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Authors: Norimasa Takasaka[1], Issara Kaweewan[1], Mayumi Ohnishi-Kameyama[2], Shinya Kodani[1,3,4]*

Affiliations: [1]Graduate School of Integrated Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529 Japan; [2] Food Research Institute, National Agriculture and Food Research Organization (NARO), 2–1-12 Kannondai, Tsukuba, Ibaraki 305-8642 Japan; [3]College of Agriculture, Academic Institute, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529 Japan; [4]Graduate School of Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529 Japan

*To whom correspondence should be addressed: Shinya Kodani, College of Agriculture, Academic Institute, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529 Japan, Tel/Fax; +81(54)238-5008, E-mail; kodani.shinya@shizuoka.ac.jp

Running title: Isolation of actinokineosin

Significance and Impact of the Study

Genome mining is a powerful tool to find new bioactive compounds from genome database. In this report, we succeeded in isolation and structure determination of a new antibacterial peptide named actinokineosin based on genome mining.

Abstract

Based on genome mining, a new antibacterial peptide named actinokineosin was isolated from a rare actinomycete *Actinokineospora spheciospongiae*. The amino acid sequence of C-terminus of actinokineosin was established by TOF-MS/MS experiment. The amino acid sequence in the macrolactam ring was determined by TOF-MS/MS analyses after cleavage with BNPS-skatole and successive trypsin treatment. As a result of antibacterial assay using paper disk, actinokineosin showed antibacterial activity against *Micrococcus luteus* at the dosage of 50 μg per disk. From the genome sequence data of *A. spheciospongiae,* the biosynthetic gene cluster of actinokineosin was found and indicated to consist of 10 genes. Among the genes, the gene *aknA* encoded precursor of actinokineosin and the genes including *aknC, aknB1,* and *aknB2* were proposed to be modification enzymes to give mature actinokineosin.

Keywords

antibacterial, lasso peptide, *Actinokineospora spheciospongia*e, biosynthesis, BNPS-skatole, TOF-MS/MS

Introduction

Lasso peptides are a group of ribosomally biosynthesized and post-translationally modified peptides with unique common knot structure in the molecule (Maksimov *et al.* 2012; Li *et al.* 2015b) . The amino group of N-terminal amino acid forms a peptide bond with β- or γ-carboxyl group of Asp or Glu in the 8th or the 9th position from N-terminus to form a macrolactam ring. The loop structure of macrolactam ring looks like "lasso" with a tail of the C-terminal linear peptide that normally locates through the ring. Lasso peptides were reported to show a wide variety of biological activities such as anti-HIV (Detlefsen *et al.* 1995), antimycobacterial (Iwatsuki *et al.* 2006), endothelin type B receptor antagonist (Morishita *et al.* 1994), and prolyl endopeptidase inhibition (Kimura *et al.* 1997).

Accumulated genome data revealed that there are many possible peptide biosynthetic genes including lasso peptides in the genomes of streptomycetes (Hranueli *et al.* 2005; Goto *et al.* 2010). Based on the accumulated genome data, genome-mining approach was recently applied to find new peptides including lasso peptides. A lasso peptide SRO15-2005 was found and identified by MALDI TOF-MS from the extract of *Streptomyces roseosporus* NRRL 15998, based on genome mining (Kersten *et al.* 2011). Recently, new lasso peptide

sviceucin was found by genome mining, and it is isolated and structure-determined using heterologous expression (Li *et al.* 2015a). A new lasso peptide chaxapeptin was isolated as an inhibitor of lung cancer cell invasion from *Streptomyces leeuwenhoekii* based on genome mining (Elsayed *et al.* 2015). These results prompted us to find new lasso peptide type compounds from streptomycetes by the genome mining approach. Based on the genome mining approach, we found new antibacterial peptide named actinokineosin from a rare actinomycete, *Actinokineospora spheciospongiae*, which was previously isolated from marine sponge tissue (Abdelmohsen *et al.* 2010; Harjes *et al.* 2014; Kampfer *et al.* 2015). The structure was determined by mass spectrometry analyses on chemical and enzymatic degraded peptides. Here we describe the isolation and structural determination of new antibacterial peptide actinokineosin.

