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The physical behaviour of food protein's supramolecular structures during freeze-drying

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ABSTRACT

Many proteins are able to self-assembly into well-defined supramolecular structures. We have previously shown that two food globular proteins with highly homologous tertiary structures and opposite electric charges, i.e. lysozyme and α-lactalbumin, interact under specific conditions of temperature and pH and generate spherical microparticles. The objective of this study was to investigate the stability of the lysozyme - α-lactalbumin microparticles during lyophilisation, one example of food processes. Solutions of protein microparticles without and with addition of protective molecules such as sucrose or matlodextrin were freeze-dried under conservative conditions in a pilot plant and samples were removed at each step of the process: after freezing, sublimation and desorption. The morphology and the particle size distribution of the protein microparticles were investigated by direct optical observation under a microscope and by using a Coulter counter. The freezing (and thawing) appeared to be the most damaging steps with the formation of clusters or agglomerates of microparticles. Addition of sucrose or maltodextrin sharply reduced the formation of particles agglomerates after freezing by limiting the cryoconcentration of the particles and trapping them in an amorphous matrix. Maltodextrin seemed to be a more efficient protective molecule than sucrose for preserving the stability of the protein microparticles during the freeze-drying process.

Keywords: lyophilisation; lysozyme, lactalbumin; microparticles

INTRODUCTION

Controlled self-assembly of biomacromolecules has recently attracted considerable attention because this process leads to new materials with potential applications in various fields such as nano-, medical, or food technologies. Molecular self-assembly is a process during which molecules placed under specific conditions spontaneously form ordered supramolecular structures through noncovalent binding. Proteins were reported to be ideal biomaterials to obtain tailor-made new nanoparticles with various architectures. Hence, throughout adequate control of their self-assembly process, a large diversity of supramolecular structures including aggregates, fibres, nanotubes or spherulites can be generated from globular proteins. Recently, we evidenced that two food globular proteins with highly homologous tertiary structures and opposite electric charges, calcium-depleted apo-α-lactalbumin (apo α-LA) and hen egg white lysozyme (LYS), interact and self-assemble to form different supramolecular structures in a temperature-dependent manner. Amorphous aggregates were observed at 5 °C while well-defined microspheres were obtained at 45 °C [11]. The assembly mechanisms and the stability of these protein supramolecular structures remain still largely ignored. Recently, it was demonstrated that ionic strength and electrostatic forces are involved in the formation of the lysozyme - α-lactalbumin microparticles and in their stability in liquid state [12]. Even if controlled interactions and self-assembly of proteins promise to be a powerful approach for generating novel supramolecular architectures, the stability of such structures during various processes needs to be investigated.

Lyophilization or freeze-drying is widely used to preserve proteins and polypeptides, which are physically and/or chemically unstable in aqueous solutions. The process involves freezing of the aqueous protein solution, followed by primary drying to remove ice by sublimation and, finally, secondary drying to remove unfrozen or sorbed water. Extensive works have been carried out on freeze-drying of proteins such as enzymes, antibodies and various therapeutic proteins [18]. The freeze-drying process generates several stresses that induce protein unfolding and denaturation. An appropriate choice of protective excipients is thus needed to inhibit freezing- and drying-induced damage and to preserve protein native conformation during the stabilization process, storage, and rehydration [4, 7, 13]. The low-molecular-weight carbohydrates, especially the disaccharides, appear as the most effective protective agents for lyophilized proteins, and two

hypotheses have been advanced to explain the mechanisms of protein stabilization by such molecules. One is the "water substitution hypothesis" proposed by Carpenter and Crowe [5], which suggests that carbohydrate molecules interact with the dried protein by hydrogen bonding in place of the removed water molecules and, hence, inhibit protein unfolding during drying. The other is "the glassy state hypothesis," which states that by trapping protein molecules in a glassy matrix, the molecular mobility of a protein containing system is greatly limited so that the rates of diffusion-controlled reactions, including protein unfolding, protein aggregation, and chemical degradation are reduced relatively to the rates occurring in a rubbery state [10, 14]. To our knowledge, no study has been reported on freeze-drying of supramolecular structures resulting from the assembly between globular proteins. Since the formation of such structures requires the modification of protein conformation and/or the formation of specific oligomers, applying the results obtained from the works on proteins freeze-drying on the protein supramolecular structures are expected to lead to original results. The objective of this study was thus to investigate the stability of the lysozyme - α-lactalbumin microparticles following a food stabilization process, lyophilisation without or in the presence of protective molecules.

