JPP Journal of Pharmacy and Pharmacology

JPP 2003, 55: 185–192

Received September 27, 2002
Accepted October 16, 2002
DOI 10.1211/002235702504
ISSN 0022-3573

Supercritical fluid processing of proteins: lysozyme precipitation from aqueous solution

Saeed Moshashaée, Mikael Bisrat, Robert T. Forbes, Éilís Á. Quinn, Håkan Nyqvist and Peter York

Abstract

Aqueous solutions of hen egg lysozyme (3% w/v) were dispersed and precipitated by a homogenous mixture of supercritical carbon dioxide–ethanol using the Solution Enhanced Dispersion by Supercritical fluid (SEDS) process. The effects of different working conditions, such as temperature, pressure and the flow rates of the solution and ethanol, on the particle-formation process were studied. The morphology, particle size and size distribution and biological activity of the protein were determined. The precipitates were examined with high-sensitivity differential scanning calorimetry (HSDSC) and high-performance cation-exchange chromatography. Particle size measurements showed the precipitates to be aggregates with primary particles of size 1–5 μm. The similarity of HSDSC data for unprocessed and processed samples indicated that the different physical forces that stabilise the native form of lysozyme are unchanged after SEDS processing. From FT-Raman spectroscopic studies secondary structural changes were observed in certain SEDS-produced lysozyme, with most processed samples displaying a slightly more disordered secondary structure than the unprocessed sample. However, SEDS samples produced at 200 bar and 40 °C exhibited negligible disturbance. Thus the SEDS process utilising aqueous solution was able to bring about size reduction of lysozyme with minimal loss of biological activity.

Eli Lilly and Company, Indianapolis, IN, 46285, USA

Saeed Moshashaée

Biovitrum AB, 112 76 Stockholm, Sweden

Mikael Bisrat

Drug Delivery Group. The School of Pharmacy, University of Bradford, BD7 1DP, UK

Robert T. Forbes, Éilís Á. Quinn, Peter York

AstraZeneca, Södertälje, 151 85 Södertälje, Sweden

Håkan Nyqvist

Bradford Particle Design, Listerhills Science Park, Bradford West Yorkshire, BD7 1HR, UK

Peter York

Correspondence: Robert T. Forbes, Drug Delivery Group, The School of Pharmacy, University of Bradford, BD7 1DP, UK.
E-mail: r.t.forbes@bradford.ac.uk

Acknowledgements: The authors thank Dr Mustafa Demirbüker for his valuable comments and Birgitta Lange for her laboratory assistance. SM thanks AstraZeneca for financial support.

Introduction

Supercritical fluids (SFs) have unique properties, since they combine liquid-like solvent power with gas-like transport properties. They have a large compressibility compared with ideal gases. This implies that a small change in temperature or pressure near the critical values will result in large changes in the fluid's density and hence the solvent power (Donsi & Reverchon 1991). Carbon dioxide is the most widely used SF, due to the favourable critical parameters (temperature 31.1 °C, pressure 73.8 bar), cost and non-toxicity.

Two principles for precipitating particles with SFs have been adapted, Rapid Expansion of Supercritical Solutions (RESS) and Supercritical AntiSolvent (SAS) or Gas AntiSolvent (GAS) precipitation. When using RESS, the sensitivity of solvent power of an SF to small changes in pressure is used to trigger a mechanical precipitation (Tom & Debenedetti 1991; Debenedetti et al 1993; Phillips & Stella 1993). However, the limitation here is that the solubility of peptides and proteins is very low in the SF. For molecules not soluble in the SF, the SAS or GAS processes can be used, as long as the SF is miscible with the liquid in which the compound is dissolved (McHugh & Krukonis 1994).

For preparation of small protein particles, techniques involving SFs (i.e., fluids used at temperatures and pressures above their critical value) have been utilised (Tom et al 1993; Yeo et al 1993, 1994). The Solution Enhanced Dispersion by Supercritical fluids (SEDS) process, in addition to the antisolvent properties of the SF, has coupled a physicomechanical function. This technique involves a continuous flow of the protein solution and the SF, co-introduced through a coaxial nozzle into a particle-formation vessel, which leads to dispersion and mixing of the protein solution, rapid super-saturation and particle nucleation and formation. It has been shown that by using different working conditions, directed changes in particle properties can be achieved (Hanna & York 1993).

