J. Microbiol. Biotechnol. (2004), **14**(3), 576–583

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Cloning, Expression, and Biochemical Characterization of dTDP-Glucose 4,6-Dehydratase Gene (*gerE*) from *Streptomyces* sp. GERI-155

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Received: October 28, 2003
Accepted: December 19, 2003

Abstract GERI-155 is a macrolide antibiotic containing two deoxyhexose molecules and shows antimicrobial activities against Gram-positive bacteria. Deoxysugar biosynthetic gene cluster of GERI-155 from *Streptomyces* sp. GERI-155 genome was cloned. Four *orfs* were identified and a putative *orf* presumed to be the dTDP glucose-4,6-dehydratase gene was designated as *gerE*. *GerE* was expressed in *E. coli* by using a recombinant expression vector pHJ1. The expressed protein was purified from *E. coli* cell lysate by using ammonium sulfate fractionation, and DEAE-sepharose CL-6B and hydroxylapatite column chromatography. The molecular mass of the expressed protein correlated with the predicted mass that was deduced from the cloned gene sequence data. The recombinant protein was a homodimer with a subunit relative molecular weight of 39,000 Dalton. It was found to have dTDP-glucose 4,6-dehydratase activity and also found to be highly specific for dTDP-glucose as a substrate. The values of K_m and V_{max} for dTDP-glucose were 32 μM and 335 nmol min^{-1} (mg protein)$^{-1}$, respectively. dTTP and dTDP were strong inhibitors of the protein. NAD^+, the coenzyme for dTDP-glucose 4,6-dehydratase, was tightly bound to the expressed protein.

Key words: dTDP-Glucose 4,6-dehydratase, GERI-155, deoxyhexose, dTDP-glucose, dTDP-4-keto-6-deoxyglucose, *Streptomyces* sp. GERI-155

Hexoses, by virtue of their universal occurrence in the glycoconjugates and complex carbohydrates of all living organisms, are a very important class of compounds. The most abundant hexoses are, of course, D-isomers of aldohexoses glucose, galactose, and mannose. However, in addition to these quantitatively most significant constituents, there is a great variety of additional, structurally-unique hexoses, which are often essential for the function of the entities containing them [11, 17, 21, 22]. Prominent among them are the deoxy- and dideoxyhexoses. A great number of antibiotics, including macrolides and anthracyclines, contain partially-deoxygenated hexose sugar components that are usually essential for the biological activity of a particular antibiotic [5]. A number of important deoxysugar biosynthetic genes have been cloned and identified, and mechanistic studies of the expressed enzymes have provided fresh insights into deoxysugar biosynthesis. Earlier examples include *in vitro* characterization of several TDP-D-glucose 4,6-dehydratase (TDPGDH; EC 4.2.1.46) from the erythromycin-producing strain *Sac. erythraea* [19], from the daunorubicin- and baumycin-producing organisms *Streptomyces* sp. C5, and from the daunorubicin-producing strain *S. peucetius* ATCC 29050 [18]. These TDPGDHs were purified to homogeneity or close to homogeneity and showed a requirement of NAD^+ as a cofactor, whereas the enzyme from *S. antibioticus* Tu99 showed a normal activity without exogenous NAD^+ [21]. Macrolide antibiotics are made in bacteria largely from simple fatty acid and glucose. GERI-155 produced by *Streptomyces* sp. GERI-155 is a macrolide antibiotic containing two deoxyhexose molecules and shows antimicrobial activities against Gram-positive bacteria [8]. The structure of GERI-155 is similar to chalcomycin and aldgamycin E, and the pathway for the biosynthesis of GERI-155 consists of a macrolide and two deoxysugars (Fig. 1).

dTDP-Glucose 4,6-dehydratase catalyzes the conversion of dTDP-glucose into dTDP-4-keto-6-deoxyglucose to produce a 6-deoxysugar. The gene that encodes dTDP-glucose 4,6-dehydratase, is also found within gene clusters which contain the biosynthetic genes of natural products

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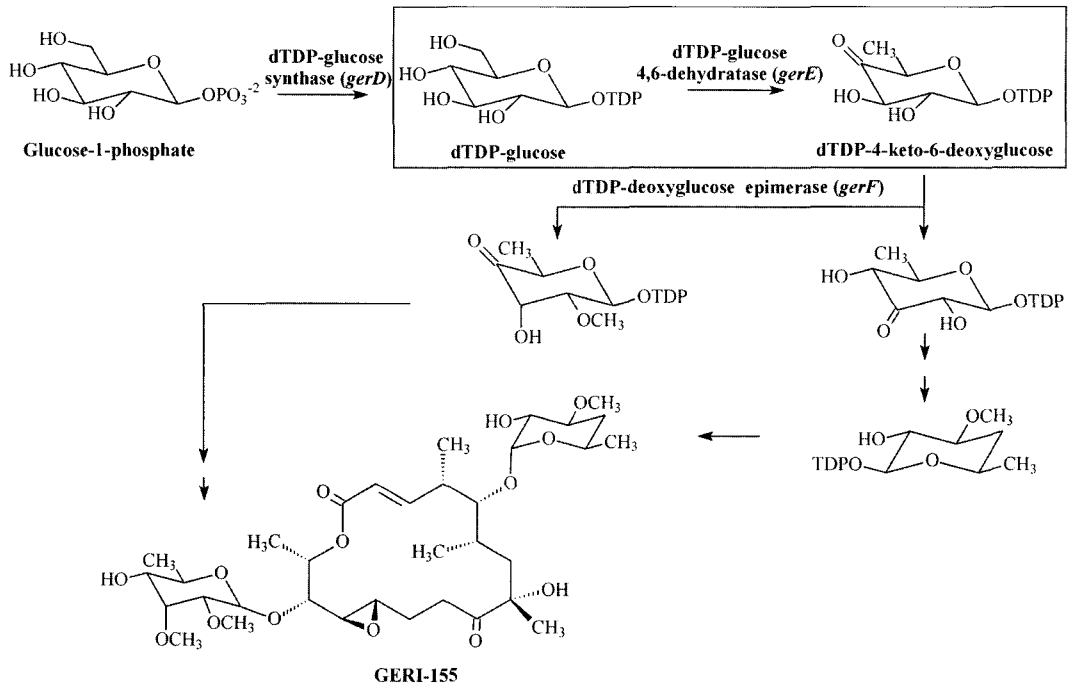

