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Production of a new sucrose derivative by transglycosylation of recombinant *Sulfolobus shibatae* β-glycosidase

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Abstract—The gene encoding β-glycosidase of the hyperthermophilic archaea *Sulfolobus shibatae* (SSG) was expressed in *Escherichia coli*. Recombinant SSG (referred to as rSSG hereafter) was efficiently purified, and its transglycosylation activity was tested with lactose as a donor and various sugars as acceptors. When sucrose was used as an acceptor, we found a distinct intermolecular transglycosylation product and confirmed its presence by TLC and high performance anion exchange chromatography (HPAEC). The sucrose transglycosylation product was isolated by paper chromatography, and its chemical structure was determined by ^{1}H and ^{13}C NMR. The sucrose transfer product was determined to be β-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-β-D-fructofuranoside with a galactose molecule linked to sucrose via a β-(1→6)-glycosidic bond.

Keywords: *Sulfolobus shibatae*; β-Glycosidase; Transglycosylation reaction; Sucrose; Sucrose transglycosylation product

1. Introduction

β-Glycosidases (β-glucoside glucohydrolases, EC 3.2.1.21) are a group of biologically important enzymes that catalyze the hydrolysis of β-glycosidic bond, linking carbohydrate residues in aryl-, amino-, or alkyl-β-D-glucosides, cyanogenic glucosides, and oligo-, or di-saccharides. Based on their amino acid sequence and folding similarities, β-glycosidases are classified into family 1 (GH1) and family 3 (GH3) of the glycosyl hydrolases,[1–3] with which the substrates were hydrolyzed while retaining an anomeric configuration via a double-displacement mechanism.[3] These enzymes are found in all living kingdoms including archaea, bacteria, fungi, plants, and animals. Recently, β-glycosidases from hyperthermophilic archaea *Sulfolobus solfataricus* (Sβgly) and *Pyrococcus furiosus* (CelB) has been extensively studied due to their stability to high temperatures and availability of 3D structure.[4–6] The gene coding for β-glycosidase from *S. solfataricus* has been cloned and expressed in *Escherichia coli*. It was found that Sβgly is extremely thermostable by showing a half-life of 48 h at 85 °C and displaying a maximal activity at 95 °C.[7] Likewise, purified CelB shows a remarkable thermostability with a half-life of 85 h at 100 °C and 13 h at 110 °C.[8] Although these two enzymes share very similar properties in thermostability, enzyme kinetics, substrate specificity, and pH optima, they show significant differences in their stability to various denaturing agents. CelB is more stable to temperature, salts, and high pH, but more sensitive to SDS than Sβgly.[4]

There is a growing interest toward β-glycosidases due to their various biological functions, including cellular

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signaling, host–pathogen interactions, and degradation of structural and storage polysaccharides, as well as a number of industrial applications such as biomass conversion, flavor enhancing, and production of biodegradable nonionic surfactants.[9–11] In general, β-glycosidases catalyze the hydrolysis of β-glycosidic bonds linking carbohydrate residues. However, the biosynthesis of glycoconjugates can occur under defined conditions. Reverse hydrolysis and transglycosylation are two different modes for the biosynthesis of glycoconjugates. Transglycosylation usually gives higher yields than reverse hydrolysis in the formation of glycoconjugates. In general, transglycosylation is known to be a kinetically controlled reaction, whereas reverse hydrolysis is thermodynamically controlled.[9] However, such reactions are under thermodynamic control if the rate of conversion of the donor into glycosyl–enzyme intermediate is slower than the subsequent attack by the acceptor and/or water. These reactions only come under kinetic control when the donor is highly activated and the rate of formation of the glycosyl–enzyme intermediate is much higher than the rates of the following steps.

