Enzyme and Microbial Technology 67 (2014) 82–86

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

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

Design of a novel chimeric tissue plasminogen activator with favorable Vampire bat plasminogen activator properties

MohammadReza Kazemali [a], Keivan Majidzadeh-A [b], Soroush Sardari [a], Amir Hossein Saadatirad [a], Vahid Khalaj [a], Najmeh Zarei [a], Farzaneh Barkhordari [a], Ahmad Adeli [a], Fereidoun Mahboudi [a,*]

[a] Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran
[b] Cancer Genetics Research Group, Breast Cancer Research Center, ACECR, Tehran, Iran

ARTICLE INFO

Article history:
Received 14 May 2014
Received in revised form 7 September 2014
Accepted 12 September 2014
Available online 22 September 2014

Keywords:
b-PA
Fibrin binding
Pichia pastoris
Catalytic activity
Plasminogen activator inhibitor-1 (PAI-1)
Tissue plasminogen activator (t-PA)

ABSTRACT

Fibrinolytic agents are widely used in treatment of the thromboembolic disorders. The new generations like recombinant tissue plasminogen activator (t-PA, alteplase) are not showing promising results in clinical practice in spite of displaying specific binding to fibrin in vitro. Vampire bat plasminogen activator (b-PA) is a plasminogen activator with higher fibrin affinity and specificity in comparison to t-PA resulting in reduced probability of hemorrhage. b-PA is also resistant to plasminogen activator inhibitor-1 (PAI-1) showing higher half-life compared to other variants of t-PA. However, its non-human origin was a driving force to design a human t-PA with favorable properties of b-PA. In the present study, we designed a chimeric t-PA with desirable b-PA properties and this new molecule was called as CT-b. The construct was prepared through kringle 2 domain removal and replacement of t-PA finger domain with b-PA one. In addition, the KHRR sequence at the initial part of protease domain was replaced by four alanine residues. The novel construct was integrated in *Pichia pastoris* genome by electroporation. Catalytic activity was investigated in the presence and absence of fibrin. The purified protein was analyzed by western blot. Fibrin binding and PAI resistance assays were also conducted. The activity of the recombinant protein in the presence of fibrin was 1560 times more than its activity in the absence of fibrin, showing its higher specificity to fibrin. The fibrin binding of CT-b was 1.2 fold more than t-PA. In addition, it was inhibited by PAI enzyme 44% less than t-PA. Although the presented data demonstrate a promising in vitro activity, more in vivo studies are needed to confirm the therapeutic advantage of this novel plasminogen activator.

1. Introduction

Plasminogen activators (PAs) are members of serine protease family that are able to lyse fibrin clot by converting plasminogen to plasmin. They are commonly used in vascular occlusive disorders like brain stroke [1–3], pulmonary embolism, venous thrombosis, myocardial infarction and cerebrovascular thrombosis [4,5]. Pathologically, when thrombosis happens, tissue plasminogen activator is secreted by the epithelial cells and acts through binding to both fibrin and plasminogen, forming a tertiary complex. Then, plasminogen is converted to plasmin causing fibrin degradation. Plasminogen activators can be categorized as medicinal products in two classes according to their specific bindings to fibrin. Streptokinase and urokinase are among nonspecific fibrin binders whereas alteplase, reteplase and tenecteplase are considered as PAs with specific fibrin binding capabilities [6,7]. Streptokinase is less commonly used due to their allergic reactions and bleeding side effects [8].

Alteplase is a 70 kDa protein which holds five domains including finger, epidermal growth factor, kringle 1, kringle 2 and protease [9]. Although this plasminogen activator demonstrates a higher fibrin affinity compared to previous PAs, it still shows some complications like hemorrhage and low half-life (3.5 min) [10]. In addition, there is no significant mortality difference between t-PA and streptokinase in clinical trials [2]. Lysine binding sites incorporated in kringle2 domain deeply conduct t-PA in attachment to fibrinogen and fibrin degradation products causing plasminemia [11]. In addition, t-PA binding to fibrin is partial and could also bind to

* Corresponding author at: Biotechnology Research Center, Pasteur Institute of Iran, Tehran 13164, Iran. Tel.: +98 21 6695 3311 20; fax: +98 21 6648 0780.
E-mail addresses: mahboudi_f@pasteur.ac.ir, mahboudi.f@gmail.com (F. Mahboudi).

http://dx.doi.org/10.1016/j.enzmictec.2014.09.005
0141-0229/

Beta-amyloids found in the cerebral vessels of old patients resulting in plasminemia [12].

