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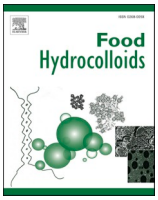

Ethanol pretreatment increases the efficiency of maltogenic α-amylase and branching enzyme to modify the structure of granular native maize starch

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ABSTRACT

A method for efficient functional modification of starch granules by thermal ethanol pre-treatment and subsequent maltogenic α-amylase (MA) and branching enzyme (BE) post-treatments is described. Ethanol pre-treatment significantly increased the swelling power of starch granules thereby increasing the MA and BE susceptibility. Ethanol pre-treated granules became shrunk and twisted after incubating in buffer. Sequential MA post-treatments remarkably increased the α-1,6 to α-1,4 ratio and the content of amylopectin short chains (DP 1–10), contributing to the low retrogradation rate. BE post-treatments significantly decreased the product yield, increased the relative crystallinity of starch granules, suggesting BE had intramolecular transglucosylation activity which altered the branch position and reduced the molecular size by forming cyclic structures. Moreover, BE post-treatments showed an α-1,6 to α-1,4 transglucosylation activity by decreasing the α-1,6 to α-1,4 ratio, especially during simultaneous MA and BE catalysis. However, in the simultaneous MA and BE post-catalysis, MA dosage was predominant by noticeably hydrolyzing amylopectin and amylose molecules and increasing the α-1,6 to α-1,4 ratio, thereby leading to the lowest digestibility and retrogradation.

1. Introduction

Starch is an important raw material utilized for various industrial applications. As a first step, most applications require a modification of starch, aiming at increasing its functionality, such as increasing the content of resistant starch (RS) or reducing the retrogradation rate, i.e. increasing the stability in the foods. For decades, enzymatic starch modification has been increasingly applied as a clean strategy for providing customised, environmental-friendly and consumer-safe products. Glucanotransferase branching enzyme (BE) and transglucosidase (TGA) catalyze transfer reactions that can lead to a retarded retrogradation rate of starch and also increase the RS content in gelatinized starch (Ao et al., 2007; Martínez, Pico, & Gómez, 2016). However for modification of granular starch, a major limiting factor remains the semi-compact and semi-crystalline structure of starch granules, which blocks the accessibility to enzymes, such as BE (Li et al., 2018) and TGA (Zhong, Keeratiburana, et al., 2021), and thus prevents efficient modification of granular starch. However, maintaining the granular structure of starch is attractive for industry, because it facilitates post-processing and reduces energy costs when compared to handling gelatinized starch.

So far, two major strategies have been introduced to increase the enzymatic susceptibility of granular starch by increasing the granule surface area, namely pre-treatment with high temperature combined with supplying very high starch concentration (Jensen, Larsen, Bandsholm, & Blennow, 2013), and using porous starch preparations (Guo et al., 2019). The former method restricts the swelling and gelatinization of granules but permits enzyme diffusion into the partly swollen granules. This approach depends on controlling the water content to conserve energy for heating and subsequent drying (Jensen et al., 2013). The latter strategy utilizes starch hydrolytic enzymes such as α- and β-amylase to produce porous starch with internal channels, thereby increasing the granular surface area and susceptibility to other modifying enzymes such as maltogenic α-amylase (MA) and TGA (Guo et al., 2019). MA is a hydrolytic enzyme with a high potential for starch modification because it does not only hydrolyse starch granules but also

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produces more branch points due to its transferase activity by trimming off external chains of the starch molecules, resulting in an increase of short amylopectin side chains (DP < 13) (Li, Li, Zhu, & Ai, 2021; Miao et al., 2014). A similar effect was observed when BE acted on gelatinized maize starch (Li et al., 2014). This is particularly interesting in the light of a recently introduced strategy for increasing the enzymatic susceptibility of starch to transglycosylases by increasing the swelling ability of starch granules via heat in aqueous ethanol, which produces so-called cold-swelling starch (Keeratiburana, Hansen, Soontaranon, Blennow, & Tongta, 2020a). So far, this strategy has only been implemented for normal rice starch (Keeratiburana et al., 2020a). However, it is unknown how MA and BE alter the molecular structure of granular starch upon ethanol treatment.

The starch source strongly influences the structure and properties of starch granules (Gregorová, Pabst, & Bohačenko, 2006; N. Singh, Singh, Kaur, Singh Sodhi, & Singh Gill, 2003; Srichuwong, Sunarti, Mishima, Isono, & Hisamatsu, 2005). For instance, when compared with rice starch, maize starch has a lower crystallinity (Huang et al., 2015; Srichuwong et al., 2005), larger granule size (Singh et al., 2003; Srichuwong et al., 2005), lower peak viscosity (Huang et al., 2015; Srichuwong et al., 2005), and a higher retrogradation rate (Jacobson, Obanni, & Bemiller, 1997). Most importantly, maize starch displays a higher enzymatic susceptibility than rice starch as tested by its higher amyloglucosidase and porcine pancreatic α-amylase susceptibility (Huang et al., 2015). Thus, we hypothesized that the effect of other glucan-acting enzymes on maize starch is different from rice starch.

In the present study, we employed combinations of ethanol pre-treatment and sequential MA/BE/MA ‡ BE post-treatment. This allowed us to explore (1) the potential of ethanol pre-treatment to increase the enzymatic susceptibility of granular native maize starch and (2) the consequences of subsequent MA and BE treatment in different combinations and enzyme concentrations on the structure and properties of granular maize starch. We hypothesized that the effect of ethanol pre-treatment on maize starch granules is different from that of rice starch, and that MA, BE, and their combinations have different effects on the structure and properties of maize starch when compared with rice starch. Our preliminary experiments showed that the enzymatic susceptibility of maize starch granules was remarkably higher than that of rice starch. To avoid the yield loss during the modification process, the experimental conditions (e.g., BE concentration) were more gentle than those applied in the rice system (Keeratiburana et al., 2020a).

2. Materials and methods

2.1. Starch and enzyme sources

Normal maize starch (NMS) (Commercial Clinton 106) was supplied by Archer Daniels Midland (ADM, Decatur, IL, USA). Maltogenic α-amylase (MA) at 41530 U/mL and *Rhodothermus obamensis* branching enzyme (BE) were provided by Novozymes (Bagsværd, Denmark). The enzyme activity, 0.001 U/mL, of BE was analyzed by a reducing end assay as described before (Zhong, Herburger, et al., 2021). Pure potato amylose (20 mg/mL) was used as substrate and 1 μg/mL BE produced 0.16 μmol/mL and 0.04 μmol/mL reducing ends with and without isoamylase debranching, respectively. Debranching isoamylase (200 U/mL) and pullulanase (700 U/mL) were supplied by Megazyme (Ireland). Pancreatin (Cat. No. P7545) and amyloglucosidase (Cat. No. A7095) were supplied by Sigma (St. Louis, MO, USA).

2.2. Ethanol pre-treatment

A protocol for ethanol pre-treatment was followed as described previously (Keeratiburana et al., 2020a) with modifications. Starch (20 g, in duplicate) was mixed with 40 mL of 50% ethanol (v/v) and incubated at 80 C for 30 min. The samples were stored at room temperature for 3 h, washed twice with 50% ethanol, and freeze-dried. All the dried samples (n ˆ 10) were mixed and gently pulverized using a pestle and mortar for further enzymatic modification. The 50% ethanol-heated samples are abbreviated as ETH.

