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Original Research Article

Evaluation of physicochemical and stability properties of human growth hormone upon enzymatic PEGylation

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ABSTRACT

Background: PEGylation is an increasingly important strategy for improving stability, pharmacokinetic and pharmacodynamic properties of proteins.
Objective: In this study, site-specific PEGylated human growth hormone (hGH) was prepared by microbial transglutaminase and physicochemical and stability properties of bioconjugates were tested.
Method: hGH was PEGylated by 20 kDa mPEG-NH_2. PEG-hGH was purified by size exclusion chromatography method and analyzed by SDS-PAGE, IEF gel and CD spectroscopy methods. Physicochemical properties, size and zeta potentials, of native and PEGylated hGH were evaluated by Dynamic Light Scattering (DLS) method. Physical and chemical stabilities were assayed at different temperatures (37, 25 and 4 °C) within three weeks by SEC- and RP-HPLC methods, respectively.
Results: By this preparation method, mono-PEG-hGH was obtained and the secondary structure was unchanged. The DLS results indicated that by PEGylation the size and zeta potentials of the protein were increased and decreased, respectively. These data indicated that PEG chain covered the protein surface. The isoelectric point (pI) of protein was not altered following PEGylation. The results of stability indicated that, the mono-PEG-hGH was considerably more stable especially in elevated temperatures as compared with the non-PEGylated one.
Conclusion: PEGylation changed the physicochemical properties of hGH and also enhanced the stability of the protein.

Introduction

Application of biopharmaceuticals, including peptides and proteins, for the treatment of different diseases is expanding rapidly. The main advantages of these drugs include enhanced specificity and activity rather than chemical drugs. However, the efficacy of the biopharmaceuticals has been influenced by some major limitations. These restrictions include low physical and chemical stabilities, and short in vivo half-time. To overcome these limitations, many strategies have been suggested such as applying novel drug delivery systems, microencapsulation and post translational modifications (PTM) (Stigsnaes et al., 2007; Payne et al., 2011; Gupta and Sharma, 2009; Khodaverdi et al., 2015). Among all of these methods, PTMs attract more attentions and extensively have been used to improve product performance. Covalent attachment of poly(ethylene) glycol (PEG) groups to proteins which commonly called PEGylation, is the most used

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approach in the field of PTMs and causes the enhancement of the protein performance (Payne et al., 2011).

The traditional approaches for PEGylation involve harsh reaction conditions and consequently provide a heterogeneous product (PEG attached randomly to different sites) along with the formation of positional isomers. Due to product heterogeneity, the PEGylated biopharmaceuticals face with challenges for FDA approval. Therefore, there is an urgent need to develop a strategy generating a homogeneous PEGylated protein drug. Microbial transglutaminase (MTGase) mediated site-specific PEGylation of proteins is recently used to site directed PEGylation (Mero et al., 2009; Mero et al., 2011; Zhao et al., 2010). By this method, alkylamine derivatives of PEG molecules can be site-specifically incorporated into glutamine residues of protein (Sato, 2002).

As mentioned above, protein instability is one of drawbacks of biopharmaceuticals and thus, stabilization of protein drugs is necessary for effective treatments. Protein instabilities are mainly divided into physical and chemical instabilities. Chemical instabilities involve processes which make or break covalent bonds and consequently generate new chemical molecules. In the cases of physical instability, the chemical composition is not altered; however, the physical structure of the protein does change. Strategies to stabilize proteins can be classified into intrinsic methods (site-directed mutagenesis and chemical modification), physical methods (e.g. lyophilization) and use of chemical additives (Manning et al., 2010; Li et al., 1995). It has previously been shown that PEGylation can enhance the protein stability (Basu et al., 2006; Treetharnmathurot et al., 2008; Grigoletto et al., 2015).

The role of PEGylation of human Growth hormone (hGH) for enhancing the protein performance was described before (Grigoletto et al., 2015; Da Silva Freitas et al., 2013; Webster et al., 2008). Additionally, in previous study, we demonstrated the effect of enzymatic PEGylation on hGH properties. The PEG-hGH showed agonist activity and also this procedure helped to maintain the proper biophysical properties of protein (Khameneh et al., 2015). Due to the desirable observed properties of PEG-hGH, evaluating other features such as physicochemical included size, zeta potential and pI of the bioconjugate was aimed.

