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Production of Stable Serum Albumin Fused Streptokinase in *Pichia pastoris*

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ABSTRACT

Streptokinase (SK) is a thrombolytic and fibrinolytic protein (47kDa), naturally produced by beta-hemolytic streptococci. Purified SK is used for many blood circulatory complications, i.e., myocardial infarction, ischemic stroke and pulmonary embolism. In this study, human albumin fusion technology has been developed to increase the half-life *in-vivo* and also invoke less immune response. We designed codon-optimized HSA-SK fusion gene, integrated into -pPICZaB vector, cloned and transferred into *Pichia pastoris* strain GS115. The transformants showing highest resistance for Zeocin™ was selected for protein expression which produced to >350mg/L HSA-SK fusion protein. Further, affinity chromatography was carried out for the purification of the HSA-SK fusion protein. SDS-PAGE, western blot, and RP-HPLC analysis were also conducted, which confirmed the 98% purity of the product with 40% yeild having a molecular weight of almost 111kDa. The purified fusion protein (HSA-SK) was also verified via western blotting by using anti-streptokinase antibodies. Clot lysis assay was used to estimate the biological activity of HSA-SK. For half-life estimation, proteolytic and thermal stability of HSA-SK was also checked.

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ST experimental design, manuscript write up. NA idea conceived (project supervisor). MAK project supervisor. MA analysis of data, manuscript writing. RA performed cloning experiments. KM reviewed the manuscript.

Key words
Streptokinase (SK), Human Serum Albumin (HSA), Fusion protein, *Pichiapastoris* expression system, Affinity chromatography

INTRODUCTION

Streptokinase (SK) is an extracellular microbial protein of 47kDa which is mostly produce by beta-hemolytic streptococci. Its blood clot lysing activity was first reported by Tillett and Garner, (1933). SK also acts as an affordable drug for the clot lysis in case of myocardial infarction, pulmonary embolism, ischemic stroke and some other circulatory disorders (Sawhney *et al*., 2016). SK performs a role similar to the tissue plasminogen activator (TPA) secreted by the endothelial lining of human blood vascular system.

Normally, clot formation is inhibited by the several inhibitors TPA and plasminogen. During clot formation, activated platelets adhere to the blood vessel walls, degranulation and aggregate. The released pro-coagulant factors from platelets stimulate production of thrombin from prothrombin which further catalyzes the conversion of fibrinogen to fibrin that adds to the platelet plug stability resulting in clot formation. Clot formation is regulated naturally by various anti-coagulant factors that induce fibrinolysis (Palta *et al*., 2014). The abnormal clot formation in the body is usually caused by interruption of this process. Currently, albumin fusion technology has been used to develop high yield of biologically active proteins like SK for the dissolution of these clots (Naseem *et al*., 2020).

SK is one of the most widely used thrombolytic drug and fibrinolytic agent which has now been added to the WHO's published model list of essential medicines (WHO, 2011). Its production is ten times cheaper as compared to TPA, hence its use is more favored in developing countries. Besides this, the pure streptokinase has short half-life and it is cleared rapidly via renal route. Despite all these shortcomings, the use of SK cannot be minimized because of its easy availability and affordable pricing. Therefore, many experimental investigations are being carried out to reduce its immunogenicity and improve the half-life by several methods, i.e., protein engineering, mutation, encapsulation, and many others. Previous studies have shown an improved half-life by inducing certain mutations in SK (Pratap and Dikshit,

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1998). Conjugation of SK in liposomes (Kim *et al*., 1998) as well as in chitosan nanoparticles (Baharifar *et al*., 2020) has also been reported.

The concentration of SK bonded to anti-streptokinase antibody has relatively shorter half-life (18 min) as compared to the half-life of plasminogen bonded SK, i.e., 80 min. SK is metabolized in liver and eliminated by kidneys. Besides the short half-life and immunogenic nature, SK is still widely used in therapeutics. Many approaches such as encapsulation, protein engineering, PEGylation, etc. have been devised to reduce the immunogenicity and increase the half-life of SK. The technology for albumin fusion is under great concern to enhance the plasma retention properties of protein-based drugs. This technology is convenient because it is highly stable and its production is very easy. This process simply requires the fusion of human serum albumin (HSA) gene with the desired therapeutic agent (protein or a peptide) followed by cloning and producing the gene product which is recombinant protein with therapeutic potential, high stability and improve half-life. The first FDA approved albumin fusion protein drug was Albiglutide which is used for diabetes. The improved stability of protein such as hirudin (Syed *et al*., 1997) and consensus interferon (Naseem *et al*., 2020) has also been reported by adopting albumin fusion technology.

