



Review

Ice structuring proteins from plants: Mechanism of action and food application

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ABSTRACT

Application of ice structuring proteins (ISPs) as a food preservative has shown potential to enhance the quality of frozen food and desserts during freezing storage, transport, and thawing. One of the main advantages of ISPs for the food industry is that they are fairly active in very low amounts, which could make these new food ingredients very cost-effective. Therefore, ISPs have gained much attention in food science and their use has become more and more feasible. ISPs have been discovered in many cold tolerating biological systems such as fish, insects and plants, which all exhibit ice recrystallization inhibition, and also in some cases, thermal hysteresis and ice nucleating activities. This review discusses cold stress and cold tolerance in plants, different mechanisms of actions of ISPs and homologies to other structurally identified proteins, which lead to the dual role of ISPs in plants as ice recrystallization inhibitors and pathogenesis-related proteins or enzymes. Ice crystallization and recrystallization (growth of larger ice crystals at the cost of “restructuring” of other ones), interaction of ice crystal and ISPs, ice recrystallization inhibition and thermal hysteresis assays, and application of ISPs in different food products are also discussed. To-date there is no data available to report toxicity or to challenge the safety of these “novel” food ingredients.

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1. Introduction

Antifreeze proteins were discovered in the early 1970s, in the blood of Antarctic fish. The temperature of Arctic and Antarctic ocean water is around -1.9°C but the high concentration of electrolytes in these fishes is only able to depress the freezing point of blood by -0.6 to -0.7°C (Hew & Yang, 1992). The analysis of the blood of Antarctic fish confirmed that colligative effects of electrolytes such as sodium chloride accounted only for 40–50% of the freezing point depression of the blood (Duman & DeVries, 1975). The compounds that were found to be responsible for the remainder of the depression of the blood temperature to lower than seawater without freezing were discovered to be small proteins. These proteins have been studied in other species and found to share the properties of depressing the freezing point of body fluids in a non-colligative way without having an effect on the melting point (Wathen & Jia, 2005). These differences between freezing and melting point are termed thermal hysteresis and are a tool to measure the effectiveness of those proteins, though the extent of the reduction of freezing point differs from protein to protein (Wathen & Jia, 2005).

Shortly after the discovery of antifreeze proteins in fish, proteins with similar activity and some with crystal growth inhibition activity were discovered in other biological systems such as animals, insects and plants (Cheng, 1998). This review focuses on plant ice structuring proteins (ISPs) and their mechanism of actions with regards to food application. It also covers areas of ice crystallization and recrystallization, interaction between ice and ISPs, and antifreeze activity assays.

2. Antifreeze proteins classification

Hew and Yang (1992) classified antifreeze proteins into two main groups: antifreeze glycoproteins (AFGPs) and antifreeze proteins (AFPs) (non-glycosylated). Antifreeze glycoproteins have been fractionated from proteins in fish blood serum (e.g., Antarctic Notothenioids and Arctic Cod) and they range in molecular mass from 2.6 kDa to 3.3 kDa (Harding et al., 2003; Tachibana et al., 2004).

Antifreeze proteins (non-glycosylated) can be either thermal hysteresis proteins (THPs) or ice structuring proteins (ISPs). These terms have been used for AFP in the literature, sometimes interchangeably. The THPs have been identified in fish, plants, insects, fungi and bacteria and they are known to prevent ice crystal formation by depressing the freezing point of water (Graether & Sykes, 2004). However, ISPs control the size, shape and aggregation of ice crystals rather than to prevent water from freezing (Clarke et al., 2002).

Ice structuring proteins, through an adsorption–inhibition mechanism, affect the growth and morphology of ice crystals (Regand & Goff, 2006). The adsorption–inhibition theory, discussed in detail in Section 7, explains that the adsorption of ISPs at the ice–solution interface causes ice surface curvature effects that results in the inhibition of ice crystal growth in fish, plants or insects (Yeh & Feeney, 1996).

AFPs from fish are classified into 4 types in the order of their discovery: type I, in winter flounders for instance, range from 3.3 to 4.5 kDa in molecular mass; type II, in herring for instance, range from 11 to 24 kDa in molecular mass; type III, in wolffish for instance, 6.5 kDa in molecular mass; and type IV, in longhorn sculpin with a molecular mass of 12 kDa (Davies & Sykes, 1997; Harding et al., 2003).

AFPs from insects, including long horn beetle and spruce budworm have been isolated, cloned, expressed and studied extensively (Graether & Sykes, 2004; Graether et al., 2003, 2001, 1999; Graham et al., 2007; Kristiansen & Zachariassen, 2005). The unique feature of insect AFPs is that they are 10–100 times more active than AFPs from fish, which allows them to survive the much colder environment (Graham et al., 2007). It has been suggested that the disulphide bonds of insect AFPs avoid structural unfolding at low temperatures (Graether & Sykes, 2004).

3. Plant ISPs

Griffith et al. (1992) were the first to report the ice structuring activity of extracts obtained from plants. Several plants (Table 1) have been investigated for ISPs (Atıcı & Nalbantoğlu, 2003; Griffith & Yaish, 2004). Ice structuring activity is present in over-wintering plants only after they have been exposed to low temperatures. Many species of perennial, over-wintering plants contain ISPs in their different parts, including seeds, stems, crowns, barks, branches, buds, petioles, leaf blades, flowers, berries, roots and tubers (Griffith & Yaish, 2004). The thermal hysteresis in plant ISPs is only 0.2 to 0.4°C (Venketesh & Dayananda, 2008) but they show strong ice recrystallization inhibition (RI) activity. For example, solutions containing winter rye ISPs exhibited RI activity at concentrations as low as $25\text{ }\mu\text{g protein/L}$ (Griffith & Ewart, 1995; Sidebottom et al., 2000). Hassas-Roudsari (2011) reported 0.42°C for the thermal hysteresis of apoplastic extracts from cold acclimated winter wheat leaves. Regand & Goff (2005) reported that sucrose solutions containing 0.13% total protein from cold-acclimated winter wheat grass extracts reduced ice crystal growth as much as 74% compared to a blank control

Table 1
Plants containing ISPs^a in their soluble extracts obtained from cold-acclimated plants^b.

Common names					
Arrow leaf plantain	Buttercup	Day lily	Heath rush	Potato	Water sedge
Antarctic hairgrass	Butterfly bush	Douglas fir	Heather	Prickly burr	Weeping forsythia
Bamboo	Cabbage	Dutchman's breeches	Jacob's ladder	Shasta fern	White oak
Barley	Carrot	Eastern cottonwood	Kale	Sheep sorrel	Winter & spring canola
Beech	Chickweed	Elder	Maple	Spurge	Winter & spring rye
Bittersweet nightshade	Chickweed	Evergreen legume	Oat	Timothy	Winter & spring wheat
Bluegrass	Crack willow	Fescue	Peach	Tussac grass	Winter cress
Brown rush	Creeping bentgrass	Garlic mustard	Perennial ryegrass	Violet	Wood aster
Brussels sprout	Dandelion	Geranium	Periwinkle	Virginia waterleaf	Wood sorrel

^a Ice structuring proteins.

