Cloning, Expression, and Characterization of UDP-glucose Pyrophosphorylase from *Sphingomonas chungbukensis* DJ77

Moon-Young Yoon, Kyoung-jin Lee, Hea-Chul Park, Sung-Ha Park,† Sang Gon Kim,‡ Sung-Kun Kim,‡ and Jung-Do Choi†,*

Department of Chemistry, Research Institute for Natural Sciences, Hanyang University, Seoul 133-791, Korea
†Department of Biochemistry, Chungbuk National University, Cheogju 361-763, Korea
**E-mail: jdchoi@cbucc.chungbuk.ac.kr*
‡Department of Chemistry and Biochemistry and Institute of Biomedical Studies, Baylor University, Waco, Texas 76798-7348, USA
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The bacterium *Sphingomonas chungbukensis* DJ77 produces the extracellular polysaccharide gellan in high yield. Gellan produced by this bacterium is widely used as a gelling agent, and the enzyme UDP-glucose pyrophosphorylase (UGP) is thought to play a key role in the gellan biosynthetic pathway. The UGP gene has been successfully cloned and over-expressed in *E. coli*. The expressed enzyme was purified with a molecular weight of approximately 32 kDa, as determined by a SDS-polyacrylamide gel, but the enzyme appears as *ca.* 63 kDa on a native gel, suggesting that the enzyme is present in a homodimer. Kinetic analysis of UDP-glucose for UGP indicates K_m = 1.14 mM and V_{max} = 10.09 mM/min/mg at pH 8.0, which was determined to be the optimal pH for UGP catalytic activity. Amino acid sequence alignment against other bacteria suggests that the UGP contains two conserved domains: An activator binding site and a glucose-1-phosphate binding site. Site-directed mutagenesis of Lys194, located within the glucose-1-phosphate binding site, indicates that substitution of the charge-reversible residue Asp for Lys194 dramatically impairs the UGP activity, supporting the hypothesis that Lys194 plays a critical role in the catalysis.

Key Words: UDP-glucose pyrophosphorylase, *Sphingomonas chungbukensis*, Extracellular polysaccharide, Site-directed mutagenesis

Introduction

The bacterium *Sphingomonas chungbukensis* DJ77 (previously identified as a *Pseudomonas* species)[1] has been isolated from contaminated freshwater sediment in Daejeon, Korea.[2] *S. chungbukensis* DJ77 has been shown to produce large quantities of extracellular polysaccharide (EPS) gellan, which can be used as a gelling agent for food and pharmaceutical use.[3] The EPS gellan is a linear tetrasaccharide composed of repeating units of β-D-glucuronic acid, β-D-glucose, and α-L-rhamnose[4] in a 2:1:1 ratio. The EPS biosynthetic pathway has been partially elucidated;[5,6] however, further investigation is needed to completely delineate the pathway. Thus cloning, expressing and characterizing the genes involved in EPS gellan biosynthesis is critical for metabolic engineering of the pathway.

Uridine diphosphate (UDP) – glucose pyrophosphorylase, also termed glucose-1-phosphate uridylyltransferase (UGP, EC 2.7.7.9) is a cytosolic enzyme that catalyzes the reversible formation of UDP-glucose and inorganic pyrophosphate from UTP and glucose-1-phosphate. In addition, this enzyme is thought to catalyze the reversible conversion of galactose to glucose by way of the Leloir pathway.[7] The *ugp* gene deficiency experiments in *E. coli* support the hypothesis that the enzyme also converts galactose to glucose.[8] UGP activity has been found in both prokaryotes and eukaryotes, although the prokaryotic and eukaryotic enzymes differ significantly. In prokaryotes, UGP is essential for the biosynthesis of several polysaccharides such as EPS, lipopolysaccharides, and capsular polysaccharides.[9,10] The enzyme product, UDP-glucose, has been known to be a precursor for the synthesis of the tetrasaccharidic unit that is involved in EPS biosynthesis[11] and so it is of keen interest to investigate the cloning and characterization of the *ugp* gene in this bacterium.

