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Full Length Research Paper

High-level expression, purification and properties of a fully active even glycosylated staphylokinase variant SakϕC from *Staphylococcus aureus* QT08 in *Pichia pastoris*

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Staphylokinase (Sak) (EC 3.4.99.22) is a 136-aa protein secreted by the lysogenic phase of *Staphylococcus aureus* and functions as potential clot dissolving agent for treating blood-clotting disorders. Sak converts the zymogen human plasminogen (hPg) to its active serine protease form, plasmin (hPm) and forms 1:1 complex with hPm, which activates other hPg molecules. In this report, we described high-level expression, purification and characterization of a fully active even glycosylated staphylokinase variant SakϕC from *S. aureus* QT08 in a eukaryotic system *Pichia pastoris* GS115. The *sak* gene of 411 bp encoding a mature staphylokinase (136 aa, 15.5 kDa) was amplified from *S. aureus* QT08 genomic DNA, inserted into the expression vector pPICZαA under the control of the *AOX1* promoter using methanol as an inducer and expressed in *P. pastoris* GS115 with a yield of 19 mg/L culture broth. The recombinant staphylokinase (rSak) was purified to homogeneity using ProBond™ Ni^{2+}-affinity chromatography with a specific activity of 20658 U/mg. The enzyme was expressed as an *N*-glycosylated protein in *P. pastoris* with a molecular mass of approximately 24 kDa on SDS-PAGE. rSak showed an optimum temperature of 30 to 37ºC and optimum pH 7.5 (phosphate buffer) and pH 8 (Tris-HCl buffer). rSak was stable in a temperature range of 15 to 37°C and pH of 4 to 9. Additives (metal ions and detergents) all inhibited slightly or strongly the rSak activity. For the first time, a fully staphylokinase from *S. aureus* was expressed in *P. pastoris* even it was glycosylated.

Key words: *Staphylococcus aureus* QT08*, Pichia pastoris,* fully active staphylokinase*,* high-level expression, purification, characterization, glycosylation.

INTRODUCTION

As a clinical treatment for myocardial infarction, activating endogenous plasminogen effectively dissolves pathologic clots and saves patient lives. Plasminogen activators, identified and cloned from human sources (tissue plasminogen activator [t-PA] and urokinase-like plasminogen activator [u-PA]) and bacterial sources (streptokinase [SK] and staphylokinase [Sak]), can catalyze the conversion of circulating plasminogen to the active protease plasmin (Verstraete, 1995).

Sak is a recently rediscovered plasminogen activator and its gene is cloned from *Staphylococcus aureus* bacteriophage 42D (Sako, 1985; Behnke and Gerlach, 1987) or lysogenic strains of *S. aureus*. Sak is a 136 amino acid protein that exhibits many desirable thrombolytic properties *in vivo*. Sak is reported not to be a proteolytic enzyme and forms a 1:1 stoichiometric

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complex with plasmin(ogen) that converts other plasminogen molecules to plasmin, a potent enzyme that degrades proteins of the extracellular matrix. The high affinity of the Sak-plasminogen complex for fibrin makes it a promising thrombolytic agent (Rajamohan et al., 2002). Sak exhibits better fibrin specificity than t-PA (without having direct affinity for fibrin) and is capable of dissolving platelet-rich clots (Collen et al., 1993). In human plasma milieu, thrombolysis with SK is highly non-fibrin-selective, whereas thrombolysis with Sak is highly fibrin-selective (Collen and Lijnen, 1994). Sak lacks any affinity for fibrin and the ability to inhibit thrombin, two functions which would supplement and potentially improve its thrombolytic potency. Both of these functions can be combined with Sak by constructing translational fusions with fibrin-binding or anti-thrombin protein domains.

So far, *sak* gene from several *S. aureus* strains has been cloned and expressed at various levels in different expression systems including *Escherichia coli* (Sako, 1985; Lee et al., 1998; Ren et al., 2008; Mandi et al., 2009), *Bacillus subtilis* (Ye et al., 1999), *Streptomyces lividans* (Cheng et al., 1998), and *Pichia pastoris* (Apte-Deshpnade et al., 2009). In a previous study, we cloned, over-expressed, purified and characterized a staphylokinase from *S. aureus* QT08 in *E. coli* BL21 (data not shown) identified as the variant Sakϕ C among 3 natural variants Sakϕ C, Sak42D, and SakSTAR which differ in only three amino acids 34, 35, and 43 in the mature protein: GGH, GRR, and SGH, respectively (Schlott et al., 1997).

