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Effects of α-glucanotransferase treatment on the thermo-reversibility and freeze-thaw stability of a rice starch gel

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Abstract

This study was designed to investigate the effect of a thermostable α-glucanotransferase (TSαGT) originated from *Thermus scotoductus* on the rheological properties, molecular weight distribution, thermo-reversibility, and freeze-thaw stability of rice starch paste. Rice starch paste samples (5%) were incubated at 70 °C with TSαGT and a commercial α-amylase, respectively, until reduction of moduli leveled off. The TSαGT-modified rice starch paste showed a yield stress. After the enzymatic treatment, both enzyme-treated pastes showed considerably low moduli with higher G'' than G', indicating a significant liquefaction. However, when stored at 4 °C, both moduli of a TSαGT-treated sample dramatically increased with significantly higher G' than G'', confirming that a solid gel structure formed. This transition was found to be reversible, when temperature fluctuated between 70 and 4 °C. The control starch gel did not show thermo-reversibility, and α-amylase-treated starch paste was incapable of forming a solid gel. The size distribution of the starch polymers, as measured by MALLS, revealed that average M_w reduced to about 5×10^5 after the TSαGT treatment, which was a significant decrease, but possibly high enough to form a solid gel matrix at low temperatures. The TsαGT-treated gel demonstrated highly improved freeze-thaw stability.

Keywords: 4-α-Glucanotransferase; Rice starch; Thermo-reversibility; Freeze-thaw stability

1. Introduction

Starch, in both its native and modified forms, is widely used in food processing and preparation. It can yield viscous dispersions, solutions, or gels, depending on concentration and temperature conditions (Rosalina & Bhattacharya, 2002). However, in the food industry the use of native starches is limited by their lack of stability under the conditions of temperature, shear, pH and refrigeration commonly applied to processed foods (Liu, Ramsden, & Corke, 1999). Furthermore, viscosities of native starch gels are often too high for industrial application (Rosalina & Bhattacharya, 2002). Recently, research has been conducted to improve the solubility and retrogradation of starches by reducing their molecular weight through chemical, physical, or enzymatic treatments (Becktel, 1959; Carrol, Boyce, Wong, & Starace, 1987; Hebeda et al., 1990). These treatments have been partly successful in solving the problems mentioned above, however, it is difficult to prevent excessive reduction in the molecular weight of starch, thus losing its inherent properties (Park, 2004). Recent studies indicate that the modification of synthetic and natural polymers with an enzyme is an environmentally friendly alternative to chemical methods using harsh conditions (Brandam, Mayer, Proth, Strehaiano, & Piaguad, 2003; Hamdi & Ponchel, 1999). Recently, genetic and enzymatic approaches for the enhancement of food functionalities have been carried out (Alexander, 1996; Davis, Supatcharee, Khandelwal, & Chibbar, 2003). The functional properties of modified starch produced by various carbohydrate enzymes are of great interest in food biotechnology area. For this reason, many attempts have been made to modify the structural properties of starch by enzymes (Park, 2004). In this study, a thermostable 4-α-glucanotransferase originated form *Thermus scotoductus* (TSαGT) was used to modify the physical and structural properties of starch. The 4-α-glucanotransferase (4αGTase) (EC 2.4.1.25) (also called

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D-enzyme) was originally found in potato tubers and known to catalyze the disproportionation of added maltooligosaccharides by intermolecular transglycosylation (Peat, Whelan, & Rees, 1956) (the transfer of maltooligosaccharides from one α-1,4 glycosidic linkage). Studies of the in vitro action of the D-enzyme (disproportionating enzyme) showed that maltooligosaccharides are effective donors with maltotriose as the smallest donor molecule, and that maltooligosaccharides and glucose serve as acceptors. A maltosyl group was the major unit transferred from donor to acceptor (Jane & Whelan, 1969). However, it was recently found that D-enzyme can use high-molecular-weight starch as both donor and acceptor molecule and can catalyze the transfer of long α-1,4-glucan chains (Takaha, Yanase, Takata, Okada, & Smith, 1996), or even highly branched cluster units of amylopecin (Takaha, 1996). Furthermore, D-enzyme catalyze the inter-molecular transglycosylation (cyclisation) of amylose to form novel cyclic α-1,4-glucans with degree of polymerization (DP) ranging from 17 to several hundreds (Takaha et al., 1996) and with amylopectin it forms cyclic glucans with a highly branched structure (Takaha, 1996). Recently, it has been reported that a 4αGTase from *Thermus thermophilus* can modify starch in a way that the modified starch gel demonstrates thermo-reversibility (Euverink & Binnema, 2003). The objectives of this study were to evaluate the properties of rice starch gel after modification with a thermostable 4αGTase (TSαGT) originated from *T. scotoductus* in terms of rheological properties, thermo-reversibility, molecular weight, and freeze-thaw stability.

