Chapter 9
Freezing and Freeze-Drying

Yrjö H. Roos, Caroline Furlong and Naritchaya Potes

Abstract Freezing of biological materials is commonly carried out to delay biochemical and chemical changes resulting in loss of activity. However, the physicochemical background of freezing is not well understood. Freeze-drying is generally aimed at preserving biological materials and bioactive components for long periods of time without the need for frozen storage. Freeze-drying relies on proper freezing and process control based on an understanding of the frozen state properties of biological materials. Such properties relate to unfrozen water and its distribution across carbohydrates, proteins and other components. Here, novel methods for deriving accurate protein hydration levels and unfrozen water content are discussed. The role of water and carbohydrate distribution in preserving the viability of micro-organisms in freezing and freeze-drying is also discussed, as this is a significant factor affecting the success of freezing and freeze-drying processes in the production of starter cultures and the preservation of probiotic bacteria.

Keywords Freezing · Freeze-drying · Glass transition · Carbohydrates · Proteins · Probiotic bacteria · Unfrozen water · Water activity

9.1 Introduction

Freezing of water is a complex process of ice formation at low temperatures, and is the fundamental principle of bioactive stabilization using freezing and freeze-drying. Freezing and frozen storage prior to freeze-drying is a process of microstructure formation for stability control of frozen and freeze-dried materials. In long-term stabilization, frozen materials need to be stored at conditions where unfrozen solutes remain in a vitrified solid state, while the vitrified solid state of frozen structures is preserved for long-term ambient storage by ice sublimation in the freeze-drying (lyophilization) process. The frozen material shows an expansion corresponding to the volume expansion of the same quantity of water converted to ice during crystallization.

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Protection of protein conformation and retention of biological functions subsequent to freezing and thawing or freeze-drying and rehydration is common in the food and pharmaceutical industries. The use of carbohydrates, particularly monosaccharides and disaccharides, as cryoprotectants to retain protein structures against freezing and dehydration damages has been of particular interest (Jorgensen et al. 2009). The phase behavior of water and the state of dissolved substances dominate the thermal properties of cryoprotectant systems. Ice formation always results in separation of a freeze-concentrated unfrozen aqueous cryoprotectant phase and vitrification of the maximally freeze-concentrated unfrozen phase over a cryoprotectant-specific temperature range (Roos and Karel 1991a, b; Luyet and Rasmussen 1968; Franks et al. 1977; Levine and Slade 1986; Pehkonen et al. 2008). The phase and state behavior of cryoprotectant–water systems may be described using state diagrams (Franks et al. 1977; Roos and Karel 1991a, b; Buera et al. 2011). While such diagrams have clarified the physicochemical properties of carbohydrate systems at low temperatures, they lack the information needed to understand carbohydrate–protein interactions in biological, food and pharmaceutical systems as well as intracellular and extracellular ice formation in cellular structures.

Although freezing and freeze-drying are well-established processes for preservation of biological materials, the present chapter highlights physicochemical properties of typical food and related materials affecting structure formation and properties of frozen and freeze-dried materials, with a particular focus on stabilization of bioactives.

9.2 Freezing and the Frozen State

The freezing of food materials is a well-established industry, as freezing stops or significantly delays microbial growth, and delays most biological processes and chemical reactions. Equilibrium freezing (referring here to ice formation and phase and state transitions below 0 °C) properties of eutectic salt solutions such as sodium chloride are well described in general physical chemistry (Atkins and De Paula 2010), but properties of sugar solutions and more complex dispersed structures are often poorly understood. Biological and food materials always contain unfrozen water at low temperatures. The term "unfrozen water" here refers to water in which interactions with solutes occur, although it is worth noting that the properties of even unfrozen pure water at temperatures within normal limits for supercooling and vitrification, i.e., −40 °C to −130 °C (*no man's land*), are poorly understood (Angell 2008, 2014). On the other hand, freezing and frozen storage of biological materials is routinely carried out using liquid nitrogen (liquid-N_2, −196 °C) and −80 °C equipment. The use of such temperatures is based on the freezing medium and equipment availability rather than scientific data for optimal material stability.

$$p_w = xp_0 \tag{9.1}$$

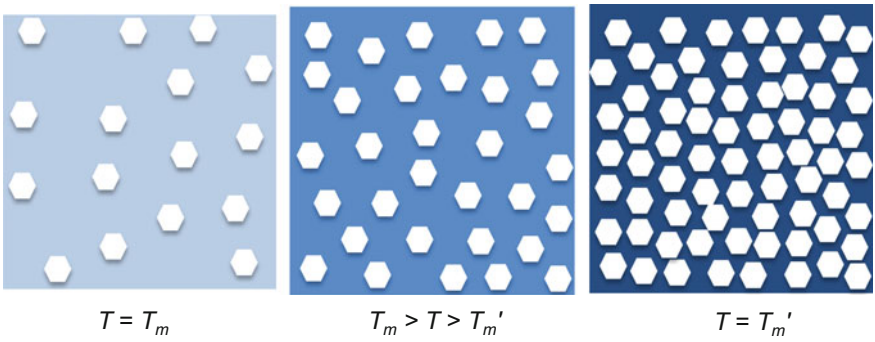

Fig. 9.1 Relative quantity of ice forming in biological materials at temperatures corresponding to equilibrium melting temperature, T_m; temperatures above the onset of ice melting in a maximally freeze-concentrated state, T_m'; and in a maximally freeze-concentrated state at T_m'

