

Cloning and characterization of phosphomannose isomerase from *shingomonas chungbukensis* DJ77

Sinh Thi Tran¹, Dung Tien Le¹, Young-Chang Kim², Malshik Shin³ & Jung-Do Choi^{1,*}

Departments of ¹Biochemistry, ²Microbiology, Chungbuk National University, Cheongju 361-763, ³Department of Food and Nutrition, Chonnam National University, Gwangju 500-757, Korea

Phosphomannose isomerase (PMI) catalyzes the interconversion of fructose-6-phosphate and mannose-6-phosphate in the extracellular polysaccharide (EPS) synthesis pathway. The gene encoding PMI in *Shingomonas chungbukensis* DJ77 was cloned and expressed in *E. coli*. The *pmi* gene is 1,410 nucleotides long and the deduced amino acid sequence shares high homology with other bifunctional proteins that possess both PMI and GDP-mannose pyrophosphorylase (GMP) activities. The sequence analysis of PMI revealed two domains with three conserved motifs: a GMP domain at the N-terminus and a PMI domain at the C-terminus. Enzyme assays using the PMI protein confirmed its bifunctional activity. Both activities required divalent metal ions such as Co²⁺, Ca²⁺, Mg²⁺, Ni²⁺ or Zn²⁺. Of these ions, Co²⁺ was found to be the most effective activator of PMI. GDP-D-mannose was found to inhibit the PMI activity, suggesting feedback regulation of this pathway. [BMB reports 2009; 42(8): 523-528]

INTRODUCTION

Shingomonas chungbukensis DJ77 was isolated from chemically contaminated freshwater sediment in Deajon (Korea) (1). This strain produces a large quantity of extracellular polysaccharide (EPS), creating a slimy and viscous old growth culture. High-pH anion exchange chromatography analysis of the EPS revealed the presence of three types of sugar: glucose, mannose and galactose. These sugars are present in a 1 : 1 : 1 ratio (personal communication).

In bacteria, EPS provides a protective capsule against dehydration, macrophages, bacteriophages, protozoa, antibiotics and other toxic compounds (i.e heavy metal ions) (2). Due to its physical properties, EPS is used widely in many applications from food processing to pharmaceutical production and

other industries (3). The application and, hence, economical value of gelling agents, including bacterial EPS, depends largely on its physico-chemical properties. The advantage of bacterial EPS over algal polysaccharide is that the physico-chemical properties of bacterial EPS can be modified by changes in culture conditions and achievable strain modifications.

For the above mentioned reasons, studies have focused on understanding the biosynthesis pathways of EPS with the aim of improving EPS production in bacteria. In *S. chungbukensis* DJ177, some enzymes and genes involved in the EPS repeating unit synthesis pathway have been identified. The *pgm* and *pmm* genes, encoding bifunctional enzymes with phosphoglucosyltransferase and phosphomannomutase activities, have been cloned and characterized (4). In addition, UDP-glucose pyrophosphorylase and UDP-glucose dehydrogenase, two enzymes involved in the pathway resulting in the synthesis of the nucleotide sugar precursors UDP-glucose and UDP-glucuronic acid were also characterized (5, 6).

Four enzymes are involved in the synthesis of the GDP-mannose precursor from glucose-6-phosphate in *S. chungbukensis* DJ77. The first enzyme, phosphoglucose isomerase catalyzes the interconversion of glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P). This enzyme stands at the branch point between glycolysis and EPS synthesis and is expected to play a key role in governing the carbon availability for EPS production in vivo. Previously, we successfully cloned, expressed and characterized this enzyme (7). The second enzyme, phosphomannose isomerase (PMI), catalyzes the interconversion of F6P and mannose-6-phosphate (M6P). The third enzyme, phosphomannomutase (PMM) catalyzes the mutation between M6P and mannose-1-phosphate (M1P), and the last enzyme, GDP-D-mannose pyrophosphorylase (GMP), converts GTP into M1P, generating GDP-D-mannose. In *Pseudomonas aureus* and *Xanthomonas campestris*, PMI and GMP activity exist in the same protein, known as the bifunctional PMI/GMP enzyme (8, 9).