The enzymatic synthesis of oligosaccharides represents an interesting alternative to the classical methods due to its regioselectivity and stereochemistry of bond formation. Two groups of enzymes, glycosyl transferases and glycosylhydrolases, are the choices for enzymatic approach in the production of oligosaccharides.[12–14] Among the glycosyl transferases, cyclodextrin glucanotransferase (CGTase) is one of the most extensively studied enzymes that participate in transglycosylation reactions to produce various oligosaccharides.[15–17] It is known that CGTase can transfer a glycosyl residue mainly to the 4-OH group of free glucose or to a glycoysl residue of glycone compounds.[18] Recently, the highly thermostable maltosyltransferase from *Thermotoga maritima* was utilized to modify daidzin, an isoflavone, to make maltosyl–daidzin via transglycosylation.[19] Maltogenic amylases (MAases) from *Bacillus stearothermophilus* and *Thermus* sp. were also applied to modify various natural compounds, such as ascorbic acid, naringin, and sorbitol, with which maltosyl residues were mainly transferred to the acceptor molecules to produce maltosyl-transfer products.[20–22] The modified products had improved oxidative stability, solubility, and bitterness compared to their original counterparts. In addition, various other enzymes including α-L-fucosidases, dextransucrase, and alternansucrase, have been reported to synthesize novel oligosaccharides via their transglycosylation activities.[23–25]

In this paper, we report the overexpression of the gene coding for β-glycosidase from hyperthermophile *Sulfolobus shibatae* JCM 8931 (SSG) in *E. coli* and provide general characteristics on the recombinant enzyme, specifically on the transglycosylation. We also describe the synthesis of a new sucrose derivative using the transglycosylation activity of rSSG and determine the structure of a newly formed sucrose transglycosylation product.

2. Results and discussion

2.1. Expression and purification of rSSG

The gene corresponding to β-glycosidase from hyperthermophile *S. shibatae* JCM 8931 was successfully amplified by PCR as described in Section 3. The absence of error in the nucleotide sequence of the PCR-generated gene was determined with BigDye Terminator Cycle Sequencing Kit for ABI377 PRISM (PerkinElmer, Boston, MA). The resulting PCR fragments were inserted into the *E. coli* expression vector, p6 × His119, to generate p6 × His119-SSG.[26] *E. coli* DH5α cells transformed with p6 × His119-SSG showed their β-glycosidase activities when aryl-glucoside (*p*NPG) was used as a substrate in cell-free extracts, implying that rSSG was successfully expressed in *E. coli*. rSSG could be efficiently purified from cell-free extracts by two steps: heat treatment at 70 °C for 30 min and Ni–NTA affinity chromatography. Furthermore, the yield of rSSG after heat treatment was 91%, suggesting that rSSG was highly thermostable and could be easily separated from most of the heat-labile *E. coli* proteins (Table 1). SDS-PAGE showed that the purified protein was present homogeneously as a single band at 57 kDa (Fig. 1). The analysis by MALDI-TOF MS of purified enzymes revealed that rSSG had molecular masses of 57,403 Da. The molecular mass of rSSG is consistent with the expected size deduced from the primary amino acid sequence of the β-glycosidase from *S. shibatae* having six-histidine tags.

Although CelB (β-glucosidases from *P. furiosus*), Sβgly (β-glucosidases from *S. solfataricus*) and rSSG showed extremely thermostable properties by exhibiting their optimal temperatures over 90 °C, the multiple sequence alignment of three thermostable β-glucosidases from *P. furiosus*, *S. solfataricus*, and *S. shibatae*, revealed that the amino acid sequence identities were extremely high (94%) within the same genus, but they were relatively low between the genus (50%). For instance, a comparison between β-glucosidases from *P. furiosus* and *S. shibatae* showed 65% sequence similarity and 50% identity over their entire length. Both thermostable β-glucosidases belong to family 1 of glycosylhydrolases, in which the typical $(\alpha/\beta)_8$ barrel fold first seen in the structure of triose phosphate isomerase is present. The three-dimensional structures of Sβgly have been solved, and revealed that the active site is placed at the center of the top face of the barrel.[27] It is connected to the surface by a radical channel, with a putative acid/base catalyst located at the end of β-strand 4 and a catalytic nucleophile near the end of β-strand 7. Although

Table 1. Steps in purification of the recombinant SSG

Purification step	Total volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)[a]	Yield (%)	Purification fold
Cell extraction	60	4055	302.5	13.4	100	1
Heat treatment	50	3698	29.5	125.3	91.2	9.4
Ni–NTA affinity chromatography	10	2486	8.5	292.5	61.3	21.8

The starting materials were 10.2 g of wet *E. coli* cells (4 L cultures).
[a] One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 μmol of *p*NP per min at 75 °C under standard assay conditions.

