Original Article

Determining Induction Conditions for Expression of Truncated Diphtheria Toxin and Pseudomonas Exotoxin A in *E. coli* BL21

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Abstract

Background: Targeted cancer therapies have played a great role in the treatment of malignant tumors, in the recent years. Among these therapies, targeted toxin therapies, immunotoxins, has improved the patient's survival rate by minimizing the adverse effect on normal tissues, whereas delivering a high dose of tumoricidal agent for eradicating the cancer tissue. Immunologic proteins such as antibodies are conjugated to plant toxins or bacterial toxins such as Diphtheria toxin (DT) and Pseudomonas exotoxin A (PEA). In this case optimizing and expressing the Diphtheria toxin and Pseudomonas exotoxin A which their binding domains are eliminated plays a crucial role in producing the desired immunotoxin

Materials and Methods*:* We expressed the truncated DT and PE toxin in a genetically modified E.coli strain BL21 (DE3). For this reason we eliminated the binding domain sequence of these toxins and expressed these proteins in an expression vector pET28a with the kanamycin resistant gene for selection. The optimization of the Diphtheria toxin and Pseudomonas exotoxin A expression were due to different IPTG concentration, induction and sonication time

Results: We observed that the optimal protein expression was gained in 4 hours and at 25°C with 0.4 mM IPTG concentration for DT and 4 hours and at 25°C with 0.5 mM IPTG concentration for PEA.

Conclusion: Our study also showed lower IPTG concentrations could result in higher protein expression. By optimizing this procedure, we facilitate the protein production which could lead to accelerate the drug development

Keywords*:* Immunotoxin, IPTG concentration, Diphtheria toxin, Pseudomonas exotoxin A

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Introduction

Cancer is one of the main reasons of death among the people worldwide and with its mysterious mechanisms it is almost inevitable to cure this malignant health problem without any adverse side effects[1]. In the recent years, targeted therapy is playing a major role in the treatment of malignant tumors[2].

Targeted toxin therapies improve the patient's survival rate by minimizing the adverse effect on normal tissues, whereas delivering a high dose of tumoricidal agent for eradicating the cancer tissue. Immunotoxins were invented for more selective binding of a specific toxin to the tumor cells. Two main parts of the immunotoxins are the targeting and killing compartments[-]. Immunologic proteins such as antibodies, growth factors or cytokines which help the immunotoxin to bind precisely to the target cells were conjugated to plant toxins such as ricin, abrin, mistletoe lectin or bacterial toxins such as Diphtheria toxin (DT) and *Pseudomonas* exotoxin A (PE)[3-6]. The potency of the immunotoxins comes from the toxin compartment whereas the specificity comes from the antibody or any other growth factors[7]. DT is a single chain protein with 535 amino acids comprising of 3 functional groups: A domain (amino acids 1-186) the catalytic domain, B domain (amino acids 389-535) the binding domain and the third domain is the transmembrane or the translocation domain (amino acids 187-388)[8]. The catalytic domain of DT inhibits the elongation factor 2 (eEF-2) by ADP-ribosylation of histidine-699[9]. This inhibition eliminates protein synthesis and induces cell apoptosis[10]. The role of the translocation domain is to internalize the toxin to the cytosol compartment[4,11]. The binding domain of the toxins is responsible for cell recognition and the attachment of the toxin to its cell receptor[12,13]. The single chain polypeptide of PE contains 613 amino acids comprising of 3 main domains. Ia domain is a binding domain, II domain is a translocation domain and the Ib domain which is a catalytic domain with the ADP-ribosylating activity which induces cell death[14,15].

In this study, we expressed truncated DT and PE toxins in a genetically modified *E.coli* strain BL21 (DE3). For this reason, The binding domain sequences of these toxins was eliminated in order to specifically target the cancerous cells and expressed these proteins in an expression vector pET28a with the kanamycin resistant gene for selection. Moreover, we optimized the expression condition of the DT and PE toxins using different IPTG concentrations, induction and sonication time.