^b Adapted and modified from Griffith and Ewart (1995); Atıcı and Nalbantoğlu (2003); Griffith and Yaish (2004).

after temperature cycling. This suggests that these proteins may act as crystallization modifiers by inhibiting the growth of ice crystals in tissues inward from the epiphytic ice nucleators or outward from the vascular bundle (Atıcı & Nalbantoğlu, 2003) but a not very strong “anti-freeze” compound as their thermal hysteresis properties are not very significant (Hassas-Roudsari, 2011; Venketesh & Dayananda, 2008). Kuiper et al. (2001) proposed a theoretical model of ryegrass ISP that partially explained the lower thermal hysteresis and better RI activities of plant ISPs compared to fish and insects ISPs due to “imperfect” ice-binding sites.

3.1. Cold stress and cold tolerance in plants (under 10 °C)

No plant has been reported to show RI activity, due to constitutive regulation and accumulation of ISPs, unless it was cold acclimated and kept at a cold temperature (Griffith & Yaish, 2004). In other words, when the plant is not cold acclimated or is brought to a warmer temperature (higher than the threshold, about 10 °C), it does not regulate ISPs, consequently, does not show RI activity.

There are complicated mechanisms involved in cold acclimation of plants. Chilling or frost temperature has direct effects on plant cells, such as increasing the fluidity of the membrane, lowering the metabolic rate and retarding the energy dissipation of cells (Beck et al., 2007). On the other hand, freezing temperatures would cause dehydration of plant cells by formation of ice crystals in the apoplast, which results in exportation of water from protoplast leading to the shrinkage of the protoplast and reduced turgor pressure. Dehydration of the cells increases the concentration of solutes inside the cells and changes the membrane potential leading to disintegration of membrane bilayer and interruption of metabolism (Beck et al., 2007).

Either low temperature by itself or the relative state of dehydration due to the cold stress are thought to act as signals to induce the gene in the plant cells to regulate ISPs (Griffith & Yaish, 2004). Another factor that has to be considered is light. In cold seasons, there is a decline in both temperature and light, which act as cues for plants to prepare for the cold or freezing temperatures by inducing or enhancing cold stress tolerance mechanisms (Winfield et al., 2010).

Upon receiving the environmental signals the genes start regulating the corresponding proteins in cells including ISPs (in case of cold and freezing stresses). As noted previously, regulation of ISPs is one of several mechanisms providing cold or freezing tolerance for the plant. However, it has to be stated here that these adaptations are not necessarily long term (Beck et al., 2007). The duration of these changes not only varies from one plant to another plant but also from one variety to another variety (Beck et al., 2007; Griffith & Yaish, 2004; Winfield et al., 2010).

3.2. Cold acclimation vs. non-acclimation

Most temperate plants, such as both spring and winter varieties of wheat, show some degree of chilling tolerance (Griffith et al., 1997). Therefore, they may show similar responses to cold, although the ability to survive cold is an extremely variable characteristic.

The ability to withstand the cold categorizes plants into chilling-sensitive and chilling-hardy plants, and also frost-tolerant and frost-intolerant plants. It is noteworthy that these categories should not be confused with the groupings ‘spring’ and ‘winter’. Spring varieties of plants do not have a requirement for an extended cold-tolerance period (several weeks) to become competent to flower. Cold varieties, on the other hand, have such a requirement (Winfield et al., 2010). However, in the case of wheat, it has sometimes been reported that winter varieties (i.e., those that require an extended cold period to flower) are harder than spring types (Limin & Fowler, 2006).

Cold acclimation is a response to low temperature in winter varieties of plants. In most cases, only 1 or 2 days of low temperatures are usually sufficient to generate cold acclimation (Sung & Amasino,

2005). On a return of a warmer environmental temperature (i.e., spring or temperature fluctuations in autumn), this capacity is rapidly lost. However, it is vital for plants to recognize that short-pronounced cold spells followed by the return of warmer temperatures are not mistaken for the end of winter. Thus, winter varieties require an extended period of cold before they become competent to flower and they tend to retain a ‘memory’ of this extended exposure to cold before flowering as temperatures rise in the spring (Sung & Amasino, 2006).

Developing cold acclimation and freezing tolerance requires the orchestration of many physiological and biochemical changes in the plants, which are at least mediated through the differential expression of many genes (Guy et al., 1985; Thomashow, 2001). As was noted earlier, low temperature per se or the relative state of dehydration, as a result of the cold temperature, is triggering those genes (Griffith & Yaish, 2004). There have been a number of studies describing gene expressions and their triggers, which were reviewed by Winfield et al. (2010).

There are also a few studies exploring the differences between cold acclimated and non-acclimated plants. Rye has been extensively studied (compared to those of other cold-tolerating plants) with regards to the characteristics of its proteins accumulated in leaves during cold-acclimation and compared to the non-acclimated rye leaves (Griffith et al., 1992, 1997; Hon et al., 1994, 1995; Marentes et al., 1993). Marentes et al. (1993) found that the levels of apoplastic proteins (some had RI activity) increased during cold acclimation but not in non-acclimated or de-acclimated rye plants. A similar observation was reported in cold acclimated canola leaves showing higher protein content than that of non-acclimated canola (Gusta et al., 2004). Similar trend was reported in cold acclimated winter wheat leaves compared to non-acclimated leaves (Hassas-Roudsari, 2011).

The extracts of non-acclimated rye leaves contained similar proteins to that of ISPs in cold acclimated rye leaves except without any RI activity (no protein identification data was provided in that study), although both proteins (from cold acclimated and non-acclimated rye leaves extracts) were similar to pathogenesis-related proteins (Hon et al., 1995; see Section 3.3). Hon et al. (1995) also reported enzymatic activity of ISPs in winter rye, concluding that the ISPs in cold acclimated rye are either subjected to post-translational modification or are encoded by different genes encoding similar pathogenesis-related proteins at low temperatures. Griffith et al. (1997) reported that winter and spring wheat and winter barley also showed high levels of accumulation of apoplastic proteins with both significant RI activity and similarity to pathogenesis-related proteins when they were cold-acclimatized.

3.3. The dual role of ISPs in plants

Ice formation in plants mostly initiates in the extracellular spaces as temperatures drop below freezing, because extracellular fluid generally has a higher freezing point than the intracellular fluid due to a lower solute concentration (Pearce & Ashworth, 1992). The accumulation of ISPs happens during cold acclimation in freeze-tolerant plants prior to the arrival of freezing conditions (Winfield et al., 2010). These proteins are apoplastic proteins since they principally release to the apoplast region and accumulate there. They comprise a diverse range of proteins obtained from different plants such as winter rye, bittersweet nightshade and carrot, all being structurally similar to pathogenesis-related proteins (Griffith & Yaish, 2004). They include β -1,3-glucanases, chitinases, thaumatin-like proteins, and a polygalacturonase inhibitor protein (Antikainen et al., 1996; Bishop et al., 2000; Griffith & Yaish, 2004; Stahl & Bishop, 2000). Chitinases, glucanases and thaumatin-like proteins belong to large gene families, the members of which have undergone extensive functional diversifications and evolutionary changes (Bishop et al., 2000; Stahl &

Bishop, 2000). Thus, they have evolved to perform more than one biological role in plants.

Pathogenesis-related proteins accumulate in the apoplast in response to infection and act together to inhibit the action of fungal enzymes by enzymatically degrading fungal cell walls. Likewise, ISPs are also released into the apoplast and form a variety of complexes with the capacity to bind to ice crystals and inhibit their growth (Griffith & Yaish, 2004; Moffatt et al., 2006; Yaish et al., 2006). Although the ice-binding domain has not been confirmed in chitinase and glucanase ISPs, it is assumed that they generally form a three-dimensional structure that presents an ice-binding surface (Yaish et al., 2006; Yeh et al., 2000). These ice-binding domains or ice-binding surfaces bind to ice crystals and inhibit ice crystal growth and recrystallization by forming hydrogen bonds or through Van der Waals forces (Griffith & Yaish, 2004; Yeh et al., 2000).