2. Materials and methods

2.1. Materials

Rice starch was purchased from Sigma Chemical Industries, Ltd (St Louis, USA). An α-amylase was provided by Novozymes, Denmark (Termamyl®), the optimum temperature and pH ranges of which were 90 °C and 7.0, respectively, as in the product data sheet provided by the manufacturer.

2.2. Cloning and purification of TSαGT

T. scotoductus strain (ATCC 27978) was purchased from American type culture collection (ATCC). Gene cloning and transformation were performed as described in Park (2004). *Escherichia coli* MC1061 [F^-, *araD*139, *recA*13, Δ(*araABC-leu*)7696, *gal*U, *gal*K, Δ*lac*X74, *rpsL*, *thi*, *hsdR*2, *mcrB*] was used as a host for DNA manipulation and transformation. Plasmids, pGNX4 and pUC119 (Sambrook, Fritsch, & Maniatis, 1989) were used for cloning and subcloning. For the purification of TSαGT, Ni-NTA affinity chromatography was used. *E. coli* MC1061 carrying the recombinant was cultured (1 L) and harvested by centrifugation at 4 °C (10,000×*g*) for 10 min. The cell pellet was resuspended in 100 ml of lysis buffer (50 mM Tris–HCl (pH 7.5), 300 mM NaCl, 10 mM imidazole) and sonicated in an ice bath using sonicater (VC-600, Sonics & Materials Inc., USA; output 4, 5 min×3 times, 60% duty). The crude cell extract was centrifuged (10,000×*g*) at 4 °C for 20 min. Two milliliters of the cell lysate was applied to 2 mL of Ni-NTA resin (QIAGEN, USA) packed in a Poly-Prep® Chromatography Column (BIO-RAD, USA). The resin was washed with washing buffer (50 mM Tris–HCl (pH 7.5), 300 mM NaCl, 20 mM imidazole) until the unbound host proteins were fully eliminated, and then the protein was eluted with elution buffer (50 mM Tris–HCl (pH 7.5), 300 mM NaCl, 250 mM imidazole). The eluted target protein was dialyzed against 50 mM Tris–HCl (pH7.5), and the purity was electrophoretiacally confirmed using SDS-PAGE. The optimum reaction temperature and pH of the TSαGT was 75 °C and 7.5, and it maintained more than 80% of its activity at the temperature and pH ranges of 50–80 °C and 5–9, respectively (data not shown).

2.3. Enzymatic modification of gelatinized rice starch

Rice starch was dispersed at concentration of 5 wt% in distilled water and heated in boiling water for 30 min while mechanically stirred. After cooling the sample to 75 °C, 10 mL of the 5% rice starch paste was incubated with 100 μL (1.38 mg/mL) of TSαGT solution at 75 °C for 4 h. For comparative study, rice starch paste was treated with α-amylase under the same condition. The enzyme mixtures were boiled for 10 min to decrease the enzyme activity, after the reduction of viscosity leveled off. For a reference, rice starch paste without enzyme was used with the same thermal history.

2.4. Measurement of flow behavior

Viscosity of the sample was determined immediately after the enzymatic reaction using a controlled strain rheometer (RheoStress RS1, Thermo Haake, Germany) using parallel plate geometry (35 mm diameter and 1 mm gap). Although the shear rate in the gap will vary with parallel plate geometry, the alternative of cone and plate was rejected because dimensions of the gap at the apex would approach that of a swollen starch granule. Flow curves with increasing shear rate (0.1–500 s^{-1}) were obtained at 4 °C. Flow behavior was estimated using a Herschel–Bulkley model, which is a general relationship to describe the behavior of non-Newtonian fluids as in Eq. (1):

$$\tau = K(\dot{\gamma})^n + \tau_0 \quad (1)$$

where τ is the shear stress (Pa), $\dot{\gamma}$ is the shear rate (1/s), K is the consistency index (viscosity, Pa·s), τ_0 is the yield stress, and n is the flow behavior index.

2.5. Measurement of dynamic viscoelasticity

The gelation kinetics and the paste/gel properties were measured using a dynamic rheological test. Freshly made samples were loaded between parallel plates in a rheometer. After trimming off the over-loaded portion of samples around plates, the open side of samples was covered with a thin

layer of silicon oil. A solvent trap was also used to minimize the water loss during the measurements. Various dynamic rheological tests were performed to characterize the enzyme-modified rice starch paste/gel. Dynamic rheological properties (G' and G'') were measured at 1% strain, which was within a linear viscoelastic region as determined through strain sweep test (data not shown), and 1 Hz of frequency except for the frequency sweep test.