The goal of this study is to evaluate the physicochemical and stability properties of PEG-hGH and compare them with those of native protein.

Material and methods

Materials

Recombinant hGH was purchased from Hospira, Adelaide PtyLtd., Australia. Microbial transglutaminase (MTGase, Activa WM) from *Streptomyces mobaraensis* was purchased from Ajinomoto (Hamburg, Germany). Amino terminating linear 20 kDa monomethoxy-poly (ethylene glycol) (PEG-NH_2,20 kDa) was purchased from NanoCS (USA). Reagents for sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) were provided by Bio-Rad Laboratories Inc. (Hercules, CA, USA). All other chemical reagents were HPLC grade and provided from Sigma Aldrich-Fluka (Sigma, Germany).

Methods

MTGase-mediated PEGylation of hGH

Firstly, mPEG–NH_2 (20 kDa) was dissolved at 40-fold molar excess to an hGH solution (1.0 mg/mL) in phosphate buffer (pH = 7.4) and MTGase was added at E/S ratio of 1:50 (w/w). The mixtures were left to stir at room temperature for 48 h and then the protein was purified by semi-preparative SEC/FPLC method. Superdex 200 10/300 GL column (Amersham) and PBS (pH 6.8) as the running buffer were used in this method. The UV detector was set at 280 nm and the corresponding peak to the conjugate PEG-hGH was collected. The solution was then concentrated under vacuum.

SDS-PAGE analysis

SDS-PAGE analysis was performed using a polyacrylamide gel (4% stacking gel, 12% running gel) prepared according to Laemmli, 1970. Each sample was diluted 1:1 (v/v) with Laemmli buffer (62.5 mM Tris–HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue) and heated for 5 min at 95 °C before loading on the gel. Ten microliter of each sample, corresponding to 3 μg of bioconjugate, was loaded in the gel electrophoresis well. The proteins bands were detected by Coomassie Brilliant Blue R 250 staining followed by 10% acetic acid–15% ethanol treatment.

Evaluation of PEG-hGH properties

The structural properties of PEG-hGH was analyzed by far-UV CD method and compared with native protein. Molecular radius and zeta potential of PEG-hGH and hGH were measured by dynamic light scattering (DLS) on a Malvern Zetasizer NS (Malvern Instruments Ltd., Worcestershire, United Kingdom) at 25 °C. The samples were centrifuged at 12,000 × *g* for 10 min before the analysis.

Isoelectric focusing gel electrophoresis

Isoelectric focusing (IEF) gel electrophoresis was performed in the pH range 3–10 using pre-cast gels and a Mini Protean III apparatus (Biorad). Each samples (hGH and PEG-hGH) at concentration of 1 mg/mL in 10 mM sodium acetate buffer, pH 4.5, were diluted 9:1 (v/v) with 50% w/w glycerol solution. A 20 mM lysine, 20 mM arginine solution was used as cathode buffer while the anode buffer was a 7 mM phosphoric acid solution. The gel was run for 1 h at 100 V, 1 h at 250 V and 30 min at 500 V. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R 250 and then treated with a water solution of 10% v/v acetic acid and 15% v/v methanol.

Stability studies

Stability studies were carried out based on the previous study (Mohammadpanah et al., 2013). Briefly, the samples (hGH and PEG-hGH) were dissolved in L-histidin buffer (10 mM and pH = 6.25) to achieve a concentration of 0.5 mg/mL and placed in three different temperatures: 37 ± 0.1 °C incubator, room temperature (25 ± 3 °C) and refrigerator (4 ± 2 °C). Then the samples were analyzed every week by size exclusion chromatography (SEC-HPLC) and reverse phasechromatography (RP-HPLC) for evaluating their physical and chemical instabilities, respectively.