MATERIALS & METHODS

Materials and Proteins' microparticles formation

Commercial lysozyme (LYS) was purchased from Ovonor and contained 95% of LYS and 3% of chloride ions. Holo α-Lactalbumin (α-LA) was purified from bovine whey by ion exchange chromatography as previously reported [8]. The resulting powder contained 95% of holo α-LA. Apo α-LA was prepared by dialysis of holo α-LA against deionised water at pH 3 during 48h at 4°C using dialysis membrane (Spectrum Laboratories, US & Canada) with a nominal cut off of 6-8000 Da in order to remove calcium ions. pH of the apo α-LA solution was adjusted to pH 7 and then freeze-dried. Solutions of the two proteins were mixed under adequate physico-chemical conditions to form microspheres [11]. The powders were separately solubilised in 30 mM Tris/HCl buffer (pH 7.5) at a final protein concentration of 0.266 mM. Equal volumes of both protein solutions previously equilibrated at 45°C were gently mixed together and the resulting solution was incubated at 45°C for 30 min. After incubation, the solution of protein microparticles was divided into three equal volumes to obtain three formulations containing no additives (control), 5% sucrose or 5% maltodextrin (DE = 6) and stored at 4°C. Powder of sucrose (Prolabo, Paris, France) or maltodextrin (Roquette, Lestrem, France) was directly added to the microparticles solution to obtain a final concentration of solute of 5%.

Freeze-drying experiments

The formulations were dispensed into 4 mL freeze–drying vials (1 mL final filled volume). The vials were placed on an aluminium tray and loaded onto the shelf of a SMH 90 freeze-dryer (Usifroid, Maurepas, France). The freeze-drying cycle consisted of freezing at –50°C (shelf cooling rate at 0.6°C/min and holding at –50°C for 2h), followed by primary drying at shelf temperature of -20°C and chamber pressure of 20 Pa and, finally, a secondary drying phase of ten hours at 25°C and chamber pressure of 20 Pa for ten hours. The heating rate of the shelf temperature applied during the primary and the secondary drying steps was fixed at 0.25°C/min. At the end of the process, the vacuum was broken by injection of dry air and the vials were rapidly stoppered and packed under vacuum in aluminium bags. That freeze-drying cycle was repeated and stopped at the end of the primary drying. After the freezing step of each freeze-drying experiments, three vials were removed to the freeze-dryer and stored at -80°C.

Protein microparticles analysis

The analysis of the microparticles was performed before freeze-drying, after freezing and thawing and after freeze-drying and rehydration. The frozen microparticles were thawed in a temperature controlled bath at 25°C for 5 min. The freeze-dried microparticles were rehydrated by addition of 1 mL of purified water. During all the analysis, the samples were kept at 4°C.

Turbidity measurements were performed using a UV-vis sprectrophotometer (Beckman Coulter, Fullerton, California, USA). Optical density was measured at 600 nm.

The spherical particles were observed using an optical microscope (Nikon Elipse E600, Nikon, Japan) equipped with a camera. Samples were put between glass slides, and structures were immediately observed at a magnification of 100 at room temperature.

The number and the size of the microparticles were determined using a Coulter Counter Z2 (Beckman Coulter, Fullerton, California, USA). Each sample was diluted before measurements to avoid detector saturation. Four diluted samples were prepared for each formulation and four measurements were performed for each diluted sample.