In this study, we cloned the gene encoding the bifunctional enzyme, PMI/GMP, expressed the enzyme in *E. coli* and characterized its functions. The data in this report will provide a better understanding of the role that the GDP-mannose synthetic pathway plays in the overall process of EPS synthesis in *S. chungbukensis* DJ77, and supply useful information for

*Corresponding author. Tel: 82-43-261-2308; Fax: 82-43-267-2306; E-mail: jdchoi@chungbuk.ac.kr

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modifying this strain to allow for higher production and the desired physico-chemical properties of EPS.

RESULTS

Gene cloning

To clone the gene encoding PMI in *S. chungbukensis* DJ77, we used the amino acid sequence *X. campestris* PMI (512 residues) (8) to search the sequence database of *S. chungbukensis* DJ77. A BlastP search revealed five significant alignments that were divided into two sequence groups. Group 1, consisting of CV365R and CS008R, was homologous with the N-terminus, while group 2, consisting of CT923R, CW660F and CW375F, aligned with the C-terminus. Sequence alignment between the two groups showed no overlapping regions; however, extended sequencing of the plasmid containing the inserted CV365R sequence confirmed the sequential order and revealed an ORF of 1,413 nucleotides, designated as *pmi*. This ORF has a GC content of 58.17% and encodes 470 amino acids (Fig. 1).

Next, the deduced amino acid sequence of *pmi* from *S. chungbukensis* DJ77 was blasted against the NCBI protein database, revealing significant homology with bifunctional PMI/GMP and PMI. In addition, this deduced amino acid sequence has 63%, 62% and 60% homology with XanB, AlgA

and AceF, respectively (8-10). The deduced amino acid sequence also contains three conserved motifs (11). Two of the motifs close to the C-terminus of sequence were found in the PMI domain, which have the consensus sequence of nine amino acids within motif 1 found at active site of *C. albicans* PMI. Motif 3 possesses a lysine residue (K173) located within the conserved VEKP sequence reported to be essential for mannose-1-phosphate binding of GDP-mannose pyrophosphorylase from *Pseudomonas aureus* (12). Furthermore, a highly conserved pyrophosphorylase signature sequence GXGXR (L)-PK, was found at the N-terminus. These data suggest that *pmi* from *S. chungbukensis* DJ77 may encode a protein with two activities, PMI and GMP.

Protein expression and purification

To express the *pmi* sequence from *S. chungbukensis* DJ77, the pET-PMI plasmid was constructed in-frame using the *pmi* sequence and then introduced into *E. coli* strain BL21 (DE3). SDS-PAGE analysis of the cell crude extract following IPTG induction revealed a prominent protein band of 50 kDa, which corresponded to the calculated molecular mass of the deduced amino acid sequence plus six histidine residues (Fig. 2). The overexpressed protein was primarily found in the insoluble fraction and was purified under denaturing conditions in the presence of sacrosyl. Refolding by extensive dialysis with 50

