Complex formation of a 4- α -glucanotransferase using starch as a biocatalyst for starch modification

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Abstract A 4- α -glucanotransferases from *Thermus thermophilus* (TT α GT) possesses an extra substrate binding site, leading to facile purification of the intact enzyme using amylose as an insoluble binding matrix. Due to the cost of amylose and low recovery yield, starch was replaced for amylose as an alternative capturer in this study. Using gelatinized corn starch at pH 9 with 36-h incubation in the presence of 1 M ammonium sulfate increased the TT α GT-starch complex formation yield from 2 to 56%. In preparative-scale production, TT α GT produced in *Bacillus subtilis* was recovered by 42.1% with the same specific activity as that of purified TT α GT. Structural and rheological analyses of the enzymatically modified starches revealed that the starch complex exhibited catalytic performance comparable to soluble TT α GT, suggesting that the starch complex can be used as a biocatalyst

for modified starch production without elution of the enzyme from the complex.

Keywords Complex formation · 4- α -glucanotransferase · Optimization · Purification · Starch modification

Introduction

4- α -Glucanotransferases (α GTases; EC 2.4.1.25), also known as amylomaltases, play important roles in maltose/maltodextrin metabolism [1]. α GTases elongate short maltooligosaccharides, including maltose, maltotriose, etc., by disproportionation to transfer a linear maltooligosaccharide to the non-reducing end of another maltooligosaccharide through α -1,4-glucosidic linkage [2]. In the presence of small maltooligosaccharides as sugar acceptors, therefore, α GTases can degrade starch and produce a series of maltooligosaccharides. In contrast, in the absence of sugar acceptors, α GTases hydrolyze amylopectin into relatively small amylopectin clusters, followed by changes in the lengths of side chains by disproportionation activity using amylose as a sugar donor [3]. The α GTase-treated starch shows different rheological and functional properties from native starch [4–6], facilitating the generation of novel modified starches as food ingredients [7, 8] as well as direct application to starch-based foods [9]. In addition, α GTases catalyze the formation of cycloamyloses with degrees of polymerization (DPs) ranging from 15 to 55 [10, 11]. As highly water-soluble cycloamyloses have a hydrophobic interior (e.g. cyclodextrins), there have been attempts to encapsulate hydrophobic pharmaceuticals and food ingredients using cycloamyloses as an alternative to cyclodextrins [12–14].

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Several safety issues must be addressed for the use of recombinant enzymes as food-grade enzymes. To resolve the safety problems, our group developed expression systems for *Bacillus subtilis*, which is a Generally Recognized As Safe (GRAS)-grade host [15]. Using an *Escherichia coli*–*B. subtilis* shuttle vector equipped with a strong promoter, a thermostable α GTase from *Thermus thermophilus* (TT α GT) was successfully produced in *B. subtilis* [16]. Because α GTases are originally cytoplasmic proteins, the recombinant TT α GT in *B. subtilis* existed in the cytoplasm. As extra peptidyl tag-fused recombinant enzymes cannot be allowed for food-grade enzymes, another purification method for intact TT α GT was requested. Given structural and ligand-enzyme binding analyses for a α GTase from *Thermus aquaticus* [17], α GTases possess extra substrate binding sites which is called surface binding site. The surface binding site of TT α GT assisted specific binding on amylose as an insoluble polymeric substrate in the absence of small maltooligosaccharides, leading to facile recovery of the resulting enzyme–substrate complex by centrifugation [18]. Although the direct affinity capture method using amylose was simple and efficient, the high price of amylose as well as the low recovery yield are drawbacks for industrial applications.

Here, starch was replaced for amylose as alternative direct capturer, but no formation of TT α GT observed. Through gelatinization of starch and optimization of the condition for complex formation, TT α GT–starch complex was prepared using the enzyme produced in *B. subtilis* on preparative scale. The structural and rheological characteristics of starches modified by free TT α GT and its complexes with either amylose or starch were investigated to compare the catalytic properties of the TT α GT–starch complex.

