MODIFICATION, EXPRESSION, AND PURIFICATION OF HYPERACTIVE ANTIFREEZE PROTEINS FROM INSECT TENEBRIO MOLITOR

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ABSTRACT

Antifreeze proteins (AFPs), also known as thermal hysteresis proteins, can protect organisms from damage caused by freezing by lowering the freezing point of fluids. AFPs can bind to small ice crystals, inhibit ice growth, and change the morphology of ice crystals. The difference between the freezing temperature and the melting temperature is termed thermal hysteresis (TH). There are different types of AFPs, such as fish AFPs (type I, II, III, and IV) and insect AFPs. These AFPs can also be classified by their thermal hysteresis activity: moderately active AFPs and hyperactive AFPs. Previous research groups successfully constructed AFP (type I, and type III) multimers, and the thermal hysteresis measurement showed that the size of multimers can affect TH activities. However, how the TH of insect AFPs will be affected by multimerization is still unknown, since they already have much higher thermal hysteresis activities than fish AFPs in nature. Making active multimers of insect AFPs can be harder than type I and type III fish AFPs because of their highly disulfide-bonded structure.

The aim of this thesis is to construct trimers of *Tenebio molitor* (TmAFP) by adding a foldon domain. The foldon domain has been added separately to N-teminus and C-terminus of TmAFP. Cold finger purification was utilized for the protein purification

process. The purified proteins were then run on protein gels, demonstrating that trimers of TmAFP were successfully made, for both the N-terminus and the C-terminus foldon domains; the TmAFPs were active, indicating proper folding.

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CHAPTER I

INTRODUCTION

1.1 What are antifreeze proteins?

Marine fish utilize sodium chloride, calcium, potassium, urea, and free amino acids in their blood to lower the freezing point of body fluids; while fish that live in extremely cold environments (such as the South and North Pole) rely on a special protein in their body to lower the freezing point of body fluid and prevent them from dying from freezing [1]. This special class of proteins is called antifreeze proteins (AFPs), also known as "thermal hysteresis proteins".

Antifreeze proteins were first discovered in Antarctic Ocean teleost fish more than forty years ago. Art DeVries [2] and his colleagues found that teleost fish can lower the freezing point of their blood to -1.9 ℃, while the normal freezing point for fish blood is -0.7 ℃ [2]. Antifreeze proteins have the ability to inhibit ice crystal growth,

thus lowering the freezing point, allowing animals, plants, and bacteria to survive in subzero temperature environments [3, 4].

Antifreeze proteins can bind to the ice surface, inhibit ice growth, and lower the freezing point of aqueous solutions. The difference between the depressed freezing temperature and the colligative melting temperature is called thermal hysteresis (TH).

Antifreeze proteins also have many practical applications. For example, AFPs can be used to improve the quality of frozen food. AFPs can also be used in cryosurgery and cryopreservation of tissues and organs [5, 6]. They can extend the storage time of tissues and organs by minimizing ice formation.

1.2 Different types of antifreeze proteins and their structures

There are many different types of AFPs, such as AFGPs (antifreeze glycoproteins) [7], fish AFPs [3], insect AFPs [8], plant AFPs [9], as well as fungi [10] and bacteria AFPs [11]. Different types of AFPs have diverse structures and ice binding affinities. In order to study the mechanism of different AFPs, it is important to understand their molecular structures.

AFGPs were found in the blood serum of some arctic fish, such as *Boreogadus saida* (polar cod) [7]. They all have the same tri-peptide unit (Alanine-Alanine-Threonine)$_n$, and a disaccharide residue attached to the threonine [1, 12]. The molecular weight has a range from 2.6kDa to 33.7kDa [1]. NMR studies [13] showed that AFGPs exist as left handed three-fold helices in solution, the disaccharide side chains provide a hydrophilic surface, which wrapped closely against the left handed three-fold helical

backbone [14, 15]. Despite these AGFPs structural studies, no three-dimensional AGFPs structure has been determined.

Fish AFPs have four types, type I, type II, type III, and type IV (Figure 1.1). Type I AFPs were found in winter flounders, longhorn sculpins, and shorthorn sculpins. They were the first AFPs which had the three dimensional structure determined [16]. They have a single, long α-helix as secondary structure, with a large portion of alanine residues [16]. Type II AFPs were found in sea raven, smelt, and Atlantic herring. Unlike type I AFPs' alanine-rich α-helix structure, type II AFPs are cysteine-rich globular protein, with limited α-helix content [17]. Type III AFPs were found in Antarctic eelpout. They are also globular proteins, neither alanine-rich nor cysteine-rich. X-ray and NMR studies [18] showed that type III AFPs have short β-strands and one α-helix [18]. Type IV AFPs were found in longhorn sculpins. They are glutamate and glutamine rich proteins, with α-helix structure [19].

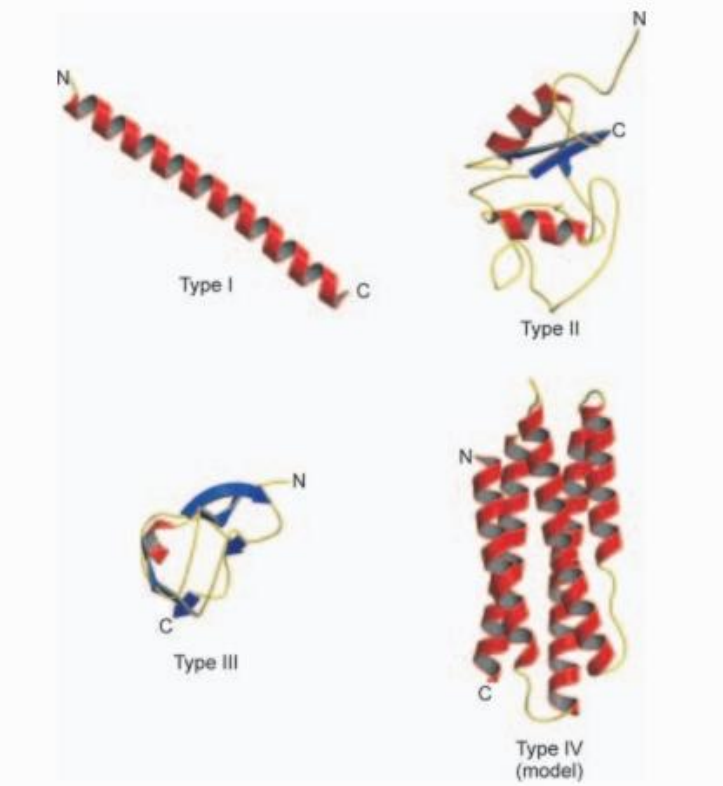

Figure 1.1: The structure of fish AFPs [20]. α-helices are shown in red, β-strands are shown in blue, and the coil structures are shown in yellow. Type I AFP is a single, long α-helix. Type II AFP is globular, with limited α-helices and β-strands. Type III AFP is also globular, with one α-helix and several β-strands. Type IV AFP is a model, based on the sequence similarity to apolipophorin [20]. Figures and captions were modified from [20] with permission of *European Journal of Biochemistry*.

There are two major types of insect AFPs, one is from spruce budworm (*Choristoneura fumiferana*), and the other one is from yellow mealworm (*Tenebrio molitor*) [20]. Insect antifreeze proteins have much higher thermal hysteresis activities than fish antifreeze proteins. Spuce budworm AFP (sbwAFP) has a left-handed β-helix structure (Figure 1.2), with 15-residue loops, form a triangular prism like shape, with

rectangular sides [21]. Its molecular weight is 9 kDa. The three sides of the triangular prism contain parallel β-sheets, and each β-sheet contains four β-strands [21]. sbwAFP has a repeat of TxT (x can be any inward pointing amino acid) motifs on one side of the protein, which constitutes the ice-binding site of sbwAFP [21].

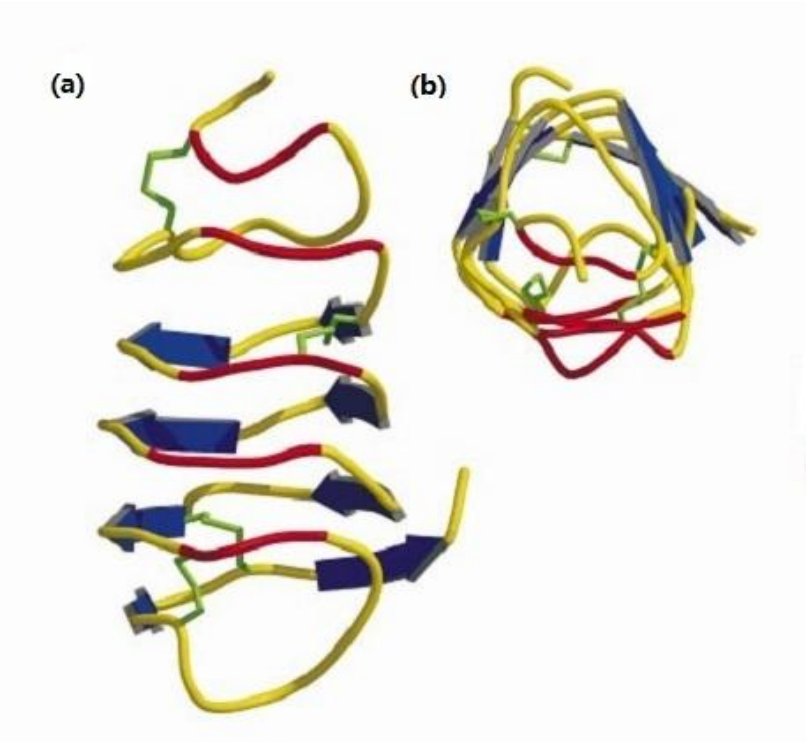

Figure 1.2: The structure of sbwAFP [21]. (a) Side view of sbwAFP structure. β-sheets are shown in blue, disulphide bonds are shown in green, and threonine-rich face coils are shown in red. (b) sbwAFP structure view down the β-helix axis. Figures and captions were modified from [21] with permission of *Nature*.

Tenebrio molitor AFP (TmAFP) has a more regular structure than sbwAFP (Figure 1.3). It has a right-handed β-helix structure, with seven 12-residue loops [22], which form a rectangular cuboid shape. Its molecular weight is 8.4kDa. The 12-residue repeats have the sequence of TCTxSxxCxxAx (x can be any inward pointing amino acid),

with high threonine and cysteine content [23]. These repeat units are highly internally disulfide-bonded, which narrows the space for the hydrophobic core and making TmAFP the narrowest β-helix [24]. Also, the spacing of the hydroxyl group of TmAFP is very similar to the spacing of the atoms in certain planes of an ice crystal.

