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Review

Stabilization of Proteins in Dry Powder Formulations Using Supercritical Fluid Technology

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Therapeutic proteins have become essential in the treatment of many diseases. Their formulation in dry form is often required to improve their stability. Traditional freeze-drying or spray-drying processes are often harmful to labile proteins and could be replaced by supercritical fluid (SCF) drying to produce particles with defined physicochemical characteristics in a mild single step. A survey of the current SCF drying processes for proteins is presented to give insight into the effect of SCF drying on protein stability and to identify issues that need further investigation. Methods used for drying aqueous and organic protein solutions are described. In particular, effects of process and formulation parameters on particle formation and protein stability are discussed. Although SCF methodology for drying proteins is still in its infancy, it can provide a serious alternative to existing drying methods for stabilizing proteins.

KEY WORDS: protein formulation; protein stability; supercritical fluid drying.

INTRODUCTION

In the past few decades, therapeutic proteins have become an important category of drugs for the treatment of life-threatening and chronic diseases. Because proteins are mostly administered by injection, protein formulations are subject to extremely stringent quality criteria. In particular, stabilization of these products is of crucial importance, because proteins can undergo a variety of chemical and physical degradation reactions (1).

In general, the long-term stability of proteins is greatly enhanced when they are stored in dry rather than liquid formulations (2).

Many commercially available protein drugs are freeze-dried formulations (3–5). Although many proteins have been stabilized successfully by freeze-drying, this technique has some serious drawbacks: it is time- and energy-consuming and therefore expensive. Besides, it often leads to incomplete recovery of the intact protein, because of process-induced degradation (i.e., during the freezing and drying phases) (4–6). Moreover, as freeze-dried procedures usually generate cakes rather than powders, lyophilization is not the drying method of choice when microparticles with defined, narrow size distributions are required (e.g., for pulmonary protein delivery) (7).

Alternative drying techniques have been explored for drying pharmaceutical protein formulations, each of which has its specific advantages and disadvantages (Table I). Among the available drying techniques, supercritical fluid (SCF) drying is especially attractive for reasons of mild process conditions, cost-effectiveness, possible sterilizing properties of supercritical (SC) carbon dioxide (SC-CO_2) (8), capability of producing microparticulate protein powders, and feasibility of scaling up.

The application of supercritical fluid (SCF) techniques for drying of proteins is relatively new: the first papers on this subject appeared at the beginning of the 1990s (9). The techniques have rapidly gained interest, resulting in several articles. The aim of this paper is to give an overview of the available literature dealing with SCF drying methods in the production of pharmaceutical protein powders. SCF processes have also been applied for making release systems for controlled delivery of proteins or other bioactive components (10–13), but this is beyond the scope of this paper.

In the following section, we explain SCF drying principles in relation to the drying of proteins from organic and aqueous solutions. Next, we give an overview of the effect of SCF drying process and formulation parameters on the formation and stability of protein powders. In the final section, we summarize the state of the art of SCF drying technology and discuss the potential of this technique for the stabilization of protein pharmaceuticals.

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ABBREVIATIONS: AP, alkaline phosphatase; DCM, dichloromethane; DMFA, *N,N*-dimethylformamide; DMSO, dimethylsulfoxide; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; LDH, lactate dehydrogenase; rhDNase, recombinant human deoxyribonuclease; rhIgG, recombinant human immunoglobulin G; SC-CO_2, supercritical carbon dioxide; SCF, supercritical fluid.

Table I. Comparison of Techniques Used for Drying of Proteins[a]

	Freezing stress	Heating stress	Process speed	Particle design	Capacity	Sterilizing properties
Freeze drying	–	+	–	–	±	–
Spray drying	+	–	+	+	+	–
Vaccum drying	+	±	–	–	±	–
Spray freezing drying	+	–	±	+	±	–
SCF drying	+	±	+	+	+	+

SCF, supercritical fluid.
[a] +, favorable; –, unfavorable.

SUPERCRITICAL DRYING TECHNOLOGY

Supercritical Fluids

A fluid is qualified as supercritical when its pressure and temperature exceed their respective critical values (Pc; Tc) (Fig. 1). Above the critical temperature, it is not possible to liquefy a gas by increasing the pressure, but the density increases continuously with increasing pressure. Other physical properties such as viscosity and diffusivity vary as well but remain gas-like. Hence, the fluid has unique thermo-physical properties under these conditions: it is able to penetrate substances like a gas and dissolve materials like a liquid (14).

Apart from their high diffusivity, up to two orders of magnitude higher than that of liquids (14), SCFs are recognized for their adjustable solvent power. The increase of process pressure results in a higher solubility as the SCF takes liquid-like solvent properties with increasing density. SCFs are remarkably good solvents for apolar compounds (15) such as cholesterol or α-tocopherol (16), but poor solvents for proteins (17).

To customize the solvent power of SCF further, modifiers such as cosolvents can be added (18). In such mixtures of three (Fig. 2) or more compounds, the solubility of one component in the others is influenced by the pressure and temperature until reaching the mixture critical point [e.g., for CO_2 containing 0.031 molar fraction of dimethyl formamide (DMFA), the mixture critical point rises to 123.1 bar and 65°C from 75.8 bar and 31.5°C for pure CO_2] (19) where compounds become fully miscible within one another (20).

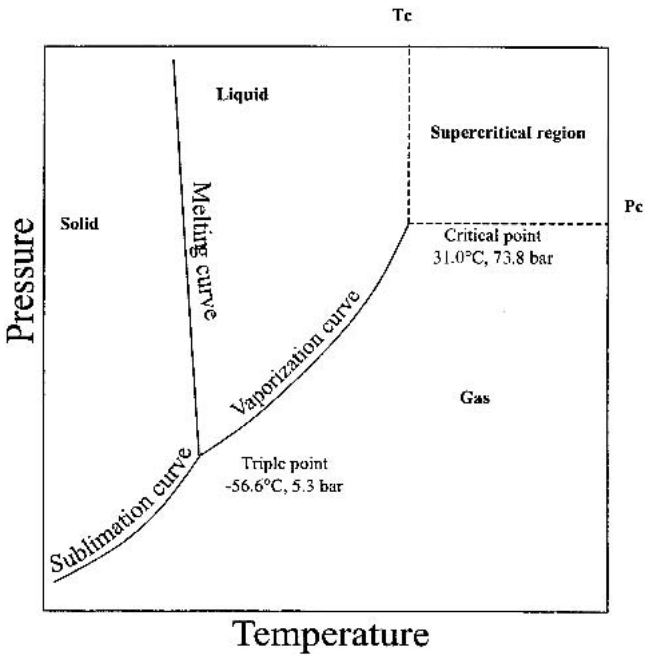

Fig. 1. Schematic pressure-temperature diagram of CO_2.

Because of their unique properties, SCFs have been used for various purposes: as reaction medium, in preparative and analytical separation techniques, in manufacturing of materials such as particles or thin films, and in drying processes.

