Molecular Cloning, Sequencing, and Biochemical Characterization of *Streptomyces rimosus* TDP-glucose 4,6-dehydratase

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TDP-(Thymidine diphosphate)-glucose 4,6-dehydratase (EC 4.2.1.46), which converts TDP-glucose to TDP-4-keto-6-deoxyglucose, is a common essential enzyme in the biosynthesis of all types of 6-deoxyhexose. Genes that encode TDP-glucose 4,6-dehydratase are found within gene clusters that contain biosynthetic genes for natural products containing 6-deoxyhexose moieties. The genes that are required for the biosynthesis of secondary metabolites, lipopolysaccharide, and O-antigen are also usually clustered in the genome in competent organisms [Kim *et al.*, 2000]. In our previous work, we therefore designed oligonucleotide primers to amplify DNA fragments of the genes for actinomycetes TDP-glucose synthase, which could then be used to efficiently amplify and isolate the genes for sugar moiety biosynthesis in actinomycetes [Hyun *et al.*, 2000]. This method was applied to the cloning of genes for TDP-glucose 4,6-dehydratase in *S. rimosus*. The expression of the cloned genes and the biochemical analysis of the expressed proteins are important steps in confirming the biological function of the cloned genes. Therefore, this report describes the overexpression of the TDP-glucose 4,6-dehydratase gene and the biochemical characteristics of the expressed protein in an *E. coli* system.

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To isolate the TDP-glucose 4,6-dehydratase gene, which generally exists near the TDP-glucose synthase gene in the 6-deoxysugar biosynthetic gene cluster, 0.3-kb PCR fragments of the *S. rimosus* TDP-glucose synthase gene were DIG-AP labeled and used to identify cross-hybridizing bands in a Southern blotting experiment with *S. rimosus* ATCC14827 genomic DNA that had been digested with several restriction enzymes. We found that the 7.0-kb *Bam*HI fragment hybridized with these homologous probes under high-stringency conditions. Based on these data, *S. rimosus* chromosomal DNA was digested with *Bam*HI. Fragments, 5-8 kb in length, were isolated and ligated into pUC18 that was previously digested with the same restriction enzyme. The resulting ligation products were used to transform *E. coli*, and colonies containing DNA that hybridized to the 0.3-kb TDP-glucose synthase probes (*S. rimosus parD* gene) were identified by colony hybridization. Screening of plasmid DNA isolated from a sub-library of 5.0-8.0 kb *Bam*HI fragments of *S. rimosus* genomic DNA yielded one positive clone that contained a 6.5-kb DNA fragment. By primer walking, we sequenced and further analyzed a 1.8 kb locus. CODON PREFERENCE analysis showed that there is one partial open reading frame (ORF) and one complete ORF in this region, which has the characteristic codon usage pattern for *Streptomyces* DNA [Bibb *et al.*, 1984]. The *parE* gene, the complete ORF, is transcribed in the same direction as the incomplete ORF, *parD*. The 954-nucleotide (nt) *parE* ORF starts with ATG at position 802 and terminates with TGA at position 1755. This ORF is predicted to encode 317-amino-acid protein with a calculated molecular mass of 34.7 kDa. The 14-nt region at the 5'-end of the first codon of the *parE* gene has a high degree of complementarity to the 3'-end of *S. coelicolor* 16S rRNA (5'-GAUCACCUCCUUUCU-3') and could serve as the ribosomal binding site [Bibb and Cohen, 1982]. The PrmE protein strongly resembles TDP-D-glucose 4,6-dehydratases from various antibiotic pathways: SgcA (65% identity, 78% similarity) from the C-1027 pathway [Liu and Shen, 2000; Liu *et al.*, 2002]; orf24 (68% identity, 77% similarity) from the ECO-02301 pathway [McAlpine *et al.*, 2005]; and GilE (66% identity, 77% similarity) from the gilvovarcins pathway [Fischer *et al.*, 2003]. Near the amino terminus, the PrmE protein has an amino acid sequence (7GGAGFIG13) that corresponds to the GXGXXG motif, which has been described as a âáâ fold with an NAD-binding motif. This nucleotide is a required cofactor for dTDP-D-glucose 4,6-dehydratase activity in *S. erythraea* and for other dehydratases in anthracycline-producing actinomycetes [Linton *et al.*,

