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Expression and characterization of recombinant rattusin, an α-defensin-related peptide with a homodimeric scaffold formed by intermolecular disulfide exchanges

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ABSTRACT

Rattusin is an α-defensin-related peptide isolated from the small intestine of rats. The primary sequence of linear rattusin is composed of 31 amino acids containing five cysteines with a unique spacing pattern. It forms a homodimeric scaffold in which the primary structure occurs in an antiparallel fashion formed by five intermolecular disulfide (SS) bonds. Rattusin is a highly potent antibiotic, which not only exhibits broad-spectrum antimicrobial activity, but also maintains its antimicrobial activity at physiological salt concentrations. Therefore, to develop new antibiotics based on rattusin, structural and functional studies of rattusin should be performed. For this purpose, large amounts of linear rattusin precursor must be obtained through appropriate preparation methods. Therefore, we established a mass production technique for linear rattusin by using recombinant protein expression and purification procedures. We verified that structure and activity of the recombinant rattusin are identical to the chemically synthesized rattusin. The described method for producing recombinant rattusin provides a high yield of rattusin, which can be used to study the biochemical and functional properties of rattusin and for the development of rattusin-based peptide antibiotics.

1. Introduction

Increased resistance to existing antibiotics has become a critical issue for human health owing to the widespread and inadequate use of existing antibiotics. Therefore, there is an urgent need to develop new types of antibiotics that can effectively inhibit antibiotic-resistant microorganisms. Antimicrobial peptides (AMP), which are found in a variety of organisms including animals, plants, insects, and amphibians, are less toxic and less allergenic to hosts; thus, they can be used as effective antibiotics against antibiotic-resistant bacterial strains [1–4]. Therefore, AMPs have become increasingly important in the development of new therapeutic agents for the prevention and treatment of infectious diseases caused by antibiotic-resistant bacteria [5,6].

Defensins are cysteine-rich AMPs that play an important role in the innate immune system through the provision of protection against infectious pathogens and the regulation of the immune response [7–10]. Mammalian defensins, which have a characteristic β-sheet structure, are stabilized by three intramolecular disulfide (SS) bonds. They exhibit a wide-range of antimicrobial activities and demonstrate a variety of defense mechanisms including immunomodulation, wound healing, toxin neutralization, and anti-cancer activity. Mammalian defensins are classified into three subfamilies, α-, β-, and θ-defensins, based on their amino acid sequences and patterns of SS bond connectivity [11] (Fig. 1). The human α-defensins (HDs), known as human neutrophil peptides (HNPs), have been isolated from neutrophils, natural killer cells, monocytes, T lymphocytes, and Paneth cells of the small intestine [12–14]. β-Defensins are produced by diverse mucosal epithelial cells. For example, human β-defensins (HβDs) are expressed in epithelial cells, the male reproductive tract, and the epididymis [10,15,16]. θ-Defensins from leukocytes of the rhesus macaque are head-to-tail cyclized defensins with three intramolecular SS bonds [17,18].

Rattusin is an α-defensin-related peptide that is abundantly expressed in Paneth cells of the distal small intestine in rats [19]. The linear rattusin peptide consists of 31 amino acids, including five cysteine residues with an unusual spacing pattern. Recently, we reported that rattusin is folded into a C_2-symmetric covalently-linked homodimer formed by five intermolecular SS bonds: four intermolecular SS bonds at the interface of the antiparallel β-sheet region and an intermolecular SS bond at the hairpin loop of the dimer [20]. The refolded dimeric rattusin exerted antimicrobial activities against gram-negative

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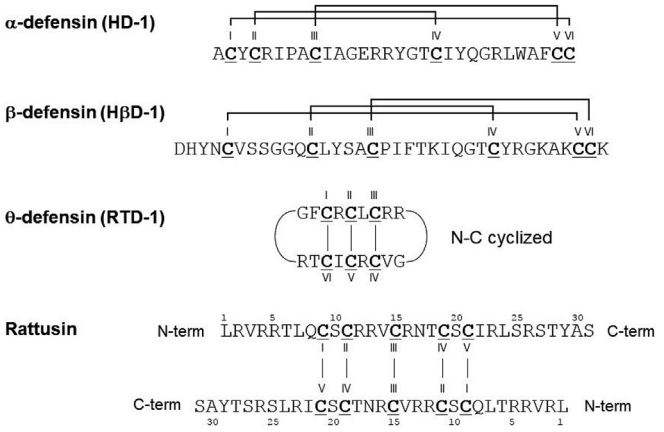

