Influence of Tableting on the Conformation and Thermal Stability of Trypsin as a Model Protein

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ABSTRACT: The objective of this study was to investigate the influence of compaction on the conformation of trypsin, its transition temperature (T_m) of unfolding, and its folding reversibility after thermal denaturation. Plain trypsin was compacted at 40–382 MPa. Pressure-induced changes in the trypsin conformation and the extent of their reversibility were determined using solid- and liquid-state IR spectroscopy together with principal component analysis and an area overlap approach. Trypsin enzymatic activity was determined by a photometric assay. Liquid-state differential scanning calorimetry was performed to determine the T_m as well as the folding reversibility after thermal denaturation of the reconstituted samples. It was found that compacted samples showed reduced activity accompanied by an altered secondary structure. Conformational changes that occur in the solid state were partially reversible upon tablet reconstitution. Aqueous-state IR spectroscopy combined with partial least squares was shown to be a powerful tool to follow irreversible structural changes and evaluate sample bioactivity. Besides its conformation, the thermal stability of trypsin was altered as a result of the applied compaction pressure, indicated by a reduced folding reversibility. In conclusion, this study reveals that tableting can have a negative impact on the biological quality of protein APIs. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:4314–4321, 2015

Keywords: FTIR; compaction; compression; multivariate analysis; partial least squares; protein structure; protein folding; principal component analysis; solid-state stability; tableting

INTRODUCTION

Most biopharmaceuticals are formulated as liquid dosage forms and are administered parenterally, as their high molecular weight and limited stability in the gastrointestinal tract hamper oral delivery.[1] However, oral administration is by far the most preferred route of administration for systemic delivery because of significantly lower manufacturing costs and improved patient compliance compared with drugs that have to be injected.[1,2] Furthermore, solid protein formulations are attractive in terms of improved long-term storage stability compared with liquid formulations because the susceptibility of protein drugs to deleterious processes such as conformational changes, aggregation, or chemical degradation is often linked to the water surrounding the biomolecules.[3,4] However, manufacturing of solid dosage forms is usually a multistep process, which includes stress that is applied to the formulation, such as friction, temperature, or pressure. In the case of biopharmaceuticals, these stress factors are often critical with respect to the stability of these drugs, as their three-dimensional structure is sensitive to environmental conditions and tends to denature during mechanical processing.[5–7] The integrity of the unique higher order structure of proteins is crucial for preserving biological activity and avoiding undesirable immune responses when ingested.[1,8,9] Thus, to successfully formulate protein drugs and to control manufacturing, it is essential to gain knowledge of the range of stress-inducing factors in which the native structure is preserved or in which the impact of processing leads to conformational changes, respectively. Depending on the process and its parameters, such structural perturbances have often been reported to be reversible upon reconstitution, for example, conformational changes induced during freeze- and spray-drying.[10–12] However, partially irreversible and even completely irreversible structural alterations have been reported to be induced during solid-state processing.[13,14]

Currently, solid-state formulation strategies for oral delivery of macromolecules, such as tablets containing surfactant-like permeation enhancers, are in advanced clinical trials and are considered attractive approaches to enhance the transport of biomacromolecules across biological membranes.[15] From this perspective, the influence of the tableting process and associated stress on the physical stability of biopharmaceuticals is of increasing interest. A negative influence of the compaction pressure on biological activity has been reported, for example, for α-amylase and catalase.[16,17] Furthermore, various studies have been performed using IR spectroscopy to monitor changes in the secondary structure of proteins upon compaction. For instance, Chan et al.[18] and Wolkers and Oldenhof[19] determined structural alterations of rhDNase and lactobacillus proteins, respectively, in a qualitative manner, whereas Meyer et al.[20] quantified pressure induced changes in the solid-state spectra of α-chymotrypsinogen and lysozyme using an area overlap approach. Furthermore, Otsuka et al.[7] applied principal component regression on the IR spectral dataset of unprocessed and compacted trypsin to evaluate its remaining enzymatic activity based on structural alterations that occur in the solid state. These studies reveal that pressure applied in the solid state can

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alter the secondary structure of proteins, although the extent of reversibility of such pressure-induced conformational changes remains unclear.

The extent of deviation from the native structure may influence product performance upon ingestion, as the conformation of proteins determines their susceptibility to interact with surrounding molecules.[21] Thus, to thoroughly evaluate tableting as a formulation process for biopharmaceuticals, it is necessary to investigate if the pressure during compaction induces changes in the susceptibility of protein APIs toward denaturation caused by the environmental conditions, such as elevated temperature during ingestion and reconstitution. In this context, the transition temperature (T_m) of unfolding, a parameter indicative for the thermal stability of proteins,[22] has been reported to be significantly reduced for proteins in the solid state as well as for reconstituted formulations as a result of stress applied during pharmaceutical processing.[5,19,23] Interestingly, Elkordy et al.[24] showed that although spray-drying leads to a decrease in the T_m of reconstituted DNase I by 5.3°C, no marked changes were found in the secondary structure of the reconstituted processed protein compared with its initial form. Nevertheless, if protein APIs are not able to maintain their native structure throughout the entire route of administration, they must at least be able to subsequently refold and thus to exert the desired biological effect. It has been shown by Forbes et al.[5] that spray-drying leads to minor changes in the T_m of trypsin, but significantly reduces the ability of the protein to regain its initial structure after thermal denaturation. To summarize, the before mentioned studies reveal that pharmaceutical processing may cause both changes in the conformation of biopharmaceuticals as well as a reduction of their conformational stability during further handling and administration.

