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Refolding of a Staphylokinase Variant Y1-Sak by Reverse Dilution

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Abstract To develop more potent thrombolytic agents with fibrinolytic and antiplatelet aggregation activity, staphylokinase (Sak) variant Y1-Sak, a recombinant mutant of the *Staphylococcus aureus* protein Sak, was constructed. Y1-Sak formed an insoluble inclusion body when overexpressed in *Escherichia coli* strain JF1125. To obtain an optimized refolding process, dilution refolding was used to optimize refolding conditions. The results revealed that additive L-arginine and refolding temperature played critical roles in the refolding of Y1-Sak. Subsequently, two refolding methods, gel filtration and reverse dilution, were investigated to refold Y1-Sak. The results indicated that the fibrinolytic activity and recovery of Y1-Sak from gel filtration were lower than those from reverse dilution. Reverse dilution refolding successfully reduced the side reaction of refolding with the help of L-arginine, and the fibrinolytic activity and recovery of Y1-Sak were significantly improved. Functional analysis revealed that refolded Y1-Sak by reverse dilution possessed fibrinolytic and antiplatelet aggregation activities. Moreover, the immunogenicity of Y1-Sak was significantly reduced.

Keywords Staphylokinase · Refolding · Gel filtration refolding · Reverse dilution refolding · RGD motif · Antiplatelet aggregation

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Abbreviations

Sak	staphylokinase
Y1-Sak	a staphylokinase variant in which N-terminal 15 amino acid residues were deleted and Ser16, Lys74, Glu75, Arg77, Lys109, Phe 111 was substituted with Lys, Ala, Ala, Ala, Arg, and Asp
rt-PA	recombinant tissue-type plasminogen activator
CV	column volumes
IBs	inclusion bodies
Gu-HCl	guanidine–HCl
RGD	Arg-Gly-Asp

Introduction

Escherichia coli is the most convenient and frequently used heterologous gene expression system. Overexpression of foreign proteins in *E. coli* often leads to formation of inclusion bodies (IBs): insoluble aggregates of misfolded proteins. These IBs can easily be isolated by centrifugation, and high purity of recombinant protein in IBs facilitates downstream purification [1–3]. However, misfolded, nonactive proteins in IBs must be solubilized and renatured into active conformation. Theoretically, unfolded protein can spontaneously fold into its native conformation following denaturant dilution. However, protein refolding is not a single reaction. The side reactions, aggregation and misfolding, can compete with the correct folding of proteins. Many methods have been developed to promote protein refolding and/or to reduce side reaction [1, 4, 5]. Traditional refolding methods, such as dialysis or dilution, have been successfully used to refold many proteins. New methods, including various chromatographic methods, are investigated to refold many proteins [4–6]. However, there is no "universal" refolding method, and a particular protein often requires specific refolding ways to reduce misfolding and aggregation. Many factors, mainly including refolding protocols and small molecular additives, could affect the rates of folding, misfolding, and aggregation as well as the stability of the intermediates. Thus, the folding conditions have to be optimized carefully to obtain a maximum yield of biologically active protein [1, 2, 7].

The plasminogen activator staphylokinase (Sak), a profibrinolytic bacterial protein, is a promising thrombolytic agent for the treatment of acute myocardial infarction [8]. However, Sak is a heterologous protein. Its administration induced neutralizing antibody formation in a majority of patients [8]. These Sak-specific antibodies preclude its readministration because they may reduce its efficacy or induce acute allergic reactions. Mutagenesis studies indicated that site-directed substitution of selected surface-exposed amino acids of the Sak molecule could significantly reduce the immunogenicity [9, 10]. Thus, a novel Sak variant Y1-Sak was constructed, in which N-terminal 15 amino acid residues were deleted and Ser16, Lys74, Glu75, Arg77, Lys109, and Phe 111 was substituted with Lys, Ala, Ala, Ala, Arg, and Asp. The deletion of the N terminus was to reduce Sak molecular weight. Then, the molecule might more easily enter the interior of thrombus and activate the plasminogen inside the thrombus [11]. After substitution mutation, Arg109, Gly110, and Asp111 composed a RGD sequence motif, which could specifically inhibit platelet aggregation [12]. Site-directed mutagenesis at Lys74, Glu75, Arg77, and Lys109 was to reduce the immunogenecity because these amino acids were associated with the immunogenecity of Sak [9]. We hoped that Y1-Sak would possess antiplatelet aggregation activity, and its

immunogenicity was reduced simultaneously. Then, the thrombolytic efficiency of Y1-Sak would be improved. However, the expression product of Y1-Sak was an IB. *In vitro* denaturation and renaturation of Y1-Sak must be performed to obtain biological activity. Two refolding methods, gel filtration and reverse dilution, were investigated to refold Y1-Sak. The results revealed that both methods were useful to refold Y1-Sak. However, reverse dilution was a more preferable method for the refolding of Y1-Sak.