2.3. Enzymatic modification of ETH starch by maltogenic -amylase, branching enzyme and their combinations

ETH starch (5 g) was mixed with 50 mL 50 mM acetate buffer (pH 5.5) containing 5 mM $CaCl_2$ and gently stirred at 50 C for 30 min. MA, BE, or MA ‡ BE was added and the reaction mixtures incubated at 50 C for 3 h. The reaction was terminated by adding 1 M NaOH to reach pH 11. After 10 min, the pH of the solutions was adjusted back to 5.5 using 2 M HCl and the samples were washed three times in 50 mM acetate buffer. Finally, samples were freeze-dried. All treatments were performed in triplicate. The dosage of enzymes and abbreviations are presented in Table 1. Three control samples were used: (1) raw normal maize starch (abbreviation: NMS); (2) ETH; (3) 3 h acetate buffer incubated ETH (abbreviation: EBUF).

2.4. Yield, swelling power, and water solubility

Yield was calculated as the ratio of the weight of treated samples after freeze-drying and the original weight (5 g). The swelling power and water solubility of starches were determined as described elsewhere (Rosell, Yokoyama, & Shoemaker, 2011). Briefly, 40 mg of the sample and 20 mL of MilliQ water were mixed and heated at 65 C for 30 min with gentle stirring. After cooling to room temperature, the sample was centrifuged at 10,000 g for 20 min. The supernatant was collected and the precipitate weighed (w_1). Swelling power (g/g) was the ratio of w_1 with the original weight (40 mg, dry basis). The supernatant was dried at 110 C overnight until the weight (w_2) was constant. The water solubility was calculated as the ratio (%) of w_2 and the original weight.

2.5. Size-exclusion chromatography - triple detector array (SEC-TDA)

The molecular structure of raw and debranched starches were analyzed by a SEC-TDA (Viscotek, Malvern, UK), equipped with tandem GS-520HQ/GS-320HQ Shodex columns attached to a TDA302 detector array (Zhong, Keeratiburana, et al., 2021). Raw starch (5 mg) was mixed with 25 μl 2 M sodium hydroxide, incubated at 4 C overnight and diluted to 5 mg/mL using distilled water, followed by heating at 80 C for 5 h with gentle stirring. The sample was diluted to 1 mg/mL with acetate buffer and centrifuged at 20,000 g for 5 min. The supernatant (100 μL) was injected onto the column and eluted with ammonium formate (10 mM) with a flow rate of 0.5 mL/min to minimize shear scission effects (Hoang et al., 2008). To analyze effects on the starch side-branches, samples (5 mg/mL) were gelatinized at 99 C for 1 h and debranched by incubating gelatinized samples with 2 μl isoamylase and 2 μl pullulanase at 40 C for 3h. The samples were diluted to 1 mg/mL, centrifuged at 20,000 g for 5 min and injected onto the column. Pure maize amylose and amylopectin without the debranching process were analyzed as standards to distinguish the amylopectin and amylose

Table 1
The experimental design of dual MA and BE treatment (n ˆ 3).

Samples	Ethanol	Buffer	MA (3h) U/g	BE (3 h) U/g
NMS	No	No	No	No
ETH	Yes	No	No	No
EBUF	Yes	Yes	No	No
EMA1	Yes	Yes	26	No
EBE1	Yes	Yes	No	1 10^5
EMB1	Yes	Yes	26	1 10^5
EMA2	Yes	Yes	104	No
EBE2	Yes	Yes	No	4 10^5
EMB2	Yes	Yes	104	4 10^5

fraction in the raw starch. The relative content of different amylopectin fractions and amylose in debranched samples was calculated as the ratio of the area under the curve of the fraction to the area of the overall curve.

2.6. High performance anion exchange chromatography-pulsed amperometric detection (HPAEC-PAD)

Gelatinization and debranching of samples (5 mg/mL) were performed as described above. Following centrifugation, the supernatant was injected onto a CarboPac PA-200 column attached to an HPAEC-PAD (Dionex, Sunnyvale, CA, USA) system (Zhong, Keeratiburana, et al., 2021). Peak integration was performed and the chain lengths distribution was calculated following detector response correction as described (Blennow, Bay-Smidt, Wischmann, Olsen, & Møller, 1998).

2.7. Wide angle X-ray scattering (WAXS)

The samples were stored in a sodium chloride humidity chamber (90% relative air humidity) for 3 days and then analyzed by using a SAXSLab instrument (JJ-X-ray, Copenhagen, Denmark) equipped with a 100 XL ‡ microfocus sealed X-ray tube (Cu-K_α radiation, Rigaku, The Woodlands Texas, USA) and a 2D 300 K Pilatus detector (Dectris Ltd, Baden, Switzerland) (Zhong, Keeratiburana, et al., 2021). The relative crystallinity of the starch samples was calculated (Brückner, 2000; Goldstein et al., 2017).

2.8. Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy

The signals representing anomeric protons of α-1,4 linkage, α-1,6 linkage, α-anomeric reducing end protons, and β-anomeric reducing end protons were analyzed by one-dimensional ^{1}H NMR spectra acquired on 500 MHz NMR spectrometers (Bruker Avance III) from Bruker (Bruker Biospin, Rheinstetten, Germany) (Zhong, Keeratiburana, et al., 2021). The starch (5 mg/mL) was gelatinized in deuterium oxide and then lyophilized and re-dissolved in deuterium oxide at 90 C for 1 h before the analysis. Areas of signals representing anomeric protons were detected and quantified using SigMa software (Khakimov, Mobaraki, Trimigno, Aru, & Engelsen, 2020).

2.9. Field emission scanning electron microscopy (FE-SEM)

The topography and morphology of the solid starch particles were monitored by FE-SEM (FEI Quanta 200) after fixing and sputter-coating granules with gold.

2.10. In vitro digestion

Digestion of raw starch and 1-day retrograded starch was done as described previously with modifications (Zhong, Herburger, et al., 2021). Starch product (100 mg) was incubated in 5 mL water and 12.5 mL sodium acetate buffer (0.1 M, pH 5.2) at 37 C for 30 min. Digestion was initiated by adding pancreatin (5 103 USP/g) and amyloglucosidase (66 U/g), and aliquots (0.1 mL) were collected at 20 and 120 min, respectively, for further analysis. The reactions were terminated by adding 1 mL 95% ethanol, samples centrifuged at 20,000 g for 5 min and the glucose content in the supernatant was quantified using the GOPOD kit (Megazyme). Rapidly digested starch (RDS) was defined as the starch digested within 0–20 min, slowly digested starch (SDS) as the starch digested within 20–120 min and the remaining residue defined as resistant starch (RS):

%RDS ˆ G20/(Initial dry mass of sample) (162/180) 100%

%SDS ˆ (G120 - G20)/(Initial dry mass of sample) (162/180) 100%

%RS ˆ %Total starch of sample on a dry basis - (%RDS ‡ %SDS)

where G20 and G120 were the total mass of glucose released from amylolysis at 20 min and at 120 min, respectively.

