Expression of human tissue plasminogen activator in the trypanosomatid protozoan *Leishmania tarentolae*

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A variety of recombinant protein expression systems have been developed for heterologous genes in both prokaryotic and eukaryotic systems such as bacteria, yeast, mammals, insects, transgenic animals and transgenic plants. Also, it has been reported that *Leishmania tarentolae*, a trypanosomatid protozoan parasite of the white-spotted wall gecko (*Tarentola annularis*), has the capability of expressing heterologous genes. Trypanosomatidae are rich in glycoproteins, which can account for more than 10% of total protein. The oligosaccharide structures of their glycoproteins are similar to those of mammals with N-linked galactose, and sialic acid residues. For a variety of reasons, including the glycosylation patterns and the secondary structures of some of these proteins, synthesis in eukaryotic system is highly preferable. In addition, formation of native disulfide bonds in complex eukaryotic proteins is tremendously important. In the present study, we tried to express the tPA (tissue plasminogen activator) gene in *L. tarentolae*. This protein is a thrombolytic agent with 527 amino acid residues. tPA possesses serine-protease activity, with 35 cysteine residues that participate in the formation of 17 disulfide bonds. We have used an expression cassette, including the α intergenic regions of *Leishmania major* and two sites at the 3′- and 5′-ends, for homologous recombination in *L. tarentolae*, in addition to antibiotic-resistant genes. Southern-blot analysis showed that the human tPA gene had been inserted into the genome of the parasite. The expression of the tPA at the mRNA and protein levels was confirmed. It was shown that the expressed tPA in this system was 70 i.u. (international units)/ml of culture media, which is much higher than levels reported previously in other systems.

Introduction

tPA (tissue plasminogen activator) is a serine protease containing 527 amino acid residues with a molecular mass of 72 kDa [1,2]. This protease consists of 35 cysteine residues that contribute in the formation of 17 disulfide bonds. tPA converts the zymogen plasminogen into plasmin, a serine protease with wide-ranging specificity that degrades the fibrin network in thrombi [3]. Thrombolysis with other plasminogen activators such as streptokinase and urokinase is coupled with systemic activation of plasminogen, which can cause indiscriminate digestion of coagulation proteins and considerably increase the risk of haemorrhage during treatment [1]. However, tPA is less likely than other proteases to cause unintended plasmin activation and internal haemorrhage. tPA is a preferable thrombolytic agent for the healing of acute myocardial infarction. It has the benefit of causing no complications such as systemic bleeding and fibrinogen exhaustion [4].

Bowes melanoma cells were initially used as a resource of tPA production for therapeutic purposes [5]. Since a reliable process for production of elevated yields of highly purified protein is vital for medical use, the manufacture of full-length rtPA (recombinant tPA) developed to a variety of prokaryotic and eukaryotic cells. Considering the low expense of production, researchers have been working on producing rtPA from bacteria, especially from *Escherichia coli* [6–8]. A broad range of studies over the last years have shown that multidisulfide polypeptides such as tPA, human tissue kallikreins, the protease inhibitors and various growth factors [5,9–19] usually do not fold properly in bacteria and accumulate in misfolded structures. Human tPA most exemplifies the challenges coupled with the production of complex polypeptides in *E. coli* [20]. tPA secreted in the periplasmic space of *E. coli* is non-functional and

Key words: expression system, *Leishmania tarentolae*, serine protease, tissue plasminogen activator (tPA), Trypanosomatidae, zymography.

Abbreviations used: BHI medium, brain/heart infusion medium; CHO cells, Chinese-hamster ovary cells; EGFP, enhanced green fluorescent protein; hyg, hygramucin resistance; i.u., international units; rhEPO, recombinant human erythropoietin; RT, reverse transcription; sat, nourseothricin resistance; tPA, tissue plasminogen activator; rtPA, recombinant tPA; ssu, small ribosomal subunit; UTR, untranslated region.

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misfolded [20]. The production of such polypeptides and other proteins with more than two disulfide bonds is complex and hence it must either be produced in superior eukaryotes that offer a good location for the creation of disulfide bonds or refolded from inclusion bodies [10,12].

