GENERATION OF LYSOZYME-LACTOSE POWDERS USING THE ASES PROCESS

Neil R. Foster[1,2], Fariba Dehghani[1,2] , Rana T. Bustami[3] and Hak-Kim Chan[3]

[1]School of Chemical Engineering and Industrial Chemistry, University of New South Wales, Sydney, 2052, Australia, Tel: 612 9385 4341, Fax: 612 9385 5966

[2]Eiffel Technologies Limited
Level 14, 50 Market Street, Melbourne, Victoria, 3000, Australia

[3]Faculty of Pharmacy, University of Sydney, Sydney, 2006, Australia

The feasibility of delivering protein to the lung has been well documented. Protein particles suitable for inhalation delivery have been successfully generated from aqueous based solutions using the modified ASES technique. However, it is necessary to protect the protein-protein interactions to minimize molecular aggregation and degradation during manufacturing and storage. Carbohydrate excipients have been found to be effective for the stabilization of spray-dried proteins.

In this study the feasibility of the precipitation of lysozyme-lactose mixture by the ASES technique was investigated. Lysozyme and lactose were used as models for the protein and the sugar, respectively. Lysozyme-lactose powders dissolved in an aqueous solution were precipitated in a pressurized collection chamber containing CO_2 modified with 20 mol % ethanol at 20°C and 155 bar. A gas energizing nozzle was used to spray the solution.

The processed Lysozyme-lactose powders (92:8) consisted of nano-sized spheres ranging from 100 to 500 nm, consistent with the morphology of lysozyme particles precipitated at the same conditions. The degree of agglomeration between lysozyme particles increased as the lactose-lysozyme ratio was increased to 45%. At higher concentrations of lactose (lactose-lysozyme ratio of 90%) precipitated powder included micron sized plates and nano-sized spheres corresponding to lactose and lysozyme, respectively. The X-ray diffraction patterns showed that powders containing 10% lactose were amorphous. However, powders containing higher levels of lactose (45%, 92%) were semicrystalline.

This study has demonstrated that the ASES technique is suitable for the generation of lysozyme-lactose powders from aqueous based solutions at a relatively low temperature. The solid state characteristics of the powders generated were a function of lactose concentration in the aqueous solutions.

INTRODUCTION

Precipitation of pharmaceuticals using gas anti-solvent techniques relies on the solubility of the solute in an organic solvent that is miscible with an anti-solvent such as carbon dioxide. The difficulty of applying gas antisolvent techniques to the processing of proteins is that they involve exposure of the protein to organic solvents, the latter being potential denaturants and very poor solvents for most therapeutic macromolecules. The ASES technique has been modified to enable the spraying of aqueous protein solutions simultaneously with an organic solvent into carbon dioxide [1] or directly into carbon dioxide modified with an organic solvent [2, 3]. Recombinant human immunoglobulin (rhIG) [3], lysozyme [2], albumin and insulin [2], trypsin [4], a therapeutic peptide antibody Fv and Fab and plasmid DNA [5] have been precipitated from aqueous based solutions. Except for rhIG, which was not obtained as stable powder, all the proteins precipitated as micron-sized particles or agglomerated nano-spheres. Depending on the protein, the biochemical integrity of the proteins micronised using the modified ASES can be a problem. Whilst the biological activity of lysozyme was almost completely recovered after reconstitution in water, other proteins were denatured to various extents [2].

During manufacturing and storage of protein powders, it is necessary to protect the protein-protein interactions to minimise degradation, especially molecular aggregation. For example, stable lyophilised protein formulations contain high proportions of excipients, such as sugars, that have the ability to protect the protein against denaturation during freezing as well as to act as a vitreous matrix that slows down the intra-and inter-molecular interactions [6]. Clark and coworkers reported the effect of carbohydrate excipients on the stabilization of spray-dried proteins [7].

In the present study, the feasibility of the generatiom of lysozyme-lactose co-precipitate from aqueous based solutions using the ASES process was investigated. Lysozyme and lactose were used as models for protein and sugar, respectively.