2.11. Pasting and dynamic gelling properties

A Rapid Visco Analyzer (RVA, Newport Scientific, Australia) was used to determine the pasting properties of starch samples (8% w/v) using ICC Standard Method No. 162. Fresh and 7-day stored (at 4 C) gelatinized starch collected after RVA analysis was subsequently analyzed by a Discovery HR-3 Rheometer (TA Instruments, New Castle, DE, USA) at 25 C.

2.12. Statistical analyses

WAXS analysis was performed once and all other analysis were carried out in duplicate. Differences were analyzed using one-way analyses of variance (ANOVAs) followed by Duncan's test ($p < 0.05$) in SPSS 18.0 (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Product yield

The EBUF treatment resulted in a 10% yield loss when compared with NMS and ETH (Table 2), which was mainly associated with the loss of granular material during the washing and drying processes, in agreement with our previous studies (Zhong, Herburger, et al., 2021; Zhong, Keeratiburana, et al., 2021). The yield of EMA1 and EBE1 dropped dramatically to 60% and 53%, respectively. We suggest that this is due to high intramolecular transfer reactions of BE based on ethanol pre-treated starch granules. It is worth noting that BE has minor effect on the yield of maize starch in a system without ethanol pre-treatment (Zhong, Herburger, et al., 2021). The decrease of the yield of EMA1 and EBE1 was likely due to the hydrolysis of α (1 → 4) linkages and the production of small soluble starch segments. A similar effect of BE on decreasing the yield was also found for sweet potato starch (Guo, 2018). In contrast, BE treatment without ethanol pre-treatment had insignificant effects on the yield of maize starch granules (Zhong, Herburger, et al., 2021), highlighting the significant potential of ethanol pre-treatment for increasing the susceptibility of granular maize starch to enzymatic modification. The yield of EMB1 was further decreased by 42% when compared with EMA1 and EBE1. After increasing the dosage of MA (EMA2), BE (EBE2), and their combination (EMB2), similar yield changes were found when compared with low dosage treatment (EMA1, EBE1, and EMB1), but the yield of these samples was lower.

3.2. Molecular size distribution of the raw starch

The amylose (AM) and amylopectin (AP) molecular pools in samples were classified by comparison with pure AM and AP standards. ETH

Table 2
Yield, swelling power and water solubility of modified granular maize starch samples.

Samples	Yield (%)	Swelling power (g/g)	Water solubility (%)
NMS	100.0 0.0^a	3.3 0.2^e	0.6 0.1^d
ETH	100.0 0.0^a	6.8 0.3^a	0.4 0.1^d
EBUF	91.0 2.5^b	6.4 0.2^b	0.3 0.3^d
EMA1	59.9 0.9^c	6.3 0.1^b	8.6 0.2^b
EBE1	53.1 0.7^d	6.0 0.1^c	0.5 0.3^d
EMB1	41.8 0.0^f	5.5 0.2^d	4.9 2.1^c
EMA2	46.3 0.7^e	5.8 0.3^c	17.0 0.7^a
EBE2	41.1 1.5^f	5.6 0.2^{cd}	3.0 0.3^c
EMB2	31.3 0.1^g	5.9 0.2^c	16.7 0.5^a

All data are means standard deviation (n ˆ 3). Values with different letters in the same column are significantly different at $p < 0.05$.

showed only a slightly higher signal than NMS in the elution volume (12.4–14.0 mL) of the AM region, whereas no difference was found in the AP region (Fig. 1 and Table S1), indicating that ETH slightly increased the molecular size of AM, possibly by forming amylose-ethanol complexes. In contrast, the signal at the same elution volume (12.4–14.0 mL) of EBUF samples decreased in comparison with ETH, implying that the proposed complexes dissociated during buffer incubation. As expected, the AP peaks of EMA1 and EMA2 shifted to a higher elution volume than EBUF, showing that higher MA dosage led to lower AP molecular size. The AP peak of EMA2-treated samples shifted to a higher elution volume than EMA1, indicating that, when applied at higher enzyme dosage, the hydrolytic activity of MA increased, corresponding to a lower yield. A significant area between 14 and 20 mL was also found for the EMA2 sample, indicating that more molecules eluted in the amylose region, which might be due to amylopectin hydrolysis. BE can cleave α-1,4-glucan chains from starch AP/AM molecules and transfer the cleaved chain segments to form an α-1,6-glucosidic linkage to the same (intramolecular transfer) or different (intermolecular transfer) molecule (Takata et al., 2010). The AP peak of EBE1 also shifted to a higher elution volume than EBUF, suggesting that BE exhibits intramolecular transfer activity when acting on ethanol pre-treated granular maize starch. In contrast, increased BE treatment (EBE2) shifted the AP peak to a lower elution volume, implying that BE at high dosage exhibited glucan transfer activity, which resulted in an increased molecular size of the products and is typical for intermolecular chain transfer. The intramolecular transfer reactions when acting on amylopectin substrate and the glucan transfer activity on amylose substrate of BE were possibly both contributing to this effect. The AP peak in the EMB1-treated samples was shifted to a higher elution volume than EMA1 and EBE1, indicating that combining MA and BE resulted in more efficient hydrolysis and/or intramolecular cyclization of AP molecules. The signal intensities in the amylose region of EMB1 were much higher than observed for the EMA1 and EBE1 treatment (Fig. 1). This effect suggests the generation of amylopectin-hydrolytic products eluting in the AM region or increased transfer activity of BE. There are mainly four possibilities to account for this effect: (1) hydrolytic cleavage of AP; (2) intramolecular transfer cyclization of AP; (3) the production of amylose molecules by α-1,4 – α-1,4 transfer (Feng et al., 2015); (4) α-1,6 – α-1,4 glucanotransferase activity. The AP peak of EMB2 resembled EM2 showing that amylopectin was not further hydrolyzed by BE. However, its AM peak was remarkably higher, mainly reflecting high cyclization or α-1,4 – α-1,4 glucanotransferase activity of BE (Feng et al., 2015; Zhong, Herburger, et al., 2021).

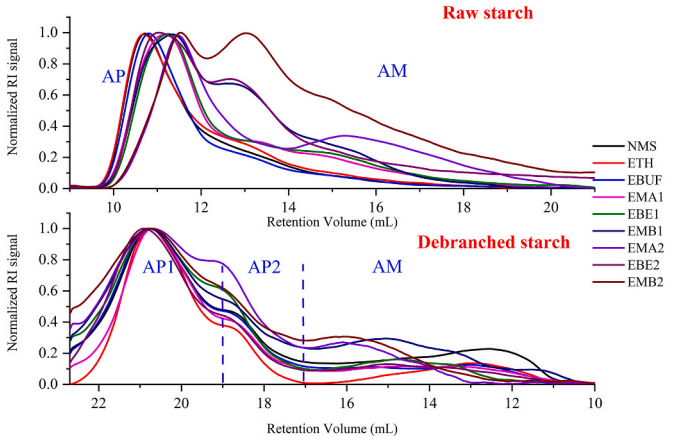


Fig. 1. Size-exclusion chromatography profiles of raw starches and debranched starches. Sample abbreviations as in Table 1.

