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Contributed Paper

Production of *Talaromyces* (*Penicillium*) *marneffei* Glyceraldehyde-3-Phosphate Dehydrogenase Recombinant Protein Expressed by *Pichia pastoris*

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ABSTRACT

Talaromyces (Penicillium) marneffei is a thermal dimorphic fungus that can cause a fatal disseminated disease in human immunodeficiency virus-infected patients. The routes of infection and factors that affect the pathogenicity of this fungus remain unclear. The previous studies demonstrated glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an adherence factor in *T. marneffei* during early phase of infection. In this study, recombinant GAPDH of *T. marneffei* was produced in a eukaryotic expression system, *Pichia pastoris*. The full-length *gpdA* gene of *T. marneffei* was amplified from cDNA clones using primers with restriction sequences. Then the amplified fragments were cloned into pPICzA plasmid and transformed into TOP10 *E. coli* competent cells. The fusion plasmids were purified, linearized and subsequently transformed into *P. pastoris* X-33 competent yeast cells by electroporation. The positive clones were then checked with PCR and sequencing. The selected clone was cultured and induced for recombinant protein expression. After purification, proteins of approximately 34 and 37 kDa were detected by coomassie blue stained gel. Only 37-kDa rGAPDH was identified by using western blot to detect his-tag. The rGAPDH was purified by using Ni-NTA reducing condition to remove the protein contamination. We have successfully cloned and produced GAPDH recombinant protein of *T. marneffei* in a yeast expression system. This protein will be further characterized for its virulence property.

Keywords: *Talaromyces marneffei*, *Penicillium marneffei*, glyceraldehyde-3-phosphate dehydrogenase, recombinant protein, *Pichia pastoris*

1. INTRODUCTION

Talaromyces (*Penicillium*) *marneffei* is the dimorphic fungus that causes the disease called penicilliosis marneffei. Most of infected patients are immunocompromised, especially HIV-infected patients who live in the endemic areas such as Southeast Asia, including North of Thailand [1]. *T. marneffei* grows as mycelial saprophytic phase and produces conidia like

the fungus of genus *Talaromyces*. In the parasitic phase, this fungus transforms to yeast phase and develops fission yeast cells in host cells or culture at 37°C. The infection probably originates by inhalation of fungal conidia from the environment [2]. Then fungal conidia convert to yeast phase in host cells and the infection disseminates to the other organs. During infection, the exposure of fungal outer membrane proteins to immune cells can elicit an immune response. Therefore the outer proteins can be candidates for developing diagnostic tool and may provide drug target for treatment.

GAPDH has been found on the cell wall, where it has different roles in host-pathogen interactions in some pathogens such as *Candida albicans*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* [3-8]. GAPDH has long been considered in molecular biology to be a housekeeping gene whose transcript level does not vary [9-10]. However, several studies have shown that the expression of *Paracoccidioides brasiliensis gapdh* gene and the cognate protein were developmentally regulated in the different growth phases of *P. brasiliensis*, with a higher expression in the yeast parasitic phase, thereby reinforcing possible new functions attributed to the enzyme. The *P. brasiliensis* GAPDH is a molecule located at the fungal cell wall, which binds components of the extracellular matrix and is capable of mediating the adherence and internalization of *P. brasiliensis* in cultured cells, suggesting its involvement in fungal pathogenesis [11]. In addition, the gene encoding GAPDH had been found to be upregulated mainly in *P. brasiliensis* recovered from infected mice [12]. In 2008, *gpdA* encoding GAPDH of *T. marneffei* was isolated and characterized. The expressions of *gpdA* in conidia and mycelial phases were higher than the yeast phase of *T. marneffei*. Furthermore *gapdA* expression was downregulated inside macrophage, suppression of this gene may leads to metabolic changes during host interaction [13]. The study by Lau and colleges has supported these results, GAPDH of *T. marneffei* was found to be upregulated in mycelial phase. Moreover, rGAPDH of *T. marneffei* was produced in *Escherichia coli*; this protein binds components of the extracellular matrix and is capable of mediating the adherence and internalization of fungi into cells, suggesting it is involved in fungal pathogenesis [14]. There have been few studies of the expression of *T. marneffei* recombinant proteins in yeast; one such protein is Mp1p, which has been used for diagnosis of infection. The authors found different immunogenic properties of Mp1P protein from *E. coli* and yeast expression systems [15-17]. As *E. coli* is a prokaryote, the inability to correctly fold the foreign eukaryotic protein and perform post-translation modifications limits the types of proteins that can be expressed. Therefore we have constructed recombinant protein in yeast system, which expressed rGAPDH protein of *T. marneffei* that can be used for further fungal pathogenesis studies.

