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Abstract Graphic

84x44mm (600 x 600 DPI)

A Thermophilic 4- α -Glucanotransferase from *Thermoproteus Uzoniensis* Retards the Long-Term Retrogradation but Maintains the Short-Term Gelation Strength of Tapioca Starch

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ABSTRACT: Gelation of starch is a process during short-term retrogradation. However, the long-term retrogradation always leads to the quality deterioration of starch-based food. In this work, a new type of modified tapioca starch (MTS) gel with maintained starch short-term gelation strength and retarded long-term retrogradation was prepared using a novel recombinantly produced and characterized 4- α -glucanotransferase (Tu α GT). In the resulting MTS exterior chains of the amylopectin part were elongated and the content of amylose was reduced, due to the disproportionation activity of Tu α GT. The retrogradation analysis demonstrated that the MTS possessed highly weakened long-term retrogradation characteristics as compared to the native starch. Most importantly, the strength of the gel formed by re-gelatinized MTS is very close to that of gelatinized native tapioca starch (NTS) when storing below 30°C. These findings provide a starting point for developing a novel method for the enzymatic modification of the starch-based gels.

KEYWORDS: *long-term retrogradation, gel properties, starch-based gel, 4- α -glucanotransferase*

INTRODUCTION

Retrogradation is an important functional attribute in starch-based foods. In this process unordered starch molecules in an amorphous phase transfer to a more ordered crystalline state.¹ This transformation can be divided into two stages, the short-term and the long-term retrogradation. Short-term retrogradation, namely gelation that is beneficial for strengthening the texture of starch-based foods, such as bread, steam bread, noodles etc., is mainly attributed to the aggregation of amylose.² However, further reassociation of amylose with amylose as well as amylopectin molecules would lead to long-term retrogradation that causes a decline in the taste of starch-based foods.³

Many studies have been conducted to inhibit or delay the long-term retrogradation of starch.^{4, 5} Among the various current treatments, enzymatic modification was preferred over chemical modification, as this provides products having the “clean-label” advantage.⁶ Reduction in amylose content or breakdown of amylose into shorter fragments by endo- and exo-acting enzymes, such as α -amylase,⁷ β -amylase⁸ and so on, represents by far the most efficient type of

approaches. However, typically enzymatic modifications also impaired the short-term retrogradation properties, resulting in weaker gel strength and poorer chewability.⁹ Another option may be to use waxy starches only containing amylopectin, but waxy starch hardly forms a solid gel during short-term retrogradation, which severely limits its application. Indeed, many studies focused on enhancing starch gel strength during short-term retrogradation by using enzymatic treatments.¹⁰ These include the action of starch debranching enzymes such as pullulanase and isoamylase hydrolyzing branch points in amylopectin with release of short amylose fragments, clearly improving the gelation property of starch.¹¹ But the long-term retrogradation of partially debranched starch became seriously disadvantageous during storage.¹⁰ Thus, the short-term retrogradation advantage and the long-term retrogradation disadvantage can co-occur and be positively correlated.

The aim to reduce the harmful effect of long-term retrogradation and keep the short-term gel formation as strong as possible, by strengthening the gelation property of amylopectin and weaken the contribution of the amylose part would be a desirable approach. As previously reported, 4- α -glucanotransferase (4 α GT, EC 2.4.1.25) belonging to glycoside hydrolase family 77 (GH77) catalyzes disproportionation reactions cleaving amylose into shorter fragments which are transferred to the non-reducing ends of amylopectin resulting in longer exterior chains.¹² 4 α GT has been successfully applied in preparation of thermo-reversible starch gels, proving the reassociation of amylose to be strongly eliminated.¹³ Thus, 4 α GT offers a potential solution to reach a better balance between short-term and long-term retrogradation.

In the present study, a new type of modified tapioca starch (MTS) gel with maintained starch short-term gelation strength and retarded long-term retrogradation was prepared using a novel 4- α -glucanotransferase (TuaGT). TuaGT, discovered from a new archebacterium *Thermoproteus uzoniensis* sharing rather low identity, i.e. maximum similarity of 64.74% with 4 α GT from *Pyrobaculum arsenaticum* DSM 13514 (GenBank accession number ABP49821.1)), was cloned and expressed in *Escherichia coli*. After the successful characterization of the produced recombinant TuaGT as a 4 α GT, tapioca starch was chosen for the enzymatic modification. The structure determination, retrogradation analysis and rheological test showed that the 4 α GT treatment indeed changed the starch structure in a remarkable way, resulting in a reduced long-term retrogradation but maintaining most of the gel strength compared to native tapioca starch. Therefore,

this novel 4 α GT from *Thermoproteus uzoniensis* might be new tool for production of modified starches of commercial value.

