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Cloning and Overexpression of Active Recombinant Fusion Streptokinase: A New Approach to Facilitate Purification

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Abstract: Streptokinase is a common fibrinolytic drug and included in the World Health Organization (WHO) Model List of Essential Medicines. Comparative clinical trails such as cost-effectiveness suggest that streptokinase can be the drug of choice for thrombolytic therapy. To reach the highest amount of the protein and production of active form of streptokinase in bacteria need to modify and optimize methods. In the present study, chromosomal DNA was extracted from *S. equisimilis* H46A and used for amplification of streptokinase gene (*skc*) (mature section: 1245 bp) by cloning into pGEX-4T-2 vector which contains a *tac* promoter. The cloning results were controlled by PCR, double digestion and sequencing. The expression level of the protein in different strain of *E. coli* was optimized and reached up to 50% of the total cell protein. The function of the fusion protein as active fibrinolytic protein was confirmed by plasmin hydrolysis of chromogenic peptidyl anilide substrate assay.

Key words: Recombinant fusion streptokinase, *S. equisimilis* H46A, cloning

INTRODUCTION

Streptokinase (SKC), a conventional fibrinolytic drug that is produced by some strains of β-hemolytic streptococcuses and included in the World Health Organization (WHO) Model List of Essential Medicines (WHO, 2005). It is used in life-threatening deep-vein thrombosis, acute myocardial infarction, pulmonary embolism, thromboembolism, thrombosed arteriovenous shunts (WHO, 2004). Streptokinase is a 45-50 Kilodalton (KD) glycoprotein that mediates the cleavage of a peptide bond in plasminogen (Pg), producing the active product plasmin, which cleaves fibrin polymers to promote thrombolysis (Hermentin *et al*., 2005). The group C strain *Streptococcus equisimilis* H46A (ATCC 12449) is the main source for isolation of the streptokinase gene (Malke *et al*., 1985). This strain does not produce erythrogenic toxins and is less fastidious in its growth requirements than the majority of group A strains (Christensen, 1945). Therefore, this strain was the source of the gene for cloning in this study. Comparative clinical trials in the treatment of acute myocardial infarction suggested that streptokinase is a cost-effective and useful thrombolytic drug (Banerjee *et al*., 2004; Gruen *et al*., 2005). Hence, several studies have been conducted focusing on production and improvement of streptokinase (Estrada *et al*., 1992; Zhai *et al*., 2003; Kumar and Singh, 2004; Kazemi *et al*., 2006; Erdogan *et al*., 2006). In the present study, the issue of purification of recombinant SKC (rSKC) is addressed, for which a new cloning approach to produce active rSKC with Glutathione S-transferase tag (GST-SKC) was introduced.

MATERIALS AND METHODS

Bacterial strains and culture condition: Strain *S. equisimilis* H46A ATCC 12449 purchased from ATCC.

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It was grown in Todd-Hewit Broth (THB) and Trypticase Soy Agar (TSA). *E. coli DH5α* were used for routine transformation and expression studies and *E. coli* BL21(DE3), *E. coli* BL21 (DE3) pLysS and *E. coli* BL21 (DE3) Codon Plus have been selected to obtain the highest level of recombinant proteins.

DNA preparation: The chromosomal DNA extraction method was altered from that previously described by Gase *et al.* (1995) in that 2 mL S. *equisimilis* H46A cultures is grown to OD of 1.0 at 600 nm then pelleted and resuspended in 120 μL ice-cold SNE lysis buffer (10% Sucrose, 0.1 M NaCl, 0.1 M EDTA. pH = 8, containing 4 mg mL^{-1} Lysozyme) and incubated for 30 min at 0°C and then 30 μL TESS lysis buffer (10 mM Tris/HCl. PH = 7.4, 1 mM EDTA, 100 mM Sodium acetate, 50 μL of 10% SDS) was added. It was incubated for 15 min at 70°C. After addition of 20 μL distilled water, the cell lysate was incubated for 30 min at 37°C with 10 μL RnaseA (1 μg $μL^{-1}$-Roche). Three microliter of proteinase K (20 mg mL^{-1}-Roche) was added to the solution and incubated at 37°C for 30 min. Phenol/chloroform extraction was carried out and upper aqueous phase transferred into a fresh microtube. Then sodium acetate (pH 5.2) equal to 0.1 volume and cooled Isopropanol equal to 2 volume of the transferred fraction were added and mixed gently. Chromosomal DNA was precipitated by centrifugation for 10 min at 14000 rpm (Eppendorf). DNA pellet washed with 70% ethanol and dried for 30 min and resuspended in 50 μL distilled water. Plasmid extraction was performed according to the alkaline lysis method (Sambrook and Russell, 2003) with slight alterations including: the volume of overnight culture which used for plasmid extraction was 3 mL instead of 1.5 mL and the 10 μL Rnase (1 mg mL^{-1}) was added to 150 μL of solution I (50 mM glucose, 25 mM Tris. HCl, 10 mM EDTA pH 8) to degrade RNA and subsequently elimination of RNase by solution II (0.2 N NaOH, 1.0% SDS) and solution III (15 mL of 5 M Potassium acetate, 2.9 mL Acetic acid, 7.125 mL DDW pH 5-5.5).

