

## Original Research Article

# Chemoselective PEGylation of Cysteine Analogs of Human Basic Fibroblast Growth Factor (hbFGF) - Design and Expression

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Received: 18 July 2014

Revised accepted: 8 September 2014

### Abstract

**Purpose:** To improve the stability and bioactivity of human basic fibroblast growth factor (hbFGF) by site-specific pegylation

**Methods:** Four new mutants of hbFGF were designed with substituted Asp68, Lys77, Glu78 and Arg81 with cysteine with the aid of bioinformatics technique, and then cloned into pET21a plasmid, transferred into *E. coli* BL21 (DE3). The expressed proteins were purified using cation exchange and heparin affinity chromatography. Cysteine analogs of hbFGF were PEGylated with 10 KDa PEG and purified using size exclusion chromatography. Mitogenic activity and resistance against denaturation agents were evaluated by MTT assay and fluorescence spectrophotometry, respectively, and the results obtained were compared with the non-PEGylated form.

**Results:** Despite greater resistance against denaturation agent (1.2 M guanidine hydrochloride for denaturation of PEGylated mutants compared with 0.8 M for non-PEGylated forms), the mitogenic activities of the four mutants Asp68, Lys77, Glu78 and Arg81 were retained at 79, 78.6, 83.3 and 75.6 %, respectively.

**Conclusion:** PEGylated hbFGF shows decreased mitogenic activity and increased resistance against denaturation agent.

**Keywords:** Bioinformatics, Fibroblast growth factor, Cysteine analog, PEGylation, Denaturation agent, Guanidine hydrochloride, Mitogenic activity

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Boline International, Open-J-Gate and Pharmacy Abstracts

## INTRODUCTION

Human basic fibroblast growth factor (hbFGF) is a polypeptide comprising of 146 up to 154 amino acids which is known for its multiple capabilities. It belongs to Fibroblast growth factor (FGF) family with 25 members that are structurally similar to each other. All members of this family activate intra membrane receptors with high

affinity [1,2]. So far different roles in the cells and tissues such as mitogenic, angiogenic [3,4]; survival properties are proposed for human basic fibroblast growth factor [5]. In addition to hbFGF's effectiveness in burn treatment [6-8], investigators have found other properties for hbFGF such as improvement in spinal injuries [9,10] and tissue repair after myocardial infarction [11-13]. Initially mitogenic role of

hbFGF on fibroblastic cells and tissues repair was considered then the function of hbFGF as neurotrophic activity in the growth and survival of brain's neuron, assisting the survival of transplanted neuron and prevention of apoptotic neuronal cells was confirmed [14]. The first commercial drug namely Trafermin is available in global market for providing numerous health benefits [15-17].

As hbFGF is a proteinaceous molecule therefore, its stability and half life are susceptible to be reduced like other proteins by proteolysis like other proteins [18,19]. PEGylation of recombinant therapeutic proteins is considered as a technique that increases the stability of a molecule [20,19], with retention of activity [21-25,20].

Random or non-specific PEGylation such as N terminal PEGylation and lysine PEGylation which belong to the first generation of PEGylation make a heterogenic low efficiency and less bioactive product. Improvement of PEGylation led to the second generation or site-specific PEGylation method which gave more stable and efficient products [26].

One of the methods used to increase the probability of binding the protein molecule under study is the employment of an activated form of polyethylene glycol(PEG maleimide) and presence of an amino acid namely cysteine where such amino acid is designed to be located at non functional and non protective sites of the protein under PEGylation. PEGylation is performed in different environmental situations like room temperate or cold room with various incubation times. PEGylation is an exothermic reaction so generally, it is recommended to perform in a cold situation. At room temperature, PEGylation incubation is shorter than cold room [19,27-29,].

Like other therapeutic proteins, hbFGF has been one of the attractive targets for PEGylation [19,29]. In the present study, by employing bioinformatics programs such as Modeller and Prosite and molecular dynamic simulation [30], the molecular structure, active site of the molecule and the protected sites of hbFGF were identified and studied. By considering points such as presence of cysteine at the surface and being away from protected sites, four mutants were designed. Such sequences were constructed, inserted into pET21a plasmids and transformed into *E. coli* BL21 (DE3).

