CHARACTERIZATION AND APPLICATION OF CARROT ANTIFREEZE PROTEIN IN MAINTAINING BACTERIAL CELL VIABILITY UNDER LOW TEMPERATURE

By

YING WANG

A thesis submitted to the

Graduate School-New Brunswick

Rutgers, the State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Master of Science

Graduate Program in Food Science

Written under the direction of

Dr. Qingrong Huang

And approved by

_____

_____

_____

_____

New Brunswick, New Jersey

May 2015

ABSTRACT OF THE THESIS

Characterization and Application of Carrot Antifreeze Protein in Keeping Bacterial Cell Viability Under Low Temperature

By

YING WANG

Thesis Director: Dr. Qingrong Huang

The ice-structuring protein (ISP) or antifreeze protein (AFP) has great potential applications in frozen food preservation. This study aims at developing a fast and economical method to prepare carrot (*Daucus carota*) AFP (DcAFP) and apply it in the preservation of bacterial cell.

The carrot AFP (*DcAFP*) was cloned and transformed into the recombinant bacteria. The recombinant protein was purified and refolded, and its second structure, as determined by circular-dichroism (CD), was found to contain approximately 60% beta sheet structure, suggesting the potential binding plane for ice and liquid water.

The inhibition of ice crystal formation by DcAFP was further demonstrated by the following three experiments: first, the recrystallization of ice crystals with the DcAFP solution was monitored by using video microscopy. By quantifying the crystal number and their average diameter, the ability of DcAFP to inhibit the recrystallization has been demonstrated. Second, bacterial cell viability under 0ºC was detected. With the addition of DcAFP, cell survival rate was 50% higher than that of the control sample. Finally, the

addition of DcAFP in the lactobacillus without any other protective agent significantly increased cell viability under 0ºC. These results suggested the potential applications of DcAFP in the preservation of probiotic foods.

ACKNOWLEDGEMENTS

The accomplishment of my Master's thesis would not be possible without the following people who helped me with their unstopped guidance, support and encouragement.

I would like to express my sincere gratitude to my advisor, Dr. Qingrong Huang, for his guidance, support and understanding throughout the time. I am very grateful to him for this opportunity despite my biochemistry background. It is my pleasure to be his student.

I would like to thank my committee members, Dr.Rong Di and Dr. Chi Tang Ho for their valuable guidance on the techniques, helpful input, and provision of lab resource.

I am very grateful for the teaching assistantship from the Office of Undergraduate Instruction, Division of Life Sciences, Rutgers University for the financial support through my course of study.

I would like to thank Dr. Michael Rogers for his support on lab instrument and useful suggestions.

All my lab mates are never forgotten. I am thankful to my friends in Dr. Huang's lab, Dr. Di's lab and in the food science building. It is a warm and active environment to work in and I have learned a lot.

My deepest gratitude goes to my family especially to my parents and my husband for their endless encouragement, support and love. This thesis work would be impossible without them.

TABLE OF CONTENTS

ABSTRACT OF THE THESIS ..... *ii*

ACKNOWLEDGEMENTS ..... *iv*

TABLE OF CONTENTS ..... *v*

LIST OF FIGURES ..... *viii*

LIST OF TABLES ..... *ix*

1. Introduction: *1*

1.1 Antifreeze proteins (AFPs) overview and classification **1**

1.1.1 Discovery of antifreeze proteins (AFPs) 1

1.1.2 Classification of AFP 1

1.1.3 Thermal Hysteresis Activity of AFPs 3

1.1.4 Ice Recrystallization Inhibition Activity of AFPs 4

1.2 Plant AFPs **6**

1.2.1 Discovery of plant AFPs. 6

1.2.2 Gene and structure 6

1.2.3 Potential applications of plant AFPs 7

1.2.4 Current Challenges 8

1.3 Probiotic applications **9**

1.3.1 Probiotic food 9

1.3.2 Lactobacillus 10

1.3.3 Challenges of probiotic foods 11

1.4 Hypothesis and objectives in our research **13**

1.4.1 Hypothesis 13
1.4.2 Objectives 13
1.4.3 Scope of research 13
1.4.4 Challenge 15
2. Materials and Methods: 16
2.1 Materials 16
2.2 Methods 16
2.2.1 Construction of the DcAFP expression plasmid 16
2.2.2 Protein expression 16
2.2.3 Protein refolding and purification 17
2.2.4 Protein analytical procedures 17
2.2.5 Circular-dichroism spectroscopy 18
2.2.6 Small Angle X-ray Scattering 18
2.2.7 Ice crystal structure characterization 19
2.2.8 In vitro antifreeze activity assay 19
2.2.9 In vitro antifreeze activity in *Lactobacillus plantarum* 20
2.2.10 Statistical analysis 20
3. Results and Discussion: 22
3.1 Amplification and identification of DcAFP 22
3.2 Expression analysis of DcAFP in *E. coli* 24
3.3 Mass spectrum result 28
3.4 Circular-dichroism spectroscopy 29
3.5 Small Angel X-ray Scattering 30

3.5 Ice recrystallization inhibition activity 32

3.6 In vivo antifreeze activity in *E.coli* cells 36

3.7 In vitro antifreeze activity in *E.coli* cells 38

3.8 In vitro antifreeze activity in *E.coli* cells with freeze-thaw 39

3.9 In vitro antifreeze activity in *Lactobacillus plantarum* cells 41

3.10 Conclusions 43

4. Future Work: 45

5. References: 48

LIST OF FIGURES

Figure 1.Structure of antifreeze proteins, from Davies PL and Sykes BD (1997), Current Opinion in Structural Biology. 2

Figure 2. Effects of winter rye antifreeze proteins on the growth of an ice crystal. 4

Figure 3.SEM (Scanning Electron Microscope) analysis of *Lactobacillus plantarum* WCFS1 cultures from van Bokhorst-van de Veen H et al. Appl. Environ.Microbiol. 2011 11

Figure 4. Small Angel X-ray Scattering (SAXS) data analysis by Kratky plot. (from Bioisis.net)...19

Figure 5. Plasmid map of carrot antifreeze protein in cloning vector pJ401. 22

Figure 6. Identification of *DcAFP* cDNA cloned into DH5α and BL21 (DE3) cells. 23

Figure 7. DcAFP recombinant protein expression. 24

Figure 8.DcAFP recombinant protein expression under IPTG series induction. 26

Figure 9. DcAFP recombinant protein purification from inclusion body and renatured by series urea elution. 27

Figure 10.MS spectrum data of DcAFP protein by MALDI-TOF/TOF. 29

Figure 11.CD spectrum of DcAFP obtained at 25°C, 0.2 mg/ml in PBS buffer. 30

Figure 12.Scattering intensity profiles of AFP under different concentrations. Scaling exponents were obtained through the best-fit in log-log plot. 31

Figure 13. Small Angle X-ray Scattering profiles of AFP by Kratky plots. 32

Figure 14. Ice morphology during recrystallization. The scale bar represented 0.1mm. 33

Figure 15. Ice crystal count of DcAFP (red) and sucrose (blue) by time points. (P value <0.05)..... 34

Figure 16. Ferret Diameter Measurement of ice crystals from DcAFP (red) and sucrose (blue) by time points (P value <0.05). 35

Figure 17. DcAFP affects bacterial survival rate under low temperature of 0°C. 37

Figure 18.Bacterial cell viability test under 0°C with and without DcAFP incubation 39

Figure 19. Stability of freeze-thaw treated bacterial cells with and without DcAFP expression. 40

LIST OF TABLES

Table 1. Ice crystalcount of DcAFP and sucrose solution by time points.……..…34

Table 2. Ice crystal size (diameter) with and without DcAFP treatment during recrystallization……....36

Table 3. Bacterial cell viability test under 0 °C with and without DcAFP expression at different time points…….……37

Table 4.Bacterial cell viability test under 0 °C with and without DcAFP incubation…..38

Table 5. Bacterial cell survival rate treated with DcAFP after freeze-thaw cycle ……40

Table 6. Survival of *Lactobacillus plantarum* with and without DcAFP treatment ….…42

1. Introduction:

1.1 Antifreeze proteins (AFPs) overview and classification

1.1.1 Discovery of antifreeze proteins (AFPs)

Antifreeze proteins (AFPs) exist in living organisms and protect them from damage in freezing condition by controlling the growth of ice. Particularly, ice crystals may penetrate cell membranes and cause fluid leakage and cell damage. While in some organisms living at the sub-zero temperature, their body fluids remain in the liquid form when the environment temperature is below their equilibrium freezing point. This phenomenon has been named as “antifreeze”, and is executed by a group of proteins-- “antifreeze proteins (AFPs)”. Antifreeze proteins have been widely found in fish, insects, microbes and plants. The first AFP was discovered in the blood of Antarctic fish that can survive in the -1.9 °C Antarctic Ocean water [1,2,3]. Shortly after the discovery of fish AFP, proteins with similar activity were found in insects and plants [4,5,6,7,8].

1.1.2 Classification of AFP

Antifreeze proteins can be classified into two main groups: antifreeze glycoproteins (AFGPs) and antifreeze proteins (AFPs) without glycosylation[3,6]. Antifreeze glycoproteins have been fractionated from proteins in fish blood serum[9]. They are carbohydrate rich with molecular weight(MW) ranges from 2.6 kDa to 3.3 kDa[10], containing an (Ala-Ala-Thr) repeating unit.

AFPs from fish are classified into four types. Type I, molecular weight range from 3.3 to 4.5 kDa, was found in winter flounders. Type II, with molecular mass range from 11 to 24 kDa, was found in herring. Type III in wolfish, was 6.5 kDa in molecular weight and Type IV in longhorn sculpins with a molecular weight of 12 kDa[6,11].

The structure of these four types of AFP has been extensively studied: Type I are alanine-rich alpha-helix proteins. Type II is cysteine-rich globular proteins, containing five disulfide bonds. Type III is globular proteins. Type IV are glutamate and glutamine-rich proteins that contain alpha-helix[6].

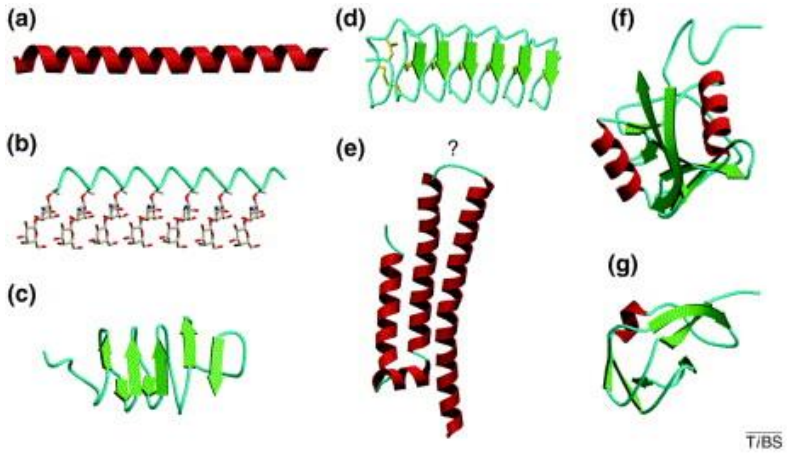

Figure 1.Structure of antifreeze proteins, from Davies PL and Sykes BD (1997), Current Opinion in Structural Biology.

Representative structures are drawn to scale for the five types of fish antifreeze proteins (AFPs) (a, b, e–g) and two insect AFPs (c, d) with helices in red, ß strands in green and coil in cyan. (In the following lists, PDB accession codes are given in parentheses.) (a–e) Repetitive AFPs. (a) Type I AFP from winter flounder (1WFA). (b) Antifreeze glycoprotein (AFGP) drawn as an extended left-handed helix with disaccharides displayed [22]. (c) Spruce budworm AFP (1EWW). (d) Tenebriomolitor AFP (1EZG). (e) Type IV AFP modeled as a helix bundle. The question mark indicates uncertainty about its repetitive character. (f, g) Non-repetitive AFPs. (f) Type II AFP from sea raven (2AFP). (g) Type III AFP from ocean pout (1MSI).

AFPs from insects have been reported to have a higher activity in preventing ice formation. Usually, they are 10-100 times more active than fish AFP, which is consistent with a severe environment for insects with much lower temperature in winter[7]. There are two types of insect antifreeze proteins, *Tenebrio* and *Dendroides* AFPs in different insect families. They are similar to one another, both have greater thermal hysteresis value, consist of varying numbers of 12 or

13 repeating units with a consensus sequence with MW of approximately 8.3 to 12.5 kDa[6,8,12,13].

1.1.3 Thermal Hysteresis Activity of AFPs

AFPs have two effects on aqueous solutions: thermal hysteresis (TH) and ice recrystallization inhibition (RI) [14,15,16]. Thermal hysteresis (TH) is the range between the melting and freezing temperature, which is used to measure the activity of AFP to lower the freezing point in preventing the ice crystal formation. At physiological concentrations, TH activity of fish AFPs is typically around 1-1.5°C. Insect AFPs have around 5°C of TH activity, which is around 10-100 times greater than those of fish AFPs [15,17].

Thermal hysteresis activity can be measured using Clifton Nanolitre Osmometer. Since used a very small volume of the sample, usually in nanoliter, the Clifton Nanolitre Osmometer is used to measure the melting point and the freezing point. Firstly, the sample on the cooling stage is cooled to a temperature of near -40 degrees (-40°C) very quickly (usually twenty seconds), which causes the sample in the sample wells to flash-freeze. Then, the sample is allowed to melt relatively slowly, under the control of the osmometer controller. The sample temperature at that point when the last ice crystal disappears is called the melting point. Finally, the sample is refrozen using deep freeze, and once again melted towards a single ice crystal. When only a single, very small ice crystal remains the sample is cooled in a controlled manner using the nanoliter control box. The solution temperature at the point when the ice crystal grows is called the freezing point. By using the osmometer, the difference between the melting point and the freezing point can be observed. In recent studies, some researchers used the Differential Scanning Calorimetry (DSC) to measure the thermal hysteresis activity, but this method has not been well established or standardized[18].