Results and Discussion

Isolation of actinokineosin

Previously a lasso peptide propeptin was isolated from *Microbispora* sp. SNA-115 (Kimura *et al.* 1997), and the peptide sequence of propeptin was determined to be GYPWWDYRDLFGGHTFISP by MS/MS analysis. We found a peptide gene (accession number: EWC60872.1) which was analogous to propeptin on the genome of *Actinokineospora spheciospongiae* (Harjes *et al.* 2014) by the blastp search (Fig. 1). The expected peptide actinokineosin was deduced to have the amino acid sequence of

GYPFWDNRDIFGGYTFIG after cleaving off leader peptide at the same position of the arrow indicated in Fig. 1. The expected mass of actinokineosin was calculated to be 2105.9 Da considering the loss of 18 Da through macrolactam ring formation of lasso peptide type biosynthesis. In order to obtain the peptide, cultivation of *A. spheciospongiae* DSM45935^T was performed with 10 l of ISP2 agar media. After 12 days of cultivation, cells of spore and aerial hyphae were harvested by steel spatula. The cells were extracted with double volume of MeOH, followed by filtration with paper filter. After condensation using rotary evaporator, the extract was subjected to open column chromatography using hydrophobic resin (CHP-20P), eluted with 10%, 60%, and 100% MeOH. The expected peptide actinokineosin was detected in 100% MeOH fraction by HPLC (Fig. S1) and ESI-MS analysis (Fig. S2). The ESI-TOF mass spectrum analysis of the new peptide gave an ion peak at *m/z* 1054.01 for $[M+2H]^{2+}$. The 100% MeOH fraction was repeatedly subjected to HPLC purification to afford pure actinokineosin.

Structure determination of actinokineosin

The molecular formula of actinokineosin was established to be $C_{103}H_{131}N_{23}O_{26}$ by the accurate mass analysis using ESI-FTCR mass spectrometry (The ion $[M+2H]^{2+}$ was observed at *m/z* 1053.9886 whose calculated value was 1053.9891). Amino acid analysis of actinokineosin gave the relative molar ratios of the constituent amino acids (4 mole of Gly, 3 mole each of Phe and Asp, 2 mole each of Ile and Tyr, and 1 mole each of Arg, Pro, and Thr).

The attempt to measure NMR spectra using DMSO-d_6 as a solvent was failed due to ambiguous broad peaks of NMR spectrum. To obtain peptide sequence, MALDI-TOF MS/MS analysis on actinokineosin was accomplished (Fig. S3). The product ions from actinokineosin by the MALDI-TOF MS/MS experiment were of *b*-series peptides, *b*9-*b*17, and of *y*-series peptides, *y*3 and *y*4 (Fig. 2a), which indicated that the sequence of IFGGYTFIG was the C-terminus tail sequence. Macrolactam ring structure was reported not to give fragment ions (Kersten *et al*. 2011), thus we proposed the structure of actinokineosin to be shown in Fig. 2a, based on the amino acid sequence of prepeptide gene. To confirm the amino acid sequence in macrolactam ring, cleavage using BNPS-skatole on C-terminus peptide bond of Trp was performed (Fontana 1972). After BNPS-skatole reaction, the cleaved actinokineosin was purified by HPLC, and the ESI-TOF mass spectrum of BNPS-skatole treated actinokineosin (BNPS-actinokineosin) gave the ion peak at *m/z* 1070.0 for $[M+2H]^{2+}$ (Fig. S4). By the reaction of BNPS-skatole the Trp residue in a peptide is oxidized and transforms to 3-oxiindole with spirolactone, which increases 32 Da (due to addition of two oxygen). The MALDI-TOF MS/MS of the cleaved actinokineosin (Fig. S5) gave the sequence of the branched peptide with two C-terminus ends (Fig. 2b) and indicated that Trp at new C-terminus was oxidized (indicated with asterisk in Fig. 2b). After the trypsin digestion of BNPS-actinokineosin, the MALDI-TOF mass spectrum showed a dominant ion at *m/z* 1771.8. The MALDI-TOF MS/MS for the ion gave the sequence of the

linear peptide with two C-terminus ends (Fig. 2c and Fig. S6). Above all, chemical structure of actinokineosin was determined to be shown in Fig. 2a. The stereochemistries of amino acids were determined by modified Marfey's method (Harada *et al.* 2001). The derivative of hydrolysate of actinokineosin was analysed by HPLC, compared with amino acid standards. As a result, all the amino acids in actinokineosin were determined to be L-form.

