Heterologous expression of cryptic biosynthetic gene cluster from Streptomyces prunicolor yields novel bicyclic peptide prunipeptin

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4 Authors: Kohta Unno[1] and Shinya Kodani[1,2*]

5 Affiliations: Graduate School of Integrated Science and Technology, Shizuoka

6 University, Shizuoka, Japan[1]; College of Agriculture, Academic Institute, Shizuoka

7 University, Shizuoka, Japan[2]

8 *To whom correspondence should be addressed: Shinya Kodani, College of

9 Agriculture, Academic Institute, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka

10 422-8529 Japan, Tel/Fax; +81(54)238-5008, E-mail; kodani.shinya@shizuoka.ac.jp,

11 ORCID:0000-0002-6792-1184

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Abstract

Recently, ω-ester-containing peptides (OEPs) were indicated to be a class of ribosomally synthesized and post-translationally modified peptides. Based on genome mining, new biosynthetic gene cluster of OEPs was found in the genome sequence of actinobacterium *Streptomyces prunicolor*. The biosynthetic gene cluster contained just two genes including precursor peptide (*pruA*) and ATP-grasp ligase (*pruB*) coding genes. Heterologous co-expression of the two genes was accomplished using expression vector pET-41a(+) in *Escherichia coli*. As a result, new OEP named prunipeptin was produced in this system. By site-directed mutagenesis experiment, a variant peptide prunipeptin 15HW was obtained. The bridging pattern of prunipeptin 15HW was determined by combination of chemical cleavage and MS experiments. As a result, prunipeptin 15HW possessed bicyclic structure with an ester bond and an isopeptide bonds. The novel ATP-grasp ligase PruB was indicated to catalyze two different intramolecular bonds (ester and isopeptide bonds).

Keywords: heterologous expression; ATP-grasp ligase; biosynthesis; ω-ester-containing peptide

1. Introduction

Ribosomally synthesized and post-translationally modified peptides (RiPPs) are a class of naturally occurring peptides that includes more than twenty subclasses such as lantibiotics (Budisa, N., 2013; Letzel, A. C. et al., 2014; Link, A. J., 2015; Li, K. et al., 2016; Sardar, D. and Schmidt, E. W., 2016; Zhang, Y. et al., 2018a; Zhong, Z. et al., 2020). Recently, new class of RiPPs: ω-ester-containing peptides (OEPs) was advocated (Lee, H. et al., 2020). The BGCs of OEPs commonly contains minimum set of two essential genes including precursor and ATP-grasp ligase coding genes. Bioinformatic analysis on bacterial genomes disclosed a total of ~1500 candidate OEPs in 12 groups (Lee, H. et al., 2020). Peptide named plesiocin was accomplished by in vitro synthesis and heterologous expression of biosynthetic genes (group 2) derived from marine myxobacterium *Plesiocystis pacifica* (Lee, H. et al., 2017; Lee, C. et al., 2020). Interestingly, plesiocin contained four repeats of a distinct hairpin-like bicyclic structure containing two ester bonds each and showed potent inhibitory activity against proteases. Peptide named thuringinin (group 3) was produced using BGC derived from *Bacillus thuringiensis serovar huazhongensis* in the same manner with plesiocin (Roh, H. et al., 2019). Like plesiocin, thuringinin had three tandemly repeating hairpin-like structures with two ester bonds each. However, the topology of ester bonds formation was

different from that of plesiocin. In addition, new OEPs (OEP4-1, OEP5-1, OEP6-1) were produced using BGCs of group 4, 5, and 6, respectively (Lee, H. et al., 2020). These OEPs mentioned above had diverse intramolecular bond formation patterns. These data indicated that intramolecular bond forming patterns are mainly dependent on the specificity of ATP-grasp enzyme of each group.

The BGCs of cyanobacterial peptides called microviridins (Ishitsuka, M. O. et al., 1990; Okino, T. et al., 1995; Shin, H. J. et al., 1996; Murakami, M. et al., 1997; Fujii, K. et al., 2000; Rohrlack, T. et al., 2003; Reshef, V. and Carmeli, S., 2006; Dehm, D. et al., 2019) were classified into group 1 of OEPs (Lee, H. et al., 2020). The biosynthesis of microviridins commonly contains two ATP-grasp ligases (MdnB and MdnC) to form two ester and one isopeptide bonds in the molecule (Philmus, B. et al., 2008; Ziemert, N. et al., 2008). The intramolecular macrocyclization mechanism of the core peptide in microviridins is utilized with genetic engineering to generate new bioactive peptides (Ziemert, N. et al., 2010; Weiz, A. R. et al., 2011; Gatte-Picchi, D. et al., 2014; Weiz, A. R. et al., 2014; Reyna-Gonzalez, E. et al., 2016; Ahmed, M. N. et al., 2017; Zhang, Y. et al., 2018b). There are many analogous BGCs of microviridin-type peptides found in the genomes of cyanobacteria (Micallef, M. L. et al., 2015), although they are mostly not explored. The distribution of BGCs of microviridin-type peptides was revealed in

