

Cloning and expression of a new recombinant thrombolytic and antithrombotic agent – a staphylokinase variant

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Received: 24 April, 2008; revised: 09 September, 2008; accepted: 18 September, 2008
available on-line: 19 November, 2008

To develop a more potent antithrombin agent with thrombolytic and antiplatelet properties, a new staphylokinase (SAK) variant was constructed. The kringle 2 domain (K2) of tissue type-plasminogen activator (t-PA) containing a fibrin-specific binding site (i), the RGD sequence (Arg-Gly-Asp) for the prevention of platelet aggregation (ii) and the antithrombotic agent – hirulog (iii) was assembled to the C-terminal part of recombinant staphylokinase (r-SAK). cDNA for the hybrid protein SAK-RGD-K2-Hirul was cloned into *Pichia pastoris* pPIC9K yeast expression vector. The introduction of K2 t-PA, the RGD sequence and hirulog into the C-terminus of r-SAK did not alter the staphylokinase activity. We observed a higher clot lysis potency of SAK-RGD-K2-Hirul as evidenced by a faster and more profound lysis of ¹²⁵I-labeled human fibrin clots. The potency of thrombin inhibition by the hirulog C-terminal part of the recombinant fusion protein was almost identical to that of r-Hir alone. These results suggest that the SAK-RGD-K2-Hirul construct can be a more potent and faster-acting thrombolytic agent with better antithrombin and antiplatelet properties compared to r-SAK and SAK-RGD-K2-Hir.

Keywords: recombinant protein, thrombolysis, thrombolytic and antithrombotic agents, antiplatelet activity, staphylokinase, hirulog, K2 domain of t-PA

INTRODUCTION

Thromboembolism is a major disease in developed countries, such as Western European countries and the USA, and is generally treated by means of thrombolytic therapy, which made application of the body's intrinsic fibrinolytic system. The incidence of coronary heart disease has been constantly increasing recently, becoming the major disease that imperils human life. As a life-threatening occurrence it often requires aggressive pharmacological treatment, thrombolytic therapy being the obvious rationale. The most common strategy of thrombolytic therapy involves activation of the fibrinolytic system by in-

fusing plasminogen activators (Collen *et al.*, 2000; Veshina *et al.*, 2006) that convert plasminogen to the proteolytic enzyme plasmin and initiate clot lysis. The large scale application of molecular biology techniques in the 1980s initiated a dynamic development of research aiming at 'improving' the properties of natural proteins, as well as engineering new proteins to be applied as thrombolytic agents. A frequent problem in thrombolytic therapy is reocclusion. The fibrinolytic system works to remove fibrin deposits from the vascular wall, but in most occluded vessels, the vascular wall is impaired because of endothelial injury as well as plaque formation. After the removal of fibrin, such vascular impairment

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Abbreviations: BSA, bovine serum albumin; C₅₀, concentration necessary for 50% clot lysis; HBS, Hepes-buffered saline; hPlg, human plasminogen; K2 t-PA, kringle 2 domain of tissue type-plasminogen activator; RGD, Arg-Gly-Asp peptide; SAK, staphylokinase; T₅₀, time necessary for 50% clot lysis; t-PA, tissue type-plasminogen activator.

results in recurrent activation of coagulation as well as platelets, and the damaged area becomes covered with platelet aggregates and a fibrin network. During thrombolysis a large amount of thrombin is released, which in turn activates the coagulation system, promotes platelet degranulation, and inhibits fibrinolysis. Thus, the benefit may be limited by incomplete reperfusion, and often by subsequent recanalization and reocclusion. To avoid the activation of thrombotic processes and early reocclusion, the thrombolytic agent is usually infused together with antithrombotic and antiplatelet agents.

Staphylokinase (SAK), an extracellular profibrinolytic 136-amino-acid bacterial protein, is at least as potent as t-PA (tissue type-plasminogen activator) in the treatment of coronary artery recanalization (Armstrong *et al.*, 2003; Moreadith & Collen, 2003). The activation of plasminogen by SAK is highly fibrin-specific, and staphylokinase mutants could have an even better benefit/cost ratio than other thrombolytic agents. The exposure of positively charged NH₂-terminal amino acids after hydrolysis by SAK is a major determinant of its plasminogen-activating potential. It enables the activation of plasminogen to plasmin on the thrombus surface without a systemic activation of the fibrinolytic system. As antibodies to SAK are expressed after administration, many SAK mutants has been examined to find molecules with lower antigenicity. Finally, PEG-SAK was tested for the treatment of acute myocardial infarction in a clinical trial (Moreadith & Collen, 2003). Hence, there is an intense search for improving its clinically useful properties (Szarka *et al.*, 1999; van Zyl *et al.*, 2000; Icke *et al.*, 2002; Lian *et al.*, 2003; Wu *et al.*, 2003).

In order to improve the thrombolytic potential of SAK as well as to introduce the antithrombotic and antiplatelet activities, the recombinant protein SAK-RGD-K2-Hir has been constructed (Szemraj *et al.*, 2005). It has the fibrin-specific properties of plasminogen activation combined with antithrombin and antiplatelet activities and was previously shown to be a more potent and faster-acting thrombolytic agent compared with standard r-SAK in an *in vitro* study (Szemraj *et al.*, 2005). To document the thrombolytic potential of SAK-RGD-K2-Hir we examined this protein in an electrically induced carotid artery thrombosis model and a stasis-induced venous model in rats (Szemraj *et al.*, 2007). A weaker thrombolytic potency in the arterial thrombosis model was due to hirudin (r-Hir) presence in molecule of SAK-RGD-K2-Hir, since SAK-RGD-K2 was the most effective in maintaining of carotid potency and reduction of arterial thrombus mass. SAK-RGD-K2-Hir was significantly more effective in reduction of venous than arterial thrombus mass in our experimental conditions. A significant prolongation of bleeding

time was observed in animals treated with proteins containing r-Hir (SAK-RGD-K2-Hir or SAK-K2-Hir). We have now constructed a new r-SAK variant with thrombolytic and antiplatelet properties and better antithrombin activity. SAK-RGD-K2-Hirul contains a fibrin-specific binding site of tissue type-plasminogen activator: the kringle 2 domain (K2), the Arg-Gly-Asp (RGD) peptide sequence for prevention of platelet aggregation, and an antithrombotic agent – hirulog (r-Hirul) fused to the C-terminal part of r-SAK.

The biological activity of the recombinant SAK-RGD-K2-Hirul protein was studied *in vitro* and compared with that of r-SAK and SAK-RGD-K2-Hir.

MATERIALS AND METHODS

Bacterial and yeast strains and plasmids. *Escherichia coli* strain BL21 (DE3) and *Pichia pastoris* GS 115 strain (Invitrogen, Groningen, Netherlands) were used as hosts for recombinant plasmids.

Recombinant plasmid PCR T7TOPO TA with either the gene for SAK, cDNA for K2-tPA or for hirudin were transformed into BL21 (DE3) competent cells. Single stranded templates of all constructs were prepared and confirmed by sequencing. Inserts from these plasmids were used for construction of recombinant plasmid pPIC 9K-SAK-RGD-K2-Hir and SAK-RGD-K2-Hirul in Multi Copy pPIC 9K yeast expression vector (Invitrogen, Groningen, Netherlands).

Reagents. All reagents used were of analytical grade. The restriction and modification enzymes, as well as *Taq* DNA polymerase came from Promega (Madison, WI, USA). Oligonucleotides, mutagenic oligonucleotides and synthetic DNA for hirulog were synthesized at IDT (Coralville, IA, USA). Human plasminogen (hPlg) and urokinase were prepared as described by Deutsch and Metz (1970) and Lijnen *et al.* (1986). CNBr-activated Sepharose 4B was purchased from Pharmacia Biotech (Uppsala, Sweden). Recombinant hirudin came from Rhein Biotech (Duesseldorf, Germany).

