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Optimization of Expression and Purification of some Model Histidine-tagged Recombinant Proteins: MiRGD, GNH, HNH, Firefly Luciferase and Human DT-Diaphorase

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ABSTRACT

Higher rates of protein expression lead to the accumulation of proteins as inclusion bodies. In contrast, the expression of soluble proteins needs to be optimized to reduce the amount of accumulated proteins. Therefore, obtaining a high amount of the desired his-tag recombinant proteins always requires a balance between expression rate and accumulation. Inducer concentration, time, and temperature of induction are the main variable in the level of soluble protein or inclusion bodies expression. In summary, if the higher concentrations of the protein are toxic or tend to be aggregated inside cells, higher ODs, lower IPTG concentrations, and shorter expression times can be effective in formation of inclusion bodies. The standard purification process can also be optimized based on the physicochemical properties, such that, in addition to increasing yield in the expression step, the activity and concentration of the desired protein is maintained in the purification step. In this study, we obtained the results of extraction of three different peptides MiRGD, GNH and HNH, and two proteins firefly mutant red-emitter luciferase, and human DT-diaphorase proteins by altering these variables on expression as well as purification conditions. Optimization of the protocols in Ni-NTA affinity chromatography of recombinant proteins with denaturants for MiRGD, GNH and HNH brought about with increase of purified protein concentrations from 0.21, 0.4, and 0.16 mg ml^{-1} in the initial attempt to 2.9, 3.4 and 2.6 mg ml^{-1} in the optimized experiment respectively. Non-denaturing optimization of purification for mutant luciferase and human diaphorase produced 4.1 and 2.6 mg ml^{-1}, respectively.

Keywords: Inclusion body, Expression, Recombinant proteins, Ni-NTA

INTRODUCTION

Protein purification includes a set of processes aiming to isolate proteins of interest from an organic complex mixture required to study the properties, function, structure, and interaction of the proteins [1]. The combination of genetic and protein engineering has revolutionized the field of biotechnology. Introduction of recombinant vectors and constructs containing genes that encode for desired peptides or proteins which are tagged by histidine (His-tag) or streptavidin (Strep-tag) in bacterial hosts and their consequent amplification and expression allows the production of proteins in large quantities [2,3], which facilitates their purification through a marked reduction in the time and the number of purification steps [1].

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However, to maximize the quantity and quality of the purified proteins, purification broadly needs to be optimized [4], which requires recognizing the physicochemical properties of each protein and adapting the standard protocol to these properties. This requirement goes so far as to determine the method of applying purification. Some properties of the proteins, such as their solubility, net charge, conformation, and binding specificity, rely on their amino acids composition, and others are expressed according to the extrinsic factors. To better explain this, if the expressed protein was not secreted and extractable from the culture medium, it would exist in soluble or aggregated (inclusion bodies) in the cytoplasm environment. Priority and preference are given to the production of protein in solution because if it accumulates in the form of inclusion bodies, its purification method is usually accompanied by denaturation and this is while some proteins do not refold

completely or correctly. Therefore, expression of some protein causes cytotoxicity or tends to precipitate, and after removing the denaturant can maintain its activity by taking the correct fold, the design conditions of the experiment would be shifted towards the formation of inclusion bodies. In the same way, all the steps of the process, especially the purification, including the buffers, temperature, and other conditions, along with the preservation of the protein and in line with its characteristics, should be adopted.

By taking the importance of the purification process into consideration, in this study, we aimed to optimize the expression and purification of three peptides, including MiRGD, GNH, and HNH, and two proteins, including the new mutated version of the red-emitting firefly luciferase and DT-diaphorase with various physicochemical properties (Table 1). Immobilized metal affinity chromatography (IMAC) with a polyhistidine tag, as the technique used in the study, emerged as a separation technique based upon molecular conformation, which frequently utilizes specific resins (Fig. 1) [5,6].