Concepts of SCF Drying of Proteins

Essentially, two concepts of using SCF for the drying of proteins have been described in the literature. In the most important concept of SCF drying, the SCF is used as an antisolvent for the protein and as a water extraction medium. Three stages in the process can be distinguished. First, the protein solution is concentrated due to the extraction of water by the SCF. Subsequently, the protein and other constituents are precipitated as a result of the increasing concentration in the solution and the dissolution of the SCF in the solution. Finally, the particles formed are dried by extraction of the remaining solvent by the SCF.

In the second concept, the SCF is dissolved at high pressure in the solution containing the protein and excipients and sprayed to atmospheric conditions. The SCF is used as propellant/effervescent agent during a low-temperature spray-drying process, enhancing the atomization process and thereby shortening the drying process.

The SCF drying processes for preparing protein or protein-containing powders based on these concepts are described in more detail below.

PROCESSING USING SCF AS DRYING MEDIUM

A number of process variants have been proposed in which a SCF is used as an antisolvent and drying medium. Variations in the drying medium, the solvent, additives, as well as variation in the operation mode have been applied. A difficulty for the reader is that authors use almost as many process names and abbreviations as there are process variants (Table II), even though the technological differences are often subtle. The main components of these processes (drying medium, solvent and operational modes) that have been used are discussed below.

Supercritical Fluids

The SCF functions as antisolvent for the protein and as extraction agent for the solvent. For the pharmaceutical in-

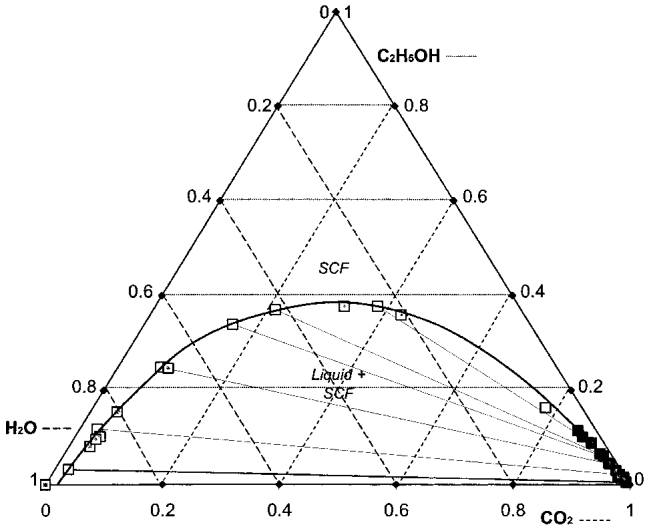

Fig. 2. Ternary phase diagram for the CO_2, C_2H_5OH, and H_2O system at 35°C and 100 bar. (□) SCF and (■) liquid molar fraction, respectively (68–70).

dustry, SC-CO_2 is appealing because it is "generally regarded as safe" by the FDA. Moreover, carbon dioxide has an easily accessible critical point (31.5°C, 75.8 bar) (Fig. 1), does not cause oxidation, is available in large quantity at high purity grade, leaves no traces behind, is inexpensive, nonflammable, environmentally acceptable, and easy to recycle or to dispose of.

Alternatives such as SC ammonia and SC ethane have been explored. The former, however, produced completely denatured proteins (18). The latter gave results comparable to SC-CO_2 when considering the particle size and improved the biological activity of insulin when compared to SC-CO_2 (21), but it is undesirable because of its high flammability.

A modifier is frequently added to the drying medium to increase the solubility of less-soluble solvents, such as water, in the SCF phase. Ethanol has been most commonly used in combination with proteins (Tables III to V), as it appears to have only limited effect on the protein stability in the process, owing to short contact times (22).

Solvent

Solvents should have a high solvent power for the protein, preferably be highly soluble in the drying medium, and be compatible with the protein. Dimethyl sulfoxide (DMSO) and DMFA have been used because they satisfy the first two requirements (9), even though they have some denaturing effect (23) and are toxic. Spherical nano-sized particles of numerous proteins were produced using DMSO. A number of solvents such as methanol, ethanol, 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), ethyl acetate, and dichloro methane (DCM) have also been used (Table III). Except for HFIP, their poor solvent power for proteins makes them poor candidates. DCM has mainly been used as solvent for protein coprecipitated with controlled release biomaterials (10,11,24).

Water is the friendliest solvent for proteins and most relevant when dealing with aqueous protein formulations, but it is poorly soluble in SC-CO_2. For the organic solvents mentioned above, the operating pressure can be chosen such that they are completely miscible with CO_2. For water, there is no such mixture critical pressure, CO_2 not being completely miscible at any pressure. To enhance the solubility of water, modifiers such as ethanol are usually added to the SCF (Fig. 2). An important issue related to water is the pH drop during the drying process (25). Without buffering, the pH may drop to 2.5–3, due to the dissolution of CO_2 in the water phase, causing a harsh environment for most proteins. Still, several processes have shown to be capable of producing protein powders from nonbuffered aqueous solution with preserved structure and activity (see Tables IV and V). If necessary, buffered solutions can be used (26,27).

Operation Mode

Antisolvent Precipitation

In this process, the SCF is added to the particle formation vessel containing the protein solution. The solute precipitates during the dissolution of the SCF in the solvent, causing a loss of solvent power and, consequently, protein supersaturation and precipitation (Fig. 3). Using this method, lysozyme, insulin, and myoglobin were precipitated from organic solutions (Table III).

The main disadvantage of this process is the lack of control of particle formation. This is especially true in batch operating conditions, because the level of supersaturation is not maintained. Moreover, extensive particle agglomeration may occur due to the low fluid viscosity of the SCF (28).

Spraying into a SCF

The antisolvent precipitation process has been improved by adding an atomization step to the initial process (Fig. 4a). The protein solution is sprayed via an atomization device into the vessel containing the SC-CO_2. Several authors have shown that this process enables producing particles with a narrow size distribution, uniform shape, and desired physicochemical characteristics (9,20). Atomization is achieved by forcing the solution through a capillary (24), orifice (9), or nozzle (7) into a SCF environment. Atomization devices will be further detailed in the section about equipment requirements.

In SCF spraying processes, the antisolvent precipitation concept remains but happens at the droplet level. This offers a favorably higher antisolvent to solvent ratio, an increase in surface area and mass transfer rate and, hence, an acceleration of the drying process to a time range of seconds. The rate is particularly fast when the operating pressure reaches the mixture critical pressure. The drying process is then controlled by mixing of miscible fluids rather than mass transfer over the interface of the droplets in the spray.

When the miscibility of the fluids is poor, particularly in systems containing water and carbon dioxide, mass transfer requires special attention. The mass transfer can be improved by increasing the drying medium to solvent ratio, decreasing the droplet size, or the relative velocity between the solvent and drying medium. Improvement of the mass transfer can be achieved efficiently by an enhanced spraying process where the protein solution is mixed together with a SCF stream at the injection point of the vessel (Fig. 4b) (20).

