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

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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, we 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. The identity of the recombinant SN-1 was verified by MALDI-TOF MS, CD and ^{1}H NMR experiments. All these data strongly indicated that the recombinant SN-1 peptide had a folding with six disulfide bonds that was identical to the native SN-1. Our findings showed 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. These assays demonstrated 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*.

Keywords

Antimicrobial peptide, Cysteine-rich, Snakin-1, *Pichia pastoris*, Over-expression, Folding, Membrane permeability

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, we 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]. We then constructed an efficient *P. pastoris* expression system for production of the recombinant SN-1. We 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, we 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 we know, this is the first report of the recombinant expression of SN-1 in *P. pastoris.*

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 with primer annealing sites (underlined) is GGTTCAAATTTTTGTGATTCAAAGTGCAAGCTGAGATGTTCAAAGGCAGGAC TTGCAGACAGATGCTTAAAGTACTGTGGAATTTGTTGTGAAGAATGCAAATG TGTGCCTTCTGGAACTTATGGTAACAAACATGAATGTCCTTGTTATAGGGACA AGAAGAACTCTAAGGGCAAGTCTAAATGCCCTTGA. 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.

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

We have picked up several 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 almost 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 we 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, we found a single peptide band at about 7 kDa (Fig. 1B). We 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, we 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, we 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.

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], we 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, we 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.

Conclusion

Our study 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.

Acknowledgement

This work was supported by JSPS KAKENHI grant number 26440072.

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Figure captions

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.

