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Original Article

Effect of site-specific PEGylation on the fibrinolytic activity, immunogenicity, and pharmacokinetics of staphylokinase

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The bacterial plasminogen-activator staphylokinase (Sak) is a promising thrombolytic agent for treating acute myocardial infarction. To effectively reduce the immunogenicity of Sak while maintaining its fibrinolytic activity, site-specific PEGylation was performed in the present study. The chemoselective cysteine PEGylation site was selected within an immunodominant region (amino acid residues 71–87) using an *in silico* approach. The PEGylated Sak variants prepared in this study showed a purity of >97.0%. PEGylation at Position 80 resulted in a Sak variant Sak(E80C-PEG) which has similar fibrinolytic activity and thermostability compared with the native recombinant staphylokinase (r-Sak). The immunogenicity of Sak(E80C-PEG) in guinea pigs was greatly reduced compared with the native r-Sak. Furthermore, preliminary pharmacokinetic results suggested that the plasma clearance of Sak(E80C-PEG) from the blood stream of rabbit was significantly decreased compared with that of r-Sak, resulting in a 2.8-fold increase of initial half-life and a 3.8-fold increase of systemic availability. In summary, these results demonstrated that site-specific PEGylation yielded a novel Sak variant Sak(E80C-PEG) with remarkable advantages over the unmodified Sak.

Keywords staphylokinase; site-specific pEGylation; immunogenicity; pharmacokinetic; fibrinolytic activity

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Introduction

Plasminogen-activator staphylokinase (Sak), a profibrinolytic bacterial protein, is a promising thrombolytic agent for the treatment of acute myocardial infarction. A pilot randomized trial has shown that recombinant staphylokinase (r-Sak) is at least equipotent for coronary artery re-canalization compared with a recombinant tissue-type plasminogen activator and is significantly more fibrin-selective than the recombinant tissue-type plasminogen activator [1]. However, the administration of this heterologous protein induces an immunological reaction in a majority of patients or even induces acute allergic reactions in some patients [2]. Thus, the reduction of immunogenicity of a Sak molecule is essential to be used as an effective clinical therapy. Furthermore, Sak has a relatively short plasma half-life. It must be administered by continuous infusion.

From the clinical point of view, Sak should be improved by reducing its immunogenicity and/or by prolonging its circulation time. PEGylation is a successful method to broaden the therapeutic uses of injected protein and peptide drugs. After conjugated with a hydrophilic polyethylene glycol (PEG) polymer, PEGylated protein often possesses a significantly increased hydrodynamic volume, which could prevent the rapid renal clearance and lead to an increased circulation time [3,4]. Furthermore, the PEG polymer can mask antigenic epitopes and/or almost affect each step of the antigen-processing pathway and result in the reduction of protein immunogenicity. To date, more than 10 PEGylated protein drugs have been approved for clinical use by US Food and Drug Administration, including PEG-adenosine deaminase, PEG-asparaginase, PEG-interferon alfa-2a, PEG-interferon alfa-2b, Pegfilgrastim, and mPEG-epoetin beta [3,4].

Over the last decade, efforts have been made to investigate PEGylation of r-Sak, but limited success has been achieved. Vanwetswinkel *et al.* [5] have selected six positions as chemoselective cysteine PEGylation site, which contained four positions (Lys96, Lys102, Lys109, and Lys135) in the core structure of Sak and two positions (Ser2 and Ser3) in the NH_2 terminal region. The results of pharmacokinetic evaluation have shown that all of the PEGylated Sak variants have a significant increase in serum half-life. However, the specific activities of the PEGylated Sak variants at Positions 96, 102, 109, and 135 have decreased by 54%, 75%, 66%, and 15%

compared with that of r-Sak, respectively. Moreover, the PEGylation at all of six selected positions have not significantly reduced the immunogenicity of the Sak analogs. Then, they further constructed PEGylated Sak variant SY161-P5, SakSTAR (S3C-P5, K35A, E65Q, K74R, E80A, D82A, T90A, E99D, T101S, E108A, K109A, K130T, K135R), which contains 12 amino acid substitutions to reduce its antigenicity and a single site-specific PEG modification at the N-terminus to improve its pharmcokinetics [6]. As SY161 possesses significantly prolonged plasma half-life, it could be administered with an intravenous bolus injection in stead of continuous infusion. The CAPTORS II clinical trial has indicated that SY161 exhibits the thrombolytic effect on patients with acute myocardial infarction, which is similar to that of conventional tissue-type plasminogen activator (rt-PA) [7]. However, the relatively high neutralizing antibody titers are still induced by a low dose of SY161, when compared with the titers elicited by a high dose of r-Sak (SakSTAR). Moreover, too many charged residues are replaced by non-charged amino acids in SY161. These substitutions obviously result in the significant reduction of the protein thermal stability [8], which often leads to the increase of protein immunogenicity. Xue *et al*. [9] modified r-Sak with 20-kDa methoxyl PEG amine at the C-terminus of Sak. A tripeptide Gly-Gly-Cys was covalently linked to r-Sak at the C-terminus and then the site-specific PEGylation was conducted at the engineered cysteine residue. The results showed that the immunogenicities of the PEGylated r-Sak variants were apparently decreased. However, PEGylation significantly decreases fibrinolytic activity of the PEGylated r-Sak variants by 38%–62% compared with that of r-Sak. Wang *et al*. performed N-terminal PEGylation of r-Sak using methoxyl PEG propionaldehyde with a molecular weight (MW) of 20 kDa [10]. Due to the presence of the competitive PEGylation between the N-terminus and the Lys residues, the obtained mono-PEGylated Sak might be the complex of the mono-PEGylated Sak modified at the N-terminus or at one of the several lysine residues of Sak. The mono-PEG-Sak retained ~38% of the biological activity when compared with r-Sak. Recently, four PEGylated Saks have been prepared by site-specific conjugation of 5/20-kDa PEG to N-terminus and C-terminus of Sak, respectively [11]. The result of docking analysis indicated that the binding affinity of the PEGylated Sak to micro-plasminogen is dependent on the length of PEG chain and the PEGylation site. The bioactivity analysis showed that the PEGylation significantly decreased the fibrinolytic activity of PEGylated r-Sak variants by 40%–70% compared with that of native r-Sak, respectively. Therefore, reducing the immunogenicity of r-Sak by PEGylation and simultaneously maintaining its fibrinolytic activity was still a pending problem up to now.

