High-Level Expression of a Biologically Active Staphylokinase in Methanol Inducible *Pichia Pastoris*

Habibollah Faraji[1], Dr. Baratali Mashkani[2], Dr. Mohammad Ramezani[3], Dr. Hamid Reza Sadeghnia[4], Dr. Fatemeh Soltani[3], Dr. Khalil Abnous[5]

[1] Department of Medical Biotechnology, [2] Department of Medical Biochemistry, [3] Pharmaceutical Biotechnology Research Center, [4] Department of Pharmacology, [5] Department of Medicinal chemistry

Introduction

Ischemic heart disease are major life-threatening issues around the world. Thrombolytic therapy is the main treatment, in which the patient's fibrinolytic system is activated by the intravenous infusion of the plasminogen activators to stimulate clot lysis. Mature staphylokinase (SAK), a 136 amino acid protein produced by certain lysogenized *Staphylococcus aureus* strains, has been recently rediscovered as a plasminogen activator. Unlike fibrin-unbounded plasmin-streptokinase complex, the free plasmin-SAK complex is rapidly neutralized by α2-antiplasmin preventing systemic degradation of fibrinogen and increased risk of severe bleeding. Furthermore, staphylokinase efficiently mediates reperfusion by lysis of platelet-rich and retracted secondary clots, on which the other thrombolytic agents such as tPA are not effective.

Materials and Methods

SAK variant of SAKϕC was codon optimized and expressed in *Pichia pastoris* strains KM71H and GS115 using pPICZα-A vector under the control of the *AOX1* promoter induced by methanol. The product was affinity purified using a Ni-IDA resin column. Biological activity of the product was determined by measuring the lysis zones on heated plasma agar plate in comparison with commercial streptokinase.

Results

Fig. 1. Outline of the rSAK inserted into pPICZαA plasmid

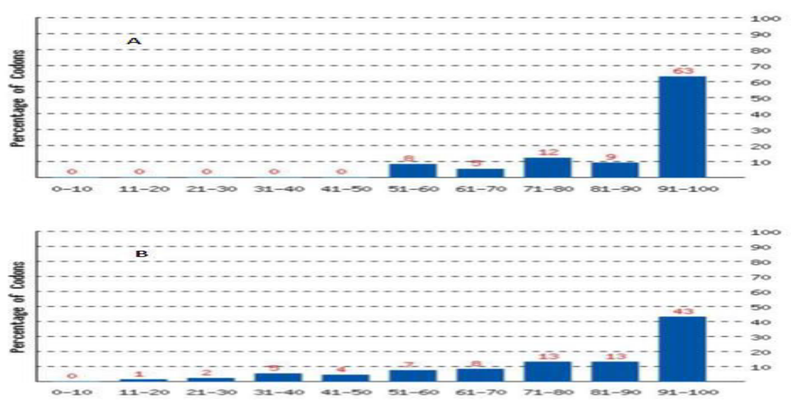

Fig. 2. The percentage distribution of the codons in the optimized rSAK (A) and native SAK (B) The codons are grouped based on their frequency in *P. pastoris*. The value of 100 is set for the codon with the highest usage frequency for a given amino acid in the expression host of interest. The analysis was performed using "Rare Codon Analysis Tool" available at http://www.genscript.com/cgi-bin/tools/rare_codon_analysis.

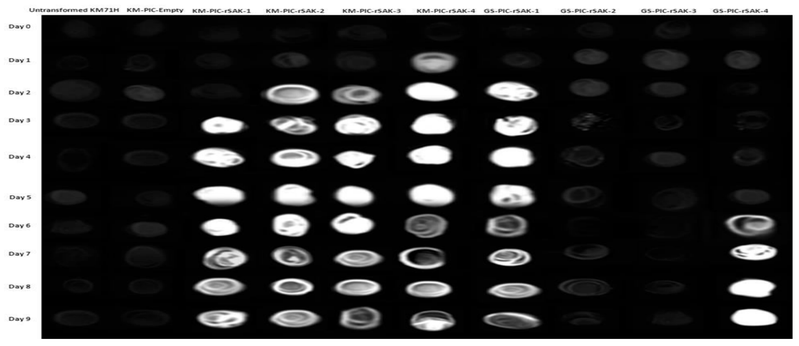

Fig. 3. Dot blot analysis of the *P. pastoris* culture supernatant. Clones were cultivated for 9 consecutive days with methanol induction. The fusion protein was probed using mouse anti-cMyc antibody and HRP-conjugated secondary anti-mouse IgG followed by visualization using enhanced chemiluminesence method. Each columns includes the supernatant collected from the culture of untransformed P. pastoris, P. pastoris transformed with empty pPICZαA vector, KM-PIC-rSAK clones#1,2,3,4 and GS-PIC-rSAK clones#1,2,3,4 , respectively.