Polymerase Chain Reaction (PCR): Primers were designed based on *Streptococcus equisimilis* H46A streptokinase gene (*skc*) (Accession No. K02986). The forward and reverse primers were 4SKCF, 5′CGCGGATCCATTGCTGGACCTGAG3′ and STK-2, 5′GCTGGATCCTTATTTGTCGTTAGGGTTATC3′ that were complementary to the sequences coding for the amino and carboxyl termini of the mature protein, respectively. Both primers have additional condos to facilitate directional cloning into the *Bam*HI restriction endonucleases sites of the pGEX-4T-2 vector. This strategy resulted in recombinant construct in which a GST domain was appended to the N-terminus of the native molecule. Reaction was performed with the Mastercycler gradient (*Eppendorf*). Amplification reaction was carried out with 1 μg of S. *equisimilis* H46A DNA (100 ng $μL^{-1}$) and 3 μL of each oligoprimers (5 μM), 2.5 μL of 10X PCR buffer, 12.8 μL Double Distilled H_2O, 1.5 μL $MgCl_2$ (25 mM), 1 μL dNTPs (5 mM), 0.2 μL of *Taq* DNA polymerase (5 u $μL^{-1}$) in a final concentration of 25 μL. The DNA was denatured in 5 min at 95°C and the samples were subjected to 29 cycles of PCR, each consisting of 30 sec of denaturtion at 94°C, 30 sec at 50°C, 45 sec of polymerization at 72°C and 7 min of the final extension at 72°C for 1 cycle (Nejadmoqaddam and Modaresi, 2005). The DNA was concentrated by ethanol precipitation. The fragment corresponding in size (1245 bp) to the *skc* gene was extracted from a low melting point agarose (Sigma, St.Louis, MO) gel. The PCR products confirmed with marker and digestion with *Hin*dIII.

Cloning of the streptokinase gene into the expression vector pGEX-4T-2: Purified PCR product was incubated with *Bam*HI for 3 h at 37°C, run on a gel agaros. The band extracted from that and purified by QIAquic® Gel Extraction kit (Cat. No. 28704). The purified product was incubated overnight at 16°C for ligation into pGEX-4T-2 vector which had been previously restricted with the same restriction enzyme and dephosphorylated with Calf intestinal alkaline phosphatase (Takara, Code: 2250A), 25 μL of efficient competent DH5α *E. coli* cells, which prepared by $CaCl_2$ procedure according to standard protocols mixed with 3 μL of ligation mix for transformation. Screening was performed with culturing of transformed DH5α on LB agar (containing 100 μg mL^{-1} ampicillin). The obtained clonies were used for plasmid preparation and for presence and orientation of the insert in the pGEX-4T-2, was confirmed by digestion with *Bam*HI (for release 1245 bp fragment) and double digestion with *Hin*dIII and *Eco*RI (for releasing about 502 bp fragment) and sequencing in both directions according to Sanger automated laser fluores system in National Research Council, Canada (NRC). The resulting plasmid was called pGEX-1.2-4T-2.

Expression test and SDS-PAGE analysis: *E. coli* BL21 (DE3), BL21 (DE3) CodonPlus and BL21 (DE3) pLysS were transformed by pGEX-1.2-4T-2. Bacteria in 5 mL LB were grown to an OD of 0.6 (A600 nm) with vigorous agitation at 37°C. Fusion protein expression was induced by adding 10 μL of (50 mM) stock solution of Isopropyl-beta-D-thiogalactopyranoside (IPTG) (final concentration 0.1 mM) and incubated for an additional 7 h. One milliliter of the liquid cultures were collected in different time points (1, 3, 5 and 7 h) after induction. They Spind down and pellet, were resuspended in 100 μL of 1x SDS sample buffer containing (Tris.base 7.5%, 2 mL SDS 10%,

