Research in Molecular Medicine

A Convenient Method for Solubilization and Refolding of Recombinant Proteins: An Experience From Recombinant Mouse Transforming Growth Factor-β 1

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ABSTRACT

Background: The production of recombinant proteins in *Escherichia coli (E.coli)* is one of the most valuable achievements in biotechnology having many therapeutic and diagnostic applications; however, the aggregation and misfolding of proteins leading to the formation of insoluble inclusion bodies is a disruptive factor in this process. Various solubilization and refolding methods could be used to improve recombinant protein conformation. In this study, we applied a dilution method with refolding buffer to produce a native form of soluble immature mouse Transforming Growth Factor-Beta 1 (TGF-β1).

Materials and Methods: The TGF-β1 complementary DNA (cDNA) which encodes the protein without the signal peptide was cloned into the pET21-b (+) vector. The target protein was expressed by the transformation of *E. coli* BL21 cells with the plasmid. The resulting inclusion bodies were diluted in lysis buffer and solubilized in refolding buffer to make a protein with native structure. The protein quantification was performed by using Bicinchoninic Acid Assay (BCA).

Results: Following Polymerase Chain Reaction (PCR) of the recombinant plasmid with T7 primers, electrophoresis and sequencing of the amplified product indicated 100% target sequence identity with the murine TGF-β1 gene. Finally, the protein solubility and immuno-reactivity were confirmed a 44 kDa protein which conducted with the anti-TGF-β1-specific polyclonal antibody by western blot. The protein quantification with the BCA method showed 30 µg/mL concentration.

Conclusion: The dilution method and refolding buffer used in this study could effectively convert aggregated immature mouse TGF-β1 to a soluble and immuno-reactive form.

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Introduction

Transforming Growth Factor-Beta 1 (TGF-β1) is a 25 kDa dimeric cytokine with intramolecular disulfide bonds that controls inflammation, immune suppression, cell proliferation, angiogenesis, wound healing, and carcinogenesis. An increase or decrease in the production of this cytokine can lead to many diseases including atherosclerosis which is a fibrotic disease of the kidney, liver, and lung [1-5]. Several studies have proven the effectiveness of exogenous TGF-β1 for therapeutic and diagnostic applications; however, the low protein yielding from natural sources makes it inapplicable in the biotechnology and pharmaceutical industries. Currently, recombinant technology is the only way to produce a large amount of TGF-β1 with high purity, solubility, and functionality [6].

There are many different expression systems to produce recombinant molecules. Escherichia coli (*E. coli*) is the oldest and most popular expression system. This system is favoured because it offers an easy, quick, and cost-effective expression of recombinant proteins [7, 8]. In bacterial cells, native protein folding is performed spontaneously and molecular chaperones prevent proteion aggregation, whereas in recombinant systems, overexpression of target proteins can result in insoluble aggregates refered to as Inclusion Bodies (IBs). Accumulation of IBs increases with the presence of cysteine residues in the target proteins, and incorrect bonding between cysteines can result in protein misfolding [9]. In this case, the industrial challenge is to produce soluble, refolded, functional proteins with native conformation [10-12].

There are much different solubilization and refolding methods such as denaturing agents, increasing pH level (≥12), and detergents usage; however, the most common method is to use high concentrations of denaturing agents. In this method, the addition of reducing agents is necessary to maintain cysteines in their reduced state and prevent disulfide bond formation [13]. Following this step, protein can be refolded by dilution, dialysis, chromatography or solid-phase refolding [14]. Dilution is the simplest method, although the final yielding is often low due to protein aggregation during the process, especially in case of cysteine-rich proteins; hence, this procedure generally uses a redox system containing thiol agents [6], in addition to chemical additives [15, 16]. In this study, we applied a dilution method using guanidine chloride and Dithiothreitol (DTT) for IB solubilization as well as a refolding buffer containing sucrose, glycerol, and glutathione to produce a soluble immature mouse TGF-β1 with correct disulfide bonds for further studies.

