

## Original Article

## Fibrinolytic Activity of Recombinant Mutant Streptokinase

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## Abstract

**Background:** Streptokinase is a bacterial protein produced by different beta hemolytic streptococci and widely used in thrombolytic treatment. The main disadvantage of using streptokinase is antibody formation which causes allergic reaction to neutralize effects of streptokinase therapy. Aim of this study was investigate of recombinant mutant streptokinase fibrinolytic activity.

**Materials and Methods:** In this study recombinant mutant streptokinase without 42 amino acids from the C terminal region was purified by affinity S-Tag column chromatography and its fibrinolytic activity was studied.

**Results:** The concentration of expressed and purified protein was 10 mg/ml. Its enzyme activity was assayed using zymography, radial caseinolytic activity and fibrin plate test methods and estimated quantitatively by casein digestion method compared to a commercial form.

**Conclusion:** It was found that this product had the more volume and more enzymatic activity.

**Keywords:** Mutant streptokinase, Zymography, Caseinolytic activity

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**Please cite this article as:** Mobarrez M, Bandehpour M, Salehmoghadam M, Kazemi B. Fibrinolytic Activity of Recombinant Mutant Streptokinase. Novel Biomed. 2015;3(2):89-93.

## Introduction

Blood clots development (thrombosis), in the circulatory system, leading to blockage of blood vessels and consequences death. Hemostatic system suppresses normal development of blood clots in normal circulation, but result of incomplete hemostasis, are pulmonary embolism, deep venous thrombosis and acute myocardial infarction<sup>1</sup>. To prevent clot formation, thrombolytic agents such as streptokinase (SK), Urokinase and tissue plasminogen activator are used<sup>2</sup>. The streptokinase (EC 22.99.4.3) is produced by strains of beta hemolytic Streptococci. The enzyme consists of a single chain polypeptide activates the plasminogen directly and its fibrinolytic activity<sup>3</sup>. The

streptokinase contains 414 amino acids (47Kda), its temperature, optimized pH and isoelectric point are 37-27°C, 7, and 4.7 respectively<sup>3-6</sup>. Plasminogen activation by streptokinase related to bacterial species<sup>7</sup>. The Streptokinase made of multi-domain structure (alfa, beta, and gamma) with different functional properties<sup>8</sup>. The  $\gamma$  domain is essential for plasminogen activation and  $\alpha$  and  $\beta$  domains involved in formation of streptokinase-plasminogen complex<sup>9</sup>. The N-terminal domain (1- 59 amino acids) with lower tendency and other amino acids (414-60) participate in plasminogen-streptokinase complex formation<sup>10</sup>. The C-terminal domain play a role in plasminogen activation<sup>11,12</sup> and Aspartic acid 41 and Histidine 48 are important in plasminogen-streptokinase connection<sup>13</sup>. Shortly after discovery of fibrinolytic

effect of streptokinase, its immunogenicity was reported<sup>14</sup>.

The main disadvantage of using streptokinase is antigenicity of this protein. The antibodies cause allergic reaction to neutralize effects of streptokinase therapy<sup>15</sup>. Five antigenic region include amino acids 1- 13, 14- 127, 1- 253, 120- 353, 353- 414 have been identified in the molecule<sup>16, 17</sup>. There is evidence about immunodominant epitope presence on the C-terminal region of natural streptokinase molecule. The mut-C42 mutant, which lacks 42 C-terminal amino acids, have been shown to lower levels of antigenicity<sup>18,19</sup>. Sera anti-streptokinase antibody level in mutant streptokinase consumers is lower than that in the normal streptokinase consumers<sup>20</sup>. We have studied mutant streptokinase fibrinolytic activity in this paper.

## Methods

The recombinant pGEMEX -1 vector contained mutant streptokinase gene<sup>21</sup> was digested by *SacI* and *BamHI* restriction enzymes and released gene subcloned into pET32a. Top10 and BL21 (DE3) *E. coli* strains were used for transformation and gene expression respectively. Recombinant protein was expressed according to previously described method<sup>22</sup>. Recombinant protein was purified by affinity chromatography using S-Tag purification kit flowed by using sodium thiocyanate<sup>23</sup>. Dialysis tubing procedure was used to remove urea and refolding the protein. Protein was dialyzed against 1x PBS buffer (containing 10 mM disodium phosphate, 150 mM sodium chloride) and concentrated using dry sucrose<sup>24</sup>.

