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Protein Expression and Purification of Romiplostim and Analysis of Its Secretory Production Using an *In Silico* Investigated Signal Peptide in *E. Coli*

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Abstract

Background: Romiplostim is a thrombopoietin receptor agonist approved for the treatment of immune thrombocytopenia. It is produced by recombinant DNA technology in *Escherichia coli*. Many researchers have studied the periplasmic or extracellular production of recombinant proteins in *E. coli* by using signal peptide sequences due to its advantages compared to intracellular production. In this study, the effect of the pelB signal peptide on Romiplostim production was analyzed.

Methods: The nucleotide sequence of Romiplostim was codon optimized for expression in *E. coli* BL21. For analysis of the effect of the pelB signal peptide, pET-22b (+) and pET-15b plasmids were used. The probability of signal peptide cleavage and pathway was predicted by using the SignalP 5.0 program, and expression, purification, and biological activity of the recombinant protein were analyzed.

Results: *In-silico* analysis predicted the correct cleavage of the pelB signal peptide. However, the experimental results showed intracellular accumulation of the protein in fusion with this signal peptide without any detectable protein band in periplasmic or extracellular spaces. The *in-vivo* experiment of purified protein without signal peptide exhibited a significant increment in platelets compared to the control group.

Conclusions: Romiplostim was expressed in *E. coli* with and without signal peptide. The latest one showed suitable *in-vivo* bioactivity. Despite the results of *in-silico* prediction, the pelB signal peptide could not transport the protein into the periplasm or extracellular environment in the experimental condition. Trying different signal peptides and more *in-silico* analysis might be helpful for the efficient secretion of the Romiplostim protein.

Keywords: *E. coli*, Romiplostim, Secretory production, Signal peptide, Thrombocytopenia.

Introduction

Immune thrombocytopenic purpura (ITP) is an autoimmune disease characterized by reduced platelet levels and bleeding (1, 2). It has been long thought to be caused due to speeded-up platelet demolition interceded by tissue macrophages that ingest and destroy antibody-coated platelets (3, 4). Treatment of ITP usually involves corticosteroids and intravenous (IV) immunoglobulin or IV anti-D immunoglobulin to mediate platelet demolition. Splenectomy is the next line of treatment in adults, but ~30–40% of cases do not respond to splenectomy or

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show a relapse (5, 6).

In 2008, the US FDA approved Romiplostim (AMG531, N-plate) for the treatment of chronic immune thrombocytopenia in patients with thrombocytopenic purpura, who showed an inadequate response to corticosteroids, splenectomy, or immunoglobulins (7, 8).

Romiplostim is a peptibody drug with no sequence similarity with human thrombopoietin (TPO) and is composed of two similar single-chain subunits. Each subunit, including the human IgG1 Fc domain, joins the C-terminus via a peptide encompassing two TPO receptor-binding domains for four entire binding sites. Romiplostim can bind to TPO receptors and stimulate platelet production (9). According to the varied sequences of amino acids in Romiplostim compared to the endogenous TPO, there is no risk of generating neutralizing antibodies that adhere to endogenous TPO (1-3). Romiplostim is now produced in *E. coli* via recombinant DNA technology (1).

Under standard laboratory conditions, *E. coli* does not secrete proteins into the extracellular space. Therefore, the presence of *E. coli* proteins in the culture medium is usually related to cell lysis or non-specific leakage. The extracellular production of recombinant proteins offers many advantages compared to cytoplasmic accumulation, including ease of isolation and purification, higher solubility, and stability, and facilitated correct disulfide bond formation (10-13).

Several methods have been used to produce secretory proteins in *E. coli*. Employing signal peptides is one of these approaches. Signal peptides are 15- to 30-unit amino acid sequences used for protein translocation through various membranes. They start transposing newly produced proteins and can be classified in the family of targeting sequences (11, 13-15).

The signal peptide functions are similar to a universal positioning system, i.e., improving the messages needed to send the target protein to the outside of the membrane (16). Since its synthesis occurs in the cytoplasm, a polypeptide competes with other transport-competent polypeptides to be exported. Various signal peptides such as ompA, pelB, malE, phoA, ompC, and lamb are generally used to secrete recombinant proteins into the extracellular medium of *E. coli* (12, 17, 18).

In this study, to evaluate the effect of pelB signal peptide in the recombinant production of Romiplostim, two constructs, one with pelB and the other without it, were designed, and protein expression, purification, and biological activity were analyzed.

