



Investigation of Supercharging as A Strategy to Enhance the Solubility and Plasminogen Cleavage Activity of Reteplase

Hooria Seyedhosseini Ghaheh¹, Mohamad Reza Ganjalikhany², Parichehreh Yaghmaei¹, Morteza Pourfarzam³, and Hamid Mir Mohammad Sadeghi^{4,*}

¹ Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran.

² Department of Biology, Faculty of Sciences, University of Isfahan, Isfahan, Iran.

³ Department of Clinical Biochemistry, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran.

⁴ Department of Pharmaceutical Biotechnology, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran.

*Corresponding author: Hamid Mir Mohammad Sadeghi, Isfahan University of Medical Sciences, Pharmaceutical University, Hezar-Jerib Ave., Isfahan, IR. Iran. Tel: +98-3137927059 / Fax: +98-3136692288 E-mail: h_sadeghi@pharm.mui.ac.ir

Background: Reteplase, the recombinant form of tissue plasminogen activator, is a thrombolytic drug with outstanding characteristics, while demonstrating limited solubility and reduced plasminogen activation. Previously, we *in silico* designed a variant of Reteplase with positively supercharged surface, which showed promising stability, solubility and activity. This study was devoted to evaluation of the utility of supercharging technique for enhancing these characteristics in Reteplase.

Objective: To test the hypothesis that reinforced surface charge of a rationally-designed Reteplase variant will not compromise its stability, will increase its solubility, and will enhance its plasminogen cleavage activity.

Materials and Methods: Supercharged Reteplase coding sequence was cloned in pDest527 vector and expressed in *E. coli* BL21 (DE3). The expressed protein was extracted by cell disruption. Inclusion bodies were solubilized using guanidine hydrochloride, followed by dialysis for protein refolding. After confirmation with SDS-PAGE and western blotting, extracted proteins were assayed for solubility and tested for bioactivity.

Results: SDS-PAGE and western blot analysis confirmed the successful expression of Reteplase. Western blot experiments showed most of Reteplase expressed in the insoluble form. Plasminogen cleavage assay showed significantly higher activity of the supercharged variant than the wild type protein ($P < 0.001$). The stability of the supercharged variant was also comparable to the wild type.

Conclusion: Our findings, i.e. the contribution of the surface supercharging technique to retained stability, enhanced plasminogen cleavage activity, while inefficiently changed solubility of Reteplase, contain implications for future designs of soluble variants of this fibrinolytic protein drug.

Keywords: Activity; Protein Engineering; Reteplase; Solubility; Surface Supercharging

1. Background

A major challenge in production and development of new generations of protein therapeutics with desirable features is the limited solubility, activity and/or stability of the pharmaceutical protein of interest (1). Reteplase (r-PA), the first third-generation thrombolytic drug, is an important example in this view; it is a recombinant form of human tissue plasminogen activator (t-PA) with ability of fibrinolysis in plasma, which is used in the treatment of acute myocardial infarction (AMI),

a dominant reason of mortality in the world (2, 3). Due to the non-glycosylated structure of Reteplase, this recombinant therapeutic protein can be produced using a bacterial host such as *Escherichia coli* (4-6). However, the relatively poor solubility of Reteplase that results in its misfolding into inclusion bodies during expression, is a limitation that particularly emerges in industrial production of Reteplase (7). In addition, due to its low half-life compared to other third generation thrombolytic drugs (5, 8), it is necessary to improve

its function by increasing its activity. Therefore, it is very beneficial to design new variants of Reteplase with enhanced characteristics in terms of solubility and activity to use the enzyme as a therapeutic drug.

Rational design approaches can be utilized to create appropriate mutations (9-11). These techniques rely on structural and functional studies of proteins to identify the mutations which are capable to improve favorable features such as activity, thermal stability and protein refolding, and to reduce the aggregation propensity (12-15).

To date, rational protein engineering has been utilized for optimal production of several therapeutic and industrial proteins. Nevertheless, the structure-function relationship studies to enhance the Reteplase properties are limited to a single *in silico* design of various single peptides to transport recombinant Reteplase to culture media (7).

In the last decade, researchers have pointed out to the effectiveness of modifying the net charge of proteins to improve their desirable traits. It has been suggested that reinforcing the net charge of protein surface (supercharging) can help resolve the solubility problem by shifting the isoelectric point of proteins and augmenting the polar interactions with water molecules (16). Engineering proteins by the supercharge technique have been shown to improve solubility, heat stability and activity of several widely used proteins such as green fluorescent protein and human enteropeptidase light chain (17, 18). In many cases, computational protein design methods have demonstrated applicability to achieve desired properties (16-18).

