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ORIGINAL RESEARCH PAPER

Cloning and expression of the sucrose phosphorylase gene from *Leuconostoc mesenteroides* in *Escherichia coli*

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Abstract The gene encoding sucrose phosphorylase (*742sp*) in *Leuconostoc mesenteroides* NRRL B-742 was cloned and expressed in *Escherichia coli*. The nucleotide sequence of the transformed *742sp* comprised an ORF of 1,458 bp giving a protein with calculated molecular mass of 55.3 kDa. 742SPase contains a *C*-terminal amino acid sequence that is significantly different from those of other *Leu. mesenteroides* SPases. The purified 742SPase had a specific activity of 1.8 U/mg with a K_m of 3 mM with sucrose as a substrate; optimum activity was at 37°C and pH 6.7. The purified 742SPase transferred the glucosyl moiety of sucrose to cytosine monophosphate (CMP).

Keywords Anticancer drug · CMP-glucose · *Leuconostoc mesenteroides* · Nucleotide glycosides · Sucrose phosphorylase

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Introduction

Sucrose phosphorylase (EC 2.4.1.7) (SPase) belongs to family 13 of the glycosyl hydrolases (Henrissat 1991), and catalyzes the reaction of sucrose and inorganic phosphate to synthesize α-D-glucose1-phosphate (G1P) and D-fructose (Lee et al. 2006). SPases occur in numerous bacteria, including *Pseudomonas saccharophila* (Doudoroff 1943; Silverstein et al. 1967), *P. puterfaciens* (Weimberg and Doudoroff 1953), *Leuconostoc mesenteroides* (Koga et al. 1991; Kawasaki et al. 1996a), *Streptococcus mutans* (Russell et al. 1988), and *Bifidobacterium adolescentis* DSM20083 (van den Broek et al. 2004). Corresponding SPase genes have been cloned from *Leu. mesenteroides* spp. (Kitao and Nakano 1992; Kawasaki et al. 1996b), *S. mutans* (Ferretti et al. 1988), *Agrobacterium vitis* (Fournier et al. 1994), and *B. adolescentis* DSM20083 (van den Broek et al.

2004). We recently reported the first isolation and molecular characterization of the sucrose phosphorylase gene (*sp1149*) (Lee et al. 2006) from *Leu. mesenteroides* B-1149.

Leu. mesenteroides DSM 20193 SPase was constitutively produced (Vandamme et al. 1987) and showed broad acceptor specificities independent of the position of the hydroxyl group. The SPase transferred the glucosyl moiety of G1P to various sugars and sugar alcohols (Lee et al. 2006), and the glucosyl moiety of sucrose to phenolic and alcoholic compounds (Lee et al. 2006). We also reported that *Leu. mesenteroides* 1149SPase produced various acceptor reaction products by transferring the glucosyl moiety of sucrose or of G1P to various acceptors such as galactose, maltose, and glucose or beta-linkage compounds such as cellobiose and gentiobiose using sucrose as a glucosyl donor (Lee et al. 2006).

Since many dextran-producing *Leuconostocs* produce more than one dextransucrases (Kim and Robyt 1995) using sucrose as substrate, different SPases would be produced depending on the *Leuconostoc* species because SPase also uses sucrose as a substrate. *Leu. mesenteroides* B-742 produces two dextransucrases with sucrose. One enzyme synthesizes a dextran containing an α-1 → 4 branch linkage, and the other synthesizes a dextran with a very high degree of single α-1 → 3 branched glucose residues. In this report, we isolated a gene (*742sp*) coding *Leu. mesenteroides* 742SPase, determined the nucleotide sequence, and constructed a sucrose phosphorylase over-producing strain. We also synthesized a CMP glucoside in which an α-glucopyranosyl residue was attached to the phosphate group of CMP (CMP-G1), which indicated that 742SPase can be used for nucleotide glycosides, which has potential as an anticancer drug.

Materials and methods

Materials

The reagents for analytical assays were purchased from Sigma. All other chemicals and reagents were of analytical grade.

Conditions of cell culture

Leu. mesenteroides B-742CB was in LW medium [0.5% (w/v) yeast extract, 0.5% (w/v) KH_2PO_4, 0.02% (w/v) $MgSO_4 \cdot 7H_2O$, 0.001% (w/v) NaCl, 0.001% (w/v) $FeSO_4 \cdot 7H_2O$, 0.001% (w/v) $MnSO_4 \cdot H_2O$, 0.013% (w/v) $CaCl_2 \cdot 2H_2O$] containing 2% (w/v) glucose at 28°C without aeration (Lee et al. 2006).

