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Cloning and Expression of Functional Full-Length Human Tissue Plasminogen Activator in *Pichia pastoris*

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Abstract Human tissue plasminogen activator (t-PA) plays a pivotal role in the treatment of acute myocardial infarction, ischemic stroke, and deep vein thrombosis. It has the benefit of generating no adverse effects such as fibrinogen depletion, systemic hemorrhage, and immunologic reactions. Human t-PA is a serine-protease enzyme containing 527 amino acid residues in five structural domains. The correct folding of t-PA requires the correct pairing of 17 disulfide bridges in the molecule. A gene encoding full-length human t-PA was cloned into pPICZαA expression vector downstream of alcohol oxidase promoter and α-mating signal sequence from *Saccharomyces cerevisiae* and flush with the kex2 cleavage site to express the protein with a native N terminus. The methylotrophic yeast, *Pichia pastoris* GS115 strain, was transformed with this cassette, and methanol utilizing (mut+) transformants were selected for production and secretion of human t-PA into culture media. SDS–PAGE and Western blot analysis showed the expressed bands of t-PA protein. Zymography test indicated suitable folding and proper function of the expressed recombinant human t-PA in conversion of plasminogen to plasmin and gelatin lysis. Amidolytic activity test showed the amidolytic activity of 1,650 IU/ml. The results of this study concluded that *P. pastoris* methylotrophic yeast can be a suitable alternative for mammalian and prokaryotic expression systems to produce t-PA.

Keywords Human tissue plasminogen activator · t-PA · *Pichia pastoris* · Cloning · Expression · Zymography · Amidolytic assay · Densitometry

Introduction

Tissue plasminogen activator (t-PA) is a preferred thrombolytic agent for the treatment of thromboembolic event [1–3]. Thromboembolism is one of the most common disorders of human blood vessels which involve many organs including myocardium, brain, lung [4], kidney, and extremities [5]. It is one of the principal etiologies of coronary artery occlusion,

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acute myocardial infarction, and ischemic stroke. Coronary artery disease is the most common cause of mortality especially in developed countries, and brain stroke is responsible for a great number of deaths and disabilities worldwide [6]. In the USA, venous thromboembolism-related deaths are estimated 300,000 annually, 34% of which are sudden fatal pulmonary embolism [7].

Although anticoagulant and antiplatelet agents are highly valuable in the prevention of such conditions, thrombolytic drugs are the most effective treatments after the formation of thromboemboli in body organs [8–11]. Compared to indirect activators of plasminogen such as *Streptokinase* and *Staphylokinase* which are non-human proteins, human t-PA as a direct activator has the benefit of causing no adverse effects such as anaphylactic reaction, fibrinogen depletion, and systemic hemorrhage [12, 13].

Tissue plasminogen activator is a polypeptide containing 527 amino acid residues. The molecule is composed of five structural domains. Near the N-terminal region is a looped finger domain followed by a growth factor domain and the two domains, kringle 1 and kringle 2. Both finger and kringle 2 domains bind specifically to the fibrin clot and facilitate t-PA protein activation. Next to the kringle 2 domain is the *serine-protease* domain in which the catalytic site is located at the C terminus of the protein. This domain is responsible for plasminogen conversion into plasmin and fibrin homeostasis. The correct folding of t-PA requires the correct pairing of multiple disulfide bridges in the molecule [14, 15].

Bowes melanoma cells were the primary source of t-PA production for medical purposes [16]. Since a steady system efficiently producing large amount of purified enzyme is needed for medical usage, the production of full-length recombinant t-PA progressed to mammalian systems especially Chinese Hamster Ovary (CHO) cells. Furthermore attracted by simplicity and cost of production, scientists have made a number of attempts to produce t-PA in *bacterial* [17] and *yeast* [18] systems. Since the native t-PA protein, has a complex structure, its production in *bacteria* encountered many problems including misfolding, inclusion body formation, lack of activity, and low production yield? Numerous methods have been proposed to overcome these problems of bacteria, but the optimum solutions are yet to come [19].

