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Title: Isolation and identification of a new lasso peptide cattlecin from *Streptomyces cattleya* based on genome mining

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Abstract

Lasso peptides are ribosomally synthesized and post-translationally modified peptides with diverse biological functions. Recent genome mining has revealed that many species of actinomycetes possibly contain biosynthetic gene clusters of lasso peptides. With genome mining for lasso peptide biosynthesis, we screened several actinomycetes for lasso peptide production using high-performance liquid chromatography (HPLC) and the electrospray ionization - mass spectrometry (ESI-MS). Consequently, *Streptomyces cattleya* was identified as a producer of a new lasso peptide named cattlecin. Analysis of cattlecin's amino acid content indicated the presence of four mole each of Asp and His, three mole each of Gly and Tyr, and one mole of Ser. Tandem mass spectrometry (MS/MS) analysis on cattlecin revealed C-terminal sequence of WHHGWYGWWDD. The peptide sequence (SYHWGDYHDWHHGWYGWWDD) was the expected amino acid sequence of cattlecin based on genome mining. As a result of MS/MS analysis, the amine residue of the first Ser was proposed to form a macrolactam ring with the β-carboxyl residue of the ninth Asp. The biosynthetic gene cluster of cattlecin comprised four genes: *catA*, *catC*, *catB1*, and *catB2*, which is typical of a lasso peptide biosynthetic gene cluster in actinomycetes.

Introduction

Lasso peptides are ribosomally synthesized and post-translationally modified peptides with biological activities, including antibacterial properties, and have the unique knot structure of a "lasso" in common (Maksimov et al. 2012). The amino group of the N-terminal amino acid of lasso peptides forms a peptide bond with β- or γ-carboxyl group of Asp or Glu in the eighth or the ninth position from the N-terminus, resulting in the formation of a macrolactam ring. The macrolactam ring appears as a loop of a "lasso," with a tail of the C-terminal linear peptide that normally locates through the macrolactam ring. Due to this unique structure, lasso peptides are stable against proteolytic degradation and high temperature. Recently, genome mining approaches have been used to identify new lasso peptides. Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF)-MS analysis identified the lasso peptide SRO15-2005 from the extract of *Streptomyces roseosporus* NRRL 15998 based on genome mining (Kersten et al. 2011). This prompted us to find new lasso peptides in *Streptomyces* spp. using the genome mining approach. Thus, we identified a new lasso peptide cattlecin in *Streptomyces cattleya*. The structure of cattlecin was analyzed by a combination of amino acid content analysis and mass spectrometry analyses. Here we describe the isolation and structural determination of cattlecin.

Materials and methods

Bacterial strains.

The microorganisms (Bacterial strains including *Streptomyces cattleya* NBRC14057, *Kutzneria albida* NBRC 13901, *Escherichia coli* NBRC 1002203, *Pseudomonas aeruginosa* NBRC 12689, *Bacillus subtilis* NBRC 13719, *Staphylococcus aureus* NBRC 100910, *Micrococcus luteus* NBRC 3333; Yeast strains including *Saccharomyces cerevisiae* NBRC 2376, *Kloeckera apiculata* NBRC 0154; fungi strains including *Aspergillus niger* NBRC 33023, *Aspergillus oryzae* NBRC 4290, *Mucor hiemalis* NBRC 9405) were obtained from the NBRC culture collection (NITE Biological Resource Center, Japan).

Isolation of cattlecin

Streptomyces cattleya was cultured using 10 L of ISP2 agar medium for 7 days at 30 °C. The aerial hyphae and spore cells on the agar surface were harvested with steel spatula. Double volume of MeOH was added to the harvested cells, followed by filtration with paper filter (Whatman No. 1, GE Healthcare Life Sciences, Little Chalfont, UK). The MeOH extract was concentrated to an aqueous residue using rotary evaporator. The aqueous residue was subjected to open column chromatography using hydrophobic resin CHP-20P (Mitsubishi Chemical, Tokyo, Japan), eluted with 10% MeOH, 60% MeOH, and 100% MeOH. The 60% MeOH fraction was subjected to high-performance liquid chromatography (HPLC) purification using ODS column (4.6 × 250 mm , Wakopak Handy-ODS, Wako Pure Chemical

Industries, Ltd., Osaka, Japan) with gradient elution from 10 to 60% MeCN containing 0.05% trifluoroacetic acid for 20 min with UV detector set at 220 nm to yield 2.0 mg of cattlecin (Retention time: 14.9 min).

