

Cloning, Expression, and Refolding of PPE17 Protein of Mycobacterium Tuberculosis as a Promising Vaccine Candidate

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What's Known

- PPE17 (RV1168c) protein is known as a potent stimulator of T cell which triggers TH1 responses in parallel with high IFN- γ release from PBMC in pulmonary tuberculosis peoples and PPD negative.

What's New

- Due to the high GC content of such PPE17 protein, high-throughput expression and correct refolding of this protein is of demand to prepare nano-vaccines.

Abstract

Background: Tuberculosis as a global health problem requires special attention in the contexts of prevention and control. Subunit vaccines are promising strategies to protect burdens of tuberculosis via increasing the BCG protection. The present study aimed to design a vaccine study by means of high-throughput expression and correct refolding of recombinant protein PPE17.

Methods: We aimed to clone, express, and refold PPE17 protein of *Mycobacterium tuberculosis* in bacterial systems as a promising vaccine candidate. The PPE17 (Rv1168c) gene was PCR amplified and inserted into pET-21b(+) vector, cloned in *E. coli* TOP10, and finally expressed in *E. coli* BL21(DE3).

Results: The expressed recombinant protein was entirely found in insoluble form (inclusion bodies). The insoluble protein was denatured in 6M guanidine-HCl and refolded by descending denaturant concentration dialysis. Moreover, the recombinant protein was purified by Ni-NTA column chromatography. The changing temperature had no effects on solubilizing protein and the maximum expression was achieved at 0.5 mM concentration of isopropyl-D-thiogalactopyranoside (IPTG) induction.

Conclusion: Bacterial expression system is a timesaving tool and refolding by descending denaturant concentration dialysis is a rapid and reliable method.

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Keywords • Rv1168c protein • Mycobacterium tuberculosis • Protein refolding • Gene expression

Introduction

Mycobacterium tuberculosis (Mtb) remains the second leading cause of death due to infective agents with an incidence rate of 1.5 million per year.¹ Subunit vaccines are considered as anticipant strategies for improving immunity to tuberculosis.² The development of subunit vaccines requires a thorough understanding of immune responses generated by potent immune stimulator proteins. The PPE proteins are unique to pathogenic mycobacteria which are absent in non-pathogenic species.³ PPE17 (RV1168c) is a surface localized protein that is upregulated in microaerophilic and anaerobic conditions, resembling macrophage environment.^{4,5} This protein is a potent stimulator of tuberculosis bacilli in the context of both innate and adaptive immunity, which was shown by high levels of IFN- γ

and IgG in pulmonary TB and BCG vaccinated populations.⁶ In the present study, the PPE17 protein of *Mycobacterium tuberculosis* was expressed and refolded as a promising vaccine candidate.

Materials and Methods

Materials

The entire Pfu DNA polymerase, 100 bp DNA ladder, 1 Kb DNA ladder, protein size marker, restriction endonucleases, and T4 DNA ligase (Thermo Scientific Life Sciences GmbH, Hilden, Germany) monoclonal anti-polyhistidine peroxidase conjugate (Sigma-Aldrich Corp., St. Louis, USA) were kept at -20°C. Ni-NTA agarose (Qiagen GmbH, Hilden, Germany), plasmid DNA extraction and gel extraction kits (Bioneer, Korea) were kept at 4°C. Cloning/expression vector pET-21b, *E. coli* Top10, and BL21(DE3) strains were already available. Isopropyl-D-thiogalactopyranoside (IPTG) and ampicillin (Pars Tous, Iran) were in demand. Dialysis membranes (Scientific Laboratory Supplies, UK) and polyvinylidene difluoride (PVDF) membranes (Amersham Bioscience, Buckinghamshire, USA) were provided. Primers for cloning PCR (Macrogen, Korea) were synthesized according to designed sequences.

Construction of Cloning/Expression Vector Containing PPE17 Gene PCR Amplification of PPE17 Gene

The Rv1168c gene of the *M. tuberculosis* H37Rv genome was used as a template for PCR amplification coding for the 346 aa protein of demand. The designed cloning primers for PCR amplification of PPE17 gene were forward primer: 5-CGACAAGCTTGATTTTCAAAATTTTCCGCCGGAGT-3 and reverse primer: 5-GATACTCGAGGCCGGCTGCGGGTGACCG-3. The embedded restriction sites for *HindIII* and *XhoI* (bolded letters) were inserted in both primers at forward and reverse directions, respectively. The specific condition for Pfu DNA polymerase activation was as follows: 94 °C for 1 minute, 57 °C for 45 seconds, 72 °C for 1.5 minutes (30 cycles), and 72 °C for 7 minutes as the final extension step. The 50 µl of buffer containing 2.5 mM MgSO₄, 250 µM dNTPs, and 2.5 U Pfu DNA polymerase was considered per reaction tube.