The freezing temperature depression of water by solutes in dilute solutions is well known, and is described by Raoult's law (Eq. 9.1), which states that the smaller the solute molecules, the larger their effect on water vapor pressure, p_w. The equilibrium melting temperature of the last ice crystals during heating of a solution with ice (i.e., freezing temperature), T_m, has the requirement of $p_i = p_w$ (where p_i is the vapor pressure of the ice). The T_m decreases with solute concentration, which for frozen systems implies that the amount of frozen water (ice) increases with decreasing temperature. Conversely, during frozen storage, temperature fluctuations can result in substantial melting–refreezing phenomena and rapid recrystallization of ice, with a consequent increase in the size of ice crystals. The temperature dependence of ice in frozen solutions is shown in Fig. 9.1. In a binary eutectic system, both the solvent water and the dissolved substance crystallize as solubility is exceeded, causing precipitation of the solute at the eutectic temperature, T_e. Eutectic freezing is unusual in complex multicomponent systems, and many biological materials show vitrification of their unfrozen water–solute phase. The glass transition temperature of the maximally freeze-concentrated unfrozen phase is known as T_g'. Ice formation in a maximally freeze-concentrated solute phase ceases at T_m' as the solute concentration approaches C_g'. A maximally freeze-concentrated material, therefore, shows an onset of the glass transition at T_g' and onset of ice melting at T_m' when measured in heating after freezing to the maximally freeze-concentrated state.

9.3 Glass Transition of Frozen Materials

Glass transition is a reversible change in the state of supercooled noncrystalline materials. The glass transition can be determined from thermodynamic properties such as heat capacity and thermal expansion of supercooled liquids as they are

heated and cooled over the glass transition temperature range (Angell 2002; Roos and Drusch 2015). Supercooled states of materials show molecular disorder and random time-dependent characteristics. The glass transition is accompanied by changes in mechanical and dielectric properties as well as other structural relaxations resulting from a change in the molecular mobility of the glass-forming substance. The structural relaxations occurring over the reversible glass transition are referred to as α-*relaxation*. The α-relaxation is typically studied in heating by monitoring the time and temperature dependence of the molecular response of the material during glass transition. The relaxation time is derived from the time dependence of the α-relaxation. The characteristic relaxation time, τ, for the solid, glassy state is 100 s, corresponding to a viscosity of 10^{12} Pa s (Angell 2002).

9.4 Carbohydrate Systems

Carbohydrates show a large variation in molecular size as components of biological materials. On the other hand, small sugars are often highly soluble and occur in large quantities in biological materials important to freezing and freeze-drying. Conversely, small sugars are the most common cryoprotectants used for preservation of starter cultures and native protein structures. Sugar systems have been studied in detail for understanding the frozen state phenomena of aqueous solutions of carbohydrates and cryoprotectant systems (Roos 1993; Corti et al. 2010; Roos and Drusch 2015).

Freezing of carbohydrate systems commonly occurs without solute crystallization, as described in Fig. 9.1. Solute crystallization is limited by the high solubility of many carbohydrates and their slow nucleation and crystal growth rates compared to that of ice formation. As a consequence, maximally freeze-concentrated states of carbohydrates are common, and are typically a prerequisite for successful freeze-drying where

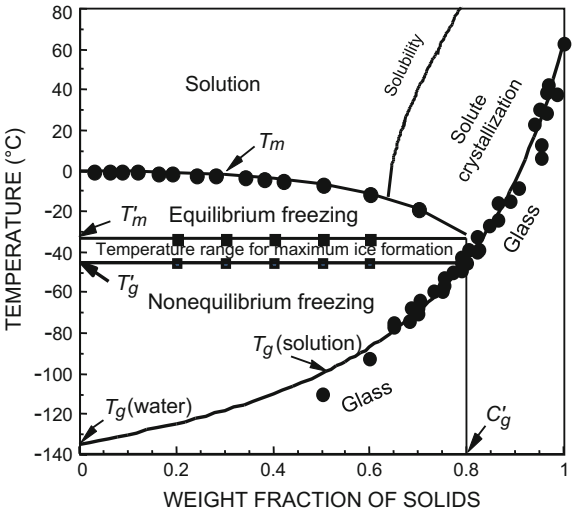

Fig. 9.2 State diagram of sucrose showing water content dependence of glass transition temperature, T_g; ice melting temperature, T_m; onset of glass transition of maximally freeze-concentrated solute phase, T_g'; and onset of ice melting in a maximally freeze-concentrated solution, T_m'

any liquid water must be part of the maximally freeze-concentrated vitrifying solute phase at temperatures lower than T_m'.