Thermal hysteresis activity can be quantified as a standard assay for antifreeze activity. However, it is seldom used for plant antifreeze proteins as their thermal hysteresis activity is relative low, usually 0.2-0.4°C, compare to those in fish with 1-1.5°C and insects with 5°C. Therefore, examining the morphology of ice crystals may be more useful for plant AFPs.

1.1.4 Ice Recrystallization Inhibition Activity of AFPs

Ice crystal morphology has been extensively studied with the winter rye antifreeze proteins. It was shown that without the antifreeze proteins, the ice crystal grows as a fattened disc. At low AFP concentrations of nano molar, the antifreeze proteins are absorbed to the prism face of crystal and form a hexagonal shape. At high AFP concentration at micro molar, ice crystals are still hexagonal, but also form columns or bipyramids. At higher AFP concentration above 100µM, ice crystals form elongated needle-like shapes[19].

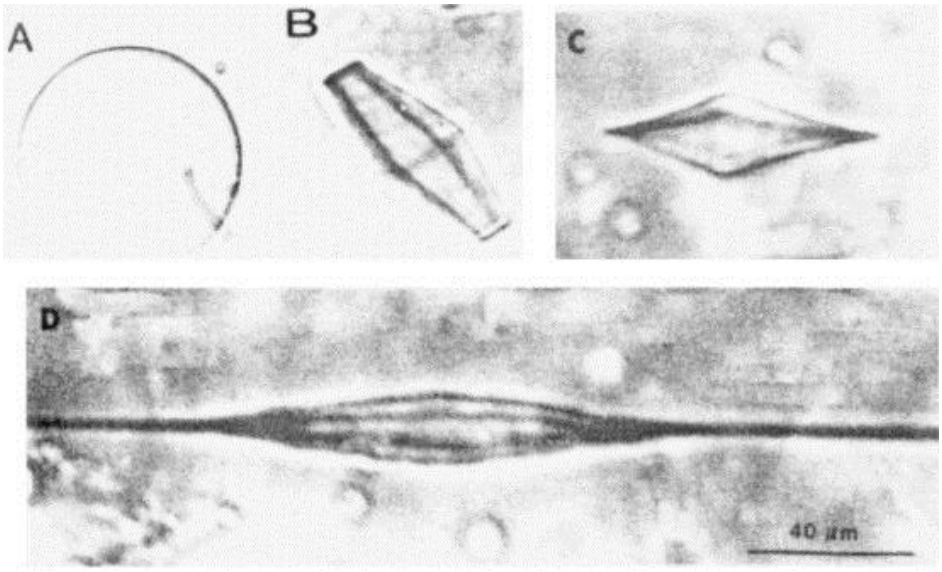

Figure 2. Effects of winter rye antifreeze proteins on the growth of an ice crystal.

(A) An ice crystal grown in water. (B) Ice crystal grown at low AFP concentrations, nM. (C) Ice crystal grown at high AFP concentrations, µM. (D) Ice crystal grown at higher AFP concentrations, above 100 µM (Griffith et al., 1992, 1997).

It has been reported that the ice-binding domains of fish and insects AFPs are flat and relatively hydrophobic. The interaction between AFP and ice are hydrophobic and the binding is stabilized by a combination of van der Waals interaction and hydrogen bonds. Unlike the fish AFP, the ice-binding domain in plant AFPs are hydrophilic due to the highly conserved asparagines. The fluorescence spectrum of carrot AFP shows that it folds into beta-helix with a hydrophobic core. This structure model suggests that plant AFPs may have the ability to bind to ice and ice nucleates and inhibits both the ice nucleation and recrystallization. But the interaction between ice and the hydrophilic domain is still unknown.

Based on the primary structure of fish, insects and plant AFPs, no consensus sequence has been found for any ice-binding domain. So it is not possible to identify a novel AFP from the sequence information.

For plant AFPs, the thermal hysteresis (TH) activity is relatively weak, only 0.2-0.4°C, but they show strong ice recrystallization inhibition (RI) activity [17,20]. This suggests that these cold-induced antifreeze proteins may act as crystallization modifiers by inhibiting the growth of ice crystals rather than depressing the freezing point of plant fluids. One possible reason for higher recrystallization activity in plant AFPs is they have multiple ice-binding domains that can interact with more than one ice surface. The AFP from perennial ryegrass (*Loliumperenne*) is predicted to fold into a beta-roll or beta-helix with two ice-binding domains located on opposite sides of the protein[21]. Plant AFPs may form oligomers that have multiple ice-binding domains and may have some activator protein present to enhance their antifreeze activity.

Antifreeze proteins have the ability to inhibit the ice crystal growth and are preferred to preservatives for cell and organ under low temperatures. First of all, AFPs depress the freezing temperature at a very low concentration without affecting the osmotic pressure. Insect AFPs can lower the freezing point with 5°C without altering the melting point. Second, AFPs adsorb onto

the surface of small ice crystals and inhibits the recrystallization to the large ice crystals. Larger ice crystals increase the possibility of physical damage to cell membrane and cytosol leakage. In addition, AFPs have the ability to interact with ice nucleates, which may inhibit the ice nucleation to block the initialization of ice crystal formation.

1.2 Plant AFPs

1.2.1 Discovery of plant AFPs.

Antifreeze proteins are found in a wide range of over-wintering plants after they have been exposed to low temperatures [4,11,22,23]. The antifreeze activity is located in the extracellular space where the ice crystallizes in cold-tolerant species. In 1992, the first plant antifreeze protein was reported in winter rye leaves. Then many plant AFPs have been isolated from species such as bittersweet nightshade (*Solanumdulcamara*), barley, ryegrass and carrot [4,14,17]. In 1998 and 1999, the cold-induced carrot AFP was reported and the corresponding gene *DcAFP* was cloned. Other AFPs from plants demonstrating antifreeze activity have also been characterized from peach, winter wheat, kale etc.

1.2.2 Gene and structure

Antifreeze proteins from plants are homologous to pathogenesis-related (PR) proteins and provide protection against psychrophilic pathogens like snow mold. The carrot *AFP* gene has similar sequence with polygalacturonase (PG) inhibitor but does not present the activity. Approximately 74% of the mature carrot AFP amino acid sequence is composed of a leucine-rich repeat (LRR) domain that is common in resistance (*R*) genes and respond or bind to foreign signals.

Most of the plants AFPs have secretory signals except the bittersweet nightshade AFP and the peach AFP. The AFPs from carrot, bittersweet nightshade and perennial ryegrass are all glycosylated, but the glycosylation is not necessary for antifreeze activity except for the bittersweet nightshades AFP.

1.2.3 Potential applications of plant AFPs

The strong ice recrystallization inhibition activity of AFPs attracts the researchers to apply in the long-term low temperature storage when it is very difficult to maintain the water in the non-frozen state[17,24,25,26,27,28,29]. Therefore, plant AFPs may have great potential application for low temperature storage by allowing the small ice granules forming but inhibiting the large ice crystals.

For agricultural production, AFPs may improve the freezing tolerance by lowering the freezing temperature of cold-sensitive crops after the frost. Moreover, AFPs could increase the shelf life of frozen fish and meat and improve the frozen food quality like dough and ice cream by inhibiting the recrystallization of ice. *Arabidopsis*, tobacco, tomato and potato have been used to study the effect of expressing the foreign AFPs in transgenic plants. When incubated with the nonacclimated winter rye suspension cells, the winter rye AFP lowered the LT50 (the lethal temperature for 50% of individual cells) by 2.5°C. Carrot AFP cDNA has been transformed into *Arabidopsis* and tobacco, and was reported to modify the ice crystal morphology and lead the transgenic plants survive at – 2°C.

AFPs can serve as the food preservative and improve the quality of frozen food during freezing storage. Concentrated carrot protein has been applied into frozen dough containing 18.3% (w/w) DcAFP and the fermentation capacity of frozen dough was evaluated. The application of carrot

protein concentration increased the retention capacity of dough and reduced the ice crystal formation.

With the winter rye extraction containing AFPs, the ice cream has significantly reduced ice recrystallization rate, and displayed a remarkably smoother texture after heat-shock storage. Commercial ice cream products with AFPs are now available on the market.

With the high recrystallization inhibition activity, plant AFPs have the potential to protect cell membrane integrity from the damage by large ice crystal formation, so they may be more useful in the low temperature storage of cells, tissues and organs. The recombinant carrot AFP (DcAFP) has been reported to protect rice suspension cells under low temperatures. At -25°C, -75°C and -180°C, the rice suspension cells with the addition of 0.1 mg/ml DcAFP had approximately 20% higher cell viability than the negative control sample with 3% glycerol[30].

1.2.4 Current Challenges

AFPs have a wide application in the frozen food storage to enhance the quality. AFPs expressed in the recombinant *E.coli* may serve as a preservative especially to conserve the cell lines. However, the future application is restricted by the high cost and high price of AFPs. Currently the purified fish AFPs are commercially available on the market, and the price is $1000 for each gram. Such an expensive cost is not applicable as an ingredient in food product. Therefore, to produce the recombinant AFPs from the prokaryotic cells or yeast is an alternative. Using molecular biology methods to incorporate *AFP* genes into genomes of some plants or fish can further lower the cost of AFPs.

Although lots of research groups have worked on the structure and function of plant antifreeze proteins especially DcAFP, the difficulty of protein expression and purification limited the study as most of the *E.coli*-expressed DcAFP are insoluble. Several methods have been applied such as

denaturing the DcAFP by urea, isolating by HPLC or affinity chromatography and renaturing using dialysis. However, these methods are time consuming, activity of purified proteins may be lowered[31,32,33,34].

In terms of food safety, plant and fish AFPs have been proved to be nontoxic when they exposed to two countries: USA, with a moderate fish consumption and Iceland, with large amount consumption, both in long term and in short term. However, the fish AFPs might induce seafood allergy. Therefore, plant AFPs, may provide a wider application than Fish AFPs.

1.3 Probiotic applications

1.3.1 Probiotic food

Probiotic microorganism was defined as “living microorganisms, which upon ingestion in certain number exert health benefits beyond inherent basic nutrition”. They become increasingly popular and play an important role in maintaining human health by maintaining the normal intestinal balance. The global market for probiotic ingredients, supplements, and foods was worth $14.9 billion in 2007 and reached US$16billion in 2008. Estimates target a total of US$19.6 billion on sales in 2013 and the global probiotics market will grow at 6.8% from 2012 to 2017 (“Probiotics Market - Global Trends & Forecasts To 2017” report from www.prweb.com , www.marketsandmarkets.com).

Probiotic food such as bio-yoghurt or drinks are well accepted as they contain live microorganisms, mainly bifidobacteria and lactobacilli, and improve the health status of the host by exerting beneficial effects in the gastrointestinal tract[35], [36]. Probiotic bacteria stimulate the growth of preferred microorganisms, crowd out potential harmful bacteria and reinforce

human body's natural defense mechanisms. Therefore, a dramatically increased demand was found both in the special and the average (general healthy) populations.

However, the viability and stability of probiotics have been a challenge in both marketing and technology for industrial producers [37,38]. First of all, the manufacturer must incorporate the specific probiotic strains into food products through various technologies such as coating, spray-drying, emulsion formulation and freeze-drying. At the meantime, no unpleasant flavors or textures are allowed in food. Secondly, products must maintain a suitable level of viable cells during the product's shelf life. For example, yogurt is under sub-zero storage condition, ice cream is frozen and probiotic cultures are freeze-dried into powder or in solid status. Finally, they must survive passage through the upper gastrointestinal (GI) tract and arrive alive at their sites, and function in the gut environment.

When developing probiotic foods, several aspects have been taken into consideration such as food safety, manufacture technology and the functions. A good probiotic strain is of healthy-human origin, has no pathogenicity and no antibiotic resistance, and remains high viability through processing and storage period. With new food technologies developed with new formulation, encapsulation and packaging, the range of food products and the probiotic strains can be expanded.

1.3.2 Lactobacillus

Lactic acid bacteria (LAB) have been used in food with a long history since the consumption of fermented milk. Members of *Lactococcus* and *Lactobacillus* are most commonly used and are generally-recognized-as-safe (GRAS), while members of the genera *Streptococcus* and *Enterococcus* contain some opportunistic pathogens.

*Lactobacillus plantarum*is a member of *lactobacillus*, naturally existing in human saliva and gastrointestinal tract. Itplays a significant role in controlling lactic fermentations. It is a gram-positive bacterium, rod-shaped, facultative anaerobic. As a member of lactic acid bacteria (LAB), it is commonly used in food fermentation and its bio-therapeutic applications have been increasingly recognized[39].

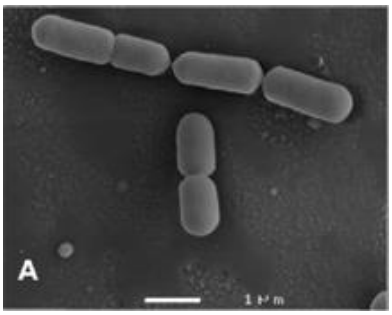

Figure 3.SEM (Scanning Electron Microscope) analysis of *Lactobacillus plantarum* WCFS1 cultures from van Bokhorst-van de Veen H et al. Appl. Environ.Microbiol. 2011

***Lactobacillus plantarum* WCFS1 was grown at 20°C and harvested during exponential phase (OD_{600} = 1.0) from MRS.**

Recently, *Lactobacillus plantarum* has been commercially prepared as a starter for production of dry and semidry sausages and pickled vegetables. Several techniques have been applied to help lactobacilli survive freezing, spray- drying with starch coating and freeze-drying. But none of them is specifically designed to help the lactobacilli undergo subzero temperature storage. Plant AFPs may act as a high-efficiency ice recrystallization inhibitor and combine with other techniques such as co-encapsulated with starch to improve their viability under low temperature.

1.3.3 Challenges of probiotic foods

Before probiotic strains can be delivered to consumers, they should be manufactured under industrial conditions, and then survive and retain their functionality during storage as frozen or

freeze-dried cultures, and in food products. To benefit human health, it has been reported that population of 10^6 -10^7colony forming unit (CFU)/g in final product are established as therapeutic quantities of probiotic cultures. In Brazil, food labeling of probiotic is required to have minimal 10^8-10^9 CFU per daily portion of food product.

