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Expression of *orf7*(*oxi* III) as dTDP-Glucose 4,6-Dehydratase Gene Cloned from *Streptomyces antibioticus* Tü99 and Biochemical Characteristics of Expressed Protein

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Abstract The gene *orf7*(*oxi* III) was expressed using an *E. coli* system in anticipation that it would encode dTDP-glucose 4,6-dehydratase which is involved in the biosynthesis of the olivose moiety of chlorothricin produced from *Streptomyces antibioticus* Tü99. The solubility of the expressed protein increased up to 20% under optimal induction conditions. The expressed protein was purified from the *E. coli* BL 21(DE3) cell lysate by a 28.5-fold purification in two chromatography steps with a 38% recovery to near homogeneity. The molecular weight and N-terminal amino acid sequence of the purified protein correlated with the predicted mass and sequence deduced from the *orf7* gene. The purified protein was a homodimer with a subunit relative molecular weight of 38,000 Dalton. The expressed protein was found to exhibit dTDP-glucose 4,6-dehydratase activity and be highly specific for dTDP-glucose as a substrate. The values of *K'm* and *V'max* for dTDP-glucose were 28 μM and 295 nmol min^{-1} (mg protein)$^{-1}$, respectively. dTTP and dTDP were strong inhibitors of this enzyme. NAD^+, the coenzyme for dTDP-glucose 4,6-dehydratase, was tightly bound to the expressed protein.

Key word: Biosynthesis, chlorothricin, deoxysugar, olivose, *orf7* dTDP-glucose 4,6-dehydratase, *Streptomyces antibioticus* Tü99

Many antibiotics, including macrolides and anthracyclines, contain a partially deoxygenated hexose sugar component that is usually essential for biological activity, and the formation of deoxygenated hexose plays a key role in the biosynthesis of these antibiotics [20, 23, 24]. dTDP-glucose 4,6-dehydratase (EC 4.2.1.46), which converts dTDP-glucose to dTDP-4-keto-6-deoxyglucose, is a common essential enzyme for the biosynthesis of all types of 6-deoxyhexose [3, 14, 24]. dTDP-glucose 4,6-dehydratase have been purified or partially purified from a wide variety of bacteria [14, 23, 24, 25]. The genes that encode dTDP-glucose 4,6-dehydratase are also found within gene clusters which contain the biosynthetic genes of natural products containing 6-deoxyhexose moieties [1, 7, 15, 21].

Chlorothricin is a macrolide antibiotic produced by *S. antibioticus* Tü99. The chemical structure of chlorothricin is an unusual macrolide containing two glycosidically-linked olivoses, one of which is attached to a modified 6-methylsalicylic acid [6] (Fig. 1). It is likely that dTDP-glucose 4,6-dehydratase is one of the enzymes involved in the biosynthesis of olivose as 2,6-dideoxyhexose. Recently, a cluster of sugar biosynthesis genes associated with a type I PKS gene was cloned from *S. antibioticus* Tü99, which was expected to encode the enzymes involved in the formation of the two olivose moieties of chlorothricin. The gene *orf7* (previously called *oxi* III) in this gene cluster showed a strong homology with the dTDP-glucose 4,6-dehydratase genes cloned from other sources (Sohng *et al.*,

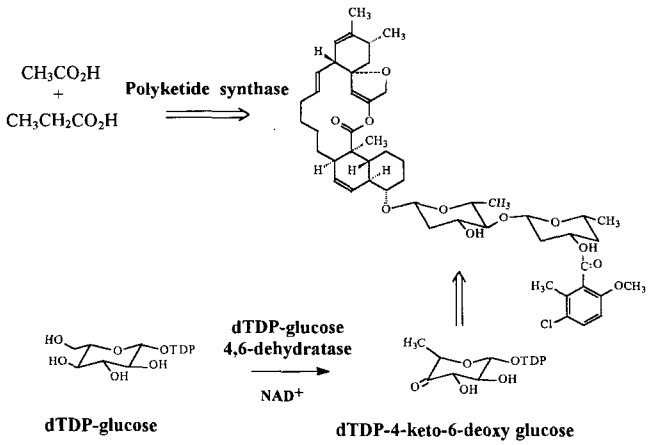

Fig. 1. Structure of chlorothricin.