MATERIALS AND METHODS

Materials. Maltotriose and maltotetraose were purchased from Sigma-Aldrich Co. Ltd (St. Louis, MO, USA). Tapioca starch was kindly provided by STARPRO Co. Ltd (Hangzhou, China, amylose content 23%). Isoamylase (from *Pseudomonas sp.*, EC 3.2.1.68, specific activity 500 U/mL), α -amylase (from *Aspergillus oryzae*, EC 3.2.1.1, specific activity 120 U/mg) and α -glucosidase (from *Aspergillus niger*, EC 3.2.1.20, specific activity 70 U/mg) were purchased from Megazyme Co. Ltd (Wicklow, Ireland). Dextran standards of 8.53×10^6 , 8.53×10^5 , 6.7×10^5 , 5×10^4 , 2.5×10^4 Da (polydispersity 1.047) were purchased from Aladdin Co. Ltd (Shanghai, China). All other chemicals were of reagent grade and purchased from China National Pharmaceutical Group Corporation (Beijing, China).

Cloning and Preparation of 4 α GT from *Thermoproteus uzoniensis* (TuaGT). Cloning of 4 α GT was conducted following the method reported by Yu et al.¹⁴ The nucleotide sequence of the 4 α GT gene from *Thermoproteus uzoniensis* (GenBank accession number WP_013679179.1) was cloned into the expression vector pET-28a (+) with kanamycin resistance gene after digestion with NdeI and HindIII to construct the recombinant plasmid. A 6 $\times$ histidine-tag was designed and inserted into the N-terminus for the purification of enzyme. The recombined plasmid was transferred into *Escherichia coli* BL21 (DE3)* for further protein production by the Beijing Liuhe Huada Gene Technology Company (Beijing, China). Strains *E. coli* DH5 α and *E. coli* BL21 (DE3) were selected as hosts for the cloning and expression of TuaGT gene, respectively.

The expression of recombined 4 α GT was carried out according to our previous work with some modifications.¹⁵ The recombinant *E. coli* BL21 (DE3)* was cultivated in LB medium containing kanamycin (50 μ g/mL) at 37°C with shaking at 200 rpm. The expression of recombined protein was performed at 18°C with shaking at 160 rpm for 24 h by adding isopropyl- β -D-thiogalactopyranoside (IPTG) to 0.4 mM when the optical density of cell suspension at 600 nm was 0.4-

0.6. The cells were collected by centrifugation ($10,000 \times g$ for 15 min) and then disrupted by sonication in an ice bath.

Purification of the Recombinant Enzyme. After incubation at 60°C for 20 min followed by centrifugation ($10,000 \times g$ for 30 min) to remove thermolabile non-target protein, the enzyme was purified from the supernatant by Ni-NTA affinity chromatography (GE Healthcare). The recombined protein was eluted by 250 mM imidazole in 20 mM Tris-HCl buffer (pH 7.5) containing 250 mM NaCl. The purified protein was analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins in each purification step were quantified by a BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China) using BSA as the standard. In total 2.4 mg of Tu α GT was purified from 2 L of culture.¹⁶

Disproportionation Activity Assay. The disproportionation activity of Tu α GT was determined following the method reported by Yu et al.¹⁷ by incubating 1% maltooligosaccharides (maltose (G2), maltotriose (G3) and maltotetraose (G4)) in 900 μL assay buffer (50 mM, pH 6.0 sodium citrate buffer) with 100 μL Tu α GT (0.2 g/mL) at 75°C 6 h. The rate of glucose released was determined by high performance liquid chromatography (HPLC) using a Shimadzu LC-20AT pump, a Rheodyne injector and a Shimadzu RID-10A detector, equipped with an Ultimate XB-NH2 column at 30°C using as mobile phase acetonitrile: ultrapure water (7:3, v/v) at a flow rate of 1 mL/min.¹⁴ One unit of disproportionation activity was defined as the amount of enzyme which released 1 μmol of glucose per minute under the above conditions.

Effects of Temperature, pH and Metal Ions on the Activity of Tu α GT. To determine the pH optimum of Tu α GT, the activity was assayed in 50 mM sodium citrate buffer (pH 4.0–5.5), phosphate buffer (pH 6.0–8.0) and glycine-NaOH (pH 8.5–10.0) at 75°C .¹⁵ The activity of the enzyme at the pH optimum was defined as 100%. Tu α GT activity was determined at different temperatures (50 – 90°C) to identify the temperature optimum at pH 6.0. To determine the thermostability, Tu α GT (0.2 mg/mL) was incubated at different temperatures (60 – 100°C) at pH 6.0 for 4 h, and the residual enzyme activity was measured at 1 h intervals. The enzyme activity without incubation was defined as 100% stability.

Different metal ions (K^{+} , Na^{+} , Cu^{2+} , Ca^{2+} , Fe^{3+} , Mg^{2+} , Mn^{2+}) were each added to assay buffer (50 mM, pH 6.0 sodium

citrate buffer) to a resulting concentration of 5 mM and Tu α GT (final concentration 0.1 mg/mL) was added to each metal ion solution and incubated at 4°C for 1 h.¹⁸ Tu α GT in buffer without metal ion was used as a control and residual activity after metal ion incubation was determined using the disproportionation activity assay.