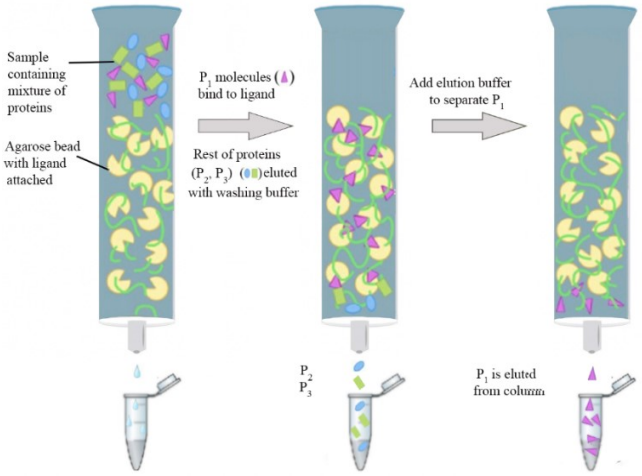

Fig. 1. Schematic diagram of affinity chromatography ([7] with modification). The desired protein (P1) in the mixture specifically was bound to ligands attached resins. Impurities (including molecules P2 and P3) were removed from the column with the Washing buffer during the washing step. Finally, by using the Elution buffer, the desired protein (P1) was eluted from the column.

MiRGD, GNH, and HNH are three synthetic nanocarriers in which a variety of functional motifs, consisting of binding and nucleic acid compacting, endosomal escaping, transmitting to the nucleus, and tissue penetration elements in a single chain of peptide were gathered through genetic engineering for efficient intracellular gene delivery purposes [8-10]. MiRGD, GNH, and HNH cationic peptides are less than 100 amino acids in length and have a net charge of +22, +22, and +21 in physiological pH, respectively. The molecular weight and the isoelectric pH of the MiRGD, GNH, and HNH are 9.7 KDa, 8.8 KDa and 10.8 KDa, and 10.93, 12.13, and 11.19, respectively [11]. These three peptides also establish extensive hydrophobic patches at their access surface [12-15]. The cationic entity of these peptides can cause bacterial toxicity; on the other hand, their high hydrophobicity creates the potential for aggregation in them. Therefore, the expression of these nanocarriers at 37 °C leads to the formation of the inclusion bodies, which can be solubilized by denaturants, purified simultaneously using Ni-NTA chromatography, and refolded on-column [10].

Table 1. Physicochemical Properties of Studied Peptides and Proteins

Peptide/protein	Molecular weight (kDa)	Isoelectric PH	Net charge	Hydrophobicity of access area[a]
MiRGD	9.7	10.93	+22	71%
GNH	8.8	12.13	+22	<71%
HNH	10.8	11.19	+21	74%
DTD	32	8.9	+4	63%
Mutant luciferase	64.5	8.2	+3	>60%

[a]The percentage of surface hydrophobicity of peptides and proteins was calculated based on their three-dimensional structural data [12-15].

Firefly luciferase is an enzyme that catalyzes the transformation of the excited-state oxyluciferin from D-luciferin in a bioluminescence reaction in the presence of Mg^{2+}, ATP, and molecular oxygen to produce the light [16]. The sensitivity and the convenience of the firefly luciferase assays have drastically increased the development and utilization of the luciferase-based biosensors, with a femtomole detection limit [17]. In a study, three mutations were introduced in the native *Lampyris turkestanicus* luciferase sequence with an isoelectric pH of 6.6, led to a shift in the light emitted from green to red. In another effort, to create a more thermally stable Red emitting luciferase, four additional mutations were introduced to the mutated version (unpublished data). Although these substitutions did not change the 64.5 KDa molecular weight of the enzyme, it has resulted in a shift in the isoelectric pH of the protein from 7 to 8.2 [18,19,11]. The difference in the net charge of the newly mutated luciferase is +3 as compared to the -1 in the mutated version [18] and -4 in the native luciferase.

DT-diaphorase (DTD) is a homodimer flavoenzyme involved in a 2-electron reduction of quinone to hydroquinone. This reaction inhibits the single electron reduction of quinones, leading to oxidative cycling of injurious radical species [20-22]. This reductase can be utilized as an important enzyme to detoxify the dietary and environmental quinones and cytotoxic quinones, in particular. Exposure of cells with a variety of potentially carcinogenic and mutagenic compounds will induce the increased activity of DT-diaphorase, resulting in greater sensitivity to drug treatment [23]. DT-diaphorase with a molecular weight of 30KDa has an isoelectric pH of about 8.9 and a net charge of +4 [11].

