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Stabilisation of proteins via mixtures of amino acids during spray drying

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ABSTRACT

Biologicals are often formulated as solids in an effort to preserve stability which generally requires stabilising excipients for proper drying. The purpose of this study was to screen amino acids and their combinations for their stabilising effect on proteins during spray drying. Catalase, as model protein, was spray dried in 1 + 1 or 1 + 2 ratios with amino acids. Some amino acids namely arginine, glycine and histidine showed good retention of catalase functionality after spray drying and subsequent storage stress. A 1 + 1 combination of arginine and glycine in a 1 + 2 ratio with catalase resulted in a tremendously good stabilising effect. Storage at high temperature/humidity also showed beneficial effects of this combination. To evaluate whether this was a general principle, these findings were transferred to an antigenic protein of comparable size and supramolecular structure (haemagglutinin) as well as to a smaller enzyme (lysozyme). Upon spray drying with the combination of amino acids it could be shown that both proteins remain more stable especially after storage compared to the unprotected protein. The combination of arginine and glycine is tailored to the needs of protein stabilisation during spray drying and may hence be utilised in dry powder formulation of biomolecules with superior stability characteristics.

1. Introduction

The biopharmaceuticals market is rapidly escalating and now is the fastest growing division of pharmaceuticals (Global, 2012). However, formulating proteins still remains a major challenge as they are only marginally stable and are prone to chemical and physical degradations such as oxidation, deamidation, hydrolysis, conformational changes, undesirable adsorption to surfaces, precipitation and aggregation (Capelle et al., 2007). Poor long-term stability due to a fragile structure has increased the demand for more stable solid protein formulations which assure storage stability and ease distribution (Arakawa et al., 2001). Water plays an important role in degradative reactions by mobilising reactants and facilitating structural changes in proteins. Hence, for effective storage, water must be removed or immobilised without disrupting the functional structure of the protein (Maltesen and van de Weert, 2008).

Solid formulations of biopharmaceuticals can improve thermostability and will prevent temperature damage promoting reduced wastage, decreased logistical and equipment requirements as well as lower costs of transportation and storage (Maa and Prestrelski, 2000; PATH, 2012). This is especially interesting for the field of vaccination, where poor vaccination rates can be a result of improper vaccine stability (Miskinis, 2007). Vaccines not requiring any refrigeration which can be stored safely at room temperature are an important goal envisioned by WHO's Global Immunisation Vision and Strategy (WHO Department of Immunization, 2005).

The most common drying method used for biomolecules is lyophilisation (freeze drying), which involves freezing a protein solution; drying it at reduced pressure to remove the majority of bulk water by sublimation and a secondary drying step by thermal desorption to produce a dry cake (Matejtschuk, 2007). However, freeze drying has some limitations such as it does not lead to a dispersible fine powder; it is a time- and energy-consuming process and can hence be very expensive (Roy and Gupta, 2004). Freeze drying can be harmful to proteins due to stress caused by freezing and also during drying which can induce conformational instability. During freezing the molecule needs to be protected by cryoprotectants from harmful effects of the forming ice crystals and a shift in pH, which may easily occur due to the formation of saturated solutions differing in salt composition from the original buffer during freezing. Afterwards, the molecule needs to be stabilised by lyoprotectants from dehydration during the removal of water. This can be achieved by an exchange of water with other hydrophilic molecules which may replace it as hydrogen bond forming partner. Another effective and versatile method for converting proteins into a dry powder is by spray drying (Schüle et al., 2008). This process involves atomisation of the protein solution via a nozzle into a drying chamber containing hot air forcing the protein solution

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Table 1
Commonly studied excipients along with their mechanism of stabilisation used for spray drying of proteins.

Protein/antigen	Excipient used for stabilisation	Possible mechanism of stabilisation	Reference
Immunoglobulin G Lactose dehydrogenase Influenza antigen	Trehalose	Glassy state stabilisation; water replacement hypothesis	Maury et al. (2005) Adler and Lee (1999) Amorij et al. (2007)
Alkaline phosphatase	Sodium carboxymethylcellulose	Glassy state stabilisation; water replacement hypothesis	Li et al. (2010)
Tissue-type plasminogen activator Human growth hormone (hGH)	Polysorbate 20	Prevention of protein adsorption to the air–liquid interface	Mumenthaler et al. (1994) Maa et al. (1998)
Oxyhaemoglobin	Sucrose	Glassy state stabilisation; water replacement hypothesis	Labrude et al. (1989)

to instantly dry into solid particles (Lee, 2002). Generally, spray drying is a gentle process due to short process time (10^{-4} s from droplet formation to the dried particle being collected in a laboratory equipment (Elversson and Millqvist-Fureby, 2005b) and low thermal stress due to the cooling of evaporation. Nonetheless, proteins may come across several stress factors during spray drying such as shear stress when the protein is atomised into fine droplets, exposure to the air/liquid interface at the droplet surface possibly causing denaturation and increased temperature when the protein comes in contact with the drying air and loses its hydration shell which may induce protein denaturation and aggregation resulting in loss of protein activity (Lee, 2002). Therefore, the protein needs effective protection and stabilisation.

