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The Effect of Signal Sequence and His-tags on the Expression and Anti-Thrombin Activity of Recombinant Hirudin

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ABSTRACT

Background: Adding signal sequence and His- tags to the expression vectors of recombinant proteins usually increased the production yield by promoting protein secretion to the periplasmic space and by facilitating the purification processes.
In this study, three different expression constructs including vectors with or without signal sequence and His-tags were designed to compare the effect of these elements on Hirudin expression and function. Hirudin is a natural anticoagulant protein produced in the salivary glands of leeches.
Methods: Hirudin variants including cytoplasmic Hirudinn without His-tag (cr-Hirudin), cytoplasmic Hirudin with His-tag (crhis-Hirudin), and periplasmic Hirudin with His-tag (prhis-Hirudin) constructs were expressed in *E.coli* BL 21(DE3). Ion exchange chromatography and ion metal affinity chromatography were applied for protein purification. The prothrombin time (PT) and activated partial thromboplastin time (aPTT) assays were performed to assess the anti-thrombin bioactivity of variants.
Results: The expression rate of prhis-Hirudin was approximately 1.6 fold higher than cytoplasmic variants (cr-Hirudin and crhis-Hirudin). The prothrombin time and activated partial thromboplastin time of crhis-Hirudin and prhis-Hirudin were similar and approximately 20 and 55 % lower than those of cr-Hirudin.
Conclusion: Applying signal peptide or His-tag increased the production yield of recombinant hirudin but had negative contributions to its activity.

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Introduction

Hirudin is a naturally produced protein in the salivary glands of blood-sucking leeches with a blood coagulant property (1). Hirudin directly effects thrombin to inhibit blood clot production (2-4). This protein is the strongest natural thrombin inhibitor which not only inhibits blood clotting but also resolves the produced clot (5, 6). Lepirudin, a safe and effective recombinant anticoagulant derivative of Hirudin, has been approved for the treatment of Heparin-induced thrombocytopenia (HIT) and anticoagulation during procedures in patients with heparin associated antiplatelet antibodies (HAAb) (7, 8).

Hirudin is a well-known strong blood coagulator so that it can inhibit coagulation of soaked blood with leech for more than 6 hours (4, 9). Researches at Kentaki University compared the function of Hirudin and heparin and showed that Hirudin does not need a cofactor for activation. Also, Hirudin affects directly on thrombin inhibition. Additionally, Hirudin has not poisoning effects and Hirudin create local anesthesia (7, 10).

There are different pharmaceutical types of Hirudin in the market which some of them produced by prokaryote recombinant technology. Expression and purification steps are some of the important bottlenecks of recombinant protein production technology regarding their complexity, yields, and costs. Using signal sequence and protein tails is one of the practical solutions for simplifying expression and purification processes (11, 12).

His-tag tail, introduced first time by Hochuli in 1987 (13), makes the advantages of specific adsorption affinity of His-tags to the immobilized certain transition metal ions (Nickel, Cobalt, or Copper) on chromatography resins (11, 12). Primary, it was supposed that the His-tags did not affect the structure or function of the target protein because of its low molecular weight, but in the following, some controversial cases were reported (14, 15).

The signal sequences mainly consist of 15 up to 40 amino acids that are added to the N-terminal of the protein sequences to export proteins from the cytosol to the outer membrane of bacterial hosts. Although, the role of the signal sequence in the navigation of protein to the outer membrane is well known (14, 15), there are few studies on its impact on the function of the proteins (16-18). Normally, signal peptides are removed by special peptidases situated in the host cell membrane (16). However, there is some doubt about correctly slicing of pre-protein by the peptidase in membrane to remove the signal peptide and the effects of inaccurate cutting of signal peptides on protein properties should be clear (18, 19).

In this study, the protein expression and anti-thrombin activity of 3 variants of recombinant Hirudin expressed with a signal sequence and His-tags, with His-tags but without signal sequence, and without signal sequence /His-tags were evaluated to assess the effects of these biotechnological tools on Hirudin function and potency.

