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Characterization and Application of a Novel Thermostable Glucoamylase Cloned from a Hyperthermophilic Archaeon *Sulfolobus tokodaii*

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Abstract A gene for a putative glucoamylase, *stg*, of a hyperthermophilic archaeon *Sulfolobus tokodaii* was cloned and expressed in *Escherichia coli*. The recombinant glucoamylase (STGA) had an optimal temperature of 80°C and was extremely thermostable with a D-value of 17 hr. The pH optimum of the enzyme was 4.5. Being different from fungal glucoamylases, STGA hydrolyzed maltotriose (G3) most efficiently. Gel permeation chromatography and sedimentation equilibrium analytical ultracentrifugation analysis showed that the enzyme existed as a dimer. STGA was stable enough to hydrolyze liquefied corn starch to glucose in 4 hr at 90°C with a yield of 95%. Comparison of the k_{cat} values for the hydrolysis and the reverse reaction at 75°C and 90°C indicated that glucose production by STGA was more efficient at 90°C than 75°C. Therefore, STGA showed great potential for application to the industrial glucose production process due to its high thermostability.

Key words: *Sulfolobus tokodaii* glucoamylase (STGA), reversion, thermostability

Introduction

Glucoamylase (1,4-α-D-glucanhydrolase; EC 3.2.1.3) is an exo-hydrolase that catalyzes release of β-D-glucopyranose from the non-reducing ends of starch, related oligosaccharides, or polysaccharides. Glucoamylase can hydrolyze α-(1,4)-glycosidic linkages and also α-(1,6)-glycosidic linkages at branch points but much less rapidly. Glucoamylase is an important enzyme in the starch processing and ethanol fermentation industries, one of the largest tonnage enzymes produced for various applications. Glucoamylase has been produced mostly from *Aspergillus niger* (1-4).

Since the last decades many enzymes from hyperthermophilic archaea have gained great attention because they are capable of catalyzing reactions under extreme conditions of high temperature or employing denaturant agents, which are unsuitable for most enzymes from mesophiles (5-14). However, although there have been many studies on fungal glucoamylases, only a few bacterial and archaeal glucoamylases have been reported (15, 16).

In the industrial starch process, whole kernels of corns are initially fractionated into semi-purified streams of protein, fiber, oil, and starch containing both amylose and amylopectin. Amylose consists of glucoses linked by α-(1, 4)-glycosidic bonds and amylopectin has chains of glucoses linked by α-(1,4)-glycosidic bonds and α-(1,6)-glycosidic bonds at the branch points. The resulting starch fraction has a pH of 4.5. The next process involves liquefaction of the semi-purified starch to malto-oligosaccharides using *Bacillus licheniformis* α-amylase (BLA) at pH 5.7 - 6.0 and 105°C in the presence of calcium for several hours. The second step in the process involves saccharification of the liquefied starch using an *Aspergillus* glucoamylase and a bacterial pullulanase, a debranching enzyme. The saccharification process takes more than 2 days and the pH and temperature conditions need to be lowered back to pH 4.5 - 4.8 and 55-60°C, respectively.

In the present study, we attempted to screen a novel hyperthermostable glucoamylase from *Sulfolobus tokodaii*, a hyperthermophilic archaeon for the purpose of application to industrial production of glucose without time and energy consuming readjustment of temperature and pH during the process. A gene encoding putative glucoamylase was cloned from the archaeon in *E. coli* and characterization of the gene product proved that it was a bona-fide glucoamylase that is extremely thermostable. Therefore, glucoamylase from *S. tokodaii* (STGA) has great potential to improve the glucose production process.

Materials and Methods

Strains and culture conditions *S. tokodaii* str. 7 was purchased from the Japan Collection of Microorganisms and cultured as described previously (10). *E. coli* MC1061 [F⁻, *araD*139, *recA*13, Ä(*araABC-leu*)7696, *galU*, *galK*, Δ*lacX*74, *rpsL*, *thi*, *hsdR*2, *mcr*B] was used as a host for DNA manipulation and transformation. The *E. coli* transformants were grown in Luria-Bertani (LB) medium [1% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, and 0.5% (w/v) NaCl] with ampicillin (100 μg /ml) or

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kanamycin (50 μg /ml) at 37°C.

