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ORIGINAL PAPER

Purification and Characterization of Recombinant Darbepoetin Alfa from *Leishmania tarentolae*

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Abstract Darbepoetin alfa is a biopharmaceutical glycoprotein that stimulates erythropoiesis and is used to treat anemia, which associated with renal failure and cancer chemotherapy. We herein describe the structural characterization of recombinant darbepoetin alfa produced by *Leishmania tarentolae* T7-TR host. The DNA expression cassette was integrated into the *L. tarentolae* genome through homologous recombination. Transformed clones were selected by antibiotic resistance, diagnostic PCRs, and protein expression analysis. The structure of recombinant darbepoetin alfa was analyzed by isoelectric focusing, ultraviolet–visible spectrum, and circular dichroism (CD) spectroscopy. Expression analysis showed the presence of a protein band at 40 kDa, and its expression level was 51.2 mg/ml of culture medium. Darbepoetin alfa have 5 isoforms with varying degree of sialylation. The UV absorption and CD spectra were analogous to original drug (Aranesp), which confirmed that the produced protein was darbepoetin alfa. Potency test results revealed that the purified protein was biologically active. In brief, the structural and biological characteristics of expressed darbepoetin alfa were very similar to Aranesp which has been normally expressed in CHO. Our data also suggest that produced protein has potential to be developed for clinical use.

Keywords Characterization · Darbepoetin alfa · *Leishmania tarentolae* · Purification

Introduction

Biopharmaceuticals are increasingly used to treat diseases such as multiple sclerosis, rheumatoid arthritis, cancer, and anemia. The use of drugs based on recombinant proteins for the treatment of anemia is more safe and efficient compared to transfusion therapy. The implementation of recombinant human erythropoietin (rhEPO) in clinical practice revolutionized the approach to the correction of anemic conditions [1, 2]. Darbepoetin alfa is a hyperglycosylated analog of EPO

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that contains two additional sialic acid containing carbohydrate chains. It has been designed to contain five N-linked glycosylation sites (Asn 24, 30, 38, 83, 88). The new carbohydrate positions have been shown to have no interference with receptor binding or any disrupting effect on the structure of protein [3]. Darbepoetin alfa has been approved in 2001 by the Food and Drug Administration (FDA) for treating anemia. The drug stimulates the bone marrow to produce more red blood cells in patients with chronic kidney failure or special types of cancer [4]. The relative molecular weight of darbepoetin alfa is 40 kDa and has a longer circulating half-life and increased in vivo bioactivity than EPO. Due to these properties, this glycoprotein can be administrated less frequently to attain a proper biological influence [5, 6]. Darbepoetin alfa is marked by Amgen under the trade name Aranesp. Therapeutic applications of darbepoetin alfa have extended interest in improving methods for its production. Production of this drug in CHO has generally a number of drawbacks including low yield, high purification cost, and possibility of product contamination [7, 8]. For these reasons, taking advantage of an alternative expression system capable of solving these problems is helpful. Among Trypanomatidae family, *Leishmania tarentolae* is a non-pathogenic parasite and has been developed as a potential eukaryotic expression host for production of recombinant biopharmaceuticals [9, 10]. *L. tarentolae* is rich in glycoproteins and its glycosylation pattern is similar to that of mammalian that includes complex-type oligosaccharides. *L. tarentolae* has simple nutrient requirements, ability for large-scale production, high growth rate, and safety for humans. These features make *L. tarentolae* interesting as an alternative to mammalian cells for production of recombinant proteins [11, 12]. Different recombinant pharmaceutical glycoproteins such as EPO [10], interferon-gamma (IFN-γ) [13], IgG [12], and many other proteins have been produced by *L. tarentolae* host. In this communication, we characterized a recombinant darbepoetin alfa expressed by *L. tarentolae* expression system.