MATERIAL AND METHODS

Expression and Purification of MiRGD, GNH, and HNH: The pET28a plasmids containing the desired gene were transformed into Escherichia coli BL21(DE3) pLysS (Novagen), cultured on agar plates with 2x YT medium (Sigma-Aldrich) containing 100 µg ml^{-1} kanamycin antibiotics as a selection marker, and then incubated overnight at 37 °C. To make the pre-culture, a single colony of each plate was suspended in 10 ml of 2x YT liquid culture containing Kanamycin antibiotics [100 µg ml^{-1}] and then incubated at 37 °C for 12 h while shaking at 220 rpm. One liter of 2x YT liquid medium containing Kanamycin antibiotic [100 µg ml^{-1}] was inoculated with 10 ml of the fresh preculture medium, incubated at 37 °C for about 3 h, and shake at 220 rpm. Upon reaching OD_{600} 0.6-0.8, the expression was induced by 1 ml of 0.8 mM IPTG and incubated at 37 °C while shaking at 220 rpm. Six and seven hours after IPTG induction, the bacteria were harvested using centrifugation (5500 rpm for 15 min), respectively, in the case of the MiRGD and HNH. The cell pellets were re-suspended in 750 µl of lysis buffer containing 50 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole, and 8 M urea (pH = 12) per 50 ml of bacterial culture medium and lysed by a probe sonicator (10 s of pulses followed by a 20 s rest for up to 30 times) while the sample was immersed in an ice bath. The lysates were centrifuged at 15000 rpm for 30 min. The resulting supernatants were centrifuged again for 15 min. It is recommended that after centrifugation, the cell lysate be passed through a 0.45 µm filter. Then, the supernatants were loaded separately into the chromatography columns (Bio-Rad) already containing Ni-NTA agarose (Qiagen) and pre-equilibrated with the lysis buffer and incubated for 1 h at room temperature. In the following, the columns were washed six times with different wash buffers (WB1-WB6, 4 ml each). The initial washing steps were common between MiRGD and HNH and included 50 mM Tris-HCl, 1 M NaCl, 15% glycerol, 5 mM imidazole, and urea in 6 (WB1), 4 (WB2), and 2M (WB3) gradients (pH = 8). The other three optimized wash buffers for MiRGD and GNH included 50 mM Tris-HCl, 1 M NaCl, and imidazole in 10 (WB4), 15 (WB5), and 20 mM (WB6) gradients (pH = 7.8). In the case of the HNH, the washing step was followed by using different buffers containing 50 mM Tris-HCl, 1 M NaCl, 15% glycerol, and imidazole in 5 (WB4), 7 (WB5), and 10 mM (WB6) gradients (pH 7.8). It should be mentioned that none of the WB4, WB5, and WB6 buffers for both of the peptides contained urea. The peptides were then eluted ten times by 10 ml of the elution buffer containing 50 mM Tris-HCl, 300 mM NaCl, and 250 mM imidazole; (pH = 7.8). Peptides undergo refolding at a concentration of less than 4 M of urea. Hence, in such conditions, the buffers and eluents should be kept on ice and preserved at -80 °C for long time storage.

The purity and relative concentration of the collected eluents were assessed by 17.5% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining and were quantized by NanoDrop (Thermo Fisher Scientific, USA).

Expression and purification of the new mutated version of firefly luciferase and DT-diaphorase:

The pET28a plasmids containing genes that encode for both mutated type of luciferase and DT-diaphorase were cloned in the *Escherichia coli* BL21 and then were cultured on 2x YT plates containing kanamycin [100 μg ml^{-1}] by overnight incubation at 37 °C. A single colony was picked and suspended in 10 ml of 2x YT liquid culture containing Kanamycin antibiotic [100 μg ml^{-1}]. The media was incubated at 37 °C for 12 h while shaking at 180 rpm. One liter of Terrific Broth (TB) liquid culture medium containing Kanamycin antibiotic [100 μg ml^{-1}] was inoculated with 10 ml of fresh preculture and then incubated at 37 °C for about 4 h while shaking at 220 rpm. Immediately after reaching OD_{600} 1-1.2, the expression of recombinant protein was induced by 1 ml of 0.1 mM IPTG, and the incubation was continued with shaking at 220 rpm at 18 °C for 18 h. Bacteria were harvested by centrifugation at 5500 rpm for 15 min and resuspended in cold lysis buffer, including 50 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole, 10% glycerol, and 1mM PMSF (pH = 7.8). Next, the cells were sonicated by a probe sonicator in 10 s burst followed by 20 s rest for 20 times while the sample was immersed in an ice bath. Next, the lysate was centrifuged at 15000 rpm for 30 min at 4 °C. The supernatants were re-centrifuged for 15 minutes at 4 °C. Then, the supernatant was loaded into a chromatography column containing Ni-NTA agarose (Qiagen) pre-equilibrated with the lysis buffer, followed by stirring with a rotary shaker at 4 °C for 10 min. It is of note that all the purification processes should be done at low temperatures. The impurities were predominantly removed by 7 ml of the lysis buffer (pH = 10). After removal of the lysis buffer, washing steps were done by using three different wash buffers (6 ml each), including 50 mM Tris-HCl, 1M NaCl, and imidazole in 10 (WB1), 20 (WB2), and 30 mM (WB3) gradients; (pH = 10). Eventually, the proteins were eluted ten times by 10 ml of an elution buffer, including 50 mM Tris-HCl, 300 mM NaCl and 250 mM imidazole (pH 7.8).

