Original Research Article

Development of lyophilization cycle and effect of excipients on the stability of catalase during lyophilization

Shantanu V Lale, Monu Goyal[1], Arvind K Bansal[1]

Departments of Pharmaceutical Technology (Formulations), [1]Pharmaceutics, National Institute of Pharmaceutical Education and Research (NIPER), Punjab, India

Abstract

Introduction: The purpose of the present study was to screen excipients such as amino acids and non-aqueous solvents for their stabilizing effect on catalase, a model protein, for lyophilization. The present study also includes optimization of lyophilization cycle for catalase formulations, which is essential from the commercial point of view, since lyophilization is an extremely costly process. **Materials and Methods:** Activity of catalase was determined using catalase activity assay. Differential scanning calorimetry was used to determine eutectic melting temperature of the frozen catalase solution, which is essential for the optimization of lyophilization cycle. **Results:** When catalase was lyophilized without excipients, it was found that about 65-78% of the initial activity of catalase was lost during the lyophilization process in a concentration dependent manner. The maximum stability of catalase during lyophilization was observed at pH 7.0. Amino acids like alanine, glycine, lysine, serine and 4-hydroxy proline showed strong stabilizing effect on catalase during lyophilization by protecting catalase activity above 95%, whereas valine and cysteine hydrochloride showed destabilizing effect on catalase. Non-aqueous solvents such as dimethyl formamide, dimethyl sulphoxide, polyethylene glycol (PEG) 200, PEG 400, PEG 600 and ethylene glycol also showed destabilizing effect on catalase during lyophilization. **Conclusions:** In order to prevent loss of catalase activity during lyophilization of catalase, use of amino acids like alanine, glycine, lysine, serine and 4-hydroxy proline in optimum concentration is highly advisable.

Key words: Cryoprotectants, freeze drying, lyoprotectants, protein stability

INTRODUCTION

Liquid protein formulations are economic and easy to manufacture but are susceptible to physical and chemical degradation.[1] Solid formulations can help in addressing these problems, as chemical reactions occurring in liquid state get drastically reduced in solid form.[1,2]

Various methods for manufacturing solid protein pharmaceuticals include freeze-drying, spray drying, spray coating, spray freeze-drying, vacuum drying, film drying, and supercritical fluid drying. Out of all these methods, freeze-drying, also called as lyophilization, is widely used due to its advantages over other drying methods.[3] There is minimal thermal damage of protein during lyophilization, since the entire process is carried out at temperature below 30°C.[3-5] This method also offers better accuracy of dose because the drug is filled in final containers in the solution form. Many protein pharmaceuticals are potent drugs having very low dose and hence microgram quantities of such potent drugs are required to be filled precisely.[6,7] Although the lyophilization possesses numerous advantages over other drying processes, it also has certain challenges.[8] The process involves inherent destabilization forces that can denature proteins e.g. cold shock, ice-water interfaces, pH changes during freezing, dehydration stress, etc.[9-11] Also, if lyophilization cycle is not optimized, the process can be highly energy demanding and time consuming.[12,13] Development of optimized lyophilization cycle and use of stabilizing excipients are adopted to overcome these challenges of lyophilization process.[14]

Excipients giving protection to protein against freezing stress are called as cryoprotectants, whereas those giving protection against drying stress are called as lyoprotectants.[15] Various excipients like sugars/polyols, polymers, surfactants, amino acids,

Address for correspondence:
Prof. Arvind K. Bansal,
Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research (NIPER),
S.A.S Nagar, Punjab -160062, India. E-mail: akbansal@niper.ac.in

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metal ions and some inorganic salts are commonly used as cryo/lyoprotectants.[16,17] Stresses during freezing and dehydration stage are different and hence it is necessary to choose a mixture of cryoprotectant and lyoprotectant when a single stabilizer does not serve both the purposes.

In this work, catalase has been selected as a model protein since it is labile to denaturation stresses of freeze-drying.[18-20] The present study includes development of lyophilization cycle for catalase solutions and determination of critical step responsible for loss of activity of catalase during lyophilization process. Effect of protein concentration and pH on the stability of catalase during lyophilization was also studied. Furthermore, excipients such as amino acids and non-aqueous solvents were screened for their stabilizing effect on catalase during lyophilization.

