Original Article

Evaluation of clinical efficacy of streptokinase by comparison with the thrombolytic agent on animal model

Avaliação da eficácia clínica da estreptoquinase por comparação com o agente trombolítico em modelo animal

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Abstract

Cardiovascular disorders, including acute myocardial infarction (AMI), often lead to blood clot formation, impacting blood circulation. Streptokinase, a cost-effective and widely available thrombolytic agent, is crucial in treating thrombosis. This study aimed to produce streptokinase from *Streptococcus pyogenes* EBL-48 and compare its efficacy with heparin in an animal model. We evaluated the clot-lysing effectiveness of streptokinase produced from *Streptococcus pyogenes* EBL-48, emphasizing its low cost and ease of production. Streptokinase was produced using pre-optimized fermentation media and purified through ion exchange and gel-filtration chromatography. *In vivo* analysis involved inducing clots in a trial animal model using ferric chloride, comparing streptokinase with heparin. Ultrasonography assessed the clot-lysing activity of streptokinase. Streptokinase (47 kDa) effectively lysed clots, proving its low cost, easy production, and minimal adverse effects. Ultrasonography confirmed its fibrinolytic efficacy. These findings highlight potential as an affordable and easily produced thrombolytic agent, particularly relevant in resource-limited settings. Streptokinase efficacy and minimal adverse effects make it a promising option for thrombolytic therapy, especially in economically constrained regions. Future studies could optimize production techniques, explore different strains, and conduct clinical trials for human validation. Comparative studies with other thrombolytic agents would enhance understanding of their advantages and limitations.

Keywords: artificial clot, cardiovascular diseases, clinical trial, *in vivo* application, thrombolytic therapy.

Resumo

Os distúrbios cardiovasculares, incluindo o infarto agudo do miocárdio (IAM), muitas vezes levam à formação de coágulos sanguíneos, afetando a circulação sanguínea. A estreptoquinase, um agente trombolítico de baixo custo e amplamente disponível, é crucial no tratamento da trombose. Este estudo teve como objetivo produzir estreptoquinase a partir de *Streptococcus pyogenes* EBL-48 e comparar sua eficácia com a heparina em modelo animal. A eficácia da estreptoquinase produzida a partir de *Streptococcus pyogenes* EBL-48 na lise de coágulos foi analisada, no qual foi possível observar seu baixo custo e facilidade de produção. A estreptoquinase foi produzida utilizando meios de fermentação pré-otimizados e purificada através de cromatografia de troca iônica e filtração em gel. A análise *in vivo* envolveu a indução de coágulos em um modelo animal experimental usando cloreto férrico, comparando estreptoquinase com heparina. A ultrassonografia avaliou a atividade de lise de coágulos da estreptoquinase. A estreptoquinase (47 kDa) lisava coágulos de forma eficaz, comprovando seu baixo custo, fácil produção e efeitos adversos mínimos. Além disso, a ultrassonografia confirmou sua eficácia fibrinolítica. Estas descobertas destacam o potencial como agente trombolítico acessível e de fácil produção, particularmente relevante em ambientes com recursos limitados. A eficácia da estreptoquinase e os efeitos adversos mínimos tornam-na uma opção promissora para terapia trombolítica, especialmente em regiões economicamente restritas. Estudos futuros poderão otimizar as técnicas de produção, explorar diferentes estirpes e realizar ensaios clínicos para validação em humanos. Estudos comparativos com outros agentes trombolíticos podem aprimorar a compreensão das suas vantagens e limitações.

Palavras-chave: coágulo artificial, doenças cardiovasculares, ensaio clínico, aplicação *in vivo*, terapia trombolítica.

1. Introduction

Thrombolytic therapy utilizes a plasminogen activator (enzyme streptokinase) which plays a vital role in dissolving blood clots for many years. It is given to patients who show signs of heart attack, to improve their chances of survival. This medication can also be used for curing blood clots in the lungs and legs (Arshad et al., 2018). A lot of experimentation is being conducted to produce this drug more efficiently workable and available in the form of a fibrinolytic agent (Mobarrez et al., 2015). The direct proteolysis mechanism is performed by tPA (tissue-type plasminogen) and urokinase while SK (streptokinase) produces a complex with plasminogen. Hence, SK is now considered the best thrombolytic agent for curing thromboembolic disease states. Due to the limited availability of angioplasty, new developments in thrombolytic therapy may provide more affordable solutions (Seyed et al., 2008).