To further improve particle characteristics, especially prevention of the agglomeration of particles, the use of emul-

Table II. SCF Drying Techniques

Technique	Name	Abbreviation
Antisolvent precipitation	Gas antisolvent	GAS (17)
Spray drying in SCF	Supercritical antisolvent	SAS (71)
	Precipitation with compressed antisolvent	PCA (72)
	Aerosol solvent extraction system	ASES (73)
	Solution enhanced dispersion by supercritical fluids	SEDS (74)
	Supercritical antisolvent precipitation with enhanced mass transfer	SAS-EM (75)
Effervescent atomization	Carbon dioxide nebulization with a bubble-dryer	CAN-BD (43)
	Depressurization of an expanded liquid organic solution	DELOS (76)

SCF, supercritical fluid.

sions and improved spraying devices were studied (29). Preparation of a water-in-oil emulsion before atomization takes advantage of the solubility of the drying medium, the SCF, in the oil phase of the emulsion. It was successful at producing a variety of protein powders, such as insulin particles of small size range (>95% of the particles having a diameter <5 μm). However, as emulsification of aqueous protein solutions with organic solvents can induce severe protein denaturation (30), one should be cautious when applying this method for the drying of proteins.

Equipment Requirements

Drying processes using a SCF clearly set other demands to the equipment than freeze drying or (freeze) spray drying. The use of high-pressure equipment requires the implementation of specific safety measures and higher investments than those for traditional process equipment. As compensation, it may also be smaller than freeze-drying and spray-drying equipment, because the process is faster. Therefore, initial capital investments for SCF drying processes can be competitive with standard processes (31).

Table III. Studies of Proteins Dried by Antisolvent Precipitation

Proteins	Solvent	Temperature (°C) Pressure (bar)	Comments: Particles size (μm) Shape Biological activity (BA)	References
Insulin	DMSO	35–50°C	1.4–8	(18, 49)
			Sperical to poorly spherical	
Insulin	MeOH		0.05–0.45	(18, 49)
			Discrete spheres	
Insulin	MeOH	35°C	0.2	(18, 49)
		109 bar	Agglomeration	
Insulin	EtOH		0.05–0.3	(18, 77)
Insulin	EA		0.3–0.7	(18, 77)
Lysozyme	DMSO	18–35°C	0.05–0.3	(18, 49)
			Spheres 10%	
			92–100% BA	
Lysozyme	DMSO	45°C	0.05–0.3	(18, 49)
			Spheres, polydisperse	
			60% BA	
Lysozyme	DMSO	10–35°C	0.2–0.3	(50)
			Spheres, amorphous	
			75% BA	
Lysozyme	DMSO DMF[a]		0.1	(18, 77)
Lysozyme	DMSO EtOH[a]		0.02–0.04	(18, 77)
Lysozyme	DMSO AA[a]		0.05 × 0.25	(18, 77)
Lysozyme	MeOH		0.01–0.05	(18, 77)
Lysozyme	EtOH H_2O[a]		0.05–1.0	(18, 77)
Myoglobin	DMSO			(18, 77)
Myoglobin	DMSO	35°C	0.05	(49)
		109 bar		
Myoglobin	MeOH		0.05–0.3	(18, 77)

[a] Modifier.

Table IV. Studies of Proteins Dried by SCF Spray Drying

Proteins	Solvent	Temperature (°C) Pressure (bar)	Comments: Particles size (μm) Shape Biological activity (BA)	References
Albumin	HFIP	37°C 82.7–96.5 bar	1.3–2.9	(64)
Albumin	H_2O EtOH[a]	20–45°C 155 bar	0.9–1.3 Spheres	(56, 57)
Alkaline phosphatase	H_2O EtOH[a]	40°C 99.8 bar	1.5 90% BA	(78)
Alkaline phosphatase	H_2O EtOH[a]	40°C 100 bar	<1 90% BA	(78)
Alkaline phosphatase	H_2O EtOH[a]	40°C 100 bar	Spheres 95–100% BA	(59)
Alkaline phosphatase, trehalose 1:10	H_2O EtOH[a]	40°C 100 bar	Needles and spheres 95–100% BA	(59)
Alkaline phosphatase, trehalose 1:2	H_2O EtOH[a]	40°C 100 bar	Spheres 95–100% BA	(59)
Carbonic anhydrase/PLG	MC+H_2O	35°C 240 bar	10–100	(60)
Catalase	H_2O+PeOH EtOH[a]		91% BA	(29)
Catalase	EtOH+H_2O	35°C 90 bar	1 Crystallized	(54)
Catalase	EtOH+H_2O	35°C 90 bar	<1 Crystallized	(53)
Chymotrypsin-PLA	MC	20.4°C 130 bar	1–2	(24)
α-Chymotrypsin	DMSO	36°C 136 bar	6.5% BA	(79)
α-Chymotrypsin	DMSO H_2O[a]	36°C 136 bar	14% BA	(79)
α-Chymotrypsin	H_2O	36°C 136 bar	0.2–0.6 Spheres 59% BA	(80)
α-Chymotrypsin	H_2O	25°C 136 bar	0.2–0.6 Spheres 91% BA	(80)
Helicobacter pylori adhesion protein A (HpaA)/PHB	MC+H_2O	50°C 180 bar		(60)
Helicobacter pylori adhesion protein A(HpaA)/PHB	MC+H_2O	35°C 240 bar		(60)
Holcus lanatus protein extract + dextran	DMSO	40°C 120 bar	Partially agglomerated	(38)
Insulin	DMSO	25–35°C 86.2 bar	1.9–3.8 Spheres	(9, 62)
Insulin	DMSO	28–46°C 90.6–142 bar	1.8–3%	(51, 63)
Insulin	DMSO	35°C 109 bar	0.05 Agglomerates	(49)
Insulin	DMFA	35°C 86.2 bar	2.5 Spheres	(9)
Insulin	HFIP	37°C 82.7–96.5 bar	1–1.9 3–6%	(64)
Insulin	H_2O EtOH[a]	45°C 155 bar	0.05–0.20 Spheres 93–95% Monomer	(56, 57)
Insulin	H_2O EtOH[a]	25°C 150 bar	8.2–11.8	(21)
Insulin	H_2O+PeOH EtOH[a]		<5 <5%	(29)
Insulin	EtOH:H_2O	35°C 90 bar	1–5 Spheres	(54)

