

Production of recombinant streptokinase from *Streptococcus pyogenes* isolate and its potential for thrombolytic therapy

Abdullah S. Assiri, ABCVM, FRCPC(C), Basiouny A. El-Gamal, MS, PhD, Elsayed E. Hafez, MS, PhD, Mohamed A. Haidara, MS, PhD.

ABSTRACT

الأهداف: إنتاج إنزيم استربتوكينيز مهندس وراثيا آمن وفعال من المعزول الممرض استربتوكوكوس بيوجينيس في فطر الخميرة ودراسة إمكانية استخدامه كعلاج لإذابة الجلطات.

الطريقة: أجريت هذه الدراسة خلال الفترة من نوفمبر 2012م إلى ديسمبر 2013م. تم عزل سلالة الاستربتوكوكوس بيوجينيس الممرضة من مسحات أخذت من حلق 45 من المرضى بالتهاب البلعوم في مستشفى عسير المركزي، أبها، المملكة العربية السعودية. وبعد التعرف على خصائصها ودراستها تم استخدام الـ DNA البكتيري لتضخيم جين الاستربتوكينيز (1200 قاعدة مزدوجة). وتم دراسة تسلسل الجين المتضخم ومن ثم كلونته في ناقل وراثي للتأكد من عمله وبعد ذلك تم نقله إلى فطر الخميرة حيث تم إنتاج البروتين الإنزيمي المهندس وراثيا والحصول عليه في صورة نقية واختبار نشاطه في إذابة الجلطات.

النتائج: أوضحت أن السلالة المعزولة هي لمعزول للاستربتوكوكوس بيوجينيس. وعند عمل الشجرة الوراثية لتلك السلالة المعزولة تبين أنها تشبه سلالات الاستربتوكوكاي بيوجينيس الأخرى المسجلة في بنك الجينات. وأوضحت دراسة تسلسل الجين المتضخم أنه جين الاستربتوكينيز. وعند عمل الشجرة الوراثية للجين المعزول تبين ارتباطه الكبير بجينات الاستربتوكينيز الأخرى المسجلة في بنك الجينات. وعند عمل الشجرة الوراثية للجين بواسطة استنتاج تسلسل الأحماض الأمينية، اتضح أن الجين المعزول يتكون من عنقود منفصل ومختلف عن العناقيد المكونة لجينات الاستربتوكينيز الأخرى التي تم دراستها وأن ذلك يرجح وجود معزول بكتيري جديد وبالتالي جين جديد. وتبين أن لإنزيم الاستربتوكينيز النقي والمهندس وراثيا والمنتج في فطر الخميرة نشاط (82%) يشابه نشاط إنزيم استربتوكينيز تجاري (81%) في إذابة الجلطات وذلك عند تركيز إنزيمي (2000 U/ml).

الخاتمة: إنزيم الاستربتوكينيز المهندس وراثيا والمنتج في فطر الخميرة فعال وله نشاط في إذابة الجلطات مشابه لنشاط إنزيم استربتوكينيز منتج تجاريا عند اختباره معمليا وذلك يرجح إمكانية استخدامه بكفاءة كعلاج في إذابة الجلطات ومحاولة إنتاجه على نطاق واسع.

Objectives: To produce an effective recombinant streptokinase (rSK) from pathogenic *Streptococcus*

pyogenes isolate in yeast, and evaluate its potential for thrombolytic therapy.

Methods: This study was conducted from November 2012 to December 2013 at King Khalid University, Abha, Kingdom of Saudi Arabia (KSA). Throat swabs collected from 45 pharyngitis patients in Asser Central Hospital, Abha, KSA were used to isolate *Streptococcus pyogenes*. The bacterial DNA was used for amplification of the streptokinase gene (1200 bp). The gene was cloned and in vitro transcribed in an eukaryotic expression vector that was transformed into yeast *Pichia pastoris* SMD1168, and the rSK protein was purified and tested for its thrombolytic activity.

