Journal of Biochemistry and Molecular Biology, Vol. 32, No. 4, July 1999, pp. 363–369

JOURNAL OF
Biochemistry
and
Molecular Biology

© BSRK & Springer-Verlag 1999

Expression of *orf8* (*chlD*) as Glucose-1-Phosphate Thymidylyltransferase Gene Involved in Olivose Biosynthesis from *Streptomyces antibioticus* Tü99 and Biochemical Properties of the Expressed Protein

Jin-Cheol Yoo*, Eun-Ha Lee, Ji-Man Han, Hee-Jae Bang and Jae-Kyung Sohng[†]
Department of Pharmacy, Chosun University, Kwangju, Korea 501-759
[†] Department of Chemistry, SunMoon University, Asan, ChungNam, Korea 336-840

Received 1 April 1999, Accepted 16 April 1999

The *orf8*(*chlD*) gene cloned from *Streptomyces antibioticus* Tü99 was overexpressed using an *E. coli* system to confirm its biological function. Induction of the *E. coli* strain transformed with recombinant plasmid pRFJ 1031 containing *orf8* resulted in the production of a 43,000 dalton protein. Glucose-1-phosphate thymidylyltransferase activity of the cell extract obtained from the transformed strain was 4–5 times higher than that of the control strain. The expressed protein was purified 18-fold from *E. coli* cell lysate using three chromatographic steps with a 17% overall recovery to near homogeneity. The N-terminal amino acid sequence of the purified protein agrees with the nucleotide sequence predicted from the *orf8* gene. The SDS-PAGE estimated subunit mass of 43,000 dalton agrees well with that calculated from the amino acid composition deduced from the nucleotide sequence of the *orf8* gene (43,000 Da). Also, the native enzyme has a monomeric structure with a molecular mass of 43,000 dalton. The purified protein showed glucose-1-phosphate thymidylyltransferase activity catalyzing a reversible bimolecular group transfer reaction, and was highly specific for dTTP and α-D-glucose 1-phosphate as substrates in the forward reaction, and for dTDP-D-glucose and pyrophosphate in the reverse reaction.

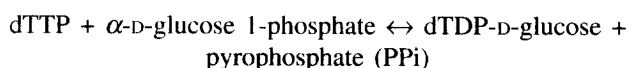
Keywords: Chlorothricin, Glucose-1-phosphate thymidylyltransferase, Olivose, *orf8*, *S. antibioticus* Tü99.

* To whom correspondence should be addressed.
Tel: 82-62-230-6380; Fax: 82-62-227-3963
E-mail: jcyu@mail.chosun.ac.kr

Introduction

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 the 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. Prominent among these are the deoxy- and dideoxyhexoses (Floss and Beale, 1989). 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. Most prominent among the deoxygenated hexoses found in antibiotics are the 2,6-dideoxyhexoses (Kessler *et al.*, 1993; Krugel *et al.*, 1993).

Given the diversity of 2,6-dideoxyhexoses encountered in various antibiotics and their biological significance, little is known about their detailed mode of formation. Numerous studies have demonstrated that the early common enzymatic steps for the biosynthesis of 2,6-dideoxyhexose found in antibiotics are the formation of dTDP-glucose from dTTP and α-D-glucose-1-phosphate by glucose-1-phosphate thymidylyltransferase (EC 2.7.7.24) (Bechthold *et al.*, 1995; Lombo *et al.*, 1997). Glucose-1-phosphate thymidylyltransferase catalyzes the following reversible bimolecular group transfer reaction:



dTTP + α-D-glucose 1-phosphate ↔ dTDP-D-glucose + pyrophosphate (PPi)

The number of genes that encode glucose-1-phosphate thymidylyltransferase were found within gene clusters which contain the biosynthetic genes of natural products containing 6-deoxyhexose moieties (Jiang *et al.*, 1991; Pissowotzki *et al.*, 1991; Merson-Davies and Cundliffe, 1994; Marolda and Valvano, 1995; Sohng *et al.*, 1998). Also, the expression of the glucose-1-phosphate thymidylyltransferase gene cloned from *Salmonella enterica* LT2 and the biochemical properties of the enzyme purified using an *E. coli* system have been reported (Lindquist *et al.*, 1993).

