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Expression of the enzymatically active legumain-like cysteine proteinase TvLEGU-1 of *Trichomonas vaginalis* in *Pichia pastoris*

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ABSTRACT

The legumain-like cysteine proteinase TvLEGU-1 from *Trichomonas vaginalis* plays a major role in trichomonal cytoadherence. However, its structure-function characterization has been limited by the lack of a reliable recombinant expression platform to produce this protein in its native folded conformation. TvLEGU-1 has been expressed in *Escherichia coli* as inclusion bodies and all efforts to refold it have failed. Here, we describe the expression of the synthetic codon-optimized *tvlegu-1* (*tvlegu-1-opt*) gene in *Pichia pastoris* strain X-33 (Mut+) under the inducible AOX1 promoter. The active TvLEGU-1 recombinant protein (rTvLEGU-1) was secreted into the medium when *tvlegu-1-opt* was fused to the *Aspergillus niger* alpha-amylase signal peptide. The rTvLEGU-1 secretion was influenced by the gene copy number and induction temperature. Data indicate that increasing *tvlegu-1-opt* gene copy number was detrimental for heterologous expression of the enzymatically active TvLEGU-1. Indeed, expression of TvLEGU-1 had a greater impact on cell viability for those clones with 26 or 29 gene copy number, and cell lysis was observed when the induction was carried out at 30 °C. The enzyme activity in the medium was higher when the induction was carried out at 16 °C and in *P. pastoris* clones with lower gene copy number. The results presented here suggest that both copy number and induction temperature affect the rTvLEGU-1 expression in its native-like and active conformation.

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1. Introduction

The TvLEGU-1 is a legumain-like cysteine proteinase (CP) from *Trichomonas vaginalis*, a protozoan parasite responsible for trichomoniasis, one of the most common non-viral sexually transmitted infections [1]. TvLEGU-1 belongs to the asparaginyl endopeptidase subfamily of family C13 of clan CD with multiple cellular locations [2–4]. This protein can be found in the Golgi complex, hydrogenosomes, and lysosomes in the cytoplasm, as well as on the parasite surface, where it plays a major role in trichomonal cytoadherence. It has been found in vaginal secretions during trichomonal infection and is one of the most immunogenic proteinases, since anti-TvLEGU-1 antibodies are detected in trichomoniasis patient sera. Hence, it is considered a potential biomarker for serodiagnosis of trichomoniasis [5,8]. In the parasite, TvLEGU-1 is synthesized as an inactive zymogen of 42.8 kDa that under appropriate redox and pH condition undergoes autocatalytic activation, where *N*-terminal and *C*-terminal pro-peptides are removed, resulting in an enzymatically active mature form of ~30 kDa [5]. The removal of the *C*-terminal pro-peptide is relevant for enzymatic activity [5–7]. In an initial effort to obtain the recombinant TvLEGU-1 protein (rTvLEGU-1) the *tvlegu-1* gene was cloned and expressed in the *Escherichia coli* system. However, all efforts to obtain this protein in its native-like conformation using a codon-optimized gene, different vectors and *E. coli* strains have failed, and in all cases, the rTvLEGU-1 was expressed as inclusion bodies [5,8]. Although we have used several refolding protocols that were successfully used with *Entamoeba histolytica* CPs expressed as inclusion bodies [9], we did not obtain rTvLEGU-1 in a native-like and active conformation, hampering structure-function studies and its further biochemical characterization. Therefore, we switched to a different recombinant expression host.

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Pichia pastoris is a yeast that can grow to high cell density and a broad range of pH and temperatures and is capable of post-translational processes. This expression system has many advantages, including a strong and tightly-controlled methanol alcohol oxidase (*AOX1*) promoter [10–13]. Additionally, several signal peptides can be used with this expression platform to ensure secretion of the recombinant protein into the culture medium and it lacks endogenous cysteine protease activity [12,14,15].

In the present study, we report the effect of gene copy number and temperature during induction on the expression of the enzymatically active rTvLEGU-1 of *T. vaginalis* in *P. pastoris* by using the codon-optimized *tvlegu-1*gene fused to the alpha-amylase signal peptide (AA) coding sequence of *Aspergillus niger*.

2. Materials and methods

2.1. Strains, plasmids, media composition, and chemicals

Recombinant DNA manipulations were carried out in the *Escherichia coli* strain DH5α growing in low salt Luria-Bertani (LB) broth (1% bactotryptone, 0.5% yeast extract, 0.5% NaCl) using Zeocin (25 μg/mL) as a selection antibiotic. The EasySelect®*Pichia* Expression kit system including the *P. pastoris* X-33 strain, pPICZB plasmid, Zeocin and the *Pichia* EasyComp™ Transformation Kit were purchased from Invitrogen (San Diego, CA. USA). All primers used were obtained from Sigma-Aldrich (St. Louis, MO. USA). The required restriction enzymes and T4 DNA ligase were obtained from New England Biolabs (Ipswich, MA. USA). Anti-His (*C*-term)-HRP antibody was purchased from Invitrogen. The stain for the flow cytometry analysis was done with the LIVE/DEAD® Yeast Viability Kit (Molecular Probes, Eugene, OR, USA). Yeast nitrogen base without amino acids was purchased from Invitrogen. Hy-Case Amino (casamino acids) was purchased from Sigma-Aldrich. All other chemicals were analytical grade and obtained from various sources.

