Cloning and Characterization of UDP-glucose Dehydrogenase from *Sphingomonas chungbukensis* DJ77

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Sphingomonas chungbukensis DJ77 has the ability to produce large quantities of an extracellular polysaccharide that can be used as a gelling agent in the food and pharmaceutical industries. We identified, cloned and expressed the UDP-glucose dehydrogenase gene of *S. chungbukensis* DJ77, and characterized the resulting protein. The purified UDP-glucose dehydrogenase (UGDH), which catalyzes the reversible conversion of UDP-glucose to UDP-glucuronic acid, formed a homodimer and the mass of the monomer was estimated to be 46 kDa. Kinetic analysis at the optimal pH of 8.5 indicated that the K_m and V_{max} for UDP-glucose were 0.18 mM and 1.59 mM/min/mg, respectively. Inhibition assays showed that UDP-glucuronic acid strongly inhibits UGDH. Site-directed mutagenesis was performed on Gly9, Gly12 Thr127, Cys264, and Lys267. Substitutions of Cys264 with Ala and of Lys267 with Asp resulted in complete loss of enzymatic activity, suggesting that Cys264 and Lys267 are essential for the catalytic activity of UGDH.

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

Introduction

The bacterium *Sphingomonas chungbukensis* DJ77 was discovered and isolated from contaminated freshwater sediment in Daejeon, Korea.[1] The *Sphingomonas* genus has members that have the ability to produce large quantities of extracellular polysaccharide (EPS) gellan, which can be used as a gelling agent for food or pharmaceutical use.[2] The EPS contains a repeating unit consisting of D-glucose, D-glucoronic acid and L-rhamnose.[3] The biosynthetic pathway for EPS has been partially elucidated,[4,5] and UDP-glucose dehydrogenase (UGDH, EC 1,1.1.22) is thought to be involved in the pathway.[6]

UGDH catalyzes the conversion of UDP-glucose to UDP-glucuronate, and the bovine enzyme and the enzyme from *Streptococcus pyogenes* have been extensively studied.[7-13] In the proposed catalytic mechanism, NAD^+ is reduced by accepting two electrons from the C-6` pro-R hydride of UDP-glucose to form NADH and an aldehyde intermediate.[9,13] The second NAD^+ is reduced by accepting two electrons from the hydride of the thiohemiacetal intermediate generated by the addition of a cysteine thiol to the aldehyde intermediate.[9,12] Consequently, the resulting thioester is hydrolyzed to generate the UDP-glucuronic acid final product. The proposed mechanism has been confirmed by X-ray crystallographic studies of the complexes of both *S. pyogenes* UGDH/UDP-xylose/NAD+ and UGDH/UDP-glucuronic acid/NAD^+.[14]

In this study, we report for the first time the cloning and overexpression of the *ugdh* gene from *S. chungbukensis* DJ77 and the purification and characterization of the corresponding protein. We found binding sites for the UDP-glucose substrate and the NAD^+ cofactor in the primary structure by *in silico* analysis. Site-directed mutagenesis and kinetic analysis were carried out to investigate the roles of the conserved sites.

Materials and Methods

Materials. A fosmid library from *S. chungubkensis* DJ77 was prepared in the laboratory of Dr. Young-Chang Kim.[1] The fosmid library was constructed using the CopyControl™ Fosmid Library Production Kit (Epicentre, Madison, WI, USA) as described previously.[15,16] Restriction enzymes and T4 ligase were purchased from Roche (Mannheim, Germany). Taq polymerase and other components for PCR were purchased from Bioneer Inc. (Daejeon, Korea). PCR primers were synthesized by Genotech (Daejeon, Korea). All other reagents were purchased from Sigma or other commercial suppliers.

