

Full Length Research Paper

Expression of recombinant Streptokinase from local Egyptian *Streptococcus* sp. *SalMarEg*

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Streptokinase (SK) is a therapeutically important thrombolytic agent. Cardiovascular disease is the first cause of adult death worldwide. In Egypt about 13% of the population die every year due to ischemic heart disease. In spite of this fact, there is no local production of cardiovascular therapeutics. We reported for the first time the expression of a recombinant SK from a local *Streptococcus* strain. When produced on industrial scale this r-SK may substantially contribute to reducing the costs of thrombolytic therapy in developing countries. In this study, a highly purified r-SK from *Streptococcus* sp. isolated from Egyptian pharyngitis patients was obtained. The isolated strain was partially identified using 16S rDNA sequencing and named *Streptococcus* sp. *SalMarEg*. It was found to be phylogenetically related to *Streptococcus pyogenes*. Analysis of the obtained sequence showed high similarity with other SK genes. The protein expression in a prokaryotic system obtained a 47-kDa SK protein that could be purified using a single-step his-tagged affinity purification chromatography, with nearly 80% recovery. The clot lytic activities of both recombinant and commercial SK were similar, thus giving the basis to scale up this SK product in order to evaluate the possibilities of its commercialization in local and/or regional markets.

Key words: Streptokinase, *Streptococcus SalMarEg*, thrombolytic agent, heterologous expression.

INTRODUCTION

Cardiovascular disease is one of the leading causes of death worldwide. According to World Health Organization (WHO) about 13% of Egyptian population die every year due to ischemic heart disease, which is considered the first cause of adult death in Egypt. Intravenous thrombolysis is the standard therapeutic approach for patients with acute myocardial infarction (Murray et al., 2010). Streptokinase (SK) is a life-saving clot-dissolving drug routinely prescribed universally in the management of

heart attack (Banerjee et al., 2004). It is now the leading fibrinolytic agent in the treatment of thromboembolic conditions (Boersma et al., 2003). It is known that the administration of a thrombolytic agent within six hours of the chest pain onset would reduce the risk of death by 40%.

SK is a protein secreted to the growth medium by many beta-hemolytic *Streptococcus* strains. SK activates plasma plasminogen to plasmin (Malke and Ferretti, 1984) causing fibrinolysis (Khil et al., 2003; Sun et al., 2004). Two plasminogen activators that occur naturally in the blood are the tissue type (t-PA) and the urokinase type (uPA). Both t-PA and uPA are trypsin-like serine proteases, which activate plasminogen directly (Coleman et al., 2005).

Currently, despite the wide use of tissue plasminogen

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activators in developed countries, SK remains essential to the management of acute myocardial infarction in developing nations (Sikri and Bardia, 2007). In Egypt, there are no specialized biotechnology firms and most pharmaceutical products are imported in bulk and then packaged in the country. Therefore, for socio-economical reasons, local biotechnological production of SK in Egypt would substantially contribute to reducing the costs of this important thrombolytic agent.

Native SK is a single chain polypeptide with a molecular weight of 47 kDa. The protein consists of 415 amino acid residues in a single polypeptide chain. Due to the pathogenicity of its natural host, the gene encoding SK has been cloned and expressed in several heterologous hosts such as *Escherichia coli* (Malke and Ferretti, 1984; Malke et al., 2000; Yazdani and Mukherjee 2002), *Bacillus subtilis* (Wong et al., 1994), *Lactococcus lactis* (Sriraman and Jayaraman, 2006), *Pichia pastoris* (Pratap et al., 2000), and *Streptomyces lividans* (Kim et al., 2010). On the other hand, hemolytic streptococci secrete several toxins that complicate the downstream purification (Pimienta et al., 2007).

Several affinity chromatography methods have been discussed for purifying SK. The earliest of such procedures used insolubilized di-isopropyl fluorophosphates (DIP) plasmin as the affinity ligand (Castellino et al., 1976). Purification through the affinity column caused a 30% decrease in the streptokinase activity. Different affinity purification methods used a monoclonal antibody ligand (Andreas, 1990; Babashamsi et al., 2009), acylated plasminogen or plasmin as the affinity ligand, or a combination of human plasminogen and monoclonal antibody against SK for chromatographic purification (Rodriguez et al., 1994). This purification method produced a preparation with about 50,000 units of activity per mg of protein and a purity of >93%. Since then, highly purified SK has become an important tool in fibrinolytic therapy.

Despite the mentioned advances, the low yield obtained in normal SK production and the pathogenicity of its natural host were the principal reasons leading to the development of a recombinant source. In this study we isolated a streptococcal strain from Egyptian pharyngitis patients and identified it as *Streptococcus* sp. *SalMarEg* using 16S rDNA sequencing. We amplified a full coding SK sequence using primers flanking the complete coding sequence of other *Streptococcus* species and cloned the product in *E. coli*. The recombinant product was then expressed in a prokaryotic expression system and obtained the purified SK protein (10 µg/ml) in a bioactive form with similar activity to commercial SK. To our knowledge, this is the first attempt to produce recombinant SK from local Egyptian species.

MATERIALS AND METHODS

Bacterial isolation, selection and culture

Throat swabs of pharyngitis patients were obtained from the Micro-

biology Department (Medical Research Institute, Alexandria University), and streaked on blood agar plate (Oxoid Ltd., Hampshire, United Kingdom) with *Streptococcus* selective media. Single colonies were streaked again on blood agar plates and incubated at 37°C overnight in order to estimate its hemolytic activity (agar plate contained 5 to 10% human blood). β-Hemolytic activity is associated with complete lysis of red blood cells. The colony with the highest hemolytic activity was inoculated in 5 ml of LB broth and incubated overnight at 37°C. The culture was subsequently inoculated into 50 ml of LB broth.