To overcome these problems, we performed several site-specific PEGylation at the new positions in this study. The chemoselective cysteine PEGylation site was selected in the immunodominant sequence 71–87 amino acid (aa), which not only contains two T-cell epitopes but also relates to two B-cell epitopes [12,13]. The site-specific PEGylation was performed by conjugation of hydroxy-PEG maleimide (HO-PEG-Mal) to the single engineered cysteine. *In vitro* assays were performed to determine the effect of PEGylation on thermostability, fibrinolytic activity, and catalytic efficiency of the PEGylated Sak variants. The effects of PEGylation on immunogenicity and pharmacokinetic properties of Sak variants were also investigated.

Materials and Methods

Materials

The expression plasmid pBV220-Sak and *Escherichia coli* strain DH5α was conserved in our laboratory [14]. Restriction endonucleases *Eco*RI and *Bam*HI were from TaKaRa Biotechnology Companies (Dalian, China). SP (sulfopropyl)-Sepharose FF, Sephadex G-50, and Q-Sepharose FF were purchased from Amersham Pharmacia (Cambridge, England). Toyopearl HW-50F was purchased from Tosoh (Tokyo, Japan). Hydroxyl-PEG maleimide was purchased from JENKEM Technology (Beijing, China). The Bio-Rad protein assay was obtained from Bio-Rad Laboratories (Hercules, USA). All other chemicals were of analytical grade.

Calculation of solvent accessible surface area (SASA) of amino acid residue within r-Sak and its variants

The SASA values of amino acid residues within r-Sak and its variants were calculated using the Parameter Optimized Surfaces (POPS) server (http://mathbio.nimr.mrc.ac.uk/wiki/POPS) [15]. The three-dimensional structure of r-Sak in monomeric state (Protein Data Bank ID 1ssn) was used to calculate the SASA values [16]. The probe radius was set at 1.4 Å. The models of Sak variants were generated by automatic modeling using the SWISS-MODEL server (http://swissmodel.expasy.org./) [17] and the structure of r-Sak in solution (Protein Data Bank ID 1ssn) was selected as the template for structural modeling. The best model was selected based on the QMEAN Z-score provided by the SWISS-MODEL server.

Construction of expression plasmids

The expression plasmids of the Sak variants including Sak(K74C), [i.e., Lys(K) in position 74 substituted with Cys(C)], Sak(R77C), and Sak(E80C) were constructed via a modified QuikChange site-directed mutagenesis method as previously described [14]. The primer sequences used in construction of Sak variants are listed in **Table 1**. Mutations were confirmed by gene sequencing.

Table 1. Sequence of primers used for Sak variant construction

Name	Mutation primer[a] (5′→3′)
Sak(K74C)	GCGACAGCATATTGTGAGTTTAGAGTAGTTG
	CAACTACTCTAAACTCACAATATGCTGTCGC
Sak(R77C)	GCATATAAAGAGTTTTGTGTAGTTGAATTAGATC
	GATCTAATTCAACTACACAAAACTCTTTATATGC
Sak(E80C)	GAGTTTAGAGTAGTTTGTTTAGATCCAAGCG
	CGCTTGGATCTAAACAAACTACTCTAAACTC

[a]The mutated sites are indicated with underline.

Expression and purification of r-Sak and its variants

The r-Sak and its variants were expressed in transformed *E. coli* DH5α, as described previously [14]. Recombinant proteins were purified employing a three-step chromatographic purification process [14]. In brief, the cell pellet was suspended in lysis buffer (20 mmol/l NaAc-HAc, 10 mmol/l EDTA, pH 5.6) and disrupted by sonication at 4°C. The homogenate was centrifuged at 8000 *g* at 4°C. The supernatant was applied to a SP Sepharose FF column (3.6 cm × 25 cm) pre-equilibrated with equilibration buffer (20 mmol/l sodium acetate buffer, pH 5.6), then eluted with 0.5 mol/l sodium chloride in equilibration buffer. The fractions containing r-Sak or its variant were pooled and applied to a Sephadex G-50 column (2.6 cm × 100 cm) pre-equilibrated with 20 mmol/l Tris–HCl buffer (pH 8.0). The active fraction obtained from the Sephadex G-50 column was subsequently applied to a Q Sepharose FF column (2.6 cm × 25 cm) pre-equilibrated with 20 mmol/l Tris–HCl buffer (pH 8.0) and eluted with 0.3 mol/l sodium chloride in 20 mmol/l Tris–HCl buffer (pH 8.0). The elution peaks were detected at 280 nm.

PEGylation of Sak variants

Prior to the conjugation reaction, the Sak variant was treated with 20-fold molar excess of dithiothreitol (DTT) for 90 min at room temperature. After removal of DTT with a Sephadex G-10 desalting column (3.6 cm × 30 cm), the reduced Sak variant in 20 mM NaAC-HAC (pH5.6) was mixed with a 5-fold molar excess of 5 kDa of linear hydroxy-PEG maleimide (HO-PEG-Mal). The reaction mixture was stirred for 60 min at room temperature, and purified with a SP Sepharose FF column (2.6 cm × 20 cm) eluted with 20 mmol/l Tris–HCl (pH 5.6) and a linear gradient of 0–500 mmol/l NaCl. The collected protein was further applied to a Toyopearl HW-50F size-exclusion column (2.6 cm × 100 cm) pre-equilibrated with 20 mmol/l Tris–HCl buffer (pH 7.6) and eluted with Tris–HCl buffer. The fractions corresponding to the PEGylated Sak variant were collected for further characterization.

