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Construction of the over-expression system of cysteine-rich plant antimicrobial peptide snakin-1

(植物由来システインリッチ抗菌ペプチド snakin-1 の大量発現系の構築)

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General Introduction

Antimicrobial peptides (AMPs), also known as host defense peptides, are important components of the innate immune system. Generally, AMPs are short peptides with a net positive charge and are ubiquitous in nature [1-3]. These multifunctional peptides are believed to act against invading pathogens by formation of membrane pores, resulting in cell leakage and ultimately, cell death [4-6]. There are three proposed mechanisms which account for peptide permeation of the membrane of the target cell, including “barrel-stave model”, “carpet model” and “toroidal-pore model” [4]. Besides membrane disruption, AMPs also target cellular processes including nucleic acid and protein synthesis, enzymatic activity, and/or cell wall synthesis [2]. Due to their natural antimicrobial properties and a low propensity for the development of bacterial resistance [7], AMPs have rapidly captured attention as a rich source of lead compounds for the discovery of novel peptide antibiotics [4,8]. For this reason, study in this field has been emphasized on screening novel AMPs.

To accomplish the extensive research on structure and mechanism of antimicrobial action, large amount of purified and bioactive antimicrobial peptide molecules are necessary. But, isolation of large amounts of AMPs from their natural sources is not practically feasible due to their low concentration in these sources [9]. Their large scale production by chemical synthesis is very costly. Therefore, recombinant DNA technique

has been employed to produce AMPs in greater amounts using different expression hosts like *Escherichia coli, Pichia pastoris, Saccharomyces cerevisiae* etc [10,11].

In chapter 1, I have utilized the *E. coli* expression system for enhanced production of recombinant SN-1. Since, *E. coli* is easy to handle, grows rapidly, and used to produce more than 80% recombinant AMPs. However, the direct expression of AMPs in *E. coli* poses some difficulties, such as their toxicity towards the expression host as well as susceptibility to protease. The expression of AMPs in the form of inclusion bodies may alleviate the problems of toxicity and instability. But, it is not easy to control the inclusion body formation of target peptides directly during the recombinant expression of AMPs. Therefore, in chapter 1, I applied the coexpression method [12] to produce potato SN-1 through the enhanced accumulation of inclusion bodies in *E. coli*. The yield of SN-1 by the coexpression method using aggregation-prone partner protein was better than that by direct expression in *E. coli* cells. After HPLC purification, I was succeeded to obtain several milligrams of functionally active SN-1. MALDI-TOF MS and NMR analysis indicated that the recombinant SN-1 produced by coexpression method had the same folding when compared to native potato SN-1. From this study, I have concluded that the coexpression method can serve as a suitable expression system for the efficient production of functionally active SN-1.

In chapter 2 of this thesis, I successfully constructed an overexpression system of SN-1 using methylotrophic yeast *Pichia pastoris* GS115. This yeast was selected because *P. pastoris* cells under the control of a methanol-induced AOX1 promoter can produce large amounts of functionally active cysteine-rich AMPs that are secreted directly into the culture media [2]. I obtained large amount of pure recombinant SN-1 (Yield = 40 mg/1L) from a fed-batch fermentation culture after purification with a cation exchange column followed by RP-HPLC. Moreover, I have confirmed the identity and disulfide connectivity of recombinant SN-1 by MALDI-TOF MS, CD and ^{1}H NMR experiments. Additionally, I examined the membrane permeability of recombinant SN-1 using *E. coli* ML35. The assay results showed that SN-1 exhibited bactericidal activity through the membrane disruption of target cells. This study provided information about efficient expression of a functionally active SN-1 in *P. pastoris.*

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Chapter 1

Enhanced expression of cysteine-rich antimicrobial peptide snakin-1 in *Escherichia coli* using an aggregation-prone protein coexpression system

1-1 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, I produced recombinant SN-1 in *Escherichia coli* by a previously developed coexpression method using an aggregation-prone partner protein. My 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, I 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. The results of this study 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.

1-2 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. In my previous report I have 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, I 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 Tomisawa *et al.*, 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, a commercially available pCOLADuet1 vector (Novagen) was utilized 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, I 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.

1-3 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, I 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, approximate 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_20/10%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.

1-4 Results

Vector construction for direct expression of SN-1

In this experiment, I 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, I 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, I 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, I 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, I was 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].

1-5 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, I 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]. I 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). I was 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. I 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, I 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, I 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, I 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, I tried to perform the dialysis using a high concentration of denatured SN-1. But I 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, I 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, I 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. I 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.

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.