In this study, albumin fusion technology has been used for the formation of human serum albumin and streptokinase (HSA-SK) fusion protein using *Pichia pastoris* vector expression system. The cloning was carried out under the control of *AOX1* promoter and α-factor secretion signal. The most Zeocin resistant strains of transformants were selected and further mass cultured for the production of high level of HSA-SK fusion protein. Protein was purified by immuno-affinity chromatography with yielding 152mg/L and purity of 98% as revealed by SDS-PAGE and HPLC. The final fusion protein formed was also tested for its biological activity, stability and enhanced half-life.

MATERIALS AND METHODS

The synthesis of HSA-SK fusion gene and HSA-SK-pPICZαB expression system was conducted via Bio Basic Inc.® Canada and Biopharmaceutical lab at NCEMB, Pakistan respectively. *E. coli* strain TOP10F', *Pichia pastoris* strain GS115, vector (pPICZαB), transformation kit (Invitrogen, USA), PCR mix, capture select human serum albumin affinity matrix, DNA markers, restriction enzymes (Fermentas Thermo Scientifific, USA), T4 ligase (Enzynomics Co. Korea), and culture media reagents (Merk Biomedical, Sigma-Aldrich, USA) were the materials used.

Construction of codon optimized HSA-SK expression cassette

The amino acid sequences of human serum albumin (UniProt: P02768) and streptokinase (Uniprot: P00779) were retrieved from the Uniprot database. For the formation of the fusion protein, the amino-terminal of SK was fused with the carboxyl-terminal of HSA through a very small five amino acid long (Gly-Gly-Gly-Gly-Ser) flexible linker. At the same time, the synthesis of a codon optimized gene was conducted from Bio Basic Inc. For the cloning of this fusion gene, a yeast transfer vector (pPICZαB) was used. The fusion gene was inserted into this plasmid vector using two restriction sites, i.e., *Xhol* and *Xbal*. Bacterial expression cells (*E. coli* strain TOP10F) were first made competent followed by the transformation process in which the recombinant vector containing the fusion gene was transferred to the competent bacterial cells. Using the Luria Bertani agar, the transformed strains were selected on it after supplementing with Zeocin in 25 µg/ml concentration. Colony PCR was conducted for the confirmation of the positive transforments. It used specific primers for the respective plasmid. Moreover, sequence analysis of the fusion gene (HSA-SK) was also done by restriction based DNA sequencing to confirm its correct orientation within the plasmid.

Transformation of Pichia pastoris *GS115 with HSA-SK-pPICZαB expression cassette*

Linearization of recombinant HSA-SK-pPICZaB was carried out by digesting the plasmid with a restriction enzyme *Sac1*. Further, it was purified via the phenol-chloroform extraction method (Estrada *et al*., 1992; Malke *et al*., 2000; Assiri *et al*., 2014). The *P. pastoris* GS115 strain was made competent using *Pichia* EasyComp™ Kit (Invitrogen, USA; Catalog No. K173001), as indicated by the manufacturer, along with certain required modifications. The transformation mixture was then allowed to grow via spread plate method, on YPD agar media (2% dextrose, 2% peptone, 1% yeast extract, and 2% agar in pH 7.0) with supplementation of 100 µg/ml Zeocin™, maintaining a sterile environment throughout the procedure. After the incubation of 3-4 days at 30°C, colonies that were most prominent were selected, and colony PCR was done to confirm their transformation with HSA-SK fusion gene.

Transformants screening based on highest Zeocin™ resistance

The seed or primary cultures were prepared by taking 5ml YPD broth media (for each positive clone) with Zeocin™ (Conc. 200 µg/µl), inoculating the media with single positive clones, and giving an overnight incubation at 30°C in a shaking incubator (210-225

rpm). Each primary culture was diluted enough up to the final OD_{600} of 0.15 in 10 ml of YPD media having different concentrations of Zeocin™ (400, 600, 800, 1000, and 1200µg/ml, respectively). All these cultures were incubated for 11 to 12 h at 30°C in a shaking incubator (210-225 rpm). While incubation 500 µl of each sample was taken at intervals, i.e. after 3, 5, 7, 9 and finally eleven hrs, for the measurement of OD_{600}. Growth curves of these measurements were plotted on the graph by taking the time intervals at X-axis and OD_{600} values on Y-axis.