When a blood clot develops in the blood vessel and movement of blood is hindered, thrombosis takes place. This process of clot formation occurs to prevent loss of blood, in case of injury. Depending on the situation, blood clotting occurs in the body even when no blood vessels are injured (Sameni et al., 2017). Blood clots induce myocardial infarction (MI), generally known as a heart attack. Over time, plaques form in the coronary arteries, which causes atherosclerosis (Tang et al., 2020).

The most well-known thrombolytic medications are tissue-type plasminogen activator, urokinase, and SK. Thrombolytic agents promote fibrin dissolution. Through the action of these drugs, plasminogen (PG) is transformed into active plasmin (PN), which degrades fibrin and releases degradation products for phagocytosis, as shown in Figure 1 (Muszbek et al., 2011). The most extensively used thrombolytic agent is SK, which is obtained from hemolytic Streptococci. The extracellular enzyme namely SK is produced by various strains of β-hemolytic streptococci (Asada et al., 2020). This enzyme containing a single polypeptide chain works indirectly to break down fibrin by turning on the circulatory plasminogen. Jackson and Tang discovered the whole amino acid sequence of SK. There are 414 amino acid residues in SK and a molecular weight of 47 kDa (Akbar et al., 2020). The pH at which the protein is most active is around 7.5, while its isoelectric pH is 4.7. Cysteine, cystine, phosphorus, conjugated carbohydrates, and lipids are absent from the protein. The structure of the SK generated by various types of streptococci varies significantly (Ali et al., 2014).

The FeCl$_3$-induced arterial thrombosis model is widely employed to evaluate the arterial effectiveness of potential antithrombotic drugs. This model aids in comprehending the cellular and molecular mechanisms involved in arterial thrombosis (Butcher et al., 2013). Recent studies reveal the intricate mechanisms by which ferric chloride induces thrombus formation. Special focus is placed on observing phenotypes in specific knockout animals within the ferric chloride models and contrasting them with other models of varying origins. Moreover, exploration is conducted to predict the pathophysiology of true atherothrombotic disease using ferric chloride models (Bock and Fuentes-Prior, 2011).

Six mature white rabbits weighing 2.4 kg were acclimated for a week before the in vivo study. The rabbit jugular vein thrombosis model was employed to evaluate the fibrinolytic activity of recombinant streptokinase (Muszbek et al., 2011). An artificial thrombus was induced, and blood flow was observed before thrombosis. Anesthesia was administered, and the external jugular vein segment was dissected. Blood was withdrawn, and a mixture of rabbit blood and human fibrinogen was injected (Tang et al., 2020).
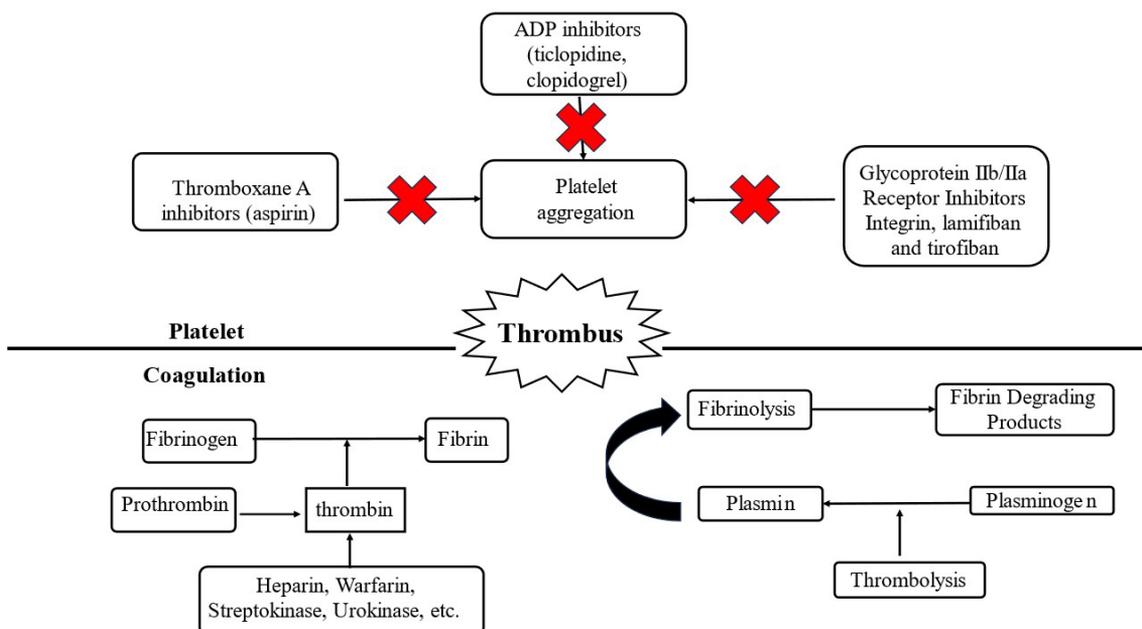
Figure 1. Antithrombotic and thrombolytic medications and the components of thrombus formation.