Figure 1A

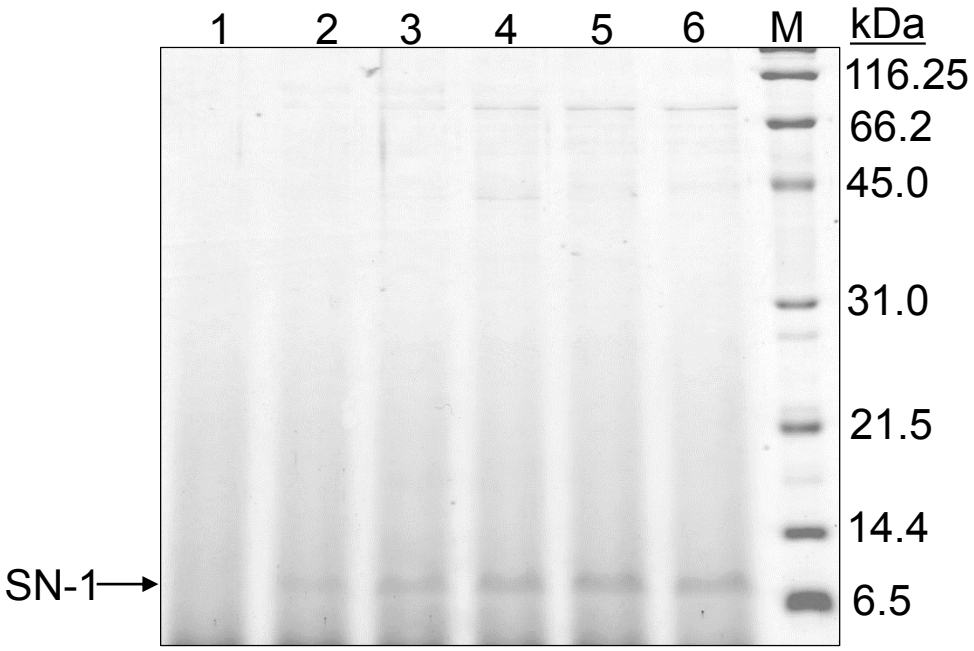


Figure 1B

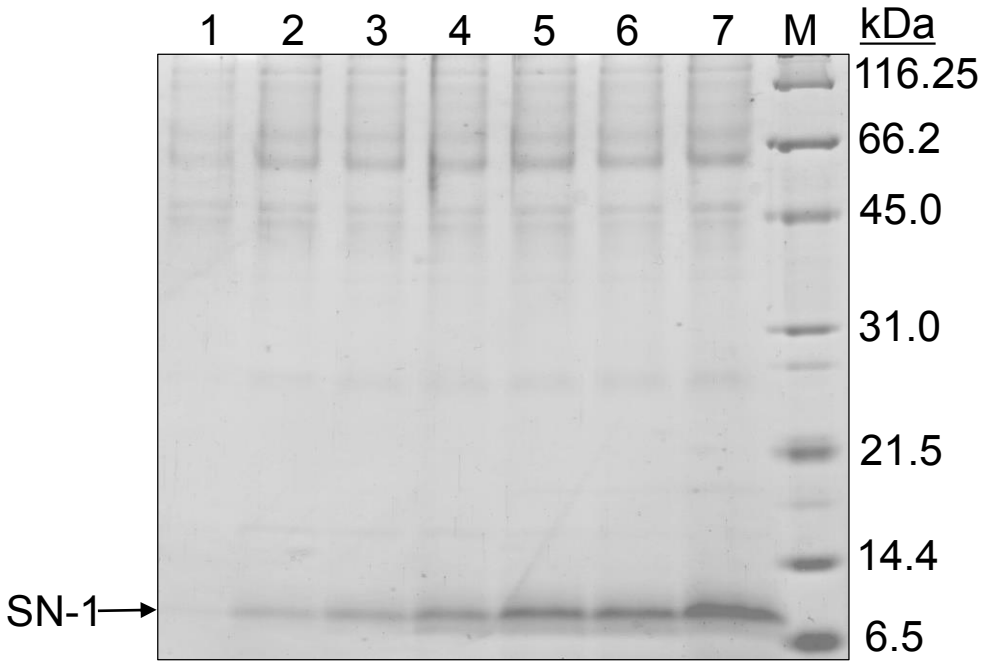


Figure 2

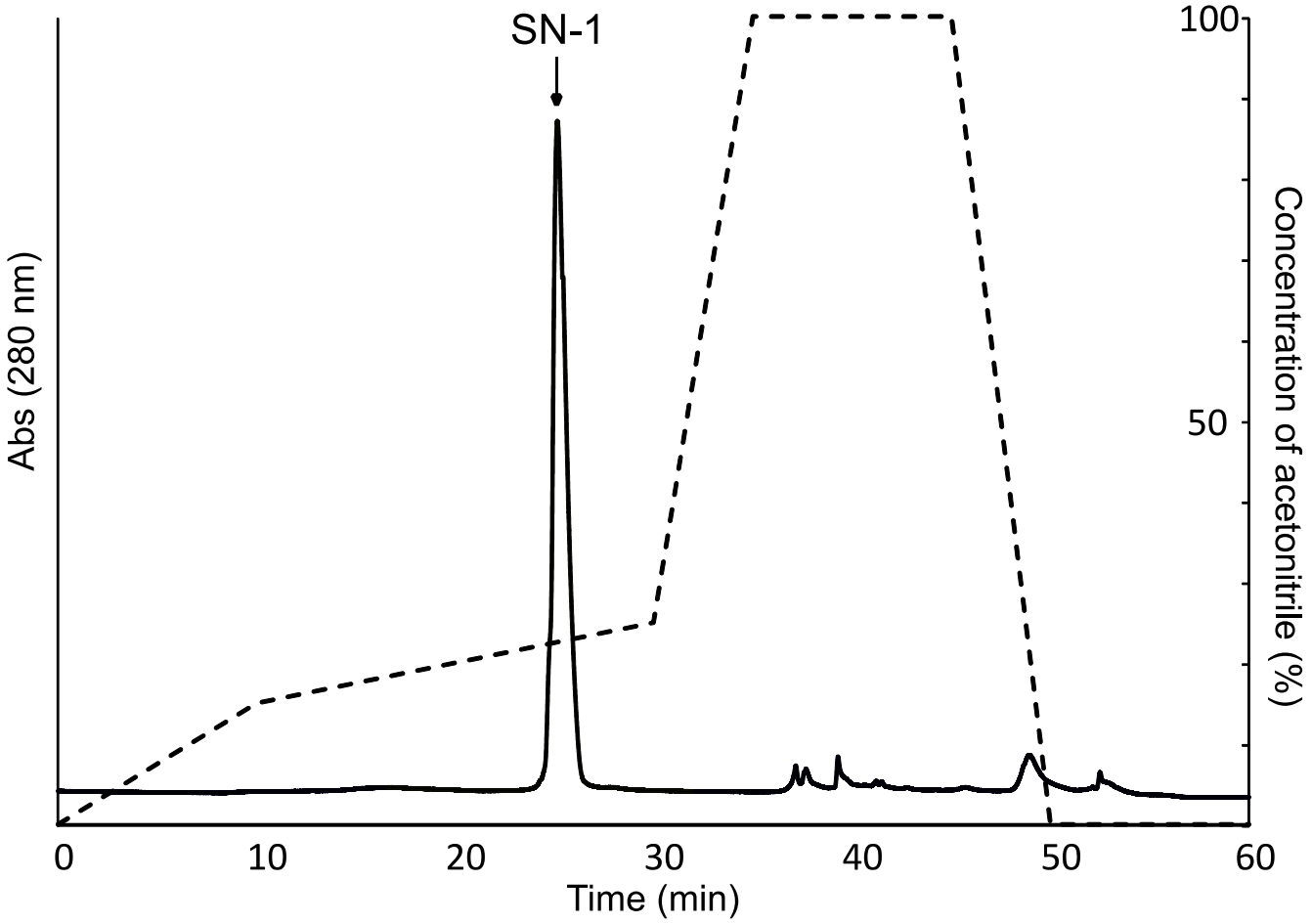

SN-1
100
50
Abs (280 nm)
Concentration of acetonitrile (%)
0
10
20
30
40
50
60
Time (min)