Expression studies of HSA-SK fusion protein

To analyze the extent of HSA-SK fusion protein expression, each primary culture was diluted in 25ml BMGY (2% peptone, 1% yeast extract, 100mM K2PO4, 1.34%YNB, 2% glycerol, and 4 x 1.05 % biotin in pH 6.0) media till the OD_{600} of the diluted culture became 0.2. This media was incubated for 24 hours at 30°C in a shaking incubator (200–225 rpm). After incubation, the culture was subjected to centrifugation at 3000 rpm for 10 min. The pellet containing cells was resuspended in 25ml of BMMY media (2% peptone, 1% yeast extract, 100mM K2PO4, 1.34%YNB, 2% methanol, and 4 x 1.05 % biotin in pH 6.0) in a 250ml flask and incubated at the same conditions for 96h along with the addition of methanol making 0.5% as final concentration. During the whole incubation period, 20µl of each culture was taken every 24 h and analyzed by 10% SDS-PAGE. The transformants showing the most increased production of HSA-SK fusion protein were then selected for the high-scale output of the desired protein.

Large scale fermentation

The large scale fermentation of the selected clone of *Pichia* GS115 was carried out by inoculating it in 500ml BMGY media in a 2L flask and incubated at 30°C in a shaking incubator (200-225rpm) till the final OD_{600} value of 6. After incubation, the culture media was centrifuged in sterile centrifugation containers at 3000rpm. The harvested cells were resuspended in 500ml of BMMY media in a 2L flask. It was incubated for 96h at 30°C in a shaking incubator (200-225rpm). During the incubation period, methanol was added at an interval of 24 h to get the final concentration of 0.5%.

Harvesting and diafiltration

After the fermentation of 96 h the culture broth was centrifuged at 9000rpm for about 20 min. The supernatant was then filtered using a 0.45µm Stericup™ filter unit (Millipore®, USA; Catalog No. AP20 075 00) via a vacuum pump. It was followed by the diafiltration of supernatant with 3L of phosphate buffer saline. Further, it was concentrated up to 50% by using a prep/ scale spiral cartridge of 5kD cutoff value via tangential flow filtration. For this, the rate flow of supernatant and PBS through the cartridge was kept 4ml/min and 1ml/min, respectively.

HSA-SK fusion protein purification

The fusion protein HSA-SK was purified via a one-step purification method using affinity chromatography column on ÄKTA Explorer system (GE Healthcare, USA). For chromatography, XK column packed with a matrix of Captureselect™ human albumin (Catalog no. 19129705 L, Thermo Scientific, USA). The column was first equilibrated, passing 10 column volumes of PBS buffer of pH 7.4 through the column at a flow rate of 5ml/min. The sample loading of diafiltred fusion protein was done on column at a flow rate of 2ml/min. Finally, the elusion was carried out using the elusion buffer (2M MgCl2, 20 mMTris and pH 7.4). All the fractions were collected for the analysis of purified protein by 10% SDS-PAGE and quantification by HPLC.

Analysis by SDS gel

The purified protein was subjected to SDS-PAGE analysis on a gel with 4% stacking and 10% resolving gel in Hoefer mini gel apparatus as mentioned by Cleveland *et al*. (1977). The loading sample was prepared by mixing 50 µl of the purified protein with 50 µl sample buffer (2x). After mixing, the sample was subjected to heat shock. For this, the sample vial was put into the boiling water for 5 min, and then vortex at 4°C for 5 min. Finally, 10µl of each sample was loaded and allowed to run in the gel, connected to a constant voltage source (110 V), for 120 min. The gel was first stained with Coomassie brilliant blue R-250 (0.25%) stain for the visualization of bands, while the de-staining was carried out by using methanol and acetic acid solution (40% methanol; 10% acetic acid).

Western blot analysis

After the appearance of purified protein band on 10% SDS-PAGE, it was transferred to Amersham™ hybond-C nitrocellulose membrane (GE Healthcare, Germany), by a semi-dry blotting process, in Hoefer blotting system (Hoffffer, USA). For this process, the nitrocellulose membrane was first blocked with skimmed milk; 5% skimmed milk (5g skimmed milk in 100 ml PBST) was applied on the membrane following its overnight incubation at 4°C. The blocked membrane was first washed with PBST three times and then incubated in a diluted solution of anti-SK monoclonal antibodies (1:500 with PBST) (ThermoFisher Catalog # PA1-85891) for 2 h at 25°C room temperature. After the incubation, the membrane was again washed three times with PBST and then incubated in a diluted solution of anti-mouse

conjugated antibody (1:10,000 in PBST) (Cat # sc-2005, Santa Cruz Biotechnologies Inc.) for one hour at room temperature. The membrane was again washed thrice with PBST, and kept in NBT/ BCIP (Thermo Fischer Scientific, Cat # 34042) substrate solution until purified protein bands appeared on it. Finally, distilled water was used for the final washing of the membrane.