Among eukaryotic systems, the *yeast*, *P. pastoris*, have been used in the production of various recombinant proteins including complex disulfide bonded ones [20]. Rapid and high-yield production of recombinant proteins driven by strong promoters like Alcohol Oxidase 1 (AOX1), low level of background secreted proteins and proper post-translational modifications of the expressed proteins have made this methylotrophic yeast a suitable candidate for production of more complex proteins [21, 22].

In this study, for the first time, GS115 strain of *P. pastoris* was used as a suitable candidate host to express full-length, active human t-PA, and secreted protein was assayed both qualitatively and quantitatively.

Materials and Methods

Strains, Plasmids, and Culture Media

Escherichia coli strain Top10 F′ and *P. pastoris* GS115 strain (Invitrogen USA) were used as hosts for recombinant plasmids. pTZ57R (Fermentas, Vinius, Lithuania), as T/A cloning vector, and pPICZαA (Invitrogen USA), as expression vector, were used in the experiments.

Buffered complex medium, containing glycerol (BMGY; Invitrogen USA), was used for growing the cells before induction, and buffered complex medium, containing methanol (BMMY; Invitrogen USA) was used as induction medium.

PCR Amplification and the Cloning of Human t-PA Gene

Full-length human t-PA (GenBank accession number 101047) was amplified using genomic DNA of purchased CHO 1-15 cell line (ATCC- CRL 9606) as PCR template and a pair of primers, containing XhoI restriction site at both ends, named Fort-PA (5′-TACTCGAGA AAAGAGAGGCTGAAGCTCAGGAAATCCATG-3′) and Revt-PA (5′-GCCTCGAGCGGTCGCATGTTGTCA-3′). To express t-PA protein with a native N terminus, the *Xho*I site at base pairs 1184–1189 of the expression vector pPICZαA (Invitrogen easy select Pichia Expression Kit) was used to clone the gene flush with the Kex2 cleavage site. So this sequence (CTCGAGAAAAGAGAGGCTGAAGCT) was included to forward primer, and PCR was used to rebuild the sequence from the *Xho*I site to the Alanine codon at nucleotide 1207 of the expression vector (Fig. 1). The t-PA gene was amplified through a high fidelity PCR based on the following thermal cycles: 2 min at 95°C for 1 cycle, 30 cycles of 1 min at 95°C, 45 s at 68°C, 2 min at 72°C, and a final extension cycle of 10 min at 72°C. Then, the amplified 1,653-bp product was cleaned up using QIAquick PCR Purification kit (Qiagen), and the amplicon was confirmed by restriction enzyme analysis.

To make an intermediate construct, an oligo A termini was added to the 3′ side of this blunt-ended amplicon using PCR in 72°C for 10 min, and the product was inserted into the pTZ57R vector using Ins T/A clone PCR Product Cloning kit (Fermentas, Vinius, Lithuania). Restriction mapping and bidirectional sequencing of the cloned fragment was performed to confirm this intermediate construct. To prepare the final construct, t-PA fragment was cut from the intermediate vector using XhoI enzyme and subsequently cloned into pPICZαA. The final construct was called pPICZαA/t-Pa and confirmed by restriction analysis and sequencing.

Transformation of GS115 Cells

Ten micrograms of *Pme* I linearized pPICZαA/t-PA plasmid was used in transformation of *GS115 cells* using an electroporation method provided by the manufacturer (Invitrogen easy select Pichia Expression Kit). The cells were prepared for electroporation through two times water and one time 1 M sorbitol washing process based on manufacturer protocol

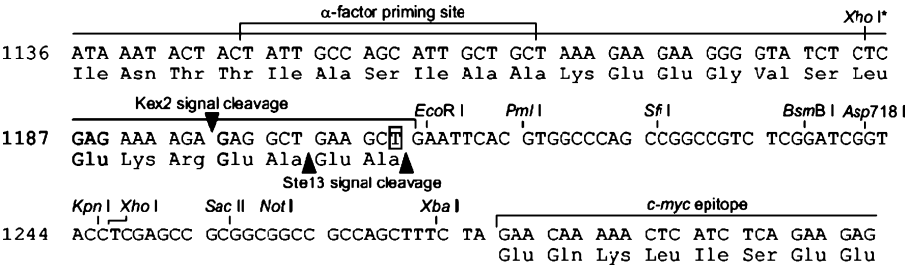

Fig. 1 Forward primer design. To express the protein with a native N terminus, the Xho I site at 1,184–1,189 bp was used to clone the gene flush with the Kex2 cleavage site. PCR was used to rebuild the sequence from the Xho I to the Alanine codon at nucleotide 1207 of the expression vector

(Invitrogen easy select Pichia Expression Kit). Transformants were selected on YPDS agar medium containing Zeocin (100 μg/ml). Genomic DNA of transformants was then extracted, and the presence of expression cassette was confirmed by PCR using t-PA-specific primers as well as primers spanning the AOX1 elements.

