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Simultaneous removal of T and B cell epitope in recombinant staphylokinase by structure-based mutagenesis of immuno-dominant Arg77 and Glu80 residues

Ruiguang Xu[1], Jintian He[1*], Kai Jia[1], Xi Chen[1], Jianwei Liu[1], Ke Zhu[2]

[1]College of Life Science, Hebei Normal University, Shijiazhuang 050016 China

[2]Shijiazhuang Pharm. Group Hebei Zhongrun Pharmaceutical Co., LTD. Shijiazhuang 050041 China

Abstract: [**Objective**] To reduce immunogenicity of recombined staphylokinase (r-Sak), site-directed mutagenesis of Arg77 and Glu80 residue was performed to simultaneously remove T and B cell epitope in r-Sak molecule. [**Methods**] The solvent accessible surface areas of residues 77 and 80 in r-Sak were used to analyze rational design of Sak mutation. The Sak mutants were expressed in *E coli* DH5α. After purified by a 3-step chromatography, their fibrinolytic activities and immunological properties were analyzed. [**Results**] Immunogenicity tests suggested that Sak induced a Th_2-type immune response. Substitution of Glu80 with alanine or serine successfully reduced its solvent accessible surface area while simultaneously removing part of the T and B cell epitope. Changing Arg77 to glutamine, asparagine, or lysine removed only part of the T cell epitope. Of six dually substituted variants, Sak(R77Q/E80A) and Sak(R77Q/E80S) variants effectively eliminated part of the B and T cell epitopes, which markedly reduced their immunogenicity.

Keywords: staphylokinase, T cell epitope, B cell epitope, immunogenicity, protein engineering

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The plasminogen activator staphylokinase (Sak), a profibrinolytic bacterial protein, is a promising thrombolytic agent for the treatment of acute myocardial infarction[1]. A pilot randomized trial has shown that recombinant staphylokinase (r-Sak) was at least equipotent with recombinant tissue-type plasminogen activator for coronary artery re-canalization and was significantly more fibrin-selective. However, administration of this heterologous protein induced Sak-specific antibodies in a majority of patients[2]. Once elicited, the presence of these Sak-specific antibodies precludes its re-administration because these would reduce its efficacy or induce acute allergic reactions. Thus, reduction of the immunogenicity of the Sak molecule is essential if Sak is to become an effective clinical therapy.

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* Corresponding author. Tel: +86-311-85895276; E-mail: he_jintian@yahoo.com

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One important method for reduction of immunogenicity of proteins is to identify B and T cell epitopes in the protein and to eliminate these by protein sequence modifications[3-4]. In the case of Sak, previous studies to lower its immunological properties have focused on removal of its B cell epitopes by site-directed mutagenesis[5]. However, even the best variant still elicited about 50% of the normal r-Sak antibody response[6].

The elimination of potential T cell epitopes by mutagenesis could also effectively reduce immune response to the protein. This strategy has been successfully used to remove T cell epitopes of recombinant erythropoietin, C1 domain of factor VIII, and monoclonal antibodies and reduced their immunogenicities[4,7]. In the case of r-Sak, five distinct immunogenic regions have been reported to be recognized by T lymphocytes from several individuals[8]. Of these five regions, the most prominent C3 T cell immunoreactive region (sequence 71-87) was comprised of two overlapping T-cell epitopes and could induce specific proliferation of T-cell clones from 90% of the donors. Alanine scanning mutagenesis of this C3 region suggested that Arg77 and Glu80 might be immuno-dominant residues of the two respective T-cell epitopes[9].

These Arg77 and Glu80 residues were also the component elements of B cell epitope 1 and epitope 3 in r-Sak, respectively[1]. Thus, the Arg77 and Glu80 residues should simultaneously be the immuno-dominant residues of overlapping T-cell and B-cell epitopes in the r-Sak molecule. Consequently, the simultaneous mutations of both the Arg77 and the Glu80 residues would be expected to have substantial impact on T-cell and B-cell epitopes of the r-Sak protein. However, few attempts have been made for simultaneous removal of B- and T-cell epitopes from a protein by mutagenesis of a few amino acid residues.

Our preliminary study suggested that substitution of Glu80 residue by alanine or serine could partially reduce immunogenicity of the r-Sak molecule[10]. In the current study, we aimed at rationally designing a combined mutation at positions 77 and 80 in r-Sak for simultaneous elimination of the associated T and B cell epitopes in the Sak molecule. Our approach was to immunize BALB/c mice with mutated proteins to determine the levels of serum IgG antibodies elicited, to evaluate the ability of the mutated proteins to bind to Sak polyclonal antibody and the extent of the proliferative response elicited in spleen cells. Cytokine production in response to Sak and its variants was also determined to characterize the type of immune response elicited and the reduction in immunogenicity of the Sak variants. The catalytic constants of plasminogen activation by Sak plasmin complexes and the fibrinolytic activity were used to evaluate the biological activity of the Sak variants.

1 Materials and Methods

1.1 Construction of expression plasmids

The expression plasmid pBV220-Sak was constructed as previously described and used as a mutagenesis template[10]. The expression plasmids of the variants including Sak(R77Q) (i.e., Arg[R] in position 77 substituted with Gln[Q]), Sak(R77K), Sak(R77N), Sak(R77Q/E80A), Sak(R77Q/E80S), Sak(R77K/E80A), Sak(R77K/E80S), Sak(R77Q/E80A), Sak(R77Q/E80S) were constructed via a modified QuikChange site-directed mutagenesis method as previously described [10]. The sequences of the oligonucleotides used as primers are listed in Table 1. Mutations were confirmed by gene sequencing.

