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

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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.

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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)

Table 1
Nucleotide sequences of primers used in diagnostic PCRs.

Primer	Sequence (5′→3′)	Annealing temp.	Amplicon size (bp)
5′odc-utr1 (aprt)			
A1304	TCCGCCATTCATGGCTGGTG	60 °C	1100
A1715	TATTCGTTGTCAGATGGCGCAC		
Blecherry- 3′odc			
A708	GGATCCACCGCATGGCCAAGTTGACCA	60 °C	2700
P1510	GTGGTGCACCCATAGTAGAGGTGC		
Darbepoetin alfa gene			
Darbo-F	ATTCTAGACGCGCCGCCG	56 °C	500
Darbo-R	AGGTACCGCGGTCGCCC		

and PCR analysis was performed under standard conditions (annealing temperature 62 °C). To purify the recombinant protein, concentrated supernatant of *L. tarentolae* culture was applied to a Ni-NTA affinity column (Qiagen, Germany) according to the manufacture's instruction. The column was washed with 3 column volumes of the 50 mM Tris–HCl buffer (pH 7.0) containing 50 mM imidazole, and then darbepoetin alfa was eluted with an elution buffer (50 mM Tris–HCl, 50 mM NaCl, 10 mM EDTA, 500 mM imidazole, pH 7.0).

2.7. In vivo bioassay test

Inbred female BALB/c mice weighting 18–22 g, and about 6–8 weeks of day were prepared from Research and Production Complex of Pasteur Institute of Iran, Karaj, Iran and housed under standardized conditions (25 °C, 12 h light/12 h darkness, humidity 50–55%) with free access to pelleted food and tap water. Each was received identical amount of purified darbepoetin alfa by subcutaneous injection (5 μg/kg in 100 μl). Peripheral blood was harvested every 24 h and heamtocrite was measured using a microhaematocrit centrifuge and Hematology Analyzer (Sysmex, Kobe, Japan).

3. Results

3.1. Design of darbepoetin alfa gene for optimal expression in L. tarentolae

The synthetic darbepoetin alfa gene was assembled based on the *L. tarentolae* codon usage. The native and codon-optimized constructs included the ORF encoding darbepoetin alfa and a C-terminal 6xHis-tag to facilitate purification. The alignment of DNA and amino acid sequences of native and designed gene were depicted (see Fig. 1). As seen, amino acid sequences were absolutely identical. The optimized gene had 79.57% of identity with respect to the wild type. To achieve the maximal resemblance with highly expressed *L. tarentolae* genes, the codon composition of synthetic gene was reprogrammed using the most frequently used codons for *L. tarentolae*. Out of 145 amino acids in the sequence of darbepoetin alfa, 56 codons were modified in codons highly preferred by *L. Tarentolae* (see Fig. 1). Upon codon optimization, codon adaptation index (CAI) increased from 0.50 to 0.99 and GC% content was changed from 56% to 58%. Additionally, within synthetic codon-optimized gene, the splice sites, polyadenylation signal, instability elements, and cis-acting sites were removed.

3.2. Construction of pLEXSYDarbo expression cassette

The native darbepoetin alfa construct was similar to the codon-optimized variant in all respect, but its results were not shown. The synthetic darbepoetin alfa genes were isolated by PCR on the pGH plasmid. A single 503 bp band was observed on agarose gel after electrophoresis. The obtained PCR product was cut with *Kpn*I and *Xba*I restriction enzymes, extracted from the gel and ligated into pLEXSY vector. The pLEXSYDarbo expression cassette includes *Kpn*I and *Xba*I cloning sites downstream of the signal peptide which originally belongs to the *Leishmania mexicana* secreted acid phosphatase (LMSAP) and a C-terminal His-tag for purification. The correct gene cloning was confirmed by restriction analysis and DNA sequencing.