Materials and Methods

Bacterial strains, plasmids, and culture media

E. coli BL21(DE3), pET-22b (+), and pET-15b were purchased from Novagen, Germany. Bacteria were cultured in a shaking incubator at 37 °C in Luria-Bertani (LB) broth medium (10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl). Ampicillin 100 µg/ml (Dana Pharmaceutical Company, Iran) was also added to the culture media.

Romiplostim and pelB signal peptide sequences

The Romiplostim sequence was obtained from DrugBank Online database at https://go.drugbank.com/ and US patent (6835809 B1) (19). The nucleotide sequence was then codon optimized for efficient expression in *E. coli* using GenScript at https://www.genscript.com and ATGme (http://atgme.org/) servers. Two restriction sites (*Nco*I/*Bam*HI) were designed at 5' and 3' ends of the sequence. The construct was then inserted into pET-15b and pET-22b (+), separately. The amino acid sequence of Romiplostim in fusion with the pelB was evaluated *in silico* by the SignalP 5.0 program (https://services.healthtech.dtu.dk/service.php?SignalP-5.0) to predict the probability of correct signal peptide cleavage and production pathway.

Expression of recombinant Romiplostim

The recombinant plasmids were separately transformed into competent *E. coli* BL21 (DE3) cells by the heat shock transformation method (20, 21). The recombinant cells were

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then cultured in the LB medium with 100 µg/ml ampicillin at 37 °C to reach the cell density to OD_{600} of ~0.8, determined by spectrophotometry. The expression of the recombinant proteins was induced by adding 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). A sample of transformed cells was cultured without IPTG induction as a control (22). After induction, bacterial cells were incubated at 37 °C under shaking (200 rpm) for 4 h and overnight.

SDS-PAGE analysis

Protein expression was evaluated after 4 h and overnight incubation for both the constructs with and without signal peptide. Bacterial cell pellets were collected by centrifugation at 10,000 RCF for 10 min. Cytoplasmic, periplasmic, and the extracellular fractions of the samples were analyzed by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) (23), and the protein bands were detected by Coomassie Brilliant Blue staining.

Periplasmic fraction preparation

The bacterial pellets were resuspended in a solution of sucrose/lysozyme containing 0.2 M Tris-HCl, 20% sucrose, 1 mM EDTA, and 0.05% lysozyme. The bacterial suspension was afterward incubated at room temperature for 15 min. Then, an equal volume of cold water was added, and the suspension was incubated on ice for another 15 min. Eventually, the periplasmic fraction was harvested by centrifugation (13000 RCF at 4 °C). The periplasmic fraction preparation protocol was also implemented at 25 °C, 30 °C, and 37 °C after induction. The pellets and supernatant were used for the analysis by SDS-PAGE.

Intracellular protein preparation through sonication

The centrifuged bacterial cells were resuspended in a buffer containing 50 mM Tris-HCl, 1 mM PMSF, 1 mM EDTA, at pH 8 after protein expression and disrupted using sonication (output= 5, duty= 50, and time=5 min). The pellets and supernatants were separated by centrifugation at 12,000 RCF for 20 min at 4 °C and analyzed using SDS-PAGE.

Purification of recombinant Romiplostim

The solution containing recombinant Romiplostim was passed through Protein A resin (Sepehr Daru Farjam Co, Iran) by 20 mM tris (pH 8). The Fc-mediated bonded peptibody was eluted with 100 mM glycine buffer (pH 2.5) (24, 25). The protein concentration was measured using the Bradford assay (26, 27).

Refolding

Refolding was done by two approaches. First, a method explained in a US patent (6835809 B1) was carried out (19, 25), in which, the solubilized combination was diluted 20 times to reach 160 mM arginine, 50 mM Tris, cysteine (3-5 mM), and urea (1-2 M) at pH 8.5. The mixture was stirred overnight at 4 °C. The recombinant Romiplostim solution was concentrated ~10-fold with Amicon Ultra-15 Centrifugal Filter (MWCO 50 kDa) (Millipore Sigma, USA), followed by 3-fold dilution in urea 1.5 M and Tris 10 mM, pH 9. Then, the refolding stage was conducted (25, 28).

The second method involved using the dialysis bag at 4 °C in PBS buffer (pH 7.5) overnight (29). The mixture was concentrated about 10-fold with Amicon Ultra-15 Centrifugal Filter (MWCO 50 kDa).