Materials and Methods

Genes designing and synthesis

The Hirudin sequence was obtained from Uniprot data bank and used for designing three gene constructs. The first construct (named rHirC1) was related to cytoplasmic recombinant Hirudin (without any signal peptide or His- tag). The second one (rHirC2) contained signal peptide and His- tag and the last (rHirC3) contained only His- tag. The constructs were synthesized in PET22b plasmid (Biomatik Company, Canada), then were transferred to *E.coli* Top 10 to amplify the plasmid and then transferred to *E.coli* BL21 (DE3) strain as expression strain (20). The Gradient PCR was applied for confirming the insertion of plasmid PET-22b (+) constructs to *E.coli* BL21 (DE3). The different types of Hirudin protein expressed by rHirC1, rHirC2, and rHirC3 gene constructs were named as cr-Hirudin (cytoplasmic recombinant Hirudin), prhis-Hirudin (periplasmic

recombinant Hirudin containing His- tag), and crhis-Hirudin (cytoplasmic recombinant Hirudin containing His-tag), respectively.

Protein expression and Purification

The Luria-Bertani (LB) broth media was used for culturing the screened E.coli BL21 (DE3) strains containing the recombinant plasmids at 37˚C. The cells were induced by IPTG (Isopropyl β-d-1-thiogalactopyranoside. The membrane of cells producing cr-Hirudin and crhis-Hirudin were disrupted by the ultrasonic method (MISONIX, USA). The nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography (Qiagene, Sweden) combined with the triton X-114 method for simultaneous endotoxin removal was used for purification of crhis-Hirudin and prhis-Hirudin through their His- tag (21, 22). The Ion-exchange chromatography method was performed for purification of not containing His-tag Hirudin (cr-Hirudin). The purity of the purified fractions was evaluated by SDS-PAGE 18%. The GelQuant.Net software was used for estimating the expression rate.

Bioactivity assay

Hirudin activity was determined and routine by PT and aPTT assays. For obtaining plasma, the blood sample was anticoagulated by adding to 0.13 M tri-sodium citrate. The mixture was centrifuged at 4 ° C (1500 ×g, 15 min) and then frozen at -20° C. For aPTT assay, 100 µl of plasma –Hirudin mixture was added to 100 µl of Dade 1 Actin 1 FSL activated PTT reagent and after 5 minutes incubation at 37 ° C, 100 µl of CaCl2 was added to the mixture and the clotting time was recorded by a coagulation timer. For PT assay, the mixture of plasma- Hirudin was added to 100 µl of PT reagent and the clotting time was recorded (23, 24).

Result

Gradient PCR

The universal primers of plasmid PET-22b (+) were used for investigating the vector transformation to *E.coli* BL21 (DE3). In this assay PET22b (+) containing rHirC2 gene and the enzymatic cutting site must have almost 500bp and those of RHirC1 and RHirC3 were slightly less (Figure 1).

Protein expression and purification

The screened strains containing recombinant plasmids were cultured, induced, and harvested. The prhis-Hirudin and crhis-Hirudin (the Hirudin variants containing His-tag) were purified by Ni-NTA affinity chromatography The Hirudin without His-tag (cr-Hirudin) was purified by ion exchange chromatography. The expression of each variant was investigated by SDS-PAGE test. The results are shown in Figures 2- 4. Semi-quantification of expression rates were calculated by using GelQuant. Net software. The expression rates about 19, 21, and 32% were estimated for cr-Hirudin, crhis-Hirudin, and prhis-Hirudin variants, respecticely.

Hirudin bioactivity determination

The anti-thrombin activity of Hirudin variants was determined by PT (prothrombin time) and aPTT (activated partial thromboplastin time) methods. The PT test evaluates the external pathway of blood coagulation and normally it lasts 12 up to 14 seconds. However, the aPTT test evaluates the internal pathway of blood coagulation and usually, it lasts between 35 to 43 seconds.