Cloning and Subcloning of Target Gene PPE17

The 1041 bp PCR product was purified through Bioneer corporation columns according to experimental procedures. The pET-21b(+) vector and the PCR products were subjected

to double digestion with *HindIII* and *XhoI*. Then, the linearized vector and insert were extracted from the gel by using gel purification kit (Bioneer, Korea). The T4 DNA ligase was engaged for the ligation of PPE17, insert into pET-21b(+) vector, and then recombinant vector was transformed into *E. coli* TOP10 cells. The transformants were screened by colony PCR method with specific primers for T7 promoter and the products were analyzed on agarose 1% gel. The recombinant plasmid pET21b-PPE17 from the positive PCR colonies was purified from cultured *E. coli* TOP10 cell and then subjected to plasmid DNA sequencing. Furthermore, this plasmid was transformed into these competent *E. coli* BL21(DE3) cells and positive colonies were identified by growing on LB agar plates containing 100 µg/ml ampicillin. For final confirmation, recombinant vector pET21b-PPE17 was extracted from these growing colonies on ampicillin agar using PrimePrep plasmid DNA extraction kit (Bioneer, Korea) and subsequently was double digested with *XhoI* and *HindIII* restriction enzymes for 16 hours at 37°C. The presence of 1041bp insert (Rv1168c gene) was confirmed by running double digested product on 1% agarose gel.

Expression and Cell Disruption

The transformed *E. coli* BL21(DE3) cells carrying the recombinant pET-21b(+) were considered for the expression of PPE17 protein. The LB broth medium, containing 100 µg/ml of ampicillin inoculated with BL21(DE3) cells, was cultured and incubated at 37 °C on 160 RPM shaker until turbidity of 0.6 at A600. Then, it was induced with 0.5 mM isopropyl-D-thiogalactopyranoside (IPTG) for 18 hours. The overnight grown (1 L) culture was harvested by centrifugation at 6,000g for 10 minutes and the supernatant was discarded. The remaining sediments (10 g) were suspended in 15 ml sonication buffer containing 50 mM KH₂PO₄, 50 mM K₂HPO₄, 150 mM NaCl, 0.5% Triton X-100, and 10% glycerol (pH 7.8). After vortexing, the sediments were sonicated for 5 minutes (pulse 1 second on/off, 80% amplitude) and the lysate was then centrifuged at 16,000 g at 4 °C for 15 minutes. The sediment and supernatant were analyzed by 12% SDS-PAGE.

Solubilizing of Inclusion Bodies

The sediments containing insoluble protein PPE17 (inclusion bodies) were re-suspended in solubilizing buffer containing 3 and 6M guanidine-HCl, 50 mM KH₂PO₄, 50 mM K₂HPO₄, 150 mM NaCl (pH 7.8), and then shook for 1 hour at room temperature. The solubilized inclusion

body was centrifuged at 16,000 g for 30 minutes at 4 °C and the supernatant was collected. Both samples from sediments and supernatants were analyzed by 12% SDS-PAGE gel.

Purification and Refolding of Protein PPE17

The cleared supernatant containing solubilized protein PPE17 was purified under denaturing conditions using Ni-NTA column chromatography. Prior to loading of cleared lysates, the column pre-equilibrated with 6M guanidine-HCl in 50 mM KH_2PO_4 , 50 mM K_2HPO_4 , and 150 mM NaCl (pH 7.8). After sample loading (0.5 ml/min rate), the column was washed with 3M guanidine-HCl in 50 mM KH_2PO_4 , 50 mM K_2HPO_4 , 150 mM NaCl, and 45 mM imidazole (pH 7.8). The protein was eluted (0.5 ml/min rate) with 500 mM imidazole in 50 mM KH_2PO_4 , 50 mM K_2HPO_4 , and 150 mM NaCl (pH 7.8). The eluted protein was collected in separate fractions (1 mL of each) and was analyzed by 12% SDS-PAGE gel.

