Generation of Fine Powders of Recombinant Human Deoxyribonuclease Using the Aerosol Solvent Extraction System

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Purpose. To investigate the feasibility of using the Aerosol Solvent Extraction System (ASES) to produce fine powders of recombinant human deoxyribonuclease (rhDNase), lysozyme-lactose and rhDNase–lactose powders from aqueous based solutions.

Methods. The ASES technique using high pressure carbon dioxide modified with ethanol or ethanol and triethylamine was used for the generation of rhDNase powders and protein-lactose powders from aqueous based solutions. Particle size, morphology, size distributions, crystallinity, and powder aerosol performance were measured. The biochemical integrity of the processed rhDNase was assessed by testing the monomer content and the degree of deamidation.

Results. RhDNase precipitated as spherical particles in the size range between 50 and 500 nm. The primary nano-sized particles were agglomerated to micron-sized clumps of particles during the precipitation process. The median particle size and the fine particle fraction were functions of the operating temperature and the nozzle system used. RhDNase was substantially denatured in the ASES process using carbon dioxide modified with ethanol as anti-solvent. However almost complete recovery of the monomer was achieved using carbon dioxide modified with ethanol-triethylamine as an anti-solvent. Lysozyme-lactose and rhDNase-lactose powders were also precipitated as agglomerated spheres using the ASES process. The powders were amorphous except for those with lactose content higher than 45%.

Conclusions. Micron-sized particles of rhDNase suitable for inhalation delivery were generated from aqueous based solutions using the modified ASES technique. The biochemical integrity of the rhDNase powder is a function of the antisolvent and the operating temperature.

KEY WORDS: ASES; rhDNase; micronization; aerosol delivery; SCF.

INTRODUCTION

Recombinant human deoxyribonuclease (rhDNase) is the first recombinant protein approved for human therapy by inhalation (1). It is available as an aqueous solution for nebulization (2,3). The low efficiency (2,3) and lack of convenience associated with the use of nebulizers make rhDNase an attractive candidate to be delivered with alternative inhalation systems such as dry powder inhalers.

Gas antisolvent techniques have been used for the production of micron-sized particles of proteins suitable for inhalation delivery, such as insulin (4–6), catalase (4,7), trypsin, and lysozyme (8,9). Catalase was precipitated as 1μm spherical and rectangular particles whereas insulin formed both agglomerated nanospheres and 1μm thick needles 5 μm in length. Temperature, solute concentration and the nature of the solvent had little effect on particle size.

Precipitation of proteins using gas anti-solvent techniques relies on the solubility of the solute in an organic solvent, the latter being miscible with an antisolvent 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 (10) or directly into carbon dioxide modified with an organic solvent (11,12). Recombinant human immunoglobulin (rhIG) (12), lysozyme (11), albumin and insulin (11), trypsin (13), and a therapeutic peptide antibody Fv and Fab and plasmid DNA (14) 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 micronized using the modified ASES can be a problem. Although the biologic activity of lysozyme has been almost completely recovered after reconstitution in water, other proteins have been denatured to various extents (11).

It is necessary to protect the protein-protein interactions to minimize degradation during manufacturing and storage, especially molecular aggregation. For example, stable lyophilized 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 (15). Clark and coworkers (1996) (16) reported the effect of carbohydrate excipients on the stabilization of spray-dried proteins.

In the present work, the modified ASES technique was used to generate fine powders of rhDNase, suitable for inhalation delivery, from aqueous solutions. The feasibility of the ASES process to produce lysozyme-lactose and rhDNase lactose powders was also investigated.

MATERIALS AND METHODS

Materials

RhDNase was provided by Genentech Inc. (USA) as an aqueous solution containing 65 mg/mL. The solution was used as received or dialyzed (Spectrum, USA, Molecular Weight Cut off 12-14 KD) against water at 5°C before the ASES processing. Microcrystalline egg white Lysozyme (crystallized (*3), dialyzed and lyophilized, lot No. 57H7045) was purchased from Sigma Chemicals Australia. Ethanol 99.7–100.0%

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ABBREVIATIONS: ASES, aerosol solvent extraction system; RhDNase, recombinant human deoxyribonuclease; VMD, volume median diameter; MMD, mass median diameter; FPM, fine particle mass; TEA: triethylamine; $D_{(0.5,V)}$, median particle size based on volume.