Clot formation and removal of clamps took 30 minutes, with confirmation through ultrasonography. Post-clot blood was drawn, and streptokinase was administered to different groups. Blood was drawn again after an hour to observe the extent of blood clot dissolution. Negative control animals received saline. The study provides insights into the fibrinolytic effects of streptokinase in the rabbit jugular vein thrombosis model (Bhardwaj and Angayarkanni, 2015).

SK proved to be a safe and effective treatment for cardiovascular disorders, and a tiny dose of SK had an effect that was comparable to the usual dose of other thrombolytic drugs (Babu and Devi, 2015). Our research suggested that SK may be used clinically to treat cardiovascular disease. This technically advanced, stable, and valid animal model was created using ultrasonography and minimally invasive surgery, and it closely matched key clinical characteristics (Aghaeepoor et al., 2019). This could aid in improving our comprehension of thrombotic diseases and the creation of preventative and remedial measures. The objective of the study is the purification of streptokinase produced from *Streptococcus pyogenes* EBL-48 and the application of ferric chloride ions to induce clot formation in an animal model. Finally, compared the clinical effectiveness of streptokinase to that of other thrombolytic drugs by applying it to research animal

2. Methodology

The entire experiment was carried out at the Enzyme Biotechnology Laboratory in the Biochemistry Department at the University of Agriculture, Faisalabad. The sample strain *Streptococcus pyogenes* EBL-48 was used for the production of SK with pre-optimized fermentation media. The preliminary steps of the research work included enzyme purification by different chromatographic techniques and enzyme quantification by performing SDS-PAGE (Cheng et al., 2016). Then purified SK was further carried forward for the animal trial investigation to assess the efficacy of the enzyme in blood clot lysis (Mahmoudi et al., 2010).

3. Streptokinase Production

The Todd Hewitt Broth Nutrient media was utilized to prepare the inoculum of *S. pyogenes* and incubated at 37°C for 24 hrs (Freydell et al., 2011). Liquid state fermentation was used to monitor the microbial growth in an Erlenmeyer flask containing 50 mL of pre-optimized production media (g/100mL: glucose, yeast extract, K_2HPO_4, KH_2PO_4, $NaHCO_3$, CH_3COONa, $MnCl_2.4H_2O$ and $FeSO_4.7H_2O$ pH 7.5).

About 1ml of inoculum was proliferated in growth media and left for incubation at 37°C for 24 hrs. After incubation, the sample was centrifuged at 10,000 rpm for a half hour and supernatant was obtained (crude enzyme).

Then at a temperature of 4°C, $(NH_4)_2SO_4$ was poured with an 80% concentration and left on the stirrer overnight. The mixture was centrifuged at a velocity of 10,000 rpm for 20 minutes at a temperature of 4°C after being agitated overnight. The precipitate was resuspended in buffer Tris-CL 50mM pH 7 before being dialyzed for 24 hours at 4°C with four buffer changes against 20 volumes of the same buffer.

4. Chromatographic Techniques for Purification

4.1. Ion-exchange chromatography

Ion exchange occurs when ions in a solution are converted into solids releasing ions of various types and with the same polarity. This implies that different ions from the solid take the place of the ions from solutions. For ionic separation of the desalted enzyme, a DEAE-cellulose ion exchange column was created. The desalted enzyme was then subjected to ion-exchange chromatography. Fifty fractions were collected in test tubes at a consistent flow rate, with continuous addition of a small buffer amount as it neared the column. Each fraction had a volume of 2 ml. Subsequently, all the fractions underwent an enzyme assay to determine enzyme activity, as shown in Figure 2.