The coagulation of human blood mixed with each of Hirudin variants was evaluated by PT and aPTT tests and the results are shown in figure 5 . The PT and PTT results of prhis-Hirudin and crhis-Hirudin were almost the same but those of cr-Hirudin were stronger.

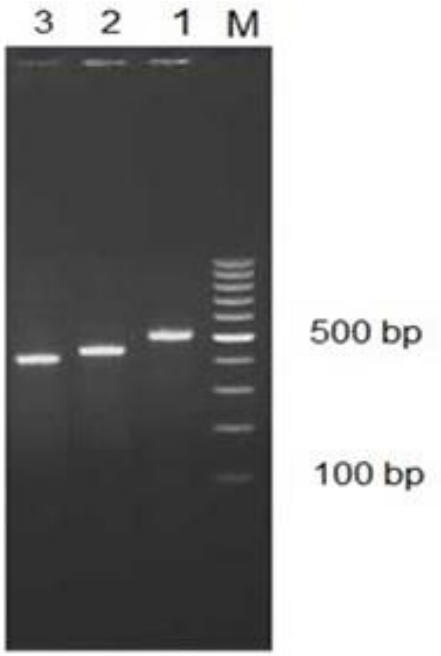

Figure 1. Gradient PCR: M: marker, 1: rHirC2 (construct containing genes of Hirudin + His- tag + signal peptide phoA), 2: rHirC3 (construct containing genes of Hirudin + His-tag), and 3: rHirC1 (construct containing only the gene of Hirudin).

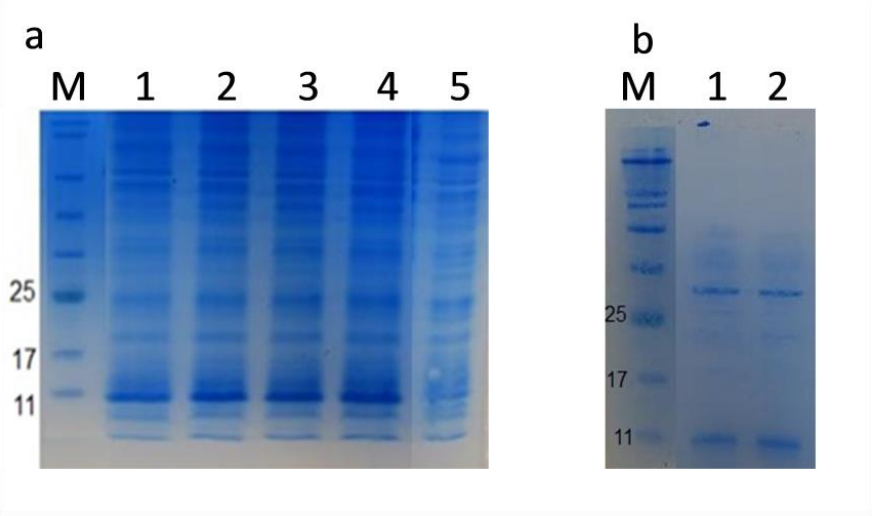

Figure 2. SDS-PAGE results of cr-Hirudin. a) Expression step. M: protein ladder (kDa), lines1 - 4: after induction, Line 5: before induction. b): Ion exchange chromatography step. M: protein ladder (kDa), lines 1 and 2: elution fractions.

