Research in Pharmaceutical Sciences, August 2020; 15(4): 331-339
Received: 29-01-2019
Peer Reviewed: 05-05-2019
Revised: 20-01-2020
Accepted: 16-08-2020
Published: 28-08-2020

School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

Original Article

Optimization of solvent media to solubilize TEV protease using response surface method

Niloufar Mohammadian[1], Hossein Mohammadian[1], Fatemeh Moazen[1], Mohammad Hosein Pakdel[2], Ali Jahanian-Najafabadi[1], and Hamid Mir Mohammad Sadeghi[1,*]

[1]*Department of Pharmaceutical Biotechnology, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.*

[2]*Department of Biotechnology, College of Agriculture, Isfahan University of Technology, Isfahan, I.R. Iran.*

Abstract

Background and purpose: Tobacco etch virus (TEV) protease, a 27 KDa protein, consists of the catalytic domain of nuclear inclusion a (NIa) protein produced by *Tobacco etch* virus. Because of its unique sequence, TEV protease is used for purging fusion tags from proteins. It also has many advantages such as stability and activity in a board range of temperature and pH and overproduction in *Escherichia coli* and these benefits make this protease valuable. Despite all these benefits, TEV protease has problems like low solubility (less than 1 mg/mL). There are methods for enhancing protein solubility and in this study, the effect of additives during cell lysis was studied.
Experimental approach: Eleven different additives that made twelve different lysis buffers were used and their effect on TEV protease solubility analyzed by Plackett-Burman and response surface methodology methods.
Findings / Results: Three best effective additives on TEV solubility (L-proline, sodium selenite, and $CuCl_2$) were selected according to software analysis and the best concentration of them was applied to optimize TEV protease solubility.
Conclusion and implications: The obtained results provided the composition of an optimum solvent for obtaining soluble TEV protease.

Keywords: Lysis buffer; Plackett-Burman method; Response surface method; Solubility; Solubilizing additives; TEV protease.

INTRODUCTION

Recombinant soluble protein production is a challenge in biotechnology and one method which can help for increasing protein solubility is using tag fusions in N/C-terminal (1). Tag fusions are protein segments that are produced along with the target protein and its expression would enhance protein solubility, expression, and efficacy (2,3). To obtain a fully functional protein, it is always advantageous to separate tags or fusion proteins which could be separated by physical or enzymatic methods (4). Tobacco etch virus (TEV) protease is an enzyme that can be used for cleavage of tag fusions recognizing sites on proteins (5,6).

TEV protease, a 27 kDa protein, consists of the catalytic part of a nuclear inclusion (NIa) protein. TEV is an unconventional serine protease (serine/histidine /aspartic acid) in which serine is replaced with cysteine. This fact explains TEV protease resistance to commonly used serine protease inhibitors (7). Its specific cleavage sequence consists of seven amino acids ENLYFQ/S and separation occurs between Q and S. This cleavage site leaves behind only one amino acid in the chain of the main protein with minimal effects on protein properties (8).

*Corresponding author: H. Mir Mohammad Sadeghi
Tel: +98-3137927059, Fax: +98-3136680011
Email: h_sadeghi@pharm.mui.ac.ir

Access this article online

Website: http://rps.mui.ac.ir

DOI: 10.4103/1735-5362.293511

TEV protease has many advantages like stability, activity in a broad range of pH (4-9) and temperatures (4-20 °C), and high levels of production in *Escherichia coli (E. coli)* (8-10). Despite these benefits, TEV protease solubility is low (less than 1 mg/mL) (5,6). Methods are available for enhancing TEV solubility like controlling expression factors, co-expression with chaperones, using solubility enhancing fusion tags, and target mutation methods (2,11-14).

A traditional method in optimizing conditions for protein production such as culture media, expression, and solubility is changing one variable at a time (15,16). This method is suitable to examine the effect of one variable on the obtained results but it consumes a lot of time and materials and also it does not consider the interaction between variables (17). Fortunately for analyzing multiple variables, there are alternative methods such as full factorial and fractional factorial (*e.g.,* Plackett-Burman) designs, response surface methodology (RSM), and Taguchi method (12,18-20). We used the Plackett-Burman method for the identification of parameters with the greatest impact on TEV protease solubility. RSM can be applied to determine the optimum conditions of lysis buffer additives for TEV solubility by simultaneously changing several variables based on a minimum number of experiments and can identify possible interactions among experimental additives (21,22).

In the present study, we decided to change lysis buffer additives and find the best condition for protein solubility with the help of Plackett-Burman and RSM methods (23,24).