Precipitation of proteins using supercritical carbon dioxide (SC-CO_2) has to date been limited to dissolving the proteins in organic solvents dimethyl sulfoxide (DMSO) and *N*, *N*-dimethylformamide (DMFA) (Yeo et al 1993, 1994; Winters et al 1996; Moshashaée et al 2000). However, significant perturbations of protein secondary structure on exposure to DMSO have been reported (Jackson & Mantsch 1991). In other studies the structural changes in SAS-processed protein observed could be linked to DMSO exposure (Yeo et al 1993; Winters et al 1996). Some loss of biological activity in SEDS-processed lysozyme has been observed at a low working pressure such as 80 bar (Moshashaée et al 2000). This could be related to prolonged exposure of precipitates to DMSO. The native form of lysozyme is more likely to exist in aqueous solutions. Additionally, it has been shown that the SF antisolvent-processed protein refolds to its native form upon reconsti-tution in aqueous solutions (Winters et al 1996). Hence, precipitation from aqueous protein solution using SF is clearly to be preferred. Lysozyme has been successfully precipitated from aqueous solutions using high-pressure-modified carbon dioxide (Bustami et al 2000) but no solid-state spectroscopic analysis of the powders was undertaken.

The extremely low solubility of aqueous solutions in SC-CO_2, and vice-versa, is a major hindrance to working with aqueous protein solutions. By using additional miscible organic solvents such as methanol or ethanol, the solubility match between carbon dioxide and water will be enhanced (Palakodaty et al 1998). The SEDS process provides the possibility of the micron-sized protein powder formation from an aqueous solution in a single equilibrium phase. In this technique the aqueous solution of protein is only contacted momentarily with the mixture of an organic solvent and SC-CO_2 (Palakodaty et al 1998). The precipitation of micro-fine protein powder from aqueous solution using SEDS has been successfully carried out earlier by different workers (Forbes et al 1998; Sloan et al 1998, 1999). In these studies, precipitation of protein occurred from aqueous solution by using SC-CO_2 mixed with ethanol immediately before dispersion of the protein solution in a three-channelled coaxial nozzle. However, the secondary structure of the SEDS-processed protein samples was not reported in these studies.

The aim of this study was to consider the precipitation of small particles of a model protein, lysozyme, with retained biological activity, from an aqueous solution using modified SC-CO_2. The effects of several parameters such as temperature, pressure and flow rates of the solution and modifier will be examined.

Materials and Methods

Materials

Lysozyme from microcrystalline hen egg white, spray-dried (Boehringer Ingelheim, Germany; Bioproducts, UK) was dissolved in distilled water. *Micrococcus lyso-*

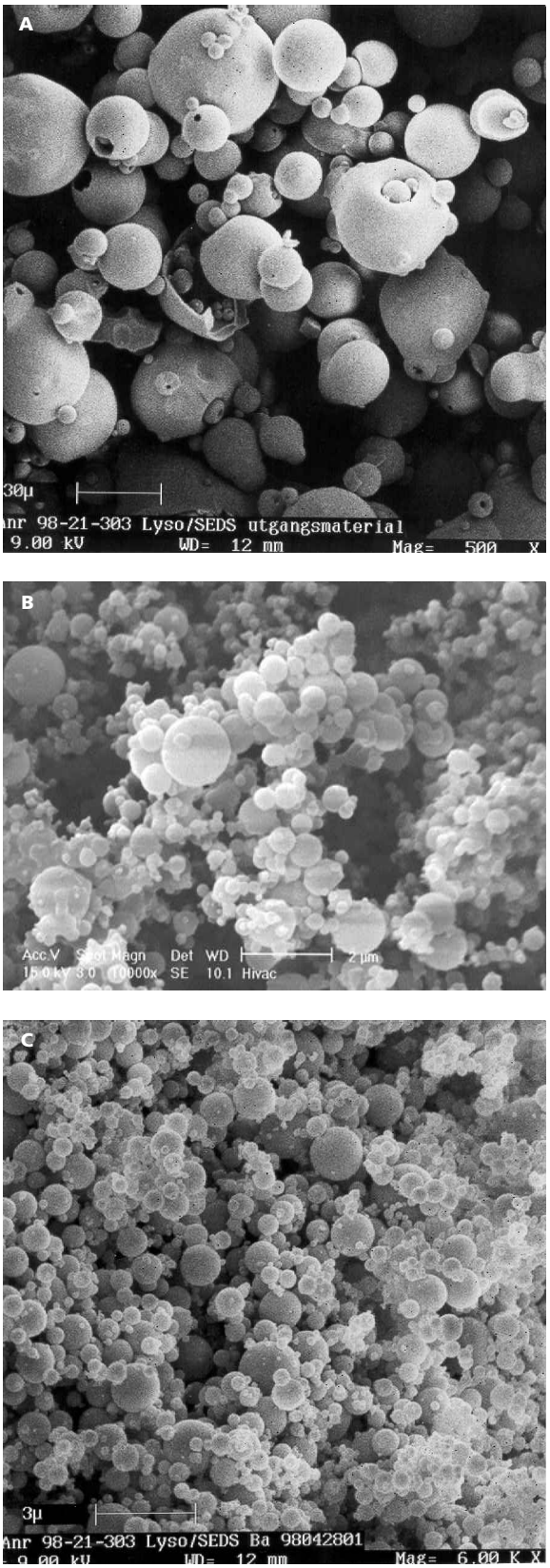

Figure 1 SEM images of untreated and SEDS-processed lysozyme particles. A. Starting material, spray-dried lysozyme; bar = 30 μm. B. Sample no. 4, bar = 2 μm. C. Sample no.1, bar = 3 μm.

deikticus (Sigma Chemical Company, St Louis MO; lot No 16H6809) was used for investigation of biological activity of lysozyme.