Materials and methods

General experiments

E. coli MC1061 [F^- , *araD139*, *recA13*, Δ (*araABC-leu*)7696, *galU*, *galK*, *lacX74*, *rpsL*, *thi*, *hsdR2*, *mcrB*] was used as the host for DNA manipulation and protein production. The recombinant *E. coli* cells were cultured overnight at 37 °C in Luria–Bertani broth [1% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, and 0.5% (w/v) NaCl] supplemented with kanamycin (20 μ g/mL). Production of the codon-optimized TT α GT (previously termed TT α GT-mut6) was carried out as described previously [16]. The protein concentration was determined according to the method of Bradford [19], using bovine serum albumin as a standard. The activity of TT α GT in solution was measured as described previously [16]. One unit of TT α GT was

defined as the amount of enzyme that degraded 1 mg/mL of amylose per minute. The activity and protein amount of the complexes were measured by subtracting those in the supernatant from those in the initial enzyme solution. The specific activity of the complex was also calculated based on the expected activity and protein amount of the complex.

Exploration of factors affecting TT α GT–starch complex formation

Upon optimizing the factors for complex formation, TT α GT purified by Ni–NTA affinity chromatography [16] was used to simplify calculation of the yield of complex formation. Starches from different cereals (rice, corn, potato, and waxy corn) and high-amylose corn starch, which contains approximately 55% amylose (Hylon-V starch; National Starch Food Innovation, Monroe, NJ, USA), were tested. After gelatinization in a boiling water bath for 30 min, five volumes of ethanol were added to the paste, followed by centrifugation at 12,000 rpm for 15 min to precipitate gelatinized starches. To facilitate complete drying, the gelatinized starch was washed with acetone to remove ethanol, followed by recovery by centrifugation and air-drying at room temperature. The complex was made using 15 mg of starch and 0.42 mg/mL of TT α GT in 0.5 mL of 50 mM Tris–HCl (pH 8.0) at 4 °C on a rocker (CR-95; FINEPCR, Gunpo, Korea) for an incubation period ranging from 6 to 48 h. After fixing the incubation time, an optimum buffer was chosen from among 50 mM Tris–HCl buffer (pH 7.0–8.0), 50 mM borate–NaOH (pH 9.0), and 50 mM glycine–NaOH (pH 9.0–10.0). Following investigation of the effect of salt, complex formation was conducted in the optimum buffer containing a salt of $MgSO_4$, $(NH_4)_2SO_4$, KCl, NaCl, and $CaCl_2$ at either 0.5 or 1 M. After centrifugation at 10,000 $\times$ g, 4 °C for 5 min, the total amount of TT α GT in the supernatant was measured by the Bradford method, and the yield of complex formation was calculated by the following equation:

$$\text{Yield of complex formation (\%)} = \frac{A_0 - A}{A_0} \times 100$$

where A_0 is the amount of TT α GT used for complex formation and A is the amount of TT α GT in the supernatant.

Preparative-scale preparation of TT α GT–starch complex

To prepare TT α GT–starch complex on a preparative scale TT α GT produced by a recombinant *B. subtilis* system was used [16]. The recombinant *B. subtilis* containing pUB–RTA–TT α GTmut6 was cultured in LB medium with 30 μ g/mL kanamycin as a selective marker for 24 h at

37 °C. The cell pellet was resuspended in 50 mM glycine–NaOH (pH 9.0) with 0.3 M ammonium sulfate. The cell extract was obtained by sonication, followed by heat treatment at 70 °C for 20 min. Then, additional ammonium sulfate (final 1.0 M) was added to the cell extract. Aliquots of 300 mL of heat-treated enzyme solution (30 U/mL) were mixed with 9 g of gelatinized corn starch powder, followed by incubation at 4 °C for 36 h. After collecting the complex by centrifugation (10,000 $\times$ g, 4 °C for 5 min), the complex pellet was washed with 600 mL of 10 mM Tris–HCl buffer (pH 8.0) three times. The resulting complex was lyophilized (PVTFD 10R; Ilsin Lab. Co., Daejeon, Korea) and used for further characterization.