Material and Methods

TGF-β1 complementary DNA synthesis

Total RNA was extracted from mouse spleen using an RNA extraction kit (Pars Tous Co., Iran) and complementary DNA (cDNA) was synthesized using oligo-dT primers and reverse transcriptase (Pars Tous Co., Iran). Then, TGF-β1 cDNA was amplified by Polymerase Chain Reaction (PCR) method without its signal peptide using TGF-β1 specific primers containing EcoRI and XhoI restriction sites. These primers were designed using Gene Runner software. The primer sequences were forward 5'GATCAGAATTCGCTCTCCACCTGCAAGACCATCG 3' (Underline indicates EcoRI restriction site) and reverse 5' GGTGCTCGAGGCTGCACTTGCAGGAGCTCAC 3' (Underline indicates XhoI restriction site). To eliminate impurities and nonspecific bands, after PCR and agarose gel electrophoresis, the desired band was purified with a DNA gel extraction kit (Pars Tous Co., Iran). The purified PCR product was digested and ligated into EcoR1- and Xho1-digested pET-21b (+) at 22° C for 4 h with T4 DNA ligase.

Gene cloning and protein expression

Afterwards, TOP10 competent *E. coli* cells prepared with the Inoue method, were transformed by the recombinant plasmid and cultured on a solid Luria-Bertani (LB) medium containing 100 µg/mL ampicillin. Colony PCR was performed using T7 primers and the products were electrophoresed. Plasmids from positive colonies were extracted using a plasmid extraction kit (Pars Tous Co., Iran) and sequenced with a bioinformatic tool (Macrogen, Korea). To express TGF-β1, competent *E. coli* BL21-CodonPlus (DE3) cells were transformed with the recombinant plasmid through heat shocking. The cells were cultured in a 3-mL LB medium containing 100 µg/mL ampicillin. After overnight incubation at 37° C, 1 mL of culture was added to 100 mL of fresh medium and grown to an optical density of 0.5 at 600 nm. Recombinant protein expression was induced by 0.2 mM Isopropyl-β-D-thiogalactopyranoside (IPTG) at 22 ° C with shaking at 150 rpm. Cells were pelleted and lysed in 50-mM KH_2PO_4 buffer (pH= 7.8, containing 10% glycerol and 0.5% Triton X-100). Supernatant and pellet fractions of the lysates were analyzed for protein expression by Sodium Dodecyl Sulfate-Polyacrylamide Ael Electrophoresis (SDS-PAGE) method.

Solubilization and refolding

To solubilize the expressed protein, the IBs were diluted in lysis buffer containing 50 µL DTT, 5 M guanidinium chloride, and 1 mM Ethylenediaminetetraacetic Acid (EDTA). The solution was sonicated on ice for 5 minutes, incubated overnight at 4° C, and then incubated at 37 ° C for 1 h. The solution was then centrifuged at 10 ° C for 10 minutes at 8000 rpm speed to remove insoluble residue. The supernatant containing solubilized protein was gradually added to the refolding buffer (containing 50 mM Tris-HCl, pH= 8.5, 0.4 M sucrose, 10% glycerol, 0.5% Triton X-100, 0.3 mM oxidized glutathione, and 3 mM reduced glutathione) and incubated at 4° C for 20 h. After incubation, the solution was centrifuged to collect the supernatant in the next process.

Protein purification and dialysis

Recombinant TGF-β1 was purified on a Ni-IDA column (Pars Tous Co. Iran). The column was first washed with 30 mL distilled water and then equilibrated with 30 mL starting buffer (containing 50 mM Tris-HCl, pH= 8.0, and 100 mM NaCl). Sodium chloride was added to the supernatant fraction of the refolded protein up to 100 mM NaCl, and this solution was added to the column with a low flow rate. The column was washed with 40 mL of starting buffer and then 100 mL of washing buffer (containing 50 mM Tris-HCl, pH= 8.0, 100 mM NaCl, and 30 mM imidazole) to remove unbound and weakly-bound components. Finally, the recombinant protein was eluted first with 500 mM imidazole and then with 1 M imidazole. The fractions were dialyzed in 150 mM Phosphate-Buffered Saline (PBS) containing 25% glycerol at 4° C overnight.