Fig. 1. The amino acid sequences of α, β, and θ-defensins with their disulfide (SS) bond connectivity shown as solid lines. Cysteine residues are indicated in bold and the order is indicated by Roman numerals. The amino acid sequence of dimeric rattusin is shown with the SS bond connectivity.

and gram-positive bacteria including antibiotic-resistant strains [19,20]. Moreover, most of the antimicrobial peptides are inactive at physiological salt concentrations, whereas rattusin maintains its antimicrobial activity at the physiological concentrations of NaCl and Mg^{2+}. These results suggested that rattusin may be a highly potent antimicrobial agent suitable for use in physiological conditions. Therefore, a high-yielding production of linear rattusin peptide is critical for the further study of rattusin for the development of new antimicrobial agents based on the rattusin molecule. In our previous study, the yield of linear rattusin peptide by using conventional Fmoc solid-phase peptide synthesis (SPPS) was very low (Fig. S1). Furthermore, the synthetic linear rattusin must be refolded to form an accurate disulfide bond structure [20], which leads to a decrease in the final yield of rattusin. Therefore, the development of an efficient synthesis method for rattusin should be developed. Here, we have presented a method for the overexpression of recombinant rattusin (rec-RTSN) by using an *E. coli* expression system. The linear rattusin peptide was expressed with a 6-histidine-tagged (6 × His-tag) fusion protein linked to the N-terminus of the rattusin sequence. The N-terminal 6 × His-tag was removed by cyanogen bromide (CNBr) cleavage. The cleaved linear rattusin was refolded in the optimized refolding conditions. The rec-RTSN was structurally and functionally identical to the chemically synthesized rattusin (syn-RTSN). The established method will be utilized for the large-scale production of rattusin and for the development of new antibiotics based on the rattusin molecule.

2. Materials and methods

2.1. Plasmid construction

The overall scheme for the cloning, expression and purification of rattusin is shown in Fig. 2. The rattusin protein was expressed using the *E. coli* expression system. The DNA encoding rattusin protein sequence was designed and chemically synthesized for *E. coli* codon preference (Fig. 3a). The synthetic rattusin DNA was amplified by PCR using the primer pair of aaacgcGGATCCATGCTGCGTGTTCGTCGTACC and aaacgcCTCGAGTTAAGACGCGTAGGTAGAACGAG. The PCR amplified DNA was digested by using the restriction enzymes BamH1 and Xho1. The enzyme-digested product was ligated to the pHIS2 vector by using the BamH1-Xho1 sites (Fig. 3b). The ligated vector (pHIS2-rattusin) was transformed into *E. coli* DH5α cells for amplification. The construct was verified by DNA sequencing.

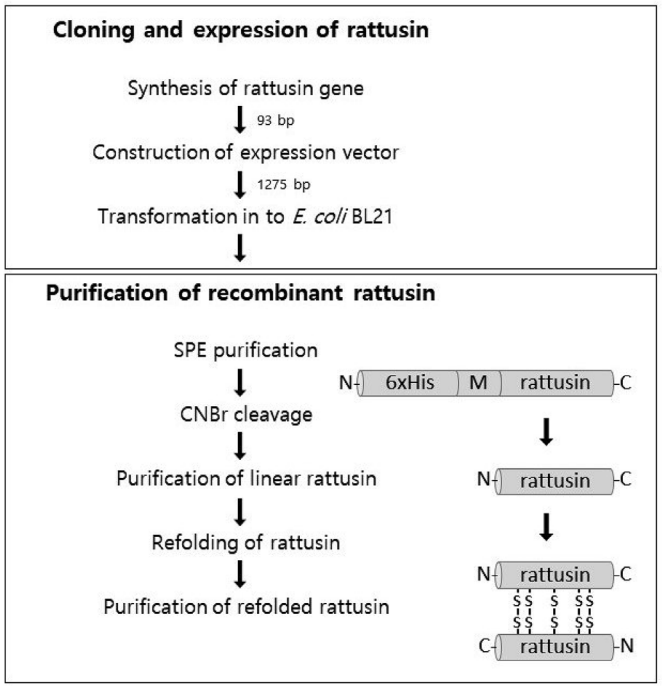

Fig. 2. The schematic diagram of the overall procedure for the expression and purification of recombinant (rec-RTSN).