1.2 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 [10]. Recombinant proteins were purified employing a similar three-step chromatographic purification process[10]. 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 0℃. The homogenate was processed by centrifugation at 10000 × g for 30 min at 4℃. After adjustment of

supernatant pH to 5.6 with 0.1 mol/L NaOH, the supernatant was applied to a SP Sepharose FF column pre-equilibrated with equilibration buffer (20 mmol/L sodium acetate buffer, pH 5.6), then eluted with 1.0 mol/L sodium chloride in equilibration buffer. The fractions containing the Sak variant were pooled and immediately adjusted to about pH 7.4, then applied to a Sephadex G-50 column pre-equilibrated with 20 mmol/L Tris-HCl buffer, pH7.4. The active fraction obtained from the Sephadex G-50 column was applied to a Q Sepharose FF column pre-equilibrated with 20 mmol/L Tris-HCl buffer, pH 7.4 and eluted with 0.3 mol/L sodium chloride in 20 mmol/L Tris-HCl buffer (pH 7.4). Purified proteins were examined by SDS/PAGE and High-performance liquid chromatography (HPLC)[10]. The methodology used to determine protein concentrations and fibrinolytic activity has been described in detail previously[10]. A qualitative limulus lysate assay kit (National Institute for the Control of Pharmaceutical and Biological Products, China) was used to determine levels of endotoxin in the r-Sak ant its variants. The sensitivity of the test reagent used is 0.25 EU/ml.

Table 1 Primer sequences used for Sak variant construction

Name	Mutation primer * (5′→3′)
Sak(R77N)	AGCATATAAAGAGTTTAACGTAGTTGAGCTCGATCCAAGCGCAAAGCTTTGCGCTTG GATCGAGCTCAACTACGTTAAACTCTTTATATGCT
Sak(R77Q)	AGCATATAAAGAGTTTCAGGTAGTTGAGCTCGATCCAAGCGCAAAGCTTTGCGCTTG GATCGAGCTCAACTACCTGAAACTCTTTATATGCT
Sak(R77K)	AGCATATAAAGAGTTTAAAGTAGTTGAGCTCGATCCAAGCGCAAAGCTTTGCGCTTG GATCGAGCTCAACTACTTTAAACTCTTTATATGCT
Sak(R77N, E80A)	GCATATAAAGAGTTTAACGTAGTTGCTTTAGATCCAAGCGCTAAGATCGAAGTCGAC TTCGATCTTAGCGCTTGGATCTAAAGCAACTACGTTAAACTCTTTATATGC
Sak(R77N, E80S)	GCATATAAAGAGTTTAACGTAGTTTCTTTAGATCCAAGCGCTAAGATCGAAGTCGAC TTCGATCTTAGCGCTTGGATCTAAAGAAACTACGTTAAACTCTTTATATGC
Sak(R77Q, E80A)	GCATATAAAGAGTTTCAGGTAGTTGCTTTAGATCCAAGCGCTAAGATCGAAGTCGAC TTCGATCTTAGCGCTTGGATCTAAAGCAACTACCTGAAACTCTTTATATGC
Sak(R77Q, E80S)	GCATATAAAGAGTTTCAGGTAGTTTCTTTAGATCCAAGCGCTAAGATCGAAGTCGAC TTCGATCTTAGCGCTTGGATCTAAAGAAACTACCTGAAACTCTTTATATGC
Sak(R77K, E80A)	GCATATAAAGAGTTTAAAGTAGTTGCTTTAGATCCAAGCGCTAAGATCGAAGTCGAC TTCGATCTTAGCGCTTGGATCTAAAGCAACTACTTTAAACTCTTTATATGC
Sak(R77K, E80S)	GCATATAAAGAGTTTAAAGTAGTTTCTTTAGATCCAAGCGCTAAGATCGAAGTCGAC TTCGATCTTAGCGCTTGGATCTAAAGAAACTACTTTAAACTCTTTATATGC

* The mutated sites are noted in bold

1.3 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 studied with chromogenic substrate S-2390 (H-D-Val-Phe-Lys-p-nitroanilide), as described previously[11]. 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℃ was determined from the change in absorbance of S-2390 at 405 nm (△A405). The kinetic constants of the plasminogen activation by complexes of Sak variants with plasmin were calculated from Lineweaver-Burk plots.

1.4 Immunization and ELISA for the detection of anti-Sak IgG

Immunogenicity of Sak was evaluated in female BALB/c mice (6 – 8 weeks old, about 20 g) purchased from the Experimental Animal Center of Hebei Province. Sixty mice randomly were randomized

into 12 groups, each consisting of 5 mice. The mice were immunized at day 0 by subcutaneous injection of 10 μg Sak or its variants, emulsified in Freund's complete adjuvant. Two booster immunizations with antigens emulsified in Freund's incomplete adjuvant were given at days 14 and 28. Blood samples were collected at day 35 and stored at −20℃ for later assay.

