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ScienceDirect
Protein Expression and Purification 52 (2007) 373–378
Protein Expression & Purification
www.elsevier.com/locate/yprep

Functional phosphoglucose isomerase from *Mycobacterium tuberculosis* H37Rv: Rapid purification with high yield and purity

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Received 6 September 2006, and in revised form 10 October 2006
Available online 25 October 2006

Abstract

Phosphoglucose isomerase (PGI) EC 5.3.1.9, is a housekeeping enzyme that catalyzes the reversible isomerization of D-glucopyranose-6-phosphate and D-fructofuranose-6-phosphate. We have previously reported expression and multistep purification of recombinant PGI from *Mycobacterium tuberculosis* using conventional methods. We now describe an improved and simplified single step approach for purification of functionally active mycobacterial rPGI. The gene encoding PGI from *M. tuberculosis* H37Rv was cloned in bacterial expression vector pET22b(+). Expression of recombinant PGI with six-histidine-tag protein was observed both in the soluble fraction and inclusion bodies. Approximately 116 mg of recombinant enzyme was purified to near homogeneity with ~80% yield from the soluble fraction of 1 L culture at shake flask level using one step Ni–NTA affinity chromatography. The specific activity of the purified six-histidine-tagged recombinant PGI (rPGI-His_6) was ~800 U/mg of protein. The apparent K_m value of the active recombinant protein followed Michaelis–Menten kinetics and was 0.27 ± 0.03 mM. K_i for the competitive inhibitor 6-phosphogluconate was 0.75 mM. The enzyme had pH optima in the range of pH 7.6–9.0 and was stable up to 55 °C. rPGI-His_6 exhibited enzyme activity almost equal to that of enzyme without histidine tag.

Keywords: Phosphoglucose isomerase; *Mycobacterium tuberculosis*; Glycolysis; Six-histidine tag

Mycobacterium tuberculosis, the causative agent of tuberculosis in humans is one of the leading causes of mortality due to a single infectious agent [1]. Over 95% cases of tuberculosis are in developing countries. The HIV/AIDS pandemic, deterioration in public health systems in developing countries and the emergence of multidrug resistance forms of tuberculosis have contributed further to the pandemic. The only TB vaccine currently available is the attenuated *Mycobacterium bovis* strain *Bacillus* Calmette–Guerin, which has been reported to have a variable protective efficacy ranging from 0 to 85% in different studies [2]. Therefore, second generation anti-TB vaccines are urgently needed for treatment of tuberculosis [3].

Mtb is a very successful pathogen that has adapted itself to survive within several hostile environments. The enzymes and pathways that are required for growth and survival under the nutritionally restrictive conditions present in the phagosome represent attractive alternative targets for new anti-TB therapies that can also target latent infection of the bacterium. Being central to the organism's survival, glycolytic enzymes may be treated as potential targets for therapeutic control of tuberculosis. Phosphoglucose isomerase plays a central role in glycolysis and gluconeogenesis. PGI belongs to the aldose–ketose family of isomerases and is active as a homo dimer. It catalyzes the reversible isomerization of D-glucopyranose-6-phosphate and D-fructofuranose-6-phosphate by promoting the transfer of proton between C1 and C2. It is a vital link between the Embden–Meyerhoff–Parnas, Entner–Duodoroff and pentose-phosphate pathways. On the basis of known crystal structures of PGI from different species it has been reported that there are significant structural differences amongst PGIs from different species which could be exploited for species-specific drug designing. In this context, it is important to produce large amounts of

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doi:10.1016/j.pep.2006.10.006

recombinant PGI of *M. tuberculosis* which might pave the way for its crystallization and functional studies. We have previously described multi-step procedure for purification of rPGI from *M. tuberculosis* H37Rv providing the final product with relatively low yields [4]. In the present study, we describe high level expression, single step efficient purification of large amounts of histidine-tagged PGI of *M. tuberculosis* and its detailed characterization.

Materials and methods

Materials

BAC genomic library of *M. tuberculosis* was obtained from Prof. Stewart Cole of the Institut Pasteur [5]. Unless stated otherwise, all chemicals were of analytical grade and purchased from Sigma Chemical Company, USA. Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs, USA. Plasmid pET22b(+) expression vector was from Novagen, USA. QIA quick spin columns, plasmid purification kit and Ni–NTA agarose beads were from Qiagen, Germany. BCA protein estimation kit was procured from Pierce, USA. *Escherichia coli* (*E. coli*)[1] DH5α and BL21 (DE3) strains were obtained from Novagen, USA.

