Original Article

Evaluation of In vivo Bioactivity of a Mutated Streptokinase

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Abstract

Background: Immunogenicity of Streptokinase, as a thrombolytic drug, has limited its clinical use. Elimination of the amino acid residues that are responsible for immunogenicity while don't affect the bioactivity of streptokinase is worthy. Recently, we modified the streptokinase through the elimination of 42 amino acids from its' C-terminal and assessed its bioactivity in vitro. In this study, bioactivity of the mutated-streptokinase determined and compared with those of commercially available streptokinase (Heberkinase) in rabbits with induced blood clot.

Materials and Methods: . Recombinant mutated streptokinase was purified and its lipopolysaccharide contained remove and evaluated by LAL test. Thrombolytic activity of drug was evaluated by rabbit jugular vein as in vivo thrombosis model. The thrombolytic property of the drug was evaluated with determining of D-dimer in plasma.

Results:. The results showed in vivo bioactivity of both truncated and commercial streptokinase ($p<0.05$). This study showed an important influence of the 42 amino acids of C-terminal in bioactivity of the streptokinase.

Conclusion: Clinical use of the r-streptokinase requires more modification to restore its' activity in vivo. This product may be a promising choice for clinical use after confirmation of its stability and non-immunogenicity.

Keywords: Thrombolysis; Recombinant streptokinase; Thrombolytic agent; Fibrinolytic activity; Jugular vein; Clot formation

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Introduction

The blood clot is formed through a complicated mechanism in which many proteins are involved. This phenomenon usually occurs after surgery, wound, myocardial infraction (MI), and so forth. After a clot formation, a healthy hemostatic system can remove blood clot from the blood circulation system. However, the removal of clot in some certain diseases such as pulmonary embolism, stroke, acute MI, or deep vein thrombosis in which the hemostatic system is failed, requires the administration of some supplementary thrombolytic agents[1].

Streptokinase and tissue plasmin activator (TPA) are

among the most important thrombolytic agents used in clinics[2]. Streptokinase is normally used in clinical thrombosis therapy[3]. This enzyme has been the first choice for acute MI treatment for more than 40 years[4]. Streptokinase is a plasminogen-activator containing 414 amino acid that is produced by several strains of β-hemolytic streptococci[5]. This thrombolytic agent makes complex with plasminogen that can activate substrate plasminogen[6].

It contains several structural domains (i.e., a-, h- and g-domains) with different functions[7]. The coiled region of the streptokinase ϒ-domain is necessary for plasminogen activation[8]. Binding the plasminogen to the lysine binding site of streptokinase causes conformational activation of plasminogen[9]. Although streptokinase is considered and applied as a promising thrombolytic agent, its immunogenicity has remained challenging[10] so that with the administration of streptokinase, the body immune system produces antibodies against it. The titer of antibody raises during the therapeutic course that ultimately causes allergic reaction[11].

Some studies have identified that the streptokinase has 5 antigenic regions containing 1-13, 14-127, 1-253, 120-353, 353-414[12]. It has been also found that the deletion of 42 amino acid from the C-terminal region of wild streptokinase leads to the production of a truncated-streptokinase with decreased immunogenicity property[13]. In a previous study, we eliminated 42 amino acids from the C-terminal region of the wild streptokinase and developed a recombinant streptokinase (r-streptokinase)[14]. Then, the bioactivity of the r-streptokinase was evaluated in vitro[15]. The objective of the present study was to investigate the bioactivity property of this modified agent *in vivo*. For this purpose, a blood clot was induced in the jugular vein of rabbits. The rate of thrombolysis was then evaluated after the intravascular administration of the r-streptokinase.

Methods

All experimental methods were approved by ethical committee of Shahid Beheshti University of Medical Sciences (Permit Number: 3.9852) with the recommendations in the Guide for the Care and Use of Laboratory Animals.

Cloning: In our previous study, the r-streptokinase (lacking 42 amino acid from C-terminal) was cloned into pGEMEX-1 expression vector with BamHI and SacI restriction enzymes[14]. The PCR product of r-streptokinase was sub-cloned into pET32a by the same restriction enzymes and transformed in BL21 (DE3) E.coli stain for protein expression.

