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Original Article

Optimized Cytoplasmic Expression of Water Soluble Human Thrombopoietin in Modified Bacterial Strain

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ABSTRACT

Background: Thrombopoietin is a glycoprotein produced by liver and kidney which is responsible for regulating the platelet production. Thrombopoietin is a key ligand with impact on regulating the self-renewal of Hematopoietic stem cells and the regulation of Megakaryocytes progenitors. Previous studies have indicated that only N-terminal domain of this protein has receptor promoting ability. The goal of this study was to express the recombinant form of the soluble Thrombopoietin by using *Rosetta gami* strain of *Escherichia coli* as the expression host and pET vectors system to produce this cytokine in a cost-effective manner.
Methods: After isolating the genomic sequence of Thrombopoietin from HepG2 cDNA, it was inserted into the pET32 vector and colonized by *DH5α*. Then, recombinant plasmids were transformed into Rosetta gami to express the recombinant protein. Expression was analyzed through SDS-PAGE and western blotting assays. Finally, the optimized time for induction and the suitable concentrations of IPTG were determined.
Results: DNA sequencing of recombinant plasmid proved that the sequence was indicative of variant 2 of Thrombopoietin. SDS-PAGE and western blotting showed a 36-kDa protein band which was compatible with the inserted sequence. Optimized time for expression was 6 h and optimized concentration of IPTG was 0.1 mM.
Conclusion: Although different methods are available to produce recombinant Thrombopoietin, low yield and costly setup are major obstacles in the production of Thrombopoietin. Also the products of usual bacterial systems are mostly inclusion bodies which is due to lacking disulfide bond formation. In this study, mutant strain of E. coli was used to provide a high level of water-soluble Thrombopoietin expression.

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Introduction

Thrombopoietin (TPO), also known as megakaryocyte growth and development factor (MDGF), is a cytokine that has attracted attention for its diverse roles on HSCs. This cytokine has the stimulating effect on megakaryocyte differentiation, and influences HSCs self-renewal through their proliferation phase. This cytokine is also important in maintaining the normal level of thrombopoiesis inside of the body. Thrombopoietin is a glycoprotein consisting of 353 amino acids with a molecular weight of 30 kDa.[1]

Human TPO gene is located on 3q27, between Chordin and RNA polymerase 2 genes.[2] Thrombopoietin gene consists of 6 exons and has 6 main splicing variants.[3] Mature TPO contains two domains; each of them consisting 2 helix bundle.[4] Its amino-terminal domain (174 amino acid) has receptor-binding and signaling

activity. While this domain of protein could support cellular proliferation,[5,6] the carboxyl-terminal domain can enhance the secretion of protein into blood stream.

Main sources of TPO secretion in the human body are liver, kidney and bone marrow. TPO expression is regulated through a negative feedback in the body.[7,8] C-mpl receptor is the main target of TPO. After binding of TPO, this receptor will dimerize,[9] and Jak-2 will be phosphorylated, resulting in Jak-stat cascade signaling.[10] Upon the receptor-ligand interaction, transcription of various transcription factors including SOCS, Bcl2, p27, HOX, and p21 will be upregulated. Each of these cytokines has their own specific influence on the HSCs.[11]

The concentration of TPO inside of human blood is very low, and despite the other blood derived proteins that could be prepared by human plasma fractionation methods,[12-14] this protein should be provided from other sources, mainly recombinant protein expression methods. Until now; a variety of expression methods have been used to express recombinant TPO, any of which having their own advantage and disadvantages.[15]

Previous studies have proven that glycosylation has a minus influence on the receptor binding ability of TPO to its receptor, but disulfide bond formation is critical to attaining tertiary structure which is necessary for receptor binding and its activation.[9]

Accordingly, using a host which is capable of forming disulfide bonds, but incapable of protein glycosylation would be appropriate to produce a functional protein.[16]

It's a concern to express soluble heterologous proteins in a bacterial host, because recombinant products of human proteins by usual bacterial system are in the inclusion body form due to inability of Disulfide bond formation.[17] In this study, *Rosetta gami (DE3)* was used as a host to produce soluble TPO. *Rosetta gami* is a mutant strain of *E. coli* which enhances disulfide bond formation.[18] This mutant strain has also been engineered to make it capable of recognizing human DNA codon usage. These adjustments resolve codon bias and post-translational modification related problems of the usual bacterial systems.[19,20]

This study had 3 main phases including (I) gene isolation and cloning of the TPO functional domain, (II) TPO expression and (III) determining the optimized expression conditions to achieve a high yield of expression through a cost-effective method.

