Protein Expression and Purification 118 (2016) 120–125

Contents lists available at ScienceDirect

Protein Expression and Purification

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

Cloning and expression of codon-optimized recombinant *darbepoetin alfa* in *Leishmania tarentolae* T7-TR

Anvarsadat Kianmehr [a, *], Raziyeh Golavar [b], Mandana Rouintan [b], Abdolkarim Mahrooz [c], Pezhman Fard-Esfahani [d], Morteza Oladnabi [e], Safoura Khajeniazi [a, f], Seyede Samaneh Mostafavi [b, **], Eskandar Omidinia [d, ***]

[a] Department of Medical Biotechnology, Faculty of Advanced Medical Technologies, Golestan University of Medical Sciences, Gorgan, Iran
[b] Department of Genetics, Islamic Azad University, Arsanjan Branch, Fars, Iran
[c] Immunogenetic Research Center, Mazandaran University of Medical Sciences, Sari, Iran
[d] Department of Biochemistry, Genetics and Metabolism Research Group, Pasteur Institute of Iran, Tehran 13164, Iran
[e] Department of Medical Genetics, Faculty of Advanced Medical Technologies, Golestan University of Medical Sciences, Gorgan, Iran
[f] Stem Cell Research Center, Golestan University of Medical Sciences, Gorgan, Iran

ARTICLE INFO

Article history:
Received 10 May 2015
Received in revised form
25 October 2015
Accepted 27 October 2015
Available online 3 November 2015

Keywords:
Darbepoetin alfa
Leishmania tarentolae
Codon optimization
Expression

ABSTRACT

Darbepoetin alfa is an engineered and hyperglycosylated analog of recombinant human erythropoietin (EPO) which is used as a drug in treating anemia in patients with chronic kidney failure and cancer. This study desribes the secretory expression of a codon-optimized recombinant form of darbepoetin alfa in *Leishmania tarentolae* T7-TR. Synthetic codon-optimized gene was amplified by PCR and cloned into the pLEXSY-I-blecherry3 vector. The resultant expression vector, pLEXSYDarbo, was purified, digested, and electroporated into the *L. tarentolae*. Expression of recombinant darbepoetin alfa was evaluated by ELISA, reverse-transcription PCR (RT-PCR), Western blotting, and biological activity. After codon optimization, codon adaptation index (CAI) of the gene raised from 0.50 to 0.99 and its GC% content changed from 56% to 58%. Expression analysis confirmed the presence of a protein band at 40 kDa. Furthermore, reticulocyte experiment results revealed that the activity of expressed darbepoetin alfa was similar to that of its equivalent expressed in Chinese hamster ovary (CHO) cells. These data suggested that the codon optimization and expression in *L. tarentolae* host provided an efficient approach for high level expression of darbepoetin alfa.

1. Introduction

Darbepoetin alfa is an engineered human erythropoietin (EPO) analog with two additional sialic acid chains. It has been designed to contain five N-linked glycosylation sites (Asn 24, 30, 38, 83, 88), two more than its natural variant. The inserted new positions have been shown to have no interference with its receptor binding ability or have no effect on the conformation of EPO [1,2]. The drug stimulates the bone marrow to produce more red blood cells. Thus it is used for treatment of anemia in patients with chronic kidney failure or special types of cancer [3]. Darbepoetin alfa with a molecular weight of approximately 40 kDa has longer circulating half-life and greater in vivo bioactivity than the EPO. Due to these properties, it can be administered less frequently to attain a proper biological influence [4]. In 2000, the darbepoetin alfa gene was constructed through the site-directed mutagenesis performed on the EPO gene and was expressed in CHO cells by Egrie and his co-workers [5]. Darbepoetin alfa was approved in 2001 by the Food and Drug Administration (FDA) for the treatment of anemia. It is marketed by the Amgen company under the trade name of Aranesp and had more than 6$ billion sales in 2006. Therapeutic applications of darbepoetin alfa have extended interests for improving and refining methods for its manufacture. Expression and production of recombinant proteins in CHO cells generally have a number of drawbacks including low yield, complex nutritional needs, high purification cost, and possibility of product contamination.

* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: kiabiotpro@yahoo.com (A. Kianmehr), samaneh.mostafavi@gmail.com (S.S. Mostafavi), e.omid8@yahoo.com (E. Omidinia).

http://dx.doi.org/10.1016/j.pep.2015.10.013
1046-5928/

Therefore, the use of an alternative expression system with the ability to solve these problems was considered. Among the Trypanomatidae family, *Leishmania tarentolae* known to be a non-pathogenic parasite, has recently been introduced and employed as a potential eukaryotic expression host [6,7]. *L. tarentolae* is rich in glycoproteins and its glycosylation pattern is similar to that of mammalian system that includes complex-type oligosaccharides [8]. Moreover, *L. tarentolae* has some advantages, including simple nutrient requirements, straightforward handling, rapid growth rate, and also potential for large scale production of recombinant proteins. These features make *L. tarentolae* an appealing and potent host for biotechnological applications [9]. To date, several successful examples of using *L. tarentolae* in the expression proteins, such as EPO [10], interferon-gamma (IFN-γ) [11], and IgG [12] were also reported. The data presented here describe the cloning and expression of recombinant darbepoetin alfa in *L. tarentolae* T7-TR secretory expression system. This is the first report on the production of darbepoetin alfa in *L. tarentolae*.

