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Cloning and characterization of phosphoglucose isomerase from *Sphingomonas chungbukensis* DJ77

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Phosphoglucose isomerase (PGI) is involved in synthesizing extracellular polysaccharide (EPS). The gene encoding PGI in *Sphingomonas chungbukensis* DJ77 was cloned and expressed in *E. coli*, and the protein was characterized. The pgi gene from DJ77 is 1,503 nucleotides long with 62% GC content and the deduced amino acid sequence shows strong homology with PGIs from other sources. The molecular masses of PGI subunit and native form were estimated to be 50 kDa and 97 kDa, respectively. Four potentially important residues (H361, R245, E330 and K472) were identified by homology modeling. The mutations, H361A, R245A, E330A, R245K and E330D resulted in decrease in Vmax by hundreds fold, however no significant change in Km was observed. These data suggest that the three residues (H361, R245Aand E330) are likely located in the active site and the size as well as the spatial position of side chains of R245 and E330 are crucial for catalysis. [BMB reports 2009; 42(3): 172-177]

INTRODUCTION

Sphingomonas chungbukensis DJ77 is a bacterial strain that produces a large quantity of extracellular polysaccharide (EPS), resulting in slimy and viscous growth cultures. The EPS synthesis in *S. chungbukensis* DJ77 is currently of particular interest in the finding of novel EPSs with unique properties and their potential application in industry. In our study, *S. chungbukensis* DJ77 was isolated from chemically contaminated freshwater sediment in Deajon, Korea (1). Several enzymes and coding genes involved in the synthesis of EPS repeating units were identified. The pgm gene encoding a bifunctional enzyme with phosphoglucomutase and phosphomannomutase activities was cloned and characterized (2). Two additional enzymes characterized were UDP-glucose pyrophosphorylase and UDP-glucose dehydrogenase, which are involved in a synthetic pathway leading to a nucleotide sugar precursor of UDP-glucose and UDP glucoronic acid (3). Recently, the pmm gene coding for phosphomannomutase has been identified (4).

Three enzymes have not yet been identified in the pathway leading to the formation of the GDP-mannose precursor from fructose-6-phosphate in *S. chungbukensis* DJ77. The first enzyme, phosphoglucose isomerase (EC 5.3.1.9), catalyzes the interconversion between glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P). This enzyme has been expected to play a key role in governing the carbon availability for EPS production *in vivo* as it intersects the point between glycolysis and EPS synthesis. The last two enzymes are phosphomannose isomerase (PMI), which catalyzes the isomerization of F6P to mannose-6-phosphate (M6P), and GDP-D-mannose pyrophosphorylase (GMP), which producces GDP-D-manose by incorporating GTP into mannose-1-phosphate. In *Pseudomonas aureus* and *Xanthomonas campestris*, PMI and GMP activity exist in the same protein known as bifunctional PMI/GMP enzyme (5, 6).

In this study, the gene encoding PGI activity from *S. chungbukensis* DJ77 was cloned, and overexpressed in *E.coli*, while a full characterization of the enzyme was performed.

RESULTS

Gene cloning

The local BlastP search of the *S. chungbukensis* DJ77 sequence database showed the presence of any significant alignments with the PGI amino acid sequence from *X. campestris*. Three significant alignments (CS090R, CR973F and FB135F) revealed that sequences in the *S. chungbukensis* DJ77 database were homologous to the C-terminus of query. To find alignment between sequences in *S. chungbukensis* DJ77 and the N-terminus of the query, sequencing was extended on a plasmid containing a *S. chungbukensis* DJ77 insert with the CR973F sequence at one end. Sequencing identified an ORF of 1,506 nucleotides which was named as pgi (Fig. 1). This ORF starts at conserved start codon ATG, preceding the putative ribosome-binding site (5'-AGAGG-3'). The GC content is high (65.67%), which is the characteristic of genes from the genus *Sphingomonas*. The deduced amino acid sequence of pgi exhibited high homology with other PGIs in the NCBI database