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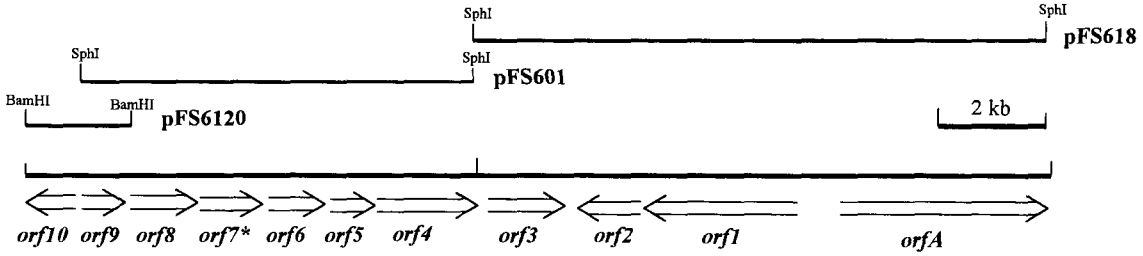

Fig. 2. Map of pFS601, pFS6180, and pFS6120, and the gene cluster of sugar biosynthesis genes associated with a type I PKS gene from *S. antibioticus* Tü99.

manuscript in preparation) (Fig. 2). The expression of the cloned genes and the biochemical analysis of the expressed proteins are important steps to confirm the biological function of cloned genes. This report describes the overexpression of the *orf7*, and the purification and biochemical characteristics of the expressed protein using an *E. coli* system.

MATERIALS AND METHODS

Materials

Polymerase chain reaction (PCR) was carried out using a GeneAmp kit (Perkin-Elmer Cetus). The Xpree™ protein expression system including pRSET plasmid and *E. coli* BL21(DE3) was purchased from the Invitrogen Corporation (San Diego, U.S.A.). Plasmids were propagated in *E. coli* XLI-Blue MRF' as described by Sambrook *et al.* [18]. All other chemicals were obtained from Sigma or United States Biochemical (Cleveland, U.S.A.). Restriction enzymes and other enzymes were purchased from Promega Biotech. (Madison, U.S.A.).

Construction of Expression Vector and Transformation

Two oligonucleotides (5'-GATCCA**CATATG**AACCTCC-TCGTCACC-3' and 5'-CGG**TGATCA**GCCATCGCG-3') were synthesizd containing an *Nde*I and *Bcl*I sites, respectively, and used as primers for the authentic *orf7* gene amplification. A PCR amplification of the *orf7* coding region was performed using pFS601 as a template and the two primers shown above (Fig. 3). After addition of 2.5 unit *pfu* DNA polymerase (Stratagene Co.), 30 cycles were performed at an annealing temperature of 57°C. The PCR product (1.0 kb) of the *orf7* gene was digested with *Nde*I and *Bcl*I (*Bam*H1-compatible end), and ligated at the *Nde*I and *Bam*HI restriction enzyme sites of the pRSET-B to produce the recombinant plasmid pRFJ4012. *E. coli* BL21(DE3) was transformed with pRFJ4012. *E. coli* BL21 (DE3), which is a lysogen of bacteriophage DE3 and carries the T7 RNA polymerase gene under the control of the inducible *lacUV5* promoter in the chromosome, was used as the host strain for the expression of the *orf7*.

DNA Sequencing

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

Measurement of dTDP-Glucose 4,6-Dehydratase Activity

A standard assay for enzyme activity was carried out in a reaction mixture of 100 mM Tris/Cl (pH 7.6), dTDP-D-glucose (1 mM), NAD^+ (1 mM), and the enzyme solution [24]. The mixture was incubated for 30 min at 3737°C. At the end of 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 318 nm. The amount of product formation was determined using an ε of 6,500 $M^{-1}L^{-1}$ for dTDP-4-keto-6-deoxyglucose [24]. 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.

Protein Concentration

Protein concentration was determined by the method of Bradford *et al.* [2] using bovine serum albumin as the standard. The estimation of protein concentration by measuring the A_{280} was useful for the routine monitoring of column fractions.

