Antifreeze Proteins and their Enhancement of Frozen Food Quality

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I. Abstract

Artificial freezing has emerged as the most important method of preventing food spoilage, which is promoted largely by the high-water content in food items. However, the quality of organic frozen foods is undermined by ice recrystallization (IR), in which larger crystals grow while smaller crystals disappear. IR is exacerbated by temperature fluctuations, slow cooling rates, and high temperature storage, which are common conditions frozen food is exposed to in several steps between manufacture and consumption. The larger crystals formed are detrimental to food quality in that they produce a coarser texture in foods eaten frozen, such as ice cream. They are also damaging to defrosted organic foods since they can rupture cell walls, causing a drip that results in a loss of nutrients. In response to this issue, antifreeze proteins (AFPs) have been extensively studied, the incentive deriving from their documented crystal growth retardation activities. This paper provides a comprehensive understanding of the mechanisms of ice recrystallization, the role of antifreeze proteins in its inhibition as well as alternative methods available to inhibit it. This understanding is crucial to the optimalization of the maintenance of food quality and the extension of its shelf-life.

II. Introduction

Significant research has been devoted to accommodating the needs of an ever-growing population. The industrialization of food production gave rise to the focus on food preservation in order to allow for bulk production and to make food available to a variety of areas via transit. The main source of food deterioration is the water contained in tissue-comprised food items (USDA, 2021). 60-80% of the mass of muscles and 90% of plant tissue is comprised of water, which enables inhabitation by microorganisms as well as chemical reactions that lead to ripening (Nesvadba, 2008). According to the Economic Research Service of the USDA, an estimated 31%

of food supply is wasted between manufacture and consumption (USDA, 2021). This enormous amount is largely attributed to spoilage which plays a role in every step of the industrial and supply processes. The economic concern of this reality notwithstanding, the environmental ramifications of masses of food waste filling landfills is also an important issue. A significant milestone in the mission of food preservation came with the invention of artificial freezing, even though there is still much improvement to be made.

Storing food at subzero temperatures helps mitigate the effects of perishability by preventing bacterial contamination and inhibiting the ripening effect. During freeze storage, liquid water within the tissue crystalizes, a process comprised of the nucleation and growth steps. In nucleation, water molecules aggregate from the melt to solidify into an embryonic ice particle, and then additional water molecules orient onto the nucleus in an organized fashion, causing the growth of the crystal (Chen et al. 2020). However, the quality of frozen foods is compromised due to the effect of ice recrystallization (IR) which occurs most pronouncedly during the temperature fluctuations that accompany freezing and thawing and during long-term storage. IR describes the phenomenon in which the growth of larger crystals is favored at the expense of smaller ice crystals. This shift in the size distribution of ice crystals is detrimental to the integrity of the product since these larger crystals can rupture cell surfaces. As the product is defrosted, these crystals melt, causing a loss in its nutrients, taste, and capacity to hold water, all of which contribute to a lower quality of the item (Gruneberg et al., 2021).

The solution to the issue of damages caused by crystallization is provided by nature, in organisms that live in cold climates. Extremophiles including some kinds of fish, bacteria, insects, and plants, are documented as having proteins that inhibit the formation and growth of ice crystals in their tissue (Griffith et al., 1995). These proteins, called antifreeze proteins (AFPs)

or ice-binding proteins (IBPs) promote survival in arctic climates by adsorbing to the surface of crystals formed in the bloodstream which could otherwise be detrimental to their cellular membranes. Utilizing the inhibition activity of these proteins as well as synthetic mimics that also display growth inhibition can be a viable way of effectively preventing the manifestation of damaging crystals and consequently enhance the storage of food.

III. Ice Recrystallization Mechanisms

Ice recrystallization proceeds through three mechanisms, as they are outlined by Ndoye *et al*. and depicted in Fig 1. The first occurs in the earlier stages of recrystallization, in which adjacent crystals fuse to form one larger crystal. The second mechanism occurs when the temperature temporarily increases, causing smaller crystals to melt preferentially, and the liquid from this melt to migrate to the lower temperature region surrounding the enduring larger crystals. The colder local temperature facilitates the organization of the water molecules onto the crystal, causing the already large crystal to increase in size. The third mechanism drives the crystals to form smoother and sharper crystals that are thermodynamically favorable and even more damaging to the surface of cells (Ndoye et al., 2015).

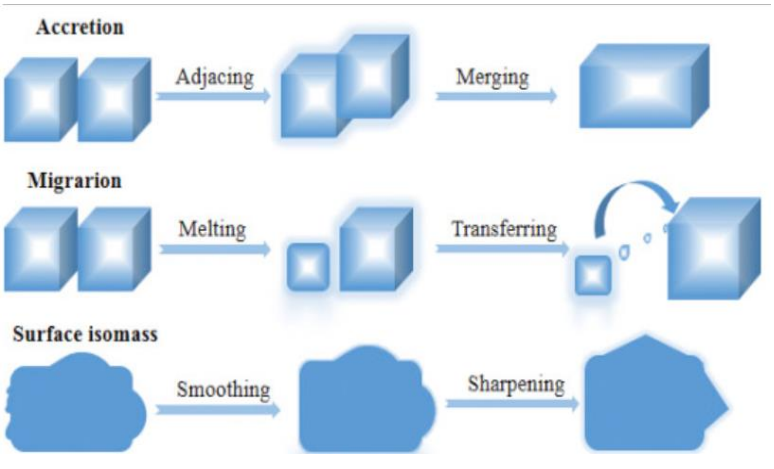

Fig 1: The three mechanisms of ice recrystallization (Ndoye, 2015)

Of the three mechanisms, the second, called migration, is most focused on in literature and is generally considered to be the most significant driving force of IR. It proceeds through Oswald Ripening, which is the result of the unequal energy states of water molecules on different parts of the crystal and is particularly sensitive to temperature fluctuations (Fig 2). Internal molecules are stabilized by interactions with neighboring molecules, and molecules on the surface are in contrast high in energy and easily detachable. Smaller crystals contain, as a result, relatively more high energy molecules than larger crystals do. An instantaneous increase in temperature causes surface molecules of these small, energetically unfavorable crystals to surpass the energy threshold and dissociate from it in order to lower the overall energy. When the colder temperature is restored, the high concentration of free water molecules in solution promotes their rapid aggregation onto the surface of the larger crystals (Ndoye et al., 2015). Thus, the evolution of the collection of crystals is driven by an environmental pressure exerted on the solution which preferentially selects for the "fittest" and most stable crystals, shifting the average crystal size such that the frequency of the larger crystals increases.

