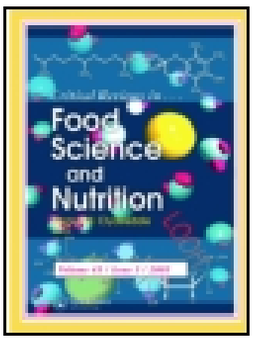

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REVIEW

Ice-binding proteins: a remarkable ice crystal regulator for frozen foods

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ABSTRACT

Ice crystal growth during cold storage presents a quality problem in frozen foods. The development of appropriate technical conditions and ingredient formulations is an effective method for frozen food manufacturers to inhibit ice crystals generated during storage and distribution. Ice-binding proteins (IBPs) have great application potential as ice crystal growth inhibitors. The ability of IBPs to retard the growth of ice crystals suggests that IBPs can be used as a natural ice conditioner for a variety of frozen products. In this review, we first discussed the damage caused by ice crystals in frozen foods during freezing and frozen storage. Next, the methods and technologies for production, purification and evaluation of IBPs were summarized. Importantly, the present review focused on the characteristics, structural diversity and mechanisms of IBPs, and the application advances of IBPs in food industry. Finally, the challenges and future perspectives of IBPs are also discussed. This review may provide a better understanding of IBPs and their applications in frozen products, providing some valuable information for further research and application of IBPs.

KEYWORDS

Antifreeze protein; freeze damage; frozen foods; ice crystal; ice-binding proteins; ice-modulators

Introduction

Water is one of the main ingredients in most foods and water freezing is a universal phenomenon in nature. The freezing process of water includes ice nucleation and ice crystals growth (Schafer 2017; Sun 2005). Ice recrystallization, a destructive process in which smaller ice crystals merge into larger ice crystals, often occurs during freezing storage. Recrystallization weakens the benefits of freezing, and it involves the change of shape, size, number, orientation, or integrity of crystals after coagulation (Elliott, Wang, and Fuller 2017; Sun 2011). When the temperature of the sample increases below the freezing point, the rate of ice recrystallization also increases. In the process of food refrigeration, controlling the recrystallization of ice and the phase transition of water are considered as the two most important methods to maintain product quality (Zhu, Zhou, and Sun 2019). The shape and size of ice crystals, as well as their location inside or outside the cell, play important roles in the physical properties and texture of frozen foods (Ayati, Hamdami, and Le-Bail 2017; Chen, Cai, et al. 2019). Thus, development of appropriate technical conditions and ingredient formulations is one of the main challenges for frozen foods manufacturers.

Ice-binding proteins (IBPs) are a kind of active proteins which widespread across biological kingdoms, and they include antifreeze proteins (AFPs) and ice nucleation proteins (INPs) (Davies 2014). The typical function of IBPs is that they can absorb on the surface of ice crystal, thus restricting the growth of ice crystals, inhibiting the recrystallization of ice crystals, and changing the morphology of ice crystals. Therefore, IBPs are also called ice structure proteins (Bayer-Giraldi et al. 2018; Cao et al. 2016; Graham and Davies 2005; Raymond et al., 2007). Recently, the theory of ice crystal adsorption-inhibition of IBPs has been used to better describe the antifreeze mechanism (Kontogiorgos et al. 2007; Liu et al. 2016). IBPs can inhibit the recrystallization of ice at a lower concentration, and can completely inhibit the growth of ice in a certain temperature range according to their own properties at a higher concentration. IBPs are generally believed to increase the surface of ice crystals by binding to the surface of ice crystals, increasing the vapor pressure and lowering the freezing point. Thus the freezing point of solution is lowered, which is the so-called Kelvin effect (Davies 2014; Hudait et al. 2018; Liu et al. 2016; Qiu, Hudait, and Molinero 2019; Raymond and DeVries 1977). IBPs have long been expected to be used in food processing and preservation due to their good control over ice crystals, and their effects of improving the nutritional value and quality of frozen foods have become one of the hot topics (Chen et al. 2017; Kashyap, Kumar, and Singh 2020; Soukoulis and Fisk 2016; Zhu, Zhou, and Sun 2019).

This review begins with an introduction to the mechanism of water freezing to ice, and discusses the damage caused by ice crystals in frozen foods systems. Secondly, we summarized the methods and technologies for production, purification and evaluation of IBPs. Importantly, the properties, characteristics, structural diversity and mechanisms of

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IBPs, and the application advances of IBPs in frozen food preservation were discussed. Finally, the challenges, future perspectives and applications of IBPs are also proposed, providing some valuable information for further research and application of IBPs.

Ice crystals

Water and ice crystals

Water is one of the major components in most organisms, accounting for about 60%–90% of their weight. Water is also widely existing in most foods and is closely related to food shelf-life, quality attributes and biological functions (Fennema 1996; Zhu, Zhou, and Sun 2019). Water freezing is a common phenomenon, the process of ice crystal formation is composed of two processes: nucleus formation and grain growth (Schafer 2017; Sun 2011). During nucleation, water molecules combine to form ordered ice particles, which helps the water molecules to arrange into ice crystals on the surface of the particles. During ice crystal growth stage, the sizes of nuclear particles increase by adding more water molecules in an orderly manner (Fennema, Powrie, and Marth 1973; Liu et al. 2016). Moreover, the morphology of ice crystals exhibits a complex dependence on temperature and supersaturation. The freezing rate, mass diffusion, heat conduction, and water molecule aggregation to ice crystals ultimately are responsible for the morphology diversity of ice crystal (Figure 1) (Libbrecht 2005; Shibkov et al., 2003; Petzold and Aguilera 2009). The final ice crystal morphology depends on the solute, temperature and ice crystal growth rate of the solution, and the growth rate is different on different ice crystal surfaces (Sun 2011).

According to the purity of water, ice nucleation takes two forms: heterogeneous nucleation or homogeneous nucleation. Heterogeneous nucleation usually occurs in non-pure water systems such as frozen foods. However, in extremely pure water (without impurities), homogeneous nucleation is more likely to occur (Sun 2011; Zhu, Zhou, and Sun 2019). In the heterogeneous nucleation process, the molecules of water are aggregated into crystals on suspended surface films, foreign particles or nucleating agents (Zhu, Ramaswamy, and Le Bail 2005). In food systems, when the temperature of the products drops to the freezing point, the atoms begin to form an ice nucleus, a tiny crystal that balances the surrounding water molecules.

As long as the formation of ice nucleus is stable, it is possible to promote the growth of ice crystals by adding molecules between the solid and liquid phases of the ice nucleus. The growth rate of ice crystals is mainly determined by the removal rate of latent heat released during the phase transition and the mass transfer rate of the solution. When the latent heat released by ice crystal growth is more effectively removed, the ice crystal growth will accelerate. It is well known that a large number of small ice crystals are formed during the quick-freezing process of foods. However, even at the same cooling rate, the sizes of ice crystals formed in different frozen foods are different. This is mainly due to the different free moisture content in different foods (Petzold and Aguilera 2009; Sun 2011).