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
```

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

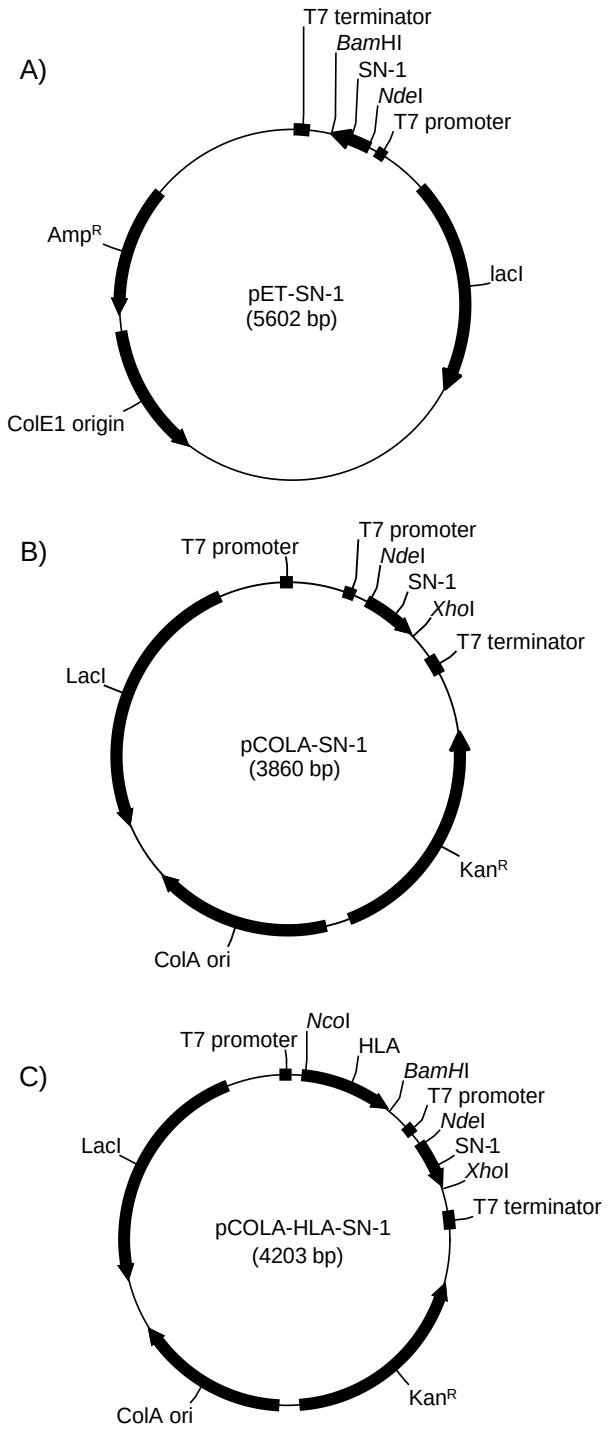


Figure 2. Schematic representation of the expression vectors pET-SN-1 (A) pCOLA-SN-1 (B), and pCOLA-HLA-SN-1 (C). SN-1, snakin-1 gene; HLA, cysteine-less human α-lactalbumin; Amp^R, ampicillin-resistance gene; Kan^R, Kanamycin-resistance gene.

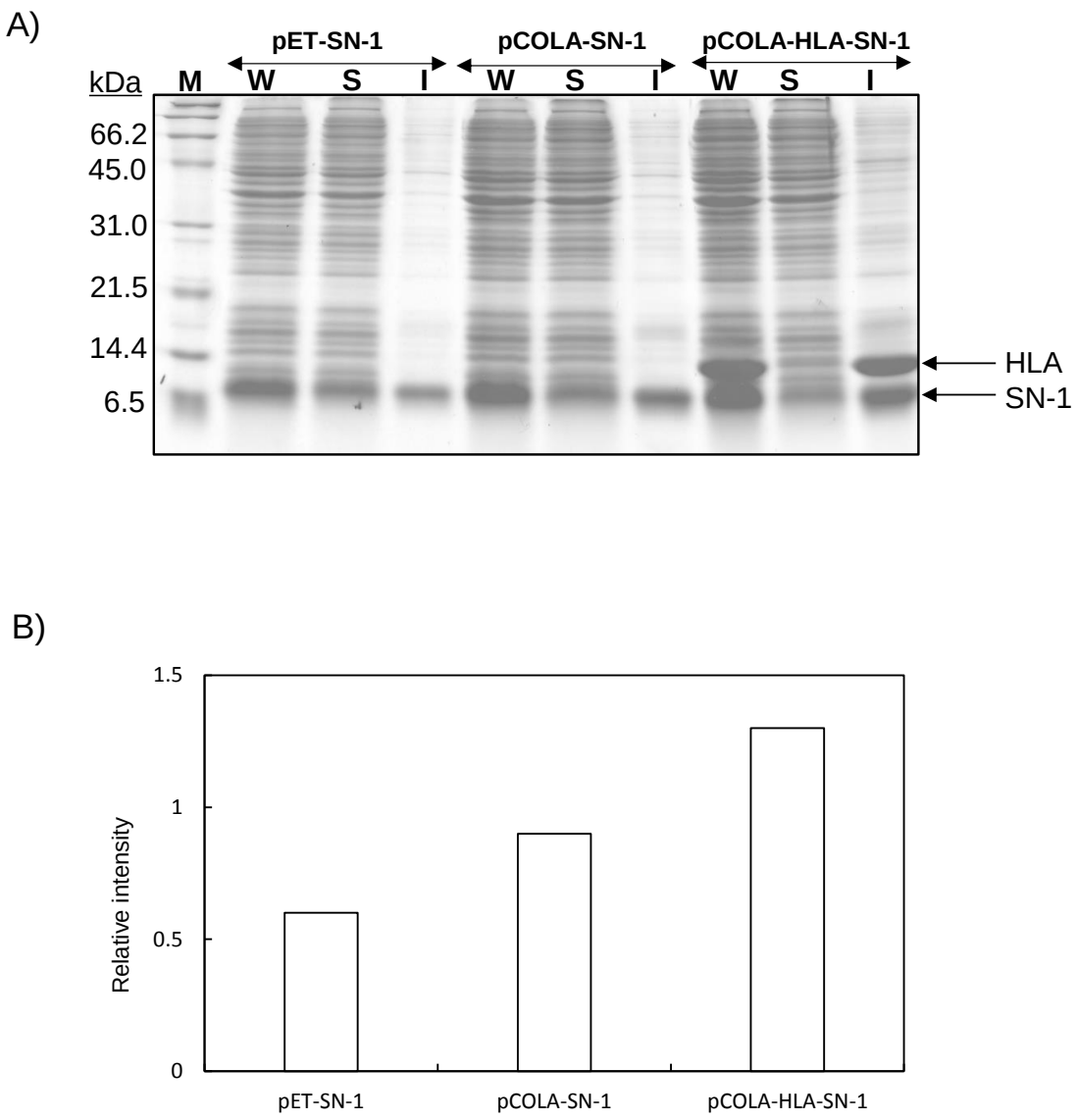


Figure 3. Effects of partner proteins on the SN-1 expression level. (A) SDS-PAGE analysis of the expression level of SN-1. Lane M, molecular weight marker; lane W, whole cell lysate; lane S, soluble portion of bacterial cell lysate; lane I, insoluble portion of bacterial cell lysate. (B) The densitometric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

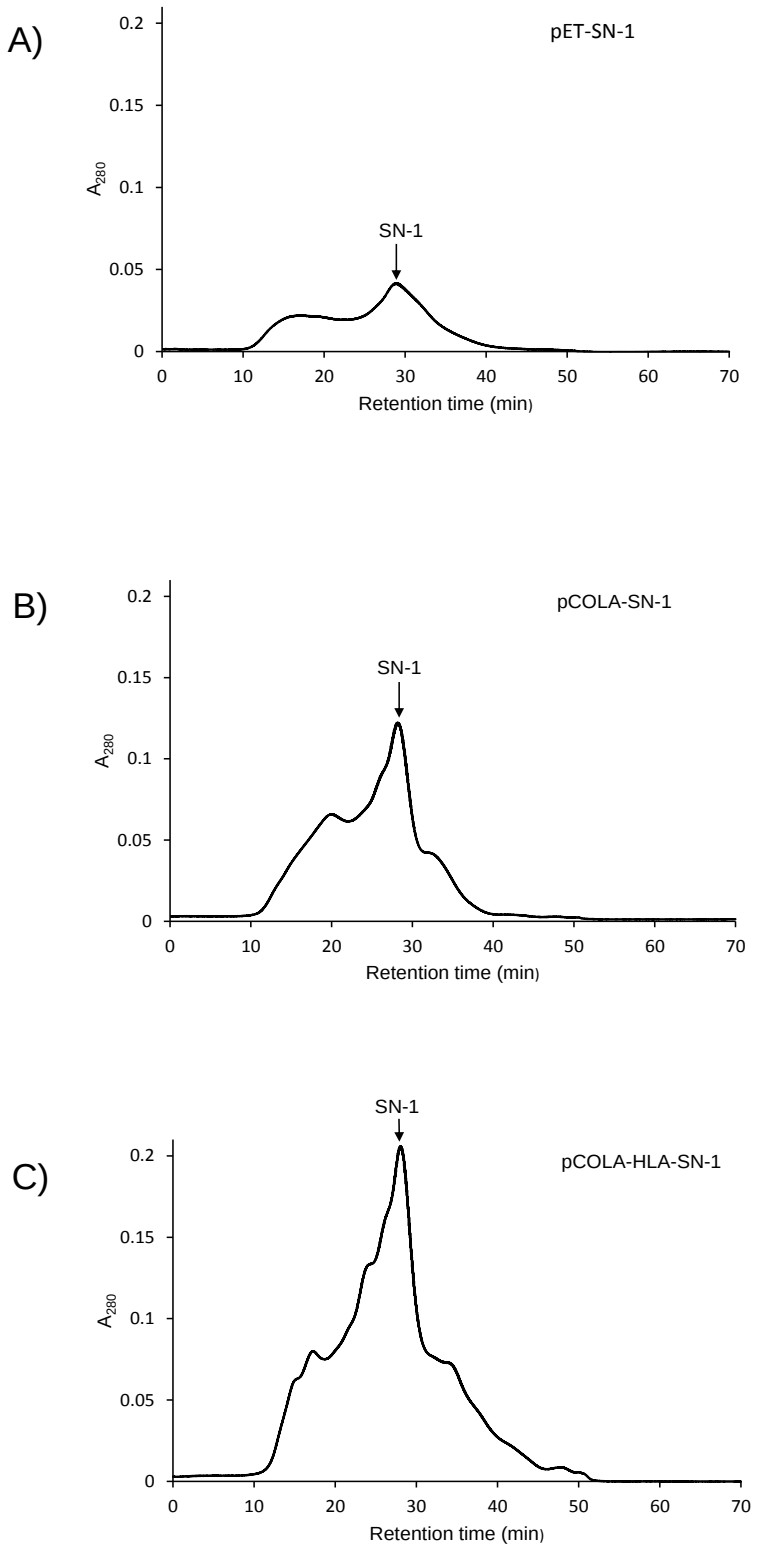


Figure 4. Cation-exchange chromatographic purification of recombinant SN-1 expressed by pET-SN-1 (A), pCOLA-SN-1 (B) and pCOLA-HLA-SN-1 (C).

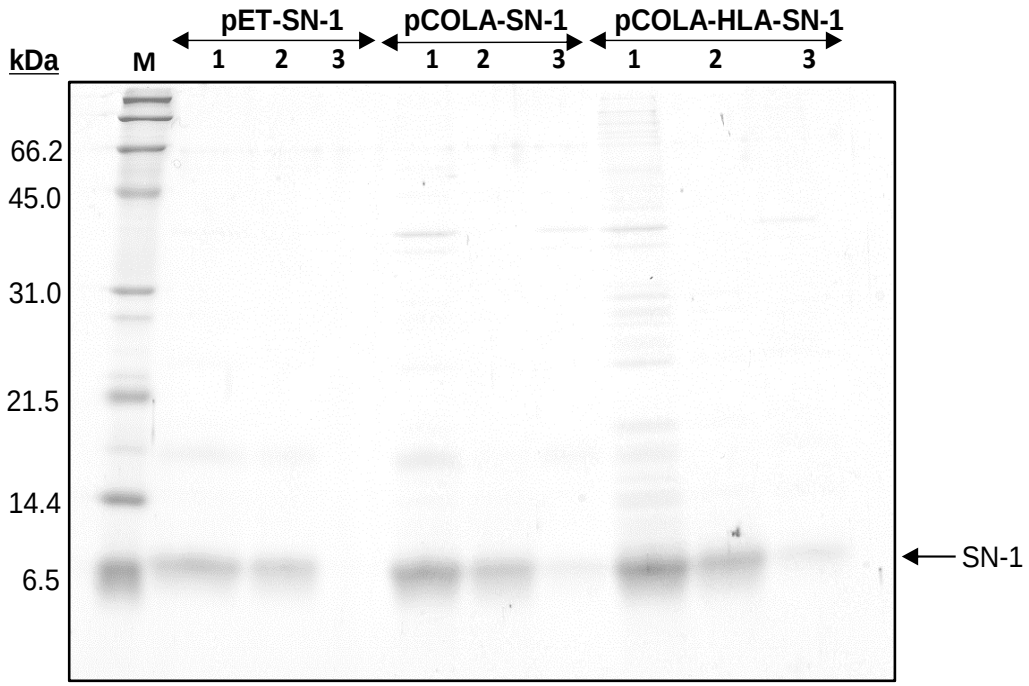


Figure 5. SDS-PAGE analysis of the refolding condition of the recombinant SN-1 peptide. Lane 1, sample containing the denatured SN-1 peptide before dialysis; lane 2, clear supernatant after overnight dialysis; lane 3, white precipitate after overnight dialysis.

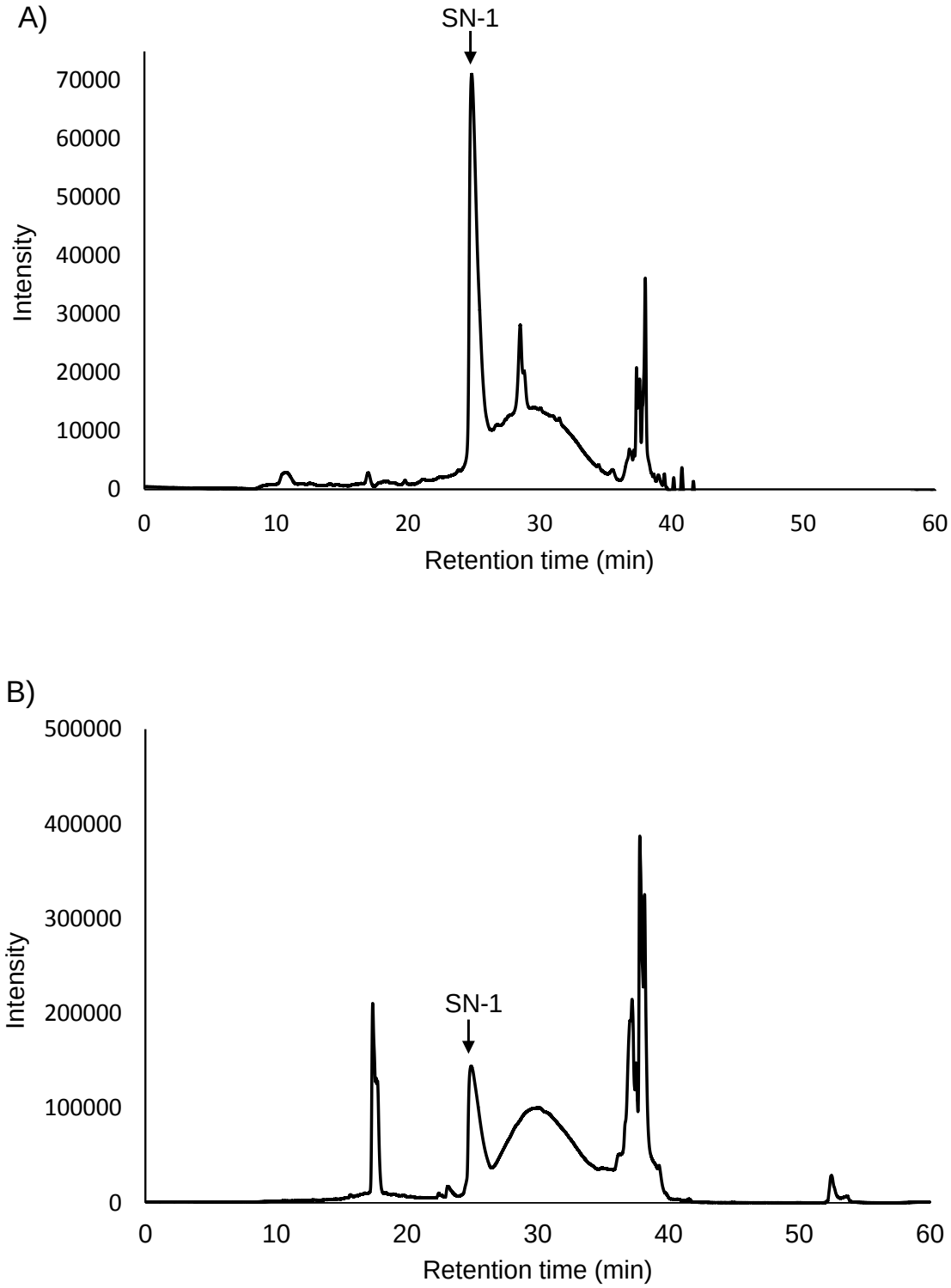


Figure 6. RP-HPLC purification of recombinant SN-1 (A) and potato native SN-1 (B).

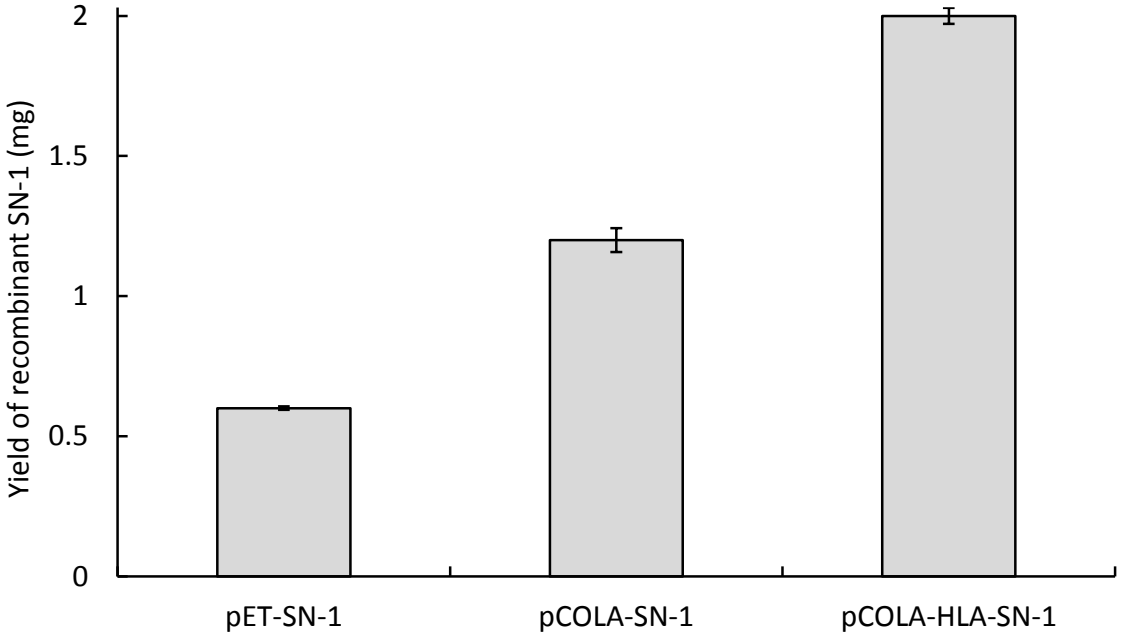


Figure 7. Final yields of the purified recombinant SN-1 produced by different expression constructs.

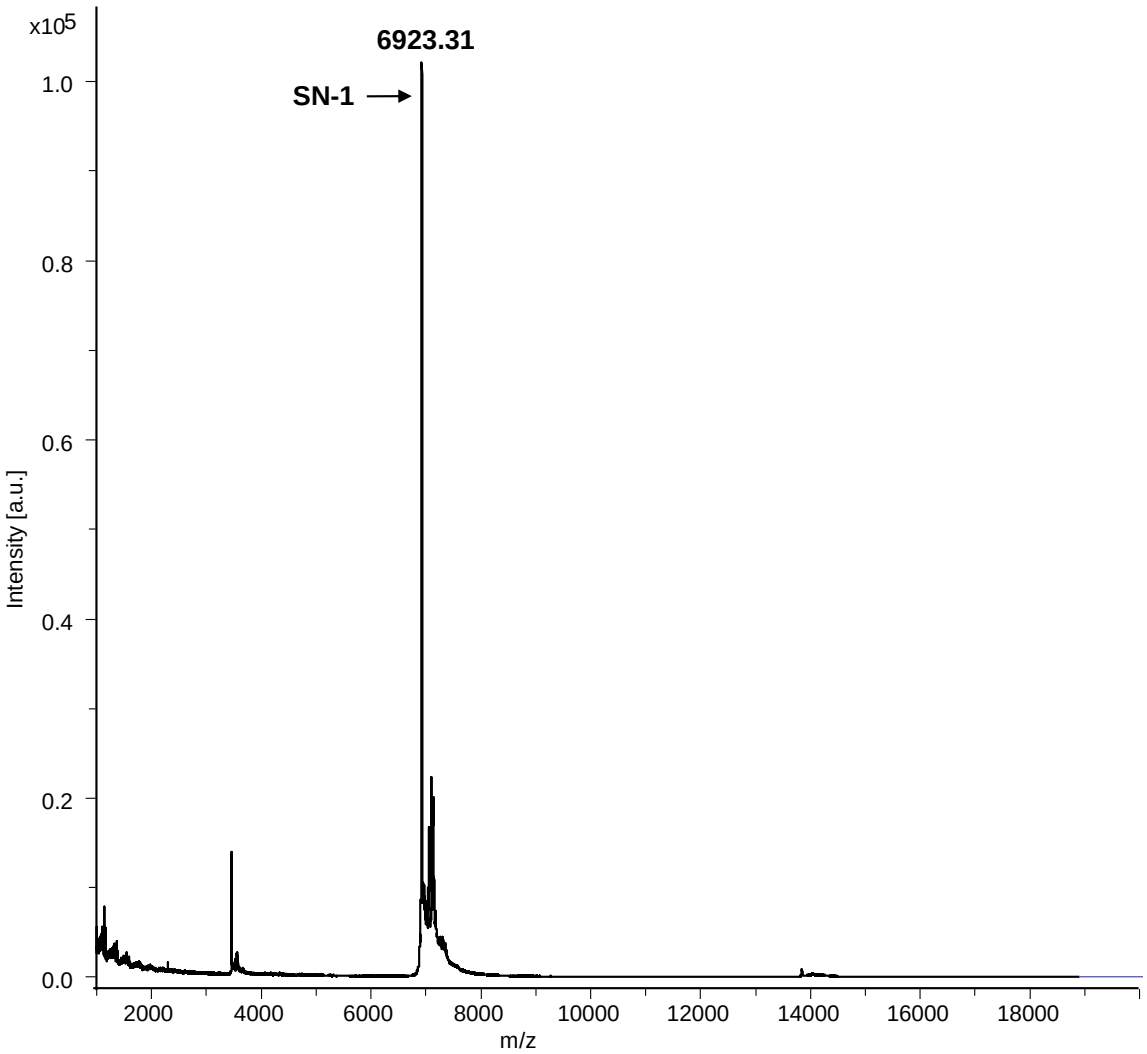
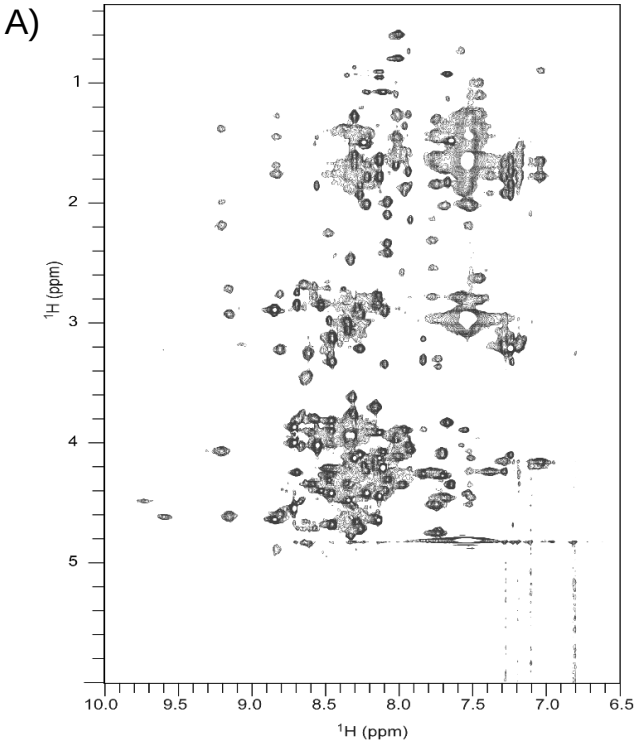


Figure 8. MALDI-TOF MS analysis of recombinant SN-1.



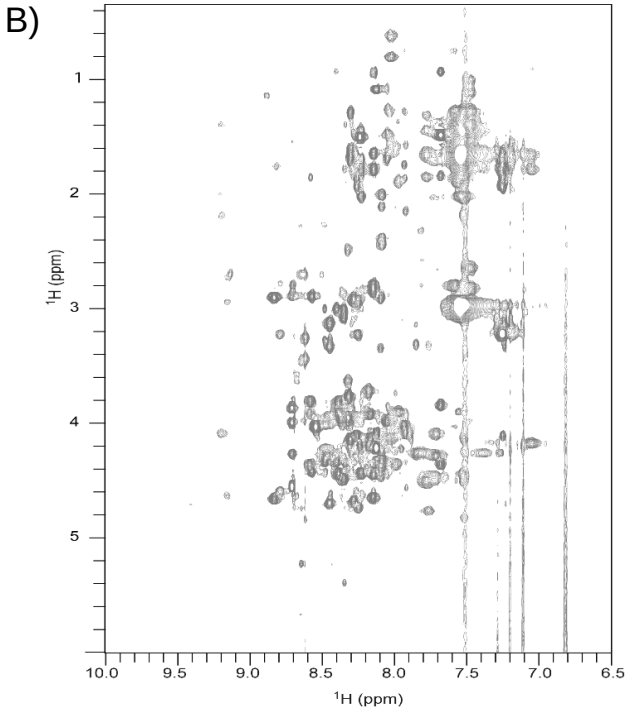


Figure 9. TOCSY NMR spectrum of recombinant SN-1 (A) and potato native SN-1 (B).

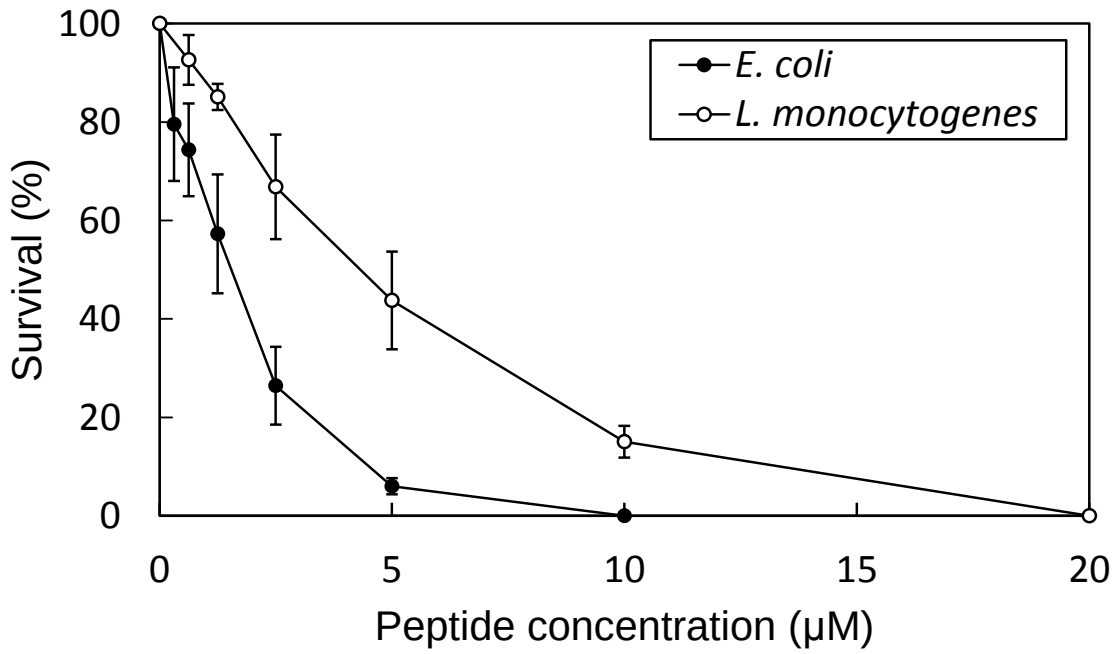


Figure 10. Antimicrobial activity of recombinant SN-1.

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Chapter 2

Expression, purification and characterization of the recombinant cysteine-rich antimicrobial peptide snakin-1 in *Pichia pastoris*

2-1 Abstract

Snakin-1 (SN-1) is a small cysteine-rich plant antimicrobial peptide with broad spectrum antimicrobial activity which was isolated from potato (*Solanum tuberosum*). Here, I carried out the expression of a recombinant SN-1 in the methylotrophic yeast *Pichia pastoris,* along with its purification and characterization. A DNA fragment encoding the mature SN-1 was cloned into pPIC9 vector and introduced into *P. pastoris*. A large amount of pure recombinant SN-1 (approximately 40 mg/1L culture) was obtained from a fed-batch fermentation culture after purification with a cation exchange column followed by RP-HPLC. I have confirmed the identity of the refolded recombinant SN-1 with six disulfide bonds by MALDI-TOF MS, CD and ^{1}H NMR experiments. I have also demonstrated that SN-1 exhibited strong antimicrobial activity against test microorganisms and produced very weak hemolysis of mammalian erythrocytes. The mechanism of its antimicrobial action against *Escherichia coli* was investigated by both outer membrane permeability assay and cytoplasmic membrane depolarization assay. From these results I have concluded that SN-1 is a membrane-active antimicrobial peptide which can disrupt both outer and cytoplasmic membrane integrity. This is the first report on the recombinant expression and purification of a fully active SN-1 in *P. pastoris*.

2-2 Introduction

Antimicrobial peptides (AMPs) are considered an essential part of the innate immune response of plants, invertebrates and vertebrates as they provide host defenses and can target a wide range of pathogenic microorganisms, including bacteria, fungi, yeast, parasites and viruses [1,2]. They are potent and bioactive, and provide a rich source of lead compounds for the discovery of promising novel antibiotics [3]. Due to their low toxicity to eukaryotic cells [4], AMPs can be widely used in the fields of pharmaceutics [3,5], cosmetology [6], veterinary medicine [7], and aquaculture [8], as well as in agricultural and food industries [9]. These peptides exhibit antimicrobial actions on target cells in various ways, such as by perturbation of the microbial cell membrane, or by inhibition of the synthesis of nucleic acids, proteins, enzymes, and cell-wall components that are essential for the survival of microorganisms [10]. Whether expressed constitutively in certain tissues or induced in response to pathogens [11,12], most AMPs share several common characteristics: they are small peptides (12-100 amino acids), have a net positive charge (+2 to +9), and are amphipathic and cysteine-rich with a disulfide bond-stabilized structure [5,13].

Many AMPs are cysteine-stabilized and these have been classified into several families [14], including cyclotides [15], defensins [16], hevein-like peptides [17], lipid transfer proteins [18], and snakins [11,19]. The snakin family is a novel plant antimicrobial