Modification of starch by TT α GT

To investigate the catalytic properties of TT α GT–starch complex, modified starches were prepared using free enzyme, TT α GT–amylose complex, and TT α GT–starch complex. One of the enzymes was added to 100 mL of a 10% (wt/v) slurry of corn starch (Samyang Genex, Daejeon, Korea) in distilled water. The final TT α GT dose was either 5 or 10 U per gram of starch. After a 120-h reaction at 70 °C, the mixture was boiled for 30 min to terminate the reaction. The modified starch product was precipitated using five volumes of 100% ethanol, and the precipitate was recovered by centrifugation at 10,000 $\times$ g for 15 min. The precipitate was washed with three volumes of acetone three times, followed by air-drying at room temperature.

High-performance size-exclusion chromatography analysis of modified starch

The molecular weight distribution of the air-dried modified starch was analyzed using high-performance size-exclusion chromatography (HPSEC). The sample (50 mg) was dissolved in 1 mL of boiling water by stirring for 30 min in a boiling water bath. The samples were filtered using a 5.0- μ m disposable membrane filter (Sigma-Aldrich, St. Louis, MO, USA) and injected into a high-pressure liquid chromatograph (HPLC) equipped with a pump (Prostar 210; Varian, Inc., Palo Alto, CA, USA) a differential refractive index detector (Prostar 355; Varian), and two SEC columns (OHpak SB-806 HQ, 8.0 mm $\times$ 300 mm and OHpak SB-804 HQ, 8.0 mm $\times$ 300 mm; Tosoh Co., Tokyo, Japan). The flow rate of the mobile phase (50 mM NaNO₃) was constant at 0.4 mL/min. The molecular weights of starches were estimated using pullulan hydrolysates (Sigma-Aldrich) as the standards.

Analysis of modified starch side chain distribution

The starch samples (10 mg) were dissolved in 50 mM sodium acetate buffer (1 mL, pH 4.5) and further treated with 1 U/mg of isoamylase (Megazyme, Chicago, IL, USA) at 40 °C for 2 h. The enzyme reactions were then terminated by boiling for 15 min, and the reaction mixtures were filtered using 0.45- μ m disposable membrane filters. The composition of the starch branched chains was analyzed using a high-performance anion-exchange chromatography (HPAEC) system (DX500; Dionex, Sunnyvale, CA, USA) equipped with a pulsed amperometric detector (ED40; Dionex). The filtrate (200 μ L) was injected and analyzed using a CarboPac PA1 analytical column (4 $\times$ 250 mm). The eluents, A and B, were 150 mM sodium hydroxide and 150 mM sodium hydroxide in 600 mM sodium acetate solution, respectively. The eluent gradients were operated at a flow rate of 1 mL/min.

Texture profile analysis of starch gel using texture analyzer

The starch gels were characterized by measuring the behavior of the samples when compressed to a large degree of deformation. Texture profile analysis (TPA) was performed using a texture analyzer (TA-XT2i; Stable Microsystems, Surrey, UK), fitted with a cylindrical aluminum probe (diameter, 20 mm). Cylindrical gel samples (35 mm diameter $\times$ 10 mm height) were compressed twice at a constant speed of 1 mm/s to 90% of the initial height of the samples and 70% strain. Textural parameters were obtained from the resulting force–deformation curves. Textural parameters were calculated from TPA graphs using the Texture Expert software. Experimental data were analyzed by one-way analysis of variance (ANOVA) and Tukey's test, and were used to establish the significance of differences in color measurements. Statistical analysis was performed using Minitab version 17.0 for Windows (Minitab Inc., State College, PA, USA).