In the last few years, we have been investigating the biosynthetic pathway of dideoxyhexose using *in vitro* enzymatic synthesis of the 2,6-dideoxyhexose moieties of chlorothricin (Sohng and Yoo, 1996). 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 group (Holzbach *et al.*, 1978) (Fig. 1). Recently, a cluster of sugar biosynthesis genes associated with a type I PKS gene was cloned from *S. antibioticus* Tü99, and was expected to encode the enzymes involved in the formation of the two olivose moieties of chlorothricin. Also, the *orf8* gene (previously called *chlD*) in this gene cluster showed strong homology with the glucose-1-phosphate thymidylyltransferase genes cloned from other sources (Sohng *et al.*, manuscript in preparation) (Fig. 2). The expression of cloned genes and biochemical analysis of the expressed proteins are important steps to confirm the biological function of the cloned genes. This report describes the overexpression of *orf8*, and the purification and some biochemical properties of the protein expressed using an *E. coli* system.

Materials and Methods

Materials Inorganic pyrophosphatase, dTMP, dTDP, dTTP, ATP, CTP, GTP, UTP, ADP-D-glucose, CDP-D-glucose, dTDP-D-glucose, UDP-D-glucose, and all sugar 1-phosphates used were obtained from Sigma Chemical Co. (St. Louis, USA). DEAE-sepharose CL-GB, Sepharose-CL-6B, and Hydroxylapatite gels were products of Pharmacia Biotech. (Uppsala, Sweden).

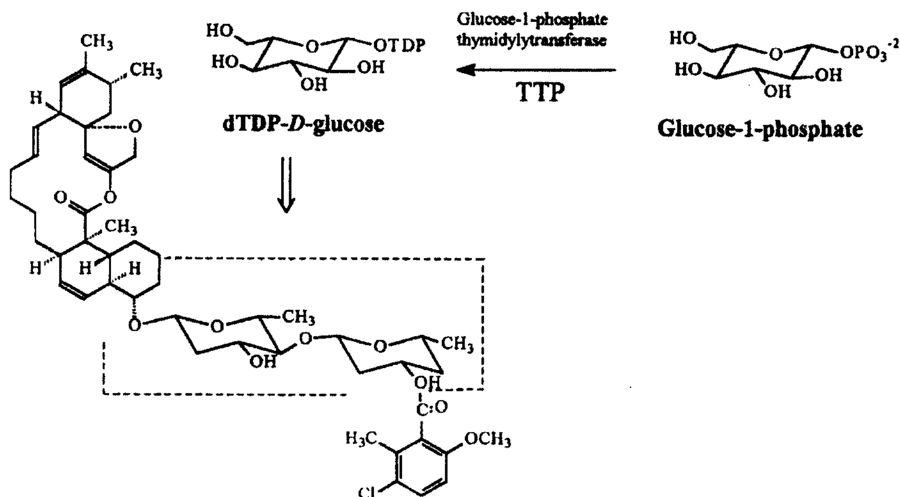


Fig. 1. Structure of chlorothricin.

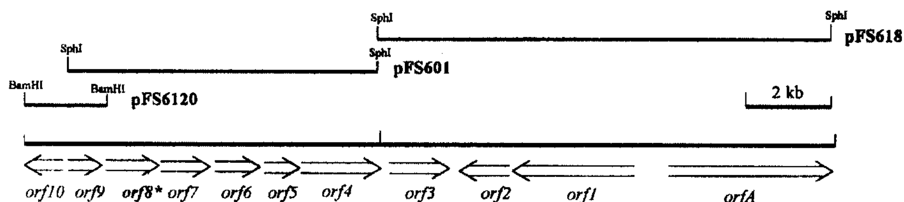


Fig. 2. Map of pFS601, pFS6180, and pFS6120, and the gene cluster of sugar biosynthesis genes associated with a type I PKS gene from *S. antibioticus* Tü99.

Polymerase chain reaction (PCR) was carried out with a GeneAmp kit (Perkin-Elmer Cetus). The XpreeTM protein expression system including pRSET plasmid and *E. coli* BL21(DE3) was purchased from Invitrogen Corporation (San Diego, USA). Plasmids were propagated in *E. coli* XLI-Blue MRF' as described by Sambrook *et al.* (1989). Restriction enzymes and other enzymes were purchased from Promega Biotech. (Madison, USA). All other chemicals were obtained from Sigma or United States Biochemical (Cleveland, USA).