Figure 3

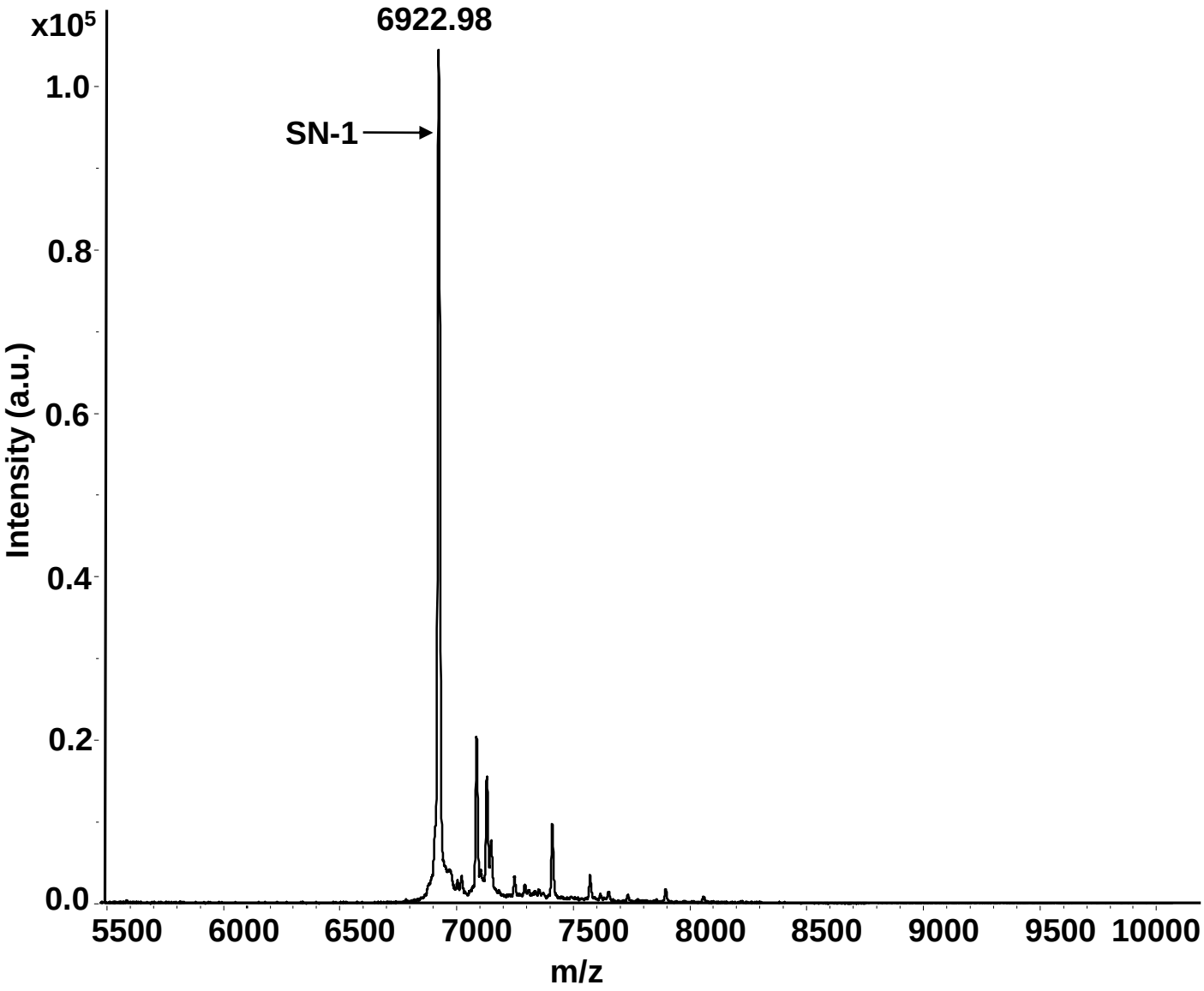

6922.98
x10^5
1.0
SN-1
0.8
0.6
0.4
0.2
0.0
Intensity (a.u.)
5500
6000
6500
7000
7500
8000
8500
9000
9500
10000
m/z