The Expression of Recombinant t-PA in GS115 Strain

A single colony of GS115 transformant was grown in 50 ml of BMGY at 30°C in a shaking incubator (250 rpm). The cells (OD_{600}=4) were harvested and resuspended in 200 ml of BMMY (OD_{600}=1.0) to induce expression at 30°C. One hundred percent methanol was added to a final concentration of 0.3% to 0.5% every 24 h to maintain induction. The growth was continued for 6 days. To consider the reproducibility of growth, many separate experiments were executed from which five experiments are shown in Fig. 2.

SDS–PAGE and Western Blotting

Supernatant of BMMY expression culture medium was harvested and then was concentrated eightfold using Amicon filtering system and a filter with 30 KD pore cut off (Millipore, USA). SDS–PAGE on 12% polyacrylamide gels and Western blotting were performed according to standard methods [23].

The eightfold concentrated supernatant of GS115 transformant's culture media before induction was used as negative control and commercial t-PA (Actylase) was used as positive control in both tests. In Western blot analysis Anti t-PA rabbit Polyclonal antibody (Apcam, USA) and conjugated goat anti-rabbit antibody (Santa Cruz, USA) were used as first and second antibodies, respectively.

Zymography

Zymography is an electrophoretic technique for the detection of enzyme activity based on SDS–PAGE that includes a substrate copolymerized with the polyacrylamide gel. Samples are prepared in the standard SDS–PAGE treatment buffer but without boiling and a reducing agent. Following electrophoresis, the SDS is removed from the gel (or zymogram) by incubation in unbuffered Triton X-100 followed by incubation in an appropriate digestion buffer for an optimized length of time at 37°C. The zymogram is subsequently

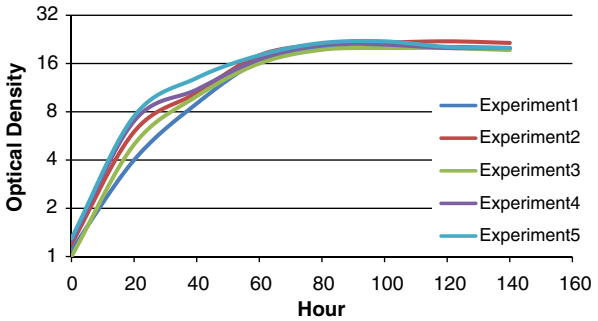

Fig. 2 Growth curve of five GS115 transformants during induction time. The induction was started at OD_{600} ~1. The culture was continued for 6 days. The average of maximum OD after 140 h was 20.18. Five distinct experiments had been done to consider the reproducibility of the growth

stained (commonly with Amido Black or Coomassie Brilliant Blue), and areas of digestion appear as clear bands against a darkly stained background where the substrate has been degraded by the enzyme [24, 25].

The test was performed as optimized by Soleimani et al. [26]. Briefly, the *Plasminogen* (Chromogenix, Italy) and *Gelatin* (Sigma, USA) were added to an 11% non-reducing polyacrylamide gel and electrophoresis was carried out at a constant current of 8 mA and at 4°C.

Following the electrophoresis, the gel slabs were shaken in 2.5% (*w*/*v*) Triton X-100 at room temperature for 1 h and then incubated in 0.1 M glycine/NaOH (pH=8.3) for 5 h at 37°C. Finally, the gels were stained by Coomassie Brilliant Blue R-250 procedure.