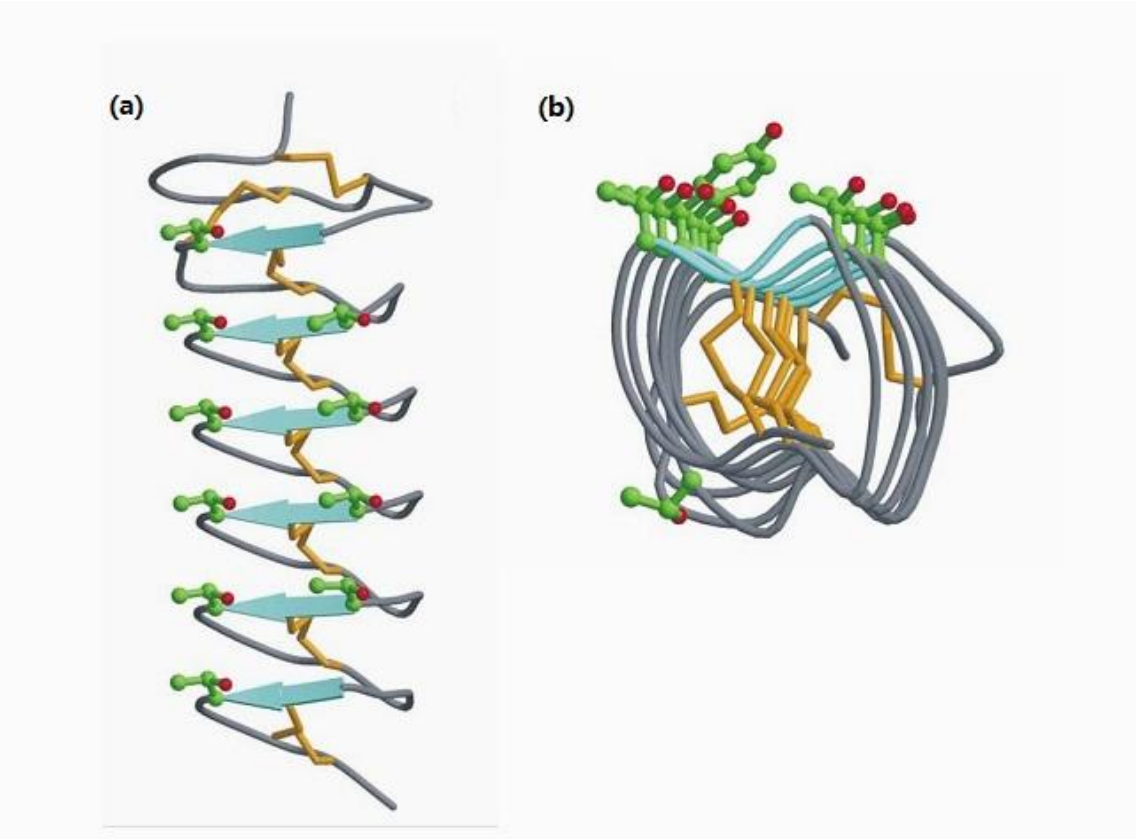

Figure 1.3: The structure of TmAFP [25]. (a) Side view of TmAFP structure. β-sheets are shown in light blue, disulphide bonds are shown in yellow, and coils are shown in grey. Threonine side chains are shown in green; with the red dots indicate the oxygen atoms. (b) TmAFP structure view down the β-helix axis. Figures and captions were modified from [25] with permission of *FEBS Letters.*

Research on plant AFPs started much later than the AFPs mentioned above. Though researchers performed experiments on more than thirty kinds of plants, few AFPs have been purified [26]. So far, the structure of plant AFPs have not been determined.

1.3 AFP and ice interactions

Different antifreeze mechanisms have been proposed to explain the interactions between antifreeze proteins and ice. Among these hypotheses, the adsorption-inhibition mechanism proposed by Raymond and DeVries [27] is still the standard model for understanding the fundamental mechanism.

The core concept of the adsorption-inhibition mechanism (Figure 1.4) is that instead of blocking the contact of water molecular and ice surface, antifreeze proteins can bind to the surface of ice, increase the curvature of the ice surface, create a "rough" surface, which is not favorable for ice crystal growth, and prevent the ice crystal growth [27]. The binding is irreversible, so the concentration dependence of thermal hysteresis activity cannot be explained by this theory.

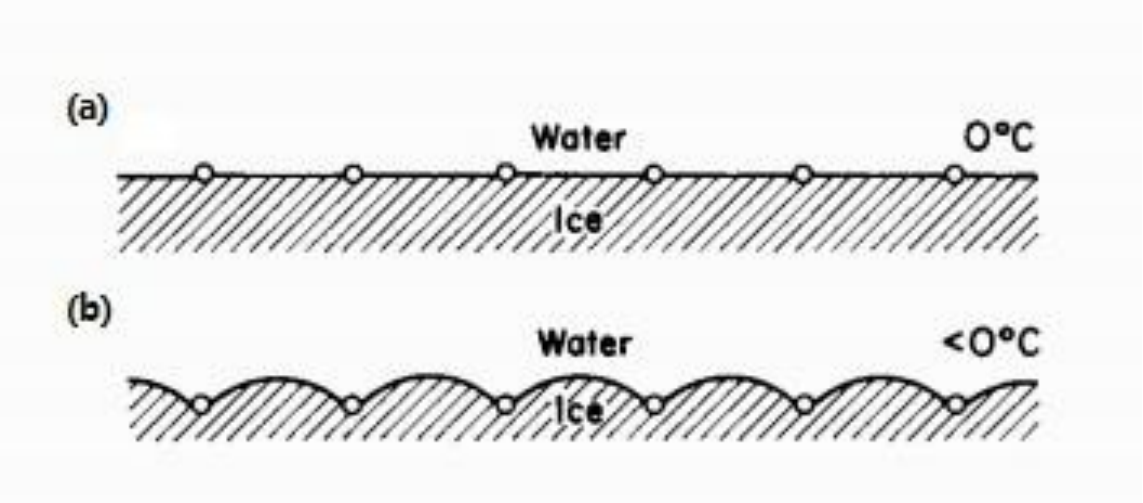

Figure 1.4: Adsorption-inhibition mechanism of AFP-ice interaction [28]. Circles indicate the AFPs and the solid lines indicate the ice crystal surface. (a) AFPs bind to the ice crystal surface at 0 ℃. (b) Under subzero environment, AFPs binding creates a "rough" surface, which is unfavorable of ice growth. Figures and captions were modified from [28] with permission of *Biophysical Journal*.

In 1983, DeVries [29] proposed a "crystal lattice matching" model. In this hypothesis, Type I AFP from *Pleuronectes americanus* has a regular distributed amphipathy α-helix, the threonine and the asparagines residues of the outer layer can bind with ice crystal prismatic plane, and prevent ice crystal growth. However, this hypothesis could not explain why the AFPs prefer to bind to ice rather than surrounding water. Also, the small number of hydrogen bonds does not fit with irreversible binding. [34]

Another model called "dipole-dipole" was proposed by Yang, Sax, Charkrabartty, and Hew [30] in 1988. They believe AFPs have dipole which is parallel to the hydrophobic groups and hydrophilic groups on the helix. This dipole can interact with the

dipole of water molecules distributed around an ice crystal. The hydrophilic side chains then provide the hydrogen bonds for ice binding interaction, while the hydrophobic side chains will inhibit ice crystal growth. This hypothesis could explain the adsorption of some α-helix AFPs, but could not be applied to non α-helix AFPs.

Knight, Driggers, and DeVries [31] proposed "crystal lattice occupy" model in 1993. This model is evolved from the "crystal lattice matching" model [29]. Hydroxyl group of AFP can occupy the position of oxygen atoms on the surface of ice crystal, then interact with neighbor oxygen atoms, and form more and more hydrogen bonds, thus AFP can irreversibly bind to ice crystal, and inhibit ice crystal growth. However, amino acid substitution experiments have weakened this hypothesis [32], and the ice binding site of AFPs has now been clearly defined as hydrophobic face [33].

In 2002, Jia and Davies [34] proposed a new model, called "surface complementation". This model is also called "receptor-ligand" model, while AFP is the receptor, and ice crystal is the ligand. Several interaction forces can affect the surface complementarity between AFP and ice crystal, such as hydrophobic interaction, van der Waals, and hydrogen bonds. These interactions require intimate surface-surface complementarity between the receptor and the ligand. The larger the interaction surface area is, the stronger the interaction force will be. Once the AFPs are in contact with the ice, the contacting surface area will extend. In order to separate AFP from ice crystal, these interact forces need to be ruptured simultaneously at the same time to release the ligand. This makes the binding of AFP to ice crystal essentially irreversible.

The recent model proposed by Kristiansen and Zachariassen [35] can explain both irreversible adsorption and concentration dependance of thermal hysteresis activity. At

different temperatures, the interactions between AFPs and ice are different. At the melting point, there is reversible equilibrium exchange of antifreeze protein molecules between solution and the water-ice interface, resulting in concentration dependant of thermal hysteresis; while at temperatures lower than melting point, antifreeze proteins interact and bind to newly formed ice crystal surface; the adsorption becomes irreversible, as described in the adsorption-inhibition model [35].

1.4 Why insect AFPs are more thermal hysteresis active than fish AFPs?

Because of the thermal buffer effect of water, marine fish usually live under environment around -2 ℃ [34]. Unlike those marine fish, insects (such as *Tenebrio molitor*, Spruce budworm) can live under much lower temperature during winter. Studies showed that 20 μM insect AFP (from spruce budworm) had a thermal hysteresis activity of 1.08 ℃, while 400 μM fish AFP (type I) only had a thermal hysteresis activity of 0.27 ℃ [21]. This confirmed that insect AFPs had much higher thermal hysteresis activities than fish AFPs.

The possible explanation of the difference between insect AFPs and fish AFPs is based on the morphology of ice crystals they produced [34, 36]. As shown in Figure 1.5 [36], ice around 0 ℃ has hexagonal ice crystal; the two hexagonal plates are the basal planes, the six sides are prism planes, and the direction perpendicular to the basal planes is the c-axis [36]. In nature, the ice crystals will bind to prism planes rather than basal planes when growing in water. The presence of AFPs changes the growth habits and morphology of this ice crystal structure [36]. Fish AFPs shape the ice crystal into a hexagonal bipyramids, and ice will grow parallel to the c-axis; the insect AFPs can bind

to both prism and basal planes, shaped the ice into columnar spicules, and ice will grow horizontally to the c-axis[36]. In conclusion, insect AFPs have the ability to prevent ice growth out of the basal plane, and this is the basis of their hyperactivity.

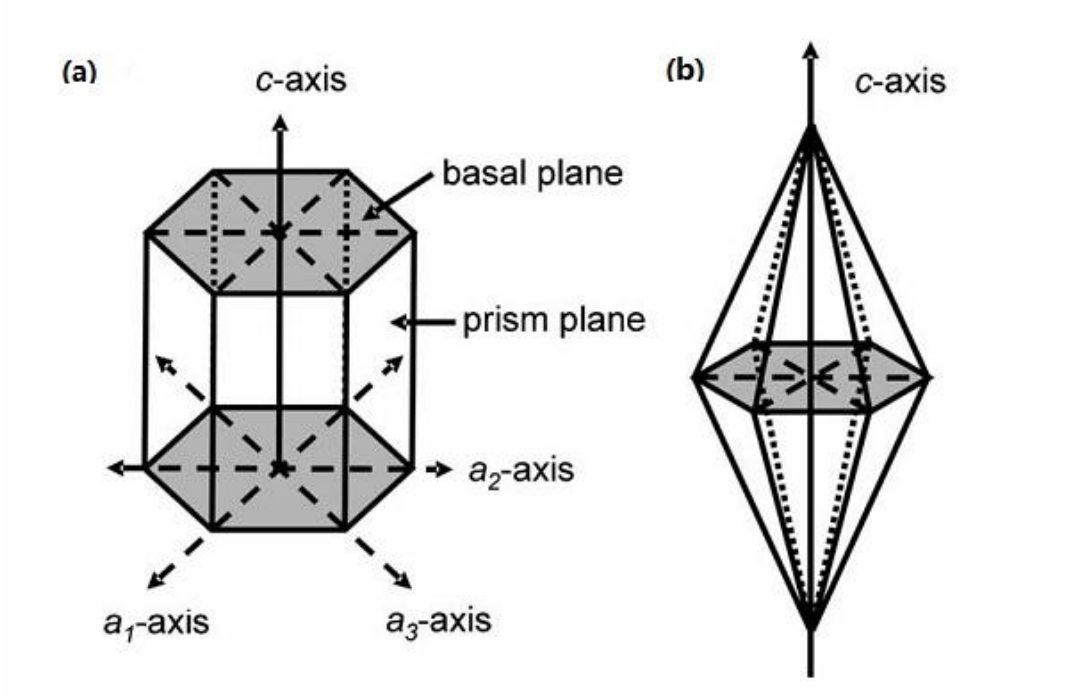

Figure 1.5: Ice Structure. (a) hexagonal ice structure with main faces and axes. (b) hexagonal bipyramids ice crystal shaped by fish AFPs [36]. Figures and captions were modified from [36] with permission of *Cryobiology*.

1.5 Previous studies on AFPs

Although antifreeze proteins exist in many creatures, fish, insects, plants, etc., only fish antifreeze proteins have been studied in detail. Insect antifreeze proteins have higher thermal hysteresis activities than other kinds of AFPs, so research on insect AFPs have important value and enormous potential.

Several research groups had success on the sequence analysis and cloning of TmAFP [24, 37, 38]. This research confirmed that TmAFP structure contains a large number of β-strands [24, 37, 38]. The hydroxyl group on the side chains of serine and threonine are the main structures that are responsible for hydrogen bond ice binding [24, 37, 38].