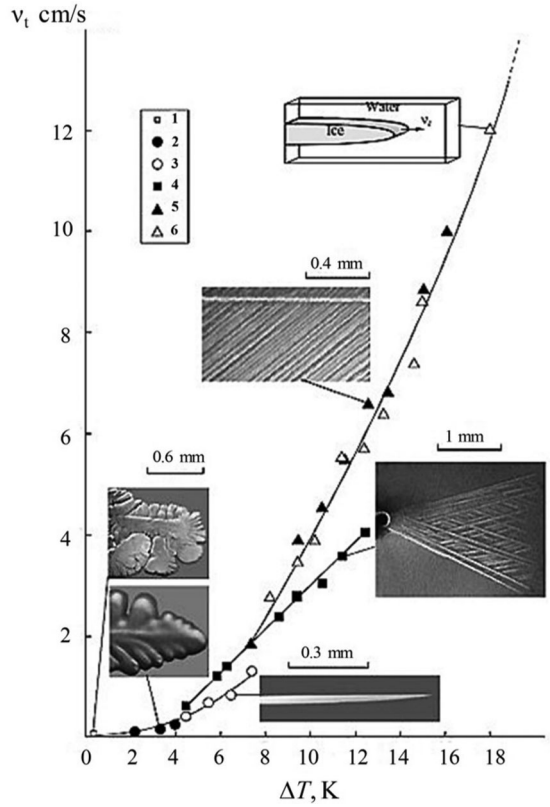

Figure 1. The ice crystal morphology diagram in supercooled water at different cooling rates. 1, 2, 3, 4, 5, 6 are dense-branching structure, dendrite, morphologically stable needle, fractal needled branch and compact needled branch, respectively. Source: Shibkov et al. (2003).

At the same time, ice crystals undergo metamorphism during the freezing process. Due to the high specific surface area and free energy of the small ice crystals, the size of the small ice crystals will increase, forming large ice crystals, that is, recrystallization occurs. Recrystallization includes melting of small ice crystals, growth of large ice crystals and fusion of ice crystals. The occurrence of recrystallization can cause the rupturing/damaging of the cell membrane and lead to cell damage, which makes the freezing technology lose its original advantage (Ayati, Hamdami, and Le-Bail 2017; Damodaran 2007; Damodaran and Wang 2017; Elliott, Wang, and Fuller 2017).

Ice crystals in food systems

During the freezing process, the chemical reaction and microbial growth in the food system are greatly reduced, which is conducive to food preservation. However, the structural disruptions associated with physical and biochemical reactions also involve in freezing processing. The formation of ice crystals is the real ringleader and should be responsible for the subsequent component changes, including lipid oxidation, endogenous enzyme activation, protein denaturation and aggregation (Dang et al. 2017; Hajj, Matta, and Sarkis 2020; Li et al. 2019; Soladoye et al. 2015; Skorpilova et al. 2019). The damage of frozen food caused by ice crystals mainly includes mechanical damage, recrystallization, cryoconcentration and freeze burn (Dalvi-Isfahan

et al. 2019). Such as, ice crystals can cause mechanical damage to cells through mechanical stress, and the damage of tissues and cell membrane structure caused by ice crystals during freezing storage will lead to the deterioration of food texture (Cai et al. 2019; Chen et al. 2020; Kashyap, Kumar, and Singh 2020; Kumari et al. 2020; Li et al. 2017). The physical changes of food during frozen storage are mainly manifested as cracking, recrystallization, water lose and migration (Zhu, Zhou, and Sun 2019). While the chemical changes during frozen storage mainly include flavor deterioration, lipid oxidation, protein denaturation, and degradation of vitamins and pigments (Kashyap, Kumar, and Singh 2020). Changes of these physical and chemical properties of frozen foods will affect the quality of the foods. For example, the size of ice particles in ice cream can ensure a smooth texture within 15–20 μm, but if the particle size of the ice crystal exceeds 40 μm, it will result in an unacceptable sense of coarse particles (Adapa et al. 2000; Damodaran and Wang 2017; Wang and Damodaran 2009).

During frozen storage and transportation, the temperature fluctuation can promote the growth of ice crystals in food. The growth rate of ice crystals is very low during lower storage temperatures, especially below the glass transition temperature (*Tg*) (Chen and Wang 2019). The *Tg* of ice cream is usually between −30 and −40 °C, depending on the sugar content (Regand and Goff 2002; Soukoulis, Rontogianni, and Tzia 2010). Above *Tg*, the greater fluidity of water molecules causes ice crystals to grow more easily. Usually, the freezing temperature of household freezers is −20 °C, and the temperature also fluctuates (Zhu, Zhou, and Sun 2019), causing the recrystallization of ice crystals to form large ice crystals, resulting in the deterioration of frozen food texture (Calanche et al. 2019; Chen et al. 2017; Kaleda et al. 2018; Tsolmonbaatar et al. 2016).

As the growth of ice crystals during the freezing process generally reduces the quality of frozen foods, new alternative technologies are still needed to control the growth of ice crystals in frozen products. For example, the growth of ice crystals can be inhibited by adding IBPs such as fish type III IBPs or *Rye* IBPs to frozen foods to increase the viscosity of samples and the mobility of water molecules (Kaleda et al. 2018), and by raising the *Tg* of food (Chen and Wang 2019; Soukoulis, Rontogianni, and Tzia 2010). The ability of IBPs to retard the growth of ice crystals suggests that IBPs can be used as a natural ice conditioner for a variety of frozen products, and have great application potential in frozen food preservation.

Methods and technologies for production, purification and evaluation of IBPs

The key technologies in the production, purification and evaluation of IBPs were shown in Figure 2. Natural IBPs are mainly found in some organisms in cold or high-altitude areas. Since DeVries first discovered the IBPs in the blood of Antarctic Marine fish in 1969, various IBPs have been discovered in fish, insects, plants, microorganisms and other variable temperature organisms.

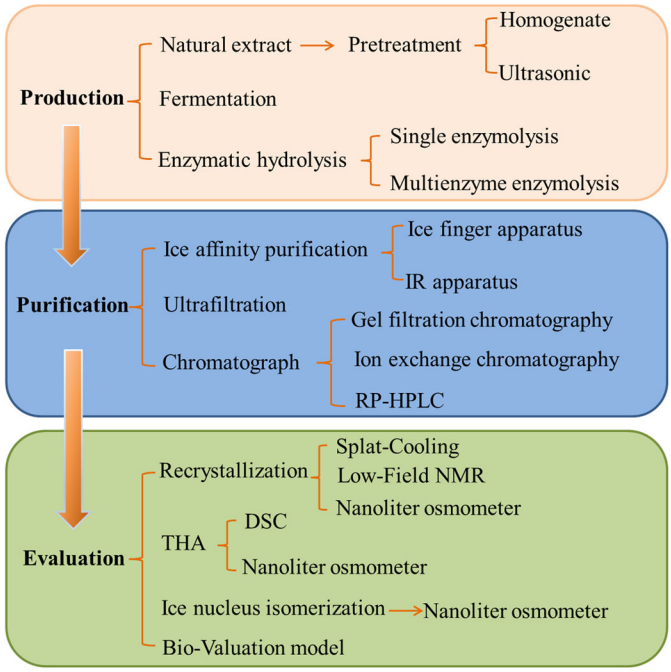

Figure 2. Flow diagram of key technologies in the production, purification, and evaluation of ice-binding proteins (IBPs). RP-HPLC, reversed phase high-performance liquid chromatography; DSC, differential scanning calorimetry; THA, thermal hysteresis activity.

Preparation of IBPs

The production methods of IBPs mainly including natural extraction purification, microbial fermentation and enzyme modification (Figure 2). Different types of AFPs are extracted in different ways. Usually, AFPs are found in biological organs or blood. For the preparation of AFPs in organs, the organs are generally homogenized, or frozen and crushed by liquid nitrogen, then homogenized with a certain volume of Tris-HCl buffer, and the crude extracts of AFPs are obtained by centrifugation, and AFPs are obtained after purification (Kashyap, Kumar, and Singh 2020; Cai et al. 2011). But, for the extraction of AFPs in blood, generally, the centrifuged blood supernatant is directly separated and purified to obtain AFPs (Cao et al. 2016). Unlike AFPs and INPs, enzymatically modified AFPs are obtained mainly from the collagen contained in the skin, scales and bones of animals through enzymatic hydrolysis (Chen, Cai, et al. 2019). The preparation process mainly includes bio-directed enzymatic hydrolysis, centrifugation, purification and identification (Damodaran and Wang 2017).

