Cloning of Tissue Plasminogen Activator cDNA in Nonpathogenic *Leishmania*

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Received: 30/May/2006, Accepted: 2/Oct/2006

Introduction: At present, most recombinant proteins are produced in prokaryotes especially *E coli*. Yeasts and CHO also are used as eukaryotic hosts. *Leishmania tarentolae*, a parasite of lizards, a member of Trypanosomatidae family is one of the new systems for expression of heterologous proteins. In this system, some of the parasitic protozoa features are used in expression of mammalian proteins.
Material and Methods: For evaluation of the protozoa for expression of human complex proteins, we cloned cDNA of *tPA* gene containing native human signal sequence. We used vectors containing 3´ and 5´ sequences of *Leishmania 18s rRNA* for integration of the vectors in *18s rRNA* gene and severe transcription.
Results: RT-PCR test showed production of specific mRNA of *tPA* gene in the recombinant cells. Southern blot analysis confirmed the cloning of t-PA in the genome of the *Leishmania*.
Conclusion: This study showed native human signal sequence mediate transport and secretion of the protein. Hence, *L. tarentolae* is the first useful biotechnologically protozoan and tPA is the most complex protein expressed in it.

Keywords: *Leishmania Tarentolae*, Tissue Plasminogen Activator, Polymerase Chain Reaction

Introduction

Expression of recombinant proteins is an important method to make proteins. Different systems including bacteria, yeasts, fungi, mammalian cells, insect cells, transgenic animals, and transgenic plants have been used as necessary. None of these numerous systems is general. All systems use independent organisms. Significant features of the protozoan biology have not yet been used for biotechnological purposes. The pathogenic protozoa of Trypanosomatidae family are well- known parasites. Members of this family infect a broad spectrum of hosts, from plants to well-developed mammals (1). Because of the importance in public heath, most studies have been performed in human Trypanosome and *Leishmania*. Polycistronic transcription, trans-splicing, and control of the gene expression at posttranslational level are among unique features of these species (2, 3). Trypanosomatidae are rich in the rate and variation of various glycoproteins, as they make over 10% of their content (4). Probably because of a parasitic life, their glycoprotein structure is usually similar to those of mammalian cells. For instance, some of these glycoproteins have complex oligosaccharides containing glucose, fucose, and sialic acid (5). Thanks to efforts of molecular parasitologists, gene manipulation methods are currently available for

Trypanosomatidae and many experiments have been performed to express heterologous genes. In all reported cases, expressed recombinant proteins were biologically active. However, these experiments have been done in human pathogenic species (6, 7, 8). Except for safety considerations, these species obviously have low growth rate and need serum for culture; so they are of limited use as expression systems. One of nonpathogenic human protozoa is *Leishmania tarentolae*, which is the parasite of *Tarentolae anulairs* lizard. This protozoan has rapid growth rate and its generation time is 4 hours at 26 ºC. Moreover, its cell density in liquid cultures can be up to 10^8 cells/ ml and it has simple nutrition needs and grows in serum-free media (9). Proteins such as human erythropoietin and green fluorescent protein have already been expressed in *L. tarentolae* (10). Recently, a variant of this protozoan has been produced which permanently expresses T7 RNA polymerase and tetracycline resistance gene. This variant is transformed permanently by T7 promoter/TET operator controlled heterologue gene and by adding tetracycline to culture media, it begins transcription.

The protein expression rate in this system is about 1% of total cell protein (11). Also it has been evident that *L. tarentolae* expression system can be used for producing isotope-labeled proteins too, which is very important in performing spectrometric researches of NMR (Nuclear Magnetic Resonance) used to study of protein structure at atomic level (12).