Table IV. Continued

Proteins	Solvent	Temperature (°C) Pressure (bar)	Comments: Particles size (μm) Shape Biological activity (BA)	References
Insulin	EtOH:H_2O	35°C 90 bar	<5 Spheres 1 × 3 Needles	(53)
Insulin, mannitol	DMSO	35°C 120 bar	21.8 Rectangular	(39)
Insulin, mannitol	EtOH:H_2O	35°C 150 bar	11.9 Rectangular	(39)
Insulin-PLA	MC	34°C 100 bar	1–5	(24)
Insulin-PLA	DMSO:MC	19–38°C 102–130 bar	1–5	(24)
Insulin, PEG, PLA	DMSO	16–22°C 130 bar	0.4–0.7 Spheres, smooth	(10)
Insulin, PEG, PLA	DMSO:DCM	37–38°C 102.5–105 bar	1–5 Spheres, agglomerated	(11)
Insulin, PEG, PLA	DMSO:DCM	19.8°C 130 bar	0.5–2 Spherical, single	(11)
Insulin, PLA	DMSO:DCM	25°C 130 bar	0.7 Spherical, single	(13)
Insulin, PEG, PLA	DMSO:DCM	25°C 130 bar	0.4–0.5 Spherical, single to network-like	(13)
Lactase	H_2O+PeOH EtOH[a]		68% BA	(29)
Lactase, 10% mannitol	H_2O+PeOH EtOH[a]		85% BA	(29)
Lysozyme	DMSO	26.6–45°C 73.4–115 bar	1–5 Loose aggregates 2.5–7.4% 88–100% BA	(51, 63)
Lysozyme	DMSO	37°C 96.5 bar	2	(7)
Lysozyme	DMSO	37°C 96.5 bar	0.3	(7)
Lysozyme	DMSO	37°C 96.5 bar	0.2–1.2	(32)
Lysozyme	DMSO	40°C 80 bar	2.5–5.3	(52)
Lysozyme	DMSO	40°C 150 bar	2.6 Agglomerated spheres	(52)
Lysozyme	H_2O EtOH[a]	40°C 99.8 bar	2 100% BA	(78)
Lysozyme	H_2O EtOH[a]	35–45°C 155 bar	0.4–0.6 Spheres 96–98%	(56, 57)
Lysozyme	H_2O EtOH[a]	55°C 150 bar	95% BA	(31)
Lysozyme	H_2O EtOH[a]	55°C 200 bar	0.8 95% BA	(67)
Lysozyme	H_2O EtOH[a]	40–50°C 100–200 bar	1.6–4.9 63–101% BA	(55)
Lysozyme	H_2O EtOH[a]	40°C 100 bar	99% <10 μm 96% BA	(59)
Lysozyme	H_2O EtOH[a]	40°C 100 bar	<1 94% BA	(35)
Lysozyme	H_2O EtOH[a]	37°C 100 bar	4.5–6.0%	(32)
Lysozyme	H_2O Organic solvent[a]		95% BA	(81)
Lysozyme, lactose	H_2O EtOH[a]	20°C 155 bar	0.1–0.2 Spheres, partially agglomerated	(40)

Table IV. Continued

Proteins	Solvent	Temperature (°C) Pressure (bar)	Comments: Particles size (μm) Shape Biological activity (BA)	References
Lysozyme, lactose	H_2O	20°C	0.1–0.5	(40)
	EtOH[a]	155 bar	Spheres, agglomerated	
Lysozyme, lactose	H_2O	20°C	<20	(40)
	EtOH[a]	155 bar	Plates, agglomerated	
Lysozyme, trehalose 1:10	H_2O	40°C	99% <10 μm	(59)
	EtOH[a]	100 bar	Needle and spheres	
			104% BA	
Lysozyme, trehalose 1:5	H_2O	40°C	92% <10 μm	(59)
	EtOH[a]	100 bar	Needle and spheres	
			96% BA	
Lysozyme, trehalose 1:1	H_2O	40°C	96% <10 μm	(59)
	EtOH[a]	100 bar	98% BA	
Lysozyme, trehalose 2:1	H_2O	40°C	97% <10 μm	(59)
	EtOH[a]	100 bar	97% BA	
Lysozyme, trehalose 4:1	H_2O	40°C	97% <10 μm	(59)
	EtOH[a]	100 bar	101% BA	
Lysozyme, PLA	DMSO+MC	19.7–20.8°C	1–2	(24)
		130 bar		
rhDNAse	H_2O	20–45°C	0.4–1.0 Spheres	(56, 57)
	EtOH[a]	155 bar	0–33% Monomer	
rhDNAse	H_2O	24–45°C	5.9–11.0 Agglomerate	(37)
	EtOH[a]	155 bar	0–40% Monomer	
rhDNAse	H_2O	20–45°C	5.5–8.5 Agglomerate	(37)
	EtOH, TEA[a]	155 bar	97% Monomer	
rIgG	H_2O	45°C	38.2–48.5% BA	(26)
	EtOH[a]	175 bar		
Therapeutic peptide	H_2O		100% BA	(81)
	Organic solvent[a]			
Trypsin	DMSO	26.6–46.5°C	5.4–7.3%	(51, 63)
		73.4–136 bar	73–88% BA	
Trypsin	H_2O	55°C	5.5	(67)
	EtOH[a]	200 bar	Spheres	
			36% BA	
Trypsin	H_2O		36& BA	(81)
	Organic solvent[a]			
Trypsin	H_2O+PeOH		80% BA	(29)
	EtOH[a]			
Trypsin, 10% mannitol	H_2O+PeOH		94% BA	(29)
	EtOH[a]			
Urease, DL:PLG	H_2O	38°C	6	(82)
	A:EA:IPA	160 bar	Discrete, spheres	

Abbreviation used in the table: A, acetone; AA, acetic acid; DCM, dichloro methane; DMFA, dimethyl formamide; DMSO, dimethyl sulfoxide; EA, ethyl acetate; EtOH, ethanol; JFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; IPA, isopropyl alcohol; MC, methyl chloride; MeOH, methanol; PeOH, n-pentanol; SCF, supercritical fluid; TEA, tri-ethylamine.

[a] Modifier.

The main design criterion for SCF drying equipment investigated to date is the atomization device. Other factors such as the residence time and vessel size and geometry have hardly been investigated so far. Various injection devices and mechanisms have been applied. In the most simple form, nozzles introduce the protein solution directly into the vessel (9). Ultrasonic nozzles (electric ultrasound generator) were shown to produce finer droplets and increase mass transfer rates, improving the drying rate (7,32). As proteins are exposed to ultrasounds only for a very short time through the nozzle, no adverse effect of the ultrasounds was observed on their activity.

To enhance the solvent-SCF mixing, as applied in enhanced spraying (Fig. 4b), coaxial nozzles (Fig. 5) containing two or three concentric annuli inlets have been used. These coaxial nozzles are constructed with (33) or without (34) a mixing chamber or with a central outlet around which are disposed a number of outer orifices (35). The outcome of a coaxial vs. a standard nozzle for processed polymer solution was the production of individual particles instead of flocks (36). Apart from the observation of a small decrease in particle size when using a coaxial ultrasonic nozzle vs. a standard coaxial nozzle (37), no vis-à-vis studies for proteins are yet available. Other alternatives are mixing devices in which impinging flows of SCF and solution are forced through an orifice (34), through a T-shaped mixer to improve the mixing

Table V. Studies of Proteins Dried by Effervescent Atomization

Proteins	Temperature (°C) Pressure (bar)	Comments: Particle size (μm) Biological activity (BA)	References
Lysozyme	32°C 103 bar	1–3 Irregular 92% BA	(27)
Lysozyme, 10% mannitol	32°C 103 bar	1–3 Irregular 95% BA	(27)
Lysozyme, 10% sucrose	32°C 103 bar	1–3 Spheres 98% BA	(27)
Lysozyme, 10% sucrose, Tween-20	32°C 103 bar	1–3 Spheres, smooth 90%	(27)
Trypsogen-sucrose	20–50°C 80–100 bar	1 Aggregates	(46)
Lactate dehydrogenase	32°C 103 bar	1–3 15% BA	(27)
Lactate dehydrogenase, 10% mannitol	32°C 103 bar	1–3 40% BA	(27)
Lactate dehydrogenase, 10% sucrose	32°C 103 bar	1–3 65% BA	(27)
Lactate dehydrogenase, 10% sucrose, Tween-20	32°C 103 bar	1–3 95% BA	(27)
RhDNase	32°C 80–100 bar	75% <3 99% BA	(44)
Ovalbumin, trehalose (50:50)		Discrete particles	(45)

(38), V-shaped mixer (39), or a coaxial nozzle in which the SCF flow induces ultrasounds causing a turbulent mixing of the phases and improving the mass transfer (40).