Cloning and nucleotide sequence analysis Chromosomal DNA of *S. tokodaii* str. 7 was isolated using a QIAmp Tissue Kit (Qiagen, Hilden, Germany). The putative glucoamylase gene was isolated by PCR (Thermocycler PE9600, Perkin-Elmer, Boston, MA) using the chromosomal DNA of *S. tokodaii* str. 7 as a template and two oligonucleotide primers (ST*Nco*I : 5'-CATAAATTC CCATGGGATATCTCAATAT AGG-3', ST*Pst*: 5'-CATT AGGTATTCTGCAGTACTTATCTGG-3') designed based on the DNA sequence in the database (17). DNA fragments were amplified by Ex-Taq™ DNA polymerase (Takara Biomedical Co., Japan) at an annealing temperature of 55°C. The resulting PCR fragment was inserted into an *E. coli* expression vector, p6xHis119, at the *Nco*I and *Pst*I restriction sites (18). The recombinant DNA was designated as p6xH(-)STG but did not carry a histidine tag fused to the cloned gene. The nucleotide sequence of the cloned gene was determined using a BigDye Terminator Cycle Sequencing kit for ABI3700 PRISM (Perkin-Elmer, Boston, MA). Other genetic manipulations were performed as described by Sambrook *et al.* (19).

Purification of glucoamylase An extract of the *E. coli* transformant harboring p6xH(-)STG was sonicated (VC-600, Sonics & Materials, Newtown, CT) and heated at 60°C for 20 min. The cell extract was centrifuged and proteins in the supernatant was fractionated with 40 - 60% (w/v) ammonium sulfate. The fraction with the highest glucoamylase activity was dialyzed against 50 mM Tris-HCl buffer (pH 7.5) and applied to a butyl-sepharose column (HiTrap 10 × 1.0 cm, Amersham Biosciences, Piscataway, NJ, USA) that was equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 1.0 M ammonium sulfate. Proteins were then eluted with a linear gradient of ammonium sulfate (1.0-0 M) in the same buffer at a flow rate of 2 ml/min. The fractions showing glucoamylase activity were dialyzed and concentrated with Amicon ultrafiltration kit (Mw cutoff 10000Da, Millipore, Waltham, Mass, USA). Purified STGA was analyzed by SDS-polyacrylamide gel according to the procedure by Laemmli (20).

Glucoamylase assay The enzyme activity of STGA was determined using the glucose oxidase/peroxidase method with slight modifications (21). The reaction mixture contained 50 μl of 1% (w/v) maltose 40 μl of 50 mM sodium acetate buffer (pH 4.5), and 10 μl of STGA. The reaction was carried out at 80°C for 10 min and was stopped by adding the same volume of 2 M Tris-HCl buffer (pH 7.5). The quenched reaction mixture (200 μl) was reacted with the glucose oxidase reagent (800 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. One unit (U) of enzyme activity was defined as the amount of enzyme that liberates 1 mmole of glucose per minute.

Thermal inactivation Purified STGA was incubated in 50 mM Tris-HCl buffer (pH 7.5) at 80°C or 90°C. Residual maltotriose hydrolyzing activity of aliquots taken at various time intervals was measured at the optimal temperature (80°C). The log of the percentage of residual activity was plotted against time in minute and the D-value of the enzyme at the particular temperature was determined.

High performance anion exchange chromatography (HPAEC) The hydrolysis products of liquefied corn starch (LCS) and the reverse reaction of glucose were analyzed using HPAEC. An analytical column for carbohydrate detection (CarboPac PA1, Dionex Co., Sunnyvale, CA) and an electrochemical detector (ED40, Dionex Co.) were used. Filtered samples were eluted with 150 mM NaOH using a linear gradient of sodium acetate from 0 to 180 mM for 30 min.

