J. Microbiol. Biotechnol. (2007), **17**(3), 454–460

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Enzymatic Characterization and Substrate Specificity of Thermostable β-Glycosidase from Hyperthermophilic Archaea, *Sulfolobus shibatae*, Expressed in *E. coli*

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Received: September 15, 2006
Accepted: October 30, 2006

Abstract Enzymatic properties and substrate specificity of recombinant β-glycosidases from a hyperthermophilic archaeon, *Sulfolobus shibatae* (rSSG), were analyzed. rSSG showed its optimum temperature and pH at 95°C and pH 5.0, respectively. Thermal inactivation of rSSG showed that its half-life of enzymatic activity at 75°C was 15 h whereas it drastically decreased to 3.9 min at 95°C. The addition of 10 mM of $MnCl_2$ enhanced the hydrolysis activity of rSSG up to 23% whereas most metal ions did not show any considerable effect. Dithiothreitol (DTT) and 2-mercaptoethanol exhibited significant influence on the increase of the hydrolysis activity of rSSG. rSSG apparently preferred laminaribiose (β1→3Glc), followed by sophorose (β1→2Glc), gentiobiose (β1→6Glc), and cellobiose (β1→4Glc). Various intermolecular transfer products were formed by rSSG in the lactose reaction, indicating that rSSG prefers lactose as a good acceptor as well as a donor. The strong intermolecular transglycosylation activity of rSSG can be applied in making functional oligosaccharides.

Keywords: β-Glycosidase, *Sulfolobus shibatae*, substrate specificity, transglycosylation reaction

A number of important commercial applications have been possible with the availability of thermostable enzymes such as bacterial α-amylases, cellulases, and DNA polymerases [1, 10, 12, 17]. Enzymes originating from hyperthermophilic bacteria and archaea show not only great thermostability, but also enhanced activity in the presence of various protein denaturants such as heat, detergent, organic solvents, and proteolytic enzymes, whereas conventional enzymes are irreversibly inactivated by these undesirable conditions [25]. Thus these extremozymes have received great scientific attention and are favorably employed in industrial applications by giving better yield under rigorously unfavorable conditions [1, 6, 12].

β-Glycosidases (β-glucoside glucohydrolases, EC 3.2.1.21) are important enzymes that are responsible for the hydrolysis of β-glycosidic linkages in aryl-, amino-, or alkyl-β-D-glucosides, cyanogenic glycosides, and oligo- or di-saccharides [4]. They play fundamental roles in the control of metabolism, including the breakdown and reassembly of cellular carbohydrates, the processing of various proteins and lipids containing oligosaccharides, and the formation of cell walls and other barrier structures that are important for the cellular defense against pathogen attack [4]. In addition, these biocatalysts have a number of industrial applications in the field of production of different chemicals not easily synthesized by chemical processes [4]. In the presence of appropriate nucleophiles other than water, the biosynthesis of glycoconjugates, so-called transglycosylation products, can occur instead of hydrolysis of substrate. Thus, the transglycosylation activity of β-glycosidases can be applied in the synthesis of a variety of biologically important compounds. These include long-chain alkyl glycosides, synthetic flavor precursors, glycosides with a spacer arm on the anomeric carbon, precursors of glycolipids, and glycosylated oligopeptides [3, 11, 13].

Sulfolobus is an extremely thermophilic archaea with optimum growth conditions of 70–80°C and pH 3. However, it is an obligate aerobic heterotroph, implying that it grows on a variety of organic carbon sources without requiring protection from the ambient atmosphere [9]. This genus includes *S. acidocaldarius*, *S. solfataricus*, *S. shibatae*, *S. metallicus*, *S. icelandicus*, *S. hakonensis*, and *S. tokodaii*. However, most physiological and biochemical researches focus on two species, *S. acidocaldarius* and *S. solfataricus*.

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Recently, three *Sulfolobus* genomes including *S. acidocaldarius*, *S. solfataricus*, and *S. tokodaii* have been completely sequenced [5, 7]. Along with the release of their genetic information, enzymes originating from *Sulfolobus* have gained great attention because of their stability at high temperatures and the ability of working in harsh conditions not suitable for mesophilic counterparts. However, the production of these enzymes from thermophiles is restricted as a consequence of the difficulty of growing large cultures of thermophiles at high temperature and the high fermentation costs involved. This difficulty can be overcome by heterologous expression of their valuable genes in mesophilic bacteria.