Efforts to produce active tPA in *Saccharomyces cerevisiae* or in insect cells have been disturbed by problems due to hyperglycosylation, poor export and inappropriate folding [9,21]. At present, CHO cells (Chinese-hamster ovary cells) have been transformed with the tPA gene to produce rtPA [22,23]. Factors such as the high price of cell-culture media, the possibility of contamination of products with viruses and prions depending on the components of animal origin and elevated purification cost are drawbacks of using mammalian systems. In recent years, scientists have recognized members of the Trypanosomatidae family as a new system for the production of heterologous proteins. Techniques of genetic manipulation and expression of heterologous genes in trypanosomatids are well established through the efforts of molecular parasitologists [24], although in all cases the heterologously expressed proteins were biologically functional. The yields for recombinant mammalian polypeptides were calculated in μg/litre of culture [25–27]. Nevertheless, the accessibility of genetic engineering techniques in along with sophisticated post-translational modifications makes members of the Trypanosomatidae family potentially attractive for biotechnological purposes [28]. Several Trypanosomatidae members exhibit fast growth rates and do not need complicated nutrient supplies.

In the present study, *Leishmania tarentolae*, a trypanosomatid protozoan parasite of the white-spotted wall gecko (*Tarentola annularis*) [29], was transfected with an expression cassette containing tPA gene. We showed that expression cassette including tPA gene integrates in *Leishmania* 18S rRNA genes through homologous recombination and transfected *Leishmania* produce biologically active tPA. Expression in *L. tarentolae* borrows a lot from finely developed and tested methods of molecular parasitology. It has been verified that *L. tarentolae* can be cultivated on an inexpensive medium in a volume of 100 litres with success densities of 4×10^8 cells/ml and 6–8 h doubling time [29].

Materials and methods

The tPA gene amplification

The CHO1-15 cell line (CRL-9606. A.T.C.C.) was cultivated in Ham's F12 medium (Gibco) supplemented with 10% (v/v) fetal calf serum (Gibco). Cells were harvested and then DNA was extracted by a Nucleon DNA Extraction kit (Amersham). The *tPA* gene was amplified by a set of primers, named FtPA 5′-AA**CCATGG**ATGCAATGAAGAGAGGGCTC-3′ and RtPA 5′-**GCGGCCGCT**CACGGTCGCATGTTG-3′ containing NcoI and NotI recognition restriction sites in each 5′-terminus respectively, which are written in boldface in the primers. Primers were designed on the basis of the human *tPA* gene sequence (GenBank® accession number I01047). The *tPA* gene was amplified by The Expand Long Template PCR System (Roche). The PCR protocol consisted of a first initial denaturation at 94 °C for 2 min, and 30 cycles were carried out of the following steps: 94 °C for 30 s, 66 °C for 60 s, 72 °C for 1.5 min, followed by 72 °C for 10 min as a final extension. The 1690 bp amplicon was cleaned up by a QIAquick PCR Purification kit (Qiagen) and the amplicon was confirmed by digestion analysis.

Cloning

The purified amplicon was cloned in pTZ57R using an InsT/Aclone™ PCR Product Cloning kit (Fermentas, Vilnius, Lithuania) following the manufacturer's procedures. The recombinant plasmid was bidirectionally sequenced. The cloned *tPA* gene was digested by NcoI and NotI restriction enzymes (Fermentas) and cloned into pFX1.4sat and pFX1.4hyg plasmids (Jena Bioscience, Jena, Germany). The recombinant pFX1.4sat-tPA and pFX1.4hyg-tPA plasmids were purified by a commercial plasmid extraction kit (Macherey-Nagel, Düren, Germany) and the presence of the tPA gene was confirmed by NcoI and NotI restriction enzymes and sequencing using M13 forward and reverse primers.