Fig. 1. The proposed biosynthetic pathway of two deoxysugars included in GERI-155.

containing 6-deoxyhexose moieties [5, 16, 22]. In most organisms that produce antibiotics containing amino/deoxy sugar moieties, the genes that encode glucose-1-phosphate thymidylyltransferase (=dTDP-glucose synthase) and dTDP-glucose 4,6-dehydratase are found within a common biosynthetic gene cluster [6, 14]. Therefore, many antibiotic biosynthetic gene clusters were cloned by using dTDP-D-glucose 4,6-dehydratase genes as probes. In this paper, we report the cloning of the deoxysugar biosynthetic gene cluster, overexpression and purification of the dTDP-glucose 4,6-dehydratase gene from *Streptomyces* sp. GERI-155, and some biochemical properties of the protein expressed, by using the *E. coli* system.

MATERIALS AND METHODS

Materials

dADP-D-glucose, dCDP-D-glucose, dGDP-D-glucose dTDP-D-glucose, UDP-D-glucose, dTMP, dTDP, dTTP, IPTG, Carbenicillin, β-Nicotinamide Adenine Dinucleotide (β-NAD), polyacrylamide, Standard proteins (blue dextrin, alcohol dehydrogenase, bovine serum albumin, carbonic anhydrase, and cytochrome C) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Trizma base, ammonium sulfate, potassium chloride, and potassuim phosphate were obtained from USB (Cleveland, OH, U.S.A.). Protein molecular weight markers for SDS-PAGE was purchased from MBI Fermentas (New York, NY, U.S.A.). DEAE-Sepharose CL-6B, Sepharose CL-6B, and Hydroxylapatite gels were supplied by Pharmacia Biotech. (Uppsala, Sweden).

Polymerase chain reaction (PCR) was carried out by using a GeneAmp kit (perkin-Elmer Cetus). *E. coli* XLI-Blue MRF was purchased from Stratagene (La Jolla, CA, U.S.A.). The Xpree™ protein expression system including pRSET plasmid and *E. coli* BL21 (DE3) was purchased from Invitrogen Corporation (San Diego, CA, U.S.A.). Restriction enzymes and other enzymes were purchased from Promega Biotech (Madison, WI, U.S.A.).

Bacterial Strains, Plasmids, and Culture Conditions

Streptomyces sp. GERI-155, obtained form the Korea Research Institute of Bioscience and Biotechnology, was grown on 1% malt extract, 0.4% yeast extract, 0.4% glucose (ISP Medium 2) at 28°C [8]. DNA manipulation was carried out by using *E. coli* XL-1 Blue MRF (Stratagene, La Jolla, CA, U.S.A.) as a host strain. *E. coli* BL21 (DE3) and pRSET-C vectors were purchased from the Invitrogen Corporation. *E. coli* strains were routinely grown on Luria-Bertani (LB) agar or liquid medium containing the appropriate antibiotics.

General Methods

General procedures for manipulating DNA were carried out according to Sambrook *et al.* [12]. Plasmids were isolated from *E. coli* by the alkaline lysis method. Restriction digestion, ligation, and other recombinant DNA

techniques were carried out according to the standard protocol.

Preparation of Cosmid Library

A cosmid library of *Streptomyces* sp. GERI-155 was prepared by using the vector pOJ446 [3]. The vector pOJ446 was digested with *Bam*HI and *Hpa*II. The chromosomal DNA isolated from *Streptomyces* sp. GERI-155 was partially digested by *Sau*3AI to produce 30- 40 kb size fragments, and then ligated into the vector [7]. The ligated DNA was packed *in vitro* by using the lamda packaging system from Stratagene as recommended by the manufacturer. The packaged phages were then transfected into *E. coli* XL1-Blue MRF. The colonies were selected in apramycin plates (50 μg/ml).

Design of PCR Primers of dTDP-Glucose 4,6-Dehydratase Gene as Probe

PCR primers of the dTDP-D-glucose 4,6-dehydratase gene were designed according to the amino acid consensus sequences found within the known five dehydratases. Based on the amino acid consensus sequences, two oligonucleotide primers (JKS-I and JKS-II) were synthesized by taking into account the preferred codon usage of *Streptomyces* having 60% G or C in the first base, 40% in the second base, and 90% in the third base of each codon [2, 20]. JKS-I was designed from the homologue site (sense, HFAAESHV: 5-CAC TTC GGY GGY GAG TCY CAC GT-3) and JKS-II from the homologue site (antisense, CSNNYGP: 5-GGY CCG TAG TTG TTY GAG CA-3, Y=G/C) [15].

