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Cloning and Characterization of Glycogen-Debranching Enzyme from Hyperthermophilic Archaeon *Sulfolobus shibatae*

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Abstract A gene encoding a putative glycogen-debranching enzyme in *Sulfolobus shibatae* (abbreviated as SSGDE) was cloned and expressed in *Escherichia coli*. The recombinant enzyme was purified to homogeneity by heat treatment and Ni-NTA affinity chromatography. The recombinant SSGDE was extremely thermostable, with an optimal temperature at 85°C. The enzyme had an optimum pH of 5.5 and was highly stable from pH 4.5 to 6.5. The substrate specificity of SSGDE suggested that it possesses characteristics of both amylo-1,6-glucosidase and α-1,4-glucanotransferase. SSGDE clearly hydrolyzed pullulan to maltotriose, and 6-*O*-α-maltosyl-β-cyclodextrin (G2-β-CD) to maltose and β-cyclodextrin. At the same time, SSGDE transferred maltooligosyl residues to the maltooligosaccharides employed, and maltosyl residues to G2-β-CD. The enzyme preferentially hydrolyzed amylopectin, followed in a decreasing order by glycogen, pullulan, and amylose. Therefore, the present results suggest that the glycogen-debranching enzyme from *S. shibatae* may have industrial application for the efficient debranching and modification of starch to dextrins at a high temperature.

Keywords: *Sulfolobus shibatae*, glycogen-debranching enzyme, amylo-1,6-glucosidase, α-1,4-glucanotransferase, hyperthermophilic enzyme

Glycogen-debranching enzymes (GDEs) play an important role in carbohydrate metabolism, working along with glycogen phosphorylase (EC 2.4.1.1) in the complete degradation of glycogen [5]. GDEs are also known to be bifunctional, possessing both transferase (oligo-1,4-1,4-glucantransferase, EC 2.4.1.25) and glucosidase (amylo-1,6-glucosidase, EC 3.2.1.33) activities within a single polypeptide chain [3, 14, 24]. GDEs have already been purified and characterized from rabbit and yeast [10, 15, 24], and a mutagenesis study of the yeast GDE revealed that the two GDE activities were independent and located at different sites in the polypeptide chain, where the oligo-1,4-1,4-glucantransferase activity was assigned to the N-terminal half and the amylo-1,6-glucosidase activity to the C-terminal half [20]. In addition, the N-terminal half of the yeast GDE was found to include four conserved sequences from the α-amylase family, whereas the C-terminal half exhibited an almost 50% identity with that of other mammalian GDEs [16, 19, 29]. GDEs are also widely distributed in various bacteria [8, 17] and archaebacteria [7, 18], as well as in mammalian cells and yeast. The molecular mass of prokaryotic GDEs ranges from 83–90 kDa because of their amino acid residues, which is approximately half the molecular mass of eukaryotic GDEs.

Various hyperthermophilic amylolytic enzymes from archaebacteria have recently attracted attention because of their extreme stability, low viscosity, and reduced microbial contamination in a reaction medium at high temperatures [2, 9, 21, 27]. So far, two hyperthermophilic GDE genes (*treX*) have been discovered in the trehalose operon from *Sulfolobus* sp. [17, 18], and the GDE from *S. solfataricus* has been expressed and characterized to exhibit the hydrolysis activity of amylo-α-1,6-glucosidase [18]. We have recently expressed and purified a hyperthermophilic GDE from the archaeon *Sulfolobus shibatae*. The enzyme exhibited several different properties to the GDE from *S. solfataricus*, including the catalytic activity, pH and temperature dependence, and metal ion effect. Accordingly, this study reports on the molecular cloning and expression of the gene encoding the *S. shibatae* GDE in *E. coli*, along with the enzymatic characteristics of the recombinant enzyme and sequence homology results.

