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The action mode of *Thermus aquaticus* YT-1 4-α-glucanotransferase and its chimeric enzymes introduced with starch-binding domain on amylose and amylopectin

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Abstract

A thermostable 4-α-glucanotransferase (TAαGT) gene isolated from *Thermus aquaticus* YT-1 had an open reading frame of 1503 nucleotides, which encoded 500 amino acid residues for a 57,969 dalton protein. The maximum activity of the TAαGT was observed at pH 7.5 and 70 °C. The enzyme catalyzed intermolecular transglycosylation of maltooligosaccharides (disproportionation) to produce linear α-1,4-glucans of various sizes. The starch-binding domains (SBD) of *Bacillus stearothermophilus* ET1 CGTase (E and DE) were introduced into the C-terminus of TAαGT to enhance the starch utilizing activity. The chimeric enzymes, TAαGT-E and TAαGT-DE, showed no difference in temperature optimum, transglycosylation activity, and amylolytic degradation pattern compared to TAαGT wild-type. However, TAαGT-DE exhibited the highest molar specific activity toward amylose. TAαGT-DE modified amylopectin molecules by its disproportionating activities to produce modified amylopectin clusters (M_w 10^5–10^6). Also, it demonstrated the ability to produce cyclo-amyloses with DP of 19 through 35 from amylose molecules.

Keywords: 4-α-glucanotransferase; *Thermus aquaticus* YT-1; Starch-binding domain (SBD); Amylose; Amylopectin; Cyclo-amylose

1. Introduction

Carbohydrates are essential components of all living organisms and the most abundant natural compounds. Starch accumulates as a complex granular structure, which composed of α-1,4 linked and α-1,6 branched glucans either in the leaf chloroplast (transient starch) or in the amyloplast (storage starch). Various enzymes acting on starch have been identified and classified into glycosyl hydrolases (EC 3.2.1.x) and glycosyl transferase (EC 2.4.x.y) on the basis of the catalytic reaction, substrate specificity, and sequence homology (IUBMB, 1992). The 4-α-glucanotransferase (EC 2.4.1.25) is a member of the α-amylase super-family (Kuriki & Imanaka, 1999; Svensson, 1994). The enzyme catalyzes the transfer of α-glucan chains from the non-reducing end of one α-glucan molecule to the non-reducing end of another (Takaha & Smith, 1999; Takaha, Yanase, Takata, Okada, & Smith, 1993).

$$(\alpha\text{-}1,4\text{-glucan})_a + (\alpha\text{-}1,4\text{-glucan})_b \leftrightarrow (\alpha\text{-}1,4\text{-glucan})_{a-x} + (\alpha\text{-}1,4\text{-glucan})_{b+x}$$

where a stands for the degree of polymerization (DP) of a donor α-1,4-glucan molecule, b represents the DP of an acceptor α-1,4-glucan molecule and x is the length of a

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glucanosyl segment transferred from the donor to the acceptor. This inter-molecular glucan transfer reaction is readily reversible, and is often called 'disproportionation'. Also, the enzyme can catalyze intramolecular glucan transfer reaction, which creates cyclic glucans (cyclo-amyloses) (Bhuiyan, Kitaoka, & Hayashi, 2003; Tachibana, Takaha, Fujiwara, Takagi, & Imanaka, 2000; Takaha & Smith, 1999; Takaha, Yanase, Takata, Okada, & Smith, 1996, 1998). This enzyme was first found in *Escherichia coli* (Monod & Torriani, 1948), and also in potato tubers (Peat, Whelan, & Rees, 1956), carrot roots, and tomato fruits (Manners & Rowe, 1969). The 4-α-glucanotransferase gene has been cloned from *E. coli* (Pugsley & Dubreuil, 1988), *Streptococcus pneumoniae* (Lacks, Dunn, & Greenberg, 1982), *Clostridium butyricum* (Goda, Eissa, Akhtar, & Minton, 1997), *Aquifex aeolicus* (Deckert et al., 1998), *Thermus aquaticus* (Terada, Fujii, Takaha, & Okada, 1999), *Thermotoga maritima* (Liebl, Feil, Gabelsberger, Kellermann, & Schleifer, 1992), and potato tubers (Takaha et al., 1993).

Both cyclodextrin glycosyl transferase (EC 2.4.1.19, CGTase) and α-amylase (EC 3.2.1.1) belong to family 13 of amylolytic enzymes, which has $(\beta/\alpha)_8$-barrel fold in the catalytic domains (Svensson, 1994). These enzymes demonstrate functional similarity, catalyzing the starch degradation by cleaving α-1,4-glycosidic bonds. Many of the amylases and related enzymes, but not all, possess, so-called, starch-binding domains (SBD) at the C-terminus except *Rizopus oryzae* glucoamylase and *Thermoactinomyces vulgaris* α-amylase, which have the SBDs at the N-terminus (Abe, Tonozuka, Sakano, & Kamitori, 2004; Ashikari et al., 1986; Christensen, Svensson, & Sigurskjold, 1999). The SBDs consisting of several β-strand segments are responsible for binding amylose and digestion of raw and granular starch (Penninga et al., 1996). It was reported that SBDs accelerate hydrolysis due to the superior-binding affinity onto starch granule and increase the chance of multiple attack of enzyme on amylose (Juge et al., 2006).

This study describes the cloning of a thermostable 4-α-glucanotransferase (TAαGT), the construction of its chimeric enzymes (TAαGT-E and TAαGT-DE) which contain the SBDs (E and DE domains) originating from *Bacillus* CGTase, and the action towards amylose, amylopectin, and starch. We hypothesized that SBDs-attached TAαGT could have improved starch-binding/-digesting capabilities as well as 4-α-glucanotransferase activity.

2. Materials and methods

2.1. Materials

2.1.1. Strains and culture conditions

Thermus aquaticus YT-1 (ATCC 25104) was purchased from American Type Culture Collection (ATCC) and cultured in the Thermus B-P medium (0.4% beef extract, 0.4% polypeptone, 0.3% K_2HPO_4, 0.1% KH_2PO_4, 2.5% agar) at 70 °C for 7 days with shaking. *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. coil* transformanants were grown in Luria–Bertani medium (1% Bacto-tryptone, 0.5% yeast extract, 0.5% NaCl) with ampicillin (100 μg/ml) at 37 °C.

