|| Volume 5 || Special Issue SBPCOE || June 2020 || ISSN (Online) 2456-0774
INTERNATIONAL JOURNAL OF ADVANCE SCIENTIFIC RESEARCH
AND ENGINEERING TRENDS

AUTO INDUCED EXPRESSION OF RECOMBINANT STAPHYLOKINSE ACHIEVED BY CHANGING CONCENTRATION OF LUREIA BERTENIA BROTH COMPONENTS

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Abstract: Development in molecular biology have greatly streamlined process of protein expression. One such development is autoinduction of recombinant proteins during fermentation step of bacterially derived protein expression. It gives ease of operation as there is no need to monitor cell growth. This system often increases cell mass along with target protein yield. Many efforts have gone into specialized media preparation to promote autoinduction of recombinant proteins. The current work focuses on changing the concentration of Luria Bertani broth components to achieve autoinduction. This phenomenon was demonstrated using staphylokinase (SAK) as representative protein. The SAK gene from S.aureus was 411 bp and yielded a protein of 15 kDa when expressed using pET21a vector in E. coli. BL21 (DE3) cells. The recombinant SAK (rSAK) was soluble in nature. Fermentation studies were carried out to optimize various parameters (media optimization, elimination of inducer, autoinduction time optimization, etc.) for maximizing the yield of rSAK. When optimal concentration of tryptone and yeast extract was used during fermentation process, the protein expression was achieved without using IPTG. The rSAK yield through autoinduction was comparable to the protein expression when inducer was used during fermentation. The protein was purified by ion exchange chromatography using single step purification. The thrombolytic activity of the purified rSAK was successfully demonstrated.

Keywords: *Staphylokinase, Autoinduction, Hyperexpression, Single step protein purification without tag.*

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I INTRODUCTION

Staphylokinase (SAK) is developing as thrombolytic agent. Staphylokinase (SAK), a 163 amino acid chain from Staphylococcus sp. is a probable alternative plasminogen catalyst [1]. Staphylokinase forms 1:1 stoichiometric complex with plasmin, which triggers conversion of plasminogen to plasmin [2,3] which aids in clump lysis by its proteolytic action [4] on fibrin, a key player in thrombus formation [5].

The high affinity of the SAK– plasminogen complex for fibrin makes it a promising thrombolytic agent. Streptokinase (SK) remains the most widely prescribed thrombolytic agent for acute myocardial infarction (AMI) Tissue plasminogen activator (t-PA), a serine protease has been extensively studied in relation to coagulation and thrombolysis [3]. SAK has higher fibrin specificity than t-PA, it makes it capable of dissolving platelet rich blood clots [4] and hence is a promising thrombolytic agent [5].

A bacterial system is the commonly used expression system for production of recombinant products such as proteins, enzymes, and antibodies. Escherichia coli DH5α is one of the most commonly used bacterial hosts for the production of bacterial recombinant protein products. A variety of recombinant products such as human growth hormone, lipase, coagulase, β- lacatmase, etc. have been produced and cultivation studies have been carried out at small scale. However, the majority of recombinant proteins are not amenable directly to large scale production processes due to various factors like optimization of seed culture conditions by refining media composition, induction time and inducer as well as different types of promoter systems are discussed in previous work[6].

Albeit it has been proven that lacUV5 promoter is tremendously torpid after cAMP- mediated depression, at the stationary phase of the growth cycle then the glucose level is low,each wild type or lacUV5 promoters come to be sensitive with cAMP-mediated depression [9]. The

auto-incitement technique is a helpful and proficient strategy. In autoinduction system, glucose is serves as the repressor of lac operon which helps in suppression of induction of lac promoter until it is available in the media. During the protein generation stage, glycerol serves as the carbon source after glucose is drained whereas lactose serves as the inducer. Consequently, heterologous gene expression is repressed during the cell growth stage and automatically induced by lactose after glucose exhaustion, resulting in little leaky expression during the initial cell growth stage, which thus diminishes cell development inhibition [10].

Recombinant proteins produced by using bacterial expression system, they are in two states. Properly folded proteins are in soluble form, while partially folded proteins tend to form inclusion bodies as they are in insoluble form. To remove out the impurities and to get theseproteins in ideal structure, they need several purification processes. The cost of these purification processes is very high and they are also very time consuming. To overcome this problem we have developed a method called as SP-bind elute method (Sepharose bind elute method). By using this method protein purification was obtained in a single step. In the previous studies, it was observed that the proteins which are purified in single step using affinity chromatography need affinity tag.These affinity tags are very costly. In this study, it was pointed out that when Sp bind elute ion exchange chromatography is used for protein purification, the need for an affinity tag is eliminated.