In this report, we describe the expression of β-glucosidase from the hyperthermophilic archaea *Sulfolobus shibatae* in *E. coli*, and the substrate specificity as well as enzymatic characteristics of the recombinant enzyme was investigated as a basic study for further application. We also compared its biochemical characteristics with other thermostable bacterial and archaeal β-glycosidases.

MATERIALS AND METHODS

Chemicals

Restriction endonucleases and other modifying enzymes such as T4 DNA ligase and *Pfu* polymerase were purchased from New England Biolabs (Beverly, MA, U.S.A.) or Promega (Madison, WI, U.S.A.). Chemicals used for the determination of β-glycosidase activity were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All chemicals used were of reagent grade.

Bacterial Strains, Plasmids, and Culture Conditions

Sulfolobus shibatae (JCM 8931) was obtained from the Japan Collection of Microorganisms (JCM, Saitama, Japan) and grown in a *Sulfolobus* medium, which contained 1 g of yeast extract in 1 l of salt base solution. The salt base solution was composed of 1.3 g of $(NH_4)_2SO_4$, 0.28 g of KH_2PO_4, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.07 g of $CaCl_2 \cdot 2H_2O$, 0.02 g of $FeCl_2 \cdot 4H_2O$, 1.8 mg of $MnCl_2 \cdot 4H_2O$, 4.5 mg of $Na_2B_4O_7 \cdot 10H_2O$, 0.22 mg of $ZnSO_4 \cdot 7H_2O$, 0.05 mg of $CuCl_2 \cdot 2H_2O$, 0.03 mg of $Na_2MoO_4 \cdot 2H_2O$, 0.03 mg of $VOSO_4 \cdot H_2O$, and 0.01 mg of $CoSO_4 \cdot 7H_2O$. The pH of the medium was adjusted to 2.0 with 10 N H_2SO_4. Cultivation of *S. shibatae* was carried out at 75°C under aerobic conditions in a shaking waterbath [8]. *Escherichia coli* BL21(DE3) [*F⁻ompT hsdS*$_B$($r_B^- m_B^-$) *gal dcm* (DE3)] and the *E. coli* expression vector pRSET-B (Invitrogen, Carlsbad, CA, U.S.A.) were used for the expression of β-glycosidase from *S. shibatae*. *E. coli* cells harboring the expression vector for β-glycosidase from *S. shibatae* were grown in Luria-Bertani (LB) medium containing 1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, supplemented with ampicillin (100 μg/ml), at 37°C in shaking incubator.

Construction of the *ssg* Gene Expression Vector

The open reading frame of the *ssg* gene was introduced into the pRSET-B *E. coli* expression vector through PCR amplification. PCR amplification was performed with *S. shibatae* genomic DNA as a template and two *ssg* gene specific primers, SSG-Ex1 (5'-GGATCCGATGTATTCATTTCCAAAAAACTTTAGG-3') and SSG-Ex2 (5'-TGGTGTCTGCAGACTTGAAAATGTCTAGTGTC-3') containing BamHI and PstI recognition sites, respectively (underlined). Two *ssg* gene specific primers were designed based on the *S. shibatae* β-glycosidase nucleotide sequence (GenBank Accession No., L47841). The 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 55°C for 1 min, extension at 72°C for 2 min, and extra extension at 72°C for 7 min. The PCR-amplified fragment was digested with BamHI and PstI and inserted into the pRSET-B vector [2]. The amplified sequence was confirmed by a BigDye Terminator Cycle Sequencing Kit for ABI377 PRISM (PerkinElmer Inc., Boston, MA, U.S.A.). The resulting *ssg* expression vector, pRB-SSG, was transformed into *E. coli* BL21 (DE3), and the transformants were selected and confirmed. In this expression vector, the polyhistidine (6×histidine) region was located in the amino-terminus of the recombinant protein for efficient purification.