67 the genome sequences of bacteria belonging to the phyla proteobacteria, bacteroidetes,
68 and cyanobacteria (Ahmed, M. N. et al., 2017). Recently, we succeeded in heterologous
69 production of new microviridin-type peptide named grimoviridin using BGC of marine
70 proteobacterium *Grimontia marina* (Unno, K. et al., 2020). Microviridin analogous
71 peptides, marinostatins, were isolated as serine protease inhibitors from a marine
72 bacterium *Algicola sagamiensis* (Imada, C. et al., 1986; Takano, R. et al., 1991).
73 Marinostatins have bicyclic structures with two intramolecular ester bonds.
74 Marinostatins lack of isopeptide bond, although the bridging pattern of two ester bonds
75 of marinostatins is similar to that of microviridins. The solution structure of
76 marinostatin was determined by NMR spectroscopy, and its mechanism of inhibition
77 was proposed based on its 3D structure (Kanaori, K. et al., 2005; Taichi, M. et al.,
78 2010). The biosynthetic gene cluster (BGC) of marinostatin was cloned in *Escherichia*
79 *coli* (Miyamoto, K. et al., 1998). Recently, new marinostatin analogue named
80 marinostatin E was produced by heterologous expression of the BGC in *E. coli* (Unno,
81 K. et al., 2021).
82 Based on these back grounds, we accomplished heterologous production of new
83 bicyclic peptide named prunipeptin and the variants using BGC of actinobacterium

84 *Streptomyces prunicolor*. Here we describe production, isolation and structure

85 determination of prunipeptin and variants.

86

2. Materials and Methods

2.1. Construction of the expression vector pET-41a-13075

The gene cluster of prunipeptin (1190 bp, *pruA* and *pruB*), optimized for *Escherichia coli* codon usage using algorism provided by Eurofins Genomics K.K. (Tokyo, Japan), was chemically synthesized and inserted into the cloning vector pEX-K4J2 by Eurofins Genomics K.K. (Tokyo, Japan) to provide pEX-K4J2-13075. The pEX-K4J2-13075 vector and the pET-41a(+) vector were double-digested with *Xba*I and *Hind*III-HF (New England Biolabs, Ipswich, MA, USA), according to the manufacturer's instructions. The two DNA products: DNA fragment (*pruA* and *pruB*) and digested pET-41a(+) were ligated using Ligation-Convenience Kit (Nippon gene Co., Ltd., Tokyo, Japan) to afford the vector pET-41a-13075. *E. coli* DH5α cells were transformed with 10 μL of the ligation mixture by chemical competence transformation, and the cells were plated on LB agar plates containing kanamycin (final concentration of 30 μg/mL). The vector pET-41a-13075 was purified using FastGene Plasmid Mini Kit (Nippon Genetics Co., Ltd.). Finally, the vector pET-41a-13075 was transformed into the expression host *E. coli* BL21(DE3) by chemical competence method, for the heterologous expression of prunipeptin.

2.2. Site directed mutagenesis on pruA gene

All site directed mutagenesis was performed by the overlap extension PCR reactions (Bryksin, A. V. and Matsumura, I., 2010). The DNA fragment containing mutation was obtained by PCR method using pET-41a-13075 as template DNA and primer sets possessing mutation (Table S1). In a PCR tube, 4.0 μL of dH_2O, 5.0 μL of 2X KAPA HiFi HotStart ReadyMix (Nippon Genetics Co., Ltd.), 0.3 μL of primer-F and primer-R (10 μM), 0.4 μL of the vector pET-41a-13075 (150 pg/μL) were combined. DNA amplification was performed according to the manufacturer's instructions. *E. coli* DH5α cells were transformed with 10 μL of the PCR mixture by chemical competence transformation, and the cells were plated on LB agar plates containing kanamycin (final concentration of 30 μg/mL). The vectors with mutation were purified using FastGene Plasmid Mini Kit (Nippon Genetics Co., Ltd.). Sequencing was carried out by Fasmac Co., Ltd. (Kanagawa, Japan).

