Protein Expression and Purification 69 (2010) 191–197

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Novel self-cleavage activity of Staphylokinase fusion proteins: An interesting finding and its possible applications

Bhaskarjyoti Prasad, Shardul S. Salunkhe, Sriram Padmanabhan *

Biotechnology R&D, Lupin Limited, Gat # 1156, Ghotawade Village, Mulshi Taluka, Pune 411042, India

ARTICLE INFO

Article history:
Received 8 July 2009
and in revised form 17 July 2009
Available online 26 July 2009

Keywords:
Staphylokinase
Self-cleavage activity
Fusion protein
Therapeutic protein
GST fusion

ABSTRACT

Staphylokinase (SAK) is reported to have a serine protease domain with no proteolytic activity unlike other plasminogen activators like tissue plasminogen activator (t-PA) and urokinase. A unique protease property of Staphylokinase was observed when SAK was expressed as a fusion protein in inducible *Escherichia coli* expression vectors. This finding was further investigated by cloning and expressing different SAK fusions, both native and N-terminal deletions, with fusion tags like glutathione *S*-transferase (GST) and signal sequence of SAK in bacterial system. While all the N-terminal SAK fusions were found to self-cleave in crude and purified preparations, the C-terminal SAK fusion was stable. The cleavage property of Staphylokinase fusion proteins, inhibited by reduced glutathione and PMSF, was independent of its thrombolytic activity and also independent on the type of host employed for its expression. The serine protease domain of the SAK gene possibly lies between 20th to 77th amino acid and serine 41 of this region appears critical for such a cleavage property.

Introduction

Staphylokinase (SAK)[1] has gained importance as a potential therapeutic thrombolytic protein and is an extra cellular protein produced by *Staphylococcus aureus* strains [1]. SAK is a bacteriophage encoded protein of ~15.0 kDa and is presently undergoing clinical trials for blood clot lysis in the treatment of thrombovascular disorders due to its ability to convert plasminogen, the inactive proenzyme of the fibrinolytic system, into plasmin.

Structurally, SAK resembles other plasminogen activators, containing a plasminogen-binding site and a serine protease domain [2], however; SAK is not a proteolytic enzyme [3]. It forms a 1:1 stoichiometric 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. Streptokinase (SK) remains the most widely prescribed thrombolytic agent for acute myocardial infarction (AMI) and has been shown to be homologous to serine proteases, with no apparent protease activity [4,5]. To assess the structural requirements for SAK/defensin interaction, six SAK mutant variants have been studied. Inactivation pattern of the tested SAK variants suggested a direct binding of defensins to serine protease-like domain of SAK [6]. Tissue plasminogen activator (t-PA), a serine protease has been extensively studied in relation to coagulation and thrombolysis [7]. Since SAK has higher fibrin specificity than t-PA, it makes it capable of dissolving platelet rich blood clots [8] and hence is a promising thrombolytic agent [9].

There are only two reports on SAK fusion protein for imparting higher thrombolytic potency using hirudin as a fusion partner in *Bacillus subtilis* system [8,10]. This study focused on identification of cleavage site of plasmin protease on SAK and reported that SAK fusion protein (with SAK at C-terminus) is an unstable protein.

Purification of SAK has been a challenge for several workers and we have recently reported a simple protocol for the same using two ion-exchange steps [11]. However, to develop alternate and easy purification strategy, we used Glutathione *S*-transferase (GST) as a fusion partner for SAK. Interestingly, when we constructed GST–SAK and purified SAK using glutathione Sepharose affinity matrix, we observed a strange phenomenon of self-cleavage of the purified GST–SAK protein upon storage at 37 °C to GST and SAK. Such an observation was, however, not seen with the SAK–GST protein. These observations led us to do additional experiments to probe into such an auto cleavage activity of SAK fusions and the results are presented in this paper.

Materials and methods

Vectors, Escherichia coli hosts and reagents

The restriction enzymes were purchased from Bangalore Genei Pvt. Ltd, Bangalore, India and New England Biolabs, USA. Glutathi-

* Corresponding author. Fax: +91 20 6659837.
E-mail address: srirampadmanabhan@lupinpharma.com (S. Padmanabhan).

[1] *Abbreviations used:* SAK, Staphylokinase; t-PA, tissue plasminogen activator; GST, glutathione *S*-transferase; SK, streptokinase; AMI, acute myocardial infarction; SDM, site directed mutagenesis; RDB, regeneration dextrose base.

1046-5928/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2009.07.011

one Sepharose 4B matrix and the vector pGEX-4T-1 vector were procured from GE Healthcare, USA while reduced glutathione was from Sigma, USA. pBAD24 vector was the kind gift from National Institute of Genetics, Japan. pMALp2X vectors was purchased from NEB, USA while the vector pET43.1 was from Novagen, USA. While BL21-A1 cells and *Pichia pastoris* kit was procured from Invitrogen, USA, the DH5α cells was purchased from Bangalore Genei Pvt. Ltd, Bangalore, India and BL21 cells were from Stratagene, USA.

Cloning and expression of SAK gene

SAK gene was amplified from a synthetic DNA obtained from GenScript, USA and cloned into the pET21a vector. The detailed protocols are described elsewhere [11].

Cloning and expression of N-terminal fusion of SAK (GST–SAK)

The mature peptide of the SAK gene was PCR amplified using primers synthesized by Sigma, USA the forward primer being 5′-CCG CCG GAA TTC CAT ATG TCA AGT TCA TTC GAC AAA GGA-3′ and the reverse primer being 5′-CCG CCG GAA TTC AAG CTT TTA TTT CTT TTC TAT AAC AAC-3′. The fragment size was 411 bp and the PCR amplification conditions were as follows: Initial denaturation of 4 min at 94 °C followed by 30 cycles consisting of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s. A final extension was done at 72 °C for 7 min.