Gene selection. From the *S. chungubkensis* DJ77 genomic DNA sequence database (http://bioinfo.chungbuk.ac.kr), we found a cu556 fosmid clone containing the *ugdh* gene. We

Abbreviations: EPS, extracellular polysaccharide; NAD^+, nicotinamide adenine dinucleotide; UGDH, UDP-glucose dehydrogenase; IPTG, isopropyl thio-β-D-galactoside; PCR, polymerase chain reaction; pI, isoelectric point; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis

determined the full length gene sequence after amplifying the fosmid clone using a primer walking method. The reverse primers used for primer walking were as follows: 5'-ACACC AGCGCAACCTTCATT-3' and 5'-GTGGGTGGAAATGA AGGGCT-3'.

Construction of expression vectors. PCR was used to amplify the gene using the forward primer 5'-CCCGGATCCTAT GAAGATCACGATGAT-3' and reverse primer 5'-TTTGGA TCCTCAGACGTTGACGCCCT-3.' The underlined sequences indicate the *Bam*HI restriction site. Genomic DNA was used as a template for PCR. The *Bam*HI-digested PCR product was inserted into a pET-15b expression vector using T4 DNA ligase. Finally, the cloned plasmid was used to transform *E. coli* BL21(DE3)pLysS. The sequence of the insert was confirmed by DNA sequencing in both directions. NCBI BLAST was used to find and compare homologous proteins to the gene product from *S. chungbukensis* DJ77.

Expression and purification of recombinant protein. Transformed BL21(DE3)pLysS cells were grown in LB medium containing 50 mg/L ampicillin at 37 °C until the optical density of the culture at 600 nm reached 0.6. The inducer IPTG was added at a final concentration of 0.1 mM, followed by an additional growth period of 5 h at 30 °C. Cells were harvested by centrifugation at 5,000 g for 20 min. The cell pellet was resuspended in a binding 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 after centrifugation at 20,000 g for 30 min. The supernatant was applied to a nickel affinity column and the protein was purified according to a protocol from Qiagen DNA Mini-Prep Kit. Purified protein was analyzed by SDS-PAGE. Native-gel electrophoresis was also used as described previously.[17] Protein concentration was estimated by the Bradford method[18] using bovine serum albumin as a standard.

Enzyme assay. The activity of UGDH was assayed based on NAD^- reduction with UDP-glucose. The activity was monitored via the increase of absorbance at 340 nm on a Beckman DU-650 spectrophotometer at 30 °C. The specific activity is the number of enzyme units per microgram of protein. One unit of enzyme activity is defined as the amount of enzyme required to reduce 1 μmol NAD^- per minute in these assay conditions. The standard reaction mixture contained 100 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, and 10 mM NAD^-, along with 0.1 - 0.3 mM UDP-glucose. To determine the kinetic parameters for NAD^-, its concentration was varied from 0.1 mM to 4.0 mM. For the pH dependent experiment, 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. A PCR mega primer method was used to produce site-specific mutations[19] in the pET15b-UGDH plasmid. The following mutagenic primers were used: G9A: 5'-GGA TCC TAT GAA GAT CAC GAT GAT CGG CAC GGC CTA TGT C-3'; G12A: 5'-GGA TCC TAT GAA GAT CAC GAT GAT CGG CAC GGG CTA TGT CGC GCT GGT G-3'; C264A: 5'-TAT CTC GGC TCC GCC TCC CCG A-3'; C264S: 5'-TAT CGC GGC TCC AGC TCC CCG A-3'; K267D: 5'-TTC CCG GAT GAC ACG CTG-3'; K267R: 5'-TTC CCG AGG GAC ACG CTG-3'; T127A: 5'-ACC TCG TCG CCG GCG CCG A-3.' The underlined sequences indicate the mutagenized sites.

Circular dichroism (CD). UGDH at 0.3 mg/mL in 10 mM potassium phosphate buffer (pH 7.5) was used to obtain CD spectra using a Jasco J-710 spectropolarimeter at 0.2 nm spectral resolution. CD data were obtained with 20-50 mdeg sensitivity, 3 units accumulation, 1-second response, and 10 nm/min scanning speed.

