International Journal of Pharmaceutics 494 (2015) 370–380

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Manipulation of lysozyme phase behavior by additives as function of conformational stability

Lara Galm, Josefine Morgenstern, Jürgen Hubbuch*

Institute of Engineering in Life Sciences, Section IV: Biomolecular Separation Engineering, Karlsruhe Institute of Technology (KIT), Karlsruhe, Germany

ARTICLE INFO

Article history:
Received 2 June 2015
Accepted 15 August 2015
Available online 21 August 2015

Keywords:
Osmolytes
Solubility line
Crystallization area
Non-native aggregation
FT-IR

ABSTRACT

Undesired protein aggregation in general and non-native protein aggregation in particular need to be inhibited during bio-pharmaceutical processing to ensure patient safety and to maintain product activity. In this work the potency of different additives, namely glycerol, PEG 1000, and glycine, to prevent lysozyme aggregation and selectively manipulate lysozyme phase behavior was investigated. The results revealed a strong pH dependency of the additive impact on lysozyme phase behavior, lysozyme solubility, crystal size and morphology. This work aims to link this pH dependent impact to a protein-specific parameter, the conformational stability of lysozyme. At pH 3 the addition of 10% (w/v) glycerol, 10% (w/v) PEG 1000, and 1 M glycine stabilized or destabilized lysozymes' native conformation resulting in a modified size of the crystallization area without influencing lysozyme solubility, crystal size and morphology. Addition of 1 M glycine even promoted non-native aggregation at pH 3 whereas addition of PEG 1000 completely inhibited non-native aggregation. At pH 5 the addition of 10% (w/v) glycerol, 10% (w/v) PEG 1000, and 1 M glycine did not influence lysozymes' native conformation, but strongly influenced the position of the crystallization area, lysozyme solubility, crystal size and morphology. The observed pH dependent impact of the additives could be linked to a differing lysozyme conformational stability in the binary systems without additives at pH 3 and pH 5. However, in any case lysozyme phase behavior could selectively be manipulated by addition of glycerol, PEG 1000 and glycine. Furthermore, at pH 5 crystal size and morphology could selectively be manipulated.

1. Introduction

The term protein aggregation describes the assembly of native or non-native protein monomers to protein multimers, i.e. aggregation characterizes both the formation of protein crystals and amorphous precipitates and includes native and non-native aggregation forms. Protein aggregation can occur through different mechanisms (Philo and Arakawa, 2009) and during different steps of a production process (Cromwell et al., 2006). However, crystallization and precipitation are also acknowledged process steps in biopharmaceutical industries either for formulation or purification purposes (Scopes, 1994). Crystalline drug formulations for example have shown significant benefits in the delivery of protein therapeutics to achieve high-concentration, high-stability, low-viscosity and controlled-release formulations (Basu et al., 2004; Jen and Merkle, 2001). Crystalline insulin formulations are market approved (Basu et al., 2004; Brange and Vølund, 1999; Vajo et al., 2001) and crystalline antibody formulations are studied, too (Yang et al., 2003). For formulated protein therapeutics, the presence of precipitates is typically considered to be undesirable because of the concern that especially non-native precipitates may lead to immunogenic reactions (Cromwell et al., 2006). The widespread opinion exists that aggregation processes are usually associated with a conformational change, i.e. partial unfolding of the proteins (Chi et al., 2003; Fink, 1998) and aggregation processes that resulted in non-native protein conformations have been observed (Dong et al., 1995; Dzwolak et al., 2003; Feng et al., 2012; Kendrick et al., 1998; Matheus et al., 2009). Moreover, aggregation processes might influence biological activity of protein therapeutics. Thus, in either case it is essential to ensure that the target protein remains in its native conformation and that biological activity is preserved despite aggregation. Thus, in cases where non-native aggregation is likely to occur aggregation needs to be prevented completely unless there are possibilities to stabilize the native conformational state. In cases where native aggregation occurs, the selective control of phase states is considered to be beneficial as sometimes either crystalline or precipitated forms are preferred e.g. due to a better bioavailability in the respective aggregate state (Vajo et al., 2001). Particular additives are thought

* Corresponding author. Tel.: +49 721 608 47526; fax: +49 721 608 4 6240.
E-mail address: juergen.hubbuch@kit.edu (J. Hubbuch).