The amplified PCR product was cloned at the C-terminal end of GST gene in pGEX-4T-1 vector as an EcoR1/EcoR1 fragment. The correctly oriented clone of GST–SAK was tested for the expression of the GST–SAK protein in DH5α cells after induction with 1 mM IPTG for 4 h at 37 °C.

Cloning and expression of C-terminal fusion of SAK (SAK–GST)

GST gene was amplified using gene specific primers using pGEX-4T-1 as the DNA template with the following PCR conditions. Initial denaturation at 94 °C for 4 min, followed by 30 cycles consisting of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min.

The forward primer used was 5′-CGC CGC GGA TCC ATG TCC CCT ATA CTA GGT TAT TGG AAA-3′ while the reverse primer used was 5′-CCG CCG CTC GAG GAA TTC TTA ACG CGG AAC CAG ATC CGA TTT TGG AGG ATG-3′. The PCR amplified GST gene was cloned as a BamH1/EcoR1 fragment into the pET21a-SAK having SAK gene as a Nde1/BamH1 fragment to generate pET21a-SAK-GST construct. Since the SAK–GST expression from this clone would require a T7 RNA polymerase expressing host, we decided to adopt the following strategy to generate a SAK–GST construct such that there is a uniform expression host for GST–SAK and SAK–GST clones. For this, an Xba1/HindIII fragment from the pET21a-SAK-GST was excised and the fragment was sub cloned into the pBAD24 vector as Nhe1/HindIII fragment. Such a clone would express SAK–GST fusion protein in DH5α cells using arabinose as the inducer.

Shake flask fermentation studies of GST–SAK and SAK–GST proteins

The clones of GST–SAK and SAK–GST were introduced into competent DH5 cells and the transformants were grown and induced with 1 mM IPTG and 13 mM arabinose, respectively, in Luria–Bertani medium with ampicillin 100 μg/ml at 37 °C for 4 h at 200 rpm.

Purification of GST fusion proteins using glutathione Sepharose 4B

The induced cell pellets were resuspended in the ratio of 0.3 g wet weight of cells in 5 ml of 10 mM Tris, pH 8.0 buffer and subjected to lysis using glass beads (425–600 μm, Sigma, USA). The bead lysed cells were centrifuged at 15,000 rpm for 15 min and the clarified supernatants containing GST–SAK and SAK–GST were taken for purification of the fusion proteins. The glutathione Sepharose 4 fastflow matrix column was equilibrated with 50 mM Tris–Cl, pH 8.0, 1% Triton X-100 and 150 mM NaCl and the bound GST fusion proteins were eluted using 10 mM reduced glutathione in 50 mM Tris–Cl, pH 8.0, unless mentioned otherwise. The purified proteins were also incubated at 37 °C before and after dialysis.

Effect of expression host on the auto catalytic cleavage of GST–SAK protein

DH5 cells are known to produce and secrete proteases like OmpT and lon. To ensure that host proteases are not responsible for such a proteolytic activity of SAK fusions, the constructs of SAK fusions, i.e. GST–SAK and SAK–GST were introduced into BL21 competent cells ($OmpT^-$ and lon^-). The cells were induced with suitable inducer, i.e. IPTG at 1 mM final concentration and 13 mM arabinose, respectively, and then the induced cells were processed. We have also purified GST–SAK in presence of 1 mM PMSF throughout the purification steps and analyzed the cleavage by SDS–PAGE.

Cloning of N19 SAK into pGEX-4T-1 vector as GST–NΔ19 SAK

PCR amplification of the NΔ19SAK was done using SAK plasmid as template with the Forward primer as 5′-CCG CCG GAA TTC GAA CCA ACA GGC CCG TAT TTG-3′ and the reverse primer as 5′-CCG CCG GAA TTC AAG CTT TTA TTT CTT TTC TAT AAC AAC-3′.

The PCR was done with denaturation at 94 °C for 4 min followed by 30 cycles of 94 °C (30 s), 57 °C (30 s) and 72 °C (30 s) with a final extension of 7 min at 72 °C. The amplicon was cloned in pGEX4T-1 vector at the EcoRI site. The clones with the gene in the right orientation with respect to the tac promoter were tested for expression of GST–NΔ19 SAK in DH5α competent cells after 1 mM IPTG induction.

Construction of SAK with its own signal peptide-full length SAK (FL-SAK)

The PCR amplification conditions for amplifying SAK gene with its own signal peptide were as follows: 30 cycles consisting of 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min. The forward primer was 5′-CCG CCG GAA TTC CAT ATG CTC AAA CGA AGT TTA TTA TTT-3′ while the reverse primer was 5′-CCG CCG GAA TTC AAG CTT TTA TTT CTT TTC TAT AAC AAC-3′. The PCR amplified FL-SAK was cloned into the pET 21a as an NdeI/HindIII fragment and transformants were screened by colony PCR and NdeI/HindIII digestion. The pET21a-FL-SAK expression was achieved in BL21-A1 cells (Invitrogen, USA) after induction with arabinose (13 mM) and the induced samples (equivalent to 10 OD_{600} nm units) were analyzed on SDS–PAGE.

Cloning and expression of NusA–SAK and MBP–SAK fusion

SAK gene was PCR amplified with the primers as mentioned in the cloning and expression section of N-terminal fusion of SAK (GST–SAK) and cloned into NusA vector pET43.1 and pMALp2X vector as EcoR1/EcoR1 fragment. Clones with SAK in the right orientation were selected for expression and self-cleavage studies in BL21-A1 and DH5 cells with arabinose (13 mM) and IPTG (1 mM) as inducers, respectively.