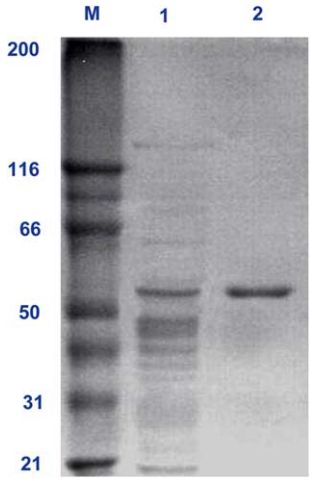


Figure 1. SDS-PAGE analysis of rSSG expressed in *E. coli*. Lane M, molecular weight standards; lane 1, crude cell extract of recombinant *E. coli* harboring p6 × His119-SSG; lane 2, recombinant SSG purified by heat treatment followed by an Ni–NTA affinity column.

similarity values within two different types of enzymes (*Pyrococcus* origin and *Sulfolobus* origin) were not high, the amino acid residues in the active site and substrate-binding site of the enzymes were well conserved. Therefore, it is expected that the enzymatic properties of rSSG are similar to those of β-glucosidases from *S. solfataricus* and *P. furiosus*.

2.2. Hydrolysis and transglycosylation activity

rSSG exhibited the highest activity in a narrow pH range between 5.0 and 6.0 with optimum of 5.0. rSSG showed its optimum temperature at 95 °C, and 80% of its activity was retained at 105 °C (data not shown). At a temperature lower than 50 °C, the enzyme lost its catalytic activity by showing less than 30% of maximal activity. Like other GH1 family β-glycosidases, rSSG showed broad substrate specificity by displaying its hydrolytic activity toward naturally occurring disaccharides (cellobiose and lactose), as well as aryl-glucoside (*p*NPG) examined by TLC.

It is known that GH1 family enzymes catalyze intermolecular transglycosylation if an appropriate acceptor molecule is present. In this transfer reaction, the donor glucoside (e.g., lactose or aryl-linked glucoside) is first hydrolyzed by β-glycosidase resulting in the formation of an enzyme–glycosyl intermediate. The intermediate is then trapped by an acceptor molecule other than water to yield a transglycosylation product.[9] Further, the transglycosylation activity and acceptor specificity of rSSG was investigated using lactose as a donor and seven different sugar or sugar alcohol acceptors. When monosaccharides (glucose and galactose) reacted with rSSG, no product was formed, implying that the reverse hydrolysis reaction was not efficient and transglycosylation is correlated with the hydrolysis reaction (data not shown). In addition, enzymatic transglycosylation of lactose into oligosaccharides was studied using β-glycosidases of the hyperthermophilic archaea *P. furiosus* (CelB) and *S. solfataricus* (SβGly).[28] Both enzymes showed a marked preference for making new β(1→3) and β(1→6) glycosidic linkages by intermolecular as well as intramolecular transfer reaction to form β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-D-Glc, β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc, β-D-Gal*p*-(1→6)-D-Glc, and β-D-Gal*p*-(1→3)-D-Glc. The accumulation of β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-D-Glc at the end of the reaction was explained by the slow degradation of β(1→6)-glycosidic