3.3. Chain lengths distribution of debranched starch

Based on our standards and previous data (Tao, Li, Yu, Gilbert, & Li, 2019; Wenwen, Tao, Gidley, Fox, & Gilbert, 2019; Zhong et al., 2020), we classified our debranched samples into three components: short amylopectin (DP 6–36), long amylopectin (DP 37–100) and amylose chains (DP > 100) (Fig. 1 and Table S1). HPAEC (described in the section below) more accurately detects the amylopectin chain lengths distribution (CLD) than does a SEC setup. Thus, in our description of SEC data we focus on the debranching and changes of amylose chains, while results for amylopectin fine structure will follow in the next section. The elution volume was negatively correlated with the molecular size of material in the amylose region. To assess the amylose structure more accurately, we divided the chains into three fractions: short amylose (elution volume 17-16 mL), medium amylose (elution volume 16-13 mL), and long amylose (elution volume 13-10 mL). When compared with NMS, ETH treatment produced a lower RI signal throughout the AM region, in agreement with SEC profiles of raw starches as described above. However, EBUF samples showed a higher signal than ETH samples in the low elution volume region (13–16 mL), suggesting the generation of higher amounts of medium-sized amylose chains by the EBUF treatment. This might be due to the weak association of medium-sized amylose-ethanol complexes under gentle heating (50 C) in buffer, and thus more medium-sized amylose molecules were dissociated and possibly re-aggregated. MA-treated samples that did not undergo ethanol pre-treatment (two-fold higher MA dosage than used in the present study) showed a degradation in the amylose region (Zhong, Keeratiburana, et al., 2021). However, treating ethanol pre-treated samples with MA (EMA1) did not affect the amylose chains, suggesting that (1) ethanol pre-treatment exerted a protecting effect on amylose hydrolysis or (2) amylopectin is more readily attacked by MA after ETH pre-treatment and thus amylopectin is the primary substrate of BE in this system. The effect of EBE1 treatment on amylose was minor and only caused a slight decrease in long amylose chains and an increase in medium amylose chains. This indicates that BE can exert hydrolysis and/or inter/intramolecular transfer of cleaved chains onto amylose chains, corresponding to a decreased yield upon BE treatment. The amylose peaks in the BE-treated samples (EBE1 and EBE2) were similar, demonstrating that increased BE dosage had no effect on the amylose structure. Interestingly, the combined MA-BE (EMB1) treatment produced higher amounts of short and medium amylose chains when compared to treatments with MA only (EMA1) and BE only (EBE1). This suggests that combining MA and BE promotes the cyclic transfer or hydrolytic cleavage of amylose molecules, which might also stem from the α-1,4 – α-1,4 transglucosylase activity of BE to extend amylopectin chains. Combined MA-BE treatment with high BE concentration (EMB2) resulted in higher amounts of short and medium amylose chains when compared with combined MA-BE treatment with low BE concentration (EMB1), showing that high BE enzyme dosage was not saturating at this low BE level.

3.4. Chain lengths distribution of debranched amylopectin

The amylopectin chain lengths distributions (CLDs) (Fig. 2A and B) showed that the ETH and EBUF treatments had insignificant effects on the amylopectin structure. Likewise, pure BE treatments (EBE1 and EBE2, Fig. 2C, D, and 2F) had limited effects on amylopectin CLD – possibly due to intramolecular transfer reactions of BE. Pure MA treatments (EMA1 and EMA2) significantly increased the relative contents of amylopectin chains with DP 1–10 and DP > 30 and decreased the chains with DP 11–29 (Fig. 2C–E). This is consistent with the exo-acting mechanism of MA as previously demonstrated using raw starch granules without ETH pre-treatment (Zhong, Keeratiburana, et al., 2021), demonstrating that the starch chains associated with the granules are successively hydrolyzed from the non-reducing end, generating mainly maltose (Bijttebier, Goesaert, & Delcour, 2007). It is worth noting that

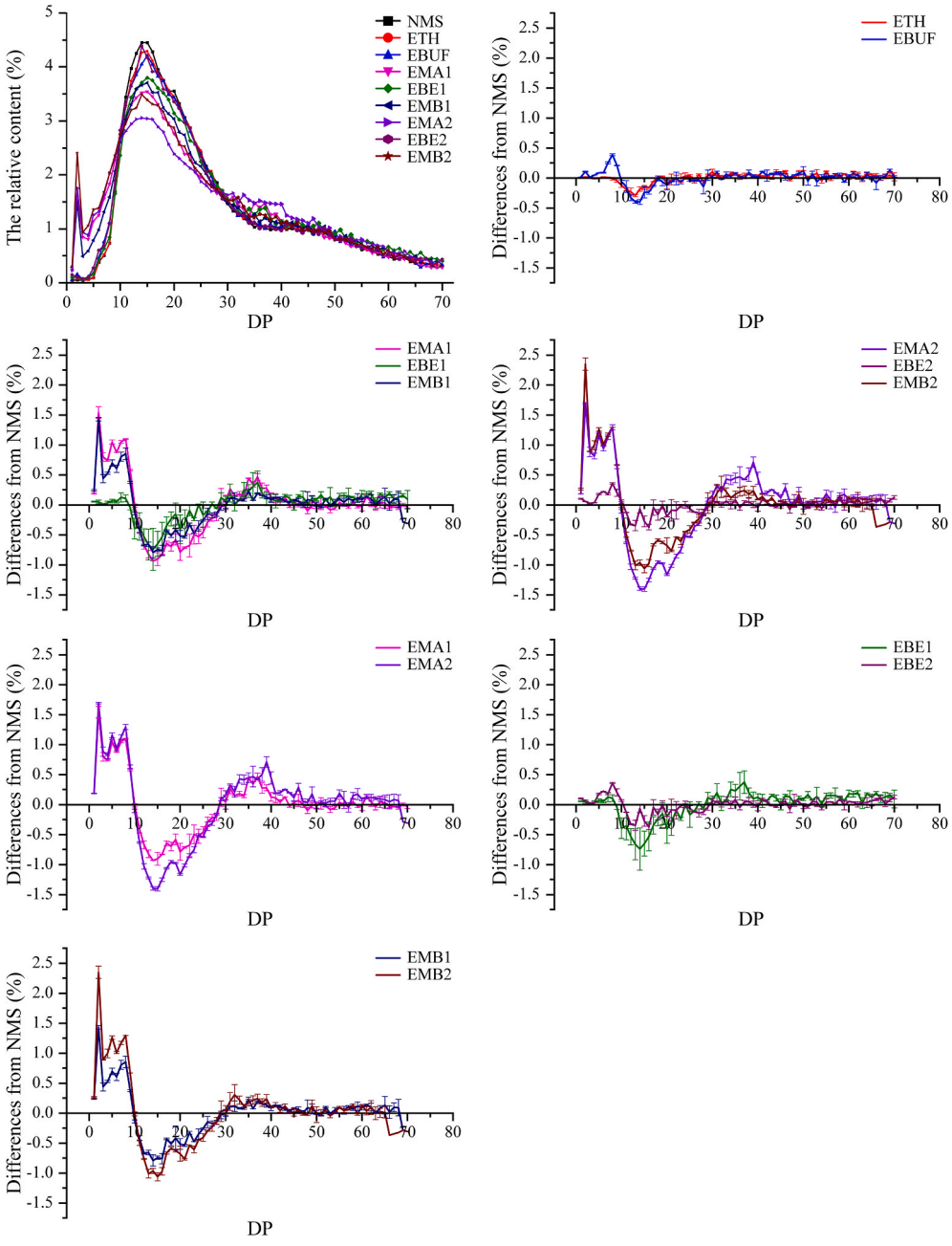


Fig. 2. Amylopectin chain lengths distributions of all samples (A) and the difference plots of treated samples compared with NMS (B–G). Sample abbreviations as in Table 1.

both treatments that combined MA-BE (EMB1 and EMB2) caused only minor increases in amylopectin chains with DP > 30 and less decreases in amylopectin chains with DP 11–29 than for the pure MA treatments (EMA1 and EMA2) (Fig. 2C and D). This again suggests that BE had negligible effects on the amylopectin CLD (Zhong, Herburger, et al., 2021).