3.3. Confirmation of integration into the expression cassette

The recombinant pLEXSYDarbo plasmid was prepared in large scale, linearized with *Swa*I restriction enzyme and transfected into *L. tarentolae* T7-TR cells by electroporation. The transfectnats were selected by plating on BHI agar medium containing 100 μg/ml bleomycin and 100 μg/ml NTC. Individual clones were selected and transferred into culture plates and then into tissue culture flasks. Integration of the expression construct into the *ssu* locus of recombinant cells was confirmed by PCR analysis which generated 1.1 (Fig. 2a) and 2.7 kbp (Fig. 2b) fragments. Amplificaltion of these fragments were not obtained in genomic DNA of the wild type parasite (Fig. 2c). Moreover, a PCR reaction with darbepoetin alfa specific primers was done. The expected PCR product size (503 bp) was only seen from transfected cells.

3.4. Expression analysis and purification of recombinant darbepoetin alfa

The secretory expression of darbepoetin alfa protein in *L. tarentolae* T7-TR host was investigated by ELISA, RT-PCR, and Western blotting techniques. The selected recombinant clone showed detectable expression data in comparison with wild type cell by ELISA (data not shown). In order to verify mRNA synthesis in *Leishmania* host, RT-PCR was performed by darbepoetin alfa specific primers. Transfected cells were indicated to have a dominant band of 503 bp on agarose gel whereas in wild cells, this fragment was not detected (see Fig. 3a). The purified protein with an expected size of 40 kDa was achieved on SDS-PAGE gel (Fig. 3b). Western blot method certainly confirmed that the band which was observed in SDS-PAGE was target protein (Fig. 3c). Furthermore, Western blotting results revealed that the expressed darbepoetin alfa was also homogeneous. The expression level in *L. tarentolae* T7-TR was determined to be 11 mg/L. The yield of purified protein from the codon-optimized construct was 64%. The procedure resulted in purification of 7.0 mg protein from 1 L of suspension culture.

3.5. Potency of produced darbepoetin alfa

The potency of recombinant darbepoetin alfa in increasing hematocrit was quantified and compared with Aranesp. The result of in vivo bioassay was depicted in Fig. 4. As can be found, the content of reticulocyte increase for both produced darbepoetin alfa in *L. tarentolae* and also Aranesp was nearly similar.

