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Cloning and Expression of Streptokinase Gene from *Streptococcus pyogenes* C1

Erman Oryasin[1*], Gamze Basbulbul[1], Ilknur Dabanoglu[2], Bulent Bozdogan[1,3]

[1] *Recombinant DNA and Recombinant Protein Center (REDPROM), Aydin Adnan Menderes University, Turkey*
[2] *Veterinary Faculty, Department of Anatomy, Aydin Adnan Menderes University, Turkey*
[3] *Medicine Faculty, Department of Medical Microbiology, Aydin Adnan Menderes University, Turkey*

Abstract

In recent years more people die each year from cardiovascular diseases (CVDs) than from any other cause and 17.7 million people died from CVDs in 2015, representing about one third of all global deaths according to the WHO. Streptokinase is a thrombolytic agent with systemic fibrinolytic effect. It is a non-enzymatic protein produced by β haemolytic streptococci and activates the fibrinolytic system indirectly. Streptokinase is used widely and routinely for treatment of acute myocardial infarctions. In this study streptokinase gene from *Streptococcus pyogenes* C1 was cloned in pET28a vector and transferred to *Escherichia coli* BL21. Expressed protein was purified by Ni-NTA column and visualized with SDS-PAGE. Streptokinase (SK) coding DNA fragment was amplified from *S. pyogenes* C1 genomic DNA by PCR. The amplification products were inserted into the cloning and expression vector pET28a(+). Recombinant pET28a(+) plasmid with recSK gene were then introduced to *E. coli* BL21 (DE3) by electroporation. The streptokinase encoded by the plasmid pET28a(+) contains the mature streptokinase with the 6X histidine-tag.

Keywords: streptokinase, fibrinolytic enzyme, expresion

Резюме

През последните години, повече хора умират всяка година от сърдечно-съдови заболявания (ССЗ), отколкото по всяка друга причина. През 2015 г., 17.7 милиона души са починали от ССЗ, което, според СЗО, представлява около една трета от всички смъртни случаи в световен мащаб. Стрептокиназата е тромболитично средство със системен фибринолитичен ефект. Това е не-ензимен белтък, произведен от β-хемолитични стрептококи, който активира фибринолитичната система индиректно. Стрептокиназата се използва широко и рутинно за лечение на остър миокарден инфаркт. В това проучване генът на стрептокиназа от *Streptococcus pyogenes* C1 беше клониран в pET28a вектор и пренесен в *Escherichia coli* BL21. Експресираният протеин се пречиства чрез Ni-NTA колона и се визуализира с SDS-PAGE. Стрептокиназната (СК) кодираща ДНК фрагмент се амплифицира от *S. pyogenes* C1 геномна ДНК чрез PCR. Продуктите за амплификация се включват в клониращия и експресионен вектор pET28a (+). След това, рекомбинантният pET28a (+) плазмид с recSK ген се въвежда в *E. coli* BL21 (DE3) чрез електропорация. Стрептокиназата, кодирана от плазмида pET28a (+), съдържа зрялата стрептокиназа с 6X хистидин-маркер.

Introduction

In recent years, the incidence of cardiovascular diseases has increased and has become the most important health problem that causes mortality and morbidity (Mohebbi *et al*., 2010). According to the World Health Organization's report, 17.9 million people died from cardiovascular diseases in 2016, representing 31% of all global deaths. Among these, 7.4 million deaths were caused by coronary heart disease, while 6.7 million were caused by heart at-

* Corresponding author: ermanoryasin@gmail.com

tacks (WHO, 2020). In Turkey, the rates of deaths due to cardiovascular diseases reached 38.5% of the total deaths during 2018 (TUIK, 2020).

Streptokinase (SK) is an important thrombolytic agent in the treatment of patients suffering from cardiovascular diseases. When streptokinase binds to circulating plasminogen (Plg), the SK-plasminogen activator complex is formed. Plasmin is an active enzyme that degrades the fibrin component to thrombin. Because of this property, streptokinase dissolves blood clots strongly and is used in the treatment of acute myocardial infarction. The enzyme is a single chain polypeptide made up of 414 amino acid residue and has a molar mass of 47 kDa (Baruah *et al.,* 2006; Nguyen *et al.,* 2014).

