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Original Research Article

The Effect of Arginine as an Anti-Aggregation Excipient on Recombinant Human Growth Hormone

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HIGHLIGHTS

- Aggregation is one of the main physical instabilities of proteins.
- Protein aggregation may result in a compromise of safety and efficacy of biopharmaceutical products.
- Arginine at the concentration of 320 mM reduced rhGH thermal/mechanical-induced aggregation.
- Arginine, in contrast to glycine, optimally decreased the formation of insoluble aggregates.

ABSTRACT

Aggregation is one of the main physical instabilities of proteins, which might occur during all steps of the manufacturing and storage of products. The presence of protein aggregates may result in the reduction of activity, induce immunologic responses and failure of therapeutic efficiency. Therefore, using additives in drug formulations is one of the essential approaches to prevent protein aggregation. The main objective of this study was to evaluate the inhibitory influence of arginine or glycine as excipients on the aggregation behavior of recombinant human growth hormone (rhGH). Two types of mechanical and thermal stresses including freeze-thaw and vortex-agitation were applied to the 1 mg/mL protein solution in PBS buffer (25 mM, pH = 7) in the presence and absence of arginine and glycine. The influence of arginine or glycine at the concentration of 320 mM on reduction of rhGH thermal/mechanical-induced aggregation was evaluated using SE-HPLC and turbidity measurement.The results of this study revealed that the monomer concentration decreased linearly; and therefore, aggregate formation was intensified with the increase in the number of freeze-thaw cycles. Moreover, it was found that a significant amount of rhGH (> 80%) was rapidly adsorbed at the walls of the vessels or converted to insoluble aggregates. Arginine decreased the insoluble aggregate formed during the freeze-thaw cycling more effectively than glycine. In addition, following the vortex-agitation stress, arginine had the optimum preventive effect in aggregate formation in contrast to glycine, which increased the formation of insoluble aggregates. The findings revealed that arginine may be a potential additive in preserving rhGH against thermal/mechanical-induced aggregation.

Introduction

Human growth hormone (hGH) is a single-chain polypeptide and one of the most vital hormones with a wide variety of physiological functions including cell proliferation and metabolism (Bidlingmaier and Strasburger 2010; Mulinacci et al.,2011; Kim et al.,2013).

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Pituitary-derived GH was first used in 1950s and since 1985, the recombinant form of human growth hormone (rhGH) has been administered to treat growth hormone deficiency, especially in children (Cazares-Delgadillo et al., 2011). Nowadays, pharmaceutical products of rhGH are one of the most economical drugs with a wide market. The global market for rhGH seems to rise in value from $1.26 billion in 2014 to reach approximately $1.88 billion by 2024 (Gohil, 2015). rhGH, as a 191 amino acids polypeptide, can be affected by physical or chemical instability mechanisms. Instability conditions in manufacturing and storage process may cause degradation of products of protein with reduced biological activity or augmented detrimental toxicity. One of the byproducts of protein instability is the aggregate form. Aggregation of rhGH could elevate the immunogenicity of its pharmaceutical product (Moore and Leppert, 1980; Fradkin et al., 2009).

Aggregation of therapeutic proteins may result in dimer or higher- order aggregates as a consequence of stresses in production or storage conditions such as thermal, agitation or chemical stresses including inappropriate pH, ionic strength, counter-ion composition and addition of some excipients such as certain antimicrobial preservatives (Maggio, 2010). Protein aggregation may result in a compromise of safety and efficacy of biopharmaceutical products (Den Engelsman et al., 2011).

Various analytical techniques have been developed to characterize the protein samples with aggregation probability such as static and dynamic light scattering, size exclusion chromatography, turbidity measurement with spectrophotometry, microscopic analysis, fluorescent spectrophotometry, circular dichroism spectroscopy, polyacrylamide gel electrophoresis (PAGE) and analytical ultracentrifugation (Maggio, 2010; Den Engelsman et al., 2011). Protein aggregates are commonly classified according to their size, reversibility, secondary/tertiary structure, covalent modification and morphology. In this respect, large and irreversible aggregates with covalent modifications will result in adverse changes of active conformation with more probability than reversible forms, and therefore, can compromise protein activity. Aggregate formation might stimulate immune response to the therapeutic protein product or cause adverse effects (Fradkin et al., 2009; Narhi et al., 2012). L-Arginine is a chemical additive, which has been applied in the refolding process of various proteins with different physicochemical characteristics (Bajorunaite et al., 2007). L-Arginine can act as an aggregation suppressor in protein purification process (Arakawa et al., 2007).

