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Abstract

Freeze-drying is widely employed in food processing to minimize quality changes that occur during drying and to extend the shelf life of food. However, proteins in food systems are susceptible to denaturation and oxidative modification even under the low-temperature conditions of this process. During the freezing stage, proteins can be destabilized through cold denaturation, freeze-concentration of solutes, and adsorption at ice–water interfaces. In the subsequent drying stage, removal of the hydration shell disrupts hydrogen bonding and hydrophobic interactions that maintain the native protein structure, promoting unfolding, secondary and tertiary structural rearrangements, and aggregation. Protein oxidation may proceed during freezing and drying, as well as after drying when the porous dried product is exposed to air, and oxidation and denaturation can promote each other. These changes can lead to losses in processing functionality, bioactivity, and enzymatic activity. To mitigate denaturation of protein during freeze-drying, lyoprotectants can be employed, which act through four proposed mechanisms: preferential exclusion, hydrogen bonding (water replacement), vitrification, and hydrophobic interactions. While lyoprotectants have been extensively studied in pharmaceutical protein formulations, studies on their application to animal-derived food proteins remain limited. This review summarizes the current understanding of the mechanisms underlying protein denaturation and oxidation during the freeze-drying of animal-derived foods and discusses lyoprotectant-mediated stabilization strategies.

Keywords: Freeze-drying, Lyophilization, Lyoprotectant, Protein denaturation, Glass transition temperature, Hydration shell

Introduction

Freeze-drying is a dehydration method in which water is removed primarily by sublimation, transitioning directly from a solid (ice) phase to the vapor phase, followed by the desorption of unfrozen, bound moisture [1]. The synonym lyophilization originates from the ancient Greek word “*lyophil*”, meaning “solvent-loving”, which reflects the dried product's ability to rapidly reabsorb solvents and regain its original state upon rehydration [2]. Because freeze-drying is performed at low temperatures, it minimizes thermal deteriorations, thereby preserving nutrients, bioactive compounds, and overall bioavailability more effectively than conventional heat-based drying methods [3]. Due to

these advantages, freeze-drying has been widely adopted for the stable drying of pharmaceutical formulations, food products, and biotechnological materials.

The freeze-drying process consists of three main stages: freezing, primary drying, and secondary drying. During the freezing stage, the free water within the food matrix crystallizes into ice, creating the solid phase necessary for subsequent sublimation [1]. Primary drying involves the removal of ice through sublimation under pressures below the triple point of water, the condition at which solid, liquid, and vapor phases can coexist in thermodynamic equilibrium [4]. Approximately 90% of the total water is typically removed during this sublimation-driven stage [5]. In the secondary drying, the remaining unfrozen, bound water is removed through desorption, reducing the final moisture content to levels that ensure long-term product stability [6].

Protein is a major nutrient and a critical determinant of quality properties in various foods. Because proteins are highly heat-sensitive, conventional thermal drying methods often lead to denaturation, aggregation, and oxidative modification. In contrast, various studies have demonstrated that freeze-drying better preserves protein conformation and functionality. Liu et al. [7] compared hot-air drying, heat-pump drying, and vacuum freeze-drying of scallop adductor muscles and reported that freeze-drying caused the least alteration in secondary structure of protein. Similarly, Wang et al. [8] investigated Clanis Bilineata Tingtauica Mell protein subjected to heat drying, vacuum drying, and freeze-drying. Freeze-dried samples exhibited the lowest surface hydrophobicity, indicating the inhibition of protein unfolding and better preservation of tertiary structure. Furthermore, freeze-drying resulted in the lowest level of disulfide bond formation, which is a marker of protein oxidation, suggesting that freeze-drying minimized oxidative cross-linking compared to other drying techniques.

However, even when freeze-drying is employed, structural and chemical alterations of proteins in food systems cannot be fully prevented. Studies reporting protein denaturation and oxidation after freeze-drying are summarized in Table 1, together with the pretreatment, processing conditions, comparator, analytical method, and key limitations of each study. Because most of these studies compared freeze-dried samples with raw or non-dried samples rather than with frozen–thawed samples, the effects of freezing, drying, and subsequent storage cannot be separated in most cases. Moreover, such studies remain limited in number, and most of them report changes in quality attributes without investigating the underlying mechanisms.

Protein destabilization can occur in both the freezing and drying stages of freeze-drying [21]. During freezing, proteins may denature as a result of solute concentration in the unfrozen phase, pH shifts, adsorption at ice–water interfaces, and cold denaturation, whereas during drying, the progressive removal of the hydration shell disrupts stabilizing interactions, leading to protein unfolding and aggregation [21]. Previous reviews have addressed these mechanisms in the lyophilization of pharmaceutical proteins [21] or in the freezing-induced denaturation and oxidation of proteins in muscle foods [22, 23], but not in the drying stage of freeze-drying in animal-derived foods. Therefore, this review integrates the mechanisms of protein denaturation and oxidation during both the freezing and drying stages with the available evidence from animal-derived foods, including meat, fish, egg, dairy, and insect products. In doing so, evidence obtained from intact foods, extracted food proteins, and pharmaceutical or model protein systems is distinguished, and the applicability of the latter to animal-derived food matrices is noted where relevant. In addition, lyoprotectant-based stabilization strategies and their practical constraints in food applications are discussed.

Freezing-induced denaturation

Freezing imposes physicochemical stresses on proteins, and consequently, structural perturbation in proteins can occur even before the drying stage begins [22]. As ice crystals form, the remaining unfrozen solution becomes increasingly concentrated, leading to changes in local solute composition, ionic strength, and pH. In addition, proteins can be displaced toward and adsorbed at ice-water interfaces, where the high interfacial energy promotes partial unfolding [24]. At sufficiently low temperature, proteins undergo cold denaturation. Although each mechanism operates through distinct pathways, their combined effects can compromise protein stability [25]. Therefore, the mechanistic understanding of these freezing-induced stresses is essential for interpreting protein behavior during freeze-drying and for designing strategies to preserve protein stability.

Freezing concentration

During the freezing of food, as ice crystals begin to form, a fraction of the water remains unfrozen, leading to the concentration of ionic solutes in the unfrozen phase [26, 27]. Protons are likewise excluded from the growing ice lattice and become concentrated in the unfrozen aqueous phase. In meat, where more than 90% of water freezes below −20 °C, a simple calculation based on the unfrozen-water fraction alone suggests an approximately tenfold increase in proton concentration [28]. However, this estimate assumes that protons are completely excluded into the unfrozen fraction and neglects buffering, changes in solute activity, temperature-dependent shifts in buffer equilibria, and salt

crystallization. Therefore, in buffered and heterogeneous meat systems, the actual pH change cannot be calculated from the unfrozen-water fraction alone. Indeed, the magnitude and even the direction of pH shifts during freezing depend on buffer composition. Kolhe et al. [29] reported that the pH of 50 mM sodium phosphate buffer decreased from 7.0 to approximately 3.4 at −30 °C owing to the crystallization of disodium phosphate, whereas histidine, acetate, citrate, and succinate buffers showed increases of less than 1 pH unit and Tris buffer an increase of approximately 1.2 units. In meat, endogenous buffering components, such as proteins and phosphates, may further modify these changes, and direct measurements of pH in the unfrozen phase of frozen meat remain limited.

Changes in pH and increased ionic strength in the unfrozen phase can act as stress factors that destabilize protein structure and promote unfolding [22]. In addition, increased ionic strength promotes charge shielding, which can facilitate self-interaction between protein molecules [26]. Zhang and Ertbjerg [28] simulated these conditions in vitro by exposing myofibrils extracted from pork to increased ionic strength and decreased pH, and observed increased surface hydrophobicity, enhanced intermolecular interactions, and greater aggregation. Although these conditions were not measured directly within frozen meat, the results suggest that freeze-concentration can contribute to protein denaturation during freezing.