The amino acid sequence of Sak showed a single potential *N*-linked glycosylation site at Asn28 which when glycosylated might be detrimental for its hPg activator activity (Miele et al., 1999). Thus, Apte-Deshpnade et al. (2009) tried to achieve a hyperexpressing, non-glycosylated, and active rSak in *P. pastoris* by integrating the *sak* gene into the *Pichia* genome and induction with methanol in the presence or absence of tunicamycin, an *N*-linked glycosylation inhibitor. The non-glycosylated rSak expressed after tunicamycin treatment showed hPg activation similar to rSak produced from bacterial expression system.

The purpose of this study was to overexpress a staphylokinase variant Sakϕ C from *S. aureus* QT08 in *P. pastoris* GS115 in an extracellular and active form. Interestingly, we found that in contrast to the study of Apte-Deshpnade et al. (2009), this staphylokinase even glycosylated showed a higher specific activity (20658 U/mg protein) than that expressed in *E. coli* (15175 U/mg protein) as non-glycosylated form (data not shown).

MATERIALS AND METHODS

Chemicals and reagents

Restriction enzymes, *Taq* DNA polymerase, and T4 ligase were supplied from Fermentas, part of Thermo Fisher Scientific Inc. (Waltham, USA); kit ProBond^{TM} Nickel-Chaleting Resin was from Invitrogen Corp. (Carlsbad, USA); 4-Chloro-1-naphthol (horseradish peroxidase color development reagent) from Bio-Rad Laboratories, Inc. (Berkeley, USA). Human plasminogen from MP Biomedicals (Santa Ana, USA), N-(p-tosyl)-gly-pro-lys 4-nitroanilide acetate salt (AAS), and anti-rabbit IgG (whole molecule)-peroxidase antibody from Sigma-Aldrich Co. (St. Louis, USA) were employed. Peptone and yeast extract were purchased from Bio Basic Inc. (New York, USA). All other reagents were of analytical grade unless otherwise stated.

Vectors, strains and culture conditions

The bacterial strain *S. aureus* QT08 (GQ247719) isolated from a patient at the Army Hospital No. 103 (Hanoi, Vietnam) was used as the source of staphylokinase (Sak) gene. *E. coli* DH5α (F–, ø80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deo*R, *recA*1, *endA*1, *hsdR*17(rk–, mk+), *phoA*, *supE*44, λ–, *thi*-1, *gyrA*96, *relA*1) and pJET1.2/blunt vector (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA) were used for DNA manipulations and amplification. *P. pastoris* host strain GS115 and pPICZαA (Invitrogen Corp., Carlsbad, USA) were used for expression of the staphylokinase. Luria-Bertani medium (LB) containing 1% (w/v) bacto tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl, at a pH of 7-7.5 was used for the cultivation of *E. coli*. LB agar contained additionally 2% (w/v) agar and 100 µg ampicillin/ml; low salt LB (1% (w/v) bacto tryptone, 0.5% (w/v) yeast extract, and 0.5% (w/v) NaCl) agar contained 25 µg zeocin/ml.

DNA manipulations

Genomic and plasmid DNA isolation was carried out by methods as previously described (Quyen et al., 2007). DNA fragments and PCR products were excised from a 0.8% agarose gel and purified by a gel extraction kit (Qiagen, Venlo, Netherlands) according to manufacturer's instruction. DNA sequencing was performed on ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems Inc., Foster City, USA). *E. coli* DH5α cells were transformed using heat shock method that has been previously described (Quyen et al., 2007).

DNA amplification and plasmid construction

Based on the nucleotide sequence of *sak*, encoding staphylokinase from *S. aureus* QT08 (FJ868207), two oligonucleotides SakF3 (5'-GCG AAT TCT CAA GTT CAT TCG ACA AAG GA-3') and SakR3 (5'-GCT CTA GAG CTT TCT TTT CTA TAA CAA C-3') were designed as primers to amplify the gene *sak* from *S. aureus* QT08 with the introduction of the *Eco*RI and *Xba*I restriction sites at 5' of forward and reverse primer, respectively. The mature Sak-coding DNA fragment (411 bp) was amplified from *S. aureus* QT08 genomic DNA by PCR with *Taq* DNA polymerase. The PCR mixture contained 2.5 µl 10× PCR buffer, 2 µl of 2.5 mM dNTP, 2 µl of 25 mM $MgCl_2$, 1 µl genomic DNA (50 ng), 0.5 µl 5 unit *Taq* polymerase, and 1 µl each primer (10 pmol), supplemented with 15 µl distillated water to a final volume of 25 µl. The thermocycler conditions were as follows: 94°C/5′; 30 cycles of 94°C/45″, 55°C/30″, 72°C/45″; 72°C/10′. The PCR products were inserted into the pJET1.2/blunt vector, resulting in pJSak and sequenced. It was followed by ligation of the *Eco*RI-*Xba*I digested pJSak products with pPICZαA linearized by the same enzymes, resulting in pPSak under the control of the *AOX1*-promoter induced by methanol and possessing the zeocin resistance marker. The staphylokinase Sak-his encoded by the plasmid pPSak contains the mature

staphylokinase fused with the 6x histidine-tag and no native leader sequence.

