Site-Directed Mutagenesis to Determine Key Amino Acid Residues in Cell Penetrating Peptides

The Freeman lab primarily focuses on the study of natural product biosynthesis, particularly in unique host organisms. Developing a protein transduction system will allow for the further study of natural product biosynthesis in a wide range of new organisms. The mechanism of protein transduction researched in this study is through the use of cell-penetrating peptides (CPPs) as carrier molecules.

CPPs are a class of molecule that are able to translocate across the cell membrane. As a result, they have the potential to be used as a carrier molecule to transport other proteins within the cell. They are typically 10-60 amino acids in size and are often positively charged.[1] Short proline-rich antimicrobial peptides (PrAMPs), specifically apidaecin-type peptides, are a well-studied class of cationic CPPs that penetrate the bacterial membrane without depolarizing it. Once inside the cell, the PrAMP binds to a subunit of the ribosome, inhibiting it from forming the full ribosome and thus halting protein translation, slowly causing the death of the cell.[2] The antimicrobial activity of these CPPs presents problems for heterologous expression in *Escherichia coli*, the most widely used host microbe in research studies. This report looks at possible ways of using site-directed mutagenesis to decouple the antimicrobial properties of PrAMPs from the cell translocation properties in order to develop a simple and effective method of both heterologous expression of CPP fusion proteins and also use them to transport cargo into host cells. A split GFP reporter assay[3] (small fragment attached to PrAMP, big fragment expressed in target cells) determined the cell penetration abilities of the PrAMP. Eight different single amino acid mutant PrAMP sequences were created and cloned into expression and cloning

cells. Table 1 below shows each mutation as well as the entire sequence of the PrAMP that was mutated.

Table 1: The top row shows the amino acid sequence of the PrAMP sequence that was mutated in this study, which was an apidaecin type PrAMP. The mutations that were constructed are also listed.

Apidaecin Primary Sequence 5’ GNNRPVYIPQPRPPHPRI 3’	
Name	**Mutation**
M01	His15Ala
M02	Arg4Ala
M03	Pro5Ala
M04	Ile8Ala
M05	Gln10Ala
M07	Tyr7Ala
M08	Pro13Ala
M09	Arg17Ala

One mutant PrAMP was successfully expressed in *E. coli* and purified using sonication and affinity chromatography.

Materials and Methods

Cloning

Alanine scanning was performed on the PrAMP using mutated PCR primers. Single amino acids were selected based on structural significance (charge, polarity, size) and mutated to

alanine. PCR products were ligated into pETDuet plasmids and transformed into a cloning strain of *E. coli* (TOP10). Upon sequence verification, successful constructs were transformed into both cloning and expression cell lines. In total, eight different mutant PrAMPs were constructed. The entire construct contained an N-terminal SUMO tag to mask the PrAMP from the expression cells as well as make the protein more soluble, but then was cleaved before assaying.