2.3. Heterologous expression of prunipeptin and prunipeptin variants

The bacterium *E. coli* BL21(DE3) harboring the pET-41a-13075 vector or the pET-41a-13075 vector with mutation was cultured using two plates of modified basal agar medium containing kanamycin (30 μg/mL, final concentration) and isopropyl-β-D-thiogalactopyranoside (0.1 mM, final concentration) at 30 °C for 24 h. The modified basal agar medium was prepared by adding the inorganic compounds (K_2SO_4, 2 g;

123 K_2HPO_4, 3 g; NaCl, 1 g; NH_4Cl, 5 g; $MgSO_4 \cdot 7H_2O$, 80 mg; $CuCl_2$, 0.5 mg;
124 $MnSO_4 \cdot H_2O$, 0.35 mg; $FeCl_3$, 0.5 mg; $CaCl_2 \cdot 2H_2O$, 0.5 mg) and 15 g agar in 1 L of
125 distilled water with adjusting pH 7.3. After autoclaving, the medium was supplemented
126 with separately sterilized glucose and yeast extract solutions at final concentrations of
127 0.25% and 0.4%, respectively. The bacterial cells were harvested with a laboratory
128 spatula and extracted by two times volume of MeOH. After centrifugation, the MeOH
129 extract was subjected to preparative HPLC using Wakopak Handy ODS column (4.6 ×
130 250 mm, Fujifilm Wako Pure Chemical Co. Osaka, Japan) with gradient mode using
131 two solvents (solvent A: H_2O containing 0.05% TFA and solvent B: MeCN containing
132 0.05% TFA). The gradient was performed by increasing percentage of solvent B from
133 14% to 24% during 20 min. UV detector was set at 220 nm.
134 *2.4. Isolation of a variant peptide prunipeptin 15HW*
135 The bacterium *E. coli* BL21(DE3) harboring the expression vector with the mutation
136 (His to Trp in *pruA*) was cultured using 1 L of modified basal agar medium containing
137 kanamycin (30 μg/mL, final concentration) and isopropyl-β-D-thiogalactopyranoside
138 (0.1 mM, final concentration) at 30 °C for 24 h. The bacterial cells were harvested with
139 a laboratory spatula and extracted by two times volume of MeOH. After centrifugation,
140 the MeOH extract was subjected to preparative HPLC using Wakopak Handy ODS

column (4.6 × 250 mm, Fujifilm Wako Pure Chemical Co.) with isocratic mode (flow rate: 1 mL/min, solvent: 17% MeCN containing 0.05% TFA). HPLC purification was performed repeatedly with the UV detector set at 220 nm to obtain prunipeptin (retention time: 14.0 min). The yield was 2.0 mg from 1 L culture.

2.5. Alkaline hydrolysis of prunipeptin 15HW

Prunipeptin 15HW (0.5 mg) was dissolved in 0.1 N NaOH solution (0.8 mL). The solution was incubated for 30 min at room temperature, followed by neutralization with addition of 1 N HCl solution (80 μL). The mixture was subjected to HPLC analysis to obtain ester opened prunipeptin 15HW. HPLC purification was performed using Wakopak Handy ODS column (4.6 × 250 mm, Fujifilm Wako Pure Chemical Co.) with gradient mode using two solvents (solvent A: H_2O containing 0.05% TFA and solvent B: MeCN containing 0.05% TFA). The gradient was performed by increasing percentage of solvent B from 18% to 23% during 20 min. UV detector was set at 220 nm.

2.6. Treatment of prunipeptin 15HW with BNPS-skatole

Prunipeptin 15HW (0.3 mg) was dissolved in 90% acetic acid (0.5 mL). BNPS-skatole (1.0 mg) was added followed by incubation at 90 °C for 1 h. The solution was evaporated using rotary evaporator. After complete removal of solvent by freeze dryer,

159 $CHCl_3$ and H_2O was added to the reaction mixture to perform two-layer partition. After
160 centrifugation (10000 rpm, for 1 min), H_2O layer was separated and subjected to HPLC
161 purification. HPLC purification was performed using Wakopak Handy ODS column
162 (4.6 × 250 mm, Fujifilm Wako Pure Chemical Co.) with gradient mode using two
163 solvents (solvent A: H_2O containing 0.05% TFA and solvent B: MeCN containing
164 0.05% TFA). The gradient was performed by increasing percentage of solvent B from
165 10% to 30% during 20 min. UV detector was set at 220 nm.

166 *2.7. Mass spectrometry experiments*

167 Accurate MS and in-source collision-induced dissociation (CID) experiments were
168 performed using LC-TOF-MS (JMS-T100LC, JEOL). The peptides were dissolved in
169 aqueous MeCN solution (10-20%). The measurements were performed following the
170 manufacturer's instructions.

