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Production of lysozyme microparticles to be used in functional foods, using an expanded liquid antisolvent process

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ABSTRACT

Expanded liquid antisolvent (ELAS) precipitation is a modification of the well-known supercritical antisolvent (SAS) process. Different from SAS, ELAS allows the micronization of water-soluble compounds using an antisolvent formed by supercritical carbon dioxide ($scCO_2$) and an organic solvent, used to improve water solubility in carbon dioxide.

In this work, ELAS was proposed for the micronization of lysozyme, an enzyme characterized by an intense antibacterial activity, using as antisolvents mixtures formed by $scCO_2$ + acetone and $scCO_2$ + isopropyl alcohol.

Microparticles of lysozyme, with a mean diameter ranging between 2.8 and 13.8 μm, varying the kind of co-antisolvent, the concentration of the enzyme in the liquid solution and the position of the operating point in the ternary diagram formed by water, carbon dioxide and the process co-antisolvent were obtained.

The obtained powders were characterized using XRD and FT-IR. These analyses showed that ELAS process did not influence solute integrity. UV–vis analysis revealed that lysozyme enzymatic activity was not significantly altered. The best results were obtained using acetone, measuring a biological activity of 95% with respect to the native protein.

1. Introduction

Functional foods are food products enriched with natural additives that can promote consumer's health and reduce the risk of some diseases (i.e., diabetes, anemia, heart attack) [1]. Their application affects different segments of food market: beverages, cereals, cheese, bakery products and meat [2]. However, the additive could alter some product characteristics, such as taste, color and texture. To avoid these adverse effects, a possible solution can be to micronize the additive [3]. Moreover, the use of nutrients and supplements at micro and nanoscales can improve their uptake, absorption and bioavailability in the body, compared to bulk equivalents [4].

Lysozyme is an enzyme widely used as food additive (as E1105) thanks to its numerous properties. In food industry, lysozyme is used as antimicrobial agent to prolong the shelf-life of cheese products, beer and malt beverages, wines and other alcoholic drinks [5]. However, to avoid that lysozyme could change the taste of foods, it has to be micronized; indeed, it has been observed [6] that particles smaller than 25 μm are not felt by the palate.

Micronization of lysozyme requires particular care, since proteins undergo to degradation [7]. Traditional techniques may induce relevant modifications in lysozyme structure with consequent loss of its characteristics [8]. Jet milling and ball milling expose the protein to high shear forces and electrostatic charge; moreover, the obtained particles are characterized by a large particle size distribution [9]. In spray drying, the final product yield is generally low and the high temperatures required to evaporate the solvent may cause the denaturation of the protein [10,11]. Lyophilization and liquid antisolvent precipitation show difficulties in solvent recovery and control of particle size distribution [10]. Freeze-drying requires long time processing, high consumption of energy and induces partial degradation of the protein due to the stress related to freezing and drying operations [12].

In this context, supercritical carbon dioxide ($scCO_2$) based techniques are of great relevance, since they can reduce process times, control accurately particle size and distribution and remove the solvent thanks to the specific characteristics of supercritical fluids, intermediate between those of liquids and gases. Supercritical fluids based techniques have been proposed for the application in several fields [13–17] and many papers based on $scCO_2$ to produce

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micro and nanoparticles are continuously proposed [18–23]. Among $scCO_2$ based processes, supercritical antisolvent (SAS) precipitation has been extensively used to produce microparticles and nanoparticles of materials belonging to various industrial categories and it has been largely studied from thermodynamic, fluid dynamic and mass transfer point of view [24–31]. This process is based on two requisites: solvent and antisolvent must be completely miscible at the process conditions; whereas, solute has to be insoluble in the mixture solvent/antisolvent. However, since water and CO_2 show a wide miscibility hole at usual SAS process conditions (temperature lower than 60 °C and pressure lower than 250 bar), hydrosoluble compounds cannot be processed by SAS. Some authors proposed the micronization of lysozyme by SAS using dimethylsulfoxide (DMSO) as solvent [9,32–36]. However, studies about the effect of solvents on proteins structure [37] showed that DMSO can induce the unfolding of proteins polypeptide chain; in particular, it has been observed [38] that, in presence of this solvent, lysozyme goes through irreversible denaturation. Some authors [9,33,34,36] measured the residual biological activity of the processed enzyme stating that it ranged between 60% and 100% of the initial value; but, none verified the presence of structural modifications related to the reduction of α-helix content, which is considered to be responsible for the loss of its properties [39].