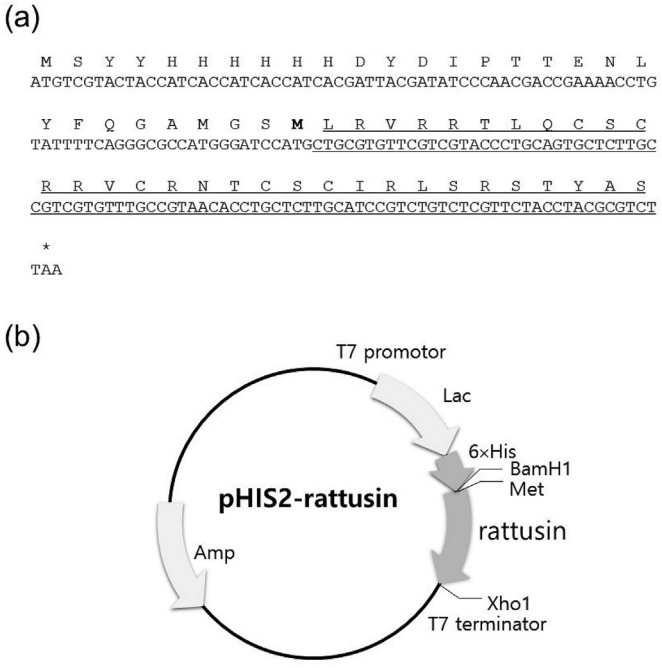

Fig. 3. Construction of the pHIS2-rattusin plasmid for rec-RTSN. (a) The synthetic DNA sequence of rattusin (underlined) with N-terminal histidine tag. The CNBr cleavage site (M) is indicated in bold. The stop codon is indicated by a star symbol. (b) The schematic representation of the expression vector pHIS2-rattusin and the position of the DNA insert coding for the mature rattusin between the BamH1 and Xho1 sites.

2.2. Expression of linear rattusin peptide

The transformed *E. coli* BL21(DE3) cells were subcultured at 37 °C in 100 mL Luria-Bertani (LB) medium with 100 μg/mL ampicillin for 4 h, transferred, and cultured in 1 L LB medium until the optical density was 0.6 at 600 nm. The expression of the 6 × His-tagged rattusin fusion protein was induced by the addition of 1 mM isopropyl β-D-

thiogalactoside (IPTG) and further cultivated for 18 h at 20 °C. The cells were harvested by centrifugation at 12,000 *g* for 10 min at 4 °C and suspended in 50 mL lysis buffer containing 2 × phosphate-buffered saline (PBS) (pH 7.4) and 2 mM dithiothreitol (DTT). The cells were lysed by sonication and the insoluble fractions were isolated by centrifugation at 30,000 *g* for 30 min at 4 °C. After centrifugation, the inclusion bodies were analyzed using 15% polyacrylamide gel electrophoresis (SDS-PAGE).