Many factors affect the survival of probiotic strains in final food product when improving food quality and extending shelf life [40,41,42,43]. These parameters include the species or strains used, method of culture preparation, storage temperature and time, pH and oxygen level, presence of protectants such as sucrose, starch and dietary fibers and type of container[44,45,46,47]. The human-orientated *Lactobacillus plantarum* was shown to be relatively acid and bile tolerant. However, *Lactobacillus plantarum*was inactivated rapidly after rapid chilling followed by storage at 0°C due to loss of membrane integrity. More than 10^5reductionswereshown after rapid chilling (200°C/min) and 0°C storage for seven days.

Fast chilling is an applicable method to preserve frozen food with original texture and maintain the probiotic strain's viability as starters. How to optimize cell survival conditions during fast chilling and 0°C storage is still being studied. A group of cold-shock proteins (CSPs) have been reported to be involved in mRNA folding, protein synthesis and freeze protection. Overproducing the cold-shock proteins improve the adaptation of *Lactobacillus plantarum* from cold shock (8°C) back to normal growth[48,49,50,51]. However, whether cold-shock proteinswork during 0°C storage remains unknown. Because 0°C storage is commonly used on market for dairy foods such as yogurt and fermented milk, the shelf life of liquid based probiotic foods will benefit greatly from the improvement of cell viability under 0°C storage condition.

1.4 Hypothesis and objectives in our research

1.4.1 Hypothesis

We hypothesize that the renatured carrot antifreeze protein (DcAFP) from insoluble fraction has ice recrystallization activity and incorporation of DcAFP will improve lactic acid bacteria (LAB) especially *Lactobacillus plantarum*'s cell viability under zero Celsius degree.

1.4.2 Objectives

Objective 1: Construct the over-expression vector of DcAFP in bacteria cells. Express and renature the DcAFP in milligram scale.

Objective 2: Characterize DcAFP by ice recrystallization assay and bacterial cell viability assay.

1.4.3 Scope of research

These two objectives were further divided into three phases as following:

Phase one: cloning the DcAFP coding cDNA into over-expression vector.

Carrot AFP (DcAFP)has a signal peptide sequence at N-terminal. This signal peptide may drive antifreeze proteins located in inter-membrane space. However, eukaryotic expression will be interrupted by signal peptides. Therefore, we constructed DcAFP coding cDNA without the N-terminal peptide sequence and gained a high expression level.

Phase two: DcAFP gene expression and protein renaturation.

Antifreeze proteins originated from plants have LRR domains, which are highly related with receptor-like proteins (RLKs). These LRR domains are possibly hydrophobic and make antifreeze proteins insoluble when expressed in bacterial cells. We applied urea to solubilize DcAFP, and desalted by the Microcon-10 kDa centrifugal filter unit at serial urea concentrations, to remove urea efficiently and rapidly. Renatured protein's activity was tested in the next phase.

Phase three: characterization of DcAFP.

1. Circular-dichroism spectroscopy

   Secondary structure of DcAFP was investigated by circular-dichroism. By observing the differential absorption of left and right circularly polarized light, molar ellipticity was calculated and the ratio of alpha-helix, beta-turn and anti-parrel was predicted by CDNN software (provided by Dr. Gerald Böhm, Institut für Biotechnologie, Martin-Luther Universität, Halle-Wittenberg, and Applied Photophysics).This data would give us some information about how the DcAFP interact with ice and water molecules.

2. Ice recrystallization inhibition activity

   Ice recrystallization inhibition activity was measured by observing ice crystals under the low-temperature microscope after an incubation stage at melting point (-6°C) in 30% sucrose solution. At this stage, tiny ice crystals melted and fused together to form large visible and countable crystals. Furthermore, recrystallization inhibition activity was quantified by counting the number of ice crystals and the average diameter from the DcAFP treated sucrose solution and control.

3. In vivo antifreeze activity assay in *E.coli* cells

   The activity of soluble DcAFP expressed in *E.coli* cells were investigated using *E.coli* DH5α cells transformed by pJ401-DcAFP vector. After IPTG induction for two hours, cells were exposed to cold treatment (0°C degree). The short induction produced a very amount of DcAFP so inclusion body was not formed.

4. In vitro antifreeze activity assay in *E.coli* cells

   This assay aimed to test renatured DcAFP's activity of recrystallization inhibition. Renatured DcAFP proteins were added into *E.coli* cells which do not have internal expression of any antifreeze proteins at 0°C degree. Cells were grown to logarithmic

phase, serial diluted by sterile saline and mixed with DcAFP. After the incubation at 0°C degree, viable cells were counted and cell viability was calculated.

5. In vitro antifreeze activity assay in *Lactobacillus plantarum*

   The denatured DcAFP was applied into *Lactobacillus plantarum* cell culture. Similar to the assay with *E.coli* cells, *Lactobacillus plantarum* cells were grown until reaching the logarithmic phase, and viable cells were counted to evaluate the cell viability.

1.4.4 Challenge

Preparation of soluble active DcAFP was challenging. A large amount of recombinant DcAFP expressed in cells could easily become insoluble, lost solubility and antifreeze activity, which limited its future application. Furthermore, the attempts to renature DcAFP will further reduce activity when DcAFP reformed back to its natural structure. The efficiency of renaturation relies on time, salt used, salt concentration and temperature. Therefore, renatured DcAFP may have various activities each time. To overcome the variability, protein expression and purification was performed numerous times, generated and experiments were performed using the same batch of DcAFP.

2. Materials and Methods:

2.1 Materials

Restrict enzymes were from Promega. Isopropyl-l-thio-beta-D-galactopyranoside (IPTG), Acryl-bis, SDS, cellysis, sucrose and urea were from Sigma-Aldrich Chemical Company (St. Louis, MO). 10 kDa Microcon ultrafiltration devices were from Millipore (Amicon).

2.2 Methods

2.2.1 Construction of the DcAFP expression plasmid

The 951-bp *DcAFP* gene was synthesized by DNA2.0 Company with 6 × His tag sequence at C-terminal on expression vector pJ401.The N-terminal primer 5'-CATATGCAGCGTTGCAACAATAACG-3' incorporates a restriction enzyme site NdeI. The C-terminal primer: 5'-CTCGAGTTAGGTACCGTGATGGTGGTGGTGATG-3' incorporates an XhoI restriction enzyme site, His-tag sequence and a stop codon. Construction was confirmed by polymerase chain reaction (PCR) and restriction enzyme digestion with NdeI/XhoI. Plasmid was transformed into *E.coli* DH5αand BL21 (DE3) cells.

2.2.2 Protein expression

E. coli BL21(DE3) cells carrying DcAFP-pJ401 plasmid were grown to OD_{600} 0.6-0.8 in luriabroth (LB) medium containing 50μg/ml Kanamycin. Cultures were induced by 0.1mM final concentration of isopropyl-l-thio-beta-D-galactopyranoside (IPTG) at 37°C for four hours. Cells were harvested by centrifugation at 4°C, 3500 revolutions per minute (rpm) for ten minutes and the cell pellet was washed with an equal volume of 50mM phosphate buffered saline (PBS) buffer (pH 8.0). Cells were centrifuged again and the pellet was stored at -70°C for future use.

2.2.3 Protein refolding and purification

Pellets was suspended in PBS buffer (pH 8.0), adding cellysis (sigma C1990) and incubated for thirty minutes at 4°C. Inclusion body was pelleted by centrifugation at 10,000 rpm for thirty minutes at 4°C, and the supernatant was discarded. The inclusion body containing DcAFP proteins was solubilized in 8M urea buffer. The suspension was centrifuged at 14,000 rpm for fifteen minutes and pellets were discarded. The solubilized inclusion bodies were purified by washing with 8M urea containing 1% Triton, centrifuged using a 10 kDa Microcon ultrafiltration device (Amicon). The solubilized DcAFP was serials desalted with 6M, 4M, 2M, 1M, 0.5M, 0.25M, 0.125M urea and then by PBS buffer three times.

Soluble DcAFP was purified using nickel-affinity resin (GE Healthcare) with thirty minutes incubation at 4°C. The unbounded fraction was washed out by ten volume of PBS with 20mM imidazole and the bound protein was eluted by PBS containing 300 mM imidazole. The salt was removed and the protein was concentrated by the 10 kDa Microcon ultrafiltration device, according to the manufacturer's instructions. The typical yield of purified recombinant protein was 10mg per liter of cultured cells.

2.2.4 Protein analytical procedures

Protein concentration was determined by the Bradford protein assay (BioRad) with a standard curve of bovine serum albumin (BSA). Sodium dodecyl sulfate (SDS)-Polyacrylamide gel electrophoresis (PAGE) was carried out according to Laemmli using the BioRad mini system and the pre-stained molecular weight marker was from Biolabs.

2.2.5 Circular-dichroism spectroscopy

Circular-dichroism (CD) measurements were performed to obtain the secondary structure information of recombinant DcAFP. The signal was recorded in PBS buffer (50 mM sodium phosphate, pH 7.4, 100 mM NaCl). Each measurement was an average of three scans in steps of 0.5 nm at 293 Kelvin degree. The differential spectrum was obtained by subtracting the buffer spectrum before conversion to molar ellipticity. The characteristic minimum at 218 nm is suggestive of a folded protein consisting predominately of beta-strands or beta-sheets. The data was analyzed by CDNN software (provided by Dr. Gerald Böhm, Institut für Biotechnologie, Martin-Luther Universität, Halle-Wittenberg, and Applied Photophysics).

2.2.6 Small Angle X-ray Scattering

DcAFP protein was dissolved in 0.1M Tris-Cl, pH 8.0 at 3 mg/ml, 1 mg/ml and 0.5mg/ml concentration. Small-angle x-ray scattering (SAXS) intensity profiles were measured at the BioCAT, 18-ID beamline of the Advanced Photon Source, Argonne National Laboratory. A flow cell of 1.5mm diameter quartz capillary was used for holding samples. The whole sample holder was maintained at 25°C in the experiments. A MICROLAB 500 Hamilton pump was used to load samples to the flow cell and to flow the samples at constant rate (10 µL/s) during X-ray exposure to minimize radiation damage. The scattering data was acquired by a single exposure of 1 second, cool-down 5 seconds and 15 iterations. The solvent background was also subtracted according to the standard procedure designed for the beam line.

The likeness of the biological macromolecule of "unfolded-ness" or "random coil", can be qualitatively assessed by a Kratky plot. The Kratky interpretation originates from Debye's scattering formulation of a Gaussian coil. Debye's equation shows that within a limited range of

data, the scattering intensities for macromolecules behaving as Gaussian-like coils will plateau in a q2 x I(q) against q plot.

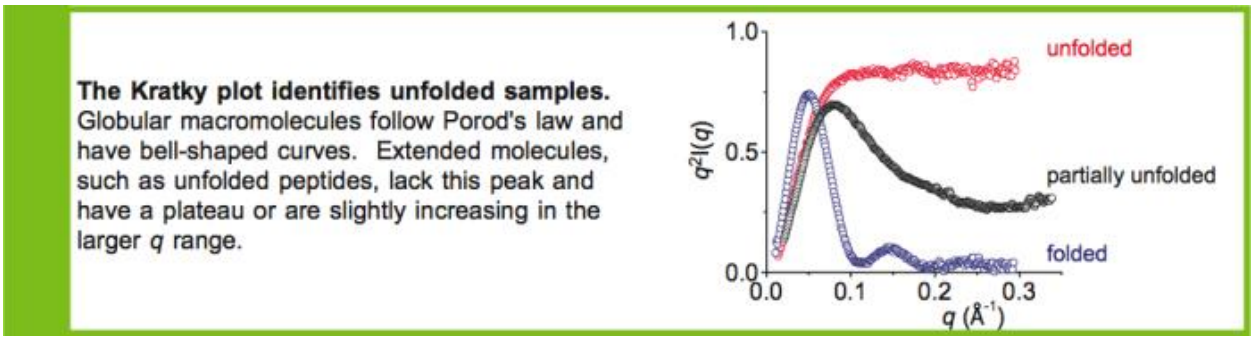

Figure 4. Small Angel X-ray Scattering (SAXS) data analysis by Kratky plot. (from Bioisis.net)

2.2.7 Ice crystal structure characterization

This assay followed the methods of Hagit Kun and Yitzhak Mastai, (2007) with some modifications.20 µl of DcAFP in 30% sucrose(final concentration 0.1 mg/ml) was frozen on a microscope slide with an average cooling rate of 5°C/min, from 25°C to -30°C on a cooling stage (Linkam system) under a light microscope. Ice crystals were observed using a 10X objective of microscope system and the images were captured during incubation at -6 °C using a video camera at 10, 20, 30, 40, 50, 60, 70 and 80 minutes.

2.2.8 In vitro antifreeze activity assay

E. coli BL21 (DE3) cells containing DcAFP-pJ401 plasmid were grown overnight at 37°C in LB media and sub-cultured (1:100) into fresh LB media, grown until the cell density reached of OD_{600} of 0.6. IPTG was added into cells at final concentration 0.1 mM and grown at 37°C for two hours until the cell density reached OD_{600} of 0.8. The BL21 (DE3) cells without IPTG induction

served as the negative control. The cells were suspended in sterilized water with a 10^8- fold gradient dilution. Diluted cells (500 µl) were transferred into 1.5 ml Eppendorf tubes and incubated at 0°C for 24, 48 and 72 hours respectively (the cells were not frozen during the treatment). The DcAFP treated samples were mixed with DcAFP to reach 0.1 mg/ml final concentration. 200 µl cells were taken from each tube, spread on LB plate with Kanamycin 50 µg/ml to prevent any possible contamination and incubated at 37°C for 16 hours. All the colonies were counted and the experiments were performed in triplicate. The number of treated colonies was divided by the number of untreated colonies to obtain cell viability rate. Three replicates were used for generating the graph.