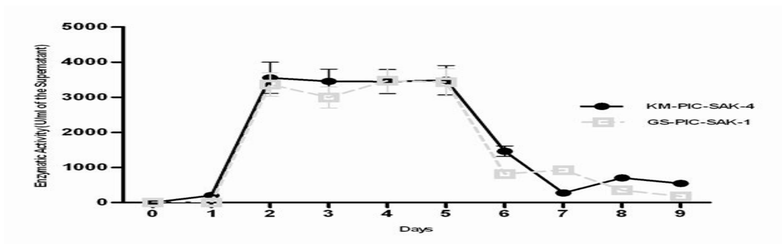

Fig. 4. rSAK expression by KM-PIC- rSAK-4 and GS-PIC- rSAK-1 clones. Samples of the supernatant were collected on 9 consecutive days. The enzyme activity (U/ml of the supernatant) was determined using the well diffusion method (the data represented as Mean±SD of three independent experiments).

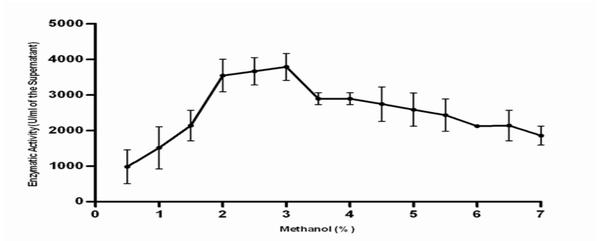

Fig. 5. Effect of methanol induction on the rSAK production by KM-PIC-rSAK-4. The rSAK expression was induced with 0.5-7% of methanol concentration for 48 h. The enzyme activity (U/mL) of the supernatant was determined using well diffusion method (the data represented as Mean±SD of three independent experiments).

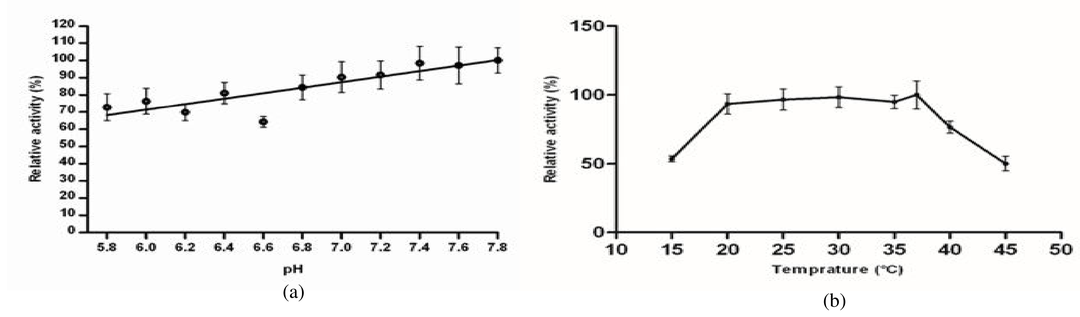

Fig. 6. The effect of pH (a) and temperature (b) on rSAK activity. The relative residual activity (%) of supernatant KM-PIC-rSAK-4 was assayed in (a) phosphate buffer (pH 5.8-7.8) (b) and different temperatures (15-45°C) incubated overnight. (The data represented as Mean±SD of three independent experiments).

