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Gene Cloning, Expression, and Characterization of Glucose-1-Phosphatase from *Enterobacter cloacae* B11

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A bacterial strain with phytase and glucose-1-phosphatase activity was isolated from seawater. The colony was identified as an *Enterobacter cloacae* strain and named *E. cloacae* B11. A gene, *agpEnB11*, coding for an intracellular acid glucose phosphatase was cloned from the strain and sequenced. It comprised 1,242 nucleotides and encoded a polypeptide of 413 amino acids. Recombinant glucose-1-phosphatase (AgpEn) was overexpressed in *Escherichia coli* and purified using Ni-NTA column under native conditions. Purified protein displayed a single band of 47 kDa on SDS-PAGE. AgpEn hydrolyzed a wide variety of phosphorylated compounds, with high activity for glucose-1-phosphate and glucose-6-phosphate. Optimum pH and temperature for enzyme activity were pH 5.0 and 50°C, respectively. Enzyme activity was stimulated by Ca^{2+} and Co^{2+}, and inhibited by Cu^{2+}.

Key words: *Enterobacter cloacae*, Cloning, Expression, Glucose-1-phosphatase, Substrate specificity

Introduction

Organic and inorganic phosphates are essential components of living organisms. They occur as orthophosphates, pyrophosphates, polyphosphates, nucleotides, sugar phosphates, and phosphorylated derivatives of other organic compounds (e.g. phytic acid). Phosphate (Pi) assimilation, storage, and mobilization are processes of particular importance owing to the vital role of Pi in energy transfer and metabolic regulation. Acid phosphatase (EC 3.1.3.2) is a key enzyme in the regulation of Pi metabolism. Acid phosphatase enzyme family is ubiquitous in nature, and organisms usually express several isozymes of acid phosphatase that differ in subcellular location and kinetic and molecular properties. Acid phosphatases often display broad substrate specificity, in contrast to their alkaline counterparts, which are more specific with respect to hydrolysable phosphorylated substrates (Duff et al., 1994). They may be extracellular or intracellular. Most extracellular acid phosphatases are relatively nonspecific enzymes involved in the acquisition of Pi from the environment (Duff et al., 1991; Duff et al., 1994; Joh et al., 1996). Intracellular acid phosphatases are undoubtedly important in the production, transport, and recycling of Pi. Phytases (*myo*-inositol hexakisphosphate phosphohydrolase, EC 3.1.3.8) are acid phosphatase enzymes that efficiently cleave phosphate moieties from phytate, thereby generating *myo*-inositol phosphates and inorganic phosphate (Mullaney and Ullah, 2003). Phytases are used as animal feed additives to improve phosphate bioavailability and to reduce the loss of phosphate and divalent cations from phytate, which is the main form of phosphate in foods and feeds of plant origin. Acid glucose-1-phosphatase (Agp, EC 3.1.3.10) cleaves primarily small monosaccharide phosphates such as glucose-1-phosphate, glucose-6-phosphate, and fructose-6-phophate, but also exhibits phytase activity (Cottrill et al., 2002). Its molecular properties have been reported from *Escherichia coli* (Pradel et al., 1990), *Enterobacter cloacae* (Herter et al., 2006), and *Citrobacter braakii* (Kim et al., 2009).

Recently, we isolated *Enterobacter cloacae* B11 from seawater, based on its relatively high phytase activity. Here, we report the cloning and sequencing of the gene *agpEnB11*, which encodes an enzyme that

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hydrolyses glucose-1-phosphate and phytate, from *E. cloacae* B11. The gene was heterologously expressed in *E. coli*, and the recombinant enzyme was purified and characterized.

Materials and Methods

Isolation and identification of phytase-producing bacteria

Seawater collected near Busan was spread onto phytase-screening plates containing 1.5% D-glucose, 0.5% calcium phytase, 0.5% KCl, 0.001% $FeSO_4 \cdot 7H_2O$, 0.01% $MnSO_4 \cdot H_2O$, and 1.5% agar adjusted to pH 7.0. The plates were incubated at 30°C for 24 h. Colonies surrounded by clear zones were selected and grown in a phytase-screening medium or marine broth 2216 (Difco, USA) at 30°C for 24 h. The strain with high phytase activity was selected. The culture supernatant and cell extract were collected by centrifugation and assayed at 37°C for phytase activity. Morphological and physiological characteristics as well as 16S rDNA sequences of the isolated colony were determined by standard procedures (Yoon et al., 1998).