2.2.9 In vitro antifreeze activity in *Lactobacillus plantarum*

Lactobacillus plantarum cells (ATCC14917) were incubated overnight in MRS medium at pH6.5 and 37°C sub-cultured (1:100) into fresh MRS media and grown until the cell density reached OD_{600} of 0.6. Cells were suspended in sterilized water with a 10^6- fold gradient dilution. The DcAFP treated samples were mixed with DcAFP to reach the final concentration 0.1 mg/ml. Diluted cells (500 µl) were transferred into 1.5ml Eppendorf tubes and incubated at 0°C for 0,1,2,3,4,5,6,7 and 8 weeks respectively (the cells were not frozen during the treatment). 200 µl cells were taken from each tube, spread on MRS agar plate and incubated at 37°C for 16 hours. All the colonies were counted and the experiments were performed in triplicate. Three replicates were used for generating the graph.

2.2.10 Statistical analysis

Results were presented as mean values± standard deviation (S.D.) and the mean values were the average of triplicates or more. Statistical significance of the differences between values was

assessed by multifactor analysis. If the P value is less than 0.05, it is considered statistically significant.

3. Results and Discussion:

3.1 Amplification and identification of DcAFP

DcAFP cDNA 951bp was synthesized by DNA2.0 (https://www.dna20.com). Gene sequence was chosen for expression from start codon ATG to the base pairs before terminator TAG. Restriction enzyme site NdeI, was added on 5' terminal, and 6X His tag was synthesized at 3' terminal, with the stop codon TAG and the XhoI site sequentially. The previous report confirmed that no intron exists in *DcAFP* from Worrall and Meyer (*Science*, 1998; *FEBS Letters*, 1999). This synthesized *DcAFP* gene was cloned into expression vector, pJ401 and transformed into *E.coli* DH5α cells.

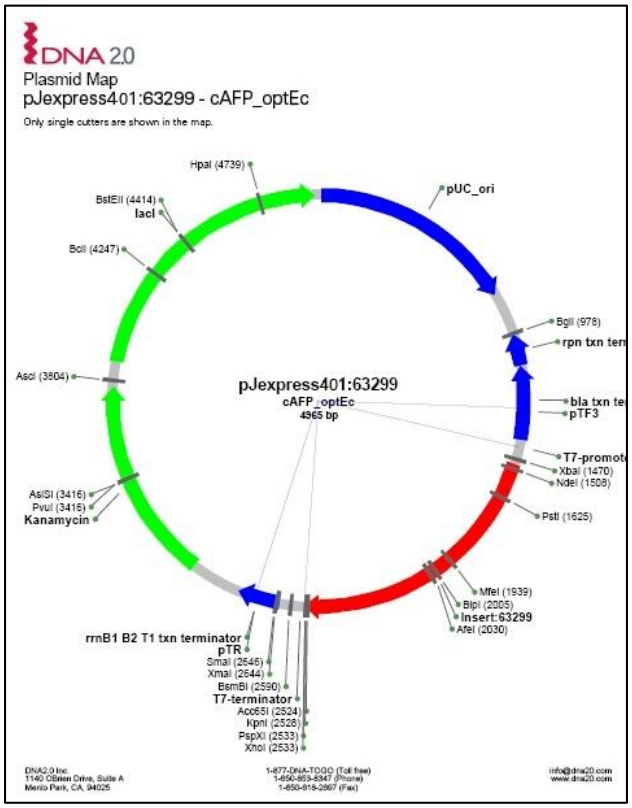

Figure 5. Plasmid map of carrot antifreeze protein in cloning vector pJ401.

The red section indicates the DcAFP coding sequence after the T7 promoter, starting from NdeI site at N-terminal and XhoI site at C-terminal.

As predicted, the 951 bp DNA fragment was amplified by PCR using 5'and 3' terminal primer pairs from DcAFP-pJ401 plasmid (Figure 6A), and the same size fragment was amplified from pJ401-DcAFP transformed *E.coli* DH5α cells. Restriction enzyme digestion using NdeI and XhoI generated a 951 bp DNA fragment from plasmid ofDcAFP-pJ401-DH5α cells (Figure 6B). This proved that transformed DH5α cells incorporated the target plasmid. After these identifications, pJ401-DcAFP plasmid was transformed into BL21 (DE3) cells for protein expression. Two clones of pJ401-DcAFP-BL21 (DE3) cells were picked randomly and identified by restriction enzyme digestions with NdeI/XhoI. Both of them had same size DNA fragment with 951 bp (Figure 6C). These two clones were used for the DcAFP recombinant protein expression and purification.

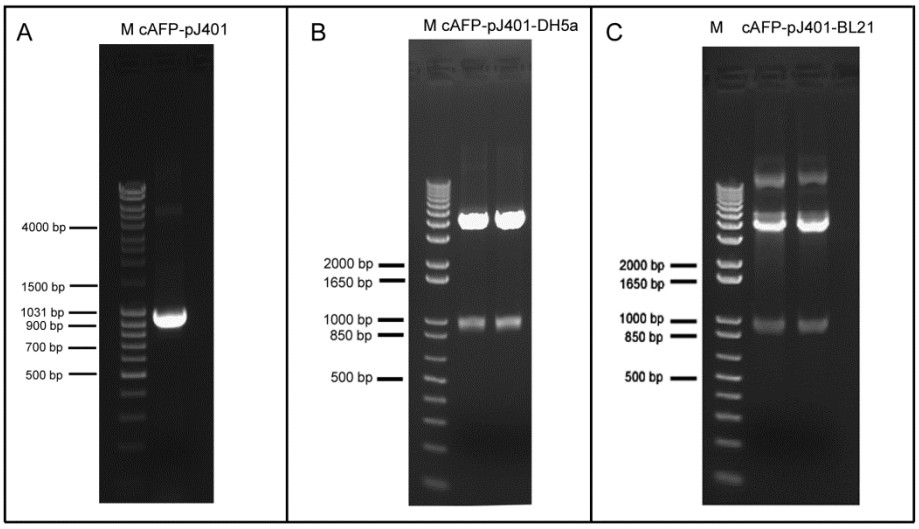

Figure 6. Identification of *DcAFP* cDNA cloned into DH5α and BL21 (DE3) cells.

A. *DcAFP* cDNA 951bp was amplified from the pJ401-DcAFP plasmid using the 5' primer with NdeI restriction sites and XhoI at 3' primer. B. pJ401-DcAFP plasmid from transformed DH5α cells was digested by restriction enzyme NdeI/XhoI. C. pJ401-DcAFP plasmid was isolated from transformed BL21 (DE3) cells and identified by restrict enzyme digestion with NdeI/XhoI. The samples in the two lanes represent two different clones. The amplified DNA was analyzed on 1% agarose gel with 1kb DNA marker.

3.2 Expression analysis of DcAFP in *E. coli*

DcAFP was expressed in *E.coli* and total proteins of IPTG-induced BL21 (DE3) cells were analyzed by SDS-PAGE. Cells were lysed by sonication and the insoluble fraction was separated from the soluble fraction by centrifugation. Both supernatants and pellets of sonicated cell lysates were investigated. After IPTG induction, a 34 kDa protein band was clearly observed on SDS-PAGE gel from the total extracts (lane 3, Figure 7), while this band was absent in the un-induced cell extracts (lane 4). Almost all of the expressed recombinant proteins were present in insoluble fraction—inclusion bodies (lane 1). This observation was consistent with the previous reports.

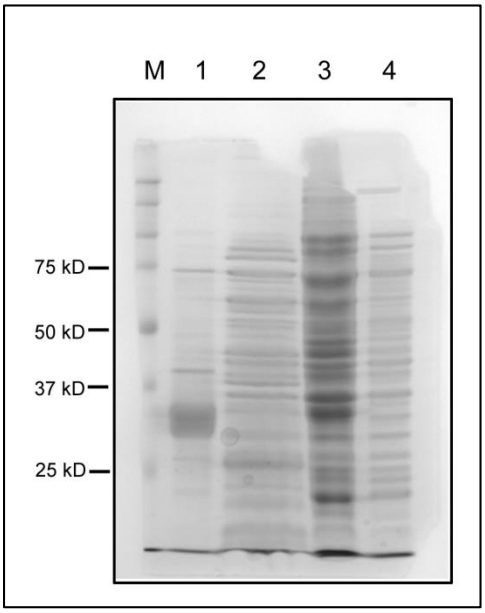

Figure 7. DcAFP recombinant protein expression.

Lane M: protein maker. 1: Pellet fraction. 2: supernatant fraction. 3: total induced protein. 4: uninduced protein. Protein samples were collected, denatured by SDS sample buffer, and analyzed on 10% SDS-PAGE gel.

DcAFP have been transformed into bacteria cells to produce large amount of recombinant proteins for structural study. In this case, we applied two methods to reduce the side-effects during induction, expression and renaturation. Firstly, reduce IPTG concentration down to the minimum level with protein expression. Secondly, optimize renature process using 10 kDa Microcon ultrafiltration devices, demonstrated in the following figure.

The first method is to minimize dosage of IPTG during induction. Therefore, the relationship between DcAFP recombinant protein expression and IPTG concentration was studied. Two final IPTG concentrations, 0.1 mM and 0.01 mM were applied in pJ401-DcAFP-BL21 (DE3) cell culture and the supernatant with pellet were analyzed on SDS-PAGE gel. From Figure7, both of the two treatments induced 34 kDa protein bands in pellet with the similar amount. It indicated that protein expression was independent of IPTG concentration at 0.1 mM and 0.01 mM. Preliminary purification was attempted from soluble fraction by binding with His-tag affinity beads and eluted using 300 mM Imidazole. However, only limited amount of protein was eluted and the 34 kDa protein bands were not observed (lane 4, Figure 8). This result was consistent with our previous figure that most of DcAFP recombinant protein was in the inclusion body.

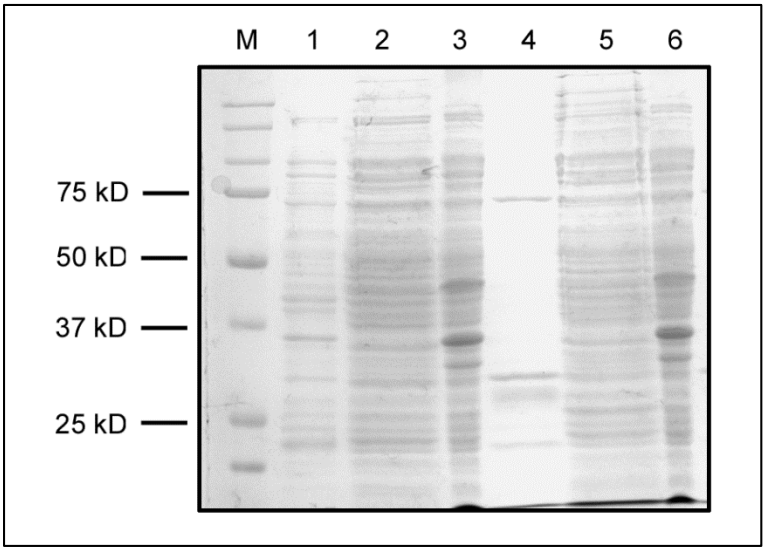

Figure 8.DcAFP recombinant protein expression under IPTG series induction.

Lane M: protein maker. 1: uninduced protein. 2: supernatant fraction under 0.01mM IPTG induction at 37°C, 3 hours. 3: pellet fraction under 0.01mM IPTG. 4: elution under 0.01mM IPTG induction. 5: supernatant fraction under 0.1mM IPTG induction at 37°C, 3 hours. 6: pellet fraction under 0.1mM IPTG induction. Protein samples were collected, denatured by SDS sample buffer, and analyzed on 10% SDS-PAGE gel.

The second method is to optimize the renaturation process using the 10 kDa Microcon ultrafiltration device (Amicon). Based on our observation above, we decided to purify DcAFP protein from pellet fraction after breakdown of cells. Pellets containing inclusion bodies of DcAFP recombinant protein were collected. After centrifugation to separate the soluble fraction, pellets were washed by PBS buffer, containing 0.1% Triton X-100 to remove the cell membrane fragments, and once more to obtain clean inclusion bodies without any detergent. Inclusion bodies were solubilized and renatured, protein sample was collected at each step and the purity was analyzed by ImageJ. As shown in Figure 9, DcAFP purity in desalted fraction was 85%, an increase from 68% in the crude pellet. Therefore, we may obtain the renatured DcAFP protein with enzyme activity retained during this mild method at serial concentrations of urea renaturation.

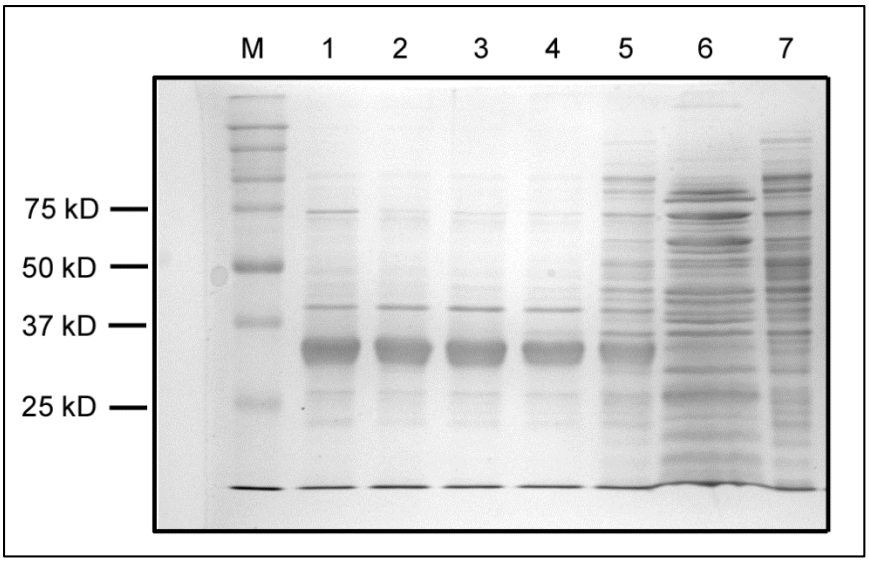

Figure 9. DcAFP recombinant protein purification from inclusion body and renatured by series urea elution. Lane M: marker. 1: denatured DcAFP. 2: soluble DcAFP in urea. 3: inclusion body washed by buffer. 4: inclusion body washed by Triton. 5: pellet fraction. 6: supernatant fraction. 7: total protein after induction.

