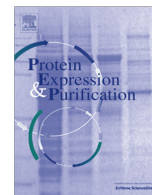
Protein Expression and Purification 92 (2013) 62–66

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep



One-step purification of soluble recombinant human 6-phosphogluconate dehydrogenase from *Escherichia coli*

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ARTICLE INFO

Article history:
Received 19 July 2013
and in revised form 13 August 2013
Available online 7 September 2013

Keywords:
Pentose phosphate pathway
6-Phosphogluconate dehydrogenase
Histidine-tag purification system

ABSTRACT

6-Phosphogluconate dehydrogenase (6PGD), the third enzyme in the pentose phosphate pathway, was recently identified as a novel target in human lung cancer. In this report, we present an expression and purification scheme of recombinant human 6PGD from *Escherichia coli*. Using a DE3 derivative strain expressing tRNAs for seven rare codons in *E. coli* called *Rosetta2 (DE3)*, a large quantity of soluble human 6PGD can be expressed with an N-terminal histidine tag and purified by a one-step purification procedure to near homogeneity without denaturants or refolding. Three to seven milligrams of purified protein could be obtained from 100 ml of culture. This recombinant human 6PGD follows classic Michaelis–Menton saturation kinetics with respect to both substrates $NADP^+$ and 6-phosphogluconate. The respective k_{cat} and K_m were comparable to those of 6PGDs purified from mammalian tissues. Using this purified 6PGD enzyme, we devised an endpoint colorimetric assay suitable for high-throughput screening for human 6PGD inhibitors.

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Introduction

The pentose phosphate pathway (PPP)[1] is a key branch of metabolism and is composed of the oxidative branch and the non-oxidative branch (Fig. 1). The oxidative PPP is thought to be one of the key sites of cellular NADPH generation and is catalyzed by three enzymes. Glucose-6-phosphate dehydrogenase (G6PD) oxidizes glucose-6-phosphate from glycolysis to form 6-phosphogluconolactone, which is hydrolyzed by 6-phosphogluconolactonase to generate 6-phosphogluconate (6PG). 6-Phosphogluconate dehydrogenase (6PGD) then oxidatively decarboxylates 6PG to yield ribulose-5-phosphate to enter the non-oxidative PPP.

Interest in identifying 6PGD inhibitors stems from the observation that 6PGD is a potential target for treating African trypanosomiasis in humans caused by a protozoan parasite called *Trypanosoma brucei* [1]. Specific inhibition of the protozoan 6PGD but not human 6PGD may provide a successful therapeutic strategy. As an overexpression and purification system for recombinant human 6PGD has not been reported to-date, inhibitors are currently assayed for their potential side effects using non-human 6PGD such as that from sheep [2–5].

Recently, we discovered that 6PGD also plays a role in human cancer. 6PGD is upregulated in human lung tumors from patients [6]. Expression knockdown of 6PGD in lung cancer cells inhibits proliferation both in vitro and in a xenograft model [6]. In addition, a previous report indicates that 6PGD activity is a prognostic marker for breast cancer [7]. Thus, inhibition of human 6PGD may be a novel anti-cancer strategy.

Here we report a bacterial system for high-level production of active human 6PGD by a one-step chromatographic procedure. A colorimetric endpoint assay for 6PGD activity suitable for future high-throughput screening was also developed.

Materials and methods

Construction of pET-16b-hm6PGD

The coding region of full-length human 6PGD (Accession number NM_002631) was amplified from human umbilical cord endothelial cells (HUVECs) with a forward primer 5′AAAACATATGGCCC AAGCTGACATCG-3′ and a reverse primer 5′AAAAGGATC CTCAGGC-ATTGTATGAC3′ using Phusion polymerase (New England Biolabs). The amplicon was fractionated by agarose gel electrophoresis and recovered using a Qiagen Gel Purification column. The coding region of 6PGD was cloned into expression vector pET16b (Novagen) between the NdeI and BamHI sites. Sequence integrity was verified by DNA sequencing (Genewiz).