Ice-water interface adsorption

An interface between ice and water is created when ice crystals are formed. At temperatures below the equilibrium melting temperature, the ice-water interface undergoes surface premelting, forming a quasi-liquid layer (QLL) characterized by liquid-like disorder. This QLL represents an interfacial state distinct from both bulk ice and bulk liquid water [30, 31]. When proteins adsorb to this layer, their native structure becomes loosened, promoting unfolding [32-34]. There are several hypotheses regarding the mechanisms that cause protein denaturation. One proposed mechanism suggests that protein denaturation arises from localized pH reduction and increased concentration within QLL, driven in part by the negatively charged surface of ice crystals, which promotes proton and cation enrichment at the ice-water interface [35]. As a result, hydrophobic amino acids such as tyrosine and tryptophan become exposed to the exterior [27], and this exposure enhances hydrophobic interactions, ultimately leading to protein aggregation. The study by Chen et al. [27] demonstrated that during the freezing of pork myofibrillar proteins, denaturation can occur as a result of protein adsorption to the ice–water interface. Additionally, Regand & Goff [36] visually confirmed through light microscopy that during freezing, milk proteins surround the ice interface, indicating direct interaction between the proteins and the ice surface.

Cold denaturation

Cold denaturation refers to denaturation that occurs not due to the formation of ice crystals during freezing, but rather as a result of the temperature drop itself [25]. While temperature-induced denaturation is often associated with high temperatures, cold denaturation can also occur at low temperatures in the opposite manner. The stability of a given protein typically shows a maximum at an intermediate temperature and decreases at both higher and lower temperatures. For example, a protein that is most stable at around 17 °C may unfold at approximately 60 °C upon heating and at approximately −20 °C upon cooling [37, 38]. However, these temperatures may vary depending on the protein and on conditions such as pH, solvent composition, and pressure, and thus may not apply to all proteins. Moreover, in food systems, cold denaturation is difficult to observe directly because it generally occurs at temperatures below the freezing point of water, where it overlaps with ice formation, freeze-concentration, and interfacial stresses. Therefore, cold denaturation may contribute to the structural changes of proteins observed after freezing, together with these ice-related stresses, but its individual contribution is difficult to distinguish experimentally.

Food proteins generally exist in water-rich environments, and water is a key factor governing both the structural stability and denaturation of proteins. The folding (F) and unfolding (U) of a protein are determined by the sum of the interactions within the protein itself and those occurring between the protein and the surrounding water molecules [39]. From a thermodynamic standpoint, the Gibbs free-energy difference between the folded (F) and unfolded (U) states of a protein is expressed as $\Delta G_F = G_F - G_U = \Delta H_F - T\Delta S_F$, and this value determines whether the protein can maintain its folded structure. When $\Delta G_F > 0$, the unfolded state is more favored, whereas when $\Delta G_F < 0$, the folded state is more favored. Among the various interactions involved in protein folding, hydrophobic interactions resulting from the entropy-driven hydrophobic effect of surrounding water are the most significant [40]. The nonpolar side chains of proteins form clusters to avoid unfavorable interactions with water molecules, thereby forming a nonpolar core [22, 41]. From a thermodynamic perspective, as a protein folds and its solvent-excluded volume decreases, the entropic freedom of surrounding water molecules increases [41]. This increase in water entropy lowers the overall free energy (ΔG), thereby playing a crucial role in stabilizing the native state of the protein [42, 43].

As liquid water is cooled, including in the supercooled or unfrozen state, its hydrogen-bond network becomes more ordered, and the hydration of exposed nonpolar groups becomes less unfavorable [44]. Consequently, the hydrophobic effect weakens at low temperatures, and exposure of nonpolar surfaces to water becomes thermodynamically less costly [23, 42, 44]. Because unfolding is accompanied by a large positive heat-capacity change (ΔCp), both the

enthalpy and entropy of unfolding decrease as the temperature decreases. Consequently, at sufficiently low temperatures, the folded state is no longer favored ($\Delta G_F > 0$), resulting in cold denaturation [37, 42]. As a result, unfolding occurs in the tertiary structure that was stabilized by hydrophobic interactions, and the formation of hydrogen bonds with water leads to a reduction in intramolecular hydrogen bonding within the protein backbone. This can cause the loss of α-helical structures, which are part of the protein's secondary structure [22]. Experimental evidence for cold denaturation of food proteins remains limited. Helmick et al. [45] reported that low-temperature conditions altered the secondary structure of pea vicilin and increased its particle size through protein–protein aggregation. However, this study was conducted on an isolated plant protein in solution, and whether comparable cold denaturation occurs in animal-derived food matrices, in which it cannot be readily separated from ice formation and freeze-concentration, has not yet been established.

Drying-induced denaturation

Water is essential for maintaining the functional folding of proteins. As explained earlier in the context of cold denaturation, the presence of water around the protein promotes the hydrophobic effect, whereby hydrophobic residues are driven into the protein interior. This effect helps preserve the folded state of the protein. In contrast, the hydrophilic amino acid residues of a protein are typically located on the exterior, where they form hydrogen bonds with water, contributing to proper folding and structural stability [46]. The water molecules that form hydrogen bonds with the protein surface are referred to as the hydration shell. This hydration shell shields electrostatic interactions and helps maintain sufficient distance between protein molecules, thereby preventing aggregation [47]. During freeze-drying, ice sublimates first, and eventually, the hydration shell surrounding the protein molecules is removed. The loss of this water, which had maintained the protein’s stable state, leads to the collapse of the native structure, denaturation, and aggregation of the protein [21, 48].

Removal of hydration shell

During the freezing process, the hydrophobic effect arising from water molecules surrounding the protein is weakened, as described above. Subsequently, progression to the drying stage removes water from the protein hydration shell, further diminishing the hydrophobic effect. As a consequence, hydrophobic interactions that stabilize the tertiary structure collapse, leading to protein unfolding [49, 50]. In aqueous systems, protein molecules typically interact only

at limited contact points, with most of the intervening space occupied by liquid water [51]. However, removal of water through freeze-drying results in the formation of voids between protein molecules, creating protein–air interfaces that generate free energy and drive structural rearrangements [51, 52]. To minimize this energetically unfavorable interface, protein molecules in the dry state tend to come into closer contact with each other [51]. In addition, proteins in the dehydrated state may donate protons to ionized carboxyl groups within their structure, thereby reducing overall charge density [21], which can further promote protein unfolding. Consistent with these mechanisms, Niu et al. [20] reported that freeze-drying of silver carp surimi increased the surface hydrophobicity of myofibrillar proteins from 51.3 to 226.2 and decreased their Ca^{2+}-ATPase activity from 23.5 to 9.0, indicating partial denaturation of myosin.

At the molecular level, the structural consequences of dehydration can be further understood by considering hydrogen bonding between amino acid residues and water molecules. Amino acid residues can act as both hydrogen bond donors and acceptors, with donor groups including amine groups (NH, NH_2, NH_3^+), hydroxyl groups (OH), and to a lesser extent thiol groups (SH), while acceptor groups include carbonyl groups (C=O), carboxylate groups (COO^-), and hydroxyl groups (OH) [53]. Upon water removal, protein-water hydrogen bonds are disrupted, promoting intermolecular hydrogen bonding between peptide chains. While the loss of hydrophobic interactions primarily destabilizes the tertiary structure, reorganization of hydrogen bonding upon dehydration governs changes in secondary structure and conformational rigidity. This transition is commonly associated with a decrease in α-helical structures and an increase in intermolecular β-sheet formation and disordered conformations [47, 54]. These intermolecular hydrogen bonds lock the protein structure, resulting in a decrease in its flexibility [49]. Such changes in secondary structure have also been reported in freeze-dried food proteins. In egg white protein hydrolysate, the freeze-dried powder contained less α-helix than the vacuum-dried powder (10.8 vs. 17.7%) [10], and in silver carp myofibrillar proteins, freeze-drying decreased the α-helix content from 36.8 to 30.4% while increasing the β-sheet content from 11.0 to 44.3% [20].