State diagrams are often constructed for binary carbohydrate–water systems to describe ice formation as well as freezing and freeze-drying properties of carbohydrate systems. The state diagram of sucrose is shown in Fig. 9.2.

9.5 Carbohydrate–Protein Systems

Protection of proteins and peptides against dehydration or freezing damage advances the survival of living organisms as well as the production and stabilization of active enzymes, pharmaceuticals and biological materials. Proteins contain hydration water in cavities and surface pores. Strong hydrogen bonding reduces the chemical potential of water molecules at hydration sites and explains the unfrozen state of hydration water at low temperature (Jansson et al. 2011; Roos and Potes 2015; Swenson and Cerveny 2015). The α-relaxation of hydration water in proteins is coupled with vitrification phenomena in cryoprotection and anhydrobiosis, particularly with the protection of protein conformation when carbohydrate protectants are used. Water molecules in amorphous carbohydrate systems retain high mobility, and they may exchange with water molecules that are hydrogen-bonded to the protein. Depending on the carbohydrate, vitrification of the hydration water and aqueous phase may occur at different temperatures, with consequent effects on protein stability (Roos and Potes 2015). In a protein–carbohydrate system, an exact quantity of water is required to exist as protein hydration water to satisfy a thermodynamic balance with a co-existing carbohydrate phase. At equilibrium, such hydration water has equal water activity a_w (equal chemical potential and vapor pressure) to that of water associated with the carbohydrate. Our studies have shown that the sugars co-existing with proteins in a freeze-concentrated solute phase give a T_g' for the unfrozen carbohydrate–unfrozen water phase. Measurement of the T_g' can be used to derive the composition of the unfrozen carbohydrate phase, while the unfrozen protein hydration water can be obtained from the water sorption data (Roos and Potes 2015). Protein hydration water in a maximally freeze-concentrated protein–carbohydrate system showed a quantity that became independent of the initial water content and the carbohydrate content, i.e., maximally freeze-concentrated carbohydrate was plasticized by a measurable unfrozen water fraction. The surrounding protein was maximally freeze-concentrated with a corresponding unfrozen hydration water fraction. A system composed of bovine whey proteins and a glucose–fructose (1:1) carbohydrate phase (GF) showed unfrozen water fractions in the maximally freeze-concentrated state with 0.164 g H_2O/g protein and 0.273 g H_2O/g GF. The data measured by Roos and Potes (2015) were used to establish a tertiary three-component state diagram shown in Fig. 9.3. The experimental quantification of the unfrozen water in the protein and carbohydrate components of cryoprotectant systems at maximum freeze-concentration as described by Roos and Potes (2015) provided a significant universal method of quantifying (i) protein hydration water and (ii) the unfrozen water in the carbohydrate phase. This

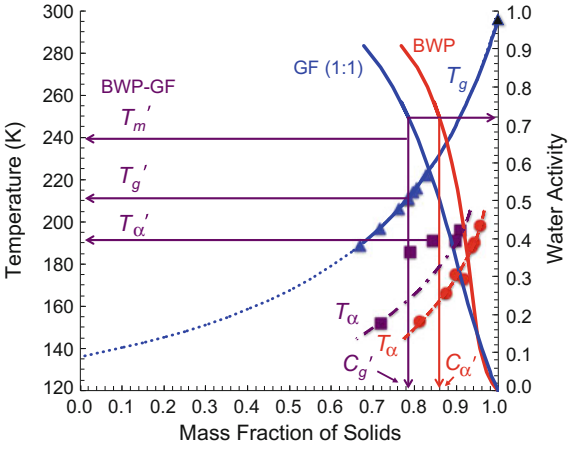

Fig. 9.3 Tertiary state diagram for bovine whey proteins (BWP) and glucose:fructose (1:1) as carbohydrates (GF). The glass transition of the GF, T_g, is found using differential scanning calorimetry at the temperature dominated by the GF phase. At maximum freeze-concentration, the glass transition, T_g', is that of the carbohydrate, while hydration water on protein shows an α-relaxation at T_α'. The water content corresponding to the glass transition of the carbohydrate, T_g, and α-relaxation of protein hydration water, T_α, can be derived from the component water sorption isotherms

is important for the understanding and use of carbohydrate protectants in freezing, lyophilization and dehydration of biological materials, and in the stabilization of bioactive components in formulation and structure engineering of food and pharmaceutical materials. It should be noted that earlier studies of protein hydration water have used a rough estimate of 0.2–0.3 g of unfrozen water/g of protein. Such estimates were made on the basis of unfrozen water measurements using differential scanning calorimetry (DSC) (Doster et al. 1986).

