Chapter 22
Responses of Living Organisms to Freezing and Drying: Potential Applications in Food Technology

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22.1 Introduction

Some living organisms can survive under extreme stresses by adapting to situations that would otherwise be lethal. They develop various mechanisms for adaptation, surviving adverse environmental conditions such as lack of water (cryptobiosis or anhydrobiosis) and freezing temperatures (cryobiosis) (Carpenter et al. 1986; Crowe et al. 1998, Watanabe et al. 2002). Since water is required to hydrate molecules (e.g., folding of proteins into active molecules cannot proceed without water), and their macromolecular structure and functionality are sensitively determined by their interactions with water, in many cases the stress is governed by interference from interaction with biomolecules (Franks 1982, 1995). At cold temperature, water freezes and forms disruptive ice crystals that can damage cellular structures. In conditions of low humidity or high osmolality, water leaves the cells, thus altering the hydration of macromolecules and their ability to participate in reactions necessary for life.

Various strategies can protect cells against extreme temperatures and dehydration, enabling their survival. Since these tolerant organisms face the same problems of protection during food preservation as their important biomolecules do, by studying the strategies used in nature, innovative procedures can be developed for use in food technology. By analyzing and interpreting these mechanisms, systems and processes can be engineered to preserve food quality during processing and to extend product shelf life (Aguilera and Karel 1997).

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22.2 Desiccation Strategies: Glass Formation and Solute-Protecting Interactions in Anhydrobiotes

Glass formation is a natural mechanism for the preservation of complex anhydrobiotic organisms, which can tolerate desiccation and survive extended periods of time in the dry state (Crowe et al. 1998). In response to dehydration the cytoplasm of desiccation-tolerant organisms forms glasses; these organisms contain large amounts of soluble nonreducing sugars and their state diagrams resemble those of simple sugar mixtures. Some of the organisms (e.g., *Artemia salina*, yeast cells, and tardigrades) accumulate α, α –trehalose, while others (pollen, seeds and resurrection plants, e.g., *Selaginella lepidophyl)* accumulate sucrose and other nonreducing β-furanosides (e.g., raffinose, stachyose, and verbascose). Other examples of living systems that exploit the formation of glass to preserve life are some species of the simple flatworm (Tunnacliffe and Lapinski 2003).

The insect *Polypedilum vanderplanki* Hint. is the largest multicellular animal known to tolerate almost complete dehydration without deterioration (Watanabe et al. 2002). Their anhydrobiotic larvae show extremely high thermal tolerance (−270°C to +102°C) and can recover soon after prolonged dehydration up to 17 years (Hinton 1960). Watanabe et al. (2002) showed that rapid accumulation of trehalose (up to 18% of dry body mass) plays a key role in the successful induction of anhydrobiosis of these larvae and that it is possible to store their individual organs, and cells from them, at room temperature under dehydrated conditions.

Crowe et al. (1998) reported that vitrification of the structure is necessary to improve enzyme and liposome stability, but specific hydrogen-bond interactions between sugars and the biomaterial are also needed. Besides forming glasses, in which kinetic restrictions for physico-chemical changes such as chemical reactions and crystallization operate, sugars develop the ability to protect proteins and membranes through hydrogen bond interactions with the active biomolecules. Dried yeast cells have been observed to have glass transition temperatures (T_g) and water sorption isotherms that are independent of the amount of trehalose. However, their viability was dramatically dependent on the quantity of disaccharides present (Cerrutti et al. 2000).

Desiccation-sensitive organisms, on the other hand, generally lose their viability during drying at water contents at which the glassy state has not yet been formed (Buitink and Leprince 2004). Besides, sugars, proteins (dehydrines), salts, and amino acids are accumulated by organisms resistant to dehydration; they act in different ways, stabilizing proteins and membranes, contributing to osmotic adjustment, or act as free radical scavengers.

The potential applications of intracellular disaccharides in animal cell biopreservation are limited by the inability of mammalian cells to synthesize or actively accumulate sugars such as trehalose (Holovati and Acker 2007). The use of liposomes as a permeabilization strategy for the intracellular delivery of trehalose has been investigated as a useful cell delivery vehicle for membrane-impermeant

biologically active compounds, including drugs, enzymes, hormones, and genetic material, and also for improved cell recovery following low-temperature exposure (Holovati and Acker 2007).

22.3 Survival in Frozen Environments: Managing the Kinetics of Ice Nucleation or Ice Crystal Growth

Many organisms exist in habitats where temperatures fall below the freezing point of water. Intracellular ice formation changes the chemical environments of biomolecules. Particularly, pH change and ionic force increase may be a cause of protein and nucleic acid damage (Franks 1982). In parallel, injuries due to mechanical changes affect the systems: large ice crystals disrupt tissue structure and thus regulation of ice crystal growth may be important for the survival of these organisms in winter. Some plants and animals have evolved to prevent the lethal effects of ice crystal formation in cells. The mechanisms by which organisms survive extreme low temperatures are (1) extracellular water crystallization (formation of crystals outside cell membrane), (2) use of antifreeze proteins (AFPs) or by the formation of a glass (Clark et al. 2007).

One of the most studied groups of organisms is the Collembola (arthropods or springtails) (Clark et al. 2007). In many freeze-tolerant insects, very potent ice nucleators are present in the hemolymph. Among vertebrates, hatchling painted turtles (*Chrysemys picta*) provide another example of "natural freeze-tolerance" (Packard and Packard 2004; Costanzo et al. 2008). In freeze-tolerant species, proteinaceous ice nucleators (INAs) trigger extracellular freezing at high subzero temperatures, either to provide cold protection from released heat of fusion or to establish a protective extracellular freezing, which drives water out of the cells, further decreasing the temperature at which intracellular ice forms (Costanzo et al. 2008).

