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Expression of Gp63 Gene from NIH Strain of *Leishmania major* in *Pichia pastoris*

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ABSTRACT

Leishmaniasis is a major infectious disease of considerable public health in more than 86 countries around the world. Several approaches toward vaccine development against this disease have been taken. Glycoprotein (gp63) is conserved among diverse species of *Leishmania* and has induced immunological responses in murine models. Therefore, this glycoprotein has been considered as a second generation vaccine for Leishmaniasis and for potential diagnostic antigen. Recombinant vaccine using gp63 in cocktail form is one of the candidates. Since, *Pichiapastoris* expression system is similar to that of the eukaryotic genes, refolding and glycosylation aspects of the expressed protein, gp63 gene from NIH strain of *L. major* cloned into *Bam*HI site of pHIL-S1 as yeast expression vector (shuttle vector). This vector carries sequences of acid phosphatase (PHO1) signal peptide from yeast. The construction transfected into the *P. pastoris* using lithium chloride method. Recombinant clones were screened on histidin minus media. The expression of rgp63 was studied by methanol after induction. The expressed recombinant protein was confirmed by Western blotting and electron microscopy. The expression level of rgp63 was more than 30%. Since the rgp63 expression in *P. pastoris* was active in SDS-PAGE gelatin gel, therefore it should be very similar to the native form. *Iran. Biomed. J. 5 (1): 39-45, 2001*

Keywords: Leishmania, Gp63, Pichiapastoris

INTRODUCTION

Leishmaniasis still remains a parasitic disease of major importance that no vaccine is available against it. Gp63 is expressed on the cell surface of the promastigote and amastigote forms of the parasite. Gp63 is encoded by multiple copies of conserved 3.1 kb gene, consisting of the 1.8 kb coding and 1.3 kb spacer regions, tandemly repeated on a single chromosome [1]. The N-terminal and C-terminal coding regions for the putative precursor peptides were deleted from the gp63 gene and replaced with translation and termination codons. The modified gp63 gene encoded mature form containing 477 amino acids [1]. This glycoprotein is one of the molecules involved in the infectivity of *Leishmania* and in uptake by macrophage receptors in mammalian cell. Gp63 gene has been expressed in different heterologous hosts, such as *E. coli* and insect cells [1, 2]. This molecule has also been used as a diagnostic antigen for leishmaniasis [3].

The initial limitations of *Saccharomyces cerevisiae* (such as hypermannosylation) as a yeast host for heterologous gene expression was studied during the last decade. Several methylotrophic yeasts (especially, *Pichiapastoris*) were selected for applications in biotechnology. Glycosylation in *P. pastoris* is different from *S. cerevisiae*. In *P. pastoris* 8-14 mannose are added to each core N-link glycosilated chain compared to 50-150 mannose in *S. cerevisiae*. Thus, the expressed proteins in *S. cerevisiae* are hyper antigenic and no suitable for therapeutic use. The strong and highly regulated promoter structures of the respective genes in *P. pastoris* such as AOX1 are available as attractive control elements for heterologous gene expression [4, 5].

Cloning and over expression of several foreign genes in *P. pastoris* have been reported since 1987,

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with high yield up to 12 g/lit for tetanus toxin fragment C [6]. Secretion of recombinant proteins may be directed by either the native or a *P. pastoris* signal sequence such as "PHO1"[7]. Another advantage of this yeast is that only small amount of *P. pastoris* endogenous proteins appear in the culture medium [8].

To study the role of post-translational modifications such as glycosylation of gp63 in biological activities, this gene was expressed in *P. pastoris*, which is a proper model for glycosylation. In this study, we expressed gp63 gene in methylotrophic yeast *P. pastoris* to provide enough glycosylated protein for future analysis.

MATERIALS AND METHODS

Gp63 gene from L.major in pPMM63. TEX3 plasmid that encoded mature form of gp63 protein and monoclonal antibody against gp63 # 235, kindly provided by professor W.R. McMaster, University of British Columbia, Vancouver, Canada.