Mass spectrometry

The electrospray ionization (ESI) TOF mass spectrum was recorded using a JEOL JMS-T100LP mass spectrometer (JEOL, Tokyo, Japan). MALDI TOF/TOF mass spectra were recorded on a 4800 plus MALDI TOF/TOF analyzer (AB SCIEX, Redwood City, CA, USA) using α-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Billerica, MA, USA) as the matrix in the positive-ion mode with an acceleration voltage of 20 kV. The mass spectrometer was tuned and calibrated using a commercially available standard peptide mixture (Peptide Calibration Standard II, Bruker Daltonics) prior to the measurement. The ESI Fourier-transform ion cyclotron resonance mass spectrum was recorded on the ApexII 70e mass spectrometer (Bruker Daltonics). The sample solution were infused into the ESI source directly at 2- 5 $\mu L \cdot min^{-1}$ using a syringe pump after dilution by 50% methanol. The accurate mass measurement in the presence of the internal standard YOKUDELNA (JEOL, Tokyo, Japan).

Amino acid analysis

Following to the previous report (Kaweewan et al. 2017), amino acid content of cattlecin was determined.

Anti-microbial test

Following to the previous report (Kaweewan et al. 2017), anti-microbial activity of cattlecin was evaluated.

Results

A lasso peptide sviceucin was recently identified by genome mining, and it was isolated and its structure determined via heterologous expression (Li et al. 2015a). Using BLAST similarity search of the amino acid sequence of sviceucin (accession number: EDY58505.1), we found five sviceucin analog peptides, as shown in Fig. 1. The leader peptide sequences (underlined in Fig. 1) that are deduced to be cleaved off during posttranslational modification had the conserved amino acid motif, indicated in boldface in Fig. 1. As a comparison of amino acid sequences of the peptide in Fig. 1, the structural peptide parts started with Cys, Ser, or Gly, and Asp was present at the seventh, eighth, or ninth position. Among the strains of possible lasso peptide producers shown in Fig. 1, *Streptomyces cattleya* and *Kutzneria albida* were cultured using ISP2 agar medium at 30°C for 7 days. The aerial hyphae and spore cells of each strain were harvested using a steel spatula. MeOH extracts of the cells were analyzed using HPLC and ESI-TOF MS (data not shown). The presence of lasso a new peptide named cattlecin was confirmed in the MeOH extract of *Streptomyces cattleya.* The ESI-TOF mass spectrum of cattlecin showed an ion peak $[M+2H]^{2+}$ at *m/z* 1344.04.

To isolate sufficient quantity of cattlecin for structural determination, *S. cattleya* was cultivated with 1 L of ISP2 agar media. After 7 days of cultivation, spore cells and aerial hyphae were again harvested using a steel spatula. The harvested cells were extracted with double the volume of MeOH, followed by centrifugation. After condensation using a rotary evaporator, the extract was purified via open column chromatography using hydrophobic resin CHP-20P (Mitsubishi Chemical), eluted with 10%, 60%, and 100% of MeOH. The 60% MeOH fraction was repeatedly purified using HPLC to acquire pure cattlecin.

The molecular formula of cattlecin was determined to be $C_{131}H_{139}N_{33}O_{32}$ via analysis using ESI-FTCR mass spectrometry ($[M+3H]^{3+}$ at *m/z* 896.3489; the calculated value was 896.3494). The amino acid content of cattlecin was analyzed as described previously (Kaweewan et al. 2017). As a result, amino acid content analysis of cattlecin gave 4 moles each of Asp and His, 3 moles each of Gly and Tyr, and 1 mole of Ser at mole rate. The attempt to measure NMR spectra using DMSO-d_6 as a solvent was unsuccessful due to ambiguous broad peaks. To determine the peptide sequence, MALDI-TOF MS/MS analysis was used as described previously (Kodani et al. 2014). The product ions formed by cattlecin as determined by MALDI-TOF MS/MS were of *b*-series peptides *b*9-*b*18 and of *y*-series peptides *y*9 and *y*10 (Fig. 2). The macrolactam ring structure was reported not to produce fragmented ions (Kersten et al. 2011), so we deduced the structure of cattlecin as shown in Fig. 2, considering the amino acid sequence of the prepeptide gene (CCB72812.1).