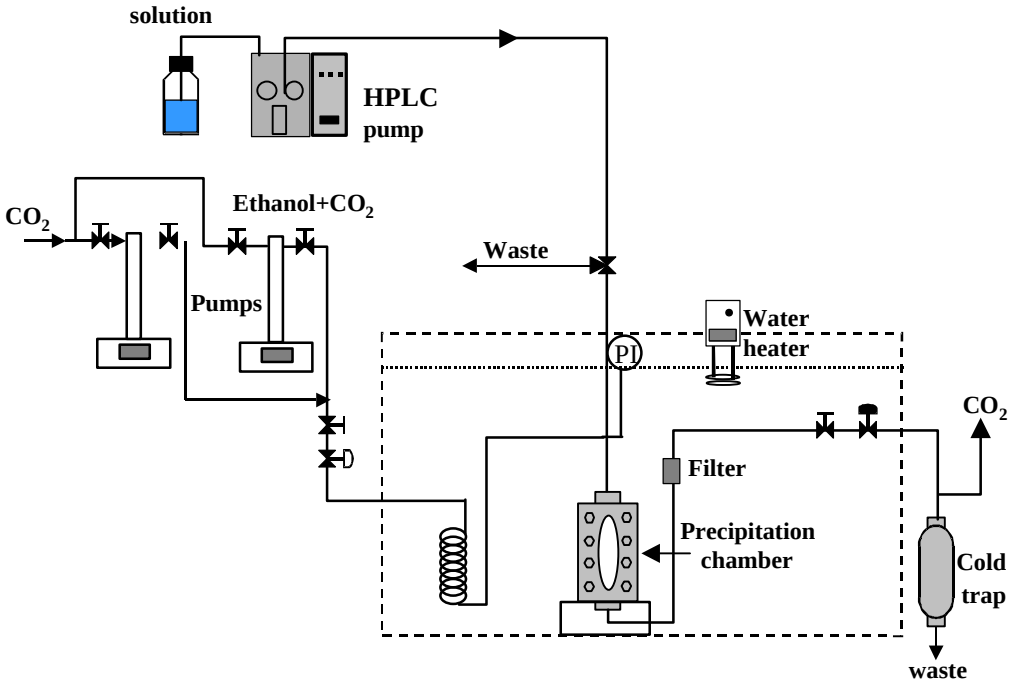
MATERIALS AND METHODS

Materials

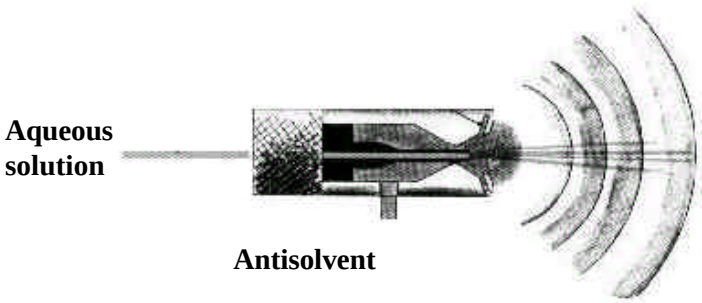
Lysozyme (crystallized (*3), dialyzed and lyophilized, lot No. 57H7045) Lactose monohydrate were purchased from Sigma Chemicals Australia. Ethanol 99.7-100.0% was purchased from Fluka Chemicals,. Liquid carbon dioxide (Industrial grade 99.5%) was purchased from BOC Gases.

Procedure

A schematic diagram of the ASES apparatus that was employed in the study is shown in Figure 1. The main parts of the apparatus include a 60 mL precipitation chamber (Jerguson sight gauge, model 13-R-32), two syringe pumps (ISCO model 260D, 500D) for the delivery of carbon dioxide and the carbon dioxide modified with ethanol and an HPLC pump (Waters, Model 510) for protein solution delivery. The delivery units were connected to a nozzle system, which was mounted on the top of the precipitation chamber. The nozzle system consisted of a specialised nozzle (Sonimist, Model 600-1, USA). The Sonimist nozzle structure included a secondary co-axial pathway surrounding the central tube for passage of the energising gas along the length of the central tube and out of the nozzle outlet (Figure 1).