PCR amplification and cloning of the SAK gene. Oligonucleotides used for PCR amplification of SAK gene from *Staphylococcus aureus* were as follows: forward 5' ATGCTCAAAGAAGTTTATTATTT 3' and reverse 5' TTTCTTTTCTATAACAACCTTTGT 3'. The amplification was accomplished by initial denaturation at 94°C for 3 min and 27 cycles at 94°C for 30 s, at 72°C for 30 s, at 58°C for 30 s and a final extension at 72°C for 10 min. The product of 491 bp was cloned into PCR T7TOPO TA vector and then recloned into *P. pastoris* Multi Copy pPIC9K at the *Sna*BI-*Eco*RI site using a forward primer 5' GG-TACGTAATGCTCAAAGAAGTTTATTATTT 3'

containing the *Sna*BI restriction site and reverse primer 5' GTGAATTCTTTCTTTTCTATAACAACCTTTGT 3' containing the *Eco*RI site. Introduction of mutations (amino acid substitutions K35A, E65Q, K74R, D82A, K130T and K135R) to the staphylokinase wild-type gene cloned in PCR T7TOPO TA vector was carried out using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and mutant oligonucleotide primers according to Collen *et al.* (1997). Nicked DNA carrying the desired mutations was transformed into Epicurian XL-Blue supercompetent cells and then recloned to pPICK 9K vector.

RNA purification, synthesis of cDNA for K2 t-PA and PCR amplification. Total cellular RNA was extracted from human endothelial cells using Trizol reagent method, a single-step purification protocol (Chomczyński & Sacchi, 1987). Polyadenylated RNA was isolated using a standard protocol on oligo dT-cellulose. Poly(A) RNA (50 ng) was then used for cDNA synthesis by SuperScript II RNase Transcriptase System (Invitrogen, Groningen, Netherlands), using 12–18 (dT) primers. cDNA was amplified with the forward 5' ATGAAGA-GAGGGCTCTGCTGTGTG 3' and reverse 5' GCTT-GGCAAAGATGGCAGCCTGCC 3' primers specific for t-PA cDNA. PCR conditions were initial denaturation 3 min at 94°C and 30 cycles of 30 s at 94°C, 30 s at 72°C and 30 s at 57°C. The reaction mix was diluted 10× and amplified again with the forward 5' AACAGTGACTGCTACTTTGGG 3' and reverse 5' GCGAAACTGAGGCTGGCTGTACTG 3' nested primers specific for K2 t-PA cDNA at the same PCR conditions. The 315 bp (K2 t-PA) product was resolved through a 6% polyacrylamide gel and after purification on Wizard columns (Promega Corp.) cloned into PCR T7TOPO TA vector.

Cloning and sequencing of SAK-RGD-K2-Hirul. Synthetic dsDNA encoding a 20-amino-acid linker peptide (ARASGRGDGGDSGRGDGGRA) containing two RGD sequences, and carrying *Eco*RI and *Avr*II sites at the ends was cloned into recombinant pPIC 9K yeast expression vector behind previously cloned staphylokinase gene and transformed into *P. pastoris* cells. The K2 t-PA cDNA previously cloned in PCR T7TOPO TA vector was then recloned into pPICK 9K yeast expression vector behind the SAK-RGD sequence in the *Avr*II–*Not*I site using the following oligonucleotides: forward 5' GCCTAGGAACAGTGACTGCTACTTTG 3' containing the *Avr*II site and reverse 5' TGCGGCCGCGGAAACTGAGGCTGGCTGTACTG 3' containing the *Not*I site. The amplification was accomplished by initial denaturation at 94°C for 3 min and 27 cycles at 94°C for 30 s, at 72°C for 30 s, at 58°C for 30 s and a final extension at 72°C for 10 min. A synthetic peptide linker was added according to Szarka *et al.* (1999); to evoke

a fusion between two domains, r-SAK and K2. The 60 bp synthetic DNA for hirulog (F-P-R-P-G-G-G-G-D-F-E-E-I-P-E-E-Y-L-Q) cloned in PCR T7TOPO TA vector was then recloned into recombinant pPIC9K yeast vector in the *Not*I site and introduced into *P. pastoris* cells. The DNA for hirulog was cloned behind SAK-RGD-K2 using the following primers: forward 5' CGCGGCCGCGGTGATTTCCCACGTCGGGA 3' and reverse 5' GGCGGCCGCTCATTGTAAATATCTTCTGG 3', both containing the *Not*I site at the ends. DNA cloned for hirulog behind SAK-RGD-K2 generated a 5-amino-acid linker RGRGD separated both (containing additional RGD sequence). Single stranded templates of all constructs were prepared and confirmed by sequencing. Recombinant plasmid pPIC 9K-SAK-RGD-K2-Hirul was digested with *Sac*I to linearize it. *P. pastoris* spheroplasts were obtained by Zymolyase digestion (Sigma Aldrich, MO, USA). *P. pastoris* spheroplasts were transformed using lithium chloride method (10³ transformants per µg DNA).

Expression and purification of SAK-RGD-K2-Hirul. The SAK-RGD-K2-Hirul fusion protein was produced in *P. pastoris* cells. The pPIC9K yeast protein expression system directs recombinant proteins to the medium provides rapid and high-yield production of recombinant proteins in a eukaryotic organism. Post-translational modification of the expressed proteins critical for their biological activity, is carried out by the host cells. Optimal expression of SAK-RGD-K2-Hirul protein was obtain by growing transformed cells in MGY medium (1.34% Yeast Nitrogen Base, 1% glycerol, 0.0004% biotin, 0.004% histidine) at 30°C for approx. 16–18 h (OD₆₀₀ 3–4). Supernatants were stored at –80°C. Recombinant protein was separated by affinity chromatography on immobilized human plasminogen coupled to CNBr-activated Sepharose 4B (Rodrigues *et al.*, 1999). For SAK-RGD-K2-Hirul detection, rabbit polyclonal anti-staphylokinase antibodies were used. During construction of the SAK-RGD-K2-Hirul recombinant plasmid the following plasmids were also obtained: r-SAK, SAK-RGD-K2-Hir, SAK-K2-Hir. The same expression and purification procedures were applied to encoded recombinant proteins: r-SAK, SAK-K2-Hir and SAK-RGD-K2-Hir, which were then used to check the biological activity *in vitro* of each part of the SAK-RGD-K2-Hirul protein. Protein was quantified by the BCA (Pierce) assay.

Disulfide bond reshuffling. The disulfide bond reshuffling of SAK-K2-Hir, SAK-RGD-K2-Hirul or SAK-RGD-K2-Hir was performed based on the method of Chang (1994), with modification. Recombinant protein samples were dialyzed and concentrated to a final concentration of 50–70 µM in 0.1 M NaHCO₃ buffer (pH 8.3) to minimize dimer formation. The reshuffling process proceeded at 4°C over-

night in a microcentrifuge tube by adding cysteine (Cys) and cystine (Cys-Cys) to final concentrations of 4 and 2 mM, respectively. The efficiency of the reshuffling process was detected by non-reducing SDS/PAGE and Western blotting.

Binding of SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir to fibrin or plasmin assayed with BIAcore system. Interactions between fibrin or plasmin and the studied proteins: r-SAK, SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir were investigated in the BIAcore X system (BIAcore AB, Uppsala, Sweden) and the measurements were carried out on the surface of a CM5 biosensor. Both fibrinogen and plasminogen were immobilized at the sensor surface by an amine coupling procedure according to the standard method described by the producer. The instrument was set to maintain a temperature of 37°C at the sensor surface.

