Journal of **A**pplied **B**iotechnology **R**eports

Original Article

Enzymatic Degradation of Organophosphate Compounds: Evaluation of High-level Production, Solubility and Stability

Gholamreza Farnoosh[1], Ali Mohammad Latifi[1*], Khosro Khajeh[2], Hossein Aghamollaei[1], Ali Najafi[3]

Abstract

The use of organophosphorus hydrolase (OPH) enzyme to degrade Chemical Warfare Agents is one of the most frequently used decontamination methods. OPH is a ~36 kDa homodimeric metalloprotein that is found in the membrane of *Flavobacterium* sp. strain ATCC 27551 and *Brevundimonas diminuta* MG and is capable of hydrolyzing a wide range of oxon and thion , such as paraoxon and parathion. OPH gene (*opd*) has been expressed in many hosts, such as bacteria, insect cells, fungi, and *Streptomyces* spp. High level and soluble expression and correct folding of each protein are of important factors. Fusion proteins, including TRX, Gb1 and MBP, are commonly used to increase solubility, folding and in some cases, stability. The present study evaluated thioredoxin (TRX) role in OPH expression level, solubility and stability by cloning the *opd* gene into pET32a and pET21a and expressing the resulting vectors in *E. coli shuffle* T7. The pET32a vector encodes a fusion protein containing TRX that is not present in the pET21a. The results revealed an increased expression level, solubility and stability in OPH produced by the pET32a-*opd* construct compared to the pET21a vector due to the presence of the TRX fusion in pET32a vector.

Keywords: Organophosphorus Hydrolase, Thioredoxin, pET32a-*opd*, High Level Production, Solubility, Stability

1. Applied Biotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
2. Department of Biochemistry, Faculty of Biologic Science, Tarbiat Modares University, Tehran, Iran
3. Molecular Biology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

*** Corresponding Author**
Ali Mohammad Latifi
Applied Biotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
E-mail: amlatifi290@gmail.com

Submission Date: 10/27/2015
Accepted Date: 12/15/2015

Introduction

Organophosphates are extremely toxic compounds that have been used as plasticizers, air fuel ingredients, chemical warfare agents, and more commonly as pesticides and insecticides. These compounds inactivate a large family of serine hydrolase. One of the most important enzymes deactivated by these compounds is Acetylcholinesterase which exists at the central nervous system. Inactivation of this enzyme results in the accumulation of acetylcholine in nervous system, the dysfunction of muscles, and damage in breathing leading to coma and finally death. Biodegradation methods, including the use of enzymes, are the most important methods for decontamination of organophosphorus chemicals [1, 2].

The Organophosphorus hydrolase enzyme (OPH) has been one of the most studied enzymes to decontaminate organophosphorus chemicals [3]. The OPH, first noticed in *Flavobacterium* strain ATCC 27551 and *Pseudomonas diminuta*. OPH, is a ~36 kDa homodimeric metalloprotein with two Zn^{2+} ions (which can be substituted with Mn^{2+}, Cd^{2+}, Co^{2+}, or Ni^{2+}) in the active site of native OPH enzyme that can degrade a broad spectrum of organophosphorus chemicals [4-6].

OPH has been expressed in many hosts including bacteria, insect cells and fungi. The expression of OPH in *Escherichia coli* is in too much higher than *P. diminuta*. *E. coli* strains produce low yields of OPH enzyme due to the low solubility and the degradation rate of proteolytic enzymes. However, in most cases the level of OPH production has been, relatively low and unstable with improper folding [1, 7-9]. Although OPH has a very large conformational stability (~40 kcal/mol), only a small volume of energy (~4 kcal/mol) needs to unfold this enzyme into its inactive form. In fact, its operational stability is much lower [10, 11].

Many strategies have been attempted to enhance OPH production yield, solubility, folding and stability, such as varying growth conditions, using fusions protein and mutation creation [1, 8]. Of course the use of an engineered host that secretes the active enzyme may also be useful to solve the problems [12]. The production of soluble and stable protein in high-level in *E. coli* strains is still a major block for researchers, and there is a need to solve it [4, 13]. To facilitate this problem, a number of fusion tags (thioredoxin, maltose binding protein, NusA, etc.) are used. Fusion tags can improve protein expression, stability and resistance to proteolytic degradation. There are many reports on an increase in the yield, solubility and stability of fused proteins when overexpressed in *E. coli* strains [12].