In this study, the protective potential of arginine for rhGH stability against aggregation events was examined. Two types of accelerated stability studies of freeze-thaw cycling and agitation stress were applied to one of the marketed rhGH formulations from LG Life Company. Aggregate formation was measured by turbidity analysis and size exclusion high performance liquid chromatography (SE-HPLC).

Materials and Methods

Materials

Recombinant human growth hormone was provided from Eutropin® (LG Life, Korea). Arginine and glycine were supplied form Merck-chemicals (Germany). Vivaspin® Turbo 4 ultrafiltration membrane was purchased from Sartorius (Germany). All other chemicals were supplied form Merck-chemicals (Germany).

Sample Preparation

First, the lyophilized powder of rhGH was reconstituted and then purified by ultrafiltration membrane, and a 2 mg/mL stock solution of rhGH in 25 mM sodium phosphate buffer (pH =7) was prepared. To obtain a 1 mg/mL rhGH containing a certain excipient, the stock solution was then mixed with PBS or buffered solutions of whether 640 mM arginine or glycine in a 1:1 ratio.

Freeze-Thaw Stress

400 µL of sample or control solution containing 1 mg/ml rhGH in PBS was transferred into a 4-mL glass vial and capped. Nine vials were entered into the freeze-thaw study. Each cycle of freeze-thaw included 20 min in ethanol bath at -85º C followed by 15 min in water bath at room temperature (23-27º C). Three vials in each step were analyzed after second, eighth and sixteenth cycles of freeze-thaw. Three control vials were kept refrigerated during the study. Then vials containing rhGH plus 320 mM arginine or glycine underwent 16 cycles of freeze-thaw (3 copies of each). All samples were analyzed for soluble and insoluble aggregates by turbidometery and Size Exclusion-High Performance Liquid Chromatography (SE-HPLC).

Agitation Stress

Vortexing stress was carried out in glass test tubes (9 cm in height, 1.5 cm in diameter) filled by 500 µL of sample on a MS3 vortex (IKA, Germany). The vortexing rate was adjusted on 3000 rpm and the duration of the test was 7, 20, 60 and 120 seconds (3 copies) for different samples. Three vials were kept at 4 º C as controls. Then, vials containing 320 mM arginine were vortexed at 3000 rpm for 7 seconds. Analysis was performed using turbidimetry or SE-HPLC.

Turbidity Measurements

The protein samples containing 1 mg/ml rhGH in

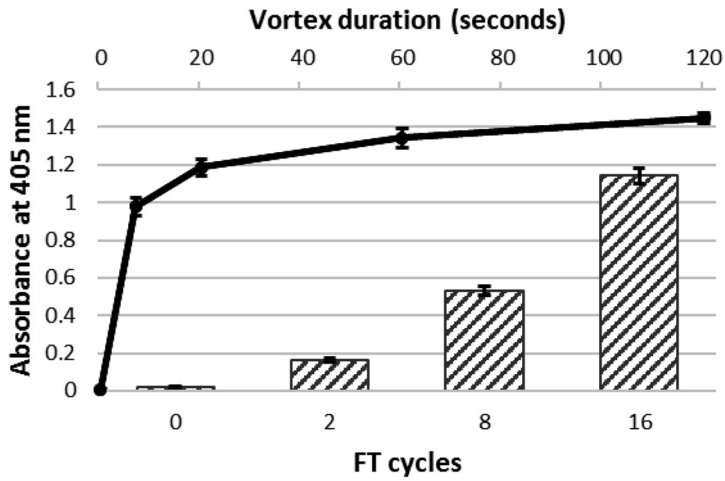

Figure 1. The Relationship between the Increase in the Number of FT Cycles on the Turbidity of rhGH Solution (boxes with oblique shades) and the Relationship between Vortex Duration and Turbidity of rhGH Solution (black line). Turbidity was determined by measuring the solution absorbance at 405 nm (n = 3, mean ± SEM).