Purification of Recombinant SSG Protein

The overexpression was performed by growing *E. coli* BL21 harboring pRB-SSG at 37°C in a shaking incubator for 12 h and inducing the recombinant SSG by adding 1 mM IPTG and incubating for 3 h at 37°C. The cell pellet was harvested by centrifugation at 10,000 ×*g* for 20 min at 4°C and completely resuspended in lysis buffer (50 mM Tris-HCl/$NaPO_4$, pH 8.0, 300 mM NaCl, buffer, and 10 mM imidazole) and disrupted by sonication. The cellular debris was removed by centrifugation at 10,000 ×*g* for 30 min at 4°C and the supernatant was heated for 30 min at 70°C to eliminate heat-labile *E. coli* proteins. After centrifugation at 10,000 ×*g* for 30 min, the supernatant was passed through a Ni-NTA affinity column (Qiagen Inc., Valencia, CA, U.S.A.) [27]. The column was washed with washing buffer (50 mM Tris-HCl/$NaPO_4$, pH 8.0, 300 mM NaCl, buffer, and 20 mM imidazole) and then the recombinant SSG was eluted with elution buffer (50 mM Tris-HCl/$NaPO_4$, pH 8.0, 300 mM NaCl, buffer, and 250 mM imidazole). Protein concentration was determined by the Bradford reagents kit (Bio-Rad, Hercules, CA, U.S.A.) with bovine serum albumin as a standard.

Determination of β-Glycosidase Activity

The hydrolysis of *p*-nitrophenyl glycoside (pNPG) was used for determining β-glycosidase activity [8, 9, 28]. Assay was carried out with 1 mM of pNPG as a substrate and enzyme in 1 ml of 100 mM sodium citrate buffer (pH 5.0). After 30 min of incubation, the reaction was terminated by

the addition of 1 ml of 100 mM NaOH solution. The color developed was detected by a spectrophotometer (UV1201, Shimadzu, Kyoto, Japan) at 410 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol (pNP) per min at 75°C under the assay conditions. The dependence of enzyme activity on pH and temperature was determined in the pH range of 3.0–9.0 and in the temperature range of 40–100°C. The buffers used were citrate buffer (pH 3.0–6.0), sodium phosphate buffer (pH 6.0–8.0), and Tris-HCl buffer (pH 8.0–9.0). Heat inactivation of the enzyme was examined at 75, 85, 90, 95, and 100°C. A total of 1 ml of enzyme was incubated at each temperature and taken at various time intervals, and its residual activity determined as described above.

Substrate Specificity

Substrate specificity of β-glycosidase was determined using various disaccharides including cellobiose, gentiobiose, isomaltose, isomaltulose, lactose, laminaribiose, maltose, melibiose, sophorose, sucrose, and trehalose. Hydrolysis activity of β-glycosidase on various substrates was determined using 5 mM of each substrate and 2 μg of enzyme in 100 mM sodium citrate buffer (pH 5.0). The reaction was performed at 75°C for 30 min, and the hydrolysis activity was measured by the glucose oxidase method with which the released glucose could be determined. A glucose detection kit (Yongdong Diagnostics, Seoul, Korea) was used for assaying glucose.

Analytical Methods

TLC analysis was performed with Whatman Silica gel K5F silica gel plates (Whatman, Kent, UK, U.S.A.). After baking a TLC plate at 110°C for 30 min, 1 to 3 μl aliquots of the reaction mixture were spotted onto a Silica gel K5F plate and developed with a solvent system of isopropanol/ethyl acetate/water (3:1:1, v/v/v). The developed TLC plate was dried in a hood and then visualized by soaking quickly into 0.3% (w/v) *N*-(1-naphthyl)-ethylenediamine and 5% (v/v) H_2SO_4 in methanol. The plate was dried and baked in an oven for 10 min to observe the reaction spots.

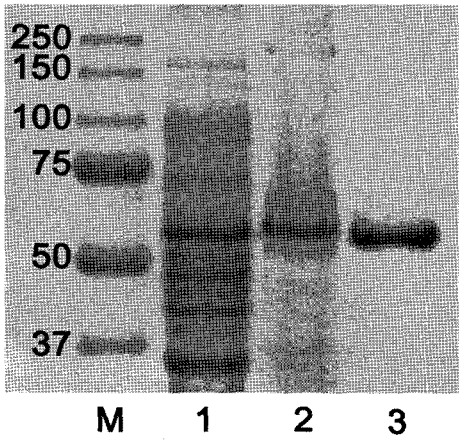

Fig. 1. SDS-PAGE analysis of rSSG expressed in *E. coli.*
Lane M, molecular weight standards; lane 1, crude cell extract of recombinant *E. coli* BL21 harboring pRB-SSG; lane 2, supernatant after heat treatment; lane 3, purified recombinant SSG using a Ni-NTA affinity column chromatography.