In present study, cloning and expression of *tPA* cDNA was used to evaluate the protozoa in production of complex proteins. tPA consists of 527 amino acids and its molecular weight is 62 kDa. tPA is a serine protease and has 5 functional domains called finger domain, epidermal growth factor domain, kringle-1 and kringle-2 domains, and serine protease domain (13, 14). It also has 35 cysteine amino acids, 17 disulfide band, three N-linked oligosaccharide chains, and one O-linked chain. It physiologically converts the plasminogen to plasmin, so beginning and intensifying the process of fibrinolysis (15). Compared to streptokinase and urokinase, tPA acts more specifically, binds stronger to fibrin clots, and mainly activates the plasminogen trapped inside clots, with minor effect on circulating plasminogen or other coagulating factors. Therefore, recombinant tPA is probably the most effective available fibrinolytic agent, while the fibrin affinity of tPA not only concentrates catalytic action, but also increases catalytic efficiency of tPA. Coadministration of heparin (as an auxiliary anticoagulant) and tPA clears up to 75% of obstructed coronary arteries in 90 min and decreases mortality 25% (16). Recombinant tPA, as a fibrinolytic drug, is used in treatment of diseases such as acute myocardial infarction, pulmonary embolism and acute ischemic stroke (17). Currently, recombinant tPA is produced using Chinese hamster ovary (CHO) cells (18, 19). The main problem of such a system is requirement for serum-containing culture media, which complicates the purification of the recombinant protein and increases the costs. So, continuous efforts are being done to improve this process and cost reduction. In this study, *tPA* cDNA was used to transfect the *Leishmania* cells using specific vectors. These vectors have sequences of 18s rRNA gene in both ends. This factor integrates vectors into this locus and the great advantage of it is abundant transcription used to produce recombinant tPA.

Material and Methods

Amplification of tPA cDNA

The CHO 1-15 (ATCC-CRL9606) cells containing cDNA of *tPA* gene were cultured in Ham's F12 (Gibco, UK) media containing FCS 10% (Gibco, Germany). After collecting cells, genomic DNA was extracted using Nucleon kit (Amersham, USA). The forward and reverse primers of *tPA* cDNA having restriction sites for NcoI and NotI enzymes, respectively, were designed and synthesized:

F-tPA: 5´AAC CAT GGA TGC AAT GAA GAG AGG GCT C-3

R-tPA: 5´GCG GCC GCT CAC GGT CGC ATG TTG-3'

These primers were designed based on *tPA* cDNA from NCBI (accession no 10047) database. The tPA cDNA containing native human signal sequence was amplified using Expand Long Template PCR System (Roche, Germany). The amplification consisted of 25 cycles as follows: denaturation, 94ºC/ 30 sec; annealing, 66 ºC/ 60 sec; extension, 68 ºC/ 90sec; and finally the reaction mixture were placed for 10 min at 68 ºC.

Cloning

Ligation reaction between the PCR product and T-vector (Fermentas, Vilnius, Lithuania) pTZ57R was done and *E. coli Top 10F´* cells were transformed. Recombinant clones were selected using white-blue screening and sequencing was made using M13 forward and M13 reverse primers for confirming gene sequence integrity. The gene was then digested using NotI and NcoI (Fermentas, vilnius, Lithuania) and subcloned in *Leishmania* specific expression vectors pFX1.4sat and pFX1.4hyg (Bioscience, Jena, Germany) (Figure 1). The recombinant vectors were used for transformation of *E. coli Top 10F´* cells and after selection of recombinant clones in ampicillin containing media, their plasmids were extracted. Selected clones containing tPA insert were confirmed using PCR analysis and enzymatic digestion.

L. tarentolae culture and transfection

L. tarentolae was cultured in Brain Heart Infusion Broth medium (DIFCO, USA) supplemented with 15 µg/ml hemin (Sigma, UK) and streptomycin (50 µg/ml and penicillin (50 IU/ml) as described by Simpson L (9). Using FX1.4 sat-tPA and pFX1.4 hyg-tPA, laboratory-cultured promastigotes were transfected by electroporation at 450 V and 450 µF capacitance (20). Solid media were used to select recombinant clones and single colonies on media surface were picked up. At first, the cells were transfected using pFX1.4 sat-tPA and recombinant *Leishmania* were selected in BHI culture media with 50 µg/ml nourseothricin antibiotic (clonNAT, Jena, Germany). After confirming the integration, the same cells were retransfected using pFX1.4 hyg-tPA in order to insert more copy number of gene. Then recombinant cells regarding both vectors were selected in solid culture media containing 50 µg/ml nourseothricin and 25 µg/ml hygromycin B (Sigma, St. Luis, USA). About 5 µg of each expression vector was digested and linearized by *SwaI* (Fermentas, Vilnius, Lithuania) for electroporation. Integration of expression cassettes into *ssu rRNA* locus was confirmed by performing PCR on genomic DNA. For this purpose, the forward primer of *sat* and *hyg* gene were selected from their vectors and the reverse primer of *ssu rRNA* locus was selected from genomic DNA. To evaluate specific *tPA* expression, recombinant cells were cultured in BHI liquid media containing 15 µg/ml hemin at 26 ºC.