Plasmid construction and bacterial strains Two oligonucleotides (5'-GACCGG**CATATG**AAGGCCCTCG-TACTG-3' and 5'-GGAGGT**TCATGA**CTGGAT-3') containing an *Nde*I and *Rca*I site were synthesized and used as primers for the authentic *orf8* gene amplification except for the start codon (the GTG of *orf8* was modified to ATG of the PCR product). PCR amplification of the *orf8* coding region was performed with pFS601 as a template and the two primers shown above (Fig. 3). After addition of 2.5 unit *pfu* DNA polymerase (Stratagene Co., La Jolla, USA), 30 cycles were performed at an annealing temperature of 57°C. The PCR product (1.2 Kb) of the *orf8* gene, having the modified start codon (GTG → ATG) of the authentic *orf8* gene was digested with *Nde*I and *Rca*I (*Nco*I-compatible end), and ligated at the *Nde*I and *Nco*I restriction enzyme sites of pRSET-B to produce the recombinant plasmid pRFJ1031 (Fig. 3). *E. coli* BL21 (DE3) was transformed with pRFJ1031. *E. coli* BL21 (DE3), which is a lysogen of bacteriophage DE3 and carries the T7 RNA polymerase gene under the control of the inducible *lac UV5* promoter in the chromosome (Studier and Hoffat, 1986), was used as a host strain for the expression of *orf8*.

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

Protein concentration Protein concentration was determined by the method of Bradford *et al.* (1976) using bovine serum albumin as the standard. The estimation of protein concentration by measuring the absorbance at A_{280} was useful for routine monitoring of column fractions.

Enzyme assay Glucose-1-phosphate thymidylyltrasferase activity was measured by following the change of concentration of dTTP and dTDP-D-glucose by HPLC analysis. The formation of dTDP-D-glucose from d-D-glucose-1-phosphate and dTTP was used in a standard assay protocol. The reaction mixture containing 15 μmol Tris·HCl (pH 8.0), 3.6 μmol $MgCl_2$, 7.2 μmol α-D-glucose-1-phosphate, 1.8 μmol dTTP, 1.8 U inorganic pyrophosphatase, and an appropriate aliquot of glucose-1-phosphate thymidylyltransferase (usually 30 μl) was incubated at 37°C in a total volume of 300 μl. Samples (30 μl) were withdrawn at timed intervals for up to 20 min, and immediately mixed with 1.0 ml 50mM potassium phosphate (pH 3.0) in order to terminate the reaction. The diluted samples were stored at 4°C until analysis by HPLC. From the integrated HPLC peak areas, the amount of TDP-D-glucose formed was calculated. One unit of enzyme activity corresponds to the formation of 1 nmol of dTDP-D-glucose per 20 min under standard assay conditions, and specific activities are reported as units per milligram of protein.

HPLC analysis A Shim-Pack CLC-DOS(M) Column (4.6 × 150 mm) equipped with a guard column (Shim-pack G-ODS (4); SHIMADZU Co.) was used for HPLC analysis. Sample or standard solutions were injected into the column and chromatograms were developed with a 200 mM potassium phosphate solution (pH 4.0). Flow rate was 1 ml/min, temperature was 25°C, and the absorbance of the effluent was recorded at 254 nm.