A scale-up study performed with an aqueous solution of lysozyme indicated that the morphology of the particles was similar when the flow rates were increased proportionally to the size of the precipitation vessel (i.e., when the residence time was kept constant) (31). In another study, the residence time was prolonged by adding a second vessel in series (21). As a result, the volumetric mean particle size of insulin was reduced from 12 μm in average diameter (based on volume) to 8 μm. However, this reduction could also be due to the equipment design presenting a constriction between the vessels, which might break up the particles.

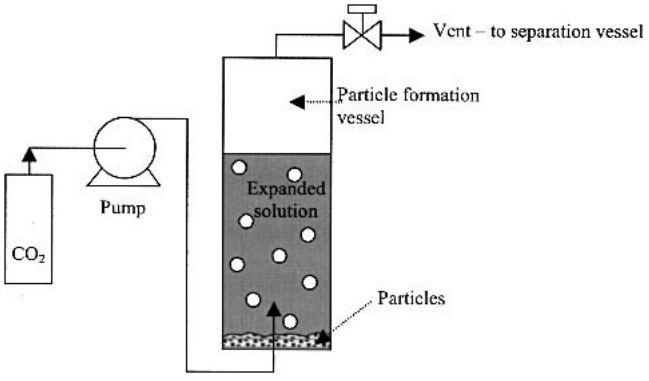

Fig. 3. SCF antisolvent process: the solution is first placed in the vessel; the drying medium, SC-CO_2, is then added by bubbling it through the solution or mixed in by stirring; following precipitation, the solvent-containing SCF is released, the product is flushed with fresh SCF until the solvent is completely removed, and then the pressure is released to recover the dry product.

Challenges associated with the industrial production of protein powders may be the atomization (larger nozzle or multiple nozzles), residual solvent (toxicity, long-term protein stability), particle recovery (efficiency, agglomeration, automatic vial filling, aseptic conditions), and recycling of process fluids and process evolution toward a continuous process (41,42).

PROCESSING USING SCF FOR EFFERVESCENT ATOMIZATION

Besides its use as drying medium, SC-CO_2 can also be used to enhance atomization at atmospheric pressure (43). In this process, the SC-CO_2 is first dissolved in the protein solution at pressures between 80 and 100 bar and temperatures between 20°C and 50°C. The solution is then sprayed to atmospheric conditions (Fig. 6). Upon spraying, the CO_2 expands, causing the breakup of the drops into finer droplets, which are then dried to powders by a flow of heated nitrogen. The authors claim that this process is able to rapidly dry proteins. This is most likely due to an improved atomization, because apart from the atomization, this process is similar to traditional spray-drying because of the analogous droplets drying conditions, conveying inherent interfacial stresses. An additional advantage of this method may be that the operating temperatures are lower than in traditional spray drying.

Protein powders (Table V) such as recombinant human deoxyribonuclease (rhDNase) (44), lysozyme, lactate dehy-

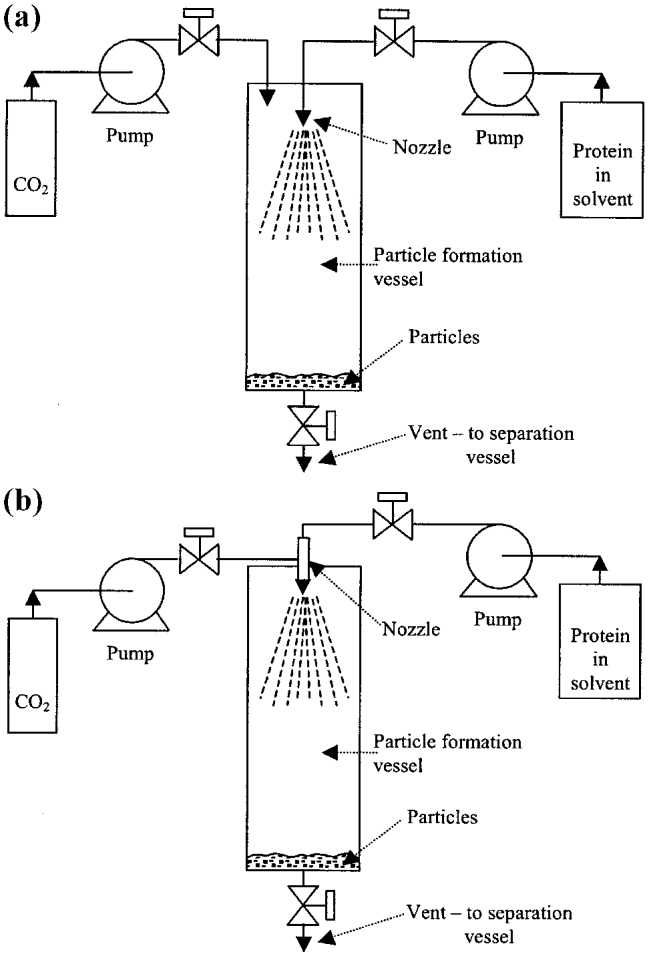

Fig. 4. Protein drying by spraying in SCF. (a) SCF spraying process: The solution is sprayed into a vessel previously filled with the SCF; as the SCF penetrates the droplets, proteins precipitate, and the drying of the protein particles is completed by the circulating SCF. (b) Enhanced spraying process: The solution is sprayed together with the SCF into a vessel previously filled with the SCF; the SCF helps the atomization as its flow rate is considerably higher than that of the solution. After drying and depressurization, the protein particles precipitated from the solution are recovered from the filter at the outlet of the particle formation vessel.

drogenase (LDH) (27), ovalbumin (45), and trypsogen (46), mostly containing excipients and less than 2% water, were produced using this technique. Effervescent atomization produces stresses similar to traditional spray-drying, which resulted in a clear drop of the LDH activity when excipients were omitted (27).

PARTICLE FORMATION AND CHARACTERISTICS

One of the major general applications of SCF processing is the production of fine particles. In the pharmaceutical field, this is particularly interesting for the development of dry-powder inhalation systems and (needle-free) dry-powder injection systems. Some reviews deal with the general concept of particle formation in SCF (20,47). In these reviews, mainly the temperature and pressure have been considered as factors determining the particle morphology. Here we also discuss the effect of processing and formulation parameters on the properties of the protein particles. Unfortunately, comparative data are very scarce, making the effect of the different process parameters difficult to quantify or even identify.