TRX is a small protein (a molecular weight about 12 kDa) found from archaebacteria to humans [14]. It has a central core with five β-sheet strands surrounded by four α-helices[15]. It is a soluble tag that assists the protein refolding, the resistance to proteolytic degradation, the high-level production of proteins and disulfide bonds

formation. TRX not only is an important factor in solubility, but in most cases it is also biological active agent. Also TRX has an inherent thermal stability [16-18]. The pET32 expression vector is one of the systems that containsTRX tag [5]. This vector is designed for cloning and high yield expression of protein sequences fused with TRX protein [19].

In this paper, we described the expression of *opd* gene at two systems; with and without TRX fusion protein (pET32a with fusion protein and pET21a without fusion protein) in engineered *E. coli shuffle* T7 to evaluate the TRX role at high-level production, solubility and stability.

Materials and Methods

Design, Optimization and synthesis of the pET-32a-opd and pET-21a-opd constructs

The *opd* gene (1008 bp) was designed and optimized for *E. coli* by JCat software [20], Gen Script's (http://www.genscript.com/cgibin/tools/rare_codon_analysis) and Mfold softwares [21], and finally was cloned into pET21a and pET32a vectors (Biomatik, Canada) as *NdeI*/*HindIII* with His tag fused at N-terminal. All the clones were confirmed by DNA sequencing (Fig. 1). At pET32a vector ,TRX is fused at N-terminal because it is more effective when located on the N-terminal of target protein [22, 23]. His tag fused at N-terminal of OPH gene because the N-terminus of OPH in a region distant from the active site (OPH active site is located at C-terminal) [24].

There are 29 amino acids at N-terminal of OPH known as peptide leader. This peptide leader was removed from the clone because it would affect OPH stability and folding. The removal of this sequence caused a major increase in the level of protein expression and stability [13, 25, 26] .

Expression optimization of opd gene in E. coli Shuffle T7

100 µl of the transformed cells was inoculated in LB Amp (80 µg/ml) plates. A single colony of transformed *E. coli Shuffle* T7 was removed and grown at 37°C in 15 ml LB broth medium with 80 mg/L ampicillin and 1 mM $ZnCl_2$ (to provide a divalent metal ion which is required for enzyme active site) at 150 rpm. Cultures were grown to an OD 600 of 0.8, and the expression of recombinant OPH was induced by adding different concentrations of IPTG (0.1 to 1 mM) at varied temperatures (14°C, 18° C, 24°C, 30°C and 37°C) for 4, 6, 8, 10, 12, 14,16, 24 and 48 h. After incubation, the cells were harvested and suspended in 20 ml of lysis buffer (20 mM Tris/HCl, pH 9.0, 200 mM NaCl) with 1 mM PMSF, 10 mM $ZnCl_2$, 0.2 mg/ml lysozyme and 0.2 mg/ml DNaseI. The cells were lysed by six 30-s sonication steps. T separate soluble and insoluble fractions, the extract were clarified by centrifugation for 20 min at 14,000 rpm, at 40°C [27, 28].

Analysis of Expression Level, Solubility and Protein Purification

Equal portions of whole-cell lysate, soluble fraction, and insoluble fraction were analyzed on 12% SDS-PAGE. To evaluate the stability, OPH purification was done with the hexa-histidine tag by Ni-NTA Colum (Qia gene).

1 ml of the Ni-NTA solution was added to soluble fraction and mixed by shaking (150 rpm) at 4°C for an overnight. The soluble fraction–Ni-NTA mixtures were spilled into a column with a bottom covered, then the bottom cap was removed and the flow was collected. Washing was done 2 times with 2 ml wash buffer (100 mM NaH_2PO_4, 10 mM Tris.HCl, 20 mM imidazole, Adjust pH to 8.0). Elution was done 2 times with 2 ml elution buffer (100 mM NaH_2PO_4, 10 mM Tris.HCl, Adjust pH to 8.0) with 150 and 250 mM imidazole. The protein concentration was determined using Bradford method. Flow, wash and elution fractions were used for SDS-PAGE analysis [29].

Activity assay and stability evaluation

For each assay, 40 µL of supernatant (OD 600 = 1.0) were added into a solution with 140 µL of Tris.HCl buffer (10 mM $ZnCl_2$, adjust pH8.5) and 20 µl of 20 mM paraoxon in 20% deionized water. The reaction was conducted in 1.5 ml eppendorf tube for 10 min at 37◦C. 100 µl of reaction medium was added in a 96-deep-well plate and the production of p-Nitrophenol (PNP) was determined by measuring the absorbance at 405 nm where PNP absorbs strongly at ambient temperature using microplate reader. A unit of enzyme activity was as one micromole of paraoxon hydrolyzed to PNP per minute, per milliliter [30].