SEC-HPLC analysis method is responsible for the amount of monomeric, dimeric and related substances of higher molecular masses including oligomer and polymer. A set of HEWLETT PACKARD HPLC (1100 SERIES) with UV detector and Alltech (Macrospher GPC 100 A, ID 7.5 mm, 300 × 7 mm, Biosep- SEC-S 2000, USA) column were used for SEC-HPLC analysis. The mobile phase was 0.063 M phosphate buffer (pH 7.0) containing 3% 2-propanol. Injection volume was 20 μl. The flow rate was 0.6 mL/min and the effluent was monitored at 214 nm.

Oxidation and deamidation are the major chemical degradation pathways for protein pharmaceuticals. Oxidized and deamidated forms (related proteins) in each samples were assessed every week by RP- HPLC. A set of HEWLETT PACKARD HPLC (1100 SERIES) with UV detector and ALTECH (Prospher 300A, 250 × 4.6 mm)

C4 column were used. Mobile phase was 1- propanol (29%), 0.05 M tris-hydrochloride buffer solution pH 7.5 (71%) and the flow rate were 0.5 mL/min. The injection volume was 20 μL, and detection was conducted at 220 nm wave length.

Statistical analysis

All tests were performed at least in triplicate. A one-way analysis of variance (ANOVA) was used for between-group comparisons, and a post-hoc analysis was applied when appropriate. Statistical evaluation was performed at the significance level 2 alpha = 0.05.

Results and discussion

In previous study the proper biological activity of PEG-hGH prepared by enzymatic procedure was demonstrated (Khameneh et al., 2015). Consequently, to find out the impact of PEGylation on physicochemical properties and stability profile of hGH, the present study was designed.

At first, PEG-hGH was prepared, purified and analyzed by SDS-PAGE method. There was no unreacted hGH in the purified protein fraction. These results were accordance with previous published data and showed that this semi-preparative method was effective (Khameneh et al., 2015).

The similarity between CD spectra of the mono-PEG-hGH and hGH was demonstrated that the PEGylation and purification processes were not destructive and the secondary structure of the protein was preserved. These results were in accordance with the literature presenting that the secondary structure of protein did not influenced by PEGylation (Da Silva Freitas et al., 2013).

The physicochemical properties of PEG-hGH and hGH were characterized by DLS method and compared with each other. This technique shows good promise approach for measuring protein particulate size distributions (Amin et al., 2014). The hydrodynamic radii (R_H) of the both protein and PEG-protein were illustrated in Fig. 1. The results showed that the size of protein increased up to 10-fold and reached approximately 10.5 nm by polymer conjugation. These data were accordance with published data which showed that polymer conjugation to protein led to increase the protein diameter (Liebner et al., 2015; Cohan et al., 2011). The R_H is a fundamental measure of molecular size and has been effectively employed to study the glomerular filtration of proteins. The increasing size of protein upon PEGylation can affect the pharmacokinetic profiles of bioconjugate. It was shown that the reducing glomerular filtration and consequently prolonging the half-life of a bioconjugate are related to increasing its R_H (Gokarn et al., 2012).

The zeta potentials of mono-PEG-hGH and hGH were −2.2 and −19.6 mV, respectively. These data revealed that the surface of the protein was covered and zeta potential of protein changed by PEGylation. Changing the zeta potential of hGH by complexing with protamine were previously described (Ablinger et al., 2012). Changing the zeta potential of protein upon PEGylation was previously described (Cohan et al., 2011). In that study, zeta potential results revealed that an increase in negative charge of protein by attachment of the PEG polymer observed (Cohan et al., 2011). The differences between the effects of PEGylation on bioconjugate charge might be due to the PEG size, PEGylation site and the number of copies of PEG chains conjugated. In our study, the glutamine residue was PEGylated, while in that study; the cysteine residue was modified by polymer. Overall, these observations indicate the effect of PEGylation on important physicochemical properties of protein.

The pI of PEGylated protein was also evaluated and compared with that of native form. The data showed that this value was not altered following PEGylation. Unchanging the pI value of protein by PEGylation was previously described (Scaramuzza et al., 2012). These results were illustrated in Fig. 2.