4.2. Gel filtration chromatography

The hydrodynamic volume and molecular size of the constituents are used in gel filtration chromatography.
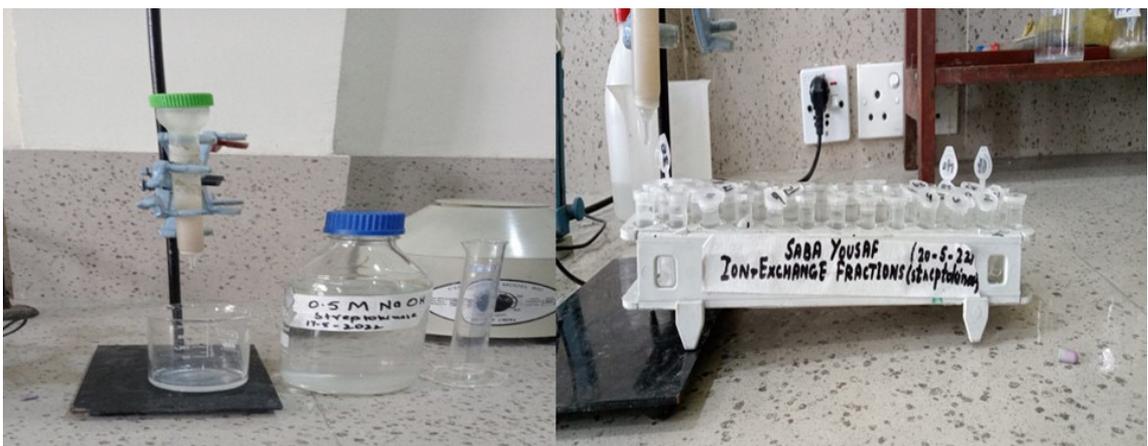
Figure 2. Fractions obtained after Ion-exchange chromatography.

As molecules move through the stationary phase, which comprises heterosporous cross-linked polymeric gel or beads, they are differentiated by solute inclusion or exclusion. A sample of approximately 0.5 ml was acquired following ion exchange chromatography, and those exhibiting the highest specific activity were selected. The sample was then applied to the packed column, and the outlet was sealed. It was allowed to permeate the column, and elution was performed using 0.1 M phosphate buffer (pH 6) at a steady drop rate. A total of 30 fractions, each with a volume of 2 ml, were collected and subsequently subjected to an enzyme activity assay, as shown in Figure 3.

4.3. SDS-PAGE analysis

According to SDS-PAGE in an electric field, a charged molecule moves in the opposite direction to the electrode. Small molecules flow more quickly because they have less resistance during electrophoresis. The streptokinase was further quantified after its purification by SDS-PAGE analysis. Two different gels were prepared i.e. resolving gel 10% and stacking gel 4% (Sheli et al., 2018).

Figure 3. Fractions obtained after Gel-filtration chromatography.

5. Enzyme Assay

The enzyme activity streptokinase using a casein digestion assay was. In this method, a mixture was prepared by combining 50 mM Tris HCl (pH 8.0), 10 mg casein, and 0.1 ml of the sample solution. The mixture was incubated at 37°C for approximately 20 minutes. After incubation, 0.4 ml of 3.3M HCl was added, followed by the addition of 2.6 ml 5% w/v Trichloroacetic acid (TCA) to stop the reaction. The resulting mixture was placed on ice for about 30 minutes and then filtered using Whatmann filter paper. The filtrate was analyzed on a spectrophotometer, and the absorbance was measured at 280 nm to determine enzyme activity.

6. In Vivo Analysis of Streptokinase

Six male adult rabbits, weighing about 2.4 kg were used for the *in vivo* experiment. One week before use, the animals were acclimated to a conventional animal house (El-Dabaa et al., 2022).

A rabbit jugular vein *in vivo* thrombosis model was used to assess the thrombolytic activity of the synthesized SK. An artificial thrombus was produced in the jugular vein specifically for this reason (Faust et al., 2015). The lignocaine HCl solution 2% (xylex) was typically used as an Intravenous injection to anesthetize the animals (Vachher et al., 2021). About 1 ml of anesthesia was injected into the site of surgery as shown in Figure 4a. When the rabbit was anesthetized, it was surgically cut using a surgical blade and an incision of about 2cm to expose the vein of the rabbit (Sameni et al., 2017). The vein was made visible using methylated spirit.