The present study was conducted based on our previous findings from computational algorithms for rational protein design on Reteplase. Using *in silico* approach, we have suggested modifications for solvent accessible residues of Reteplase in order to design supercharged variants with improved solubility while retaining or enhancing its activity (19). To the best of our knowledge, this is the first investigation of the effect of enhanced charging on Reteplase properties, where the results of computational techniques are validated through experimental methods.

2. Objective

The present study aimed to investigate the effect of supercharging on solubility, activity and stability of Reteplase by replacing selected surface amino acids with charged ones. We evaluate our computational findings and test three hypotheses: 1) supercharged variant of Reteplase is more soluble than the wild type, hence it has less propensity to aggregate as inclusion

bodies; 2) plasminogen cleavage activity of the S7 r-PA variant is higher than that of the wild type; 3) the stability of Reteplase will not be compromised due to surface supercharging.

3. Material and Methods

In our previous simulation study, we hypothesized the supercharging technique as an effective method for improving Reteplase stability, solubility and activity. We tested this hypothesis by replacing appropriate surface residues of r-PA variants with arginine (R) to increase the net charge on the protein surface. Four supercharged variants of r-PA were designed this way. Computational molecular modeling studies suggested the supercharging technique to be an effective method for improving the above-mentioned biochemical and biophysical properties of the protein. The S7 variant of Reteplase (with N113R, A284R, E291R, E212K and F288R substitutions) demonstrated the best profile in terms of structural stability, solubility and biological activity, and was selected as the most appropriate candidate for experimental study (19). The current work was devoted to experimental validation of favorable features which can be introduced in Reteplase through the designed supercharging mutations.

3.1. Gene Construction

The supercharged variant which demonstrated the highest stability and solubility in the molecular modeling phase (S7) was cloned and expressed to assess its plasminogen activation effect. The coding sequence of S7 r-PA variant was optimized for expression in *E. coli* BL21 (DE3) strain. One 6x-His-tag coding sequence was added to the 5' end of the gene. The sequence was synthesized by Made Biocompany (Tehran, Iran), and sub-cloned into the pDest527 expression plasmid using *HindIII* and *XbaI* restriction enzymes. All these stages were performed for the cloning of wild type Reteplase as the control for biological assay.

3.2. Expression of Supercharged Reteplase

Reteplase was expressed in *E. coli* BL21 (DE3) as the most widely used host for target gene expression. Transformation was done with recombinant pDest527 containing Reteplase insert using heat shock procedure, followed by selection of transformed colonies according to resistance to 100 $\mu\text{g}\cdot\text{mL}^{-1}$ ampicillin which was obtained from Sigma, Germany.

For this stage, one colony of recombinant *E. coli* BL21 (DE3) was inoculated into 5 mL of Luria-Bertani (LB) medium containing 100 $\mu\text{g}\cdot\text{mL}^{-1}$ ampicillin and incubated at 4 \times g for 16 h at 37 °C. These overnight

cultures were used to inoculate (10% v/v) 150 mL of fresh LB medium. The cells were incubated at 37 °C until they reached the exponential phase (an OD₆₀₀ nm of 0.4-0.6). In the following, the expression of Reteplase was induced by the addition of 1 mM IPTG. At the end of expression time, cells were harvested by centrifugation at 7000 ×g for 10 min at 4 °C and the final product was stored at -20 °C in order to be used for further studies.

3.3. Western Blot Analysis

To confirm the accuracy of the expression of recombinant Reteplase, western blot analysis was carried out. The bacterial lysate specimen was separated by 12% SDS-PAGE gel electrophoresis, then transferred to a nitrocellulose membrane (Sigma, Steinheim, Germany). The membrane was incubated at 4 °C overnight with blocking buffer containing 3% non-fat dry milk in Tris buffer (pH 8.8), afterwards it was incubated at room temperature with anti-His tag HRP conjugated antibody (Invitrogen, USA; 1:1000 in TB-Tween buffer [0.1 %]) for 1.5 h, followed by three times of 10min washing with wash buffer (TB-Tween buffer, pH 8.8). Finally, the protein bands were visualized using DAB solution (3, 3-diaminobenzidine, Sigma, Germany).

