Original Article

Expression and purification of toxic anti-breast cancer p28-NRC chimeric protein

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Abstract

Background: Chimeric proteins consisting of a targeting moiety and a cytotoxic moiety are now under intense research focus for targeted therapy of cancer. Here, we report cloning, expression, and purification of such a targeted chimeric protein made up of p28 peptide as both targeting and anticancer moiety fused to NRC peptide as a cytotoxic moiety. However, since the antimicrobial activity of the NRC peptide would intervene expression of the chimeric protein in *Escherichia coli*, we evaluated the effects of two fusion tags, that is, thioredoxin (Trx) and 6x-His tags, and various expression conditions, on the expression of p28-NRC chimeric protein.

Materials and Methods: In order to express the chimeric protein with only 6x-His tag, pET28 expression plasmid was used. Cloning in pET32 expression plasmid was performed to add both Trx and 6x-His tags to the chimeric protein. Expression of the chimeric protein with both plasmids was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis following optimization of expression conditions and host strains.

Results: Expression of the chimeric protein in pET28a was performed. However, expression yield of the chimeric protein was low. Optimization of culture conditions and host strains led to reasonable expression yield of the toxic chimeric protein in pET32a vector. In cases of both plasmids, approximately 10 kDa deviation of the apparent molecular weight from the theoretical one was seen in SDS-PAGE of purified chimeric proteins.

Conclusions: The study leads to proper expression and purification yield of p28-NRC chimeric protein with Trx tag following optimizing culture conditions and host strains.

Key Words: Anticancer peptide, apparent molecular weight, chimeric protein, NRC, p28

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INTRODUCTION

Cancer is one of the leading causes of death in the entire world, and breast cancer is the most common reason of cancer death in women aged 20–59.[1] Conventional chemotherapeutics for cancer treatment include restrictions such as damaging healthy growing cells and development of drug resistance.[2] Moreover,

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they do not affect dormant and slow-growing cells, and some of these drugs cause secondary malignancies in the body.[3] Anticancer therapy aims to find an approach to selectively kill cancer cells and restrict side effects to the minimum, unaffected by common mechanisms of chemo-resistance and kill dormant and slow-growing cells.[4] Targeted therapy is a promising method in cancer treatment. Targeted therapy enhances efficacy of drugs and reduces their side effects.[5] Peptide can be used for tumor targeting and also directly as a drug.[6] Peptides have higher affinity/specificity to their targets and lower toxicity (degradation products of peptides are amino acids) profiles in comparison to small molecules.[7] Combining of a tumor targeting peptide and a killer peptide as a chimeric protein can be used for targeted therapy in cancer treatment.[8,9]

p28 peptide is the amino acids 50–77 of azurin that can preferentially penetrate cancer cells. After internalization, this peptide is able to inhibit cancer cell proliferation by stabilizing p53 protein.[10] NRC, a pleurocidin family of cationic antimicrobial peptides, can kill multiple breast cancer cell lines. Furthermore, intratumoral administrations of NRC kill breast cancer cells grown as xenografts in NOD SCID mice.[11] The combination of p28 and NRC peptides as a chimeric protein may increase their efficiency in specific killing of breast cancer cells.

Escherichia coli expression system is preferred for recombinant protein production for laboratory investigations due to advantages such as fast growth, large number of cloning vectors, inexpensive medium, genetically different host strains, and well-characterized genetics.[12-15] Despite the many benefits of recombinant protein production in *E. coli*, this expression system has problems such as low expression levels or even lack of expression in case of toxic peptides and proteins. However, recombinant production of toxic proteins faces potential difficulties due to their cytotoxicity for bacterial hosts. Different strategies have been employed for reducing their cytotoxicity and increasing their stability. Expression of toxic proteins with a carrier protein is a way that used to eliminate toxicity problems. Thioredoxin (Trx) tag has been used to increase solubility and reduce protein toxicity.[16] Application of different host strains and optimization of expression condition are other strategies that can be used to eliminate protein toxicity problems.[17] NRC peptide is an antimicrobial peptide, and it may intervene in recombinant production of the chimeric protein. Various mechanisms have been proposed for killing of the antimicrobial peptides, but the main target is the cytoplasmic membrane. The electrostatic interaction between these peptides and negatively charged bacterial cell membrane leads to pores formation and membrane damage.[11] For this reason, the expression of the chimeric protein was investigated in two different plasmids containing Trx and 6x-His tag.