Nucleotide Sequencing and Analysis

Nucleotide sequences were determined by the dideoxy chain termination method by using the automatic laser (fluorescence sequencers). Computer-aided sequence analysis was completed with the DNASIS software package (version 2.1, 1995; Hitachi Software Engineering), and database searches were performed with the BLAST program on the server of the National Center for Biotechnology Information (NCBI).

Cloning of dTDP-Glucose 4,6-Dehydratase (*gerE*) Gene and Construction of Expression Vector

gerE was amplified from the cloned GERI-155 biosynthetic gene cluster by using two oligonucleotide primers complementary to the sequence at each end of the gene. The primers DH-1 (5'-GAAAGGCGGATCCAGGATGC-GC-3') and DH-3 (5'- GCGGCGAAGCTTCCGCTCATC-3') contain *Bam*HI and *Hin*dIII sites, respectively. The PCR condition was performed on a Tachne thermocycler (Eppendorff) by using a Pre-Mix™-Top kit (Bioneer, Daejeon, Korea). The PCR product was isolated from low melting agarose, purified, digested with *Bam*HI and *Hin*dIII, and cloned to expression vector pRSETC. The resulting plasmid, pHJ1, was transformed to *E. coli* BL21 (DE3). *E. coli* BL21 (DE3) was used as a host strain for the expression of the GerE protein.

DNA Sequencing

Nucleotide sequencing of the PCR product was carried out directly on single- and double-stranded templates, by using M13, pBluescript, and several synthetic oligonucleotides.

Protein Determination

Protein concentration was determined by the method of Bradford [4] using bovine serum albumin as a standard. Estimations of protein concentration by measuring the A_{280} was used for routine monitoring of column fractions.

Assay for dTDP-D-Glucose 4,6-Dehydratase

Assay for dTDP-D-glucose 4,6-dehydratase activity was carried out in a reaction mixture of 100 mM Tris (pH 7.6), dTDP-D-glucose (1 mM), NAD^+ (1 mM), and enzyme solution. The mixture was incubated for 30 min. At the end of the incubation period, the reaction was terminated by adding 0.1 N of NaOH and incubated for 20 min at 37°C. Extinction differences at 318 nm were measured between reaction mixture and control reaction mixture. The amount of product formation was determined by using ε of 6,500 $M^{-1}l^{-1}$. One unit of enzyme activity corresponds to the formation of 1 μmol of product per hour under the assay condition, and the specific activities are reported as units per milligram of protein.

Overexpression and Purification of Recombinant Protein

Transformed cells were grown in LB broth (600 ml) containing carbenicillin (100 μg/ml) to OD_{600} of 0.8 at 25°C and then IPTG was added to a concentration of 0.4 mM. After a further 3 h growth at 25°C, the cells were harvested by centrifugation at 5,000 ×*g* for 5 min, and resuspended into the standard buffer (25 ml) consisting of 50 mM Tris/HCl (pH 7.6), 1 mM EDTA, 1 mM $MgCl_2$, and 1 mM DTT. The cells were disrupted by ultrasonicator and then centifuged at 15,000 ×*g* for 30 min. The supernatant is referred to as the crude extract (step 1). The crude extract fraction was brought to 25% saturation with ammonium sulfate powder and then centrifuged at 15,000 ×*g* for 30 min. The pellet was discarded and the supernatant was brought to 80% saturation with ammonium sulfate powder. This second precipitate was collected by centrifugation and dissolved in the standard buffer solution (10 ml) and dialyzed against the same buffer (step 2). The enzyme solution from step 2 (13.5 ml) was applied to a column of DEAE-Sepharose CL-6B (2.0×25 cm) preequilibrated with the standard buffer. After washing with 120 ml of the standard buffer containing 200 mM KCl, the enzyme was eluted with a linear gradient of 200 mM to 800 mM potassium

chloride in 500 ml of the standard buffer and active fractions were combined (step 3). Ammonium sulfate powder was added to the enzyme solution (51 ml) from step 3 to give a 30% saturation and then centrifuged for 30 min. The resulting supernatant was brought to 60% saturation with ammonium sulfate powder, then the precipitate was collected by centrifugation. The precipitated proteins were redissolved into the buffer consisting of 5 mM potassium phosphate (pH 7.0) and 1 mM DTT, and dialyzed against the same buffer (step 4). The enzyme solution from step 4 (4 ml) was applied to a column of hydroxylapatite (1.5×25 cm) preequilibrated with 5 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT. The column was then washed with 100 ml of the same buffer. A 300 ml of linear gradient of 5- 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT was then used to elute the enzyme. The active protein solution (12 ml) eluted from 10 mM to 15 mM potassium phosphate buffer was combined and stored at - 85°C.