Liquid CO_2 (AGA, Sweden), 99.9% purity, was used as the SF. Ethanol 99.5% was used as modifier.

Precipitation of protein micropowders

The SEDS apparatus and process has been described previously (Palakodaty et al 1998). Lysozyme was dissolved in filtered water in different concentrations and then pumped by a Jasco PU-980 (Japan) through a two-channelled coaxial nozzle into a 100-mL particle-formation vessel (Thar-Designs, USA). SC-CO_2 was modified by mixing and dissolving ethanol in SC-CO_2 by a Valco (USA) T-piece before entering the nozzle. The pressure inside the particle-formation vessel was controlled by a back-pressure regulator (Jasco 880–81, Japan), and an air-heated oven controlled the temperature of vessel. Methanol, ethanol, acetone and isopropanol as organic solvents facilitate the extraction of water into the SF, which results in the precipitation of protein particles. Of these solvents, which we have used to modify SC-CO_2 to increase the region of miscibility of water in SF, ethanol was preferred in this study due to its lower toxicity and less deleterious effect on the protein.

The particles were collected on a steel frit with a pore size of 0.5 μm and from the wall of the particle-formation vessel, while the mixture of the solvent and modified CO_2 left the apparatus through the filter. After complete delivery of the protein solutions, pure CO_2 was flushed through the vessel to remove any residual solvent from the particles. After a slow depressurisation of the system, the protein particles were collected, weighed, placed in airtight cans and stored at $-18\,^{\circ}C$.

The working parameters of interest to precipitate lysozyme using the SEDS process were: pressure and temperature inside the particle-formation vessel and flow rates of the modifier and the solution (Table 1).

Morphology and particle size distribution measurements

Photomicrographs were taken using a scanning electron microscope (Philips XL30 ESEM, Netherlands) equipped with a GSE-detector. The samples were sputter-coated with 15 nm gold (Bal-Tec SCD 005) before examination.

A Coulter Counter Multisizer II (Beckman Coulter, Inc., US) was used to determine the particle size distribution of the precipitates. The particles were suspended in 10% ammonium thiocyanate in methanol and sonicated before the analysis. Combination of these methods offers a possibility to study the size of the primary particles as well as the ease of dispersion of any agglomerates.

Determination of protein structure and physicochemical characterisation of lysozyme samples

FT-Raman

Raman spectroscopy was performed on solid samples using a Bruker IFS 66 spectrometer (Germany) with an FRA 106 FT-Raman module attachment equipped with an Nd3+ :YAG laser emitting at a wavelength of 1064 nm. Solid samples were analysed in stainless-steel sample cups. The laser power was approximately 200 mW. All spectra were the average of 200 scans and 4000 scans at 8 cm^{-1} resolution. A liquid-nitrogen-cooled germanium detector with an extended spectral bandwidth was used over the wavenumber range 3600–400 cm^{-1}. Wavenumber positions are accurate to better than $\pm 1\,cm^{-1}$. All experiments were performed at room temperature ($25 \pm 1\,^{\circ}C$).

Table 1 Working conditions for the lysozyme precipitation from aqueous solution and biological activity data.

Sample no.	Pressure (bar)	Temp. (°C)	Flow rate (mL min^{-1})		Biological activity
			Modifier	Solution	
1	100	40	1.5	0.1	94 (0.02)
2	100	50	2	0.1	86 (0.1)
3	100	50	2	0.1	88 (0.1)
4	200	40	2	0.1	96 (0.1)
5	200	50	1.5	0.1	90 (0.1)
6	100	40	2	0.2	No particles produced
7	100	50	1.5	0.2	No particles produced
8	200	40	1.5	0.2	No particles produced
9	200	50	2	0.2	No particles produced
10	200	50	2	0.1	80 (0.1)
11	200	40	1.5	0.1	101 (0.2)
12	150	45	1.75	0.15	65 (0.2)
13	150	45	1.75	0.15	63 (0.1)
14	150	50	1.5	0.1	Not measured

Biological activity is presented as mean (± s.d.) of 3 measurements.