Several stabilising excipients have been studied which can be added to a protein solution prior to spray drying and shall prevent degradation during processing and subsequent storage (van der Walle, 2011). Disaccharides such as sucrose and trehalose are commonly used along with surface active agents for limiting the denaturation of proteins (Adler et al., 2000). Table 1 summarises commonly used excipients along with their mechanism of stabilisation.

Amino acids are well-known stabilisers for biomolecule formulations (Hasija et al., 2013). Histidine is often used as a buffering agent and also as an antioxidant in several formulations (Hasija et al., 2013). Glycine has also been used a buffering agent and as a bulking agent during lyophilisation (Pikal-Cleland et al., 2002). Arginine has been used as a solubilising agent during purification steps and as an excipient highly effective in suppressing protein–protein interaction and therefore minimising protein aggregation. Hydrophobic interaction with proteins is a potential mechanism by which arginine suppresses aggregation of proteins in solution (Das et al., 2007). In solid state, amino acids are reported to stabilise proteins by direct binding to protein molecules via hydrogen bonding or ion–dipole interactions (Hasija et al., 2013). A handful of amino acids are also reported to form amorphous solids upon drying (Mattern et al., 1999). Recently, a complex mixture of five to seven amino acids in combination with other molecules such as saponins has been shown to have excellent stabilising properties for biomolecules being immobilised and dried on surfaces (Tscheliessnig et al., 2012). A list of several marketed formulations of biomolecules containing amino acids is summarised in Table 2.

Table 2
Marketed protein therapeutics containing amino acids as a stabilising excipient (Adler and Lee, 1999; Hasija et al., 2013).

Amino acid	Marketed products	Mechanism of stabilisation
L-Arginine	TNKase®, Activase®	Suppressing aggregation; interaction with protein
Glycine	Nutropin®, Synagis®	Buffering agent
L-Glutamate	Streptase®, RabAvert®, Flumist®	Specific interaction with protein
L-Histidine	Herceptin®, BeneFIX®, Gardasil®	Buffering agent; antioxidant

Various mechanisms have been put forward that can (at least in parts) explain the lyoprotecting effects of different excipients (Elversson and Millqvist-Fureby, 2005a):

(a) *Water replacement hypothesis* (Carpenter et al., 1994; Allison et al., 1999): Proteins usually bind to many water molecules in solution. As water is removed during drying, the excipient forms hydrogen bonds with the protein to create a water-like environment around the protein and strengthen the existence of its tertiary structure.
(b) *Glassy state stabilisation* (Green and Angell, 1989; Chang and Pikal, 2009): Stabilisers form a glassy matrix on drying in which the protein is molecularly dispersed and its molecular mobility is limited. Due to the amorphous character of the glass it enables the formation of hydrogen bonds with proteins allowing the excipients to act as a water substitute.
(c) *Reducing surface adsorption/concentration*: Surfactants reduce the concentration of protein at the surface of the drying phase due to their own surface activity thereby preventing interfacial-induced denaturation (Maa et al., 1998).

Protection during drying is needed in both freeze drying and spray drying, whereas particular attention should be given to stabilisation during droplet formation in spray drying. Here, increased shear stress is imposed on the formulation and surface area is largely increased, which emphasises the importance of stabilisation mechanism (c) in this process.

In this study, the potential of different amino acids as stabilising excipients for biomolecules, here the model protein catalase, in spray drying and subsequent storage was systematically evaluated. Further, the best composition was selected to prove its stabilising capacity for a small enzyme, lysozyme, and the influenza antigen haemagglutinin in spray drying.

2. Materials and methods

2.1. Materials

Bovine liver catalase was purchased from Sigma–Aldrich (St. Louis, MO, USA) as a lyophilised powder with an activity of 2000–5000 units/mg protein. Lysozyme was also purchased from Sigma–Aldrich (St. Louis, MO, USA) as a crystalline powder with an activity of 70,000 units/mg. The amino acids L-arginine (Arg), glycine (Gly), L-histidine (His), L-lysine (Lys), L-leucine (Leu), L-methionine (Met), L-phenylalanine (Phe), L-tryptophan (Try) and L-tyrosine (Tyr) were also obtained from Sigma–Aldrich (St. Louis, MO, USA). Trehalose dihydrate was obtained from British sugar plc (Peterborough, UK). All reagents used were of analytical grade.

Pandemrix influenza vaccine (GlaxoSmithKline) was a generous gift for research purposes by the government of Brandenburg, Germany. The vaccine contains an (H1N1)v (split virion, inactivated) A/California/7/2009 (H1N1)v like strain (x-179a) at a