A vital factor determining the winter survival of winter cereals is the plant resistance to low temperature diseases, such as snow mold or powdery mildew (Griffith & Ewart, 1995). Interestingly, some ISPs have also retained their capacity to interact with pathogens and are thought to provide preventive defense mechanisms against psychrophilic (cold loving) fungi. Fungi, psychrophilic or not, can be a serious problem in the cultivation of forage and cereal crops (Hoshino et al., 2009).

It has been reported that winter cereals are more resistant to fungal diseases after developing cold tolerance (Griffith & Ewart, 1995). For instance, an improved resistance to disease in winter barley was correlated with inoculation of plant with mildew as well as the appearance of pathogenesis-related proteins after exposing the plant to the low temperature (Tronsmo et al., 1993). The thaumatin-like protein in extracts from cold-induced winter wheat showed an anti-fungal activity against snow mold (Kuwabara et al., 2002).

Wheat protein extracts have also been reported as an efficient, non-toxic cryopreservative for mammalian cells and provide long-term (4 years) protection for primary hepatocytes (Grondin et al., 2008; Hamel et al., 2006). Rye proteins did not act as cryoprotectants; rather, they interact directly with ice and modify the recrystallization process (Griffith et al., 2005).

3.4. Molecular mass and homology of ISPs in some plants

Ice structuring proteins from plants are relatively small, varying in their homologies to some known proteins (Griffith & Yaish, 2004; Hon et al., 1994; Huang & Duman, 2001; Meyer et al., 1999; Smallwood et al., 1999; Wisniewski et al., 1999).

Three ISPs were isolated from bittersweet nightshade with molecular masses of 67, 47 and 29 kDa (Huang & Duman, 2001). The ISP in bittersweet nightshade with 67 kDa molecular mass was identified with homology to proteins that are transcription factors to regulate the production of pathogenesis-related proteins in plants. The 47 and 29 kDa bittersweet nightshade ISPs were homologous with chitinase-like proteins and class I chitinase-like proteins, correspondingly (Huang & Duman, 2001).

Isolation and identification of ISP in carrot revealed that this protein has a sequence similar to the family of polygalacturonase inhibitor proteins with a molecular mass of 36 kDa, although it does not inhibit polygalacturonase (Meyer et al., 1999; Smallwood et al., 1999).

Peaches contain ISP that is homologous with the 60 kDa dehydrins (Wisniewski et al., 1999). Dehydrins are a family of proteins that are regulated by some environmental stresses including low temperature or dehydration.

The isolation of ISPs has been reported in some other plants including *Ammopiptanthus mongolicus* and perennial ryegrass with no homology reported to any known family of proteins and molecular masses of 50, 28 and 29 kDa, respectively (Griffith & Yaish, 2004).

Apoplastic extracts of cold acclimated winter rye leaves contain six ISPs, ranging in size from 16 to 35 kDa (Hon et al., 1994). The 35 kDa and 32 kDa ISPs in winter rye showed homology to β -1,3-endoglucanase. The other 35 kDa ISP in winter rye and the 28 kDa ISP had homology to class I endochitinase and class II endochitinase, respectively. The ISPs with molecular masses of 25 and 16 kDa were similar to those thaumatin-like proteins in winter rye (Hon et al., 1994). Yu and Griffith (1999) reported that the winter rye ISPs form oligomeric complexes and enhance the RI capacity of these proteins by blocking the binding of water over a larger area of the ice surface.

Winter wheat seeds contain ISP complexes ranging from 15 to 30 kDa (Xu et al., 2009). Apoplastic extracts of cold acclimated winter wheat leaves were reported to contain ISPs ranging in size from 12 to 39 kDa (Atıcı & Nalbantoğlu, 2003). It has been thought that ISPs in winter wheat are homologous to thaumatin-like proteins (Hassas-Roudsari, 2011; Venketesh & Dayananda, 2008).

4. Ice crystallization and recrystallization

Ice crystallization and recrystallization refer to the phenomena of phase transition of water from liquid to solid (Goff, 2005). The key difference is that in ice crystallization ice crystals are formed primarily upon nucleation whereas in ice recrystallization the existing large

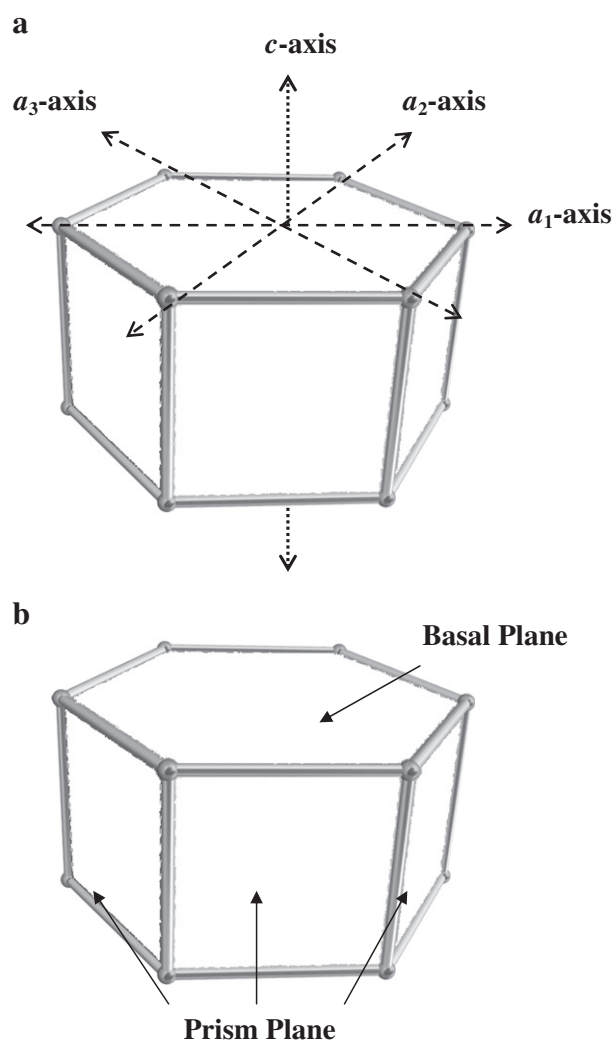


Fig. 1. The hypothetical morphology of an ice crystal; a) three symmetric a -axes lay in one plane and a lone c -axis lay in a plane perpendicular to a -axes plane b) the plane with a -axes is called basal plane and the plane with c -axis is called prism plane.

ice crystals grow at the cost of vanishing small ice crystals without the nucleation process involved (Sahagian & Goff, 1996).

4.1. Structure of ice

Ice is the outcome of the special arrangement of water molecules in a hydrogen bonding network wherein each water molecule forms four hydrogen bonds with its nearest neighboring water molecules (tetrahedral arrangement). In this crystal form, three symmetric *a*-axes lay in one plane and a lone *c*-axis lay in a plane perpendicular to *a*-axes plane (Fig. 1a). The plane with *a*-axes is called basal plane and the plane with *c*-axis is called prism plane (Wathen & Jia, 2005) (Fig. 1b).