The purpose of this study was to improve the expression and purification of the toxic chimeric protein p28-NRC. There are various strategies to increase expression of toxic peptides and proteins in *E. coli* such as using fusion tags and modification of expression conditions.[18] In this study, we cloned the toxic chimeric protein gene in pET32a to examine the possibility of protein expression with Trx tag. Furthermore, the possibility of expression of the toxic chimeric protein by optimizing expression conditions and using different host strains was investigated with cloning of the toxic chimeric protein gene in pET28a.

MATERIALS AND METHODS

Bacterial strain, plasmids, and culture conditions

E. coli strains TOP10, BL21(DE3) and BL21(DE3) pLysS, and pET-28a and pET-32a plasmids were obtained from Novagen (Madison, WI). *KpnI*, *XhoI*, and *NdeI* restriction enzymes, *T4* DNA Ligase, GeneJet Gel Extraction kit, and *GenJet plasmid Preparation* kit were obtained from Thermo scientific (MD, USA). Luria-Bertani (LB) medium was purchased from HiMedia (Mumbai, Maharashtra, India). Ni-NTA resin was provided by GenScript (Piscataway, USA). All recombinant DNA manipulation procedures were performed according to standard molecular biology techniques.[19]

Gene constructs preparation

In order to construct the chimeric protein, a rigid linker, including $(PA)_5P$ was used. Codon optimization of the coding sequence containing p28, linker, and NRC sequences for expression in *E. coli* was performed using the http://eu.idtdna.com/CodonOpt website. In addition, nucleotide sequences for supplying the chimeric protein with an N-terminal enterokinase digestion site were added to the 5' end of the codon optimized fragment. Figure 1 shows the schematic presentation and amino acid sequences of the optimized fragment. Finally, the DNA fragment was *de novo* synthesized by Nedayefan Company (Tehran, Iran) and supplied in the pGE plasmid.

Cloning of the chimeric protein coding sequence in pET32a and pET28a plasmids

Primer pairs FPP32Fr and FPP32Rv [Table 1] were used for polymerase chain reaction (PCR) amplification of the synthesized fusion construct. Following amplification, the PCR products were gel

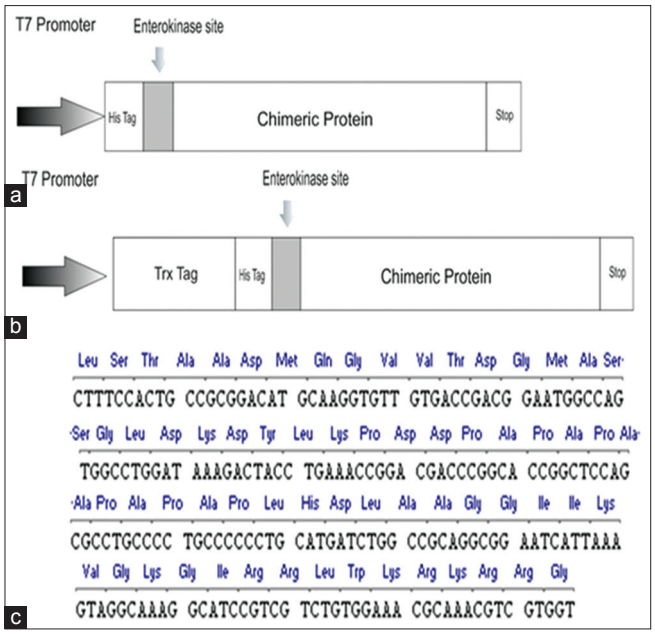

Figure 1: Schematic representation of the chimeric protein. (a) The chimeric protein in pET28 (b) the chimeric protein in pET32 (c) the DNA and amino acid sequence of the chimeric protein