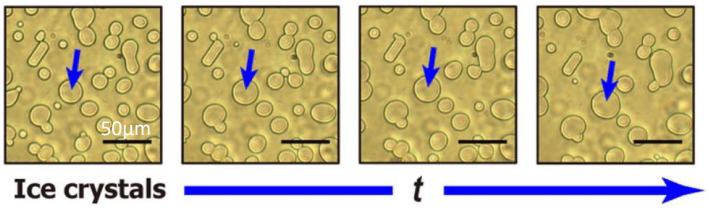

Fig 2: The disappearance of small crystals and subsequent increase in large crystal diameter over time. The large crystal visible gaining in size is denoted by the blue arrow, and the size bar represents 50µm (Rahman et al., 2019)

The fitness of these crystals comes at the expense of that of the organic tissue. Cell damage following crystallization of ice is induced by a variety of pathways, as listen by Tomares (Fig 3). Firstly, extracellular crystals have sharp edges that can tear through the plasma membrane of cells, a phenomenon facilitated by recrystallization. Additionally, crystals formed

intracellularly can damage structures inside the cell and trigger a cascade of signals culminating in cell death. Lastly, generation of crystals from the melt increases the osmolarity of the extracellular fluid since solute particles are largely excluded from the lattice structure. This exerts an osmotic pressure on the cells and causes their subsequent dehydration as water escapes the cell to reestablish osmotic equilibrium (Tomares, 2018).

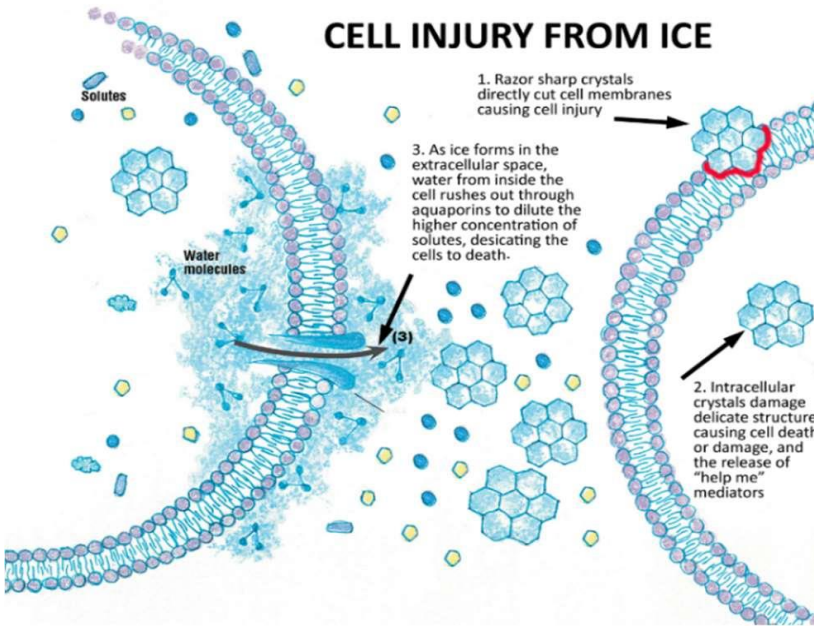

Fig 3: mechanisms of crystal-induced cell damage (Tomares, 2018)

IV. Ice Growth Inhibition by Antifreeze Proteins

In living organisms, this problem is addressed by antifreeze proteins, which have been found to inhibit the growth of ice crystals in arctic species and species occasionally exposed to subzero climates. Specifically, three aspects of the activity of these proteins is causing thermal hysteresis (TH) in the ice crystals, ice recrystallization inhibition (IRI), and changing the morphology of crystals formed from the melt. TH is the gap created between the apparent freezing and melting temperatures of the crystals. This effect is valuable since the crystal will maintain its size at higher degrees of supercooling, stabilizing it across a temperature range

below when it would normally freeze and cause damage. Similarly, IRI displays the ability of AFPs to promote smaller, less harmful crystals instead of exhibiting the shift to larger crystals that occurs through the mechanisms listed above. This activity of AFPs has been shown to successfully reduce adverse hypothermic effects in arctic organisms in which they are upregulated (Venketesh et al., 2008).

V. Adsorption Mechanism

The mechanism of inhibition caused by AFPs has emerged from the evidence of several studies to involve binding onto the ice surface. TH activity, for example, is understood to occur through the Kelvin effect, in which the adsorption of AFPs to the crystal surface causes the surface to grow as a curved interface into the liquid. The growth of the crystal is forced to proceed in the gaps between the proteins, yielding a curved surface rather than the usual flat one (Fig 4). Subsequent growth becomes less thermodynamically favorable since new water molecules will join onto a high energy surface that is less stabilized by neighboring molecule. The resulting crystals require a higher degree of supercooling to grow while their melting temperatures are unchanged, which creates the measurable parameter of thermal hysteresis (Kuiper et al., 2017).

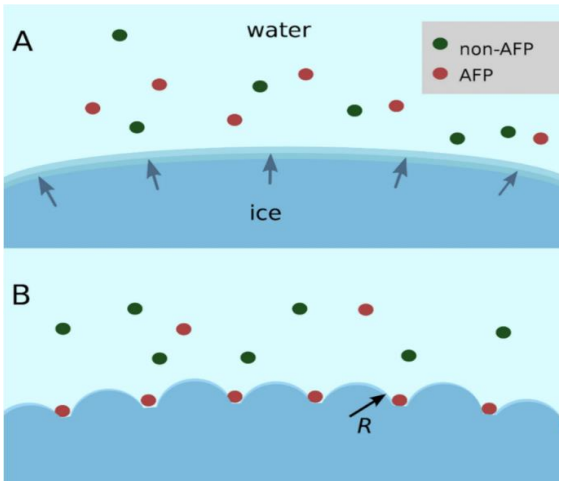

Fig 4: curvature on crystal surface induced by AFP adsorption (Kuiper et al., 2015)

An additional study found that while ice crystals normally grow as planar disks at temperatures just below 0°C, in the presence of AFPs in sufficiently high concentrations, the morphology of these crystals evolves into a faceted, needle-like shape (Griffith, 1995). The physical inhibition caused by AFPs on the crystal face creates a facet parallel to that plane, and the crystal instead grows along the opposing axis, as is demonstrated in Fig 5.