Materials and Methods

DNA polymerase enzymes were purchased from Thermofisher Corporation (U.S.A). Gel extraction, DNA purification, and plasmid purification kits were obtained from Qiagen Company (Germany). Ampicillin, chloramphenicol, and kanamycin were supplied from Sigma Aldrich Company (U.S.A), and size markers were purchased from Thermo scientific company (Lithuania).

Hep-G2 cell line was chosen as a source for TPO gene isolation. This cell line was cultured in DMEM-f12 media. Guanidinium thiocyanate-phenol-chloroform extraction method was performed to harvest total cell RNA. Hep-G2 RNA was used as a template in the synthesis of cDNA through RT-PCR reaction.[21]

In order to isolate designated sequence, specific primers were designed for PCR reactions. TPO primers also had restriction sites embedded in their sequence (Nco1 at forwarding primer and Xho1 in reverse primer) which was located at their edges to make inserting the sequence in linear plasmid possible.

TPO-Forward primer:
CATGCCATGGTAAGCCCGGCTCCTCCTGC

TPO-reverse primer:
CCGCTCGAGGAGCTCGTTCAGTGTGAGGAC

At this study, pET-32 was used as both cloning and expression vector. Nco1 and Xho1 are restriction sites were chosen because they could provide convenience in expression and purification of the target protein. Due to choosing these sites of restriction, the final product will be His-tag, S-tag and Trx-tag fusion proteins.[22-24]

For inserting the sequence of interest into the vector, pET32 plasmid and the PCR products were double digested with the same enzymes for 4 h at room temperature. Then, ligation performed by using T4 DNA ligase for 16 h at 4oC.

As the cloning host, *DH5α* strain of *E. coli* was used. For making the competent bacterial cells for receiving the recombinant plasmid, the chemical competency method by cold CaCl2 was carried out. For this purpose, 1.5ml of the fresh bacterial culture (600 nm OD=0.5) was centrifuged (8000g, at 4oC) for 5 min. The provided sediment was suspended in 1ml of ice-cold CaCl2 (0.1 M) and was placed on ice for 30 min. The suspension was centrifuged with the same condition of the previous step. After discharging the supernatant, 670 µL of the ice-cold CaCl2 was added. Bacterial pellets were re-suspended in the mixture and placed on ice for another 30 min. At the end, the suspension was centrifuged again at the same condition and after discharging the supernatant, the pellet was re-suspended in 200 µL of ice-cold CaCl2.

For transforming the ligation products into chemical competent *DH5α*, thermal shock was used. For this step, the ligation products were added to 100 µL of competent *DH5α* suspension and as a positive control, 1 µL of undigested vectors were used. The suspensions were placed on ice for 30 min, and after that placed on 42 oC bain-marie for 1 min. After one min, the suspensions immediately transferred on ice for two min.

After thermal shock, 1mL of LB media was added to the bacterial and suspension incubated at 37 oC for 45 min. Following this step, the suspensions centrifuged (2700g, 4 oC) for 10 min, and after discharging the extra supernatant, the pellets re-suspended in 200 µL of LB media and transferred on LB-agar plates. Ampicillin was used as a selection marker in the LB-agar to discriminate recombinant colonies from those that have not received the vector.

Recombinant colonies were subcultured and used as template for colony PCR assay. Recombinant plasmids were extracted from colonizing the host and further analyzed with PCR to make sure of recombinant plasmid. Recombinant plasmids were sequenced through the chain termination method by using universal T7 terminator

primer.[25]

In order to express recombinant TPO, *Rosetta gami (DE3)* strain of *E.coli* was used.[26] This strain is resistant to chloramphenicol, tetracycline and kanamycin.[27] Chemical competency by using cold CaCl2 and thermal shock (similar to the method used for *DH5α*) was applied to transfer recombinant plasmid into the expression host. For this stage, Ampicillin, Tetracycline, Chloramphenicol, and kanamycin were added to the medium for discriminating colonies of *Rosetta gami* with the recombinant vector. At this culturing condition, only those bacteria resistant to all these antibiotics could survive.