Substrates Specificity of Tu α GT. The substrate specificity of Tu α GT was determined by incubating with glucose, maltose, maltotriose, or maltotetraose as substrate (1%, w/v) in 50 mM sodium citrate buffer at 75°C and pH 6.0 for 6 h. To determine the type of glycosidic linkage of the product, 0.5 unit of α -amylase (from *Aspergillus oryzae*) and 1 unit of α -glucosidase (from *Aspergillus niger*) were added to the obtained product, followed by incubation at 40°C and pH 5.0 for 1 h. The formed products were analyzed by HPLC using a Shimadzu LC-20AT pump, a Rheodyne injector and a Shimadzu RID-10A detector, equipped with an Ultimate XB-NH2 column at 30°C using as mobile phase acetonitrile: ultrapure water (7:3, v/v) at a flow rate of 1 mL/min.¹⁴

Preparation of Modified Tapioca Starch (MTS). Enzymatic modification of tapioca starch was performed following the method reported by Lee et al.¹⁹ Dry starch powder (6%, w/v) was suspended in 20 mM sodium citrate buffer (pH 6.0), followed by gelatinization at 121°C for 30 min while stirring. The modification was carried out by incubating with 0.12 mg Tu α GT per 1 g of starch at 75°C for 8 h, and terminated by heating at 100°C for 30 min. The modified starch was precipitated by adding three volumes of ethanol and dried at 40°C for 8 h after collecting by centrifugation (5000 rpm for 10 min).

Amylose Content. The amylose content of tapioca starch during the modification was determined by Megazyme K-AMYL kit method.²⁰ Concanavalin A was used to precipitate the amylopectin by complex formation. After being hydrolyzed by a glucoamylase/ α -amylase mixture, the amylose content was obtained as calculated from the converted glucose quantified using the GOPOD method.

Retrogradation Properties. Retrogradation of native tapioca starch (NTS) and MTS was examined by measuring the melting onset (T_o), peak (T_p), conclusion (T_c) temperature and melting enthalpy (ΔH) using a DSC7000 calorimeter (HITACHI, Japan). Three milligrams of NTS and MTS were mixed with 6 μ L deionized water and hermetically sealed

132 in an aluminium pan.²¹ The samples were placed at room temperature (25°C) overnight and then tested from 20 to 100°C
133 at a rate of 10°C/min with an empty aluminum pan as reference. Melting enthalpy (ΔH) was calculated from the area of
134 the main endothermic peak and expressed in terms of J/g of dry starch using the equipment software.

135 **Chain Distribution of Debranched Starch.** Chain distribution of starch was determined according to our previous
136 work with some modifications.²² High performance anion exchange chromatography with pulsed amperometric detection
137 (HPAEC-PAD) was used to analysis the chain length distribution of starch. Two mg/mL dry solid (w/v) starch was
138 suspended in 50 mM sodium acetate buffer (pH 4.5), followed by gelatinization on a boiling water bath for 30 min. The
139 gelatinized starch was debranched by incubation with 2 U of isoamylase per 1 mg of starch at 42°C for 12 h. The obtained
140 reaction mixtures were analyzed by HPAEC-PAD (ICS-5000+, Thermo Fisher Scientific, USA). Samples (20 μ L) were
141 injected onto a CarboPac PA-200 column at 0.6 mL/min flow rate. The average DP was calculated from the corrected
142 values of the relative content of each chain.²³

143 **Molecular Weight of NTS and MTS.** Size Exclusion Chromatography-Multi-Angle Laser Light Scattering-Refractive
144 Index Detector (SEC-MALLS-RI) was used to analysis the molecular weight of NTS and MTS samples according to the
145 previous work.²⁴ The calibration of the instrument was carried out with dextran standards (8.53×10^6 , 8.53×10^5 , 6.7×10^5 ,
146 5×10^4 , 2.5×10^4 Da, polydispersity 1.047, Aladdin, China) which were solubilized in MilliQ water (1 mg/mL) and treated
147 on a boiling water bath for 1 h. Dry starch (5 mg/mL) was suspended in a mixture of DMSO and ultrapure water (9:1,
148 v/v), followed by gelatinization on a boiling water bath for 1 h and shaking every 10 min until the solution becomes clear
149 and free of floc. The gelatinized starch was incubated at 30°C in a shaker with 250 rpm for 48 h to disrupt the starch
150 granules. Then the samples were re-boiled and filtered through a 0.45 μ m filter (MAISINUO, China). Filtrate (100 μ l)
151 was injected on a tandem column (Ohpak SB-804 HQ, Ohpak SB-806 HQ) using 0.1 M NaNO₃ (0.02% NaN₃) as mobile
152 phase at a flow rate of 0.6 mL/min. The column temperature was set at 50°C. Data obtained from the MALLS and RI
153 detectors were analyzed by ASTRA software version 5.3.4 (Wyatt Technologies).