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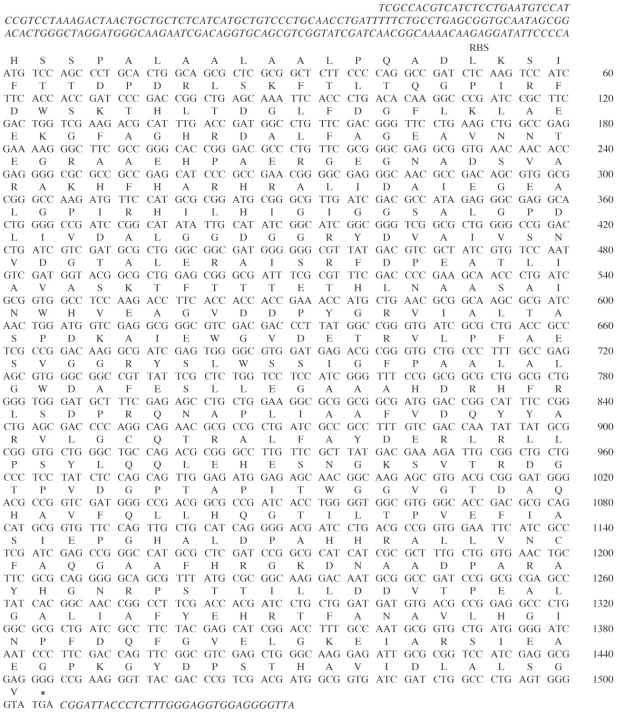

Fig. 1. The complete nucleotide sequence and deduced amino acid sequence of PGI from *S. chungbukensis* DJ77.

using the BLASTP program. Especially, PGI from *Zymomonas mobilis*, showed the highest similarity with *S. chungbukensis* DJ77 pgi with 76% similarity and 64% identity. These data indicate that pgi may code for PGI from *S. chungbukensis* DJ77.

Protein expression and purification

The pgi sequence once inserted into a pGEX-4T-1 expression vector and transformed into the *E. coli* strain BL21 (DE3) was expressed as GST-fusion ptotein that upon IPTG induction produced a prominent protein band at the position corresponding to the sum of GST and deduced molecular mass of PGI on a SDS-PAGE of cell lysate (not shown). Purified protein by GST-affinity column seemed to to be homogeneous as indicated by a single band on SDS-PAGE. Thrombin-treated protein exhibited the expected size of 50 kDa (Fig. 2).

To determine the native structure of PGI from *S. chungbukensis* DJ77, we performed a native-PAGE. The estimated molecular mass of the PGI protein based upon standard proteins of native PAGE was about 97 kDa (not shown). This value appears twice as large as that of the calculated molecular mass from amino acid sequence of PGI, suggesting that native PGI protein from *S. chungbukensis* DJ77 is dimer.

Kinetic properties

The phosphoglucose isomerase activities of the fusion and GST-cleaved PGI proteins from *S. chungbukensis* DJ77 were

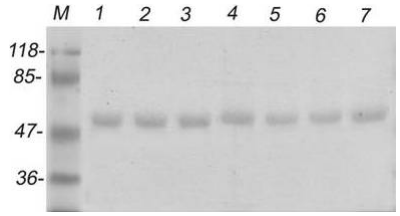

Fig. 2. SDS-PAGE of the wild type and mutant PGIs. Each sample was electrophoresed on 11% polyacrylamide gel containing SDS. M, marker; 1, wild type PGI; 2, R245A; 3, H361A; 4, E330A; 5, K472A; 6, R245K and 7, E330D.

Table 1. Kinetic parameters of the wild type and the mutant PGIs

Mutants and wild type PGIs	Km (mM)	Vmax (mM/min/mg)	Kcat (s^{-1})	Kcat/Km (s^{-1}/mM)
Wild type PGI	0.35	103.37	53.77	152.85
H361A	0.48	0.20	0.18	0.37
K472A	0.39	2.55	2.05	5.21
E330A	0.62	0.25	0.22	0.36
R245A	0.31	0.22	0.19	0.63
R245K	0.45	0.06	0.05	0.11
E330D	0.39	0.23	0.20	0.51

similar when measured in the reverse direction from F6P to G6P. The enzymatic activity of the *S. chungbukensis* DJ77 PGI protein was dependent upon the F6P concentration, followed the Michealis-Menten pattern and was stable in the broad range of pH 6.5 to 9.5. The apparent values of Km and Vmax for F6P were 0.35 mM and 103 mM/min/mg, respectively (Table 1). The calculated Kcat based on the deduced molecular mass of PGI (51 kDa) was 53.77s^{-1}. Both erythrose-4-phosphate (E4P) and phosphogluconate acid (6PGA) inhibited enzymatic activity by 50% in the presence of 0.6 mM E4P and 0.5 mM 6PGA , respectively.

Homology model of *S. chungbukensis* DJ77 PGI

The pair wise alignment of the amino acid sequences of *S. chungbukensis* DJ77 PGI and rabbit PGI (PDP accession number, 1HOX) is highly similar (38% identity), allowing us to build a homology model in order to visualize the structure of *S. chungbukensis* DJ77 PGI. The homology model is constructed using Swiss-Model (not shown) based upon the X-ray structure of rabbit PGI, which produced two models (monomer and dimer form). Results from a structure validation server, Whatcheck, validated the fidelity of the models. All residues in the model were found in the favorable region of Ramachandran plot, indicating a good overall conformation (not shown).