These dehydration-induced structural alterations expose hydrophobic residues on the protein surface, which in turn facilitates aggregation through hydrophobic interactions between exposed hydrophobic residues [21, 49]. Additionally, the removal of hydrogen bonds previously formed between amino acids and water leaves charged groups, either positively or negatively charged, unneutralized. The interaction between these oppositely charged groups can further promote protein aggregation [47, 52]. In the study by Han et al. [9], myosin from freeze-dried meat batter showed lower intrinsic tryptophan fluorescence intensity and a blue-shifted emission maximum compared with that from

frozen–thawed batter, indicating unfolding during drying. In addition, the freeze-dried batter showed a lower absolute zeta potential and a larger particle size than raw batter, indicating protein aggregation [9]. The extent of these drying-induced changes depended on the preceding freezing conditions. Freezing at −70 °C produced smaller pores than freezing at −20 °C, and after freeze-drying, myosin from the batter frozen at −70 °C showed higher surface hydrophobicity and a greater blue shift of tryptophan fluorescence, whereas no such differences were observed between the corresponding frozen–thawed batters [9]. The authors attributed this to the larger ice–solute and air–water interfacial areas formed by small ice crystals, at which proteins can adsorb and unfold during drying, as well as to the tendency toward a slower drying rate at −70 °C [9].

Dehydration-induced aggregation is also reflected in reduced protein solubility. Myofibrillar protein solubility was lower in freeze-dried pork than in frozen–thawed pork (65.3 vs. 72.8 mg/g) [17], and protein solubility of mealworm larvae decreased from 53.2 to 40.7% after freeze-drying, although it remained higher than after heat-based drying [19]. In contrast, freeze-drying did not reduce the solubility of whey protein concentrate [12], indicating that the extent of dehydration-induced aggregation differs among food protein systems.

Oxidation

Protein oxidation is one of the factors that can cause structural changes. A schematic illustration of oxidation occurring during freeze-drying is presented in Figure 1. Protein oxidation is initiated mainly by reactive oxygen species and radicals rather than by molecular oxygen itself, and in muscle foods these species are generated through transition metals such as iron via the Fenton reaction, heme proteins such as myoglobin, and lipid peroxidation [55, 56]. Protein oxidation primarily targets sulfur-containing amino acids (cysteine and methionine) and aromatic amino acids (tyrosine, phenylalanine, tryptophan, and histidine), and the oxidation of these residues increases structural instability in proteins [57]. The sulfur-containing amino acid, cysteine, is oxidized to form unstable intermediates such as cysteine thiyl radicals and sulfenic acid, and sulfenic acid can react with another thiol to form a disulfide bond [58]. Another sulfur-containing amino acid, methionine, is oxidized to methionine sulfoxide and can be further oxidized to methionine sulfone [59, 60]. Oxidation of methionine to methionine sulfoxide eliminates the hydrophobic interactions between its sulfur atom and the aromatic rings of tryptophan, phenylalanine, and tyrosine, which can perturb the three-dimensional folding of the protein. These perturbations may expose normally buried residues, so that surface hydrophobicity increases as a secondary consequence of methionine oxidation [61]. Among the aromatic amino acids, tryptophan is oxidized to form N-formylkynurenine [62]. The oxidation of tryptophan likewise increases the polarity

of this hydrophobic residue [63]. Another aromatic amino acid, tyrosine, forms tyrosyl radicals upon oxidation [64]. Two tyrosyl radicals can couple to form 3,3′-dityrosine, in which both phenolic hydroxyl groups are retained, leading to protein–protein cross-linking [65]. Protein carbonyls, the most commonly used marker of protein oxidation in freeze-dried foods, are formed mainly through the metal-catalyzed oxidation of lysine, arginine, and proline side chains, yielding α-aminoadipic semialdehyde from lysine and γ-glutamic semialdehyde from arginine and proline, which together account for approximately 70% of the total carbonyls in oxidized animal proteins [55, 56]. In addition, carbonyl groups can be introduced into proteins indirectly when reactive carbonyl compounds derived from lipid peroxidation, mainly α,β-unsaturated aldehydes, covalently bind to amino acid side chains [56]. These oxidative modifications lead to the formation of disulfide bonds, the loss of hydrophobic interactions, the formation of carbonyl groups, and protein–protein cross-linking, which can destabilize the secondary and tertiary structures of proteins and lead to their denaturation.

In the context of freeze-drying, such oxidative processes can be further exacerbated by structural changes induced during the process, and oxidation may proceed at several stages. During freezing, dissolved oxygen and prooxidants such as free iron and myoglobin become concentrated in the unfrozen phase, which may accelerate oxidation reactions [9]. During drying, although most of the oxygen is removed under vacuum, residual oxygen may remain within the matrix, and the removal of water itself can promote oxidation. Labrude et al. [66] reported that freeze-drying converted approximately 50% of oxyhemoglobin into methemoglobin, whereas freeze–thawing alone caused little oxidation. In their study, hemoglobin desiccated under vacuum was more oxidized than hemoglobin that remained frozen (39.2% vs. 14.1%), and comparable oxidation occurred when hemoglobin was dried in air (42.6%), indicating that the loss of hydration water, rather than the vacuum itself, was responsible for the oxidation of heme iron. Because metmyoglobin can be activated by H_2O_2 into hypervalent species that initiate protein oxidation [55], such dehydration-induced oxidation of heme proteins may also be relevant to freeze-dried muscle foods, although this requires further investigation. After drying, the product is exposed to air during unloading, storage, and rehydration. Because the removal of ice crystals leaves pores within the matrix, oxygen can then more readily reach its inner regions, which may make the protein more susceptible to oxidation [67]. Messina et al. [68] suggested that, in freeze-dried bovine muscle, the larger number of smaller pores formed at lower freezing temperatures increased the surface area exposed to oxygen and possibly enhanced oxygen penetration, which was associated with greater color changes, including a decrease in redness attributed to metmyoglobin formation. In addition, the residual moisture of the dried product may influence oxidation during storage. Messina et al. [68] also suggested that the low water activity of freeze-dried bovine

muscle (0.18–0.23) may accelerate oxidation, which is consistent with the dehydration-induced oxidation of heme iron reported by Labrude et al. [66]. Oxidation and denaturation can be mutually reinforcing. Protein oxidation can induce structural denaturation, and proteins that have partially unfolded during drying may in turn become more susceptible to oxidation. However, conformational changes do not necessarily result from or lead to oxidation.

Experimental studies have reported increased protein oxidation after freeze-drying. Lee et al. [17] found that freeze-dried pork exhibited a higher protein carbonyl content than frozen–thawed pork, and Han et al. [9] also observed a higher carbonyl content in freeze-dried meat batter than in frozen–thawed meat batter. Increased protein oxidation after freeze-drying has also been reported in other food matrices. Bordignon et al. [16] reported that freeze-drying of whey protein isolate dispersions doubled the protein carbonyl content relative to the untreated powder (4.24 vs. 2.09 nmol/mg protein), and Kröncke et al. [19] found that freeze-dried mealworm larvae contained more 4-HNE–protein adducts than fresh larvae (111.0 vs. 39.5 μg/mL). The latter finding suggests that lipid-derived reactive aldehydes can also modify proteins during or after freeze-drying. Lipid oxidation has likewise been observed after freeze-drying. In the same study, Han et al. [9] reported higher TBARS values in freeze-dried meat batter than in frozen–thawed meat batter, and Lenaerts et al. [18] found that the peroxide value of freeze-dried mealworm larvae increased to 125.1 meq O_2/kg fat, whereas it was below the detection limit in fresh larvae, together with a higher p-anisidine value (3.46 vs. 1.89). These indices mainly reflect lipid oxidation, and their relationship with protein oxidation requires further investigation. Taken together, these studies on meat, dairy, and insect products consistently indicate that freeze-drying is accompanied by increased oxidation. However, in the studies on whey protein and mealworms, untreated or fresh samples were used as controls, so the effects of freezing and drying could not be distinguished. Moreover, the specific stage at which oxidation occurs, such as drying, unloading, storage, or rehydration, and the contribution of oxygen diffusion through the porous structure remain to be clarified.