Anti-Sak IgG of each serum sample was determined by ELISA. Polystyrene microtiter plates were coated overnight at 4℃ with 100 μl of 0.1 mg/ml Sak or its variants in coating buffer (50 mmol/L sodium carbonate buffer pH 9.6) and rinsed with PBST (0.01 mol/L PBS containing 0.05% Tween 20). After blocking with 5% (w/v) skim milk powder at 37℃ for 2 h, the microtiter plates were washed three times with PBST. A 100 μl sample of the diluted serum (1/5000) elicited with Sak or its variants was loaded and incubated at 37℃ for 1 h. After three washes with PBST, 100 μl of horseradish peroxidase-labeled goat anti-rabbit polyclonal immunoglobulin, diluted 1∶2000, was added and incubated for 1 h at room temperature. After three washes with PBST, the bound enzyme conjugate was detected by addition of 100 μl 3,3,5,5-Tetramethylbenzidine (substrate) to the plate wells. The reaction was stopped 10–20 min later using 50 μl of 2 mol/L H_2SO_4. Absorbance at 450 nm was measured on a microplate reader (Bio-Rad 550).

1.5 Assay for the ability of r-Sak or its variants to bind to polyclonal anti-sera elicited by r-Sak or its variants in mice

The specific binding ability of Sak variants to polyclonal anti-sera elicited in mice by immunization with r-Sak was determined by the indirect ELISA, as described above, except that the primary antibody was mouse anti-r-Sak serum.

To determine the binding ability of r-Sak to antisera raised against Sak variants, the diluted serum samples (1/5000) were reacted with 0.1–1 mg/mL of immobilized r-Sak for 1 h. The residual antibody within unabsorbed plasma was then reacted with immobilized Sak variants and detected by 3,3,5,5-Tetramethylbenzidine.

1.6 Spleen cell proliferation assays and ELISA for the detection of cytokines

Female BALB/c mice were immunized s.c. with 10 μg r-Sak or its variants in alum on days 1, 3, and 10. Spleen cells were aseptically isolated on day 15. The culture medium was RPMI 1640 minimal essential medium supplemented with 10% inactivated fetal calf serum. Proliferation tests were performed as follows. In brief, 1.0×10^6 cells/well were incubated in quadruplicate in 96-well microtiter plates with antigens at 100 μl of 100 μg/ml for 3 days in a humidified 5% CO_2 incubator at 37℃. Then, cell proliferation was estimated by a Cell Counting Kit-8 (CCK-8) (Dojindo Laboratory Co., Ltd., Kumamoto, Japan). 10 μL of of the cell count reagent, WST-8 was added to all wells and incubated for 4 h at 37℃. Absorbance at 450 nm was determined with a Model 550 Microplate Reader (Bio-Rad Laboratories (UK) Ltd.).

To determine cytokine production, spleen cells were cultured in culture medium alone or in culture medium containing 100 μg/ml r-Sak or its variants for 24–48 h. The concentration of interleukin (IL)-4 and interferon (IFN)-γ in 36 h culture supernatants were assayed by cytokine-specific ELISA according to the manufacturer's protocol (R&D Systems, Minneapolis, USA). OD was measured at 450 nm on a Model 550 Microplate Reader (Bio-Rad Laboratories (UK) Ltd.). Results were expressed as means ± standard deviation (SD) and are representative of three independent experiments (n = 3 mice per experiment).

1.7 The solvent accessible surface areas (SASAs) of position 77 and 80 within r-Sak and its variants

The solvent accessible surface areas (SASAs) of position 77 and 80 within r-Sak in a microplasmin-staphylokinase-microplasmin (μPlm · Sak · μPlm) complex (Protein Data Bank ID 1BUI) and in monomeric state (Protein Data Bank ID 1ssn) were calculated using the POPS server[12-14]. 1BUI was chosen since the r-Sak molecule existed within a

ternary complex of r-Sak and μPlm, which was a proteinase-cofactor-substrate complex in action structure. The probe radius was set at a 1.4 Å. The models of Sak variants were generated by automatic modeling using the SWISS-MODEL server and the structure of r-Sak in solution (Protein Data Bank ID 1ssn) was selected as the template for structural modeling[15]. The SASAs of position 77 and 80 within Sak variants were also calculated using the POPS server.

2 Results

2.1 Rational design of Sak variants

The tertiary structure of r-Sak in a ternary μPlm · Sak · μPlm complex (Protein Data Bank ID 1BUI) and in monomeric state (Protein Data Bank ID 1ssn) showed that sequence 76 – 81 comprised a β-strand. Comparison of the SASA of the Glu 80 residue in a μPlm · Sak · μPlm complex with that in the monomeric state revealed the Glu80 residue to be a highly surface-exposed residue (SASA > 80 $Å^2$) and the interaction between r-Sak and μPlm had little effect on its SASA (Table 2). Thus, Glu 80 appeared to have little influence on the interaction between r-Sak and μPlm. The Glu80 residue was therefore designed to be mutated to small size residues; i.e. alanine or serine. After the Glu80 residue was mutated to alanine or serine, the SASAs of position 80 significantly decreased from more than 110 $Å^2$ to about 50 $Å^2$ or 60 $Å^2$, respectively (Table 2). For the Arg 77 residue, its SASA decreased significantly from 163.10 $Å^2$ to 57.46 $Å^2$ after r-Sak formed a ternary complex with μPlm (Table 2). Therefore, Arg 77 might have an important influence on protein conformation and biological activity of r-Sak. To maintain fibrinolytic activity of the Sak variants, the Arg77 residue was mutated to glutamine, asparagine, or lysine residues that most closely resembled its size. After the Arg77 residue was mutated to glutamine, asparagine, or lysine, the SASA of position 77 was still greater than 80 $Å^2$ (Table 2).