Materials and Methods

Construction of Darbepoetin Alfa Expression Cassette

Codon-optimized darbepoetin alfa gene was synthesized and cloned into the pGH cloning vector (BIONEER, Korea). Codon optimization was done by the on-line program Optimizer (http://genomes.urv.es/OPTIMIZER). The DNA expression cassette was constructed using components of pLEXSY_I-blecherry3 vector (Jena Bioscience, Cat. No. EGE-1410). The synthetic gene was amplified by polymerase chain reaction (PCR) 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 *Xba*I and *Kpn*I enzymes, respectively. PCR was performed by thermal cycler (Eppendorf, Germany) under 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, and 72 °C for 10 min as final extension. PCR product (503 bp) was digested with *Xba*I and *Kpn*I, and ligated into a digested pLEXSY_I-blecherry3 expression vector. The resultant expression construct (pLEXSYDarbo) was purified and verified by restriction digestion and DNA sequencing. Then, pLEXSYDarbo plasmid was digested with *Swa*I restriction enzyme and the 6000 bp fragment containing darbepoetin alfa gene was gel purified.

Production of Transgenic *L. tarentolae*

Leishmania tarentolae T7-TR (Jena Bioscience, Jena, Germany) was grown as a static suspension in Brain Heart Infusion (BHI) broth medium containing 5 μg/ml hemin, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml hygromycine at pH 7.2 and 26 °C. 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 10 μg of the linearized expression cassette and electroporated using an Bio-Rad Gene Pluser 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]. To confirm the homologs recombination integration into the *odc* locus of *L. tarentolae* genome, diagnostic PCRs were performed. Genomic DNA from 2 ml of a dense culture was prepared by conventional phenol/chloroform extraction. The PCR reactions will result in a characteristic fragment for each PCR (Table 1), which is not observed in control reactions where the template is the genomic DNA from the wild-type parasite.

Expression and Purification of Recombinant Protein

For secretory expression, 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 measurement of fluorescence at 590 nm (excitation) and 620 nm (emission) [12, 15]. 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 three 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).

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

SDS-PAGE electrophoresis under reducing and non-reducing conditions was performed in a 12 % gel, followed by staining with Coomassie brilliant Blue R-250 [16]. Recombinant *L. tarentolae* cells were harvested from a 3-day cell culture by centrifugation at 3500 rpm for 20 min and supernatant of the cell culture was removed and concentrated. The precipitated sample was dissolved in 1X 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.

Isoelectric Focusing

Isoelectric focusing (IEF) was performed in a gel with a pH gradient from 3.0 to 10.0 using GE Healthcare (Multiphor II, MultiTemp IV, EPS 3501 XL) with the following modes: prefocusing at a voltage of 800 V, amperage of 30 mA, and power of 25 W for 45 min; entering of proteins into the gel at 600 V, 30 mA, and 25 W for 45 min; and separation of proteins at 1600 V, 30 mA, and 25 W for 60 min. After the separation of proteins, the gel was placed in a fixation solution containing 0.7 M trichloroacetic acid and 0.16 M sulfosalicylic acid and incubated for 40 min at room temperature. Then, the gel was washed in a solution containing 8 % acetic acid and 25 % ethanol and subjected to staining as described above.

Ultraviolet–Visible spectrum

The ultraviolet–Visible (UV–Vis) absorption spectrum was measured from 200 to 700 nm via UV–visible spectrophotometer (Shimadzu 1601 PC, Japan) with cuvette length of 1 cm at room temperature. Aranesp was also used as positive control in spectrum analysis.

Circular Dichroism Measurement (CD)

The CD spectrum was collected with the JASCO J-820 Circular Dichroism Spectropolarimeter in phosphate buffer saline (PBS) (10 mM, pH 7.4) containing 0.3 mg/ml of darbepoetin alfa and Aranesp at room temperature. The ellipticity value [θ] was obtained in millidegree (mdeg) directly from the instrument. The cell path length of 1 cm was utilized for the range 190–260 nm. The scanning speed was 100 nm per min. In assay, five scans were made and the no-protein spectrum was subtracted. For a comparative study, Aranesp was used as positive control.

Potency Test

Inbred female BALB/c mice weighting 18–22 g, and about 6–8 weeks of day were prepared from the Research and Production Complex of Pasteur Institute of Iran, Karaj, Iran, and housed under standardized conditions with free access to pelleted food and tap water. Each one received identical amount of purified darbepoetin alfa and Aranesp (Amgen, Thousand, CA, USA) as positive control by subcutaneous injection (5 μg/kg in 100 μl). Peripheral blood was harvested every 24 h and hemtocrit was

Table 1 The primers used in diagnostic PCRs

Gene	Primer	Sequence ($5'\rightarrow3'$)	Annealing temp. (°C)	Amplicon size (bp)
5′odc-utr1 (aprt)	A1304	TCCGCCATTCATGGCTGGTG	60	
5′odc-utr1 (aprt)	A1715	TATTCGTTGTCAGATGGCGCAC	60	1100
blecherry- 3′odc	A708	GGATCCACCGCATGGCCAAGTTGACCA	60	
blecherry- 3′odc	P1510	GTGGTGCACCCATAGTAGAGGTGC	60	2700

measured using a microhematocrit centrifuge and Hematology Analyzer (Sysmex, Kobe, Japan).

