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Improving Pertuzumab production by gene optimization and proper signal peptide selection

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ABSTRACT

Using proper signal peptide and codon optimization are important factors that must be considered when designing the vector to increase protein expression in Chinese Hamster Ovary (CHO) cells. The aim of the present study is to investigate how to enhance Pertuzumab production through heavy and light chain coding gene optimization and proper signal peptide selection. First, CHO-K1 cells were transiently transfected with whole-antibody-gene-optimized, variable-regions-optimized and non-optimized constructs and then we employed five different signal peptides to improve the secretion efficiency of Pertuzumab. Compared to the native antibody gene, a 3.8 fold increase in Pertuzumab production rate was achieved with the whole heavy and light chain sequence optimization. Although an overall two fold increase in monoclonal antibody production was achieved by human albumin signal peptide compared to the control signal peptide, this overproduction was not statistically significant. Selected signal peptides had no effect on the binding of Pertuzumab to the ErbB2 antigen. The combined data indicate that human albumin signal peptide along with whole antibody sequence optimization can be used to improve Pertuzumab production rates. This sequence was used to produce Pertuzumab producing CHO-K1 stably transfected cells. This result is useful for producing Pertuzumab as a biosimilar drug.

1. Introduction

There are a large variety of expression systems for heterologous protein production, including bacteria, mammalian cell lines, fungi, and plants. Among these systems, the ability of mammalian cell lines including Chinese hamster ovary (CHO), NS0, Sp2/0, HEK293, and PER.C6, to perform post-translational modifications (PTMs), especially glycosylation, has made them suitable hosts for recombinant protein production. Within mammalian expression platforms, CHO cells have become the host of choice for production of most marketed recombinant protein pharmaceuticals [1–4].

Despite the ability to perform PTMs, there are many limitations on the use of CHO cells as the host cell line for heterologous protein production, including slow growth, low productivity, and relatively high production timing and costs [2,5]. In order to reduce the production cost, it is strongly important to improve the production yield, which in turn influenced by both cell line development and cell culture process optimization [6–8]. There are several factors affecting the cell line productivity including the nature of the expression vector, vector transfection method, and host cell line [9,10]. Using strong promoter, proper signal peptide, and gene of interest (GOI) codon optimization are important factors that must be considered when designing the vector to increase protein expression [11].

In order to significantly improve the expression levels of heterologous genes in CHO, optimization of gene coding-sequence, is an effective strategy [9,12]. There are several algorithms, which optimize a variety of parameters including preferred codon usage, removal of splicing sites, improving mRNA stability by optimizing GC content, elimination of direct repeats and secondary structure elements, removal of RNA instability motifs, and evaluating the interaction of codon and anti-codon [9,13–15]. These parameters are critical to improve transcription, translation, and protein folding and therefore can strongly increase the expression levels

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It has been demonstrated that the efficient expression of heterologous proteins depends on the selection of appropriate signal peptides in order to transport of the synthesized protein into the endoplasmic reticulum (ER) for correct processing and secretion [17]. Signal peptides from different species can properly and efficiently recognized by the CHO cells, and the native signal peptide is not necessarily the best one [18,19]. To date several studies have been conducted that examined the effectiveness of different signal peptides on protein production in CHO cells [17,19,20].

Pertuzumab, which is approved by FDA in 2012 for the treatment of metastatic breast cancer, is a humanized monoclonal antibody that targets subdomain II of the ErbB2 extracellular domain, prevents ErbB2 from homo- and heterodimer formation, and inhibits ErbB2 function [21,22]. ErbB2 homo- or heterodimerization with other members of HER family results in intracellular domain autophosphorylation and activation of signaling pathway and cell proliferation [23]. Some types of cancer, including breast and ovary cancer, undergoing ErbB2 gene amplification or overexpression [22,24–26].

In this study, the effect of gene optimization and different types of signal peptides on the production of Pertuzumab mAb in CHO-K1 cell line were investigated by means of production capacity and product quality.

2. Materials and methods

At first, the effect of gene optimization on the production of Pertuzumab was evaluated and then the best antibody producer gene sequence was selected and fused to five different signal peptides.

2.1. Gene optimization study

Here we investigated the effect of gene optimization on Pertuzumab mAb production in CHO-K1 cells by comparing the native Pertuzumab gene sequence to a sequence-optimized variant generated by GenScript's Multi-parameter Gene Optimization algorithm, OptimumGene™ (NJ, USA). Genes encoding the native Pertuzumab heavy chain (HC: **1S78_F**) and light chain (LC: **1S78_E**) (Non Optimized: NO), and whole Pertuzumab codon optimized HC and LC (Fully Optimized: FO) were synthesized. Then the DNA fragments encoding the variable regions of the codon optimized HC and LC were fused to the constant regions of native Pertuzumab HC and LC, respectively, by overlap PCR (Variable Optimized: VO).