Molecular Weight Determination

The subunit molecular weight and purity of the enzyme samples were determined by SDS-PAGE as described by Laemmli [9] using the following standards: β-galactosidase (116,000), bovine serum albumin (66,200), ovalbumin (45,000), lactate dehydrogenase (35,000), restriction endonuclease *Bsp*981 (25,000), β-lactoglobulin (18,400), lysozyme (14,400). The separating and stacking gels were 10% and 5% polyacrylamide, respectively. The molecular mass of the native purified enzyme was determined by using gel filtration on Sepharose CL-6B (1.4×80 cm). Chromatographic runs with the purified enzyme and the following protein standards calibrated the column: Alcohol dehydrogenase (150,000), bovine serum albumin (66,200), carbonic anhydrase (31,000), and blue dextrin (void volume). The reference and sample proteins were applied in a volume of 1.5 ml and eluted at a constant flow rate of 6 ml/h. The apparent molecular mass of the enzyme was estimated from a plot of *Ve/Vo* (elution volume) against the logarithm of the molecular mass of the standard proteins.

Kinetics, and Substrate and Inhibitor Specificity

K_m and V_{max} were determined for dTDP-D-glucose from Lineweaver-Burk double reciprocal plots of initial rates of enzyme reactions. For the kinetics as a function of TDP-D-glucose concentration, NAD^+ was in excess (1 mM) and the concentration of TDP-D- glucose varied from 20 μM to 200 μM. To determine NAD^+ requirement for enzyme activity, TDP-D-glucose was in excess (1 mM) and the concentration of NAD^+ varied from 0 mM to 1.0 mM.

Inhibition reactions were carried out by using 2 mM of inhibitor (TTP, TDP, or TMP). Alternative substrates (dADP-D-glucose, dGDP-D-glucose, dCDP-D-glucose, UDP-D-glucose, or dTDP-D-glucose) were used at a concentration of 1 mM.

RESULTS

Cloning of the Deoxysugar Biosynthetic Gene Cluster

In order to clone the deoxysugar biosynthetic gene cluster of GERI-155 from *Streptomyces* sp. GERI-155 genome, the genomic DNA library was screened by colony hybridization using the dehydratase-PCR product as a probe [15]. Twelve colonies (pGERI-1~12) were screened as positively hybridized clones. pGERI-5 cosmid DNA was hydrolyzed with *Bam*HI and the fragments were separated in 0.5% agarose for Southern blot analysis. A 4-kb *Bam*HI-fragment was hybridized with a ^{32}P-labeled dehydratase-PCR product probe. Therefore, the 4-kb *Bam*HI fragment was subcloned into pGEM vector to give pGERI-54 (Fig. 2). The 4-kb fragment inserted in pGERI-54 was sequenced and analyzed to identify the open reading frame. Four *orfs* were identified and a putative *orf* supposed to code the dTDP-glucose 4,6-dehydratase gene was designated as *gerE* (Fig. 2).

The *gerE* has an ATG translation initiation codon and a TGA stop codon (Fig. 3). GerE, as deduced from its nucleotide sequences is composed of 323 amino acids with molecular mass of 37,000 Da. The overall G+C content of the coding sequence is 69%, which is typical for the genus *Streptomyces*. The comparison of the deduced amino acid sequences with known sequences in the database showed identities of 66%, 63%, 61%, and 58% to the sequences of the AprE from *Streptomyces tenebrarius*, SgcA from *Streptomyces globisporus*, NbmH from *Streptomyces narbonensis*, and GraE from *Streptomyces violaceoruber*, respectively (Fig. 4).

Heterologous Expression of *gerE* Gene in *E. coli*

The *gerE* gene was amplified by PCR using the designed primers. The 22-mer region of the modified forward primer was designed to contain a *Bam*HI site. The reverse

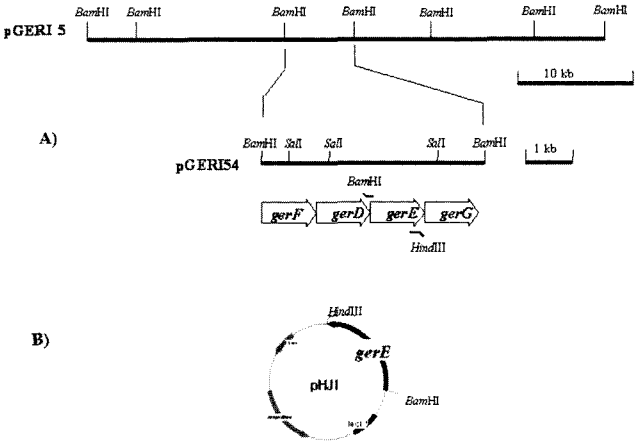

Fig. 2. (A) Map of pGERI5 and pGERI54. (B) Recombinant plasmid pHJ1 containing the *gerE* gene.