2. MATERIALS AND METHODS

2.1 Strains and Culture Conditions

T. marneffei gpdA cDNA cloned in *E. coli* XL1 blue was maintained in Luria-Bertani (LB) medium and collected the plasmids by using the GeneJET plasmid extraction kit (Thermo Scientific, USA). *E. coli* strain TOP10 was cultured on LB agar and a single colony was used for competent cell preparation with the TransformAid bacterial transformation kit (Thermo Scientific). *Pichia pastosis* X-33 was cultured in YPD broth at 30°C with shaking overnight. After the growth reached to OD_{600} to 1.3-1.5, the cells was centrifuged at 1500 × g for 5 min, 4°C and washed twice

with ice-cold sterile water. Then the cell pellet was washed with 1M ice-cold sorbitol, resuspended again with 1M sorbitol and kept on ice until use for transformation.

2.2 Cloning and Transformation of *gpdA* to *P. pastoris*

The complete coding sequence of *gpdA* was amplified by polymerase chain reaction (PCR) from cDNA clone using specific primers. Forward and reverse primers, FgapE (5'-AGCTGAATTCATGGTTACC AAGGTTGATCA-3') and RgapN (5'-ATGCGGCCGCCTAATGATGATGAT GATGATGAGCGTTGCCGTCGACCT -3') were used for the amplification of *gpdA*. The underlined sequences in FgapE and RgapN primers encode for *Eco*RI and *Not*I restrict sites, respectively. PCR was performed in 25 µl reaction mixture containing cDNA, 1× proof reading PCR buffer, 200 µM of each dNTP, forward and reverse primers and 0.02 U proof reading Platinum® Taq DNA polymerase (Invitrogen, USA) and subjected to an initial 94°C for 5 min for denaturation, followed by 35 cycles of 95°C for 20 sec, 60°C for 10 sec and 72°C for 60 sec, with final extension at 72°C for 10 min in an automatic Thermocycler (GeneAmp PCR System 2700, Applied Biosystems, Singapore). The PCR products were cut by restriction enzymes and then cloned into the pPICzA plasmid (Invitrogen). The plasmids were transformed into *E. coli* TOP10 competent cells by chemical method (Thermo Scientific). Transformed bacteria were plated on LB medium with 100 µg/ml zeocin (Invitrogen) at 37°C. The positive clones were checked for *gpdA* insertion by PCR with specific primers. The selected clone was cultured in LB broth and isolated for *gpdA*-pPICzA plasmid. Approximately 5 µg of purified recombinant plasmid was linearized with *Mss*I restriction enzyme and transformed in to *P. pastoris* strain X-33 competent yeast cells by electroporation. The positive recombinant yeast clones were selected on yeast peptone dextrose sorbitol (YPDS) agar with 200 and 400 µg/ml of zeocin incubated at 30°C, for 24-48 h. The selected recombinant clone was isolated and checked for insertion by colony-PCR. The positive clones were determined for methanol utilization (Mut) phenotype. The positive yeast colonies were cultured on minimal media with dextrose (MMD) and minimal media with methanol (MMH) at 30°C for 2 days.