MATERIALS AND METHODS

Preparation of expressed protein

One hundred mL of lysogeny broth (LB) culture media containing 100 mL ampicillin (100 µL/mL), 10 mL *E. Coli* BL21 (DE3) (pRK93 plasmid and MBP-TEV genome) were mixed entirely and were fixed in shaking incubator at 180 rpm for 3 h at 37 °C until they reached the exponential phase (OD_{600} nm of 0.6). Subsequently, isopropyl β-d-1-thiogalactopyranoside (IPTG, 100 mL into 100 mL culture media, 1µL/mL) was added to induce protein production and was incubated for another 3 h. Subsequently, the culture media was divided into different tubes (1 mL in each tube), then centrifuged at 10000 rpm for 1 min and the pellet was stored at -70 °C.

Preparation of lysis buffers using Plackett-Burman designed table

The combination of various lysis buffers designed according to the Plackett-Burman are represented by Table 1. Buffer was composed of tris 2 mM (pH 8), NaCl 500 mM, glycerol 10%, natrium azide 0.025%, $MgCl_2$ 10 mM based on SSGCID's (Seattle structural genomics center for infectious disease) general lysis buffer plus additives. Trehalose (0.75 M-5.0%), glycine betaine (10 mM-1 M), mannitol (0.5 M-1.0%), sodium citrate (0.1 M), proline (100 mM-0.5 M), L-arginine (75 mM-375 mM), xylitol (5.0% or 1 M), sodium selenite (10 mM), dipotassium phosphate (100 mM), $CuCl_2$ (10 mM), cetrimonium bromide (0.01-0.5%) were chosen as additives to enhance TEV protease solubility among 14 additives with best effect on protein solubility (23). Underlined amounts are concentrations that have the most effect on protein solubility and were used as our high level amount and the low level amount was set at zero. Lysis buffers were prepared in 10 mL volumes and pH was set at 8 (stored at 4-8 °C).

Extraction of TEV protease from E. coli in designed lysis buffers

Five hundred µL of lysis buffer was added to the pellet that was stored at -70 °C (12 lysis buffers, each plate one buffer). After dispersing plate in buffer completely, the solution was transferred to micro smash tubes containing glassy pearls and all tubes were centrifuged in micro smash centrifuge (TOMY, Japan) at 4500 rpm for 1 min, then these tubes were put on ice for 2 min. This procedure was repeated 5 times. Subsequently, 150 mL of each solution was set aside as total samples and the residual solutions were centrifuged for achieving soluble samples. The centrifuge was completed in two phases at 4 °C, first at 5000 rpm for 15 min, and second at 12000 rpm for 5 min. The obtained solutions were labelled as soluble samples (about 250 µL).

Table 1. Plackett-Burman designed lysis buffers. This table shows eleven additives with zero and one code in each twelve recommended lysis buffers. 1 represents the existence of additive and 0 represents that the additive does not exist in lysis buffer. The amount of each variable shows in the text.

Run	Trehalose	Glycine betaine	Mannitol	Sodium citrate	Proline	L-Arginine	Xylitol	Sodium selenite	Dipotassium phosphate	CuCl2	Cetrimonium bromide
1	1	1	0	1	1	1	0	0	0	1	0
2	1	1	1	0	0	0	1	0	1	1	0
3	1	1	0	0	0	1	0	1	1	0	1
4	0	1	0	1	1	0	1	1	1	0	0
5	0	0	0	0	0	0	0	0	0	0	0
6	1	0	1	1	0	1	1	1	0	0	0
7	0	0	0	1	0	1	1	0	1	1	1
8	1	0	1	1	1	0	0	0	1	0	1
9	0	1	1	1	0	0	0	1	0	1	1
10	1	0	0	0	1	0	1	1	0	1	1
11	0	1	1	0	1	1	1	0	0	0	1
12	0	0	1	0	1	1	0	1	1	1	0

Quantification of extracted protein in total and soluble samples using gel electrophoresis

The samples were prepared for loading into sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Loading buffer (×5) was added to each tube and incubated in a water bath for 5 min at 100 °C. Then a 15% SDS-gel was prepared and samples (total and soluble) were loaded (20 mL) to the gel and electrophoresed at 70 mA, for 4 h. Afterwards, the gel was stained with Coomassie blue followed by destaining. TEV protease protein band was identified using the pre-stained protein ladder (Thermo Fisher Scientific, MA, USA). Band intensity measured by ImageJ 1.50e software (National Institutes of Health, USA). This software is capable of analyzing and assigning a number to protein band intensity. These numbers were imported to Plackett-Burman software to determine the most effective additives on TEV protease solubility. In the next step, we used the RSM (Box-Behnken) method (18,19) to design lysis buffers based on the most effective additives. In this method, software studied additive concentrations on 3 levels: high level, low level, and middle level. Eventually, the most effective concentration of additives was selected for preparing the lysis buffer to optimize TEV solubility. Validation of this method was tested in the laboratory.