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[1] *Abbreviations used:* G6PD, glucose-6-phosphate dehydrogenase; HUVECs, human umbilical cord endothelial cells; IPTG, isopropyl β-D-1-thiogalactopyranoside; $NADP^+$, nicotinamide adenine dinucleotide phosphate, oxidized; NADPH, nicotinamide adenine dinucleotide phosphate, reduced; NBT, nitroblue tetrazolium; 3PG, 3-phosphoglycerate; 6PG, 6-phosphogluconate; 6PGD, 6-phosphogluconate dehydrogenase; PMS, phenazine methosulfate; PMSF, phenylmethylsulfonyl fluoride.

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http://dx.doi.org/10.1016/j.pep.2013.08.017

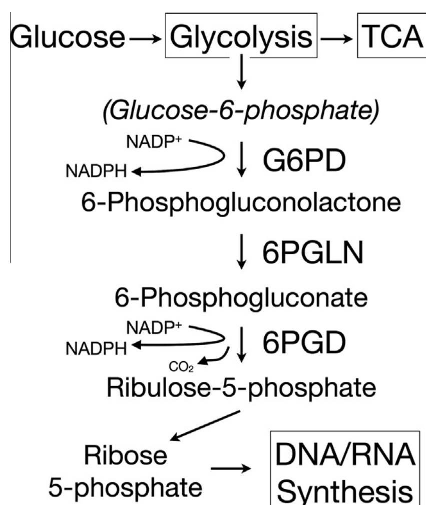

Fig. 1. Schematic of the pentose phosphate pathway (PPP). Glucose-6-phosphate from glycolysis enters the PPP through an oxidative step catalyzed by G6PD to generate 6-phosphogluconolactone. This intermediate is hydrolyzed by 6PGLN to form 6-phosphogluconate, which is oxidatively decarboxylated by 6PGD to yield ribulose-5-phosphate.

Expression and purification of recombinant human 6PGD

pET-16b-hm6PGD was transformed into Rosetta™ 2(DE3). A single colony was used to inoculate 25 ml of LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. After growing overnight at 37 °C, the culture was centrifuged at 6000 RPM for 30 min. The cell pellet was resuspended in 10 ml fresh LB medium. A portion of this suspension (5 ml) was used to inoculate 100 ml LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. The culture was grown at 37 °C with vigorous shaking until OD_{600nm} reached 0.7. IPTG was then added to 0.1 mM. The culture was grown at 37 °C with vigorous shaking for an additional 4 h. Cells were then collected by centrifugation and frozen at −20 °C. The following steps were carried out at 4 °C. The cell paste was resuspended in a low salt Lysis Buffer (50 mM NaH_2PO_4/30 mM NaCl/10mM Imidazole/100 μM $NADP^+$/0.5 mM PMSF/1.4 mM β-mercaptoethanol) supplemented with a protease inhibitor cocktail (SIGMA P8849; 1 ml per 20 g cell paste). After 30 min, egg white lysozyme (Sigma L6876) was added to 0.1 mg/ml. The mixture was incubated for 2 h, followed by a 1-h Benzonase treatment (Sigma E1014-25KU; 1 μl stock/ml lysate). After adjusting NaCl to 300 mM by adding 3 M NaCl stock, the lysate was incubated for 1 h and then centrifuged at 14,000 RPM for 30 min. The clarified lysate was applied by gravity to a 1-ml Ni–NTA column pre-equilibrated with 10 ml Lysis Buffer (50 mM NaH_2PO_4/300 mM NaCl/10 mM Imidazole/100 μM $NADP^+$/0.5 mM PMSF/1.4 mM β-mercaptoethanol). The flow-through fraction was re-loaded onto the column twice to ensure maximum binding. The column was washed successively with 10 ml of Lysis Buffer and Wash Buffer (50 mM NaH_2PO_4/300 mM NaCl/20 mM Imidazole/100 μM $NADP^+$/0.5 mM PMSF/1.4 mM β-mercaptoethanol). Recombinant 6PGD was eluted with 10 ml of Elution Buffer 1 (50 mM NaH_2PO_4/300 mM NaCl/250 mM Imidazole/100 μM $NADP^+$/0.5 mM PMSF/1.4 mM β-mercaptoethanol) and Elution Buffer 2 (50 mM NaH_2PO_4/300 mM NaCl/500 mM Imidazole/100 μM $NADP^+$/0.5 mM PMSF/1.4 mM β-mercaptoethanol). The two eluates were combined and concentrated using a Centricon-Plus (MW cutoff 5000) to ∼500 μL. The sample was reconstituted to 15 ml with 1X PBS/100 μM $NADP^+$/0.5 mM PMSF/1.4 mM β-mercaptoethanol). This process was repeated twice. The final concentrate was diluted with equal volume of 80% glycerol. Purified 6PGD was stored at −20 °C in aliquots.