M	P	V	I	L	C	G	G	S	G	T	R	L	W	P	L	S	R	R	S	60	
ATG	CCC	GTC	ATC	CTC	TGC	GGG	GGG	TCT	GGA	ACT	CGG	CTC	TGG	CCG	CTG	TCC	CGG	CGG	TCA		
F	P	K	Q	F	A	R	L	L	D	D	E	S	L	F	Q	A	T	A	R		
TTT	CCA	AAG	CAG	TTT	GCT	CGA	CTG	CTT	GAC	GAC	GAG	AGC	CTG	TTC	CAG	GCG	ACA	GCC	CGG	120	
R	V	L	G	P	A	F	D	R	P	I	I	V	T	G	E	P	F	R	F		
CGC	GTT	CTC	GGT	CCC	GCT	TTC	GAT	CGG	CCC	ATC	ATT	GTG	ACG	GGC	GAG	CCA	TTC	CGC	TTC	180	
T	I	V	E	Q	L	A	A	L	G	V	K	P	D	G	V	L	I	E	P		
ACG	ATA	GTG	GAG	CAA	CTG	GCG	GCC	CTG	GGC	GTG	AAA	CCT	GAC	GGG	GTG	TTG	ATT	GAA	CCG	240	
E	A	R	N	T	A	P	A	V	L	A	A	A	L	W	L	R	D	R	H		
GAG	GCA	CGT	AAC	ACG	GCG	CCT	GCA	GTT	CTT	GCC	GCC	GCA	CTG	TGG	CTT	CGC	GAT	CGG	CAT	300	
P	E	A	L	M	L	V	M	P	S	D	H	Q	I	A	D	V	A	S	F		
CCT	GAA	GCG	CTG	ATG	CTC	GTG	ATG	CGG	TCC	GAT	CAT	CAG	ATC	GCG	GAC	GTC	GCG	TCA	TTT	360	
H	A	A	I	E	A	A	K	P	R	A	L	A	G	D	I	V	T	F	G		
CAT	GCG	GCC	ATC	GAG	GCG	GCG	AAA	CCG	CGG	GCG	TTG	GCG	GGT	GAT	ATC	GTC	ACA	TTT	GGT	420	
I	V	P	S	H	A	E	T	G	Y	G	Y	L	E	L	K	P	G	E	L		
ATA	GTC	CCT	TCT	CAC	GCT	GAA	ACG	GGA	TAT	GGC	TAT	CTG	GAG	TTG	AAA	CCA	GGG	GAG	CTA	480	
N	V	S	G	P	Q	E	L	R	Q	F	V	E	K	P	D	A	E	R	A		
AAC	GTC	AGC	GGC	CCA	CAG	GAA	CTG	CGG	CAG	TTT	GTA	GAA	ARG	CCA	GAC	GCA	GAA	AGG	GCC	540	
S	L	M	I	E	A	A	V	N	F	L	W	N	A	G	I	F	L	F	S	V	
TCT	TTA	ATG	ATC	GAG	GCT	GGT	AAT	TTC	CTG	TGG	AAT	GCC	GGC	ATC	TTC	TTA	TTC	TCG	GTT	600	
Q	A	I	V	D	A	Y	S	A	H	A	S	A	M	S	A	A	V	A	S		
CAG	GCG	ATC	GTT	GAC	GCT	TAT	TCG	GCG	CAT	GCT	TCC	GCT	ATG	TCA	GCA	GCC	GTC	GCG	TCG	660	
S	L	A	A	A	R	L	D	L	D	F	T	R	L	E	P	E	P	W	R		
TCG	CTG	GCT	GCT	GCG	CGG	CTG	GAT	CTT	GAC	TTC	ACT	CGG	CTG	GAG	CCG	GAA	CCC	TGG	CGT	720	
G	V	E	S	I	S	I	D	Y	A	I	M	E	K	I	S	N	L	A	V		
GGG	GTA	GAA	AGT	ATC	TCG	ATC	GAT	TAT	GCG	ATT	ATG	GAG	AAG	ATC	TCC	AAT	CTC	GCA	GTG	780	
M	P	L	T	A	G	W	S	D	L	G	G	W	A	S	V	W	N	E	G		
ATG	CCG	CTC	ACG	GCT	GGA	TGG	TCT	GAT	CTC	GGT	GGT	TGG	GCA	AGC	GTG	TGG	AAC	GAA	GCC	840	
R	Q	D	A	V	G	N	V	C	S	N	D	V	T	A	I	D	C	S	G		
CGC	CAG	GAC	GCA	GTA	GGA	AAT	GTT	TGT	TCG	AAC	GAT	GTG	ACG	GCG	ATA	GAC	TGT	TCT	GGA	900	
T	L	L	R	T	E	G	E	G	L	H	L	V	G	I	G	L	Q	D	M		
ACG	CTT	CTC	AGG	ACA	GAA	GGG	GAA	GGG	CTA	CAT	CTT	GTC	GGT	ATC	GGC	CTG	CAA	GAC	ATG	960	
I	A	V	A	M	P	D	A	V	L	V	A	P	M	S	E	S	Q	R	V		
ATC	GCG	GTC	GCT	ATG	CCG	GAC	GCC	GTA	CTC	GTT	GCG	CCT	ATG	TCT	GAA	AGC	CAA	CGT	GTC	1020	
G	E	A	V	A	L	L	R	E	N	G	V	K	Q	A	N	M	S	N	R		
GGC	GAG	GCG	GTG	GCT	CTG	TCT	CGG	GAA	AAT	GGG	GTG	AAA	CAG	GCG	AAC	ATG	TCC	AAC	CGC	1080	
D	L	R	P	W	G	W	Y	E	S	I	A	S	G	D	R	F	Q	V	K		
GAT	CTT	CGG	CCA	TGG	GGC	TGG	TAC	GAG	AGT	ATC	GCT	TCG	GGA	GAT	CGC	TTC	CAG	GTC	AAA	1140	
R	I	M	V	R	P	G	A	G	S	L	S	L	Q	S	H	H	R	A	E		
CGA	ATC	ATG	GTG	AGG	CCA	GGG	GCG	TCC	CTT	TCC	CTG	CAA	AGC	CAT	CAC	CAC	CGC	GCC	GAG	1200	
H	W	I	V	V	A	G	T	A	Q	V	T	I	E	D	Q	I	S	L	I		
CAC	TGG	ATT	GTT	GTC	GCC	GGC	ACG	GCT	CAG	GTC	ACT	ATC	GAG	GAC	CAG	ATA	TCC	CTG	ATC	1260	
T	E	N	Q	S	I	Y	V	P	L	G	A	R	H	R	L	E	N	T	G		
ACG	GAG	AAC	CAG	TCG	ATC	TAC	GTT	CCT	TTG	GGA	GCC	AGG	CAT	CGG	CTG	GAA	AAC	ACC	GGG	1320	
K	L	P	V	L	L	I	E	V	Q	T	G	A	Y	L	G	E	D	D	I		
AAG	CTG	CCC	GTG	CTT	CTC	ATC	GAG	GTC	CAA	ACC	GGC	GCT	TAT	CTC	GGA	GAA	GAC	GAT	ATC	1380	
I	R	Y	A	D	V	Y	A	R	N	*											
ATC	CGC	TAC	GCT	GAT	GTA	TAT	GCG	AGG	AAT	TGA										1413	