Alternatively, some organisms will die if frozen and avoid freezing by maintaining their body fluids in the liquid state even at extremely low subzero temperatures. Freeze-avoiding species increase their supercooling potential by removing ice nucleators and accumulating polyols. Terrestrial invertebrates and polar marine fish stabilize their supercooled state by means of noncolligatively-acting AFPs. Fish, insects, and some plants that live in Arctic regions have evolved to produce AFPs, which inhibit the growth of ice crystals by adsorption to the ice surface (Zachariassen and Kristiansen 2000). These organisms (specifically insects) also have the mechanisms to inactivate INAs, minimizing the risk of inoculation by ice and INAs. Antinucleating protein of bacterial origin inhibits the fluctuation of ice nucleus formation via foreign particles (Kawahara 2002).

Many nonfreezing tolerant arthropods and insects use freeze avoidance, but others, such as the Arctic springtail *Onychiurus arcticus* or some insects, use the strategy of protective dehydration (Clark et al. 2007). In protective dehydration,

loss of water occurs across a diffusion gradient between the super-cooled cell fluids and ice in its surroundings, such that freezing point depression always exceeds the environmental temperature, and eventually the organisms lose sufficient water and desiccate to ensure that a freezing event cannot occur. Studies have shown with the springtail *O. Arcticus* or the freshwater snail, *P. canaliculata*, that exposure to subzero temperatures and low water vapor pressure induces extensive dehydration through a highly permeable cuticle; in combination with rapid synthesis and accumulation of membrane/protein cryoprotectant trehalose and some low molecular weight compounds, cold hardiness is enhanced (Clark et al. 2007; Matsukura et al. 2008). As a result, water in the cells freezes at −40°C (Zachariassen et al. 2004).

Table 22.1 summarizes the main strategies that allow some living organisms to survive adverse conditions, showing representative examples of each group. It should be noted that some species can switch between freeze avoidance and freeze tolerance, depending on prevailing physiological and environmental conditions (Costanzo et al. 2008).

22.4 Some Theoretical Considerations

Before interpreting different strategies depicted in previous sections, some theoretical concepts should be introduced. Removal of water, either by drying or freezing, induces supersaturation of cytosolic components, leading to an increase in cohesive forces between molecules and restriction of molecular mobility within cytoplasm. Although both drying and freezing are considered to promote damage by hydric stress, different protective mechanisms operate in each process (Carpenter et al. 1986).

The strategies for avoiding the above-mentioned injuries caused by dehydration and freezing can be analyzed through simplified temperature-composition state diagrams (shown schematically in Fig. 22.1), in arbitrary units, but they are generically designed by approximating the composition of cell cytosol, similar to that also observed for seeds and different tissues (Levine and Slade 1992; Buitink and Leprince 2004; Espinosa et al. 2006; Matiacevich et al. 2006).

In such supplemented diagrams the curves corresponding to equilibrium conditions (liquidus, T_m, and solubility, T_s curves) and T_g of systems are plotted as a function of water content (w). Since different curves in Fig. 22.1 delimit regions where main dynamic changes could happen as a consequence of phase/state changes (Levine and Slade 1992), this kind of diagram can predict whether systems are under thermodynamic or kinetic control, for given composition-temperature conditions, provided thermal history of samples is known (Levine and Slade 1986; Roos and Karel 1991a; Levine and Slade 1992). Of particular interest for analysis of involved phenomena are the formation of glasses and freezing characteristics of systems.

Table 22.1 The main strategies allowing some living organisms to survive adverse conditions; involved mechanisms and representative examples

Type of organism	Mechanisms	Action	Involved solutes		Examples
Anhydro-biotes	Glass formation at low water content	Reduce molecular mobility, protective hydrogen bond interactions	Protective non-reducing sugars Proteins: dehydrines	Trehalose	*Artemia salina*, yeast cells (*Saccharomyces cerevisiae*), tardigrades, flatworm, insects (larvae of chironomid *Polypedilum vanderplanki)*, rotifers (*Philodina roseola*); resurrection plants (*Selaginella spp.)*
				sucrose, raffinose, stachyose	pollen, orthodox seeds, resurrection plants (*Selaginella lepidophyl*)
Freeze-tolerant	Promote extracellular water crystallization	Decrease supercooling, increase ice nucleation temperature, decrease freezing time	Adaptive INAs		Insects, arthropods or springtails (snow flea), vertebrates (hatchling painted turtles)
Freeze-avoiding	Inhibit growth of ice crystals by adsorption to ice surface	Increase supercooling, decrease freezing temperature	AFPs (non-colligative)		Polar marine fishes, terrestrial invertebrates, insects, arthropods, nematodes, plants, fungi
	Inactivation of ubiquos INAs	Inhibit fluctuation of ice nucleus formation by a foreign particle	Proteins, polymers		Antarctic bacteria (*Pseudomonas fluorescens*)
	Vitrification at high water content	Reduce molecular mobility	Low molecular weight compounds: polyols		freshwater snail (*P. canaliculata*), fly (*Eurosta solidaginis*), frog (*Rana sylvatica*)