Purification of IBPs

Due to the specific ice affinity adsorption of IBPs, as ice crystal grown, IBPs molecule gradually bound to the surface of the ice crystal and was separated from other solutes to achieve separation and purification. Wu et al. (2015) designed an ice affinity purification system based on the specific adsorption between IBPs and ice crystals. This system controls the temperature through the pipeline connected to it, cools it to the surface to adsorb a thin layer of ice, and the AFP in the solution can be specifically adsorbed on the ice

surface to separation and purification. This method can increase the concentration of IBPs several times, which is more efficient and faster than traditional chromatography separation. The ice-enriched mixture after ice extraction can be purified by ultrafiltration and chromatography to obtain IBPs with a relatively concentrated molecular mass distribution to further improve the purity of IBPs.

Evaluation of IBPs

AFP has the characteristics of the wide source and structural diversity, large differences in antifreeze activity. Therefore, accurate antifreeze activity detection and evaluation methods are particularly important. The existing methods for the detection and evaluation of antifreeze activity are mainly quantitative or qualitative methods derived from thermal hysteresis activity (THA) and ice crystal recrystallization inhibition principle.

The methods based on thermal hysteresis include nanoliter osmometer, differential scanning calorimetry (DSC), and low-field nuclear magnetic resonance (LF-NMR). While the methods based on recrystallization mainly including splat-cooling, nanoliter osmometer (Figure 2). Nanoliter osmometer is a method to observe the growth of ice crystals through a microscope using a nanoliter osmometer system, which mainly composed of a capillary loading system, refrigeration system, camera system and computer image processing system (Takamichi et al. 2007). The principle of DSC to determine THA is mainly to determine the actual crystallization starting temperature by measuring the changes in the endothermic and exothermic in the crystallization process of the system. The measurement results of DSC are more objective and accurate, and the accurate ice crystal content in the samples can also be obtained (Chen and Wang 2019; Wang et al. 2020). The ice recrystallization inhibition (IRI) capability of IBPs is generally performed by the splat cooling technique, which generates ice crystals by rapid freezing and then detects the growth of ice crystals by microscopy at low temperatures (Wu et al. 2015). The THA of antifreeze peptides can make the freezing point of solution lower than the melting point. To know exactly the antifreeze peptides on melting characteristics, the influence of antifreeze peptides on the frozen liquid melting process could be analyzed by LF-NMR imaging (Chen, Cai, et al. 2019). Considering that when the cells are in an environment below the physiological freezing point, the ice crystals generated in the extracellular or intracellular environments will cause mechanical damage to cells, induce apoptosis and accelerate cell death (Cao et al. 2016; Chen et al. 2020; Davies 2014; Graham and Davies 2005). Given this, the antifreeze activity of IBPs can be evaluated by determining the viability of cells after cold stress (Wang et al. 2020; Wang et al. 2014).

Structural diversity and mechanism of IBPs

IBPs are widespread across biological kingdoms, and their functions include thermal hysteresis, freeze tolerance and ice adhesion. Since the first discovery of IBPs in 1969 (DeVries and Wohlschlag 1969), they have been discovered in marine fish, arthropods, plants, bacteria and fungi (Table 1). Therefore, according to the different sources of IBPs, it can be divided into fish IBPs, insect IBPs, plant IBPs, bacterial IBPs and fungal IBPs (Davies 2014; Hudait et al. 2019; Liu et al. 2016). Moreover, according to the different ways of generating IBPs, they can also be divided into AFPs, INPs and enzyme-modified antifreeze proteins (EMAFPs).

Antifreeze proteins

AFPs were first discovered due to observations of fish living in the Arctic. They are completely functional when the temperature is below or close to the freezing point of fish plasma (DeVries and Wohlschlag 1969). Through the analysis of the blood of Antarctic fish, it was found that the effect of sodium chloride and other electrolytes on the freezing point of fish plasma accounted for only about 40%–50%. The remaining decrease in freezing point was caused by small proteins in the serum (Cao et al. 2016; DeVries and Wohlschlag 1969). These proteins can significantly lower the freezing point of fish blood below that of seawater without significantly altering plasma osmotic pressure (Cao et al. 2016; Liu et al. 2016). This finding rapidly provoked great interest amongst researchers. Since then, AFPs with different structures have been identified from marine fish serum, insects, microorganism and plants in cold regions (Figure 3), all of which have a non-collateral reduction of the freezing point of body fluids without affecting the melting point of these body fluids, although the degree of reduction varies from protein to protein. (Cao et al. 2016; Graham and Davies 2005; Hudait et al. 2019; Provesi et al. 2019; Raymond et al., 2007; Sidebottom et al. 2000). The crystal structure of AFPs is diverse, including coil, polyproline type II coil, α-helix, β-sandwich and β-sheet (Figure 3).

AFPs can be divided into glycoproteins and non-glycoproteins according to the fact that whether they are connected with carbohydrates (Meister et al. 2018). For convenience, non-glycoproteins are usually called AFPs, while glycoproteins are called AFGP. Structural studies on AFGP and AFPs have been performed for decades. Alanine (Ala) was found to be the main amino acid in AFGP and the most common AFPs (type I), with approximately 67% of each family. However, the structure of AFGP and AFPs are quite different. AFGP mainly consists of tripeptide repeats of Ala-Ala-Thr with one of them being glycosylated (Meister et al. 2018). Some AFGP species replace alanine (Ala) with proline (Pro) after some threonines with the disaccharide groups extending from threonine (Thr) residues. AFGP molecules are lack of α-helical structure. At the same time, AFPs (type I) are almost the secondary conformation of α-helix, with no ordered peptide repeats (Meister et al. 2018). This demonstrates that the special function of AFPs is closely related to its unique structure.

Fish AFPs are the most widely studied. The characteristics and X-ray crystal structures of these AFPs were shown in Table 1 and Figure 3, respectively (Sun 2005). Fish AFPs are categorized into AFGP, type I AFP, type II AFP, type III

Table 1. Sources, structural characteristics and activities of ice-binding proteins (IBPs).

Category	IBPs	Species	MW (kDa)	Secondary structure	Tertiary structure	Activity	References
Marine fish	Type I AFPs	*Winter flounder*	2.6–33.0	α-Helical	100% Helical	TH, IRI	Liepinsh et al. 2002
	Type II AFPs	*Herring*	3.3–4.5	β-Sheet	Globular c-type lectin fold	TH, IRI	Arai et al. 2019; Cai et al. 2019
	Type III AFPs	*Wolffish*	6.5–14.0	β-Sandwich	Globular	TH, IRI	Kumari et al. 2020
	Type IV AFPs	*Longhorn sculpin*	12.3	α-Helix	Four-helix bundle	TH, IRI	Deng and Laursen 1998
	AFPGs	*Antarctic notetheniid*	2.6–33	Expanded	PPII helices	TH, IRI	Mochizuki and Molinero 2018
Arthropods	sfAFP	*Snow flea*	6.5 or 15.4	β-helix	PPII helices	TH, IRI	Graham and Davies 2005
	MpdAFP	*Microdera punctipennis dzungarica*	30	β-Sheet	Expanded	TH, IRI	Liu et al. 2016
	RiAFP	Rhagium inquisitor	13.5	β-Sandwich	β-solenoid fold	TH, IRI	Hakim et al. 2013
	TmAFP	Tenebrio molitor	9–14	β-Helical	Regular β-Helical	TH, IRI	Liou et al. 2000
	sbwAFP	*Spruce Budworm*	9–27	unknown	unknown	TH, IRI	Leinala et al. 2002
	cfAFP	*Choristoneura fumiferana*	9–12	β-structure	Globular	TH, IRI	Qin et al. 2007
	DaAFP	*Dendroides canadensis*	9–14	β-Helical	Regular β-Helical	TH, IRI	Halwani et al. 2014
Plants	AsAFP	*Avena sativa*	14.4–97.4	unknown	unknown	TH, IRI	Zhang et al. 2016
	LpAFP	Lolium perenne	13	β-Helical	Left-handed β-roll	TH, IRI	Middleton et al. 2012
	DcAFP	*Daucus carota*	36.8	unknown	unknown	TH, IRI	Zhang, Zhang, and Wang 2007
microorganism	MpAFP	Marinomonas primoryensis	34	Ca^{2+}-β-helix	Anchored clathrate	TH, IRI	Garnham, Campbell, and Davies 2011
	TisAFP	Typhula ishikariensis	28	β-Helix	β-helical	TH, IRI	Cheng et al. 2016

TH, thermal hysteresis; IRI, ice recrystallization inhibition.

