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Author(s)	Kuddus, Md. Ruhul; Yamano, Megumi; Rumi, Farhana; Kikukawa, Takashi; Demura, Makoto; Aizawa, Tomoyasu
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Enhanced Expression of Cysteine-Rich Antimicrobial Peptide Snakin-1 in *Escherichia coli* Using an Aggregation-prone Protein Coexpression System

Md. Ruhul Kuddus*

Graduate School of Life Science, Hokkaido University, Sapporo, Hokkaido 060-0810, Japan
Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Dhaka, Dhaka 1000, Bangladesh

Megumi Yamano*

Graduate School of Life Science, Hokkaido University, Sapporo, Hokkaido 060-0810, Japan

Farhana Rumi

Graduate School of Life Science, Hokkaido University, Sapporo, Hokkaido 060-0810, Japan

Takashi Kikukawa

Graduate School of Life Science, Hokkaido University, Sapporo, Hokkaido 060-0810, Japan
Global Station for Soft Matter, Global Institution for Collaborative Research and Education, Hokkaido University, Sapporo, Japan

Makoto Demura

Graduate School of Life Science, Hokkaido University, Sapporo, Hokkaido 060-0810, Japan
Global Station for Soft Matter, Global Institution for Collaborative Research and Education, Hokkaido University, Sapporo, Japan

Tomoyasu Aizawa

Graduate School of Life Science, Hokkaido University, Sapporo, Hokkaido 060-0810,

Japan
Global Station for Soft Matter, Global Institution for Collaborative Research and Education, Hokkaido University, Sapporo, Japan

*Contributed equally to this study
Correspondence concerning this article should be addressed to: Dr. Tomoyasu Aizawa at aizawa@mail.sci.hokudai.ac.jp

Abstract

Snakin-1 (SN-1) is a cysteine-rich plant antimicrobial peptide and the first purified member of the snakin family. SN-1 shows potent activity against a wide range of microorganisms, and thus has great biotechnological potential as an antimicrobial agent. Here, we produced recombinant SN-1 in *Escherichia coli* by a previously developed coexpression method using an aggregation-prone partner protein. Our goal was to increase the productivity of SN-1 via the enhanced formation of insoluble inclusion bodies in *E. coli* cells. The yield of SN-1 by the coexpression method was better than that by direct expression in *E. coli* cells. After refolding and purification, we obtained several milligrams of functionally active SN-1, the identity of which was verified by MALDI-TOF MS and NMR studies. The purified recombinant SN-1 showed effective antimicrobial activity against test organisms. Our studies indicate that the coexpression method using an aggregation-prone partner protein can serve as a suitable expression system for the efficient production of functionally active SN-1.

Keywords: Recombinant expression, antimicrobial peptide, snakin-1, inclusion body, Escherichia coli

Introduction

Cationic antimicrobial peptides (AMPs) are an important component of the host innate immune system against invading pathogens. Most AMPs share common characteristics—namely, small size and a strong cationic and amphipathic nature.[1] They have been isolated from a wide range of sources, including plants, insects, microbes, lower vertebrates and mammals.[2] In plants, the majority of AMPs are cysteine-rich,[3] which leads to the formation of multiple disulfide bonds and contributes to the compact peptide structure. Cysteine-rich plant AMPs are divided into several families, including the defensins, hevein-like peptides, knottin-type peptides, lipid transfer proteins, thionins, α-hairpinins and snakin families.[4] Among these, the peptides of the snakin family are perhaps the most characteristic plant AMPs; they contain conserved cysteine residues that are involved in the formation of six disulfide bonds that act as bridges to maintain the tertiary structure of the peptides.[5]

Snakin-1 (SN-1) is a cationic AMP which was first isolated from potato (*Solanum tuberosum*).[5] SN-1 is reported to possess significant activity against a wide range of both plant[5,6] and human[7] pathogens. Our recent report demonstrated that SN-1 is a membrane-active AMP that can kill targets by membrane disruption without being toxic

to mammalian cells.[8] According to X-ray crystallographic analysis of SN-1,[9] its 3-D structure is characterized by two short helices (α1 and α2) forming a helix-turn-helix, an additional helical section consisting of a short 3_{10}-helix, two rigidly held loops and six disulfide bonds between Cys^{5}-Cys^{30}, Cys^{9}-Cys^{26}, Cys^{13}-Cys^{22}, Cys^{29}-Cys^{62} , Cys^{33}-Cys^{49}, and Cys^{35}-Cys^{47}. The disulfide bonds are thought to confer this peptide with strong thermal, chemical and proteolytic stability. The second snakin peptide, snakin-2 (SN-2), is a 66-amino-acid-long AMP which was also isolated from potato tubers.[10] Although the amino acid sequence of SN-2 is only 38% identical to that of SN-1, both snakin peptides exhibit a similar spectrum of antimicrobial activity.[10] These peptides induce prompt aggregation of pathogens, although this response does not correlate with their inhibitory activity.[6,10] The overexpression of snakin peptide genes can confer broad-spectrum resistance to a wide variety of invading phytopathogens in crops.[11,12]

Since the first isolation and identification of snakin peptides from potato tubers,[5,10] many researchers have tried to prepare these peptides using a variety of methods, such as recombinant expression systems and chemical synthetic methods. At first, SN-1 was produced as insoluble inclusion bodies in the periplasm of *E. coli* cells using a pelB leader sequence.[6] Although, in this work it was designed to produce the correctly folded SN-1 peptide in the oxidizing compartment of *E. coli* periplasm, but complete disulfide

bridge formation was not successful, may be due to over production of SN-1 as well as its complicated structure. As a result, misfolded SN-1 was aggregated as insoluble inclusion bodies in the periplasmic space of *E. coli* host cell. Several years later, SN-1 and SN-2 peptides were produced by using a combination of solid-phase synthesis and chemical ligation.[13] Herbel *et al*. succeeded in expressing the SN-2 peptide from tomato (*Solanum lycopersicum*) in *E. coli* as a thioredoxin fusion protein.[14] Recently, we constructed the *Pichia pastoris* expression system for the production of a bioactive SN-1 peptide.[8]

Among these methods, the *E. coli* expression system is the most often utilized for AMPs production,[15,16] largely because *E. coli* is easy to handle, is inexpensive and grows rapidly. The formation of inclusion bodies in the *E. coli* cell cytoplasm is often considered as a suitable technique for heterologous expression of AMPs.[17–19] Although protein expression in the form of inclusion bodies is generally considered undesirable, it has proven beneficial in a number of biotechnological applications.[20–22] The major advantages of inclusion bodies are: i) they provide a rich source of relatively pure recombinant protein; ii) they can be easily isolated from the hosts that express them; and iii) they are resistant to proteolytic attack by cellular proteases. Therefore, inclusion body formation would be a useful strategy for the efficient production of recombinant

AMPs in *E. coli* hosts.[23,24] In general, it is not easy to control the inclusion body formation of target peptides directly during the recombinant expression of AMPs. To better control this process, fusion expression using insoluble carrier proteins such as the ketosteroid isomerase,[25] PurF fragment,[26] PaP3.30[27] and TAF12 histone fold domain[28] is used to form inclusion bodies of target AMPs in the *E. coli* cell cytoplasm, since these carrier proteins readily form inclusion bodies. However, in insoluble fusion protein systems, chemical cleavage is necessary to remove the fusion protein tags, because enzymatic cleavage is not suitable in denaturing condition for the solubilization of fusion proteins.[29] But, the major drawback of using chemical reagents is their undesirable side reactions with target peptides.[23]

Recently we developed a coexpression method[30,31] that enhanced the inclusion body formation of the target peptide by coexpression of an aggregation-prone protein as a partner protein. The charge of the partner protein was previously shown to affect the inclusion body formation by the target peptide, with an oppositely charged partner protein being considered most suitable for efficient formation of inclusion bodies of the target peptide.[30] Thus, negatively charged aggregation-prone partner protein can effectively enhance the inclusion body formation of positively charged antimicrobial peptides. To construct a coexpression system, we utilized a commercially available

pCOLADuet1 vector (Novagen) as coexpression vector. Recombinant peptides produced by this method can easily be purified from their partner proteins without the need of enzymatic or chemical cleavage. Therefore, in this study, we applied this coexpression method to produce potato SN-1 through the enhanced accumulation of inclusion bodies in *E. coli*. In this approach, the coexpression of an aggregation-prone protein (partner protein) was expected to enhance the inclusion body formation of the target peptide and to protect the newly expressed protein from proteolytic degradation by protease.