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MATERIALS AND METHODS

Strains, Plasmids, Media, and Chemicals

The *S. shibatae* ATCC 51178 was purchased from the American Type Culture Collection and cultivated as described previously [17]. *E. coli* strain MC1061 [F⁻ *araD139 recA13 Δ(araABC-leu)7696 galU galK ΔlacX74 rpsL thi hsdR2 mcrB*] was used as the host for the gene manipulation and protein expression. Plasmid p6xHis119 was used as the cloning and expression vector with a hexahistidine tag [13]. The *E. coli* transformants were cultured at 37°C in a Luria-Bertani (LB) medium supplemented with ampicillin (100 μg/ml). The glycogen, amylose, rice starch, maltooligosaccharides, and 6-*O*-α-maltosyl-β-cyclodextrin (G2-β-CD) were all obtained from Sigma Chemical Co, and the amylopectin was from Fluka (Buchs, Switzerland). All the other chemicals used were of reagent grade.

PCR Cloning of the SSGDE Gene from *S. shibatae*

The chromosomal DNA of *S. shibatae* was isolated using the spool method [6]. The nucleotide sequence of the SSGDE gene in the archaeon was originally retrieved from the GenBank with the BLAST program [1], and recognized as *treX* encoding a putative glycogen-debranching enzyme (Swiss-Prot Accession Number Q8NKQ3). The gene was amplified with a PCR using *Pwo* DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany) and the genomic DNA of *S. shibatae* as the template. The oligonucleotide primers for the 5- and 3-flanking ends of the gene were designed as 5'-ATAAATCTAGACATGTC-GATATTCTTC-3' (forward) and 5'-TGTGCAAGCTTTC-ATAGTTCTGTCCTC-3' (reverse), which contained an XbaI and HindIII restriction site, respectively (underlined). The PCR amplification was perfomed according to a previous method with annealing at 55°C [22]. The 2.16-kb amplified DNA fragment was subsequently digested with XbaI and HindIII and ligated into the expression vector p6xHis119 to finally construct p6xH119-SSGDE. The nucleotide sequence of the PCR-generated gene was determined using a BigDye terminator cycle sequencing kit for the ABI 377 Prism (Perkin-Elmer, Norwalk, U.S.A.). The other genetic manipulations were carried out according to Sambrook and Russell [23]. The sequence analysis of the DNA and deduced amino acids was performed using the DNASIS (v7.0, Hitachi Software, Tokyo, Japan) and CLUSTAL programs [25].

Expression and Purification of Recombinant Enzyme

The *E. coli* MC 1061 harboring p6xH119-SSGDE was constitutively able to produce the recombinant enzyme without isopropyl thiogalactoside (IPTG) induction. After the *E. coli* transformant was grown in an ampicillin-supplemented LB broth overnight, the cells were harvested by centrifugation (7,000 ×*g* for 30 min at 4°C) and resuspended in a 50 mM sodium phosphate lysis buffer (pH 8.0) containing 300 mM NaCl and 10 mM imidazole. An extract of the crude recombinant protein was obtained by sonication (VC-600, Sonics & Materials, Newtown, CT, U.S.A.) and heated at 70°C for 30 min. After removing the precipitant by centrifugation, the supernatant was collected and the recombinant protein purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity column chromatography (Qiagen, Hilden, Germany) [22]. The purified protein was then concentrated by ultrafiltration (Millipore Co., Bedford, MA, U.S.A.) after dialysis against a 50 mM sodium phosphate buffer (pH 6.5) and used for further investigation. The purity and molecular mass of the recombinant protein were estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using a 10% (w/v) acrylamide gel.

Enzyme Assay

The GDE activity was determined by measuring its amylo-1,6-glucosidase activity using G2-β-CD as the substrate. A reaction mixture (140 μl) containing 10 mM G2-β-CD and the enzyme was incubated under standard conditions at 70°C in a 50 mM sodium acetate buffer (pH 5.5) for 30 min, and then 1 N HCl (10 μl) was added to the reaction tube to stop the reaction. The amount of reducing sugars liberated was spectrophotometrically measured using the copper-bicinchoninate method [7], where one unit of the enzyme activity was defined as the amount of enzyme that released 1 μmol of maltose per min under the assay conditions. The protein concentration was determined according to the Bradford method [2].