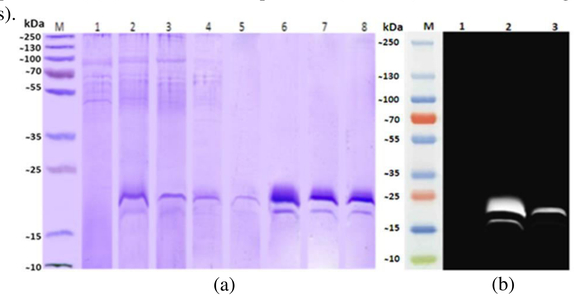

Fig. 7. The SDS-PAGE analysis of rSAK protein. (Panel a) lane M: protein marker (10-250 kDa), lane 1: KM-PIC-Empty as negative control, lane 2: culture supernatant of KM-PIC-rSAK-4 on day 2, lane 3: culture supernatant of GS-PIC-rSAK-1 clone on day 2, lane 4: flow-through from first wash; lane 5: flow-through from second wash; Lane 6, 7, and 8: purified protein elutes. (Panel b) Western blotting analysis of rSAK expressed in *P. pastoris*. Lane M: protein marker (10-250 kDa), lane 1: negative control, lane 2: purified protein of KM-PIC-rSAK-4 clone, lane 3: culture supernatant of KM-PIC-rSAK-4 clone.

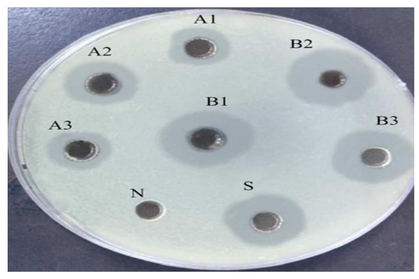

Fig. 8. Enzymatic activity (clear zone) of rSAK determined using well diffusion method. crude culture supernatant of rSAK was loaded in the wells in triplicates (A1, A2, A3) along with the purified rSAK (B1, B2, B3); S: streptokinase (10000 U) as the positive control; N: culture supernatant of KM-PIC-Empty as the negative control.

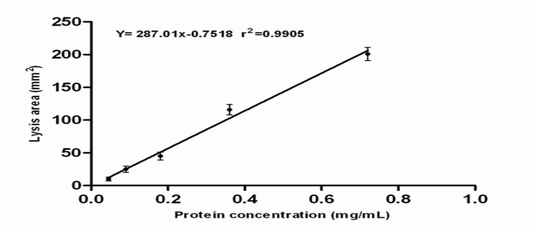

Fig. 9. Concentration dependency of rSAK activity. The areas of clear zones were plotted against the various rSAK protein concentrations (0.72, 0.36, 0.18, 0.09 and 0.045 mg/mL). The data represented as Mean±SD of three independent experiments.

Table 1: Purification parameters of rSAK obtained from the supernatant of KM-PIC-rSAK-4. The enzyme total activity (U) was determined according to the well diffusion method.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor
Culture supernatant	189047	21	9002	100	1
Ni-IDA resin column	106054	5.04	21042	56.1	2.34

Conclusions

It was concluded that *P. pastoris* is a proper host for expression of biologically active and endotoxin-free rSAK, due to its high expression and low protein impurity in culture supernatant. The purified enzyme had high specific activity and can be used for further thrombolytic therapy studies, both in vitro and in vivo.

References

1. Pulicherla, K.K.; Kumar, A.; Gadupudi, G.S.; Kotra, S.R.; Rao, K.R. In vitro characterization of a multifunctional staphylokinase variant with reduced reocclusion, produced from salt inducible E. coli GJ1158. Biomed Res Int. 2013, 2013, 297305.
2. Apte-Deshpnade, A.; Mandal, G.; Soorapaneni, S.; Prasad, B.; Kumar, J.; Padmanabhan, S. High-level expression of non-glycosylated and active staphylokinase from Pichia pastoris. Biotechnol Lett. 2009, 31 (6), 811-7.
3. Ram, K.S.; Jb, P.; Kumar, A.; Rao, K.S.; Pulicherla, K. Staphylokinase: a boon in medical sciences–review. Mintage journal of Pharmaceutical & Medical Sciences. 2013, 2 (2), 28-34.
4. Nguyen, T.H.T.; Quyen, D.T. High-level expression, purification and properties of a fully active even glycosylated staphylokinase variant SakϕC from Staphylococcus aureus QT08 in Pichia pastoris. African Journal of Microbiology Research. 2012, 6 (9), 2129-2136.