TmAFP has the TxT (T stands for threonine, and x can be any inward pointing amino acid) face, which constitutes the ice binding site. Mutations with a long side chain may inhibit the TxT face ice binding interaction. Researchers mutated the threonine residues in the TxT sequence to tyrosine, leucine, and lysine [39]. Experiment data showed that the mutation of threonine to tyrosine caused the thermal hysteresis activity lost by 90%; the mutation of threonine to leucine caused about the same loss in thermal hysteresis activity as to tyrosine; while the mutation of threonine to lysine caused only 25% loss in thermal hysteresis activity [39]. Further NMR showed that after mutation, TmAFP were still β-helical and well-folded, verifying that the mutation only changed the surface properties of the protein, not the structure of protein [39]. These data led the researchers to believe that the amount of thermal hysteresis loss by mutation may be affected by the size of the substituted residue [39].

A research group from Japan [40] constructed intramolecular dimer, trimer, and tetramer of the type III fish antifreeze proteins. The results showed that by making multimers, the thermal hysteresis activity can be increased (Figure 1.6). The increase is in greater with more domains, and is also concentration dependant.

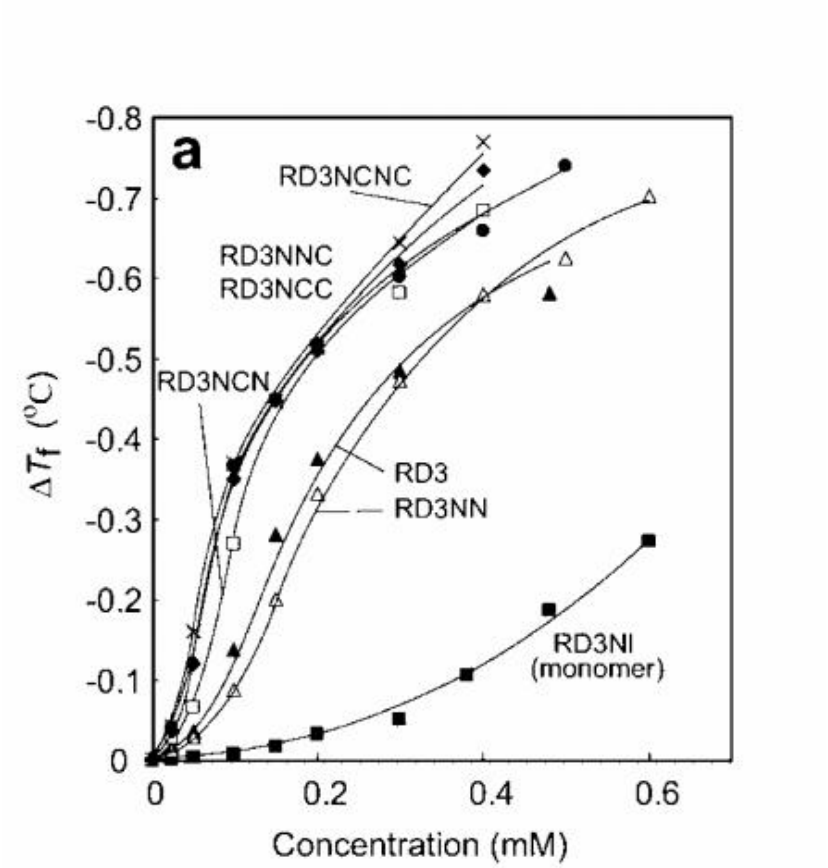

Figure 1.6: Thermal hysteresis measurement results for monomer and multimers of type III fish AFPs [40]. RD3NI is the monomer, RD3 and RD3NN are dimers, RD3NCN, RD3NNC, RD3NCC are trimers, and RD3NCNC is tetramer. Figures and captions were modified from [40] with permission of *Journal of Biological Chemistry*.

Another approach for creating multimers has been done by Özge Can [41]. In his research, trimers of type I and type III fish antifreeze proteins were constructed by adding a foldon domain on C-terminus. The study showed that this structure modification can significantly change the thermal hysteresis activity for those two types of antifreeze proteins.

As shown in Figure 1.7 (a), the presence of foldon domain lowered the thermal hysteresis activity of type I AFP (T1D). The reason may be the presence of foldon causes these hydrophobic residues to be closer, and prefer to interact with each other, rather than interact with surrounding solvent which would allow it to bind to the ice surface [41].

Figure 1.7 (b) shows the difference of thermal hysteresis activity between monomer type III AFP (R3D) and trimer type III AFP. It is obviously that by adding foldon domain, the TH activity increases significantly, especially at low concentration. The explanation for this different concentration dependence may be the different binding patterns of single and multi-domain. Single domain AFPs can reversibly adsorb to the ice crystal surface with adsorption and desorption rate k_a and k_d respectively (Figure 1.8) [42]; while multi-domain AFPs have multi-step binding process. Take a two-domain AFP as an example (Figure 1.8), the adsorption is a two-step process: adsorption (k_{a1}) and desorption (k_d) of the first domain; adsorption (k_{a2}) and desorption (k_d) of the second domain [42]. After adsorption of the second domain, these two domains must both desorb at the same time for the protein to detach from the ice crystal, while a single domain can detach when the one domain desorbs. This two-step binding process increases the surface coverage for a given concentration, thus increase the thermal hysteresis. Figure 1.9 shows the difference of surface coverage (θ) between one and two-domain type III AFPs at different concentration [42]. At low concentration, the surface coverage of two-domain type III AFP is much higher than one domain; when the concentration is increased, the difference become less significant [42].

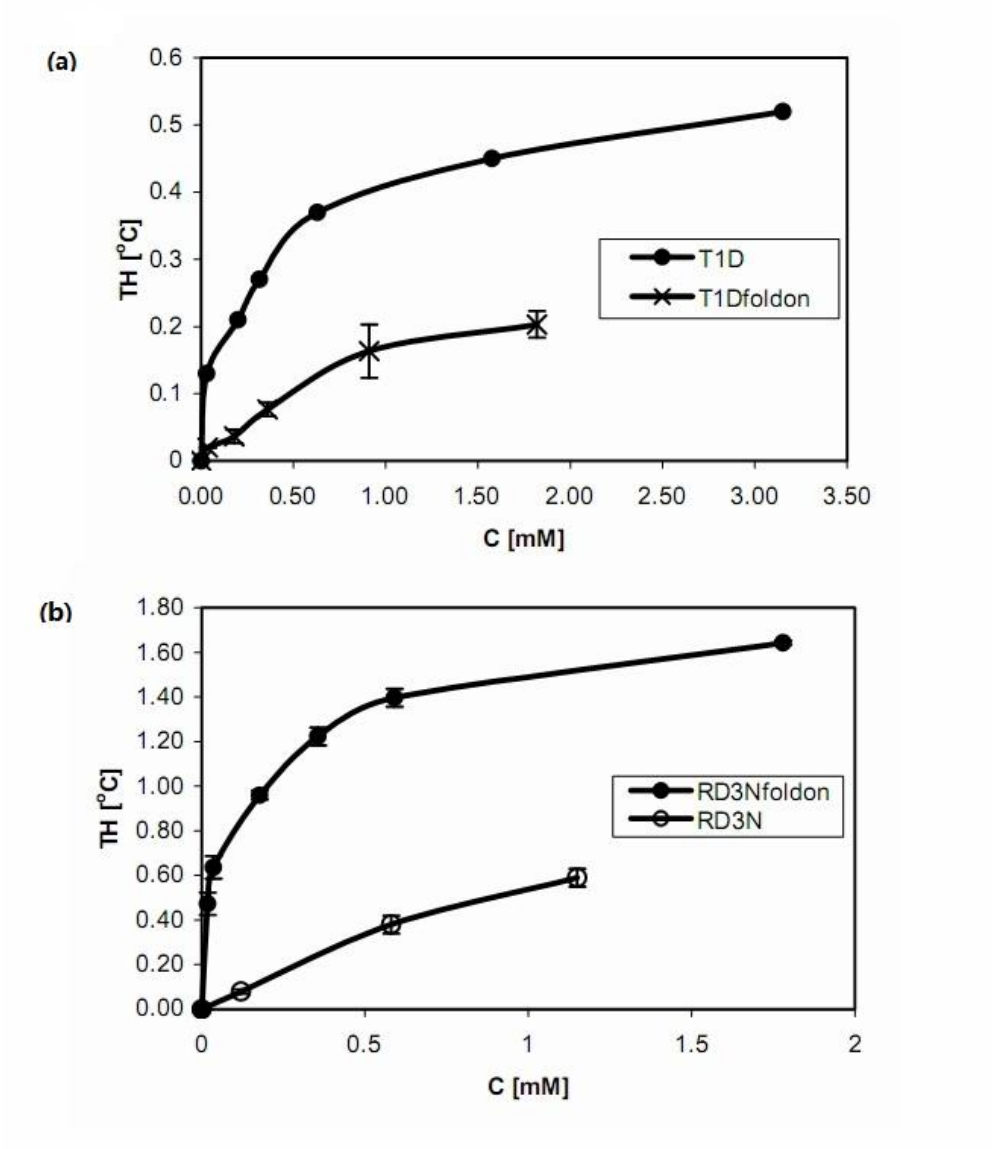

Figure 1.7: Thermal hysteresis measurement results for trimers of type I and III fish AFPs [41]. (a) comparison of thermal hysteresis activity for monomer type I fish AFPs and trimer type I AFPs, (b) comparison of thermal hysteresis activity for monomer type III fish AFPs and trimer type III AFPs. Figures and captions were modified from [41] with permission of *Özge Can.*

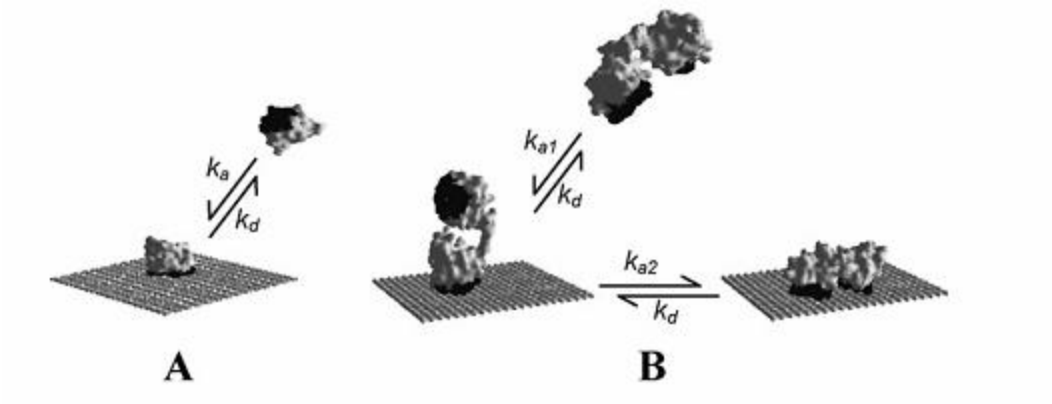

Figure 1.8: Scheme for one and two-domain protein adsorption to an ice lattice [42]. Figures and captions were modified from [42] with permission of *Journal of Colloid and Interface Science*.