Effects of pH and Temperature on SSGDE Activity and Stability

To determine the optimal pH and pH range for enzyme stability, the glucosidase activity of SSGDE was compared using G2-β-CD in the following 50 mM buffers with various pHs: sodium citrate (pH 2.0 to 3.5), sodium acetate (pH 4.0 to 6.0), sodium phosphate (pH 6.0 to 7.0), Tris-HCl (7.0 to 8.0), and glycine-NaOH (8.5 to 11.0). To examine the pH stability, the enzyme (0.1 mg/ml) was first incubated in the buffers with various pHs for 1 h at 25°C, and then the remaining activity was measured under the standard conditions described above. The optimal temperature for SSGDE activity was determined in the 50 mM sodium acetate buffer (pH 5.5) using a range from 40 to 95°C. To determine the thermal stability, the enzyme (0.1 mg/ml) was preincubated for 1 h in the standard buffer at temperatures ranging from 60 to 100°C. After taking a sample and chilling it immediately in an ice-water bath, the residual activity was determined under the standard conditions.

Effect of Metal Ions and Organic Solvents on SSGDE Activity

The effect of metal ions and organic solvents on the enzyme activity was investigated using $MnCl_2$, $CuSO_4$, $NiCl_2$, $FeCl_2$, $CoCl_2$, $MgCl_2$, $CaCl_2$, $BaCl_2$, and $AlCl_3$, plus dimethylsulfoxide (DMSO), dimethylformamide (DMF), methanol, and ethanol, respectively. The enzyme was incubated in the absence and presence of 5 mM multivalent cations or a 10% (v/v) organic solvent in the standard buffer for 1 h at 25°C. Immediately after the preincubation, an appropriate aliquot was taken and the GDE activity measured under the standard conditions.

Hydrolytic Patterns of SSGDE

The reducing value assay above [12] was also used to determine the glucosidase activity with 1% (w/v) amylose, amylopectin, rice starch, glycogen, and pullulan under the standard conditions at 70°C. The hydrolysis pattern of maltooligosaccharides (G4, G5, G6, and G7), glycogen, pullulan, and G2-β-CD by the enzyme was also analyzed by thin-layer chromatography (TLC) on Whatman K5F silica gel plates (Whatman, Kent, U.K.) with isopropyl alcohol:ethyl acetate:water (3/1/1, v/v/v) as the solvent system. After irrigating, the TLC plate was dried and visualized by dipping in a solution containing 0.3% (w/v) *N*-(1-naphthyl)-ethylenediamine and 5% (v/v) H_2SO_4 in methanol and heating at 110°C for 10 min [22].

Determination of Kinetic Parameters

The kinetic parameters of the SSGDE activity for the hydrolysis of maltopentaose (G5), G2-β-CD, and pullulan were determined with various concentrations ranging from 2 to 40 mg/ml in the standard buffer at 80°C. An aliquot (100 μl) was taken periodically from the reaction mixture and the reaction quenched by placing the aliquot in an ice-water bath. The amount of reducing sugars produced from the above substrates was measured using the same method. The kinetic data were transformed to Lineweaver-Burk plots using Duggleby's software [7], producing K_m and V_{max} values.