Identification of the *Streptococcus* sp. *SalMarEg*

Bacterial genomic DNA was extracted from an overnight bacterial culture using QIAGEN DNA extraction kit according to the manufacturer's protocol. The 16S rDNA was amplified from the bacterial genomic DNA using a 16S rDNA universal primer pair: 1155F: 5'-AAC TGG AGG AAG GTG GGG AT-3' (gb| HQ658085.1) R: 5'-AGG AGG TGA TCC AAC CGC A-3' according to Innis et al. (1990). PCR was performed in a total volume of 50 µl containing 5 µl of 5× Green Go Taq flexi buffer (Promega, USA), 5 µl of 5× colorless Go Taq flexi buffer (Promega, USA), 5 µl MgCl₂ (25 mM) (Promega, USA), 2 µl of dNTP mixture (10 mM of each) (BIORON, Germany), 50 ng bacterial genomic DNA as template, 4 µl of each primer (20 pmol/µl) and 2 U Taq polymerase (5 U/µl) (Promega, USA). The volume was completed to 50 µl with sterile ddH₂O. The PCR thermal profile used to amplify 16S rDNA was as follows: initial denaturation step at 95°C for 3 min, followed by 35 cycles of amplification with denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min ending with extension at 72°C for 10 min. PCR amplification was performed using a thermocycler (MJ Research, model PTC-200 Peltier, USA). Purification of the amplified 16S rDNA fragment was performed using QIAquick PCR purification kit according to manufacturer's instructions. Sequencing of the 16S fragment was carried out by MACROGEN, Korea using the 16S rDNA forward primer. Alignment of 16S rDNA sequence was conducted using CLUSTAL W (1.83) multiple sequence alignment software (Thompson et al., 1994; Larkin et al., 2007). Phylogenetic tree and molecular evolutionary analyses were performed according to NCBI nucleotide BLAST, genbank database and generated by CLC Genomics workbench-3.

Amplification, cloning and sequencing of the SK gene

A specific SK primer pair flanking the complete coding sequence was designed using DNA star software from about 218 DNA sequences representing genes from different streptokinase species selected from GenBank: 1F 5'-TAC TAA CGA CCT GGA CTC AC -3' and 1248R 5'-TAT TGG GAT TGC TG TTT ATT-3' (*Streptococcus dysgalactiae* subsp. *equisimilis* strain 04/04 streptokinase gene, complete coding sequence (gb| HQ629621.1)). Gene amplification was carried out using standard PCR as described above, however with annealing temperature of 51°C. The amplified PCR product was purified using the EzWay™ Gel Extraction kit according to the manufacturer's protocol then cloned into pCR® 2.1-TOPO® cloning vector in Top 10 *E. coli* strain using TOPO TA Cloning Kit® (Invitrogen™, USA). The insert was verified by DNA sequencing (MACROGEN, Korea) using the following forward primer 442F 5'-GGT TTA GTT CAT GTA TCG-3'.

Expression of SK gene

The cloned SK gene was released from the pCR® 2.1-TOPO® vector using *EcoRI* restriction enzyme, and purified using EzWay™ Gel Extraction kit (KOMBIOTECH, Korea) and ligated to

the linearized prokaryotic expression vector pPROEX™ HT according to the manufacturer's protocol. The pPROEX™ HT Prokaryotic Expression System was designed for expression of foreign proteins fused to a 6 × histidine sequence (His)₆ for affinity purification (Life Technologies, Inc. by QIAGEN, Inc., USA) (Hoffman and Roeder, 1991). *E. coli* strain DH5α competent cells were used in this study for subcloning of r-SK. Expression of r-SK was induced using Isopropyl-β-D-1-thiogalactopyranoside (IPTG) as follows: a single colony of sub-cloning transformation reaction was inoculated into 2 ml of LB media containing 100 µg/ml ampicillin. The culture was incubated overnight at 37°C. 10 ml of LB media containing 100 µg/ml ampicillin were inoculated with 0.1 ml of overnight culture and allowed to grow at 37°C. When the culture reached an A₅₉₀ of 0.5 to 1.0, 1 ml was removed and centrifuged for 1 min in a microcentrifuge (Hittich MIKRO 20, Germany). The supernatant was discarded and pellets resuspended in 100 µl of PBS and designated as (the uninduced sample). The remaining culture was induced by addition of IPTG to a final concentration of 0.6 mM and incubated as mentioned before. 1 ml culture was removed after 1, 2, 3 h after induction and OD measured at A₅₉₀. The cells were centrifuged as described above and the pellets resuspended in 100 µl of PBS and designated (the induced sample). 20 µl of each induced and non induced samples were mixed with an equal volume of 2X SDS sample buffer, boiled and were analyzed by SDS-polyacrylamide gel electrophoresis.

Affinity purification of 6X Histidine-tagged r-SK

The expressed r-SK fused to 6x His residue has a strong affinity against Ni-NTA resin matrix (QIAGEN, Inc., USA) (Hoffman and Roeder, 1991) that specifically binds His-tagged proteins. The induced bacterial cells were collected and resuspended in 4 volumes of lysis buffer [50 mM Tris-HCl (pH 8.5 at 4°C), 100 mM KCl, 5 mM β-mercaptoethanol, 1 mM PMSF]. The suspension was lysed by sonication, the cell debris removed by centrifugation and the supernatant transferred to a fresh tube (crude supernatant). Affinity purification was performed according to the manufacturer's protocols (QIAGEN NI-NTA affinity purification kit). The column was equilibrated with column binding buffer. The crude sample was loaded onto the column, which was washed with 10 volumes of binding buffer, then 2 volumes of wash buffer, followed by 2 volumes of binding buffer. The bound proteins were eluted with elution buffer (Life Technologies, Inc. by QIAGEN, Inc., USA).

Determination of r-SK concentration

Protein concentration was estimated in the elution fraction by Bradford assay. A standard curve was made using bovine serum albumin (BSA) with different concentrations (0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5 and 20 µg/ml). 40 µl of dye stock were 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 r-SK was calculated from the standard curve (Bradford, 1976).

Solubilization and renaturation of r-SK inclusion bodies

In this study, r-SK was expressed as insoluble inclusion bodies (inactive form), which required renaturation as follows: the inclusion pellets were solubilized in 8 M urea buffer at pH 8. The mixture was incubated at 25°C for 1 h before the insoluble parts were removed by centrifugation. The solution was then diluted with phosphate buffer (pH 10.7) for SK renaturation. The solution was transferred for dialysis against the buffer [20 mM Tris/HCl pH 8.0, 50 mM NaCl,

1 mM EDTA] at 4°C overnight. The folded SK was then assayed as thrombolytic agent. Using this procedure, approximately 3.0 mg of enzymatically active SK was obtained from 1 g wet weight.