The PGI model of *S. chungbukensis* DJ77 and rabbit PGI X-ray structure exhibited high similarity regarding overall structure (Fig. 3). Furthermore, conservation was observed between the PGI model of the DJ77 and the corresponding important

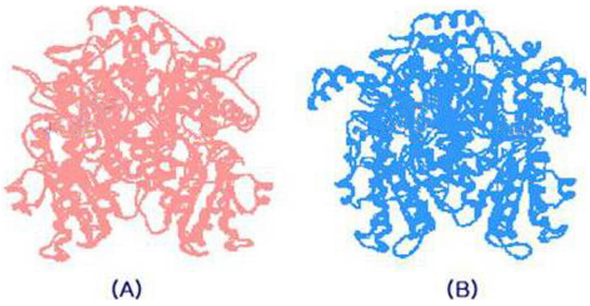

Fig. 3. Comparison of *S. chungbukensis* DJ77-PGI model (A) and X-ray structure of rabbit PGI (B).

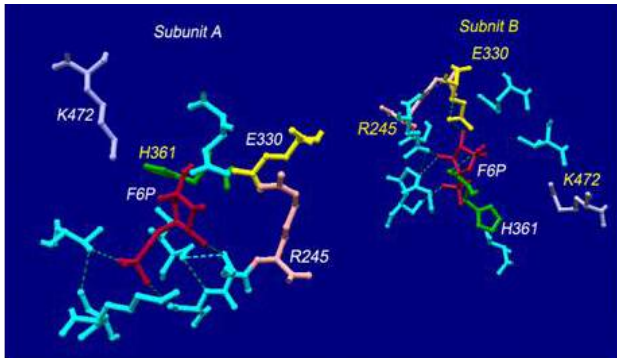

Fig. 4. The structure at the active site of the PGI enzyme from *S. chungbukensis* DJ77.

catalytic residues in rabbit PGI. Four amino acid residues (R245, E330, H361, K472) of *S. chungbukensis* DJ77 were also conserved (Fig. 4).

Active site residues of PGI

To investigate the active site of the *S. chungbukensis* DJ77 PGI protein, four residues (R245, E330, H361 and K472) were subjected to mutagenesis and assessed for structural and activity changes. The procedure used for wild type protein purification was applied and all were found in soluble fraction. The purified proteins further showed homogeneity on SDS-PAGE (Fig. 2). No differences were observed in the secondary structures of the mutants compared to wild type protein when determined by CD spectroscopy, suggesting that mutagenesis did not affect overall structure (not shown).

Target residues were individually substituted by alanine, which produced hundred folds decrease in Vmax except K472A; however, no significant difference was found in Km value (Table 1). For example, a decrease of 426 fold was observed in the activity of R245A mutant compared to wild type PGI protein, however, the Km value remained similar. These data suggest that these residues are located in active site of PGI and are essential for catalysis. To further investigate the role of R245 and E330, mutants R245K and E330D were created. The activity of both mutants showed dramatic reduction (hundreds fold) in comparison to wild type protein (Table 1), indicating that the proper spatial position of side chains of R245 and E330 are crucial for catalysis of PGI from *S. chungbukensis* DJ77.

DISCUSSION

Phosphoglucose isomerase catalyses the isomerization of G6P to F6P and intersects the point between the glycolysis and EPS precursor synthesis. The gene coding for this enzyme was identified in *S. chungbukensis* DJ77 and then cloned and characterized. Amino acid sequence analysis of the *S. chungbukensis* DJ77-PGI protein revealed two highly conserved PGI superfamily motifs, including the sequence patterns (LIVM)-G-G-R-(FY)-S-(LIVM)-X-(STA)-(STA)-(LIVM)-G and (FY)-D-Q-XG-VE-X-X-K, which are documented as signature PGI patterns by the ExPASy Molecular Biology Center at the University of Geneva (http://expasy.hcuge.ch/sprot/prosite.html). The pgi gene was first introduced to a pET-15 expression vector and expressed as a 6X His fusion protein because no extra cleavage step was required to remove the 6XHis upon purification. However, an alternative approach to expression was required as the protein was present as an inclusion body at various culture and induction conditions. The protein was fused with GST in the pGEX-4T-1 expression system to make a GST-PGI fusion protein soluble. This allowed the facilitation of protein purification under native conditions.