Purification of dTDP-Glucose 4,6-Dehydratase

Transformed cells were grown in an LB broth (1 l) containing carbenicillin (100 mg/ml) to OD_{600} of 1.8-2.0 at 30°C, and then IPTG was added to a concentration of 0.2 mM. After a further 3.0 h growth at 30°C, the cells were harvested by centrifugation at 15,000 g for 10 min, and resuspended into a standard buffer (80 ml) consisting of 50 mM Tris-HC1 (pH 7.6), 1 mM EDTA, 1 mM $MgCl_2$, and 1 mM DTT. The cells were disrupted by an ultrasonicator and then spun down at 15,000 g for 30 min. This 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 then discarded and the supernatant was brought to 60% saturation with ammonium

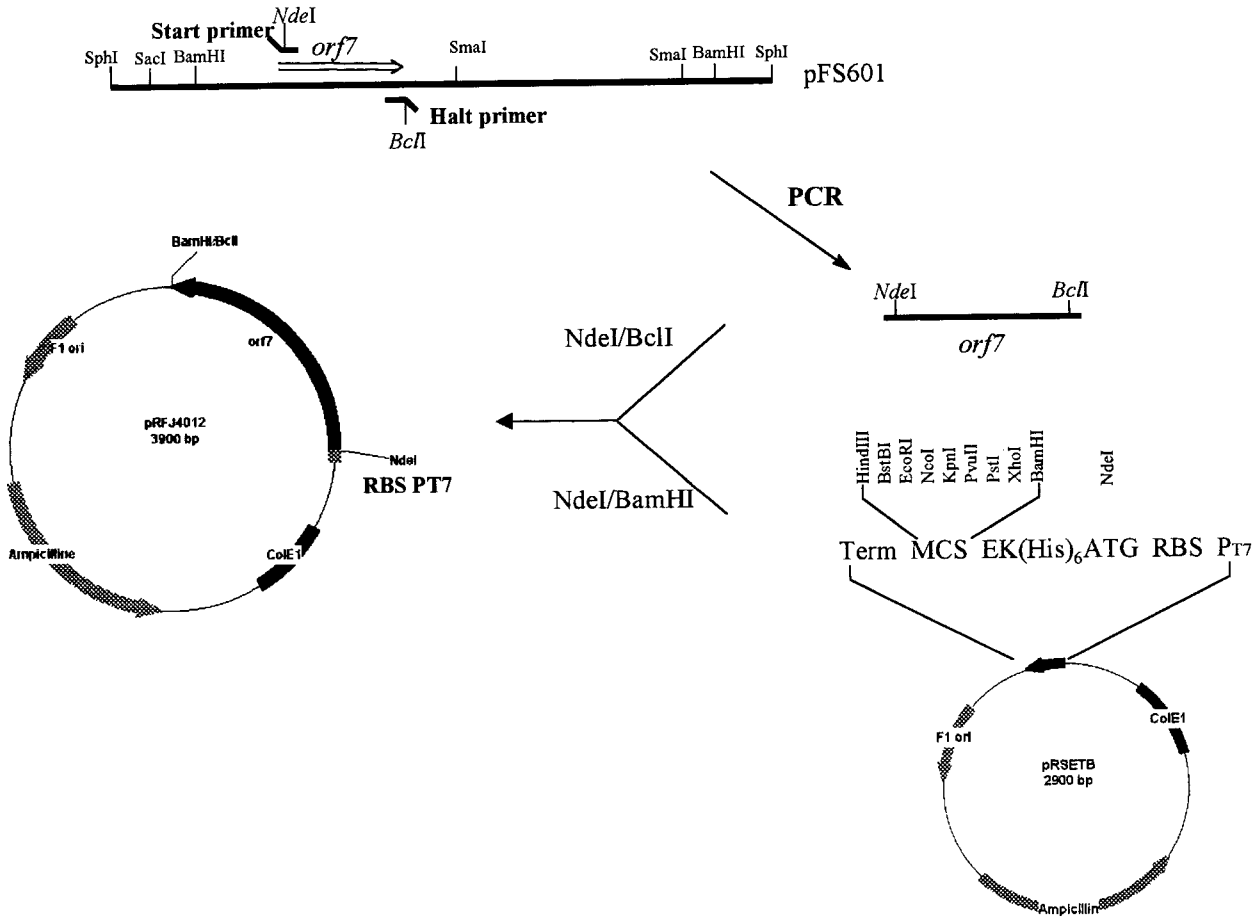

Fig. 3. Construction of the expression vector from pRSET-B and the amplified *orf7* DNA fragment.

