

Protein DPI production: discriminating destabilising influences during spray drying

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Summary

Protein formulations are especially challenging since proteins tend to aggregate or denaturise easily, resulting in a possible function loss. Most protein drugs are parenteral solutions having several disadvantages; an alternative would be a dry powder formulation for inhalation. Being a dry powder the drug should be more stable and, since sterility is not necessary for inhalation, easier to formulate. In this study, chymotrypsin as model protein and mannitol as stabilising excipient were investigated to examine the influence of the drying process in a Nano Spray Dryer B-90 HP on the protein structure. The focus laid on the impact of the different stress factors during the stages of spray drying on the protein. During pumping shear forces and adsorption to tubing material, during atomisation shear forces and ultrasonic sound and during drying, air-liquid-interfaces, thermal stress and dehydration occur. All stress factors can possibly lead to denaturation or aggregation. To detect structural changes the chymotrypsin activity assay and the measurement of changes in the fluorescence emission spectra of the fluorescent dye anilinonaphthalene-8-sulfonic-acid were used. It was found that mannitol stabilises the protein against high temperatures. Different results occurred for the stages of spray-drying. The pumping process did not cause structural changes. Atomisation, however, seems to influence the protein structure, although the enzyme activity did not change. The drying did not only influence the protein structure but also caused a small decrease in enzyme activity. Further studies will be conducted to find the best production parameters to get a stable product.

Key Message

To achieve stable protein DPI products, it is important to identify destabilising factors during production to address these with stabilising excipients. Individual stages in spray drying caused different structural changes, drying even decreased enzyme activity. Mannitol stabilised the protein against heat, whereas for other stress factors excipient selection is ongoing.

Introduction

Nowadays, protein drugs are usually formulated as solutions for parenteral application. The formulation is especially challenging because the drug has to be sterile, isotonic and isohydric but also needs a stabilising environment for the protein. Usually those formulations have to be stored cool. Proteins being sensitive drugs are likely to undergo structural changes during production and storage [1]. This could influence the drug's effect. Proteins in dry powder formulations are less prone to aggregate or denaturise [1]. This can lead to longer shelf life and may make cooling unnecessary [1]. A possible formulation would be a dry powder formulation for inhalation (DPI).

Pulmonary delivered protein drugs like Dornase alfa against cystic fibrosis are especially important for the local treatment of respiratory diseases, since the protein drug's distribution to the lung from systemic circulation is often limited [2]. However, pulmonary protein delivery can also be suitable for systemic targeting for protein drugs with a good ability to cross the blood-air barrier [2]. Furthermore, these formulations do not need to meet the criteria for parenteral use, therefore making protein stabilisation easier. For protein DPI production, spray drying is a suitable formulating option. However, during different stages of spray drying, stress factors occur and can be challenging for the proteins' structural stability. At first, the pumping process exposes the protein to possible adsorption stress at tubing-liquid interfaces and shear forces. During atomisation more shear forces occur. In case of nebulisation an additional stress factor would be ultrasonic frequency. Last, during drying, air-liquid-interfaces, thermal and dehydration stresses occur. All these could lead to aggregation or denaturation [3].

In general, sugars, like trehalose, are known stabilising excipients against heat and dehydration for protein drugs. This effect seems to rely on the hydrogen bonds between sugar and protein, mostly based on the theory of replacing the molecular water or causing a steric hindrance [3].

To investigate these phenomena, a study utilising the peptidase chymotrypsin, a model protein with a molecular weight about 25 kDa and good analytical properties, and mannitol, a not reducing sugar alcohol as excipient for inhalation, was performed.

Experimental methods

For discrimination of the possible destabilising influences during spray drying, the performed study investigated the different stress factors separately. For all stress factors, protein-stabilising properties of mannitol were also evaluated. The first part of the study, a thermal stability study in liquid, investigated the influence of storage at elevated temperature. Second, the protein was exposed to the different steps of spray drying: pumping, atomisation and drying (Table 1). For this, a construction was built allowing collection of the samples directly after atomisation to investigate the influence of atomisation. Furthermore, protein solutions were spray-dried to study the dehydration in heat. To investigate the influence of pumping on the protein, the remaining solution, after pumping through the tubing system during spray-drying, was also collected.

