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Constitutive expression, purification and characterization of a phosphoglucomutase from *Fusarium oxysporum*

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ABSTRACT

The phosphoglucomutase gene from a wild type *Fusarium oxysporum* strain (F3), was homologously expressed, under the control of the constitutive promoter of *gpdA* of *Aspergillus nidulans*. The transformant produced elevated levels of phosphoglucomutase activity compared to the wild type, a fact that facilitated the subsequent purification procedure. The enzyme (*Fo*PGM) was purified to homogeneity applying three anion exchange and one gel filtration chromatography steps. The native enzyme revealed a monomeric structure with a molecular mass of 60 kDa, while the isoelectric point was 3.5. *Fo*PGM was active in pH ranged from 6.0 to 8.0, with an optimum using 3-(N-morpholino)propanesulfonic acid buffer at 7.0, while loss of activity was observed when phosphate buffer was used in the above mentioned pH range. The optimal temperature for activity was 45 °C but the enzyme became unstable at temperatures above 40 °C. *Fo*PGM requires the presence of a divalent cation for its function with maximum activity being obtained with Co^{2+}. The apparent K_m for Co^{2+} was found to be 10 μM. The enzyme was also active with other divalent metal ions such as Mn^{2+}, Mg^{2+}, Ni^{2+} and Ca^{2+} but to a lesser extent. The following kinetic constants were determined: v_{max}, 0.74 μmol $mg_{protein}^{-1}$ min^{-1}; k_{cat}, 44.2 min^{-1}; K_m(G1P), 0.10 mM; K_m(G1,6diP), 1.03 μM; k_{cat}/K_m(G1P), 443 mM^{-1} min^{-1} and k_{cat}/K_m(G1,6diP), 42,860 mM^{-1} min^{-1}. The enzyme was considered to follow a Ping Pong substituted enzyme or enzyme isomerization mechanism.

1. Introduction

Phosphoglucomutase (PGM; EC 5.4.2.2) represents a key enzyme in carbohydrate metabolism, where it catalyzes the reversible transfer of a phosphate group between C-1 and C-6 of glucose *via* glucose-1,6-diphosphate (G1,6diP). PGM is the link between glucose 6-phosphate (G6P), produced by anaerobic or aerobic glycolysis, and glucose 1-phosphate (G1P), produced by various glycan phosphorylases. These phosphorylated sugars may enter several different catabolic pathways, to yield energy (ATP) or reducing power (NADPH), or enter anabolic pathways, leading to the synthesis of UDP-glucose, a sugar donor for the production of glucose-containing polysaccharides. Hence PGM plays a key role in directing the metabolism towards polymer synthesis or catabolic pathways, a fact that makes it an ideal target for the protein engineering of micro-organisms to redirect their metabolic flux [1].

PGM is widespread in living organisms from bacteria to humans and includes two types of similar but distinguishable enzymes, an independent phosphoglucomutase (PGM) and a bi-functional protein, phosphomannomutase/phosphoglucomutase (PMM/PGM), that catalyzes the phosphoryl group-converting reaction for both phosphoryl glucose and phosphoryl mannose. Biochemical properties of PGMs and PGMs/PMMs of different origin have been investigated from: *Acetobacter xylinum* [1], *Bacillus subtilis* [2], *Bacillus cereus* [3], *Clostridium thermocellum* [4], *Lactococcus lactis* [5,6], *Micrococcus lysodeikticus* [3], *Pseudomonas aeruginosa* [7], *Pyrococcus horikoshii* OT3 [8], *Saccharomyces cerevisiae* [9], *Sphingomonas chungbukensis* [10], *Thermococcus kodakaraensis* [11], *Zea mays* [12], human and animal tissues [13–18].