AFP and type IV AFP according to their amino acid composition and structure (Wierzbicki et al. 2000). Table 1 shows that the most widely studied are type I AFPs with a glycine-rich-helical peptide consisting of 36-44 amino acid residues (3300-5000 Da). In some cases, type I AFPs are composed of 11 amino acid residue repeats. AFGP consists of a helical structure in which Ala-Ala-Thr repeat unit contains disaccharides in its threonine side chain (Figure 3). Type II and type III AFPs are non-helical molecules consisting of non-repeating amino acid sequences. Type II AFPs are large-sized proteins composed of about 120 amino acid residues (13,000 Da) and are cysteine-rich proteins composed of reverse turns, helices and β-sheets (Figure 3). Type III AFPs are medium-sized proteins (65+ residues, ca. 7000 Da) and lack the major amino acid types. Furthermore, the type IV AFPs are folded from the similar length of four amphoteric helices into a four-helix bundle, containing about 60% α-helix at 1 °C. These relatively large AFPs composed of about 108 amino acid residues (12,299 Da) and have about 22% similarities to some apolipoproteins, some of which are known to form helix bundle structures (Wierzbicki et al. 2000). So far, all analyzed fish AFPs contain amino acids that can attach to the ice surface (Davies 2014; Qiu, Hudait, and Molinero 2019).

THA is the gap between melting and freezing point (Figure 4a), which is an important indicator for identifying the activity of AFPs, and the function of AFPs is to reduce the growth point of ice crystals and modify the growth rate and ice habit (Arai et al. 2019; Kristiansen and Zachariassen 2005; Li, Liang, and Li 2009). When AFPs adsorbs to the surface of ice crystals, the ice surface increases (Figure 4b), which makes the freezing point of solution decrease, but slightly elevated the melting point (Knight 2000). This THA can be explained by the Kelvin effect, because from a thermodynamic point of view, it is more difficult to add water to a curved ice surfaces than to a flat one (Davies 2014; Knight 2000). It is widely believed that AFPs work by combining with ice surface to interfere with the proliferation of water molecules on the crystal surface. The adsorption of different AFPs on the surface of different ice crystals showed different preference (Hakim et al. 2013; Hudait et al. 2019). Moreover, these AFPs bind to the ice crystal through a specific plane to reduce the freezing point of the solution in a non-colligative manner, thus reducing the damage to the ice crystal. The decrease of solution freezing point depends on the concentration of AFP and is mainly realized by adsorption-inhibition. A two-step hypothesis was proposed for the binding of AFPs to ice crystals. AFPs molecules are bound to the ice surface individually at low concentrations, while they exert their maximum antifreeze function in a manner of intermolecular cooperation at high concentrations (Bayer-Giraldi et al. 2018). A model of ice crystal growth inhibition was proposed in Figure 5. This model assumed that the AFP molecular patches or aggregates are tightly bound to the ice surface. Therefore, the ice lattice can only grow in the space between the AFP molecules, thus reducing the stability of the ice-water

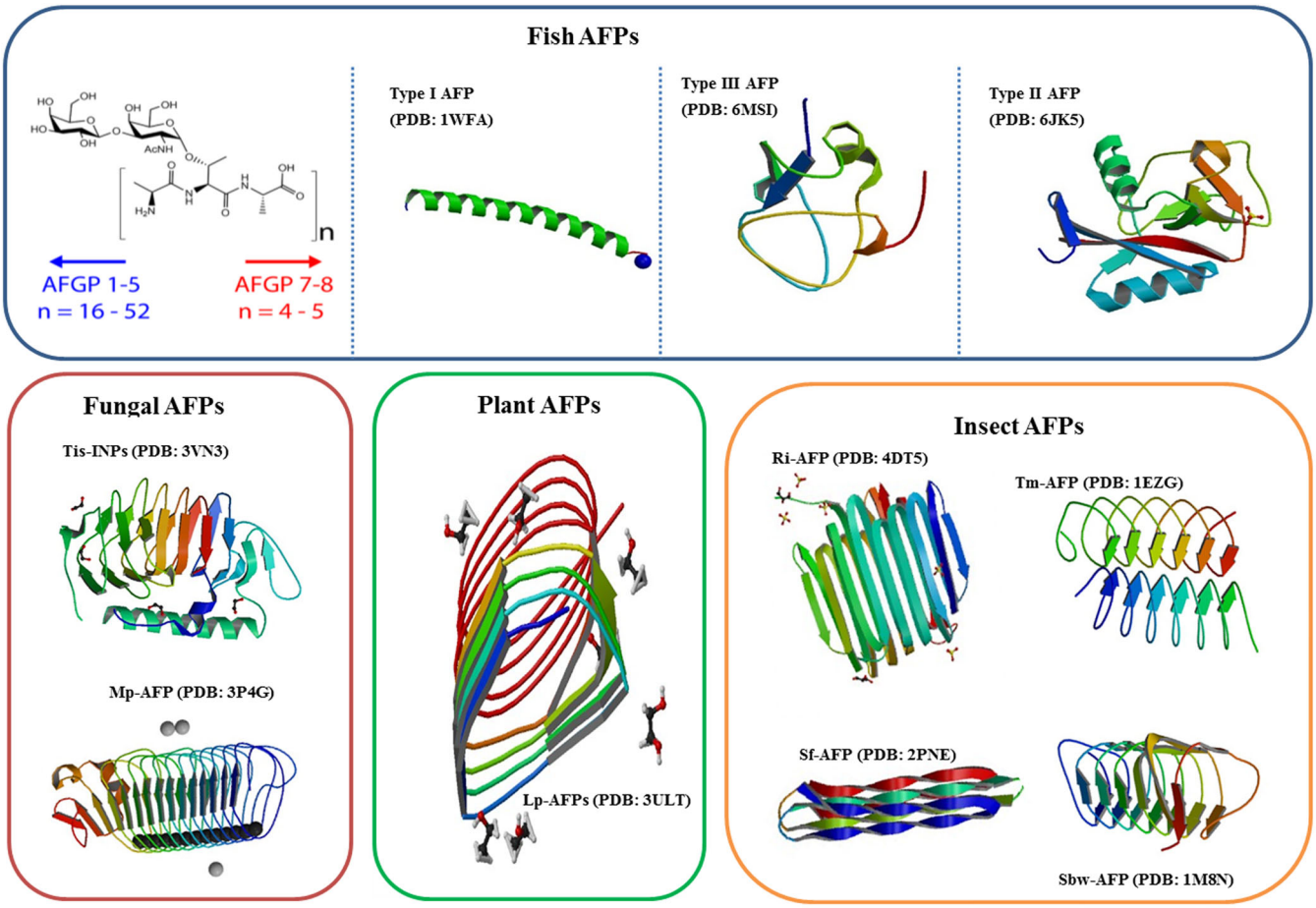

Figure 3. X-ray crystal structures of ice-binding proteins (IBPs). The crystal structures are obtained from the Protein Data Bank (PDB, https://www.rcsb.org/structure/). AFGP, antifreeze glycoprotein; Tis-INPs, Typhula ishikariensis ice-binding protein; Mp-AFP, Marinomonas primoryensis antifreeze protein; Lp-AFP, Lolium perenne antifreeze protein; Ri-AFP, Rhagium inquisitor antifreeze protein; Sf-AFP, *Snow flea* antifreeze protein; Tm-AFP, Tenebrio molitor antifreeze protein; Sbw-AFP, *Spruce budworm* antifreeze protein.