Glass transition–induced structural collapse

During freeze-drying, ice located closest to the external surface sublimates first. As a result, a clear boundary is established between regions where ice has been removed and regions in which ice crystals remain [69]. Ice crystals within the interior subsequently sublimate and migrate outward through the pores formed by ice removal. During primary drying, the product temperature should be maintained 2–3 °C below the allowable limit. For amorphous

matrices, this maximum allowable temperature corresponds to the glass transition temperature of the maximally freeze-concentrated solution (T'_g) or the collapse temperature (T_c) [70]. Below this temperature, the viscosity reaches approximately 10^{12} Pa·s or higher [71]. In many formulations, T_c is located within 1–2 °C of T'_g [70]. However, the two parameters are conceptually distinct: T'_g is the glass transition temperature of the maximally freeze-concentrated amorphous phase, whereas T_c is an operational collapse temperature under the relevant drying conditions [72, 73]. Moreover, depending on sample composition, T_c can be up to approximately 20 °C higher than T'_g, and the difference between T'_g and T_c may increase with increasing protein concentration [70, 74]. During freezing, a concentrated amorphous solution (CAS) is formed as a matrix between ice crystals. Although the CAS still contains water, this matrix exhibits solid-like behavior due to its extremely high viscosity at temperatures below T'_g or T_c [69]. However, when primary drying is conducted at temperatures above T_c (or T'_g), the viscosity of the CAS decreases and the matrix becomes fluid-like. As a result, surface tension within the wetted CAS matrix induces cake (pore) collapse, pore coalescence, and the formation of holes within the cake structure, particularly in regions adjacent to the ice–vapor interface [69, 73]. When the viscosity of the unfrozen solute phase decreases to below approximately 10^7 Pa·s, viscous flow can occur in the dried pore structure near the sublimation front, which still contains a high moisture content. This phenomenon can lead to structural collapse during freeze-drying [73, 75]. When such structural deformation occurs, drying efficiency decreases and nonuniform drying is promoted [69, 76]. In addition, the final product may exhibit a reduction in internal specific surface area, an increase in residual moisture content, decreased solubility, reduced protein stability, loss of textural integrity, and loss of volatile compounds [69, 73, 77]. Therefore, accurate determination of T'_g (or T_c) is critical, and maintaining the product temperature 2–3 °C below this threshold is considered a stable operating condition during freeze-drying. Although celery is not a protein-based food, Karathanos et al. [78] investigated changes in viscosity as a function of initial freezing temperature and the extent of final product collapse during freeze-drying of celery. Their results showed that viscosity decreased at temperatures above the glass transition temperature (−45 °C), and no further decrease in viscosity was observed above −28 °C. The extent of collapse was quantified by calculating the ratio of the specific bulk volume of samples frozen and freeze-dried at higher temperatures (V_{bs}) to the specific bulk volume of samples frozen at −55 °C and subsequently freeze-dried (V0). Experimentally, celery samples frozen at −28 °C prior to freeze-drying exhibited significantly lower V_{bs}/V_0 values compared with samples frozen at lower temperatures, indicating a greater degree of structural collapse. These findings demonstrate that conducting freeze-drying at temperatures above T_c (or T'_g) promotes matrix fluidization, which leads to structural collapse during the freeze-drying process. In animal-derived foods, however, information on glass

transition behavior, associated physical changes, and the direct relationship between T'_g and freeze-drying remains very limited. In addition, data describing volume changes and collapse induced by process conditions during freeze-drying are scarce [76]. Therefore, systematic studies on freeze-drying and glass transition phenomena in protein-based food materials are required. During secondary drying, T_g of the amorphous phase increases sharply as the moisture content decreases, which makes structural collapse unlikely [79]. Nevertheless, if the shelf temperature is increased for secondary drying before all residual ice has been removed, collapse or eutectic melting may still occur during this stage [73, 77]. Accordingly, accurate determination of the end point of primary drying, defined by a critical residual moisture content, is essential. Patel et al. [77] demonstrated that using comparative pressure measurement with a Pirani gauge to identify the completion of primary drying can effectively reduce such structural deformations.

Loss of protein functional properties

The oxidation and structural denaturation discussed above can lead to a decline in the functional and physiological properties of proteins. In food proteins, when unfolding occurs and hydrophobic residues become exposed, leading to aggregation, processing functionalities such as solubility, gelling capacity, emulsifying capacity, and foaming capacity may decrease, depending on the extent of unfolding and aggregation. However, partial unfolding or controlled aggregation may also maintain or even improve emulsifying, foaming, or gelling properties, so the consequences depend on the target property. In bioactive proteins, the biological activity that depends on the native structure may be reduced, and in the case of enzymes, structural changes in the active site can reduce catalytic activity.

The following studies serve as examples of such functional losses. In the Antarctic krill protein study by Lin et al. [14], freeze-dried samples showed inferior functional properties, such as solubility, emulsifying capacity, and foaming capacity, compared with spray-dried samples, suggesting that structural changes during freeze-drying can reduce functionality. Haas et al. [12] reported that freeze-drying of whey protein concentrate decreased the concentrations of lactoferrin and immunoglobulin G, indicating that their native structure was partially altered during freeze-drying. Notably, under their processing conditions, freeze-drying did not retain these heat-labile proteins better than spray-drying, despite its considerably lower processing temperature. Although this reduction in immunoreactivity does not by itself establish a loss of biological activity, it shows that structural modification of bioactive proteins can occur even under freeze-drying conditions. Finally, Leite et al. [15] reported that freeze-drying ruminant milk, particularly ovine milk, reduced elastase activity, which was attributed to structural damage to the proteins. Although freeze-drying is a method that can reduce protein denaturation compared with other drying techniques, it can still induce

protein oxidation and secondary and tertiary structural denaturation through processes such as freeze-concentration, interfacial adsorption, and removal of the hydration shell, which, depending on the processing conditions and the target property, can reduce processing functionality, bioactivity, and enzymatic activity. Therefore, when processing food proteins by freeze-drying, it is necessary to optimize process conditions, such as the freeze-drying process and the addition of lyoprotectants, taking these denaturation mechanisms into account.

Lyoprotectant

To minimize protein denaturation during the freeze-drying process, the use of lyoprotectants is recommended [48]. A schematic illustration of the mechanisms of lyoprotectants is presented in Figure 2. When lyoprotectants are used, their protective effect is primarily attributed to their ability to form hydrogen bonds between the protein and the protectant, thereby stabilizing and preserving protein structure during drying [80]. In addition, various mechanisms such as preferential exclusion, vitrification, and hydrophobic interactions further contribute to the stability of proteins during the freeze-drying process [21]. In the pharmaceutical field, commonly used lyoprotectants include sugars, polyols, surfactants, and amino acids [48]. The lyoprotectants utilized in pharmaceutical formulations are summarized in Table S1. Typically, nonreducing sugars such as sucrose and trehalose, widely employed as cryoprotectants in food systems, are also among the most frequently used lyoprotectants in protein formulations [81, 82]. However, for protein-based foods, only a limited number of studies have reported the use of lyoprotectants during freeze-drying. Because the type and concentration of lyoprotectant required for effective protection vary depending on the protein, it is necessary to experimentally identify the most suitable lyoprotectant for each protein-based food [21]. To determine the appropriate lyoprotectant, it is first essential to understand the underlying mechanisms by which the protectant stabilizes the protein. There are various perspectives regarding the mechanisms involved, and different lyoprotectants may exhibit distinct stabilization mechanisms. The following section introduces the mechanisms that have been elucidated to date.