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

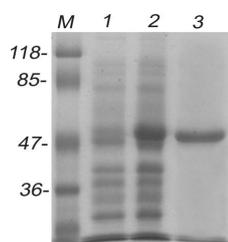


Fig. 2. SDS-PAGE analysis of purified PMI. M, marker (in *kDa*); 1, total cell extract before induction; 2, crude cell extract upon IPTG induction; 3, purified PMI protein.

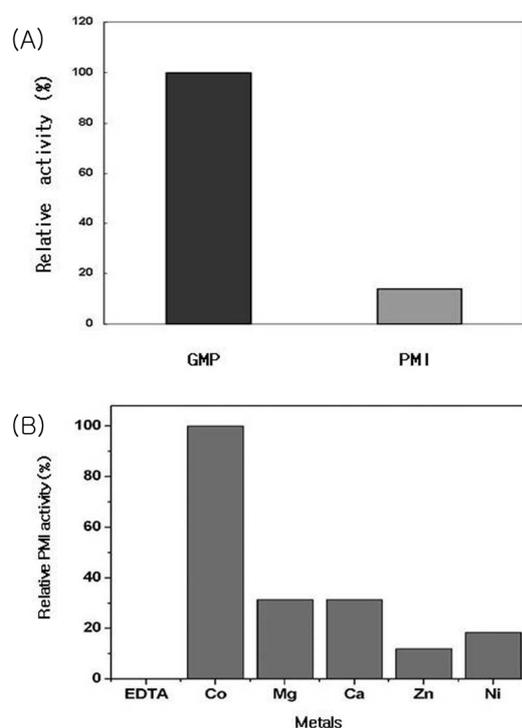


Fig. 3. Enzyme Activity. (A) Relative activity of GMP and PMI. (B) Metal activation of PMI activity. Purified protein was assayed for PMI activity in the presence of various metal cofactors. The assays were performed by adding the chloride form of each metal to the reaction mixture at a final concentration of 1 mM. The data presented are representative of three replicates.

mM Tris buffer (pH 8.0) did not re-precipitate the protein. The purified protein appeared homogeneous upon SDS-PAGE analysis (Fig. 2).