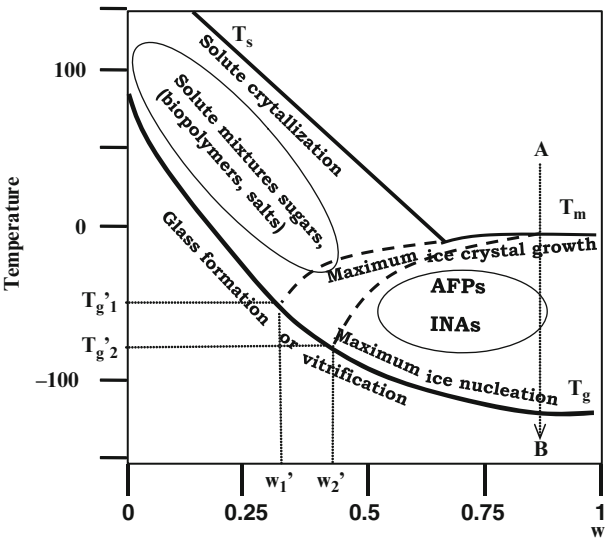

Fig. 22.1 Supplemented state diagram showing the equilibrium curves (solubility, T_s; ice melting, T_m) and nonequilibrium glass transition curve (T_g). The main expected changes are indicated, as are the mechanisms for avoiding damage (see text)

Amorphous glasses are formed by a continuous process in which no interface or solidification front is involved. Their structure is comparable to that of a liquid (some short-range order is observed, but no long-range order), but their properties are those of a solid. Glasses are characterized by a critical temperature T_g, above which most physical changes (including solute crystallization) result from sharp increase in molecular mobility, which occurs above their T_g (Levine and Slade 1986; Roos and Karel 1991a, b, c; Slade and Levine 1991). As shown in Fig. 22.1, T_g of a material is a function of the relative proportion of its glass-forming components and water content. Inclusion of T_g curve in supplemented state diagrams (corresponding to nonequilibrium transition) allows including the concept of time in which a certain event will take place or changes, which will be kinetically delayed/inhibited under certain conditions.

In region below T_m, as ice separates out during freezing, solute concentration of unfrozen phase in contact with ice increases. The combined effects of lowering temperature and increasing concentration impose molecular mobility restrictions on systems. Concentration of the unfrozen phase increases as temperature decreases. The point at which the liquidus curve, T_m, intercepts T_g curve defines the point (T_g'; w_g'), which represents the particular temperature/solute concentration below which water crystallization is kinetically inhibited. w_g' represents the lowest mass fraction of water that can be obtained by solute concentration in amorphous matrix due to ice crystallization. T_g' is the glass transition temperature of maximum ice-concentrated solution (Fig. 22.1). It should be noted that material composition will affect T_g' and w_g', thus affecting the amount of water remaining unfrozen in the

matrix ($w_g'_1$ and $w_g'_2$ in Fig. 22.1) and temperature range at which the process should be performed (Fig. 22.1). Due to predominantly nonequilibrium conditions of naturally-occurring frozen or dehydrated systems, they are subjected to dynamical (time-dependent) changes. Thus, the extent of damage will be related to the mentioned variables, to the rate and temperature of cooling, and should be taken into account in the design of processes and system formulation.

Freezing is a process of ice crystallization from supercooled water. In this process, water should undergo a stage of ice nucleation, followed by growth of ice. Nucleation is the initial stage and one of the most important steps toward creating ice. Without this step, ice would never occur in supercooled water. Nucleation can be regarded as a kinetic process for ice nuclei to overcome a kinetics barrier, the so-called nucleation barrier under a given thermodynamic driving force, which is proportional to the supercooling.

Ice growth is thermodynamically favored in water at temperatures below 0°C. However, ice nucleation is not kinetically favored in pure water and can remain liquid up to nearly −40°C, the homogeneous nucleation temperature (T_h), if free of ice nucleating species. A supercooled system is that in which no crystallization has occurred, even if it is below the liquidus curves (T_s and T_m in Fig. 22.1). Ice nucleation at temperatures greater than T_h is induced by heterogeneous INAs (Wowk and Fahy 2002). Almost all organic and inorganic substances can catalyze ice formation (i.e., serve as ice nuclei) at temperatures between −15°C and −40°C.

The degree of supercooling is the thermodynamic impulsive force for crystallization, defined as:

$$\Delta T = T_m - T \tag{22.1}$$

where T is actual subzero temperature and T_m is equilibrium melting point of ice.

The transient ice-like embryos formed by aggregation of water molecules is subjected to continuous fluctuation in size due to incorporation of new molecules and detachment of others. For a given temperature, there will be a critical radius that defines the minimum size a nucleus can have to be a stable crystal. Heneghan et al. (2002) defined the supercooling point, which is often approximated in biological studies as the temperature at which, on an average, 50% of samples are frozen. The supercooling capacity of an organism, also frequently mentioned in literature, is thus inversely related to this supercooling point: the lower the supercooling point (i.e., the lower the ice crystallization temperature), the higher the supercooling capacity of an organism. Supercooling is limited by heterogeneous nucleation in the presence of solid impurities. Certain compounds can serve as nuclei at temperatures as high as −6°C, and the wide variety of impurities present in natural systems makes homogeneous nucleation impossible. Ice nucleation preferentially occurs on these impurities, such as mold walls or cell membranes (nonadaptive INAs); since they are points with high excess energy, they lower the energy required to form the interface between the existing phase and new phase. Removing heterogeneities is one effective way of decreasing temperature at which ice forms, i.e., increasing difficulty of freezing. Some proteins have the function of

inhibiting nucleating activities of nonadaptive INAs. The critical radius decreases with decreasing temperature below T_m, and the rate of nucleation would increase with temperature below T_m. This effect is limited by decrease in molecular mobility at lower temperatures; the curves showing actual variation of nucleation frequency with temperature, crystal growth, and overall crystallization are shown as full lines in Fig. 22.2a, b.