DNA isolation and gene cloning

Genomic DNA from *Leu. mesenteroides* B-742 was prepared as described previously (Kim et al. 2000). Routine DNA manipulations were performed according to the protocol of Maniatis et al. (1989). Plasmid DNA was isolated from an overnight culture of *E. coli* using the alkaline lysis method. For extraction of chromosomal DNA and plasmid DNA from agarose gels, AccuPrep Gel Purification kit (Bioneer Co., Daejeon, Republic of Korea) was used. Competent *E. coli* DHα cells, prepared according to the procedure of Cohen et al. (1973), were transformed with plasmid DNA using the $CaCl_2$ method (Cohen et al. 1973). For cloning of *742sp*, three PCR steps were used. First, two degenerate primers were designed from conserved amino acid sequences from other bacterial SPases (GenBank accession numbers AF065394, AAK004742, AB029313, AJ401152, AJ303085, and AY795566): SP1F: 5′-ATGGAAA TTCAAAACAAAGCAA-3′ and SP1R: 5′-TAATAA ATTTGTGGAATACC-3′. These primers have been used in the amplification of a 1,173-bp fragment of *Leu. mesenteroides* 1149, *sp1149* (Lee et al. 2006). The PCR mixture contained 10 mM Tris/HCl (pH 8.5), 50 mM KCl, 3 mM $MgCl_2$, 2 mM of each deoxyribonucleotide triphosphate (dNTP), 0.25 mg genomic DNA from *Leu. mesenteroides* B-742, and 10 pmol of each primer. After incubation for 5 min at 94°C, 1 μl *Taq* DNA polymerase (Takara, Japan) was added, followed by 25 cycles of denaturation (94°C for 30 s), annealing (54°C for 30 s), and elongation (72°C for 30 s). The PCR fragment was ligated into pGEM-T Easy vector (Promega, USA) for DNA sequencing, followed by the amplification of 5′- and 3′-regions using the thermal asymmetric interlaced (TAIL) PCR method (Liu and Whittier 1995). To obtain 5′- and 3′-end of *742sp*, the secondary primer

set was designed based on sequence of the first PCR product. The nucleotide sequence of the initial clone facilitated the design of internal primers for the 5′-end of gene (SP2F, 5′-AGATATTTATCAGATT AAT-3′) and the 3′-end of gene (SP2R, 5′-TAATCA GAAGGAGCGAAGCC-3′). TAIL PCR needs various sizes of DNA as templates. Different restriction enzymes were treated in partial against *Leu. mesenteroides* chromosomal DNA, and *Bam*HI and *Pst*I treatments resulted good templates for TAIL PCR (data not shown). After partial digestion with *Bam*HI and *Pst*I, self-ligated *Leu. mesenteroides* B-742 chromosomal DNA was used as a template in PCR, according to the protocols described above. The amplified fragment was inserted into pGEM-T Easy vector and sequenced. Finally, the whole 742SPase gene was amplified by PCR using chromosomal DNA from *Leu. mesenteroides* B-742 as a template and two oligonucleotide primers (SP1F, as above, and E3R: 5′-TTATTTGTTTTGTAAGACTGTCT-3′) derived from the nucleotide sequences of the 5′- and 3′-termini. The amplified gene was inserted into pGEM-T Easy vector and cloned using *E. coli* DHα.

Overexpression of *742sp* and purification of recombinant 742SPase

To express *742sp*, PCR was performed to introduce a *Bam*HI site at the initiation codon (5′-ACAGGATC CATAACTATGGAAATTCAAA-3′) and a downstream *Kpn*I site (5′-CTTCTGAATTCTTTTCTTA TTTGTTTTGT-3′). The PCR fragment was ligated into the *Bam*HI and *Kpn*I sites of the pRSETA vector (Invitrogen, The Netherlands) predigested with *Bam*HI and *Kpn*I. The ligated vector was then transformed into *E. coli* BL21(DE3)pLysS (Invitrogen, USA). *E. coli* BL21(DE3)pLysS carrying *p742sp* was grown in 50 ml Luria–Bertani (LB) liquid medium containing 50 μg ampicillin/ml and 1 mM IPTG at 28°C for 6 h. The purification of recombinant 742SPase was performed using the same procedure as that used for 1149SPase (Lee et al. 2006).

