MICRONIZATION OF LYSOZYME USING SUPERCRITICAL ASSISTED ATOMISATION

G. Della Porta, A. Spada, E. Reverchon*

* Dipartimento di Ingegneria Chimica ed Alimentare, Università di Salerno,
Via Ponte Don Melillo, 84084 Fisciano (SA), Italy
Tel +39089964116 fax +39089964057
Email ereverchon@unisa.it

Lysozyme is an enzyme responsible for breaking down the polysaccharide walls of many kinds of bacteria.

Supercritical Assisted Atomisation (SAA) was used to produce Lysozyme microparticles. This innovative process is based on the solubilization of supercritical carbon dioxide in a water solution containing the enzyme; then, the ternary mixture is sprayed through a nozzle, and, as a consequence of the enhanced atomization, enzyme microparticles are formed. Different SAA process parameters were explored: solubilization temperature and pressure, precipitation temperature and enzyme concentration in the liquid solution. The effect of these parameters on the morphology, particle size and particle size distribution was studied. Controlled particle sizes in micronic and submicronic ranges with a narrow particle size distribution was obtained. Enzyme activity was monitored after SAA processing.

INTRODUCTION

Lysozyme is an enzyme with anti-bacterial activity that is normally present in various body fluids. Particularly, it is responsible for breaking down the polysaccharide walls of many kinds of bacteria and, thus, it provides some protection against infections. Indeed, bacteria build a tough skin of carbohydrate chains, interlocked by short peptide strands, that braces their membrane against the cell's high osmotic pressure. Lysozyme breaks these carbohydrate chains, destroying the cell walls: as a consequence the bacteria burst under their own internal pressure.

Lysozyme is used to increase the natural defenses of the body against bacterial infections. The pharmaceutical use of lysozyme encompasses applications such as oto-rhino-laryngology and in ophthalmology (eye drops and solutions for the decontamination of contact lenses). Today advanced solid-state drug formulations for aerosol delivery system included lysozyme in the aerosolized mixture.

Conventional methods for micronic particle generation usually do not provide an efficient control of the particle size and, sometimes, produce thermal degradation or contamination. Several Supercritical Fluids (SF) based micronization processes were proposed to overcome these limitations. The most studied are: the Rapid Expansion of Supercritical Solutions (RESS) [1, 2]**,** the Particles Generation from gas Saturated Solutions (PGSS) [3, 4] and the Supercritical AntiSolvent precipitation (SAS) [5-8]. An innovative supercritical fluid assisted micronization was recently proposed [9-12]. In this process a thermostated packed contactor is used to obtain a continuous near-equilibrium solubilization of supercritical carbon dioxide (SC-CO_2) in the liquid solution. The solution, formed in the contacting device, is sprayed into the precipitator at atmospheric pressure. A two steps atomization is obtained: the primary droplets produced at the outlet of the injector are further

divided in secondary droplets due to SC-CO_2 expansion from the inside of the primary ones. SAA technique has several advantages; among them: mild processing conditions and the possibility of use water as liquid solvent. Therefore, proteins and enzymes can be successfully micronized without denaturation due to high temperature exposure or solubilization in organic solvent.

The aim of this work was the Lysozyme micronization by SAA to verify the performance of these techniques. Different SAA process parameters were studied to reach a good particle size control: saturator pressure and temperature, precipitation temperature and enzyme concentration. The effects of these process parameters were monitored on: particles morphology, size and size distribution. Enzyme activity was also evaluated after SAA processing.

I - EXPERIMENTAL APPARATUS

SAA apparatus consists of two high-pressure pumps (mod. 305, Gilson) that deliver the liquid solution and the CO_2 to a heated bath (Forlab mod. TR12, Carlo Erba) and then to the saturator. The saturator is a high-pressure vessel (I.V. 50 cm^3) loaded with stainless steel perforated saddles, which assures a large surface contact between liquid solution and CO_2, and allows the dissolution of the gaseous stream in the liquid solution. Residence times in the saturator can vary from several seconds to minutes at the ordinary process condition. The mixture obtained in the saturator is sprayed through a 80 μm injection nozzle into the precipitator.

