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Properties of a Novel Thermostable Glucoamylase from the Hyperthermophilic Archaeon *Sulfolobus solfataricus* in Relation to Starch Processing

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A gene (*ssg*) encoding a putative glucoamylase in a hyperthermophilic archaeon, *Sulfolobus solfataricus*, was cloned and expressed in *Escherichia coli*, and the properties of the recombinant protein were examined in relation to the glucose production process. The recombinant glucoamylase was extremely thermostable, with an optimal temperature at 90°C. The enzyme was most active in the pH range from 5.5 to 6.0. The enzyme liberated β-D-glucose from the substrate maltotriose, and the substrate preference for maltotriose distinguished this enzyme from fungal glucoamylases. Gel permeation chromatography and sedimentation equilibrium analytical ultracentrifugation analysis revealed that the enzyme exists as a tetramer. The reverse reaction of the glucoamylase from *S. solfataricus* produced significantly less isomaltose than did that of industrial fungal glucoamylase. The glucoamylase from *S. solfataricus* has excellent potential for improving industrial starch processing by eliminating the need to adjust both pH and temperature.

Starch is the reserve carbohydrate source in plant cells and is one of nature's major renewable resources. As well as being a major component of most of the world's staple foods, it is used in many industries including textile, pharmaceutical, and food and candy manufacture. Starch consists mainly of two homopolysaccharides, amylopectin and amylose. Amylopectin is an α-(1,4)-linked D-glucose polymer with ca. 5% of α-(1,6)-linked branches, whereas amylose is an essentially α-(1,4)-linked linear D-glucan with, at most, a negligible amount of branches. The major use of starch in the food industry is for the production of glucose, glucose syrups, and high-fructose corn syrups. In addition, the resulting glucose can be utilized to produce other products, such as ethanol, amino acids, or organic acids (2).

The production of glucose from starch is a multistage process involving different microbial enzymes in successive enzymatic steps. Two key enzymes are a thermostable bacterial α-amylase and a fungal glucoamylase (19). These enzymes catalyze the conversion of starch to glucose. In the first liquefying step, starch slurry is gelatinized and liquefied at pH 6 and 95 to 105°C by thermostable α-amylase. Subsequently, in the saccharification step, the liquefied maltodextrin is further converted to glucose by fungal glucoamylase, working optimally at a pH 4.5 and 60°C (16). There are two major drawbacks to using fungal glucoamylase in the starch industry. First, the optimal temperature of fungal glucoamylase is much lower than that of microbial α-amylase, resulting in the need for a cooling step and a low rate of catalysis. Second, at relatively high glucose concentrations, fungal glucoamylase condenses glucose into various small maltooligosaccharides, mainly maltose (G2) and isomaltose (IsoG2), depending on the source of the enzymes. The final glucose level of the saccharification step decreases due to the formation of di- or trisaccharide by-products via this reverse reaction (19).

In recent decades, many archaeal enzymes originating from hyperthermophilic archaea have attracted great attention because they are able to function under extreme conditions unsuitable for mesophilic enzymes, including high temperatures and the presence of denaturing agents (9, 11). Highly thermoactive α-amylases from hyperthermophilic archaea of the genera *Sulfolobus*, *Pyrococcus*, and *Thermococcus* have been described (3–5, 14). In addition, a couple of glycosyl hydrolases from *Sulfolobus* were studied. Two thermophilic enzymes involved in the production of trehalose, trehalosyl dextrin-forming enzyme and trehalose-forming enzyme, were highly expressed, purified, and characterized (6). Also, the α-glucosidase gene from *Sulfolobus solfataricus* was characterized and found to be closely homologous to its mammalian counterpart (20). The optimal pH for glycogen hydrolysis of the recombinant α-glucosidase gene at the intracellular pH of the organism suggests a unique role for the *S. solfataricus* α-glucosidase in carbohydrate metabolism. Unlike α-amylase, glucoamylase is very rare in hyperthermophilic archaea, although some glucoamylases from thermophilic anaerobic bacteria have been purified and characterized (1, 3, 8).

In this study, we cloned the glucoamylase gene (*ssg*) from

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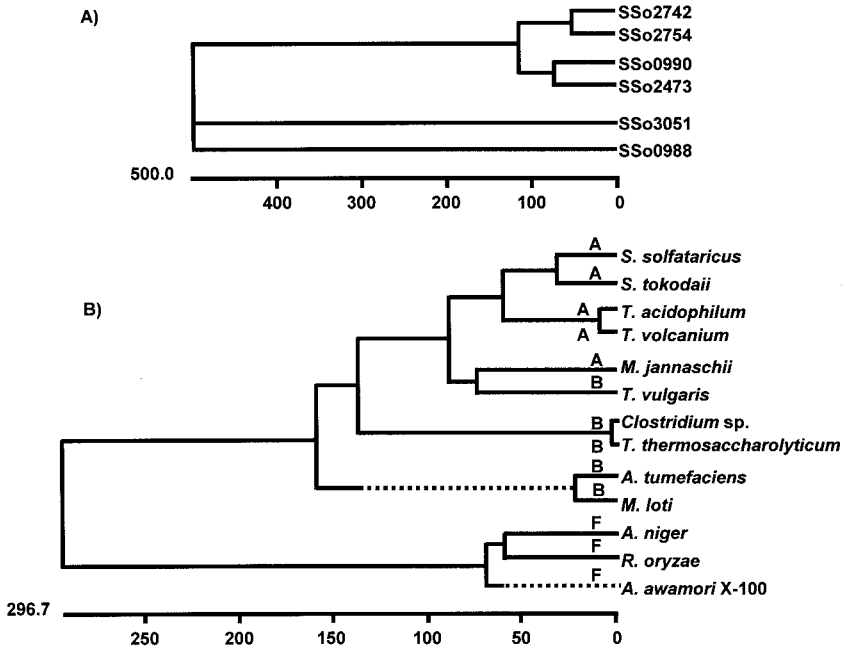