RESULTS & DISCUSSION

Optical microscopy

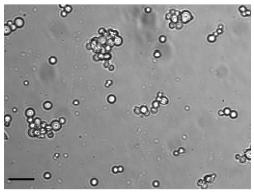
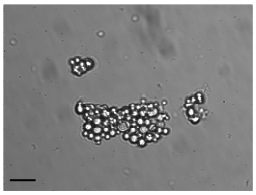
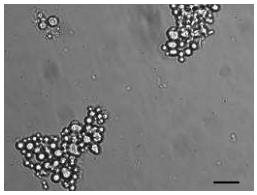
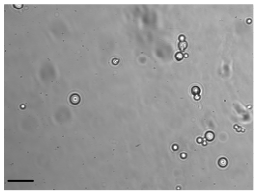
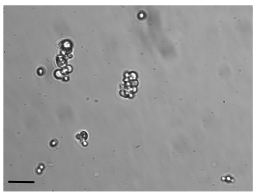
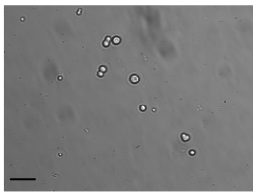
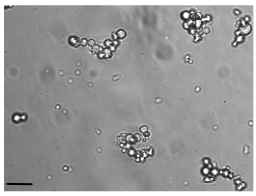
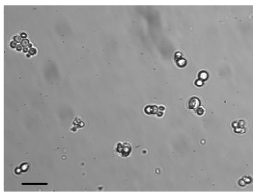
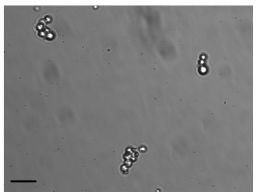
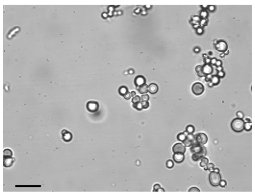
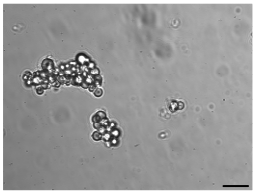
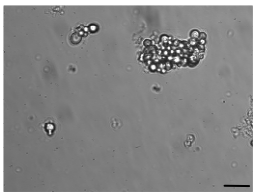
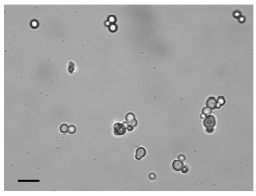
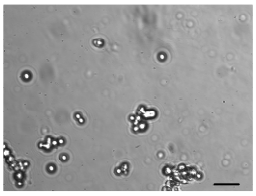
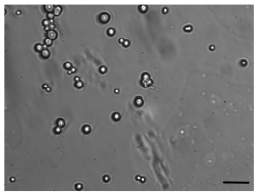
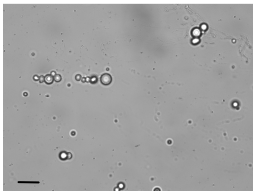
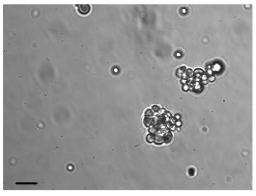
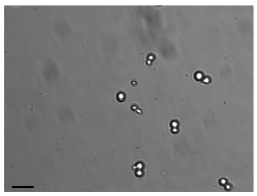
	Before freeze-drying	After freezing	After sublimation
Without additive Control			
With sucrose			
With maltodextrin			
	Before freeze-drying	**After freezing**	**After desorption**
Without additive Control			
With sucrose			
With maltodextrin			

Figure 1. Optical microscopic observations of the protein microparticles after each step of the freeze-drying process for the three formulations studied (containing no additive, 5% sucrose or 5% maltodextrin). Scale bar = 10 µm.

The micrographs of the proteins microparticles solutions obtained after each step of the freeze-drying process (before freeze-drying, after freezing, after sublimation (primary drying) and after desorption (secondary drying)) are shown in Figure 1 for the three formulations studied. These figures illustrate the qualitative evolution of the morphology of the microparticles during the freeze-drying process. Before freeze-drying, separated microparticles with spherical and regular shape were observed whatever the formulation considered. The diameter of the microparticles varied between 4 and 6 µm. However, different physical behaviours of the microparticles were obtained at the various steps of the freeze-drying process. In the absence of protective molecule, formation of large clusters of microparticles was observed after freezing and thawing. The diameters of these clusters could reach up to 50 µm. Those large clusters of microparticles remained after the sublimation and the desorption steps of the process. The size of the clusters appeared to have slightly decreased after the desorption step of the process where the unfrozen water was removed and the hydrogen-bonds between water and proteins were broken.
When adding sucrose or maltodextrin to the protein microparticles solution, the formation of clusters after freezing and thawing was sharply reduced. Separated and regular shape microparticles were observed after the sublimation and the desorption steps. Slightly smaller clusters of microparticles can be noticed after freezing and thawing for the samples of "sublimation experiment" compared to the samples of the "desorption experiment". Freezing and especially ice nucleation are stochastic processes. Even if the cooling rate was controlled during the experiments, variation in ice nucleation temperatures could be obtained resulting in different ice crystals morphology and size [17].