Fibrinogen, used for the immobilization at a concentration of 10 µg/ml in 10 mM sodium acetate buffer, pH 5.0, was converted immediately to fibrin by enzymatic digestion with thrombin (2 U/ml, 15 min, 5 µl/min). The digestion process was clearly observed as a time-dependent decrease in resonance response. The elution of the released fibrinopeptides continued until the baseline stabilized. The studied recombinant proteins were subsequently injected at a series of concentrations: 100, 50, 25, and 12 µg/ml and contact with fibrin was permitted for 2 min. During this period the complexes were formed and the appropriate resonance response was recorded. After that, the dissociation of the formed complexes was recorded and analyzed. The surface of the sensor chip was regenerated after each experiment by the injection of 10 µl of 5 mM HCl.

Plasminogen was also used for immobilization at a concentration of 10 µg/ml in 10 mM sodium acetate buffer, pH 5.0. The immobilized hPlg was allowed to convert to plasmin under constant flow (5 µl/min) of HBS buffer (0.01 M Hepes, 0.13 M NaCl, pH 7.4). This process was observed as a time-dependent decrease in the resonance response during several tens of minutes until the baseline stabilized. Then the studied proteins were applied onto the sensor at a concentration of 10 µg/ml and allowed to interact with plasmin for 4 min to permit the formation of complexes. Cessation of injection resulted in dissociation of the formed complexes. Both association and dissociation processes, observed as changes in the resonance response, were recorded and used for further calculations. The kinetic parameters k_a and k_d and kinetic constants K_A and K_D were then calculated using the BIAcore evaluation software. The molecular masses of the recombinant proteins were taken into consideration to compare binding of the recombinant constructs at equimolar concentrations.

Activation of human plasminogen by SAK-RGD-K2-Hirul. The activity of r-SAK, SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir, revealed as conversion of hPlg to plasmin (hPlm), was measured using a coupled assay. A solution of 5×10^{-9} M hPlg in 50 mM Hepes buffer, pH 7.4, containing 0.01% Tween 80, was supplemented with 5×10^{-9} M of recombinant proteins, and then the chromogenic substrate S2251 (Chromogenix, Uppsala, Sweden) was added to a final concentration of 0.9×10^{-3} M. The release of *p*-nitroanilide was monitored continuously for 1 h at 37°C on a microplate reader (EL 340 BIO-TEK Instr.) at 405 nm according to Miele *et al.* (1999). The kinetics of plasminogen activation was determined as described by Vanwetswinkel *et al.* (2000) using various concentrations of hPlg (125×, 250×, 500×10^{-9} M). Generated plasmin was measured with 0.3 mM S2403 substrate (Chromogenix) at different time intervals (0–10 min).

Fibrinolytic, fibrinogenolytic and plasminogen activation properties of recombinant proteins. The fibrinolytic and fibrinogenolytic properties of r-SAK, SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir were determined in human plasma using fibrin labeled with (iodine-125) ^{125}I -fibrin clot according to Collen *et al.* (1996). After the addition of ^{125}I -labeled human fibrinogen (1 mg/ml) to normal human pooled plasma, it was clotted with thrombin (2 U NIH/ml) and 35 mM CaCl_2 according to Collen *et al.* (1996). The lysis of ^{125}I -fibrin-labeled plasma clots by SAK-RGD-K2-Hir was monitored by measuring changes in clot turbidity at 25°C. An equieffective concentration of SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir required to obtain a 50% clot lysis (C_{50}) within 2 h at 37°C was determined from plots of percent lysis *versus* the concentration of test compound required to obtain a 50% clot lysis.

Fibrin clot lysis analysis was performed in a microtiter plate at room temp. using a Titertek Twin-reader (Flow Laboratories) to monitor the changes in turbidity at 405 nm. Fibrin clots were obtained according to Wu *et al.* (2003). The wells were loaded with thrombin (1 NIH unit/ml), human fibrinogen (1 mg/ml) in HBST buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 0.1% Tween 80) and 10 mM CaCl_2 and the mixtures incubated for 3 h at room temp. The formed clots were washed with HBST buffer, and then layered with a solution containing 1.5×10^{-6} M hPlg and r-SAK, SAK-RGD-K2-Hir, or SAK-RGD-K2-Hirul at varying concentrations (25×, 50×, 100×10^{-3} M). After 30 min incubation the clots were washed, layered with 1.5 µM hPlg in HBS buffer (20 mM Hepes, pH 7.4, 150 mM NaCl) and the clot lysis was monitored until completion.

Clot lysis activity of recombinant proteins in a circulating plasma system *in vitro*. Thrombolytic activity of r-SAK was compared with that obtained

for SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir in the circulating plasma system containing ^{125}I -labeled thrombus, as described by Matsuo *et al.* (1990). ^{125}I -labeled fibrinogen (2×10^5 c.p.m.) was used to obtain the radioactive plasma clot. The radioactive thrombus was then transferred to the circulating plasma system. Recombinant staphylokinase, SAK-RGD-K2-Hir or SAK-RGD-K2-Hirul were injected to the system at a final concentration of 5, 1.25 or 0.625 $\mu\text{g}/\text{ml}$ and left at 37°C for 6 h. Plasma samples were taken before the injection, and at 1 h intervals during the experimental time. The degree of clot lysis was estimated from the radioactivity released and the recordings were expressed as the percentage of the original clot radioactivity. The total radioactivity recovered from the remaining plasma clot and circulating plasma was expressed as the percentage of the radioactivity in the original clot.

Antithrombin activity of SAK-RGD-K2-Hirul. The antithrombin activity of SAK-RGD-K2-Hirul *versus* SAK-RGD-K2-Hir and r-Hir was determined by the degree of chromogenic substrate S-2238 cleavage inhibition (Chromogenix, Sweden) (the inhibition of the amidolytic activity of human thrombin by r-Hir). Then, 1.5×10^{-4} M r-Hir, SAK-RGD-K2-Hir or SAK-RGD-K2-Hirul was incubated with 2.1×10^{-7} M thrombin in 50 μl Tris-buffered saline, containing 0.1% BSA, pH 7.4, for 1 h at 37°C . The thrombin activity was measured by the increase of absorbance at 405 nm in a microplate reader (EL 340 BIO-TEK Instr.) during its incubation with 300 μM substrate S-2238 for 10 min at 37°C (Riesbeck *et al.*, 1998). The hirulog activity was also confirmed by the thrombin clot assay. Fifty microliters of thrombin (2×10^{-13} M) was added to 50 μl of r-Hir, SAK-RGD-K2-Hir, or SAK-RGD-K2-Hirul (0 \times , 0.15 \times , 0.3 \times , 0.6 \times and 1×10^{-12} M) in an assay buffer containing 0.12 M NaCl, 0.01 M sodium phosphate, 0.01% NaN_3 , 0.1% BSA, pH 7.4. After 5 min of mixing at room temp., 100 μl of human plasma diluted 1:10 was added and mixed for 20 s. The turbidity of the clot was monitored in a microplate reader (EL 340 BIO-TEK Instr.) at 405 nm at 0, 15, 30 and 60 min.