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and triethylamine (98%) were purchased from Fluka Chemicals, while sodium sulphate was from Ajax Australia. Lactose monohydrate, sodium phosphate, sodium chloride analytical grade, HEPES buffer 99.5%, and potassium phosphate monobasic were all of analytical grade and were purchased from Sigma Chemicals. Liquid carbon dioxide (Industrial grade 99.5%) was purchased from BOC Gases.

Apparatus and Precipitation Process

A schematic diagram of the ASES apparatus that was used in the study is shown in Fig. 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, or ethanol containing triethylamine (TEA), 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 either of a capillary tube (Peek tube, 50 μm i.d. and 1.59 mm o.d., SGE) inserted into a stainless steel tube (2.16 mm i.d. and 3.18 mm o.d., Altech) (11), or a specialized nozzle (Sonimist, Model 600-1, USA), which included an elongated body presenting a central tube which served as the primary spray nozzle for the dispersion of the aqueous solution. The Soni-

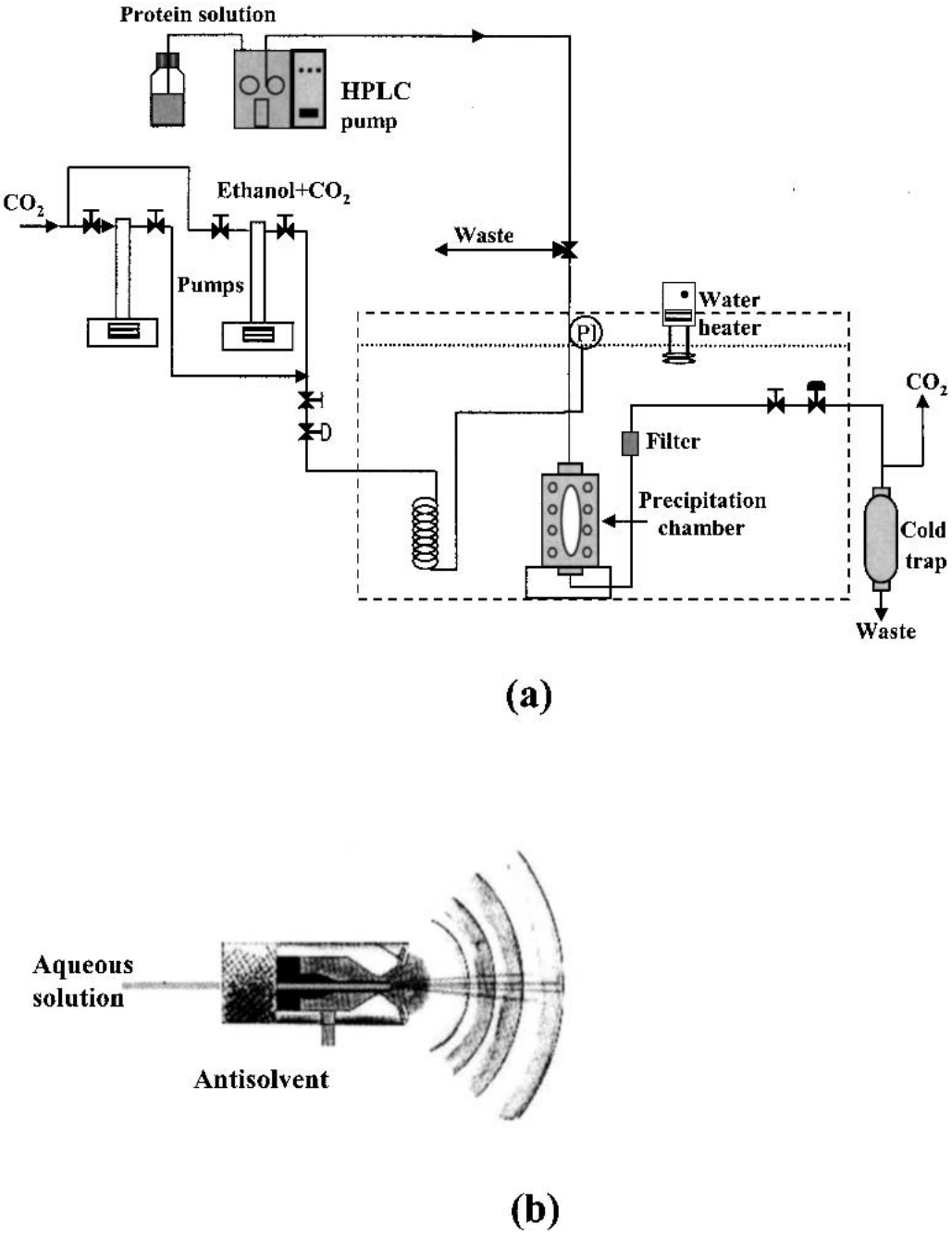

Fig. 1. Schematic diagram of the modified ASES apparatus that was utilized in the experiments (a), gas-energizing nozzle (b).

mist nozzle structure also included a secondary co-axial passageway surrounding the central tube for passage of the energizing gas along the length of the central tube and out of the nozzle outlet (see Fig. 1).

Modified carbon dioxide was prepared by mixing carbon dioxide with ethanol or ethanol containing triethylamine in the syringe pump as previously described (17). The mole fraction of ethanol and TEA in the antisolvent was kept at 0.2 and 0.0012, respectively. During the start-up stage, the chamber was first pressurized with carbon dioxide to attain a pressure of 50–80 bar to avoid phase separation of the carbon dioxide-ethanol mixture on 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 and the desired processing pressure of 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 (0.4 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 depressurized and the powder was collected, sealed in airtight containers, and stored in a freezer (–18°C) for future characterization.

