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PREPARATION OF INHALABLE PROTEIN PARTICLES BY SCF-EMULSION DRYING

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In this study, an new patented emulsion drying technique using high pressure carbon dioxide was employed for the generation of fine protein particles. Water-in-oil (W/O) emulsion droplets were sprayed into a continuous feed of high pressure carbon dioxide. Protein particles precipitated as a result of expansion of the droplets and removal of water by the CO_2-organic solvent mixture. Model enzymes (trypsin, catalase and lactase) and insulin were precipitated using this new process as micron-sized particles. Stabilized formulation of proteins incorporating buffer salts, sugars and possibly surfactants can be obtained and bio-activity is preserved as shown on several enzymes (catalase, trypsin, lactase). Moreover, *in vivo* tests of insulin demonstrated complete recovery of biological activity after processing.

INTRODUCTION

Macromolecules, notably proteins and peptides, are large fragile compounds that are usually administered only by injection. The pulmonary administration of dry powder of proteins and peptides receives more and more attention as an alternative to injection as it provides a direct route to the blood circulation, increasing patient compliance with a minimum of discomfort and pain. Aerosol delivery of therapeutic drugs requires a narrow particle size distribution (1-5 µm) without re-agglomeration during storage and structural stability during both processing and storage [1].

Supercritical processes have already been used for the generation of micron-sized particles of proteins and peptides [2]. The literature reports protein micronization using anti-solvent processes, following the pioneering work of Debenedetti et al [3-5] who prepared insulin particles in 1991-92. Two anti-solvent techniques were developed: The classical anti-solvent process consisting in expanding/pulverizing a solution of the bio-molecule in an organic solvent with SCF CO_2 [6,7], or according to the SEDS process, pulverizing an aqueous solution into SCF CO_2 added with a co-solvent [8,9]. However, the main difficulty arising for protein processing with these two techniques is related to the exposure of the protein to organic solvents that often results in a significant bio-activity decrease [6-9]. Another process is being developed by Sievers [10-12], consisting in a CO_2-assisted aerosolization of a protein aqueous solution; the aerosol is further dried by contact with a stream of inert gas warmed at a temperature between 25 and 75°C.

Separex-Lavipharm is now developing another route to extract water from aqueous solution using SCF CO_2 through a patented process [13]. In this new technique, an aqueous solution of active is emulsified into a polar organic solvent, often in presence of a surfactant. This emulsion (possibly a micro-emulsion) is then pulverized into a supercritical fluid that extracts the solvent and water, leading to a dry powder of particles consisting in the active mixed with other compounds dissolved in the aqueous medium (salts, sugars, etc.). This process is

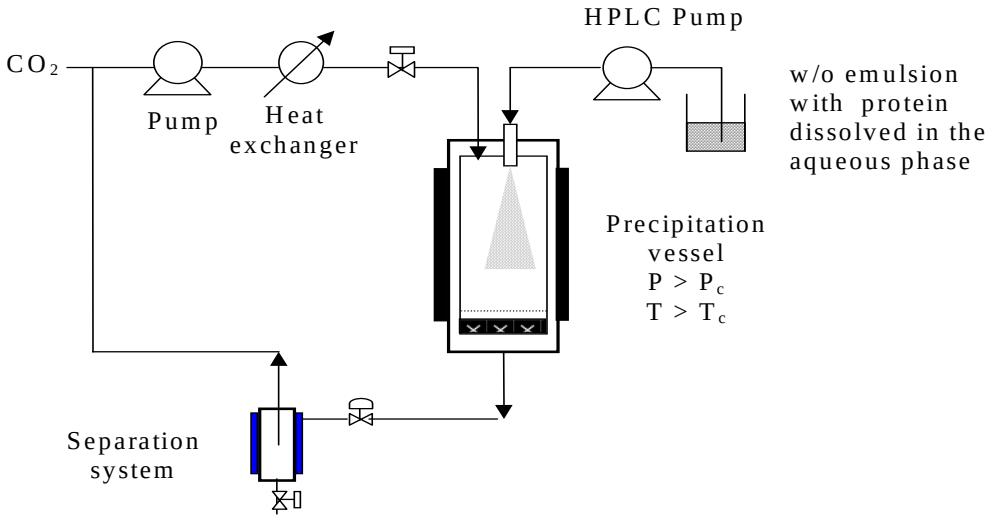
particularly adapted to bio-molecules pulverization as they only have a limited contact with the organic phase and are protected from denaturation by the presence of surfactant. Moreover, this new technique can be applied to a large range of active molecules and stabilizing agents.