Results and Discussion

Cloning and Expression of *S. shibatae* GDE (SSGDE)

A gene encoding a putative glycogen-debranching enzyme was cloned from *S. shibatae*. The open reading frame (*treX*), located in the trehalose operon of the *S. shibatae* B12 genome, was expected to encode a single polypeptide of 718 amino acid residues with a predicted molecular mass of 83,234 Da (http://www.expasy.org/tools/pi_tool.html). A 2.16-kb PCR product was amplified and inserted into an expression vector, generating the recombinant plasmid p6xH119-SSGDE. The construct was sequenced to confirm the fidelity of the amplification and transformed into the *E. coli* strain MC1061. The expression of the gene was efficiently performed, thereby constitutively producing the recombinant SSGDE in the ampicillin-resistant host. The recombinant enzyme was successfully purified by a convenient two-step procedure. The cell extract was heated at 70°C for 20 min to remove considerable amounts of the *E. coli* proteins. The histidine-tagged recombinant SSGDE was then efficiently purified to homogeneity by Ni-NTA affinity chromatography. The purified SSGDE showed a single protein band on the SDS-PAGE gel with an estimated molecular mass of over 80 kDa (Fig. 1), which was similar to the deduced molecular mass mentioned above.

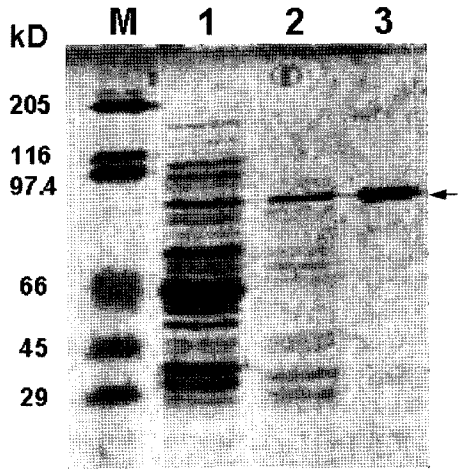

Fig. 1. Molecular mass estimation of recombinant SSGDE using SDS-PAGE.
Lane M, standard proteins; myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa); lane 1, cell extract after sonication; lane 2, supernatant after heat treatment (70°C, 30 min) and centrifugation; lane 3, purified SSGDE (arrow) after Ni-NTA affinity chromatography.

Sequence Analysis of SSGDE

A multiple amino acid sequence alignment (http://www.ncbi.nlm.nih.gov/BLAST) of SSGDE revealed a high homology with four conserved regions of GDEs from other microorganisms and the α-amylase family (Table 1). The deduced amino acid sequence of SSGDE only shared an overall sequence identity with the GDEs from *Sulfolobus acidocaldarius* (74%, Swiss-Prot Accession Number O05152), *S. solfataricus* (95%, P95868), *E. coli* (42%, P15067), and isoamylase from *Flavobacterim* sp. (34%, O32611). In particular, SSGDE had 34 different amino acid residues from the GDE from *S. solfataricus*, all of which were located outside of the conserved regions. For the overall sequence identity, SSGDE did not exhibit a significant homology with any of the eukaryotic GDEs or enzymes belonging to the α-amylase family in Table 1. However, SSGDE did have strictly matched and conserved

Table 1. Comparison of amino acid residues in conserved regions of SSGDE and the α-amylase family.

Enzyme	Conserved regions			
	I	II	III	IV
SSGDE	286- **D** V V Y N H	359- G F **R** F **D** L A	396- L I A **E** P W	466- Y V T S **H D**
EGDE	261- **D** I V L N H	332- G F **R** F **D** L A	368- L I A **E** P W	438- L V T A **H D**
YGDE	224- **D** I V F N H	531- G F **R** I **D** Y C	561- V V A **E** L F	665- M D C T **H D**
HGDE	198- **D** V V Y N H	505- G V **R** L **D** N C	535- V V A **E** L F	605- M D I T **H D**
F-ISOAMY	326- **D** V V Y N H	406- G F **R** F **D** L A	455- L I A **E** P W	528- F V V A **H D**
K-PUL	619- **D** V V Y N H	690- G F **R** F **D** L M	720- F F G **E** G W	846- Y V S K **H D**
A-AMY	117- **D** V V A N H	202- G L **R** I **D** T V	227- C I G **E** V L	292- F V E N **H D**
A-GT	217- **D** V W T N P	282- E L **R** L **D** H F	330- F I A **E** D L	340- Y T S T **H D**
B-GBE	238- **D** W V P G H	304- G F **R** V **D** A V	348- M I A **E** D S	414- L P F S **H D**
		*	*	*

