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-glucuronate, 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*<sub>m</sub> and *V*<sub>max</sub> 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. 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. The EPS contains a repeating unit consisting of D-glucose, D-glucoronic acid and L-rhamnose. The biosynthetic pathway for EPS has been partially elucidated, and UDP-glucose dehydrogenase (UGDH, EC 1.1.1.22) is thought to be involved in the pathway.

UGDH catalyzes the conversion of UDP-glucose to UDP-glucuronate, and the bovine enzyme and the enzyme from *Streptococcus pyogenes* have been extensively studied. In the proposed catalytic mechanism, NAD<sup>+</sup> is reduced by accepting two electrons from the C-6' pro-R hydride of UDP-glucose to form NADH and an aldehyde intermediate. The second NAD<sup>+</sup> 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. 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<sup>+</sup> and UGDH/UDP-glucuronic acid/NAD<sup>+</sup>.

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<sup>+</sup> 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. chungbukensis* DJ77 was prepared in the laboratory of Dr. Young-Chang Kim. The fosmid library was constructed using the CopyControl<sup>TM</sup> Fosmid Library Production Kit (Epicentre, Madison, WI, USA) as described previously. 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. chungbukensis* DJ77 genomic DNA sequence database (http://bioinfo.chungbuk.ac.kr), we found a cu556 fosmid clone containing the *ugdh* gene. We

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**Abbreviations:** EPS, extracellular polysaccharide; NAD<sup>+</sup>, 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 for primer walking were as follows: 5'-ACACC AGCGCAACCTTCATT-3' and 5'-GTTGGGTGGAAATGAA GGCT-3'.

**Construction of expression vectors.** PCR was used to amplify the gene using the forward primer 5'-CCCCGATCCCTAT GAAATCACTATGATGAT-3' and reverse primer 5'-TTTGGGA TCCTAAGACTTTGACGCCCT-3'. The underlined sequences indicate the BamHI restriction site. Genomic DNA was used as a template for PCR. The BamHI-digested PCR product was inserted into a pET-15b expression vector to produce site-specific mutations in the pET15b-ugdh plasmid. The following mutagenic primers were used: GAT CAC GA T GAT CGG CAC GGG CTA TGT CGC G-3'; C264S: 5'-TA T CGC GGC TCC AGC AGGGCT-3'; C264A: 5'-TAT CTC GGC TCC GCC AGCGCAACCTTCATT-3'; K267: 5'-ATT GCT GAT CAC GCG TCT TCT CTA TGT CTA TGT-3'; K267R: 5'-TAT CTC GGC TCC GCC AGCGCAACCTTCATT-3'; K267D: 5'-TAT CTC GGC TCC GCC AGCGCAACCTTCATT-3'; T127A: 5'-ACC TCG TCC 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...
Cloning and Characterization 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, 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 aromaticivorans DSM 12444, Agrobacterium tumefaciens str. C58, Sinorhizobium meliloti, and Rhodopseudomonas palustris CGA009 have 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 expressed and purified. Within the conserved catalytic site GG SCFPKDT, Thr172, Cys264, and Lys267 were mutated, 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.

<table>
<thead>
<tr>
<th>Variant</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (mM/min/mg)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$/mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.18</td>
<td>0.97</td>
<td>1.59</td>
<td>24.65</td>
</tr>
<tr>
<td>C264A</td>
<td>No enzymatic activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C264S</td>
<td>No enzymatic activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K267D</td>
<td>No enzymatic activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K267R</td>
<td>0.41</td>
<td>N.D.*</td>
<td>0.03</td>
<td>0.46</td>
</tr>
<tr>
<td>T127A</td>
<td>0.54</td>
<td>N.D.</td>
<td>0.22</td>
<td>3.41</td>
</tr>
<tr>
<td>G9A</td>
<td>N.D.</td>
<td>1.69</td>
<td>0.25</td>
<td>3.87</td>
</tr>
<tr>
<td>G12A</td>
<td>N.D.</td>
<td>6.25</td>
<td>0.39</td>
<td>6.04</td>
</tr>
</tbody>
</table>

*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.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Product</th>
<th>Identities (%)</th>
<th>Positives (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Novosphingobium aromaticivorans DSM 12444</td>
<td>Predicted UGDH</td>
<td>321/433 (74)</td>
<td></td>
</tr>
<tr>
<td>Magnetospirillum magnetotacticum MS-1</td>
<td>Predicted UGDH</td>
<td>319/433 (73)</td>
<td></td>
</tr>
<tr>
<td>Agrobacterium tunefaciens str. C58</td>
<td>Predicted UGDH</td>
<td>314/433 (72)</td>
<td></td>
</tr>
<tr>
<td>Sinorhizobium melloti</td>
<td>Predicted UGDH</td>
<td>310/433 (71)</td>
<td></td>
</tr>
<tr>
<td>Rhodopseudomonas palustris CGA009</td>
<td>Predicted UGDH</td>
<td>309/433 (71)</td>
<td></td>
</tr>
<tr>
<td>Rhizobium leguminosarum</td>
<td>Predicted UGDH</td>
<td>301/433 (69)</td>
<td></td>
</tr>
<tr>
<td>Zymomonas mobilis subsp. mobilis ZM4</td>
<td>Predicted UGDH</td>
<td>301/433 (69)</td>
<td></td>
</tr>
</tbody>
</table>

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's $K_m$ values (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 variant was due to a change in the secondary structure of the protein. The CD spectra of the C264A variant showed a significant decrease in the negative peak at 222 nm, which is characteristic of the $eta$-sheet structure. This suggests that the loss of enzymatic activity is due to a change in the secondary structure of the protein.
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,\(^{14}\) 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 GXXGXXG motif is known to be involved in NAD\(^+\) binding.\(^{1,22}\) The X-ray structure of S. pyogenes UGDH\(^{14}\) suggests that the motif serves as a connection loop between a \(\beta\)-sheet (\(\beta\)1) and an \(\alpha\)-helix (\(\alpha\)1). 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 (UDGH) contributes to EPS synthesis.

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