Results

To the best of our knowledge, the UGDH from *S. chungbukensis* DJ77 has not yet been characterized in the literature. We performed primer walking sequencing starting with a designed reverse primer, and eventually obtained the full length 1.3 kb *ugdh* gene. The DNA sequence was confirmed by sequencing analysis. Based on the DNA sequence, UGDH is expected to contain 440 amino acids, beginning with a methionine residue arising from the start codon ATG. The calculated mass and pI of the protein are 46.4 kDa and 5.49, respectively.

The gene encoding UGDH was cloned into a pET-15b(+) expression vector. We used the *E. coli* BL21(DE3)pLysS strain to overexpress the gene. UGDH was produced with a hexahistidine tag and purified to homogeneity. The enzyme appeared as a single band with a molecular mass of approximately 46 kDa on Coomassie Blue stained SDS-PAGE, which is in good agreement with the calculated mass (Figure 1). We used native gel electrophoresis to determine the molecular mass of the protein without SDS detergent. The results indicated dimer formation in the native gel with a molecular mass of 102 kDa, which corresponds to the mass of the two monomers (Figure 2).

An activity assay for UGDH was carried out with the two possible substrates, UDP-glucose and NAD^+. Figure 3 shows the results of the enzyme activity assay under two different conditions: varied UDP-glucose concentrations with fixed NAD^- concentration, and varied NAD^+ concentrations with

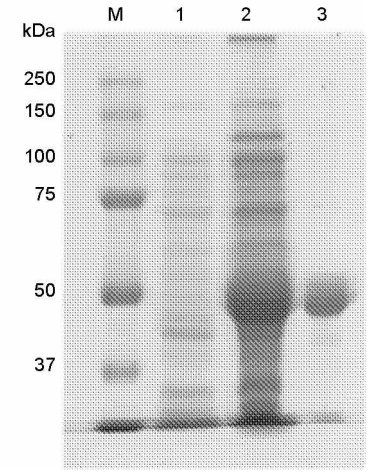

Figure 1. SDS-PAGE gel of UGDH. M: molecular markers; Lane 1: crude cell extract from *E. coli* BL21(DE3)pLysS without IPTG; Lane 2: crude cell extract from *E. coli* BL21(DE3)pLysS with IPTG; Lane 3: His-tag purification of UGDH.

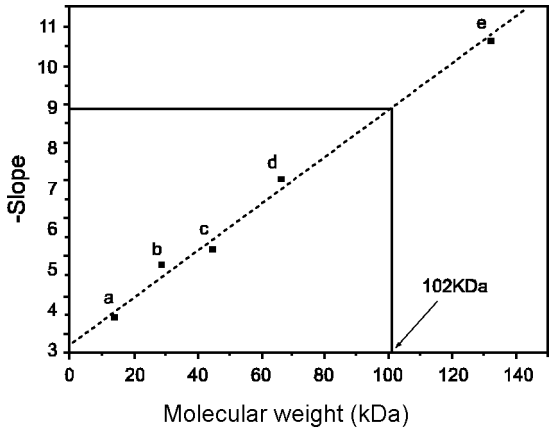

Figure 2. Molecular weight determination of the active forms of UGDH by non-denaturing gel electrophoresis. The non-denatured protein molecular weight markers were alpha-lactalbumin (a), carbonic anhydrase (b), chicken egg albumin (c), bovine serum albumin monomer (d), bovine serum albumin dimer (e).

fixed UDP-glucose concentration. Hydrophobic curves were determined for both conditions, and the curves fit well into the Michaelis-Menten equation. This equation was used to determine the kinetic parameters of the protein. The K_m values of the enzyme for UDP-glucose and NAD^+ were 0.18 mM and 0.97 mM, respectively. V_{max} was determined to be 1.59 mM/min/mg from kinetic analysis. Table 1 summarizes the kinetic parameters. The optimal pH of 8.5 (Figure 4), determined from pH analyses over the range of 5.5 to 9.5 with the use of different buffers, was used for all the kinetic experiments.

-UDP-glucuronic acid is a known inhibitor of UGDH[13], so we expected that UDP-glucuronic acid would inhibit the cloned gene product. Therefore, an inhibition assay was carried out with various inhibitor concentrations, and decreased activity was observed as the concentration of the inhibitor increased (Figure 5). The IC_{50} was determined to be 2.17 mM.

