

Technical report

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Production and differential activity of recombinant human wild-type G6PD and G6PD_{Viangchan}

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Abstract

Background: Glucose-6-phosphate dehydrogenase (G6PD) is essential to produce reduced nicotinamide adenine dinucleotide phosphate, which is required to protect cells against oxidative stress. G6PD deficiency is a genetic variation that may lead to hemolysis with potential consequences, such as kidney failure, and patients often experience low quality of life.

Objectives: To establish a simple, efficient, and optimized method to produce a G6PD_{Viangchan} variant and characterize the phenotypes of recombinant human wild-type G6PD and G6PD_{Viangchan}.

Methods: *G6PD* was amplified by polymerase chain reaction (PCR) from a human cDNA plasmid, and the gene for G6PD_{Viangchan} was amplified by initiating a mutation at location 871 (G>A) through site-directed mutagenesis. Protein expression and western blotting were conducted after successful cloning. The enzymatic activity of both proteins was assessed spectrophotometrically after purification.

Results: Both amplicons were successfully cloned into a pET26b(+) expression vector and transformed into *Escherichia coli* BL21 (DE3) cells for overexpression as C-terminally histidine-tagged recombinant proteins. Western blotting confirmed that both proteins were successfully produced at similar levels. The enzymes were purified by immobilized metal (Co) affinity chromatography. Postpurification assay of enzyme activity revealed about 2-fold differences in the levels of specific activity between the wild-type G6PD (155.88 U/mg) and G6PD_{Viangchan} (81.85 U/mg), which is consistent with earlier reports. Analysis in silico showed that the coding change in G6PD_{Viangchan} has a substantial effect on protein folding structure.


Conclusions: We successfully cloned, expressed, and purified both wild-type G6PD and G6PD_{Viangchan} proteins. Such a protocol may be useful for creating a model system to study G6PD deficiency disease.

Keywords: phenotype; glucose-6-phosphate dehydrogenase Viangchan, human; cloning; protein expression; glucosephosphate dehydrogenase deficiency

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Human *G6PD* is a constitutive gene that is required to maintain basic cellular functions, and is expressed in all cell types. Expression of *G6PD* produces glucose-6-phosphate dehydrogenase (G6PD) that converts the glucose-6-phosphate to 6-phosphoglucono-d-lactone and simultaneously reduces nicotinamide adenine dinucleotide phosphate (NADP⁺) to NADPH [1]. NADPH plays an important role to protect cells against oxidative stress by maintaining a normal intracellular reduced state [2, 3]. The G6PD and NADPH pathway is the only source of reduced glutathione (GSH) in red blood cells (RBCs). Reduced GSH plays a vital role in detoxification of H₂O₂, a toxic reactive oxygen species (ROS) and byproduct of cellular metabolism in aerobic organisms [4]. NADPH produced in both the pentose phosphate pathway and the antioxidant GSH, is necessary for the continuous removal of ROS in the erythrocytes. A person with G6PD deficiency is usually asymptomatic provided oxidative stress is not triggered by oxidative agents. Oxidative agents produce higher levels of free ROS in RBCs. GSH is predominantly active in cells, but without G6PD, it remains present in the cells in an oxidized form and is unable to eliminate ROS [5]. Consequently, accumulated free radicals will form Heinz bodies and bind to the RBC membrane. This binding will further damage the cell membrane, and the cell is likely to undergo lysis.

G6PD deficiency is the most common genetic enzyme deficiency with a prevalence of approximately 400 million people worldwide [6, 7]. To date, there is no cure for G6PD deficiency, and it is a chronic lifelong condition. Treatment is often focused on managing the hemolysis or lifelong blood transfusion. Although mortality resulting from G6PD deficiency is very low or rare, in severe cases, the deficiency can lead to kidney failure or death. G6PD deficiency has also been linked to various types of cancers, tumors, and metabolic diseases and patients often experience low quality of life.