3.4. Preparation and Extraction of Reteplase Inclusion Bodies

Cell pellets obtained after induction of about 150 mL culture, were suspended in 13 mL buffer (50 mM Tris, 25% sucrose, 10 mM DTT and 1 mM NaEDTA). Then, the bacteria were lysed by sonication (3 cycles of 30 seconds and 3 cycles of 15 seconds with intensity of 0.5 Amplitude) (20). The pellets were resuspended in lysosyme, DNAase and MgCl₂ with slowly vortex. Following that, lysis buffer was added and it was shortly vortexed again, mixed and incubated at room temperature for 45 min. After adding NaEDTA and MgCl₂, the solution was rested for 30 min. Subsequently, NaEDTA was added and centrifugation was carried out for 25 min at 4 °C (13000 ×g). Afterwards, the pellets were resuspended in Triton buffer and sonication was carried out as mentioned above. This procedure was repeated twice with no Triton buffer (50 mM Tris, 100 mM NaCl, 1 mM NaEDTA and 1 mM DTT).

3.5. Solubilization and Refolding of Reteplase

In order to solubilize the extracted inclusion bodies, resuspension in solubilizing buffer (25 mM Tris, 10 mM NaEDTA, 6 M guanidine HCl, 1mM glutathione reductase, 0.1 mM Glutathione oxidized, 0.5 mol.L⁻¹ L-Arginine, 0.01% Tween and 1 mg.mL⁻¹ bovine serum

albumin) was performed and after that, the samples were incubated for 24 h at 22 °C in the mentioned buffer. The reducing agent and buffer components were separated by dialysis against 0.1 mol.L⁻¹ Tris-HCL and 1 mM NaEDTA (pH=7) at 4 °C. This stage was repeated every one hour for two times at 4 °C and finally overnight.

3.6. Reteplase Activity Assessment

Plasminogen activation to plasmin by Reteplase was detected by Human t-PA Chromogenic Activity Kit (AssayPro, USA) according to its instructions. At the first stage, an assay mix containing assay diluents (60 µL), plasminogen (10 µL) and plasmin substrate (10 µL) per each sample or standard was prepared. Then, 80 µL of this mixture was added to the supplied 96-well plate. Eventually, after adding 20 µL of tPA standard or samples (including wild type Reteplase or S7 variant in the same concentration as 180 µg.mL⁻¹) to each well, the plate was incubated at 37 °C in a humid incubator and the absorbance was measured at 405 nm in various time intervals for each sample. This stage was repeated for 20 µL of refolding buffer as blank. The activity test was performed in three independent experiments with 2 repeats for each compound as well as different standards. SPSS software (version 16; Chicago, IL, USA) was used for statistical analysis. Repeated measures analysis of variance (ANOVA) was used to determine the differences between groups.

4. Results

4.1. Confirmation of the S7 Reteplase Gene Clone

Digestion of recombinant pDest527 plasmid containing the mutant Reteplase (S7) with *Xba*I and *Hind*III created a band in about 1289 bp in 0.8% agarose gel electrophoresis, which is equal to the size of Reteplase gene as shown in **Figure 1A**.

4.2. Expression of Reteplase

At the final stage of expression of the supercharged Reteplase (S7) with IPTG (1mM) induction, the expression with an estimated protein size of ~43 kDa was confirmed in SDS-PAGE (**Fig. 1B**). In order to investigate the soluble expression level of S7 Reteplase, the supernatant and bacterial pellets were treated with anti-His HRP conjugated antibody as western blotting procedure. A band was observed approximately in 43 kDa in pellet sample but not in supernatant, showing that almost whole of the expressed protein was in insoluble (inclusion body) form (**Fig. 1C**).