Results: The *Streptococcus pyogenes* strain was isolated and its DNA nucleotide sequence revealed similarity to other *Streptococcus pyogenes* in the Gene bank. Sequencing of the amplified gene based on DNA nucleotide sequence revealed a SK gene closely related to other SK genes in the Gene bank. However, based on deduced amino acids sequence, the gene formed a separate cluster different from clusters formed by other examined genes, suggesting a new bacterial isolate and accordingly a new gene. The purified protein showed 82% clot lysis compared to a commercial SK (81%) at an enzyme concentration of 2000 U/ml.

Conclusion: The present yeast rSK showed similar thrombolytic activity in vitro as that of a commercial SK, suggesting its potential for thrombolytic therapy and large scale production.

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From the Department of Internal Medicine (Assiri), the Department of Clinical Biochemistry (El-Gamal), the Department of Physiology (Haidara), College of Medicine, King Khalid University, Abha, Kingdom of Saudi Arabia, and the City of Scientific Researches and Applied Biotechnology (Hafez), Borg El-Arab, Alexandria, Egypt.

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Address correspondence and reprint request to: Professor Abdullah S. Assiri, Department of Internal Medicine, College of Medicine, King Khalid University, Abha, Kingdom of Saudi Arabia. E-mail: aassiri@yahoo.com

There has been increasing interest in the use of thrombolytic therapy with fibrinolytic (thrombolytic) agents for the treatment of various circulating disorders, for example, pulmonary embolism, deep venous thrombosis, and myocardial infarction.^{1,2} These disorders are increasingly becoming the leading causes of mortality wide world.³ Among the commonly used fibrinolytic agents in thrombolytic therapy are urokinase (UK), tissue type plasminogen activators (TPA), and streptokinase (SK). These agents are collectively named as plasminogen activators, as they convert the enzymatically inactive plasminogen (PG) to an active protease, plasmin, which dissolves the fibrin clots and solubilizes the degradation products that can be removed by phagocytosis, and thus help the restoration of blood flow through the occluded vessels.³ Unlike UK and TPA, SK has no enzymatic activity of its own, but it has an indirect PG activity property, by first forming a high affinity 1:1 stoichiometric complex with PG or plasmin. The resultant activator complex is a highly specific protease that changes PG molecules to proteolytically active plasmin.⁴ Streptokinase was the first drug introduced as a therapy for acute myocardial infarction more than 40 years ago.⁵ It is now one of the leading fibrinolytic agents in the treatment of thrombotic conditions,⁶ and is included in the WHO Model List of Essential Medicines.⁷ Currently, despite the wide use of TPA in developed nations, SK remains essential to the management of MI and other thrombotic events in developing countries.⁸ Streptokinase (E.C.3.4.99.22) is an extracellular single chain, non-enzymatic, monomeric protein, consisting of 440 amino acids, including a 26-amino acid N-terminal single peptide, which is cleaved during secretion to give the mature 414 amino acids production residues of 47 KDa molecular weight.^{9,10} Streptokinase has been produced by many strains of β -hemolytic streptococci isolated from the upper respiratory tract. Streptokinase produced by different groups of streptococci differs greatly in structure.¹¹ However, due to its bacterial origin, the natural (native) SK might have a potential antigenic capacity.¹² This can be avoided by the use of rSK that has been produced by recombinant DNA technology,¹³⁻¹⁵ and found to have similar thrombolytic activity as that of the native SK.¹⁶ Indeed, rSK has been

extensively used in clinical trials in myocardial infarction and proved to be an effective and safe thrombolytic agent.¹⁷ The aim of the present study is the production of a purified recombinant streptokinase from pathogenic *Streptococcus pyogenes* isolate by its expression in yeast, using recombinant DNA technology, and evaluation of its thrombolytic activity *in vitro*.