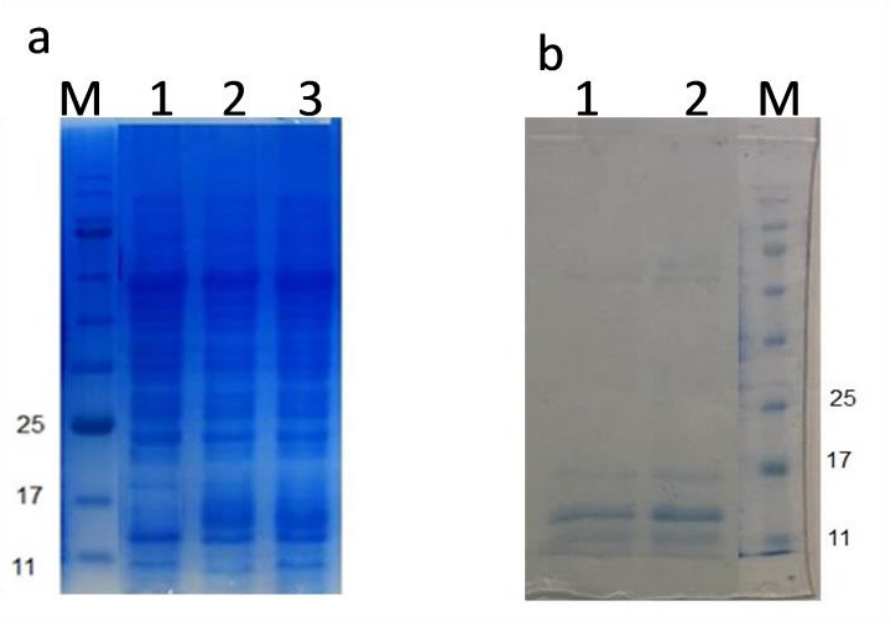

Figure 3. SDS-PAGE results of crhis-Hirudin. a) Expression step. M: protein ladder (kDa), line 1: before induction, Lines 2 and 3: after induction. b): Ni-NTA Affinity chromatography step. Lines 1 and 2: elution Fractions, M: protein ladder (kDa).

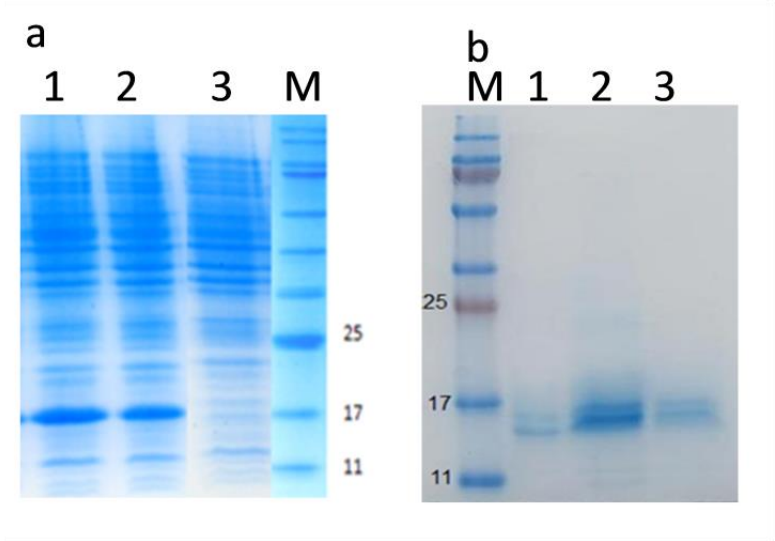

Figure 4. SDS-PAGE results of prhis-Hirudin. a) Expression step. Lines1 and 2: after induction, Line 3: before induction, M: protein ladder (kDa). b): Ni-NTA Affinity chromatography step. M: protein ladder (kDa), Lines 1 to 3: elution Fractions.