Preparation of gp63 gene. pPMM63. TEX3 plasmid was obtained from *E. coli* strain BL21 (DE3), and separated from plysS plasmid on the basis of their size differences confirmed by antibiotic resistance markers. pPMM63: TEX3 and plysS plasmids containing ampicillin and chloramphenicol resistence genes, respectively [1, 9].

Construction of expression vector. Modified gp63 gene was isolated with *Bam*HI restriction enzyme and subcloned into the pHIL-S1 shuttle vector [Invitrogen, San Diego] under control of AOX1 promoter. The new construct, pS1-gp63, was linearized by *Bgl*II and then transformed into the *P. pastoris* strain KM71 by lithium chloride method [9].

Transformants were selected on Histidin minus media after the incubation at 30°C for 3 days. Total DNA of *P. pastoris* transformants was extracted by the standard method [6, 7]. Primarily, the cell wall of the yeast was digested by litycase, then disrupted by 10% SDS followed by extraction with phenol-chloroform-isoamyl alcohol (P.C.I extraction). Finally, genomic DNA of yeast was recovered by ethanol precipitation.

To confirm the integration of gp63 gene into the chromosomal DNA of the *P. pastoris*, PCR and Southern blot analysis were used. PCR was performed with the primers 5′-GGT CGC ACA TCA AGA TGC –3′ (PCR-3) and 5′- CGG TGC TCA GCT CAA CTC –3′ (PCR-4). Amplification was carried out using 100 pmol of each primers, 0.5 U Taq DNA polymerase (Cinnagen, Co. Iran), and PCR mix containing 0.25 mMdNTPs. Primary denaturation was carried out at 94°C for 5 min as hot start. Denaturation was performed at 94°C for 1 min, followed by annealing at 55°C for 1min, and extention at 72°C for 1min for 30 cycles with a final extention at 72°C for 10 min [7, 9].

Expression of gp63 gene. To remove inhibitory effect of other carbon sources, the recombinant *P. pastoris* strain KM71was cultured in BMGY medium, (containing 1% yeast extract, 2% peptone, 0.34% yeast nitrogen base, 1% ammonium sulfate, 4×10^{-5}% biotin, 100mM Potassium phosphate pH 6, and 1% glycerol) in order to obtain OD of 6 at 600 nm [9]. After acquiring BMGY turbidity to OD_{600} = 6, the cells were harvested by centrifuge and transferred into the BMMY medium (the same as BMGY medium but with 0.5% methanol instead of 1% glycerol) for induction [7, 9]. In this system, the inducer was %1 methanol, which was added into the medium from day 2 up to 7 day.

The level of expression was checked by 12% SDS-PAGE. To confirm the expression of the rgp63, Western blot analysis was used using mAb # 235 [6] and sera of the challenged mice with *L. major* for the electron microscopy, and for the indirect fluorescence (conjugated) antibody (IFA).

Protease activity. Protease activity of rgp63 expressed in the *P. pastoris* was assayed by SDS-PAGE gelatin gel (10% SDS-PAGE with 0.1% gelatin). To visualize the protease activity, the gel was rinsed in TBS-Zn buffer, the gel was washed with 2.5% Triton-X100 in TBS-Zn buffer, followed by overnight incubation in TBS-Zn buffer at 37°C. Finally, the gel was stained by Coomassie Brillian Blue [2].

RESULTS

In this study, *P. pastoris* strain KM71 his4- [auxotrophic for histidine] was used as the host and the pHIL-S1 with 5′-promoter and 3′- transcription terminator was used as an expression vector. Gp63 gene was subcloned in *Bam*HI site of MCS from pHIL-S1 (Fig. 1). This construct was named pS1-gp63. This vector carries the genes for histidinol dehydrogenase required for selection of the