Methods

Bacterial strains and growth conditions: *E. coli* BL21 was used as host for the expression of truncated *Diphtheria* toxin and *Pseudomonas* exotoxin A. Cultures were grown in LB medium supplemented with kanamycin (50 µg/mL, Sigma). IPTG (SinaClone) was added to Luria Broth **(**LB) as an inducer.

Gene design and vector construction: The protein sequences of *Diphtheria* toxin and *Pseudomonas* exotoxin A were searched at the Uniprot site. The binding domain sequences of these toxins were eliminated. The synthesis of pET28a expression vector encoding truncated DT and PE were ordered to Shinegene Company. The nucleotide sequences were optimized for *E. coli* codon usage. The optimized sequences of the toxins were subcloned with *BamHI* and *XhoI* into pET28a expression vector with kanamycin resistant gene. The finalized toxin sequences consisted of 379 amino acids (42 KD) for DT and 360 amino acids (40 KD) for PE.

Transformation: The transformation of the expression vector pET28a to the *E.coli* bacteria BL21 (DE3) was fulfilled by incubating the bacteria with the expression vector comprising of the truncated diphtheria toxin and pseudomonas exotoxin A, separately, on ice for 30 minutes and heat/shock treatment for 30 seconds. 250µl LB agar was added to the transformed bacteria and it was shacked for 45 minutes at 37°C. To form single bacteria colonies 50 µl of the transformed bacteria was placed on a LB agar plate with Kanamycin and it was incubated at 37°C overnight. Colonies, which grew up on the plate, were the ones with the pET vector containing the Kanamycin resistant gene.

Diphtheria Toxin Production and Optimization: Each colony on the LB plate was inoculated in 10 ml LB Broth medium (0.2 gr LB Broth in 10 ml distilled water) with 10 µl Kanamycin and incubated for 16h at 37°C in a bacterial shaker. 400 ml LB broth medium plus 400 µl Kanamycin was prepared after 16h of incubation for high yield production of the diphtheria toxin protein. 4 ml of the old medium was added to the 400 ml LB Broth medium that makes 1% of the completely new medium. The optical density of the bacteria reached between 0.4 and 0.6 after 3 hours of incubating at 37°C in a bacterial shaker. Optimization of the protein expression is due to variable Isopropyl

(IPTG) concentrations, induction and sonication time. The bacteria were induced with 0.4, 0.6, 0.8 and 1 mM IPTG in different induction times of 2,4,6 and 8 hours at 2 different induction temperatures of 25 and 30°C. After inducing the bacteria with IPTG in the selected induction time and temperature, the solution was centrifuged at 4000 rpm for 15 min. The desired pellets of bacteria were dissolved in 8 ml of lysis buffer containing 50 mM Tris. Hcl (pH 8) and 150 mM Nacl. Finally the cells were sonicated on ice (10 × 15s) with 15s intervals and centrifuged at 5000 rpm for 20 min at 4°C. The supernatant containing the proteins were stored at -20°C.

Pseudomonas exotoxin A Production and Optimization: The procedures, which were done for the production and optimization of the Diphtheria toxin, were applied on *Pseudomonas* exotoxin A. In this case, the bacteria were induced with 0.5 and 1 mM IPTG in different incubation times of 2, 4, 6 and 8 hours at 25°C and 30°C incubation temperatures.

SDS-Page and western blot analysis: To obtain the best induction IPTG concentration, time and temperature, sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) analysis was used. Proteins with different IPTG concentrations, time and temperatures were separated on a SDS-PAGE.

Briefly, Protein samples were mixed with 5x loading buffer (0.5 M Tris, Glycerol, SDS, Bromophenolblue with 0.5% ethanol and 2-Mercaptoethanol) with 1:1 ratio and boiled for 5 minutes just before loading and then equal amount of protein samples were subjected to 12% SDS-PAGE. Gels were analyzed by staining with Coomassie brilliant blue. The densities of protein bands were calculated using Image J software. Proof of *Diphtheria* toxin and *Pseudomonas* exotoxin A existence was done by western blotting analysis. For this matter, equal amount of protein samples were subjected to 12% SDS-PAGE and transferred to PVDF membranes. Then the blots were blocked overnight at 4°C in the blocking solution TBST (20mM Tris-Hcl pH 7.6, 100 mM NaCl and 0.1% tween) containing 3% skimmed milk and then incubated with anti his-tag HRP conjugated antibody diluted to 1:1500 in TBST with 1% skimmed milk. Our Diphtheria toxin and pseudomonas exotoxin A proteins were detected using an ECL western blotting kit followed by exposure to X-ray film.