2.3 Expression and Purification of rGAPDH

The yeast positive clone was cultured in buffered glycerol-complex medium (BMGY) yielding OD_{600} to 2-6 and then *AOX*1 promoter was induced for protein expression by adding 0.5 or 1% methanol in buffered methanol-complex medium (BMMY) for 4 days. The rGAPDH protein was collected from the cell pellet by Mini-Bead Beater 8 homogenization (BioSpec, USA) with NPI-10 buffer and using batch purification of polyhistidine-tagged fusion proteins under native conditions following the manufacturer's instructions (Macherey-Nalgel, USA). 100 µl of nickel-nitrilotriacetic acid (Ni-NTA) agarose was pipetted to 1.5 ml centrifuge tube and gel was sedimented by centrifugation at 500 × g for 5 min. Then the supernatant was discarded. 500 µl of NPI-10 was added to equilibrate the gel, the tube was inverted to mix, centrifuged and the supernatant was discarded. 500 µl of cell lysate was added to the equilibrated gel and the mixture was gently mixed for 60 min, centrifuged and supernatant was discarded. The gel was washed 2 times with 500 ml of NPI-20. Then proteins were eluted by adding 50 µl of NPI-250 to the sediment gel, gently mixing for 2 min at room temperature to

liberate the polyhistidine-tagged fusion protein from the gel and the gel was sediment by centrifugation. The eluted protein was carefully pipetted into a new tube and the eluting step repeated twice (elute 1-3) and then proteins were stored at -80°C. For decontamination of other proteins, the concentration of imidazole was increased to 20 µg/ml in the binding step. The purified proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with coomassie blue.

2.4 Western Blot Analysis of rGAPDH

Western blot was performed by direct detection of his-tagged fusion proteins using chromogenic Ni-NTA conjugates system (Kirkegaard & Perry Laboratories, Inc., USA). After SDS-PAGE, proteins were transferred to nitrocellulose membrane and blocked with 1% BSA blocking solution for 1 h at room temperature with gentle agitation. 1/2,000 diluted nickel-horseradish peroxidase (Ni-HRP) conjugate was added directly into blocking solution and incubated for 1 h at room temperature with gentle agitation. Membranes were washed 3 times with 1× tris-buffered saline with tween 20 (TBST) for 5 min each with gentle rocking. Then 3,3',5, 5'-tetramethylbenzidine (TMB) substrate was added directly to the membrane and the reaction was allowed to develop for 3-5 min. The membrane was rinsed for 10-30 sec in water to stop the reaction and allow the membrane to air dry.

2.5 Proteins Identification

Liquid Chromatography Mass Spectrometer (LC-MS/MS) for proteins identification was performed by Proteomics Service Center, Mahidol University, Thailand.

3. Results and Discussion

3.1 PCR and Cloning

Approximately 1 Kb of *T. marneffei gpdA* was amplified from cDNA clone by PCR with specific primers, cloned in to pPICzA plasmid and transformed into TOP10 *E. coli* competent cells. The positive clones were checked with PCR (Figure 1) and the sequences confirmed with *AOX*1 primers sequencing. The putative 337 amino acid sequences of amplified fragment have 100 percent identities to glyceraldehyde-3-phosphate dehydrogenase (GpdA) of *T. marneffei* ATCC 18224 (XP_002151104) with 6 histidine residues at C-terminus (data not shown). The selected clone was cultured and the recombinant plasmid was purified for further cloning in yeast.