Table 1 Overview of evaluated samples

Sample name	Evaluated sample	Temperatures
Thermal stability study	protein concentration: 1.5 mg/mL/ 3.0 mg/mL/ 7.5 mg/mL in 15 mL centrifuge tubes (Sarstedt AG & Co. KG; Nümbrecht, Germany)	50°C for 1 d, 4 d, 7 d, 14 d
Pumped	Solution in the tubing system after spray-drying	Inlet: 120°C
Atomised	Atomised in construction outside of the spray dryer	Room temperature
Spray-dried	collection of the spray-dried product	Inlet: 120°C, Outlet: ca. 45°C

Thermal stability study: Different concentrations of chymotrypsin (lyophilised α -chymotrypsin from bovine pancreas, Sigma-Aldrich, Traufkirchen, Germany) were dissolved in a saturated (25 mg/mL) mannitol (Parreck® M DPI, Merck KGaA, Darmstadt, Germany) solution in milliQ water or pure milliQ water. Triplets of samples containing either 100 μ L mannitol- and chymotrypsin solution or containing 100 μ L pure chymotrypsin solution were analysed (Table 1). The samples were analysed with the chymotrypsin activity assay.

Nano spray dryer: Spray drying was performed with the Nano Spray Dryer B-90 HP (Büchi Labortechnik AG, Essen, Germany). The spray dryer mainly consists of the drying chamber including a high voltage particle collector for the product. The feed solution is pumped in a circle through the piezo electric spray head (medium membrane, produced droplets: medium mass diameter of 5-8 μ m, frequency: 125 Hz, pumping rate: 100%, spray rate: 80%), only parts of the solution are sprayed at the time. Triplets of samples containing a concentration of 4.0 mg/mL chymotrypsin or samples containing a concentration of 4.0 mg/mL chymotrypsin and 4.0 mg/mL mannitol in milliQ water were treated different ways (Table 1). The protein concentration of the pumped and atomised samples was determined using the Pierce-BCA assay kit by Thermo Fischer Scientific, Waltham, USA. As standard a 2.0 mg/mL chymotrypsin solution was used for the calibration curve. The samples were diluted to a concentration about 1 mg/mL. 25 μ L of each sample and each dilution were pipetted in triplicate into a 96 well plate and 200 μ L of the reacting agent containing cupric sulphate in an alkaline buffer were added. The plate was incubated for 30 min at 37 °C and analysed at 562 nm with a Tecan Spark® plate reader (Tecan Trading AG, Männedorf, Switzerland).

Chymotrypsin enzyme activity assay: A chymotrypsin enzyme activity assay similar to the one described by Worthington Biochemical Corporation, Lakewood, USA was performed [5]. Prior to the measurement, the sample was diluted to an estimated concentration of 2.75 U/mg with 0.001N HCl. For the assay, 50 μ L of the prepared sample, 50 μ L of 0.001 N HCl and 2.9 mL of a freshly prepared mixture of 135 mL 0.08 M Tris-HCl buffer pH 7.8 containing 0.1 M calcium chloride (Merck KGaA) and 126 mL 0.00107 M Benzoyl-L-tyrosine ethyl ester (BTEE) (Sigma Aldrich) in 63% V/V methanol/ milli-Q-water were mixed in a quartz cuvette. The activity was calculated via measuring the increasing absorption at 256 nm for 5 minutes using the kinetics function of the UV-Vis-spectrometer (UV-1280 by Shimadzu, Kyoto, Japan) which directly calculated the $\Delta A_{256}/\text{min}$. With this using the following equation (964 being the extinction coefficient of BTEE and concentration being the chymotrypsin concentration in the reaction medium in mg/mL), the enzymatic activity of chymotrypsin in U/mg was calculated.

$$\text{enzyme activity U/mg} = \frac{1000 * \Delta A_{256}}{964 * \text{concentration}}$$