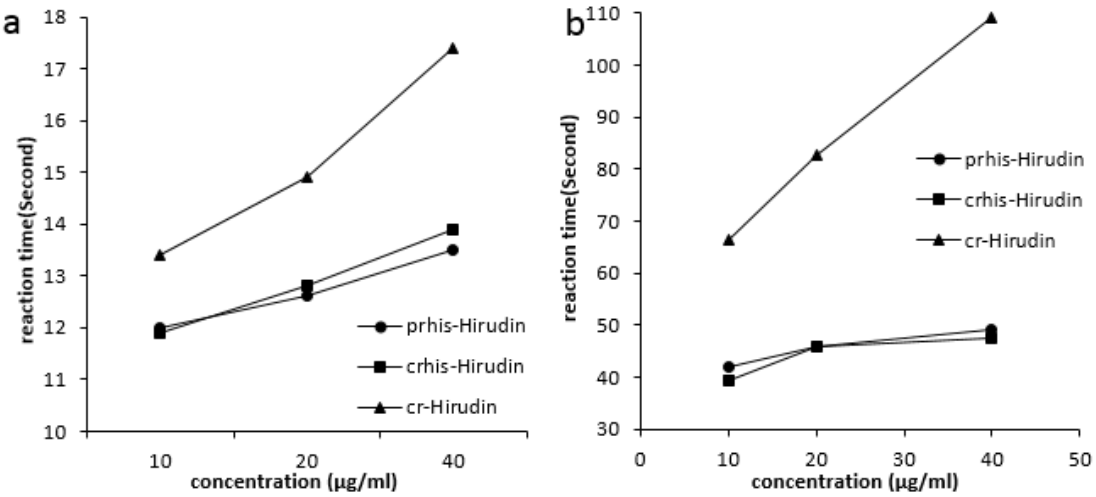

Figure 5: The activity of different expressed types of Hirudin by PT (a) and PTT (b) assays at different protein concentrations.

Discussion

Hirudin naturally is extracted from the Para pharyngeal gland of the medicinal leech. Several derivatives and recombinant types of Hirudin have been presented to the market (25). Hirudin makes a strong complex with thrombin to inhibit the transformation of fibrinogen to fibrin as well as thrombin-induced platelet aggregation (26).

Recombinant forms of Hirudin mostly are produced in *E.coli* because of the fast-growing and easy culturing. Signal peptide and His-tag tail are some of the most useful tools of recombinant technology to facilitate the expression and purification steps of recombinant proteins. Although signal peptide normally is removed by specialized membrane-associated signal peptidase (16) its impacts on the biological activity of synthesized precursor proteins are not clear. Significant differences between folding rate (27-29) or stability (30, 31) of precursors and mature proteins have been reported.

In the case of Hirudin, as it can be observed in figures 5-a and 5-b, the bioactivity of the secreted Hirudin was less than the non-signal peptide ones. The PT of crhis-Hirudin and prhis-Hirudin were approximately similar (13.9 and 13.5 seconds at 40 µg/ml) and about 20% less than the prothrombin time of cr-Hirudin. The activated partial thromboplastin times of crhis-Hirudin and prhis-Hirudin were the same and about 55% less than that of cr-Hirudin of In opposite of our result, the experiment performed by Goder et al on bovine pre-adrenodoxin (padx) and bovine adrenodoxin (adx) showed equal activity but reducing some physic-chemical properties include heat capacity and transition temperature in padx (19). It was proposed that the N-terminal positive charge of signal peptide made a hydrophobic region on the protein which may interact with other hydrophobic regions of the protein that affected its bioactivity (31).

The His-tag tail has been used for purification by immobilized metal affinity chromatography (IMAC) (32). In some proteins, the tag help folding and stability (33) and believed that His-tag has not interfered with protein function (33, 34) but nowadays there are some convert assumptions (35, 36). In a previous study, Dongxin et al in 2016 established two His-tag tails on N-terminal

and C-terminal of zinc finger protein ZNF191 (243-368) and compared their properties. Their finding showed that each His-tag gave a unique and different structure and folding to the zinc finger protein (37). The same experiment by William T. Booth and coworkers showed that His-tag tails affected differently the thermal stability of proteins and for some of them, positive contribution and for others, negative contribiutions were observed. Comparing the protein expression rate of different variants of hirudin (figures 2 to 4) revealed that the expression rate of native cytoplasmic variants (cr-Hirudin and crhis-Hirudin) were similar (around 20%). However, the periplasmic variant expressed by signal peptide showed a higher expression rate (approximately 32%).