Figure 4

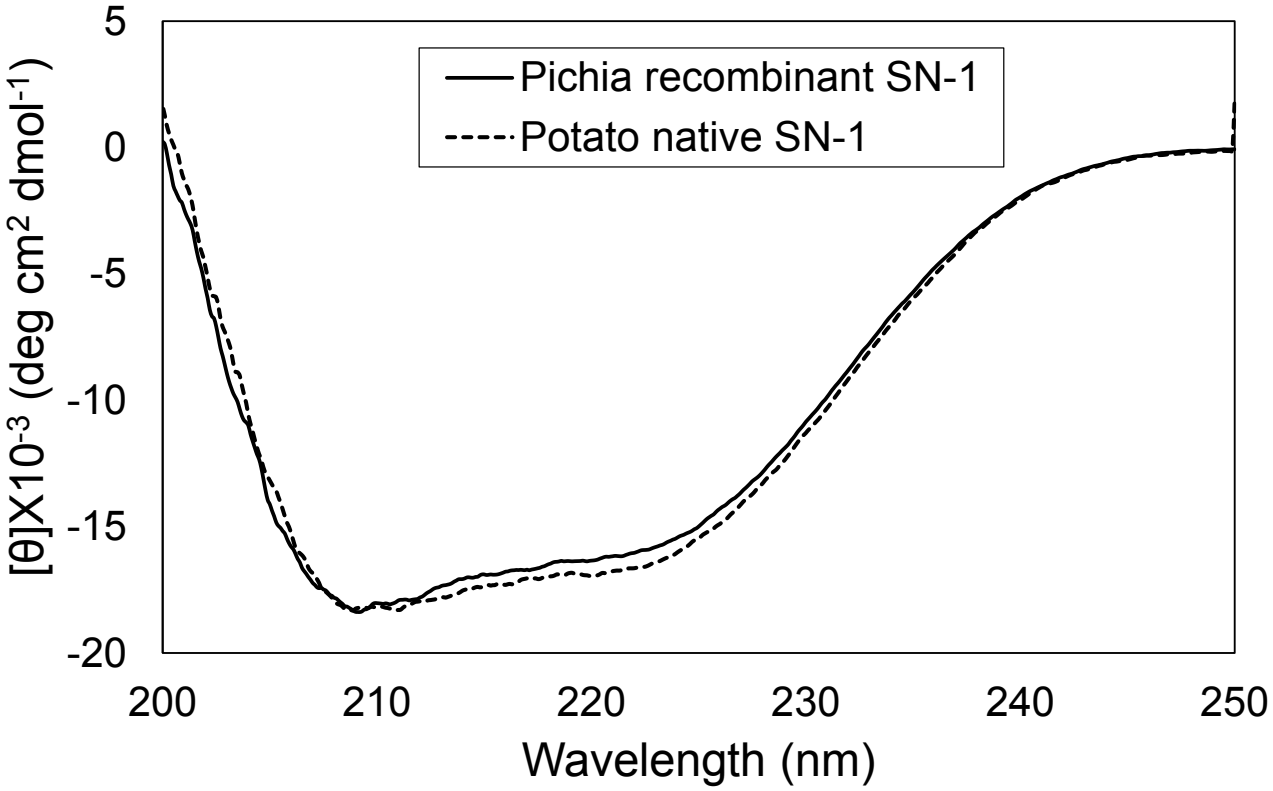

Pichia recombinant SN-1
Potato native SN-1
[θ]X10-3 (deg cm2 dmol-1)
5
0
-5
-10
-15
-20
200
210
220
230
240
250
Wavelength (nm)