2.2. Plasmid construction

All HC coding genes were cloned into pBudCE4.1 expression vector (Invitrogen, NY, USA) at *Sal*I/*Xba*I restriction site and LC coding genes at *Not*I/*Xho*I site to generate pBud-NO, pBud-FO, and pBud-VO constructs. Plasmid DNA was amplified in *E. coli* and purified using PureLink® HiPure Plasmid Filter Maxiprep Kit (Invitrogen, NY, USA).

2.3. Transient expression

The expression level of NO, FO, and VO sequences were compared in transient transfections in adherent CHO-K1 cells, which was a generous gift from Prof Fazel Shokri (Tehran University of Medical Sciences, Tehran, IRAN). CHO-K1 cells were routinely maintained in the RPMI 1640 supplemented with 10% FBS (both from Gibco, Life Technologies, Grand Island, NY, USA). For transient transfection, CHO-K1 cells were transfected with 20 μg of each pBud-NO, pBud-FO, and pBud-VO plasmids together with 6 μg pCMV6-AC-GFP vector (OriGene Technologies, Herford, Germany) using Gene Pulser Xcell (Bio-Rad, CA, USA) (Capacitance: 950 μF, Voltage: 300 V and Resistance: ∞), and immediately transferred to T-25 culture flasks in the RPMI 1640 containing 10% FBS and incubated for 48 h at 37 °C in a 5% CO2 incubator. Co-transfection of the pCMV6-AC-GFP vector was done to normalize the transfection efficiency. Transfection was performed in triplicates.

2.4. Quantification of secreted Pertuzumab by ELISA

At 48 h post-transfection, supernatant from NO, FO, and VO T-25 flasks were collected for Pertuzumab concentration analysis using sandwich Enzyme-Linked ImmunoSorbent Assay (ELISA) and Pertuzumab purification using HiTrap Protein G HP column (GE Healthcare, Uppsala, Sweden). Briefly, a 96-well ELISA plate (PolySorp, NUNC™, Denmark) was coated with 50 μL of rabbit anti-human Immunoglobulin G (IgG) (prepared in our laboratory) in Bicarbonate/carbonate coating buffer (100 mM), incubated overnight at 4 °C and blocked with PBS supplemented with 0.05% Tween 20 (Bio-Rad) and 3% nonfat skim milk. Diluted cell supernatant samples were added to the plates and were incubated for 1.5 h at room temperature. Perjeta (Roche, Welwyn Garden City, UK) was used as a positive control to create a standard curve. Plate was washed 3 times with PBS/0.05% Tween 20. HRP-conjugated, goat anti-human IgG antibody (1:60000; Sigma, MO, USA) was added and plate incubated for 1 h at 37 °C and washed as above. Then 50 μL of TMB substrate (Invitrogen) was added per well and incubated at room temperature for 10 min, and the reaction was stopped by the addition of 50 μL of 0.25 M sulfuric acid. Plate was read on a standard absorbance microplate reader (Biochrom anthos 2020, Cambridge, UK) at 450 nm.

The CHO-K1 transfected cells were also collected for analysis of GFP fluorescent intensity to determine transfection efficiency using a FACS Calibur (BD Biosciences, NJ, USA). Then the expression level of Pertuzumab was normalized to GFP expression, according to Ho et al. [27].

2.5. Purification of Pertuzumab by affinity chromatography

Purification was performed using a HiLoad Pump P-50 (GE Healthcare, Uppsala, Sweden) and UV detection at 280 nm. 0.2 μm filtered cell culture supernatant was loaded on a HiTrap Protein G HP column (GE Healthcare). Sample was injected at a flow rate of 0.2 mL/min in binding buffer (20 mM sodium phosphate (Merck, Darmstadt, Germany), pH 7.0) followed by an elution buffer of 0.1 M glycine-HCl (Merck), pH 2.7. Eluted samples were neutralized using 1 M Tris pH 9.0 (Merck) and then buffer exchanged using dialysis tubing (Sigma). Purified Pertuzumab was quantified by sandwich ELISA.

2.6. Western blotting of purified Pertuzumab

To prove the authenticity of specific binding of purified Pertuzumab mAbs to the ErbB2 extracellular domain, western blot analysis in non-reducing condition and flow cytometric analysis were performed according to Amiri et al. [28]. In brief, 50 ng of the recombinant extracellular part of ErbB2 (SinaBiotech, Tehran, Iran) was loaded on 12.5% Native-PAGE gel. Separated proteins were transferred to polyvinylidene difluoride (PVDF) (GE Healthcare) membranes using the Trans-Blot® Turbo™ Blotting System (Bio-Rad, CA, USA). Membranes were blocked in PBS supplemented with 0.15% Tween 20 (Bio-Rad) and 5% nonfat skim milk, overnight at 4 °C. A total of 10 μg/mL of each purified Pertuzumab mAbs and Perjeta (as a positive control) were added at room temperature for 1.5 h on a shaker and then incubated for 1 h in HRP-conjugated,