Bovine Serum Albumin micronization from aqueous solutions using Expanded Liquid Antisolvent process

V. Prosapio, E. Reverchon, I. De Marco*
Department of Industrial Engineering, University of Salerno, Via Giovanni Paolo II 132, 84084, Fisciano (SA), ITALY
*idemarco@unisa.it Fax: +39-89-964057

ABSTRACT

Bovine Serum Albumin (BSA) solubilized in water was successfully micronized using a modification of the supercritical carbon dioxide antisolvent process, named Expanded Liquid AntiSolvent (ELAS) precipitation. In the ELAS process, the antisolvent is constituted by supercritical carbon dioxide + an organic liquid (isopropanol in this case) and the operating point is positioned in the expanded liquid region.

We obtained sub-microparticles and microparticles of BSA, varying the position of the operating point.

The powders were characterized by differential scanning calorimetry, X-ray diffractometry and HPLC analyses.

Keywords: Expanded liquid antisolvent process, hydrosoluble proteins, bovine serum albumin.

INTRODUCTION

Supercritical AntiSolvent (SAS) has been successfully used to obtain microparticles and nanoparticles of various materials, changing the organic solvent and the operating conditions [1, 2, 3, 4]. The condition of miscibility limits the applicability of the SAS process to hydrophobic compounds, considering that water solubility in carbon dioxide is very limited at the usual SAS conditions (40-60 °C and 100-250 bar) [5]. However, several categories of pharmaceutical products, proteins and enzymes are water-soluble; therefore, to successfully process these compounds, a modification of the SAS process is required.

An interesting opportunity could be to add an organic solvent, completely miscible with SC-CO_2 to form a supercritical mixture in which water miscibility is largely enhanced or complete. Recently, De Marco and Reverchon [6] proposed the antisolvent processing of bovine serum albumin (BSA) using CO_2 + ethanol mixtures, but the operating range of antisolvent composition was extended to expanded liquid conditions and, accordingly, named this process ELAS (Expanded Liquid AntiSolvent) precipitation.

In this work, bovine serum albumin (BSA) was still used as a model protein compound but isopropanol was used, in place of ethanol, in order to extend the applicability of the technique to other co-antisolvents; the effect of the operating point on morphology and dimensions of the precipitates was investigated.

MATERIALS AND METHODS

Materials.

BSA (fraction V, 66 kDa, ɣglobulin free, purity 99%), distilled water (H_2O), isopropanol (iPRO, purity 99.8%) have been purchased from Sigma-Aldrich. CO_2 (purity 99%) has been purchased from SON (Naples, Italy). All materials were used as received.

Analytical methods

Samples of the precipitated material were observed by a Field Emission Scanning Electron Microscope (FESEM, mod. LEO 1525, Carl Zeiss SMT AG, Oberkochen, Germany). Powders were dispersed on a carbon tab previously stuck to an aluminum stub (Agar Scientific, United Kingdom); then, were coated with gold-palladium (layer thickness 250Å) using a sputter coater (mod. 108 A, Agar Scientific, Stansted, United Kingdom).

Particle size distributions (PSDs) of the powders were measured from FESEM photomicrographs using the Sigma Scan Pro image analysis software (release 5.0, Aspire Software International Ashburn, VA). Approximately 1000 particles, taken at high enlargements and in various locations inside the precipitator, were analysed in the elaboration of each particle size distribution. Histograms representing the particle size distributions were fitted using Microcal Origin Software (release 8.0, Microcal Software, Inc., Northampton, MA).

Thermograms of BSA were obtained using a differential scanning calorimeter (DSC, mod. TC11, Mettler-Toledo, Inc., Columbus, USA) using Mettler STARe system. Fusion temperature and enthalpy were previously calibrated with indium standard materials (melting point 156.6 ◦C, enthalpy of fusion 28.52 J/g). BSA powder samples (5 ± 0.5 mg), prepared in duplicates, were accurately weighed, crimped into an aluminium pan and heated from 35 to 100 °C at 2.5 °C/min under a nitrogen purge (50 mL/min). After the end of the first heating round, the protein sample was cooled to 35 °C, and rescanned after 5 min stabilization time at 35 °C.

