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Expression and Intein-Mediated Purification of Novel Staphylokinase SakSTAR with Reduced Immunogenicity and Antiplatelet and Antithrombin Activation

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Abstract

In an effort to combine the benefits of fibrinolytics, such as staphylokinase (Sak), with those of thrombin inhibitors for the prevention of vessel reocclusion after vascular injury, we produced chimeric protein with plasminogen activator and thrombin-inhibiting properties. This fusion protein was a construct consisting of Sak (SakSTAR) lengthened about 36 amino acids from the C-terminus end of hirudin. We inserted 16 point mutations into the sequence of the gene encoding SakSTAR for reduced antibody binding from 50% to about 17% and inserted two RGD sequences for antiplatelet activity. The inhibition rate of platelet aggregation was 27%. Moreover, we proposed an efficient method of expression and purification in which we used 16 mg/L of an *Escherichia coli* strain of this novel fusion protein and retained full biologic activities toward plasminogen and thrombin.

Index Entries: Staphylokinase; plasminogen activator; intein-mediated purification; hirudin; thrombin.

Introduction

Thrombolytic complication of cardiovascular diseases is the main cause of death and disability for these diseases. Consequently, thrombolysis favorably influences the outcome of such life-threatening diseases as myocardial infarction *(1)*.

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Staphylokinase (Sak), a 136 amino acid profibrinolytic bacterial protein, is a promising thrombolytic agent. It forms a 1:1 stoichiometric complex with plasminogen that activates other plasminogen molecules *(2)*. The Sak gene has been cloned from the bacteriophages *sak*ϕC and *sak*42D as well as from the *sak*STAR genomic DNA of lysogenic *Staphylococcus aureus* strain. Only four differences in amino acid codons were found in the coding regions of the *sak*ϕC, *sak*42D, and *sak*STAR genes. Amino acid 61 is Ser in SakSTAR but Gly in Sakϕ C and Sak42D. In addition,, amino acid 63 is Gly in SakSTAR and Sakϕ C but Arg in Sak42D. Furthermore, histidine (70) residue in SakSTAR and in Sakϕ C was replaced by Arg in Sak42D *(3,4)*.

Recent reports on Sak-related research *(5–10)* have demonstrated the growing interest in the development of Sak-based plasminogen activators. In addition, Sak is presently undergoing clinical trials *(11–13)* as a potential drug for thrombolytic treatment. Although Sak proves successful in the lytic therapy of thromboembolic disorders, such as acute myocardial infarction, reocclusion of the damaged blood vessels (high procoagulatory potential with increased thrombin liberation at the injured site of the vessel) frequently reduces the therapeutic effect *(14)*. Another problem is that the infusion of Sak results in a high titer of the neutralizing specific IgG, which would predict therapeutic refractoriness on repeated administration *(15)*.

In this article, we report on studies concerning fusion proteins consisting of the plasminogen activator SakSTAR Sak and part of the C-terminus of hirudin specifically blocking the FRE of thrombin. Moreover, on the basis of previous experiments the following mutations were inserted into the SakSTAR gene, reducing the immunogenic properties of the protein: K35A, E65Q, K74R, D82A, S84A, T90A, E99D, T101S, E108A, K130T, K135R, K136T. Two sequences of RGD were also inserted, according to Su et al. *(16)*.

Materials and Methods

Amplifications and Point Mutations in Sequence of Gene Encoding Sak

To find and amplify the *sak*STAR gene, the nested-polymerase chain reaction (PCR) method was employed. The external primers were as follows: SN1 (5′GGAGGAAGCGCCATGCTCAAAAGAAG3′) and SN2 (5′CTGATTAGGTGGGGAAGAACCATTCGTG3′) The annealing temperature was 55°C (60 s). Internal primers were as follows: SK1 (5′TT ACGAATTCGGTACATATGTCAAGTTCATTCGACAAAG GAA3′) and SK2 (5′TTATTTCTTTTCTATAACAACCTTTGTA3′). The annealing temperature was 65°C (60 s). Genomic DNA was derived from methicillin-susceptible *Staphylococcus aureus* staphylococci isolated with an A&A Biotechnology set from patients with osteitis using lysostaphin (DNA-Gdańsk II).