interface surface. As a result, the aggregation of water molecules to the ice crystal surface is inhibited, thus inhibiting the growth of crystals (Halwani et al. 2014; Hudait et al. 2019; Liu et al. 2016; Mochizuki and Molinero 2018). Furthermore, AFPs tend to bind to ice prismatic surfaces when they are adsorbed onto the ice surface. The preferred binding of AFPs can be explained by their dipole properties (Davies 2014; Liu et al. 2016; Middleton et al. 2012). This dipole property suggests that AFPs have dipoles parallel to its helical hydrophilic and hydrophobic groups. The dipole of AFPs interacts with the dipole production of water molecules around the ice core, and the dipole acts as the initial driving force for AFPs to interact with the ice crystal. The amphiphilic particle-helix provides the interacting hydrogen bond through the hydrophilic side chain, while the hydrophobic side chain prevents the growth of the ice crystal (Zhang, Jin, et al. 2018). Under these interactions, AFPs can specifically adsorb to the prismatic surface of ice, which changes the growth habits of ice (Garnham, Campbell, and Davies 2011; Hudait et al. 2019).

Ice nucleation proteins

Ice nucleation activator (INA) is a foreign particle promoting ice nucleation during freezing. As previously reported, the first step in the formation of ice crystals during freezing is to activate the ice nucleus, that is, to enable the ice nucleus to form ice crystals. So far, INPs are usually secreted by INA bacteria that facilitate ice nucleation. Obviously, the ice nucleation is achieved by arranging water molecules along a 48 amino acids repeated domain. These repeated domains are composed of 16 residues containing the conserved octamer AGYGSTxT (Kawahara 2002; Kumaki et al. 2008).

There exist miscellaneous biogenic INAs in bacteria and fungal (Davies 2014; Kawahara 2002). INA with the highest activity is the INPs from some ice nucleation bacteria (Kawahara 2002). The function of INA bacterial is to reduce the supercooling point and catalyze ice nucleation. The typical freezing curves are shown in Figure 6. As shown in the figure, the addition of INPs can significantly reduce the supercooling point, and the time the ice nucleation occurs is greatly advanced, thus shortening the freezing time. At present, the most common types of INA bacteria producing INA are genera *Erwinia*, *Pseudomonas* and *Xanthomonas*. INAs are also found in some strains of fungi and fusarium (Rubiolo 2000). These bacteria can promote the formation of ice crystals at −2 to −3 °C, causing frozen damage to many crops (Kawahara 2002). However, not all-natural strains can produce INA, so the strain that can produce INA is called INA^+, otherwise, it is called INA^-. Sun (2005) established a method to isolate cell-free ice nucleus from *Erwinia herbicola*

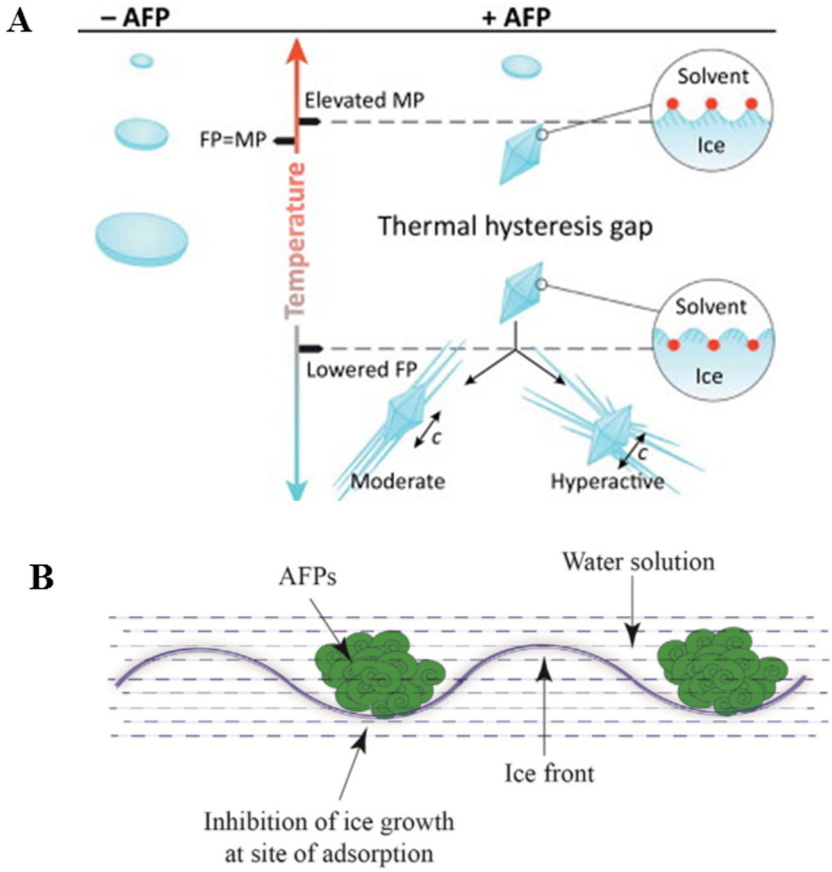

Figure 4. (a) Thermal hysteresis; (b) Schematic representation of AFPs adsorbs and inhibits ice growth on the ice surface. FP, freezing point; MP, melting point. Source: Davies (2014); Kar and Bhunia (2015).

M1 and conducted a series of studies. The results indicated that these cell-free INA were related to extracellular vesicles. The phenotype of ice nucleated bacteria was sensitive to sulfhydryl-modifying and proteases, suggesting that ice nucleus activity requires proteins (Block and Worland 2001). Phospholipids are also essential for *Pseudomonas syringae* to express the ice nucleus activity. The activity of INP was lost after being treated with phospholipase C, and then recovered after natural lipid Phosphatidylethanolamine was added. These results indicated that, in order to obtain and maintain a structure capable of achieving ice catalysis, INPs had a requirement for lipids to reconstruct a physiological hydrophobic environment similar to that existing in vivo (Sun 2005). It is summarized that the INPs from INA bacteria have a similar primary structure consisting of three distinguishable domains, namely, a C-terminal single sequence domain, an N-terminal single sequence domain, and a highly repeating domain in the central. Moreover, the central domain is rich in glycine, alanine, threonine, and serine (Kun and Mastai 2007; Ning, Xiang, and Choy Leong 2003). INA bacterial has potential applications in frozen food industry, and addition of INA bacteria to solid model foods is an example (Zhang, Wang, and Chen 2010).

Enzyme-modified antifreeze proteins

Previously, it has been reported that the products with antifreeze property can be obtained by enzymatic modification of gelatin with protease (Damodaran 2007). The EMAFPs discussed below has also been classified as antifreeze peptides, which are obtained from gelatin by hydrolysis at specific enzyme cutting site. Gelatin is a unique protein rich in nature, which is a water-soluble and polyphase mixture of high molecular weight proteins hydrolyzed from collagen, mainly composed of glycine (33%) and proline/hydroxyproline (33%). Most of the currently reported EMAFPs have a specific amino acid sequence length, and their molecular weight is generally less than 3000 Da. EMAFPs with a molecular mass of less than 3000 Da exhibit higher antifreeze activity, possibly because EMAFPs with smaller molecular chains may form stronger colloidal helix, which are more likely to bind to ice nuclei with oxygen atoms in the prism face of ice nuclei. While, EMAFPs greater than 3000 Da have a lower ability to inhibit the growth of ice crystals, possibly because of the lower hardness of EMAFPs with long molecular chains. In addition, steric hindrance prevents the correct accumulation of peptides on the surface of the ice core prism (Du and Betti 2016; Li et al. 2017; Wang et al. 2014; Wu et al. 2015).