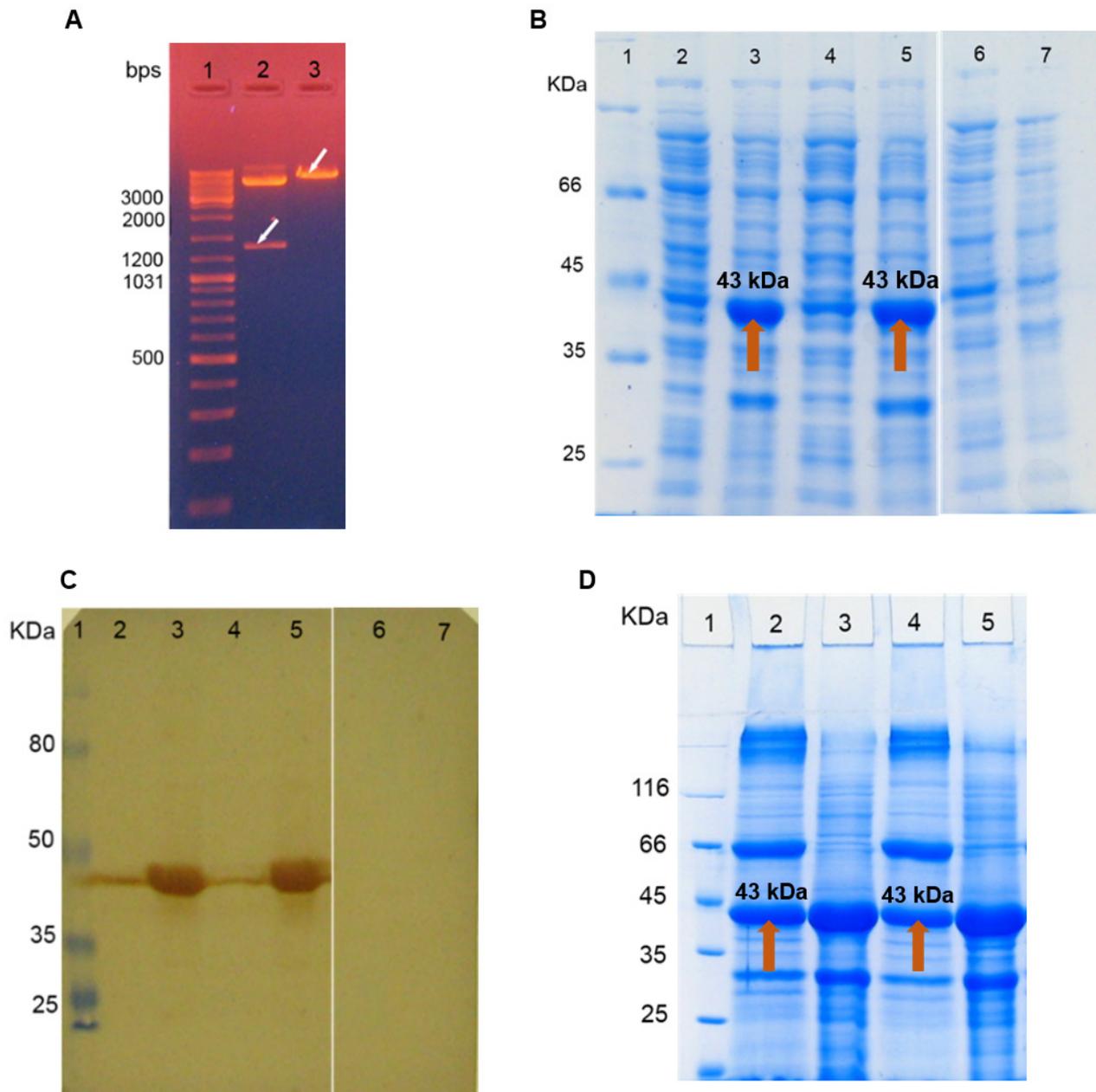


Figure 1. **A)** Agarose gel electrophoresis of pDest527 containing supercharged Reteplase coding sequence: Lane 1, Standard molecular weight marker; Lane 2: Digested recombinant pDest527; Lane 3: pDest527 without digestion. **B)** SDS-PAGE analysis of wild-type and S7 Reteplase expression: Lane 1, Standard molecular weight marker; Lane 2: Supernatant of induced *E. coli* BL21 (DE3) cells containing pDest527-wt (wild type) Reteplase; Lane 3: Pellet of induced *E. coli* BL21 (DE3) cells containing pDest527-wt Reteplase; Lane 4: Supernatant of induced *E. coli* BL21 (DE3) cells containing pDest527-S7 Reteplase; Lane 5: Pellet of induced *E. coli* BL21 (DE3) cells containing pDest527-S7 Reteplase; Lane 6: Supernatant of induced *E. coli* BL21 (DE3) cells containing BL21; Lane 7: Pellet of induced *E. coli* BL21 (DE3) cells containing BL21. **C)** Western blot analysis for evaluation of Reteplase expression: Lane 1, Pre-stained standard molecular weight marker; Lane 2: Supernatant of induced *E. coli* BL21 (DE3) cells containing pDest527-WT Reteplase; Lane 3: Pellet of induced *E. coli* BL21 (DE3) cells containing pDest527-WT Reteplase; Lane 4: Supernatant of induced *E. coli* BL21 (DE3) cells containing pDest527-S7 Reteplase; Lane 5: Pellet of induced *E. coli* BL21 (DE3) cells containing pDest527-S7 Reteplase; Lane 6: Supernatant of *E. coli* BL21 (DE3) cells (negative control); Lane 7: Supernatant of *E. coli* BL21 (DE3) cells (negative control). **D)** SDS-PAGE of the proteins extracted from inclusion bodies and refolded form: Lane 1, Standard molecular weight marker; Lane 2: Refolded wild type; Lane 3: Purified inclusion body of wild type; Lane 4: Refolded supercharged (S7) Reteplase; Lane 5: Purified inclusion body of supercharged Reteplase.