In the case of the luciferase protein, bioluminescence intensity was recorded by a luminometer (Bertold detection system, Germany). The assay was done by mixing 100 μl of a stock containing 2.5 mM Tris-HCl buffer (pH 7.8), 5 mM $MgSo_4$, 2 mM ATP, and 20mM luciferin with 100 μl of a solution containing the purified mutant luciferase.

The purity and relative concentration of the collected eluents were assessed using 15% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining and were quantized by NanoDrop (Thermo Fisher Scientific, USA).

The comparison of the main factors in the initial and optimal conditions of the experiment is briefly mentioned in Table 2.

RESULTS

Optimizing of Expression and purification of MiRGD, GNH, and HNH. The primary attempts for the expression and purification of peptide nanocarriers according to the initial protocol resulted in achieving small concentrations of MiRGD, GNH, and HNH peptides at 0.21, 0.4, and 0.16 mg ml^{-1}, respectively. The obtained bands of MiRGD, GNH, and HNH peptides on the SDS-PAGE (Fig. 2a), is also indicative of their low concentration; consistent with the low amount of peptides estimated by the NanoDrop; and the presence of a large amount of impurities in collected eluents, as well. Measurements of the concentrations of peptides obtained by the optimal purification protocol show, respectively, 2.9 mg ml^{-1}, 3.4 mg ml^{-1}, and 2.6 mg ml^{-1} for MiRGD, GNH, and HNH peptides obtained from 1lit media culture. The SDS-PAGE image in Figs. 2b, 2c, and 2d also verify a significant increase in the amount of the peptides. The purity of the bands related to the desired peptides was also more than 95%.

Optimizing of Expression and Purification of the Mutant Red-emitting Luciferase

The first attempt for the expression and purification of the protein was performed according to the initial protocol for the native firefly luciferase. However, no protein was observed in the collected eluents. As shown in Fig. 3a, the samples pertaining to the supernatant samples collected after centrifugation of the cell lysates contain relatively high

Table 2. Overview of the Main Factors in the Initial and Optimal Experiments' Conditions

Experiments conditions	Initial experiment for MiRGD,GNH and HNH peptides	Optimized experiment for MiRGD, GNH and HNH peptides	Initial experiment for DTD and mutant luciferase proteins	Optimized experiment for DTD and Mutant luciferase proteins
Desired OD_{600} to Induce	0.8-1	0.6-0.8	0.8-1	1-1.2
Concentration of inducer (IPTG)	0.6	0.8	0.6	0.1
Incubation temperature after induction	22 °C	37 °C	22 °C	18 °C
Incubation time after induction (h)	14	6-7	14	18
Salt concentration in wash buffer	300 mM	1 M	300 mM	1 M
Imidazole concentration in wash buffer	20, 30 and 40 mM	10, 15 and 20 mM/ 5, 7 and 10 mM	20, 30 and 40 mM	10, 20 and 30 mM
The pH of wash buffer	7.8	7.8	7.8	10
The volume of used washing buffer	35 ml	24 ml	35 ml	25 ml