RP-HPLC analysis

Liquid chromatography system from Shimazdu with C18 column from Dicovery (15cm x 4.6mm and 5µm particle size) was used for RP-HPLC analysis of Streptokinase and HAS-SK. Mobile phase A was prepared by addition of 1 mL trifluoro acetic acid, 300 mL of acetonitrile and remaining water up to 1L while composition of mobile phase B was 1 mL trifluoro acetic acid, 800 mL of acetonitrile and 199 mL of water with flow rate of 1 mL per min. 50µl (1mg per mL) of reference standard streptokinase from Merck Millipore (Cat No. Y0000868: European Pharmacopoeia (EP) Reference Standard) and purified streptokinase sample were injected and eluted by gradient elution; 28% B 0 min, 33% B 5 min s, 37% B 10 min, 43% B 10 min, 60% B 10 min, 60% B 2 min, 28% B 50 min, 28% B 60 min.

Biological activity assay of HSA-SK fusion protein

The evaluation of the biological activity of the purified HSA-SK fusion protein was done by clot lysis assay. Percentage lysis of the purified HSA-SK was carried out by taking 1ml fresh blood, from a healthy person, in twelve pre-weighed Eppendorf and incubating at 37°C for 1 h. The blood plasma was removed while leaving the blood clots behind in each Eppendorf of all the 12 Eppendorfs, five were treated with different concentrations of standard streptokinase, and the other five were treated with varying concentrations of the purified HSA-SK. In contrast, the rest of the two were treated with elusion buffer. After mixing, all the eppendorfs were incubated again at 37°C for 1 h. After the incubation period, the supernatant of all the tubes was discarded, leaving behind the lysed blood clots. The Eppendorf with treated blood clots were again weighed. The percentage lysis of the standard as well as purified HSA-SK was measured and compared by using the following formula:

Percentage lysis = 100-{[(W3- W1)/(W2-W1)]*100}

Where W1 is the weight of empty eppendorf, W2 is the weight of eppendorf containing untreated blood clots and W3 is the weight of eppendorf containing treated blood clots.

HSA-SK fusion protein proteolytic stability test

The proteolytic stability of the fusion protein (HSA-SK) was determined by comparing the anti-trypsin activity of HSA-SK and the standard SK. Trypsin solution of concentration 100mg/ml was prepared in ammonium bicarbonate buffer. The fusion protein (HSA-SK) and the standard streptokinases were separately mixed with trypsin by keeping the ratio of protein to trypsin 30:1. The reaction mixtures were incubated at 37°C for 15 h. During the incubation period, 50 µl samples from both solutions were taken out along with the addition of 20% trifluoro acetic acid (TFA) after an interval of one hour. All the samples were analyzed by RP-HPLC to detect the change in peaks with the passing time. The rate constant for this reaction was also calculated by assuming the reaction to be in first order kinetics.

HSA-SK fusion protein thermal stability test

A relative comparison of the thermal stability of purified fusion protein with the standard SK was also evaluated by placing both of them in a water bath at temperature 50°C and measuring the turbidity of sampled for every 5 h at 340nm.

RESULTS AND DISCUSSION

HSA-SK-pPICZαB expression cassette in P. pastoris *GS115*

The fusion gene (HSA-SK) was cloned in the expression vector pPICZαB between *XhoI* and *XbaI* restriction sites downstream to α-secretion signal. Restriction digestion and DNA sequencing of the resulting expression cassette (HSA-SK-pPICZαB) were conducted to confirm the in-frame ligation and the sequence of the fusion gene in the vector. HSA-SK-pPICZαB diagram was also designed *insilico*, using SnapGene® cloning tool (Fig. 1). The competent *P. pastoris* cells GS115 were transformed with the expression cassette, after its linearization via *Sal1*. The transformed cells were incubated on 100 µg/ml Zeocin™ containing YPD media for 3 days at 30°C to allow the prominent growth of transformants, further confirmed by colony PCR.