Enzyme activity assay

One unit of sucrose phosphorylase activity was defined as the amount of enzyme that caused the reduction of 1 μmol $NADP^+$ per min under the above assay conditions (Silverstein et al. 1967; Koga et al. 1991; Lee et al. 2006). The amount of protein was determined by the Bradford method (1976) using bovine serum albumin as standard.

Results and discussion

Cloning and nucleotide sequence of *742sp*

A 1,100 bp PCR product of the 742SPase gene was obtained using a sense primer (SP1F) of the 1149 SPase gene and an antisense primer (SP1R), and primers for the thermal asymmetric interlaced (TAIL)-PCR method were designed (Liu and Whittier 1995) to amplify a 300 bp fragment and a 500 bp fragment of the 5′- and 3′-end regions of the *742sp* gene, respectively. Finally, the whole *742sp* (about 1,500 bp) was amplified using two oligonucleotide primers of the 5′- and 3′-terminal sequences. The ORF of *p742sp* is composed of 1,458 bp, encoding 485 amino acid residues and these sequences are given in Supplementary Figure 1. The sequence has been submitted to GenBank under accession number (EU012451). The theoretical molecular mass and isoelectric point (pI) were calculated to be 55.3 kDa and pH 9.09, respectively. The derived amino acid full sequence showed 89, 66, 66, 66, and 83% identities to SPases from *Leu. mesenteroides* ATCC 12291 SPase (GenBank accession number, D90314), *Streptococcus mutans* UA159 SPase (AE014929), *S. pneumoniae* TIGR4 SPase (AE007480), *Lactococcus acidophilus* SPase (AY172020), and *Leu. mesenteroides* B-1149 SPase (GenBank accession number, AY795566), respectively. Interestingly, from amino acid number 428 to the *C*-terminal end, the similarity and identity of 742SPase was different from other bacterial SPases. 742SPase showed 68, 78% amino acid similarity and 46, 60% amino acid identities with *Leu. mesenteroides* ATCC 12291 (Genbank No. D90314) and *Leu. mesenteroides* B-1149 SPase (Genbank No. AY795566), respectively. However, the potential catalytic amino acid residues (Asp-196, Glu-237, Asp-295) were located in the conserved sequences of *Leu. mesenteroides* SPases (Fig. 1), and it was suggested that the 742SPase belongs to glucoside hydrolase family 13 (Mario and Bernd

	I	II	III
LM	181–TLEDMVKHGANLIRLDAFAYAVKK—204	232–AEILPEIHEHYSI—244	284–MKQFTTLDTHDGIGVVDARD—304
1149SP	181–TLEDMVKHGANLIRLDAFAYAVKK—204	232–AEILPEIHEHYSI—244	284–MKQFTTLDTHDGIGVVDARD—304
742SP	181–TLEDMVQHGANLIRLDAFAYAVKK—204	232–AEILPEIHEHYTI—244	284–MKQFTTLDTHDGIGVVDARD—304
SM	178–TIENLAANGCDLIRLDAFAYAVKK—201	229–AEILPEIHEHYTI—244	281–MKQFTTLDTHDGIGVVDVKD—301
SP	178–TIQHLASNGCDLIRLDAFAYAVKK—201	229–TELLPEIHEHYSI—244	281–MKQFTTLDTHDGIGVVDVKD—301
LA	178–TLRALIDHGADIIRLDAFAYAVKK—201	229–AMILPEIHEHYSM—244	281–MKQFTTLDTHDGIGVVDARD—301

Fig. 1 Alignment of highly conserved sequences in SPase catalytic domains. LM = *Leu. mesenteroides* ATCC12291 SPase (D90314); 1149SP = *Leu. mesenteroides* B-1149 SPase (AY795566); 742SP = *Leu. mesenteroides* B-742 SPase (EU012451); SM = *S. mutans* UA159 SPase (AE014929); SP = *S. pneumoniae* TIGR4 SPase (AE007480); LA = *Lact. acidophilus* SPase (AY172020). The catalytic amino acid residues are shaded

2007). Replacement of Asp by Ala (D196A) of *Leu. mesenteroides* SPase altered the kinetic mechanism of the transfer of glucosyl to and from phosphate (Alexandra and Bernd 2006), while site-directed replacement of Asp-295 by Asn (D295N) and Glu (D295E) decreased the catalytic activity of *Leu. mesenteroides* SPase and resulted in a disruptive character of the binding site (Mueller and Nidetzky 2007). More information on various *Leu. mesenteroides* SPase genes is useful for understanding the reaction mechanism and analyzing the molecular diversity among SPases, and can be used for the synthesis of unique transglycosylation products.