Table 2 shows an amino acid comparison of *S. chungbukensis* DJ77 UGDH with homologous proteins. The UGDH amino acid sequences from *Novosphingobium aromaticivoans* DSM 12444, *Agrobacterim tumefaciens* str. C58, *Sinorhizobium meliloti*, and *Rhodopseudomonas palustris* CGA009 have a high sequence homology (i.e., more than 70% sequence identity) with the gene product that we used for cloning, supporting the hypothesis that the gene produces the UGDH protein from *S. chungbukensis* DJ77. *Rhizobium leguminosarum* and *Zymomonas mobilis* subsp. mobilis ZM4 also have a high amino acid sequence homology with the cloned gene product. The putative conserved UGDH catalytic active site (GGSCFPKDT) and cofactor NAD^+-binding site (GXXGXX XG) were found in the proposed *S. chungbukensis* DJ77 UGDH by sequence alignment (Figure 6), providing further evidence that the gene encodes UGDH.

To verify the conserved catalytic active site and NAD^+-binding site identified by an *in silico* amino acid alignment, a series of site-directed variants were generated using the same method of gene expression and protein purification that was carried out for the wild-type enzyme. As was the case for the wild-type enzyme, all three variants were successfully ex-

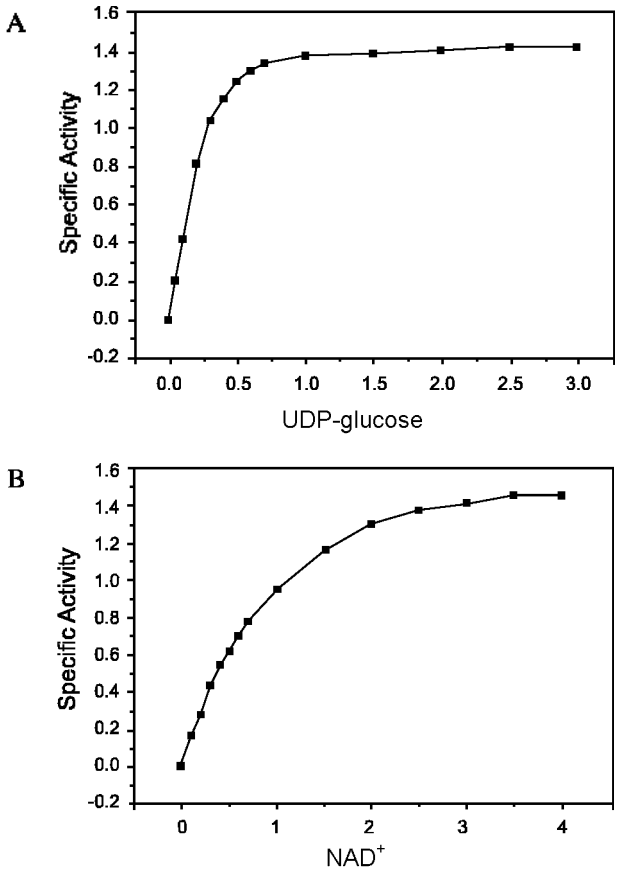

Figure 3. Wild type UGDH demonstrates kinetics for (A) specific activity (μmol/min/mg) of UDP-glucose and (B) specific activity of NAD^+. Steady state kinetic assay was monitored by the increased absorbance at 340 nm through the reduction of NAD^+. The reaction buffer contained 100 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, and 10 mM NAD^+.