Refolding was done with descending denaturant concentration dialysis with some modification.⁷ The denatured protein was dialyzed against 1 L of freshly made PBS containing 0.5 M guanidine-HCl and 20% glycerol (pH 7.4). The concentration of denaturant was then decreased steadily by pumping in 3 L of PBS containing 20% glycerol at the rate of 50 ml/h. SDS-PAGE analysis on 12% gel revealed a 36 kDa protein as demand.

Protein Quantification Assay

BCA (bicinchoninic acid) method was used to determine the protein concentration. Glycerol and guanidine-HCl should be removed before BCA assay since these interfere with the chemicals. The 1:10 dilution of the sample protein in DW was mixed with 100 μl 4 mM sodium deoxycholate and after 10 minutes at RT, 100 μl of 4.5 M trichloroacetic acid was added. The suspension was then centrifuged at 3,000 g for 15 minutes. After discarding the supernatant, the pellet was mixed with 1 ml of 3:1 Diethyl Ether-ethanol solution and was centrifuged at 9,000 g for 45 minutes. The pellet was air-dried and dissolved in 100 μl of DW. The 1 mg/ml bovine serum albumin (BSA) was used as the standard protein.

Western Blotting of PPE17

Following electrophoresis on 12% SDS-PAGE gel, the refolded protein was blotted onto polyvinyl difluoride (PVDF) membrane in western blot transfer equipment (Bio-Rad, USA). Then, the gel was covered with transfer buffer containing Tris-HCl (25 mM), glycine (190 mM), and methanol (20%). The transfer was

accomplished in a cold room at a constant current of 300 mA for 15 minutes. The blocking was achieved with 2% BSA in PBS overnight at 4 °C and then rinsed three times with PBS and probed with the monoclonal anti-polyhistidine (mouse IgG2a isotype) peroxidase conjugate antibody at 1:2000 dilutions (PBS containing 1% BSA) for 2 hours at RT. The blot was rinsed four times with PBS and applied to chemiluminescence substrate according to manufacturer's recommendations. The chemiluminescent signals were captured by a CCD camera-based imager (G: BOX Chemi XRQ, UK) and the target protein band intensity was read by an image analysis software (GeneTools).

Results

PCR Amplification and Cloning of PPE17 Gene

The 1041-bp ORF, coding for PPE17 protein of *M. tuberculosis* H37RV, which terminated on both sides by restriction sites for endonucleases, *Hind*III, and *Xho*I were amplified by high-fidelity pfu DNA polymerase (figure 1). The plasmid pET-21(b)+ was extracted from *E. coli* TOP10 and both inserts and vectors were double digested prior to ligation by T4 ligase (figure 2). The linearized cloning/expression vector pET-21(b)+ was cloned with double digested PPE17 insert to form recombinant vector plasmid. The recombinant vector was then transformed into *E. coli* TOP10 and the cloning process was confirmed by colony PCR method (figure 3). The recombinant vector from positive colonies was extracted and the existence of insert was proved by sequencing (data not shown). The results from the blasting of sequenced fragment had a sequence identity of at least 98% (data not shown). The presence of PPE17 gene was then

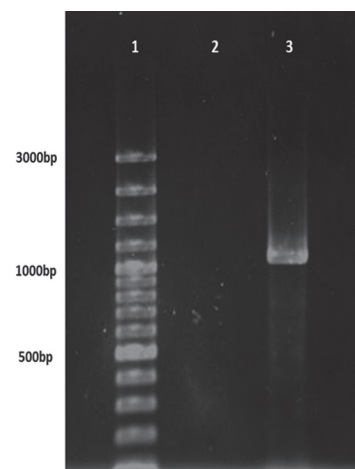


Figure 1: PCR amplification of *M. tuberculosis* PPE17 gene shows a 1041 bp sequence. Lane 1: 100 bp plus DNA ladder; Lane 2: Negative control; Lane 3: PPE17 gene.

confirmed by double digestion of transformed *E. coli* BL21 (DE3) plasmids with restriction enzymes *Hind*III and *Xho*I (figure 4).