It is evident from the previous studies (Nordén *et al*., 2011; Naseem *et al*., 2020) that the highest resistance toward an antibiotic Zeocin™ indicates the production of the high level of protein of interest in the transforments (*P. pastoris*). It can be considered a criterion to screen out the clone producing highest level of fusion protein (HSA-SK) among all the 6 clones. For this purpose, all of the six selected clones were allowed to grow in YPD media containing different concentrations of Zeocin™ antibiotic, i.e., 400µg/ml, 600µg/ml, 800µg/ml and 1200 µg/ml respectively and incubated. While incubation, optical density for each culture was a measure to plot the growth curves of all clones. Figure 2 shows that clone 1, among

the six selected clones, showed the best growth rate in all Zeocin™ concentration.

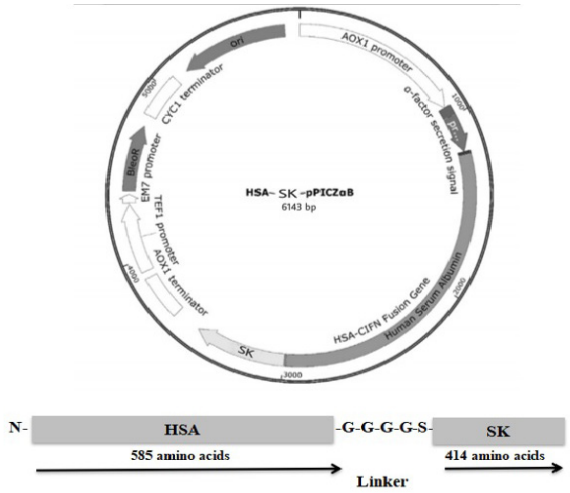

Fig. 1. Codon optimized HSA-SK gene in pPICZαB expression cassete (B) HSA-SK fusion gene graphical demonstration.

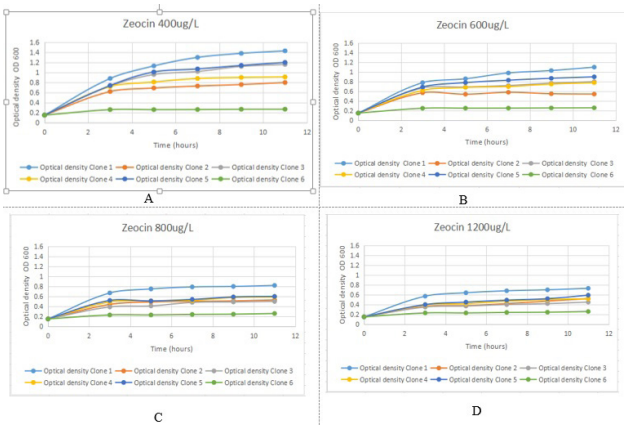

Fig. 2. The productivity curves of transformed *P. pastoris* GS115 strains at different time intervals in gradually increasing Zeocin™ concentration i.e., A. 400 µg/µl B. 600 µg/µl C. 800 µg/µl and D. 1200 µg/µl concentration of Zeocin™.

Expression and purification of HSA-SK fusion protein

The fusion protein expression analysis of each clone was also carried out. The primary culture biomass of each selected clones was first subjected to 4 days incubation in BMMY broth media. The incubated cultures for all the respective clones were separately centrifuged and their supernatants were checked for the presence of fusion protein HSA-SK concentration via SDS-PAGE analysis. Figure 3A represents the expression extent of HSA-SK fusion protein in the form of band, stained by Coomassie brilliant blue (R-250). The lane 1 does not show the formation of any band because its respective culture microbe contained an empty vector pPICZαB i.e., without the instructions of any protein formation. The bands appeared at almost 113kDA which is approximately equal to the theoretical molecular weight of HSA-SK. Among all the six clones, the clone 1 showed highest results for gel scanning assay by Image J software (NIH, USA) as indicated in Figure 3B. It can be estimated by the results that resistance toward increasing Zeocin™ concentration is directly proportional to the fusion protein expression, reported by previous studies as well (Werten *et al*., 1999; Nordén *et al*., 2011).