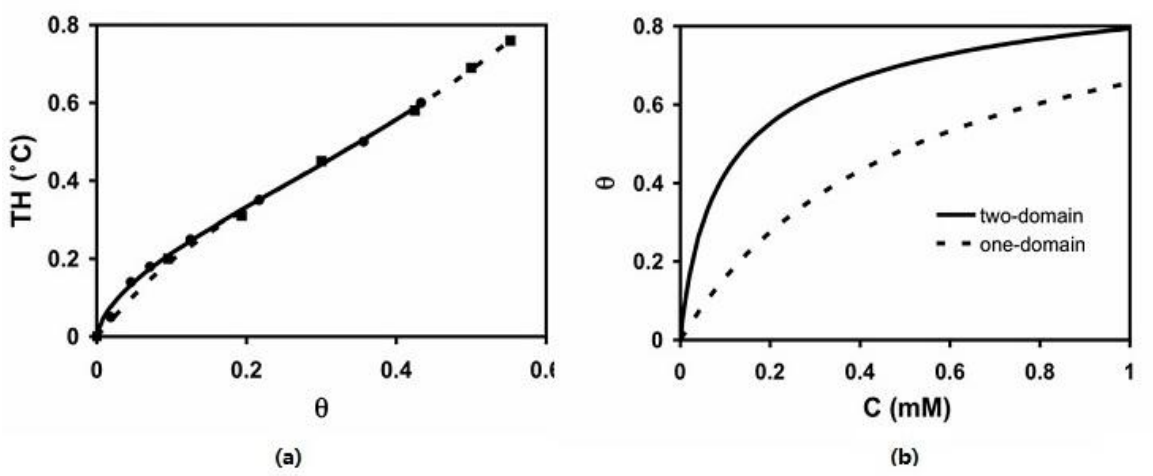

Figure 1.9: Relationship among thermal hysteresis, surface coverage and concentration. (a) Relationship between thermal hysteresis and surface coverage (θ) for type III AFP; (b) relationship between surface coverage (θ) and concentration for one and two-domain type III AFP [42]. Figures and captions were modified from [42] with permission of *Journal of Colloid and Interface Science.*

1.6 Scope of this thesis

As mentioned above, antifreeze proteins have many practical applications, and potentials to be used in numerous areas. Creating a superior antifreeze protein may have the potential to increase the thermal hysteresis activity of AFPs.

Previous studies [40, 41] showed that multimer constructs of type III AFP can increase the thermal hysteresis activities, especially at low concentrations. While the presence of a foldon domain causes a decrease in TH activity for type I AFP. As introduced previously, insect antifreeze proteins have much higher thermal hysteresis activities in nature than fish antifreeze proteins. On the other hand, constructs of multimers of TmAFP have a much more complicated structure compared to fish AFPs, since they are highly disulfide-bonded, so it is a challenge to make TmAFP multimers, in order to investigate how the size of multimers can affect the thermal hysteresis activity.

. In this thesis, insect antifreeze protein from *Tenebrio molitor* (TmAFP) has been used. Foldon domains are added to N-terminus and C-terminus of TmAFP separately. Cold finger purification is utilized to purify the foldon constructs of TmAFP and demonstrate they have thermal hysteresis activity. The purified proteins were then run on protein gels to prove they form trimers. Thermal hysteresis measurements are not included in this thesis, but could be an aim for further study.

CHAPTER II

EXPERIMENTAL

This section describes the experimental steps, including synthesis of the genes, as well as the expression and purification of the proteins.

Plasmids containing genes for the TmAFP proteins were provided by Dr. Deborah Fass's research group [23] from Department of Structural Biology of Weizmann Institute of Science in Israel. This research group [23] constructed the fusion proteins, named as MBP-His6-TEV-TmAFP and His6-eGFP-TmAFP. The genes were used to add a foldon domain to both N terminus and C terminus of TmAFP separately, with a linker.

2.1 Synthesis of the genes

Origami was used as the cloning and expression host strain, and pET25b was used as the expression plasmid (Novagen).

The DNA sequence for TmAFP is 252 bp:

TmAFP (shown as a single piece of DNA from 5' to 3'):

CAGTGCACCGGTGGTGCTGACTGCACCAGCTGCACCGCTGCTTGCACCGGTTGCGGTAA

CTGCCCGAACGCTGTTACTTGCACTAACAGCCAGCACTGCGTTAAAGCTACCACCTGCA

CCGGTAGCACCGACTGCAACACCGCTGTTACCTGCACTAACTCTAAAGACTGCTTCGAA

GCTCAGACCTGCACCGACTCTACCAACTGCTACAAAGCTACCGCTTGCACCAACTCTAC

CGGCTGCCCGGGTCAC

This corresponds to the TmAFP amino acid sequence:

QCTGGADCTSCTAACTGCGNCPNAVTCTNSQHCVKATTCTGSTDCNTAVTCTNSKDCFE

AQTCTDSTNCYKATACTNSTGCPGH

The restriction enzyme recognition map of TmAFP (Figure 2.1) was checked to make sure there will not be any repeated cut site in the primers designed for further experiment, which include NdeI, SfiI, and EcoRI restriction enzymes. All of the custom primers were purchased from Invitrogen.

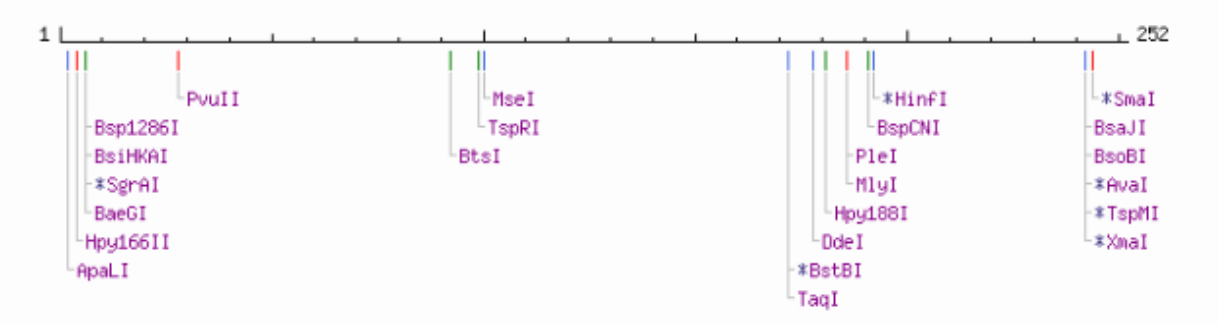

Figure 2.1: The restriction enzyme recognition site map of TmAFP. This map was generated by using NBEcutter v2.0, provided on New England Biolabs website [43].

2.1.1 Foldon-linker-TmAFP

In order to add the foldon and linker sequences on the TmAFP, several cut sites need to be introduced to the system. A set of primers (named as TmAFP-Sfi-Forward, and TmAFP-Reverse) were designed to amplify the TmAFP part out from the His6-eGFP-TmAFP, as well as add restriction enzyme recognition sites in during the PCR process. The sequences for the primers were shown below; the yellow highlighted parts indicate the restriction enzyme recognition sites, while the grey highlighted part matches the TmAFP.

TmAFP-Sfi-Forward (shown as a single piece of DNA from 5' to 3'):

NdeI *SfiI*

ATTTTTCATATGAGCAAAGGGCCGGGCTGGCCACAGTGCACCGGTGGTGCTGACTGCAC

C

TmAFP-Reverse (shown as a single piece of DNA from 5' to 3'):

EcoRI

TAAAGAATTCTTAGTGACCCGGGCAGCCGGTAGAGTTGGT

The PCR amplifications were carried out in 50 µl solution, which included 5 µl forward primer, 5 µl reverse primer, 2 µl template DNA, 1x PCR master mix (Promega) and nuclease free water (Promega). A detailed PCR protocol is given in Appendix (A.1).

The amplified DNA contains the sequence below, with NdeI and SfiI recognition sites at the N-teminus and EcoRI recognition site at the C-teminus. The yellow highlighted parts indicate the recognition sites, and the grey highlighted part is the TmAFP.

PCR amplification product (shown as a single piece of DNA from 5' to 3'):

NdeI *SfiI*

ATTTTTCATATGAGCAAAGGGCCGGGCTGGCCACAGTGCACCGGTGGTGCTGACTGCAC

CAGCTGCACCGCTGCTTGCACCGGTTGCGGTAACTGCCCGAACGCTGTTACTTGCACTA

ACAGCCAGCACTGCGTTAAAGCTACCACCTGCACCGGTAGCACCGACTGCAACACCGCT

GTTACCTGCACTAACTCTAAAGACTGCTTCGAAGCTCAGACCTGCACCGACTCTACCAA

CTGCTACAAAGCTACCGCTTGCACCAACTCTACCGGCTGCCCGGGTCACTAAGAATTCT

TTA *EcoRI*

The amplified DNA was then run on 2% agarose gel with 1x TAE buffer at 80 V, for about 30 minutes. A bright band around 300 bp was observed under UV light, and purified from the gel using a gel extraction kit from Qiagen. The purified DNA sample was used as a ligation insert later. Detailed agarose gel purification protocol was given in Appendix (A.2).

The insert DNA and the expression vector pET25b were double digested by using restriction enzymes NdeI and EcoRI. The double digestions were carried in a 50 µl solution, with 2 µl NdeI, 2 µl EcoRI, 10 µl DNA, 5 µl buffer EcoRI, and 31 µl nuclease free water. The reaction was carried out in a 37 ℃ water bath for 3 hours. The double digested DNA were then run on 2% agarose gel with 1x TAE buffer at 80 V again, and purified by using the same gel extraction kit as mentioned above. The purified samples were ready for the ligation and transformation.

Ligations were done in a 21 µl solution, with 1 µl expression vector, 5 µl insert DNA, 10 µl quick ligase buffer, 1 µl quick ligase enzyme, as well as nuclease free water. The mixed solution was then leaved at room temperature for 5 minutes. A 10 µl aliquot

of the ligation product was then transformed into origami competent cells on agar plates. The agar plates were made of LB (Lysogeny broth) media, agar, and ampicilin.

After overnight transformation, colonies were selected for an overnight culture with 5 ml LB media. The plasmid DNA was then purified from the overnight cultures by using a miniprep kit from Qiagen. PCR was used with T7 forward and reverse primers for sample screening. After PCR, samples were run on 2% agarose gel with 1x TAE buffer at 80 V one more time, for pre-screen, and then sent for DNA sequencing. The DNA sequencing was carried out with T7 universal primer at Cleveland Clinic Foundation. At this point, the TmAFP has been ligated into a pET25b vector, and was used as vector for later experiment.

The foldon sequence was added after a linker which was used to give space between the domains. The linker DNA was designed by Özge Can [41]. It was formed from two complimentary oligonucleotides annealed to give SfiI compatible overhang on both N-teminus and C-teminus. To ligate the linker with the TmAFP, a single digestion was done on the TmAFP-pET25b vector. A single digestion was done in a 50 µl solution, with 2 µl SfiI, 10 µl DNA, 5 µl buffer 4, 1x BSA, and nuclease free water. The mixed solution was held in a 50 ℃ dry bath for 2.5 hours. After the single digestion, the samples were run on 2% agarose gel with 1x TAE buffer at 80 V, followed by the gel purification.

Unlike with double digestion, a dephosphorylation step is required for single digestion. This step can remove the 5’ phosphate group from DNA and prevent the self ligation of the single digested DNA. Dephosphorylation was carried out by adding 1/10 volume antarctic phosphatase reaction buffer and 1 µl antarctic phosphatase enzyme

(New England Biolabs). The mixed sample was then kept at 37 ℃ in a water bath for 1 hour and then 65 ℃ dry bath for 5 minutes.

The linker insert was produced by annealing 4 µl of each complimentary oligonucleotide in 21 µl quick ligase buffer and 21 µl nuclease free water, for a total volume of 50 µl. Detailed annealing protocol was given in Appendix (A.3).

Linker (the yellow highlighted part indicate the SfiI compatible overhang):

TGGCCGGACGGTACCACCTCCAAAGGC

CCGACCGGCCTGCCATGGTGGAGGTTT

The vector (TmAFP-pET25b) and the insert linker were then ligated, and the ligated samples were transformed into origami competent cells by using the same method as mention previously.

After transformation, colonies were picked for overnight culture with 5 ml LB media, and plasmid DNA were purified from the overnight culture by miniprep. The samples were screened by PCR and agarose gel, and sent for DNA sequencing.