Characterizing enzymatic activity

Purified protein from *S. chungbukensis* DJ77 revealed both PMI and GMP activity, as indicated by the increasing of OD₃₄₀ of enzyme assay mixtures upon the addition of protein (Fig. 3A). The protein required divalent metal ions for catalysis, as in-

dicated by the lack of PMI and GMP activity in the presence of EDTA. Various metal ions (Mg, Ca, Co, Ni, Zn) were found to activate PMI; addition of the Co²⁺ ion resulted in the highest activity and addition of Mg²⁺ only activated the GMP activity (Fig. 3B and data not shown). The pH-dependent profile of this protein in both the PMI and GMP assay showed a bell shape and an optimum pH of 8.0 (data not shown). Inhibition of the PMI activity was tested using several potential inhibitors (GTP, M1P, GDP-mannose and phosphopyrophosphate) at a concentration of 0.5 mM. Only the presence of GDP-mannose caused a decrease in PMI activity (data not shown).

DISCUSSION

The clustering of individual genes encoding for enzymes involved in the same metabolic pathway provide a significant advantage over disparate localization since expression of these genes can be controlled by a single regulatory element. The association of individual enzymes into multienzyme complexes also offers several advantages. For example, a catalytic event taking place on one enzyme can have a direct influence on other enzymes in the same complex, meaning that a single regulatory site can control the activity of all enzymes in the complex. For certain enzyme complexes, the direct transfer of reactants from one active site to another can support efficient metabolism. It seems that the most highly evolved enzyme forms are multifunctional enzymes in which gene fusion has incorporated related catalytic activities into a single polypeptide. Several bifunctional enzymes have been characterized (4, 9, 10).

Phosphomannose isomerase and GDP-mannose pyrophosphorylase catalyze two noncontiguous reactions in the pathway leading to the expression of GDP-mannose, a proposed precursor of EPS produced by *S. chungbukensis* DJ77 (7). The high background of endogenous enzymes in *S. chungbukensis* DJ77 cell extracts prevented us from determining the actual presence of PMI and GMP activity. Therefore, the amino acid sequence of PMI from *X. campestris* was used to search for the *pmi* gene in the *S. chungbukensis* database (13). Search results, and subsequent sequencing results allowed us to identify an ORF of 1,413 nucleotides with very high homology to bifunctional PMI/GMP enzymes in the protein database. In addition, analysis of the deduced amino acid sequence revealed three conserved motifs (Fig. 4) (11). Two motifs close to the C-terminus of the sequence were found in the PMI domain, which contains the consensus sequence of nine amino acids within motif 1 found at the active site of *C. albicans* PMI. Motif 3 was shown to possess a lysine residue (K173) located within the conserved VEKP sequence that is reportedly essential for mannose-1-phosphate binding of GDP-mannose pyrophosphorylase from *Pseudomonas aureus* (12). Furthermore, the N-terminus contains the highly conserved pyrophosphorylase signature sequence, GXGXR (L)-PK, which is believed to comprise at least a portion of the activator-binding site of the large