Enzyme activity was calculated according to: Absorbance at 405 nm × Volume of assay × Dilution factor/Extinction coefficient of PNP × Volume of enzyme × Time [31].

To evaluate OPH stability, we used two independent methods; thermal and proteolytic. For thermal stability evaluation, pure OPH produced by two constructs was incubated at 600c for 0, 10, 20, 30, 40, 50 and 60 minutes, and then was assayed based on the above protocol [32].

Evaluation of proteolytic stability is a useful biochemical method which can give information about protein structure and conformational changes [33, 34]. In an analysis of the proteolytic stability between the two forms, pure OPHs was incubated at 400°C for one hour then was treated with trypsin (100 µg/ml) with 1:10 trypsin/OPH ratio at 20 mM Tris buffer, pH 8.0 and 37°C. For OPH activity assay after proteolytic cleavage, aliquots of the reaction were removed at specific time points (5, 10, 30, and 60 minutes) then trypsin activity was stopped by adding 0.1mM PMSF (phenylmethylsulfonyl fluoride). To remove the PMSF, the aliquots were dialyzed against 50mM Tris then were assayed according to the assay protocol immediately [15, 35-42]. The concentrations of pure OPH obtained from both constructs were the same.

Results

Optimum condition for soluble expression

The effects of temperature, IPTG concentration and duration of incubation were investigated. The best condition for OPH expression at both constructions were 18°C, 30°C and 37°C, IPTG 0.3 mM and 7 hrs.

Evaluation of OPH Solubility by SDS Page Analysis

SDS-PAGE of whole-cell lysate, soluble and insoluble fractions obtained from both constructs showed two distinct bands at anticipated monomeric molecular weight (~35 kDa for pET 21a-*opd* construct and ~46 kDa for pET32a-*opd* construct). The results of SDS page analysis revealed increased OPH expression level and solubility in the pET32a-*opd* construct compared to the pET21a vector.

As can be seen in Figure 2, OPH solubility increased in the pET32a-*opd* construct compared to the pET21a vector due to the presence of the fusion with TRX in pET32a (This is known at the three temperatures and IPTG concentrations). Of course, despite using the different conditions of temperature, time and IPTG concentration, the majority of this protein was in the insoluble fractions in both systems. It is suggested to take denaturation refolding.

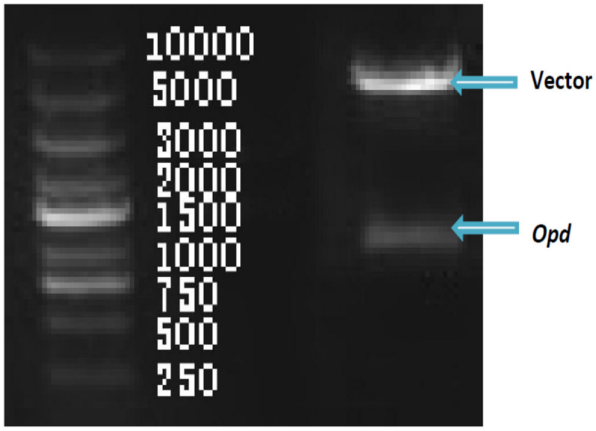


Figure 1. The electrophoresis of *opd* gene and vector**.** The *opd* gene and vector are shown.

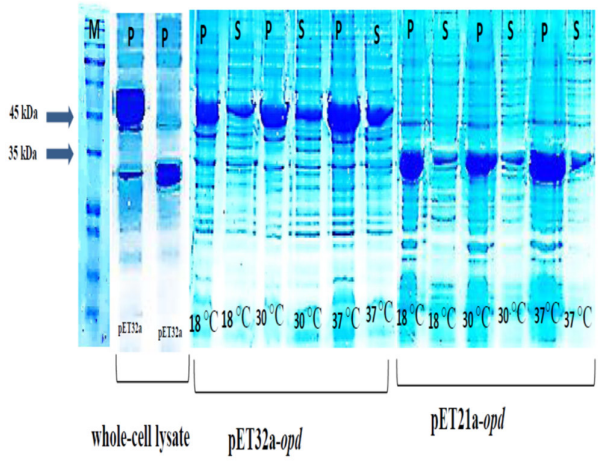


Figure 2. SDS-PAGE analysis of pET21a-*opd* and pET32a-*opd* whole-cell lysate, soluble and insoluble fractions expressed in *E. coli shuffle* T7 cells with 0.3 mM IPTG for 7 h at 18°C, 30°C and 37°C. **M**: Molecular weight Marker; **P:** Pellet; **S:** soluble.