Evolution of the number and the size of the protein microparticles during the freeze-drying process

The evolution of the number and the size (mean diameter) of protein microparticles after each step of the freeze-drying process are displayed in Figure 2 and 3, respectively. Without protective molecules, the number of microparticles sharply decreased after freezing and thawing. Only 20% to 40% of the initial microparticles number were recovered. Such decrease of microparticles was due to the formation of large clusters exhibiting a diameter higher than 10 µm observed with the microscope but not detected by the Coulter counter. The increase of the mean diameter of the microparticles by 11-13% confirms the latter hypothesis. After thawing, the solution was stored several days at 4°C and the microparticles clusters were still observed after 10 days of storage. After the sublimation and the desorption steps, the number of microparticles slightly increased to 42% and 58%, respectively. Such increase could be ascribed to the dissociation of clusters and that dissociation phenomenon seemed more pronounced when the product was rehydrated after the desorption step. In the same way, the mean diameter of the microparticles was lower after the desorption step than after the freezing step, whereas it remained constant after the freezing and the sublimation steps.
In the presence of protective molecules, sucrose or maltodextrin, higher number of microparticles was recovered after the freezing step as compared to the control sample. However the results were variables between the both freeze-drying experiments: 100% (sublimation experiment) and 70% (desorption experiment). The formation of some microparticles clusters observed after freezing under the microscope in the case of the "desorption experiment" was confirmed by the increased of the mean diameter by 7-9%. After freezing and thawing, the samples were stored a few days at 4°C. After 4 days at 4°C, dissociation of the protein microparticles was observed and the number of microparticles compared to the initial concentration before freeze-drying reached 90% for both protective molecules. Rehydration of the formulation after the sublimation and the desorption steps resulted in higher recovery of microparticles number. When the process was stopped after the removal of ice by sublimation, 100% of the initial microparticles number was recovered and no change of the mean diameter of the particles was observed for both formulations. However, when the process was stopped after the removal of unfrozen water by desorption, few differences were noticed between the both protective molecules. With maltodextrin, no change of the number and mean diameter were observed whereas slight decrease of the number recovered (from 102% to 87%) and increase of the mean diameter (by 6% compared to the mean diameter before freeze-drying) were observed with sucrose indicating the formation of some microparticles clusters.

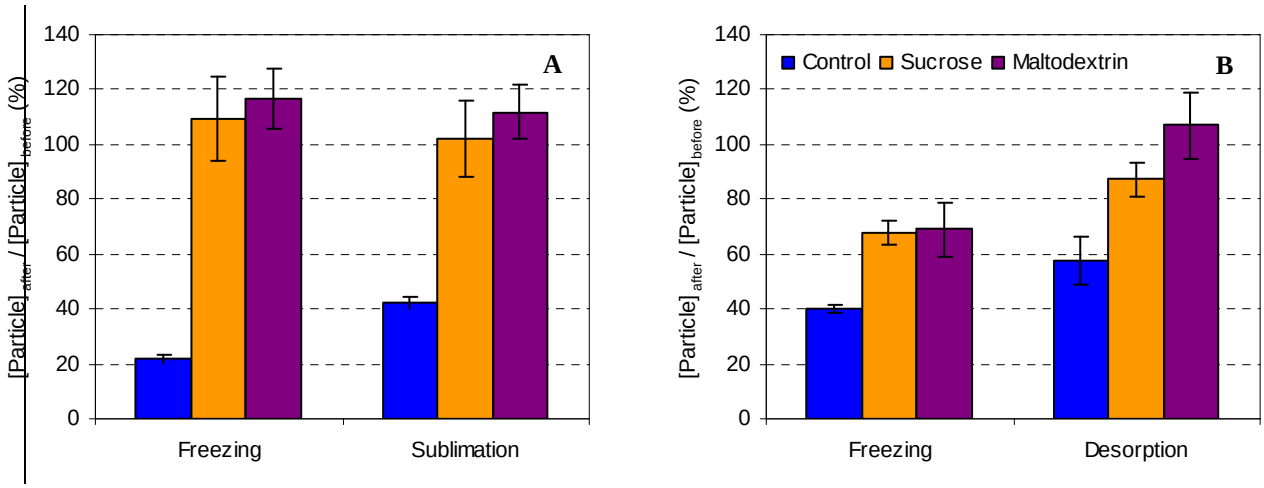


Figure 2. Evolution of the number of the protein microparticles after each step of the freeze-drying process measured by the Coulter counter. The number of microparticles before the freeze-drying process was considered as reference. A. Experiment where the process was stopped at the end the sublimation step. B. Experiment where the process was stopped at the end of the desorption step