Amino acids are numbered from the N-terminal end. Invariant amino acids are in bold. Asterisks indicate catalytic amino acid residues. SSGDE, *S. shibatae* glycogen-debranching enzyme (GDE) from (Swiss-Prot Accession Number Q8NKQ3); EGDE, *E. coli* GDE (P15067); YGDE, *Saccharomyces cerevisiae* GDE (Q06625); HGDE, human GDE (P35573); F-ISOAMY, *Flavobacterim* sp. isoamylase (O32611); K-PUL, *Klebsiella aerogenes* pullulanase (P07811); A-AMY, *Aspergillus oryzae* α-amylase (P10529); A-GT, *Aquifex aeolicus* 4-α-glucanotransferase (O66937); B-GBE, *Bacillus stearothermopilus* glycogen-branching enzyme (P30538).

amino acids in common with the GDEs and other enzymes above, based on a sequence alignment with the α-amylase family enzymes. The amino acid residues Asp-363, Glu-399, and Asp-471 in SSGDE, which are known to play a catalytic role in the active site of the α-amylase family, were perfectly invariable in all the enzymes compared. A search of conserved domain databases (CDDs), including Smart and Pfam (http://www.ncbi.nlm.nih.gov/BLAST), revealed a considerably high sequence homology between the SSGDE N-terminal part (about 120 residues) and the isoamylase N-terminal and the SSGDE middle part (about 300 residues) and α-amylase. It is generally known that the N-terminus of isoamylase may be related to the immunoglobulin or fibronectin type III superfamilies, and involved in oligomeric interaction. According to the predicted tertiary structure based on the primary sequence (http://www.sbg.bio.ic.ac.uk/~3dpssm), SSGDE was also composed of three domains, including the N-, C-terminal domains, and central catalytic $(\beta/\alpha)_8$-barrel domain that commonly exists in the α-amylase family. This predicted structure of SSGDE strongly resembles that of isoamylase, neopullulanase, oligo-1,6-glucosidase, and the N-terminal half of yeast GDE. In addition, Trp-401, the most likely candidate for the binding of an α-1,6-glucosidic branched substrate to the enzyme [28], was only conserved in the prokaryotic GDEs, isoamylase, and pullulanase. For the eukaryotic GDEs, the spacing between the conserved regions I and II was more than four times longer than that for SSGDE and the other amylases compared. Although the meaning of the differences in the length of the spacing in relation to the enzyme specificity has not yet been defined, it has been previously suggested that the spacing between the conserved regions in the α-amylase family may reflect the chemical nature of the reaction catalyzed and the product specificity of the enzyme [11].

pH and Temperature Dependence of Recombinant SSGDE

The pH range at which SSGDE remained active and stable was determined using G2-β-CD as the substrate. The maximum SSGDE activity was observed at pH 5.5 (Fig. 2A), whereas the activity was maintained within a narrow range