2.3. Purification of linear rattusin peptide

The inclusion body containing 6 × His-tagged rattusin fusion protein was washed with washing buffer containing 1% (v/v) Triton X-100 in 1 × PBS (pH 7.4). The washed pellet of 6 × His-tagged fusion protein was dissolved in 8 M urea buffer containing 20 mM Tris-HCl (pH 8.0) and 150 mM DTT. The 6 × His-tagged fusion protein was purified by using a solid phase extraction (SPE) C18 cartridge. The sample bound to the resin was eluted with 60% acetonitrile (ACN). The fractions containing 6 × His-tagged fusion protein were identified by using SDS-PAGE and liquid chromatography-mass spectrometry (LC-MS) and then lyophilized. The Met residue between the 6 × His-tag and rattusin sequence provides a cleavage site for CNBr cleavage (Fig. 3a). To remove the 6 × His-tag, the lyophilized 6 × His-tagged fusion protein powder was dissolved in 70% (v/v) formic acid. CNBr was added to the solution at up to 100-fold molar excess over the Met residues. The solution was incubated at 25 °C for 24 h. After incubation, three volumes of water were added to the reaction mixture and the mixture was lyophilized. The lyophilized peptide was dissolved in 20 mM Tris-HCl (pH 8.0) buffer with 150 mM DTT and then incubated at 25 °C for 24 h to reduce the rattusin peptide. The reduced linear rattusin was purified by preparative reverse phase-high performance liquid chromatography (RP-HPLC) (Shimadzu, 6AD) with a C18 column (20 × 250 mm). The elution was performed by using a linear gradient of ACN (20–26% over 12 min) at a flow rate of 10 mL/min. The elution was monitored by UV absorbance at 230 nm. The fractions containing linear rattusin were collected and lyophilized.

2.4. In vitro oxidative refolding and purification of rattusin

The purified linear rattusin was refolded in refolding buffer, which contained 5% ACN, 0.1 mM peptide, 10 mM cysteine, 1 mM cystine, 50 mM NaCl, and 20 mM Tris-HCl (pH 8.0), at 4 °C for 24 h. The refolding process of rattusin was monitored by LC-MS. The rattusin protein was purified by preparative RP-HPLC with a C18 column. The purity and molecular mass of refolded rattusin were confirmed by LC-MS analysis.

2.5. Determination of minimal inhibitory concentration (MIC) of rec-RTSN

The MIC of rec-RTSN was performed in sterile 96-well 200 μL plates by the following methods. One hundred microliter aliquots of *E. coli* cell suspension (4×10^6 CFU/mL in 1% peptone) were added to 100 μL of the sample solutions (two-fold serial dilutions in 1% peptone). After incubation for 16 h at 37 °C, the MIC was determined by visual examination to identify the lowest concentration of the peptide solution with no *E. coli* cell growth. The *E. coli* strain KCTC 1682 was procured from the Korea Collection of Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology.

2.6. Circular dichroism (CD) spectroscopy

The CD spectrum of the rec-RTSN was measured on J-810 spectropolarimeter (JASCO). The 50 μM samples were dissolved in 10 mM sodium phosphate buffer (pH 7.0) with 100 mM NaCl. The spectrum was accumulated from 260 nm to 190 nm from three scans in a quartz cell with a 0.1-cm path length.

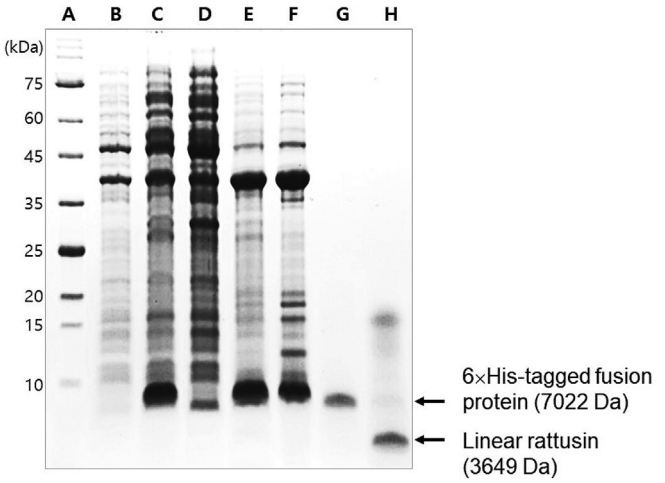

Fig. 4. The profile of the rec-RTSN expression in *E. coli*. Coomassie blue-stained 15% SDS-PAGE shows the following: lane A, molecular weight markers (kDa); lane B, extract of uninduced *E. coli* containing the pHIS2-rattusin expression vector; lane C, extract of induced *E. coli* containing the vector; lane D, soluble protein fraction; lane E, insoluble protein fraction; lane F, pellet washings after 1% Triton X-100; lane G, purified 6 × His-tagged rattusin fusion protein by SPE; lane H, CNBr cleavage of the purified protein.