Purification of glucose-1-phosphate thymidylyltransferase Transformed cells were grown to an OD_{600} of 0.4 at 30°C in LB broth (1 L) containing carbenicillin (100 mg/ml) and then IPTG was added to a concentration of 0.4 mM. After a further 3.0 h growth at 30°C, cells were harvested by centrifugation at 10,000 × *g* for 10 min, and then washed twice with cold buffer A consisting of 50 mM Tris·HCl (pH 7.6), 1 mM EDTA, and 10 mM $MgCl_2$. The washed cells were resuspended in buffer B consisting of 20 mM Tris·HCl (pH 8.0), 1 mM $MgCl_2$, and 22% glycerol, and then disrupted ultrasonically while kept on ice. Cellular debris was removed by centrifugation at 15,000 × *g* for 30 min. The supernatant was referred to as the crude extract (step 1). The crude extract fraction (25 ml) was applied to a DEAE sepharose CL-6B column (2.5 × 33 cm) that had been pre-equilibrated with buffer B. After washing with 160 ml of buffer B, the enzyme was eluted with a linear gradient of 0.0 to 0.5 M NaCl in 800 ml of buffer B. Enzyme activity was detected in fractions eluted at 0.3–0.35 M NaCl and active fractions were combined (37 ml) (step 2). The enzyme solution from step 2 was diluted two-fold in modified buffer B (without 22% glycerol), and then brought to 35% saturation with ammonium sulfate powder and centrifuged at 15,000 × *g* for 30 min. The pellet was discarded and the supernatant was brought to 70% saturation with ammonium sulfate powder. This second precipitate was collected by centrifugation and dissolved in a small volume of buffer C consisting of 20 mM potassium phosphate buffer (pH 7.0) and 22% glycerol, and dialyzed against the same buffer (step 3). The enzyme solution from step 3 (1.2 ml) was applied to a sepharose CL-6B column (1.5 × 80 cm) previously equilibrated with buffer C. The enzymes were eluted with same buffer at a flow rate of 6 ml/h. Active fractions were combined (10 ml) and diluted with the same volume of 22% glycerol (step 4). The enzyme solution from step 4 was applied to a column of hydroxylapatite (1.5 × 27 cm) preequilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 22% glycerol. The column was then washed with 50 ml of the same buffer. A linear gradient composed of 90 ml of 10 mM and 90 ml of 120 mM potassium phosphate buffer (pH 7.0) containing 22% glycerol was then used to elute the enzyme at a flow rate of 6.4 ml/h. Glucose-1-phosphate thymidylyltransferase was eluted at a gradient concentration of about 100 mM potassium phosphate. The fractions containing enzyme activity were pooled (13 ml) and concentrated using a centricon (Amicon, Inc.), and then stored at −85°C.

Molecular weight determination The subunit molecular weight and purity of the enzyme samples were determined by SDS-polyacrylamide gel electrophoresis as described by Laemmli *et al.* (1970) using standard molecular weight markers (Bio-Rad Co.). Separating and stacking gels were composed of 11% and 5% polyacrylamide, respectively. The molecular weight of the native purified enzyme was determined using gel filtration on a

Sepharose CL-6B column (1.5 × 80 cm). Chromatographic runs with the purified enzyme and the following protein standards calibrated the column: β-amylase (200,000), alcohol dehydrogenase (150,000), bovine serum albumin (66,200), carbonic anhydrase (31,000), and blue dextran (void volume). The reference and sample proteins were applied in a volume of 1.5 ml and eluted at a constant flow rate of 6.4 ml/h. The apparent molecular mass of the enzyme was estimated from a plot of V_e/V_o (elution volume) against the logarithm of the molecular mass of the standard proteins.

Amino-termination analysis The SDS-PAGE-purified enzyme was transferred to a PDVF membrane (Bio-Rad Co., Hercules, USA). The N-terminal sequence was then sequenced with an Applied Biosystems 470A protein sequencer at the Department of Biochemistry, University of Washington.

Substrate and inhibitor specificity The substrate specificity of the purified enzyme for various nucleoside triphosphates (2.0 mM) and sugar 1-phosphates (6.0 mM) in the forward reaction, and for nucleotide sugars (2.0 mM) and pyrophosphate (6.0 mM) in the reverse reaction was studied using the standard assay system in which inorganic pyrophosphatase was omitted. Inhibition reactions were carried out using 2 mM and 5 mM inhibitor concentrations (ATP, CTP, GTP, UTP, TDP, or TMP).

Results and Discussion

Expression of *orf8* in *E. coli* Constructed *orf8* expression vector, pRFJ1031 (Fig. 3), was resequenced to confirm the absence of mutations and authenticate the *orf8* sequence containing the modified start codon (GTG → ATG), and then used to transform the expression host *E. coli* BL21 (DE3). Cultivation and induction of the transformed *E. coli* BL21(DE3)/pRFJ1031 strain resulted in production of the 43,000 dalton protein (Fig. 4). This protein band was intensified by a prolonged induction time to 3 h. The same protein band was absent in the cell-free extracts obtained from IPTG-induced cells harboring pRSET-B. The molecular mass was in accordance with the predicted molecular mass (43,000 Da) of the Orf8 protein (Fig. 4). High-level expression of many genes cloned from *Streptomyces* in an *E. coli* system often leads to the formation of inclusion bodies, very dense aggregates of insoluble proteins (Schein and Noteborn, 1988; Kil and Chang, 1998). Overexpression of *orf8* in *E. coli* at normal culture temperature (37°C) resulted in the formation of an inclusion body. Solubility of the expressed protein increased 20–30% by lowering the cultivation temperature to 30°C (data not shown). After IPTG induction, the glucose-1-phosphate thymidylyltransferase activity of the crude cell extract obtained from the cells transformed with pRFJ1031 showed 3–4 times higher activity than that of cells transformed with pRSET-B as a control.