MATERIALS AND METHODS

Materials

Bovine liver catalase (lyophilized powder, 2,000-5,000 units/mg protein), bovine serum albumin (lyophilized powder), alanine, arginine, cysteine hydrochloride, glycine, 4-hydroxy proline, histidine, lysine, serine, threonine, and valine were purchased from Sigma Chemical Co. (St. Louise, USA). Hydrogen peroxide solution (30%), dimethyl formamide (DMF), dimethyl sulphoxide (DMSO), ethylene glycol, polyethylene glycol (PEG) 200, PEG 400, PEG 600 were purchased from Merck Specialities Pvt. Ltd. (Mumbai, India). All the excipients used in the experiment were of analytical grade. Dialysis membrane (Mol wt cut-off 12000-14000 Da) was purchased from Himedia Laboratories Pvt. Ltd. (Mumbai, India)

Methods

Biuret assay

The protein was dialyzed using dialysis membrane before preparing the formulations. Concentration of catalase was determined by biuret method. Bovine serum albumin (BSA) was used as a standard protein and was dissolved in 50 mM phosphate buffer (pH 7.0) at different concentrations ranging from 100-2500 μg/ml. BSA solutions were then mixed with biuret reagent and absorbance was measured at 540 nm. Dialyzed catalase solution (0.5 ml) was mixed with biuret reagent and absorbance was recorded at 540 nm on ELISA plate reader (μQuant, New Delhi, India). Concentration of catalase solution was calculated based on calibration curve of BSA.

Activity assay of catalase

Hydrogen peroxide solution (5% H_2O_2) (2.9 ml) was taken in a spectrophotometer cell. Catalase solution in 50 mM phosphate buffer (0.1 ml) was added to it and mixed thoroughly. It was then immediately placed in the cuvette holder. The decrease in absorbance of H_2O_2 was recorded at 240 nm using single beam Beckmann UV/visible spectrophotometer (DU 640, Beckman Coulter, Brea, USA). The time taken for absorbance of H_2O_2 to decrease from 0.45 to 0.40 absorbance units was determined using kinetic mode of the spectrophotometer. Units of biologically active catalase were determined by following formula[21,22]

$$\text{Units/ml} = \frac{(3.45)(\text{df})}{(\text{t})(0.1)}$$

where, 3.45 corresponds to the decomposition of 3.45 μM of H_2O_2 in a 3.0 ml reaction mixture producing a decrease in the absorbance of H_2O_2 from 0.45 to 0.40 absorbance units; df is the dilution factor; t is the time in min required for the absorbance of H_2O_2 to decrease from 0.45 to 0.40 absorbance units; 0.1 is the volume (in ml) of catalase solution taken for the experiment.

Differential scanning calorimetry

Eutectic melting temperature of frozen catalase solution (T_{eu}) was determined using differential scanning calorimeter (TA Q2000, TA Instruments, Delaware, USA). Catalase solution in 50 mM phosphate buffer (3.4 mg) was placed in T_{zero} aluminium pan and sealed hermetically. Empty T_{zero} aluminium pan was taken as a reference. The sample was equilibrated for 2 min at 20°C. It was then frozen in a stepwise manner, initially up to 0°C at a rate of 5°C/min and then up to -50°C at a rate of 1°C/min. Freezing rate was set at 1°C/min to simulate the conditions in the freezing step of lyophilization cycle. Sample was kept at -50°C for 30 s and then heated to 30°C at a rate of 10°C/min. The thermogram was analysed using Universal® software (version 4.5A, TA Instruments, Delaware, USA).[23] The temperature of onset of melting in heating phase of DSC was noted and taken as T_{eu} of the catalase solution in frozen state.

Freezing study of catalase

Different concentrations of catalase ranging from 0.1-6.0 mg/ml were prepared and kept in a bench top lyophilizer (Virtis Bench Top Lyophilizer, New York, USA) at -50°C for 10 h. Thereafter, the frozen samples were thawed at 25°C for 6 h.