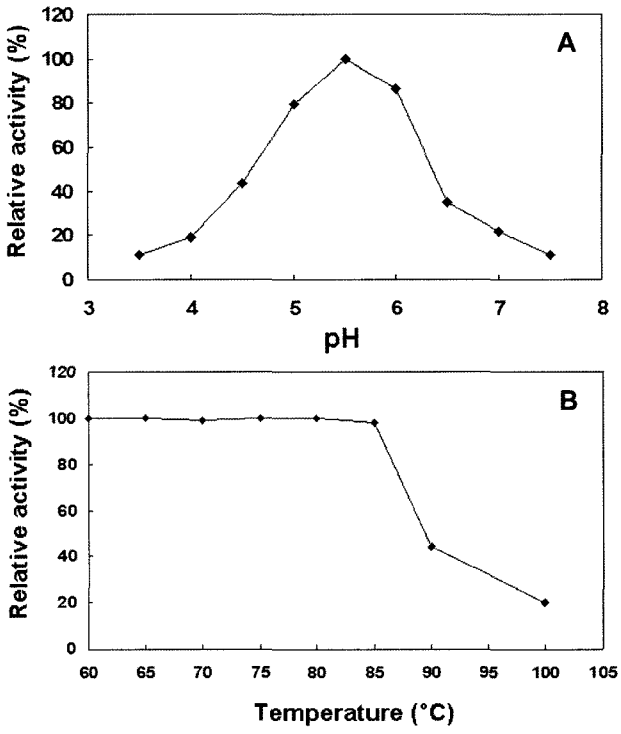

Fig. 2. Optimum pH for activity (**A**) and thermal stability (**B**) of SSGDE.

A. Values in ordinate are shown as percentages of maximum specific activity (100%) observed at pH 5.5. **B.** Values are shown as percentages of specific activity (100%) observed at 60°C.

of between pH 4.5 and 6.5. The optimal temperature for the enzyme was 85°C. As expected, the enzyme exhibited a remarkable thermal stability, retaining its full activity after 60 min of incubation at 85°C (Fig. 2B). About 20% of the enzyme activity remained after 60 min of incubation at 100°C. When compared with the GDE from *S. solfataricus* P2, the optimum pH for SSGDE was right-shifted by 0.5 units, and included a much higher stability in an acidic pH from 3 to 6.5, yet lower stability in an alkaline pH from 9 to 10. In addition, SSGDE had a higher optimal temperature by 10°C [8].

Effect of Metal Ions and Organic Solvents on SSGDE Activity

The activity of SSGDE was slightly activated or inhibited by 5 mM of the metal ions tested (Table 2). Among them, Mn^{2+}, Fe^{2+}, and Ba^{2+} increased the enzyme activity by 41–62% compared with the original activity, whereas Cu^{2+}, Ni^{2+}, and Al^{3+} decreased the activity by 22–27%. EDTA had no effect on the activity. The SSGDE activity was also inhibited 8–27% in the presence of 10% (v/v) organic solvents. The dependence of SSGDE on the metal ions tested was not so different from that of *S. solfataricus* GDE, except for Cu^{2+} and Ni^{2+}. The isoamylase activity of *S. solfataricus* GDE was shown to be strongly inhibited by 70–80% in the presence of Cu^{2+} and activated up to 120–130% by Ni^{2+} [8].

Catalytic Properties of Recombinant SSGDE

As expected for the sequence homology analysis, SSGDE exhibited amylo-1,6-glucosidase activity. It cleaved an α-1,6-branched linkage in pullulan and glycogen, producing a maltotriose and maltooligosyl chain, respectively (Fig. 3). The enzyme also showed its catalytic activity as a kind of 4-α-glucanotransferase. As a transferase, GDE generally attacks α-1,4-D-glucans to transfer a segment of the maltooligosyl chain to a new 4-position in another α-1,4-D-glucan acceptor, which can produce a glucose and different sizes of α-1,4-D-glucan [20]. When using G4, G5, and G6 as the substrate, SSGDE transferred certain sizes of chain, mainly ranging from G2 to G4, to produce α-1,4-glucan products elongated by the specified size of the maltooligosyl chains, rather than a disproportionated product sequentially differentiated by the glucosyl moiety. However, when G7 was used as the substrate, the enzyme clearly exhibited disproportionated hydrolysis products. In addition, its transferring activity was too low to produce larger maltooligosaccharides than the substrate used. According to the 6 h time course of the reaction with G5 (Fig. 4A), SSGDE clearly preferred the transfer of a maltotriosyl chain to G5, thereby leaving G2 increased and accompanying the hydrolysis to G1 and G4. For the 3 h time course reaction with G2-β-CD (Fig. 4B), SSGDE catalyzed not only the cleavage of the α-1,6-branched linkage between G2 and β-CD, but also the transfer of the maltosyl residue cleaved to the 4-position of the maltosyl moiety in another G2-β-CD, resulting in the formation of G4-β-CD. A minimal amount of G6-β-CD was also produced in a prolonged reaction (data not shown). Therefore, the above results confirmed that SSGDE contained the bifunctional activities of amylo-1,6-glucosidase and α-1,4-1,4-transferase, including α-1,4-hydrolyzing activity. Interestingly, this was the first time α-1,6-1,4-transferase activity was identified with a G2-β-CD substrate. This kind of transferase activity has not previously been reported