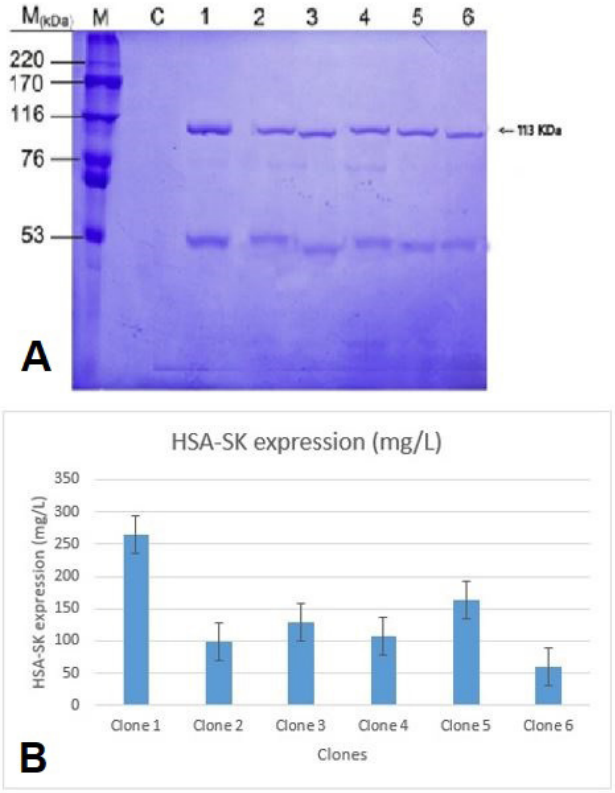

Fig. 3. The expression analysis of six HSA-SK-pPICZαB transformant clones of *P. pastoris* GS115 using 10% SDS-PAGE. Lane M shows the protein marker, Lane C contain a control (*P. pastoris* GS115 strain transformed with a simple pPICZαB vector), and lanes 1-6 indicate HSA-SK-pPICZαB expression vector containing *P. pastoris* GS115 clones. The bands lie quite close to the expected HSA-SK value with clone 1 representing comparatively thicker band. B, A band intensity based estimation of HSA-SK fusion protein by analyzing the high definition image of SDS PAGE stained by CBB stain.P. pastoris clone 1 represented the highest expression level of HSA-SK gene i.e., more than 350mg/L.

Table I. Purification scheme of HSA-SK fusion protein.

Purification step	Volume (ml)	Protein Conc. (mg/L)	Total protein (mg)	Yield (%)	Bioactivity (IU/ml)	Total bioactivity	Specific activity (IU/mg)	Purity (%)
Supernatant	1000	0.22	220	88	N.D	N.D	N.D	-
Dial filtration and concentration	487	0.44	214.28	85.6	0.5 X 10^6	2.5 X 10^8	N.D	70
Affinity purification	80	1.03	82.4	40	2.0 X 10^6	1.59 X 10^8	2.0X 10^6	98.6

The protein concentration was determined via gel densitometry analysis and quantitative RP-HPLC.The purity was calculated by RP-HPLC and SDS-PAGE.

The selected clone, giving a higher expression level of HAS-SK, was allowed to ferment at a larger scale, i.e., in a large shake flask. During the incubation period, methanol was kept on adding in the media till 0.5% final concentration. The incubated broth culture was then centrifuged, and its supernatant was filtered via a 0.45um Stericup™ filter unit followed by its concentration up to 50% via dia-filtration using prep/scale spiral cartridge. Further purification and concentration were carried out by a highly specific Captureselect™ human albumin affinity matrix (Eifler *et al*., 2014).

We were able to retrieve 80mg/L of HSA-SK fusion protein with 40% recovery as quantified by RP-HPLC. (Table I, Fig. 4A).

The purified fractions of fusion protein HSA-SK appeared in the form of a single prominent peak lying in almost similar to the estimated range indicating the purified HSA-SK. The extent of purity was precisely estimated by RP-HPLC as a result of which a single peak was observed at a retention time of 10.7 min with 100% purity (Fig. 4B). This sharp peak indicated the highest purity level of the purified HSA-SK. The verification of the purified fusion protein was carried out via Western blot analysis by using anti-streptokinase antibodies (ThermoFisher Catalog # PA1-85891) for detection purpose as shown in Figure 4C.

In vitro *stability analysis of HSA-SK fusion protein*

Many previous studies on recombinant proteins reveal that such proteins have a very short half-life, when injected into the bloodstream, prone to degrade by serum protease very swiftly. The stability of fusion protein can be maintained by adding a linker chain between both participant proteins, as mentioned by Zhao *et al*. (2008). However, the most significant strategy to stabilize recombinant proteins is Albumin fusion technology. The presence of human serum albumin residue with the recombinant protein helps to enhance its stability and serum level half-life. The stability of HSA-SK was compared with the standard SK on the basis of trypsin digestion assay. Both samples were digested with trypsin, and their RP-HPLC peaks were observed at different time intervals. Figure 5 indicates the proteolytic stability of the fusion protein (HSA-SK) after comparing the anti-trypsin activity of HSA-SK and the standard SK. Purity of SK and HSA-SK was confirmed by RP-HPLC (Fig. 5) as well as SDS-PAGE (Fig. 4A). Rate constant determination for this reaction was based on the assumption that both reactions follow first order kinetics. Improvement in stability was assessed by rate constant. Figure 5E showed a clear decrease in rate constant from 0.90 × 10^{-2} min^{-1} to 0.39 ×10^{-2} min^{-1} of HSA-SK fusion protein. This change in stability can be attributed to the fusion of albumin residue fused with the recombinant SK. PEGylated streptokinase (Sawhney *et al*., 2016) which shows 1h increased stability compared to conventional SK. However, our product show in serum stability up to 6h which is six times than PEGylated SK.