In previous studies when the fermentation capabilities of *Fusarium oxysporum* have been evaluated under both aerobic and anaerobic conditions, the profile of phosphorylated sugars indicated high levels of accumulated glucose-1,6-diphosphate (G1,6diP) during glucose and cellulose fermentation, which might indicate a reduced activity of PGM and hence potential difficulties of glucose to be channeled towards cell wall biosynthesis [19,20]. These results combined with the key role of PGM in metabolic flux regulation, led us to pursue the purification and characterization of the native

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enzyme from the wild type *Fusarium oxysporum* strain F3 [21], a fact that is expected to facilitate the evaluation of future results in fermentations of native and engineered *F. oxysporum* strains.

We took advantage of the recently released genome sequence of *F. oxysporum* (Broad Institute of Harvard and MIT, http://www.broad.mit.edu) where one gene was annotated as PGM namely *foxg_00670.2*. In the present study, the *foxg_00670.2* gene was homologously expressed in the wild type *F. oxysporum* strain F3, under the control of the constitutive promoter of *gpdA* of *Aspergillus nidulans* and the recombinant protein (*Fo*PGM) was purified and fully characterized.

2. Materials and methods

2.1. Microorganism strains, plasmids and media

The wild type strain F3 of *Fusarium oxysporum* [21] maintained on PDA (Potato Dextrose Agar) stock cultures at 4 °C, was used. Plasmid pBluescript SK II was obtained from Stratagene (La Jolla, CA, USA). Plasmid pCSN44 was obtained from the Fungal Genetics Stock Center (University of Missouri, Kansas City, USA). Plasmids were propagated in *Escherichia coli* strain DH5α. For plasmid isolation and purification the Macherey-Nagel (Bethlehem, PA, USA), GmbH Nucleospin® Plasmid purification kit was used.

Minimal medium (MM1) used for DNA isolation and protoplasts preparation consisted of ($g\,L^{-1}$): $NaNO_3$, 3.0; KCl, 0.3; $MgSO_4{\cdot}7H_2O$, 0.3; KH_2PO_4, 0.5; $ZnSO_4$, 0.05; H_3BO_3, 0.03; $MnCl_2{\cdot}4H_2O$, 0.01; $FeSO_4{\cdot}7H_2O$, 0.01; $CoCl_2{\cdot}6H_2O$, 0.005; $CuSO_4{\cdot}5H_2O$, 0.005; $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.003; Na_2EDTA, 0.1; glucose, 10.0.

Minimal medium (MM2) used during transformant growth for isolation consisted of ($g\,L^{-1}$): sucrose, 200; KH_2PO_4, 1; $MgSO_4{\cdot}7H_2O$, 0.5; KCl, 0.5; $NaNO_3$, 2; glucose, 20; $FeSO_4$, 0.01; agar, 4 or 15 as noted in text.

Growth of the transformed *F. oxysporum* strains in liquid cultures was conducted in mineral medium MM3 that had the following composition ($g\,L^{-1}$): $(NH_4)_2HPO_4$, 10.0; KH_2PO_4, 2.0; $NaH_2PO_4{\cdot}2H_2O$, 15.6; $MgSO_4{\cdot}7H_2O$, 0.3; $CaCl_2{\cdot}2H_2O$, 0.3; glucose, 20. The pH of the medium was adjusted to 7.0. The inoculum was prepared by transferring 5 mL mycelia and spore suspension of *F. oxysporum* from a 6-day-old culture, grown on PDA slopes at 30 °C, to 250-mL Erlenmeyer flasks each containing 100 mL of the above mentioned mineral medium. The fungus was grown at 30 °C in an orbital shaker operating at 200 rpm for 48 h and served as a preculture. Cultures were prepared in 3-L Erlenmeyer flasks containing 600 mL of the above medium supplemented with glucose ($20\,g\,L^{-1}$). The Erlenmeyer flasks were inoculated with 10% (v/v) of the above mentioned preculture. Growth was allowed to proceed for 48 h, at 30 °C on an orbital shaker (200 rpm).

All media were heat sterilized at 121 °C for 20 min. In liquid media, carbon sources were sterilized separately.