Materials and methods

Bacterial strains, plasmids, media, antibiotics

E. coli DH5α was used as a host strain for cloning and for preparing template plasmids. *E. coli* BL21 (DE3) was used as an expression host in combination with the pET22b(+) and pCOLADuet1 vector (Novagen) for expression of SN-1. Luria-Bertani (LB) [Bacto-tryptone 1% (w/v), yeast extract 0.5% (w/v), NaCl 1% (w/v)] was used as a culture medium. Ampicillin (50 μg/mL) and kanamycin (20 μg/mL) were used as selectable markers for pET22b(+) and pCOLADuet1 vector, respectively.

Vector construction

The SN-1 gene (GenBank accession no. GU137307) fragment was amplified by PCR with a set of primers using synthetic oligonucleotide as a template (Table 1). The purified PCR product was digested and then ligated to the pET22b(+) vector by using *Nde*I and *Bam*HI sites to remove the pelB leader sequence. The ligated vector (pET-SN-1) was introduced into *E. coli* DH5α and the presence of the SN-1 gene in the vector was analyzed by colony PCR and DNA sequencing.

Next, as a first step in vector construction for the coexpression system, the SN-1 gene fragment was again amplified by PCR with a set of primers using this pET-SN-1 vector template (Table 1). The PCR-amplified product was ligated to the pCOLADuet1 vector by using *Nde*I–*Xho*I sites, and the resulting pCOLA-SN-1 vector construct was analyzed by colony PCR and DNA sequencing. The pCOLA-SN-1 vector construct was also introduced into *E. coli* cells for the direct expression of SN-1.

For the coexpression technique, we selected a cysteine-less mutant of aggregation-prone human α-lactalbumin (GenBank accession no. NM002289)[31] as a partner protein. The cDNA of cysteine-less human α-lactalbumin (HLA) was synthesized by Eurofins MWG Operon. In this mutant, all eight cysteine residues in human α-lactalbumin were replaced with serine. The PCR-amplified partner protein gene fragments (HLA) were

digested using restriction enzymes, and then subcloned into the pCOLA-SN-1 vector by using *Nco*I-*Bam*HI sites. In this experiment, the pCOLA-SN-1 vector containing the HLA gene was named pCOLA-HLA-SN-1. The clone sequence was confirmed by capillary sequencing.

Evaluating the effect of the partner protein on the SN-1 expression level

E. coli BL21 (DE3) cells were transformed with the various expression constructs (pET-SN-1, pCOLA-SN-1, pCOLA-HLA-SN-1). The transformant cells were grown at 37°C in 5 mL of LB medium until the OD_{600} reached 1.0-1.2, then induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and further cultivated for 4 h. The cells were harvested by centrifugation at 15,000 rpm for 5 min at 4°C, then lysed using Bugbuster protein extraction reagent (Novagen). The extraction was carried out according to the manufacturer's instructions. The inclusion bodies were isolated by centrifugation at 15,000 rpm for 5 min at 4°C and analyzed by SDS-PAGE. During sample preparation for SDS-PAGE analysis, appropriate volume of SDS-PAGE buffer was added to the same final volume for each sample *i.e.*, soluble fraction, insoluble fraction and whole cell lysate. And then equal volume of sample was applied to each

well to compare the yields of target SN-1 peptide. The intensity of SN-1 bands was quantified by densitometry.

Expression of recombinant SN-1 peptide

The *E. coli* expression host harboring the pET-SN-1, pCOLA-SN-1, and pCOLA-HLA-SN-1 vectors was cultured overnight at 37°C in LB medium supplemented with one of the selectable markers. After the overnight preculture, the culture medium was inoculated in 1L of LB medium containing the appropriate marker. The cells containing three types of expression constructs were grown separately under the same culture conditions (37°C, 120 rpm) to an OD_{600} of 1.0-1.2. Then, 1 mM IPTG was added to induce peptide expression, and the cells were cultivated for another 4 h and harvested by centrifugation (6,000 rpm, 4°C, 10 min). The harvested cell pellets from 1L of bacterial culture were resuspended in lysis buffer (20 mM Tris–HCl, 1 mM EDTA, pH 8.0) and lysed by sonication. The mixture was centrifuged at 7,500 rpm for 30 min at 4°C to separate the soluble supernatant and the insoluble pellet fraction containing inclusion bodies. Next, the inclusion bodies were solubilized in solubilization buffer (8 M urea, 50 mM glycine-NaOH, 300 mM β-mercaptoethanol, 5

mM EDTA, pH 8.65) to prepare completely reduced and unfolded SN-1 and then subjected to overnight rotation at room temperature.

Purification of denatured SN-1 peptide

After centrifugation at 7,500 rpm for 10 min at 20°C, the clarified supernatant was filtered (0.45 μM) and applied to a 5 ml prepacked HiTrap SP HP cation-exchange column (GE Healthcare) pre-equilibrated with equilibration buffer (8 M urea, 50 mM glycine-NaOH, 300 mM β-mercaptoethanol, 5 mM EDTA, pH 8.65). The column was washed with the same equilibration buffer to remove impurities, and the bound SN-1 peptide was eluted at room temperature at a flow rate of 1 ml/min with a linear gradient of 0-40% equilibration buffer with 1M NaCl.

Refolding and purification

The eluted peptides were collected and dialyzed three times against refolding buffer (20 mM Tris–HCl, pH 8.0) at 4°C to remove urea and β-mercaptoethanol. After dialysis, the white sedimentation composed of the misfolded peptides was isolated by centrifugation (7500 rpm for 20 min) from a clear supernatant containing the refolded SN-1 peptide. Then both the clear supernatant portion of folded SN-1 and white precipitate of

misfolded SN-1 were analyzed by SDS-PAGE. For sample preparation, SDS-PAGE loading dye (2x) was added to the clear supernatant portion. Similarly, twice volume of dye (1x) was used to solubilize the white precipitate of misfolded SN-1. Then, equal volume of both supernatant portion and solubilized portion of precipitate was applied to each well of SDS-PAGE gel to compare the amount of folded and misfolded SN-1. The final purification of correctly folded SN-1 was achieved by RP-HPLC on a Cosmosil 5C18-AR-300 column (Nacalai Tesque). The peptide was eluted using a linear gradient of 15-25% acetonitrile with 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The yield of SN-1 was determined by measuring the absorbance at 280 nm. The purified recombinant SN-1 was lyophilized and stored at −30°C.

Purification of potato native SN-1

The native SN-1 peptide from potato tuber was extracted and purified according to the procedures published previously[5,8] with some modifications.

MALDI-TOF MS analysis of recombinant SN-1

MALDI-TOF mass spectrometry was carried out using a Bruker Autoflex Speed mass spectrometer (Bruker Daltonics). The peptide sample was mixed with sinapic acid matrix on a MALDI sample plate, and then the plate was air-dried and loaded onto the spectrometer for analysis.