Yeast transformation and screening

The plasmid pPSak linearized with *Sac*I was then transformed into *P. pastoris* GS115 according to the manufacturer's instructions for the EasySelect™ Pichia Expression Kit (Invitrogen Corp., Carlsbad, USA). Transformants were screened on YPDS [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 1 M sorbitol, 2% (w/v) agar] plates containing zeocin at a final concentration of 100 µg/ml. The presence of the *sak* gene in the transformants was confirmed by PCR using yeast genomic DNA as template and *sak*-specific primers. Clones that showed the right size of the PCR product and zeocin resistance were selected for expression.

Gene expression

P. pastoris transformants were grown in 20 ml of YP medium [1% (w/v) yeast extract, 2% (w/v) peptone] supplemented with 1% (w/v) glycerol at 30°C with agitation at 220 rpm until an $OD_{600\ nm}$ reached 5 to 6. The cell pellet was harvested by centrifugation at 4500 rpm for 5 min. For *AOX1* promoter-controlled expression of Sak, the cell pellet was resuspended in 25 ml of YP medium supplemented with 1% (v/v) methanol and every 24 h to maintain induction. Cultivation was performed at 30°C and 220 rpm. The culture supernatant was collected periodically to detect Sak activity.

Purification of recombinant Sak

The purification of the recombinant His-tagged rSak was carried out according to the manufacturer's instructions (Invitrogen Corp., Carlsbad, USA). The culture supernatant containing rSak was harvested from 25 ml culture by centrifugation at 8000 rpm and 4°C for 5 min and 10 ml of culture supernatant was applied to a ProBond™ Ni^{2+}-affinity column and rinsed with wash buffer. The rSak was eluted with 5x 1 ml of native elution buffer.

Home-made production of anti-rSak serum

2 New Zealand rabbits (Central Company of Veterinary Vaccine, Vetvaco, Hanoi, Vietnam) were used for anti-rSak antibody production. Preimmunized blood samples were taken from the ear veins of each rabbit. For the initial immunization, rabbits were immunized subcutaneously with purified rSak expressed from *E. coli* (200 µg per rabbit) emulsified in Freund's complete adjuvant. Subsequent booster injections were given every week with 200 µg purified rSak in Freund's incomplete adjuvant. Ten days after the fourth immunization, the rabbits were sacrificed. The sera were collected from blood, portioned into Eppendorf tubes, 1 ml per tube and stored in deep freezer at -80°C.

Western blot analysis

Western blot analysis was carried out according to the protocol described in "Protein Methods" (Bollag et al., 1996). The PVDF membrane was embedded in the primary antibody (anti-rSak) serum solution diluted to 1:1000 in 1% (w/v) nonfat milk and incubated in the secondary anti-rabbit IgG (whole molecule)-peroxidase antibody solution diluted to 1:10000 in 1% (w/v) nonfat milk. Finally the membrane was colored with 4-chloro-1-naphthol (HRP color development reagent).

Electrophoresis analysis and protein concentration

The homogeneity and molecular mass of the staphylokinase were determined by 12.5% SDS polyacrylamide gel electrophoresis (Laemmli, 1970) with Biometra equipment (Göttingen, Germany). Proteins were visualized by staining with Coomassie Brilliant Blue R-250. Protein concentrations were estimated by the Bradford assay with bovine serum albumin as standard (Bradford, 1976).

rSak assay

The Sak activity was determined by using a coupled plasminogen-chromogenic substrate according to the method as previously described (Hernandez et al., 1990). The Sak activity was assayed by mixing 2 µl plasminogen (0.025 U/µl) in 10 µl of 20 mM Tris-HCl buffer pH 7.5 and 10 µl of appropriate dilution of rSak (30 ng) in 50 mM potassium phosphate buffer (pH 7) and incubated at 37°C for 30 min. The plasminogen*Sak complexes were mixed with 40 µl of 1 mM chromogenic substrate AAS in 50 mM potassium phosphate buffer (pH 7) and incubated at 37°C for 15 min. The reaction was stopped by adding 10 µl of 0.4 M acetic acid and the absorbance was measured at 405 nm. One unit (U) of Sak is defined as one unit of standard streptokinase from Sigma-Aldrich Co. (St. Louis, USA) liberating a standard clot of fibrinogen, plasminogen, and thrombin at pH 7.5 and 37°C for 10 min. The units of Sak were calculated by using a standard curve of pure standard streptokinase (Sigma-Aldrich Co., St. Louis, USA).