3.5. Crystalline structure of starch

WAXS data (Fig. 3) revealed transformation of the crystalline allomorph from an A-type in NMS – characterized by two peaks at 2θ of 15 and 23 and an unresolved doublet at 2θ 17 and 18 (Cheetham & Tao, 1998) – to the typical V_h-type allomorph in ETH, showing two strong peaks at 2θ of 13 and 20 (Buléon, Colona, Planchot, & Ball, 1998). This is in agreement with the effect of ethanol on starch granules found in previous studies (Chen, Dai, & Gao, 2020; Choi, Baik, & Kim, 2017). This allomorph transition might stem from the dissociation of double helices in starch granules during heating, followed by the association of amylopectin and amylose chains with alcohol (Jane, Craig, Seib, & Hoseney, 1986). ETH samples also contained small amounts of A-type crystals, reflected as minor WAXS peaks at 2θ of 15 , 17 and 18 . From ETH to EBUF treatment, the crystalline allomorph was altered to a mixture of the B-type allomorph and the V_h-type allomorph with strongest peaks at 2θ of 17 and minor contributions at 2θ of 5 , 13 and 20 (Buléon et al., 1998). This might be due to the partial dissociation of V_h-type complexes when incubating ETH samples in buffer, accompanied by the re-association of amylose and amylopectin chains. All enzyme-treated samples showed the same crystalline allomorph as found for EBUF, demonstrating that MA and BE had no effect on the crystalline type of ethanol pre-treated granular starch systems. In

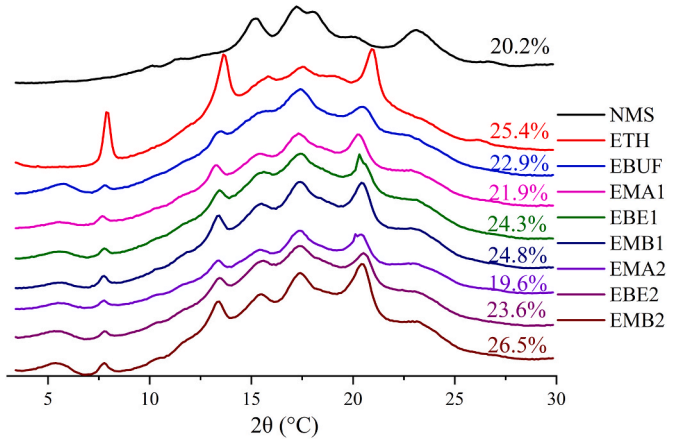


Fig. 3. Wide-angle X-racy scattering profiles of samples. Sample abbreviations as in Table 1.

contrast, when omitting ethanol pre-treatment, simultaneous treatment with MA and BE transformed the crystalline allomorph of NMS from the A-type to the B-type allomorph (Zhong, Herburger, et al., 2021). In the present study, EMAs and EBEs treatments significantly affected the relative crystallinity of starch (Fig. 4). MA treatments (EMA1 and EMA2) decreased the crystallinity of the EBUF samples. This in agreement with our previous study using native starch granules as substrate without ETH pre-treatment (Zhong, Keeratiburana et al., 2021) and likely due to the production of more short branch chains precluding the formation of crystalline aggregates (Hizukuri, 1985). In contrast, BE treatments (EBE1 and EBE2) slightly increased the crystallinity, confirming that BE can increase the B-type allomorph of granular NMS without ethanol pre-treatment by its α-1,6 – α-1,6 transfer reaction (Zhong, Herburger, et al., 2021); i.e., BE altered the positions of branches thereby producing a crystalline structure with less defects. As expected, MA ‡ BE treatments (EMB1 and EMB2) further increased the relative crystallinity of EBUF when compared with BE treatments only (EBE1 and EBE2). This shows that MA pre-treatments can assist BE in rearranging the chain assembly and promote crystallization of chain segments, increasing the crystallinity of the starch granules, possibly by decreasing the starch granular stability.

3.6. Morphology of starch

Significant effects on the starch granule morphology were found for the EBUF samples as judged by EF-SEM (Fig. 4). ETH treatment maintained the integrity and spherical/ellipsoidal shape of the NMS granules, whereas EBUF treatment produced aggregated and irregular/cubical granules (Fig. 4). This might be caused by the shrinkage of granules after incubation in buffer followed by the drying process (Singh & Singh, 2003). I.e., the evaporation of water inside starch granules induced the formation of deformed and twisted granules (Chen et al., 2020). The