Analysis of gel properties using a rheometer

The gelation kinetics were measured by a dynamic rheological test. Freshly made samples were loaded between 20-mm parallel plates in a rheometer (AR1500ex; TA Instruments Ltd., New Castle, DE, USA). Dynamic rheological properties were measured at 1% strain and a frequency of 1 Hz. To determine the experimental gelation kinetics, the storage modulus (G') of the modified starch pastes was measured continuously at 4 °C as a function of time over a period of about 10 h.

Results and discussion

Optimization of TT α GT–starch complex formation

To investigate the binding affinity of TT α GT to starch, five raw starches—corn, wheat, potato, waxy, and a high-amylose starch (Hylon-V)—were tested. Although incubation for 2 h was sufficient for the formation of a complex with amylose [18], there was no successful binding of TT α GT on raw starches for 12 h of incubation. Based on the remaining enzyme activities in the supernatants, the binding yields were <3% of the TT α GT included in the experiment (Fig. 1A). Gelatinization of starches improved the binding yields of TT α GT (Fig. 1B). Ethanol precipitation was better than freeze-drying as a method for recovery of gelatinized starch; the binding yields were 10.55 and 5.04%, respectively (Fig. 1B). Hylon V with the highest content of amylose (ca. 55%) was shown to be the best starch for complex formation, followed by corn starch (Fig. 1C). Due to the high cost of Hylon V, gelatinized corn starch was chosen as the matrix for the TT α GT–starch complex.

Although a 2-h incubation is sufficient when using amylose for complex formation, it seemed that a longer incubation time was needed to reach equilibrium for the binding of TT α GT to starch. The recovery yields continued

to increase in a time-dependent manner until 36 h (Fig. 1D). As the difference between yields at 36 and 48 h was negligible, the optimum incubation time was determined as 36 h.

With the exception of the borate–NaOH buffer, the buffers at pH 9.0 exhibited higher yields than the other buffer solutions (Fig. 1E). Of the buffers at pH 9.0, glycine–NaOH buffer (yield, 29.84%) was chosen as the optimum buffer for complex formation. The reasons for the improved binding at pH 9.0 and lack of improvement by the borate–NaOH buffer at pH 9.0 were unclear.

Next, we investigated the effects of salt on complex formation. At concentrations of the test salts above 1 M, TT α GT was markedly precipitated (data not shown). Therefore, the experiments were conducted at 0.5 and 1 M MgSO₄, (NH₄)₂SO₄, CaCl₂, KCl, and NaCl. After calculating the amount of enzyme bound on the complex, the amounts of precipitated proteins at the tested concentration (usually <10% of the enzyme used) and the remaining TT α GT in the supernatants after complex formation were determined and subtracted from the original amount of TT α GT used for binding. Of the salts tested, the divalent salts [MgSO₄ and (NH₄)₂SO₄] showed improvement of the yields, and a salt concentration of 1 M was better than 0.5 M (Fig. 1F). Therefore, 1 M (NH₄)₂SO₄ was chosen as the best salt concentration. The incubation conditions were

Fig. 1 Optimization of condition for formation of TT α GT–starch complex. (A) Complex formation yields for different raw starches; (B) complex formation yields for drying method after gelatinization of corn starch; (C) complex formation yields for different starches after gelatinization, followed by ethanol precipitation; (D) effect of

incubation time in 50 mM Tris–HCl buffer/pH 8.0; (E) effect of buffers (white bars, 50 mM Tris–HCl; black bars, 50 mM Glycine–NaOH); (F) effect of salts in 50 mM Glycine–NaOH buffer/pH 9.0 (lined bar, no salt; white bars, 0.5 M of each salt; gray bars, 1 M of each salt) on the complex formation yields

fixed at 50 mM glycine–NaOH buffer (pH 9.0) containing 1 M $(\text{NH}_4)_2\text{SO}_4$ for 36 h at 4 °C.