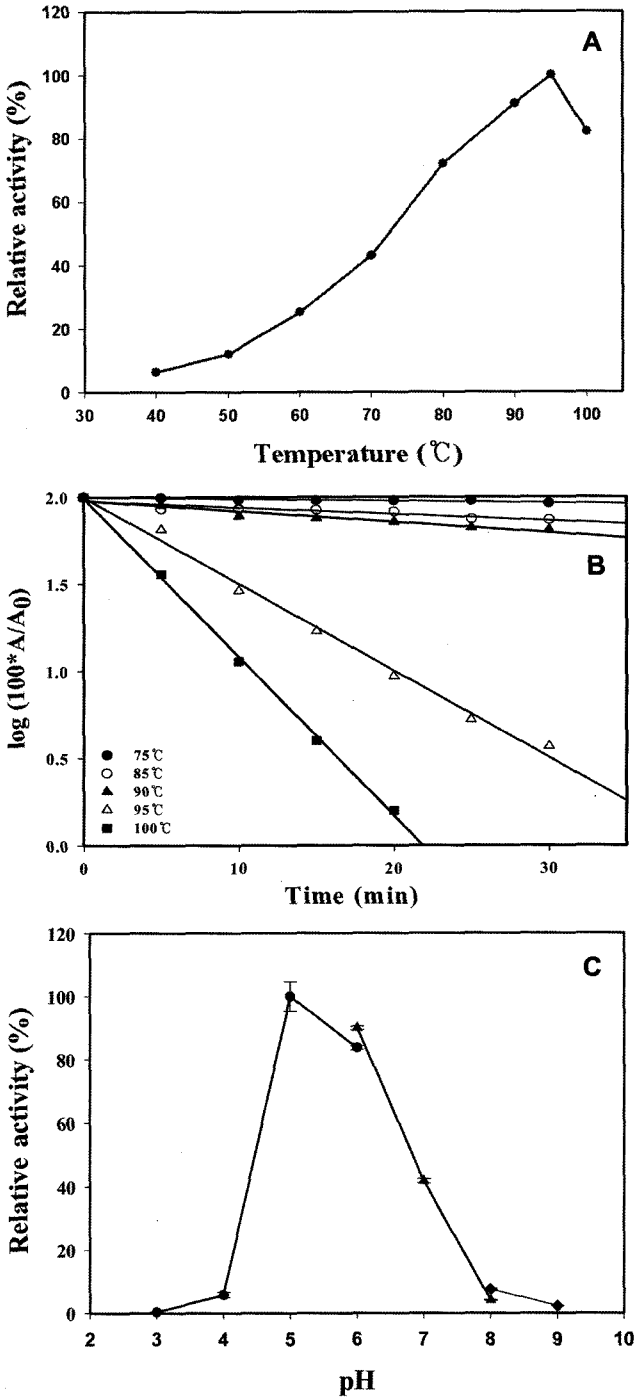

Fig. 2. Effect of temperature (**A** and **B**) and pH (**C**) on the activity of rSSG.
The buffers used (**C**) were citrate buffer (pH 3.0–6.0), sodium phosphate buffer (pH 6.0–8.0), and Tris-HCl buffer (pH 8.0–9.0).