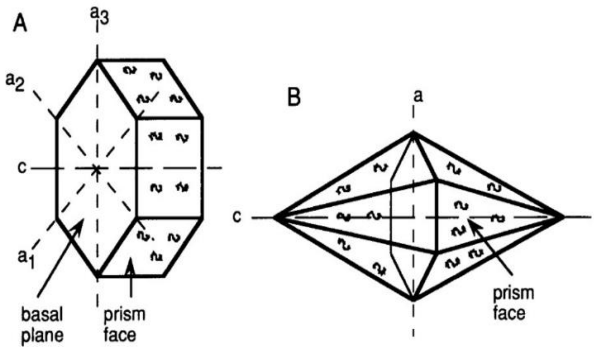

Fig 5: crystal growth along its axis. (B) shows the characteristic growth of a crystal acted upon by antifreeze proteins (Griffith 1995)

When ice crystals grow in pure water, growth proceeds along axis a1, a2, and a3, parallel to the basal plane (Fig 5). The binding of AFPs to the prism faces instead causes elongation of the crystals along the c-axis, and the crystal appears faceted (Griffith 1995). This evidence supports the postulated mechanism of the Kelvin effect whereby the direct binding of AFPs to the ice surface obstructs growth in that direction. The selective binding of AFPs to the crystal planes point to a specific affinity of the proteins for the ice surface.

A study used fluorescent labeling to demonstrate that antifreeze glycoproteins (AFGPs), a type of AFP isolated from fish, adsorb irreversibly to ice. The authors studied the binding mechanism by labeling AFGPs with fluorescent dyes. They then measured the intensity of the fluorescent signal on the crystal when in AFGP solution and after the solution was exchanged with water. They found that the intensity of fluorescence in the crystal interface persisted after solution exchange, giving evidence that the direct interaction of these proteins at the crystal surface is a crucial element of the activity of the antifreeze protein (Meister et al., 2018).

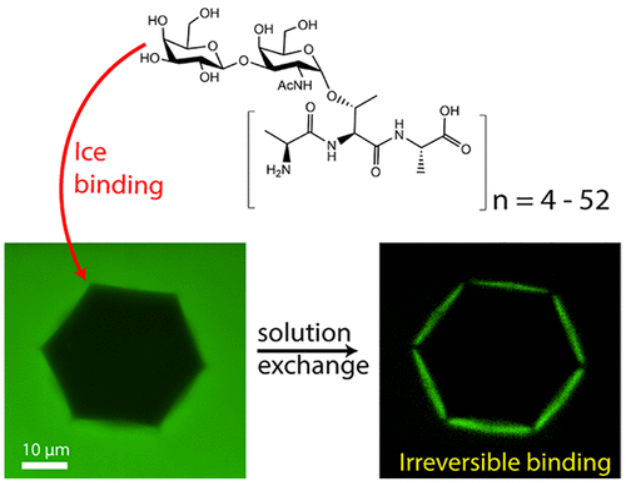

Fig 6: fluorescent imaging of crystal in AFGP solution (left) and after solution exchange with water (right). Fluorescence on crystal surface after solution exchange is visible. The structure of the AFGP is depicted above, with a peptide polymer of *n* monomers bound to a carbohydrate (Meister et al., 2018).

The structure-function relationship of AFPs is accepted to involve hydrophobic and hydrophilic regions; however, the mechanism by which these regions interact with the ice is not fully elucidated. It was suggested to be the result of hydrogen bonding between hydrophilic residues that are repeated in the proteins and the ice face (Knight et al., 2001). Later research substituted hydrophobic residues for the residues in the hydrophilic domain and observed unaltered ice binding activity (Haymet et al., 1999). This indicated that it is the complementarity of the face to the protein, hydrophobic, and van der Waals interactions – not hydrogen binding – that is the driving force for the AFP adsorption mechanism.

Further research elucidated the binding of AFPs to be attributed to the presence of ordered molecules on its exterior surface. In this study, the authors investigated the binding of an AFP from the spruce budworm, which is naturally exposed to extreme cold, and visualized its mechanical structure through molecular dynamics simulation. Their results indicated that the ice binding surface of the protein fold consists of adjacent threonine residues that are associated with water molecules arranged in an ordered lattice. The protein interacts with the ice through the affinity of these ordered waters on its ice-binding site and inhibits its growth (Kuiper et al., 2015). This arrangement is depicted in figure 5 below:

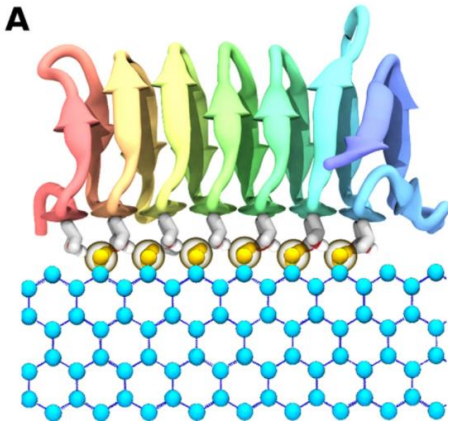

Fig 7: the interaction of AFPs with ice in the spruce budworm. Ice molecules are depicted by cyan balls, and the ordered water associated with the protein's threonine residues (white sticks) are given in yellow (Kupier et al., 2015).

The above discussion elucidates the binding mechanism of AFPs onto the surface of ice, but it is important to note that while IRI involves this effect, the exact mechanism of IRI is as of yet not known (Knight et al., 1998).

VI. **Classification of Antifreeze Proteins**

In nature, antifreeze proteins play a vital role in organisms that are constantly or sporadically exposed to subzero temperatures. AFPs were first found and isolated from fish (DeVries et al., 1970), and since then it has been discovered that fish contain several different types of antifreeze proteins, namely antifreeze glycoproteins (AFGP)s and three types of antifreeze proteins. AFPGs have disaccharides attached to the threonine residues, Type I AFP is alpha-helical and contains many alanine residues, type II is larger and has a tertiary structure with disulfide bridges and reverse turns. This is in contrast to type III AFPs, which are reported to have less defining characteristics (Davies et al., 1990).

Chen *et al*. visualized the role of AFPs in the cold acclimatization of fish with scanning electron microscopy (SEM). Antifreeze proteins were isolated from the scales of tilapia fish and applied to bacterial cells exposed to subzero temperatures. Their results demonstrate the protective characteristics of AFPs in preventing cellular damage. In the untreated group, cells were seen to be ruptured and deformed, while the surface of the treated cells were visibly intact (Chen et al., 2019).