Commonly used fibrinolytic agents in thrombolytic therapy are streptokinase, urokinase (UK) and tissue type plasminogen activator (TPA). These agents are often referred to as plasminogen activators and they convert enzymatically inert plasminogen in the fibrinolytic system to plasmin. Plasmin dissolves fibrin clots by breaking them down to soluble parts and these products are removed by phagocytes (Kunamneni *et al*., 2007). Unlike urokinase and TPA, SK doesn't have a proteolytic activity alone; SK becomes active if only it makes a complex with circulatory plasminogen or plasmin (Banerjee *et al*., 2004).

Streptokinase has a higher *in vivo* half-life compared to urokinase and plasminogen activator. Since streptokinase has a bacterial origin and non-specific mechanism of action, side effects and risks are higher in thrombolytic therapies than urokinase and plasminogen activator. However, streptokinases are preferred in developing countries because of their cost-effectiveness and short therapy times (Kunamneni *et al*., 2007).

In this study, a potential therapeutic recombinant streptokinase (recSK) was obtained by cloning streptokinase gene from *Streptococcus pyogenes* C1.

Materials and Methods

Plasmids, bacterial strains, and culture conditions

The bacterial strain *S. pyogenes* C1 was used as the source of the streptokinase gene (*ska*). *Escherichia coli* BL21 (DE3) and the vector pET28a(+) were used for DNA manipulations, amplification and expression of SK. Tryptic soy agar (TSA) was used for cultivation of *E. coli* BL21 (DE3).

DNA amplification and plasmid construction

DNA extracted from S. pyogenes C1 using the Insta Gene Matrix DNA extraction kit (Bio-Rad, Hercules, CA), according to the manufacturer's recommendations. SK-coding DNA fragment was amplified from *S. pyogenes* C1 genomic DNA by PCR. Based on the nucleotide sequence of the *ska* from S. pyogenes strain (GenBank: Z48617), SKF-BamHI GGCGGATCCATGAAAAATTACTTAT and SKR-XhoI GCCTCGAGGTTTGTCATTAGGGTT were designed as primers for introduction of the underlined BamHI and XhoI restriction sites, respectively. The PCR mixture contained 5 μL 10x PCR buffer; 1 μL of 2.5mM dNTP; 4 μL of 25mM $MgCl_2$; 2μL genomic DNA; 0.3 μL 5 unit Taq polymerase, and 0.4 μL each primer (100 pmol), supplemented with 36.9 μL distillated water to a final volume of 50 μL. The DNA was amplified using the following protocol: held at 94 ºC for 5 min, followed by 35 cycles of denaturation (94 ºC for 30 s), annealing (52ºC for 30) and extension (72 ºC for 1.5 min), with a single final extension of 8 min at 72 ºC. Using 1% agarose gel bands were separated for 30 min at 100 V, stained with Safe View (ABM, Canada), and visualized PCR products under UV. Then, the PCR products amplified from the genomic DNA with the primer pair SKF-BamHI and SKR-XhoI were digested with the BamHI and XhoI enzymes. Digested amplification products were inserted into the cloning and expression vector pET28a(+) digested with the same enzymes by ligation. Recombinant pET28a(+) plasmid with *ska* gene were then introduced into *E. coli* BL21 (DE3) by electroporation and selected on tryptic soy agar media supplemented with 50 mg/L kanamycin. Recombinant plasmid was confirmed by plasmid isolation using a Qiagen Plasmid Midi kit. The recombinant streptokinase had 6X histidine-tag because of the expression plasmid pET28a(+) (Fig. 1).

Expression of recombinant streptokinase

E. coli BL21 (DE3) bacterial cells were used as expression hosts for plasmid pET28a(+) containing recombinant streptokinase (recSK). The culture of transformants was done at 37°C and until an optical density (OD) at 600 nm reached 0.5-0.6 (for approximately 3h); then IPTG (isopropyl-β-D-thio-galactoside) was added for induction. The culture was continuously incubated at 37°C with agitation at 200 rpm for 3–6h induction. Cells were harvested by centrifugation at 5000 rpm for 10min at 4°C and were used for protein purification (Sambrook, 2006).