The stability of biotherapeutics in solutions is of crucial importance due to the fact that protein stability can affect the protein function (Ciorba et al. 1997). Various factors affect the protein stability in solutions such as pH, storage temperatures and also solution buffers (Manning et al. 2010). In the case of hGH, the effect of different solution buffers on hGH stability was studied and the data showed that the histidine buffer (10 mM and pH = 6.25) provides maximum stability (Mohammadpanah et al., 2013). Consequently, proteins in both native and PEGylated forms were dissolved in the same buffer and their physical and chemical stabilities were investigated to find out the role of PEGylation in protein stabilization. To this end, proteins were kept in three different temperatures and analyzed by SEC-HPLC and RP-HPLC methods every week.

The SEC-HPLC results exhibited the physical stability of proteins and forming the higher molecular weight of proteins such as aggregated or dimer forms. The degradation percentages of protein monomer at different times and temperatures were illustrated in Fig. 3. As illustrated in Fig. 3.A, the attachment of PEG to hGH has slowed the kinetics of monomer degradation during the storage time, especially in elevated temperatures and the degradation

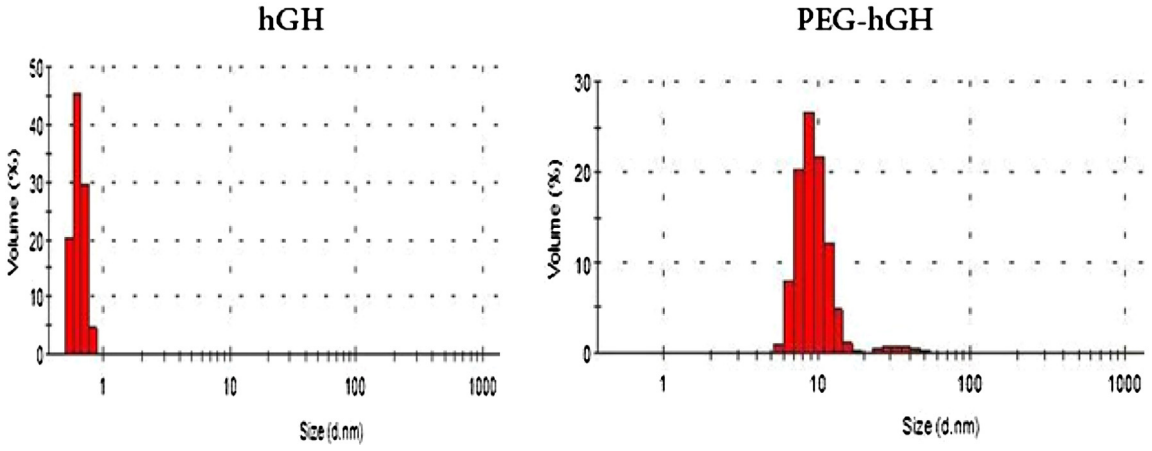

Fig. 1. Comparison between size of human growth hormone (hGH) and PEGylated human growth hormone (PEG-hGH) obtained by DLS method.

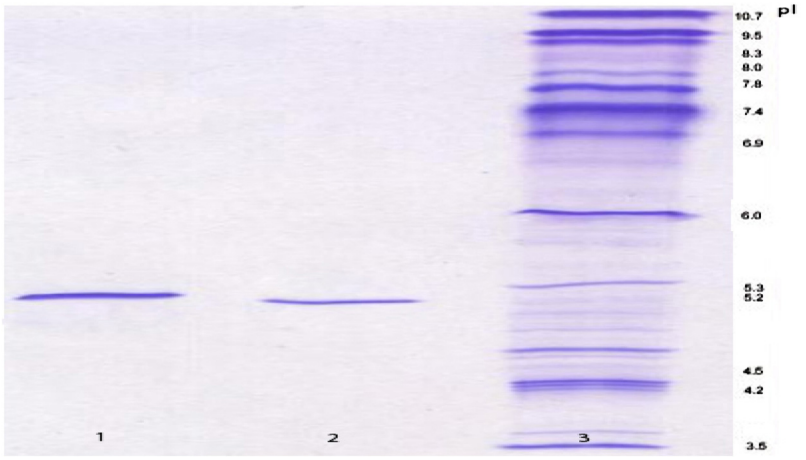

Fig. 2. Isoelectric focusing (IEF) of proteins carried out on a pH 3–10 pre-casted gel. Line 1, human growth hormone; line 2, PEGylated human growth hormone; line 3, standard proteins of different pI.