2.1.2. Chemicals and enzymes

Amylose and amylopectin from corn starch, rice starch, and maltoologosaccharides (G1 through G7) were purchased from Sigma–Aldrich Co. (St. Louis, MO). Isoamylase isolated from *Pseudomonas amyloderamosa* was purchased from Hayashibara Biochemical Laboratories Inc. (Okayama, Japan). β-Amylase from sweet potato was purchased from Sigma–Aldrich Co. (St. Louis, MO). Cyclo-amylose standards with DP ranging from 21 to 33 were purchased from Ezaki Glico Co.(Osaka, Japan).

2.2. Cloning of 4-α-glucanotransferase gene

The DNA probe for 4-α-glucanotransferase gene (*taαgt*) cloning was prepared by PCR amplification with two oligonucleotide primers, primer 1: 5′-GGTGGCTCGAGGACT ACGCCC-3′ and primer 2: 5′-CCCTGGGCTGGTACC GCACGGCC-3′, which were designed on the basis of known DNA sequence of *T. aquaticus* 33923 4-α-glucanotransferase (Terada et al., 1999). The genomic DNA of *T. aquaticus* YT-1 was digested with restriction enzymes. The resulting fragments were separated on a 0.8% agarose gel, transferred onto a Hybond N^+ membrane, and hybridized with the PCR product (about 750 bp) labeled using ECL labeling kit (Amersham Bioscience, Sweden). In order to acquire clones containing the *taαgt* gene, colony hybridization was carried out by following the procedure of Sambrook, Fritsch, and Maniatis (1989) using ECL DNA labeling and detection kit (Amersham Bioscience, Sweden). The structural *taαgt* gene was isolated by PCR using the putative clone as a template and primers, primer 3: 5′-GCTAAAGCTTTTTAAGACCCCGG-3′ and primer 4: 5′-ATCTTAGGCATATGGAGCTTCCTAG-3′. The resulting PCR products (about 1520 bp) were digested with *Nde*I and *Hin*dIII, and inserted into the *E. coli* expression vector p6 × His119, creating p6 × HisTAαGT (Fig. 1). The nucleotide sequence of PCR-generated gene was determined using BigDye™ Terminator Cycle Sequencing kit for ABI3700 PRISM (Perkin-Elmer, Norwalk, Conn.).

2.3. Construction of chimeric genes

In order to remove the termination codon (TAA) of TAαGT gene and make new restriction enzyme site, *Eco*RI, which would be a linker between the TAαGT gene and starch-binding domains (SBDs), the TAαGT gene was amplified by PCR using primers, primer 3: 5′-GCTAAAG CTTTTTAAGACCCCGG-3′ and primer 5: 5′-CCCGGG GAATTCCGTCCTCCCCG-3′. The E-domain (321 bp) and DE-domains (570 bp) from SBDs of *Bacillus stearothermophilus* ET1 CGTase were isolated, by using forward

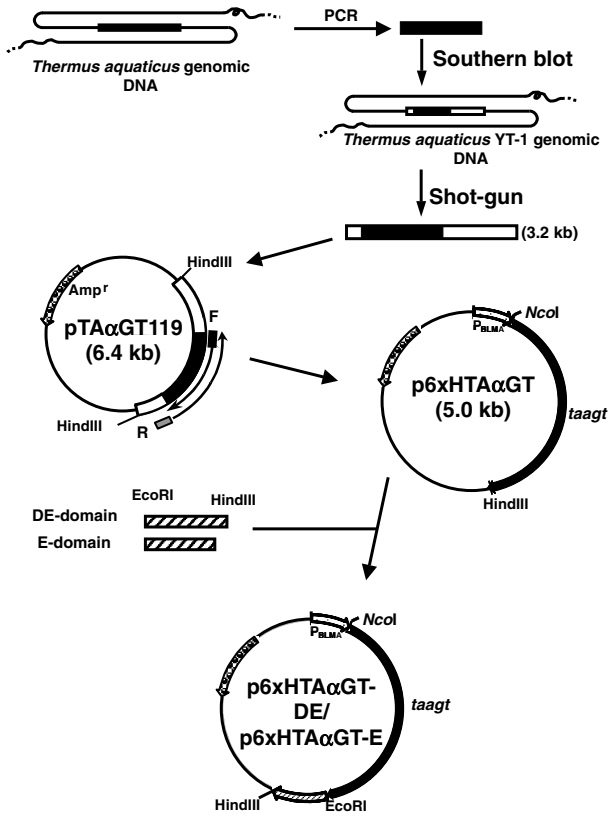

Fig. 1. Strategy for construction of pTAαGT119 and SBD (E and DE) attachment.

primers, primer 6: 5′-TATAGTGAATTCGAAAGTGT GCCA-3′ for E-domains and primer 7: 5′-TTTGAAGAA TTCACGAATGACCAA-3′ for DE-domains, and reverse primer, primer 8: 5′-GATACAAGGAAGCTTCAGCT ATTCTTCG-3′. The PCR products of TAαGT digested with *Nde*I/*Eco*RI, and that of E- and DE-domains cleaved with *Eco*RI/*Hin*dIII, were ligated into the *Nde*I/*Hin*dIII-cut p6 × His119 vector, creating p6 × HisTAαGT-E and p6 × HisTAαGT-DE recombinant (Fig. 1).

2.4. Purification of enzymes

The *E. coli* transformant carrying recombinant plasmid (p6 × HisTAαGT, p6 × HisTAαGT-E, or p × 6 HisTAαGT-DE) was cultivated in LB medium containing ampicillin (100 μg/ml). The cells were harvested by centrifugation and resuspended in lysis buffer [50 mM Tris–HCl buffer (pH 7.5) containing 300 mM NaCl and 10 mM imidazole]. The cell extract was obtained by sonication (VC-600, Sonics & Materials, Newtown, Conn.) and heated at 65 °C for 20 min. After centrifugation, the supernatant was passed through a nickel–nitrilotriacetic acid (Ni–NTA) column (Qiagen, Hilden, Germany). The column was washed with 50 mM Tris–HCl buffer (pH 7.5) containing 300 mM NaCl and 20 mM imidazole followed by the elution with 50 mM Tris–HCl buffer (pH 7.5) containing 300 mM NaCl and 250 mM imidazole. The purified protein was dialyzed against 50 mM Tris–HCl buffer (pH 7.5). For further purification of chimeric enzymes containing SBDs, the dialyzed enzyme solution was loaded into amylose affinity column (New England BioLab, USA) and washed with the washing buffer [50 mM Tris–HCl (pH 7.5) and 200 mM NaCl]. The chimeric protein was eluted with elution buffer [50 mM Tris–HCl (pH 7.5), 200 mM NaCl and 10 mM maltose]. The eluted enzyme was dialyzed against 50 mM Tris–HCl (pH 7.5). The molecular mass of the recombinant enzyme was estimated by SDS–PAGE using a 10% gel according to Laemmli (1970).

