Enzymatic Characterization of a Thermostable 4-α-Glucanotransferase from *Thermotoga neapolitana*

Kyoung-Hwa Choi[†], Ja Yeong Seo[†], Jieun Kim, and Jaeho Cha*

Department of Microbiology, Pusan National University, Busan, 609-735, Republic of Korea

The gene encoding 4-α-glucanotransferase (*mgtA*) from *Thermotoga neapolitana* was cloned and expressed in *Escherichia coli* in order to investigate whether this enzyme was capable of producing cycloamylose for industrial applications. MgtA was purified to homogeneity by HiTrap Q HP and Sephacryl S-200 HR column chromatographies. The size of the enzyme as determined by SDS-PAGE was about 52 kDa, which was in good agreement with its deduced molecular mass of 51.9 kDa. The optimal temperature and pH for the activity of the 4-α-glucanotransferase was found to be 85°C and 6.5, respectively. The enzyme hydrolyzed the 1,4-α-glucosidic bonds in oligomeric 1,4-α-glucans and transferred oligosaccharides (maltotriose being the shortest one) to acceptor maltodextrins. However, the enzymes had no activity against pullulan, glycogen, and other di- or trioligosaccharides with rare types of α-bond. MgtA is distinguished from 4-α-glucanotransferase from *Thermotoga maritima* in that it can convert maltotriose into maltooligosaccharides. The treatment of glucoamylase after the reaction of MgtA with maltotriose, maltotetraose, maltopentaose, or maltohexaose as sole substrate revealed that MgtA yielded linear maltooligosaccharides instead of cycloamylose.

Key words : 4-α-Glucanotransferase, glycosyl hydrolase family 13, thermostability, *Thermotoga neapolitana*

Introduction

Maltodextrin glycosyltransferase or 4-α-glucanotransferase (EC 2.4.1.25) (GTase) catalyzes glucan transfer from one α-1,4-glucan to another α-1,4-glucan or to glucose. Since this enzyme was first found in *Escherichia coli* [13], homologues have been known in many bacterial species [2,6,7,22]. A similar enzyme was also present in plants and is called disproportionating enzyme (D-enzyme) [19]. Based on the characterization of GTases, it has been thought that the substrates of these enzymes are maltooligosaccharides. Most of GTases produce linear maltooligosaccahrides. However, D-enzyme can use amylose as a substrate to produce cyclic α-1,4-glucans (cycloamylose) with degree of polymerization ranging from a minimum of seventeen to a few hundred via intramolecular transglycosylation (cyclization) reaction [21]. The cycloamyloses have specificities for guest molecules different to those of cyclodextrins which are cyclic oligosaccharides composed of 6–8 glucose units, because each has different dimensions and tertiary structures.

Therefore, there is a great potential for the application of cycloamyloses in the chemical, pharmaceutical, and food industries to safely achieve the solubilization, increased stability, or altered reactivity of molecules with which they can form inclusion complexes [12,20,24]. Recently, the GTases from thermophilic bacteria or archaea have also known to produce cycloamylose [8,9,18].

Thermotoga is a rod-shaped, gram-negative hyperthermophile capable of growth at up to 90°C. Two closely related species, *T. maritima* and *T. neapolitana*, are obligatory heterotrophs growing on polymeric carbohydrates, starch in particular. Although the GTase from *T. maritima* was cloned and reported to have disproprotionating activity with maltotetraose [10], it is not clear whether the enzyme can produce cycloamylose in addition to linear maltooligosaccharides. In this paper, we demonstrate that GTase from *T. neapolitana* cannot produce cycloamylose. Furthermore, this enzyme is distinguished from GTase from *Thermotoga maritima* in that it can convert maltotriose into maltooligosaccharides while the enzyme from *T. maritima* cannot use maltotriose as a sole substrate.

*Corresponding author
Tel : +82-51-510-2196, Fax : 82-51-514-1778
E-mail : jhcha@pusan.ac.kr
[†]These authors contributed equally to the work.