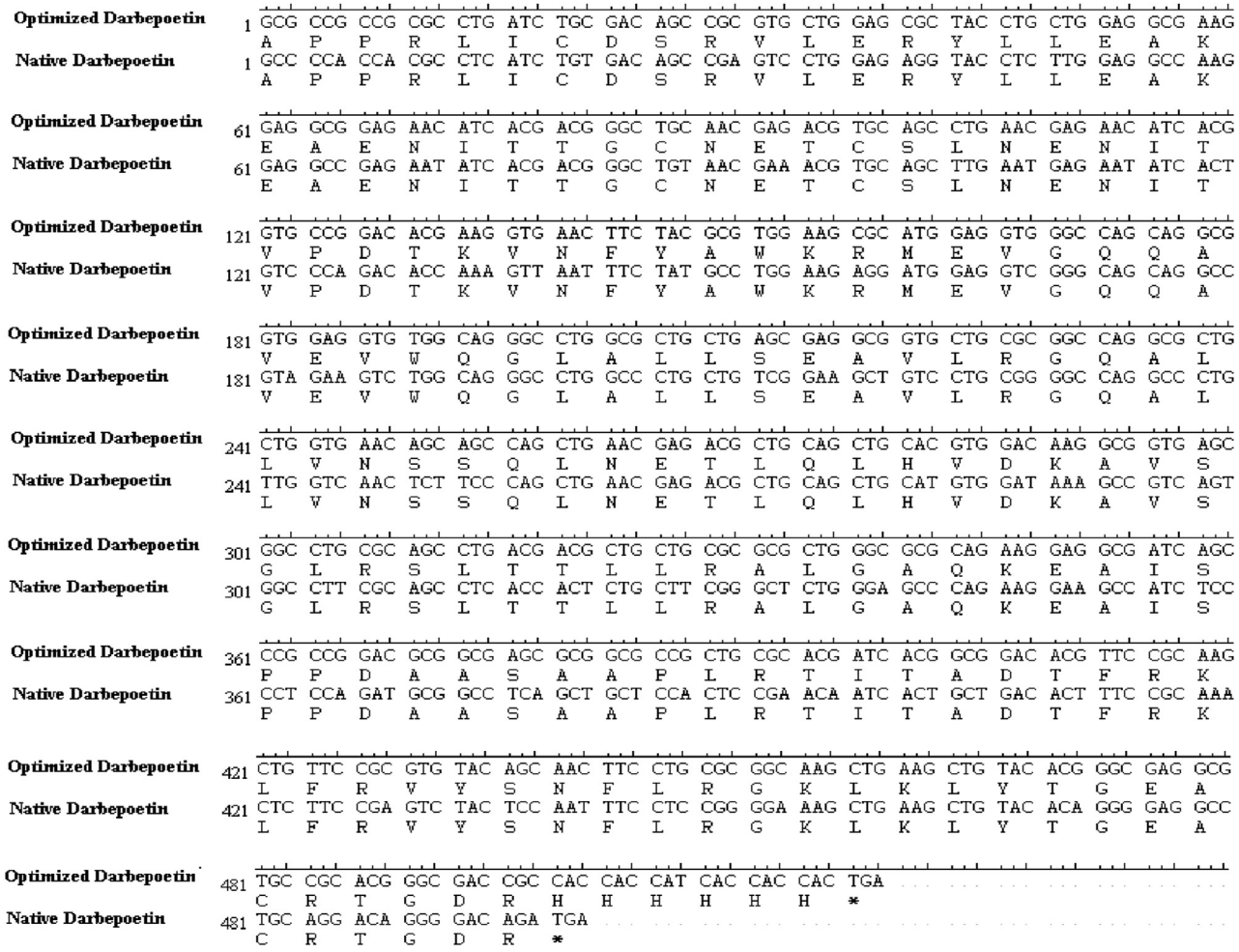

Fig. 1. DNA and amino acid sequence alignment of darbepoetin alfa native gene and its synthetic variant designed for optimal expression in *L. tarentolae* host.

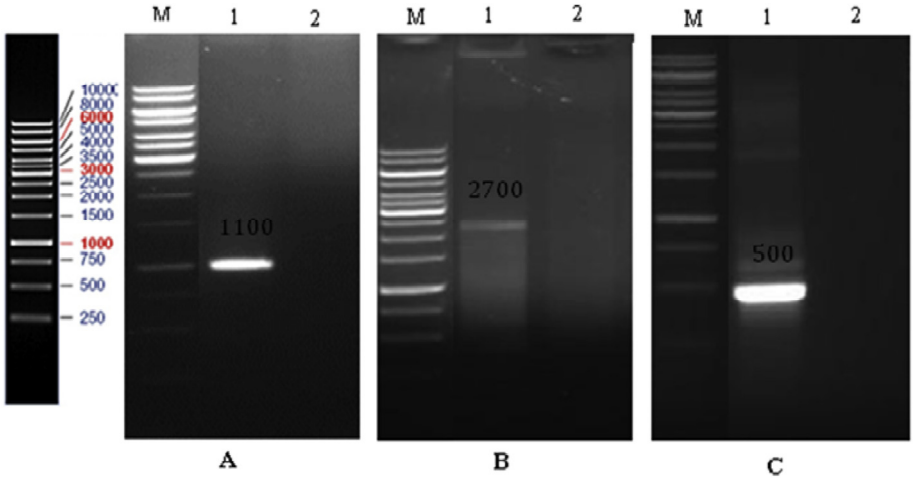

Fig. 2. (A) Diagnostic PCR on genomic DNA of recombinant *L. tarentolae* cells with A1304 and A1715 primers. Lane M: DNA molecular weight marker; Lane 1: 1.1 kbp PCR product; Lane 2: Wild type parasite (B) Diagnostic PCR on genomic DNA of recombinant *L. tarentolae* cells with A708 and P1510 primers. Lane M: DNA molecular weight marker; Lane 1: 2.7 kbp PCR product; Lane 2: Wild type parasite (C) Diagnostic PCR on genomic DNA of recombinant *L. tarentolae* cells with Darbo-F and Darbo-R primers. Lane M: DNA molecular weight marker; Lane 1: 500 bp PCR product; Lane 2: Wild type parasite.