DNA amplification and sequencing

The colony that produced the phytase activity was identified as an *Enterobacter* strain, and thus we used the primers Enf1 (5′-ATGAAAACGCTATTTCTTC ATTTAT-3′) and Enr1 (5′-TTATCGGTTTATATCAG CCATG-3′), derived from the DNA sequence of *Enterobacter* sp. 638 glucose-1-phosphatase/inositol phosphatase (GenBank accession no. YP_001176245). The PCR conditions were as follows: 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final elongation step of 72°C for 10 min. The PCR product was purified, ligated into pGEM-T easy vector (Promega, Madison, WI), and sequenced.

Construction of the expression plasmid

For construction of the *E. coli* expression vector, the glucose-1-phosphtase gene containing the signal peptide was amplified by PCR using a sense *Nco*I-linker primer (5′-GGGCCATGGTTATGAGAAAAG CACTAC-3′) and an antisense *Xho*I-linker primer (5′-GGGCTCGAGTTTTGCCGCGTCGTTCAT-3′). The amplified product was cloned into the *Nco*I and *Xho*I sites of expression vector pET22b (Novagen, Madison, WI). The constructed plasmid was designated pEAGP.

Purification of the recombinant protein

Escherichia coli BL21 (DE3) transformed with pEAGP was cultivated in LB medium containing ampicillin (100 μg/mL) at 37°C. When the optical density at 600 nm reached 0.6, isopropylthiogalactoside (IPTG; 1 mM) was added, and the cultures were further incubated at 30°C for 4 h. The *E. coli* cells were then harvested and ruptured by ultrasonic cell lysis. The soluble proteins were recovered from the cell extract by centrifugation (10,000×*g* for 20 min) and loaded onto a nickel-nitrilotriacetic (Ni-NTA) column. After washing with 60 mM imidazole, 500 mM NaCl, and 50 mM Tris-HCl buffer (pH 7.9), the bound glucose-1-phosphatase was eluted using 1000 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl buffer (pH 7.9) and dialyzed against 50 mM Tris-HCl buffer (pH 8.0), for characterization of its biochemical properties.

Analytical methods

Phytase and glucose-1-phosphatase activities were assayed as previously described (Kim et al., 2003). The concentration of released inorganic phosphate was measured by reference to a K_2HPO_4 standard. Acid phosphatase activity was determined by the liberation of *p*-nitrophenol from *p*-nitrophenyl phosphate in accordance with published methods (Bolton and Dean, 1972; Owen et al., 1992). One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmole of phosphate or nitrophenol per min at 37°C. Protein concentration was determined by the BCA method using a protein assay kit (Sigma).

Determination of pH and temperature optima

pH optimum for hydrolysis of gucose-1-phosphate was determined using standard assay conditions with the following buffers (0.1 M): glycine/HCl (pH 2-3.5), sodium acetate/acetic acid (pH 3.5-6), Tris/acetate (pH 6-7), Tris/HCl (pH 7-9), and glycine/ NaOH (pH 9-10). For pH stability, the enzyme was preincubated at 37°C in the various pH buffers for 1 h, and the remaining activity was measured by standard assay. Temperature optimum was determined using the standard assay at temperatures of 30-70°C. For temperature stability, the enzyme was preincubated at various temperatures for 30 min, and the remaining activity was measured by standard assay.

Substrate specificity and metal effect

Substrate specificities were determined for different phosphate-containing compounds, including *p*-nitrophenol phosphate (*p*NPP), ADP, ATP, glycerol-

phosphate, glucose-1-phosphate, glucose-6-phosphate, fructose-1-phosphate, fructose-6-phosphate, mannose-6-phosphate, and phytic acid, at a concentration of 2 mM. The effects of metal ions and EDTA were determined by measuring glucose-1-phosphate activity in the presence of 5 mM Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}, K^{+}, or EDTA. The test system without addition of metals or EDTA was used as the control.

Results and Discussion

Isolation and identification of phytase-producing bacteria

Using a selective medium, phytase-producing bacterial strains were isolated from seawater samples collected near Busan. From among them, the high phytase-producing (48 U/mL) strain B11 was selected. This strain was a Gram negative bacterium with high catalase activity and could grow under anaerobic conditions. Table 1 shows the results of biochemical tests for this strain, performed as described in Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). On the basis of the 16S rDNA sequence (data not shown) and biochemical characteristics, strain B11 was identified as an *Enterobacter cloacae* and named as *E. cloacae* B11.

