Original Article

Effect of Twine-arginine Translocation-signaling Fusion System and Chaperones Co-expression on Secretory Expression of Somatropin

Mohammad Reza Bagherinejad, Hamid Mir-Mohammad Sadeghi, Daryoush Abedi, Fateme Moazen, Mohammad Rabbani

From the Department of Pharmaceutical Biotechnology, School of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran

Abstract

Background: Twine-arginine translocation (TAT) system is one of the exporting systems in *Escherichia coli* which could transport fully/semi-correctly folded proteins outside the reductive cytoplasmic space. In combination with co-expression with a chaperone system, the correctly folded proteins could be transported to oxidative periplasmic space and culture media to pass the main limitations in *E. coli* expression system such as misfolding and inclusion body formation. **Materials and Methods:** To study the effectiveness of signaling sequences and chaperone co-expression on the translocation of expressed protein, somatropin was selected as the target. Two common signal sequences in TAT system (TorA and SufI) were added at the N-terminal of somatropin and the cassettes were co-expressed in *E. coli* BL21 (DE3) by a chaperone team including DnaK/J-GrpeE. **Results:** The expression pattern studies including Western blotting and sodium dodecyl sulfate polyacrylamide gel electrophoresis confirmed that somatropin is expressed in two cassettes. However, the pattern was different for two signaling sequences. **Conclusion:** The results confirmed that the approach of using TAT-signaling sequences and co-expression with the chaperone team could enhance translocation of protein to periplasmic space and culture media compared to control groups. Western blotting results showed that the signal sequence TorA could transport more expressed proteins to the periplasmic space and culture media in comparison with SufI. However, there was a considerable amount of human growth hormone in the cytoplasm which could not be transported outside the cytoplasmic space.

Keywords: *Co-expression, DnaK/J-GrpE, growth hormone, signal sequence, SufI, TorA, twine-arginine translocation*

Introduction

Although *Escherichia coli* expression system has several advantages in the expression of heterologous proteins for therapeutic applications, lack of eukaryotic posttranslational modifications, misfolding, and inclusion body formation are the main disadvantages of this system. The improper conformation could make the expressed proteins biologically inactive and to undergo proteolytic degradation or inclusion body formation.[1] In reductive cytoplasmic space, intra- or inter-molecular disulfides bonds could not be correctly formed as well. Therefore, inactive aggregate form of the proteins is accumulated in cytoplasm and further solubilizing/refolding steps should be used in downstream process to obtain the correctly folded and active molecules. Therefore, engineering of transporting and folding pathways could address these limitations to produce more biologically active form of the proteins.[2]

In the folding pathway engineering, co-expression of protein of interest with chaperones could improve the yields of soluble proteins. DnaK/J-GrpE is one of the most active chaperone teams in *E. coli* which help in correct folding of the proteins.[3] It is supposed that DnaK could bind specifically to the twin arginine translocation (TAT) leader sequence during translation and synthesis of the protein and prevent misfolding in combination with DnaJ. ATP hydrolysis by DnaK is also stimulated by its interaction with DnaJ. GrpE acts as a nucleotide exchange factor. Thus, the team is capable of tightly regulating the normal housekeeping functions and stress-related functions.[4]

By targeting the proteins to the outside of the cytoplasmic space, the downstream process in industrial production of therapeutic proteins could be simplified, affordable, and cost-efficient. Fusing

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Address for correspondence:
Prof. Mohammad Rabbani, Department of Pharmaceutical Biotechnology, School of Pharmacy, Isfahan University of Medical Sciences, Isfahan 81746-73461, Iran. E-mail: rabbani@pharm.mui.ac.ir

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the N-terminus of the target protein with appropriate signal sequences transports the proteins to the oxidative environment of the periplasm and culture media which could help in the correct formation of disulfide bond as well. The most common translocation systems in *E. coli* are Sec-system, signal recognition particle-dependent pathway, and TAT system.[5,6] TAT pathway has a unique feature in transferring correctly fully/semi-folded proteins. Therefore, it could be considered as an internal quality control system for exporting the correct-folded proteins.[7]

In the current study, the effects of co-expression of DnaK/J-GrpE chaperone team and fusing with TAT signal sequences were investigated simultaneously on the proper folding and exporting of somatropin as target protein. Combination of these two approaches is supposed to reduce the inclusion body formation and transporting the proteins outside the cytoplasmic space.

