Large Scale Production of Soluble Recombinant Staphylokinase Variant from Cold Shock Expression System using IPTG Inducible *E. coli* BL21(DE3)

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Abstract

Staphylokinase and its variant (Staphylokinase-Hirulog), the third generation thrombolytic molecule plays an significant role in treating the acute myocardial infarction and stroke which are leading causes of morbidity and mortality across the globe. In refolding of the staphylokinase and its variant from industrially feasible pET28a+, gradual decrease in concentration of urea was used to solubilise the protein. But urea is a chaotropic denaturant, which unravel the structure of proteins by destabilizing internal, non-covalent bonds between atoms. In this study, we selected the expression system to have the increased soluble fraction of sak and its variant from IPTG inducible expression host Escherichia coli BL21(DE3). The expression level and solubility of recombinant sak and its variant from cold shock expression vector pCOLDI was induced at the low temperature (15°C) was highly contrast to the induction process at 37 °C from pET28a+, which gives insoluble fractions. Recombinant sak and its variant were expressed primarily as soluble protein using pCOLDI, later purified with Nickel-chelating resin (Ni-NTA) and the quantity of the purified protein was 913 mg/L. Soluble fractions of purified sak variant having the fibrinolytic activity of 21,825 U/ml and specific anti-thrombin activity of 1,200 ATU/mg. For the first time soluble fractions was achieved instead of inclusion bodies without compromising the quantity and activity of the protein and working with urea was excluded.

***Keywords:** Staphylokinase-hirulog, urea, IPTG, soluble form, pCOLD I, fibrinolytic and anti-thrombin activity*

1. Introduction

Ischaemic heart diseases and cerebro vascular diseases are the leading causes of death and disability around the globe. 12.8 % of mortalities are by ischaemic heart diseases. Blood clot dissolving agents are used to dissolve the thrombus has gained lot of importance in present day research. Now a day's clinically approved clot busters like t-

PA, Urokinase (UK), Streptokinase (SK), APSAC, Tenecteplase, Reteplase are in use for the patients who were alleged from acute myocardial infarction and stroke, because the use of alternative thrombolytics are still in process of getting approval [1]. On the repeated administrations of the sk, immune responses are predictable and leads to anaphylaxis and also on the other hand reduced efficacy for clot lysis was observed on booster doses. Hence researchers looked into other alternatives.

Mature staphylokinase (mSAK) devoid of signal peptide is an extracellular bacterial protein consists of 136 amino acids is a promising blood clot dissolving agent [2]. It is potent as t-PA (tissue type–plasminogen activator) in the treatment of coronary artery recanalization [3, 4]. Sak is a potent and safe thrombolytic agent. It dissolves the clot in a fibrin-specific manner without disturbing the systemic fibrinogen and plasma proteins [5, 6]. Sak is not an enzyme and it is a profibrinolytic agent that forms a 1:1 stoichiometric complex with plasminogen, converts to plasmin. Later the plasmin activates other plasminogen molecules to form plasmin [7]. Staphylokinase induces fibrinolysis without fibrinogen depletion with elevated fibrinolytic activity compared with other plasminogen activators like streptokinase, urokinase and tissue plasminogen activator [8]. Compared to other thrombolytics, staphylokinase (sak) could be relatively inexpensive and can be scaled up into large amounts for industrial production [9].

The expressed sak–hirulog was accumulated as inclusion bodies from pET28a+ and to obtain the active molecule, preliminary the sak variant was refolded [10]. Most of the bacterial therapeutics are produced in the form of inclusion bodies. In the process of solubulization, the activity of the protein may decrease drastically. On the other hand, while using, patients are alleged from severe complications. So, the present study aimed at cloning and high level expression of soluble recombinant staphylokinase–hirulog using cold shock expression system from IPTG inducible expression host *E. coli* BL21(DE3).

2. Material and Methods

2.1. Microbial Cultures and Plasmids

E.coli strains like DH5α and BL21 (DE3) were used in the present study. The former culture was used as the maintenance host and the later was used for the expression of the heterologous recombinant fusion protein. DH5α and BL21 (DE3) were procured from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. Cold shock expression vector pCold I was procured from TAKARA Biosciences, Japan and pET28a+ were procured from Novagen. All media ingredients were procured from Fischer Scientifics, Mumbai. Restriction enzymes like *Bam*HI *E.co*RI and *Hind*III were procured from Fermentas. The oligos were procured from Sigma-Aldrich, Bangalore.