Materials and Methods

Strains and plasmids

Thermotoga neapolitana KCCM41025 was obtained from the Korean Culture Center of Microorganism (KCCM) and was cultivated under anaerobic conditions as previously described [23]. The *Escherichia coli* strains BL21 (DE3) and DH5 α were used as the hosts for protein production and cloning, respectively. *E. coli* transformants were grown in Luria-Bertani (LB) medium (bacto-tryptone 10 g/l, yeast extract 5 g/l, NaCl 5 g/l) containing kanamycin (30 μg/ml) at 37°C. Plasmid pET-29b (Novagen, Inc., San Diego, CA, USA) was used as a cloning and expression vector.

Chemicals

The chemicals used for the determination of GTase activity were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were reagent grade. The DNA primers and plasmid miniprep kit were prepared by Genotech (Yusung, Korea). Prime Taq premix was purchased from GeNet Bio (Nonsan, Korea). Dialysis tubing Spectra/Por 4 (MWCO 12-14 kDa) was purchased from Spectrum Laboratories, Inc. (Houston, Texas, USA).

Construction of the *mgtA* gene expression vector

The open reading frame of the *mgtA* (*T. neapolitana* KCCM41025) gene was introduced into the pET29b *E. coli* expression vector through PCR amplification. PCR amplifications of *mgtA* was performed with *T. neapolitana* KCCM41025 genomic DNA as a template, and two *mgtA* gene-specific primers, TN-EcoRV (5′-AGGG<u>GATATC</u>AT GATAGGCTACCAGATCTACGT-3′) and TN-XhoI (5′-TCA T<u>CTCGAG</u>TCAAATCACCTCCGTGCT-3′) containing EcoRV and XhoI recognition sites (underlined). The *mgtA* gene-specific primers were designed based on the *T. neapolitana* putative GTase nucleotide sequence (GenBank Accession No., CAA08864). The conditions for PCR were as follows: one cycle at 94°C for 5 min, 30 cycles at 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min, with an extra extension at 72°C for 7 min. An amplified 1.3 kbp DNA fragment was digested with the same restriction enzymes as mentioned above, and inserted into the pET29b vector. The amplified sequence was confirmed by a BigDye terminator cycle sequencing kit and the ABI Prism 3100 genetic analyzer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The resulting expression vector, pET-MgtA was transformed into the *E. coli* strain BL21(DE3), and the transformants were selected and confirmed.

Expression and purification of the recombinant MgtA

The transformants were grown in LB medium containing kanamycin (30 μg/ml) overnight at 37°C for small-scale culture. The overnight culture was inoculated into fresh medium at 37°C until the optical density (A_{600}) reached 0.4, and protein expression was induced by 0.2 mM IPTG. After incubation for 4 hr or overnight, the cell pellet was collected by centrifugation at 8,000× g for 10 min at 4°C. The drained pellet was stored at -80°C or subjected to subsequent cell disruption. The harvested cells were disrupted by a French press in buffer A (50 mM Tris-HCl, pH 7.0). The suspension of disrupted cells was centrifuged at 27,000× g for 30 min and the supernatant fraction was heat-treated at 80°C for 30 min followed by centrifugation. The supernatant was loaded onto a HiTrap Q column (GE Healthcare) equilibrated in buffer A and the bound protein was eluted with a linear gradient of NaCl (0 to 1.0 M in the same buffer). The protein solution was concentrated using a Centricon-10 filter from Amicon (Millipore, Bedford, MA, USA) and dialyzed against buffer B (50 mM Tris-HCl, pH 7.5, 200 mM NaCl). The dialyzed solution was loaded on a HiPrep Sephacryl S-200 HR 16/60 column (GE Healthcare) and eluted with buffer B. The purity of the recombinant protein was confirmed by SDS-PAGE. Protein concentration was determined using a Bio-Rad protein assay system (Bio-Rad, Hercules, USA) with bovine serum albumin as the standard.