Methods. Experimental design. This involved the following steps: 1) Isolation of *Streptococcus pyogenes* from throat swabs collected from pharyngitis patients. 2) Amplification of the SK gene using bacterial DNA. 3) Cloning of the SK gene and its *in vitro* transcription in eukaryotic expression vector that was transformed into yeast. 4) Purification and characterization of the produced rSK protein. 5) Testing of the purified rSK protein for its thrombolytic activity *in vitro* compared with a standard commercial SK.

This study was conducted at the College of Medicine, King Khalid University, Abha, Saudi Arabia from November 2012 to December 2013. Throat swabs from 45 patients with pharyngitis were collected in the Microbiology Department, Asser Central Hospital, Abha, Kingdom of Saudi Arabia. Purification and characterization of the bacterial isolate was carried out by the hemolytic test as described by Owens et al.¹⁸ Thus, the hemolytic activity was examined on blood agar plates (Oxoid Ltd., Hampshire, United Kingdom), and then the plates were incubated at 37°C overnight. β -hemolytic activity is associated with complete lysis of red blood cells. A colony with the highest hemolytic activity was selected and reinoculated in 5 ml lysogeny broth medium. The culture was incubated overnight at 37°C with shaking at 200 rpm.

Bacterial genomic DNA was isolated by extraction from an overnight bacterial culture using QIAGEN DNA extraction kit (Qiagene, Hilden, Germany), according to the manufacturer's procedures. The bacterial genomic DNA was subjected to polymerase chain reaction (PCR) amplification using 16S rRNA universal primers to amplify around 420 bp of the variable region according to Hafez and Elbestawy.¹⁹ The PCR amplification was carried out using the SK primers that amplify the region between the base 24 and the base 1225; forward (5'-GTGGATCCATCGAGGGAAGGATTGCTGGGTATGAATGGCTG-3') and the reverse primer (5'-TGCTGCAGTTATTTGTCTTTAGGGTTATC-3') according to Cook et al.²⁰ The PCR reaction was performed in a total volume of 50 μ l; 5 μ l 10X Taq polymerase buffer, 2 μ l 4 dNTPs mixture 2.5mM, 4 μ l genomic DNA, 4 μ l of each primer (20pm/ μ l), 2U Taq polymerase (5U/ μ l), and the volume was completed

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to 50 µl with sterile ddH₂O. The PCR amplification conditions were: initial denaturation step at 95°C for 3 minutes (min), followed by 35 cycles consisting of denaturation at 95°C for one min, annealing at 51°C for one min, and extension at 72°C for one min ending with a final extension at 72°C for 10 min, and then the reaction was held at 4°C. We used a Perkin Elmer PE 9700 thermal cycler (PerkinElmer, Waltham, MA, USA).

The PCR product (1200 bp) was subjected to DNA sequencing using the automated DNA sequencer (Applied Biosystems, Foster City, USA). Sequence analysis was carried out using the CLUSTAL W 1.4 program (<http://www.genome.jp/tools/clustalw/>). The nucleotide sequences were analyzed with the BLAST database (<http://www.ncbi.nlm.nih.gov>) and the DNA sequence was submitted into the Gene bank under accession number: JQ844573.1. Cloning of amplified PCR products was carried out by a transferase activity based cloning protocol by using TOPO TA Cloning® with pCR® 2.1-TOPO® Cloning vector and a TOP 10 *E-coli* strain (Invitrogen, Oregon, USA). Recombinant plasmids were subjected to DNA isolation, the resultant plasmid DNA was digested using *Bam*H1 (Promega, Madison, USA) restriction enzyme, and ligated to the linearized prokaryotic expression pPROEX HT (Life technologies (Invitrogen, Oregon, USA). Streptokinase purification was carried out by Ni-NTA resin matrix (Qiagen, Research Biolabs, Malaysia). The induced bacterial cells were pelleted and resuspended in 4 volumes of lysis buffer (50mM Tris-HCl, [PH 8.5 at 4°C], 5mM 2-mercaptoethanol, one mM phenylmethylsulfonyl fluoride [PMSF]). The suspension was sonicated until 80% of the cell was lysed. The cell debris was removed by centrifugation; the supernatant was removed to a new tube (crude supernatant). Affinity purification was carried out according to the protocols outlined by Life Technologies (Invitrogen, Oregon, USA). Solubilization and renaturation of the SK protein were achieved according to the procedures developed by Martin et al.²¹ Thus, inclusion pellets were solubilized in 8M urea buffer (pH 8). The urea mixture was incubated at 25°C for one hour before the insoluble molecules were removed by centrifugation. The urea solution was then diluted in a high pH buffer (pH 10.7) for renaturation of SK. After the solubilization in 8 M urea, the inclusion body solution was diluted with phosphate buffer pH 10.7, the solution was incubated at 25°C for one hour and then adjusted to pH 8; and incubation was continued at 25°C for one hour. The solution was transferred for dialysis against buffer (20 mM Tris/HCl pH 8.0, 50 mM NaCl, one mM EDTA) at 4°C overnight. The