sulfate powder. This second precipitate was collected by centrifugation and dissolved in the standard buffer solution and dialyzed against the same buffer (step 2). The enzyme solution from step 2 (10 ml) was applied to a column of DEAE-Sepharose CL-6B (2.5×32 cm) preequilibrated with the standard buffer containing 50 mM potassium chloride. After washing with 500 ml of the same equilibration buffer, the enzyme was eluted with a 1,800 ml linear gradient of 50 mM to 600 mM potassium chloride in the standard buffer and the active fractions were combined (50 ml) (step 3). Ammonium sulfate powder was added to the enzyme solution from step 3 to produce 33% saturation and then centrifuged for 30 min. The resulting supernatant was brought to 53% saturation with ammonium sulfate powder, and 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 then dialyzed against the same buffer (step 4). The enzyme solution from step 4 (4 ml) was applied to a column of hydroxylapatite (1.5×20 cm) preequilibrated with a 5 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT. The column was then washed with 60 ml of the same buffer. A linear gradient between 90 ml of the 5 mM and 90 ml of the 40 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT was then used to elute the enzyme. The active protein fractions (13 ml) eluted from the 10 mM to 15 mM potassium phosphate buffer were combined and stored at -85°C.

Molecular Weight Determination

The subunit molecular weight and purity of the enzyme samples were determined by SDS-PAGE electrophoresis as described by Laemmli *et al.* [11] using the standard molecular weight marker (Bio-Rad Co.). The separating and stacking gels were 11% and 5% polyacrylamide, respectively. The molecular weight of the purified native enzyme was determined using gel filtration on a Sepharose CL-6B column (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 (66,000), carbonic anhydrase (31,000), and blue dextran (void volume). The flow rate was 6 ml/h.

Kinetics, Substrate, and Inhibitor Specificity

The *K'm* and *V'max* values were determined for dTDP-glucose and NAD^+ from the Lineweaver-Burk double reciprocal plots of the enzyme reactions with a various range of dTDP-glucose and NAD^+ concentrations. Inhibition reactions were carried out using a 2 mM inhibitor (TTP, TDP, or TMP). To determine substrate specificity, TDP-glucose, ADP-glucose, GDP-glucose, CDP-glucose, or UDP-glucose were used at 1 mM.

Preparation of Apoenzyme

Apoproteins were prepared by the method of He *et al.* [5]. The 0.5 ml (250 mg protein) purified enzyme solution was dialyzed against 1 l of the 50 mM potassium phosphate buffer (pH 7.6) containing 1 mM DTT, 1 mM EDTA, and 2 M potassium bromide. The buffer was changed three times over a period of 2 days. The extent of the NAD^+ washout was determined on the basis of the loss of enzyme activity in the absence of exogenous NAD^+. After NAD^+ was completely removed, the dialysis bag containing the apoenzyme was transferred to 1 l of the 50 mM potassium phosphate buffer (pH 7.6) containing 1 mM DTT. The dialysis was continued for 2 days with three changes of buffer to remove potassium bromide.

Fluorescence Spectroscopy of Enzymes

The concentrations of native enzyme and prepared apoenzyme were adjusted to 76 mg/ml. A fluorescence measurement was carried out using a Shimazu RF-5301PC spectrofluorometer. Emission spectra were obtained by exciting the samples at 360 nm (5 nm slit width) and monitoring the emissions from 350 to 600 nm (15 nm slit width).

Amino-Termination Analysis

The purified enzyme on the SDS-PAGE was transferred to a PDVF membrane (Bio-Rad Co.) and the N-terminal sequence was determined using an Applied Biosystems 470A protein sequencer. This analysis was carried out at the Department of Biochemistry, University of Washington.