Temperature and pH optimum

The pH and temperature optimum of rSak were determined by measuring the activity, as described above, using a 50 mM acetate buffer (pH 4 to 6), a 50 mM potassium phosphate buffer (pH 5.5 to 7.5), and a 50 mM Tris-HCl buffer (pH 7.5 to 9.5) at 37°C for 30 min, and in the temperature range of 20 to 45°C at a pH 7 for 30 min, respectively.

Temperature and pH stability

For the determination of temperature and pH stability, purified rSak, 7 ng for each reaction, was preincubated in 50 mM potassium phosphate buffer (pH 8) at different temperatures 15 to 80°C for 0 to 8 h, and under various pH conditions, with 50 mM acetate at pH 4, potassium phosphate at pH 6 to 7 and Tris-HCl at pH 8 to 9, at 37°C for 0 to 8 h, respectively. The residual activity was then determined as above mentioned.

Effect of metal ions, EDTA and detergents

Purified rSak, 7 ng for each reaction, was incubated with 2.5 mM and 25 mM of various metal ions (Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}, Ag^{+}, and K^{+}) and EDTA and in 10% (w/v) of different detergents (Tween 80, Tween 20, SDS, and Triton X-100) at 37°C for 60 min. The residual activity was then determined.

All measurements were carried out in triplicate with the resulting values being the mean of the cumulative data obtained.

Glycosylation prediction

The glycosylation prediction was analyzed by using the glycosylation prediction program NetOGlyc-3.1 (www.cbs.dtu.dk/services/NetOGlyc/) for the *O*-glycosylation site

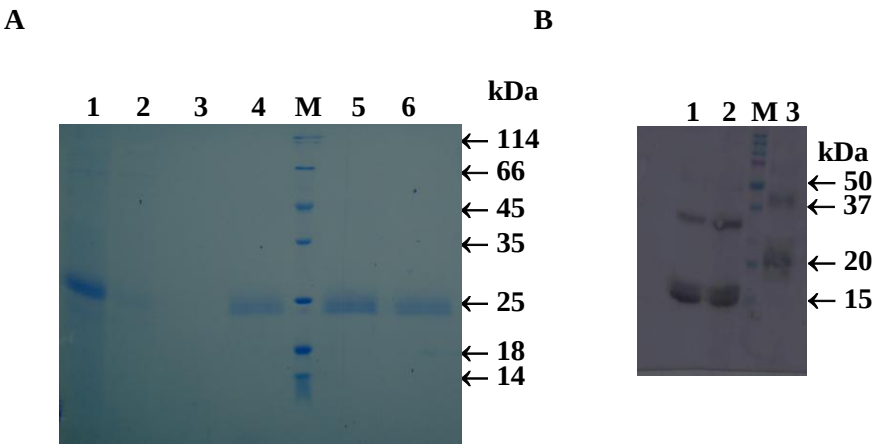

Figure 1. SDS-PAGE analysis of rSak samples (A). Lane 1, *P. pastoris* GS115/pPSak culture supernatant of rSak; Lane 2, through-column sample; Lane 3, column-washing; Lane 4-6, eluates 1, 2, and 3; Lane M, standard proteins (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA). Western blot (B) of rSak expressed in *E. coli* (lane 1, 2) and expressed in *P. pastoris* (lane 3) and Lane M, standard proteins (Bio-Rad Laboratories, Inc., Berkeley, USA).

and the glycosylation prediction program NetNGlyc-1.0 (www.cbs.dtu.dk/services/NetNGlyc-1.0/) for the *N*-glycosylation site. The residue is predicted as glycosylated if the prediction score is >0.5 (Julenius et al., 2005).

