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Engineering, expression and purification of a chimeric fibrin-specific streptokinase

Mohammad Naser Taheri [a, b, c], Abbas Behzad-Behbahani [a, c], Gholamreza Rafiei Dehbidi [a, c], Saeede Salehi [a, b, c], Sedigheh Sharifzadeh [a, c, *]

[a] Department of Medical Biotechnology, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran
[b] Student Research Committee, Shiraz University of Medical Sciences, Shiraz, Iran
[c] Diagnostic Laboratory Sciences and Technology Research Center, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran

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ABSTRACT

Streptokinase is a valuable fibrinolytic agent used to cope with myocardial infarction and brain stroke. Despite its high efficiency in dissolving blood clots, streptokinase (SK) has no specificity in binding fibrin, causing some problems such as internal bleedings following its administration. To make streptokinase fibrin specific and limit the fibrinolytic process to the clot location, we engineered a chimeric streptokinase by fusing the fibrin binding Kringle 2 domain of tissue plasminogen activator (TPA) to the streptokinase N-terminal end.

The chimeric SK construct (KSK) with inserted Kringle 2 domain was cloned into pET28a expression vector. The expression of recombinant protein was carried out in *Escherichia coli* origami (DE3) and confirmed by SDS-PAGE and Western blotting analyses. We used the chromogenic substrate S-2251 method to assess the specific activities of the chimeric and control wild-type proteins. Then, the two proteins were added in amounts with equal activity to fibrin clots of identical size. Finally, the supernatant above the fibrin clots was collected and subjected to the chromogenic assay to analyze the specificity of the chimeric protein.

The specific activities of the chimeric and wild-type proteins were found to be 0.06 U/mg and 0.07 U/mg, respectively. Because of the binding of the chimeric protein to fibrin, the mean specific activity was significantly lower in the KSK supernatant (0.01) compared with the control (approximately 0.06) ($p < 0.05$).

Our in vitro results indicate that the chimeric streptokinase protein has strong fibrin-specific activity compared to the wild-type protein. However, further *in vivo* studies are needed to evaluate its potential fibrinolytic effects.

1. Introduction

The human hemostatic system contains a wide variety of enzymes that regulate the formation of blood clots and its reverse process called thrombolysis. However, under some conditions, the hemostatic system fails to remove unwanted blood clots. This may lead to the blockage of vital arteries carrying nutrients and oxygen to important organs including the heart and brain. Therefore, the blockage of such arteries may lead to myocardial infarction and brain stroke [1,2]. Streptokinase and tissue plasminogen activator (TPA) are among the most important thrombolytic agents used to cope with myocardial infarction. Such thrombolytic agents bind an inactive zymogen called plasminogen and convert it to its active form, plasmin. Finally, Plasmin binds fibrin and degrades it, resulting in clot disintegration [3].

Streptokinase is a protein produced by β-hemolytic streptococcal bacteria of the Lancefield groups A, C and G. *Streptococcus equisimilis* H46A (ATCC 12449) which belongs to group C, was for the first time isolated from human and has been used for streptokinase production [4]. It is less fastidious in its growth requirements, does not produce erythrogenic compounds and yields the most active streptokinase [1].

* Corresponding author. Present address: Diagnosis Laboratory Sciences and Technology Research Center, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran.
E-mail address: sharifsd@suma.ac.ir (S. Sharifzadeh).

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Streptokinase is not a fibrin specific thrombolytic protein and consists of a single chain of 414 amino acids [5]. Although it is not a protease and has no intrinsic enzymatic activity, SK forms a complex with plasminogen and converts it to the active plasmin form [1,6].

On the other hand, TPA is a fibrin-specific protein and acts only on fibrin clots; thus, the risk of internal bleeding is less. As a fibrin-specific protein, tissue plasminogen activator (TPA) is a serine protease consisting of 527 amino acids and 5 domains. Among them, Kringle 2 domain of TPA, with 87 amino acid residues and 3 disulfide bonds, is the most important for binding to fibrin [7–10].

Large-scale production of recombinant SK is more cost-effective than TPA. However, the streptokinase inability to bind fibrin makes it a less desirable thrombolytic drug than TPA because it produces more fibrinolysis and causes internal bleeding [1].

To make streptokinase fibrin-specific and limit its thrombolytic activity to the clot location, we designed a chimeric protein construct of streptokinase with fusion of TPA kringle-2 domain to the N-terminal end of the streptokinase. The chimeric and wild-type proteins were expressed in *Escherichia coli* and then purified. Finally, the fibrin binding specificity of the chimeric protein was evaluated.

2. Materials and methods

2.1. Strains and reagents

All the restriction enzymes (NdeI, BamHI and XhoI), T4 DNA ligase and the protein marker were purchased from Thermo Fisher Scientific (Grand Island, NY). The primers and synthetic gene were obtained from Bioneer (South Korea). Anti-his-tag antibody was purchased from sigma (St. Louis, MO). The chromogenic substrate S-2251 was purchased from the Chromogenix (Milan, Italy). Thromboplastin was purchased from Thermo Fisher Scientific (Grand Island, NY). The agarose gel extraction kit was purchased from Bioneer (South Korea). The pET28a expression vector was purchased from Iran's Pasteur Institute. DH5α *E. coli* was used for cloning and plasmid extraction purposes. The BL21 (DE3) and origami (DE3) strains of *E. coli* were employed as hosts for expression of the wild-type and chimeric constructs, respectively.