NMR spectroscopy

Freeze-dried recombinant SN-1 and potato native SN-1 peptide were dissolved in a mixture of 90%$H_2$0/10%D_2O and adjusted to pH 3.0. NMR experiments were performed on a Bruker Avance III HD 600 MHz instrument. All spectra were processed using Bruker Topspin 3.4 software.

Microbicidal assay

The antimicrobial activity of refolded SN-1 was determined against Gram-positive *Listeria monocytogenes* (ATCC 19111) and Gram-negative *E. coli* ML35 (ATCC 43827). The minimal bactericidal concentrations (MBCs) were determined by colony forming unit (CFU) assay.[8] Bacteria growing exponentially (OD_{600} = 0.4-0.6) at 37°C were collected by centrifugation, washed, resuspended in sterile water and diluted in sterile water. Then bacteria (10^5-10^7 cfu/ml) were incubated with recombinant SN-1 for

1 h in a shaker incubator at 37°C, and the surviving bacteria were counted as cfu/ml after overnight growth on tryptic soy agar plates. Each assay was repeated three times.

Results

Vector construction for direct expression of SN-1

In this experiment, we first constructed a pET-SN-1 vector (Figure 2A) for the direct expression of SN-1 in the form of insoluble inclusion bodies in *E. coli* BL21(DE3). For preparation of the pET-SN-1 expression construct, the purified PCR product of the SN-1 gene was successfully ligated to the pET expression vector. Similarly, a pCOLA-SN-1 vector (Figure 2B) was also constructed for direct expression of SN-1 in *E. coli* cells. The *E. coli* transformants, upon the induction of IPTG, successfully produced the recombinant SN-1 in the form of insoluble inclusion bodies. In both cases a small amount of SN-1 was also detected in the soluble portion of the bacterial lysate. Although the amount of SN-1 directly expressed by the pET vector was low, surprisingly, the productivity could be increased in the same *E. coli* host cells simply by replacing the pET vector with pCOLA vector (Figure 3).

Construction of coexpression plasmid and its effect on SN-1 expression

To produce a high-yield SN-1 peptide through the formation of inclusion bodies, we exploited the principle of the coexpression method[30,31] using the same pCOLA vector as coexpression plasmid. The pCOLA vector is designed for the coexpression of two target genes from a single plasmid in *E. coli*. In order to construct a coexpression plasmid, the aggregation-prone HLA partner gene was subcloned into the first multiple cloning site of the pCOLA vector, and the SN-1 gene was subcloned into the second multiple cloning site of the pCOLA vector (Figure 2C). By coexpression of the HLA partner gene, the production of SN-1 in the form of an inclusion body was markedly increased as compared to the production via direct expression of SN-1 using the pET and pCOLA vectors (Figure 3). The results of SDS-PAGE analysis demonstrated that the soluble part contained a moderate amount of SN-1 produced directly by both the pET and pCOLA vectors, but in the case of the coexpression method, the expressed SN-1 peptide was exclusively present in the insoluble part in the form of inclusion bodies (Figure 3).

Purification of recombinant SN-1

Due to their opposite charges, recombinant SN-1 can be easily purified from its partner HLA by cation-exchange chromatography without enzymatic or chemical cleavage. The yield of SN-1 was increased the most by coexpression of HLA (Figure 4). After

cation-exchange chromatography, the denatured SN-1 peptide was refolded by dialysis. Using pET-SN-1expression system, only a clear supernatant solution was found in the dialysis bag after overnight dialysis. But, in case of pCOLA-SN-1 and pCOLA-HLA-SN-1 expression vector, we observed a small amount of white precipitate along with clear supernatant solution in the dialysis bag. The SDS-PAGE analysis indicated that the folded SN-1 was exclusively present in the clear supernatant portion. However, a tiny amount of misfolded SN-1 peptide was present in the white precipitate (Figure 5). Finally, the refolded SN-1 was purified by using a reverse-phase HPLC column. The retention time of SN-1 was found to be from 24.0 to 26.0 min (Figure 6A), which was identical to that of potato native SN-1 (Figure 6B). Although compared with the native SN-1 peptide sequence, the recombinant SN-1 was introduced one methionine residue at the amino terminal but this methionine residue was likely to be cleaved by methionine endopeptidase enzyme present in *E. coli* expression host cell.[32,33] Therefore, methionine residue may have no effect on the retention time of recombinant SN-1. After a two-step purification procedure, we finally obtained 0.6 and 1.2 mg of pure recombinant SN-1 by direct expression using the pET and pCOLA vectors, respectively. On the other hand, by the coexpression method, we were able to obtain about 2.0 mg of correctly folded SN-1 from 1L of bacterial culture (Figure 7).

Characterization of recombinant SN-1 by mass spectrometry and NMR

The purified recombinant SN-1 was subjected to MALDI-TOF MS to determine its molecular weight. The MALDI-TOF MS peak had a molecular mass of 6923.31 Da (Figure 8), which is consistent with the theoretical $[M+H]^+$ value, 6923.00 Da, of the SN-1 peptide with six disulfide bonds. This result indicates that recombinant SN-1 does not contain amino terminal methionine residue due to the cleavage by methionine endopeptidase enzyme. The TOCSY NMR spectrum of recombinant SN-1 was also identical with the native one (Fig. 9A, 9B). Because in NMR experiments, chemical shifts of peptides are quite sensitive to tertiary structure, not only the disulfide bridge pattern but also tertiary structures of recombinant SN-1 were most likely to be identical to those of the native one.

Antimicrobial activity of recombinant SN-1

Recombinant SN-1 produced by the coexpression method showed strong antimicrobial activity against the tested organisms. As shown in Figure 10, it exhibited strong activity against *E. coli* and *L. monocytogenes*, with MBC values of 10.0 and 20.0 μM, respectively, indicating that the Gram-negative bacterium (*E. coli*) was more sensitive than the Gram-positive bacterium (*L. monocytogenes*). These results are in close

agreement with those published previously for both the native SN-1[7] of potatoes and the recombinant SN-1 derived from *P. pastoris*.[8]

Discussion

Recently, snakin peptides have attracted much attention in the field of agricultural biotechnology due to their potent antimicrobial activities against a wide range of bacterial and fungal phytopathogens, which suggests their potential importance as crop protection agents.[6,11,34–36] However, because further structural and functional analyses are required, there is need of an efficient method to produce high yields of functional snakin peptides.

In this study, we attempted to develop such a method by selecting a snakin peptide, SN-1, as the target peptide. Protein SN-1 is a peptide of 63 amino acid residues (MW-6922.00 Da) which contains a short, central hydrophobic stretch (residues 25 to 30) in its structure.[5] We first attempted to produce recombinant SN-1 by direct expression as an inclusion body using two expression vectors, pET and pCOLA. The amount of SN-1 peptide produced by the pCOLA vector was markedly increased compared to the amount by direct expression with the pET vector (Figure 7). We were

not able to clarify why this simple vector change induced an increased level of SN-1 expression even in the absence of a coexpression partner protein. We speculated that the enhanced productivity of SN-1 was due to differences in the antibiotic-resistance gene for selection or the origin of the replication gene between the pET and pCOLA vectors.