Identification of protein band locale in electrophoresis gel using western blot method

Western blot is an analytical technique used in molecular biology for the detection of a specific protein in a complex mixture of protein (25). In this study, we used colourimetric (3,3'-diaminobenzidine, DAB method) and enhanced chemiluminescent (ECL method) detection for visualization of TEV protease.

DAB method: briefly, after electrophoresis of our samples on an SDS-gel, they were transferred (25 w, 0.4 A, 1 h) to a nitrocellulose paper by Bio-Rad system (Trans-Blot, USA). Subsequently, the nitrocellulose paper was incubated in 3% skim milk (3 g skim milk in 100 mL tris-buffered saline (TBS) (NaCl 125 mM, tris 25 mM pH 8.0) overnight followed by washing with enough TBST (TBS + Tween® 80 0.1%) 3 times for 10 min and then adding 5 mL of conjugated antibody to 10 mL TBST and shacking for 2 h. When this procedure was completed, the nitrocellulose paper was washed 3 times for 10 min with TBST. Finally, 9 mg DAB was added to 15 mL TBST and shook to dissolve DAB, then nitrocellulose paper was incubated in this solution and shook in the presence of 50 mL of H_2O_2 until the appearance of protein bands.

For further investigation, we used nitrocellulose paper that was washed 3 times with TBST after shaking with a conjugated antibody. All the steps were performed in a dark

room. First, detection reagent (125 mL per 1 cm^2 of paper) was added and the paper was put into a cassette. A sheet of film was placed on to the paper and the cassette was closed for 10 sec and then the film was immersed into the stabilizing solution, deionized water, and appearance solution, respectively. Protein bands were detected and were sharper and more intense than the DAB method, so small quantities of proteins could be detected.

RESULTS

Confirmation of TEV protease protein by western blot

Western blot analysis of the obtained gels demonstrated the existence of TEV protein (27 KDa, Fig. 1).

Analysis of TEV protease solubility in the designed lysis buffers

Analysis of TEV protease protein band intensity with Plackett-Burman method

The extraction of TEV protease in the presence of different lysis buffers (Table 1) was performed and total/soluble samples were loaded on SDS-gel (Fig. 2). Protein bands were analyzed by Image J software. The results of protein band intensity are shown in Table 2.

Table 2. Results of *Tobacco etch* virus (TEV) protease protein band intensity performed by Image J software.

Samples	Total	Soluble
1	30281.3	2917.6
2	32827.6	2788.4
3	33474.4	1255.3
4	23614.2	2655.1
5	26864.2	5763.4
6	22984.4	2473.7
7	20310.3	1569.4
8	22988.3	1417.9
9	165687.5	7048.4
10	46516.8	14745.8
11	44846.2	7968.6
12	41760.6	8483.5

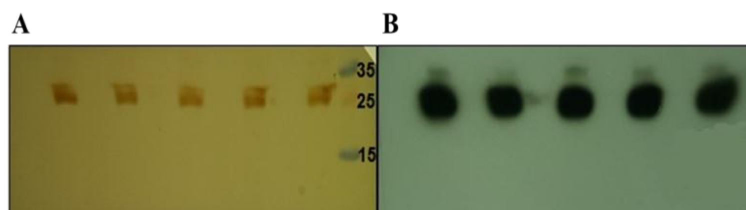

Fig. 1. Results of TEV protease western blot of (A) DAB and (B) ECL methods. The same sample was loaded in different lanes and the obtained size matched the size of TEV protease protein. TEV, *Tobacco etch* virus; DAB, 3,3'-diaminobenzidine; ECL, enhanced chemiluminescence.

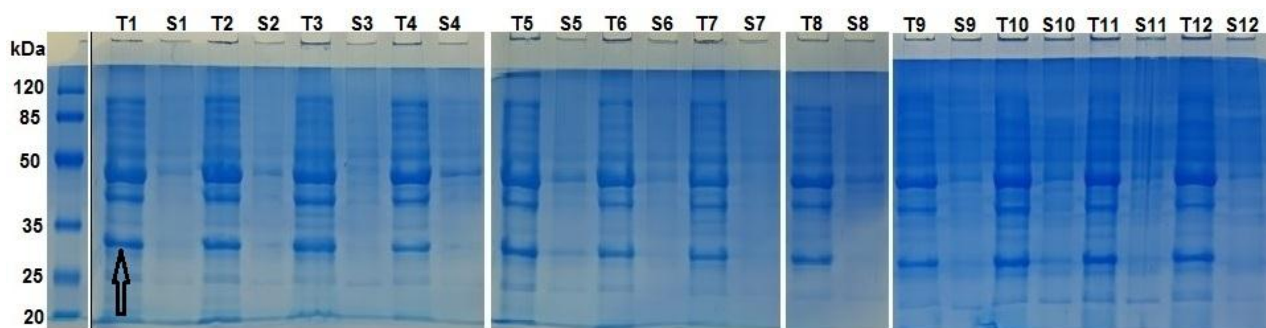

Fig. 2. SDS-PAGE of TEV protease extraction in the designed lysing buffer in Table 1. T shows the total sample and S shows the soluble sample. Numbers correspond to the number of lysis buffers in Table 1. TEV protease protein band is marked by an arrow. SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TEV, *Tobacco etch* virus.