HPLC

High-performance cation-exchange chromatography (Lachrome, Merck-Hitachi) was carried out to compare the precipitates with the commercial as-supplied lysozyme, as well as to determine purity and any process-induced conformational changes. A conformational change should alter the hydrophobicity of the protein and thus affect the retention time of the sample compared with the starting material (Williams & Dunker 1981). The separations were performed with a mobile phase containing 0.02 M phosphate buffer and 0.5 M sodium chloride at pH 7.5. The column employed was a TSK-gel SP-NPR (35 mm × 4.6 mm i.d.) from TosoHaas (Labkemi, Sweden). The lysozyme solutions consisted of 0.5 mg mL^{-1} in the phosphate buffer.

Biological activity assay

The biological activity of the products was determined by studying the hydrolysis of β-1,4 glycosidic linkages between *N*-acetylglucosamine and *N*-acetylmuramic acid in bacterial cell walls, caused by the lysozyme. A bacterial suspension was prepared by adding 20 mg of *Micrococcus lysodeikticus* (Sigma Chemical Company, St Louis, MO; lot no. 16H6809) to 100 mL of a phosphate buffer, pH 7 and 0.06 M (Winters et al 1996). The lysozyme solution consisted of 0.1 mg mL^{-1} of the sample in phosphate buffer. The enzyme solution (10 μL) was added to a cuvette with the bacterial suspension and diluted to an initial absorbance of 0.800 ± 0.010. The rate at which the absorbance at 450 nm decreased over 4 min was determined spectrophotometrically with a Perkin Elmer lambda 2S (Norwalk, US) spectrometer. The rate of lysis of the precipitates was compared with that of the starting material (Verhamme et al 1988).

Results and Discussion

Biological activity

Using an experimental design approach, we have previously shown (Moshashaée et al 2000) that working pressure had the most significant effect on the biological activity of lysozyme when produced by SEDS from DMSO. In this paper we probe the benefit of using SEDS to form particles from an aqueous solution. Based on this earlier study, the flow rate of carbon dioxide was 30 mL min^{-1}, the concentration of lysozyme in the solution was 3% w/v and a 0.2-mm nozzle was used for each run. The other relevant working conditions for each run, along with biological activity data of samples, are presented in Table 1. Thus, because of our previous work, the number of experimental variables is reduced, as is the number of experimental runs. The SEDS process was highly reproducible in that the biological activity measured for two separate batches (2 and 3) differed by only 2%.

From Table 1 it is clear that pressure is not the major factor governing product quality, since, in some SEDS-treated runs (nos 6–9, with low ethanol–water levels), excess water was not completely removed by the SC-CO_2. Because water remained in the particle-formation vessel, no particles could be collected. In this situation the mixture of water–ethanol–CO_2 is not in a homogeneous phase at equilibrium and thus the protein might exist below the binodal curve in the vapour + liquid phase. Hence the concentration of lysozyme in the water-rich phase did not achieve supersaturation, and, in turn, no precipitation occurred. For those samples that did produce particles, the data presented in Table 1 reveals that the retained biological activity of the samples varied between 63 and 101%. In contrast to our previous study (Moshashaée et al 2000), at a temperature of 50 °C, no obvious correlation between the working conditions and the level of activity is found when the working pressure chosen is either 100 or 200 bar. The lowest biological activity is observed at 150 bar and 45 °C, and appears to be related to the ratio of ethanol–water in the ternary system, since this ratio is the lowest used that formed a precipitate. The highest biological activity is observed at 200 bar and 40 °C, which, in line with theory, is the most likely working condition to form particles from a single homogeneous phase between water–ethanol and supercritical CO_2. The data in Table 1 also illustrate the benefit of using water for the formation of protein powders, in that the loss of biological activity from aqueous precipitates is less than those for precipitates from organic solution (Moshashaée et al 2000). A further benefit of the aqueous component is that activity appears to be less sensitive to pressure than when DMSO is used as the solvent (Moshashaée et al 2000). Given that samples 6–9 did not form precipitates and that samples 12 and 13 had the lowest activity, the key factor for the formation of protein powder from aqueous solution with high biological activity in this study seems to be the composition of water–ethanol and carbon dioxide. This means that the particles should be formed in a single equilibrium phase, while removing the water and organic modifier from the particle-formation vessel.

Morphology and size distribution

Typical SEM photomicrographs of the starting material and representative SEDS precipitates from aqueous solutions are shown in Figure 1 and particle size data for selected working pressures are presented in Table 2. The starting material (spray-dried lysozyme) presents a wide particle-size distribution and non-uniform shape, while the SEDS-processed particles are homogenous in size and more spherical in shape. SEM and Coulter Counter data show that in general, the particle size of SEDS-processed products have decreased significantly compared with the starting material. The SEDS process produced primarily spherical particles in the 1–5 μm range. Thus, SEM images show no significant effect of the working pressure on particle size and morphology with Coulter data indicating that at both lower and higher pressures the samples are readily dispersible to primary particles. Since the concentration of CO_2 is constant and relatively high to the solution flow in all experimental runs and the diffusion of ethanol in carbon

Table 2 Coulter Counter Multisizer data (volume statistics) for SEDS-processed lysozyme particles from water solutions.

