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Ectoine and hydroxyectoine inhibit thermal-induced aggregation and increase thermostability of recombinant human interferon Alfa2b

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ABSTRACT

This study is to investigate whether ectoines (ectoine and hydroxyectoine) can reduce aggregation of rhIFNα2b in aqueous solutions on thermal stress. The effect of thermal stress condition on the stability was therefore investigated using size exclusion-high performance liquid chromatography (SE-HPLC), different spectroscopic measurements, dynamic light scattering (DLS), electrophoresis, and differential scanning calorimetry (DSC). All experiments were performed in a sodium phosphate buffer system (100 mM, pH 7). The protein samples (100 μg/ml) were incubated at 50 °C for 14 days in the absence or presence (1, 10, 20, and 100 mM) of ectoines. In summary, thermal-induced aggregation was reduced in the presence of ectoines, regardless of the ectoines concentration in different periods of incubation time by analyzing with SE-HPLC and turbidity measurement. The inhibitory effect of ectoines on the aggregation was shown by other techniques used. The optimal ectoines concentration was 10 mM for aggregation reduction, so samples containing of 10 mM of ectoines were selected for further evaluation. Secondary structural and conformational stability increased in presence of ectoines as measured by far-UV circular dichroism and fluorescence spectroscopy, respectively. DSC showed slight increase in T_m of interferon in the presence of ectoines. Hydroxyectoine had superior protein-stabilizing properties than ectoine. In conclusion, this study demonstrates that ectoine and hydroxyectoine are highly effective excipients which can significantly reduce the thermal-induced aggregation of rhIFNα2b at low concentration.

1. Introduction

Stabilization of protein molecules and achievement of an acceptable shelf-life are the most challenging obligations in the development of biopharmaceutical formulations. Protein aggregation, occurred in almost all steps of manufacturing process, is one of the most common and critical manifestations of protein instabilities and has significant influence on the efficacy and safety of a biological product. More importantly, protein aggregates may have immunogenicity or cause cellular toxicities.

Commercialized products of recombinant human interferon alpha (rhIFNα) are good examples in this regard. There are some reports that indicate the aggregation of IFNα products could subsequently cause their immunogenicity (Bonetti et al., 1994; Braun et al., 1997) and that the level of anti-rhIFNα2 antibodies was related to the presence of aggregates, associated with the loss of efficacy (Hochuli, 1997; Palleroni et al., 1997). Many factors have been identified and reported in the literature affecting the process and rate of the protein aggregation such as changes in pH, temperature, and ionic strength, presence of cosolvents, and other additives (Mahler et al., 2009; Wang et al., 2010). It has been found that IFNα2 subtype has a tendency to form multiple partially unfolded states whose conformations are sensitive to temperature. These partially unfolded states can play an important role in the aggregation and therefore long-term stability of IFNα2 in solution (Sharma and Kalonia, 2003).

Aggregation suppressing additives, including sugars, polyols, surfactants, salts, metal ions, and amino acids, have been used extensively to stabilize proteins (Fesinmeyer et al., 2009; Maity et al., 2009; Pavisic et al., 2010; Wei et al., 2007) and they still remain the most reasonable option for combating this problem. Due to the great diversity in protein sequence, structure and, consequently, routes of degradation, no single formulation strategy can be generally applied to all proteins and an appropriate formulation should be developed for each therapeutic protein. Hence, it is a continuing process to search for novel excipients to stabilize protein formulations.

Stabilization of proteins can be inspired by nature. Extremophilic microorganisms are good examples in this regard that have pursued a diversity of smart strategies for survival under the environmental extremes like high (or low) temperature, excessive pressure, and severe salt concentrations. A specific set of intracellular solutes is found in these extremophiles that can protect not only cells but also proteins

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and other labile molecules from the harmful effects of environmental stress (Lentzen and Schwarz, 2006). These compatible solutes from extremophiles are also termed extremolytes. Particularly, ectoines (ectoine and its hydroxy derivative, hydroxyectoine) as the first extremolytes that are produced in a large scale, have been studied extensively. Protection of cells, enzymes and DNA by ectoines against the effect of heat, freezing, freeze–thawing, drying, oxidative damage, proteolysis and desiccation has been reported (Ablinger et al., 2012; Andersson et al., 2000; Goller and Galinski, 1999; Kolp et al., 2006; Lentzen and Schwarz, 2006; Lippert and Galinkski, 1992; Malin and Lapidot, 1996; Van-Thuoc et al., 2013). While the market for these compatible solutes has appeared in areas such as food processing (Hinrichsen et al., 1994) or fermentation of microorganisms (Zhang et al., 2008), also ectoines can be used as potential substances with manifold benefits for skincare in the cosmetic market (Graf et al., 2008; Heinrich et al., 2007). Moreover these applications have promoted a growing interest in using these solutes as potential therapeutic agents for certain diseases (Abdel-Aziz et al., 2013; Kanapathipillai et al., 2005; Sydlik et al., 2009; Sydlik et al., 2013).

In the present study, the protective nature of ectoines for maintaining alpha interferon stability during heat-induced aggregation was assessed, which to the best of authors' knowledge has not been studied so far. Ectoine (Ect) and hydroxyectoine (EctOH) were chosen because they can be produced in an industrial scale with high quality standards (Sauer and Galinski, 1998), have no toxic side effects (Kanapathipillai et al., 2005), and have potential to be used as therapeutic or preventive agents stabilizing biopharmaceutical formulations.