peptide family which shows good similarity with the members of the gibberellic acid stimulated transcript (GAST) and gibberellic acid stimulated in *Arabidopsis* (GASA) protein families in *Arabidopsis* [20]. These cysteine-rich peptides are widely distributed among plant species, including potato, tomato, avocado, petunia, French bean, gerbera, strawberry, maize, soybean, pepper, rice, Arabidopsis, and alfalfa [20–23]. All snakin peptides are characterized by twelve conserved cysteine residues that are involved in the formation of six disulfide bonds [19]. These disulfide bonds are thought to be important for maintaining their tertiary structure and also responsible for their biological activity [13,24,25]. In previous studies, snakin peptides from potato (*Solanum tuberosum*) were found to exert strong antimicrobial activity against phytopathogens [11,19,24,26] and animal pathogens [27]. However, little is known about their mechanism of action against target organisms.

Snakin-1 (SN-1) is a cysteine-rich, highly basic (pI = 8.97) and small peptide of 63-amino acid residues in length (MW-6922 Da). The amino acid sequence of SN-1 is GSNFCDSKCKLCSKAGLADRCLKYCGICCEECKCVPSGTYGNKHECPCYRDKK NSKGKSKCP [19,22]. Computational modeling indicates that the 3-D structure of the SN-1 peptide is composed of two long α-helices with six disulfide bonds and shows a small degree of structural similarity with thionins [25]. Until now, however, neither experimental information related to the 3-D structure nor the complete disulfide bridge

pattern of the snakin family has been published. The second snakin peptide, snakin-2 (SN-2), was also isolated from potato tubers [11]. 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 activity against microorganisms [11]. Both cause rapid aggregation of pathogens, although this response is not directly related with their antimicrobial activity [11,26]. Transgenic plants over-expressing the SN-1 and SN-2 genes have been shown to exhibit increased resistance to a wide range of invading phytopathogens [28,29].

In a previous study, an *E. coli* expression system was constructed and a recombinant SN-1 peptide was obtained in bacterial cells in the form of insoluble inclusion bodies which were unfolded and biologically inactive [26]. Similarly, chemical synthesis has been used to produce disulfide-rich SN-1 and SN-2 peptides [24] in an inactive, unfolded state that required laborious oxidative refolding and purification techniques to recover the functionally active peptides. Recently, the SN-2 peptide from tomato (*Solanum lycopersicum*) was successfully expressed in *E.coli* as a thioredoxin fusion protein in the active form, although the yield was approximately 1 mg/L [21]. To accomplish extensive structural and functional analysis, high-level expression of snakin peptide in the functionally active form is necessary.

Therefore, the aim of the current study was to construct an efficient expression system in order to obtain a large amount of biologically active SN-1 peptide. To meet this goal, I

selected methylotrophic yeast *P. pastoris* as an excellent alternative to the *E. coli* expression system for successful production of a disulfide-rich SN-1 peptide. This yeast was chosen in part because *P. pastoris* cells under the control of a methanol-induced AOX1 promoter can produce large amounts of functionally active cysteine-rich AMPs [30,31] that are secreted directly into the culture media [32]. I then constructed an efficient *P. pastoris* expression system for production of the recombinant SN-1. I investigated the antimicrobial activity of the recombinant SN-1 against several microorganisms, including Gram-positive and Gram-negative bacteria and yeast. As a first step in elucidating the mode of action of this peptide, I investigated its effect on both the outer and cytoplasmic membrane of *E. coli* cells. Our results demonstrated that SN-1 functions as a cationic antimicrobial peptide, exerting its antimicrobial effects via perturbation of the cell membrane. As far as I know, this is the first report of the recombinant expression of SN-1 in *P. pastoris.*

2-3 Materials and methods

Strains and plasmid

E. coli DH5α was used as the host strain for vector construction. The vector pPIC9 plasmid and *P. pastoris* GS115 strain (Invitrogen) were used in cloning and expression, respectively.

Construction of recombinant plasmid

A DNA fragment encoding SN-1 was amplified by PCR with a set of primers using synthetic oligonucleotide as a template. The DNA sequence of SN-1 is GGTTCAAATTTTTGTGATTCAAAGTGCAAGCTGAGATGTTCAAAGGCAGGACT TGCAGACAGATGCTTAAAGTACTGTGGAATTTGTTGTGAAGAATGCAAATGTG TGCCTTCTGGAACTTATGGTAACAAACATGAATGTCCTTGTTATAGGGACAAG AAGAACTCTAAGGGCAAGTCTAAATGCCCTTGA. The primers were 5′-GTTAACTCGAGAAAAGAGGTTCAAATTTTTGTGATTCAAAGTGC-3′ (forward primer), and 5′-CGGAATTCTCAAGGGCATTTAGACTTGCC-3′ (reverse primer), with the *Xho*I site and *Eco*RI site underlined, respectively. The purified PCR product was digested with *Xho*I and *Eco*RI, and ligated to the pPIC9 plasmid vector by using *Xho*I–*Eco*RI restriction sites. The ligated vector (pPIC9-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 (3100-Avant Genetic Analyzer; Applied Biosystems/Hitachi).

Transformation of P. pastoris and expression of the recombinant SN-1

The pPIC9-SN-1 construct was linearized with *Sal*I and introduced into the competent *P. pastoris* GS115 strain by electroporation according to the manual of the *P. pastoris* expression kit v.3.0 (Invitrogen). A pPIC9 vector with no SN-1 gene was also linearized and introduced into *P. pastoris* cells as a negative control. Recombinant His^+ yeast clones were obtained from MD plates (1.34% YNB, 4×10^{-5}% biotin, 2% dextrose, 1.5% agar).

