

SUPERCRITICAL DRYING OF LYSOZYME FROM AQUEOUS SOLUTION

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Hen egg lysozyme in aqueous solution was dried using the supercritical anti-solvent principle. The lysozyme solution was sprayed in a vessel together with supercritical carbon dioxide and ethanol to produce a stable powder. Operating conditions, such as the flow rates and the protein concentration, were modified to minimize the use of ethanol, which could denature proteins. The particle size and morphology were observed by scanning electron microscopy, the water content by Karl-Fisher titration, and the protein structure by circular dichroism. A free-flowing lysozyme powder containing between 4.5 % and 6.0 % water could be produced at a pressure and temperature as low as 100 bar and 37°C. Depending on the CO₂ and ethanol flow rates during drying, different primary particle sizes were obtained.

INTRODUCTION

A lot of effort is currently being put towards developing stable peptide or protein based pharmaceutical products as recombinant DNA technology advancements make the production of such highly specific compounds available [1]. The stability of these components is such that a number of excipients must be used to extend their shelf-life or to limit damages caused by their processing [1,2]. Dehydration, rehydration and storage stability are the main stresses occurring in any drying process, but other stresses such as ice crystal formation, and high temperatures can be avoided using supercritical fluid drying instead of conventional freeze drying or spray drying [2]. Successful drying of proteins is a challenging operation as it requires – together a low residual water content and a specific range of the particle size – that the protein structure is maintained so the protein will retain its function.

A number of supercritical techniques are available [3] but only a limited number of them is applicable to proteins. Several studies have been reported on supercritical drying of proteins from organic solvents [3], such as dimethyl sulfoxide. However, the use of organic solvents is preferably avoided because many pharmaceutical proteins are incompatible with these solvents. Gas Anti-Solvent (GAS) crystallisation [4], Aerosol Solvent Extraction System (ASES) [5,6], and Solution Enhanced Dispersion by Supercritical fluids (SEDS) [7,8] are the main supercritical drying techniques that have been used with proteins in aqueous solution. These techniques are known for their ability to produce small particles. Particle sizes published are in the range of 0.78 µm [9] to 5 µm [8]. As a means for drying, however, the technique can be improved. Water contents were around 10 wt.% [7,9], which is too high for a sufficient long-

time stability. The aim of the present study is to investigate whether the water content can be lowered while minimizing the use of the co-solvent (ethanol) by varying the operation conditions in a basic set-up.

MATERIALS AND METHODS

Materials

Lyophilised powder of lysozyme from chicken egg white (product code 62971; lot number 418426/1) was purchased from Fluka and conserved according to their instructions until use. Technical grade ethanol (100%) was used. Carbon dioxide (grade 3.5) was purchased from Hoek Loos, Schiedam, The Netherlands.

Supercritical drying set-up

Mixing of the various flows was performed in two consecutive T-mixers. First the ethanol was mixed with the carbon dioxide. This mixture was subsequently mixed with the lysozyme solution. The lysozyme solution was sprayed into the second T-mixer via a 100- μm nozzle. The pressure was maintained at 100 bar, and flow rates and concentrations were modified according to the experimental design (Table 1). The lysozyme solution, ethanol and carbon dioxide were all maintained at 37°C before and after the nozzle. The lysozyme solution and ethanol were added using syringe pumps (Isco). The supercritical CO₂ was added using a positive displacement pump (Williams). The pressure in the vessel was controlled using the exit valve. The dry protein powder was collected in a 1-litre vessel and recovered once the pressure was released.