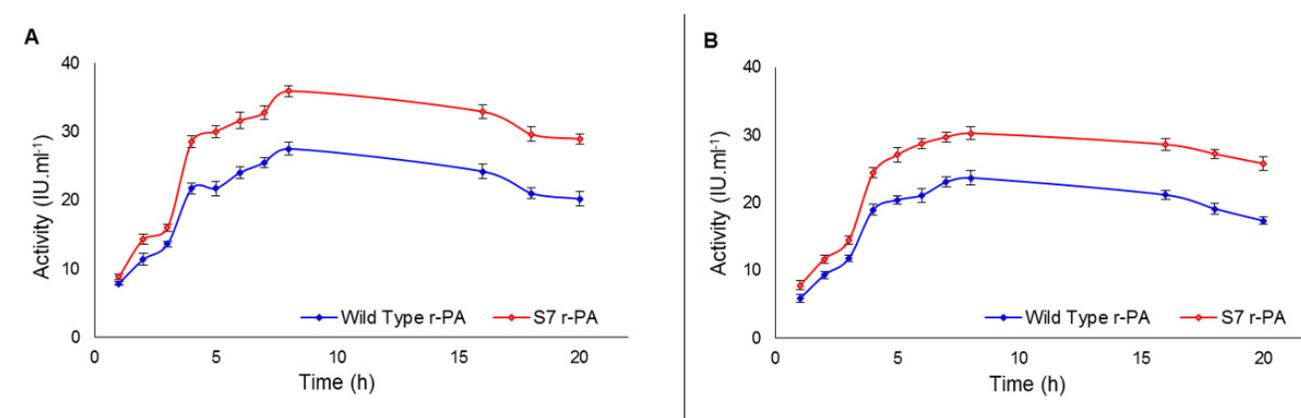


Figure 2. Comparison of the plasminogen activator properties of wt r-PA and S7 r-PA forms. (A) Immediately after dialysis, and (B) two weeks after dialysis. n = 3; Error bars represent SD; P value < 0.001.

4.3. Extraction, Solubilization and Refolding of Reteplase Inclusion Bodies

After large-scale expression of S7 Reteplase as insoluble form, bacterial cells were collected and disrupted by sonication. Isolated Reteplase inclusion bodies was performed by washing with buffers containing detergent and high speed centrifugation. Based on our results, 6 M guanidine HCl (pH 7.8) was found to be the best solubilizing agent. As shown in **Figure 1D**, solubilization of inclusion bodies, refolding and dialysis of Reteplase analyzed using SDS-PAGE electrophoresis showed the presence of a prominent band of about 43 kDa. Furthermore, a band approximately in about 66 kDa, was observed in SDS-PAGE which can be attributed to the dimeric form of S7 Reteplase created in the refolding stage (**Fig. 1D**).

4.4. Bioactivity Assays of Supercharged Reteplase

The activity of the refolded enzyme was evaluated using standard t-PA enzyme activity kit. The change in the absorbance of chromogenic substrate at 405 nm has a direct relation to the Reteplase enzymatic activity. As shown in **Figure 2A**, the plasminogen cleavage activity of supercharged Reteplase has increased significantly over time in both groups ($P < 0.001$, for both groups) and it was significantly higher in S7 variant than that of the wild type ($P < 0.001$). The maximum activity of both wild-type and supercharged Reteplase variants was reached at the 8th hour after dialysis (27.5 IU.mL⁻¹ and 35.93 IU.mL⁻¹, respectively). Furthermore, two weeks after dialysis, the trends of decreasing activity were similar for the wild type and S7 variants, indicating unchanged stability of S7 compared to the wild type. After 2 weeks of dialysis, the supercharged variant

showed significantly increasing trend over time in both groups ($P < 0.001$, for both groups) and higher activity was seen in S7 variant than the wild type during the surveyed time ($P < 0.001$). (**Fig. 2B**).

5. Discussion

This study was devoted to experimentally evaluate the properties of a computational model of Reteplase. Previously, we employed several *in silico* methods to design new surface-supercharged variants of Reteplase, and in this study we expressed our engineered enzyme in the cytoplasm of *E. coli* BL21 (DE3) under optimal conditions utilizing pDest527, a vector containing the powerful T7 promoter and a polyhistidine tag (6). In comparison with periplasmic production of Reteplase, the cytoplasmic expression has been revealed as a more appropriate system yielding high amounts of protein (4, 5, 22).