In this work, we report the cloning and expression of the *ugp* gene and purification of UGP from *S. chungbukensis* DJ77. Amino acid alignment, kinetic analysis and site-directed mutagenesis experiments were carried out to characterize the enzyme.

Experimental Section

Materials. *S. chungubkensis* DJ77 and its fosmid library were prepared in the laboratory of Dr. Young-Chang Kim.[2] Fosmid library construction was carried out according to methods described previously,[12,13] using CopyControl™ Fosmid Library Production Kit (Epicentre, Madison, USA). The restriction enzymes and T4 ligase were purchased from Roche (Mannheim, Germany), while *Taq* polymerase and other components for PCR were purchased from Bioneer Inc.

Abbreviations: EPS, extracellular polysaccharide; $NADP^+$, nicotinamide adenine dinucleotide phosphate; UGP, UDP-glucose pyrophosphotase; IPTG, isopropyl thio-beta-D-galactoside; PCR, polymerase chain reaction; pI, isoelectric point; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis

(Daejeon, Korea). PCR primers were synthesized by Genotech (Daejeon, Korea).

Gene selection. Based on the *S. chungubkensis* DJ77 genomic database (http://bioinfo.chungbuk.ac.kr), we found a cu292 fosmid clone that contains the *ugp* gene. Using the fosmid clone, we determined the whole gene sequence with the primer walking method. The reverse primers used were: 5'-GGTCACGAAGATCAGATGCT-3', 5'-GAGATCCAGCTGACCGATG-3', 5'-GTCTGAAACGGGGTAAGCCA-3', and 5'-TGTCACTGCGCATCACGTTG-3'.

Construction of expression vectors. PCR amplification was done using the following forward primers: (5'-AAGGATCCCATGTCCTACAAACCGATTC-3') and (5'-TTGGATCCTCAGGCCGCGGCGGCAAT-3'), where the underlined sequences indicate the *Bam*HI restriction site. Genomic DNA was used as a template for PCR. The *Bam*H I-digested PCR products were inserted into the pET-15b expression vector using T4 DNA ligase. Finally, *E. coli* BL21(DE3)pLysS was transformed with the cloned plasmid. The sequence of the insert was confirmed by DNA sequencing in both directions.

Expression and purification of recombinant protein. The transformed *E. coli* strains BL21(DE3)pLysS were grown at 37 °C in LB medium containing 50 mg/L ampicillin, until the optical density of the culture at 600 nm reached 0.6. The inducer IPTG was added to the culture at a final concentration of 0.1 mM, followed by growth for 5 h at 30 °C. Cells were harvested by centrifugation at 5,000 g for 20 min. The cell pellet was resuspended in buffer (50 mM Tris, pH 8.0, 500 mM NaCl). The cell suspension was lysed by sonication on ice, and the soluble fraction was obtained by centrifugation at 20,000 g for 30 min. The supernatant was applied to a GE HP high trap nickel affinity column. The protein purification procedure was conducted as described by the manufacturer's protocol provided from Qiagen, and the purified protein was analyzed on SDS-PAGE. Native-gel electrophoresis was done, with methods described previously.[14] Protein concentration was estimated by the Bradford method.[15] using a bovine serum albumin standard.