RT-PCR: The cells were cultured as mentioned and incubated at 26 ºC for 72 hr. Total RNA was extracted from 3×10^8 cells using TRIzol reagent (Gibco, UK) as follows. After washing the cells, the sediment was dissolved in 1.5 ml TRIzol, centrifuged for 10 min at 12000*g* and 4ºC, and kept at room temperature for 5 min. Then 0.2 ml chloroform was added for each ml TRIzol used and after shaking, it was kept at room temperature for 15 min. It was centrifuged for 15 min at 12000*g* and 4ºC, and the aqueous phase was collected. Then 0.75 ml isopropanol was added to the mixture and after keeping at room temperature for 10 min, it was centrifuged for 10 min at 12000*g* and 4ºC. RNA sediment was washed using ethanol 75% and finally dissolved in 30µl RNase-free (DEPC-treated) water. The cDNA was synthesized using Single-Stranded cDNA Synthesis kit (Fermentas, Vilnius, Lithuania) as recommended by manufacturer: 1 µl oligo dT (50 pmol/µl) was added to 1-5 µg total RNA and the reaction volume was increased to 11µl using RNase-free water. The reaction mixture was kept at 70ºC for 5 minutes and then cooled rapidly on ice. Then 2 µl of 10 mM dNTPs, 2 µl RNase inhibitor, and 4µl 5x reaction buffer were added and the volume was increased to 19µl using RNase-free water. The reaction mixture was kept at 37ºC for 5 min and then 40 units of Moloney murine leukemia virus reverse transcriptase enzyme added. The

reaction mixture was kept at 37ºC for 60 min and at 70 ºC for 10 min, and finally freezed at -70ºC. RT-PCR was performed on 3µl cDNA using *tPA, sat, hyg* genes primers.

Zymography: The zymography was performed as described by Heussen C with a little change (21). Using acrylamide-bisacrylamide (1:30), 12% polyacrylamide gel containing SDS was prepared and copolymerized with 0.1% gelatin and plasminogen (10 µg/ml) (Chromogenix, Italy). Two percent stacking gel was prepared free of plasminogen and gelatin. In order to prepare specimens, the cells were cultured and incubated at 26 ºC for 72 hr as mentioned. Then, the cell culture media was used for zymography. To maintain exact configuration of protein and serine protease activity, all specimens were electrophoresed using non-reducing (2-ME-free) conditions and constant current of 8 mA at 4ºC. In order to omit SDS, the gel was first washed using Triton X100 (2.5%) for 1 hr at room temperature with shaking and then washed using distilled water. The gel was placed in 0.1 M glycine buffer (PH=8.3) at 37ºC for 3 hr, and then stained with Coomassie blue R-250 0.1% dissolved in two parts methanol, one part acetic acid, and six parts distilled water for 30 min. The gel was destained using the same solution but without dye. The zymography was done using two other methods to precisely analyze the expressed recombinant tPA. Firstly, the zymography was done using plasminogen-free gels, and in second method, the specimens were incubated with polyclonal anti-tPA antibody for one hr at 37ºC before zymography.

Measurement of amidolytic activity: The Chromolize tPA Assay kit (Biopool, Ireland) was used as described by the manufacturer for quantitative measurement of the amidolytic activity of recombinant tPA expressed in transfected *Leishmania* culture media. Biologically active tPA converts the plasminogen into active plasmin, which lyses the polypeptide amid bands (amidolytic activity). In this way, produced plasmin makes color by affecting choromogenic substrate which is directly related to specimen tPA and is detected by measurement of light absorbance at 405 nm. A 1:100 dilution of cell culture media and the same conditions as described for preparation of zymography specimens were used in this test. Standard tPA solutions of 0, 0.5, 1, 1.5, 2 IU/ml were used to prepare the standard curve.