Results

Cloning and sequencing of truncated DT and PE in expression vector: *Diphtheria* toxin and *Pseudomonas* exotoxin A binding domains were eliminated. The nucleotide and protein sequences of the truncated *Diphtheria* toxin were shown in figure 1 and 2 and truncated *Pseudomonas* exotoxin A sequences were shown in figure 3 and 4. The truncated *Diphtheria* toxin and *Pseudomonas* exotoxin A were cloned into a pET28a prokaryotic expression vector and transformed into *E. coli* BL21 (DE3). Formation of single bacteria colonies was due to overnight incubation at 37°C.

GGATCCATGGGTGCTGACGACGTTGTTGACTCTTCTAAATCTTTCGTTATGGAAAAC
TTCTCTTCTTACCACGGTACCAAACCGGGTTACGTTGACTCTATtCAGAAgGGTATCC
AGAAACCGAAATCTGGTACCCAGGGTAACTACGACGACGACTGGAAAGGTTTCTAC
TCTACCGACAACAAATACGACGCTGCTGGTTACTCTGTTGAtAAtGAAAACCCGCTGT
CTGGTAAAGCTGGTGGTGTTGTTAAAGTTACCTACCCGGGTCTGACCAAAGTTCTGG
CTCTGAAAGTTGACAACGCTGAAACCATCAAAAAAGAACTGGGTCTGTCcCTGACCG
AACCGCTGATGGAACAGGTTGGTACCGAAGAATTCATCAAACGTTTCGGTGACGGT
GCTTCTCGTGTTGTTCTGTCaCTGCCGTTCGCTGAAGGTTCTTCTTCTGTTGAATACAT
CAACAACTGGGAACAGGCTAAAGCTCTGTCTGTTGAACTGGAAATCAACTTCGAAA
CCCGTGGTAAACGTGGTCAGGACGCTATGTACGAATACATGGCTCAGGCTTGCGCTG
GTAACCGTGTTCGTCGTTCTGTTGGTTCTTCTCTGTCTTGCATCAACCTGGACTGGGA
CGTTATCCGTGACAAgACCAAgACCAAAATCGAATCTCTGAAAGAACACGGTCCGAT
CAAAAACAAAATGTCTGAATCTCCGAACAAAACCGTTTCTGAAGAAAAAGCTAAAC
AGTACCTGGAAGAATTCCACCAGACCGCTCTGGAACACCCGGAACTGTCTGAACTG
AAAACCGTTACCGGTACCAACCCGGTTTTCGCTGGTGCTAACTACGCTGCTTGGGCT
GTTAACGTcGCTCAaGTTATCGACTCTGAAACCGCTGAtAACCTtGAAAAAACCACCG
CTGCTCTGTCTATCCTGCCGGGTATCGGTTCTGTTATGGGTATCGCTGACGGTGCTGT
TCACCACAACACCGAAGAAATCGTTGCTCAGTCTATCGCTCTGTCTTCTCTGATGGTT
GCTCAGGCTATCCCGCTGGTTGGTGAACTGGTTGACATCGGTTTCGCTGCTTACAAC
TTCGTTGAATCTATCATCAACCTGTTCCAGGTTGTTCACAACTCTTACAACCGTCCG
TAA**CTCGAG**

Figure 1. Optimized DNA sequence of truncated DT. *BamHI* and *Xho1* restriction sites, used for cloning in pET28a vector, are shown in bold.