## EXPERIMENTAL

### Bioinformatics analyses

The primary sequence of human basic fibroblast growth factor was obtained from the Uniprot database (Accession No P09038). 3D structures of cysteine analogs were generated by homology modeling using MODELLER version 9v13 and a crystal structure of FGF2 (PDB code: 1BFG, chain A, 1.6 °A) with high score and lower e-value and maximum sequence identity served as a template. For each cysteine analog, 10,000 molecules were generated and structure quality was validated by PSVS ([http://psvs-1\\_4-dev.nesg.org/](http://psvs-1_4-dev.nesg.org/)). The best structural model was chosen for further studies. The stability of modeled analogs was examined by MD simulation. GROMACS 4.6.5 package and GROMOS96 53a6 force field were used on LINUX architecture for energy minimization and MD simulations. All simulations were performed at normal pressure (1 bar) and temperature (300 K) for 5 ns.

A literature review was performed to find conserved residues; based on this, sequences 66-96 was the best region for manipulation and replacement with cysteine. The 3D structures of selected analogs were generated and screened based on the basis of protein energy according to the internal scoring function of Modeller. Four analogs (Asp 68, Lys77, Glu78 and Arg81) were chosen based on minimum protein energy and minimum Root-Mean-Square Deviation (RMSD) from native bFGF in the final average structure, for experimental analysis. The quality of modeled analogs was verified by ramachandran plot.

Superimposed native bFGF and Cysteine analogs were selected (Asp 68, Lys77, Glu78, Arg81). MD analysis was performed for all analogs in 5 ns, and model stability and average structure calculations were evaluated using the plot of RMSD versus time.

### Construction, expression and purification of cysteine analogs of hbFGF

The resultant sequences were constructed (Biomatik Corporation, Canada), and cloned into pET21a plasmid, then transferred to *E. coli* BL21 (DE3) by using One Shot® BL21(DE3) Chemically Competent *E. coli* (Invitrogen). The engineered *E. coli* BL21 (DE3) was grown in LB broth (Sigma-Aldrich) till reaching an optical density (OD600 nm)  $\approx$  0.6 - 1.0. The induction of hbFGF was carried out with 1 mM Isopropyl  $\beta$ -D-1-thiogalactopyranoside [IPTG] (Thermo Scientific, Fermentas) at 30 °C for 4 h. The cells

were harvested by centrifugation at 2000 rpm, 4 °C for 20 min. They were suspended in phosphate buffer, passed through high pressure homogenizer and cell debris was separated by centrifugation. The expressed proteins were confirmed by SDS PAGE 12 % and Western blot (semi dry) analysis.

The supernatant containing hbFGF were purified by Fast Purification liquid chromatography FPLC (Bio-Rad) in two steps using cation exchange chromatography (Sartobind S membrane: Sartorius Co) and heparin affinity chromatography (HiTrap, GE Health Care Life Sciences), both at a flow rate of 1 ml/min. The resultant peaks were subjected to SDS-PAGE 12 % to identify the target protein. The collected peak was buffer exchanged and concentrated to reduce the salt concentration with Vivaspin sample concentrator 10 KDa (GE Health Care Life Sciences). Anion exchange column (Sartobind Q membrane: Sartorius Co) was applied at a flow rate of 1 ml/min for endotoxin reduction to desired level where it does not affect the biological activity assay.

### PEGylation of purified cysteine analog hbFGF

The expressed and purified cysteine analogs hbFGF proteins were subjected to PEGylation using PEG-maleimide 10 KD (Jenkem) under nitrogen gas in a cold room for 24 h. The PEGylated forms were separated from non-PEGylated proteins by size exclusion chromatography using a Hiload 16/600 Superdex 75 prep grade column (GE Health care life sciences) at a flow rate of 1 ml/min, analyzed by 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis [SDS-PAGE]. Stability of PEGylated and non-PEGylated forms of hbFGF cysteine analog was studied by incubating them in the presence of denaturing agent such as guanidine HCl at 37 °C for 24 h. The effect of such reagent was determined by fluorescence spectrophotometry (Luminescence spectrometer Perkin Elmer LS 50 B). PEGylated cysteine analogs of hbFGF were used for MTT assay for biological activity test. The results were compared with non-PEGylated hbFGF.

### Biological activity test

MTT assay was used to evaluate the biological activity of the samples. Balb/c 3T3 cells from mouse embryo tissue were cultivated in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) containing 10 % FBS (Gibco). About 10000 cells/ml per well were added to plastic 96 well plates and cultured at 37°C for 2 h in a humidified 5 % CO<sub>2</sub> - 95 % air atmosphere. The

cells were treated with different concentrations of hbFGF mutants. The assay was going on using the MTT exclusion dye which reacts only with viable cells. observed Optical density (OD) measured by ELISA reader shows the amount of the alive cells. The range of sample concentration was from 15 pg/ml up to 2000 pg/ml. The MTT assay was used for PEGylated form too. The results were compared with bFGF standard curve.