Materials and Methods

Materials

E. coli strain JF1125 and expression vector pLY-4 was kindly provided by Professor Xinyuan Liu, Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China. Sephadex G-50 and Q-Sepharose FF were purchased from Amersham Pharmacia Biotech. The Bio-Rad Protein Assay was obtained from Bio-Rad Laboratories. All other chemicals used were of analytical grade.

Construction of Expression Plasmids

All routine deoxyribonucleic acid (DNA) manipulations including plasmid purification, restriction enzyme digest, ligation, and agarose gel electrophoresis were performed essentially according to the protocols described by Maniatis et al. [13].

The expression plasmid pST-Sak was previously described [10]. The mutant fragment Y1-Sak was generated by polymerase chain reaction (PCR) using the primers: forward primer 5′-CGC*GAATTC*ATG**AAA**TATTTTGAACCAAC-3′, reverse 5′-GCG*GGATCC* TTATTTCTTTTC-3′, mutagenic primer 1 for Y1-Sak, 5′-GTCTTTCCCTATAACA GAA**CGC**GGT**GACGT***G**GTACC*AGATTTATCAGAGC-3′, and mutagenic primer 2, 5′-CATTAGATGCGACAGCATAT**GCGGCA**TTT**GCG**GTAGTT*GAGCTC*GATCCAAGCG CAAAG -3′. The mutated sites are noted in bold. The restriction sites for *Sac*I, *Kpn*I, *Eco*RI, and *Bam*HI are italicized, respectively. The PCR product, which included the full length of the Y1-Sak-coding region, was ligated to plasmid ply-4 at the *Eco*RI and *Bam*HI site. Then, the expression plasmid pST-Y1-Sak was obtained. The sequence analysis confirmed that the resulting gene was identical to the designed.

Expression of Y1-Sak

Inoculum was prepared by streaking sterile Luria–Bertani agar plates containing 50 μg of ampicillin per milliliter with cells obtained from the glycerol stock culture. A single colony from the plates was used to inoculate 5 ml of the Luria–Bertani/ampicillin medium and grown for 8 h at 30 °C and 200 rpm in a gyratory shaker. Then, 0.5 ml of this culture was transferred aseptically to 500 ml of the Luria–Bertani/ampicillin medium and cultured for another 8 h under the same conditions. Subsequently, the cells were transferred into 25 l of M_9 medium supplemented with a mixture of 2% (*w*/*w*) glucose and 2% (*w*/*w*) casamino acids in a 30-l fermentor [14]. This culture was grown at 30 °C until the culture turbidity (optical density at 600 nm) reached 3.5. Then, the culture temperature rose swiftly from 30 °C to 42 °C, and the cells were grown at 42 °C for 2.5 h before harvest. Cells harvested from a 30-l fermentor were then either frozen at −20 °C or processed immediately.

Isolation and Purification of Inclusion Bodies

In a typical preparation, 50 g cell pellet was resuspended in 1,000 ml disruption buffer (50 mM phosphate-buffered saline [PBS], 5 mM ethylenediamine tetraacetic acid [EDTA], pH 7.4) and homogenized with high-pressure homogenizers (JHG-Q54-P70; Shanghai Zhangyan Light Industry Equipments). A total of three to five passes were performed to obtain at least 95% cell disruption. The precipitates were collected by centrifugation at 10,000×*g* and 4 °C for 30 min. SDS-PAGE analysis showed that Y1-Sak was mainly in the pellet (data not shown). Therefore, Y1-Sak was expressed mostly in the form of IBs.

Fifteen grams of crude IBs were washed three times with 50 ml disruption buffer containing 0.5% Triton X-100 in using a homemade homogenizer. The purified IBs were stored at −20 °C.

Solubilization (Denaturation) of Inclusion Bodies

Ten grams of IBs were dissolved in 200 ml of dissolved buffer containing 6 M guanidine–HCl (Gu-HCl), 50 mM phosphate buffer (PB), 100 mM NaCl, and 5 mM EDTA, pH 7.4, at room temperature for 60 min with gentle stirring. Insoluble material was removed by centrifugation at 10,000 rpm and 4 °C for 30 min. The supernatant containing 10.0 mg/ml protein was immediately subjected to dilution or size-exclusion chromatography.

Protein Refolding by Dilution

Process optimization on the renaturation of Y1-Sak was carried out using the dilution method. The effects of various factors, including pH, temperature, initial concentration of Y1-Sak, and small molecular additives, on the refolding efficiency of Y1-Sak were studied in detail. In brief, a 0.1-ml sample of isolated and dissolved IBs (10 mg/ml in 6 M Gu-HCl) was diluted 100-fold in the PB and kept at 15 °C without stirring for 1 h.

