Generation of Micro-Particles of Proteins for Aerosol Delivery Using High Pressure Modified Carbon Dioxide

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Purpose. To investigate the feasibility of using the Aerosol Solvent Extraction System (ASES) to generate microparticles of proteins suitable for aerosol delivery from aqueous-based solutions.

Methods. The ASES technique using high- pressure carbon dioxide modified with ethanol was utilised for the generation of microparticles of proteins (lysozyme, albumin, insulin and recombinant human deoxyribonuclease (rhDNase)) from aqueous solutions. Particle size, morphology, size distributions and powder aerosol performance were examined. The biochemical integrity of the processed proteins was assessed by testing the level of molecular aggregation using size exclusion chromatography and by bioassay technique for lysozyme.

Results. Proteins were precipitated as spherical particles ranging in size from 100 to 500 nm. The primary nano-sized particles agglomerated to form micron-sized particles during the precipitation process. The median size of the particles was a function of the operating conditions. In-vitro aerosol performance tests showed that the percent fine particle mass (< 5μm) was approximately 65%, 40% and 20% for lysozyme, albumin and insulin, respectively. Negligible loss in the monomer content or biological activity was observed for lysozyme. Insulin exhibited slight aggregation and 93% of the monomer was retained after processing. Albumin was affected by processing and only 50–75% of the monomer was retained compared with 86% in the original material. However, rhDNase was substantially denatured during processing as shown by the significantly reduced monomer content.

Conclusions. Micron-sized particles of lysozyme, albumin and insulin with satisfactory inhalation performance were successfully generated from aqueous solutions using the modified ASES technique. The biochemical integrity of the processed proteins was a function of the operating conditions and the nature of the individual protein.

KEY WORDS: ASES; proteins; micronisation; aerosol delivery; SCF.

INTRODUCTION

The feasibility of protein delivery to the lung to achieve local or systemic effects has been well-documented (1). The size requirement of the particles (1–5 μm) and the structural stability of the protein are among the essential criteria for aerosol delivery of therapeutic proteins.

Conventional techniques for producing such micron-sized particles include grinding, jet milling, liquid-phase anti-solvent precipitation, freeze-drying and spray drying. Unfortunately, these processes often incur thermal and chemical degradation, involve the use of excessive solvent with subsequent disposal problems, result in high levels of solvent residues, inter-batch particle size variability, broad size distribution and low yield.

Alternative techniques utilising fluids near or above the critical point as a solvent or anti-solvent have been developed in recent years (2–4). Pharmaceutical processing with dense gases is a relatively new and efficient approach for the preparation of high purity micronised particles with defined morphological structures in a one step operation. Carbon dioxide is commonly used as the dense gas in these applications as it is nonflammable, inexpensive, has a moderate critical temperature (31.1°C) and is relatively non-toxic.

Two dense gas methods have been considered for the production of solid particles. The first method is known as the Rapid Expansion of Supercritical Solution (RESS), and involves preparing a solution of the material of interest in a dense gas and expanding it through a nozzle (2). Whilst providing a very effective method for producing fine particles, the applicability of the RESS method is limited by the low solubility of proteins in dense carbon dioxide. The second method, known as the gas anti-solvent process, involves rapidly precipitating solutes from a solution, using a dense gas as an anti-solvent. The anti-solvent expands the solution, thereby decreasing the dissolution power of the solvent, and eventually resulting in the precipitation of the solute (3). Gas anti-solvent processes have been utilised for the generation of micron-sized particles in two modes. The first mode, known simply as the gas anti-solvent process (GAS) involves the gradual addition of anti-solvent to the organic solution containing the solute until the precipitation occurs (4). The second mode, known as the Aerosol Solvent Extraction System (ASES), involves introducing the organic solution of the solute through a capillary nozzle into a flowing dense gas stream (5). Gas anti-solvent processes have been utilised for the generation of micron-sized particles of insulin, lysozyme, tyrosine, peroxidase and myoglobin (4,6–8). The difficulty of applying these techniques to the processing of proteins is that they involve exposure of the protein to organic solvents, the latter being potential denaturants (9) and very poor solvents for most therapeutic macromolecules. In order to overcome this limitation York and co-workers have developed a new supercritical fluid technique, referred to as Solution Enhanced Dispersion by Supercritical Fluid (SEDS), which involves using a special coaxial nozzle (10). The nozzle facilitates the generation of bioactive micro-particles of proteins from aqueous solutions, the advantage of which is that water is the native and favourable medium for protein. Sloan *et al.* (11) utilized the SEDS technique for the production of micron-sized particles of lysozyme and trypsin. Lysozyme biological activity was recovered upon reconstitution in water but a significant loss of the biological activity of trypsin was reported.

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ABBREVIATIONS: ASES, aerosol solvent extraction system; rhDNase, recombinant human deoxyribonuclease; RESS, rapid expansion of supercritical solutions; GAS, gas anti-solvent technique; SEDS, Solution enhanced dispersion by supercritical fluid; VMD, volume median diameter; MMD, mass median diameter; FPM, fine particle mass.

