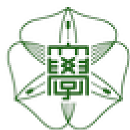
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学　位　論　文　内　容　の　要　旨

博士の専攻分野の名称　博士（ソフトマター科学）　氏　名　宋　雨遅

学　位　論　文　題　名

Efficient recombinant production of mouse-derived cryptdin family peptides by a novel facilitation strategy for inclusion body formation
（新規封入体形成促進技術によるマウス由来 cryptdin ファミリーペプチドの組換え体の効率的生産）

Antimicrobial peptides (AMPs) are the primary factors of innate immunity and are the frontline of biological defense mechanisms against infection. Most AMPs are positively charged and are thought to exhibit microbicidal activity through a membrane-disrupting mechanism due to their interaction with negatively charged microbial membranes. A number of AMPs hold promise as new drugs owing to their potent bactericidal activity and because they are often refractory to the development of drug resistance. Cryptdins (Crps) are a type of AMP, also known as α-defensins, found in mouse intestinal Paneth cells, comprising six isoforms containing three sets of disulfide bonds. It has been reported that Crps contribute to the antibacterial barrier function of the small intestinal mucosa, and the selective activity of Crps may be related to the composition of the intestinal microbiota in vivo and homeostasis of the entire intestine. Furthermore, low Crp levels are thought to be closely related to dysbiosis, which is an abnormality of the intestinal microflora, and the various diseases it induces. Although Crp4 is actively being investigated, there have been few studies to date on the other Crp isoforms. The level of gene expression of different Crps in various positions in the small intestine differs, and their dissimilar characteristics indicate that different isoforms appear to have specific roles in the small intestine. Based on these backgrounds, it is very meaningful to better understand the Crp family. A better understanding of Crps requires them to be produced in a more efficient manner.

In chapter 1, I produced large amounts of most of the Crps by a novel strategy to promote inclusion body formation. In previous study, co-expression of Crp4 with the aggregation-prone protein human α-lactalbumin (HLA) was used to promote the formation of stable inclusion bodies in order to avoid the degradation of Crp4 during recombinant expression in *Escherichia coli*. Using this method, the production of Crp4 and Crp6 by the BL21 strain was effective, but the expression of other Crp isoforms was not as efficient. I considered two possible reasons why this co-expression system did not result in enhanced expression. First, when ribosomes synthesized the different Crp isoforms, the synthesis levels were different, leading to significant differences in the final amount produced. The second possibility is that there was a difference in the efficiency of inclusion body formation, even though there was not much difference in the level of ribosome synthesis for each peptide. Therefore, I first verified the amount of each Crp isoform synthesized by ribosomes using the reconstituted cell-free expression system. The results of a cell-free system study showed that except for Crp5, the expression levels of Crp1, Crp2, and Crp3 were comparable to those of Crp4 and Crp6. This suggests that the reason why

low levels of insoluble granules were previously obtained with Crp1, Crp2, and Crp3 in the co-expression system with HLA in *E. coli* may be due to a limitation of the efficiency of their formation once synthesized, while for Crp5, the actual amount synthesized by the ribosomes may be limiting. Based on these results, I sought to promote the formation of insoluble granules by forming non-natural disulfide cross-links between HLA and Crps using Origami B, strain which is an expression host with an oxidative internal environment. As expected, using the Origami B strain greatly improved the expression levels of Crp1, Crp2, and Crp3. Subsequently, Crps and HLA were successfully separated by solubilizing inclusion bodies in a solubilization buffer with the addition of reducing agents and urea, and cation exchange chromatography. Refolding was performed by dialysis, then the product obtained after dialysis could be purified by reverse-phase HPLC. Each purified Crp isoform could be separated into two or three peaks, which is thought to be due to modification of the N-terminal methionine by a formyl group. Then I used acid hydrolysis to deformylate Crps with N-terminal formyl group. After exploring the conditions, 0.6 M hydrochloric acid was considered as the optimum condition. The proportion of successful deformylation for each Crp isoform exceeded 70%. Deformylation resulted in a significant increase in the production of each Crp isoform, with yields reaching at least 3 mg/L of medium.

In Chapter 2, I studied the secondary structure and antibacterial activity of Crps prepared in the previous chapter. First, to obtain information on the steric structures of the Crps, CD spectra were measured under different conditions. In order to investigate reduced Crps, which have recently attracted attention for their activity *in vivo*, I also prepared Crps completely reduced with DTT. CD spectra were measured in 10mM PBS, highly hydrophobic (40% TFE) and membrane-mimetic (10 mM SDS) environments, fully reduced Crps, compared to the spectrum in 10mM PBS, showed a very large structural change, but for the Crps prepared in the previous chapter, no significant changes in the spectra were observed. This suggests that all prepared Crps had a stable secondary structure due to disulfide cross-linking and that their steric structures were stable in various environments. The bactericidal activity of Crps against Gram-positive bacteria (*Staphylococcus aureus* and *Listeria monocytogenes*) and Gram-negative bacteria (*E. coli* and *Salmonella enterica*) was analyzed. Crp4 exhibited the strongest antimicrobial activity against Gram-negative bacteria, followed by Crp3 and Crp2. Crp1 and Crp6 exhibited the weakest activities. However, Crp4 exhibited very low activity against Gram-positive bacteria, but the other four Crps all exhibited very strong bactericidal activity. These trends clearly indicate that each Crp isoform has a very different antimicrobial spectrum.

In this study, I established a novel and efficient method for the production of the cryptdin family of cysteine-containing antimicrobial peptides. Additionally, I found that there were notable differences in the antibacterial activities of the various Crp family members. The expression system established in this study is expected to provide new insights regarding the mechanisms underlying the different antibacterial activities of the Crp family of peptides.