Shake-flask cultivation of P. pastoris

A single positive transformant colony was grown at 30°C for about 25 h in a 1.0 L shaking flask containing 100 ml of BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin and 1% glycerol) until OD_{600} = 2. The cells were harvested by centrifugation at 3000*g* for 5 min and resuspended in 300 ml BMMY (1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin and 2% methanol) to induce expression of SN-1. Methanol was added every 24 h to a final concentration of 2.0% (v/v) during the 120 h induction period. The expression of SN-1 was determined by SDS-PAGE analysis.

High-density fermentation of recombinant P. pastoris

High-density fermentation of *P. pastoris* was performed with a 5.0 L jar fermenter (MBF-500; Tokyo Rikakikai), according to standard procedures [33], with slight modifications. A single colony from a positive transformant was incubated in 5 ml of YPD medium overnight at 30°C. About 400 μl of an overnight cell culture of the *P. pastoris* expression strain was inoculated into 200 ml BMG medium (1% YNB, 4×10^{-5}% biotin, and 1% glycerol, 100 mM potassium phosphate buffer, pH 6.0) and was cultured for 24 h at 30°C, and then the resulting culture was inoculated into 2.4 L of an initial medium containing 3.8% glycerol, 840 ml of 10× basal salts, 10 ml PTM1, and 10 ml of 0.02% biotin in the jar fermenter. During all of the fermentation steps in the jar fermenter, the temperature and agitation rate were maintained at 30°C and 800 rpm, respectively. The pH was maintained at 5.0 by adding 10% (v/v) ammonia water prior to inoculation. The temperature was adjusted by adding cooled recycled water. An antifoaming agent (10% propylene glycol) was delivered to control foaming. When the glycerol batch phase was completed, as indicated by the sudden increase in the level of dissolved oxygen, then the glycerol fed-batch phase was initiated by delivery of 300 ml of a second medium (50% glycerol, 3.6 ml PTM1, 3.6 ml of 0.02% biotin in 300 ml medium) at a rate of 30 ml/h. Within 7 h of the second medium feeding, a third medium (900 ml methanol, 10.8 ml PTM1, 10.8 ml of 0.02% biotin) was started at a 3 ml/h feed rate. The feed was

gradually increased until the maximum feed rate (15 ml/h), which was maintained for 36 h. During the methanol induction phase, 1 ml samples were taken, and the cell pellets and supernatants were collected by centrifugation at 20000*g* for 1 min for the *P. pastoris* cell density and protein assays. At the end of the fermentation process, the cell mass was harvested by centrifugation (14000*g*, 4°C, 30 min) and the peptide containing the supernatant was separated from the yeast pellet. The expression level of recombinant SN-1 was determined by SDS-PAGE.

Purification of recombinant SN-1

Both the pH and conductivity of the supernatant containing expressed peptide were adjusted as 6.0 and 5.3 mS/cm, respectively. The filtered product was loaded onto a 5 ml pre-packed HiTrap SP HP cation exchange column (GE Healthcare) pre-equilibrated with buffer (50 mM potassium phosphate, pH 6.0). The column was then washed with the same buffer and the peptide was eluted with a linear gradient of 15-65% elution buffer (50 mM potassium phosphate and 1M NaCl, pH 6.0). The eluted recombinant SN-1 was analyzed by SDS-PAGE. Fractions containing SN-1 were mixed, filtered and finally purified by RP-HPLC on a Cosmosil 5C18-AR300 column (Nacalai Tesque). The elution was carried out with a linear gradient of 15-25% acetonitrile with 0.1% trifluoracetic acid. The final yield of SN-1 was estimated by measuring the absorbance at 280 nm. The

purified recombinant SN-1 was lyophilized and stored at -30°C.

Purification of native potato SN-1

The native SN-1 peptide from potato tuber was extracted and purified according to the procedures published previously [19] with some modifications. Frozen tuber material (500 g) was ground to a fine powder in liquid nitrogen with a sample mill, and washed once with 1.0 L wash buffer (0.1 M Tris-HCl, 10 mM EDTA, pH 7.0) and twice with 2 L of distilled water. After centrifugation, the resulting pellet was extracted with 500 ml of extraction buffer (0.1 M Tris-HCl, 1.5 M LiCl and 10 mM EDTA, pH 7.0) at 4°C for 1 h. The slurry was then centrifuged (12000*g*, 15 min) and the supernatant was dialyzed against 15 L of H_2O with a dialysis membrane (MWCO-3500) overnight. The crude peptide solution was filtered and loaded onto a 5 ml pre-packed HiTrap SP HP cation exchange column pre-equilibrated with buffer (50 mM acetate buffer, pH 5.0) and washed with 30% elution buffer (50 mM acetate buffer and 1M NaCl, pH 5.0). The peptide was eluted with a linear gradient of 30-60% elution buffer. Then the sample was filtrated, loaded onto a Cosmosil 5C18-AR300 column, and purified by RP-HPLC.

Characterization of recombinant SN-1 by mass spectrometry

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.

CD spectroscopy analysis