At this point, the linker sequence is incorporated at the N-terminus of TmAFP in pET25b vector. The linker-TmAFP has a sequence as following, the yellow highlighted part indicate the recognition sites, the grey highlighted part is the TmAFP, and the blue highlighted part is the linker.

linker-TmAFP (shown as a single piece of DNA from 5' to 3'):

NdeI *SfiI*

CATATGAGCAAAGGGCCGGGCTGGCCGGACGGTACCACCTCCAAAGGCTGGCCACAGTG

CACCGGTGGTGCTGACTGCACCAGCTGCACCGCTGCTTGCACCGGTTGCGGTAACTGCC

CGAACGCTGTTACTTGCACTAACAGCCAGCACTGCGTTAAAGCTACCACCTGCACCGGT

AGCACCGACTGCAACACCGCTGTTACCTGCACTAACTCTAAAGACTGCTTCGAAGCTCA

GACCTGCACCGACTCTACCAACTGCTACAAAGCTACCGCTTGCACCAACTCTACCGGCT

GCCCGGGTCACTAAGAATTCTT

EcoRI

The next step is to introduce the foldon sequence. As shown above, the linker-TmAFP sequence has NdeI recognition site and SfiI recognition site at the 5', while the foldon domain has NdeI recognition site and BglI recognition site. SfiI recognizes the interrupted palindromic sequence 5'-GGCCNNNN^NGGCC-3' (N can be any base, ^ indicates the cleave), BglI recognizes the interrupted palindromic sequence 5'-GCCNNNN^NGGC-3' (N can be any base, ^ indicates the cleave); after digestion, BglI cut DNA can have a compatible cohesive end with an SfiI cut DNA [44].

The linker-TmAFP was double digested with NdeI first at 37 ℃ for 2 hours, then SfiI was added to the solution, and digest at 50 ℃ for another 2 hours. The foldon was double digest with NdeI and BglI at 37 ℃ for 3 hours. The double digested samples were then ligated and transformed into origami competent cells.

After transformation, colonies were picked for overnight culture with 5 ml LB media, and plasmid DNA were purified from the overnight culture by miniprep. The samples were screened by PCR and agarose gel, and sent for DNA sequencing.

At this point, gene for the foldon-linker-TmAFP sequence has been produced. The yellow highlighted part is the start codon, the green highlighted part is the foldon, the blue highlighted part is the linker, and the grey highlighted part is the TmAFP.

foldon-linker-TmAFP (shown as a single piece of DNA from 5' to 3'):

ATGGGCCACGGCGTGGGTTACATCCCGGAAGCTCCGCGTGACGGTCAGGCTTACGTTCG

TAAAGACGGTGAATGGGTTCTGCTGTCTACCTTCCTGGGGCCGG

GCTGGCCGGACGGTACCACCTCCAAAGGCTGGCCACAGTGCACCGGTGGTGCTGACTGC

ACCAGCTGCACCGCTGCTTGCACCGGTTGCGGTAACTGCCCGAACGCTGTTACTTGCAC

TAACAGCCAGCACTGCGTTAAAGCTACCACCTGCACCGGTAGCACCGACTGCAACACCG

CTGTTACCTGCACTAACTCTAAAGACTGCTTCGAAGCTCAGACCTGCACCGACTCTACC

AACTGCTACAAAGCTACCGCTTGCACCAACTCTACCGGCTGCCCGGGTCAC

The foldon-linker-TmAFP amino acid sequence is as below. The green highlighted part is the foldon, the blue highlighted part is the linker, and the grey highlighted part is the TmAFP.

MGHGVGYIPEAPRDGQAYVRKDGEWVLLSTFLGPGWPDGTTSKGWPQCTGGADCTSCTA

ACTGCGNCPNAVTCTNSQHCVKATTCTGSTDCNTAVTCTNSKDCFEAQTCTDSTNCYKA

TACTNSTGCPGH

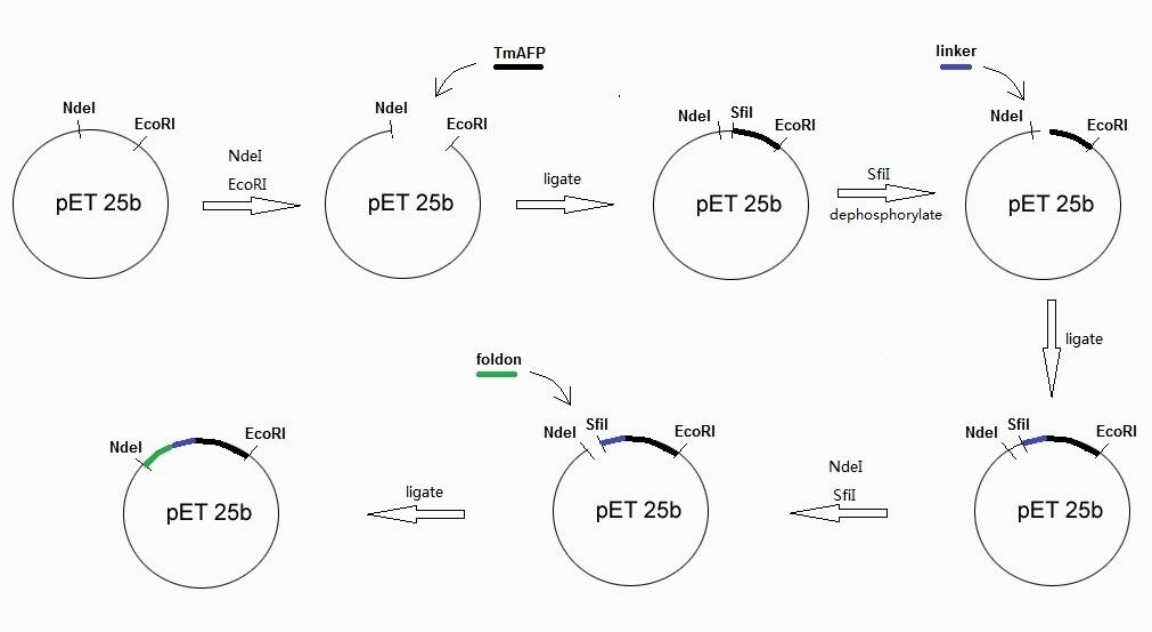

Figure 2.2: Schematic diagram of the synthesis of the gene for foldon-linker-TmAFP. Foldon, linker, TmAFP are shown in green, blue and black, respectively.

2.1.2 TmAFP-linker-foldon

The process for making TmAFP-linker-foldon is much easier than making foldon-linker-TmAFP. The liker-foldon system had been designed and produced previously by Özge Can [41].

As similar to making foldon-linker-TmAFP, several recognition sites need to be introduced first. Another set of primers have been designed for the PCR amplification, named as TmAFP-forward, and TmAFP-Sfi-reverse. The sequences for the primers are shown below; the yellow highlighted parts indicate the recognition site, while the grey highlighted parts match the TmAFP.

TmAFP-forward (shown as a single piece of DNA from 5' to 3'):

NdeI

ATTTTTTCATATGCAGTGCACCGGTGGTGCTGACTGCACC

TmAFP-Sfi-reverse (shown as a single piece of DNA from 5' to 3'):

SfiI

TAAAAAAGGCCAGCCCGGCCCGTGACCCGGGCAGCCGGTAGAGTTGGT

The PCR amplification was carried out in 50 μl solution, which included 5 μl forward primer, 5 μl reverse primer, 2 μl template DNA, 1x PCR master mix (Promega), and nuclease free water (Promega).

The amplified DNA contains the sequence as follows, with NdeI recognition site at the N-teminus and SfiI recognition site at the C-teminus. The yellow highlighted parts indicate the recognition sites, and the grey highlighted part is the TmAFP.

PCR amplification product (shown as a single piece of DNA from 5' to 3'):

NdeI

ATTTTTCATATGCAGTGCACCGGTGGTGCTGACTGCACCAGCTGCACCGCTGCTTGCAC
CGGTTGCGGTAACTGCCCGAACGCTGTTACTTGCACTAACAGCCAGCACTGCGTTAAAG
CTACCACCTGCACCGGTAGCACCGACTGCAACACCGCTGTTACCTGCACTAACTCTAAA
GACTGCTTCGAAGCTCAGACCTGCACCGACTCTACCAACTGCTACAAAGCTACCGCTTG
CACCAACTCTACCGGCTGCCCGGGTCACGGGCCGGGCTGGCCTTTTTTA

SfiI

The amplified DNA was then run on 2% agarose gel with 1x TAE buffer at 80 V, for about 30 minutes. A bright band can be observed under UV light, with a size around the expected size of 285 bp. Gel purification was preceded after agarose gel

electrophoresis by using a gel extraction kit. The purified DNA sample was used as an insert later.

The insert (TmAFP) and the vector (linker-foldon) were then double digested with NdeI at 37 ℃ for 2 hours first, SfiI was then added into solution, and digested at 50 ℃ for another 2 hours. The double digested DNA were then run on 2% agarose gel with 1x TAE buffer at 80 V again, and purified by gel extraction. The purified samples were then ligated and transformed into origami competent cells.

After overnight growth, colonies were selected for an overnight culture with 5 ml LB media. The plasmid DNA was then purified from the overnight cultures by miniprep. The samples were screened by PCR and agarose gel, and sent for DNA sequencing.

The TmAFP-linker-foldon sequence is shown below. The yellow highlighted part is the start codon, the green highlighted part is the foldon, the blue highlighted part is the linker, and the grey highlighted part is the TmAFP.

TmAFP-linker-foldon (shown as a single piece of DNA from 5' to 3'):

ATGCAGTGCACCGGTGGTGCTGACTGCACCAGCTGCACCGCTGCTTGCACCGGTTGCGG
TAACTGCCCGAACGCTGTTACTTGCACTAACAGCCAGCACTGCGTTAAAGCTACCACCT
GCACCGGTAGCACCGACTGCAACACCGCTGTTACCTGCACTAACTCTAAAGACTGCTTC
GAAGCTCAGACCTGCACCGACTCTACCAACTGCTACAAAGCTACCGCTTGCACCAACTC
TACCGGCTGCCCGGGTCACGGGCCGGGCTGGCCGGACGGTACCACCTCCAAAGGCTGGC
CGGGTTACATCCCGGAAGCTCCGCGTGACGGTCAGGCTTACGTTCGTAAAGACGGTGAA
TGGGTTCTGCTGTCTACCTTCCTG

The code for the TmAFP-linker-foldon amino acid sequence is as below. The green highlighted part is the foldon, the blue highlighted part is the linker, and the grey highlighted part is the TmAFP.

MQCTGGADCTSCTAACTGCGNCPNAVTCTNSQHCVKATTCTGSTDCNTAVTCTNSKDCF

EAQTCTDSTNCYKATACTNSTGCPGHGPGWPDGTTSKGWPGYIPEAPRDGQAYVRKDGE

WVLLSTFL

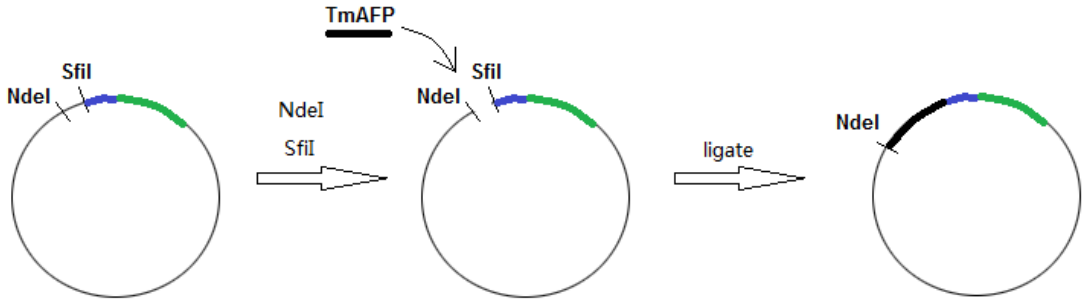

Figure 2.3: Schematic diagram of the synthesis of the gene for TmAFP-linker-foldon. Foldon, linker, TmAFP are shown in green, blue and black, respectively.

2.2 Protein expression and purification

Protein expression was carried out in 1 L LB media with 100 µl ampicilin. Three 10 ml overnight cultures of foldon-linker-TmAFP, TmAFP-linker-foldon, and His6-eGFP-TmAFP respectively in LB media were used to inoculate three 1L LB expression cultures. Cultures were shaken at 37 ℃. Optical density was measured during this time by using UV spectrophotometer (Thermo Scientific Biomate 3) at 600 nm. After the optical density reached 0.6, 120 mg isopropyl-β-D-thiogalactopyranoside (IPTG) (from Fisher Scientific) was added to each culture to induce. Expression cultures were then sent

to shake at 15 ℃ for 48 hours. Before induction and periodically during the 48 hour expression, 1 ml samples were taken to monitor expression by protein gel electrophoresis.