Results

Cloning of DNA Expression Cassette into *L. tarentolae*

The synthetic darbepoetin alfa gene was isolated by PCR on the pGH plasmid. A single 503 bp band was observed on agarose gel after electrophoresis (Fig. 1a). The obtained PCR product was cut with *Xba*I and *Kpn*I restriction enzymes, extracted from gel and ligated into pLEXSY_I-blecherry3 vector. The pLEXSYDarbo expression cassette comprise *Xba*I/*Kpn*I cloning site downstream from the signal peptide of *L. mexicana* secreted acid phosphatase (LMSAP) and a C-terminal His-tag for purification. The correct gene cloning was confirmed by restriction analysis (Fig. 1b). The recombinant plasmid was prepared in large scale, linearized with *Swa*I restriction enzyme and transfected into *L. tarentolae* T7-TR cells by electroporation. The transfectants were selected by plating on BHI agar medium containing 100 µg/ml hygromycin, 100 µg/ml bleomycin, and 100 µg/ml NTC. Individual clones were selected and transferred into culture plates and then into TC 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 strains .

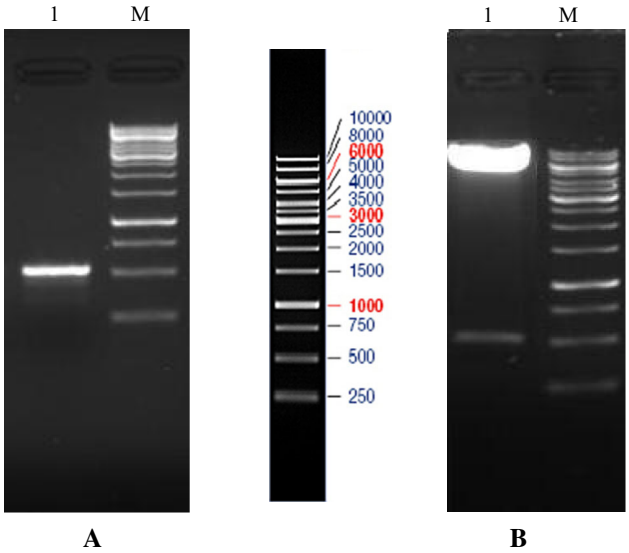

Fig. 1 **a** PCR amplification. *Lane M* DNA molecular weight marker; *Lane 1* PCR product (503 bp). **b** Restriction enzyme analysis. *Lane M* DNA molecular weight marker; *Lane 1* pLEXSYDarbo digested with *Xba*I and *Kpn*I, 503 bp band related to the insert fragment

Expression of Recombinant Darbepoetin Alfa

The recombinant *L. tarentolae* expressed darbepoetin alfa protein that was detectable by SDS-PAGE and Western blotting. The DTT-reduced and non-reduced darbepoetin alfa migrated at approximately 40 kD by SDS-PAGE (Fig. 3a). Western blot method certainly confirmed that the band which was observed in SDS-PAGE was the desired protein (Fig. 3b). Furthermore, Western blotting revealed that the obtained darbepoetin alfa was also homogeneous.

Analysis of Produced Darbepoetin Alfa

For further analysis, IEF was used for estimation of the glycosylation profile of recombinant protein. According to IEF result (Fig. 4), darbepoetin alfa contained 5 isoforms, while Aranesp consisted of 10 isoforms. Figure 5 shows UV–Vis spectra of darbepoetin alfa and Aranesp. As can be found, there was not any noticeable difference in UV–Vis spectrum of these proteins. This result primarily indicated that darbepoetin alfa possess a structural similarity to Aranesp. CD is a powerful tool for the analysis of secondary structures and conformation. The CD spectrum in far UV region (190–260 nm) is related to alpha helix and beta sheet content of a protein. Figure 6 shows the CD spectra of darbepoetin alfa and Aranesp. As observed, produced protein in *L. tarentolae* had a CD spectrum similar to Aranesp. These data indicated that the secondary structure of darbepoetin alfa was not altered.