The exposed jugular vein is then treated for 3-5 minutes with a few drops of 20% $FeCl_3$-saturated filter paper. This was followed by placing the filter paper on the rabbit vein (Nitzsche et al., 2015). When the blood had coagulated, the filter paper was removed as shown in Figure 4b.

Figure 4. a) Injecting anesthesia into rabbit muscles. **b)** Vein exposed surgically. **c)** Ferric chloride application on vein exposed. **d)** Clot formation on rabbit vein.

7. Ultrasonography

Doppler ultrasounds are noninvasive diagnostic procedures that evaluate blood flow via your blood vessels by reflecting high-frequency sound waves (ultrasound) off of moving red blood cells. Blood flow is monitored using this technique.

8. Drug Administration

Three groups of rabbits (each group has two rabbits) labeled as blank, sample, and comparative were injected with different solutions respectively.

8.1. Blank rabbit

The rabbits labeled as blank were injected with 2ml PBS saline (phosphate buffered saline) after the clot had formed. Then its blood flow was monitored using ultrasonography (Kikkert et al., 2014).

8.2. Sample rabbit

The rabbits marked as sample rabbits were injected with 2ml of the sample SK (EBL-48). After the sample injection, its blood flow velocity was observed on ultrasonography (Banerjee et al., 2004).

8.3. Comparative rabbit

Now the rabbits labeled comparative rabbits were given the injection of commercially available Heparin IV (4000 U/kg) of about 1ml. Its comparative blood flow movement was determined by its ultrasonography images (Bera et al., 2015).

9. Ultrasound for Blood Velocity Analysis

To verify the induced thrombosis, ultrasonography was used to monitor the blood flow velocity inside the jugular vein. The difference between the frequencies of ultrasonic waves that are transmitted and reflected is influenced by the blood flow through the channel (Zia, 2020). Ultrasound revealed that a clot had formed in the jugular vein on the application of $FeCl_3$ ions. Further, differences in blood flow with the application of sample drugs were also observed, as shown in Figure 5a and 5b.

10. Statistical Analysis

ANOVA was used to conclude the overall enzyme activity after different stages of purification including crude enzyme, ion-exchange, and gel-filtration chromatography supernatants and the statistical analysis of data supports their high significance (Mohammed, 2022). It also concludes that the purified enzyme under preoptimized conditions and techniques worked well to boost streptokinase efficiency. Hence results proved that streptokinase produced from *Streptococcus pyogenes* EBL-48 strain is effective in clot lysis with the advantage of being inexpensive, easily produced, and least adverse effects even (Shamsi and Zahedi, 2017). Results are demonstrated in Tables 1 and 2 below.
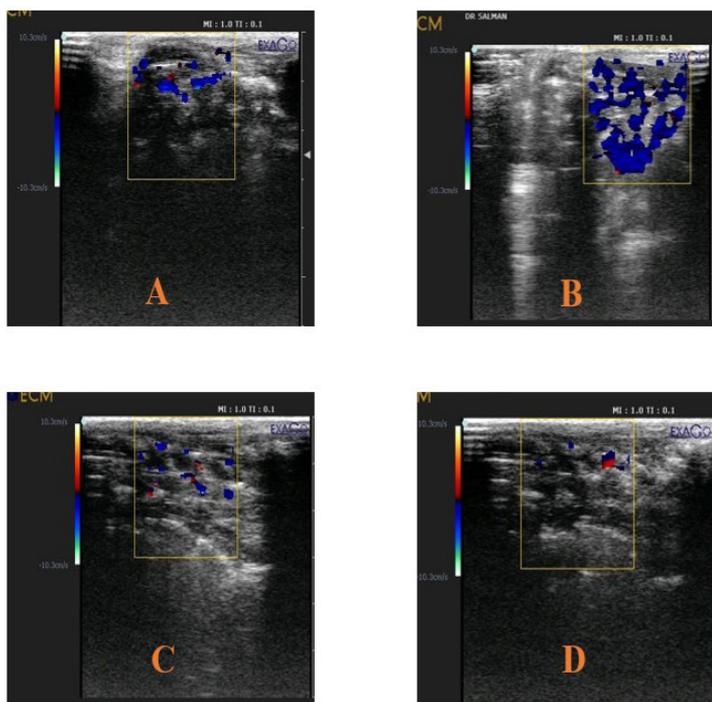
Figure 5. A) Ultrasound demonstrating blood velocity after thrombus induction. **B)** Blood velocity after sample injection of Streptokinase. **C)** Blood flow after heparin injection. **D)** Blood movement after saline injection.