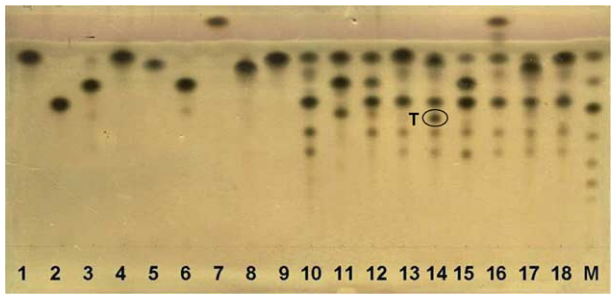
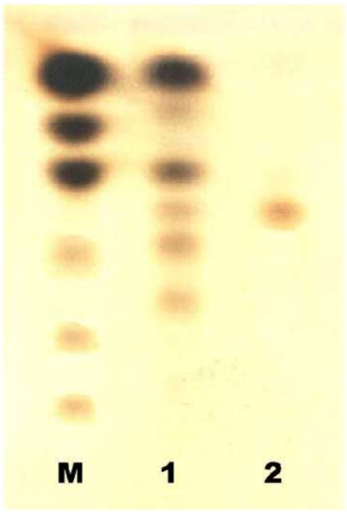


Figure 2. TLC analysis of transglycosylation reaction with rSSG and various sugar and sugar alcohols. Lane 1–9, standard molecules; glucose (lane 1), lactose (lane 2), cellobiose (lane 3), fructose (lane 4), sucrose (lane 5), maltose (lane 6), rhamnose (lane 7), sorbitol (lane 8), and mannitol (lane 9). Lane 10–18 are the transglycosylation reaction mixture with lactose (donor) and various sugar or sugar alcohols (acceptors) by the recombinant SSG. Lane 10, lactose only (30%, w/v); lane 11, cellobiose only (30%, w/v); lane 12, lactose (15%, w/v) and cellobiose (15%, w/v); lane 13, lactose (15%, w/v) and fructose (15%, w/v); lane 14, lactose (15%, w/v) and sucrose (15%, w/v); lane 15, lactose (15%, w/v) and maltose (15%, w/v); lane16, lactose (15%, w/v) and rhamnose (15%, w/v); lane 17; lactose (15%, w/v) and sorbitol (15%, w/v); lane18, lactose (15%, w/v) and mannitol (15%, w/v); lane M, maltooligosaccharides (glucose to maltoheptaose).

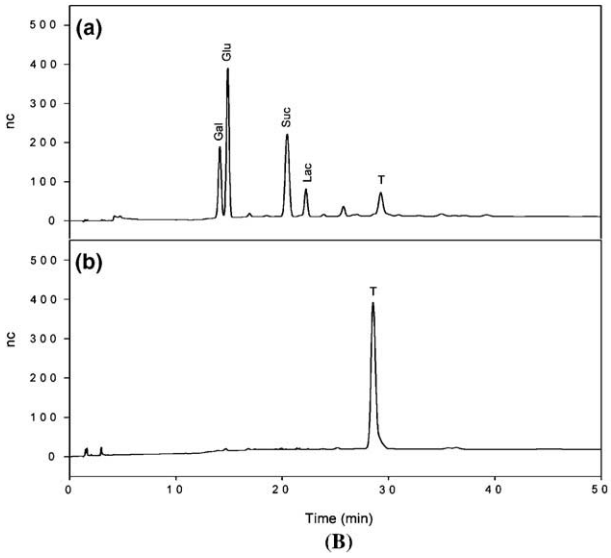
linkages over β(1→3)-glycosidic linkages. Two major transfer products were produced when rSSG reacted with lactose only (Fig. 2, lane 10). These products appeared in the same positions as those observed during the reaction with *S. solfataricus* β-glycosidase and lactose, using TLC and HPAEC analyses (data not shown), suggesting that these products were β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-D-Glc and β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc.[28] Unlike the lactose reaction, rSSG produced only one major transglycosylation product in the cellobiose reaction (Fig. 2, lane 11). Intermolecular transglycosylation between lactose (a donor) and various sugars and sugar alcohols (acceptor molecules) was observed (Fig. 2). Transglycosylation products observed in the lactose-only reaction were also formed in all reactions as major products, implying that rSSG shows strong acceptor specificity toward lactose. Among the tested sugars, a distinct transglycosylation product was observed in sucrose and lactose reaction (Fig. 2, lane 14). In fact, the reaction between lactose as a donor and sucrose as an acceptor exhibited relatively higher yield of transglycosylation products than any other transglycosylation reactions. The yield was approximately 20% on the basis of sucrose.