I - MATERIALS AND METHODS

Different proteins and peptides were used as model molecules to evaluate the process efficiency. Catalase, trypsin and bovine zinc-insulin were obtained from Sigma and used without further purification. Lactase was a gift from Biocatalysts (Wales).

The protein is dissolved in an appropriate aqueous medium and emulsified with n-pentanol containing a surfactant. The resulting emulsion is then pulverized through a nozzle into a flow of supercritical CO_2 as shown on picture 1. The contact between the emulsion droplets and the supercritical fluid leads to the extraction of the aqueous phase by the fluid mixture resulting in protein precipitation.

Figure 1 : Schematic representation of the emulsion drying process



Samples of the powders precipitated were observed by Scanning Electronic Microscopy (JEOL JSM-T 330A) whereas particle size distribution was measured using laser diffraction particle size analyzer (Horiba LA 920). Enzymatic activities of the different proteins after SCF-treatment were determined using the USP methods. For insulin samples, bio-activity was measured by measuring blood glycaemia of diabetic rats after injection of reconstituted solutions of SCF-processed insulin.

II - RESULTS

Several aqueous solutions of enzymes and insulin were processed according to the emulsion-drying process and the particle characterization leads to the following results :

- *Enzyme particle size:*

The following SEM pictures show that it is possible to prepare dry particles of different proteins from aqueous solutions emulsified in n-pentanol. The residual moisture content, measured in trypsin and insulin samples, was found to be less than 5wt %, similar to the starting material one.

Figure 2 : Insulin particles produced by emulsion SCF-drying

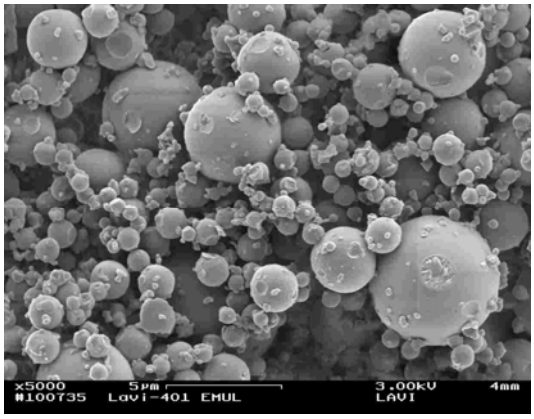


Figure 3 : Catalase particles produced by emulsion SCF-drying

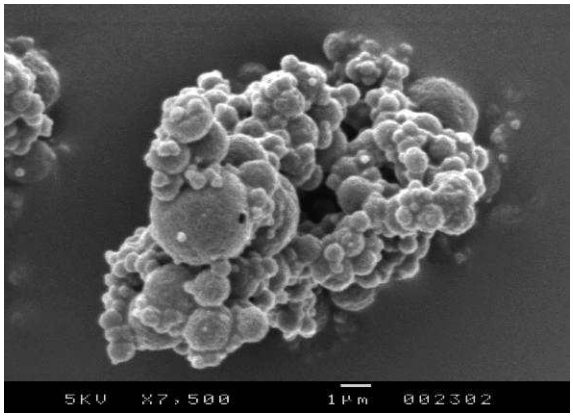
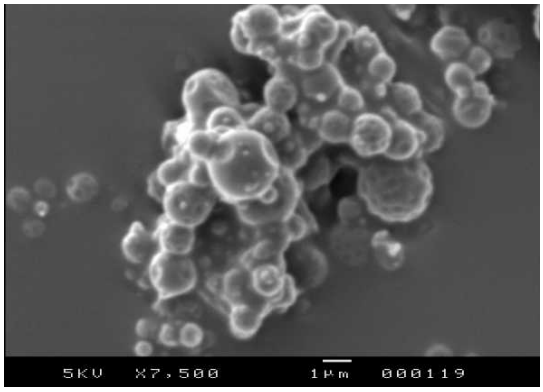