Preparation of catalase solutions for freeze-drying

Various concentrations of catalase ranging from 0.1-8.0 mg/ml were prepared in 50 mM potassium phosphate buffer (pH 7.0) in order to study the effect of protein concentration on catalase stability. In case of study involving effect of excipients on catalase stability, samples containing 4 mg/ml catalase and excipients with concentration range 0.1 to 5.0% (w/v) were prepared. For pH stability studies, 4 mg/ml catalase solution was used. Buffer solutions ranging from pH 3 to 11 were prepared as per Indian Pharmacopoeia (4th Edition, 1996). Each sample solution (2 ml) was placed in a 5 ml glass vial. Aluminium foil was wrapped on top of each vial. Pin holes were made on the aluminium foil for escape of water vapour during lyophilization. These vials were then placed in the lyophilizer.

Freeze-drying procedure

Freeze-drying was performed in bench top lyophilizer (Virtis Bench Top Lyophilizer, New York, USA). Vials containing catalase solution were frozen to -60°C at a rate of 1°C/min in

a stepwise manner and were kept at -60°C for 2 h. Primary drying was carried out for 12 h at -35°C that is far below the eutectic melting temperature of catalase solution. End of primary drying was identified as the point where product temperature equalled the shelf temperature. Primary drying was carried out for additional 120 min to ensure complete drying. Secondary drying for catalase solutions was carried out at 30°C for 7 h. End of secondary drying was determined based on residual moisture content in the product. At regular intervals, vials were removed from the lyophilizer and residual moisture content was determined on infrared moisture balance. Secondary drying was continued until moisture content in lyophilized cake was found to be less than 2%. Vacuum during primary drying and secondary drying was kept at 500 and 200 mTorr, respectively. After lyophilization, the glass vials were sealed and stored in a dessicator at 2-8°C until further analysis. The process parameters used for the lyophilization of catalase are shown in Table 1.

Table 1: Lyophilization cycle parameters used for catalase solutions

Lyophilization steps	Temperature (°C)	Time (min)	Vacuum (mTorr)	Ramp (R)/ Hold (H)
Freezing				
	10	10	-	R
	0	10	-	R
	-10	10	-	R
	-25	15	-	R
	-40	15	-	R
	-60	120	-	H
Primary drying				
	-40	20	500	R
	-35	720	500	H
Secondary drying				
	0	30	200	H
	10	10	200	H
	15	10	200	H
	25	10	200	H
	30	420	200	H

Residual moisture analysis

The moisture content was determined using infrared (IR) moisture balance (PM 480 Delta Range, Mettler-Toledo, Columbus, USA). Lyophilized cake (10 mg) was taken on IR moisture balance and percent moisture loss was determined.

RESULTS AND DISCUSSION

Development of lyophilization cycle

Primary drying

Primary drying temperature and primary drying time are the important parameters in primary drying stage.

Primary drying temperature

Selection of primary drying temperature is dictated by the eutectic melting temperature (T_{eu}) or collapse temperature (T_{col}) of the solution. Primary drying temperature is always kept few degrees below that of T_{eu} or T_{col} temperature to avoid melting of the frozen cake, which would otherwise result in collapse of the cake and improper drying of the same.[24-27]

DSC study of catalase solution was carried out to determine critical temperature (T_{eu} or T_{col}) of the solution. Dialyzed catalase solution showed crystallization peak during freezing. This peak corresponds to crystallization of phosphate buffer salts along with water. Since frozen solution of catalase system is in crystalline state, glass transition temperature of frozen system (T_g') was not observed. As shown in Figure 1, the exothermic peak corresponds to spontaneous freezing of catalase solution. In heating run, the transition of melting of the formed ice is represented by T_{eu} followed by complete melting of ice represented by broad endothermic peak. In heating run, T_{eu} of the frozen system was observed at -18.01°C [Figure 1], which is close to the reported collapse temperature of frozen catalase solution (-15°C).[28] Hence, for lyophilization studies, primary drying should be carried out below -18.01°C. With addition of excipients, T_{eu} of the catalase solution can shift towards lower temperature.