Modified caseinolytic assay for streptokinase

In order to demonstrate streptokinase activity, *E. coli* BL21 (DE3), bacteria containing plasmids with and without *recSK* gene were streaked

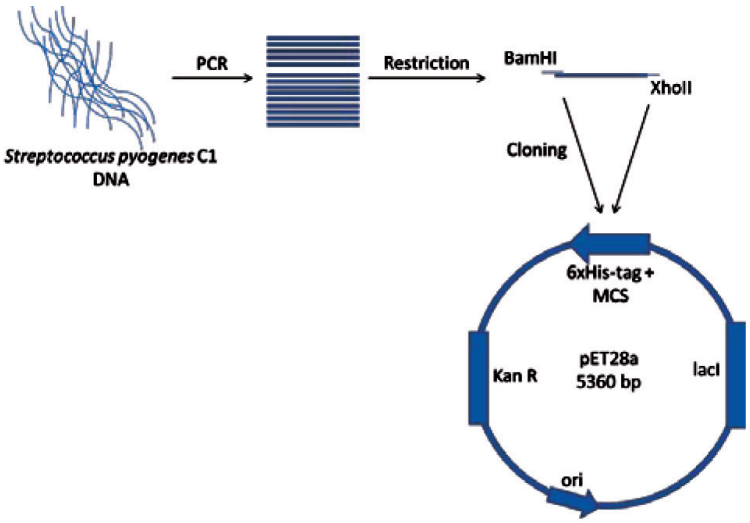

Fig. 1. Structure of pET28a(+) expression plasmid. Amplified fragments of the ska gene were digest with BamHI and XhoI restriction enzymes and ligated into the vector pET28a(+).

on tryptic soy agar (TSA) plate. After bacterial cultivation, 5 ml of mixture containing 1 ml of skim milk, 1 ml of human blood plasma and 1% bacteriological agar were poured and incubated at 37°C for 16-18 hours.

Purification of recombinant streptokinase and protein electrophoresis

Bacteria from a 100 mL culture were harvested by centrifugation and suspended in 10 mL of RIPA lysis buffer (1X) with 1 tablet proteinase inhibitor (Thermo A32959) to purify RecSK. Then, the samples were placed in the homogenizer device (Tehtnica Millmix 20) and processed for 25 seconds at 30 frequencies 5 times. Homogenized cells were centrifuged at +4°C for 5 minutes at 10 000 rpm. The supernatant was taken and used as protein extract. In order to purify the recombinant streptokinase containing 6X His-tag, the resulting supernatant was loaded onto the Ni-NTA column on a low pressure chromatography device (Biologic LP, Bio-Rad). The bound recombinant protein was eluted with elution buffer containing 8 M Urea, 100 mM NaH_2PO_4, 100 mM Tris-HCl, pH 4.5. The recombinant streptokinase (RecSK) was visualized by 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE).

Results

A single band of expected size (1323 bp) was obtained by PCR using the primers SKF-BamHI and SKR-XhoI in the genomic DNA of *S. pyogenes* C1 strain (Fig. 2). The recombinant plasmid (pET28a+*ska*) was confirmed by gene sequencing. The nucleotide sequence of the cloned fragment (*ska*) displayed >99% identity with the streptokinase nucleotide sequence in GenBank: WP_030127337.1.

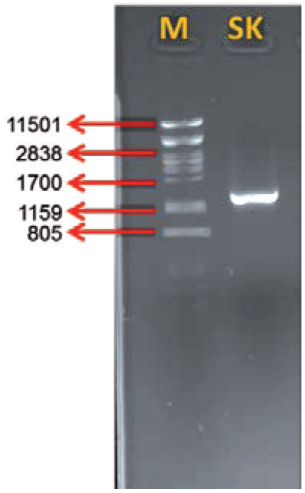

Fig. 2. Agarose gel electrophoresis of the PCR amplified fragment of the *ska* gene in *S. pyogenes* C1. M: Lambda-PstI DNA Marker, SK: PCR product of the *ska* gene (1323bp).