In the present work, high pressure carbon dioxide modified with ethanol was employed as an anti-solvent in the ASES process to precipitate the model proteins, lysozyme and albumin, and the therapeutic proteins, Recombinant human deoxyribonuclease (rhDNase) and insulin from aqueous solutions. rhDNase is a commercially available protein approved for administration by nebulisation as an inhalation aerosol to patients with cystic fibrosis (12). rhDNase has to be given as inhalation aerosols as it acts locally in the lung. Insulin is given to diabetic patients by self-injection, intramuscularly or subcutaneously. However, such injections are associated with relatively slow systemic availability of insulin. In addition, there is generally low compliance with the use of injections and nebulisers. Hence these proteins would be attractive candidates for processing with the ASES technique to produce micron-sized powders for inhalation delivery.

MATERIALS AND METHODS

Materials

rhDNase was provided by Genentech Inc. (USA) as an aqueous solution containing 65 mg/mL. The solution was dialyzed against water before the ASES processing. Microcrystalline egg white lysozyme (crystallized (×3), dialyzed and lyophilized, lot No. 57H7045), low endotoxin bovine insulin (lyophilized powder of 28.5 USP units/mg, lot No. 47H0573), albumin; bovine fraction V (approximately 99%, lot No. 974H984) and Micrococcus Luteus were purchased from Sigma chemicals and were used as received. Ethanol 99.7–100.0% was purchased from Merck. Sodium phosphate, sodium sulphate (APS Ajax), sodium chloride analytical grade, HEPES buffer 99.5%, and potassium phosphate monobasic were all of analytical grade and were purchased from Sigma. Protein reagent kit was purchased from BIO-RAD. Liquid carbon dioxide (Industrial grade 99.5%) was purchased from BOC Gases.

Apparatus and Precipitation Process

A schematic diagram of the 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 coaxial nozzle arrangement, which was mounted on the top of the precipitation chamber. The nozzle consists of a capillary tube (Peek tube, 50μm i.d. and 1.59 mm o.d., SGE) inserted into a stainless steel tube (2.16 mm i.d. and 3.18mm o.d., Altech). The aqueous protein solution and the anti-solvent were delivered through the inner and the outer tube, respectively. The coaxial nozzle enables simultaneous spraying of the aqueous solution and the anti-solvent and facilitates both optimum dispersion of the aqueous solution and mixing of the two streams.

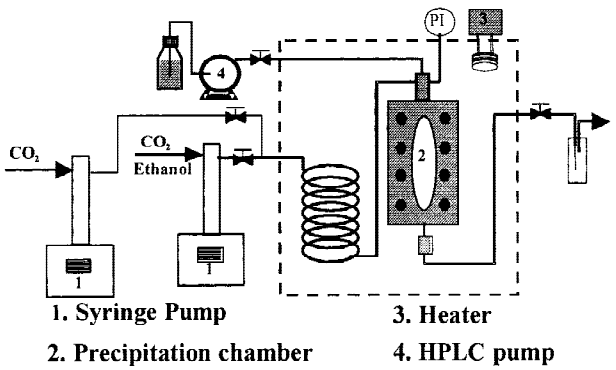

Fig. 1. Schematic diagram of the apparatus used in the precipitation process.

Modified carbon dioxide was prepared by mixing carbon dioxide with ethanol in the syringe pump as previously described in detail (13). During the start-up stage, the chamber was first pressurised with carbon dioxide to attain a pressure of 80–90 bar to avoid phase separation of the carbon dioxide-ethanol mixture upon delivery into the chamber. The modified carbon dioxide was then delivered into the precipitation chamber at the desired processing pressure. The 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 and sprayed into the chamber. The pressure drop through the nozzle was adjusted to approximately 50 bar by a metering valve. Modified carbon dioxide was fed continuously to the chamber. The flow rate of the antisolvent was controlled by a metering valve placed at the exit.

In the precipitation region, sub-millimeter liquid droplets were sprayed into a continuous feed of high pressure modified carbon dioxide. Protein particles precipitated after the expansion of, and extraction of, the water from the droplets. 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 future characterisation.

The operating conditions, the flow-rate ratio of the aqueous feed and the antisolvent, and the modifier mole fraction were selected to produce a homogenous mixture of CO_2-ethanol-water in the precipitation chamber. The mole fraction of ethanol in the antisolvent was kept at 0.2 and a volumetric flow rate ratio of feed to antisolvent of 0.4/12 was used in the process. The flow rate of the aqueous solution was adjusted to achieve a pressure drop of 50 bar across the nozzle to provide atomisation of the spray.

Smaller droplets are generated when solutions are sprayed at a higher pressure drop (14). The high flow rate of the antisolvent facilitated dispersion and mixing of the aqueous spray mist across the chamber resulting in higher rates of water extraction from the droplets. The experimental conditions used are listed in Table 1.