To confirm the correct folding of recombinant SN-1, the peptide sample was added to 1 mm pathlength quartz cuvettes and the CD spectra were taken using a J-725 spectropolarimeter (Jasco). Freshly prepared SN-1 samples (50 μM) were dissolved in PBS (pH 7.4). Spectral scans were recorded over a wavelength range from 250 to 200 nm, with a step resolution of 0.1 nm and scanning speed of 50 nm/min at room temperature (25°C), a response time of 1.0 sec, a bandwidth of 1.0 nm, and an average of 12 scans under a nitrogen atmosphere. All measurements were averaged and converted to the mean residue ellipticity.

NMR spectroscopy

Freeze-dried recombinant SN-1 powder was dissolved in a mixture of 90% H_2O/10% D_2O at pH 3.0. NMR experiments were performed on a Bruker Avance III HD 600 MHz instrument. All spectra were processed using NMRpipe and NMR draw [34].

Growth inhibition test of recombinant SN-1 against filamentous fungi

Fungal spores of the filamentous fungus *Fusarium oxysporum* f. sp. *lycopersici* (JCM 12575) were collected from 8-day-old cultures grown at 25°C on potato dextrose agar plates. Spore suspensions (10^4 spores/ml) in 25 μl of potato dextrose broth were placed into the wells of 96-well microtiter plates containing two-fold serial dilutions of the recombinant SN-1 dissolved in 50 μl of sterile water. The plates were incubated at 25°C for 40 h and the growth was recorded by measuring in a microplate reader at 600 nm. Controls were treated in the same manner except that the peptide was omitted. For calculation of the inhibition rates, the absorbance of the control was considered to represent 100% growth.

Microbicidal assay of purified recombinant SN-1

The minimum microbicidal concentrations (MMCs) of purified SN-1 peptide were determined against several microorganisms by a colony forming unit (CFU) assay. Antibacterial assays were performed with three strains of bacteria, i.e., Gram-positive *Listeria monocytogenes* (ATCC 19111), Gram-negative *Salmonella enterica* Serovar Typhimurium (ATCC 13311), and *Escherichia coli* ML35 (ATCC 43827), as well as two fungi strains, i.e., *Pichia pastoris* GS115 (ATCC 20864) and *Candida parapsilosis* (ATCC 22019). Bacteria growing exponentially (OD_{600} = 0.4-0.6) in tryptic soy broth

(TSB) were collected by centrifugation, washed, resuspended in sterile water, and diluted in sterile water. Briefly, an assay mixture consisting of 100 μl peptide of various concentrations and an equal volume of diluted cell suspension (10^5-10^7 cfu/ml) were incubated at 37°C for 1 h. After incubation, the reaction mixtures were diluted 100 times in sterile water and 200 μl of the diluted sample was plated on tryptic soy agar plates. The plates were incubated at 37°C for 35 h (*L. monocytogenes*) or 15-20 h (*S. enterica* and *E. coli)*. Then the colonies were counted and the minimum bactericidal concentrations (MBCs) were determined for each test strain. Fungal strains, *i.e., P. pastoris* and *C. parapsilosis*, were grown in YPD medium (1% yeast extract, 2% peptone, 2% dextrose). Various concentrations of peptide were incubated with an equal volume of fungi suspension at 30°C for 1 h. The minimum fungicidal concentrations (MFCs) were determined after 48 h of incubation at 30°C, by counting the number of colonies developed on a Sabouraud agar plate. In this experiment, two-fold serial dilutions of purified recombinant SN-1 were prepared in potassium phosphate buffer (10 mM, pH 6.0) with a gradient concentration of 0, 0.312, 0.625, 1.25, 2.50, 5.00, 10.0, 20.0 μM. The results were expressed as the mean ± SD of three individual experiments.

Outer membrane permeability assay of recombinant SN-1

The ability of the recombinant SN-1 peptide to permeabilize the outer membrane was

determined by measuring the uptake of the fluorophore 1-N-phenylnaphthylamine (NPN) [35]. An overnight culture of *E. coli* was diluted in TSB and grown to OD_{660} = 0.6-0.8. The bacterial cells were harvested, washed twice in buffer (10 mM HEPES, 20 mM glucose, pH 7.4) and resuspended in the same buffer to an OD_{660} of 0.8. NPN was added to a final concentration of 5 μM in a 1 cm quartz cuvette containing 2 ml of cell suspension and the fluorescence was measured using a fluorescence spectrophotometer (Hitachi, F2000) with an excitation wavelength of 355 nm and emission wavelength of 407 nm. The increase in fluorescence due to partitioning of NPN into the outer membrane of *E. coli* was measured with the MBC (10 μM) and half-MBC (5 μM) of recombinant SN-1 peptide. Melittin (10 μM) was used as a positive control and the results were expressed as the mean ± SD of three individual experiments.

Cytoplasmic membrane depolarization assay of recombinant SN-1

The ability of the recombinant SN-1 to depolarize the bacterial cytoplasmic membrane was determined using a membrane potential-sensitive fluorescent dye $diSC_3(5)$ and standard melittin (10 μM), as described previously [36]. Briefly, *E. coli* cells in mid-log phase were washed twice in buffer (10 mM HEPES, 20 mM glucose, 0.2 mM EDTA, pH 7.4) and then resuspended in the same buffer. The bacterial suspension was incubated with $diSC_3(5)$ for 20 min at 37°C to allow for dye uptake. The change in fluorescence

intensity was recorded after addition of the MBC and half-MBC of SN-1 peptide using a fluorescence spectrophotometer with excitation and emission wavelengths of 622 and 670 nm, respectively. The results were expressed as the mean ± SD of three individual experiments.