Expression and Purification of r-streptokinase: The transformed bacterial cells, *Escherichia coli* strain BL21 (DE3), were cultured in LB broth medium until reaching the mid-log phase. Thereupon, 1mM isopropyl-1-thio-β-D galactopyranoside (IPTG), as an expression-inducing agent, was added to the medium and the cells were incubated for 5 hours at 37ºC. After the incubation time, the cells were harvested, washed and the protein was purified using affinity chromatography with S.Tag resin. The samples were then dialyzed in PBS for 1 hour. The final concentration of the r-streptokinase in the samples has been increased by dry sucrose.

SDS-PAGE and Western Blotting: The purified r-streptokinase was characterized and detected by SDS-polyacrylamide gel electrophoresis and western blotting. Briefly, the cell lysate was collected and mixed with protein lysis buffer/5 µl PMSF and centrifuged in 4000 rpm for 5 minutes. The supernatant was discarded and the pellet was mixed with 20 µL deionized water. After adding 20 µl 2-Mercaptoethanol, the sample was heated in 85ºC for 5 minutes. The samples were run in 10% SDS – Polyacrylamid gel[16] and observed after staining with 1% (wt./vole) Coomassie Brilliant blue R-250. To detect the r-streptokinase with specific antibody, the gel was transferred to nitrocellulose sheet, blocked with Skim milk in PBS at 4 C ° for 24 hours and washed with PBS containing 0.05 % Tween-20. The samples were incubated and then detected by antibody.

Bacterial Endotoxin Assay: The endpoint chromogenic limulus amebocyte lysate (LAL) is a qualitative test for the measurement of gram-negative bacterial endotoxin, which is called also lipopolysaccharide (LPS)[17]. This test is based on the capability of a serine protease enzyme in the digestion of LPS leading to clot formation. Briefly, the purified r-streptokinase was mixed with the LAL and incubated at 37°C (±1°C) for 10 minutes. A substrate solution was added to the LAL-sample (Cambrex

LAL) and incubated for 6 minutes at 37°C (±1°C). The samples were examined for clot formation.

Removal of LPS: LPS has a strong antigenicity and the injection of this agent into the body stimulates immune system profoundly that ultimately can lead to allergic reactions. Therefore, the full removal of this agent is critical for the *in vivo* administration of the r-streptokinase. One ml of the purified r-streptokinase was mixed with one ml Triton X-114 and centrifuged in 1000 rpm for five minutes. The last step was repeated three times. The supernatant was recollected and remixed with the LAL-sample to confirm the full removal of the LPS.

***In Vivo* Study**

Rabbit Housing: *The in vivo* study was performed on six adult male New Zealand White rabbits weighing 2.4±0.3 kg (mean±SD). The animals were purchased from Pasteur institute of Iran and acclimatized in an animal house under standard conditions for one week prior to use. All studies were performed under the approval of the laboratory of the Animal Care and Use Committee on Animal Investigations[18].

Surgery: Rabbit jugular vein was used as an in vivo thrombosis model to evaluate the fibrinolytic activity of recombinant streptokinase. For this purpose, the artificial thrombus in the jugular vein was induced as explained by Collen *et al*,[19]. The in vivo study performed in this study is illustrated in Figure 1. Briefly, the blood samples were taken from the marginal ears of rabbit for the measurement of D-dimer before inducing thrombosis (Figure 1a). Animals were then generally anesthetized with ketamine HCl (35 mg/kg IM) (Alfamime; Alfasan, Woerden, Holland) and xylazine (5 mg/kg IM) (Rompun; Bayer, Leverkusen, Germany). After the general anesthesia, an external jugular vein was exposed and carefully dissected from the main bifurcation of the external jugular vein and the facial vein (Figure 1b). When bleeding was stopped, two clamps were placed on both sides of the vein proximally and distally to isolate a vein segment (Figure 1c). Then, the closed vein was emptied of all blood by suction via the catheter. Volumetric syringe was used for the measurement of the volume of the segment by the injection of saline. Approximately 100 µl of human fibrinogen (10 mg fibrinogen mixed with 500 µl PBS) aspirated in a 1.0-ml syringe was mixed with a volume of fresh rabbit blood. Then, 0.5 mg human thrombin (Sigma, 100 UN) was mixed with 1ml PBS 1x, and 100 µL was injected into external jugular vein quickly (Figure 1d). It took 30 minutes that blood clot was formed and both vessel clamps were removed. At the end of the study, to follow the guidelines of animal welfare and prevent any sufferance, rabbits were euthanized with an overdose of intravenous sodium phenobarbital. The clot formation in the jugular vein was confirmed by Doppler sonography.