2.5. Assay for 4-α-glucanotransferase activity

The activity of 4-α-glucanotransferase was determined by measuring the optical change in iodine-staining during the conversion of amylose by the enzyme (Liebl et al., 1992). The enzyme reaction mixture containing 250 μl of 0.2% (w/v) amylose, 50 μl of 1% (w/v) maltose, 600 μl of 50 mM Tris–HCl buffer (pH 7.5), and 100 μl of enzyme solution was incubated at 70 °C for 10 min. The reaction was stopped by boiling for 10 min. Aliquots (0.1 ml) were mixed with 1 ml of Iodine solution [0.02% (w/v) iodine and 0.2% (w/v) potassium iodide], and the absorbance at 620 nm was measured immediately with an Ultraspec 4000 UV/Visible spectrophotometer (Amersham Bioscience, UK). One unit of 4-α-glucanotransferase activity was defined as the amount of enzyme which degrades 0.5 mg/ml of amylose per min under the assay conditions used. The protein concentration was determined according to the method of Bradford (1976) using Bovine serum albumin as a standard.

2.6. Thin-layer chromatography (TLC)

Five units of 4-α-glucanotransferase was incubated with 1% (w/v) of each maltooligosaccharide (glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, or maltoheptaose) in 50 mM Tris–HCl buffer (pH 7.5) at 70 °C for 6 h. After the reaction, the sample was boiled for 10 min to stop the enzyme reaction and centrifuged to remove the denatured enzyme. The reaction products were analyzed by using thin-layer chromatography (TLC). The samples were spotted onto a Whatman K5 TLC silica gel plate (Fisher Scientific, Chicago, IL), and the plate was irrigated with a solvent mixture of isopropyl alcohol : ethyl acetate : water (3:1:1, by volume) at 25 °C. The carbohydrates on the TLC plate were detected by dipping the dried plate into methanol solution containing 0.3% (w/v) *N*-(1-naphthyl)-ethylendiamine and 5% (v/v) sulfuric acid, followed by heating at 110 °C for 10 min (Robyt & Mukerjea, 1994).

2.7. Enzymatic modification of amylopectin and rice starch

Amylopectin or rice starch (5%, w/v) was completely dissolved in 90% dimethyl sulfoxide. The solution was

diluted with 50 mM Tris–HCl buffer (pH 7.5) and then added with 0.1 U of 4-α-glucanotransferase per mg amylopectin (rice starch) to make the final amylopectin (rice starch) concentration of 0.5%. The enzyme reaction was carried out in a water bath at 70 °C and sampled at selected time interval up to 12 h. The reaction was terminated by boiling for 10 min. In order to remove free sugars in the reaction mixture, the sample was mixed with three-fold volume of ethanol and followed by centrifugation at 12,000*g* for 15 min. The precipitant was dried at room temperature and re-dissolved in deionized water for further analyses.

2.8. Production of cyclo-amylose

Amylose (2%, w/v) was dissolved in 90% dimethyl sulfoxide. The reaction mixture containing 0.5% amylose, 0.1 U of 4-α-glucanotransferase per mg amylose, and 50 mM Tris–HCl buffer (pH 7.5) was incubated at 70 °C for 12 h, The reaction was terminated by boiling for 10 min. Aliquots (5 ml) was incubated with β-amylase (350 U) at 37 °C for 3 h to hydrolyze linear glucans. After the reaction was stopped by boiling for 10 min, the β-amylase-resistant glucans were separated by precipitating it with ten-fold volume of ethanol and then lyophilized. The sample was dissolved in deionized water for HPAEC and MALDI-TOF MS analysis.

2.9. High performance anion exchange chromatography (HPAEC)

For the analysis of branch chain length distribution of enzyme-treated amylopectin and rice starch, samples were debranched by using isoamylase (Hayashibara Biochemical Laboratories Inc., Okayama, Japan) in 25 mM NaOAC buffer (pH 4.3) at 60 °C for 72 h. The reaction mixture was boiled for 5 min to stop the debranching reaction. The branch chain length distribution was analyzed by using high-performance anion-exchange chromatography (HPAEC) system (Dionex-300, Dionex, Sunnyvale, CA) with an electro-chemical detector (ED40, Dionex). A CarboPac™ PA-1 anion-exchange column (250× 4 mm, Dionex) and a guard column were used for the separation of debranched samples. After column was equilibrated with 150 mM NaOH, the sample was eluted with varied gradients of 600 mM sodium acetate in 150 mM NaOH at a flow rate of 1 ml/min. The applied gradients of sodium acetate was as follows: a linear gradient 10–30% for 0–10 min, 30–40% for 10–16 min, 40–50% for 16–27 min, 50–60% for 27–44 min, 60–64% for 44–60 min. As for the cyclo-amylose analysis, a linear gradient of sodium acetate from 0 to 100% was used over 70 min.

2.10. SEC-MALLS-RI

The molecular weight distribution of modified amylopectin was analyzed by using size exclusion chromatography (SEC), multi-angle laser light scattering (MALLS) (Dawn DSP, Wyatt Technology, St. Barbara, CA), and refractive index (RI) detector (Waters 410). A TSK G5000 SEC column (7.8 × 600 mm, Tosoh Co., Tokyo, Japan) with a guard column (TSK PWH, Tosoh Co., Tokyo, Japan) was used at room temperature. The flow rate of mobile phase (0.15 M $NaNO_3$ containing 0.02% NaN_3) was 0.4 ml/min. The weight-average molecular weight (M_w) of sample was calculated using a ASTRA V4.90.07 software (Wyatt Technology) with the Berry extrapolation method for curve fitting and the d*n*/d*c* value of 0.146 ml/g.