General Principles of Particle Formation

Modifying the pressure and/or temperature has a tremendous effect on the SCF density. As the fluid flow characteristics and the breakup of the spray are strongly affected by the density of the medium, these parameters are expected to have a strong effect on the resulting properties of the particles. The temperature is also expected to affect the solubility of the protein and the antisolvent in the solvent, and the solubility of the solvent in the antisolvent, thus modifying the precipitation kinetics and crystal growth (48).

As mentioned earlier, the solubility of organic solvents in CO_2 is much larger than the solubility of water. This influences the drying rate and the precipitation and crystallization dynamics (e.g., nucleation rate, diffusion, phase homogeneity, etc.). For instance, particles produced from DMSO are usually of nanoscale size and from aqueous solutions result in micrometer-size clusters (Table IV), because the high solubility of DMSO and SC-CO_2 results in a rapid achievement of the supersaturation conditions and a faster precipitation, reducing the opportunity for crystal growth. Therefore, the process parameters for drying from organic and aqueous solutions will be discussed separately.

Particles from Organic Solutions

It has been observed in antisolvent precipitation that increasing the lysozyme concentration resulted in smaller particles (49). This technique generally yields nano-sized particles, except for insulin in DMSO, which formed clusters up to 8 μm in diameter. Mixing during the precipitation phase stimulated the formation of spherical amorphous particles. The increase of temperature was the parameter causing the main effect, inducing agglomeration of particles (50).

When spraying is used in combination with antisolvent precipitation, temperature, pressure, nozzles, and organic solvents have shown some effect on the particle formation from organic solvents. Within the temperature (25°C and 35°C) and concentration ranges (5 and 15 mg/ml) explored for lysozyme dissolved in DMSO, the particle morphology was relatively insensitive to the temperature and protein concentration (9). A temperature increase from 35.5°C to 45°C resulted in the formation of larger primary particles (51). Increasing the process pressure from 80 to 150 bar resulted in an increase of the particle agglomeration (52).

Improving the droplet formation process by using ultrasounds resulted in a substantial reduction of the lysozyme particle size from 2 μm to 0.3–0.4 μm (7,32). For unknown reasons, precipitation of catalase from 90% ethanol resulted in crystallized particles instead of typical amorphous formations (53,54).

Particles from Aqueous Solutions

Similar observations concerning the effect of nozzles, pressure, temperature and concentration were made in aqueous systems. The use of a coaxial ultrasonic nozzle vs. a standard coaxial nozzle resulted in the production of smaller particles (from 68% (w/w) to 61% of particles >10 μm) (37). Increasing the pressure for lysozyme processing from 100 to

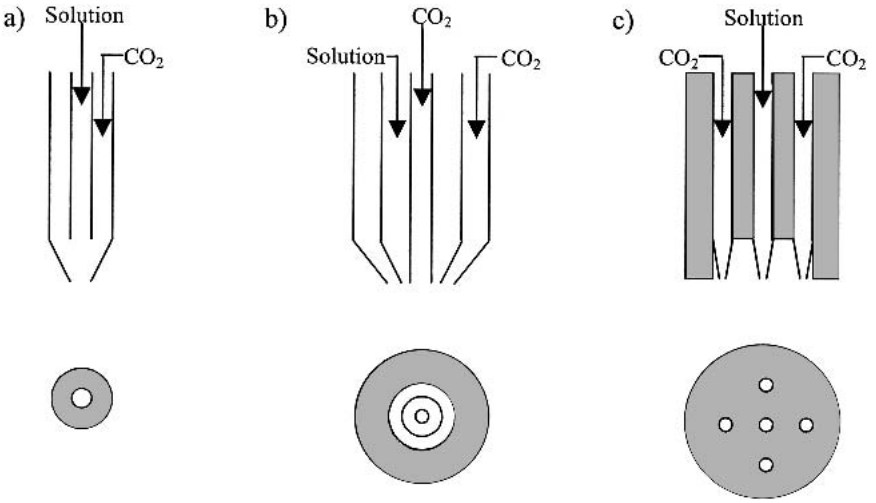

Fig. 5. Coaxial nozzles (a) 2-way, concentric annuli, with internal chamber, (b) 3-way, concentric annuli, without internal chamber, (c) 2-way with outer orifices.

200 bar resulted in the production of 90% of the particles being smaller than 5.2 μm in diameter instead of smaller than 10.5 μm. The higher diffusion coefficient of water in SC-CO_2 at increased pressure, favoring nucleation over crystal growth, was suggested to be responsible for the smaller particle size (55). The same phenomena could explain that an increase of temperature caused a decrease in particle size for lysozyme and rhDNase (from 35°C to 45°C), and for albumin (bovine) (from 20°C to 35°C) (56). Increasing the lysozyme, insulin, or albumin concentrations (15 to 50, 10 to 30, or 15 to 80 mg/ml, respectively) resulted in a decrease of particle size (56). It was observed for various proteins that lowering the relative flow rate of SC-CO_2 to the solvent and modifier produced smaller particles (31). Because of the poor water solubility in SC-CO_2, an increase in the protein solution flow rate, while maintaining constant drying medium and modifier flow rates, could impede the precipitation. An ethanol mole fraction of 0.2 or higher in the SCF was required to precipitate proteins (57).

Particles from Formulated Proteins

Excipients, such as sugars, in aqueous solutions affect not only the protein stability, but also the particle formation and morphology. As excipients are often used in a larger concentration than the therapeutic protein itself, their precipitation characteristics (e.g., solubility, crystallization) will drive the precipitation dynamics and the morphology of the product. Lactose had the tendency to crystallize and caused phase separation and the appearance of particle families (amorphous spherical protein particles and lactose crystals), unless used in less than 10% (w/w solids) concentration with lysozyme (37,40). This problem is not restricted to SCF processes as it was previously reported for traditional spray-drying of mixtures, such as trehalose:trypsinogen (58). Increasing the lactose fraction from 15% (w/w) to 35% (w/w) with rhDNase resulted in more agglomeration of the amorphous particles (37).

Formulations containing sucrose resulted in spherical particles with smooth surfaces. In contrast, SCF drying of mannitol-containing formulations resulted in less homogeneous particles, even for a solid fraction of only 10% (w/w) (29), which is far below values typically used in freeze drying (6). The lower solubility of mannitol and its propensity to form crystals might have affected the particle morphology.

Higher contents of trehalose in lysozyme/trehalose or alkaline phosphatase (AP)/trehalose mixtures (1:10 w/w) resulted in powders with two populations of particles: needle-shaped and spherically shaped particles. The lower trehalose content powders (1:2 w/w protein to sugar) showed the spherical particles population only (59).

Surfactants were used in some cases because they were essential to the process, like in the preparation of emulsions (29,60) or to smooth the particle surface in effervescent atomization (27).