2.2. Construction of Sak Variant and Cloning

Total genomic DNA was isolated from S*taphylococcus aureus* and the primers were designed according to the information from GenBank data base (gi 21283614). The oligos were designed to get the mature staphylokinase (420 bp) from the full length gene by truncating the signal peptide with the help of PCR techniques with a set of 24 nt forward primer: CGC**GGATCC**TCAAGTTCATTCGAC and 27 nt reverse primer: CCC**GAATTC**TTTCCTTTCTATAACAAC. The PCR program was set to be 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 61 °C for 1 min, 72 °C for 1 min) and final extension of 72 °C for 15 min. The N–terminus of the mature sak was created with *Bam*HI and C–terminus was created with *Eco*RI.

Hirulog (FPRPGGGGNGDFEEIPEEYL), the synthetic gene codes for antithrombin activity contains 60 bp (5' TTTCCGAGACCGGGCGGCGGCGGCAATGGCGATTTTGAGGAG ATTCCAGAGGAGTACTTG 3') was constructed with the overlapping strategy using

a set of 48 nt forward primer 5' CCC**GAATTC**TTTCCGAGACCGGGCGGCGGCGGCAATGGCGA TTTTGAG 3' and 39 nt reverse primer 5' GGG**AAGCTT**CTAAAACTCCTCTAAGGTC TCCTCATGAAC 3'. The N–terminus of the hirulog was created with *Eco*RI and C–terminus was created with *Hind*III.

On ligation, the N and C–terminus of the construct (staphylokinase – hirulog) was created with *Bam*HI and *Hind*III respectively for easy ligation into the MCS region of highly expressible and easy purification vectors like pET28a+ and pCOLD I with T4 DNA ligase. Further, the ligation samples were transformed into maintenance host. The ligation reaction was confirmed by ligation PCR with gene specific forward primer and vector specific reverse primers and by transformation into *E.coli* DH5α.

2.3. Expression and Activity Analysis of Sak Variant

The heterologous staphylokinase variant expressed from the chosen bacterial host system. The recombinant bacterial expression system *E. coli* BL21(DE3) was grown in LB medium with appropriate antibiotic of kanamycin (50µg/ml) for pET28a+ vector, when the cell density reached between 0.8 to 1.0, the BL21(DE3) was induced with 1mM IPTG and incubated at 37 ^{0}C for 4 hours. A different strategy was followed for expression of chimeric protein from bacterial culture holds the recombinant pCOLD I. When the cell density reaches to 0.8 – 1.0, the culture was refrigerated at 15^{0}C for 30 minutes, later induced with 1mM IPTG and incubated at 15 ^{0}C for 24 hours. After induction, both the uninduced and induced samples of pET28a+ and pCOLDI were pelletted down and suspended in PBS (pH 8.0). The protein profiles were analyzed on 12% SDS-PAGE against a standard protein marker.

2.4. Soluble form of the Protein

In order to know the soluble form of the protein, after induction, 50 ml of the two induced cell cultures (Msak–Hirulog pET28a+ *E. coli* BL21(DE3) and Msak-Hirulog pCOLDI *E. coli* BL21(DE3) were pelleted down separately and STE buffer (100 mM NaCl, 10 mM Tris–HCl, 1 mM EDTA) was added to the pellets and mixed well. The samples were centrifuged at 4 ^{0}C at 10,000 rpm for 10 min and supernatants obtained were transferred into a microfuge tube labelled as S–1. In continuation, NTE buffer was added to the pellet and subjected to sonication (10 cycles on time, 10 cycles off time and 5 min of total time). The sonicated samples were centrifuged at 10,000 rpm for 10 min at 4 ^{0}C and the supernatant was transferred into a fresh microfuge tube labelled as S–2. NTET buffer (100 mM NaCl, 10 mM Tris–HCl, 1 mM EDTA, 1 % Triton X–100 and 2 M Urea) was added to the pellet and mixed well, centrifuged to obtain the supernatant and pellet. This supernatant was labelled as S–3 and solubulizing buffer (50 mM NaH_2PO_4, 20 mM Tris, 100 mM NaCl and 8 M Urea) was added to this pellet and labelled as S–4. Protein analysis was done with the help of 12 % separating gel to all the tubes labelled tubes S1, S2, S3 and S4 of four samples.