Immunogenicity analysis

The dialyzed proteins were electrophoresed by 12.5% SDS-PAGE and transferred to the Polyvinylidene Difluoride (PVDF) membrane by electroblotting. The membrane was blocked with 2% Bovine Serum Albumin (BSA) in PBS at 4 ° C overnight. Subsequently, the polyclonal anti-TGF-β1 biotin-conjugated antibody (diluted 1:500 in 1% BSA) was added at room temperature for 2 h and detected with a 1:20000 diluted horseradish peroxidase streptavidin in 1% BSA solution. Antibody binding was visualized by chemiluminescence substrates.

Protein concentration and quantification

Bicinchoninic Acid Assay (BCA) was used in order to quantify the concentration of recombinant protein. All samples and reagents were prepared according to the manufacturer's instruction (Pars Tous Co. Iran) and tested in a duplicate way. The optical density of samples was detected at 630 nm and the final concentration was obtained by comparing the standard curves.

Results

Amplification, cloning and sequencing of cDNA coding mouse TGF-β1

Amplification of TGF-β1 cDNA with the forward and reverse primers showed a 1084 bp PCR product. Am-

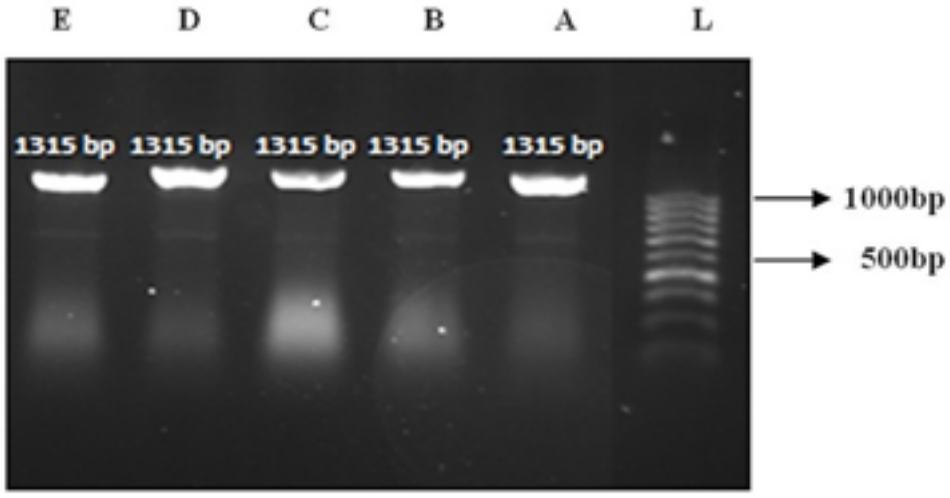

Figure 1. Agarose gel electrophoresis of mouse TGF-1b PCR product obtained from transformed TOP10 cells with T7 primers. Lane L: 100 bp ladder; Lanes A to E: TGF-β1 PCR products with T7 primers.