Point mutations were inserted using a PCR method; Table 1 presents the starter sequences and annealing temperatures. Polymerase *Pwo* (DNA Gdansk II) was used in the experiments in a quantity of 0.5 μL in 25 μL of reaction mixture.

Table 1
Starter Sequences Used in PCR Introducing Changes in Nucleotide Sequence Encoding Sak

Primers	Sequence (5′-3′)[a]	Point mutation
SAKI	ATGTCAAGTTCATTCGACAAAGGAAAATAT	—
SAKlr	ATGAGGGGATAGCAATTCATTTCCTGCAGTATCACCTCGAGTCAC	G31R, V32G, K35A
SAK2n	GGAAATGAATTGCATTCCCCTCATTATGTCGAG	—
SAK2r	CTTTGCCGCTTCTATAACAACCGTTGTAATTAAGTTGAATCCAGG	K130A, K135R, K136T, ∇K137
SAK3n	TCATATGTCCAATGGGCATTAGATGGGCGAGCATATAGAGAG	G65Q, A70G, T71R, K74R
SAK3r	GGCTGGGGCTAATTCAACTACTCTAAACTCTCTATATGCTCTCCCATCTAATGCCCA	D82A, S84A, K74R, A70G, T71R
Normal1	AAAGACGAATCGAAGTCTTTCCCTATAACAGCAGCAGGTTTT	E99D, T101S, El08A, K109A
RNormal	AAAACCTGCTGCTGTTATAGGGAAAGACTTCGATTCGTCTTT	E99D, T101S, El08A, K109A

[a]The underlined regions are responsible for the changes in the gene encoding Sak.

Construction of Plasmids

pSAKTX and pSAKTXm

Both plasmids were constructed on the basis of the pTXB1 plasmid (New England Biolabs), using *Nde*I and *Spe*I restrictases. The pSAKTX plasmid introduced sequences encoding SakSTAR Sak, while the pSAKTXm plasmid had sequences encoding SakSTARm Sak with the inserted point mutations.

Each product of PCR reaction carried with individual primers from Table 1 was cloned into the pUC19 plasmid (Invitrogen), using the *Sma*I restrictase.

pBAD TOPOHH

A plasmid containing a sequence encoding hirudin was obtained using the following megaprimers: H1 (5′GTCGTCTACACTGATTGTACT GAATCTGGTCAAAACTTGTGTTTGTGTGAAGGTTCTAACGTTTGTG GTCAAGGTAACAAGTGTATTTTGGGTTCTGACGGTGAAAAGAA CCA3′) and H2 (5′TTGCAAGTACTCCTCTGGAATCTCCTCAAAAT CACCATCGTTATGAGATTGTGGCTTTGGAGTACCTTCACCAGTAAC ACATTGGTTCTTTTCACCGTCAGAACCCAAAATAC3′) (Sigma, St. Louis, MO). A reaction mixture containing 8 µL each of primer H1 and H2 (10 µ*M*), 10 µL of dNTPs (2 m*M*), 1 µL of *Taq* polymerase (Fermentas), 5 µL of 10X concentrated buffer (Fermentas), and 18 µL of H_2O was incubated for 30 min at 72ßC. Primers R2 (5′-TTGCAAGTACTCCTCTGGAATCTCCTC-3′) and F2 (5′-GTCGTCTAC ACTGATTGTACTGAATCTGG-3′) were used to amplify the gene encoding hirudin; primers were added at 60°C for 60 s, and the pBAD TOPO plasmid (Invitrogen) was used as the vector into which the obtained gene was cloned, becoming a source of the hirudin-coding sequence.

