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ORIGINAL RESEARCH PAPER

Cloning and expression of truncated form of tissue plasminogen activator in *Leishmania tarentolae*

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Abstract An expression cassette containing kringle 2 and serine protease domains (K2S), tissue plasminogen activator (tPA), together with a signal sequence derived from *Leishmania tarentolae* and two fragments of the small subunit ribosomal RNA locus, was introduced into *L. tarentolae*. The transfected cells produced recombinant K2S (rK2S) protein extracellularly with serine protease activity. Expression and enzyme activity of rK2S in the supernatant was 930 i.u./ml. The specific activity of purified rK2S was 7.4 U/mg of protein. Replacement of the human signal sequence tPA with the signal sequence derived from *Leishmania* increased the secretion of recombinant protein up to 30 times.

Keywords Homologous recombination · *Leishmania tarentolae* · Tissue plasminogen activator · Zymography

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Introduction

Tissue plasminogen activator (tPA) is a serine protease containing 527 amino acid residues with a molecular mass of 72 kDa (Pennica et al. 1983). The finger domain (F), the epidermal growth factor domain (EGF), two kringle domains (K1 and K2), and the *C*-terminal serine protease domain (S) are five functional domains of this protein. tPA is a fibrin-specific plasminogen activator (Rouf et al. 1996) with no risk of extensive fibrinogen depletion and concomitant bleeding risks (Ellis and Brener 2004). tPA is thus a superior thrombolytic agent for treating acute myocardial infarction and pulmonary embolism but its clinical efficiency and pharmacokinetic properties should be improved (Baruah et al. 2006). Kringle 2 and serine protease domains (K2S) is a 39 kDa derivative of tPA with K2S (Mattes 2001). The hepatic clearance of K2S is reduced and its plasma half-life is increased to 14–18 min (versus 3–4 min with native tPA) (Nordt and Bode 2003). Insertion of K2S sequence into *Escherichia coli* leads to the formation of inactive inclusion bodies that must be purified and refolded to achieve activity (Kohnert et al. 1992). In recent years, scientists have recognized some members of the *trypanosomatidae* family, as new systems for the production of heterologous protein (Clayton 2002; Georgiou and Valax 1996; Hockney 1994; Orlando et al. 2007). *Leishmania tarentolae* is a non-pathogenic lizard-infecting example (Orlando et al. 2007).

The aim of this study was to introduce an expression cassette containing K2S form of tPA into the small subunit ribosomal (ssu) locus of 18s rRNA genes of *L. tarentolae*. In addition, we evaluated whether transfected *Leishmania* could produce recombinant K2S (rK2s) protein in active form.

Materials and methods

Amplification and cloning of K2S gene

The K2S fragment of tPA gene was amplified by PCR using pTZ57R-tPA plasmid as a template (Gift) with forward primer, F-K2S (5′-ACAGGCGCCTCTTACC AAGGAAACAGTGACTGCTAC-3′) containing a *Kas*I site, 1–3 amino acids codon sequence and reverse primer, and R-K2S (5′-ACACTCGAGTCA CACCACCACCACCACCACCGGTCGCATGTTG TCACG-3′) containing a *Xho*I site and a stop codon. Primers were designed on the basis of the human tPA gene sequence (GenBank accession number 101047). An Eppendorf DNA thermal cycler and Expand Long Template PCR System were used for amplification of the desired fragment in 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 68°C and extension for 1 min at 72°C, followed by 72°C for 10 min as a final extension. The PCR product was cloned in pGEM-T Easy vector (T-A cloning, Promega Co) using manufacturer's procedures yielding the recombinant plasmid pGEM-T vector-K2S. The recombinant plasmids were confirmed by digestion analysis with *Kas*I and *Xho*I restriction enzymes and sequencing using M13 forward and reverse primers. The insert was removed by *Kas*I and *Xho*I digestion and cloned into the *Kas*I–*Xho*I insertion site of *Leishmania* expression vector pF_4splmsapx1.4hyg (Jena Bioscience, Jena, Germany) to create the recombinant pF_4splmsapx1.4hyg plasmid. The presence of the K2S gene in pF_4splmsapx1.4hyg-K2S plasmid was confirmed by *Kas*I and *Xho*I restriction enzymes.