3. Results and discussion

3.1. Cloning and expression of rattusin

The pHIS2-rattusin plasmid was constructed as an N-terminal 6 × His-tagged rattusin fusion protein with a methionine residue between the 6 × His-tag and rattusin sequence for CNBr cleavage. The 6 × His-tagged rattusin fusion protein was expressed in inclusion bodies (Fig. 4, line C), which was most likely because the rattusin could not be folded into a soluble form with the correct disulfide bond connectivity inside the *E. coli* cells. The inclusion bodies containing the 6 × His-tagged rattusin fusion protein was solubilized in the 8 M urea buffer containing 150 mM DTT to reduce the scrambled SS bonds. The solubilized 6 × His-tagged rattusin fusion protein was purified by using an SPE cartridge. The purified fusion protein appeared as a single band on the SDS-PAGE gel (Fig. 4, line G). The theoretical mass of the fusion protein, 7021.9 Da, was confirmed by LC-MS analysis (Figs. 5(a) and S2(a)).

3.2. CNBr cleavage and oxidative refolding

The purified 6 × His-tagged rattusin fusion protein was dissolved in a solution of 70% formic acid for the CNBr cleavage reaction. The fused protein band at approximately 7 kDa disappeared and a band at approximately 3.5 kDa appeared after incubation (Fig. 4, line H). The reaction mixture was lyophilized and dissolved in Tris buffer containing DTT to obtain the reduced form of linear rattusin. The linear rattusin was purified by RP-HPLC and confirmed by LC-MS (Figs. 5(b) and S2(b)). The yield of linear rattusin was approximately 20 mg from 1 L of culture. After purification, the linear rattusin was refolded in a redox buffer system containing cysteine and cystine. As the rattusin was easily precipitated in low salt buffers, we included NaCl to enhance the solubility of the rattusin peptide. The linear rattusin contains eight arginines without any negatively charged residues (Fig. 1), which makes the peptide highly basic. Similar to rattusin, imperatoxin A (IpTxa), a neurotoxin peptide stabilized by three intramolecular SS bonds, is a highly basic peptide with +8 net charge at a neutral pH. In our previous report, IpTxa was also observed to refold at a high salt concentration with a high refolding yield [21,22]. Both rattusin and IpTxa form large basic surface areas on the molecule [20,21]. The results indicated that the addition of salt into a refolding buffer can protect the