Gel electrophoresis and Western blotting

Protein samples were prepared and run on 12% SDS-polyacrylamide gel in the presence of pre-stained molecular weight markers (14 to 116.0 kDa) (Jena Bioscience, Germany). Proteins were then transferred to a nitrocellulose membrane (Costa, Bio Blot, Canada) for 1 h at 0.3 A, and detection was performed using BCIP/NBT liquid Substrate System (Sigma, USA).

Enzyme activity assays

Zymography

Samples were run on 12% of PAGE with PBS but water in the separating gel was replaced by human plasma diluted (1:5). After running, the gel was soaked in 1% Triton X100 for 2 h. The solution was changed every 1 h to remove SDS for renaturation of the enzyme. The gel was then washed three times with tap water and soaked in universal buffer pH 8 for 2 h at 37°C. The gel was then stained with Coomassie blue stain overnight (0.25%, w/v) in methanol-acetic acid-water 5:1:4 (v/v/v) followed by destaining (Laemmli, 1970).

Quantitative assay for determination of r-SK activity

R-SK activity was estimated as units/mg using a simple quantitative colorimetric method. A standard curve was made using commercial SK with different known concentrations diluted from a stock of (1 mg/ml) as follows: (1:1, 1:2, 1:3, 1:4, 1:5, 1:6, and 1:7). The principle of the assay is based on the release of Coomassie brilliant blue R-250 dye from the clot on applying SK as thrombolytic agent. 30 µl of freshly prepared Coomassie brilliant blue R-250 (1 mg/ml) was added to 200 µl of the diluted human plasma (1:5, v/v) and mixed. 100 µl of thrombin (Sigma, USA) (2.5 units/ml) were then added to facilitate the formation of a plasma clot. Subsequently, 1 ml of commercial SK standard (Sigma, 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 microtiter wells and the absorbance at 540 nm was determined as a measure of clot lysis as shown in Supplementary Figure 1 (Mao and Tucci, 1991).

Comparison of clot lysis activity of commercial and r-SK

Venous blood was drawn from 20 healthy people without a history of oral contraceptive or anticoagulant therapy. About 500 µl of blood were transferred to previously weighed Eppendorf tubes arranged in two groups: one group for r-SK and the other for commercial SK and incubated at 37°C for 45 min. After clot formation, serum was completely removed and each tube was again weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of the tube alone). 100 µl of r-SK along with various dilutions in sterile distilled water were added to the tubes (0.01, 0.008, 0.006, 0.004, 0.002 g/ml) (n=20 for each dilution). Water was also added to the other pre-weighted tubes containing clots to serve as negative thrombolytic control. The same dilutions were done with commercial SK group as positive control in order to assess the effectiveness of both SKs as thrombolytic agents. All the tubes were then incubated at 37°C for 90 min and observed for clot lysis. After incubation, the fluid obtained was removed and tubes were

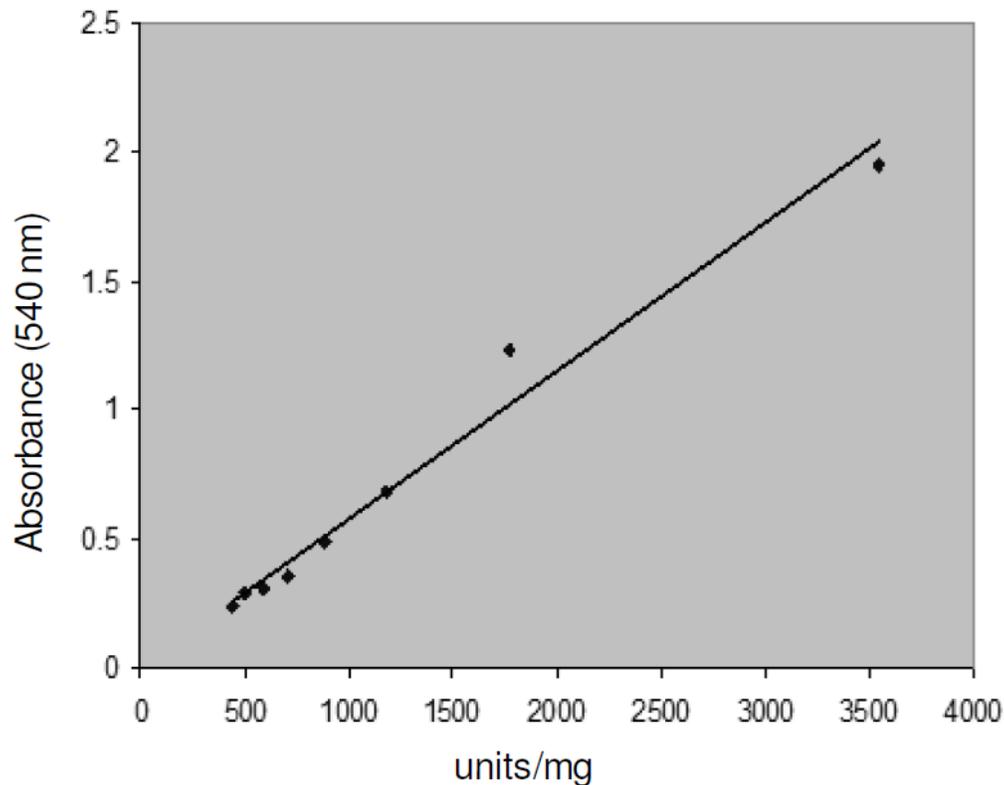


Figure 1. Quantitative colorimetric assay for determination of r-SK activity. A standard curve was made using commercial SK with different known concentrations diluted from a stock of 1 mg/ml. These were 3550, 1775, 1183, 887, 710, 591 and 444 units/mg, respectively. X-axis represents streptokinase (units/mg) dissolved in 1 ml buffer and y- axis represents the absorbance at 540 nm.

again weighed. Difference obtained in weight taken before and after clot lysis was expressed as mean percentage of clot lysis. The test was repeated three times for each dilution of r-SK and commercial SK in the blood samples of the 20 different healthy people (Prasad et al., 2006).

Ethical approval

Informed consent from pharyngitis patients and healthy volunteers were collected and approved by the ethics committee of the medical research institute, Alexandria University, Egypt and, therefore the study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

RESULTS

Isolation and identification of *Streptococcus* sp.