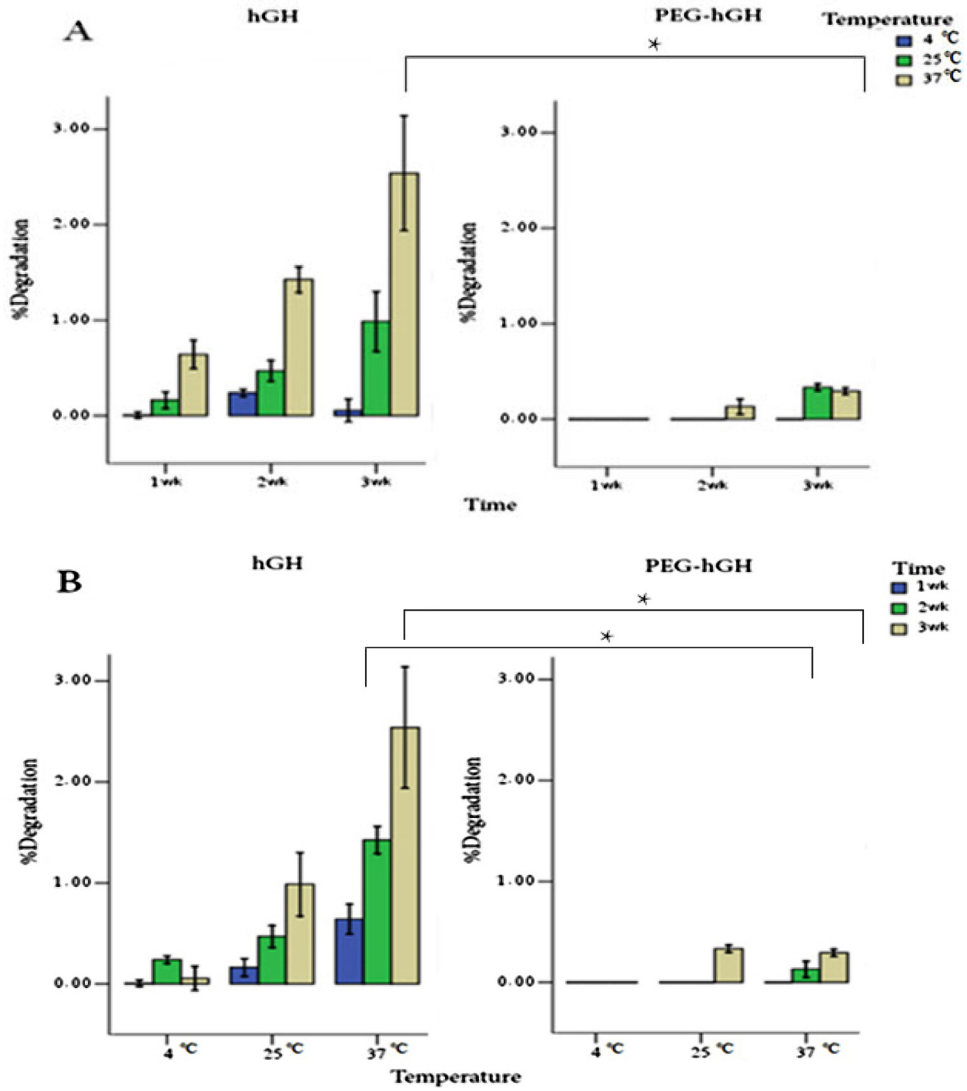

Fig. 3. Physical stability profile of human growth hormone (hGH) and PEGylated human growth hormone (PEG-hGH). (A) The effect of time on the stability profile of the proteins at different temperatures. (B) The effect of temperature on the stability profile of the proteins at different times.
* = Statistically significant as compared hGH and PEG-hGH.

rates were statistically significant. These observations were in accordance with previously published data (Rajan et al. 2006; Liebner et al. 2015). Liebner et al. (2015) exhibited that PEGylation leads to an improved physical stability and reduction in protein loss.