Hydrolysis and reversion kinetics The kinetic parameters of STGA were established as follows; an appropriate concentration of enzyme (10 μl) was mixed with various concentrations of maltose in 50 mM acetate buffer (pH 4.5). The hydrolysis reaction was carried out at 75°C or 90 °C. To determine the initial rate of the enzyme activity, aliquots were taken at every 30 seconds for duration of 2.5 min and immediately quenched with 100 μl of 2 M Tris-HCl buffer (pH 7.5) to stop the reaction. The amount of glucose in the samples was measured by the GOD-POD method modified slightly for the micro plate reader (505 nm; EL340i, Anachem, Luton, UK). Kinetic parameters were determined by Lineweaver-Burk plots.

For reversion kinetic analysis, 30% (w/v) glucose solution was reacted with 0.06 U of STGA. Aliquots of the reaction mixture were taken at regular time intervals and placed in an ice bath to stop the reaction. The reaction products were then analyzed using HPAEC and the peaks were identified by comparison with standards. The initial rates of the reverse reaction were then calculated as described above.

Gel permeation chromatography and analytical ultracentrifugation A Superdex 200 HR column (10 × 300 mm) was used for the estimation of the apparent molecular mass of STGA at room temperature. The enzyme (100 μl) was applied to the column equilibrated with 50 mM Tris-HCl buffer (pH 7.0), and eluted with the same buffer at a flow rate of 0.4 ml/min. Beta-amylase (200 kDa), alcohol dehydrogenase (ADH; 150 kDa), bovine serum albumin (BSA; 66 kDa), and carbonic anhydrase (29 kDa) were used as molecular weight standards.

Sedimentation equilibrium measurements were performed at 20°C using a Beckman Optima XL-A analytical ultracentrifuge equipped with a four-hole rotor with standard six channel cells at a rotor speed of 10,000 × g. The absorbance-versus-radius distributions A(r) were recorded at 280 nm. These were evaluated using the nonlinear regression method provided by Sigma Plot software (SPSS Inc., USA). The general equation used for fitting the A(r) data was:

$$A(r) = \sum_i A_i(r) = \sum_i A_i(r_o) \exp\left[iM_1 1 - (\bar{v} \cdot \rho_o)\omega^2 (r^2 - r_o^2)/2RT \right]$$

where i denotes number of protomers per oligomer; A_i, the absorbance of the corresponding species;n, the partial specific volume of the protein calculated as described by Zamyatnin (22) and assumed to be independent of the state of oligomerization; ρ_o the solvent density w the angular velocity of the rotor and r_o is the fixed radial position. Protein concentration used in analytical ultracentrifugation analysis was between Δ0.3 - 0.35 absorbance at 280 nm, corresponding to 0.2 mg/ml.

Results and Discussion

Cloning and expression of STGA in *E. coli* A gene encoding STGA was amplified by PCR and ligated into the plasmid p6xHis119. The resulting clone, p6xH(-)STG, carried a gene of 1845 bp encoding 615 amino acids for a protein with a predicted molecular mass of 71,426 Da. Bacterial glucoamylases consist of two domains, a catalytic domain and a N-terminal domain, but the role of the N-terminal domain is not clear yet (23, 24). The predicted amino acid sequence of STGA (gi : 15921050) shared about 55% homology (N-domain, 41%; catalytic domain, 66%) to glucoamylase from *S. solfataricus* (SSGA) which exhibited typical characteristics of bacterial glucoamylase (25). STGA showed limited homology of 30 % and less than 20% to bacterial and fungal glucoamylases, respectively. STGA maintained conserved amino acids that are important for the activity of glucoamylases.