154 **Rheological Properties of NTS and MTS.** Rheological Properties of NTS and MTS was determined according to our

previous work²¹ and the method reported by van der Maarel et al.¹² with some modifications. Dry starch powder (6%, w/v) was suspended in deionized water, followed by gelatinization at 90°C for 30 min while stirring. The resulting starch solutions were transferred to a disposable petri dish (40 mm diameter) with a plastic wrap covered on the bottom and stored at 4°C for 7 days before the dynamic rheological tests. Dynamic rheological data on NTS and MTS were obtained using a Rheometer (TA Instruments, Waters LLC, USA) using a parallel-plate system (40 mm diameter) at a gap of 1000 μm . Each sample was transferred to the rheometer plate with the plastic wrap at the desired temperature and then the plastic wrap was removed slowly. The surplus sample was wiped off with a spatula. Oscillation amplitude was obtained over an oscillation strain range of 0.1–100% at room temperature (25°C) to detect the linear viscoelastic range. Dynamic shear data were obtained from frequency sweeps over the range of 0.1–100 rad/s in the linear viscoelastic range at room temperature (25°C). The effect of temperature (5–90°C) on storage modulus (G') and loss modulus (G'') was studied under steady increasing speed (10°C/min).

Statistical Analysis. The data were expressed as means of triplicate determinations. The statistical significance was assessed with one-way analysis of variance (ANOVA) using SPSS 20.0 (SPSS Inc., Chicago, USA) for windows program. p value <0.05 was considered to be statistically significant throughout the study.

RESULTS AND DISCUSSION

Sequence and Phylogenetic Analyses of Tu α GT. The target protein sequence of *Thermoproteus uzoniensis* WP_013679179.1 was aligned with known 4 α GT sequences using MEGA6 software (Figure 1A). The Tu α GT sequence shared three conserved regions, three catalytic site key amino acid residues, and a characteristic 250s loop with the enzyme homologues. Asp282 in conserved region I is the putative catalytic nucleophile, Glu323 in conserved region II the proton donor, and Asp382 of the conserved region III is transition state stabilizer.²⁵

Studies have shown that the 250s loop, which is highly conserved in 4 α GTs from GH77, but not in 4 α GTs from GH13 and GH57, is a unique sequence to these 4 α GT.²⁶ The 250s loop forms a lid on the substrate binding groove at the +1, +2

and +3 subsites.^{27, 28} Experiments have shown that the 250s loop has a role in both hydrolysis and transglycosylation activity.²⁷ A BLAST comparison to the gene sequences in the NCBI database revealed that the Tu α GT sequence only possessed up to 64.74% similarity to known 4 α GTs. α -amylase, pullulanase, and 4 α GT from different sources were selected to construct the phylogenetic tree (Figure 1B). The results illustrate that Tu α GT of *Thermoproteus uzoniensis* was more closely related to other 4 α GTs. Together the phylogenetic tree and the BLAST sequence alignment supported the preliminary conclusion that the recombinantly produced enzyme (Tu α GT) was a 4 α GT.

Purification and Characterization of Tu α GT. After heat treatment and Ni-NTA purification, a single protein band corresponding to molecular weight of about 57 kDa was obtained by SDS-PAGE, consistent with 57.9 kDa predicted by ExPasy website (Figure. 2A). As seen from Figure 2B and 2C, optimal pH and reaction temperature of Tu α GT were 6.0 and 75°C. Tu α GT maintained more than 80% activity at 60~80°C, indicating that Tu α GT is a thermophilic enzyme. In Figure 2D is shown that after 4 h of heat treatment Tu α GT maintained > 80% of the activity even at 80°C. The activity, however, decreased significantly at 90°C with a half-life of 38.5 min compared to 690 min at 75°C. When incubated under 100°C for 30 min, Tu α GT completely lost enzyme activity. Tu α GT is a highly thermostable enzyme in agreement with its host strain *Thermoproteus uzoniensis* being alive above 90°C in the crater.²⁹ To determine the influence of metal ions on activity, Tu α GT was incubated with metal ions at 4°C for 1 h (Figure 2E). Cu²⁺, Mg²⁺, and Fe²⁺ significantly reduced the enzyme activity, while Ca²⁺ slightly activated Tu α GT and Mn²⁺, Na⁺, and K⁺ hardly affected the activity of Tu α GT.

Substrate and Reaction Specificity of Tu α GT. To analyze substrate and reaction specificity, the recombinant Tu α GT was incubated with various substrates including glucose, maltose, maltotriose or maltotetraose and the products were analyzed by HPLC. As can be seen from Figure 3 A, no product was formed with glucose as substrate, indicating that Tu α GT was inactive on glucose alone. However, when maltose, maltotriose or maltotetraose were used as substrates, glucose was released by Tu α GT in all cases. Importantly, a series of products with higher degree of polymerization (DP) than of the substrate were formed, demonstrating that Tu α GT has disproportionation activity as reported for other

4 α GTs.³⁰ To determine whether the product of the disproportionation activity consisted of α -1,4 linkages, α -amylase and α -glucosidase were used to hydrolyse the product. The two different enzymes both specifically hydrolyze α -1,4 linkages with formation of glucose when used in combination. As can be seen from Figure 3 B, after the hydrolysis by α -amylase and α -glucosidase, only glucose was detected from the product, confirming that Tu α GT has strong reaction specificity on forming α -1,4 linkage by disproportionation. The smallest substrate of Tu α GT is maltose, which is similar to that reported for 4 α GT from *Thermus aquaticus*.³¹ In conclusion, Tu α GT has strong potential to act on and produce larger maltodextrins and modified starch molecules.