Ice grows at a different rate in each plane; the growth in the prism plane direction is much faster than in the basal plane direction. This difference in the growth rate is due to the different configuration of water molecules and hydrogen bonding opportunities in each place, which provides diverse surface characteristics (Wathen & Jia, 2005). Temperature plays a major role on the growth rates. The morphology of ice crystals at temperatures just below 0 °C appears as circular disks (Fig. 2a) and at lower temperatures looks more irregular (Fig. 2b).

4.2. Mechanism of ice crystallization

When water or any solution is cooled, molecular motions slow down and the solution might remain in a liquid phase even when the temperature is lower than its equilibrium freezing point. This is called undercooling or supercooling (Goff, 2005). The melting point

of a solution is the temperature that the last ice crystal melts upon the slow heating of the frozen solution (Zachariassen & Kristiansen, 2000). Solutions undercool to varying degrees until they reach the point of spontaneous freezing (nucleation point), at which time crystallization begins (Zachariassen & Kristiansen, 2000). Crystallization involves two major steps: nucleation, which is the formation of a stable crystal nucleus (embryo), and propagation of ice crystals by growth of the nucleus (Goff, 2005).

Nucleation generally occurs around a foreign molecule (heterogeneous nucleation) or through a process by which molecules form nuclei spontaneously during intrinsic fluctuations (homogeneous nucleation) in the case of absolute pure water (Sahagian & Goff, 1996). Heterogeneous nucleation occurs at higher temperatures than homogenous nucleation (Zachariassen & Kristiansen, 2000).

The formation of small stable nuclei takes place when the molecular motion of the solution decreases upon the reduction of the temperature and molecules in the liquid phase deposit on a growing cluster (embryo). A higher rate of heat removal or more undercooling results in smaller and more stable nuclei, accordingly, more numerous ice crystals are formed since each nucleus promotes the formation of one ice crystal (Goff, 2005; Sahagian & Goff, 1996). In contrast to nucleation, a slow rate of heat removal or low undercooling increases the rate of crystal growth, which causes fewer and larger ice crystals (Goff, 2005).

4.3. Mechanisms of ice recrystallization

Recrystallization of ice occurs when the temperature fluctuates within the subzero range through different mechanisms such as disappearance of smaller ice crystals and the growth of larger ice crystals. These are more potent to cause physical damage to tissues and cells than smaller ones (Griffith & Ewart, 1995).

There are numerous types of recrystallization processes described in the literature (Fennema, 1973; Goff, 2005; Zaritzky, 2006). Iso-mass, migratory and accretive recrystallization are the types that are most common (Fig. 3). The reduction in surface free energy is the thermodynamic driving force of all these mechanisms (Sutton & Wilcox, 1998).

4.3.1. Iso-mass recrystallization

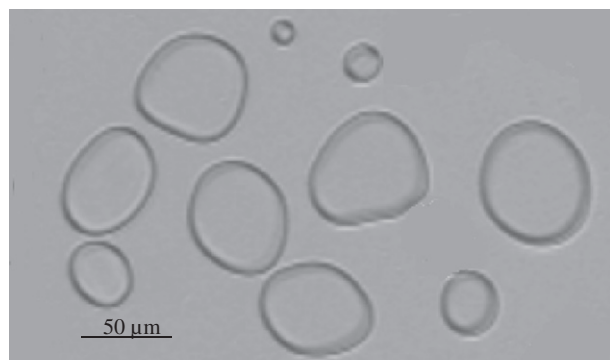
This process is also called rounding off and involves the conversion of the ice crystals with irregular surfaces to smoother ones (Fig. 3a). Ice crystals with sharper surfaces are less stable due to a higher radius of curvature of higher specific energy, therefore, they become flatter over time (Goff, 2005). This process lowers the surface energy of ice crystals with irregular shapes and large surface-to-volume ratios through a change in surface or internal structure of ice by adapting a more compact structure (Fennema, 1973). Surface diffusion of the water molecules may initiate this change in ice crystal structure (Zaritzky, 2006).

4.3.2. Migratory recrystallization

In most ISP-related literature, migratory recrystallization is described as the ice recrystallization mechanism (Atıcı & Nalbantoğlu, 2003; Griffith & Ewart, 1995; Griffith & Yaish, 2004; Venketesh & Dayananda, 2008; Winfield et al., 2010; Zachariassen & Kristiansen, 2000). This type of recrystallization refers to the growth of large crystals at the expense of smaller ones. Smaller ice crystals melt and disappear more rapidly than those of larger ones due to their higher free surface energy as a result of a low radius of curvature and large surface-to-volume ratio (Zaritzky, 2006). At constant temperature and pressure, migratory recrystallization is also called Ostwald ripening (Fennema, 1973).

When temperature fluctuates, migratory recrystallization can also be described as melt–refreeze recrystallization and categorized to melt–diffuse–grow and melt–shrink–regrow recrystallization

a) -4°C



b) -10°C

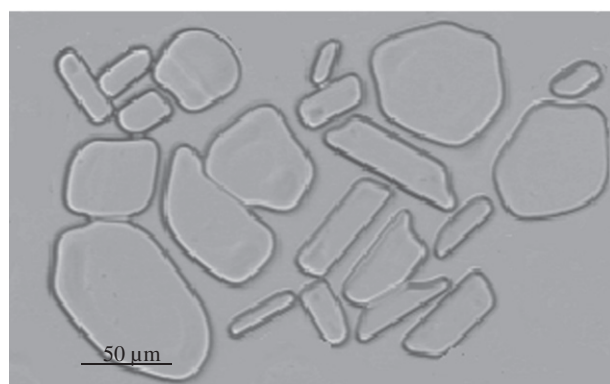


Fig. 2. Brightfield images obtained from 23% sucrose solution at freezing temperatures show a) circular disk shaped ice crystals at -4 °C and b) irregular shaped ice crystals from fast freezing, which have not yet ripened to their lowest energy state.

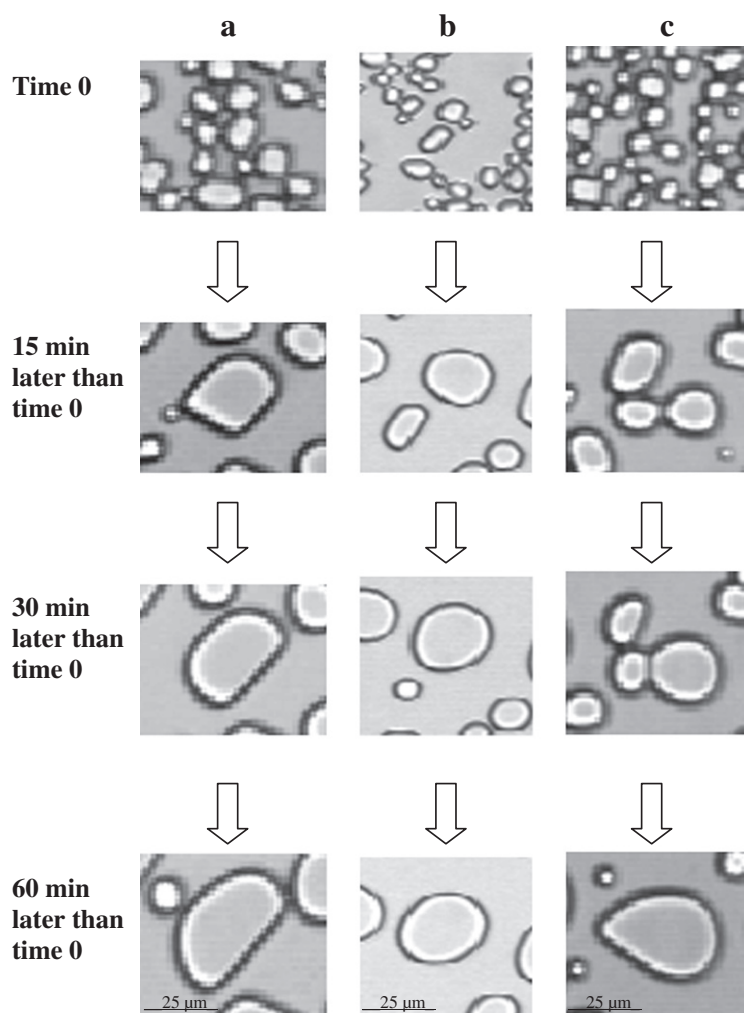


Fig. 3. Brightfield images obtained from 23% sucrose solution at -4°C , at time 0, 15, 30 and 60 min later than time 0. Images indicate the progress of mechanisms of recrystallization over time: a) isomass; b) migration; and c) accretion.