Purification and characterization of STGA During the purification of STGA from *E. coli* harboring p6xH(-)STG, the cell extract was treated with heat at 60°C for 20 min to eliminate heat labile host proteins. STGA was purified by 62.3 folds by the purification process and the yield was 35.7% (Table 1). The estimated molecular mass of purified STGA was about 60 kDa, which was much smaller than the predicted molecular mass (Fig. 1). Similar abnormal mobility on SDS-PAGE gels have previously been observed among hyperthermophilic starch-degrading enzymes (26-28), probably due to incomplete denaturation of the proteins by the loading buffer.

The optimal temperature and pH of the recombinant glucoamylase were determined using maltotriose(G3) as a substrate at various temperatures, 60-90°C, and pHs, 4.0-8.0 (Fig. 2). STGA showed it's highest activity at pH 4.5 and 80°C. The temperature optimum is quite high compared to those of fungal glucoamylases that are used in starch industry. Glucoamylase from *A. niger* has an optimal temperature of 65°C and the activity is barely detected at temperature above 75°C (1, 2, 29). STGA was also active at 90°C retaining over 90% of the activity at the optimal temperature.

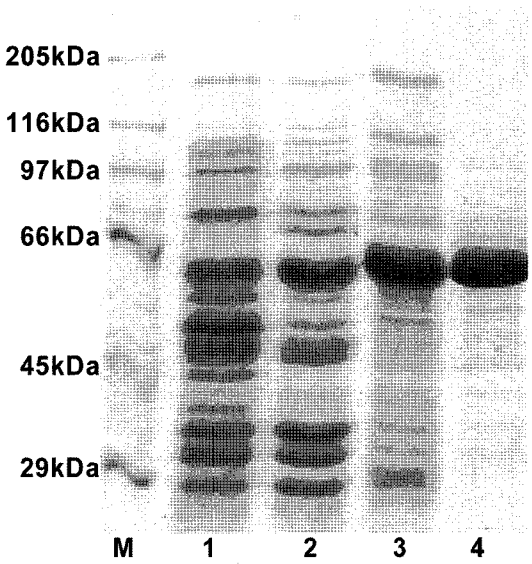

Fig. 1 SDS-PAGE of purified STGA. Lane M represents size marker; lane 1, cell extract; lane 2, cell extract heat treated at 60 °C; lane 3, ammonium sulfate fractionation; lane 4, STGA eluted from a butyl-sepharose column.

Oligomeric state of STGA in solution was investigated by GPC using a Superdex 200HR 10/30 column. The apparent molecular mass calculated by comparison with the elution time of standard proteins was 99.2 kDa (Fig. 3A), which was larger than the predicted molecular mass. To verify the GPC, the apparent molecular mass of STGA was estimated by sedimentation equilibrium analytical centrifuge analysis. The apparent molecular mass of STGA determined by sedimentation equilibrium analytical ultracentrifugation was 115 kDa (Fig. 3B). These results indicated that STGA exists as dimeric form in 50 mM Tris-HCl (pH 7.0). Fungal glucoamylases have been known to function in monomeric state, while prokaryotic glucoamyase from *Thermoanaerobacterium thermosaccharolyticum* and archaeon *S. solfataricus* is present as dimer and tetramer in solution, respectively (23-25).

Substrate specificity of STGA Substrate specificity of the enzyme was determined using various substrates such as maltose (G2), maltoheptaose (G7), isomaltose (IsoG2), and soluble starch. Thin layer chromatography analysis of a-glucans hydrolyzed by STGA demonstrated undoubtedly that both α-(1,4) and α-(1,6) glycosidic linkages were hydrolyzed by STGA (data not shown). The enzyme preferentially attacked α-(1,4) glycosidic linkages to α-(1,6) glycosidic linkages and hydrolyzed maltooligosaccharides more readily than starch. STGA exhibited the

Table 1. Purification steps of recombinant STGA

	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Cell extract	100	299	455	0.70	-	1.00
Heat treatment	94	322	68.9	2.82	100	7.12
Ammonium sulfate fractionation	13	215	16.9	11.43	71.0	19.3
Butyl-sepharose	10	107	2.50	56.3	35.7	62.3