Structure Analysis of the Modified Tapioca Starch (MTS) Compared with Native Tapioca Starch (NTS). To confirm the activity of Tu α GT on starch, tapioca starch as one of the widely used starches was selected as substrate. After the reaction with Tu α GT, the structure of the product was elucidated by determining the chain length distribution, molecular weight and amylose content. The chain length distribution of native tapioca starch (NTS) and modified tapioca starch (MTS) was compared by difference profile values (Figure 4A). As shown previously chains of amylopectin are defined as A, B and C-chains.³² C-chain is the main chain with the reducing end residue and consists of more than 60 glucose residues. B-chains are divided into B₁-, B₂-, and B₃-chains, all being side chains linked to the C-chain via α -1,6-bonds and containing 13–24, 25–36, and more than 37 glucose residues, respectively.³³ A-chains are side chains linked to B-chains which have 6–12 glucose residues. The modification of tapioca starch by Tu α GT had a very important effect on the structural properties. The content of chains in the MTS with DP <10 (A-chains) decreased significantly, and chains with DP >10 (B-chains) showed different degrees of increase in length. The content of chains of DP 10–30 (B-chains) clearly increased. According to the previous studies,¹² this feature indicated that the exterior chains of amylopectin were elongated, this structure therefore would not fit with the regular definition of A, B and C-chains and probably also represent increases in the molecular weight (M_w) of amylopectin as confirmed by the SEC-MALLS-RI analysis (Figure 4B). Before Tu α GT treatment, two typical peaks of amylopectin (Peak 1) and amylose (Peak 2) were observed in NTS, of which Peak 2 (amylose) decreased significantly after the modification, indicating that Tu α GT converted a larger part

of the amylose in the tapioca starch. Accordingly, the elution volume of amylopectin moved from 13 mL to 12.5 mL indicating an increase in M_w and using the ASTRA software. M_w of amylopectin (Peak 1) and amylose (Peak 2) was calculated using the ASTRA software (Table 1). It can be seen that M_w of NTS amylopectin significantly increases from 2.486×10^7 Da to 3.214×10^7 Da in MTS. Besides, the M_w of amylose decreased from 1.642×10^7 Da to 0.692×10^7 Da. These results indicated the transfer of shorter fragments from amylose to amylopectin. However, the significantly decreased M_w of NTS from 2.872×10^7 Da to 2.317×10^7 Da may be related to the cyclization reaction. The amylose degraded was not only transferred to amylopectin, but also forming large-ring cyclodextrin.²⁴ The amylose content was quantified enzymatically and gradually decreased from 23.5% to a low level of about 9% remaining essentially constant after 4 h (Figure 4C) in agreement with the SEC profile (Figure 4B). Combined with the SEC-MALLS-RI data, chain distribution of amylopectin, and results obtained in previous studies, it was concluded that a broader amylopectin cluster with higher M_w was formed by the Tu α GT modification.³⁴

Retrogradation Properties of MTS in Comparison to NTS. The long-term retrogradation of MTS was estimated by DSC (Table 2, Figure 5). The changes in onset temperature (T_o), peak temperature (T_p), conclusion temperature (T_c) and melting enthalpy (ΔH) values reflect the crystallinity and composition of starches, indicating the tightness of recrystallized amylose and amylopectin crystals.³⁵ The T_o , T_p , T_c of MTS showed a significant decrease comparing with that of NTS. (Table 2, Figure 5A), indicating that less hydrogen bonding occurs around the initially generated crystalline structure and a looser crystalline structure was formed which was easier to uncoil, probably due to the reduced amylose content (Figure 4C).³⁶ At the same time the ΔH of NTS increased significantly with increasing storage time, while MTS showed a much lower ΔH compared to NTS. Generally, it is more likely to retrograde for starches which contain more amylose or less branched, longer glucan chains. The content and structure of amylose played a key role in the retrogradation of starch as the crystalline element formed by amylose only is similar to that formed in starch.³⁷ Consequently, after modification by Tu α GT, ΔH values of NTS decreased from 1.482 J/g and 6.817 J/g to 0.851 J/g and 4.274 J/g, respectively (Table 2, Figure 5B) when stored for 1 d and 21 d. Importantly, the ΔH of MTS at 7 d (1.876 ± 0.07 J/g) is almost similar to that of

NTS (1.482 ± 0.04 J/g) at 1 d, which means that the long-term retrogradation of tapioca starch was impaired dramatically after short-term retrogradation. According to the results, the long-term retrogradation problem of the starch gel was partially overcome, indicating that the MTS has the potential to be used in some foods with short shelf-life.