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 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 (18). 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 (19) and a value of 1.25 g/mL was found.

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 particle bounce.

A mass of 4–6 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. 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 plus the filter, divided by the total mass recovered in the device, mouthpiece and the impactor.

Biochemical Integrity of rhDNase Powders

The biochemical integrity of rhDNase was assessed using molecular aggregation and deamidation testing. The biologic activity of the rhDNase was not tested as it has been confirmed in earlier studies that there is a correlation between the biologic activity of rhDnase and the percentage of monomer using molecular aggregation studies.

Molecular Aggregation Studies

The biochemical integrity of the processed rhDNase molecules was assessed using size exclusion chromatography. The monomer and the concentrations of high molecular weight protein in the processed powders were determined using a Protein-Pak 300SW column (Waters, USA)

The powder sample was dissolved in deionized water by gentle shaking for 10 min to assure complete dissolution. The supernatant was filtered through a 0.45 μm membrane filter after centrifugation for 5 min at 15,000 rpm, and then injected into the HPLC system. The mobile phase consisted of 0.5 mM HEPES buffer, 150 mM sodium chloride and 1 mM calcium chloride adjusted to pH 7. The percent of monomer and 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 rhDNase.

Deamidation Testing

The percent deamidation of rhDNase during ASES processing was determined using tentacle cation exchange chromatography on a Waters HPLC system. The mobile phase consisted of 1 mM calcium chloride and 0.1% acetate at pH 4.7 ± 0.2. The percent deamidated (residue Asn 74 to Asp 74) was determined on Fractogel EMD SO_3 25–40 μm of 50 × 100 mm length (E Merck Separations, USA) at a flow rate of 0.5 mL/min. A linear pH gradient of 0 to 0.7 nM NaCL over 30 min was used and the absorbance was monitored at 280 nm. The % deamidation was determined by comparing the peak area of deamidated fraction to the total peak area, which is the sum of the deamidated, and the non-deamidated peak areas.