Transfection and cultivation of *L. tarentolae*

L. tarentolae was cultivated in BHI (brain/heart infusion) broth medium (Difco) supplemented by 15 μg/ml haemin (Sigma, Littlehampton, West Sussex, U.K.). Transfections were performed by electroporation of *in-vitro*-cultivated promastigotes [28]. Single colonies were selected on solidified BHI media containing 50 μg/ml nourseothricin (clonNAT, Jena, Germany) or 25 μg/ml hygromycin B (Sigma, St. Louis, MO, U.S.A.) or both. Approx 5 μg of the expression plasmids was digested with SwaI (Fermentas) for each electroporation. Integration of the expression cassette into the ssu (small ribosomal subunit) locus of 18S rRNA was confirmed by genomic PCR using the following primers: sat (nourseothricin resistance) forward primer 5′-CCTAGTATGAAGATTTCGGTGATC-3′, hyg (hygromycin resistance) forward primer 5′-CATGAAAAAGCCTGAACTCACCGCG-3′ and reverse ssu primer 5′-CTGCAGGTTCACCTACAGCTAC. For transcription and expression assays, transformed *L. tarentolae* cells were cultivated at 26 °C in suspension cultures in BHI medium supplemented with 5 μg/ml haemin.

RNA extraction and RT (reverse transcription)–PCR

A total of 10^8 cells was subjected to total RNA extraction. Using TRIzol® reagent (Gibco), cDNA synthesis was

performed by a First Strand cDNA Synthesis kit (Fermentas). Transcription accuracy was checked by amplifications of 1960, 500 and 1000 bp of the tPA, sat and hyg mRNAs respectively.

Zymography

An 11% resolving polyacrylamide gel (Sigma) was co-polymerized with plasminogen (Chromogenix, Milano, Italy) and gelatin (Sigma). The 4% stacking gel was prepared without plasminogen and gelatin. Electrophoresis was performed at 4°C at a constant current of 8 mA. The residual SDS in the gel slab was removed in 2.5% (w/v) Triton X-100 with shaking at room temperature (25°C) for 1 h. Then, the gel slab was incubated in 0.1 M glycine/NaOH (pH 8.3) for 5 h at 37°C. Finally, the gel slab was stained and destained using the Coomassie Brilliant Blue R-250 procedure. The location of the peptide harbouring enzymatic activity was not stained by the dye, in contrast with the blue-stained background.

Southern-blot analysis

Southern-blot analysis was performed using an ECF Random Prime Labelling and Signal Amplification System (Amersham) according to the manufacturer's instructions. Briefly, *Leishmania* genomic DNA was extracted and digested by NdeI and HindIII restriction enzymes (Fermentas). Digestion products were size-fractionated on 0.8% agarose gels, transferred on to nylon membrane (Biosupport Membrane) and cross-linked to membranes by UV irradiation (UV cross-linker; Hoefer). DNA was prehybridized at 63°C for 30 min in 5 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), 0.1% SDS and 5% dextran sulfate in 1:20 dilution of liquid blocking solution containing 1% BSA. Hybridization was performed at 63°C overnight with fluorescein-labelled probes. Blots were washed once with 1 × SSC and 0.1% SDS, for 15 min at 63°C, and once with 0.5 × SSC and 0.1% SDS for 15 min at 63°C. The membrane was incubated at room temperature for 1 h in the alkaline phosphatase-conjugated anti-fluorescein 5000-fold in freshly prepared 0.5% BSA (Sigma). Detection reagent was added to the membrane and bands were detected by autoradiography.

Amidolytic activity test

A test kit for the detection of tPA activity was purchased from Biopool. Biopool's Chromolyze tPA is a BIA (biofunctional immunosorbent assay) intended for quantitative determination of human tPA. The sample tPA was captured by antibodies on the microtest wells. The sp-322 monoclonal antibody used allows excellent tPA recovery at pH 5.9 without inhibiting tPA activity. After washing, the tPA substrate consisting of plasminogen, a plasma-sensitive chromogenic substrate and tPA activity promoters was added. Samples were read at 405 nm. The various dilutions of each sample were assayed. The assay procedure was carried out according to the Chromolyze tPA manual (Biopool).