Conclusion

In this study, 3 variants of Hirudin expressed with different gene constructs including, constructs with a signal sequence and His-tag, Hirudin with His-tag, and Hirudin without signal sequence / His-tag were designed and produced. According to the results, Hirudin without signal sequence and His-tag (cr-Hirudin) has more activity than two others. However, the expression rate of periplasmic Hirudin was more than the cytoplasmic ones.

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Ethics approval and consent to participate

Not needed.

Conflict of interest

The authors declare no competing financial interest.

References

1. Lemke S, Müller C, Lipke E, et al. May salivary gland secretory proteins from hematophagous leeches (*Hirudo verbana*) reach pharmacologically relevant concentrations in the vertebrate host? *PloS one* 2013; **8**(9):e73809.
2. Tertrin C, De La Llosa P, Jutisz M. Effect of chemical modification of hirudin on its inhibition of the enzymatic activity of thrombin. *Bull Soc Chim Biol* 1967; **49**(12):1837.
3. Markwardt F. Hirudin; an inhibitor of blood coagulation from medical leeches. *Blut* 1958; **4**(3):160.
4. Landmann H. Hirudin and other thrombin inhibitors from blood-sucking animals. *Folia Haematol (Leipz)* 1972; **98**(4):437.
5. Hallak O, Shams SA, Broce M, et al. Similar success rates with bivalirudin and unfractionated heparin in bare-metal stent implantation. *Cardiovasc Intervent Radiol* 2007; **30**(5):906-11.
6. Herman K, Vaszily M, Szentgyörgyi L, et al. Anticoagulation with hirudin for repeat cardiac surgery in a patient with heparin induced thrombocytopaenia. *Magy Seb* 2008; **61**:67-70.
7. Mureebe L. Direct thrombin inhibitors: alternatives to heparin. Vascular 2007;15(6):372-5.
8. Corral-Rodríguez MaAn, Macedo-Ribeiro S, Barbosa Pereira PJ, et al. Leech-derived thrombin inhibitors: from structures to mechanisms to clinical applications. *J Med Chem* 2010; **53**(10):3847-61.
9. Markwardt F. Hirudin; an inhibitor of blood coagulation from medical leeches. *Blut* 1958; **4**(3):160-1.
10. Piascik P. Medicinal leeches: ancient therapy is a source of biotech drugs. *J Am Pharm Assoc*

