International Journal of Biological Macromolecules 109 (2018) 311–322

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Preferential exclusion mechanism by carbohydrates on protein stabilization using thermodynamic evaluation

Nam Ah Kim, Ritu Thapa, Seong Hoon Jeong*

College of Pharmacy, Dongguk University-Seoul, Gyeonggi 410-820, Republic of Korea

ARTICLE INFO

Article history:
Received 9 November 2017
Received in revised form 7 December 2017
Accepted 16 December 2017
Available online 16 December 2017

Keywords:
Protein stability
Carbohydrates
Preferential exclusion

ABSTRACT

Carbohydrates are widely used as additives for biopharmaceutical formulations, but the mechanisms by which they confer stability to and their applicability on protein stability remain undiscovered. Herein, we aimed to elucidate these mechanisms, by studying the thermodynamic changes using isothermal titration calorimetry and micro-differential scanning calorimetry. Furthermore, conventional biophysical analyses, namely circular dichroism, dynamic light scattering, and size-exclusion chromatography, were used to investigate the beneficial effects of carbohydrates on protein stability. Four representative carbohydrates (sucrose, fructose, mannitol, and trehalose) were evaluated at three different concentrations on etanercept, a fusion protein. Consequently, sucrose and trehalose increased the exothermic enthalpy while mixing together with protein along with different concentrations. The results were consistent with those of size-exclusion chromatography after accelerated storage. Nevertheless, non-covalent specific interactions between proteins and carbohydrates could not be detected. However, significant increases in exothermic enthalpy were observed due to the carbohydrates, indicating preferential exclusion of water molecules around etanercept moieties. This energy was maximal at the highest concentration of sucrose and trehalose (i.e., 250 mM). Thus, these carbohydrates consistently exhibited a beneficial effect on the aggregation and conformational stability of etanercept. Based on such findings, the stabilizing mechanism of carbohydrates is proposed herein.

1. Introduction

Proteins have marginal stability and are, consequently, susceptible to detrimental effects from even slight environmental changes [1]. Even small perturbations in the structures of therapeutic protein can lead to the loss of drug potency and may elicit an immune response *in vivo*, due to the formation of protein aggregates. Enhancing or maintaining the stability of therapeutic proteins has been one of the main challenges for formulation scientists [2,3]. Consequently, various strategies have been aimed at overcoming stability issues and producing commercially available drugs with appropriate formulations [4]. Among the different approaches investigated, adding excipients into the protein solution is a conventional method to maintain or enhance protein stability and stabilize the drug product. These excipients are primarily categorized into stabilizers or osmolytes (carbohydrates), surfactants, aggregation suppressors, antioxidants, and preservatives [5,6]. During drug formulation, certain compatibility tests are typically performed by adding combined excipients, which are selected based on critical quality attributes (CQA) of target proteins. Ultimately, stability tests involving physicochemical stresses that affect the protein stability should be confirmed with and without excipients, thereby achieving a preliminary protein formulation based on the target protein's CQA. Of the many physicochemical stresses that affect protein stability, the major ones are heat, shaking, and oxidation [7–9].

Optimizing the formulation requires understanding the broad spectrum of stresses affecting protein stability, access to relevant analytical equipment, and expertise in an extensive repertoire of biophysical analysis methods. Several previous studies have interpreted the thermodynamic stability on protein folding, using micro-differential scanning calorimetry (μDSC) [10–13]. Evidently, the stability of target proteins significantly varied with protein concentration, pH, buffer, buffer concentration, and the excipients. Recently, the generation of nitrogen oxide species (NO_x) by arginine and related compounds was shown to suppress protein aggregation but enhance fragmentation at high temperature [14]. The demonstration exhibited several functions of arginine related its chemical structure although it has guanidinium group which could act as a protein denaturant [15]. Of several excipients, sugars, polyols, and

* Corresponding author.
E-mail address: shjeong@dongguk.edu (S.H. Jeong).

https://doi.org/10.1016/j.ijbiomac.2017.12.089
0141-8130/

carbohydrates, act as promising stabilizing agents in protein formulations. Nevertheless, the mechanism of their stabilizing effect on proteins remains to be fully elucidated. Understanding the mechanisms of protein stabilization afforded by carbohydrates, will accelerate the design of appropriate biopharmaceutical formulations with enhanced storage stability either in the liquid or solid state [16–20].

Souillac et al. [21] demonstrated the secondary structure rearrangement of two therapeutic proteins during lyophilization, was inhibited by freeze-drying in the presence of carbohydrates (sucrose, mannitol, 69 and 503 kDa dextran, and trehalose, separately), as observed by using Fourier transform infrared spectroscopy. In this instance, it was proposed that the modifications to the secondary structure of the freeze-dried proteins could be attributed to a water replacement mechanism, i.e., the loss of hydrogen bonds during secondary drying (removal of absorbed/bound water) [21]. In the presence of carbohydrate, however, the secondary structure was replenished by hydrogen bonds formed between the polar groups on the surface of the protein and carbohydrate moieties [21].