11. Results

Different streptokinase samples were used for this experiment. The findings revealed that the purity, enzyme activity, and composition of the various samples of SK varied significantly as shown in Table 1. Our present concluded that it has a 47 kDa molecular weight. The manufacturing, purification, and *in vivo* application of streptokinase enzyme with increased quantity, improved purity, and increased activity were the goals of this study. To do this, bacteria were first cultured in a pre-optimized growth medium under optimal circumstances. Next, ion-exchange chromatography and gel filtration chromatography were performed on the enzyme. According to the study's findings, the purification of the enzyme is what allows for a recovery rate of 75%. According to this investigation, the maximum activity of the crude enzyme was 4.25 U/ml and its specific activity was 2.61 U/mg. Later, the enzyme-specific activity was determined by ion exchange to be around 4.14 U/mg, and on gel filtration chromatography (Sephadex G-150), the enzyme-specific activity was determined to be 5.18 U/mg as shown in Table 2. It was concluded that SK has a 47kDa molecular weight. This investigation aimed to look into the bioactivity of the *Streptococcus pyogenes* EBL-48 strain produced in the Enzyme Biotechnology Laboratory (EBL), Department of Biochemistry at the University of Agriculture-Faisalabad. A blood clot was created in the jugular vein of rabbits for this reason. The streptokinase generated was given intravenously to the animals, which were compared to those who got a comparative thrombolytic drug and normal saline as positive and negative controls, respectively. The ability of medicines to thrombolysis was determined by analyzing blood flow visuals in ultrasound scans.

12. Discussion

Purification results were assessed using ANOVA and obtained the following results. Due to the size disparities between the protein components, gel filtration can be used to separate them (Mahmoud et al., 2020). The resolved protein is then passed through an absorbent bed and an uncharged gel to recover it (Vellanki et al., 2013). The purification results are demonstrated in Figure 6 present below.

Table 1. Analysis of variance of *Streptococcus pyogenes* EBL-48 after purification.

Source	Degree of Freedom	Sum of squares	Mean squares	F-value	P-value
Precipitation step	1	38.74	38.735	32.90	0.0000[*]
Error	28	32.97	1.177		
Total	29	71.70			

*Highly significant (P<0.01).

Table 2. Summary of purification of *Streptococcus pyogenes* EBL-48.

Purification steps	Protein content (mg/ml)	Activity (U/ml)	Specific activity (U/mg)	Fold Purification	Recovery %
Crude	1.625	4.25	2.615385	1	100
Ammonium sulfate	1.56	6.004	3.848718	2.53	90
Ion-exchange	1.365	5.65	4.142125	5.15	83.3
Gel-Supernatant	1.158	6.004	5.184801	9.45	75.3
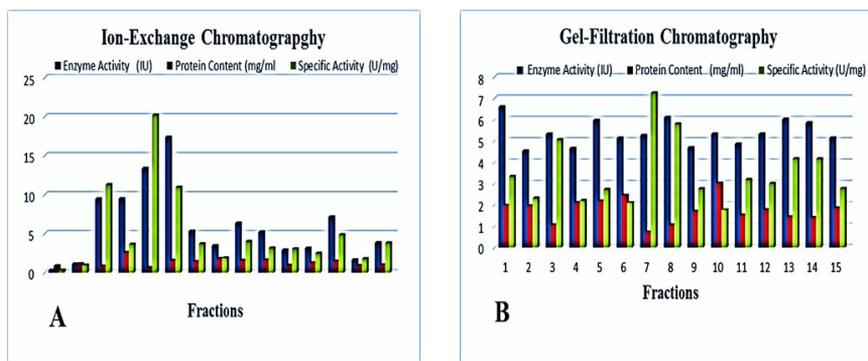
Figure 6. A) Graph representing enzyme activity, protein, and specific activity after Ion exchange chromatography. **B)** Graph representing enzyme activity, protein, and specific activity after Gel-filtration chromatography.