2.3. Purification and structural determination of sucrose transfer product

To determine the structure of the sucrose derivative product formed by the rSSG transglycosylation reaction, the transfer product was purified by paper chromatography. After two irrigations, a major sucrose transglycosylation product was successfully separated from the other carbohydrates. The isolated major transfer product gave a single spot and peak on TLC and HPAEC analyses, respectively (Fig. 3A and B). The molecular mass of the purified major sucrose transfer product was estimated to be 504 Da by MALDI-TOF MS analysis. The molecular mass corresponded to the calculated molecular mass of monoglycosyl–sucrose, implying that the major sucrose transfer product was glycosyl–sucrose. ^{1}H and ^{13}C NMR analyses were carried out to determine the glycosidic linkage between the glycosyl group and sucrose. Assignment of the NMR data to each sugar including glucose and galactose was performed through a comparison of the chemical shifts in the ^{13}C NMR spectrum with those listed previously.[29,30] Chemical shifts in the major sucrose transfer product in ^{13}C NMR were compared with those of sucrose (Table 2). The position for the terminal galactose to be linked at the glucose was determined through glycosylation shift of the C-6 signal of glucose. The new compound showed 18 carbon signals containing those of a terminal galactose, indicating that the galactose was connected to the sucrosyl moiety. The chemical shifts of C-6 and C-5 in the glucose unit of sucrose largely



(A)



(B)

Figure 3. TLC (A) and HPAEC (B) analyses of the purified sucrose transglycosylation product. (A) TLC analysis, lane M, maltooligosaccharides (glucose to maltohexaose); lane 1, the transglycosylation reaction mixture between lactose and sucrose; lane 2, the purified major transfer product. (B) HPAEC analysis, (a) the transglycosylation reaction mixture between lactose and sucrose and (b) the purified major transfer product.

changed from 60.16 to 68.65 ppm (−8.49 ppm) and from 72.45 to 71.64 ppm (+0.81 ppm), respectively, for the major sucrose transfer product, confirming that the transferred galactosyl group was connected to C-6 in the glucose unit of sucrose. In addition, ^{1}H NMR analysis revealed that the galactosyl residue of lactose was transferred to C-6 in the glucose unit of sucrose by β-anomeric configuration based on the coupling constant (J = 7.6 Hz) of the galactose anomeric proton signal observed at 4.22 ppm. Based on our results, the structure of the major sucrose transfer product was defined as β-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-β-

Table 2. ^{13}C (100 MHz) and ^{1}H NMR (400 MHz) data for sucrose and its transfer products (units: ppm)

Carbon atoms		^{13}C NMR		^{1}H NMR	
		Sucrose	Sucrose transfer product	Sucrose	Sucrose transfer product
Fructose	1	62.43	62.61		
	2	103.75	103.72	4.01 (d, J = 8.8 Hz)	4.01 (d, J = 8.0 Hz)
	3	76.43	76.37		
	4	74.03	73.99		
	5	81.43	81.41		
	6	61.38	61.39		
Glucose	1′	92.24	92.09	5.21 (d, J = 4.0 Hz)	5.21 (d, J = 3.6 Hz)
	2′	71.13	71.06		
	3′	72.62	72.51[a]		
	4′	69.27	69.37		
	5′	72.45	71.64		
	6′	60.16	68.65	3.61 (2H, s)	3.46 (2H, s)
Galactose	1″		103.57		4.22 (d, J = 7.6 Hz)
	2″		70.97		
	3″		72.79[a]		
	4″		68.79		
	5″		75.22		
	6″		61.13		

[a] The signals are changeable with each other.

D-fructofuranoside, in which a galactose molecule was linked to sucrose via β-(1→6)-glycosidic linkage. This structure is similar to the molecular structure of raffinose, α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-β-D-fructofuranoside, but differs in the anomeric configuration of the glycosidic linkage between galactose and sucrose. α-D-Galactopyranosyl sucroses are found in plants, and as a group are nearly as abundant as sucrose. Raffinose, having an α-D-Gal*p* attached to O-6 of the glucose unit of sucrose is ubiquitous, and both umbelliferose and planteose are well distributed in seeds, roots, stems, and fruits. However, in general, β-D-galactopyranosyl sucroses have not been found in nature.