RESULTS

Expression of *orf7* in *E. coli*

The constructed *orf7* expression vector, pRFJ4012 (Fig. 3), was resequenced to confirm no mutation and authenticate the *orf7* sequence, and then used to transform the expression host *E. coli* BL21 (DE3). Cultivation and induction of the transformed *E. coli* BL21(DE3)/pRFJ4012 strain resulted in the production of the 38,000 Dalton protein (Fig. 4). This protein band was intensified by a prolonged induction time of up to 3 h. The same protein band was not present in the cell free extracts obtained from IPTG-induced cells harboring pRSET-B. The molecular mass was in accordance with the predicted molecular mass (38,000 Da) of the Orf7 protein (Fig. 4). The expressed protein was partially solubilized in the cell lysate (about 20%) and most of the expressed protein was in an insoluble form (data not shown). After IPTG induction, the dTDP-glucose 4,6-dehydratase activity of the cell-extract obtained from the cells transformed with pRFJ4012 was seven times higher than that of cells transformed with pRSET-B, which as the control exhibited a relatively high level of the same enzyme activity.

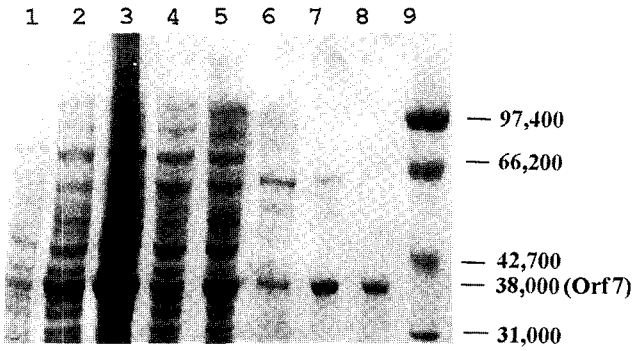

Fig. 4. SDS-PAGE analysis of dTDP-glucose 4,6-dehydratase from *S. antibioticus* Tü99.
Lane 1 and 2, total cell extract of *E. coli* BL21(DE3)/pRFJ4012 (control/induction); Lane 3, Pellet; Lane 4, Crude extract; Lane 5, 1st Ammonium sulfate; Lane 6, DEAE-Sepharose; Lane 7, 2nd Ammonium sulfate; Lane 8, Hydroxylapatite; Lane 9, SDS-PAGE standards: Phosphorylase b (97,000), bovine serum albumin (66,000), ovalbumin (42,000), and carbonic anhydrase (31,000).

Purification and Molecular Weight

The expressed protein in the cell-free extract was purified 28.5-fold with a yield of 38% to near homogeneity through ammonium sulfate fractionation, DEAE-Sepharose column chromatography, and hydroxylapatite chromatography (Table 1). The molecular weight of purified protein was determined to be about 76,000 Da by gel filtration (Fig. 5). The protein showed a single protein band with a molecular weight of about 38,000 Da upon SDS-PAGE (Fig. 4). These data suggest that the expressed protein is a homodimer with the same subunit.

Table 1. Scheme for purification of dTDP-glucose 4,6-dehydratase from *S. antibioticus* Tü99.

Purification step	Protein (mg)	Total activity (μmol/h)	Specific activity (μmol/h/mg)	Purification factor	Recovery (%)
Crude extract	1024	1094	1.07	1	100
1st Ammonium sulfate	766	1306	1.64	1.5	119
DEAE-Sepharose CL-6B	66	794	11.86	11.1	73
2nd Ammonium sulfate	26	586	20	18.7	54
Hydroxylapatite	14	412	30.44	28.5	38