N_2 is taken from a cylinder, heated in an electric heat exchanger (mod. CBEN 24G6, Watlow) and sent to the precipitator to assist the liquid droplets evaporation. The precipitator is a stainless steel vessel (I. V. 3 dm^3) operating at atmospheric pressure. A stainless steel frit at the bottom of the precipitator allows the powder collection and the gaseous stream flow out. A condenser located after the precipitator is used to recover the liquid solvent. The SAA layout is schematically reported in **Figure 1**. Further details were published elsewhere [10-12]. All SAA experiments runs were performed in replicates.

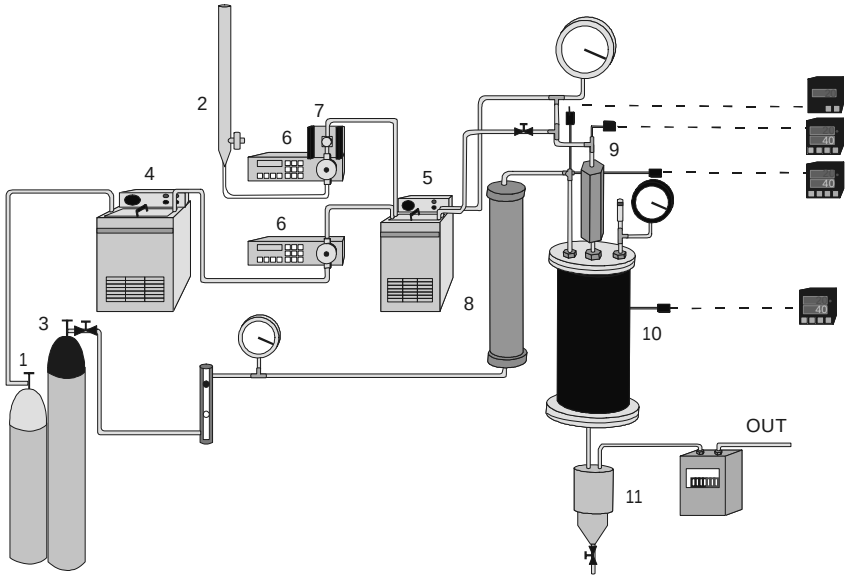


Figure 1 Schematic representation of the SAA apparatus: 1) CO_2 cylinder; 2) liquid solution; 3) N_2 cylinder; 4) cooling bath; 5) heating bath; 6) high pressure pumps; 7) dampener; 8) heat exchanger; 9) saturator; 10) precipitator; 11) condenser.

II - MATERIALS AND METHODS

Lysozyme from chicken egg white (LYS, purity 95%.) was supplied by Sigma (Milano, Italy). Water (H_2O, HPLC grade) was supplied by Carlo Erba Reagenti (Italy). CO_2 (purity 99.9%) was purchased from SON (Naples, Italy). The solubility of LYS in water was measured at room temperature and is 25 mg/mL**.** Untreated LYS consisted of irregular crystals with particle sizes of some millimiters (see Figure 2b). All products were used as received.

Powder morphology and Particle size distribution

Powders, sampled at different height in the precipitation chamber, were observed by Scanning Electron Microscope (SEM, mod. 420, LEO). Powders were dispersed on a carbon tab previously stuck to an aluminum stub (Agar Scientific, UK) and coated with gold-palladium (layer thickness 250Å) using a sputter coater (mod. 108A, Agar). At least 20 SEM images were taken for each run to verify the powder uniformity.

The particle size (PS) and the particle size distribution (PSD) were evaluated from SEM images using the Sigma Scan Pro Software (rel. 5.0, Jandel Scientific); more than 1000 particle diameters were considered in each PSD calculation. Histograms representing the PSDs were calculated by Microcal Origin Software (rel. 5.0, Microcal Software Inc.). The histograms were best fitted using Log-Norm curves giving a fair good representation of the non-symmetric distributions obtained.

Enzyme activity

The biological activity was monitored studying the hydrolysis of β-1,4 glycosidic linkages between N-acetylglucosamine and N-acetylmuramic acid in bacterial cell walls. A bacterial suspension of Micrococcus lysodeikticus (concentration of 0.25 mg/mL) in 67 mM phosphate buffer (p.H. 6.6) and a lysozyme solution of 4 μg/mL in phosphate buffer were used. The rate at which the absorbance at 450 nm decreased over 4 min was measured spectrophotometrically using a Cary 50 spectrophotometer (Varian, Milano).