Transfection and cultivation of *L. tarentolae*

L. tarentolae was cultivated in brain–heart infusion (BHI) broth medium supplemented with 5 μg of hemin/ml at 26°C. Transfection was carried out by electroporation of in vitro cultivated promastigotes at 10^8 cells/ml. Recombinant clones were selected as single colonies on solidified BHI medium containing 50 μg hygromycin B/ml (Sigma) as selective antibiotic. For electroporation, ~10 μg of the expression plasmid was digested with *Swa*I for linearization and integration into the ssu locus. Integration of the expression cassette into the ssu locus of 18s rRNA was confirmed by PCR on genomic DNA with hyg (hygromycin resistance) forward primer 5′-CATGA AAAAGCCTGAACTCACCGCG-3′ and reverse ssu primer 5′-CTGCAGGTTCACCTACAGCTAC-3′.

Purification and evaluation of the expression of recombinant protein with western blotting

Recombinant protein was expressed as a secretory hexa-histidine-tag fusion protein in the supernatant of transformed *L. tarentolae* culture. Recombinant protein containing the histidine tag was affinity purified using Ni–NTA resin (Qiagen) according to the manufacturer's protocol with some modifications. Briefly, the supernatant was passed through a FPLC column containing 15 ml Ni–NTA resin, washed with buffer A containing 20 mM imidazole, 300 mM NaCl, and 50 mM sodium phosphate buffer followed by elution of the bound material with linear gradient buffer B (2–100%) containing 300 mM imidazole, 300 mM NaCl and 50 mM sodium phosphate buffer at 60 min. The purified recombinant protein was shown by SDS-PAGE analysis to be free of any significant amount of *Leishmania* protein. For western blotting, 30–35 μg culture supernatant of transformed cell or 5 μg purified rK2S was separated on 12% (v/v) SDS-PAGE gel and blotted onto a nitrocellulose membrane. The membrane was blocked 2 h at room temperature with 5% (v/v) skim milk in PBS containing 0.05% Tween 20 and incubated for 2 h with 1/1,000 dilution of rabbit anti-tPA antibodies (Abcam). After washing, the membrane was incubated in a 1/3,000 dilution of horse radish peroxidase conjugated goat anti-rabbit IgG (Abcam). Then, reactive bands were detected by peroxidase/3.3′ diaminobenzidine substrate.

Zymography

Zymography analysis was performed for assay of the plasminogenolytic activity of rK2S protein.

The SDS-PAGE gels were copolymerized with plasminogen (Chromogenix, Milan, Italy) and gelatin as sequential substrates for detecting proteolytic bands. After electrophoresis at 4°C at a constant current of 8 mA, the gels were washed with 2.5% Triton X-100 for 1 h with shaking at room temperature (25°C) to remove the SDS. The gels were then incubated in 0.1 M glycine/NaOH (pH 8.3) for 5 h at 37°C. Finally, the gels were stained and destained using Coomassie Brilliant Blue R-250. The location of the peptide possessing enzymatic activity was revealed as clear zone on a blue background.

Determination of amidolytic activity

Biopool's Chromolize tPA assay kit was used to determine the amidolytic activity of rK2S. [Chromolize tPA is a bio-functional immunosorbent assay intended for the quantitative determination of human tPA.] The assay started by adding the samples to the microtest wells which contains antibodies. The wells were washed with mild detergent and the sample was captured by antibodies on the microtest wells. The substrate consisting of plasminogen and a plasmin-sensitive chromogenic substrate was added. The amidolytic activity was calculated from the increase of absorbance at 405 nm.