Amidolytic Activity Assay and Densitometry

In Amidolytic activity measurement by Chromolize t-PA Assay kit (Biopool, Australia), the sample t-PA is captured by antibodies on microtest wells. The SP-322 monoclonal antibody in this kit allows excellent t-PA recovery without inhibiting t-PA activity. After washing, the t-PA substrate, consisting of plasminogen, a plasmin-sensitive chromogenic substrate, and t-PA activity promoters, is added. The amount of developed color which is proportional to the amount of t-PA activity in the sample is measured using spectrophotometer at 405 nm of absorbance (A_{450}). The t-PA activity standard provided in this kit contains human t-PA and is calibrated against the international standard for t-PA. The expressed t-PA activity is determined by interpolation from standard curve that is plotted A_{450} against each 0, 0.5, 1.0, 1.5, and 2 IU/ml standard (Fig. 6). Various dilutions of the sample were assayed from which 1/1,000 dilution is represented in Table 1 and Fig. 6.

Densitometry on SDS–PAGE gel was done using Quantity One software to measure the total expression of t-PA secreted into the culture media.

Results

Cloning of t-PA cDNA

Restriction enzyme analysis of pPICZαA showed successful subcloning of t-PA gene downstream of Kex2 cleavage site. The size of expression construct was 5,178 bp and digestion of this construct with Bgl II restriction enzyme resulted in two fragments sized 3,935 and 1,243 bp. Also, the Xho I digest of the construct resulted in two fragments sized 3,531 and 1,647 bp that confirmed cloning and correct orientation of t-PA gene into pPICZαA expression vector. Final sequencing of the construct

Table 1 Dilution factor and related absorbance and activity for each standard and sample test in this study.

Sample (dilution)	Milli-absorbance at 405 nm	t-PA activity (IU/ml)
t-PA standard (0)	30	0
t-PA standard(1/4)	230	0.5
t-PA standard(2/4)	440	1.0
t-PA standard(3/4)	620	1.5
t-PA standard(4/4)	840	2.0
Culture supernatant(1/1,000)	700	1.65

confirmed the presence and correct orientation of insert into the vector (data not shown). The expression construct fused to α-mating secretion signal correctly and contained a yeast consensus sequence (A/YAA/TAATGTCT).

Transfection of *P. pastoris*

The result of PCR on genomic DNA extracted from GS115 colony which had been transfected with final expression vector (pPICZαA/t-PA) showed that pPICZαA/t-PA expression cassette is inserted successfully into GS115 genome. The presence of expression cassette in the genome was confirmed again by PCR using 5′ and 3′ AOX oligonucleotides (data not shown). Ten colonies of positive transformants were selected for the expression of recombinant human t-PA.

Production and Secretion of Human t-PA

Expression of recombinant human t-PA was processed successfully by methanol induction. The induction was performed by changing the culture media from BMGY to BMMY. One hundred percent methanol was used to a final concentration of 0.3–0.5% every 24 h, and the highest expression was resulted in 0.5% of methanol. The growth curve of five different *GS115* transformants which was prepared according to the optical density measurement (OD_{600} nm) is illustrated in Fig. 2. Maximum growth was obtained on the 5th day of the induction in all transformants. The average of maximum OD after 140 h was 20.18.

SDS–PAGE and Western Blotting

In SDS–PAGE, the expressed band of t-PA was clearly detected through comparing positive and negative controls (Fig. 3). Lane 4 in Fig. 3 shows the expressed band of t-PA (illustrated by arrow) in eightfold concentrated supernatant of GS115 transformant culture media after 6 days of induction. The band related to t-PA protein expressed by GS115 transformant is shown by the arrow. Lanes one and two in this figure show the protein marker and commercial t-PA (Actylase) as positive control, respectively, and lane 3 shows eightfold concentrated supernatant of GS115 transformant culture media before induction as