re-optimized gene was constructed to avoid *Eco*RI and *Apa*I site in the middle of the sequence. So, the gene was cloned into pPICZA by *Eco*RI and *Apa*I, and the open reading frame (ORF) was fused with C terminal HIS-tag. The vector was linearized with the double digestion of 100 µg of the vector DNA using 100 U of each restriction enzyme. The reaction was incubated at 37°C for 3 hours. Phenol/chloroform extraction was performed for the reaction to diminish any protein residues in the digestion reaction. The purified linear DNA vector was ligated with a double amount of the amplified gene DNA using the T4 DNA ligase enzyme (Bio-Rad, Hercules, CA., USA) and the reaction lasted for one hour at room temperature. The ligation reaction was purified from the protein and also was examined with the vector's forward and reverses primers to assure that the vector contains the insert.

The yeast *Pichia pastoris* (*P. pastoris*) SMD1168 was used for the production of recombinant proteins (Invitrogen, Oregon, USA). The yeast strain was cultivated in yeast peptone dextrose (YPD) medium (2% [w/v] glucose, 2% [w/v] Bacto-Peptone (Difco), and 1% [w/v] Bacto-Yeast extract, and the culture was incubated at 30°C. Transformation of *P. pastoris* was carried out according to the method described by Gietz et al.²² The rSK was expressed in a *P. pastoris* SMD1168 strain that was transformed with pPICZA as described above. The expressed protein was purified from the culture medium on a metal chelating affinity column. To eliminate trace contaminants, gel filtration chromatography was performed. Finally, SK yield was approximately 1.6 mg per 6 L of culture supernatant. The protein content of purified rSK was determined by standard Bradford assay.²³ Different concentrations of bovine serum albumin (BSA) were used to make a standard curve by which the real concentration of the resultant protein was determined. The BSA concentrations used for curve construction were; 0, 2, 5, 8, 11, 12, 15, 17, and 20 µg/ml). Approximately 40 µl of dye stock was mixed with 160 µl of protein standard solution or samples to be assayed and incubated for 10 min. Absorbance was then measured at 595 nm in a spectrophotometer (Perkin Elmer, Lambda EZ 201, USA). The total protein content of purified rSK was calculated from the standard curve. The protein molecular mass of purified SK expressed in yeast was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) 12% (polyacrylamide gel) using mini-electrophoresis protein cell (Bio-Rad, Hercules, CA., USA). The molecular size of the resultant protein was compared with the standard molecular markers in the 216 kDa to 14 kDa range.