Administration of Drug: After blood clot formation, the blood sample was collected through marginal ear vein in each group and coded as pre-drug administration. The r-streptokinase (500 µl estimated 4000 U/kg, but not determined) was injected into the marginal ear vein (Figure 1e). The animals that received normal saline were considered as negative control group. The third group was injected with commercial streptokinase (Heberkinase) at the dose of 4000 U/kg. After 1 hour (Figure 1f), the blood sample was obtained from each group (was coded as after drug administration) and transferred into the tube supplemented with sodium citrate, as an anticoagulant agent. The samples were centrifuged in 5000 rpm for 5 minutes and the plasma was collected for the measurement of D-dimer level.

Results

The results obtained from PCR were analyzed and confirmed by 1% agaros gel electrophoresis as indicated in Figure 2, as lanes 1 and 2 indicates the bands of DNA ladder marker (ranging from 1-10kbp) and r-streptokinase plus universal primers (1116 bp), respectively (Figure 2).

SDS-PAGE: The collected bacterial samples were lysed by lysis buffer and subjected to electrophoresis on 10% SDS-PAGE. The samples were stained by Coomassie Brilliant Blue R250 in order to detect the presence of the r-streptokinase. The stained SDS-PAGE is shown in Figure 3, as lanes 1, 2 and 3 indicate the lysate of BL21 containing r-streptokinase (KDa: 42 kDa for recombinant streptokinase) 5 hours after induction by IPTG, the lysate of BL21 without induction and the lysate of BL21 without plasmid, respectively (Figure 3).

Doppler Ultrasound: Blood flow velocity within the jugular vein was measured by Doppler ultrasound in order to confirm the induced thrombosis. The difference in frequency between transmitted and reflected ultrasound depends on the velocity of blood in the vessel. However, the intensity of the reflected Doppler signals depends on some other factors such as size of particles in the blood and their acoustic impedance[20]. As described above, the flow velocity was expected to increase after clot formation. The flow velocity in the jugular vein was recorded by Doppler ultrasound before and after thrombosis induction. As it can be observed in Figure 4, the flow velocity augmented approximately 25% after 1 hour of thrombosis induction (Figure 4).