Sample no.	Temp. (°C)	Pressure (bar)	D(10%) (μm)	D(50%) (μm)	D(90%) (μm)
1	40	100	1.26	3.34	10.51
2	50	100	1.33	4.91	12.35
3	50	100	0.76	1.61	10.32
4	40	200	1.05	1.90	5.19
14	50	150	1.01	2.78	7.40

D(10%), 10% of particles undersize; D(50%), 50% of particles undersize; D(90%) 90% of particles undersize.

dioxide (and vice-versa) occurs rapidly, the particle nucleation, growth and subsequently the morphology of particles is thought to be primarily dependent on the diffusion coefficient of water in carbon dioxide. Change in the composition of carbon dioxide, ethanol and water for the SEDS samples that formed precipitates are relatively small in these experimental domains and thus the rate-determining step for particle formation is changing insignificantly and consequently the particle size and the morphology of the SEDS treated particles are similar (Figure 1).

The Multisizer Coulter Counter, with an aperture tube of 30 μm, only measured particles in the range 0.6–15 μm, thereby excluding the smaller particles present in the samples, as seen on the SEM images. Coulter-Counter-determined size measurements for some SEDS-processed samples with primary particles in the 1–5 μm range, at different pressures and temperatures, are shown in Table 2. At both lower and higher pressures (100–200 bar) the samples are readily dispersible to primary particles and consequently measurable by the Coulter Counter (Table 2). It is interesting to note that the particle size and size distribution of spray-dried lysozyme used as the starting material for this investigation could not be measured with the Coulter Counter due to the blockage of the measuring cell. Interestingly, and yet yielding high retained biological activity, the particle size distribution is narrower at a working pressure of 200 bar (Table 2). Such a size range would be suitable for formulating protein particles into respiratory drug-delivery systems.

Determination of protein structure and physicochemical characterisation of lysozyme samples

While particle size and retained biological activity may be important product properties, the solid-state configuration of the proteins is also of interest. Raman spectroscopy has been applied to study supercritically precipitated lysozyme from DMSO (Winters et al 1996). This technique was used to investigate the lysozyme precipitates formed by the SEDS process from aqueous solutions. Results are shown in Figures 2, 3 and 4. In Figure 2 the spectra of lysozyme following SEDS processing are compared with the spectrum of lysozyme before SEDS and are in close agreement with published spectra (Lord & Yu 1970). Although it

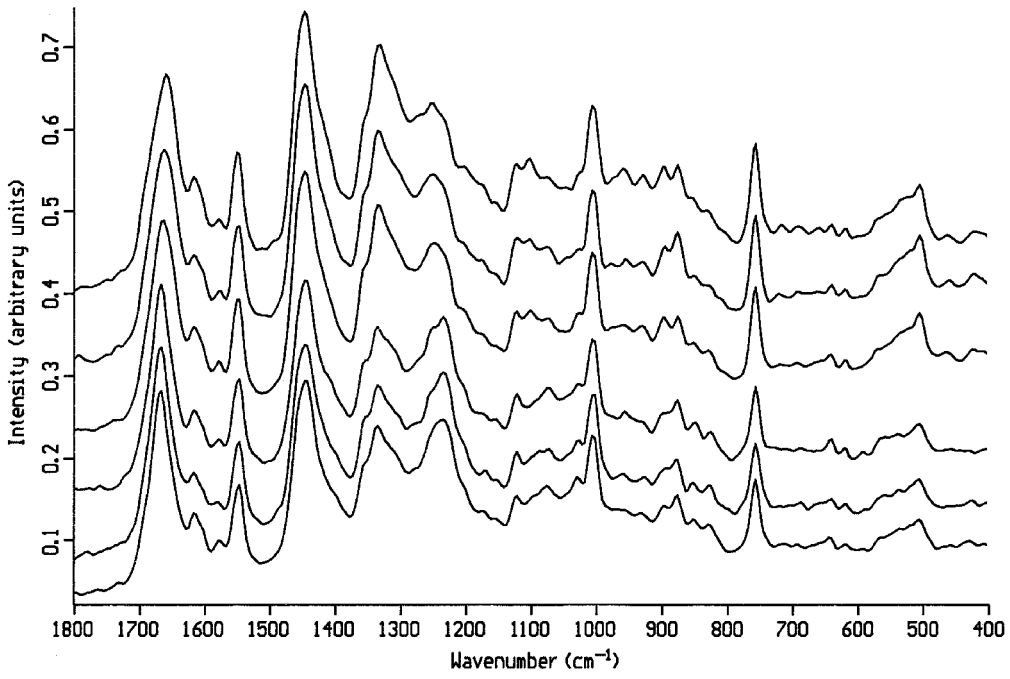

Figure 2 FT-Raman spectra of unprocessed (starting material) and SEDS-processed lysozyme samples over the wavelength range 1800–400 cm^{-1}. From top to bottom: unprocessed lysozyme, sample no. 4, sample no.11, sample no. 12, sample no. 12 (repeated run) and sample no. 1 (Table 1).