The kinetic properties of purified rSK were detected as following: Human plasma (as a substrate) was prepared from blood samples collected into 0.1% volume of 3.8% sodium citrate. The plasma euglobulin fraction was prepared by 10-fold dilution of citrated plasma and acidification to pH 5.0 using 1% acetic acid after standing for one hour at 4°C. Diluted plasma was centrifuged at 3500 rpm for 15 min at 4°C and the euglobulin precipitate was dissolved in 0.1 M Tris-HCl buffer, pH 7.4. Ninety-six well-microtiter plates were used to determine the euglobulin lysis time. Control samples were prepared by adding 20 µl human thrombin (10 IU/ml) and 30 µl 0.1 M Tris-HCl buffer, pH 7.4, to individual wells. Test samples were prepared by adding 10 µl thrombin, 10 µl buffer, and 20 µl SK solution. Clot formation was initiated by the addition of 150 µl of the plasma euglobulin fraction. The turbidity in the wells was measured as absorbance at 340 nm every 30 seconds for 20 min using a SPECTR Nano Microplate Spectrophotometer (BMG Labtec, Ortenberg, Germany). Effect of different concentrations of human plasma as a SK enzyme substrate, on maximum velocity (V_{max}) and Michaelis constant (K_m) of purified rSK were detected.

The yeast recombinant SK activity was determined using the colorimetric method as follows: A standard curve was made using the standard enzyme (Sigma, St. Louis, MO, USA) with different known concentrations diluted (1 mg/ml) as follows: (1:1, 1:2, 1:3, 1:4, 1:5, 1:6, and 1:7). The release of Coomassie brilliant blue R-250 dye from the clot when the enzyme was used as thrombolytic agent is the main factor for measuring the activity.²⁴ Approximately 20 µl of freshly prepared Coomassie brilliant blue R-250 with a concentration of one mg/ml was added to 200 µl of diluted human plasma (1:5, v/v) and mixed. A 100 µl of thrombin (Sigma, St. Louis, MO, USA, 2.5 units/ml) were then added to facilitate the formation of a plasma clot. Subsequently, one ml of commercial SK standard (Sigma, St. Louis, MO, USA) (0.1 mg/ml) was used as a plasminogen activator to initiate clot lysis. The mixture was incubated for 10 min at 37°C. During the course of lysis, 100 µl of soluble material were transferred to microliter wells and the absorbance at 540 nm was determined as a measure of clot lysis.

The thrombolytic activity of the purified yeast rSK compared with a commercial streptokinase (Sigma, St. Louis, MO, USA) was determined by the method described by Mao and Tucci²⁴ using a colorimetric assay for measuring the lysis of a plasma clot. Thirty µl of freshly prepared Coomassie brilliant blue R-250 (one mg/ml) were mixed with 200 µl of diluted human

plasma (1:6). The thrombin (Sigma, St. Louis, MO, USA) (2.5 units/ml) was used to compare the activity of the resultant enzyme and 100 µl were used for formation of a plasma clot. Different concentrations (200-2000 U/ml) of commercial streptokinase standard (Sigma, St. Louis, MO, USA) and the purified yeast rSK were used as a plasminogen activator to initiate clot lysis (in vitro). During the course of lysis, 100 µl of soluble material were transferred to spectrophotometer cuvette (2 ml) and the clot lysis was measured at O.D.₅₄₀. One unit of rSK was defined as one unit of standard SK, which liquefies a standard clot of fibrinogen, plasminogen, and thrombin at 37°C, and pH 7.5 for 10 min.

Results. The SK species were isolated from swabs collected from local pharyngitis patients. Out of the swabs from 45 pharyngitis patients, we isolated streptococcus species from one culture containing 35 colonies of high β-hemolytic activity. The isolate with the highest β-hemolytic activity was selected and its genomic DNA was extracted for molecular taxonomy purposes by partial sequencing of the 16S rDNA gene using PCR. Figure 1A shows PCR amplification of the 16S rRNA gene of the obtained *S. pyogenes* revealing a DNA sequence closely related to *S. pyogenes*. The sequence of the bacterial isolate was deposited in Gene Bank (*S. pyogenes* strain SAE) under accession number JQ844573.1. Figure 1B shows the phylogenetic tree of *S. pyogenes* isolate that reveals a great similarity with other 20 different strains presented on the gene bank. This figure shows that the examined 21 bacterial strains were grouped into 2 main clusters. The first cluster contains 13 different strains while the second cluster contains