171 *2.8. Protease inhibitory assay*

172 Protease inhibitory assays (trypsin, chymotrypsin, and elastase) were performed
173 following to the previous report (Unno, K. et al., 2020).
174

3. Results

3.1. Analysis of the BGCs of OEP in group 11

In the group 11 of OEPs, 534 BGCs were indicated to be distributed over actinobacteria, mainly in streptomycetes (Lee, H. et al., 2020). The BGCs of the group 11 commonly contain of two genes including a precursor coding gene and ATP-grasp ligase coding gene. The alignment of amino acid sequences of precursor coding genes of streptomycetes was shown in Fig. 1. Normally, a precursor peptide has leader peptide region to *N*-terminus and core peptide region at *C*-terminus. The leader peptide region is required for recognition of modification enzyme. After modification on core peptide region, the leader peptide region is cleaved off. The peptides seemed to have several conserved motifs (QPFALNYARPA, LNYAR, PYXYD, QLNVXAA) in leader peptide region of *N*-terminus. In precursor encoding gene, the core peptide region commonly had the consensus RE(L/V)GTTTSTAGSKTHFDD at *C*-terminus. To obtain new OEP biosynthesized by this class of BGC, the BGC of S*treptomyces prunicolor* was chosen to be a target for cloning and co-expression in *Escherichia coli*.

3.2.Production of prunipeptin by heterologous expression

The BGC of *S. prunicolor* included a precursor encoding gene *pruA* (accession number: WP_019056920.1) and ATP-grasp ligase encoding gene *pruB* (accession

193 number: WP_019056921.1). The DNA sequence of each gene was optimized for
194 expression according to codon usage of *E. coli*, and DNA sequence was chemically
195 synthesized (Fig. S1). The DNA sequence (1190 bp) including *pruA* and *pruB* was
196 integrated into expression vector pET-41a(+) to give pET-41a-13075 (Fig. S2). The
197 vector pET-41a-13075 was cloned in *E. coli* DH5α. The vector pET-41a-13075 was
198 transformed into *E. coli* BL21(DE3). The bacterium *E. coli* BL21(DE3) possessing the
199 vector pET-41a-13075 was cultured on modified basal agar medium containing
200 kanamycin and isopropyl-β-D-thiogalactopyranoside (IPTG) at 30 °C for 24 h. The cells
201 were harvested with a laboratory spatula and extracted with double volume of MeOH
202 and the MeOH extract was analyzed by HPLC and ESI-MS. The transformant was
203 indicated to produce several analogous peptides (Fig. S3 and S4). Among them, we
204 isolated the highest yielding peptide named prunipeptin. The accurate ESI-MS analysis
205 of prunipeptin showed ion peak at $[M+2H]^{2+}$ *m/z* 866.3945 (the calculated *m/z* value
206 was 866.3947), which indicated the molecular formula of $C_{73}H_{110}N_{20}O_{29}$ (Fig. S5). In-
207 source CID analysis on prunipeptin (Table S2 and Fig. S6) indicated amino acid
208 sequence of ELGTTT at *N*-terminus (Fig. 2A) by *y* series ions (*y11–y16*). We proposed
209 that prunipeptin had the amino acid sequence of ELGTTTSTAGSKTHFDD with
210 removal of two unit of H_2O, considering the molecular formula. Since prunipeptin had

two units of Asp at *C*-terminus, the two units of Asp were expected to form ester or isopeptide bonds with Ser, Thr, or Lys. We speculated that the cyclic structure was the reason why fragmentation was not observed in the sequence of STAGSKTHFDD by in-source CID analysis.

3.3.Production of prunipeptin variants by site directed mutagenesis experiments

To determine amino acid residue involved in ester or isopeptide bond formation in prunipeptin, site directed mutagenesis experiment (Bryksin, A. V. and Matsumura, I., 2010) to the vector pET-41a-13075 was performed to substitute 7^{th} Ser, 8^{th} Thr, 11^{th} Ser , 12^{th} Lys, 13^{th} Thr with Ala in prunipeptin, respectively (Fig. 3). In the case of substitution of 8^{th} Thr, 11^{th} Ser, and 13^{th} Thr, the Ala-substituted prunipeptin was detected by HPLC and ESI-MS (Fig. S7 and S8) in the extract of transformant harboring the vector with mutation (Fig. 3). In the case of one amino acid substitution of 7^{th} Ser, 12^{th} Lys, 16^{th} Asp, and 17^{th} Asp, the expected peptide was not detected by HPLC (Fig. S7) in the extracts of transformants (Fig. 3). With the site directed mutagenesis experiments, one of intramolecular bonds was designed not to be formed. We speculated that the site directed mutagenesis experiments (7^{th} Ser, 12^{th} Lys, 16^{th} Asp, and 17^{th} Asp) leaded to production of incompletely modified peptide or non-modified peptide degradable by endogenous proteases of *Escherichia coli*. Thus, the

229 amino acid residue of 7th Ser and 12th Lys were proposed to form ester or isopeptide
230 bond with 16th or 17th Asp, although bridging pattern was not clear.