Table 1: Primers and restriction enzymes used for cloning of chimeric protein in pET28 and pET32

Primer name	Sequence	Primer features
FPP32Fr	5'-GGGGTACCGATGATGATGATAAACTG-3'	*KpnI* site, start codon
FPP32Rv	5'-CCGGCTCGAGTCACAGATGATCCAGC-3'	*XhoI* site, stop codon
FPP28Fr	5'-GGAATTCCATATGGATGATGATAAAC-3'	*NdeI* site, start codon
FPP28Rv	5'-CCGCTCGAGTCACAGATGATC-3'	*XhoI* site, stop codon

purified and digested with *KpnI* and *XhoI* restriction endonucleases and ligated into the similarly digested ends of the pET32 plasmid, being in-frame with the upstream Trx and 6x-His tag coding sequences.

For cloning of the fragment into the pET28a plasmid, the primer pairs FPP28Fr and FPP28Rv primers [Table 1] were used for PCR amplification of the fragment. Subsequently, the PCR products were gel purified and double digested with the *NdeI* and *XhoI* restriction endonucleases and cloned into the similarly digested ends of the pET28a plasmid. Finally, the cloning fidelity in both plasmids was authenticated by DNA digestion with the mentioned enzymes and subsequently by DNA sequencing.

Expression and purification of the chimeric protein

BL21(DE3)PlysS *E. coli* cells containing authenticated recombinant pET28-chimeric or pET32-chimeric plasmids were overnight cultivated in the LB medium supplemented with 50 μg/ml kanamycin (Sigma, Germany) or 100 μg/ml ampicillin (Sigma, Germany) in case of the pET28-chimeric or pET32-chimeric plasmids, respectively. Then, the inoculation was performed at a ratio of 1–50 and the cultures were incubated at 37°C and 220 rpm until OD_{600} reached 0.5–0.6. Afterward, the expression was induced with various concentration of IPTG and also various induction time lengths. In addition, the expression was also investigated by cultivation of the cells in the terrific broth (TB) medium as an enriched bacterial culture medium. Following each induction period, cells were harvested by centrifugation at 5000 rpm for 10 min and stored at –80°C until use. Expression of the chimeric protein was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. In addition, for authentication of the observed bands, Western blot analysis was performed. In this regard, protein bands were separated on 15% SDS-PAGE and wet-transferred to activated polyvinylidene difluoride membranes. Then the membranes were blocked with tris-buffered saline-Tween 20 (TBST) buffer containing 3% skim milk for 90 min. Afterward, the membranes were washed three times with the TBST buffer and incubated with HRP-conjugated anti His-tag antibody (Sigma, USA) for 90 min at room temperature. Finally, the membranes were washed 3 times with the TBST buffer and the bands were visualized with diaminobenzidine. In order to purify the expressed protein, the bacterial cell pellets were resuspended in 15 ml of lysis equilibration buffer (LE buffer) containing 50 mM NaH_2PO_4, 300 mM NaCl (pH 8.0) for purification under native condition, or in 15 ml of phosphate-buffered saline (PBS) pH 8 for purification under denaturing conditions. Then, the mixtures were sonicated on ice and subsequently centrifuged at 10,000 × *g* for 15 min. The collected supernatant and cell pellets were used for purification of 6x-His tagged chimeric protein under native and denaturing conditions, respectively.

Purification of the 6x-His tagged chimeric protein was performed using High-Affinity Ni-NTA Resin (GenScript, USA) according to the manufacturer's instructions. For purification under native condition, the collected supernatant was applied to Ni-NTA column. Then, the column was washed with the LE buffer containing different concentrations of imidazole, and finally, the desired protein was eluted with the native elution buffer (LE buffer supplemented with 250 mM imidazole). For, purification under denature condition, the collected cell pellets were Solubilized in denaturant buffer containing 100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea (pH 8), and incubated for 60 min at room temperature. Then, the mixture was centrifuged at 12,000 rpm for 30 min to remove any remaining insoluble materials. Afterward, the supernatant was loaded on Ni-NTA column, and the column was washed with the denaturant buffer

supplemented with different concentrations of imidazole, and finally the desired chimeric protein was eluted with the denaturant buffer containing 250 mM imidazole. Both purification products were subjected to dialysis against PBS buffer to remove undesired unwanted reagents.