2.5. Purification of Recombinant Sak Variant

The chimeric protein carrying an N–terminal 6x His tag was expressed in *E. coli* BL21(DE3) and purified using affinity chromatography with Ni2+ - resin under hybrid conditions [11]. In order to purify chimeric protein, 800 mg wet cells were obtained from a freshly grown 100 ml culture in LB and LB medium without NaCl, containing the appropriate antibiotics. The cells were harvested and centrifuged at 10,000 rpm for 10 min at 4 °C and the pellet attained was suspended in 12 ml of guanidinium lysis buffer containing 7 M guanidine HCl, 22 mM sodium phosphate, 510 mM NaCl with pH 7.8 at 4°C for 1 hr. The cell lysate was again centrifuged at 13000 rpm for 10 to 15 min at 4 °C.

A volume of 10 ml cell lysate was purified on a column containing 2 ml resin equilibrated with denaturing binding buffer, incubated for 45 min at room temperature with gentle hand shaking for several times. The column was washed with 6 ml of denaturing wash buffer and twice with 6 ml of native wash buffer. The bounded protein was eluted with 5 ml of native elution buffer and analysed on 12 % separating gel against to protein marker.

2.6. Fibrinolytic Activity of Soluble Sak–Hirulog

Fibrinolytic activity of soluble staphylokinase variant was determined on a fibrin plate containing 0.8 % (w/v) agarose, 0.8 NIH U/ml of thrombin, 12 nM of hPg and 0.5 mg/ml of bovine fibrinogen. 25 micro liters of samples were added to the wells on the plate and incubated at 37 °C for 12 h. Specific activities of staphylokinase and its variant were obtained from the standard curve. The diameter of digested fibrin on the plate was considered as the measurement of fibrinolytic activity of the soluble form of the enzyme. The international units (U/ml) of the enzyme activity of protein was measured by the mean diameter of the zone of clearance around the well according to the standard curve plotted using the other thrombolytic protein streptokinase.

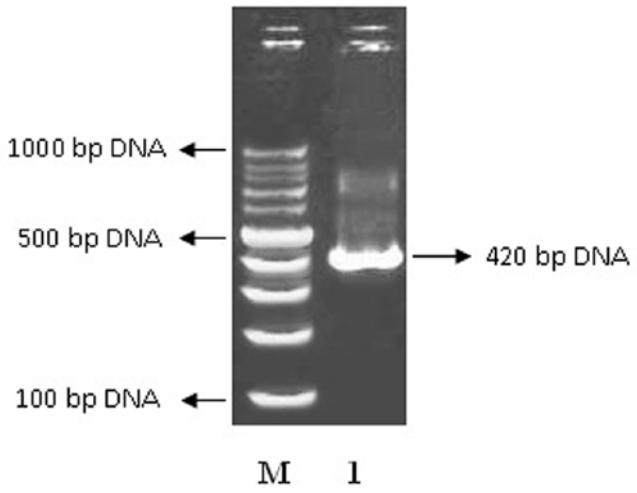
2.7. Antithrombin Activity of Sak–Hirulog

The antithrombin activity of Sak–hirulog was determined by the degree of chromogenic substrate S-2238 cleavage inhibition. Then, 1.5×10^{-4} mSak - hirulog was incubated with 2.1×10^{-7} M thrombin in 50 µl Tris–buffered saline, containing 0.1 % BSA, pH 7.4, for 1 hr at 37 ^{0}C. The thrombin activity was measured by the increase of absorbance at 405 nm in a microplate reader, during its incubation with 300 µM substrate S-2238 for 10 min at 37 ^{0}C [12]. The hirulog activity was also confirmed by the thrombin clot assay. 50 µl of thrombin (2 × 10–13 M) was added to 50 µl of r–Hir, mSak–Hir, in an assay buffer containing 0.12 M NaCl, 0.01 M sodium phosphate, 0.01 % NaNO3, 0.1 % BSA, pH 7.4. After 5 min of mixing at room temp., 100 µl of human plasma diluted 1: 10 was added and mixed for 20 sec. The turbidity of the clot was monitored in a microplate reader (EL 340 BIO-TEK Instr.) at 405 nm at 0, 15, 30 and 60 min.