After shaking at 15 ℃ for 48 hours, cultures were transferred into centrifuge tubes and centrifuged at 14000 rpm in a microcentrifudge for 20 minutes, supernatant was discarded, and the bacteria pellets were kept in freezer for further purification. Samples before induction and after expression were centrifuged at 14000 rpm in a microcentrifudge for 5 minutes, and the supernatant were discarded. Pellets was then re-suspended in 100 μl BPER (Thermo Scientific) and centrifuged again to separate soluble and insoluble fractions. The new pellets after re-suspension and re-centrifuge were considered to be the insoluble fraction, while the supernatant were considered to be the soluble fraction. The pellets were then re-suspended in 100 μl one more time for protein gel analysis. Samples (20 μl) from both soluble and insoluble fractions were run 12 % Bis-TRis gel (Invitrogen) at 80 V for 2 hours with 1x MES buffer. Then the gel was stained with GelCode Blue staining reagent (from Thermo Scientific), shaken for about 2 hours, and shaked overnight with GelCode Blue staining reagent been replaced by double distilled water. It was observed that all the proteins were primarily in the soluble fractions. Detailed protein expression protocol was given in Appendix (A.5).

Foldon-linker-TmAFP, TmAFP-linker-foldon, and His6-eGFP-TmAFP samples were purified by cold finger purification. Frozen bacteria pellets from expression were re-suspended with 33 ml PBS, and sonicated. Detailed sonication protocol was given in Appendix (A.4). After sonication, samples were diluted with double distilled water to a total volume of 80 ml. During the purification process, ice freezes onto a cold finger, the AFP is purified since antifreeze proteins can selectively adsorb to the ice while other

proteins do not. The cold finger purification apparatus (Figure 2.2) [41] includes a cold finger, which was connected to a temperature controlled water bath (VWR Scientific Model 1156). The water bath was filled with anti-freeze. Samples were placed in a cone-shaped glass and insulated with polystyrene foam. A small magnetic stirrer was placed in the glass to ensure good mixing during purification.

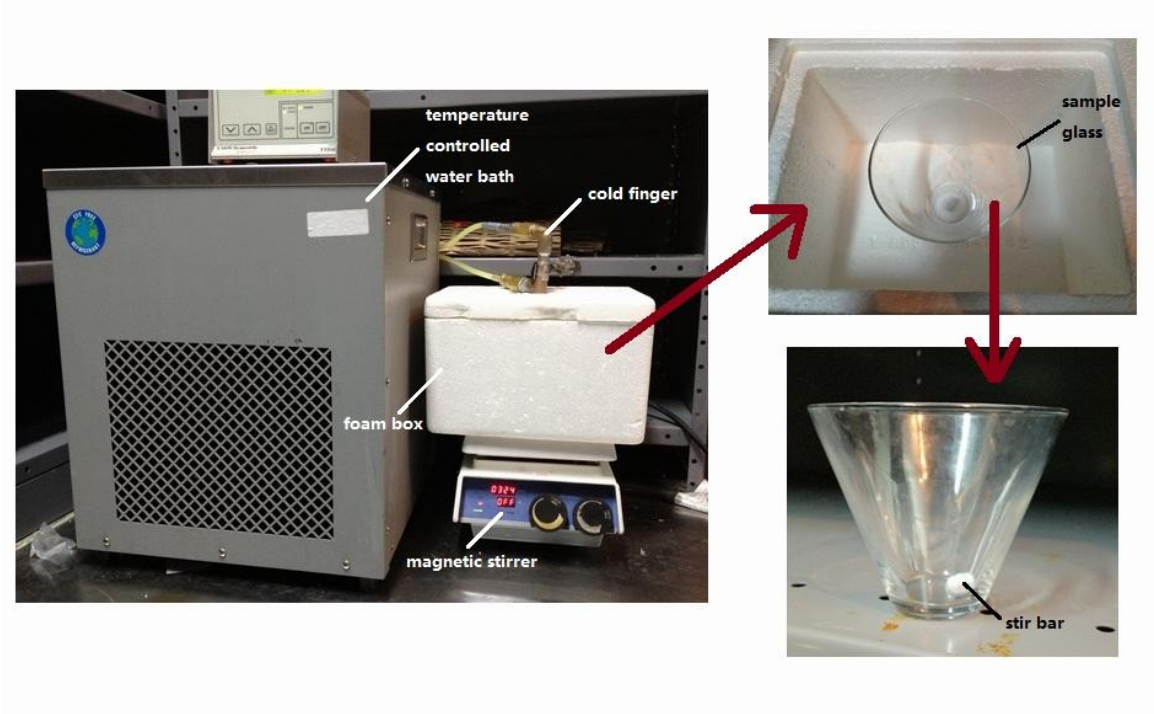

Figure 2.4: Cold finger purification apparatus.

The general method for cold finger purification was shown in Figure 2.3 [45]. The temperature controlled water bath was pre-set at -0.5 ℃. To start the purification, the cold finger was seeded with a thin layer of ice by insert into a beaker, containing ice cold double distilled water with pieces of ice, for 10 minutes. After seeding, the cold finger was then inserted into the pre-chilled sample solution. The temperature of the water bath

was lowered gradually at 0.1 ℃ every 30 minutes. Ice forms around the cold finger; the ice fraction was then removed from unfrozen fraction, and washed with double distilled water. It was then placed in a clean beaker for melting, and collected in falcon tubes for further measurement. For a good purification, ice will appear clear. Detailed cold finger purification protocol was given in Appendix (A.6).

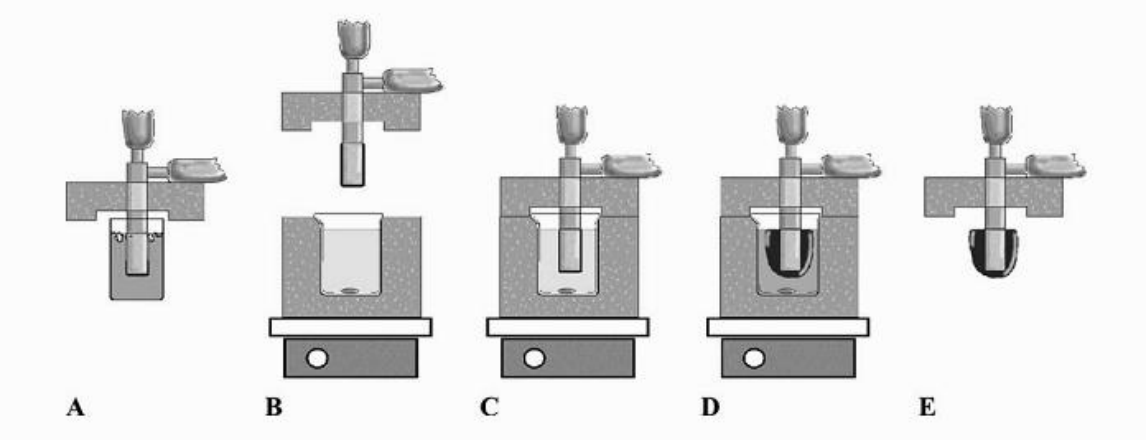

Figure 2.5: General method for cold finger purification [45]. A) seeding the cold finger with ice cold distilled water; B) a thin layer of ice was formed on the cold finger; C) cold finger was inserted into sample solution; D) clear ice formed around cold finger; E) ice was removed from unfrozen fraction. Figures and captions were modified from [45] with permission of *Biochemical and Biophysical Research Communications*.

Samples taken before and after purification were then checked by protein gel electrophoresis (12% Bis-Tris gel, Invitrogen).

CHAPTER III

RESULTS

3.1 Synthesis of genes

3.1.1 Synthesis of plasmid contains foldon-linker-TmAFP genes

The plasmid containing the foldon-linker-TmAFP genes was sent for sequencing at Cleveland Clinic Foundation and the expected sequence below was returned. The yellow highlighted part is the NdeI recognition site, the green highlighted part is the foldon, the blue highlighted part is the linker, and the grey highlighted part is the TmAFP.

Foldon-linker-TmAFP shown as a single piece of DNA from 5' to 3'):

GAAGGAGATATACATATGGGCCACGGCGTGGGTTACATCCCGGAAGCTCCGCGTGACGG

TCAGGCTTACGTTCGTAAAGACGGTGAATGGGTTCTGCTGTCTACCTTCCTGGGGCCGG

GCTGGCCGGACGGTACCACCTCCAAAGGCTGGCCACAGTGCACCGGTGGTGCTGACTGC

ACCAGCTGCACCGCTGCTTGCACCGGTTGCGGTAACTGCCCGAACGCTGTTACTTGCAC

TAACAGCCAGCACTGCGTTAAAGCTACCACCTGCACCGGTAGCACCGACTGCAACACCG

CTGTTACCTGCACTAACTCTAAAGACTGCTTCGAAGCTCAGACCTGCACCGACTCTACC

AACTGCTACAAAGCTACCGCTTGCACCAACTCTACCGGCTGCCCGGGTCAC

The corresponding code for the foldon-linker-TmAFP amino acid sequence is below. The green highlighted part is the foldon, the blue highlighted part is the linker, and the grey highlighted part is the TmAFP.

MGHGVGYIPEAPRDGQAYVRKDGEWVLLSTFLGPGWPDGTTSKGWPQCTGGADCTSCTA

ACTGCGNCPNAVTCTNSQHCVKATTCTGSTDCNTAVTCTNSKDCFEAQTCTDSTNCYKA

TACTNSTGCPGH

This sequencing result confirmed that foldon-linker-TmAFP has been successfully made.

3.1.2 Synthesis of plasmid containing TmAFP-linker-foldon genes

The plasmid containing the TmAFP-linker-foldon genes was sent for sequencing at Cleveland Clinic Foundation, and the expected sequence below was returned. The yellow highlighted part is the NdeI recognition site, the green highlighted part is the foldon, the blue highlighted part is the linker, and the grey highlighted part is the TmAFP.

TmAFP-linker-foldon shown as a single piece of DNA from 5' to 3'):

GAAGGAGATATACATATGCAGTGCACCGGTGGTGCTGACTGCACCAGCTGCACCGCTGC

TTGCACCGGTTGCGGTAACTGCCCGAACGCTGTTACTTGCACTAACAGCCAGCACTGCG

TTAAAGCTACCACCTGCACCGGTAGCACCGACTGCAACACCGCTGTTACCTGCACTAAC

TCTAAAGACTGCTTCGAAGCTCAGACCTGCACCGACTCTACCAACTGCTACAAAGCTAC

CGCTTGCACCAACTCTACCGGCTGCCCGGGTCACGGGCCGGGCTGGCCGGACGGTACCA

CCTCCAAAGGCTGGCCGGGTTACATCCCGGAAGCTCCGCGTGACGGTCAGGCTTACGTT

CGTAAAGACGGTGAATGGGTTCTGCTGTCTACCTTCCTG

The corresponding code for the TmAFP-linker-foldon amino acid sequence is below. The green highlighted part is the foldon, the blue highlighted part is the linker, and the grey highlighted part is the TmAFP.

MQCTGGADCTSCTAACTGCGNCPNAVTCTNSQHCVKATTCTGSTDCNTAVTCTNSKDCF

EAQTCTDSTNCYKATACTNSTGCPGHGPGWPDGTTSKGWPGYIPEAPRDGQAYVRKDGE

WVLLSTFL

This sequencing result confirmed that TmAFP-linker-foldon has been successfully made.

3.2 Expression

Samples before induction and from expression were run on 12 % Bis-Tris protein gel (Invitrogen) with 1x MES buffer for 2 hours. Low-range molecular weight marker was used in the gel electrophoresis.

3.2.1 Expression of foldon-linker-TmAFP

Figure 3.1 presents the 48 hour expression of foldon-linker-TmAFP in origami. The molecular weight of foldon-linker-TmAFP is 13905.19 Da, as calculated based on the amino acid code sequence, through protein molecular weight calculator provided by PIR (Protein Information Resource) [46].