Somatropin (human growth hormone [hGH]) is one of the most important therapeutic proteins playing role in a variety of biological functions. The endogenous hGH is a nonglycosylated molecule; therefore, the recombinant forms of hGH have been extensively produced in industrial scale in prokaryotic expression systems. Inclusion body formation, low-yield recovery in biological activity, and high cost of purification have forced researchers to explore the secretory expression of somatropin.[8-11]

Materials and Methods

Chloramphenicol and ampicillin were purchased from Sigma Chemical Co. (USA) and the culture media (Nutrient and Luria agar/broth) from Oxoid (Hampshire, UK). All chemicals and reagents were obtained from Merck (Darmstadt, Germany) and were of analytical grade.

Plasmids and bacterial strains

DNA polymerase (PFU polymerase) and endonucleases were purchased from Fermentas (Thermo Scientific, Lithuania). The constructs were synthesized by Biomatik (Canada) using *E. coli* coding optimization platform. Qiagen Gel Extraction Kit and Qiagen Miniprep Kit were used for purification from agarose gels and plasmid preparation, respectively. Chaperone plasmid set was obtained from TaKaRa (Japan). The expression vector, pET-22(b), was purchased from Novagen (Madison, USA). The primers were synthesized and purified by Tag Copenhagen A/S (Denmark). All the genetic materials and bacterial strains are listed in Table 1.

Cloning

The sequence of somatropin was obtained from www.drugbank.ca and checked with the United State Pharmacopeia for confirmation. Following codon optimization, the sequences were ordered for chemical synthesis in fusion with the selected signal sequences. Two signal sequences, TorA and SufI, were used for the synthesis of recombinant expression cassettes. Following synthesis, the cassettes were

Table 1: Genetic materials and bacterial strains used in the current study and their source

Item	Description/sequence (5'→3')
Primer forward I	CATATGAACAACAACGATCTGTTTTCA
Primer forward II	CATATGTCACTGAGTCGTCGCCA
Primer reverse	CTCGAGTTAGTGGTGGTGGTGGT
TorA	Atgaacaacaacgatctgtttcaggcgagccgccgccgctttctggcgcagctgggcggcctgaccgtggcgggcgaggctgctgggcccgagcctgctgacccgcgccgcgccgaccgcggcgcaggcgggcagc
SufI	atgagcctgagccgccgccagttattcaggcgagcggcattgcgctgtgcgcgggcgggtgccgctgaaagcgagcgcggcgggcgggcagc
Somatropin	tttccgaccattccgctgagccgcctgtttgataacgcgatgctgcgcgcgcatcgcctgcatcagctggcgtttgatacctatcaggaatttgaagaagcgtatattccgaaagaacagaaatatagctttctgcagaacccgcagacc
	agcctgtgctttagcgaaagcattccgacccgagcaaccgcgaagaaacccagcagaaaagcaacctggaactgctgcgcattagcctgctgctgattcagagctggctggaaccggtgcagtttctgcgcagcgtgtttgcgaacagc
	ctggtgtatggcgcgagcgatagcaacgtgtatgatctgctgaaagatctggaagaaggcattcagacctgatgggccgcctggaagatggcagcccgcgcaccggccagatttttaacagacctatagcaaatttgataccaacagc
	cataacgatgatgcgctgctgaaaactatggcctgctgtattgctttcgcaaagatatggataaagtggaaaccttctgcgcattgtgcagtgccgcagcgtggaaggcagctgcggcttt
pET-22(b)	T7 promoter, His-tag, Amp^R, PelB
pKJE7	pACYC ori, araB, Cm^R
pGEM-B1	pBR322 origin, lacZ_a reporter, Amp^R
pGEM-cassette I	Cassette I, pBR322 origin, lacZ_a reporter, Amp^R
pGEM-cassette II	Cassette II, pBR322 origin, lacZ_a reporter, Amp^R
pMRB I	pET-22(b)-TorA-hGH-Histag, AmpR
pMRB III	pET-22(b)-SufI-hGH-Histag, AmpR
DH5α	F^-, lacZ (lacZYA-argF), *deoR*, *recA1*, *endA1*, *hsdR17*
BL21 (DE3)	F^-, *ompT*, $hsdS_B$ ($r_B^- m_B^-$) *gal dcm* (DE3)

cloned to pET-22(b) using NdeI and XhoI endonuclease. The first recombinant plasmid, pET-22b-TorA-hGH (pMRB I), contained TorA as signal sequence and the second pET-22b-SufI-hGH (pMRB II) including signal sequence SufI. His-tag was used at the C-terminus of somatropin to enhance the purification and Western blotting. The constructs were checked and confirmed by polymerase chain reaction (PCR) and digestion. All the techniques used for the manipulation of genetic materials were performed as described by Sambrook and Russell.[12]