Results

Subcloning of tPA gene

A PCR reaction with tPA primers on genomic DNA of CHO1-15 cells resulted in a band with the expected size of 1690 bp product (results not shown). The desired PCR product (tPA gene) was subcloned into two expression vectors, pFX1.4sat and pFX1.4hyg, containing antibiotic resistance gene, nourseothricin and hygromycin B respectively (Figure 1). The expression vectors containing tPA gene were introduced to *L. tarentolae* by electroporation. Three types of transformed cells were obtained: cells transformed with pFX1.4sat-tPA cassette, cells transformed with pFX1.4hyg-tPA cassette and cells transformed with both pFX1.4sat-tPA and pFX1.4hyg-tPA cassettes. Integration of tPA (1690 bp), sat (500 bp) and hyg (1000 bp) in the genomic DNA of the recombinant cells was confirmed by PCR analysis (Figure 2). Furthermore, to verify the right orientation of the integrated pFX1.4sat-tPA cassette, a PCR reaction using a forward sat and reverse ssu primers was performed on both wild-type cells and cells transformed with pFX1.4sat-tPA cassette. The expected 2.3 kb band was only observed from transformed cells indicating the right orientation of pFX1.4sat-tPA cassette (Figure 2). The same procedure was performed on genomic DNA of cells transformed with pFX1.4hyg-tPA cassette by using forward hyg and reverse ssu primers. The desired bands, 2.8 kb, were observed on the agarose gel (Figure 2, last lane).

Southern blotting

For evaluation of expression cassettes integration in 18S rRNA locus, we performed Southern-blot analysis. Genomic DNA was digested with NdeI. In this analysis, fluorescein conjugated tPA was used as probe. The probe detected no band in genomic DNA of wild *Leishmania* (Figure 3, lane 6) but detected a clear band in genomic DNA of pF4X1.4sat-tPA- and pFX1.4hyg-tPA-transfected *Leishmania* in one locus (Figure 3, lanes 2 and 3 respectively). While both alleles of the *Leishmania* were transfected with constructs containing tPA genes, higher-intensity bands representing tPA were observed (Figure 3, lanes 4 and 5). These results confirmed integration of pF4X1.4sat-tPA and pF4X1.4hyg-tPA into both alleles of the 18 S rRNA gene.

tPA expression assays

To verify tPA mRNA synthesis, RT–PCR was performed on total mRNA extracted from transformed cells using sat and hyg primers. The desired bands with the expected size of 1960 bp (tPA), 500 bp (sat) and 1000 bp (hyg) were obtained

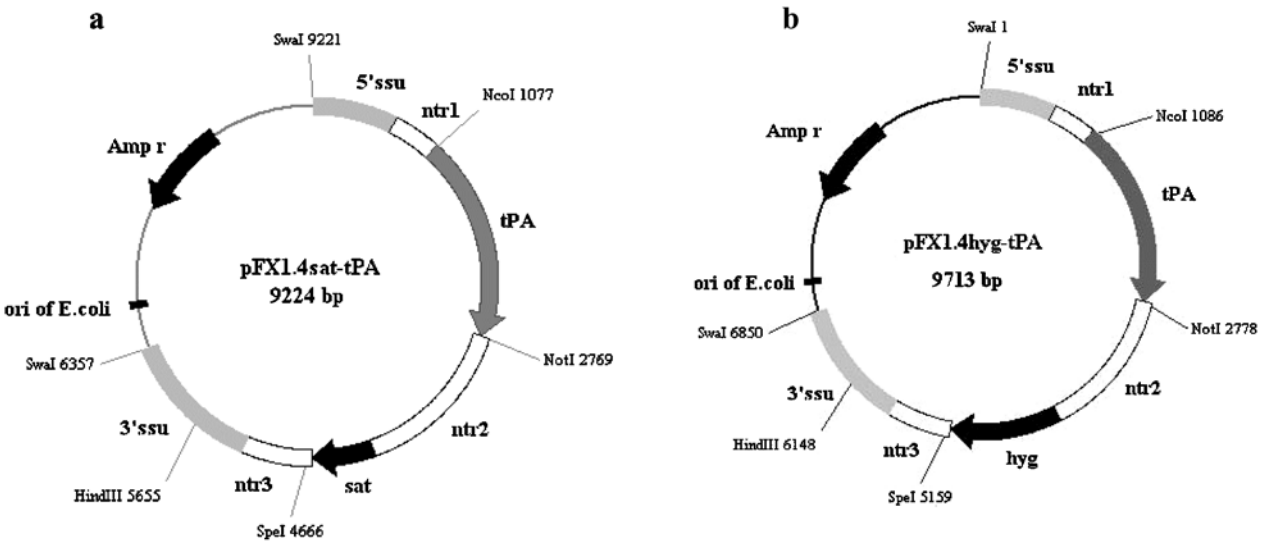

Figure 1 Restriction map of the cassette expression used for transfection of *L. tarentolae*