(Fig. 3b). In melt–diffuse–grow, upon increasing of temperature, the smallest ice crystals completely melt and increase the liquid phase of the solution. As soon as the temperature drops, water from liquid phase deposits on the surface of larger crystals, therefore, the number of ice crystals decreases (Fennema, 1973). In melt–shrink–regrow, the number of ice crystals remains constant since ice crystals partially melt during warming and regrow during cooling. Melt–diffuse–grow behavior is more anticipated at higher freezing temperatures during temperature fluctuation since smaller ice crystals melt faster at those temperatures due to their lower resistance towards melting (Franks, 1985). Smallest crystals are thermodynamically more potent towards shrinking or melting, hence, the largest ice crystals would grow in order to minimize surface area and free energy. The net result of this process is a decline in the total number of ice crystals and an increase in the mean size of crystals (Goff, 2005).

4.3.3. Accretive recrystallization

The fusion of two or more neighboring ice crystals together results in fewer and larger ice crystals (Fig. 3c). The mechanism of accretive recrystallization is thought to be surface diffusion without any movement of the crystals (Zaritzky, 2006). Recrystallization through accretion takes place at a high ice phase volume since the movement of ice crystals towards each other is thought to be unlikely. At the point of contact between ice crystals (neck), high concentration gradients occur due to the transportation of material into that region (Goff,

2005). In some cases, networks of crystals form through accretion recrystallization (Sutton & Wilcox, 1998). Ice crystal accretion is generally followed by rounding off to provide a more spherical shape in ice crystals to reduce surface free energy as high-curvature surfaces become planar (Goff, 2005).

As noted above, migratory recrystallization has been addressed in ISP studies as the only mechanism of ice recrystallization, which does not truly express the total phenomena. All above mechanisms occur, at the same time, when ice recrystallizes. Therefore, authors suggest that the recrystallization concept could be reworded to “growth of larger ice crystals at the cost of restructuring of other ones”.

5. Interaction between ice crystals and ISP

The nature of ISP–ice binding has been studied for decades. Early studies have shown that the interaction of ISPs and ice is different than those of other solutes, also the way those proteins affect the freezing process is dissimilar to other solutes (Wathen & Jia, 2005). However, it is noteworthy that AFPs may interact with ice formation in addition to ice recrystallization.

There are three known behavior of ISPs and AFPs when it comes to interaction with ice crystals. These include interactions with ice nucleation properties, thermal hysteresis and ice recrystallization inhibition.

5.1. Interactions with ice nucleators

Some bacteria use strong ice nucleating agents such as proteins to nucleate ice formation on the surface of fruits and plants at just below 0 °C. Ice formation would cause injuries in the plant and make the nutrients available to the bacteria (Zachariassen & Kristiansen, 2000). Ice nucleating proteins have been subdivided into three types: ice nucleating proteins that nucleate water at or above −5 °C (Type I), ice nucleating proteins with nucleating activity at temperatures between −5 °C and −7 °C (Type II), and proteins that are nucleating ice below −7 °C (type III) (Cochet & Widehem, 2000; Venketesh & Dayananda, 2008).

It has been shown that some AFGPs bind to ice nucleating proteins of bacteria and inhibit the activity of those proteins, hence, result in antifreeze activity in the same manner as they do with ice crystals (Griffith & Yaish, 2004). The interaction of these two proteins not only inactivated the nucleating activity due to binding of the proteins to each other but also enhanced the antifreeze action (Duman et al., 1993).

5.2. Thermal hysteresis

The AFPs, including THPs and to a much lower extent some ISPs, decrease the freezing point of water without significantly altering the equilibrium melting point in a non-colligative way. The thermal hysteresis of those proteins, particularly THPs, was a thousandfold greater than expected for their molar concentrations in the solutions (DeVries, 1983; Venketesh & Dayananda, 2008; Zachariassen & Kristiansen, 2000).

Thermal hysteresis activity has been one of the routine methods to measure the activity of AFPs or even ISPs, however, higher concentrations of protein are required to exhibit this activity compared to other methods such as RI assays (Griffith & Yaish, 2004). It has been reported that AFPs or ISPs work additively with colligative agents such as sugars, salts and amino acids to a certain concentration of AFP, where the activity saturates and a plateau is reached; a further increase in the concentration of AFPs does not result in a further thermal hysteresis activity (Yeh & Feeney, 1996). Thaumatin-like proteins were reported to work as “enhancers” for thermal hysteresis activity (Wang & Duman, 2006).

Thermal hysteresis in plant ISPs is low and only between 0.2 °C and 0.4 °C compared to those of fish AFPs, which show a thermal hysteresis between 0.6 °C and 1.5 °C (Ewart et al., 1999; Fletcher et al., 2001; Hassas-Roudsari, 2011; Venketesh & Dayananda, 2008).

5.3. Ice recrystallization inhibition

Ice recrystallization was discussed extensively in Section 3.4. In ISP related literature, migratory recrystallization (i.e., larger crystals growing larger at the cost of smaller ones, which disappear) has been considered as the main recrystallization phenomenon that occurs most rapidly at temperatures just below the freezing point (Atıcı & Nalbantoğlu, 2003; Griffith & Ewart, 1995; Griffith & Yaish, 2004; Venketesh & Dayananda, 2008; Winfield et al., 2010; Zachariassen & Kristiansen, 2000).

Extremely low concentrations of ISPs, as low as 100 µg/L and 10^{−8} M, are effective in RI activity, while these concentrations are too low for the exhibition of any thermal hysteresis activity (Griffith & Yaish, 2004; Yeh & Feeney, 1996). ISPs from ryegrass exhibited a very low thermal hysteresis activity but a high RI activity compared to those of fish ISPs (Sidebottom et al., 2000). Solutions containing plant ISPs exhibited RI activity at concentrations as low as 25 µg protein/L (Griffith & Ewart, 1995; Sidebottom et al., 2000). It has been reported that sucrose solutions containing 0.13% total protein from winter wheat crude extracts containing ISPs reduced ice crystal growth by 74% compared to control after temperature cycling (Regand & Goff, 2005). The RI activity occurrences were

reported by fish, insects and plant ISPs (Atıcı & Nalbantoğlu, 2003; Venketesh & Dayananda, 2008; Winfield et al., 2010; Zachariassen & Kristiansen, 2000) showing that RI activity may be a shared characteristic between all ISPs but not thermal hysteresis or ice nucleator binding activities, therefore, it has become a common tool to measure antifreeze activity.