Roos and Potes (2015) showed that a separate α-relaxation of the protein hydration water and of the bulk GF were signs that hydration water molecules were primarily hydrogen-bonded to protein molecules, and that hydration water provided a bridge for hydrogen bonding of the protein to the continuous GF phase of the system. Proteins as large molecules were considered as dispersed particles within the carbohydrate phase. At maximum freeze-concentration, a "syrup" with 2.5 mol of water per mol of hexose unit is known to remain unfrozen in sugar solutions (Roos and Karel 1991a, b), while the whey protein isolate (WPI)–GF shows an increased 2.75 mol H_2O/mol GF, implying an increase in hydrogen bonding towards the protein hydration interface. At low temperatures and under lyophilization conditions, proteins with carbohydrate cryoprotectants exist with unfrozen hydration water exposed to either a solid (glassy) or liquid (rubbery) unfrozen aqueous carbohydrate phase. A liquid carbohydrate phase causes significant osmotic stress which is not present in the vitrified state of the carbohydrate. According to Roos and Potes (2015), hydrated protein particles within cryoprotectant solutions become encapsulated by the vitrifying carbohydrate phase. Such a

mechanism explains the retention of protein conformation in freezing and dehydration by freeze-drying. In other dehydration processes, glass formation around hydrated protein by a protectant carbohydrate can also explain the stabilization of protein conformation and structure.

9.6 The Freeze-Drying Process

Freeze-drying (lyophilization) involves the freezing of water to ice and establishment of drying conditions where sublimation of ice to water vapor is accelerated and the vapor is condensed as ice on a cold surface. Such conditions can be established by lowering the temperature or using vacuum equipment to lower the pressure of a drying chamber to control the temperature of the ice.

The freeze-drying process is described in Fig. 9.4. A typical process uses vacuum equipment to lower the pressure of the drying chamber to $p < 1$ mbar. At such conditions, the ice temperature, T_{ice}, is a direct function of the surrounding pressure. On the other hand, refrigeration equipment is used to lower the temperature of a condensing surface, T_c, to below the temperature of the sublimating ice. This allows for a condition where the vapor pressure of the ice, p_{ice}, of the drying material is higher than that found on the condenser surface, p_c. Hence, the driving force for sublimation is defined by the vapor pressure difference, $\Delta p = p_{ice} - p_c$. Obviously, an increase in Δp accelerates the freeze-drying process.

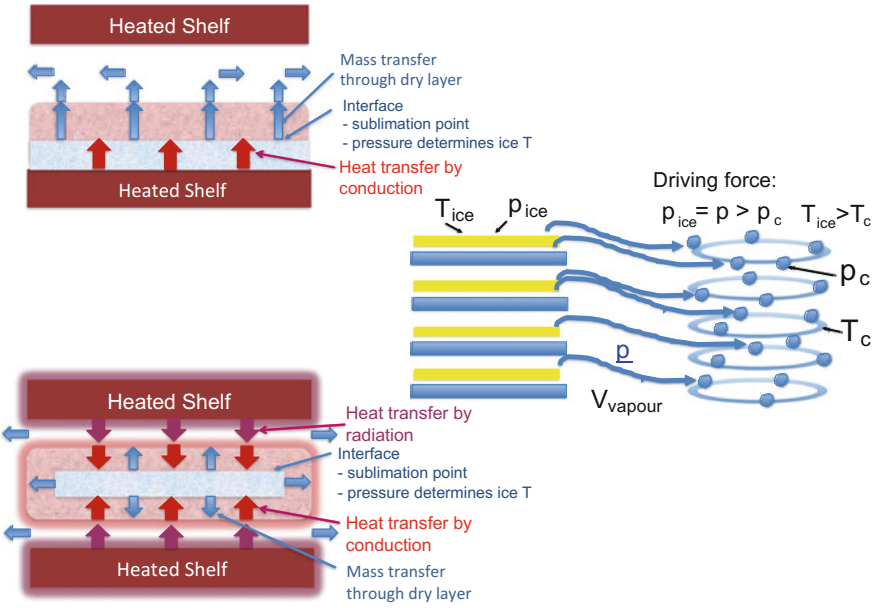

Fig. 9.4 The principle of freeze-drying (*right*) and heat transfer and mass transfer to accelerate and control freeze-drying when by conduction (*left top*) or radiation (*left bottom*)

Solutes in the water phase of biological materials affect the freezing of water, and they each have a specific temperature for maximum freeze-concentration and corresponding onset temperature of ice melting, T_m'. A prerequisite for freeze-drying is the solid state for the sublimating ice, and T_m' thus provides a critical temperature for sublimation in freeze-drying. Successful freeze-drying requires that the temperature for sublimation is lower than the T_m' of the water–solutes phase to avoid melting during sublimation. The ice temperature is affected by the chamber pressure and also by the heat and mass transfer within the material undergoing freeze-drying. Heat supplied to the sublimating ice can accelerate freeze-drying, while mass transport inside the drying particle controls the internal pressure of the drying particle (Fig. 9.4). This also causes large differences between freeze-drying processes using radiation and contact heat transfer. As described in Fig. 9.4, during freeze-drying, ice is an excellent heat conductor (heat transfer by conduction through an ice layer on a heated shelf), while an already dried porous layer of a biological material is an efficient insulator for heat transfer (radiation from heated plates and heat conduction to sublimation interface within a drying particle).