Construction of Gene with a Sequence Encoding Fusion Protein Consisting of SakSTARm Sak and C-terminus End of Hirudin

The sequences encoding fusion protein consisting of SakSTARm Sak modified by point mutations and the C-terminus end of hirudin were obtained through the hybridization of the two products of PCR. The first product was obtained during PCR with the use of the following primers: H1 (5′CCTGGATTCAACTTAATTACAACGGTTGTTATAGAAGCAGC GAAGTCCTGGGCTCTGACGGCGAAAAAAACCAGTGCG-3′) and H2 (5′CCTATTACTGCAGGAA TTCTTCCGGGA-3′). The template used in the reaction was the pBAD TOPOHH plasmid with the hirudin-coding sequence. The second product was obtained during PCR using SAK1 and SAK2r primers (Table 1), and the template used in this reaction was the pSAKTXm plasmid. The product of the hybridization was cloned into a pUC19 plasmid with the use of *Sam*1 restrictase.

pTYBStaHir Plasmid

The pTYBStaHir plasmid was constructed on the basis of the pTYB12 plasmid (New England BioLabs) with the use of *Bam*HI restrictase. Cloning of the sequence encoding fusion protein was enabled by the following primers: TOTAL1 (5′-TTTTGGATCCCAGGTTGTT GTACAGAATGCTT CAAGTTCATTCGACAAAGGAAAATAT3′) and TOTAL2 (5′-TTATTC CGGATCCTCACCTATTACTGCAGGTATTCTTCCGGGA-3′). The template used in the PCR was the pUC19 plasmid with a sequence encoding SAKHE fusion protein.

Overexpression and Purification of SAKHE Fusion Protein

E. coli ER2566 strain was transformed using the recombinant plasmids pSAKTX, pSAKTXm, and pTYBStaHir, enabling the expression of the gene under the control of the T7 promoter. Production was carried out in Luria-Bertani medium with the addition of ampicillin (final concentration of 100 μg/mL). After reaching an OD_{600} of 0.6 by the culture, the overexpression of Sak was induced by the addition of isopropyl-β-D-thiogalactopy-runoside (IPTG) to a final concentration of 0.5 m*M*. Cultivation was continued at 15°C for 24 h. The bacterial cells were suspended in 10 mL of buffer I (0.605 g of 10 m*M* Tris, 7.312 g of 250 m*M* NaCl, H_2O distilled to 500 mL) and subjected to sonification in a SONIPREP 150 device in four 30-s cycles with 1-min intervals after each cycle. The obtained lysate was applied onto a chromatographic column containing chitin (1 g of chitin in 15 mL of cell lysate). Native proteins from *E. coli* were rinsed out from the column with buffer I. After immobilization of recombinant protein, the column was filled with buffer II (0.242 g of 20 m*M* Tris, 0.272 g of 50 m*M* NaCl, 0.770 g of 50 m*M* dithiothreitol [DTT], H_2O distilled to 100 mL), and the autohydrolysis reaction of the product was continued for 72 h at 4°C. The concentration of the obtained protein fraction was determined according to the Bradford method.

Determination of Activity of Fusion Protein: Preparation of Platelet-Poor Plasma

A solution of blood in citrate buffer in a 1:9 ratio by volume was spun at 10,000 rpm for 15 min, and the precipitated leukocytes and erythrocytes were discarded. The collected supernatant minus the platelet-rich plasma (PRP)was again spun at 6000 rpm for 20 min. The platelet-poor plasma (PPP) supernatant was used in the research on Sak activity.

Determination of Proteolytic Activity of Fusion Protein: Fibrin Clot Lysis Study

Fibrin clots were formed by adding human thrombin (Sigma) (to 0.6 NIH units/mL) and $CaCl_2$ (to 20 m*M*) to human fibrinogen (Sigma) (1 mg/mL final concentration) in HEPES-buffered saline (HBS) (0.1 *M* HEPES,

0.13 *M* NaCl, pH 7.4). Immediately after mixing, 100-µL aliquots of the polymerizing fibrin solution were pipetted to the walls of a microtiter plate. Clot formation was allowed to proceed for 3 h at room temperature. A 100-µL solution containing freshly mixed human plasminogen (1.5 µL; Sigma) and varied concentrations of purified SakTXm and SakHE in HBS was lay-ered on each clot. The changes in clot turbidity with time were monitored by measuring changes in the absorbance at 405 nm and 25°C.