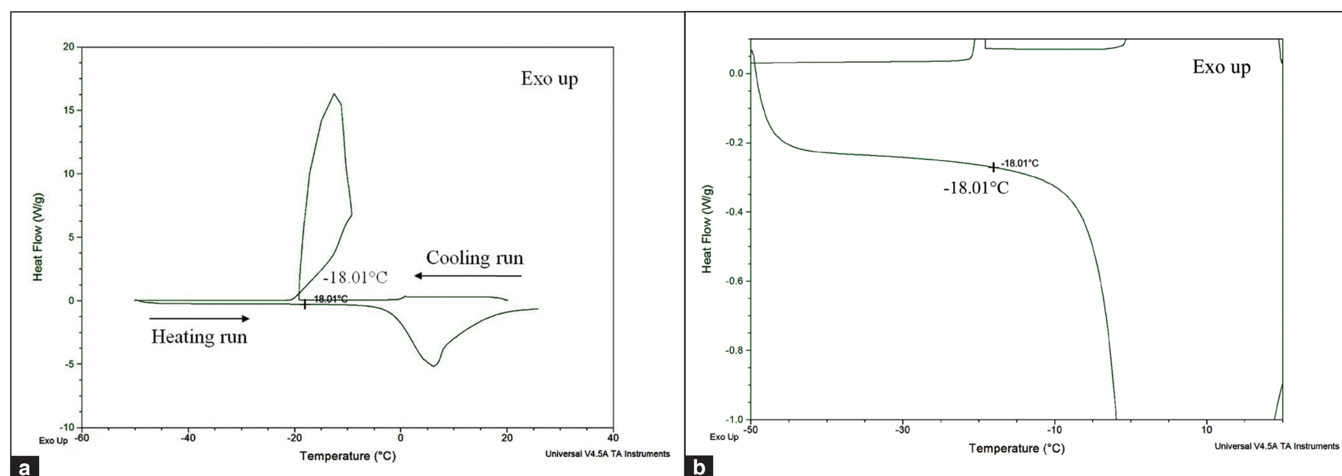

Figure 1: DSC profile of catalase (4 mg/ml, pH 7.0) illustrating thermal events occurring during initial freezing followed by heating of sample in DSC with eutectic melting temperature (T_{eu}) of catalase solution. (a) DSC thermogram of catalase solution showing T_{eu} of -18.01°C. (b) Expanded view of T_{eu} from DSC thermogram of catalase solution

Therefore, in order to keep lyophilization cycle constant for all excipients, primary drying was carried out at -35°C, well below T_{eu} of catalase solution.

Primary drying time

Inadequate primary drying can cause melting of ice and collapse of the cake resulting in improper drying of the product. Hence, it is very important to determine time required for completion of primary drying. Based on product and shelf temperature, primary drying time was found to be 10 h. In order to ensure complete drying, primary drying was carried for additional 120 min.

Secondary drying

Two parameters are important while optimizing secondary drying-secondary drying temperature and secondary drying time.

Secondary drying temperature

Higher the secondary drying temperature, faster will be desorption of water and lesser time will be required for secondary drying to be completed.[29] Secondary drying temperature is dictated by stability of the protein. For heat labile proteins, secondary drying temperature is kept at 25°C or below. For other molecules secondary drying temperature at 30°C or 35°C can be applied. Catalase molecule has been reported to be stable up to 50°C with no loss of activity,[30] hence, the process of secondary drying at 30°C presents no harm to catalase.

Secondary drying time

Residual moisture required in final product dictates the time required for secondary drying. Complete removal of moisture is not recommended since protein molecules require some hydration for their stability. Residual moisture required for stability differs from protein to protein and thus secondary drying time also varies.[31-33] In case of catalase formulations, after completion of 7 h secondary drying, all samples were found to contain residual moisture in the range of 1-2% (w/w).

Vacuum

Degree of vacuum in the lyophilization chamber governs the rate of transfer of effective heat between shelf and product. A moderate increase in vacuum in the chamber causes more effective heat transfer leading to increase in drying rate.[5] Keeping higher vacuum in secondary drying (200 mTorr) as compared to vacuum in primary drying (500 mTorr) facilitated faster water desorption during secondary drying.