4. Discussion

Darbepoetin alfa, a synthetic and hyperglycosylated form of human erythropoietin, has an extended circulating half-life and thus a higher in vivo bioactivity than EPO [15,16]. *L. tarentolae* is a unicelluar non-pathogenic parasite of white spotted wall gecko, *Tarentola annularis*, which have recently attracted a huge attention as a feasible eukaryotic expression system for the expression of

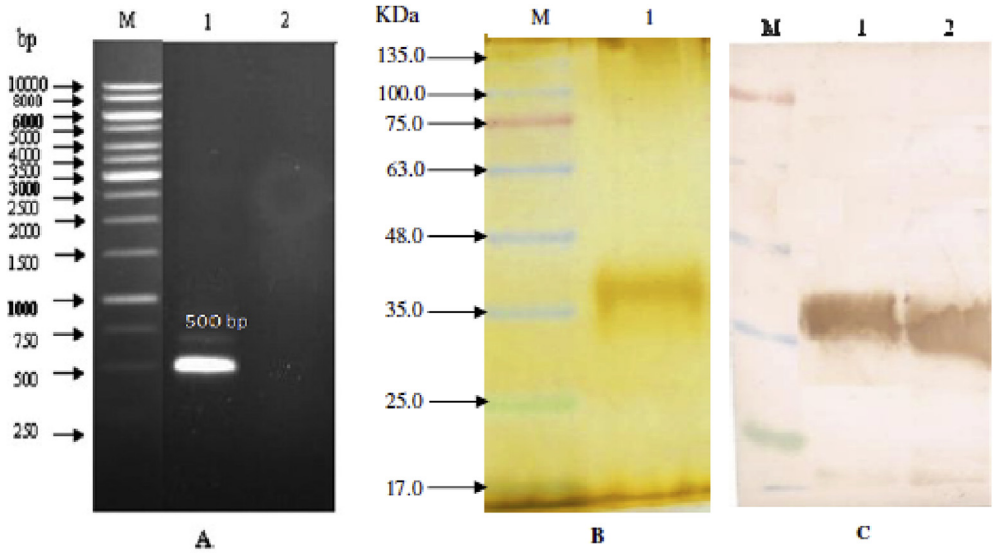

Fig. 3. Expression analysis of recombinant darbepoetin alfa produced in *L. tarentolae* T7-TR by RT-PCR, SDS-PAGE and Western blotting methods. (A) RT-PCR. Lane M: DNA molecular weight marker; Lane 1: wild parasite as negative control; Lane 2: transfected cell with darbepoetin alfa expression cassette. (B) SDS-PAGE. Lane M: protein marker; Lane 1: purified darbepoetin alfa. (C) Western blot. Lane M: protein marker; Lane 1: transfected cell with codon-optimized darbepoetin alfa; Lane 2: transfected cell with native darbepoetin alfa.

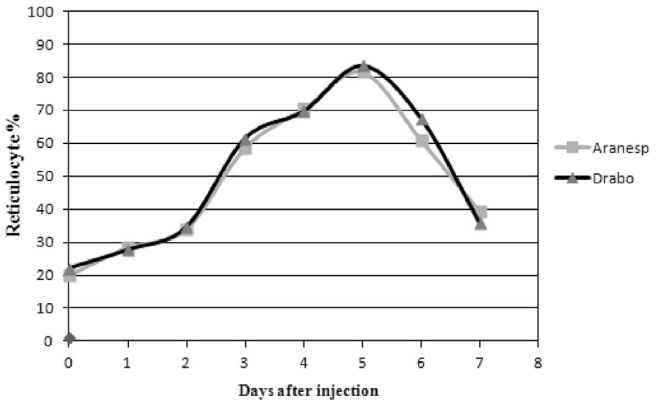

Fig. 4. In vivo bioactivity of recombinant darbepoetin alfa. Blood samples were collected daily and reticulocytes were detected.