FIG. 1. (A) Phylogenetic analysis of six open reading frames (SSo0990, SSo0988, SSo2473, SSo2742, SSo2754, and SSo3051) encoding products homologous to carbohydrate-hydrolyzing enzymes, such as glucoamylase and glycoside hydrolase. (B) Phylogenetic analysis of glucoamylases of various microbial origins, including *S. solfataricus* (gi:15897867), *S. tokodaii* (gi:15921050), *Thermoplasma acidophilum* (gi:16081473), *Thermoplasma volcanium* (gi:13541259), *Methanococcus jannaschii* (gi:1592211), *Thermoactinomyces vulgaris* (gi:8777462), *Clostridium* sp. (gi:216417), *Thermoanaerobacterium thermosaccharolyticum* (gi:3243238), *Agrobacterium tumefaciens* (gi:15890159), *Mesorhizobium loti* (gi:14024312), *Aspergillus niger* (gi:30025851), *Rhizopus oryzae* (gi:1168453), and *Aspergillus awamori* var. X-100 (gi:1389841). A, archaea; B, bacteria; F, fungi.

S. solfataricus, a hyperthermophilic archaeon, and expressed it in *Escherichia coli*. The properties of the recombinant glucoamylase were characterized, and its possible application in starch processing was examined by comparison with a fungal glucoamylase used in the starch industry.

MATERIALS AND METHODS

Strains and culture conditions. *S. solfataricus* P2 was purchased from the Japan Collection of Microorganisms and cultivated as described previously (7). *E. coli* MC1061 [F$^-$ *araD139 recA13* Δ(*araABC-leu*)*7696 galU galK* Δ*lacX74 rpsL thi hsdR2 mcrB*] was used as a host for DNA manipulation and transformation. The *E. coli* transformants were grown in Luria-Bertani medium (1% [wt/vol] Bacto-Tryptone, 0.5% [wt/vol] yeast extract, 0.5% [wt/vol] NaCl) with ampicillin (100 μg/ml) or kanamycin (50 μg/ml) at 37°C.

Cloning and nucleotide sequence analysis. Chromosomal DNA of *S. solfataricus* P2 was isolated using a QIAmp tissue kit (Qiagen, Hilden, Germany). The putative glucoamylase gene was isolated by PCR (Thermocycler PE9600; Perkin-Elmer, Boston, Mass.) using the chromosomal DNA of *S. solfataricus* as a template and two oligonucleotide primers (SSNdeI [5′-GAAAGGCATTTCC ATATGAGAGTTTCCTCC-3′] and SSHindIII [5′-GGGGATCAAGCTTGGG TTATATATGG-3′]) designed from the chromosomal DNA sequence. DNA fragments were amplified by Vent DNA polymerase (New England Biolabs, Beverly, Mass.) at an annealing temperature of 55°C. The resulting PCR fragment was inserted into the *E. coli* expression vector pGNX4 (15) and digested with NdeI and HindIII, creating pGNX4SSGA. The nucleotide sequence of the PCR-generated gene was determined using a BigDye Terminator cycle-sequencing kit for ABI3700 PRISM (Perkin-Elmer). Other genetic manipulations were performed as described by Sambrook et al. (21).

Purification of recombinant glucoamylase. An extract of the *E. coli* transformant harboring the glucoamylase gene in pGNX4SSGA was obtained by sonication (VC-600; Sonics & Materials, Newtown, Conn.) and heated at 70°C for 10 min. After the precipitant was removed by centrifugation, the supernatant was collected and applied to a butyl-Sepharose column (10 by 1.0 cm) equilibrated with a 50 mM Tris-HCl buffer (pH 7.5) containing 1.0 M ammonium sulfate. It was then eluted with a linear gradient of ammonium sulfate (1.0 to 0 M) in the same buffer at a flow rate of 2 ml/min. The fractions showing enzymatic activity were dialyzed and concentrated. The sample was loaded on a Superdex 200 HR10/30 column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated in 50 mM Tris-HCl buffer (pH 7.5) and eluted with the same buffer at a flow rate of 0.4 ml/min.

Determination of enzyme activity. The enzyme activity of two glucoamylases, *S. solfataricus* glucoamylase (SSG) and *Aspergillus niger* glucoamylase (AMG; Novozymes, Bagsværd, Denmark), was determined using the glucose oxidase-peroxidase method with slight modifications (10). The reaction mixture contained 100 μl of 1% (wt/vol) maltose, 80 μl of 50 mM sodium acetate buffer (pH 6.0), and 20 μl of SSG; it was incubated at 80°C for 10 min. The reaction conditions for the AMG assay were the same as those for SSG, except for the reaction temperature (60°C) and pH (pH 4.5). The SSG reaction was stopped by putting the mixture in an ice-water bath, while the AMG reaction was stopped by boiling for 5 min. The quenched reaction mixture (100 μl) was reacted with the glucose oxidase reagent (900 μl) from a glucose-E kit (Youngdong Pharmaceutical Co., Seoul, Korea) at 37°C for 30 min, and the absorbance was measured at 505 nm. The equivalent of hydrolyzed glycosidic bonds was quoted as the glucose equivalent, using a glucose calibration curve. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 μmol of glucose per min.

Analysis of the anomeric configuration of glucose by ^{1}H-NMR. To examine the anomeric configuration of the hydrolysis product formed by SSG, 1% (wt/vol) maltotriose was incubated with SSG in a nuclear magnetic resonance (NMR) tube. The pattern of the ^{1}H-NMR spectrum was observed at 80°C. The spectrum was obtained with a JNM LA-400 FT NMR spectrophotometer (JEOL Ltd., Tokyo, Japan) using the heteronuclear multiple-bond connectivity mode.