G6PD deficiency was first found in African Americans and thought to be restricted to one ethnic group. Subsequently, it was found that G6PD deficiency is an X-linked genetic disorder that is more common in men and boys than women and girls. In 1961, it became evident that the disorder was not exclusive to people with African ancestry, but also affects people with Southern European and the Middle Eastern ancestry. In 1967, the World Health Organization (WHO) published a standard method to measure the G6PD enzyme activity [8] and later, in 1971, the G6PD deficiency variants classification according to the severity of the phenotypes was published. By 1988, more than 370 variants had been described [9]. Currently, more than 500 variants are described. The prevalence of G6PD deficiency in Malaysia is around 3.1% in the male population and is most common

among Malays and Malaysian Chinese, and less common among those with Indian ancestry [10, 11]. A small study comprising 87 people resulted in similar findings, but with a higher prevalence of 4.59% [12]. The most common types of variants in the Malay ethnic group are G6PD_{Viangchan} (37.2%), followed by G6PD_{Mediterranean} (26.7%), and G6PD_{Mahidol} (15.1%) [13, 14]. The G6PD_{Viangchan} variant carries a point mutation at position 871 (G>A) [15].

In humans, the Viangchan-type variant can reduce G6PD expression by up to 80%–90%, which is based on biochemical characterization of enzyme partially purified from the RBC of those with the Viangchan-type variant [15] and this severe deficiency is classified as a WHO G6PD Class II variant [16], with <10% of wild-type G6PD activity, and has clinical implications, such as intermittent hemolysis.

A number of different G6PD deficient variants have been produced in heterologous expression systems, such as G6PD_{Yucatan}, G6PD_{Nashville}, G6PD_{Valladolid} and G6PD_{Mexico City} [17], G6PD_{Volendam} [18], G6PD_{Plymouth} and G6PD_{Mahidol} [19], G6PD_{Wisconsin} [20, 21], G6PD_{Fukaya} and G6PD_{Campinas} [21], G6PD_{Viangchan} [22, 23], and G6PD_{Zacatecas} and G6PD_{Vanua-Lava} [23]. Although G6PD_{Viangchan} has previously been cloned, produced, and characterized previously [22, 23], different template source/vectors, expression systems, methods, conditions, and parameters were used in each study, indicating the diversity of techniques that can be used to study the gene and enzyme. Previously, direct comparisons have been made between different recombinant human G6PD, such as between G6PD_{Viangchan} and G6PD_{Viangchan + Mahidol} [22], G6PD_{Zacatecas}, G6PD_{Vanua-Lava}, and G6PD_{Viangchan} [23], and between wild-type G6PD and 5 other G6PD variants (excluding Viangchan) [24].

In the present study, we aimed to clone, produce, and compare protein production and enzyme activity of both the wild-type G6PD and the G6PD_{Viangchan} variant in parallel, to establish a simple, efficient, and optimized method to produce the G6PD variant. Such a protocol may be useful to create a model system to study G6PD deficiency disease.

Methods

G6PD amplification

G6PD was amplified by polymerase chain reaction (PCR) using pOTB7 cloning vector as a template, which was purchased from the Mammalian Gene Collection (MGC) of cDNA clones (Dharmacon). Restriction sites *Nde*I and *Xho*I were inserted (as underlined) in the PCR primers sequence 5'-TTTCATATGGCAGAGCAGGTGG-3' (forward) and

3'-CCCCACAAGCTCGAGAAA-5' (reverse). PCR was performed under the following conditions: denaturation at 95°C for 5 min, amplification at (95°C for 15 s, 60°C for 30 s, and 68°C for 90 s) for 25 cycles, and 10 min of extension at 72°C. The PCR Master Mix comprising 1 × Pfx amplification buffer, 0.3 mM dNTP mixture, 1 mM MgSO₄, 0.3 μM of primer mix, and 1 U of Platinum Pfx DNA polymerase (Invitrogen) was used to produce approximately 1.5 kb amplicon. PCR was performed by using an Applied Biosystems Veriti 96-Well Thermal Cycler (ThermoScientific).