## RESULTS

### Bioinformatics results

The sequences under study through whose mutants were made up, were blasted by Protein Data Base and the most aligned sequence (> 98 % alignment) belonged to fibroblast growth factor variant bearing the number P09038 [FGF2-Human] 1BFG (R090p38).

The sequence under study contains 9 amino acids less than the sequence 1BFG (R090p38) which contains 154 amino acids located at the beginning of the sequence. These two sequences differ at amino acids numbers 21, 96 and 97 where our sequence alanine, serine and alanine were replaced with phenylalanine, cysteine and valine respectively. Our constructed sequences along with the file bearing suffix PDB related to 1BFG (R090p38) were subjected to Modeller software with the aim of increasing accuracy and sensitivity of the resultant information, thus 10,000 molecules were generated and the molecule corresponding to the lowest value of probability density function was selected.

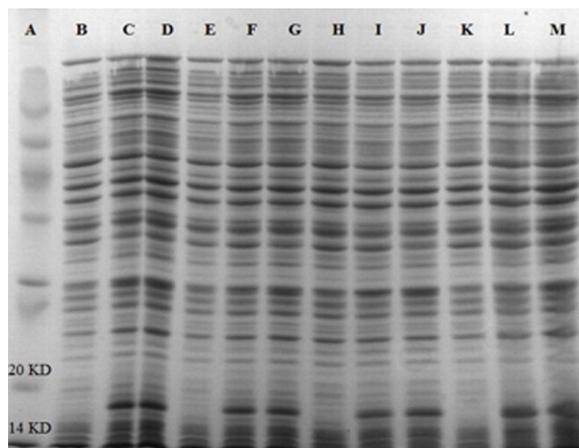
In continuation by using multiple sequence alignment and prosite software, the protected sites in this family and also the available motifs were extracted in order to make convergence comparison through the related literature [31-33]. The results are as follows: the binding receptor sites include amino acids 25-69 and 94-121 where amino acids 31-51 and 107-116 play fewer roles in binding. The binding site of this protein/molecule to heparin includes the sequence 94-121,104-147 and 106-142. The regions 128-138 create a loop-like area that plays a major role in binding to heparin. Finally, the regions 66-96 were selected as the best amino acid regions to substitute the available amino acids with cysteine. The first selected mutant was made by substitution of asparlate 68 with cysteine. The quality of models was checked using discrete optimized protein energy (DOPE),

PROCHECK software and ramachandran plots indicating that more than 93% residues were located in the favoured region and no residue was present in the disallowed region (Table 1). Other three mutants were obtained in a similar manner.

The RMSD of the backbone atoms of the protein over the course of simulation is usually used as a measure of the conformational stability of the protein. RMSD of analog proteins with native hbFGF were compared using NAMD software indicating similarity between native BFGF and Asp 68, Lys77, Glu78, Arg81 analogs.

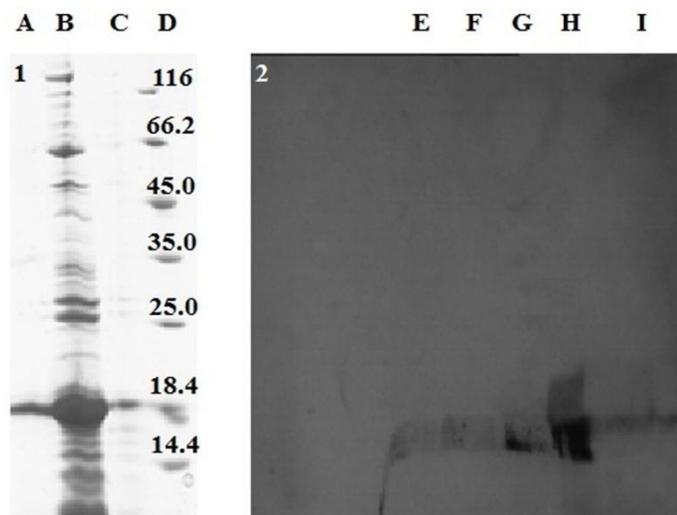
### Experimental results

The engineered bacteria containing the gene of interest in their plasmid were allowed to grow in LB medium; induction was achieved with 1 mM IPTG. Protein expressions of various mutants were compared at 4 and 6 h after induction and no significant differences were detected (Figure 1).