Enzyme assays

The assay mixture containing 0.05% amylose, 20 mM citrate-phosphate buffer (pH 7.0) and enzyme was incubated at 80°C. Samples (0.1 ml) withdrawn at each time interval were mixed with 1 ml 0.02% iodine/potassium iodide solution and the absorbance at 620 nm was measured immediately with a spectrometer. One unit of enzyme activity was arbitrary defined as the amount of enzyme which causes a change in absorbance of 1 in 15 min under the above conditions. The assay was reproducible and linear for enzyme concentrations causing absorbance differences up to 0.5. The dependence of enzyme activity on pH and temperature was determined in the pH range of 5.0-10.0 and in a temperature range of 50 to 100°C. The buffers used were citrate (pH 4.5-5.0), citrate-phosphate (pH 5.0-7.0), Hepes

(pH 7.0-8.5), and borate (pH 8.5-10.0). For the temperature optimum, activity was assayed at pH 7.0.

Analytical methods

The reaction mixture (0.2 ml) containing the enzyme (0.15 unit), 1% maltooligosaccharides, and 20 mM citrate-phosphate buffer (pH 7.0) was incubated at 80°C for overnight. The enzyme reaction was stopped by chilling on ice. The hydrolysis products were separated by thin-layer chromatography on aluminum plates coated with silica gel (0.2 mm, type 60, Merck), in the *n*-butanol-ethanol-water (5:3:2, v/v/v) mixture. The plate was dried in a hood and then visualized by soaking it rapidly in a diphenyl-amine-aniline-phosphate reagent (a mixture of 0.4 g diphenylamine, 0.4 ml aniline, 3 ml 85% phosphoric acid, and 20 ml acetone). The plate was dried and baked in an oven for 10 min to observe the reaction spots. The amino acid sequence data was analyzed with ExPASy proteomics server (http://ca.expasy.org/tools/protparam.html). Homology searches in GenBank were performed using a BLAST program.

Results and Discussion

Cloning and sequence analysis of the *mgtA* gene from *T. neapolitana*

PCR was used to clone the GTase (*mgtA*) gene from *T. neapolitana*, as described in Materials and methods. The PCR product for the entire open reading frame (ORF) of the *mgtA* gene was cloned into the pET29b vector and sequenced. The ORF encoded a protein comprising 442 amino acids with a predicted molecular weight of 51.9 kDa and an estimated isoelectric point of 5.51. The deduced amino acid sequence of MgtA was most homologous with *T. maritima* 4-α-glucanotransferase (84% identity). *T. neapolitana* MgtA together with *T. maritima* 4-α-glucanotransferase belong to the family 13 glycosyl hydrolase (GH13) α-amylase. Based on amino acid sequences, 4-α-glucanotransferases have assigned to GH 13, 57, and 77 [5]. GH13 and GH77 belong to the α-amylase superfamily, sharing a similar folding and having the same catalytic mechanism [11], while GH57 is a separate enzyme family [25]. Therefore, it is consistent that MgtA from both *Thermotoga* sp. showed a higher homology to α-amylases than to maltodextrin glycosyl-transferases of other organisms.

Expression and purification of recombinant MgtA

E. coli BL21 (DE3) cells harboring pET-MgtA exhibited 4-α-glucanotransferase activity in cell-free extracts after IPTG induction, implying that MgtA was successfully expressed in *E. coli*. A three-step simple purification including a heat treatment, HiTrap Q and Sephacryl S-200 chromatographies was used to efficiently purify the enzyme. SDS-PAGE analysis showed that the purified protein was homogeneous, with a molecular mass of about 52 kDa (Fig. 1). The molecular mass of MgtA was determined to be about 56 kDa by gel filtration chromatography with a Sephacryl S-200 HR 16/60 column, indicating that the putative MgtA is monomeric enzyme. Approximately 20 mg of pure MgtA could be purified from 1 liter of cell culture (Table 1). The enzyme contains two internal cysteine residues and the aliphatic index was 82.83. The high aliphatic index and possible disulfide bond corresponds well with its thermostability.