Expression and Solubilization of Recombinant Protein PPE17

SDS-PAGE analysis of transformed *E. coli* BL21 (DE3) expression resulted in an insoluble ~36 kD protein in the form of inclusion bodies (IB), as expected. Figure 5 shows the high expression of 0.5 mM IPTG induced cultures compared with 0.2 mM IPTG and non-recombinant BL21 (DE3). There was no obvious increase in protein expression on IPTG concentration over 0.5 mM (data not shown). There was a low expression of supernatant bacterial protein equals to 36 kDa recombinant protein PPE17. Solubilization of

expressed protein was not affected by changing the incubation temperatures at 18, 22, and 27°C for 18 hours (figure 6). Protein PPE17 was solubilized under denaturant condition in 6 M guanidine-HCl. Our data showed that IBs were insufficiently solubilized by 3 M guanidine-HCl; however, the entire IBs were nearly solubilized in 6 M concentration of guanidine-HCl (data not shown).

Purification and Refolding of Denaturant Protein PPE17

The 6×His-tagged recombinant protein PPE17 was purified through affinity chromatography in denaturant condition using

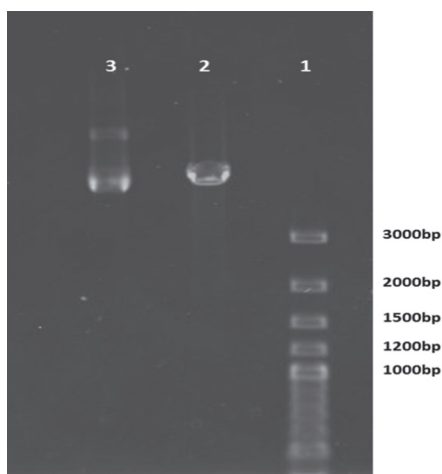


Figure 2: Double digestion of recombinant pET-21b(+) cloning vector by *Hind*III and *Xho*I restriction enzymes shows a single band representing linear plasmid and two tandem bands representing circular and supercoiled plasmids. Lane 1: 100 bp plus DNA ladder, Lane 2: Digested vector, Lane 3: Undigested pET-21b(+).

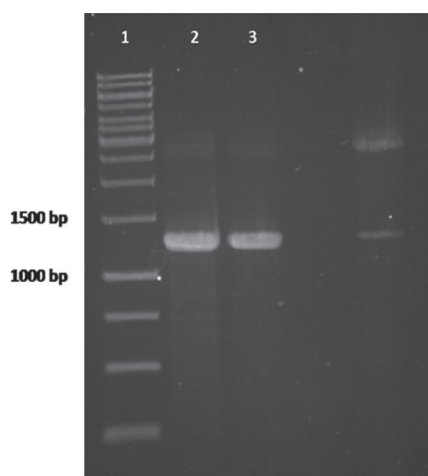


Figure 3: PCR on recombinant *E. coli* TOP10 colonies harboring pET21(b) proves the existence of PPE17 gene. Lane 1: 1 kb DNA ladder; Lanes 2-3: PCR products (1041 bp).

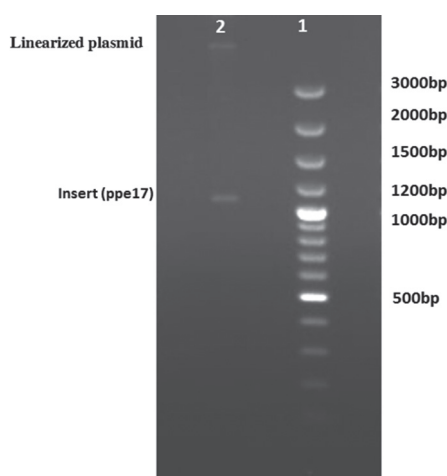


Figure 4: Post-digestion of recombinant pET21(b)+ cloning vector by *Hind*III and *Xho*I restriction enzymes shows 1041 bp segment of interest. Lane 1: 100 bp plus DNA ladder; Lane 2: PPE17 gene and linearized plasmid.

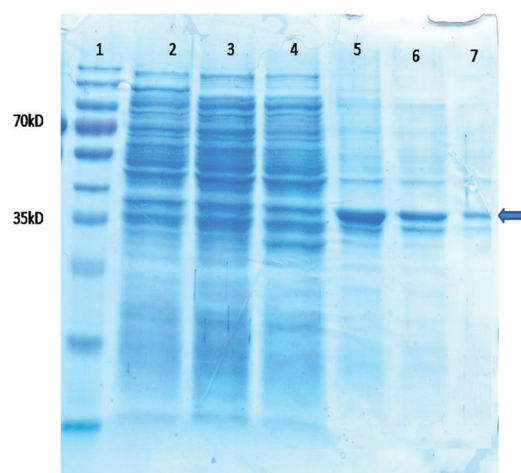


Figure 5: Optimization of recombinant PPE17 expression by different IPTG concentrations shows a 36 kD band protein resembling PPE17 protein in variable values at supernatant. Lane 1: Protein molecular weight marker; Lanes 2-4: Bacterial supernatant induced with 0.5, 0.2, and 0 mM IPTG at 37 °C; Lane 5: Bacterial sediment with 0.5 mM IPTG; Lane 6: Bacterial sediment with 0.2 mM IPTG; Lane 7: Bacterial sediment without IPTG.