Fluorescence emission spectra changes: To investigate if the protein structure changed, the changes in the emission spectra of the proteins fluorescence were detected using a Tecan Spark® plate reader. The emission spectra from 400 to 600 nm of the extrinsic fluorescence of a saturated solution in water (about 0.015 mg/mL) of the fluorescent dye anilinonaphthalene-8-sulfonic acid (ANS) using an excitation wavelength of 375 nm were recorded. ANS is almost nonfluorescent in water but interacts with the

hydrophobic parts of a protein leading to an increase in the intensity of the emission. An increase in hydrophobicity caused by changes in the tertiary structure or protein aggregation can lead to a blue shift and an even higher intensity [4]. The atomised and pumped samples were measured without further treatment; the spray-dried samples were dissolved in milliQ water to a theoretical concentration of 4.0 mg/mL chymotrypsin. Also a sample of the native protein as positive control and of the denatured (100°C for 3 h) protein as negative control were analysed at a concentration of 4.0 mg/mL.

Results

The performed study investigated the impact the stress factors during pumping, atomisation and spray drying had on the samples either containing only chymotrypsin or containing additionally mannitol as stabiliser. Additionally, the stability of different samples containing chymotrypsin or chymotrypsin and mannitol were examined in a thermal stability study. The results show differences in the destabilising properties of the different stages of spray-drying such as a stabilising potential of mannitol.

Thermal stability study: Figure 1 shows the results of the thermal stability study in liquid. Compared to the samples containing only chymotrypsin the samples containing additionally mannitol show a higher activity after all storage times at 50°C. The activity decreases with increasing protein concentration of the samples, an effect that can be seen for both the pure chymotrypsin samples and for the samples containing additionally mannitol.

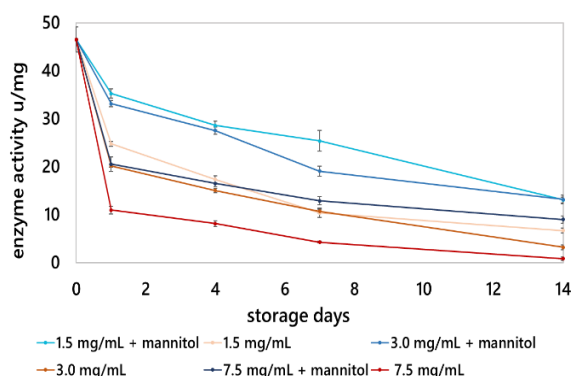


Figure 1: Enzyme activity after storage in 50° C for 1 d, 4 d, 7 d and 14 d, 1.5/ 3.0/ 7.5 mg/mL being the chymotrypsin concentration, the samples with mannitol are saturated with mannitol; n= 3; error bars= 95% CI

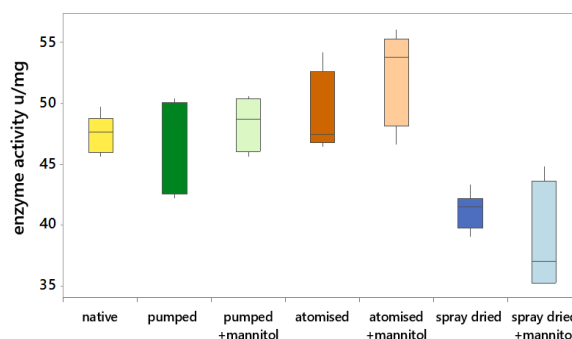


Figure 2: Enzyme activity after processing with the nano spray dryer compared to the native protein; showing the distribution of the 9 measurements (n=3 with performed measurements in triplicate), with the whiskers showing maximum and minimum, the outer lines of the box the first and third quartile and the inner line the median

Spray drying process: The enzyme activity was also determined for the chymotrypsin and chymotrypsin & mannitol samples after pumping, atomisation and drying. Compared to the original activity the pumped and atomised samples did not change significantly in activity whereas a small activity decrease was found in the spray-dried samples (Figure 2).