Recently, lactosucrose, β-D-galactopyranosyl-(1→4)-α-D-glucopyranosyl-β-D-fructofuranoside, has been produced by enzymatic transfer of the fructosyl residue of sucrose to lactose.[31] Lactosucrose is known to be resistant to digestion in the stomach and small intestine. It is selectively utilized by intestinal *Bifidobacterium* species resulting in a significant induction of growth of these bacteria in the colon. Therefore, under physiological conditions, lactosucrose acts on the intestinal microflora as a growth factor for *Bifidobacterium* species.[32,33] Likewise, there is a strong possibility that the transglycosylation compound produced by rSSG, which has a distinct glycosidic linkage between galactose and sucrose, may have similar functionality as lactosucrose, implying that β-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-β-D-fructofuranoside can be used as a dietary supplement and in functional foods in the food industry. In conclusion, this work shows that the sucrose transfer product, β-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-β-D-fructofuranoside, formed by the transglycosylation reaction with lactose, sucrose, and rSSG is a new sucrose derivative. Studies on the functional roles of this compound, including prebiotic effects, and anticarcinogenic effects are in progress.

3. Experimental

3.1. Bacterial strains

S. shibatae (JCM 8931) was obtained from JCM (Japan Collection of Microorganisms) and grown under aerobic conditions in a medium with the following composition (in g/L): yeast extract, 1.0; casamino acids, 1.0; $(NH_4)_2SO_4$, 1.3; KH_2PO_4, 0.28; $MgSO_4{\cdot}7H_2O$, 0.25; $CaCl_2{\cdot}2H_2O$, 0.07; $FeCl_2{\cdot}4H_2O$, 0.02; $MnCl_2{\cdot}4H_2O$, 1.8×10^{-3}; $Na_2B_4O_7{\cdot}10H_2O$, 4.5×10^{-3}; $ZnSO_4{\cdot}7H_2O$, 2.2×10^{-4}; $CuCl_2{\cdot}2H_2O$, 0.5×10^{-4}; $Na_2MoO_4{\cdot}2H_2O$, 0.3×10^{-4}; $VOSO_4{\cdot}H_2O$, 0.3×10^{-4}; $CoSO_4{\cdot}7H_2O$, 0.1×10^{-4}. The pH was adjusted to 3.5 using 10 N H_2SO_4. Cultivation of *S. shibatae* was carried out at 75 °C in a shaking water bath. *E. coli* DH5α cells used as a host for cloning and expression studies were grown in Luria-Bertani (LB) medium containing 1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, supplemented with ampicillin (100 μg/mL).

3.2. Enzymes and chemicals

Restriction endonucleases and modifying enzymes, such as T4 DNA ligase and *Pfu* DNA polymerase were purchased from New England Biolabs (Beverly, MA) or Promega (Madison, WI). The QIAquick Gel Extraction

kit used for the purification of PCR products or DNA restriction fragments was obtained from Qiagen (Valencia, CA). For sugar analysis, a silica gel K5F thin-layer chromatography (TLC) plate from Whatman (Kent, UK) was used. The determination of β-glycosidase activity was performed with *p*-nitrophenyl β-D-glucopyranoside (*p*NPG) and *p*-nitrophenol (*p*NP) obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of reagent grade and purchased from Sigma Chemical Co.