RESULTS AND DISCUSSION

Expression of a fully active rSak even glycosylated

In a previous study, we cloned, over-expressed, purified and characterized a staphylokinase from *S. aureus* QT08 in *E. coli* BL21 (data not shown) identified as the variant SakϕC among three natural variants SakϕC, Sak42D, and SakSTAR which differ in only three amino acids 34, 35, and 43 in the mature protein: GGH, GRR, and SGH, respectively (Schlott et al., 1997). The DNA fragment (411 bp) encoding the mature staphylokinase (Sak) truncated 27 N-terminal amino acids from *S. aureus* QT08 was inserted into pPICZαA vector at the *Eco*RI and *Xba*I sites resulting in the recombinant plasmid pPSak which was linearized with *Sac*I and introduced into *P. pastoris* by transformation.

P. pastoris GS115/pPSak transformants were grown in YP medium and after 48 h of methanol induction, the culture supernatants were used for enzyme activity assay. The transformant showing the highest Sak activity was used for enzyme production, purification, and characterization. rSak was purified from the culture supernatant of *P. pastoris* GS115/pPSak (Figure 1A, lane 1) by affinity chromatography with Ni^{2+}-ProBond™ resin to the homogeneity on SDS-PAGE with a molecular mass of approximately 24 kDa (Figure 1A, lane 4, 5, 6). The Western blot analysis with the rabbit serum produced by the injection with the purified rSak expressed in *E. coli* also reconfirmed that the rSak expressed in *P. pastoris* had a molecular mass of >20 kDa (Figure 1B, lane 3), whereas the molecular mass of the rSak expressed in *E. coli* was approximately 15.5 kDa (Figure 1B, lane 1,2).

The higher molecular mass of the rSak, compared with the expected size (15.5 kDa) calculated from its deduced amino acid sequence, might be due to the glycosylation of rSak. The glycosylation prediction program NetOGlyc-3.1 revealed that no serine or threonine was predicted as the *O*-glycosylation site in the rSak sequence, with a G-score of less 0.286. If the G-score is >0.5, the residue is predicted as glycosylated; the higher the score, the more confident the prediction (Julenius et al., 2005). However, the rSak sequence was predicted to be *N*-glycosylated (Asn-Xaa-Ser/Thr sequins) in the asparagine position 28 of the rSak sequence (NVT) using the glycosylation prediction program NetNGlyc-1.0 with a predicted potential of 0.8312 (threshold 0.5). Recombinant proteins derived from *Pichia* have predominantly *N*-linked glycosylation with little *O*-linked glycosylation (Grinna and Tschopp, 1989; Cregg et al., 1993). The *N*-glycosylation pathway of the yeast *P. pastoris* required $Gal_2GlcNAc_2Man_3GlcNAc_2$*N*-glycans to modify its glycoproteins (Jacobs et al., 2008). The glycosylation prediction for our rSak agreed quite well with other studies that the Sak protein had a single site for *N*-glycosylation at the Asn28 residue, when expressed in *P. pastoris* (Miele et al., 1999).

The 6xhis tag has a molecular mass of 0.839 kDa, thus the difference in the molecular mass (6-8 kDa) here demonstrated clearly a glycosylation. It is surprising that in our study practically 100% of the expressed Sak was glycosylated. No non-glysocylated form of rSak (15.5 kDa) was found in the supernatant and also in the eluate on SDS-PAGE (Figure 1A, lane 1, 4, 5, 6) and on Western blot (Figure 1B, lane 3). The Western blot

Table 1. Purification procedure of rSak from the culture supernatant of *P. pastoris* GS115/pPSak.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor
Culture supernatant	6321±19	0.583±0.024	10847±32	100	1
Ni^{2+}-ProBond™ resin column	3857±90	0.187±0	20658±48	61	1.9

showed 2 forms of rSak, monomeric (>20 kDa) and dimeric protein (>40 kDa) (Figure 1B, lane 3). Our finding was completely different to other reports, (Miele et al., 1999), using the same *P. pastoris* strain obtained a mix of glycosylated and non-glycosylated forms with a ratio of 5 to 6. Apte-Deshpnade et al. (2009) obtained also most glycosylated form of rSak with a molecular mass of approximately 18 kDa that was expressed in *P. pastoris* without treatment of tunicamycin as an *N*-linked glycosylation inhibitor.

Surprisingly, two glycosylated rSaks [GenBank Accession No. E02873 (Mandi et al., 2009), and FJ868207 (in this study)] expressed in *P. pastoris* GS115 showed a different molecular mass of 18 kDa (Apte-Deshpnade et al., 2009) and 24 kDa, respectively, although their amino acid sequences (136 amino acids) showed 100% identity (data not shown). Another glycosylated staphylokinase variant SakSTAR expressed in *P. pastoris* GS115 using the pPIC9K vector also showed a molecular mass of approximately 18.5 kDa (Miele et al., 1999). The only explanation might be that the 18 and 24 kDa rSak was expressed in *P. pastoris* GS115 using a multiple integration vector pPIC9K and a normal expression vector pPICZαA, respectively.