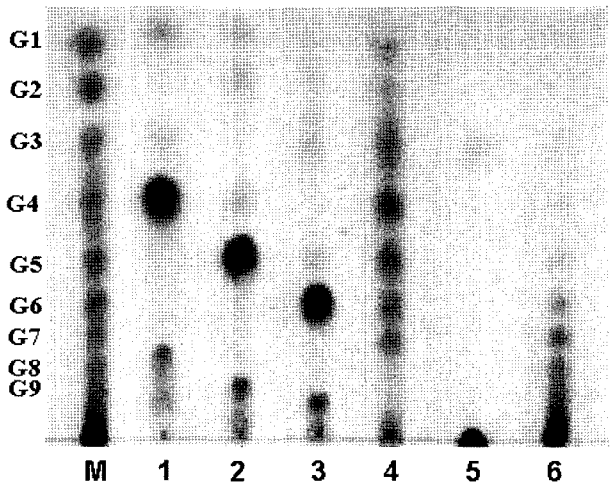

Fig. 3. TLC analysis of SSGDE reaction with various substrates. Lane M, maltooligosaccharide standards (G1–G9); lane 1, maltotetraose; lane 2, maltopentaose; lane 3, maltohexaose; lane 4, maltoheptaose; lane 5, pullulan; lane 6, glycogen.

Table 2. Effect of metal ions and organic acids on SSGDE activity.

Metal ions or organic solvents	Relative activity (%)
None	100
Mn^{2+}	162.2
Cu^{2+}	78.3
Ni^{2+}	76.8
Fe^{2+}	140.6
Co^{2+}	115
Mg^{2+}	117.2
Ca^{2+}	127.3
Ba^{2+}	141.7
Al^{3+}	73.2
EDTA[a]	101.7
DMSO	80.8
DMF	72.9
Methanol	91.6
Ethanol	83.3

Reaction was carried out in the absence and presence of metal ions at a final concentration of 5 mM or 10% organic solvent (v/v), respectively.
[a]EDTA (5 mM) was added as a chelating agent.