Since pET32 has lac operon (lac operator sequence),[28] Isopropyl β-D-1-thiogalactopyranoside (IPTG) was used to induce expression of recombinant protein. For this step, bacterial colonies with recombinant plasmid were grown in Ampicillin, Tetracycline, Chloramphenicol, and kanamycin containing LB broth media. After 3 h of culturing, IPTG with the concentration of 1 M was used to induce the expression of recombinant TPO.[29]

Upon 4 h of induction by IPTG, 2 samples, each 1 mL were taken from 2 different colonies and were sedimented. Tris-HCl buffer was added to the samples and sonication performed to lyse the bacteria. In order to prevent the action of bacterial proteases, mixture protease inhibitor was added to the sample and all the preparation steps performed on the ice.

To examine the expression of recombinant fusion TPO, the SDS-PAGE assay performed on the bacterial lysate. Electrophoresis assay performed with 12% SDS-PAGE gel, then samples further examined through western blotting. Monoclonal anti-His-tag antibodies were used for analyzing the expression of His-tag containing recombinant Thrombopoietin. These antibodies were conjugated with horseradish peroxidase (HRP) and 3,3'-diaminobenzidine (DAB) was used as a substrate for HRP.

In order to inspect the optimized time for large-scale production, 3 different samples were taken at 2, 4 and 6 h post induction. Another parameter that should have been determined was the optimized concentration of IPTG for inducing TPO expression.[30,31] Different concentrations of IPTG ranging from 0.1 to 1 M were introduced into culture media and the production of recombinant protein was inspected.

Results

After amplification of TPO sequence through PCR reaction using Pfu enzyme and specific designed primers, the product was electrophoresed on agarose gel. Through this stage, a sharp and specific band between 500 and 600 base pairs was witnessed, compatible with the sequence of interest. The band was dissected and purified from agarose gel and used for further processing.

Cloning hosts of *E. coli (DH5α)* were transformed with ligation product of TPO sequence. Colony PCR assay showed that 2 colonies harbor the designated sequence (Figure 1).

Recombinant plasmids were sequenced and aligned with refseq-TPO sequence from gene bank (NCBI). The result

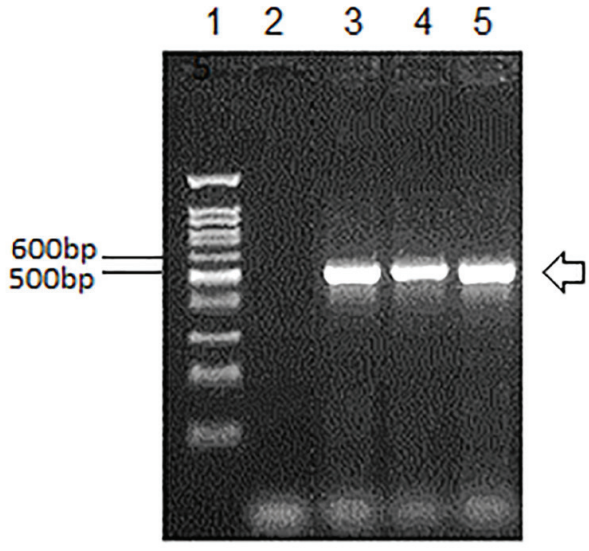

Figure 1: Colony PCR assay; 1- 100 bp DNA ladder, 2- Negative control, 3-Positive control (cDNA template), 4- Colony 1, 5- Colony 2

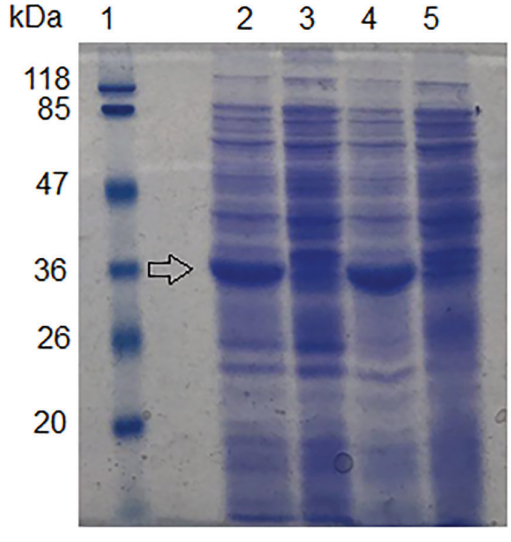

Figure 2: SDS-PAGE of bacterial lysate. 1- Protein size marker, 2-Colony 1 after 4 h of induction, 3-Colony 1 Non-induced after 4 h, 4-Colony 2 after 4 h of induction, 5- Colony 2 Non-induced after 4 h.

was identical to TPO splicing variant 2, consisting of 519 bp. After transforming recombinant vector into *Rosetta gami*, and induction by IPTG, protein expression was analyzed by SDS-PAGE analysis. It showed expression of a 36-kDa protein which is a fusion of TPO along with supportive tags (Figure 2).