HPAEC analysis. The hydrolysis products of maltohexaose (G6) and the reverse reaction of glucose were analyzed using high-performance anion-exchange chromatography (HPAEC). An analytical column for carbohydrate detection (CarboPac PA1; Dionex Co., Sunnyvale, Calif.) and an electrochemical detector (ED40; Dionex Co.) were used. Filtered samples were eluted in 150 mM NaOH, using a linear gradient of sodium acetate from 0 to 180 mM for 30 min.

TLC analysis. A thin-layer chromatography (TLC) plate (K5F silica gel 150Å; Whatman) was activated for 30 min in an oven at 110°C. Prepared samples were spotted on the plate, which was then developed in a TLC chamber at room temperature using *n*-butanol–acetic acid–water (5:3:1, vol/vol/vol) as the solvent. The plate was air dried and soaked with the solvent with 3 g of *N*-(1-naphthyl)

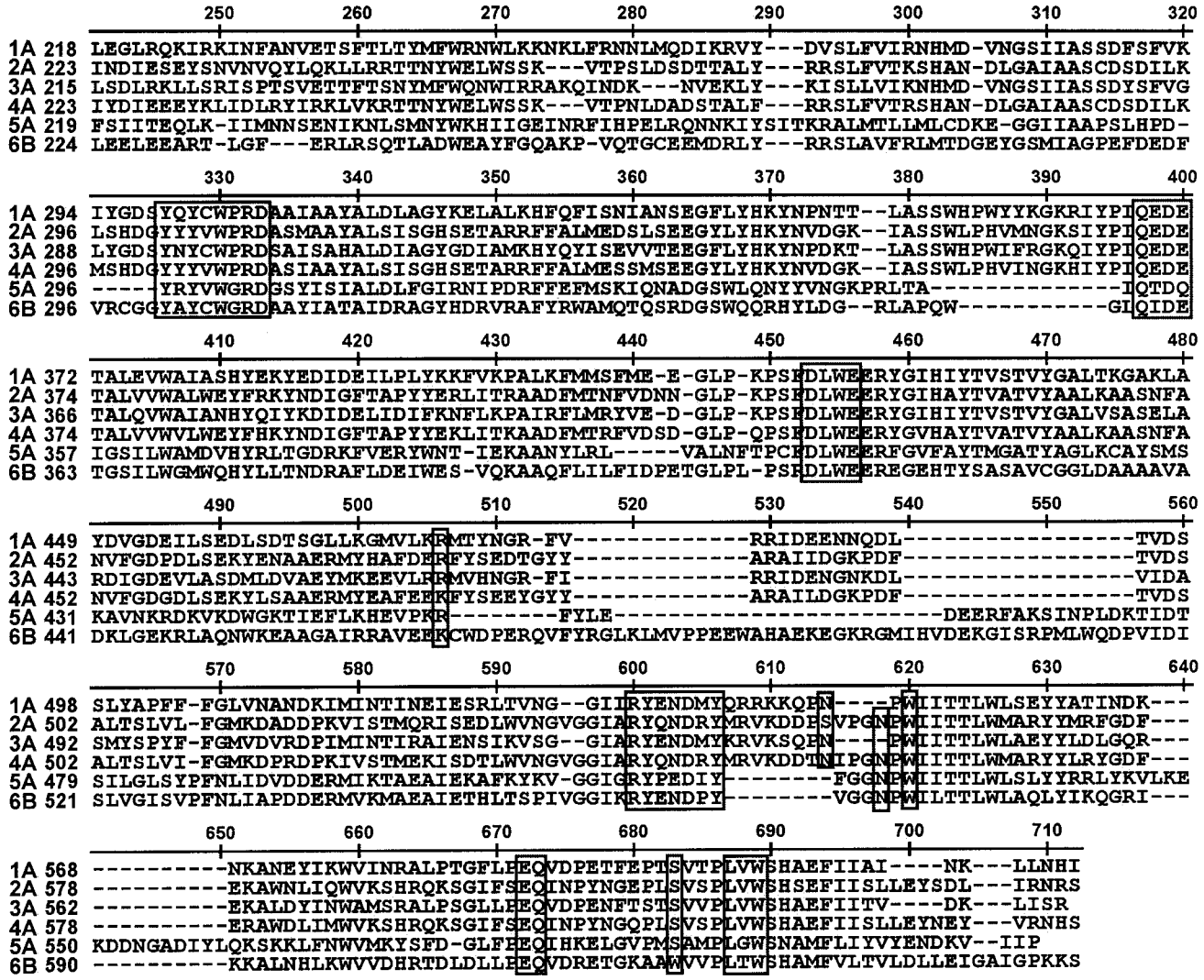

FIG. 2. Multiple-sequence analysis of glucoamylase homologs from *S. solfataricus* with known glucoamylases. The conserved amino acid residues are boxed. 1A, *S. solfataricus* (gi:15897867); 2A, *Thermoplasma acidophilum* (gi:16081473); 3A, *S. tokodaii* (gi:15921050); 4A, *Thermoplasma volcanium* (gi:13541259); 5A, *Methanococcus jannaschii* (gi:1592211); 6B, *Thermoactinomyces vulgaris* (gi:8777462).

ethylenediamine and 50 ml of concentrated H_2SO_4 per liter of methanol. The plate was then dried and placed in an oven at 110°C for 10 min.