3.3. Construction of SSG expression plasmids

Genomic DNA of *S. shibatae* was efficiently purified using a genomic DNA purification kit (Promega Co., Madison, WI). The gene corresponding to SSG was obtained by PCR using two primers designed based on the *S. shibatae* β-glycosidase nucleotide sequence (Genbank accession number, L47841). PCR reaction was performed with *S. shibatae* genomic DNA as a template and two oligonucleotides, SSgly1 (5′-CATATGTATTCATTTCCAAAAAACTTTAGG-3′) and SSgly4 (5′-TGGTGTCTGCAGACTTGAAAATGTCTAGTGTC-3′), containing *Nde*I and *Pst*I recognition sites (underlined), respectively. The single *Nde*I site located inside of the *ssg* gene was removed by the SOE method[34] with two primers, SSgly2 (5′-TCTTTATTCCATCGTATGCT-3′) and SSgly3 (5′-CTCATGCTAGAGCATACGAT-3′). The standard conditions for PCR were as follows: one cycle of denaturation at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 2 min, and extra extension at 72 °C for 7 min. The PCR product of SSG was cloned into T-easy vector (Promega Co., Madison, WI) and the recombinant plasmids were digested with *Nde*I and *Pst*I. The nucleotide sequence of the PCR-generated gene was determined by BigDye Terminator Cycle Sequencing Kit for ABI377 PRISM (PerkinElmer Inc., Boston, MA). The resulting fragments were inserted into p6 × His119 vector treated with the same restriction enzymes to yield p6 × His119-SSG. In this vector, the *ssg* gene was controlled constitutively by *Bacillus licheniformis* maltogenic amylase promoter.[26] *E. coli* DH5α was transformed with the resulting recombinant plasmids for efficient expression.

3.4. Purification of recombinant SSG

Purification of recombinant His_6-tagged SSG was performed using an Ni–NTA affinity column chromatography. *E. coli* transformants harboring p6 × His119-SSG were cultured at 37 °C in a shaking incubator for 18 h and harvested by centrifugation at 10,000*g* for 20 min at 4 °C. The cell pellet was thoroughly suspended in lysis buffer [50 mM NaH_2PO_4, 300 mM NaCl, buffer, 10 mM imidazole (pH 8)] and disrupted by sonication in an ice bath. The cell lysate was centrifuged at 10,000*g* for 30 min at 4 °C to pellet down the cellular debris. The supernatant was incubated at 70 °C for 30 min to denature heat-labile proteins of *E. coli*, which were later removed by centrifugation at 10,000*g* for 30 min at 4 °C. The supernatant was filtered and then passed through a Ni–NTA affinity column (Qiagen Inc., Valencia, CA). In order to elute rSSG, the samples were washed twice with washing buffer [50 mM NaH_2PO_4, 300 mM NaCl, buffer, 20 mM imidazole (pH 8)] followed by elution using a buffer composed of 50 mM NaH_2PO_4, 300 mM NaCl, buffer, 250 mM imidazole (pH 8.0). The protein from the eluted fraction was dialyzed to remove the excess imidazole. The purity of rSSG was checked by SDS-PAGE using 10% (w/v) polyacrylamide gels and Coomassie brilliant blue R-250 for staining. Protein concentration was determined using the Bradford reagents kit (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard.[35]

3.5. Enzyme assay

β-Glycosidase activity was determined using *p*NPG at a 1 mM final concentration in a 30 min assay. Assays were performed in 1 mL of 100 mM sodium citrate buffer (pH 5.0) containing *p*NPG and enzyme solution. After preincubation of the substrate solution at 75 °C for 5 min, the reaction was initiated by 10 μL (20 ng) of the diluted enzyme solution and continued for 30 min before being terminated by the addition of 1 mL of 100 mM NaOH solution. The developed color was detected spectrophotometrically at 410 nm (UV1201, Shimadzu, Kyoto, Japan) and expressed in μmol of *p*NP using a standard graph prepared under the same conditions. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of *p*NP per min at 75 °C under the assay conditions described above.

3.6. Transglycosylation activity

Transglycosylation was monitored using either cellobiose or lactose as a donor, and galactose, fructose, sucrose, maltose, rhamnose, sorbitol, or mannitol as an acceptor. A 1 mL reaction mixture containing 20 U/mL of SSG, 15% (w/v) donor, and 15% (w/v) acceptor molecules in a 100 mM sodium citrate buffer (pH 5) was incubated at 75 °C for 12 h. Samples of 20 μL were withdrawn at different time intervals (1–12 h) and applied to TLC or HPAEC to detect transglycosylated products.