The purity of Sak variant and its PEG conjugate was examined by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) as previously described [14]. The protein purity was also detected by size-exclusion high-performance liquid chromatography (SEC-HPLC). Briefly, the purified protein was assayed by applying the samples to a Thermo Scientific BioBasic SEC-300 size-exclusion column (7.8 mm diameter × 150 mm height) using a Shimadzu LC-10Avp HPLC instrument (Shimadzu, Kyoto, Japan). A solution of 50 mmol/l phosphate buffer with 100 mmol/l sodium chloride (pH 7.4) was used as the mobile phase running at a flow rate of 0.5 ml/min. Absorbance was recorded at 280 nm. Protein concentrations were measured by the Bio-Rad protein assay kit using BSA as a standard.

The fibrinolytic activities of r-Sak and its variants were measured by radial caseinolytic assay as described elsewhere [14]. A qualitative limulus lysate assay kit (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China) was used to determine the levels of endotoxin in r-Sak and its PEGylated variants. The sensitivity of the test reagent used is 0.25 EU/ml.

The kinetic constants of human plasminogen activation by the complexes of Sak variants with plasmin

The kinetic constants of human plasminogen activation by complexes of Sak variants with plasmin were measured with chromogenic substrate S-2390 (H-D-Val-Phe-Lys-*p*-nitroanilide), as described previously [18]. Briefly, equimolar mixtures of Sak variants and plasminogen were preincubated for 30 min in phosphate buffer (50 mmol/l, pH 7.4). The conversion of plasminogen to plasmin was completed for all mixtures during the preincubation. These complexes (final concentration 10–40 nmol/l) were then mixed with plasminogen (final concentration 0.25–10 μmol/l). And the plasmin generated at 37°C was determined from the change in the absorbance of S-2390 at 405 nm (ΔA405). The kinetic constants of the plasminogen activation by complexes of Sak variants with plasmin were calculated from the Lineweaver–Burk plots.

Thermostability of Sak(E80C-PEG)

The purified r-Sak and Sak(E80C-PEG) were diluted to a concentration of 1 mg/ml in 0.15 mol/l sodium chloride,

0.05 mol/l phosphate buffer (pH7.4) and incubated at 37°C and 56°C in a polypropylene tube. At various time points, aliquots were removed and the residual activity of r-Sak and Sak(E80C-PEG) was determined by the fibrinolytic-activity assay as described previously [14].

Immunization and enzyme-linked immunosorbent assay for the detection of anti-Sak IgG

Immunogenicity of r-Sak and Sak(E80C-PEG) was evaluated in guinea pigs (6–8 weeks old, ~200 g) purchased from the Experimental Animal Center of Hebei Province (Shijiazhuang, China). The animals were housed under conventional laboratory conditions and were fed with commercial food and water *ad libitum*. All animal studies were performed at Hebei Normal University in accordance with Institutional Animal Care and Use Committee guidelines. Sixteen guinea pigs were randomized into two groups, each consisting of eight guinea pigs. The guinea pigs were immunized by subcutaneous injection of 5000 IU/kg r-Sak or Sak(E80C-PEG) on days 1, 3, and 5, respectively. Blood samples were collected on days 0, 7, 14, 21, 28, and 35 after final immunization and stored at −20°C for the subsequent assay. Guinea pig antibodies were detected by an enzyme-linked immunosorbent assay (ELISA) method. Briefly, microtitre plates were coated with 1 μg/well r-Sak or Sak(E80C-PEG) and rinsed with phosphate buffered saline (PBS, pH 7.4). One hundred microliter serially diluted antiserum was added to the wells and blocked with 1% BSA and incubated for 1 h at 37°C. The secondary antibodies, goat anti-guinea pig IgG-horseradish peroxidase (HRP) (Santa Cruz, Santa Cruz, USA), were diluted at 1 : 4000, and 100 μl was added to each well. 3, 3, 5, 5-Tetramethylbenzidine (TMB) was used as a chromogenic substrate and the absorbance at 450 nm was measured using a microplate reader (Bio-Rad 550, Bio-Rad). The washing buffers did not contain detergents such as Tween 20 or Tween 80. The relative concentration of antibody in serum was expressed as antibody titer. The sample was considered as positive if its absorbance is twice as much as the absorbance of the control well (PBS only).

Pharmacokinetic analysis

The pharmacokinetics of r-Sak and Sak(E80C-PEG) were evaluated in New Zealand rabbits with a mean body weight of 2.0 ± 0.2 kg purchased from the Experimental Animal Center of Hebei Province in China. Rabbits (six per group) were injected intravenously with 50 000 IU/kg of r-Sak or Sak(E80C-PEG) solution through the marginal ear vein. After injection, 0.4 ml of blood samples was withdrawn through a femoral artery catheter at 1, 2.5, 5, 10, 20, 30, 45, 60, 90 min after injection. The obtained blood samples were centrifuged at 1400 *g* for 15 min. About 0.2 ml of plasma samples were obtained and stored at −40°C until analysis.

Concentrations of r-Sak and Sak(E80C-PEG) in plasma were measured by a sandwich ELISA method as described below. The pharmacokinetic analysis of the data was performed using the Practical Pharmacokinetic Program 3P87 specially developed by the Chinese Pharmacological Society. The pharmacokinetic data were fitted to a two-compartment model. The goodness of fit of the models was assessed by the Akaike Information Criterion value. The following pharmacokinetic parameters were determined: initial half-life ($t_{1/2}\alpha$), terminal half-life ($t_{1/2}\beta$), plasma clearances (Clp), and area under the curve (AUC).