OPH purification

The purification results at SDS-PAGE analysis showed two bands of ~46 kDa and ~35 kDa. By comparing the two bands, pET32a-opd band was stronger than pET21a-opd band, revealing an increase in the expression level and solubility (Fig. 3). The concentration of protein was determined using Bradford method (0.1 mg/ml for pET- 32a-opd construct and 0.04 mg/ml for pET- 21a-*opd* construct).

Activity assay and stability evaluation

By investigating the activity of produced OPH in solution fractions against specific substrate (paraoxon), most protein was actively expressed in samples from the pET32a-opd construct (Fig. 4). This increase in activity due to the strong bands in the SDS-PAGE was predictable (Fig. 2).

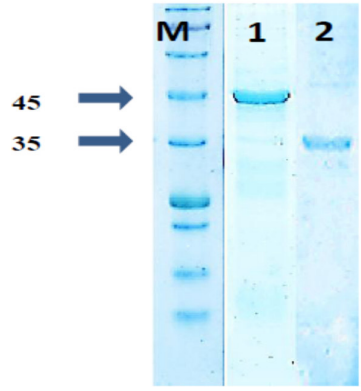


Figure 3. SDS-PAGE analysis of purified proteins. **M**: Molecular weight Marker; **1:** OPH with TRX, **2:** without TRX.

Thermal stability was determined by measuring residual enzymatic activity after incubation at 600c temperature for different times (Figure 5a). To determe proteolytic stability, residual activity was measured after incubation with trypsin for different times (Figure 5b). The results were indicated the stability of pET32a-*opd* samples in comparison with of pET21a-opd samples (Fig. 5).

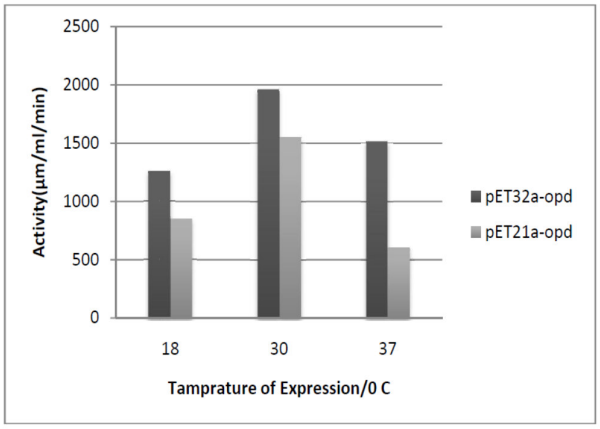


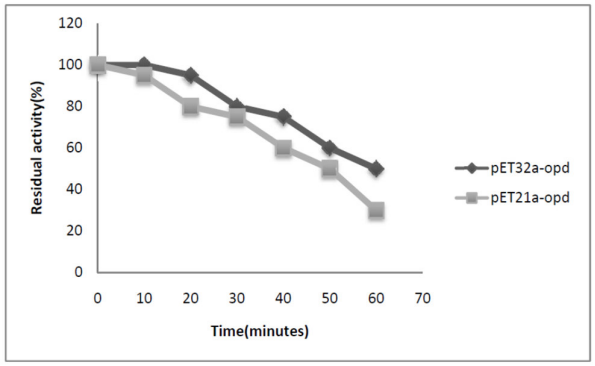
Figure 4. Activity assay of soluble fractions at pET32a-*opd* and pET21a-*opd* enzymes. The compression of activity at three temperatures, the most activity was observed in sample expressed at 30°C and the lowest in sample expressed at 37°C.

Discussion

E. coli strains produce low yields of OPH enzyme due to the low solubility and stability of this protein [43]. However, in most cases, the production level and stability of OPH has been relatively low and the protein is

degraded by proteases. The production of soluble and stable protein in *E. coli* strains is still a major block for researchers, and there is a need to improve the folding of produced recombinant protein in *E. coli* [13, 24, 26, 44].