The expressed proteins were further analyzed by western blot (Figure 3). Results indicated that only the recombinant protein bands had the capability to react with HRP conjugated anti-His-tag antibodies.

Appropriate induction time was investigated at 2, 4 and 6 h after induction (Figure 4). It was witnessed that 6 h after induction of protein expression has the highest level of TPO expression and could be considered as the optimized time for TPO production.

Optimized IPTG concentration for inducing TPO

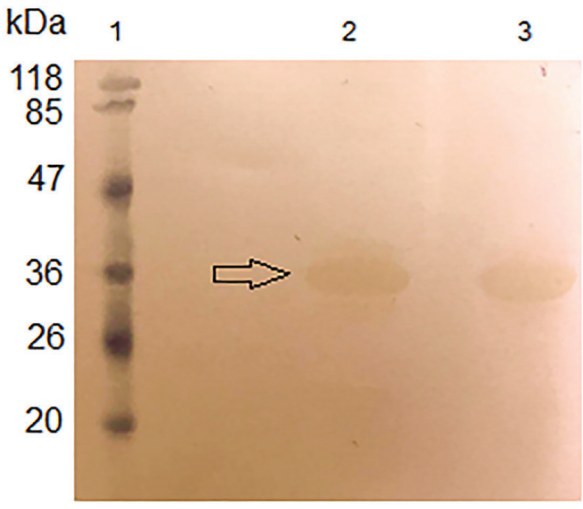

Figure 3: Western blot analysis of Thrombopoietin expression (Semi-dry electro blotter). 1-Protein size marker, 2- Expression induced colony 1, 3-Expression induced colony 2.

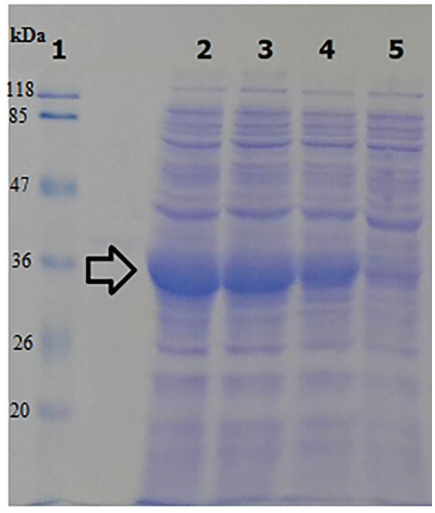

Figure 4: SDS-PAGE electrophoresis of the recombinant TPO expression by Rosetta gami at different times upon induction. 1-Protein ladder, 2- Six h post induction, 3- four h post induction, 4-Two h post induction, 5- Before induction.

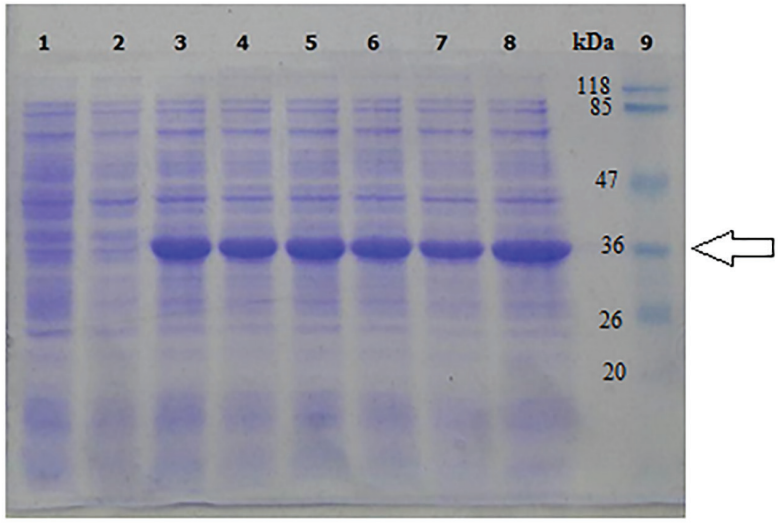

Figure 5: Determining optimized IPTG concentration for induction of recombinant TPO functional domain four h after induction. 1-Rosetta gami without the rec-plasmid, 2- Rosetta gami before induction, 3- Expression induction with 0.1 mM, 4- Induction with 0.2 mM, 5- Induction with 0.4 mM 6- Induction with 0.6 mM, 7- Induction with 0.8 mM, 8- Induction with 1 mM, 9-Protein lader

expression was another parameter to be determined. It was investigated at 0.1 to 1 Mm concentration (Figure 5). The visual evaluation showed that 0.1 mM of IPTG concentration provides satisfying levels of protein expression.