Cloning of wild-type G6PD/pET26b(+)

The PCR product and pET26b(+) (Novagen) were digested using *Nde*I (New England Biolabs) and *Xho*I (New England Biolabs) restriction enzymes for 2 h at 37°C in CutSmart Buffer (New England Biolabs). The digested products were purified by electrophoresis in 1% agarose gel (Figure 1) and ligated at 22°C for 15 min. The ligation mix was transformed into DH5α Competent Cells (New England Biolabs) and

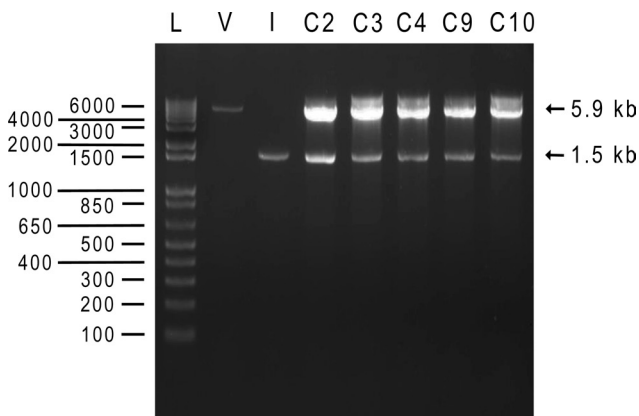


Figure 1. Agarose gel electrophoresis showing purified 5.9 kb vector pET26b (lane V), the PCR-amplified glucose-6-phosphate dehydrogenase gene (*G6PD*) 1.5 kb insert (lane I), and the plasmid DNA isolated from positive transformation clones (lanes C2, C3, C4, C9, and C10), which was digested using *Nde*I and *Xho*I. Lane L is a 1 Kb Plus DNA Ladder (Thermo Fisher Scientific; catalog No. 10787018). DNA (100 ng) was quantified spectrophotometrically using Nanodrop 2000C Spectrophotometer (Thermo Scientific) and mixed with 1× DNA Gel Loading Dye (Thermo Fisher Scientific) before loading onto a 1% agarose gel (7 cm × 10 cm) and separated by electrophoresis at 120 V for 45 min in 1 × TAE buffer (40 mM Tris-acetate and 1 mM EDTA at pH 8.3) in a Mini-Sub cell GT horizontal gel electrophoresis system (Bio-Rad). The separated DNA was stained with 0.5 μg/mL of ethidium bromide, and visualized under ultraviolet light at 300–360 nm. The isolated plasmid DNA produced 2 products when digested indicating successful cloning of *G6PD* into the vector. The purified products were used in the subsequent ligation reaction.

plated on kanamycin-resistance selective agar plates followed by incubation overnight at 37°C.

Production of G6PD variant

The mutation was initiated in wild-type *G6PD*/pET26 (+) plasmid using a QuickChange Site-Directed Mutagenesis Kit (Agilent). Primers were designed using software: (<http://www.genomics.agilent.com/primerDesignProgram.jsp>) that is, 5'-CTGAGATGCATTCAACATCTTGACCTTCTCATCACG-3' (forward) and 5'-CGTGATGAG-AAGGTCAAGATGTTGAAATGCATCTCAG-3' (reverse). The PCR mixture comprised 50 ng DNA template, 125 ng forward primer, 125 ng reverse primer, 0.3 mM dNTP mixture, 1 × reaction buffer, 2.5 U of PfuUltra HF DNA Polymerase (Agilent) and water, in a final volume reaction of 25 μL. The PCR cycling parameters used were 1 cycle of denaturation at 95°C for 30 s followed by 12 cycles of amplification at 95°C for 30 s, 55°C for 1 min, and 68°C for 4 min. The PCR product (of approximately 1.5 kb) was digested for 1 h using 1 U of *Dpn*I (10 U/μL) (ThermoScientific). The digested product was then transformed into XL1-Blue Competent Cells (Agilent) and then plated on chloramphenicol-resistance (Sigma-Aldrich) selective agar plates for overnight incubation at 37°C. Positive plasmid was sequenced to confirm the mutated site.

Overexpression of G6PD

Genes for wild-type *G6PD* and *G6PD*_{Viangchan} were transformed into *Escherichia coli* BL21 (DE3) competent cells for protein expression. At log phase, OD_{600nm} 0.8, the culture was induced with 0.1 mM and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and grown at 16°C, 25°C, and 37°C for 3, 5, 12, and 16 h. Cell lysate was centrifuged at 4,000 rpm for 10 min using an Eppendorf Benchtop 5424 Microcentrifuge with an FA-45-11 rotor. The pellets were resuspended with lysis buffer (0.1 M Tris-HCl, pH 7.6, 3 mM MgCl₂, 0.5 mM EDTA free protease inhibitor cocktail (Merck), and 0.1% β-mercaptoethanol). The cells were sonicated on ice with 10 short pulses (10 s) followed by pauses (30 s). The suspension was centrifuged at 15,000 rpm using an Eppendorf Benchtop 5424 Microcentrifuge with an FA-45-11 rotor for 15 min at 4°C. Supernatants were collected as soluble protein. Protein concentration was determined using a Pierce bicinchoninic acid protein assay kit (Thermo Fisher Scientific) with bovine serum albumin as the protein standard, before sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western

blotting. All G6PD proteins expressed were C-terminally tagged with histidine (His-Tag).