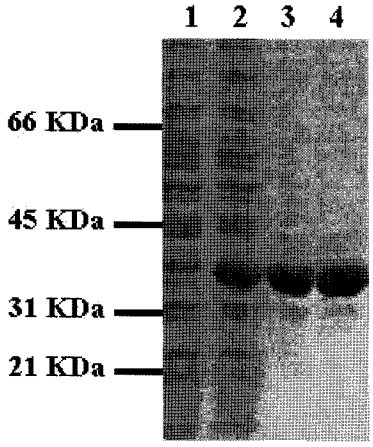

Fig. 1. SDS-PAGE of purified wild-type PrmE enzymes. Lane 1, non-induced cell-free extracts; Lane 2, IPTG-induced cell-free extracts of clone pHCG1213; Lane 3, 4; purified wild-type PrmE.

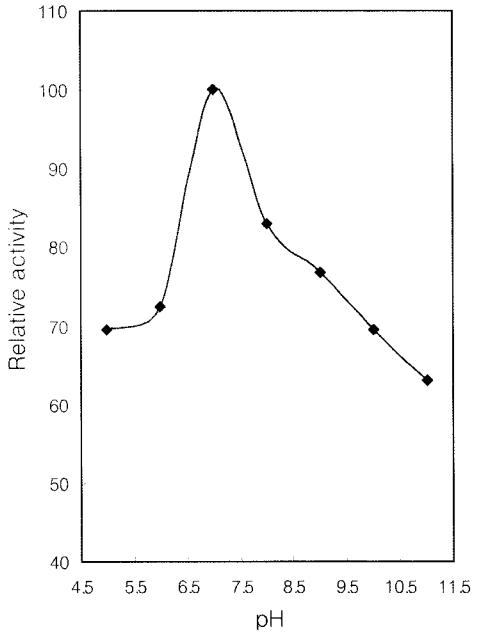

Fig. 2. Effects of pH on enzyme activity. A standard enzyme activity assay was performed in a reaction mixture of 100 mM Tris/Cl (pH 7.6), dTDP-D-glucose (1 mM), NAD (1 mM), and enzyme solution. The mixture was incubated for 30 min at 37°C. At the end of this incubation, the reaction was terminated by adding 0.1 N NaOH and incubated for 20 min at 37°C. The extinction differences between reaction and control reaction mixtures were measured at 340 nm.

1995; Lombo *et al.*, 1997]. Also, the high affinity of NAD for the enzyme is thought to be due to its interaction with the ε-amino group of Lys-152, as in the dehydratases and epimerase [Gerratana *et al.*, 2001]; this lysine residue is also conserved in PrmE. The nucleotide sequence reported here was deposited in the GenBank database under accession number AF144042.

To express ParE protein, PCR was performed with plasmid template pSH1201 (*parDE* locus in pUC18) and primers designed to introduce the *Nde*I (PrmE-*Nde*I; 5'-ccttcccccatatgaagacc-3') and *Eco*RI (PrmE-*Eco*RI: 5'-atcacacgccgaattcgtgt-3') restriction sites flanking ParE. The final reaction volume was 50 mL and contained 100 pmol of each primer, 50 ng of pSH1201 plasmid DNA, 0.25 mM dNTP, 4 pmol of each oligonucleotide, 2 units of Taq polymerase, and 1 × buffer (Takara, Kyoto, Japan). Amplification was performed in a thermal cycler (Model 480, Perkin-Elmer, Foster City, CA) by denaturing the samples at 94°C for 3 min, subjecting them to 30 cycles of denaturing (98°C, 20 sec) and annealing (68°C, 1 min), and then a final elongation at 72°C for 10 min. [Hyun and Suh, 1999]. An expected 0.96-kb PCR product with *Nde*I and *Eco*RI sites was recovered by 1% agarose gel electrophoresis and ligated into pT7Blue (Novagen, Madison, WI) to produce pSH1211. This supercoiled recombinant vector was digested with *Nde*I and *Eco*RI and ligated into the restriction-digested pET28a expression vector to yield pSH1213.