The action mechanism of EMAFPs to inhibit the growth of ice crystals may be similar to that of AFPs and AFGP (Damodaran 2007; Zhang, Liu, et al. 2018). Generally, the primary structure of EMAFPs has the characteristics of -Gly-Pro(Hyp)-X- tripeptide repeats and X could be any one of the twenty amino acid residues. Moreover, Gly usually occupies the first position of the tripeptide repeat sequence

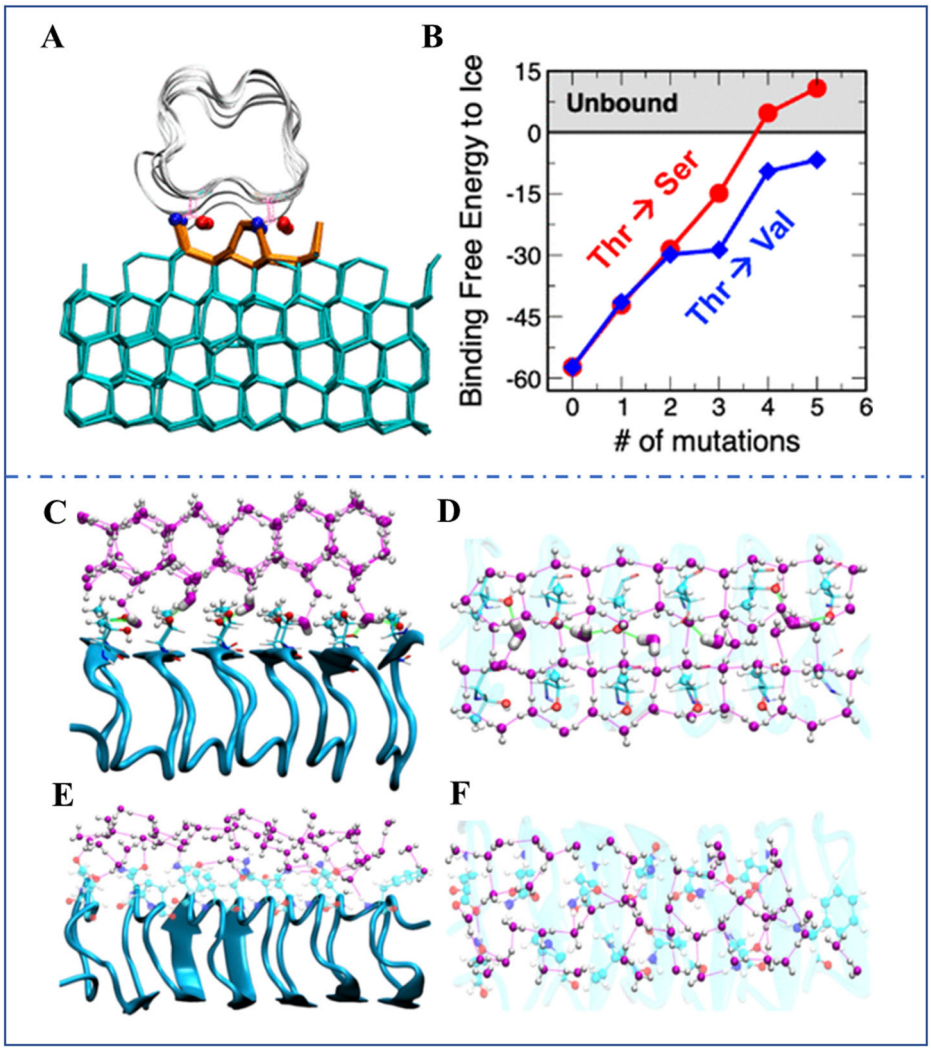

Figure 5. The simulation of the mechanism of ice-binding proteins. (a) The binding model between IBPs and ice crystal. (b) Ice crystal binding energy. (c) Side view and (d) top view of the hexagonal ice-like water molecules (magenta–white spheres) atop the icebinding face. Note the trapped water molecules (magenta–white sticks) on the ice-binding face with the methyl (cyan–white spheres) and hydroxyl (red–white spheres) groups. (e) Side view and (f) top view of the disordered water molecules atop the non–ice-binding face of the MpdAFP with various residues (carbon, nitrogen, oxygen, and hydrogen atoms are represented in cyan, blue, red, and white spheres). Source: Hudait et al. (2019); Liu et al. (2016).

in the primary structure of EMAFPs. Although the characteristics of this tripeptide repeats are different from the -(Ala-Ala-Thr)$_n$- found in AFGP (Griffith and Ewart 1995), they are very similar to the snow fleas AFP (*sf*AFP), of which the -(Gly-X-X)n- tripeptide repeats (Graham and Davies 2005). However, *sf*AFP contains disulfide bonds, which is essential for their antifreeze activity (Celik et al., 2010), EMAFPs do not contain any sulfur-containing amino acids. Nevertheless, the primary structure of *sf*AFP and EMAFPs are surprisingly similar, suggesting that the inhibitory effect of EMAFPs on ice crystal growth may be related to -Gly-Pro(Hyp)-X- tripeptide repeats.

In addition, based on the amino acid sequence characteristics of EMAFPs, researchers further use molecular dynamics simulation technology to construct a theoretical model of the interaction between EMAFPs and the molecular surface of ice nucleus. The results show that the mechanism of action between EMAFPs and ice crystals obeys the surface hydrophilic-complementary mode. Briefly, a collagen antifreeze peptide with a specific amino acid length and structure forms hydrogen bonds with water molecules within the ice crystal prism face, and then the peptides bind to the surface of the ice nucleus by hydrogen bonds. Generally, peptide chains with a relative molecular mass of less than 2000 Da are sufficiently hydrophilic and flexible. Meanwhile, antifreeze peptides rich in alkyl side chains such as Pro and Ala residues can provide partial non-polar environment, which can stabilize the interaction between hydrogen bonds and counter the competition of hydrogen bond interactions between ice crystals and water, exhibiting a significant IRI activity (Wu et al. 2015).

Advance applications of IBPs in frozen food preservation

Cryopreservation technology is one of the most frequent and effective methods for long-term storage of food (Dalvi-Isfahan, Hamdami, and Le-Bail 2016; Schafer 2017). Nevertheless, during freezing and frozen storage of frozen food, ice crystals will cause the rise of local osmotic pressure

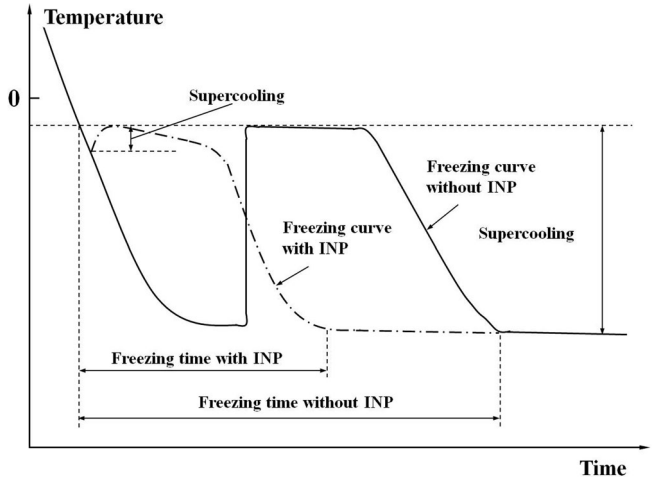

Figure 6. Typical freezing curves with and without addition of ice nucleation protein (INP). Addition of INP can significantly reduce the supercooling point, and the time when ice nucleation occurs is greatly advanced, thus shortening the freezing time. Source: Sun (2011).

of the products, change the properties of the components in the cell, and result in the degeneration of starch and protein. In the process of thawing, several problems such as loss of drip, soft rot or loss of original form frequently occur (Schafer 2017; Sun 2011). The causes of these problems are relevant to food material handling, freezing methods, packaging, freezing storage conditions and thawing means. The most crucial factor is the recrystallization caused by temperature fluctuations in the freezing storage process (Ayati, Hamdami, and Le-Bail 2017; Elliott, Wang, and Fuller 2017). Hence, how to control the growth of ice crystals is a burning issue to be settled in the processing, transportation and storage of frozen food (Chen, Cai, et al. 2019). Owing to the security and the ability to actively suppress ice crystal recrystallization, IBPs has extensive application prospect in food freezing and refrigeration (Table 2).