X-ray diffractograms were recorded using an X-ray powder diffractometer (model D8 Discover; Bruker, USA) with a Cu sealed tube source. The measuring conditions were: Ni-filtered CuKα radiation, λ= 1.54 Å, 2θ angle ranging from 5° to 50° with a scan rate of 0.3 s/step and a step size of 0.028°.

The influence of ELAS processing on the protein degradation was evaluated using HPLC (model 1200 series; Agilent Technologies Inc.) analyses. The elution was obtained using a TSK-Gel G3000SWXL column (Tosoh Biosciences, LLC, Montgomeryville, PA). The column was equilibrated at a flow rate of 0.5 mL/min with a mobile phase consisting of phosphate buffered saline (PBS) at pH 7.2. The drug was dissolved in water, filtered with a 0.2 μm membrane syringe and detected at 214 nm with a retention time of 25 min. All chromatographic analyses were carried out at room temperature.

ELAS apparatus and procedures

The ELAS laboratory apparatus consists of a diaphragm high-pressure pump (Milton Roy, model Milroyal B) used to deliver carbon dioxide, an HPLC pump (Gilson, model 805) used to deliver the co-antisolvent, and a diaphragm high-pressure pump (Milton Roy, mod. Milroyal D), used to deliver the aqueous solution. The pump that delivers CO_2 possesses a cooling head to avoid cavitation. The pre-mixer is a high-pressure vessel with an internal volume of 35 cm^3, loaded with stainless steel perforated saddles, which ensures a large contact surface between co-antisolvent and CO_2. A cylindrical vessel with an internal volume of 500 cm^3 was used as the precipitation chamber. The precipitation chamber was electrically heated using thin band heaters. The pressure in the chamber was measured by a test gauge manometer (Salmoiraghi, model SC-3200) and regulated by a micrometering valve (Hoke, model 1315G4Y) located at the exit (bottom) of the chamber. The aqueous solution was delivered to the precipitator through a thin wall stainless steel nozzle. A second collection

chamber located downstream the precipitator, operating at a lower pressure (18–20 bar) was used to recover the mixture of water and co-antisolvent. The pressure in this chamber was regulated by a backpressure valve (Tescom, model 26-1723-44). At the exit of the second vessel a rotameter and a dry test meter were used to measure the CO_2 flow rate and the total quantity of CO_2 delivered, respectively. The co-antisolvent was, then, recovered using a rotary evaporator.

An experiment usually begins delivering supercritical CO_2 at a constant flow rate to the pre-mixer and to the precipitation chamber, until the desired pressure is reached. Then, the co-antisolvent is pumped to the pre-mixer, where it is put in contact with CO_2 forming an expanded liquid solution. Once reached stable flow rates, temperature and pressure conditions in the precipitation chamber, water is sent through the nozzle in the precipitator to obtain steady state composition conditions of the fluid phase during the solute precipitation. Then, the flow of water is stopped and the aqueous solution is delivered through the nozzle at the given flow rate, producing the precipitation of the solute. At the end of the aqueous solution delivery, two washing steps were performed: in the first one, the mixture co-antisolvent + CO_2 continues to flow to wash the chamber to eliminate water residues for a time t_1 and, in the second one, CO_2 alone continues to flow to eliminate co-antisolvent residues for a time t_2. At the end of this second washing step, CO_2 flow is stopped and the precipitator is depressurized down to atmospheric pressure.

RESULTS

The operating conditions were chosen on the basis of the previous experiments conducted on this protein [6]: therefore, ELAS experiments were carried out at a pressure of 150 bar, a temperature of 40 °C, a BSA concentration in water of 20 mg/mL, a CO_2 flow rate of 10000 mL/min and an injector of 100 μm in diameter. The effects of the operating point was investigated. The high pressure phase equilibria diagrams for the systems CO_2/iPRO/H_2O, adapted from literature data [7], at 100 bar and 40 °C is reported in Fig. 1 with the indication of the operating points investigated. In Table 1, a list of the experiments performed at all the operating conditions, the mole fractions, the morphology obtained, the mean diameter (m.d.) and the standard deviation (s.d.) is reported.