Table 2 The solvent accessible surface areas (SASAs) of positions 77 and 80 within r-Sak and its variants

Name	SASAs of position 77 ($Å^2$)	SASAs of position 80 ($Å^2$)
r-Sak in solution	163.10	127.19
r-Sak in μPlm·Sak·μPlm complex	57.46	112.56
Sak(R77N)	94.97	126.33
Sak(R77Q)	130.41	126.25
Sak(R77K)	163.74	126.31
Sak(E80A)	179.81	50.31
Sak(E80S)	179.86	60.18
Sak(R77N, E80A)	94.96	50.31
Sak(R77N, E80S)	94.96	60.17
Sak(R77Q, E80A)	130.42	50.31
Sak(R77Q, E80S)	130.36	60.12
Sak(R77K, E80A)	163.68	50.31
Sak(R77K, E80S)	163.65	60.14

2.2 Mutation, expression and purification of r-Sak and its variants

Nine Sak variants including three single point mutations at position 77 and six combined mutations at position 77 and 80, were successfully constructed using a modified QuikChange site-directed mutagenesis, as described in the materials and methods. After DNA sequences were confirmed by gene sequencing, the expression plasmids were expressed at high levels in *E. coli* strain DH5α (Figure 1). Densitometric scanning of SDS/PAGE gels stained by Coomassie blue showed that the target protein accounted for more than 40% of the total cellular protein. The soluble and insoluble fractions of *E. coli* cell lysate were separated by centrifugation and analysed by SDS/PAGE to determine the nature of Sak variants expressed in *E.*

coli. The results showed that all of Sak variants were expressed as soluble proteins in the cytoplasm (Data not shown).

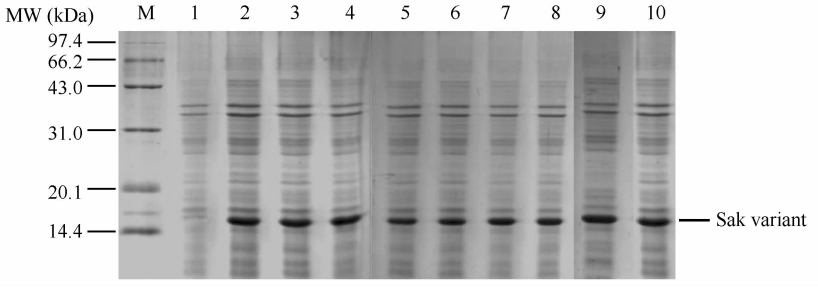

Fig. 1　SDS-PAGE analysis of expression of Sak variants in *E. coli* DH5α. Lane M, molecular mass standards; lane 1 total proteins of DH5α/pBV220-R77K before induction; lane 2 – 10, total proteins of DH5α/pBV220-R77K, DH5α/pBV220-R77N, DH5α/pBV220-R77Q, DH5α/pBV220- R77K/E80A, DH5α/pBV220-R77K/E80S, DH5α/pBV220-R77N/E80A, DH5α/pBV220- R77N/E80S, DH5α/pBV220-R77Q/E80A, DH5α/pBV220-R77Q/E80S, at 2.5 h after induction, respectively.

All of Sak variants were subsequently purified by a three-step chromatographic purification process. The first column was a SP Sepharose FF column which was used to capture the target protein in the supernatant. The elution fraction containing Sak variant from SP Sepharose FF column was further processed through a Sephadex G-50 column. Minor impurities presented in fraction obtained from Sephadex G-50 column were effectively removed by a Q Sepharose FF column. All of Sak variants were successfully purified by this three-step chromatographic process. Purification runs resulted in final product yields, ranging from 40% to 60% and yielded up to about 20.0 mg/g wet cells. The purified Sak variants showed single bands on SDS/PAGE when at least 10 μg of proteins were loaded onto each lane (Figure 2). High-performance liquid chromatography analysis showed that the Sak variants gave single symmetrical peaks with purities > 97% on an analytical HPLC column. No significant peak of impurity was found on the RP-HPLC spectrum (data not shown). The residual endotoxin content ranged between less than 2.0 and 0.3 EU/mg. Together with two single point mutations Sak (E80A) and Sak (E80S) obtained in this laboratory previously[10], all

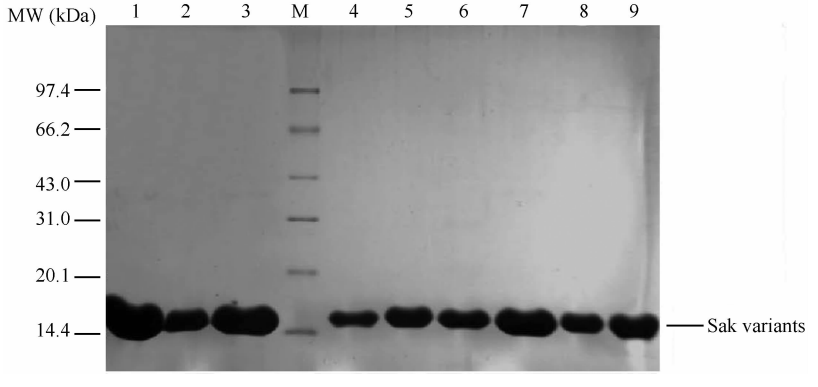

Fig. 2　SDS-PAGE analysis of the purity of Sak variants. Lane M, molecular mass standards; lane 1 – 9, Purified Sak (R77K), Sak (R77N), Sak (R77Q), Sak (R77Q/E80A), Sak (R77Q/E80S), Sak (R77K/E80A), Sak (R77K/E80S), Sak (R77Q/E80A), Sak(R77Q/E80S), respectively. The amount of protein applied to each lane was about 10 – 20 μg.

of these Sak variants were used to evaluate the impact of mutations at position 77 and 80 on the immunogenicity and fibrinolytic activity of Sak molecule.