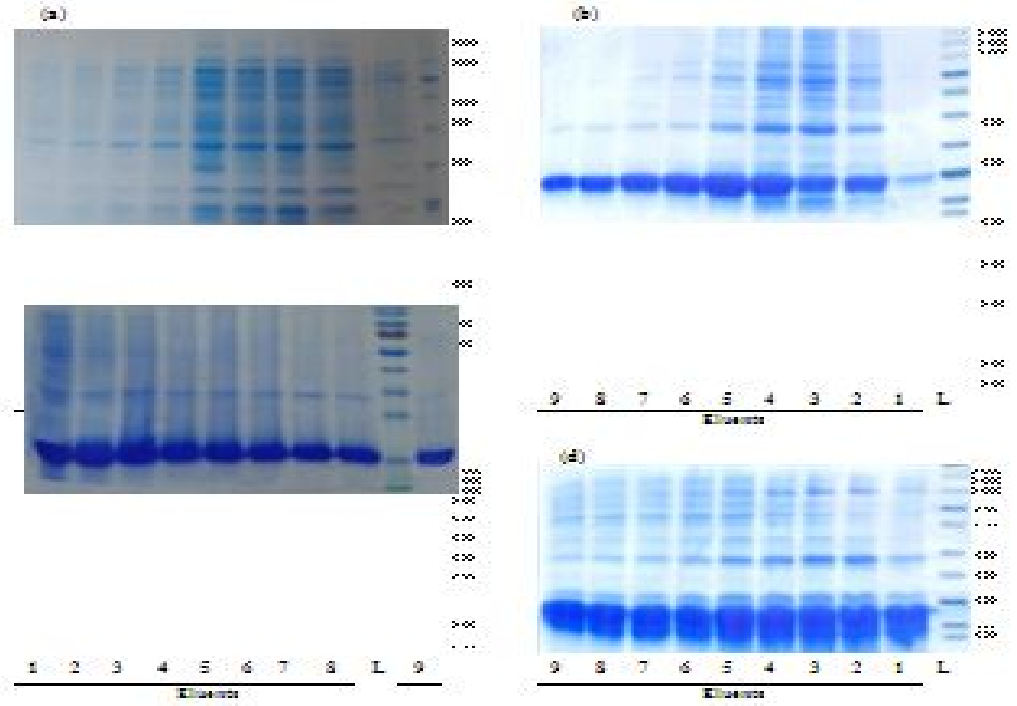

Fig. 2. Analysis of expressed MiRGD, GNH, and HNH in the Expression and Purification initial and optimal protocols; The purity and relative concentration of the purified carriers were analyzed by 17.5% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. The molecular weight of MiRGD, GNH, and HNH are 9.7KDa, 8.8KDa, and 10.8KDa. Note that due to cationic nature of these peptides, they place in a higher molecular weight position. a) MiRGD peptide eluents pattern obtained from the initial experiment, (Here, the observed concentration of eluents is 2.17 mg/ml obtained from 750 ml media culture); b) MiRGD peptide eluents pattern obtained from optimal experiment c) GNH peptide eluents pattern obtained from the optimal experiment, (Here, the observed concentration of eluents is 1.7 mg/ml obtained from 500 ml media culture); d) HNH peptide eluents pattern obtained from optimal experiment. "L" in the images stands for Protein Ladder, and the numbers are the sequences of collected Eluents.