Our supercharged Reteplase variant showed increased plasminogen cleavage activity over that of the wild type protein. In fact, augmenting the charged groups on protein surface leads to the strengthening of connection of water molecule clusters to the surface of protein which are essential for enzyme activity (23). In addition, several studies revealed that the number of arginine residues on the protein surface significantly affects the protein stability and function by increasing the number of hydrogen bonds and electrostatic interactions (13, 24, 25). It is suggested that the surface arginine substitutions improve the structural stability and perhaps enhance the stability of catalytic residues, which correlates to its enhanced activity. In agreement with our results, Turunen and colleagues indicated that introducing of arginine residues into xylanase

surface led to the stability and functional properties improvement (25).

Shafiee and colleagues reported that the activity of Reteplase expressed in *E. coli* TOP10 under optimal conditions was reduced in comparison to commercial Reteplase (Retelase®), which could be attributed to the presence of His-tag (3). In contrast with Shafiee *et al.*, the activity of our product is higher than that of wild type. In our study, the His-tag sequence was placed in the N-terminal which was not in close proximity of the active site. In agreement with this approach, Aslantas and Surmeli indicated that adding His-tag sequence can influence the electronic structure of active site and the activity of the protein. Hence, they suggested that the His-tag located in N-terminus is the optimal choice for isolation and purification of proteins (26).

Despite the fact that modifying the protein surface charge and removing surface hydrophobic residues can help improve refolding and solubility and prevent aggregation of proteins (13, 17, 18), in our study, the high amount of expressed Reteplase was accumulated in cytoplasmic space in the form of inclusion bodies. The production of inclusion bodies is preferred in many cases since they can accumulate in higher quantities than soluble proteins, and the recombinant product can be isolated in a highly concentrated and purified state (21, 27). However, due to their insoluble nature, optimizing their purification and refolding process is critical in order to prevent formation of inactive aggregates (3, 28). Optimal conditions for solubilization are unique for each protein (29). In the case of highly charged proteins, due to the reinforcement of structural interactions, guanidine hydrochloride is a more appropriate chaotropic agent than urea (8, 30), hence it was used here for dissolving the supercharged Reteplase inclusion bodies. We also used arginine and oxidizing/reducing glutathione in refolding buffer, as the interaction of Arg with tryptophan side chains can suppress protein aggregation (31, 32). Despite these strategies, the proportion of solubilized protein still remains low. This may be due to the unequal distribution of mutations between the two functional domains of Reteplase, because the serine protease domain is bulkier and offers more sites amenable to mutation, than the Kringle II domain. As an alternative explanation, the proposed substitutions may have been inefficient due to larger stabilization of the aggregated form (15). Whatever the reason, our observation revokes the notion of supercharging as a strategy for enhancing the Reteplase solubility, which is an implication for future studies.

The supercharge technique can augment the protein

stability as shown by other works. Miklos *et al.*, for example, applied this technique to structural design of thermo-resistant antibodies, and reported this method to be useful in enhancing the resistance to thermal inactivation due to induced aggregation, as well as in potentiating the antibody affinity (14)(29). Stability analyses in our study using the wild type as reference, showed comparable or higher stability of the supercharged Reteplase. The trend of decreasing activity of the new Reteplase variant with time was similar to that of the wild type form. Furthermore, fortnight storage of the enzyme led to no change in the descending trend, compared to wild type r-PA, indicating retained storage stability of the protein.

6. Conclusion

Overall, our data indicated that Reteplase surface charge modification can be an effective strategy to increase the protein activity through non-destabilizing substitution of residues to charged ones. The observations of our study, i.e. preserved stability, enhanced plasminogen cleavage activity, while inefficiently changed solubility of Reteplase, suggest implications for further study and design of new soluble variants of this fibrinolytic protein drug, with the purpose of treating acute myocardial infarction.

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Authors' contributions

H.M.M.S., M.R.G., P.Y., and M.P. were involved in designing and supervised the research. H.S.H.G., carried out the experiments and wrote of the manuscript. All authors analyzed the data, discussed the results and contributed to the final manuscript.

Conflict of interest

The authors declare that there is no conflict of interest.