(**a**) pFX1.4sat-tPA. (**b**) pFX1.4hyg-tPA. Abbreviations: 5′ssu, 5′-portion of the small subunit of *L. tarentolae* rRNA gene; ntr1, a 0.4 kb fragment with the *camA* (calmodulin A) splice acceptor of *L. tarentolae*; tPA, tPA cDNA; ntr2, a 1.8 kb fragment of the intragenic region between *camA* and *camB* (calmodulin B) genes of *L. tarentolae*; ntr3, a 1.7 kb fragment of 3′-UTR of the *L. major* dihydrofolate reductase gene; 3′ssu, 3′-portion of the small subunit of rRNA gene.

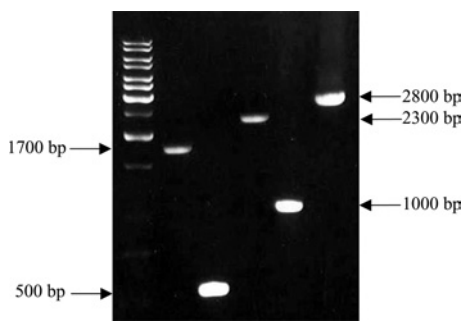

Figure 2 PCR on genomic DNA of *Leishmania* transfected with pFX1.4sat-tPA and pFX1.4hyg-tPA

Lane 1, size markers; lane 2, tPA gene; lane 3, sat gene; lane 4, a 2.3 kb band indicating integration of pFX1.4sat-tPA cassette into the ssu locus; lane 5, hyg gene; lane 6, a 2.8 kb band indicating integration of pFX1.4-tPA cassette into the ssu locus. Primers used for the PCR were: sat forward primer 5′-CCTAGTATGAAGATTTCGGTGATC, hyg forward primer 5′-CATGAAAAAGCCTGAACTCACCGCG and reverse ssu primer 5′-CTGCAGGTTCACCTACAGCTAC. Conditions of the PCR reaction were the same as that for tPA gene except that extension time was 2 min.

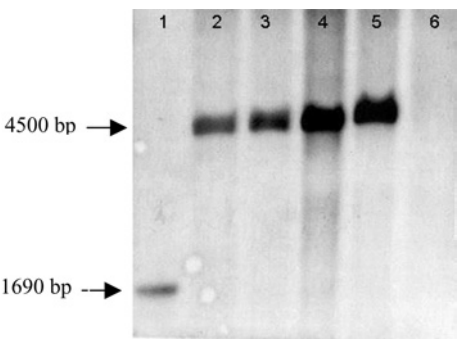

Figure 3 Southern-blot analysis with the tPA probe

Southern-blot analysis of the genomic DNA of wild-type *Leishmania* (lane 6), of the genomic DNA of transfected *Leishmania* with pF4X1.4sat-tPA only (lane 2), of the genomic DNA of transfected *Leishmania* with pFX1.4hyg-tPA only (lane 3), of the genomic DNA of transfected *Leishmania* with both pF4X1.4sat-tPA and pFX1.4hyg-tPA (lanes 4 and 5). Lane 1 is the PCR product of t-PA as the positive control.

(Figure 4). Using the zymography test the presence of secreted tPA in culture media and its serine-protease activity were evaluated. The plasminogen was co-polymerized and immobilized with gelatin in the polyacrylamide. Both the culture media of transformed *Leishmania* and non-transformed *Leishmania* were analysed under non-reducing conditions in order to preserve the correct conformation and activity of the serine protease domain. The transparent areas on the gel, indicating serine-protease digestion of plasminogen, were only observed in culture media of transformed *Leishmania* (Figure 5, lane 3). Also, culture media from both transformed and non-transformed cells were run on SDS-containing gel without plasminogen. To assess the nature of clear zones, each sample was incubated with function-blocking anti-(human tPA) polyclonal antibodies for 60 min at 37 °C. As we expected, clear zones related to the serine protease domain were not visualized (Figure 5, lane 4).