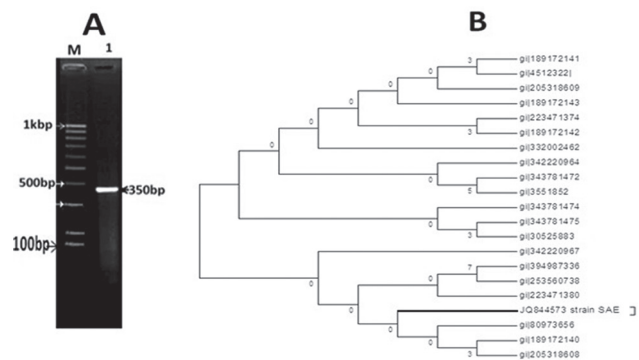


Figure 1 - Photograph showing the A) polymerase chain reaction (PCR) amplification of the 16S ribosomal RNA (rRNA) gene of the selected *S. pyogenes* using specific PCR. B) The phylogenetic tree of the new *S. pyogenes* isolate compared with other *S. pyogenes* presented in Gene Bank. The phylogeny was constructed based on the DNA nucleotide sequence of the 16S rRNA gene. The Mega 4 program was used for construction of the phylogeny.²¹

9 strains including the new one. The 2 clusters were divided into different subclusters, and each subcluster was divided into different groups. In case of the second cluster; 2 subclusters were observed; subcluster one contains only the strain (gi|34220967), but subcluster 2 was grouped into 2 main groups; group one contains strains (gi|394987336, gi|2525883, and gi|223471380) and the second group was divided into 2 main subgroups; subgroup one contains the new strain (JQ844573.1) and subgroup 2 contains the strains (gi|80973656, gi|189172140 and gi|205318608). **Figure 2A** shows the phylogenetic tree of the new SK gene based on the nucleotide DNA sequence that was found to be closely related to the SK genes presented in the Gene Bank with different percentages. This new SK showed a 100% similarity with the gene (gi|1872136) with (French isolate). **Figure 2B** shows the phylogenetic tree of the new SK gene based on the deduced amino acids that were different from that observed by the nucleotide sequence. The amino acids sequence of the isolated gene is closely related to (gi|407876). The protein content of the purified rSK as detected by standard Bradford assay indicated a yield of 0.267 µg/ml.

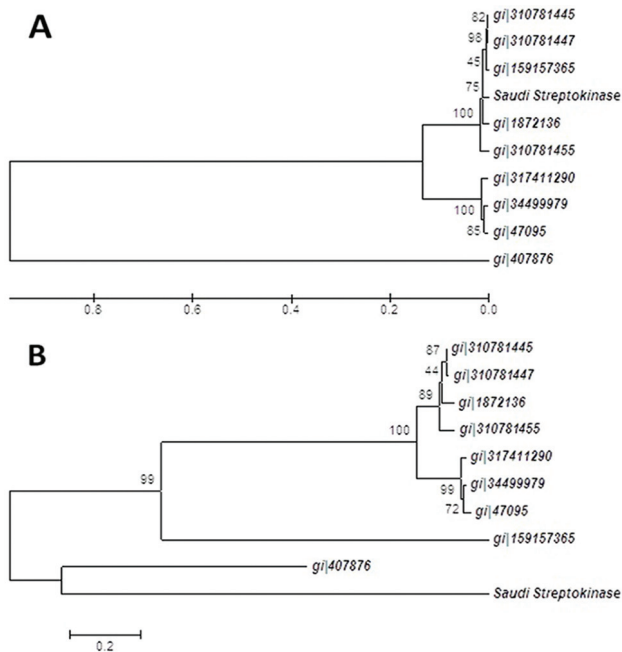


Figure 2 - Phylogenetic construction showing A) The phylogenetic tree for the streptokinase gene compared with the others listed in the Gene bank. The phylogeny was constructed using Mega 4 program based on the nucleotide DNA sequence.²² B) The phylogenetic tree for the streptokinase gene compared with others listed in Gene bank. The phylogeny was constructed using Mega 4 program based on the deduced amino acids sequence.²¹