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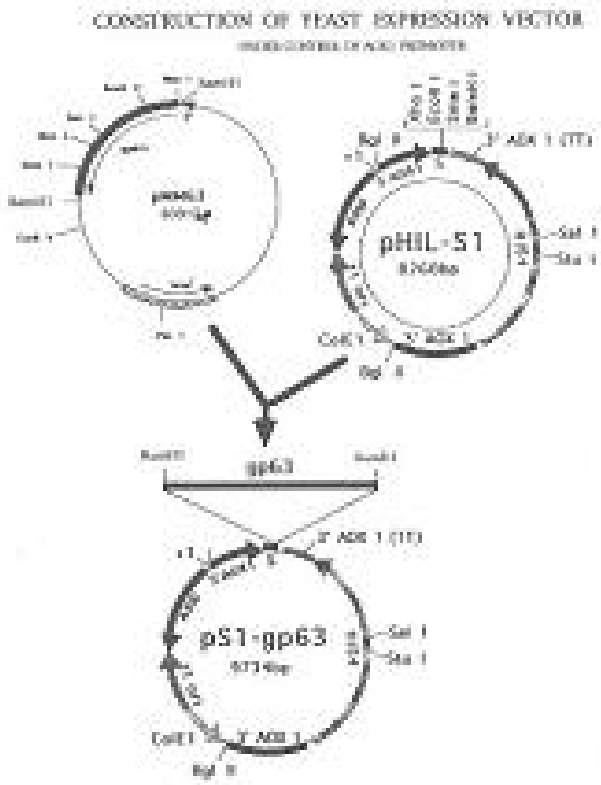

Fig. 1. Schematic diagram of subcloning modified gp63 gene in *Bam*HI site in MCS of pHIL-S1 (pS1-gp63).

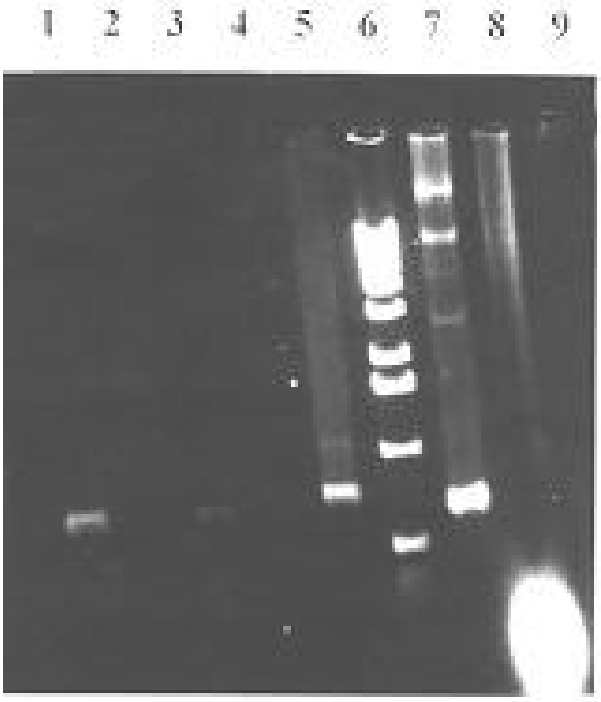

Fig. 2. PCR analysis of integrated gp63 gene into the chromosomal DNA of the yeast using specific primers. Lane 1 clone No. 26; lane 2, clone GS-1; lane 3, clone No. 110; lane 4, chromosomal DNA of *P. pastoris* transformed with pHIL-S1 only; lane 5, PCR product of irrelevant gene as DNA size marker with 734 bp; lane 6, 1Kb DNA Ladder; lane7, pUC plasmid containing gp63 gene (positive control), lane 8, pUC plasmid; lane 9, pHIL-S1 plasmid.

transformants, and the 3′-end of the AOX1 [alcohol, methanol, oxides] gene required for integration of the expression cassette into the yeast genome. This vector is containing the PHO1 secretion signal sequence in downstream of the it's AOX1 promoter to which the foreign DNA can be used for secretion expression or targeting membrane proteins. To integrate gp63 gene into the chromosomal DNA of *P. pastoris*, linearized pS1-gp63 plasmid No. 14 was used.