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GCGACGCTCACCGGTTGATCACCCCCTCCCCCACACAGCACGGCAGAAAGGCAGATCAAGG
ATGCGCGTCCTGGTGACCGGGGCGGCGGGCTTCATCGGCTCGCACTTCGTCAGACAGCTC 60
M  R  V  L  V  T  G  A  A  G  F  I  G  S  H  F  V  R  Q  L
CTGTCGGGCTCCTATCCGGAGCTGGCCGACGCCCACGTCCTCTCGCTGGACAAGCTCACG 120
L  S  G  S  Y  P  E  L  A  D  A  H  V  L  S  L  D  K  L  T
TACGCCGGGAACACCGAGAACCTCGCCGAGGTCGCCGGCCACCCCCGTCACACCTTCCTG 180
Y  A  G  N  T  E  N  L  A  E  V  A  G  H  P  R  H  T  F  L
CACGGCGACATCTGCGACCGGTCCACGGTCACCCAGGCCCTGCGCGGCATCGACCTGGTG 240
H  G  D  I  C  D  R  S  T  V  T  Q  A  L  R  G  I  D  L  V
GTGCATTTCGCCGCCGAATCGCACGTGGACCGCTCGATCACGGACTCCGCCGCCTTCATC 300
V  H  F  A  A  E  S  H  V  D  R  S  I  T  D  S  A  A  F  I
ACCACCAACGTGCTCGGGACGCACACACTGCTCCGGTCGGCGCTTGAGGCGGGCGTGAGC 360
T  T  N  V  L  G  T  H  T  L  L  R  S  A  L  E  A  G  V  S
CGGTTCGTCCACGTCTCCACCGACGAGGTGTACGGTTCGATCCCCGAGGGCTCATCGGCC 420
R  F  V  H  V  S  T  D  E  V  Y  G  S  I  P  E  G  S  S  A
GAAACCGACCCCCTGGACCCGAACTCCCCCTACGCGGCGTCCAAGGCCTCCTCCGACCTC 480
E  T  D  P  L  D  P  N  S  P  Y  A  A  S  K  A  S  S  D  L
CTGGCGCTCGCCTTCCACCGCACACACGGCCTCGACGTCCGGGTGACGCGCTGCTCCAAC 540
L  A  L  A  F  H  R  T  H  G  L  D  V  R  V  T  R  C  S  N
AACTACGGCCCCCATCAGCACCCCGAAAAGGTCGTCCCGCTCTTCGTGACCCACTTGCTG 600
N  Y  G  P  H  Q  H  P  E  K  V  V  P  L  F  V  T  H  L  L
GAGGGCCTGCGCCTTCCCCTGTACGGCGACGGCCTCCACCGGCGCGACTGGCTGCACGTG 660
E  G  L  R  L  P  L  Y  G  D  G  L  H  R  R  D  W  L  H  V
GACGACCACTGCCGGGGCATCGCCATGGTCGCCGGCCGGGGAAGGGCCGGAGAGGTGTAC 720
D  D  H  C  R  G  I  A  M  V  A  G  R  G  R  A  G  E  V  Y
AACATCGGTGGCGGTACGGAACTGTCCAACGTCGACCTCACCCGTCGTCTTCTGGGCGTC 780
N  I  G  G  T  E  L  S  N  V  D  L  T  R  R  L  L  G  V
TTCGGCGCCGACTGGTCCGTGGTGGACAGGGTGCCGGACCGGGCGGCGCACGACCGTCGC 840
F  G  A  D  W  S  V  V  D  R  V  P  D  R  A  A  H  D  R  R
TACTGCGTGGACACCCGGAAGATCACCGAGGAGCTGGGATGGGCTCCCCGGGTCGCCTTC 900
Y  C  V  D  T  R  K  I  T  E  E  L  G  W  A  P  R  V  A  F
GAGCGCGGGCTCGCGGAGACCGTCGACTGGTACCGGGACAACGGCACGTGGTGGAAGGCG 960
E  R  G  L  A  E  T  V  D  W  Y  R  D  N  G  T  W  W  K  A
CTGAAACCGTGA                                                  972
L  K  P  *
AACCGTGACGCCCGGAAGCGGATCACGCATGAGCGGAGGGTCCGCCGCGACCGCCGCCGGC
TCCACCGACGCGGGCGACCTCTGGCTGCGCCG
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Fig. 3. Nucleotide sequence of *gerE* and its deduced amino acid sequence.

primer contained a *Hind*III site. The PCR product (1 kb) obtained was cloned to an expression vector pRSETC to construct pHJ1. The pHJ1was resequenced to confirm no mutation and used to transform the expression host *E. coli* BL21 (DE3), which harbors a prophage carrying the gene for the T_7 RNA polymerase under control of the *lac UV5* promoter. Induction with IPTG in cells transformed with pHJ1 resulted in the production of the 39,000 Da protein. This protein band was intensified by a prolonged induction time of 3 h (Fig. 5). The same protein band was absent in the cell-free extracts obtained from IPTG-induced cells harboring pRSET-C. The molecular mass was in accordance with the predicted mass (39,000 Da) of the protein expressed from the *gerE* gene plus flanking sequence of the expression vector.

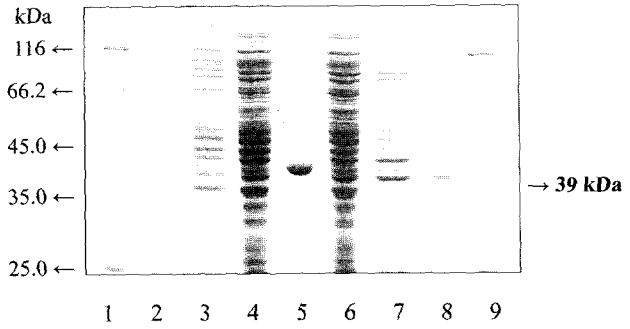

Fig. 5. Purification fractions and molecular weight determination of the dTDP-glucose 4,6-dehydratase by SDS-PAGE.
Lanes 1 & 9: SDS-PAGE standard marker; Lanes 2 & 3: *E. coli* BL21 (DE3) pHJ1 (Control/Induction). Lanes 4 & 5: Crude extract and pellet; Lane 6: 1st Ammonium sulfate fractionation (25-80%). Lane 7: Fraction of DEAE-sepharose CL-6B chromatography. Lane 8: Fraction of hydroxylapatite chromatography.