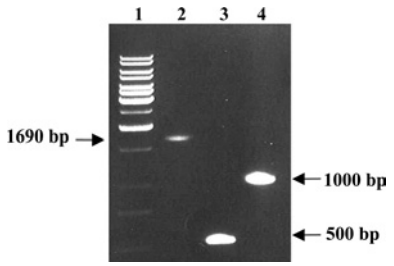

Figure 4 RT–PCR on total RNA isolated from pFX1.4sat-tPA- and pFX1.4hyg-tPA-transformed *Leishmania*

Lane 1; size markers; lane 2, tPA; lane 3, sat; lane 4, hyg.

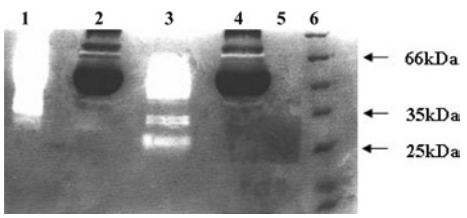

Figure 5 Zymography results

Lane 1, standard tPA (actilyse); lane 2, standard tPA + anti-tPA polyclonal antibody; lane 3, supernatant of *Leishmania* transformed with pFX1.4sat-tPA; lane 4, supernatant of *Leishmania* transformed with pFX1.4sat-tPA + anti tPA polyclonal antibody; lane 5, supernatant of wild-type *Leishmania*; lane 6, protein molecular-mass markers.

Table 1 Amidolytic activities

Sample (dilution)	tPA activity (i.u./ml)	$10^3 \times A_{405}$
tPA standard		
Undiluted	0	30
1:4	0.5	230
2:4	1.0	440
3:4	1.5	620
4:4	2.0	840
Culture supernatant		
Singly transfected *Leishmania* (1:100)	0.3	160
Doubly transfected *Leishmania* (1:100)	0.7	320

Amidolytic activity measurement

Serine protease converts plasminogen into plasmin, which subsequently digests the chromogenic substrate to a colour product with a maximum absorbance at 405 nm. The specific activity of the culture media of transformed *Leishmania* cells was measured (Table 1). The culture supernatant of cells transformed with pFX1.4sat-tPA showed an enzymatic activity of 30 i.u. (international units)/ml, while the enzymatic activity of the cells transformed with both pFX1.4sat-tPA and pFX1.4hyg-tPA was 70 i.u./ml.

Discussion

In the present study, *L. tarentolae* was transfected with an expression vector containing the tPA gene. One of the reasons for using Trypanosomatidae species is that the protein-coding genes can be integrated into the 18 S rRNA locus, which is transcribed by RNA polymerase I and, since RNA processing is uncoupled from DNA transcription, the gene of interest can be largely transcribed [30–32]. Such a system developed for *L. tarentolae* would be invaluable for a large number of applications, including the expression of toxic or unstable proteins [24]. In trypanosomatid protozoa, polycistronic mRNAs are post-transcriptionally sliced into individual mRNAs through a trans-splicing and polyadenylation procedure within the intergenic regions [33]. Since gene regulation in Trypanosomatidae happens mainly post-transcriptionally through intergenic UTRs (untranslated regions), the selection of such UTRs is critical for the structure of a well-constructed vector [30]. In order to produce high level of tPA protein, pFX1.4sat-tPA and pFX1.4hyg-tPA vectors contain three UTRs: ntr1, ntr2 and ntr3. The origins of UTRs are as follows: ntr1, partial UTR of calmodulin A gene from *L. tarentolae*; ntr2, complete UTR of calmodulin BA gene from *L. tarentolae*; and ntr3, hydrofolate reductase thymidylate synthase locus from *L. major*. Our expression cassette consisting of sequences of 5′- and 3′-ssu can be properly integrated into the ssu rRNA gene, which is powerfully transcribed by RNA polymerase I [34]. The expression cassettes were introduced to the genome of *Leishmania* that results in the integrated form rather than the episomal form, which expresses lower amount of recombinant protein [24].

We used the native human signal sequence of tPA gene. To evaluate the efficiency of different signal sequences, Breitling et al. [24] replaced the native signal sequence of rhEPO (recombinant human erythropoietin) with a signal sequence derived from the secreted acid phosphatase of *L. mexicana*. This study revealed that using *Leishmania*-derived signal sequence yielded considerably better secretion of rhEPO from transformed *L. tarentolae* [24]. We had quite a high expression with native human signal peptide of tPA gene to the extent that we did not replace it with a signal peptide derived from a protein of *Leishmania*. Our results indicated that human signal peptide not only could perform the ER (endoplasmic reticulum) translocation and secretion in *Leishmania*, but was also normally cleaved off during this procedure.