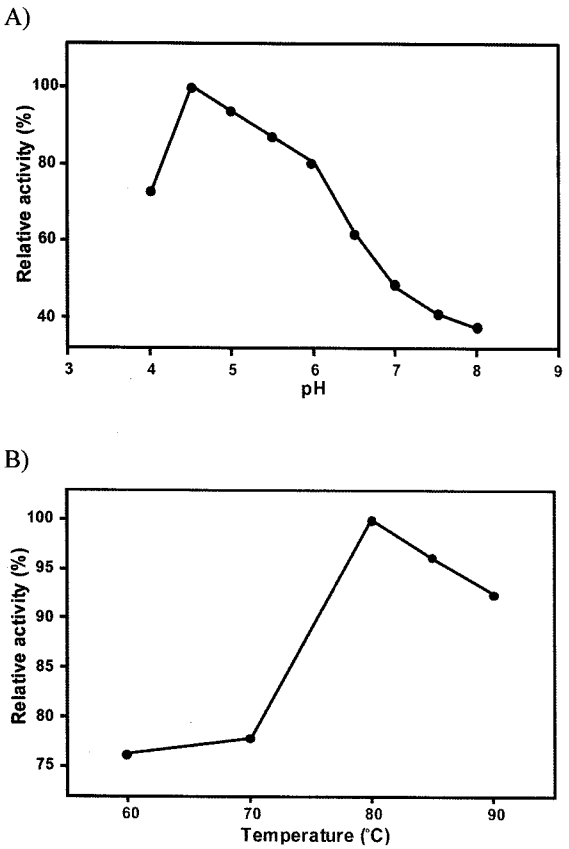

Fig. 2. The optimal pH (A) and temperature (B) of recombinant STGA. The relative activity was determined using G3 as substrate. For the pH range of 4.0- 6.0, sodium acetate buffer was used; 6.5- 8.0, sodium phosphate buffer.