Preferential exclusion

Preferential exclusion or preferential interaction refers to whether a protein preferentially interacts with water or with the added protectant when a protectant is present during freezing. When sugars, amino acids, or polyols are used as excipients, they are preferentially excluded from the vicinity of the protein, resulting in preferential hydration of

the protein [21, 49]. During this process, a greater number of water molecules and fewer additive molecules are present on the protein surface compared to the bulk composition [21]. The protein is surrounded by a hydration layer, with the additives existing beyond this layer. Because the excipients are preferentially excluded from the protein surface, they do not directly bind to or shield the protein. Instead, their exclusion makes the exposure of additional protein surface, as occurs upon unfolding, thermodynamically unfavorable [21, 48]. For a protein to unfold, it must exclude the additive from a larger surface area, which increases the free energy of unfolding required for the transition [83, 84]. This increase in the free energy required for unfolding stabilizes the native structure of the protein [21]. As a result of the preferential exclusion effect, protein unfolding caused by freezing-induced stresses can be reduced. However, this mechanism operates in solution and during freezing, while the unfrozen phase remains liquid. It does not account for protein stabilization in the dried state, which is instead explained by the water replacement and vitrification mechanisms described below.

Hydrogen bonding

The water replacement theory proposes that lyoprotectants help preserve the native structure of proteins during drying by forming hydrogen bonds with the protein in place of water molecules [21, 85]. Lyoprotectants capable of forming hydrogen bonds with proteins, such as sugars, polyols, and amino acids, are representative examples that function through this mechanism. As described in the previous section (Removal of hydration shell), the removal of hydrogen bonds between the polar groups of the protein surface and water during freeze-drying causes denaturation of the secondary and tertiary structures. When a lyoprotectant is used during the freeze-drying process, the excipient forms hydrogen bonds with the polar groups on the protein surface, thereby replacing the water molecules [86]. In the case of disaccharides, hydroxyl groups (–OH) are primarily involved in hydrogen bond formation [87]. These interactions mimic the hydrogen-bonding network originally formed between the protein and water [48]. Through this process, the formation of intra- or intermolecular hydrogen bonds among protein molecules during drying is inhibited, thereby enhancing the structural stability of the protein [21]. Providing spectroscopic evidence for the water replacement mechanism, Pieters et al. [88] monitored the amide A/II band frequency by near-infrared spectroscopy (NIRS) during freeze-drying of IgG formulations containing sucrose, and found that the amide A/II frequency remained unchanged despite continued water removal during secondary drying. This indicates that nearly all external amide groups of the protein had formed hydrogen bonds with sucrose molecules, suggesting that sucrose effectively replaced water molecules bound to the protein backbone through direct hydrogen bonding. Corroborating these

findings, Allison et al. [6] compared the apparent moisture content predicted from carboxylate band absorbance with the actual moisture content measured by Karl Fischer titration in freeze-dried lysozyme formulations containing sucrose or trehalose, and demonstrated that the carboxylate band at 1580 cm^{-1} was maintained in the dried state despite residual moisture levels below 4%. This indicates that disaccharides directly replaced water molecules by forming hydrogen bonds with the protein's carboxylate groups, rather than simply trapping water around the protein.

In general, carbohydrates with smaller molecular weights are known to have lower steric hindrance, allowing them to form closer hydrogen bonds with proteins [85]. This was further supported by Pieters et al. [88], who demonstrated that PVP, a high-molecular-weight polymer, failed to decrease the amide A/II frequency during freeze-drying of IgG formulations, whereas sucrose, a low-molecular-weight disaccharide, effectively formed hydrogen bonds with the protein backbone, suggesting that molecular size and the associated steric hindrance are critical determinants of hydrogen bond formation between excipients and proteins. The close contact formed between the excipient and the protein through hydrogen bonding also reduces the local mobility of reactive regions within the protein [87, 89]. In a related observation, Choi et al. [90] demonstrated through molecular dynamics simulations of 13 disaccharides that trehalose formed an average of 2.8 long-lived hydrogen bonds per MD step with water molecules, which was significantly higher than the average of 2.1 for the other sugars, attributing this to the conformational rigidity and anisotropic hydration unique to the α,α-(1→1) glycosidic linkage of trehalose. Because this study examined trehalose–water rather than trehalose–protein interactions, it indicates the strong hydration capacity of trehalose but does not directly demonstrate water replacement at the protein surface.

Vitrification

Vitrification refers to the process by which lyoprotectants immobilize proteins within a rigid glassy matrix, thereby minimizing molecular mobility and preventing denaturation, aggregation, and oxidation during freezing and drying [48, 91]. This effect is particularly effective for carbohydrates with a high T'_g, such as trehalose [72]. In the glassy state, the extremely high viscosity slows down conformational relaxation and conformational substates interconversion of proteins, thereby enhancing their stability [21]. Additionally, the restricted molecular mobility acts as a crowding agent that reduces the likelihood of protein aggregation, helping to preserve the native structure [48]. Therefore, using a lyoprotectant that can increase T'_g allows the drying process to be conducted at the same temperature while reducing physicochemical deterioration.

Even after drying, the low moisture content greatly reduces molecular mobility in the glassy state, but it is not completely eliminated, and residual translational motion below T_g can still contribute to degradation over long storage periods [92]. In the pharmaceutical field, where this principle has been studied in detail, a common rule of thumb holds that the glass transition temperature should exceed the storage temperature by at least ~50 °C for such residual mobility to become negligible over a product's shelf life [92, 93], although equivalent criteria have not yet been established for food proteins. Therefore, maintaining a high T_g after drying by using a lyoprotectant is advantageous for post-drying storage stability.

Glass type can be classified into two types: fragile and strong glasses. In the case of fragile glasses, the viscosity increases sharply with decreasing temperature, which makes the formation of fragile glasses more favorable for protein stability [71, 94]. Sucrose and trehalose are representative additives that form fragile glasses [94]. In general, carbohydrates with larger molecular weights tend to more easily form amorphous glasses and exhibit higher T_g values. However, their increased steric hindrance can interfere with hydrogen bond formation with proteins [85]. Consistent with this, a larger oligosaccharide (inulin) provided weaker stabilization than the smaller disaccharide trehalose at comparable glass transition temperatures, attributed to the larger molecule's reduced ability to closely fit the protein surface and form hydrogen bonds [93]. Therefore, when selecting an additive, it is important to balance both the ability to form a stable glassy matrix and the capacity to establish hydrogen bonds with the protein [21].

Hydrophobic interactions

Among lyoprotectants, polymers such as polyethylene glycol (PEG) and nonionic surfactants such as Tween-80 can enhance protein stability through hydrophobic interactions with the protein. Nonionic surfactants possess hydrophobic segments that can bind to the exposed hydrophobic regions of unfolded proteins, thereby preventing protein aggregation [95]. Additionally, by adsorbing onto the hydrophobic regions exposed on the surface of denatured proteins, they can modulate surface tension and local hydrophobic interactions, facilitating protein dissociation and refolding [96]. This stabilizing effect has been experimentally demonstrated during lyophilization. Ji et al. [95] reported that adding Tween-80 to a marine lysozyme formulation markedly improved the retention of enzymatic activity after freeze-drying and storage, whereas the unprotected enzyme showed substantial activity loss. They attributed this to hydrophobic interactions in which the surfactant's hydrophobic segment targets the exposed hydrophobic regions of the unfolded protein and suppresses self-association. Similarly, polymers such as PEG can interact with nonpolar amino acid residues in the hydrophobic regions of proteins, helping to prevent aggregation [48].