Enzyme kinetics

The kinetic constants of purified 6PGD were determined by using a published method developed for rabbit 6PGD purified from mammary gland [8]. Briefly, data were collected using a Beckman Coulter DU730 Life Science UV/VIS spectrophotometer. Reactions were followed by measuring the increase in absorbance at 340 nm due to the appearance of NADPH (10-s intervals). To determine K_m and k_{cat} for $NADP^+$, 6-phosphogluconate (6PG) was kept at 1 mM in 1x Reaction Buffer (83 mM Tris HCl pH 7/16 mM $MgCl_2$) with $NADP^+$ varied from 1 to 200 μM. Reactions were initiated by the addition of recombinant 6PGD (8 nM). To determine K_m and k_{cat} for 6PG, $NADP^+$ was kept at 1 mM in 1x Reaction Buffer with 6PG concentration varied from 12.5 to 3200 μM. Absorbance at 340 nm was converted to NADPH concentration using an extinction coefficient of 5100 M^{-1} cm^{-1} at 340 nm, which was determined empirically using pure NADPH (Sigma) dissolved in 1x Reaction Buffer.

Colorimetric end point assay

To facilitate future high-throughput screening, an end-point assay for 6PGD was developed based on a published protocol [9]. NADPH produced by the 6PGD-catalyzed reaction reacts with phenazine methosulfate (PMS) and nitroblue tetrazolium (NBT) to yield dark-blue formazan observable spectrophotometrically at 580 nm. The optimized conditions are: 50 mM Tris HCl pH 7.0, 300 μM 6PG, 300 μM $NADP^+$, 100 μM NBT, 1 μM PMS, 0.4% glycerol, and 5 nM 6PGD at room temperature for 30 min. Data were collected using a Beckman Coulter DTX880 Multimode Detector.

Results and discussion

Construction of expression plasmids harboring human 6PGD with an N-histidine tag

The coding sequence of 6PGD gene was amplified from total cDNAs of human endothelial cells and cloned into pET16b to generate pET16b-hm6PGD (Fig. 2). The resulting construct would produce full-length human 6PGD (Accession number AAA75302) with a MGHHHHHHHHHHSSGHIEGRH tag at the N-terminus. This tag contains a factor Xa cleavage sequence (IEGR), which can be used to remove the peptide by factor Xa treatment if desired.

Production and purification of human 6PGD

pET16b-hm6PGD was transformed into Rosetta™ 2(DE3) for protein production. This strain is a modification of BL21 (DE3) and carries a plasmid that harbors coding sequences of seven tRNAs for rare codons (AUA, AGG, AGA, CUA, CCC, GGA, and CGG). This approach was independently verified to dramatically increase the success rate of expressing human proteins in bacteria [10]. We first determined the time-course for IPTG-induced 6PGD expression in this host (Supp. Fig. 1) and found that 4 h of induction at 37 °C yielded maximum amount of 6PGD expression. Next, a series of small-scale expression was performed to determine the optimal IPTG needed for induction. We found that IPTG concentration could be lowered to 0.1 mM without compromising the level of 6PGD induction (data not shown). Therefore, induction with 0.1 mM IPTG at 37 °C for 4 h was chosen for subsequent experiments.

Next, we determined whether this recombinant 6PGD was overexpressed as a soluble protein. After induction, the cell paste was frozen overnight at −20 °C. Cells were lyzed gently by resuspending cells in a hypotonic buffer followed by sequential addition of lyzsozyme and Benzonase. With this treatment, over 50% of re-

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