3. Results and Discussion

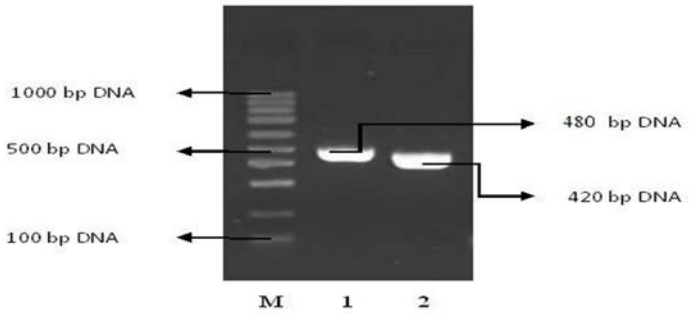
3.1. Generation of Chimeric Staphylokinase and Production of r – DNA

The PCR amplified Msak gene was confirmed on 1.2 % agaorse gel against to the low range DNA ladder. A product of 420 bp DNA (Figure 1) was observed. The desired product size of 480 bp DNA (Figure 2) of constructed Msak–Hirulog was identified against low range DNA ladder with gene specific primers. A size of 797 bp and 699 bp (Figure 3) products were observed from pET28a+ and pCOLDI against 100 bp DNA ladder with vector specific primers.



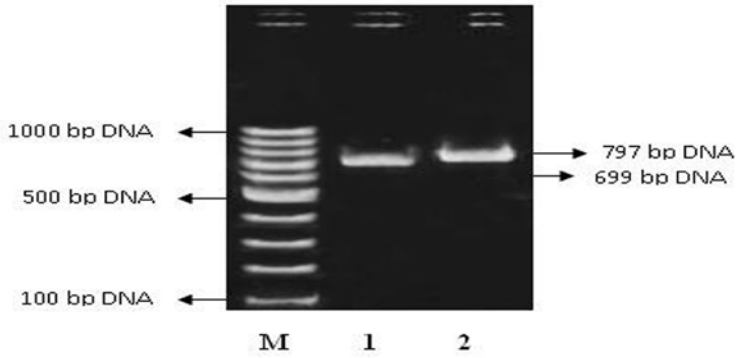
M = Low range DNA ladder
1 = Mature staphylokinase gene

Figure 1. PCR Amplified Mature Staphylokinase Gene against 100 bp DNA Ladder



M = low range DNA ladder
1 = Construct (mature staphylokinase + hirulog gene)
2 = Mature staphylokinase gene

Figure 2. Analysis of Chimera and Staphylokinase Gene against to 100 bp DNA Ladder



M = Low range DNA ladder
1 = 699 bp ligation product from pCOLD I (498 bp sak variant + 201 vector DNA)
2 = 797 bp ligation product from pET28a+ (498 bp sak variant + 299 vector DNA)

Figure 3. Confirmation of Recombinant DNA's against to 100 bp DNA Ladder

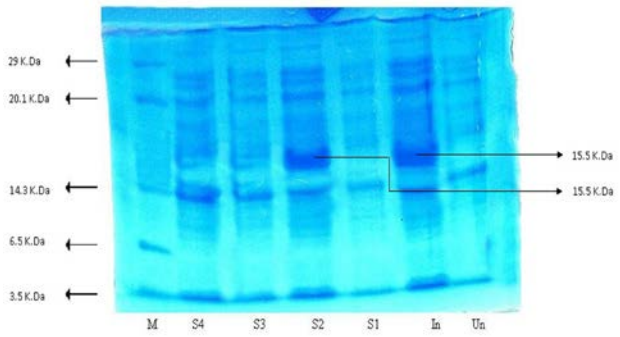
3.2. Expression and Purification of Sak–Hirulog

After confirmation of r-DNA, *E. coli* expression host BL21 (DE3) was used for the better expression of the constructed recombinant Sak–hirulog. The sak variant gene containing colonies were picked up and grown up to 0.6–0.8 O.D with appropriate antibiotic and induced the recombinant cultures of pCOLD I and pET28a+ BL21(DE3) with appropriate quantity of inducer from 1M IPTG. For recombinant pCOLD I, when the OD reaches to 0.6 – 0.8, the culture was refrigerate at 15 ^{0}C for 30 min, after then induced with 1 mM IPTG.