pressed and purified. Within the conserved catalytic site GG SCFPKDT, Thr172, Cys264, and Lys267 were mutagenized, and the effects of these amino acid substitutions on kinetic parameters were investigated. As K_m value is related to binding affinity for the substrate UDP-glucose, we measured K_m values of these variants (i.e., Thr172, Cys264, and Lys267) for only UDP-glucose. The uncharged polar side chain of Thr172 was changed to the non-polar side chain of Ala in order to probe the effects of removing the oxygen atom of the side chain. A significant increase in the K_m value for UDP-glucose was observed for this variant when compared to the wild-type UGDH (0.54 mM for T172A mutant *vs.* 0.18 mM for wild-type). Also, a substantial decrease in V_{max} for the T127A mutant was observed, indicating that turnover decreased considerably. At position Cys264, the polar side chain was mutated to Ala and Ser. No enzymatic activity was observed for either the C264A or C264S mutant, indicating that Cys264 is essential for catalytic activity. The charge-reversed substitution in the K267D variant also abolished activity of the enzyme, suggesting that Lys267 may play a critical role in the catalytic mechanism. To probe the effect of the side chain charge at this position, Lys267 was mutated to Arg as a positive control. K267R showed some catalytic activity, with an increased K_m

Table 1. Kinetic properties of UGDH variants.

	K_m(mM)		V_{max}	k_{cat}	k_{cat}/K_m
	UDP-glucose	NAD^-	(mM/min/mg)	(s^{-1})	(s^{-1}/mM)
Wild-type	0.18	0.97	1.59	24.65	136.9
C264A		No enzymatic activity			
C264S		No enzymatic activity			
K267D		No enzymatic activity			
K267R	0.41	N.D.*	0.03	0.46	1.12
T127A	0.54	N.D.	0.22	3.41	6.31
G9A	N.D.	1.69	0.25	3.87	2.28
G12A	N.D.	6.25	0.39	6.04	0.96

*N.D. stands for Not Determined. The activity of UGDH was assayed based on NAD^- reduction with UDP-glucose at 30 °C, pH 8.5.

Table 2. Comparison of *S. chungbukensis* DJ77 UGDH with homologous proteins.

Organism Product	Identities (%) Positives (%)
Novosphingobium aromaticivorans DSM 12444	321/433 (74)
Predicted UGDH	368/433 (84)
Magnetospirillum magnetotacticum MS-1	319/433 (73)
Predicted UGDH	358/433 (82)
Agrobacterium tumefaciens str. C58	314/433 (72)
Predicted UGDH	354/433 (81)
Sinorhizobium meliloti	310/433 (71)
Predicted UGDH	362/343 (83)
Rhodopseudomonas palustris CGA009	309/433 (71)
Predicted UGDH	351/433 (81)
Rhizobium leguminosarum	301/433 (69)
Predicted UGDH	355/433 (81)
Zymononas mobilis subsp. mobilis ZM4	301/433 (69)
Predicted UGDH	360/433 (83)

value and significantly decreased V_{max} value. This supports the conclusion that the amino acid identity at this position is critical, and Lys267 is likely to be involved in the enzymatic mechanism. Table 1 includes the kinetic parameters obtained from the mutagenic studies.

To test the possibility that the GXXGXXXG site is involved in NAD^- binding, the first two Gly residues were changed to Ala (G9A and G12A). The V_{max} values for both variants were decreased by more than 70 % from the wild-type enzyme V_{max} value. The difference in the decreased V_{max} values was not significant between the two variants, while the difference in K_m values between the two was substantial. The G9A mutant's K_m value for NAD^- was 1.69 mM, and the K_m value of the G12A mutant was 6.25 mM. While both the G12A and G9A variants have higher K_m values than the wild-type enzyme (0.97 mM), the G12A mutant, with its significantly higher K_m value, demonstrates that the Gly at this position plays a particularly important role in NAD^- binding. It should be mentioned that given that the GXXGXXXG site is related to NAD^- containing, we determined K_m values of G9A and G12A for NAD^+.