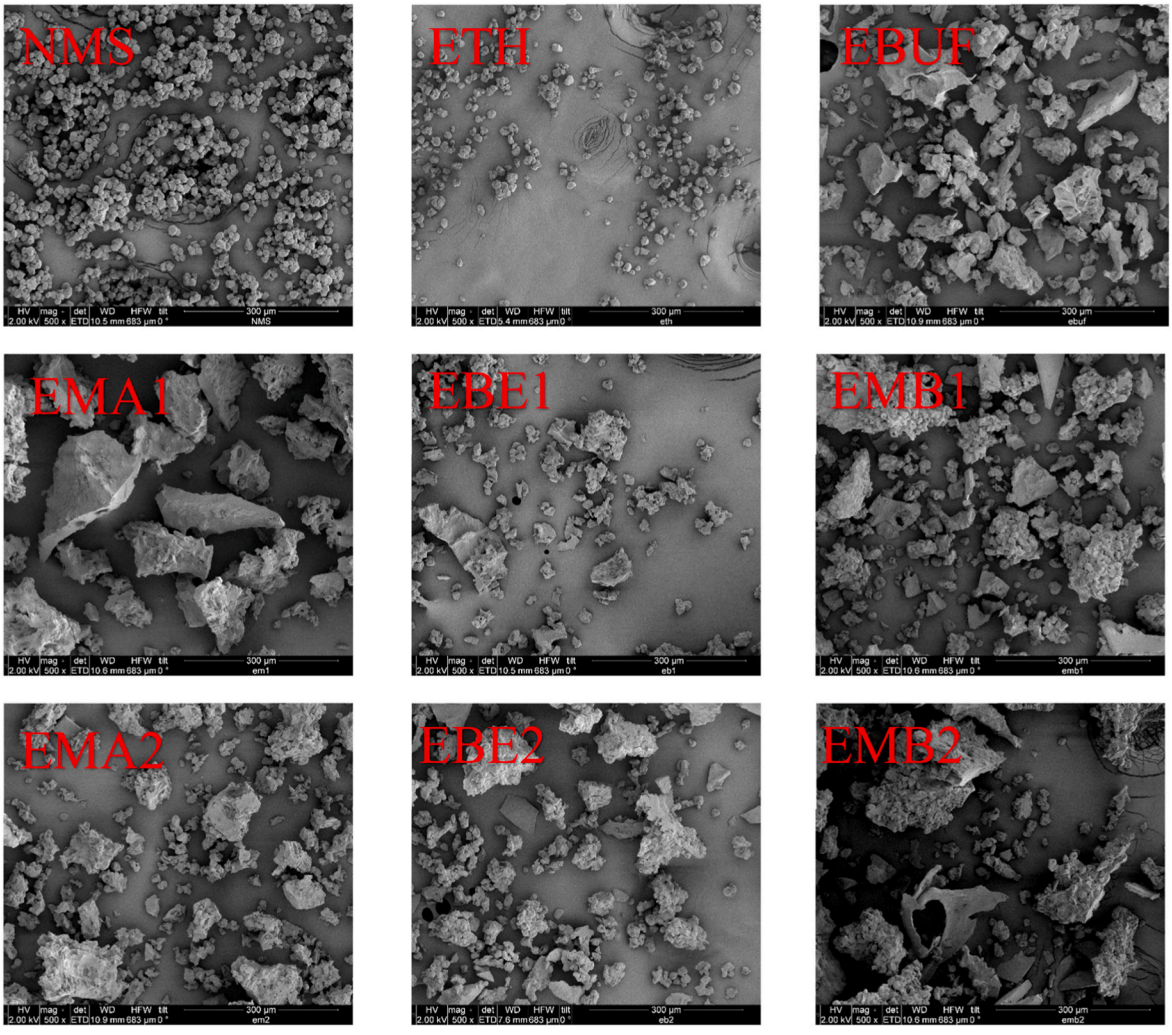


Fig. 4. FE-SEM images of differently treated granular starch. Sample abbreviations as in Table 1.

transformation of a regular granule shape in ETH to an irregular shape in EBUF also indicates the further decrease of granular stability of maize starch. As judged by EF-SEM (Fig. 4), further enzymatic treatments had insignificant effects on the morphology of the samples.

3.7. The -1,6 to -1,4 linkage ratio

Four types of anomeric protons, repesenting α-1,4 linkage, α-anomeric reducing end protons, α-1,6 linkage, and β-anomeric reducing end protons, were quantified from the ^{1}H NMR spectra (Fig. 5). As expected, ETH and EBUF had no effect on the signals of these protons, whereas MA alone (EMA1 and EMA2) significantly increased the relative ratio of α-1,6 to α-1,4 linkages (Fig. 5). This increase can be an effect of both the α-1,4 hydrolytic activity of MA releasing soluble maltooligosaccharides from the granules and α-1,4 – α-1,6 transfer reactions of MA. BE alone (EBE1 and EBE2) showed a slight decrease of α-1,6 to α-1,4 linkage ratio as for the NMS, ETH and EBUF controls, indicating the possibility of α-1,6 – α-1,4 transfer reaction of BE. The molecular profiles of raw starches, shown in the SEC-TDA section, demonstrated an increase in the amylopectin molecular size for EBE2 when compared to EBE1, suggesting possible intermolecular transfer reaction of BE in this ETH system. Furthermore, the WAXS profiles showed that EBE treatments increased the starch crystallinity, reflecting that BE also alters the amylopectin internal structure to accommodate chain re-assembly, possibly by changing the positions of branch points to promote a more ordered crystalline structure (α-1,6 – α-1,6 glucan transfer activity). Hence, we suggest that EBEs mainly exhibited intramolecular transfer reactions and α-1,6 – α-1,6 glucan transfer activity on starch granules, and relatively low α-1,6 – α-1,4 transfer activity. Combined MA and BE (EMB1 and EMB2) treatments reduced the α-1,6 to α-1,4 linkage ratio when compared with MA alone samples (EMAs), suggesting an α-1,4 – α-1,4 or α-1,6 – α-1,4 glucan transfer activity of BE, which is based on MA pre-treatment as reported before (Sorndech et al., 2015; Zhong, Herburger, et al., 2021). Interestingly, a previous study showed that MA→BE sequential treatments of native starch granules without ETH pre-treatment mainly increased the amount of long amylose chains (Zhong, Herburger, et al., 2021), whereas the present data show that the simultaneous MA and BE treatment increased the content of short and medium amylose chains (Fig. 1). This might be explained by BE preferably acting on amylose chains during the combined MA and BE modification (Zhong, Herburger, et al., 2021), whereas BE also acted on the amylopectin chains in the present study, as EBE samples exhibited a decreased amylopectin molecular size. Hence, the changes of starch

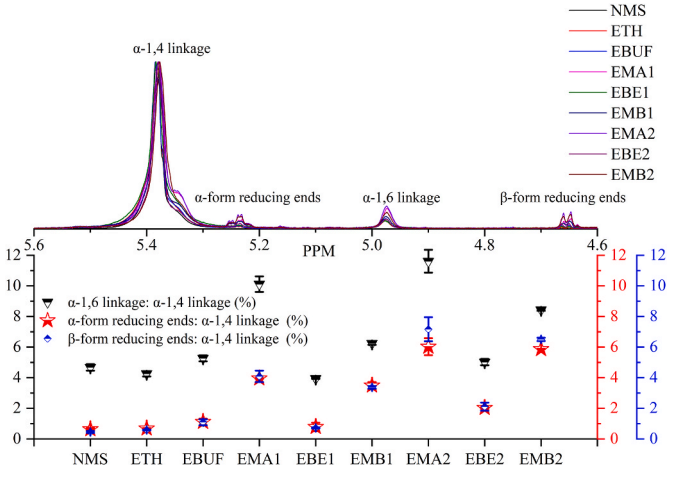


Fig. 5. One-dimensional ^{1}H NMR spectra of starch acquired in deuterium oxide and the percentage ratios of α-1,6 linkage, α-anomeric reducing end protons, and β-anomeric reducing end protons to α-1,4 linkage. Sample abbreviations as in Table 1.

structures induced by modification are determined by the parental starch. We found both the α- and the β-form of reducing ends: α-1,4 linkage ratios were increased by MA, substantiating the hydrolytic activity of MA in these granular catalytic systems.

Our previous study (Zhong, Keeratiburana, et al., 2021) hypothesized that the signals of α- and β-reducing ends might contain additional superimposed signals due to transfer reactions or specific strain on the glucose ring as an effect of very intense signals hiding potential additional signals. The reducing end signals were decreased by the combined MABE treatments. We offer three possible explanations for this observation: (1) the products of MA treatment were further hydrolyzed by BE, became more soluble, and were washed out during the sample preparation process; (2) MA products were adopted as efficient substrates for BE (Sorndech et al., 2015); (3) the catalytic competition between MA and BE prevented the transfer action of MA (Sorndech et al., 2015). We suggest that the second one is most plausible, because it explains why EMB2 showed similar AP peak and much higher AM area than EMA2. I. e., the increase in molecules in the amylose region in the EMB2 sample is not due to a significant hydrolytic activity of BE of amylopectin but due to its glucanotransferase activity of BE on MA-hydrolyzed products. Finally, our previous data on BE modified semi-gelatinized starch suggest an α-1,4 producing glucanotransferase activity of BE for specific solid state catalyses (Jensen et al., 2013).