Reverse reaction. Glucose (30% [wt/vol]; Showa Chemical Co., Tokyo, Japan) in 50 mM sodium acetate buffer (pH 6.0) containing various amounts of SSG was incubated at 80°C for 11 days. Similarly, AMG was reacted with 30% (wt/vol) glucose in 50 mM sodium acetate buffer (pH 4.5) at 60°C. The amounts of enzymes used were 7.2, 14.4, and 28.8 U per ml of reaction mixture. The products of the reverse reaction were analyzed using HPAEC.

Production of glucose from G6. In the presence of G6 (5%) as a substrate, enzyme reactions with either SSG (5.45 U/ml) or α-glucosidase (4.0 U/ml) were performed with 50 mM sodium acetate buffer (pH 6.0) at 80°C. For AMG (5.45 U/ml), the reaction mixture containing 5% of G6 was incubated in 50 mM sodium acetate buffer (pH 4.5) at 60°C. The resulting reaction products were analyzed using HPAEC.

RESULTS

Cloning and expression of glucoamylase from *S. solfataricus* in *E. coli*. Analysis of the genome of the hyperthermophilic archaeon *S. solfataricus* revealed the presence of six open reading frames (SSo0990, SSo0988, SSo2473, SSo2742, SSo2754, and SSo3051) homologous to those encoding carbohydrate-hydrolyzing enzymes such as glucoamylase and glycoside hydrolase. Among those, two genes (SSo0988 and SSo3051) were analyzed as encoding α-amylase and α-glucosidase, respectively. A phylogenetic tree showed that the other four genes

TABLE 1. Purification steps of recombinant SSG

Step	Vol (ml)	Total activity (U)	Total amt of protein (mg)	Sp ac (U/mg)	Yield (%)	Fold purification
Cell extraction	450	936	1,345	0.696	100	1.00
Heat treatment	425	787	279	2.82	84	4.05
Butyl-Sepharose	25	150	13.13	11.43	16	16.4
Superdex 200 HR 10/30	4	43.94	0.78	56.3	4.6	80.9

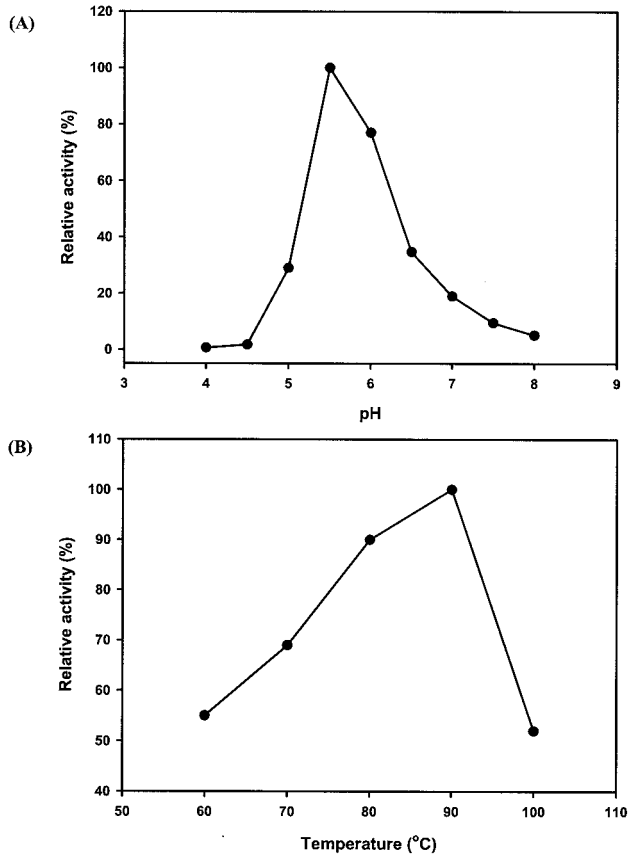

FIG. 3. Optimal pH (A) and temperature (B) of recombinant SSG. The relative activity of SSG (20 μl; 2.5 μg/ml) at the specified pH or temperature was determined in Universal buffer. G3 (1% [wt/vol]; 100 μl) was used as the substrate, and the enzyme reaction proceeded for 10 min.

(SSo0990, SSo2473, SSo2742, and SSo2754) clustered away from the first two genes (Fig. 1A). A BLAST search revealed that these genes were highly homologous to those encoding glucoamylases from various microorganisms (Fig. 1B). However, the multiple-sequence analysis of these putative glycoside hydrolases with known microbial glucoamylases showed that only one gene (SSo0990; *S. solfataricus* glucoamylase [*ssg*]) maintained the conserved amino acid residues which are important to the enzyme activity (Fig. 2). This gene encodes 622 amino acids with a putative molecular mass of 65 kDa. The *ssg* gene (1.87 kb) that resides in the genome of *S. solfataricus* P2 was amplified by PCR as described in Materials and Methods. The amplified gene was inserted into pGNX4, an expression vector for *E. coli* (15). The resulting plasmid was designated pGNX4SSGA; in this plasmid, the expression of the *ssg* gene was under the control of *tac* promoter. Finally, the expression of the *ssg* gene in *E. coli* was successful and the recombinant thermostable enzyme was stably maintained during expression.

Purification and characterization of recombinant glucoamylase. As is customary in the purification of thermostable enzymes, the soluble fraction of the cell extract was heated at 70°C for 10 min to eliminate considerable amounts of heat-labile host proteins from the extract (19). The heat-treated sample was purified further by using butyl-Sepharose and gel permeation chromatography. The glucoamylase was purified 80-fold with a yield of about 4.6% from the cell extract (Table 1). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the final preparation revealed a single band with a molecular mass of 65 kDa, which is consistent with the expected size deduced from the primary amino acid sequence of the glucoamylase.