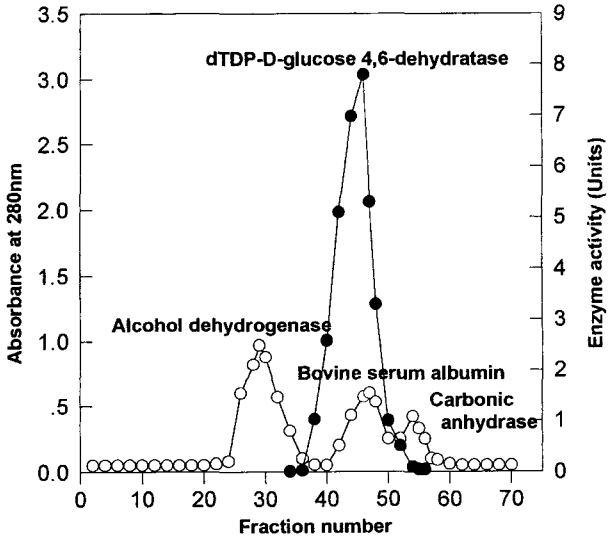

Fig. 5. Molecular weight estimation of native enzyme by gel permeation chromatography on Sepharose CL-6B column (1.4× 80 cm).

Chromatographic runs with the purified enzyme and the following protein standards calibrated the column: alcohol dehydrogenase (150,000), bovine serum albumin (66,000), carbonic anhydrase (31,000), and blue dextran (void volume). The flow rate was 6 ml/h. (● : protein concentration; ○ : enzyme activity).

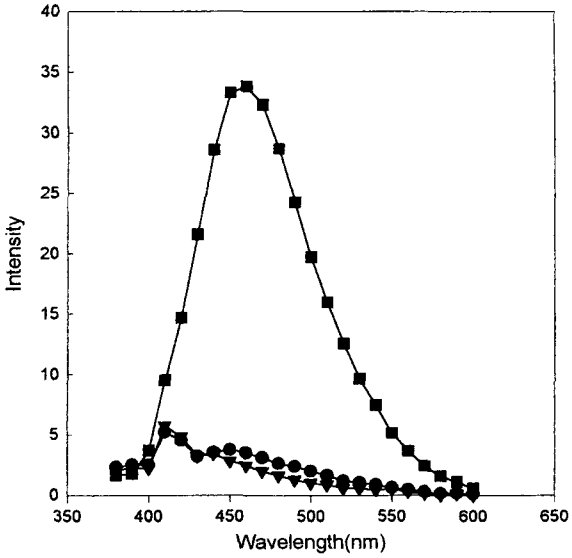

Fig. 6. Fluoresence spectra of dTDP-glucose 4,6-dehydratase from *S. antibioticus* Tü99.

The native dTDP-glucose 4,6-dehydratase (76 mg/ml, ■) and dTDP-glucose 4,6-dehydratase (76 mg/ml, ●) was treated with KBr to remove NAD^+ from the holo enzyme and reequilibrated with 50 mM potassium phosphate buffer (pH 7.6). ▲ : Buffer solution (50 mM potassium phosphate buffer, pH 7.6). The excitation wavelength was set at 360 nm and the emission of fluorescence between 350 nm and 600 nm was examined. The emission slit was 15 nm.

Amino-Terminal Sequencing

The N-terminal amino acid sequence of the expressed protein was determined by automated Edman degradation for the first 14 amino acids of the hydroxylapatite-purified protein and determined as Met-Asn-Lys-Lys-Val-Thr-Gly-Ala-Ala-Gly-Phe-Ile-Gly-Ser. This sequence of the purified protein correlated with the predicted sequence deduced from the *orf7*.

Substrate Specificity and Some Biochemical Properties

Only dTDP-glucose was used as a substrate by expressed enzyme. ADP-glucose, GDP-glucose, CDP-glucose, and UDP-glucose were not used as substrates at 1 mM concentration. The *K'm* and *V'max* values for dTDP-glucose were determined as 28 μM and 295 nmol/min. mg protein, respectively (Table 2). The *K'm* for NAD^+ was

Table 2. Comparison of the characteristics of dTDP-glucose 4,6-dehydratase from *S. antibioticus* Tü99 with other sources [23].