Plasminogen Activation Reaction

A mixture of plasminogen (1.5 µ*M*) and SakSTAR or SAKHE (final concentration of 20 µg/mL) was incubated at 37°C for 5 min in 0.1 *M* phosphate buffer (pH 7.5) containing 0.1% bovine serum albumin (BSA) and 0.01% Tween-80. The change in absorbance was recorded at different time intervals after the addition of chromozyme PL (1.2 m*M*).

Establishment of Antithrombin Properties

Activated partial thromboplastin time (APTT) and prothrombin time (PT) were determined semiautomatically using a coagulometer (STA; Stago) applying the manufacturer's reagents and procedures.

Antiplatelet Aggregation Assay

Three hundred microliters of PRP plus 10 µL of protein in saline was incubated in an aggregometer at 37°C for 15 min. A total of 5 µL of adenosine 5N-diphosphate (final concentration of 5 µ*M*) was added, and the change in light transmission was recorded over 5 min. Transmission was set at 100% for PPP and 0% for PRP.

Results

Amplification of Gene Encoding Staphylokinase

The proposed nested-PCR system consisting of the SN1 and SN2 external primers and SK1 and SK2 internal primers enabled the finding of the gene as well as a very efficient amplification of the gene encoding SakSTAR Sak, which is a part of the genomic DNA of the staphylococcus, and not a DNA fragment of bacteriophage.

Insertion of Point Mutations into Sequence Encoding Sak Through PCR Method to Reduce Antigenicity and Antiplatelet Aggregation

Sixteen mutations were inserted into the gene encoding Sak in order to reduce its antigenic properties while retaining its function as plasmino-gen activator. The choice of the particular changes in the gene sequence was made according to Laroche et al. *(13)*, Su et al. *(16)*, and Chen et al. *(18)*. Fourteen of the mutations concerned three nonoverlapping immunodominant epitopes of SakSTAR that were mapped by a competitive antibody-binding study.

Table 2
Effects of Sak and Variants in Platelet Aggregation Assay and Antibody-Binding Test[a]

Antibody-binding test	
Protein sample	Antibody absorption (%)
SakSTAR	95
SakSTARm	17
SakHE	17

Antiplatelet aggregation		
Protein sample	Max. aggregation (%)	Inhibition (%)
Saline	58 ± 8	—
SakSTAR	53 ± 4	8
SakSTARm	42 ± 6	27
SakHE	41 ± 4	26

[a]Data are expressed as the mean ± SD of three independent samples ($p < 0.05$).

Insertion of the mutations caused the reduction in antibody binding from 50 to 30% *(13,17)* to about 17%. The additional insertion of RGD and DGR sequences in appropriate areas of the plasminogen activator, i.e., in regions 31–33 and 69–71 (the most external regions of the protein) equipped the fusion protein with additional antiplatelet aggregation properties. SakHe can inhibit platelet aggregation, and at a concentration of 25 μg/mL which of this Sak the inhibition rate of platelet aggregation was 27% (Table 2).

Protein Expression and Construction of Expression Vector Containing Sequence Encoding SakHE Sak Fusion Protein Containing C-terminus End of Hirudin

New England Biolabs developed a series of pTYB12 expressive plasmids, suitable for expression of recombinant protein combined with fusion domains: intein- and chitin-binding domain (CBD).