Discussion

Thrombopoietin is a cytokine that has attracted attention due to its diverse roles on HSCs.6 This cytokine has stimulating effect on megakaryocyte differentiation,[32] and poses a critical influence on self-renewal of HSCs.[33] Since a complete understanding of Thrombopoietin and different aspects of its influence is calling for further studies, it's quite an issue to have a satisfactory resource of this cytokine.

Due to its scarce amount in human plasma, it's almost impossible to harvest Thrombopoietin through blood fractionation methods,[34-36] which leaves no alternative except using biotechnological methods to produce recombinant TPO. So far, lots of attempts have been made on cloning and expression of this cytokine, but they mostly suffer from downsides which result in increased costs of recombinant TPO. An optimized expression method to produce water soluble recombinant protein in a cost effective manner is an important issue.

Previously many attempts have been dedicated for producing recombinant TPO and different expression systems have been used including both prokaryotic and eukaryotic ones. Desired expression systems are those which could have the highest yield of production while they could be considered as cost effective.

In a study by Hou et al. the *E. coli* expression system was used for recombinant production of TPO-153. At that study it was reported that the genomic sequence of TPO was synthesized according to the human codon usage. Also the expression product of TPO-153 were inclusion bodies which required further processing to achieve active form of the protein.[37]

Apart from the bacterial codon bias, one major problem using unmodified *E.coli* expression host systems is lack of capability for disulfide bonding formation.[38] These bacteria usually express recombinant proteins in the form of inclusion bodies, which causes a budget burden.

In another study, Foster et al. isolated the human TPO gene from fibroblast DNA genomic library and inserted the sequence of TPO into pZGTPO-124 vector. By using LipofectAMINE, they transfected the Hamster BHK 570 cells and isolated the stable TPO producing cell lines with methotrexate. The final products were biologically active.[39] In 2000, Kaszubska W, et al. used Chinese hamster ovary (CHO) cells for expression of full length TPO. In their study, the final products were having the glycosylation sites and were biologically active.[40]

The studies that were using eukaryotic host did not require the synthesizing of optimized sequence, but it should be kept in mind that bacterial hosts provide a higher yield of expression compared with the eukaryotic ones. It is worthy to mention that full length expression could reduce the overall yield of expression by the bacteria. On the other side, as it was mentioned earlier, the glycosylation and full length expression are not necessary for the activation of the C-mpl receptor.

Guo et al. reported using pET32a (+)/TPO for expression of recombinant TPO. In their study, the *Origami (DE3)* which is an engineered strain of *E. coli*, was used for expression of recombinant TPO. This strain is capable of producing disulfide bonds, but cannot recognize human codon usage and for that the sequence of interest needs to be synthesized. It was shown by their study that the expression system was effective for producing the recombinant TPO and the final recombinant products were constituting more than 40 percent of the total protein produced by the host cells.[41]

In the current study, the Hep G2 cell line cDNA was used to isolate TPO sequence. In comparison with previous prokaryotic studies, in the current study the requirement for synthesizing optimized sequence of TPO was bypassed, because the *Rosetta gami* strain could realize the human codon usage. Also the final products were expressed in the water soluble form that could reduce the cost of protein production.

SDS-PAGE and western blotting by His-tag monoclonal antibody confirmed that specified sequence has been expressed by the bacterial host. Inserting His-tag sequence was performed to ease protein purification by Ni-column.[42] This adjustment also was helpful in detection protein by western blotting.

Conclusion

Expressing recombinant human Thrombopoietin by using bacterial systems is an important issue. A still remaining concern for production of this recombinant protein by such a system is its inability to form disulfide bonds on the grounds of the oxide cytoplasm condition of bacteria which renders the final products as inclusion bodies. The new approach of using modified bacterial hosts is preferable for producing recombinant proteins like TPO that do not necessarily need glycosylation sites. Adopting both *Rosetta gami* as a host and pET32 as a vector resulted in a high production of the recombinant soluble TPO in the cytoplasm of the bacteria.

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Conflict of Interest: None declared.

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