Protein Expression and Purification 114 (2015) 128–135

Contents lists available at ScienceDirect

Protein Expression and Purification

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

Optimizing conditions for production of high levels of soluble recombinant human growth hormone using Taguchi method

Marzieh Savari [a], Sayyed Hamid Zarkesh Esfahani [b,*], Masoud Edalati [c], Davoud Biria [d]

[a] University of Isfahan, Isfahan, Iran
[b] Department of Biology, Faculty of Sciences, University of Isfahan, Isfahan 81746-73695, Iran
[c] Department of Pathology, Medical School, Isfahan University of Medical Sciences, Hezar Jerib Street, Isfahan 81746-73695, Iran
[d] Department of Biotechnology, Faculty of Advanced Sciences and Technologies, University of Isfahan, Isfahan, Iran

ARTICLE INFO

Article history:
Received 25 January 2015
and in revised form 7 June 2015
Accepted 9 June 2015
Available online 4 July 2015

Keywords:
Human growth hormone
Soluble protein
Taguchi software
Escherichia coli

ABSTRACT

Human growth hormone (hGH) is synthesized and stored by somatotroph cells of the anterior pituitary gland and can effect on body metabolism. This protein can be used to treat hGH deficiency, Prader–Willi syndrome and Turner syndrome. The limitations in current technology for soluble recombinant protein production, such as inclusion body formation, decrease its usage for therapeutic purposes. To achieve high levels of soluble form of recombinant human growth hormone (rhGH) we used suitable host strain, appropriate induction temperature, induction time and culture media composition. For this purpose, 32 experiments were designed using Taguchi method and the levels of produced proteins in all 32 experiments were evaluated primarily by ELISA and dot blotting and finally the purified rhGH protein products assessed by SDS–PAGE and Western blotting techniques. Our results indicate that media, bacterial strains, temperature and induction time have significant effects on the production of rhGH. The low cultivation temperature of 25 °C, TB media (with 3% ethanol and 0.6 M glycerol), *Origami* strain and a 10-h induction time increased the solubility of human growth hormone.

1. Introduction

The production of recombinant proteins in *Escherichia coli* has become of great importance for many biological applications and fields of research [23]. Because *E. coli* has several advantages such as the ease of use and the low cost, it has been exploited for the production of a variety of therapeutic proteins [6]. The growth hormone a polypeptide hormone secreted by the somatotroph cells in the anterior hypophysis of the vertebrates, is a cardinal factor for linear growth in childhood and normal body composition in adults. This protein is an important anabolic cytokine hormone and is involved in metabolism of proteins, carbohydrates, lipids and mineral metabolism as well as in growth, development and immunity. The structure of GH was shown to consist of a single chain peptide of 191 amino acids and having a molecular weight of 22 kDa that contains two disulfide bridges. Recombinant human growth hormone (rhGH) is administered as a pharmaceutical protein in the treatment of variety of diseases and to large numbers of patients. It was used as a therapeutic agent against growth-retarded GH deficiency (GHD), Turner syndrome and End-stage renal disease in children and also used against SHOX (short stature homeobox gene) deficiency and AIDS-associated wasting in adults [4,11,13]. Using of pituitary-derived hGH has been prohibited when its association with Creutzfeldt–Jakob disease was proved [2]. Although recombinant DNA technology has presented as a safe way of producing numerous products of rhGH in various heterologous systems, improper folding of the overexpressed proteins has been as an important limitation for the production of recombinant proteins in *E. coli* as well as formation of insoluble protein aggregates (inclusion bodies) in vivo. Therefore, solubilization and renaturation of inclusion bodies are significant parametric issues in the efficient production of pharmaceutical proteins [14,18]. The additional protein refolding step is an important process to achieve soluble recombinant proteins from inclusion bodies, but the process of protein refolding from *E. coli* inclusion bodies is not straightforward and predictable. In order to achieve sufficient amounts of recombinant proteins from insoluble refractile bodies in *E. coli*, it usually requires large volumes of bacterial growth media to culture sufficient numbers of *E. coli* cells and also needs large volumes of buffers to solubilize the inclusion bodies, and this additional step in the protein production increases its costs [24,26]. To save time,

* Corresponding author.
E-mail addresses: Marzieh.savari@gmail.com (M. Savari), s.h.zarkesh@sheffield.ac.uk (S.H. Zarkesh Esfahani), masoud_edalati@hotmail.com (M. Edalati), d.biria@ast.ui.ac.ir (D. Biria).