Transformation

Following plasmid preparation, an electroporation platform (GenePulser II, BioRad, USA) was used for co-transformation of the recombinant pET-22(b) and pKJE7 to the expression host strain, *E. coli* BL21 (DE3). The electroporation system was set at 25 μF, 2.5 kV, and 200 Ω. The pulse duration was 4.8 ms. Following recovery of the cells, selective plates containing ampicillin 20 μg/ml and chloramphenicol 34 μg/ml were spotted and streaked out with the tube content and incubated at 37°C for 24 h.

Growth conditions

A single colony from the streaked plate was inoculated in 10 ml LB and incubated at 37°C and 200 rpm overnight. This culture at OD 0.6 was used as inoculum for 50 ml media containing ampicillin and chloramphenicol as the selective marker. Following induction by IPTG and L-Arabinose at the final concentration of 1 mM and 4 mg/ml, respectively, the cultures were re-incubated at 37°C and 200 rpm for 4 h. As control, the same procedure was applied for cassette including somatropin without any signaling sequence and chaperone.

Expression study

After 4 h of induction, samples from cytoplasm, periplasm, and culture media were prepared as follows to study the effect of signal sequence and chaperone on translocation of hGH.

Exporting the target protein to the culture media

The media were harvested by centrifugation at 7000 g, 4°C for 15 min, to separate the clarified supernatant from biomass. The supernatant was used for studying the secretory expression of somatropin to culture media.

Exporting the target protein to the periplasmic space

Cold osmotic shock method was carried out for the extraction of the periplasmic content of pellets. One ml of hypertonic solution which containing sucrose 25% w/v, ethylenediaminetetraacetic acid 5 mM, and Tris-HCl 50 mM was added to the biomass. Mixtures were shaken on ice for 60 min. Following centrifugation, the supernatant was collected and kept at −20°C for protein analysis. Then, 1 ml of hypotonic solution ($MgCl_2$ 5 mM) was added to the pellet and resuspended and shaken for 60 min. The cell suspension was centrifuged again at 7000 g, 4°C for 15 min. The supernatant was kept at −20°C. The supernatant from two steps of centrifugation was mixed and used as periplasmic sample.

Cytoplasmic fraction

The pellet collected by centrifugation from the previous step was resuspended on phosphate buffer (100 mM, pH 7.2). Following sonication, the suspension was centrifuged at 7000 g, 4°C for 15 min, and the supernatant was collected and kept at −20°C as cytoplasmic fraction.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting analyses

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 15% (w/v) acrylamide-resolving gel with 5% stacking as described by Laemmli (1970). Proteins were stained by Coomassie brilliant blue R-250. Western blotting was performed using diaminobenzidine and anti-hGH monoclonal antibody to confirm the expression of hGH.

Results

Expression cassettes

The recombinant plasmids and bacterial hosts used in the current study are shown schematically in Figure 1. Following synthesis and sequence approval of the cassettes including signaling sequence and somatropin, the cassettes were amplified by PCR using the primers that were designed in this study [Figure 2]. The optimum conditions for the PCR reaction were determined through gradient method. The amplified fragments were 738 and 699 bp, respectively, for cassette I and II. The insert in control group containing somatropin with no signaling sequences was 599 bp. Following digestion by Nde I and Xho I, the insert fragments were ligated to pET 22 b. The recombinant plasmids were co-transformed to *E. coli* BL 21 (DE3) with pKJE7 plasmid from TaKaRa kit containing DnaK/J-GrpE chaperone team [Figure 1].