Table 1. Biochemical characteristics of strain B11

Characteristics	Result
Gram staining	-
Enzymatic activity of	
β-galactosidase	+
arginine dihydrolase	+
lysine decarboxylase	-
ornithin decarboxylase	+
urease	-
tryptophan deaminase	-
cytochrome oxidase	-
gelatinase	-
Citrate utilization	+
H_2S production	-
Indole production	-
Acetone production (V-P test)	+
Acid form	
glucose	+
mannitol	+
inositol	+
sorbitol	+
rhamnose	+
sucrose	+
melibiose	+
amygdalin	+
arabinose	+

+, positive reaction; -, negative reaction.

Cloning and sequencing of the acid glucose-1-phosphatase

Previously, acid glucose-1-phosphatase-related phytase has been reported in *E. cloacae* (Sajidan, 2002). To clone the gene encoding an enzyme that hydrolyzes glucose-1-phosphate and phytate from *E. cloacae* B11, PCR was performed with designed primers and using genomic DNA of the isolated *E. cloacae* B11 as the template. A 1,242-bp amplified fragment was cloned into pGEM-T easy vector and sequenced. This fragment, designated as *agpEnB11* (GenBank accession no. GU109476), encoded a protein of 413 amino acids (Fig. 1). The deduced polypeptide had a molecular weight of 45.94 kDa and a theoretical isoelectric point of 5.42. It contained a variation of the active site motif, RH*N*XRXP instead of RH*G*XRXP, which is characteristic of histidine acid phosphatase (Van Etten et al., 1991; Mitchell et al., 1977). It also contained a C-terminal HD motif at residues 311-312. To date, only one *E. cloacae* glucose-1 phosphatase gene, *agpEn*, has been reported, by Herter et al. (2006). The difference between *agpEnB11* and *agpEn* is an exchange of several amino acid acids: I96V, P103A, E105D, T106S, S178T, A294T, D298E, H333Q, E378Q, E389K, and D410N. The amino acid sequence alignment with available bacterial acid glucose phosphatases was performed to generate a phylogenetic tree. Deduced amino acid sequence of *agpEnB11* displayed 97% identity with that of *E. cloaca,* and 90-79% identity with other glucose-1-phosphatases from *Enterobacter* sp., *Citrobacter* sp., *E. coli*, *Shigella* sp., *Klebsiella* sp., and *Salmonella* sp. Deduced amino acid sequence of *agpEnB11* showed a close relationship to the enterobacteria *E. coli*, *Shigella*, *Salmonella, Enterobacter, and Klebsiella* acid glucose phosphatases. It could be distinguished from the HAPs/phytase group by sequence similarity. Fig. 2 presents the phylogenetic tree, showing the relationships among bacterial glucose-1-phosphatases and HAPs based on amino acid sequences.

Expression of acid glucose phosphatase

Constructed plasmid pEAGP was transformed into *E. coli* BL21 (DE3), and recombinant protein expression was induced with 1 mM IPTG for 4 h at 30°C. Cells were collected by centrifugation and disrupted by sonication for protein purification. The recombinant protein represented 20% of the total protein expressed in *E. coli* and provided a good yield of soluble and active protein. Recombinant glucose-1-phosphatase (AgpEn) was purified by Ni-NTA column chromatography under native conditions.

