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Cloning and Overexpression of 4-α-Glucanotransferase from *Thermus brockianus* (TBGT) in *E. coli*

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Abstract A gene corresponding to 4-α-glucanotransferase (αGTase) was cloned from the thermophilic bacterium *Thermus brockianus*. The nucleotide sequence analysis showed that the αGTase gene is composed of 1,503 nucleotides and encodes a polypeptide that is 500 amino acids long with a calculated molecular mass of 57,221 Da. The deduced amino acid sequences of *Thermus brockianus* αGTase (TBGT) exhibited a high level of similarity to the amino acid sequence of αGTase of *Thermus thermophilus* (86%), but low level of homology to that of *E. coli* (26%). The TBGT gene was overexpressed in *E. coli* BL21, and the corresponding recombinant enzyme was efficiently purified by Ni-NTA affinity chromatography. The enzymatic characteristics revealed that optimal pH and temperature were pH 6 and 70°C, respectively. Most interestingly, TBGT reacted with small oligosaccharides, especially maltotriose, to form various maltooligosaccharides by using its disproportionation activity

Key words: Amylomaltase, disproportionation, 4-α-glucanotransferase, *Thermus brockianus*, transglycosylation

4-α-Glucanotransferase (amylomaltase, disproportionating enzyme, αGTase hereafter; EC 2.4.1.25) is found in various bacteria and plants [11, 8]. αGTase was observed in many microorganisms including *Aquifex aeolicus* [2], *Bacillus subtilis* [15], *Escherichia coli* [14], *Pyrococcus kodakaraensis* [17], *Streptococcus mitis* [22], *Thermococcus litoralis* [5], and *Thermotoga maritima* [10]. Although many αGTases can be separately classified into three glycoside hydrolase families (GHF), GHF 13, 57, and 77, most of the bacterial αGTases belong to family GHF 77, which shows very similar catalytic properties to plant disproportionating enzymes (D-enzyme) [11]. It catalyzes not only an intermolecular transglycosylation in which a segment of an α-1,4-D-glucan is transferred to a new 4-position in an acceptor in glucose or another α-1,4-D-glucan, but also an intramolecular transglycosylation with which cyclic α-1,4-glucans (cycloamylose) are produced [2, 16].

Cycloamylose is a class of cyclic oligomers of glucose with α-1,4-glycosidic linkages. Different from cyclodextrins, which are cyclic oligosaccharides composed of 6–8 glucopyranoside units, cycloamylose has a degree of polymerization ranging from a minimum of 17 to a few hundreds [8]. Such high molecular weight cyclic compound was assumed to form a single helical V-amylose conformation and a toroidal shape, with an anhydrophilic channel-like cavity [4]. As expected from their channel-like cavity in the V-amylose helix, cycloamyloses can form inclusion complexes with various inorganic [9] and organic molecules [18]. There might be a correlation between the geometries of the V-helices defined by the number of glucoses per turn and the nature of the enclosed guest molecules. Recently, it was found that cycloamylose has an artificial chaperone property on protein refolding toward three different enzymes (citrate synthase, carbonic anhydrase B, and lysozyme), which have no structural homology among them [12]. It is of great interest to produce cycloamylose for industrial applications.

Here, we describe the isolation of αGTase from the thermophilic bacterium *Thermus brockianus* JCM11602 (hereafter, TBGT), and the enzymatic characteristics of its recombinant enzyme were determined to apply for the production of cycloamylose. *T. brockianus* JCM11602 was obtained from Japan Collection of Microorganisms (JCM,