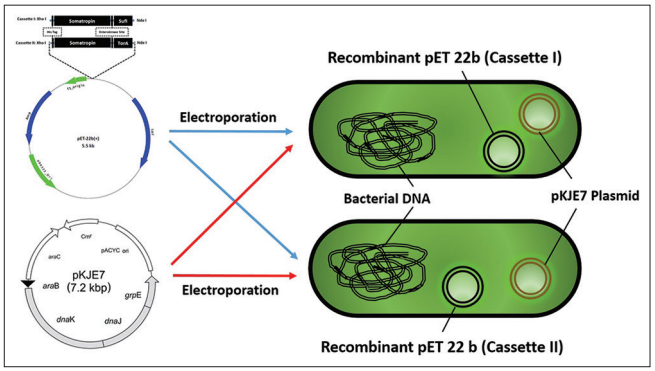

Figure 1: pET-22b(+) was used for expression of target genes. Cassettes including the signal sequence and somatropin were synthesized and ligated to pET-22b(+) using appropriate endonuclease. TorA was used for cassette I and II which was synthesized by the SufI signal sequence. Enterokinase site was also added between the signal

Expression study

The SDS-PAGEs following expression of the two recombinant plasmids and the control group are shown in Figures 3-5 which represent the control, cassette I, and cassette III results, respectively. Western blotting of expression pattern of cassette I is shown in Figure 6. The results showed that using signal sequences and chaperone co-expression could export the target protein, somatropin, from cytoplasm to periplasmic space and culture media [Figures 4-6] compared to control [Figure 3]. Although using signaling sequences could transport the targeted protein outside the cytoplasm, most of the proteins were accumulated in cytoplasm. The presence of protein in the periplasmic space and culture media supports the hypothesis that TAT signaling sequence could transport the protein to the outside of the cell. A quantitative study should be performed to compare the efficiency of the two cassettes and evaluate the feasibility of this approach for secretory production of recombinant protein. However, it

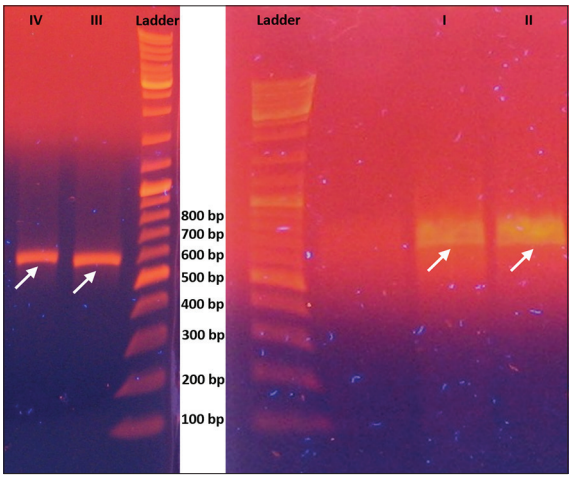

Figure 2: The size of inserts and control was cloned to pET-22b for transformation to *Escherichia coli* DH5α. Following plasmid preparation, digestions were carried out with appropriate endonucleases (NdeI and XhoI). The cassette sizes were 738, 699, and 591 bp for cassettes I and II and control, respectively. L: DNA Ladder Mix, I: The insert of cassette II (SufI with human growth hormone), II: The insert in cassette I (TorA and human growth hormone), III: control (somatropin)

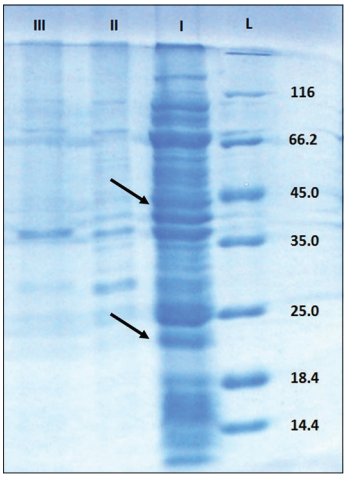

Figure 3: Expression pattern of the control group. The cassette containing somatropin without signaling sequence/DsbA. The periplasmic solutions were pooled and loaded on the gel as one fraction. The target protein, somatropin, with molecular weight 22 kDa was accumulated in the cytoplasm (Lane II and III). Although expression system is capable of producing proteins in the absence of signaling sequence/DsbA (Lane I), the proteins form inclusion body in the cytoplasm. L: Protein molecular weight marker, I: Cytoplasmic fraction, II: Periplasmic fraction following the osmotic shock, III: Secretory fraction to culture media. The molecular weight of somatropin is 22 kDa. The band around 45 kDa is supposed to be the dimer form of target protein according to Western blotting result