SDS-PAGE and western blotting

Proteins (30 μ g) were loaded into wells in a 5% polyacrylamide stacking gel on top of a 12.5% polyacrylamide resolving gel (8.3 cm \times 7.3 cm) and separated for 90 min at 180 V in 1 \times running buffer (Tris-glycine buffer with 10% SDS) using a Mini-Protean Tetra cell system (Bio-Rad). The proteins in the gel were stained with Coomassie Blue R-250 staining solution for about 30 min and destained using distilled water.

For western blotting analysis, the protein in an unstained gel was transferred to a polyvinylidene difluoride (PVDF) membrane in a semidry manner using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (catalog No. 170-3940; Bio-Rad) in transfer buffer (containing 25 mM Tris, 192 mM glycine, 20% (v/v) methanol (pH 8.3)) at 18 V for 1 h. Non-specific binding to the membrane was then blocked with 5% skimmed milk in phosphate buffered saline (PBS) for 1 h at room temperature. Subsequently, the membrane was incubated with polyclonal rabbit anti-G6PD (1:1,000 dilution; Cell Signaling Technologies, catalog No. 8866S), polyclonal rabbit anti-6 HisTag (1:1,000 dilution; Abcam, catalog No. ab14923) and polyclonal rabbit anti- β -actin (1:1,000 dilution; Abcam, catalog No. ab8227) primary antibodies, overnight at 4 $^{\circ}$ C. The primary antibodies were diluted in PBS with 5% skimmed milk and 0.1% Tween-20. After washing several times in PBS, the membrane was incubated with goat anti-rabbit IgG (H and L) secondary antibody conjugated to horseradish peroxidase (HRP) (1:10,000 dilution; Abcam catalog No. ab6721). β -Actin protein served as a loading control to show that equal amounts of protein was loaded into the wells. Benchmark Pre-Stained Protein Ladder (Invitrogen, catalog No. 10748010) was used to provide size markers. Finally, enhanced chemiluminescence (ECL) Western Blotting Detection Reagents (GE Healthcare) were used to detect the protein bands and the image was viewed and documented using a Versadoc Imaging system (Bio-Rad).

Purification of Recombinant G6PD

G6PD was purified using a HisTALON Gravity Column Purification kit (TaKaRa Clontech). As a starting material, we used 10 g of cell pellet. The pellet was suspended in 5 mL of lysis buffer and incubated on ice for 15 min. The suspension was sonicated on ice with 10 short pulses (10 s) followed by pauses (30 s) and was centrifuged at 10,000 \times g for 20 min at 4 $^{\circ}$ C.

The supernatants were collected and loaded onto the column, which had been equilibrated in washing buffer. The eluate was collected in 1 mL microcentrifuge tubes. After several washes, the proteins were eluted using 8 mL of elution buffer (containing 150 mM imidazole). Enzyme activity was measured using a G6PD activity colorimetric assay kit (BioVision, catalog No. K757). The amount of NADH that was generated between time points was quantified at OD_{450nm}. One unit (U) of G6PD activity was defined as the amount of enzyme that catalyzes the conversion of 1.0 μ mol of glucose-6-phosphate to 6-phosphoglucono- δ -lactone and 1.0 μ mol of NAD⁺ to NADH per min at 37 $^{\circ}$ C.

Mutation in silico

The wild-type G6PD structure (PDBID: 2HB9) was retrieved from the RCSB Protein Data Bank was subjected to point mutation in silico using the UCSF Chimera Rotamer tool [25]. Valine 291 was changed to methionine (V291M; G6PD_{Viangchan}). The side chain rotamer of the mutated residue was selected based on the Dynameomics rotamers library [26].