1 mL Glycerol, 2 mg Bromophenol blue, 25 μL 2ME). The cells were lysed using a strong vortexing, heated at 100°C for 5 min, spind down and the supernatants were applied to SDS-12% polyacrylamide gel electrophoresis. Proteins were stained with Coomassie blue and the fusion protein band was visualized by destaining with methanol: acetic acid solution (Sambrook and Russell, 2003). In order to analysis of the GST-SKC Molecular Weight (MW), the distance migrated by the recombinant molecule to that migrated by the marker Bio-Rad, Cat. No. 161-0309 (Retention factor-Rf) was determined by ruler and the semilogaritmic curve was plotted with respect to the inverse relation between Rf and MW. The MW found on the vertical axis of the curve by plotting a straight line to Rf of the band on horizontal axis.

Determining SKC activity in GST-SKC: After identification of the fact that 5th h culture is the time of the maximum expression level and the protein had the strongest expression in BL21 (DE3) pLysS, this host was used for measurement of streptokinase activity. For this purpose cells from 5 mL of original culture at 37°C after 5 h were harvested by centrifugation at 5000 rpm and the bacterial pellet was resuspended in 1 mL of lysis buffer (20 mM Tris-Cl (pH 8.0), 100 mM NaCl, 8 M urea). The resuspended bacterial cells were then disrupted with a Sonoplus HD 2070 sonicator (Bandeln, Berlin) twice with MS 73 microprobe at 70% full power for a 5 min -20% cycle. Cell debris were precipitated by centrifugation at 14,000 rpm for 30 min and the supernatant used for the Streptokinase activity. Activity was measured by plasmin hydrolysis of chromogenic peptidyl anilide substrate (S-2251), according to Kulisek *et al.* (1989) procedure with slight modification: In a total volume of 131.5 μL containing 1.5 μL of 50 mM Tris-HCl (pH 7.4) and 30 μL of Pg (0.2 mg mL^{-1}), the reaction was initiated by adding of the 15 μL sample of clarified sonicated extract, incubated for 15 min at 37°C and added 75 μL of S-2251 substrate (5 mg mL^{-1}), again incubated for further 10 min at 37°C. The reaction was stopped by adding 10 μL of acetic acid (0.4 N) and monitored at 405 nm.

RESULTS

Isolation of *skc* gene (1245 fragment) by PCR: The *skc* gene was isolated by the Polymerase Chain Reaction (PCR) from *Streptococcus equisimilis* group C ATCC 12449 (Fig. 2). Analysis of *skc* gene (1245 fragment) sequence (Malke *et al.*, 1985) by Webcutter 2.0 program (http://rna.lundberg.gu.se/cutter2/) revealed a *Hin*dIII restriction site which divided it into two fragments of lengths 498 and 747 bp nucleotides. Restriction analysis

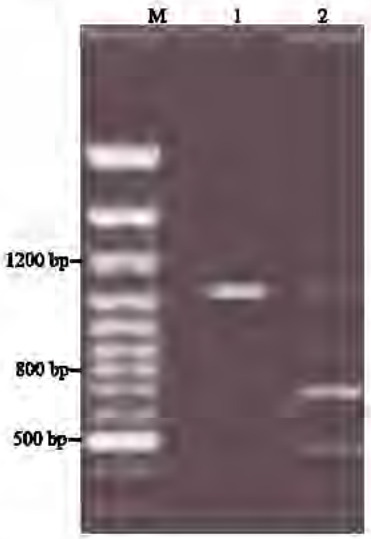

Fig. 1: PCR products confirmation test with digestion by *Hin*dIII. M: DNA marker, Lane 1: 1245 bp fragment (PCR products of the gene), Lane 2: the gene fragment digested by *Hin*dIII

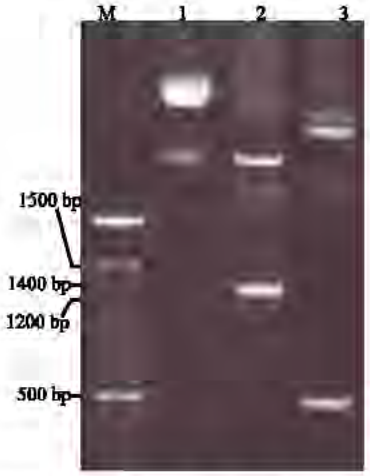

Fig. 2: Confirmation of the clone. M: DNA marker, Lane1: pGEX-1.2-4T-2 clone (undigested), Lane 2: pGEX-1.2-4T-2 digested with *Bam*HI and released 1245 bp fragment (skc gene) and Lane 3: pGEX-1.2-4T-2 digested with *Hin*dII/*Eco*RI and released 498 bp fragment (3´Region of the Gene and Part of Multiple Cloning Site (MCS) the Vector)

of the PCR product from clone pGEX-1.2-4T-2 by *Hin*dIII showed consistent results with the gene sequence and was confirmed by the expected fragments (Fig. 1).