### Fibrinolytic activity assay

#### Zymography

A polyacrylamide gel (12% separating and 5% stacking) was prepared and kept in the refrigerator for 18 hours. Loading buffer without 2ME was used for electrophoresis. Electrophoresis was performed using 1x PBS at 4°C. Gel was flood in 1% Triton X100 for 2 hours to eliminate SDS (SDS causes enzyme denaturation). The gel washed with water for three times and incubated in 1x PBS pH 8 at 37°C for 2 h. Enzyme activity was determined after staining with coomassie brilliant blue R250 (w/v 0.25%)<sup>25,26</sup>.

### Radial Caseinolytic activity

A mixture contained 1.2% agarose, 1% skim milk, 100 microliter of human plasma and 1xPBS was prepared. Some wells with 5 mm diameter were created at appropriate intervals using sterile Pasteur pipette. Five mg (100 microliter) of commercial drug streptokinase as standard and 5 mg (100 microliter) of purified recombinant protein were poured in wells and incubated for 12 hours at 37°C. Clear zone around the sample and standard wells were created by lysed casein. Zones were measured and compared sample with standard commercial drug streptokinase<sup>27</sup>.

### Fibrin plate method

A mixture of 2.5 ml soluble fibrinogen (containing 1.5% bovine fibrinogen in 20 mM Tris-HCl buffer pH 4.7), 0.5 ml of thrombin solution (1 U / ml in 20 mM Tris-HCl buffer pH 4.7), 2.5 ml of 1% agarose and 100 microliter human plasma was plated and some well created. Five mg of commercial drug streptokinase as standard and 5 mg of purified protein were poured in wells and incubated for 12 hours at 37°C. Created cleared zone around the sample and standard wells were reviewed and interpreted<sup>28</sup>.

### Casein digestion method

Streptokinase activity determined by the casein digestion method which is based on the activation of tyrosine released from casein digestion after plasminogen activation<sup>29</sup>. Streptokinase activity was determined based on the modified previously described method. Briefly; a mixture of 0.1 mg casein, 1.5 mg of purified recombinant mutant streptokinase, 500 microliters of 50 mM Tris-HCl pH 8 was prepared and incubated at 37°C for one hour. Then one ml trichloro acetic acid (TCA) and 130 microliter of 3.3 M HCl was added and placed on ice for 30 min. and centrifuged, the supernatant removed, and diluted at the ratio 1/4<sup>30,31</sup>. Absorbance of protein was measured at 280 nm. Streptokinase enzyme activity was calculated by a standard curve<sup>32</sup>.

## Results

Recombinant streptokinase gene was sub cloned into pET32a expression vector and transformed into *E. coli* BL21 (DE3) strain. Recombinant protein was expressed and purified by affinity chromatography S-Tag system. Expressed and purified protein was refolded and its fibrinolytic activity was studied.

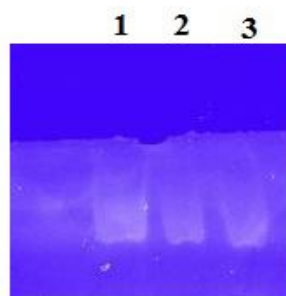


Figure 1. Fibrinolytic activity assay by zymography procedure on the 12% acrylamide gel; Lane 1- Control (commercial streptokinase), Lane 2 and 3- Recombinant-mutant streptokinase.

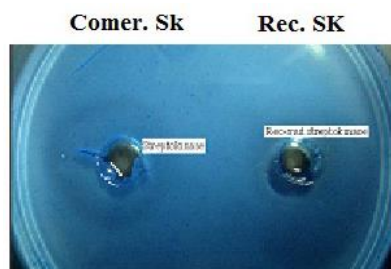


Figure 2. Streptokinase qualitative activity by Radial Caseinolytic activity.

