Investigation of Histidine Stabilizing Effects on LDH During Freeze-Drying

ANAS AL-HUSSEIN,[1] HENNING GIESELER[1,2]

[1]Division of Pharmaceutics, Freeze Drying Focus Group, University of Erlangen, Erlangen 91058, Germany

[2]GILYOS GmbH, Wuerzburg 97076, Germany

ABSTRACT: The objective of this study was to investigate the effect of histidine on the stability of the model protein lactate dehydrogenase (LDH) during freeze-drying. Several parameters were varied including pH of the bulk solution, histidine concentration, and performance of an annealing step during freezing. First, histidine was used as a buffer in the protein formulations and compared with "conventional" potassium phosphate and citrate buffer systems. For this purpose, sucrose or mannitol was used as stabilizers. Second, the possibility of using histidine as both buffer and stabilizer (cryoprotectant and lyoprotectant) in the protein formulations was evaluated with focus on protein stability and the physical state of histidine in the final product, in addition to cake elegance. Protein stability was evaluated both functionally by measuring the activity recovery of the model protein LDH after freeze-drying and structurally by analyzing the protein secondary structure. LDH showed improved stability in histidine buffer in comparison with other buffers. Protein stability and the tendency of histidine to crystallize during freeze-drying were pH dependent. Annealing destabilized LDH and resulted in a decrease of the activity recovery. However, the extent of protein destabilization caused by annealing appears to be also pH dependent. © 2012 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 102:813–826, 2013

Keywords: freeze drying/lyophilization; stability; proteins; excipients; physical characterization; buffers

INTRODUCTION

Advances in biotechnology over the last decades resulted in an increasing number of therapeutic proteins. Protein-based products are likely to represent four of the five top-selling drugs globally by 2013.[1] The maintenance of protein stability and efficacy in the dosage form presents a great challenge to the pharmaceutical industry. Because of their limited stability in an aqueous environment, proteins often need to be converted into solid state to achieve an acceptable shelf life as pharmaceutical products.[2] The most commonly used method for manufacture of solid protein pharmaceuticals is freeze-drying (lyophilization).[3,4] However, the freeze-drying process generates many stresses during both freezing and drying, which may cause the loss of protein bioactivity. Therefore, a range of excipients can be added to the protein formulation to overcome these stresses and to improve protein stability during freeze-drying and storage. This goal is commonly achieved by using amorphous excipients to serve as protein stabilizer during both freezing (cryoprotectant) and drying (lyoprotectant).[3,4] One of the most widely accepted protein stabilization mechanisms during freezing is preferential exclusion, which means that the excipient is preferentially excluded from the surface of the protein. Thereby the free energy required for denaturation is increased and the native structure of the protein is stabilized.[5] During the drying phase, the protein is stabilized by the water replacement mechanism and by the formation of a viscous glassy state. The water replacement mechanism involves the formation of hydrogen bonds between a protein and an excipient to satisfy the hydrogen-bonding requirement of polar groups on the protein surface.[6] The formation of an amorphous viscous gla: ying and the corresponding ext

Correspondence to: Henning Gieseler (Telephone: +49-931-90705678; Fax: +49-931-90705679; E-mail: info@gilyos.com)

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also increase protein stability by retarding protein denaturation.[7,8]

Disaccharides such as sucrose and trehalose are widely used as protein stabilizers, and these sugars have been extensively studied in the literature to investigate their stabilizing effect on proteins during freeze-drying. Several amino acids are frequently cited as being suitable excipients for freeze-drying of proteins. Glycine, for example, is widely used as crystalline bulking agent in freeze-dried formulations,[9] whereas other amino acids such as lysine and arginine have been described as possible buffers in protein formulations.[10] Arginine in combination with phosphoric acid was reported to exert a stabilizing effect on lactate dehydrogenase (LDH) during freeze-drying.[11] In contrast to sugars, amino acids can also function as buffers, and therefore, provide more choices/flexibility for the design of proteins formulations. Histidine is one of the amino acids that can be used in protein formulations to function as both buffer and protein stabilizer. Histidine, which has three ionization sites on the molecule's carboxyl, imidazole, and amino group with pK_1 of 1.9, pK_2 of 6.1, and pK_3 of 9.1, has been used as a buffer especially in the pH range 5–7.[12]