A



B

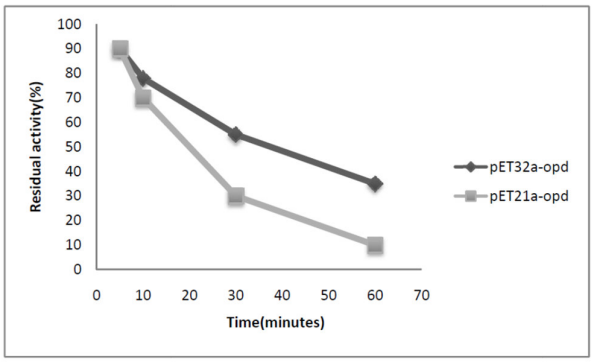


Figure 5. Thermal and proteolytic stability curves for pET32a-*opd* and pET21a-*opd* enzymes. A**)** The thermal stability of the enzymes was determined by monitoring residual enzymatic activity after incubation for 0 to 60 minutes at 60°C temperature and B) proteolytic stability the enzymes was incubated at 40°C for one hour then was treated with 20mM trypsin for 5, 10, 30, and 60 minutes. Enzymatic activity was then assayed using standard enzyme assay. Both diagrams indicated the stability of produced OPH by pET32a-*opd* construct compared to the pET21a vector.

To enhance OPH expression level, solubility and resistance to proteolytic degradation, the use of fusion proteins and engineered hosts (include *E. coli shuffle* T7, *E. coli* Rosetta-gami and etc.) are of the most important methods [12]. Fusion tags can improve protein expression, resistance to proteolytic degradation and solubility [45].

There are reports on an increase in the solubility and the stability of fused proteins when overexpressed in engineered *E. coli* strains. According to the studies, TRX, Gb1 and MBP were most efficient tags in giving a soluble protein [12, 46]. TRX is a soluble tag assisting protein refolding which needs a reducing condition and is resistant to proteolytic degradation [47].

In this study, we described the expression of opd gene at two systems; with and without TRX fusion protein (pET32a with fusion protein and pET21 without fusion protein) in engineered *E. coli* strain (shuffle T7) to evaluate the TRX role at expression level, solubility and stability.

In evaluating the expression level and the solubility of produced enzymes, the results of SDS page analysis and assay reflected an increase in the expression level and the solubility of OPH produced by pET32a-*opd* construct, which were higher than OPH produced by pET21a-*opd* construct, which were due to the presence of TRX fusion protein in pET32a. These results were similar to previous studies in the TRX role at the high level production and increased solubility [18, 19, 48].

Although solubility of produced OPH by pET32a-opd construct was increased compared to the pET21a vector but the majority of this protein was found in the insoluble fractions that is suggested to take denaturation refolding. Of course fusion proteins such as TRX increases the yield of soluble proteins after refolding steps [49].

The results of thermal and proteolytic experiments showed stability improved at OPH produced by the pET32a-*opd* construct compared to the pET21a vector because of the presence of TRX. TRX has a central core with five β-sheet strands surrounded by four α-helices and contains a *cis*-proline located in beta-strand 4 that interacts with cysteines in the active site and is essential for both stability and function [15, 50, 51]. The TRX structure also has a very tight fold so that >90% of its primary sequence is involved in secondary structure [52]. It probably acts as a scaffold for OPH and causes resistance to protease and so thermal stability [51]. The previous studies confirm our results on the thermal and proteolytic stability that is related to the present of TRX [15, 50, 51, 53].

Conclusion

In summary, we have described the first application of the utility of TRX as an increasing agent of the expression level, moderate solubility and stability of recombinant OPH enzyme. In particular, a significant correlation was made between OPH expression level, moderate solubility, stability and TRX. Meanwhile, the use of the engineered host (*E. coli shuffle* T7) was played an important role in strengthening it.

Acknowledgements

This study is extracted from a PhD's thesis which approved and supported by Applied Biotechnology Research center, Baqiyatallah University of Medical Sciences, Tehran, Iran. The authors would like to thanks the staff of these research centers for their kind help.

References

1. Farnoosh, G., Latifi, A. M., A review on engineering of organophosphorus hydrolase (OPH) enzyme. *J Appl Biotechnol Rep*, 2014, Vol. 1, pp. 1-10.
2. Latifi, A. M., Khodi, S., Mirzaei, M., Miresmaeili, M., Babavalian, H., Isolation and characterization of five chlorpyrifos degrading bacteria. *Afr J Biotechnol*, 2014, Vol. 11, pp. 3140-3146.
3. Harvey, S. P., Kolakowski, J. E., Cheng, T. C., Rastogi, V. K., Reiff, L. P., DeFrank, J. J., Raushel, F. M., Hill, C., Stereospecificity in the enzymatic hydrolysis of cyclosarin (GF). *Enzyme Microb Technol*, 2005, Vol. 37, pp. 547-555.