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1 ATGAGAAAAGCACTACTGGCACTGGCAGTCGCCGGTTCCATTTCAGCAACGTTTGGCGTGCAGGCACAAGAGACGCCGGAAGGGTATCAG
  M R K A L L A L A V A G S I S A T F G V Q A Q E T P E G Y Q                                  30
91 CTGGAGCAAGTTTTAATCATGAGTCGCCATAACCTGCGCGCACCGCTCGCCAACAACGGCAGCGTGCTGGAGCAGTCCACGCCGAAACAG
  L E Q V L I M S R H N L R A P L A N N G S V L E Q S T P K Q                                  60
181 TGGCCAGAGTGGGAGGTACCGGGTGGTCAGCTCACCACCAAAGGCGGCGTGCTGGAAGTCTATATGGGTCATTACATGCGCGAGTGGCTG
  W P E W E V P G G Q L T T K G G V L E V Y M G H Y M R E W L                                  90
271 GCGCAGCAGGGGATGATAAAGACTGGAGAGTGCCCGCCAGCGGAGACCGTTTATGCATATGCCAATAGCCTGCAGCGTACCGTTGCGACT
  A Q Q G M I K T G E C P P A E T V Y A Y A N S L Q R T V A T                                 120
361 GCGCAATTCTTCATTACCGGCGCGTTCCCGGGGTGCGATGTGCCTGTGCATCATCAGGAAAAAATGGGCACGATGGATCCCACCTTTAAT
  A Q F F I T G A F P G C D V P V H H Q E K M G T M D P T F N                                 150
451 CCGGTCATTACCGATAACTCGCCTGAGTTCCGCGAAAAAGCGCTGAAGGCGATGGAGACCGAGCGGCAGAAAATGCAGCTTTCAGAAAGC
  P V I T D N S P E F R E K A L K A M E T E R Q K M Q L S E S                                 180
541 TATAAGCTGCTGGAGCAGATGACGAACTACGCCGATTCGCCGTCCTGCAAAGAGAAAAAAGTCTGCTCGCTGGCGGACGCGAAAGATACG
  Y K L L E Q M T N Y A D S P S C K E K K V C S L A D A K D T                                 210
631 TTCAGTGCCGACTATGAAAAAGAGCCAGGCGTGTCCGGTCCGCTGAAAGTGGGTAACTCGCTGGTGGATGCGTTCACGCTGCAATATTAC
  F S A D Y E K E P G V S G P L K V G N S L V D A F T L Q Y Y                                 240
721 GAAGGTTTCCCGGCTGATCAGGTGGCCTGGGGTGAGATCAAGACTGACCAGCAGTGGCGTGTGCTGTCGAAGCTGAAAAACGGCTATCAG
  E G F P A D Q V A W G E I K T D Q Q W R V L S K L K N G Y Q                                 270
811 GACTCGCTGTTTACCTCCACCGAGGTGGCGCAAAACGTCGCCAAACCGCTGGTGAAATATATTGATAAAGCGCTGGTCACCGATCAGGCG
  D S L F T S T E V A Q N V A K P L V K Y I D K A L V T D Q A                                 300
901 AAAGCGCCTAAAATCACCCTGCTGGTGGGGCATGATTCGAACATTGCTTCGCTGCTGACCGCGCTGGATTTCAAACCGTATCAACTCCAC
  K A P K I T L L V G H D S N I A S L L T A L D F K P Y Q L H                                 330
991 GATCAGCACGAGCGCACGCCAATTGGCGGCAAAATAGTCTTCCAGCGCTGGCATGACAAAAACGCTAACCAGGAACTGATGAAAATTGAG
  D Q H E R T P I G G K I V F Q R W H D K N A N Q E L M K I E                                 360
1081 TATGTCTACCAGAGCTCAGAGCAGCTGCGAAATGCCAGCGTGCTGTCGCTGGAATCCCCCGCGCAGCGTGTGACGCTGGAGCTGGAAGGC
  Y V Y Q S S E Q L R N A S V L S L E S P A Q R V T L E L E G                                 390
1171 TGCCCGGTGGATGCTAACGGCTTCTGCCCGGTTGATAAGTTTAATGCGGTGATGAACGACGCGGCAAAATAA
  C P V D A N G F C P V D K F N A V M N D A A K *                                             413
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Fig. 1. The nucleotide and deduced amino-acid sequences of the *agpEnB11* gene. Residue numbers for nucleotide and amino acid are indicated in the left and right of each row, respectively. Putative signal sequence is underlined. The conserved regions related to the enzymatic activity sites are boxed.