Once initiated, crystal growth is slowed and often terminated with increasing viscosity (MacKenzie 1977). Because the free energy barrier of three-dimensional

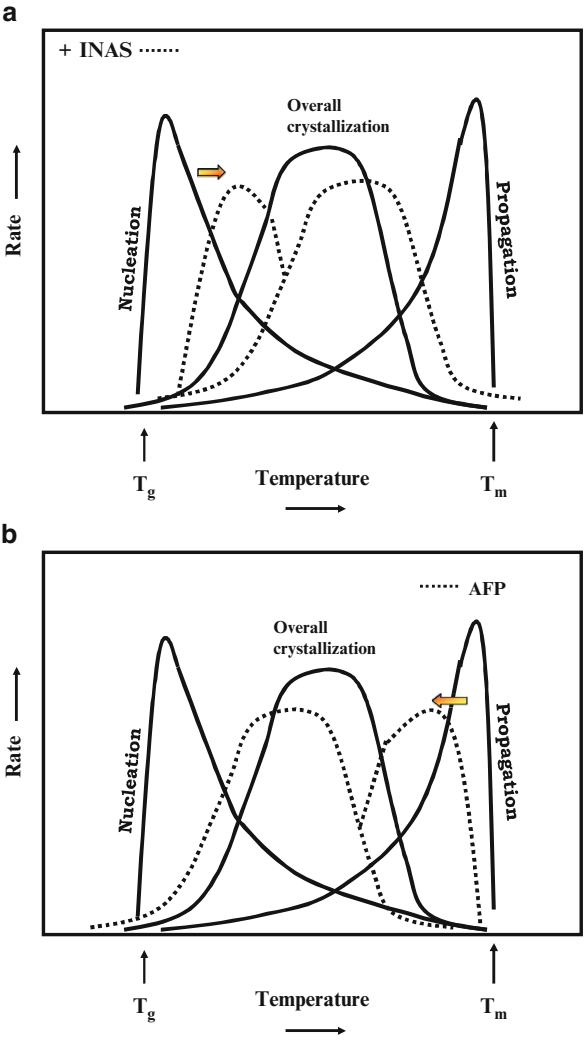

Fig. 22.2 Schematic curves showing ice nucleation and crystal growth rates, and overall crystallization rate (*full lines*) and their potential modification (*dotted lines*) by the presence of (**a**) ice nucleating agents (*INAs*) or (**b**)antifreeze proteins (*AFPs*)

nucleation is much higher than that of growth, in the case of ice crystals, growth normally becomes much easier than nucleation: once ice nuclei are formed, the rapid growth rate leads to instant freezing (Du et al. 2003). To control nucleation systematically, the upper size needs to be controlled.

22.5 Involved Mechanisms

22.5.1 Avoidance of Solids Crystallization in Supercooled State

Anhydrobiotes are dehydrated systems that have the ability to form glasses and can be located in the low **w** region of Fig. 22.1. Amorphous glasses are metastable materials in a nonequilibrium state, as these systems are located below the saturation curve, T_s, in the temperature-composition state diagram (Fig. 22.1), in which the stable form is crystal. However, in the glassy state, most structural changes occur very slowly and only small motions of molecules, mainly rotational motions of side chains and vibrations, may occur (Levine and Slade 1986; Slade and Levine 1991). Thus, crystallization of amorphous solids is kinetically delayed if sample remains in the glassy state, below T_g. Since direct interaction of sugars with molecules imbedded in the glassy matrix will prevent protein denaturation and lead to optimal preservation (Arakawa and Timasheff 1982), it is important that sugars remain in a noncrystalline state, either glassy or supercooled. It should be noted that when solute crystallization occurs, composition of the noncrystalline phase changes, and often an increase of deteriorative reaction rates is observed (Buera et al. 2005).

In the case of seeds, embryos and plant tissues, presence of several oligosaccharides has been related to seed longevity due to protection given to proteins and membranes, and to prevention of sucrose crystallization (Obendorf 1997; Murthy et al. 2003).

The influence of different yeast (e.g., *Saccharomyces cerevisiae*) cellular fractions was studied in an attempt to gain knowledge on the feasibility of trehalose crystallization in yeast cells. Certain constituents of *S. cerevisiae* cells inhibited/delayed trehalose crystallization upon humidification at high R.Hs. (Espinosa et al. 2006). This reveals that cellular structures naturally have components that act in delaying or inhibiting sugar crystallization in the interior of cells. Although crystallization of sugars in the cytoplasm has been considered as a cause of viability loss, no evidence of sugar crystallization in cells or tissues was ever reported (Buitink and Leprince 2004; Espinosa et al. 2006; Matiacevich et al. 2006). In fact, sugar crystallization has not been evident in any published, seed, fruit, or tissue thermogram during rewarming of systems from below to above glass transition temperature.