The monomer degradation (expressed as% degradation) on storage at different temperatures were quantified by SEC-HPLC (Fig. 3.B). In the case of hGH, storage at 4°C did not result in any measurable degradation, however, following storage at 25 °C and 37 °C, the hGH exhibited the most pronounced change, in monomer degradation and the degradation amounts showed statistically significant differences. In mono-PEG-hGH formulations, there was a measurable but not pronounced decrease in percentage of degradation at each temperature. The monomer loss was due to generate low molecular weight species from the PEG portion of bioconjugate (Abed et al., 2014). These results were previously confirmed by Bhatnagar et al., 2011 study.

Additionally, by PEGylation the amount of higher molecular weight protein species in the solution was reduced (Fig. 4). Therefore, it can be deduced that PEGylation suppressed protein aggregation in the solution. These findings, previosuly described by Rajan et al. (2006). They showed that PEGylation prevents aggregation of GCSF and the stability is vastly improved in comparison to the native protein. In another study, the stability of mono-PEG-IFN was investigated and the results indicated that PEGylation leads to a decrease in the aggregation amount and loss of monomers (Abed et al., 2014). The chromatograms of physical stability are illustrated in Fig. S.1. These results, overall, indicate that the conjugation of polymer to hGH allowed to obtain a considerable reduction in protein aggregation compared to the non-PEGylated form. These results were in agreement with the