2.3 Fibrinolytic activities and catalytic efficiency of r-Sak and its variants

The fibrinolytic activity of Sak variants was determined with a radial caseinolytic assay and the results are listed in Table 3. The Sak(R77N), Sak(R77N/E80A) and Sak(R77N/E80S) variants had significantly reduced specific activity, while the other variants maintained or increased 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(E80S) (0.055 $\mu mol/L^{-1} \cdot s^{-1}$), Sak(R77Q/E80A) (0.050 $\mu mol/L^{-1} \cdot s^{-1}$), or Sak(R77Q/E80S) (0.056 $\mu mol/L^{-1} \cdot s^{-1}$) was comparable to that of r-Sak (0.064 $\mu mol/L^{-1} \cdot s^{-1}$) (Table 3). The catalytic efficiency k_{cat}/K_m of Sak(R77N) (0.012 $\mu mol/L^{-1} \cdot s^{-1}$) and Sak(R77N/E80A) (0.025 $\mu mol/L^{-1} \cdot s^{-1}$) was significantly reduced compared to that of r-Sak (0.064 $\mu mol/L^{-1} \cdot s^{-1}$) (Table 3).

Table 3 Fibrinolytic activities and catalytic efficiency for plasminogen activation of r-Sak and its variants

Name	Purity/%[b]	Specific activity/(HU/mg)	K_m/(μmol/L)	k_{cat}/(s^{-1})	Catalytic efficiency/(k_{cat}/K_m)/($\mu mol/L^{-1}s^{-1}$)
r-Sak	97.5	8.4×10^4	4.80	0.31	0.064
Sak(R77Q)	97.3	9.4×10^4	ND[a]	ND[a]	ND[a]
Sak(R77K)	97.2	7.8×10^4	ND[a]	ND[a]	ND[a]
Sak(R77N)	98.1	4.6×10^4	6.90	0.086	0.012
Sak(E80S)	97.7	10.1×10^4	3.8	0.21	0.055
Sak(R77N/E80A)	97.3	7.1×10^4	3.54	0.088	0.025
Sak(R77N/E80S)	98.2	7.1×10^4	ND[a]	ND[a]	ND[a]
Sak(R77Q/E80A)	96.9	8.7×10^4	5.21	0.26	0.050
Sak(R77Q/E80S)	98.5	10.3×10^4	6.37	0.36	0.056
Sak(R77K/E80A)	97.6	8.1×10^4	ND[a]	ND[a]	ND[a]
Sak(R77K/E80S)	97.9	10.2×10^4	ND[a]	ND[a]	ND[a]

[a] ND, not determined; [b] Protein quantities were determined by HPLC analysis.

2.4 Immunogenicity of the Sak mutants

The immunogenicity of Sak variants, as determined by serum IgG antibody levels following immunization of female BALB/c mice with r-Sak and its variants, is shown in Figure 3. All three single point mutations at position 77 induced lower amount of specific IgG antibody than r-Sak ($p < 0.05$). Of the six combination variants, Sak(R77Q/E80A) and Sak(R77Q, E80S) induced less amount of specific IgG antibody than r-Sak, respectively ($p < 0.05$). The other four combination variants, including Sak(R77N/E80A), Sak(R77N/E80S), Sak(R77K/E80A) and Sak(R77K/E80S), elicited slightly lower amounts of antibodies compared with r-Sak. However, these differences were not statistically significant ($p > 0.05$).

2.5 Binding of Sak variants to anti-Sak polyclonal serum

Indirect ELISA detected specific binding abilities of Sak variants to polyclonal anti-serum elicited with r-Sak, as shown in Figure 4. All six combination variants bound significantly less anti-Sak polyclonal antibodies than did r-Sak ($p < 0.05$). Three single-site variants at position 77 bound similar amounts of anti-Sak polyclonal antibodies to that bound by r-Sak ($p > 0.05$).

2.6 Binding of r-Sak to anti-sera elicited by Sak variants

To determine whether amino acid substitution generated new epitopes that were not present in r-Sak, antisera raised against Sak variants were assayed for antibody binding to r-Sak. The antibodies induced by the Sak variants could be completely bound by r-Sak

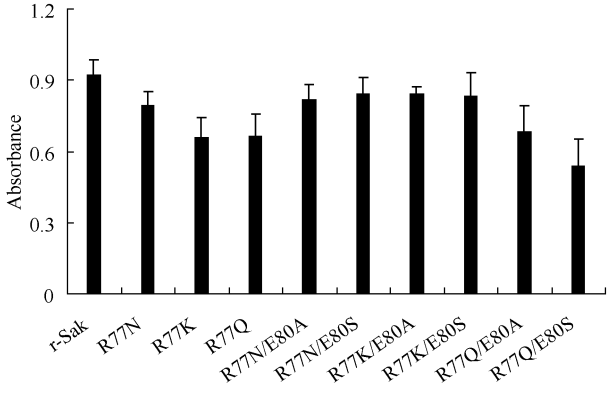

Fig. 3　ELISA for the detection of Anti – Sak IgG induced by r – Sak and Sak variants in BALB/c mice. Ninety – six – well microplates were coated with r – Sak and its variants, and incubated with serum samples to determine the relative amount of IgG in the serum. The data are the means ± SD from five separate experiments.