The effect of a mixture of several sugars on crystallization and stability of embedded enzymes was analyzed in freeze-dried model systems. In trehalose-lactose systems, the time to crystallization of lactose increased when trehalose

was added (Mazzobre et al. 2001). The addition of raffinose also had a retarding effect on sucrose crystallization (Buera et al. 2005). The onset temperature for trehalose or sucrose crystallization, determined by DSC, increased when raffinose or lactose was added and was even avoided during the run if enough of the second sugar was present (Mazzobre at al. 2001). Buera et al. (2005) showed that the presence of proteins delayed sugar crystallization and, in parallel, sugars retarded protein denaturation**.** Santagapita et al. (2008) have also reported delay of trehalose crystallization with the presence of polymers or salts. Because of this, when simple sugars or polyol matrices are designed to protect biomolecules, a common practice in pharmaceuticals or food ingredient formulations, a second excipient is needed.

22.5.2 Increase of Extracellular Ice Nucleation Rate: INAs

Many biological systems promote extracellular heterogeneous nucleation by the presence of a variety of INAs, which may be either incidental or adaptive. Incidental INAs (e.g., cell walls or dust particles) promote heterogeneous nucleation only as a normally unwanted side effect. Adaptive INAs are generally lipoproteins of about 30 kD; they reduce supercooling capacity by increasing supercooling temperature to as little as $-1°C$, nucleating ice crystals between cells. The amino acids within proteins form templates for ice, which serve as embryos for ice formation. Among the most efficient INAs in nature are ice nucleating proteins found on the surface of certain bacteria, such as *Pseudomonas syringae*, *Erwinia herbicola*, and *Pseudomonas fluorescens* (Kawahara 2002), commonly found on plant leaves, and other above-ground plant parts. Such proteins must assume a rigid, ice-like conformation larger than 10 nm and be able to aggregate. A thin layer of ice can always form on the surface of an INA. However, this will not lead to spontaneous ice growth unless the INA is of a certain critical size above which free ice growth will occur. Consequently, a larger INA gives a smaller required supercooling and a higher T_n. As they have the tendency to form protein aggregates of large spatial extent (larger than 10 nm) (Kajava and Lindow 1993), they act as strong heterogeneous ice nuclei in dewdrops, and ice crystals thus formed grow and break plant tissues, causing frost injury to host plants (Lindow 1983). The effects of INAs on nucleation kinetics, and further crystal growth, are schematized in Fig. 22.2a. It can be seen that supercooling point is decreased in the presence of INAs and consequently, supercooling capacity of organisms is decreased.

In natural settings, inhibition of bacterial INA is of considerable interest for environmental ice control, particularly in the prevention of frost damage to crops (Wowk and Fahy 2002). Ingestion of these ice-nucleating bacteria caused an abrupt decrease in supercooling capacity of the Colorado potato beetle (*Leptinotarsa decemlineata Say*), a freeze-intolerant species that overwinters as adults in shallow, terrestrial burrows (Costanzo et al. 1998). The ice-nucleating-active bacterium *Pseudomonas syringae* was employed as pest control in grain silos, to increase

mortality of the granary weevil *Sitophilus granarius* (L.) and saw-toothed grain beetle *Oryzaephilus surinamensis* (L.) (Mignon et al. 1998).

22.5.3 Inhibition of Ice Crystal Growth: AFP

Freeze avoidance is often achieved by a high capacity for supercooling, minimizing the risk of inoculation by ice and INAs. At low temperatures, many plants and microorganisms produce colligative protectants such as proline or sucrose. However, some organisms are also capable of producing AFPs and antifreeze glycoproteins (AFGPs), protecting them from the negative effects of freezing. AFPs and AFGPs were first identified in 1970 in Antarctic fish varieties that can survive in seawater colder than the freezing temperature of their blood; AFPs and AFGPs were then found in microorganisms, insects, plants, and nematodes. Their molecular weight ranged between 2,600 and 33,000 Da. Although they were thought to act by colligative effect, decreasing melting point of water, it was later observed that they act by avoiding growth of ice crystals, having a low effect on colligative properties.

AFPs act at concentrations in the order of 10^{-5} M and inhibit ice crystal growth by adsorption-inhibition. The nuclear magnetic resonance, x-ray structure, and many spectroscopic studies with AFPs have been helpful in determining the structure-function relationship. Mutational studies have indicated the importance of hydrophobic residues in ice binding (Venketesh and Dayananda 2008). Ice crystals can grow along six a-axes, all in the same plane, or along the c-axis, which is perpendicular to the plane of the six a-axes. Ice crystal growth at temperatures close to T_m typically occurs along the a-axes, which accounts for the typical hexagonal shape of snowflakes. The c-axis growth results in needle-like ice crystals, which are potentially damaging (Davies and Hew 1990). The morphology of ice crystals will depend on the planes in which growth inhibition is performed by AFP. AFPs typically found in Arctic fish specifically adsorb to basal ice (a-axis), growing planes and avoiding propagation, and behave as "structural ice inhibitor agents" (Pertaya et al. 2007a, b); ice crystal growth occurs in bipyramidal shape. By contrast, AFPs from Arctic insects typically inhibit c-axis growth, forming brown flat discs.

All AFPs tested to date show both antifreeze activity and inhibition of ice recrystallization, suggesting a common mechanism for these two effects mediated through ice binding. To visualize the binding of AFP to ice, Pertaya et al. (2007a, b, 2008) labeled the spruce budworm AFP with enhanced green fluorescent protein (GFP) and observed the AFP-GFP molecules directly on ice crystals using confocal microscopy. Melt-growth-melt sequences in low concentrations of AFPs revealed that the same general behavior of an apparently rotated crystal, observed in pure ice under high pressure, is reproduced in ice under the influence of AFP at ambient pressure and temperatures near 0°C.