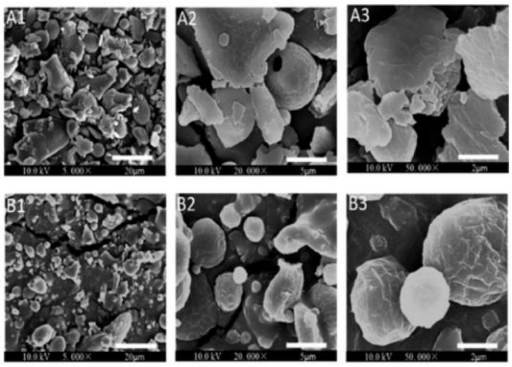

Fig 8: SEM images of S. thermophilus cells after freezing. A1, 2, and 3 represent the 5000-, 20000-, and 50000-fold magnification of the control group. B1, 2, and 3 indicate the same magnifications for the treated cells. Scale bars represent 20, 5, and 2 µm respectively for the three magnifications (Chen *et al.,* 2019).

Since then, AFPs have also been found in bacteria, fungi, plants, and invertebrates (Griffith et al., 1995). It is interesting to note the presence of antifreeze proteins across a wide spectrum of cold-adapted species. Antifreeze proteins serve a similar function across these organisms, and the mechanism of their function is also similar, yet they vary wildly in their crystalline structure. While they all function to inhibit ice growth, there are discrepancies between the proteins' specific activities. This is the result of convergent evolution, whereby each species develops independent adaptations to a similar problem resulting in the development of

diverse proteins that inhibit ice growth through a similar mechanism(Goodsell, 2019). Crystallization of ice from different animal groups gives rise to evidence of the separate evolutionary pathways that led to their similar acclimatization to cold climates, as is depicted below (Fig 9). All of these proteins exhibit an ice binding face, giving evidence to the conservation of mechanism between them. In fact, the binding region of these proteins and several others are all rich in threonine residues and have been shown to associate with ice with their clathrate structures (Goodsell, 2019).

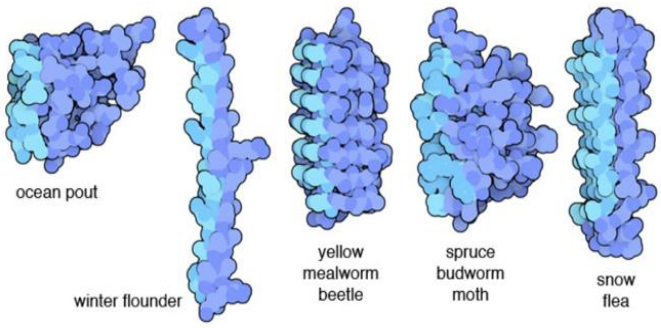

Fig 9: crystal structure of ice binding proteins from several different organisms. Ice binding face is shown in light blue (Goodsell, 2009).

However, the aspect of ice growth inhibited by this mechanism of binding is somewhat different across organisms, corresponding to their individual adaptive needs. For example, arctic fish have several AFPs with high potency in terms of their TH activity. This is vital because their climate creates the need for internal ice crystals to be stabilized over very high degrees of supercooling in order to prevent their growth. In contrast, plant AFPs demonstrate relatively low TH, but even very low concentrations of the protein are sufficient to inhibit ice recrystallization, as Bredow *et al*. demonstrated in a species of annual grass. This also correlates with the species'

habitat conditions since the temperatures are higher and subject to fluctuations, both of which facilitate IR. Furthermore, the authors prove that the proteins in this particular species exhibited an additional role of inhibiting the activity of ice-nucleating proteins (INPs) which are produced by bacteria that inhabit that species (Bredow et al., 2018). INPs facilitate the initial stage of crystal formation by acting as the seed upon which water molecules accumulate. It is proposed that AFPs bind to INPs in a similar manner that they do authentic crystal surfaces, adsorbing to their surface and blocking the development of the crystal (Griffith 1995). Indeed, in a species of rye grass, addition of 1 mg/mL of IRI proteins depressed the temperature of ice nucleation significantly in the presence of higher concentrations of INPs (Bredow et al., 2018). This property is crucial in this species, since the plants are inhabited by INP producing microorganisms that stimulate the formation of damaging crystals. The microbes can then feed from the nutrients that escape the ruptured cells while the organism experiences both the damage to its tissue as well as the loss of those nutrients. Therefore, the adaptation of AFPs in such organisms also serves as a mechanism defending against parasitic bacteria and retaining cellular integrity.

VII. Measuring Ice Recrystallization Inhibition

A crucial aspect of the evaluation of IRI activity is its accurate quantification. IRI efficiency is defined as the lowest concentration for which the amount of inhibitor present influences the rate of recrystallization (Budke *et al.,* 2019). The recrystallization rate is determined analytically by observing the change in the radial size of the crystal with respect to time. The equation relating crystal size and elapsed time is put forth as $r^3 = r_0^3 + kt$, where r_0 represents the initial radius size, r represents its size after time has passed, and k represents the recrystallization rate (Hagiwara *et al.,* 2006). Budke *et al.* described a method of observing these parameters by

inserting the sample with the liquid and IR inhibitors onto a photomicroscope. 2 µL of the sample were placed in between coverslips onto a cold stage in the microscope and cooled to -50 °C at a rate of 20 °C per minute, causing nucleation and crystallization of the ice. Then the sample was heated at 10 °C per minute to the desired annealing temperatures of -6, -8, and -10 °C , at which point the sample was annealed, and ice grain sizes were observed with the passage of time (Budke *et al.*, 2009).

The measurements of grain sizes over the course of annealing are used to determine the recrystallization rate and the IRI activity. A study evaluated the IRI efficacy of a variety of AFPs by recording a 40-minute video of a frozen sample and measuring the crystal radius at different points in time from snapshots of the recording. Plotting r^3 against time, the authors derived a linear graph for which the slope represents recrystallization rate k. The relationship between k and the inhibitor concentration indicated the efficacy of the inhibitor, given by the inflection point of the curve, which is the initial point of concentration dependency (Fig 10) (Rahman *et al.*, 2019).