The optimal temperature and pH of the recombinant SSG were determined using G3 as a substrate at various temperatures (60 to 100°C) and pH values (pH 4.0 to 8.0). The recombinant SSG had its optimal temperature at 90°C and pH at 5.5. The enzyme displayed 90% of its maximum activity at 80°C, but the relative activity was greatly reduced at 100°C (Fig. 3). The enzyme maintained its activity in a very narrow pH range (pH 5.5 to 6.0). It was observed that SSG cleaved the α-(1,4) glycosidic bond sequentially from the nonreducing end and completely hydrolyzed 4-nitrophenyl-α-D-maltopentaoside into glucose and 4-nitrophenol within 15 h (Fig. 4). Gel permeation chromatography revealed that the molecular mass of SSG was about 250 kDa, suggesting that the enzyme is present as a tetramer (Fig. 5A). Moreover, sedimentation equilibrium analysis of glucoamylase by analytical ultracentrifugation confirmed that the enzyme existed as a tetramer (Fig. 5B).

Substrate specificity of the recombinant glucoamylase of *S. solfataricus*. Glucoamylases liberate β-D-glucose from their substrates, while α-glucosidase generally releases α-D-glucose. To elucidate the anomeric configuration of the product formed by hydrolysis by SSG, the pattern of the ^{1}H-NMR spectrum at 80°C was investigated. The β-anomeric form appeared at 5.0 to 5.1 ppm at the early reaction time, and its amount gradually increased as the reaction continued (Fig. 6). In the meantime, the amount of the α-anomeric form (5.6 to 5.7 ppm) was slightly increased due to the equilibrium reaction between the α and β anomers. This reaction pattern was clearly visible compared to that of α-glucosidase (data not shown). The increasing rate of the quantifiable area of the corresponding β-anomer peaks was much higher for SSG, again compared to α-glucosidase. This clearly indicated that the recombinant glucoamylase liberates β-glucose from its substrate.

The substrate specificity of the enzyme was determined us-

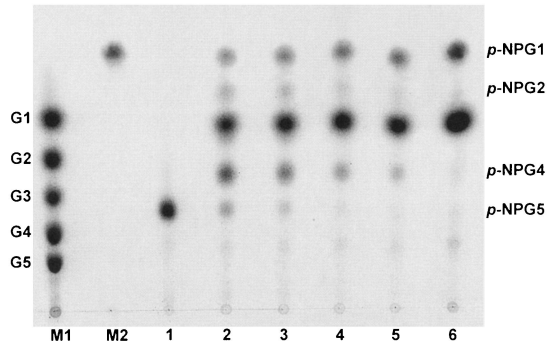

FIG. 4. The change of the hydrolysis product of *p*-nitrophenyl-α-maltopentaoside (*p*-NPG5) by SSG as a function of reaction time. Lanes M1, maltooligosaccharide standards (glucose to maltopentaose); M2, *p*-nitrophenyl-α-glycoside (*p*-NPG1) standard; 1 to 6, the hydrolysis product of *p*-NPG5 at different reaction times (0, 0.5, 1, 2, 3, and 15 h, respectively).