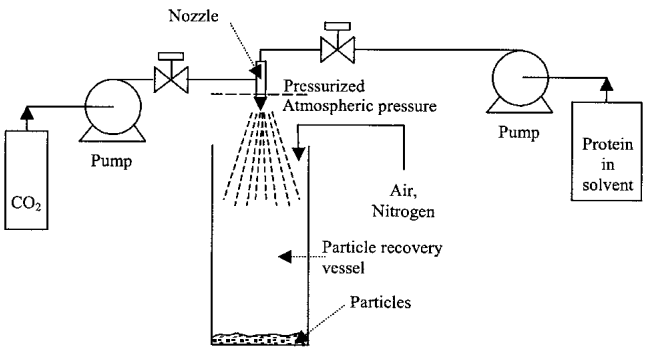

Fig. 6. Effervescent atomization: The solution is sprayed together with the SCF into a vessel under atmospheric pressure. The SCF helps the atomization by breaking up the solution jet as it expands. The particles can then be recovered from the vessel.

PROTEIN STABILITY

There are only a limited number of published articles in which protein structure and stability issues are addressed for material dried in SCF. Therefore, it is difficult to derive general relationships between processing and formulation parameters on the one hand and protein stability on the other. However, it may be possible to extrapolate some general concepts from the freeze-drying literature. In freeze-drying, pre-

serving protein structure during processing is a crucial step in achieving long-term stability of proteins. As in freeze-drying, protein stability during SCF drying is likely to be affected by process parameters and formulation. As proteins dried by SCF processes are exposed to stress conditions that are different from freeze-drying stresses, it can be expected that protective mechanisms against SCF process-induced protein degradation are also different. On the other hand, simply designing a formulation that allows the protein to survive the process still does not assure stability during long-term storage. However, as a start, one can assume that the long-term stability of proteins formulations dried with SCF is dependent on similar formulation parameters as those known for freeze-dried protein products (e.g., low residual moisture levels and Tg values exceeding the highest temperature encountered during shipment and storage) (61).

Following the chronological development of SCF drying of proteins, we will first discuss studies on the drying of proteins from organic solvents, next the drying of nonformulated proteins from aqueous solution, and finally the drying of (aqueous) protein formulations.

An overview of the relevant literature on protein stability in SCF drying processes is given in Tables III–V. The studies mentioned below all refer to SCF spraying in SCF unless otherwise mentioned.

Proteins Dried from Organic Solvents

Proteins that were dissolved in organic solvents and dried by spraying in SCF often show increased β-sheet content and concomitant loss of α-helicity, as detected by a shift of the amide I band in Raman and FTIR spectra of dried powder. This was reported for insulin dried from DMSO or DMFA (9,62); for insulin, lysozyme, and trypsin dried from DMSO (51,63); and for insulin dried from HFIP (64,65). HFIP was used because halogenated alcohols are helix formers, increasing the amount of ordered structure in the protein. Moreover, HFIP is much more volatile than DMSO and, hence, should facilitate the removal of residual solvent. Although there were changes in the structure of dried proteins, after dissolution in water, Raman and FTIR spectra of all proteins mentioned above were similar to those of aqueous nontreated proteins, indicating that the conformational changes were reversible upon reconstitution (51). Moreover, insulin powders recovered essentially full biological activity on reconstitution in water (9).

Regarding the structural changes induced by SCF drying, the question arises: What are the most critical process parameters affecting protein structure? First of all, the extent to which structural perturbations in the solid state were induced during processing were protein-specific: lysozyme < trypsin < insulin (51). Some of the changes might be attributed to exposure of the proteins to organic solvent (62). On the other hand, higher operating temperatures (9,51) and pressures led to more extensive β-sheet–mediated intermolecular interactions in the precipitates (51), indicating that the drying process itself also contributes to conformational changes. The recovered biological activity also varied with the drying conditions applied, but it was not possible to find obvious correlations between precipitation conditions, structural changes in the solid state, and recovered biological activity upon reconstitution for insulin, lysozyme, or trypsin (51). Upon redissolution in water, lysozyme sprayed in SCF regained between 88 and 100% of its biological activity and trypsin regained between 69% and 94% of its biological activity. The varying recovery illustrates that process parameters do have an effect on the protein activity. Then again, insulin samples processed over a wide range of operating conditions recovered essentially complete biological activity upon reconstitution, which is in agreement with the work of Yeo *et al.* (9). In another investigation, process conditions (pressure, solution concentration, and solution flow rate) had little effect on the structure of insulin precipitated by spraying in SCF (65).

Increasing the operating temperature in the range tested led to a slightly greater structural perturbation of insulin (62). On the other hand, it was found that within the temperature (25°C and 35°C) and concentration ranges (5 and 15 mg/ml) explored, there was no significant difference between the biological activity of processed and unprocessed product (9).

A discrepancy between recovered secondary structure and preserved biological activity was also observed for lysozyme dried from DMSO under different process conditions: temperature, pressure, protein concentration, and flow rates of SC-CO_2 and protein solution were varied (52). DSC and high-performance cation-exchange chromatography indicated that the drying process did not cause major denaturation of lysozyme, but the retained biological activities of the samples varied between 44% and 100%, depending on the experimental conditions. The operating pressure (80–150 bar) was the most critical parameter affecting the biological activity: the higher the pressure, the more biological activity was retained. In a follow-up study, it was suggested that some loss of biological activity at a low working pressure of 80 bar could be related to prolonged exposure of precipitates to DMSO (55).

Proteins Dried from Aqueous Solution

Therapeutic proteins are usually produced in aqueous rather than organic solution. Therefore, and because organic solvents may harm the native protein structure, SCF drying of aqueous protein solutions is of particular interest for pharmaceutical applications.

Traditional drying techniques were compared to SCF drying using aqueous solutions of lysozyme (66). The recovered enzymatic activity of samples dried by spraying in SCF (95.0%) was better than for spray-dried (85.3%) or freeze-dried (89.3%) samples. The water content depended on the drying method used, but there was no clear relationship between water content and recovered biological activity. When the same SCF drying process was applied to aqueous solutions of trypsin, less than 40% of the enzymatic activity was recovered (67). This is in agreement with previously reported protein-specific effects of SCF drying (51).

Drying of lysozyme, albumin, insulin, and rhDNase caused considerable destabilization of some of these proteins (56). Whereas lysozyme and insulin retained almost full monomer content, 97% and 93% respectively, albumin and especially rhDNase were affected to a much larger extent by processing. For albumin, the monomer content was reduced from 86% in the original material to 50–75% after drying. For rhDNase, at most 33% of monomer was recovered. Although there was no clear general effect of process temperature (20–55°C) or protein concentration (10–80 mg/ml) on the stability of the proteins, it was reported for rhDNase that with increas-

ing temperature (20–45°C), recovery of the monomer decreased from 33% to 0%. Likely, optimal process parameters are protein specific and have therefore to be established for each individual protein.