231 *3.4.Structure determination of prunipeptin 15HW*

232 Analysis of amino acid sequence of ring structure in peptide is difficult, because it
233 normally does not give fragmentation by MS/MS experiment. And ring structure of
234 peptide often gives resistance against proteases. Normally, reagents for chemical
235 cleavage such as BNPS-skatole are used to open the ring structure. For structure
236 determination of cyclic peptides such as actinokineosin (Takasaka, N. et al., 2017) and
237 achromosin (Kaweewan, I. et al., 2017), reaction using BNPS-skatole was indicated to
238 be efficient to open the ring structures by cleaving peptide bond at *C*-terminus of Trp.
239 To introduce Trp into the ring structure, substitution of His to Trp was performed by
240 site directed mutagenesis experiment of the plasmid pET-41a-13075 (Fig. 3). The
241 transformant harboring the vector with mutation for substitution (His to Trp) produced
242 the expected peptides (Fig. S9) including one amino acid substituted prunipeptin
243 variants named prunipeptin HW (amino acid sequence: ELGTTTSTAGSKTWFDD)
244 and its truncated peptide named prunipeptin 15HW (amino acid sequence:
245 GTTTSTAGSKTWFDD). The molecular formula of peptides was confirmed by
246 accurate ESI-MS, which indicated both peptides had two units of H_2O removal from

247 expected molecular formula of the linear peptides as same as prunipeptin (Fig. S10).
248 Unexpectedly, the transformant harboring vector with mutation for substitution (His to
249 Trp) produced prunipeptin 15HW with the yield of about 5 times larger amount
250 compared to that of prunipeptin (Fig. S11). For structure determination, prunipeptin
251 15HW (2.0 mg) was isolated from 1 L culture of the transformant (Fig. S12). NMR
252 analysis was performed using DMSO-d_6 as solvent, however it was difficult to analyze
253 due to broadness of NMR spectrum. The in-source CID Analysis on prunipeptin 15HW
254 (Table S3 and Fig. S13) indicated the sequence of GTTT for *N*-terminus (Fig. 2B). The
255 cyclic part of prunipeptin 15HW did not give fragmentation as same as prunipeptin. To
256 determine isopeptide bond pattern, alkaline hydrolysis was performed on prunipeptin
257 15HW. As a result, ester cleaved prunipeptin 15HW was obtained by HPLC (Fig. 2C
258 and S14A). To determine bridging pattern, In-source CID Analysis on ester cleaved
259 prunipeptin 15HW was performed (Table S4 and Fig. S15). As a result, ion series
260 *y6*+H_2O–*y14* was detected, which indicated the amino acid sequence of GTTTSTAGS
261 at *N*-terminus (Fig. 2C). In addition, an ion *b14* was detected, that indicated the
262 isopeptide bond between 10^{th} Lys and 14^{th} Asp. To determine the amino acid sequence
263 in the ring (KTWFD), treatment of BNPS-skatole was performed (Fontana, A., 1972;
264 Crimmins, D. L. et al., 2005). As a result, Trp *C*-terminus cleaved prunipeptin 15HW

was obtained (Fig. 2D and S14B). By the reaction of BNPS-skatole the Trp residue in a peptide is oxidized and transforms to 3-oxiindole with spirolactone (Fontana, A., 1972). By the in-source CID Analysis, ions of *y8–y11*, *b10*, *b11*, and *y'5* were detected (Table S5 and Fig. S16), which confirmed the structure of Trp *C*-terminus cleaved prunipeptin 15HW (Fig. 2D). The bridging pattern of prunipeptin 15HW was determined to have ester bond (5^{th} Ser and 15^{th} Asp) and isopeptide bond (10^{th} Lys and 14^{th} Asp), as shown in Fig. 2B. The bridging pattern of prunipeptin was also proposed to be as shown in Fig. 2A.

3.5.Protease inhibitory assay of prunipeptin 15HW

As other cyclic peptides microviridins (Ishitsuka, M. O. et al., 1990; Okino, T. et al., 1995; Shin, H. J. et al., 1996; Murakami, M. et al., 1997; Fujii, K. et al., 2000; Rohrlack, T. et al., 2003; Reshef, V. and Carmeli, S., 2006; Dehm, D. et al., 2019) were reported to possess inhibitory activity against proteases, we accomplished protease inhibitory assay against trypsin, chymotrypsin, elastase using prunipeptin 15HW. As a result, prunipeptin 15HW showed no inhibitory activity against all testing proteases at the concentration of 65 μM (Table S6).