Considering that the cattlecin-analogous peptide sviceucin (Li et al. 2015a) contains an isopeptide bond between the amine residue of Cys1 and the β-carboxyl residue of Asp8, we assumed that cattlecin may also possess a similar topology in terms of biosynthesis.

The antibiotic paper disk method was performed as an anti-microbial test for cattlecin against many microorganisms as described previously (Kaweewan et al. 2017). The testing microorganisms included bacterial strains, such as *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus,* and *Micrococcus luteus*; yeast strains, such as *Saccharomyces cerevisiae* and *Kloeckera apiculata*; and fungal strains, such as *Aspergillus niger*, *Aspergillus oryzae*, and *Mucor hiemalis*. However, cattlecin, at a dose of 50 μg/disk, did not show anti-microbial activity against any of the testing microorganisms.

Discussion

Cattlecin was a highly hydrophobic peptide that contained total 8 hydrophobic amino acids (5 mole of Trp and 3 mole of Tyr) in 20 amino acid residues. In addition to that, cattelcin contained 4 units of Asp in the molecule, which gave cattlecin the characteristic of acidic peptide at biological pH. To find similar peptide to cattlecin, blastp search using core peptide amino acid sequence of cattlecin (SYHWGDYHDWHHGWYGWWDD) was performed. However, no similar peptide was found in the database. Although leader peptide sequence was conserved among lasso peptides of actinomycetes (Fig. 1), the core peptide sequences were

very diverse.

A lasso peptide microcin J25 was isolated from *Escherichia coli* (Salomon and Farias 1992), and its biosynthesis has been well studied as a model for lasso peptide biosynthesis. The biosynthetic gene cluster of microcin J25 consists of four genes, including a precursor peptide (gene A: McjA), two maturation enzymes (gene B: McjB and gene C: McjC), and an ATP-binding cassette transporter (gene D: McjD) in one gene cluster within the region of approximately 4.5 kbp (Solbiati et al. 1996). Normally, lasso peptide biosynthetic genes of proteobacteria have the same set of the above-mentioned four genes, although some gene clusters lack the transporter gene D (Li et al. 2015b). The protein McjC is involved in the formation of the macrolactam ring, and the function of protein McjB was found to be cleaving of the leader peptide from the prepeptide, as deduced via in vitro experiments (Yan et al. 2012). In actinomycetes, lasso peptide biosynthetic genes consist of a similar gene set, except that a maturation enzyme B is encoded by split-B genes: gene B1 and gene B2 (Li et al. 2015b). So far, biosynthetic gene clusters of lasso peptides, such as lariatin, (Inokoshi et al. 2012) SRO15-2005 (Kersten et al. 2011), lassomycin (Gavrish et al. 2014), sviceucin (Li et al. 2015a), and streptomonomicin (Metelev et al. 2015), have been identified. By referring to such lasso peptide biosynthetic genes (Inokoshi et al. 2012), we found the possible biosynthetic gene cluster of cattlecin, which consists of four genes: *catA* (accession number: CCB72812.1, 44 aa), *catC* (CCB72813.1, 605 aa), *catB1* (CCB72814.1, 67 aa), and