RESULTS

Construction of recombinant pET32-chimeric and pET28-chimeric expression plasmids

The synthesized fusion constructs was amplified with each of the specific sets of primers [Table 1] and subsequently were cloned into the pET32 or pET28 expression plasmids. The fidelity of cloning was confirmed by digestion of the obtained recombinant pET28-chimeric with *NdeI* and *XhoI* enzymes, and digestion of the obtained recombinant pET32-Fusion by *XhoI* and *KpnI* enzymes. In both cases, releasing a band of about 200 bp confirmed the correctness of cloning. Finally, DNA sequencing authenticated the fidelity of cloning.

Expression and purification of chimeric protein in pet32a-chimeric and pET28a-chimeric clones

Following authentication of the cloning of the chimeric protein gene in both pET28 and pET32 vectors, the recombinant vectors were used to transform *E. coli* BL21(DE3) cells and subjected to the induction of expression as mentioned in the materials and methods section. Since the chimeric protein could be toxic for the expression host because of its antimicrobial moiety, i.e. NRC peptide, various expression conditions were examined to improve the chimeric protein yield.

Expression and purification of the chimeric protein by the pET28-chimeric recombinant plasmid was performed. In this case, no sign of expression was observed when the BL21(DE3) strain was used as expression host. However, when BL21(DE3) pLysS host was used, a faint band was observed on SDS-PAGE pertaining to low expression yield of the chimeric protein in this case. Different parameters such as induction time length (1, 2, 4, 8 h, and overnight), IPTG concentration (0.1, 0.5, 1, and 2 mM), and temperature (25, 30, 37°C) were examined when using BL21(DE3)pLysS to optimize the chimeric protein expression yield. The most appropriate parameters showed to be induction time length of 1 h, IPTG 1 mM, and 37°C temperature. Changing the culture medium from LB to TB enriched medium and also induction at higher cell density (OD_{600} ~ 0.9) improved expression yield. The results obtained from optimizing the expression conditions and its western blot depicted in Figure 2a and 2b after purification. However, although the theoretical molecular weight

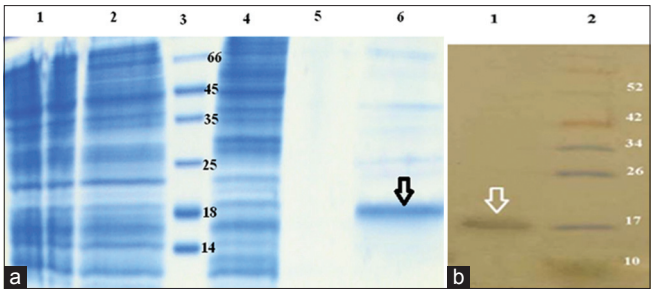

Figure 2: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot of the expressed chimeric protein purification in pET28 using BL21(DE3)PlysS. (a) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of 6x-His-chimeric protein purification. From lane 1–6 is supernatant, apply supernatant to column, protein standard marker (26610 Fermentase), 40 mM imidazole wash, 60 mM imidazole wash, and 250 mM imidazol elution. (b) Western blot analysis of purified 6x-His-chimeric protein. Lane 1, western blot of purified 6x-His-chimeric protein, Lane 2, protein standard marker (310007 DeLuxe)

(MW) of 6x-His-chimeric protein was expected to be about 11 kD, but its apparent MW in SDS-PAGE and Western blotting was about 19 KD.