Potency Result

The result of in vivo bioassay is depicted in Fig. 7. As can be found, the content of reticulocyte increases for produced darbepoetin alfa by *L. tarentolae* (22) and Aranesp was similar.

Discussion

Darbepoetin alfa is an analog of human EPO, which has an increased biological half-life due to the increased degree of glycosylation when compared to that of the native EPO. It has higher in vivo stability, and the time of its biological half-life is three times higher than that of EPO. Because of these properties, the use of darbepoetin alfa in medical practice allows less frequent administration to patients [17, 18]. It has been shown that *leishmaina* protozoan can be applied as an alternative to express foreign genes with the eukaryotic source [19]. *L. tarentolae* is a non-pathogenic parasite and has been developed as a potential eukaryotic expression host. This host is rich in

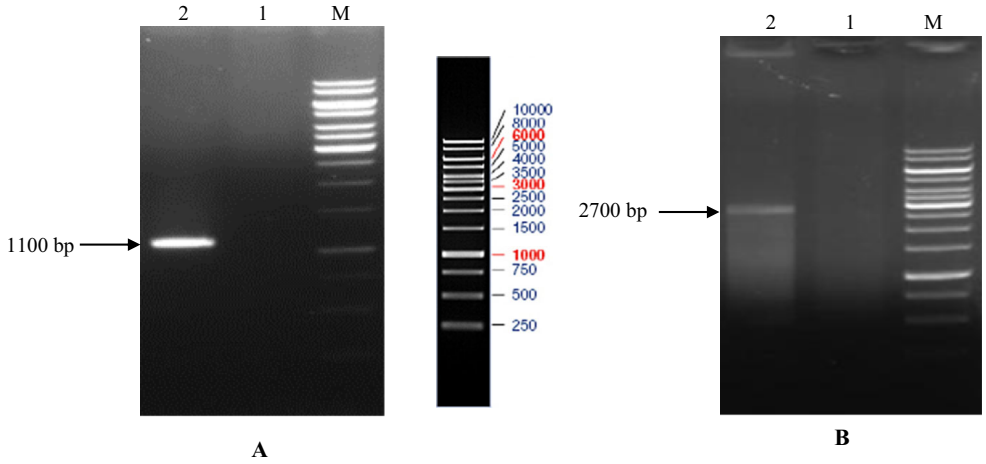

Fig. 2 **a** Diagnostic PCR with A1304 and A1715 primers. *Lane M* DNA molecular weight marker; *Lane 1* 1.1 kbp PCR product; *Lane 2* wild-type parasite **b** Diagnostic PCR on genomic with A708 and P1510 primers. *Lane M* DNA molecular weight marker; *Lane 1* 2.7 kbp PCR product; *Lane 2* wild-type parasite

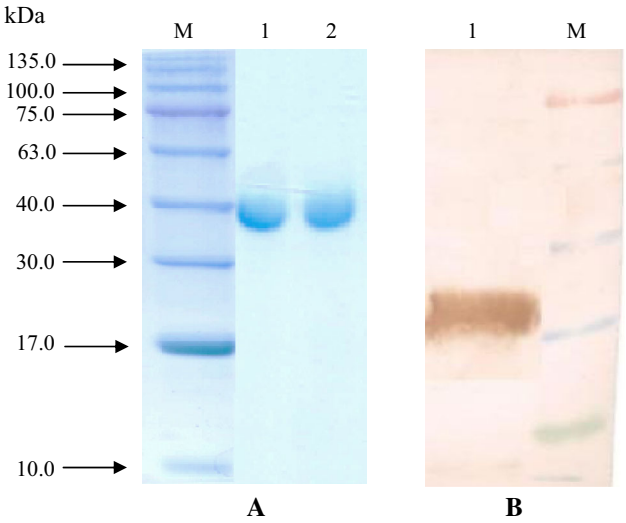

Fig. 3 Expression analysis. **a** SDS-PAGE. *Lane M* protein marker; *lane 1* purified darbepoetin alfa under non-reducing condition; *Lane 2* purified darbepoetin alfa under reducing condition. **b** Western blot. *Lane M* protein marker; *lane 1* transfected cell with darbepoetin alfa

