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Impact of additives on the formation of protein aggregates and viscosity in concentrated protein solutions

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ABSTRACT

In concentrated protein solutions attractive protein interactions may not only cause the formation of undesired aggregates but also of gel-like networks with elevated viscosity. To guarantee stable biopharmaceutical processes and safe formulations, both phenomenons have to be avoided as these may hinder regular processing steps. This work screens the impact of additives on both phase behavior and viscosity of concentrated protein solutions. For this purpose, additives known for stabilizing proteins in solution or modulating the dynamic viscosity were selected. These additives were PEG 300, PEG 1000, glycerol, glycine, NaCl and ArgHCl. Concentrated lysozyme and glucose oxidase solutions at pH 3 and 9 served as model systems. Fourier-transformed-infrared spectroscopy was chosen to determine the conformational stability of selected protein samples. Influencing protein interactions, the impact of additives was strongly dependent on pH. Of all additives investigated, glycine was the only one that maintained protein conformational and colloidal stability while decreasing the dynamic viscosity. Low concentrations of NaCl showed the same effect, but increasing concentrations resulted in visible protein aggregation.

1. Introduction

Increasing titers in fermentation (Chon and Zarbis-Papastoitsis, 2011) and the trend towards highly concentrated formulations (Shire et al., 2004) require biopharmaceutical downstream processing to cope with concentrated protein solutions. Their tendency to form protein aggregates and high viscosity impacts judgment on developability and manufacturability – pumping, filtration or chromatography – of the target molecule as well as its syringeability (Shire et al., 2004; Jezek et al., 2011; Guo et al., 2012). From a molecular point of view, the aggregation tendency and viscosity of a protein solution are governed by attractive protein interactions. Depending on the physicochemical nature of the protein surface, the complexity of attractive protein interactions may lead to various aggregation mechanisms and result in differing aggregate morphology. The multimers formed through assembly of native or non-native protein monomers can be reversible or irreversible, visible or invisible as well as soluble or insoluble (Mahler et al., 2009). At high concentrations, not only electrostatic interactions but also short-range van der Waals, hydration, hydrophobic and steric interactions influence protein aggregation which may either result in the formation of dense aggregates or spacious networks with elevated viscosity (Wang, 1999; Liu et al., 2005; Chari et al., 2009). Thus, in order to preserve the colloidal stability of concentrated protein solutions and guarantee reliable processing and safe formulations, attractive protein interactions resulting in aggregate formation as well as high viscosity need to be prevented (Shire, 2009; Patro and Przybycien, 1996). This can be achieved by manipulating protein interactions through the addition of additives which either induce changes in proteins' conformational or colloidal stability in solution (Shire et al., 2004). The specific impact of additives on protein interactions can strongly vary and is usually dependent on additive type, additive concentration, protein type, protein concentration and pH.

Their impact on the formation of protein aggregates was already extensively investigated for solutions with low protein concentration. PEGs (Kozer et al., 2007), sugars (Arakawa and Timasheff, 1982), polyols (Vagenende et al., 2009) and amino acids (Arakawa et al., 2007) were found to have a stabilizing impact due to preferential interactions (Arakawa and Timasheff, 1985). The effect of salts on protein aggregation is more complex. Their impact depends on complex ionic interactions with the protein surface. Salts can either stabilize, destabilize or have no effect on protein aggregation depending on the type and concentration of salt. At low concentrations, salts were shown to stabilize due to electrostatic shielding of attractive forces (Hamada et al., 2009).

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The impact of additives on concentrated protein solutions was considered by investigating their dynamic viscosity. Salts and amino acids were published to have a lowering effect on this parameter. Du and Klibanov (2011) found so-called hydrophobic salts to have a strong decreasing impact on concentrated bovine serum albumin and γ-globulin solutions. Inoue et al. (2014a) showed amino acids, such as glycine and argine, to decrease the dynamic viscosity of concentrated bovine and human serum albumin solutions.

Influencing protein interactions due to changes in the physicochemical nature of the protein surface, pH has been shown to additionally influence the impact of additives (Kohn et al., 1997). Galm et al. (2015) published PEG 1000, glycerol, and glycine to either have an impact on changes in protein conformation or in protein solubility. A pH-dependent impact of additives on dynamic viscosity was published investigating the gelation of soy proteins with sugar and $CaCl_2$ (Alvarez et al., 2008).

Hence, until now, either the impact of additives on the formation of protein aggregates at low protein concentrations or the dynamic viscosity of concentrated protein solutions was examined. However, for concentrated protein solutions different aggregation mechanisms can either lead to the formation of dense protein aggregates or spacious networks with high viscosity (Liu et al., 2005; Wang et al., 2010).

As a consequence, this work aims to provide a picture of the impact of additives on attractive protein interactions in concentrated protein solutions by investigating the impact on the formation of aggregates as well as viscosity. For this purpose, additives known to stabilize protein aggregation as well as additives known to modulate the viscosity of concentrated protein solutions were selected. These additives, namely PEG 300, PEG 1000, glycerol, glycine, sodium chloride (NaCl), and arginine hydrochloride (ArgHCl) were examined at different pH values. Changes in protein interactions depending on pH and the impact of selected additives on the protein conformation were evaluated by Fourier-transformed-infrared (FT-IR) spectroscopy. The formation of visible aggregates and changes in dynamic viscosity of each sample were determined by phase behavior experiments and microrheological measurements.