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Saitama, Japan) and grown in Castenholz medium (tryptone 1 g, yeast extract 1 g, and Castenholz basal salt solution 100 ml in 1 l) at 65°C under aerobic conditions [21, 3, 6]. The chromosomal DNA of *T. brockianus* JCM11602 was efficiently purified using a genomic DNA purification kit (Promega Co., Madison, WI, U.S.A.). The gene corresponding to αGTase was obtained by PCR using two primers, designed based on the conserved sequence among various bacterial αGTases. PCR reaction was performed with *T. brockianus* JCM11602 genomic DNA as a template and two degenerate oligonucleotides, TB1 [5'-ATG GAG CTT CCN (C/A)G(C/G) GC(T/C) T(T/A)(C/T) GG-3'] and TB2 [5'-TAC CG(G/C) CTC CGG TGC CC(G/C) ACC (G/C)AN ATC-3'] and *Pfu* DNA polymerase (Stratagene, La Jolla, CA, U.S.A.). 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 50°C for 1 min, extension at 72°C for 2 min, and extra extension at 72°C for 7 min. The PCR product of the TBGT gene was cloned into T-easy vector (Promega Co.) and the nucleotide sequence of the PCR-generated gene was determined by BigDye Terminator Cycle Sequencing Kit for ABI377 PRISM (PerkinElmer Inc., Boston, MA, U.S.A.). Based on the preliminary Blast sequence alignment, it was revealed that the cloned gene was highly homologous to the various αGTases, implying that the right gene was cloned. The whole nucleotide sequence of the cloned gene was determined and deposited to the GenBank database. The nucleotide sequence and deduced amino acid sequence data are available from the GenBank database with an accession number of DQ083509. The nucleotide and amino acid sequence analyses, including an open reading frame search, molecular weight calculation, and homology search, were performed using the DNASIS software (Hitachi software, Tokyo, Japan). The open reading frame of the TBGT gene is 1,503 bp long and encodes 501 amino acid residues with a calculated molecular weight of 57,286 Da and pI 5.3. The overall G+C content of *tbgt* was 66 mol%, but the G+C content of the third positions of codons was 84 mol%. Based on the deduced amino acid sequences, *T. brockianus* αGTase exhibited high levels of homology with the αGTases from *Thermus* sp. (86% identity with *Thermus thermophilus* and 84% identity with *Thermus scotoductus*) as expected, but low level of homology with various other plants and bacterial αGTases including *Solanum tuberosum* (43% identity), *Arabidopsis thaliana* (41% identity), *Synechococcus* sp. (51% identity), *Rhodopirellula baltica* (45% identity), *Aquifex aeolicus* (48% identity), *E. coli* (28% identity), and *Lactococcus lactis* (28% identity).

To determine the enzymatic characteristics of TBGT, the *tbgt* was overexpressed in *E. coli* BL21. The open reading frame of TBGT was introduced into the pRSET-B *E. coli* expression vector (Invitrogen, Carlsbad, CA, U.S.A.) through PCR amplification. The TBGT-specific primers were GT-Ex15'-GGA TCC GAT GGA GCT TCC TTG CGC TTA TGG T-3' and GT-Ex15'-GAA TTC TTA AAC CCT GCC CGT GGC CTC CGC-3'. The PCR amplified fragment was digested with BamHI and EcoRI and subcloned into the pRSET-B vector. The amplified sequence was confirmed by BigDye Terminator Cycle Sequencing Kit for ABI377 PRISM (PerkinElmer Inc.). The resulting TBGT expression vector, pRBTBGT, was transformed into *E. coli* BL21, and the transformants were selected and confirmed. The overexpression was performed by growing *E. coli* BL21 harboring pRBTBGT and inducing TBGT by adding 1 mM IPTG at 37°C for 3 h. The transformants exhibited strong GTase activity from the assay based on the glucose oxidase [13], meaning that the expression of TBGT was successful. To purify the recombinant TBGT, the cell pellet was resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, and pH 8.0). Cells were disrupted by sonication for 10 min on ice. Crude extract of the recombinant enzyme was purified by Ni^{2+}-NTA affinity column chromatography followed by heat treatment at 65°C for 30 min. As shown in Table 1, heat treatment was very efficient to remove heat-labile *E. coli* endogenous proteins. SDS-PAGE analysis (Fig. 1) also showed that TBGT is highly expressed in *E. coli* and efficiently purified by Ni^{2+}-NTA affinity chromatography [1]. Finally, TBGT was purified homogeneously, with 67% yield. The purified enzyme was subjected to the MALDI-TOF/MS analysis to confirm the molecular mass (Fig. 1). The detected molecular mass of the recombinant TBGT (61,034 Da) was approximately fit to the estimated molecular mass (61,034 Da). The discrepancy between the molecular mass of native TBGT (57,286 Da) and that of the recombinant TBGT was caused by the additional amino acids (33 amino acids) originated from the expression vector, including six histidines that was inserted during the subcloning process for overexpression.