3.8. Swelling power and water solubility

Ethanol pre-treatment doubled the swelling capacity of NMS (Table 2). This strong increase implies that ETH enhances the enzymatic susceptibility by increasing the granular surface area, in agreement with the general effects of cold swelling starch (Jane et al., 1986; Majzoobi & Farahnaky, 2021). However, the swelling power was continuously decreased from EBUF, to low-dosage enzymatic treated samples (EMA1, EBE1, EMB1), to high-dosage enzymatic treated samples (EMA2, EBE2, EMB2), showing that buffer incubation, enzyme treatments, and further increase of the enzyme dosage all decreased the swelling capability of maize starch granules. High amylopectin content in starch promotes granular swelling (Vamadevan & Bertoft, 2015) and correspondingly, our data suggests that degradation of amylopectin molecules reduced the swelling capacity (Keeratiburana et al., 2020a; Zhong, Keeratiburana, et al., 2021).

Single MA or BE treatments both showed that these enzymes reduce the molecular size of starch chains when acting on granules as reflected by the decrease of yield. However, MA-only treatment significantly increased the water solubility of samples, whereas BE treatment was less effective (Table 2), indicating that EMA samples contained more soluble compounds than EBE samples. This corresponds well with the hydrolytic activity of MA as demonstrated by NMR. EMB1 showed a significantly lower water solubility than EMA1, indicating that the effect of BE on retarding MA-catalyzed hydrolysis is possibly due to production of a crystalline structure with less defects and increased structural stability as discussed above. However, the water solubility of EMB2 and EMA2 did not show significant differences, implying that the hydrolytic effect of MA was dominant as the dosage of MA and BE increased.

3.9. Pasting profiles

ETH pre-treatment generated starch functionality with suppressed peak viscosity and reduced setback (Fig. 6) which is a desirable stable functionality in industry, typically achieved by chemical cross-linking. Excessive enzymatic modification retained this profile but further decreased the viscosity. Buffer treatment (EBUF) decreased the viscosity of ETH, possibly due to the dissociation of V_h-type crystals, which induce a further disordering of the crystalline region. The hydrolytic activity of MA and the intramolecular activity of BE in the ETH system both had an effect on disrupting the granular structure, reflected as yield loss and lower swelling capacity, resulting in a lower viscosity of the

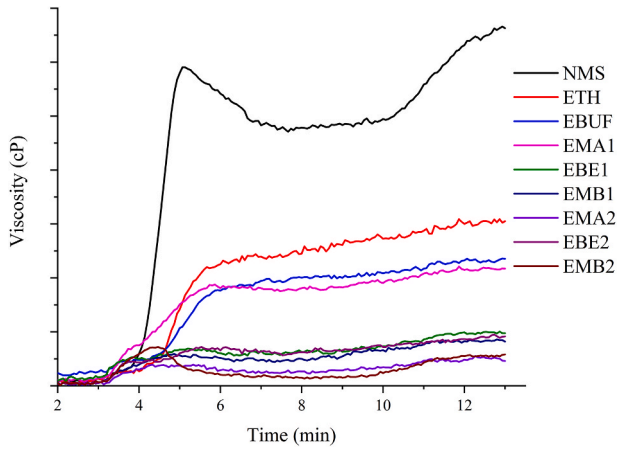


Fig. 6. RVA pasting profiles of granular samples. Sample abbreviations as in Table 1.

enzymatically treated samples when compared to the EBUF samples. A similar result was found for rice starch (Keeratiburana et al., 2020a), suggesting that the effect of MA and BE on pasting properties of ethanol pre-treated granular starch is not species-specific.

3.10. In vitro digestion

The hydrolytic digestibility was analyzed for both the raw granular starch systems and for gelatinized and for 1-day retrograded systems (Table 3). In the raw starch system, the content of rapidly digested starch (RDS) increased considerably by the ethanol treatment (ETH), reflecting the effectiveness of ethanol pre-treatment to disrupt the crystalline structure of granules, which increases their amylolytic susceptibility (Zhang, Dhital, Haque, & Gidley, 2012). For the buffer treatment (EBUF), the RDS content was not altered but the content of resistant starch (RS) increased significantly, while the slowly digested starch (SDS) content decreased correspondingly. As mentioned above, treatment with ethanol and buffer incubation caused V_h-type crystals to dissociate and B-type crystals to form. Hence, the digestibility data here suggest that the newly formed B-type crystals exhibited a higher amylolytic resistance than V_h-type crystals. In agreement with our previous data on modified rice granules (Keeratiburana et al., 2020a; Zhong, Herburger, et al., 2021), we also found that MA treatment increased the amylolytic resistance of granular starch, i.e., the sum of SDS and RS contents in both EMA1 and EMA2 were higher than those of the EBUF samples. It is worth mentioning that low dosage of MA (EMA1) led to an increase in SDS, while higher MA dosage (EMA2) increased the RS content, demonstrating that MA dosage is important for enzymatic resistance of the product. A major reason for these results might be a continuous increase of the α-1,6 to α-1,4 ratio from EBUF to EMA1 to EMA2 samples (Fig. 5), which would prevent the amylolytic effect of amyloglucosidase (Ao et al., 2007) by increasing the steric hindrance for this hydrolase. Increasing BE dosage stepwise increased the amylolytic susceptibility of the starch granules, reflected by a decrease in RS and an increase in SDS. We suggest this was attributed to the intramolecular transfer activity of BE. In contrast, combined MA ‡ BE treatments increased the amylolytic resistance of starch granules as documented by the increased RS content found in the series from EBUF to EMB1 and EMB2 treatments. We suggest that BE utilized the hydrolysis products generated by MA as a substrate to generate new amylose chains (Fig. 1), thereby increasing amylose re-association during the AM-catalyzed reactions.

Table 3
Digestion parameters of raw starches and retrograded starches.