The sandwich ELISA assay for r-Sak and its PEGylated variant in plasma

The content of r-Sak and Sak(E80C-PEG) in plasma was determined using a sandwich ELISA method as described by Collen *et al*. [13]. Briefly, the anti-Sak IgG fraction of polyclonal antiserum was obtained by immunizing rabbits with r-Sak. Polystyrene microtitre plates were coated with 2 μg/well IgG fraction for 24 h at 4°C in a humid box and blocked with 1% BSA at 37°C for 2 h. One hundred microliters of diluted samples were added to the wells and incubated at 37°C for 1 h. The anti-Sak IgG-HRP conjugate was prepared as described by Nakane and Kawaoi [19]. One hundred microliters of the anti-Sak IgG-HRP conjugate, diluted to 0.4 μg/ml in PBS (pH7.4) was applied to the wells and incubated at 37°C for 2 h. Then the bound enzyme conjugate was detected with TMB (substrate). The washing buffers were PBS and did not contain detergents such as Tween 20 or Tween 80.

Statistical analysis

The differences between two groups were evaluated statistically using analysis of variance followed by the Newman–Keuls test. $P < 0.05$ was considered to be statistically significant. All analysis of the data was performed with a Statistica statistical software package (version 6.0, StatSoft, Inc., Tulsa, USA).

Results

Selection of a position for site-specific PEGylation using an *in silico* protocol

The hydrophilic amino acids within the immunodominant region at amino acid positions 71–87 contained Lys74, Glu75, Arg77, Glu80, and Asp82 residues. To select a suitable position for site-specific PEGylation, the SASA values of these residues and the replaced cysteine residues within r-Sak were calculated. And the results are summarized in **Table 2**. It was obvious that the Glu75 and Asp82 residues were covered within Sak molecule (SASA < 80 Å^2). The SASA of the replaced cysteine residue at positions 75 and 82 were also relatively low (SASA < 30 Å^2). Therefore, the

Table 2. The solvent accessible surface area (SASA) of amino acid residue within the immunodominant sequence 71–87 of r-Sak and its variants

Residues	Residue SASA in monomer (Å^2)
K74 in r-Sak	123.26
C74 in Sak(K74C)	48.92
E75 in r-Sak	60.55
C75 in Sak(E75C)	23.64
R77 in r-Sak	163.10
C77 in Sak(R77C)	39.72
E80 in r-Sak	127.19
C80 in Sak(E80C)	58.16
D82 in r-Sak	57.98
C82 in Sak(D82C)	28.71

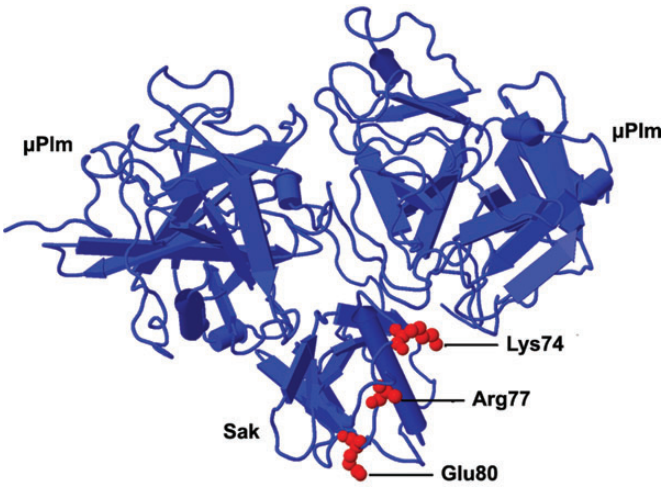

Figure 1. The three-dimensional structure of microplasmin–staphylokinase–microplasmin (μPlm•Sak•μPlm) ternary complex (Protein Data Bank ID 1BUI) and sites of cysteine substitution Amino acid residues targeted for cysteine substitution are shown in space filling mode.

Glu75 and Asp82 residues were firstly excluded. The Lys74, Arg77, and Glu80 residues were highly surface-exposed residues within r-Sak (SASA > 80 Å^2). Moreover, except the Lys74 residue, both Arg77 and Glu80 residues were far away from the interaction interfaces between Sak and μPlm (**Fig. 1**). Therefore, Lys74, Arg77, and Glu80 were selected for further investigation.

Construction and purification of the PEGylated Sak variants

The expression plasmids of Sak(K74C), Sak(R77C), and Sak(E80C) were successfully constructed using a modified QuikChange site-directed mutagenesis as described in the Materials and Methods section. After transformation into

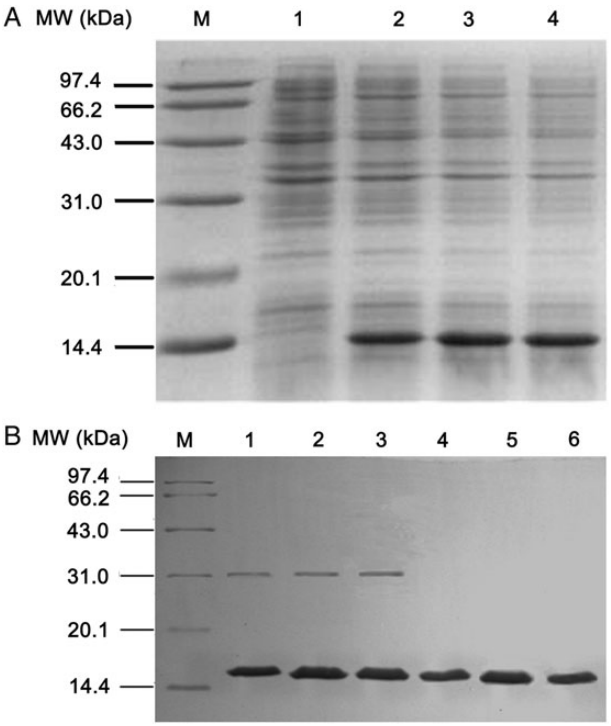

Figure 2. SDS–PAGE analysis of Sak analogs (A) SDS–PAGE analysis of expression products from the engineering bacteria. Lane M, molecular mass standards; lane 1, total proteins of DH5α/pBV220-Sak(K74C) before induction; lanes 2–4, total proteins of DH5α/pBV220-Sak(K74C), DH5α/pBV220-Sak(R77C) and DH5α/pBV220- Sak(E80C) at 2.5 h after induction, respectively. (B) SDS–PAGE analysis of the purified Sak variants. lane 1–3, Sak(K74C), Sak(R77C), and Sak(E80C) under non-reducing conditions, respectively; lane 4–6, Sak(K74C), Sak(R77C), and Sak(E80C) under reducing conditions, respectively.