catB2 (CCB72815.1,143 aa) from the genome sequence of *S. cattleya*. Among the lasso peptides of actinomycetes, the function of modification genes in lariatin has been well studied (Inokoshi et al. 2012; Iwatsuki et al. 2009). Based on the similarity of each cattlecin gene to lariatin biosynthetic genes, *larA*, *larB*, *larC*, and *larD*, we proposed the functions of the genes as shown in Fig. 3. The gene catA encodes a precursor of cattlecin, and *catB1*, *catB2*, and *catC* encode modification enzymes that produce the mature lasso peptide. The gene *catC* encodes a putative asparagine synthetase possibly responsible for the formation of the Gly1–Glu9 isopeptide bond, which showed high similarity to larB, as determined via a BLAST homology search (28% identity; 45% positive matches). The gene *catB1* showed a high similarity to *larC* as seen via BLAST homology search (37% identity; 53% positive matches). Although *larC* has been indicated to be essential for lariatin biosynthesis, as deduced by a gene-disruption experiment (Inokoshi et al. 2012), its function remains unclear. BLAST homology search also showed high similarity between *catB2* and *larD* (43% identity; 55% positive matches). We deduce that *catB2* may be involved in the processing of the leader peptide because the sequence possesses the Cys-His-Asp catalytic triad, which is essential for serine protease similar to *larD* (Inokoshi et al. 2012). The gene for mature peptide exporter corresponding to *larE* was not found in/near the biosynthetic gene cluster of cattlecin.

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Figure legends

Figure 1. Amino acid sequences of lasso prepeptide genes similar to sviceucin

Figure 2. A) Proposed structure of cattlecin; proposed amino acid sequence was indicated by bald letter and B) MS/MS analysis on cattlecin

Figure 3. Biosynthetic gene cluster of cattlecin

Fig.1

Streptomyces sviceus (EDY58505.1, sviceucin)	MTSTDEL**YE**A**P**ELI**E**I**G**DYA**ELT**RCVWGGDCTDFLGCGTAWICV
Streptomyces cattleya(CCB72812.1, cattlecin)	MTESIEA**YE**P**P**MLV**E**V**G**SFA**ELT**RSYHWGDYHDWHHGWYGWWDD
Streptomyces sp. HPH0547 (EPD94850.1)	MESMEHT**YE**P**P**ALT**E**L**G**DFG**ELT**QCLPSGDCPDFLGCGRAIWCI
Kutzneria albida (AHH97260.1)	ME-AQDL**YE**P**P**AVV**E**I**G**DYA**ELT**MGGVGTVFDTWGLTPIP
Kibdelosporangium sp. MJ126-NF4 (EL12831.1)	MIHDDEI**YE**V**P**TLV**E**V**G**EFT**ELT**LGIPFGFGCPDYMHMLTPYAC
Kibdelosporangium sp. MJ126-NF4 (EL12830.1)	MIKDDEI**YE**V**P**TLV**E**V**G**DFA**ELT**LGLPWGCPNDLFFVNTPFAC

Letters with underline:leader peptide; bald letters: conserved amino acids

Figure 1. Sugai et al.