Purification of dTDP-D-Glucose 4,6-Dehydratase

The enzyme was purified from cells of *Streptomyces* sp. GERI-155 as described in Materials and Methods, and the results of a typical purification are shown in Table 1. Two enzymes from *E. coli* and GerE from *Streptomyces* sp. GERI-155 were separated at the hydroxylapatite column chromatography step (Fig. 6). About 180-fold purification of the enzyme from *Streptomyces* sp. GERI-155 was achieved with a yield of 15%. The enzyme preparation

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GerE    MRVLVTGAAGFIGSHFVRQLLSGSYPELADAHVLSLDKLTYAGNTENLASVAGHP--RHT  58
SgcA    MRMLVTGGAGFIGSQFVRATLHGELPGSEDARVTVLDKLTYSGNPANLTSVAAHP--RYT  58
AprE    MRVLVTGGAGFIGSHYVRELLGGAYPAFADAEVVVLDKLTYAGNEANLAPVADAAGSRLR  60
NbmH    MRLLVTGGAGFIGSHFVRQLLAGAYPDVPAGEVIVLDSLTYAGNRANLAPVEADP--RLR  58
graE    MRLLVTGAAGFIGSHYVREILAGSYPESDDVHVTVVDRLTYAGRRDNLP--EHHE--RLD  56

GerE    FLHGDICDRSTVTQALRGIDLVVHFAAESHVDRSITDSAAFITTNVLGTHTLLRSALEAG  118
SgcA    FVQGDTVDPRVVDEVVAGHDVIVHFAAESHVDRSIDTATPFVTTNVLGTQTLLEAALRHG  118
AprE    FVRGDICDAELVRTLMTGVDVVVHFAAESHVDRSILGATEFVMTNVLGTQTLLQAAREAG  120
NbmH    FVHGDIRDADLLARELRGVDAVVHFAAESHVDRSLAGASVFIGTNVQGTQTLLQCALDNG  118
graE    FVHGDICDRDLLDRVLPGHDAVVHFAAESHVDRSLTGPGEFVRTNVMGTQQLLDAALHAG  116

GerE    VSRFVHVSTDEVYGSIPEGSSAETDPLDPNSPYAASKASSDLLALAFHRTHGLDVRVTRC  178
SgcA    VGRFVHVSTDEVYGSIASGSWTEDTPLAPNVPYAASKASSDLMALAWHRTRGLDVVVTRC  178
AprE    VGKFVHVSTDEVYGSIDSGSWPEDHPLEPNSPYSASKASSDLLARAFHRTHGLPVCVTRC  180
NbmH    VGRVVHVSTDEVYGSIDSGSWNESSPLEPNSPYAASKASSDLVARAYHRTYGLDVRITRC  178
graE    VDRVLHVSTDEVYGSLDSGTWTETDSPLLPNSPYAASKASTTWSAAPTTVRHGLDVRITRC  176

GerE    SNNYGPHQHPEKVVPLFVTHLLEGLRLPLYGDGLHRRDWLHVDDHCRGIAMVAGRGRAGE  238
SgcA    TNNYGPYQYPEKVIPLFVTNILDGLRVPLYGDGAHRRDWLHVSDHCRAIQMVMNSGRAGE  238
AprE    SNNYGPYQFPEKVIPLFVTNLMDGRPVPLYGDGLNVRDWLHVDDHCRGIQLVVEGGRPGE  240
NbmH    CNNYGPYQHPEKLIPLFVTNLLDGGTLPLYGDGANVREWVHTDDHCRGIALVLAGGRAGE  238
graE    SNNYGPRQHPEKLIPNFVTRLLTGRQVPLYGDGRNVREWLHVDDHCRALQLVLTKGRAGE  236

GerE    VYNIGGGTELSNVDLTRRLLGVFGADWSVVDRVPDRAAHDRRYCVDTRKITEELGWAPRV  298
SgcA    VYHIGGGTELSNEELTGLLLTACGTDWSCVDRVADRQHDRRYSLDITKIRQELGYEPLV  298
AprE    VYNIGGGTELTNRELTERLLAVMGADWSMVEQVPDRQHDRRYSVDITKISTELGYRPRV  300
NbmH    VYHIGGGRELTNRELTGILLDSLGADWSSVRRVADRQHDLRYSLDGGKIERELGYRPQI  298
graE    IYNIGGGSGMSNREMTARLLDLLGADWDMVRHVEDRLGHDFRYAIDDSKIRELGYAPRW  296

GerE    AFERGLAETVDWYRDNGTWWKALK----------                          322
SgcA    AFEDGLAATVKWYHENRSWWQPLKEAAGLLDAVG----------                 332
AprE    PFEEGLARTVAWYRENRSWWEPLKRRAALAAR----------                   332
NbmH    APADGLARTIRWYRENRDWWEPLKATAPQPPATAPQPPATAVGVSA               344
graE    SIESGLGAVVDWYRDHPDFWRAPAS----------                          321
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Fig. 4. Multiple sequence alignments of known dTDP-glucose 4,6-dehydratase. SgcA [10], AprE, NbmH (AF521878), GraE [1].
The deduced amino acid sequence was compared. Identical residues are highlighted in gray.