Several studies have already referred to a stabilizing effect of histidine on proteins during freeze-drying. Although most of the stability studies deal with in-process instability of proteins, there were some (long-term) storage-stability studies. Osterberg et al.[10] described the development of a stable freeze-dried formulation for recombinant factor VIII-SQ (r-VIII SQ) without the addition of albumin. The authors found that a combination of sucrose, nonionic surfactant (polysorbate 80), crystalline bulking agent (sodium chloride), and L-histidine preserve factor-VIII activity during freeze-drying and storage. It was also reported that L-histidine, besides functioning as a buffer, also had a stabilizing effect on r-VIII SQ during freeze-drying and storage. However, it is important to underline that the stabilizing effect of histidine was not studied in depth and not delineated and differentiated from the stabilizing effect of other stabilizers used in the same formulation. Cleland et al.[13] used histidine as a buffer for the freeze-drying of a monoclonal antibody, rhuMAb HER2. The authors compared the stability profile of rhuMAb HER2 formulated at 25 mg/mL in either 5 mM succinate (pH 5) or 5 mM histidine (pH 6) in the presence of other excipients. They found that in the absence of sugar, a greater extent of aggregation was observed in the histidine formulation than in the succinate formulation. Chen et al.[14] found that the increase of the histidine concentration from 4 to 6 mM reduced the soluble-aggregate levels of a human anti-IL8 monoclonal antibody (ABX-IL8) upon freeze-drying. The authors used multiple excipients systems consist-

ing of glycine, glutamic acid, mannitol, and polysorbate 20. Furthermore, the freeze-dried monoclonal antibody trastuzumab (Herceptin®) produced by F. Hoffmann-La Roche (Basel, Switzerland) is formulated with histidine.

Overall, the reported studies about the stabilizing effect of histidine on proteins during freeze-drying are still limited, and all of these studies did not differentiate between the buffering effect of histidine and the other possible stabilizing effects of this amino acid on the proteins. Furthermore, literature does not provide data regarding the influence of histidine on proteins in the absence of other excipients that are regularly included in protein formulations. The presence of such excipients might complicate the identification of the effect of histidine on protein stability during freeze-drying.

The scope of the present study was to investigate the influence of histidine on the stability of a model protein LDH for concentrations relevant for use as a buffer and as a stabilizer. Histidine buffer was compared with other common buffers (potassium phosphate and citrate). Furthermore, the ability to use histidine as a sole excipient in protein formulation was investigated. Moreover, the effect of pH and histidine concentration in the pre-freeze-dried bulk solution on both protein stability and the elegance of the final freeze-dried product was investigated. Because the amorphous state of the stabilizer is an essential property for the stabilization of protein during freeze-drying, the physical state of histidine in freeze-dried samples with and without annealing was analyzed and correlated with the protein stability. LDH was selected for this study because of its well-documented labile nature and sensitivity to the stresses generated during freeze-drying.[15] A comparably low protein concentration of 15 µg/mL was employed to avoid protein self-protection, which is present at high concentrations.[6,16]

MATERIALS

l-Lactate dehydrogenase type II from rabbit muscle (11.4 mg protein/mL; 1150 units/mg) was used as aqueous suspension in ammonium sulfate and purchased from Sigma–Aldrich (Munich, Germany). L-Histidine, sodium pyruvate, and β-nicotinamide adenine dinucleotide (NADH) were also obtained from Sigma–Aldrich at analytical grade. Sucrose was obtained from Fluka Analytical (Buchs, Switzerland) and D-mannitol was purchased from Riedel-de Haën (Seelze, Germany). Potassium dihydrogen phosphate (KH_2PO_4) and citric acid were obtained from Carl Roth (Karlsruhe, Germany) to prepare phosphate and citrate buffers, respectively. The pH of formulations containing histidine was adjusted to 4, 5, 6, and 7

Table 1. Overview of the Studied Formulations and the Buffers in Each Part of this Study