The course of the ligation process of DNA vector—plasmid pTYB12 and DNA insert—enabled the application of TOTAL1 and TOTAL2 starter sequences in PCR. In the sequence of the pTYB12 plasmid, there are two recognition sites of *Bam*HI restriction endonuclease, which was employed to create complementary sticky ends in the particles of the DNA vector and the DNA insert. Digestion of the DNA plasmid with this enzyme causes the removal of a DNA fragment (95 bp), including a fragment of gene encoding the C-terminal part of the intein. Hence, an analogical fragment of the gene was inserted into the DNA sequence of the insert. Ligation of the DNA insert and the DNA vector thus enabled the introduction of a gene encoding

a mature form of SakHE into the plasmid as well as reconstruction of the intein gene. Use of the pTYB12 plasmid enables expression of SakHE Sak without the additional amino acid residues at the N- and C-terminus ends of the protein. This ensures retention of the biologic activity of the enzyme and enables its use in clinical tests. SakHE Sak fusion protein combined with a CBD and intein is produced in the cells. The CBD enables protein purification on chitinous beads, while the presence of intein enables separation of the fusion domains from the enzyme and obtainment of the desired protein with free N- and C-terminus ends.

Expression of SakHE Sak was conducted in *E. coli* ER2566 cells. The culture was induced at an OD_{600} of 0.6 using IPTG at a concentration of 0.5 m*M*. Attempts to induce the culture at lower ODs entirely inhibited the expression of protein, while inducing cultures of higher ODs did not improve the efficiency of the expression. Attempts to obtain the protein at temperatures higher than 15°C also resulted in failure. An efficient expression of SakHE Sak was obtained when after the addition of the inductor the temperature of the bacterial culture was decreased to 15–17°C. After 18 h of incubation, the pellet (about 3 g wet wt of cells) was resuspended in 20 mL of lysis buffer A (20 m*M* Tris-HCl, pH 7.0; 500 m*M* NaCl; 1 m*M* EDTA). All subsequent steps were performed at 4°C. The cells were disrupted by sonification, and the insoluble debris was removed by ultracentrifugation for 1 h at 260,000*g*. The clear lysate was applied directly onto a column containing 10 mL of chitin beads (New England BioLabs) preequilibrated with 10 vol of buffer A. After loading, the column was washed with 10 bed vol of buffer A. The column buffer was exchanged with cleavage buffer (20 m*M* Tris-HCl, pH 7.0; 500 m*M* NaCl; 1 m*M* EDTA; 50 m*M* 2-mercaptoethanol) by quickly washing the column with 3 bed vol. The flow was then stopped and the column was left at 4°C for 72 h. Sak elution was carried out by continuing the column flow with buffer A. The eluted fraction was dialyzed into a buffer containing 20 m*M* Tris-HCl, pH 7.5; 100 m*M* KCl; 0.1% Triton X-100; 0.1 m*M* EDTA; 1 m*M* DTT; and 50% glycerol. The Sak content of the fraction was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and protein concentrations were determined using the Bradford dye-binding assay (Bio-Rad) and BSA as the standard. The material applied to the chitin column was cleaved and eluted with almost 100% efficiency. We obtained 16 mg of purified Sak from 1 L of induced culture (Fig. 1).

Antithrombotic and Fibrinolytic Activities

Figure 2 shows the typical changes in clot turbidity. As presented, SakSTAR-mediated clot lysis began with a longer lag phase than SakHE. The time required for 50% clot lysis ($T_{50\%}$) was insignificantly longer than that required by SakHE. In this assay, we proved that our alterations in SakSTAR including all the proposed mutations and the extension of Sak by the C-terminus end of hirudin did not affect fibrin clot lysis activity. Along