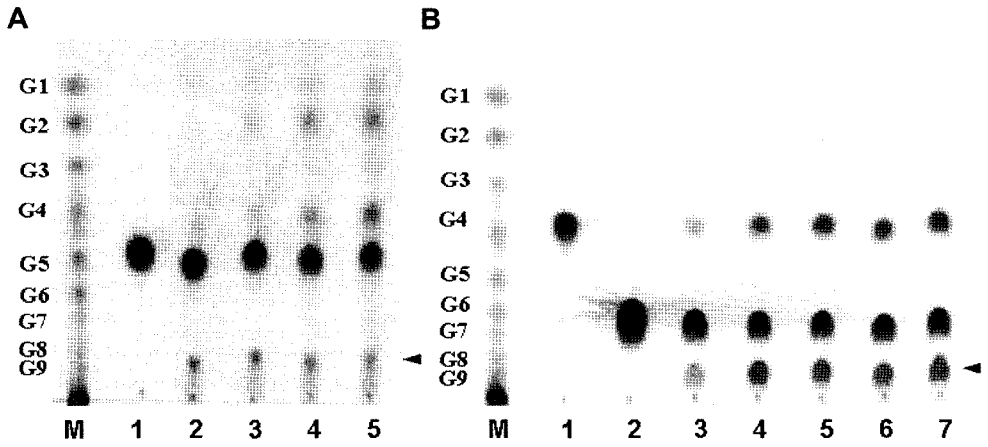

Fig. 4. TLC analysis of the time course of SSGDE reaction with maltopentaose (**A**) and maltosyl-β-cyclodextrin (**B**).
A. Lane M, maltooligosaccharide standards (G1–G9); lane 1, 0 min; lane 2, 60 min; lane 3, 120 min; lane 4, 240 min; lane 5, 360 min. **B**. Lane 1, β-cyclodextrin; lane 2, 0 min; lane 3, 10 min; lane 4, 20 min; lane 5, 30 min; lane 6, 90 min; lane 7, 180 min. Arrows indicate the transfer product produced from each substrate.

for the *S. solfataricus* GDE, which only demonstrated isoamylase activity cleaving an α-1,6-linkage. Reportedly, although yeast GDE cannot hydrolyze the α-1,6-linkage of G2-β-CD, it can hydrolyze the α-1,6-linkage of 6-*O*-α-glucosyl-β-CD (G-β-CD) into glucose and β-CD [11]. In fact, yeast GDE has a two-fold molecular weight compared with SSGDE, and is known to include transferase activity in its N-terminal half and amylo-1,6-glucosidase in its C-terminal half [15, 20]. Consequently, this means that SSGDE efficiently includes both activities within a single polypeptide that is half the size of yeast GDE. Furthermore, it is likely that the bifunctional activity of SSGDE is located at a single active site.

The glucosidase activity of SSGDE was compared with several polysaccharide substrates (Table 3). Amylopectin was the most preferentially hydrolyzed, with the others hydrolyzed in the order of rice starch, glycogen, pullulan, and amylose. This tendency of SSGDE was somewhat different to that of *S. solfataricus* GDE [8]. For glycogen and pullulan, SSGDE exhibited about 74 and 37% of glucosidase activity, respectively, on amylopectin, whereas *S. solfataricus* GDE exhibited less than 1% and no activity, respectively [2]. In fact, the *Sulfolobus* sp. GDE encoded by *treX* plays a role in degrading the branched linkage in glycogen, and provides a maltodextrin from which maltooligosyltrehalose synthase (*treY*) and maltooligosyltrehalose trehalohydrolase (*treZ*) cooperatively synthesize a trehalose in the microorganism [18]. It was thus very reasonable that SSGDE exhibited a significant substrate specificity for glycogen when compared with amylopectin. The kinetic parameters for SSGDE were determined with G5, G2-β-CD, and pullulan using Lineweaver-Burk plots. The K_m value of SSGDE for G2-β-CD was 12.23 mM, whereas the value for G5 was about 20 times higher at 246.3 mM. When assuming that the smallest Mw for pullulan was 1,000, the K_m value for pullulan was less than about 19 mM. The V_{max}/K_m values of SSGDE for G5, G2-β-CD, and pullulan were 1.29×10^{-6}, 13.6×10^{-6}, and more than 13.2×10^{-6} s^{-1}, respectively. As such, these results indicated that the α-1,6-branched structure was kinetically favorable for substrate binding to the active site of SSGDE.

Table 3. Relative activity of SSGDE with various polysaccharide substrates.

Substrates	Relative activity (%)
Amylopectin	100
Rice starch	82.4
Glycogen	73.5
Pullulan	36.8
Amylose	16.9

In conclusion, the hyperthermophilic GDE from *S. shibatae* was cloned and expressed in *E. coli*. The recombinant SSGDE exhibited amylo-1,6-glucosidase and α-1,4-glucanotransferase activities when employing various substrates, such as amylopectin, glycogen, pullulan, amylose, maltooligosaccharides, and 6-*O*-α-maltosyl-β-CD. The enzyme also had an acidic optimum pH and better stability in an acidic region. Furthermore, when considering its very high thermostability, SSGDE would appear to be an excellent candidate for application in industrial processing to degrade and modify the branching points of starch at a high temperature.

Acknowledgments

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