A series of CD spectra were obtained in order to determine whether the loss of enzymatic activity observed for the C264A

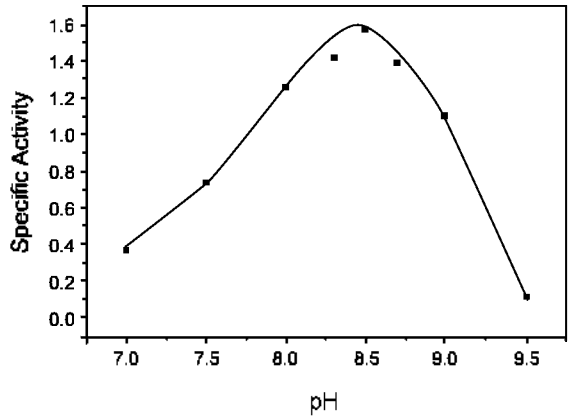

Figure 4. The pH-dependent specific activity (μmol/min/mg) of UGDH activity. The buffers and pH range used in the assay were as follows: MEPS (5.5 - 6.5), MOPS (6.5 - 8.0) and TAPS (8.0 - 9.5).

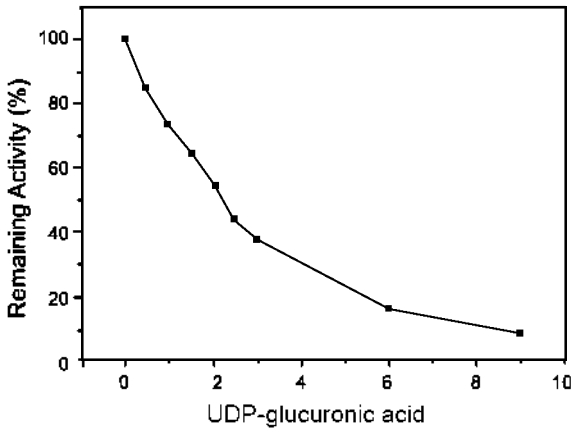

Figure 5. Inhibition of UGDH by UDP-glucuronic acid. The concentration of UDP-glucuronic acid was varied from 0 - 9 mM. The reaction buffer contained 100 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, and 10 mM NAD^+.

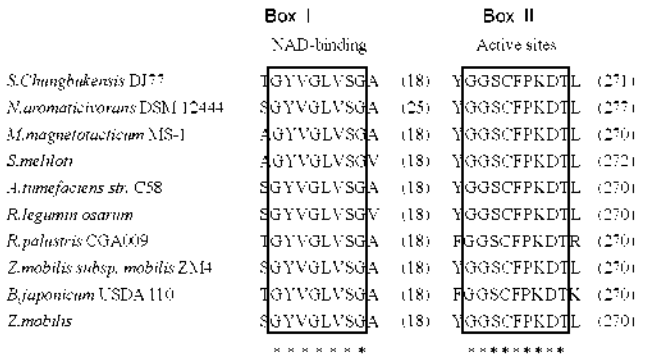

Figure 6. Multiple amino acid sequence alignment of the two characteristic consensus sequences present in 10 proteins with UGDH activity from different organisms. Boxes I and II show the functional domains exhibiting the highest similarity among the polypeptides. The amino acid sequences shown in boxes I and II are known to be critical for UGDH activity. The box I motif contains the NAD-binding site of the enzyme; the box II motif contains the active site of the enzyme. Asterisks below the sequences indicate conserved residues. Numbers of intervening amino acid are given in parentheses.

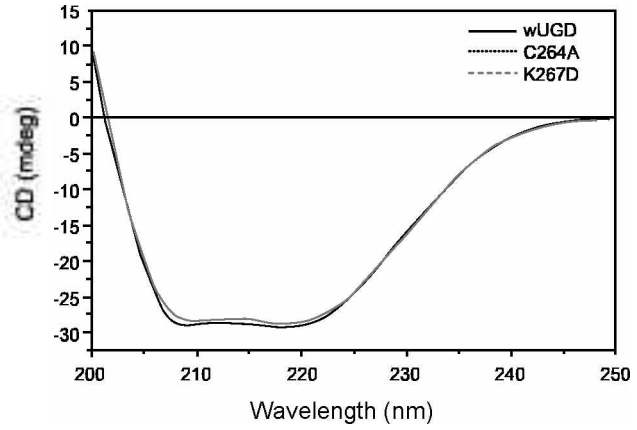

Figure 7. Far-UV CD spectra of wtUGDH, C264A and K267D mutants. Each protein was present at a concentration of 0.3 mg/mL in 10 mM potassium phosphate buffer (pH 7.5).

and K267D variants was due to a conformational change of the site-specific variants. The CD spectra were measured for the wild-type enzyme and variants in the far-UV region, ranging from 200 nm to 250 nm. No significant differences in the CD spectra between the wild-type enzyme and the variants were found (Figure 7). These results suggest that the activity loss is not likely to be due to a conformational change in the enzyme.