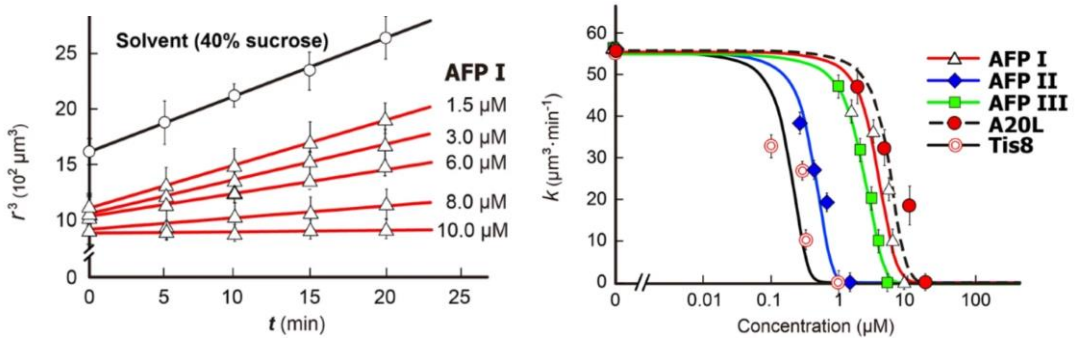

Fig 10: graphical depiction of relationship between cubic radius and time (left) and between reaction rate and inhibitor concentration (right) (Rahman *et al.,* 2019).

VIII. Recrystallization in Foods Thawed and Frozen

The high-water content in food creates the problem of spoilage by way of chemical reactions and microbe infestation. This issue can be effectively eliminated by storing the food in subzero temperatures, causing the water inside it to freeze into crystals. The effects of freeze storage in food needs to be evaluated as they relate to frozen and defrosted food as opposed to food eaten frozen. Food that is thawed should ideally be comparable to unfrozen food in freshness, texture, and nutrient composition. However, degradation of the item quality can come from freezing for a number of reasons, all of which are most relevant to cellularly comprised food products. First of all, ice takes up a 10% larger volume than does liquid water, so the formed crystals exert pressure on the particles that make up the product (Nesvadba, 2008). In organic matter, the exerted pressure can weaken the membranes and walls of the cells, causing the soggy texture observed in, for example, frozen and thawed strawberries. Additionally, the freezing process generates an abnormally high concentration of extracellular electrolytes, causing cellular dehydration. Upon thawing, the capacity of the cells to reabsorb the water is often non optimal, resulting in “drip loss” and a reduction in the turgor pressure and, consequently, in the quality of the food (Nesvadba, 2008).

The process of freezing food for storage occurs in three steps. First, the product is cooled to its freezing point. Then, the temperature is decreased until the liquid freezes into solid. Nucleation describes the initial aggregation of waters from the melt into the embryonic crystal, which is followed by growth as additional waters gather onto the crystal surface. Finally, the temperature is lowered until the desired storage temperature is reached. It is during and subsequent to this final stage that recrystallization is observed. Migration and Oswald ripening

occur mostly in later stages of storage until the smaller crystals disappear in favor of larger ones (Zhu et al., 2019).

The most important factor in post-freezing quality reduction is the cellular drip loss brought on by membrane damage due to crystal growth and recrystallization. This points to the main issue of quality deterioration being in the storage of the food rather than in the initial process of its freezing. In storage, extracellular crystals grow and puncture the membranes while simultaneously increasing the osmotic pressure outside the cell, facilitating water loss. In animal cells, this causes shriveling and collapsing of the cells. In plant items, recrystallization causes a reduction in turgor pressure, and the membrane of the cell is pulled in from the cell wall. These effects are summarized in fig 11, and they translate into compromised texture, flavor, and firmness in the cellular food product.

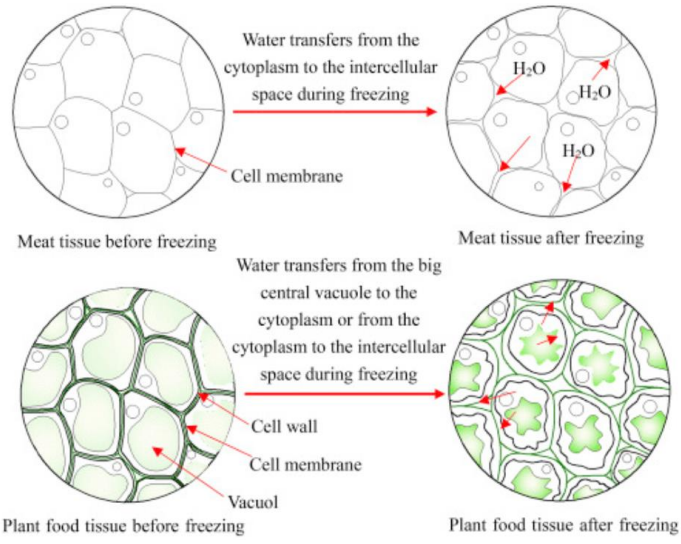

Fig 11: damage to structure of cells of fresh meat and plant food tissue after freezing (Li et al., 2018).

One technique that has been shown to improve food quality after thawing is through rapid freezing, which does not allow sufficient time for accumulation onto large crystals nor for water

to escape from the cell. It also causes small crystals to form within the cells, which undermine the pressure gradient imposed by extracellular crystals (Nesvadba, 2008). However, appreciable effects of rapid freezing is more difficult to achieve for the large quantities of product that are industrially produced. In addition, this does not address the issues that arise during prolonged storage which, as mentioned, are a central force in post-thaw deterioration. Consequently, research interest has been devoted to developing processes to ensure optimal product quality under normal processing conditions.

In foods eaten frozen, such as ice cream, low temperature causes liquid waters in the solution to freeze into solid, which is nucleation. Crystallization is the continued growth of the crystals, and their size distribution of contributes to the overall texture of the product. Long durations of storage and temperature fluctuations, both of which are common in the processing and export of ice cream, cause the crystals in the ice to shift to a larger average size. Larger crystals generate a coarse and grainy texture, while smaller, homogeneously spread crystals yield a soft and smooth texture (Cook et al., 2010).

In a study that investigated ice crystals in ice cream, Ndoye and Alvarez demonstrated that the size of crystals in ice cream increased as the duration and temperature of the storage were increased (Fig 12). It is assumed that this effect occurs because providing the liquid phase molecules with more kinetic energy facilitates their migration to larger crystals, a primary mechanism of recrystallization. By lowering the temperature, the solution is both more viscous and lower in energy. Additionally, crystal size was directly correlated with the amplitude of temperature fluctuations applied to the sample, and this correlation is exacerbated at higher storage temperatures (Fig 13).

