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Molecular Cloning of the Gene 1355SPase Encoding a Sucrose Phosphorylase from the Bacterium *Leuconostoc mesenteroides* B-1355

[1,2]Emad Abd El-Moniem Abada, [2]Mohamed El-Sayied Osman, [3]Jin-Ha Lee and [3]Doman Kim
[1]Laboratory of Developmental Genetics, Department of Life Science,
Hanyang University, Seoul 133-791, Republic of Korea
[2]Department of Botany and Microbiology, Faculty of Science, Helwan University, Ain Helwan, Cairo, Egypt
[3]School of Biological Science and Technology, Research Institute for Catalysis and Institute of Bioindustrial Technology, Chonnam National University, Gwangju 500-757, Republic of Korea

Abstract: In this study, the sucrose phosphorylase gene 1355SPase was cloned by PCR amplification from the chromosomal DNA of *Leuconostoc mesenteriodes* NRRLB-1355. The sequence analysis showed that this gene was composed of 1517 bp nucleotides, with one major open reading frame of 1494 bp encoding a polypeptide of 497 amino acid residues. The amino acid sequences of this gene is almost identical except two residues, to those gene previously reported from other bacteria. The purified 1355SPase showed an apparent MW of 56.6 kDa and specific activity of 2 U mg^{-1} for sucrose. The effects of temperature and pH on the enzyme activity were also determined. The optimum temperature and pH were 37°C and 6.7, respectively. To our knowledge, this is the first study reporting the activity and the full length sequence of 1355SPase.

Key words: Anticancer, food ingredients, glycoside hydrolases, *Leuconostoc mesenteroides*, sucrose phosphorylase

INTRODUCTION

Sucrose phosphorylase (SPase) catalyzes the conversion of sucrose and phosphate into α-D-glucose-1-phosphate (Glc-1-P) and D-fructose (Kitao and Nakano, 1992; Vandamme *et al*., 1987). According to the systematic sequence-based classification of glycoside hydrolases (GH) and glycosyltransferases (GT) enzymes (Henrissat, 1991; Weimberg and Doudoroff, 1953), SPase belongs to GH13 family and often referred to as α-amylase family. Sucrose phosphorylase has plenty of uses in biotechnological production of sucrose from starch (Kogure *et al*., 1997; Silvertein *et al*., 1967). Moreover, SPase can be used for determination of inorganic phosphate in clinical analysis (Maestre *et al*., 2001; Tedokon *et al*., 1992), sucrose concentrations in sugar transport by plants (Birnberg and Brenner, 1984) and for the determination of sucrose concentration in flow-injection method without interference from glucose or fructose in sucrose electrode (Kitao *et al*., 2000; Kitao and Sekine, 1994). Additionally, it can be employed for one-pot enzymatic galactosyltransferase assays.

Leuconostoc mesenteroides produce SPase constitutively and it has a significant advantage in the microbiological production of sucrose phosphorylase, because the crude extract of this organism had much higher total and specific activities than that of *Pseudomonas saccharophila* or *Pseudomonas putrefaciens* (Mieyal and Abeles, 1972).

It was reported that SPase from *L. mesenteroids* has broad acceptor specificity and transfers the glucosyl moiety of sucrose to phenolic or alcoholic OH groups of various substances (Kitao and Nakano, 1992; Kitao and Sekine, 1992, 1994). In addition, acceptor products by SPase also have various potential applications including anticarcinogenic materials, probiotics, antibiotic substitutes and food ingredients (Kim *et al*., 2000; Kitao *et al*., 1993, 2000; Van Den Broek *et al*., 2004). Since different kinds of glucansucarases are produced by different strains or species of *Leuconostoc*, it would be of great interest to study the SPases from different *Leuconostoc* sp. This in turn is of pivotal importance to analyze the molecular diversity among SPase and to construct the new SPase having unique transglucosylation characteristics. Here, we report the molecular cloning and biochemical characterization of the SPase from *Leuconostoc mesenteroids* B-1355.