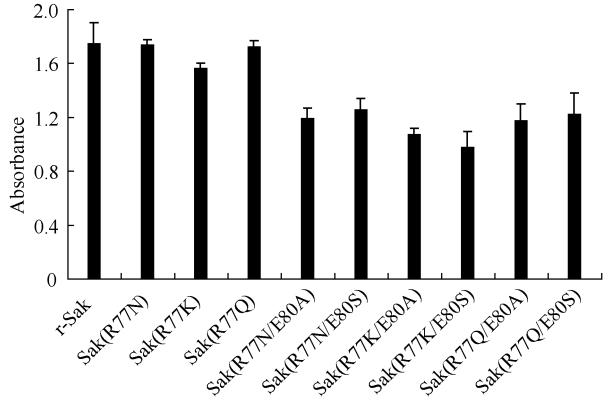

Fig. 4　ELISA for the detection of specific binding ability of Sak variants to polyclonal anti – serum elicited with r – Sak. All Sak mutants retained partial ability to bind with anti – Sak polyclonal antibodies. The data are the means ± SD from five separate experiments.

(data not shown).

2.7　Proliferative responses of T cells from BALB/c mice to r-Sak and its variants

Proliferative responses of T cells from BALB/c mice to r-Sak and its variants were expressed as a proliferative index and are summarized in Figure 5. The ability of all of the single-site and combined variants to stimulate proliferation of T cells was significantly lower than that of r-Sak ($p < 0.05$). Compared with r-Sak, the proliferative index of two combined variants, Sak (R77Q/E80A) and Sak (R77Q/E80S), decreased by 51.4% and 61.8%, respectively.

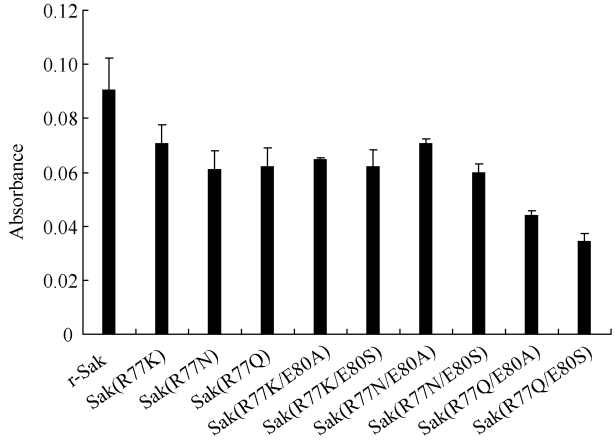

Fig. 5　Proliferative responses of spleen cells from BALB/c mice upon stimulation with r – Sak and its variants. The data are means ± SD from four separate experiments.

2.8　Cytokine production in response to r-Sak and its variants

To determine the secretion levels of cytokine elicited by r-Sak and its variants, spleen cell culture supernatants were collected after 36 h of stimulation and INF-γ and IL-4 levels in culture supernatants were further examined. As shown in Figure 6, IL-4 secretion elicited by Sak variants was significantly lower than that elicited by r-Sak ($p < 0.05$). No INF-γ was detected following elicitation by either r-Sak or its variants (Data not shown).

3　Discussion

Since Arg77 and Glu80 residues are overlapping components of the B and T cell epitopes in the r-Sak molecule, mutagenesis of the Arg77 and Glu80 residues would be important for molecular design of any novel Sak with reduced immunogenicity. In designing our Sak variants, biochemical and structural information of both the substituting amino acids and the r-Sak molecule were carefully considered.

Highly exposed surface residues (SASAs > 70 Å^2) have been reported to often supply significant energy to the binding between antigen and antibody, through polar interactions or hydrogen bonds[16]. Onda et al. found that after mutating highly exposed surface residues (>70 Å^2) in *Pseudomonas* Exotoxin PE38 to small amino acids (Ala, Gly, or Ser), the SASAs of

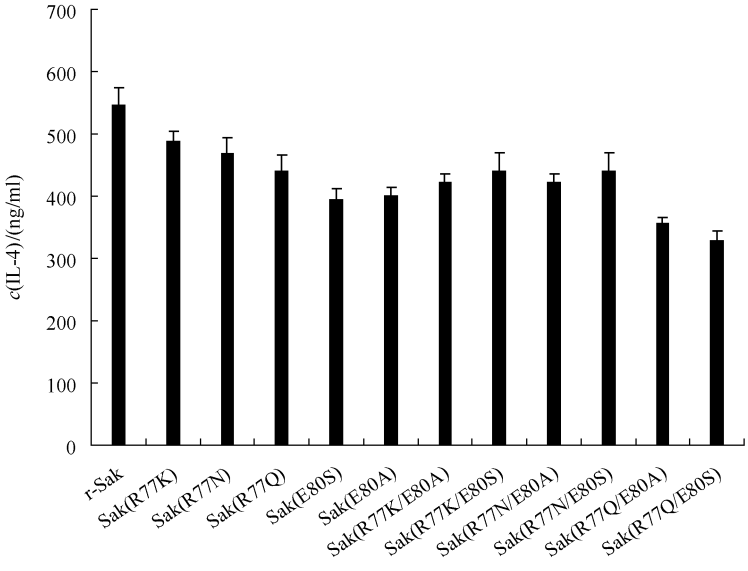

Fig. 6 IL-4 production by spleen cell cultures in response to r – Sak and its variants. Data are represented as means ± SD (n = 3) and are representative of three independent experiments.