Antibacterial activity of actinokineosin

The antibacterial assay using paper disk (6 mm in diameter) was accomplished against microorganisms (Bacterial strains including *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus, Micrococcus luteus*; Yeast strain including *Saccharomyces cerevisiae*; fungal strains including *Aspergillus niger*, and *Aspergillus oryzae*) following a previous report (Kodani *et al.* 2012). At the dosage of 50 μg per disk, actinokineosin showed inhibitory zones with diameter of 8 mm against *M. luteus* (Fig. S7). Actinokineosin did not show inhibitory activity against the other testing microorganisms at the same dosage.

Gene cluster of biosynthesis for actinokineosin

A lasso peptide microcin J25 is isolated from *Escherichia coli*, which is regarded as the archetype of lasso peptides (Salomon and Farias 1992). Its biosynthetic gene cluster consisted 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 about 4.5 kbp (Solbiati *et al.* 1996). Normally lasso peptide biosynthetic genes in proteobacteria have a same set of this four genes, although the transporter gene is optional (Li *et al.* 2015b). The protein McjC was reported to get involved in formation of macrolactam ring, and the function of the protein McjB was assigned to be cleaving off the leaderpeptide from prepeptide by in vitro experiments (Yan *et al.* 2012). In actinobacteria, lasso peptide biosynthetic genes consist of a similar gene set, except that a maturation enzyme gene B have split-B genes including gene B1 and gene B2 (Li *et al.* 2015b). So far, biosynthetic genes clusters of lasso peptide of actinomycetes were identified for lariatin (Inokoshi *et al.* 2012), SRO15-2005 (Kersten *et al.* 2011), lassomycin (Gavrish *et al.* 2014), sviceucin (Li *et al.* 2015a), chaxapeptin (Elsayed *et al.* 2015) and streptomonomicin (Metelev *et al.* 2015). By reference to these lasso peptide biosynthetic genes, we assigned the putative biosynthetic gene cluster for actinokineosin, which consisted of 10 genes including *aknA* (accession number: EWC60872.1, 39 aa), *aknC* (EWC60871.1, 480 aa), *aknB1* (EWC60870.1, 88 aa), *aknB2* (EWC60869.1,127 aa), *aknD4* (EWC60873.1, 586 aa), *aknD3* (EWC60874.1, 237 aa), *aknD2* (EWC60875.1, 253 aa), *aknD1* (EWC60876.1, 257aa), *aknR* (EWC60877.1, 258 aa), and *aknS* (EWC60879.1, 259 aa), as shown in Fig. 3. The gene *aknA* encoded precursor of actinokineosin and the genes including *aknC*, *aknB1*, and *aknB2* were proposed to be modification enzymes to give mature lasso peptide. The gene *aknC* encoded putative asparagine synthase possibly responsible for

formation of the Gly1-Glu9 amide bond. The split genes B including *aknB1* and *aknB2* were present next to the gene *aknC*. The genes *aknD1* to *D4* seemed to get involved in transport/immunity of actinokineosin. The gene *aknD4* encoded putative ABC transporter that had two essential functional domains including membrane domain at *N*-terminus and ATPase domain at *C*-terminus. The gene *aknD3* encoded ATPase domain of ABC transporter and the genes *aknD2*and *aknD1* encoded membrane domain of ABC transporter, which may presumably function as counterpart to *aknD3*. The two genes *aknR* and *aknS* encoded regulatory protein and sensor kinase protein of two component regulatory system. The set of regulatory protein and sensor kinase was found in the biosynthetic gene clusters of sviceucin, siamycin I, and aborycin (Li *et al.* 2015a), so there may be a similar regulatory system among these lasso peptides. Above all, the biosynthetic gene cluster of actinokineosin was proposed as shown in Fig. 3, although the gene disruption experiments are needed to confirm the function of the biosynthetic genes.

Materials and Methods

Bacterial strains.

The actinomycete *Actinokineospora spheciospongiae* DSM45935^{T} was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany). The microorganisms (Bacterial strains including *Escherichia coli* NBRC102203^{T}, *Pseudomonas aeruginosa* NBRC12689^{T}, *Bacillus subtilis* NBRC13719^{T}, *Staphylococcus*

aureus NBRC100910^{T}, and *Micrococcus luteus* NBRC3333^{T}; Yeast strain including *Saccharomyces cerevisiae* NBRC2376; fungi strains including *Aspergillus niger* NBRC33023^{T} and *Aspergillus oryzae* NBRC4290) were obtained from the NBRC culture collection (NITE Biological Resource Center, Japan).