The ANS fluorescence emission spectra differed for the investigated samples; the atomised and the spray-dried samples differed from the native protein (Figure 3).

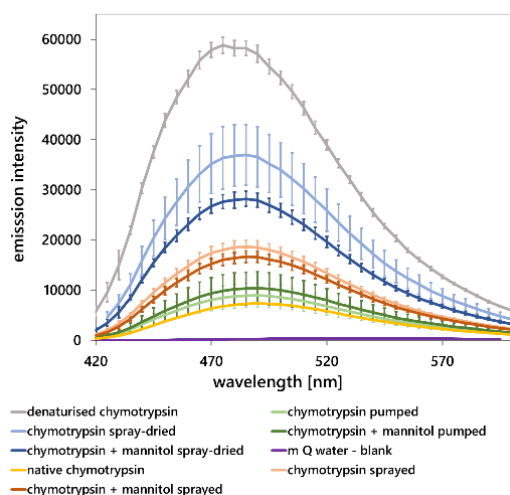


Figure 3: Extrinsic fluorescence of the fluorescence dye ANS after processing with the nano spray dryer compared to native and denatured chymotrypsin; n= 3; error bars= S

Discussion

The results of the thermal stability study of samples containing chymotrypsin or chymotrypsin and mannitol in liquid clearly indicate a stabilising effect of mannitol against heat induced denaturation of chymotrypsin during storage. This is probably caused by stabilising hydrogen bonds with the protein. Furthermore, self-destructing properties of the peptidase chymotrypsin seem likely due to the activity decrease for the samples with higher protein concentration [6].

Processing with the nano spray dryer seems to have an overall impact on the protein structure. An effect of the pumping process could not be found, possibly due to the stress factors here being least intense. While the atomisation by itself did not cause a decrease in enzyme activity, shear forces and ultrasonic sound influenced the protein structure as seen in the fluorescence assays. The blue shift and the intensity increase can be explained by an increase of the protein's hydrophobicity and so of the binding sites for ANS. This can be caused by a structural change leading to either aggregation or denaturation. However, it is possible that this effect is either reversible by dilution prior to the enzyme assay or does not influence the protein's overall activity. For the spray-dried samples, a small decrease in the enzyme activity was found which is attributed to the dehydration process. For the spray-dried samples with and without mannitol, an increase in the intensity and a blue shift of the maximum of the fluorescence emission indicate an increase of the proteins' hydrophobicity. During spray-drying a combination of tubing-liquid interfaces, shear forces, ultrasonic stress, air-liquid-interfaces, thermal and dehydration stress occur. They all possibly lead to aggregation or denaturation and thus a decrease or effect loss of a protein drug.

However, since no structural changes occurred during pumping and only minor changes occurred during atomisation, it seems likely that heat and dehydration had the highest effect on the protein structure. Adding mannitol did not preserve the protein structure during atomisation under the examined conditions. A small stabilising effect during drying seems likely, since more pronounced changes in the fluorescence emission spectra for the pure protein samples were found. These observations seem logical, since small sugars being structurally related to mannitol are known to be good stabilisers against heat and dehydration stress but less so against shear forces [3]. It is also possible that the mannitol concentration used in this setting was not high enough to show additional protection during atomisation. It had been shown that mannitol was able to stabilise the protein as thermoprotectant against heat-induced loss of activity in solution, however a clear stabilising effect of mannitol could not be seen in the product. This emphasises the need of additional or alternative excipients, like amino acids or polysorbates, to stabilise the protein as a lyoprotectant during the dehydration step.

Conclusion

Although mannitol showed good stabilising effects during the thermal stability study in liquid, only smaller effects during drying and no effect during the atomisation occurred. The atomisation as well as the drying process influenced the protein structure of chymotrypsin. However, drying had the major impact. Further studies will be conducted using different mannitol concentrations and extending the study to further excipients. Furthermore, different spray drying settings and other model proteins will be investigated to get a deeper understanding of destabilising processes and the rational use of stabilisers for protein drug production by spray drying.

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