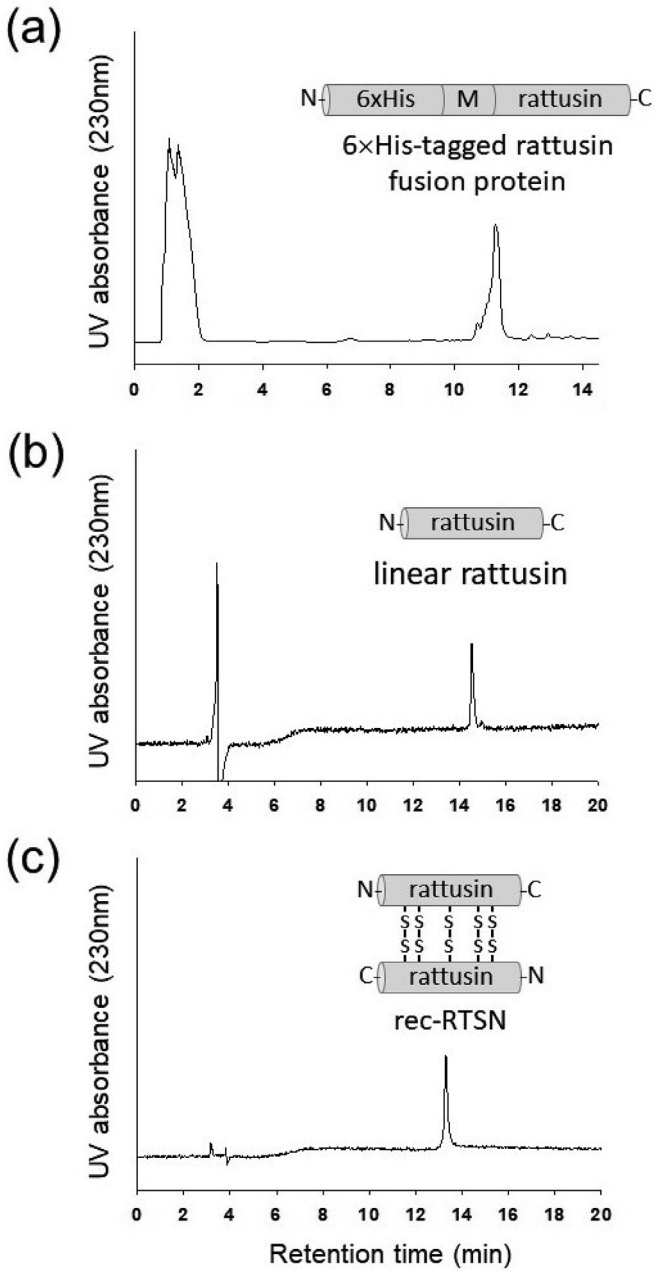

Fig. 5. RP-HPLC analyses of rattusin peptides. (a) The 6 × His-tagged rattusin fusion, (b) linear rattusin, and (c) refolded rattusin. The schematic fragments of rattusin peptides are shown on the RP-HPLC peaks.

aggregation of highly basic peptides during the refolding process and enhance the yield of refolded products. After refolding, the rattusin peptide was purified and the final product displayed a single peak at a mass of 7289.4 m/z, consistent with the dimeric form of the peptide with five intermolecular disulfide bonds (Figs. 5(c) and S2(c)).

3.3. Characterization of rec-RTSN

Rec-RTSN was eluted as a single peak at approximately 13.5 min in RP-HPLC (Fig. 6). The syn-RTSN used for the structure and functional studies in our previous research [20] had the same retention time and the same mass as rec-RTSN. Moreover, the mixture of rec-RTSN and syn-RTSN was eluted as a single peak with a single mass, which indicated that they were identical molecules. To obtain the structural characteristics of rec-RTSN, we measured the CD spectrum (Fig. S3a).

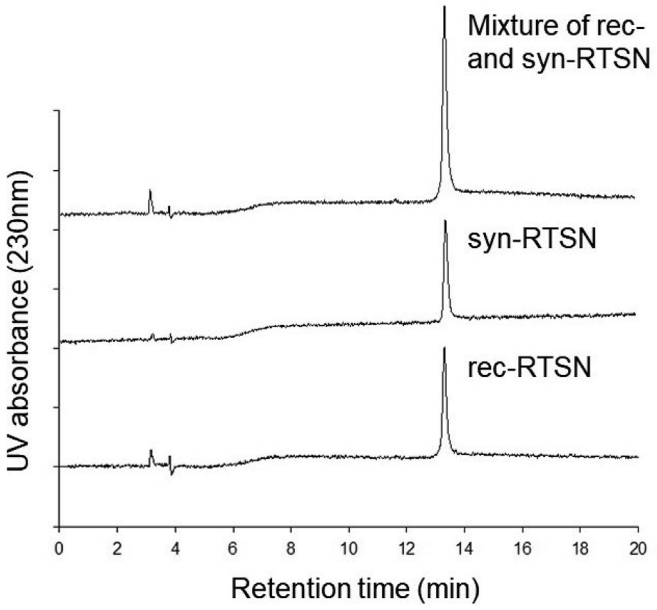

Fig. 6. The comparison of the RP-HPLC chromatographic behavior of recombinant and chemically synthesized rattusin. RP-HPLC chromatograms were recorded by using an analytical RP-HPLC equipped with a 4.6 × 250 mm C18 silica column. The peptides were eluted with a linear gradient of 5–45% buffer B over 20 min at a flow-rate of 1 mL/min.