Isolation of actinokineosin

Actinokineospora spheciospongiae DSM45935^{T} was cultured using 10 l of ISP2 agar medium for 12 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 100% MeOH fraction was subjected to HPLC purification using ODS column (4.6 × 250 mm , Wakopak Handy-ODS, Wako Pure Chemical Industries, Ltd., Osaka, Japan) with gradient elution from 30 to 50% MeCN containing 0.05% trifluoroacetic acid for 20 min with UV detector set at 220 nm to yield 5.0 mg of actinokineosin (Retention time: 18.1 min).

Amino acid analysis

The amino acid content of actinokineosin was determined by HPLC analysis of PTC-derivatized amino acids (Heinrikson and Meredith 1984). For hydrolysis, actinokineosin (0.2 mg) was incubated in 100 µl of 6 mol l^{-1} HCl at 110 °C for 16 h. Aliquot of 40 µl of hydrolyzed sample, or aliquot of 10 µl of amino acid mixture standard solution (Wako Pure Chemical Industries, LTD.) was evaporated by a freeze dryer. Aliquots of 20 µl of ethanol/water/triethylamine (2/2/1, v/v/v) were added to each sample and evaporated by a freeze dryer. For PTC-derivatization of amino acids, aliquots of 50µl of ethanol/water/triethylamine/phenylisocyanate (7/1/1/1, v/v/v/v) were added to each sample, and derivatization was accomplished by incubation at room temperature for 20 min. After evaporation using a freeze dryer, 1.0 ml of PTC-derivatized amino acid mobile phase A (60 mmol l^{-1} CH_3COONa aqueous solution pH 6.0/MeCN, 6:94) was added to dissolve each sample. The PTC-derivatized samples were analyzed using an HPLC system (PU980 system, JASCO, Tokyo, Japan). The conditions for HPLC analysis were following: HPLC column, Wakopak Handy-ODS (4.6 mm × 250 mm; Wako Pure Chemical Industries, Ltd.); mobile phase A (60 mmol l^{-1} CH_3COONa aqueous solution pH 6.0/MeCN, 6:94); mobile phase B (60 mmol l^{-1} CH_3COONa aqueous solution pH 6.0/MeCN, 60:40); flow rate, 1 ml min^{-1}; gradient of mobile phase B, 5% to 65% from 0 to 30 min (linear gradient); UV detector, 254 nm.

Mass spectrum experiments

The electrospray ionization mass spectrum (ESI-MS) was recorded using a JEOL JMS-T100LP mass spectrometer (JEOL, Tokyo, Japan). The matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF)/TOF mass spectrometer (4800 plus MALDI TOF/TOF analyzer, AB SCIEX, CA, USA) was tuned and calibrated by a commercially available standard peptide mixture (Peptide Calibration Standard II, Bruker Daltonics) prior to the measurement in the positive-ion mode. The α-cyano-4-hydroxycinnamic acid (Bruker Daltonics, MA, USA) was used as the matrix. The electrospray ionization (ESI) Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrum was obtained on the ApexII 70e mass spectrometer (Bruker Daltonics) for the sample solution infused into the ESI source directly. The accurate mass measurement in the presence of the internal standard YOKUDELNA (JEOL, Tokyo, Japan).

BNPS-skatole reaction

Actinokineosin (0.4 mg) was subjected to cleavage reaction by adding 100 μl of BNPS-skatole solution (2 mg ml^{-1} acetic acid) and 10 μl of distilled water. The solution was incubated at 90 °C for 1 hour. Acetic acid was completely evaporated using rotary evaporator. After evaporation, 300 μl of chloroform and 500 μl of distilled water was added to the reaction mixture. After two-layer partition, water-soluble layer was transferred to a new micro tube. After adding 500 μl of MeOH, the reaction mixture was analyzed by an HPLC system. The conditions for HPLC analysis were following; HPLC column, Wakopak

Handy-ODS (4.6 mm × 250 mm; Wako Pure Chemical Industries, Ltd.); mobile phase A, MeCN containing 0.05% trifluoroacetic acid; mobile phase B, H_2O containing 0.05% trifluoroacetic acid; flow rate, 1 ml min^{-1}; gradient of increasing the mixing rate of mobile phase A from 20% to 60% for 20 min (linear gradient); UV detector, 220 nm.