Protein Refolding by Size-exclusion Chromatography

Size-exclusion refolding was performed using a column (2.5 cm diameter×100 cm long) packed with Sephacryl S-100 gel media. Prior to sample application, the chromatographic apparatus was cooled to the chosen temperature. The Sephacryl S-100 column was first loaded with 0.75 column volumes of pH 7.4 buffer containing 0.2 M Gu-HCl followed by a gradient of 0.25 column volumes from 0.2 to 6 M Gu-HCl. When the sample was applied, the top of the column was in 6 M Gu-HCl, whereas from 0.25 column volumes downward, the Gu-HCl concentration was 0.2 M. After application of the sample and an additional small volume of high Gu-HCl buffer, the Y1-Sak was eluted with PB pH 7.4 containing 0.2 M Gu-HCl. As the proteins in the sample are excluded from the beads, they will flow through the column at a higher running speed than Gu-HCl, thus passing gradually from the denaturing to refolding buffer. The speed of this transition is governed by the flow rate. Thus, the effect of the flow rate on recovery of active Y1-Sak was investigated in the range of 0.5 to 2.5 ml/min. In addition, the effect of sample volume on the recovery of active fragment was studied in the range of 5.0 to 30.0 ml. In control experiments, the column was operated without the Gu-HCl gradient, and all other parameters were kept constant (sample volume 15 ml [10.0 mg/ml in 6 M Gu-HCl] and flow rate 1.5 ml/min).

The refolded protein was dialyzed against 0.02 M PB (pH 7.4) after size-exclusion chromatography. Then, the refolded protein was further purified by a Q Sepharose FF column

(2.5 cm diameter×10 cm long) pre-equilibrated with 10 mM PB, pH 7.4. After loading, the column was prewashed with about 1 bed volume of buffer to elute a small amount of unbound impurities. Then Y1-Sak was eluted with a linear gradient of 0–0.3 M NaCl in 20 mM PB (pH 7.4). The elution flow rate was 40 cm/h. The elution peak was detected at 280 nm. The elution peak was present at about 0.08 M NaCl, and elution fractions (120 ml) were collected.

Protein Refolding by Reverse Dilution

Protein refolding by reverse dilution was divided into two steps. First, 1,200 ml refolding buffer (0.2 M L-arginine, 1.0 mM EDTA, and 20 mM PB, pH 7.4) was added into a 50-ml sample of dissolved IBs (about 40 mg/ml protein) at a rate of 25 ml/min with mild mixing. The refolding process was done at a temperature of 15.0 °C. When the first dilution process was finished, agitation was continued for 20 min to provide enough protein-folding time. Then, 1,250 ml refolding buffer was added to further dilute the denaturant concentration. Aggregation during refolding was measured on the turbidity represented by absorbance at 450 nm. The refolded protein solution was subsequently concentrated by ultrafiltration (polyethersulfone ultrafiltration membranes, 5 kDa cutoff, Millipore), and the buffer of refolded protein solution was exchanged with PB (pH 7.4). About 250 ml concentrated solution was finally obtained.

In the purification of Y1-Sak, freshly *in vitro* refolded protein solution was loaded onto a Sephadex G-50 gel column (7.5 cm diameter×100 cm long) pre-equilibrated with 5 CV of 20 mM PB (pH 7.4). The elution flow rate was 20 cm/h. Fractions were pooled directly on the basis of the continuous chart record of the effluent. The active fraction obtained from the Sephadex G-50 column was applied further to a 300-ml Q Sepharose FF column pre-equilibrated with 10 mM PB (pH 7.4). The other processes were the same as mentioned above.

Protein Characterization

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under nonreducing conditions using 15% polyacrylamide gels and staining with Coomassie Blue R-250. Gel analysis (the thickness of bands and their absorbance) was carried out in ImageMaster VDS apparatus (Pharmacia Biotech) using ImageMaster VDS Software 2.0. Protein concentrations were determined by the Bradford protein assay (Bio-Rad) using bovine serum albumin as the standard.

High-Performance Liquid Chromatography–Mass Spectrometry

Experimental work was performed using a Waters Alliance liquid chromatography-mass spectrometry (MS) System. Chromatography for separation and determination of Y1-Sak was carried out by applying the samples to a Waters reversed-phase C18 column (3.5 μm particle size; 2.1 mm diameter×100 mm height) using a Waters 2690 Alliance high-performance liquid chromatography (HPLC) instrument. Samples were eluted with a 0.1% formic acid in water/0.1% formic acid in methanol gradient (100:0 [*v*/*v*]–0:100) in 30 min at a flow rate of 0.2 ml/min. Sample room and column temperatures were set at 10 and 20 °C, respectively. Mass spectra of the eluted Y1-Sak were obtained using a Micromass ZQ mass spectrometer equipped with an electrospray ionization (ESI) source. The following instrumental parameters were used for the ESI detection of Y1-Sak in scanning mode: scan range, 800–1,500 *m*/*z*; capillary voltage, 40 V; ion source voltage, 3.8 kV.

Circular Dichroism Spectroscopy

Circular dichroism (CD) spectra were recorded in a quartz cell with an optical path of 0.1 cm by using a Jasco J-715 spectropolarimeter (Tokyo, Japan) at a scan speed of 50 nm/min. Samples contained 13 μM protein in 5 mM PB, pH 7.4. Spectra from five scans recorded at 25 °C were averaged. The baseline was corrected by subtracting the spectra of the respective buffers collected under identical conditions. Molar ellipticity [θ] was calculated according to the formula $[\theta]=\theta\times 100/(nlc)$, where n represents the number of amino acids in the protein, l represents the path length in centimeters, and c represents the concentration in mmol/l. The secondary structure of the peptide was estimated from spectral simulations based on reference CD spectra of Yang et al. [15].

