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

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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 *Sphingomonas 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 Co^{2+}, Ca^{2+}, Mg^{2+}, Ni^{2+} or Zn^{2+}. Of these ions, Co^{2+} 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

Sphingomonas 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 phosphoglucomutase 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

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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 posses 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 auresus* (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

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 M   P   V   I   L   C   G   G   S   G   T   R   L   W   P   L   S   R   R   S
ATG CCC GTC ATC CTC TGC GGG GGG TCT GGA ACT CGG CTC TGG CCG CTG TCC CGG CGG TCA     60
 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 CCG 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 AAG CCA GAC GCA GAA AGG GCC    540
 S   L   M   I   E   A   G   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 GGC    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 CTT CGG GAA AAT GGG GTC 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   S   L   S   L   Q   S   H   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
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Fig. 1. The complete nucleotide sequence and deduced amino acid sequence of PMI from *S. chungbukensis* DJ77.

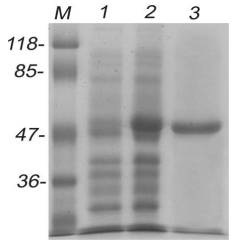

Fig. 2. SDS-PAGE analysis of purified PMI. M, marker (in *kDa*); 1, total cell extract before induction; 2, crude cell extract upon IPTG induction; 3, purified PMI protein.

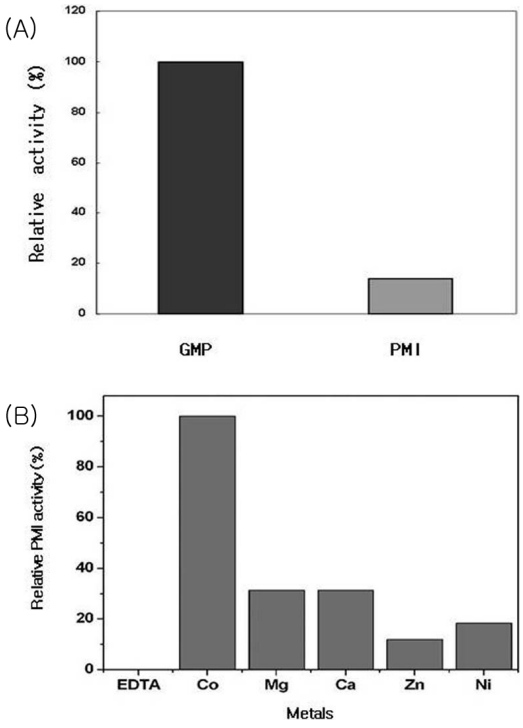

Fig. 3. Enzyme Activity. (A) Relative activity of GMP and PMI. (B) Metal activation of PMI activity. Purified protein was assayed for PMI activity in the presence of various metal cofactors. The assays were performed by adding the chloride form of each metal to the reaction mixture at a final concentration 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 OD_{340} of enzyme assay mixtures upon the addition of protein (Fig. 3A). The protein required divalent metal ions for catalysis, as indicated 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 Co^{2+} ion resulted in the highst activity and addition of Mg^{2+} 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).

Phosphomanose 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

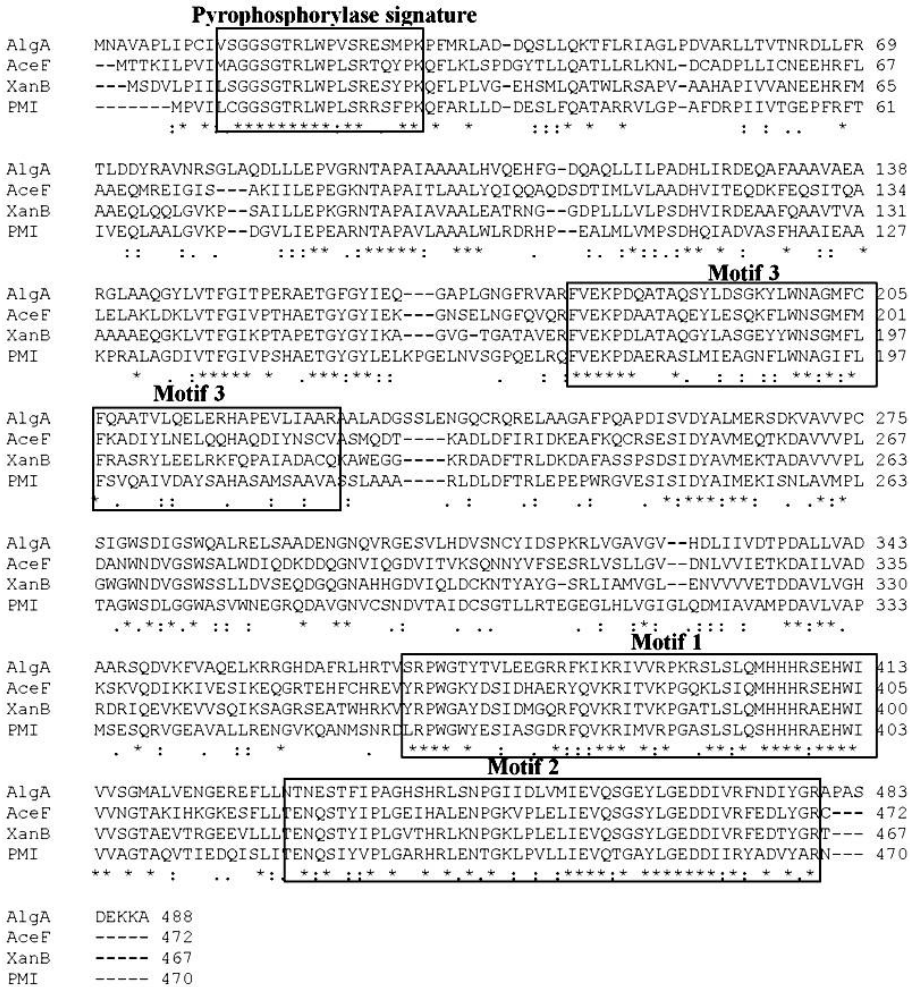

Fig. 4. Amino acid sequence alignment and motif analysis of bifunctional PMI/GMP from *S. chungbukensis* DJ77 (Identical residues are indicated by an asterisk, similar residues are indicated by dot. AlgA, AceF, XanB and PMI protein are known to be bifunctional enzymes from *P. aureus, Acetobacter, X. 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 s primer and genomic DNA as a template. Primer PMI-A: 5'-TACTGTCCCTCTATGCCCGTCAT C-3' and primer PMI-B: 5'-GATCGTTCACTTCCGCGCATACC 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 OD_{600} 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 OD_{340} 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 $MgCl_2$, 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 $MgCl_2$, 0.1 mM ADP, 2 mM pyrophosphophate, 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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