Zymography analysis for evaluation of the serine-protease activity of tPA was performed (Figure 5). Zymography is comparable with ELISA, as levels of less than 10 pg of matrix metalloproteinase have been detected on gelatin zymograms [35]. Zymography of culture supernatants of transformed *Leishmania* with pF4X1.4sat-tPA and pF4X1.4hyg-tPA, and both showed three distinct bands, corresponding to 25, 30 and 65 kDa. The lower-molecular-mass bands may have resulted from limited proteolysis of the tPA [36,37].

To verify that the clear zones are caused by tPA function, a zymography analysis using co-polymerized PAGE

without plasminogen was performed. Our samples did not create clear zones in gels without plasminogen, showing that clear zones have specifically resulted from tPA function (results not shown). This result was in agreement with the finding of another study demonstrating that plasminogen-independent protease could be detected and analysed by excluding plasminogen from the zymography gel [35]. Moreover, to further confirm that the functioning protein in the present study is exclusively tPA, we added function-blocking polyclonal antibody against tPA to both samples and positive control prior to zymography. In this analysis, clear zones on the gel were not created, confirming the exclusive presence of tPA. This is due to formation of an antigen–antibody complex that blocks the serine-protease activity of tPA [39].

To ensure that a higher level of tPA production is achieved, two expression cassettes containing different selection markers were integrated into the ssu locus. Doubly transfected *Leishmania* (with pF4X1.4sat-tPA and pF4X1.4hyg-tPA) produced more tPA than singly transformed cells. Correlation between the number of integrated gene copies and level of protein expression was confirmed by using a Chromolyze tPA kit (Table 1). The tPA enzymatic activity of culture media of singly transformed *Leishmania* (pFX1.4sat-tPA) was 30 i.u./ml, whereas this activity was 70 i.u./ml in the culture supernatant of doubly transformed *Leishmania* (pFX1.4sat-tPA and pF4X1.4hyg-tPA). If we consider the specific activity of rtPA to be almost 4×10^5 i.u./mg, the amount of expression should be 0.17 μg/ml. This amount is much higher than enzymatic activity of truncated tPA (7 i.u./ml) produced in *E. coli*, although it is not enough to be visualized by SDS/PAGE [38]. Also, in this system the direct correlation between the copy numbers of integrated *egfp* (enhanced green fluorescent protein) gene and the expressed protein has been shown [24]. In one study Qiu et al. [20] produced active tPA in *E. coli*. After purification, approx. 180 μg of tPA with a specific activity almost that of the authentic protein is obtained per litre of culture in a high-cell-density fermentation [20]. Recombinant human tPA has been expressed in a cell line derived from the *Drosophila melanogaster* (fruitfly). The rtPA isolated from this cell line was equal to that produced in CHO cells. It had a specific activity (4.1×10^5 i.u./mg) similar to rtPA from CHO (5.4×10^5 i.u./mg).

Here, we have shown that *L. tarentolae* can be utilized for significant production of human rtPA. In this system, rtPA shows normal biological activity and proper folding. Clear zones on PAGE of zymography analysis show the serine-protease activity of the tPA produced. Performing a bioimmunoassay analysis using the Chromolyze tPA kit demonstrated amidolytic activity, bioactivity and a natural immunological response to the recombinant protein. Our results were also in accordance with earlier observations that *Trypanosoma* and *Leishmania* species are able to produce active mammalian cytokines such as IL-2 (interleukin 2) and IFN (interferon) [25,27]. Breitling et al. [24] produced various proteins in *L. tarentolae*. In the case of EGFP, the clone with the maximum expression produced recombinant EGFP at approx. 30 mg/l of suspension culture. Other proteins included both cytosolic proteins (T7 RNA polymerase, Cu/Zn superoxide dismutase, proto-oncogene Myc etc.) and membrane-associated proteins such as the small GTPase Rab7. The obtained expression levels were of the similar array of amount as EGFP. All proteins analysed were biologically active and, in the case of Rab7, modified with isoprenoid moieties [24].

Acknowledgments

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