2.11. Matrix assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry

The Molecular masses of cyclo-amylose standard (Ezaki Glico, Osaka, Japan) and cyclo-amylose produced by the 4-α-glucanotransferase were determined by using a MALDI-TOF mass spectrometer (Voyager™-DE, Perceptive Biosystem, Framingham, USA). A cyclo-amylose sample (1 μg/ml) was mixed with a matrix (2,5-dihydroxybenzoic acid, 5 pmol/μl). The mixture (1 μl) was applied to the probe tip and dried until homogeneous crystals formed. The sample was desorbed/ionized from the probe tip under the proper condition (Grid voltage; 87, Grid wire voltage: 0.3, Delayed extraction: 300 ns, and Laser: 2000).

3. Results and discussion

3.1. Cloning of T. aquaticus YT-1 4-α-glucanotransferase (TAαGT) gene

The 4-α-glucanotransferase gene (*taαgt*) was isolated from *T. aquaticus* YT1 by following the procedure shown in Fig. 1. This TAαGT gene had an open reading frame of 1503 nucleotides, which encoded a single polypeptide of 500 amino acids with an estimated molecular mass of 57,969 Da. The nucleotide sequence and deduced amino acid sequence data are available from EMBL/GenBank databases under the Accession No. AY459352. The predicted amino acid sequence of TAαGT shared 87% identity with that of 4-α-glucanotransferase from *T. aquaticus* 33,923 (Terada et al., 1999) and 40% identity with potato D-enzyme (Takaha et al., 1993), but showed a low level of homology with *T. maritima* 4-α-glucanotransferase (Liebl et al., 1992). It contained four highly conserved regions, which is characteristic of α-amylase super-family 13.

3.2. Construction of chimeric enzymes (TAαGT-E and TAαGT-DE)

The domain E of CGTase has been reported to be involved in binding to raw starch and accelerate its hydrolysis (Penninga et al., 1996; Svensson, Jespersen, Sierks, & MacGregor, 1989). However, its exact function is remained to be clarified. Also, the function of D-domain, which is connected to $(\beta/\alpha)_8$-barrel catalytic domains, is unknown.

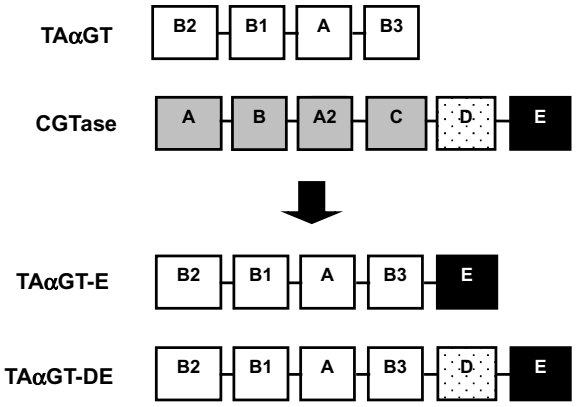

Fig. 2. Construction of TAαGT-E and TAαGT-DE. Black boxes symbolize the domains of TAαGT from *T. aquaticus* YT1 and CGTase from *B. stearothermophilus* ET1.

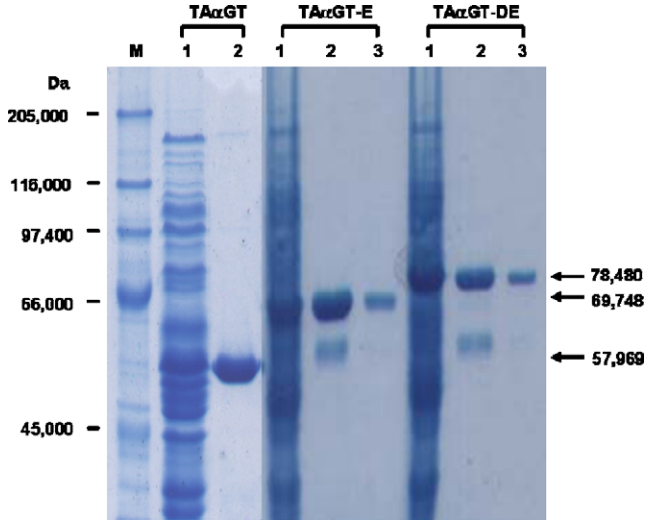

Fig. 3. SDS–PAGE analysis of the purified enzymes. Lane M, protein size standards; lane 1, cellular proteins from crude extract (p6 × HisTAαGT, p6 × HisTAαGT-E and p6 × HisTAαGT-DE); lane 2, purified 6 × His-tagged TAαGT, TAαGT-E, and TAαGT-DE after Ni–NTA column chromatography; lane 3, purified TAαGT-E and TAαGT-DE after amylose affinity column chromatography.

Since 4-α-glucanotransferase does not possess E-domain, its starch-binding and -digesting abilities are considered to be weak. Therefore, starch-binding domains of CGTase originating from *B. stearothermophilus* ET1 (Lee, 1997) were introduced into the C-terminus of TAαGT by following the strategy shown in Fig. 2, as an effort to improved its starch-utilizing ability. Two chimeric enzymes TAαGT-E, which was composed of TAαGT and E-domain of CGTase, and TAαGT-DE, which consisted of TAαGT and both D and E domains of CGTase, were constructed. TAαGT-E and TAαGT-DE had 615 and 698 amino acids, respectively. The molecular weight of TAαGT-E and TAαGT-DE deduced from their nucleotide sequences were 69,748 and 78,480 Da, respectively.

3.3. Characteristics of TAαGT, TAαGT-E, and TAαGT-DE

The recombinant TAαGT was efficiently purified by Ni–NTA affinity chromatography after heat treatment at 65 °C for 20 min. The chimeric proteins, TAαGT-E and TAαGT-DE, were successfully purified by sequential two-step chromatography using Ni–NTA and amylose affinity column after heat treatment. The molecular masses of purified enzymes were around 57, 69, and 78 kDa for TAαGT, TAαGT-E, and TAαGT-DE, respectively, as estimated from SDS–PAGE analysis (Fig. 3).