4. Lu, H. D., Wheeldon, I. R., Banta, S., Catalytic biomaterials: engineering organophosphate hydrolase to form self-assembling enzymatic hydrogels. *Protein Eng Des Sel*, 2010, Vol. pp. 559-566.
5. Wu, C. F., Valdes, J. J., Bentley, W. E., Effects of in situ Cobalt ion addition on the activity of a GFP-OPH fusion protein: The fermentation kinetics. *Biotechnol Prog*, 2001, Vol. 17, pp. 606-611.
6. Khodi, S., Latifi, A. M., Saadati, M., Mirzaei, M., Aghamollaei, H., Surface display of organophosphorus hydrolase on E. coli using N-terminal domain of ice nucleation protein InaV. *J Microbiol Biotechnol*, 2012, Vol. 22, pp. 234-8.
7. Xu, G., Zheng, W., Li, Y., Wang, S., Zhang, J., Yan, Y., Biodegradation of chlorpyrifos and 3, 5, 6-trichloro-2-pyridinol by a newly isolated *Paracoccus* sp. strain TRP. *Int Biodeter Biodegr*, 2008, Vol. 62, pp. 51-56.
8. Kang, D. G., Lim, G. B., Cha, H. J., Functional periplasmic secretion of organophosphorous hydrolase using the twin-arginine translocation pathway in *Escherichia coli*. *J Biotechnol* 2005, Vol. 118, pp. 379-385.
9. Karpouzas, D. G., Singh, B. K., Microbial degradation of organophosphorus xenobiotics: metabolic pathways and molecular basis. *Adv Microb Physiol*, 2006, Vol. 51, pp. 119-225.
10. Armstrong, C. D., Elucidating the chemical and thermal unfolding profiles of organophosphorus hydrolase and increasing its operational stability, 2007, Texas A&M University.
11. Grimsley, J. K., Scholtz, J. M., Pace, C. N., Wild, J. R., Organophosphorus hydrolase is a remarkably stable enzyme that unfolds through a homodimeric intermediate. *Biochem*, 1997, Vol. 36, pp. 14366-14374.
12. Markland, K., Methodology for high-throughput production of soluble recombinant proteins in *Escherichia coli.* MSc Tesis, 2007, The Swedish Centre for Bioprocess Technology.
13. Dumas, D. P., Caldwell, S. R., Wild, J. R., Raushel, F., Purification and properties of the phosphotriesterase from Pseudomonas diminuta. *J Biol Chem*, 1989, Vol. 264, pp. 19659-19665.
14. Štefanková, P., Perečko, D., Barák, I., Kollárová, M., The thioredoxin system from *Streptomyces coelicolor*. *J Basic Microbiol*, 2006, Vol. 46, pp. 47-55.
15. Pedone, E., Saviano, M., Rossi, M., Bartolucci, S., A single point mutation (Glu85Arg) increases the stability of the thioredoxin from *Escherichia coli*. *Protein Eng*, 2001, Vol. 14, pp. 255-260.
16. Smith, P. A., Tripp, B. C., DiBlasio-Smith, E. A., Lu, Z., LaVallie, E. R., McCoy, J. M., A plasmid expression system for quantitative *in vivo* biotinylation of thioredoxin fusion proteins in *Escherichia coli*. *Nucleic Acids Res*, 1998, Vol. 26, pp. 1414-1420.
17. Kwok, W., Attempted Cloning of Escherichia coli csg into flavin reductase or thioredoxin in the pET32a (+) vector to assess disulfide bond effects on amyloid deposits formation. *J Exp Microbiol Immunol*, 2004, Vol. 6, pp. 72-76.
18. Lavallie, E. R., DiBlasio, E. A., Kovacic, S., Grant, K. L., Schendel, P. F., McCoy, J. M., A thioredoxin gene fusion expression system that circumvents inclusion body formation in the E. coli cytoplasm. *Nat Biotechnol*, 1993, Vol. 11, pp. 187-193.
19. Kim, Y. V., Gasparian, M. E., Bocharov, E. V., Chertkova, R. V., Tkach, E. N., Dolgikh, D. A., Kirpichnikov, M. P., New strategy for high-Level expression and purification of biologically active monomeric TGF-beta1/C77S in *Escherichia coli*. *Mol Biotechnol*, 2015, Vol. 57, pp. 160-171.
20. Grote, A., Hiller, K., Scheer, M., Münch, R., Nörtemann, B., Hempel, D. C., Jahn, D., JCat: a novel tool to adapt codon usage of a target gene to its potential expression host. *Nucleic Acids Res*, 2005, Vol. 33, pp. W526-W531.