Here, we report the cloning of the SAK gene from Staphylococcus aureus under T7 promoter based vector (pET21a) along with optimization of its expression in E. coli. BL21(DE3) using optimized autoinduction parameters. We have achieved purification of bacterial derived rSAK to homogeneity using single step ion-exchange chromatography and the activity is reported in this communication.

II .MATERIALS AND METHODS

Synthetic SAK gene was obtained from GenScript,(USA). Host strainBL21 (DE3) cells were procured from (--)India. pET21a vector DNA was from Invitrogen. DH5α cells from (--) all PCR reagents and restriction enzymes were procured from (Thermo Scientific).NaCl, Na2HPO4, D-glucose, glycerol, IPTG are from Thermo Fisher and agarose from Hi media, PCR product purification kit and SDS–PAGE reagents were from Qiagen, Sigma and Merck. Fermentation media were from Hi-Media and the ion-exchange resins (Q-Sepharose and SP- Sepharose) were gifted from Lupin Pharma(India).

2.1 Design of oligonucleotides, PCR amplification and cloning

Oligonucleotides were designed with suitable restriction enzyme sites. The forward primer was with a NdeI site and the reverse primer with a HindIII site. The Forward primer sequence was (PCD8): 5' CCG CCG GAA TTC CAT ATG TCA AGT TCA TTC GAC 3' while the reverse primer sequence was (PCD9): 5' CCG CCG GAA TTC AAG CTT TTA TTT CTT TTC TAT AAC 3'. The mature peptide of the SAK gene was amplified by PCR performed in 50ul volume by initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 56°C for 30 s and 72 °C for 30 s. After a final extension at 72 °C for 7 min, the PCR product was gel purified using PCR purification kit(--) reagents. The purified PCR product, after digestion with NdeI and HindIII, was ligated to pET21a vector digested with the same set of enzymes, for 16 h at room temperature. The ligation mix was then transformed into competent cells of E.coli DH5α prepared using Hanahan's method [19] and the recombinant clones were screened by colony PCR for SAK gene using SAK specific primers.The PCR program was set as mentioned before. Further, the cloned plasmid was sequenced by Sanger's method to reconfirm the presence of the cloned gene.

Post confirmation, the cloned plasmid was transformed into competent E.coli BL21(DE3) cells (expression host) using CaCl2 method[19].

2.2 SAK expression studies at shake flask level

The colonies of pETSAK were then inoculated separately in 10ml Luria-Bertani broth (LB-10 g/L Tryptone, 5 g/L Yeast extract, 5 g/L NaCl) with ampicillin (100 µg/ml) and and incubated at 37 °C with shaking at 160 rpm. The cultures were induced with 1 mM IPTG at A600 =0.8 for 4 h at 37 °C. The induced cultures were then subjected to bead lysis to separate soluble and insoluble fractions. Both the fractions were later run on a 12% SDS–PAGE gel and the gel visualized after Coomassie G-250 staining [18].

LB medium was used for cultivation of the seed from the stock culture. Two media were tested for

production of SAK at shake flask level LB and optimal medium (25 g/L Yeast extract, 14 g/L Tryptone, and 5g/L NaCl).

2.3 Identifying the inducing component of the Optimal LB media for autoinduction phenomenon

All the genes under study exhibited expression in E.Coli.BL21(DE3) cells in modified LB media without addition of any additional specific inducer. Individual constituents from modified LB media were tested in LB to determine the component responsible for such an auto induction phenomenon. Here we carried out autoinduction of protein expression in modified 1% LB medium that containing 1.5%, 2%,2.5%,3% and 4% YE and 1.2%,1.4% and 1.6% Tryptone. The optimum protein yield was observed by studying the expression profile in BL21(DE3) cells and was analyzed by SDS-PAGE in 2.5% yeast extract and 1.4% tryptone. The auto induction standard media was prepared with this observation, which contained 2.5% YE, 1.4% Tryptone and 0.5% NaCl.

2.4 Auto induction time optimization

Transfer 2.5ml of overnight inoculum (pETSAK in standard LB) in 50 ml of optimized media supplemented with 50ul of ampicillin and was incubated for 2 hours.An aliquote of 12 ml sample was collected after 4, 8, 12 and 24 hrs of incubation. The samples were centrifuged at 15000 rpm for 10 min. The cell pellet was re- suspended in 1.5ml cold lysis buffer (10mM Tris-Cl pH 8.0) and disrupted by bead lysis in an ice-bath. After lysis, the pellet and supernatant were separated by centrifugation and were loaded on 12% SDS-PAGE gels for visualization of induced protein.