The maximum activity of TAαGT was observed at pH 7.5 and 70 °C, when amylose was used as a substrate. TAαGT exhibited a high thermal stability, retaining its 90% activity after 120 min of incubation at 80 °C. Time spent for 10-fold decrease of TAαGT activity was determined to be 210 and 29 min at 85 °C and 90 °C, respectively (data not shown). Many benefits such as higher substrate concentration, limited risk of bacterial contamination, increased reaction rate, and lower cost in enzyme purification are produced by using thermally stable enzyme and running reaction at an elevated temperature (Vieille & Zeikus, 2001).

In order to confirm the identity of the 4-α-glucanotransferase overexpressed in *E. coli*, the action pattern of the purified TAαGT was investigated using maltooligosaccharides (glucose to maltoheptaose) as substrates (Fig. 4). Transglycosylation products (glucose and maltooligosaccharides) were generated from all of maltooligosaccharides tested except glucose. Larger maltooligosaccharides (maltotetraose, maltopentaose, maltohexaose, and maltoheptaose) were more effective substrates than smaller molecules (maltose and maltotriose). These results suggested that TAαGT catalyzes the transfer of glucose units from one 1,4-α-glucan to another and requires at least maltose

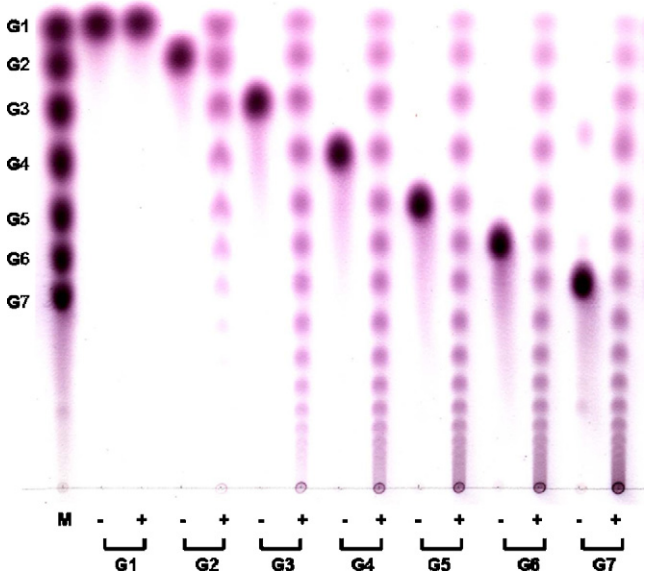

Fig. 4. TLC analysis of reaction pattern of TAαGT on maltooigosaccharides. Lane M, maltooligosaccharide standards; reactions on maltooligosaccharides (glucose to maltoheptaose) with (+) or without (−) enzyme.

Table 1
Molar specific activities of TAαGT, TAαGT-E, and TAαGT-DE

Enzyme	Molecular weight[a]	Molar specific activity (U/nmol)	Relative specific activity
TAαGT	57,969	5.38 ± 0.11	1
TAαGT-E	69,748	5.65 ± 0.20	1.1
TAαGT-DE	78,480	9.61 ± 0.38	1.8

0.05% (w/v) amylose was used as a substrate.
[a] Molecular weight was deduced from the amino acid sequence.

unit for the 'disproportionation' reaction. Palmer, Ryman, and Whelan (1976) reported that 4-α-glucanotransferase from *E. coli* can transfer glucose or larger units. Also, it was reported that, unlike other known 4-α-glucanotransferase, the smallest donor molecule for *T. maritima* 4-α-glucanotransferase was maltotetraose, and glucose did not function as an acceptor nor did it appear as a reaction product (Liebl et al., 1992).

The chimeric enzymes (TAαGT-E and TAαGT-DE), which had additional starch-binding domains, showed the same transglycosylation pattern with maltooligosaccharides, and their optimal temperature and pH were essentially the same as those of TAαGT (data not shown). Table 1 showed molar specific activities (enzyme activity per unit mole of protein) of TAαGT and its chimeric enzymes toward amylose. TAαGT-E exhibited similar molar specific activity with TAαGT, whereas the activity of TAαGT-DE was almost twice as high as that of TAαGT and TAαGT-E. Ohdan, Kuriki, Takata, Kaneko, and Okada (2000) have reported that chimeric enzymes consisting of α-amylase from *Bacillus subtilis* and starch-binding domains from *Bacillus* CGTase showed higher raw starch-binding and digesting abilities but no differences in amylolytic pattern, transglycosylation ability, and effects of pH and temperature on stability and activity. Giardina et al. (2001) reported that the SBD has two binding sites for maltoheptaose: the site 1 is involved in the initial recognition of the chain and the site 2 promotes the tighter binding and leads to the circularization of an amylose chain. They observed that, when the SBD binds to an amylose chain, a novel two-turn helical amylose complex structure was produced. The function of D-domain from CGTase is difficult to be clarified from the above result. However, the increase in the molar specific activity of TAαGT-DE suggested that the D-domain might assist the binding of E-domain to starch.

Among TAαGT and its chimeric enzymes (TAαGT-E and TAαGT-DE), TAαGT-DE, the most effective starch digester, was selected and further examined on its characteristics in terms of the modification of starch molecules.

3.4. Modification of amylopectin using TAαGT-DE

Amylopectin solution was treated with TAαGT-DE, and its digesting process was monitored in terms of SEC elution profile (Fig. 5). As reaction time increased, the first fraction (elution volume, Ve: 25–30 ml) in the elution profile, which corresponds to the amylopectin macromolecules, gradually decreased in height and shifted to right. In the meantime, the second fraction (Ve: 30–45 ml) and the third fraction (Ve: 45–55 ml) gradually developed. After 40 min, the second fraction started to decrease, while the third fraction continuously increased. The weight average molecular weight (M_w) of amylopectin was measured during the enzymatic degradation using SEC-MALLS-RI system as presented in Fig. 6. The molecular weight of amylopectin in the literature varies from several millions to several hundred millions depending on solubilization methods and measurement techniques. The measured M_w of undigested amylopectin (0 min) was 4.4×10^8 g/mol, which is similar to those reported by Hanselmann, Ehrat, and Widmer (1995) (3.6×10^8) and Klavons, Dintzis, and Millard (1997) (4.12×10^8). The M_w rapidly decreased at the beginning of the reaction and the decrease slowed down afterward. Interestingly, the average molecular weight reached to several hundred thousands and did not decrease significantly even after 12 h of digestion (Fig. 6). The average M_w of amylopectin digested for 12 h with TAαGT-DE was 7.1×10^5, which corresponds to about 4000 glucose units. The final elution profile (12 h) indicated a bimodal distribution, even though those fractions were overlapped to a large extent. The average molecular weight of the earlier fraction in the profile was 1.1×10^6, and that of the latter fraction was 1.5×10^5, with the approximate