Our research rationale was to use serial dilution combined with the ultrafiltration device, and obtain the renatured protein in a mild and rapid way, in order to maximize the refolded protein activity. There are two methods to renature insoluble DcAFP. The first method involved denaturing DcAFP by 8 M urea, using His-tag affinity resin to bind DcAFP, then gradually renatured by 4 M, 2 M, 1 M, 0.5 M urea and PBS buffer, finally with PBS buffer containing imidazole to elute. Although this method purified His-tagged DcAFP, it was time-consuming, required good temperature control during chromatography to keep protein activity and needed one more step to remove the imidazole.

The second method used HPLC to isolate denatured DcAFP, analyzed molecular weight by SDS-PAGE and dialyzed to remove organic solvents. This method required HPLC equipment for separation, was time-consuming and temperature control is difficult. Renatured DcAFP have been

reported to have antifreeze activities, and improved cell viability of rice suspension in cold treatment.

In comparison to these methods, our process was expedited and facilitated temperature and salt control. Our renature step was time efficient, accomplished in one hour and easy to set working temperature. No dialyses were needed, and we avoided protein precipitation on membrane from rapid urea concentration drop. Furthermore, the Microcon ultrafiltration device is multi-functional: it is able to remove salt, balance a sample with its new buffer system and remove unwanted small molecular proteins all at the same time. Therefore, we assume that our renatured DcAFP may regain the antifreeze activity through this efficient method.

3.3 Mass spectrum result

To further prove the protein identity, the DcAFP band was cut from the SDS-PAGE gel and sent to Mass spectrum center for mass spectrum analysis. In Figure 10, the blue amino acid sequence matched the peptide sequence from the database. All the major peaks matched the predicted carrot antifreeze proteins, suggesting a high confidence.

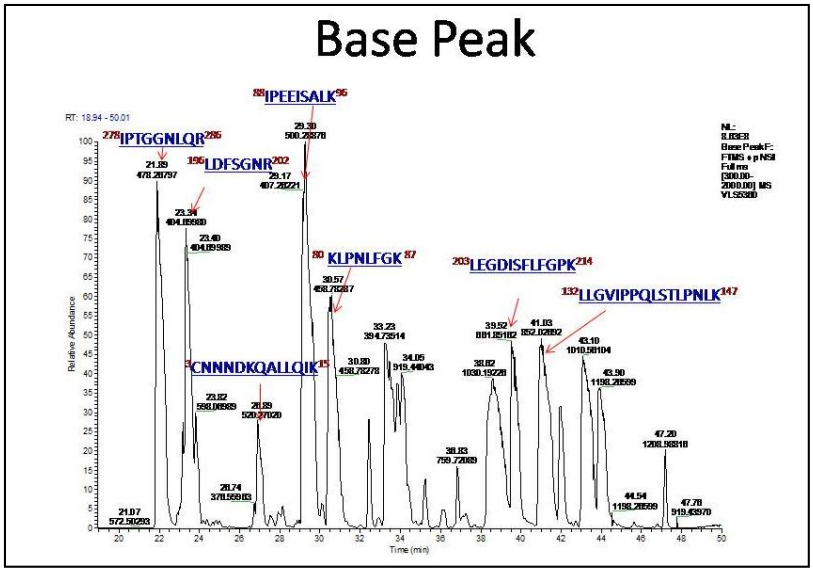

Figure 10.MS spectrum data of DcAFP protein by MALDI-TOF/TOF.

The aligned peptide sequences were displayed corresponding with the m/z peaks labeled with the amino acid numbers.

3.4 Circular-dichroism spectroscopy

To characterize the secondary structure of DcAFP, we used circular dichroism spectroscopy (CD) taken at 25°C. The results of CD measurements are shown in Figure 11. DcAFP reveals an anti-parallel beta sheet secondary structure with 44.40 % anti-parallel, 21.50% beta turn and 13.60% alpha-helix.

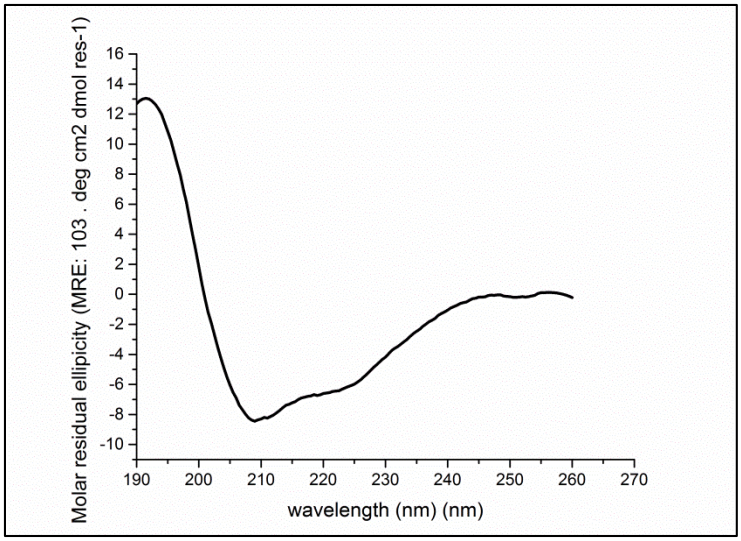

Figure 11.CD spectrum of DcAFP obtained at 25°C, 0.2 mg/ml in PBS buffer.

This spectrum behavior was consisted with Zhang's fluorescence spectrum result in that DcAFP has a beta-roll structure and may provide more information for putative binding planes for ice and water. This beta-sheet-dominated secondary structure is different from the alpha-helix-dominated structure in fish AFPs, suggesting the functional difference between plant and fish AFPs.

3.5 Small Angel X-ray Scattering

Figure 12 shows the scattering intensity profiles of DcAFP at three different concentrations in logarithmic scale. The fractal dimension in the intermediate Q range provides the contour shapes of proteins in solutions. It is 1 for a rod-like distribution, and 2 for a random coil with an isotropic Gaussian distribution.

Two typical regions of fractal dimensions (Df) were examined. At small Q range, fractal dimensions is about 1.7, which indicates an aggregation of DcAFP proteins is like a polymer chain with excluded volume in solvent, or a diffusion-limited aggregation, or a typical fractal

dimension of protein according to their structure[52,53]. In large Q range, the fractal dimension of 3.0 indicates a symmetric association (in 3-D) of groups in protein structure.

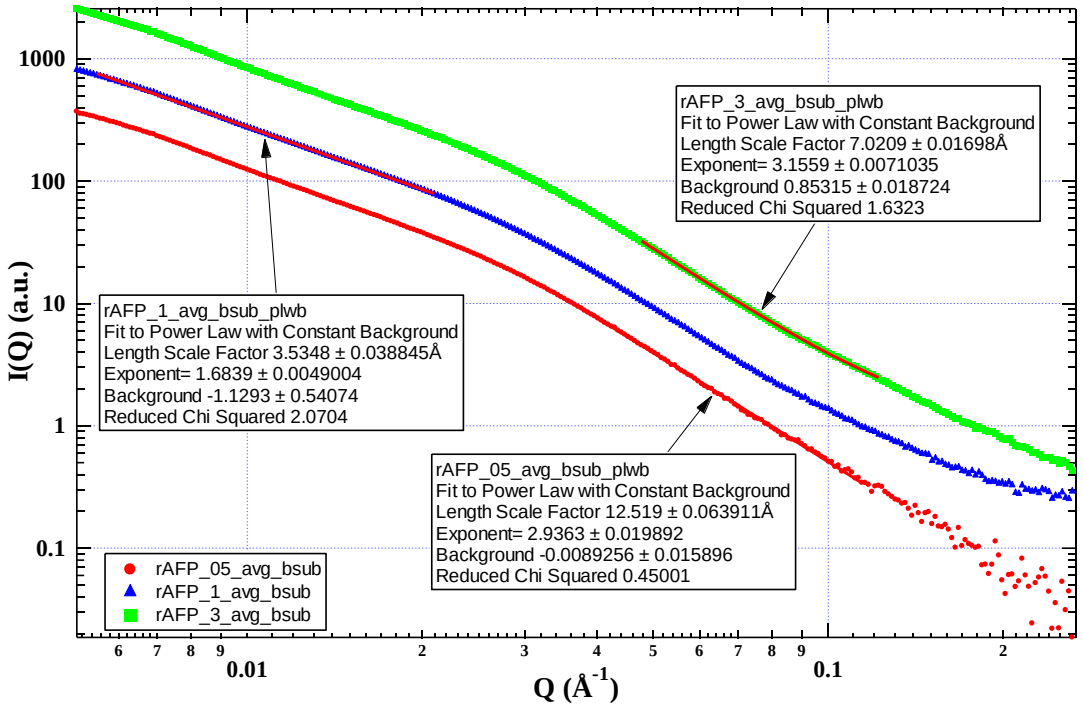

Figure 12.Scattering intensity profiles of AFP under different concentrations. Scaling exponents were obtained through the best-fit in log-log plot.

Furthermore, the scattering profile was further investigated by Kratky plot. As shown in Figure 13, all the DcAFPs remain folded. The length of the principal axis of the protein is around 9 nm, slightly decrease to 8.7 nm and 8.4 nm with concentration increase from 0.5 mg/ml to 1 mg/ml and 3 mg/ml respectively.

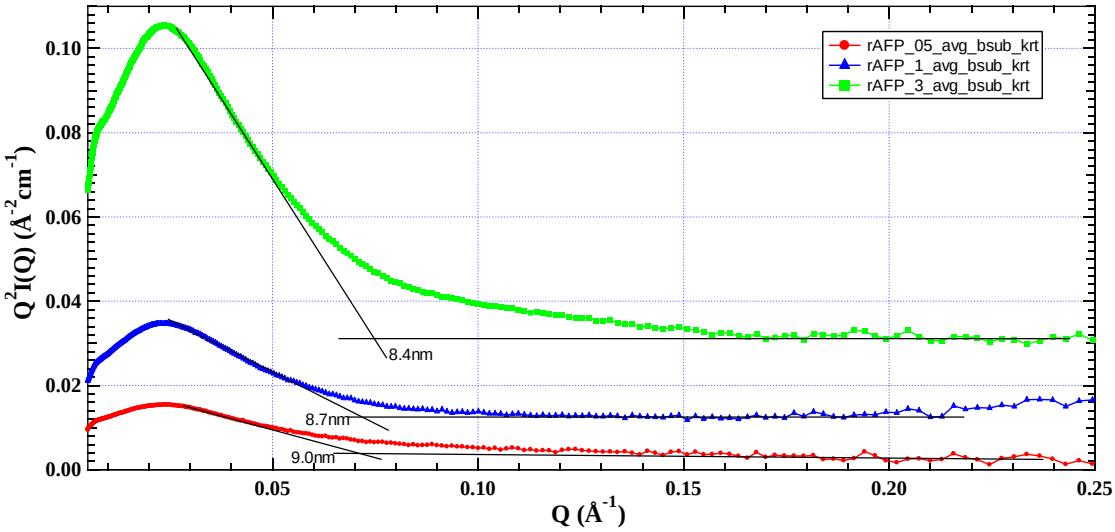

Figure 13. Small Angle X-ray Scattering profiles of AFP by Kratky plots.

3.5 Ice recrystallization inhibition activity

AFPs can interact with ice surfaces, change crystal morphology and inhibit crystallization. The effects of DcAFP on crystal formation were determined by cryomicroscopy. Small ice crystals created by flash-freezing the solution and their growth were monitored microscopically. In Figure 14, the appearance of ice crystals were showed in a control sample (30% sucrose) without DcAFP and with DcAFP following 80 minutes incubation at -6°C. In 30% sucrose solution, ice crystals formed at -18°C. Ice crystallization with0.5mg/ml DcAFP showed crystals formation occurred at -25°C with tiny crystals (left panel). This crystallization inhibition indicated strong interaction of DcAFP with ice surfaces and low surface tension.

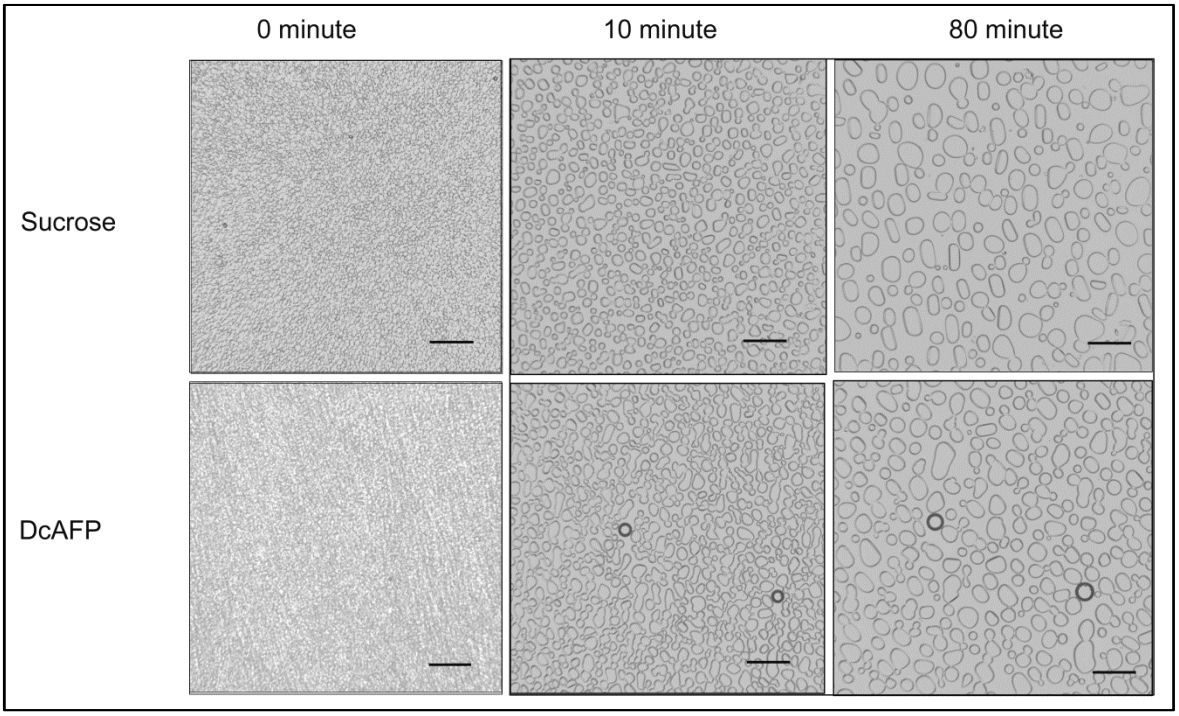

Figure 14. Ice morphology during recrystallization. The scale bar represented 0.1mm.