(a)



(b)

Figure 1: (a) Schematic diagram of the ASES apparatus, (b) gas energizing nozzle

Modified carbon dioxide was prepared by mixing carbon dioxide with ethanol in the syringe pump as previously described [8]. During the start-up stage, the chamber was first pressurised with carbon dioxide to attain a pressure of 50-80 bar to avoid phase separation of the carbon dioxide-ethanol mixture upon delivery into the chamber. The modified carbon dioxide was then continuously delivered into the precipitation chamber at a constant flow rate of 12 mL/min at the desired processing pressure at 155 bar. The flow rate was controlled by a metering valve located at the exit, and operating temperature was controlled to within ± 0.1°C with a

temperature controlled water bath. Once the desired temperature and pressure had been achieved in the chamber, the aqueous solution containing the protein was pumped at a constant flow rate (0.2 mL/min) and sprayed into the chamber. After precipitation, the ethanol and water residues were flushed out by feeding carbon dioxide at the operating pressure and temperature. The chamber was then gradually depressurised and the powder was collected, sealed in airtight containers, and stored in a freezer (-18°C) for characterisation.

Particle Characterization

The morphology of the processed particles was examined using a scanning electron microscope (Joel JSM 6000F, Tokyo, Japan). Samples were mounted on metal plates and gold coated using a sputter coater (Edwards. UK) under vacuum prior to analysis.

Powder crystallinity was assessed by an X-ray powder diffractometer (Siemens D5000, Hamburg, Germany). The powders were packed on a glass sample plate and irradiated by Cu Kα radiation at 40 KV and 30mA with an angular increment of 0.05°/s and an increment count time of 2 seconds.

The percentage content of lysozyme in the processed powders was determined by measuring the absorbance of an aqueous solution of the processed powder at 280 nm against a known concentration of lysozyme solution.

RESULTS AND DISCUSSION

Lactose was successfully precipitated using CO_2 as an antisolvent modifed with 20 mole % ethanol at 20°C and 155 bar. Scanning electron micrographs have shown that lactose precipitated as thin plates smaller than 20 μm (Figure 2). Lactose was previously precipitated from aqueous based solutions using a specialised nozzle utilizing the SEDS technique [9]. The morphology ranged from thin large bands to large agglomerated chunks by varying the flow rate of carbon dioxide.

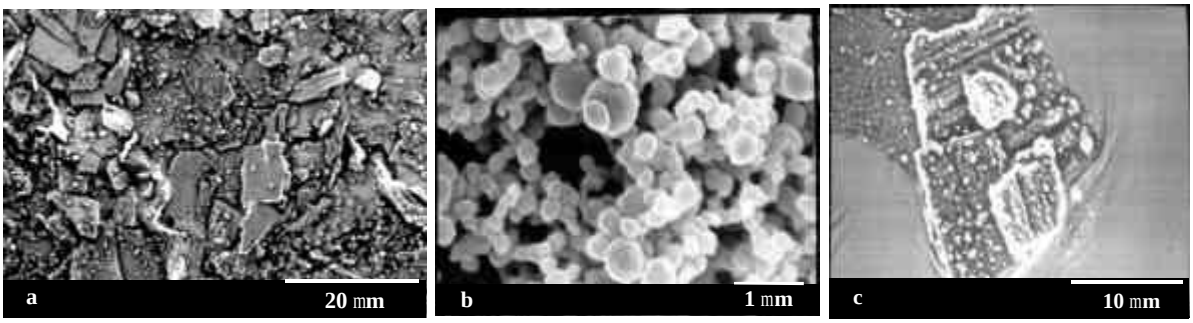


Figure 2: The SEM images of (a) lactose, lysozyme-lactose mixture (b) 92:8 (c) 10:90 precipitated from aqueous solution by the ASES technique using CO_2 modified with ethanol at 20°C and 155 bar as an anti-solvent.