Purification and physical characterization of glucose-1-phosphate thymidylyltransferase The expressed protein in cell-free extract was purified 18-fold with a yield of 17% to near homogeneity, through DEAE-Sepharose column chromatography, ammonium sulfate fractionation,

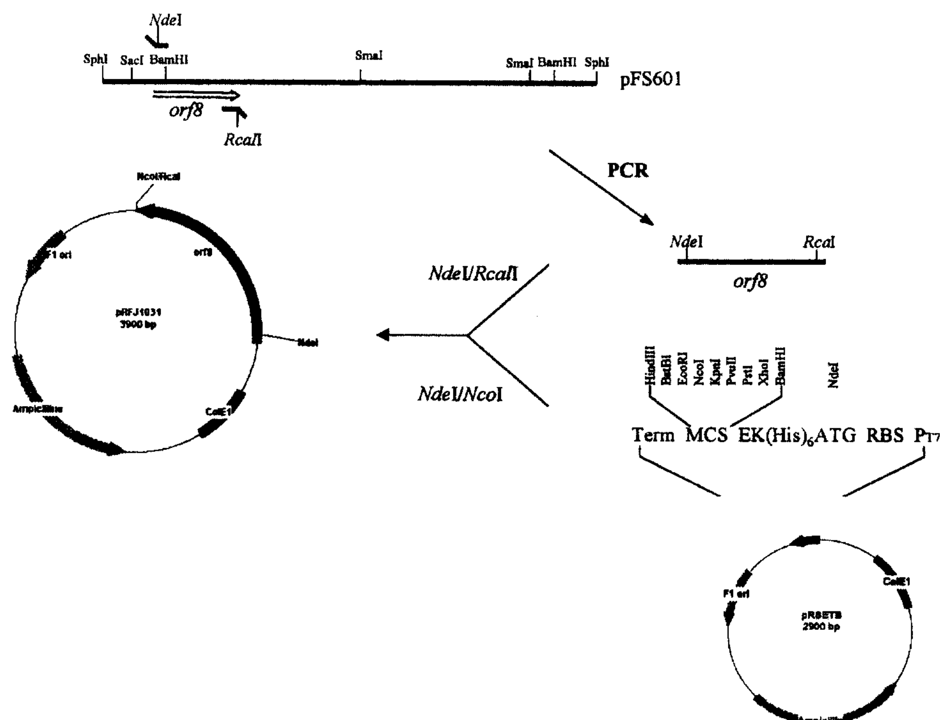


Fig. 3. Construction of the expression vector, pRFJ 1031, from pRSET-B and amplified *orf8* DNA fragment.

Fig. 4. SDS-PAGE analysis of glucose-1-phosphate thymidylyltransferase from *S. antibioticus* Tü99. Lanes 1 and 2, total cell extract of *E. coli* BL21(DE3)/pRFJ1031 (control/induction); Lane 3, Pellet; Lane 4, Crude extract; Lane 5, DEAE-Sepharose; Lane 6, Ammonium sulfate; Lane 7, Sepharose CL-6B; Lane 8, Hydroxylapatite; Lane 9, SDS-PAGE standards.

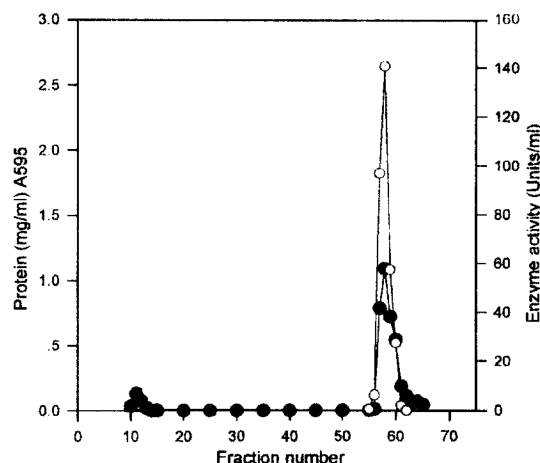


Fig. 5. Hydroxylapatite column chromatography step in the purification of glucose-1-phosphate thymidylyltransferase. Pooled enzyme solution from the ammonium sulfate fractionation step was separated by a column of hydroxylapatite (1.5 × 27 cm). The effluent was collected as 1.6 ml fractions, and the flow rate was 6.4 ml/h. (●; protein concentration, ○; enzyme activity)