Ice recrystallization was mimicked by increasing the platform temperature from freezing point to -6°C by 10°C/min and incubated at -6°C. Video camera was used to capture the images. ImageJ software was used to analyze ice crystal numbers and the average diameter at each time point. Each field of view was a 1mm×1mm square area and was fixed for each sample during the incubation time. Ferret Diameter was chosen to represent crystals size. Due to the ice crystal inhibition activity, the number of ice crystal is expected to be higher and crystal size to be lower. Three independent replicates were analyzed and the result is shown in Figure 15.

The first time point was named as “0 minute”, the moment when temperature increased from freezing point to -6°C after rapid thawing. In Figure 14, small ice crystals were formed in sucrose sample, but were not visible or countable in DcAFP sample with smaller size, indicating a slow thawing process.

At the ten minutes point, the ice crystal number was similar in sucrose and DcAFP. During this incubation, ice recrystallization occurred, small crystals thawed and merged into big ones. The ice crystal number in sucrose quickly went down to 356 at the time point of twenty minutes, and DcAFP treated sample had 425 ice crystals. The same trend was observed during the incubation of eighty minutes (Figure 15).

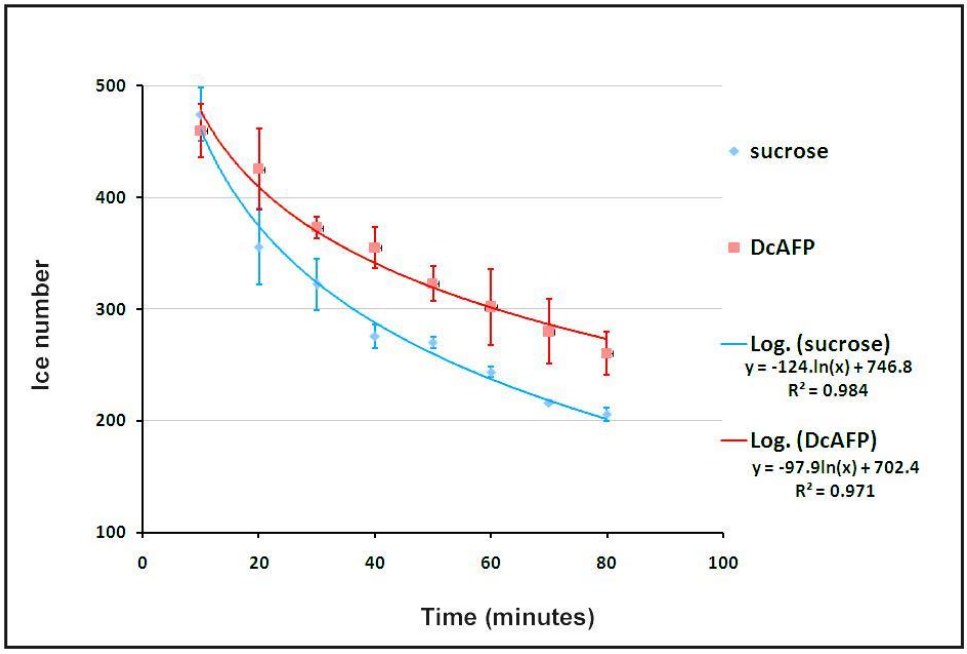

Figure 15. Ice crystal count of DcAFP (red) and sucrose (blue) by time points. (P value <0.05)

Table 1. Ice crystalcount of DcAFP and sucrose solution by time points with the standard deviation.

Time	Ice Countin Sucrose	Ice Count with DcAFP
10 minute	474.33±24.01	459.67±24.01
20 minute	356±33.95	425.33±36.35
30 minute	322.33±23.11	373±9.85
40 minute	276.01±10.53	355±18.36
50 minute	270.33±5.03	322.67±15.56
60 minute	243.66±4.51	302±33.86
70 minute	216.33±2.09	280±28.80
80 minute	205.66±6.05	260.33±19.14

Here we show the best fit from the logarithm model with highest R square value. The coefficient from the sucrose solution was -124.4, significantly different from DcAFP's, -97.91. From the data shown above, recrystallization velocity (slope of the trend line on graph) in DcAFP was smaller than the control sample, indicating slower recrystallization procedure.

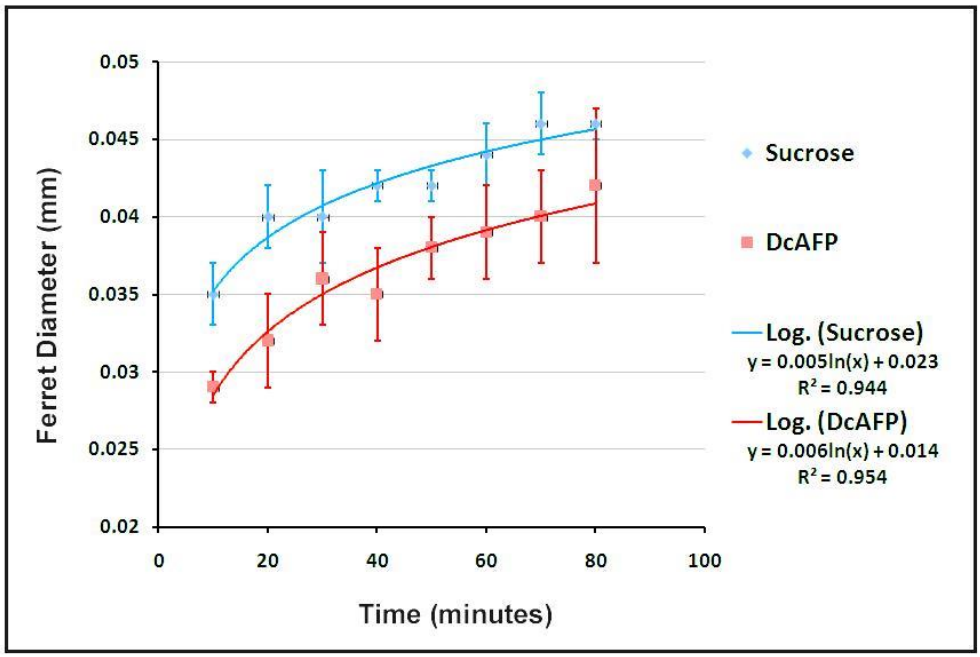

Figure 16. Ferret Diameter Measurement of ice crystals from DcAFP (red) and sucrose (blue) by time points (P value <0.05).

Ferret Diameter was measured to represent crystal size. As shown in Table 2 and Figure 16, velocity from DcAFP and sucrose control were similar. The logarithm model fitted with the highest R square value of 0.9442 and 0.9544 for sucrose and DcAFP. The coefficient was 0.005 for sucrose and 0.006 for DcAFP, suggesting no significant difference. Because of the lack of observation for rapid thawing during the first ten minute, further investigation was required.

With this limitation, the ice recrystallization velocity in DcAFP is similar with sucrose during the stable stage at -6°C (slope from Figure 16). This result suggests that DcAFP may act both in rapid thawing and stable stage (after ten minutes).

Table 2. Ice crystal size (diameter) with and without DcAFP treatment during recrystallization

Time	Ice Diameterin Sucrose	Ice Diameter with DcAFP
10 minute	0.035±0.002mm	0.029±0.001mm
20 minute	0.040±0.002mm	0.032±0.003mm
30 minute	0.040±0.003mm	0.036±0.003mm
40 minute	0.042±0.001mm	0.035±0.003mm
50 minute	0.042±0.001mm	0.038±0.002mm
60 minute	0.044±0.002mm	0.039±0.003mm
70 minute	0.046±0.002mm	0.040±0.003mm
80 minute	0.046±0.001mm	0.042±0.005mm

3.6 In vivo antifreeze activity in *E.coli* cells

DcAFP activity evaluation is crucial both in vivo and in vitro. Since DcAFP was renatured from insoluble fraction, the in vivo activity was tested with basal level of soluble protein is a good beginning.

Low temperature storage (0°C) induces cell injury mainly due to ice crystal formation, recrystallization from small to large crystals, and the rupture of membrane structure. AFPs may bind to ice surface and increase bacterial cell survival rate by inhibiting ice crystal formation. Therefore, the higher survival rate, better the antifreeze activity. The survival rate was calculated using this following equation:

$$Survival\ rate\ (\%) = \frac{\text{CFU of survival cells after cold treatment}}{\text{CFU of original cells without cold treatment}} \times 100$$

In Figure 17, bacteria cells without any induction served as negative control. The results showed that with induced DcAFP in bacteria cells, cold-resistant activity was conferred, and 24 hour treatment displayed more significant increase of cell survival rate from 41% to 60%.

Table 3. Bacterial cell viability test under 0˚C with and without DcAFP expression at different time points

Survival rate %	0 hour	24 hour	48 hour	72 hour
With DcAFP	100%	59.81%	31.78%	28.04%
STDEV%	0.46%	0.51%	0.23%	0.23%
Without DcAFP	100%	41.13%	21.27%	17.02%
STDEV%	0.38%	0.34%	0.26%	0.18%

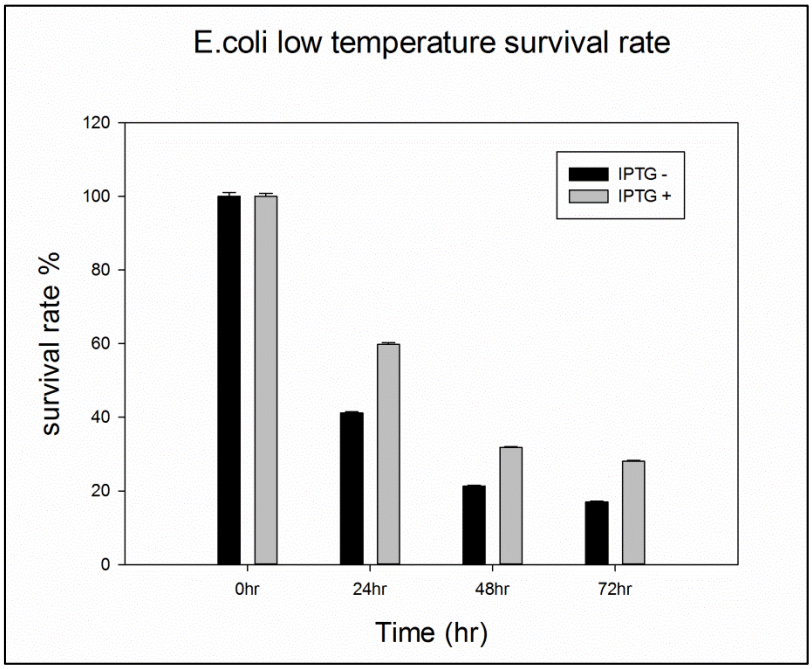

Figure 17. DcAFP affects bacterial survival rate under low temperature of 0˚C.

The CFU (Colony forming units) ratios before and after cold treatment (% viability) are expressed as a function of time with cold treatment. Error bars represent the standard deviations from the means (n=3). Black series: no IPTG induction or DcAFP expression cells. Gray series: after IPTG induction and DcAFP expressed cells. (P value <0.05)

This improvement of survival rate was consistent with the previous report of DcAFP in rice suspension cells. Although it was not as dramatic as the logarithm-reduction in food pathogens, this survival enhancement was reasonable considering our experiment conditions as followings:

Firstly, to limit the variables in experiments, our culture system was very basic. There was an only saline and no organic compound or broth-in order to see the basic responses in bacterial cells. It might be possible that without optimal growth conditions, bacterial cells may function differently than normal.

Secondly, our DcAFP level was relatively low from using induced cells. The better performance was predicted after enrichment or accumulation of DcAFP in bacterial cell system.

3.7 In vitro antifreeze activity in *E.coli* cells

To further validate the protein activity, we performed in vitro assay with additional 0.3 mg/ml DcAFP under 0°C cold storage condition for 24 hours. Cell survival rate with DcAFP was 35 %, higher than 18 % in control samples.

Table 4.Bacterial cell viability test under 0°C with and without DcAFP incubation after 24 hours

Survival rate %	0 hour	24 hour
With DcAFP	100%	35.23%
STDEV%	0.46%	0.14%
Without DcAFP	100%	18.18%
STDEV%	0.38%	0.35%

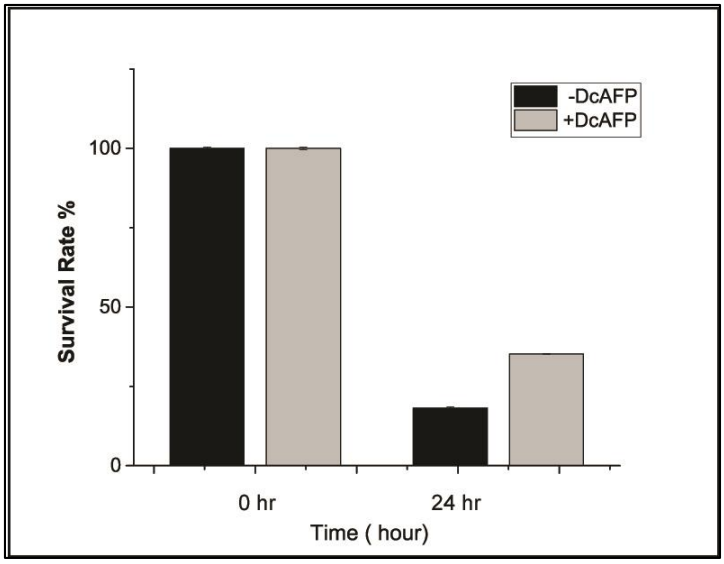

Figure 18.Bacterial cell viability test under 0˚C with and without DcAFP incubation after 24 hours.