Enzyme assay. The activity of UGP was assayed by previously described methods.[16,17] The UGP-catalyzed glucose-1-phosphate was converted to glucose-6-phosphate by the enzyme phosphoglucomutase (purchased from Roche Co.). The glucose-6-phosphate was then used as substrate for glucose-6-phosphate dehydrogenase (purchased from Roche Co.) with $NADP^+$ used as a cofactor. The UGP activity was monitored via the increase of NADPH concentration by the reduction of $NADP^+$, and the absorbance at 340 nm was detected using a Beckman DU-650 spectrophotometer at 30 °C. The standard reaction mixture for UGP activity assay contained 100 mM Tris-HCl (pH 8.5), 2 mM pyrophosphate, 10 mM $MgCl_2$, 10 mM $NADP^+$, 1 unit of phosphoglucomutase, and 1 unit of glucose-6-phosphate dehydrogenase, along with 0.05 - 1.2 mM UDP-glucose.[14,18-20] To study the effect of changing pH, 100 mM MES (pH 5.5 - 6.5), 100 mM MOPS (pH 6.5 - 8.0) and 100 mM TAPS (pH 8.0 - 9.5) were used.

Site-directed mutagenesis. The PCR mega-primer method was used to produce site-specific mutations in UGP.[21] The pET15b-ugp plasmid was used for the site-directed mutagenesis with the mutagenic primers (K194D: 5'- GTG GAA GAT CCC GCT CCA-3'; K194R: 5'-GTG GAA AGG CCC GCT -3'), where the underlined text indicates the bases where the mutation was introduced.

Results and Discussion

The fosmid library of *S. chungbukensis* DJ77 was used to find the *ugp* gene, which was predicted to be in the cu292 fosmid clone. PCR was used to amplify the full gene from the genomic DNA, and the PCR products were cloned into the expression vector pET15b (pET-15b-ugp). The inserted gene sequence was confirmed by DNA sequencing. Overexpression of the gene using an *E. coli* system was successful, and the 6x His-tagged enzyme was purified to homogeneity. The enzyme displayed one band on SDS-PAGE with Coomassie Blue staining, and the molecular weight was estimated to be approximately 32 kDa, which is consistent with a calculated value of 32.1 kDa (Figure 1).

Amino acid sequence homology analysis, using translation data based on the *ugp* gene sequence of *S. chungbukensis* DJ77, shows a high degree of amino acid sequence homology with the *ugp* gene of other organisms, particularly *S. paucimobilis*. This homology is likely due to both organisms belonging to the same genus. The UGP from the bacteria *Zymononas* and *Novosphingobium* also show high amino acid sequence similarity with that of *Sphingomonas*. UGPs from other bacteria (*e.g.*, *Gluconacetobacter*, *Rhodopseudomona*, *Brucella*, and *Bradyrhizobium*) show fairly high similarity (58 - 60% identity) in the amino acid sequence as well. Table 1 summarizes the amino acid sequence homology analysis.

A multiple amino acid sequence alignment of UGP enzymes across several bacteria including *S. chungbukensis* DJ77 was carried out to determine conserved regions that may play an

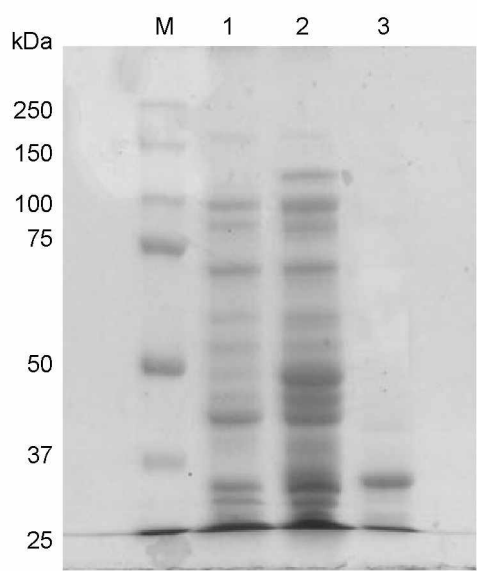

Figure 1. SDS-PAGE analysis of UGP. M, molecular markers; Lane 1, crude cell extract from *E. coli* BL21 (DE3)pLysS without IPTG; Lane 2, crude cell extract from *E. coli* BL21 (DE3)pLysS with IPTG; Lane 3: UGP purified by His-tag purification.

Table 1. Comparison of *S. chungbukensis* DJ77 UGP with other homologous proteins.

