



## SPRAY FREEZE-DRYING OF PROTEINS: CAUSES OF INACTIVATION OF TRYPSINOGEN

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### Introduction

Recent investigations show spray freeze-drying (SFD) to be a suitable technique for the preparation of protein-loaded dry powders. To reveal the mechanism of protein inactivation during the SFD process, aqueous trypsinogen solutions were subjected to isolated steps of the SFD process. In this way, the steps crucial for protein stability were detected. Trypsinogen concentration in the particle surface was examined by ESCA.

### Experimental Methods

#### Materials

Bovine pancreas trypsinogen (MW 24,000), lyophilized and dialyzed, was used as received (Sigma). Trehalose-dihydrate, polyoxyethylene-sorbitan monooleate (Polysorbate 80) and N-benzoyl arginine ethylester (BAEE) also were obtained from Sigma.

#### Spray Freeze Drying

An ultrasonic nozzle (120 kHz) was used for atomization. The solution was sprayed into a metal bowl filled with liquid nitrogen (LN<sub>2</sub>) which was stirred continually. On completion of spraying, the bowl was immediately transferred to a lyophilizer (Christ) which had been pre-chilled to -45°C and a drying program was started.

#### Trypsinogen Activity Assay

To determine residual enzymatic activity of the processed trypsinogen, the protein was at first activated by reduction to trypsin. Trypsin activity was then calculated from the absorbance decrease at  $\lambda = 253$  and 25°C using BAEE as a substrate.

#### Size Exclusion Chromatography (SEC) and Gel Electrophoresis (SDS-PAGE)

SEC was performed on a BioSep-SEC-3000 column using a 0.1M phosphate buffer pH 6.8 as mobile phase (flow rate 0.5ml/min). Samples of

10 $\mu$ l were analyzed at a concentration of 2-5mg/ml and detected photometrically at  $\lambda = 280$ nm. For SDS-PAGE, samples were separated on a 12% poly-acrylamide gel under non-reducing conditions using a mini protean II unit (Biorad). After fixing, the gels were silver stained.

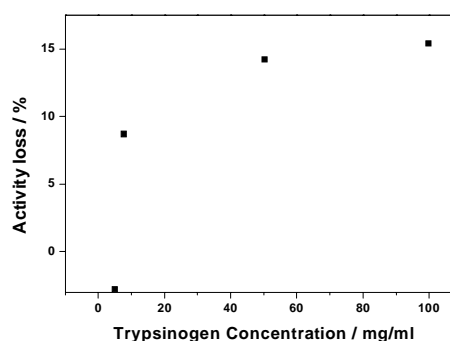
#### Electron Spectroscopy for Chemical Analysis (ESCA)

With ESCA, the atomic composition of the near surface region (10nm) of a solid can be detected quantitatively.

### Results and Discussion

#### Trypsinogen Stability during the Process

Pure trypsinogen loses about 15% of its residual activity during the SFD process. Freeze- or spray-drying the protein results in a comparable activity loss [1]. The combination of both methods in SFD was therefore expected to show a higher degree of inactivation.



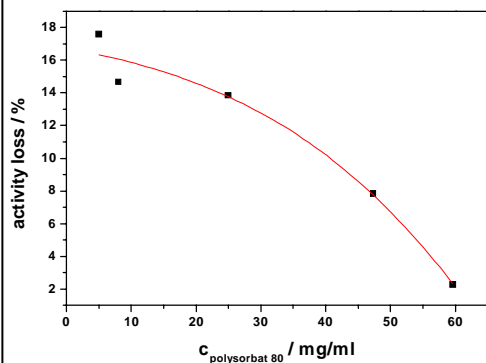
**Fig 1:** Loss of trypsinogen activity in aqueous solutions after nebulization

The isolated steps of the process were therefore examined for their influence on protein stability. Aqueous trypsinogen solutions were either a) nebulized into a beaker using the ultrasonic nozzle, b) nebulized into LN<sub>2</sub> and thawed, or c) frozen at -80°C and thawed.