After induction the cell pellets from recombinant pCOLDI BL21 and pET28a+ BL21 were suspended in PBS and protein expression profiles were resolved on 12 % separating gel (Figure 4). 1mM IPTG induction in BL21(DE3) was identified as the best inducer concentration. The induction time of 4 hours was identified as the optimal time for the inducer for the maximal production of recombinant protein. All protein expressions were analyzed by running on 12 % SDS-PAGE and recombinant protein bands were observed against to the standard protein marker. *E. coli* expression host systems are extensively used in the production of recombinant proteins.

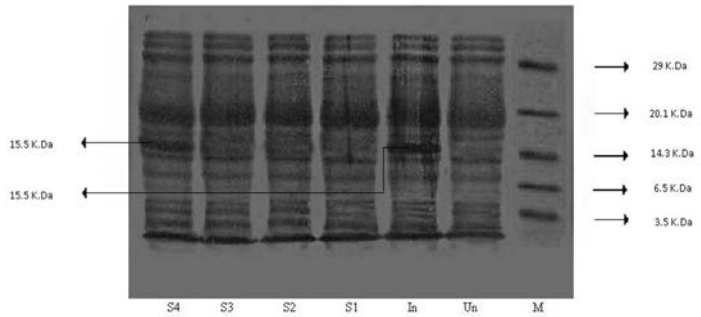
Even though there is very good expression from pET28a+ (Figure 5), but due to its insoluble form pCOLDI is preferred, pCOLDI BL21 producing chimeric protein at low temperatures (Cold-shock expression vector pCOLDI was designed to perform efficient protein expression utilizing the promoter derived from *cold shock promoter* A gene). To get the protein in soluble form with reasonable cost, industries require cold shock expression vectors to reduce the production cost.

Sak expression was carried out in different expression host systems like *E.coli* (13, 14, 15), *Bacillus subtilis* (16), *Streptomyces lividans* (17). In this study the yield of recombinant sak and sak variant (mSAK-hirulog) were estimated as 957mg/L and 913 mg/L. The expression of recombinant staphylokinase as inclusion bodies from *Escherichia coli* BL21 was producing 20 mg/L (18), 200 mg/L fermentation broth (6), 300 mg/L (20) 70 to 500 mg/L (21) and 2.8 g/L of fermentation broth (22).



M = Protein marker
S4 = Fraction 4
S3 = Fraction 3
S2 = Soluble fraction 2
S1 = Soluble fraction 1
In = Induced culture of sak-hirulog pCold I BL21 (DE3)
Un = Uninduced culture of sak-hirulog pCold I BL21 (DE3)

Figure 4. SDS PAGE Analysis of Sak Variant from pCold I



M = Marker protein
S4 = soluble fration 4
S3 = soluble fraction 3
S2 = soluble fraction 2
S1 = soluble fraction 1
In = Induced culture of sak-hirulog pET28a+ BL21 (DE3)
Un = Uninduced culture of sak-hirulog pET28a+ BL21 (DE3)

Figure 5. SDS PAGE Analysis of Sak Variant from pET28a+

3.3. Fibrinolytic and Antithrombin Activity

The fibrinolytic activities of SAK and soluble sak-hirulog (Figure 6) were measured as 21,910 U/ml and 21,825 U/ml respectively. There was no significant difference in the fibrinolytic activity of SAK and its variant. Hence, it suggests the chimeric protein mSAK-hirulog having the same fibrinolytic activity like mature staphylokinase even after addition of hirulog even expressed in soluble form. The antithrombin activity of chimera protein was about 1,200 anti thrombin activity units (IU/mg). Chromogenic assay results shown the 1.8 X 10^{-3} M of mSAK- hirulog was sufficient to inhibit the action of thrombin.