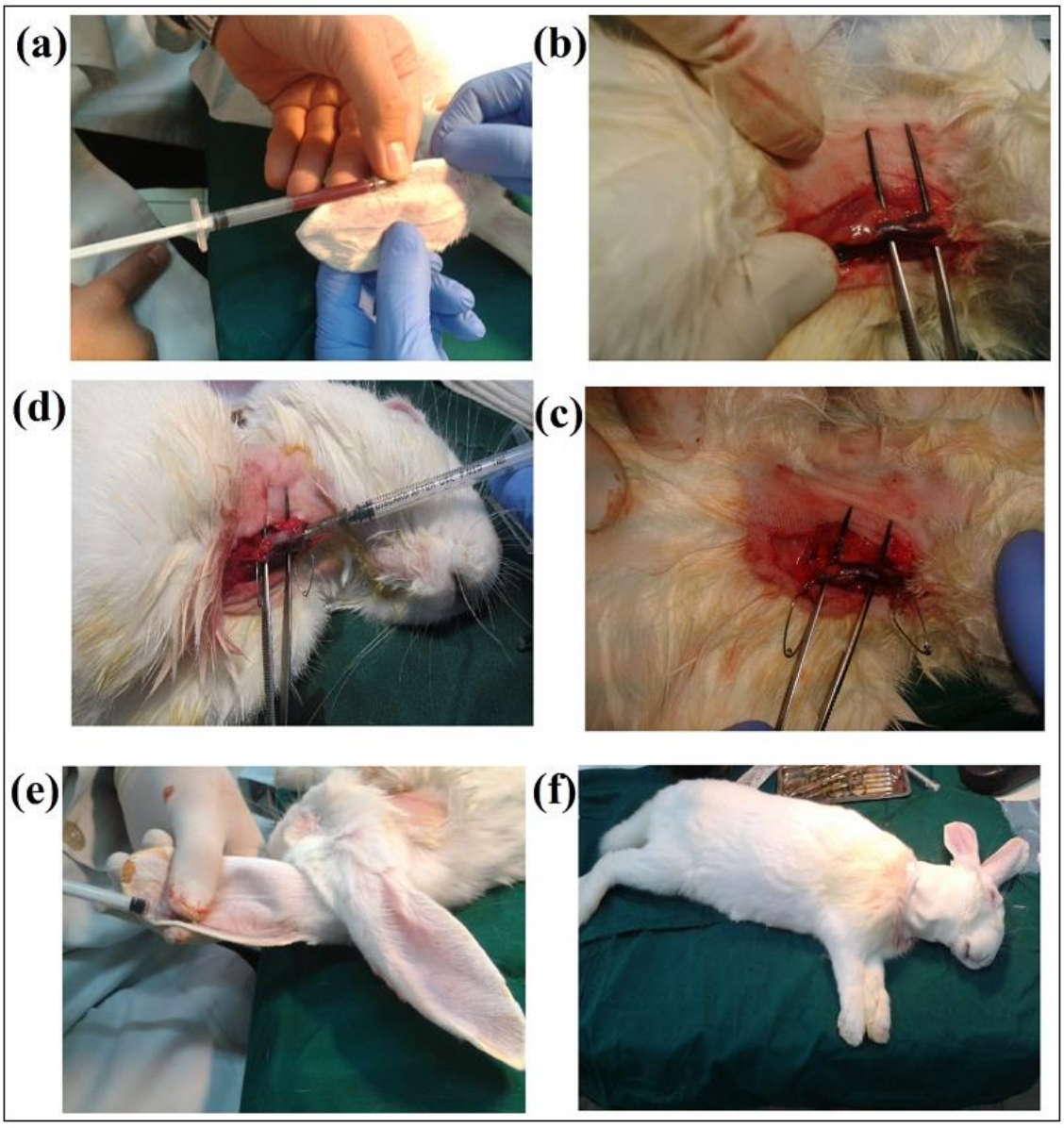

Figure 1. Summary of surgery and administration of truncated streptokinase *in vivo.* a) Collection of blood samples from marginal vein of rabbits' ear. b) Jugular vein was exposed and separated from the facial and bifurcation vein. c) Two Clamps were placed on both sides of the vein (proximally and distally) to isolate a vein segment, then the closed vein was emptied of all blood by suction via the catheter. d) Injection of fibrinogen and thrombin for the formation of blood clot through jugular vein. e) Administration of rSTK and commercial STK through marginal ear vein of rabbit. f) The animal was maintained under anesthesia for 1 hour to allow the thrombolysis of drug.

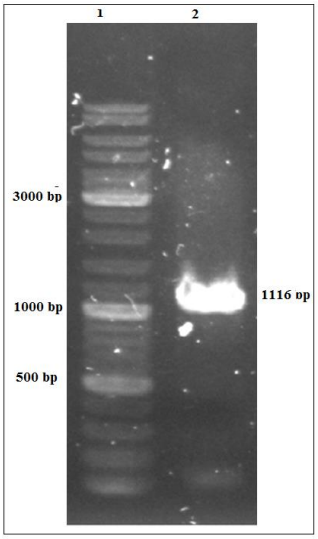

Figure 2. 1 percentage agaros gel electrophoresis. Lane 1: DNA ladder marker, Lane 2: rSTK PCR product (1116 bp).