Biological activity

Twenty healthy normal female BALB/c mice (7–9 weeks of age; 25-30 g live weight) were obtained from animal breeding center of the Shiraz University of Medical Sciences, Shiraz, Iran. Mice were divided into case and control groups. Recombinant Romiplostim (50 µg/kg) and normal saline (2.5 mL/kg) were subcutaneously (sc) injected into control and case groups on day 0. A hematology analyzer (Sysmex KX-21, Japan) evaluated the whole platelet factors on days 0, 4, 7, 9, and 17 through lateral tail vein (25).

The ethics committee on the use and care of experimental animals at Shiraz University of Medical Sciences approved the experimental

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procedures of the current investigation (Approval Code: 23453). The ARRIVE guidelines for experimental animals use and care was also followed.

Statistical Analysis

Data are represented as mean±SE (n=5). A One-Way ANOVA with a Tukey post *hoc* test was used to assess the significance of the obtained data. A P< 0.05 was considered statistically significant.

Results

Analysis of Romiplostim and pelB sequences

The complete DNA sequence of the Romiplostim protein was synthesized after optimization. Both constructs were confirmed by DNA sequencing (data not shown). The probability and cleavage site according to SignalP 5.0 program is given in Table 1 and Figure 1.

Table 1. The *in-silico* prediction of the cleavage site of pelB signal peptide in fusion with Romiplostim protein.

Signal peptide	Cleavage probability	Cleavage site
pelB	0.9972 (Sec/SPI)	Between aa 22 and 23

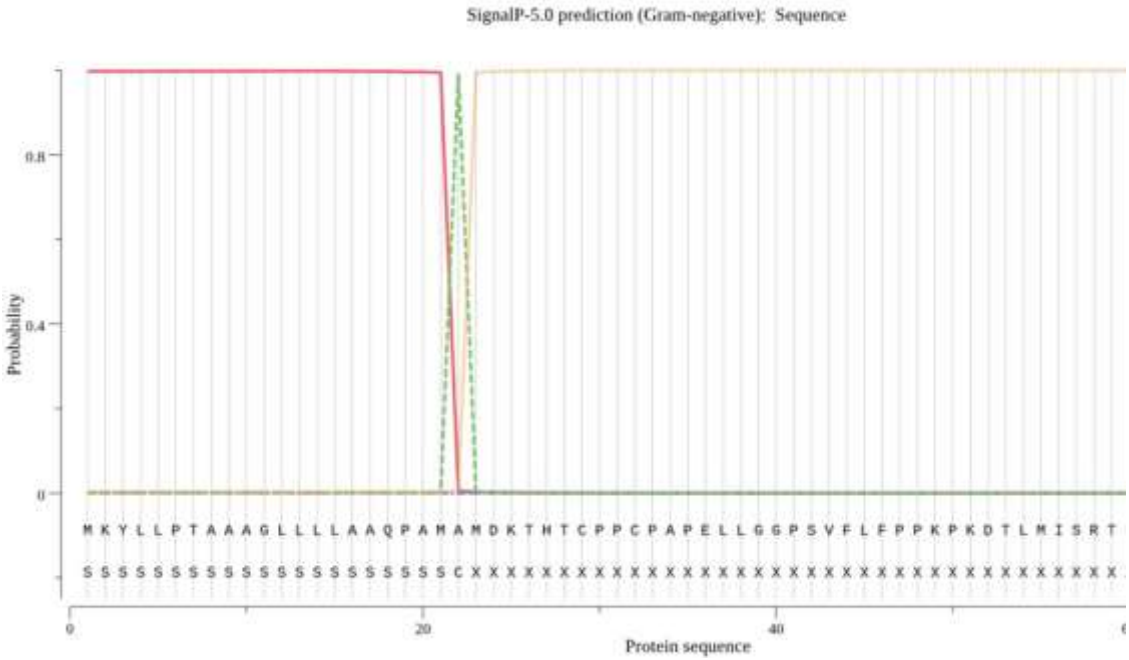

Fig. 1. Sequence analysis of the Romiplostim with pelB signal peptide by the SignalP 5.0 server. Signal peptide cleavage site was predicted between aa 22 and 23

Cytoplasmic, periplasmic, and extracellular protein expression analysis

The SDS-PAGE analysis of Romiplostim expression with or without signal peptide was performed on the intracellular fraction after sonication and solubilization. The results showed corresponding protein bonds with the expected size of the Romiplostim single strand (30 kDa) for the construct without signal peptide (pET15b). The protein band from the expression of the construct with pelB signal peptide (pET22b), showed a larger protein size, which indicated the signal peptide was not separated (Fig. 2). The SDS-PAGE analysis of the recombinant Romiplostim with pelB signal peptide (pET22b) revealed no protein band in the expected size in the culture medium or the periplasmic space of the recombinant cells. The results were the same after the examination of periplasmic or extracellular expression at different temperatures of 25 °C, 30 °C, and 37 °C after overnight induction (data not shown).