Trypsin treatment on BNPS-skatole treated actinokineosin

The 25 μl of solution of BNPS-actinokineosin (0.2 mg ml^{-1} in 50 % acetonitrile) was mixed with the 50 μl of the trypsin solution in which 1 μg of trypsin (Sigma-Aldrich Japan, Proteomics grade, Tokyo) in 10 μL of 50 mmol l^{-1} acetic acid solution, 450 μl of 0.1 mol l^{-1} ammonium hydrogen carbonate and 450 μl of distilled water were included. The digestion was performed overnight at 37°C, and the reaction mixture was applied to MS analyses.

Modified Marfey method

Actinokineosin (1.5 mg) was subjected to acid hydrolysis at 110 °C for 16 h with 6 mol l^{-1} HCl, and the hydrolysate was dried by a freeze-dryer and resuspended in H_2O (200 μl). To the hydrolysate, 50 μl of a solution of *N*α-(5-fluoro-2,4-dinitrophenyl)-L- leucinamide (L-FDLA, Tokyo Chemical Industry Co., LTD, Tokyo, Japan) in acetone (10 mg ml^{-1}) and 200 μl of 1 mol l^{-1} $NaHCO_3$ solution were added, after which the mixture was incubated at 80 °C for 3 min. The reaction mixture was cooled, neutralized with 2 mol l^{-1} HCl (100 μl), and diluted with MeCN (1 ml). Each standard amino acid was also derivatized with L-FDLA and D-FDLA in the same manner. About 20 μl of each solution of FDLA derivatives was subjected

to HPLC analysis with C18 column (4.6 × 250 mm, Wakopak Handy ODS, WAKO). The DAD detector (MD-2018, JASCO, Tokyo, Japan) was used for detection of the amino acid derivatives accumulating the data of the absorbance from 220nm to 420 nm. The HPLC analysis was performed at a flow rate of 1 ml min^{-1} using solvent A (distilled water containing 0.05% TFA) and solvent B (MeCN containing 0.05%TFA) with a linear gradient mode from 0 min to 50 min, increasing percentage of solvent B from 20% to 60%. The retention times (min) of L- or D-FDLA derivatized amino acids in this HPLC condition were following; L-Arg-D-FDLA (19.6 min), L-Arg-L-FDLA (23.0 min), L-Thr-L-FDLA (24.6 min), L-Asp-L-FDLA (26.2 min), L-allo-Thr-L-FDLA (26.6 min), L-Asp-D-FDLA (27.3 min), L-allo-Thr-D-FDLA (27.9 min), L-Pro-L-FDLA (30.5 min), L-Thr-D-FDLA (30.9 min), L-Tyr-L-FDLA (33.2 min), L-Pro-D-FDLA (34.3 min), L-Tyr-D-FDLA (35.8 min), L-allo-Ile-L-FDLA (37.8 min), L-Ile-L-FDLA (38.1 min), L-Trp-L-FDLA (39.2 min), L-Phe-L-FDLA (40.0 min), L-Trp-D-FDLA (43.5 min), L-Phe-D-FDLA (47.0 min), L-Ile-D-FDLA (48.3 min), L-allo-D-FDLA (48.3 min).

Antibacterial assay

Paper disk diffusion assay (6 mm paper disk in diameter, thick type) was performed to evaluate antibacterial activity of actinokineosin following the guideline of Clinical and Laboratory Standards Institute. Actinokineosin was dissolved in DMSO at the concentration of 10 mg ml^{-1}. The test microorganisms including *E. coli, P. aeruginosa, B. subtilis, S.*