Ni-NTA resin. The SDS-PAGE analysis of eluted proteins (10 separate 1 ml fractions) has shown efficient results in elutions 3-6 and downward results in fractions 7-10 (data not shown). Refolding of PPE17 protein was achieved by descending denaturant concentration dialysis against PBS. Finally, protein aggregation occurred in guanidine-HCl concentrations lower than 125 mM. Figure 7 shows a 36 kDa protein of interest after purification and refolding.

Western Blotting Analysis

The evaluated 6×His-tagged recombinant protein PPE17 was confirmed by mouse anti-polyhistidine antibody. Results from western blotting have revealed a 36 kD protein of demand, as illustrated in figure 8.

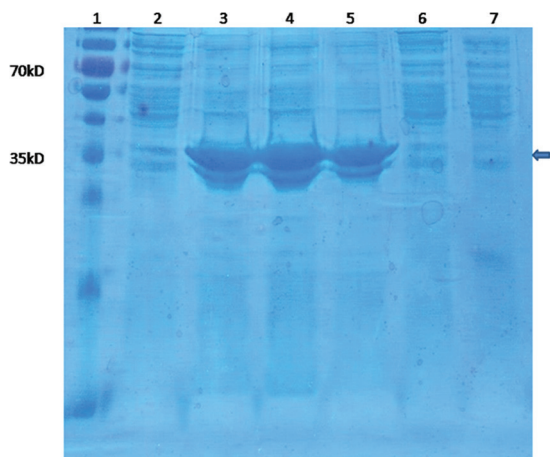


Figure 6: Expression of recombinant PPE17 protein under different temperatures has no effects on solubilization. Lane 1: Protein molecular weight marker; Lanes 2, 6, 7: Bacterial supernatants at 22, 25, and 27°C; Lanes 3-5: Bacterial sediments at 22, 25, and 27°C.

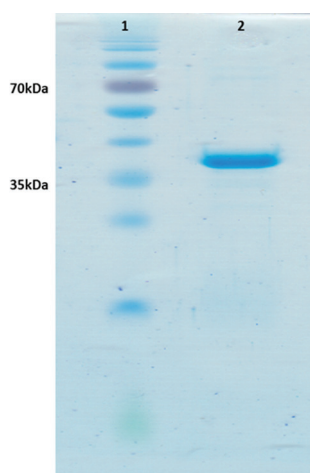


Figure 7: SDS-PAGE analysis of purified and refolded protein PPE17 shows a single band. Lane 1: Protein molecular weight marker; Lane 2: 36 kD protein of PPE17.

Estimation of Protein Concentration and Yield of Expression

The protein concentration of final refolded protein was 850 µg/ml using bicinchoninic acid (BCA) assay. The yield of expression was ~4.5 mg/L of bacterial cultures.

Discussion

Considering some inefficiencies of currently available BCG vaccine, there has been an increasing need for the development of a promising vaccine with high reliability against *tuberculosis*.⁸ Due to the lack of or down regulation of some proteins with dominant immunogenicity in BCG strains, the subunit vaccines have been investigated for improving BCG-induced immunity.⁹ In this regard, many of the cell components of *M. tuberculosis* have been investigated for subunit-based vaccine design.¹⁰⁻¹² Since the subunit vaccine proteins do not need any post-translational modifications, we had to use *E. coli* as expression hosts for the expression of PPE17 recombinant protein. Bacterial expression systems, contrary to other hosts (e.g. yeasts), have shorter growth time. Hence, the contamination of bacterial cultures is less likely. As mutations are common in gene amplifications by such well-known bacterial DNA polymerase, PCR amplification of target gene PPE17 was investigated by high fidelity *pfu* DNA polymerase with 3' to 5' exonuclease proofreading activity to minimize the mutation rates to less than 10% from similar amplifications performed with *Taq* DNA polymerase.¹³ There is a Pwo polymerase by which blunt-ended PCR products are directly usable for ligation without purification, as was done in the present study.¹⁴ The blunt-ended PCR products have restriction sites at both