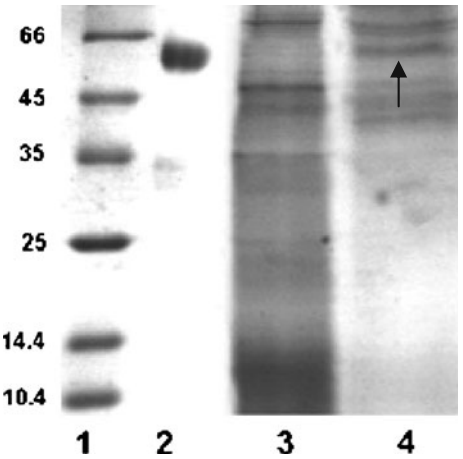

Fig. 3 SDS–PAGE of eightfold concentrated supernatant of GS115 transformants culture media. *Lane 1* Protein marker. *Lane 2* Commercial t-PA (Actylase) as positive control. *Lane 3* Eightfold concentrated supernatant of GS115 transformant culture before induction as negative control. *Lane 4* Eightfold concentrated supernatant of GS115 transformant culture after 6 days induction. The expressed t-PA protein is shown by the *arrow*

negative control. The protein bands under 30 kD are not present in SDS–PAGE gel due to filtering the supernatant using 30-kD filter.

Figure 4 shows the result of Western blot analysis in which lane 1 shows the expressed band of t-PA by GS115 transformants and lanes 2 and 3 show negative and positive controls, respectively. Supernatant of GS115 transformant culture media before induction was used as negative control and commercial t-PA (Actylase) was used as positive control.

Zymography

In Zymography test, the plasminogen and gelatin were co-polymerized and immobilized with a *non-reducing* polyacrylamide gel. The supernatant of culture media of transformed *P. pastoris* before and after induction were applied to evaluate the activity of t-PA (Fig. 5). The transparent regions on the gel which was observed only in transformed *P. pastoris* (lane 4 of Fig. 5), indicated that the plasminogen is digested by serine-protease activity of t-PA and derived plasmin resulted in gelatin hydrolysis. We also evaluated the activity of supernatant from GS115 transformant incubated with function blocking anti-human t-PA antibody. As predicted, a major reduction of transparent band was occurred due to inactivation of t-PA by this antibody (Fig. 5, lane 6). Commercial t-PA (Actylase) was used as positive control (Lanes 2 and 3 of Fig. 5). Supernatant of non-transformed GS115 culture media was used as negative control (Lane 5 of Fig. 5).

Amidolytic Activity Measurement and Densitometry

We used another assay to quantify how much t-PA was being made. In *Amidolytic activity measurement*, the activity of expressed recombinant human t-PA was measured using Chromolize t-PA Assay Kit as cited in "Materials and Methods." In this test, the specific anti-t-PA antibodies will capture the protein, and after a washing step, the recovered t-PA will convert the plasminogen into the plasmin. In the final step, plasmin will convert a chromogenic substrate to a color product with a maximum absorbance at 405 nm. Measurement was performed using the procedure described by the manufacturer (Fig. 6 and

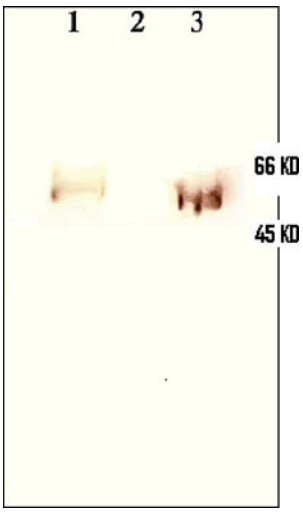

Fig. 4 Western blot analysis on supernatant of GS115 transformant culture media. Anti t-PA rabbit Polyclonal antibody and conjugated goat anti-rabbit antibody were used as first and second antibodies, respectively. *Lane 1* Eightfold concentrated supernatant of GS115 transformant culture after 5 days induction. *Lane 2* Eightfold concentrated supernatant of GS115 transformant culture before induction as negative control. *Lane 3* Commercial t-PA (Actylase) as positive control