2. Materials and methods

2.1. Materials

Recombinant human interferon alpha2b (rhIFNα2b) was kindly gifted by Pasteur Institute of Iran (Tehran, Iran). All chemicals used for electrophoresis experiments were purchased from Merck (Darmstadt, Germany). Ultrapure ectoine and hydroxyectoine were obtained from Biomol GmbH (Homburg, Germany). Sodium chloride, sodium dodecyl sulfate (SDS), di-sodium hydrogen phosphate anhydrous and sodium dihydrogen phosphate 1-hydrate were purchased from Merck (Darmstadt, Germany). RPMI 1640 medium, neonatal calf serum, penicillin, streptomycin, L-glutamine, and naphthalene black were purchased from Sigma (Germany). All solutions were prepared with sterile deionized water (Milli-Q; Millipore Corporation, USA). The buffers were filtered through 0.2 μm membranes prior to use.

2.2. Methods

2.2.1. Sample preparation

To evaluate the protein stability at elevated temperatures, samples of rhIFNα2b were diluted to 100 μg/ml in 100 mM sodium phosphate buffer at pH 7.0. The solutions were incubated (IFE 500 incubator, Memmert, Germany) at 50 °C for 14 days. In order to investigate the exteremolytes efficacy, some samples were co-incubated with 1, 10, 20 or 100 mM of Ect or EctOH.

2.2.2. Size Exclusion-High Performance Liquid Chromatography (SE-HPLC) analysis

A Knauer High Performance Liquid Chromatograph (Berlin, Germany), equipped with an isocratic pump 1000, a degasser manager 5000, a UV detector 2500 (set at 214 nm), a TSK gel G3000 SW XL column (7.8 mm ID × 300 mm, 5-μm particles and 250 Å pore size; Tosoh Bioscience Corporation, Tokyo, Japan) and a guard column, was used for SE-HPLC analysis by the method previously reported by Diress et al. (2010). The samples were eluted using a mobile phase consisting of 100 mM sodium phosphate (pH 7.0), 150 mM NaCl, and 0.05% (w/v) SDS at 25 °C under isocratic elution of 0.5 ml/min. Percentages of recovered fractions were calculated based on SE-HPLC peak areas relative to the total peak area in SE-HPLC of non-incubated rhIFNα2b.

2.2.3. Turbidimetric analysis

Turbidity measurements were carried out at 350 nm with a ScanDrop® 250 UV–VIS spectrophotometer (Analytik Jena AG, Jena, Germany) using a 10 mm path length quartz cell. Phosphate buffer (100 mM) was used as a blank.

2.2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

All experiments were performed using a separating gel containing 12.5% (w/v) acrylamide and 0.1% SDS, and a stacking gel containing 3% (w/v) acrylamide and 0.1% SDS in the Omni PAGE mini vertical gel electrophoresis unit (Cleaver Scientific, Warwickshire, UK)·Gels were run under non-reducing condition (sample buffer without β-mercaptoethanol) at 200 V at room temperature. Protein bands were visualized by coomassie staining.

2.2.5. Dynamic light scattering (DLS)

A Malvern Zetasizer Nano ZS (Worcestershire, UK) laser light scattering system, equipped with a Nano ZS® software for data acquisition and analysis, was used for particle size measurements.

2.2.6. Far-UV circular dichroism (far-UV CD)

CD measurements were carried out using an AVIV 215 Spectropolarimeter (Aviv Biomedical, NJ, USA). The far-UV CD studies were performed on protein samples using a 1 mm path length quartz cell with a scan speed of 20 nm/min from 185 to 260 nm. The spectra were an average of three measurements and the buffer spectrum was subtracted from the protein spectra. All scans were normalized for the concentration and number of amino acid residues in order to convert the obtained ellipticities into molar ellipticities. The percentage of secondary structure was obtained using the CDNN CD Spectra Deconvolution Software (Version 2.1).

2.2.7. Intrinsic fluorescence

Fluorescence scannings of the protein solutions (100 μg/ml) were carried out using a CARY Eclipse Fluorescence Spectrophotometer (Varian, Australia) in 10 mm path length quartz cell at 25 °C. The fixed excitation wavelength was 295 nm and fluorescence emissions were collected within the wavelength range of 300–450 nm. The excitation and emission slit widths were 5 nm. The spectra were an average of three measurements and the buffer spectrum was subtracted from the protein spectra.

2.2.8. Differential scanning calorimetry (DSC)

A CSC 6100 Nano DSC II Calorimeter (Calorimetry Sciences Corporation, USA) equipped with platinum capillary cells (0.3 ml) was used for DSC analysis. Thermograms were obtained from 35 to 90 °C at a scan rate of 2 °C/min. All solutions were degassed prior to DSC runs. The buffer background was subtracted from each sample scan and each thermogram was an average of three scans. T_m, the midpoint transition temperature at which folded and unfolded molecules are equally populated, was then determined using CpCalc™ (Calorimetry Sciences Corp.) software.

2.2.9. Biological activity

IFN antiviral activity was assayed by the inhibition of the cytopathic effect (CPE) produced by encephalomyocarditis virus (EMCV) on Hep-2 cells (ATCC No. CCL23), as reported in European pharmacopoeia. Cell monolayers in 96-well microtiter plates were incubated for 24 h at 37 °C, under 5% CO_2 and 95% humidity, with IFN samples (diluted in RPMI 1640 medium with 10% neonatal calf serum, 10,000 IU/ml penicillin, 10 ng/ml streptomycin, and L-glutamine 200 mM). Virus (3×10^7 PFU/ml) was then added to each well and incubation proceeded under the same conditions until CPE (90% cell lysis) was evident (approx.