Catalytic properties of MgtA

The pH-activity profile of the enzyme obtained with amylose showed maximum activity around pH 6.5. In the pH range of 6.0–8.0, the enzyme showed relatively high activity, >80% of the maximum activity (Fig. 2A). The highest GTase activity was detected at 80°C (Fig. 2B). Previously, *T. neapolitana* 4-α-glucanotransferase was reported and the optimum pH and temperature as well as substrate specific-
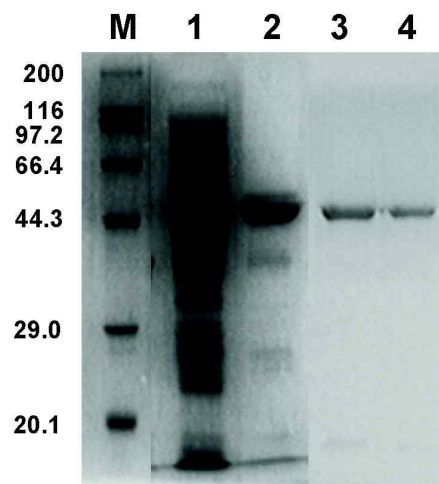

Fig. 1. SDS-PAGE analysis of recombinant MgtA. The purified MgtA was subjected to SDS-PAGE on 12% polyacrylamidegel and stained with Coomassie Brilliant Blue R-250. Lane M, molecular mass marker; lane 1, cell-free extracts; lane 2, heat-treated extracts; lane 3, Hi Trap Q purified; lane 4, Sephacryl S-200 purified.

Table 1. Purification of MgtA

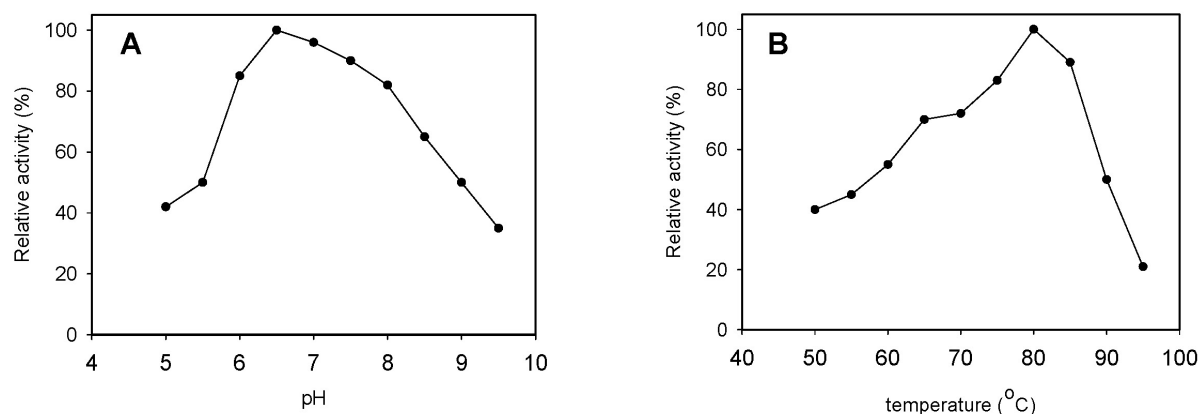| Fraction	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor (-fold)	Yield (%)
Cell-free extract	520	260	0.50	1.0	100
Heat-treated	120	100	0.83	1.7	39
Hi Trap Q	71	84	1.19	2.4	32
Sephacryl S-200	20	80	4.00	8.0	31

Fig. 2. Effects of pH (A) and temperature (B) on the enzyme activity of MgtA. The assay was performed at 80°C with amylose as a substrate. For the temperature optimum, activity was assayed in a temperature range of 50 to 100°C and pH 7.0.