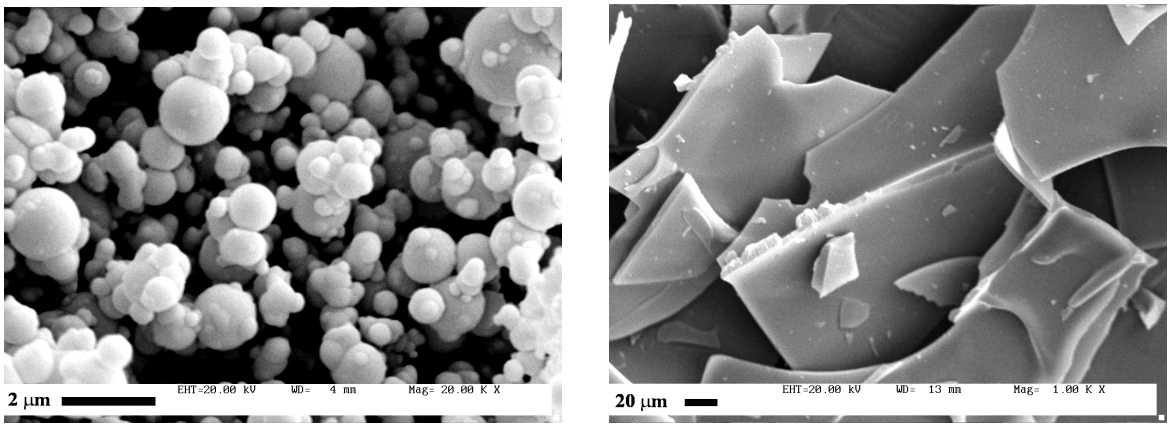
III - RESULTS AND DISCUSSION

In this work only water was used as the liquid solvent. Indeed, as discussed in the introduction, the possibility to use water as liquid solvent is one of the advantages of the innovative SAA technique.

The solubilization of supercritical CO_2 in the water solution is carried out inside the saturator (see SAA apparatus) and is one of the key parameters controlling the efficiency of the process. The high-pressure vapor- liquid equilibrium (VLE) system of the binary system water/CO_2 define the conditions at which is possible the formation of a homogeneous mixture in the saturator. These conditions are relevant since we want to obtain a solution, saturated by gas, before its atomization through the nozzle. Data on high pressure VLEs for the binary system water/supercritical CO_2 were found in the literature [13]. Therefore the operating conditions were selected to produce the formation of a single phase in the saturator and assuming that the presence of the enzyme does not modify the miscibility behavior of the water/CO_2 system. However, solubility of water in SC-CO_2 is very low at ordinary operating condition; as a consequence, CO_2 in excess with respect to the expected solubilization concentration was used. This last condition also assures pressurization of the saturator. According with these considerations, the saturator operating conditions were set at 90 bar and at 85°C. The flow ratio (R) between CO_2 and liquid solution was regulated at 1.8 w/w (liquid flow rate = 6.5 g/min; CO_2 flow rate = 11.7 g/min).

The temperature selection in the precipitation chamber was performed with the scope of minimizing temperature stress on the treated drug and to assist droplets evaporation. The optimum temperature value was found at 55°C.

The morphology of particles obtained in all these experiments was spherical with well-defined and non-coalescing micronic and sub micronic particles; it can be observed in a SEM image reported in **Figure 2a**. In the same Figure a SEM image of the untreated enzyme is reported for comparison (**Figure 2b**).