Table 3. Effect of additives on *Tobacco etch* virus (TEV) protease solubility. The second column shows the percentage of impact on solubility, and this percentage could be positive or negative. The third column shows the amount of positive or negative impact on solubility. Three additives with the best effect on protein solubility (according to software analysis) are proline, $CuCl_2$, and sodium selenite.

Additives	Percent	Quantity
Trehalose	2.88	-1314.93
Glycine betaine	4.46	-1636.73
Mannitol	0.08	+212.33
Sodium citrate	24.28	-3820.51
Proline	13.81	+2881.69
L-Arginine	4.39	-1625.17
Xylitol	1.31	+885.81
Sodium selenite	9.36	+2372.74
Dipotassium phosphate	23.91	-3791.31
$CuCl_2$	11.86	+2669.86
Cetrimonium bromide	3.68	+1487.28

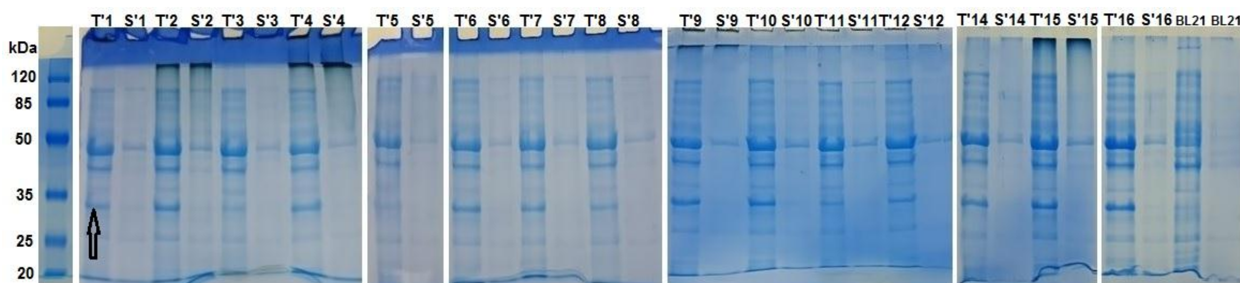

Fig. 3. SDS-PAGE of the extracted proteins (*Tobacco etch* virus protease) in lysis buffers designed by RSM method to determine the best concentration of three additives chosen by Plackett-Burman method. T' and S' show total samples and soluble samples, respectively. Numbers correspond to runs in Table 5. BL21 cells that were not transformed with any plasmids were used as the negative control. SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RSM, response surface methodology.

Table 4. Most effective additives on *Tobacco etch* virus (TEV) protease solubility based on Plackett-Burman analysis with three levels of concentrations, low, high and middle ones.

Additives	Concentrations (mM)		
	Low	Middle	High
Proline	100	300	500
Sodium selenite	5	10	15
$CuCl_2$	5	10	15

Protein band intensity of soluble samples obtained from Image J software was entered to Design Expert software and results were analyzed and reported in Table 3. Three best additives with a positive effect on TEV protease solubility (according to software analysis) were selected and used for subsequent experiments i.e. optimization of TEV protease solubility using best additive concentration.

Analysis of TEV protease protein band intensity with RSM method

In this step, the three most effective additives on TEV protease solubility were used to design new lysis buffers.

Table 5. Lysis buffers that designed by response surface methodology (Box-Benhken) method.

Run	Proline (mg/mL)	Sodium selenite (mg/mL)	$CuCl_2$ (mg/mL)
1	100	15	10
2	500	5	10
3	100	10	5
4	500	10	15
5	300	5	15
6	300	15	15
7	300	10	10
8	500	15	10
9	100	10	15
10	300	10	15
11	500	10	5
12	300	15	5
13	300	5	5
14	100	5	10
15	300	10	10

In the RSM method (Box-Benkhen), each additive studied in three levels; high level, low level, and middle level. Additives concentrations are shown in Table 4 and designed lysis buffers with these three additives are shown in Table 5.

Table 6. Analysis of protein band intensity by ImageJ software in step 2.