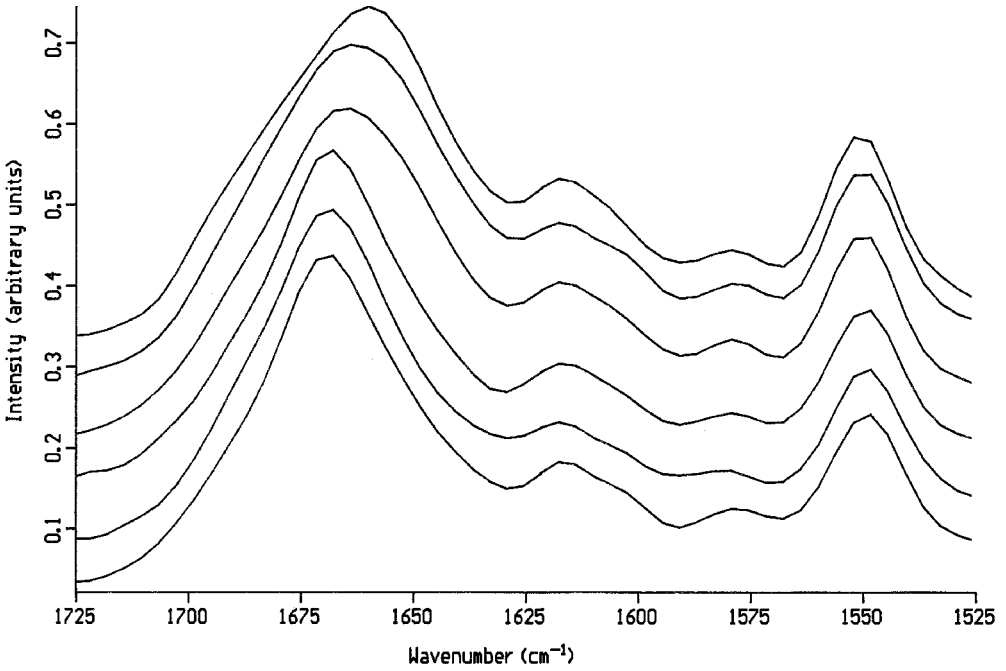

Figure 3 FT-Raman spectra of the amide I band of lysozyme over the wavelength range 1525–1725 cm^{-1}. From top to bottom: unprocessed lysozyme, sample no. 4, sample no. 11, sample no. 12, sample no. 12 (repeated run) and sample no. 1 (Table 1).

can be seen that the spectra are generally very similar, as would be expected for samples of the same molecular composition, there are some subtle differences between the different samples.

Lysozyme is a protein primarily exhibiting an α-helical structure with a low amide I band at 1660 cm^{-1} (Williams & Dunker 1981). Figure 3 shows the amide I region ($\sim$1660 cm^{-1}) of the FT-Raman spectra of SEDS-pro-