E. coli strain DH5α, all of Sak variants were expressed at high levels as soluble proteins in the cytoplasm (**Fig. 2A**).

Sak(K74C), Sak(R77C), and Sak(E80C) were successfully purified by a three-step chromatographic process. Purification runs resulted in final product yields, ranging from 30% to 50%. The purified Sak variants showed single bands on reducing SDS–PAGE when at least 10 μg of proteins were loaded onto each lane (**Fig. 2B**, lane 4–6). The fibrinolytic activity of Sak variants was determined with a radial caseinolytic assay and the results are listed in **Table 3**. Sak(E80C) possessed a slightly higher fibrinolytic activity (11.9×10^4 HU/mg) while both Sak(K74C) and Sak(R77C) had a significantly lower specific activity (5.3×10^4 and 7.4×10^4 HU/mg) than r-Sak (10.0×10^4 HU/mg). The mutation of Lys74 to cysteine resulted in a 47% decrease in specific activity compared with r-Sak. Thus, Sak(K74C) was excluded from further PEGylation investigation.

The non-reducing and reducing SDS–PAGE analysis was used to detect the formation of intermolecular disulfide bonds of Sak cysteine variants. Minor dimers were present in the non-reducing condition (**Fig. 2B**, lanes 1–3) and disappeared in the reducing condition (**Fig. 2B**, lanes 4–6).

Table 3. Characterization of r-Sak and its variants[a]

Name	Purity/%[b]	Specific activity/ ($\times 10^4$ HU/mg)	K_m/(μmol/l)	k_{cat}/(s^{-1})	Catalytic efficiency /k_{cat}/K_m [(μmol/l)$^{-1}$ s^{-1}]
r-Sak	97.5 ± 0.31	10.0 ± 0.39	1.26	0.031	0.024
Sak(K74C)	97.3 ± 0.23	5.3 ± 0.56			
Sak(R77C)	97.3 ± 0.29	7.4 ± 0.62			
Sak(R77C-PEG)	98.2 ± 0.45	7.0 ± 0.49	1.44	0.013	0.005
Sak(E80C)	97.7 ± 0.21	11.9 ± 0.37			
Sak(E80C-PEG)	98.5 ± 0.36	11.8 ± 0.61	1.31	0.024	0.018

[a]Data are mean ± SD of three experiments performed in duplicate.
[b]Protein quantities were determined by SEC-HPLC analysis.

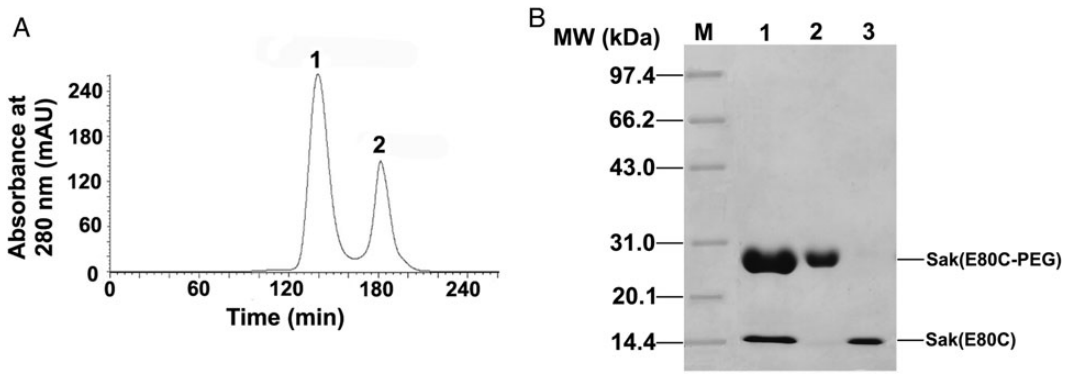

Figure 3. Purification of Sak(E80C-PEG) by size-exclusion gel filtration chromatography (A) Elution profile of Sak(E80C-PEG) from a preparative Toyopearl HW-50F column. (B) SDS−PAGE analysis of the elute fractions. Lane M, molecular mass standards; lane 1, fraction eluted from SP-Sepharose FF cation-exchange chromatography; lane 2, fraction corresponding to elution peak 1; lane 3, fraction corresponding to elution peak 2.

The results indicated that Sak(K74C), Sak(R77C), and Sak(E80C) formed the protein dimers, respectively, which should be mediated by intermolecular disulfide bonds between Sak cysteine variants. Therefore, the purified Sak variants were reduced to monomers with DTT prior to the conjugation reaction. In DTT-treated Sak analogs, there was a single introduced free cysteine residue available for PEG conjugation. The maleimide group of HO-PEG-Mal may react with the sulfhydryl group to form a stable thioether bond and yield Sak(R77C-PEG) and Sak(E80C-PEG) under ambient temperature. To minimize the side reaction between the amine group and the maleimide group of mPEG-Mal, the PEGylation process was performed under slightly acidic conditions (pH 5.6) [20].

Sak(R77C-PEG) and Sak(E80C-PEG) were purified through SP Sepharose FF and Toyopearl HW-50F chromatography. Attempts to purify the PEGylated Sak variants by ion exchange chromatography using SP Sepharose FF failed to produce satisfactory purification, suggesting that PEGylation at Positions 77 and 80 did not alter the electrophilic property of r-Sak. However, the free PEG could be removed from the crude reaction mixture by SP Sepharose FF chromatography. After PEGylation, the size of PEGylated Sak variants increased significantly, allowing the Sak(R77C-PEG) and Sak(E80C-PEG) to be purified by size-exclusion chromatography. As shown in **Fig. 3A**, Toyopearl HW-50F chromatography successfully separated the Sak(E80C-PEG) from Sak(E80C). SDS−PAGE analysis indicated that Peak 1 corresponded to Sak(E80C-PEG) and Peak 2 corresponded to Sak(E80C) (**Fig. 3B**).