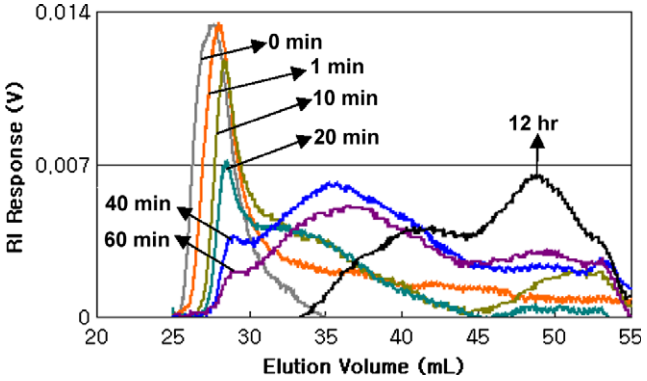

Fig. 5. The change of SEC elution profile of amylopectin during enzymatic degradation by TAαGT-DE.

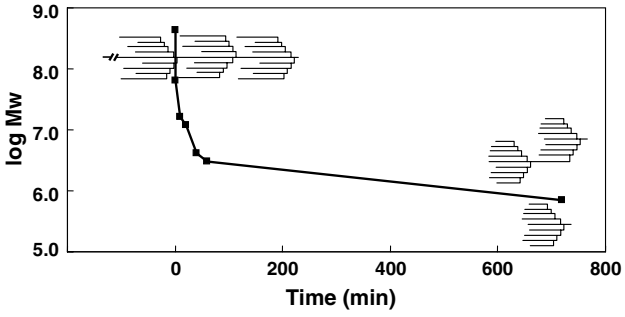

Fig. 6. The change of weight-average molecular weight (M_w) of amylopectin modified by TAαGT-DE.

corresponding glucose units of 6000 and 800, respectively. The amylopectin-TAαGT-DE reaction product was isolated by precipitating with three fold volume of ethanol, and its branch chain length distribution was observed using HPAEC after treating with isoamylase (Fig. 7). Before debranching, it was confirmed that free linear glucans were absent in the isolated reaction product (Fig. 7c). As presented in Fig. 7b, the TAαGT-DE- treated amylopectin contained more amounts of shorter branches compared to control amylopectin (Fig. 7a), which agrees well with Takaha, Yanase, Takata, Okada, and Smith (1998). Above results indicated that the enzyme product was composed of branched polymers, whose size is hundred to thousand times smaller than that of amylopectin, with shortened branch chains. Considering the fact that average chain length of amylopectin is only about 25 DP (Hoseney, 1994) and adopting the model for amylopectin structure proposed by French (1984), the above significant reduction of molecular weight is possible only when inner chains (probably C or B chains) of amylopectin are cleaved in addition to the outer chain trimming. As a result, small amylopectin clusters with shortened branch chain would be generated. The reason why the final product of TAαGT-DE treatment contained multi-fraction (Fig. 5, 12 h) is not clear with presented data. However, the earlier fraction in Fig. 5 (12 h) might be considered as associations of small cluster units. Takaha et al. (1998) have proposed a model for the transglycosylation of a whole amylopectin cluster unit from a lager cluster to another cluster in potato D-enzyme (disproportionating enzyme). Adopting this model, it could be postulated that larger amylopectin clusters in the earlier fraction in Fig. 5 (12 h) is retained without further reduction in size due to the enzyme's certain rate of cluster transfer activity. However, further study will be required to elucidate the cluster transfer of TAαGT-DE. The suggested structural change of amylopectin molecules after TAαGT-DE treatment is presented schematically in Fig. 6.

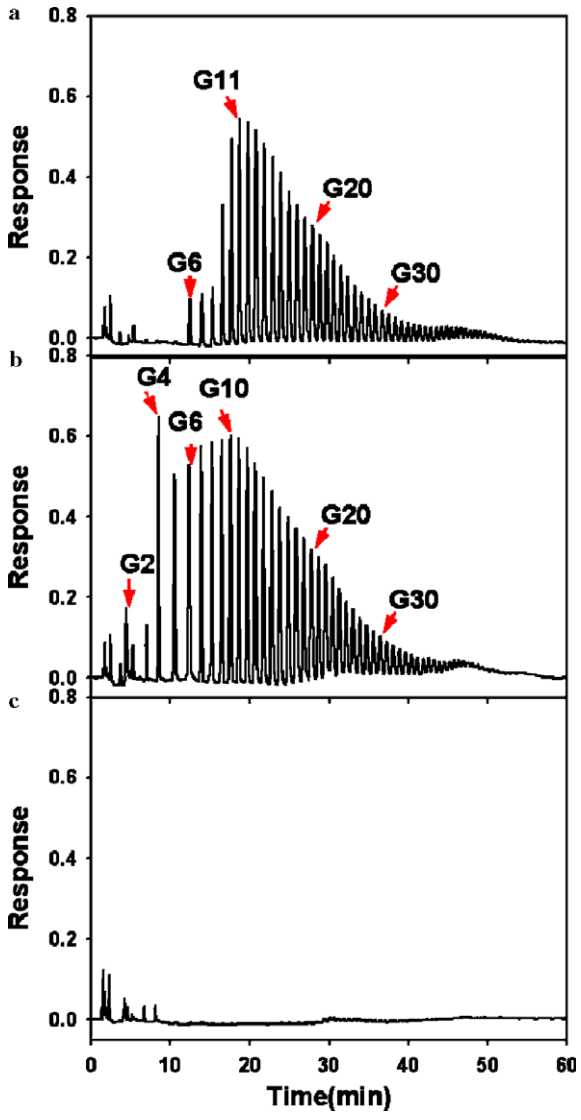

Fig. 7. HPAEC analysis for branch chain length distribution of amylopectin. (a) amylopectin control treated with isoamylase, (b) TAαGT-DE-modified amylopectin treated with isoamylase, and (c) TAαGT-DE-modified amylopectin separated by ethanol precipitation before isoamylase treatment.