**Fig1:** SDS PAGE expressed proteins. Lane A: marker; lanes B-D: Asp68 before induction, 4 and 6 hours after induction; lane E-G: similar time for Lys77; lanes H – J: similar times for Glu78 and lane K-M: similar times for Arg81

Since the results of the four mutants are similar, only the results of cysteine hbFGF analog Asp68 are presented. Figure 2 shows SDS-PAGE (12%) and western blot analysis confirmation of Asp68.



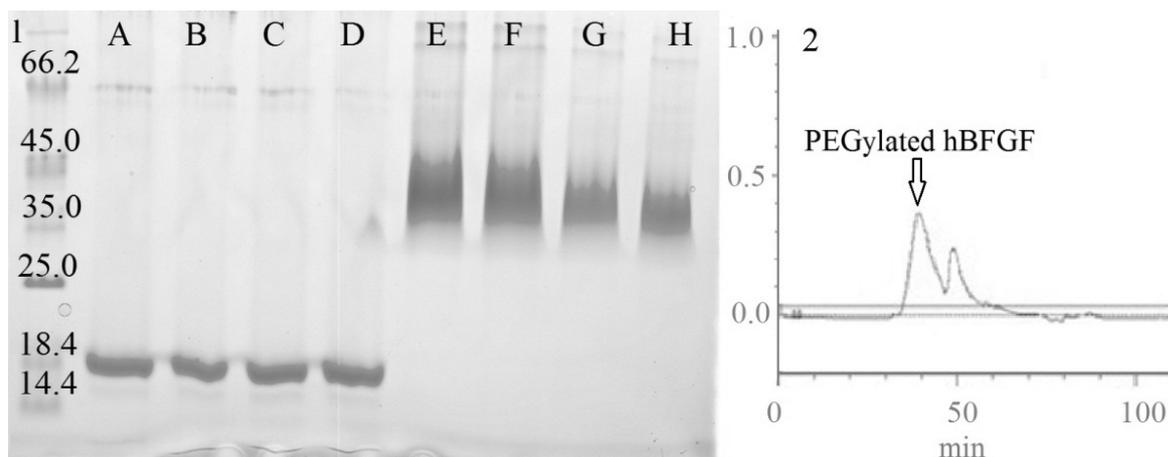
**Fig 2:** SDS PAGE of 2 steps purification and Western blot of mutated forms and the standard hbFGF: 2-1) Lane A: Heparin affinity peak; Lane B: fraction 2 of cation exchange, Lane C: fraction 1 of cation exchange; Lane D: Marker; 2-2) western blot of: Lane E: Asp68; Lane F: Lys77; Lane G: Glu78; Lane H: Arg81

The purified 4 mutants were used for MTT assay to determine their biological activity using balb/c 3T3 clone A 31. The MTT results showed the similarity of the 4 mutants to the normal variant (data not shown).

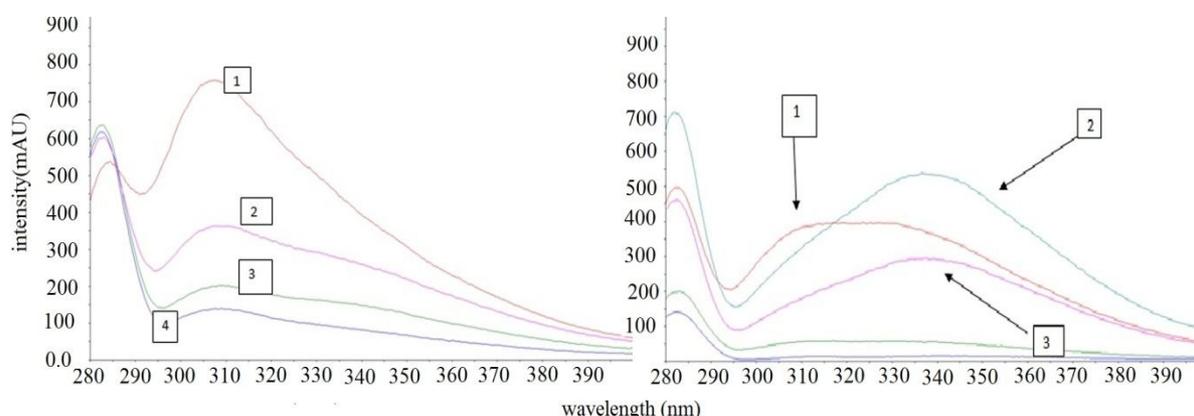
PEGylation of the purified proteins were performed in the dark, in a cold room under a stream of nitrogen gas using PEG-maleimide 10 kD. PEGylated forms were separated from the non-PEGylated hbFGF with size exclusion chromatography and further were confirmed by SDS-PAGE 12 % (Figure 3).