PARTICLE CHARACTERIZATION

Particle Morphology

A scanning electron microscope (Joel JSM 6000F, Tokyo, Japan) was used to examine the morphology of the particles produced. Samples were mounted on metal plates and

Table 1. Characteristics of Protein Powders Generated at 155 Bar Using ASES Process

Batch no.	Temperature °C	Protein conc. (mg/ml)	PSD[a] D(v,50%), D(v,90%) μm		FPM[a] %	Monomer %
Lysozyme original			51.1 (9.9)	110.1 (14.4)		
Lys1	45	15	3.5 (0.4)	12.3 (2.0)	51.5 (3.8)	97.0
Lys2	45	50	3.1 (0.1)	10.5 (0.6)	62.9 (2.2)	96.1
Lys3	35	15	9.0 (1.7)	31.6 (9.2)	37.5 (2.6)	96.4
Lys4	35	50	4.5 (0.5)	15.5 (2.4)	51.2 (2.5)	97.9
Lys5	35	15	3.9 (0.5)	13.6 (1.3)	57.3 (0.5)	ND
Lys6	35	15	10.7 (0.8)	30.7 (4.0)	30.0 (3.4)	ND
Lys7	45	50	2.9 (0.1)	9.0 (0.8)	63.1 (3.4)	96.5
Lys8	35	50	4.8 (0.3)	15.1 (1.5)	46.5 (1.6)	ND
Lys9	35	15	4.1 (0.4)	14.1 (2.5)	55.5 (3.0)	ND
Insulin Original			11.9 (1.1)	19.1 (2.5)		
Ins1	45	10	16.4	51.9	ND	
Ins2	45	30	9.6 (0.5)	33.1 (2.0)	21.9 (2.4)	94.6
Ins3	45	30	11.8 (1.6)	38.5 (3.5)	20.3 (1.5)	92.7
rhDNase						
Dor1	45	10	5.9 (0.3)	14.3 (1.5)	ND	0
Dor2	35	10	8.4 (2.0)	22.5 (5.9)	ND	14.0
Dor3	20	20	10.6 (1.1)	27.8 (3.5)	ND	33.2
Albumin original						86.0
Alb1	45	15	8.5 (1.1)	22.0 (2.6)		ND
Alb2	35	15	18.2 (4.0)	50.3 (12.2)		ND
Alb4	20	50	7.5 (0.8)	21.0 (2.7)	30.8 (5.6)	77.3
Alb5	35	50	6.4 (0.4)	15.0 (1.3)		ND
Alb6	45	80	5.0 (0.4)	15.2 (1.4)	45.4 (5.0)	70.7
Alb7	55	50	11.6 (3.3)	32.8 (6.5)		49.2

Note: In all the experiments 20 mole % ethanol was used except in Lys5 and Lys9, and Lys6 for which 25 mole % and 30 mole % were used, respectively. The flow rate ratio of the aqueous solution to the antisolvent was approximately 0.4/12. ND—not determined. PSD—particle size distribution based on volume (Laser Diffraction using Malvern Mastersizer). [a:] mean (SD) of 3 determinations except for insulin (mean of 2).

gold coated using a sputter coater (Edwards. UK) under vacuum prior to analysis.

Particle Sizing

Particle size distributions of the processed powders were determined using laser diffraction (Mastersizer, Malvern, UK) as previously described (15). The size distribution was expressed in terms of the volume median diameter (VMD), which is the diameter below which 50% by volume of the particles reside. The volume median diameter is directly related to the mass median diameter (MMD) by the density of the particles. The true density of the powders was determined by the buoyancy method (16) and values of 1.25 g/mL for albumin, lysozyme and rhDNase, and 1.5 g/mL for insulin, were used.

Aerosol Performance Studies

In-vitro testing of the aerosol performance of the protein powders was assessed using a 5-stage Marple-Miller Impactor (Model 160, MSP corporation, USA) with cut-off diameters of 10.0, 5.0, 2.5, 1.25, and 0.625 μm calibrated at a flow rate of 60 L/min. The stages were previously coated with propylene glycol/methanol (50/50) to prevent particles bounce.

A mass of 2–6 mg of the powder was weighed into a gelatin capsule (size 3, Park Davis, Australia) and was immediately dispersed by a powder inhaler device (Dinkihaler®, Aventis) into the Marple-Miller impactor at 60 ± 2 L/min using a vacuum source (ERWEKA GmbH, Germany). The mass collected on each stage was determined using UV spectrophotometry (HITACHI, U2000, Japan) at 280 nm and 275 nm for lysozyme and insulin, respectively. The mass of albumin was determined by colorimetric assay using a special kit for protein analysis (Dc, BIO-RAD). The respirable fraction was expressed in terms of percent fine particle mass <5μm (% FPM), which is defined as the total mass collected on stages 3,4,5 and the filter divided by the total mass recovered in the device, mouthpiece and the impactor.

Molecular Aggregation Studies

The biochemical stability of the processed protein molecules was assessed using size exclusion chromatography. The monomer and the high molecular weight protein contents in the processed protein powders were determined using a Protein-Pak 125 column (Waters) for insulin and lysozyme and a Protein-Pak 300SW column (Waters) for rhDNase and albumin.