Characteristic	*S. antibioticus* Tü99	*Streptomyces* sp. C5	*S. peucetius*	*Sac. erythrea*	*E. coli*
M.W. of native enzyme (Da)	76,000	78,000	72,000	72,000	78,000
M.W. of subunits (Da)	38,000	39,000	36,000	36,000	39,000
Enzyme structure	Homodimer	Homodimer	Homodimer	Homodimer	Homodimer
Activity in absence of NAD^+	+[a]	-	-	-	+[a]
Optimum pH for activity	7.6~7.8	7.6	7.8	7.5	8.0~8.5
Kinetics					
K'm (TDP-D-glucose)	28 μM	31 μM	35 μM	34 μM	70 μM
V'max (TDP-D-glucose)[b]	295	309	201	433	7000
K'm (NAD^+)	120~250 μM	19 μM	20 μM	19 μM	100~200 μM
Inhibition by					
TTP(2 mM)	79%	37%	40%	77%	ND
TDP(2 mM)	85%	62%	58%	90%	ND
TMP(2 mM)	12%	25%	23%	11%	ND

[a] NAD^+ tightly bound to the enzyme, removable only with hard conditions [6, 25].
[b] nmol/min/mg protein.
ND: not determined by cited authors [25].

120–250 μM (Table 2). The optimum pH and temperature were 7.6–7.8 and 37°C, respectively. TDP and TTP strongly inhibited the dTDP-glucose 4,6-dehydratase activity. With 2 mM TDP and TTP, the dTDP-glucose 4,6-dehydratase activity of the expressed protein was only 15% and 21% of the control value, respectively (Table 2).

NAD^+ as a Cofactor

The native enzyme showed fluorescence at 450 nm, which is a typical characteristic of NAD^+. However, prepared apoenzyme showed no fluorescence at 450 nm and was catalytically inactive without exogenous NAD^+ (Fig. 6). It retained full activity in the presence of excess NAD^+ (data not shown).

DISCUSSION

The high level expression of many genes cloned from the Bacteria in an *E. coli* system, frequently resulted in the formation of inclusion bodies, which were very dense aggregates of insoluble proteins [12, 19]. The overexpression of *orf7* in *E. coli* resulted in the formation of an inclusion body under normal induction conditions. However, the solubility of the expressed protein increased up to 20% by modulating induction timing (at a late exponential growth phase; OD_{600}, 1.8–2.0), and lower cultivation temperature [30] and inducer concentration (0.1–0.2 mM IPTG).

The function of the expressed protein from *orf7* can be identified by the following criteria: (1) substrate specificities, (2) kinetic parameters, and (3) molecular structure. The expressed protein only used dTDP-glucose as a substrate, and was inhibited by dTDP and dTTP. Also, the *K'm* and *V'max* values for dTDP-glucose were similiar (28 μM and 295 nmol/min/mg, respectively) to those of dTDP-glucose 4,6-dehydratase from other *Streptomyces* strains (Table 2). In contrast, the *K'm* and *V'max* values for the *E. coli* enzyme [25] were 70 μM and 7,000 nmol/min/mg, respectively. The *V'max* value for *E. coli* enzyme was 20 times higher than that for the *Streptomyces* enzymes (Table 2). Finally, the expressed protein was a homodimer, a common feature of dTDP-glucose 4,6-dehydratases from microbial sources [23]. Accordingly, it was clearly confirmed that the expressed protein from the *orf7* gene was dTDP-glucose 4,6-dehydratase highly specific for dTDP-glucose as a substrate.

The dTDP-glucose 4,6-dehydratase from *S. antibioticus* Tü99 showed a normal activty without exogenous NAD^+, and the *K'm* value for NAD^+ was higher than that of other *Streptomyces* strains, yet similar to that of the *E. coli* enzyme (Table 2). These results suggest that NAD^+ coenzyme for dTDP-glucose 4,6-dehydratase is tightly bound to the expressed enzyme from *orf7*, similiar to the *E. coli* enzyme. This suggestion was confirmed by comparing the fluoresence of native- and apo-dTDP-glucose 4,6-dehydratase from *S. antibioticus* Tü99 (Fig. 6). The NAD^+ binding domain is known to be a β α β motif that corresponds to the region of the protein interacting with the adenosine pyrophospharyl moiety, and shows a homology within this conserved binding region that includes a glycine-rich phosphate binding loop, GXGXXG [13]. The N-terminal amino acid peptide of Orf7 has a β α β motif and exhibits the sequence motif Gly-Ala-Ala-Gly-Phe-Ile-Gly (GXXGXXG) (Fig. 7A). Also, the high affinity of NAD^+ to the enzyme is suggested to be due to the interaction of the ε-amino group of Lys-148, in accordance with the dehydratases and epimerase [5, 22]. This lysine residue is also conserved in dTDP-glucose 4,6-dehydratase