1997; **3**:285-6.
11. Khoo KM, Chang CF, Schubert J, et al. Expression and purification of the recombinant His-tagged GST-CD38 fusion protein using the baculovirus/insect cell expression system. *Protein Expr Purif* 2005; **40**(2):396-403.
12. Croci C, Brandstätter JH, Enz R. ZIP3, a new splice variant of the PKC-ζ-interacting protein family, binds to GABAC receptors, PKC-ζ, and Kvβ2. *J Biol Chem* 2003; **278**(8):6128-35.
13. Hochuli E, Döbeli H, Schacher A. New metal chelate adsorbent selective for proteins and peptides containing neighbouring histidine residues. *J Chromatogr A* 1987; **411**:177-84.
14. Mason AB, He Q-Y, Halbrooks PJ, et al. Differential effect of a His tag at the N-and C-termini: functional studies with recombinant human serum transferrin. *Biochemistry* 2002; **41**(30):9448-54.
15. Chant A, Provatopoulou X, Manfield IW, et al. Structural and functional characterisation of the DNA binding domain of the *Aspergillus nidulans* gene regulatory protein *AreA*. *Biochim Biophys Acta -Proteins Proteom* - 2003; **1648**(1-2):84-9.
16. Blobel G, Dobberstein B. Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. *J Cell Biol* 1975; **67**(3):835-51.
17. Milstein C, Brownlee G, Harrison TM, et al. A possible precursor of immunoglobulin light chains. *Nature New Biol* 1972; **239**(91):117.
18. Lee H, Chi S-W, Kang M, et al. Stability and folding of precursor and mature tryptophan-substituted ribose binding protein of *Escherichia coli*. *Arch Biochem Biophys* 1996; **328**(1):78-84.
19. Goder V, Beckert V, Pfeil W, et al. Impact of the Presequence of a Mitochondrium-Targeted Precursor, Preadrenodoxin, on Folding, Catalytic Activity, and Stability of the Proteinin Vitro. *Arch Biochem Biophys* 1998; **359**(1):31-41.
20. Asgari S, Mirzahoseini H, Karimipour M, et al. Rational design of stable and functional hirudin III mutants with lower antigenicity. *Biologicals* 2015; **43**(6):479-91.
21. Reichelt P, Schwarz C, Donzeau M. Single step protocol to purify recombinant proteins with low endotoxin contents. *Protein Expr Purif* 2006; **46**(2):483-8.
22. Asgari S, Ebrahim-Habibi A, Mahdavi M, et al. Therapeutic protein deimmunization by T-cell epitope removal: antigen-specific immune responses in vitro and in vivo. *Acta Pathol Microbiol Immunol Scand* 2017; **125**(6):544-52.
23. Greinacher A, Warkentin TE. The direct thrombin inhibitor Hirudin. *Thromb Haemost* 2008; **100**(05):819-29.
24. Maraganore JM, Chao B, Joseph ML, et al. Anticoagulant activity of synthetic Hirudin peptides. *J Biol Chem* 1989; **264**(15):8692-8.
25. Kobsar A, Koessler J, Kehrer L, et al. The thrombin inhibitors hirudin and Refludan activate the soluble guanylyl cyclase and the cGMP pathway in washed human platelets. *Thromb Haemost* 2012; **107**(03):521-9.
26. Pitt B. Cardiovascular Therapeutics: A Companion to Braunwald's heart disease. *Circulation* 1997; **96**(3):1041-2.
27. Liu G, Topping TB, Cover WH, et al. Retardation of folding as a possible means of suppression of a mutation in the leader sequence of an exported protein. *J Biol Chem* 1988;263(29):14790-3.
28. Diamond DL, Strobel S, Chun SY, et al. Interaction of SecB with intermediates along the folding pathway of maltose-binding protein. *Protein Sci* 1995; **4**(6):1118-23.
29. Raffy S, Sassoon N, Hofnung M, et al. Tertiary structure-dependence of misfolding substitutions in loops of the maltose-binding protein. *Protein Sci* 1998; **7**(10):2136-42.
30. Chun S-Y, Strobel S, Bassford P, et al. Folding of maltose-binding protein. Evidence for the identity of the rate-determining step in vivo and in vitro. *J Biol Chem* 1993; **268**(28):20855-62.
31.

32. Beena K, Udgaonkar JB, Varadarajan R. Effect of signal peptide on the stability and folding kinetics of maltose binding protein. *Biochemistry* 2004; **43**(12):3608-19.
33. Porath J, Carlsson J, Olsson I, et al. Metal chelate affinity chromatography, a new approach to protein fractionation. *Nature* 1975; **258**(5536):598.
34. Gräslund S, Nordlund P, Weigelt J, et al. Protein production and purification. *Nat Methods* 2008; **5**(2):135.
35. Nilsson B, Forsberg G, Moks T, et al. Fusion proteins in biotechnology and structural biology. *Curr Opin Struct Biol* 1992; **2**(4):569-75.
36. Majorek KA, Kuhn ML, Chruszcz M, et al. Double trouble—Buffer selection and H is-tag presence may be responsible for nonreproducibility of biomedical experiments. *Protein Sci* 2014; **23**(10):1359-68.
37. Deller MC, Kong L, Rupp B. Protein stability: a crystallographer's perspective. *Acta Crystallogr Sect F: Struct Biol Commun* 2016; **72**(2):72-95.
38. Zhao D, Huang Z. Effect of his-tag on expression, purification, and structure of zinc finger protein, ZNF191 (243-368). *Bioinorg Chem Appl* 2016; **2016**:1-6.