Accordingly, we applied a previously developed coexpression method[30,31] using pCOLA vector to coexpress target SN-1 peptide and partner proteins. In some studies, the coexpression of an insoluble partner protein has been reported to enhance the inclusion body formation of the target peptide. Tomisawa *et al.* succeeded in expressing a large amount of cysteine-rich AMPs such as antibacterial factor-2 and the mouse α-defensin, cryptidin-4 in *E. coli* cells as inclusion body by coexpression of aggregation-prone partner protein.[30,31] Saito *et al.* reported the enhanced expression of somatomedin C by coexpression of insulin-like growth factor I.[37] Similarly a potent antimicrobial peptide, buforin IIb was successfully produced by coexpression of human gamma interferon.[38]

In the current work, we selected cysteine-less human α-lactalbumin (HLA) as an anionic (p*I* 4.7) coexpression partner protein for overexpression of the cationic SN-1 peptide (p*I* 8.97). Previously a study was carried out to evaluate the effect of cysteine residue of the partner protein on the inclusion body formation of the target peptide using cysteine-less

partner protein.[31] The result of that study prompted us to use cysteine-less human α-lactalbumin (HLA) as partner protein because it induced a greater increase in the expression level of target gene than the partner protein with cysteine residues. In our study, we confirmed that the coexpression of HLA by pCOLA vector markedly enhanced the expression level of SN-1. Coexpression of an aggregation-prone partner protein can enhance the formation of inclusion bodies of target peptides and protect them from proteolytic degradation. Thus the coexpression method enhances the expression level of the target peptide as an inclusion body.

Recombinant SN-1 was efficiently separated from the HLA partner by one-step cation-exchange chromatography because the charge of SN-1 is opposite to that of HLA. Then, the denatured SN-1 peptide was refolded by a standard dialysis procedure. Initially, we tried to perform the dialysis using a high concentration of denatured SN-1. But we did not succeed in obtaining a large amount of refolded SN-1 under this refolding condition. Therefore, the concentration of the unfolded SN-1 peptide solution was adjusted to approximate 0.5 mg/ml by spectrophotometric method at 280 nm. After dialysis and RP-HPLC purification, we obtained 2.0 mg of correctly folded SN-1 from 1L of bacterial culture, while about 0.2 mg of pure native SN-1 was directly isolated from 1.0 Kg of potato tubers.[8]

In summary, we have constructed an efficient system for the overexpression of SN-1 as inclusion bodies in *E. coli*. The expression level of SN-1 was enhanced by coexpression of an anionic partner protein. We purified the expressed SN-1 and confirmed that its six disulfide bonds were correctly formed by MALDI-TOF MS and NMR studies. The results suggest that the present coexpression technique using an aggregation-prone partner protein may provide an easy and low-cost strategy for the large-scale production of AMPs.

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Figure legends

Figure 1. The nucleotide sequence and its corresponding amino acid sequence of SN-1 (A) and partner gene HLA (B).

Figure 2. Schematic representation of the expression vectors pET-SN-1 (A) pCOLA-SN-1 (B), and pCOLA-HLA-SN-1 (C). SN-1, snakin-1 gene; HLA, cysteine-less human α-lactalbumin; Amp^R, ampicillin-resistance gene; Kan^R, Kanamycin-resistance gene.

Figure 3. Effects of partner proteins on the SN-1 expression level. (A) SDS-PAGE analysis of the expression level of SN-1. Lane M, molecular weight marker; lane W, whole cell lysate; lane S, soluble portion of bacterial cell lysate; lane I, insoluble portion of bacterial cell lysate. (B) The densitometric intensity data of insoluble portion (I) for the coexpression method are expressed in relation to those for the direct expression method.

Figure 4. Cation-exchange chromatographic purification of recombinant SN-1 expressed by pET-SN-1 (A), pCOLA-SN-1 (B) and pCOLA-HLA-SN-1 (C).

Figure 5. SDS-PAGE analysis of the refolding condition of the recombinant SN-1 peptide. Lane 1, sample containing the denatured SN-1 peptide before dialysis; lane 2, clear supernatant after overnight dialysis; lane 3, white precipitate after overnight dialysis.

Figure 6. RP-HPLC purification of recombinant SN-1 (A) and potato native SN-1 (B).

Figure 7. Final yields of the purified recombinant SN-1 produced by different expression constructs.

Figure 8. MALDI-TOF MS analysis of recombinant SN-1.

Figure 9. TOCSY NMR spectrum of recombinant SN-1 (A) and potato native SN-1 (B).

Figure 10. Antimicrobial activity of recombinant SN-1.

Table

Table 1. Sequence of primers used in this study

Name	Primer sequence[a] (from the 5' end to 3' end)	Restriction site
A) pET-SN-1 vector construction		
Primers for the SN-1 gene	F= GGAATTCCATATGGGTTCAAATTTTTGTGATTCAAAGTGC	*Nde*I
	R= GCGGATCCTCAAGGGCATTTAGACTTGCCCTTAGA	*Bam*HI
B) pCOLA-SN-1 and pCOLA-HLA-SN-1 vector construction		
Primers for the SN-1 gene	F= GGAATTCCATATGGGTTCAAATTTTTGTGATTCAAAGTGC	*Nde*I
	R=GTTAACTCGAGTCAAGGGCATTTAGACTTGCC	*Xho*I
Primers for the partner HLA gene	F = GAATTCCCATGGGCAAGCAATTCACAAAATCTGAG	*Nco*I
	R= CGGGATCCTTACAACTTCTCAGAAAGCCAC	*Bam*HI

a. Restriction sites are underlined.

Fig.1

A)

```
ATG GGT TCA AAT TTT TGT GAT TCA AAG TGC AAG CTG AGA TGT TCA AAG GCA GGA CTT GCA
Met Gly Ser Asn Phe Cys Asp Ser Lys Cys Lys Leu Arg Cys Ser Lys Ala Gly Leu Ala

GAC AGA TGC TTA AAG TAC TGT GGA ATT TGT TGT GAA GAA TGC AAA TGT GTG CCT TCT GGA
Asp Arg Cys Leu Lys Tyr Cys Gly Ile Cys Cys Glu Glu Cys Lys Cys Val Pro Ser Gly

ACT TAT GGT AAC AAA CAT GAA TGT CCT TGT TAT AGG GAC AAG AAG AAC TCT AAG GGC AAG
Thr Tyr Gly Asn Lys His Glu Cys Pro Cys Tyr Arg Asp Lys Lys Asn Ser Lys Gly Lys

TCT AAA TGC CCT TGA
Ser Lys Cys Pro End
```

B)

```
ATG GGC AAG CAA TTC ACA AAA TCT GAG CTG TCC CAG CTG CTG AAA GAC ATA GAT GGT TAT
Met Gly Lys Gln Phe Thr Lys Ser Glu Leu Ser Gln Leu Leu Lys Asp Ile Asp Gly Tyr

GGA GGC ATC GCT TTG CCT GAA TTG ATC TCT ACC ATG TTT CAC ACC AGT GGT TAT GAC ACA
Gly Gly Ile Ala Leu Pro Glu Leu Ile Ser Thr Met Phe His Thr Ser Gly Tyr Asp Thr

CAA GCC ATA GTT GAA AAC AAT GAA AGC ACG GAA TAT GGA CTC TTC CAG ATC AGT AAT AAG
Gln Ala Ile Val Glu Asn Asn Glu Ser Thr Glu Tyr Gly Leu Phe Gln Ile Ser Asn Lys

CTT TGG TCT AAG AGC AGC CAG GTC CCT CAG TCA AGG AAC ATC TCT GAC ATC TCC TCT GAC
Leu Trp Ser Lys Ser Ser Gln Val Pro Gln Ser Arg Asn Ile Ser Asp Ile Ser Ser Asp

AAG TTC CTG GAT GAT GAC ATT ACT GAT GAC ATA ATG TCT GCC AAG AAG ATC CTG GAT ATT
Lys Phe Leu Asp Asp Asp Ile Thr Asp Asp Ile Met Ser Ala Lys Lys Ile Leu Asp Ile

AAA GGA ATT GAC TAC TGG TTG GCC CAT AAA GCC CTC TCT ACT GAG AAG CTG GAA CAG TGG
Lys Gly Ile Asp Tyr Trp Leu Ala His Lys Ala Leu Ser Thr Glu Lys Leu Glu Gln Trp

CTT TCT GAG AAG TTG TAA
Leu Ser Glu Lys Leu End
```