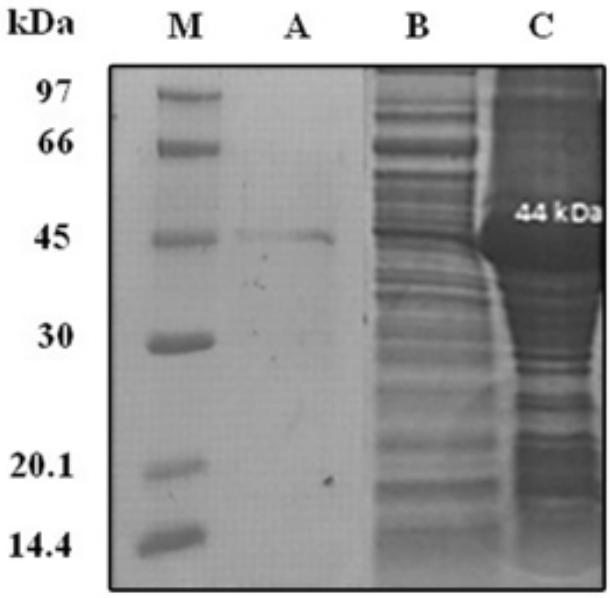

Figure 2. SDS-PAGE results of purified mouse TGF-β1 and cell lysate supernatant and pellet.

Lane M: Protein marker; Lane A: Purified protein after refolding; Lane B: Supernatant fraction of cell lysate before refolding; Lane C: Pellet fraction of cell lysate before refolding.

plification of recombinant pET-21b (+) from the transformed TOP10 cells with T7 primers added 231 bp to the 1084 bp TGF-β1 PCR product, resulting in a 1315 bp band (Figure 1). The PCR fragment was sequenced and shown to be 100% homologous with mouse TGF-β1 precursor in the NCBI database.

Protein purification, immunoblotting and quantification

Supernatants and cell pellets of bacterial lysates were examined by SDS-PAGE method (Figure 2). The recombinant TGF-β1 was migrated at 44 kDa; however, as it was expected, most of the induced protein was found

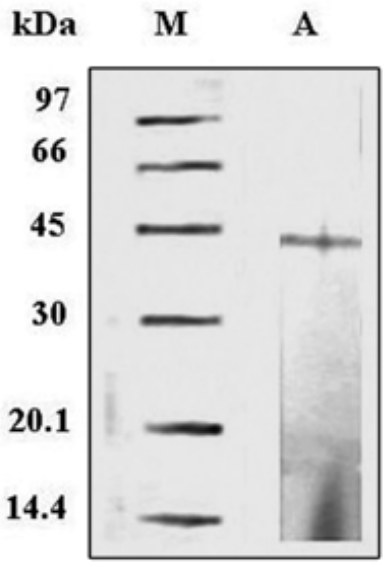

Figure 3. Western blot of purified recombinant TGF-β1.

Lane M: Protein marker; Lane A: 44 kDa TGF-β1 with anti- TGF-β1 antibody.

in IBs. After solubilization and refolding, the purified TGF-β1 protein was migrated as a 44 kDa band (Figure 2). Western blotting with an anti-TGF-β1 antibody verified the immunological reactivity of the 44 kDa band (Figure 3). The final concentration of recombinant protein measured by the BCA method showed 30 µg/mL optical density (OD)630= 0.414.

Discussion

In this study, we produced a soluble immature mouse TGF-β1 that aggregated as IB when expressed in *E. coli*. There are many different solubilization and refolding methods such as increasing the pH level (≥12), denaturing agents, and using detergents. The most common method is to use high concentrations of denaturing agents such as guanidinium chloride or urea to disrupt the protein structure. In this method, the addition of reducing agents such as 2-mercaptoethanol or DTT is necessary to maintain cysteines in their reduced state and prevent disulfide bond formation [13, 17]. Following this step, protein can be refolded by dilution or dialysis methods [14]. Dilution method is the simplest one, although the final yielding is often low due to protein aggregation during the process, especially in case of cysteine-rich proteins. Hence, this procedure generally uses a redox system containing thiol agents such as cysteamine, cysteine, or glutathione [6], in addition to chemical additives including sucrose, glycerol, and amino acids [15, 16].