We isolated local *Streptococcus* species using swabs from Egyptian pharyngitis patients, which were routinely streaked on blood agar plates. Out of the swabs from five pharyngitis patients, we isolated *Streptococcus* sp. from one culture containing about 26 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 amplification and partial sequencing of the 16S rDNA gene (ca 350 bp) using PCR. Sequence analysis revealed that the selected isolate belongs to the *Streptococcus* genus and the partial sequence was submitted to GenBank under the accession No. FJ490631. Nucleotide BLAST and phylogenetic analysis of the submitted sequence revealed that it may be a new *Streptococcus* isolate, and it was subsequently called *Streptococcus* sp. *SalMarEg*. *Streptococcus* sp. *SalMarEg* had 99% identity with *Streptococcus pneumoniae*, *Streptococcus intermedius*, *Streptococcus sanguinis*, *Streptococcus anginosus* and 98% identity with *Streptococcus mitis* and *Streptococcus suis*.

SK gene amplification, cloning and subcloning into prokaryotic expression vector

The SK gene was amplified from the genomic DNA of *Streptococcus* sp. *SalMarEg* using specific SK primers flanking the complete coding sequence of other *Streptococcus* sp. (Figure 2A, Lane 2). The PCR product(1000-1500 bp) was excised from the gel, purified

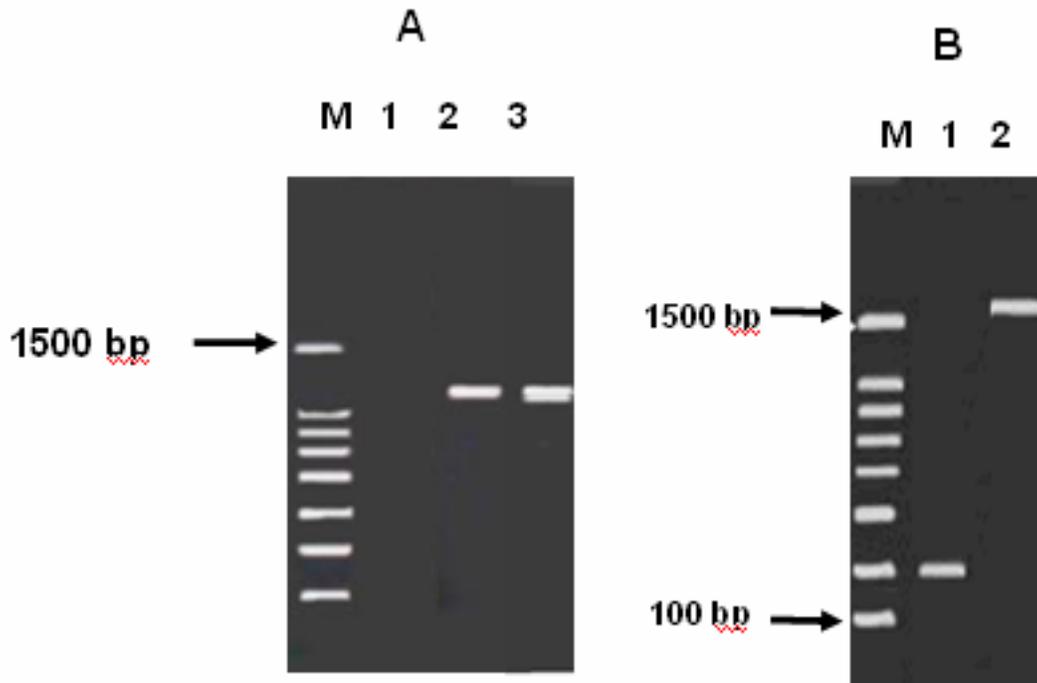


Figure 2. Cloning of SK gene from *Streptococcus* sp. *SalMarEg*. One percent agarose gel showing (A) lane M, DNA ladder marker (100 bp) (Biron, Germany); Lane 1, negative control of PCR reaction (contains water); lane 2, PCR amplification product of SK gene (~1320 bp) from the genomic DNA of *Streptococcus* sp. *SalMarEg*.; lane 3, SK insert amplified from TOPO cloning vector using SK specific primers after cloning the SK PCR product. (B) PCR product amplified from TOPO cloning vector using M13 primers. Lane M, DNA ladder marker (100 bp) (Biron, Germany); lane 1, PCR amplification of TOPO cloning vector containing no SK insert; lane 2, PCR amplification of TOPO cloning vector containing SK insert.

and cloned in the pCR 2.1-TOPO plasmid vector. We screened for the SK insert using streptokinase-specific primers, which yielded the expected 1320 bp band (Figure 2A, lane 3), as well as with M13 universal primers, which yielded a 1520 bp band that included an additional 200 bp representing the M13 vector sequence (Figure 2B, Lane 1). Next, we digested the plasmid DNA with *EcoRI* to release the insert, which was sub-clonedTM into the prokaryotic expression vector pPROEXTM HT. The transformed bacterium was subjected to gene induction using IPTG in the culture medium.

Sequencing of the recombinant SK and phylogenetic analysis

In order to confirm our results, we sequenced the r-SK using SK forward primers and submitted the obtained partial sequence into GenBank database under the accession No. FJ490630. We translated the r-SK nucleotide sequence using ExPASy tool (Albar et al., 2007) and compared its amino acid sequence with that of SK from *Streptococcus equisimilis*, *Streptococcus uberis* and *Streptococcus pyogenes* using CLUSTAL W (1.83)

multiple sequence alignment software and Mega4 software (Tamura et al., 2007). The identity scores were 82, 25.6 and 90% in the aligned regions, respectively (Figure 3). We then used NCBI-BLAST for nucleotide sequence alignment and used the results to construct a phylogenetic tree using CLC Genomics Workbench-3 software (Figure 4).