Fig. 2

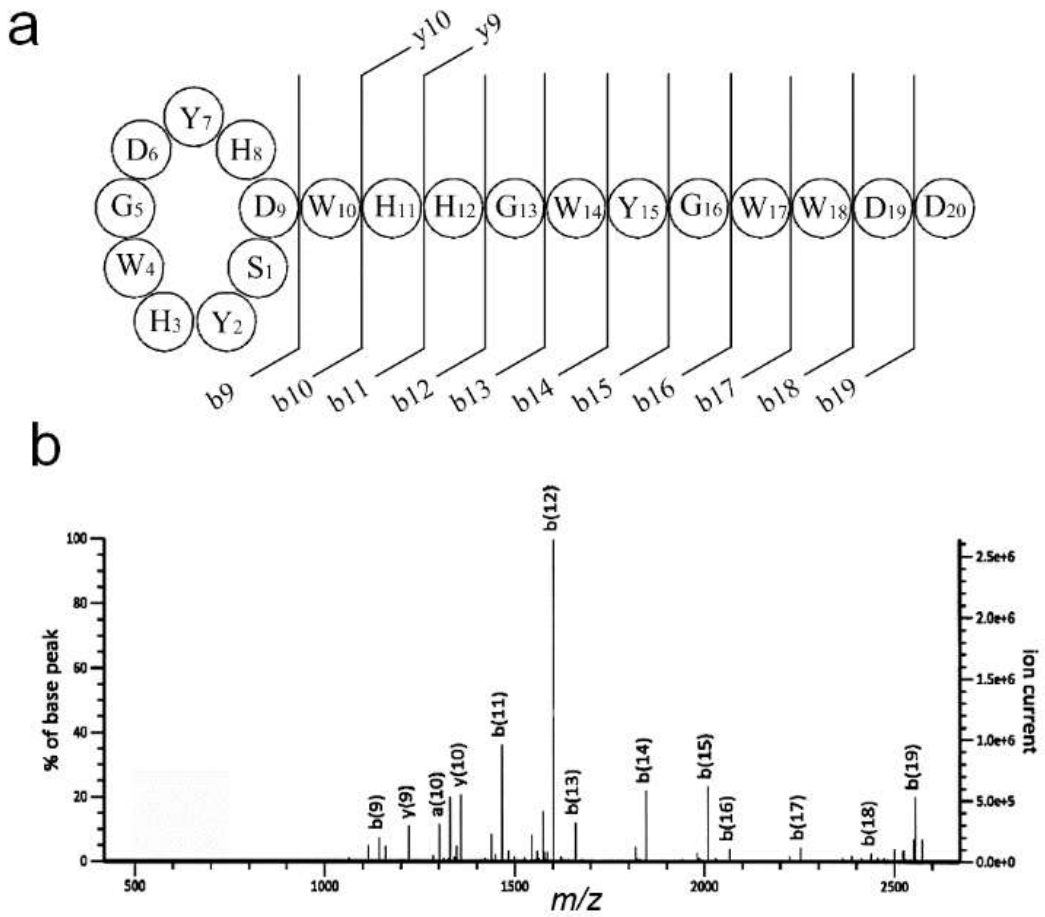

Figure 2. Sugai et al.

Fig. 3

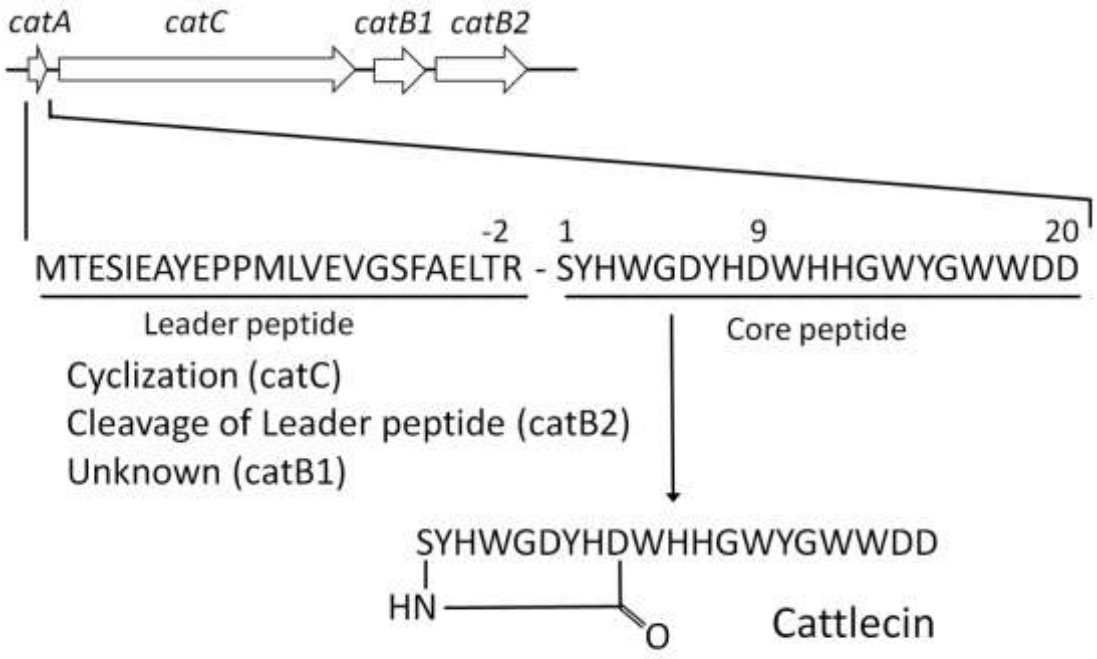

Figure 3. Sugai et al.