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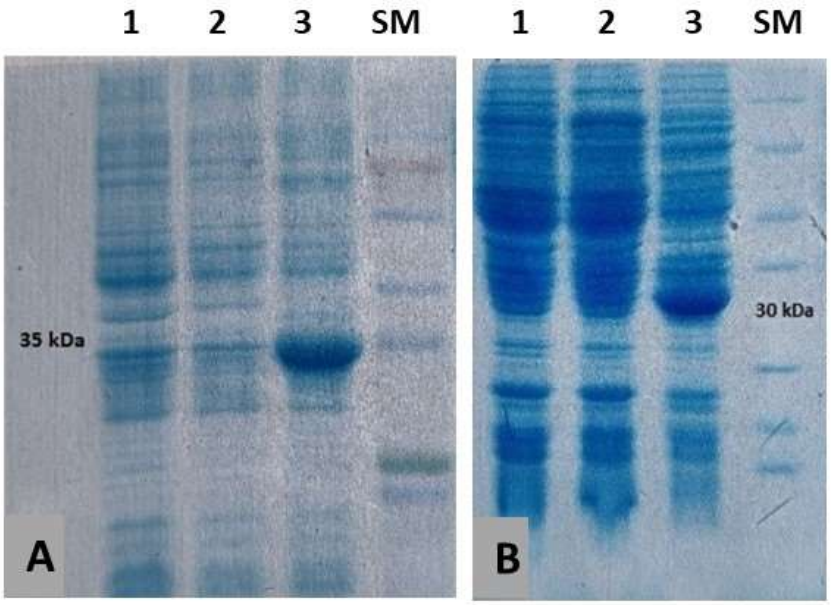

Fig. 2. SDS-PAGE analysis of Romiplostim expression with (A) and without (B) signal peptide after sonication and solubilization.
Lane 1: *E. coli* BL21 without transformation, lane 2: Recombinant cells before induction with IPTG, lane 3: Recombinant cells after induction with IPTG, SM: protein size marker (Sinaclon)

Purification of recombinant Romiplostim

Protein purification was performed for the recombinant protein without the signal peptide. The results showed a single band of purified protein after protein A affinity purification on SDS-PAGE (Fig. 3). The purified protein was approximately 30 kDa in size, corresponding to Romiplostim monomer, similar to the commercially available drug product (Nplate®).

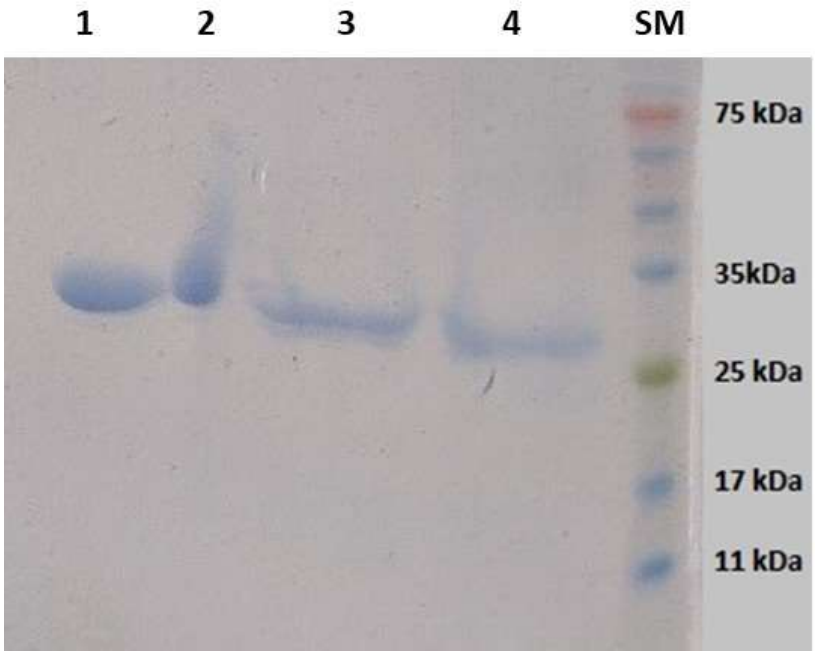

Fig. 3. SDS-PAGE analysis of the construct without signal peptide after protein A affinity purification. Lane 1 and 2: Nplate®, Lane 3 and 4: purified recombinant Romiplostim, SM: protein size marker (Sinaclon).