http://dx.doi.org/10.1016/j.pep.2015.06.006
1046-5928/

operations and laboratory equipment, it is essential to obtain soluble recombinant protein in its native form without refolding from inclusion bodies. Several experimental approaches have been extended to inhibit forming these aggregates, which include the use of variety of cultivation conditions and parameters related to the host. Folding of many proteins into a native conformation depends on the formation of stable disulfide bonds, therefore these disulfide bonds have an important role in protein folding and without them proteins accumulate in inclusion bodies [3]. The high reducing potential of the cytoplasmic compartment of *E. coli* decreases the production of properly folded proteins. The disulfide bond formation in the cytoplasm is enhanced in the *Origami* and *Rosetta-gami* strains from Novagen. These bacterial strains with disruption in *gor* and/or *trxB*, which encode thioredoxin reductase and glutaredoxin, overcome this limitation [27]. Another approach to enhance the production of soluble recombinant proteins in their native forms is to express them at low temperature conditions. Generally, expression of proteins in *E. coli* growing at low temperature conditions increased the stability and correctly folding of the protein, which is due to the strong temperature dependence of hydrophobic interactions that determine inclusion body formation [20]. On the other hand, the activity and expression of a number of *E. coli* chaperones are increased at lower growth temperatures. It has also been observed that temperature reduction leads to the low activity of heat shock proteases which are induced during over-expression [5,9,15]. In addition to temperature shifts, the heat-shock response has also been induced by a variety of stress conditions, for example harmful substances such as ethanol [16]. Chemical chaperones are low molecular weight compounds including glycerol and ethanol which have been used to prevent the formation of aggregates into inclusion bodies during invitro folding. Glycerol due to the enhancement of hydrophobic interactions can act as a stabilizer of protein conformation [1,12]. Recently, invitro studies in transfected cells demonstrated that glycerol can correct the defective folding of delta F508 CFTR in cystic fibrosis by stabilizing an otherwise unstable intermediate in CFTR biosynthesis [21]. To increase the efficacy for obtaining high levels of soluble form of recombinant human growth hormone, in this study, using Taguchi's method, we evaluated the effects of different temperatures, IPTG concentrations, induction periods, *E. coli* strains, glycerol and ethanol, on the recombinant human growth hormone expression levels. Taguchi's method allows the accurate optimization of a process by performing the minimum numbers of experiments possible.

2. Materials and methods

2.1. E. coli strains, plasmids and media

The *E. coli* strains used in this study are *Origami* (DE3, Novagen) and *Rosetta-gami* (DE3, Novagen) harboring pTrcHis-Topo (Invitrogen # Cat: K4410-01) for hGH expression. The vector contains of a strong trc promoter, the lacO sequence, and the structural gene for rhGH. Bacteria were grown in Luria–Bertani (LB) [10 g/l Bacto-tryptone, 5 g/1 Bacto yeast extract, 5 g/l NaCl and pH 7.0 with NaOH] (Merck-Germany) and Terrific Broth (TB) [12 g/1 Bacto-tryptone] (Merck-Germany), 24 g/1 Bacto yeast extract (Merck-Germany), 2.31 g KH_2PO_4, 12.54 g K_2HPO_4, glycerol 60% (0.6 M ≈ 73 ml, 1.2 M ≈ 147 ml), (Aplichem).

2.2. Design of experiments

The goal of designing of experiment (DOE) in Taguchi's method is to achieve maximum significant information with the least numbers of experiments. In Taguchi designs, a measure of robustness used to identify control factors that reduce variability in a product or process by minimizing the effects of uncontrollable factors (noise factors). In Taguchi method, the word "optimization" implies "determination of BEST levels of control factors". In turn, the BEST levels of control factors are those that maximize the Signal-to-Noise (S/N) ratios [10]. Taguchi experimental design (Minitab 16) a standard array L32, was used to examine five following factors: temperature (°C) in four levels, concentration (mM) of IPTG in four levels, induction period in four levels (h), media including ethanol and glycerol (ml) in four levels and strains of *E. coli* in two levels (Table 1). The subscript in L32 indicates the number of experimental runs. The thirty-two experiments were carried out in five different parameters in mixed levels (Table 2). Thirty-two experiments were repeated three times under the identical experimental conditions.

2.3. Protein preparation and analysis by dot blotting, SDS–PAGE and Western blotting

For the analysis of the total expression levels of rhGH, 1 ml culture volumes were taken at different times after induction. Cell supernatants were prepared from culture samples by centrifugation at 4000 × rpm for 20 min. To obtain cell lysate, the pellet of 1 ml cell culture was resuspended in 100 μl of Phosphate buffered saline (PBS, 1×, pH 8.0). The cell mixture was incubated in −20 °C for 30 min, followed by incubation at 37 °C for 15 min. This cycle of slow freezing/thawing were repeated three times. The suspension of disrupted cells was centrifuged at 4000 × rpm for 20 min, and the supernatant (the fraction of soluble cellular proteins) was collected. The supernatants were used for protein analysis. Proteins in supernatants were detected by dot blotting and also were separated by SDS/PAGE and Western blotting using nitrocellulose membrane (Porablot, Germany) 10A7 antibody against hGH (gift from professor Richard Ross, Sheffield University) diluted 1:1000 as the primary antibody and sheep anti-mouse IgG conjugated to HRP (Amersham Pharmacia Biotech) diluted 1:1000 as the secondary antibody were used for dot blotting and Western blotting. Recombinant hGH proteins on the membrane were visualized with one-step TMB (3, 3_, 5, 5_-tetramethylbenzidine) blotting substrate (Cyto Matin Gene, Company, Iran). Samples of supernatant under denaturizing conditions (heated at 95 °C for 5 min) mixed with 2-Mercaptoethanol loaded on SDS–PAGE gel (with the 12% acrylamide concentration of the resolving gel).

Table 1
Factors (parameters) and levels in Taguchi experimental design.

	Levels			
Temperature	18	25	30	37
Concentration of IPTG	0.05	0.1	0.5	1
Induction period	3	6	10	18
Strains of *E. coli*		*Rosetta-Gami*	*Origami*	
Culture media	LB	TB containing 0.6 M glycerol	TB containing 1.2 M glycerol	TB containing 0.6 M glycerol and 3% ethanol