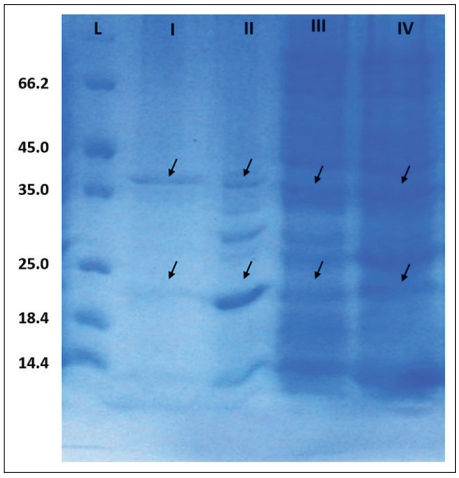

Figure 4: Expression pattern of somatropin when using TorA as signaling sequence and co-expression. L: Protein molecular weight marker, I: Secretory fraction to culture media, II: Periplasmic fraction following osmotic shock with a hypotonic solution, III: Periplasmic fraction following osmotic shock with a hypertonic solution, IV: Cytoplasmic fraction. Although there is a nice and sharp band regarding the somatropin in periplasmic solution (lane II), the secreted protein to culture media was low. The molecular weight of somatropin is 22 kDa. The band around 45 kDa is supposed to be the dimer form of target protein according to Western blotting result

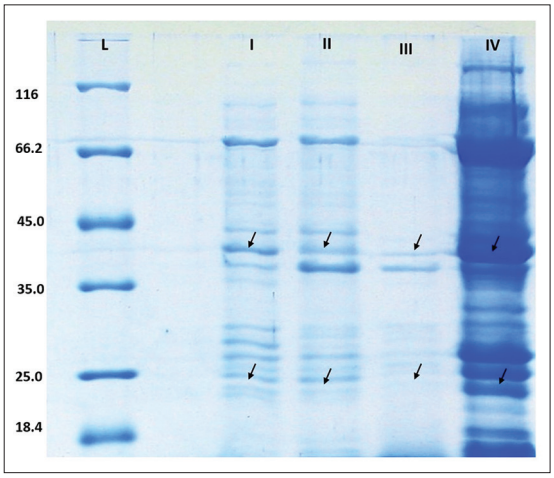

Figure 5: Expression pattern of somatropin using SufI as signaling sequence. L: Protein molecular weight marker, I: Periplasmic fraction following osmotic shock with a hypertonic solution, II: Periplasmic fraction following osmotic shock with a hypotonic solution, III: Secretory fraction to culture media, IV: Cytoplasmic fraction. Although the target protein is exported to periplasmic space and outside the cell, most of the proteins are still located in the cytoplasm. The molecular weight of somatropin is 22 kDa. The band around 45 kDa is supposed to be the dimer form of target protein according to Western blotting result

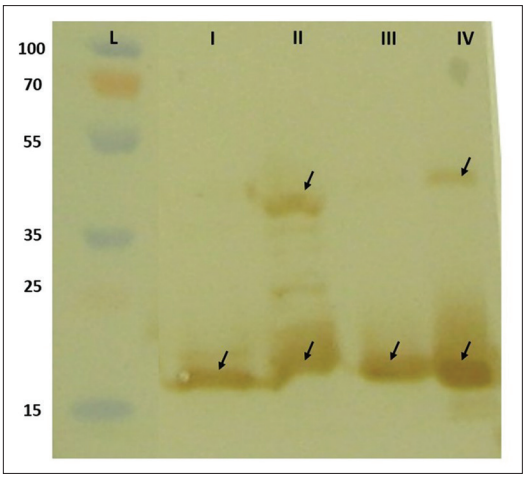

Figure 6: Western blot of the expression pattern of somatropin when using TorA as signaling sequence. L: Protein molecular weight marker, I: Cytoplasmic fraction, II: Secretory fraction to culture media, III: Periplasmic fraction following osmotic shock with a hypertonic solution, IV: Periplasmic fraction following osmotic shock with a hypotonic solution. Although the target protein is exported to periplasmic space and outside the cell, most of the proteins are still located in the cytoplasm. The hypertonic solution is eluted with more protein compared to the hypotonic solution. The molecular weight of somatropin is 22 kDa. The band around 45 kDa is the dimer form of target protein

seems TorA could secrete more proteins according to the Western blotting results.