ity was measured [1]. However, the optimum pH and temperature were slightly different with our results and the detailed properties of the reaction products such as the molecular structure were not discussed. MgtA converts maltotriose as a substrate into maltooligosaccharides of 2-7 glucose units in length as products (Fig. 3). Glucose was not detected as a product. It also showed high disproportionating activities for various maltooligosaccharides longer than maltotriose. However, MgtA does not convert glucose and maltose taken individually or in combination implying that they cannot act as donor substrates. The enzymes had no activity against pullulan, glycogen, and other di- or tri-oligosaccharides with rare types of α-bond such as α-1,2, α-1,3, and α-1,6 glycosidic linkages (data not shown). There is a slight difference of *T. neapolitana* MgtA compared to *T. maritima* MgtA in that *T. maritima* MgtA cannot use maltotriose as a donor molecule. Previously, other 4-α-glucanotransferase-type enzymes have been described and some of these transfer only maltodextrinyl segments but not glucosyl moieties (potato D-enzyme), while others transfer both glucosyl and maltodextrinyl units (*E. coli* amylomaltase, *Streptococcus bovis* transglucosylase, and *Pseudomonas stutzeri* amylomaltase) [14,15,17]. The *Thermotoga* enzymes belong to the former group of transferases. It is clearly distinct from the latter class of en-
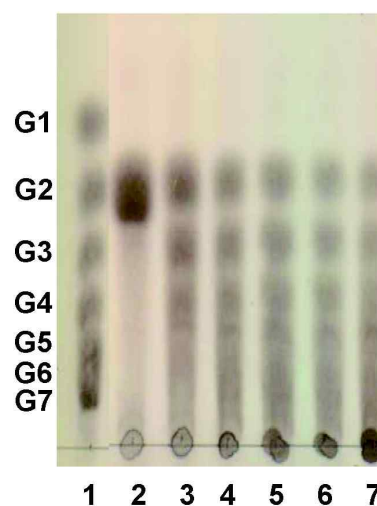
Fig. 3. Thin-layer chromatograms of reaction products formed from the activity of MgtA with various sizes of maltooligosaccharides. Reaction mixtures (0.2 ml) containing 1% substrate in 20 mM citrate-phosphate buffer (pH 7.0) and 0.15 U of enzyme was incubated at 80°C for overnight. Lane 1, standard maltooligosaccharides (G1, glucose; G7, maltoheptaose); lanes 2-7, each substrate (maltose through maltoheptaose) was used.

zymes since it displayed no significant glucosyl-tranfer activity. *Thermotoga* GTases also differ clearly from most other similar enzymes in that it seems neither to utilize glucose as an acceptor nor to liberate glucose during trans-

fer reactions.

Thermus GTases as well as potato D-enzyme have ability to produce cycloamylose to be useful for industrial applications. In order to confirm whether the disproportinating products (various sizes of maltooligo-saccahrides) produced by *T. neapolitana* MgtA are linear or cyclic form, the reaction products were treated by exo-acting glucoamylase which hydrolyzes both α-1,4 and α-1,6-linkages in starch to produce glucose from the non-reducing end of the substrate. If the products were linear glucans, they would be completely broken down to glucose by the enzyme. If not, they should be resistant to glucoamylase. As shown in Fig. 4, the maltooligosaccharides composed of 2-7 glucose units are degraded mostly into glucose. This result strongly suggests that MgtA catalyzes the intermolecular transglycosylation of maltooligo-saccharides (G3 to G7) to produce linear form of maltooligo-saccharides not cyclic form.