4. Discussion

In the biosynthesis of microviridin B, the two ATP-grasp ligases, MdnB and MdnC forms two ester bonds and one isopeptide bond in the molecule (Ziemert, N. et al., 2008; Hemscheidt, T. K., 2012). The function of MdnC was determined to be the formation of two ester bonds, and the other modification enzyme, MdnB, was shown to function as a ligase for isopeptide bond (Li, K. et al., 2016). In the biosynthesis of microviridin, two enzymes are individually responsible for formation of two different bonds (ester and isopeptide bond). Interestingly, ATP-grasp ligase of the group 5 intramolecularly catalyzes two ester bonds and one isopeptide bond to give OEP5-1 (Lee, H. et al., 2020). In the present study, we indicated that ATP-grasp ligase PruB also had bifunctional function to form one ester and one isopeptide bonds in the molecule. The bridge formation patterns of OEP5-1 and prunipeptin showed low similarity. The amino acid sequences of PruB and ATP-grasp ligase for OEP5-1 (accession number: GAJ78971.1) were compared by Blastp search. As a result, the amino acid sequences of two proteins showed low similarity (25 % similarity) and no conserved region was found on alignment (Table S7).

The reaction mechanism of ATP-grasp ligase MdnC was studied based on the structure obtained by X-lay crystallography (Li, K. et al., 2016). The X-lay

crystallography analysis of MdnC indicated that leader sequence was recognized by pocket between a helix α7 and a hairpin β9β10. The core peptide region is recruited into the active pocket close to ATP-binding site. In the core peptide, phosphorylation of side chain carboxyl residue of Asp/Glu is occurred by phosphate transfer from ATP. The hydroxyl group of Thr or Ser attacks the mixed carboxylate-phosphate anhydride to generate the ester bond. The same mechanism was proposed in the case of other OEPs (Lee, H. et al., 2020). ATP-grasp ligases in the groups 3, 4, and 6 catalyzes formation of esters to give the peptides, thuringinin, OEP4-1, and OEP6-1 from precursor peptides. Interestingly, ATP-grasp ligases in groups 3, 4, and 6 exerted ability of forming an intramolecular isopeptide bond when the core peptide sequence in the precursor was swapped to that of other groups (Lee, H. et al., 2020). However, the mature peptides derived from the swapped core peptides possessed the noncanonical bridging pattern of isopeptide bond formation. The diverse topologies of intramolecular bridges of groups of OEPs were summarized in Fig. 4A. In the present study, prunipeptin (OEP in group 11) had novel bridging pattern with ester and isopeptide bonds, which indicated that ATP-grasp ligase PruB possessed novel reaction specificity of ligation on the substrate. We proposed that PruB also function in the same manner with other ATP-grasp ligases. The phosphorylation of side chain carboxyl residue of two Asp residues in the precursor

peptide were occurred by phosphate transfer from ATP (Fig. 4B). The hydroxyl group of Ser and amino residue of side chain of Lys function as nucleophile to the mixed carboxylate-phosphate anhydride, resulting in formation of the ester and isopeptide bonds, respectively (Fig. 4B). The ring topology of OEPs was indicated to be governed by both the core sequence and the ATP-grasp enzyme (Lee, H. et al., 2020). The mechanism of ATP-grasp ligase to exert this specificity on the topology is not clarified so far.

In conclusion, we produced new bicyclic peptide named prunipeptin and variant peptides. The peptide prunipeptin contained one ester and isopeptide bond in the molecule. The bridging pattern of ester and isopeptide bonds is novel, which indicated that ATP-grasp ligase PruB possessed unique specificity on formation of each bond. To clarify the mechanism of formation of two different bonds by one enzyme, X-lay crystallography experiment of the enzyme is needed.

Author contribution statement

KU carried out the experiments and wrote the manuscript. SK designed the experiments and wrote the manuscript. All the authors gave intellectual input and critically revised the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Fig. 1. Alignment of amino acid sequences of precursor peptides in the group 11 of OEPs, bold letters indicates conserved amino acids.

Fig. 2. In-source CID analysis on (A) prunipeptin, (B) prunipeptin 15HW, (C) ester cleaved prunipeptin 15HW, (D) Trp *C*-terminus cleaved prunipeptin 15HW

Fig. 3. Amino acid substitution experiments in prunipeptin by site directed mutagenesis in the gene *pruA,* +: production of prunipeptin variant, −: no production

Fig. 4. (A) Bridging patterns of OEPs. Line indicates ester bond. Botted line indicates isopeptide bond. (B) Proposed macrocyclization mechanism in prunipeptin biosynthesis.