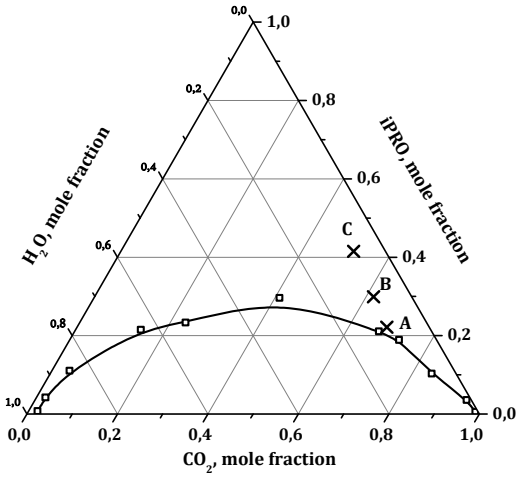

Figure 1: High pressure phase equilibria for the systems carbon dioxide/isopropyl alcohol/water, at 100 bar, 40 °C, adapted from literature data [7]. A, B, C indicate some characteristic conditions used in the following of this work.

Table 1: ELAS experiments performed on BSA (MP= microparticles; SMP= sub-microparticles).

Co-antisolvent	$Q_{co\text{-}ant}$ (mL/min)	Q_{sol} (mL/min)	x_{CO2}	x_{H2O}	$x_{co\text{-}anti}$	Operating Point	Morphology	m.d. (μm)	s.d. (μm)
iPro	10	1	0.685	0.094	0.221	A	MP	4.75	2.78
	15	1	0.617	0.084	0.299	B	MP	4.05	2.92
	25	1	0.514	0.071	0.415	C	SMP	0.93	0.37

In correspondence of an isopropyl alcohol flow rate of 10 mL/min, the mole fractions of the fluid components are: $x_{CO2} = 0.685$, $x_{iPro} = 0.221$, $x_{H2O} = 0.094$; the operating point is indicated with the letter A in the high pressure phase equilibria diagram for the system CO_2/iPro/H_2O reported in Figure 1. In correspondence of these conditions, BSA precipitated as microparticles (with a mean diameter of about 4.75 μm) as it is possible to observe from the FESEM image in Figure 2a. Increasing the isopropyl alcohol flow rate at 15 mL/min, the mole fractions of the fluid components were: $x_{CO2} = 0.617$, $x_{iPro} = 0.299$, $x_{H2O} = 0.084$ (point B in Figure 1): microparticles were obtained (with a mean diameter of about 4.05 μm), as shown in Figure 2b. Afterwards, it was used an isopropyl alcohol flow rate of 25 mL/min ($x_{CO2} = 0.514$, $x_{iPro} = 0.415$, $x_{H2O} = 0.071$; point C in Figure 1), getting smaller microparticles (the mean diameter is equal to 0.93), as illustrated in Figure 2c.