active recombinant biopharmaceuticals. It was indicated that this host has some unique advantages including higher specific growth rate compared to mammalian cells, simple and low cost nutrient needs, the least required biosafety level for humans, possibility to introduce several copies of a foreign gene into the parasite genome, and production of recombinant proteins with a mammalian-like N-glycosylation pattern. These advantages, besides the possibility of constitutive or inducible protein production, make the *L. tarentolae* a highly potential host for heterologous expression of recombinant proteins [17–20]. In the present study, we established a heterologous secretory expression system for high yield production of recombinant darbepoetin alfa in *L. tarentolae* T7-TR. However, the presence of rare codons could hamper the expression of darbepoetin alfa, due to inefficient translation in the host. A strategy to improve the codon translation efficiency by *L. tarentolae* was the construction of a codon-optimized synthetic gene. Therefore, we optimized codons of darbepoetin alfa according to the codon usage of *L. tarentolae* to achieve the highest possible level of expression. After codon optimization, CAI raised from 0.50 to 0.99 and the GC% content underwent a change from 56% to 58%, indicating optimal adaptation and suggesting efficient translation. Higher CAI value and GC content which was closer to that of *L. tarentolae*, also provided a good estimation for high level expression. Expression analysis showed the presence of a protein band at 40 kDa. Ultimately, reticulocyte experiment results displayed that the activity of optimized darbepoetin and that of Aranesp was identical. Therefore, all the obtained data suggested an acceptable functional and structural profile for recombinant codon-optimized darbepoetin alfa which is comparable to those of the native protein. In 2002, Breitling et al. reported the study on expression of EPO in *L. tarentolae* [10]. Although they took advantage of the native sequence, the expression level was achieved to be 30 mg/L of suepension culture. The reported expression level for proteins in *L. tarentolae* usually varied between 0.1 and 30 mg/L. For example, the expression level for green fluorescent protein (GFP) [22], IFN-γ [11], and tissue plasminogen activator (t-PA) [21] were reported to be 30.0, 9.5, and 0.17 mg/mL, respectively. In this study a codon-optimized ORF was used that gave an expression level of 11 mg/L of culture medium. This was more than double what was reported in CHO cells (4.5 mg/L). The expression of native darbepoetin alfa gene was also measured to be 6 mg/L of culture medium. Hence, it can be concluded that the codon optimization could be adopted as a good approach to enhance production yield and, as a result to diminish the production cost of the protein, i.e. higher protein yield from the same conditions. What is more, the lower expression of darbepoetin alfa in comparison with the EPO in *L. tarentolae* may be rooted in the lower number of gene copies integrated into the host's genome. Based on the reports of Jena Bioscience, there are numerous examples of highly expressed genes with native codons and thus, using optimized synthetic gene does not always guarantee a higher expression level. It is proper to mention that production yield of the recombinant protein could be enhanced by further optimization of expression conditions such as culture method (static or agitated suspension cultures) and induction parameters (tetracycline concentration, time point of induction and induction time) using one-variable-at-a-time studies or statistical techniques e.g. response surface methodology (RSM) [23,24]. We are currently pursuing this subject in our laboratory.

5. Conclusion

Taken together, we generated a recombinant *L. tarentolae* strain

expressing the codon-optimized darbepoetin alfa. Our results suggested that *L. tarentolae* can be used as a host for production of hyperglycosylated proteins like darbepoetin alfa and that still codon optimization might have a boosting effect for protein expression in *L. tarentolae*. The presented work is the first report on secretory expression of codon-optimized darbepoetin alfa in *L. tarentolae* T7-TR.

Acknowledgments

This project was financially supported by the Pasteur Institute of Iran. The authors wish to express their deep gratitude to all who provided support, especially enzyme technology lab. of Metabolic and Genetic Research Group, Pasteur Institute of Iran.

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