The crystallinity of lactose may change when processed by the ASES technique. The X-ray diffraction analysis confirmed that lactose is semicrystalline and the degree of crystallinity remained after processed by the ASES technique as shown in Figure 3.

Lysozyme-lactose powders were precipitated using the gas-energising nozzle at 20°C and 155 bar using various weight ratios of lysozyme/lactose in the aqueous feed (Table 1). Lysozyme–lactose ASES processed powders containing 92.5 wt% lysozyme consisted of nano-sized spheres ranged from 100 to 500 nm as shown in the typical SEM images (Figure 2). Powders containing high levels of lactose (44 wt% and 89.5 wt%) were more agglomerated than powders containing 7.5 wt% lactose. Lysozyme-lactose powders containing 90% lactose exhibited two particle morphologies, micron-sized plates and nano-sized spheres corresponding to lactose and lysozyme, respectively (Figure 2). The X-ray diffraction analysis confirmed that powders containing less than 10% lactose were amorphous while powders containing higher than 44 wt% lactose were semicrystalline (Figure 3).

Table 1: Characteristics of Lysozyme-lactose powders precipitated by the ASES process at 20°C and 155 bar using gas-energising nozzle.

Lysozyme:lactose Conc. mg/mL	**Size and morphology**	**Lysozyme content %**	**Crystallinity**
0:50	Thin plates <20 µm particles < 1 µm	0	semicrystalline
50:9.3	Agglomerated nanospheres 100-200 nm	92.5	amorphous
50:80	Agglomerated nanospheres 100-500	56.0	semicrystalline
50:100	Agglomerate nanospheres Plates < 20 µm	10.5	semicrystalline

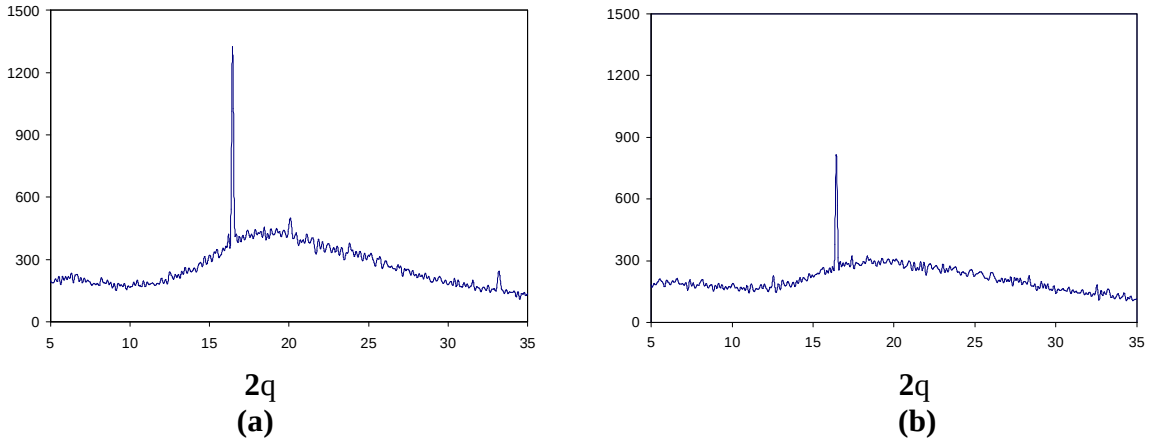


Figure 3: X-ray diffraction pattern of ASES processed powders of (a): lactose (b): lysozyme-lactose (56:44).

CONCLUSIONS

The ASES process has been shown to be a feasible method for the production of micron-sized particles of lysozyme-lactose co-precipitate from aqueous based solutions at a relatively low temperature. Powders containing low levels of lactose were amorphous. However, agglomerated semicrystalline powders were produced at high levels of lactose.

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