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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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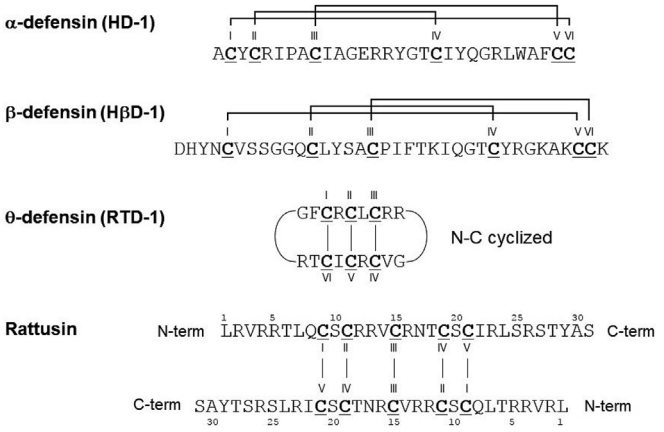

Fig. 1. The amino acid sequences of α, β, and θ-defensins with their disulfide (SS) bond connectivity shown as solid lines. Cysteine residues are indicated in bold and the order is indicated by Roman numerals. The amino acid sequence of dimeric rattusin is shown with the SS bond connectivity.

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.

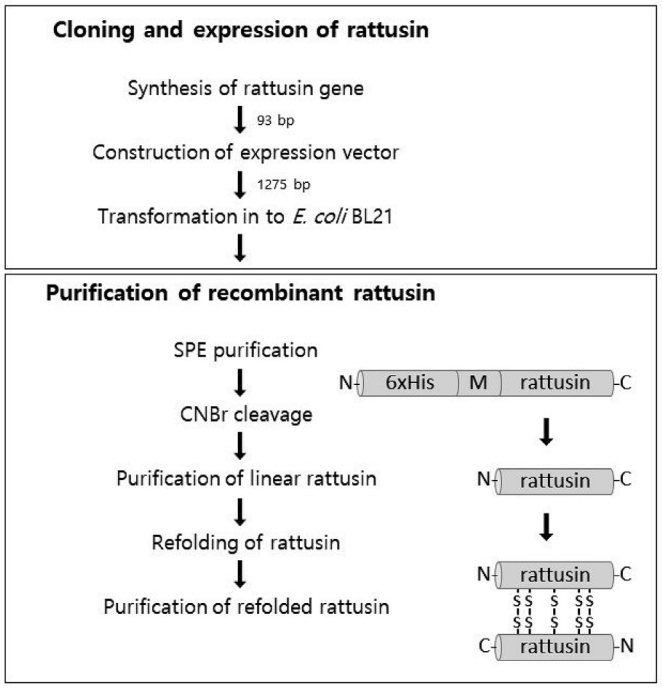

Fig. 2. The schematic diagram of the overall procedure for the expression and purification of recombinant (rec-RTSN).

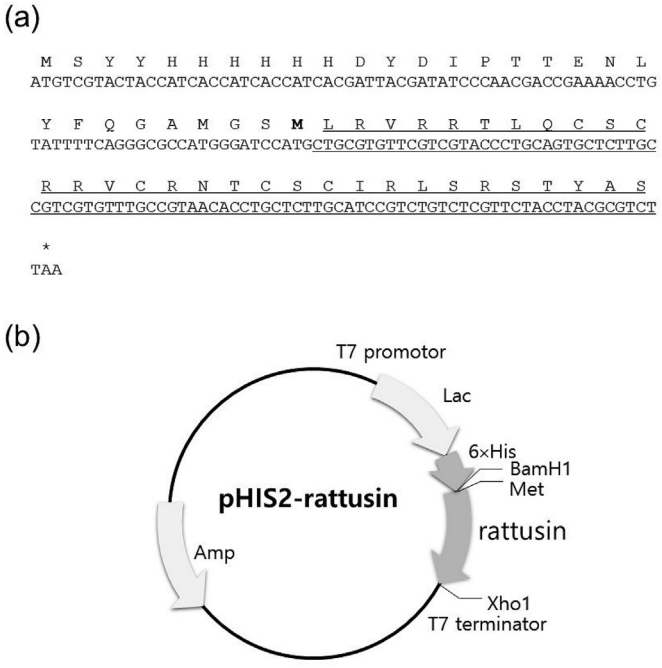

Fig. 3. Construction of the pHIS2-rattusin plasmid for rec-RTSN. (a) The synthetic DNA sequence of rattusin (underlined) with N-terminal histidine tag. The CNBr cleavage site (M) is indicated in bold. The stop codon is indicated by a star symbol. (b) The schematic representation of the expression vector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