The overall goal of the present study was to investigate the influence of the tableting process on the integrity of trypsin as a model protein API to assess the suitability of tableting as formulation strategy for biopharmaceuticals. Trypsin has been chosen as a model protein in this study as it is a well-characterized biomolecule,[25–29] and has already been used as model to investigate the influence of pharmaceutical processing on the stability of biomolecules.[5] Furthermore, Trypsin is sufficiently stable in the solid state at room temperature to allow compaction experiments. In addition, the plain trypsin powder shows a sufficiently high compactability to be compressed to tablets. The first aim of this study was to determine pressure-induced conformational changes of trypsin as well as their reversibility upon reconstitution. For this purpose, IR spectroscopy was used, as it is a fast and accurate technique to determine the conformation of proteins irrespective of their hydration state.[30–32] The second aim of the study was to evaluate the relationship between irreversible conformational characteristics and activity, potentially allowing for the structural characteristics obtained by liquid-state IR spectroscopy to be used as a surrogate marker for activity. The determination of the fraction of irreversible structural changes is assumed to allow a more accurate forecast of protein enzymatic activity based on spectroscopic data compared with the quantification of both[7] reversible and irreversible changes in the solid state. This approach may be attractive for simplifying activity determinations. The third aim of this study was to determine whether tableting affects the thermal stability of trypsin, characterized by the T_m and the folding reversibility after thermal denaturation.

MATERIALS AND METHODS

Materials

Bovine trypsin [United States Pharmacopoeia (USP) grade] in a lyophilized form was purchased from Biozym (Hamburg, Germany). Benzoyl-L-arginine ethyl ester hydrochloride (BAEE) was purchased from AppliChem (Darmstadt, Germany). Hydrochloric acid, monobasic sodium phosphate, and sodium hydroxide were all of analytical grade and obtained from Roth (Karlsruhe, Germany). Deionized water was obtained from a Millipore purification system (Merck Millipore, Schwalbach, Germany).

Methods

Preparation of Tablets

Plain trypsin powder was gently ground by mortar and pestle, sieved (125 μm mesh), and stored at 21°C and a relative humidity of 45% for at least 48 h prior to compaction. Tableting was performed at predefined compaction pressures (40–382 MPa) on a Fette E XI instrumented single punch eccentric press (Fette Compacting, Schwarzenbek, Germany), equipped with 10 mm flat-faced punches. Tableting was carried out at a compaction speed of 16 rpm in single stroke mode by filling 100 mg of the powder manually into the die.

Assay of Trypsin Activity

To evaluate the effect of compaction on trypsin, enzymatic activity of unprocessed and compressed enzyme powder was determined by a constant rate UV-photometric assay under steady-state conditions according to the USP monograph "Crystallized Trypsin." Tablets were gently ground by mortar and pestle and the remaining powder was dissolved in 10 mM hydrochloric acid. Protein solution (200 μL) was mixed with 3.0 mL of BAEE substrate solution. The absorbance at 253 nm was measured at 30 s intervals for 5 min. Trypsin activity of all samples expressed as a percentage of the mean initial activity of the trypsin powder [activity (%)] was determined from the slope of the absorbance profiles using Equations (1) and (2).

$$\text{activity}\,(\%) = \frac{\text{units}_s}{\text{units}_i} \times 100 \tag{1}$$

$$\text{units}_s = \frac{(A_1 - A_2)}{0.003 \times T \times W} \tag{2}$$

where units_s is the trypsin activity, in USP units, of the respective samples, units_i is the mean initial trypsin activity of unprocessed trypsin powder, A_1 is the absorbance straight-line final reading, A_2 is the absorbance straight-line initial reading, T is the elapsed time, in minutes, between the initial and final readings, and W is the weight, in mg, of trypsin in the volume of the sample solution.

FTIR

Infrared spectra of unprocessed trypsin and ground trypsin tablets were recorded using a Tensor 37 FTIR spectrometer (Bruker, Ettlingen, Germany) equipped with a nitrogen-cooled MCT detector. The instrument was continuously purged with almost dry and carbon dioxide-free air (double column air dryer, SDAT-670/420; DRUMAG GmbH, Bad Saeckingen, Germany)