X-ray Powder Diffraction

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 30 mA with an angular increment of 0.05°/s and an increment count time of 2 s.

RESULTS AND DISCUSSION

In the present study, carbon dioxide modified with ethanol or with ethanol-TEA was used as an anti-solvent for the

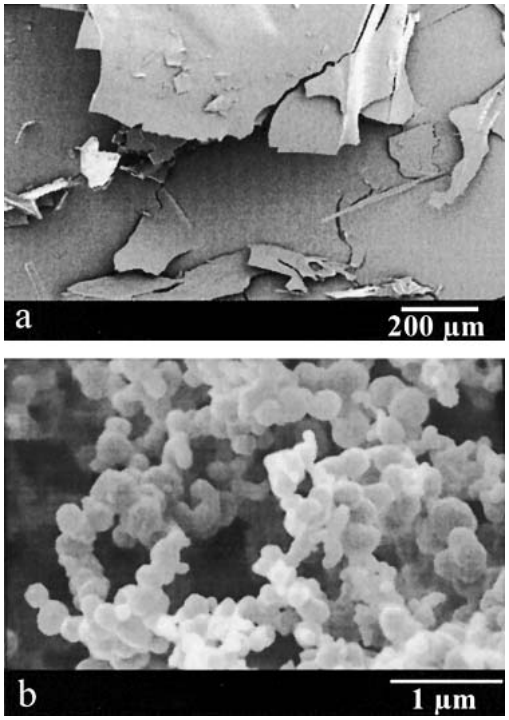

Fig. 2. SEM images of rhDNase, freeze-dried (a), and ASES processed (b).

generation of rhDNase powders and powder co-precipitates with lactose suitable for inhalation delivery. The effect of process parameters, antisolvent, and nozzle system on the characteristics of the rhDNase processed powders was assessed. Suitable operating conditions for dense gas processing of aqueous based rhDNase solutions and rhDNase-lactose solutions using the ASES technique have been determined.

RhDNase Powders

Deoxyribonuclease was precipitated as nano-sized spherical particles ranging in diameter from 100 to 500 nm compared with the freeze-dried powders, which consisted of large irregular particles of a broad particle size distribution (Fig. 2). Typical SEM images of freeze-dried and ASES rhDNase particles processed at 20°C and 155 bar are depicted in Fig. 2. The primary particles of the ASES processed powders tended to agglomerate during the precipitation process to form micron-sized clumps of particles. The results are in agreement with particle size distribution studies using laser diffraction, which showed that the median particle size of the processed powders is in the micron-size range (Table I). Agglomeration of protein particles in the ASES process has been previously reported. Insulin, albumin, lysozyme, and myoglobin form agglomerates of primary particles—a result suggested to be a consequence of the high degree of agitation and frequency of particle collisions in the precipitation chamber (4,7,8,11).

Effect of Process Parameters

Effect of Temperature

The effect of temperature on the median particle size and the biochemical integrity was studied in the range 20°C to 45°C. Although the processing temperature in this range had no apparent effect on the size and morphology of the particles, it had a significant effect on the biochemical integrity of the rhDNase powders (see Table I). Powders of rhDNase 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 40% monomer was recovered at 20°C compared with 0% recovery at 45°C using carbon dioxide modified with ethanol as antisolvent. Clearly, the lower the temperature the more stable was the product. Although the peak onset of thermal denaturation of rhDNase is reported at about 50°C (20), it seems that the onset of thermal denaturation of rhDNase in the ASES process occurs at lower temperature (35°C).