The powder samples were dissolved in deionised water by gentle shaking for 10 minutes to assure complete dissolu-

tion. The supernatant was filtered through a 0.45μm membrane filter after centrifugation for 5 minutes at 15,000 rpm, and then injected into the HPLC system. The mobile phase consisted of 50 mM sodium phosphate buffer with 150 mM-sodium sulphate adjusted to pH 6.24 for lysozyme, 50 mM of sodium phosphate buffer at pH 3.0 with 300 mM sodium chloride for insulin, 0.5 mM HEPES buffer, 150 mM sodium chloride and 1mM calcium chloride adjusted to pH 7.0 for rhDNase, and 200 mM isotonic phosphate buffer pH 7.4 for albumin. The percent of the monomer and the soluble aggregates were determined by comparing the peak area of both the monomer and the high molecular weight soluble species in the sample with the peak area of a standard containing the same concentration of the protein. The percent of insoluble aggregates was estimated from the difference in the total peak area between the sample and the protein standard solution.

Lysozyme Biological Activity

Lysozyme biological activity against Micrococcus Luteus was measured turbidimetrically at 450 nm in 66 mM potassium phosphate buffer at a pH of 6.24 (17). In brief, the rate of bacterial lysis in a suspension containing 0.1 mL of bacterial suspension (of 0.15% in 66 mM potassium phosphate buffer) and 2.5 mL of protein solution (15 μg/mL in potassium phosphate buffer) was determined. The rate at which the absorbance of the suspension reduced at 450 nm was determined and was found to be linear and proportional to the concentration of the protein in the suspension.

Residual Content of Solvents

The residual content of the solvents was measured by thermogravimetric analysis. Samples of about 2–3 mg were placed in platinum pans and heated at a rate of 10°C/min under a nitrogen purge in a thermogravimetric analyser (TGA 2950, TA Instruments) up to 100°C and were held isothermally at 100°C for 20 min. Data analysis was carried out on a TA data station using the software Thermal Solutions Release 2.5.

Ethanol residue in the powder was traced by nuclear magnetic resonance spectroscopy (Varian Gemini, 300MHz N.M.R). A mass of the protein powder (50mg) was dissolved in one mL of D_2O. Ethanol response in the sample was measured against a standard ethanol solution of 50 ppm in D_2O.

RESULTS AND DISCUSSION

In the present study, the utilisation of carbon dioxide modified with ethanol as an antisolvent for the precipitation of micron-sized particles of proteins suitable for aerosol delivery has been investigated. The effect of process parameters, including temperature, ethanol fraction in the antisolvent and protein concentration on the aerosol performance and the biochemical integrity of the processed powders were assessed. Suitable operating conditions for dense gas processing of aqueous-based proteins using the ASES technique have been determined.

Particle Sizing and Morphology

All proteins precipitated as nano-sized spherical particles, ranging in diameter from 100 to 500 nm (Figure 2). The primary particles tended to agglomerate during the process to form micron-sized clumps of particles. Particle size distribution studies (using the Malvern MasterSizer) showed that the median particle size of processed protein powders is in the micron-sized range (Table 1). Insulin was the most agglomerated powder of all proteins studied. Agglomeration of protein particles in the ASES process has been previously reported (4,6–8). Thiering *et al.* (7) have reported that extensively agglomerated insulin and myoglobin particles were produced using ASES and rapidly expanded GAS processing and suggested that this was a result of the high degree of agitation and frequency of particle collisions in the precipitation chamber.

Process parameters including operating temperature, protein and ethanol concentrations in the antisolvent were found to influence the extent of agglomeration of the primary particles and consequently the median particle size of the processed protein powders. The effect of operating temperature was studied in the range of 20°C–45°C. Whilst processing at higher temperatures in this range had no impact on the morphology of the particles, the powders produced were less agglomerated and exhibited smaller median size as determined by laser diffraction (Table 1). The result is consistent with the enhanced mass transfer of the antisolvent into the water droplet, and the lower density of the antisolvent, both of which could induce a higher degree of supersaturation which resulted in higher nucleation rate with correspondingly less agglomeration. In addition, the solubility of water in dense carbon dioxide increases at higher temperature (18); thus water is more effectively extracted from the droplet by the antisolvent which may result in less agglomeration of the primary particles and consequently agglomerates with smaller median size (measured by laser diffraction) were produced.