Corresponding Author: Emad Abd El-Moniem Abada, Laboratory of Developmental Genetics, Department of Life Science, Hanyang University, Seoul 133-791, Republic of Korea
Tel: +82-02-2220-0954 Fax: +82-02-2220-4474

MATERIALS AND METHODS

Strains and bacterial cultures: *L. mesenteroides* NRRLB-1355 was grown aerobically at 28°C for 48 h in a 2 L jar containing 1 L of LWG medium consisting of 2% (w/v) glucose, 0.5% (w/v) yeast extract and peptone, 2% (w/v) K_2HPO_4 and 1% (v/v) mineral solution (2% (w/v) $MgSO_4 \cdot 7H_2O$, 0.1% NaCl, 0.1% $FeSO_4 \cdot 7H_2O$, 0.1% $MnSO_4 \cdot H_2O$ and 0.13% $CaCl_2 \cdot 2H_2O$) (Kim and Robyt, 1995; Kim *et al*., 2000).

Molecular cloning of 1355SPase: Chromosomal DNA from *L. mesenteroides* B-1355 was isolated as previously described (Kim and Robyt, 1995; Maniatis *et al*., 1989). The 1355SPase gene was amplified by PCR (Ex Taq DNA polymerase, 1 x Ex Taq buffer, dNTP mixture; Takara, Japan) using 10 ng of the genomic DNA and 10 pmol of synthetic oligonucleotide primers. The following two pairs of oligonucleotide primers was used SP1F, 5′-ATGGAAATTCAAAACAAAGCAA-3′; SP1R, 5′-TAGTAGATTTGTGGAATACC-3′. For amplification of the full length Spase1355, SP-EF, 5′ ACAGGATCCATAACTATGGAAATTCAAA-3′ and the antisense primer SP-ER 5′-TTTAAGAATTCATATATTTAAAATTACAATTG-3′: were constructed (Lee *et al*., 2006). After thirty cycles of amplification, (initial melting at 94°C for 5 min, denaturation at 94°C for 0.5 min, annealing at 57°C for 1 min and elongation at 72°C for 1.5 min), the 1355SPase PCR products were checked for the right size by using 1% agarose gel electrophoresis and purified by using (Qiagen, Germany). The purified PCR products were subcloned into pGEM-T Easy vector (Promega, USA). *E. coli* DH5α was transformed with this plasmid and transformants were selected by blue-white selection on LB medium-agar plates supplemented with 50 μg mL^{-1} ampicillin, 50 μL 100 mM IPTG and 100 μL of 20 mg X-Gal ml L^{-1}. After growing the transformants for 16 h at 37°C in LB medium supplemented with 50 μg mL^{-1} ampicillin, the cells were harvested by centrifugation (10,000 x g, 5 min, 4°C). The plasmid was isolated from the *E. coli* cells using QIAprep Spin Miniprep Kit (Qiagen, Germany) and was used for nucleotide sequence analysis of the 1355SPase gene. The full length SPase DNA sequence was analyzed by Korea Basic Science Institute (KBSI, Gwangju, Republic of Korea) by using an automated DNA sequencer. The sequence has been submitted to GenBank under accession number (EU081843).

Construction of plasmid for 1355SPase expression: The Full length SPase 1355 PCR fragment was then ligated into the corresponding sites of pRSETA vector (Invitrogen, USA) predigested with *Bam*HI and *Kpn*I. The ligated vector was then transformed into *E. coli* BL21 (DE3) pLysS (Invitrogen). *E. coli* BL21 (DE3) pLyss harboring the 1355SPase plasmid was grown at 37°C in 10 mL LB medium supplemented with 50 μg mL^{-1} ampicillin until OD_{600} reaches 0.4-0.6 was obtained. The culture was diluted with 50 mL fresh medium and incubated with shaking incubator (135 rpm) at 37°C until an OD_{600} of 0.4-0.6 was reached. 1 mM IPTG was added and the culture was kept in shaking incubator for an additional 24 h at 6°C. The cells were then harvested by centrifugation (15,000 x g, 10 min, 4°C), suspended in 200 μL of phosphate buffer (50 mM, pH 6.8) and disrupted by sonication. Cell debris was removed by centrifugation (15,000 x g, 10 min, 4°C) and the supernatant was used for further study.

Purification and polyacrylamide gel electrophoresis of recombinant enzyme: The N-terminal, 6-histidyl tagged protein was purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography (6 x His tag; Qiagen). The protein concentration of the supernatant was determined using a protein assay kit (Bio-Rad) (Bradford, 1976) and the homogeneity of the purified enzyme was confirmed by 10% SDS-PAGE.