Preparative-scale production of TT α GT–starch complex

When the complex was prepared on a large scale for further experiments, TT α GT produced in recombinant *B. subtilis* was used [16]. In order to remove heat-labile proteins from *B. subtilis*, heat-treatment of the cell extract carried out at 70 °C, pH 7 for 20 min that was determined previously [16]. Unexpectedly, the heat treatment had no effect at pH 9.0, which is the optimum pH for complex formation (data not shown). To improve the heat treatment effect, the cell extracts were prepared using glycine–NaOH buffer (pH 9) with different concentrations of ammonium sulfate. Among the conditions tested, heat treatment of the cell extract in glycine–NaOH buffer (pH 9) with 300 mM ammonium sulfate showed the best performance, and the heat-treated cell extract exhibited specific activity similar to that at pH 7 (73.12 and 72.07 U/mg, respectively, Table S1 in supplementary information). At ammonium sulfate concentration >300 mM, severe reduction of the recovery yield of TT α GT was observed due to co-precipitation during heat treatment (data not shown). The recovery yield of the heat-treatment of TT α GT was 71% that was lower than the expected because TT α GT is stable under the condition of the heat-treatment (half-life at 70 °C = 265 min) [18]. The reason for the low yield would be the heat inactivation of amylose-degrading enzymes from the host cell, such as a branching enzyme [20] and a α GTase [21]. Indeed, there was amylose-degrading activity in the cell extract of the host cell, but no detectable activity after the heat-treatment at pH 9 in the presence of 300 mM ammonium sulfate (Data not shown).

To determine the ratio of TT α GT to starch, the heat-treated cell extract was concentrated by ultrafiltration, followed by serial dilution with the same buffer. Then, ammonium sulfate was added to the samples to adjust the ammonium sulfate concentration to 1 M. The total activity of the complex increased over the amount of TT α GT added, up to 30.1 U for 15 mg of starch, whereas the recovery yield decreased from 52.2 to 29.6% over the amount of TT α GT added (Fig. 2), which was consistent with results reported previously for complex formation using amylose [18]. As starch in the complex will be used as the substrate to produce modified starch, the yield is more important than the activity per starch. Therefore, the dosage of the enzyme for starch complex formation was determined at 50% of the recovery yield: 15.26 U of TT α GT for 15 mg of starch.

To obtain the complex at preparative scale, the reaction conditions were increased by 600-fold (9 g of gelatinized

Fig. 2 Effect of TT α GT dosage on formation of TT α GT–starch complex. The complex formation was conducted at the optimal condition; 50 mM Glycine–NaOH buffer (pH 9) containing 1 M ammonium sulfate. Total bound TT α GT in the complex, the complex formation yield, and specific activity of the complex were represented by closed circles, open circles, and open triangles, respectively

starch and 300 mL of heat-treated TT α GT solution with 30.52 U/mL and 71.01 U/mg of protein), resulting in a complex formation yield of 46.5% based on the heat-treated cell extract, which can be translated to 33.2% based on the cell extract (Table S1). The activity (U per mg of complex) and the specific activity (U per mg of proteins on the complex) of the complex were 0.47 U/mg of complex and 119.5 U/mg of protein, respectively. Notably, the specific activity and the purity of TT α GT in the complex were almost same as that of TT α GT purified by Ni–NTA chromatography (Table S1 and Fig. S1).

Secondary binding was performed to recover TT α GT in the supernatant. Due to the influence of the TT α GT concentration on complex formation yield, the supernatant was concentrated by ultrafiltration. Unexpectedly, we lost almost half of the enzyme during concentration. The same ratio of gelatinized corn starch (2.4 g of starch) to the volume of concentrated supernatant solution (80 mL and 30.84 U/mL) was then added. The activity, specific activity, and complex formation yield of the secondary complex (0.47 U/mg of complex, 133.2 U/mg of protein, and 46.2% based on the concentrated supernatant, respectively) were almost the same as those of the primary complex (Table S1). Consequently, the final recovery yield was 42% based on the cell extract through binding twice.