In the above-mentioned studies, the consequences of SCF drying from aqueous solutions on protein secondary structure were not reported. The Raman spectra of lysozyme dried under different process conditions, temperature, pressure, and flow rates of the protein solution and cosolvent (ethanol), showed some disturbance of the secondary structure (+4 cm^{-1} to +9 cm^{-1} shift of the amide I band) (55). This study differs from previous reports (51,62), because here the smallest shift (+4 cm^{-1}) in amide I band region was obtained at the highest working pressure (200 bar). The biological activity ranged between 63% and 101%, and a fairly good correlation between the magnitude of the peak shift and the biological activity was observed. Still, it is not clear which process parameter was most detrimental to protein stability. As compared with a previous study where lysozyme was dried from DMSO (52), the activity of dried lysozyme from an aqueous solution appeared to be less sensitive to operating pressure. It was suggested that a key factor for the formation of intact dry proteins was the ratio of water, ethanol and CO_2 in a single homogeneous phase (Fig. 2). When this criterion was not fulfilled, loss of bioactivity was observed (the lowest biological activity at 150 bar and 45°C). In contrast, the highest activity was observed at 200 bar and 40°C, which, according to the authors, is the most likely working condition to form particles from a single homogeneous phase (55).

Formulated Proteins

From stability data on numerous protein formulations and experience with other drying techniques, it is to be expected that preservation of protein structure and function during processing and long-term storage should be achieved by adding stabilizers (2).

An aqueous formulation of recombinant human immunoglobulin G (rhIgG) containing sucrose, sodium chloride, sodium citrate, and Tween 20 was sprayed in SCF (26). After drying and rehydration, UV and SEC profiles were indistinguishable from those of the reference standard. However, the retained antigen-binding activity, as assessed by ELISA, was unexpectedly low (<50%). According to the authors, the activity loss was likely to be a reflection of subtle alterations induced in sensitive rhIgG regions. Unfortunately, the stabilizing effect of the individual excipients was not investigated, and no comparison was made with unformulated protein.

The importance of formulation for preserving the structure and activity of lysozyme and LDH during effervescent atomization of aqueous protein solutions was illustrated in a study by Sellers *et al.* (27). In the absence of excipients, lysozyme was observed to undergo perturbations of its secondary structure, as observed by FTIR measurements of the dried protein. In the presence of sucrose, these structural changes were minimized. Addition of Tween 20 did not improve the retention of its native secondary structure in the dried solid. Upon reconstitution, all lysozyme powders (with or without excipients) regained almost complete activity, and SEC profiles were similar to those of nontreated protein. In the absence of stabilizers, LDH suffered irrecoverable loss of activity (only 15% recovered) on reconstitution. LDH was stabilized during dehydration by the addition of 10% (w/w) sucrose, and almost complete preservation of activity was achieved with the further addition of Tween 20.

Stable dry protein powders by coprecipitation of AP or lysozyme with trehalose were produced by spraying in SCF (59). It was demonstrated that each stabilizer/protein couple has an optimal stabilizer/protein ratio among the different ratios tested, ranging between 0:1 and 10:1 (w/w). The retained enzymatic activity was between 95% and 100%, and it was shown, by measuring enzymatic activity, that AP/trehalose coprecipitated particles were more stable under different storage conditions than the equivalent freeze-dried product.

In another study, insulin/mannitol formulations were dried in order to improve insulin absorption via lung administration. SCF drying was compared with conventional spray drying. No insulin degradation during these processes was observed by HPLC and SDS-PAGE, but the hypoglycemic effect of SCF dried powder was increased after intratracheal administration in rats, compared with spray dried powder. This might be partly attributed to the observed difference in the dissolution rates (39).

SUMMARY AND PERSPECTIVES

Considering the examples from the literature described herein, it is clear that SCF drying of proteins is still in its infancy, but at the same time holds great promises for protein stabilization. Similar to other drying methods, the quality of proteins dried with SCF depends on the operating conditions, the formulation, and the nature of the protein, as schematically depicted in Fig. 7. The design of an effective SCF drying process includes the selection of an appropriate drying technique (such as spraying in SCF), drying medium, modifiers, protein solvent, and applied pressure and temperature, which together must lead to a stable protein product that is safe to administer to patients. Regarding protein stability, resistance to SCF drying processing is clearly protein dependent, but process conditions like temperature, pressure, and composition of the SCF–cosolvent–solvent mixture are important as well. In terms of particle formation, the selection of an appropriate atomization device is primordial, as well as the optimization of the temperature, pressure, and composition of the protein solution to obtain uniform particle populations of predefined size and morphology.

Most likely, several key process parameters have to be optimized simultaneously for achieving a pharmaceutically acceptable dried protein product (Fig. 7). A complicating factor is that optimization of particle characteristics may require process conditions that do not lead to the most stable protein product and *vice versa*. Moreover, the optimal process conditions are likely to depend on the protein species and composition of the formulations. Clearly, further research is required to identify and predict the main stress factor(s) during the SCF drying process in order to rationally optimize the process in conjunction with the formulation. Moreover, studies of other therapeutically relevant proteins are necessary to gain insight into the general applicability of SCF drying of proteins. Such studies are warranted because of the continuous growth in number and volume of biotechnology products used in therapy and the limited drying capacity currently available. The possibility to obtain large amounts of high-

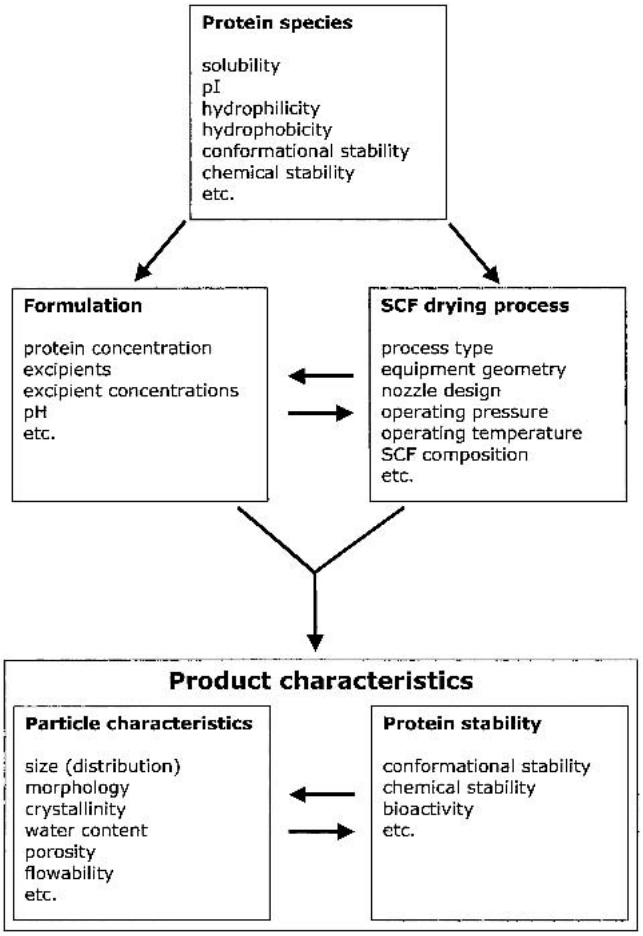

Fig. 7. Schematic illustration of interrelationships between protein species, SCF drying process, and formulation in SCF drying, all of which can affect product characteristics.

quality protein powders in a single step can make SCF drying a serious alternative to existing drying techniques for the stabilization of pharmaceutical proteins.

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