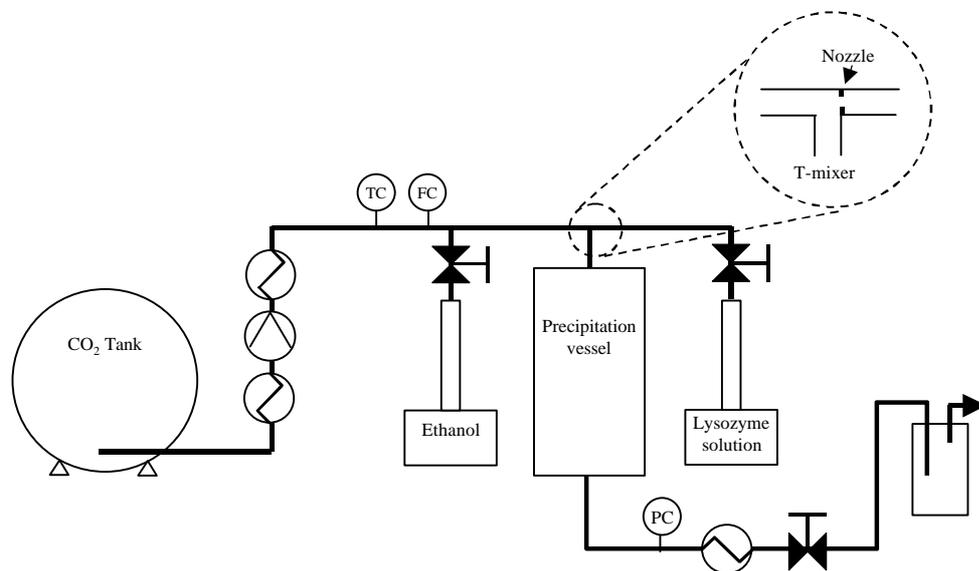


Figure 1 : Basic scheme of the experimental set-up

Analytical techniques

Scanning electron microscopy (Jeol JSM-5400) images were used to examine the morphology of particles. Karl-Fisher titration was performed with a Mitsubishi MCI moisture meter model CA-05 as per manufacturer instructions. A DSM 1000 circular dichroism spectrometer from OLIS was used to record CD spectra.

RESULTS AND DISCUSSION

A series of four experiments were done in which the CO₂ flow and the ethanol flow rate were varied (Table 1). All experiments yielded a fluffy free-flowing powder. Increasing the CO₂ flow rate resulted in a smaller primary particle size (Figure 2). This observation was valid for both ethanol flow rates selected and is in accordance with data reported by Gilbert and co-workers [8]. The decrease in primary particle size at the high CO₂ flow rate can be explained by the formation of smaller droplets during spraying as well as the higher driving force for dehydration, resulting in a faster precipitation of the proteins, limiting potential growth of primary particles. For a selected CO₂ flow rate, increasing the ethanol flow rate resulted in smaller particles, presumably because the ethanol rich phase causes a faster nucleation and limits the growth.