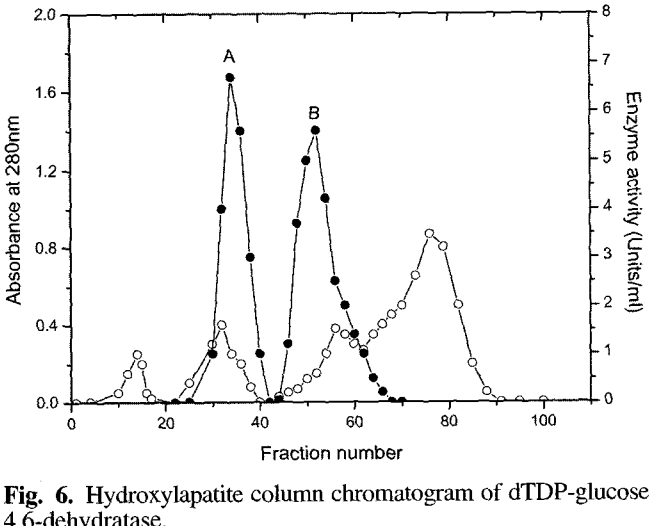

Fig. 6. Hydroxylapatite column chromatogram of dTDP-glucose 4,6-dehydratase.
Pooled enzyme solution from the 2nd ammonium sulfate fractionation step was separated by a column of hydroxylapatite (1.5×25 cm) (A peak: Expressed protein of *gerE*; B peak: Enzyme from *E. coli*).

Table 1. Scheme for purification of dTDP-D-glucose 4,6-dehydratase from *Streptomyces* sp. GERI-155.

Purification step	Total protein (mg)	Total activity (μmol/h)	Specific activity (μmol/h^{-1}/mg)	Purification factor	Recovery (%)
Crude extract	2,796	137.3	0.05	1	100
1st Ammonium sulfate	1,657	83	0.05	1	61
DEAE-sepharose CL-6B	500	73.5	0.15	3	54
2nd Ammonium sulfate	187	33.4	0.18	3.6	25
Hydroxylapatite	2.21	20.25	9.16	187	15

obtained at the final step of purification was homogeneous by the criteria of SDS-PAGE (Fig. 5).

Molecular Weight of dTDP-D-Glucose 4,6-Dehydratase

The molecular weight of the purified enzyme was estimated as 78,000 Da by comparing its rate of filtration through gel permeation chromatography column of Sepharose CL-6B with the rates of standard proteins (Fig. 7). In order to characterize the enzyme subunits, the purified enzyme was subjected to SDS-PAGE. The molecular weight of dTDP-D-glucose 4,6-dehydratase determined was estimated at 39,000 Da by comparing its migration position with the standards (Fig. 5). These results indicate that dTDP-D-glucose 4,6-dehydratase from *Streptomyces* sp. GERI-155 has a homodimeric structure.

Kinetics, Substrate Specificity, and Some Biochemical Properties

The K_m and V_{max} for TDP-D-glucose of expressed enzyme was determined to be 32 μM and 335 nmol/min. mg, respectively (Table 2). In the absence of NAD^+ in the enzyme reaction mixture, the expressed enzyme showed about 50% enzyme activity compared to the standard reaction [NAD^+ was in excess (1 mM)] (Table 3). Only dTDP-D-glucose was used as a substrate by expressed protein. dADP-D-Glucose, dGDP-D-glucose, dCDP-D-glucose, or UDP-D-glucose was not used as a substrate at 1 mM concentration. The optimum pH and temperature of the enzyme reaction were 7.4 and 37°C, respectively (Table 2).

Inhibition of Expressed Enzyme by Nucleotides

Inhibition was determined by including dTTP, dTDP, or TMP in standard enzyme reaction mixtures. With 2 mM-TDP and TTP, enzyme activities of expressed protein were 10% and 50%, respectively, compared to the control (Fig. 8).

DISCUSSION

High level expression of genes cloned from *Streptomyces* in *E. coli* system can lead in many cases to the formation of inclusion bodies, very dense aggregates of insoluble proteins [13, 21]. Overexpression of *gerE* in *E. coli* resulted in the formation of inclusion body at a normal induction condition. However, factors such as induction at late

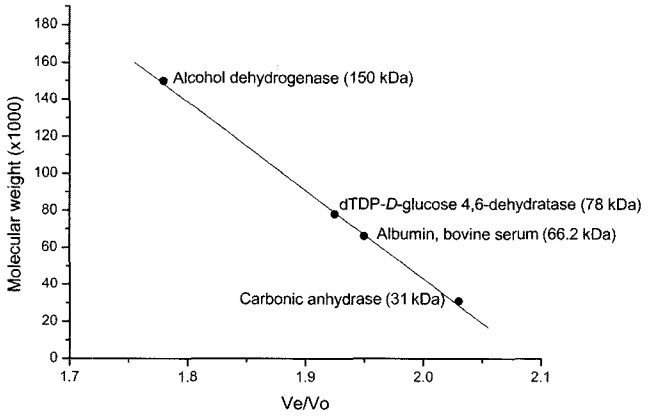

Fig. 7. Calibration curve for molecular weight estimation of native enzyme by gel permeation chromatography on Sepharose CL-6B column (1.4×80 cm).
Chromatographic runs with the purified enzyme and the following protein standards were used to calibrate the column: alcohol dehydrogenase (150,000), bovine serum albumin (66,200), carbonic anhydrase (31,000), and blue dextran (void volume).