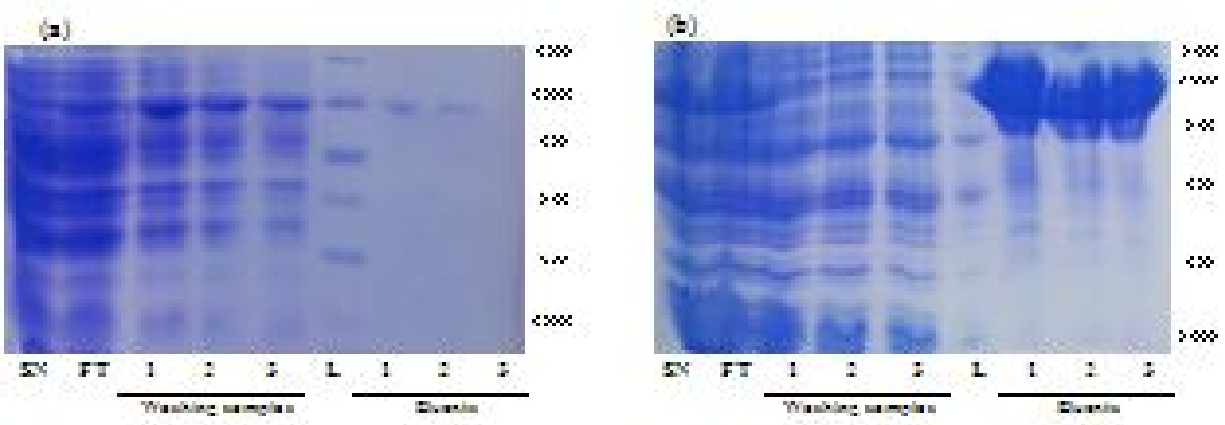

Fig. 3. Analysis of expressed Mutant Red Emitted Luciferase in the Expression and Purification initial and optimal protocols; the purity and relative concentration of the purified carriers were analyzed by 15% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. The molecular weight of the Luciferase is 64.5 KDa) Mutant Luciferase eluents pattern obtained from initial experiment; b) Mutant Luciferase eluents pattern obtained from the optimal experiment, (Here, the observed concentration of eluents is 1.7 mg/ml obtained from 500 ml media culture). "SN", "FT" and "L" in the images stand for supernatant, Flow-through, and Protein ladder words, respectively. The numbers are the sequences of collected washing samples and Eluents.

amounts of protein. But, along with the progress of the wash process and increase in the imidazole concentration, the amount of the obtained protein decrease remarkably in such a way that no protein will be left in the eluents. This is suggestive of the loss of luciferase protein during the washing process. It is of note that the excreted proteins during the wash step even did not show full activity. Despite non-negligible impurities, the luminescence counts of the first three wash collections were measured 9, 11, and 10 × 10^6 RLU/sec, respectively.

After optimization of the expression and purification, not only the amount of the protein in the eluents was sharply increased than the initial protocol, the luminescence counts pertaining to the ten obtained eluents were measured more than the reader's high limit of 25 × 10^6 RLU/sec. The concentration of the obtained luciferase protein in the first eluent was estimated at 4.1 mg ml^{-1} obtained from 1lit media culture by NanoDrop. Moreover, the image of SDS - PAGE in Fig. 3b verifies the presence of condensed and pure bands of the desired protein for the eluents. It is also notable that no protein was wasted during the washing process and the increase in imidazole in the washing process had no adverse effect on the loss of protein from the column.

Optimizing of Expression and Purification of DT-Diaphorase

Same as the initial protocol for the luciferase purification, the expressed diaphorase was wasted significantly during the purification step, as shown by the SDS-PAGE image in Fig. 4a. However, after modification of both expression and purification processes, the obtained protein collected in the elution step showed a remarkable increase in terms of concentration and purity. The concentration of the obtained Diaphorase protein in the first eluent fraction was estimated at 2.6 mg ml^{-1} obtained from 1lit media culture.

DISCUSSION

MiRGD, GNH, HNH Purification

Overexpression of recombinant proteins in bacterial hosts often leads to their accumulation as inclusion bodies [24]. The accumulation accelerates at higher temperatures due to the intense temperature dependence of related hydrophobic interactions. Furthermore, expression under strong promoter systems and utilization of a higher amount of the inducer in the mid-log phase of growth (*i.e.*, OD_{600} 0.6-0.8) often leads to overexpression of the recombinant