Figure 5

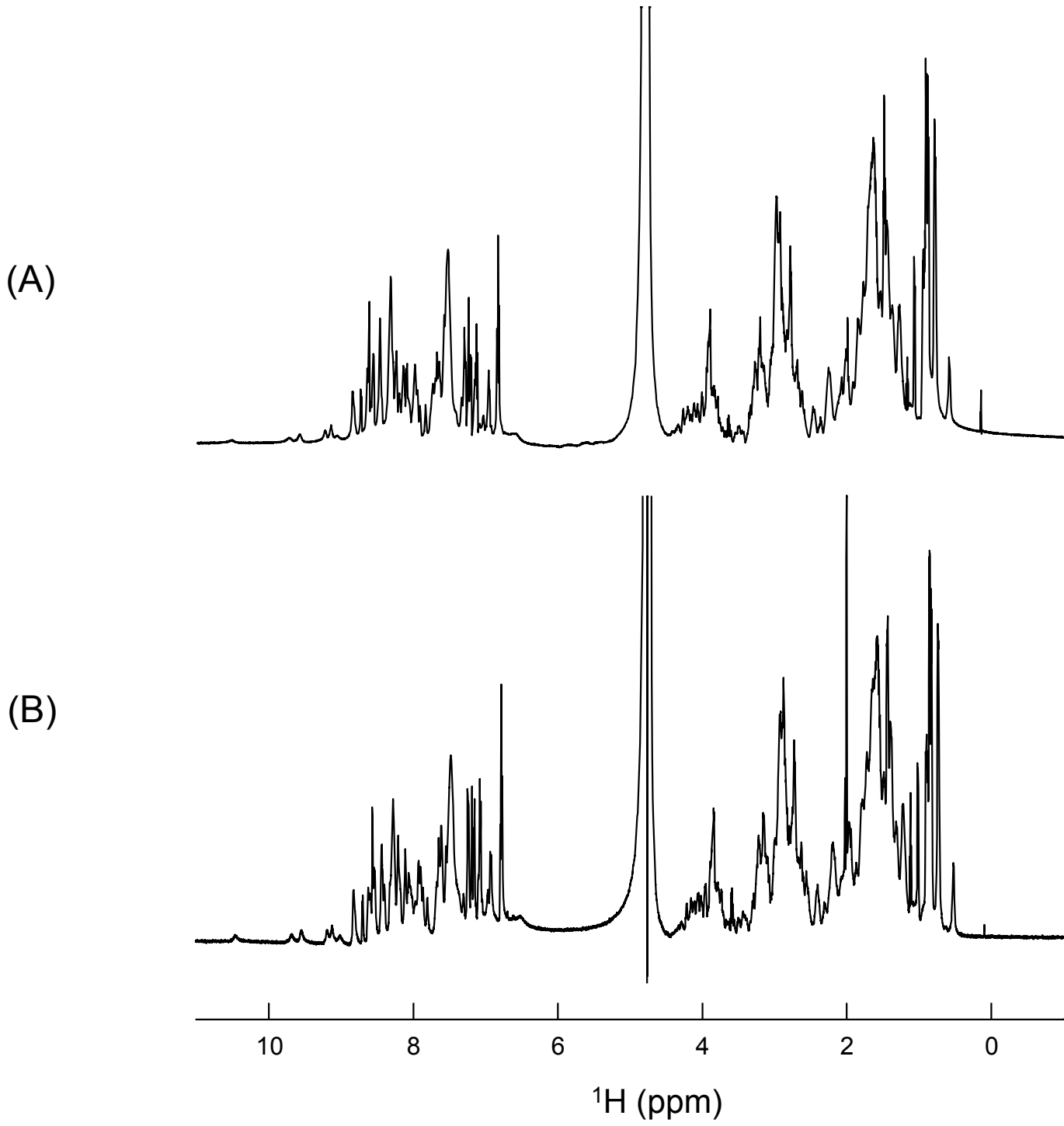


Figure 6

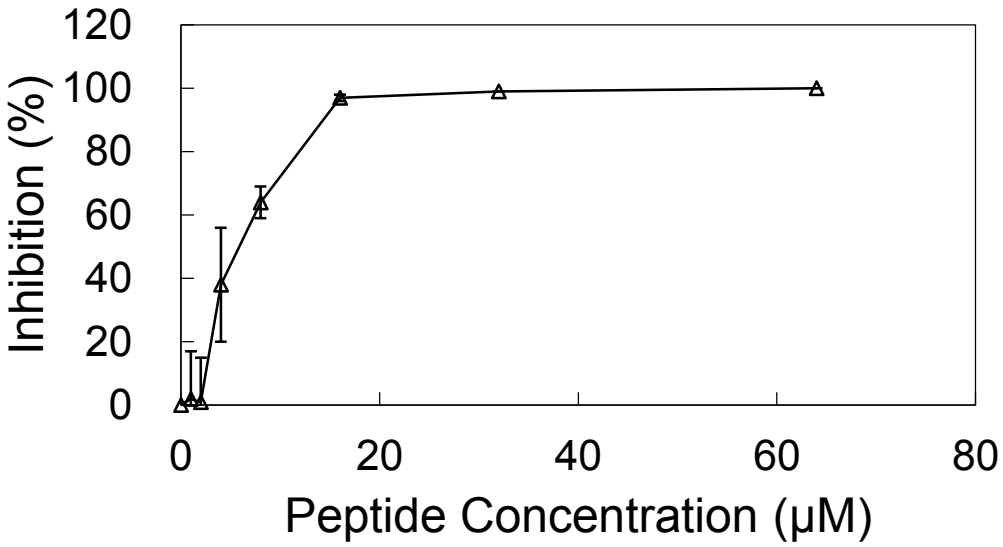

120
100
80
60
40
20
0
Inhibition (%)
0
20
40
60
80
Peptide Concentration (μM)