21. Zuker, M., Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res*, 2003, Vol. 31, pp. 3406-3415.
22. Malhotra, A., Tagging for protein expression. *Methods Enzymol*, 2009, Vol. 463, pp. 239-258.
23. Dyson, M. R., Shadbolt, S. P., Vincent, K. J., Perera, R. L., McCafferty, J., Production of soluble mammalian proteins in *Escherichia coli*: identification of protein features that correlate with successful expression. *BMC Biotechnol*, 2004, Vol. 4, pp. 32-36.
24. March, J., Rao, G., Bentley, W., Biotechnological applications of green fluorescent protein. *Appl Microbiol Biotechnol*, 2003, Vol. 62, pp. 303-315.
25. DeFrank, J.J., Harvey, S.P., Rastogi, V.K., Cheng, T.-c., Penet, C.S., *E*nzymatic Decontamination: From Concept To Commercialization, 2004, DTIC Document.
26. Richins, R. D., Mulchandani, A., Chen, W., Expression, immobilization, and enzymatic characterization of cellulose-binding domain-organophosphorus hydrolase fusion enzymes. *Biotechnol Bioeng*, 2000, Vol. 69, pp. 591-596.
27. Sani, M. R., Moghaddam, M. M., Aghamollaei, H., Hassanpour, K., Taheri, R.A., Farnoosh, G., Investigation of caspase-1 activity and interleukin-1beta production in murine macrophage cell lines infected with *Leishmania major*. *Asian Pac J Trop Med*, 2014, Vol. 7S1, pp. 70-73.
28. Cheng, T., Harvey, S. P., Chen, G. L., Cloning and expression of a gene encoding a bacterial enzyme for decontamination of organophosphorus nerve agents and nucleotide sequence of the enzyme. *Appl Environ Microbiol*, 1996, Vol. 62, pp. 1636-1641.
29. Davis, G.D., Harrison, R.G., Discovery of new fusion protein systems designed to enhance solubility in *E. coli*, in *E. coli* Gene Expression Protocols. 2003, Springer. p. 141-154.
30. Briseño-Roa, L., Oliynyk, Z., Timperley, C.M., Griffiths, A.D., Fersht, A.R., Highest paraoxonase turnover rate found in a bacterial phosphotriesterase variant. *Protein Eng Des Sel*, 2011, Vol. 24, pp. 209-211.
31. Gulzar, S., Amin, S., Kinetic studies on β-Galactosidase Isolated from Apricots (Prunus armeniaca kaisa). *Am J Plant Sci*, 2012, Vol. 3, pp. 636-645.
32. Asad, S., Torabi, S. F., Fathi-Roudsari, M., Ghaemi, N., Khajeh, K., Phosphate buffer effects on thermal stability and H2O2-resistance of horseradish peroxidase. *Int J Biol Macromol*, 2011, Vol. 48, pp. 566-570.
33. Hubbard, S. J., The structural aspects of limited proteolysis of native proteins. *BBA-Protein Struct Mol Enzymol*, 1998, Vol. 1382, pp. 191-206.
34. Fontana, A., Polverino de Laureto, P., De Filippis, V., Scaramella, E., Zambonin, M., Probing the partly folded states of proteins by limited proteolysis. *Fold Des*, 1997, Vol. 2, pp. 17-26.
35. Ruiz-Argüello, M. B., Martín, D., Wharton, S. A., Calder, L. J., Martín, S. R., Cano, O., Calero, M., García-Barreno, B., Skehel, J. J., Melero, J. A., Thermostability of the human respiratory syncytial virus fusion protein before and after activation: implications for the membrane-fusion mechanism. *J Gen Virol*, 2004, Vol. 85, pp. 3677-3687.
36. Khangembam, B. K., Yvr, K. S., Chakrabarti, R., Purification and characterization of trypsin from the digestive system of carp Catla catla (Hamilton). *Int Aquatic Res*, 2012, Vol. 4, pp. 1-12.
37. Luchini, A., Espina, V., Liotta, L. A., Protein painting reveals solvent-excluded drug targets hidden within native protein–protein interfaces. *Nat Commun*, 2014, Vol. 5, pp. 1-6.
38. Chen, Y., Bienkowski, M., Marnett, L., Controlled tryptic digestion of prostaglandin H synthase. Characterization of protein fragments and enhanced rate of proteolysis of oxidatively