Rheological Properties of MTS Comparing with NTS. On the basis of the success in the reduction of long-term retrogradation, the rheological properties of MTS were compared with those of NTS (Figure 6). The firmness of the gel depends on the extent of linear viscoelastic range.³⁸ In the present study, the strain independence of storage modulus (G') and loss modulus (G'') for all samples, indicated a strain selected for measurement from 0.1% to 5% was within a linear viscoelastic range (Figure 6A). Hence, all the following tests were conducted at 1% strain. Complex viscosity (η^*), defined as the ratio of the shear stress over shear rate obtained from a dynamic test,³⁹ was detected with angular frequency (ω) from 0.1 rad/s to 100 rad/s (Figure 6B). With the increase of angular frequency, the complex viscosity of NTS and MTS gels experienced an extremely similar decrease from nearly 60 Pa·s to 0.35 Pa·s (data not shown). This decrease in complex viscosity is often referred to as shear thinning, which is also understood as a transition from disordered phase to ordered phase.⁴⁰

Changes in the G' and G'' of NTS and MTS solutions determined during heating from 5°C to 90°C were presented in Figure 6C. During the heating from 5°C to 30°C, G' and G'' of both NTS and MTS solutions remained almost identical with G' of NTS gels at nearly 8.7 Pa and G' of MTS gels at nearly 8.3 Pa (Table 3). Also, the higher value of G' than G'' indicates the formation of a “weak-gel”⁴¹ after storage at 4°C for 7 days. Hence, the data above show that the rheological properties of NTS and MTS solutions were highly similar in the investigated temperature range (5–30°C).

With the continuous increase of temperature, a slight reduction was noted with the G' and G'' of NTS solution. By contrast, both G' and G'' of the MTS solution experienced a dramatic decline of G' from 8.01788 Pa at 30°C to 0.98834 Pa at 90°C and G'' from 6.0395 Pa at 30°C to 1.65962 Pa at 90°C. Besides, a specific point of intersection (flow point) was observed between the G' and G'' of MTS solution when the temperature increased up to 65°C, indicating that a transition from weak gel to a true polymer solution happened in the MTS solution.⁴² This transition means that a valuable

thermal-reversibility was observed in the MTS gel comparing with NTS gel. Thermo-reversibility means that the gel maintains gelation status when the temperature is under gelation temperature and return back to solution status when the temperature is above gelation temperature. As is noted above, after the modification by Tu α GT, the amylose fragments were transferred onto non-reducing ends of amylopectin. The degradation of amylose (Figure 4C) leads to decreased amount of double helix formed by amylose which is too strong to be broken. Also, a shorter double helix formed among extended chains of amylopectin can be easily broken compared with the double helix formed by amylose, which possibly explains why the MTS gel was thermo-reversible.⁴³ Moreover, it can be concluded that the temperature of the flow point of the MTS solution is similar to that of gelatinization of granular tapioca starch (67.4°C).⁴⁴ Hence, the MTS can be processed similarly to the native starch although the MTS was prepared after a gelatinization process.

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Conflict of interest

The authors declared that they have no conflicts of interest to this work.

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted, named “A Thermophilic 4- α -Glucanotransferase from *Thermoproteus Uzoniensis* Retards the Long-Term Retrogradation but Maintains the Short-Term Gelation Strength of Tapioca Starch”.

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395

396 **FIGURE CAPTIONS**

397

398 **Figure 1** Bioinformatics analysis of Tu α GT. (A) Comparison of amino acid sequence in three characteristic conserved regions (I, II
399 and III) and the 250s loop of different 4 α GT. The three putative catalytic residues were emphasized in red. 4 α GT from *Thermoproteus*
400 *uzoniensis* (WP_013679179.1, putative) 4 α GT from *Thermanaerotherrix daxensis* (WP_054521162.1, putative), 4 α GT from
401 *Meiothermus ruber* (WP_013013404.1, putative), 4 α GT from *Oceanithermus profundus* (WP_013457929.1, putative), 4 α GT from
402 *Anaerolinea thermolimosa* (WP_062192608.1, putative), 4 α GT from *Thermus filiformis* (AKR04336.1, characterized), 4 α GT from
403 *Eubacterium* sp. CAG:76 (CDF09158.1, characterized), 4 α GT from *Eubacterium* sp. CAG:38 (CDE37197.1, characterized), 4 α GT
404 from *Eubacterium* sp. CAG:192 (CDB13236.1, characterized), 4 α GT from *[Eubacterium] eligens* ATCC 27750 (ACR71403.1,
405 characterized). (B) Phylogenetic tree of 4 α GTs and related enzymes was constructed using the neighbor-joining method (MEGA7.0
406 software) from sequences of the following enzymes: α -amylase from *Salmonella enterica* (GAS16060.1), α -amylase from
407 *Enterobacteriaceae* (WP_042311668.1), pullulanase from *Vibrio cholerae* (AWB75151.1), pullulanase from *Lactobacillus*
408 (OSY79198.1) and 4 α GTs (NCBI number noted above). Tu α GT was marked with a red box.