Fig.2

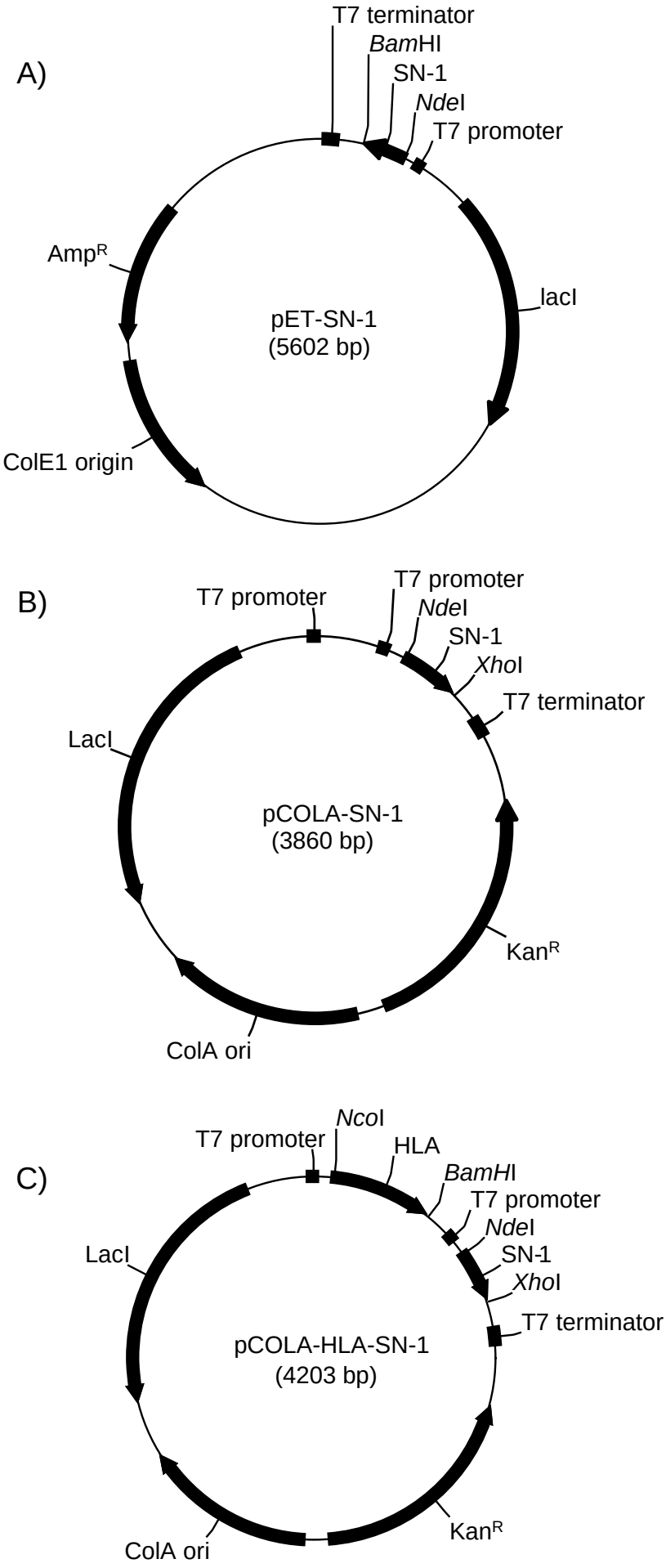
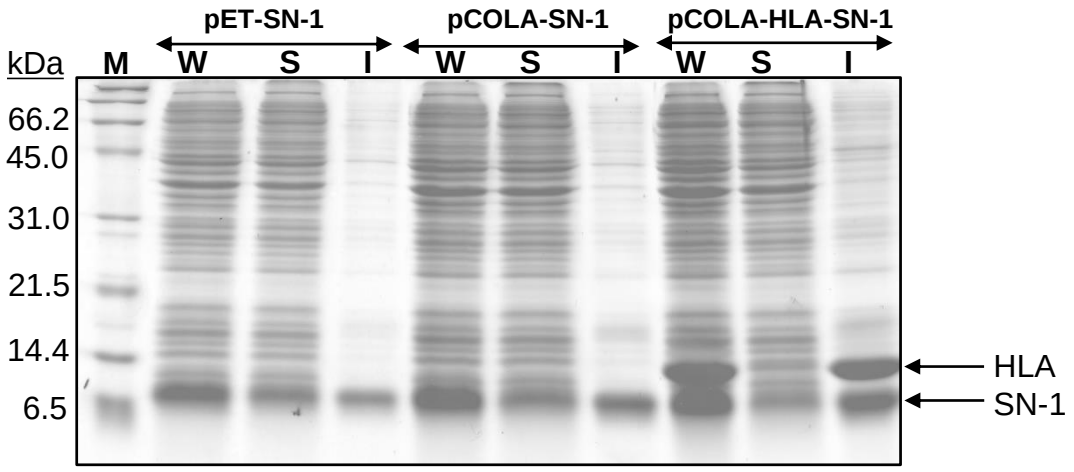

A)
T7 terminator
BamHI
SN-1
NdeI
T7 promoter
AmpR
lacI
pET-SN-1
(5602 bp)
ColE1 origin
B)
T7 promoter
T7 promoter
NdeI
SN-1
XhoI
T7 terminator
LacI
pCOLA-SN-1
(3860 bp)
KanR
ColA ori
C)
NcoI
T7 promoter
HLA
BamHI
T7 promoter
NdeI
SN-1
XhoI
T7 terminator
LacI
pCOLA-HLA-SN-1
(4203 bp)
KanR
ColA ori

Fig.3

A)


pET-SN-1
pCOLA-SN-1
pCOLA-HLA-SN-1
kDa
M
W
S
I
W
S
I
W
S
I
66.2
45.0
31.0
21.5
14.4
6.5
HLA
SN-1

B)

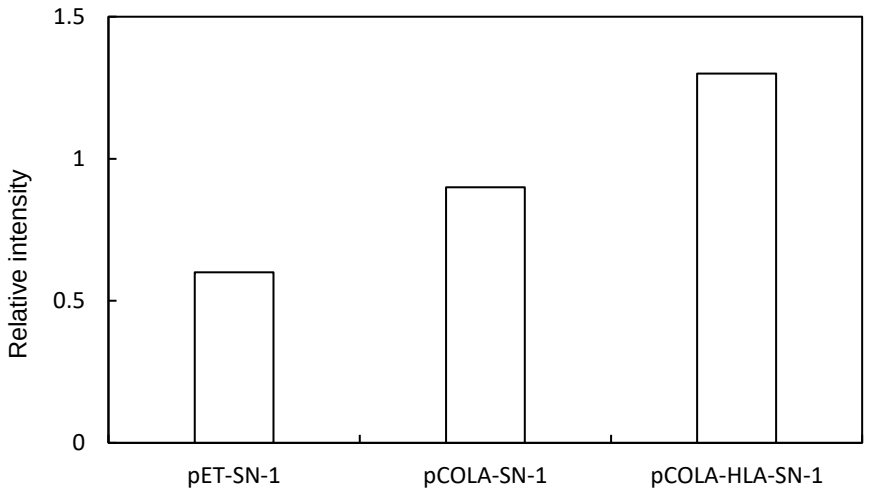
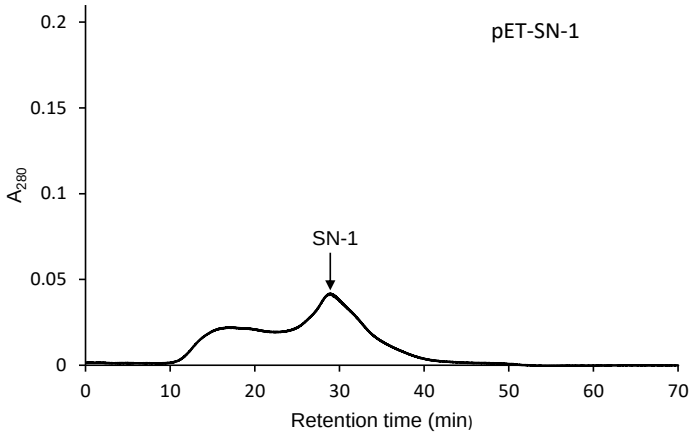