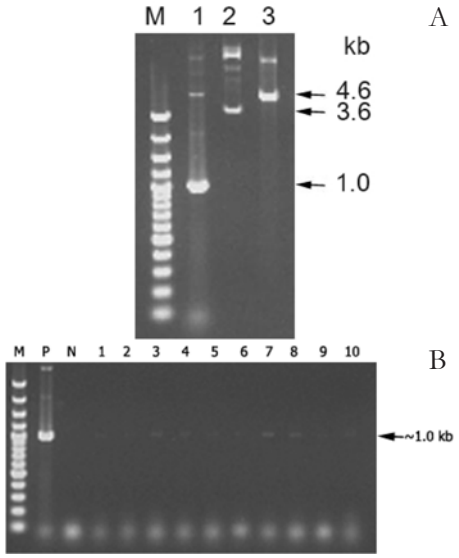

Figure 1. (A) Agarose gel electrophoresis to confirm insertion and plasmid size. Lane M: marker, 1: PCR product of *gpdA*, 2: pPICzA plasmid, 3: *gpdA*-pPICzA plasmid. (B) Colony-PCR amplification with *gpdA* specific primers for positive clone screening. Each clone was picked and suspended in 10 µl sterile water then heat at 80°C for 10 min. 1 µl of supernatant was used as the template for PCR. Lane M: marker, P: positive control (*gpdA* PCR product), N: negative control (X-33), 1-10: selected yeast colonies on YPDS-zeocin agar.

3.2 Cloning in Yeast Chromosome

The positive recombinant yeast cells were selected on YPDS agar with 200 and 400 µg/ml of zeocin. After selection, the pPICzA-*gpdA* insertion was checked by colony-PCR with specific primers. Approximately 1 Kb of PCR products were amplified from positive yeast clones (Figure 1B). The positive clones were determined for methanol utilization (Mut) phenotype. The PCR positive yeast colonies were cultured on minimal media with dextrose (MMD) and minimal media with methanol (MMH). After 2 days of incubation at 30°C, Mut^+ strains grew normally on both plates, while Mut^S strains grew normally on the MMD plate but slow or no growth on the MMH plate (data not shown). The Mut^+ clones, which were single crossover recombination at the *AOX*1 locus, were selected.

3.3 Yeast Culture and Protein Expression

After protein induction, the total protein lysate was extracted from the aliquot cell pellet at day 0-4. The 37 kDa rGAPDH could be detected by SDS-PAGE at day 4 after induction with both 0.5 and 1% methanol (Figure 2). Then cell pellet was collected from day 4 and purified for poly-histidine recombinant protein by Ni-NTA agarose. The expected positive rGAPDH band of 37 kDa was found (Figure 3). After purification, 2 major bands of proteins could be detected, 34 and 37 kDa. The protein expression was increased with an extended period of incubation; however more contaminated proteins could be detected. Therefore yeast culture for a maximum of 4 days was used for protein extraction and purification.

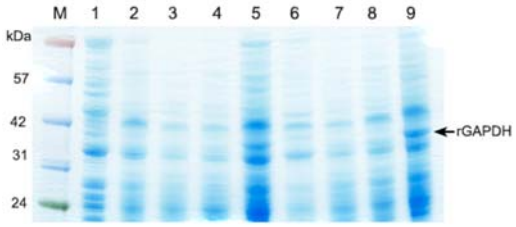

Figure 2. Expression of rGAPDH can be detected with SDS-PAGE after 4 days induction with 0.5 or 1% methanol (arrow). M: marker, lane 1: day 0, lane 2-5: day 1-4 with 0.5% methanol, lane 6-9: day 1-4 with 1% methanol.

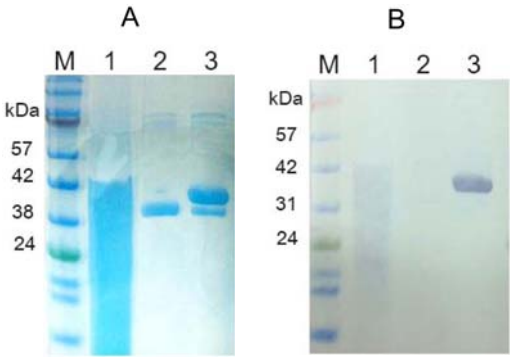

Figure 3. Western blot analysis of recombinant proteins expressed by *P. pastoris*. After SDS-PAGE analysis, protein bands were detected with coomassie blue staining (A) and transferred to nitrocellulose membrane, his-tag fusion proteins were directly detected with chromogenic Ni-labeled system (B). Lane M: marker, 1: *P. pastoris* protein lysate, 2: negative control protein (BSA) and 3: Ni-NTA purified rGAPDH.