409

410 **Figure. 2** Enzymatic properties of Tu α GT. (A) SDS-PAGE of the recombinant Tu α GT. Lane M: Molecular mass standard, Lane G1:
411 Purified Tu α GT after a two-step purification of heat treatment at 60°C for 20 min followed by Ni-NTA chromatography; Lane G2:
412 Cellular proteins from crude extract; (B) Effect of pH on activity of Tu α GT. Maximum Tu α GT activity was set to 100%. (C) Effect of
413 temperature on Tu α GT activity. Maximum Tu α GT activity was set to 100%. (D) Thermostability of Tu α GT was determined in the
414 temperature range 60–100°C. (E) The effect of metal ions on the activity of Tu α GT.

415

416 **Figure. 3** Identification of disproportionation activity of Tu α GT. (A) Substrate specificity and product profiles of Tu α GT analyzed by
417 HPLC. Black curve: standard mixture (G1 to G7 represent glucose to maltoheptaose); red curve: reaction mixture using glucose as
418 substrate; blue curve: reaction mixture with maltose as substrate; green curve: reaction mixture with maltotriose as substrate; purple

419 curve: reaction mixture with maltotetraose as substrate. (B) Product produced by Tu α GT hydrolysed by α -amylase and α -glucosidase.
420 Black curve: standard mixture (G1 to G7 represent glucose to maltoheptaose); red curve: product hydrolysed by α -amylase and α -
421 glucosidase using reaction mixture with glucose as substrate of Tu α GT; blue curve: product hydrolysed by α -amylase and α -glucosidase
422 using reaction mixture with maltose as substrate of Tu α GT; green curve: product hydrolysed by α -amylase and α -glucosidase using
423 reaction mixture with maltotriose as substrate of Tu α GT; purple curve: product hydrolysed by α -amylase and α -glucosidase using
424 reaction mixture with maltotetraose as substrate of Tu α GT.

425

426 **Figure. 4** The changes of tapioca starch structure by Tu α GT modification. (A) difference value in chain length distribution using
427 HPAEC-PAD, the bars above zero indicate a decline of the content of chains with this DP in the MTS compared with NTS, the bars
428 below zero indicate an increase of the content of chains with this DP in the MTS compared with NTS. (B) Molecular weight using
429 SEC-MALLS-RI (elution volumes of dextran standards of 8.53×10^6 , 8.53×10^5 , 6.7×10^5 , 5×10^4 , 2.5×10^4 Da are shown as black dots),
430 (C) The amylose content during the modification of tapioca starch determined using the Megazyme K-AMYL kit, and (D) a possible
431 reaction mechanism of 4 α GT on amylose and amylopectin.

432

433 **Figure. 5** Retrogradation properties of NTS and MTS. (A) Retrogradation thermograms of NTS and MTS with different storage time.
434 (B) Melting enthalpy (ΔH) of NTS (black solid column) and MTS (white open column) with different storage time.

435

436 **Figure. 6** Comparison of rheological properties of MTS and NTS. (A) detecting the zone of linear viscoelasticity of NTS and MTS
437 with strain from 0.1% to 100%, (B) detecting the frequency dependence of complex viscosity (η^*) for NTS and MTS with angular
438 frequency (ω) from 0.1 rad/s to 100 rad/s, and (C) detecting the temperature dependence of G' , G'' for NTS and MTS with temperature
439 from 5°C to 90°C.

440

441 **Table 1** Weight-average molecular weight of tapioca starch during modification

442 ^a Tests were performed in triplicate. Mean $\pm$ standard deviation values in the same column for each sample followed by different letters
 443 are significantly different ($p < 0.05$) by Duncan's test.

Sample	$M_w \times 10^7$ (g/mol)		
	Peak 1 (amylopectin)	Peak 2 (amylose)	Peak 1 + Peak2
NTS	$2.486 \pm 0.191b$	$1.642 \pm 0.105a$	$2.872 \pm 0.281a$
MTS-1 h	$2.749 \pm 0.162b$	$1.331 \pm 0.149b$	$2.716 \pm 0.147ab$
MTS -8 h	$3.214 \pm 0.213a$	$0.692 \pm 0.092c$	$2.317 \pm 0.172b$

444

Table 2 The retrogradation properties (onset temperature (To), peak temperature (Tp), conclusion temperature (Tc) and melting enthalpy (ΔH)) of NTS and MTS stored from 1 d to 21 d measured by DSC

^a Tests were performed in triplicate. Mean $\pm$ standard deviation values in the same column for each sample followed by different letters are significantly different ($p < 0.05$) by Duncan's test.