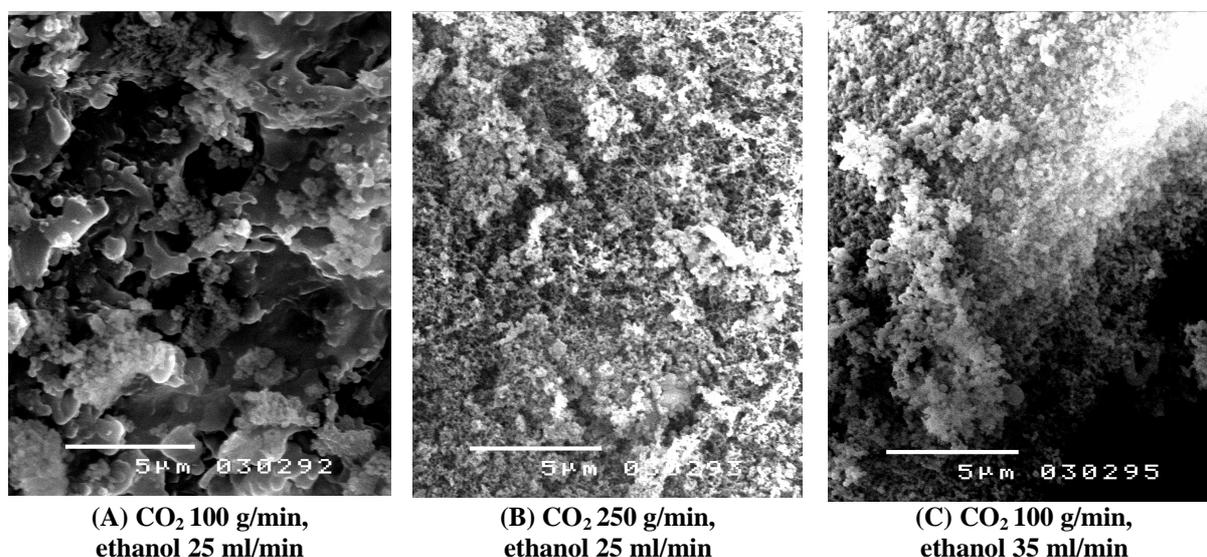


Figure 2 : Scanning electron micrographs of lysozyme particles

The water content of the particles decreased with increasing CO₂ flow rate (Table 1). Because of the low solubility of water in CO₂, the substantial increase of the amount of CO₂ used to remove the water coupled with smaller droplets/particles are likely to be responsible for the decrease of the water content. The water content of samples was independent of the ethanol flow rate (Table 1). A lower water content was expected when using a higher ethanol flow rate due to the higher solubility of water in the ethanol rich CO₂ phase. However, increasing the ethanol flow rate may also have led to a faster protein precipitation. Precipitation at the gas-liquid interface may have slowed down the diffusion of the water from the droplets, resulting in a higher water content.

Table 1 : Experimental conditions and water content

Conditions	CO ₂ (g/min)	Ethanol (ml/min)	Water content (%)
A	100	25	5.96
B	250	25	4.46
C	100	35	6.04
D	250	35	4.48

CD spectra

Near and far UV CD spectra (Figure 3) showed that the secondary and tertiary structure of the treated lysozyme were not significantly affected by the supercritical drying process and subsequent rehydration.

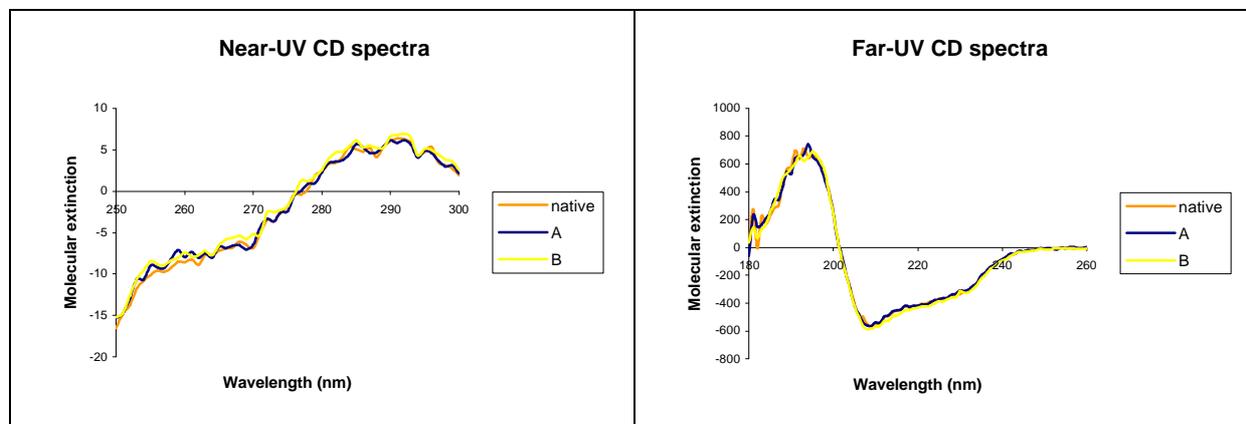


Figure 3 : CD spectra of lysozyme

CONCLUSION

The proposed supercritical spray-drying process is a promising technique for drying proteins. It is a simple, single-step and fast technique. This study shows that protein particles could be prepared with preservation of the protein conformation. Water contents achieved were reduced to 4.5% - 6.0%, which is lower than previous published data of 10% and near the expectations of about 2% usually obtained through freeze-drying.

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