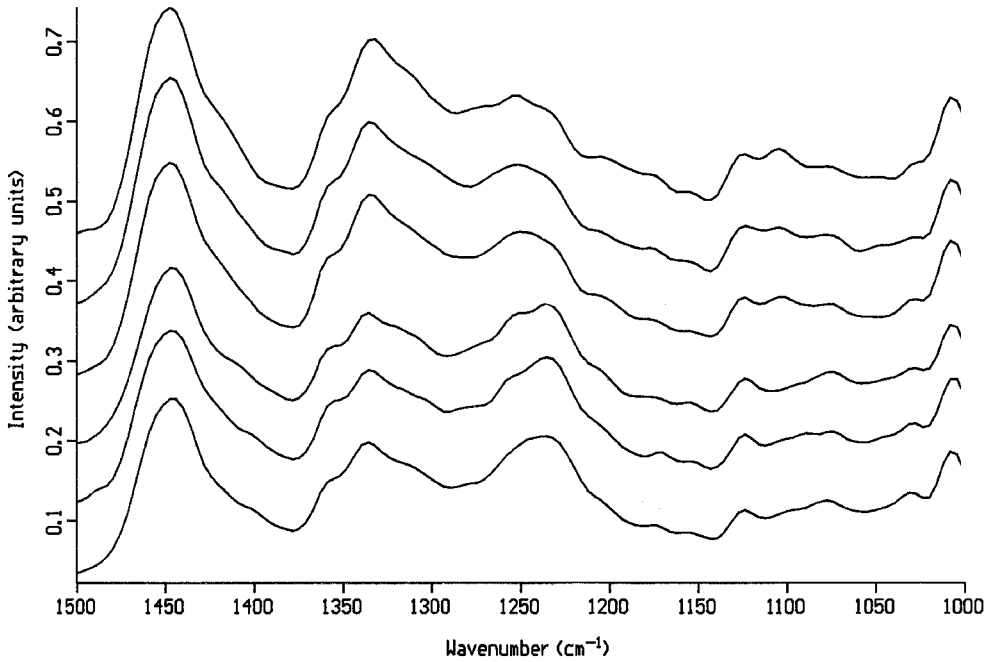

Figure 4 FT-Raman spectra of the amide III region of lysozyme samples. From top to bottom: unprocessed lysozyme, sample no. 4, sample no. 11 sample no. 12, sample no. 12 (repeated run) and sample no. 1. (Table 1).

cessed lysozyme in the solid state. The spectrum shows a shift in peak position to a higher wavenumber ($+4\,cm^{-1}$ to $+9\,cm^{-1}$) on going from the unprocessed sample to the Raman-analysed processed samples (200, 150 and 100 bar pressure, 40–45 °C). This increase in wavenumber position is consistent with a decrease in the extent of hydrogen bonding and a change in secondary structure. Winters et al (1996) reported that the overall features of SAS-precipitated lysozyme from DMSO were present in the Raman spectra and noted that their amide I band data indicated that higher pressures and temperatures lead to more extensive β-sheet-mediated intermolecular interactions in the precipitates. In our study, the least shift ($+4\,cm^{-1}$) in wavenumber, in the amide I region, is obtained with the highest working pressure (200 bar). This means that the increase in wavenumber position and consequently decrease in the extent of hydrogen bonding and a change in secondary structure is least for the samples precipitated at 200 bar.

Changes in the amide III region at around $1250\,cm^{-1}$ (Williams et al 1980), shown in Figure 4, also indicate that changes in secondary structure have occurred. In this instance, the broad amide III band shows a general downward shift in wavenumber position and broadening after sample processing compared with the starting material. The changes in the positions of the amide I and amide III bands are consistent with a change towards a more disordered secondary structure. Further major differences between the unprocessed and processed samples can also be observed in the composition of the spectra, notably the C-N stretching modes υ(C-N) between 1050 and $1150\,cm^{-1}$ and the C-C stretching modes υ(C-C) between 900 and $930\,cm^{-1}$ (Figure 2). The disulfide bond is a covalent linkage, which provides more stability than the other side-chain bonds (hydrogen bonds, hydrophobic bonding, van der Waals' interactions and ionic interactions). The S-S vibrational stretching band lies in the 500–$550\,cm^{-1}$ range and the prominent vibrational band of lysozyme occurs at $507\,cm^{-1}$ corresponding to a gauche-gauche-gauche configuration. Similarly, on spectral analysis of Figure 2, no change is observed in this region even at pressures up to 200 bar.

Summarising the Raman data, a good correlation between an increase in peak shift in the solid-state and solution-state biological activity was observed. An exception to this trend was the high activity of sample 1 despite the greatest peak shift. It is possible that the perturbations in the solid state for this sample were recovered on dissolution or that those that were maintained were not related to the active site. Data from the high-performance cation-exchange chromatography (data not shown) indicated no conformational changes in the precipitated lysozyme samples and therefore suggests that refolding of lysozyme occurred on dissolution of the solid precipitates. It is possible that Raman was sensitive to picking up the more likely formation of aggregates at 100 bar due to the increased risk for crossing the boundary from a single equilibrium phase to the liquid + vapour phase region.

Conclusions

This study has shown that the modified SEDS process can be utilised to precipitate protein particles of lysozyme of 1–5 μm size, with retained tertiary and secondary structure and biological activity. The advantage of using the modified SEDS process, albeit not at a full commercial scale yet, is the ability to precipitate protein from aqueous solution without deterioration of the quality of the products under certain operating conditions.

A comparison of the Raman spectra of lysozyme before and after processing using SEDS indicates that a minor disturbance of the secondary structure of processed samples has occurred in SEDS-processed samples; however, this disturbance is negligible at the working pressure of 200 bar and 40 °C.

The biological activity of aqueous processed lysozyme particles ranged between 63% and 101%. The working conditions associated with high pressure (200 bar) and 40 °C gave products with totally retained biological activity. The general conclusion of this work is that production of pure micron size-protein powder from an aqueous solution using the modified SEDS process is possible. Through careful choice of operating parameters, the biological activity and the secondary and tertiary structure of the SEDS-processed lysozyme are not affected using the SEDS process. Since results from Raman spectroscopy are obtained from solid-state samples and biological activity from the solution form, working at 200 bar and 40 °C brings about micron-size powder with 100% retained biological activity and secondary structure in solid and solution forms. This indicates that an optimum working condition has been identified for the micron-sized protein precipitation from an aqueous solution using SEDS. In contrast, an optimum condition could not be determined with lysozyme precipitated from DMSO by using SEDS (Moshashaée et al 2000).