Quantification of clot-bound thrombin. Cross-linked fibrin clots were prepared by adding human thrombin (Sigma) at final concentrations from 0.1 to 2 NIH units/ml to 4 mg/ml human fibrinogen (Sigma) in Hepes-buffered saline (HBS, 0.02 M Hepes, 0.13 M NaCl, pH 7.4) containing 20 mM CaCl_2 at room temp. After mixing, 100 μl of the polymerizing solution was transferred to a microtiter plate (Lian *et al.*, 2003). The clots were formed at room temp. for 2 h. Two sets of fibrin clots were formed. The first set of fibrin clots was washed with 100 μl of HBS followed by careful removal of the washing buffer. This step was repeated five times until no thrombin activity in the wash buffer was detected. The second

set of clots were unwashed and used as the control to determine the ratio of thrombin incorporated into the fibrin clot. One hundred microliters of 560 μM thrombin-specific chromogenic substrate (*N*-(*p*-tosyl)-Gly-Pro-Arg-*p*-nitroanilide, Sigma) was then added to each well and the color development was monitored over a 10-min period at 37°C in the kinetic mode at 405 nm using a plate reader (EL 340 BIO-TEK Instr.). Thrombin activity was expressed as the initial rate of color development (mA/min).

Thrombin inhibition assays. These assays were performed under two conditions with hirudin and its derivatives (SAK-RGD-K2-Hir and SAK-RGD-K2-Hirul) as inhibitors. The first series was performed with thrombin freely in solution, while the second series was determined with clot-bound thrombin. For the first variant, increasing amounts of hirudin or its derivatives (final concentrations from 0 to 30 nM) were incubated with thrombin (final concentration: 1 NIH unit/ml) at room temp. in HBS containing 0.2 mg/ml BSA (Lian *et al.*, 2003). The reaction was started by the addition of 50 μl of 560 μM thrombin-specific chromogenic substrate to the thrombin/hirudin mixture (50 μl). Thrombin activity was determined as described above. IC_{50} values of hirudin and its derivatives were then determined. To monitor the inhibition of the clot-bound thrombin activity by hirudin and its derivatives, each washed fibrin clot was incubated with 100 μl of either HBS or inhibitors (75 nM). After incubation at room temp. for 1 h, liquid was carefully removed without disturbing the clot, and the clot was then washed with HBS to remove any unbound inhibitors. Finally, chromogenic substrate was added to each clot and the rate of color development (at 405 nm) was measured. The activity of the non-inhibited clot-bound thrombin was plotted over the thrombin concentrations used to form the clot. The amount of clot-bound thrombin inhibited by the inhibitor was determined as the differences of thrombin activities between the clots treated with the inhibitor and the clots treated with HBS.

Inhibition of platelet aggregation by SAK-RGD-K2-Hirul. Blood platelets were isolated using a modified washing procedure, a modified Mustard method (Mustard *et al.*, 19770, with modifications according to Walkowiak *et al.*, 1989). The platelet washing procedure was repeated twice the platelets were suspended (4×10^8 platelets/ml) in Hepes/Tyrode buffer (136 mM NaCl, 5.5 mM dextrose, 2 mM MgCl_2 , 0.47 mM NaHCO_3 , 2.7 mM KCl and 10 mM Hepes, pH 7.3). The platelets were counted by photometric method (Walkowiak *et al.*, 2000). The isolated platelets were incubated for 20 min at room temp. with either SAK-RGD-K2-Hirul, SAK-RGD-K2-Hir (1.5 \times , 7.5 \times , 15 \times , 30×10^{-6} M) or reference compounds at equimolar concentrations: RGD

(Sigma Bio Sciences) and SAK-K2-Hir prior to their stimulation with 1 µg/ml of collagen type I (CHO-NO-LOG, Havertown, PA, USA). The aggregation test was performed in a dual channel aggregometer (APACT Labor, Ahrensburg, Germany) at 37°C for 10 min under continuous stirring (1000 r.p.m.). The results were recorded with a dual channel plotter. Aggregation results were expressed as percent inhibition of platelet aggregation.

Statistics. Data presented in figures and tables are the means ±S.D. of duplicate determinations from 3 to 4 independent experiments. One-way analysis of variance (ANOVA) was used where applicable, and the differences with *P* values less than 0.05 were considered statistically significant. Non-parametric analysis of variance (Kruskal-Wallis test) and the all-pairwise comparisons Conover-Inman test were used as the inference tests.

RESULTS

Expression and purification of SAK-RGD-K2-Hirul

Chimeric proteins SAK-RGD-K2-Hir, r-SAK, SAK-K2-Hir and SAK-RGD-K2-Hirul were expressed in *P. pastoris* cells, purified from the medium and characterized by Western blotting using polyclonal anti-SAK antibodies (Figs. 1A and B). SAK-RGD-K2-Hir and SAK-RGD-K2-Hirul were purified on immobilized human plasminogen coupled to CNBr-activated Sepharose (Fig. 1A). SAK-RGD-K2-Hir and SAK-RGD-K2-Hirul were purified to homogeneity for the analysis of their specific biological activity and compared with r-SAK. Total protein concentration in the medium was 35 µg/ml, of which secreted SAK-RGD-K2-Hirul was 8.21 µg/ml (approx. 25% of whole amount medium proteins).

Reshuffling of disulfide bonds in SAK-RGD-K2-Hirul

P. pastoris strains has been shown to produce biologically active secretory proteins with disulfide bonds. The K2-tPA protein has two pairs of disulfide bonds, but in the case of hirudin there are three pairs of disulfide bonds and the cysteine residues involved in their formation are not arranged in a sequential manner. When purified SAK-RGD-K2-Hir and SAK-RGD-K2-Hirul proteins were analyzed by Western blotting, additional faster bands were observed under non-reducing SDS/PAGE. In contrast, after reduction, all forms of recombinant proteins migrated as a single band with the apparent molecular mass of 40 kDa for SAK-RGD-K2-Hirul and 42.5 for SAK-RGD-K2-Hir (not shown). These data

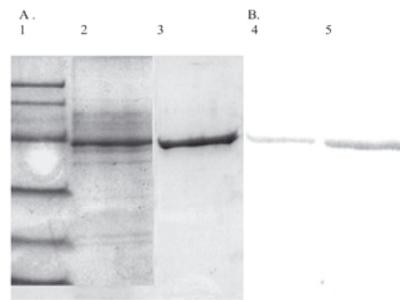


Figure 1. Purification of recombinant protein SAK-RGD-K2-Hirul expressed in *Pichia pastoris*.

A. 10% SDS/PAGE of preparation of SAK-RGD-K2-Hirul. Lane 1, protein calibration mixture from Biorad (97400, 66200, 45000, 31000, 21000, 14400, 6500); lane 2, recombinant protein SAK-RGD-K2-Hirul (approx. 41000) in growth medium; lane 3, purified preparation of the recombinant protein SAK-RGD-K2-Hirul. **B.** Western blot analysis with polyclonal anti-staphylokinase antibodies corresponding to gel in **A**.

indicated that the recombinant proteins SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir had the correct pairing of cysteine residues.

Kinetic assays on BIAcore

The ability of the recombinant proteins to bind fibrin was assayed with fibrin clots immobilized on the biosensor was performed. We observed significant differences in the binding kinetics of these proteins, i.e., an approximately three-fold stronger fibrin binding by SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir in comparison with r-SAK. The binding parameters calculated from the association and dissociation phases of the reaction curves are presented in Table 1. The lower K_D values for SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir show a higher affinity and binding strength of these proteins to fibrin as compared with those of r-SAK. It means that the higher affinity and binding strength of these proteins to fibrin must be associated with the presence of the K2 domain of t-PA. We observed no differences between the parameters obtained for SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir.