Energy minimization

The G6PD_{Viangchan} variant was subjected to energy minimization in silico to optimize the initial geometry of the protein structure by removing possible steric clashes. The energy minimization was performed using the Gromacs simulation package (version 4.6.7) [27].

Molecular docking of NADP⁺ to G6PD WT and

G6PD_{Viangchan} variant monomer

To understand the impact of mutation on the molecular interaction between G6PD_{Viangchan} and the structural NADP⁺, flexible small molecule and rigid protein docking was performed using AutoDock (version 4.2) [28]. Initially, the wild-type G6PD PDB file was cleaned by removing the existing NADP⁺ coordinate lines to create an “apoform” wild-type G6PD protein PDB file to be docked with the NADP⁺ structure using docking. The nonpolar hydrogen atoms were merged and total Kollman and Gasteiger charge were added to the protein. We ensured that there were no nonbonded atoms in the protein. Kollman and Gasteiger partial charges were also assigned to the NADP⁺ and all torsions were allowed to rotate during docking. Residues of the structural NADP⁺ binding site were specified based on the details obtained from previous work [29].

A grid box was assigned around the selected active site to cover the entire protein with a dimension of $60 \times 60 \times 60$. The Lamarckian Genetic Algorithm (LGA) option was set to 150 runs with default parameter settings. The best conformation with the lowest docking energy was selected from the docking search. The interaction of structural NADP⁺ and wild-type G6PD including hydrogen bonds and hydrophobic interaction was analyzed using LigPlot+ (version 2.1) [30]. The same docking simulation approach was used for G6PD_{Viangchan}.

Results

Cloning, protein expression, and enzyme activity

Mammalian Gene Collection (MGC) clones are commercially available sequence-validated full-length protein-coding cDNA clones for human, mouse, and rat from Dharmacon (GE Healthcare). pOTB7, which was used as the PCR template, contains human G6PD cDNA (GenBank RNA accession No. NM_001042351.2). The full-length of the gene contains 2295 bp nucleotides that include 128 bp upstream of *ORF* and 620 bp downstream sequences after the stop codon. The full-length coding region is 1548 bp long and can be translated into G6PD isoform B (515 amino acids). The forward and reverse primers were designed to carry restriction enzymes *NdeI* and *XhoI*, respectively. In addition, the reverse primer was also designed to remove the stop codon of the gene, which includes the His-Tag sequence. The size of the amplicon is 1,545 bp and the PCR product was cloned into the pET26b expression vector. Plasmid DNA were isolated from positive transformation colonies. Digestion with restriction enzymes *NdeI* and *XhoI* produced both vector and insert of the correct size, indicating successful G6PD gene cloning (Figure 1). Both pET26b+G6PD (wild type and Viangchan) were transformed into *E. coli* BL21 (DE3) competent cells for protein expression, which was induced by 0.1 mM and 1 mM IPTG. IPTG (1 mM) produced protein expression is 2-fold higher than 0.1 mM (data not shown). It was observed from SDS-PAGE that the protein expression level increased equally when the cells were incubated at different temperatures (16 °C, 25 °C, and 37 °C) (data not shown). SDS-PAGE showed optimum soluble G6PD expression when induced by incubation with 1 mM IPTG at 25°C for 16 h. The purified protein was run on 12.5% SDS-PAGE to confirm its molecular mass. The molecular mass of the protein is 59 kDa (Figure 2), which is similar to the mass previously reported for G6PD [5]. Western blotting showed a distinct single band indicating immunoreactivity of target proteins to anti-G6PD, anti-6 × His-Tag, and