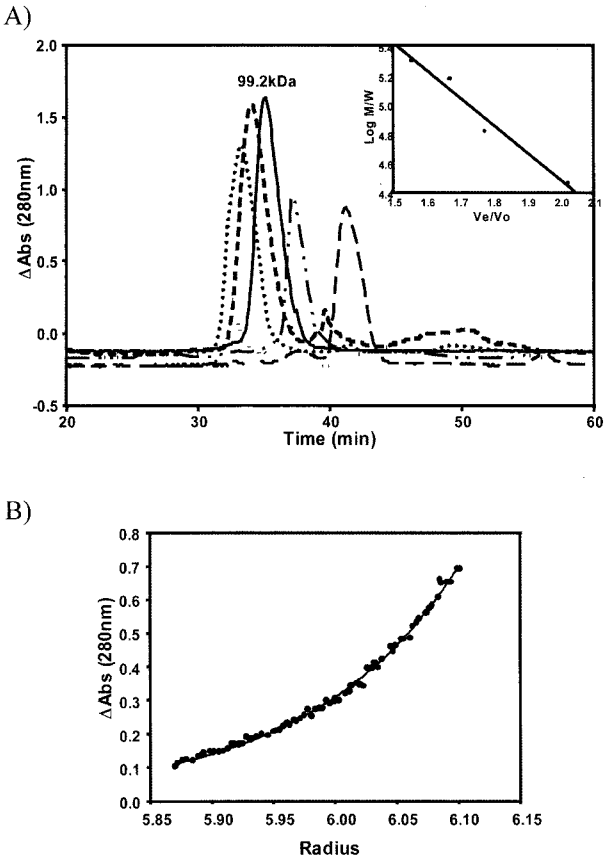

Fig. 3 Gel permeation chromatography (A) and sedimentation equilibrium analytical ultracentrifugation (B) of STGA. (A) The enzyme was eluted with 50 mM Tris-HCl (pH 7.0) through a Superdex 200 HR10/30 column. Four kind of standard molecules were used as size marker. ——, STGA; ···, beta-amylase, 200kDa; – – –, alcohol dehydrogenase, 150kDa; – · · –, bovine serum albumin, 66kDa; — — —, carbonic anhydrase, 29,000kDa. The calculated molecular weight of STGA from amino acid sequence is 71kDa. The inset shows the line fitting the plot of the elution time *versus* the logarithm of the molecular weight of the size markers. (B) A sedimentation equilibrium measurement was performed at 20°C in 50 mM sodium phosphate (pH 5.0). The values of the two variables, the absorbance at 280 nm *versus* radial positions, were obtained. The apparent molecular mass of STGA was calculated.

highest hydrolysis activity toward maltotriose (G3), while significantly less hydrolyzing activity toward maltotetraose (G4) and G2. The relative activity of STGA decreased further when longer maltooligosaccharides were used as substrates in contrast to fungal glucoamylases that hydrolyze starch more efficiently than maltooligosaccharides. The substrate specificity of STGA was more similar to that of α-glucosidase that is also known to hydrolyze maltooligosaccharides efficiently to glucose in α-D-glucopyranose form (14, 29). On the other hand, SSGA, the most closely related glucoamylase to STGA at the amino acid sequence level was demonstrated to liberate β-D-glucopyranose from its substrate in a manner similar to that of fungal glucoamylases (25). Also some bacterial glucoamylases have been reported to prefer short maltooligosaccharides to starch (14, 24). These results indicated that the enzymatic properties of STGA, fungal glucoamylases and α-glucosidases are clearly different from each other.

Thermostability of STGA Thermostability of STGA was investigated by thermal inactivation analysis at 80°C and 90°C. STGA had a D-value of 17 hr at 80°C and 13 hr at 90°C in a protein concentration of 0.0075 mg/ml (data not shown). The half-life of *A. niger* glucoamylase at 60°C was 63.4 hr, but the enzyme activity was detected for only a few minutes at 75°C and 85°C (29). STGA is remarkably more thermostable than glucoamylase from *A. niger* at all

Table 2. Substrate specificity of STGA[a]

Substrate	Specific activity (U[b]/mg protein)
G2	20.4
G3	171.6
G4	69.0
G5	52.5
G6	22.2
G7	18.1
IsoG2	ND[c]
SS	ND

[a]Reaction rates were determined at 75°C for 10 min; Each reaction mixtures contained 100 ml of 1% substrate, 80 μl of 50 mM sodium acetate buffer (pH 4.5), and 20 μl of purified STGA.
[b]The amount of glucose(μmole) produced from the enzyme reaction per minute. In the case of G2, the unit obtained was divided by 2.
[c]Activity was not detected.

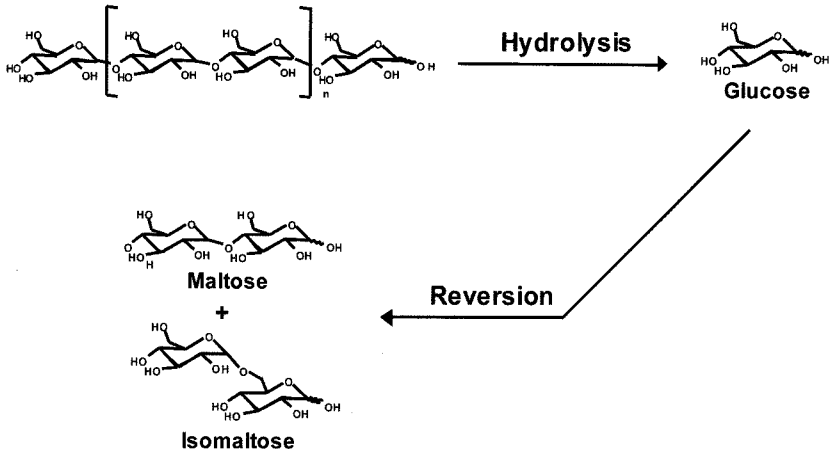

Fig. 4 Hydrolysis and reverse reaction producing IsoG2 and maltose from glucose by glucoamylase.

temperatures investigated. This property makes the enzyme more competitive in the saccharification process by avoiding a drastic cooling of the system. Moreover, since the recombinant enzyme can operate for extended time at high temperatures, higher substrate solubility and the sterility of bioreactor are guaranteed during the whole process employing STGA.