2.5Protein purification by Ion exchange chromatography and its analysis

Proteins were further purified by Ion Exchange Chromatography using SP bind elute method.

2.5.1. SP Bind Elute method:

The soluble cell lysate was centrifuged at 14,000 rpm for 20 min to remove the cellular debris and the supernatant was used as the starting material for purification studies. The supernatant was dialyzed against 50 mM citric acid-phosphate buffer, pH 5.0 overnight in cold and loaded onto a cation exchanger (SP–Sepharose). The column was eluted by a linear gradient of 0.2–0.8N NaCl. Elution of the bound proteins was carried out by using a linear gradient of NaCl solution (0.4–1 N). The purity of rSAK was checked by loading the protein on a 12% SDS–PAGE gel and visualizing it using silver staining.

2.6 Protein Activity

Anticoagulant activity was checked by adding purified enzyme in to a eppendorf tube containing human blood and result was observed. To check the its thrombolytic activity, the enzyme was added into the coagulated blood and lysis of blood clot was observed.

III.RESULTS

3.1 Design of oligonucleotides, PCR amplification and cloning.

The SAK gene was amplified in PCR using an annealing temperature of 56°. A custom synthesis SAK gene was used as a template for this reaction. An amplicon of 411bp was observed(Fig.1A) and negative control did not show any reaction.

This amplicon was digested with the enzyme NdeI ,HindIII and purified.The pET21a vector digested with same set of the enzymes was ligated to this amplicon and transformed into E.Coli. DH5α cell line, upon transformation luxurious growth was observed on the positive control plate but negative control did not have colonies, on the V+I plate 12 colonies was observed out of this 4 well isolated colonies was screen using colony PCR of SAK gene.

The amplicon of SAK gene was observed in all four reactions(Fig.1B) out of which high intensity band was observed from colony no.2(pETSAK2), hence this colony was selected for expression studies.

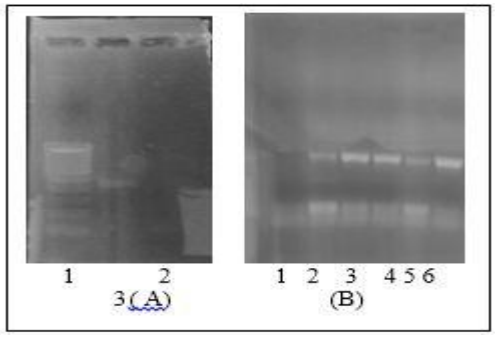

Fig.1(A). PCR amplification of SAK gene.

Lane 1:100bp marker. Lane 2:SAK PCR product ,Lane 3:Negative control Fig.1(B).Colony PCR of SAK gene. . Lane 1:Negative,Lane2: 1stcolony, Lane3:2nd colony ,Lane 4: 3rdcolony, Lane5: 4thcolony, Lane 6: Reference of SAK PCR product.

3.2 SAK expression studies at shake flask level

The plasmid of pETSAK2 was transformed into E.Coli BL21(DE3). A 10 mL shake flask level fermentation of pETSAK2 BL21(DE3) was carried out using optimal media. The soluble and insoluble fraction of the cellular protein obtained upon bead lysis were loaded on a 12% SDS PAGE gel. A hyper expressed protein band of ~15kDa was observed in both the protein samples. Thus the SAK gene was cloned successfully and gave a hyper expression

3.3 Identifying the inducing component of Optimal LB medium for autoinduction phenomenon.

Yeast extract is an yield defined nutrient component that probably contents trase amounts of lactose which can serve as an inducer for protein expression in recombinant organism hence it is expected that an increase the concentrations of YE in the medium would affect protein expression.

The expression was studied in optimal media containing 1.5%,2% 2.5%, 3% , 4% among this best expression of rSAK was observed that 2.5% YE[(Fig3.3 A).

The rSAK protein expression was also studied at various concentrations of tryptone viz.1.2%, 1.4%, 1.6%. the highest intensity of protein expression was observed 1.4% (Fig3.3B).