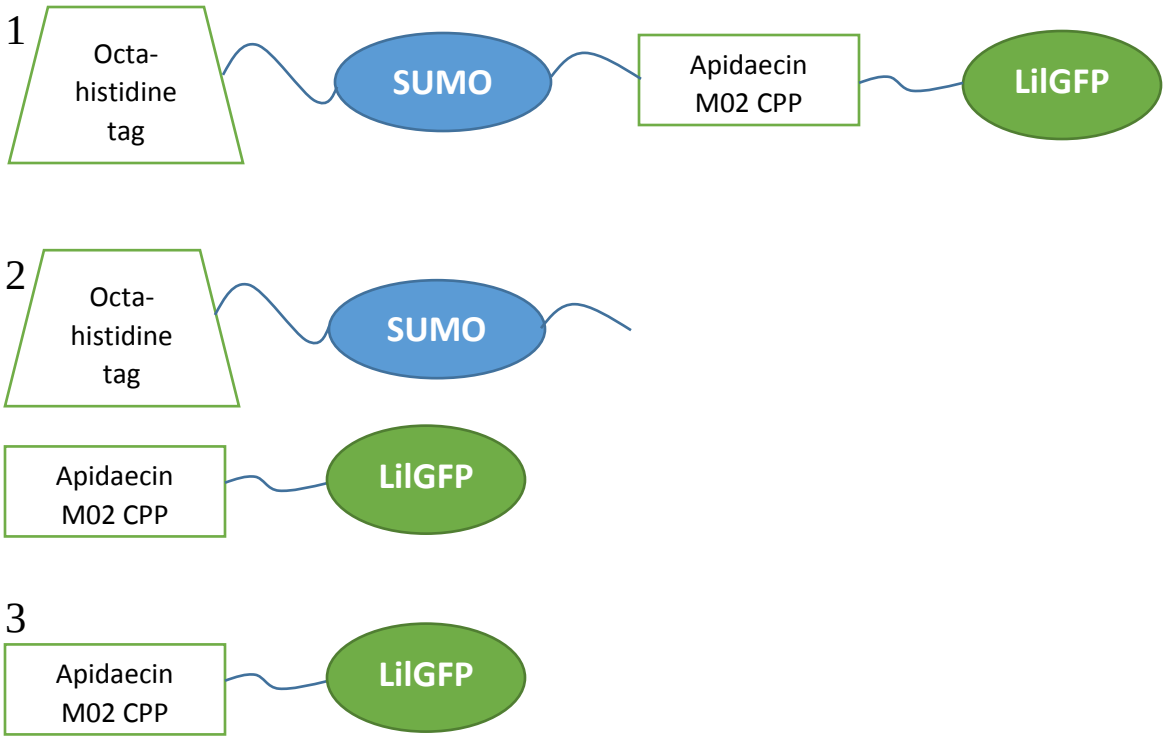
Protein Purification

Overnight cultures of BL21 A1 expression cells were grown in LB+amp shaking cultures at 37C. A 10% inoculation was done into TB+amp cultures, which were grown to an OD600 of 2.70 in a 37C shaker, at which point protein expression was induced with IPTG and 20% L-arabinose. After three hours of growth cells were harvested by snap freezing in liquid nitrogen. Proteins were purified using sonication and standard Ni-NTA affinity chromatography using the N-terminal octa-histidine. After sonication in lysis buffer containing 50mM NaPhosphate, 300mM NaCl, and 5mM imidazole, the protein was still insoluble, so the pellet was used for further purification. The pellet was then resuspended in the same lysis buffer above but with the addition of 0.1% triton-X100 to remove cell debris. This was centrifuged again and the pellet was resuspended in lysis buffer containing 6M urea and 25mM imidazole to unfold the protein and prevent non-specific binding to the Ni-NTA resin. After centrifugation, the supernatant was then used for the Ni-NTA affinity chromatography purification. Five elutions were performed using a buffer containing 250mM imidazole to displace the protein from the resin. Gel samples were collected at several steps to monitor the location of the protein throughout the purification process. After purification, proteins were concentrated by buffer exchange at 4C using a dialysis buffer containing 50mM NaPhosphate, 300mM NaCl, 5mM imidazole, 2mM TCEP, and 2mM $MgCl_2$. After dialysis, protein was further purified using aquacide II sand at 4C.

Results

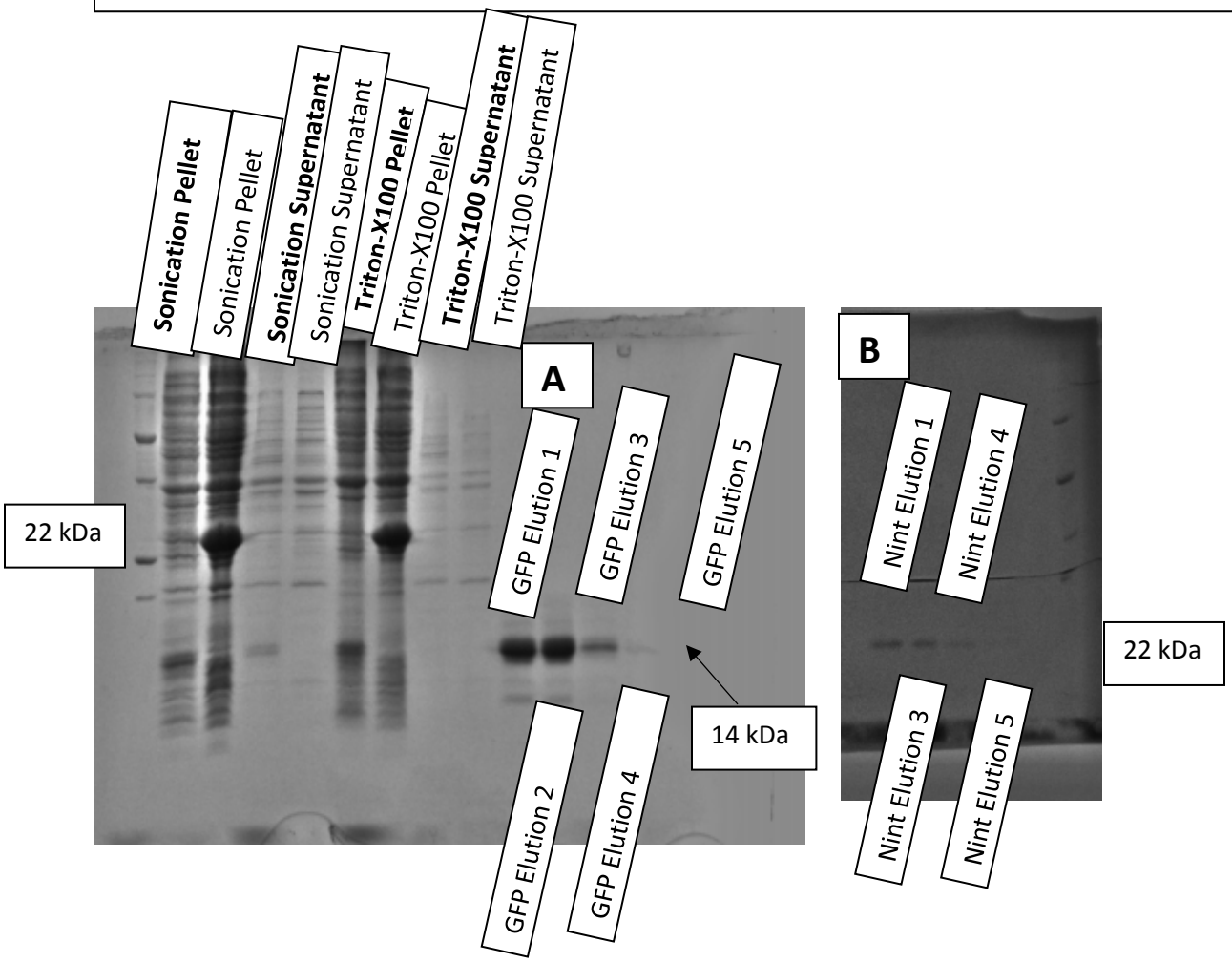
Eight different mutant PrAMP sequences were created with single amino acid changes and verified using sequencing data. Each mutant constructed was cloned into a cloning and expression cell line. The M02 mutated PrAMP, shown in Table 1, was purified using affinity chromatography. The SUMO solubility tag was cleaved so that the final protein contained only the PrAMP attached to the little fragment of GFP. Figure 1 below shows the processing sequence the protein underwent.