S = 20 micro liters of standard streptokinase
A = 10 micro liters of standard streptokinase
B = 20 micro liters of soluble fraction of sak
C = 20 micro liters of soluble fraction of sak – hirulog
D = 20 micro litres of insoluble fractions of sak – hirulog
E = 10 micro liters of purified soluble fractions of sak – hirulog

Figure 6. Fibrinolytic Activity of Sak and Sak-Hirulog from pCOLD I and pET28a+

The nature of fibrinolytic activity of the soluble protein and its variant on fibrin plates was similar to that of sak and its variant produced from pET28a+. The fibrinolytic activity

was higher than the commercially available streptokinase and hence it has got prospective use in the treatment of myocardial infarction. Staphylokinase, the fibrinolytic protein may play a very important role in saving the humankind from blood clot related diseases and less side effects. So, in future it would be ideal drug which passes the toxicological tests and clinical trials.

4. Conclusion

Now a days thrombolytic therapy requires an ideal drug against to thrombosis. The recombinant bacterial protein produced by r-DNA technologies has more scope in the healing of cardio and cerebrovascular diseases. As developments in molecular cloning, chimeric staphylokinase has been produced in soluble form in order to minimize drawback of native staphylokinase as inclusion body from industrially feasible vector pET28a+.

Hence in the present study the soluble form of the sak variant was achieved from cold shock expression system with retained fibrinolytic property like staphylokinase and the variant is also shown the antithrombin activity. The purified protein having the molecular weight of 15.5 kDa on 12 % SDS-PAGE. Still lot of research have to be done to minimize the risks during thrombolytic therapy. In future it will be ideal drug for the patients who need thrombolytic therapy.

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References

[1] M. L. Simoons, A. P. Maggioni, G. Knatterud, J. D. Leimberger, P. Dejaegere, R. Van Domburg, E. Boersma, M. G. Franzosi, R. Califf and R. Schröder, “Individual risk assessment for intracranial haemorrhage during thrombolytic therapy”, Lancet, vol. 342, **(1993)**, pp. 1523-8.

[2] D. Collen and H. R. Lijnen, “Review: Staphylokinase, a fibrin-specific plasminogen activator with therapeutic potential?”, Blood, vol. 84, **(1994)**, pp. 680-686.

[3] P. W. Armstrong, J. Burton, S. Pakola, P. G. Molhock, A. Betriu, M. Tendera, C. Bode, A. Adgey, F. Bar, A. Vahanian and F. Van der Werf, “Collaborative angiographic patency trial of recombinant staphylokinase (Captors II)”, Am Heart, vol. 146, **(2003)**, pp. 484-488.

[4] R. W. Moreadith and D. Collen, “Clinical development of PEGylated recombinant staphylokinase (PEG-SAK) for bolus thrombolytic treatment of patients with acute myocardial infarction”, Adv Drug Deliv Rev., vol. 55, **(2003)**, pp. 1335-1345.

[5] D. Collen, F. De Cock and J. M. Stassen, “Comparative immunogenicity and thrombolytic properties toward arterial and venous thrombi of streptokinase and recombinant staphylokinase in baboons. Circulation”, vol. 87, **(1993)**, pp. 996-1006.

[6] B. Schlott, M. Hartmann, K. Gührs, E. Birch-Hirschfeid, H. D. Pohl, S. Vanderschueren, F. Van de Werf, A. Michoel, D. Collen and D. Behnke, “High yield production and purification of recombinant staphylokinase for thrombolytic therapy”, Biotechnology, vol. 12, **(1994)**, pp. 185-189.

[7] H. R. Lijnen, B. Van Hoef, F. D. Cock, K. Okada, S. Ueshima, O. Matsuo and D. Collen, “On the mechanism of fibrin-specific plasminogen activation by staphylokinase”, J. Biol. Chem., vol. 266, **(1991)**, pp. 11826-11832.