Gp63 gene was subcloned under the control of AOXI promoter. Dot–blotting, PCR (Fig. 2), and Southern-blotting showed that gp63 gene was integrated into the yeast chromosomal DNA. Two specific primers [PCR3 and PCR4] were designed for internal regions of gp63 gene with 700 bp size. No DNA bands were found in negative controls.

Chromosomal DNA of the *P. pastoris* transformed with pHIL-S1 alone was used as control. Two plasmids pUC and pHIL-S1 were used as negative template DNA. As it is shown, clear band of 700 bp, is seen in the PCR product with genomic DNA of the clone No. 26 (Fig. 2, lane 1). The same size band is seen in the positive control (Fig. 2, lane 7).

Four positive clones were selected and cultured in the media as described in the materials and methods. The expression of rgp63 in induction media, cell pellet and supernatant of the sonicated cells was studied by 12% SDS-PAGE. The results are shown in Figure 3. The sonicated *P. pastoris* cells [strain KM7I transformed with pHIL-S1] were centrifuged and the supernatant was used as the negative control. The rgp63 has been expressed by selected clone No. 26 (Fig. 3, lane 5). No band was seen with the pellet of the same disrupted cells in this region.

Western blot analysis of using monoclonal antibody 235 is shown in Figure 4. The supernatant of the sonicated cells of clone No. 26 expressed in *P. pastoris* was specifically reacted with antibody against gp63. No reaction was seen with sample prepared from negative control with the same procedure.

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Fig. 3. SDS-PAGE analysis of expressed rgp63 in the supernatant of sonicated cells of *P. pastoris*. Lane1, uninduced form of clone No. 26; lane 2, rgp63 expressed in *E. coli*; lane 3, clone GS-1; lane 4, clone No. 110*;* lane 5,clone No. 26.

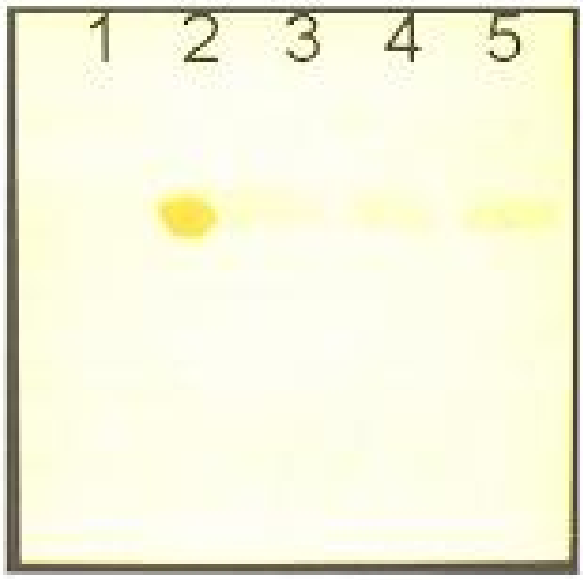

Fig. 4. Western blotting of the expressed rgp63 in the supernatant of the sonicated cells of the *P. pastoris.* Lane1, uninduced form of clone No. 26; lane 2, rgp63 expressed in *E. coli*; lane 3, clone GS-1; lane 4, clone No. 110*;* lane 5, clone No. 26.

The level of expressed rgp63 was optimized and determined during a period of 6 days after daily induction with %1 methanol. The expression level by clone No. 26 was more than the others. The highest level of expression by this clone was shown after 5 days induction, and was 32.9% of the total yeast proteins. In this experiment, rgp63 expressed in *E. coli* was used as the positive control and after

Fig. 5. Expression of rgp63 by clone no. 26 of the *P. pastoris* after induction. Lanes 1-5, after 1-5 days induction respectively; lane 6, rgp63 expressed in *E. coli*; lanes 7 and 8, (before and after induction of) *P. pastoris* transformed with pHIL-S1 only.

induction of *P. pastoris* strain KM71, carrying pHIL-S1 as negative control. The level of expressed rgp63 was increased up to 5 days after daily induction (Fig. 5).