Conditions which must be satisfied during a successful freeze-drying process are shown in Fig. 9.5. A preferably external pre-freezing step of a material must ensure that the freezing step produces a desired size of ice crystals and surrounding external wall of unfrozen solids phase thickness (Harnkarnsujarit et al. 2012). Thereafter, the ice temperature must be lowered to well below the T_m', with corresponding ice vapor pressure of $p_{ice} < p_m'$, to ensure that $T_{ice} < T_m'$ applies during loading of the freeze-dryer until proper freeze-drying conditions for maintaining $T_{ice} < T_m'$ have been established. During any condition of $T_{ice} > T_m'$ corresponding to $p_{ice} > p_m'$, a liquid water phase appears and results in viscous flow within the unfrozen water–solutes phase. Such conditions lead to shrinkage and collapse of the structure of the drying material, and often to poor dehydration and loss of quality.

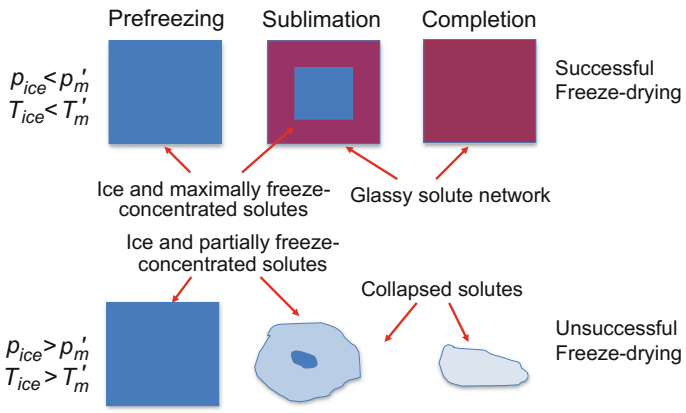

Fig. 9.5 Control of a freeze-drying process using information on ice melting properties to maintain ice sublimation at temperatures below the onset temperature of ice melting, T_m'

9.7 Freezing and Freeze-Drying of Microbes

The survival of starter cultures and preservation of bioactive micro-organisms, particularly lactic acid bacteria, are of fundamental importance in preserving viable probiotic cultures for food use, as well as in the development of dehydrated foods with probiotic activity. Frozen culture concentrates of probiotics exhibit maximal survival in liquid nitrogen (Smittle et al. 1974), while freeze-drying is one of the most successful and convenient methods of preserving microbial cultures (Berny and Hennebert 1991; Carvalho et al. 2003).

The major loss of cell viability due to freezing is attributed mainly to poor freezing or freeze-drying conditions that affect ice crystal formation, high osmotic stress due to high concentrations of internal solutes of cells, leading to membrane damage, and macromolecular denaturation and the removal of water, which affect the properties of many hydrophilic biological macromolecules (Thammavongs et al. 1996). Franks et al. (1991) showed that the activity of proteins was substantially reduced during freeze-drying, and was further reduced by subsequent storage unless the dried material was kept below −20 °C, and ideally at −80 °C. Such finding suggested that stability of protein materials may be achieved at conditions supporting vitrification of protein hydration water and associated low mobility of macromolecular components (Roos and Potes 2015).

Cryoprotectant molecules used in freezing and freeze-drying are often small sugars such as glucose and sucrose, while lactose may be also present depending on the fermentation media. Skim milk and/or combinations of sucrose, trehalose and dextran are most commonly used as cryoprotectants (Hubalek 2003). Sugars exhibit typical glass-forming properties of carbohydrates, and they have a significant impact on freezing, freeze-drying and storage characteristics. Such properties were reported for common sugars by Roos (1993), and are useful in the selection of cryoprotectant systems and freezing and freeze-drying conditions. Reconstituted skim milk solids are among the most useful suspension media for the freezing or freeze-drying of starter cultures due to their cryoprotective effects on cells (Abraham et al. 1990), and supplementing skim milk solids with different cryoprotectants may increase the cryoprotective effect (King and Lin 1995). A large number of compounds have been assessed for use as cryoprotectants, including skim milk solids, glycerol, mannitol, sorbitol, trehalose, sucrose, maltose, lactose, fructose, glucose, betaine, monosodium glutamate, honey, and amino acids and their salts.

A typical protectant should provide cryoprotection of probiotic bacteria cells during freezing and freeze-drying processes and ease of rehydration after long-term storage (Costa et al. 2000). Protectants present in a suspending medium have two main modes of action in preserving the viability of freeze-dried cells. Firstly, a glass-forming carbohydrate will provide mechanical support during storage. Secondly, the living cells are biochemically protected against damage during the drying process and subsequent storage (Berny and Hennebert 1991). Stabilization by small carbohydrate molecules has also been explained using the "water replacement hypothesis", which refers to their capacity to form hydrogen bonds

with components of biological structures in the absence of water (Crowe et al. 2001). The ability of skim milk to protect freeze-dried microorganisms is thought to be due to its capacity to stabilize the cell membrane constituents and to create a porous structure in the freeze-dried materials (Selmer-Olsen et al. 1999).