Cloning and expression of *skc* gene: Cloning confirmation and the gene orientation were carried out using *Bam*HI

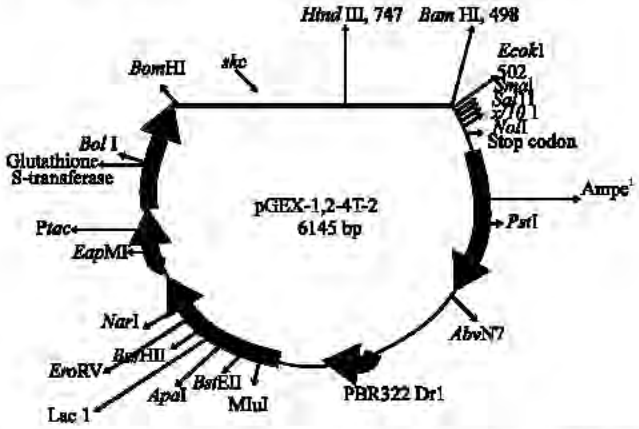

Fig. 3: Schematic diagram of pGEX-1.2-4T-2 clone. *skc* gene was cloned under control of strong and regulable *tac* promoter which was named pGEX-1.2-4T-2

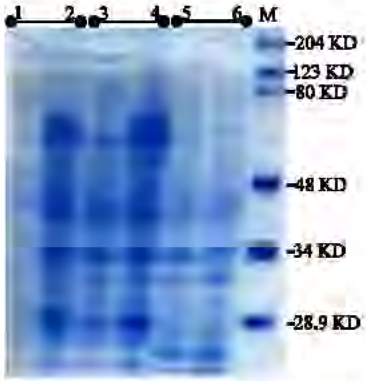

Fig. 4: Comparison of GST-SKC protein in different hosts. Each pair of lanes shows before and after induction for hosts. Lanes 1 and 2, 3 and 4, 5 and 6 are BL21(DE3), BL21 (DE3) plysS and BL21 (DE3) CodonPlus, respectively. M: molecular weight marker

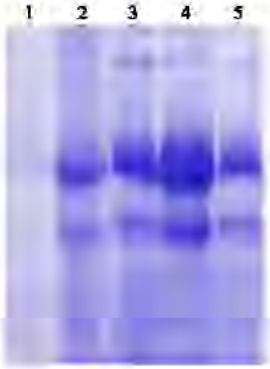

Fig. 5: Optimization of the protein product. Lane 1: BL21 (DE3) plysS containing pGEX-4T-2 (negative control), Lanes 2, 3, 4 and 5: BL21 (DE3) plysS containing pGEX-1.2-4T-2 after 1, 3, 5 and 7 h incubation, respectively

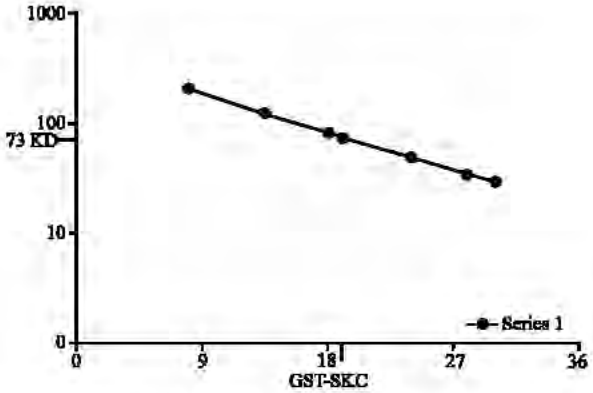

Fig. 6: Determination of the accurate Molecular Weight (MW) of GST-SKC band on SDS-PAGE. vertical axis: MW (KD), Horizontal axis: Rf related to 204, 123, 80, 48, 34, 28/9 KD of markers and the GST-SKC are 8, 13.5, 18, 24, 28, 30 and 19 mm, respectively. The molecular weight of GST-SKC coincided with a point on the vertical axis found to be about 73 KD

and *Hin*dIII/*Eco*RI digestion and double digestion, respectively (Fig. 2). The clone sequencing showed insertion of the *skc* gene in the vector (Fig. 3). The pGEX-1.2-4T-2 vector was introduced into three types of *E. coli*, BL21 BL21codonplus and BL21pLysS with different genotypes for expression a level analysis.