Results

Cloning of K2S gene

The K2S gene was amplified by PCR using forward and reverse primers and a single 1100 bp band was seen on agarose gel after electrophoresis. The K2S gene was cloned into the *Kas*I/*Xho*I site of expression vector (Fig. 1), containing signal sequence derived from the secreted acid phosphatase of *Leishmania mexicana* and the antibiotic resistance gene (Fig. 2A). Then expression cassette containing K2S gene was flanked by two fragments of the small subunit rRNA locus (5′ssu and 3′ssu) for double homologous recombination and was introduced into *L. tarentolae* by electroporation. The integration of the expression cassette into the ssu locus was confirmed by diagnostic PCR. For this purpose, primer pairs, including on primer hybridizing within the expression cassette (hyg forward primer), and one primer hybridizing to ssu sequence (ssu reverse primer) which is not present on the plasmid were used. The expected 2.8 kb band was only observed from transformed cells indicating the proper integration of expression cassette into the ssu locus in the genomic DNA. The desired band was not obtained in control reactions where the template was the genomic DNA from the wild type cells (Fig. 2B).

Expression of rK2S protein in *L. tarentolae*

Purification of recombinant protein was performed by affinity chromatography and fractions were analyzed by SDS-PAGE (Fig. 3A). A desired band was at 40 kDa, which is slightly higher than the native mature protein due to the addition of a hexa-his-tag sequence. The presence of rK2S in the culture supernatants and in the fractions of purification was determined by western blotting (Fig. 3B).

The sharp clear zones on the blue background of the zymography gel indicated the activity of rK2S serine protease which observed only in the culture supernatant of transformed *L. tarentolae* (Fig. 4).

The culture supernatant of transformed *L. tarentolae* with secretory expression vector pF_4splmsapx1.4hyg containing K2S gene, showed an enzymatic activity of 931 i.u./ml. The specific activity of purified rK2S was calculated as 7.43 U/mg of protein.

Discussion

The K2S gene was cloned into a vector for high level protein expression by integration of the expression cassettes into the ssu rRNA gene of *L. tarentolae*, which is strongly transcribed by RNA polymerase I (Misslitz et al. 2000). Western blotting analysis by using a rabbit anti-tPA antibodies showed that transfected *Leishmania* produce rK2S protein extracellularly in supernatant culture medium; but this protein was absent in supernatant culture of untransfected cells. Our study showed serine protease activity of the produced rK2S. We tested it according to Heussen and Dowdle (1980) demonstrating that plasminogen-independent proteases could be detected and analyzed by omitting plasminogen from the zymography gel.