In the current study, the guanidinium chloride, DTT and EDTA were used for solubilizing the IBs. Guanidinium chloride is a chaotropic agent that denatures proteins; DTT inhibits the formation of disulfide bonds by preserving cysteines in their reduced state; and EDTA is a chelating agent that prevents metal-catalyzed air oxidation of cysteines [18]. Following solubilization, the concentrated guanidinium chloride must be removed due to its interference with correct protein folding and aggregation. However, at low concentrations, guanidinium chloride stabilizes protein structure; hence; it is included in the refolding buffer [19, 20]. Then, glutathione, sucrose, glycerol, triton X-100, and Tris were included in the refolding buffer due to their beneficial roles in the renaturation process. Glutathione in oxidized and reduced forms improves oxidation of thiol (-SH) groups and isomerization (reshuffling) of disulfide bonds. Umetsu et al. demonstrated the role of glutathione in refolding of the single-chain Fv fragment [21].

Glycerol decreases hydrophilic interactions and increases protein stabilization [22, 23]. In a study about the effect of different polyols on citrate synthase refolding, glycerol was the most effective one in enhancing the citrate synthase refolding yield and preserving complete enzymatic activity [24]. In addition, sucrose as a stabilizer, changes the environmental condition by binding to intermediates or interacting with the hydrophobic side chains of molecules to prevent protein aggregation [25]. A study examined the effect of osmolytes as chemical chaperones on the refolding and reactivation of aminoacylase and lipase, and revealed the significantly higher influence of sucrose and glycerol in this process compared to other agents [26, 27]. Tris and Triton X-100 can increase renaturation yields [28, 29]. All these compounds are contributed to disulfide bond formation.

One of the most important steps in the cloning process is the selection of appropriate vectors. Vector selection depends on the promoter wich influence solubilization of recombinant protein in *E. coli* host [30]. In this study, we used the pET-21b (+) vector. The main reason for choosing this vector was the existence of a smaller tag (6-Histidine tag (His-tag)) in this vector, in comparison with other pET vectors such as pET-32a (+) [31]. Since pET-32a (+) contains a long fusion tag which adds 109-aa, this large size can interfere with the activity of recombinant proteins. Moreover, in the pET-21b(+) vector, IPTG can be used to induce lac repressor-regulated promoters which can influence the yield of desired protein [32]. Protein expression can be regulated by optimizing the concentration of IPTG, because some proteins were required to be induced slowly with lower IPTG concentrations, while others need induction with higher levels of IPTG [33]. Since optimum concentration can be gained empirically by varying the IPTG concentration between 0.01-5 mM, the concentration of IPTG was achieved at 0.2 mM through examining different concentration.

Conclusion

Although there is still no global process for solubilization and refolding of recombinant proteins, simple and effective procedures are available. The method used in this study included guanidinium chloride, DTT, sucrose, glycerol, and glutathione. It was concluded that these materials can allow the folding of soluble immature mouse TGF-β1 as a disulfide-bonded protein with immunological activity.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles were considered in this article. The participants were informed about the purpose of the

research and its implementation stages; they were also assured about the confidentiality of their information; Moreover, They were allowed to leave the study whenever they wish, and if desired, the results of the research would be available to them.

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Authors contribution's

Experiments and draft preparation: Fahimeh Maleki, Kazem Mashayekhi, Malihe Moghadam and Seyedeh Elham Badiee Kheirabadi; Data analysis, review and editing: Mohammad Javad Mousavi; Conceptualization, review and editing: Mojtaba Sankian.

Conflict of interest

The authors declared no conflict of interests.