References

- Kamionka M. Engineering of therapeutic proteins production in *Escherichia coli*. *Curr pharm biotechnol*. 2011;**12**(2):267-274. doi.org/10.2174/138920111794295693 pmid: 21050165
- Shafiee F, Moazen F, Rabbani M, Sadeghi HMM. Expression and activity evaluation of reteplase in *Escherichia coli* TOP10. *J Param Sci*. 2015;**6**(3):58-64. doi.org/10.17795/jjnpp-16676
- Shafiee F, Moazen F, Rabbani M, Sadeghi HMM. Optimization of the expression of reteplase in *Escherichia coli* TOP10 using arabinose promoter. *Jundishapur J Nat pharm prod*. 2015;**10**(1). doi.org/10.17795/jjnpp-16676 pmid: 25866712
- Rosano GL, Ceccarelli EA. Recombinant protein expression in *Escherichia coli*: advances and challenges. *Front in microbiol*. 2014;**5**:172. doi.org/10.3389/fmicb.2014.00172 pmid:24860555
- Khodabakhsh F, Zia MF, Moazen F, Rabbani M, Sadeghi HMM. Comparison of the cytoplasmic and periplasmic production of reteplase in *Escherichia coli*. *Prep Biochem Biotechnol*. 2013;**43**(7):613-623. doi.org/10.1080/10826068.2013.764896 pmid:23768109
- Fathi-Roudsari M, Akhavian-Tehrani A, Maghsoudi N. Comparison of three *Escherichia coli* strains in recombinant production of reteplase. *Avicenna J Med Biotechnol*. 2016;**8**(1):16. pmid: 26855731
- Mousavi P, Mostafavi-Pour Z, Morowvat MH, Nezafat N, Zamani M, Berenjian A, *et al*. In silico analysis of several signal peptides for the excretory production of reteplase in *Escherichia coli*. *Curr Proteomics*. 2017;**14**(4):326-335. doi.org/10.2174/1570164614666170809144446
- Esmaili I, Sadeghi HMM, Akbari V. Effect of buffer additives on solubilization and refolding of reteplase inclusion bodies. *Res Pharm Sci*. 2018;**13**(5):413. doi.org/10.4103/1735-5362.236834 pmid:30271443
- Van Den Berg S, Löfdahl P-Å, Härd T, Berglund H. Improved solubility of TEV protease by directed evolution. *J Biotechnol*. 2006;**121**(3):291-298. doi.org/10.1016/j.jbiotec.2005.08.006 pmid: 16150509
- Schweiker KL, Makhatadze GI. A computational approach for the rational design of stable proteins and enzymes: optimization of surface charge-charge interactions. *Methods Enzymol*. 2009;**454**:175-211. doi.org/10.1016/s0076-6879(08)03807-x pmid:19216927 pmid:19216927
- Poluri KM, Gulati K. Rational Designing of Novel Proteins Through Computational Approaches. *Protein Eng Tech*. 2017;**61**-83. doi.org/10.1007/978-981-10-2732-1_3
- Savardashtaki A, Sharifi Z, Hamzehlou S, Farajollahi MM. Analysis of immunoreactivity of heterologously expressed non-structural protein 4B (NS4B) from hepatitis C virus (HCV) genotype 1a. *Iran J Biotechnol*. 2015;**13**(4):32. doi.org/10.15171/ijb.1321 pmid:28959307
- Mosavi LK, Peng Zy. Structure-based substitutions for increased solubility of a designed protein. *Protein Eng*. 2003;**16**(10):739-745. doi.org/10.1093/protein/gzg098 pmid:14600203
- Miklos AE, Kluwe C, Der BS, Pai S, Sircar A, Hughes RA, *et al*. Structure-based design of supercharged, highly thermoresistant antibodies. *Chem Biol*. 2012;**19**(4):449-455. doi.org/10.1016/j.chembiol.2012.01.018 pmid:22520751
- Cabrita LD, Gilis D, Robertson AL, Dehouck Y, Rooman M, Bottomley SP. Enhancing the stability and solubility of TEV protease using in silico design. *Protein Sci*. 2007;**16**(11):2360-2367. doi.org/10.1110/ps.072822507 pmid:17905838
- Shaw KL, Grimsley GR, Yakovlev GI, Makarov AA, Pace CN. The effect of net charge on the solubility, activity, and stability of ribonuclease Sa. *Protein Sci*. 2001;**10**(6):1206-1215. doi.org/10.1110/ps.440101 pmid:11369859
- Simeonov P, Berger-Hoffmann R, Hoffmann R, Sträter N, Zuchner T. Surface supercharged human enteropeptidase light chain shows improved solubility and refolding yield. *Protein Eng Des Sel*. 2010;**24**(3):261-268. doi.org/10.1093/protein/gzq104 pmid:21084283