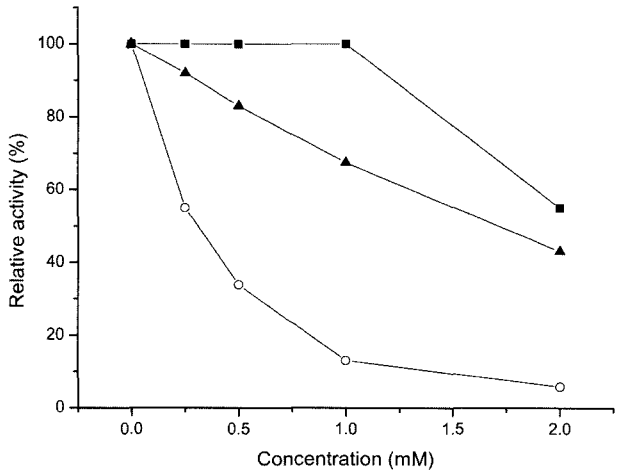

Fig. 8. Inhibitory effects of TTP, TDP, and TMP on the enzyme activity of expressed dTDP-D-glucose 4,6-dehydratase (●: TMP; ○: TDP; ▼: TTP).

Table 2. Comparison of the characteristics of dTDP-D-glucose 4,6-dehydratase from *Streptomyces* sp. GERI-155, *Streptomyces antibioticus* Tu99, *Streptomyces* sp. C5, *Steptomyces peucetius*, *Saccharopolyspora erythraea*, and *Escherichia coli* (Yoo *et al.*, 1999).

Characteristic	*Streptomyce* sp. GERI-155	*S. antibioticus* Tü99	*S.* sp. C5	*S. peucetius*	*Sac. erythraea*	*E. coli*
M_r of native activity (Da)	78,000	76,000	78,000	72,000	72,000	78,000
M_r of subunits (Da)	39,000	38,000	39,000	36,000	36,000	39,000
Enzyme structure	Homodimer	Homodimer	Homodimer	Homodimer	Homodimer	Homodimer
Otimum pH (Kinetics)	7.4	7.6 7.8	7.6	7.8	7.5	8.0 8.5
K_m (TDPG)	32 μM	28 μM	31 μM	35 μM	34 μM	70 μM
V_{max} (TDPG)[b]	335	295	309	201	433	7,000

exponential growth phase (OD_{600}, 0.8), lowering the cultivation temperature (25°C), and inducer concentration (0.4 mM IPTG), can lead to an increase in solubility of expressed protein upto 20% (data not shown).

We showed here that the GerE protein is dTDP-D-glucose 4,6-dehydratase. The enzymatic activity of GerE was clearly identified by the following criteria: (1) substrate specificities, (2) kinetic parameters, (3) conezyme requirement. To determine the substrate specificity of the expressed enzyme, dADP-D-glucose, dGDP-D-glucose, dCDP-D-glucose, dUDP-D-glucose, and dTDP-D-glucose were tested as a substrate. Among the substrates tested, only dTDP-D-glucose showed enzyme activity. Thus, it was confirmed that the expressed protein was dTDP-D-glucose 4,6-dehydratase specific for dTDP-D-glucose. Due to the high specificity of the protein, we examined the inhibitory role of dTTP, dTDP, and dTMP on the enzyme activity. dTTP and dTDP inhibited enzyme activity of the expressed dTDP-D-glucose 4,6-dehydratase at 2 mM (Fig. 8). In particular, dTDP had the greatest inhibitory effect. With 1 mM and 2 mM-TDP, enzyme activity of expressed protein were 16% and 10% of the value for the control, respectively. With 2 mM TDP, *Streptomyces* sp. C5, *Streptomyces peucetius*, and *Streptomyces antibioticus* enzyme activities were 38%, 42%, and 15%, respectively [21]. The average K_m values of dTDP-D-glucose 4,6-dehydratases from *Streptomyces* was 32 μM, whereas for *E. coli*-derived enzyme, K_m was 70 μM. The V_{max} value for *E. coli*-derived enzyme exhibited a 20-fold higher value than that for *Streptomyces*-derived enzyme. The expressed protein, Ger E, showed high affinity to NAD^+ (Table 3). In the absence of NAD^+ in the reaction mixture, the expressed enzyme showed approximately 50% of the enzyme activity. It was reported that dTDP-D-glucose 4,6-dehydratase (ChlE) from *S. antibioticus* Tü99 showed a high affinity level for NAD^+, and NAD^+ was bound to the enzyme tightly [21]. On the other hand, other *Streptomyces* and *E. coli* enzymes showed a low affinity [17, 21]. Therefore, it seems that expressed protein, Ger E, contains NAD^+ in its catalytic site.

Table 3. Effect of NAD^+ on the enzyme relative activities of *Streptomyces* sp. GERI-155 and *E. coli* dTDP-D-glucose 4,6-dehydratase.

Concentration of NAD^+ (mM)	Relative activity (%)	
	Streptomyces sp. GERI-155	*E. coli*
0	46.1	0
0.25	74.9	28.1
0.5	82.3	34.8
1.0	100	100

Acknowledgments

This work was supported by the Korea Science and Engineering Foundation (KOSEF: Project No. R01-2000-000-00151-0) and by a research fund from Chosun University, 2002.

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