The calculated parameters of the binding and subsequent dissociation of SAK-RGD-K2-Hir, SAK-RGD-K2-Hirul and SAK to human plasmin immobilized on the biosensor are shown in Table 2. The kinetic association rate constants (k_a), the apparent affinity equilibrium constants (K_A) and appropriate dissociation parameters for the binding of the derivatives of r-SAK to plasmin are similar to those of r-SAK alone, showing their comparable affinity to plasmin. The obtained results confirmed the biological activity of staphylokinase in the SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir hybrid proteins. The

Table 1. Binding parameters of r-SAK, SAK-RGD-K2-Hir and SAK-RGD-K2-Hirul to fibrin

	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_A (M^{-1})	K_D (M)
r-SAK	$1.64 \pm 0.42 \times 10^4$	$1.59 \pm 0.05 \times 10^{-2}$	$1.07 \pm 0.29 \times 10^6$	$9.53 \pm 2.34 \times 10^{-7}$
SAK-RGD-K2-Hir	$4.72 \pm 1.31 \times 10^4$	$1.28 \pm 0.08 \times 10^{-2}$	$3.56 \pm 1.12 \times 10^6$	$2.91 \pm 1.12 \times 10^{-7}$
SAK-RGD-K2-Hirul	$4.96 \pm 0.10 \times 10^4$	$1.02 \pm 0.01 \times 10^{-2}$	$3.79 \pm 0.11 \times 10^6$	$2.62 \pm 0.07 \times 10^{-7}$

Kruskal-Wallis test and all-pairwise comparisons Conover-Inman test revealed significant differences between the parameters K_A and K_D obtained for r-SAK compared to recombinant proteins containing K2 domain (K_A : r-SAK vs. SAK-RGD-K2-Hir $P = 0.0018$, SAK-RGD-K2-Hirul $P = 0.0017$ and K_D : r-SAK vs. SAK-RGD-K2-Hir $P = 0.0036$, SAK-RGD-K2-Hirul $P = 0.0024$), whereas differences between parameters obtained for two chimeric variants were not significant.

Table 2. Binding parameters of r-SAK, SAK-RGD-K2-Hir and SAK-RGD-K2-Hirul to plasmin

	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_A (M^{-1})	K_D (M)
r-SAK	$8.58 \pm 0.27 \times 10^4$	$7.93 \pm 0.08 \times 10^{-3}$	$1.20 \pm 0.11 \times 10^7$	$10.45 \pm 0.24 \times 10^{-8}$
SAK-RGD-K2-Hir	$1.02 \pm 0.11 \times 10^5$	$7.61 \pm 0.14 \times 10^{-3}$	$1.37 \pm 0.13 \times 10^7$	$7.42 \pm 0.09 \times 10^{-8}$
SAK-RGD-K2-Hirul	$1.01 \pm 0.09 \times 10^5$	$7.65 \pm 0.08 \times 10^{-3}$	$1.34 \pm 0.23 \times 10^7$	$7.43 \pm 0.05 \times 10^{-8}$

Analysis of variance with ANOVA test revealed non-significant difference of parameters obtained for r-SAK when compared with other recombinant proteins. Differences between parameters obtained for two chimeric proteins are also not significant.

formation of complexes of SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir with plasmin proceeds similarly to that of r-SAK-plasmin suggesting that addition of K2-tPA and hirulog did not change the staphylokinase activity.

Fibrinolytic, fibrinogenolytic and plasminogen activation properties of recombinant proteins

The concentration of r-SAK, SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir causing 50% fibrinogen degradation after 2 h in normal human plasma in the absence of fibrin are shown in Table 3. The SAK-RGD-K2-Hir and SAK-RGD-K2-Hirul proteins exhibited a higher clot lysis activity as compared to r-SAK. We did not observe differences between the clot lysis activity of SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir. The presented values were determined from four independent experiments. Using fibrin clot lysis assay the introduction of the K2 domain to SAK-RGD-Hirul and SAK-RGD-K2-Hir changed the speed of fibrin degradation as compared to r-SAK. The analysis of T_{50} value (time required for 50% clot lysis) car-

ried out at 50 mM r-SAK, SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir concentrations showed a significant difference (Fig. 2). The T_{50} values for r-SAK were 121 ± 4.1 min (clot not washed, average from five experiments) and 182 ± 3.1 min (clot washed), while the T_{50} values for SAK-RGD-K2-Hirul were 94.2 ± 2.8 min (clot not washed) and 116 ± 2.6 min (clot washed) and 104 ± 4.1 min (clot not washed) and 118 ± 3.1 min (clot washed) for SAK-RGD-K2-Hir. The ratio of T_{50} for r-SAK:SAK-RGD-K2-Hirul was 1.28 (clot not washed) and 1.56 (clot washed), r-SAK:SAK-RGD-K2-Hir 1.16 (clot not washed) and 1.54 (clot washed). We observed a significant increase of thrombus lysis rate by SAK-RGD-K2-Hirul as compared with r-SAK (from 22.15% to over 36.3%) and SAK-RGD-K2-Hir (from 14% to over 35.2%). The kinetic constants: catalytic rate constant (k_{cat}) and Michaelis constant (K_m) for human plasminogen activation by the SAK-RGD-K2-Hirul-plasmin and SAK-RGD-K2-Hir-plasmin complexes were calculated from Lineweaver-Burk plots (Table 3); they were similar for SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir and comparable to those of r-SAK.

Table 3. Fibrinolytic and amidolytic properties of r-SAK, SAK-RGD-K2-Hir and SAK-RGD-K2-Hirul *in vitro*

	Fibrinolytic potency C_{50} [$\mu g/ml$]	Fibrinogenolytic potency C_{50} [$\mu g/ml$]	Plasminogen activation K_M [μM]	Plasminogen activation k_{cat} [s^{-1}]
r-SAK	0.24 ± 0.03	33 ± 3	2.11 ± 0.08	1.85 ± 0.09
SAK-RGD-K2-Hir	0.13 ± 0.02	38 ± 2	2.24 ± 0.12	1.89 ± 0.12
SAK-RGD-K2-Hirul	0.12 ± 0.01	38 ± 1	2.12 ± 0.12	1.83 ± 0.13

C_{50} indicates the concentration of variant SAK required for 50% clot lysis or 50% fibrinogen breakdown in 2 h. Analysis of variance of fibrinolytic potency with ANOVA test performed for r-SAK/SAK-RGD-K2-Hirul ($P < 0.001$) and r-SAK/SAK-RGD-K2-Hir ($P < 0.001$) showed significant difference between these groups, but differences between parameters obtained for two chimeric proteins are not significant ($P = 0.061$). Fibrinogenolytic potency and kinetic constants K_m and k_{cat} were not significantly different between compared proteins.

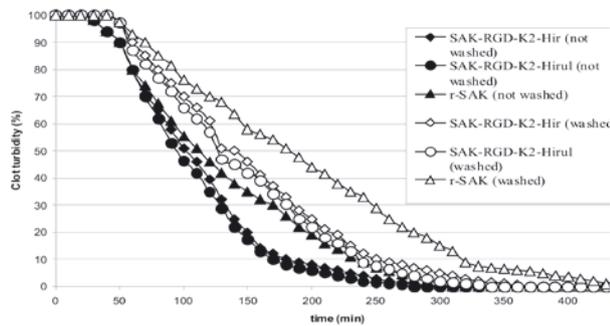


Figure 2. Turbidimetric fibrinolysis of recombinant proteins r-SAK, SAK-RGD-K2-Hir and SAK-RGD-K2-Hirul. Turbidity time course of fibrin clot lysis by r-SAK, SAK-RGD-K2-Hir and SAK-RGD-K2-Hirul at 50 mM in a solution containing 1.5 μ M human plasminogen. Clots were incubated with r-SAK, SAK-RGD-K2-HIR or SAK-RGD-K2-Hirul for the entire period for 30 min, then were washed with buffer.

