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Title of Doctoral Dissertation

Production and analysis of recombinant antimicrobial peptides by fusion expression with calmodulin
（カルモジュリンとの融合発現によるリコンビナント抗菌ペプチドの生産と解析）

Antibiotic resistance is a growing global health concern. It occurs when bacteria become resistant to the antibiotics used to treat them, making it difficult to treat infections. Antibiotic resistance can lead to longer hospital stays, higher medical costs, and increased mortality. It is a major public health issue, as it can spread quickly and affect people of all ages. As antibiotic resistance continues to increase, it is important to understand the causes and take steps to prevent it. In addition to tweaking the antibiotics themselves, finding another antimicrobial substance to replace them is also a solution. Antimicrobial peptides (AMPs) are interesting candidates.

AMPs are small cationic peptides with amphiphilicity that are produced by a variety of organisms, including bacteria, fungi, plants, and animals. They are an important part of the innate immune system, providing the first line of defense against invading pathogens. AMPs are highly effective against a wide range of microorganisms, including bacteria, fungi, and viruses. They are also known to have anti-inflammatory and immunomodulatory properties. AMPs are an attractive alternative to traditional antibiotics, as they are less likely to induce resistance in bacteria.

To produce large quantities of AMPs for basic research, recombinant expression is generally considered. Compared with chemical synthesis, recombinant expression is cost-effective and suitable to produce isotope-labeled peptides for use in nuclear magnetic resonance (NMR) studies. But overexpression of AMPs can be a difficult process due to the complexity of the peptide structure and the need for precise control of the expression levels. AMPs are typically expressed in a heterologous system, such as bacteria or yeast, and require careful optimization of the expression conditions to ensure that the peptide is expressed at the desired levels. Additionally, the peptide must be purified and characterized to ensure that it is active and stable. Finally, the expression system must be monitored to ensure that the peptide is not toxic to the host organism. Degradation and toxicity are two main obstacles. In the most common *Escherichia coli* expression systems, the use of fusion proteins to increase the solubility of the target AMPs and simplify purification has become a common approach. In recent years, using calmodulin (CaM) as a fusion protein label to produce alpha-helical peptides has become a novel research direction. Compared to traditional fusion proteins such as glutathione-S-transferase (GST), maltose binding protein (MBP), and thioredoxin (Trx), CaM is reported that expression systems solve the problems of hydrolysis and host-toxicity of AMPs.
CaM is a calcium-binding protein that exists in all eukaryotic cells. It is involved in a wide range of cellular processes, including signal transduction, gene expression, and muscle contraction. CaM binds to calcium ions and changes its conformation, allowing it to interact with other proteins and activate or restrict them. Many AMPs with amphiphilic helical structures can be well bound to Ca^{2+}-CaM, and the AMPs are protected while limiting their toxicity. Many kinds of AMPs have been expressed in the past.

The first objective of this study was cecropin P1 (CP1) from a nematode, an AMP from the cecropin family. CP1 has a strong antibacterial function and shows a helical structure in co-solvent. The Trx expression system was used to produce CP1, but due to its strong toxicity, the expression level was very low. In this study, a large amount of isotope-labeled CP1 was produced using the CaM expression system. The three-dimensional structure of CP1 and its analogs in the membrane mimetic environment was discovered by NMR. DNA binding experiments showed that

CP1 has DNA binding ability, and it may be the cause of toxicity. The C-terminal of CP1 plays an important role in its DNA-binding ability.

Another subject of this study is fowlicidin-1 (Fow), from the cathelicidin family of chicken. Due to its toxicity, recombinant expression of Fow has never been reported in the past, even though two other members from the same family have been successfully expressed. Even CaM does not limit its toxicity well. In this study, a new co-expression system was developed, in which additional CaM was added during the expression process, and the toxicity of Fow was successfully limited. I found that fusion proteins play a major role, assisted by co-expressed CaM. Fusion protein still has a certain bactericidal function, but in the presence of excessive CaM, Fow lost the bactericidal function. I produced the isotope-labeled Fow with an optimized novel CaM expression system. The secondary structural analysis showed that Fow showed a random structure in water and a helical structure in the presence of co-solvent.

This study presents the performance of the CaM expression system in the production of toxic AMPs. Isotope-labeled products were also produced, which provides a method for many protein NMR researchers to obtain experimental samples. The activity of CP1 outside membrane reaction was also understood through the analysis. A new method was also found to reduce the activity of AMPs by additional CaM interaction with AMPs. There will be more research on the CaM expression system in the future, making it a good alternative to adapt to the recombinant expression of various peptides and proteins.