1.5
1
0.5
0
Relative intensity
pET-SN-1
pCOLA-SN-1
pCOLA-HLA-SN-1

Fig.4

A)


pET-SN-1
SN-1
A_{280}
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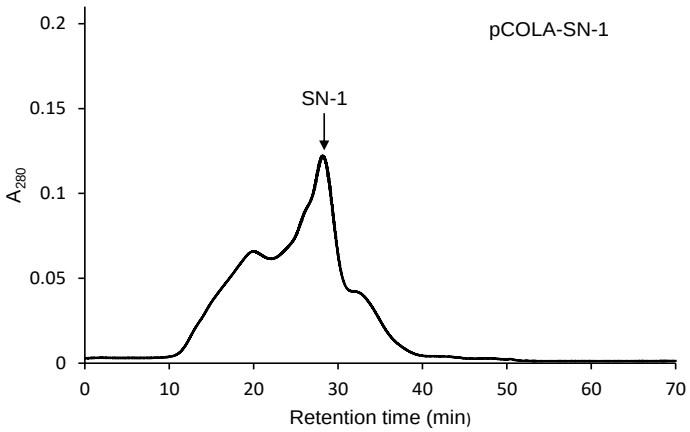
0.15
0.1
0.05
0
0
10
20
30
40
50
60
70
Retention time (min)

B)


pCOLA-SN-1
SN-1
A_{280}
0.2
0.15
0.1
0.05
0
0
10
20
30
40
50
60
70
Retention time (min)

C)

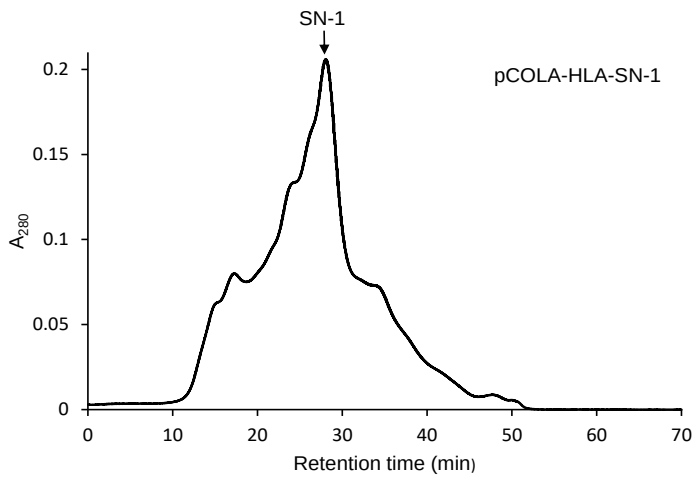

SN-1
pCOLA-HLA-SN-1
A_{280}
0.2
0.15
0.1
0.05
0
0
10
20
30
40
50
60
70
Retention time (min)