Confirmation of bacteria colonies containing pET 28a vector with the truncated DT and PE gene were due to plasmid purification done by Vivantis Miniprep kit (Malaysia). DNA sequencing analysis confirmed

MGADDVVDSSKSFVMENFSSYHGTKPGYVDSIQKGIQKPKSGTQGNYDDDWKGFYST
DNKYDAAGYSVDNENPLSGKAGGVVKVTYPGLTKVLALKVDNAETIKKELGLSLTEPL
MEQVGTEEFIKRFGDGASRVVLSLPFAEGSSSVEYINNWEQAKALSVELEINFETRGKRG
QDAMYEYMAQACAGNRVRRSVGSSLSCINLDWDVIRDKTKTKIESLKEHGPIKNKMSE
SPNKTVSEEKAKQYLEEFHQTALEHPELSELKTVTGTNPVFAGANYAAWAVNVAQVID
SETADNLEKTTAALSILPGIGSVMGIADGAVHHNTEEIVAQSIALSSLMVAQAIPLVGELV
DIGFAAYNFVESIINLFQVVHNSYNRP-Stop codon

Figure 2. Protein sequence of truncated DT. Methionine followed by 1 to 385 amino acid residues of DT.

GGATCCATGCACCACCACCACCACCACGGTGGTTCTCTGGCTGCTCTGACCGCTCACCAGGCTTGCCA
CCTGCCGCTGGAAACCTTCACCCGTCACCGTCAGCCGCGTGGTTGGGAACAGCTGGAACAGTGCGGT
TACCCGGTTCAGCGTCTGGTTGCTCTGTACCTtGCaGCTCGTCTGTCTTGGAACCAGGTTGACCAGGTT
ATCCGTAACGCTCTGGCTTCTCCGGGTTCTGGTGGTGACCTGGGTGAAGCTATCCGTGAACAGCCGGA
ACAGGCTCGTCTGGCTCTGACCCTGGCTGCcGCTGAATCTGAACGTTTCGTTCGTCAGGGTACCGGTAA
CGACGAAGCTGGTGCTGCTTCTGCTGACGTTGTTTCTCTGACCTGCCCGGTTGCTGCTGGTGAATGCG
CTGGTCCGGCTGACTCTGGTGAACGCTCTGCTGGAACGTAACTACCCGACCGGTGCTGAATTCCTGGGT
GACGGTGGTGACATCTCTTTCTCTACCCGTGGTACCCAGAACTGGACCGTTGAACGTCTGCTGCAGGCT
CACCGTCAGCTGGAAGAACGTGGTTACGTTTTCGTTGGTTACCACGGTACCTTCCTGGAAGCTGCTCA
GTCTATCGTTTTCGGTGGTGTTCGTGCTCGTTCTCAGGACCTGGACGCTATCTGGCGTGGTTTCTACATC
GCTGGTGACCCGGCTCTGGCTTACGGTTACGCTCAGGACCAGGAACCGGACGCTCGTGGTCGTATCCG
TAACGGTGCTCTGCTGCGTGTTTACGTTCCGCGTTCTTCTCTGCCGGGTTTCTACCGTACCGGTCTGACC
CTGGCTGCTCCGGAAGCTGCTGGTGAAGTTGAACGTCTGATCGGTCACCCGCCGCTGCGTCTGGACGC
TATCACCGGTCCGGAAGAAGAAGGTGGTCGTCTGGAAACCATCCTGGGTTGGCCGCTGGCTGAACGT
ACCGTTGTTATCCCGTCTGCTATCCCGACCGACCCGCGTAACGTTGGTGGTGACCTGGACCCGTCTTCTA
TCCCGGACAAAGAACAGGCTATCTCTGCTCTGCCGGACTACGCTTCTCAGCCGGGTAAACCGCCGCGT
GAAGACCTGAAATAA**CTCGAG**

Figure 3. Optimized DNA sequence of truncated PE. *BamHI* and *Xho1* restriction sites, used for cloning in pET28a vector, are shown in bold.