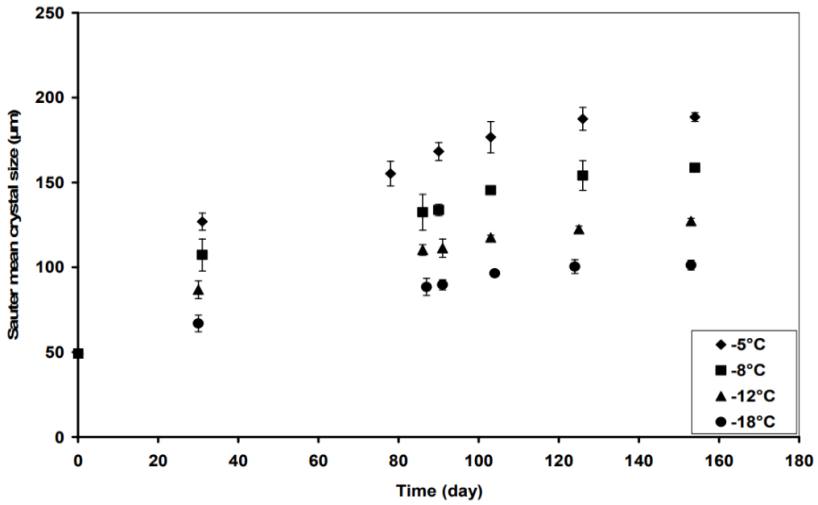

Fig 12: crystal size as a function of duration and temperature of freeze storage (Ndoye et al., 2015)

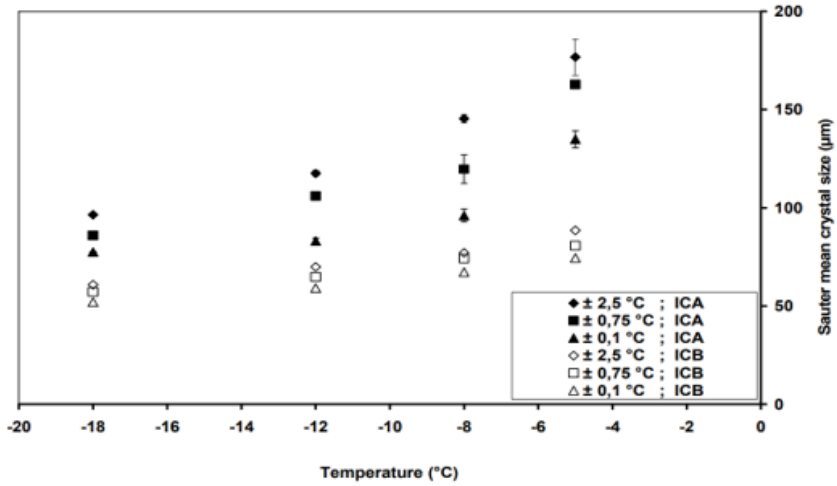

Fig 13: crystal size as a function of amplitudes of temperature fluctuations (Ndoye et al., 2015)

A similar study found that ice cream stored at -13.3°C became grainy after only 2 weeks of storage, while ice cream stored at -26.1 °C maintained a creamy texture for 16 weeks (Earl et al., 1960).

IX. Antifreeze Proteins in Food

Since rapid freezing is not always feasible for mass-produced food items, there has been significant incentive to find alternative methods of controlling crystal growth in frozen food. The application of antifreeze proteins to food products has been presented as an effective mechanism for controlling the proliferation of crystals and, notably, reducing recrystallization. The benefits of antifreeze proteins in food have ramifications both in fresh food stored by freezing and in foods meant to be eaten frozen.

In foods thawed after freeze storage, antifreeze proteins have proved to effectively enhance product quality by preventing the deterioration brought on by recrystallization. AFPs were applied to meat before it was frozen and annealed at -20 °C. The authors visualized the meat tissue with scanning electron microscopy and observed smaller crystal size in the treated versus untreated meat (Payne et al., 1994). A later study assessed the benefits of antifreeze proteins in meat products by injecting the proteins into lambs prior to their slaughter. The researchers found in subsequent IRI assays that the size of the crystals in the tissue after freezing were significantly smaller than those in the control group (Payne et al., 1995).

Similarly, AFPs exhibit quality enhancement in products intended to be consumed in their frozen state, such as ice cream, ice pops, and sorbets. In frozen desserts, the motivation is to counter IR to eliminate large, texturally unfavorable crystals. Manufacturing companies often utilize the ice binding properties of AFPs or synthetic polymers in order to achieve softer ice cream even during prolonged shelf-life. The proteins stabilize the smaller crystals during freezing such that they do not grow despite their low storage temperature (Flores et al., 1999).

Antifreeze proteins are also beneficial in helping to minimize fat content in ice cream. Fat droplets dispersed throughout the ice cream cause it to have a creamier texture, so reducing fat content is detrimental to its textural quality. However, by using ice-binding proteins, the texture of the food is improved by having more abundant and smaller crystals (Zhang *et al.*, 2016). As a result, fat-free or low-fat ice cream products can be produced with textures comparable to those of high fat content, so that product is both creamier and more temperature stable (Crilly, 2007).

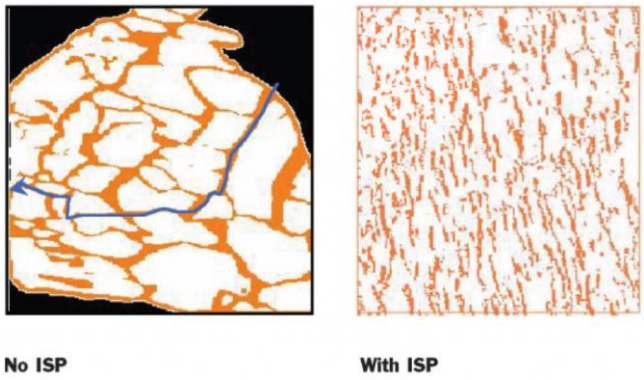

Fig 14: smaller crystal size observed in sample to which IBPs are applied (image on right) compared to the control sample (left) (Crilly, 2007).

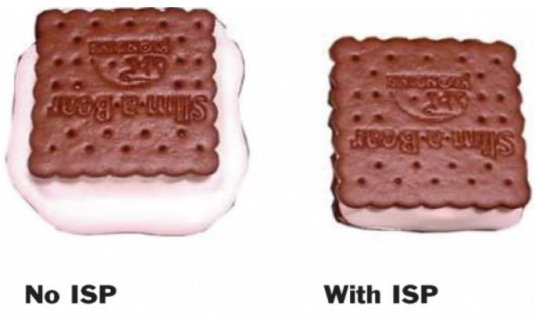

Fig 15: the quality difference between a low-fat ice cream with (left) and without (right) IBPs (Crilly, 2007).