To overcome the limitations of SAS process, some authors tried to micronize proteins using other supercritical fluid based techniques [40]. The obtained powders were characterized by a good biological activity but they did not produce nanoparticles. Other authors used a modification of SAS in which the antisolvent is formed by a mixture of $scCO_2$ and an organic solvent. Foster and co-workers [41,42] added ethanol or ethanol + dimethylstriethylamine to $scCO_2$ to micronize different proteins, including lysozyme, obtaining irregular and aggregated particles. Bouchard et al. [43,44] added methanol, ethanol, acetone or 2-propanol to $scCO_2$ to process lysozyme; but, they obtained regular particles only using very small quantities of water solutions (the water mole fraction was around 0.006), that imply the possibility to process only very small quantities of solute; moreover, the measure of the solvent residue was not reported.

Recently, De Marco and Reverchon [45] proposed a new supercritical based technique, in which the antisolvent is formed by CO_2 and a polar organic solvent (called co-antisolvent), that operates at expanded liquid conditions (ELAS, expanded liquid antisolvent). An expanded liquid is a liquid containing $scCO_2$; it maintains the liquid state, but shows a very reduced surface tension and hybrid properties between the ones of $scCO_2$ and the selected liquid solvent. Considering high pressure phase equilibria for the system CO_2 + co-antisolvent + H_2O, in correspondence of the ternary mixture critical point (MCP), the three liquids are completely miscible [46]. Decreasing the co-antisolvent concentration, the mixture exists in the two-phase region: a carbon dioxide rich phase (supercritical conditions) and a co-antisolvent rich phase (expanded liquid conditions) [41]. Operating in this way, it is possible to process homogeneous mixtures containing large mole fractions of water, increasing the concentration of solute that can be processed. ELAS process has already been successfully used to micronize bovine serum albumin (BSA) [45,47], using ethanol, acetone or isopropyl alcohol as co-antisolvents.

Therefore, to overcome the limitations of the previously discussed processes, in this work, the micronization of lysozyme using ELAS to obtain precipitates with regular morphology, good biological activity, unaltered conformational structure and negligible solvent residue was proposed. The effects of the kind of co-antisolvent and of the operating parameters on morphology, particle size and particle size distribution are investigated. Various analytical methods are used on the processed powders to verify how ELAS process influences lysozyme integrity.

2. Materials and methods

2.1. Materials

Lysozyme (chicken egg white), *Micrococcus lysodeikticus* (lyophilized cells), distilled water (H_2O), acetone (AC, purity 99.5%) and isopropyl alcohol (iPrOH, purity 99.5%) were supplied by Sigma–Aldrich (Italy). CO_2 (purity 99%) was purchased from SON (Italy). All materials were used as received.

2.2. ELAS apparatus and procedure

In Fig. 1, a scheme of the ELAS laboratory plant used in this work is reported. The apparatus is equipped with a diaphragm high-pressure pump (Milton Roy, model Milroyal B) used to deliver carbon dioxide, equipped with a cooling head to avoid cavitation, an HPLC pump (Gilson, model 805) used to deliver the co-antisolvent (AC or iPrOH), and a diaphragm high-pressure pump (Milton Roy, mod. Milroyal D), used to feed the aqueous solution. 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 inner volume of 500 cm^3 was used as the precipitation chamber. The aqueous solution was delivered to the precipitator through a thin wall stainless steel nozzle with the diameter of 100 μm. A second vessel located downstream the precipitator, operating at a lower pressure (18–20 bar) was used to recover the mixture of water and co-antisolvent. Then, the co-antisolvent was separated from water using a vacuum evaporator.

An ELAS experiment usually starts delivering supercritical CO_2, at a constant flow rate fixed at 20 g/min, to the pre-mixer and to the precipitation chamber, until the desired pressure (150 bar) is reached. Then, the co-antisolvent (AC or iPrOH) is pumped to the pre-mixer, where it is put in contact with CO_2 forming an expanded liquid solution. When stable flow rates, temperature (40 °C) and pressure conditions in the precipitation vessel are reached, water is sent through the 100 μm diameter nozzle to obtain steady state composition conditions of the fluid phase during the solute precipitation. Afterwards, the flow of water is stopped and lysozyme aqueous solution is delivered at a flow rate equal to 1 mL/min through the nozzle, producing the precipitation of lysozyme in form of amorphous particles. At the end of the aqueous solution delivery, the precipitator is purged first with the mixture co-antisolvent + CO_2 to eliminate water residues (for a time t_1 equal to 45 min) and, afterwards, with CO_2 alone to eliminate co-antisolvent residues (for a time t_2 equal to 90 min). Finally, CO_2 flow is stopped and the precipitator is depressurized down to atmospheric pressure. All the experiments were performed in triplicates.

2.3. Analytical methods

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

Particle size distribution (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 analyzed in the elaboration of each particle size distribution. Histograms representing particle size distributions were fitted