The effects of temperature and pH on the activity and stability of the recombinant TBGT were analyzed by the glucose oxidase method. The purified TBGT exhibited its

Table 1. Purification table of TBGT expressed in *E. coli*.

Step	Vol (ml)	Total protein (mg)	Total activity (Unit)	Specific activity (U/mg)	Yield (%)	Purification fold
Cell extract	2.95	23.03	45,992	1,997	100	1
Heat treatment	2.54	2.169	40,991	18,899	89	9
Ni^{2+}-NTA affinity chromatography	1.74	0.438	30,981	70,734	67	35

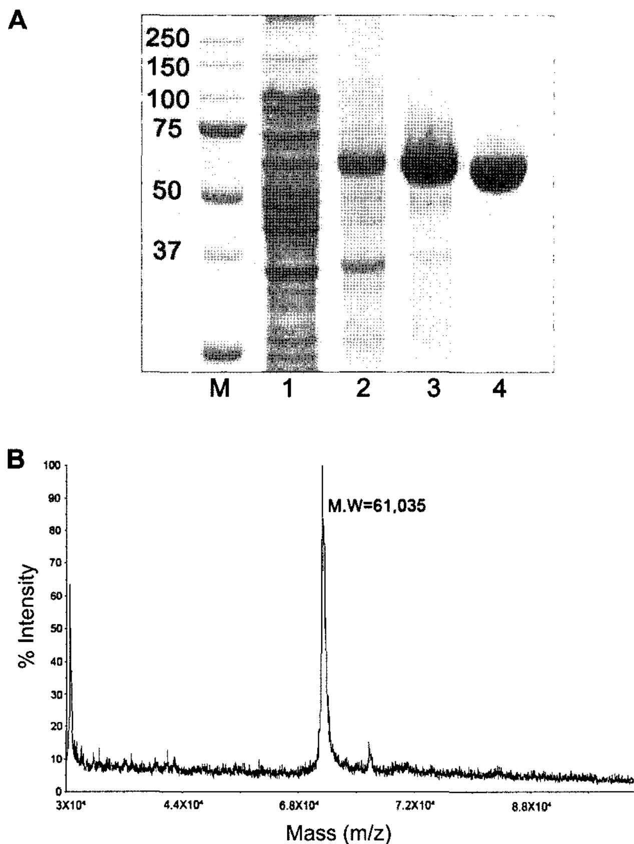

Fig. 1. SDS-PAGE and MALDI-TOF MS analyses of TBGT expressed in *E. coli*.

A. The gel was stained with Coomassie Brilliant Blue after SDS-PAGE analysis. Lane 1, *E. coli* BL21 cell extract (control); lane 2, Cell extract of *E. coli* BL21 harboring pRBTBGT; lane 3, same as lane 2 but after heat treatment; lane 4, purified TBGT using Ni-NTA affinity column chromatography; lane M, the molecular standard marker (Bio-Rad, Hercules, CA, U.S.A.). **B**. MALDI-TOF MS analysis of recombinant TBGT expressed in *E. coli*. The molecular mass was determined to be 61,034 Da.