the residues were greatly diminished and B cell epitopes were abolished in the mutant protein[17]. At the same time, alanine could be used to replace immunodominant residues of T cell epitopes and could successfully eliminate the related T cell epitope[9,18]. Comparing the SASA of r-Sak with those of its variants, mutation of Glu80 to alanine or serine within r-Sak led to significant decreases in the SASAs. Thus, in order to eliminate the related B cell epitope 1 and T cell epitope in r-Sak, small amino acids (Ala and Ser) were selected to substitute for the Glu80 residue.

The Arg77 residue in monomeric r-Sak was a highly exposed surface residue (SASAs > > 80 Å^2), but not in a ternary μPlm · SAK · μPlm complex (SASAs = 57.46 Å^2). The ternary complex was a proteinase-cofactor-substrate complex and r-Sak was within the action structure. Thus, Arg 77 was considered to have important influences on protein conformation and biological activity of r-Sak. The results from alanine scanning mutagenesis suggested that alanine substitution at position 77 would have substantial influences on the fibrinolytic activity of r-Sak[11]. To maintain this activity in the Sak variants, glutamine, asparagine, and lysine were selected to substitute for the Arg 77 residue. After the Arg77 residue was mutated to glutamine, asparagine, or lysine, position 77 remained a highly exposed surface residue (SASA > > 80 Å^2). The aim of these mutations was to provide effective removal of part of T cell epitope within Sak molecule, since these amino acids had been successfully used to remove the T-cell epitope in the C1 domain of factor VIII[19].

Biological activity analysis of these mutations showed that, apart from the Sak(R77N), Sak(R77N/E80A) and Sak(R77N/E80A) variants, all variants maintained the fibrinolytic activity of the r-Sak molecule. The values for catalytic constants of plasminogen activation by Sak-plasmin complexes further confirmed that the catalytic efficiencies of Sak(E80S), Sak(R77Q/E80A), and Sak(R77Q/E80S) were comparable to that of r-Sak, while the catalytic efficiencies of Sak(R77N) and Sak(R77N, E80A) were substantially decreased.

Immunization of BALB/c mice clearly showed that substitution mutations at position 77 and 80 led to a reduction in immunogenicity of Sak variants, as detected by levels of serum IgG antibodies elicited in mice, the ability of the variants to bind to anti-Sak polyclonal antibody, and the stimulation of specific proliferation of T cells in BALB/c mice. Cytokine

production in response to Sak variants also indicated that the Sak molecule could effectively stimulate lymphocytes to secrete substantial levels of IL-4 but not INF-γ. IL-4 is known as a Th2-specific cytokine, which regulates the IgG subclasses and induces IgE production in splenic B cells[20]. R-Sak clearly induced a Th_2-type immune response, but the significant decrease in this response elicited by Sak (E80S) and Sak(E80A) illustrated that substitution of Glu80 by alanine and serine effectively eliminated one of strong T cell epitopes in the Sak molecule. These present results provided further support for our previous suggestion that mutation of Glu80 to alanine and serine could simultaneously removed parts of the T and B cell epitopes of r-Sak[10]. When the Arg77 residue was substituted by glutamine, asparagine, or lysine, the abilities of Sak (R77N), Sak (R77Q) and Sak (R77K) to bind to anti-Sak polyclonal antibody were comparable to that of r-Sak, while their ability to stimulate proliferation of T cells from BALB/c mice was lower than that of r-Sak. Levels of IL-4 elicited by Sak(R77N), Sak(R77Q), and Sak(R77K) were significantly lower than that elicited by r-Sak ($p < 0.05$), suggesting that mutation of Arg77 had little influence on the B cell epitope of the Sak molecule, but effectively removed part of the T cell epitope. Of the six combination Sak variants, Sak(R77Q/E80A) and Sak(R77Q/E80S) induced less amount of specific IgG antibody than r-Sak, respectively. The T cell proliferative index of Sak (R77Q/E80A) and Sak (R77Q/E80S) was reduced by 51.4% and 61.8%, respectively, compared with r-Sak, which was clearly lower than the corresponding single site mutant ($p < 0.05$). The T cell proliferative index of Sak(R77Q/E80A) and Sak(R77Q/E80S) was also lower than the corresponding single site mutant Sak(E80S) and Sak (E80A) ($p < 0.05$)[10]. The IL-4 secretion elicited by Sak variants was significantly lower than that elicited by r-Sak ($p < 0.05$). The IL-4 secretion elicited by Sak(R77Q/E80A) and Sak(R77Q/E80S) was significantly lower than the corresponding single site mutant including Sak (E80S) and Sak (E80A) ($p < 0.05$). These results suggested that the combined mutant Sak (R77Q/E80A) and Sak (R77Q/E80S) could more effectively destroyed T cell epitopes within r-Sak than single site mutant including Sak(E80S) and Sak(E80A). The ability of Sak variants to bind to anti-r-Sak polyclonal serum was also significantly decreased. These results suggested that parts of the T and B cell epitopes were effectively removed after simultaneous mutation of Arg77 and Glu80 to glutamine and alanine or serine, respectively. The immunogenicity of Sak (R77Q, E80A) and Sak (R77Q, E80S) was significantly reduced when compared with that of r-Sak. Furthermore, the antibodies induced by the Sak variants could be completely bound by r-Sak, indicating that the amino acid substitutions did not generate any neoepitopes in the Sak variants. Of course, the present results were only obtained in single strain of mice. The helper T-cell epitopes of BALB/c mice are greatly different from those of other strains of mice and Human T helper cell epitopes. Thus, mice and human T lymphocytes with different MHC haplotype should be further applied to evaluate the reduction of immunogenicity of combination variants Sak (R77Q, E80A) and Sak(R77Q, E80S).