inactivated enzyme. *J Biol Chem*, 1987, Vol. 262, pp. 16892-16899.
39. Ahmad, F., Coleman, S. K., Kaila, K., Blaesse, P., Cold-adapted protease enables quantitation of surface proteins in the absence of membrane trafficking. *Biotechniques*, 2011, Vol. 50, pp. 255-257.
40. Medel, V., Palma, R., Mercado, D., Rebolledo, R., Quiroz, A., Mutis, A., The Effect of Protease Inhibitors on Digestive Proteolytic Activity in the Raspberry Weevil, Aegorhinus superciliosus (Guérin)(Coleoptera: Curculionidae).
41. Katona, L. I., Ayalew, S., Coleman, J. L., Benach, J. L., A bactericidal monoclonal antibody elicits a change in its antigen, OspB of Borrelia burgdorferi, that can be detected by limited proteolysis. *J Immunol*, 2000, Vol. 164, pp. 1425-1431.
42. Zeinoddini, M., Khajeh, K., Hosseinkhani, S., Saeedinia, A.R., Robatjazi, S.M., Stabilisation of recombinant aequorin by polyols: activity, thermostability and limited proteolysis. *Appl Biochem Biotechnol*, 2013, Vol. 170, pp. 273-280.
43. Grimsley, G. R., Shaw, K. L., Fee, L. R., Alston, R. W., Huyghues-Despointes, B. M., Thurlkill, R. L., Scholtz, J., Pace, C., Increasing protein stability by altering long-range coulombic interactions. *Protein Sci*, 1999, Vol. 8, pp. 1843-1849.
44. Das, K.M., Banerjee, S., Shekhar, N., Damodaran, K., Nair, R., Somani, S., Raiker, V. P., Jain, S., Padmanabhan, S., Cloning, soluble expression and purification of high yield recombinant hGMCSF in Escherichia coli. *Int J Mol Sci*, 2011, Vol. 12, pp. 2064-2076.
45. Costa, S. J. M. d., Development of a novel fusion system for recombinant protein production and purification in *Escherichia coli*. 2013, Doctoral Thesis.
46. Woestenenk, E. A., Hammarström, M., van den Berg, S., Härd, T., Berglund, H., His tag effect on solubility of human proteins produced in Escherichia coli: a comparison between four expression vectors. *J Struct Funct Genomics*, 2004, Vol. 5, pp. 217-229.
47. Xiong, C., O'Keefe, B.R., Botos, I., Wlodawer, A., McMahon, J.B., Overexpression and purification of scytovirin, a potent, novel anti-HIV protein from the cultured cyanobacterium *Scytonema varium*. *Protein Express Purif*, 2006, Vol. 46, pp. 233-239.
48. Hamedifar, H., Salamat, F., Saffarion, M., Ghiasi, M., Hosseini, A., Lahiji, H., Nouri, Z., Arfae, H., Mahboudi, F., A Novel approach for high level expression of soluble recombinant human parathyroid hormone (rhPTH 1-34) in *Escherichia coli*. *Avicenna J Med Biotechnol*, 2013, Vol. 5, pp. 193.
49. Sachdev, D., Chirgwin, J.M., Solubility of proteins isolated from inclusion bodies is enhanced by fusion to maltose-binding protein or thioredoxin. *Protein Express Purif*, 1998, Vol. 12, pp. 122-132.
50. Saarinen, M., Gleason, F.K., Eklund, H., Crystal structure of thioredoxin-2 from Anabaena. *Structure*, 1995, Vol. 3, pp. 1097-1108.
51. Canali, E., Bolchi, A., Spagnoli, G., Seitz, H., Rubio, I., Pertinhez, T. A., Müller, M., Ottonello, S., A high-performance thioredoxin-based scaffold for peptide immunogen construction: proof-of-concept testing with a human papillomavirus epitope. *Sci Rep*, 2014, Vol. 4, pp. 15-20.
52. LaVallie, E.R., McCoy, J.M., Gene fusion expression systems in *Escherichia coli*. *Curr Opin Biotechnol*, 1995, Vol. 6, pp. 501-506.
53. Nazari-Robati, M., Khajeh, K., Aminian, M., Mollania, N., Golestani, A., Enhancement of thermal stability of chondroitinase ABC I by site-directed mutagenesis: an insight from Ramachandran plot. *Biochim Biophys Acta*, 2013, Vol. 1834, pp. 479-86.