Studied Aspect	LDH (μg/mL)	Buffer Concentration	pH		Nonbuffer Solute Concentration	
Histidine as a buffer (comparison with other buffers)	15	Citrate (10, 50, and 150 mM)	7.3	(Only buffer)	Sucrose, S (10 mg/mL)	Mannitol, M (10 mg/mL)
	15	Histidine (10, 50, and 150 mM)	7.3	(Only buffer)	Sucrose, S (10 mg/mL)	Mannitol, M (10 mg/mL)
	15	Phosphate (10, 50, and 150 mM)	7.3	(Only buffer)	Sucrose, S (10 mg/mL)	Mannitol, M (10 mg/mL)
Histidine as a stabilizer (comparison with other solutes)	15	Potassium phosphate (10 mM)	7.3		Sucrose, S (100 mM)	
	15		7.3		Mannitol, M (100 mM)	
	15		7.3		Histidine, H (100 mM)	
	15		7.3		(Only buffer)	
Histidine as buffer and stabilizer (histidine-concentration effect)	15	Histidine	7.3		Histidine (2 mg/mL)	
	15		7.3		Histidine (5 mg/mL)	
	15		7.3		Histidine (10 mg/mL)	
	15		7.3		Histidine (20 mg/mL)	
	15		7.3		Histidine (35 mg/mL)	
Histidine as buffer and stabilizer (effect of solution pH)	15	Histidine	4		Histidine (20 mg/mL)	
	15		5		Histidine (20 mg/mL)	
	15		6		Histidine (20 mg/mL)	
	15		7		Histidine (20 mg/mL)	
	15		8		Histidine (20 mg/mL)	

with hydrochloric acid and to 8 with sodium hydroxide, whereas potassium hydroxide was used to adjust the pH in the formulations containing KH_2PO_4 as a buffer. Sodium hydroxide was used also to adjust the pH when citrate buffer was used. Potassium hydroxide, sodium hydroxide, and hydrochloric acid were purchased from Sigma–Aldrich. Vials (2 mL) were purchased from SCHOTT forma vitrum (Müllheim, Germany). FluroTec® 13-mm stoppers were obtained from West Pharmaceutical Services (Eschweiler, Germany). To detect the possible pH shifts in the studied formulations during freezing, a universal indicator solution was purchased from Sigma–Aldrich.

The range of formulations that were freeze-dried and analyzed is illustrated in Table 1. The experiments were structured into four parts to evaluate the effect of histidine on LDH when applied as a buffer and as a stabilizer. First, histidine was used as a buffer in three different concentrations and compared with two other common buffer systems. The buffer formulations were also freeze-dried with addition of sucrose and mannitol. Second, histidine was used as a stabilizer and compared with sucrose and mannitol. The same buffer system was used for all combinations and was also freeze-dried without addition of stabilizers for reference. In the third and fourth part, formulations containing only LHD and histidine in different concentrations and pH levels were investigated under consideration of the physical state.

METHODS

Preparation of Enzyme Solutions

The LDH suspension was dialyzed with a special membrane (Spectra/Por® membrane, Spectrum Laboratories, Rancho Dominguez, California) with a molecular weight cut off (MWCO) of 12–14 kDa (molecular weight of LDH: 140 kDa). Dialysis was performed against potassium phosphate buffer (pH 7.3) at 5°C overnight. The obtained enzyme solution was concentrated with an Amicon Ultra-15 centrifugal filter device (MWCO 30 kDa; Millipore Corporation, Billerica, Massachusetts) in a centrifuge (Minifuge RF, Heraeus Sepatech GmbH, Osterode, Germany). Protein concentration was determined spectrophotometrically at 280 nm. Aliquots of dialyzed LDH and excipients solution were mixed in glass vials to obtain 0.5-mL samples with a final concentration of 15 μg/mL enzyme.

Freeze-Drying Process Conditions

Individual formulation containing LDH (0.5 mL) was filled into 2-mL vials, and the vials were subsequently semistoppered. Freeze-drying experiments were performed using a FTS Lyostar™ II (SP Scientific, Gardiner, New York). Samples were loaded onto the shelves at room temperature, frozen using a 1°C/min shelf cooling rate down to−40°C, and maintained at this temperature for 1 h. Annealing (when applied)

Table 2. Obtained Glass Transition Temperature (T'_g) for the Studied Formulations