503 Figure 1

Fig. 1 Unno&Kodani

506 Figure 2

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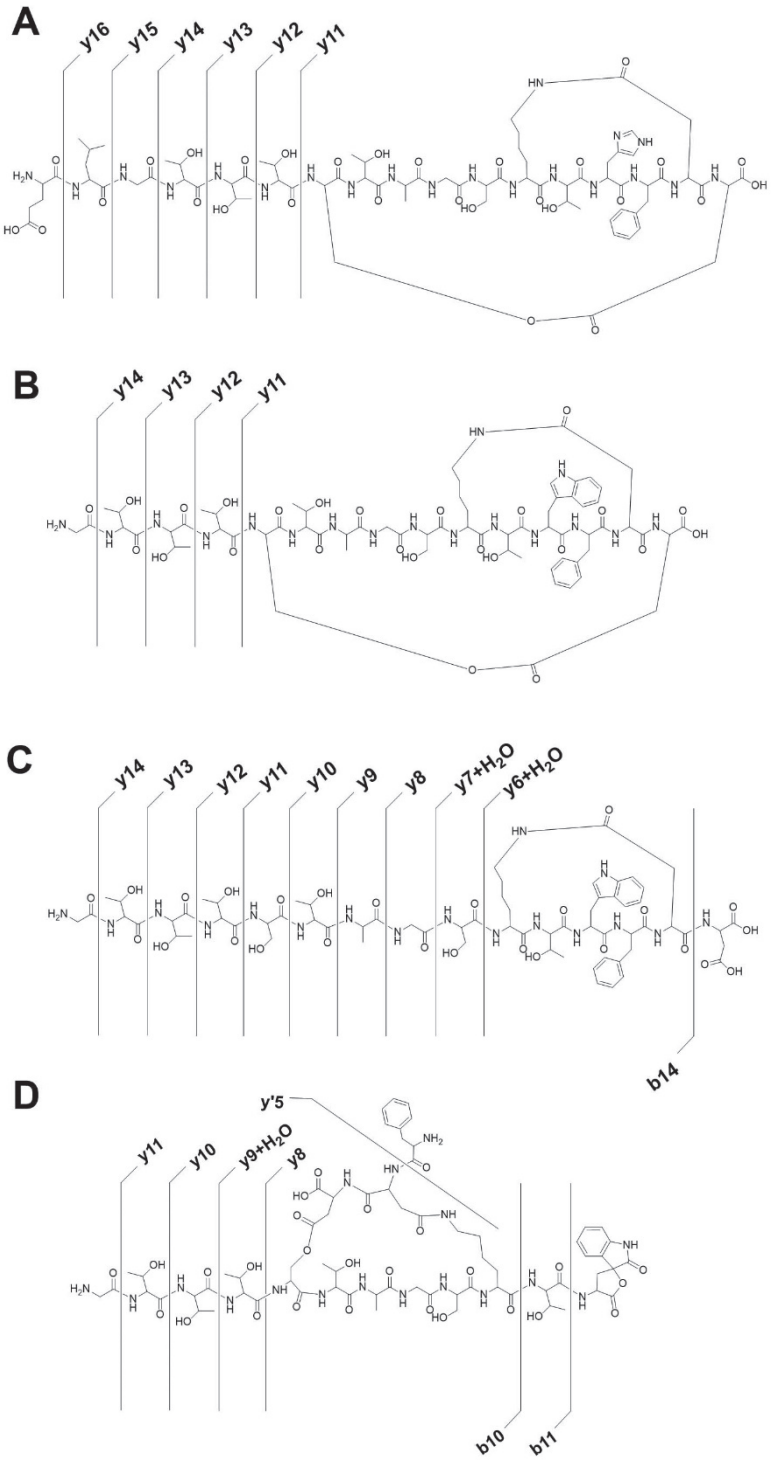