RESULTS AND DISCUSSION

Enzymatic Properties of SSG

E. coli BL21 (DE3) cells harboring pRB-SSG, the *ssg* expression vector, exhibited their β-glycosidase activities in cell-free extracts after IPTG induction, implying that rSSG was successfully expressed in *E. coli*. A two-step simple purification including a heat treatment and a Ni-NTA affinity chromatography was efficiently used to purify the rSSG. Importantly, heat treatment of cell extracts at 70°C for 30 min eliminated most heat-labile *E. coli* proteins (Fig. 1, lane 2) as previously reported in the expression of other heat-stable protein [21, 26]. SDS-PAGE analysis showed that the purified protein was homogeneous, with a molecular mass of 57 kDa (Fig. 1, lane 3). The rSSG showed its optimum temperature at 95°C, and still retained more than 80% of its maximum activity at 100°C (Fig. 2A). However, it only maintained less than 50% of its maximum catalytic activity at lower than 70°C. Thermal inactivation of SSG was studied in the temperature range between 70°C and 100°C (Fig. 2B). rSSG showed that its half-life of enzymatic activity at 75°C was 15 h, whereas it drastically decreased to 3.9 min at 95°C. Owing this result, the enzyme activity assay was carried out at 75°C although it exhibited its maximal activity at 95°C. The effect of pH on the enzyme activity of rSSG was studied in the range of pH 3.0–9.0 at 75°C. The highest enzymatic activity of rSSG was found in a narrow range of pH 5.0–6.0 with its optimum pH at 5.0 (Fig. 2C). Interestingly, rSSG showed less than 10% of its maximal activity at below pH 4.0, although the original microorganism, *S. shibatae*, has its optimal growth pH at 3.5. The enzymatic properties of β-glycosidases from various thermophilic microorganisms are compared in Table 1. Various β-glycosidases originated from thermophilic microorganisms showed their optimal enzymatic activity at very high temperature, from 70°C to 105°C. They also maintained their maximal activity at pH 5–7 (Table 1). Interestingly, however, the recombinant β-glycosidase (*bglB*) of *Thermotoga maritima* expressed in *E. coli* was optimally active at pH 5.0 at 85°C whereas it exhibited higher activity at pH 3.5 at a lower temperature condition (70°C) [16]. Many β-glycosidases exist as oligomeric states; for example, β-glycosidases of *Pyrococcus* and *Sulfolobus* are found to be as tetramers, whereas that of *Thermotoga maritima* is present as a dimer [14, 15, 19, 23]. In the gel permeation chromatography analysis, the rSSG was eluted as a peak with a retention volume corresponding to a molecular mass of about 240 kDa (data not shown), suggesting a tetrameric structure.

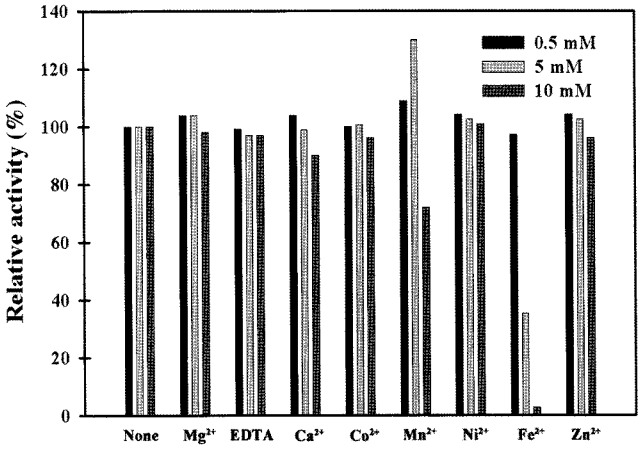

Fig. 3. Effect of various metal ions on the activity of rSSG.

Effect of Metal Ions and Reducing Agents on the Activity of rSSG

The effects of various metal ions and reducing agents on rSSG activity were investigated. Most metal ions did not show any considerable effect on the hydrolysis activity of rSSG, implying that the β-glycosidase from *S. shibatae* did not require any divalent metal ion for its activity. Unusually, the enzyme activity was enhanced 23% by the addition of 10 mM of $MnCl_2$, whereas 1 or 5 mM of $MnCl_2$ did not affect the enzyme activity (Fig. 3). Different from metal ions, reducing agents such as dithiothreitol (DTT) and 2-mercaptoethanol exhibited significant influence on the hydrolysis activity of rSSG. The activity was increased in

Table 1. The enzymatic properties of β-glycosidases from various thermophilic microorganisms.

Organism	Enzyme properties				References
	Optimal temperature (°C)	Optimal pH	Molecular mass (kDa)	Oligomeric state	
Sulfolobus shibatae	95	5	57	Tetramer	This study
Sulfolobus solfataricus	95	6.5	57	Tetramer	[23]
Pyrococcus furiosus	102–105	5	58	Tetramer	[19]
Pyrococcus kodakaraensis	100	6.5	56	Tetramer	[14]
Thermus thermophilus	90	6.5–7.0	49	-	[10]
Thermus nonproteolyticus	90	5.6	49	-	[27]
Thermotoga maritima	85 (70)	5.0 (3.5)	81	-	[16]
	90	6.0–6.2	47	Dimer (tetramer, octamer)	[15]
Thermotoga neapolitana	95	5–7	56.2	-	[21]

