International Journal of Biological Macromolecules xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Preparation, characterization and molecular modeling of PEGylated human growth hormone with agonist activity

Bahman Khameneh[a], Mahmoud Reza Jaafari[b,**], Mohammad Hassanzadeh-Khayyat[c], AbdolReza Varasteh[d], JamshidKhan Chamani[e], Mehrdad Iranshahi[f], Hamid Mohammadpanah[b], Khalil Abnous[c], Mohammad Reza Saberi[g,*]

[a] Department of Food and Drug Control, Students Research Committee, Mashhad University of Medical Sciences, Mashhad, Iran
[b] Nanotechnology Research Center, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran
[c] Pharmaceutical Research Center, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran
[d] Allergy Research Center, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[e] Department of Biology, Faculty of Sciences, Islamic Azad University-Mashhad Branch, Mashhad, Iran
[f] Biotechnology Research Center, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran
[g] Medical Chemistry Department, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran

ARTICLE INFO

Article history:
Received 12 May 2015
Received in revised form 17 June 2015
Accepted 18 June 2015
Available online xxx

Keywords:
Human growth hormone
PEGylation
Microbial transglutaminase
Biological activity
Characterization
Molecular simulation

ABSTRACT

In this study, site-specific PEGylated human growth hormone (hGH) was prepared by microbial transglutaminase, modeled and characterized. To this end, the effects of different reaction parameters including reaction media, PEG:protein ratios, reaction time and pH value were investigated. PEG-hGH was purified by size exclusion chromatography method and analyzed by SDS-PAGE, BCA, peptide mapping, ESI and MALDI-TOF–TOF mass spectroscopy methods. Biophysical and biological properties of PEG-hGH were evaluated. Molecular simulation was utilized to provide molecular insight into the protein–receptor interaction. The optimum conditions that were obtained for PEGylation were phosphate buffer with pH of 7.4, 48 h of stirring and PEG:protein ratio of 40:1. By this method, mono-PEG-hGH with high reaction yield was obtained and PEGylation site was at Gln-40 residue. The circular dichroism and fluorescence spectrum indicated that PEGylation did not change the secondary structure while tertiary structure was altered. Upon enzymatic PEGylation, agonistic activity of hGH was preserved; however, Somavert®, which is prepared by chemical PEGylation, is an antagonist form of protein. These data were confirmed by the total energy of affinity obtained by computational protein–receptor interaction. In conclusion, PEGylation of hGH was led to prepare a novel form of hormone with an agonist activity which merits further investigations.

1. Introduction

In previous decades, biologically derived pharmaceuticals have been developed widely and rapidly [1]. Despite their advantages, protein drugs suffer from some major limitations such as instability or short *in vivo* half-life [2]. These limitations can be overcome by various approaches like applying novel drug delivery systems, microencapsulation and especially post-translational modification (PTM) [3].

Conjugation with polyethylene glycol (PEG), PEGylation, represents as one of the most versatile PTM strategies which enhance the performance of protein drugs. PEGylation can be performed by different approaches including random and site-specific PEGylation [4]. Random PEGylation is usually achieved by derivatization of primary amino groups of lysine residues in the polypeptide chain of proteins and often produces heterogeneous bioconjugates due to non-specific conjugation at multiple sites (positional isomers). Site-directed PEGylation exhibits a number of advantages over random PEGylation, most importantly the removal of these positional isomers [4,5].