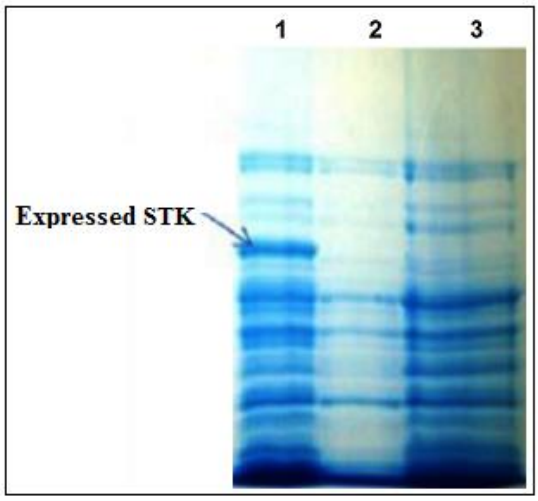

Figure 3. 10 percentage SDS-PAGE. Lane 1: Lysate of BL21 contains rSTK 5 hours after induction. Lane 2: Lysate of BL21 without induction. Lane 3: Lysate of BL21 without plasmid.

D-Dimer Test: D-dimer measurement is a moderate to highly sensitive diagnostic tool for the presence of DVT (Venous Thromboembolism) or PE (Pulmonary Embolism) [21]. For each experimental group, the level of D-dimer in plasma was measured before thrombosis induction and 1 hour after drug administration. For this purpose, two different antithrombotic agents were evaluated for their effects on the generation of thrombus. Separate groups of animals were used for each drug treatment and control. The control group received normal saline, r-streptokinase at the dosage 4000 IU/Kg and Heberkinase (Heb) at the same dosage, respectively. The blood samples were taken before clot formation and one hour after thrombosis induction. Figure 5 shows the significant difference before and after clot formation in each group. The level of D-dimer after the administration of the r-streptokinase and Heb was found to be significantly more than that in the control group. On the other hand, Heb group indicated an increased level of D-dimer in comparison with the r-streptokinase experimental group (Figure 5).

Discussion

Streptokinase as a bacterial enzyme, displays partial immunogenicity in the human body. Therefore, after the administration of streptokinase, the human body immune system is stimulated leading to the production of antibody (Ab) against streptokinase. The high titer of anti-streptokinase in the body may lead to both severe immune reactions during the streptokinase therapy and neutralization of streptokinase activity[17]. Although other thrombolytic agents such as UK and TPA are available[22], but they

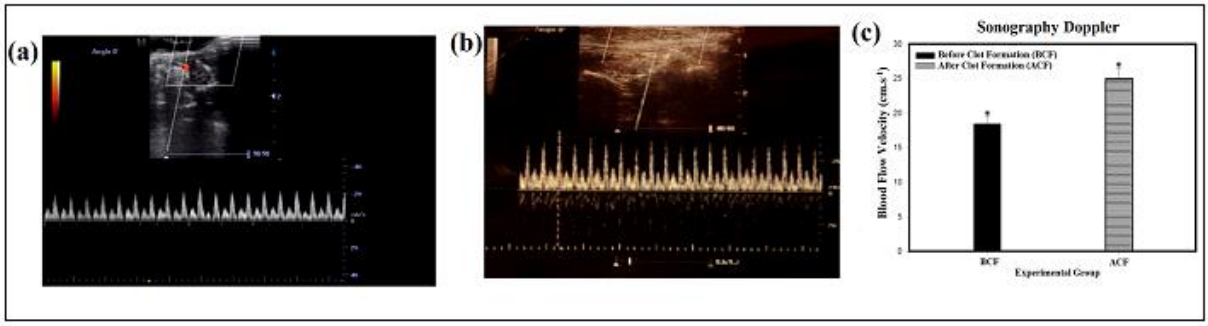

Figure 4. Doppler ultrasound results. A) Flow velocity before thrombosis induction 18.5 cm/s. B) Flow velocity after thrombosis induction 25 cm/s. C) Comparison between the flow velocities before and after clot formation.

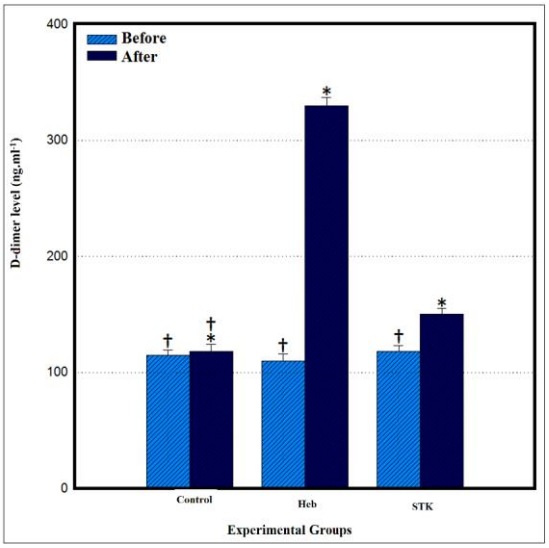

Figure 5. D-dimer level in three experimental groups (Control: injected with normal saline, Heb: injected with Heberkinase injection, rSTK: injected with sterptokinase.
*** Significant difference (p<0.05).**
† Insignificant difference (p>0.05).