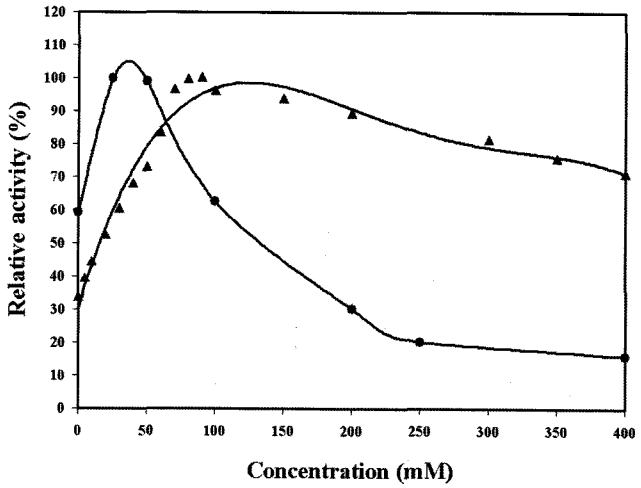

Fig. 4. Effect of the concentration of DTT (●) and 2-mercaptoethanol (▲) on the activity of rSSG.

Table 2. Substrate specificity of *Sulfolobus shibatae* rSSG.

Substrate	Linkage of glycosyl group	Glucose released from hydrolysis reaction (mM)[a]
Sophorose	β(1→2) Glc	4.28 (63.7%)
Laminaribiose	β(1→3) Glc	6.72 (100%)
Cellobiose	β(1→4)Glc	2.06 (30.7%)
Gentiobiose	β(1→6) Glc	2.56 (38.1%)
Melibiose	β(1→6) Gal	ND[b]
Lactose	β(1→4) Gal	0.95 (14.1%)
Trehalose	β(1→1) Glc	ND
Maltose	β(1→4) Glc	ND
Isomaltose	β(1→6) Glc	ND
Sucrose	β(1→2) Fru	ND
Isomaltulose	β(1→6) Fru	ND

[a]Hydrolysis activity of β-glycosidase on various substrates was determined using 5 mM of each substrate and 2 μg of enzyme in 100 mM sodium citrate buffer (pH 5.0). The reaction was performed at 75°C for 30 min, and the hydrolysis activity was measured by the glucose oxidase method.
[b]ND, not detected.

proportion to the concentration of 2-mercaptoethanol, up to 200 mM, and maintained its enhanced activity up to 400 mM concentration (Fig. 4). DTT was also very effective in increasing enzyme activity up to 50 mM. However, the positive effect of DTT was gradually reduced as the DTT concentration is higher than 50 mM. α-Glucosidases (AglA) of the hyperthermophilic bacterium *Thermotoga maritima* and *Thermotoga neopolitana* have the unusual property of requiring NAD^+, Mn^{2+}, and reducing agents such as DTT and 2-mercaptoethanol for its activity [21, 24]. As mentioned above, the activity of rSSG was also positively affected by both Mn^{2+} and reducing agents, whereas the significant effect of NAD^+ was not observed. It can be assumed that a reducing agent may protect the enzyme from being oxidized at high temperature as exemplified in the crystal structure of AglA. Lodge *et al.* [20] suggested that the strong reducing conditions were required to reduce the oxidized active site cysteine residue.

Substrate Specificity of rSSG

The substrate specificity of rSSG was investigated using various disaccharides containing different glycosidic linkages between monosaccharides (Table 2). rSSG actively hydrolyzed substrates having β-glycosidic linkage such as sophorose (β1→2Glc), laminaribiose (β1→3Glc), cellobiose (β1→4Glc), gentiobiose (β1→6Glc), and lactose (β1→4Gal), as expected from its nature as a β-glycosidase. According to the production of hydrolysis product glucose, the enzyme evidently preferred laminaribiose (β1→3Glc), followed by sophorose (β1→2Glc), gentiobiose (β1→6Glc), and cellobiose (β1→4Glc). There was no significant difference in the hydrolysis of cellobiose (β1→4Glc) and lactose (β1→4Gal), although those disaccharides had β1→4 glycosidic linkages between two different glycosides (glucose and galactose, respectively). With regard to α-D-glycosides, it was inactive on degrading various α-D-glycosides including melibiose (α1→6Gal), maltose (α1→4Glc), isomaltose (α1→6Glc), sucrose (α1→2Fruc), and isomaltulose (α1→6Fruc). Substrate preference of β-glycosidases from various hyperthermophilic microorganisms such as *Pyrococcus furiosus* and *T. neopolitana* are different from that of *S. shibatae* [21, 22]. β-Glycosidase of *Pyrococcus furiosus* displayed its substrate specificity in the order of laminaribiose, cellobiose, sophorose, and gentiobiose [22], whereas that of *T. neopolitana* showed its substrate preference in the order of sophorose, laminaribiose, cellobiose, and gentiobiose [21]. This finding implies that substrate specificity is fully dependent on the source of enzyme. Interestingly, all enzymes undoubtedly prefer laminaribiose (β1→3Glc). Comparison of the substrate specificity on various disaccharides verified that rSSG is a typical glycosidase specific for β-D-glycosides.