Clot-lysis activity of recombinant proteins

To evaluate the thrombolytic potency of the recombinant proteins, the degree of thrombolysis induced by different amounts of recombinant staphylokinase, SAK-RGD-K2-Hirul or SAK-RGD-K2-Hir (5, 1.25 and 0.125 μ g/ml) was studied in circulating human plasma containing 127 I-labeled plasma clot (Fig. 3). Dose-dependent lysis of 127 I-labeled fibrin human clots submerged in human plasma was obtained for r-SAK, SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir. The speed of thrombolysis of radioactive thrombus by SAK-RGD-K2-Hirul or SAK-RGD-K2-Hir was double that of r-SAK. Fifty percent thrombolysis of

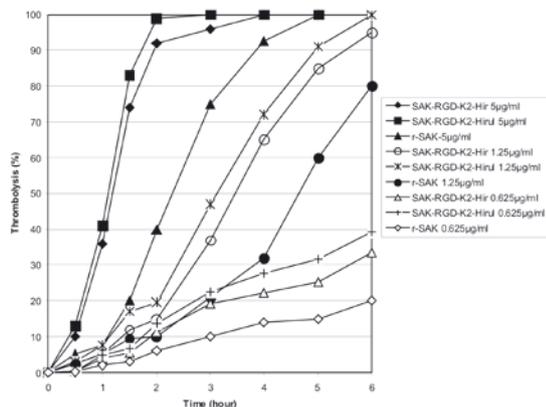


Figure 3. Clot lysis activity of recombinant proteins: SAK-RGD-K2-Hirul, SAK-RGD-K2-Hir and r-SAK.

The extent of thrombolysis of radioactive thrombus was expressed as percentage of original clot radioactivity released upon incubation with varying concentrations of recombinant staphylokinase, SAK-RGD-K2-Hirul or SAK-RGD-K2-Hir. Data represent means \pm S.D. for triplicate experiments. There are statistically significant differences in thrombolytic activity between SAK-RGD-K2-Hirul, SAK-RGD-K2-Hir and r-SAK obtained for each tested concentration of recombinant proteins ($P < 0.001$).

the original clot was obtained in 1.06 ± 0.07 h for 5 μ g/ml SAK-RGD-K2-Hirul and 1.17 ± 0.05 h for SAK-RGD-K2-Hir, while the same thrombolytic effect for 5 μ g/ml by r-SAK was observed in 2.18 ± 0.11 h which shows their higher thrombolytic potency. Thrombolysis was slightly faster for SAK-RGD-K2-Hirul than for SAK-RGD-K2-Hir with varying concentration of recombinant proteins (5 μ g/ml by 12%, 1.25 μ g/ml – 14%, and 0.125 μ g/ml – 15%).

Antithrombotic activity of SAK-RGD-K2-Hirul

To provide evidence that the recombinant SAK-RGD-K2-Hirul protein binds to thrombin, its ability to inhibit the activity of thrombin using amidolytic assay was investigated (Fig. 4). Our results show that inhibition of the thrombin catalytic activity by SAK-RGD-K2-Hirul was almost identical to that by recombinant hirudin and slightly higher (several percent) than that by SAK-RGD-K2-Hir. In the chromogenic assay 150 μ M SAK-RGD-K2-Hirul, similarly to r-Hir, was sufficient to inhibit the action of thrombin significantly, while r-SAK alone was unable to inhibit the amidolytic activity of thrombin. It could suggest that the recombinant fusion protein contains an active binding site of hirudin, thus inhibiting thrombin catalytic activity.

These results were confirmed by the fibrin clot assay, when human plasma was used as a source of fibrinogen. SAK-RGD-K2-Hirul protein at different concentrations (0.15, 0.3, 0.6 and 1 pM) similarly to r-hirudin, inhibited completely the clotting activity of thrombin. As shown in Fig. 5 the addition of thrombin increased the turbidity due to the fibrin clot formation.

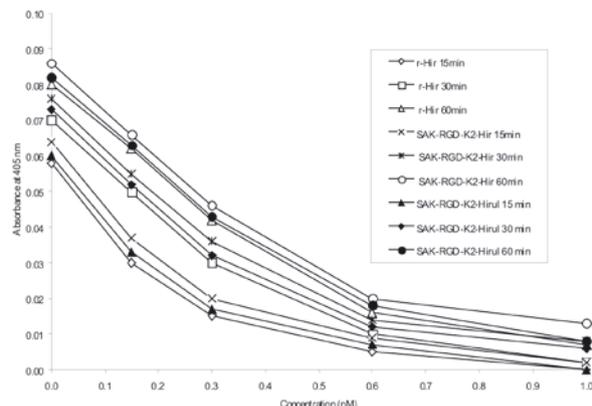


Figure 4. Inhibition of antithrombotic activity by recombinant recombinant proteins SAK-RGD-K2-Hirul, SAK-RGD-K2-Hir or r-hirudin.

Thrombin (0.2 pM) was incubated with r-Hir, SAK-RGD-K2-Hirul or SAK-RGD-K2-Hir (at 0, 0.15, 0.3, 0.6, 1 pM). Turbidity of clot was monitored by microplate reader at 0, 15, 30, 60 min. Data represent means \pm S.D. for triplicate experiments. No differences in antithrombotic activities between proteins analyzed at a given concentration ($P = 0.065$).

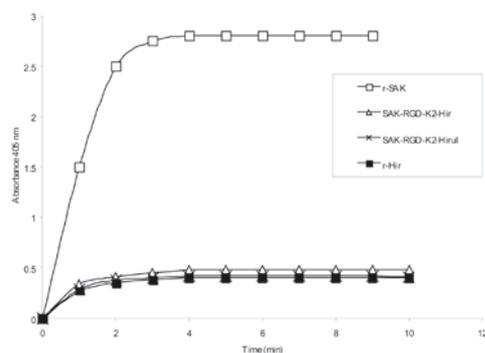


Figure 5. Antithrombotic activity of the recombinant proteins r-hirudin, r-SAK, SAK-RGD-K2-Hir and SAK-RGD-K2-Hirul expressed as inhibition of thrombin amidolytic activity.

Thrombin (210 nM) activity was determined with substrate S-2238 (300 μ M) for 10 min at 37°C in a microplate reader (EL340BIOTEK Intr.). Recombinant proteins were at 150 μ M. Data represent means \pm S.D. for triplicate experiments. There are no significant statistic differences in antithrombotic activity between SAK-RGD-K2-Hir, SAK-RGD-K2-Hirul and r-Hir obtained for each concentration of recombinant proteins ($P=0.062$).

Quantification of clot-bound thrombin and thrombin inhibition assays

Thrombin binds to fibrin directly through an interaction at its anion-binding exosite. Washed fibrin clots formed in the presence of different amounts of thrombin were used in this study. The concentration of hirudin, SAK-RGD-K2-Hir or SAK-RGD-K2-Hirul of 75 nM was selected because the clinical doses of SAK used in thrombolysis are usually in the range of 64–192 nM in circulation (Collen & van de Vert, 1993). After several washes to remove any unbound hirudin, SAK-RGD-K2-Hir or SAK-RGD-K2-Hirul, thrombin activity within these fibrin clots was determined (Fig. 6A). When the clots were formed with low doses of thrombin (up to 1.5 NIH units/ml), SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir were equally effective. For fibrin clots formed in the presence of higher levels of thrombin (1.5–3 NIH units/ml) SAK-RGD-K2-Hirul was by around 5% more effective than SAK-RGD-K2-Hir in inhibition of clot-bound thrombin. This is so probably because of the smaller size of hirulog *versus* hirudin so that it can diffuse more easily into the interior of the clot. The differences of thrombin activities between the clots treated with hirudin, SAK-RGD-K2-Hirul, SAK-RGD-K2-Hir and HBS (control) showed a thrombin dose-dependent inhibition mediated slightly better (5%) by SAK-RGD-K2-Hirul compared with SAK-RGD-K2-Hir (Fig. 6B).