2.6. Freeze-thaw stability

Freeze-thaw stability was measured according to the procedure of Jobling, Tayal, Jeffcoat, & Schwall (2001). After enzymatic modification, about 0.5 mL of each sample was placed into 1.5 mL Eppendorf tubes and the exact weight of the sample in each tube was measured. The sample tubes were frozen-stored at −70 °C overnight, and then thawed for 60 min in a 22 °C water bath. The frozen-thawed sample was then centrifuged at 8000 × *g* for 10 min at 18 °C. The free liquid was decanted and the weight of the remaining gel was determined. The ratio of the free-liquid weight to the initial sample weight (expressed as a percentage) was used as a measure of syneresis. The remaining tubes were placed back at −70 °C for additional freeze-thaw cycles and the analysis was repeated.

2.7. SEC-MALLS-RI

Starch paste samples were freeze-dried, ground in a mortar, and passed in a 200 mesh. The sample powder was re-dissolved in 1 N NaOH, diluted with distilled water, and neutralized with 1 N HCl to make 0.5% solution. After autoclaving (121 °C) it for 20 min, the sample was filtered using a 5 μm disposable syringe filter, and injected into SEC-MALLS-RI system.

The size exclusion chromatography (SEC) system consisted of a pump (Waters 510), an injection valve (Model 7010, Rheodyne) with a 200 μL sample loop, a guard column (TSK PWH, Tosoh Corp.), SEC column (G5000 PW, 7.8 × 600 mm, Tosoh Co., Tokyo, Japan), a multi-angle laser light scattering detector (MALLS) (Dawn DSP, Wyatt Technology, St Barbara, USA) and a refractive index detector (RI) (Waters 410). The column was kept at room temperature. The flow rate of mobile phase (0.15 M $NaNO_3$ containing 0.02% NaN_3) was 0.4 mL/min. Calculation of molecular weight was performed using a Astra 472 software (Wyatt Technology, St Barbara, USA) with the Berry extrapolation method.

3. Results and discussion

3.1. Modification of gelatinized rice starch suspensions with enzymes

Freshly gelatinized rice starch paste samples were cooled to 75 °C and incubated with enzymes between rheometer plates set at 75 °C. The enzymatic degradation process was monitored by measuring dynamic rheological properties (G' and G'') as a function of time. Fig. 1 wrepresents control rice starch paste (a), rice starch paste with TSαGT (b), and rice starch paste with α-amylase (c) during the incubation period. The elastic modulus (G') and the viscous modulus (G'') of control starch paste remained constant during the test period (Fig. 1(a)) showing elastic behavior with G' greater than G''. In contrast, rice starch paste with 4αGTase and α-amylase showed a dramatic change in rheological properties