Determination of lyophilization step majorly responsible for destabilization of catalase

Lyophilization process involves two denaturation stresses namely freezing stress and drying stress. Some proteins are stable towards freezing stress while they denature in the drying phase of lyophilization, and vice versa. There are some proteins which denature during both freezing as well as drying phase.[29] Determination of stress which is mainly responsible for destabilization of protein during lyophilization is necessary for formulation optimization since it affects the choice of stabilizing agents. For proteins that denature during freezing phase, cryoprotectants like sugars, non-aqueous solvents, surfactants, salts and amines must be used, whereas for proteins that denature during drying phase, lyoprotectants like sugars, polymers, amino acids must be used.[5] Thus, two studies - freezing and freeze-drying were carried out to determine the major stress responsible for loss of activity of catalase during lyophilization.

Freezing study of catalase

No significant change in residual activity of catalase (concentration range 0.1-6.0 mg/ml) was observed after freezing at -50°C for 10 h. Maximum activity retention of about 100% was observed at catalase concentration of 0.5 mg/ml, whereas minimum activity retention was found to be about 80% at catalase concentration of 3 mg/ml. [Figure 2a].

These observations are in accordance with previous reports.[19,28] Shikama *et al.* reported that pure catalase retained 80% of its

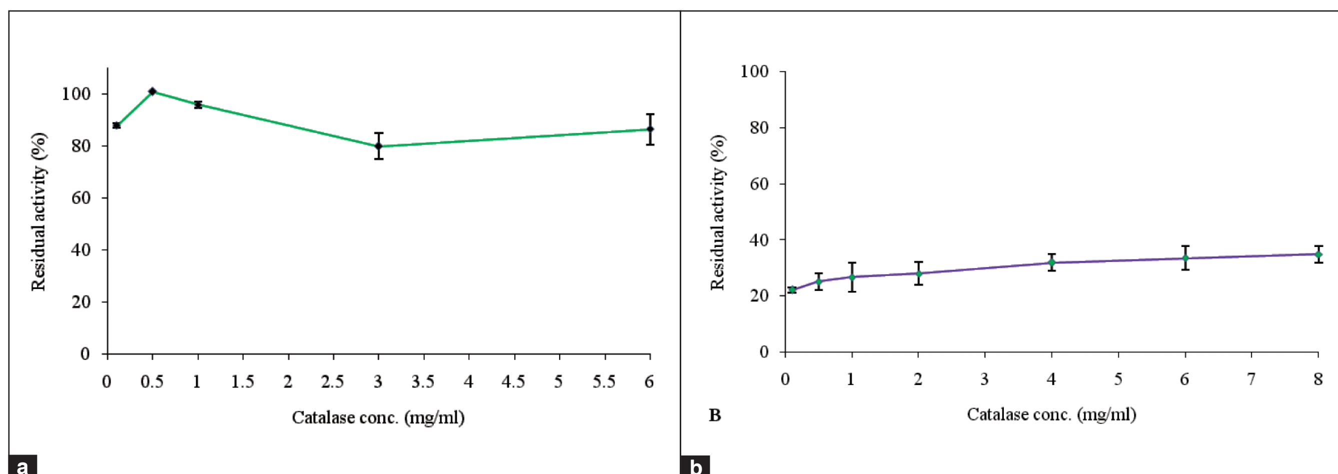

Figure 2: Determination of lyophilization step mainly responsible for activity loss of catalase during lyophilization. (a) Graph showing effect of freeze thawing on the stability of catalase. (Mean ± SD, n = 3) (b) Graph showing effect of catalase concentration on its activity during freeze drying (Mean ± SD, n = 6)

initial activity after freezing.[19] Similarly, Nail *et al.* reported that only 15% activity of catalase was lost during freezing.[28] Hanafusa *et al.* has reported that freezing caused about 40% activity loss, but large conformational changes in the catalase molecule was not observed after freezing, i.e. enzymatic activity of catalase was affected by freezing without the change in the protein conformation.[18] The conformational stability of catalase against freezing was explained by Hanafusa *et al.* by the hypothesis that in case of catalase, a globular protein, hydrophobic bonds are formed in the interior of the molecule and there is relatively smaller surface area of protein contacting water when compared to rod-shaped proteins. Hence, partial dehydration process like freezing will be less effective in disturbing hydration shell of catalase.[18]