Table I. Characteristics of rhDNase Powders Generated at 155 bar Using the ASES Process

T°C	RhDNase conc. mg/mL	Formula	Anti-solvent	$D_{(0.5,V)}$ µm	% M	% IA	FPM %
45	10	DD	ETOH-CO_2	5.9	0	88.7	ND
35	10	DD	ETOH-CO_2	8.4	14.0	82.3	ND
20	20	DD	ETOH-CO_2	10.6	33.2	65.5	ND
20	20	DD	ETOH-CO_2	11.0	42.5	55.5	ND
20	50	DD	ETOH-T-CO_2	7.5	93.0	2.0	20.5
20	50	DD	ETOH-T-CO_2	6.5	93.5	1.7	19.6
35	45	DD	ETOH-T-CO_2	6.4	46.5	52.3	ND
35	45	DD	ETOH-T-CO_2	8.5	59.0	40.3	29.2
35	65	D	ETOH-T-CO_2	5.9	76.0	15.4	ND
35	65	D	ETOH-T-CO_2	6.2	80.8	9.4	ND
20	65	D	ETOH-T-CO_2	7.3	97.5	0	21.0
20	65	D	ETOH-T-CO_2	6.0	ND		30.0
20	65	D	ETOH-T-CO_2	5.5	ND		33.0

Note: EtOH, ethanol; CO_2, carbon dioxide; T, triethylamine; DD, dialysed rhDNase solution; D, rhDNase solution, $D_{(0.5,V)}$, median particle size based on volume; M, monomer content; IA, insoluble aggregates and FPM, fine particle mass. All experiments were performed using the coaxial nozzle except the last two experiments.

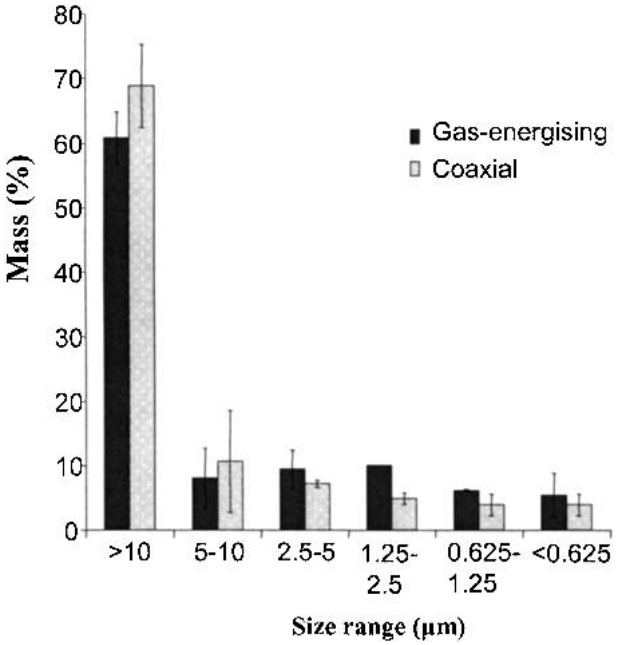

Fig. 3. Aerodynamic particle size distribution of rhDNase generated at 20°C and 155 bar using coaxial nozzle and gas-energizing nozzle.

The aerosol performance of rhDNase powders generated in the temperature range of 20 to 45°C using carbon dioxide modified with ethanol was not assessed due to the denaturation of the protein.

Effect of Antisolvent

The antisolvent seemed to have a significant effect on the stability of the rhDNase molecules during the ASES precipitation process from aqueous solutions. Substantial denaturation of rhDNase occurred in the carbon dioxide during the ASES process even at operating temperature as low as 20°C (see Table I). The biochemical integrity of the rhDNase was influenced by the low pH (2.5–3) of the aqueous medium in contact with the supercritical carbon dioxide (21). The result is consistent with previous reports concerning rhDNase molecule aggregation to high molecular weight proteins in acidic environments (2). It is concluded that the acidity of the precipitation environment in the ASES process significantly affected the biochemical stability of rhDNase. Thus carbon dioxide alone is not a suitable antisolvent for the generation of stable powders of rhDNase in the ASES process.

The detrimental effect of carbon dioxide on the biochemical integrity of proteins has also been previously reported (12). Precipitation of recombinant human immunoglobulin from aqueous based solutions using carbon dioxide modified with ethanol as antisolvent in an ASES process resulted in 50% loss in the activity of the protein (12).