Biochemical characterization of 742SPase

His-tagged 742SPase was purified by Ni-NTA affinity column chromatography. The purified 742SPase showed a band of 59.3 kDa (including His tags of 4 kDa) on SDS-PAGE (Fig. 2). Its molecular mass was close to the SPases of *Leu. mesenteroides* ATCC 12291 (55 kDa) (Koga et al. 1991) and *Leu. mesenteroides* B-1149 SPase (56.1 kDa) (Lee et al. 2006). The K_m value for the SPase from *Leu. mesenteroides* B-742 is 3 mM and 285 μmol/min ml^{-1} with sucrose as a substrate, which is half that of 1149SPase (Lee et al. 2006).

The optimum temperature and pH of 742SPase were 37°C, measured over 30 min, (Fig. 3a) and pH 6.7 (Fig. 3b). Thermostability was shown at temperatures up to 37°C (Fig. 3c), and pH stability was shown between pH 6.0 and pH 7.5 (Fig. 3d). The activities were decreased sharply at pH values above and below pH 6.7. These characteristics are similar to previous reports of *Leu. mesenteroides* SPases (Kawasaki et al. 1996a; Lee et al. 2006).

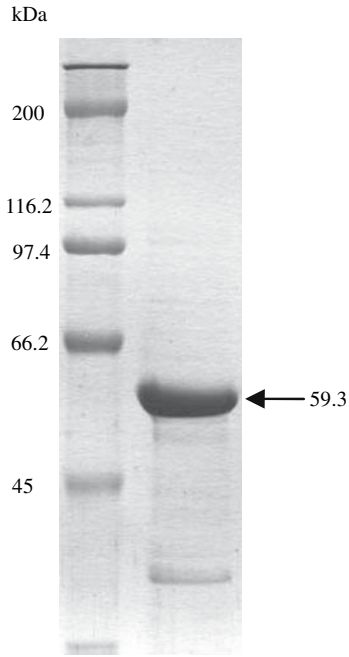

Fig. 2 SDS-PAGE analysis of purified 742Spase. Proteins were separated on 12% (w/v) SDS-PAGE and stained with Coomassie Brilliant Blue R-250. The following molecular weight markers were used: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45 kDa)

Glucosylation of CMP

After reaction using 742SPase with CMP and sucrose, one product was identified via TLC analysis (Fig. 4a, lane 5) as CMP-G1 (Fig. 4b). The *Rf* value of the CMP-G1 was 0.37 ± 0.01. Nucleotide analogues, the derivatives of the natural nucleotides found as building blocks of DNA and RNA, are effective in the clinical treatment of human cancers or

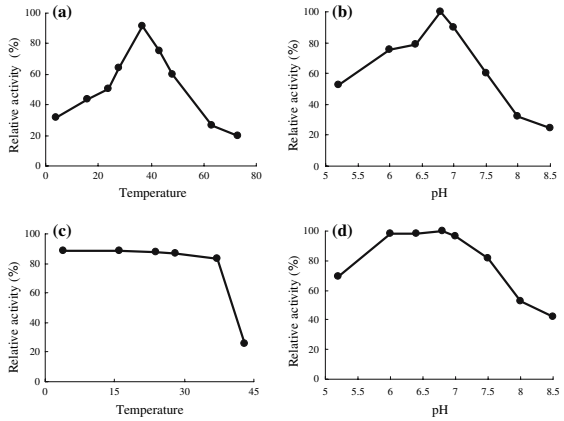

Fig. 3 Biochemical properties of 742SPase. (**a**) Optimum temperature, (**b**) optimum pH, (**c**) thermostability, (**d**) pH stability. Relative activity is expressed as a percentage of the maximum activity. Optimum temperature of the purified 742SPase (1.8 U/mg) was measured between 20 and 70°C for 10 min in 20 mM potassium phosphate buffer (pH 6.8). For the thermostability determination, the enzyme was incubated between 20 and 70°C for 1 h in 20 mM potassium phosphate buffer (pH 6.8). Optimum pH of enzyme was determined over 30 min using the buffer solutions of 20 mM citric acid Na_2HPO_4/ buffer (pH 5–7), 20 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 7–8), and 20 mM glycine/NaOH buffer (pH 8–10) at 37°C