SDS-PAGE of the distribution pattern of somatropin expressed using cassette II showed that the protein is highly accumulated in periplasmic space compared to control and cassette I.

Discussion

Inclusion bodies formation is one of the limitations in the expression of heterologous proteins in *E. coli*. Additional solubilization and refolding steps before purification is the method to solve this issue which impose more cost and lower yield of production process.[13] Accordingly, there is a huge interest in the secretory expression of therapeutic proteins to the periplasmic space or culture media.[14] Different strategies could be used to solve these problems such as fusing the protein with signaling peptide and co-expression with chaperone proteins which help in proper folding of the proteins. We designed the current study to investigate the effect of these strategies in secretory expression of somatropin.[15]

Using signaling sequences is one of the most practically accepted approaches which could transport the proteins to the more oxidative environment outside the cytoplasm space.[16] This method could offer several advantages including more biologically active form of the protein, proper disulfide bond formation, and improve product quality by lowering the contamination with nucleic acids and lipopolysaccharide and host cell proteins. There are different transport systems in *E. coli* such as Sec-system and TAT pathway. The signal sequence in Sec-system is studied very well and there are many reports in this regard.[17,18] However, TAT pathway which is found recently is another promising system to export the protein of interest to the outside of the cytoplasm. The highly distinctive features of this system are that correctly folded proteins could be exported to the periplasmic space. Therefore, it is some kind of quality control system which could actively distinguish the correctly folded active form of the proteins from inactive ones. For example, fusion of green fluorescent protein (GFP) to a Sec-specific signal sequence resulted in periplasmic localization of GFP in an inactive form.[17] However, fusion of GFP to a TAT signal peptide efficiently exports GFP to the periplasm in an active form.[19,20] This study also proposes the great potential of the TAT pathway for industrial production of therapeutic proteins.[19] According to our result, in comparison with previous works, it could be supposed that the capacity of TAT system is acceptable for industrial applications; however, more quantitative studies should be performed for confirmation. Some studies have also confirmed that the export capacity is increased if the TAT ABC genes are overexpressed.[21] There are some studies which use TAT pathway to export different kinds of heterologous therapeutic proteins such as somatropin, interferons, and single-chain fragment antibodies in active form. TAT pathway is supposed to be the only effective method for export of active proteins to periplasm. In the current study, we could not do any bioassay to determine the activity of the somatropin. However, it is planned to study in further works.

Culture conditions such as temperature, pH, and culture media compositions have also effect on the export of the proteins to the outside of the cells. Choi and Lee showed lowering of the induction temperature could significantly solubilize most of the inclusion bodies of somatropin under optimized culture conditions in periplasmic space[13] However, we could not find any significant difference in the expression of proteins in the culture media and periplasmic space by reducing the culture temperature from 37°C to 30°C.

Other parameter which is very important for efficient secretory expression of recombinant proteins is the sequence of the signaling peptide. OmpA, STII, Penicillinase, PhoA, LTB, NPR, DsbA, pelB, and natural hGH signal peptide are examples of the signal sequences used in different studies.[5] Zamani *et al.* have predicted the effect of different signal sequences on the efficiency of the system by *in silico* methods.[22] However, their ranking system was not approved by the *in vitro* studies. In our study, the Western blotting result could propose that TorA is more active in transporting the heterologous protein to the outside of the cytoplasm. It has already planned to confirm this item through ELISA study.

Conclusion

The current study showed that fusing the heterologous protein with TAT-signaling peptides and co-expression with

chaperones could export somatropin to the outside of the cytoplasmic space and reduce the inclusion body formation. The study demonstrated that TorA signal sequence was more active in transporting the target protein to the culture media compared to SufI as it was confirmed in previous study which was performed by DsbA as signaling sequences.[23] These data approved previous work regarding the potential of the TAT pathway for industrial applications as well. However, most of the proteins form inclusion body and quantitative study and bioassay methods should be used in further studies to confirm the potential use of this method in industrial scale.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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