Samples	Total	Soluble
1	22541.46	3868.79
2	41455.61	4140.34
3	13198.02	2123.62
4	35939.79	1255.51
5	11095.12	1385.41
6	38933.47	1945.31
7	34758.18	2062.48
8	19611.17	2245.13
9	41792.61	2820.79
10	37770.77	3554.21
11	9979.81	1287.06
12	20675.43	550.85
13	20420.87	697.58
14	20420.14	1447.99
15	31363.20	1353.16

Protein was extracted in lysis buffers based on concentrations shown in Table 5 and the obtained solutions (total and soluble) were loaded on SDS-PAGE (Fig. 3) to analyze protein band intensity by Image J software. The results of protein band analysis are shown in Table 6.

Optimization of additives concentration in lysis buffer to obtain TEV soluble

Finally, in the last step, the most effective additives on TEV protease solubility were used to optimize protein solubility. The optimized lysis buffer was prepared with proline, sodium selenite, and $CuCl_2$ (the concentrations were 100, 5, and 5 mM, respectively). It is shown in Table 7. Also, an optimized lysis buffer designed by Plackett-Burman is shown in Table 8.

Proteins extracted in lysis buffers designed by RSM and Plackett-Burman (data is shown in Tables 7and 8) and obtained solutions (total and soluble) loaded on SDS-PAGE. The results of this procedure and the results of protein band intensity analysis are shown in Fig. 4 and Table 9, respectively. The reason why we used Plackett-Burman again, is explained in the discussion section.

Table 7. Optimized lysis buffer suggested by Box-Benhken method.

Proline (mM)	Sodium selenite (mM)	$CuCl_2$ (mM)	Intensity	Desirability
100	5	5	4195.020	1.000

Table 8. Optimized lysis buffer designed by Plackett-Burman method. The concentration of variables is molar.

Trehalose	Glycine betaine	Mannitol	Sodium citrate	Proline	L-Arginine	Xylitol	Sodium selenite	Dipotassium phosphate	$CuCl_2$	CTAB	Band intensity	Desirability
0.256	0	0	0	0.5	0	1	0.005	0	0.005	0.01	12692	0.848

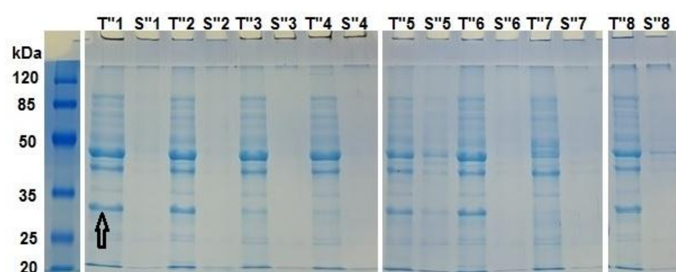

Fig. 4. SDS-PAGE image of step 3, optimization of additives concentrations to make TEV-protease more soluble after extraction. T" and S" show total and soluble samples, respectively. T"4 and S"4 are protein band extracts that their lysis buffer was designed by RSM method (Table 8), T"5 and S"5 are protein bands extracts that its lysis buffer was designed by Plackett-Burman method. Numbers 6, 7 and 8 show protein extraction in the presence of lysis buffer without additives, BL21 bacteria without TEV-protease genome, and extraction of protein in the presence of PBS as lysis buffer, respectively. SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RSM, response surface methodology; TEV, *Tobacco etch* virus protease.