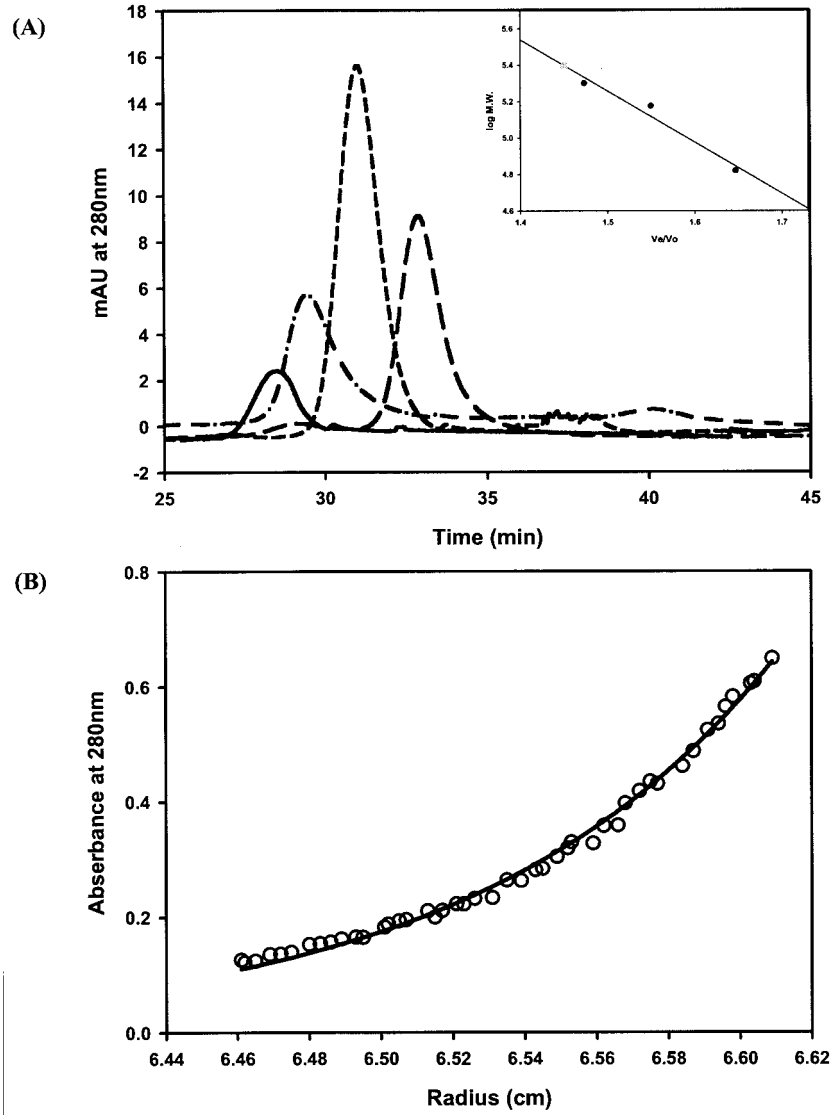

FIG. 5. Gel permeation chromatography (A) and sedimentation equilibrium analytical ultracentrifugation (B) of SSG. (A) The proteins were eluted with 50 mM Tris-HCl buffer (pH 7.5) through a Superdex 200 HR10/30 column. ——, SSG; –·–·–, β-amylase, 200kDa; -------, alcohol dehydrogenase, 150kDa; – – –, bovine serum albumin, 66kDa. The inset shows the line fitting the plot of the elution time versus the logarithm of the molecular weight of the size markers. mAU, milli-absorbance units. (B) A sedimentation equilibrium measurement was performed at 20°C with the sample in a Beckman Optima XL-A analytical ultracentrifuge at a rotor speed of 8,000 rpm. The values of the two variables, the absorbance at 280 nm versus radial positions, were obtained. The apparent molecular weight of SSG was calculated.

ing various α-glucans (G2 to G7, IsoG2, and soluble starch). Analysis of the hydrolyzed products by SSG with TLC clearly demonstrated that both α-(1,4) and α-(1,6) glycosidic linkages were hydrolyzed (data not shown). Usually, bacterial glucoamylases efficiently hydrolyze maltooligosaccharides such as G2, G3, and G4. By contrast, fungal glucoamylase hydrolyzes starch more effectively than it hydrolyzes maltooligosaccharides. The glucoamylase from *S. solfataricus* hydrolyzed G3 much more efficiently than it hydrolyzed any other maltooligosaccharide (Table 2). A significant decrease in the hydrolyzing activity was observed when G2 and G4 were used as substrates. The relative activity decreased further when longer maltooligosaccharides were used in the hydrolysis reaction. The *S. solfataricus* glucoamylase preferentially attacked α-(1,4)

TABLE 2. Reaction rate of SSG for various substrates[a]

Substrate	Reaction rate (μmol/min)
G2	57.9 ± 2.90
G3	388 ± 12.3
G4	79.6 ± 13.0
G5	32.6 ± 2.35
G6	26.6 ± 1.11
G7	17.5 ± 0.55
IsoG2	1.15 ± 0.24
SS	0.68 ± 0.01

[a] Reaction rates were determined at 80°C for 10 min. Each reaction mixture contained 100 μl of 1% substrate, 80 μl of 50 mM sodium acetate buffer (pH 6.0), and 20 μl of purified SSG.

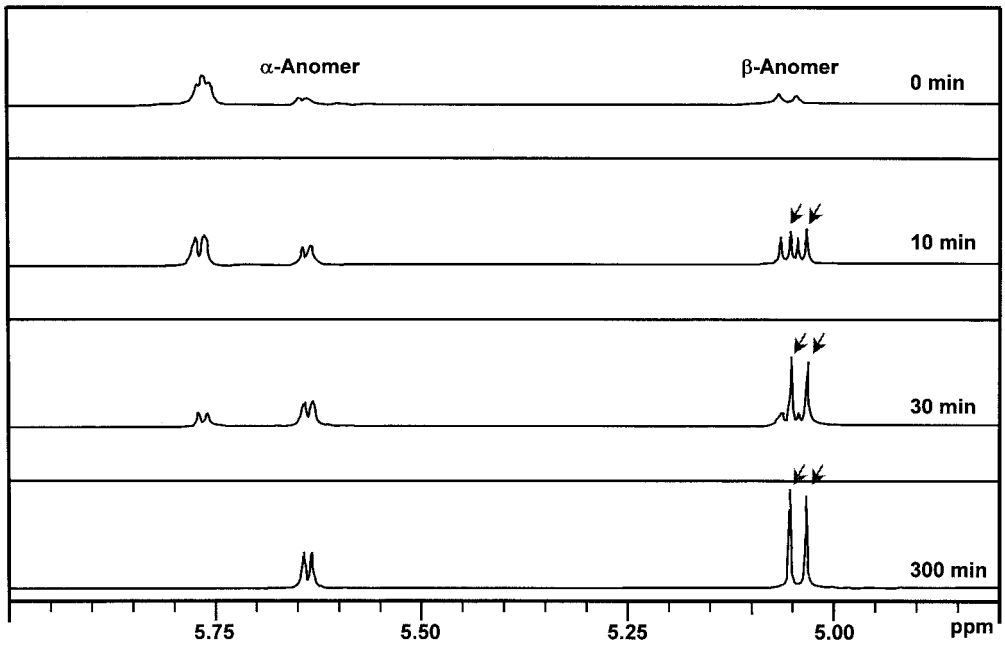

FIG. 6. ^{1}H-NMR spectrum of G3 hydrolysates by SSG at different reaction times. The arrows in the NMR spectrum indicate the corresponding peaks of the β-anomeric form.

glycosidic linkages rather than α-(1,6) glycosidic linkages, as judged by comparison of the relative hydrolyzing activity of G2 and IsoG2 (Table 2).