Molecular weight changes of modified starch by free TT α GT and the complexes

To compare the catalytic performance for starch modification, corn starch paste was treated with free enzyme, TT α GT–amylose complex, and TT α GT–starch complex

Fig. 3 Molecular weight changes of enzyme-treated starches. The samples were taken at 1 h (solid lines) and 120 h (dashed lines). (A) Free TT α GT of 5 U per gram starch; (B) TT α GT-starch complex of 5 U per gram starch; (C) TT α GT-amylose complex of 10 U per gram starch

Fig. 4 Side chain distribution profiles of enzyme-treated starches. Black bars for no enzyme treatment; gray bars for 5 U of TT α GT-starch complex per gram starch; dark gray bars for 10 U of TT α GT-starch complex per gram starch; light gray bar for free TT α GT of 5 U of complex per gram starch

(5 U/g starch). After a 120-h reaction with enzymes, the changes in the molecular weight distribution of the enzyme-treated corn starches were analyzed by size exclusion chromatography. In the case of treatment of free enzyme, the peak representing amylopectin was shifted to the late elution time, suggesting that the massive amylopectin was hydrolyzed to smaller amylopectin clusters ranging from 2.12×10^5 to 4.72×10^4 Da (Fig. 3A). In the case of TT α GT-amylose complex (10 U/g starch), the amylopectin peak moved slightly, implying that the huge amylopectin structure was still maintained (Fig. 3B). In contrast, the molecular weight distribution profile of the starch modified by TT α GT-starch complex (10 U/g starch) was nearly the same as that by free TT α GT (Fig. 3C). These results suggest that the performance of the starch complex was better than the amylose complex, and as good as the free enzyme. As the performance of the TT α GT-amylose complex was not comparable with the free enzyme, the amylose complex was discarded in further experiments.

Change in side chain distribution of the modified starches

In HPAEC analysis of the branched chain length distributions of the native starch (black bars in Fig. 4), very short

side chains ($DP \leq 7$) were rare, and short side chains ($8 \leq DP \leq 10$) were present at a low ratio (<2%). Medium side chains ($11 \leq DP \leq 30$) comprised the majority of the side chains, and the ratios of the long side chains ($DP \geq 30$) were less than 2%. The TT α GT-treatment yielded the typical changes in the side-chain distribution pattern of starch by intra- and intermolecular disproportionation by the enzymes [22]: markedly increased populations of the very short side chains and nearly doubling of the long side chains ($DP \geq 35$), as well as reduced populations of the intermediate chains ($11 \leq DP \leq 30$) (Fig. 4). The starch treated with 5 U of the complex possessed the smaller populations of the very short ($DP < 6$) and the very long ($DP > 40$) side chains and the higher populations of the side chains with DP ranging from 11 to 19 than the others (Fig. 4). These results suggested that the complex exhibited less efficient catalytic power compared to the same unit of free TT α GT.

Texture profile analysis of starch gels made of modified starches

TPA was performed to determine the textural properties of the starch gels made of native and modified starches. The gels made of 10% [w/v] native starch or modified starch pastes were prepared at 4 °C overnight. After enzyme