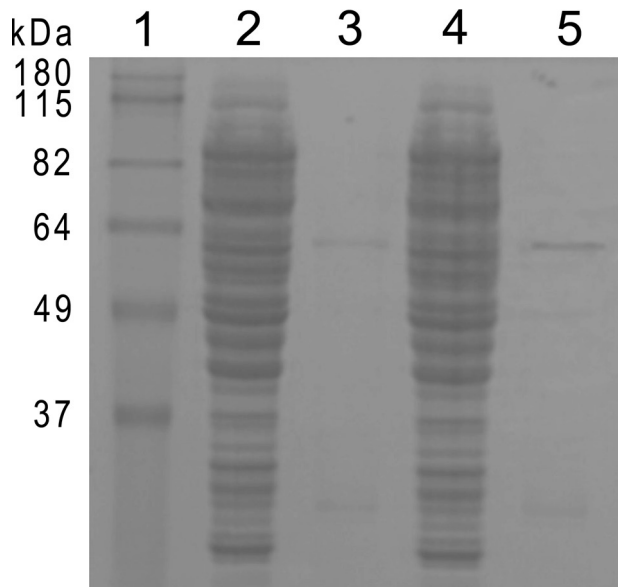


Figure 2. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) of unpurified and purified glucose-6-phosphate dehydrogenase (G6PD). After electrophoresis, the proteins in the 12.5% polyacrylamide resolving gel were stained with Coomassie Blue R. The purified G6PD has a molecular mass of 59 kDa. Lane 1: Benchmark Pre-Stained Protein Ladder (Invitrogen, catalog No. 10748010). Lane 2: unpurified wild-type protein. Lane 3: purified wild-type protein. Lane 4: unpurified Viangchan variant protein. Lane 5: purified Viangchan variant protein).

anti- β -actin antibodies (Figure 3). A total yield of 1.32 mg and 0.82 mg of protein were obtained for wild-type and G6PD_{Viangchan}, respectively, per 1 L of culture. Enzyme activity of 205.76 U for wild-type G6PD and 67.12 U for G6PD_{Viangchan} were detected. The total specific activity for wild-type G6PD was 155.88 U/mg whereas for G6PD_{Viangchan} it was 81.85 U/mg.

Bioinformatics analysis and 3D modeling

The genomic sequence variation from G>A results in an amino acid substitution of valine by methionine and the protein variation exists at amino acid position 291. The residue is located at alpha helix loop 11 and is highly exposed to solvent. The alpha strand is connected to the Rossmann fold, which is characterized by an alternating motif of β -strand- α -helix- β -strand secondary structures. The initial β - α - β fold is the most conserved segment of the Rossmann fold, and is responsible for FAD, NAD⁺, and NADP⁺ binding [31, 32]. It is assumed that the mutation might disrupt the orientation of the fold indirectly. A simple docking by using AutoDock revealed that the variant residues do not alter the NADP⁺ ligand binding site or the G6PD directly, but is located nearby. The binding energy obtained for NADP⁺ toward wild-type G6PD was -7.7 kJ/mol

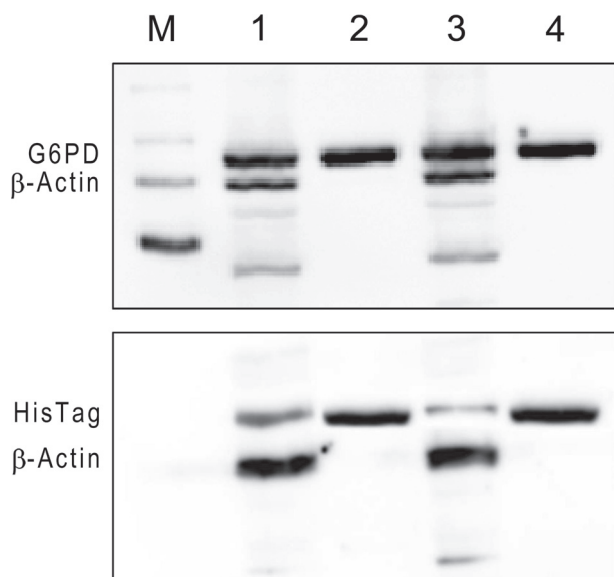


Figure 3. Western blot analysis of lysates containing unpurified and purified glucose-6-phosphate dehydrogenase (G6PD) protein using anti-G6PD (upper panel), anti-6 × HisTag (lower panel) and anti-β-actin antibodies (both panels). A distinct single band is visible indicating immunoreactivity of target proteins to anti-G6PD, and anti-6 × His-Tag antibodies. No band is visible when purified G6PD protein was incubated with anti-β-actin antibody indicating the G6PD protein was purified successfully. Lane M: Benchmark Pre-Stained Protein Ladder (Invitrogen, catalog No. 10748010; markers shown are 82, 64, 49, 37 kDa). Lane 1: unpurified wild-type enzyme. Lane 2: purified wild-type enzyme. Lane 3: unpurified Viangchan variant. Lane 4: purified Viangchan variant).

and that for $G6PD_{Viangchan}$ was -7.6 kJ/mol. The $NADP^+$ ligand is located at the center of the Rossmann fold (**Figure 4**). The wild-type structure interacts with more amino acids than the variant structure, which results in the reduced protein production. Ligand-residue analysis by using LigPlot+ also shows the $NADP^+$ interacts with almost 80% of the residues that exist within the Rossmann fold and indicates that the mutation might affect the orientation of the fold, resulting in reduced binding of the $NADP^+$ ligand.

