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Investigation of excipients on the denaturation of proteins during freezing and freeze drying

J. U. ANEKWE, R. T. FORBES, R. WILLSON* AND P. YORK

*Drug Delivery Group, Postgraduate Studies in Pharmaceutical Technology, The School of Pharmacy, University of Bradford, Bradford BD7 1DP, and *SmithKline Beecham Pharmaceuticals, New Frontiers Science Park, Harlow, Essex CM19 5AW*

The inherent chemical and physical instability of most proteins can cause problems during their formulation, purification and storage. Often proteins are formulated as freeze dried products to achieve long term stability. Additives including sugars, amino acids and surfactants have been added to formulations to prevent inactivation, on exposure to freezing and dehydration stresses Carpenter et al (1993). However, excipient selection for the protection of proteins is often empirical due to the lack of a full understanding of the stresses and mechanisms involved.

In this study, we present a two stage approach to examine the effect of both freezing and freeze-drying induced stresses on the activity of two model proteins, as influenced by several stabilising excipients. The excipients Polyethylene glycol (PEG, 6000 MW), Polyvinyl pyrrolidine (10,000 MW) and Dextran (42,000 MW), were chosen to examine separately their capacity to protect proteins during the freezing stage alone, and during freeze drying. The model proteins were β-galactosidase from *Aspergillus oryzae* (Sigma) and Hen Egg Lysozyme (HEL) (Boehringer), and additives were studied in the concentration range from 1 to 10%w/v. Protein solutions were prepared as described by Izutsu et al (1993), and the concentration was determined by the Lowry method. The additives were then added individually to the protein solutions to provide a final protein concentration of 20μg ml^{-1}. Freeze dry experiments were performed using a Dura Stop MP Lyophiliser (FTS, USA). Freeze thaw experiments were performed by immersing the solutions in liquid nitrogen for 1 minute, transferring to the pre-cooled lyophiliser at -45°C for 12 hours, and thawing at room temperature. The effect of the additives was estimated by protein activity measurements, and were expressed as percentage of initial activity retained after freezing or freeze drying. Assay of β-galactosidase and HEL were carried out using 2-nitrophenyl-β-D-galactopyranoside and *Micrococcus lysodiekiticus* respectively as substrates.

On freeze drying, in the absence of excipients enzymatic activity recovered was greatly reduced, to approximately 30% of original value in the case of β-galactosidase. In the presence of all the excipients, residual activity was further reduced than compared to with protein alone. On freeze thawing, in the absence of additives both proteins retained between 70-80% of their inital activity. On addition of excipients retained activity was again less than with the protein alone, with the exception of PEG 10%w/v for which almost full enzyme activity was recovered after freeze-thawing. For all excipients, overall activity retained was higher on freezing than compared to freeze drying, with DSC measurements indicating excipient crystallisation on freeze drying.

This method clearly demonstrates the usefulness of PEG in the protection of proteins against freeze-induced damage. Work is currently in progress to apply the two-stage method presented, to mixed systems in which a second component can be tested for it's capacity to stabilise dried proteins, independent of it's ability to provide cryoprotection.

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