In conclusion, we have presented evidence that mutagenesis of the Arg77 and Glu80 residues of r-Sak could effectively remove parts of the T- and B-cell epitopes within the r-Sak molecule. The SASAs of positions 77 and 80 within r-Sak and its variants were used for rational design of single-site and combination variants. Nine Sak variant proteins were obtained by site-directed mutation. Investigation into the immunogenicity of Sak and its variants suggested that Sak induced a Th_2-type immune response and that substitution of immuno-dominant residues could reduce the immune response elicited by the Sak molecule without affecting its fibrinolytic function. Of Sak variants investigated, combination variants Sak(R77Q, E80A) and Sak(R77Q, E80S) effectively eliminated parts of the T cell epitopes and B cell epitopes in the Sak molecule and their immunogenicity (allergenicity) remarkably decreased compared with r-Sak. The

fibrinolytic activity and kinetic constants of Sak(R77Q, E80A) and Sak(R77Q, E80S) were comparable to those of r-Sak. Our findings provided a strategy for simultaneous elimination of T and B cell epitopes in r-Sak without affecting its therapeutic fibrinolytic activity.

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Arg77 和 Glu80 定点突变同时去除葡激酶中的 T 和 B 细胞抗原表位

徐瑞光[1]，贺进田[1*]，贾锴[1]，陈希[1]，刘建伟[1]，朱科[2]

[1]河北师范大学生命科学学院，石家庄　050016

[2]石家庄制药集团河北中润制药技术有限公司，石家庄　050041

摘要：【目的】对葡激酶的 T 和 B 细胞抗原表位重叠的关键氨基酸 Arg77 和 Glu80 进行定点突变以降低葡激酶的免疫原性。【方法】基于 Arg77 和 Glu80 的溶剂可及表面积设计葡激酶的突变体；突变体在大肠杆菌 DH5α 中进行表达。经过三步层析法纯化后，分析突变体的纤溶活性和免疫原性。【结果】免疫学实验提示，葡激酶导致 Th_2 免疫反应；Glu80 突变为丙氨酸和丝氨酸减少了溶剂可及表面积，同时去除了部分 T 和 B 细胞抗原表位；Arg77 突变为天冬酰胺、谷氨酰胺和赖氨酸仅去除了部分 T 细胞抗原表位；6 个组合突变体中，Sak(R77Q/E80A)和 Sak(R77Q/E80S)有效去除了部分 B 和 T 细胞抗原表位，降低了葡激酶的免疫原性；Sak(R77Q/E80A) and Sak(R77Q/E80S)的纤溶活性和催化效率与 r-Sak 相当。

关键词：葡激酶，T 细胞抗原表位，B 细胞抗原表位，免疫原性，蛋白质工程

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* 通信作者。Tel：+86-311-85895276；E-mail：he_jintian@yahoo.com

作者简介：徐瑞光(1984－)男，内蒙古包头人，硕士，主要从事分子生物学研究。E-mail：xuruiguang_1984@163.com

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本刊为了加强对综述投稿的管理，在 2010 年 10 月 30 日召开的《微生物学报》第九届编委会第四次会议上，讨论通过了对综述类投稿的要求，增加了作者数量和第一作者的要求。2003 年 4 月本刊出台了对综述类投稿的具体要求(6 条)，坚持实行“Mini Review”的原则，在此基础上又增加了 2 条。下面就是最新修订的具体要求，欢迎大家根据要求踊跃投稿。感谢大家对《微生物学报》的支持！

《微生物学报》综述文章投稿要求

2010 年 11 月修订

为了避免篇幅庞大、罗列文献、内容空泛、缺乏观点，力求内容更加新颖、并更具可读性，本刊对综述类投稿提出以下几点要求。

1. 本刊主要刊登微型综述(mini review)，来稿字数最好控制在 5000 字以内(不包括参考文献)。
2. 综述的选题要有新意，对读者及同行确有一定的启发作用和参考价值。
3. 参考文献应控制在 40 篇以内，近 3 年发表的文献不少于 10 篇。
4. 应结合文献扼要评述国内外学者在本领域的研究进展，不要泛泛罗列文献，只述不评。
5. 应结合自己的研究工作，就该研究领域存在的问题和解决的途径提出自己的观点。
6. 综述文章不同于研究报告，规定作者的数量应不多于 3 人。
7. 要求文章的第一作者提供一份不少于 200 字的研究经历的简介。
8. 欢迎投送“能够反映国际研究热点、对学科发展有指导意义”的述评类文章。