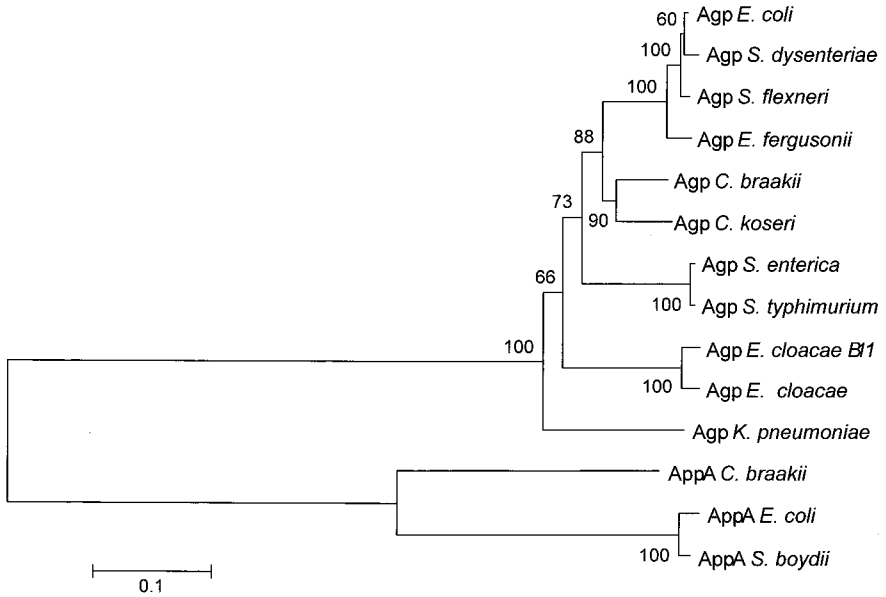

Fig. 2. Phylogenetic analysis of the *agpEnB11* and other bacterial acid phosphatases. Bootstrap values (%) from analysis of 1,000 bootstrap replicates are given at the respective nodes. The phylogenetic tree was constructed using the neighbor-joining method in MEGA 3.1. GenBank accession numbers of proteins are as follows: Agp *Enterobacter cloacae* B11, GU109476; Agp *Citrobacter braakii,* FJ716800; Agp *Escherichia coli*, NP_415522; Agp *Escherichia fergusonii*, NC_011740.1; Agp *Shigella dysenteriae*, N_P007606.1; Agp *Shigella flexneri*, NC_004741.1; Agp *Citrobacter koseri*, YP_001453610; Agp *Salmonella enterica*, ZP_02658556; Agp *Salmonella typhimurium*, NC_003197.1; Agp *Enterobacter cloacae*, AJ783768.1; Agp *Klebsiella pneumoniae*, NC_011283.1; AppA *Citrobacter braakii,* AAS45884; AppA *Escherichia coli,* YP_001744188; AppA *Shigella boydii,* YP_001880822.

Purified protein gave a single band of 47 kDa on SDS-PAGE, in accordance with its predicted molecular mass. Western blots revealed a specific band corresponding to the 6× His-tagged fusion protein of AgpEn (Fig. 3). Specific activity of the recombinant AgpEn was 128 U/mg at pH 5.0 and 37°C.

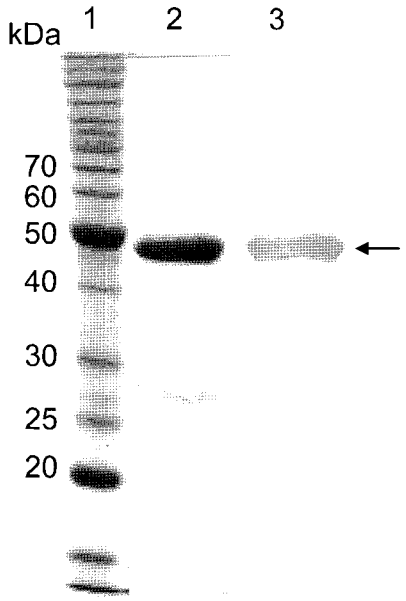

Fig. 3. SDS-PAGE and western blot analysis of the AgpEn. Lane 1, molecular weight markers; lane 2, AgpEn protein purified after performing Ni-NTA spin column; lane 3, western blot of the purified AgpEn protein after performing Ni-NTA spin column. Arrow indicates the acid glucose phosphatase.

Effects of pH and temperature

Effects of pH and temperature on the activity of AgpEn were examined with glucose-1-phosphate as a substrate. The purified enzyme exhibited optimal activity at approximately pH 5.0. The enzyme was stable at pH 3.5 to 10.0, but rapidly lost activity above pH 8.0 (Fig. 4A). The effect of temperature on AgpEn activity was examined at temperatures ranging from 20°C to 60°C. The optimal temperature for enzyme activity was 50°C. To test thermostability, AgpEn activity was measured after incubation at various temperatures for 30 min. The enzyme was stable at up to 50°C, but was rapidly inactivated at 55°C (Fig. 4B).

Substrate specificity

Substrate specificity of AgpEn is shown in Fig. 5. The enzyme showed high activity toward glucose-1-phosphate and glucose-6-phosphate, and was also active toward other monosaccharide sugar phosphates such as glucose-6-phosphate, mannose-6-phosphate, fructose-1-phosphate, and fructose-6-phosphate. Activity toward phytate and the general phosphatase substrate *p*-nitrophenyl phosphate was also detected. Compared with the preferred substrate glucose-1-phosphate, the phytase activity of AgpEn was 16%, which was 6.6-fold that of acid glucose phosphatase from *Citrobacter braakii* (Kim et al., 2009).