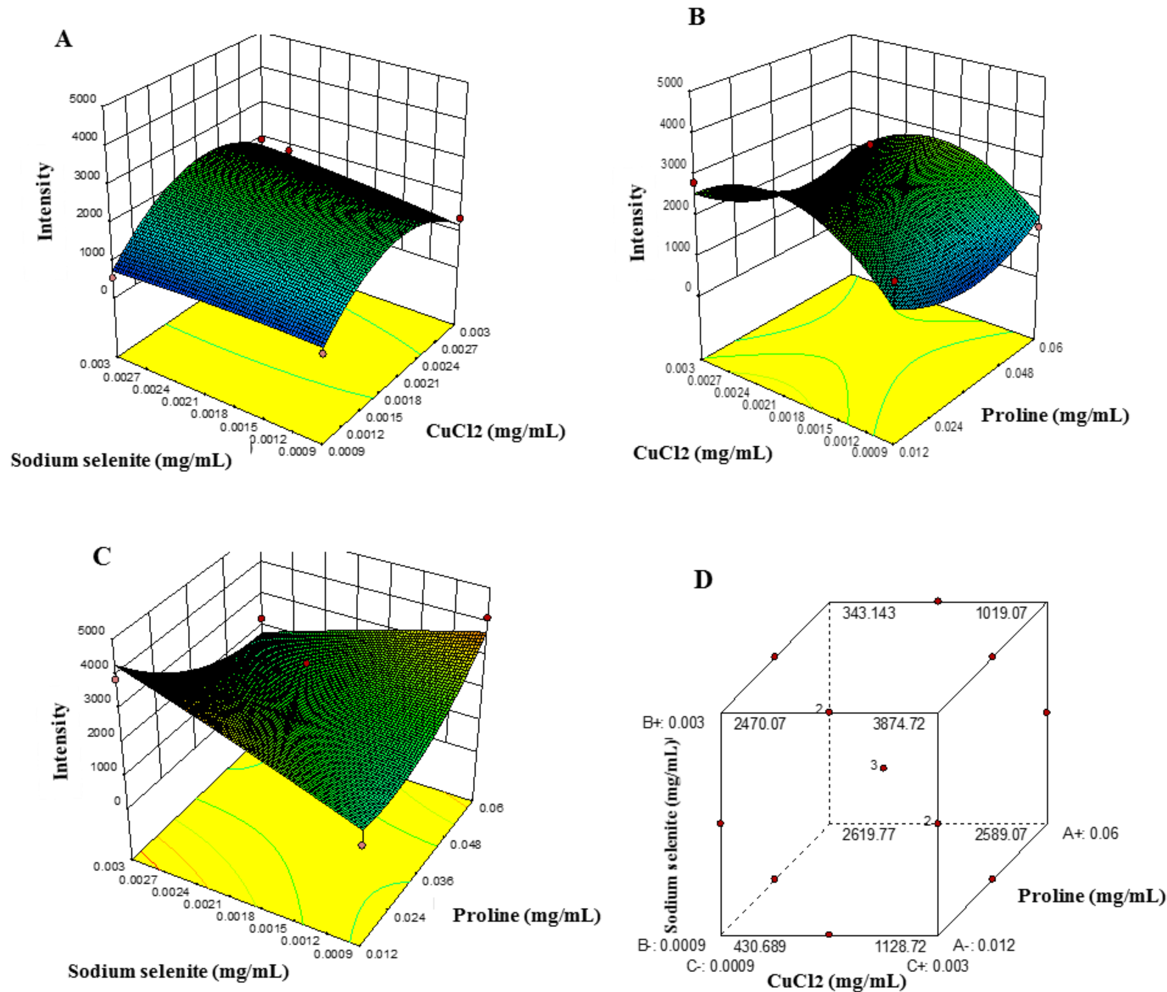

Fig. 5. 3D charts of protein band intensity (soluble part) in the Box-Benhken method showing the interaction between two factors by keeping other factors constant. (A) Sodium selenite and CuCl2, (B) proline and CuCl2, (C) proline and sodium selenite, (D) cube chart of intensity in the presence of all three variables

DISCUSSION

Tag fusions are peptide segments that are added to proteins for various reasons such as enhancing protein expression, solubility and efficacy, facilitation of refolding, and prevention of proteolysis (1,26). Also after protein production and extraction, it is important to detach the protein tag to obtain a functional native protein. One of the enzymes used for this purpose is TEV protease that has great benefits which make it worthy to study more and more (27,28). Despite its benefits, TEV protease has some deficiencies such as low solubility. Controlled expression, co-expression with chaperones, and changes in lysis buffer additives are suggested methods for enhancing its solubility. Most studies on enhancing TEV protease solubility were conducted by adding fusion tags to the protein and mutations on TEV protease DNA sequence (6,29), while few studies examined the effect of host expression and conditions of culture media like temperature and IPTG concentration (24,30). On the other hand, some studies have used additives and small molecules for enhancing protein solubility. It has been recently reported that when trehalose or sorbitol or L-arginine added to a growth culture, two insoluble proteins appear soluble (31). One proposed theory is that protein is produced in a form that host could use it to survive, and the functional form of most proteins is their soluble form. Because of the difference in the extraction environment as compared to the cytosol, proteins may turn to functionally inactive inclusion bodies (23). Therefore, the selection of suitable lysis buffers can help to avoid inclusion body production or aggregation after extraction.

In the present study, eleven additives with the best effect on recombinant protein solubility were selected to study their effects on TEV protease solubility (23). First, all eleven additives were tested. Then three more effective additives on TEV solubility were studied to obtain the best concentration of them (according to TEV solubility enhancement) in lysis buffer. Our results demonstrated that proline had the most positive effect, about 13.81%, on TEV solubility and its higher concentrations were even more effective on protein solubility.

Results in step 2 showed a significant decrease in protein band intensity, and it does not mean that L-proline, sodium selenite, and $CuCl_2$ have not any effect on protein solubility, it means that additives interaction and effectiveness on each other and extracted protein may not correlate with analyzing by statistical software equations (Fig. 5). So, we returned to step 1 (Plackett-Burman method) and optimized conditions according to this method (all eleven additives were involved in this method). The outcome of Plackett-Burman design was obtaining some soluble protein.

