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Arginine as a protein stabilizer and destabilizer in liquid formulations

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ABSTRACT

Even though arginine monohydrochloride (ArgHCl) is a useful additive for protein stabilization, its mechanism is not yet fully elucidated. Moreover, there is a concern that ArgHCl may be a protein denaturant since it decreases transition melting temperature (T_m) of certain proteins. It contains a guanidinium group, a critical structure for denaturating activity of guanidine hydrochloride (GndHCl). Effects of ArgHCl, GndHCl, and creatinine on a model protein, etanercept, were examined by biophysical analyses including DLS, DSC, FT-IR, microviscometer, and SEC. Accelerated storage stability of the protein was examined in the absence and presence of H_2O_2 at different incubation temperatures with pH monitoring. ArgHCl reduced protein aggregation and retained monomer, but increased fragmentation at high temperature. T_m1 and T_m2 of the protein increased with ArgHCl, but slight decrease (>1 °C) in T_m3 was observed. GndHCl and creatinine decreased all three T_ms. In the presence of heat and H_2O_2, the effect of ArgHCl was significantly decreased compared to GndHCl and creatinine. In addition, it accelerated the loss of monomer and increased fragmentation with decreasing pH. ArgHCl differed from GndHCl in the mode of physical interaction with the protein, due to its unique balance of three steric functional groups (guanidinium, carboxylic acid, and carbon aliphatic straight chain). In contrast, ArgHCl acted as a protein denaturant at high temperature since NO_x generated from the amine group at the 3-carbon aliphatic straight chain and it is supported by GndHCl which did not change the pH nor accelerated the monomer loss after oxidation by H_2O_2 at high temperature.

1. Introduction

One of the most challenging tasks in the development of protein formulation is to deal with physical and chemical instability of proteins, which is one of the major reasons why protein formulations are mostly administered through injection (Manning et al., 2010; Wang, 1999). Consequently, the investigation of enhancing protein stability in aqueous state is necessary to obtain susceptible protein stability and a shelf life required for economic viability. However, achieving this goal is particularly challenging because proteins are only marginally stable and are susceptible to degradation (Chi et al., 2003; Manning et al., 2010).

Chemical instabilities involve processes that make or break covalent bonds, inducing new chemical entities. Physical instabilities are those in which the chemical composition is unaltered, but the physical state of the protein changes. This includes denaturation, aggregation, precipitation (i.e., insoluble aggregate formation), and adsorption (Cleland et al., 1993; Chi et al., 2003; Frokjaer and Otzen, 2005; Manning et al., 2010). These are the major challenges confronting pharmaceutical scientists involved in the development of proteins as well as bio-engineered medicines. Successful protein formulation development depends on a thorough understanding of their physicochemical and biological characteristics, immunogenicity, and pharmacokinetic properties (Frokjaer and Otzen, 2005).

Among physical instabilities, protein aggregation is particularly problematic since it occurs routinely during manufacturing processes (refolding, purification, sterilization) and even shipping processes. Aggregation can occur even under thermodynamically favorable conditions and in the absence of stresses. In addition, aggregation is often irreversible, and these aggregates contain high levels of non-native, intermolecular β-sheet structures (Dong et al., 1995; Kim et al., 2015a, 2015b; Yang et al., 2004). Aggregation behaviors, such as onset, aggregation rate, and the final morphology of the aggregate state (i.e., precipitates or fibrils) have been elucidated to be dependent strongly on the properties of a protein's solution environment. Extensive investigation has been performed previously with regard to various protein concentration, pH, types of buffer, buffer concentration, aggregation suppressors, sugars, and amino acids (Kim et al., 2014a, b,c; Lim et al., 2014, 2015). It has become evident that the stability of

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proteins significantly varies with solution environment and can be enhanced by various pharmaceutical excipients, including amino acids.

The effect of amino acids is protein-dependent in each protein formulation. However, a specific amino acid, arginine, is known to be highly effective in suppressing protein aggregations and can be suitable for increasing the shelf life of proteins as a universal reagent (Arakawa and Tsumoto, 2003; Baynes et al., 2005; Lange and Rudolph, 2009). Addition of arginine monohydrochloride (ArgHCl) to protein solution led enhanced recovery of the proteins by suppressing protein folding intermediates, without imparting any stabilizing effect on the native structure itself. In summary, ArgHCl does enhance the solubility of proteins and suppresses aggregation (Arakawa et al., 2007, 2006; Arakawa and Tsumoto, 2003; Baynes et al., 2005; Reddy et al., 2005; Shiraki et al., 2002, 2004; Tischer et al., 2010; Tsumoto et al., 2005, 2004). Nevertheless, the mechanism underlying the ArgHCl effect remains unclear, even though great progress has been made over the years.

On the other hand, there is a concern that ArgHCl may be a protein-denaturant, which may limit the expansion of its application based on the work exhibiting that the activity and stability of certain enzymes are perturbed by arginine (Xie et al., 2004; Yancey et al., 1982). They interpreted the observed effects of arginine in terms of denaturing property by the guanidinium group, which makes guanidine hydrochloride (GndHCl) a strong denaturant. In addition, ArgHCl decreases the transition temperatures of certain proteins, proposing that arginine does not stabilize proteins, but reduces aggregation and increases reversibility of thermal unfolding (Arakawa and Tsumoto, 2003).