The effect of the ethanol concentration in the antisolvent on the particle size of lysozyme was investigated. At low ethanol concentrations (≤10 mole %), a second liquid water rich phase was formed at both 35°C and 45°C and 155 bar and precipitation could not be induced (feed to antisolvent flow rate ratio of 0.4/12). Highly agglomerated particles in a low yield were produced at 45°C and 155 bar using 15 mole % ethanol. Formation of the second liquid phase was not observed at higher concentrations of ethanol in CO_2, and lysozyme precipitated when ≥20 mole % of ethanol in CO_2 was used. These results are consistent with the phase behaviour of ethanol-water-carbon dioxide ternary system (19–21). Gilbert and Paulaitis (20) have demonstrated that at the critical point of the ternary mixture (plait point), the three liquids are completely miscible. For example, at 35°C and 136 bar, the equilibrium concentrations of the components (mole fraction basis) at the critical point are 0.194, 0.082, and 0.724 for ethanol, water, and carbon dioxide, respectively. At lower ethanol concentrations, the mixture exists in the two-phase region: a carbon dioxide rich phase and an ethanol rich phase. The phase separation facilitated the washing of the protein with the ethanol rich phase. At higher concentrations of ethanol (e.g. 30 mole %) the mixture exists in the ethanol rich liquid region (Figure 3), and lysozyme was precipitated as fused particles with no distinct morphology (Figure 2b). This could be due to the poor diffusivity and mass transfer of the ethanol rich liquid phase into the water droplets. Although the addition of ethanol to CO_2 influences the phase behaviour, mass transfer properties and the jet break up in the system, it is not

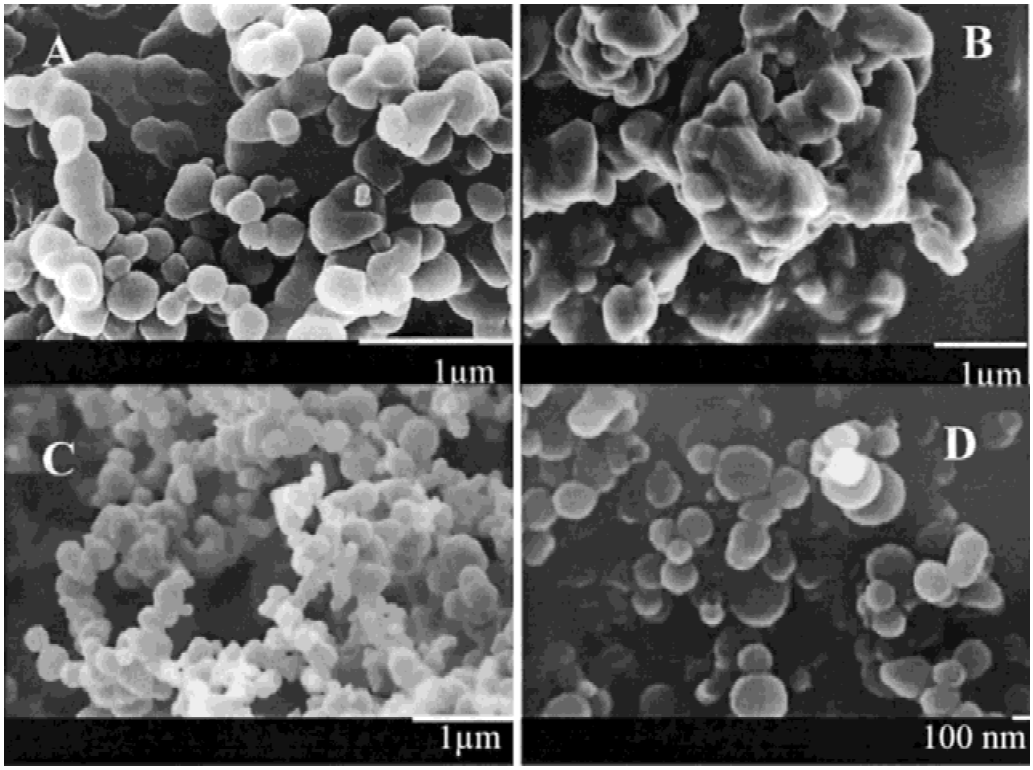

Fig. 2. SEM images of processed proteins, (A) lysozyme at 45°C, 155 bar, and 20 mole % ethanol, (B) lysozyme at 35°C, 155 bar, and 30 mole % ethanol, (C) rhDNase at 20°C, 155 bar, and 20 mole % ethanol, (D) insulin at 45°C, 155 bar, and 20 mole % ethanol.

clear which factor was dominant at each concentration of ethanol. However, the results show that at 35°C, 155 bar, and lysozyme concentration of 15 mg/mL, less agglomerated particles with smaller particle size measured by laser diffraction were produced using 25 mole % ethanol in CO_2. At these conditions, the ternary mixture of ethanol-water-CO_2 exists in the one-phase region and close to the plait point in the ternary phase diagram (Figure 3). It is proposed that at a condition close to the critical point (plait point), the water is extracted more efficiently from the droplets containing lysozyme resulting in less agglomeration in the precipitate and smaller median size.

The effect of protein concentration on lysozyme particle size was investigated in the range 5 mg/mL to 50 mg/mL. Precipitation did not occur immediately upon spraying dilute solutions of lysozyme (5 mg/mL) at 45°C and 155 bar. This indicated that supersaturation was not attained immediately and highly agglomerated particles with a low yield were produced. Less agglomerated Lysozyme and albumin powders with smaller median particle size measured by Malvern were produced with more concentrated protein solutions as shown in Table 1. The rate of nucleation is dependent on the degree of supersaturation achieved in the system, which in turn is a function of the mass transfer rate of the anti-solvent into the droplet and the concentration of the solute. Thus the higher the concentration of the solute within the droplet, the higher the nucleation rate and consequently the lower the degree of agglomeration (22). Hence the median particle size of the agglomerates (measured by laser diffraction) is smaller at higher protein concentrations (Table 1). The result is consistent with those reported in a previous study, which showed that at low protein concentration highly agglomerated particles were produced (7). Similar observations were reported by Benedetti et al. where less agglomerated particles of HYAFF-11 were produced at high solute concentrations (23).