viral diseases. Flurouracil (5-FU) is one of the most effective chemotherapeutic agents for colorectal adenocarcinoma (Tai et al. 2003). Among its derivatives, one derivative, 5-fluoro-2′-deoxyuridine 5′-phosphate, inhibits thymidylate synthase, resulting in the inhibition of thymidine nucleotide synthesis (Gmeiner 2005). Another derivative, 5-fluorouridine triphosphate, is incorporated into RNA and interferes with RNA function. Here, we synthesized the CMP-glycoside, and with an understanding of the properties and characteristics of 742SPase, this information can be used for further improvement of the synthesis of nucleotide-glycosides. Further experiment for understanding about possible anti-tumor activity of CMP-glucoside will be designed and conducted.

Conclusion

We report the purification of sucrose phosphorylase, isolation and characterization of corresponding gene (*742sp*) from *Leu. mesenteroides* 742CB. The information of SPase genes from various *Leu. mesenteroides* is

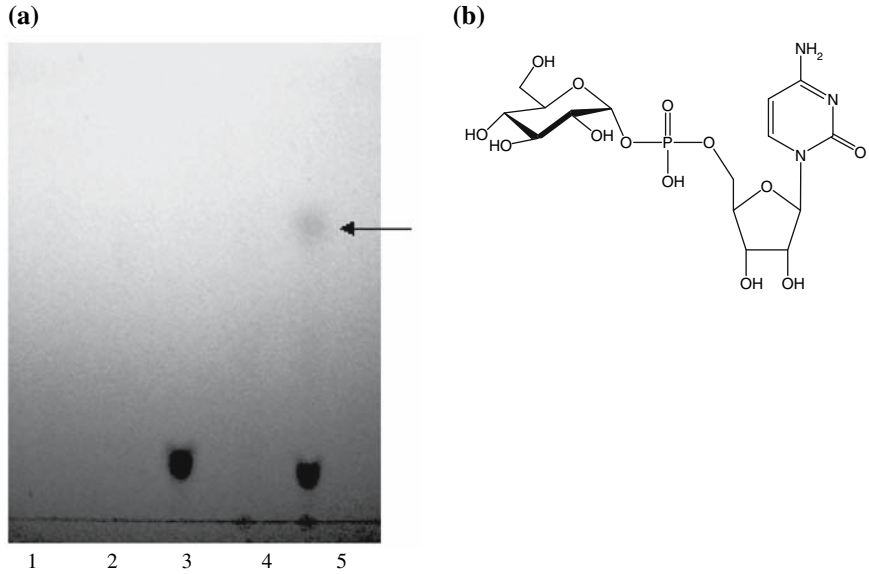

Fig. 4 TLC of the glucansucrase acceptor reaction digests (**a**) and structures of CMP-G1 (**b**). (**a**) The reaction mixture (80 ml) in 100 mM HEPES buffer (pH 6.8) was consisted of 25 mM CMP, 250 mM sucrose, and 742SPase (0.5 U/ml). It was incubated at 28°C for 3 h, and then boiled for 5 min to halt the enzyme reaction. One µl of the reaction mixture was run on a Silica gel 60 F_{254} TLC plates, developed with nitromethane/ 1-propanol/water (2:5:1.5, by vol). The developed plate was then dried and visualized under UV light (254 nm). (**b**) A model 1525 HPLC system, connected to a 7.8 mm × 300 mm i.d. μ-Bondapak C_{18} Column and a model 2487 UV detector at 280 nm, were employed in the isolation of the CMP-G1. And the structure of CMP-G1 was confirmed NMR analysis. Lane 1, fructose; lane 2, sucrose; lane 3, CMP; Lane 4, enzyme reaction digest (without CMP); lane 5, enzyme reaction digest with CMP. Arrows indicate CMP acceptor reaction products

useful to analyze the molecule diversity among SPases and it can be used to make various transglucosylation products, such as nucleotide glycosides which may have potentials as anticancer drugs, having different linkages and characteristics.

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