optimum temperature at 70°C. Although it retained 80% of its catalytic activity at 60°C, it lost most of its enzymatic activity above 80°C (Fig. 2). At the optimal temperature (70°C) for activity, the half-life of enzyme was 10.3 h. As expected, the half-life of TBGT decreased with increasing reaction temperature, as the half-life dramatically decreased to 6 min at 80°C. Some heat-stable GTases have been reported from various thermostable bacteria and archaea including *T. aquaticus* (T_{opt} at 70°C, 20), *Thermococcus litoralis* (T_{opt} at 90°C, 5), and *Pyrobaculum aerophilum* (T_{opt} at 95°C, 7). These thermostable GTases including TBGT are potential applications in processes requiring high temperature, such as the formation of cycloamylose and thermoreversible gel formation [7]. The enzyme showed its catalytic activity in the broad range pH between pH 4–8 with an optimum pH of 6. Various substrates were incubated with the purified TBGT at 70°C and pH 6. The reaction contained 40 μl of various substrates (0.25%), 50 μl of 50 mM sodium citrate

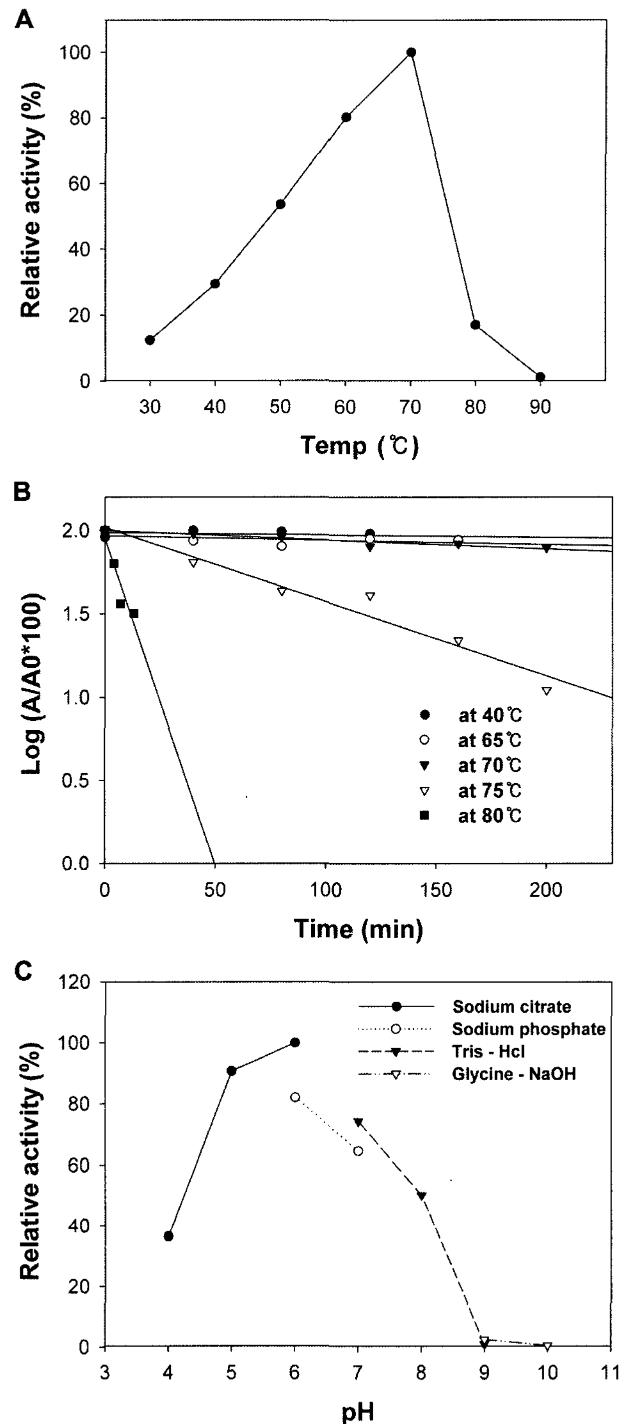

Fig. 2. Effects of temperature and pH on the activity of purified recombinant TBGT expressed in *E. coli*.