Sepharose CL-6B gel permeation chromatography, and hydroxylapatite chromatography (Table 1). In the final hydroxylapatite chromatography step, coincidental elution of enzyme activity with protein is shown in Fig. 5. The purified enzyme revealed a specific activity of 53.67 units/mg of protein. The specific activity and purification fold of the enzyme solution obtained from the hydroxylapatite chromatography step were actually lower than that of the enzyme solution obtained from Sepharose CL-6B gel permeation chromatography (Table 1). It seems likely that glucose-1-phosphate thymidylyltransferase was inactivated by the hydroxylapatite gel (Ca^{2+}-phosphate) and/or potassium phosphate. Because of this, the enzyme solution obtained from the hydroxylapatite chromatography step was reequilibrated with Tris buffer and quickly stored at −85°C.

The protein showed a single protein band on SDS-PAGE, with a molecular weight of about 43,000 Da (Fig. 4.). Also, the molecular weight of the native purified protein was determined to be about 43,000 Da by gel filtration (Fig. 6). This data confirmed that the expressed protein has a monomeric structure. The molecular weight of the subunit obtained from *S. antibioticus* Tü99 was somewhat larger than that obtained from *S. enterica* LT2 (31,000 Da) (Lindquist *et al.*, 1993). The pH and temperature optimum of the enzyme were around pH 7.0–8.0 and 35–37°C, respectively.

N-Terminal sequencing The N-terminal amino acid sequence of the expressed protein was determined by automated Edman degradation for the first 10 amino acids of the hydroxylapatite-purified protein and found to be as met-lys-ala-leu-val-leu-ala-gly-gly-ser-. This sequence correlates with the predicted sequence deduced from the *orf8* gene.

Substrate and inhibitor specificity of glucose-1-phosphate thymidylyltransferase The substrate specificity of the purified enzyme for various nucleotide triphosphates and sugar-1-phosphates in the forward

Table 1. Scheme for purification of glucose-1-phosphate thymidylyltransferase from *Streptomyces antibioticus* Tü99.

Purification step	Total protein (mg)	Total activity (Units)	Specific activity (Units/mg)	Yield (%)	Purification (Fold)
Crude extract	639.2	1943	3.04		1
DEAE-sepharose CL-6B	35.89	518	14.43	100	4.8
Ammonium sulfate	6.93	300	43.29	58	14.2
Sepharose CL-6B	4.2	287	68.3	55	22.5
Hydroxylapatite	1.6	86	53.67	16.9	17.7

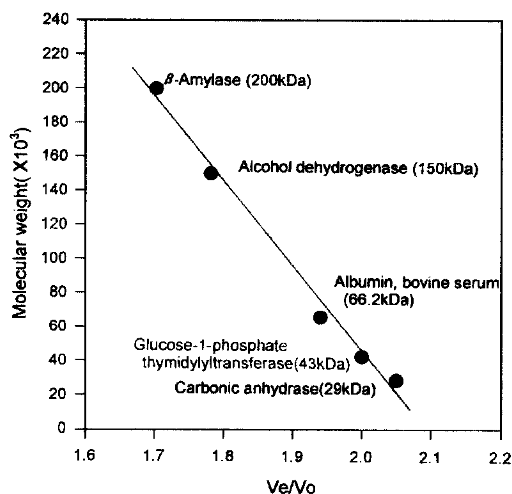


Fig. 6. Calibration curve for molecular weight estimation of native enzyme by gel permeation chromatography on Sepharose CL-6B column (1.4 × 80 cm). Chromatographic runs with the purified enzyme and the following protein standards calibrated the column: β-amylase (200,000), alcohol dehydrogenase (150,000), bovine serum albumin (66,200), carbonic anhydrase (29,000), and blue dextran (void volume).