Fig.5

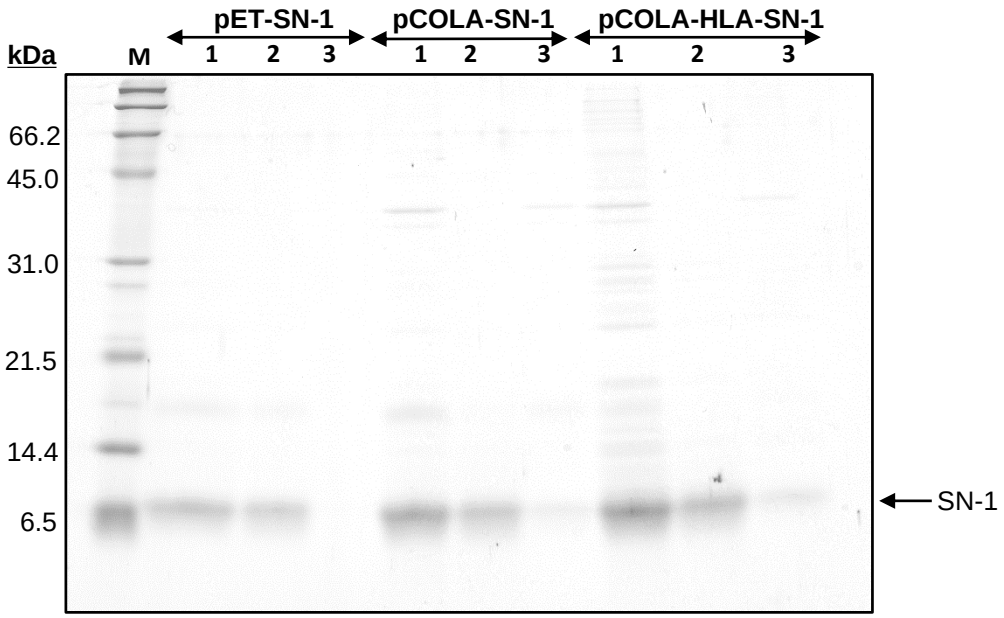

pET-SN-1
pCOLA-SN-1
pCOLA-HLA-SN-1
kDa
M
1 2 3 1 2 3 1 2 3
66.2
45.0
31.0
21.5
14.4
6.5
SN-1

Fig.6

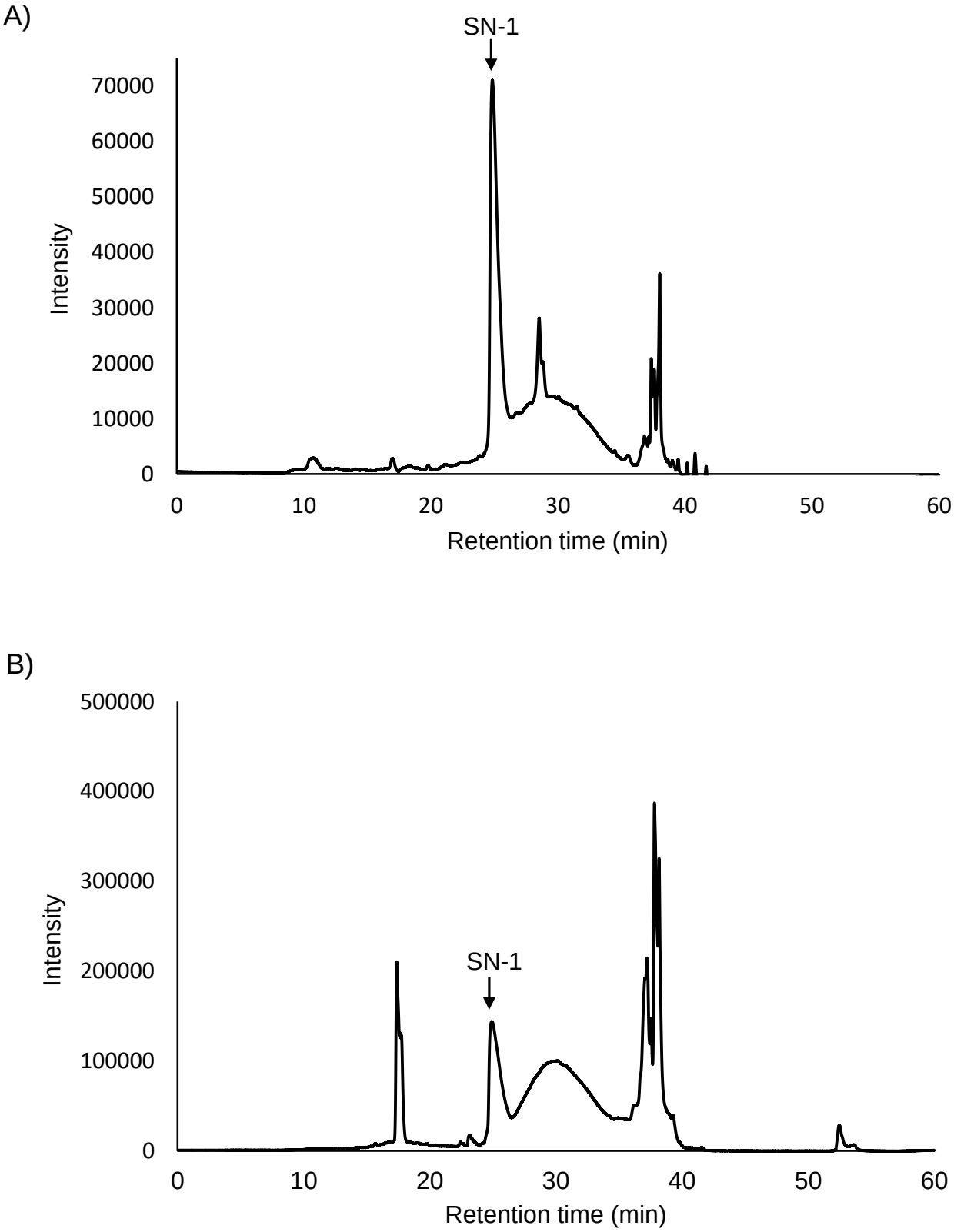

A)
SN-1
70000
60000
50000
40000
30000
20000
10000
0
Intensity
0
10
20
30
40
50
60
Retention time (min)
B)
500000
400000
300000
200000
100000
0
Intensity
SN-1
0
10
20
30
40
50
60
Retention time (min)