Modification of the ethanol-carbon dioxide mixture with TEA significantly improved the biochemical integrity of rhDNase in the ASES process. A substantial improvement in the stability of the processed powders was achieved. Almost complete recovery of the monomer content of rhDNase (97%) was achieved after processing of rhDNase solution at 20°C using ethanol-TEA-CO_2 as an antisolvent (see Table I).

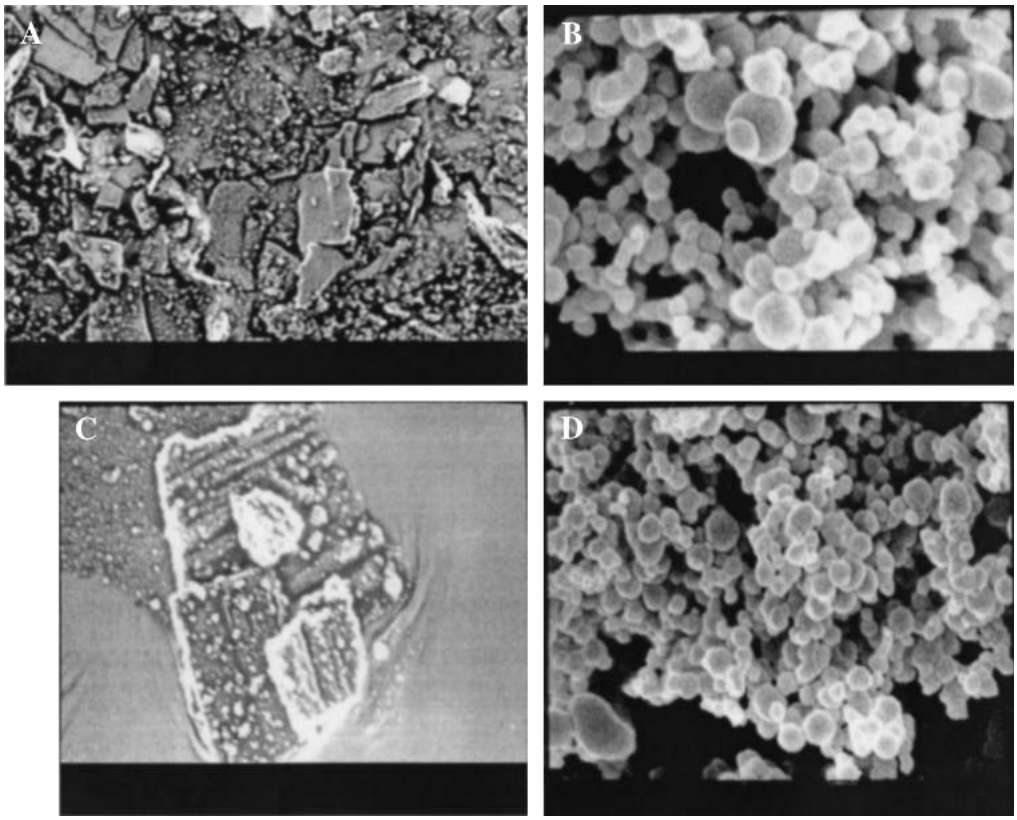

Fig. 4. SEM images of ASES processed powders of lactose (a), lysozyme-lactose (92:8) (b), lysozyme-lactose (10:90) (c), rhDNase-lactose (65-35) (d)

In addition, the deamidation testing of the processed powder showed less than 1% change in the deamidated fraction after the ASES processing.

Triethlyamine is not listed among the Class I solvents that should be avoided in pharmaceutical processing according to the published guidelines CPMP/ICH/283/95. Moreover, because of the high solubility of ethanol and TEA and the efficient washing stage in the ASES process, the amount of residual solvent in the final product would be very limited. Nuclear magnetic resonance spectroscopy was used to trace the residual TEA and indicated a level of less than 10 ppm in a standard 50 mg/mL sample in D_2O solution.