While AFPs lower the freezing point, the melting point is unaltered. The separation of melting and freezing temperature is usually referred to as thermal hysteresis (TH), and temperature of ice growth is referred to as hysteresis freezing point. The hysteresis is the result of an adsorption of AFPs to crystal surface. This causes ice to grow as convex surface regions between adjacent adsorbed AFPs, thus lowering temperature at which the crystal can visibly expand (Kristiansen and Zachariassen 2005). Within the resulting TH gap, ice crystals appear to be kinetically stable, neither growing nor melting. The level of TH is directly related to intrinsic activity of specific AF(G)P and to their concentration. Results by Evans et al. (2007) showed that when AF(G)P are dissolved in salt solutions, such as NaCl, at concentrations they could encounter in nature, there is a synergistic effect on TH that is positively related to salt concentration. This enhancement could have resulted from the hydration shell of dissolved ions, which reduces freezable water. Alternatively, salt could influence the hydration shell surrounding AF(G)P, increasing protein surface area available to adsorb to ice/water interface (Evans et al. 2007).

Five AFP types have been isolated from fish, six from plants, and two from insects; five bacteria produce AFPs and show no related structures, varying in protein size and specific activity (Davies and Hew 1990; Brush et al. 1994; Davies et al. 2002; Gilbert et al. 2004). The AFPs found in certain insects (which allow them to survive winters at temperatures as low as −30°C) are up to 100 times more powerful than similar proteins in fish (Graether et al. 2000). Although TH activity of plant AFPs is low compared to that of fish and insects, their inhibition of ice recrystallization is comparable or even greater (Smallwood et al. 1999). The effect of AFP on kinetic control of ice crystallization is shown schematically in Fig. 22.2b (dotted lines).

22.5.4 Vitrification at High Water Content (Liquid N and/ or with Concentrated Solutes)

The alternative route to remove ice crystallization is rapid cooling into the glassy state, or vitrification (line A–B in Fig. 22.1). Vitrification as a cryopreservation method has many primary benefits, such as no ice crystal formation through increased speed of temperature conduction, which provides a significant increase in cooling rates. This can eliminate structural damage during freezing, but mass transfer rates limit the sample size in which this can be achieved and thawing is never rapid enough to prevent crystallization from causing extensive damage (Lillford and Holt 2002). It should be noted that the maximum ice nucleation occurs just above T_g and the maximum ice-crystal growth-rate occurs just below T_m (Fig. 22.1). Thus, the many nuclei formed when cooling can cause massive ice growth when rewarming. This leads to devitrification. The main concern with cooling is the maximum nucleation range near T_g. The addition of a cryoprotectant may allow cooling up to the T_g region without forming any nuclei, if cooling is

quick enough. If nuclei are formed, the rapid cooling and high viscosity near T_g in the presence of cryoprotectant will not allow the nuclei to grow very much and thus prevents them from being harmful. Increasing cryoprotectant concentrations lowers both T_h and T_m, but the effect is more dramatic on T_h than T_m. Enough cryoprotectant to lower T_m will lower temperature of nucleation to T_g, thereby delaying nucleation. Northern frogs use glucose as a cryoprotectant (Mietchen et al. 2008). When temperatures drop, the liver of these frogs produces large amounts of glucose, which a special form of insulin allows to enter into their cells in large quantities. Except for the heart and brain, much of the frog's body freezes. The two disaccharides that most protect proteins and cell membranes against chilling, freezing, and dehydration are sucrose and trehalose.

Dissolved polymers form glasses readily once they are freeze-concentrated. Below T_g, no further ice forms, and crystal growth rates and sintering of ice are kinetically slowed to almost zero. The damage caused by the amount of ice present and temperature at which it is stabilized against growth and/or recrystallization should be balanced (Lillford and Holt 2002). Increasing speed of thermal conduction and decreasing concentration of cryoprotectant are challenges to overcome with vitrification methods. The convenience of vitrification could push development of this technique beyond most presently common clinical uses for embryos and tissue preservation (Kattera and Chen 2006).

22.5.5 The Problem of Recalcitrant Seeds

Orthodox seeds undergo a programmed desiccation at termination of their development. Vitrification of cytoplasm components in orthodox seeds is proposed to be advantageous for germplasm stability, and their transitions may affect seed viability (Walters 2004). The cytoplasmic glass of seeds is composed mainly of sugars (sucrose and oligosaccharides comprise over 10–20% of dry weight), high molecular weight oligosaccharides, and proteins. As storage temperature or water content increases, seeds undergo glass-to-liquid transition (T_g), resulting in an increase in molecular mobility. As T_g is a function of water content, whether seed tissues are in the glassy state or not, it will depend on both seed water content and storage temperature (Buitink and Leprince 2004; Matiacevich et al. 2006). Investigations have led to the suggestion that seeds induce glass formation as water content is depressed, thus limiting deteriorative reactions (Murthy et al. 2003). The seed's tolerance to desiccation (orthodox) can be attributed to the presence of the soluble carbohydrates raffinose and sucrose in the cytoplasmasm of embryo cells. In contrast, recalcitrant seeds (*Araucaria angustifolia*, palm) do not tolerate a reduction in water below a relatively high level without loss of viability. Conventional storage techniques are thus not applicable to these seeds and cryopreservation is the only feasible alternative for their long-term storage. Panza et al. (2006) have observed that at necessary water contents (or relative humidities (R.Hs.)) at

which embryos of recalcitrant *Araucaria angustifolia* seeds retain their viability (at and above R.H. 85%), water freezes upon cooling at subzero temperatures. Their high-freezable water content promotes injuries, and their conservation at low-temperatures represents a challenge. Thus, recalcitrant seeds are good models for analyzing the impact of freezing rate, storage time, and temperature on degree of injury. If plant tissues are exposed to rapid cooling only minor damage occurs (−196°C). Seeds with lower amounts of frozen water would be more easily cryopreserved. The preservation of recalcitrant seeds could be improved by vitrification, either by dehydration, after imbibition of embryos with protecting agents, or by freezing, avoiding ice formation, but this is an area of further research, of interest in biodiversity preservation, for example.