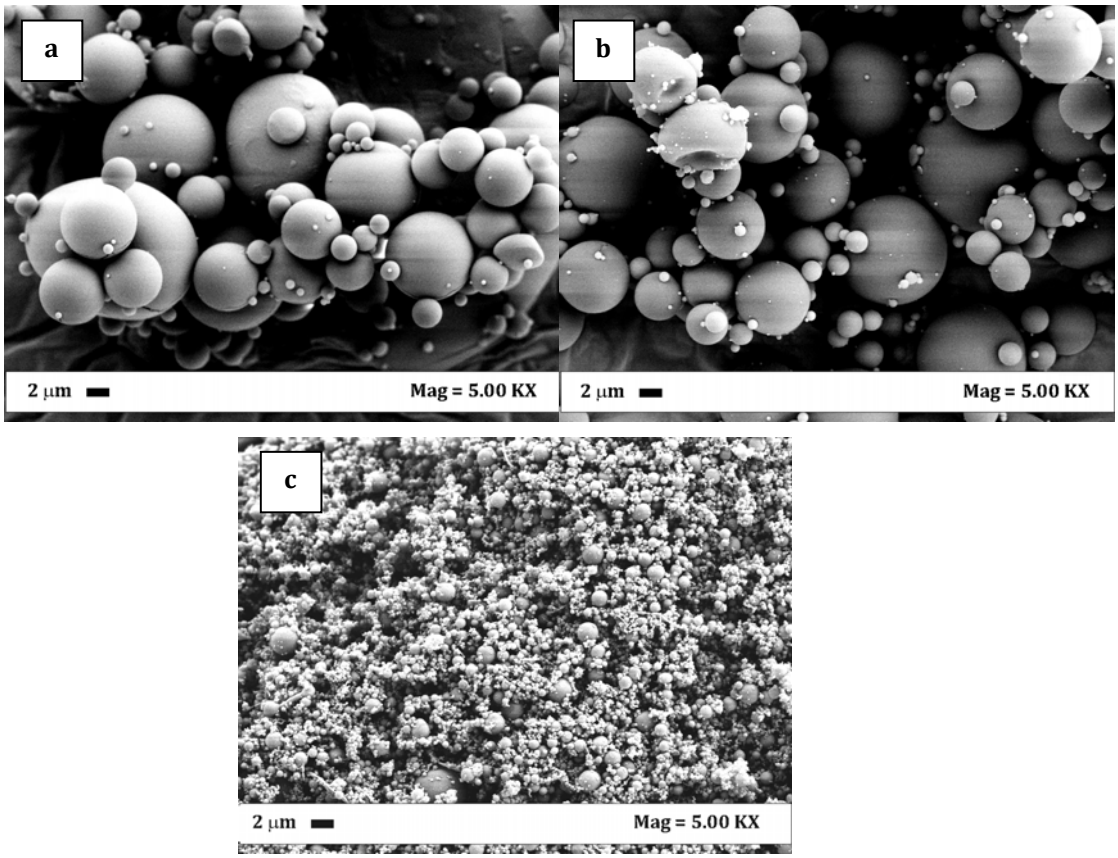

Figure 2: FESEM images of BSA particles obtained using an isopropyl alcohol flow rate of: (a) 10 mL/min; (b) 15 mL/min; (c) 25 mL/min.

A comparison between the volumetric cumulative particle size distributions (PSDs) of the BSA particles obtained at different co-antisolvent flow rates is reported in Fig. 3. Increasing the isopropyl alcohol flow rate, that corresponds to an increase of the isopropyl alcohol mole fraction and a decrease of the carbon dioxide mole fraction, the mean size of the particles decreases and the PSD becomes narrower. However, it is possible to note that no variations can be detected for the particles obtained at co-antisolvent flow rates equal to 10 and at 15 mL/min.

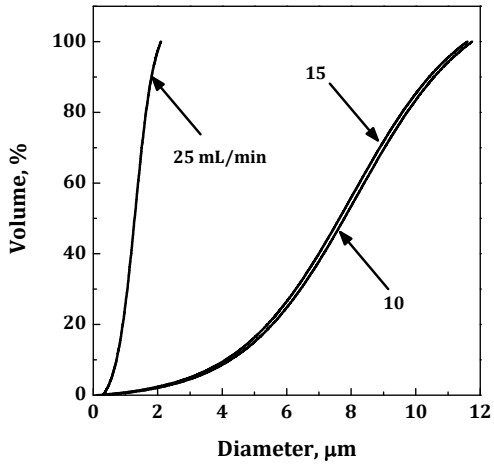

Figure 3: Volumetric cumulative PSDs of the BSA particles obtained at different iPRO flow rates.

DSC and XRD analyses were performed to evaluate if the ELAS process modified the structure of the compound. In both the cases, traces of native and processed BSA are similar, as it is possible to observe from DSC thermograms and XRD spectra, reported in Fig. 4. These analyses confirm that the temperature and the pressure used in the ELAS process did not alter the product.

.