3.4 Proteins Identification

After purification with Ni-NTA, 34 and 37 kDa protein bands were cut from SDS-PAGE gel and their protein identifications were performed by mass spectrometry. The amino acid sequences analysis showed most identity with

mitochondrial alcohol dehydrogenase of *P. pastoris* [gi254568544] and glyceraldehyde 3-phosphate dehydrogenase GpdA of *T. marneffei* [gi212541899], It's possible that during purification with Ni-NTA agarose, yeast alcohol dehydrogenase could bind to rGAPDH protein and both proteins were eluted from Ni-NTA agarose. The alcohol dehydrogenase of *P. pastoris* was likely linked to rGAPDH via disulfide bonds therefore we could detect 2 protein bands after analysis with SDS-PAGE. The contaminated proteins should be removed before further study.

3.5 Purification of rGAPDH

Protein decontamination was done by increasing the concentration of imidazole to 20 µg/ml for purified fusion protein in the Ni-NTA agarose binding step. High binding strength buffer could reduce contaminated proteins but could not remove all of them (Figure 4A). Then 2-mercaptoethanol was used in the binding step to break disulfide bonds of proteins. The results showed a single band protein of 37 kDa however the binding of rGAPDH to Ni-NTA was also reduced under this condition (Figure 4B). Although rGAPDH from *P. pastoris* expression system has the same molecular weight as in the *E. coli* system, the post-translation modifications such as protein folding, disulfide bonds formation and immunogenic properties will need further investigation.

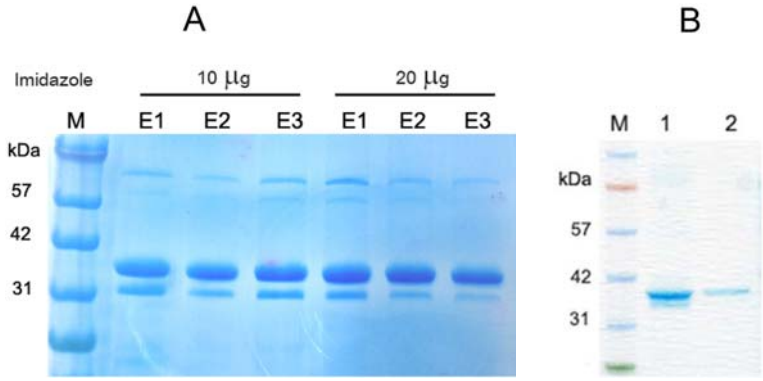

Figure 4. SDS-PAGE of rGAPDH protein purification. (A) 34 and 37 kDa were purified by Ni-NTA agarose; M: marker, E1-3: elute 1-3 with 10 and 20 µg imidazole containing-binding buffer, respectively. (B) rGAPDH purification in reducing condition; M: marker, 1: proteins from Ni-NTA agarose, 2: protein from Ni-NTA with 2-mercaptoethanol.

4. Conclusions

In this study, the GpdA recombinant protein of *T. marneffei* was produced by using a yeast expression system. The value of the *P. pastoris* expression system are (i) it has a strong and inducible promoter that can be used for high yield protein production (ii) it is capable of generating post translation modification similar to eukaryotic proteins (iii) can be designed with an a-factor signal sequence for production of secreted proteins that are easy to purify. Therefore, *P. pastoris* expression system offers significant advantages over *E. coli* expression systems for production of heterologous eukaryotic proteins [18]. This system can be applied for production of other interesting proteins of *T. marneffei*; it may provide the necessary

post-translational modifications, such as glycosylation, to produce a protein that may be structurally closer to its native form. Further, rGAPDH will be immunized into experimental animals to produce polyclonal antibodies for study of phases and metabolic changes during infection.

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