Freeze-drying of catalase

Freeze-drying of catalase at concentrations ranging from 0.1 to 8.0 mg/ml caused significant loss of activity at all concentrations. At 0.1 mg/ml catalase concentration, about 22% of initial activity was retained after freeze-drying, whereas at 8.0 mg/ml catalase concentration, about 35% of catalase activity was retained [Figure 2b]. These observations are similar to those reported by Shikama *et al.* wherein pure catalase retained only 30% of its initial activity after freeze-drying.[19] Tanaka *et al.* also reported retention of 35% activity after freeze-drying of catalase.[20] According to Hanafusa *et al.*, after freeze-drying of catalase, catalase molecule dissociates into either 3.8 S (monomer) or 5.8 S (dimer) subunits during freeze-drying, and also exhibits conformational change and unfolding.[18] According to Tanford *et al.*, after lyophilization, the reduced activity of catalase is attributed to its dissociation into monomer and dimer subunits.[34]

Water plays a vital role in binding the subunits of catalase molecule i.e. retaining the quaternary structure required for its biological activity. Freeze-drying dehydrates the protein more than freezing alone, causing destruction of hydrophobic bonds and disruption of intramolecular hydrogen bonds. This removal of hydration shell disturbs the quaternary structure of catalase.[18] From freezing and freeze-drying study, it can be concluded that drying phase of the lyophilization is mainly responsible for the loss of activity of catalase during lyophilization since catalase retained most of its biological activity during freezing phase.

Effect of catalase concentration on its stability during lyophilization

Having established that drying phase plays a critical role in stability of catalase during lyophilization, the effect of catalase concentration on the stability was further evaluated. Plot of residual activity versus concentration showed increased stability of catalase with increase in catalase concentration [Figure 2b]. The results obtained are in good agreement with the literature reports that higher protein concentration retains higher activity during lyophilization.[5] Mechanism of concentration-dependent protein stabilization can be explained by a number of hypotheses. With increase in protein concentration, there is steric repulsion of neighbouring protein molecules which prevents unfolding of other protein molecules during lyophilization. Also, due to higher protein concentration, the finite surface area of ice-water interface formed upon freezing limits the amount of protein to be denatured at the interface and thus the remaining protein molecules in the bulk survive the unfolding process.[35]

Appearance of cake and reconstitution time

Lyophilized samples were visually inspected for appearance of cake. At lower concentrations of catalase, cake had diffuse appearance due to presence of low solid content that prevented structural rigidity required for the cake formation. Bulking agent can be added to improve cake formation. Higher concentration of catalase showed improved structure. All the lyophilized cakes were checked for time of reconstitution. Cakes were porous and got easily reconstituted (less than 5 s) on addition of distilled water.

Effect of pH on stability of catalase during lyophilization

pH stability profile of catalase indicated a bell shaped relationship between residual activity and pH after lyophilization [Figure 3]. pH 7.0 was found to retain highest catalase activity during lyophilization. Catalase activity was observed to be practically zero at extreme pH conditions of 3.0, 4.0, 5.0, 10.0, and 11.0.

The state of ionisation of amino acid residues in the active site of catalase is pH dependent. Since catalytic activity relies on a specific state of ionisation of these residues, enzyme activity is pH dependent. Catalase, being an ampholyte, ionizes to give both anions and cations. At pH below its isoelectric point (pI 5.4), it does not exhibit its catalytic activity because it is present mainly in the form of cations which are catalytically inactive. Catalytic activity of catalase is assumed to be because of anion which can increase above its Isoelectric pH up to pH 9.0.[36] The order of stability of catalase at different pH values during lyophilization was observed as- pH 7>8>6>9. Therefore, pH 7.0 was selected for further studies.