The positive recombinant plasmid was transformed into the host, *E. coli* BL21 (DE3). The difference in the production of streptokinase in the recombinant bacterium relative to the control bacterium (not containing the recSK gene) was demonstrated by the modified caseinolytic activity test as shown in Fig. 3.

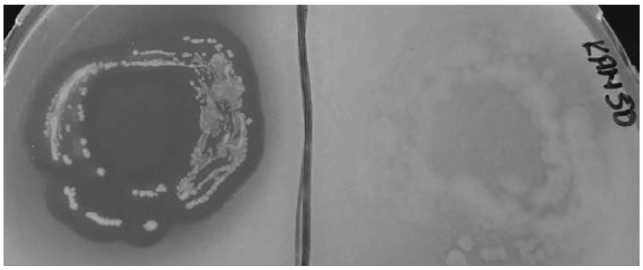

Fig. 3. Modified caseinolytic assay for streptokinase. C1-SK: containing the *ska* gene (pET28a+*ska*); pET28a(+):not containing *ska*

The addition of IPTG induced the overexpression of approximately 50.75 kDa molecular weight recombinant protein. The expressed protein was purified successfully via affinity chromatography using Ni-NTA resin (Fig. 4).

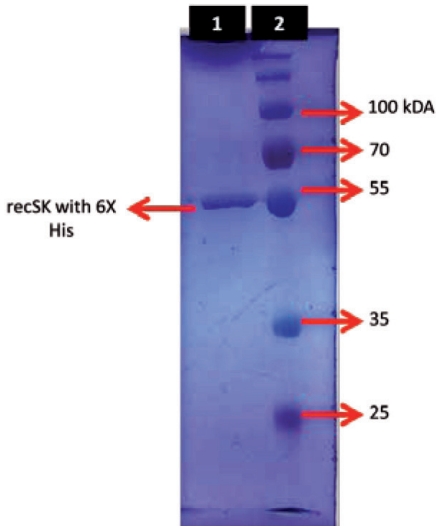

Fig. 4. Analysis of recombinant protein by SDS-PAGE. 1: recSK protein purified from *E. coli* BL21 (DE3), 2: Protein marker (PageRuler, ThermoFisher)

Discussion

Streptokinase is an important thrombolytic agent in the treatment of cardiovascular diseases. It is widely used in the treatment of acute myocardial infarction due to its ability to dissolve blood clots strongly (TUIK, 2020). *E. coli* is widely used in research and industrial production of recombinant proteins. *E. coli* has been shown to be suitable for recombinant production of streptokinase protein with a high yield (Baneyx, 1999).

In 1984, Malke *et al*., using the bacteriophage Lambda vector L47 from *Streptococcus equisimilis* strain, created a recombinant phage bank carrying the *E. coli* streptokinase gene (Malke, 1984). In 1998, Perez *et al*. produced and purified the recombinant streptokinase using *E. coli* K12 strain. Streptokinase protein was obtained in 99% purity by hydrophobic interaction chromatography (Zhang *et al*., 1999).

Many chemical modifications are used to increase the half-life and plasminogen activation of the circulating streptokinase and to reduce or eliminate its immunogenicity. These targets were to some degree achieved by mutated and produced streptokinases (Banerjee *et al.,* 2004).

Streptokinase is cost-effective for thrombolytic therapy in clinical use. Through bacterial fermentation, low-cost streptokinase can be produced in abundance. Cloning the streptokinase gene into non-pathogenic microorganisms enables the production of recombinant streptokinase.

In our study, the streptokinase gene amplified from *S. pyogenes* C1 was cloned into pET28a(+) vector and transferred to *E. coli* BL21 (DE3). High expression of the recombinant protein recSK by inducing the expression vector was targeted. The strain producing the recSK protein was registered as a potential strain for industrial streptokinase production in the recombinant library of Recombinant DNA and Recombinant Protein Application and Research Center (REDPROM) of Adnan Menderes University.

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