Chimeric protein expression and purification were assessed by pET32-chimeric plasmid harboring clones. The parameters including IPTG concentration (0.1, 0.5, 1, and 2 mM), induction time length (1, 2, 4, 8 h, and overnight), and temperature (25, 30, 37°C) were examined to optimize expression yield of the chimeric protein. Finally, the most appropriate combination of expression conditions were shown to be IPTG concentration of 0.5 mM, post induction time 4h at 37°C and in BL21(DE3)pLysS host strain [Figure 3]. The expressed chimeric protein was then subjected to native and denatured purification steps. However, native purification procedure showed to be an effective method, but the denature purification method did not yield any desirable amount of the expressed protein (data not shown). Again, although the theoretical MW of 6x-His-Trx-chimeric protein is about 26 kDa, but its apparent MW in SDS-PAGE and Western blotting was about 36 KDa.

DISCUSSION

Here we tried to express the chimeric protein p28-NRC with His tag; therefore, cloning and expression of the chimeric protein was performed in pET28a. The first expression test of chimeric protein was done in 37°C, induction time 3 h, and IPTG 1 mM conditions using BL21(DE3) host strain. The test did not lead to good result and there was no visible purification band in the soluble fractions on SDS-PAGE (data not shown). Afterward, we tried to change the conditions to optimize the chimeric protein expression. There is not a general rule for optimal expression of recombinant proteins in *E. coli*.

Depending on the nature of proteins, there are different strategies for expression optimization[20] and several factors have to be optimized for expression of heterologous proteins.[21,22] First, the growth rate of BL21(DE3) contained pET28a-chimeric protein was investigated. Slower growth of BL21(DE3) strain containing the pET28a-chimeric plasmid in comparison with BL21(DE3) strain contain pET28a empty vector could be due to the leaky expression of toxic chimeric protein. Leaky expression is a drawback in toxic peptide and protein expression and leads to plasmid instability that ultimately reduces or eliminates protein expression.[23] Co-expression of T7 lysozyme in strains like BL21(DE3) pLysS can solve this problem. Studies have shown that this strain can be effective in improving protein expression.[24] In this study, the use of BL21(DE3) pLysS leads to eliminate the slow growth problem. Often, protein toxicity appears after reaching the threshold of host cell tolerance.[25] Hence, post induction time is an important factor in expression of toxic peptide and protein. In addition, degradation due to low protein half-life and nonstructured peptides can be problematic in protein expression.[26] For these reasons, post induction time was studied. The best result was obtained 1 h after 1 mM IPTG induction. The yield of the chimeric protein due to the short post induction time was low. High-cell density IPTG induction can lead to increased yield of protein expression.[27] TB is a rich medium that contained high amount of tryptone and yeast extracts.[28] Studies have shown that TB can increase the cell density and yield of protein expression.[29] TB culture media were used to increase cell density. Besides, induction was performed in OD 0.9. These changes lead to increased yield of the chimeric protein expression. The best result was obtained by post induction time 1 h, IPTG 1 mM and using BL21(DE3)pLysS in TB medium [Figure 3]. These optimizations resulted in the increased yield of the chimeric protein. However, the yield of the chimeric protein was as low as 1 mg/L. Therefore, expressing of the chimeric protein was studied by Trx tag.

Cloning and expression of the chimeric protein were performed in pET32a which adds 160 amino acids including the Trx and 6x-His tags, and enterokinase cleavage site to the N-terminal site of the expressed protein [Figure 1]. It has been shown the fusion tags such as Trx can be used for toxic protein expression in *E. coli*.[30,31] In this study, expression and purification of the chimeric protein was successfully done by pET32a with Trx tag [Figure 3]. Then, the Trx-6x-His-chimeric protein was purified using IMAC chromatography on Ni-NTA agarose in native condition and analyzed by SDS-PAGE [Figure 4a]. Expression and purification of the chimeric protein confirmed by Western blot analysis [Figure 4b]. Other studies reported the expression of the antimicrobial peptides successfully using Trx-tag as a fusion partner.[26] After optimization, the yield of protein was 9 mg/L that is much higher than expressing the chimeric protein only with His tag. This result is an agreement with previous studies that showed Trx tag can lead to toxic protein expression and increasing of the protein yields.[25,27] Purification of the chimeric protein in the denatured state did not lead to a good result that could be the due to charged amino acids in chimeric protein structure and thus its high solubility and solubility improving features of Trx Tag(data not shown).[25]