Figure 7A

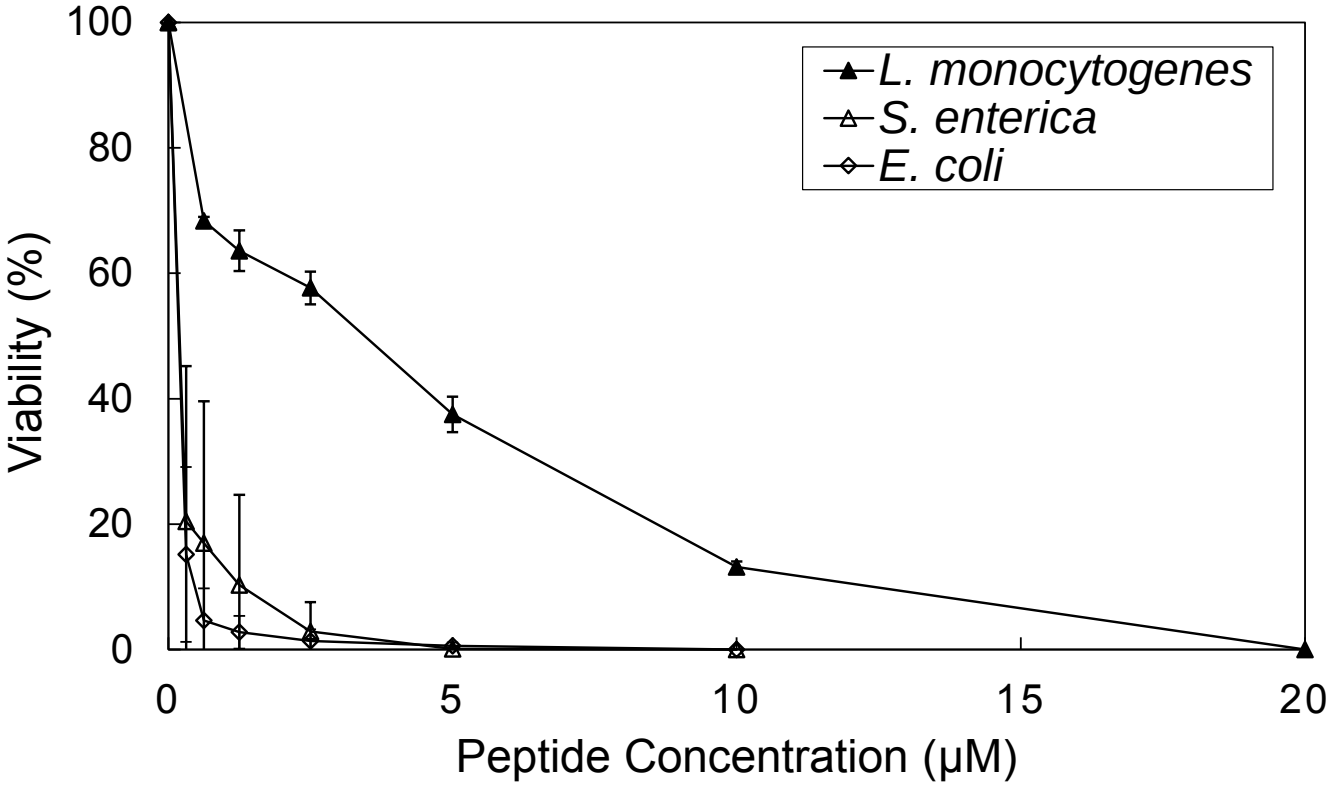

100
80
60
40
20
0
Viability (%)
0
5
10
15
20
Peptide Concentration (μM)
L. monocytogenes
S. enterica
E. coli

Figure 7B

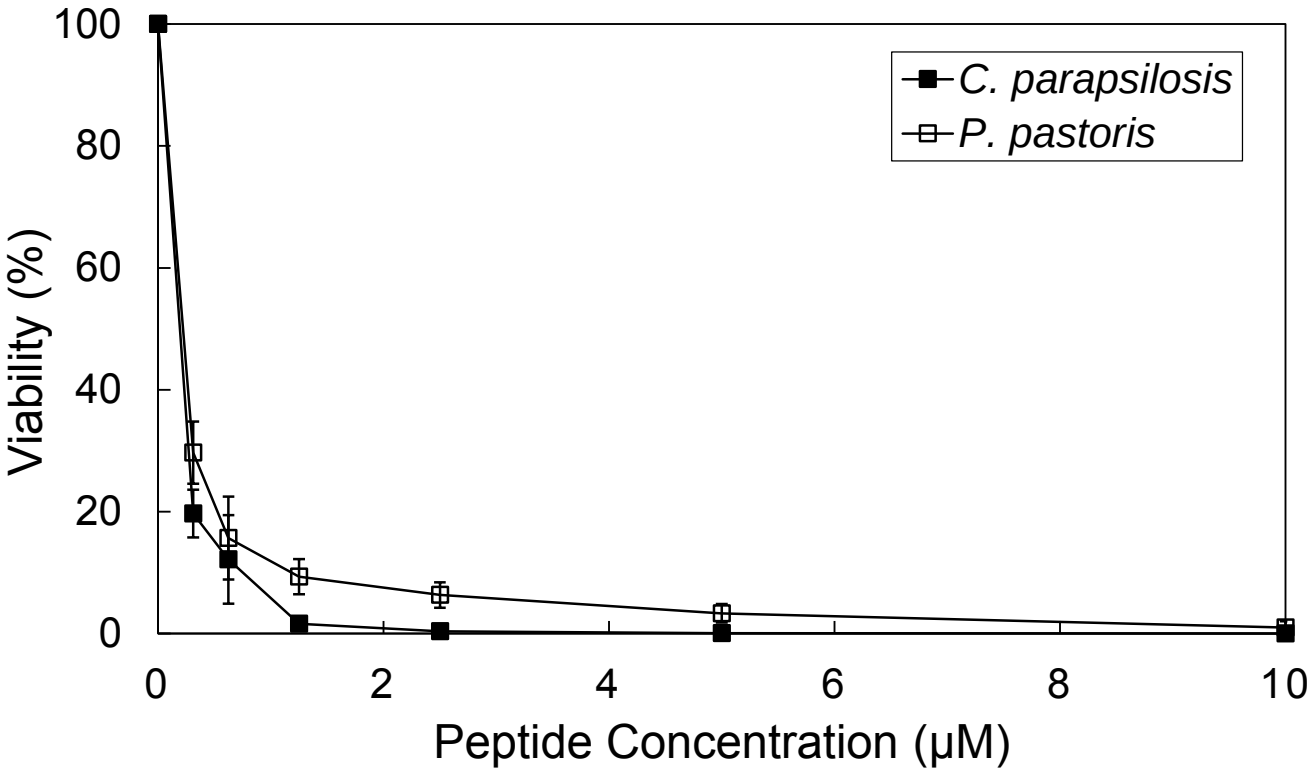

100
80
60
40
20
0
Viability (%)
0
2
4
6
8
10
Peptide Concentration (μM)
C. parapsilosis
P. pastoris