Error bars represent the standard deviations from the means (n=3). Black series: cells without additional DcAFP. Gray series: DcAFP treated cells. (P value <0.05)

This result showed that adding renatured DcAFP into the bacterial cell culture improved cell viability from 18% to 35%, suggesting partial activity gained through renaturation. This data provided us a foundation using renatured protein into a novel system: lactobacillus. In addition, optimizing the renature procedure for higher activity recovered is feasible in the future.

3.8 In vitro antifreeze activity in *E.coli* cells with freeze-thaw

A freeze-thaw cycle from -80˚C to 25˚C to IPTG-induced *E.coli* cells with soluble DcAFP was examined. In a cycle from -80˚C to 25˚C in five minutes, few control cells survived (Figure 19). However, even with induced DcAFP, the survival rate did not increase significantly. Based on our previous results from renatured DcAFP, the activity was lower than the native form, so the freeze-thaw cycle treatment to renatured DcAFP was not performed.

This drastic temperature shift from -80°C to 25°C in a short time led to the cell membrane turning into liquid when ice crystals burst. During this process, cell membrane was easily broken and there was no mild procedure to recover their double-layer structure. From the result, the presence of DcAFP can prevent the damage so no obvious improvement on bacterial cell survival rate was observed.

Table 5. Bacterial cell survival rate treated with DcAFP after freeze-thaw cycle from room temperature, 25°C to -80°C.

Survival rate %	0 Cycle	1 Cycle
With DcAFP	100%	4.73%
STDEV%	0.25%	0%
Without DcAFP	100%	2.17%
STDEV%	0.08%	0.47%

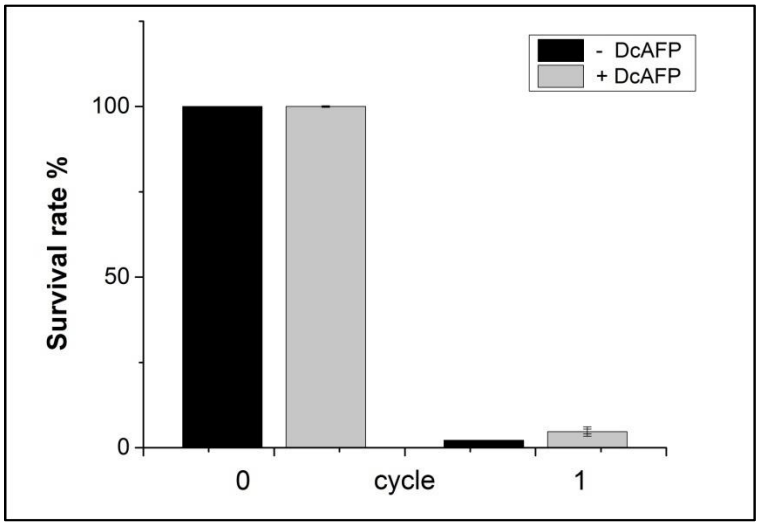

Figure 19. Stability of freeze-thaw treated bacterial cells with and without DcAFP expression.

Error bars represent the standard deviations from the means (n=3).Black series: no IPTG induction or DcAFP expression cells. Gray series: after IPTG induction and DcAFP expressed cells. (P value <0.05)

3.9 In vitro antifreeze activity in *Lactobacillus plantarum* cells

The effects of renatured DcAFP on *lactobacillus plantarum* cells were examined. As reported in Figure 20, the DcAFP affected cell viability of Lactobacillus *plantarum* was compared with the control sample.

DcAFP treated cells had higher viability in the first and fourth week than the control cells. During the first and second week, improved cold stress adaptation was found. Similar adaptation was reported in *Lactobacillus plantarum* with heat-shock and cold-shock proteins and monitored by OD_{600} value.

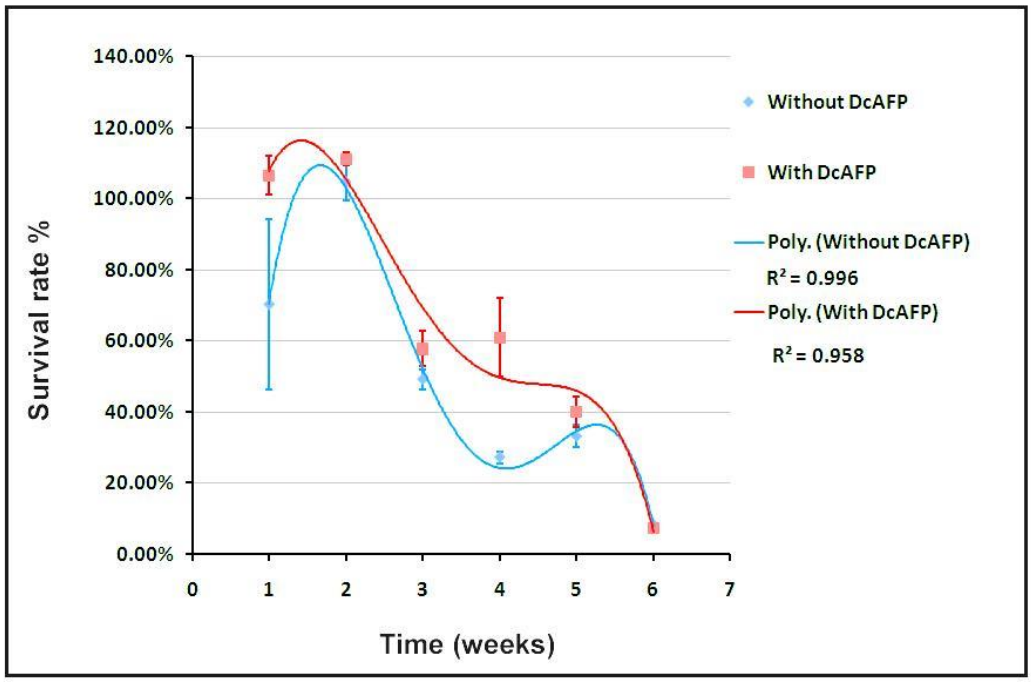

Figure 20.Survival of *Lactobacillus plantarum* with and without DcAFP treatment under cold tolerance (0˚C).

Red line: viability of DcAFP treated cells, black line: viability of control cells. Data presented were the mean value of three replicates with their standard deviations indicated by vertical bars.

Table 6.Survival of *Lactobacillus plantarum* with and without DcAFP treatment under cold tolerance (0˚C).

Time	Cell Viability without DcAFP	Cell Viability with DcAFP
Week 1	70.28%±24.05%	106.60%±5.48%
Week 2	104.67%±5.08%	111.14%±1.89%
Week 3	49.20%±2.89%	57.88%±5.01%
Week 4	27.30%±1.59%	61.07%±11.15%
Week 5	33.16%±3.14%	40.23%±4.31%
Week 6	8.32%±0.52%	7.59%±0.52%

An improved cold tolerance was observed at fourth week with DcAFP treatment. DcAFP cell viability was 60% compare to control cells' 30%. After five weeks, viability went down in both samples. This loss of DcAFP function might be caused by protein denaturation without any protection or buffering. The data fit a polynomial model with the highest R square value was 0.9961 and 0.9581 for sucrose and DcAFP samples. The coefficient from this model was quite different with -0.0379 and -0.0213 for non-DcAFP and DcAFP treated cells, respectively.

From this result, the function of DcAFP in keeping cell viability during the cold tolerance has been investigated. It suggested that DcAFP may function well in keeping cell viability in the cold-sensitive strains. Application of various protections may be involved with DcAFP treatment in the future to improve cold-tolerance. Buffering systems such as PBS and Tris-Cl may be applied to keep protein activity during long time storage. In addition, biopolymer or small molecular compound may help such as sucrose, glycerol or polysaccharide. They could increase

the viscosity of cell culture suspension, remaining liquid phase without frozen. Therefore, cell viability with DcAFP treatment might be further improved.

3.10 Conclusions

DcAFP was expressed in recombinant bacteria cells, renatured using serial dilution and 10 kDa Microcon ultrafiltration device (Amicon). DcAFP protein was identified by SDS-PAGE and mass spectrum.

The circular dichroism spectra revealed that the major structure in DcAFP was 44.40% beta-sheet and 21.50% beta turn. This is the first report that DcAFP's secondary structure has been revealed using circular dichroism, suggesting a potential ice-binding plane. The protein's aggregation is similar with a polymer chain and the length of the principal axis of the protein is around 9 nm by using Small Angle X-ray Scattering.

This study gave a good example on quantifying the recrystallization inhibition activity (RI) by image analysis. Dynamic change has not been reported by quantifying crystal number and diameter. By measuring these two parameters during incubation, ice recrystallization process can be examined as a continuous alteration. The number of ice crystals dropped from 475 to 280 per millimeter square with DcAFP, and from 490 to 200 without DcAFP. The number was almost 30% higher than the control sample crystals after 80 minutes. The diameter of crystals increased from 0.028mm to 0.041mm with DcAFP, while the amount without DcAFP increased from 0.035mm to 0.046mm .The result indicated that ice recrystallization was inhibited with DcAFP present.

In this study, the function of DcAFP has been investigated in both bacterial *E.coli* and lactic acid cells. In *E.coli* cells, soluble DcAFP expressed in cell and renatured DcAFP were applied to cell culture in saline without any preservatives at $0^{\circ}C$.

After 8 hour, cell viability difference became obvious. At 24 hour, cell viability difference was remarkable. Based on this observation, the 24 hour time point was chosen for further study with best DcAFP performance to protect cells under low temperature.

Renatured DcAFP showed that antifreeze activity improved cell viability at 24 hour .The application of recombinant DcAFP on *lactobacillus* under zero degree was investigated. Unlike *E.coli*, *lactobacillus plantarum* has better tolerance under cold stress. So we applied six weeks treatment and found the best storage time was during the first and fourth week. Cells with DcAFP displayed a higher viability with 106.60 % and 61.07 % compare to 70.28 % and 27.30 % respectively at the first and fourth week.

Lagged effective time of DcAFP on *lactobacillus* was observed as an interesting phenomenon. This difference may due to different cell structures. *E.coli* is Gram-negative rod-shaped bacterium, cell membrane is composed of a thin layer of peptidoglycan between the outer membrane and inner membrane, so it is relatively easy to be stained and allow the penetration of secret protein. In contrast, lactobacillus cells are Gram-positive with a thick cell wall surrounded by many layers of peptidoglycan. This thick layer of peptidoglycan may delay the penetration time for protein on membranes. Current research has been done on constructing the genetic modified *lactobacillus* by transforming the vector with DcAFP gene. However, the lactobacillus cells are resistant to Kanamycin, which is the selective gene in the DcAFP vectors. Therefore, the lactobacillus cell selection test needs to be done in order to differentiate the transformed cells.

In summary, the addition of DcAFP can efficiently improve cell survival rate on lactobacillus suggest their potential application in probiotic food formulation.

4. Future Work:

This research has investigated the efficient method to produce DcAFP, revealed the secondary structure and application in maintaining bacterial cell viability. The future research on DcAFP can be in the following areas:

1) Improve the DcAFP productivity and activity:

DcAFP attracted many research interests. It is still unclear how to produce renatured functional DcAFP in efficiently. Our method used here recovered partial protein activity but can be improved in the future in the following areas: cell culture condition, purification procedure and using yeast as an expression system to obtain secreted proteins.

The optimal cell culture condition may affect correct protein folding with high expression level. This study applied normal condition as 37°C with 200rpm to produce the maximum product of insoluble fraction. If lower temperature applied to bacterial cells with 150-180 rpm, cell replication rates with protein expression rates will be lower; this may increase the probability of expressing soluble proteins.

Purification procedure can be further optimized. Our method shortened the purification time, but the trace amount of salt ion might not be fully removed. Therefore, multiple washing with buffer or water would eliminate urea in final product and higher refolded protein ratio will be predicted.

The last improvement on protein expression is using yeast cell as an expression system especially for secretive protein or membrane protein. *Pichia pastoris* and *Saccharomyces cerevisiae* have been widely used to produce secreted functional heterologous protein [54,55] . Once the target protein has been secreted into culture, further purification will be much easier than the one from broken cell which has large amount of unwanted proteins and cell membrane fragments.

However, the activity of target protein after secretion from yeast cell still need to be monitored carefully[56].

2) Structure characteristics of DcAFP:

To better understand the morphology and interaction of DcAFP, fluorescence tag labeled DcAFP protein will be used. Our Circular Dichroism data showed that DcAFP has a beta-structure dominated profile, indicating an interaction layers to bind ice and water. However, the ice binding sites have not been discovered. Based on the *DcAFP* sequence, it is convenient to add the fluorescent label such as *GFP* (Green Fluorescence Protein) with *DcAFP* gene for co-expression. Through measuring the fluorescence intensity and mapping the location, dynamic detail of DcAFP binding with ice crystals can be easily observed and further quantified at different time points.

At the same time, the truncation of *DcAFP* gene based on our construction is applicable. Key functional domain will be examined and deleted by mutagenesis from *DcAFP* gene sequence. The truncated protein will be expressed and their binding affinity and activity to inhibit ice crystal recrystallization will be determined following the antifreeze assay applied in this research. If the truncated protein loses most of activity, it indicates the truncated piece is the key or active domain of DcAFP.

3) Application of DcAFP in food systems:

AFPs have been approved by FDA as GRAS (Generally Recognized As Safe) and applied into ice cream to reduce ice crystal size, and bring a more smooth oral feel after the freeze-thaw cycle during storage and shipping[15,57,58,59,60]. AFPs used in these applications were fish-orientated AFPs of type I and III.