The purified rSak showed a specific activity of 20658 U/mg with a purification factor of 2 and a yield of 61% (Table 1). Another finding was that the glycosylated rSak expressed in *P. pastoris* GS115 showed a high activity of 20658 U/mg with N-(p-tosyl)-gly-pro-lys 4-nitroanilide acetate salt (AAS) as substrate in this study and 2.5 U/mg with val-leu-lys-*p*-nitroanilide (S2251) as substrate in the report of Apte-Deshpnade et al. (2009), even the activity of the non-glycosylated rSak was also low 95 U/mg, 8263 and 217 times lower than rSak in our study, respectively. Amount of rSak protein that released 1 mM p-NA per min was considered as one unit (U) of rSak in both studies.

In contrast to the rSak expressed in *P. pastoris* GS115, the recombinant Sak expressed from *E. coli*/pET21a-Sak clone showed a specific activity of 2197420 U/mg protein with S2251 as substrate (Mandi et al., 2009), 145 times higher than rSak from *S. aureus* QT08 expressed in *E. coli*/pET21a-Sak (15175 U/mg protein with AAS as substrate) (in our study, data not shown). In another study, the glycosylated staphylokinase variant SakSTAR did not show any activity with S2251 as substrate, however, the non-glycosylated SakSTAR displayed a high level of plasminogen activator activity (Miele et al., 1999).

The yield of the rSak production by *P. pastoris* in our study (19 mg/L) was quite lower in comparison to that in other studies [1 g/L (Apte-Deshpnade et al., 2009) or 33-50 mg/L (Miele et al., 1999)]. The prokaryote expression system *E. coli* could produce Sak at a high level of gram-scale (Lee, 1996; Mandi et al., 2009) or at a middle level of hundred-milligram-scale (Schlott et al., 1994a; 1997) even at a low level of 10 mg scale (Lee et al., 1998). In a previous study, we obtained a high yield of 300 mg Sak per liter of *E. coli* culture broth (data not shown). In the prokaryote expression system *Bacillus*, Sak production could reach hundred-milligram-scale (Ye et al., 1999; Kim et al., 2001) or lower level of ten-milligram-scale (Behnke and Gerlach, 1987; Gerlach et al., 1988).

Temperature and pH optimum

The rSak from *S. aureus* QT08 was active in a broad temperature range of 20 to 45°C and had optimum temperature of 30 to 37ºC (Figure 2A) and optimum pH of 7.5 (phosphate buffer) and pH 8 (Tris-HCl buffer) (Figure 2B). This agreed with our previous result for the rSak from *S. aureus* QT08 produced in *E. coli*. Its optimum temperature and pH was 30-37^{o}C and pH 7 (phosphate buffer) and pH 9 (Tris-HCl buffer), respectively. Our study result was also coincident with the report on the wild type Sak (Vesterberg and Vesterberg, 1972), the optimal pH value for the Sak activity was 8.5 in Tris-HCl buffer (pH 7.6 to 9.2) and 7.5 in phosphate buffer (pH 6.8 to 7.7).

Temperature and pH stability

The rSak from *S. aureus* QT08 expressed in *P. pastoris* was stable at a temperature range from 15 to 37°C, after incubation for 4 h, the rSak activity retained over 80% in comparison to the original activity (Figure 3A) and over 40% after incubation for 8 h. In our previous study, the activity of the in *E. coli* expressed rSak also retained over 80% of the original activity after incubation for 4 h (data not shown). The rSak from *S. aureus* QT08 showed a similar profile in the temperature stability to the wild type Sak (Fujimura et al., 1974; Schlott et al., 1994b). The activity of the wild type Sak from *S. aureus* after heating at 60°C for 2 h decreased to 11% of that of the original sample (Fujimura et al., 1974). Both wild type Sak42D