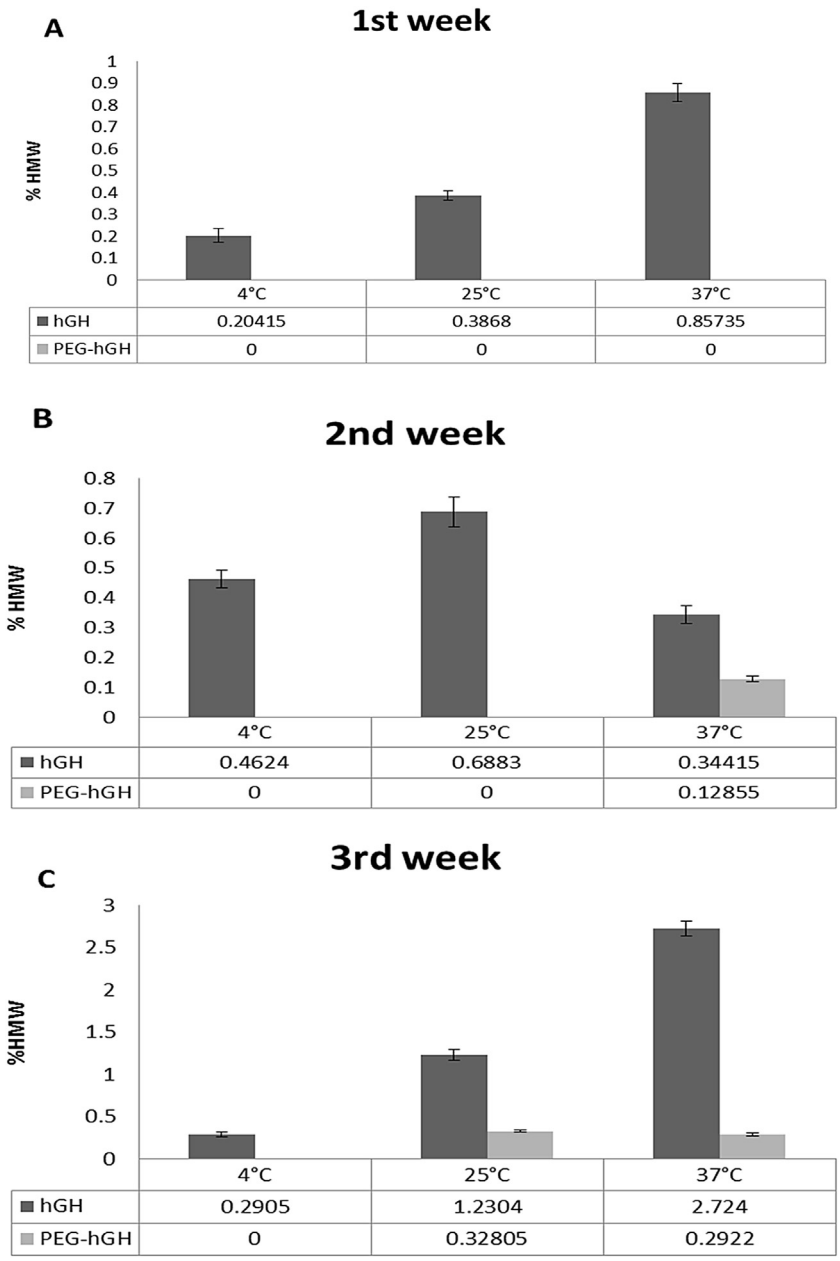

Fig. 4. Percentages of higher molecular weight species (%HMW) in protein solutions which was evaluated by size exclusion chromatography method. A) data in the first week of analysis, B) data in the second week of analysis, C) data in the third week of analysis. hGH: human growth hormone and PEG-hGH: PEGylated hGH.