Tissue plasminogen activator is also inhibited by plasminogen activator inhibitor-1 (PAI-1) which declines its half-life. Therefore, scientists focus on the design of better thrombolytic agents [13]. Tenecteplase is another form of t-PA in which changing PAI-1 interaction site from KHRR to AAAA resulted in more resistance to PAI-1 [10]. Reteplase and pamiteplaseare other forms of t-PA with improved resistance to inhibitors and prolonged half-life [14].

Desmoteplase (b-PA) is a plasminogen activator which was primarily isolated from the saliva of vampire bat *Desmodus rotundas* [15]. b-PA is a 54 kDa serine protease containing finger, EGF, kringle1 and protease domains with 14 cysteine bonds and 2 *N*-glycosylation sites. Pharmacological and pharmacokinetic studies show that b-PA is more potent than other plasminogen activators. The higher fibrin specificity of b-PA is mainly related to its structural properties [16,17]. As a priority over t-PA, b-PA is activated only in the presence of fibrin. The greater affinity of finger domain to fibrin and the lack of Kringle 2 domain increase the catalytic activity of b-PA 100,000 times [6]. As a result of its non-human origin, b-PA may show some important disadvantages like immunogenicity. In some reports, b-PA induced antibody has been detected in animal models. Among PAs desmoteplase is more prone to antigenicity following streptokinase and anistreplase [6].

In the present study, we designed a new form of plasminogen activator with conserved sequence of human t-PA and the main advantage of b-PA. For this purpose, the finger domain of t-PA was replaced with desmoteplase counterpart. Furthermore, PAI-1 recognition site was modified to prolong half-life through preventing PAI interaction with this novel molecule (Fig. 1). *Pichia pastoris is* an expression system suitable for high-level expression of proteins with cost-effective media culture. So in the present study, *Pichia pastoris* (GS115, his-) was chosen to express our chimeric t-PA.

2. Materials and method

2.1. Strains, reagents and culture media

Pichia pastoris (Invitrogen) strain GS115 as the expression host and pPICZαA (Invitrogen) as the expression vector were used for heterologous protein expression. *Escherichia coli* strainTop10F'(Invitrogen) cells were used in standard cloning procedures. Buffered complex medium, containing glycerol (BMGY; Invitrogen) and buffered complex medium containing methanol (BMMY; Invitrogen) were used during protein expression in *Pichia pastoris*. Restriction enzymes were purchased from Fermentase and other reagents were obtained from standard commercial sources.

2.2. Construction of chimeric t-PA (CT-b) expression vector

A novel CT-b construct contained the finger domain of b-PA and the growth factor, kringle 1 and protease domains of human t-PA was designed. The PAI-1 interaction site (KHRR correspondent to amino acids 213–216 of CT-b) was also replaced by AAAA sequence. To express the final product with a native N terminus, a sequence containing XhoI restriction site and Kex2 recognition site was added to the 5′ end of the designed construct. To facilitate the purification of the novel recombinant protein, a polyhistidine (6X) tag was added to the downstream of CT-b construct. The final construct was codon optimized according to the *Pichia pastoris* codon usage and synthesized by Generay Biotech (Shanghai, China).

The synthesized gene construct was cloned into pPICZαA using XhoI/XbaI sites. The recombinant plasmid was confirmed through PCR, restriction mapping and bidirectional sequencing.

2.3. Transformation of P. pastoris

Five to ten microgram of SacI linearized pPICZ-CT-b plasmid was electroporated into *Pichia pastoris* according to Invitrogen instruction.1 ml of YPD medium was added to the electroporated cells and the cells were allowed to recover for 2 h at 30 C at 250 RPM. Transformants were plated on YPDS plates containing 200, 500 and 1000 μg/ml Zeocin and the zeocin-resistant transformants were isolated. The presence of expression cassette was confirmed by colony PCR using the specific primers of CT-b (CT-b _f: 5′ GTTGCCTGCAAGGATGAGATCACACAAATG-3′ and CT-b _r5′-TGGTCTCATGTTATCTCTGATCCAGTCCAAATA-3′).