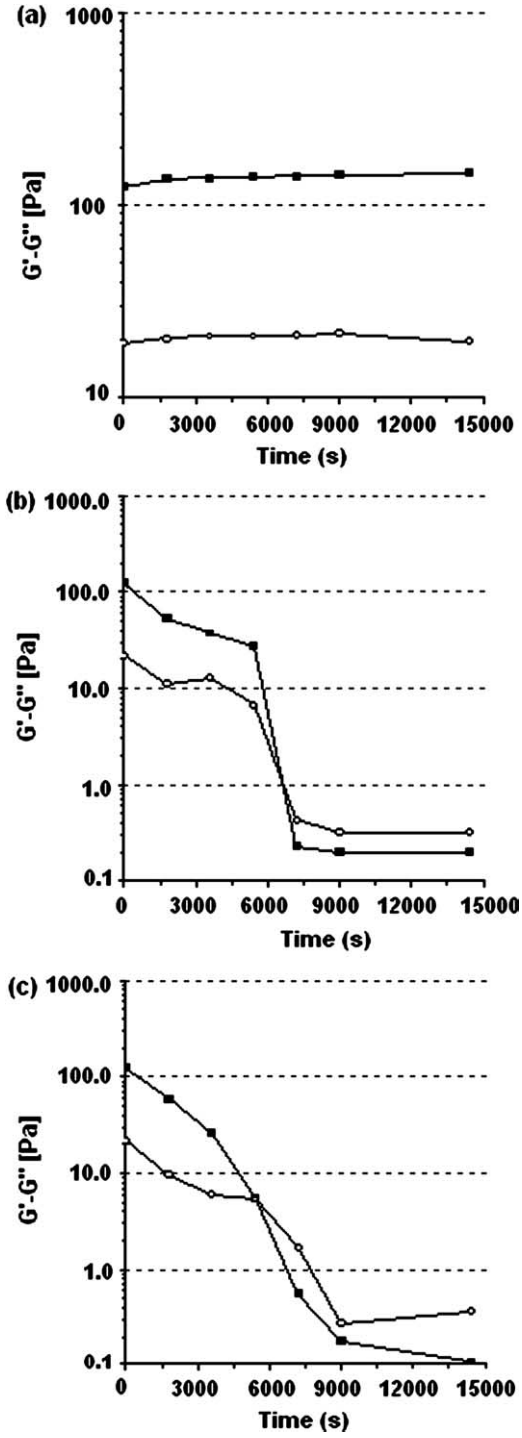

Fig. 1. Changes in elastic modulus (G', solid symbols) and viscous modulus (G'', open symbols) with incubation time at 75 °C. (a) 5% Rice starch suspension (b) with TSαGT and (c) with α-amylase. Oscillatory strains (1%) were applied at 1 Hz.

during incubation. In the case of rice starch paste with TSαGT (Fig. 1(b)), G′ and G″ decreased gradually during the initial period of incubation followed by an abrupt decrease after 1.5 h. The dynamic rheological properties remained constant after 2.5 h. The elastic and viscous modulus crossed over at around 2 h of incubation so that the viscous modulus became greater than the elastic modulus, which indicates that the material behavior changed from solid-like to liquid-like. Rice starch paste incubated with α-amylase (Fig. 1(c)) showed a somewhat gradual decrease in G' and G'' until both values leveled off. The crossover of G' and G'' was also observed at similar time or a little earlier compared to the case of TSαGT. At the end of the incubation period, there was only a small difference in G' and G'' between the two types of enzymes. Based on the result of moduli decrease, the end point of enzymatic reaction was determined as 4 h, from which the rheological properties of rice starch paste were no longer altered.

3.2. Flow behavior

After the enzymatic reaction, flow curves of the modified rice starch paste samples were obtained as a function of shear rate (0.1–500 s^{-1}) at 4 °C, and compared with that of the unmodified rice starch paste. As shown in Fig. 2, enzyme treatments resulted in a significant change in flow behavior of rice starch paste. While unmodified rice starch paste exhibited a typical shear-thinning behavior, the α-amylase-modified starch paste demonstrated nearly Newtonian-like behavior. Interestingly, TSαGT-modified rice starch paste exhibited a different behavior from other two samples,

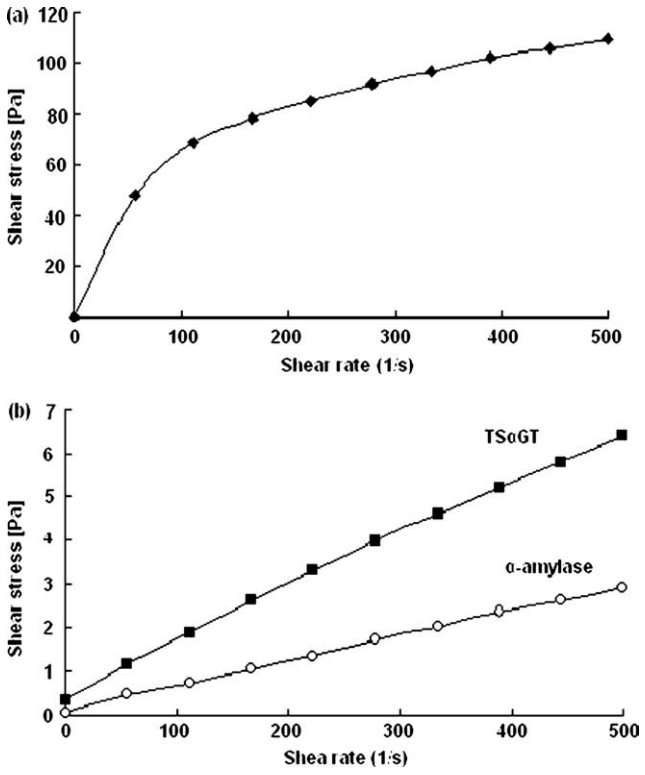

Fig. 2. Flow curves 5 °C, after the viscosity had reached constant values. (a) 5% Rice starch suspension, (b) with α-glucanotransferase and with α-amylase.