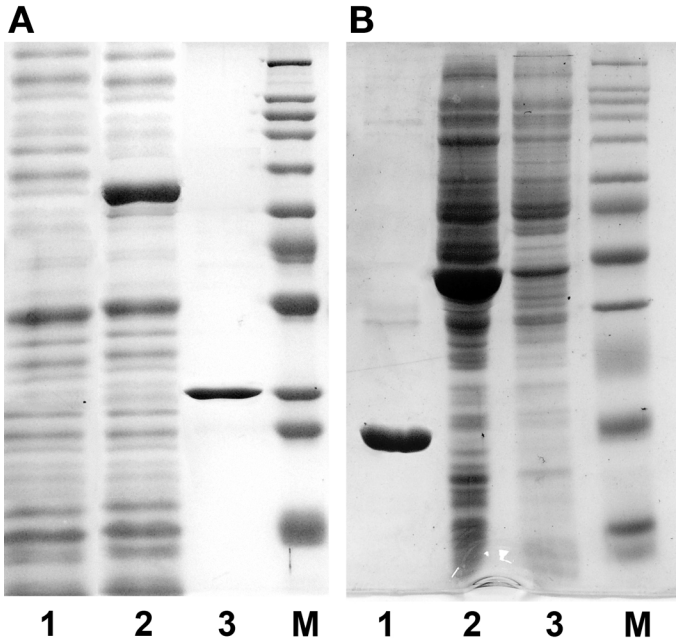

Fig. 1. SakHE Sak expression in pTYB12 system/*E. coli* ER2556: **(A)** SDS-PAGE gel stained with Coomassie blue; **(B)** SDS-PAGE visualized by silver staining. Lane 1 in (A) and lane 3 in (B), *E. coli* cell lysate ER2556; lane 2 in (A) and lane 2 in (B), *E. coli* cell lysate ER2556 after IPTG induction; lane 3 in (A) and lane 1 in (B), purified SAKHE Sak. M, model of molecular mass of Sigma proteins (205, 116, 97, 84, 66, 55, 45, 36, 29, 24, 20, 14.2, and 6.5 kDa).

with the increased concentration of SakSTAR and SakHE, the time required for 50% clot lysis was obviously shorter in both cases (Fig. 2).

The specific plasminogen-activating activities of SakHE were determined with a plasminogen-coupled chromogenic assay. PL chromozyme is a peculiar substrate for obtaining plasmin during the activation of plasminogen by Sak. To ensure the conditions in which the whole plasminogen could be used to create complexes with Sak, recombinant protein was added in excess to the mixture assay. The activation of plasminogen in the presence of SakHE occurred faster with a marked lag phase as opposed to SakSTAR, in which case plasminogen activation occurred exponentially with time. This result, as shown in Fig. 3, indicated that changes inserted in SakHE recombinant protein improve fibrinolytic activities in relation to Sak STAR. To establish the anticoagulant activities of SakHE, we routinely employed APTT and PT to monitorthe anticoagulant effect. The control times were APTT 32 ± 2 and PT 13.2 ± 0.3. As can be seen in Fig. 4, SakHE induced lengthening of PT and APTT more gradually with similar prolongations up to 20 μg/mL; thereafter APTT was less sensitive to the recombinant protein while PT continued to respond to the increasing peptide

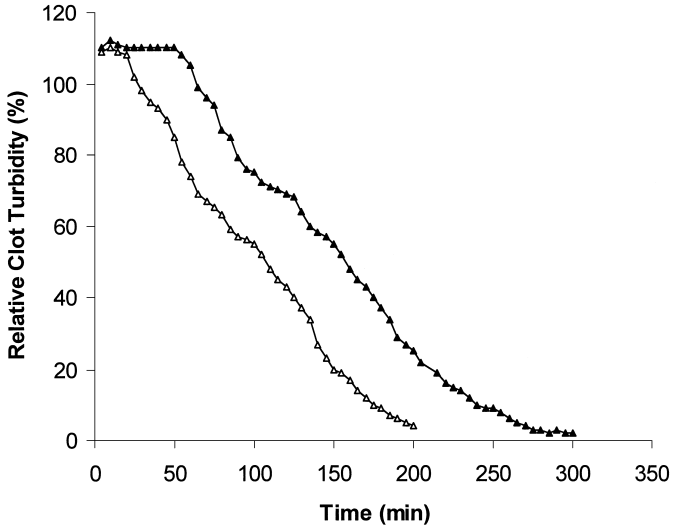

Fig. 2. Representative curve showing time course of fibrin clot lysis. SakTXm or SakHE was used at 50 n*M*. The decrease in absorbance at 405 nm with time was used to calculate the relative clot turbidity at different time points. The clots were incubated with either SakTXm (▲) or SakHE (△).