Discussion

Here, we report successful cloning of the full-length of *G6PD* into the pET26b (+) system. The recombinant plasmid was inducibly expressed in *E. coli* BL21 (DE3) strain cultured at 25°C, by 1 mM IPTG for 16 h. The bacterial expression system was selected because of the ease of protein production, it is relatively less expensive, and had a longer shelf life than other expression systems [22]. Despite successful protein production, the yield of wild-type G6PD protein obtained was low compared with the amount published by others [19, 21, 23, 24, 33].

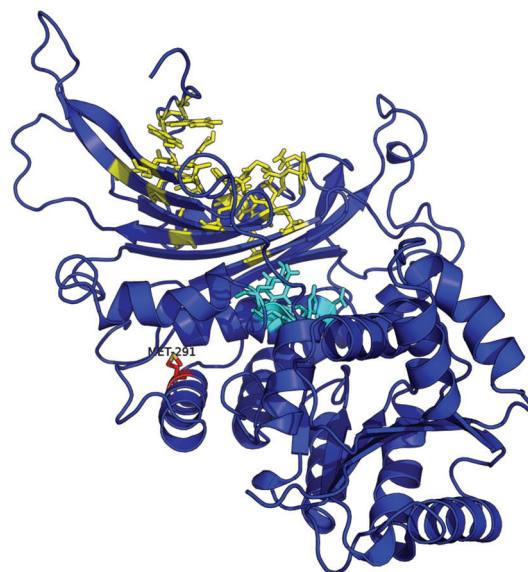


Figure 4. Three-dimensional structure of glucose-6-phosphate dehydrogenase ($G6PD_{Viangchan}$) obtained through mutation in silico. The wild-type G6PD structure (PDBID: 2HB9) was retrieved from the RCSB Protein Data Bank and subjected to mutation in silico using the UCSF Chimera Rotamer tool [25]. Valine 291 was changed to methionine (in red) ($G6PD_{Viangchan}$). The side chain rotamer of the mutated residue was selected based on the Dynameomics rotamers library [26]. The energy minimization was performed on the $G6PD_{Viangchan}$ variant (V291M) using the Gromacs simulation package (version 4.6.7) [27]. The molecular docking was performed using AutoDock (version 4.2) [28]. The yellow region represents the structural nicotinamide adenine dinucleotide ($NADP^+$) binding site and green region represents substrate binding site.

This may be due to the different downstream purification methods used in the other studies, such as anion exchange chromatography and protein recovery by using Amicon ultra centrifugal filter devices, which could influence the yield. In addition, different temperatures, kits, or tools used to assess the enzyme activity could have contributed to the variance in the enzyme activity level as we used 37 °C (in accordance with the protocol specified by the manufacturer), while most other studies used 25 °C as is the standard international unit (IU). In the present study, the recombinant proteins were purified using immobilized metal affinity chromatography using HisTALON resin charged with cobalt, which binds to Histidinetagged proteins with higher specificity than nickel-charged resins. Gómez-Manzo et al. [23] produced recombinant G6PD protein using a heterologous *E. coli* expression system, and purified the enzyme by using anion exchange column chromatography. Activity was assayed spectrophotometrically by monitoring the reduction of $NADP^+$ at 340 nm at 25°C. They obtained 230 IU/mg for wild-type G6PD and 228 IU/mg for $G6PD_{Viangchan}$. Boonyeun et al. [22] also produced $G6PD_{Viangchan}$ using an *E. coli* expression system and purified it using an anion exchange

column, and assayed G6PD activity using a method similar to that used by Gómez-Manzo et al. They obtained 80.1 IU/mg for wild-type G6PD and 51.9 IU/mg for G6PD_{Viangchan}. Nevertheless, the specific enzyme activity we obtained was consistent with these earlier works (Table 1). The specific activity for wild-type G6PD was 155.88 U/mg and for the Viangchan enzyme was 81.85 U/mg and therefore about 2-fold lower than that for the wild-type G6PD protein. G6PD_{Viangchan} is a class II variant [16], with less than 10% residual activity of the wild-type G6PD [15], whereas we obtained approximately 53% of wild-type G6PD activity in the mutant protein. Although the specific activity of our recombinant enzyme is higher than that might expected from the WHO classification, this classification is based on G6PD activity from the biochemical characterization of the enzyme from the blood, and is therefore influenced by other various genetic, temporal, and environmental factors [34, 35].