Table 1
Flow behavior parameters for Herschel–Bulkley equation (Eq. (1))

Samples	n[a]	K[b]	σ_0[c]	r^2
5% Rice starch	0.1978	51.86	0	0.99
With TSαGT	0.8019	0.0438	0.3352	0.99
With α-amylase	0.9189	0.0097	0	0.99

[a] Flow behavior index.
[b] Consistency index (viscosity) (Pa·s^n).
[c] Yield stress (Pa).

showing a yield stress. TSαGT-modified rice starch paste acted similarly as the Bingham plastic. Possibly, different enzymatic reactions towards rice starch between α-amylase and TSαGT with different enzymatic products of different size and shape caused the different rheological behaviors. The parameters obtained for the Herschel–Bulkley model (Eq. (1)) are summarized in Table 1. The apparent viscosity decreased significantly after the treatment with both enzymes.

3.3. Gelling properties of enzyme-modified rice starch

In order to determine the experimental gelation period, dynamic rheological properties of rice starch paste were continuously measured at 4 °C after the enzymatic reaction (data not shown), and 24 h was found to be sufficient time for the gel set.

Fig. 3 represents the dynamic moduli of control and enzyme-modified rice starch paste before and after gelation. The figures on the left correspond to G' and G'' of rice starch paste immediately after the enzymatic reaction (for control sample, only a corresponding heat treatment), and figures on the right represent those after 24 h at 4 °C. All the measurements were performed at 4 °C. At 0 h, both enzyme-treated samples (Fig. 3(b) and (c)) showed a low moduli with higher G'' than G', compared to those of the control sample (Fig. 3(a)), indicating a significant liquefaction of starch paste upon enzyme treatment. After 24 h at 4 °C (right figures), both moduli of control (Fig. 3(a)) and TSαGT-treated (Fig. 3(b)) samples increased significantly increased with much higher values of G' than G'', whereas those of α-amylase-treated sample (Fig. 3(c)) did not change significantly. These results indicated that a relatively rigid gel structure formed in the control and TSαGT-treated samples, whereas α-amylase-treated rice starch paste remained in the liquid state. Interestingly enough, the gel formed from significantly liquefied TSαGT-treated rice starch paste showed a much higher G' and greater difference between G' and G'' compared to those of control rice starch paste. The frequency sweep result (Fig. 4) for control and TSαGT-treated rice starch gel clearly reveals a higher rigidity of TSαGT-treated starch gel compared to the control gel. Both gels showed a lack of frequency dependency and large difference between G' and G'' along the whole frequency range tested, confirming that solid gel structure formed. It is known that acid treatment of starch causes partial hydrolysis of starch chains, resulting in much