The major challenge for frozen food manufacturers is to develop appropriate techniques to control or inhibit the growth of ice crystals during food storage. IBPs have always been considered as a natural cryoprotectant for frozen food because of the ability to inhibit ice crystal growth and reduce ice recrystallization (Zhu, Zhou, and Sun 2019). Adding IBPs to frozen food is one of the key technologies to control or inhibit the growth of ice crystal for extending shelf life during the storage of frozen food. IBPs can preserve the nutritional value and quality of frozen foods even at lower concentrations. Inhibiting ice recrystallization can help preserve the texture of frozen foods by reducing the formation of large ice crystals in frozen foods such as ice cream. Thereby, IBPs have broad application prospects in inhibiting ice recrystallization and reducing freezing point during food freezing (Feeney and Yeh 1998; Zhan et al. 2018; Zhu, Zhou, and Sun 2019).

Methods of introducing IBPs into food products

When designing refrigeration systems for foodstuffs that involve IBPs, the key consideration that must be underlined is how to add IBPs into the food. Since IBPs work outside the cell, they can be directly applied to food by mechanical methods, for example, soaking, injection, vacuum infiltration or mixing (Provesi et al. 2019; Song et al. 2019; Zhang et al. 2016). This allows IBPs to be introduced into various foodstuffs, including cell-based and non-cell-based foodstuffs. Except for mechanical methods, IBPs could be genetically introduced into an organism or could be synthesized by genetic or chemical methods and added to the food (Liu et al., 2018a; Qin et al. 2007; Zhang, Liu, et al. 2018).

Application in frozen meat products

Freezing technology is important for long term preservation of muscle products such as processed surimi and meat. However, mechanical damage caused by ice crystals would lead to quality deterioration. As mentioned above, immersing the meat in a solution with IBPs can significantly decrease the size of ice crystal and reduce tissue damage caused by freezing (Zhu, Zhou, and Sun 2019). The recrystallization inhibitory activity of IBPs also shows good effect on improving the quality of frozen meat, because ice crystals are easy to form in cells during the slow freezing process. It is easy to cause cell rupture and result in nutrient loss during thawing.

Cai et al. (2019) investigated the effect of AFPs combined with CS@Fe3O4 nanoparticles on quality in bream. The results showed that soaking the bream in the AFPs solution before freezing could reduce the drip loss and maintain the fiber microstructure of the bream meat. Compared with the control groups, the secondary and tertiary structures of the fish proteins in AFPs groups tended to be stable, the viscoelasticity and thermal stability of bream meat was improved, the degree of protein oxidation and aggregation was reduced. Moreover, when AFPs is added during freezing, the oxidation degree and structure of myofibrillar protein are improved after freeze-thaw treatment (Cai et al. 2020). Moreover, before freezing at −20 °C, the muscles of cattle and sheep were immersed in a solution containing 1 mg/mL type I AFP or AFGP. Results showed that the ice crystal size was significantly reduced. Even a low concentration of AFGP (0.5 mg/mL) can significantly inhibit the growth of ice crystals, because the hyperactive AFPs from fish contained multiple ice binding sites (Graham et al. 2008). Injecting AFPs into living animals also has a positive effect on meat quality during freezing and thawing. Payne and Young (1995) investigated the effect of AFGP on the quality of frozen meat before slaughter, intravenous injection of AFGP isolated from Antarctic cod in lambs slaughtered at different times, and meat samples were stored at −20 °C for 2-16 weeks after vacuum-packaging. Results showed that AFGP injection at 1 or 24 h before slaughter could reduce the size of ice crystals and drip loss. Particularly, when 0.01 μg/kg of AFGP was injected at 24 h before slaughter, the ice crystals in meat samples were the smallest.

Application in frozen fruits and vegetables products

Cell components of plant tissues or physical disruption to cell morphology, such as the moisture loss and cell wall fracture, is the most adverse impact of freezing (Upadhyay, Ghosal, and Mehra 2012). The cell integrity can be

Table 2. Application of ice-binding proteins (IBPs) in frozen food preservation.

Category	Application	IBPs	Source	Concentration	References
Frozen meat	Red sea bream	Type II AFPs	*Herring*	0.1% (wt/wt)	Cai et al. 2020
	Myofibrillar protein	Type II AFPs	*Herring*	0.05% (wt/wt)	Cai et al. 2019
	Lamb meat	AFGP	*Antarctic notetheniid*	0.5 mg/mL	Graham et al. 2008
	Lamb	AFGP	*Antarctic notetheniid*	0.01 μg to 10 mg/kg liveweight	Payne and Young 1995
Vegetables	Green beans	AFPs HrCHI4	*Hippophae rhamnoides*	0.1 mg/mL	Kashyap, Kumar, and Singh2020
	Star fruit	*Da*AFPs	*Drimys angustifolia*	0.1 mg/mL	Provesi et al. 2019
	Cucumber	*Tm*AFP	*Tenebrio molitor*	1.0 mg/mL	Song et al. 2019
	Carrot	*Tm*AFP	*Tenebrio molitor*	1.0 mg/mL	Song et al. 2019
	Zucchini	*Tm*AFP	*Tenebrio molitor*	1.0 mg/mL	Song et al. 2019
	Onion	*Tm*AFP	*Tenebrio molitor*	1.0 mg/mL	Song et al. 2019
Frozen dough	Dough	EMAFPs	Pig skin collagen	0.1%–0.5% (wt/wt)	Cao et al. 2020
	Gluten proteins	DaAFP	*Daucus carota*	0.5% (wt/wt)	Liu et al. 2018a
	Gluten proteins	TmAFP	*Tenebrio molitor*	0.5% (wt/wt)	Liu et al. 2018a
	Gluten proteins	FiAFP	*Epinephelus coioides*	0.5% (wt/wt)	Liu et al. 2018a
	Frozen dough	EMAFPs	Pig skin collagen	1% (wt/wt)	Chen et al. 2017
	Dough	rCaAFP	*Daucus carota*	0.5% (wt/wt)	Liu et al. 2018b
Ice cream	Ice cream	Type III IBPs	*HPLC12*	3 mg/mL	Kaleda et al. 2018
	Ice cream	Rye IBPs	*Rye*	6 mg/mL	Kaleda et al. 2018
	Ice cream	EMAFPs	Fish collagen	4% (wt/wt)	Damodaran and Wang 2017
	Ice cream	AsAFP	*Avena sativa L*	0.1% (wt/wt)	Zhang et al. 2016
	Ice cream	EMAFPs	Chicken collagen	1 mg/mL	Du and Betti 2016
	Ice cream	EMAFPs	Sericin	NA	Wu et al. 2015
	Ice cream	EMAFPs	Bovine collagen	4.0% (wt/wt)	Wang and Damodaran 2009
	Ice cream	EMAFPs	Collagen	NA	Damodaran 2007
Cryobiology	*S. thermophilus*	EMAFPs	*Tilapia* scales	1 mg/mL	Chen et al. 2020
	S. thermophilus	EMAFPs	*Tilapia* scales	1 mg/mL	Chen, Cai, et al. 2019
	S. thermophilus	EMAFPs	Pig skin collagen	5 mg/mL	Chen, Cai, et al. 2019
	L. lactis	*sf*AFP	*Snow flea*	NA	Zhang, Jin, et al. 2018
	S. cerevisiae	Proline	Baker's yeast	NA	Tsolmonbaatar et al. 2016
	L. lactis	EMAFPs	Sericin	1.0 mg/mL	Li et al. 2017
	L. bulgaricus	EMAFPs	Shark skin	250 μg/mL	Wang et al. 2014
	Roe	ZP AFPs	*Notothenioid* fishes	6 mg/mL	Cao et al. 2016
	Rat smooth muscle cells	*DaAFP*	*Daucus carota*	0–3 mg/mL	Halwani et al. 2014