Fibrinolytic Activity Assay

The fibrinolytic activity was measured by a radial caseinolytic assay [14]. In brief, casein plates were prepared in dishes containing 1.0% agarose, 2.7% (*w*/*v*) dried skimmed milk powder, and 15 μg/ml plasminogen. Equal-diameter wells were cut for the test samples. After 10 μl of standard Sak or Y1-Sak solution was added into each well, the plates were kept in a moister chamber at 37 °C for 10–12 h. The diameter of the halo around the well was measured and used to calculate the fibrinolytic activity. Standard Sak obtained from the National Institute for the Control of Pharmaceutical and Biological Products was used to determine fibrinolytic activity of the prepared Sak. Every ampoule bottle contains 1,000 U of wild-type Sak (wt-Sak).

Antiplatelet Aggregation Assay

Human platelet-rich plasma (PRP) pooled from healthy donors was used to perform the platelet aggregation assays [10]. First, 200 μl of PRP plus 5 μl of Y1-Sak in 0.02 M PBS (pH 7.4) was incubated in a MPG-3E aggregometer (Shanghai Sylong) at 37 °C for 5 min. A total of 5 μl of adenosine diphosphate (final concentration 5 μM) was added, and the change of light transmission was recorded over 3 min. Transmission was set at 100% for platelet-poor plasma and 0% for PRP.

Immunogenicity of Sak Variants in Guinea Pigs

The comparative antigenicity of wt-Sak versus Y1-Sak was studied with guinea pigs. The guinea pigs were randomized into two groups, each with six guinea pigs. Immunization was carried out by subcutaneous injection of 50 μg/kg wt-Sak and Y1-Sak at days 0, 3, and 5, respectively. Intravenous injection of 50-μg/kg samples was performed to the immunized guinea pigs at the third week. Allergic reactions in the guinea pigs could be observed immediately.

Results

Optimization of Y1-Sak Expression and Extraction

To increase the protein production and save the time of fermentation, conditions of the expression were optimized. After the culture temperature rose from 30 to 42 °C, the growth of bacteria would continue for about only 1 h. The expression level of Y1-Sak increased

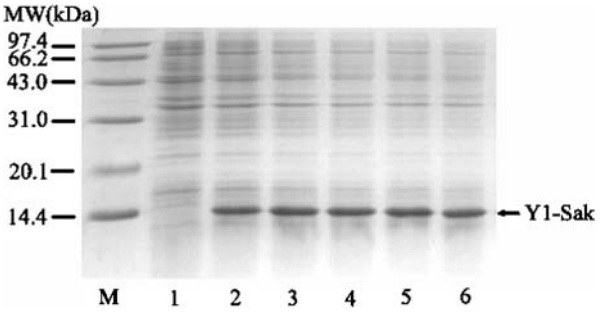

Fig. 1 SDS-PAGE analysis of expression of Y1-SAK in *E. coli* JF1125 at different induction time. *Lane M*: molecular mass standards, *lane 1*: total proteins of JF1125/pST-Y1-SAK before induction, *lane 2–6*: total proteins of JF1125/pST-Y1-SAK at 0.5, 1.0, 1.5, 2.0, and 2.5 h after induction, respectively

swiftly during the stage (Fig. 1, lanes 2, 3). Subsequently, the bacteria growth decreased progressively, while the expression level of Y1-Sak increased continuously (Fig. 1, lanes 4, 5). The maximum expression level was obtained after temperature induction at 42 °C for 2.0–2.5 h. Densitometric scanning of SDS-PAGE gels stained with Coomassie Blue revealed that Y1-Sak accounted for more than 50% of the total cell protein (Fig. 1, lane 5). The final cell yield was 9 g wet bacteria per liter culture.

Isolation of Y1-Sak Inclusion Bodies

After the bacterial cells were lysed by sonication, the expression product was analyzed by SDS-PAGE. The results showed that Y1-Sak mainly existed in the precipitates and the supernatant almost contained no target protein (data not shown). Thus, Y1-Sak was expressed mostly in the form of IBs. The crude IBs generally contain many impurities such as DNA, lipopolysaccharide, and membrane proteins. These impurities can affect the efficiency of refolding [2, 3, 7]. Before refolding of Y1-Sak, a washing process was performed as described in "Materials and Methods." After being washed three times, Y1-Sak accounted for more than 85% of total proteins in the IBs (Fig. 2).