Thermal stability analysis of HSA-SK fusion protein

Thermal stability comparison between the fusion protein as well as the standard proteins was carried out to check their ability of aggregation at respective temperature. Both proteins i.e., HSA-SK and standard SK were heated at 50°C, and OD was checked at regular intervals. Figure 6 shows an abrupt and massive increase in the OD of standard SK while the purified HSA-SK remained stable while heating for more than 200 min. As the temperature increases, the hydrophobic regions of protein interact with each other and results into the aggregation and structure deformation of protein. However, the albumin protein residue masks the hydrophobic patches to suppress the hydrophobic interaction and aggregation. Hence, the fusion of albumin protein helps in aggregation suppression, thermal stability enhancement and biological activity assessment.

Biological activity of HSA-SK fusion protein

The typical function of the streptokinase drug is to dissolve blood clots formed in the blood vessels. To check biological activity, both standard streptokinase Merck Millipore (Cat No. Y0000868: European Pharmacopoeia (EP) Reference Standard) and purified albumin fused streptokinase HSA-SK were subjected to clot lysis. The results reported HSA-SK with an improved biological activity as the percentage lysis caused by it was 43% and that of standard streptokinase was 33% (Table II).

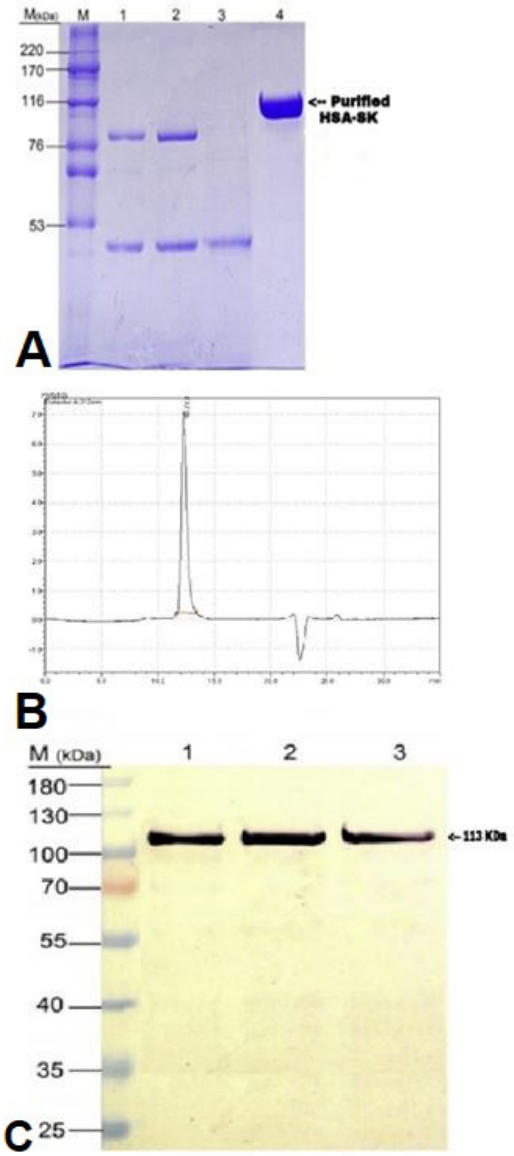

Fig. 4. (A) SDS-PAGE analysis of purified HSA-SK as a result of one step chromatographic purification by capture select human serum albumin affinity marix. Here Lane M represents protein markers of high molecular weight, Lane 1 contain proteins from broth supernatent, Lane 2 contain protein from diafiltered supernatent, Lane 3 represents protein from flow through and finally, Lane 4 represents the final eluted fusion protein HSA-SK. (B) Chromatogram is showing the analysis of purified HSA-SK fusion protein by RP- HPLC. 210 nm detector was used for the absorbance of protein and chromatogram showed a single sharp peak at retention time 10.7 min. (C) Western blot analysis of purified fusion protein HSA-SK where Lane M contain marker protein, Lane 1 represents a band of purified HSA-SK, Lane 3 has a band of non-reduced HSA-SK and Lane 3 shows a band of HSA-SK standard.