Characterization of the PEGylated Sak variants

SDS−PAGE analysis confirmed that Sak(R77C-PEG) and Sak(E80C-PEG) showed single bands under non-reducing and reducing conditions (**Fig. 4A**). They migrated with apparent MWs of ~29-kDa. HPLCanalysis showed that Sak(R77C-PEG) and Sak(E80C-PEG) gave single symmetrical peaks with purities >97% on an analytical SEC-HPLC column (**Fig. 4B,C**). Similar results were obtained using an RP-HPLC column (data not shown). The residual endotoxin content ranged between <2.0 and 0.3 EU/mg. The substitution, PEGylation, and purification strategies yielded homogeneous PEGylated Sak analogs.

The fibrinolytic activity of PEGylated Sak variants was determined with a radial caseinolytic assay and the results are listed in **Table 3**. Sak(R77C-PEG) had significantly

reduced specific activity, while Sak(E80C-PEG) maintained specific activity. Plasminogen activation by Sak–plasmin complexes obeyed the Michaelis–Menten equation and the kinetic constants were calculated from linear Lineweaver–Burk plots. The catalytic efficiency k_{cat}/K_m of Sak(E80C-PEG) [0.018 $(\mu mol/l)^{-1}\ s^{-1}$] was comparable with that of r-Sak [0.024 $(\mu mol/l)^{-1}\ s^{-1}$], while Sak(R77C-PEG) [0.005 $(\mu mol/l)^{-1}\ s^{-1}$] had a significantly reduced catalytic efficiency (Table 3). As Sak(E80C-PEG) retained the bioactivity of Sak, it was selected for further thermostability, immunogenicity, and pharmacokinetic studies.

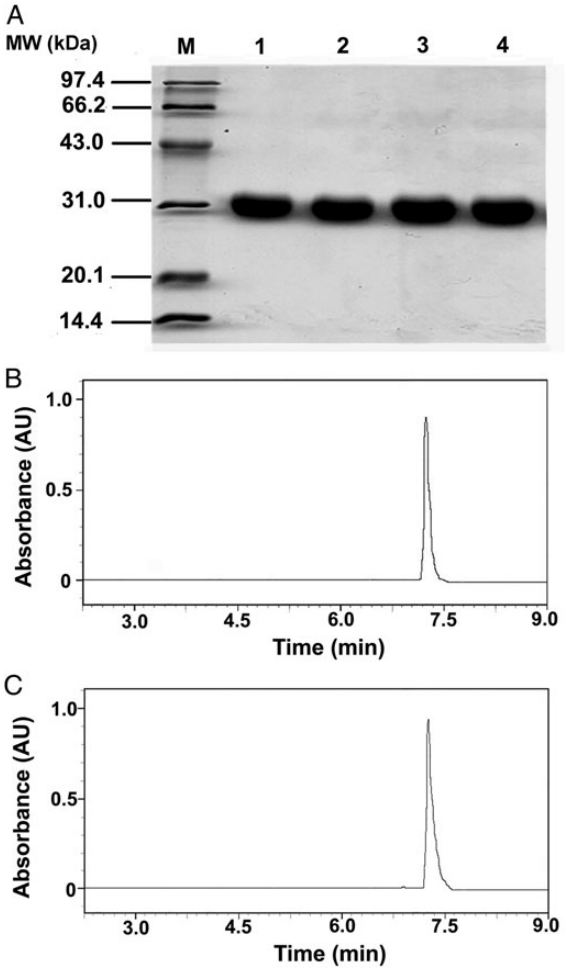

Figure 4. Analysis of PEGylated Sak analogs (A) SDS–PAGE analysis of PEGylated Sak analogs. Lane M, molecular mass standards; lanes 1 and 2, Sak(R77C-PEG) and Sak(E80C-PEG) under non-reducing conditions, respectively; lanes 3 and 4, Sak(R77C-PEG) and Sak(E80C-PEG) under reducing conditions, respectively; (B) SEC-HPLC pattern of Sak(E80C-PEG); (C) SEC-HPLC pattern of Sak(R77C-PEG).

Thermostability of Sak(E80C-PEG)

The effect of temperature on the stability of r-Sak and Sak(E80C-PEG) was investigated at physiological pH. Both r-Sak and Sak(E80C-PEG) were incubated at 37°C for 120 h and at 56°C for 24 h and then the residual activity of the samples at different time points was determined by the fibrinolytic-activity assay. As shown in **Fig. 5A**, r-Sak and Sak(E80C-PEG) retained similar fibrinolytic activity at 37°C for 120 h. However, Sak(E80C-PEG) lost ~15.0% of its activity, while r-Sak lost only 7.2% of its activity after 24 h at 56°C (**Fig. 5B**). The results indicated that thermal stability of Sak(E80C-PEG) was slightly decreased when compared with r-Sak.

Immunogenicity of Sak(E80C- PEG)

After repeated administration with r-Sak and Sak(E80C-PEG), respectively, the sera of the guinea pigs were collected and the antiserum titers were assayed by ELISA (**Fig. 6**). A low titer of antibodies (<300) were detected in the two groups at the end of the first and second week after immunization. However, r-Sak subsequently elicited high antibody titers in the guinea pigs, which rapidly rose to 30 000, 40 000, and 40 000 at the end of the third, the fourth and the fifth week, respectively. Sak(E80C-PEG) elicited significantly lower antibody titers ($P < 0.05$) compared with r-Sak, which were 2000, 2500, and 2500 at the end of the third, fourth and fifth week, respectively. These results indicated that Sak(E80C-PEG) had greatly reduced immunogenicity in guinea pigs.