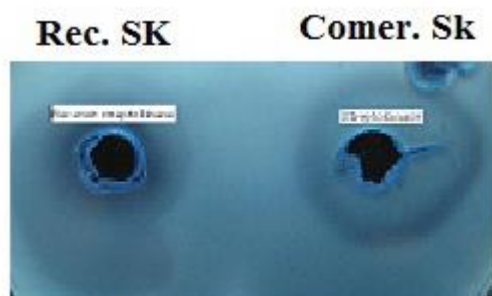


Figure 3. Streptokinase qualitative activity by Fibrin plate method.

Figure 1 shows electrophoresis and zymography of purified protein on 12% SDS-PAGE gel. Protein concentration was 10 mg/ml. It was found that the more volume, more enzyme activity.

#### Radial Caseinolytic activity

Streptokinase qualitative activity was assessed using radial caseinolytic activity. Commercial streptokinase began to lysis casein and a halo with a diameter of more established at one hour, while the recombinant streptokinase start to lysis casein at 5 hours and a cleared zone with a diameter of less

developed (Figure 2).

#### Fibrin plate method

Streptokinase qualitative activity was assessed by Fibrin plate method. Commercial streptokinase started to lysis casein and a halo with a diameter of more established at two hours, while the recombinant streptokinase started to lysis casein at 5 hours and a cleared zone with a diameter of less developed (Figure 3).

#### Casein digestion method

Digested casein absorbance at 280 nm was 0.888 and compared with a standard curve<sup>32</sup>.

## Discussion

Clots in the circulatory system can cause blockage of blood vessels, brain-cardiac MI and ultimately lead to death. In such cases, thrombolytic or fibrinolytic agents such as streptokinase, tissue plasminogen activator and eurokinase, as blood clot-busting drugs are prescribed<sup>3,33</sup>. Among the three mentioned above drugs, streptokinase is cheaper than others. Streptokinase, a beta hemolytic streptococcus extracellular enzyme is a single polypeptide chain, composed of 414 amino acids with three structural domains, alpha, beta and gamma<sup>34,35</sup>. The gamma domain is essential for plasminogen activation. Alfa and beta domains are involved in the formation of streptokinase-plasminogen complex<sup>9,36</sup>. Complex formation by streptokinase and plasminogen will hydrolysis the Arginine 561 and Valine 562 bond and convert plasminogen to plasmin. Plasmin can also cause degradation of soluble fibrin clots<sup>37</sup>. Non-human source streptokinase is immunogenic and stimulates the immune system. Production of neutralizing antibodies is leading to a massive allergic reaction<sup>3,33</sup>. There were some researches to reduce the immunological effects of streptokinase<sup>17,38-40</sup>. Seyed et al. were prepared mutant recombinant streptokinase through genetic manipulation<sup>22</sup>. Bandehpour et al. showed that the OD of the antigen -antibody reaction by natural streptokinase in streptokinase consumers as chemotherapy is three times more than that of antigen-antibody reaction by mutated antigen. In other words, removal of the C-terminal 42 amino acids of the streptokinase reduces the immunogenicity<sup>20</sup>. Similar findings have been reported by Torrens et al.<sup>38,39</sup>. These results are similar to results of Arabi et al.<sup>41</sup>.

They showed that less antibody by truncated streptokinase, whereas fibrinolytic activity is even higher, although clot lysis is slower<sup>41</sup>. Streptokinase does not directly digest the blood clot, but it involved in the blood clots digestion by plasminogen activation. Nihalani believed that N-terminal amino acids of streptokinase are involved in plasminogen activation<sup>10</sup>. There is no problem for plasminogen activation of the studied mutant streptokinase because its N-terminal amino acids are intact. Tharp *et al.* replaced basic (Arg253, Lys256, and Lys257) and acidic (Glu249, Glu262, and Glu263) residues of 250-loop of the streptokinase beta-domain with alanine, and proposed that streptokinase-plasminogen complex is formed when plasminogen identified streptokinase through Arg253, Lys256, and Lys257<sup>42</sup>. Mutated streptokinase contained Arg253, Lys256, and Lys257 and could plasminogen - streptokinase complex.

## Conclusion

We concluded that mutant streptokinase has enzyme activity and was found that more volume and more enzymatic activity.

## Acknowledgment

This study was financially supported by Vice Chancellor for Research of Shahid Beheshti University of Medical Sciences through grant no 3.9852. It has done in Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran. The authors wish to thank the directors.

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