aureus, M. luteus were inoculated onto nutrient agar medium (Wright 1933). The test microroganisms including *S. cerevisiae, A. niger*, and *A. oryzae* were inoculated onto ISP2 agar medium (Shirling and Gottlieb 1966). Paper disks with 50 μg of actinokineosin (5 μl, 10 mg ml^{-1} solution in DMSO) were placed onto the surface of the agar medium, and paper disk with DMSO (5 μl) was used as a negative control. After incubation for 2 days at 30 °C, the diameter of the inhibitory zone was measured for evaluation of antibacterial activity. Tetracycline was used as a positive control reagent against the test microorganisms including *E. coli, P. aeruginosa, B. subtilis, S. aureus,* and *M. luteus.* At the dosage of 50 μg per disk, tetracycline showed inhibitory zones with diameter of 18, 8, 20, 35, and 21 mm against *E. coli, P. aeruginosa, B. subtilis, S. aureus,* and *M. luteus*, respectively. Cycloheximide was used as a positive control reagent against the test microorganisms including the test microroganisms including *S. cerevisiae, A. niger*, and *A. oryzae.* At the dosage of 50 μg per disk, cycloheximide showed inhibitory zones with diameter of 26 mm against *S. cerevisiae*. At the dosage of 200 μg per disk, cycloheximide showed inhibitory zones with diameter of 11 and 18 mm against *A. niger*, and *A. oryzae*, respectively.

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Conflict of interest

The authors declare no conflict of interest associated with this manuscript.

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Figure legends

Fig. 1. Alignment of amino acid sequence of propeptin and actinokineosin precursor peptide gene

Fig. 2. a) TOF-MS/MS analysis on actinokineosin, b) TOF-MS/MS analysis on cleaved actinokineosin with BNPS-skatole, c) TOF-MS/MS analysis after successive trypsin treatment on cleaved actinokineosin with BNPS-skatole

Fig. 3. Gene cluster for biosynthesis of actinokineosin (prepeptide: *aknA*, modification: *aknC*, *aknB1*, *aknB2*, transport/immunity: *aknD1*, *aknD2*, *aknD3*, *aknD4*, two component regulatory system: *aknR*, *aknS*)