Likewise, Regand and Goff demonstrated that ice binding proteins from winter wheat grass significantly reduce ice recrystallization (Regand et al., 2006). Another study microscopically observed the effect of AFP addition to frozen products. The authors treated the nonfrozen food items with the antifreeze proteins, froze the foods at -80°C, and annealed them at -6 to -8°C for extended amounts of time. They observed significantly larger crystal size in the frozen products to which AFPs were not applied that were seen in AFP-treated foods (Warren et al., 1992). Another study proved the effectiveness of AFPs in reducing recrystallization by observing a reduction in individual crystal size with AFP addition, and quantified its IRI by plotting the reaction rate against the protein concentration. The paper also brings evidence to support crystal aggregate formation as an additional activity of AFPs in that may contribute to enhanced ice cream texture (Kaleda *et al.,* 2017).

In cold acclimated organisms, AFPs are located outside of the cells. This suggests that incorporation of AFPs into food items to cause crystal growth retardation can be achieved through non-invasive means like mixing or soaking. Supercooled stabilization of fish tissue was achieved through injection of AFPS (Fletcher et al., 1986), and vacuum-filtration addition proved successful in depressing nucleation temperatures in plants (Cutler et al., 1989). In addition, the above-mentioned experiment in which AFPs were administered to lambs incorporated the proteins via injection (Payne et al., 1995).

Another interesting possibility for AFP incorporation is by genetic modification. The transfer of the gene producing AFP I in flounder into salmon caused them to express the AFP I gene but without major IRI effect (Hew et al., 1992). In 1991, a study was conducted transfecting tomatoes with the *afa3* gene which encodes an antifreeze protein in winter flounder. The study gained public interest, and the tomato termed the “fish tomato” of the “antifreeze tomato”.

However, the transgenic tomato did not display slower crystal growth than did the control, although it did spark significant controversy from anti-GMO activists (Hightower et al., 1991).

X. Additional Techniques to Inhibit Recrystallization

In addition to the use of biological proteins, several techniques to attenuate recrystallization have been suggested. Delgado *et al.* used scanning electron microscopy to visualize the damage caused to cells in strawberries that were systematically frozen and thawed. They observed an adverse effect on cell structure when the rate of temperature change decreased, as is demonstrated in Figure 16. Cells cooled at a rapid rate were analogous in integrity to the unfrozen control group, while cells cooled slowly appeared misshapen and deformed and lost nutrients and water.

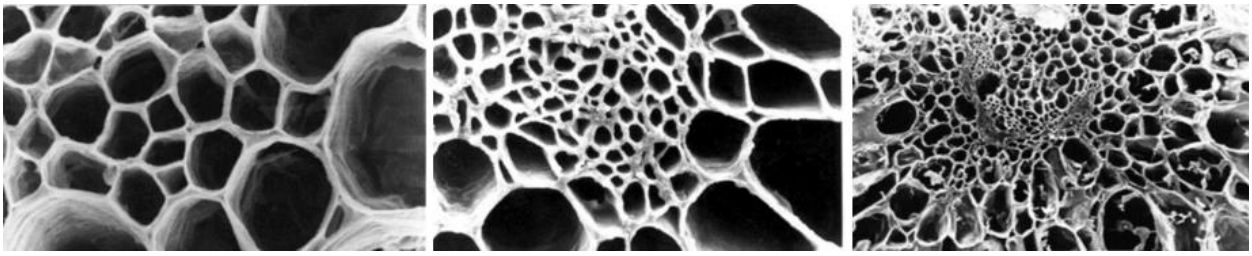

Fig 16: In order from left to right: the visualized membranes of strawberry cells that were untreated, subject to rapid cooling (2.43°C/min), and cooled slowly (.82°C/min) (Delgadlo et al., 2005).

Their results demonstrate the role that processing conditions play in the quality preservation of frozen products. The degradation of the cell membrane compromises its permeability, resulting in a loss of flavor, hydration, color, and nutrients, which in turn yields a lower quality food product.

Decreasing the storage temperature helps mitigate recrystallization by hindering the rate of the migration of the waters. When frozen material is stored for a prolonged amount of time, higher temperatures increase the migration rate, so the crystals grow larger (Zhu et al., 2019). This is in contrast to ice crystals while they are freezing, which grows faster at lower temperatures. Storage at low temperature deprives the liquid molecules of the energy needed to travel to and orient onto the larger crystals, so the average crystal size will remain relatively smaller (Zhu et al., 2019).

Another possibility is to control the magnitude of temperature fluctuations. This also has an effect on the migratory mechanism, since the smaller crystals melt preferentially with sudden heat increases and then aggregate onto the more stable crystals once supercooling is reestablished. Gutiérrez et al., found by analyzing frozen tilapia samples at different temperature fluxes that the size of the crystals developed asymptotically, meaning that they reached a maximal value, which was set by the magnitude of fluctuation and was independent of the initial ice grain size (Gutiérrez et al., 2017). This confirms the pivotal role of the temperature consistency in crystal distribution.

In addition to manipulating the freezing conditions, novel technologies have emerged in order to inhibit recrystallization of ice. Tironi *et al*. demonstrated that cooling water at very high pressures promotes their maintenance in the liquid phase, since the freezing point of a substance is depressed with an increase in environmental pressure. The resulting liquid is supercooled and highly unstable, so the alleviation of the pressure induces very rapid crystal formation. The average sizes of these crystals were found to be smaller and more homogenously distributed (Tironi et al., 2010). Since then, other researchers have attempted to exploit the pressure-dependence of ice phases; however, a reported setback of this technique is that it causes

alterations in the properties of the membrane and can destroy cells when the applied pressure exceeds a threshold tolerance (Li et al., 2018).

Another strategy used by manufacturers is the addition of stabilizers in ice cream. Smooth ice cream has ice crystals with that average 10 to 20 μm in size, but recrystallization due to nonideal storage conditions causes the size distribution of crystals to shift upward and exceed this average size, which is perceived by the consumer as less creamy. Manufacturing companies commonly add stabilizers to increase the viscosity of the liquid, hindering migration of molecules to form larger crystals. These stabilizers also create a cross-linked gel that stabilizes the crystals across temperature fluctuations (Akhtar et al., 2005). During the manufacture process, ice cream is also whipped at a high frequency to homogenously disperse the ice crystals. Even distribution also contributes to a more appealing texture (Flores et al., 1999).