Purification of r-SK and Western blot analysis

pPROEXTM HT expression vector expresses recombinant proteins fused with 6x His residues. His-tagged recombinant proteins have a great affinity towards Ni-NTA matrix. In this study, the high affinity concentration of the recombinant protein enabled single-step purification by one step His-tagged affinity purification chromatography, with nearly 80% recovery. For the assessment of r-SK purity, washing and elution fractions were examined using 12% SDS-PAGE (Figure 5A). All samples to be examined were pre-heated prior to running on gel, and about 30 μ l of each sample were loaded. The purified r-SK-6-His-Tag was then immunoblotted and detected using anti-His-Tag antibody, which gave a 47

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          10      20      30      40      50      60
equisimilis  MKNYLSFGMFFALLFALTFFGTVNSVQAIAGPEWLLDR----PSVMNSQLVVSVAGTVEGTN
pyogenes    MKNYLSFGMFFALLFALTFFGTVKPVQAIAGPEWLLDR----PSVMNSQLVVSVAGTVEGTN
uberis      MKKWFLLMLLLGI FGCATQPSK-VAAITGYDSDYYARYIDPDENKITFAINVDGFVEGSN
SalMarEg    --PNQVLCKMQLVQRIMSKRNTTITHEKKNQYKLOQN----LLILDMLYRLLPSTLMMISHQ
          :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :
Prim.cons.  MKNYLSFGMFFALLFALTFFGTV23VQAIAGPEWLLDRRYIDPSVMNSQLVVSVAGTVEGTN
          70      80      90      100     110     120
equisimilis  QDISLKF FEIDLTSRPAHG GKTEQGLSPKSKPFATDSGAMPHKLEKADLLKAIQEQLIAN
pyogenes    QEISLKF FEIDLTSGPAQG GKTEQGLSPKSKPFATNKGAMPHKLEKADLLKAIQEQLIAN
uberis      QEILIRGIHHLTD-----QNQK-----I VTKAELDAIRHQMVLL
SalMarEg    FSKILMYSKHSLSATPS----HPKM-----YWLKHKAFSTKAIQITSFLN
          .  :.  .  *:  :  :  :  :  :  :  :  :  :  :  :  :  :
Prim.cons.  QEISLKF FE2DLTS3PA2G GKTEQGLSPKSKPFAT22GAMPHKLEKADLLKAIQEQLI2N
          130     140     150     160     170     180
equisimilis  VHSMDDY FEVIDFASDATI TDRNGKVYFADK--DGSVTLPTQP VQEFLLKGHVVRVPYKE
pyogenes    VHSMDGY FEVIDFASDATI TDRNGKVYFADR--DGSVTLPTQP VQEFLLSGHVRVRVPYQP
uberis      QLDYS--YELVDFAPDAQL LTRDRRLLFANRNFEESV SLEDT-IQEYLLKGHVILRKRVE
SalMarEg    RHSSQG-YFPYNTNGS---R---VYLPCQ-----IPDQ-----SCNREQAYQNDNK
          .  .  :  :  *:  :.  :  :  *  :  :  :  :  :  :  :  :  :
Prim.cons.  VHSMDGY 2EVIDFASDATI TDRNGKVYFADRNF3SVTLPT2QP VQEFLLKGHVVRVPY4E
          190     200     210     220     230     240
equisimilis  KPVQNOAKSVDVE YTVQFT PLNPD DFKPVLKDTKLLKTLAIGDTVTSQELLAQAQSILM
pyogenes    KAVHNSAERVNVNVEVSVFVSETGDLDFTPLLRNQYHLTTLAVGDSLSSQELAAIAQFILS
uberis      EPITHPTETANIE YKVQFATKDG EFHPLPIFVDYG--EKHIGEKLTSD EFRKIAEEKLL
SalMarEg    TGLKKETKNTDLI SEKYYI LKKGEE PYDFLRLY-----CLYVLVD SLLTVSKEIY
          :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :
Prim.cons.  KPV4N4224V2VE YEVQF44K4G24DF4P4LRD4Y2L2TLAIGD42TS2ELLAIAQ4IL4
          250     260     270     280     290     300
equisimilis  ESHPDYTIYERDS SIVTHDKD-IFR TILPMDQEFTYRVKNREQAYEINKKSG---LNEEI
pyogenes    KKYPDYIITKRDS SIVTHDND-IFR TILPMDQEFTYHIKDREQAYKANSKTG---IVEKT
uberis      QSYPDYIMIDQKEYTIIKHNSLGLPRYYSYPDDFSYEIQDRQRIMAKDPKSGK--ELGET
SalMarEg    KEYGYA EKTISVALSGVDGEQSKSKSATIPKEWNATAITVVEYSSSNSYSRCSTLLCSG
          :  :  :  *  .  :  :  :  :  :  :  :  :  :  :  :  :  :
Prim.cons.  KSYPDY4I44RDS SIVTHD 4D2IFR TILPMD2QEFTY4IKDREQAY44NSKSG2STLLEET
          310     320     330     340     350     360
equisimilis  NNTDLISEKYYVLKKGKPYDPFDRSHLKLFTIKYVDVNTMELLKSEQLLTASERNLDFR
pyogenes    NNTDLISEKYYVLKKGKPYDPFDRSHLKLFTINYVDVNTMELLKSEQLLTASERNLDFR
uberis      QSIDNVFEKYLITKKSYP-----
SalMarEg    FESHKIRTEYSLLVN-----
          .  .  :  :  *:  :  :  :  :  :  :  :  :  :  :  :
Prim.cons.  NNTDLISEKYYVLKKGKPYDPFDRSHLKLFTI2YVDVNTMELLKSEQLLTASERNLDFR
          370     380     390     400     410     420
equisimilis  DLYDPRDKAKLLYNNLDAFGIMDYTLTGKVEDNHDDTNRIITVYMGKRPEGENASYHLAY
pyogenes    DLYDPRDKAKLLYNNLDAFGIMDYTLTGKVEDNHNDTNRIITVYMGKRPEGENASYHLAY
uberis      -----
SalMarEg    -----
          Prim.cons.  DLYDPRDKAKLLYNNLDAFGIMDYTLTGKVEDNH2DTNRIITVYMGKRPEGENASYHLAY
          430     440     450
equisimilis  DKDRYTEEEREVYSYLRYTGTPIPDNPNDK
pyogenes    DKDRYTEEEREVYSYLRYTGTPIPDNPNDK
uberis      -----
SalMarEg    -----
          Prim.cons.  DKDRYTEEEREVYSYLRYTGTPIPDNP2DK

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Figure 3. Comparison of the amino acid sequence of r-SK from *Streptococcus* sp. *SalMarEg*. to that of SK from *S. equisimilis*, *S. uberis* and *S. pyogenes* using CLUSTAL W (1.83) multiple sequence alignment software and Mega4 software (Tamura et al., 2007). The identity (*): 10 is 2.22%, strongly similar; (:): 47 is 10.44%, weakly similar; (.) : 28 is 6.22% and different: 365 is 81.11%.