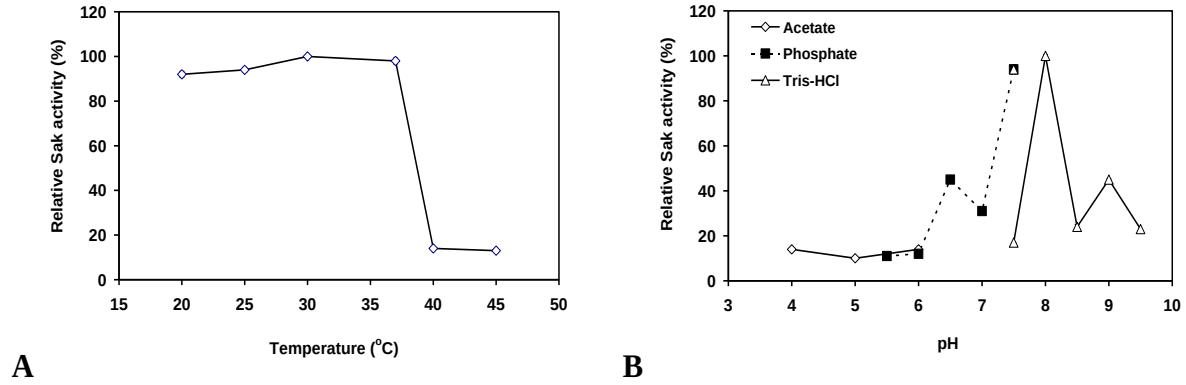

Figure 2. Temperature (A) and pH (B) optimum of rSak.

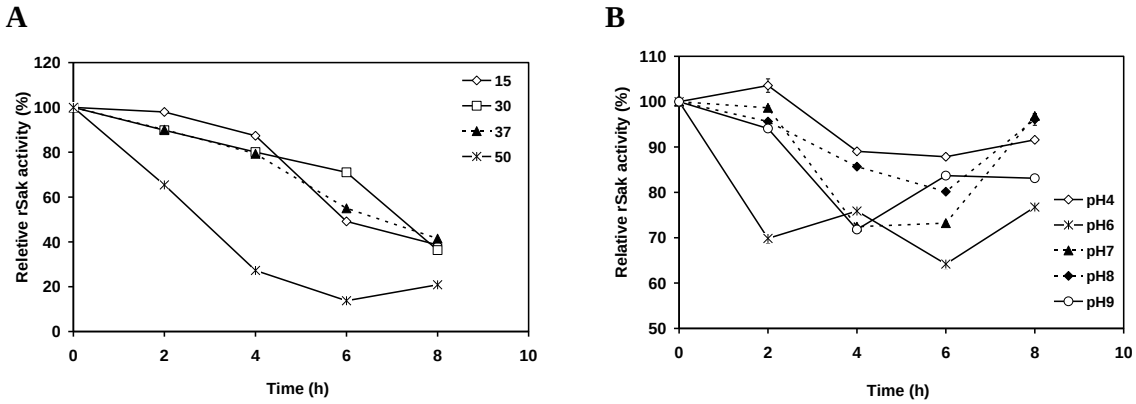

Figure 3. Temparature (A) and pH (B) stability of rSak.

and SakSAR showed a different thermal stability, Sak42D was inactivated more rapidly while SakSAR gradually inactivated for several hours at high temperature (70°C) (Schlott et al., 1994b).

The rSak from *S. aureus* QT08 expressed in *P. pastoris* showed pH stability at a pH range of 4 to 9. The residual rSak activity expressed in *P. pastoris* and in *E. coli* (data not shown) retained over 60% in comparison to the original activity after treatment at pH 4 to 9 for 8 h (Figure 3B) and over 40% after treatment at pH 7 to 9 for 8 h (in our previous study, data not shown), respectively. The staphylokinase in the crude supernatant was stable between pH 2 and 11 for 24 h at 5°C (Davidson, 1960).

Effect of metal ions and EDTA

The addition of metal ions and EDTA at the concentration of 2.5 mM showed no or slightly inhibitory effect on enzyme activity (Table 2) whereas the addition of metal ions and EDTA at the higher concentration of 25 mM inhibited the rSak expressed in *P. pastoris* completely (Cu^{2+}, Mg^{2+}, and Zn^{2+}) or strongly (EDTA, Ag^{+}, Ca^{2+}, Co^{2+}, and Ni^{2+}) except for Fe^{2+} (Table 2). These results were coincident with previous results for the rSak expressed in *E. coli* (data not shown*) that the rSak activity was reduced strongly by the addition of Ag^{+}, Co^{2+}, and Ni^{2+}, and completely lost by the addition of Cu^{2+} and Zn^{2+}.