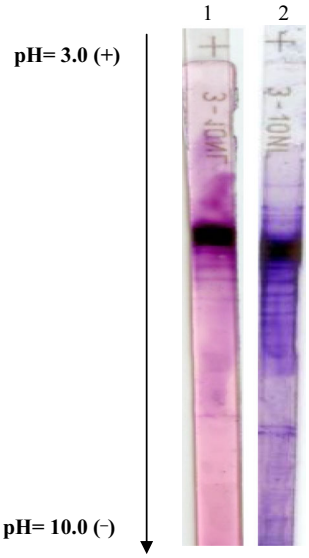

Fig. 4 Isoelectrohoretic separation of isoforms. *Lane 1* shows the produced darbepoetin alfa in *L. tarentolae*; *Lane 2* shows the drug Aranesp

glycoproteins and its glycosylation pattern is similar to the mammalian that include complex-type oligosaccharides [20]. Additionally, it offers several advantages such as simple nutrient requirements, ability for large-scale production, and fast growth [9]. These features make *L. tarentolae* interesting for biotechnological applications. The goal of this research was to characterize the produced darbepoetin alfa from *L. tarentolae* T7-TR. The codon-optimized gene was cloned into the genome of *L. tarentolae*. Expression analysis showed and confirmed the presence of a protein band at 40 kDa. The reported expression level for proteins in *L. tarentolae* is usually varied between 0.1 and 30 mg/l. For example, the expression level for green fluorescent protein (GFP) [9], interferon-gamma (IFN-γ) [13], and human coagulation factor VII [21] has been achieved to be 30.0, 9.5 and 1 mg/ml, respectively. In this study, a codon-optimized ORF was used which resulted in 51.2 mg/ml of culture medium. This result was more than what has been reported in CHO cell (43.0 mg/ml) [7]. Hence, it can be concluded that the codon optimization of target gene resulted in a good approach to enhance protein yield. For further study, the purified protein was also structurally characterized. Primarily, the isoform composition of target protein was

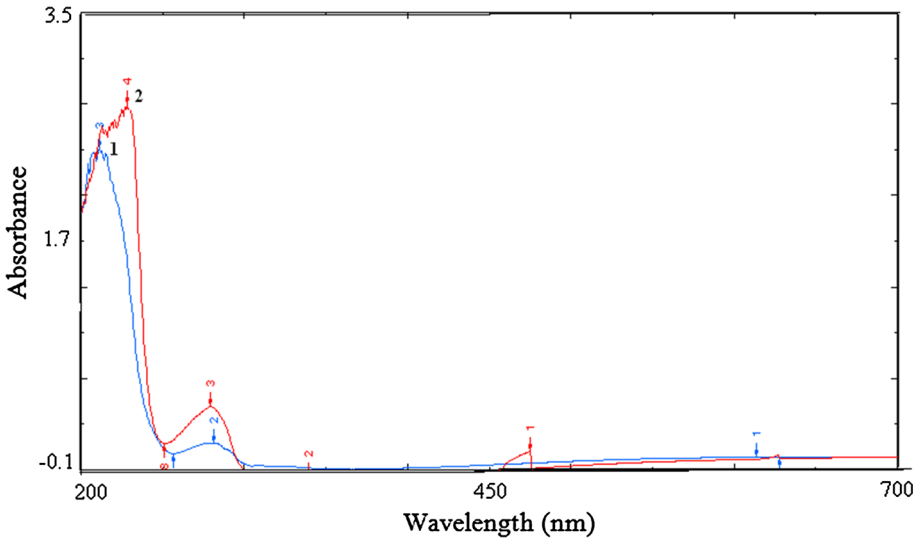

Fig. 5 UV–Vis absorption spectra of darbepoetin alfa *1* and Aranesp *2*. Aranesp was used in parallel as positive control. The concentration of both protein samples was 0.2 mg/ml. The medium was phosphate buffer saline (PBS), 10 mM, and pH 7.4