Figure 8

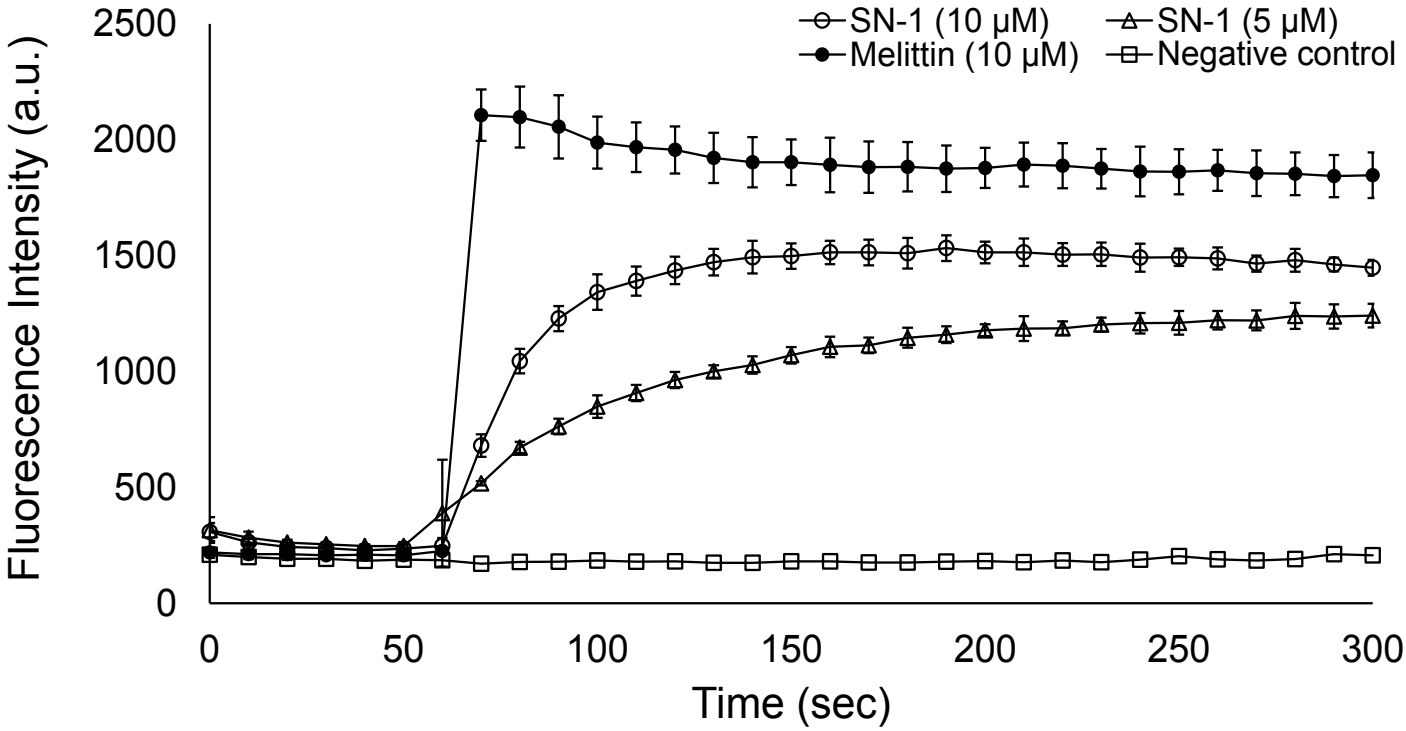

SN-1 (10 μM)
SN-1 (5 μM)
Melittin (10 μM)
Negative control
Fluorescence Intensity (a.u.)
2500
2000
1500
1000
500
0
0
50
100
150
200
250
300
Time (sec)