The purified enzyme from *S. chungbukensis* DJ77 was characterized for activity. The apparent Km and Vmax values were similar to those of rabbit and Bacillus PGI (7). However, the pH dependence profile was not bell-shaped, thereby differing from that of previous reported PGIs (8). The *S. chungbukensis* DJ77 PGI enzyme was inhibited by two specific PGI family inhibitors, 6PGA and E4P, the second of which caused 50% loss in enzyme activity at a concentration of 0.6mM, a figure much higher than that of rabbit PGI and yeast PGI (2 μM) (9).

According to the X-ray structure of rabbit PGI, four residues (R272, E357, H388 and K518) were reported to be essential for catalysis and the roles in the PGI mechanism was also established (10, 11). It were reported that H388 and K518 participated in the opening and closure of substrate ring, that the positive charge of R272 helps stabilize the negative charge of the intermediate product and that E357 was proved to directly catalyze the isomerization. Mutational analysis of Bacillus PGI supported the prominent role of the corresponding histidine and glutamic acid residues; however, the role of corresponding lysine was not emphasized (9, 12, 13). In this study, in order to investigate the active site of the *S. chungbukensis* DJ77 PGI protein, the residues (R245, E330, H361 and K472) once aligned with the above catalytic residues from rabbit PGI were subjected to site-directed mutagenesis. All mutants maintained secondary structure but simultaneously demonstrated dramatic decrease in enzymatic activity, except Km which only chang-

ed slightly. These results suggest that these residues were indeed located in the active site of the *S. chungbukensis* DJ77 PGI protein and were involved in catalysis rather than substrate binding. This finding is in agreement with our results regarding the PGI homologous model, which also established the position of these residues in the active site. The effect of the K472A mutation was lower than that of other target residues (only about 50 fold decrease in Vmax) and the distance from K472 to F6P in the model was found to be farther. This may suggest that K472 has a less prominent role in the PGI catalysis mechanism and plays a similar role in the Bacillus mechanism (9, 12, 13).

MATERIALS AND METHODS

Materials

The bacteria strain *S. chungbukensis* DJ77 and sequencing plasmids (pCR973 and pCV365) were kindly provided by Prof. Young Chang Kim (Chungbuk National University, Cheongju, Korea). LB Broth and LB agar were purchased from Difco. Restriction enzymes and T4 DNA ligase were obtained from Roche. Taq and Pfu polymerase were purchased from Bioneer (Korea). Thrombin protease and epoxy-activated Sepharose 6P was purchased from Amersham, and Probond His-Tag resin was obtained from Invitrogen. All enzymatic assay reagents were obtained from Sigma. Other chemicals were of the highest grade from commercially available sources.

Gene cloning

To clone *S. chungbukensis* DJ77 gene coding for PGI, the genomic DNA sequence database of *S. chungbukensis* DJ77 (http:// bioinfo.chungbuk.ac.kr) was screened using the corresponding gene from *X. campestris*. The obtained homologous sequence fragments were aligned with each other using multiple Clustal W in Bioedit program (14). The unknown sequences were determined by sequencing extension in ABI 3700 automatic DNA sequencer (Macrogen). In order to clone the complete pgi sequence, PCR was performed in a total reaction solution of 50 µl containing 1U Pfu polymerase, 1X reaction buffer, 2.5 mM dNTP, 10 pmol of each specific primer and genomic DNA as template. Primer PGI-A: 5'- CAATGTCC AGCCCTGCACTG -3' and primer PGI-B: 5'-TAATCCGTCATA CCCCACTCAG -3' were used to amplify pgi gene. The PCR program repeated 30 cycles and each cycle consisted of 1 min at 94°C, 45 sec at 58°C and 1 min at 72°C. PCR products were cloned into a pGEM-T vector and further sequencing was carried out to confirm the correct sequences.