2. Material and methods

To investigate the impact of additives on attractive protein interactions in concentrated protein solutions, the phase behavior and the dynamic viscosity were determined. The additives selected were PEG 300, PEG 1000, glycerol, glycine, NaCl, and ArgHCl. Concentrated lysozyme and glucose oxidase solutions at pH 3 and 9 served as model system. Changes in secondary structure of these proteins were investigated for selected additives. This section presents the preparation of the buffers as well as the additive and protein solutions applied in this study. It also contains information about the methods, such as the examination of structural changes by FT-IR spectroscopy, the phase behavior experiments, and the microrheological measurements.

2.1. Buffers and protein solutions

All buffers had an ionic strength of 100 mM. The respective components for pH 3 were citric acid (Merck KGaA, Darmstadt, Germany) and sodium citrate (Sigma–Aldrich, St. Louis, MO, USA). For pH 9, BisTris propane (Molekula Limited, Newcastle upon Tyne, UK) was used. The additives investigated were PEG 300, PEG 1000, glycine (Sigma–Aldrich), glycerol (Alfa Aesar®, Ward Hill, MA, USA), NaCl, and ArgHCl (Merck KGaA). For each additive, a stock solution was prepared. These additive solution contained the buffer components at the respective pH and an additive concentration of 0.6 M for PEG 300, PEG 1000, glycerol, or 1 M for glycine, NaCl and ArgHCl. The pH of the buffers and additive solutions was determined with a five-point calibrated pH meter (HI-3220, Hanna® Instruments, Woonsocket, RI, USA) equipped with a SenTix® 62 pH electrode (Xylem Inc., White Plains, NY, USA) and corrected by titration of NaOH or HCl (Merck KGaA) with an accuracy of ±0.5 pH units. After titration, the buffers were filtered with 0.2 μm membranes consisting of cellulose acetate (Sartorius AG, Göttingen, Germany) for pH 3 and Supor® Polyethersulfone (PES) (Pall Corporation, Port Washington, NY, USA) for pH 9. Each solution was first used 24 h after preparation. They were stored at room temperature and regularly checked for constant pH. Lyophilized lysozyme (Hampton Research, Aliso Viejo, CA, USA) and glucose oxidase (Sigma–Aldrich) were weight in and dissolved in the respective buffer without additive. The protein solutions were filtered with 0.2 μm syringe filters (cellulose acetate for pH 3, PES for pH 9 (VWR, Radnor, PA, USA)). Production related salts were removed by size exclusion chromatography with a HiTrap Desalting column (GE Healthcare, Uppsala, Sweden) on an ÄKTAprime™ plus chromatography system (GE Healthcare). Afterwards, the solutions were concentrated with Vivaspin® centrifugal concentrators (Sartorius AG). For lysozyme at pH 3 and 9, the protein stock solution had a concentration of 360 mg/mL. For glucose oxidase at pH 3, a concentration of 100 mg/mL, and at pH 9, a concentration of 260 mg/mL was reached. These protein concentrations were determined by a NanoDrop™ 2000c UV–Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The respective extinction coefficients were $E^{1\%}(280\,nm) = 22.00\,L\,g^{-1}\,cm^{-1}$ for lysozyme and $E^{1\%}(280\,nm) = 12.00\,L\,g^{-1}\,cm^{-1}$ for glucose oxidase. The samples with constant protein concentrations of 180 mg/mL for lysozyme, 50 mg/mL for glucose oxidase at pH 3 and 130 mg/mL at pH 9 were prepared by mixing the correct volume of buffer, protein stock solution and additive solution.

2.2. FT-IR spectroscopy

Changes in the conformational stability of selected protein samples were determined by FT-IR spectroscopy. This measurement was performed with a Nicolet™ iS5 and an iD7 ATR detector (Thermo Fisher Scientific). The absorbance of each sample was scanned 150 times with a spectral resolution of 2 cm^{-1} from 3500 to 1000 cm^{-1}. Background spectra at the respective additive concentration and pH were recorded with 256 scans. All measurements were conducted in duplicate with a sample volume of 5 μL. The OMNIC software (Thermo Fisher Scientific) was used for recording and processing of the FT-IR spectra. Processing steps were atmospheric suppression to delete the impact of water vapor bands and the calculation of the second derivative to investigate the protein conformation. For the formation of the second derivative Savitzky–Golay with 25 points and third polynomial order was applied. The wavenumber bands relevant for the conformational stability of a protein lie within a range of 1700–1600 cm^{-1} and can be assigned to α-helix (1658–1650 cm^{-1}), β-sheet (1695–1670 cm^{-1} and 1640–1620 cm^{-1}) and random coil (1650–1640 cm^{-1}) (Byler and Susi, 1986; Dong et al., 1997).

2.3. Phase behavior experiments

The formation of visible protein aggregates for the samples investigated in this study was determined by phase behavior experiments. Therefore, 30 μL of each sample were pipetted on MRC Under Oil 96 Well Crystallization Plates (SWISSCI AG, Neuheim, Switzerland) and sealed with Duck® Brand HD Clear sealing tape (ShurTech® brands, Avon, OH, USA) to avoid evaporation. The plates were incubated in the automated chrystallographer RockImager