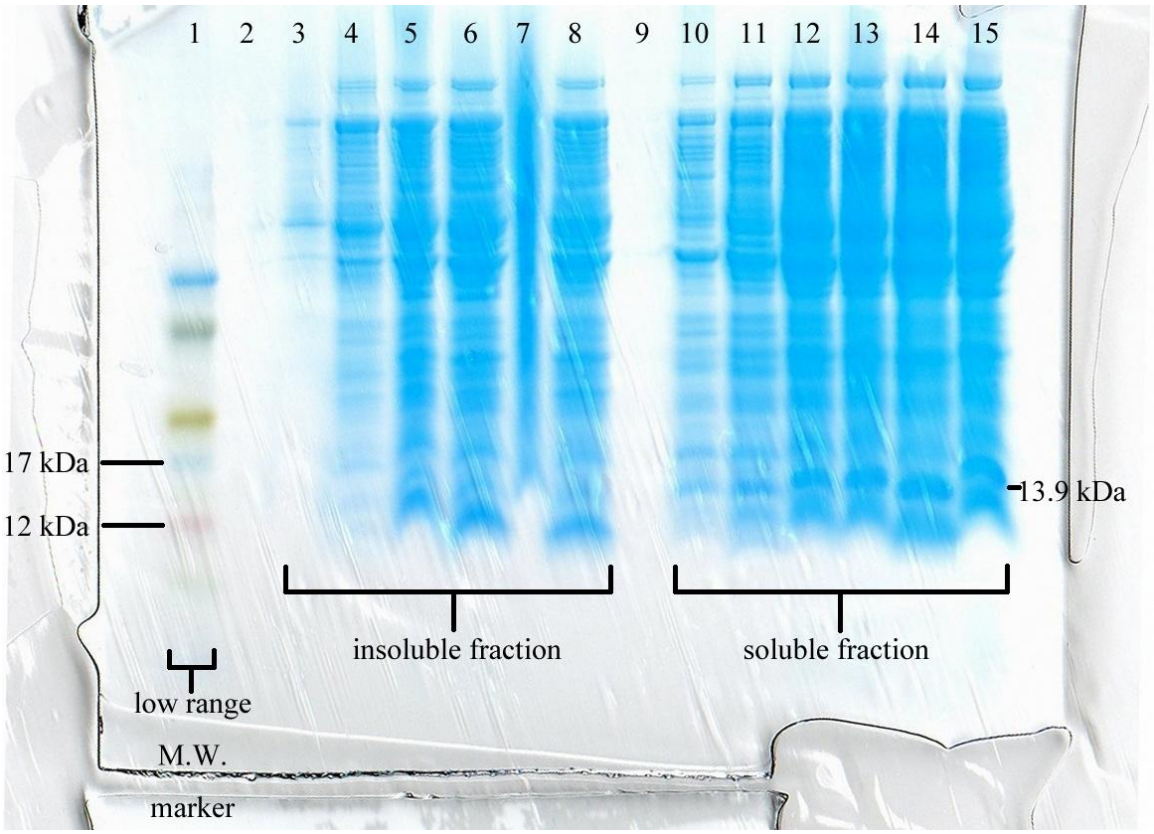

Figure 3.1: 12 % Bis-Tris gel of foldon-linker-TmAFP expression process. Lane 1 is the low range molecular weight marker. Lane 2 and lane 9 are blank. Lane 3 to lane 8 are the insoluble fraction, while lane 3 is sample before induction, lane 4 to lane 8 are samples taken during expression, taken at 5h, 19h, 30h, 43h, and 48h. Similarly, lane 10 to lane 15 are the soluble fraction, with the same order as the insoluble fraction.

It can be observed that foldon-linker-TmAFP is accumulated in the soluble fraction. Compared to the low-range molecular weight marker, the size is around 14 kDa, consistent with the expected molecular weight of foldon-linker-TmAFP of 13.9 kDa.

3.2.2 Expression of TmAFP-linker-foldon

Figure 3.2 presents the 48 hour expression of TmAFP-linker-foldon in origami. The molecular weight of TmAFP-linker-foldon is 13554.80 Da, as calculated through protein molecular weight calculator [46].

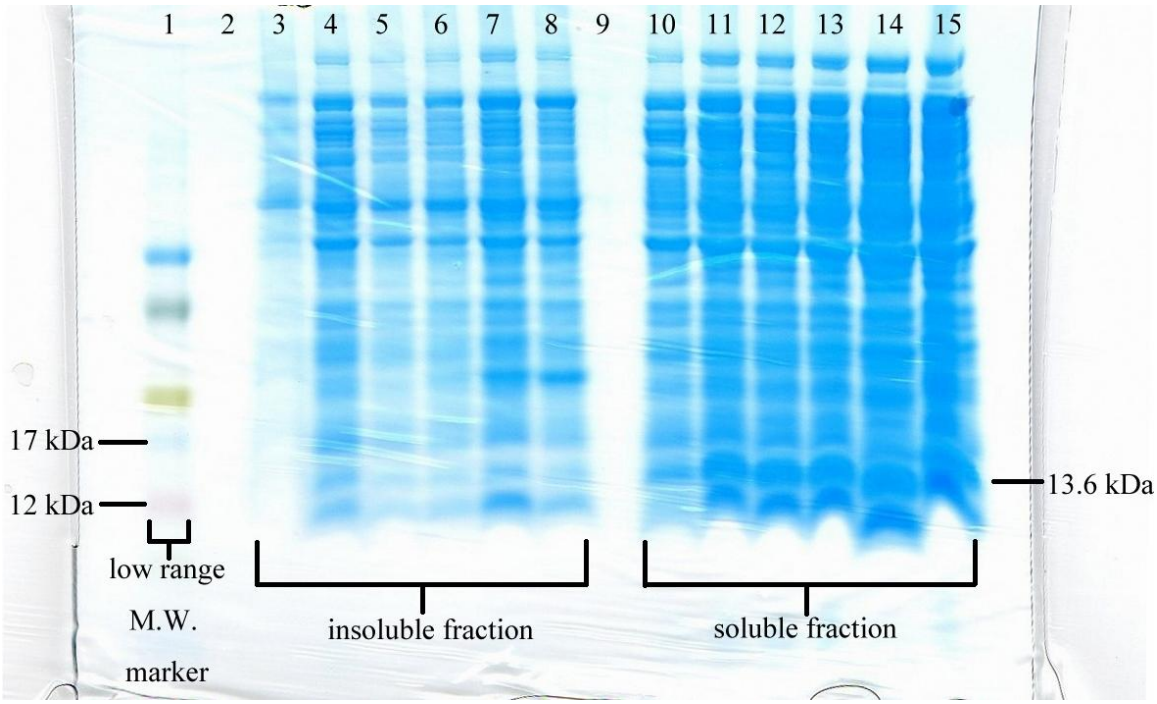

Figure 3.2: 12 % Bis-Tris gel of TmAFP-linker-foldon expression process. Lane 1 is the low-range molecular weight marker. Lane 2 and lane 9 are blank. Lane 3 to lane 8 are the insoluble fractions, while lane 3 is sample before inducion, lane 4 to lane 8 are samples taken during expression, taken at 5h, 19h, 30h, 43h, and 48h. Similarly, lane 10 to lane 15 are the soluble fraction, with the same order as the insoluble fraction.

It can be observed that TmAFP-linker-foldon also accumulated in the soluble fraction. Compared to the low-range molecular weight marker, the size is around 14 kDa, consistent with the expected molecular weight of TmAFP-linker-foldon of 13.6 kDa.

3.2.3 Expression of His-eGFP-TmAFP

Figure 3.3 presents the 48 hour expression of His-eGFP-TmAFP in origami. The molecular weight of His-eGFP-TmAFP is 19609.64 Da, as calculated through protein molecular weight calculator [46].

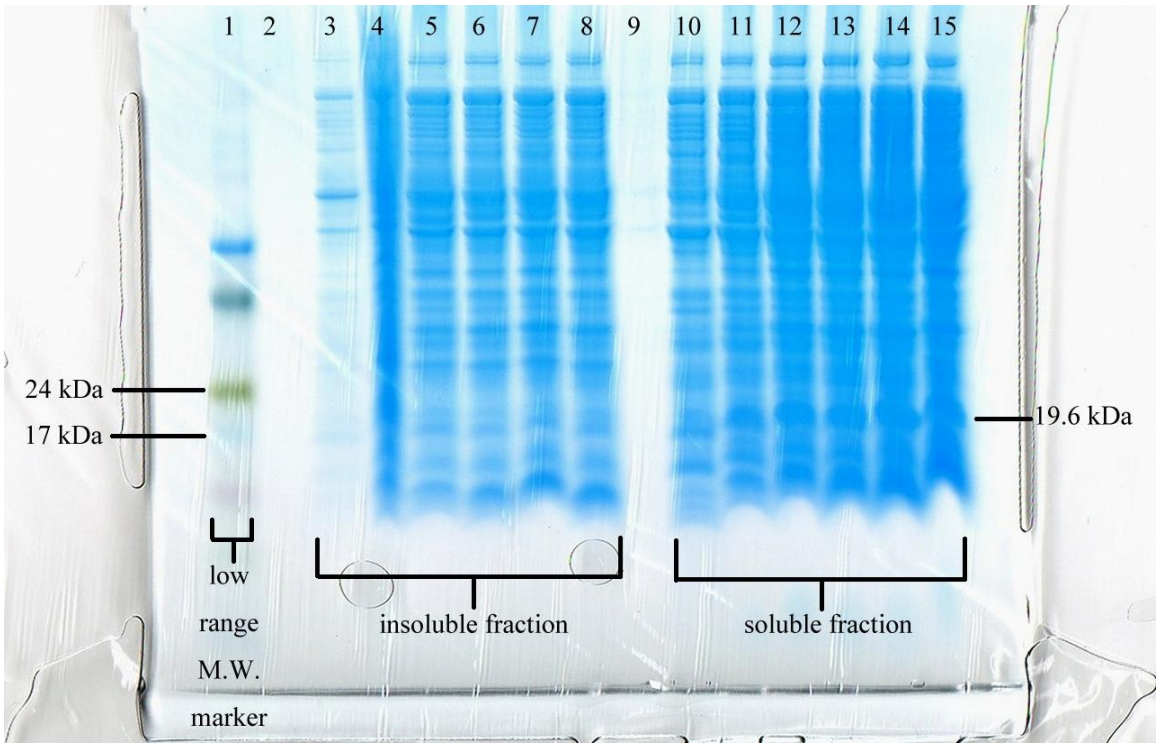

Figure 3.3: 12 % Bis-Tris gel of His-eGFP-TmAFP expression process. Lane 1 is the low-range molecular weight marker. Lane 2 and lane 9 are blank. Lane 3 to lane 8 are the insoluble fractions, while lane 3 is sample before induction, lane 4 to lane 8 are samples taken during expression, taken at 5h, 19h, 30h, 43h, and 48h. Similarly, lane 10 to lane 15 are the soluble fraction, with the same order as the insoluble fraction.

It can be observed that His-eGFP-TmAFP accumulated in the soluble fraction. Compared to the low-range molecular weight marker, the size is around 20 kDa, consistent with the actual molecular weight of His-eGFP-TmAFP of 19.6 kDa.

3.3 Purification

Foldon-linker-TmAFP, TmAFP-linker-foldon, and His6-eGFP-TmAFP samples were purified by cold finger purification. During the purification process, samples will grow on a cold finger since antifreeze proteins can selectively adsorb to the ice while other proteins cannot.

Figure 3.4 presents the cold finger purification of foldon-linker-TmAFP, TmAFP-linker-foldon, and His6-eGFP-TmAFP. As mentioned before, the molecular weight of foldon-linker-TmAFP, TmAFP-linker-foldon, and His6-eGFP-TmAFP are 13.9 kDa, 13.6 kDa, and 19.6 kDa.