(A)

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                1                                                  48
Orf7    .........M NLLVTGAAGF IGSRYVHHCW NSAAAEPA. ..IHVLEQAH LTRRPRQRPR
OxiI    .........M RLAGHGGAGF IGA.FVRTLL DGRLPRPRD VPVTULDKLT YAGHRDNLP.
GraE    .........M RLLVTGAAGF IGSHYVREIL AGSYPESDD VHVTVVDRLT YAGRRDNLP.
StrE    .....MALTT HLLVTGAAGF IGSQYVRTLL GPGGP..PD VVVTAIDALT YAGNPDNLAA
RfbB1   .........V KILITGGAGF IGSAVVRHII KNT.....Q DTVVNIDKLT YAGNLESLSD
RfbB2   .........M KILVTGGAGF IGSAVVRHII NNT.....Q DSVVNVDKLT YAGNLESLAD
RfbB3   ...MQTANKK TILVTGGAGF IGSAVVRHII RNT.....Q DSVVNLDKLT YAGNLESLTD
RfbB4   .........M TWLVTRGAGF IGANFVLEAV SRG..... IRVVNLDALT YAGNLNTLAS
GalE1   .........M RVLVTGGSGY IGSHTCVQLL QNG..... HDVIILDNL. CNSKRSVLPV
GalE2   .........M RVLVIGGSGY IGSHTCVQLL QNG..... HDVVILDNL. CNSKRSVLPV
GalE3   .........M AILVTGGAGY IGSHTVVELL NVG..... KEVVVLDNL. CNSSPKSLER
RfbG    MIDKNFWQGK RVFVTGHTGF KGSWLSLWLT EMGAIVKGY ALDAPTVPSL FEIVRINDL.
AscB    MINNSFWQGK RVFVTGHTGF KGGWLSLWLQ TMGATVKGY SLAPPTVPSL FETARVADG.
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(B)

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      148
NSPYSASKASSDLL
NSPYSASKASSTCS
NSPYAASKASTTWS
NSPYSASKASGDLL
SSPYSASKASSDHL
SSPYSASKASSDHL
SSPYSASKASSDHL
NSPYSASKAASDHL
QSPYGKSKLMVEQI
QSPTGKSKLMVEQI
TNPYGTSKLMVEQI
YDPYSNSKGCAELV
YDPYSNSKGCAELV
```

Fig. 7. NAD^+ binding domain in β α β motif (A) and Comparison of NAD^+ binding domain of 2,3-hydroxy group of the nicotiamide ribosyl moiety in epimerase with dehydratase (B).

Orf7: dTDP-glucose 4,6-dehydratase (dTDPDH) from *S. antibioticus* Tü99; OxiI: dTDPDH from *S. antibioticus* Tü99 [20]; GraE: dTDPDH from *S. violaceoruber* Tü22 [1]; StrE: dTDPDH from *S. griseus* [10]; RfbB1: dTDPDH from *Salmonella serovar typhimurium* LT [7]; RfbB2: dTDPDH from *Shigella flexneri* [16]; RfbB3: dTDPDH from *Neisseria meningitidis* [4]; RfbB4: dTDPDH from *Xanthomonus campestris* [9]; GalE1: UDP-glucose 4-epimerase (UDPE) from *E. coli* [15]; GalE2: UDPE from *Salmonella typhimurium* [15]; GalE3: UDPE from *S. lividins* [15]; RfbG: dCDP-glucose 4,6-dehydratase (dCDPDH) from *Salmonella typhimurium* [7]; and AscB: dCDPDH from *Y. pseudotuberculosis* [8].

(Orf7) from *S. antibioticus* Tü99 (Fig. 7B). Further research is being conducted on how Lys-148 and the motif affect the NAD^+ binding affinity, using the site-directed mutagenesis and a kinetic study of various mutated enzymes.

Acknowledgments

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