GGSLAALTAHQACHLPLETFTRHRQPRGWEQLEQCGYPVQRLVALYLAARLSWNQVDQVIRNA
LASPGSGGDLGEAIREQPEQARLALTLAAAESERFVRQGTGNDEAGAASADVVSLTCPVAAGEC
AGPADSGDALLERNYPTGAEFLGDGGDISFSTRGTQNWTVERLLQAHRQLEERGYVFVGYHGT
FLEAAQSIVFGGVRARSQDLDAIWRGFYIAGDPALAYGYAQDQEPDARGRIRNGALLRVYVPRSS
LPGFYRTGLTLAAPEAAGEVERLIGHPPLRLDAITGPEEEGGRLETILGWPLAERTVVIPSAIPTDPR
NVGGDLDPSSIPDKEQAISALPDYASQPGKPPREDLKstop Codon

Figure 4. Protein sequence of truncated PE. Methionine followed by 1 to 366 amino acid residues of PE.

constructed plasmid.

Optimization of protein expression using different IPTG concentrations and induction time and temperature: Proteins with different IPTG induction concentrations, time and temperature were separated by SDS-pages. The densities of each desired protein bands were calculated using Image J software (USA). For truncated DT, we observed that the optimal protein expression was gained in 4h, 0.4 mM IPTG concentration at 25°C. The next best protein expression was observed in 4h, 0.6 mM IPTG concentration at 25°C. The expression ratios were calculated compared to control and presented below the figures (Figure 5; A-D). For truncated PE, we concluded that the best expression rate occurred in 0.5 mM IPTG concentration no matter of time and temperature in comparison of 1mM IPTG concentration. In addition, we observed that the optimal protein expression was gained in 4h, 0.5 mM IPTG concentration at 25°C. The next best protein expression was observed in 2h, 0.5 mM IPTG concentration at 25°C. The expression ratios were calculated compared to control and presented below the figures (Figure 7; A-B). To confirm the identity of the *Diphtheria* toxin and *Pseudomonas* exotoxin A, western blot analysis was done with two best induction procedures. For DT 0.4 and 0.6 mM IPTG concentrations after 4 hours of induction in 25°C (Figure 6) and for PE 0.5 mM IPTG concentrations after 2 and 4 hours in 25°C (Figure 8) showed two sharp bonds compared to control.

Discussion

Prokaryotic expression systems such as bacterial expression systems are desirable and economical