Hemolytic assay of recombinant SN-1

The hemolytic activity of recombinant SN-1 was determined by the released hemoglobin from suspensions of fresh sheep erythrocyte as reported previously [37]. Fresh sheep red blood cells (sRBCs) were washed three times with PBS (pH 7.4) by centrifugation (2000*g*, 5 min) and resuspended in the same buffer. Aliquots (50 μl) of peptide solution in PBS at various concentrations were added to 50 μl of a 4% (v/v) sRBC suspension and incubated for 1 h at 37°C. The samples were centrifuged and supernatant (50 μl) was added to 150 μl of PBS in a 96-well plate. Absorbance was measured at 405 nm using a microplate reader and 0% and 100% hemolysis was determined in PBS and 0.1% (w/v) Triton X-100, respectively. Melittin at different concentrations was used as a positive control. The results were expressed as the mean ± SD of three individual experiments.

2-4 Results

Construction of recombinant plasmid pPIC9-SN-1 and transformation of *P. pastoris*

The SN-1 peptide sequence was amplified by PCR and verified by agarose gel electrophoresis. The PCR product encoding 63 amino acids was digested with *Xho*I and *Eco*RI, and subsequently cloned into pPIC9 expression vector with the secretion signal of the α-mating factor peptide. The resulting recombinant pPIC9-SN-1 plasmid was verified by DNA sequencing. After linearization with *Sal*I, pPIC9-SN-1 plasmid was introduced into the genome of *P. pastoris* GS115 competent cells by electroporation.

Expression and purification of recombinant SN-1

I have picked up some colonies of recombinant *P. pastoris* for small scale expression trial and the amount of recombinant SN-1 peptide was determined by SDS-PAGE. The level of expression was the same for all transformants. Among these, a single transformant was selected for fermentation at the shaker flask level, and the expression level of SN-1 was determined by SDS-PAGE. As shown in Fig. 1A, a prominent band at about 7 kDa was observed starting at about 24 h of methanol induction, with its yield increasing over the course of the induction.

After confirming the SN-1 expression at the shake flask level, in order to obtain a high yield I conducted high density fermentation in a 5-L jar fermenter starting with 2.4 L of

basal salt medium supplemented with trace salts and glycerol. The total induction time with methanol was 48 h. The wet cell weight reached 317 g/L after 48 h of induction. By SDS-PAGE analysis of *Pichia* fermentation culture, I found a single peptide band at about 7 kDa (Fig. 1B). I also observed that the peptide expression started 6 h after methanol induction and increased to the maximum level at 48 h after induction. These results indicated that the secreted expression of SN-1 in *P. pastoris* was successful.

At the end of fermentation, the *P. pastoris* culture medium was harvested by centrifugation. As a first step in the purification, the filtrate culture containing the recombinant SN-1 peptide was applied to a cation exchange column and the bound peptides were eluted as described in the Materials and Methods section. The fractions containing the expressed SN-1 peptide were collected and further purified by using a reverse-phase HPLC column, and the retention time of SN-1 was found to be from 24.0 to 26.0 min (Fig. 2). A large amount (approximately 40 mg/1L *P. pastoris* culture) of pure recombinant SN-1 was obtained by using this two-step purification process. The purified SN-1 was freeze dried and stored at -30°C for further analysis. For purification of the native SN-1, about 0.2 mg of pure native SN-1 was obtained from 1 kg of potato tubers.

Characterization of recombinant SN-1

The purified recombinant SN-1 was subjected to MALDI-TOF MS to determine its

molecular weight. The MALDI-TOF spectrum of the recombinant SN-1 (Fig. 3) exhibited the expected peak, corresponding to $[M+H]^+$, at *m/z* 6922.98, which was identical to the theoretical average mass, 6923.00, of the SN-1 peptide with six disulfide bonds. The CD spectrum of recombinant SN-1 showed a broad negative band with dual peaks at around 208 and 220 nm (Fig. 4), which is identical to that of the potato native SN-1. The ^{1}H NMR spectrum of recombinant SN-1 was also identical with the native one (Fig. 5A, 5B). Because it is well known that NMR chemical shifts of peptides are quite sensitive to tertiary structure, both the disulfide bridge pattern and tertiary structures of recombinant SN-1 were most likely to be identical to those of the native one.

Growth inhibitory activity of recombinant SN-1 against filamentous fungi

To determine whether the recombinant SN-1 was biologically functional, I performed an inhibition assay against a phytopathogen filamentous fungi, *F. oxysporum*. As shown in Fig. 6, *F. oxysporum* was highly susceptible to recombinant SN-1 and spore germination was completely inhibited with 60 μM of SN-1. This result was in accordance with the findings described in previous reports for the potato native SN-1 and chemically synthetic SN-1 [19,24].

Microbicidal assay of recombinant SN-1