In order to obtain the GRAS level DcAFP, the following work will be incorporating DcAFP gene into yeast cell and produce functional proteins. Yeast can be genetically modified to have a secret signal to obtain secreted AFPs[61]. Walker et al reported that AFPs with disulfide bonds and threonine-rich with glycosylation can be expressed and secreted in a yeast expression system (*Pichiapastoris*), but the ice crystal inhibition activity is low. Fish AFPs expression with disulfide bonds without potential threonine glycosylation can be produced successfully[56].

The synergistic impacts of DcAFP with other molecules such as polysaccharide to improve probiotic cell viability can be studied. In our research, DcAFP without buffer or biopolymers has been shown to improve cell viability. During storage period, DcAFP may lose their function by denaturation. If optimal conditions such as pH, salt concentration and applied to DcAFP with bacterial cell mixture, cell viability is expected to be further improved.

Furthermore, the viability of lactic bacteria, *Lactobacillus rhamnosus* has been reported to be improved with the presence and interactions with dietary fibers during freeze-drying and storage[47]. Therefore, dietary fibers and polysaccharide such as starch can be combined with DcAFP by encapsulating the lactic bacteria cells to provide a functional barrier to harsh environment and maintain cell viability.

5. References:

1. Fletcher GL, Kao MH, Fourney RM (1986) Antifreeze peptides confer freezing resistance to fish. Canadian Journal of Zoology 64: 1897-1901.
2. Goddard SV, Kao MH, Fletcher GL (1992) Antifreeze Production, Freeze Resistance, and Overwintering of Juvenile Northern Atlantic Cod (Gadus morhua). Canadian Journal of Fisheries and Aquatic Sciences 49: 516-522.
3. Davies PL, Hew CL (1990) Biochemistry of fish antifreeze proteins. The FASEB Journal 4: 2460-2468.
4. Hon WC, Griffith M, Chong P, Yang DSC (1994) Extraction and Isolation of Antifreeze Proteins from Winter Rye (Secale cereale L.) Leaves. Plant Physiol 104: 971-980.
5. Hon WC, Griffith M, Mlynarz A, Kwok YC, Yang DSC (1995) Antifreeze Proteins in Winter Rye Are Similar to Pathogenesis-Related Proteins. Plant Physiol 109: 879-889.
6. Davies PL, Sykes BD (1997) Antifreeze proteins. Current Opinion in Structural Biology 7: 828-834.
7. Duman J, Xu L, Neven L, Tursman D, Wu D (1991) Hemolymph Proteins Involved in Insect Subzero-Temperature Tolerance: Ice Nucleators and Antifreeze Proteins. In: Lee R, Jr., Denlinger D, editors. Insects at Low Temperature: Springer US. pp. 94-127.
8. Wu D, Duman J (1991) Activation of antifreeze proteins from larvae of the beetle Dendroides canadensis. Journal of Comparative Physiology B 161: 279-283.
9. Osuga DT, Feeney RE (1978) Antifreeze glycoproteins from Arctic fish. Journal of Biological Chemistry 253: 5338-5343.
10. Tachibana Y, Fletcher GL, Fujitani N, Tsuda S, Monde K, et al. (2004) Antifreeze Glycoproteins: Elucidation of the Structural Motifs That Are Essential for Antifreeze Activity. Angewandte Chemie 116: 874-880.
11. Atıcı Ö, Nalbantoğlu B (2003) Antifreeze proteins in higher plants. Phytochemistry 64: 1187-1196.
12. Wu D, Duman J, Cheng C-H, Castellino F (1991) Purification and characterization of antifreeze proteins from larvae of the beetle Dendroides canadensis. Journal of Comparative Physiology B 161: 271-278.
13. Graether SP, Kuiper MJ, Gagne SM, Walker VK, Jia Z, et al. (2000) [beta]-Helix structure and ice-binding properties of a hyperactive antifreeze protein from an insect. Nature 406: 325-328.
14. Worrall D, Elias L, Ashford D, Smallwood M, Sidebottom C, et al. (1998) A Carrot Leucine-Rich-Repeat Protein That Inhibits Ice Recrystallization. Science 282: 115-117.
15. Clarke CJ, Buckley SL, Lindner N (2002) Ice Structuring Proteins--A New Name for Antifreeze Proteins. Cryoletters 23: 89-92.
16. Jia Z, Davies PL (2002) Antifreeze proteins: an unusual receptor–ligand interaction. Trends in Biochemical Sciences 27: 101-106.
17. Hassas-Roudsari M, Goff HD (2012) Ice structuring proteins from plants: Mechanism of action and food application. Food Research International 46: 425-436.
18. Hassas-Roudsari M, Goff HD (2012) A New Quantitative Method to Measure Activity of Ice Structuring Proteins Using Differential Scanning Calorimetry. Cryoletters 33: 117-124.
19. Griffith M, Antikainen M, Hon W-C, Pihakaski-Maunsbach K, Yu X-M, et al. (1997) Antifreeze proteins in winter rye. Physiologia Plantarum 100: 327-332.
20. Griffith M, Yaish MWF (2004) Antifreeze proteins in overwintering plants: a tale of two activities. Trends in Plant Science 9: 399-405.
21. Middleton AJ, Brown AM, Davies PL, Walker VK (2009) Identification of the ice-binding face of a plant antifreeze protein. FEBS Letters 583: 815-819.

22. Antikainen M, Griffith M, Zhang J, Hon WC, Yang DSC, et al. (1996) Immunolocalization of Antifreeze Proteins in Winter Rye Leaves, Crowns, and Roots by Tissue Printing. Plant Physiol 110: 845-857.
23. Meyer K, Keil M, Naldrett MJ (1999) A leucine-rich repeat protein of carrot that exhibits antifreeze activity. FEBS Letters 447: 171-178.
24. Fan Y, Liu B, Wang H, Wang S, Wang J (2002) Cloning of an antifreeze protein gene from carrot and its influence on cold tolerance in transgenic tobacco plants. Plant Cell Reports 21: 296-301.
25. Boonsupthip W, Lee T-C (2003) Application of Antifreeze Protein for Food Preservation: Effect of Type III Antifreeze Protein for Preservation of Gel-forming of Frozen and Chilled Actomyosin. Journal of Food Science 68: 1804-1809.
26. Regand A, Goff HD (2006) Ice Recrystallization Inhibition in Ice Cream as Affected by Ice Structuring Proteins from Winter Wheat Grass. Journal of Dairy Science 89: 49-57.
27. Zhang C, Zhang H, Wang L (2007) Effect of carrot (Daucus carota) antifreeze proteins on the fermentation capacity of frozen dough. Food Research International 40: 763-769.
28. Zhang D-q, Shun-Yang D, Shao-Gang F, Zheng-Jun G, Qing-Yun H, et al. Application of Engineered Carrot Antifreeze Protein in the Cryopreservation of Rice Cells by Adsorbing into Ice surface to Inhibit Recrystallization; 2009. pp. 1-4.
29. Gerez CL, Font de Valdez G, Gigante ML, Grosso CRF (2012) Whey protein coating bead improves the survival of the probiotic Lactobacillus rhamnosus CRL 1505 to low pH. Letters in Applied Microbiology 54: 552-556.
30. Capicciotti CJ, Doshi M, Ben RN (2013) Ice Recrystallization Inhibitors: From Biological Antifreezes to Small Molecules.
31. Yeh C-M, Kao B-Y, Peng H-J (2009) Production of a Recombinant Type 1 Antifreeze Protein Analogue by L. lactis and Its Applications on Frozen Meat and Frozen Dough. Journal of Agricultural and Food Chemistry 57: 6216-6223.
32. Kwan AHY, Fairley K, Anderberg PI, Liew CW, Harding MM, et al. (2005) Solution Structure of a Recombinant Type I Sculpin Antifreeze Protein. Biochemistry 44: 1980-1988.
33. Zhang D-Q, Liu B, Feng D-R, He Y-M, Wang J-F (2004) Expression, purification, and antifreeze activity of carrot antifreeze protein and its mutants. Protein Expression and Purification 35: 257-263.
34. Muryoi N, Sato M, Kaneko S, Kawahara H, Obata H, et al. (2004) Cloning and Expression of afpA, a Gene Encoding an Antifreeze Protein from the Arctic Plant Growth-Promoting Rhizobacterium Pseudomonas putida GR12-2. J Bacteriol 186: 5661-5671.
35. Cruz AG, Antunes AEC, Sousa ALOP, Faria JAF, Saad SMI (2009) Ice-cream as a probiotic food carrier. Food Research International 42: 1233-1239.
36. Mattila-Sandholm T, Myllärinen P, Crittenden R, Mogensen G, Fondén R, et al. (2002) Technological challenges for future probiotic foods. International Dairy Journal 12: 173-182.
37. Collins JK, Thornton G, Sullivan GO (1998) Selection of Probiotic Strains for Human Applications. International Dairy Journal 8: 487-490.
38. Salminen S, von Wright A, Morelli L, Marteau P, Brassart D, et al. (1998) Demonstration of safety of probiotics — a review. International journal of food microbiology 44: 93-106.
39. van Bokhorst-van de Veen H, Abee T, Tempelaars M, Bron PA, Kleerebezem M, et al. (2011) Short- and Long-Term Adaptation to Ethanol Stress and Its Cross-Protective Consequences in Lactobacillus plantarum. Applied and Environmental Microbiology 77: 5247-5256.
40. Nualkaekul S, Deepika G, Charalampopoulos D (2012) Survival of freeze dried Lactobacillus plantarum in instant fruit powders and reconstituted fruit juices. Food Research International 48: 627-633.

41. Ferraz JL, Cruz AG, Cadena RS, Freitas MQ, Pinto UM, et al. (2012) Sensory Acceptance and Survival of Probiotic Bacteria in Ice Cream Produced with Different Overrun Levels. Journal of Food Science 77: S24-S28.
42. Knorr D (1998) Technology aspects related to microorganisms in functional foods. Trends in Food Science & Technology 9: 295-306.
43. Shah NP (2000) Probiotic Bacteria: Selective Enumeration and Survival in Dairy Foods. Journal of Dairy Science 83: 894-907.
44. Nualkaekul S, Charalampopoulos D (2011) Survival of Lactobacillus plantarum in model solutions and fruit juices. International journal of food microbiology 146: 111-117.
45. Talwalkar A, Kailasapathy K (2004) A Review of Oxygen Toxicity in Probiotic Yogurts: Influence on the Survival of Probiotic Bacteria and Protective Techniques. Comprehensive Reviews in Food Science and Food Safety 3: 117-124.
46. Saarela M, Virkajärvi I, Alakomi H-L, Sigvart-Mattila P, Mättö J (2006) Stability and functionality of freeze-dried probiotic Bifidobacterium cells during storage in juice and milk. International Dairy Journal 16: 1477-1482.
47. Saarela M, Virkajärvi I, Nohynek L, Vaari A, Mättö J (2006) Fibres as carriers for Lactobacillus rhamnosus during freeze-drying and storage in apple juice and chocolate-coated breakfast cereals. International journal of food microbiology 112: 171-178.
48. Fiocco D, Capozzi V, Goffin P, Hols P, Spano G (2007) Improved adaptation to heat, cold, and solvent tolerance in Lactobacillus plantarum. Applied Microbiology and Biotechnology 77: 909-915.
49. Derzelle S, Hallet B, Ferain T, Delcour J, Hols P (2003) Improved Adaptation to Cold-Shock, Stationary-Phase, and Freezing Stresses in Lactobacillus plantarum Overproducing Cold-Shock Proteins. Applied and Environmental Microbiology 69: 4285-4290.
50. Wouters JA, Rombouts FM, Kuipers OP, de Vos WM, Abee T (2000) The Role of Cold-Shock Proteins in Low-Temperature Adaptation of Food-Related Bacteria. Systematic and Applied Microbiology 23: 165-173.
51. Cao-Hoang L, Dumont F, Marechal PA, Gervais P (2010) Inactivation of Escherichia coli and Lactobacillus plantarum in relation to membrane permeabilization due to rapid chilling followed by cold storage. Archives of Microbiology 192: 299-305.
52. Johansen D, Trewhella J, Goldenberg DP (2011) Fractal dimension of an intrinsically disordered protein: Small-angle X-ray scattering and computational study of the bacteriophage λ N protein. Protein Science 20: 1955-1970.
53. Fitzkee NC, Rose GD (2004) Reassessing random-coil statistics in unfolded proteins. Proceedings of the National Academy of Sciences of the United States of America 101: 12497-12502.
54. López-García B, Moreno AB, San Segundo B, De los Ríos V, Manning JM, et al. (2010) Production of the biotechnologically relevant AFP from Aspergillus giganteus in the yeast Pichia pastoris. Protein Expression and Purification 70: 206-210.
55. Driedonks RA, Toschka HY, van Almkerk JW, Schäffers IM, Verbakel JMA (1995) Expression and secretion of antifreeze peptides in the yeast Saccharomyces cerevisiae. Yeast 11: 849-864.
56. Tyshenko MG, d'Anjou M, Davies PL, Daugulis AJ, Walker VK (2006) Challenges in the expression of disulfide bonded, threonine-rich antifreeze proteins in bacteria and yeast. Protein Expression and Purification 47: 152-161.
57. Clemmings JF, Huang VT, Rosenwald DR, Zoerb HF (1997) Method of making ice cream. US Patent 5,620,732.
58. Byass LJ, Darling DF, Doucet CJ, Fenn RA, Lillford PJ, et al. (2000) Frozen food product. US Patent 6,096,867.

59. Crilly JF, Russell AB, Cox AR, Cebula DJ (2008) Designing Multiscale Structures for Desired Properties of Ice Cream. Industrial & Engineering Chemistry Research 47: 6362-6367.
60. Daniel A, Fenn RA, Oldroyd JR (2002) Ice confection. US Patent 6,436,460.
61. Barney MC, Bower PA, Chicoye E, Cronan CL, Huige N, et al. (1998) Method of expressing antifreeze proteins in yeast. US Patent 5,849,537.