are more expensive than streptokinase. Many efforts were made to overcome the immunogenicity of streptokinase. The detection and elimination of antigenic residues form streptokinase were considered as an efficient strategy in order to minimize its immunogenicity. One of the concerns arisen from such a strategy is that the deletion of some regions in streptokinase may affect its bioactivity. The determination of a region responsible for the immunogenicity of streptokinase is worthy. In 1995, Parhami-Seren et al.[23] found three major antigenic regions in streptokinase through mapping the anti-streptokinase antibody from the human sera, containing regions 1-253 and 120-352 (containing distinct, non-overlapping epitopes). Parhami-Seren et al.[23] reported that two other epitopes in streptokinase constructed by amino acids 1-13 and 353-414 were not antigenic in all humans. In our previously published work, a region in the C-terminal of the streptokinase containing 42 amino acids was eliminated. The activity of the truncated streptokinase was then determined in vitro. According to our results, the elimination of 42 aa from the C-terminal of native streptokinase did not affect the activity of enzyme in vitro[14]. Our experiment showed that Iranian consumer streptokinase serum does not respond to mutant streptokinase in comparison to Heberkinase by ELISA method[25]. The purpose of the present study was to investigate the bioactivity of the same truncated streptokinase in vivo. For this purpose, a blood clot was induced in the jugular vein of New Zealand white rabbits. The r-streptokinase was administered intravascular and compared with the animals that received Heberkinase (a commercial Streptokinase) and normal saline as positive and negative controls, respectively. The thrombolysis property of drugs was quantified through the determination of D-dimer level in rabbit blood plasma.

Many studies have been conducted to develop a strategy for the induction of thrombus in animal models[24]. For instance, Clozel *et al,*[25] induced blood clot formation in rabbits' jugular veins through thrombin and radioactive fibrinogens. Then, the level of degraded radioactive fibrinogen, representing the rate of thrombosis, was measured by using a gamma counter. In this study, the thrombus induced in the jugular vein of rabbit using fibrinogen and thrombin. The rate of thrombolysis was measured one hour after drug administration. The determination of thrombolysis in vivo is challenging due to limitations in the direct visualization of plaque and thrombus. Johnstone *et al*,[26] used MRI to determine the exact location of thrombus formation in the rabbit model. In this study, the exact location of thrombus and the relationship between the thickness of the aortic stenosis and thrombus formation were defined by Doppler sonography.

According to our results, streptokinase infusion increased the levels of D-dimers. Results showed that the recombinant streptokinase displayed a detectable fibrinolytic activity when compared with before drug administration and negative control samples. In line with these observations, the infusion of 4000 IU of r-streptokinase provoked the extensive systemic activation of the fibrinolytic system. According to the D-dimer results, r-streptokinase significantly increased the level of D-dimer in rabbits' plasma in comparison with negative control. Furthermore, the level of D-dimer in Heberkinase group had a significant increase in comparison with r-streptokinase. This decrease in the bioactivity of the r-streptokinase indicates the potential of the 42 amino acids of C-terminal in its bioactivity. It is important to

note that the synthesized r-streptokinase requires further modifications to restore its full activity invivo and make it an efficient thrombolytic agent for clinical use. Since it was reported that the C-terminal of streptokinase is critical for its immunogenicity, the synthesized enzyme may be a promising choice for clinical use after the optimization of its full bioactivity.

Conclusion

Efficacy of C-terminal truncated-streptokinase in the elimination of blood clot was evaluated in rabbits. Our data indicated that the r-streptokinase had bioactivity invivo, but less than that in commercial streptokinase (Heberkinase).

Acknowledgment

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