Figure 3 shows SDS-PAGE for the purified recombinant protein isolated from the yeast compared with the standard commercial SK (Sigma, Saint. Louis, MO, USA), in addition to the protein markers (ranged from 216-14 kDa). Results revealed that the obtained recombinant protein was in the expected molecular weight range when compared with the standard one (46 kDa). Here, we purified the proteins from our own clones (no. 66) and the results were confirmed by specific PCR and sequencing as well. All results revealed that the expressed gene is the SK gene, and the validity of the PCR is 80% whereas the validity of the serology is 44%. Kinetics properties of the purified rSK showed a k_m value of 111.8 mg/ml, and a V_{max} of 208.3 U/min/ml. The biological activity (which represents the activity of rSK in units per ml of total volume, U/ml) and specific activity (which refers to the activity of rSK per mg of total protein, U/mg) were calculated. The calculated values for the biological activity, which gave the best clot lysis (82%) was 2000

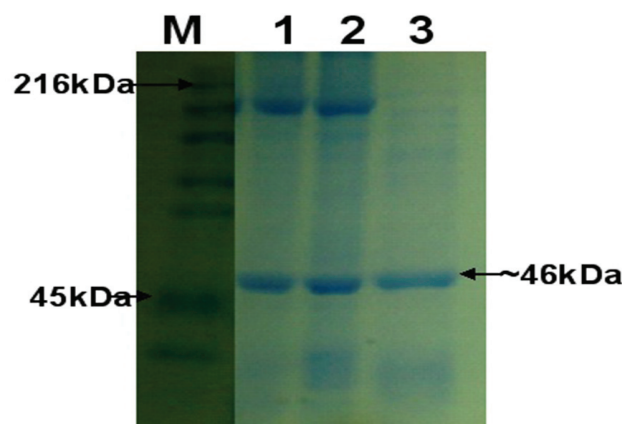


Figure 3 - Sodium dodecyl sulfate polyacrylamide gel electrophoresis for the partially purified and the purified recombinant streptokinase. M: High range protein marker. Lanes; 1 and 2: partially purified protein. Lane 3: purified protein.

Table 1 - Thrombolytic activity of the recombinant streptokinase (yeast) compared with a commercial SK (Sigma, Saint Louis, MO, USA).

Enzyme concentration (U/ml)	Mean clot lysis (%)	
	Yeast recombinant SK	Commercial SK
200	51±2.08	49±1.53
400	56±1.73	53±2.31
600	66±1.73	64±2.08
800	72±2.9	68±2.31
1000	83±2.00	79±1.73
2000	82±2.08	81±2.03

Data are expressed as Mean±SEM. Results are mean of 3 experiments performed in duplicate. SK - streptokinase

U/ml, corresponding to a specific activity of 7.49×10^6 U/mg of the purified protein.

Table 1 shows the thrombolytic activity of our yeast rSk compared with a commercial SK (Sigma, Saint. Louis, MO, USA) at different concentrations of both enzymes. Our purified rSK showed a (82%) clot lysis compared to that of the commercially available SK (81%) at an enzyme concentration of 2000 U/ml.