Figure 4 : Lactase particles produced by emulsion SCF-drying



- *Bio-activity of the processed enzymes*

As shown on table 1, the residual activity was found higher than 80% for nearly all the samples, demonstrating a satisfactory preservation of the enzymatic activity. This confirms that the new process is particularly adapted to fragile bio-molecules drying and it is to be noticed that trypsin activity was very well preserved, meanwhile 60% loss of activity was reported when using the anti-solvent SEDS process [9]. Moreover, the stabilizing power of saccharides, both during the drying process and storage, was verified on two enzymes: trypsin and lactase, as described in the literature [14-16]. This demonstrates that this emulsion process can simultaneously dry the protein and its stabilization agents, leading to an homogeneous mixture of the components present in the starting aqueous solution.

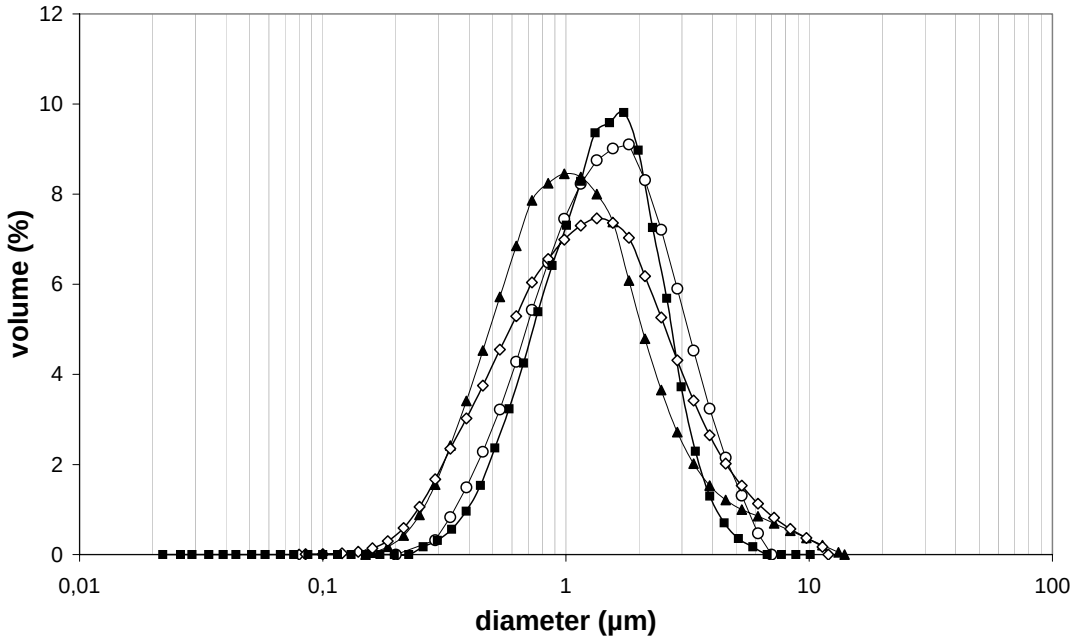
Table 1 : Residual enzymatic activity of different proteins after emulsion SCF-drying

Protein	*Residual activity (%)*
Lactase	68
Lactase + mannitol 10%	85
Trypsin	80
Trypsine + mannitol 10%	94
Catalase	91

- *Insulin particle size distribution*

Particle size distributions of different bovine insulin samples after emulsion drying are presented on figure 5: for all samples, more than 95% of the particles have a diameter below 5 µm, and the mean diameter can be tuned by changing the process parameters.