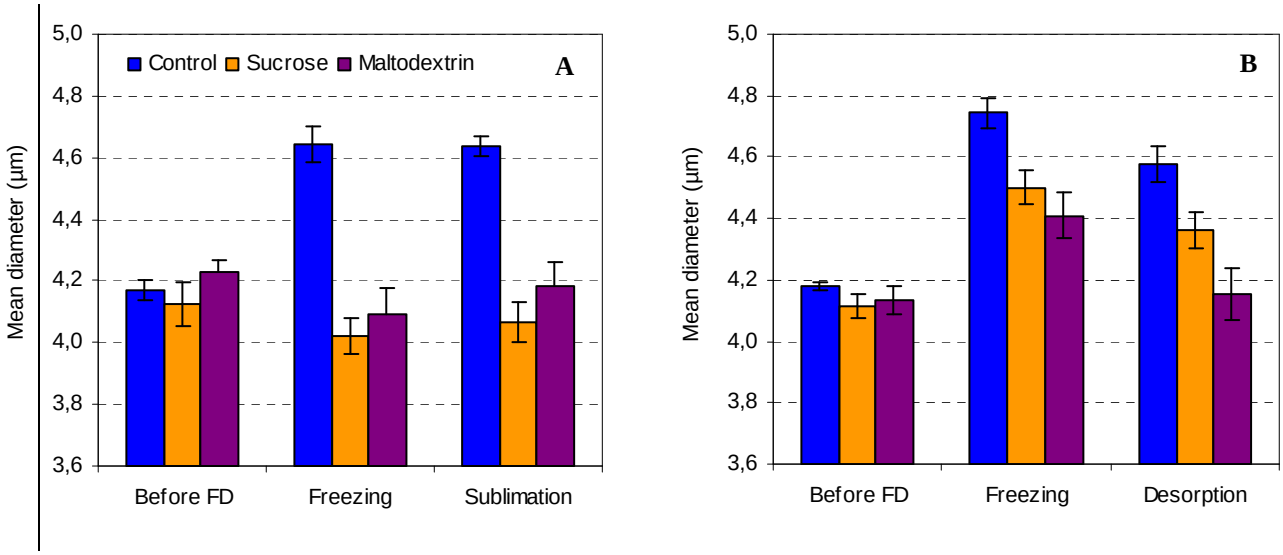


Figure 3. Evolution of the mean diameter of the microparticles determined by the Coulter counter after each step of the freeze-drying process. A. Experiment where the process was stopped at the end the sublimation step. B. Experiment where the process was stopped at the end of the desorption step

In summary, the main damaging steps of the freeze-drying process appears to be the freezing and the desorption steps. Freezing is the first stage of freeze-drying and is the stage where most of the water is removed from microparticles and excipients. The system is separated into multiple phases and the interfaces between ice and microparticles form. Freezing often induces many destabilizing stresses on protein: protein adsorption at the ice-water interface, cryoconcentration of the solutes and increase of the ionic strength [2, 6, 9, 16]. The formation of ice results in a high increase of solute concentration in the remaining phase and thus in the enhancement of the interaction between microparticles leading to their agglomeration or fusion. When adding sucrose or maltodextrin, the protein microparticles are more dispersed in the concentrated phase limiting their agglomeration. The desorption step consists of the removal of the unfrozen water with the breakage of the hydrogen bonds between the proteins and the water. This step seems to cause some particles agglomeration only when sucrose was added before processing. Sucrose is able to establish hydrogen bonds with proteins when the unfrozen water is removed [1, 3, 5, 15]. These specific interactions between sucrose and proteins might explain the decrease of the number of microparticles and the increase of their mean diameter observed after the desorption step.

CONCLUSION

The stability of well-defined supromolecular structure resulting from the self-assembly properties of lysozyme and α-lactalbumin were investigated during the freeze-drying process. Protective molecules such as sucrose or maltodextrin are required for preserving the protein microparticles and inhibiting the formation of agglomerates by trapping them in an amorphous matrix.