6. Antifreeze activity assays

There are two main assays to measure the antifreeze activity of ISPs *in vitro*: thermal hysteresis and RI measurements.

The thermal hysteresis assay measures the difference in temperature between freezing and melting point of a solution in the presence of ISP (Zachariassen & Kristiansen, 2000). Thermal hysteresis *in vitro* is measured by using a nanoliter osmometer. In this assay, a very low amount (submicroliter volumes) of ISP solution is injected in a cylindrical well drilled in a metal plate which has been filled already with an oil droplet (to hold the surface tension) in a cold stage. The chamber temperature is controlled by a Peltier device and the sample is viewed under a microscope. The growth and shrinking of the ice crystals during the measurement can be recorded by video photography and studied (Fletcher et al., 2001).

There has been some inaccuracy and inconsistency with thermal hysteresis measurements with direct microscopic observation of crystal growth in a sample. In this method there is no accurate way of recording the freezing point, and a secondary onset temperature of crystallization in a solution with an ice nucleus, since they are judged by the unassisted visual assessment (Lu et al., 2002). Samples after measurement cannot be re-used for other applications. Inserting samples in a metal plate and also finding a proper crystal to observe for the assay can be a very time-consuming and tedious process.

Hansen and Baust (1988) proposed a method using a differential scanning calorimeter (DSC) to measure the thermal hysteresis activity of fish AFPs. In this method AFP containing samples were suspended in oil in the DSC pan. This method brought a high level of accuracy into thermal hysteresis measurements since DSC is able to record melting and freezing temperature of samples, meticulously. One of the limitations of the proposed method was using an oil environment, which is not only very different than the natural condition but also interferes with AFP and thus may affect the results. Thermal hysteresis of AFPs was assayed using DSC in aqueous solution directly obtained from plants (Lu et al., 2002), on the basis of Hansen and Baust's method. This method provided a more repeatable and reliable tool for studying thermal hysteresis of plant AFPs. Data from our lab also showed consistency and accuracy of this method (Hassas-Roudsari, 2011).

DSC has the advantage of using plant extract solution without any specific preparation and also samples can be recovered and reused for other purposes such as RI activity. The amount of sample that is needed for this assay could be as low as 1 µL, however, thermal hysteresis activity is concentration-dependent. Since DSC instruments are computerized devices, they are very easy to program to explore different conditions and parameters. Although it is possible to calculate the volume of the ice in the sample, this assay does not provide any data for ice crystal sizes. High cost of a DSC instrument can be one of the disadvantages of using this method.

The ice recrystallization inhibition assay measures the gradual changes in the size of ice crystals in a solution at constant or fluctuating temperature. There are several publications on conducting this assay. In one of the earliest methods, a thin layer of ice was formed immediately when an aqueous solution of ISP was expelled from a height of 3 m onto a liquid nitrogen-cooled metal plate, the so-called plate test (Knight et al., 1988). This method was modified by Smallwood et al. (1999) who snap-froze the sample between two cover slips in a beaker of dry ice. In both methods, frozen samples were analyzed using a microscope. Smallwood et al. (1999) modified

this method in which they snap-froze the sample between two cover slips in a beaker of dry ice-cooled. One the main disadvantages of these methods is that the sample is unrecoverable after analysis and cannot be stored for other applications or to be analyzed simultaneously.

In the most recent method introduced by Regand and Goff (2005), a sample containing ISP and sucrose, to act as a solute to depress the freezing point and create an unfrozen phase in equilibrium with the ice, is placed on a slide and then cooled quickly to a very low temperature (e.g., -50°C) in the chamber controlled by a cold stage device to provide rapid nucleation. Afterwards, the chamber temperature is increased (still in freezing range), cycled between -10°C and -4°C and held at each temperature for a certain amount of time to let the ice crystals grow. The large ice crystals grow and the smaller ice crystals disappear during the process. After temperature fluctuations, a solution containing ISP shows smaller ice crystals compared to a control solution (without ISP). The process is monitored using a microscope and can be recorded by camera so the pictures can be analyzed through computer software for obtaining quantitative results.

The original method takes about 4 h to be finished. We were able to shorten the method to 30 min, which makes it very suitable for a quick qualitative analysis (Hassas-Roudsari, 2011). This method requires less than 5 μL of sample for each run with the downside of un-recovery of samples, due to the presence of sucrose.

In a method called “RI end point”, serial dilutions of samples were prepared for detecting the RI activity of a sample to the point of concentration that the RI activity is no longer detectable (Tomczak et al., 2003). This method has the advantage of preserving the samples for further analysis and also viewing them simultaneously. All RI assays require a lower concentration of ISP for detection compared to DSC methods.

Very recently, Hassas-Roudsari and Goff (in press) proposed a quick qualitative method using DSC based on RI assay. This method measures the ISP activity rate in an isothermal condition over 60 min. In this method, ISP containing samples show exothermic thermograms at certain concentration and time ranges. Thermograms of blank samples are not exothermic. This method eventually can be applied for any ISP or AFP containing samples in their own aqueous solvent, i.e., in a buffer or water based extract solution. This method is very precise and quick and does not require intense laboratory work or observation. The amount of sample can be as low as 1 μL . This method can be combined with thermal hysteresis studies using previously described DSC methods. A few limitations of this method are that samples cannot be recycled and the working concentration range needs to be identified. This assay does not provide any data for ice crystal sizes.

7. Mechanisms of action of ISPs

Restriction of crystal growth by ISPs is through a generally accepted mechanism by which ISPs bind to the surface of an ice crystal and interfere with the normal propagation of the ice crystal surface by inhibiting the growth in the areas where ISPs cover the ice surface (Raymond & DeVries, 1977; Venketesh & Dayananda, 2008; Wen & Laursen, 1993). Such inhibited growth in some regions of the ice surface while other regions are still growing results in surface curvature of the ice crystal and consequently, an increase in the surface-area-to-volume ratio beyond the point that is thermodynamically favorable for spontaneous ice growth to occur. Therefore, ice crystal growth will be terminated. This mechanism is called “Kelvin (Gibbs–Thomson) effect” or the “button-mattress theory” (Yeh & Feeney, 1996).

In a solution without ISPs, crystal growth along a-axes results in the expansion of basal planes of ice crystals (Fig. 4a). In solutions containing ISPs, ice growth is often along the c-axis giving rise to prism faces perpendicular from the basal plane or in directions that are not along basal faces (Grandum et al., 1999; Knight et al., 1991,

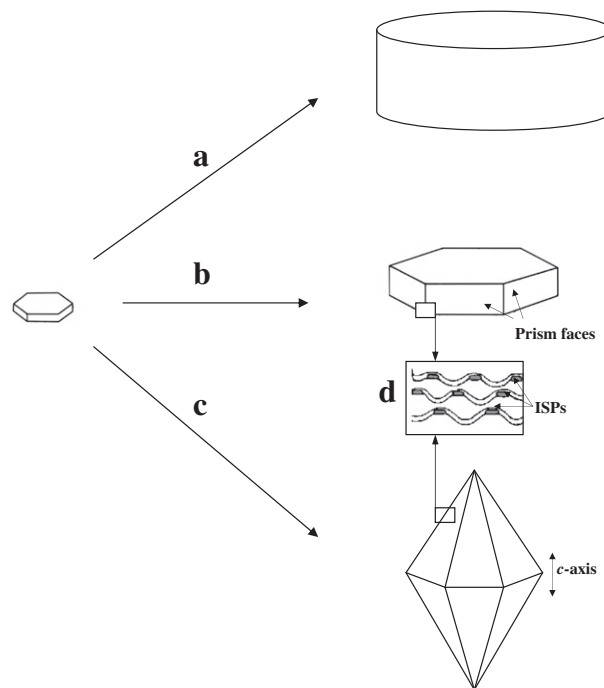


Fig. 4. Ice crystal growth a) in a solution without ISPs; b) in a dilute solution of ISPs which is absorbed to the prism face, forming a crystal that is hexagonal in shape; c) in a concentrated solution of ISPs, shaping an ice crystal into a hexagonal bipyramid; d) Inhibition of crystal growth by ISPs through “Kelvin effect” by which ISPs (gray dots) bind to the surface of an ice crystal and interfere with the normal propagation of the ice crystal surface by inhibiting the growth in the areas where ISPs cover the ice surface.