To determine that the parent streptokinase, which was isolated from its natural form, has the same molecular mass and to determine the molecular mass of the isolated streptokinase, SDS-PAGE was done. Our findings can be contrasted with those (Mohammed, 2022) who claim that native streptokinase exhibits a 47 kDa band on SDS-PAGE, and compared urokinase and streptokinase (Benyahia et al., 2020). The first thrombolytic medication authorized for use in treating acute myocardial infarction was SK (Shi et al., 1994). We decided to hunt for novel SK-producing microorganisms because of the growing potential of SK applications (Burck et al., 1990). Furthermore, the exponential increase of SK applications in a variety of fields during the previous few decades needs an expansion in both qualitative and quantitative enhancement (Torréns et al., 1999). The bioactivity of the *Streptococcus pyogenes* EBL-48 strain was evaluated. A blood clot was created in the jugular vein of rabbits for this reason. The animals received lab-produced SK and were then contrasted with those who received normal saline as a positive control and a comparative thrombolytic medication as a negative control. By examining blood flow images from ultrasound scans, it was possible to assess the thrombolytic potential of various drugs (Banerjee et al., 2004).

To formulate a method for inducing thrombus in animal models, numerous investigations have been carried out. The rabbit was prepared for *in vivo* study to check the bioactivity of SK in the rabbit model (Sameni et al., 2017). For instance, Ferric chloride ($FeCl_3$) ions were used to cause blood clots to develop in the jugular veins of rabbits. In this work, a 20% ferric chloride solution was used to create a thrombus in a rabbit jugular vein (Mican et al., 2019). The rate of thrombolysis was measured one hour after medication delivery. Due to restrictions in the direct observation of plaque and thrombus, thrombolysis *in vivo* determination is difficult (Nikitin et al., 2021). Ultrasonography was used in this investigation to pinpoint the specific site of the thrombus and determine the connection between the thickness of the aortic stenosis and thrombus formation (Risser et al., 2022). Our findings show that the infusion of SK caused the dissolution of the blood clot induced. When compared to samples taken before and after the medicine was administered, the results demonstrated that the produced SK (*Streptococcus pyogenes* EBL-48) displayed a detectable fibrinolytic activity. According to these findings, the widespread systemic activation of the fibrinolytic system was induced by the infusion of 4000 IU of SK (Taheri et al., 2016). The ultrasound results showed that, in comparison to the other thrombolytic agents, SK considerably dissolved artificially induced thrombus much better (Zia, 2020). Results showed that SK made from the *Streptococcus pyogenes* EBL-48 strain is effective in clot lysis and has the advantages of being low cost, simple to make, and with few side effects. The results proved that blood velocity decreased after enzyme introduction into rabbits' veins, which means that streptokinase produced in the lab is effective like other thrombolytic agents. But this enzyme synthesized in the lab is cheaper and easily produced which provides the easy availability of a thrombolytic medication for CVD patients.

12.1. Limitations and prospects

Although native streptokinase-based thrombolytic treatment is effective, it can cause allergic reactions when administered. Certain immunogenic responses have been observed. The creation of genetically engineered streptokinase should receive greater attention in the future to increase its half-life in blood circulation and decrease immunogenicity. This research paved the way for future efforts to commercialize the lab-prepared SK for cardiac patients. This could provide a lot of ease to people who cannot afford much to treat fatal conditions like stroke, myocardial infarction, etc. In prospect, this produced enzyme shall be commercialized for the benefit of people so that more and more patients can be cured of heart diseases at a lower expense.

13. Conclusion

Streptokinase is used when the blood circulation in the human body is disrupted by the development of blood clots, which lead to major cardiac problems and diseases including acute myocardial infarction. Due to its low cost, ease of availability, and widespread use in developing nations, SK is the most preferred agent. Streptokinase therapy is most effective for heart attacks because it is naturally produced by using various strains of Streptococci from groups C, A, and G. The manufacturing and purification of SK enzyme with increased quantity, improved purity and increased activity made it the best choice for thrombolytic therapy. It also concludes that the purified enzyme under preoptimized conditions and techniques worked well to boost SK efficiency. Hence results proved that SK produced from *Streptococcus pyogenes* EBL-48 strain is effective in clot lysis with the advantage of being inexpensive, easily produced, and least adverse effects. This study has shown that a tiny dose of the thrombolytic drug SK may be used clinically to treat cardiovascular disease (CVD). This could help improve our understanding of thrombotic diseases and the creation of preventative and remedial measures. According to the ultrasonography findings, SK significantly and more effectively dissolved artificially produced thrombus than the other thrombolytic drug. The *Streptococcus pyogenes* EBL-48 strain of SK was shown to be effective in clot lysis and to be inexpensive, straightforward to manufacture, and somewhat safe, according to the results.

Acknowledgements

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