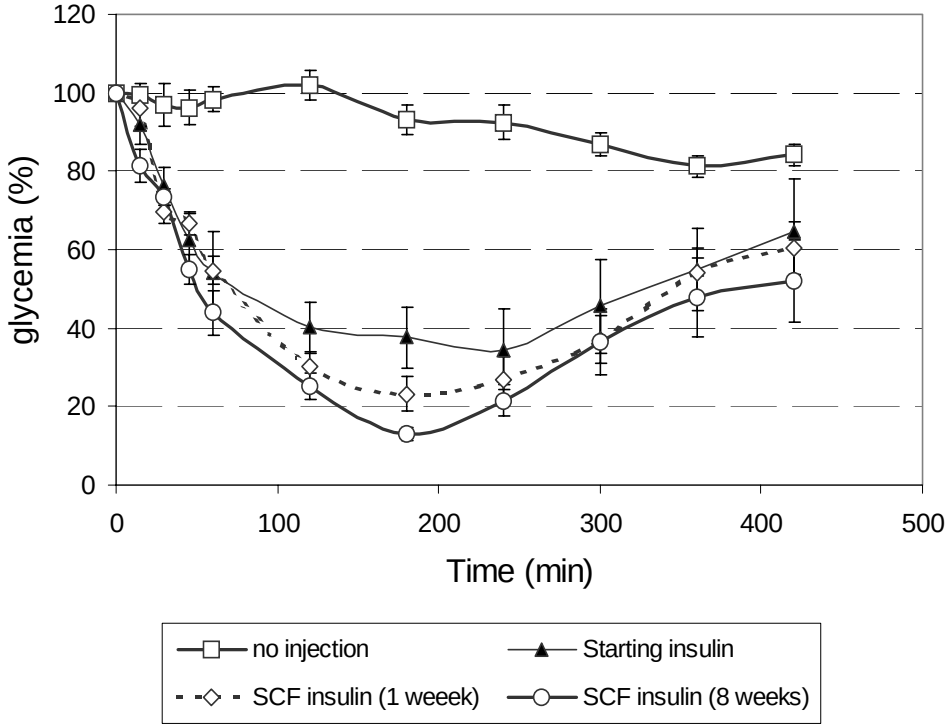
Figure 5: Particle size distribution of insulin samples after emulsion drying



- *Insulin Biological activity*

The insulin particles were injected into diabetic rats after conservation during one week and eight weeks respectively. As seen on figure 6, the effect on glycaemia was at least as good as with the original insulin, demonstrating that bio-activity does not decrease during SCF processing and further storage. Surprisingly, it seems that insulin bio-activity is increased after CO_2 treatment as it was already observed by Foster et al. [17]. This phenomenon is not already explained but it can be linked to a modification of the molecule aggregation in the aqueous medium after reconstitution.

Figure 6 : Glycaemia (ratio to origin value) after subcutaneous injection of 10 U/kg to diabetic rats



CONCLUSION

A new process based on W/O emulsion drying with supercritical CO_2 was designed to produce fine powders of bio-molecules directly from aqueous solutions. Experimental results demonstrated several key-advantages:

- Particles generated with this process are generally spherical with a diameter between 1 and 5µm which fits the specifications required for inhalation,
- Bio-molecules drying is realized at low temperature without direct contact between the fragile molecules and organic solvents, preserving bio-activity,
- Stabilizers or excipients can be mixed with the bio-molecules in the emulsion to realize in a one-step precipitation and pre-formulation.

Process application to several therapeutic bio-molecules and scale-up in compliance with GMP [18] is now on-going.

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