NA, relevant information was not available in the original references.

destroyed after the freeze-thaw cycle, which leads to the dehydration and deterioration of the overall quality (Chen, Cai, et al. 2019). As a result, the tissue of fruits and vegetables become soggy (Kashyap, Kumar, and Singh 2020). The main reason of deterioration of the overall quality is recrystallization caused by temperature fluctuation. IBPs can inhibit the recrystallization of ice at a lower concentration, and can completely inhibit the growth of ice in a certain temperature range according to its own properties at a higher concentration.

Kashyap, Kumar, and Singh (2020) reported that the green beans soaked in AFP HrCHI4 (0.1 mg/mL) from *Hippophae rhamnoides* could avoid electrolytic leakage and drip loss during freezing. The texture analysis and SEM further validated structural maintenance. Furthermore, through the analysis of volatile components, it was found that the fresh degree of beans treated with HrCHI4 was better than control groups after freeze-thaw. Similarly, the star fruits (*Averrhoa carambola*) vacuum infiltration or immersion with *Da*AFPs (*Drimys angustifolia*) could retain their drip loss constant after 15 days and maintain the firmness of star fruit after being stored at $-20 \pm 2\,^{\circ}C$ for 60 days (Provesi et al. 2019). Moreover, Song et al. (Song et al. 2019) investigated the effect of *Tm*AFP (*Tenebrio molitor*) on vegetables including cucumber, carrot, zucchini, and onion. Results indicated that vegetables treated with *Tm*AFP (*Tenebrio molitor*) displayed a higher quality and stable structure characteristics after after storage at $0\,^{\circ}C$ for 13 days.

Application in frozen dough

The physicochemical properties and network structure of gluten protein will be destroyed by ice crystals during freezing process, leading to the deterioration of the quality of frozen dough. AFP could be used as a beneficial additive to frozen dough. The effects of concentrated carrot (*Daucus carota*) AFP on texture properties of frozen dough and volatile compounds of breadcrumbs were investigated. Researchers found that the sensory quality and texture profile analysis results of bread after adding AFP were similar to those of the control group (Zhang, Zhang, and Wang 2007). Meanwhile, the addition of AFPs has certain benefits in maintaining bread volume. Results of texture property analysis showed that the hardness of the dough was steadier and softer than that of the control group when AFP was added during frozen storage, which may be due to the lower freezing water content (Liu et al., 2018b). Chen et al. (2017) demonstrated that addition of AFPs weakened the influence of the freeze-thaw treatment on water mobility, influenced the water distribution in frozen dough and improved the water holding capacity of the dough. Furthermore, Solid phase micro-extraction (SPME) GC–MS analysis showed that the addition of AFP had no negative effect on the volatile compounds in bread crumbs, and the Trans Caryophyllene gave bread crumbs a pleasant aroma similar to *Michelia alba DC* (Zhang et al. 2008).

Application in frozen dairy products

Controlling the formation and recrystallization of ice crystals is very important to maintain the stability of frozen dairy products during frozen storage (Soukoulis and Fisk 2016). The formation of ice crystals determines the growth of ice crystals during the hardening and storage of frozen dairy products, which affects the sensory qualities of ice cream such as creaminess, roughness and moisture and hardness. While, the recrystallization of ice crystals determines the texture and structural stability of the ice cream during the freezing process (Singo and Beswa 2019). From the mechanical point of view, adding IBPs can control the size of ice nucleus, inhibit the growth of ice crystal in sucrose solution, and improve the stability under static storage conditions (Soukoulis and Fisk 2016).

One study was using a microscope with a cold stage to investigate the effect of fish type III IBPs on the ice crystal structure modification in ice cream (Kaleda et al. 2018). In this experiment, a 3 μL drop of ice cream mix (with or without IBPs) was placed between cover slip and microscope slide, and was placed onto a Linkman cryostage with a holding temperature. The ice crystal sizes of ice cream at different holding temperatures were investigated, and the results showed that adding 3 mg/mL recombinant type III IBPs could significantly improve the hardness and the stability of ice cream during melting. Similarly, Zhang et al. (2016) investigated the cryoprotective activities of the oat AFPs (AsAFPs) on ice cream. Results found that the THA of AsAFPs was as high as 1.24 °C at a concentration of 15 mg/mL. Moreover, the glass transition temperature of ice cream increased from −29.14 to −27.74 °C with 0.1% AsAFPs, and the melting resistance of this ice cream was dramatically improved at same concentration. Moreover, Damodaran and Wang (2017) found that EMAFPs could also significantly inhibit ice recrystallization in ice cream. Based on the studies of the cryoprotective effect of IBPs on ice cream, IBPs could be used as a natural ice conditioner for ice cream to reduce the deteriorate caused by frozen and temperature fluctuation, improving the stability of frozen dairy products.

Conclusions and future perspectives

Ice nucleation and recrystallization often cause great damages in frozen foods. By inhibiting the growth of ice crystals and reducing the recrystallization of ice, IBPs have been considered as a natural choice for improving the nutritional value and quality of frozen foods in the food processing industry. IBPs with unusual effects on the growth of ice are a variety of small proteins found in diverse organisms that grow in extreme cold environments. Cellular damage in meats and ice crystals in other frozen foods can be reduced due to the ability of AFPs to inhibit ice recrystallization. Food products which are vulnerable to severe freezing damage may be maintained in super-cooled states with AFPs at sub-freezing temperatures. With the discovery and in-depth study of various IBPs, three key problems restricting the research and application of natural IBPs in the field of frozen food have become increasingly prominent. Firstly, the amount of naturally occurring IBPs is very limited, and it is very expensive to purify the trace IBPs in living organisms. Secondly, the synthetic IBPs faces the problem of high cost, and the chemically synthesized IBPs has the problem of food safety. In addition, due to the special structure of AFGP, it is almost impossible to prepare by chemical synthesis. Thirdly, similar to most proteins, IBPs prepared by genetic engineering also face the problems of low expression and loss of activity. Although the yield of the prokaryotic expression system is relatively high, the prokaryotic expression system is easy to form inclusion bodies, resulting in low activity or even loss of the expression products. Moreover, some of the prokaryotic expression system products contain endotoxin, which may cause food safety problems.

IBPs are handled extracellularly, and current methods of introducing IBPs into food are mainly mechanical means. In theory, if the gene of IBPs could be successfully transferred to an organism, both the original one and its offspring would be able to resist the cold. However, despite the high returns, this technique is susceptible to troubles at some stages. There are many obstacles that must be overcome in the process of gene transfer, these include appropriate targeting of DNA, correct and full expression of mRNA and protein, appropriate protein folding and environmental stability, appropriate sensitivity to protease digestion, correct distribution in vivo, and extracellular protein targeting. All of these factors mainly depend on the selected target genes and the compatibility of IBPs in the new environment. However, the success of this technique is limited among the onging studies introducing IBPs into organisms at the DNA level. The enzymatic modifications of AFPs are very promising, such as modified collagen peptides by papain have antifreeze activity. The purpose of further studies is to increase the levels of EMAFPs and accelerate further application in frozen foods or food products. However, EMAFPs are restricted in their application, and a lot of research work is needed.

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