In this study, we tried to introduce a suitable lysis buffer for the extraction of TEV protease and reduce inclusion body production and aggregation after extraction. However, software forecast (Plackett-Burman and RSM) did not prove validity but it seems that about 1.18% (ratio of protein band intensity in the soluble sample to total sample in the original buffer without any additives) of the protein was found as soluble and this ratio in the optimized lysis buffer designed by Plackett-Burman method and not RSM was about 27.68%, a 2345.76% improvement of solubility.

CONCLUSION

In conclusion, the designed experiments in the present study were successful in providing a lysis buffer composition that could enhance the solubility of the obtained TEV protease. Further studies on the production of this recombinant protease to obtain a reasonable amount of the soluble protein are ongoing.

ACKNOWLEDGMENTS

The content of this paper is extracted from the Pharm. D thesis submitted by Niloufar Mohammadian which was financially supported by the Vice Chancellery of Research of Isfahan University of Medical Sciences through Grant No. 396905.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest in this study.

AUTHORS' CONTRIBUTION

H. Mirmohammad Sadeghi and H Mohammadian designed the study. N Mohammadian, H. Mohammadian, F. Moazen, and M. Pakdel contributed to the experiments and the results were verified by N. Mohammadian and H. Mohammadian. The results were analyzed by H. Mohammadian and H. Mirmohammad Sadeghi. N. Mohammadian and H. Mohammadian prepared the first draft of the manuscript. All the authors read the manuscript and it was finalized by A. Jahanian-Najafabadi and H. Mirmohammad Sadeghi. H Mirmohammad Sadeghi and A. Jahanian-Najafabadi finalized the manuscript corresponding to the comments raised by the reviewers and editor.

REFERENCES

1. Terpe K. Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. Appl Microbiol Biotechnol. 2003;60(5):523-533.
   DOI: 10.1007/s00253-002-1158-6.
2. Esposito D, Chatterjee DK. Enhancement of soluble protein expression through the use of fusion tags. Curr Opin Biotechnol. 2006;17(4):353-358.
   DOI: 10.1016/j.copbio.2006.06.003.
3. Sun C, Liang J, Shi R, Gao X, Zhang R, Hong F, *et al*. Tobacco etch virus protease retains its activity in various buffers and in the presence of diverse additives. Protein Expr Purif. 2012;82(1):226-231.
   DOI: 10.1016/j.pep.2012.01.005.
4. Lundbäck AK, van den Berg S, Hebert H, Berglund H, Eshaghi S. Exploring the activity of tobacco etch virus protease in detergent solutions. Anal Biochem. 2008;382(1):69-71.
   DOI: 10.1016/j.ab.2008.07.018.
5. 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: 10.1110/ps.072822507.