The similar phenomena were also observed in a decrease in catalytic activity of the wild type Sak treated with 20 mM of the metal ions (Vesterberg and Vesterberg, 1972). The addition of 20 mM of Ag^{+} and Cu^{2+} completely inhibited the Sak activity. Other metal ions showed a Sak inhibitory activity of 98-70% in order of Zn^{2+}> Co^{2+}> Mn^{2+} > Ca^{2+} > Pb^{2+} while Na^{+} and Mg^{2+} did not show any significantly inhibitory activity (Vesterberg and Vesterberg, 1972). Mg^{2+} showed an opposite effect on the rSak activity in this study (25 mM) and the wild type Sak activity studied by Vesterberg and Vesterberg (1972) (20 mM) whereas Ag^{+}, Ca^{2+}, Cu^{2+}, Co^{2+}, and Zn^{2+} had a same effect. The similar results were also found in the report that Cl^{-}, Na^{+}, K^{+}, and Ca^{2+} affected the rate at which plasminogen was activated by rSak. In almost all cases, a decrease of the initial velocity of activation was observed (Yarzábal et al., 1999).

Table 2. Effect of metal ions on the rSak activity.

Additives[a]	Residual activity (%)[b]	
	2.5 mM	25 mM
$AgNO_3$	89.58 ± 0.61	9.82 ± 0.29
$CaCl_2$	86.53 ± 0.55	12.44 ± 0.17
$Co(NO_3)_2$	101.51 ± 0.43	8.76 ± 0.34
$CuSO_4$	73.40 ± 1.46	0.00 ± 0.00
$FeSO_4$	88.81 ± 0.73	98.19 ± 0.24
KCl	93.11 ± 0.12	51.01 ± 0.00
$MgCl_2$	93.24 ± 0.67	0.00 ± 0.00
$NiCl_2$	85.37 ± 1.10	10.54 ± 0.09
$ZnSO_4$	90.19 ± 1.10	0.00 ± 0.00
EDTA	98.28 ± 1.95	16.34 ± 0.18
Control (no additive)	100	

[a] The final concentration of additives (EDTA or inorganic salt) in the reaction mixture was 25 mM and 2.5 mM;
[b] Relative activity was expressed as a percentage of the control reaction (100% rSak activity was 30620 U/mg).

Table 3. Effect of detergents on the rSak activity.

Detergents[a]	Residual activity (%)[b]
Control (no additive)	100
SDS	0 ± 0
Tween 20	9.16 ± 0.48
Tween 80	17.70 ± 0.10
Triton X-100	97.88 ± 0.68

[a] The final concentration of detergent in the reaction mixture was 10%.
[b] Relative activity was expressed as a percentage of the control reaction (100% rSak activity was 27404 U/mg).

The sodium salt of EDTA at concentrations higher than 10 mM showed an inhibitory effect on the Sak activity, but at lower concentrations no inhibitory effect on the enzyme activity (Vesterberg and Vesterberg, 1972). It agreed with our study that at the concentration of 2.5 mM, EDTA showed no significantly inhibitory effect (2%) on the enzyme activity, at the concentration of 25 mM, inhibited 84% of the enzyme activity.

Effect of detergents on rSak activity

The enzyme activity was completely inhibited by the addition of SDS, strongly by the addition of Tween 20 and Tween 80, whereas the addition of Triton X-100 showed no effect (Table 3). SDS and Triton X-100 showed the same effect on the rSak activity expressed both in *E. coli* (data not shown) and in *P. pastoris* (this study), whereas Tween 20 and Tween 80 showed an opposite effect, a slightly inhibitory effect on the rSak expressed in *E. coli* (data not shown) but a strongly inhibitory effect on the rSak expressed in *P. pastoris* (Table 3). So far, there have been no reports of the effect of detergents on the Sak activity.

The study on the influence of metal ions, detergents, temperature and pH on the Sak activity was rather complicated since, in fact, two reactions including the activation of plasminogen to plasmin by Sak (first), and the digestion of substrate by plasmin (second) occur simultaneously in one assay. By adding metal salts simultaneously with the substrate it was possible to observe the influence of this on the fibrinolytic activity, but it was not possible to isolate the influence of additives on the activation of plasminogen to plasmin by Sak. Therefore, additives had an inhibitory effect on plasmin and on the total activity (Vesterberg and Vesterberg, 1972).

Conclusions

In conclusion, we have reported on high-level expression of a fully active even glycosylated staphylokinase from *S. aureus* QT08 in *P. pastoris* and purification of the rSak protein using 6xhis-tag. Temperature and pH optimum for the rSak was 30 to 37°C and pH 7.5 (phosphate buffer) and pH 8 (Tris-HCl buffer), respectively. The rSak was stable in a temperature range of 15 to 37°C and pH 4 to 9. Additives (metal ions and detergents) all exhibited a slightly or strongly inhibitory effect on the rSak activity.

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