Protein expression and purification

To overexpress the pgi gene in *E. coli*, pgi was inserted after the GST sequence in a pGEX-4T-1 vector, creating the plasmid pGEX-PGI. Two primers used were PGI-F: 5'-TAAGGATCCAG TCCTGCACTGGCA-3' (BamHI); primer PGI-R: 5'-AAACTCGA GGTTAACACCACTCA-5' (XhoI). The pGEX-PGI construct was transformed into *E. coli* BL21 (DE3) and transformed cells were grown at 37°C until OD_{600} level of 0.6-0.7. PGI expression was induced by 0.4 mM isopropyl-D-thiogalactoside (IPTG) at 25°C and cells were harvested after 5-hour induction by centrifugation at 4,500 g for 10 minutes. PGI was purified according to the GST fusion purification protocol provided by the manufacturer, Amersham. Briefly, the cell pellets were suspended with the 1X PBS buffer containing protease inhibitor (1 mM phenyl-methylsulfonyl fluoride). The cell suspension was then lysed by sonication on ice. The homogenate was centrifuged at 20,000 g for 30 minutes and supernatant was applied onto the GSH-coupled Sepharose 6B column. Unbound proteins were removed by washing with the 1X PBS before the GST-PGI fusion was eluted with 50 mM Tris-HCl containing 15 mM GSH. To obtain the cleaved PGI, the fusion PGI was treated with thrombin (10 U/mg protein) at 4°C overnight. Additional chromatography seperated the GST protein from the cleavage mixture.

Proteins in both crude cell extracts and purified samples were analyzed on SDS-PAGE. The molecular mass standard (Biorad) was used to estimate the molecular mass of the target proteins.

Mutagenesis of PGI

Site-directed mutagenesis of PGI was performed directly on the pGEX-PGI construct using the PCR megaprimer method (15). The first PCR series was carried out with primer B (PGI-B) paired individually with each mutagenic internal primer. The mutagenic primers were as follows.

H361A, 5'-GCAG**GCA**GCGGTGTTCCAGT-3'
R245A, 5'-TGGGCGGT**GCT**TATTCGCTCTG-3'
R245K, 5'-TGGGCGGT**AAA**TATTCGCTCTG-3'
E330A, 5'-TGGAGATG**GCG**AGCAACG-3
E330D, 5'-TGGAGATG**GAC**AGCAACG-3
K472A, 5'-GCAATCTC**CGC**GCCCAGCT-3

The DNA fragments obtained were subjected to a second PCR with the universal primer A (PGI-A). However, the order of universal primer used in the second PCR of mutant K472A was reversed. These PCR products were double digested with the restriction enzymes BamHI and XhoI and cloned into pGEX- 4T-1. Overexpression and mutant protein purification followed the same procedure with wild type PGI.

Native PAGE

The native molecular mass of PGI protein was determined by native PAGE using non-denatured protein molecular mass marker kit (Sigma), including α-lactatalbumin (14.2 kDa), carbonic anhydrase (29.0 kDa), albumin of chicken egg (45.0 kDa), monomer and dimer albumin of bovine serum (60.0 kDa and 132.0 kDa) (not shown), as described by Hedrick and Smith (16).

Enzyme assays

All assays measuring enzyme activity were based on the level

of $NADP^+$ reduction in coupled reaction systems at 37°C. Increase in OD_{340} was recorded on Beckman DU-650 spectrophotometer. The PGI enzyme activity was determined by coupled glucose 6-phosphate dehydrogenase (ZWF) method (17). The standard assay solution contained 50 mM HEPES (pH 7.5), 0.5 mM $NADP^+$, 1 unit of ZWF, PGI and varied amount of substrate (fructose-6-phosphate). The kinetic parameters (V-max, Km) were determined by fitting the data in to the equation (1).

$$v = Vmax/[1 + Km/(S)] \quad (1)$$

In this equation, v is the reaction velocity, Vmax is the maximum velocity, Km is the Michaelis-Menten constant and (S) is the substrate concentration. Protein concentration was estimated by the Bradford method using bovine serum albumin as a standard.

The optimum pH was determined using MES, MOPS, HEPES, TAPS, and CAPS at 50 mM each. Erythrose 4-phosphate and 6-phosphogluconate acid were tested for potential inhibitory effect on PGI activity.

Circular dichroism of wild type and mutant PGIs

The protein concentration was adjusted to 0.35 mg/ml in 10 mM potassium phosphate, pH 7.4. All CD spectra were recorded over the range of 190-250 nm on a Jasco J-710 spectropolarimeter set at 20-50 mdeg sensitivity, 0.2 nm resolution, 3 units accumulation, one-second response and at a scanning speed of 20 nm/min. Samples were assayed in a 1 mm path length cylindrical quartz cell.

Homology modeling of PGI

Homology modeling of PGI structure was conducted essentially similar to that reported for tobacco acetohydroxy acid synthase (18). Briefly, the PGI sequence from *S. chungbukensis* DJ77 and rabbit were aligned on Bioedit program. The resulting alignment was examined and adjusted manually and then submitted for automatic modeling at Swiss-Model server (19, 20). A homodimer model of *S. chungbukensis* DJ77-PGI was obtained. A single round of energy minimization was done with the GROMOS96 implemented on Deep View. Structural illustration was created from coordinate files with Deep View (19).

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