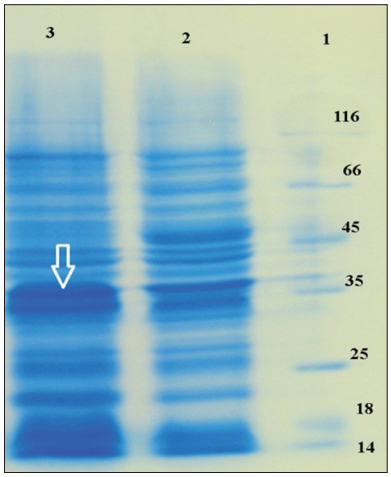

Figure 3: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the expressed chimeric protein in pET32 using BL21(DE3) PlysS. Lane 1, weight marker. Lane 2, represents total bacterial lysate of noninduced bacteria. Lane 3 represents induced bacterial lysate

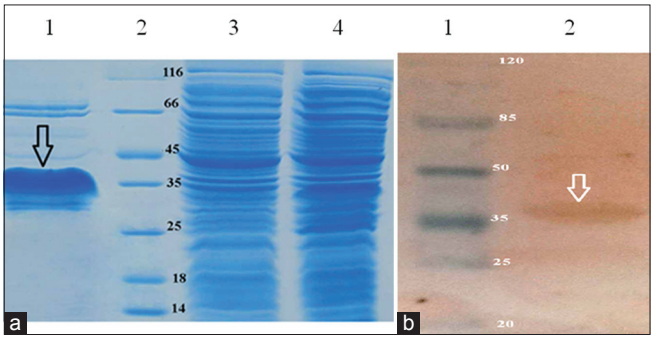

Figure 4: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot of the expressed chimeric protein purification in pET32 using BL21(DE3)PlysS. (a) sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of purification. From Lane 1 to 4 is purified chimeric protein from Ni affinity chromatography, protein standard marker (26610 Fermentase), apply supernatant to column and supernatant, respectively. (b) Western blot analysis of purified Trx-6x-His-chimeric protein. Lane 1, protein standard marker (26612 Fermentase), Lane 2, Western blot of purified Trx-6x-His chimeric protein

In both clones, approximately 10 kDa deviation of the apparent molecular weight from the theoretical one was seen in SDS-PAGE of purified chimeric protein. Deviating apparent molecular weight is also seen in other studies.[32-34] Structural rigidity and kinks caused by high proline content may decrease electrophoretic mobility of the protein. Gel shifting due to high content of proline residue in protein structure has been shown in other studies.[35,36] The selected linker in the chimeric protein structure has six residue of Proline. Therefore, the high proline content of the selected linker can be a possible reason for the deviation of apparent molecular weight of the chimeric protein. Previous studies have shown that hydrophilic proteins have slow migration in SDS-PAGE. The SDS binds to proteins by hydrophobic interactions. Hydrophilic proteins bind to the lower amount of SDS and migrate slower in SDS-PAGE.[37] In addition, more interaction of hydrophilic proteins with water leads to increase of their hydrodynamic size and slow migration in SDS-PAGE.[38] More than 29% of the amino acids in the chimeric protein, without any tag, are charged. Hence, other reasons for this deviation can be high content of charged amino acids in the chimeric protein structure.

CONCLUSION

In conclusion, in the present study, we cloned the coding sequence of p28-NRC chimeric protein in both pET28a and pET32a plasmids for high-yield expression of this hypothetic cancer-specific cytotoxic protein. Although the yield of expression mediated by the pET28-chimeric protein was low, it was expressed at a desirable amount by the pET32-chimeric plasmid. Since the high amount of the designed chimeric protein is needed for evaluation of its cytotoxicity; *in vitro* and *in vivo*, this study was performed to set up an optimized system for its high-yield expression. Further studies on removing the fusion tags, Trx and 6x-His, and cytotoxic evaluation of the chimeric protein are undergoing.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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