Enzyme activity assay: A sucrose phosphorylase activity was assayed as described by Lee *et al*. (2006). The glucose-1-phosphate from sucrose and P_i were determined by coupling the reduction of $NADP^+$ in the presence of phosphoglucomutase (Sigma-P3397, USA) and glucose-6-phosphate dehydrogenase (Sigma-G5760). The standard assay medium contained 50 mM potassium phosphate buffer (pH 6.8), 140 mM sucrose, 0.09 mM EDTA-2Na, 15 mM $MgCl_2$, 0.36 mM of $NADP^+$ (Sigma-N0505), 0.0015 mM of glucose-1,6-bisphosphate (Sigma-G7137), 20 U of phosphoglucomutase, 20 U of glucose-6-phosphate dehydrogenase and the 1355SPase (20 μL) in a final volume of 3.3 mL. Increase in absorbance of NADPH at 340 nm was measured at 25°C. One unit of sucrose phosphorylase activity was defined as the amount of enzyme that reduced one μmol of $NADP^+$ per min under the above assay conditions (Ferretti *et al*., 1988; Kim and Robyt, 1995). Molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1} was used for the calculation of enzyme activity.

Physical conditions affecting the enzyme activity: The effect of temperature and pH on the stability and activity of enzyme activity were performed according to Lee *et al*. (2006).

Analysis of enzyme reaction with oligosaccharides: The enzyme reaction of α-linked oligosaccharides was carried out at 30°C in 60 mM potassium phosphate buffer (pH 6.4) containing 100 mM sucrose. The reaction mixture (100 μL) consisting of the substrate and partially purified enzyme from recombinant *E. coli* in the buffer was allowed to react for 1 h, then the reaction products were analyzed using TLC (Mieyal and Abeles, 1972) with two ascents of nitromethane/water/1-propanol 2:1.5:5 (v/v/v). The carbohydrates were visualized by dipping the TLC plate into a solvent mixture of 0.5 (W/V) α-naphthol and 5% (w/v) sulfuric acid in methanol and heating at 120°C for 10 min.

RESULTS AND DISCUSSION

Cloning and sequencing of the 1355SPase gene: The PCR amplification of 1355SPase was carried out using a sense primer (SP1F) of the 1149SPase (Lee *et al*., 2006) and an antisense primer (SP1R) resulted in a fragment 976 bp. Then, the whole 1355SPase gene, 1517 bp, was amplified by PCR using the chromosomal DNA as a template and two oligonucleotide primers SP-EF and SP-ER (Fig. 1). Based on computer analysis one major open reading frame of 1494 bp, encoding 497 amino acid residues was identified. The deduced amino acid sequence of 1355SPase was compared with other SPases gene sequences by using database homology search using BLAST (http://www.ncbi.nlm.nih.gov) (Fig. 2). Present results show that amino acid sequence of 1355SPase was almost identical to those of glucosidase and 1149SPase from other *L. mesenteroides* strain (as discussed later in the text). The sequence of 1355SPase has been submitted to GenBank under accession number (EU081843). The molecular mass of the 497 amino acid protein was calculated to be 56.6 kDa.

Production and purification of 1355SPase: The full-length 1355SPase PCR fragment was ligated into the corresponding sites of pRSETA vector (Invitrogen) predigested with *Bam*HI and *Kpn*I. The *E. coli* cells BL21(DE3)pLys harboring this plasmid were cultivated and the expression of 1355SPase was induced by the addition of IPTG. In order to characterize the enzymatic properties of 1355SPase, His-tagged 1355SPase was purified using Ni-NTA affinity column chromatography. The MW of the purified enzyme was detected by SDS-PAGE (Fig. 3). The purified 1355SPase from *E. coli* lysate showed more than 95% homogeneity on SDS-PAGE as a band of 60.6 kDa (including His tags of 4 kDa).

There was good agreement between the actual size of the protein and the predicted size calculated from the ORF. The apparent MW of 1355 SPase showed similar size to SPases of *L. mesenteroides* NRRLB-1149 (56.1 kDa) (Lee *et al*., 2006), *L. mesenteroides* ATTC 12291 (55 kDa) (Koga *et al*., 1991), *S. mutans* (55.7 kDa) (Ferretti *et al*., 1988), but larger than the SPase of *Clostridium pasteurianum* (36.5 kDa) (Silvertein *et al*., 1967).