Interestingly, sugar composition within microbial cells cannot be assumed to be the same as that of a cryoprotectant medium. Sugar metabolism involves sugar uptake into the cell and breakdown into simpler products that are easily metabolized for energy. In lactic acid bacteria, there are two systems for the transport and metabolism of lactose. The two methods for transporting lactose into the cells are as follows (Walstra et al. 1999):

1. A phosphoenol pyruvate-dependent phosphotransferase system (PEP/PTS): As lactose is transported into the cell, it is transformed to lactose-P. Once inside the cell, phospho-β-galactosidase (P-β-gal) hydrolyzes the lactose-P to glucose and galactose-6-P. Glucose is further converted to glucose-P, and both phosphated sugars are further metabolized.
2. An ATP-dependent permease system: Lactose is transported into the cell as described above, and hydrolyzed by β-galactosidase or lactase into glucose and galactose. Glucose is converted to glucose 6-P. In galactose-fermenting bacteria, galactose is converted to glucose-6-P by the Leloir pathway.

Galactose is not metabolized if enzymes of the Leloir pathway are not present (i.e., *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*). Therefore, galactose present in such probiotic cells is either secreted or converted to (exo) polysaccharides (Walstra et al. 1999). Obviously, fermentation conditions and cell composition at the time of freezing may have an important effect on cell viability in both freezing and freeze-drying, as intracellular sugar composition may show large variations. Fonseca et al. (2000) found that the larger the surface area of the cell, the greater the membrane damage, owing to extracellular ice crystal formation during freezing. Water content as such is also an important factor affecting the viability of bacteria during freeze-drying and subsequent storage (Zayed and Roos 2004).

Crowe et al. (1984) reported that the role of trehalose in the stabilization of dry biological membranes was by hydrogen bonding to the polar head group of the phospholipid membrane. In addition to possible hydrogen bonding to cell membranes, trehalose and sucrose as glass formers play a role in protecting cell membranes from fusion and breakage (Crowe et al. 1984). Such carbohydrates also stabilize proteins against denaturation during freezing and freeze-drying (Crowe et al. 1988; Franks et al. 1991). Zayed and Roos (2004) demonstrated that the capacity of lactic acid bacteria (LAB) to metabolize trehalose did not seem to confer greater resistance following use as a protectant during the freeze-drying process. Pehkonen et al. (2008) also showed that maximum ice formation during pre-freezing to satisfy the condition $T_{ice} < T_m'$ was more important than the use of lactose, trehalose or their mixture as cryoprotectant for *Lactobacillus rhamnosus* GG. Post-freeze-drying conditions require maintenance of the glassy state of cryoprotectants and possible cell constituents which typically require storage in hermetic packages in the absence of water, at almost "zero" water content

and relative humidity of approximately 0 % (Pehkonen et al. 2008). Pehkonen et al. (2008) reported slightly improved stability for *L. rhamnosus* GG when freeze-dried with trehalose or lactose–trehalose compared to lactose. Such differences in storage stability with various cryoprotectants may depend primarily on the intracellular sugar composition, which may be strongly affected by cell activity in the cryoprotectant system under pre-freezing conditions (Pehkonen et al. 2008).

9.8 Intracellular Sugar Composition

Although there is limited knowledge regarding the intracellular sugar composition of living cells during fermentation and freezing, we analyzed sugar utilization by selected probiotic bacteria to evaluate the effects of cryoprotectants on their intracellular sugar composition for freezing and freeze-drying applications. *L. acidophilus* was able to ferment several sugars, while *L. casei* fermented lactose, trehalose, glucose and galactose. *L. bulgaricus* grew successfully in maltose, lactose and glucose. *Lactobacillus viridescens* showed positive results for maltose and glucose, whereas *Lactobacillus sakei* gave positive results for such sugars except lactose (Table 9.1).

We further studied the sugar composition of *Lactobacillus acidophilus* and *Lactobacillus casei* cells grown with skim milk and various sugars containing growth media. Probiotic cultures were washed and sonicated to remove extracellular sugars and release cell contents, respectively. Sonicated *L. acidophilus* cells showed little or no maltose uptake, and glucose concentrations remained low. These data showed that upon entering the cell, maltose was immediately hydrolyzed into two glucose moieties and utilized by *L. acidophilus*. A significant accumulation of maltose within cells was found for *L. casei*.