**Table 1:** Structural validation of model structure generated from Modeler by PSVS

Sample name	Most favoured regions (%)	Allowed regions (%)	Generously allowed regions (%)	Disallowed regions (%)
Wild bFGF	96.7	3.3	0.0	0.0
N68C	93.4	6.6	0.0	0.0
K77C	94.2	5.8	0.0	0.0
E78C	93.4	5.8	0.8	0.0
G81C	94.2	5.8	0.0	0.0



**Fig. 3:** Size exclusion chromatography and SDS-PAGE of PEGylated and non-PEGylated forms of mutated proteins after size exclusion chromatography. (3-1): Lane A: Asp 68; Lane B: Lys77; Lane C: Glu78; Lane D: Arg81; Lane E: PEGy Asp68; Lane F: PEGy Lys77; Lane G: PEGy Glu78; Lane H: PEGy Arg81.(3-2) Size exclusion chromatogram



**Fig 4:** Fluorescence spectroscopy of four native mutants and Asp68 in steps of natural and denature forms. Left: nature forms of 1: Asp 68, 2: Lys77; 3: Glu78; 4: Arg81. Right: Asp68: 1: nature form; 2 and 3: denature forms in two different concentrations

Both PEGylated and non-PEGylated forms of purified hbFGF were converted to their denatured forms in the presence of guanidine HCl at 37 °C for 24 h. It was observed that PEGylated human basic fibroblast growth factor and non PEGylated forms of hbFGF were denatured at 1.2 and 0.8 M concentrations of the guanidine HCl denaturant respectively. Figure 4 shows the position of hbFGF in native, semi denatured and denatured forms using fluorescence spectrophotometry.

Finally the PEGylated forms of hbFGF were subjected to biological assay in comparison with Standard bFGF which revealed that 79, 78.6, 83.3 and 75.6 percent of mitogenic activity was retained respectively in mutants Asp68, Lys77, Glu78 and Arg81.

## DISCUSSION

PEGylation is a well-known method for improving the stability and efficiency of therapeutic proteins [34-36]. PEGylation of hbFGF have done by a limited research group. Some of them tried to conjugate PEG molecules to native hbFGF [28] and the others used site-specific method [19,29]. Mutants were created by replacing four amino acids with cysteine (Asp68, Lys77, Glu78, Arg81) through bioinformatics' prediction, and were expressed at a high level in *E. coli* BL21 (DE3). We used two chromatographic method which include cation exchange and heparin affinity to achieve more than 95 percent purity. In addition, an anion exchange chromatography was further used to reduce endotoxin before MTT assay. Bioactivity of the 4mutants was same and equal to standard hbFGF. Purified cysteine analog hbFGF was PEGylated using PEG-maleimide

(10 KDa) in the dark and in the cold room under nitrogen gas for 24 h.

PEGylated and non-PEGylated proteins were separated using size exclusion chromatography and their fractions were run on 12 % SDS PAGE. PEGylated proteins were larger than the expected size (Figure 3), probably due to the more extended structure of PEG-protein conjugate [19]. Our result shows that bioactivity of PEGylated hbFGF was less than the non-PEGylated form. The reduction in bioactivity of PEGylated protein had been reported by other researchers [28,19,29] and there is a one theory that PEGylation makes the protein more stable but may shield the active site of protein partially [19]. Stability of hbFGF is measured using different methods such as heat-stability [29], resistance to denaturing agent like guanidine hydrochloride (GuHCl) and or urea using fluorescence spectroscopy [37]. We used resistance to guanidine hydrochloride (GuHCl) method and showed that the PEGylated hbFGF increased stability up to 1.2 molar GuHCl compared with 0.8 molar for the non-PEGylated mutant.

## ACKNOWLEDGEMENT

This project was financially supported by the Pasteur Institute of Iran via the sponsorship of the PhD thesis of Shahin Hadadian.

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