The CD spectrum of rec-RTSN showed a negative minimum at approximately 203 nm, which was indicative of β-sheet structural characteristics found in other cysteine-rich β-strand containing peptides [21,23,24]. Moreover, the CD signal of unfolded rattusin exhibited a typical random coil conformation with a negative minimum at less than 200 nm (Fig. S3a). The CD spectrum analysis of rec-RTSN indicated that rec-RTSN contained approximately 32% of the strand content as analyzed by DichroWeb server, consistent with the NMR structure analysis of rec-RTSN (Fig. S3b). We previously reported that the three-dimensional structure of rattusin is composed of two antiparallel β-stands that form a cylindrical array similar to that of β-sandwich folds [20]. To compared the structural characteristics of rec- and syn-RTSN, we measured 2D TOCSY NMR spectra and superimposed them (Fig. S4). The number of peaks and their chemical shifts are almost identical, which clearly indicates that the three-dimensional structure of rec-RTSN is identical to that of syn-RTSN.

To assess the biological function of rec-RTSN, the antimicrobial activity of rec-RTSN was examined by the determination of its MIC. Rec-RTSN inhibited the growth of *E. coli* at the minimum concentration of 8 μM, the same value as syn-RTSN [20], which indicated that rec-RTSN was both chemically and functionally identical to syn-RTSN.

3.4. Significance of rec-RTSN

Chemical synthesis by SPPS is a common methodology for the preparation of a variety of peptides. For example, small, cysteine-rich peptides can be synthesized by SPPS and refolded *in vitro* with acceptable yields. However, with longer polypeptides, the synthesis yields become very low owing to the aggregation of the growing peptide within the reaction solvent during peptide synthesis. Although linear rattusin is not a long peptide (31 amino acids), the yield of linear rattusin by SPPS was very low (below approximately 1%; Fig. S1). Moreover, a refolding process is required for rattusin as it forms a dimeric structure with five intermolecular SS bonds. Therefore, owing to the required additional preparation steps, the final synthesis yield of the synthetic rattusin was significantly reduced.

E. coli has been used for the production of various bioactive peptides, such as hormones, neurotoxins, and antimicrobial peptides

[25–28]. The *E. coli* expression system is also suitable for the production of uniformly or partially stable isotope-labeled peptides, which are required for structural and biophysical NMR spectroscopy. However, the expression of antimicrobial cysteine-rich peptides, such as rattusin, has been challenging owing to the cytotoxicity to host cells, susceptibility to degradation or aggregation, and low expression levels [29,30]. Therefore, fusion proteins are used for the biological expression of antimicrobial peptides to reduce the cytotoxicity to host cells and enhance product stability and expression levels [28,31–33]. The expressed peptide is released from the fusion protein by chemical or enzymatic cleavage to obtain an intact product.

In the study, we used the 6 × His-tag as a fusion partner to induce a high level of the expression of rattusin peptide in inclusion body, which may enhance the stability and prevent host cytotoxicity of the peptide inside the cell. The fusion partners are chemically or enzymatically cleaved to release the desired product. In general, as enzymatic cleavage cannot be used for insoluble protein aggregates, we utilized CNBr, which cleaves the carboxyl side of a methionine residue in acidic conditions. The insoluble 6 × His-tagged rattusin fusion protein was efficiently solubilized in 70% formic acid. The 6 × His-tag was completely cleaved by the CNBr digestion reaction. The recombinant proteins or peptides can have additional residues at N- or C-terminus after the removal of the fusion protein, which can affect the biological activity or molecular structure of the desired recombinant proteins or peptides. The rec-RTSN does not contain any extra residues in its sequence after CNBr cleavage. The linear rattusin was refolded into a homodimer formed by five intermolecular SS bonds. After refolding procedure, the final yield of the expression and purification of rec-RTSN was over 10 mg from 1 L culture. These results suggested that the established recombinant method was much more effective and advantageous for a large production yield and the subsequent future study of rattusin than the chemical synthesis.

We have developed an *E. coli* expression system and purification method of rattusin, which provides a high yield production of rattusin and also enables site-directed mutagenesis and stable isotope labeling to analyze the structure-activity relationships of rattusin.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pep.2018.02.006.

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