The microbicidal activity of the recombinant SN-1 was determined against a series of microorganisms by colony forming unit assay. Much like the potato native and *E. coli*-derived recombinant SN-1 [19,26], *P. pastoris*-derived recombinant SN-1 exhibited significant antimicrobial activity against the Gram-positive bacteria, Gram-negative bacteria, and fungi tested, with MMCs between 5 and 20 μM (Fig. 7A, 7B). Our recombinant SN-1 peptide showed strong activity against human pathogen *L. monocytogenes*, which was previously reported for potato native SN-1 [27]. Furthermore, SN-1 showed more potent microbicidal activity against Gram-negative bacteria, i.e., *S. enterica* and *E. coli*, with MBCs between 5-10 μM. Similarly, it also exhibited microbicidal activity against *C. parapsilosis* and *P. pastoris*, with MFC values of 5 and 10 μM, respectively, indicating that the yeast strains were sensitive to SN-1.

Permeabilization of outer membranes

The outer membrane permeability of recombinant SN-1 was determined by performing an NPN uptake assay using intact *E. coli* cells. NPN is a useful fluorescent probe that fluoresces weakly in an aqueous environment but strongly in the hydrophobic environment of an injured cell membrane [35]. The results showed that SN-1 rapidly permeabilized the outer membrane of *E. coli* in a concentration-dependent manner, based on the increase in NPN fluorescence (Fig. 8). These results suggested that SN-1

possesses outer membrane permeability.

Depolarization of the cytoplasmic membrane

The membrane depolarization activity of recombinant SN-1 was determined using a membrane potential-dependent fluorescent dye, $diSC_3(5)$. Depolarization of the cytoplasmic membrane by the SN-1 peptides was monitored over a period of 300 sec. As shown in Fig. 9, the depolarization of the cytoplasmic membrane of *E. coli* cells by SN-1 was also concentration-dependent. Compared with standard melittin, the test peptide SN-1 could efficiently depolarize the cytoplasmic membrane at the same molar concentration. These results indicated that SN-1 is a membrane-active antimicrobial peptide that can depolarize the cytoplasmic membrane potential of *E. coli* cells.

Hemolytic assays of recombinant SN-1

Some cationic antimicrobial peptides have been reported to show toxicity against mammalian cells [38,39]. In order to examine the cytotoxicity of SN-1 to the mammalian cell membrane, I measured the percentage of hemolysis of sheep erythrocytes at various concentrations of peptide (from 0 to 128 μM). SN-1 had little hemolytic effect at any concentration, whereas melittin showed 100% hemolysis at 10 μM (Fig. 10). These results indicate that SN-1 has more activity against microbial cells compared to

mammalian erythrocytes.

2-5 Discussion

SN-1 is a cysteine-rich small cationic peptide that contains six intramolecular disulfide bridges. It is well known that the expression of cysteine-rich AMPs such as snakin peptides in *E. coli* cells is a significant challenge because the formation of disulfide bridges in the expressed protein is inefficient, leading to incorrect folding and destabilization of the tertiary structure [40,41]. These limitations of the *E. coli* expression system prompted us to select the methylotrophic *P. pastoris* strain in order to develop a method for the large-scale preparation of a disulfide-rich SN-1 containing about 20% cysteine in its amino acid sequence. Like chaperone, the protein disulfide isomerase (PDI) enzyme in the *P. pastoris* endoplasmic reticulum plays a pivotal role in the folding process of newly synthesized proteins by the formation of disulfide bonds [42]. Due to their efficient folding, the *P. pastoris*-derived recombinant AMPs can, in some cases, exhibit stronger activity than the same peptides produced in *E. coli* [43,44]. In our experiments, the expression of the SN-1 gene in *P. pastoris* by the pPIC9 plasmid vector resulted in the large-scale production (about 40 mg/L of culture) of a recombinant SN-1 peptide with six disulfide bridges. After purification, the *P. pastoris*-derived recombinant SN-1 was confirmed to be identical to the native one by MALDI-TOF MS, CD and NMR studies (Fig. 3, 4, 5). In particular, the identity of the NMR spectra of native and recombinant SN-1 clearly suggested that the correct native disulfide bonds and the

tertiary structure were formed in recombinant SN-1. Furthermore, the good dispersion of the chemical shifts of amide protons indicated that both SN-1s formed a folded structure. In distinction to the previous report [26], I have successfully constructed an expression system to produce a large amount of bioactive SN-1 peptide without the need of any renaturation process.

Numerous reports have documented the strong antimicrobial activities of snakin peptides against a wide range of microorganisms [11,19,21,24,26,27]. Enhanced resistance to pathogen infections has also been reported in plants that express snakin genes [28,29]. Snakin peptides are thus considered important candidates for producing biotic stress tolerance in crops. Until now, however, there has been no detailed information about their mode of action. In order to determine how cysteine-rich cationic SN-1 exerts its microbicidal action on target cells, I selected *E. coli* ML35 as a model organism. The outer membrane of Gram-negative bacteria acts as a permeability barrier which is composed of negatively charged phospholipids [45]. Generally, cationic AMPs having positive charges are electrostatically attracted to the negatively charged outer microbial membrane and subsequently cause a disruption of the inner membrane through pore formation, resulting in release of the cellular contents and cell death [10]. Thus, the outer and cytoplasmic membrane permeability assays were performed to detect the target site of SN-1. NPN is a fluorescent probe that can not cross intact cell membranes, but