22.6 Applications in Food Technology

The implications of glass formation and glass transitions in food technology have been extensively analyzed since the pioneering work by Levine and Slade in the 1980s (Levine and Slade 1986, 1992), based on polymer science approaches. Afterwards, many scientific publications evolved (Roos 1995). Parallel advances in the area of preservation mechanisms of living organisms under extreme conditions promoted the study of sugar properties in relation to their protective effects on labile biomolecules (Arakawa and Timasheff 1982; Crowe et al. 1998; Clegg 2001). Fundamental research areas were also focused on dynamic aspects related to glass-forming properties of sugars, and on molecular interactions that sugars are capable of developing in hydrogen bonding.

According to the discussed aspects, possible strategies to avoid sugar crystallization for food and ingredient formulation in low water content systems may include vitrification or sugar crystallization delay in supercooled media, which can be achieved by combinations with biopolymers, salts, or other sugars. Recently, Leinen and Labuza (2006) and Belcourt and Labuza 2007) successfully suppressed sucrose recrystallization by addition of raffinose to the formulation of cotton candy and soft cookies, respectively, which consequently improved technological properties of products.

The efficiency of freezing and resulting food quality is affected by two important factors: supercooling (cooling of liquid below its freezing point, without freezing) and nucleation (initiation of crystallization of liquid water into solid ice) (Li and Lee 1995). Both phenomena are modified by the presence of two kinds of proteins showing opposite behavior (Hew and Yang 1992; Li and Sun 2002), which provide enormous technological potential in industrial processes (Lillford and Holt 2002).

INAs, on the one hand, decrease supercooling and increase nucleation temperature (T_n), thus decreasing freezing time and forming a large number of ice crystals with a dendritic structure (Feeney and Yeh 1998; Li and Lee 1995; Li and Sun 2002). AFPs, on the other hand, increase supercooling, generate very small ice crystals, lower freezing temperature, and retard recrystallization in frozen storage.

Lillford and Holt (2002) and Li and Sun (2002) have reviewed the main potential applications of INAs and AFPs. The beneficial effects of INAs are manifested in decreasing energy cost and crystal size, favoring quality improvements and increased shelf life. Bacterial ice nucleators were employed in the freezing of meat (Payne et al. 1994), and in products difficult to concentrate or freeze-dried products, such as fruit juices or pastes (Li and Lee 1995). The management of ice nucleation and crystallization times can also be employed for the development of new products. For example, freeze-texturing of protein products may generate foods with special characteristics (Watanabe and Arai 1994).

AFPs behave both as antinucleators and as growth inhibitors, preventing heterogeneous nuclei from growing (Lillford and Holt 2002). One of the most interesting applications is the performance of AFP after ice is nucleated or in conditions where ice crystals would normally grow and sinter (Griffith and Ewart 1995). The presence of AFPs in these products may inhibit ice crystal growth at fluctuating subzero temperatures during storage or transportation, thus preserving food quality and decreasing operative costs.

AFPs are natural products that form part of a normal diet through their occurrence at high concentrations in food fish and vegetables (Smallwood et al. 1999). Simple natural extracts that are purified or partially purified are likely to meet consumer acceptance. The AFPs from vegetables have a number of potential applications. For example, Zhang et al. (2007a) improved the texture of frozen dough and the effect of volatile compounds in crumb foods by the addition of a concentrated carrot extract containing carrot AFP. AFP is efficient at relatively low concentration, but the cost of extraction from natural sources restricts their use only to applications with very high added value. Strategies for improving AFP production from natural sources can be developed. Naturally-occurring "antifreeze" has been found in carrots, winter wheat, and rye (Li and Lee 1995). Desjardins et al. (2007) showed that the thermal regimen of wolffish can be manipulated to enhance their tolerance to subzero temperatures and also their ability to produce AFP. Abundant AFPs could also be generated as by-products in processing certain fish species for food (Griffith and Ewart 1995).

Alternatively, AFP genes can also be introduced into microorganisms used to produce frozen yogurt (Yeh et al. 2008). Recent advances in biotechnology and control of heterologous gene expression may make them potentially accessible in high quantities. The multiple forms of AFPs synthesized within each organism, and demonstrated possibilities for useful modification through protein engineering, make it feasible to choose the most appropriate AFP, with a suitable level of activity for addition to a particular product or expression in a particular plant or animal (Griffith and Ewart 1995; Li and Lee 1995; Lillford and Holt 2002). Further, the ability to modify the rate and shape of crystal growth and to protect cellular membranes during lipid-phase transitions have resulted in identification of a number of potential applications of AFGPs as food additives, and in the cryopreservation and hypothermal storage of cells and tissues (Harding et al. 2003).