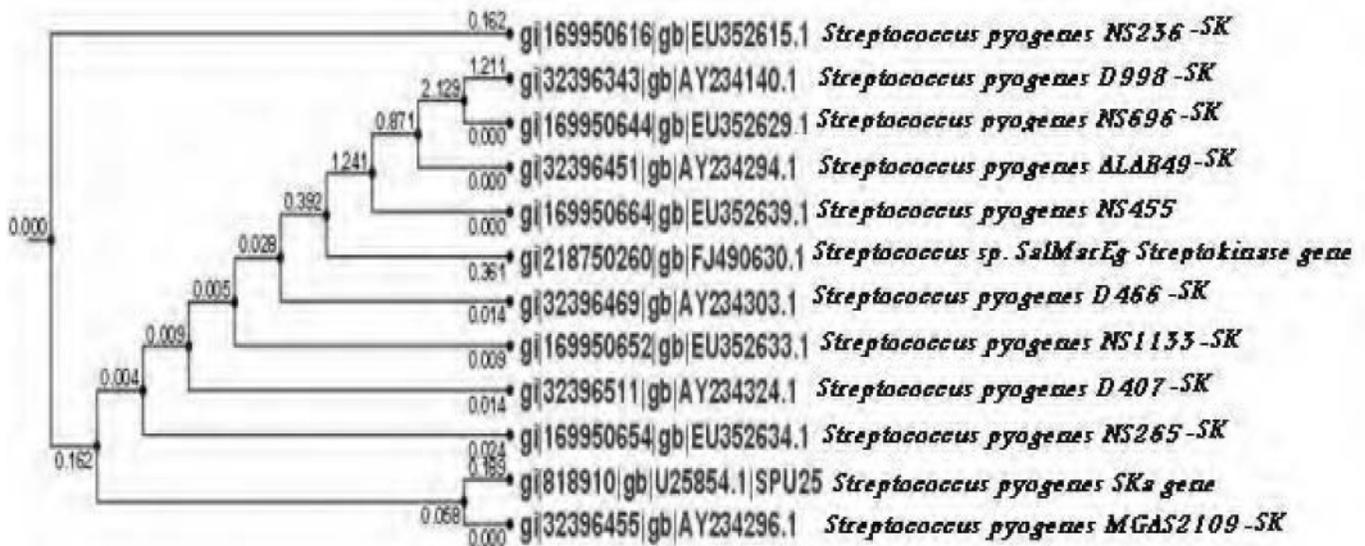


Figure 4. Phylogenetic tree of *Streptococcus* sp. *SalMarEg* SK gene partial codons generated by CLC Genomics workbench-3 according to NCBI nucleotide BLAST and GenBank database.

kDa band (Figure 5B).

Detection of r-SK activity

Zymography

The renaturation efficiency of r-SK was evaluated using zymography. A SK substrate (plasminogen) replaced water in the separating gel in SDS-PAGE. Different concentrations of r-SK were loaded onto the gel and different activities were obtained as shown in Figure 6A.

Colorimetric assay

A colorimetric assay for SK activity quantification was performed in order to estimate the units (U) of enzyme per mg of recombinant protein. According to the standard curve from serial dilutions of a commercial SK stock, results revealed that 1 mg r-SK contained about 2950 U (Figure 1).

Comparison of clot lysis activity of r-SK and commercial SK according to the *in vitro* model study

To determine the *in vitro* activity of r-SK to lyse blood clots, five different concentrations of r-SK (0.01, 0.008, 0.006, 0.004 and 0.002 g/ml) for each of the 20 blood samples were used. Each experiment was repeated three times. Mean percentage of clot lysis was calculated for each concentration: these were 72.6, 64.2, 62.9, 59.2, 56.7% for the five different r-SK dilutions, respectively,

and 2.2% for the negative control (water). These results were almost similar to those of the commercial SK (Figure 6B). It was observed that 2000 U/ml of the commercial native SK gave the same activity as 1904 U/ml of the r-SK herein reported.

DISCUSSION

Isolation and identification of *Streptococcus* sp.

In this study, SK isolated from local *Streptococcus* sp. *SalMarEg* was efficiently produced in a recombinant bioactive form. It is worthy to mention that the binding of plasminogen by pathogenic Group C streptococci isolated from human, horses, and pigs is specific to the homologous host (McCoy et al., 1991). For this reason, we isolated *Streptococcus* sp. from human origin. It has been previously reported that SK activity associated with the plasminogen activator secreted from *S. uberis* is different from that of *S. pyogenes* (Lancefield group A) and *S. equisimilis* (Lancefield group C) strains, as it is able to activate bovine but not human plasminogen. It also differs from that produced by Lancefield group E strains because of its inactivity against porcine plasminogen (Ward and Leigh, 2004).

The isolated *Streptococcus* strain in this study was molecularly identified based on 16S rDNA gene sequence. In this work, the amplified SK gene from the selected isolate's genomic DNA was cloned into an expression vector. Previous attempts were made to clone SK gene included *in vitro* cloning by PCR using specific SK primers and restriction digestion of the gene from the whole genome (Malke and Ferretti, 1984). In this study,