Effects of metal ions and chemical reagent

Effects of various compounds on the activity of the purified acid glucose phosphatase were examined (Table 2). Both Ca^{2+} and Co^{2+} stimulated the activity,

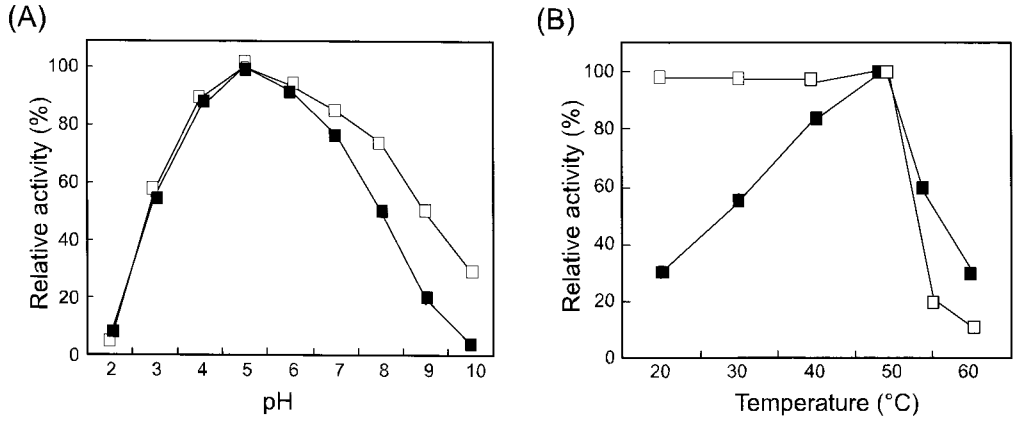

Fig. 4. Effect of pH (A) and temperature (B) on the AgpEn activity and stability. (A) Enzyme assay was performed at 37°C in various pH buffers. For the pH stability, the enzyme was preincubated at 37°C in various pH buffers for 1 hour and the remaining activity was measured as in standard assay. ■, optimal pH; □, pH stability. (B) Enzyme assay was performed at various temperatures in 0.1 M sodium acetate buffer (pH 5.0). For the temperature stability, the enzyme was preincubated at various temperatures for 30min and the remaining activity was measured as in standard assay. ■, optimal temperature; □, temperature stability.

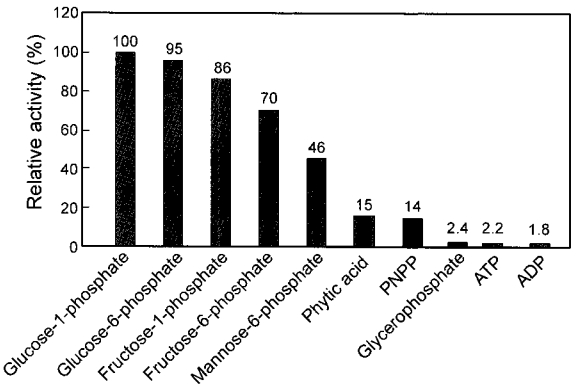

Fig. 5. Substrate specificity of the purified AgpEn. The enzyme activity assay with various phosphorylated compounds was done as standard assay described above using 2 mM of each substrate. Hydrolysis rate of glucose-1-phosphate was taken as 100%.

Table 2. Effect of various compounds on the activity of the purified AgpEn

Compounds (5 mM)	Relative activity (%)
None	100.0
$CaCl_2$	130.9
$CoCl_2$	125.2
$CdCl_2$	107.9
$FeSO_4$	103.6
KCl	103.2
$MnSO_4$	101.2
$NiSO_4$	99.1
$ZnCl_2$	98.8
$MgCl_2$	92.0
$CuSO_4$	75.1
EDTA	112.2

whereas Cu^{2+} inhibited it. EDTA, a chelating reagent, had no effect on the activity, suggesting that AgpEn is not a metalloprotein and that a sulfhydryl group does not participate in the catalytic reaction or active conformation of the enzyme. These properties appear to be typical of plant and microbial acid phosphatases (Hass et al., 1991; Ullah and Cummins, 1987, 1988; Yoshida et al., 1989).

In this study, we cloned, expressed, and characterized an acid glucose phosphatase from *E. cloacae* B11 that has high specificity toward glucose-1-phosphate and glucose-6-phosphate. Studies on the localization and regulation of expression by glucose and phosphate are needed to obtain more detailed information about the enzyme.

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