated peptide. However, subsequent incubation with FlaP resulted in inefficient cleavage in comparison with fully modified FlaA (Figure 2C). Similarly, substitution of all of the Ser and Thr residues with Ala ($FlaA_{S/T\rightarrow A}$) did not allow for efficient cleavage of the leader. These results indicate that ring formation is crucial for the recognition by the protease, as the linear dehydrated peptide was not processed. However, the partial cleavage of $FlaA_{S/T\rightarrow A}$ and $FlaA_{C8A,C21A}$, in contrast to the complete absence of proteolytic activity observed for unmodified FlaA, suggests that polar hydroxyl and thiol side chains play some role in preventing hydrolysis of the nonprocessed peptide.

To gain a better understanding of the structural features required for recognition by FlaP, in the next step we substituted Ser and/or Thr residues in either the N- or C-terminal parts with Ala ($FlaA_{S10A,S14A,S17A}$ and $FlaA_{S1A,T2A,T4A}$). Interestingly, depriving the peptide of the N-terminal ring formation capabilities by this approach had very little effect on the FlaP activity, whereas preventing the formation of the C-terminal ring hampered efficient cleavage (Figure 2C). Inspired by this result, we synthesized two truncated mutants, $Fla_{\Delta(1-13)}$ and $Fla_{\Delta(9-22)}$, which from the core peptide contain only the N- or C-terminal partial sequences, respectively. While both peptides were modified by FlaKC, we were surprised to find that $Fla_{\Delta(1-13)}$ was cleaved much more efficiently than $Fla_{\Delta(9-22)}$. Further MS/MS investigation revealed that the $Fla_{\Delta(9-22)}$ mutant did not contain a ring structure (Figures S8 and S9). Altogether, these results indicate that FlaP is able to recognize the presence of rings and even discriminates between N- and C-terminal rings. Moreover, incubation of the leader peptide alone did not result in the cleavage, additionally proving the importance of the cyclized core peptide for proteolysis.

We further tested in more detail the importance of the C-terminal region of FlaA for protease recognition by making single Ser → Ala substitutions ($FlaA_{S14A}$ and $FlaA_{S17A}$). MS/MS analyses of the mutant peptides incubated with FlaKC revealed that despite full dehydration they were devoid of C-terminal rings (Figures S10 and S11). Nevertheless, both peptides after

modification were cleaved by the protease FlaP with decreased efficiency relative to fully modified FlaA (Figure 2B). We propose that the enhanced cleavage of the $FlaA_{S17A}$ and $FlaA_{S14A}$ mutants relative to $FlaA_{S10A,S14A,S17A}$ results from a partial spontaneous cyclization. This observation also further highlights the importance of the C-terminal part for proteolytic processing.

Finally, we tested whether the Pro residue in the leader is mandatory for processing by FlaP. We synthesized four different single-substitution mutants, Pro(−12) → Ala/Gly/Val/Lys. All four mutants were efficiently modified by the synthetase FlaKC (Figures S12−14). As shown in Table S3 in the SI, FlaP tolerates nonpolar substitutions by Ala, Gly, and Val. Interestingly, the introduction of Val resulted in additional cleavage sites, as this peptide was cleaved not only between Val(−12) and Gly(−11) but also between Gly(−11) and Tyr(−10) and Tyr(−10) and Gly(−9) (Table S3). Likewise, the substitution with sterically more demanding and positively charged Lys did not inhibit the proteolysis but instead shifted the cleavage site further in the direction of the C-terminus. Hence, cleavage was observed only between Gly(−11) and Tyr(−10) and between Tyr(−10) and Gly(−9) (Table S3 and Figure S14). These results indicate that FlaP has a relaxed specificity toward residues other than Pro and also displays significant flexibility in the positioning of the peptide relative to the P′1−P1 site.

In summary, we have identified for the first time a protease (FlaP) involved in the removal of the leader peptide of a class III lanthipeptide. This is also the first prolyl oligopeptidase involved in the maturation of a lanthipeptide. Further studies, including in vitro reconstitution of the biosynthesis of the novel lanthipeptide flavipeptin, enabled a detailed characterization of FlaP substrate specificity. Remarkably, the protease does not process linear and dehydrated peptide. It was shown that FlaP requires the presence of intramolecular rings; in particular, the C-terminal ring plays the most important role. Importantly, our results indicate for the first time that factors other than size and sequence context of the cleavage site might be involved in POP substrate recognition.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures and MS data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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