reaction and nucleoside diphosphate sugars in the reverse reaction was examined. In the forward reaction, the highest activity was obtained with the combination of dTTP and α-D-glucose-1-phosphate (Table 2). Exchange of α-D-glucose 1-phosphate by α-D-galactose-1-phosphate yielded an 8% relative activity in combination with dTTP. With α-D-mannose-1-phosphate or α-D-glucosamine-1-phosphate sugar donors, no significant activity was observed. Neither was any significant activity observed when ATP, CTP, GTP, or UTP were used as nucleotide substrates. In the reverse reaction, the highest activity was obtained with dTDP-D-glucose and pyrophosphate (Table 2). Exchange of dTDP-D-glucose by ADP-D-glucose yielded a 7% relative activity. With CDP-D-glucose and UDP-D-glucose as nucleotide sugars, no significant activity was observed. For the glucose-1-phosphate thymidylyltransferase purified from *S. enterica* LT2, it was found that α-D-glucosamine-1-phosphate and UTP were used efficiently as a sugar donor and a nucleotide substrate, respectively, in the forward reaction (Lindquist *et al.*, 1993). Also, it was reported that glucose-1-phosphate thymidylyltransferase of *S. enterica* LT2 used UDP-D-glucose and pyrophosphate efficiently as substrates in the reverse reaction (Lindquist *et al.*, 1993). These data suggest that glucose-1-phosphate thymidylyltransferase from *S. antibioticus* Tü99 has a narrower range of substrate specificity than that of the glucose-1-phosphate thymidylyltransferase from *S. enterica* LT2. With 5 mM ATP and CTP, the glucose-1-phosphate thymidylyltransferase activity of the expressed protein was only 23% and 34% of the control value, respectively (Table 3).

According to the enzyme activity and substrate specificity of the purified protein, it was clearly confirmed that the expressed protein of the *orf8* gene cloned from *S. antibioticus* Tü99 is a glucose-1-phosphate thymidylyltransferase catalyzing a reversible bimolecular group-transfer reaction. Also, this enzyme was highly specific for dTTP and α-D-glucose-1-phosphate in the forward reaction, and for dTDP-D-glucose and pyrophosphate in the reverse reaction.

It was reported that glucose-1-phosphate cytidylyltransferase and glucose-1-phosphate thymidylyltransferase purified from *S. enterica* LT2 catalyze reversible

Table 2. Substrate specificity of glucose-1-phosphate thymidylyltransferase from *Streptomyces antibioticus* Tü99*.

	Substrate A (2 mM)	Substrate B (2 mM)	Relative activity
Forward reaction	ATP	α-D-glucose-1-phosphate	<0.001
	CTP	α-D-glucose-1-phosphate	<0.001
	GTP	α-D-glucose-1-phosphate	<0.001
	UTP	α-D-glucose-1-phosphate	<0.001
	TTP	α-D-glucose-1-phosphate	1.00
	TTP	α-D-galactose-1-phosphate	0.08
	TTP	α-D-mannose-1-phosphate	<0.001
	TTP	α-D-glucosamine-1-phosphate	<0.001
Reverse reaction	ADP-D-glucose	Pyrophsphate	0.07
	CDP-D-glucose	Pyrophsphate	0.02
	TDP-D-glucose	Pyrophsphate	1.00
	UDP-D-glucose	Pyrophsphate	0.02

*Purified glucose-1-phosphate thymidylyltransferase was incubated with substrates in 300 μl of 50 mM Tris HCl, pH 8.0, containing 12 mM $MgCl_2$.

Table 3. Effect of various nucleotides on the activity of glucose-1-phosphate thymidylyltransferase from *Streptomyces antibioticus* Tü99*.

Nucleotides tested	Relative remaining activity (%)	
	2 mM	5 mM
TTP	100	100
ATP	108	23
CTP	109	34
GTP	116	109
UTP	110	111
TDP	117	119
TMP	97	49

*Nucleotides were added to the standard forward reaction mixtures.

bimolecular group-transfer reactions by a ping-pong mechanism (Lindquist *et al.*, 1993; Lennart *et al.*, 1994). Detailed kinetic studies are currently being conducted to elucidate the reaction mechanism of glucose-1-phosphate thymidylyltransferase from *S. antibioticus* Tü99 .

Acknowledgments The authors wish to acknowledge the financial support of the Korea Research Foundation made in the program year of 1997.

References

Bechthold, A., Sohng, J. K., Smith, T. M., Chu, X. and Floss, H. G. (1995) Identification of *Streptomyces violeceoruber* Tü22 genes involved in the biosynthesis of granaticin. *Mol. Gen. Genetics* **248**, 610–620.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

Floss, H. G. and Beale, J. M. (1989) Biosynthetic studies on antibiotics. *Angewandte Chemie* **28**, 146–177.