Formulations[a]		T'_g (°C)[b]
Only histidine formulations[c]	pH 4	−49.66
	pH 5	−49.12
	pH 6	−40.80
	pH 7	−35.50
	pH 8	−36.85
Sucrose formulations[d]	In phosphate buffer	−34.07
	In citrate buffer	−33.25
	In histidine buffer	−30.80
Mannitol formulations[d]	In phosphate buffer	−32.35
	In citrate buffer	−33.27
	In histidine buffer	−38.58

[a]LDH concentration in all studied formulations is 15 µg/mL.
[b]Measurements were performed in triplicate ($n = 3$). All standard deviations were <1.4°C.
[c]Histidine concentration is 20 mg/mL.
[d]pH 7.3.

was conducted by ramping the shelf temperature from −40°C to −20°C at 1°C/min and keeping this shelf temperature for 4 h for thermal treatment. Then, the product was frozen back to −40°C and kept at this temperature for 1 h. For all formulations, primary drying was performed by controlling the shelf temperature at −30°C and the chamber pressure at 80 mTorr for 25 h. The selected shelf temperatures for annealing and primary drying were selected on the basis of the glass transition temperatures (T'_g) of the studied formulations, which were determined by differential scanning calorimetry (DSC). It is well known that annealing should be carried out at temperature above T'_g of the formulation.[3] The selected annealing temperature in this study (−20°C) was higher than the measured glass transition temperatures of all studied formulation. The glass transition temperatures (T'_g) of the studied formulations are reported in Table 2. The conditions used are very conservative for conventional freeze-drying, and the rationale behind the use of such experimental parameters is (1) to avoid any impact of the primary drying phase on the physicochemical state of the mixture and (2) to verify that differences in protein stability between different formulations did not arise from differences in drying conditions.

Lastly, secondary drying was carried out at the same chamber pressure applied during primary drying but increasing the shelf temperature to +40°C at a ramp rate of 0.1°C/min and maintaining this temperature for 4 h. The shelf temperature and duration of secondary drying were selected to obtain final products with relatively low and comparable residual moisture content to ensure that differences in protein stability between different formulations are not attributed to different residual moisture contents. Throughout this study, product temperatures during the cycle were measured using calibrated 36 gauge

T-Type copper/constantan thermocouples from Omega (Omega Engineering, Stamford, Connecticut). Each thermocouple was introduced through a stopper and positioned bottom-center in the vial to achieve both representative temperature monitoring as well as accurate endpoint detection of the time point when no ice was left in the product.

Assay to Assess the Enzymatic Activity

Enzymatic-activity recovery was used in this study as an indicator for the functional stability of LDH. LDH catalyzes the interconversion of pyruvate to lactate with concomitant interconversion of NADH to NAD⁺. The decomposition of NADH was measured by the decrease in absorption at 340 nm. A 10-mm quartz cuvette was placed into a Lambda 25-UV/Vis spectrometer (PerkinElmer, Rodgau, Germany) connected to a computer system (WinLab V 5.0 software, PerkinElmer). The rate of absorbance decrease is directly proportional to LDH activity in the sample. Activity was measured before freeze-drying and after reconstitution of the freeze-dried product. The enzyme activity was defined as 100% in the solution prior to freezing, and the remaining activity of LDH in the reconstituted freeze-dried samples was expressed as percentage of the original activity before freezing. All assays were performed in triplicate. Both the mean value and the standard deviation were calculated. The variation coefficient of this assay ranged between 4.3% and 4.7%.

Turbidity Measurements

The turbidity of solutions was measured to quantify protein denaturation that led to the formation of insoluble protein aggregates. Turbidity values were obtained by UV-spectroscopy measurements at 350 nm.

Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy (FTIR) was employed to evaluate potential changes in protein secondary structure after freeze-drying. The evaluation was executed through comparison of second-derivative spectrum of untreated LDH before freeze-drying with second-derivative spectra of LDH obtained after the reconstitution of freeze-dried samples. FTIR spectra of samples containing LDH were obtained using a Nicolet Magna IR 550 FTIR spectrometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts). The apparatus was constantly purged with dry air. Samples were measured in a temperature-controlled CaF₂ window with a fixed sample-layer-thickness of 5.6 µm. The water spectrum was subtracted from the sample spectrum using the Nicolet Omnic software (Thermo Scientific, Waltham, MA, USA). A subtraction of the background

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