Starch processing: comparative study with an industrial glucoamylase. The saccharifying abilities of SSG and two other enzymes were evaluated using gelatinized soluble starch as a substrate at 80°C. The α-amylase from *B. licheniformis* (Termamyl; Novo Nordisk) and the pullulanase from a strain of *Thermotoga maritima* were included to enable faster, more complete degradation of the substrate by the saccharifying enzymes. The maximum glucose yield (89.4% with 0.9% IsoG2 as the substrate) was obtained with the saccharification of the 17-h SSG reaction at 80°C. This value is comparable to the final glucose yield obtained using fungal glucoamylase (16). The final yield of G1 increased to 99% when 5% G6 was used as the substrate (Table 3). When the reaction was allowed to continue, however, the G1 level decreased due to the increased reversion rate, forming primarily the disaccharides G2 and IsoG2. Comparing the hydrolytic activity of SSG with that of other enzymes, we found that α-glucosidase cloned from *S. solfataricus* (20) produced less glucose (90%) and three

TABLE 3. Production of glucose from 5% (wt/vol) G6[a]

Enzyme	Concn (U/ml)	pH	Temp (°C)	Composition of product[b]	
				G1	IsoG2
SSG	5.45	6.0	80	99.3	0.33
α-Glucosidase (*S. solfataricus*)	4.0	6.0	80	90	1.1
AMG	5.45	4.5	60	99.3	0.67

[a] The enzyme reactions producing glucose from G6 were performed for 24 h in 50 mM sodium acetate buffer at the designated pHs and temperatures.
[b] The composition of the resulting products was analyzed by HPAEC as described in Materials and Methods.

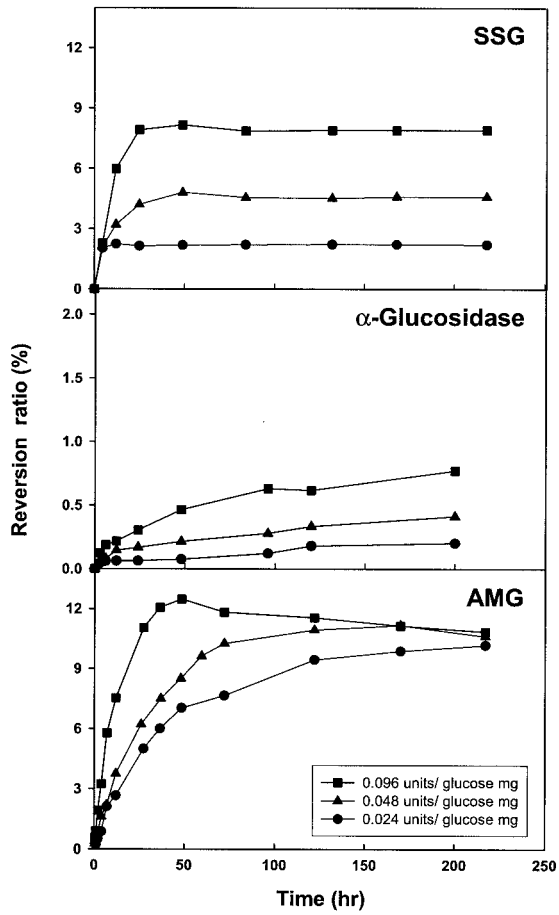

FIG. 7. Reversion of glucose into isomaltose by SSG, α-glucosidase, and AMG.

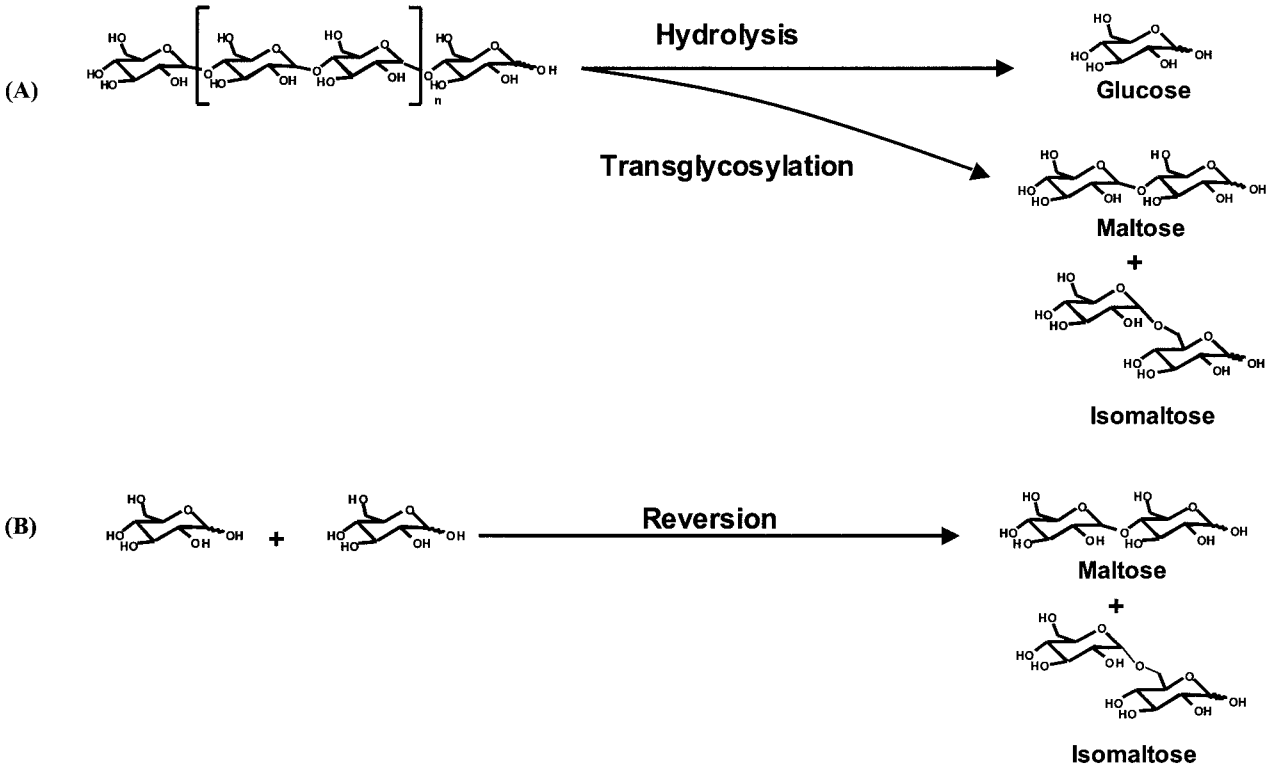

FIG. 8. Two distinct mechanisms of G2 and IsoG2 productions by glucoamylase action. (A) Hydrolysis and transglycosylation reaction; (B) reverse reaction by glucoamylase.

times as much IsoG2 (1.1%) while AMG produced the same amount of glucose and twice as much IsoG2 (0.67%) (Table 3).