The expression analysis by electron microscopy are shown in Figure 6. Secretion of dense protein regions has been seen by induced *P. pastoris* containing gp63 gene, but have not seen the same regions in induced *P. pastoris* without gp63 gene.

Immune electron microscopy and IFA analysis with the same antibodies used in Western blotting are performed with ultra thin sections of the clone No. 26. The results of these experiments have shown that the dense protein regions inside the induced transformant of *P. pastoris* (clone No. 26) are related to the rgp63 (data not shown).

The activity of the rgp63 expressed in *P. pastoris* was determined in %10 SDS-PAGE gelatin gel.

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Final concentration of gelatin in this gel was %0.1. Results are shown in Figure 7. In this study, native gp63 extracted from *Leishmania major* and methaloproteinase (commercial) were used as the positive controls, denatured form of native gp63, rgp63 expressed in *E. coli*, and *P. pastoris* without gp63 gene were used as the negative controls. Gelatin was digested with methaloproteinase as positive control at the beginning of lane 4. Digested regions by rgp63 expressed in *P. pastoris* [clone 26] was the same size as the regions digested with native gp63 as the positive control. This phenomenon was also observed with two other clones, Gs-1 and 110. Digested regions were not seen in the boiled native gp63 and the *P. pastoris* without gp63 gene.

The proteinase activity of rgp63 expressed in yeast was inhibited when it was treated with iodoacetamide and EDTA. These findings show that rgp63 expressed in *P. pastoris* is more similar to the native gp63 than rgp63 expressed in *E. coli*.

DISCUSSION

P. pastoris has several advantages over *E. coli* as a heterologous recombinant gene expression system. *P. pastoris* performs some posttranslational modifications such as refolding and glycosylation on heterologous eukaryotic proteins [4]. Glycosylation can affect the stability and activation of recombinant protein [10].

Although, expression in baculoviruse-insect cell, as an eukaryotic expression system, enables preparation of the recombinant proteins in reasonable amounts, but with post-translational modifications, some of them such as isoprenylation are deficient and incomplete. This change is important for studies of at least some aspects of recombinant protein functions [2, 11].

We cloned gp63 coding gene under the control of AOX1 promoter with PHO1 secretion signal sequence in downstream of the AOX1 promoter. Recent evidences suggest that the PHO1 signal sequence might be modified to include KEX2-like processing sites for efficient cleavage to occur [12, 13].

The expression of gp63 gene in *P. pastoris* was confirmed by SDS-PAGE, Western blotting, immune electron microscopy, and IFA.