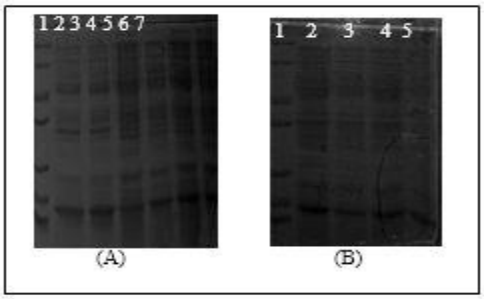

Fig.2A)The effect of concentration of YE and tryptone.

Panel (A) The effect of concentration of YE . M: medium molecular weight marker (14–116kDa),lane 1:Marker, lane 2: Std, lanes 3:1% YE, Lane 4: 1.5% YE, Lane 5: 2%YE,Lane 6: 2.5% YE, Lane 7:4% YE. Panel (B) The effect of concentrations of tryptone. lane 1:M,lane 2: 1% tryptone, lane 3: 1.2% tryptone, lane 4: 1.4% tryptone, lane 5: 1.6% tryptone.The effect of different concentration of yeast extracts and tryptone.

3.4 Auto induction time optimization.

A shake flask level fermentation of pETSAK BL21(DE3) was set, using the optimized media component concentrations (2.5% YE, 1.4%tryptone,0.5% NaCl) eight such experiments was started and each of this was stoped at varied autoinduction time viz. 2hours,3hours,4hours,5hours,6hours,7hours,8hours,10hours. The rSAK protein obtained after cell lysis was elecrophoresed on 12% SDS PAGE gel. Five hours of autoinduction time(fermentation duration) was found to give the highest level protein expression(Fig 3A&B).

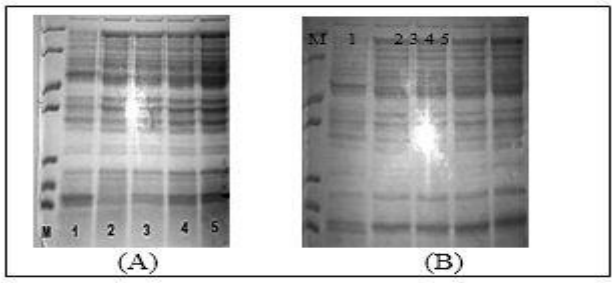

Fig.3.The effect of time in autoinduction.

Panel(A) Lane M:M, lane 1: IPTG Induced, lanes 2: 2hrs autoinduced,Lane 3:.3hrs autoinduced, Lane 4: 4hrs autoinduced, Lane 5: 5hrs autoinduced. Panel (B) Lane M: M, Lane 1: IPTG Induced, Lane 2: 6hrs autoinduced, Lane 3: 7hrs autoinduced, Lane 4: 8 hrs autoinduced, Lane 5: 10hrs autoinduced.

3.5 Protein purification by Ion exchange chromatography and its analysis.

The rSAK protein was purified from the soluble fraction obtained upon bead lysis pETSAK BL21(DE3) culture. SP bind elute chromatography was utilize this purpose as explained in the method (Sec.2.5.1). the purified fractions were loaded on 12% SDS PAGE gel along with mid range protein marker. 85-90% protein purification was observed on elution with 0.8N NaCl using sapharose cation exchange column(Fig.4)

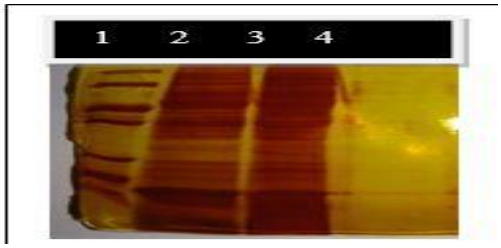

Fig 4.The protein purification.
Lane1:Marker,Lane2:Load,Lane3:Flow through,Lane 4:SP Bind-Elute

3.6 Protein Activity

The activity of purified protein was confirmed using clot prevention assay and thrombolytic assay. In the first case freshly drawn human blood (devoid of any anticoagulant)was added to varying quantities of the purified enzyme (Fig 5A), the time required for the blood to clot was noted in each case. He result have been displayed in (Table no.1)

In the thrombolytic asset a drop of freshly drawn blood was added in four eppendorf tube and was allow to coagulate, post coagulation varied volumes of purified enzyme(10ul,20ul,40ul)were added in three tubes were as one tube without enzyme served as negative control(Fig.5B) The time required for the purified enzyme to lysed blood coagulam was noted down in each case the result are displayed in (Table no.2).

In the clot prevention assay it was observed that as the quantity of purified enzyme increased, the time required for clot formation increased.in the thrombolytic assay as the enzyme concentrations increased time required for clot lysis decreased,thus the enzyme purification was efficient and yielded a highly potent enzyme.