E. coli BL21 (DE3) cells harboring pSH1213 or pET28a (control) plasmids were grown at 37°C in LB medium to an A_{600} of 0.6. Expression was induced by adding IPTG (isopropyl-thio-β-D-galatoside) to a final concentration of 1 mM; growth was then allowed to continue for 4 h. As determined by SDS-PAGE, a 34-kDa protein was produced by induction of T7-polymerase in the strain carrying pSH1213. However, this protein was not induced in the strains carrying the vector only (Fig. 1). PrmE protein was purified as the His-tagged fusion protein.

The following enzymatic characteristics of dTDP-glucose 4,6-dehydratase from *S. rimosus* were examined: (1) substrate specificity, (2) kinetic parameters, (3) optimum pH, and (4) optimum temperature. A standard enzyme activity assay was performed in a reaction mixture of 100 mM Tris/Cl (pH 7.6), dTDP-D-glucose (1 mM), NAD (1 mM), and enzyme solution. The mixture was incubated for 30 min at 37°C. At the end of this incubation, the reaction was terminated by adding 0.1 N NaOH and incubated for 20 min at 37°C. The extinction differences between reaction and control reaction mixtures were measured at 340 nm. Product formation was determined using an ε of 6,500 M^{-1} L^{-1} for dTDP-4-keto-6-deoxyglucose. One unit of enzyme activity corresponds to the formation of 1 μmol of product per hour under standard assay conditions, and the specific activities are reported as units per milligram of protein. The dTDP-glucose 4,6-dehydratase activity of the purified protein was examined with ADP-glucose, CDP-glucose, GDP-

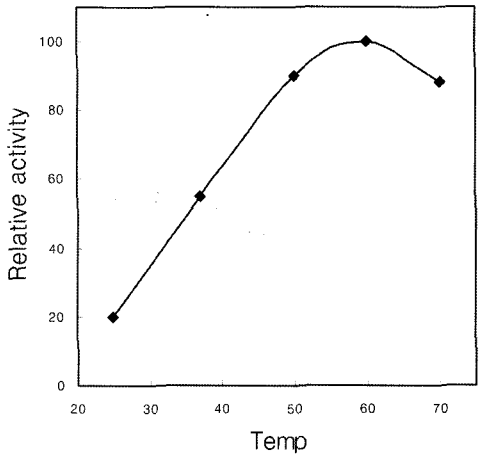

Fig. 3. Effects of temperature on enzyme activity. dTDP-glucose 4,6-dehydratase activity was assayed at various temperatures. Although the enzyme showed activity over an unusually broad range of temperatures, its apparent temperature optimum was 60°C.

glucose, and TDP-glucose (Sigma Co.). Among these possible substrates, only TDP-glucose served as a substrate. The K_m and V_{max} values were determined for dTDP-glucose from the Lineweaver-Burk double-reciprocal plots of the enzyme reactions over a range of dTDP-glucose concentrations. The K_m and V_{max} values for dTDP-glucose were determined as 11.24 mM and 0.67 mM/min.

Figure 2 shows the dTDP-glucose 4,6-dehydratase activity at various pH values. The optimum pH was about 7.5 in Tris-Cl buffer; almost all actinomycetes dTDP-glucose 4,6-dehydratases have been reported to have similar pH optima. dTDP-glucose 4,6-dehydratase activity was also assayed at various temperatures. Although the enzyme showed activity over an unusually broad range of temperatures, its apparent temperature optimum was 60°C (Fig. 3).

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