25 mM phosphate buffer pH 7 were analyzed for aggregate formation in the absence and presence of 320 mM L-arginine monohydrochloride. Turbidity was measured by sample absorbance at 405 nm after each stress condition. A sample of 0.15 mL was run for each measurement using UV/visible nano-spectrophotometer of ScanDrop 250 (Analytik Jena AG, Germany).

Size Exclusion-High Performance Liquid Chromatography (SE-HPLC)

Alteration in the profile of monomers, dimers and soluble aggregates were analyzed by a set of TSKgel G3000PWXL column and guard column (Tosoh Bioscience Co., Japan) using a LC-10AD VP liquid chromatograph (Shimadzu co., Japan) at room temperature. A mobile phase of 63 mM PBS containing 3% propan-2-ol and a flow of 0.4 mL/min were considered. Furthermore, 20 µL of sample centrifuged at 7500 rpm was injected into the system and tracked by a UV-detector at 214 nm.

Results and Discussion

Effect of Freeze-Thaw Cycling on rhGH Aggregation

In Freze-Thaw (FT) study of rhGH samples, increase in the number of cycles resulted in a linear elevation (r2 = 0.9974) of absorbance at 405 nm as a marker of insoluble aggregates from 0.0231 (± 0.0062) in control samples to 1.1414 (± 0.0742) in 16-cyc samples (Fig. 1). No lag-phase was observed and the test showed repeatable results. The turbidity difference of 0, 2, 8 and 16 cycles were strongly significant ($p<0.05$). Chromatographic data revealed a steady linear significant reduction in monomer recovery percentage from 100.0 (± 1.2) to 83.1 (± 3.2) in 16-cycle FT samples (Fig. 2). No noteworthy changes were observed in dimers, but the fraction of soluble aggregates to the total protein decreased four folds permanently on early cycles of FT (data not shown).

During freeze-thaw cycling, adsorption of rhGH monomers on glass-liquid, ice or salt crystals-liquid and other interfaces initiated aggregation procedure. Adsorption occurred steadily with a constant rate and introduction of sticky hydrophobic patches on rhGH surface and promoted self-assembly of monomers on the interfaces, resulting in formation of aggregates. Subsequently, aggregated proteins detached from the surface and returned to the liquid phase, making the solution turbid. Moreover, pH alterations during freezing might change the microenvironment of rhGH monomer (Philo and Arakawa 2009). In phosphate buffers, these changes occur toward acidic condition (Bhatnagar et al., 2007). Since pI of rhGH is around 5 (Wilhelmsen et al., 2010), freeze-thaw cycling may reduce repulsive forces and mediate protein aggregation.

Effect of Vortexing on rhGH Aggregation

Vortexing rhGH solution at 3000 rpm caused a drastically rapid turbidity formation, particularly within the first 20 seconds. After 7 and 120 seconds of vortexing, absorbance raised to 0.9778 and 1.4474, respectively (Fig. 1). SE-HPLC results revealed a huge loss of monomers in initial seconds followed by a plateau phase. Seven seconds of vortexing resulted in 53.6% reduction in monomer

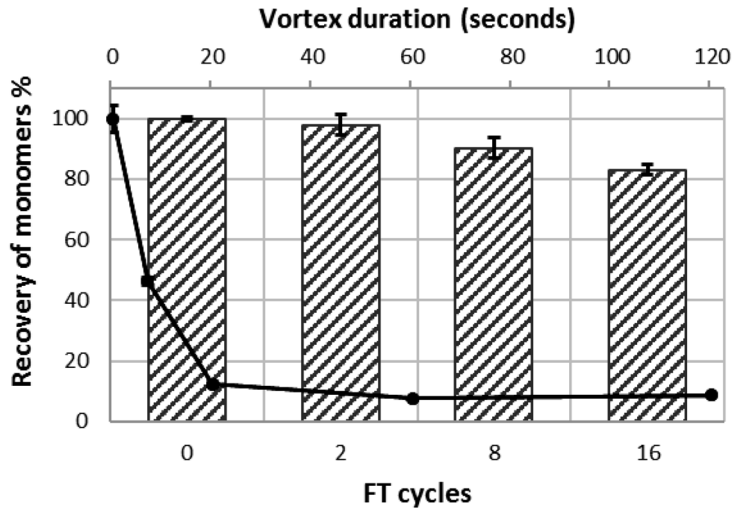

Figure 2. Percent of Recovered Monomers from rhGH Solution after 0, 2, 8, and 16 Cycles of FT (boxes with oblique shades) and Percent of Recovered Monomers after 0, 7, 20, 60, and 120 Seconds of Vortexing (black line). Monomer content were analyzed using SE-HPLC, and recovery fraction were calculated by dividing AUC of monomers in the stressed sample to AUC of monomers in the control sample (n = 3, mean ± SEM).