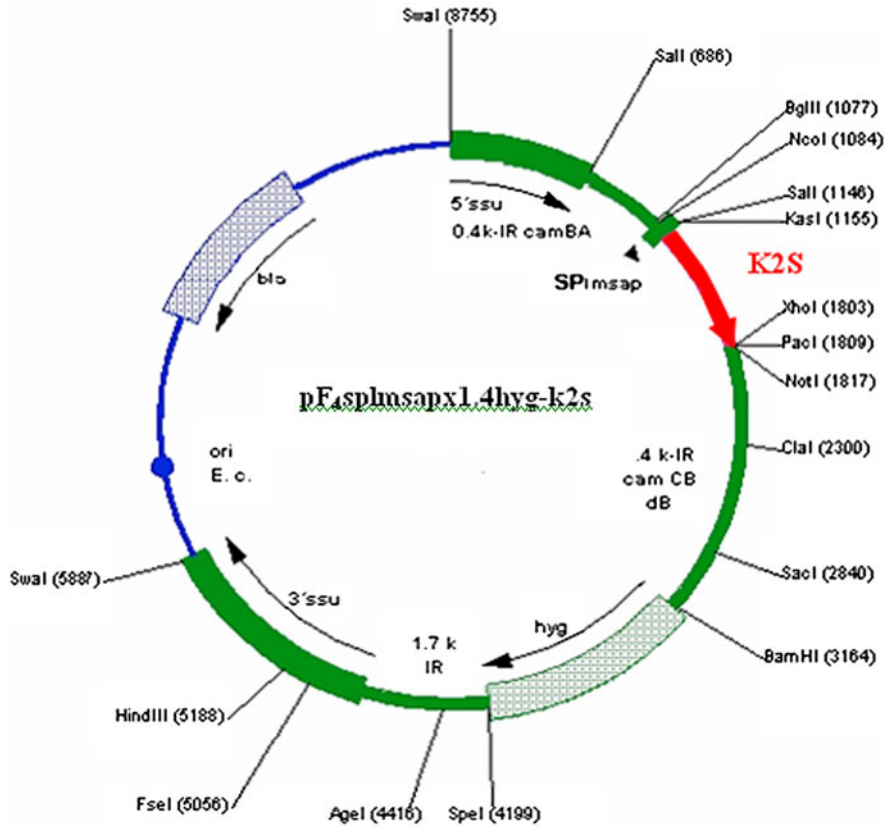

Fig. 1 Map of the expression construct used for transfection of *L. tarentolae*. *Abbreviations*: 5′ ssu and 3′ ssu are regions of the small subunit of *L. tarentolae* rRNA gene for homologous recombination into the host chromosome following linearization of the expression plasmid with *Swa*I. 0.4k-IR camBA, 1.4k-IR camCB and 1.7k-IR are optimized gene-flanking non-translated regions providing the splicing signals for posttranscriptional mRNA processing for expression of target and marker genes in the *L. tarentolae*. SP designates the signal peptide of *L. mexicana* secreted acid phosphatase. Hyg marker gene for selection with hygromycin

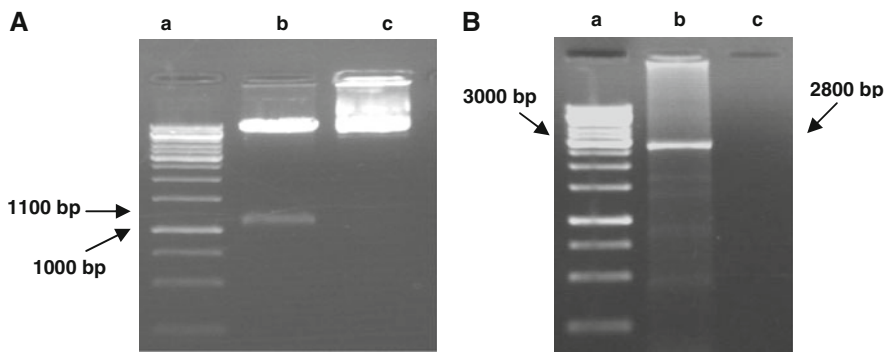

Fig. 2 Cloning of K2S gene. **A** Restriction enzyme analysis for confirm cloning of K2S gene in pF_4splmsapx1.4hyg plasmid. *Lane a* size marker, *lane b* digestion pF_4splmsapx1.4hyg-K2S plasmid with *Kas*I/*Xho*I restriction enzymes produce expected 1100 bp band that confirmed cloning of K2S gene into pF_4splmsapx1.4hyg plasmid, *lane c* undigest pF_4splmsapx1.4hyg-K2S plasmid as a negative control. **B** Diagnostic PCR on genomic DNA of transfected and wild type cells. *Lane a* size marker, *lane b* a 2.8 kb band was only observed from transfected *L. tarentolae* cells indicating the integration of expression cassette into the ssu locus, *lane c* wild type cells as a negative control

Manosroi et al. (2001) produced rK2S in *E. coli* with an activity of 7 i.u./ml. The enzyme activity for rK2S produced in transfected *L. tarentolae* was 931 i.u./ml. To evaluate the efficiency of the signal sequence we replaced the native signal sequence of human tPA with the signal sequence derived from secreted acid phosphatase of *L. mexicana*. Soleimani et al. (2007) used the native human signal sequence