Fig.7

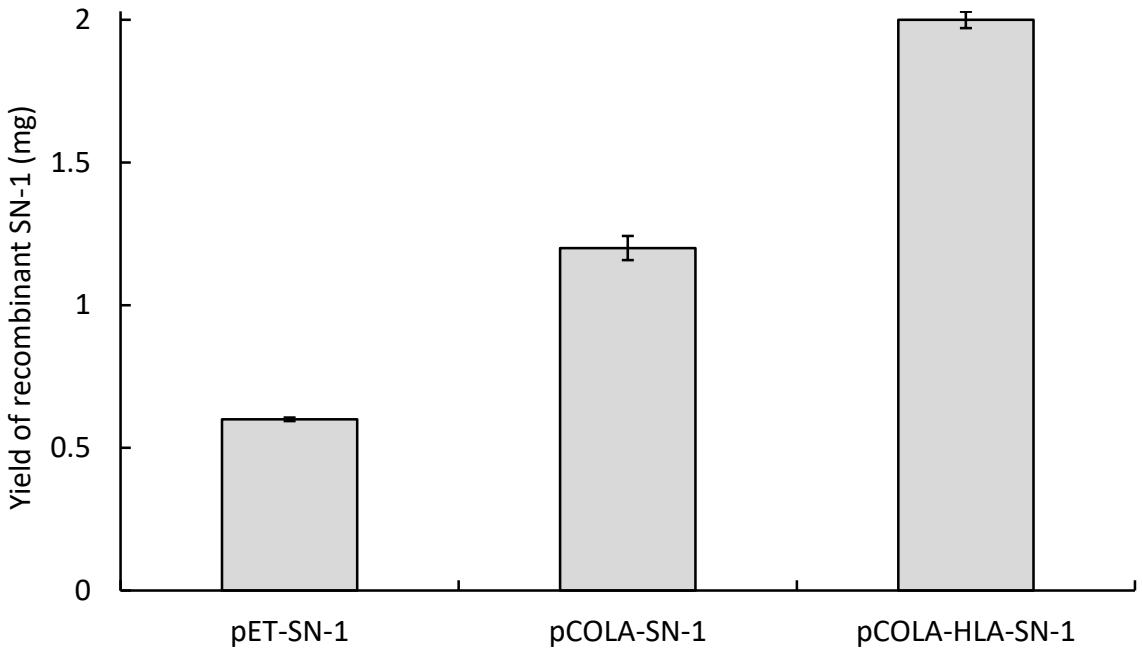

Yield of recombinant SN-1 (mg)
2
1.5
1
0.5
0
pET-SN-1
pCOLA-SN-1
pCOLA-HLA-SN-1

Fig.8

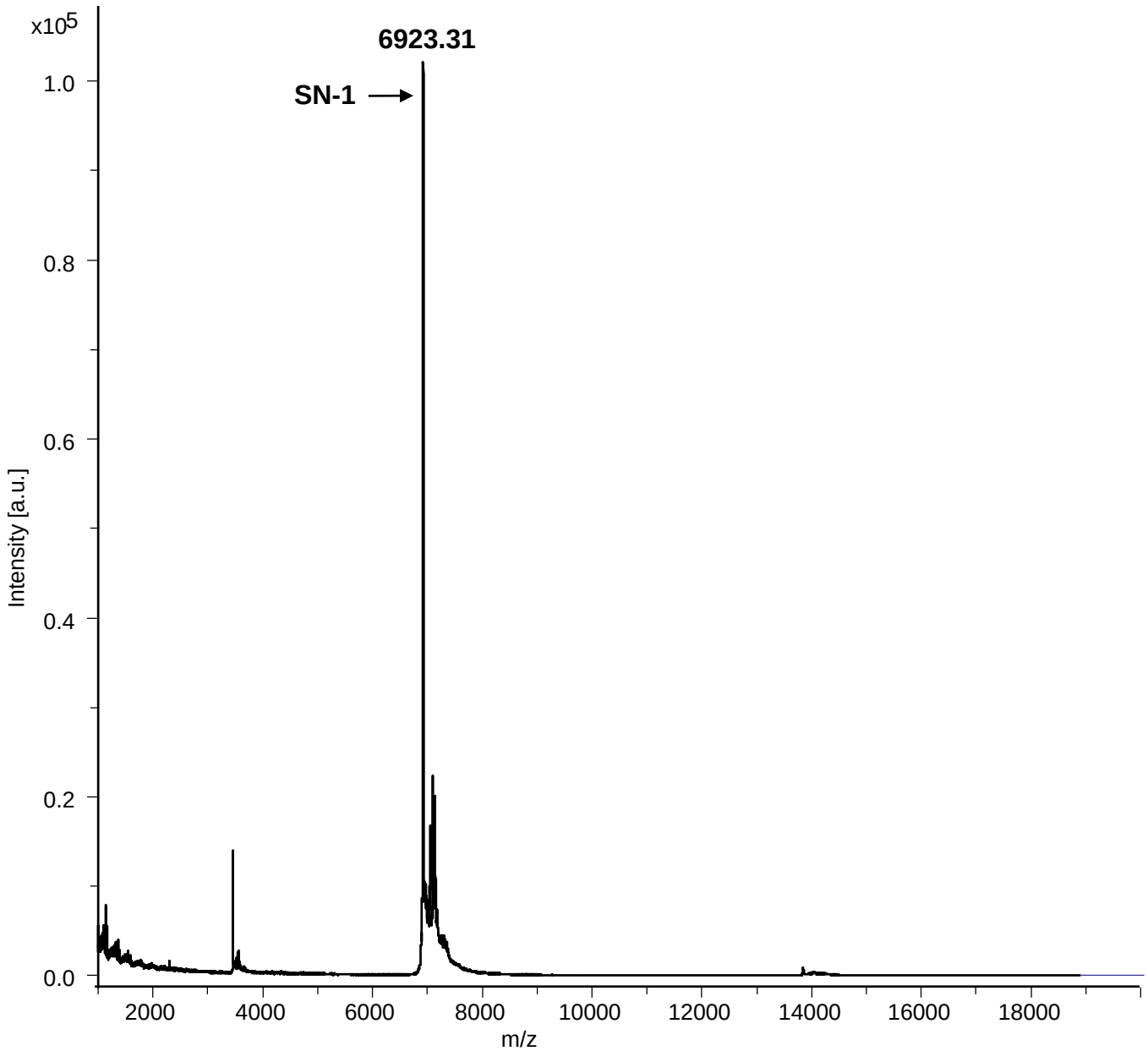
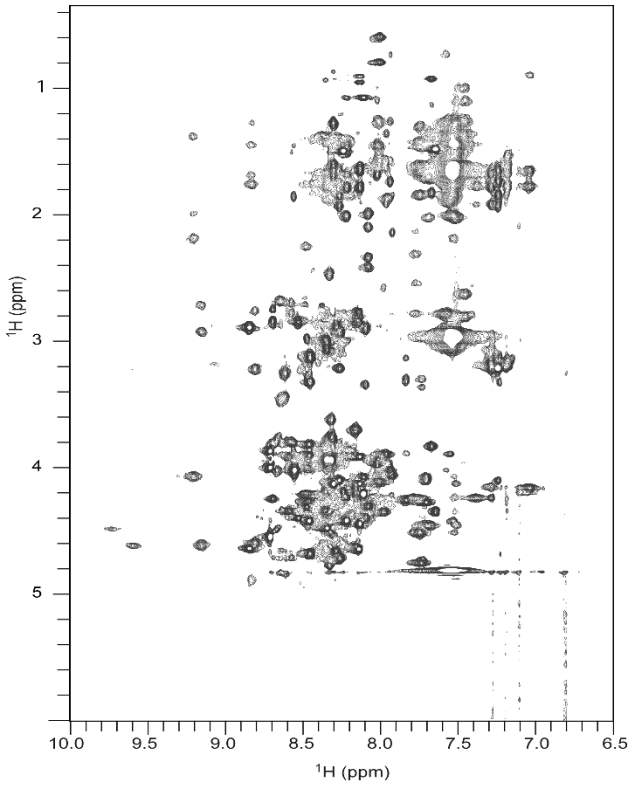

x10^5
1.0
0.8
0.6
0.4
0.2
0.0
Intensity [a.u.]
6923.31
SN-1
2000
4000
6000
8000
10000
12000
14000
16000
18000
m/z

Fig.9

A)


1
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3
4
5
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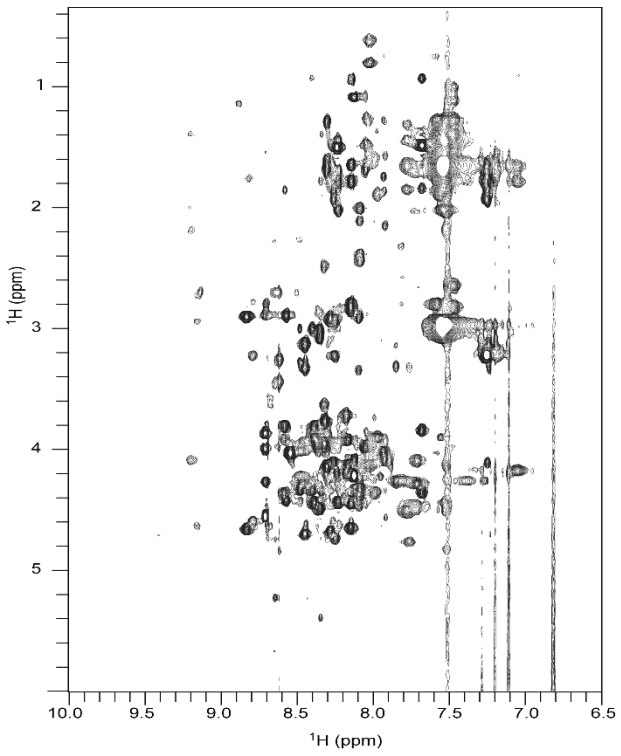
9.0
8.5
8.0
7.5
7.0
6.5
^{1}H (ppm)

B)


1
2
3
4
5
^{1}H (ppm)
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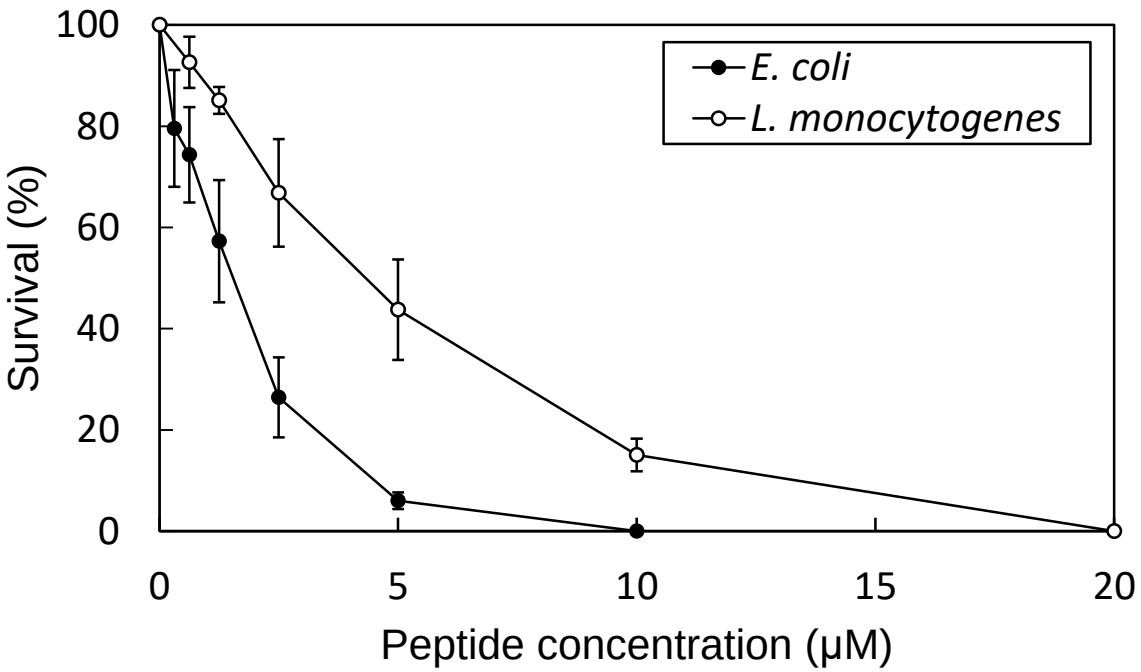
9.0
8.5
8.0
7.5
7.0
6.5
^{1}H (ppm)

Fig.10


100
80
60
40
20
0
Survival (%)
0
5
10
15
20
Peptide concentration (μM)
E. coli
L. monocytogenes