Fig. 1

bold letter: conserved amino acid
arrow: cleavage position

Fig. 2

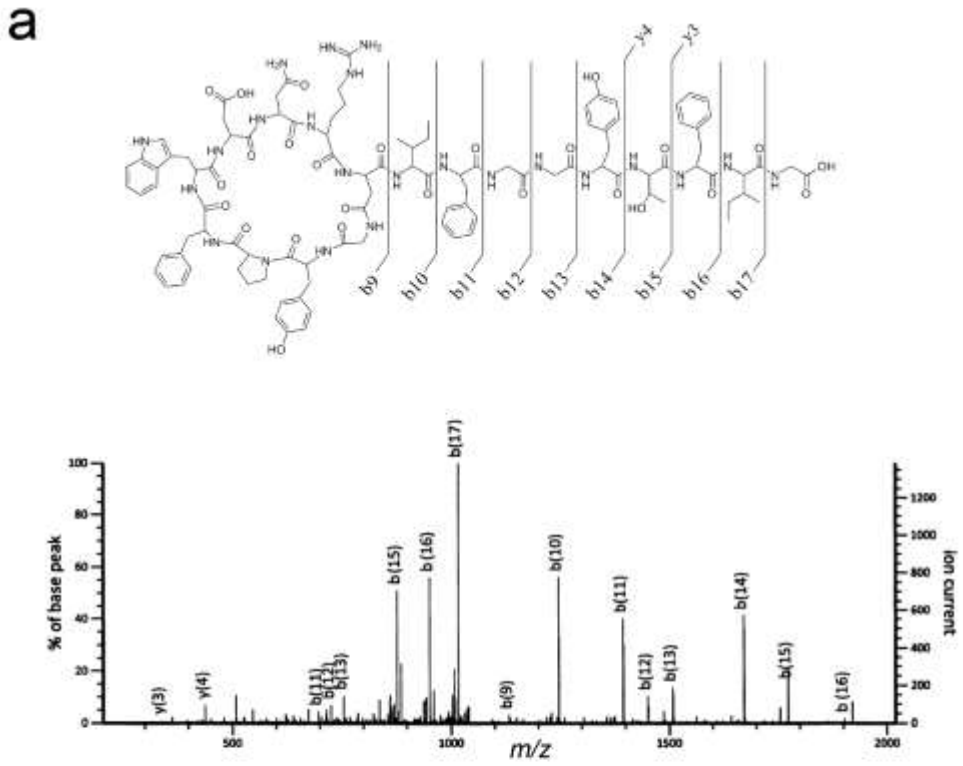
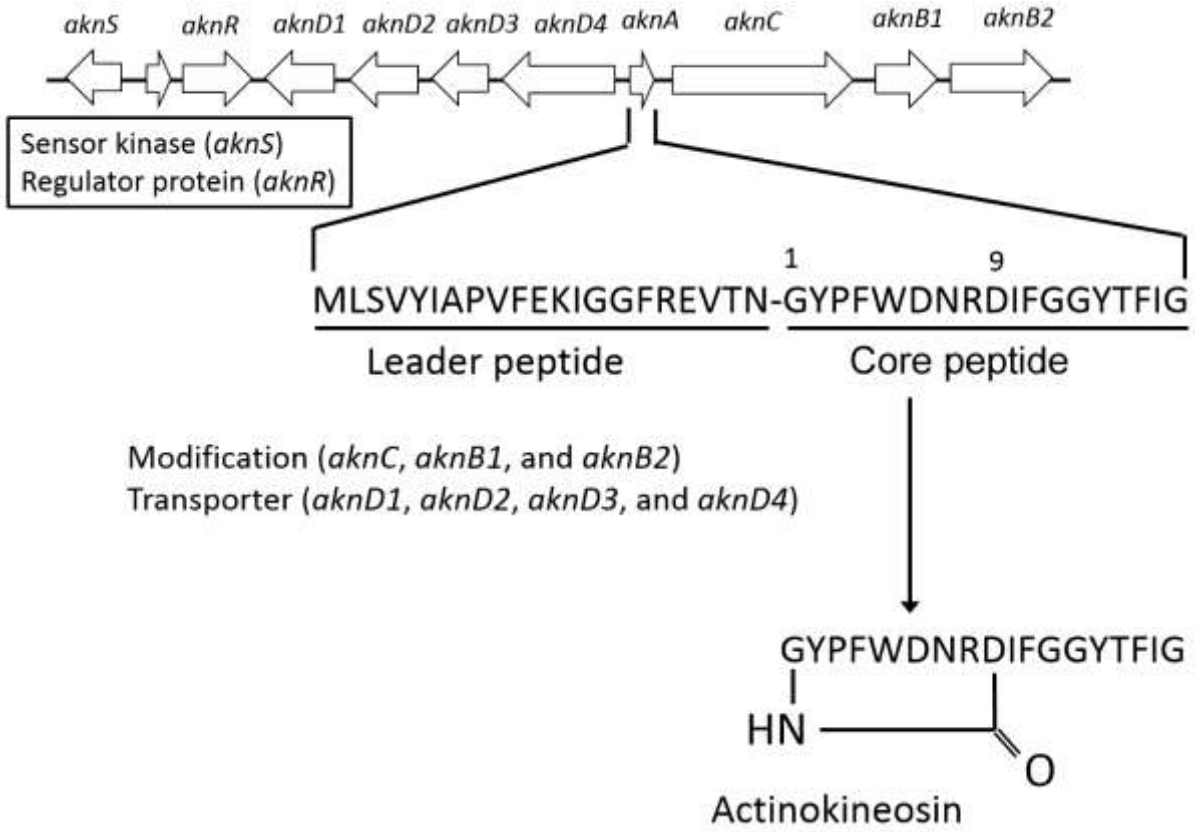

a
b9
b10
b11
b12
b13
b14
b15
b16
b17
y4
y3
% of base peak
ion current
m/z
b(17)
b(15)
b(16)
b(10)
b(11)
b(14)
b(12)
b(13)
b(9)
y(3)
y(4)
b(2)
b(3)
b(4)
b(5)
b(6)
b(7)
b(8)
b(9)
b(10)
b(11)
b(12)
b(13)
b(14)
b(15)
b(16)
b
N-terminus
C-terminus
C-terminus
c
N-terminus
C-terminus
C-terminus

Fig. 3


aknS
aknR
aknD1
aknD2
aknD3
aknD4
aknA
aknC
aknB1
aknB2
Sensor kinase (aknS)
Regulator protein (aknR)
1
9
MLSVYIAPVFEKIGGFREVTN-GYPFWDNRDIFGGYTFIG
Leader peptide
Core peptide
Modification (aknC, aknB1, and aknB2)
Transporter (aknD1, aknD2, aknD3, and aknD4)
GYPFWDNRDIFGGYTFIG
HN
O
Actinokineosin