Organism Product	Identities (%) Positives (%)
Sphingomonas paucimobilis UDP-glucose pyrophosphorylase	217/291 (74) 248/291 (85)
Zymomonas mobilis UTP-glucose-1-phosphate uridyltransferase	183/289 (63) 226/289 (78)
Novosphingobium aromaticivorans DSM12444 UDP-glucose pyrophosphorylase	184/265 (71) 235/292 (80)
Gluconacetobacter xylinus UDP-glucose pyrophosphorylase	167/284 (58) 215/284 (75)
Rhodopseudomona palustris CGA009 UTP-glucose-1-phosphate uridylyltransferase	168/277 (60) 209/277 (75)
Brucella suis 1330 UTP-glucose-1-phosphate uridylyltransferase	166/279 (59) 201/279 (72)
Bradyrhizobium japonicum USDA110 UTP-glucose-1-phosphate uridylyltransferase	167/277 (60) 199/277 (71)

important role in the UPG enzyme activity. Figure 2 shows the results of the multiple sequence alignment comparison of UGP enzymes from various organisms. Two important conserved regions were found in the amino acid sequence using the CLUSTAL W program (Figure 2). The N-terminal region of the UGP contains the GXGTRXLPATK motif, which is highly conserved amongst bacterial UGP enzymes. It has been suggested that this domain is the activator-binding site.[22,23] The other conserved domain amongst bacterial UGPs was found in the amino acid sequence shown in Figure 2, which is believed to be the glucose-1-phosphate binding site.[22] We also found a conserved lysine residue in the binding site, which is likely to play a critical role in the enzymatic catalysis.[22]

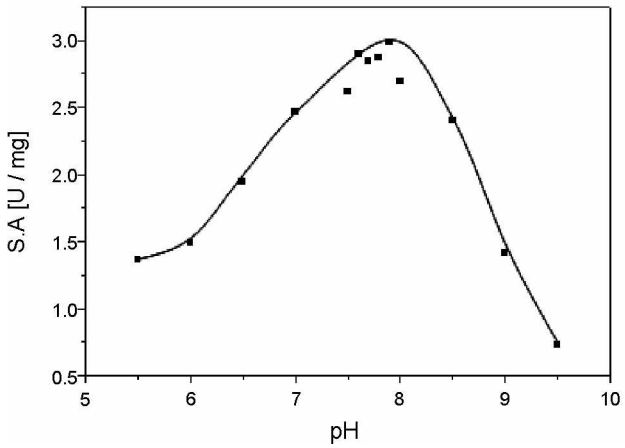

Figure 3. Optimizing the reaction pH for UGP activity. The pH of the assay solution MES (5.5 - 6.5), MOPS (6.5 - 8.0) and TAPS (8.0 - 9.5) buffers.

It was also important to determine the optimal pH for UGP activity. To do so, we carried out the enzyme assay over a pH range from 5.5 to 9.5. Figure 3 shows the results of the enzyme activity as a function of pH, indicating maximum activity is obtained at pH = 8.0.

To determine whether the enzyme has multiple subunits, we used a native gel, and the results are shown in Figure 4. In the denaturing gel (SDS-PAGE), the estimated mass of the monomer is 32 kDa; however, the native gel displays a band corresponding to an estimated mass of 63.17 kDa (results not shown). This result provides evidence that the protein forms a homodimer.