On atomization of trypsinogen solutions, the

observed activity loss is concentration dependent (Fig. 1). A higher protein concentration in the bulk solution leads to a greater amount of adsorption at the air-liquid interface during spraying, which in turn causes unfolding and greater inactivation of the protein.

Addition of Polysorbate 80 to the bulk solution decreases the activity loss on atomization (Fig. 2). Complete amelioration of activity loss requires a surfactant/protein weight ratio of approximately 1:1. For the other individual process steps, no activity loss could be detected, as shown in Tbl. 1.

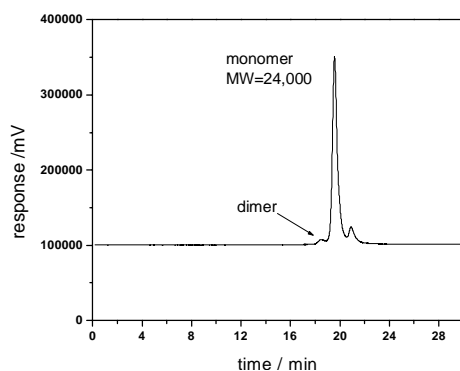


**Fig. 2:** Influence of Polysorbate 80 on residual activity of atomized trypsinogen solution

Step	Description	Residual activity / %	Trypsinogen conc. mg/ml
a)	Nebulization	83.3	50
b)	Nebulization into LN2	100.4	50
c)	Freezing / thawing	101.2	50

**Tbl. 1:** Residual trypsinogen activity after isolated process steps

Recall that trypsinogen loses about 15% activity on SFD. The loss in activity occurring on atomization must therefore either not occur on spraying into LN2 or is reversed during droplet freezing. There is no activity loss detectable for step b). Since freeze/thawing of the trypsinogen



**Fig. 3:** Chromatogram of pure SFD trypsinogen

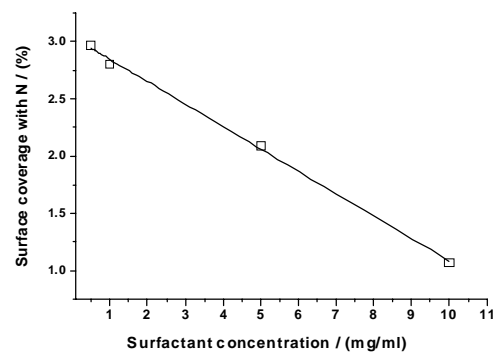
solution does not affect protein stability, the 15% activity loss on freeze drying must be related to removal of water from the region of the protein molecule.

#### Aggregation Status of the Protein

Both SEC and SDS-PAGE detected aggregates in the SFD trypsinogen samples. These could be the cause of the reduced activity measured by the enzymatic assay. The chromatogram obtained for SFD trypsinogen shows one peak representing the protein monomer and a second one showing a dimer fraction (Fig. 3). The untreated protein only contains monomeric protein (data not shown), as was also found for the nebulized trypsinogen solutions. With SDS-PAGE, only the SFD protein shows an aggregated fraction (not shown), not the samples obtained by the isolated process steps.

#### ESCA

The protein presence in the surface of SFD protein/sugar particles is a result of its surface activity and adsorption to the liquid/air interface on spraying. Fig. 4 shows how addition of Polysorbate 80 depletes the solid particle surface of protein. This occurs however at a much lower surfactant/protein weight ratio than seen in Fig. 2 for prevention of inactivation on pure nebulization.



**Fig. 4:** Influence of surfactant on N-surface coverage

#### Conclusions

The mechanism of trypsinogen inactivation during the process steps of SFD is more complex than expected from observations of freeze- and spray-drying the protein. Although trypsinogen is surface active, atomization into LN2 surprisingly causes no loss in activity or change in aggregation status. Protein unfolding appears to be reversed during spray droplet freezing.