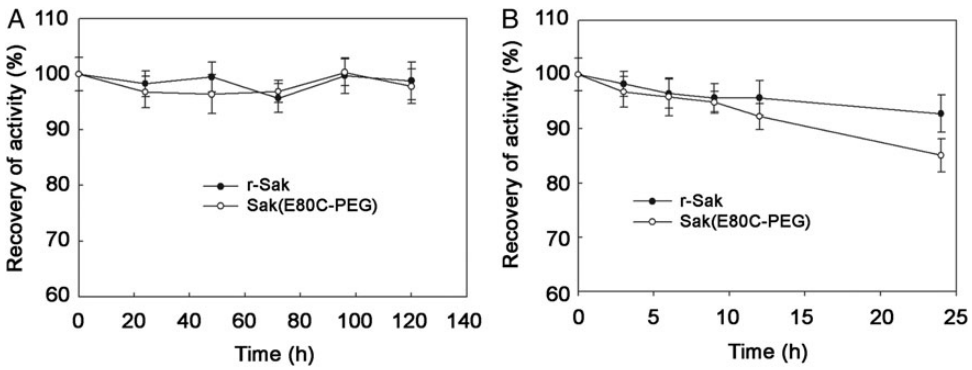

Figure 5. Thermostability of r-Sak and Sak(E80C-PEG) Samples were incubated in 0.15 mol/l sodium chloride, 0.05 mol/l phosphate buffer, pH 7.4 at 37°C (A) and 56°C (B). The concentration of r-Sak and Sak(E80C-PEG) was 1 mg/ml.

Pharmacokinetics of Sak(E80C- PEG)

Figure 7 shows the *in vivo* r-Sak and Sak(E80C-PEG) concentration−time profiles after bolus intravenous injection of r-Sak and Sak(E80C-PEG) solution in rabbits. The pharmacokinetic parameters derived from plasma disappearance curves are shown in **Table 4**. PEGylation at Position 80 could significantly extend the *in vivo* circulatory half-life and decrease plasma clearance of PEGylated Sak variant. Sak(E80C-PEG) showed a significantly longer initial distribution phase $t_{1/2}\alpha$ (6.93 min) and a terminal elimination phase $t_{1/2}\beta$ (38.38 min), when compared with t1/2α (2.47 min) and t1/2β (8.18 min) for r-Sak ($P < 0.05$). The system availability (AUC) of Sak(E80C-PEG) (86.49 μg × min/ml) was also significantly increased when compared with that of r-Sak (22.96 μg × min/ml) ($P < 0.05$). The plasma clearances of Sak(E80C-PEG) (11.56 ml/min) was significantly slower than that of r-Sak (43.56 ml/min) ($P < 0.05$). These results were consistent with the previous study by Vanwetswinkel *et al*. [5].

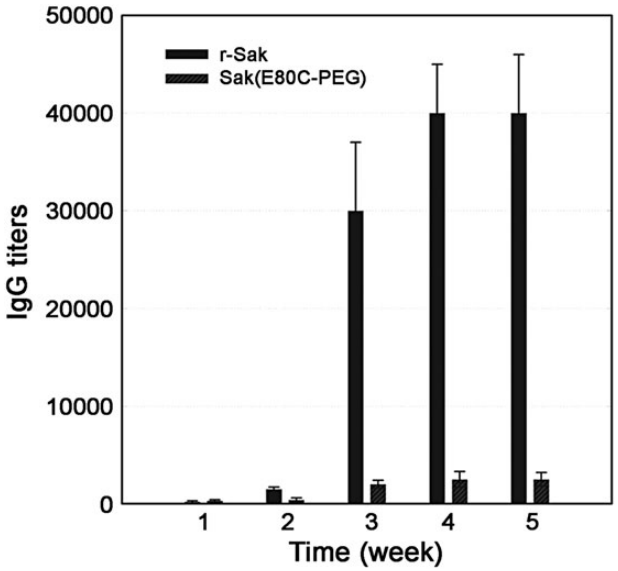

Figure 6. Titers of antibodies elicited by the native r-Sak and Sak(E80C-PEG) in immunized guinea pigs Data are means ± SD of eight experiments performed in duplicate.

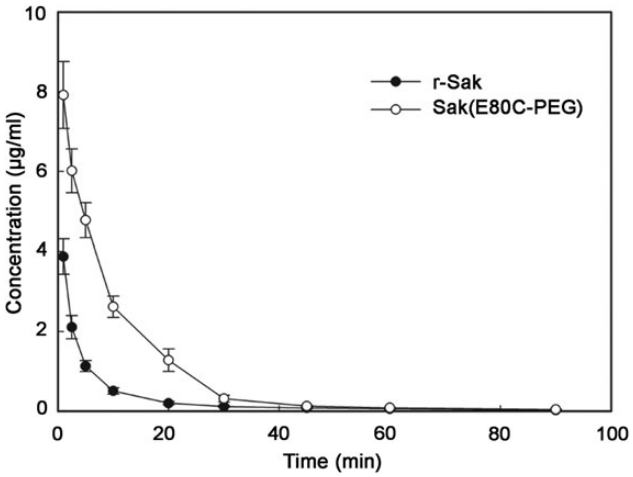

Figure 7. Pharmacokinetic profile of the PEGylated Sak variant Sak(E80C-PEG) and the native r-Sak New Zealand rabbits were intravenously injected with r-Sak or its conjugate solution at a dose of 50 000 IU/kg. Data are means ± SD of six experiments performed in duplicate.

Discussion

Since native Sak does not contain cysteine residue, free cysteine residue can be introduced by genetic engineering. To identify a suitable site from Positions 71−87 aa for cysteine replacement and PEGylation without affecting the fibrinolytic activity of Sak, some key factors need to be considered, including (i) the introduced cysteine residue must be accessible for covalent PEG conjugation [21,22]; because of the hydrophobic nature of the cysteine side chain, cysteine is most frequently buried inside the protein structure and therefore only partially accessible to PEGylation reagents. Thus, highly exposed hydrophilic residues (SASAs > 80 $Å^2$) should be firstly selected. (ii) The introduction of PEG chain must not affect the complex formation between Sak and plasminogen. PEG chains can shield the protein surface and influence the interactions between Sak and plasminogen that are responsible for the fibrinolytic activity. Then, the PEGylated position had better be far away from the interaction interfaces between Sak and plasminogen.