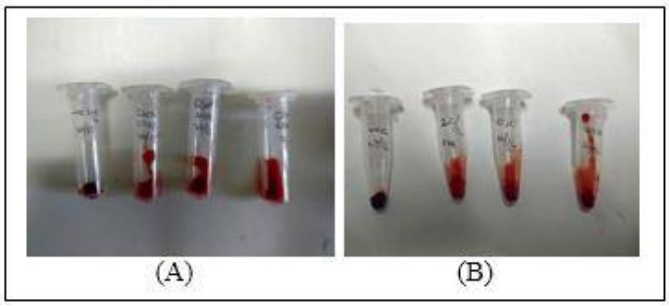

Fig 5.The Staphylokinase activity assay.

Fig 5(A). Protein activity using human blood fibrin. a) set 1 – assay to check clotting time; tube 1 - negative, tube 2to4: 5µl, 10 µl, 20 µl enzyme action respectively. (B) set 2 – assay to check enzyme potency; tube 1- negative, tube 2 to 4- 10 µl, 20 µl, 40 µl enzyme action respectively.

Table No. 1. Set 1 – clot prevention assay

Enzyme	Clotting Time
Positive control	1.44 mins
5 µl	11.32 mins
10 µl	21 mins
20 µl	More than 1 hour

Table No.2. Set 2–Enzyme potency/clot lysis assay

Enzyme	Enzyme potency
Negative control	Blood clot
10 µl	5.10 mins
20 µl	3 mins
40 µl	1.45 mins

IV. DISCUSSION

E.Coli. is most commonly used expression system to get higher yield of recombinant proteins because it is very easy to handle and is economical. The pET-21a vector offers T7 phage based expression system suitable for recombinant protein expression in bacterial host. Although, the T7 promoter is strong promoter and T7 RNA polymerase is highly processive and specific, this system has some limitations due to leaky expression of target proteins, in the absence of an inducer.

Fu et al. [7] has shown that the inclusion of high amount of YE increases the yield of recombinant protein expression, without any inducer addition and the reasons for such observations have not been clear till date.

As it was seen that pET21a-SAK recombinant could give autoinduction of protein in presence of optimal LB medium(Fig.4)], it was essential to optimize the time required for the optimal expression of the protein which will match with the expression of induced proteins in LB medium . The protein was present in the soluble fraction which indicates that it is maintaining it's native structure. When expression host BL21(DE3) carrying recombinant SAK was allowed to grow in modified LB medium in the absence of inducer and the protein expression was checked at various time points, it was observed that, the expression was initiated after 3 hours. Interestingly, the recombinant protein expression increased along with time and after 5 hours it was comparable to induced protein with IPTG.

To check the thrombolytic activity of expressed proteins they were purified with the help of ion- exchange chromatography. Chen et al. have described the purification of SAK using three column chromatographic steps namely cation, gel filtration and anion exchangers [17]. Ren et al. [11] protocol describes the use of expensive affinity chromatography matrices like Ni2+ agarose and enterokinase cleavage for rSAK purification. All the described protocols were expensive, laborious and cumbersome to use at large scale manufacturing. This

prompted us to develop novel, simple, cost-effective and scalable steps for purification of rSAK.

The cation exchange chromatography performed using the Sepharose resin was found to give 85- 90% protein purification in a single step. Furthermore this protein was found to be highly active (as confirmed by the clot prevention and thrombolytic assays). Thus here we report for the first time the single step purification of rSAK using cation exchange chromatography.

V. CONCLUSIONS

The key feature of the study is expression of the recombinant protein in pET21a system without inducer and with optimal LB medium which is not been reported earlier with Staphylokinase. Yeast extract and Tryptone are responsible for the induction of the protein in the absence of the inducer. Monitoring of the cell growth is not required in the autoinduction. The cost of fermentation will be significantly reduced as the requirement for IPTG has been eliminated.The major achievement of this research was that, the obtained recombinant enzyme could be purified upto 85-90% in single step purification without using affinity chromatography. This helped in developing a cost effective downstream process for the purification of Staphylokinase enzyme. The resultant recombinant enzyme was found to be highly active and showed anticoagulant and thrombolytic activity. This correlates to the practical application of the enzyme in healthcare and hospitals where the enzyme is used to lyse the blood clot and hence allow the blood flow. The therapeutic potential of the developed recombinant staphylokinase was established successfully.

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