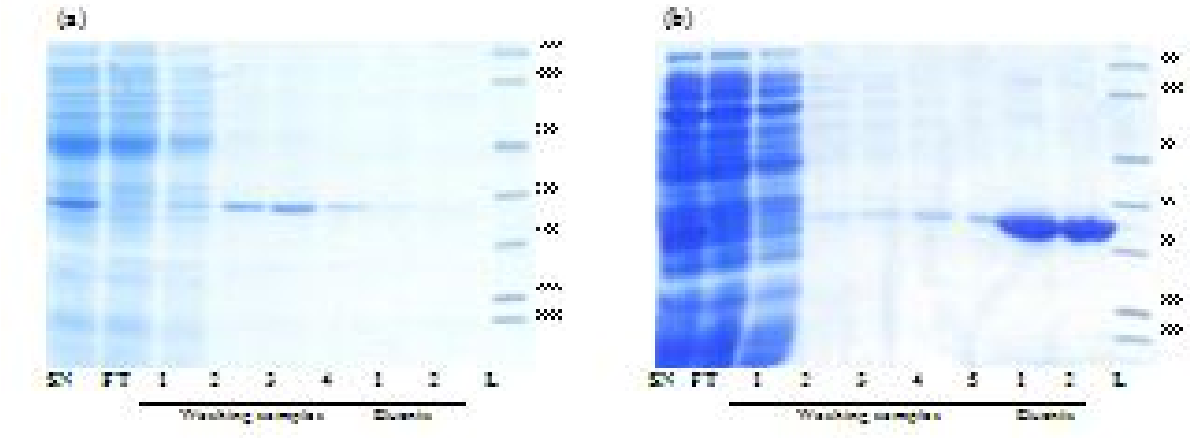

Fig. 4. Analysis of expressed DT-Diaphorase in the Expression and Purification initial and optimal protocols; The purity and relative concentration of the purified carriers were analyzed by 15% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. The molecular weight of the Luciferase is 32 KDa) DT-Diaphorase eluents pattern obtained from initial experiment; b) DT-Diaphorase eluents pattern obtained from the optimal experiment, (Here, the observed concentration of eluents is 1.3 mg ml^{-1} obtained from 500 ml media culture). "SN", "FT" and "L" in the images stand for Supernatant, Flow-through, and Protein ladder words, respectively. The numbers are the sequences of collected washing samples and Eluents.

protein [25]. Such conditions may adversely affect the protein quality control system in bacteria and result in the aggregation of the folded and misfolded proteins. Decreased bacterial cytosolic space, lack of post-translational modification system along with the reduced activity and expression of some *E. coli* chaperones at temperatures above 30 °C are among factors that may contribute to the formation of inclusion bodies [24]. As shown in Fig. 2a, the bands pertaining to the eluted MiRGD peptide under the initial experiment condition were so weak and associated with impurities. One of the possible reasons for observation of the high amounts of the impurities can be the low expression level of the peptide, which causes a part of the existing capacity to bind to the Ni-NTA to be occupied by non-specific binding of non-histidine-tagged proteins. To overcome this issue, some factors related to the expression elevation, such as IPTG induction in proportional OD_{600} and its concentration, incubation time, and temperature, were adjusted according to the physicochemical properties of the MiRGD GNH and HNH peptides aiming to maximize the production of the inclusion bodies. It means, in case if the higher concentrations of the protein are toxic or tend to be aggregated inside cells, higher ODs, lower IPTG concentrations, and shorter-expression times can be effective.

Moreover, solubilization and refolding of inclusion bodies can be adjusted according to their functional and structural properties, which mainly depends on the ionic strength, temperature, time, presence, and concentration of reducing agent and ratio of denaturant to protein which mainly depends on the ionic strength, temperature, time, presence and concentration of reducing agent and ratio of denaturant to protein [24,26]. The denaturing agent used in this study to solubilize inclusion bodies was Urea. As mentioned in the results section, for MiRGD, GNH and HNH, the initial purification protocol was associated with protein loss along with the washing steps. To prevent this, according to the pattern of supernatant containing the expressed peptides as well as the pattern of extruded peptides in the washing step on SDS-PAGE, we made changes in the protocol, especially by modification of the values and concentrations of the wash buffer. Most proteins, especially those with a pI above 9 or less than 5 are more soluble at high salt concentrations. Hence, it is recommended to use NaCl at a final concentration of at least 500 mM for the lysis and wash buffers to reduce the amount

of impurities by weakening of the unwanted interactions between the non-his-tagged proteins and Ni-NTA resins. Also, adding 10-15% of glycerol to Ni-NTA buffer can contribute to protein stability and solubility [27]. The binding strength of the histidine repeat with the column resins relies on the physicochemical properties of the tagged protein. In some cases, the target proteins bind weakly to the IMAC column. So, even a subtle increase in the imidazole concentration can result in remarkable extrusion of the protein from the column during washing steps due to the competitive effects. Therefore, in the optimized protocol, the imidazole concentration in the wash buffers was decreased significantly to prevent wasting protein [28]. According to this fact, most impurities were removed in the early stages of washing using a set of 3 different buffers containing a decreasing gradient of urea and similar concentration of the imidazole, which were common for all three peptides. Consequently, to increase the purity of the desired peptide and to reduce nonspecific binding of the non-histidine-tagged proteins with the column, three other buffers lacking urea and with an increasing imidazole gradient were used. It of note that due to the greater sensitivity of HNH to the imidazole concentration than the MiRGD and GNH, the imidazole concentrations in the HNH-specific wash buffers W4, W5, and W6 were relatively lower. Comparing Figs. 2a and 2b reveals that the MiRGD peptide bands obtained under the optimal expression and purification conditions are obviously thicker and purer than the corresponding bands for the initial experiment. The qualitative assessment of the bands related to the GNH and HNH shown in Fig. 2c and 2d support the positive impacts of the expression and purification optimization on target protein yield and level of unwanted contaminations. Moreover, quantitative assessment of the purified proteins verifies the desirable consequences of the modification to the protocol on the efficacy of both protein expression and purification.