Apart from these hydrophobic interactions, nonionic surfactants are amphiphilic molecules that preferentially locate at interfaces, such as the ice–water interface formed during freezing and the air–water interfaces of air bubbles trapped between ice crystals, thereby limiting protein adsorption at these interfaces and the resulting unfolding [31, 32]. Arsiccio and Pisano [31] further suggested that surfactants may surround proteins near the ice surface and form protein–surfactant complexes, which can suppress both unfolding and aggregation.

Lyoprotectant used in protein-based foods

There are a limited number of studies that have employed lyoprotectants during the freeze-drying of protein-based foods. Furthermore, most of the published research has primarily utilized surimi as the model system, resulting in limited diversity among the available studies. In the case of surimi, it is common to add 4% (w/w) sorbitol, 4% (w/w) sucrose, and 0.2% (w/w) polyphosphate as cryostabilizers. Accordingly, experiments have been conducted to determine whether these additives also contribute to stabilization during freeze-drying. In the study conducted by Shaviklo et al. [97], saithe surimi was prepared and supplemented with 25 g/kg (w/w) sucrose and 2 g/kg (w/w) sodium tripolyphosphate as lyoprotectants to evaluate their protective effects. The experimental results showed that the samples containing lyoprotectants exhibited higher protein solubility after freeze-drying compared to those without lyoprotectants. In addition, greater water-holding capacity and gel-forming ability were observed in the lyoprotectant-treated samples. These findings indicate that sucrose and polyphosphate, which are commonly used in surimi production, provide not only cryoprotective effects during freezing but also stabilizing effects during freeze-drying. There are studies that have described the protective effects of different additives. In the experiment conducted by Ramadhan et al. [98], red tilapia surimi was freeze-dried to compare the effects of conventional surimi additives, 4% (w/w) sorbitol, 4% (w/w) sucrose, and 0.2% (w/w) polyphosphate, with those of trehalose at various concentrations (2%, 4%, 6%, and 8%). The experimental results showed that the use of trehalose led to a higher content of myofibrillar proteins after freeze-drying compared to the samples containing conventional additives. In addition, the rehydration ability of the trehalose-treated samples was also superior to that of samples treated with the conventional additives. These findings suggest that trehalose effectively protects proteins from the stresses induced during drying and freezing processes. One study explored the potential of various additives to enhance the stability of surimi during freeze-drying by increasing the T'_g. In the study by Ohkuma et al. [99], the effects of sorbitol, glucose, sucrose, and trehalose on the glass transition of frozen surimi prepared from carp were investigated, with commercial cod surimi used as a comparator. Two glass transitions, T'_{g1} and T'_{g2}, were observed. Although their origin is not completely understood,

T'_{g1} has been suggested to correspond to the glass transition of the maximally freeze-concentrated phase, whereas T'_{g2} corresponds to an ante-melting temperature at which viscous flow initiates in the amorphous region a few degrees below the onset of ice melting [99]. The results showed that as the sugar content increased (from 16.9% to 87.0% on a dry matter basis), T'_{g1} increased correspondingly, with trehalose and sucrose exhibiting the most pronounced rise.

In addition to surimi, studies have also examined the effects of lyoprotectants on other protein-based foods, such as mechanically recovered broiler meat. Kijowski and Richardson [100] compared mechanically recovered broiler meat samples that were freeze-dried with the addition of 4% sorbitol, 4% sucrose, and 0.25% sodium tripolyphosphate to those without any additives. The experimental findings demonstrated that the samples containing lyoprotectants exhibited higher protein solubility after freeze-drying. Moreover, the amount of water expelled under pressure was lower in the lyoprotectant-treated samples. These results indicate that the use of lyoprotectants effectively protected the broiler meat from damage induced by the drying process. Although the use of lyoprotectants reduced protein denaturation after freeze-drying in the mentioned studies, a decline in quality was still observed when compared to the pre-freeze-drying state. Given the limited number and diversity of studies applying lyoprotectants to protein-based foods, further research is needed to identify lyoprotectants suitable for each specific protein material in order to more effectively mitigate denaturation caused by the freeze-drying process.

However, several practical constraints must be considered when lyoprotectants are applied to protein-based foods. First, sugars and polyols are typically added at several percent, which can impart sweetness and increase the caloric content of the product. For example, the commercial cryoprotectant used in surimi, a mixture of 4% sucrose and 4% sorbitol, has been criticized for its sweetness and high caloric content, which do not meet the preferences of consumers seeking low-sugar and low-calorie products [101]. Low-sweetness alternatives, such as polydextrose, lactitol, and trehalose, have also been evaluated to address this issue [102–104]. Second, sugars with reducing carbonyl groups can bind to the free amino groups of lysine and arginine through the Maillard reaction, and Nopianti et al. [102] attributed the reduced whiteness of sugar-containing surimi gels to this reaction. Holm et al. [105] also noted that the reducing nature of most disaccharides other than trehalose, sucrose, and sucralose limits their suitability as general lyoprotectants, and Haas et al. [12] detected a higher proportion of Maillard-modified peptides in freeze-dried whey protein concentrate than in the liquid feed. Third, cost is an important consideration. Sucrose and sorbitol have been widely used in surimi partly because of their low cost and low tendency to cause the Maillard reaction [104], whereas the broader use of novel cryoprotectants, such as natural polysaccharides, protein hydrolysates, and antifreeze peptides,

is limited by their high production cost [101]. Finally, lyoprotectants used in foods must be permitted food ingredients or additives, and some are subject to labeling requirements. For example, egg white used in surimi products is an allergen that must be declared on the label, and the use of beef plasma protein has been restricted because of concerns about bovine spongiform encephalopathy [104]. Accordingly, the selection of lyoprotectants for protein-based foods should consider not only their protective efficacy but also their sensory impact, chemical stability during storage, cost, and regulatory acceptability.

Conclusion

During the freeze-drying of animal-derived foods, protein denaturation can occur in both the freezing and drying stages. In the case of freezing, protein stability is reduced during the temperature decrease and the formation of ice crystals. In the drying stage, the removal of water molecules that were involved in hydrogen bonding with proteins leads to alterations in their secondary and tertiary structures. Of these mechanisms, drying-induced structural changes and oxidation have been directly observed in animal-derived foods, whereas cold denaturation, glass transition–induced collapse, and the stabilizing mechanisms of protectants have been established mainly in pharmaceutical or model systems.

To reduce protein denaturation during freeze-drying, two primary strategies can be employed: the optimization of the freeze-drying process and the use of cryoprotectants or lyoprotectants. Process optimization involves maintaining the product temperature below T_c or T'_g during primary drying to prevent collapse of the dried structure and raising the shelf temperature during secondary drying to minimize drying time, both of which help preserve protein stability. Protectants can interact with proteins by substituting for water molecules or by preventing protein–protein interactions, thereby reducing denaturation after freeze-drying. Because the physicochemical properties differ among food proteins, the selection of suitable protectants and the optimization of freeze-drying conditions should be tailored to each specific food system. Concurrently, to bridge the gap between pharmaceutical concepts and practical food processing, future research should evaluate food-grade protectants that can improve storage stability while considering their effects on sensory quality, cost, and regulatory acceptability.

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Tables and Figures

Table 1. Summary of studies reporting protein denaturation, protein oxidation, or related changes in animal-derived food proteins after freeze-drying.