2.2. DNA constructs

To amplify the gene encoding streptokinase (1245 bp in size), PCR was performed on genomic DNA of *Streptococcus equisimilis* H46A. The forward and reverse primers had BamHI and XhoI restriction sites at their 5′ ends (Table 1). The resulting amplicons were purified by agarose gel extraction kit and inserted into the pET28a vector using T4 DNA ligase according to the manufacturers' protocol. Correct construction of the subsequent plasmid with inserted SK sequence (pET-SK) was confirmed by colony PCR assay, restriction enzyme analysis, and nucleotide sequencing method.

The sequence of Kringle 2 domain from human TPA with restriction sites for NdeI and BamHI at its 5′ and 3′ termini was commercially custom-synthesized. For optimal expression in *E. coli,* the Kringle 2 sequence was also codon-optimized according to the *E. coli* codon usage. For the stability, correct folding, and optimal biological activity of kringle-2 domain, a serine-glycine (SG) linker sequence of 10 amino acids was also designed at the 3′ end of the sequence. The Kringle 2 domain was fused to the N-terminal end of the streptokinase through the SG liker sequence (Fig. 1). The fragment was then amplified from the synthetic sequence, using pfu DNA polymerase and Kringle 2 specific primers (Table 1). The amplified fragment was gel-purified by agarose gel extraction kit and digested using NdeI and BamHI restriction enzymes. The double digested fragment was then inserted into the pET-SK construct to make the chimeric streptokinase construct (pET-KSK). The resulting chimeric construct was verified using PCR and restriction analysis.

Table 1
The primer sequences used in polymerase chain reaction. The restriction enzyme sites are underlined.

Primer name	Primer sequence (5′ − 3′)
SK-F	aaggaaGGATCCATTGCTGGACCTGAGTGGCTG
SK-R	tggttaCTCGAGTTATTTGTCGTTAGGGTTATC
Kringle F	tggcatCATATGAATAGTGACTGCTAC
Kringle R	gtacttGGATCCTGAGCCCGATCCTGAA

Keys: F = forward primer, R = reverse primer, SK = streptokinase.

2.3. Expression of the chimeric and wild-type proteins

The wild-type and chimeric proteins were expressed in BL21 (DE3) and origami (DE3) strains of *E. coli*, respectively. The transformed BL21 (DE3) and origami (DE3) strains were grown on LB agar plates with 70 μg/mL kanamycin and incubated overnight at 37 °C. A single colony from each plate was inoculated into 5 mL of LB with 70 μg/mL kanamycin and grown overnight at 37 °C, 200 rpm. Two hundred microliters of the starter culture was transferred into 200 mL of fresh LB medium containing 70 μg/mL kanamycin and 1% glucose. The cells were grown at 37 °C, 200 rpm until reaching the desired cell population for induction of expression (OD_{600} = 0.6). Then, protein expression was induced using 1 mM isopropyl-β-*d*-thiogalactopyranoside (IPTG) at 37 °C, 200 rpm for 4 h. The cells were harvested by centrifugation at 5000*g* for 7 min at room temperature.

2.4. Verification of protein expression using SDS-PAGE and Western blotting

The expression of chimeric and wild-type proteins in pre- and post-induction samples was analyzed using SDS-PAGE and Western blotting. The bacterial cells, harvested in the previous step, were resuspended in native binding buffer (NaH2PO4 100 mM, Tris-HCl 10 mM, 3 M urea, 1 mM PMSF, pH 8.0) and disrupted using sonication. The resulting lysate was centrifuged at 15000 g for 10 min at 4 °C. Twenty microlitters of the SK or KSK crude extract was mixed with 20 μl of Laemmli buffer and boiled at 100 °C for 5 min. The samples were loaded onto 12% (v/v) resolving, 0.8% (v/v) stacking gels and run at 80 V for 120 min. The gel was stained using Coomassie Brilliant Blue and the chimeric (57 kDa) and wild-type (50 kDa) proteins were detected.

For Western blotting, the protein bands were transferred to PVDF membrane using the semidry protocol at 25 A for 15 min. The membrane was blocked using 2.5% (w/v) skim milk for 2 h and washed 3 times, using PBS containing 0.02% (v/v) tween. The blot was probed with a horseradish peroxidase-conjugated anti-his-tag antibody (diluted 1:1000) while being shaked at room temperature. It was then washed 3 times in PBS (containing 0.02% (v/v) Tween-20 and 2.5% (w/v) skim milk). Finally, the protein bands of interest were visualized using diaminobenzidine (DAB) solution as

Fig. 1. The design of a chimeric fibrin-binding streptokinase. Kringle 2 domain from TPA was fused to the N-terminal end of streptokinase through a serine-glycine linker sequence to make it a fibrin-specific protein.