The activity of TBGT was assayed by the standard method as described in the text. **A**. Effects of temperature on the activity of TBGT were determined from 40 to 90°C. **B**. Heat stability of TBGT was determined from 40 to 80°C. Residual activities after preincubation at 40°C (●), 65°C (○), 70°C (▼), 75°C (▽), and 80°C (■) in the absence of substrate were measured as functions of the incubation time. **C**. Effects of pH on the activity of TBGT were determined using sodium citrate (pH 4–6, ◆), sodium phosphate (pH 6–7, ◇), Tris-HCl (pH 7–9, ▼), and glycine-NaOH (pH 9–10, ▽) buffers.

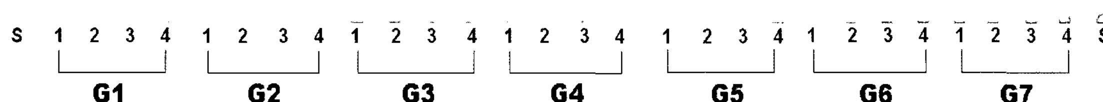

Fig. 3. TLC analysis of the reaction products formed from the activity of TBGT with maltooligosaccharides.
Reaction mixtures (100 μl) containing 1% (w/v) each substrate (G1 to G7) in 20 mM sodium citrate buffer (pH 6) and no enzyme (lanes 1), 0.1 U of enzyme per ml (lanes 2), 0.5 U of enzyme per ml (lanes 3), or 1 U of enzyme per ml (lanes 4), were incubated at 70°C for 6 h. Lanes S are standard maltooligosaccharides from G1 to G7.

buffer (pH 6), and 10 μl of enzyme solution. The reaction product analysis was performed by thin-layer chromatography (TLC). An aliquot (1 μl) of the reaction mixture was spotted onto a Silica gel K5F plate (Whatman, Kent, U.K.) and developed with a solvent system of n-butanol/acetic acid/water (3:1:1, v/v/v) in a TLC developing tank. Ascending development was repeated twice at room temperature. The plate was allowed to air-dry in a hood and then developed by being soaked rapidly in 0.3% (w/v) *N*-(1-naphthyl)-ethylenediamine and 5% (v/v) H_2SO_4 in methanol. The plate was dried and placed in a 110°C oven for 10 min to visualize the reaction spots.

The reaction was specific to α-1,4-glucans, since no reaction was detected in various other substrates including cellobiose, lactose, sucrose, gentibiose, and trehalose (data not shown). However, all maltooligosaccharides used [maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6), and maltoheptaose (G7)], except glucose (G1), were effective substrates for TBGT. Various maltooligosaccharides of different length were produced from each reaction (Fig. 3). At the high enzyme concentration (1U), transglycosylation products (maltooligosaccharides and G1) were produced from all of the maltooligosaccharides tested. However, at a low enzyme concentration (0.1U), no transglycosylation products were obtained from small substrates such as G2, G3, and G4, indicating that TBGT catalyzed transglycosylation of maltooligosaccharides with high substrate specificity toward maltooligosaccharides longer than G4. When relatively long maltooligosaccharides (G5, G6, and G7) were used as a substrate, high molecular mass oligosaccharides were observed at the spotting point of the TLC plate. Takaha *et al.* [19] reported similar enzymatic activities of the D-enzyme of potato tubers on maltooligosaccharides. In the case of the D-enzyme, G2 was not detected after enzyme reaction with G3, G4, and G5, and no transglycosylation reaction of G2 was observed, meaning G2 was a poor substrate for the D-enzyme. However, GTase from *Thermococcus litoralis* produced a significant amount of G2, as detected in the reaction mixture in the same reactions [5]. In this study, it is obvious that TBGT catalyzed transglycosylation of G2 with high concentration of enzyme, but the G2 concentration observed in the transglycosylation reaction was negligible (Fig. 3), implying that there are some differences in the reaction mechanisms of those three enzymes although they shared general GTase activities. Further research on the reaction mechanism and application of TBGT are in progress.

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