Aerosol Properties

The aerosol performance of the processed protein powders was a function of the operating conditions. Generally, powders with larger fine particle fraction were produced at

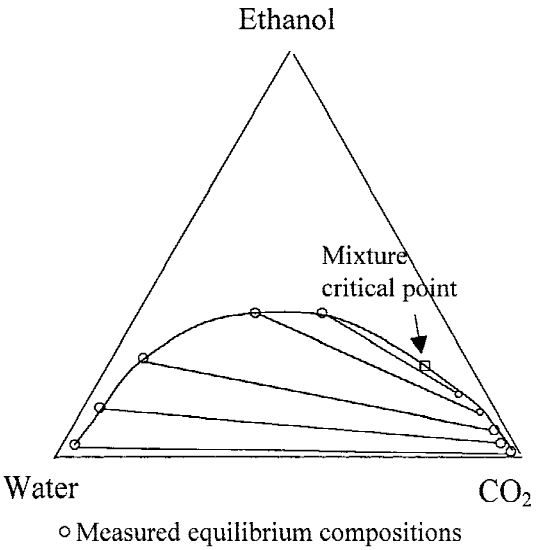

Fig. 3. Ternary phase diagram for ethanol-water-CO_2 at 35°C and 136 bar (ref. no. 21).

higher operating temperature and higher protein concentrations. The fine particle mass for powders processed at 45°C was about 65%, 40%, and 20% for lysozyme, albumin, and insulin, respectively. The aerodynamic particle size distributions of lysozyme, insulin and albumin processed at 45°C (lys7, Alb6, and Ins2) are shown in Figure 4. Large variations in the aerosol performance of the processed proteins indicated that the solute has an effect on the phase behaviour of the mixture and on the mass transfer process, which ultimately affects the degree of particle agglomeration and ultimately the physical characteristics of the processed powders.

The fine particle fraction in dry powder aerosols is generally a function of the inhalation device (16), inspiratory flow rate (24) and dry powder formulation (25). In this study the powders were dispersed at 60 L/min with a high efficiency inhaler (Dinkihaler®) which comprises a small cylindrical swirl chamber with two tangential air inlets. Whilst changing the device or the flow rate would affect the fine particle fraction, it is clear that insulin has a relatively low fine particle fraction compared with other proteins processed under the same conditions in this study and with protein powders processed by spray-drying (15).

The particle size distribution curves of both the original powder (determined by laser diffraction) and the aerosol generated by the Dinkihaler® (determined by impaction) for Lys7 and Alb4 are presented in Figure 5. Apparently, both distributions are close to each other, which indicates that both represent the size distribution of the agglomerates and not the primary particles and the powder was sufficiently dispersed by the Dinkihaler® to generate the respirable agglomerates.

The aerosol performance of the rhDNase powders was not assessed because the protein was substantially denatured during processing.

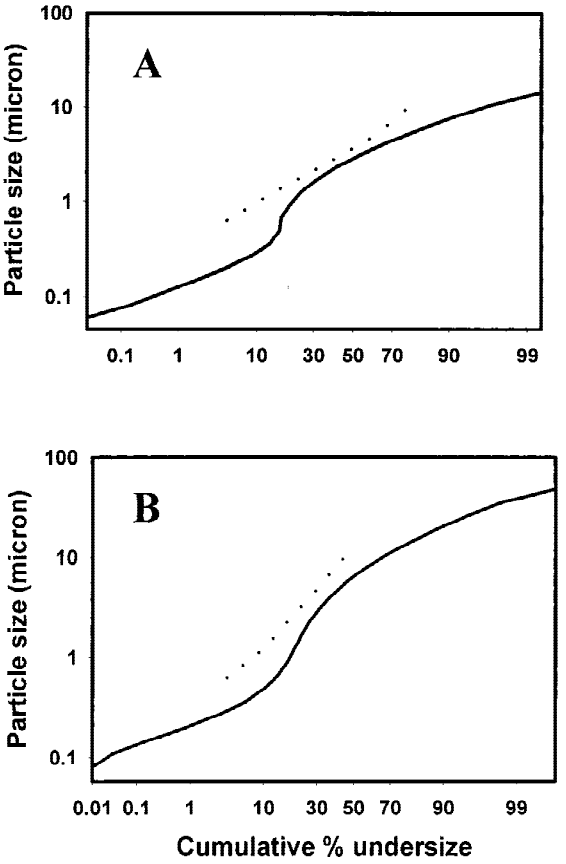

Fig. 5. Comparison of particle size distribution of ASES processed protein powders: (A) Lys 7 and (B) Alb4 before (__) and after dispersion using the Dinkihaler® at 60 L/min (. . .) n = 3.