Results

Cloning of *tPA* cDNA: PCR was performed on the DNA extracted from CHO 1-15 cells using primers containing restriction sites for NcoI and NotI. A single 1690 bp band was seen on agarose gel after electrophoresis. This band was extracted from the gel and ligated with pTZ57R T-vector. The correct gene cloning was confirmed by PCR (showing a 1690 bp band) and DNA sequencing. BLAST assessment of obtained sequence showed 100% similarity to human *tPA* mRNA. In order to assess the relationship between integrated gene copies in *18s rRNA* locus and protein expression rate, this gene was sub-cloned in pFX1.4hyg and pFX1.4sat expression vectors; *tPA* gene was cut out of the pTZ57R-tPA by NcoI and NotI enzymes and extracted from the gel. In addition, the pFX1.4hyg and pFX1.4sat vectors were digested using the same enzymes and electrophoresed on agarose gel. This enzymatic digestion cut out the 0.7kb fragment from the 7.5kbp pFX1.4sat and 8kbp pFX1.4hyg. The ligation reaction was prepared between *tPA* gene and 7.5kbp and 8kbp fragments. After transformation of *E. coli Top10F´* cells, the recombinant clones in LB agar media containing ampicillin were selected. The pFX1.4sat-tPA and pFX1.4hyg-tPA clones were confirmed using enzymatic digestion with NcoI and NotI (showing a 1690 bp band) and PCR analysis (showing a 1690 bp band). Then, about 5 µg of above vectors was digested using SwaI, and a 2.9kbp fragment (a piece of *E. coli* vector) and a 6.3kbp pFX1.4sat-tPA and 6.8kbp pFX1.4hyg-tPA were obtained. The larger band was extracted from the gel and electroporated into *L. tarentolae*. Recombinant colonies were seen in solid culture media plates after 5-10 days. PCR

nalysis of DNA extracted from these cells showed the presence of *tPA, sat,* and *hyg* genes. PCR analysis using the forward primers of *sat* and *hyg* genes and the reverse primer of *18s rRNA* gene showed 2.3kb and 2.8kb fragments respectively, and confirmed cassette integration in *18s rRNA* gene (Figures 2 and 3).