References

Bustami, R. T., Chan, H. K., Dehghani, F., Foster, N. R. (2000) Generation of micro-particles of proteins for aerosol delivery using high pressure modified carbon dioxide. *Pharm. Res.* **17**: 1360–1366

Debenedetti, P. G., Tom, J. W., Kwauk, X., Yeo, S.-D. (1993) Rapid expansion of supercritical solutions (RESS): fundamentals and applications. *Fluid Phase Euilibria* **82**: 311–321

Donsi, G., Reverchon, E. (1991) Micronization by means of supercritical fluids: possibility of application to pharmaceutical field. *Pharm. Acta Helv.* **66**: 170–173

Forbes, R. T., Sloan, R., Kibria, I., Hollowood, M. E., Humphereys, G. O., York, P. (1998) Production of stable protein particles: a comparison of freeze, spray and supercritical drying. *Proc. IchE Word Congress on Particle Technology 3*. Brighton, UK, pp 180–184

Hanna, M., York, P. (1993) Method and apparatus for the formation of particles. European patent No. 9313642.2

Jackson, M., Mantsch, H. H. (1991) Beware of proteins in DMSO. *Biochem. Biophys. Acta* **1078**: 231–235

Lord, R. C., Yu, N. T. (1970) Laser excited Raman spectroscopy of biomolecules. *J. Mol. Biol.* **50**: 509–524

McHugh, M., Krukonis, V. (1994) *Supercritical fluid extraction. Principle and practice*. 2nd Edition, Butterworth-Heinmann, USA

Moshashaée, S., Bisrat, M., Forbes, R. T., Nyqvist, H., York, P. (2000) Supercritical fluid processing of proteins I: Lysozyme precipitation from organic solution. *Eur. J. Pharm. Sci.* **11**: 239–245

Palakodaty, S., York, P., Pritchard, J. (1998) Supercritical fluid processing of materials from aqueous solutions: the application of SEDS to lactose as a model substance. *Pharm. Res.* **15**: 1835–1843

Phillips, E. M., Stella, V. J. (1993) Rapid expansion from supercritical solutions: application to pharmaceutical processes. *Int. J. Pharm.* **94**: 1–10

Sloan, R., Hollowood, M. E., Ashraf, W., Humphreys, G. O., York, P. (1998) Proceeding of the 5th Meeting on Supercritical Fluids, Nice, France, Tome 1, p. 275

Sloan, R., Tservistas, M., Hollowood, M. E., Sarup, L., Humphreys, G. O., York P., Ashraf, W., Hoare, M. (1999) Proceeding of the 6th Meeting on Supercritical Fluids-Chemistry and Materials, Nottingham, UK, pp 169–174

Tom, J. W., Debenedetti, P. G. (1991). Particle formation with supercritical fluids — a review. *J. Aerosol. Sci.* **22**: 555–584

Tom, J. W., Lim, G.-B., Debenedetti, P. G., Prud'homme, R. K. (1993) Supercritical fluid engineering science: fundamental and applications, *ACS Symp. Series* **514**: 238–257

Verhamme, D., Storck, J., Racchelli, L., Lauwers, A. (1988) Lysozyme (N-acetylmuramyl $\beta(1\rightarrow4)$ glycanohydrolase EC 3.2.1.17). *Int. Pharm. J.* **2**: 168–171

Williams, R. W., Dunker, A. K. (1981) Determinations of the secondary structure of proteins from the amide I band of the laser Raman spectrum. *J. Mol. Biol.* **152**: 783–813

Williams, R. W., Cutrera, T., Dunker, A. K., Peticolas, W. L. (1980) The estimation of protein secondary structure by laser Raman spectroscopy from the Amide III intensity distribution. *FEBS Lett.* **115**: 306–308

Winters, M. A., Knutson, B. L., Debenedetti, P. G., Sparks, H. G., Przybycien, T. M., Stevenson, C. L., Prestrelski, S. J. (1996) Precipitation of proteins in supercritical carbon dioxide. *J. Pharm. Sci.* **85**: 586–594

Yeo, S.-D., Lim, G.-B., Debenedetti, P. G., Bernstein, H. (1993) Formation of microparticulate protein powders using a supercritical fluid antisolvent. *Biotechnol. Bioeng.* **41**: 341–346

Yeo, S.-D., Debenedetti, P. G., Patro, S. Y., Przybycien, T. M. (1994) Secondary structure characterization of microparticulate insulin powders. *J. Pharm. Sci.* **83**: 1651–1656