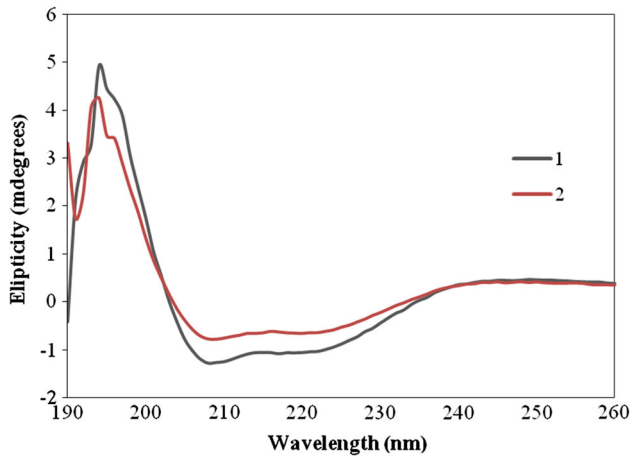

Fig. 6 CD spectra of darbepoetin alfa *1* and Aranesp *2* in the far UV region (190–260 nm). The concentration of both protein samples was 0.3 mg/ml. The medium was phosphate buffer saline (PBS) 10 mM and pH 7.4. Each point is the mean of four independent measurements. Temperature 20 °C

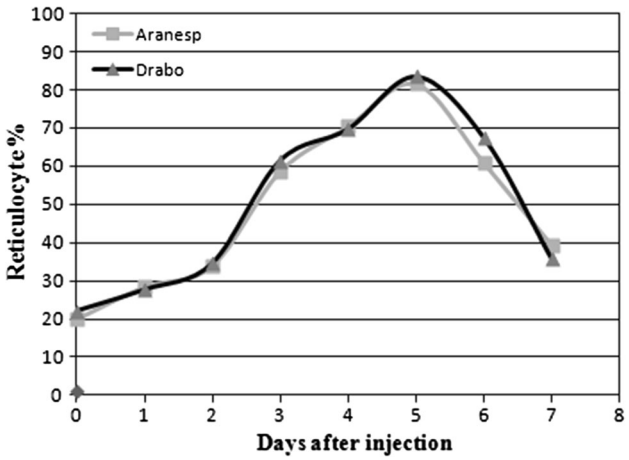

Fig. 7 Potency test result. Aranesp was used in parallel as positive control

studied (Fig. 4). The comparative drug, Aranesp, which has been produced in CHO host contained 10 isoforms of the protein, while darbepoetin alfa consisted of 5 isoforms. The differences in isoform profiles between the darbepoetin alfa from *L. tarentolae* and CHO represented distinct glycosylation, which originated from cell lines. In other words, expressed darbepoetin alfa was exceptionally more homogenous than darbepoetin alfa in CHO. Similar profiles have also been found in other LEXSY-produced protein including IFN-γ and EPO suggesting that it's a common feature of all recombinant glycoproteins produced in *L. tarentolae* expression system (LEXSY) [9]. Based on the further structural analysis using UV–Vis and CD spectra, it was proven that the darbepoetin alfa from *L. tarentolae* had similar structure to analogous protein expressed in CHO. The DTT-reduced and non-reduced darbepoetin alpha migrated approximately at the same MW by SDS-PAGE. The obtained result was contradictory to our knowledge that darbepoetin alpha contains two disulfide. It might be possible that expressed protein was not properly folded through production in *L. tarentolae* host. However, this hypothesis was rejected by the CD data (Fig. 6) that target protein had native folding and secondary structure. Previously, Breitling and coworkers reported the expression of EPO in *L. tarentolae* [10]. But, no information about reducing SDS-PAGE has been given in their paper; therefore, we cannot compare our result with them. Accordingly, in order to further characterize the disulfide linkages of darbepoetin alfa, MALDI-TOF mass spectroscopy analysis can be carried out. Reticulocyte experiment results showed that the biological activity of recombinant darbepoetin was similar with that of Aranesp.

Therefore, it can be concluded that the difference in glycosylation profile did not change the potency of drug. In summary, the results presented here demonstrate that expressed darbepoetin alfa in *L. tarentolae* was structurally similar to its original drug which has been previously produced in CHO.

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Compliance with Ethical Standards

Ethical Statement This article does not contain any studies with human participants performed by any of the authors.

Human and Animal Rights All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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