The possible reverse reaction catalyzed by SSG was compared to that of an industrial fungal glucoamylase, AMG. Various amounts of enzymes were reacted with 30% G1 (1.67 M), and the formation of reversion products, mainly IsoG2, was monitored. As shown in Fig. 7, the amounts of the reversion products increased in proportion to the amount of enzyme used. The amount of IsoG2 produced by SSG increased at the beginning of the reaction and was maintained at a constant level after 24 h of reaction. The reverse reaction pattern of AMG is quite different from that of SSG since the amount of IsoG2 gradually increased up to 125 h of reaction. At a high AMG concentration (0.096 U/mg of glucose), rehydrolysis of the reversion product was observed after 50 h of reaction due to the relatively high concentration of the reversion product and the hydrolysis activity of AMG. At the same concentration, SSG had a much lower reverse reaction rate than AMG did. For instance, 2% IsoG2 produced by the reverse reaction was observed with SSG (0.024 U/mg of glucose) whereas up to 10% IsoG2 appeared under the identical reaction conditions with AMG. Even at higher enzyme concentrations, SSG produced less IsoG2 than AMG did. Of the enzymes used in this study, α-glucosidase (*S. solfataricus*) had the lowest reversion yield from G1 to IsoG2—less than 1% even with the highest concentration of the enzyme (0.096 U/mg of glucose).

DISCUSSION

Industrial starch processing uses fungal glucoamylases isolated from *A. niger* or closely related species in the saccharification step. These enzymes have pH and temperature optima at pH 4.0 and 60°C, respectively. Identification of a thermostable glucoamylase that can act at the pH and temperature of the liquefaction step, pH 5.5 to 6.0 and 95 to 105°C, could improve the starch conversion process. SSG exhibited its maximum activity at 90°C and pH 5.5, conditions that are very close to the industrial operating conditions of starch liquefaction. This implies that SSG would be suitable for improving the enzymatic two-step liquefaction-saccharification conversion process by eliminating the need to adjust the pH and temperature to the optima for the fungal glucoamylase.

Although numerous glucoamylases from fungi and yeasts have been studied (24), relatively few bacterial glucoamylases have been detected to date. Glucoamylases from some *Clostridium* species have been described (12, 18). These anaerobic bacteria are thermophilic and produce glucoamylases that are optimally active at 60 to 70°C and pH 5 to 6. Recently, heat- and acid-stable glucoamylases from extremophilic archaea were identified and characterized (22). The purified enzymes from *Thermoplasma acidophilum*, *Picrophilus torridus*, and *P. oshimae* were optimally active at pH 2.0 and 90°C. These archaeal glucoamylases, like the fungal glucoamylase, have higher substrate specificity for larger molecules, such as starch, amylopectin, and glycogen, than for the smaller maltooligosaccharides. By contrast, SSG showed a substrate preference for

smaller molecules rather than large polysaccharides. Of the maltooligosaccharides, G3 is the most favorable substrate for SSG. The substrate preference of SSG is more likely to be related to that of α-glucosidase than to that of typical glucoamylases, since α-glucosidase converts maltodextrins, especially G2, to glucose. However, the enzyme obviously liberated β-D-glucose from the substrate during the hydrolysis reaction, indicating that SSG can be distinguished from α-glucosidase, which releases α-D-glucose. Glucoamylases isolated from the hyperthermophilic archaea *Methanococcus jannaschii* and *Thermoactinomyces vulgaris* exhibited substrate specificity similar to that of SSG (23). Determination of the three-dimensional structures of these enzymes followed by comparison of their active sites with those of their fungal counterparts should explain the differences in the substrate specificity of these glucoamylases more clearly.

Two distinct mechanisms of action of glucoamylase lead to the accumulation of G2 and IsoG2 in the maltodextrin- (or glucan)-hydrolyzing process (Fig. 8). During the hydrolysis of α-(1,4)-linked glucan, G2 and IsoG2 are concurrently produced by the transglycosylating action of glucoamylase (Fig. 8A). Comparing the G6-hydrolyzing activity, it was noticed that SSG produced less transglycosylated IsoG2 than the other enzymes did. Particularly, α-glucosidase (*S. solfataricus*) accumulated much more IsoG2 during the hydrolysis reaction.

The industrial processing of starch requires high concentrations of solids (>30%) in order to be economical; this results in a high glucose concentration. Under this high-glucose condition, glucoamylase is likely to form reversion products (Fig. 8B). G2 is produced in the early stage of the reverse reaction, but it does not increase in amount since equilibrium occurs at approximately 1 to 2%. However, IsoG2, an α-(1,6)-linked reversion product, tends to accumulate in the reaction mixture at the expense of glucose. When the reverse reaction of SSG was compared with that of the industrial enzyme (AMG), it was found that SSG produced many fewer reversion products. Interestingly, the α-glucosidase from *S. solfataricus* showed even lower reversion activity (<1%) (Fig. 7). However, this enzyme is not suitable for industrial application, since it has a very low hydrolytic activity toward large maltodextrins. The enzyme dose and the reaction temperature and time can be adjusted to minimize the reverse reaction (13). Even industrial fungal glucoamylase can be engineered to have decreased reversion activity. From this point of view, SSG is an excellent candidate for application in industrial starch processing, not only because of its optimal activity at high temperature and pH 6.0 but also because of its low levels of transglycosylation and reversion activity.

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