Sample	Pretreatment	Freeze-drying condition	Comparator	Analytical method	Direction / magnitude of change (significance)	Key limitation	Ref.
Pork meat batter	Batter (75% pork, NaCl, nitrite, phosphate, soy protein); frozen at −20 or −70 °C for 16 h	5 mTorr; condenser −100 °C; dried until core temperature reached 25 °C	FT batter frozen at the same temperature; raw batter	ANS surface hydrophobicity; intrinsic Trp fluorescence; DNPH carbonyl; TBARS; DLS particle size; ELS zeta potential	vs. FT: ↓ Trp fluorescence and blue-shifted λmax at both temperatures ($P < 0.05$); ↑ surface hydrophobicity at −70 °C only ($P < 0.05$; n.s. at −20 °C); ↑ carbonyl and TBARS ($P < 0.05$). vs. raw: ↑ particle size and ↓ absolute zeta potential ($P < 0.05$)	Drying at 25 °C (T_g not measured) cannot be separated from sublimation; values reported in figures only	[9]
Egg white protein hydrolysate	Papain hydrolysis (1%, 90 min), with or without	Pre-frozen at −20 °C for 12 h; 24 h; condenser −55 °C	Vacuum-dried hydrolysate (70 °C, 0.13	FTIR (amide I)	Untreated FD vs. VD: α-helix 10.8 vs. 17.7%; β-sheet 35.9 vs. 32.0%; β-turn 35.4 vs. 27.8%;	Comparator is another drying method;	[10]

Sample	Pretreatment	Freeze-drying condition	Comparator	Analytical method	Direction / magnitude of change (significance)	Key limitation	Ref.
	hydrodynamic cavitation		bar); no non-dried control		random coil 17.9 vs. 22.6% ($P < 0.05$)	hydrolysate rather than intact protein	
Pork (M. longissimus thoracis)	Frozen in liquid-nitrogen vapor to −80 °C; stored at −80 °C for ≤ 5 d	3.5 Pa; collector −49 °C; 24 h; rehydrated at 25 °C for 2 h	Raw; FT (thawed in 35 °C water)	Modulated DSC	Myosin ΔH: 0.81 (FD) vs. 0.96 (raw) vs. 0.12 J/g (FT), all different ($P < 0.05$); actin ΔH 0.99 vs. 1.03 J/g (raw), n.s.	Measured after rehydration; residual ΔH does not directly quantify the extent of prior denaturation	[11]
Whey protein concentrate	Pasteurized liquid whey; ultra- and diafiltration (20–35% solids)	Pilot scale; < 13.3 Pa; product −40 °C for ≤ 30 h; final product 30 °C	Liquid feed (non-dried); spray-dried WPC	ELISA (LF, IgG); pH 4.6 solubility; SDS-PAGE	vs. feed: ↓ immunoreactive LF and IgG ($P < 0.05$). vs. spray-dried: n.s. Solubility not different from feed; no loss of SDS-PAGE band intensity	ELISA reflects immunoreactivity, not biological activity; n = 3 lots	[12]
Egg white	Pre-frozen in a freezer for 3 h	Microwave freeze-drying: 503 W; 150 Pa	Non-dried egg white (0 h)	FTIR (amide I); gel texture	α-Helix 20.5% (0 h) → 18.8% (5 h) ($P < 0.05$); gel hardness	Microwave heating confounds the effect of freeze-drying;	[13]

Sample	Pretreatment	Freeze-drying condition	Comparator	Analytical method	Direction / magnitude of change (significance)	Key limitation	Ref.
					increased, reaching a maximum at 5 h	small magnitude of change	
Antarctic krill protein	Alkali extraction–isoelectric precipitation; phosphorylated with sodium tripolyphosphate	Frozen at −18 °C for 24 h; 20–30 Pa; −46 °C	Spray-dried and hot-air-dried (55 °C); no non-dried control	DSC; FTIR; solubility; emulsifying and foaming properties	FD vs. spray-dried: ΔH 80.5 vs. 210.8 J/g; solubility 292 vs. 714 mg/g; EAI 8.8 vs. 9.6 m^2/g; foaming activity 32.4 vs. 41.5% ($P < 0.05$); all higher than hot-air-dried	No non-dried control; phosphorylated protein; FTIR changes not quantified	[14]
Ovine milk	Raw or heated (63 °C/30 min or 75 °C/15 s); frozen at −20 °C for 24 h	1 mbar; 72 h; condenser −40 °C	Non-dried counterpart	Fluorogenic substrate assays (elastase, plasmin, cathepsin D)	Elastase activity ↓ 68% (raw) and ↓ 40% (75 °C/15 s) ($P < 0.05$); cathepsin D ↓ in raw milk ($P < 0.05$)	Structural damage was proposed but not measured	[15]
Whey protein isolate	Commercial WPI dispersed in water (20	< 100 μmHg; −55 °C; 72 h	Untreated WPI powder	DNPH carbonyl; dityrosine fluorescence;	Carbonyl 4.24 vs. 2.09 nmol/mg protein ($P < 0.05$); ↑ dityrosine fluorescence (no	Control was an already-dried commercial	[16]

Sample	Pretreatment	Freeze-drying condition	Comparator	Analytical method	Direction / magnitude of change (significance)	Key limitation	Ref.
	mg/mL) for 30 min			free SH; zeta potential	statistical test); free SH and zeta potential n.s.	powder; WPI heated before freeze-drying showed lower carbonyl (2.75 nmol/mg) than WPI freeze-dried alone	
Pork (front leg)	Vacuum-packed cubes frozen at −20 °C for 2 d	5 mTorr; room temperature; 7 d	FT pork (−20 °C for 30 d; thawed at 4 °C for 48 h)	DNPH carbonyl; ANS surface hydrophobicity; Trp fluorescence; protein solubility	Carbonyl 2.14 vs. 1.31 nmol/mg ($P < 0.05$); surface hydrophobicity and Trp fluorescence n.s.; myofibrillar protein solubility 65.3 vs. 72.8 mg/g ($P < 0.05$)	Different frozen-holding times (2 vs. 30 d); drying at room temperature; timing of oxidation unresolved	[17]
Mealworm (*Tenebrio molitor*)	Live or blanched (40 s) larvae frozen at −21 °C	52 h	Fresh and blanched larvae; microwave-dried larvae	Peroxide value; p-anisidine value	Peroxide value 125.1 meq O_2/kg fat vs. below LOD (fresh, microwave-dried); p-anisidine	Lipid oxidation only; protein oxidation not measured	[18]

Sample	Pretreatment	Freeze-drying condition	Comparator	Analytical method	Direction / magnitude of change (significance)	Key limitation	Ref.
					value 3.46 vs. 1.89 (fresh) ($P < 0.05$)		
Mealworm (*Tenebrio molitor*)	Live larvae frozen at −21 °C for 48 h	24 h; condenser −50 °C	Fresh larvae; vacuum-, microwave-, fluidized-bed-, and rack-oven-dried larvae	4-HNE–protein adducts (ELISA); protein solubility	4-HNE 111.0 vs. 39.5 μg/mL (fresh), highest among drying methods ($P < 0.05$); protein solubility 40.7 vs. 53.2% (fresh) ($P < 0.05$), higher than microwave-, fluidized-bed- and rack-oven-dried but lower than vacuum-dried (49.7%) samples	Lipid-derived marker; direct protein oxidation markers not measured	[19]
Silver carp myofibrillar proteins	Commercial frozen surimi	Not reported; powder stored at room temperature	Non-dried surimi; vacuum spray-dried powder	Ca^{2+}-ATPase; ANS surface hydrophobicity; CD; solubility; emulsifying properties	Ca^{2+}-ATPase activity 23.5 → 9.0; surface hydrophobicity 51.3 → 226.2; solubility 0.62 → 0.21 g/g ($P < 0.05$); α-helix 36.8 → 30.4%; emulsion stability index 66 → 167 min (↑, $P < 0.05$)	Freeze-drying conditions and storage time not reported; commercial surimi may contain cryoprotectants	[20]

FD, freeze-dried; FT, frozen–thawed; VD, vacuum-dried; ANS, 1-anilinonaphthalene-8-sulfonate; Trp, tryptophan; DNPH, 2,4-dinitrophenylhydrazine; TBARS, 2-thiobarbituric acid reactive substances; DLS, dynamic light scattering; ELS, electrophoretic light scattering; FTIR, Fourier transform infrared spectroscopy; DSC, differential scanning calorimetry; ΔH, denaturation enthalpy; ELISA, enzyme-linked immunosorbent assay; LF, lactoferrin; IgG, immunoglobulin G; EAI, emulsifying activity index; SH, sulfhydryl; LOD, limit of detection; 4-HNE, 4-hydroxy-2-nonenal; CD, circular dichroism; n.s., not significant. Values and significance are as reported in the original studies; ↑ and ↓ indicate increases and decreases relative to the comparator.