Reverse reaction of STGA Two distinct mechanisms of glucoamylase lead to accumulation of G2 and isomaltose (IsoG2) in the maltodextrin (or glucan)- hydrolyzing process (Fig. 4). During hydrolysis of α-(1,4)-linked glucan, G2 and IsoG2 are concurrently produced by the condensation action of glucoamylase. The industrial process of starch processing involves high concentrations of dissolved solids. Glucoamylase is likely to undergo reverse reaction at high concentration of glucose. G2 is produced in the early stage of the reverse reaction but it does not increase in amount since the equilibrium exists at approximately 1-2%. IsoG2, a reversion product containing an α-(1,6)-glycosidic linkage, tends to be accumulated at the expense of glucose.

The reverse reaction catalyzed by STGA was investigated by reacting different amounts of the enzyme with 30% glucose and reversion products, especially IsoG2, were monitored. The amount of IsoG2 increased as the reaction proceeded until the equilibrium was reached at 90°C in 2 hr, while the reaction continued for more than 7 hr at 75°C (data not shown). Kinetic analysis of STGA was carried out as shown in Table 3. The k_{cat} value for hydrolysis of maltotriose at 90°C was 2 times higher than that of reaction at 75°C, whereas the k_{cat} value for the reverse reaction at 90°C increased by 1.7 fold compared with the k_{cat} at 75°C. The results suggested that the process at 90°C is more favorable to obtain higher yield of glucose production than at 75°C.

Saccharification of liquified corn starch using STGA The capability of STGA saccharifying liquefied corn starch (LCS) in 50 mM soium acetate (pH 4.5) was evaluated at 75°C and 90°C. The enzyme concentrations used were 0.004 U/mg of LCS and 0.006 U/mg of LCS for 75°C and 90°C, respectively. Pullulanase from *Thermotoga maritima* (25) purified in the laboratory was added simultaneously (0.01 U/mg of LCS) for a faster and more efficient degradation of the substrate at 75°C, but not at 90°C. A maximal yield of about 95% was obtained in 8 hr and 4 hr of reaction at 75°C and 90°C, respectively (Table 4). These values are comparable to the final glucose yield obtained by fungal glucoamylase (1, 2). However, saccharification by STGA was much faster than fungal glucoamylase whose reaction takes 24 - 60 hr to attain the maximal yield of 95%. The maximum glucose yield using SSGA was 89.4% in 17 hr of reaction at 80°C (25). When the reaction was allowed to continue, the amount of glucose decreased considerably due to the reverse reaction that favors production of IsoG2 and G2 at high glucose concentrations (data not shown). In saccharification of starch, excess glucoamylase is required to obtain a reasonable rate, but excess glucoamylase also leads to formation of reversion products that lower the final DE value. From the results, STGA seemed to have a great potential to improve the industrial starch saccharification process.

Table 3. Catalytic constant (k_{cat}) of STGA for hydrolysis and reverse reaction

	$k_{cat}(s^{-1})$	
Temp(°C)	Hydrolysis*	Reversion
75	24.8	0.14
90	49.6	0.25

*Maltotriose was used as a substrate.

Table 4. Saccharification of liquefied corn starch using STGA

	Reaction products[a]	
Reaction time (hr)	Glucose (%)	Isomaltose (%)
0.5	70.2	0.0
1.0	86.2	1.2
3.0	94.7	2.4
5.0	94.9	3.6
6.0	94.4	4.6

[a]Reaction was carried out in 50 mM sodium acetate buffer (pH 4.5) at 90°C by STGA (0.006U/mg LCS).

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