1993; Raymond & DeVries, 1977) (Fig. 4b,c). Consequently, the morphology of ice crystals affected by ISPs is similar to hexagonal bipyramids or needles. It has also been found that crystal shape and size are strongly dependent on the undercooling in the liquid surrounding the crystal (Grandum et al., 1999). The a-axes, c-axis and different planes of an ice crystal are shown in Figs. 3 and 4. This preferential affinity of ice faces and ISPs became a point of interest in much research and was demonstrated through ice-etching experiments (Knight et al., 1991). In this experiment a single pure ice crystal is grown slowly in a dilute ISP containing solution and ISPs bind to the particular planes of ice that they have affinity to, over time, and those planes are the ones that become visible. This experiment explained that ISPs do not bind to random planes across the ice surface. It is noteworthy that ice-ISP binding efficacy is not only concentration dependent but also diffusion rate dependent. It has been reported that at lower ISP concentration, the same freezing point depression can be achieved as at higher concentration, by applying a very gentle cooling rate to allow more time for ISP diffusion to occur (Wathen & Jia, 2005).

The positioning of suspected ice-binding motifs in some ISPs demonstrated regularities while other ISPs show very little structure in common. There are two techniques most frequently used to study the activity of ISPs in terms of interaction between active ice-binding motifs and ice, knowing that it is still not possible to directly look at the molecular interactions occurring between ISPs and ice. These techniques are mutagenesis and computational modeling (Venketesh & Dayananda, 2008; Wierzbicki et al., 2007).

The first model was based on hydrogen bonding with ice surfaces to understand the ice binding mechanism of ISPs (DeVries, 1983). It focused on hydrophilic amino acids, i.e., regularly spaced threonine residues of fish type I ISP from winter flounder as a primary mechanism for ice binding. A few other experiments such as ice-etching experiments (Knight et al., 1991; Yang et al., 1998) and computer docking simulations (Chen & Jia, 1999) supported this hypothesis.

However, the certainty of this assumption was questioned when, surprisingly, some other mutagenesis experiments on a different surface of fish type I ISP from winter flounder revealed that hydrophobicity is also very critical for ice binding (Baardsnes et al., 1999). In these mutagenesis experiments, hydrophobic alanine residues replaced with hydrophilic threonine residues demonstrated weak interactions with the ice surface.

In the current most widely accepted model, called the “snug fit” model, the hydrophobic surfaces of ISPs are essential for the ice binding mechanism (Harding et al., 1999; Jia & Davies, 2002). In this model, nonpolar side chains of amino acids (e.g., methyl groups) fit in regular spacing on the surface of ice, i.e., ice cages, grooves, or hexagonal rings, through favorable van der Waals and/or hydrophobic interactions (Jorov et al., 2004; Yang et al., 1998). Hydrogen bonds may support these contacts; i.e., Damodaran (2007) proposed that formation of hydrogen bonds between an ice crystal and gelatin hydrolysate is inhibiting ice crystal growth. However, hydrogen bonding is not necessarily essential for ice binding. In some literature, contribution of van der Waals contacts is assumed to be prior to the hydrogen bonds (Davies et al., 2002). In a very recent proposed mechanism, an “anchored” ice-like lattice to the backbone of the ISP was identified as the exact match to both the primary prism and basal planes of ice (Garnham et al., 2011). This suggests that ISPs form their binding site on the ice before binding to it. This mechanism is called the “anchored clathrate” mechanism (Garnham et al., 2011). They explained that the ISPs order surface water molecules into an ice-like lattice through the hydrophobic effect. This lattice is attached to the ISP by hydrogen bonds. These anchored ice-like waters bind to the ice during formation of the ice, resulting in ice-ISP binding at a specific plane, or planes, of ice (Garnham et al., 2011).

Another factor that has been examined as a contributing factor towards the activity of ISPs is “flatness”, which has also been suggested to play an important role in a snug fit model (Jia & Davies, 2002; Yang et al., 1998). At the molecular level, the ice binding surface is crammed with projections, cavities and other major discontinuities that extremely disturb antifreeze activity as demonstrated by site-directed mutagenesis (Jia & Davies, 2002). Therefore, relative flatness on the ice binding surface is one of the important elements for a snug fit model.

There is still much debate whether binding of ISP to ice is reversible or not (Celik et al., 2009; Garnham, 2011; Knight, 2000; Venketesh & Dayananda, 2008; Wathen & Jia, 2005). Some propose that binding of ISP to ice needs to be virtually irreversible for ice crystal growth inhibition to occur. Computational modeling calculated an adsorption free energy of 2–3 kJ/mol (Wierzbicki et al., 2007). This study took the dynamic nature of ice (i.e., liquid water) into account, a parameter that has been neglected by many other studies. They did not find a structural match between ISPs and ice; hence they proposed that ISPs do not bind to the ice directly. Instead, ISPs accumulate at the interfacial region with their hydrophobic face oriented toward the ice/water interface to minimize the interfacial energy of interaction with the ice surface. This mechanism is similar to that of a hydrophobic solvation effect (Despa et al., 2004) suggesting that ISPs are “poisoning” the interfacial region by facilitating the ordering of liquid water into a specific surface of ice at its hydrophobic face as originally proposed by Haymet et al. (1998) and reported by many others (Dalal et al., 2001; Houston et al., 1998; Madura et al., 2000). In the “kinetic pinning” mechanism, proposed by Sander and Tkachenko (2004), the interaction between ISPs and ice c-axis of an ice crystal is assumed as a dynamic process and this mechanism also supports the hypothesis that ISPs interfere with the growth of an ice surface at the ice/water interface. Celik et al. (2009) argued that ISP-ice binding is irreversible, which causes a “superheating” phenomenon by which retained superheated ice crystals are stable for hours above their equilibrium melting point (up to 0.44 °C). However, it is still unclear how ISPs remain intact to the surface of ice

crystals when ice crystals partially melt. Either ISP detach from the surface of ice (e.g., due to melting) and re-attach quickly to the surface or ISPs exist in the form of a network around an ice crystal so at least a section of the “net” is intact while other sections are disconnected. Both assumptions may be explained theoretically by the anchored clathrate mechanism as the ice-like water attached to ISPs act as strong ISP-ice or ISP-ISP connectors.

The degree of ice crystal growth inhibition varies from one ISP to another. In a computational model, the interactions between hundreds of ISPs and dynamic ice crystals with millions of water molecules were investigated (Wathen et al., 2003). According to this study, the differences in ice crystal growth inhibition are attributable to the different planes of ice to which the ISPs prefer to bind rather than the strength of ISP-ice interaction.