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References

[1] Gressner AM, Weiskirchen R, Breitkopf K, Dooley S. Roles of TGF-beta in hepatic fibrosis. Front Biosci. 2002; 7(1):d793-807. [DOI:10.2741/A812]

[2] Epstein FH, Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor β in human disease. N Engl J Med. 2000; 342(18):1350-8. [DOI:10.1056/NEJM200005043421807] [PMID]

[3] Bartram U, Speer CP. The role of transforming growth factor β in lung development and disease. CHEST J. 2004; 125(2):754-65. [DOI:10.1378/chest.125.2.754] [PMID]

[4] Tian M, Neil JR, Schiemann WP. Transforming growth factor-β and the hallmarks of cancer. Cell Signal. 2011; 23(6):951-62. [DOI:10.1016/j.cellsig.2010.10.015] [PMID] [PMCID]

[5] Mathuriya AS. Magnetotactic bacteria: Nanodrivers of the future. Crit Rev Biotechnol. 2016; 36(5):788-802. [DOI:10.3109/07388551.2015.1046810]

[6] Misawa S, Kumagai I. Refolding of therapeutic proteins produced in Escherichia coli as inclusion bodies. Biopolym-Pept Sci Sect. 1999; 51(4):297. [DOI:10.1002/(SICI)1097-0282(1999)51:43.0.CO;2-I]

[7] Demain AL, Vaishnav P. Production of recombinant proteins by microbes and higher organisms. Biotechnol Adv. 2009; 27(3):297-306. [DOI:10.1016/j.biotechadv.2009.01.008] [PMID]

[8] Abdi A, Hosseinpour M, Mashayekhi K, Mousavi MJ, Badiee Kheirabadi SE, Sankian M. Optimization of cloning conditions for high-level production of recombinant mouse interleukin-2 in escherichia coli. Res Mol Med. 2019; 7(1):16-25. [DOI:10.18502/rmm.v7i1.5256]

[9] Berkmen M. Production of disulfide-bonded proteins in Escherichia coli. Protein Expr Purif. 2012; 82(1):240-51. [DOI:10.1016/j.pep.2011.10.009] [PMID]

[10] Middelberg AP. Preparative protein refolding. Trends Biotechnol. 2002; 20(10):437-43. [DOI:10.1016/S0167-7799(02)02047-4]

[11] Wingfield PT, Palmer I, Liang SM. Folding and purification of insoluble (inclusion body) proteins from Escherichia coli. Curr Protoc Protein Sci. 2001; 6:6.5. [DOI:10.1002/0471140864.ps0605s00] [PMID]

[12] Singh A, Upadhyay V, Upadhyay AK, Singh SM, Panda AK. Protein recovery from inclusion bodies of Escherichia coli using mild solubilization process. Microb Cell Fact. 2015; 14(1):41. [DOI:10.1186/s12934-015-0222-8] [PMID] [PMCID]

[13] Kaur J, Kumar A, Kaur J. Strategies for optimization of heterologous protein expression in E. coli: Roadblocks and reinforcements. Int J Biol Macromol. 2018 Jan 1;106:803-22. [DOI: 10.1016/j.ijbiomac.2017.08.080] [PMID]

[14] Tsumoto K, Ejima D, Kumagai I, Arakawa T. Practical considerations in refolding proteins from inclusion bodies. Protein Expr Purif. 2003; 28(1):1-8. [DOI:10.1016/S1046-5928(02)00641-1]

[15] Wetlaufer DB, Branca PA, Chen GX. The oxidative folding of proteins by disulfide plus thiol does not correlate with redox potential. Protein Eng. 1987; 1(2):141-6. [DOI:10.1093/protein/1.2.141] [PMID]

[16] Lilie H, Schwarz E, Rudolph R. Advances in refolding of proteins produced in *E. coli*. Curr Opin Biotechnol. 1998; 9(5):497-501. [DOI:10.1016/S0958-1669(98)80035-9]

[17] Peach M, Marsh N, Miskiewicz EI, MacPhee DJ. Solubilization of proteins: The importance of lysis buffer choice. Methods Mol Biol. 2015; 1312:49-60. [DOI:10.1007/978-1-4939-2694-7_8] [PMID]

[18] Clark EDB. Protein refolding for industrial processes. Curr Opin Biotechnol. 2001; 12(2):202-7. [DOI:10.1016/S0958-1669(00)00200-7]