XI. Synthetic polymers

Although AFPs have proven to be effective, isolating and extracting these proteins from living organisms is time and cost inefficient. These proteins can be purchased from commercial manufacturers, but the cost is $50 USD for a gram of protein on average, which is reasonable for purposes of research but not in large-scale product manufacture. The high cost of the proteins is largely credited to the difficulty of synthesizing them, and although small amounts of AFPs are sufficiently potent to inhibit IR, the costs of their implementation outweigh the benefits more often than not (Feeney et al., 1998). This consideration drove the development of synthetic polymers that would reproduce the inhibition activity of AFPs without their inefficient production. As early as 1995, researchers observed the inhibition activity of synthetic polymers on growth and, most notably, on recrystallization. Knight *et al*. demonstrated the IRI activity of

poly(L-histidine), poly(L-hydroxyproline) and poly(vinyl alcohol), all of which were active in experimental concentrations below 1 mg/mL (Knight et al., 1995).

An additional study published in 2009 quantified the activity of several different polymers with documented or postulated IRI capabilities. They also demonstrated the effect of the polymer size on IRI by applying PVA of different molecular weights to the sample and measuring its mean largest grain size (MLGS) after annealing. As evidenced by their data, higher molecular weight correlates with enhanced IRI activity in the case of IRI. A summary of these results is depicted in the figure below (Fig 17):

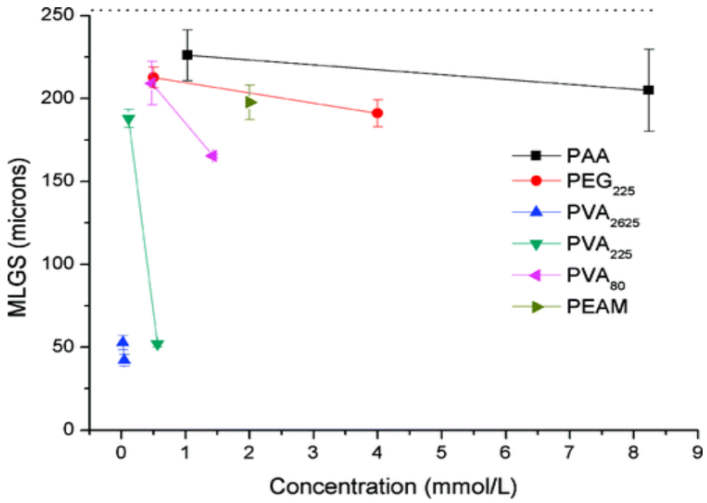

Fig 17: mean largest grain size measurements (MLGS) for synthetic polymers poly(acrylic acid) (PAA), poly(2-aminoethyl methacrylate) (PAEM), poly(vinyl alcohol) (PVA), and poly(ethylene glycol) (PEG) (Gibson et al., 2009).

In contrast to PVA, PAA was proved by this data to be less potent as an IR inhibitor. This finding was corroborated by another study that found polyacrylic acid to be weakly active relative to the IRI of PVA (Fig 18) (Inada et al., 2003).

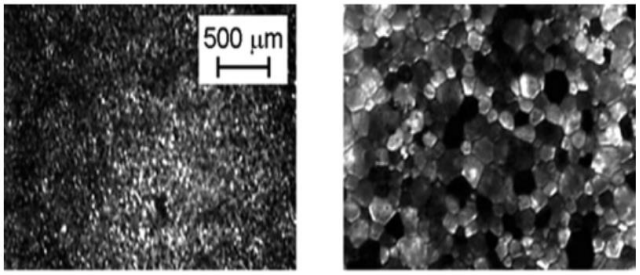

Fig 18: size of ice crystals after annealing with PVA (a) and PAA (b) (Inada et al., 2003)

Additional studies have proved polyvinyl alcohol (PVA) to have IRI activity analogous to antifreeze protein. When applied in the same molar concentration, PVA was found to inhibit IRI to the same extent as did AFP type I and at concentrations as low as 5 x 10-7 mol/L (Inada et al., 2003). However, it should be noted that by mass, the quantity of PVA required to achieve comparable IRI is much higher. Nonetheless, economic and efficiency considerations compensate for this setback.

XII. Conclusion

Early efforts to alleviate the exorbitant food waste caused by spoilage stimulated the development of artificial freezing methods. This development did indeed reduce the amount of spoiled food, but the quality of many frozen products was compromised, largely due to ice recrystallization. IR is understood to be primarily driven by thermodynamic considerations, as high energy crystals are replaced by larger, stabler ones. IR is an especially prevalent issue since it is exacerbated in those conditions which normally accompany product manufacture, such as temperature fluctuations, slow cooling rates, and prolonged storage. Efforts to develop methods of interfering with this process led to heightened research interest in ice binding proteins.

AFPs inhibit crystal growth by adsorbing directly to the ice surface, where they introduce curvature onto the surface, causing non-colligative freezing point depression that results in crystal growth inhibition. The mechanism by which binding occurs is thought to be through direct, irreversible adsorption to the crystal planes. The ordered waters associated with AFPs are important to the complementarity of the crystal planes to which the AFP binds preferentially. This adsorptive mechanism is relatively conserved across species and across organisms, although the evolutionary origins of the proteins are independent of one another. This explains the wide variation in their crystalline structures and the discrepancy in their species-specific activities.

The use of AFPs in frozen food is proved to preserve the integrity of fresh food stored in freezers and in food consumed frozen. By adsorbing to crystal faces, it prevents crystals from rupturing cells and causing drip loss even when stored for long periods of time. Similarly, it promotes the maintenance of small and evenly dispersed crystals in ice cream, which translated to enhanced texture and can replace fat content in the product. Other methods of achieving a favorable texture includes high cooling rates, whipping of the cream in the freezing process, addition of stabilizers, and high pressure freezing. In addition, research is underway to identify synthetic polymers that exhibit comparable ice retarding properties to provide a more economical additive for mass produced food. Poly (vinyl alcohol) has emerged as one such promising option as a cheaper shelf-life prolongation agent.

The ramifications of research on AFPs are far-reaching. In addition to improving a process that reduces spoilage and world hunger, antifreeze incorporation offers potential benefits in improving the tolerance of cold-exposed crops, preservation of tissues and organs, and perhaps in treating hypothermia. These proteins are especially interesting because of the

versatility derived from their independent evolutions, their potency, and their unique qualities, as well as the multitudes of benefits they may offer.

XIII. References

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