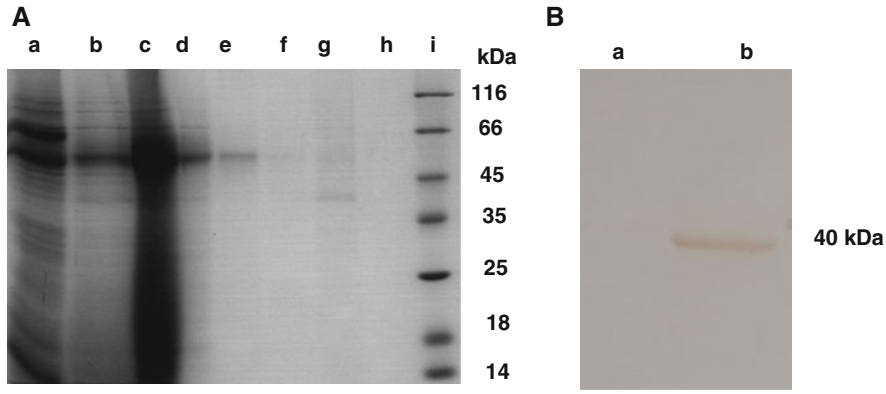

Fig. 3 Analysis of K2S gene expression in transfected *L. tarentolae*. **A** SDS-PAGE analysis of culture supernatant of transfected *L. tarentolae*. *Lane a* culture supernatant of wild type *L. tarentolae, lane b* culture supernatant of transfected *L. tarentolae*, *lane c* FT1 fraction of purification, *lane d* FT2 fraction of purification, *lane e* W1 fraction of purification, *lane f* W2 fraction of purification, *lane g* E1 fraction of purification and Ni–NTA purification recombinant K2S protein, *lane h* E2 fraction of purification, *lane i* molecular weight markers. **B** Western blot analysis of culture supernatant of transfected cells and purified recombinant protein using anti-tPA antibody. *Lane a* culture supernatant of wild type cells blotted with anti-tPA antibody as negative control, *lane b* culture supernatant of *L. tarentolae* transfected with pF_4splmsapx1.4hyg-K2S expression vector containing K2S gene blotted with anti-tPA antibody

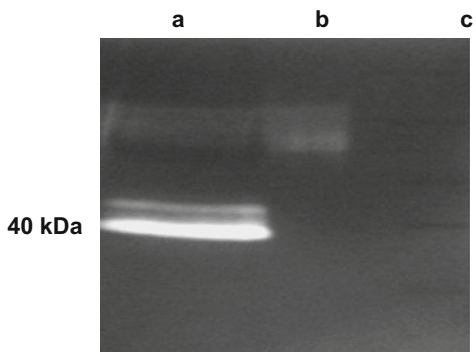

Fig. 4 Zymography analysis of culture supernatant of transfected and wild type cells. *Lane a* culture supernatant of *L. tarentolae* transfected with pF_4splmsapx1.4hyg-K2S, *lane b* culture supernatant of wild type *leishmania*, *lane c* molecular weight markers

of tPA gene for cloning this gene in *L. tarentolae* and reported an enzyme activity of 30 i.u./ml. Our findings were consistent with the observation of Breitling et al. (2002) that replacement of the native human signal sequence with the signal sequence derived from *Leishmania* increased considerably secretion of recombinant protein.

In the current study, deletion of the three *N*-terminal domains did not have any influence on the active site of the serine protease domain and the biological activity properties of the kringle 2 and protease domains. These data were in accordance with the findings of Manosroi et al. (2001), too.

On the other hand, we showed that *L. tarentolae* can be utilized for significant production of rK2S; the recombinant human K2S protein produced in *L. tarentolae* cells shows normal biological activity and proper folding. The *Leishmania* expression system has also been successfully used to produce many other proteins including active mammalian cytokines such as IL-2 and IFN-gamma in previous studies (La Flamme et al. 1995; Tobin et al. 1993; Breitling et al. 2002).

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