2. Materials and methods

2.1. Hosts, plasmid and chemicals

L. tarentolae T7-TR strain, pLEXSY-I-blecherry3 vector (Cat.-No. EGE-1410), in addition to brain heart infusion (BHI) medium and all required materials were purchased from Jena Bioscience (Jena Bioscience, Jena, Germany). All chemicals were also obtained from Sigma–Aldrich (St. Louis, USA). Darbepoetin alfa with the trade name of Aranesp was prepared from Amgen (Thousand Oaks, CA, USA).

2.2. Codon optimization and gene synthesis

The DNA sequence of darbepoetin alfa was obtained from a published sequence [5,13] and used to gene design. Codon optimization was done by the online program Optimizer (http://genomes.urv.es/OPTIMIZER) based on the codon usage table of *L. tarentolae* (http://www.kazusa.or.jp/codon). Native and codon optimized darbepoetin alfa genes were synthesized and cloned into the pGH cloning vector (BIONEER, Korea).

2.3. Construction of expression plasmid for darbepoetin alfa

The expression cassette was constructed using components of pLEXSY-I-blecherry3 vector (Jena Bioscience, Cat. No. EGE-1410). The native and synthetic codon optimized darbepoetin alfa genes were amplified by polymerase chain reaction (PCR) technique from the pGH plasmid. The forward and reverse primers, which contained restriction sties in 5′terminus (underlined) were; Darbo-forward: 5′-ATTCTAGACGCGCCGCCG-3′and Darbo-reverse: 5′-AGGTACCGCGGTCGCCC-3′. The restriction sites in forward and reverse primers correspond to *Kpn*I and *Xba*I enzymes, respectively. PCR was performed by thermal cycler (Eppendorf, Germany) under standard protocol consisted of a 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 56 °C for 1 min, 72 °C for 40 s and 72 °C for 10 min as a final extension step. The obtained PCR product (503 bp) was digested with *Kpn*I and *Xba*I, gel purified and ligated into a digested pLEXSY-I-blecherry3 expression vector. Then the ligation reaction was transformed into *Escherichia coli* TOP10. The resultant expression construct (pLEXSYDarbo) was purified from the recombinant colonies using an alkaline lysis method (Qiagen Plasmid Maxi Kit), verified by restriction enzyme digestion and DNA sequencing.

2.4. Transfection of L. tarentolae T7-TR

Initially, *L. tarentolae* T7-TR was grown as a static suspension in BHI broth medium containing 5 μg/ml hemin, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml hygromycin at pH 7.2 and 26 °C. Afterwards, pLEXSYDarbo plasmid was digested with *Swa*I restriction enzyme and the 6000 bp fragment containing darbepoetin alfa gene was gel purified. For transfection, log-phase parasites with OD_{600} = 2.0 resuspended in 400 μl of ice-cold electroporation buffer (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose, pH 7.5), mixed with approximately 15 μg of the linearized expression cassette and then electroporated using a Bio-Rad Gene Pulser at 500 μF, 450 V and two pulses. The electroporated promastigotes were incubated in BHI broth medium at 26 °C for 24 h without any drug. Selection of single colonies was done by growth on solidified BHI medium containing 100 μg/ml nourseothricin (NTC) and 100 μg/ml bleomycin [14].

2.5. Confirmation and screening of transfectants by diagnostic PCRs

To confirm the homologues recombination integration of the cassette containing darbepoetin alfa into the *odc* locus of *L. tarentolae* T7-TR genome in transfectant strains, different diagnostic PCRs were performed. For this purpose, primer pairs including one primer hybridizing within the expression cassette and one primer hybridizing to an *odc* sequence not present on the plasmid were applied. The information related to the primers is shown in Table 1. Genomic DNA from 5 ml of a dense culture (OD approx. 2–3) was prepared by conventional phenol/chloroform extraction. The PCR reactions resulted in a characteristic fragment for each PCR (Table 1), which was not observed in control reactions. Diagnostic PCR, including darbepoetin alfa-specific primer was also performed.

2.6. Expression and purification

For secretory expression of recombinant protein, transfected *L. tarentolae* cells were grown in BHI medium supplemented with hemin, penicillin, streptomycin, bleomycin, and NTC at 26 °C as static suspension culture in TC flasks. The T7 driven transcription was induced with 10 μg/ml tetracycline for 72 h after inoculation. The induction was also monitored by the measurement of fluorescence at 590 nm (excitation) and 620 nm (emission). Expression of recombinant darbepoetin alfa was confirmed by ELISA, reverse-transcription PCR (RT-PCR) and Western blotting. Recombinant *L. tarentolae* cells were harvested from a 3-day cell culture by centrifugation at 3500 rpm for 10 min and supernatant of the cell culture was removed and concentrated. The precipitated sample was dissolved in 1X SDS polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and then boiled for 5 min. Samples from both wild and transgenic *L. tarentolae* was separated on 12% (w/v) SDS-PAGE gel. For western blot, the resolved proteins were transferred to the PVDF membrane using a wet blotting system and incubated with TBST solution containing 3% bovine serum albumin (BSA) for overnight at 4 °C. The membrane was washed three times with TBST and reacted with anti-EPO antibody (2 μg; rabbit polyclonal antibody to EPO; Abcam, UK) as the first antibody for 2 h at room temperature. After three washes, goat anti-rabbit horseradish peroxidase (HRP)–conjugated IgG was added and incubated for 1 h at room temperature. The band of target protein was detected using 3, 3′-Diaminobezidine (DAB) and H_2O_2. For RT-PCR reaction, RNA samples were extracted according to the manufacturer's instructions (Jena Bioscience, Jena, Germany). The cDNA synthesis was done through cDNA synthesis kit (Premix kit, BioNEER, Korea)