6. Tropea JE, Cherry S, Waugh DS. Expression and purification of soluble His(6)-tagged TEV protease. Methods Mol Biol. 2009;498:297-307. DOI: 10.1007/978-1-59745-196-3-19.
7. Waugh DS. TEV Protease FAQ. National Cancer Institute, Macromolecular Crystallography Laboratory. 2010. Available from: https://mcl1.ncifcrf.gov/waugh_tech/faq/tev.pdf.
8. Blommel PG, Fox BG. A combined approach to improving large-scale production of tobacco etch virus protease. Protein Expr Purif. 2007;55(1):53-68. DOI: 10.1016/j.pep.2007.04.013.
9. Jenny RJ, Mann KG, Lundblad RL. A critical review of the methods for cleavage of fusion proteins with thrombin and factor Xa. Protein Expr Purif. 2003;31(1):1-11. DOI: 10.1016/s1046-5928(03)00168-2.
10. Phan J, Zdanov A, Evdokimov AG, Tropea JE, Peters HK, Kapust RB, *et al*. Structural basis for the substrate specificity of tobacco etch virus protease. J Biol Chem. 2002;277(52):50564-50572. DOI: 10.1074/jbc.M207224200.
11. de Marco A, Deuerling E, Mogk A, Tomoyasu T, Bukau B. Chaperone-based procedure to increase yields of soluble recombinant proteins produced in *E. coli*. BMC Biotechnol. 2007;7:32-40. DOI: 10.1186/1472-6750-7-32.
12. Papaneophytou CP, Kontopidis G. Statistical approaches to maximize recombinant protein expression in *Escherichia coli*: a general review. Protein Expr Purif. 2014;94:22-32. DOI: 10.1016/j.pep.2013.10.016.
13. Sørensen HP, Mortensen KK. Soluble expression of recombinant proteins in the cytoplasm of *Escherichia coli*. Microb Cell Fact. 2005;4(1):1-8. DOI: 10.1186/1475-2859-4-1.
14. Sørensen HP, Mortensen KK. Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. J Biotechnol. 2005;115(2):113-128. DOI: 10.1016/j.jbiotec.2004.08.004.
15. Czitrom V. One-factor-at-a-time versus designed experiments. Am Stat. 1999;53(2):126-131.
16. Frey DD, Engelhardt F, Greitzer EM. A role for "one-factor-at-a-time" experimentation in parameter design. Res Eng Des. 2003;14:65-74. DOI: 10.1007/s00163-002-0026-9.
17. Noguère C, Larsson AM, Guyot JC, Bignon C. Fractional factorial approach combining 4 *Escherichia coli* strains, 3 culture media, 3 expression temperatures and 5 N-terminal fusion tags for screening the soluble expression of recombinant proteins. Protein Expr Purif. 2012;84(2):204-213. DOI: 10.1016/j.pep.2012.05.011.
18. Salehinia J, Sadeghi HMM, Abedi D, Akbari V. Improvement of solubility and refolding of an anti-human epidermal growth factor receptor 2 single-chain antibody fragment inclusion bodies. Res Pharm Sci. 2018;13(6):566-574. DOI: 10.4103/1735-5362.245968.
19. Akbari V, Sadeghi HM, Jafarian-Dehkordi A, Chou CP, Abedi D. Optimization of a single-chain antibody fragment overexpression in *Escherichia coli* using response surface methodology. Res Pharm Sci. 2015;10(1):75-83.
20. Xie L, Hall D, Eiteman M, Altman E. Optimization of recombinant aminolevulinate synthase production in *Escherichia coli* using factorial design. Appl Microbiol Biotechnol. 2003;63(3):267-273. DOI: 10.1007/s00253-003-1388-2.
21. Bezerra MA, Santelli RE, Oliveira EP, Villar LS, Escaleira LA. Response surface methodology (RSM) as a tool for optimization in analytical chemistry. Talanta. 2008;76(5):965-977. DOI: 10.1016/j.talanta.2008.05.019.
22. Mandenius CF, Brundin A. Bioprocess optimization using design-of-experiments methodology. Biotechnol Prog. 2008;24(6):1191-1203. DOI: 10.1002/btpr.67.
23. Leibly DJ, Nguyen TN, Kao LT, Hewitt SN, Barrett LK, Van Voorhis WC. Stabilizing additives added during cell lysis aid in the solubilization of recombinant proteins. PLoS One. 2012;7(12):e52482,1-13. DOI: 10.1371/journal.pone.0052482.
24. Miladi B, Bouallagui H, Dridi C, El Marjou A, Boeuf G, Di Martino P, *et al*. A new tagged-TEV protease: construction, optimisation of production, purification and test activity. Protein Expr Purif. 2011;75(1):75-82. DOI: 10.1016/j.pep.2010.08.012.
25. Mahmood T, Yang PC. Western blot: technique, theory, and troubleshooting. N Am J Med Sci. 2012;4(9):429-434. DOI: 10.4103/1947-2714.100998.
26. Costa S, Almeida A, Castro A, Domingues L. Fusion tags for protein solubility, purification and immunogenicity in *Escherichia coli*: the novel Fh8 system. Front Microbiol. 2014;5:63,1-20. DOI: 10.3389/fmicb.2014.00063.
27. Puhl AC, Giacomini C, Irazoqui G, Batista-Viera F, Villarino A, Terenzi H. Covalent immobilization of tobacco-etch-virus NIa protease: a useful tool for cleavage of the histidine tag of recombinant proteins. Biotechnol Appl Biochem. 2009;53(3):165-174. DOI: 10.1042/BA20080063.
28. Cesaratto F, Burrone OR, Petris G. Tobacco Etch Virus protease: A shortcut across biotechnologies. J Biotechnol. 2016;231:239-249. DOI: 10.1006/abio.1994.1060.
29. van den Berg S, Löfdahl PA, Härd T, Berglund H. Improved solubility of TEV protease by directed evolution. J Biotechnol. 2006;121(3): 291-298. DOI: 10.1016/j.jbiotec.2005.08.006.
30. Xiao J, Burn A, Tolbert TJ. Increasing solubility of proteins and peptides by site-specific modification with betaine. Bioconjug Chem. 2008;19(6):1113-1118. DOI: 10.1021/bc800063k.
31. Singh LR, Dar TA, Rahman S, Jamal S, Ahmad F. Glycine betaine may have opposite effects on protein stability at high and low pH values. Biochim Biophys Acta. 2009;1794(6):929-935. DOI: 10.1016/j.bbapap.2009.02.005.