Conversely, the theory of preferential exclusion is also a well-known hypothesis, attributing the mechanism of protein stabilization by carbohydrates, to the repulsive forces between the protein chain and carbohydrate molecules [6,22]. Supportive to this hypothesis is that protein-water interactions are more plausible than protein-excipient interactions [5]. Carbohydrates may act by altering the solvent arrangement rather than interacting directly with proteins, thereby stabilizing the protein structure, and the strength of this interaction is proportional to the surface area of the protein exposed to the solvent [17].

In this study, the mechanism by which carbohydrates confer stability to proteins was initially explored, using isothermal titration calorimetry (ITC) and micro-differential scanning calorimetry (μDSC). Supportively, ITC can be used to detect the interaction and binding affinity between a protein and its surroundings, whereas μDSC is used to investigate the conformational stability of proteins [23]. In addition, other biophysical methods, including circular dichroism (CD), size-exclusion chromatography (SEC), and dynamic light scattering (DLS) are also performed in this study, to evaluate the results simultaneously with the calorimetry data. Etanercept has been chosen as a model drug, and its stability was compared in the presence of four different carbohydrate excipients, namely sucrose, fructose, mannitol, and trehalose, all of which have different chemical structures.

2. Materials and methods

2.1. Materials and sample preparation

Etanercept is a fusion protein of two soluble human 75 kDa tumor necrosis factor-α receptors (TNFR) linked to the Fc portion of immunoglobulin G1 (IgG1). The commercial product of etanercept is Enbrel®, which was supplied by Seoul National University (Seoul, Korea). The molecular weight is 51234.9 g/mol with 934 amino acids. All the reagents used in the experiment were of analytical grade. Sodium phosphate monobasic dihydrate, sodium phosphate dibasic dihydrate, sucrose, and mannitol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fructose and trehalose were procured from Junsei Chemical (Tokyo, Japan) and Ferro Pfanstiehl Laboratories, Inc. (Waukegan, USA), respectively.

5 mg/mL of etanercept was dialyzed at 4 °C in 25 mM phosphate buffer at pH 7.4 for 24 h, with and without carbohydrates (0, 62.5, 125, and 250 mM, respectively). The Cellu Sep® H1 dialysis membrane (Membrane Filtration Products, Seguin, TX, USA) had a molecular weight cut-off of 5000 Da. The external dialysis medium (sink) was exchanged every 8 h, and the desired pH was confirmed after the dialysis. All the samples were degassed under vacuum before biophysical analyses.

2.2. Isothermal titration calorimetry (ITC)

A 350 μL aliquot of the dialyzed protein solution (devoid of carbohydrates) was placed in a Nano ITC instrument (TA Instruments, DE, USA) sample cell, and 50 μL carbohydrate solution was loaded into the injection syringe provided with the device. Incremental titration was performed, placing 20 injections of 2.5 μL aliquots of excipient into the sample cell. 200 s gap was maintained between two successive injections, to allow thermal equilibration. The sample cell was stirred at 300 rpm throughout the experiment. Before data acquisition, all samples were filtered through a 0.45 μm membrane (Hyundai Micro Co. Ltd, Korea) and were degassed with a vacuum degasser supplied with the apparatus. The raw data was processed using the Nano Analyze software provided with the instrument.

2.3. Differential scanning calorimetry (μDSC)

Microcal VP-DSC calorimeter (Northampton, MA, USA), with two tantalum cells for the sample and the reference (i.e., 0.5147 cm^3), respectively, was used to investigate the thermal stability of etanercept in the presence and absence of the carbohydrates. The samples, containing 2.5 mg/mL etanercept, were scanned from 15 to 120 °C at the heat increment rate of 75 °C/h. All samples and the buffer were filtered and degassed before each measurement. The baseline for each thermogram was obtained through buffer screening. The captured baseline was subtracted from the sample thermogram, to achieve the final thermogram of etanercept. The obtained thermogram compensation curves were evaluated using the Microcal LLC DSC plug-in for the Origin 7.0 software package, provided with the equipment. These results were fitted to a multistate model with three transitions, to calculate the transition melting temperature (T_m).

2.4. Far-UV circular dichroism (CD)

CD measurements were performed using the Chirascan-plus spectrometer (Applied Photophysics, Surrey, UK), equipped with a TC125 temperature controller (Quantum Northwest, Spokane, USA) and a temperature probe. The samples were measured after dilution by 20%, to avoid excessive absorbance values (> 2.0). All samples and buffers were degassed before each measurement. The samples were loaded into a quartz cell of 0.02 cm path length (Hellma Analytics, Müllheim, Germany). Before sample measurements, the buffer baselines were achieved at the scanning rate of 2 °C/min, while increasing the temperature from 20 to 90 °C at the far-UV wavelength region of 190–260 nm at a resolution of 1 nm. Sample spectra were also measured under the same conditions. Data were processed using a Pro-Data viewer and CDNN software provided with the equipment, to obtain a CD value and secondary structural content data.

2.5. Dynamic light scattering (DLS)

Particle size distribution of each sample was determined using Zetasizer Nano ZS90 (Malvern Instruments, Worcestershire, UK). 1 mL of each sample was loaded into a disposable sizing cuvette (Sarstedt, Nümbrecht, Germany) and placed in the sample holding chamber of the equipment. The samples were equilibrated to 15 °C for 120 s before measurement. After equilibration, each measurement was acquired continuously five times at 30 s intervals. Once the measurements were obtained, zeta-average size, particle size