Table 1 Textural properties of native and TT α GT-modified starch gels

Enzyme	Hardness	Adhesiveness	Springiness	Cohesiveness	Gumminess	Chewiness	Resilience
No enzyme	560.87 $\pm$ 71.18 ^a	19.21 $\pm$ 4.73 ^a	0.91 $\pm$ 0.02 ^a	0.24 $\pm$ 0.02 ^a	133.40 $\pm$ 17.58 ^a	121.31 $\pm$ 18.51 ^a	0.06 $\pm$ 0.00 ^a
Complex (5 U/g starch)	23.79 $\pm$ 0.75 ^b	17.18 $\pm$ 3.41 ^a	0.29 $\pm$ 0.07 ^c	0.08 $\pm$ 0.01 ^c	1.80 $\pm$ 0.16 ^b	0.52 $\pm$ 0.17 ^b	0.02 $\pm$ 0.00 ^b
Complex (10 U/g starch)	25.18 $\pm$ 0.81 ^b	34.73 $\pm$ 2.18 ^b	0.30 $\pm$ 0.02 ^{bc}	0.09 $\pm$ 0.01 ^{bc}	2.27 $\pm$ 0.17 ^b	0.68 $\pm$ 0.08 ^b	0.02 $\pm$ 0.00 ^b
Free TT α GT (5 U/g starch)	21.84 $\pm$ 2.76 ^b	36.45 $\pm$ 9.70 ^b	0.41 $\pm$ 0.07 ^b	0.12 $\pm$ 0.02 ^b	2.54 $\pm$ 0.67 ^b	1.08 $\pm$ 0.47 ^b	0.02 $\pm$ 0.00 ^b

Values followed by the same letter in the same textural property are not significantly different ($p < 0.05$)

Fig. 5 Gelation kinetics of starches modified by TT α GT-starch complex of 5 U per gram starch (**a**), TT α GT-starch complex of 10 U per gram starch (**b**), and free TT α GT of 5 U per gram starch (**c**); (**B**) magnified graph of (**A**) over time to 40 min

treatment, the hardness of the modified starches markedly decreased without no significant differences, resulting in the same situations in gumminess (hardness $\times$ cohesiveness) and chewiness (hardness $\times$ cohesiveness $\times$ springiness) that are mostly affected by the hardness (Table 1). In the case

of the other parameters of TPA (adhesiveness, springiness, and cohesiveness), the starch treated with 5 U of the complex were significantly different, albeit not so much, from those with 5 U of the free enzyme. The starch modified by 10 U of the complex displayed the intermediate values for these TPA parameters, but not significantly compared to the starches by 5 U of the complex or the free enzyme. These results suggested that the double dose of the complex yielded modified starch as free TT α GT did.

Effects of TT α GT treatment on starch gel formation

The gelation kinetics of hydrocolloid solutions can be analyzed by monitoring the changes in shear modulus at a constant frequency and temperature [23–25]. To monitor the changes in shear modulus at a constant frequency and temperature, the storage moduli (G') of 10% starch paste treated with soluble TT α GT and the starch complex were monitored as a function of time for 20 h at 4 °C immediately after the enzyme reaction. Regardless of type and amount of enzyme, all starch pastes showed a period of exponential G' increase (a, b, and c in Fig. 5). Trigger points for exponential G' increase depending on the enzyme treatment level and the final G' are inversely proportional to the amount of TT α GT used [5]. Indeed, the gelation of starch pastes by 10 U of the complex yielded a lower final G' value (a in Fig. 5A) and a delayed trigger point for exponential G' (a in Fig. 5B) than that by 5 U of the complex (b in Fig. 5A, B). Although free TT α GT (5 U) showed an intermediate trigger point (17 min, c in Fig. 5B) compared to 5 and 10 U of the starch complex (23 and 13 min of the trigger points, respectively), the overall profile for the starch modified by 10 U of the complex was similar to that by 5 U of free TT α GT.

In conclusion, the destruction of the crystalline starch by gelatinization as well as the addition of divalent salts improved the binding yield of the enzyme. Also, the optimum pH for binding was not the optimum pH for the catalysis by the enzyme. It would therefore be interesting

to carry attempts to apply these factors for recovery of proteins and enzymes with binding affinity to other glycans, such as cellulose, chitin and so on. Although TT α GT is simply purified by the complex formation with starch and the complex can be used as a biocatalyst for starch modification, the reduced catalytic performance of the complex and the moderate recovery yield are still challenging. Also, this process needs several chemicals that would be harmful for human health. Therefore, the process for complete removal of these reagents should be added for application of TT α GT-starch complex in industrial fields.

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