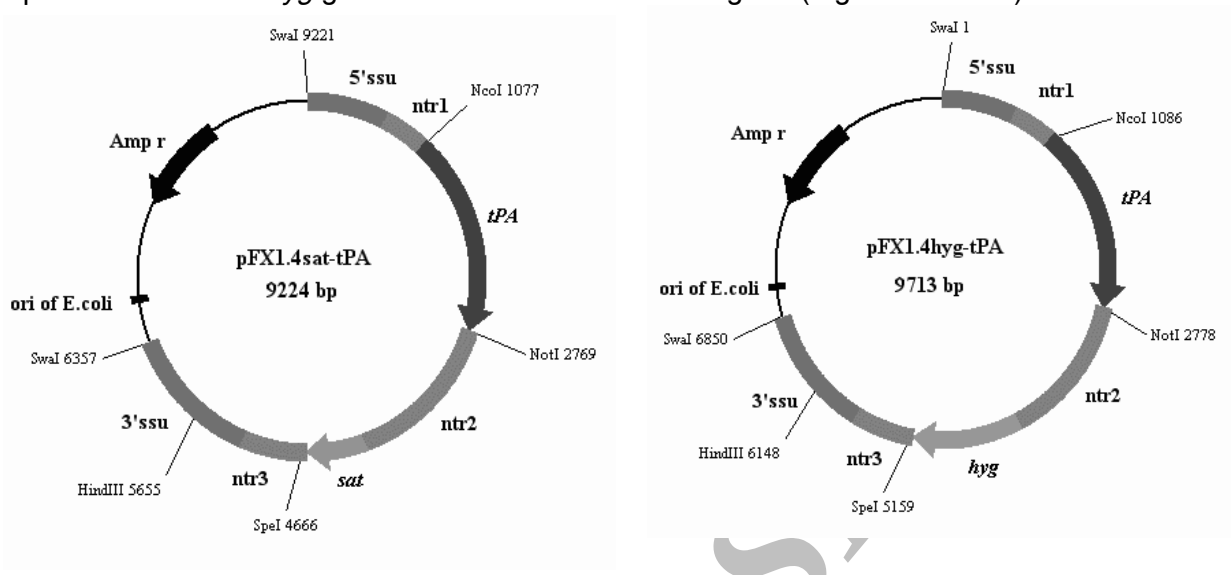

Figure 1. Restriction map of vectors used for transfection

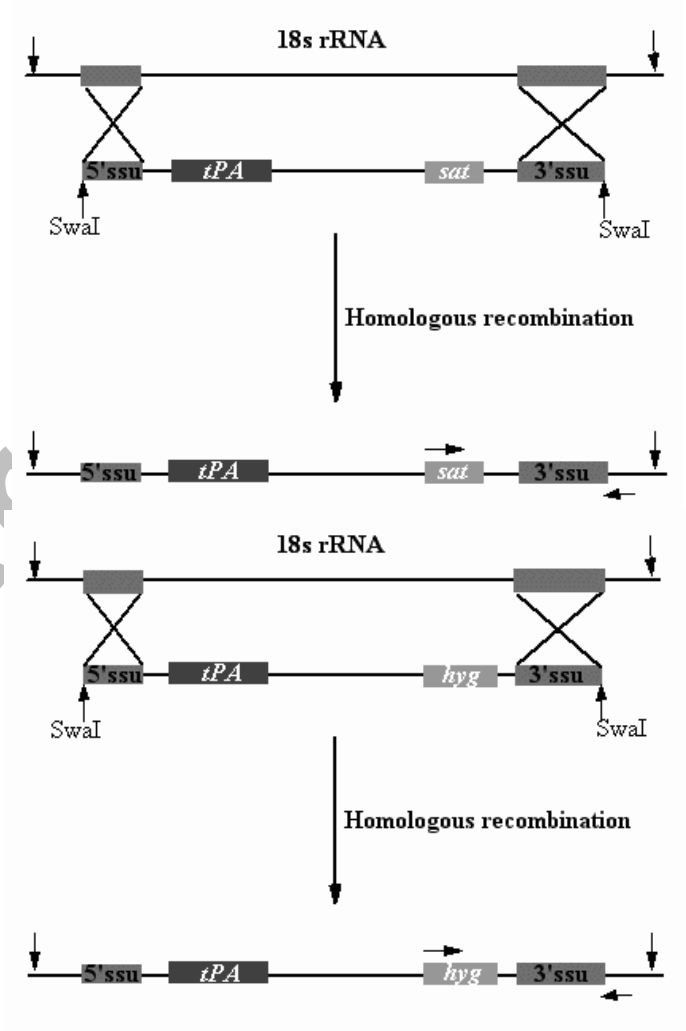

Figure 2. Integration of vector pFX1.4 sat-tPA (up) and vector pFX1.4hyg-tPA in 18s rRNA locus (down)

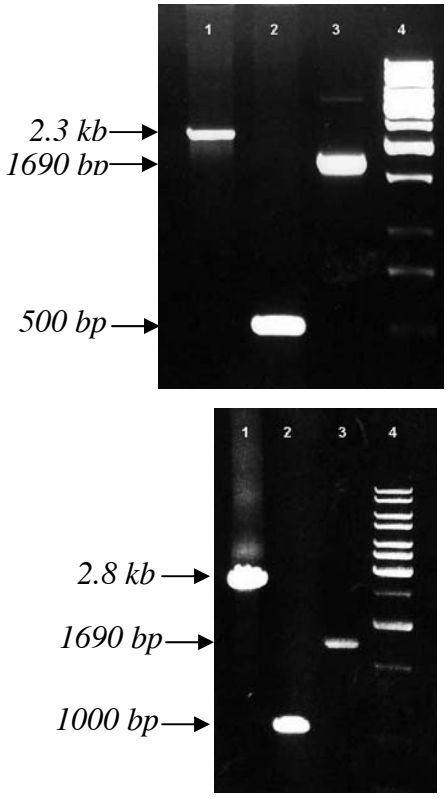

Figure 3. PCR analysis of the genomic DNA extracted from the cells transformed with pFX1.4sat-tPA (up). Lane 1: 2.3 kbp; lane 2: sat gene; lane 3: tPA gene; and lane 4: DNA size marker. Results of PCR analysis after second transfection with pFX1.4 hyg-tPA (down). Lane 1: 2.8kbp; lane 2: hyg gene; lane 3: tPA gene; and lane 4: DNA size marker.

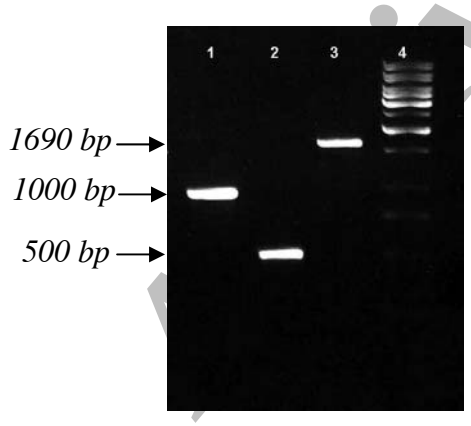

Figure 4. RT-PCR on total RNA isolated from transformed cells with pFX1.4 sat-tPA and pFX1.4hyg-tPA. Lane 1: hyg; lane 2: sat; lane 3: tPA; and lane 4: DNA size marker.