Figure 1: The figure below shows the process of expression, purification, and cleavage of the M02 mutated apidaecin construct. Step 1 shows the entire construct that was expressed in BL21 A1 cells. This construct was purified using the standard Ni-NTA affinity chromatography purification using the octa-histidine tag. Step 2 shows the result of cleavage of the SUMO tag using the bdSENP1 protease, which cleaved immediately before the N-terminus of the CPP. Step 3 shows the result of a reverse purification, in which the SUMO tag was bound to Ni-NTA resin by the octa-histidine tag, and the apidaecin M02 CPP + LilGFP peptide was collected in the flow-through.



Gel samples were collected from the cell pellet and supernatant after the sonication and triton-X100 treatment steps, as well as from each elution. The gels are shown below in Figure 2.

Figure 2: The gels below track the protein through the purification process. Samples were taken after sonication, triton-X100 treatment, and after each elution. Labels in bold text describe samples from Su001LilGFP M02 construct purification, while unbolded text labels indicate Su001Nint M02 construct samples. The Su001Nint M02 construct is the same construct as the Su001LilGFP M02 construct described in Figure 1 except with an N-terminal split intein replaced for the small GFP fragment.



The gels in Figure 2 show that the protein of interest in each case was in fact in the pellet in both the initial centrifugation and after the triton-X100 step, which is what was expected. It also

shows that there was a lot of protein recovered in the first couple elutions, with less and less protein being purified with each consecutive elution.

Conclusions

Several mutant apidaecin PrAMPs have now been cloned into expression cells. In the future, the rest of the mutated PrAMPs could be purified and tested for both cell penetration ability as well as antimicrobial activity. Mutations that hinder antimicrobial activity but maintain cell penetration could potentially be combined to create a PrAMP sequence that is able to penetrate the cell but then not kill the cell, making it a useful tool in delivering cargo including natural products and other peptides into the cell for further study. In this manner, it would be possible to study a single biosynthetic pathway using several different heterologous hosts because a simple method would be available to transfer all intermediates of the pathway to each host through the utilization of CPPs as carriers.

I would like to thank the UROP program and University of Minnesota for providing me with this opportunity to gain research experience in a novel field and advance my knowledge of the field of biochemistry. I would also like to thank Dr. Michael Freeman, Fredarla Miller, and the rest of the Freeman lab for their help and guidance throughout the entirety of this project.

Works Referenced

1. Zorko M, Langel Ü. 2005. “Cell-penetrating peptides: mechanism and kinetics of cargo delivery,” Advanced Drug Delivery Reviews, 57(4):529.
2. Krizsan A, Prahl C, Goldbach T, Knappe D, Hoffman R. 2015. “Short proline-rich antimicrobial peptides inhibit either the bacterial 70S ribosome or the assembly of its large 50S subunit,” Chembiochem, 16(16):2304.

3. Cabantous, S., Terwilliger, T. C. & Waldo, G. S. Protein tagging and detection with engineered self-assembling fragments of green fluorescent protein. *Nat. Biotechnol.* **23,** 102–107 (2005).

4. Milech, N. et al. 2015. “GFP-complementation assay to detect functional CPP and protein delivery into living cells,” Scientific Reports, 5:18329.