The branch chain length distribution of rice starch was also examined with and without enzyme treatment as presented in Fig. 8. The experimental procedure was exactly same as that for amylopectin, and the absence of free linear and cyclic glucans was confirmed after isolation by ethanol (threefold volume) precipitation before debranching (Fig. 8c). In the case of rice starch, which contains approximately 20% of amylose, the amount of longer branch chains (>DP 25) apparently increased as well as that of shorter branch chains after TAαGT-DE treatment (Fig. 8b) compared to control rice starch (Fig. 8a). The increase in the amount of longer branch chains was not observed in the case of TAαGT-DE-treated amylopectin (Fig. 7b). Kaper, van der Maarel, Euverink, and Dijkhuizen (2004) claimed the transfer of α-glucan from amylose to amylopectin to partly elongate amylopectin side chains after the treatment of starch with 4-α-glucanotransferase from *Thermus thermophilus*. However, the increase could also be attributed to shortened and branched amylose, even though the branched portion of amylose is much less compared to that of amylopectin in starch. Elucidation of amylose's exact whereabout after TAαGT-DE treatment is remained for future study. Lee, Kim, Park, and Lee, 2006 reported the formation of thermoreversible gel after the treatment of rice starch with 4-α-glucanotransferase from *Thermus scotoductus*. The formation of amylopectin clusters with re-organized side chains might be responsible for the formation of thermoreversible gel, which can not be obtained from popular starch hydrolyzing enzymes, such as α-amylase.

3.5. Production of cyclo-amylose

It has been reported that potato D-enzyme catalyzes the intramolecular transglycosylation (cyclization) of amylose,

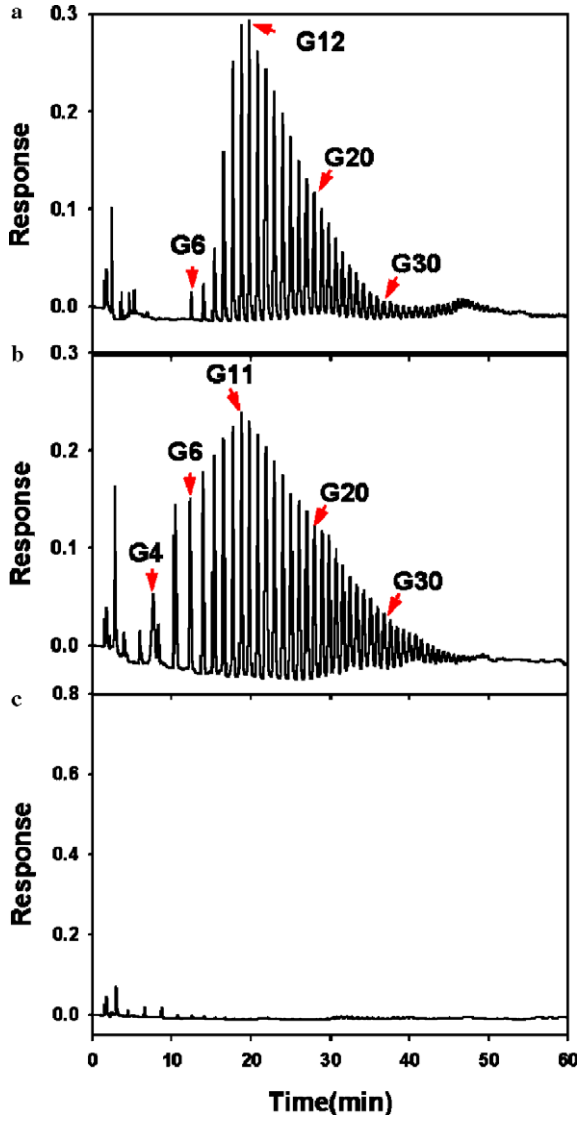

Fig. 8. HPAEC analysis for branch chain length distribution of rice starch. (a) rice starch control treated with isoamylase, (b) TAαGT-DE-modified rice starch treated with isoamylase, and (c) TAαGT-DE-modified rice starch separated by ethanol precipitation before isoamylase treatment.

yielding cyclic α-1,4-glucans (cyclo-amyloses) (Takaha et al., 1996). These highly water-soluble cyclo-amyloses are known to have the ability to form inclusion complexes with inorganic (Kitamura, Nakatani, Takaha, & Okada, 1999) and organic molecules (Takaha & Smith, 1999), and have numerous potential applications including food and drink compositions, food additive compositions, infusion solutions, adhesive compositions, inclusion materials, adsorption materials, anti-retrogradation agents for starch, and starch substitutes for biodegradable plastics.

The ability of TAαGT-DE to produce cyclo-amyloses from amylose molecules was examined using HPAEC (Fig. 9) and MALDI-TOF MS (Fig. 10). After the TAαGT-DE treatment, amylose molecules (Fig. 9a) degraded into low molecular weight glucans (Fig. 9b). Linear maltooligosaccharides produced by the intermolecular transglycosylation of TAαGT-DE were hydrolyzed into maltose by treating with β-amylase. The β-amylase-resistant glucans were then separated by ethanol precipitation. HPAEC analysis demonstrated that the β-amylase-resistant glucans (Fig. 9c) was eluted at the similar elution time with cyclo-amylose standard (DP 21–33) (Fig. 9d). In order to confirm that the β-amylase-resistant glucans were cyclic forms, the molecular weights of the β-amylase-resistant glucans were measured using MALDI-TOF MS (Fig. 10) The DP of the β-amylase-resistant glucans ranged from about 19 to 35 and their molecular weights agreed well with theoretical values for cyclic glucans, which confirmed the conclusion that TAαGT-DE catalyzed the formation of cyclo-amyloses from amylose molecules.