Galactose, glucose and lactose content in both *L. acidophilus* and *L. casei* varied depending on the length of fermentation, as shown in Fig. 9.6. Lactose was readily accumulated over time in the cells of both strains. Lactose uptake more than quadrupled from 5 min to 1 h after inoculation of *L. casei* into 15 % (w/w) lactose solution, and the intracellular lactose concentration reached 10 g/100 g of solids at

Table 9.1 Sugars fermented by *Lactobacillus* species

Strains	*L. acidophilus*	*L. casei*	*L. bulgaricus*	*L. sakei*	*L. viridescens*
Sugars					
Sucrose	+	−	−	+	−
Lactose	+	+	+	−	−
Maltose	+	−	+	+	+
Trehalose	+	+	−	+	−
Glucose	+	+	+	+	+
Galactose	+	+	−	+	−

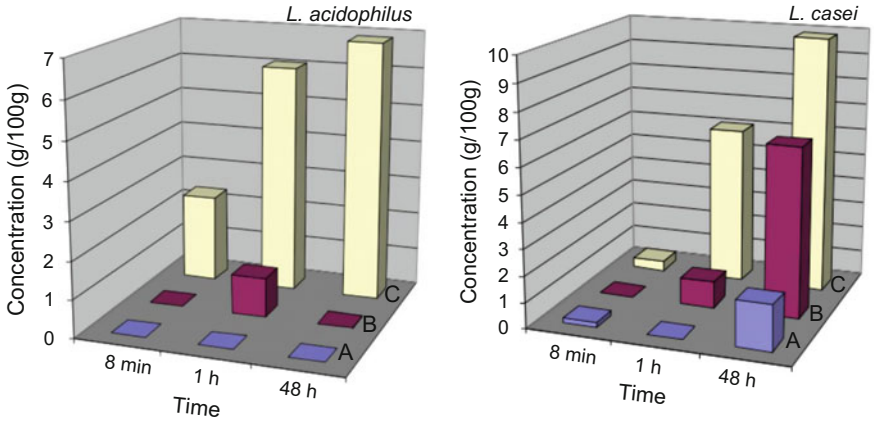

Fig. 9.6 Intracellular galactose (*A*), glucose (*B*) and lactose (*C*) content in *L. acidophilus* and *L. casei* cells during growth in 15 % (w/w) lactose solution

48 h. In addition to the high uptake of lactose, its hydrolysis to glucose and galactose in *L. casei* cells was confirmed by high concentrations of intracellular monosaccharides, especially glucose. The glucose concentration increased steadily with time to a little over 6/100 g of solids, and the galactose reached a concentration of 1.7/100 g of solids at 48 h (Fig. 9.6).

Lactose uptake by the *L. acidophilus* cells was similar to that of the *L. casei* cells, but the accumulated concentration was not as high, with a maximum concentration of only 6.8/100 g of solution accumulating after 48 h. The concentration of glucose within the *L. acidophilus* varied.

Our study showed greater disaccharide utilization by *L. acidophilus* than by *L. casei* while variations in intracellular composition implied significant differences in intracellular freezing and freeze-drying properties. Sonication of the cells at different time points showed that lactose was accumulated almost immediately by *L. acidophilus*, and that its concentration was proportional to the length of time of immersion in the media. However, *L. casei* showed uptake of a larger concentration of lactose after 48 h than *L. acidophilus*, and also showed a much greater increase in monosaccharide formation. The other disaccharides showed a similar pattern, with the exception of maltose. The maltose concentration within the cells was quite low and remained constant up to 48 h; however, accumulation within the *L. casei* cells after 7 days suggested that maltose uptake was directly proportional to utilization needs, and only accumulated over time in a saturated environment.

9.9 Conclusions

Freezing and freeze-drying are important technologies for the stabilization of bioactive materials used in the food industry as ingredients or by consumers in foods and supplements. The effects of freezing have been studied in biological materials, but little information is available on the properties of water and ice in complex biological materials at low temperatures. A better understanding of complex carbohydrate–protein–lipid systems in freezing and freeze-drying is needed in order to improve the stability of bioactive materials during freezing, thawing, drying and reconstitution.

References

Abraham A, De Antoni GL, Anon MC (1990) Microbiology of starter cultures. Cryobiology 27:336–342

Angell CA (2002) Liquid fragility and the glass transition in water and aqueous solutions. Chem Rev 102:2627–2650

Angell CA (2008) Insights into phases of liquid water from study of its unusual glass-forming properties. Science 319:582–587

Angell CA (2014) Supercooled water. Two phases? Nat Mater 13:673–675

Atkins P, De Paula J (2010) Atkin's physical chemistry, 9th edn, 972 p. Oxford University Press, Oxford

Berny JK, Hennebert GL (1991) Viability and stability of yeast cells and filamentous fungus spores during freeze-drying: effects of protectants and cooling rates. Mycologia 83:805–815

Buera MP, Roos YH, Levine H, Slade L, Corti HR, Auffret T, Angell CA (2011) State diagrams for improving processing and storage of foods, biological materials and pharmaceuticals. Pure Appl Chem 83:1567–1617

Carvalho AS, Silva J, Ho P, Teixeira P, Malcata FX, Gibbs P (2003) Protective effect of sorbitol and monosodium glutamate during storage of freeze-dried lactic acid bacteria. Lait 83:203–210

Corti HR, Angell CA, Auffret T, Levine H, Buera MP, Reid DS, Roos YH, Slade L (2010) Empirical and theoretical models of equilibrium and non-equilibrium transition temperatures of supplemented phase diagrams in aqueous systems. Pure Appl Chem 82(5):1065–1097

Costa E, Usall J, Teixido N, Garcia N, Vinas I (2000) Effect of protective agents, rehydration media and initial cell concentration on viability of *Pantoea agglomerans* strain CPA-2 subjected to freeze-drying. J Appl Microbiol 89:793–800