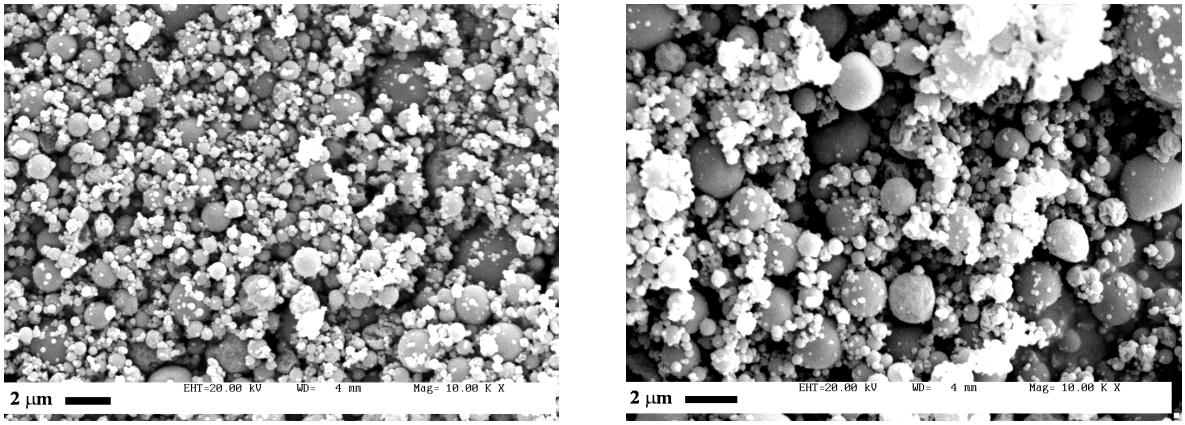


a) **b)**

Figure 2 a-b SEM image of LYS precipitated by SAA from water (b) and of untreated enzyme (b).

Fixed the operating pressure, temperature and flow rate ratio, systematic experiments were performed varying the drug concentration in the water solution injected. Particularly, the enzyme concentration was varied from 10, 15 and 20 mg/mL to explore the effect of this process parameter on size and distribution of the precipitated powders. The morphology of particles obtained in all these experiments was spherical with well-defined and non-coalescing micronic particles.

Some examples of SEM images of LYS particles are reported in **Figure 3a-b** and are referred to particles obtained in the experiments performed at 15 and 20 mg/mL LYS in water, respectively. SEM images were reported with the same enlargement (10 KX); therefore, they allow a qualitative evaluation of the enlargement of particles size as the solute concentration increases.



a) **b)**

Figure 3 a-b SEM image of LYS precipitated by SAA from water; concentration of the liquid solution 15 (a) and 20 mg/mL (b).

SEM images were studied using an image analysis software (as described in the Methods section) to measure the particle size distributions. The results are illustrated in the diagram reported in **Figure 4,** where distributions based on the number of particles are plotted. They are asymmetric and were described using a log-norm distribution. The mode (the most frequent particle size) varies from 0.22 to 0.34 and to 0.67 μm, when the solute concentration varies from 10 to 15 and 20 mg/mL, respectively. A moderate enlargement of the distributions can be also observed when the solute concentration is increased.

Enzyme activity was retained for the 95% after SAA processing.

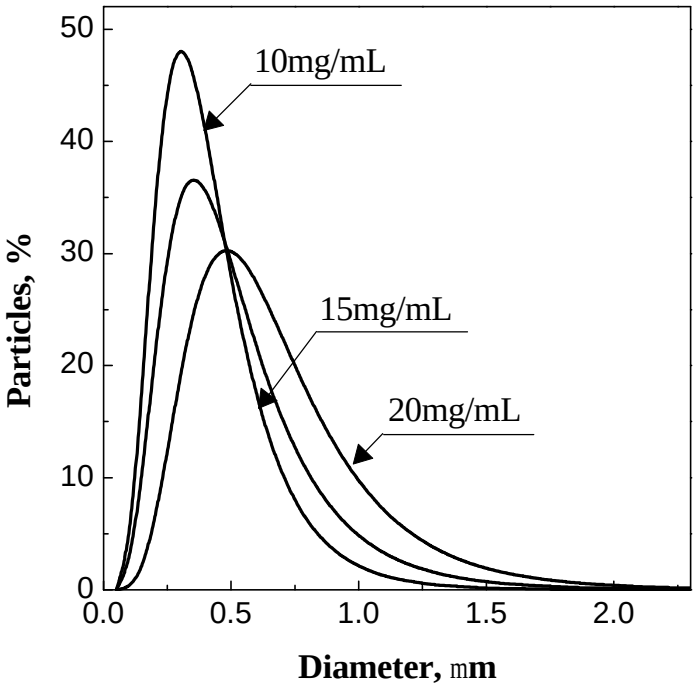


Figure 4 Distributions based on the number of LYS particles, produced by SAA from H_2O varying solute concentration from 10 to 20 mg/mL.

CONCLUSIONS

Supercritical Assisted Atomization is a promising technique for enzyme micronization, as demonstrated in the case of Lysozyme. Indeed, LYS microparticles were successfully obtained as a result of the enhanced atomization at relatively mild operating conditions. A fine particle size control was reached and LYS particles with mean diameters ranging from about 0.2 to 0.6 microns were produced with a narrow particle size distribution. The effect of LYS concentration in the liquid solution revealed the possibility of particle size tailoring by SAA in dependence of the target dimensions requested.

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