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Refolding

In the first method, in which arginine, Tris, cysteine, and urea were used, a 30 kDa band was observed, indicating non-dimerization. However, in the second method, the single-chain structure was immersed in the PBS buffer overnight in a dialysis bag. As a result, the 60 kDa band was seen on the SDS-PAGE, which was related to the dimeric structure of Romiplostim (Fig. 4).

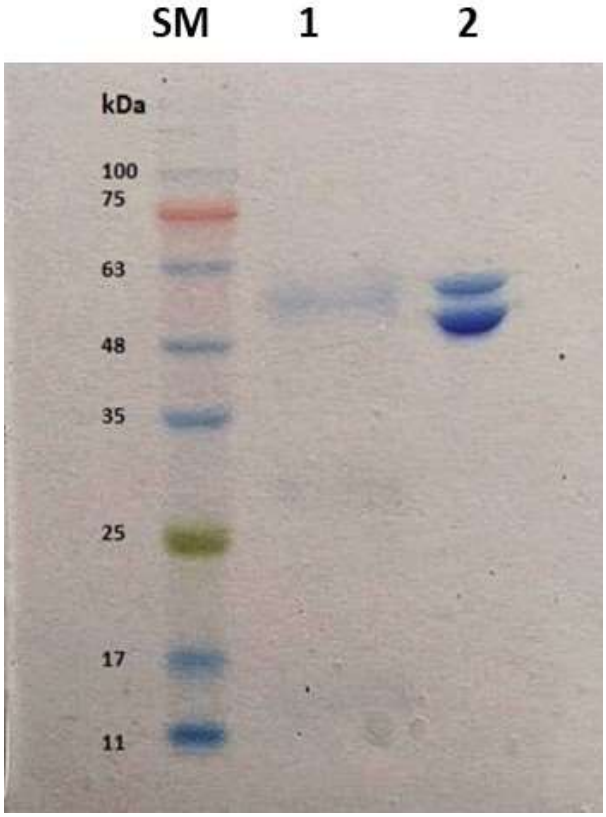

Fig. 4. SDS-PAGE analysis of purified Romiplostim with a dimerized structure. Non-reducing sample buffer was used. Lane 1: Recombinant Romiplostim after purification and refolding (60 kDa), lane 2: Nplate®, SM: protein size marker (Sinaclon).

Biological activity

Based on the data obtained, the platelet count in the group injected with recombinant Romiplostim showed an almost four-fold increase on day 4 compared to the control group. On days 7, 9, and 17, it fell back to the level of day 0 (Fig. 5).

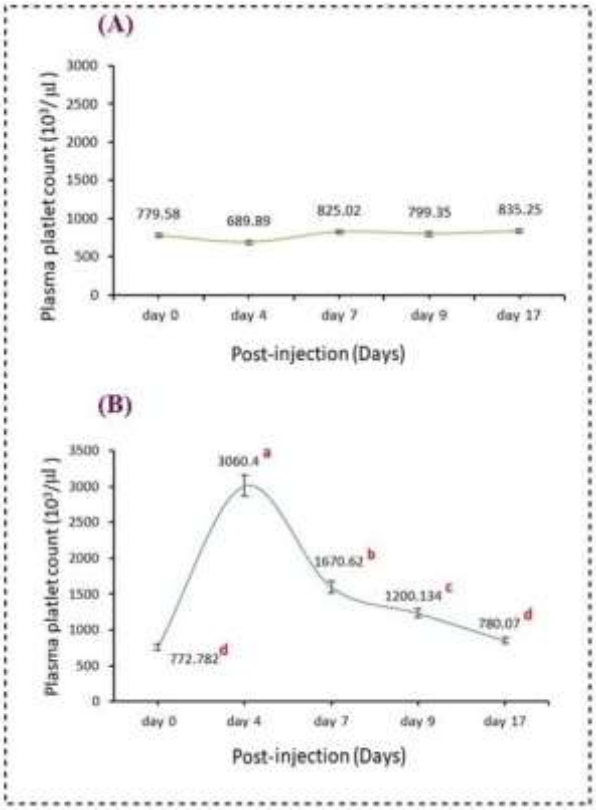

Fig. 5. Platelet counts in normal saline (control group; 2.5 ml/kg, s.c, Panel A) and recombinant Romiplostim-treated group (50 µg/kg, s.c Panel B). A comparison of platelet counts between control and romiplostim-treated group (recombinant Romiplostim) was performed on days 0, 4, 7, 9, and 17.
Data are represented as mean ± SEM (n = 5).
Data sets with various alphabetical superscripts are significantly different (P< 0.01).