http://dx.doi.org/10.1016/j.ijpharm.2015.08.045
0378-5173/

to stabilize the proteins' native state, for example stabilize the protein against thermal denaturation, and thus might be used to prevent non-native aggregation processes. According to Harries and Rösgen (2008) particular additives influence protein solubility as well, resulting in a manipulation of the protein phase behavior, i.e. protein aggregation might be completely inhibited or protein phase states (e.g. crystallization, precipitation) might be selectively changed. Frequently used additives are polymers (polyethylene glycol, PEG) and osmolytes. Osmolytes are low molecular weight additives, that can be grouped into the major categories of free amino acids and derivates (e.g. glycine), polyols and uncharged sugars (e.g. glycerol), methylamines, and urea (Yancey, 2001). The impact of these additives on protein stability is described to be due to a preferential binding or a preferential exclusion of the additives from the proteins' local domain. In cases where they are preferentially excluded from the proteins' local domain they are known to stabilize the proteins' native state (Arakawa and Timasheff, 1982, 1983, 1985a,b; Lee and Lee, 1981; Timasheff and Arakawa, 1988; Webb et al., 2001), The impact of additives on protein solubility is according to Harries and Rösgen (2008) not as easy as predicting protein stability and no general models exist. Though, the mode of action of osmolytes and PEG on protein stability is as well not as easy as it might sound since for some additives stabilizing as well as destabilizing effects have been observed. Parameters that strongly influence the stabilizing or destabilizing character of additives are the additive concentration, the molecular weight of the additive, and the solvent pH. PEG, dimethylglycine, and betaine for example have been found to stabilize proteins up to a certain concentration and destabilize them for higher concentrations (Arakawa and Timasheff, 1985b; Santoro et al., 1992). High molecular weights of PEG have been found to be destabilizing as well (Lee and Lee, 1981), whereas high molecular weight polyols stabilize proteins better than their low molecular weight counterparts (Poddar et al., 2008). Additive impact as function of solvent pH is even harder to generalize. According to Singh et al. (2011), polyols have a higher potency to stabilize the proteins' native state at low pH, whereas methylamines are described to act most stabilizing at neutral pH and destabilizing at low pH and amino acids were found to stabilize the native state of proteins almost independent of the pH (Macchi et al., 2012). This pH dependent osmolyte action has been related to the chemical nature of the osmolytes, e.g. the pK_a values (Granata et al., 2006; Natalello et al., 2009; Singh et al., 2009), but could not explain all pH dependent observations (Kaushik and Bhat, 2003). In contrast, there are indications that the pH dependent mode of action of additives might have its origin in the nature of the proteins instead (Kaushik and Bhat, 2003; Macchi et al., 2012). It is for example known that proteins at extreme pH values are conformationally unstable, i.e. prone to at least partial unfolding (Wang et al., 2010). Thus, our work aims to find a link between the pH dependent mode of action of additives and the conformational stability of a model protein in its initial state without additive. To the best of our knowledge up to now there are no investigations on the pH dependent mode of action as a function of conformational stability of proteins in their initial state without additive. Furthermore, this publication examines if the additives impact protein solubility and thus protein phase behavior. Lysozyme from chicken egg white was studied as a model protein, but the presented approaches can easily be transferred to other biopharmaceutical proteins. Lysozyme was investigated at pH 3 and pH 5. Sodium chloride was added as precipitant to induce phase transitions of lysozyme (e.g. crystallization and precipitation) in order to study the phase behavior of lysozyme. In the following the term binary describes lysozyme in aqueous sodium chloride solutions ranging from 0 M to 2.5 M sodium chloride. This was also referred to as the initial state of lysozyme above. The term ternary in the following describes lysozyme in aqueous sodium chloride solutions ranging from 0 M to 2.5 M sodium chloride and with a constant additive concentration. Lysozyme conformational stability, solubility, and phase behavior in these ternary systems (with additive) will be compared to lysozyme conformational stability, solubility, and phase behavior in the binary systems (without additive), i.e. to lysozymes' initial state. Glycerol and glycine as additives were chosen as representatives of two osmolyte classes and PEG 1000 as additive beyond the osmolyte class. Fourier-transformed-infrared (FT-IR) spectroscopy was applied to monitor lysozyme conformation and to account for non-native conformational changes. This allows to evaluate the impact of the additives on conformational stability and their potency to stabilize or destabilize the proteins' native state. Ternary phase diagrams, consisting of lysozyme, sodium chloride and the respective additive as solution components, were generated and compared to binary ones, consisting of lysozyme and sodium chloride. The comparison of the phase diagrams reveals information about how strong the additives manipulate lysozyme phase behavior, i.e. if they completely prevent aggregation, delay it or if they can be used to selectively control phase states, e.g. transfer former precipitated to crystalline phase states. The phase diagrams additionally allow to experimentally determine lysozyme solubility in cases where crystallization occurs as the supernatant of a crystalline solution is saturated and the lysozyme concentration in the supernatant thus reflects lysozyme solubility (Asherie, 2004; Howard et al., 1988; Retailleau et al., 1997). Experimentally determined solubility data points are fitted to an empirically found equation, resulting in continuous solubility lines. Comparison between the binary and ternary systems gives the additive impact on lysozyme solubility lines.

Altogether this publication aims to elucidate the potential origin of pH dependent additive action and tries to expand the basic knowledge on additive impact on protein solubility and phase behavior.

2. Materials and methods

2.1. Materials

The used buffer substances were citric acid (Merck, Darmstadt, Germany) and sodium citrate (Sigma–Aldrich, St. Louis, MO, USA) for pH 3 and sodium acetate (Sigma–Aldrich, St. Louis, MO, USA) and acetic acid (Merck, Darmstadt, Germany) for pH 5. PEG 300 and PEG 1000 were obtained from Sigma–Aldrich (St. Louis, MO, USA). Sodium chloride as well as glycine were purchased from Merck (Darmstadt, Germany), glycerol was from Alfa Aesar (Ward Hill, MA, USA) and lactose from Carl Roth GmbH & Co., KG (Karlsruhe, Germany). Hydrochloric acid and sodium hydroxide for pH adjustment were obtained from Merck (Darmstadt, Germany). pH adjustment was performed using a five-point calibrated pH-meter (HI-3220, Hanna Instruments, Woonsocket, RI, USA). All buffers were filtered through 0.2 μm cellulose acetate filters (Sartorius, Goettingen, Germany).

Lysozyme from chicken egg white was purchased from Hampton Research (Aliso Viejo, CA, USA). The lysozyme solutions were filtered through 0.2 μm syringe filters with cellulose acetate membranes (VWR, Radnor, PA, USA) previous to further desalting via size exclusion chromatography. Size exclusion chromatography was conducted using a HiTrap Desalting Column (GE Healthcare, Uppsala, Sweden) on an AEKTAprime plus system (GE Healthcare, Uppsala, Sweden). A subsequent protein concentration step was performed using Vivaspin® centrifugal concentrators (Sartorius, Goettingen, Germany) with PES membranes and molecular weight cutoffs of 3 kDa.