3.7. Analytical methods

The detection and identification of hydrolysis and transglycosylation products produced after enzyme reaction

were done by TLC and HPAEC analyses. TLC analysis was performed as follows. The reaction products were spotted on Whatman K5F silica gel plates (Whatman) activated at 110 °C for 30 min. Aliquot (1 μL) of the reaction mixture was spotted onto a Silica gel K5F plate and developed with a solvent system of isopropanol/ethyl acetate/water (3:1:1, v/v/v) in a TLC developing tank. Ascending development was repeated twice at room temperature. The plate was allowed to air-dry in a hood and then developed by being soaked rapidly in 0.3% (w/v) *N*-(1-naphthyl)-ethylenediamine and 5% (v/v) H_2SO_4 in MeOH. The plate was dried and placed in an 110 °C oven for 10 min to visualize the reaction spots. For the HPAEC analysis, the reaction mixtures were filtered using a 0.45 μm filter and analyzed on a Carbopak PA1 column (0.4 × 25 cm; Dionex Co., Sunnyvale, CA) connected to a Dionex model DX-600 system with ED50 electrochemical detector (Dionex Co., Sunnyvale, CA). Separation of various transglycosylation products was achieved isocratically using eluent A (16 mM NaOH) followed by application of a linear gradient from 10 up to 40 min using eluent B (200 mM NaOH) at a flow rate of 1.0 mL/min. The yield of various sucrose transglycosylation products was calculated from the reduction of the area of sucrose peak in HPAEC analysis. Since sucrose itself is resistant to the hydrolysis activity of β-glycosidase, the decrease of the amount of sucrose implies the formation of sucrose transglycosylation products.

3.8. Purification of the transfer product

The transglycosylation reaction was performed as described in Section 3.6. About 0.5 mL of the transglycosylation reaction mixtures were loaded onto BioGel P2 (Bio-Rad, Hercules, CA) column (1.5 × 110 cm), and eluted with deionized water at a flow rate of 3 mL/h, collecting 1.0 mL fractions. This procedure was repeated several times to fractionate the entire reaction mixture. The composition of the fractions was analyzed by TLC as described above. Preparative paper chromatography was carried out to further purify the sucrose transglycosylation product by multiple descending techniques.[36] About 250 μL of the concentrated enzyme reaction products, which were fractionated by BioGel P2 column chromatography, were loaded onto a Whatman 3 MM paper (23 × 55 cm). Initially, the paper was irrigated with 3:1:1 (v/v/v) of isopropanol/ethyl acetate/water for 24 h. After air-drying the paper, a second irrigation was performed with *n*-propanol and water (65:35, v/v) for 24 h. The spots on the paper were located using a $AgNO_3$ reagent to verify the separation of purified carbohydrates.[36] The paper was sectioned and eluted with deionized water, and then the purified carbohydrate was concentrated to 1 mL by rotary evaporation and used for the analysis.

3.9. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis

The MALDI-TOF MS spectrum was obtained with a Voyager-DE MALDI-TOF MS (Perseptive Biosystems, Framingham, MA) in linear mode. α-Cyano-4-hydroxy-cinnamic acid (Sigma Chemical Co.) was used as a matrix and des-Arg^1-bradykinin was used for calibration. Purified sample (1 M) and α-cyano-4-hydroxy-cinnamic acid (10 mg/mL) were dropped onto a sample plate and thoroughly dried. Then the sample plate was placed in a Voyager-DE Biospectrometry workstation, operating at 20 kV acceleration voltage.

3.10. NMR analysis

About 20 mg of sucrose and about 20 mg of the purified sucrose derivative were exchanged three times with D_2O and were dissolved in 0.5 mL of pure D_2O, and then placed into 5 mm NMR tubes. ^{13}C and ^1H NMR spectra of sucrose and a sucrose derivative produced by SSG were recorded at 100 and 400 MHz with a Varian Inova AS 400 MHz NMR spectrometer (Varian, Palo Alto, CA). Samples were dissolved in D_2O at 24 °C with tetramethylsilane (TMS) as a chemical shift reference. NMR data for the purified sucrose derivative are given in Table 2.

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