Crowe JH, Crowe LM, Chapman D (1984) Preservation of membranes in anhydrobiotic organisms: the role of trehalose. Science 223:701–703

Crowe JH, Crowe LM, Carpenter JK, Rudolph AS, Winstrom CA, Spargo BJ, Anchordoguy TJ (1988) Interactions of sugars and membranes. Biochim Biophys Acta 947:367–384

Crowe JH, Crowe LM, Oliver AE, Tsvetkova N, Wolkers W, Tablin F (2001) The trehalose myth revisited: introduction to a symposium on stabilization of cells in the dry state. Cryobiology 43:89–105

Doster W, Bachleitner A, Dunau R, Hiebl M, Lüscher E (1986) Thermal properties of water in myoglobin crystals and solutions at subzero temperatures. Biophys J 50:213–219

Fonseca F, Beal C, Corrieu G (2000) Method for quantifying the loss of acidification activity of lactic acid starters during freezing and frozen storage. J Dairy Res 67:83–90

Franks F, Asquith MH, Hammond CC, Skaer HB, Echlin P (1977) Polymeric cryoprotectants in the preservation of biological ultrastructure I. Low temperature states of aqueous solutions of hydrophilic polymers. J Microsc (Oxford) 110:223–238

Franks F, Hatley RHM, Mathias SF (1991) Material science and the production of shelf-stable biologicals. Biopharmaceuticals 4:38–42

Harnkarnsujarit N, Charoenrein S, Roos YH (2012) Microstructure formation of maltodextrin and sugar matrices in freeze-dried systems. Carbohydr Polym 88:734–742

Hubalek Z (2003) Protectants used in the cryopreservation of microorganisms. Cryobiology 46:205–229

Jansson H, Bergman R, Swenson J (2011) Role of solvent for the dynamics and the glass transition in proteins. J Phys Chem B 115:4099–4109

Jorgensen L, Hostrupb S, Horn Moeller E, Grohganz H (2009) Recent trends in stabilising peptides and proteins in pharmaceutical formulation—considerations in the choice of excipients. Expert Opin Drug Deliv 6:1219–1230

King VAE, Lin HJ (1995) Studies on the effect of protectants on *Lactobacillus acidophilus* strain dehydrated under controlled low-temperature vacuum dehydration and freeze-drying by using response surface methodology. J Sci Food Agric 68:191–196

Levine H, Slade L (1986) A polymer physico-chemical approach to the study of commercial starch hydrolysis products (SHPs). Carbohydr Polym 6:213–244

Luyet B, Rasmussen D (1968) Study by differential thermal analysis of the temperatures of instability of rapidly cooled solutions of glycerol, ethylene glycol, sucrose and glucose. Biodynamica 10(211):167–191

Pehkonen KS, Roos YH, Song M, Ross RP, Stanton C (2008) State transitions and physicochemical aspects of cryoprotection and stabilization in freeze-drying of *Lactobacillus rhamnosus* GG (LGG). J Appl Microbiol 104:1732–1743

Roos Y (1993) Melting and glass transitions of low molecular weight carbohydrates. Carbohydr Res 238:39–48

Roos YH, Drusch S (2015) Phase transitions in foods, 2nd edn 366 p. Academic Press, Inc., San Diego

Roos YH, Karel M (1991a) Applying state diagrams to food processing and development. Food Technol 45(66):68–71, 107

Roos YH, Karel M (1991b) Amorphous state and delayed ice formation in sucrose solutions. Int J Food Sci Technol 26:553–566

Roos YH, Potes N (2015) Quantification of protein hydration, glass transitions and structural relaxations of aqueous protein and carbohydrate-protein systems. J Phys Chem B 119:7077–7086

Selmer-Olsen E, Birkeland SE, Sorhaug T (1999) Effect of protective solutes on leakage from and survival of immobilized *Lactobacillus* subjected to drying, storage and rehydration. J Appl Microbiol 87:429–437

Smittle RB, Gilland SE, Speck ML, Walter Jr (1974) Relationship of cellular fatty acid composition to survival of *Lactobacillus bulgaricus* in liquid nitrogen. J Appl Microbiol 27:738–741

Swenson J, Cerveny S (2015) Dynamics of deeply supercooled interfacial water. J Phys: Condens Matter 27:033102

Thammavongs B, Corroler D, Panoff JM, Auffray Y, Boutibonnes P (1996) Physiological response of *Entercoccus faecalis* JH2-2 to cold shock: growth at low temperatures and freezing/thawing challenge. Lett Appl Microbiol 23:398–402

Walstra P, Geurts TJ, Noomen A, Jellema A, van Boekel MAJS (1999) Dairy technology: priniciples of milk properties and processes. Marcel Dekker Inc., New York, pp 325–362

Zayed G, Roos YH (2004) Influence of trehalose and moisture content on survival of *Lactobacillus salivarius* subjected to freeze-drying and storage. Process Biochem 39:1081–1086