Discussion. Streptococcus species were isolated from swabs collected from local pharyngitis patients. Out of the swabs from 45 pharyngitis patients, we isolated streptococcus species from one culture containing 35 colonies of high β -hemolytic activity. The isolate with the highest β -hemolytic activity was selected, and molecular identification was carried out using the 16S rRNA gene²⁵ and the sequence analysis revealed that the obtained DNA sequence closely related to *Streptococcus pyogenes*. The sequence of the bacterial isolate was deposited in Gene bank (*S. pyogenes* strain SAE) under accession number JQ844573.1. When the phylogenetic tree was constructed for the new *S. pyogenes* isolate, it showed a great similarity with 20 other different strains presented in the database of the Gene bank. This new strain showed close similarity to the previous 2 strains, which were isolated from France and India in a respective manner. Walker et al²⁶ studied the relationship of his isolates, and the other *S. pyogenes* isolates presented in Gene bank based on the variable region of the 16S- rRNA gene (423bp). They used 30 different *S. pyogenes* and found that the examined 30 bacterial isolates were grouped into 3 main clusters. Conversely, Kalia and Bessen²⁷ found strong linkage disequilibrium between *S. pyogenes* strains (which may have different alleles of SK) and they suggested that the phenotype might contribute to increased bacterial fitness during skin infection.²

Streptokinase is a single chain, 414 amino acid proteins, composed of 3 distinct domains. For isolation of the SK gene from the identified bacterium by using specific primers, amplicon with molecular size (1100bp) was obtained. When the DNA sequence was performed for the PCR amplicon using Blast (NCBI), results showed that the sequence was closely related to the SK genes presented in Gene bank with different percentage. The phylogenetic tree was constructed based on the nucleotide DNA sequence. Our SK was closely related to the gene (gi|1872136) with a similarity of 100% (French isolate). The deduced amino acids showed results different from that observed by the nucleotide sequence. Figure 2B showed that the

amino acids sequence of the isolated gene is closely related to (gi|407876). The same results were obtained by Svensson et al²⁸ when phylogenetic trees were constructed based on the activity of the resultant SK that was isolated from the *S. pyogenes* strains. Phylogenetic studies on the sequence of 104 divergent SK revealed only 2 main sequence clusters (cluster type-1 and 2) with the presence of smaller sub-clusters for cluster type-2 sequences.²⁴

The recombinant yeast containing the SK gene inserted in pPICZA eukaryotic expression vector was *in vitro* transcribed. The yeast filtrate was subjected to protein purification, and the purified protein was separated on 12% SDS PAGE and a protein with molecular weight 46 KDa was observed. The results obtained in this study were consent with that obtained by Keramati et al²⁹ who isolated the SK gene from *S. equisimilis* bacteria and transformed it into *E. coli*, and the resultant protein showed a molecular weight of 47 KDa. Molae et al³⁰ reported the isolation of a purified recombinant SK with a molecular weight around 46 KDa. The protein content of the purified rSK as detected by the standard Bradford assay indicated a yield of 0.267 ug/ml. Kinetic properties of the purified rSK showed a k_m value of 111.8 mg/ml, and V_{max} of 208.3 U/min/ml indicating a high affinity of the enzyme to the substrate and high purity. The present purified yeast rSK was tested for its thrombolytic activity and compared with the commercially existing Sigma SK. Our purified rSK showed a 82% clot lysis compared to the commercially available SK (81%) at an enzyme concentration of 2000 U/ml.

The present purified yeast rSK was tested for its thrombolytic activity and compared with the commercially existing Sigma SK. Our purified rSK showed a 82% clot lysis compared to the commercially available SK (81%) at an enzyme concentration of 2000 U/ml.

In conclusion, the present rSK produced in yeast showed high *in vitro* thrombolytic activity comparable to standard commercially SK, suggesting its potential for use in thrombolytic therapy and its production on a large scale. *In vitro* experiments have contributed much to our understanding of the mechanisms of different therapeutic agents, but their value in predicting the effectiveness of treatment strategies in clinical trials has remained controversial. Clinical trials are required to prove the efficiency of this new recombinant streptokinase as a thrombolytic agent. In addition, we plan to study the cost effectiveness of production of this new rSK on a large commercial scale.

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