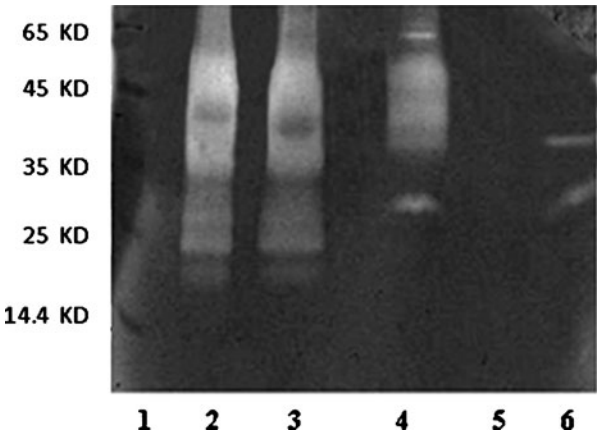

Fig. 5 Zymography. The *Plasminogen (as substrate of t-PA)* and *Gelatin (as substrate* of plasmin) were added to the 11% *nonreducing* polyacrylamide gel and electrophoresis was carried out. Following electrophoresis, the zymogram was stained, and areas of digestion of gelatin appeared as *clear bands* against a darkly stained background where the gelatin had been degraded by the action of t-PA on plasminogen. *Lane 1* Protein marker (Dark bands). *Lanes 2*, *3* Commercial t-PA (Actylase) as positive control (both lanes are the same). *Lane 4* Supernatant of GS115 culture media. Transparent region in the gel indicates the degradation of gelatin by plasmin. *Lane 5* Supernatant of non-transformed GS115 culture media as negative control. *Lane 6* A major reduction of transparent band due to inactivation of t-PA by polyclonal anti t-PA antibody

Table 1). Considering the dilution factor (1/1,000) of the sample, final t-PA activity was calculated 1,650 IU/ml in culture supernatant harvested in the 5th day of induction.

Total amount of t-PA expression after 140 h was nearly 10 mg/L based on densitometric results, and the proportion of active form of the t-PA was estimated 35±2%.

Discussion

Tissue plasminogen activator is a popular target protein for biotechnology industry due to its wide application in medical practice. Wild-type *prokaryotic systems* have been generally

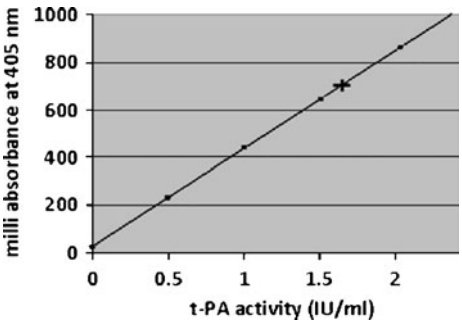

Fig. 6 Sample calibration curve. Amidolytic sample calibration curve. Standard curve that is plotted the spectrophotometric absorbance at 405 nm against each 0, 0.5, 1.0, 1.5, and 2.0 IU/ml t-PA standard (Chromolize t-PA Assay kit, Biopool, Australia). The activity of recombinant t-PA produced in this study is shown by cross sign in the plot; 1/1,000 diluted supernatant was used in activity measurements

unsuccessful in the expression of complex proteins due to their reducing environment of the cytoplasm [27], resulting in the necessity to refold disulphide-bonded proteins from inclusion bodies or to secrete the proteins into the periplasmic space [28]. A number of efforts have been made to overcome these problems of prokaryote species especially solubility of heterologous proteins in cytoplasm, but there are still some problems [19, 29]. Although *E. coli*-expressed full-length t-PA [19] has not become commercial yet, a deletion mutant of t-PA called Reteplase in which the finger epidermal growth factor and kringle-1 region were deleted has been produced in this bacterial host [12]. One of the most important disadvantages of this form is its weaker affinity for fibrin compared to full-length t-PA (Actylase), causing more fibrinogen depletion, which results in higher frequency of bleeding complications [30].

The expression of t-PA in *yeast*, *Saccharomyces cerevisiae*, has been reported in some papers with variable amounts, but molecular weight heterogeneity has been observed. Meanwhile, in spite of using either yeast or human secretion signals, extracellular secretion into the culture medium of *S. cerevisiae* did not occur, and the protein was detected just in cells following cell lysis [18]. Although large-scale cell lysis methods are demonstrated, protein purification from cellular debris complicates the process and increases the cost of production.

Upshall et al. reported the expression of recombinant t-PA in the filamentous *fungus*, *Aspergillus nidulans*, in which the product glycosylated less than native t-PA, and the yield of production was approximately 100 μg/L of culture medium [31]. Wiebe et al. reported the expression of t-PA in *Aspergillus niger* up to 25 mg/L, with less than 1% active protein [32].

Oka et al. produced t-PA in an *Insect* cell line, Drosophila melanogaster, with a specific activity near to t-PA from CHO but the carbohydrate side chains of the two products were different [33].