2.2. Isolation of the phosphoglucomutase gene

Genomic DNA was isolated from *F. oxysporum* F3 conidia inoculated in liquid minimal medium and allowed to grow for 24 h at 29 °C and 150 rpm. Mycelia were harvested, frozen in liquid nitrogen and genomic DNA was isolated as described by Sambrook et al. [22]. The phosphoglucomutase gene (*pgm*) was isolated from the *F. oxysporum* F3 genomic DNA, by PCR, using the following primers that were constructed according to the annotated phosphoglucomutase gene sequence from the *Fusarium oxysporum* genome (FOXG_00670.2, Broad Institute, MIT):

5′-TA-GCGGCCGC-ATGGGCGTCCAAACTGTTG-3′
5′-AT-ACTAGT-TTAGGTCTTGACATCAGGCTCG-3′

The above procedure resulted in a PCR product comprised of the *F. oxysporum pgm* between the *Not*I and *Spe*I restriction sites (underlined in above primer sequences).

2.3. Plasmid construction

Initially, plasmid pBlPGM was constructed on a pBluescript SK II template by cloning the open reading frame (ORF) of *F. oxysporum* F3 PGM (above) between the promoter of *gpdA* of *A. nidulans* and the terminator of *trpC* of *A. nidulans*. The *gpdA* promoter was inserted between the *Sac*II and *Not*I sites and the *trpC* terminator between the *Spe*I and *Sma*I sites of the pBluescript SK II polylinker. Consequently, the *pgm* was inserted between the *Not*I and *Spe*I sites of the polylinker, yielding plasmid pBlPGM. Following this, a 2.4 kb fragment carrying the hygromycin resistance gene, isolated from plasmid pCSN44 following digestion with *Sal*I, was ligated into *Xho*I digested plasmid pBlPGM. The final resulting plasmid pBlPGM-hyg was used for the transformation of the wild type *F. oxysporum* strain F3.

2.4. Protoplast preparation and transformation

F. oxysporum F3 protoplasts were prepared according to Tilburn et al. [23] with certain modifications. Conidia were harvested from a 4-day PDA culture and filtered through muslin. 10 mL of liquid minimal medium were inoculated with 10^8 conidia and incubated for 17 h, at 26 °C and 150 rpm. Conidia were collected with centrifugation at 5000 rpm for 5 min and diluted to 10 mL of 1.2 M $MgSO_4$, pH 5.8. 0.2 g of Glucanex (Novozymes A/S, Bagsvaerd, Denmark) was added, and the solution was incubated for 1.5 h at 100 rpm and 30 °C. 10 mL of 0.6 M Sorbitol and 10 mM MOPS, pH 6.3, were added gently and the solution was centrifuged at 3000 rpm. Protoplasts were collected from the interface, purified with a solution of 1 M Sorbitol, 10 mM MOPS, pH 6.3 and diluted to a final concentration of 10^8 protoplasts per mL with a solution of 1 M Sorbitol, 10 mM MOPS and 40 mM $CaCl_2$, pH 6.3.

Plasmid pBlPGM-hyg was diluted in a 10 mM, Tris buffer, pH 7.5, supplemented with 1 mM EDTA and 40 mM $CaCl_2$. Sixty μL of appropriately diluted plasmid solution (approx. 1 μg plasmid DNA) were mixed with 100 μL protoplast solution and following 20 min incubation on ice, 160 μL PEG4000 60% (w/v, in 1 M Sorbitol, 10 mM MOPS, pH 6.3) was added. The mixture was incubated at room temperature for 15 min and then diluted and purified in 1 M Sorbitol, 10 mM MOPS and 40 mM $CaCl_2$. The above mixture was mixed in MM2 supplemented with $4\,g\,L^{-1}$ agar and poured on Petri dishes containing solid minimal medium (MM2 with $15\,g\,L^{-1}$ agar) supplemented with hygromycin at a concentration of $50\,\mu g\,mL^{-1}$. Transformants were isolated after 7 days of incubation in 25 °C.