Protein Refolding by Dilution

Dilution is a commonly used method for optimization of folding conditions. In this work, the results of refolding condition optimization indicated that small molecular additive L-arginine and refolding temperature played critical roles in increasing refolded protein activity and the specific activity. Low temperature (4 °C) could inhibit the aggregation of

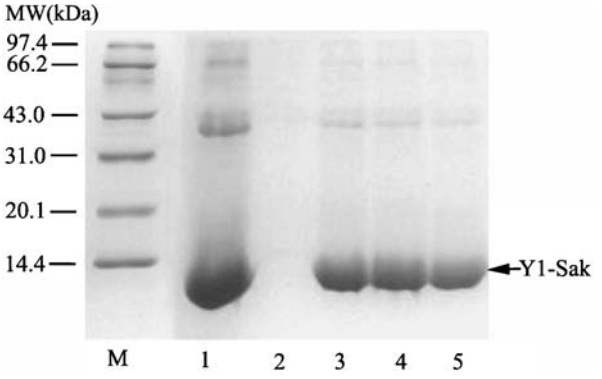

Fig. 2 SDS-PAGE analysis of IB samples. *Lane M*: Molecular mass standards, *lane 1*: IB sample before washing, *lane 2*: the supernatant after washing, *lane 3–5*, IB sample after washing one to three times, respectively

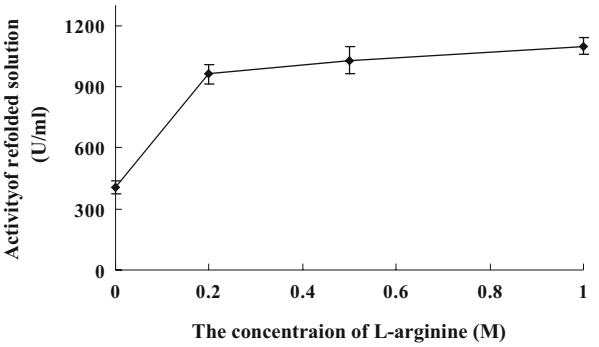

Fig. 3 The effect of L-arginine on the refolding of Y1-Sak. Dilution process was performed as described in "Materials and Methods"

Y1-Sak. However, the refolded protein-specific activity was low (14.0 kU/mg). The optimal refolding temperature was 15 °C, and the specific activity of refolding Y1-Sak was more than 30 kU/mg at 15 °C. When temperature was more than 25 °C, the high temperature-promoted aggregation and white protein precipitates were observed in the refolding solution. The activity of the refolded protein reduced significantly (lower than 20 kU/mg). Thus, the subsequent refolding process was carried out at 15 °C.

It was reported that L-arginine could be used to improve refolding of many proteins. Thus, the effect of L-arginine on the refolding of Y1-Sak was investigated. As shown in Fig. 3, 0.2 M L-arginine could significantly promote refolding of Y1-Sak. A high concentration of L-arginine had no obvious effect on the protein refolding.

Other conditions were also optimized by dilution. However, pH and initial protein concentration had little effects on refolding efficiency. Thus, the refolding buffer used in the subsequent experiments contained 0.2 M L-arginine, 1.0 mM EDTA, and 20 mM PB, pH 7.4. Even if refolding of Y1-Sak by dilution was performed at the optimized condition, the refolding yields ranged from 40% to 50% and specific activity from 30 to 35 kU/mg when the purity of refolded Y1-Sak was about 85%. These results were not satisfactory. Thus, new methods were investigated to refold Y1-Sak.

Renaturation of Y1-Sak Using Size-exclusion Chromatography

Size-exclusion chromatography could reduce denaturant concentration necessary to initiate protein refolding and aggregate removal in a single process. Thus, it was investigated to refold many kinds of proteins [5]. A column of Sephacryl S-100 was chosen as a gel medium for Y1-Sak refolding. At first, a sample of dissolved IBs was directly subjected to

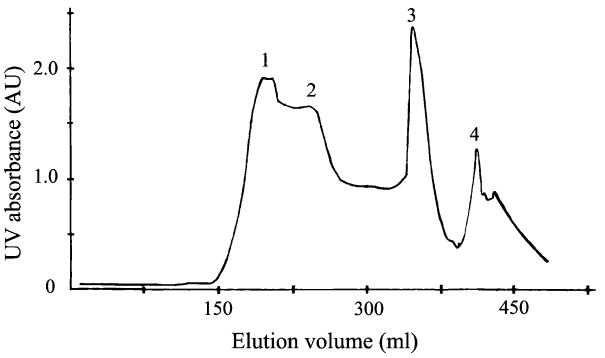

Fig. 4 Elution profile of refolded Y1-Sak after passage through a Sephacryl S-100 column equilibrated with a combination of a descending Gu-HCl gradient. A 20-ml sample containing 200 mg was applied to the column. Gu-HCl gradient was made according to "Materials and Methods"