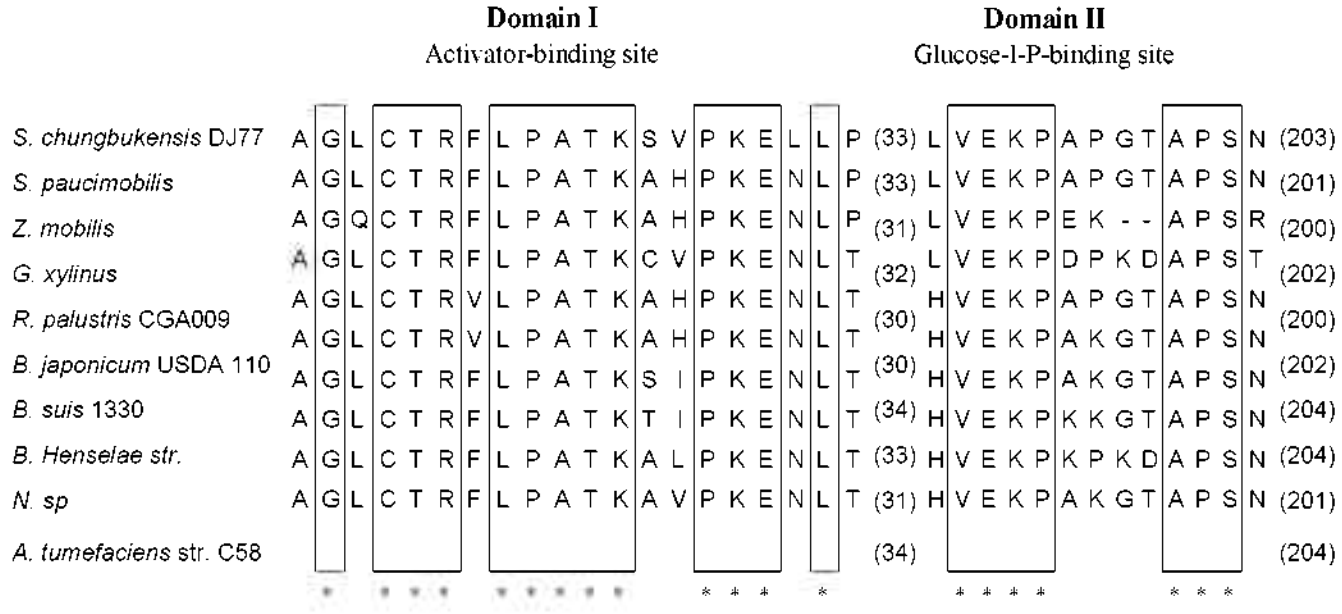

Figure 2. Multiple alignment of the two characteristic consensus amino acid sequences. The domain I motif contains the activator-binding site of the enzyme, the domain II motif contains a glucose-1-P-binding site of the enzyme. Numbers of intervening amino acid are given in parentheses. *S. chungbukensis* DJ77, *Sphingomonas chungbukensis* DJ77; *S. paucimobilis*, *Sphingomonas paucimobilis*; *Z. mobilis*, *Zymomonas mobilis*; *G. xylinus*, *Gluconecetobacter xylinus*; *R. palustris* CGA009, *Rhodopseudomonas palustris* CGA009; *B. japonicum* USDA 110, *Bradyrhizobium japonicum* USDA 110; *B. suis* 1330, *Brucella suis* 1330; *B. henselaestr*, *Bartonella henselae*; *N. sp*, *Novosphingobium species*; *A. tumefaciens* str. C58, *Agrobacterium tumefaciens* str. C58

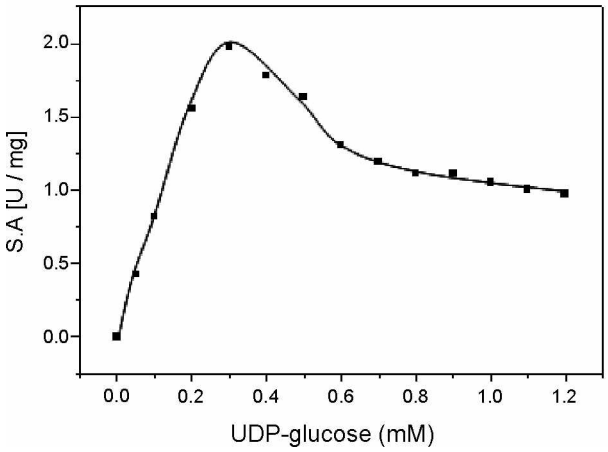

Figure 4. Kinetic analysis of UGP. Purified UGP was incubated with varied concentrations of UDP-glucose, and the enzyme activity was determined.