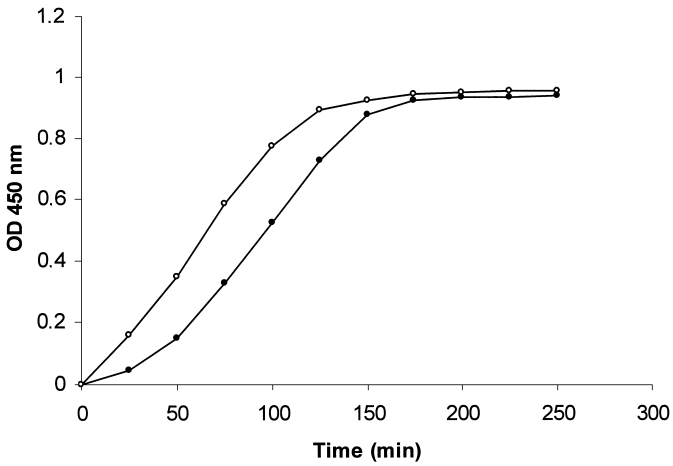

Fig. 3. Pattern of plasminogen activation by recombinant SakHE (○) and SakTXm (●). Human plasminogen (1.5 µL) was incubated with SakTXm or SAKHE (0.5 µ*M*) in the presence of 1.2 m*M* chromozyme PL at 25°C in 0.5 *M* Tris-Cl, 0.1 *M* NaCl (pH 7.4) containing 0.1% BSA and 0.01% Tween-80. The rate of Pm generation was followed by monitoring the absorption at 405 nm at different time intervals.

concentrations. PT was prolonged to two and three times the control time at approx 20 and 50 µg/mL, whereas APTT required 21 and >100 µg/mL to reach two and three times the control time (Fig. 4).

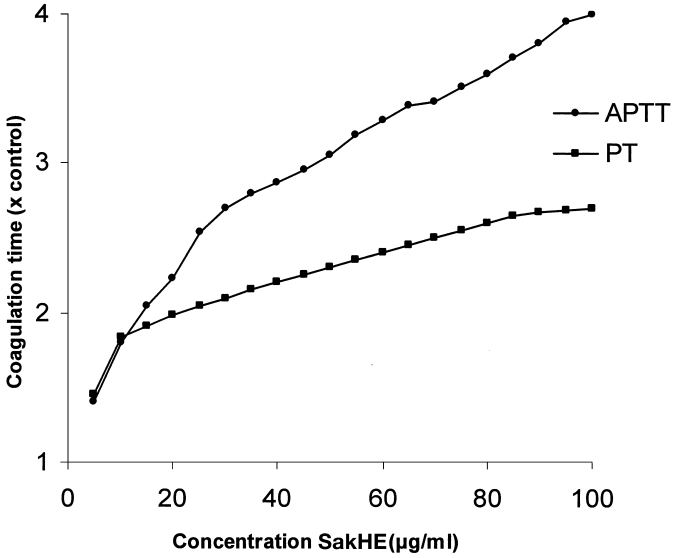

Fig. 4. Anticoagulant effect of SakHE. The effect of SakHE on the thrombin time PT and APTT was assessed by adding 0.05 mL of SakHE to 0.1 mL of citrated plasma approx 2 min before initiating activation and coagulation.

Discussion

The search for the new thrombolytic medicaments is based on the creation of more effective, safer drugs that do not cause complications and are easy to apply. The synthesis of fusion proteins is a feasible way to design multifunctional compounds of pharmacologic interest. One of the most promising drugs is a bacterial protein, Sak, which has shown high selectivity toward fibrin, one of the components of a clot. This property reduces the side effects of healing such as bleeding *(19)*.