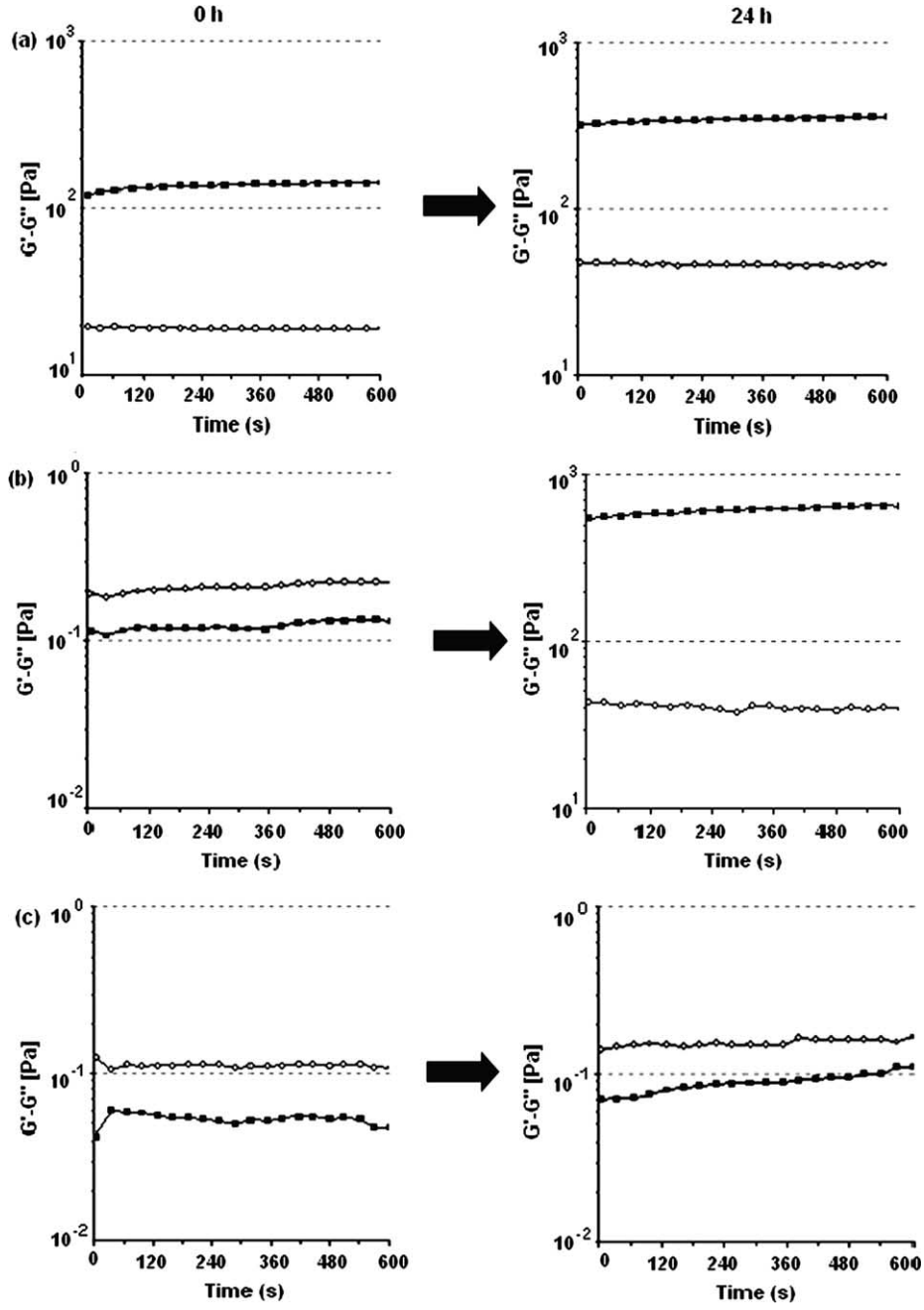

Fig. 3. Changes in the rheological properties of 5% rice starch paste after 24 h at 4 °C. (a) Control rice starch, (b) with TSαGT, and (c) with α-amylase. Oscillatory strains (1%) were applied at 1 Hz.

lower paste viscosity compared to native starch. However, when it cools acid-treated starch chains tend to associate with each other more easily and thus form a more rigid gel (Hoseney, 1994). Based on this fact, it could be postulated that TSαGT treatment reduces the molecular weight of rice starch chains at a certain level, which is small enough to significantly decrease viscosity of the paste, but large enough to form a rigid gel through re-association. In the case of α-amylase treatment, chain cleavage seems to be too severe for remaining chains to re-associate. Whether only a chain size is important structures of the modified chains are also involved, are not clear at this moment.

3.4. Thermo-reversibility of TSαGT-treated rice starch gel

After the gel formed at 4 °C, its melting behavior were characterized by heating it from 4 to 70 °C at 1 °C/min followed by holding it at 70 °C while G' and G'' were measured continuously (Fig. 5). In the case of control rice starch gel (Fig. 5(a)), both moduli did not change significantly during heating and holding at 70 °C, indicating that it retains its rigidity at 70 °C. However, TSαGT-treated rice starch gel showed an obvious melting behavior as presented in Fig. 3(b). During heating, G' and G'' started to decrease at around 40 °C and continuously decreased with increasing temperature. The

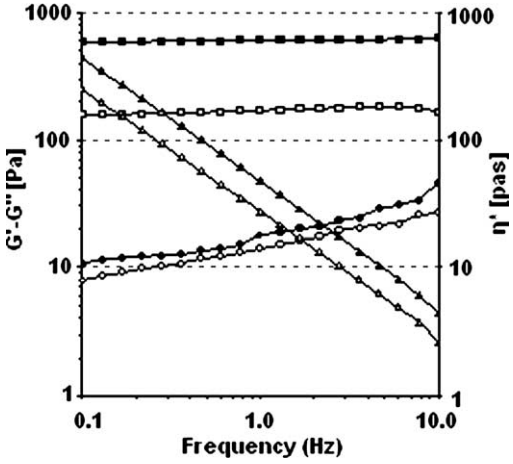

Fig. 4. Mechanical spectra (1% strain, 4 °C), showing the frequency-dependence of G' (square), G'' (circles) and η^* (triangles) for 5% rice starch gels without (open symbols) and with (filled symbols) α-glucanotransferase after incubated at 4 °C for 24 h.

difference between G' and G'' also gradually decreased. At 70 °C, both moduli remained the same for the initial 20 min and then continuously decreased thereafter. Finally, the G' and G'' curves crossed over at around 1 h so that G'' became greater than G' afterward, indicating an obvious liquefaction of gel structure. The heating and cooling cycle (heating at 70 °C and cooling at 4 °C) was repeated and G' was measured at the end of heating and cooling steps as depicted in Fig. 6. Although there was a slight change in G' as the cycle repeated, TSαGT-treated rice starch gel exhibited almost perfect thermoreversibility between 4 and 70 °C. Euverink and Binnema (2003) also claimed the formation of thermoreversible starch gel after the treatment of potato starch with 4-α-glucanotransferase from *T. thermophilus*. This result suggest considerable potential for TSαGT-modified rice starch gel in numerous industrial applications.