Discussion

Human *tPA* cDNA containing complete coding and signal sequences were used to assay the secreting capacity of *L. tarentolae* for complex mammalian proteins. mRNAs are transcribed as polycistronic precursors in this protozoan and other Trypanozomatidae protozoa and then processed to single mRNAs through trans-splicing and polyadenylation using intergenic regions (24). In these species, gene expression regulation occurs primarily at RNA level and may be affected by intergenic regions (24, 25). This means that intergenic regions have signals for trans-splicing. Therefore, it is necessary to select such untranslated regions (UTR) in the structure of well-organized vectors (26). pFX1.4sat and pFX1.4hyg have three UTRs: ntr1, ntr2, and ntr3. The origin of ntr1 is a fragment of *calmodulin A* UTR in *L. tarentolae*, while the origin of ntr2 is complete *calmodulin BA* UTR, and the origin of ntr3 is complete *hydrofolate reductase tymidilate synthase* sequence of *L. major* (Figure 1). These expression vectors have 5′ and 3′ sequences of small ribosomal subunit of *Leishmania* encompassing above gene. So, they produce high levels of specific transcripts. In this way, high-level gene transcription occurs using RNA polymerase I (27) (Figure 2). In this study, human *tPA* cDNA containing native signal sequence was used. Breitling *et al.* studied signal sequence effect on production of recombinant human erythropoietin in *L. tarentolae*. They replaced human native signal sequence with that of *acid phosphatase* of *L. mexicana* and concluded *L. tarentolae* transformed with such vectors produce more recombinant erythropoietin than cells transformed with previous vectors (10). Our results showed that mammalian signal peptide transports tPA correctly in *L. tarentolae* too and causes its secretion into cell culture media. Zymography on specimens of the cells transformed by pFX1.4sat-tPA, pFX1.4hyg-tPA, or both showed three clear distinct zones, weighting 25, 30, and 65 kDa on the polyacrylamide gel. These specimens produced no clear zones on plasminogen-free gels. This shows that production of clear bands on plasminogen-containing zymogram gels is specific tPA activity. Heussen, the inventor of this method, has shown that plasminogen-independent proteases may be assayed and detected by omitting the plasminogen of gelatin polyacrylamide mix (21). Then, to assay exact structure of produced recombinant

proteins, the specimens were complexed with tPA-blocking polyclonal antibody before zymography. There was no clear band in this phase. This primarily showed that formation of clear bands on zymogram is exclusively because of produced recombinant tPA and other proteases like Leishmania serine protease are of no concern. Moreover, it was shown that the recombinant tPA has also a normal immunologic activity, which makes a complex with anti-tPA antibody and stops its activity. Hosomi *et al.* used this method to confirm normal catalytic activity of tPA and found the same results (28). In this study, *tPA* cDNA was used to evaluate *L. tarentolae* for production of complex heterologous proteins and after integration of expressing vectors in small ribosomal subunit locus, a high amount of recombinant protein was obtained. This expression is reproducible and increases by increasing the *tPA* copy number. The relationship between integrated gene copy number and protein expression rate was studied using quantitative measurement of amidolytic activity. Amidolytic activity was 30 and 70 IU/ml in the specimen of transformed cells by pFX1.4sat-tPA and pFX1.4hyg-tPA, respectively. This activity is more than reported ones in other systems. For example, enzymatic activity of *E. coli*-produced tPA has been reported as 7 IU/ml (29). Other researchers have integrated one or more copies of *green fluorescent protein* gene in *18s rRNA* locus using these vectors and obtained a direct relationship between the number of integrated copies and protein expression rate (10). tPA has 35 cycteine amino acids which participate in formation of 17 disulfide bands, and so it seem necessary to study the pattern of disulfide bands in tPA expressed in *L. tarentolae*. Certainly, integration of disulfide bands plays a major role in correct three-dimensional structure and biological activity of the protein and we indirectly confirmed it by showing the biological activity of recombinant tPA. Similarly, other researchers have shown *Leishmania* species to be able to produce recombinant mammalian proteins such as interleukin-2 and gamma interferon with normal activities (6, 30).

Conclusion

L. tarentolae has many advantages like requirement for inexpensive culture media, serum independency, rapid multiplication, growth at room temperature, possibility of posttranslational modifications like mammals, and etc. In addition, our results show this protozoan is able to express complex recombinant proteins such as tPA, having normal biological activity. So, it may be reasonable to study its application in production of recombinant pharmaceutical proteins.

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