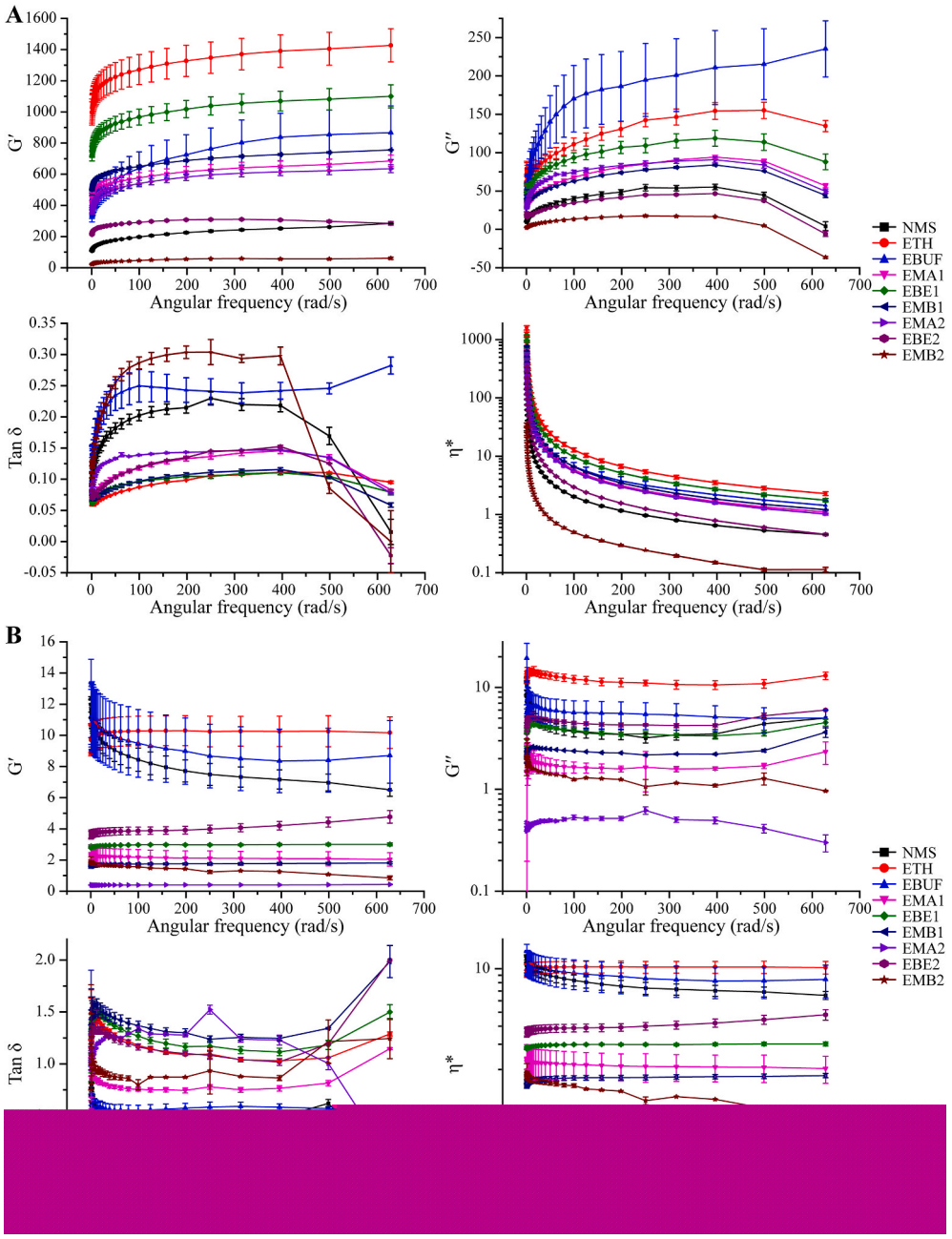
Samples	Native			Retrograded		
	RDS (%)	SDS (%)	RS (%)	RDS (%)	SDS (%)	RS (%)
NMS	30.7	46.3	22.9	69.3	18.5	12.3
	0.6^{d}	0.9^{a}	1.4^{e}	3.4^{c}	5.6^{a}	2.1^{de}
ETH	68.7	23.3	8.0	71.3	14.9	13.9
	0.0^{a}	1.3^{b}	1.3^{h}	1.6^{bc}	2.0^{a}	0.4^{d}
EBUF	67.5	6.6	25.8	80.5	8.4	11.4
	1.8^{a}	0.5^{f}	1.3^{d}	1.5^{a}	1.1^{c}	0.4^{e}
EMA1	61.2	17.6	21.1	67.5	6.2	26.3
	1.5^{b}	1.3^{c}	0.1^{f}	1.9^{c}	1.8^{c}	0.1^{b}
EBE1	65.8	11.1	23.0	75.3	3.4	21.2
	0.2^{a}	1.2^{d}	1.5^{e}	1.7^{b}	1.8^{d}	0.0^{c}
EMB1	61.8	10.9	27.3	66.1	4.9	29.0
	0.8^{b}	0.3^{d}	0.4^{c}	1.4^{c}	0.3^{d}	1.7^{a}
EMA2	60.8	8.7	30.5	60.2	11.2	28.7
	0.4^{b}	1.6^{e}	1.3^{b}	1.0^{d}	0.4^{b}	1.4^{a}
EBE2	62.0	17.5	20.5	73.4	7.0	19.6
	0.9^{b}	0.8^{c}	0.1^{g}	1.6^{b}	0.1^{c}	1.7^{c}
EMB2	56.6	4.6	38.8	59.3	11.3	29.3
	1.3^{c}	0.1^{g}	1.2^{a}	1.4^{d}	0.3^{b}	1.8^{a}

All data are means standard deviation (n ˆ 3). Values with different letters in the same column are significantly different at $p < 0.05$. RDS: rapidly digested starch; SDS: slowly digested starch; RS: resistant starch.

The digestibility of retrograded starch is predominantly related to compact structures formed during the reorganization of starch chains during the retrogradation process (Sajilata, Singhal, & Kulkarni, 2006). ETH exhibited a similar molecular structure of starch as NMS (Figs. 1 and 2), and as expected, the content of RDS, SDS, and RS in retrograded ETH was similar to that of the retrograded NMS (Table 3). EBUF showed a minor degradation of the amylopectin molecules (Fig. 1), which may have hindered amylopectin reorganization and the following amylolytic resistance. Accordingly, EBUF treatment resulted in a higher RDS content and lower SDS and RS content. Similar to the effect of MA on raw starch system without ETH pre-treatment (Zhong, Keeratiburana, et al., 2021), EMAs increased the amylolytic resistance of samples related to an increased α-1,6 to α-1,4 ratio. However, in the gelatinized starch system (Ao et al., 2007), molecular movement and reorganization is more dynamic when compared with raw starch. As a consequence, we found that RS increased moderately for the low MA enzyme dosage (EMA1) product, but for the low BE product (EBE1), a remarkable increase in RS was observed implying that the effect of BE was primarily due to rearrangement of branch positions in amylopectin and a resulting increase in the structural stability of the starch granules. The SEC profiles of the raw EBUF and EBE1 samples (Fig. 1) showed that the latter had a higher signal at elution volume 13–20 mL, suggesting that EBE1 contained more low molecular weight material (mainly short and medium amylose chains and/or hydrolyzed/cyclic amylopectin segments). Due to their high mobility, we suggest that these molecules were the main cause of the increased RS content observed (Gong, Cheng, Gilbert, & Li, 2019). Increasing the dosage of BE (EBE2) further increased the amylolytic resistance as indicated by an increased SDS content, while the RS content remained the same. This might be due to the further increase of relatively short amylose molecules. The combined MABE treatment (EMB1) showed a higher RS content than the single enzyme treatments (EMA1 and EBE1), demonstrating the potential of simultaneous MA and BE catalysis to increase the amylolytic resistance of starch. We suggest that this was mainly due to a further increase of relatively short linear molecules. Interestingly, EMB2 showed a similar RS content and higher SDS than the lower BE dosage (EMB1), implying an even higher content of these short chain or chain segments, which accelerated the formation of RS.

3.11. Effects on paste rheology

The rheological behavior of fresh (1-day) (Fig. 7A) and 7-day stored starch pastes (Fig. S1) were analyzed to derive four characteristic parameters: storage modulus (G^{0}), loss modulus (G^{00}), loss tangent (G'/G^{00}; tan δ), and the modulus of complex viscosity (η*). We found classical gel-behavior for all starch pastes (G^{0}> $G^{00}$and tan δ < 1).



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