The V291M variant residue of G6PD_{Viangchan} is located some distance from the structural binding site of NADP and the substrate (Figure 3). This indicates that the mutation does not directly disrupt the catalytic site of the protein. NADP⁺ plays an important role in maintaining the dynamic behavior of the active enzyme [36]. Therefore, to gain a clearer insight toward the anomaly that exists as a consequence of the point mutation, we performed simple docking simulation. The similar binding energy for both wild-type G6PD and the Viangchan variant indicates that V291M does not alter the

binding site of structural NADP⁺. Furthermore, comparison of molecular interaction of NADP⁺ between wild-type G6PD and the Viangchan variant indicates that NADP⁺ forms a higher number of hydrogen bonds with G6PD_{Viangchan}, which suggests that NADP⁺ binding with G6PD_{Viangchan} is more stable than it is with the wild-type enzyme. Therefore, the docking analysis has elucidated that the inactivity of G6PD_{Viangchan} does not result from the destabilization caused by structural NADP⁺. A more detailed computational analysis using molecular dynamics simulation could provide a better understanding of the fundamental impact of the V291M variant that results in the G6PD_{Viangchan} variant.

Conclusions

We successfully cloned, expressed, and purified both wild-type G6PD and G6PD_{Viangchan} proteins. Although the reduction in G6PD activity obtained in the variant was lower than expected, the specific enzyme activity of the recombinant protein obtained was consistent with the activity achieved by others. Such a protocol may be useful for creating a model system to study G6PD deficiency disease, which would be useful for future understanding of the molecular mechanisms underlying the observed clinical phenotypes, and could be valuable in the quest to develop new therapies for this disease.

Table 1. Specific enzyme activity (U/mg) of heterologous glucose-6-phosphate dehydrogenase

Variant	Roos et al. [18]	Wang et al. [20]	Huang et al. [19]	Wang and Engel [21]	Gómez-Manzo et al. [33]	Gómez-Manzo et al. [17]	Gómez-Manzo et al. [23]	Boonyuen et al. [22]
Wild-type G6PD	210	180	182	210	224	224	230	228
Amsterdam	–	–	–	95	–	–	–	–
Volendam	36	–	–	–	–	–	–	–
Wisconsin	–	–	–	–	178	–	–	–
Nashville	–	130	–	–	–	103	–	–
Yucatan	–	–	–	–	–	132	–	–
Valladolid	–	–	–	–	–	92	–	–
Mexico City	–	–	–	–	–	175	–	–
Mahidol	–	–	177	–	–	–	–	141
Fukaya	–	175	–	–	–	–	–	–
Champinas	–	160	–	–	–	–	–	–
Plymouth	–	–	187	–	–	–	–	–
Viangchan	–	–	–	–	–	–	228	107
Zacatecas	–	–	–	–	–	–	58	–
Vanua-Lava	–	–	–	–	–	–	182	–

G6PD, glucose-6-phosphate dehydrogenase.

Author contributions. LM, MAY, NMY and ISI contributed substantially to the conception and design of this study. LM contributed substantially to the acquisition of data. LM, MAY, NMY, BHY and ISI analyzed and interpreted the data. LM and ISI drafted the manuscript. All authors contributed substantially to its critical revision and approved the final version submitted for publication.

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Conflicts of interest statement. The authors have each completed and submitted an International Committee of Medical Journal Editors Uniform Disclosure Form for Potential Conflicts of Interest. None of the authors has anything to disclose.

Data sharing statement. Data deposition (for the variant structure) is made in the BioModels repository of mathematical models of biological and biomedical systems found at <https://www.ebi.ac.uk/biomodels/MODEL1911200001>. Other data is available from the authors for noncommercial purposes on reasonable request.

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