P. pastoris was grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) using Zeocin (100 μg/mL) as a selection antibiotic. *P. pastoris* transformants were routinely grown at 30 °C either in YPD broth or in buffered minimal glycerol medium (BMGY) [1% yeast extract, 2% peptone, 1% glycerol, 1.34% yeast nitrogen base (YNB) without amino acids, 4×10^{-5}% biotin, 100 mM potassium phosphate, pH 6.0]. The induction medium used in this study was the buffered methanol minimal medium supplemented with casamino acids and EDTA (BMMH-CA-EDTA) [1.34% YNB without amino acids, 4×10^{-5}% biotin, 100 mM potassium phosphate, pH 6.0, 1% (w/v) casamino acids, 5 mM EDTA and 0.5% methanol] added every 24 h and 0.01% antifoam 204 (Sigma-Aldrich).

2.2. Synthesis of the codon-optimized tvlegu-1 gene

The 1167 bp full-length sequence of the *tvlegu-1* gene (GenBank accession number AY326446; TVAG_426660) [2] was analyzed for codon optimization according to the codon usage of *P. pastoris* using proprietary algorithms that replace rare codons, problematic mRNA structure, and various cis-elements in transcription and translation (GenScript, Piscataway, NJ. USA). Additionally, the *Hind*III (AAGCTT), *Bsa*I (GGTCTCN), *Nde*I (CATATG), *Xho*I (CTCGAG), *Sac*I (GAGCTC), *Eco*RI (GAATTC), *Eco*RV (GATATC), *Xba*I (TCTAGA), *Kpn*I (GGTACC), *BamH*I (GGATCC), *Not*I (GCGGCCGC) and *Pst*I (CTGCAG) restriction enzyme sites were avoided in the optimized sequence for cloning purposes. The full-length codon-optimized *tvlegu-1* gene (*tvlegu-1-opt*) (GenBank accession number KX812517; Supplementary Fig. 1) was delivered as a pUC57-*tvlegu-1-opt* plasmid.

2.3. Construction of the pPICZB-alpha-amylase signal peptide (pPICZB-AA) plasmid

The pPICZB expression vector containing the coding sequence for the secretion signal peptide derived from the alpha-amylase gene from *A. niger* (AA) was constructed as follows. The phosphorylated 5′ end complementary primers (Table 1) were annealed in a thermocycler using the program shown in Table 2. Then, the hybridized complementary primers that contain the *Eco*RI-*Kpn*I compatible overhangs were cloned into the same restriction sites on the pPICZB plasmid to create pPICZB-AA. The ligation product was transformed into *E. coli* DH5α using heat shock at 42 °C. Transformants were selected on LB-Zeocin agar plates. Positive colonies were screened by restriction analysis of plasmid DNA, and the orientation and fidelity of the cloned sequence were verified by DNA sequencing. The coding sequence for the alpha-amylase signal peptide contains the consensus sequence corresponding to the Kozak sequence (5′-GAAACG**ATG**-3′) from the native AOX1 gene, acting as a start signal for translation [16,17].

2.4. Cloning of the tvlegu-1-opt gene into the pPICZB-AA plasmid

The pUC57-*tvlegu-1* plasmid was used as the template for PCR amplification of the *tvlegu-1-opt* gene using the tvlegu1F (5′-GGGGTACCCAATGTTTTGCCTTCTTCAACTTGCTAGATGTGACAG-3′) and tvlegu1R (5′-TTGCGGCCGCACAAATGGCGTCAA-TAGCTGCTTTAATG-3′), where *Kpn*I and *Not*I restriction sites are underlined. PCR amplification was performed in a reaction mixture containing *Pfx* DNA polymerase (1 U) (Invitrogen), $MgCl_2$ (2 mM), dNTP mix (200 μM), primers (10 pmol each) and DNA template (50 ng) in a thermocycler (MyCycler™, Bio-Rad). The PCR program was set for initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. A final extension was performed at 72 °C for 10 min. The PCR product was analyzed by agarose gel electrophoresis and purified using the QIAquick® Gel Extraction Kit (Qiagen) following the manufacturer's instructions. The pPICZB-AA expression vector and the purified DNA fragment were digested with *Kpn*I and *Not*I restriction enzymes. After digestion and gel purification, the DNA fragment was ligated into the pPICZB-AA expression vector by T4 DNA ligase. The ligation product was transformed into the *E. coli* DH5α strain, transformants were selected on LB-Zeocin agar plates as before, and positive colonies were screened by restriction analysis of the plasmid DNA. The orientation and fidelity of the cloned sequence were verified by DNA sequencing.

2.5. Transformation of P. pastoris strain X-33 with the expression plasmid pPICZB-AA- tvlegu-1-opt

The recombinant plasmid (5 μg) was linearized by the *Sac*I restriction enzyme and transformed into competent *P. pastoris* strain X-33 cells according to the *Pichia* EasyComp™ Transformation Kit instructions. The transformants were selected for the ability to grow on YPD (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) plates containing Zeocin (100 μg/mL).

2.6. PCR analysis and extent of Zeocin resistance in P. pastoris transformants

Direct PCR was undertaken on transformant yeast colonies to confirm the integration of plasmid DNA into the genome using the *AOX*1_F (5′-GACTGGTTCCAATTGACAAGC-3′) and *AOX*1_R (5′-GCAAATGGCATTCTGACATCC-3′) primers, as described in the EasySelect™ *Pichia* Expression kit manual. To determine the extent of

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