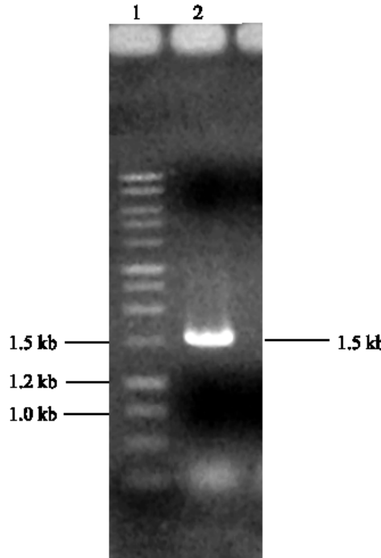

Fig. 1: Amplified PCR product of the 1355SPase gene from *Leuconostoc mesenteriodes* B-1355. 1 kb DNA, (lane 1), PCR product of 1355SPase gene, (Lane 2)

Enzymatic properties of 1355SPase: The enzyme characterizations, such as the optimum pH and temperature, thermostability and pH stability were investigated. The Purified 1355SPase showed similar activity to those have reported before with other *Leuconostoc* spp. SPase (Lee *et al*., 2006). The optimum temperature and pH were 37°C and 6.7, respectively (data not shown).

Substrate specificity against oligosaccharides: Sucrose phosphorylase (sucrose: orthophosphate, α-D-glucosyltransferase, EC 2.4.1.7) has a phosphorolytic activity such as a reversible conversion of sucrose and inorganic phosphate into α-D-glucose 1-phosphate (G 1-p) and D-fructose (Mieyal and Abeles, 1972) and catalyzes transglucosylation. This enzyme has high substrate specificity toward glycosyl donors; only sucrose, G 1-P and glucose 1-fluoride were able to transfer their glucose moiety, whereas it has broad substrate specificity to glycosyl acceptors (Kim *et al*., 2000). The 1355SPase was reactive when sucrose was used as a

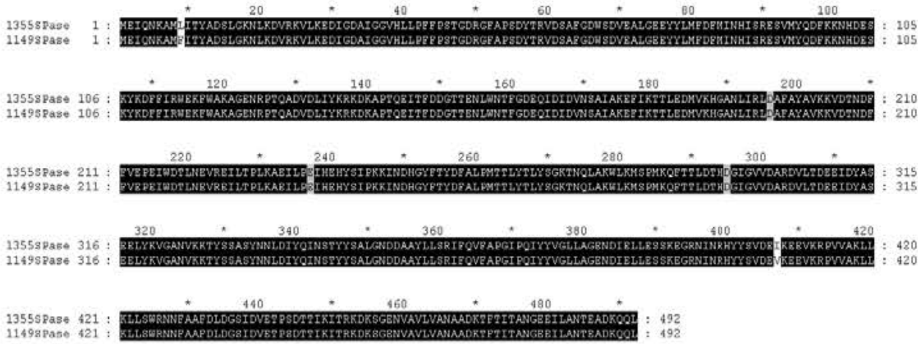

Fig. 2: Amino acid sequence alignment of SPases. 1355SPase, *Leuconostoc mesenteroides* subsp. mesenteroides NRRLB-1355 SPase (EU081843) and *Leuconostoc mesenteroides* subsp. mesenteroides NRRLB-1149 SPase (AY795566). The amino acid residues differences showed by white boxes, while the catalytic amino acid residues are gray boxes