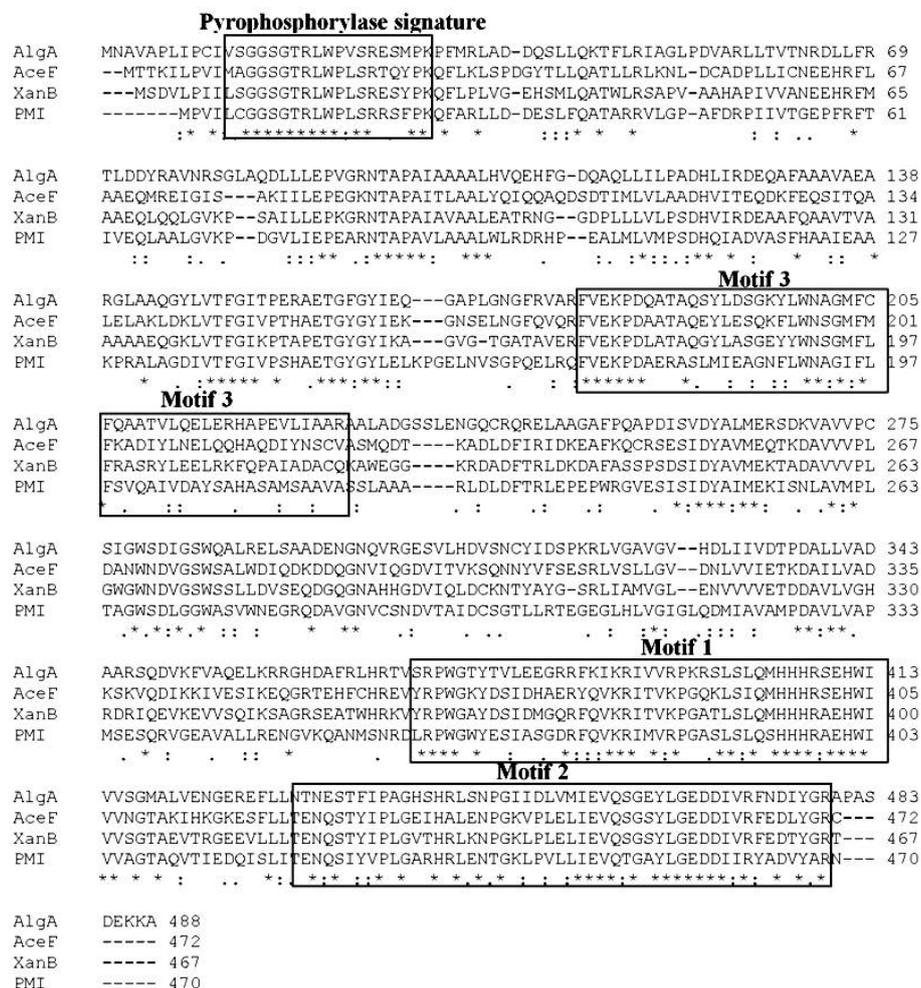


Fig. 4. Amino acid sequence alignment and motif analysis of bifunctional PMI/GMP from *S. chungbukensis* DJ77 (Identical residues are indicated by an asterisk, similar residues are indicated by dot. AlgA, AceF, XanB and PMI protein are known to be bifunctional enzymes from *P. aureus*, *Acetobacter*, *X. campestris* and *S. chungbukensis* DJ77, respectively).

family of bacterial XDP-sugar pyrophosphorylase. These analyses suggest that the pmi sequence from *S. chungbukensis* DJ77 encodes a bifunctional enzyme possessing PMI and GMP activity.

Overexpression of the postulated pmi gene from *S. chungbukensis* DJ77 in a pET-15b expression system resulted in the formation of inclusion bodies and we were unable to improve the solubility of the expressed protein by modifying the culture and induction conditions. Fusing the expressed protein with GST and NusA and co-expressing the protein with chaperon proteins was also unsuccessful. Finally, the 6Xhis fusion system was selected and the recombinant protein was purified under denaturing conditions. Among common denaturing agents (urea, guanidine hydrochloride, sarcosyl), sarcosyl gave the highest purified yield. In addition, protein amounts recovered using sarcosyl were sufficient to generate an active protein using the subsequent refolding process.

The refolded protein exhibited low PMI and GMP activity

(data not shown); however, the presence of both activities confirmed our prediction of the function of the *S. chungbukensis* DJ77 pmi sequence. A possible explanation for the low activity of the obtained protein may be that a large portion of the refolded protein was not active. Another possible cause of the low activity may be that the enzyme favors the forward reaction, while the enzyme assays used in this study were determined in the reverse direction. Similar to other reported bifunctional PMI/GMP proteins, the protein from *S. chungbukensis* DJ77 required divalent metal ions for both activities. The Co^{2+} ion was the most effective activator of PMI activity. The order of metal activators of *S. chungbukensis* DJ77 PMI differed from that of *P. aeruginosa* AlgA (5, 14) and *Helicobacter pylori* PMI/GMP protein (15). GMP activity was only activated by the Mg^{2+} ion, a situation also found in other bifunctional PMI/GMPs (14). The PMI/GMP protein catalyzes a non-sequential reaction in the GMD-mannose pathway; therefore, all intermediates in the pathway may interact with the en-

zyme and act as regulators of enzyme activity. The inhibition of PMI feedback by GDP-mannose was first reported in *H. pylori* (15); however, there was no evidence of PMI inhibition by the intermediate product in alginate producing *P. aeruginosa* (5). In this study, we found that the PMI activity of the protein from DJ77 was also inhibited by GDP-mannose. It is believed that this inhibition contributes to the regulation of GDP-mannose synthesis in *S. chungbukensis* DJ77.