Enzymatic site-specific PEGylation *via* microbial transglutaminase (MTGase) has been introduced as a promising approach. Various proteins were PEGylated by this method including, human growth

* Corresponding author at: School of Pharmacy, Mashhad University of Medical Sciences, P.O. Box 91775-1365, Mashhad, Iran. Tel.: +98 513 8823255; fax: +98 513 8823251.
** Co-corresponding author at: School of Pharmacy, Mashhad University of Medical Sciences, P.O. Box 91775-1365, Mashhad, Iran. Tel.: +98 513 8823255; fax: +98 513 8823251.
E-mail addresses: Jafarimr@mums.ac.ir (M.R. Jaafari), Saberimr@mums.ac.ir (M.R. Saberi).

http://dx.doi.org/10.1016/j.ijbiomac.2015.06.037
0141-8130/

hormone (hGH), granulocyte colony-stimulating factor (GCSF), salmon Calcitonin (sCT) and apomyoglobin (apoMb) [6–8].hGH is a 22 kilo dalton (kDa) protein that is used therapeutically to treat humans with growth hormone deficiency. Currently hGH is administered as daily SC injections. The main drawbacks of hGH as other biopharmaceuticals are short *in vivo* half-time and protein instabilities. Thus, developing longer-acting versions of hGH has been ongoing for many years in order to reduce the number of injections and increase protein stability. Ongoing work includes PEGylation, albumin conjugation and preparing depot formulations. Among them, PEGylation attracts more attention and effectively overcomes the limitations [7,9–11]. The current available PEGylated form of hGH is Somavert®, which is prepared by chemical PEGylation, and is an antagonist form of protein and is used for treatment of acromegaly [12]. Consequently, preparing the PEGylated forms of hGH which have agonistic activity is desirable.

This study aimed to prepare the PEGylated form of hGH with agonistic activity. To this end, hGh was PEGylated by enzymatic site-specific method *via* MTGase and various conditions such as reaction time, pH and PEG:protein ratio were tested to find out the optimized preparation conditions. The PEG-hGH was purified by size exclusion chromatography (SEC–HPLC) method and characterized by SDS-PAGE, BCA, peptide mapping, electronspray ionization (ESI) and MALDI-TOF–TOF mass spectroscopy methods. Biophysical properties were evaluated by CD and fluorescence spectroscopy methods and biological activity was demonstrated with cell proliferation assay. Molecular simulation was utilized to provide molecular insight into the protein–receptor interaction.

2. Materials and methods

2.1. Materials

Recombinant hGH was purchased from Hospira, Adelaide Pty Ltd., Australia. Microbial transglutaminase (MTGase, Activa WM) from *Streptomyces mobaraensis* was purchased from Ajinomoto (Hamburg, Germany). Amino terminating linear 20 kDa monomethoxy-poly(ethylene glycol) (PEG20kDa-NH_2) was purchased from NanoCS (USA). N-α-carbobenzoxy-L-glutaminylglycine and L-glutamic acid γ-monohydroxamate were provided by Sigma (Saint Louis, MO, USA). Reagents for sodium dodecyl sulfate polyacryl amide gel electrophoresis (SDS-PAGE) were provided by Bio-Rad Laboratories Inc. (Hercules, CA, USA). The PRL-dependent rat T-lymphoma cell line, Nb2-11 cells, was obtained from the European Collection of Cell Cultures. All other chemical reagents were of analytical grade and provided by Sigma Aldrich-Fluka (Sigma, Germany).

2.2. Methods

2.2.1. Determination of MTGase activity in reaction media

The effect of different concentrations of Tween 20, Tween 80, poloxamer 188, methanol (MeOH) and ethanol (EtOH) on mTGase activity was determined by adding one of these co-solvents or surface agents to a MTGase solution in 0.1 M phosphate buffer, pH 7. The enzyme activity was assayed with the specific substrate, CBZ-n-glutaminylglycine (Z-Gln-Gly), as previously described by Scaramuzza et al. [13]. Briefly, to a test tube containing 500 μL of 0.1 M phosphate buffer pH 7.0, 0.1 M hydroxylamine and 30 mM of Z-Gln-Gly, a predetermined volume of each reaction media was added in order to achieve a final volume of 1 mL. Several test tubes were prepared for each condition (*e.g.* different co-solvents or surface agents at certain concentration). 50 μL of MTGase (30 mg/mL) was added and incubated for 10, 60, 120 and 180 min at 37 °C after which, 0.5 mL of ferric chloride (5%, w/v) and trichloracetic acid (12%, w/v) were added; finally the absorbance was measured at 525 nm. The enzyme activity in phosphate buffer was assumed as 100% which was confirmed in other reaction media based on phosphate buffer. The concentration of co-solvents was specified as percent of "v/v."