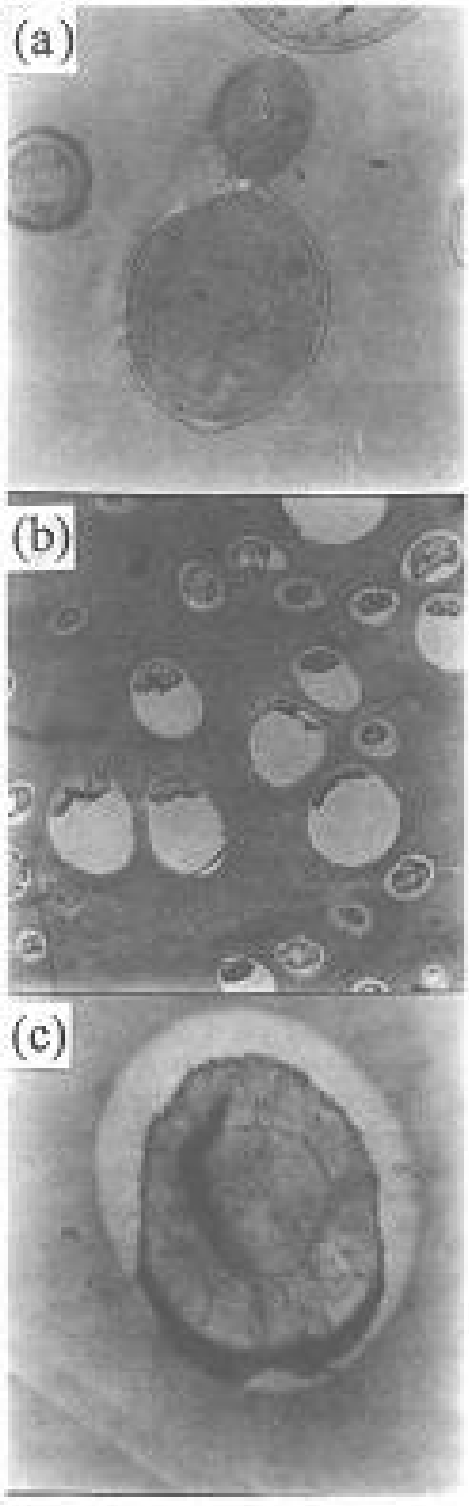

Fig. 6. Expression analysis of rgp63 after induction by electron microscopy: (a) negative control, induced *P. pastoris* without gp63 gene (magnification; X12000). (b) induced *P. pastoris* containing gp63 gene (clone No. 26 (magnification; X3000). (c) induced *P. pastoris* containing gp63 gene (clone No. 26, magnification; X20000).

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Fig. 7. Activity analysis of rgp63 expressed in *P. pastoris* on SDS-PAGE gelatingel. The supernatant of sonicated cells in lane 1, *P. pastoris* transformed with pHIL-S1 only; lane 2, clone No. 110;lane 3,clone No. 26;lane 4, methaloproteinase (commercial);lane 5, concentrated induction medium of clone No. 26; lane 6, rgp63 expressed in *E. coli*; lane 7, boiled native gp63; lane 8, native gp63; lanes 9 and 10, clone GS-1.

We observed two protein bands of 53 and about 63 kDa in the SDS-PAGE. Both of these bands were reacted with anti-gp63 antibodies. The 53 kDa band produced in *P. pastoris* is probably the unglycosylated form of the rgp63, because it is similar to the rgp63 that produced in *E. coli* [1]. It seems that the heavier protein band may be the glycosylated form of rgp63. As shown in Figures 3-5, the heavier band is located above the rgp63 expressed in *E. coli*. When this band treated with endoglycosidase F its molecular weight decreased to 53 kDa (data not shown). These findings are similar to the expression of the "testicular angiotensin converting enzyme (ACEt) produced in *P. pastoris.* A heterogeneity in size was due to the different pattern of glycosylation as observed in ACEt produced in *P. pastoris* and Hella-cell [13]. The heterogeneity may be due to overloading of the secretion and glycosylation machinery of the *P. pastoris* previously reported by Angela *et al.* [14]. This finding was similar to the expression of the tissue factor expressed in the *P. pastoris* as three discrete forms, which appeared SDS-PAGE as three bands in the range of 37-54 kDa [14]. These bands were all recognized by an anti-tissue factor monoclonal antibody. They also showed that three forms of human tissue-factor observed were due to the different levels in glycosylation of the proteins [14]. The same results were seen when encoding sequence (for producing a 9-kDa protein) of plant non-specific lipid transfer protein [LTP] expressed in the *P. pastoris*. Production of LTP in shake-flask and in a fermentor led to the synthesis of two major species of LTP in the range of 10 and 14 kDa [15].

It is notable that rgp63 expressed in *E. coli* did not digest the gelatin. Thus, the recombinant gp63 produced in *P. pastoris* is very similar to the native form and it seems like a good substitution for the native gp63 in the vaccination studies.

In conclusion, we have shown that the rgp63 can be produced at high levels by *P. pastoris*. The expressed rgp63 had glycosylated and unglycosylated forms with different molecular weights in comparison with the rgp63 expressed in *E. coli*. It was functional on SDS-PAGE gelatin gel. The level of expression was in the range of other heterologous protein expressed in *P. pastoris* and reported 14% [15] to 80% [14] of the secreted proteins or 27% of the total cell proteins [15].

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