Discussion

The gene encoding UGDH was isolated and cloned into an expression vector and successfully expressed and purified in the *E. coli* system. Strong evidence confirming the identity of this gene product was obtained using a variety of experiments. The observation that the enzyme activity requires UDP-glucose as substrate and NAD^+ as a cofactor suggests that the gene product is UGDH. In addition to the enzyme activity assay, the high degree of amino acid sequence homology with UGDH from other organisms also indicates that the gene product is UGDH from *S. chungbukensis* DJ77.

Once the putative catalytic and cofactor binding sites were identified by amino acid alignments, we were able to design a series of mutagenic experiments to provide additional evidence for the identity of the gene product. Site-directed mutagenesis of the conserved catalytic site suggested that Cys264 and Lys267 are essential for enzymatic activity. Because there is no three dimensional structure available for *S. chungbukensis* DJ77 UGDH, we cannot definitively state that the two amino acid residues are involved in substrate binding. However, the model structure of human UGDH,[20] based on the three dimensional structure of *S. pyogenes* UGDH (Protein Data Bank code 1DLI),[14] shows a ternary complex formed by human UGDH, UDP-Glucose, and NAD^+ at the active site. In this complex structure, Cys276 (corresponding to Cys264 for *S. chungbukensis* DJ77 UGDH) forms a hydrogen bond with UDP-Glucose, while Lys279 (corresponding to Lys267 for *S. chungbukensis* DJ77 UGDH) makes a hydrogen bond with NAD^+. Sommer and colleagues[20] used site-specific mutation experiments to show that Cys276 is essential for the activity of human UGDH, while Lys279 is dispensable. Our experiments on *S. chungbukensis* DJ77 UGDH and enzyme kinetic measurements showed that both Cys and Lys are essential for enzymatic activity, suggesting that the location of Lys267 for *S. chungbukensis* DJ77 UGDH is different from that of human UGDH Lys279. However, the X-ray structure of *S. pyogenes* UGDH shows the amino group of Lys204 forming a hydrogen bond with UDP-glucose, suggesting that Lys204 of *S. pyogenes* UGDH serves as a general base in the mechanism.[14] The Lys of the *S. chungbukensis* DJ77 UGDH could also function as a general base, which would account for the loss of enzymatic activity that we observed when this position was mutated.

While the variants from the substitutions made at Cys264 and Lys267 show no enzymatic activity, site-directed mutagenesis at Thr127 produced an enzyme with some activity. This result implies that Thr127 is not critical for catalytic activity, but still plays a role in the activity of the enzyme. The X-ray structure for *S. pyogenes* UGDH indicates that Thr118, corresponding to Thr127 in *S. chungbukensis* DJ77 UGDH, can form a hydrogen bond with a water molecule, and may play a role in the catalytic mechanism as a general base. This residue might function by enhancing the nucleophilicity of the water molecule, which could account for the significant decrease in activity observed for the T127A variant.

The GXGXXG motif is known to be involved in NAD^+ binding.[21,22] The X-ray structure of *S. pyogenes* UGDH[14] suggests that the motif serves as a connection loop between a β-sheet (β1) and an α-helix (α1). The substitutions we made in the first two glycines of the motif provide evidence that a complex with NAD^+ is mediated by the glycines. This shows that *in silico* analysis can provide valuable information that can guide us in our experimental approaches.

This research provides the groundwork for further investigation of the enzyme and its function. Disruption of the gene in *S. chungbukensis* DJ77 would be the next step to explore the possibility that the gene product (UGDH) contributes to EPS synthesis.

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