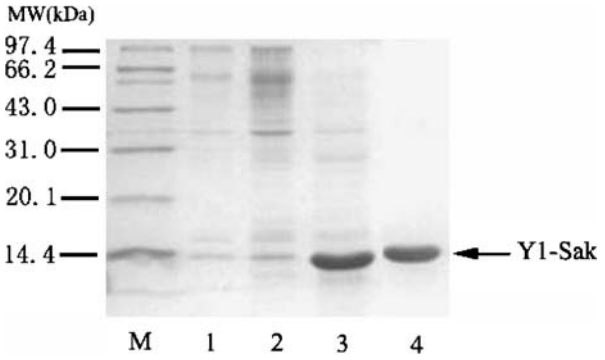

Fig. 5 SDS-PAGE analysis of fractions refolded by size-exclusion chromatography and purified by ion-exchange chromatography. *Lane M*: molecular mass standards, *lane 1–3*: peaks 1–3 at Fig. 4, respectively, *lane 4*: Y1-Sak purified by Q Sepharose FF

size-exclusion chromatography pre-equilibrated with the refolding buffer. It was discovered that a large amount of white precipitate was observed in the elution solution when elution flow rate was high (2.0 ml/min). However, if the elution flow rate was decreased (0.5 ml/min), the specific activity of protein decreased simultaneously from 35 to 16 kU/mg, although the precipitates disappeared. Therefore, a gradient of Gu-HCl was constructed in Sephacryl S-100 gel column as described in "Materials and Methods."

The elution profile of refolded Y1-Sak through the Sephacryl S-100 column was presented in Fig. 4. The fractions from size-exclusion chromatography and subsequent purification were analyzed by SDS-PAGE as illustrated in Fig. 5. The results revealed that peak 3 from the size-exclusion chromatography contained mainly target protein (Fig. 5, lane 3). Peaks 1 and 2 contained a little amount of Y1-Sak derived possibly from protein aggregates (Fig. 5, lanes 1, 2). Then, the active fraction obtained from the Sephacryl S-100 column was further applied to a Q Sepharose FF column. The purified Y1-Sak showed a single band in SDS-PAGE analysis (Fig. 5, lane 4). The effect of flow rate on the recovery of active Y1-Sak was investigated in the range 0.5 to 2.5 ml/min. The results were summarized in Table 1. The flow rate played a critical role in the refolding process. The higher the flow rate, the lower was the protein recovery. However, the specific activity of Y1-Sak exhibited a reverse tendency.

Although the refolding of Y1-Sak by size-exclusion chromatography successfully obtained protein activity, the specific activity of refolded Y1-Sak was relatively low. Thus, refolding of Y1-Sak by reverse dilution was further investigated.

Renaturation of Y1-Sak Using the Reverse Dilution Method

Based on the results obtained above, larger-scale protein refolding by reverse dilution was performed under the optimized conditions (0.2 M L-arginine, 0.05 M PB, pH 7.4, 15 °C).

Table 1 Effect of flow rate on activity of Y1-Sak using gel filtration with Gu-HCl gradients[a].

Flow rate (ml/min)	Specific activity (kU/mg)	Recovery of Y1-Sak (%)
0.5	26.3	21.1
1.0	35.1	20.5
1.5	45.2	17.8
2.0	43.6	16.5
2.5	39.8	14.2

[a] The purity of samples used was more than 95% determined by HPLC analysis.

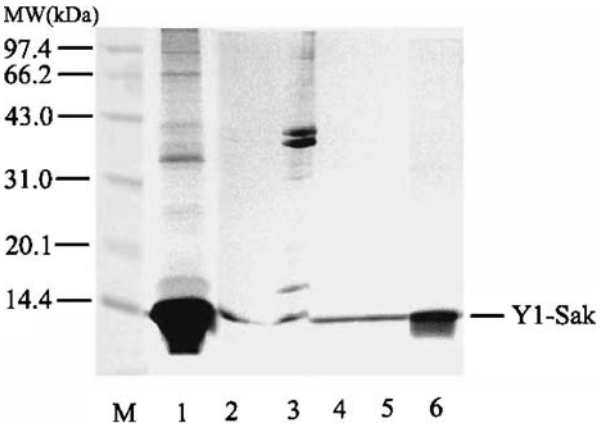

Fig. 6 SDS-PAGE analysis of fractions refolded by reverse dilution and purified by ion-exchange chromatography. *Lane M*: molecular mass standards, *lane 1*: total proteins of Y1-Sak IBs, *lane 2*: fraction after reverse dilution, *lane 3*: precipitate after concentration of ultrafiltration, *lane 4*: supernatant after concentration of ultrafiltration, *lane 5*: fraction purified by Sephadex G-50, *lane 6*: fraction purified by Q Sepharose FF

The resulting protein solution in a large volume was then concentrated by ultrafiltration. To prevent denaturation of the refolded protein, the residue denaturant (Gu-HCl) was reduced simultaneously by buffer exchange during the ultrafiltration process. The concentrated solution was subsequently purified by chromatography on Sephadex G-50 and Q Sepharose FF. The SDS-PAGE analysis of the fractions during refolding by reverse dilution and purification is presented in Fig. 6. Some white precipitates were observed during the ultrafiltration process. SDS-PAGE analysis indicated that the precipitates were mainly of the impurity proteins (Fig. 6, lane 3). Some target proteins appeared at the precipitates (Fig. 5, lane 3). They might be derived from unfolded or misfolded Y1-Sak. The main fraction obtained showed one target band on SDS-PAGE analysis after purification by gel filtration and ion-exchange chromatography.