MATERIALS AND METHODS

Materials

The bacterial strain *S. chungbukensis* DJ77 and sequencing plasmids (pCR973 and pCV365) were prepared by Prof. Kim Young Chang (Chungbuk National University). 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. Thrombin protease and epoxy-activated Sepharose 6P were purchased from Amersham. Invitrogen provided the Probond His-Tag resin. All enzymatic assay reagents were obtained from Sigma. All other chemicals used in this study were of the highest grade available from commercial sources.

Gene cloning

To clone the gene encoding PMI from *S. chungbukensis* DJ77, the corresponding gene from *X. campestris* was used to screen the genomic sequence database of *S. chungbukensis* DJ77 (<http://bioinfo.chungbuk.ac.kr>). The obtained homologous sequence fragments were aligned using multiple ClustalW in the Bioedit program (16). The unknown sequences were determined by sequencing extension in an ABI 3700 automatic DNA sequencer (Macrogen). To clone the complete pmi sequences, PCR was conducted in a reaction with a total volume of 50 µl containing 1U Pfu polymerase, 1X reaction buffer, 2.5 mM dNTP, 10 pmol of each primer and genomic DNA as a template. Primer PMI-A: 5'-TACTGTCCCTCTATGCCCGTCAT C-3' and primer PMI-B: 5'-GATCGTTCACTCCGCGCATACC T-3' were used to amplify the gene. The PCR program consisted of 30 cycles of the following: 1 min at 94°C, 45 sec at 58°C and 1 min at 72°C. PCR products were cloned into the pGEM-T vector and sequenced to confirm the correct sequences.

Protein expression and purification

To overexpress the pmi gene, the full open reading frame of the pmi sequence was inserted into the pET-15b vector. Two specific primers, PMI-F: 5'-TTAGAATTCATGCCAGTCATCCT CTG-3' (EcoRI), and PMI-R: 5'-GATCTCGAGTCAAATTTCTCG CCATATAC-3' (XhoI), were used to amplify the insert. The resulting construct, pET-PMI, was then transformed into *E. coli* BL21 (DE3). Next, the transformants were cultured at 37°C until reaching an OD₆₀₀ of 0.8. Next, expression was induced by adding 0.1 mM IPTG and the culture continued at 18°C

overnight. The cells were then harvested by centrifugation and the pellet was suspended in binding buffer containing 50 mM Tris-Cl (pH 8.0), 0.5 mM NaCl and 1.5% sarcosyl. Next, the cell suspension was sonicated on ice, and the cell debris removed by centrifugation at 4°C and 20,000 g for 30 min. The obtained supernatant was then loaded onto a Probon Nickel Chelating Resin column. The column was rinsed with washing buffer (Tris-HCl (pH 8.0), 0.5 mM NaCl, 20 mM imidazole and 1% sarcosyl) and the protein was recovered with elution buffer (Tris-HCl (pH 8.0), 0.5 mM NaCl, 250 mM imidazole and 0.5% sarcosyl). Finally, the recovered protein was extensively dialyzed in 50 mM Tris buffer (pH 8.0) at 4°C overnight.

Protein from the crude cell extracts or the purified samples were analyzed by SDS-PAGE. The molecular mass standard (Biorad) was used to estimate the molecular mass of the target proteins.

Enzyme assays

All assays were based on the reduction of NADP⁺ in coupled reaction systems at 37°C and increases in OD₃₄₀ were measured using a Beckman DU-650 spectrophotometer.

The pH optimum was determined using MES, MOPS, HEPES, TAPS, and CAPS at 50 mM each.

PMI: The enzyme activity was measured following the conversion of M6P to F6P coupled to exogenous PGI and ZWF using the method described by Gill *et al.* (17), with some modifications. A typical assay mixture with a total volume of 1 ml contained 50 mM Tris-Cl (pH 8.0), 5 mM MgCl₂, 1 mM NADP⁺, 1 U of PGI, 1 U of ZWF, enzyme and 1 mM of M6P.

GMP: The enzyme activity was measured according to a previously described method (17, 18). The assay mixture contained 50 mM Tris-Cl (pH 7.5), 10 mM MgCl₂, 0.1 mM ADP, 2 mM pyrophosphate, 0.5 U of each hexokinase, nucleoside 5'-diphosphate kinase, ZWF, 0.8 mM glucose and 1 mM NADP⁺. The reaction was initiated by adding enzyme and 0.1 mM GDP-D-mannose.

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