CHO cells have been a suitable candidate of producing t-PA, but in general, high cost of cell culture media, probable viral and prion contamination, clearance rate, and purification problems are the most noticeable disadvantages of mammalian hosts for the production of complex proteins [34].

In recent years, some strains of *Tripanosomatiae* family have been recognized as an alternative host for the production of heterologous proteins. Soleimani et al. used *Leishmania tarentolae* to express full-length t-PA and produced 70 IU/ml of functional enzyme [26].

Previous studies have shown that the yeast, *P. pastoris*, has the potential of expressing proteins with limited disulfide bonds. A fragment of thrombomodulin, coagulation protease (Factor XII), and recombinant kringle 2 domain of t-PA, with three disulfide bonds, are among such proteins that have been synthesized in *P. pastoris* before [20].

Rapid and high-yield production of recombinant proteins driven by strong promoters like AOX1, low level of background secreted proteins, and low cost of production has made us to use a methanol utilization positive strain of this methylotrophic yeast for cloning and the expression of full-length human t-PA cDNA in secretory form and to characterize the quantity and function of the expressed protein.

Although the available *Pichia* expression systems are efficient and easy to use with well-defined process protocols, varying degrees of process optimization is required to achieve maximum production, as well as maximum activity of the heterologous protein. In fact, yield and activity are often dependent upon the physical parameters of the culture vessel, like pH, temperature, and O_2 availability, and they are dependent on the residual concentrations of methanol [35]. In this study, 100% methanol was used to a final

concentration of 0.3–0.5% every 24 h, and the highest expression was made in 0.5% of methanol. Curvers and colleagues' results showed that when methanol concentration increases more than a critical point, a selective pressure will be imposed against product formation leading to the enrichment of nonproducing mutants [36].

Foreign genes may be cloned in *P. pastoris* vectors to align them in the correct reading frame with either the native secretion signal for the protein of interest, the *S. cerevisiae* factor prepro-peptide or the *P. pastoris* acid phosphatase (*PHO1*) signal sequence [37]. The signal sequence used in this study was the α-mating signal sequence from *S. cerevisiae*. The *S. cerevisiae* factor prepro-signal is the most widely used and successful secretion signal, in some cases being better than the leader sequence of the native heterologous protein [38].

In this study, *Zymography* was used to evaluate the proper folding, serine-protease activity and quantity of the recombinant t-PA and *Amidolytic activity measurement* was used to consider the amidolytic activity of secreted protein and measuring the amount of expression. From the quantification point of view, zymography is comparable with ELISA, as levels of less than 10 pg of matrix metalloproteinase have been detected on gelatin zymograms [39]. Meanwhile, since it is a non-reducing PAGE, there is a possibility to evaluate the folding and also the function of the enzyme. The zymography of culture supernatant of transformed *P. pastoris* showed clear zone of hydrolysis comparing positive and negative controls. To verify that the functioning protein is exclusively t-PA, function blocking antibody against t-PA was added to the sample and was loaded in another column of the test (lane 6 of Fig. 5). This process due to formation of antigen–antibody complex, confirmed the exclusive presence of t-PA in the sample [40]. The low-molecular-mass bands may be due to limited proteolysis of the protein [41].

The total amount of t-PA expression in this study was 10 mg/L. In Chromolyze test, the protein represented suitable amidolytic activity and the amount of active enzyme secreted in culture medium was 1,650 IU/ml which was nearly 35% of the total recombinant protein. Comparing this with the enzymatic activity of produced t-PA in *E. coli* (3–7 IU/ml), CHO (50–500 IU/ml) [34, 42], *A. niger* (up to 25 mg/L, with less than 1% active protein) [32], *A. nidulans* (100 μg/L) [31], or *L. tarentolae* (70 IU/ml) [26], the yield of production in this host looks promising for the production of this very important enzyme.

Here, we have presented that GS115 strain of *P. pastoris* can be a suitable alternative for the production of full-length functional human recombinant t-PA. In this host, t-PA stated proper folding and biologic activity. Bio-immunoassay analysis of chromolyze represented amidolytic activity and reasonable quantity of the expressed enzyme and white bands on PAGE of Zymography analysis showed appropriate serine-protease activity.

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