2.4. Small-scale expression in P. pastoris

A single colony of CT-b positive transformants was grown in YPD broth overnight. Subsequently, the cells were diluted to an OD600 of 1 in 10 ml of BMGY and grown for 3 days. When the culture optical density was reached to an OD600 of 10, the cells were harvested by centrifugation and cultivated in 10 ml of BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 0.5% methanol). The culture was induced for 72 h at 30 °C with shaking (225 rpm). To maintain the induction process; absolute methanol was added every 24 h. The culture supernatant was finally collected and stored at −80 °C. Quantity One software (Bio-Rad, USA) was used for densitometry [18] of the SDS-PAGE gels to measure the expressed protein CT-t-PA in comparison to total protein.

2.5. CT-b activity assay

Chromogenic activity kit (Assaypro, USA) was used to assess the biological activity. Briefly, plasminogen (40 μg/ml) and plasmin substrate (0.4 mM) were mixed gently and then CT-b (20 μl) was added to the mixture. The whole reaction was kept at room temperature for 1 h, and the absorbance was read by spectrophotometer at 405 nm. Soluble fibrin (80 μg/ml) was added where needed. The biological activity standard curve was plotted using standard t-PA as suggested by manufacturer. All assays performed in triplicates.

2.6. Purification

The culture supernatant was dialyzed against PBS buffer using Medicell MWCO 12–14,000 Da dialysis bags (Medicell International Ltd., England). Ni-NTA purification column (Amersham–Pharmacia Quarry Bay) was used for CT-b purification. The binding buffer containing 10 mM Na_2HPO_4, 300 mM NaCl, and 10 mM imidazole was applied to the column. The washing step was processed exploiting washing buffer containing 10 mM Na_2HPO_4, 300 mM NaCl, and 20 mM imidazole. Finally, elution was done using 10 mM Na_2HPO_4, 300 mM NaCl, and 400 mM imidazole at pH 8.8 according to the manufacturer instruction.

2.7. SDS-PAGE and Western blotting

Sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) and western blot analysis were carried out according to standard methods. Protein bands were separated on SDS gel and transferred to nitrocellulose membrane using a semi-dry blotting system (Biorad, USA). A polyclonal rabbit anti-human t-PA antibody (Abcam, USA) was used as the primary antibody and a HRP labeled goat anti-rabbit antibody (Santa Cruz, USA) was used as the secondary antibody. The antigen–antibody complexes were visualized by DAB staining.

2.8. Fibrin binding assay

The CT-b binding activity was assessed as described before [19,20]. To prepare fibrin clot the bovine thrombin (0.5 μ/ml) in buffer (0.05 M Tris–HCl, pH 7.4, 0.12 M NaCl. 0.01% Tween 80, 1 mg/ml bovine serum albumin) was mixed with different concentrations of fibrinogen (0–0.3 mg/ml) (Sigma–Aldrich, USA) and incubated for 30 min at 37 °C. Then, CT-b or full-length t-PA was added in equal units (3000) and incubated for 30 min at 37 °C. Centrifugation (15 min, 13,000 rpm, 4 C; sigma 202 MD) was performed to remove existing clots. The amount of enzyme bound to fibrin was calculated from the difference of the total amount of enzyme and free enzyme in the supernatant, as determined by ELISA.

2.9. PAI-1 resistance assay

Resistance of CT-b to PAI-1 enzyme was assessed according to previous studies [21,22]. Full-length t-PA and CT-b (3000 IU/ml) were incubated with different concentrations of Human PAI-1 (0 to 100 μg/ml) (Sigma–Aldrich, USA) at 25 °C for 1 h. Then, residual activity measurement was performed by AssayPro kit as described above.

3. Results

3.1. Preparation of CT-b expression construct

To express a high level of novel plasminogen activator, CT-b, the codon optimized synthetic gene was successfully cloned in pPICZAα plasmid [23]. Restriction mapping and sequence analysis of recombinant vector confirmed the correct cloning procedures (data not shown).