2.2.2. Structural properties of hGH in reaction media

2.2.2.1. Circular dichroism (CD) measurements. Far-UV CD spectra were measured on a Jasco J815 spectropolarimeter (Jasco, Tokyo, Japan) equipped with a Jasco 2-syringe titrator under constant nitrogen flush at room temperature. The instrument was controlled by Jasco Spectra Manager™ software. The scanning rate, bandwidth, and response were set at 50 nm min^{-1}, 1 nm and 2 s, respectively. The instrument was calibrated using d-10-camphorsulfonic acid. A quartz cuvette with a path length of 1 mm was used. Measurements of native hGH were made in each reaction media at a concentration of 0.1 mg/mL (Table S.2).

For exploring changes in secondary structure, far-UV CD spectra were obtained over a wavelength range of 190–240 nm with an average of 5 scans. The data at each wavelength were averaged for 5 s. The sample cell path length was 1 mm.

2.2.2.2. Conformational investigation. Fluorescence measurements were performed on a spectrofluorometer Model F-2500 (Hitachi, Japan) with a 150 W Xenon lamp, a 1.0-cm quartz cell and a thermostat bath. The widths of excitation and emission slit were set at 5.0 nm. The excitation wavelength was set at 295 nm, and the emission wavelength was recorded between 300 nm and 600 nm.

Synchronous fluorescence spectroscopy was also carried out by simultaneously scanning the excitation and emission monochromators. The spectra only showed the microenvironment of tyrosine and tryptophan residues of hGH when the wavelength intervals ($\Delta\lambda$) were 15 nm and 60 nm, respectively. The spectra of light scattering (RLS) were recorded by scanning both the excitation and emission monochromators of a common spectrofluorometer with $\Delta\lambda = 0$ nm and from 220 nm to 600 nm with slit widths of 5 nm for the excitation and emission

2.2.3. MTGase-mediated PEGylation of hGH

Firstly, mPEG20kDa-NH_2 was dissolved at 10-fold molar excess to a hGH solution (1.0 mg/mL) in different reaction media and MTGase was added at E/S ratio of 1:50 (w/w). The mixtures were left to stir at room temperature for 24 h and then analyzed by SDS-PAGE and the best reaction medium was chosen for the subsequent steps. Q3

To optimize the reaction yield, different parameters were tested including PEG:protein ratios (5:1, 10:1, 20:1, 40:1 and 60:1), reaction times (24, 48, 72, 96, 120 and 144 h) and the pH of the reaction. After the reaction period, the mixture was analyzed by SEC–HPLC using a GF-250 column (4.6 mm × 250 mm; Agilent Technologies, Palo Alto, CA) eluted with 63 mM phosphate buffer, pH 7 and 3% 2-propanol at a flow rate of 0.6 mL/min. The UV detector was set at 214 nm and the corresponding peak to the conjugate PEG-hGH was collected and the solution was concentrated under vacuum and dialyzed against phosphate buffer 50 mM (pH 7.0).

Bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Pierce Protein Research Products, Rockford, USA) was used to measure the PEG-hGH concentrations. The determining accuracy of the kit was 0.1–2.0 mg/mL. Due to the absence of reaction between PEG and BCA reagents, the concentration of the samples was measured in terms of hGH equivalents.

2.2.4. SDS-PAGE analysis

SDS-PAGE analysis was performed using a polyacryl amide gel (4% stacking gel, 12% running gel) prepared according to Laemmli [14]. Each sample (0.1 mL) was diluted 1:1 v/v with Laemmli buffer