Three amino acid residues containing Lys74, Arg77, and Glu80 were selected as a chemoselective cysteine PEGylation site according to these guiding principles. Biological activity analysis indicated that both Sak(E80C) and Sak(R77C) retained the specific activity of r-Sak while Sak(K74C) possessed an obviously decreased specific activity. This might be due to its spatial position, which was close to the interaction interfaces of Sak and μPlm. Mutation of

Table 4. Pharmacokinetic parameters of Sak(E80C-PEG) and r-Sak ($n = 6$)

Name	$t_{1/2}\alpha$ (min)	$t_{1/2}\beta$ (min)	AUC (μg min ml^{-1})	CLp (ml/min)
r-Sak	2.47 ± 0.27	8.18 ± 0.95	22.96 ± 3.41	43.56 ± 3.27
Sak(E80C-PEG)	6.93 ± 0.56	38.38 ± 2.39	86.49 ± 9.36	11.56 ± 0.86

Lys74 to Cys74 might have some influence on protein conformation of r-Sak.

The maleimide group of HO-PEG-Mal may react with single sulfhydryl group in Sak cysteine variant and yielded Sak(R77C-PEG) and Sak(E80C-PEG). After purified with size-exclusion chromatography, the PEGylated Sak variants with high purity were obtained. The fibrinolytic-activity assay and the kinetic constant assay showed that Sak(E80C-MP5) maintained the fibrinolytic activity of r-Sak. Molecular dynamics simulation had indicated that the PEG chain loosely folded on the surface of the PEGylated Sak variant in an irregular form [11]. The steric shielding effect of PEG might interfere with the interaction between Sak and plasminogen and decrease the bioactivity of Sak. However, PEGylation did not significantly decrease the specific activity of Sak(R77C-PEG) and Sak(E80C-PEG) compared with Sak(R77C) and Sak(E80C), respectively. The results confirmed our speculation that Positions 77 and 80 in the Sak molecule are far away from the interaction interfaces between Sak and plasminogen. In addition, the 5 Kd-PEG used in the present work is relatively short and the steric shielding effect of PEG could not affect the interaction between Sak and plasminogen. There was no doubt that the steric shielding effect of 20 Kd-PEG should be much greater than that of 5 Kd-PEG. While the 20 Kd-PEG was used to modify Sak, it should more severely influence the interaction between Sak and plasminogen. As a result, the bioactivity of the PEGylated Sak decreased more significantly [9–11].

Thermal stability is a very important property of biopharmaceutics, which can significantly influence product development and storage [23]. Modification of the Sak molecule with PEG might influence protein thermostability [22,24]. After PEGylation, the thermal stability of Sak(E80C-PEG) slightly decreased when compared with r-Sak.

An important advantage of PEGylation is the reduction or elimination of protein immunogenicity [4,24,25]. The immunogenicity studies indicated that Sak(E80C-PEG) had greatly reduced immunogenicity in guinea pigs. Some researchers thought that immunogenicity of protein was associated with protein aggregate [26]. However, aggregates of Sak(E80C-PEG) were not observed in either SDS–PAGE or SEC-HPLC analysis. Thus, the reduction of immunogenicity of PEGylated Sak should not be related to protein aggregation. On the contrary, the immunogenicity reduction of Sak(E80C-PEG) should be attributed to the steric hindrance yielded by PEG chain, which affect or prevent each step of the antigen-processing pathway [4,25].

Several studies have claimed that the PEG moiety of PEGylated therapeutic proteins itself might be immunogenic and could induce anti-PEG antibodies [27]. Further researches have indicated that the methoxy group within methoxy-PEG modified proteins played a critical role in the development of anti-PEG antibody with high affinity [27]. Moreover, these anti-PEG antibodies might contribute to the loss of efficacy of methoxy-PEG modified conjugates [28]. Thus, HO-PEG-Mal was selected to prepare the PEGylated Sak variants in the present study.

The pharmacokinetic data indicated that Sak(E80C-MP5) had a better pharmacokinetic profile compared with r-Sak following intravenous bolus injection. MW of attached PEG could significantly influence the plasma half-life of PEGylated Sak variants. As PEG MW increased from 5 to 20 kDa, initial half life of the PEGylated Sak variants increased from 4.0–4.8 min to 20 min [5]. A PEGylated Sak variant SY161-P5 conjugated with 5-kDa PEG exhibited a thrombolytic effect similar to that of recombinant tissue-type plasminogen activator in a pilot trial and the CAPTORS II clinical trial [7]. The results suggested that 5-kDa PEG-conjugated Sak variant was suitable for the treatment of acute myocardial infarction. Thus, 5-kDa PEG was selected to modify r-Sak in the present work.

The improved pharmacokinetic properties of Sak(E80C-PEG) suggested that a much lower dose of the PEGylated Sak might be used to obtain the same clinical benefit as with the native r-Sak, which could minimize potential toxic or immune responses to the drug. Furthermore, extension of plasma half-life would facilitate developing the conjugate as a single bolus formulation [7,29].

In summary, the PEGylated Sak variant Sak(E80C-PEG) developed in this study showed retained fibrinolytic activity, significantly reduced immunogenicity, and improved pharmacokinetic properties relative to the native r-Sak. Surface-exposed hydrophilic amino acid residues within the immunodominant sequence 71–87 were selected, replaced by cysteine, and PEGylated by conjugation of HO-PEG-Mal to the single engineered cysteine. The PEGylation site is critical for the preservation of the fibrinolytic activity and simultaneous reduction of immunogenicity of the Sak molecule. In conclusion, the site-directed PEGylation improved the biological, immunological, and pharmacokinetic properties, leading to significant advance in the conversion of a bacterial-derived protein to a useful therapeutic drug.

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