The *tac* promoter was induced as described in the materials and methods and the maximum GST-SKC expression was obtained in *E. coli* BL21 (DE3) pLysS (Fig. 4). The expression induced by IPTG. The highest level of the protein was observed 5 h after induction (Fig. 5). The expression rate in the soluble fraction was about 50% of the total cell protein which determined by densitometric scanning of the stained SDS-PAGE (Fig. 5).

Streptokinase activity assay and analysis of the fusion GST-SKC molecular weight: The activity of recombinant SKC was determined by chromogenic substrate (S-2251) assay, in comparison to commercial recombinant Streptokinase (Heberkinasa from Heber Biotec, Cuba). Streptokinase activity was estimated more than 15000 units mL^{-1} of LB-ampicilin media. The MW of GST-SKC was found to be 73 KD (Fig. 6).

DISCUSSION

The purpose of the present study was cloning of *S. equeisimilis* H46A *streptokinase* gene using new vector and hosts to produce active recombinant fusion streptokinase in order to facilitate purification. The aforementioned protocols allowed us to produce a recombinant fusion streptokinase at levels of about 50% of total protein using stable pGEX-1.2-4T-2 clone and *tac* promoter in the optimized condition. This level is favorable compared to previous studies that used pEKG-3 vector with *trp* promoter and *E. coli* K-12 strain W3110 (Estrada *et al*., 1992) or pKK 223-3 vector with *taq* promoter and *E. coli* JM105 (Avilan *et al*., 1997).

Different *E. coli* strains derived from BL21 (DE3) were used for comparative protein expression studies. The expression levels of the recombinant protein (GST-SKC) in these strains are significantly different as showed in Fig. 4. These strains are deficient in the known cytoplasmic protease gene products, such as Lon, OmpT, DegP or HtpR, in order to give high level expression of fusion proteins (Sugimura and Higashi, 1988). Therefore, the highest expression in BL21 (DE3) plysS could be due to proteases deficiency in favor of streptokinase.

An interesting finding in this study was that 0.1 mM of IPTG could produce the maximum amount of the recombinant protein compared to the previous studies of Lee *et al*. (1997) and Avilan *et al*. (1997), which used 1 mM and 0.5 mM of IPTG for production of the recombinant streptokinase, respectively.

It has been reported that fusion of 6x His (Caballero *et al*., 1999) and Maltose Binding Protein (MBP) tags (Sazonova *et al*., 2001) to the N-terminal of streptokinase dose not affect streptokinase activity. The results of present study support the mentioned studies.

The activity of recombinant streptokinase was measured and it was about 15000 units mL^{-1} of culture which is 3 fold higher than what has been previously obtained (Ko *et al*., 1995).

The presence of 26 hydrophobic amino acids as a signal peptide at the N-terminal of streptokinase facilitates its secretion by streptococcus (Malke *et al*., 1985). The insert of pGEX-1.2-4T-2 clone had no signal peptide. It is reported that the recombinant streptokinase expressed in *E. coli* can be secreted in the absence of signal peptide (Avilan *et al*., 1997).

Recombinant streptokinase purification is a major problem. Several schemes have been described for recovery and purification of streptokinase (Banerjee *et al*., 2004). However, recombinant streptokinase with GST tag which can bind to glutathione shortens the purification to a Single-step (Smith and Johnson, 1988). In addition, it can cause reduction of denaturing rate, maintaining the physiologic pH and reducing the amount of buffers and solutions.

Accordingly to present study it is speculated that GST tag which has added to the N-terminus of the recombinant streptokinase may increase the stability of the recombinant protein. Another study has showed that a modification on N-terminus of the streptokinase has increased its stability (Gase *et al*., 1996).

Moreover, some alterations were performed during cloning to obtain better results. For example, using PCR primers with *Bam*HI restriction site in both side of the amplified fragment allowed the *skc* gene to be cloned in various expression vectors exactly next to GST sequence with minimal amino acid residue added to N-terminus of SKC protein which could affect the function.

This product (pGEX-1.2-4T-2) can be used for rapid *skc* gene subcloning in different vectors, direct transformation into other expressive host cells, mutant streptokinase production in order to reduce its immunogenicity and optimizing other protein expression factors.

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