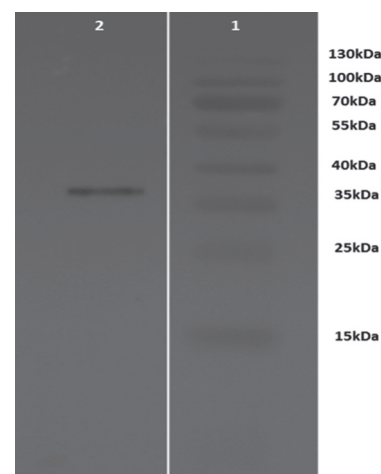


Figure 8: Western blotting analysis of recombinant protein PPE17 shows a single band of 36 kD protein of demand. Lane 1: Protein molecular weight marker; Lane 2: Western blot of the purified recombinant PPE17 protein.

ends for *Hind*III and *Xho*I restriction enzymes, which enabled insertion into plasmid vector pET-21b(+). Since the two restriction enzymes have propensity to act in a wide-spectrum of buffered conditions, double digestion of insert and vector was accomplished in the same buffer conditions to avoid any adverse reactions. In the present study, PPE17 amplified gene was ligated into the prokaryotic cloning/expression vector pET-21b(+). The pET-21b(+) is a 5442 bp transcription vector with a C-terminal His-tag and N-terminal T7 promoter, being designed for cloning/expression in bacterial systems. In a study by Azimnia et al., pJET ϵ and pET-22b(+) vectors were used for cloning and expression, respectively.¹⁵ Meanwhile, in the present study, to avoid any possible errors on insertion of PPE17 into vectors, the pET-21b(+) vector was used for cloning and expression in a single-step. The 6 \times His-tagged sequence, designed upstream of the PPE17 gene, is used to enable purification by metal chelating chromatography as with the Ni-NTA column, and western blotting investigation by using anti-His tag monoclonal antibodies. In pET-21b(+) vector system, the IPTG inducible lac-operon together with T7 promoter is designed to high recombinant protein expressions. The recombinant plasmid pET-21b(+) was transformed to *E. coli* BL21(DE3), which is a specific bacterial strain for the expression of target genes under regulation of T7 promoter. The inserted PPE17 gene, which was cloned downstream of T7 promoter showed maximum expression under induction by IPTG. In many studies, 0.1-1 mM concentration of IPTG is considered as the highest.^{16,17} In the present study, experimental optimizations have revealed that high concentrations of IPTG over 0.5 mM have no obvious effect on the level of protein expression. According to previous studies, low temperature was an effective way to solubilizing expressed proteins to which correct folding of proteins is desired.^{18,19} However, the results obtained from optimization at different temperatures in the current study revealed that decreasing temperature conditions had no obvious effects on solubilizing recombinant protein PPE17. Generally, high concentration of denaturants (e.g. urea and guanidine-HCl) are used to solubilize recombinant proteins.²⁰ Urea is a known denaturant with higher safety than guanidine-HCl, which is preferred for solubilizing bioactive proteins, but its efficiency to solubilize denatured proteins is lower than guanidine-HCl.²¹ Nevertheless, in the study by Wang et al., the yield of unfolded protein from treating with 8M urea is 20 times higher than with guanidine-HCl.¹⁴ However, the results from the present study showed that guanidine-HCl as denaturant has acceptable efficiency in

solubilizing IBs. Refolding by gel filtration methods resulted in the prevention of aggregation, as was shown in a study by Batas et al.²² However, we found that, in order to refold protein by dialysis, complete separation of denaturant led to the aggregation of recombinant protein. In this regard, the presence of low concentrations of chaotropes in refolding of denatured proteins is considered as a noteworthy procedure.²³ Based on our data, refolding by dialysis in the presence of refolding buffer containing 0.5 M guanidine as additive is an acceptable method in high-throughput protein refolding process. However, inability to complete the separation of guanidine from refolded protein is a negligible limit.

Conclusion

The PPE17 protein of *M. tuberculosis* was expressed and refolded in the presence of guanidine-HCl as a mild additive. Compared with the other expression hosts, bacterial system is a timesaving tool with low technical problems and refolding of denatured proteins by descending denaturant concentration dialysis is a rapid and reliable method.

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Conflict of Interest: None declared.

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