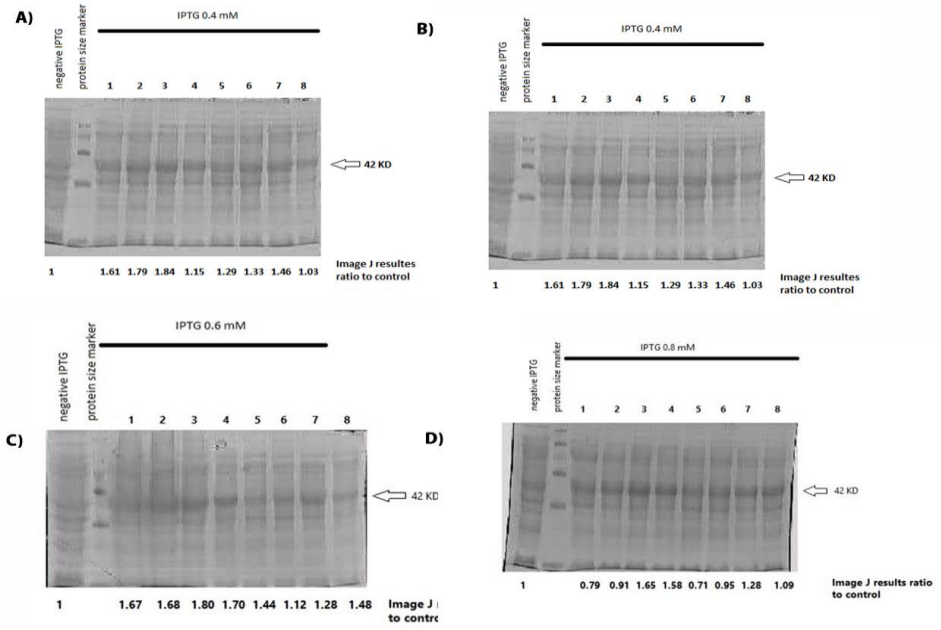

Figure 5. SDS-Page analysis of different protein expression levels in different IPTG concentrations, induction time and temperature. 1: 2h induction at 25˚C, 2: 2h induction at 30˚C, 3: 4h induction at 25˚C, 4: 4h induction at 30˚C, 5: 6h induction at 25˚C, 6: 6h induction at 30˚C, 7: 8h induction at 25˚C, 8: 8h induction at 30˚C.
(A) Diphtheria toxin expression levels in 0.4 mM IPTG concentration, 25˚C and 30˚C induction temperature and four different time inductions such as 2,4,6 and 8 hours.
(B) Diphtheria toxin expression levels in 0.6 mM IPTG concentration, 25˚C and 30˚C induction temperature and four different time inductions such as 2,4,6 and 8 hours.
(C) Diphtheria toxin expression levels in 0.8 mM IPTG concentration, 25˚C and 30˚C induction temperature and four different time inductions such as 2,4,6 and 8 hours.
(D) Diphtheria toxin expression levels in 1 mM IPTG concentration, 25˚C and 30˚C induction temperature and four different time inductions such as 2,4,6 and 8 hours.

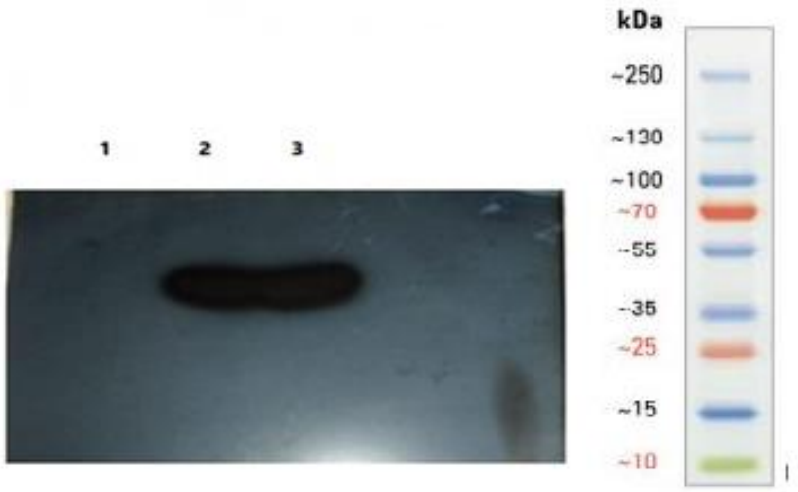

Figure 6. Western blots analysis of the two best induction procedures. Confirmation of Diphtheria toxin existence by western blot analysis. 1: Control 2: 4h of 0.4 mM IPTG induction at 25˚C 3: 4h of 0.6 mM IPTG induction at 25˚C.