recovery (Fig. 2). Dimeric content underwent 3.3 to 4.6-fold increase from 7 to 120 seconds, and soluble aggregates experienced a decrease from 0.4% in the control sample primarily and then grew to 1.0% in 120-second samples.

Vortexing is associated with high interfacial shear stresses; however, other mechanisms are also involved in agitation-induced aggregation of proteins. Agitation is accompanied with cavitation, shock waves, highly turbulent flow conditions, extreme pressures and temperature in the liquid phase, which may result in the generation of hydroxyl and hydrogen radicals and thus leading to the formation of protein aggregates (Mahler et al., 2009). Our experiment revealed that aside from insoluble aggregate formation, a substantial amount of rhGH absorbed on glass surface during vortexing, which emphasized the role of shear stress on monomer loss.

Effect of Arginine and Glycine on rhGH Aggregate Formation during Freeze-Thaw Cycling

Turbidity measurement at 405 nm revealed that both arginine and glycine were efficient in lowering the amount

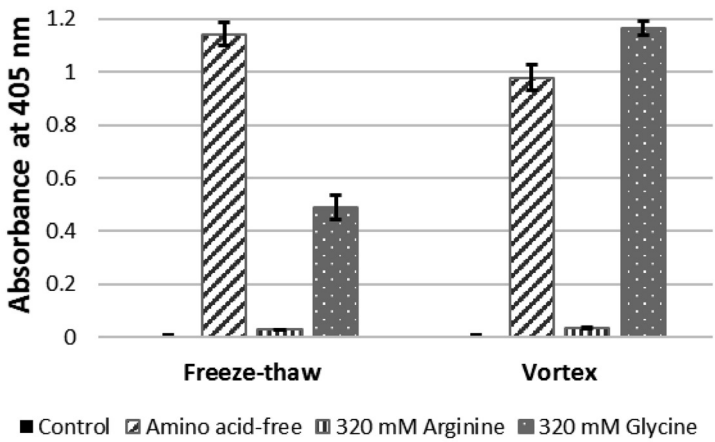

Figure 3. The Effect of Arginine and Glycine on Formation of Insoluble Aggregates in rhGH Solution during FT or Vortex Stress. Not Stressed Control Samples (black boxes), Stressed Amino Acid-free Samples (boxes with oblique shades), Samples Containing 320 mM Arginine (vertically shaded boxes) and Samples Containing 320 mM Glycine (spotted gray boxes) (n = 3, mean ± SEM).

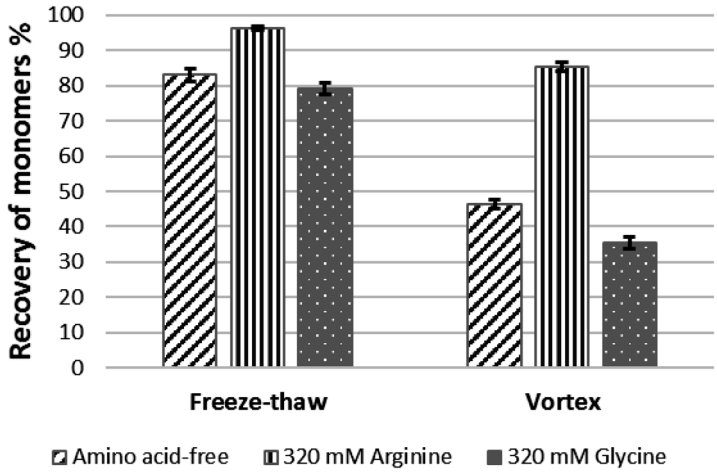

Figure 4. The Effect of Arginine and Glycine on the Percent of Recovery of Monomers in rhGH Solution during FT or Vortex Stress. Stressed Amino Acid-free Samples (boxes with oblique shades), Samples Containing 320 mM arginine (vertically shaded boxes) and Samples Containing 320 mM Glycine (spotted gray boxes) (n = 3, mean ± SEM).

of insoluble aggregate formation. While glycine could decrease absorbance to 0.4898 (± 0.0792) compared to 1.1414 (± 0.0742) in amino acid-free samples, arginine strongly inhibited insoluble aggregation by an absorbance of 0.0295 (± 0.0004), which was statistically similar to control samples kept refrigerated (Fig. 3).