Biochemical Stability of ASES Processed Proteins

Lysozyme powders generated using the ASES technique at 45°C and 35°C showed almost complete recovery of the biological activity. The moisture content of the processed lysozyme powder was found to be 6.3 wt. % (Lys7) and 7.8 wt. % (Lys8) compared with the 8.5 wt. % in the starting material. Ethanol was not detected in either batch. The result is consistent with a previous study, which reported that lysozyme powders processed at 55°C using the SEDS technique experienced no significant loss of biological activity (11). In addition, molecular aggregation studies have shown that lysozyme experienced no significant loss in the monomer content (Table 1).

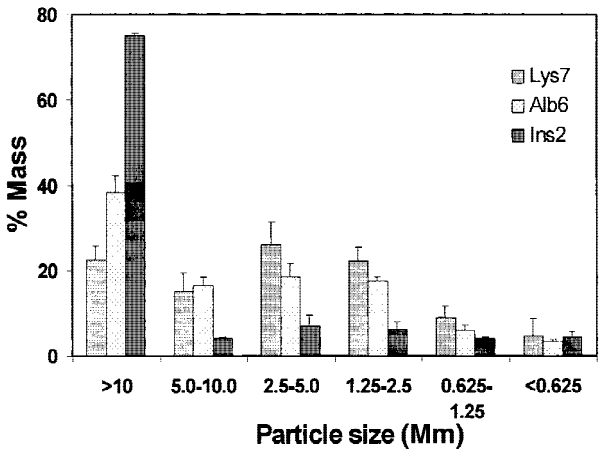

Fig. 4. Aerodynamic particle size distribution of protein powders processed at 45°C. (Lys7 and Alb6 n = 3 and Ins2 n = 2).

A small degree of aggregation to form high molecular weight protein was observed for insulin and almost 93% of the protein was retained as monomer after processing. The result is in good agreement with a previous study, which showed that the structural changes of insulin produced in the ASES precipitation process are reversible upon reconstitution of the processed protein powder in water (6).

The albumin starting material consisted of 86% monomer and 14% soluble high molecular weight proteins. Processing of albumin at 55°C resulted in significant loss in the biochemical integrity. Considerably more activity was retained when powders were processed at 20°C (Table 1). In a previous study on conformational changes of albumin by heat treatment, the structural changes of albumin at pH 2.8 were completely reversible in the temperature range of 2–65°C (26). However, the carbon dioxide mediated ASES process has some impact on the biochemical integrity of the albumin at temperatures lower than 65°C.

rhDNase was substantially denatured by the ASES process as shown by the molecular aggregation studies (Table 1). Powders processed at 45°C were totally aggregated. Reducing the operating temperature from 45°C to 20°C resulted in significant improvement in the stability of the product. About

33% monomer was recovered at 20°C compared with 0% recovery at 45°C. Chan *et al.* (27) reported that rhDNase in the liquid state thermally denatured with a peak onset at about 50°C. It appears that the onset of thermal denaturation of rhDNase occurs at lower temperatures in the ASES process. The other factor that influences the biochemical integrity of the protein is the pH of the aqueous medium in contact with supercritical carbon dioxide, reported to be between 2.5–3 (28). The rhDNase molecules tend to aggregate to high molecular weight proteins in acidic environments (29). It is concluded that the acidity of the precipitation environment and the operating temperature in the ASES process significantly affect the biochemical stability of rhDNase.

In conclusion, the results demonstrate that the modified ASES technique is suitable for the production of micron-sized particles of model and therapeutic proteins for aerosol delivery. The effect of the ASES process on the biochemical integrity of the macromolecules is protein-dependent.