Lyophilization of catalase in the presence of excipient(s)

Excipients from different classes such as amino acids and non-aqueous solvents were screened for their stabilizing/destabilizing

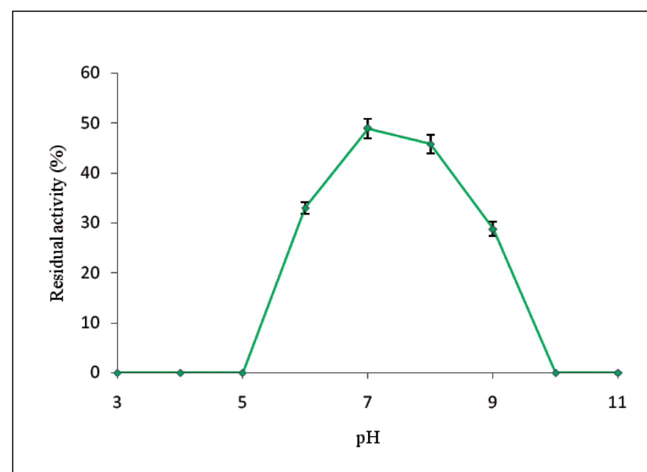

Figure 3: Graph showing the pH-activity relationship for catalase (4 mg/ml) during lyophilization. (Mean ± SD, n = 4)

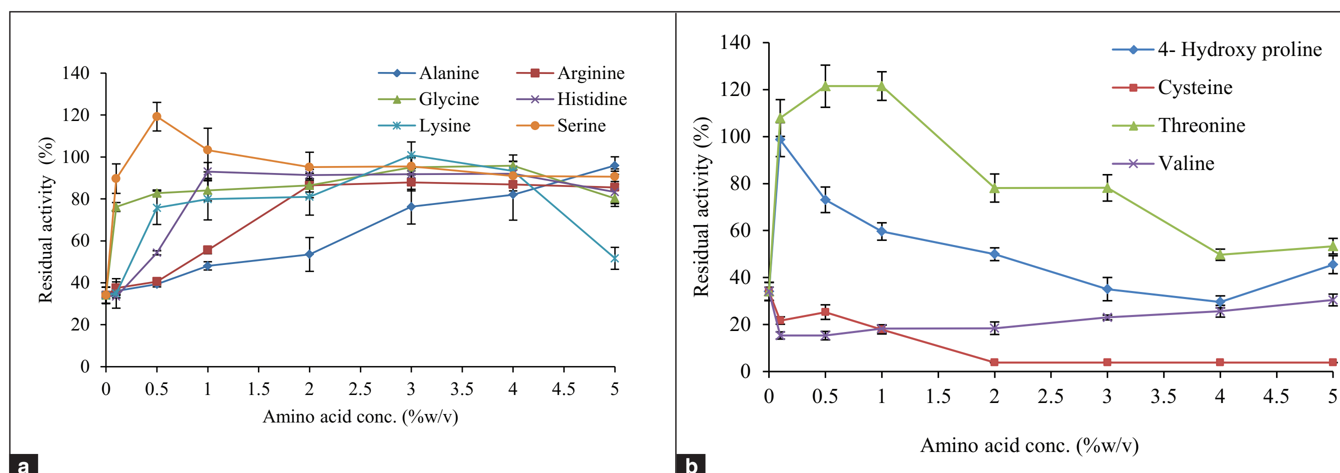

Figure 4: Effects of amino acids on catalase (4 mg/ml) during lyophilization. (a) Graph showing stabilizing effect of alanine, glycine, serine, arginine, histidine and lysine on catalase. (b) Graph showing destabilizing effect of cysteine and valine at all concentrations and effect of 4-hydroxy proline and threonine on catalase. (Mean ± SD, n = 4)

effect on catalase activity during lyophilization. None of the excipients interfered during the activity assay of catalase.

Amino acids

Amino acids are widely reported to act as cryoprotectants as well as lyoprotectants and are used in many commercial protein formulations.[37-40] Amino acids used for the study were neutral amino acids such as alanine, cysteine, glycine, serine, 4-hydroxy proline, threonine, and valine and basic amino acids such as arginine, histidine, and lysine. Effect of these amino acids on the stability of catalase is shown in Figure 4. Alanine, glycine, serine, arginine, histidine, and lysine showed concentration-dependent stabilizing effect [Figure 4a]. 4-hydroxy proline and threonine showed stabilizing effect on catalase only at lower concentrations. At higher concentrations, they showed destabilizing effect on catalase. Cysteine and valine both showed less activity than the control sample after lyophilization within the concentration range studied [Figure 4b], indicating lack of protective effect.