This purification process was repeated three times at the pilot scale for an overall evaluation. The main parameters, such as yield, purity, and elution profiles of the three tests, were highly reproducible. Purification runs resulted in final product yields ranging from 30% to 35% and a purity of more than 95%. The specific activity of purified Y1-Sak reached 62 kU/mg. The key results from the process of reverse dilution refolding are summarized in Table 2.

Table 2 Results of each step used for refolding of Y1-Sak by reverse dilution[a].

	Weight (g)	Total proteins (mg)	Total activity (kU)	Specific activity (kU/mg)	Recovery (%)	Purity[b] (%)
Washed inclusion bodies	10.3	2,100	–	–	100	80
Fractions after reverse dilution		1,520	6.5×10^4	43	72.4	85
Fraction after ultrafiltration		1,159	5.0×10^4	43	55.2	85
Sephadex G-50		819	4.4×10^4	54	39.0	92
Q Sepharose FF		564	3.5×10^4	62	32.2	96

[a] 50 g of wet weight cells was used in this preparation.

[b] Protein purity was estimated from densitometric measurements using 15% SDS-PAGE or determined by HPLC analysis.

Identification of Purified Y1-Sak

The target proteins derived from the two refolding methods, gel filtration and reverse dilution, were identified, respectively. On SDS-PAGE, the purified Y1-Sak showed a single band with an apparent molecular mass of 14 kDa (Fig. 5, lane 4 and Fig. 6, lane 6). To identify the homogeneity of the refolded Y1-Sak, HPLC-MS analysis was performed as described in "Materials and Methods." The results revealed that the refolded Y1-Sak by reverse dilution gave a single symmetrical peak and had purity greater than 95% (Fig. 7a). The molecular mass of refolded Y1-Sak by reverse dilution could be calculated from the

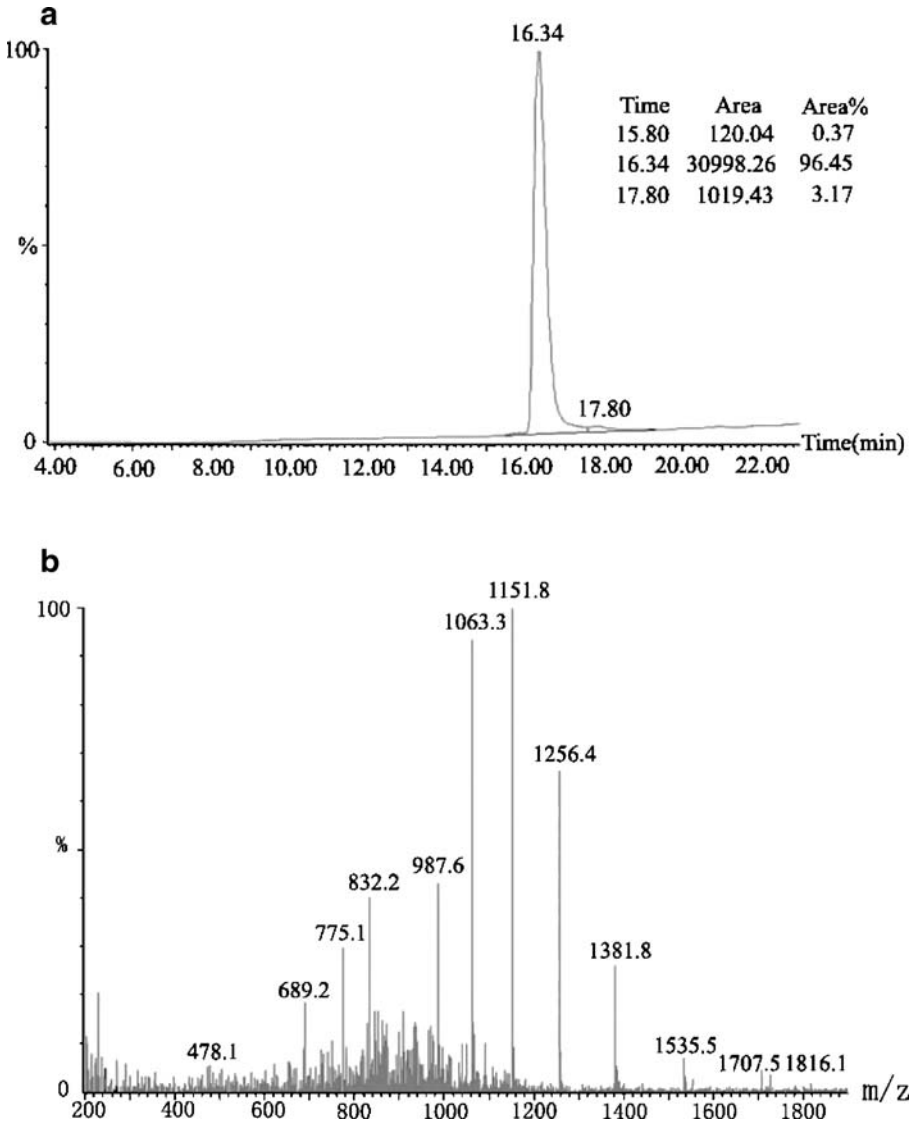

Fig. 7 HPLC-MS analysis of purified Y1-Sak. The concentration of Y1-Sak was 1 mg/ml. All LC parameters and MS conditions were as described in "Materials and Methods." **a** LC profile, **b** MS spectrum