The variant of SakHE Sak that we introduced may be seen as a summary of the most interesting works concerning this protein that have been published in the last 5 yy. In the majority of the articles concerning fusion proteins with fibrinolytic properties, the role of the plasminogen activator is played by Sak, usually Sak42D. For our experiments, we chose SakSTAR Sak, which is currently undergoing numerous clinical tests. We inserted 16 point mutations into the sequence of the gene encoding SakSTAR. Laroche et al. *(13)* introduced Sak (code SY155) containing the following mutations: T90A, K35A, K130A, K135R, G65Q, D82A, S84A, K74R, E99D, T101S, E108A, K109A. On the basis of their study, this variant has been selected for further development toward fibrin-selective thrombolytic therapy in patients with thromboembolic disease *(13)*. The Sak that we proposed contains all the aforementioned mutations except the T90A change, which caused a drastic decrease in protein activity and thermostability. In addition, on the basis of

the work by Chen et al. *(18)*, we inserted two additional mutations, K136T and K137, preventing the formation of protein dimers, which influence its immunogenicity. Moreover, we equipped the recombinant protein with two RGD sequences proposed by Su et al. *(16)*. In our first variant of the Sak, the RGD sequence was placed in the G31, V32, D33 region and was formed through the replacement of glycine 31 by arginine and valine 32 by glycine without substitution of asparagine in position 33. Consequently, RGD is placed before serine 34 and alanine 35 residues. Both these amino acids participate in forming protein coil, which means that the exposed RGD sequence appears to be the closest to the external environment. The second DGR sequence was introduced into the Asp69, Ala70, Thr71 region, through the replacement of alanine 70 by glycine and threonine 71 by arginine using asparagine 69 present in this region. As in the case of the first investigated sequence, the RGD sequence was formed in the place of the protein coil. SakHE Sak reduced antibody binding from 50% to about 17%, and when the two RGD sequences for antiplatelet activity were inserted, the inhibition rate of platelet aggregation was 27%.

A problem resulting from the administration of fibrinolytic drugs is reocclusion of the clot. The therapy of thromboembolic disorders can be improved by the application of compounds that simultaneously dissolve a thrombus and inhibit the *de novo* formation of blood clots. These goals can be achieved by the combination of a thrombin inhibitor and a plasminogen activator. For our experiments, we chose hirudin, a 65 amino acid residual peptide found in medicinal leeches, which is the most potent inhibitor of thrombin found in nature. It acts by forming a tight, stoichiometric complex with thrombin. Newly available recombinant forms of hirudin are very effective in inhibiting arterial thrombosis in experimental models and are currently under clinical evaluation *(19–21)*. The N-terminus end of hirudin interacts with the active center of thrombin and the C-terminus end with the FRE of thrombin. Thrombin is one of the known proteases that contain the FRE domain. The 10 final amino acids of hirudin are responsible for the interaction with the FRE of thrombin. In our research, we decided to use the C-terminus end of hirudin because of its functions as well as its simple linear structure, which in our experiments became the extension of Sak, endowing it with a new quality. We have empirically proved that to obtain antithrombin properties of protein, 36 amino acids from the C-terminus end of hirudin must be employed. A smaller amount of amino acids—10, 15, 20, and 25—attached to SakTXm Sak also prolongs the PT and APTT times together with minor decreases or increases in fibrinolytic activity, yet only the structure of 36 amino acids attached directly to Sak ensures its prolonged retention of both activities at a temperature of 37 and 45°C. Furthermore, the method of obtaining protein that we have proposed is efficient, inexpensive, and not time-consuming. It enables easy obtainment of the protein in the native form with free C- and N-terminus ends without using additional proteolytic enzymes. We believe that SakHE Sak

summarizes many works concerning this problem, but it may also constitute new and convenient starting material for further research on Sak as an element playing the role of a plasminogen activator in fusion proteins, as well as further research on reducing its immunogenicity and research in *Staphylo-coccus aureus* diagnostics.

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