Holzbach, R., Pape, H., Hook, D., Kreutzer, E. F., Chang, C.-J. and Floss, H. G. (1978) Biosynthesis of the macrolide antibiotic chlorothricin: Basic building blocks. *Biochemistry* **17**, 556–560.

Jiang, X.-M., Neal, B., Santiago, F., Lee, S. J., Romana, I. K. and Reeves, P. R. (1991) Structure and sequence of the *rfb* (O-antigen) gene cluster of *Salmonella serovar* typhimurium (strain LT2). *Mol. Microbiol.* **5**, 695–713.

Kessler, A. C., Haase, A. and Reeves, P. R. (1993) Molecular analysis of the 3,6-dideoxyhexose pathway genes of *Yersinia pseudotuberculosis* serogroup IIA. *J. Bacteriol.* **175**, 1412–1422.

Kil, M. W. and Chang, S. I. (1998) Expression in *E.coli*, purification, and characterization of the tobacco sulfonylurea herbicide-resistant recombinant acetolactate synthase and its interaction with the triazolopyrimidine herbicides. *J. Biochem. Mol. Biol.* (formerly *Korean Biochem. J.*) **31**, 287–295.

Krugel, H., Schumann, S., Hanel, F. and Fiedler, G. (1993) Nucleotide sequence analysis of five putative *Streptomyces griseus* genes, one of which complements an early function in daunorubicin biosynthesis that is linked to a putative gene cluster involved in TDP-daunosamine formation. *Mol. Gen. Genetics* **24**, 193–202.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (London) **227**, 680–685.

Lennart, L., Kaiser, R., Reeves, P. R. and Lindberg, A. A. (1994) Purification, characterization, and high performance liquid chromatography assay of *Salmonella* glucose-1-phosphate cytidylyltransferase from the cloned *rfb F* gene. *J. Biol. Chem.* **269**, 122–126.

Lindquist, L., Kaiser, R., Reeves, P. R., and Lindverg, A. A. (1993) Purification, characterization and HPLC assay of *Salmonella* glucose-1-phosphate thymidylyltransferase from the cloned *rfbA* gene. *Eur. J. Biochem.* **211**, 763–770.

Lombo, F., Siems, K., Brana, A. F., Mandez, C., Bindseil, K. and Salas, J. A. (1997) Cloning and insertional inactivation of *Streptomyces argillaceus* genes involved in the earliest steps of biosynthesis of the sugar moieties of the antitumor polyketide mithramycin. *J. Bacteriol.* **179**, 3354–3357.

Marolda, C. L. and Valvano, M. A. (1995) Genetic analysis of the dTDP-rhamnose biosynthesis of the *E. coli* Vw187(O7:K1) rfb gene cluster: Identification of functional homologs of *rfbB* and *rfbA* in the *rff* cluster and correct location of the *rffE* gene. *J. Bacteriol.* **177**, 5539–5546.

Merson-Davies, L. A. and Cundliffe, E. (1994) Analysis of five tylosin biosynthetic genes from the *tylIBA* region of the *Streptomyces fradiae* genome. *Molecular Microbiology* **13**, 349–355.

Pissowotzki, K., Mansouri, K. and Piepersberg, W. (1991) Genetics of streptomycin production in *Streptomyces griseus*: Molecular structure and putative function of genes *strELMB2N*. *Mol. Gen. Genetics* **231**, 113–123.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schein, C. H. and Noteborn, M. H. M. (1988) Formation of insoluble recombinant proteins in *E. coli* is favoured by lower growth temperature. *Bio/technology* **6**, 291–294.

Sohng, J. K. and Yoo, J. C. (1996) Cloning, sequencing and expression of dTDP-D-glucose 4,6-dehydratase gene from *Streptomyces antibioticus* Tü99, a producer of chlorothricin. *J. Biochem. Mol. Biol.* (formerly *Korean Biochem. J.*) **29**, 183–191.

Sohng, J. K., Oh, T. J. and Kim, C.H. (1998) Method for cloning biosynthetic genes of secondary metabolites including deoxysugar from actinomycetes. *J. Biochem. Mol. Biol.* (formerly *Korean Biochem. J.*) **31**, 484–491.

Studier, F. and Hoffatt, B. A. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189**, 113–130.