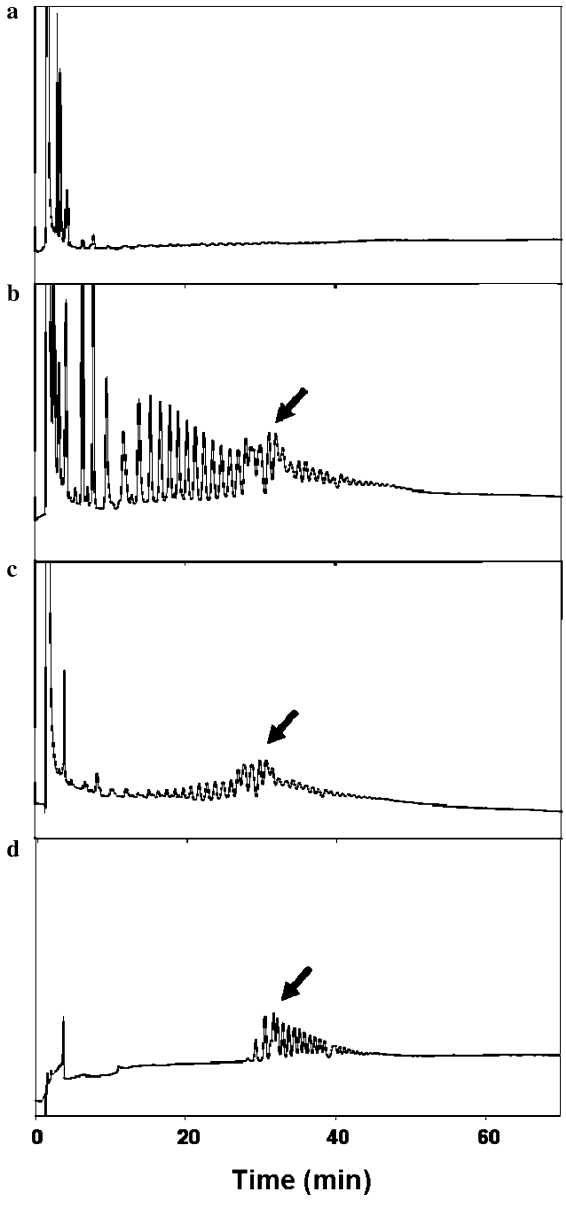

Fig. 9. HPAEC analysis for TAαGT-DE-treated amylose. (a) amylose, (b) TAαGT-DE-modified amylose, (c) TAαGT-DE-modified amylose after β-amylase treatment followed by ethanol precipitation, and (d) cyclo-amylose standard. An arrow in each graph indicates a group of cyclo-amylose peaks.

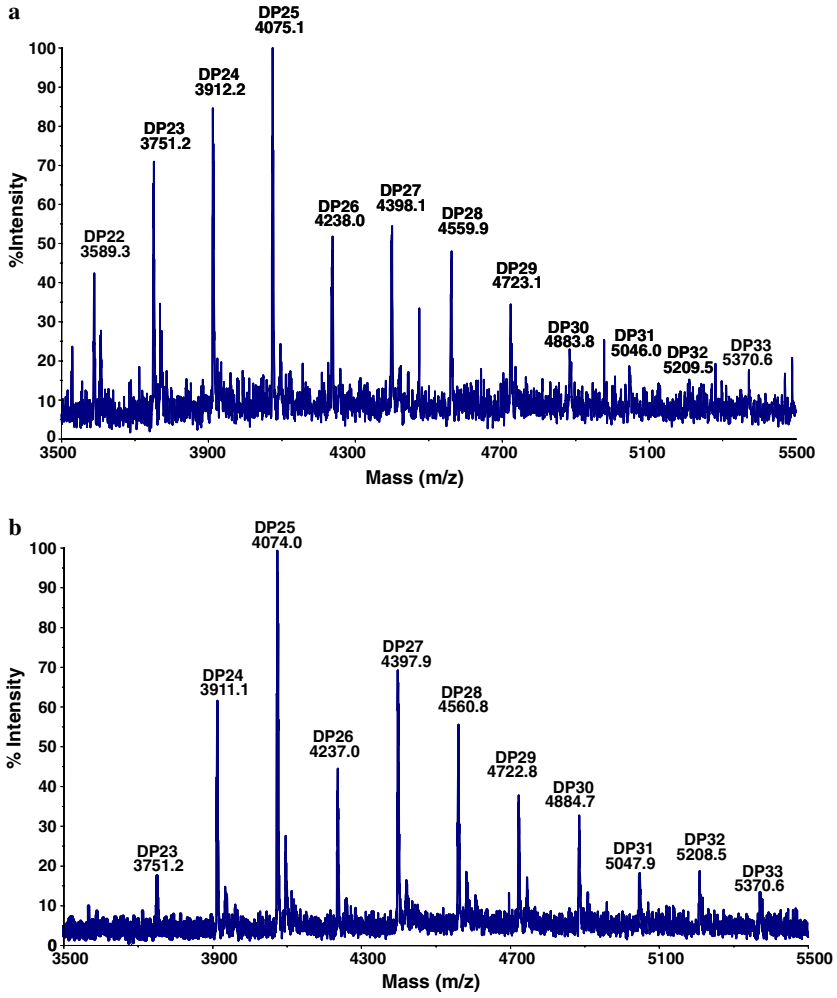

Fig. 10. MALDI-TOF MS analysis for cyclo-amylose. (a) cyclo-amylose produced by TAαGT-DE and (b) cyclo-amylose standard.

4. Conclusions

Most of the glucoamylases and CGTases from various species contain starch-binding domains (SBDs) in the C- or N-terminus, which is known to aid starch molecule degradation (Giardina et al., 2001). This report describes the properties of a 4-α-glucanotransferase from *T. aquaticus* YT-1 (TAαGT) and its chimeric enzymes (TAαGT-E and TAαGT-DE) containing SBDs from CGTase in the C-terminus, especially in utilizing amylose and amylopectin molecules. TAαGT-DE showed enhanced molar specific activity toward amylose. TAαGT-DE reassembled amylopectin molecules by its disproportionation activity to produce modified amylopectin clusters. Also, it demonstrated the ability to form cyclo-amyloses by manipulating amylose molecules. Based on its versatility in utilizing starch molecules, this chimeric protein would be highly useful for many starch-related industries.

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