[8] S. Vanderschueren, L. Stockx, G. Wilms, H. Lacroix, R. Verhaeghe, J. Vermyle and D. Collen, “Thrombolytic therapy of peripheral arterial occlusion with recombinant staphylokinase”, Circulation, vol. 92, **(1995)**, pp. 2050-2057.

[9] D. Behnke and D. Gerlach, “Cloning and expression in Escherichia coli, Bacillus subtilis and Streptococcus sanguis of a gene for staphylokinase-A bacterial plasminogen activator”, Mol gen genet., vol. 210, **(1987)**, pp. 528-534.

[10] A. Cheng, G. Song, H. B. Su, M. Yu, Y. Y. Li and H. Y. Song, “The renaturation and purification of RGD-Staphylokinase by gel filtration”, Chinese journal of biotechnology, vol. 18, no. 6, **(2002)**, pp. 693-7.

[11] G. Zhong, Y. U. Aiping, B. Shi, Y. Liu and C. Wu, "Pilot-Scale production and purification of a staphylokinase based fusion protein over-expressed in Escherichia coli", Front, Biol, China, vol. 4, no. 1, **(2009)**, pp. 75-81.
[12] K. Riesbeck, D. Chen, G. Kemball-Cook, J. H. Mc Vey, A. J. T. George, E. Tuddenham, A. Dorling and R. Lechler, "Expression of hirudin fusion proteins in mammalian cells-A strategy for prevention of intravascular thrombosis", Circulation, vol. 98, **(1998)**, pp. 2744-52.
[13] M. Moussa, M. Ibrahim, M. El Ghazaly, J. Rohde, S. Gnoth, A. Anton, F. Kensy and F. Mueller, "Expression of recombinant staphylokinase in the methylotrophic yeast Hansenula polymorpha", BMC Biotechnology, vol. 12, **(2012)**, pp. 96.
[14] D. Ren, D. Li, W. Yang, Y. Li, X. Gou, B. Liang, B. Li and Q. Wu, "Novel preparation protocol for the expression and purification of recombinant staphylokinase", Biotechnol, Appl. Biochem., vol. 51, **(2008)**, pp. 9-13.
[15] T. Sako, "Overproduction of staphylokinase in Escherichia coli and its characterization", Eur. J. Biochem, vol. 149, **(1985)**, pp. 557-563.
[16] R. Q. Ye, J. H. Kim, B. G. Kim, S. Szarka, E. Sihota and S. L. Wong, "High-level secretory production of intact, biologically active staphylokinase from Bacillus subtilis", Biotechnol, bioeng., vol. 62, **(1999)**, pp. 87-96.
[17] Y. Cheng, Y. Li, B. Liu and L. Guo, "Cloning and secretory expression of staphylokinase in Streptomyces lividans", zhongguo yi xuekexue yuan xuebao, vol. 20, **(1998)**, pp. 428-432.
[18] S. J. Lee, C. Kim, D. M. Kim, K. H. Bae and S. M. Byun, "High level secretion of recombinant staphylokinase into periplasm of Escherichia coli", Biotechnol, Lett., vol. 20, **(1998)**, pp. 113-116.
[19] T. Nguyen and D. Thi Quyen, "Cloning, high-level expression, purification and characterization of a staphylokinase variant, sakØc, from staphylococcus aureus QT 108 in Escherichia coli BL2", African Journal of Biotechnology, vol. 11, no. 22, **(2012)**, pp. 5995-6003.
[20] B. Schlott, K. H. Gührs, M. Hartmann, A. Röcker and D. Collen, "Staphylokinase requires NH_2-terminal proteolysis for plasminogen activation", J. Biol. Chem., vol. 272, **(1997)**, pp. 6067-6072.
[21] N. Mandi, S. Soorapaneni, S. Rewanwar, P. Kotwal, B. Prasad, G. Mandal and S. Padmanabhan, "High yielding recombinant staphylokinase in bacterial expression system: Cloning, expression, purification and activity studies", Protein Exp. Purif., vol. 64, **(2009)**, pp. 69-75.

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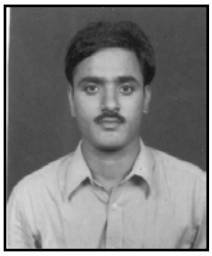

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