Antiplatelet activity of SAK-RGD-K2-Hirul

In the next set of experiments we investigated the interaction of recombinant staphyloki-

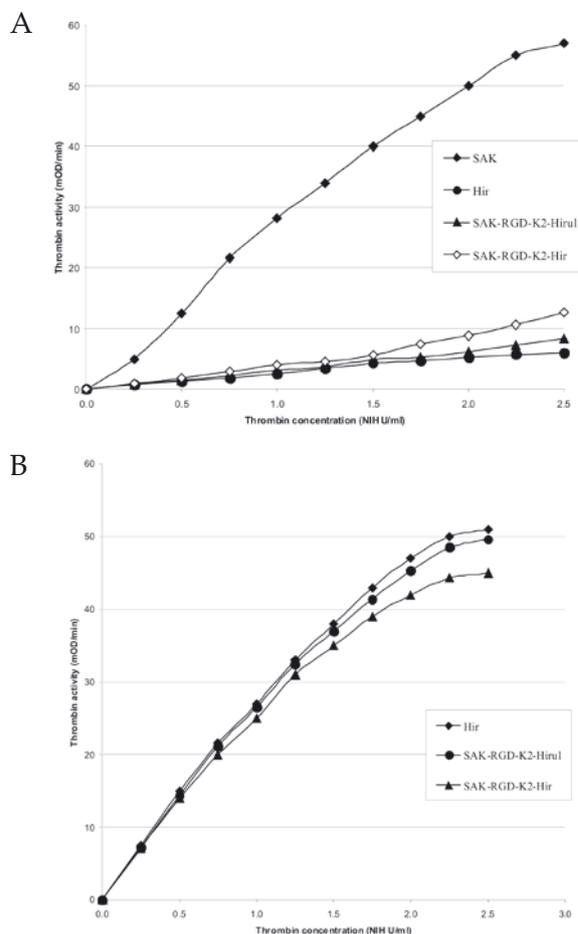


Figure 6. Inhibition of clot-bound thrombin activity by rSAK, Hir, SAK-RGD-K2-Hir and SAK-RGD-K2-Hirul.

A. Clot-bound thrombin activity from washed clots was determined in the presence of hirudin, r-staphylokinase, SAK-RGD-K2-Hir and SAK-RGD-K2-Hirul. **B.** The amount of clot-bound thrombin activity inhibited was determined as the differences of thrombin activities between the clots treated with the hirudin, SAK-RGD-K2-Hir or SAK-RGD-K2-Hirul and the clots treated with HBS.

nase, SAK-RGD-K2-Hirul, SAK-RGD-K2-Hir, SAK-RGD-K2-Hir and RGD-peptide with human platelets *in vitro*. A low inhibitory effect of r-SAK-K2-Hir on the aggregation of washed platelets was obtained as compared with that of SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir (Fig. 7). A significant inhibition of platelet aggregation was obtained at 1.5–30 nM of SAK-RGD-K2-Hirul. The platelet disaggregation was comparable with RGD-peptide alone (Fig. 7). A similar inhibitory effect on platelet aggregation was detected at 1.5–7.5 nM of SAK-RGD-K2-Hirul, SAK-RGD-K2-Hir and RGD sequence, whereas at concentrations of 15–30 nM a diminished inhibitory effect was observed by, respectively 19%, 38.3% for SAK-RGD-K2-Hir, 26%, 44.4% for SAK-RGD-K2-Hirul and 32%, 51% for RGD. Recombinant SAK-K2-Hir, SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir did not affect the ADP and collagen-induced aggregation (not shown).

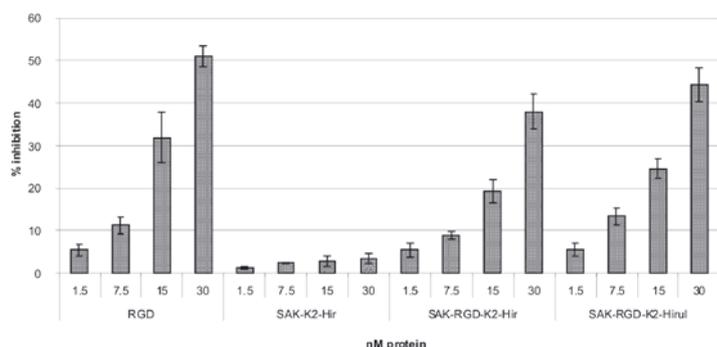


Figure 7. Comparison of percentage inhibition of platelet aggregability assessed in the aggregation test.

Aggregation of washed human platelets was induced by thrombin (0.1 U/ml). The means \pm S.E.M. came from the three separate experiments.

DISCUSSION

A frequent problem in thrombolytic therapy is reocclusion. The fibrinolytic system works to remove fibrin deposits from the vascular wall, but in most occluded vessels, the vascular wall is impaired because of endothelial injury as well as plaque formation. After the removal of fibrin, such vascular impairment again results in activation of coagulation as well as platelets, and the damaged area becomes covered with platelet aggregates and a fibrin network. As we have no agent that can promote regeneration of the damaged vascular wall, reocclusion is a serious problem in thrombolytic therapy. To avoid reocclusion, an anticoagulant, such as heparin or low-molecular-weight heparin has been used as adjunctive therapy (Arjomand *et al.*, 2004) in some clinical trials. In other cases, an antiplatelet, such as aspirin, or a GPIIb/IIIa blocker, are used.

The ideal thrombolytic agent should, therefore, possess high affinity for fibrin and express high plasminogen activator activity on the fibrin (thrombus) surface, as well as antiplatelet and anticoagulant activity at the damaged vascular surface. After administration into the circulation, it should be delivered specifically to the thrombus (*via* its high affinity for fibrin) and generate plasmin on the thrombus. The plasmin digests fibrin and restores blood flow. This ideal thrombolytic agent then acts to block platelet activation and coagulation at the newly exposed vascular surface, thereby preventing reocclusion. In our previous *in vitro* study (Szemraj *et al.*, 2005) we described the biological activity of a new compound with four domains: (i) a plasminogen activator domain derived from SAK, (ii) a fibrin affinity domain containing a K2 motif derived from t-PA, (iii) an antiplatelet domain with Arg-Gly-Asp (RGD) sequences, (iv) an anticoagulant domain derived from hirudin. That newly developed compound apparently possessed all the required features of an ideal thrombolytic agent, and indeed exhibited rapid and potent clot lysis as well as antiplatelet and anticoagulant activities. Interestingly, the anti-

platelet activity of the compound was comparable to that of the RGD sequences alone, which may indicate that the RGD sequences contained between the SAK and K2 moieties in the new agent lie on the surface of the molecule.

In our *in vivo* study (Szemraj *et al.*, 2007) we tried to document the thrombolytic activity of SAK-RGD-K2-Hir after bolus administration in arterial and venous thrombosis models in rats. In order to see how the thrombolytic effects of SAK-RGD-K2-Hir depend on its molecule structure, the recombinant proteins SAK-K2-Hir and SAK-RGD-K2 were studied. Although SAK-RGD-K2-Hir caused arterial thrombus lysis, there was no significant reduction of thrombus mass in comparison with r-SAK-treated group. The main end points of arterial thrombolysis consisted of the frequency and rate of arterial recanalization and the persistence of patency. We observed that arterial patency after SAK-RGD-K2-Hir was not significantly greater in comparison to that after r-SAK or t-PA. The time to reperfusion after SAK-RGD-K2-Hir injection was also very similar in comparison to r-SAK-treated group. In the venous thrombosis model, however, SAK-RGD-K2-Hir was significantly more effective in lysis of thrombus than r-SAK.