3.5. Molecular weight of TSαGT-treated rice starch

The weight average molecular weight (M_w) of TSαGT-treated rice starch was measured using a SEC-MALLS-RI system. As shown in the SEC elution profile (data not shown), at the end of 4 h of reaction, amylase in the amylopectin bimodal profile in control rice starch converged into a single profile at lower molecular weight region. The calculated M_w of TSαGT-treated rice starch was 4.5×10^5, which corresponds to about 2500 glucose units, whereas that of control rice starch was 5.3×10^7 (Table 2). These relatively bulky molecules obtained after the TSαGT treatment seem to be mainly

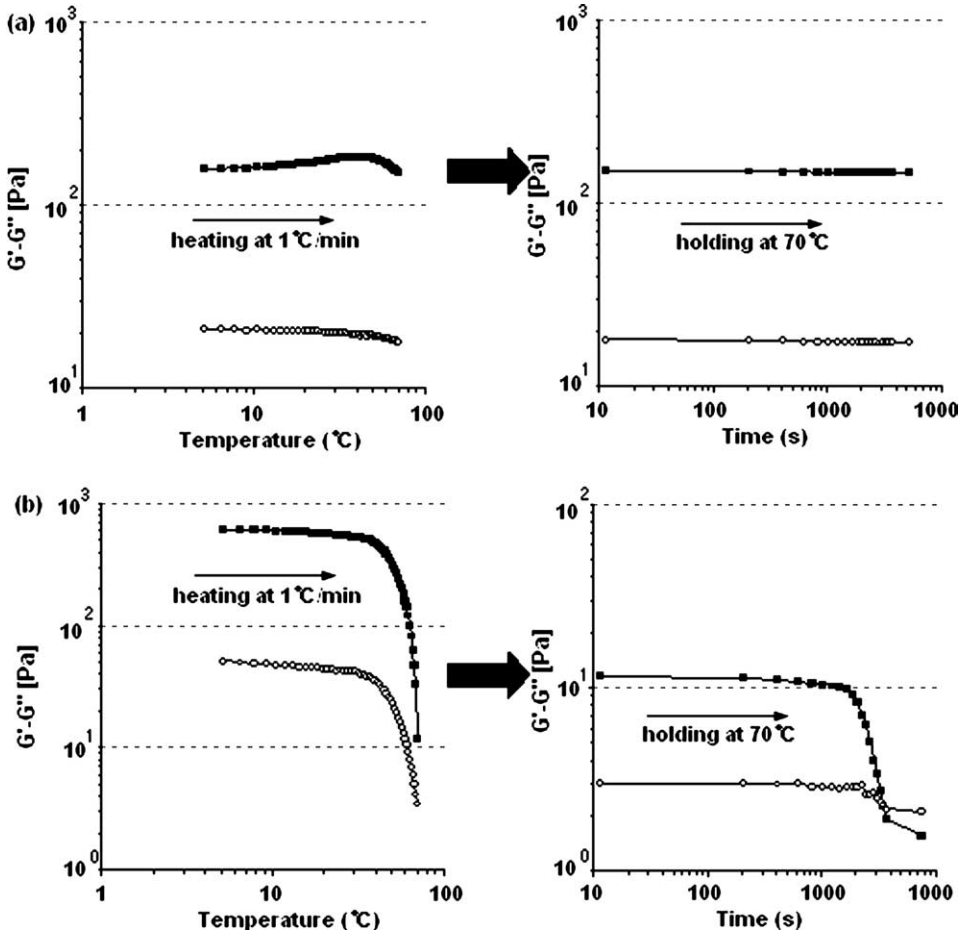

Fig. 5. Temperature dependence curves of G' (■) and G'' (○) for 5% rice starch gels (a) without and (b) with α-glucanotransferase. The measurements were performed from 5 to 70 °C for 1 h at a constant frequency of 1 Hz and at a constant strain of 1%.