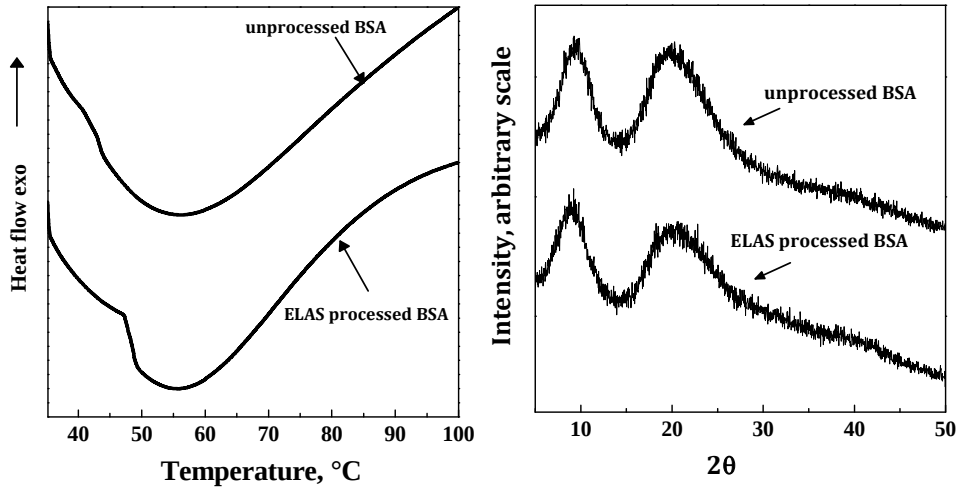

Figure 4: DSC thermograms and XRD spectra for unprocessed and ELAS processed BSA.

HPLC analyses were performed on BSA powders processed using isopropyl alcohol as co-antisolvent, to study how much the protein was degraded by the process. HPLC traces of

native BSA and BSA microparticles obtained using isopropyl alcohol showed similar peaks for processed and native BSA (the peak was detected at 214 nm in correspondence of 24 min), but the intensity of the processed BSA peak with respect to the one detected for the untreated BSA was equal to 90%, indicating a very low degradation of the protein, that, in every case, is smaller than the degradation obtained in the case of the traditional processing with organic solvents at low temperature.

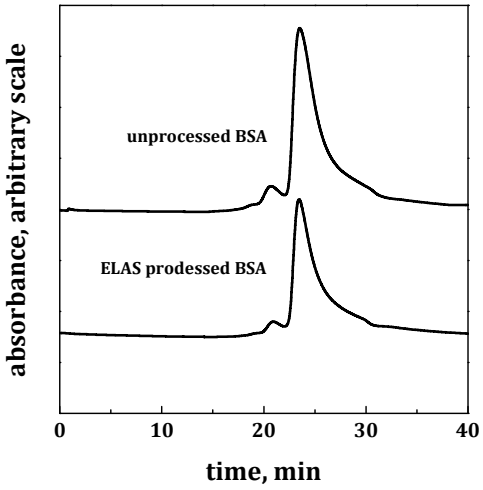

Figure 5: HPLC traces for unprocessed and ELAS processed BSA

CONCLUSION

The ELAS process demonstrated to be very efficient in producing microparticles of Bovine Serum Albumin (BSA) of different dimensions, properly varying the operating point within the ternary mixtures CO_2 + organic solvent + water; in all the experiments, the yield of the process was larger than 90 %.

It was verified that there is a link between the position of the operating point with respect to the vapor liquid equilibria and the morphology of the precipitates: increasing the co-antisolvent mole fraction, the mean size of the particles decreased and the particle size distribution become narrower.

Further experiments are required to gain additional information about different proteins that can be processed and about the detailed mechanisms involved in particles precipitation.

REFERENCES:

[1] A. SHARIATI, C.J. PETERS, Recent developments in particle design using supercritical fluids, Current Opinion in Solid State and Materials Science, Vol. 7, **2003,** p. 371.
[2] E. REVERCHON, G. DELLA PORTA, P. PALLADO, Supercritical antisolvent precipitation of salbutamol microparticles, Powder Technology, Vol. 114, **2001**, p. 17.
[3] E. REVERCHON, I. DE MARCO, G. DELLA PORTA, Tailoring of nano- and micro-particles of some superconductor precursors by supercritical antisolvent precipitation, Vol. 23, **2002**, p. 81.
[4] E. TORINO, I. DE MARCO, E. REVERCHON, Organic nanoparticles recovery in supercritical antisolvent precipitation, J. of Supercritical Fluids, Vol. 55, **2010**, p. 300.
[5] A.N. SABIRZYANOV, A.P. IL'IN, A.R. AKHUNOV, F.M. GUMEROV, Solubility of water in supercritical carbon dioxide, High Temperature, Vol. 40, 2002, p. 203.
[6] I. DE MARCO, E. REVERCHON, Supercritical carbon dioxide + ethanol mixtures for the antisolvent micronization of hydrosoluble materials, Chemical Engineering Journal, Vol. 187, **2012**, p. 401.
[7] B.-S. CHUN, G. T. WILKINSON, Ternary phase equilibria of the iso-propanol+water+carbon dioxide system at high pressure, Korean J. of Chemical Engineering, Vol. 16, **1999**, p. 189.