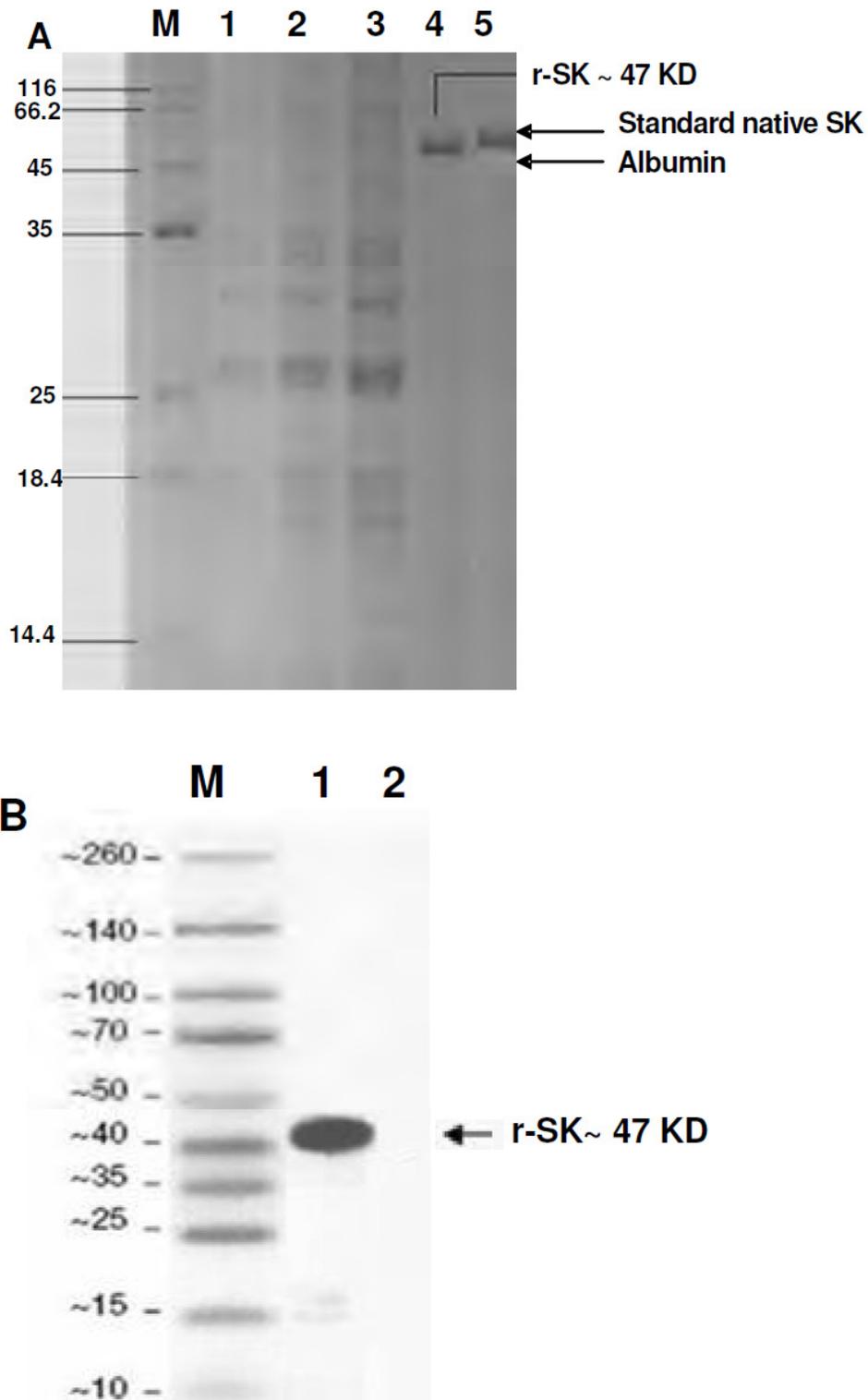


Figure 5. Affinity purification of r-SK. (A) 12% SDS-PAGE showing affinity purification fractions of r-SK; Lane M, protein molecular weight marker 14.4 to 116 kD; Lane 1, uninduced *E. coli*; Lane 2, IPTG-induced *E. coli* for r-SK expression; Lane 3, washing fraction of affinity purification; Lane 4, elution fraction (r-SK); lane 5, standard native SK (Sigma, USA). (B) Western blot analysis of purified r-SK. Lane M; Spectra™ Multicolor Broad Range Protein Ladder (10-260 kD) (Fermentas, Canada); Lane 1, r-SK purified fraction detected by QIA Express anti His-Tag detection system (ALP labeled); Lane 2, negative control.