The differences in the thrombolytic potency of SAK-RGD-K2-Hir in the two models may be related to the different composition of arterial and venous clot. Whereas the venous thrombus is mainly composed of a fibrin mesh enriched in red blood cells, in the arterial thrombus a high amount of structural blood elements dominates over the rather small amount of fibrin, which is the main target of the investigated recombinant proteins. The presence of dominating morphotic blood elements participating in arterial thrombus may constitute a peculiar spheric barrier for thrombolytic enzymes and underlie the limited accessibility of the protein to fibrin.

Our *in vitro* studies showed that the potency of thrombin inhibition by the hirudin part of the recombinant SAK-RGD-K2-Hir protein was the same as that of r-Hir alone (Szemraj *et al.*, 2005). In this *in vivo* study we demonstrate that

SAK-RGD-K2-Hir is more effective in the venous-type thrombolysis model. Other researchers also suggested that the antithrombotic effect of direct thrombin inhibitors is more pronounced in the venous study (Bakker *et al.*, 1993). The thrombin inhibitory potential of the recombinant protein was revealed by findings of a dose-dependent prolongation of APTT and TT. This strongly suggested that the antithrombin activity depends on the r-Hir part of the SAK-RGD-K2-Hir molecule in our *in vivo* conditions. It was also shown that anticoagulants markedly inhibit platelet deposition in the venous-type thrombolysis model but they have little effect on platelet deposition onto collagen surface (Bakker *et al.*, 1993).

In the present study, we report the construction of a new recombinant protein SAK-RGD-K2-Hirul, a more potent antithrombin agent consisting of four functional modules: SAK, RGD, K2-tPA and Hirulog. Staphylokinase mutant (K35A, E65Q, K74R, D82A, K130T, K135R) (Collen *et al.*, 1997; Szemraj *et al.*, 2005), the first element of the chimera reveals properties similar to staphylokinase alone. The results obtained from three independent analyses of the interaction of staphylokinase and staphylokinase as a part of hybrid proteins with plasmin are similar to the results obtained by Okada *et al.* (2000). Those results demonstrated that the addition of RGD-K2-Hirul and RGD-K2-Hir to staphylokinase did not lead to an increase in K_m for the substrate plasminogen and that the SAK-plasmin enzyme complexes display an amidolytic activity comparable to that of the SAK-plasmin complex. The K2 domain of t-PA is involved in fibrin-dependent plasminogen activation, lysine binding and fibrin binding (Bakker *et al.*, 1993; Horrevoets *et al.*, 1994). The addition of the K2 domain resulted in a significant change of the biological properties of the protein. Our results indicate a higher binding affinity of SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir for intact fibrin. We observed an approximately three times stronger fibrin binding to SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir in comparison with r-SAK. A decreased K_D value for SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir shows a higher affinity and binding strength of these proteins to fibrin as compared with r-SAK. Introduction of K2-tPA to r-SAK increased the thrombolytic and fibrinolytic efficiency of staphylokinase. Thrombolysis of radioactive thrombus by the recombinant proteins SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir was by 51% (SAK-RGD-K2-Hirul) or 46.3% (SAK-RGD-K2-Hir) faster compared to that by r-SAK. Lower concentrations of SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir were required to obtain a 50% clot lysis in 2 h *versus* r-SAK, which documents their higher potential in *in vitro* clot lysis. Analysis of the T_{50} value (time nec-

essary for 50% clot lysis) carried out at 50 mM of r-SAK and SAK-RGD-K2-Hirul revealed a significantly higher clot lysis rate for SAK-RGD-K2-Hirul protein as compared to that for r-SAK (by 22.15% to 36.5% for SAK-RGD-K2-Hirul, 14% to 35.2% for SAK-RGD-K2-Hir). The effect of contraction on clot lysis time by SAK-RGD-K2-Hirul was similar to that obtained by Wu *et al.* (2003) for a chimera containing the K1 domain of plasminogen. The addition of the K2-tPA domain significantly decreased the concentration of SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir necessary to reach the C_{50} value compared with r-SAK. These results showed that recombinant proteins SAK-RGD-K2-Hirul and SAK-RGD-K2-Hir had similar biological activities as r-SAK, but their fibrin specificity was significantly increased.

The addition of the RGD sequence to recombinant staphylokinase resulted in the protein acquiring the ability to prevent platelet aggregation, the main component of the forming clot. We observed an effective block of platelet-platelet interaction comparable with that of the RGD sequence alone, while staphylokinase alone failed to inhibit platelet aggregation. It may result from chance plasmin formation (lack of α_2 -antiplasmin) causing degradation of fibrinogen and other proteins involved in the aggregation process. This phenomenon was not observed in platelet-rich plasma, confirming the results presented by others (Horrevoets *et al.*, 1994). We introduced three RGD sequences (two in the 20-amino-acid linker peptide and another in the five amino-acids linker RGRGD separating SAK-RGD-K2 and hirulog) in the recombinant SAK-RGD-K2-Hirul protein. The efficiency of blocking the platelet-platelet interaction by SAK-RGD-K2-Hirul was around 7% better than that of SAK-RGD-K2-Hir and comparable to that of the RGD sequence alone. These results showed that the recombinant protein SAK-RGD-K2-Hirul containing the RGD sequences possesses the ability to block platelet aggregation and hence should be more effective in clot lysis than SAK-K2-Hir.

Hirudin, which is the most potent and specific thrombin inhibitor, blocks the thrombin-dependent platelet activation without influencing the aggregation effect brought about by various factors. The weaker thrombolytic potency in the arterial thrombosis model was the result of hirudin (r-Hir) presence in the molecule of SAK-RGD-K2-Hir (Szemraj *et al.*, 2007). We have constructed a new SAK variant — SAK-RGD-K2-Hirul with hirulog instead hirudin. The addition of the smaller-size hirulog instead of hirudin as a part of the recombinant protein resulted in a strong interaction with thrombin. We obtained similar K_D values of the order of picomoles, similarly as for hirudin alone

(not shown). Our results show that inhibition of the thrombin catalytic activity by SAK-RGD-K2-Hirul was almost identical to that by recombinant hirudin and by several percent higher than by SAK-RGD-K2-Hir. Also for fibrin clots formed in the presence of higher levels of thrombin SAK-RGD-K2-Hirul was by approx. 5% more effective than SAK-RGD-K2-Hir in inhibition of clot-bound thrombin.

In conclusion, our data indicate that recombinant protein SAK-RGD-K2-Hirul is a more potent and faster-acting clot-dissolving agent with better antithrombin and antiplatelet properties compared to r-SAK and SAK-RGD-K2-Hir. The animal model of venous and arterial thrombosis was used to verify the biological activity of recombinant protein SAK-RGD-K2-Hirul *in vivo* and has demonstrated the efficacy of RGD-SAK in the lysis of platelet-rich coronary blood clots and in prevention of reocclusion after thrombolysis. The ongoing studies on SAK-RGD-K2-Hirul fusion protein will provide further details about their therapeutic potential from animal models of thrombosis *in vivo*.

Acknowledgements

This work was supported by a research project of the Foundation for the Development of Polish Pharmacy and Medicine – Polpharma.

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