MS spectrum (Fig. 7b). The calculated molecular mass of 13,809 Da is shown in Table 3, a value that was in accordance with the theoretical molecular mass of 13,796 Da. The difference of 13 mass units was within the accuracy of this method. The similar results of HPLC-MS analysis of Y1-Sak from gel filtration are summarized in Table 3.

Characterization of the Refolded Y1-Sak

The CD spectra of Y1-Sak from reverse dilution refolding and Y1-Sak from gel filtration refolding were similar to that of wt-Sak. Analysis of the far-ultraviolet CD spectra showed that Y1-Sak from reverse dilution and protein from gel filtration refolding comprised a similar content of β-sheet, α-helix, and turns and coil (Table 3). It was suggested that the secondary structure of Y1-Sak from different refolding methods have no significant differences with wt-Sak.

Furthermore, Immunogenicity of Y1-Sak in guinea pigs and its antiplatelet aggregation activity were assayed as described previously [10]. Researches on the allergic reactions in the guinea pigs by intradermal injections of Y1-Sak and wt-Sak revealed that 100% of the guinea pigs of the wt-Sak group (six of six) suffered extreme allergic reaction, anaphylactic shock, or death, while none of the Y1-Sak group (zero of six) suffered extreme allergic reaction (Table 3). These data showed that Immunogenicity of Y1-Sak was significantly lower than wt-Sak ($P<0.05$). The analysis of antiplatelet aggregation activities indicated that the inhibition rate of Y1-Sak was 15.2% at a final concentration of 2 μM (Table 3). The inhibitory effects of the variant were significant, compared with that of wt-Sak ($P<0.05$).

Discussion

Several factors would affect refolding of proteins from IBs, which include methods to remove the denaturant, refolding conditions, and small molecule additives [1–3, 5, 7]. Thus, optimization of folding conditions should be first performed. Dilution of the solubilized protein directly into the refolding buffer is the most commonly used method in small-scale refolding studies because of its simplicity. Refolding conditions of Y1-Sak was optimized by dilution refolding in this work. Results revealed that L-arginine plays a critical role in increasing refolded protein activity and the specific activity of Y1-Sak is greatly affected by refolding temperature. However, the recovery and specific activity of Y1-Sak was relatively low even if refolding was performed at optimized conditions. On the basis of the refolding

Table 3 Characterization of the refolded Y1-Sak by reverse dilution and by gel filtration.

		wt-Sak	Y1-Sak	
			Reverse dilution	Gel filtration
Purity (%)		95.2	96.0	95.6
Specific activity (kU/mg)		95	62	45
Molecular mass calculated from MS		–	13,809.4	13,806.4
Content of second structure (%)	α-helix	18.5	18.5	19.2
	β-sheet	36.4	38.2	34.2
	turns and coil	45.2	43.3	46.6
Inhibition rate of platelet aggregation (%, $n>3$)		2.3±1.5	15.2±4.5	
Allergic reactions ($n=6$)		100%	0	

condition optimization, two denaturant removal methods, gel filtration and reverse dilution, were used to refold Y1-Sak. Refolding by a gel chromatographic process is attractive because it is easily automated using commercially available preparative chromatography systems and can often be combined with simultaneous partial purification [5]. However, fibrinolytic activity and recovery of refolded Y1-Sak was still low when gel filtration was used to refold Y1-Sak (Table 1). The reverse dilution process was then investigated to refold Y1-Sak. During reverse dilution, both the denaturant and protein concentrations decreased simultaneously when the refolding buffer was added into an unfolded protein solution. Nevertheless, protein concentration is relatively high, and the proteins easily form aggregates at an intermediate denaturant concentration. It was fortunate that no protein precipitates were observed during reverse dilution refolding of Y1-Sak. The recovery and activity of Y1-Sak were significantly improved.

After site-directed mutation, Y1-Sak lost the 16 amino acids at the N terminus of wt-Sak. Moreover, Ser16, Lys74, Glu75, Arg77, Lys109, and Phe 111 was substituted with Lys, Ala, Ala, Ala, Arg, and Asp, respectively. A RGD motif was then produced in the Y1-Sak. The analysis of antiplatelet aggregation activity indicated that Y1-Sak possessed certain inhibitory function on platelet aggregation. Moreover, the immunogenicity of Y1-Sak was obviously reduced. A suitable *in vivo* animal model should be applied to evaluate their thrombolytic efficacy.

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