Fig. 2. Unno&Kodani

510 Figure 3

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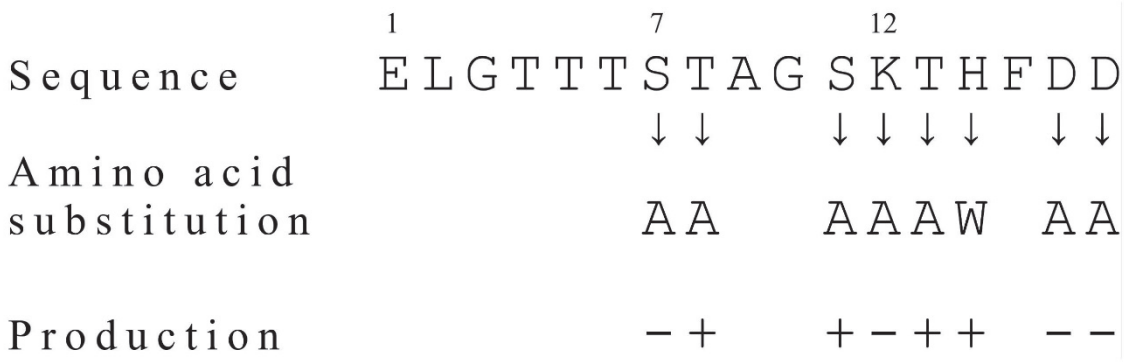

Fig. 3. Kodani et al.

513 Figure 4

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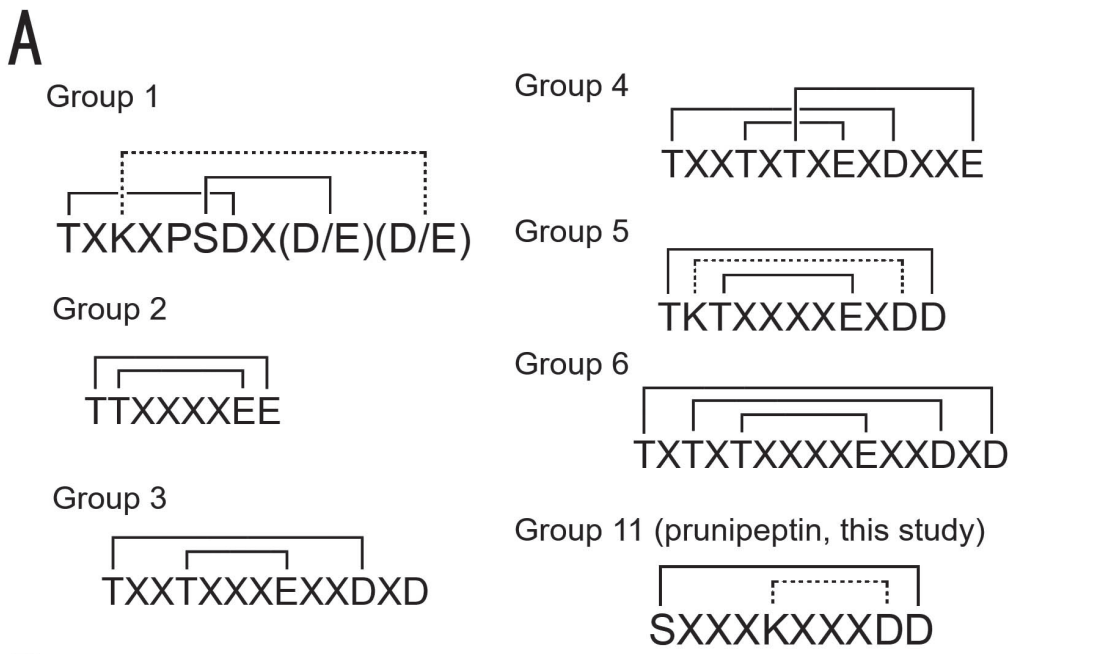

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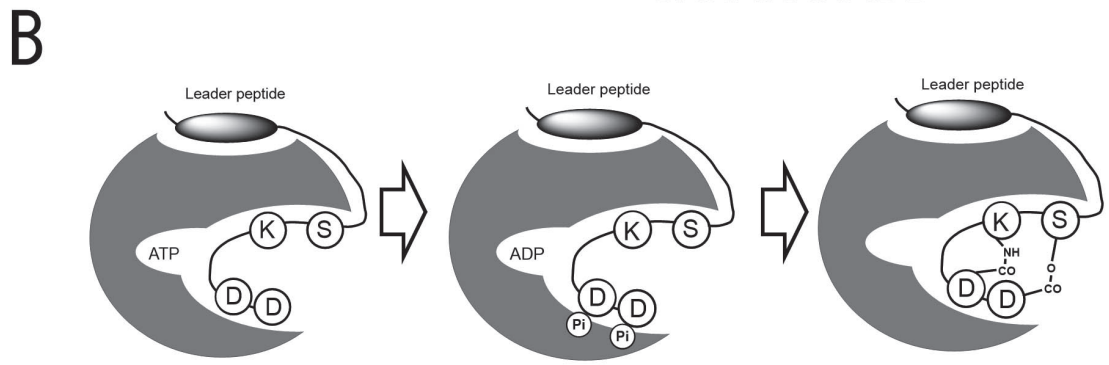

Fig. 4 Unno&Kodani