The mechanism by which these amino acids stabilize catalase during lyophilization may vary. It can be attributed to suppression of pH changes resulting from crystallization of buffer salts during freeze-drying.[41] The amino acids tend to bind some amount of water around protein molecules thus helping to maintain their three dimensional conformation. According to water replacement hypothesis, amino acids have the ability to form hydrogen bonds with the catalase molecules. They replace water molecules around the catalase and help in maintaining its three dimensional structure.[18,39] Hydrogen bonding or ion–dipole interactions occurring between amino acid and catalase may favour its stability during lyophilization. Protonated amino acids probably form good hydrogen bonding with catalase because of the presence of protonated nitrogen. Amino acids showing stabilizing effect on catalase probably form amorphous matrix, which restricts catalase mobility, inhibiting catalase unfolding, and aggregation.[39]

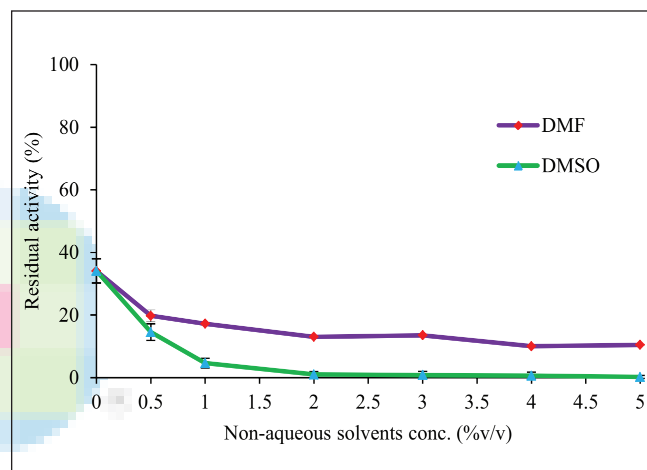

Figure 5: Destabilizing effect of non-aqueous solvents on residual activity of catalase (4 mg/ml) during lyophilization (Mean ± SD, n = 3).

Non-aqueous solvents

In general, non-aqueous solvents denature proteins, but certain non-aqueous solvents have shown cryoprotective effect on few proteins during freezing.[42] Polyethylene glycol 400 protected rabbit muscle lactate dehydrogenase (LDH) during freezing.[43] Ethylene glycol also protected LDH from denaturation on freeze-thawing.[29] Polyethylene glycol showed cryoprotective effect on LDH and phosphofructokinase (PFK) but was unable to protect these proteins during freeze-drying.[44] Glycerol, DMSO, and ethylene glycol have shown cryoprotective effect on catalase during freezing.[19,38,45]

In our study, these solvents were investigated for their effect on catalase during freeze drying. Non-aqueous solvents selected for this study were DMF, DMSO, ethylene glycol, PEG 200, PEG 400, and PEG 600. Catalase solutions containing DMF and DMSO within the concentration range studied, showed less activity than the control indicating their destabilizing effect [Figure 5], whereas ethylene glycol, PEG 200, PEG 400,

and PEG 600 showed complete loss of protein activity (data not shown).

CONCLUSIONS

The effect of freeze-drying on the stability of catalase was investigated. Lyophilization of catalase resulted in 65-78% loss of initial activity depending on its concentration. Drying phase of the lyophilization cycle was found to be major force responsible for the destabilization of catalase. The optimum pH for catalase formulation during freeze drying was found to be 7.0. Amino acids like alanine, glycine, serine, and lysine stabilized catalase up to more than 95% while arginine and histidine stabilized catalase up to ~90% during lyophilization. Threonine and 4-hydroxy proline stabilized catalase at lower concentrations while at higher concentration they showed destabilizing effect on catalase. Valine and cysteine and non-aqueous solvents- DMF, DMSO, ethylene glycol, PEG 200, PEG 400, and PEG 600 showed destabilizing effect on catalase during lyophilization. Thus, in order to prevent loss of catalase activity while lyophilizing catalase, use of amino acids like alanine, glycine, lysine, serine, and 4-hydroxy proline in optimum concentration is highly advisable.

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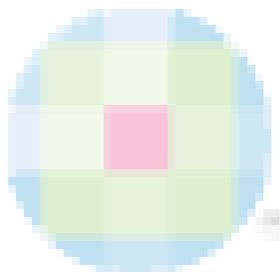

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