Transglycosylation

Transglycosylation activity was demonstrated using either cellobiose or lactose as a substrate. At low concentration of substrate, hydrolysis activity was dominant whereas a considerable amount of transglycosylation product was observed in high substrate concentration. TLC analysis revealed that two distinct transfer products were formed when lactose was used as a substrate (Fig. 5). However, one apparent transfer product spot was observed in the reaction with cellobiose. This transfer product was assumed to be β-D-Glc*p*-(13)-β-D-Glc*p*-(14)-D-Glc according to the location of the spot in TLC, but it needs to be confirmed. In fact, there were a couple of minor transfer products in both reactions. The analysis of the lactose transglycosylation products of β-glycosidases of *Pyrococcus furiosus* and *Sulfolobus solfataricus* revealed that both enzymes clearly preferred making new β(1→6) and β(1→3) glycosidic bonds, forming β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-D-Glc and β-D-

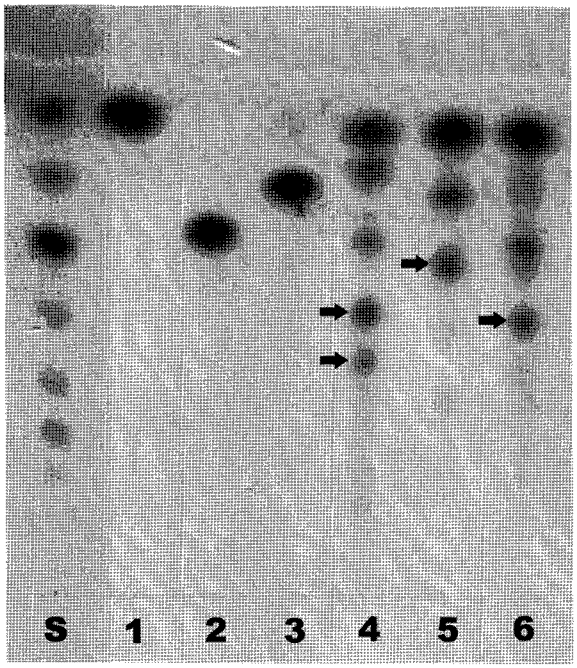

Fig. 5. TLC analysis of transglycosylation reaction with rSSG and substrates (lactose and cellobiose).
Lanes 1 to 3 are standard molecules; lane 1, glucose; lane 2, lactose; lane 3, cellobiose. Lanes 4 to 6 are the transglycosylation reaction mixture; lane 4, lactose only (30%, w/v); lane 5, cellobiose only (30%, w/v); lane 6, lactose (15%, w/v) and cellobiose (15%, w/v). Lane S is the maltooligosaccharides standard including glucose to maltoheptaose. Major transglycosylation products are marked by arrows.

Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc [22]. Therefore, two major transfer products in the lactose transglycosylation reaction of rSSG seemed to be 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. In the transglycosylation reaction with lactose and cellobiose (Fig. 5, lane 6), most of the cellobiose was hydrolyzed, whereas lactose was still remained in the reaction mixture, indicating that rSSG preferred lactose as an acceptor and cellobiose as a donor. This strong intermolecular transglycosylation activity of rSSG can be applied to make oligosaccharides having β(1→6) and β(1→3) glycosidic linkages that might work as a functional carbohydrate.

Acknowledgment

This work was supported by the Biogreen 21 Project (20050401-034-683-006-03-00) of the Rural Development Administration.

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