Based on the substrate specificity and the identification of the reaction products by MgtA, the prediction of the physiological role of the MgtA in *Thermotoga neapolitana* may be possible. Only a few bacteria and archaea possess
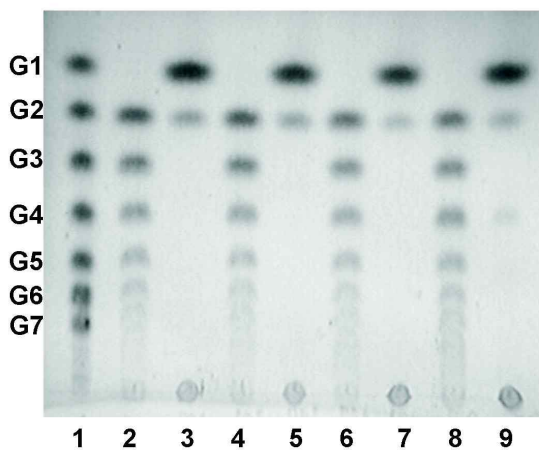

Fig. 4. Thin-layer chromatograms of reaction products of MgtA after glucoamylase treatment. Reaction mixtures (0.2 ml) containing 1% substrate in 20 mM citrate-phosphate buffer (pH 7.0) and 0.15 U of enzyme was incubated at 80°C for overnight and then the reaction products were treated with 1 U of glucoamylase at 50°C for overnight. Lane 1, standard maltooligosaccharides (G1, glucose; G7, maltoheptaose); lanes 2, 4, 6, and 8, without glucoamylase treatment; lanes 3, 5, 7, and 9, with glucoamylase treatment. Lanes 2 and 3, maltotraose; lanes 4 and 5, maltopentaose; lanes 6 and 7, maltohexaose; lanes 8 and 9, maltoheptaose.

GTases. In *E. coli*, the enzyme is essential for growth on short maltooligosaccharides, which are converted into glucose and longer oligosaccharides. The glucose enters the glycolysis pathway, while the longer maltooligosaccharides are substrates for maltodextrin phosphorylase, yielding glucose 1-phosphate which can be used in various metabolic pathways [4,16]. Orthologs of 4-α-glucanotransferase (TM0364) and maltodextrin phosphorylase (TM1168) for the synthesis and degradation of α-glucan are found in *T. maritima* [3]. Therefore, MgtA might act like a 4-α-glucanotransferase from *E. coli*. Further efforts should be followed to elucidate the role of this enzyme for the synthesis and breakdown of transported maltooligosaccharides from environments.

Acknowledgement

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초록 : *Thermotoga neapolitana* 유래 내열성 4-알파-글루칸전이효소의 효소적 특성

최경화† · 서자영† · 김지은 · 차재호*
(부산대학교 미생물학과)

Thermotoga neapolitana 유래 내열성 4-알파-글루칸전이효소(MgtA)가 산업적 응용성을 지닌 싸이클로아밀로스를 생산할 수 있는지를 검사하기 위하여 그 유전자를 클로닝하고 대장균에서 발현시켰다. MgtA는 HiTrap Q와 Sephacryl S-200 분배 크로마토그래피를 이용하여 순수한 형태로 정제되었으며, SDS-PAGE를 통하여 분자량이 약 52 kDa으로 아미노산서열로부터 계산된 분자량과 일치하였다. 효소활성의 최적 pH와 온도는 6.5와 85℃였으며 알파1,4결합을 갖는 글루칸의 1,4결합을 효율적으로 가수분해함과 동시에 작은 크기의 올리고당을 말토덱스트린에 전이하는 전이활성을 가지고 있었다. 그러나 플루란, 글리코겐 및 1,4결합 이외의 다른 알파결합을 갖는 글루칸에는 활성을 나타내지 않았다. MgtA는 말토트리오스를 말토올리고당으로 전환할 수 있는 능력에서 그렇지 못한 *Thermotoga maritima* 의 효소와 구별할 수 있었으며, 반응 후 glucoamylase의 처리결과로부터 그 전이산물이 싸이클로아밀로스 대신에 긴 연쇄상의 말토올리고당을 생산하는 효소로 확인할 수 있었다.