2.5. DNA and RNA manipulations

Southern blot analysis was conducted as follows: Genomic DNA from wild type and transformant *F. oxysporum* strains was digested by *Eco*RI overnight and fractionated on a 1% agarose gel. The DNA was then transferred to a nitrocellulose membrane and hybridized overnight at 65 °C with 32P-labeled probes [22]. The probes were prepared by random primer labeling of the *F. oxysporum* PGM coding region. The membrane was washed at 65 °C, successively in 2×, 2×, 1× SSC solution (15 mM sodium citrate, 150 mM NaCl) containing 0.1% SDS for 30 min each and then exposed to X-ray film at −80 °C for 3 h.

For RNA isolation, conidia were inoculated in liquid minimal medium (MM1) and incubated for 20 h at 28 °C and 150 rpm. Mycelia were then harvested, frozen in liquid nitrogen and RNA was extracted according to Sambrook et al. [22]. For northern analysis, total RNA was fractionated in 1% agarose, 10 mM orthophosphate buffer and transferred to nitrocellulose membrane (Porablot NY plus, Macherey – Nagel GmbH). Hybridization conditions were as described in Southern analysis.

2.6. Crude enzyme preparation

Biomass from a 48 h liquid culture of the *F. oxysporum* selected transformant was collected with a continuous flow centrifuge at 4 °C. If not immediately processed, biomass was stored at −80 °C. 50 g of wet mycelium was suspended in 250 mL of 50 mM imidazole buffer pH 7 (pre-cooled at 4 °C) supplemented with 5 mM $MgCl_2$, 1 mM DTT, 10% (v/v) glycerol and 2.5 mL of protease inhibitor cocktail (Sigma Chemical Co., St Louis, MO, USA). The resulted suspension was homogenized in a household blender (1 min at maximum speed).

Disruption of the mycelia was completed using a laboratory sonicator model VC 600, (Sonics and Materials, Inc., Newtown, CT, USA) in 100 mL batches under continuous cooling. At 1 min intervals an aliquot (approx. 0.5 mL) was pooled from the sonication vessel and filtered (0.45 μm filter cartridges, Millipore Corporation, Billerica, MA, USA). PGM activity and total protein content was determined in the filtrate, in order to monitor the release of the intracellular material. Based on the above results, a total sonication time of 10 min was selected for maximum release of PGM activity (data not shown).

Following sonication, the crude extract was collected by centrifugation for 40 min, at 54,000 × *g* and 4 °C, concentrated and desalted in an Amicon ultrafiltration apparatus (Amicon chamber 8400 with membrane Diaflo PM-10, exclusion size 10 kDa), (Millipore Corporation, Billerica, MA, USA) and used for further purification.

2.7. Enzyme purification

All chromatographic steps were performed at 4 °C. The buffer used throughout purification was 20 mM imidazole buffer, 5 mM $MgCl_2$, 1 mM DDT, 10% (v/v) glycerol, pH 6.0 (buffer A).

The concentrated sample was loaded onto a Q-Sepharose column (Sigma Chemical Co.) (5 cm i.d., 30 cm length), equilibrated with buffer A. The column was first washed with 1410 mL of buffer A, followed by two successive linear gradients from 0 to 100 mM NaCl in 1200 mL of buffer A and then from 100 to 200 mM NaCl in 300 mL of buffer A, at a flow rate of $600\,mL\,h^{-1}$. Fractions (10 mL) containing PGM activity were pooled, desalted and concentrated using an Amicon ultrafiltration apparatus (Amicon chamber 8400 with membrane Diaflo PM-10, exclusion size 10 kDa) (Millipore Corporation).

The concentrated sample from the previous step was loaded onto a Q-Sepharose column (Sigma Chemical Co.) (5 cm i.d., 30 cm length), equilibrated with buffer A. The column was first washed with 1580 mL of buffer A, then a linear gradient from 0 to 56 mM NaCl in 1120 mL of buffer A, was applied, followed by isocratic elution of 56 mM NaCl in 1370 mL at a flow rate of $600\,mL\,h^{-1}$. Fractions (10 mL) containing PGM activity were pooled, desalted and concentrated using an Amicon ultrafiltration apparatus.