22.7 Future Prospects

The study of delay/inhibition of sugar crystallization in supercooled liquids at low water content may increase the range of applications of sugar as excipients for food ingredient formulation for specific purposes. Currently, relative long-term storage of living cells in the high water content range can be done only in cryoprotectant solution at extremely low subzero temperatures, and most of the time media formulations are performed on an empirical basis. A standardized vitrification protocol for cryopreservation on a scientific basis has yet to be defined, taking into account the many variables that can profoundly influence its effectiveness and potential to improve survival rates of vitrified cells (e.g., type and concentration of cryoprotectant; temperature of vitrification solution at exposure; duration of exposure to final cryoprotectant before plunging into LN2).

Liquid-to-solid nucleation of a supercooled aqueous solution seems to be a simple phenomenon, but it remains a poorly understood process of evolution of a metastable state to its final equilibrium state. Inputs from fundamental research in this area are also needed (Heneghan et al. 2002). Careful design of experiments for measuring temperatures and times of nucleation and crystal growth should be developed (Chapsky and Rubinsky 1997; Heneghan et al. 2002; Du et al. 2003; Zachariassen et al. 2004; Yin et al. 2005).

The presence of ice surfaces was reported as a cause of freeze-induced perturbations of protein native fold, but these interactions are poorly understood. Gabellieri and Stambrini (2006) reported that a binding method using a fluorescence probe may find practical utility in testing the effectiveness of various additives employed in frozen or freeze-dried protein formulations. Research work in this area would be helpful in the development of "ice managing" additives. Besides, the quantitative relationships between the activity of INAs and AFPs and variable parameters that affect the measurement of activity should be established to elucidate the mechanism and organize the research findings.

Fundamental research also on freeze tolerance mechanisms may allow identification of secondary metabolic products that prepare organisms for stress from ice formation, osmotic dehydration or freeze concentration; further, molecules that have applications in industrial settings could be produced in large quantities once their functions are elucidated (Feeney and Yeh 1998; Lillford and Holt 2002). The effect of proteins and polysaccharides involved in INA inactivation should also be further investigated to explore their potential applications (Kawahara 2002; Wowk and Fahy 2007).

Although many advances have been achieved in the last 10 years, additional research is still needed to define the relationship between molecular structure and the function of INAs and AFPs. The developments of infrared thermography, microscopic and magnetic resonance spectroscopy, and imaging techniques for cryobiological investigations were demonstrated as powerful tools for interpretation of mechanisms of action for different AFPs (Wisniewski et al. 2001; Pertaya et al. 2007a, b, 2008; Mietchen et al. 2008), and are expected to further contribute to advances in these areas.

The availability of ice nucleators or antinucleators for food uses involves development of purification protocols of potential AFPs and INAs and effective ways for their characterization (Kawahara et al. 2006; Zhang et al. 2007b). Besides being nontoxic, nonpathogenic, and environmentally safe, they should be palatable (Lillford and Holt 2002). Sensory evaluation should thus be performed on processed products with these additives before being commercialized.

The chemical synthesis of AFPs is an attractive alternative to their difficult isolation and purification from natural sources, and this would permit quality control and mass production (Matsumoto et al. 2006). The successful synthesis of small AFGPs using solution methods and solid-phase chemistry provides the opportunity to perform key structure-activity studies that would clarify important residues and functional groups required for activity (Harding et al. 2003). The possibility of production using different molecular biological techniques, which will help increase the yield, is also dealt with (Venketesh and Dayananda 2008).

Improving the efficiency of AFPs by the presence of simple molecules normally present in foods, such as electrolytes (Du and Liu 2008), provides another new insight into the antifreeze mechanism of AFPs on ice crystallization.

AFPs can display both protective and cytotoxic actions, and both nucleation of ice and inhibition of ice crystal growth; they have also been shown to either stabilize or disrupt the membrane, depending on several factors (membrane composition, type, and concentration of AFP) (Wang 2000; Tomczak et al. 2001). At certain levels, AFP-ice interactions may induce the complexes to aggregate, promoting ice nucleation, and loss of ability to inhibit recrystallization. Although at low concentrations AFPs can be employed very effectively to maintain texture and flavor of frozen foods, it should be noted that bipyramidal or spicular ice crystals, which grow in solutions containing high concentrations of AFP, are so detrimental for cell survival that they are proposed as an important tool in cryosurgery (Matsumoto et al. 2006). These properties indicate that careful consideration of concentrations and type of AFP used require further study in order to understand the mechanisms by which they occur and their potential usefulness in food technology.

The use of gene transfer to generate food organisms that produce AFPs needs to be carried out. Since one important consideration is that of consumer acceptance of transgenic products, transfer of genes from INA^+ bacteria to other microorganisms commonly used in production of foods (Yeh et al., 2008), or AFP genes from cereal grains (e.g., winter wheat or rye) to plants for production of more freezing-resistant fruits, is expected to be better accepted by consumers than the use of fish AFPs or bacterial INAs (Lillford and Holt 2002). The addition of INAs or AFPs to food products could involve significant modification in formulation and handling procedures. Thus, a further analysis of phase/state diagrams is necessary before large-scale food production.

The relatively high cost of freezing processes currently used in the food industry and the search for products with special structures could be the major impulse toward new innovations in freezing techniques. Additional work to analyze cost-benefit, balancing the energy savings, and product-quality improvements, is essential before final commercialization of application in the food industry (Lillford and

Holt 2002). The discussed aspects are examples of how the tools from cryobiology may stimulate those innovations.

It is interesting to note that the different aspects referred to in this chapter show the synergistic interaction of multidisciplinary interfaces of physics, chemistry, medicine, biotechnology, food science and technology, pharmacy, and biology, which are faced with preservation of living organisms such as embryos and cells, structures like liposomes, as well as labile molecules, such as enzymes, antibodies, and hormones.

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