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REFERENCES

[1] Allison S.D., Chang B., Randolph T.W. & Carpenter J.F. 1999. Hydrogen bonding between sugar and protein is responsible for inhibition of dehydration-induced protein unfolding. Archives of Biochemistry and Biophysics, 365(2), 289-298.

[2] Arakawa T., Prestrelski S.J., Kenney W.C. & Carpenter J.F. 2001. Factors affecting short-term and long-term stabilities of proteins. Advanced Drug Delivery Reviews, 46(1-3), 307-326.

[3] Carpenter J.F., Arakawa T. & Crowe J.H. 1991. Interactions of stabilizing additives with proteins during freeze-thawing and freeze-drying. Developments in Biological Standardization, 74, 225-239.

[4] Carpenter J.F., Chang B.S., Garzon-Rodriguez W. & Randolph T.W. 2002. Rational design of stable lyophilized protein formulations: theory and practice. Pharmaceutical Biotechnology, 13, 109-133.

[5] Carpenter J.F. & Crowe J.H. 1989. An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. Biochemistry, 28(9), 3916-22.

[6] Carpenter J.F., Izutsu K. & Randolph T.W. 1999. Freezing- and drying-induced perturbations of protein structure and mechanisms of protein protection by stabilizing additives. In Rey L. & May J.C. (Eds), Freeze-drying/Lyophilization of pharmaceutical and biological products. Marcel Dekker, New York, USA, pp. 123-160.

[7] Carpenter J.F., Prestrelski S.J. & Arakawa T. 1993. Separation of freezing- and drying- induced denaturation of lyophilized proteins using stress-specific stabilization. Archives of Biochemistry and Biophysics, 303(2), 456-464.

[8] Caussin F., Famelart M.H., Maubois J.L. & Bouhallab S. 2003. Mineral modulation of thermal aggregation and gelation of whey proteins: From β-lactoglobulin model system to whey protein isolate. Lait, 83, 353–364.

[9] Chang B.S., Kendrick B.S. & Carpenter J.F. 1996. Surface-induced denaturation of proteins during freezing and its inhibition by surfactants. Journal of Pharmaceutical Sciences, 85(12), 1325-1330.

[10] Franks F. 1991. Freeze-drying: from empiricism to predictability. The significance of glass transitions. Development in Biologicals Standardization, 74, 9-19).

[11] Nigen M., Croguennec T., Renard D. & Bouhallab S. 2007. Temperature affects the supramolecular structures resulting from α-lactalbumin–lysozyme interaction. Biochemistry, 46(5), 1248–1255.

[12] Nigen M., Croguennec T. & Bouhallab S. 2009. Formation and stability of α-lactalbumin–lysozyme spherical particles: Involvement of electrostatic forces. Food Hydrocolloids, 23, 510–518.

[13] Passot S., Fonseca F., Alarcon-Lorca M., Rolland D. & , Marin M. 2005. Physical characterisation of formulations for the development of two stable freeze-dried proteins during both dried and liquid storage. European Journal of Pharmaceutics and Biopharmaceutics, 60; 335-348.

[14] Pikal M.J. 1999. Mechanisms of protein stabilization during freeze-drying and storage: The relative importance of thermodynamic stabilization and glassy state relaxation dynamics. In Rey L. & May J.C. (Eds), Freeze-drying / Lyophilisation of pharmaceutical and biological products. Marcel Dekker, New York, USA, pp. 161-198.

[15] Prestrelski S.J., Tedeschi N., Arakawa T. & Carpenter J.F. 1993. Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers. Biophysical Journal, 65(2), 661-71.

[16] Sarciaux J., Mansour S., Hageman M.J. & Nail S.L. 1999. Effects of buffer composition and processing conditions on aggregation of bovine IgG during freeze-drying. Journal of Pharmaceutical Sciences, 88(12), 1354-1361.

[17] Searles J.A., Carpenter J.F. & Randolph T.W. 2001. The ice nucleation temperature determines the primary drying rate of lyophilization for samples frozen on a temperature-controlled shelf. Journal of Pharmaceutical Sciences, 90(7), 860-871.

[18] Wang W. 2000. Lyophilization and development of solid protein pharmaceuticals. International Journal of Pharmaceutics, 203, 1-60.