Effect of Nozzle Design

The effect of the nozzle system used on the median particle size and the aerosol dispersion of the ASES processed powders was investigated using two nozzle arrangements, the coaxial and the gas-energizing nozzle. The aerodynamic particle size distribution of the powders produced using both nozzles is depicted in Fig. 3. Processing of rhDNase at 20°C and 155 bar using the gas-energizing nozzle produced powders having smaller median size and higher fine particle fraction compared with powders produced by the coaxial nozzle arrangements. The effect of the gas-energizing nozzle on the particle formation in the ASES process was attributed to the introduction of high frequency waves that speed up the atomization process and increase mass transfer rates so that nucleation occurs before coalescence of droplets. The gas-energizing nozzle might produce smaller droplets and more efficient mixing in the precipitation cell. Both effects may induce higher mass transfer for the anti-solvent into the droplets with correspondingly more efficient water extraction resulting in less agglomerated powders.

Protein-Lactose Powders

Lactose is used in most dry powder inhalers as a carrier to facilitate flow and dispersion of the powder. In the present study, the lactose was prepared as fine particles for inhalation. The long-term effect of fine lactose particles in the lung is unknown, but the acute effect is expected to be similar to that of mannitol (i.e., causing bronchoconstriction in sensitive subjects) if a sufficient amount is inhaled (22).

Lactose was successfully precipitated using CO_2 as an antisolvent modified 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 (Fig. 4). The X-ray diffraction pattern showed that ASES processed lactose powder was semicrystalline (Fig. 5).

Lactose was previously precipitated from aqueous based solutions using a specialized nozzle utilizing the SEDS technique (23). The morphology ranged from thin large bands to large agglomerated chunks by varying the flow rate of carbon dioxide.

In the present study, the effect of the ASES process conditions on the characteristics of lactose was not investigated as the precipitation of lactose-protein powders was performed under the optimum process conditions for the precipitation of proteins. Lysozyme is readily available and was used as a model protein to allow initial optimization of the experimental conditions to be carried out.