Sample	T _o (°C)	T _p (°C)	T _c (°C)	ΔH (J/g)
NTS-1 d	49.87 $\pm$ 0.01a	51.43 $\pm$ 0.02b	62.92 $\pm$ 0.04c	1.482 $\pm$ 0.04g
MTS-1 d	48.56 $\pm$ 0.04b	50.97 $\pm$ 0.01d	61.04 $\pm$ 0.05d	0.851 $\pm$ 0.07h
NTS-7 d	48.14 $\pm$ 0.12c	51.99 $\pm$ 0.07a	64.21 $\pm$ 0.021a	2.641 $\pm$ 0.17e
MTS-7 d	46.39 $\pm$ 0.09d	50.12 $\pm$ 0.04e	63.41 $\pm$ 0.08b	1.876 $\pm$ 0.07f
NTS-14 d	44.19 $\pm$ 0.06e	51.43 $\pm$ 0.11b	64.17 $\pm$ 0.18a	4.892 $\pm$ 0.06b
MTS-14 d	42.27 $\pm$ 0.07g	50.86 $\pm$ 0.04d	63.34 $\pm$ 0.04b	3.162 $\pm$ 0.01d
NTS-21 d	43.96 $\pm$ 0.01f	52.14 $\pm$ 0.09a	62.98 $\pm$ 0.06c	6.817 $\pm$ 0.07a
MTS-21 d	41.14 $\pm$ 0.04h	51.22 $\pm$ 0.15c	60.12 $\pm$ 0.02e	4.274 $\pm$ 0.12c

Table 3 The storage modulus (G') and loss modulus (G'') of NTS and MTS at temperature ramp from 5°C to 90°C

^a Tests were performed in triplicate. Mean $\pm$ standard deviation values in the same column for each sample followed by different letters are significantly different ($p < 0.05$) by Duncan's test.

Temperature (°C)	G' -NTS (Pa)	G'' -NTS (Pa)	G' -MTS (Pa)	G'' -MTS (Pa)
5	8.77 $\pm$ 0.2a	5.57 $\pm$ 0.14a	8.32 $\pm$ 0.21a	6.16 $\pm$ 0.23a
10	8.67 $\pm$ 0.22a	5.49 $\pm$ 0.23a	8.32 $\pm$ 0.19a	6.16 $\pm$ 0.22a
15	8.65 $\pm$ 0.18a	5.48 $\pm$ 0.22a	8.31 $\pm$ 0.12a	6.15 $\pm$ 0.16a
20	8.64 $\pm$ 0.19a	5.44 $\pm$ 0.16ab	8.28 $\pm$ 0.14a	6.07 $\pm$ 0.12ab
25	8.61 $\pm$ 0.17a	5.42 $\pm$ 0.12ab	8.27 $\pm$ 0.23a	6.07 $\pm$ 0.02ab
30	8.57 $\pm$ 0.14a	5.35 $\pm$ 0.02ab	8.01 $\pm$ 0.22a	6.04 $\pm$ 0.12ab
35	8.56 $\pm$ 0.22a	5.27 $\pm$ 0.12ab	7.5 $\pm$ 0.18b	5.72 $\pm$ 0.11b
40	8.47 $\pm$ 0.21a	5.18 $\pm$ 0.11abc	6.99 $\pm$ 0.19c	5.36 $\pm$ 0.14c
45	8.33 $\pm$ 0.19ab	5.07 $\pm$ 0.2abcd	6.34 $\pm$ 0.17d	4.89 $\pm$ 0.23d
50	8.14 $\pm$ 0.12b	4.96 $\pm$ 0.22bcd	5.49 $\pm$ 0.14e	4.51 $\pm$ 0.22e
55	7.9 $\pm$ 0.14bc	4.86 $\pm$ 0.18cde	4.67 $\pm$ 0.22f	3.95 $\pm$ 0.18f
60	7.7 $\pm$ 0.23c	4.74 $\pm$ 0.14de	3.74 $\pm$ 0.12g	3.64 $\pm$ 0.19f
65	7.48 $\pm$ 0.13cd	4.59 $\pm$ 0.22e	2.98 $\pm$ 0.23h	3.21 $\pm$ 0.22g
70	7.31 $\pm$ 0.16de	4.59 $\pm$ 0.21e	2.22 $\pm$ 0.12i	2.74 $\pm$ 0.18h
75	7.21 $\pm$ 0.12de	4.49 $\pm$ 0.19e	1.71 $\pm$ 0.06j	2.4 $\pm$ 0.14i
80	7.1 $\pm$ 0.12def	4.52 $\pm$ 0.12e	1.25 $\pm$ 0.06k	2.07 $\pm$ 0.09ij
85	6.95 $\pm$ 0.11ef	4.53 $\pm$ 0.14e	1.08 $\pm$ 0.05k	1.79 $\pm$ 0.08jk
90	6.83 $\pm$ 0.12f	4.5 $\pm$ 0.12e	0.99 $\pm$ 0.06k	1.66 $\pm$ 0.09k

Figure 1 Bioinformatics analysis of TuαGT.

Figure. 2 Enzymatic properties of TuαGT.

Figure. 3 Identification of disproportion activity of TuαGT.

Figure. 4 The changes of tapioca starch structure by TuαGT modification.

Figure. 5 Retrogradation properties of NTS and MTS.

Figure. 6 Comparison of rheological properties of MTS and NTS.

472

TOC graphic

473

474

475 Highlights

- 476 1. A novel thermophilic 4- α -glucanotransferase was characterized.
- 477 2. The structure of tapioca starch was dramatically modified by Tu α GT.
- 478 3. The short-term gelation ability and gel strength of tapioca starch was maintained after modification.
- 479 4. Long-term retrogradation of modified starch was partially retarded.