Therefore, is ArgHCl a protein-denaturant or is arginine aggregation suppressor? In order to answer this question, it should be observed in physical and chemical aspects. There are certain demonstrations of a non-enzymatic pathway for the generation of nitric oxide (NO) by the reaction of hydrogen peroxide and ArgHCl (Mukherjee, 2013; Nagase et al., 1997). ArgHCl is the precursor of NO, and it can be synthesized not only in the presence of NO synthase (NOS), but also can be synthesized by the oxidation of ArgHCl with hydrogen peroxide. Consequently, if the reaction takes place in protein formulations, the effect of NO derived from ArgHCl on protein stability must be elucidated. Because NO can generate a hydroxyl radicals that can have destabilizing effects on protein and also affect the human body (Beckman et al., 1990; Hogg et al., 1992). In the present study, the effect of ArgHCl on protein in solution is investigated using various biophysical methods with the model protein, etanercept. In addition, the oxidation of ArgHCl, GndHCl, and other pharmaceutical excipients are also investigated with hydrogen peroxide to observe whether there is NO_x generation.

2. Materials and methods

2.1. Materials and sample preparation

Etanercept is selected as a model protein which is a fusion protein of two soluble human 75-kDa tumor necrosis factor-α (TNF-α) receptors linked to the Fc portion of immunoglobulin G1 (IgG1). The commercial product of the model protein is Enbrel® and is supplied by Seoul National University. The molecular weight is 51,234.9 g/mol with 934 amino acids.

After etanercept was dissolved in water for injection, it was dialyzed for 24 h at 4 °C in a Cellu Sep® H1 cellulose membrane having a MW cut-off of 5000 Da (Membrane Filtration Products, Seguin, TX, USA). 25 mM Phosphate buffer at pH 7.4 with excipients was used as a medium, and the resulting protein solutions were used for dilution, if necessary. Monitoring of pH was performed during dialysis and oxidation test with a pH meter (Metrohm, Zurich, Switzerland). Sodium phosphate monobasic dihydrate, sodium phosphate dibasic dihydrate, L-arginine monohydrochloride, guanidine hydrochloride, and creatinine were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Hydrogen peroxide was purchased from Junsei Chemical (Tokyo, Japan).

2.2. Dynamic light scattering (DLS)

Electrostatic interactions and hydrodynamic sizes of prepared etanercept solutions with various excipients were investigated by a Zetasizer Nano ZS90 apparatus (Malvern Instruments, Worcestershire, UK). All the measurements were done at a temperature of 15° C. 1 mL of each prepared solution was loaded into a disposable sizing cuvette (Sarstedt, Numbrecht, Germany) for Z-average size, hydrodynamic size, and polydispersity index (PDI). In addition, a disposable capillary cell (Malvern Instruments, Worcestershire, UK) was adopted for the measurement of zeta potential. Each measurement was repeated 5 times continuously with an interval of 10 s. Corresponding factors above were achieved from the autocorrelated function using Zetasizer software version 7.11 (Malvern Instruments, Worcestershire, UK).

2.3. Differential scanning calorimetry (DSC)

DSC measurements were carried out using a VP-DSC Microcalorimeter (Microcal, Northampton, MA, USA) having 0.51471 cm^3 twin cells for the sample and reference solutions. Before the DSC measurements, the prepared solutions were degassed under vacuum with stirring. Dialysis medium were used as a reference to achieve the baseline thermogram. Thermal analysis of prepared protein solution was done at a scan rate of 1 °C/min from 20° C to 120° C. The final thermogram was obtained by subtracting the baseline thermogram from the sample thermogram. The final thermograms were processed using the Microcal LLC DSC plug-in for the Origin 7.0 software package provided with the equipment. The final thermograms were fitted to a multistate model with three evident thermal transitions to calculate the transition melting temperature (T_m).

2.4. Size-exclusion high performance liquid chromatography (SEC)

Various concentrations of etanercept and prepared samples were analyzed using an Agilent high performance liquid chromatography system (Agilent HPLC 1260, Santa Clara, CA, USA) with a diode array detector at an ultraviolet wavelength of 280 nm UV absorbance spectra with a TSK-GEL G3000SWXL SEC column (TOSOH Bioscience, King of Prussia, PA, USA). To separate soluble etanercept based on size, a mobile phase containing 100 mM sodium phosphate (pH 6.8) and 100 mM NaCl was used at a flow rate of 0.5 mL/min. The injection volume was 20 μL. The peak areas for multimers and dimers were combined to calculate the total amount of soluble aggregates. The difference in the total area of etanercept (sum of all SEC peaks in each chromatogram) at any time point versus time zero is defined as the formation of insoluble aggregates (Barnard et al., 2011; Manikwar et al., 2013). The percentage of each species (soluble aggregation, monomer, and fragmentation) remaining relative to the total area at time zero was calculated and plotted against incubation time and temperature using the following equation:

$$\%\ \text{Remaining} = (a_t \div A_0) \times 100$$

where a_t is the area of individual species on any given time and A_0 is the total area of all the species at time zero. The error bars for soluble aggregation, monomer, and fragmentation represent standard deviation (SD) of three individual measurements.