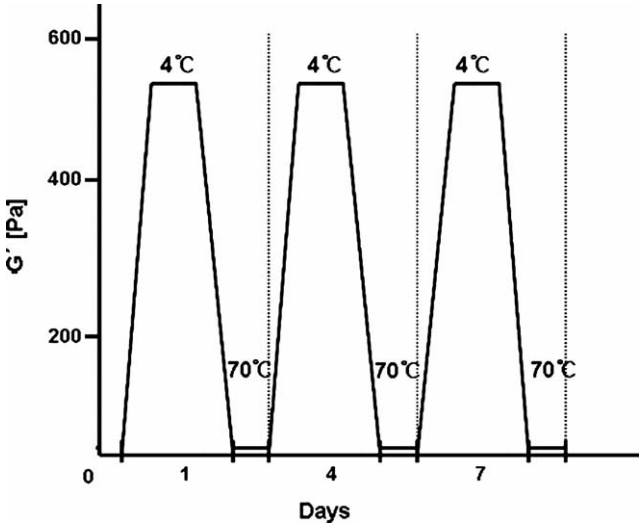

Fig. 6. Changes of storage modulus (G'; 1 Hz, 1% strain; 5 °C) with various freeze-thawing (thermo-reversible cycles).

amylopectin cluster units or modified amylopectin clusters re-organized by disproportionation, which have been proposed by Takaha, Yanase, Takata, Okada, and Smith (1998). They have suggested a model for the formation of cycloamyloses and amylopectin cluster units, from the results with potato D-enzyme (disproportionating enzyme) and waxy corn starch. They reported that the enzyme-treated waxy corn starch contains more amounts of shorter branches. The formation of the relatively high M_w amylopectin clusters with re-organized side chains as well as lack of long amylose chains might be responsible for the formation of thermoreversible gel, which cannot be obtained from conventional starch hydrolyzing enzymes, such as α-amylase.

3.6. Freeze-thaw stability

Freeze-thaw stabilities of starch pastes were assessed by measuring liquid released during freezing/thawing cycle. The effect of freeze-thawing on the amount of syneresis in starch gel is presented in Fig. 7. Control starch gel showed a considerable amount of syneresis, discharging more than 40% of total weight within the first two cycles. This is a typical case for starch gel (Jobling, et al., 2002). However, TSαGT-treated starch gel showed dramatically increased free-thaw stability, maintaining 10% initial syneresis during all four cycles of freeze-thawing. The use of native starch in food processing is often limited due to its tendency to retrograde and subsequent syneresis (Rosalina & Bhattacharya, 2002). It is generally accepted that the long unbranched amylose chains have a greater tendency to retrograde than the highly branched and much shorter amylopectin chains (Park, 2003). Based on these facts, significant reduction of long chain amylose and modification of amylopectin side chain caused by TSαGT treatment gives the improved freeze-thaw stability.

Table 2
Weight average molecular weight (M_w) of control rice starch and TSαGT-treated rice starch

Samples	Weight average molecular weight (M_w)
Control rice starch	5.3×10^7
TSαGT-treated rice starch	4.5×10^5

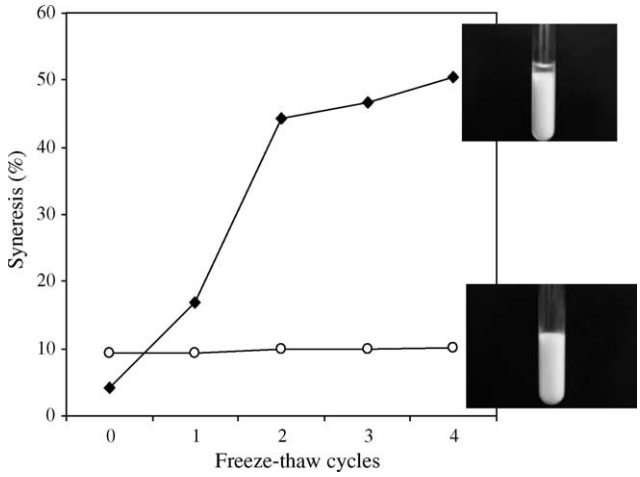

Fig. 7. Freeze-thaw stability. The percentage syneresis (the amount of water as a proportion of the initial sample weight) is shown at each cycles of freeze-thaw and centrifugation.

4. Conclusions

A thermostable α-Glucanotransferase (TSαGT) originated from *T. scotoductus* having both hydrolyzing and disproportionating activity modified rice starch paste in a unique way TSαGT-treated rice starch paste demonstrated a yield stress. Upon cooling, TSαGT-treated rice starch paste formed a gel structure even more rigid than that of native starch. This gel showed thermo-reversibility between 4 and 70 °C, which was not observed in control rice starch gel. The α-amylase-treated rice starch failed to form a rigid gel. The enzyme's unique way of modification produced relatively bulky starch chains ($M_w \approx 7\times10^5$) with re-organized chain distributions, which was possibly responsible for the reversible gel formation. The reduction of long chain amylose and the modification of amylopectin side chain by TSαGT gave much improved freeze-thaw stability of the enzyme-treated rice starch gel.

Acknowledgements

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