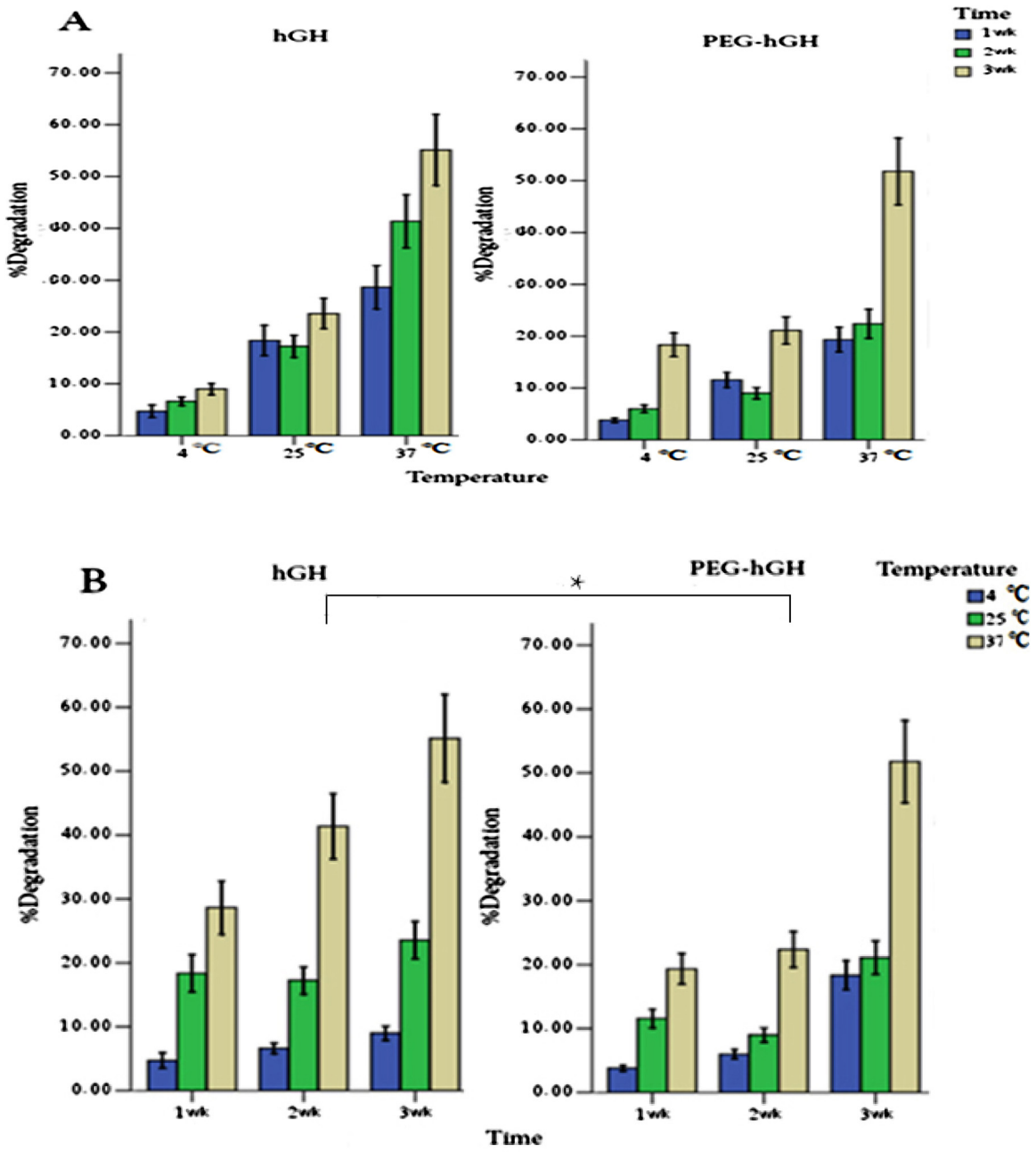

Fig. 5. Chemical stability profile of human growth hormone and PEGylated human growth hormone. (A) The effect of temperature on the stability profile of the proteins at different times. (B) The effect of time on the stability profile of the proteins at different temperatures.
* = Statistically significant as compared hGH and PEG-hGH.

results of previous studies. The improved stability of proteins following PEGylation is due to the steric hindrance of PEG chain around the protein and consequently prevents close contact between the protein moieties (Trivedi et al., 2012; Natalello et al., 2012; Holm et al., 2014; Pasut, 2014).

The chemical instability was also investigated in present study and the results were illustrated in Fig. 5. The chemical stability of hGH upon PEGylation was improved but not as well as physical stability. PEG-hGH did not prevent protein degradation. On the contrary, the amount of degraded form of PEG-hGH seemed to be more than unmodified hGH when kept at 4 °C, but this effect was not statistically significant ($P > 0.05$). At 4°C, PEGylation neither positively nor negatively effects on the chemical stability of hGH. This observation was in line with previously published data, which showed that there is no significant difference between insulin and PEG-insulin monomer loss in metal-catalyzed oxidation (Torosantucci et al., 2011). The results also indicated that following PEGylation, hGH shows the time-dependent manner in stability profile (Fig. 5.A). However, at elevated temperatures, the instability of protein was drastically increased and PEGylation led to decrease the degradation amounts of hGH.

To our knowledge, there are a few study indicated that the effect of PEGylation on chemical stability of bioconjugates. One

possible reason for reducing the efficacy of PEGylation for improving the chemical stability with respect to the physical ones is hydrophilic properties of PEG molecule. Deamidation of protein is a hydrolytic reaction and requiring only water to form byproducts (Wright, 1991). Additionally, in pervious study, it was shown that PEG polymer was covered the surface of hGH (Khameneh et al., 2015). Due to the hydrophilicity and water absorption character of PEG molecule, by this structure the microenvironment of methionine residue might be changed and water molecules surrounded this residue. Consequently, it can be assumed that in this condition the chemical instability of hGH was slightly increased. However, at elevated temperatures the chemical stability was increased. The enhancement of chemical stability might be caused by reducing the interaction of protein–protein interaction following PEGylation. It should be mentioned that the influence of PEGylation on interaction of water molecules with protein residues was previously described (Payne et al., 2011).

The chemical stability of protein was less improved compared to physical stability. However, in the case of human growth hormone, the chemical instability was less important in comparison with physical one. It was shown that by oxidation of methionine residues of growth hormone, the biological activity was not influenced and maintained (Glaser and Li, 1974; Houghten et al., 1977).

Conclusion

In this study, the physicochemical properties and stability of liquid form of mono-PEG-hGH were investigated. The results of physicochemical properties obtained by DLS method indicated that PEGylation led to increase the size and decrease the surface charge by covering protein surface. As seen in the IEF gel, the pI of protein was not altered upon PEGylation. This study also highlighted the advantages of PEGylation on biotherapeutic stability. Both type of proteins, hGH and mono-PEG-hGH, were kept at different temperatures (37, 25 and 4 °C). Mono-PEG-hGH was considerably more stable toward elevated temperatures compared to the others. In this regard, PEGylation improved the stability of hGH and deserving further investigations for its efficacy in animal models.

Conflict of interest

All authors have declared that there is no conflict of interest in this study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jab.2016.06.002.

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