Construction of pgi-His$_6$ clone

PCR amplification of the *pgi* gene was carried out using gene specific primers that were designed using the genome sequence of *M. tuberculosis* H37Rv (forward 5′-CCCCATATGACCTCCGCGCCAATC-3′ and reverse 3′-CAAACTCGAGGCCCGCGCGGCCACGTTC -5′). *Nde*I and *Xho*I sites (underlined) were introduced in the forward and reverse primers, respectively, for convenient cloning in expression vector pET22b(+). Genomic DNA isolated from BAC genomic library of *M. tuberculosis* was used as template. Standard conditions of PCR were used for 30 cycles; denaturation at 94 °C for 1 min, annealing at 60 °C for 2 min and extension at 72 °C for 1 min with a final extension at 72 °C for 7 min in GeneAmp PCR system (Perkin-Elmer, USA). The amplified product, purified using Qiagen mini columns (Qiagen, Germany) as per manufacturer's instructions, was digested with *Nde*1 and *Xho*1, and cloned into plasmid pET22b(+) digested with the same enzymes. The ligation mixture was used to transform *E. coli* BL21 (DE3) cells (Novagen, USA) and selected on LB-Agar plates containing ampicillin (100 μg/ml). Recombinant colonies were analyzed by restriction digestion with *Xho*I and *Nde*I for the release of the insert and confirmed by automated DNA sequencing (Applied Biosystem Model 393A).

Expression of rPGI-His$_6$ in E.coli

Escherichia coli BL21 (DE3) cells transformed with the recombinant plasmid were grown in 250 ml Luria-Bertini medium containing 100 μg/ml ampicillin at 37 °C. At A_{600} of 0.5, the culture was induced with isopropyl-β-D-thiogalacto-pyranoside (IPTG) to a final concentration of 1 mM. The culture was grown for additional 8 h at 37 °C. Cells were harvested by centrifugation at 6500*g* for 15 min. The cell pellet (1.3 g) was resuspended in 12.5 ml of sonication buffer (50 mM NaH_2PO_4, and 300 mM NaCl, pH 7.8) and the cells disrupted by sonication (1 min pulse on followed by 30 s pulse off for 30 cycles; MISONIX, USA). The lysate was centrifuged at 12,000*g* for 20 min at 4 °C, The pellet (insoluble fraction) was resuspended in 4 ml of buffer. For analytical purpose, equal volumes of different fractions were analyzed by SDS–PAGE for protein expression.

Purification of rPGI-His$_6$ and Western blot analysis

All protein purification steps were performed at 4 °C. Ni–NTA agarose beads were added to the sonication supernatant (1 ml per liter of culture) and allowed to bind for 1 h with constant shaking on an end-to-end rotor. Following the addition of Ni–NTA agarose beads, all centrifugation steps were performed at 1200*g* in a swing out rotor for 2 min. Flow through was discarded and non-specifically bound proteins were removed by washing the beads twice in 15 ml of Tris-phosphate buffer (0.01 M Tris, 0.1 M sodium dihydrogen phosphate, pH 7.8). The recombinant protein was eluted in 12.5 ml of 250 mM imidazole (in Tris-phosphate buffer, pH 7.8).

For Western blotting, purified rPGI-His$_6$ was electrophoresed on 12% polyacrylamide gel and transferred onto nitrocellulose membrane. The blot was probed with Ni–NTA HRPO (horse radish peroxidase) conjugate and developed with 3,3′-diaminobenzidine (DAB).

Determination of enzyme activity

Phosphoglucose isomerase activity was determined as described previously [4] by monitoring the increase in absorbance due to the reduction of $NADP^+$ to NADPH at 340 nm. The assay mixture in a total volume of 1 ml consisted of 0.1 mM Tris-chloride buffer (pH 7.6), 2 mM EDTA, 0.5 mM $NADP^+$, 1 mM fructose-6-phosphate, and 1 U glucose-6-phosphate dehydrogenase. The reaction mixture was incubated at 25 °C for 10 min and the reaction was initiated by the addition of an aliquot of the purified recombinant enzyme. The reaction was followed for 5 min. The activity was measured by monitoring the change in the absorbance at 340 nm using spectrophotometer Lambda25 (Perkin-Elmer, USA).

One unit of PGI activity is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of fructose-6-phosphate to glucose-6-phosphate per minute under the above assay conditions.

[1] *Abbreviations used*: *E. coli*, *Escherichia coli*; IPTG, isopropyl-β-D-thiogalacto-pyranoside; DAB, 3,3′-diaminobenzidine.