[19] Hevehan DL, De Bernardez Clark E. Oxidative renaturation of lysozyme at high concentrations. Biotechnol Bioeng. 1997; 54(3):221-30. [DOI:10.1002/(SICI)1097-0290(19970505)54:33.0.CO;2-H]

[20] Orsini G, Goldberg M. The renaturation of reduced chymotrypsinogen A in guanidine HCl. Refolding versus aggregation. J Biol Chem. 1978; 253(10):3453-8. [PMID]

[21] Umetsu M, Tsumoto K, Hara M, Ashish K, Goda S, Adschiri T, et al. How additives influence the refolding of immunoglobulin-folded proteins in a stepwise dialysis system spectroscopic evidence for highly efficient refolding of a single-chain fv fragment. J Biol Chem. 2003; 278(11):8979-87. [DOI:10.1074/jbc.M212247200] [PMID]

[22] Alibolandi M, Mirzahoseini H. Chemical assistance in refolding of bacterial inclusion bodies. Biochem Res Int. 2011; 2011:631607. [DOI:10.1155/2011/631607] [PMID] [PMCID]

[23] Kohyama K, Matsumoto T, Imoto T. Refolding of an unstable lysozyme by gradient removal of a solubilizer and gradient addition of a stabilizer. J Biochem Biotechnol. 2010; 147(3):427-31. [DOI:10.1093/jb/mvp184] [PMID]

[24] Mishra R, Seckler R, Bhat R. Efficient refolding of aggregation-prone citrate synthase by polyol osmolytes: How well are protein folding and stability aspects coupled? J Biol Chem. 2005; 280(16):15553-60. [DOI:10.1074/jbc.M410947200] [PMID]

[25] Yamaguchi H, Miyazaki M. Refolding techniques for recovering biologically active recombinant proteins from inclusion bodies. Biomolecules. 2014;4(1):235-51. [DOI:10.3390/biom4010235] [PMID] [PMCID]

[26] Kim SH, Yan YB, Zhou HM. Role of osmolytes as chemical chaperones during the refolding of aminoacylase. Biochem Cell Biol. 2006; 84(1):30-8. [DOI:10.1139/o05-148] [PMID]

[27] Akbari N, Khajeh K, Ghaemi N, Salemi Z. Efficient refolding of recombinant lipase from Escherichia coli inclusion bodies by response surface methodology. Protein Expr Purif. 2010; 70(2):254-9. [DOI:10.1016/j.pep.2009.10.009] [PMID]

[28] Kim CS, Lee EK. Effects of operating parameters in in vitro renaturation of a fusion protein of human growth hormone and glutathione S transferase from inclusion body. Process Biochem. 2000; 36(1):111-7. [DOI:10.1016/S0032-9592(00)00185-0]

[29] Daugherty DL, Rozema D, Hanson PE, Gellman SH. Artificial chaperone-assisted refolding of citrate synthase. J Biol Chem. 1998; 273(51):33961-71. [DOI:10.1074/jbc.273.51.33961] [PMID]

[30] Gopal GJ, Kumar A. Strategies for the production of recombinant protein in Escherichia coli. Protein J. 2013; 32(6):419-25. [DOI:10.1007/s10930-013-9502-5] [PMID]

[31] Novy R, Berg J, Yaeger K, Mierendorf R. pET TRX fusion system for increased solubility of proteins expressed in *E. coli*. Innovations. 1995; 3:7-9.

[32] Grabski A, Mehler M, Drott D. The overnight express autoinduction system: high-density cell growth and protein expression while you sleep. Nat Methods. 2005; 2(3):233-5. [DOI:10.1038/nmeth0305-233]

[33] Sambrook J, Russell D. Molecular Cloning, A Laboratory Manual, 3rd edition. New York: Cold Spring Horbor laboratory Press; 2001.

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