Mutant of Red-emitting Firefly Luciferase and Human DT-Diaphorase Purification

To enhance the level of the protein expression and to reduce the amount of the protein loss, through the formation of the inclusion bodies, due to the fact that here the desired proteins are purified from soluble proteins, some modifications were done in the primary protocol. An approach to reduce the *in vivo* aggregation of recombinant proteins consists of cultivation at lower temperatures. This strategy was effective in improving the solubility of a number of proteins, including bacterial luciferase. A direct consequence of the temperature reduction is the partial elimination of heat shock proteases that their expression typically can be induced under overexpression conditions [25]. In a study, the specific enzymatic activity in the culture medium incubated at 4°C was 180-fold higher than in the cultures incubated at 37 °C showed, and it was suggested that lower temperatures are beneficial for proper folding of the recombinant proteins in the *E. coli* cytoplasm [25]. Moreover, a lower concentration of the inducer molecules can contribute to a higher yield of soluble protein. This results in the reduction of cellular protein concentration, which favors folding. However, these conditions can attenuate the bacterial growth rate and lead to a decrease in the amount of biomass. The other factor is the induction of protein expression in the late log phase of culture not only can cause a remarkable increase in the amount of the expressed proteins but also enhances the yield of soluble protein. Most protocols recommend using inducers for the expression of recombinant proteins. It is while they lack enough data about the expression in late log phase cultures and late log phase culture has been described as a mean for the induction of higher cell densities which can compensate the low level of protein expression. Importantly, the data presented here demonstrate that the increased yield of soluble protein from late log phase culture induction was not merely due to the greater number of cells, and late log phase cells sequestered relatively less of the total expressed protein in the form of inclusion bodies. Likely, the late log phase cells have undergone a metabolic and growth shift that may attenuate the response to the foreign and potentially toxic proteins. Alternatively, reduced growth rates may be associated with a reduced rate of protein synthesis; hence, less aggregation within inclusion bodies occurred [29]. Induction was also performed at higher concentrations of IPTG in OD_{600} 1-1.2, and since TB media culture is rich in nutrients and buffered and can sustain higher cell densities, the incubation time was hence longer. It is worth mentioning minimizing the duration of the purification process and working in low

temperatures helps the activity of the desired enzymes to be maintained considerably. We also showed that adjustment of the pH and concentrations of the wash buffer's ingredients, such as imidazole, significantly reduce the protein extrusion through the washing process. This can be explained by the fact that in the pH conditions close to the isoelectric pH of proteins, the binding strength of the proteins to the column resins will be attenuated. Hence, at the beginning of the washing process, the lysis buffer with a pH of 10 was used as the first wash buffer to remove most of the impurities from the column and then followed by three more buffers with imidazole gradients. The pH of a solution determines the physical properties of the proteins, such as charge, stability, binding to other proteins based on the pKa values of their amino acids. One way to keep them stable and active is to use appropriate buffer systems. Here, the lysate was actually buffered by using the Tris buffer; otherwise, it can often be more acidic than be realized. Buffers used for Ni-NTA need to be at pH above 7.4. Otherwise, the His-tags due to protonation will not efficiently bind to the column beads. Also, protein would be stable and charged at a different pH of its isoelectric pH. As a result, pH should be adjusted at least 1 and preferably 1.5 to 2 points away from the protein's isoelectric pH. The most unexpected finding of this study pertains to a significant increase in the strength of the protein-resin attachment by changing the pH of the wash buffer compared to the primary conditions, which resulted in the prevention of protein extrusion from the column during the wash steps (shown in Figs. 3b and 4b). It is while in the primary protocol there almost no protein was observed in the wash collections (as shown in Figs. 3a and 4a). Here, we can conclude with certainty that the optimization of the protocol was effective according to the physicochemical properties of the mentioned proteins.

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