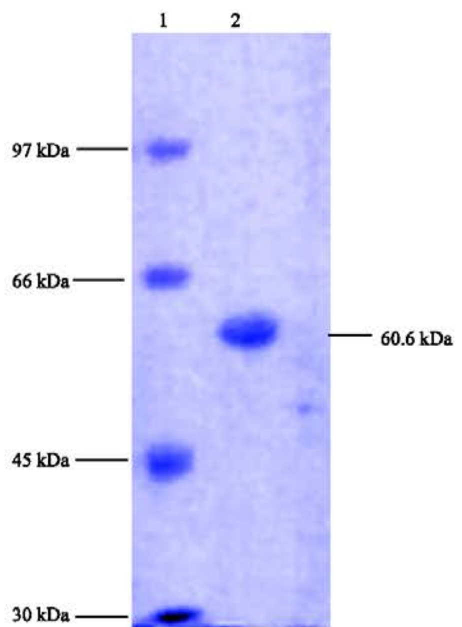

Fig. 3: SDS-10% PAGE and molecular weight determination of purified 1355SPase. Molecular weigh marker (lane 1) and Purified 1355SPase (lane 2)

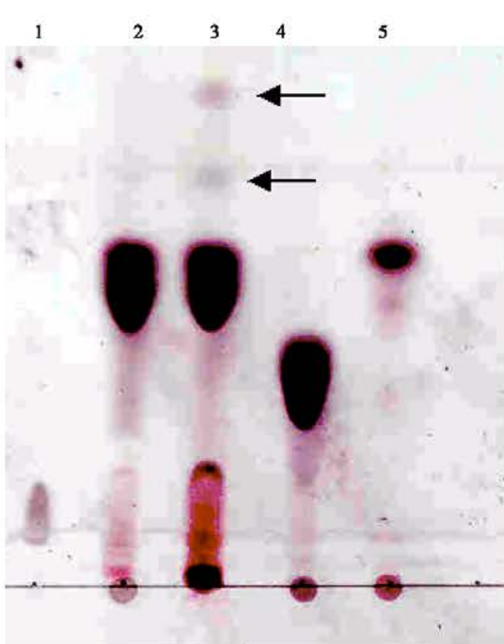

Fig. 4: TLC of the reaction products from crude and purified sucrose phosphorylase with sucrose. Glucose-1-phosphate standard (lane 1), crude enzyme reaction with sucrose (lane 2), purified enzyme reaction with sucrose (lane 3), sucrose standard (lane 4), fructose standard (lane 5) and arrows indicates the reaction by product

substrate. As a result, glucose, fructose and nature two kinds of by-products were detected as reaction products on TLC plate (Fig. 4). While, it was not reactive with the disaccharide sugar melibiose (galactose + glucose), the trisaccharide, melezitoze (glucose + turanose) and raffinose (glucose + fructose + glucose) (data not shown). Therefore, the product of 1355SPase gene expressed in *E. coli* seems to posse's transglucosylation activity in addition to sucrose hydrolyzing activity.

These results were agreed with those of Kitao *et al.* (1995) as the transglucosylation activity of sucrose phosphorylase, from *Leuconostoc mesenteroides*, was reactive for sucrose in the presence of glucose 1-phosphate but not for melibiose, melezitoze and raffinose. Moreover, our results show that the amino acid sequence

of 1355SPase was almost identical (including C-terminal end) to those of 1149SPase, except two amino acid residues. While the amino acid No. 9 is Leucine (L) for 1355SPase, its correspondence is (phenylalanine) (F) for 1149SPase (Lee *et al*., 2006). In addition, the amino acid No. 407 is (Isoleucine) (I) for 1355SPase, its correspondence is (Valine) (V) for 1149SPase. However, the potential catalytic amino acid residues (Asp-196, Glu-237, Asp-295) were located in the conserved sequences of *L. mesenteroides* SPases (Fig. 2) and it was suggested that the 1355SPase belongs to glucoside hydrolase family 13 (Lee *et al*., 2008). Replacement of Asp by Ala (D196A) of *L. mesenteroides* SPase altered the kinetic mechanism of the transfer of glucosyl to and from phosphate, while site-directed replacement of Asp-295 by Asn (D295N) and Glu (D295E) decreased the catalytic activity of *L. mesenteroides* SPase and resulted in a disruptive character of the binding site (Mueller and Nidetzky, 2007). The identity of 1355SPase and 1149SPase is sufficient for each to serve the same biochemical purpose; therefore we call each a SPase. Also, we tried to amplify other SPase gene from the strain *L. mesenteriodes* B-1299, but no PCR product was obtained (data not shown) and it is probably because of variation between N and C-terminal end among various *L. mesenteroides*.

More information on various *L. mesenteroides* SPases 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. Here we report for first time the activity and sequence of 1355SPase.

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