Investigation of Histidine Stabilizing Effects on LDH During Freeze-Drying

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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

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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 glass during freeze-drying and the corresponding extremely high viscosity

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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 consisting 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