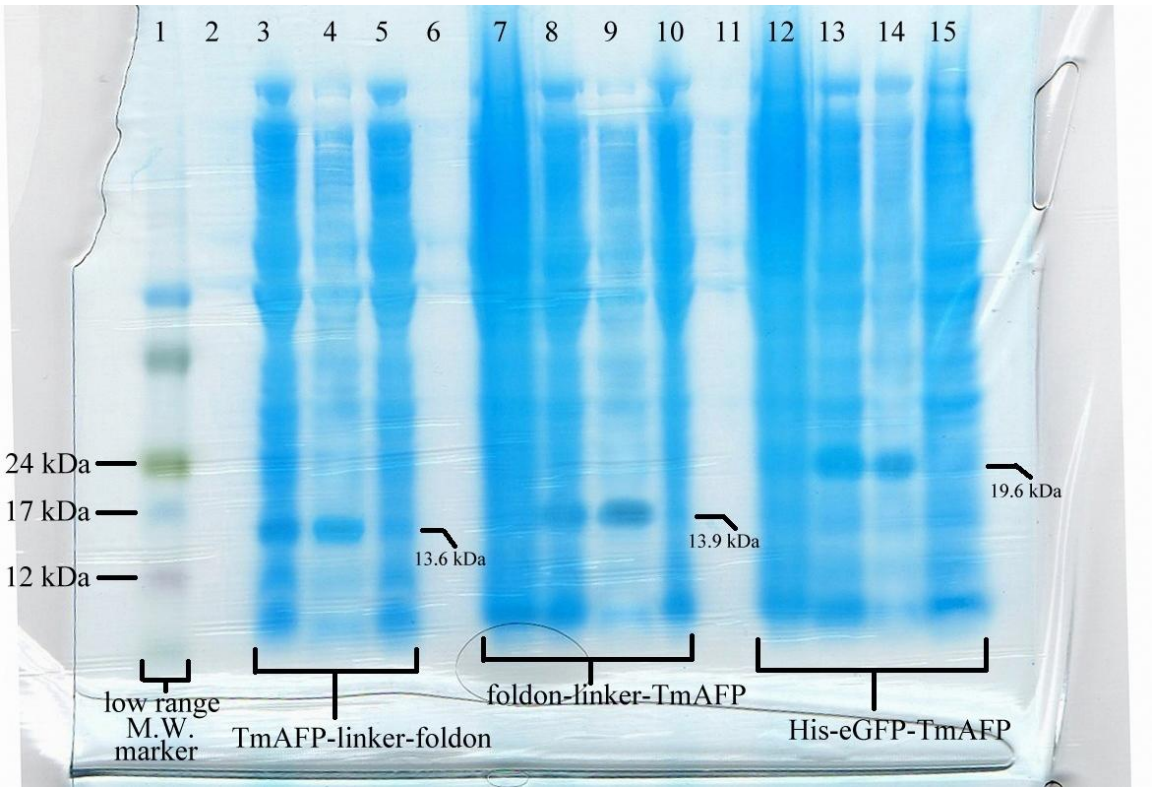

Figure 3.4: A 12 % Bis-Tris gel of cold finger purification for TmAFP-linker-foldon, foldon-linker-TmAFP, and His-eGFP-TmAFP. Lane 1 is the low-range molecular weight marker. Lane 2, lane 6, and lane 11 are blank. Lane 3 to lane 5 are TmAFP-linker-foldon, while lane 3 is the diluted unpurified sample, lane 4 is the cold finger purified sample (frozen fraction), and lane 5 is the unfrozen fraction after purification. Lane 7 to lane 10 are foldon-linker-TmAFP, while lane 7 is the undiluted unpurified sample, lane 8 is the dilute unpurified sample, lane 9 is the cold finger purified sample (frozen fraction), and lane 10 is the unfrozen fraction after purification. Similarly, lane 12 to lane 15 are His-eGFP-TmAFP, with the same order as lane 7 to lane 10.

It can be observed that before purification, the samples all contain antifreeze protein and other proteins; after purification; antifreeze proteins are in the frozen fraction

by binding to cold finger, while other proteins remain in the unfrozen fraction. It can be concluded from the gel results that all the antifreeze proteins can be purified by cold finger purification, which indicates they bind to the ice.

Further experiments were done to test the folding of TmAFP-linker-foldon and foldon-linker-TmAFP. Purified samples of TmAFP-linker-foldon and foldon-linker-TmAFP were boiled at 90 ℃ for 5 minutes, and both boiled and unboiled samples were then run on 12 % Bis-Tris gel with full range molecular weight marker.

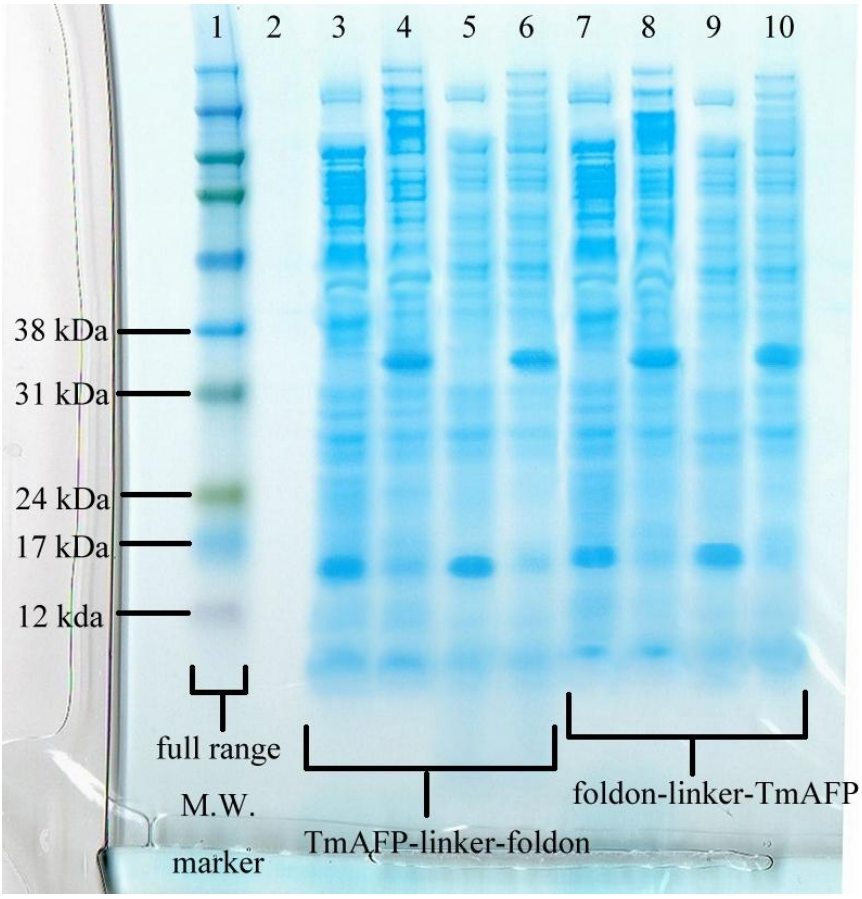

Figure 3.5: A 12 % Bis-Tris gel of purified TmAFP-linker-foldon and foldon-linker-TmAFP. Lane 1 is the full-range molecular weight marker. Lane 2 is blank. Lane 3 to lane 6 are TmAFP-linker-foldon, while lane 3 is the boiled purified sample, lane 4 is the unboiled purified sample, lane 5 is the boiled purified sample with additional centrifuge, and lane 6 is the unboiled sample with additional centrifuge. Similarly, lane 7 to lane 10 are foldon-linker-TmAFP, with the same order as lane 3 to lane 6.

As can be seen in Figure 3.5, boiling can completely disrupt the folding of the foldon domain. The boiled samples have molecular weights similar as monomers, 13.6 kDa for TmAFP-linker-foldon and 13.9 kDa for foldon-liner-foldon. The unboiled

samples have molecular weights three times larger than the monomers. Also, additional centrifuge can help to get rid of unwanted insoluble proteins.

CHAPTER IV

CONCLUSION

In this thesis, foldon was successfully added on both the N-teminus and the C-terminus of *Tenebrio molitor* antifreeze protein. Both foldon-linker-TmAFP and TmAFP-linker-foldon samples were expressed in origami and purified by cold finger purification.

The structure modification of adding foldon and linker is very similar to previous studies done on type I and type III antifreeze proteins by Ozge Can [41]. Due to the high disulfide bond content in *Tenebrio Molitor* antifreeze protein, the structure is much more complex, but still can be produced.

TmAFP with foldon can be folded into trimer, heat treatment can completely disrupt the foldon structure, and the samples will run as monomers on the gel electrophoresis.

This thesis does not include thermal hysteresis measurement and comparison which needs to be done to quantify the effect of trimer further.

We have confirmed that foldon can be added to both the N-teminus and the C-teminus of *Tenebrio molitor* antifreeze protein, and that they are still active after cold finger purification. As an extension of this research, thermal hysteresis measurement needs be performed for foldon-linker-TmAFP and TmAFP-linker-foldon, to determine the effect of different position for foldon. A comparison should also be made between trimer TmAFPs and monomer TmAFP at different concentrations, to determine whether the foldon constructs increase the thermal hysteresis activity, which has already been demonstrated for type III AFP, particularly at low concentration.

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APPENDICES

APPENDICES

A.1 PCR protocol

Reaction is prepared in PCR tubes, the total volume is 50 µl:

- 1 µl template primer
- 5 µl forward primer
- 5 µl reverse primer
- 25 µl PCR master mix
- 25 µl nuclease free water

Place PCR tubes in thermocycler.

Sample program:

- 2 minutes at 95 ℃ for melting the DNA.
- 30 cycles of:
    - 1 minute at 72 ℃ for annealing the primers to the DNA.
    - 30 seconds at approximately 5 ℃ below the melting point of the primers for primer extension.
    - 1 minute at 72 ℃ for the primer extension.
- Refrigeration at 4 ℃ for several hours until the sample is removed from the thermocycler.

A.2 Agarose gel electrophoresis protocol

Preparing agarose gel:

- Mix 1.1 g agarose (molecular biology grade) with 75 ml 1x TAE buffer in a beaker.
- Stir and heat until clear liquid is formed.
- Add 7.5 µl ethidium bromide and mix by swirling.
- Pour the solution in a cassette and place the appropriate combs for sample injection.
- Allow it to cool down to room temperature.
- Remove the combs and place the gel in gel box.
- Fill the gel box with 1x TAX buffer.

A.3 Annealing protocol

Annealing sample is prepared in PCR tubes, the total volume is 50 µl:

- 4 µl forward primer
- 4 µl reverse primer
- 21 µl quick ligase buffer
- 21 µl nuclease free water

Place PCR tubes in thermocycler.

Sample program:

- 2 minutes at 95 ℃.
- Ramp cool to 25 ℃ over a period of 45 minutes.
- Refrigeration at 4 ℃ for several hours until the sample is removed from the thermocycler.

A.4 Sonication protocol

Sample solution preparation protocol:

- Resuspend the pellets with PBS, the total volume should not exceed 35 ml.
- Transfer the sample into 50ml Falcon tubes.
- Chilled on ice for 15 minutes.
- Place the Falcon tube into clean beaker containing ice and water.

Sonicator clean and tune protocol:

- Wash sonicator probe with ethanol and distilled water.
- Turn on sonicator, press “tune”.
- Turn the amplitude control knob to 3, 6, 10, respectively, adjust the bar graph of the screen to be under 10 by rotating the tuning knob.
- Turn the amplitude control knob back to 0.
- Press “stop” to end the tuning cycle.
- When finished using the sonicator, wash the probe with ethanol and distilled water again.

A.5 Protein expression protocol

Protein expression for *Tenebrio molitor* AFP:

- Make 5 ml starter culture (100 μg ampicilin per ml) and grow cells at 37 ℃ overnight.
- Pour starter culture into 1L LB (Lysogeny broth) with ampicillin and grow at 37 ℃ until the optical density reaches 0.6 at 600 nm UV length.
- Induce protein expression with 120 mg IPTG.
- Continue grow the culture at 15 ℃ for 48 hours.
- Centrifuge the cell culture and freeze the pellet.

A.6 Protein purification protocol

Cold finger purification for *Tenebrio molitor* AFP:

- Set water bath to -0.5 ℃, insert the cold finger into a clean beaker which contains distilled water and pieces of ice.
- After a thin layer of ice has formed on the cold finger, change the water beaker into sample solution glass.
- Lower the temperature at the rate of -0.1 ℃ every half hour until reaches -2 ℃.
- Wash ice fraction with distilled water.
- Set cold finger to 1 ℃, let the ice fraction detach from the cold finger.
- Let the ice fraction melt, and collect the solution.