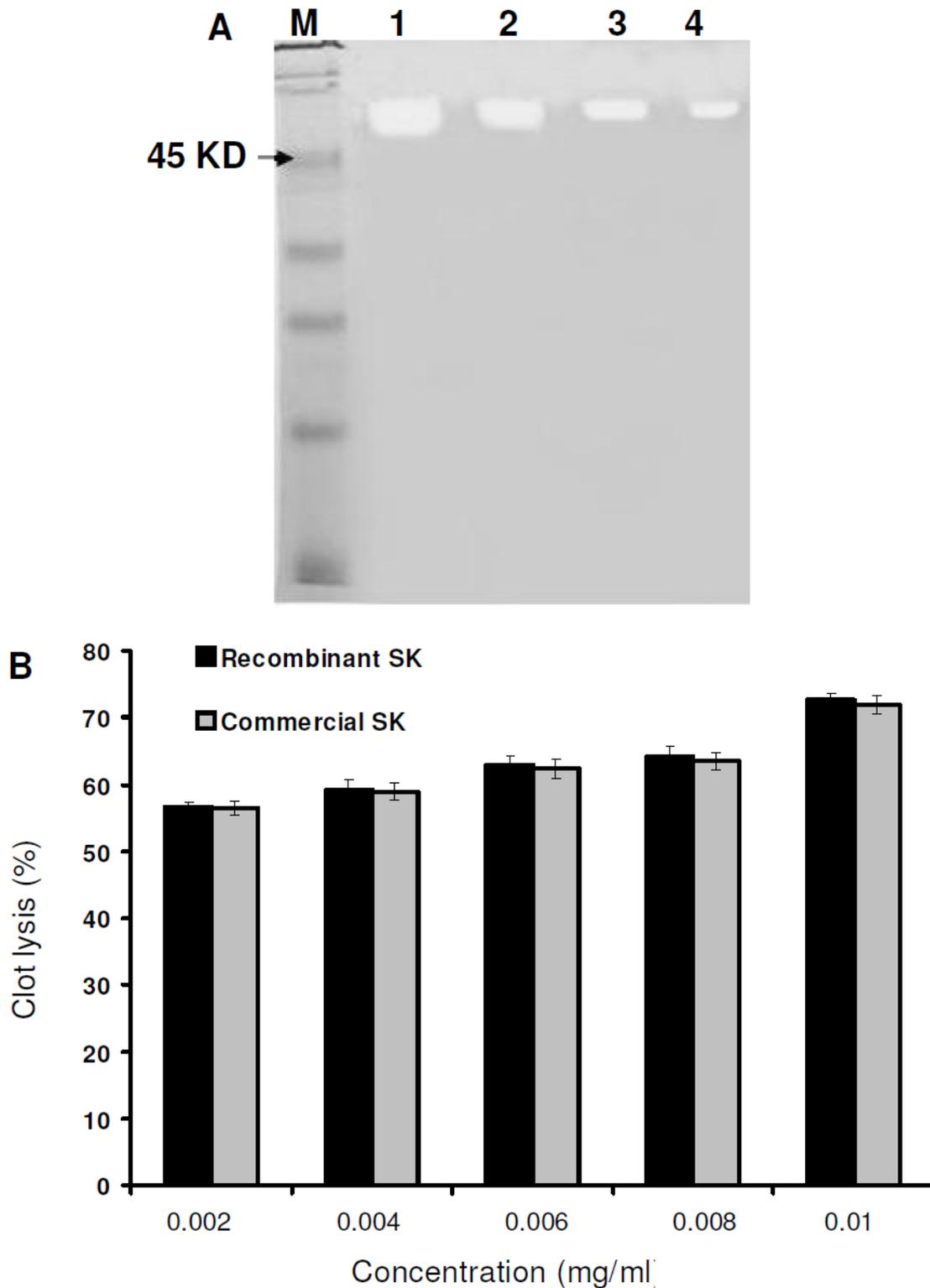


Figure 6. r-SK activity. (A) r-SK activity by zymography. Different concentrations of r-SK were run on 12% SDS-PAGE in 1X electrophoresis buffer, stained with coomassie blue R-250 (Alliance bio, USA) and photographed. Lane M, protein molecular weight marker 14.4 to 116 KD (Jena Bioscience, Germany); lane 1, 2, 3 and 4 each represents 50, 40, 30, and 20 μ l from 0.1 mg/ml of renatured r-SK. (B) Clot lytic activity of r-SK. histogram showing mean \pm SD of clot lytic activities of different concentrations of r-SK in comparison to the same concentrations of commercial standard SK (Sigma, USA) using blood samples from normal subjects (n=20). SK activity represents the average of three replicates.

the r-SK protein (fused with 6X His-Tag) expression was induced by IPTG and the obtained r-SK was subsequently purified. In general, two different strategies for the production of enzymatically active SK were reported. In the first strategy, SK is expressed as inclusion bodies without its signal sequence followed by purification, solubilization, and renaturation to obtain an active preparation. In the second strategy, enzymatically-active SK is secreted into the culture medium. The design of the expression strategy depended on process economics, regulatory requirements and the end-use of the purified protein (such as biochemical studies, structural characterization, and commercial usage as an industrial enzyme). In this study, we used the second strategy as it supports the biochemical characterization and semi-industrial approach for the production of r-SK in Egypt.

Sequencing of the recombinant streptokinase and phylogenetic analysis

Among the currently known SKs, *S. uberis* seems to be the least conserved, since the degrees of identity at the nucleotide level for six other streptokinases from serological Groups A, C, and G range between 80 and 98% (Frank et al., 1995). In line with this observation, no similarity between SK from *Streptococcus* sp. *SalMarEg* and SK from *S. uberis* was found at the nucleotide level by BLAST searching.

The fact that streptococci with different host specificities produce SK that shows considerable sequence diversity but conserved plasminogen activation potential indicates that generation of plasmin activity is important for the pathogenesis of these bacteria. On the other hand, the high degree of amino acid sequence diversity also indicates that only a low degree of sequence constraint is needed for the ability of SK to activate plasminogen. In this study, we used *E.coli* DH5 α strains for the high level expression of r-SK. Mahmoudi et al. (2010) used *E.coli* BL21 (DE3) *plysS* strain, which is deficient in cytoplasmic protease gene products and, therefore, gives high level expression of fusion proteins (Sugimura and Higashi, 1988). Other hosts have certain limitations. For example, *B. subtilis* produces a number of extracellular proteases, which recognize and degrade heterologous proteins. In addition the recombinant vector is largely unstable inside the competent *Bacillus* strain.

Purification and activity of r-SK

The possibility to obtain high concentrations of the r-SK enabled the single-step purification by His-tagged affinity purification chromatography, with nearly 80% recovery. To our knowledge, this would be the first report on a highly pure r-SK in a single-step purification process. Zhang et al. (1999) obtained 49% recovery and 99%

purity of r-SK using two-step purification by ion exchange followed by gel filtration chromatography. Ignatova et al. (2000) reported purification of excretory r-SK with 51% recovery and about 97.8% purity.

Several affinity chromatography methods have been discussed for purifying SK (Castellino et al., 1976; Liu et al., 2000). It was concluded that downstream processing costs can be considerably reduced and yields of pure recombinant protein is enhanced by reducing the number of processing steps and increasing the recovery of protein product in each step. Inclusion bodies, refolding and single-step purification achieves this goal. The whole process of expression and purification involves a minimal set of procedures and is very useful for economic production of proteins with therapeutic significance such as SK on a large scale.

In this study, r-SK was induced as inclusion bodies in a small scale. 10 μ g/ml was obtained. Expression of r-SK as inclusion bodies provides a large quantity of the enzyme that can be easily recovered and refolded (Azuaga et al., 2002; Balagurunathan et al., 2008). The production of recombinant protein as insoluble inclusion bodies accumulates about 25% of total cell protein compared to the soluble secretory recombinant protein, which accumulates up to 15% of total cell protein (Perez et al., 1998).

In this work, we found that 1 mg r-SK contained about 2950 U. Other studies reported that when r-SK was expressed as secreted into the medium or under shake-flask cultivation method, about 8 to 100 U/ml and 55 U/mg were obtained, respectively (Malke and Ferretti, 1984). In this study, we found that 2000 U/ml of commercial native streptokinase gave the same activity as 1904 U/ml of our r-SK using an *in vitro* colorimetric model. Most of the *in vitro* methods that are currently applied to study thrombolysis have certain limitations. Some involve complex computational and mathematical skills that give only theoretical prediction of the outcome and most are expensive to be performed in a laboratory. The assay used in this study is easy and cost effective (Prasad et al., 2006).

The importance of substitution of expensive imported SK by locally produced low cost r-SK is reinforced by the notion that not all commercial SK preparations are effective (Quriel, 2002). This was demonstrated in a comparative study by Hermentin et al. (2005), in which they compared the activities of 16 different SK preparations and found that only three fulfilled the minimum requirements of the European Pharmacopoeia (Hermentin et al. 2005; Thimme, 2005).

Conclusion

This work introduces a novel strategy for the production of purified, efficient and low cost r-SK for pharmaceutical applications. The ongoing further step after the successful

production of bioactive r-SK is to scale up the production process using fermentation technology. This would represent the first time to develop r-SK in Egypt.

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