enters through disrupted outer membranes and exhibits increased fluorescence [35]. Thus, the increase in the NPN fluorescence of SN-1 (Fig. 8) indicated that the peptide had the ability to penetrate the bacterial outer membrane in a dose-dependent manner. This suggests that micromolar ranges of SN-1 peptides have the capacity to destroy the outer membrane. In addition, the membrane activity of SN-1 was also determined by an inner membrane permeability assay using $diSC_3(5)$ dye as the indicator of membrane potential. When this dye was added to the *E. coli* cell suspensions for incubation, a large portion of $diSC_3(5)$ was taken up and concentrated in the cytoplasmic membrane of *E. coli* cells, where it bound to the lipid rich intracellular components [36]. Thus, $diSC_3(5)$ quenched its own fluorescence. If the cytoplasmic membrane is damaged by peptide or other agents, $diSC_3(5)$ dye will be released into the surrounding medium, causing a rapid increase in fluorescence [36]. In our study, SN-1 peptide induced different levels of depolarization of the cytoplasmic membrane in a dose-dependent manner (Fig. 9). In these two kinds of fluorescence probe experiments, the SN-1 peptides rapidly permeabilized the outer and inner membrane of *E. coli* at concentrations lower than the MBC. These results suggest that SN-1 exhibited bactericidal activity against microbes by disrupting their cell membrane.

In conclusion, I have demonstrated for the first time that the *P. pastoris* expression

system can be used for the large-scale production of a correctly folded and biologically active SN-1 peptide with its six disulfide bridges. The results of our biological experiments suggested that SN-1 is a membrane-active AMP that exhibits strong bactericidal activity against a wide range of microbes. This study provides information about producing a functionally active SN-1 in *P. pastoris* in high yield, which is important for further structural and functional analysis.

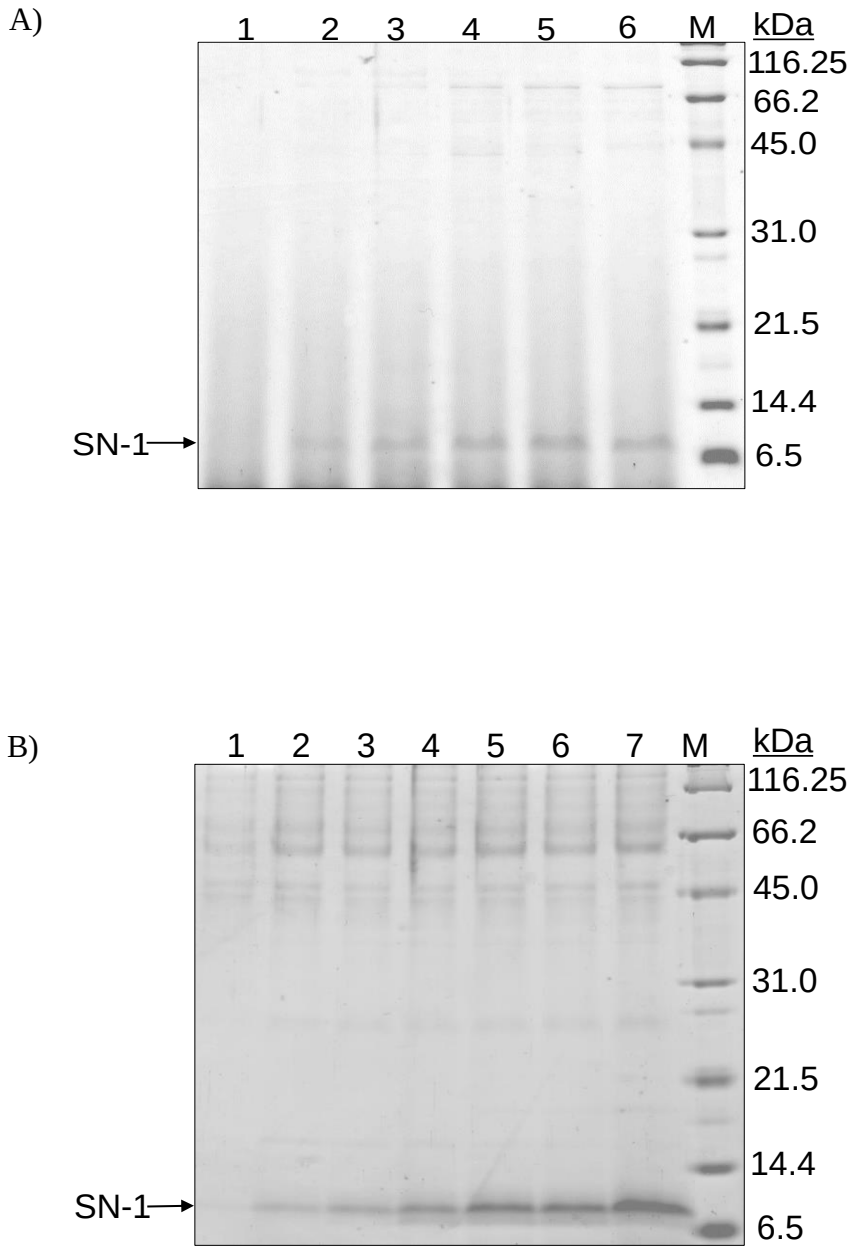

Figure 1. (A) SDS-PAGE analysis of recombinant SN-1 in fermentation supernatants from *P. pastoris* in shaker flask cultures. Lanes 1-6: a total of 10 μl of supernatant samples taken at 0, 24, 48, 72, 96, 120 h of induction, respectively. *Lane M*, protein molecular weight marker. (B) Production of recombinant SN-1 in yeast using high density cultivation. SDS-PAGE analysis of SN-1 secreted into the fermentation broth of *P. pastoris*. Lanes 1-7: a total of 10 μl of supernatant samples taken at 0, 6, 12, 24, 36, 41,

48 h of induction, respectively.

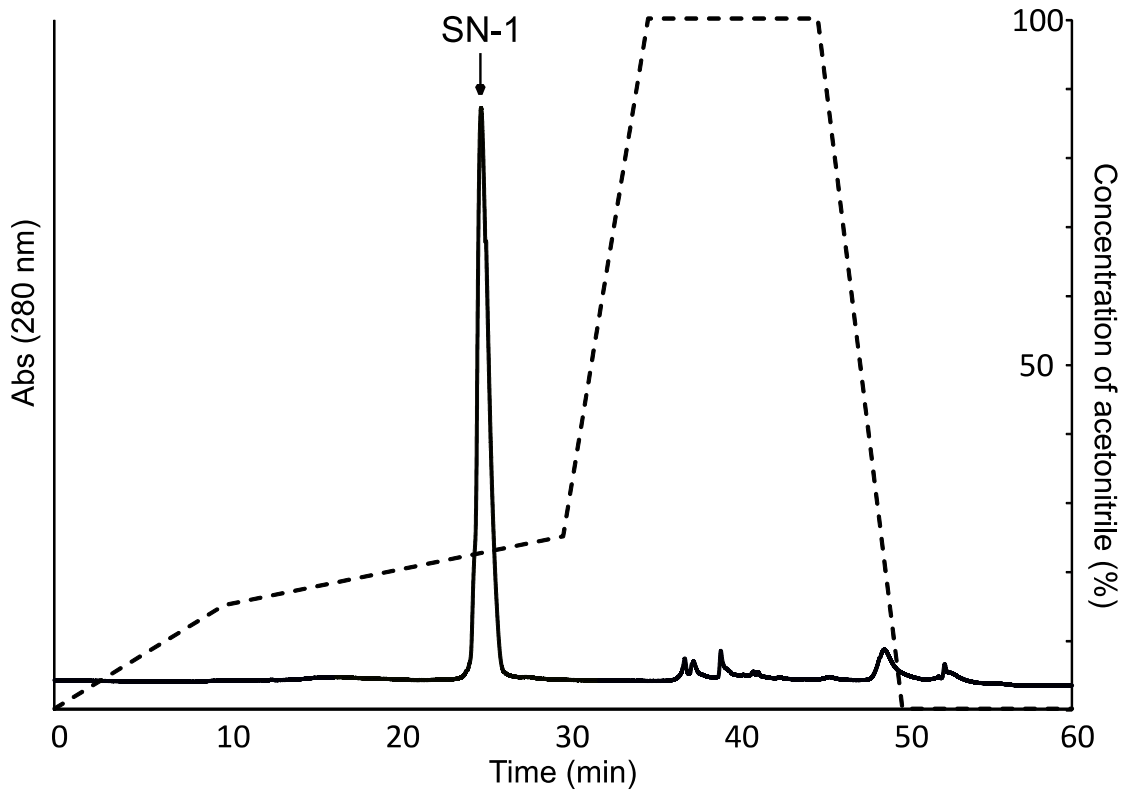

Figure 2. Purification of recombinant SN-1 by an RP-HPLC C18 column that was eluted with a linear gradient of 15-25% acetonitrile with 0.1% TFA.

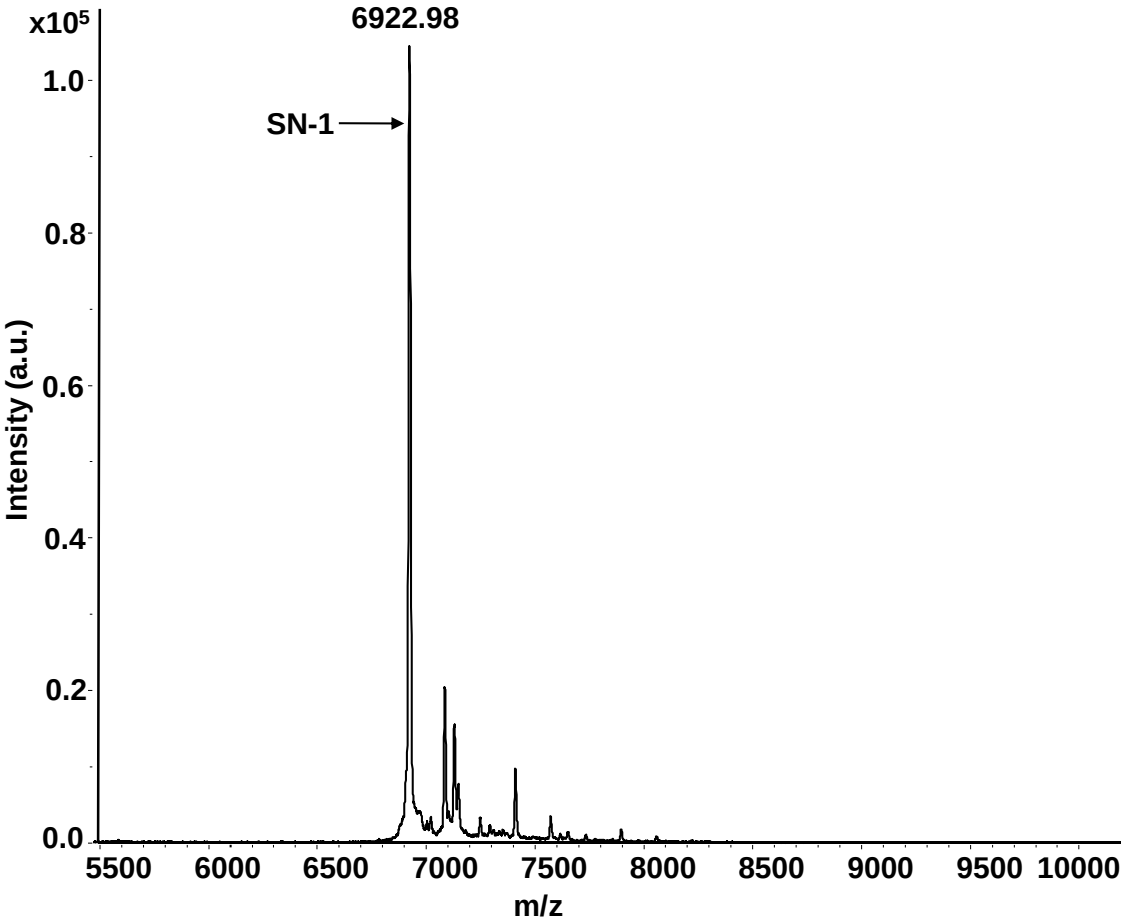

Figure 3. MALDI-TOF mass analysis of the purified recombinant SN-1

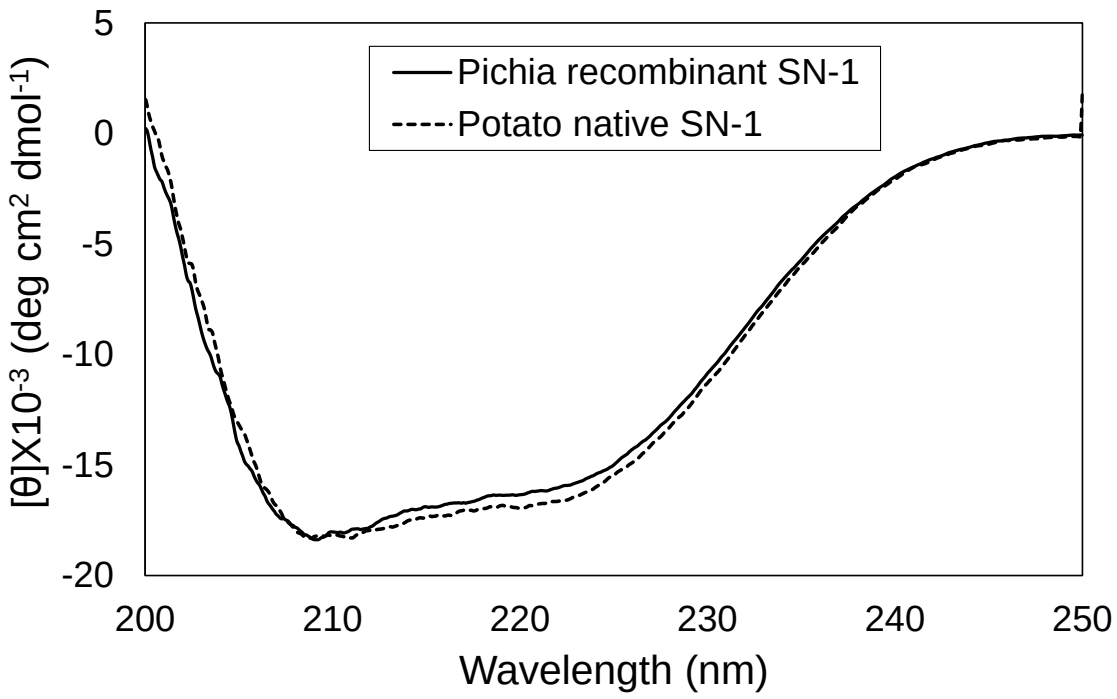

Figure 4. CD spectra of recombinant SN-1 (solid line) and potato native SN-1 (dotted line). The peptide samples were dissolved in PBS (pH 7.4) at 25°C. The mean residue ellipticity was plotted against the wavelength.

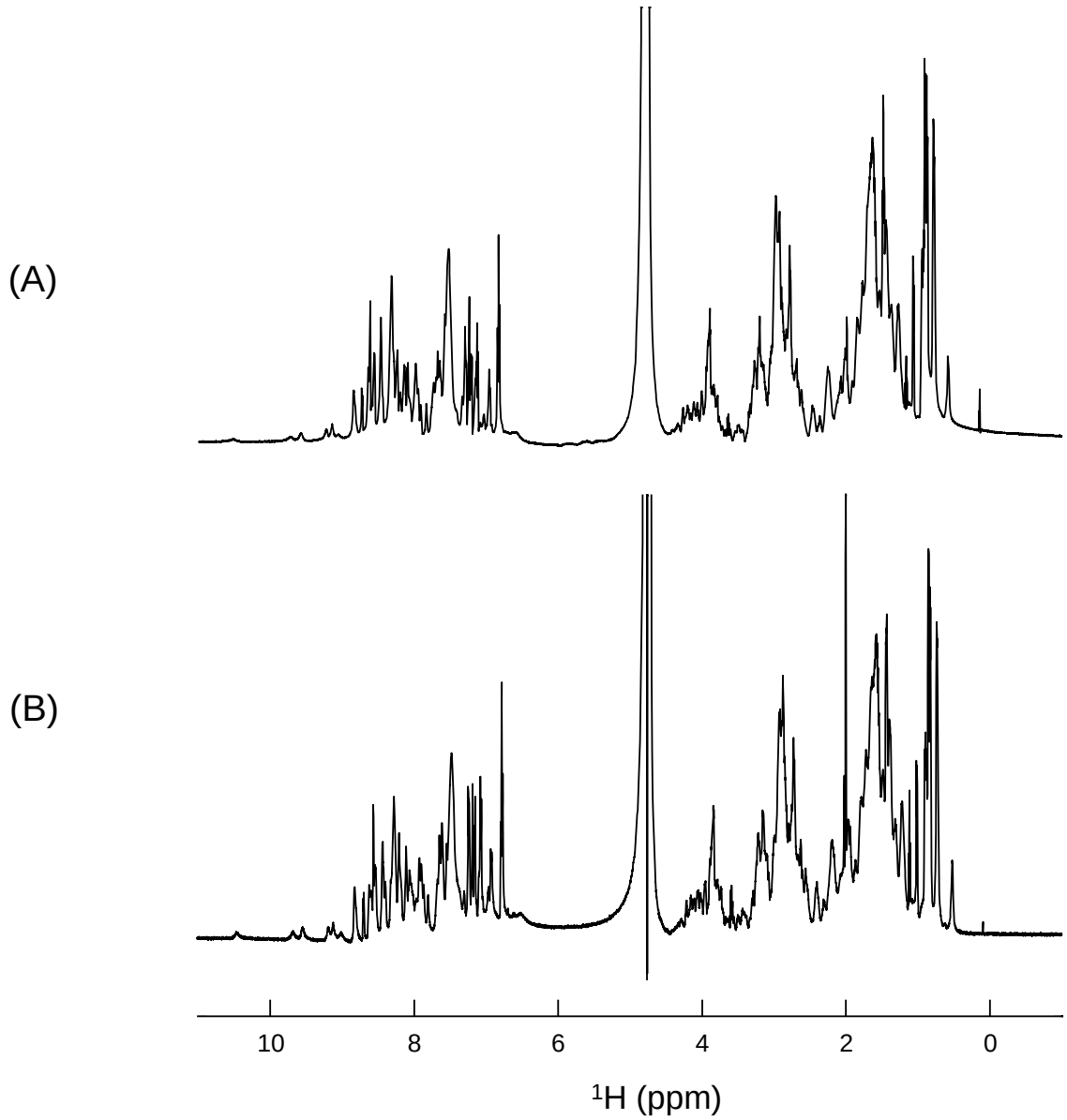

Figure 5. NMR analysis of the recombinant SN-1. The ^{1}H NMR spectrum of recombinant SN-1 (A) and potato native SN-1 (B) at 25°C, pH 3.0.

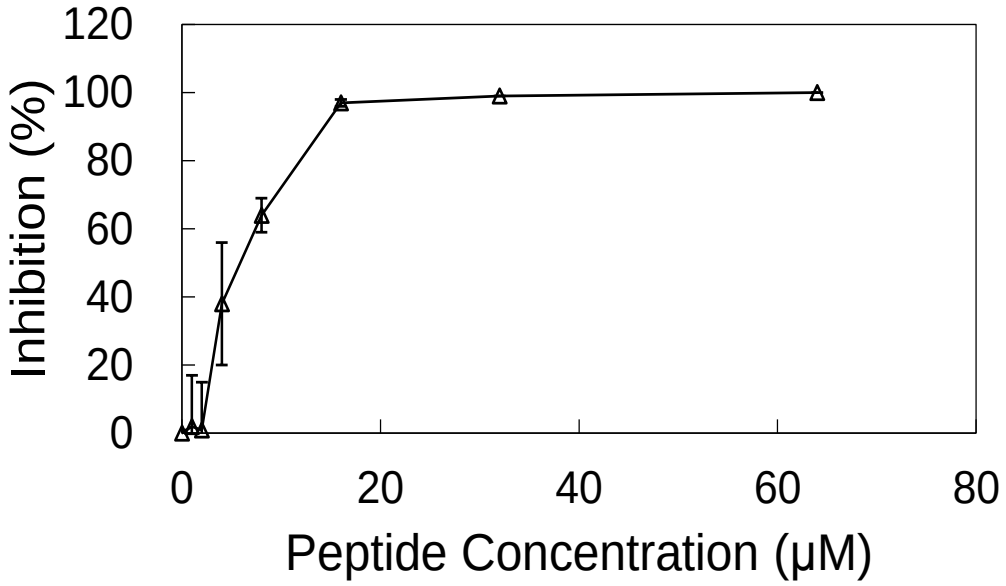

Figure 6. Growth inhibitory effect of the recombinant SN-1 against a filamentous fungi, *F. oxysporum*. The results were expressed as the mean ± SD of three individual experiments.

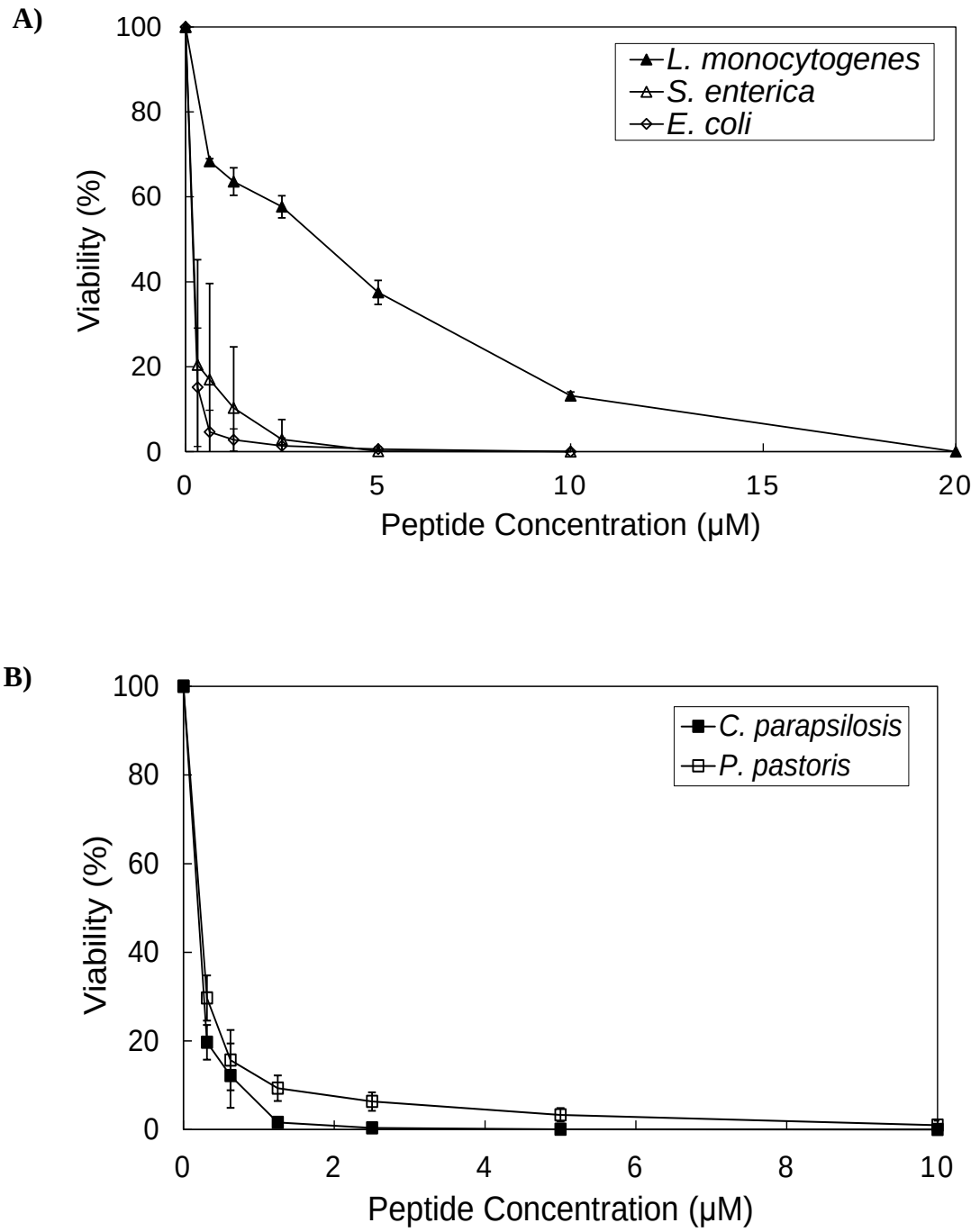

Figure 7. Microbicidal activity of the recombinant SN-1 against bacteria *L. monocytogenes*, *S. enterica*, *E. coli*, (A) and the yeast strains *C. parapsilosis* and *P. pastoris* (B). The results were expressed as the mean ± SD of three individual experiments.

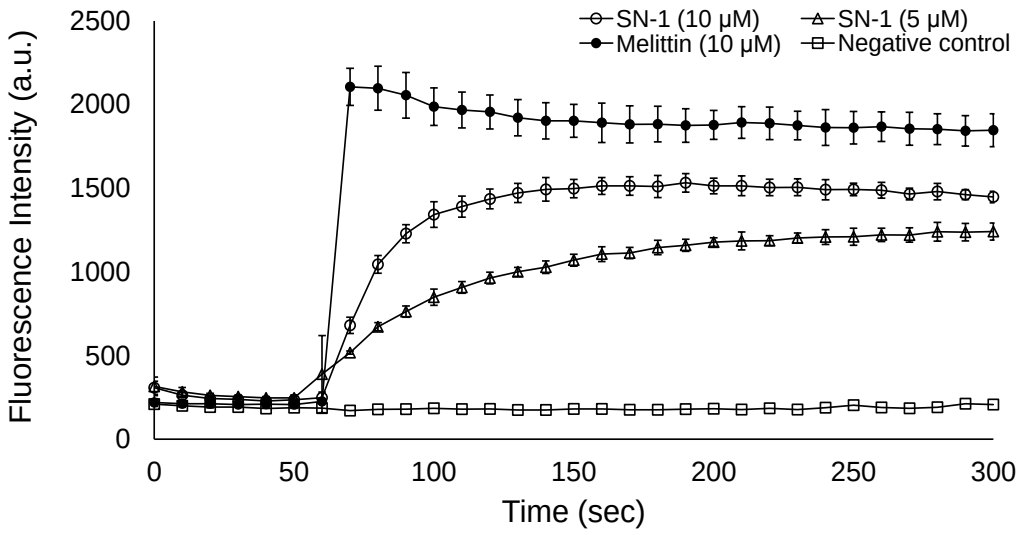

Figure 8. Outer membrane permeability was determined by an NPN uptake assay in the presence of the MBC and half-MBC of recombinant SN-1 peptide. The results were expressed as the mean ± SD of three individual experiments.

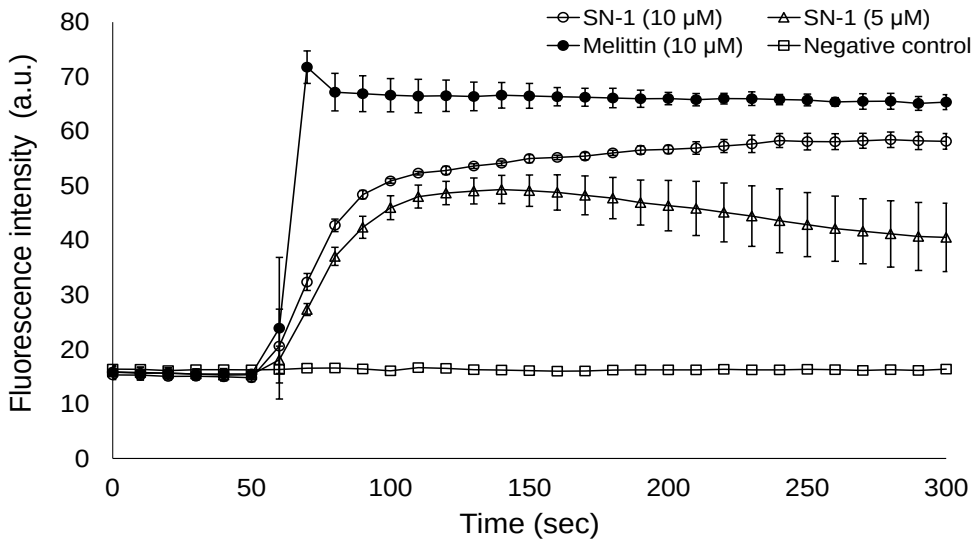

Figure 9. Cytoplasmic membrane depolarization of *E. coli* was detected using $diSC_3(5)$ dye. The MBC and half-MBC of the recombinant SN-1 peptide were added at t = 60 sec to monitor the changes in fluorescence. The results were expressed as the mean ± SD of three individual experiments.

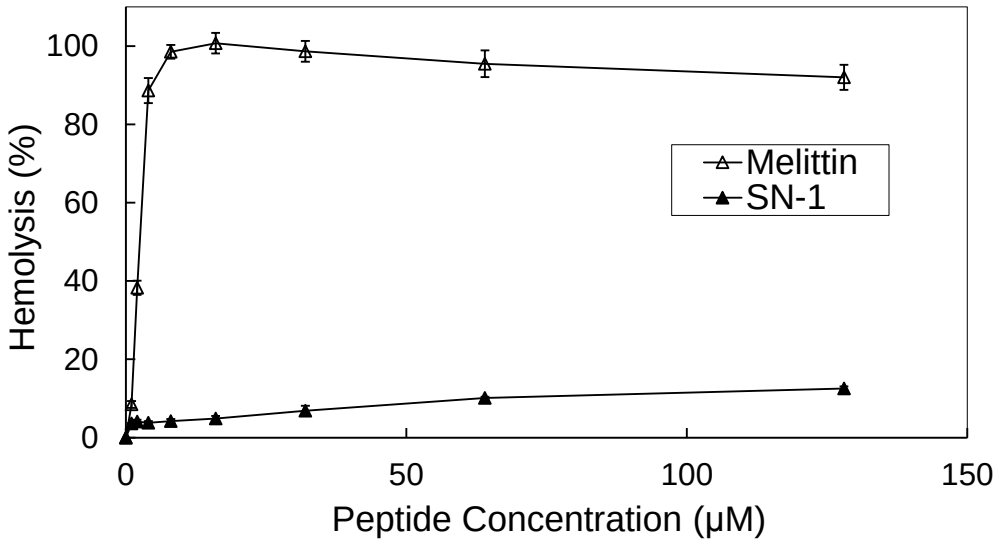

Figure 10. Hemolytic activity of recombinant SN-1. Hemoglobin release was monitored by measuring the absorbance of the supernatant at 405 nm. Hemolysis induced by 0.1% (w/v) Triton X-100 was defined as 100%. Melittin was used as a positive control. The results were expressed as the mean ± SD of three individual experiments.

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