Sample analysis by SE-HPLC confirmed the positive influence of arginine on monomer content of the rhGH solution where it saved 96.3% (± 0.9) of monomers versus 83.1% (± 3.2) monomers recovered from amino acid-free samples ($p = 0.005$). On the opposite side, glycine showed poor effect in monomer salvage. Recovery of monomers in samples containing 320 mM glycine was almost 4% less than amino acid-free samples, which was statistically insignificant ($p = 0.26$) (Fig. 4).

One proposed mechanism of protein stabilization by amino acids is preferential exclusion. Some solutes increase surface tension of water, which is unfavorable in protein-aqueous phase interface. In addition, repulsive forces between functional groups of the solute and the protein chain might push solute molecules away from protein surface. In this method, a hydrated shell encompasses the protein and isolates it in a compact conformation, forcing hydrophobic parts to be buried in the core of the protein and preventing protein from denaturation, unfolding or aggregation (Arakawa and Timasheff 1983, Arakawa and Timasheff 1985). However, arginine seems to share a unique mechanism of action beyond preferential exclusion. The side chain of arginine resembles guanidine hydrochloride –a known denaturing agent- which is able to interact with all amino acid residues of protein chain. Nevertheless, arginine represents higher volume of exclusion and surface tension, and therefore, it is unable to denature proteins and the sum of these opposite forces endows a special ability to arginine.

Effect of Arginine and Glycine on rhGH Aggregate Formation during Vortexing

Arginine performance in vortexing stress was promising. While 3000 rpm vortexing for 7 seconds severely produced insoluble particles in rhGH solution, adding 320 mM arginine could completely suppress aggregation with an absorbance of 0.0344 (± 0.0027). Glycine, recording the absorbance of 1.1648 (± 0.0465) at 405 nm, conversely deteriorated particle formation, but insignificantly ($p = 0.051$) (Fig. 3).

SE-HPLC data ascertained the positive role of arginine in rhGH stabilization against agitation stress. Addition of arginine to rhGH solution unexpectedly improved monomer recovery whereas glycine might have destabilized the protein monomers. Monomer recovery percentage for arginine, glycine and amino acid-free samples were 85/3% (± 2.1), 35/5% (± 2.8) and 46/4% (± 2.1), respectively (all differences were significant) (Fig. 4).

It is thermodynamically favorable that arginine binds to protein residues. Since proteins offer a larger surface in monomeric form rather than aggregated or adsorbed form, the equation shifts toward monomeric units which is accompanied by lower level of Gibbs free energy (Tsumoto et al., 2005; Arakawa et al., 2007). Overall, presence of arginine in rhGH solution protected this protein against aggregation as well as adsorption on the glass surface during both freeze-thaw test and high shear vortex-agitation.

On the other hand, protein destabilization by

osmolytes –like what was observed in glycine case- was reported in some experiments, but it is mechanistically unknown. However, we are aware that the preferential binding of the osmolyte with the protein that originates from a favorable interaction between the osmolyte and protein side chains, favors denatured state; and denaturing osmolytes accumulate or bind at the surface and promote unfolding (Singh et al., 2011).

According to the results, arginine did not show a denaturing effect on rhGH, but it unexpectedly improved protein stability and monomer recovery.

Conclusion

In this study, the effect of arginine and glycine on the aggregation of recombinant human growth hormone was examined during freeze-thaw and agitation stresses. Arginine, through its unique chemical structure, successfully overcame the harsh accelerated studies and stabilized rhGH to a great extent, whereas glycine –the typical amino acid based excipient- appeared to be destabilizing rather than stabilizing. According to the literature review, this was the first report on the positive effect of arginine (or any other amino acids) on the stabilization of proteins during agitation stress. Nevertheless, further studies are necessary to confirm this effect.

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Competing Interests

The authors declare no competing interest.

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