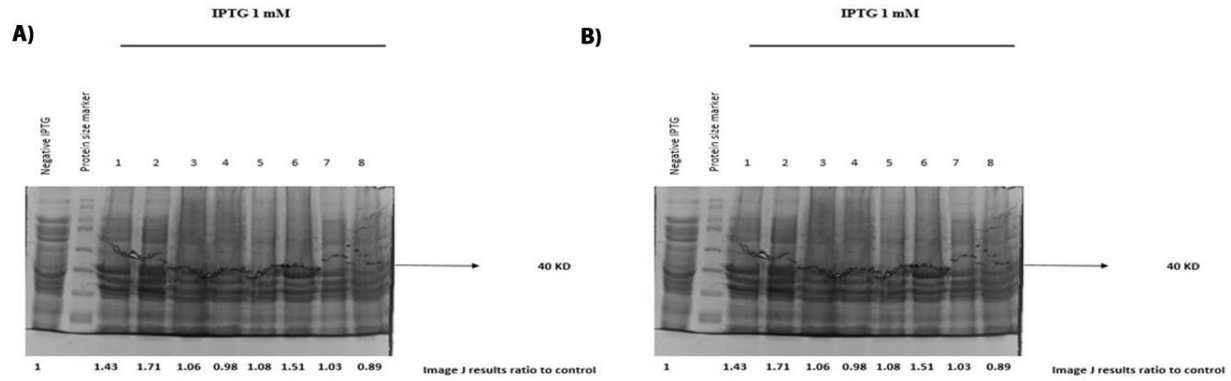

Figure 7. SDS-Page analysis of different protein expression levels in different IPTG concentrations, induction time and temperature for truncated PE. 2: 2h incubation for 25˚C 3: 4h incubation for 25˚C 4: 6h incubation for 25˚C 5: 8h incubation in 25˚C 6: 2h incubation for 30˚C 7: 4h incubation for 30˚C 8: 6h incubation for 30˚C and 9: 8h incubation for 30˚C .
A: PE expression levels in 1 mM IPTG concentration, 25˚C and 30˚C induction temperature and four different time inductions such as 2,4,6 and 8 hours.
B. PE expression levels in 0.5 mM IPTG concentration, 25˚C and 30˚C induction temperature and four different time inductions such as 2,4,6 and 8 hours.

instruments in producing recombinant proteins such as toxins with no binding domain sequences[16,17]. *E. coli* is the most bacterial expression system used in producing recombinant proteins for its beneficial superiorities such as, simple high cell density efforts, easy exogenous DNA transformation and reasonable culture media[18]. *E. coli* BL21 (DE3) is a suitable strain for producing recombinant proteins because of its deficiency in coding protease genes that can degrade expressed proteins[19,20]. Induction temperature and incubation time are two most important factors that influence the protein expression in *E. coli* BL21[21]. 25˚C and 30˚C induction temperatures are lower temperatures than 37˚C used in most protein expression protocols in order to reduce the inclusion body formations. In addition, higher temperatures could lead to plasmid loss[19]. Our results showed that 25˚C is the best induction temperature for expressing truncated DT and PE and the best induction temperature for reducing the inclusion body formations as inclusion bodies could lead to protein misfolding and malfunction. The other crucial factor that can influence the expression of the DT and PE proteins is the IPTG concentration. Ramirez *et al*, showed that low IPTG concentrations can lead to

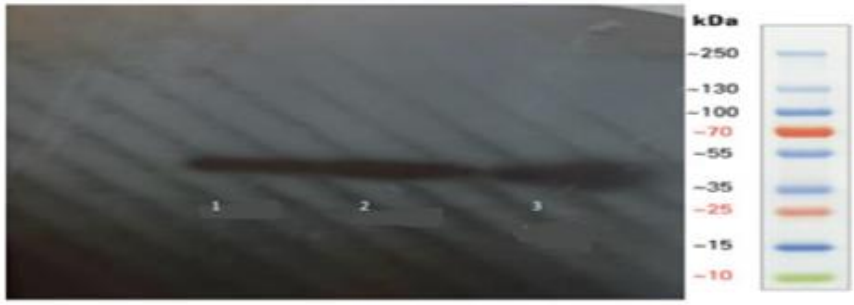

Figure 8. Western blots analysis of the two best induction procedures. Confirmation of truncated PE existence by western blot analysis. 1: Control 2: 2h of 0.5 mM IPTG induction at 25˚C 3: 4h of 0.5 mM IPTG induction at 25˚C.

lower protein yield and higher inducer concentrations can result in toxic effects[20]. Whereas our results showed that lower concentrations of IPTG such as 0.4 mM for DT and 0.5 mM for PE has higher protein expression rates in contrast to higher IPTG concentrations, which would result in toxic effects, aligned with Ramirez studies. Overall, we investigated that the lower the induction temperature and inducer concentration the higher the recombinant protein expresses.

On the other hand, high yield of functional recombinant protein synthesis has the key role in producing economical biopharmaceutical drugs. By optimizing this complex procedure, we will simplify the protein production which could accelerate the drug development[21].

Conclusion

Accordingly, we present here the optimized condition for the production of truncated *Diphtheria* toxin and *Pseudomonas* exotoxin A in *E.coli* BL21 strain (DE3). We concluded that various IPTG concentrations in different induction times and temperatures vary the protein yield production and with SDS-Page method, at the end the best optimizing conditions were proved.

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