To support the hypothesis that the gene product is UGP, enzyme activity experiments were carried out *via* measurements designed to monitor the increase in the absorbance at 340 nm with increased NADPH concentration. To examine the effects of substrate concentration on enzymatic activity, the enzyme assay was conducted over a wide range of UDP-glucose concentrations. Figure 4 shows the results of the enzyme activity assay as a function of UDP-glucose concentration. The enzyme activity was higher at low substrate concentration (0 - 0.4 mM) and was decreased at higher concentrations of the substrate (> 0.4 mM), indicating substrate inhibition occurs at higher concentrations. Therefore, we conclude that a UDP-glucose concentration of 0.4 mM is optimal for enzyme activity. Kinetic data was collected for the conversion of UDP-glucose to glucose-1-phosphate. The K_m and V_{max} values of the enzyme were measured at pH = 8.0 at a UDP-glucose concentration of 0.4 mM, and values of 1.14 mM for K_m and of 10.09 mM/min/mg for V_{max} were determined.

We performed site-directed mutagenesis experiments to determine whether the conserved amino acid, Lys194, is involved in the enzyme activity. A charge-reversal mutagenic substitution was carried out at Lys194 by mutation to Asp, and no enzymatic activity of K194D was detected. This result indicates that Lys194 plays a critical role in the enzyme mechanism. In addition, we also substituted an Arg for Lys194 (which is a conserved substitution of one positive charged side chain for another). The K_m and V_{max} values for the Arg-substituted enzyme however, were significantly lower than those of the wild-type enzyme, suggesting that the proximity of the Lys residue to the substrate is essential for catalysis. Table 2 summarizes the kinetic data for wild-type UPG and its variants.

To date, a K_m value of 7.5 μM for *S. paucimobilis* ATCC 31461[11] is the highest value known to these authors, and the lowest known is 3.2 mM for *Acetobacter xylinum*.[24] Thus, our K_m value of 1.14 mM for *S. chungbukensis* UGP lies within the range for comparable systems. It should be noted that the K_m value cannot be a conclusive value for the affinity between the substrate and the enzyme, but no information about binding constants for the enzyme is available, so we included the K_m values as affinity values.

Table 2. Kinetic parameters of the wild type and variant UGPs.

	K_m (mM)	V_{max} (mM/min/mg)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1}/mM)
Wild-type	1.14	10.09	27.04	23.71
K194D		No enzymatic activity		
K194R	0.51	1.77	4.74	9.29

We observed substrate inhibition, and to our knowledge, this kind of inhibition has not been reported for any UGP enzymes. The mechanism of the substrate inhibition was related to a second, non-active site binding on the enzyme, which resulted in a decrease in enzyme activity at higher substrate concentrations. In this study we used a coupled method in which other molecules may act as the second substrate; however, to identify the other molecules, further investigation is needed.

The Lys194 is located within the VEKP (Val-Glu-Lys-Pro) motif, which is found in many bacterial enzymes, and is predicted to be essential for substrate binding or catalysis.[22] Our site-directed mutagenesis data show that substitution of the charge-reversible residue Asp for Lys194 within the consensus sequence motif dramatically impairs the UGP activity, supporting the hypothesis that the Lys194 plays a critical role in the catalysis.

In conclusion, it has been determined by gel electrophoresis, amino acid alignment, kinetic analysis, and site-directed mutagenesis experiments that our gene product is highly likely to be the UGP enzyme. As understanding UGP is essential to EPS biosynthesis, future study should examine the role of UGP in the biosynthetic pathway. This could be done by disrupting the gene in *S. chungbukensis* DJ77, and then evaluating the contribution of this enzyme to EPS biosynthesis in this bacterium.

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