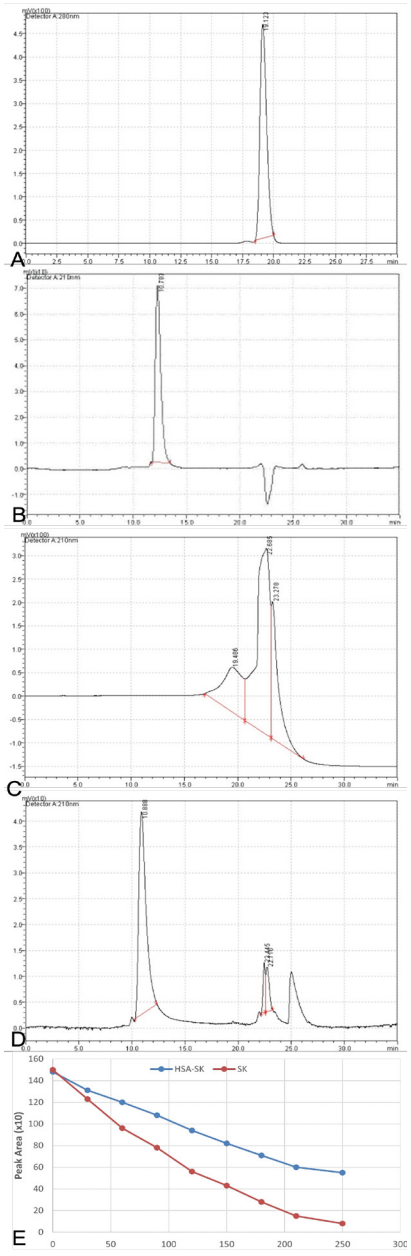

Fig. 5. Indirect serum stability response of SK vs HSA-SK. (A) RP-HPLC analysis of purified Streptokinase showed single peak at 19 min; (B) RP-HPLC analysis of purified HSA-SK showed single peak at 10 min; (C) RP-HPLC analysis of SK taken after 4 hours of trypsin digestion showed that simple SK was almost completely digested in 4 hours; (D) RP-HPLC analysis of HSA-SK taken after 4 hours of trypsin digestion showed partial digestion in 4 hours and HAS-SK is more stable as compared to simple SK; (E) Tryptic digestion of both samples was performed and the difference in peaks was observed by RP-HPLC. HSA-SK showed decrease in rate constant for tryptic digestion as compared to SK.

Table II. Biological activity analysis by clot lysis assay.

Sample	W1	W2	W3	W3-W1	W2-W1	Percentage
SK Standard	0.94	1.356	1.217	0.272	0.416	33%
Purified HSA-SK	0.94	1.404	1.20	0.26	0.464	43%

Albumin fused streptokinase clearly showed enhanced biological activity.

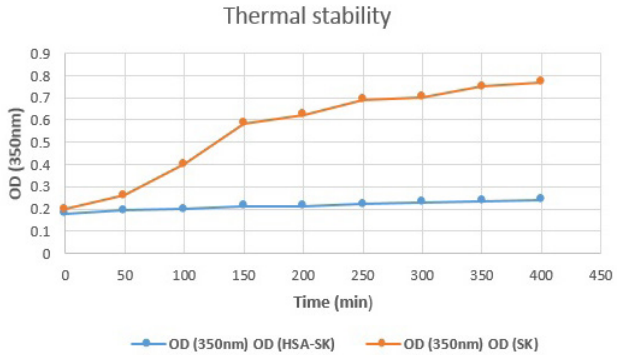

Fig. 6. HSA-SK fusion protein thermal stability testing indicates higher stability of HSA-SK as compare to the standard SK protein.

CONCLUSION

The main objective of this research study was to improve the serum stability half-life of Streptokinase protein using albumin fusion technology and attain a high secretion level of the target protein from *P. pastoris*. We successfully fused the albumin residue with Streptokinase and attained its potential expression level in *P. pastoris.* One step affinity-based chromatography recovered maximum products of the fusion protein (HSA-SK). Moreover, the functional capabilities of HSA-SK also proved it to be more biologically stable and active compared to the native standard SK in the parameters of temperature, trypsin digestion and biological activity. It is the first ever reported study on production of highly stable streptokinase in *P. pastoris* with yield of 40% and purity 98%.

Statement of conflict of interest

The authors have declared no conflict of interest.

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