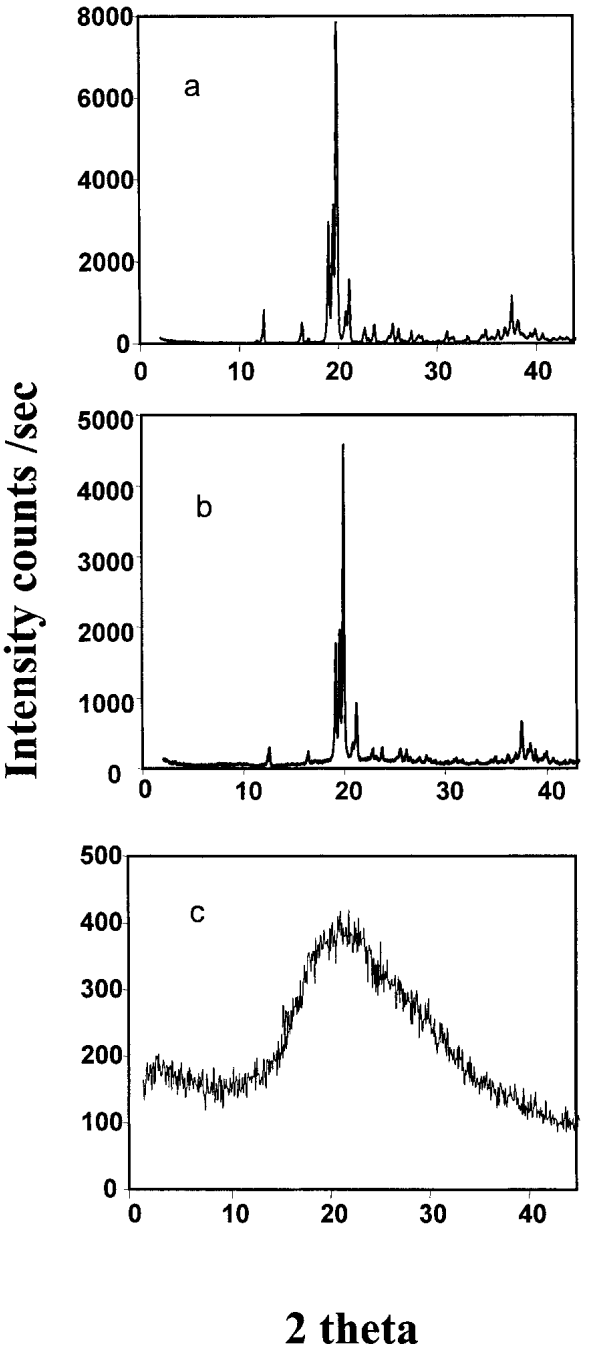

Fig. 5. X-ray diffraction pattern of ASES processed powders of lactose (a), lysozyme-lactose (56:44) (b), and rhDNase-lactose (92:8) (c).

Lysozyme-lactose powders were precipitated using the gas-energizing nozzle at 20°C and 155 bar from various weight ratios of lysozyme and lactose in the aqueous feed (Table II). Lysozyme–lactose ASES processed powders containing 92.5 wt% lysozyme consisted of nano-sized spheres ranged from 100–500 nm as shown in the typical SEM images (see Fig. 4). 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 about 90% lactose exhibited two particle morphologies, micron-sized plates and nano-sized spheres corresponding to lactose and lysozyme, respectively (see Fig. 4). The X-ray diffraction

Table II. 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 nm	56.0	semicrystalline
50:100	Agglomerate nanospheres plates <20 μm	10.5	semicrystalline

patterns showed that powders containing 10% lactose were amorphous. However, powders containing higher than 44 wt% lactose were semicrystalline (see Fig. 5).

Generation of rhDNase-lactose powders from aqueous solutions was achieved using ethanol-TEA in carbon dioxide as antisolvent and spraying the solution through the gas-energizing nozzle. The ASES processed powders of rhDNase-lactose (15 wt% lactose) consisted of smooth spherical particles ranging in size from 50–500 nm as shown in the SEM images (see Fig. 4). The absence of diffraction pattern in the X-ray diffraction peaks of the powders indicated the amorphous nature of the product. The fine particle fraction of the rhDNase powder containing 15 wt% lactose was similar to that of rhDNase powders prepared under similar conditions (30.5 wt. %). Increasing the lactose mass fraction in the sprayed solution to 35 wt% resulted in the production of amorphous highly agglomerated powders with lower fine particle mass compared to rhDNase powders containing 15 wt% lactose (20.0 wt. % vs. 30.5 wt. %). High concentrations of lactose in the sprayed solutions might increase the viscosity of the droplets resulting in low mass transfer rates of the antisolvent into the droplets with correspondingly less efficient water extraction and consequently higher coalescence and agglomeration.

The generation of rhDNase-lactose powders have been previously reported using the spray drying technique (18,24). The spray-dried powders, with and without the excipient lactose, appear as smooth spherical particles (24). Both types of powders (pure rhDNase powders and rhDNase—lactose powders containing up to 40 wt% lactose—note: present study was 44% semi-crystalline) were amorphous as shown in the X-ray diffraction patterns. The results are consistent with our findings in the present study using the ASES technique for the generation of protein powders.

CONCLUSIONS

The ASES process has been shown to be a feasible method for the production of micron-sized, stable powders of rhDNase, suitable for inhalation, from aqueous based solutions. The biochemical integrity of the rhDNase powder was a function of the anti-solvent and operating temperature. The gas-energizing nozzle produced rhDNase powders with higher fine particle mass compared with those produced by a coaxial nozzle. The technical feasibility of the generating protein-lactose powders from aqueous solutions using the ASES process has been demonstrated. However, agglomerated powders were produced at high levels of lactose.

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