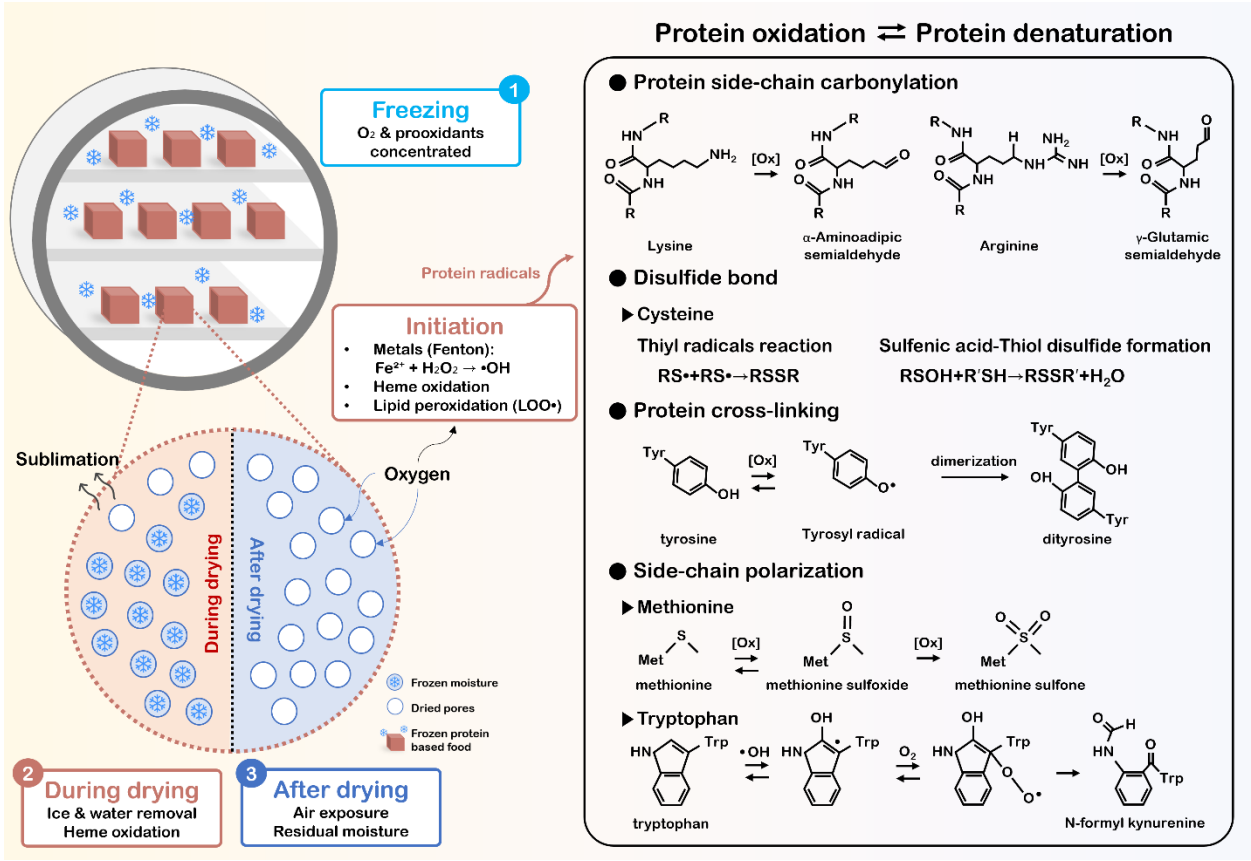
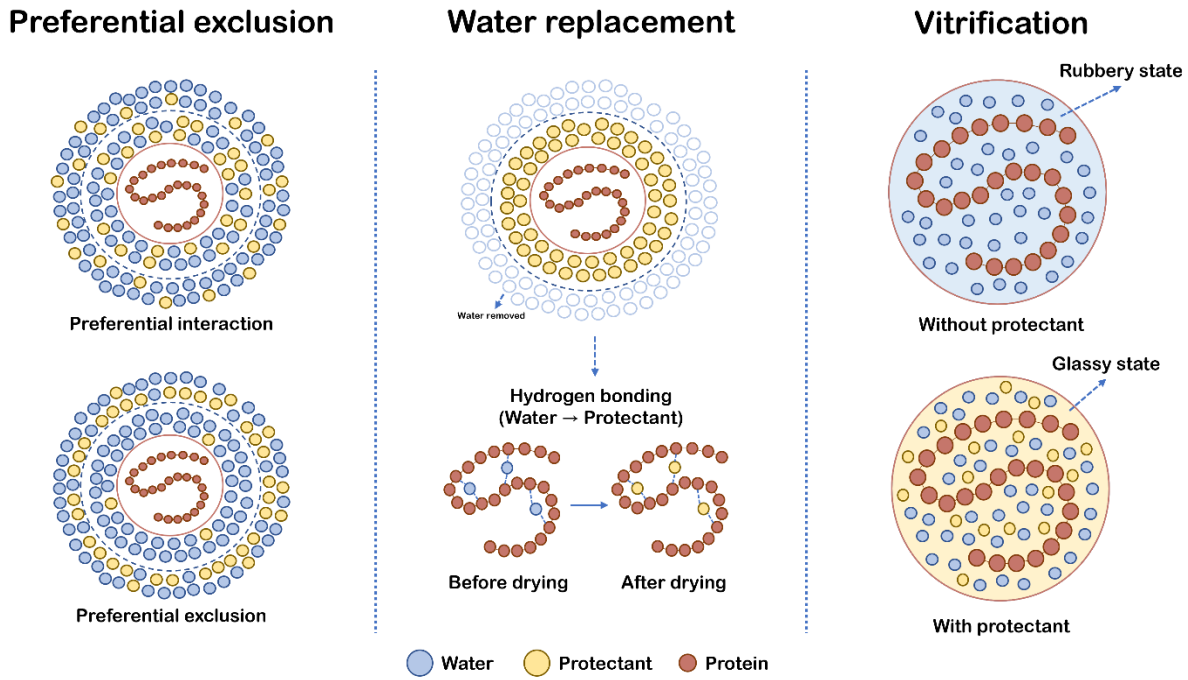


Figure 1. Proposed bidirectional relationship between protein oxidation and denaturation during freeze-drying.

Oxidation may proceed during freezing, drying, and after drying. Protein radicals are initiated by transition metals via the Fenton reaction, by heme proteins, and by lipid peroxidation, rather than by molecular oxygen itself. Oxidation and denaturation can promote each other, although conformational changes do not necessarily result from, or lead to, oxidation.



826

827 **Figure 2. Schematic illustration of lyoprotectant-mediated protein stabilization mechanisms: preferential exclusion, water replacement, and vitrification.**

828 Three of the four proposed mechanisms are shown. Preferential exclusion operates mainly in the solution and frozen states. Hydrophobic interactions, including
829 interfacial protection by surfactants, are described in the text but are not illustrated.
830