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REFERENCES

1. A. L. Adjei and P. K. Gupta. *Inhalation delivery of therapeutic peptides and proteins,* M. Dekker, New York, 1997.
2. D. W. Matson, R. C. Petersen, and R. D. Smith. Production of powders and films by the expansion of supercritical solutions. *J. Mater. Sci.* **22**:1919–1926 (1987).
3. P. M. Gallagher, M. P. Coffey, and V. J. Krukonis. Gas antisolvent recrystallisation of RDX: Formation of ultra-fine particles of difficult-to-comminute explosives. *J. Supercrit. Fluids* **5**:130–138 (1992).
4. S. Yeo, G. Lim, P. G. Debenedetti, and H. Bernstein. Formation of microparticulate protein powders using a supercritical fluid antisolvent. *Biotechnol. Bioeng.* **41**:341–346 (1993).
5. B. W. Muller and W. Fisher. Method and apparatus for the manufacture of a product having a substance embedded in a carrier. *US patent* 5, 043 280, 1991.
6. M. A. Winters, B. I. Knutson, P. G. Debenedetti, H. G. Sparks, T. M. Przybycien, C. I. Stevenson, and S. J. Prestelski. Precipitation of protein in supercritical carbon dioxide. *J. Pharm. Sci.* **85**:586–594 (1996).
7. R. Thiering, F. Dehghani, A. Dillow, and N. R. Foster. The influence of operating conditions on the dense gas precipitation of model proteins. *J. Chem. Techol. Biotechnol.* **75**:12–24 (2000).
8. P. G. Debenedetti, J. W. Tom, S. D. Yeo, and G. B. Lim. Application of supercritical fluids for the production of sustained delivery devices. *J. Controlled release* **24**:27–44 (1993).
9. P. Huang, A. Dong, and W. S. Caughey. Effects of dimethyl sulfoxide, glycerol, and ethylene glycol on secondary structures of cytochrome c and lysozyme as observed by infrared spectroscopy. *J. Pharm. Sci.* **84**:387–392 (1995).
10. M. H. Hanna, and P. York. Method and apparatus for the formulation of particles using a supercritical fluid, *European patent* 706421B1, 1998.
11. R. Sloan, H. E. Hollowood, G. O. Hupreys, W. Ashraf, and P. York. Supercritical fluid processing: Preparation of stable protein particles. *Proceedings of the fifth meeting of supercritical fluids,* Nice, France, 1998.
12. D. C. Cipolla, A. R. Clark, H. K. Chan, I. Gonda, and S. J. Shire. Assessment of aerosol delivery system for recombinant human deoxyribonuclease. *S.T.P. Pharma. Sciences* **4**:50–60 (1994).
13. F. Dehghani, T. Wells, N. J. Cotton, and N. Foster. Extraction and separation of lanthanides using dense carbon dioxide modified with tributyl phosphate and di (2-ethylhexyl) phosphoric acid, *J. Supercrit. Fluids* **9**:263–272 (1996).
14. A. H. Leferbvre. *Atomisation and sprays.* Hemisphere Publishing Corporation, 1989.
15. H.-K. Chan, A. Clark, I. Gonda, M. Mumenthaler, and C. Hsu. Spray dried powders and powder blends of recombinant human deoxyribonuclease (rhDNase) for aerosol delivery. *Pharm. Res.* **14**:431–437 (1997).
16. N. Y. K. Chew and H.-K. Chan. Influence of particle size, air flow, and inhaler device on the dispersion of mannitol powders as aerosols. *Pharm. Res.* **16**:1098–1103 (1999).
17. A. E. El Nimr. A standard method for determination of lysozyme activity via controlling the kinetics of substrate-enzyme interactions. *Drug Dev. Ind. Pharm.* **11**:653–662 (1995).
18. M. B. King, A. Mubarak, J. D. Kim, and T. R. Bott. The mutual solubilities of water with supercritical and liquid carbon dioxide. *J. Supercrit. Fluids* **5**:296–302 (1992).
19. R. Wiebe and V. L. Gaddy. Vapour phase composition of carbon dioxide-water mixtures at various temperatures and at pressures to 700 atmosphere. *J. Am. Chem. Soc.* **63**:475–48 (1941).
20. M. L. Gilbert and M. E. Paulaitis. Gas-liquid equilibrium for ethanol-water-carbon dioxide mixtures at elevated pressures. *J. Chem. Eng. Data* **31**:296–298 (1986).
21. J. S. Lim and Y. Y. Lee. Phase equilibria for carbon dioxide-ethanol-water system at elevated pressures. *J. Supercrit. Fluids* **7**:219–230 (1994).
22. S. Palakodaty and P. York. Phase behavioral effects on particle formation processes using supercritical fluids. *Pharm. Res.* **16**: 976–985 (1999).
23. L. Benedetti, A. Bertucco, and P. Pallado. Production of micronic particles of biocompatible polymer using supercritical carbon dioxide. *Biotech. Bioeng.* **53**:232–237 (1997).
24. K. J. Smith, H.-K. Chan, and K. F. Brown. Influence of flow rate on aerosol particle size distributions from pressurised and breath-actuated inhalers. *J. Aerosol Med.* **11**:231–245 (1998).
25. D. L. French, D. A. Edwards, and R. W. Niven. The influence of formulation on emission, deaggregation and deposition of dry powders for inhalation. *J. Aerosol Sci.* **27**:769–783 (1996).
26. K. Takeda, A. Wada, K. Yamamoto, Y. Moriyama, and K. Aoki. Conformational change of bovine serum albumin by heat treatment. *J. Protein Chem.* **8**:653–659 (1989).
27. H.-K. Chan, K. L. Au-Yeung, and I. Gonda. Effects of additives on heat denaturation of rhDNase in solutions. *Pharm. Res.* **13**: 756–761 (1996).
28. K. Toews, R. Shroll, and C. M. Wai. pH-Defining equilibrium between water and supercritical carbon dioxide. Influence of SFE of organics and metal chelates. *Anal. Chem.* **67**:4040–4043 (1995).
29. D. Cipolla, I. Gonda, K. C. Meserve, S. Weck, and S. J. Shire. Formulation and aerosol delivery of recombinant DNA derived human deoxyribonuclease. In: J. L. Cleland and R. Langer (eds.), *Protein Formulations and Delivery,* ACS Symposium series 567, American Chemical Society, Washington DC, pp. 322–342 (1993).