Figure 9

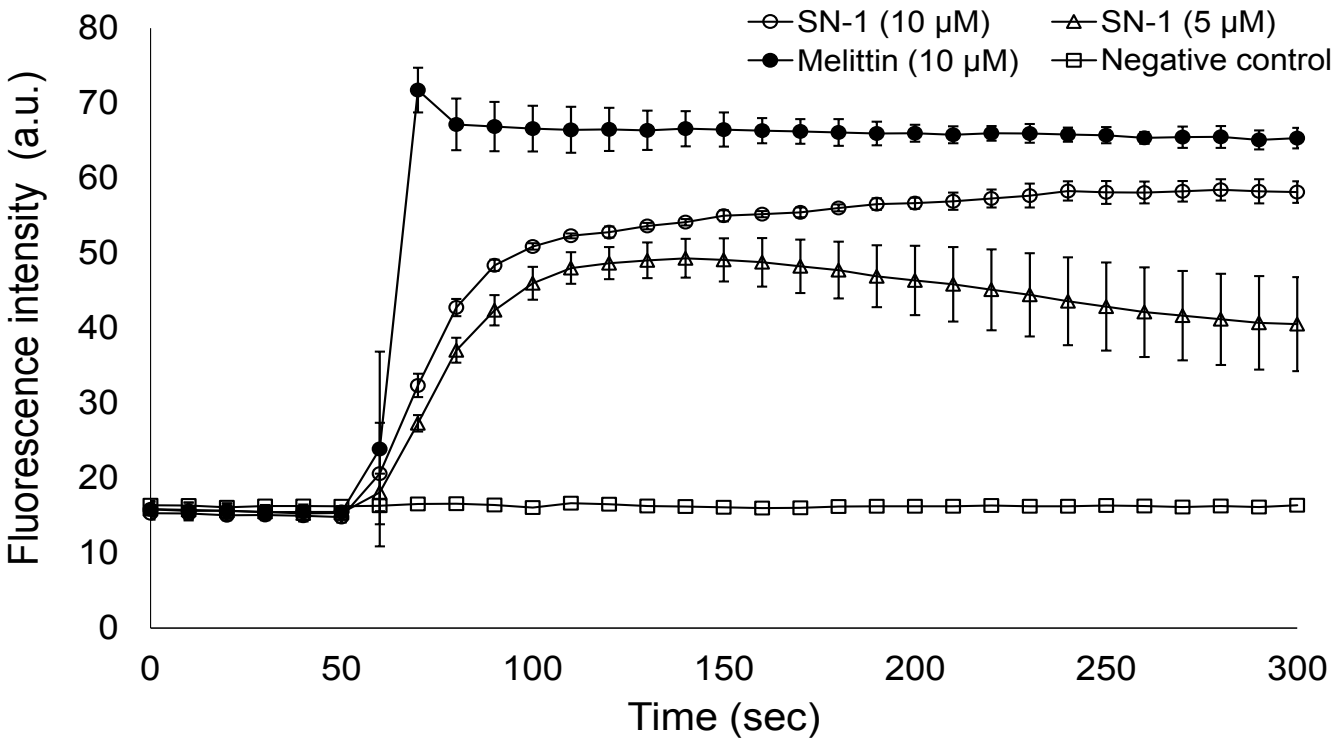
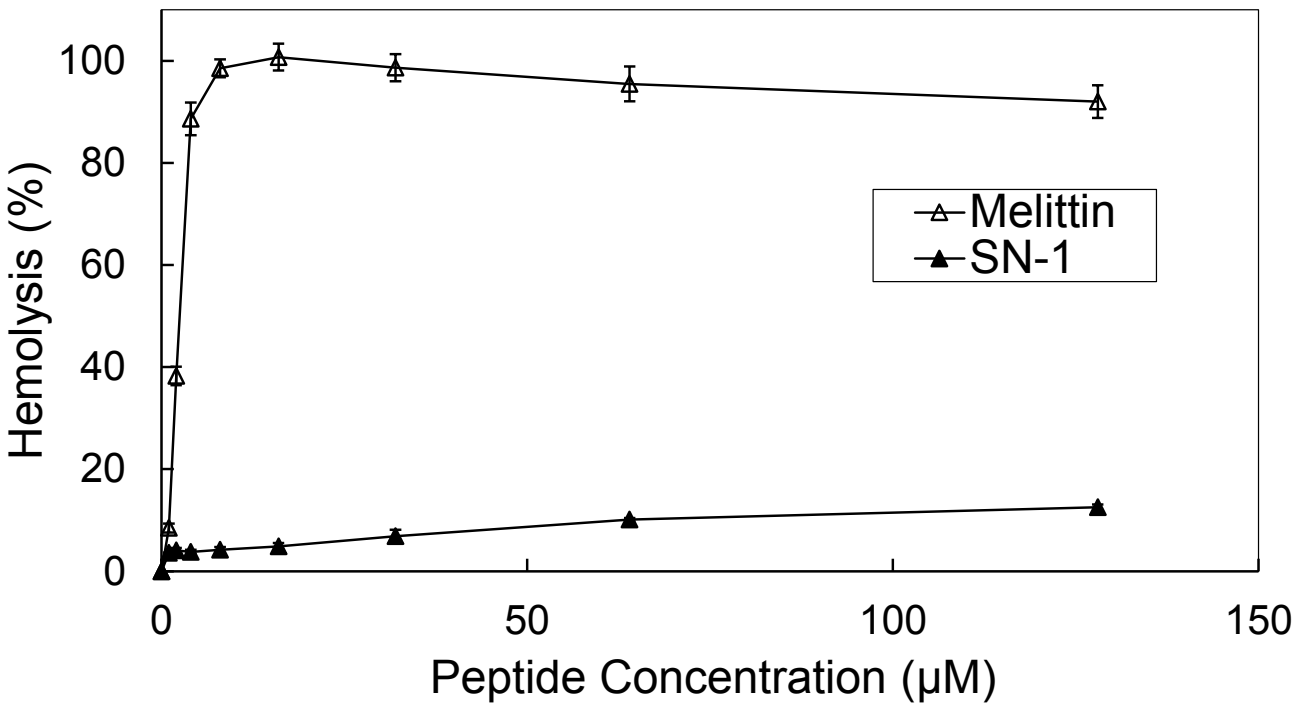

SN-1 (10 μM)
SN-1 (5 μM)
Melittin (10 μM)
Negative control
Fluorescence intensity (a.u.)
Time (sec)
0
10
20
30
40
50
60
70
80
0
50
100
150
200
250
300

Figure 10


Hemolysis (%)
100
80
60
40
20
0
0
50
100
150
Peptide Concentration (μM)
Melittin
SN-1