Ice growth is anisotropic; therefore, growth rates differ from one direction to another. Also, the ice binding plane(s) in conjunction with the particular orientation of ISPs on that plane determine the amount of ice surface curvatures (Wathen & Jia, 2005). Supporting this finding, Liu and Li (2006) reported that the level of ISP activity is governed by the affinity and specificity of ISPs for ice surfaces, as well as surface coverage. The enormous activity of some hyperactive ISPs is correlated with multiple binding sites and the large combined surface area of these regions (Graham et al., 2007). Garnham et al. (2011) proposed that moderately active ISPs are able to order the (ice-like) water molecules that allow them to absorb to multiple planes of ice excluding the basal plane. Whereas hyperactive ISPs (e.g., insects or fish ISPs) are able to order (ice-like) water molecules in a more versatile way that they will be adsorbed to both prism and basal planes of ice.

8. Applications of ISPs in food

Some ISPs naturally exist in many foods consumed as part of the human diet (Atıcı & Nalbantoğlu, 2003; Crevel et al., 2002; Griffith & Ewart, 1995; Griffith & Yaish, 2004). Cold-water fishes have been one of the main sources of AFP in the human diet. A 200 g of ocean pout contains 180 to 420 mg of AFP Type III, while the same portion of cod provides 42 to 196 mg of AFGP (Crevel et al., 2002).

Food technology also could benefit enormously from isolated ISPs by inhibition of ice crystal growth during freezing storage, transport, and thawing, consequently preventing quality loss such as deterioration in food texture, i.e., by reducing cellular damage and also the loss of nutrients by reducing drip (Griffith & Ewart, 1995; Feeney & Yeh, 1998; Wathen & Jia, 2005). In many freeze-tolerant organisms, ISPs are the means to protect cells extracellularly. This indicates that ISPs can be added to food by physical means such as mixing, injection, soaking, coating, spraying, or vacuum infiltration.

One of the applications of ISPs in frozen foods involves inhibition of recrystallization of ice in ice cream. This technique has been applied in ice cream, where ice crystal growth was very small compared to the control (Goff et al., 2002; Regand & Goff, 2003). Commercial ice cream products with ISPs are now available in the market (Crilly et al., 2008).

Freezing and thawing can cause damage to frozen meat, where large ice crystals may form intracellularly and burst the cell, resulting in drip and loss of nutrition during thawing. In a study where bovine muscles were soaked in a solution of type I AFP or AFGP (from the Winter Flounder and Antarctic Cod, respectively, up to 1 mg/mL) prior to freezing at −20 °C, ice crystals were significantly reduced in size (Payne et al., 1994). In another study, the effect of pre-slaughter administration of AFGP from Antarctic Cod on lambs and the quality of meat after slaughtering and freezing were investigated (Payne & Young, 1995). They injected AFGP intravenously into lambs at various times prior to slaughtering, vacuum packed the meat samples after slaughtering and stored at −20 °C for 2 to 16 weeks. It was reported that injection of AFGP, at either 1 h or 24 h before slaughter,

reduced drip loss and ice crystal size. At 24 h before slaughter, crystals were smallest in the lambs injected to a final concentration of 0.01 µg AFGP per kg weight of the animal. Yeh et al. (2009) reported that frozen meat treated with type I AFP (produced and secreted by *Lactococcus lactis*, a food-grade microorganism of major commercial importance) showed less drip, less protein loss, and a high score on juiciness by sensory evaluation. The expression of ISPs in lactic acid bacteria during fermentation has been patented in the past since these bacteria are used for production of yogurt, sauerkraut, pickles, fermented sausages, and miso and thus no purifying AFP prior to addition to food is required (Fletcher et al., 1997; Venketesh & Dayananda, 2008).

Frozen storage is one of the solutions to enhance the short shelf life of conventional bread dough, although several problems need to be considered such as deterioration of the bread quality by a reduced volume, a weakened structure, a poor texture, and a decrease in the proportion of gas content (Ribotta et al., 2001). Application of ISPs has been reported to improve the quality of frozen dough through enhancing the gassing rate and total gas production in frozen sweet dough (Panadero et al., 2005). Concentrated antifreeze protein from carrots has been shown to improve the quality of the dough by maintenance of loaf volume and improving the softness of the dough during frozen storage (Zhang et al., 2007). Incorporation of ISP from winter wheat decreased the rate of structural deterioration in the gluten–ice structure during the recrystallization stage (Kontogiorgos et al., 2008). In a different study, frozen dough treated with type I AFP (produced and secreted by a food-grade *L. lactis*) showed better fermentation capacity than untreated frozen dough and after baking the breads acquired the same consumer acceptance as fresh bread (Yeh et al., 2009). However, authors did not mention the effect of ISP on the quality of frozen and thawed bread. In a recent study, the addition of ISP (0.5%, w/w) to corn and wheat starch gels improved freeze-thaw stability in terms of syneresis and hardness (Li et al., 2010).

In terms of food safety, several ISPs that exist in edible parts of plant or some fish have been in the human diet for thousands of years. However, introduction of novel proteins into foods may be a concern for individuals sensitive to that protein (Lehto et al., 2010; Lopata & Lehrer, 2009).

8.1. Toxicity

Crevel et al. (2002) reviewed short and long term exposure to ISPs using examples of the USA, a country with moderate fish consumption, and Iceland, a country where fish are consumed in large amounts. Based on the history of consumption, they concluded that the fish and plant ISPs are not toxic. Their functional characteristics would not cause any obvious adverse toxicological properties; these proteins have not been affiliated with any known toxic effect due to fish consumption, either in the short or long term. The acute exposure can be described as consumption of a typical portion of fish (estimated as 200 g) and, therefore, the digestion of fish AFPs and long-term exposure is a concept that can be explained by estimated data on fish catches and consumption (Crevel et al., 2002).

8.2. Allergy

Seafood allergy (i.e., allergy to fish protein) has been known and thoroughly investigated (Lopata & Lehrer, 2009). Thus far, there has been no report on the role of ISPs in fish as potential food allergens. The results from a study on evaluation of the general health and immunogenicity of ISP Type III on healthy volunteers who consumed the protein for a period of 8 weeks showed that ISP does not have any effect on general health (Crevel et al., 2007). In plants, it has been reported that some thaumatin-like proteins are associated

with baker's respiratory allergy (Lehto et al., 2010), although the antifreeze activity of these proteins was not explored in that study.

9. Conclusion

The anchored clathrate mechanism has been the most recent proposed mechanism to explain the mechanism of action of ISPs, as well as intensity of the activity. There has not been extensive food application for ISPs to date but there is very promising outlook for this new food ingredient. No data has been reported that challenges the safety of ISPs from plant sources.

Application of ISPs as a food preservative would enhance the quality of frozen foods and desserts during freezing storage, transport, and thawing. One of the advantages of ISPs for the food industry is that they are fairly active in such a low amount, which could make these “novel” food ingredients very cost effective. However, commercial supply of isolated ISPs may limit application.

Even though our knowledge about ISPs is not advanced enough to define the exact mechanism(s) of action, it has reached the level of understanding of the fundamental structures to be able to “invent” new ISP(s) or ice structuring peptides. Gelatin hydrolysate (small peptides, 2000–5000 kDa) produced by papain action showed inhibitory activity on ice crystal growth in ice cream mix (Damodaran, 2007). More studies are required to explore the possibility of special processing (e.g., enzymatic reaction) on safety and nutritional capacity of convenient food products, such as milk and meat, as new sources of ISPs.

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