Discussion

Considering the obvious benefits of extracellular production of recombinant proteins, this research was aimed to produce Romiplostim and examine the effect of the pelB signal peptide on Romiplostim protein production and secretion in *E. coli*. Thus, two Romiplostim constructs were designed, one with and the other without pelB signal peptide. Since many studies have shown that optimized codons considerably enhance mammalian gene expression in *E. coli* (30, 31), the constructs used for Romiplostim production were codon optimized and transformed into *E. coli* host cells.

Following induction, Romiplostim protein

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was observed in the cytoplasmic fraction of the construct without signal peptide with a major protein band at about 30 kDa. In the case of Romiplostim with the pelB sequence, the results of SDS-PAGE revealed a protein band with a higher molecular weight than the construct without signal peptide. Nevertheless, there was no protein band in the periplasmic or extracellular spaces of both constructs with and without signal peptide.

There are also several records of using pelB signal peptide with other proteins in publications. In a study by Chung et al., for inducing human granulocyte colony-stimulating factor (hG-CSF) secretion in *E. coli*, the gene encoding mature hG-CSF was fused to the gene encoding the pelB signal peptide, and the fed-batch fermentation of the recombinant *E. coli* was carried out. No secretion of hG-CSF and no processing of the signal peptide from the fusion protein was observed (32). In another study, pelB signal peptide mediated the extracellular expression of *Thermobifida fusca* cutinase in *E. coli* (33). In a study by Shi et al., enhanced extracellular production of PETase, a biocatalyst for Poly (ethylene terephthalate) (PET) hydrolysis, was reported in *E. coli* via engineering of the pelB signal peptide by mutation (34).

In the case of Romiplostim, the results showed that despite the *in-silico* prediction, pelB signal peptide was not properly cleaved from the N-terminus of the recombinant protein, and no protein band of interest was observed in the periplasmic or extracellular spaces.

A successful secretion process usually needs an optimal balance between all the secretory cascades (35). Ineffective secretion may be due to various factors such as the structural properties of the signal peptide, incongruity among the target protein and the signal peptide, impotence to cross over the membrane and achieve the periplasmic space, or inadequate equilibrium among the valency of the export machinery and the speed of protein synthesis (36).

Another important factor affecting secretion is knowing the features of the target protein, which in many cases, leads researchers to use *in-silico* study and/ or the trial-and-error methods (37). In the case of Romiplostim, the analysis of the protein in fusion with other signal peptides along with consideration of culture media and other conditions could be helpful in determining the possibility of its secretion in *E. coli* (38).

The overexpression of recombinant proteins in *E. coli* mostly leads to the production of insoluble proteins known as inclusion bodies. The *in-vitro* refolding of these inclusion body proteins is essential to retain their biological activity, which could be confirmed through *in-vivo* studies. In this investigation, recombinant Romiplostim without signal peptide was efficiently purified using protein A resin and refolding process. Purification by protein A is done through binding to the FC part of the protein. However, any change in this sequence such as binding of a signal peptide can prevent the purification process (24). According to the results of the mentioned study, the platelet value was induced almost four folds on day 4, in the group treated with purified recombinant Romiplostim compared to the control group. However, platelet enhancement was temporary and almost returned to the level of day 0 on day 7 (Fig. 5). The biological activity of the expressed recombinant Romiplostim was confirmed suggesting the appropriate refolding of the protein.

Recombinant Romiplostim was expressed intracellularly in *E. coli*, and its biological activity was shown in mice. Despite the favorable predictions of the primary *in-silico* analysis, fusion of pelB signal peptide did not result in periplasmic or extracellular production of Romiplostim protein. Thus, *in-silico* and *in-vitro* studies on other signal peptides and investigating culture media and expression conditions are suggested as potentially promising approaches in future studies.

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Conflicts of Interest
The authors declare no conflict of interest.

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