- Der BS, Kluwe C, Miklos AE, Jacak R, Lyskov S, Gray JJ, *et al*. Alternative computational protocols for supercharging protein surfaces for reversible unfolding and retention of stability. *PLoS One*. 2013;**8**(5):e64363. doi.org/10.1371/journal.pone.0064363 pmid:23741319
- Ghaheh HS, Ganjalikhany MR, Yaghmaei P, Pourfarzam M, Sadeghi HMM. Improving the solubility, activity, and stability of reteplase using in silico design of new variants. *Res Pharm Sci*. 2019;**14**(4):359. doi:10.4103/1735-5362.263560 pmid:31516513
- Pourhadi M, Jamalzade F, Jahanian-Najafabadi A, Shafiee F. Expression, purification, and cytotoxic evaluation of IL24-BR2 fusion protein. *Res Pharm Sci*. 2019;**14**(4):320. doi.org/10.4103/1735-5362.263556 pmid: 31516508
- Sadeghi HMM, Dormiani K, Khazaie Y, Rabbani M, Moazen F. Cloning and expression of a human tissue plasminogen activator variant: K2S in *Escherichia coli*. *Pakistan J Biol Sci*. 2007;**10**:946-949. doi.org/10.3923/pjbs. pmid:19069895
- Ramos C, Abreu P, Nascimento A, Ho P. A high-copy T7 *Escherichia coli* expression vector for the production of recombinant proteins with a minimal N-terminal His-tagged fusion peptide. *Braz J Med Biol Res*. 2004;**37**(8):1103-1109. doi.org/10.1590/s0100-879x2004000800001 pmid:15273812
- Verma R, Mitchell-Koch K. In Silico studies of small molecule interactions with enzymes reveal aspects of catalytic function. *J Catal*. 2017;**7**(7):212. doi.org/10.3390/catal7070212 pmid: 30464857
- Sokalingam S, Raghunathan G, Soundrarajan N, Lee S-G. A study on the effect of surface lysine to arginine mutagenesis on protein stability and structure using green fluorescent protein. *PLoS One*. 2012;**7**(7):e40410. doi.org/10.1371/journal.pone.0040410 pmid:22792305
- Turunen O, Vuorio M, Fenel F, Leisola M. Engineering of multiple arginines into the Ser/Thr surface of *Trichoderma reesei* endo-1, 4- β -xylanase II increases the thermotolerance and shifts the pH optimum towards alkaline pH. *Protein Eng*. 2002;**15**(2):141-5. doi.org/10.1093/protein/15.2.141 pmid:11917150
- Aslantas Y, Surmeli NB. Effects of N-Terminal and C-Terminal Polyhistidine Tag on the Stability and Function of the Thermophilic P450 CYP119. *Bioinorg Chem Appl*. 2019;2019. doi.org/10.1155/2019/8080697 pmid:31320891
- Ramón A, Señorale M, Marín M. Inclusion bodies: not that bad.... *Front Microbiol*. 2014;**5**:56. doi: 10.3389/fmicb.2014.00056 pmid:24592259
- Latifi AM, Khajeh K, Farnoosh G, Hassanpour K, Khodi S. The Cytoplasmic and periplasmic expression levels and folding of organophosphorus hydrolase enzyme in *Escherichia coli*. *Jundishapur J Microbiol*. 2015;**8**(12). doi.org/10.5812/jjm.17790 pmid:26870308
- Hou Q, Bourgeas R, Pucci F, Rooman M. Computational

- analysis of the amino acid interactions that promote or decrease protein solubility. *Sci Rep.* 2018;**8**(1):14661. doi.org/10.1038/s41598-018-32988-w
30. Rashid F, Sharma S, Bano B. Comparison of guanidine hydrochloride (GdnHCl) and urea denaturation on inactivation and unfolding of human placental cystatin (HPC). *Protein J.* 2005;**24**(5):283-292. doi.org/10.1007/s10930-005-6749-5 pmid:16284726
31. Tsumoto K, Umetsu M, Kumagai I, Ejima D, Philo JS, Arakawa T. Role of arginine in protein refolding, solubilization, and purification. *Biotechnol Prog.* 2004;**20**(5):1301-1308. doi.org/10.1021/bp0498793 pmid:15458311
32. Yamaguchi H, Miyazaki M. Refolding techniques for recovering biologically active recombinant proteins from inclusion bodies. *Biomolecules.* 2014;**4**(1):235-251. doi.org/10.3390/biom4010235 pmid:24970214