Food Hydrocolloids 120 (2021) 106904

Contents lists available at ScienceDirect

Food Hydrocolloids

journal homepage: www.elsevier.com/locate/foodhyd

Sequential maltogenic α-amylase and branching enzyme treatment to modify granular corn starch

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ARTICLE INFO

Keywords:
Starch
Maltogenic α-amylase
Branching enzyme
Solid state modification

ABSTRACT

Due to the semi-crystalline structure of native starch granules, enzymatic modification of these solid, raw, entities by branching enzyme (BE) is limited. Here, we describe a method to efficiently modify starch by BE after maltogenic α-amylases pre-treatment. This pre-treatment produced pores at the starch granule surface, which decreased the granular yield, but increased the branching degree in starch molecules. BE post-treatments recovered the yield, increased the content of long amylose chains, and the starch crystallinity. WAXS analysis showed that BE transformed the unresolved doublet peak at 2θ 17° and 18° to a strong peak at 2θ 17°, i.e. transformed the granules from the A-type to a mixed A-, B-type allomorph. Syneresis of starch gels increased with increasing BE concentrations and increased the content of slowly digested starch in retrograded starch preparations. Rheology data demonstrated that low and medium BE concentrations produced starch gels with higher G′ and G″ after storage for 1day, whereas high BE concentrations reduced both G′ and G''. Our data demonstrate the potential of clean, enzyme-based protocols using sequential addition of starch active enzymes for post-harvest modification of raw starch granules to obtain clean and functional starch.

1. Introduction

Maize starch is widely used to produce food, feed, fuel, materials and chemicals. An efficient utilization of starch for industrial applications usually requires its modification, which typically includes modulation of the digestibility and retrogradation of starch. The three main types of starch modification entail physical, chemical and/or enzymatic treatments. In recent years, enzymatic modification is gaining in importance as it provides a more environmentally friend and tailored strategy to produce desired starch products. The compact structure of intact, raw starch granules limits their modification via enzymes. In particular, the internal parts of the granules are hardly accessible and thus enzymatic modification is typically carried out on gelatinized starch. However, maintaining the solid state of the granule without gelatinization is highly attractive for industry, because it avoids handling expensive and energy-demanding issues associated with gelatinization and the resulting highly viscous and unstable systems (Zhong et al., 2021).

Starch branching enzyme (BE; EC 2.4.1.18), belonging to the glycoside hydrolase family 57 (GH57), or family 13 (GH13) transfers oligosaccharides of an (1,4)- α-D-glucan chain to a primary hydroxyl group on the 6 position of an adjacent glucan chain (Jensen, Larsen, Bandsholm, & Blennow, 2013). Several studies have reported that BE produces highly branched glucans when acting on preparations of gelatinized starch (Ao et al., 2007; Le et al., 2009; Lee et al., 2008), extruded starch (Martínez, Pico, & Gómez, 2016) and on semi-solid-state starch (Jensen, Larsen, et al., 2013). In contrast, the effect of BE on raw starch is only poorly understood. As shown before, potato starch granules are a poor substrate for BE (Guo, Deng, Lu, Zou, & Cui, 2019) and as expected, our preliminary data suggest that the same is true for maize starch granules.

Maltogenic α-amylase (MA; EC 3.2.1.133) from GH13 exhibits both *exo*-activity (releasing successive maltose in the α-configuration) and *endo*-glucanase activity and can, due to its structural similarity to cyclodextrinases (CGTases), both transglycosylate and hydrolyze

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https://doi.org/10.1016/j.foodhyd.2021.106904
Received 17 February 2021; Received in revised form 16 April 2021; Accepted 16 May 2021
Available online 19 May 2021

maltotriose at high substrate concentration (Christophersen, Otzen, Noman, Christensen, & Schäfer, 1998). Our previous study documented that pretreatment of granular starch with MA at sub-gelatinization temperatures creates pores and internal channels in starch granules, which allows transglucosidase (TGA; EC 3.2.1.20) from GH31 to act upon (Zhong et al., 2021). TGA produces a new α-(1 → 6) bond by transferring glucosyl residue from α(1 → 4)-glucans (donor) to malto-oligosacharides (acceptor) (Ota, Okamoto, & Wakabayashi, 2009).

Accordingly, combining MA and BE could provide a route to explore the effect of BE on granular native maize starch. We hypothesized that the pretreatment of starch granules with MA increases the activity of BE when acting on granular starch similar to what we observed with TGA. Our preliminary data from size exclusion chromatography (SEC) suggest that BE-modified the starch molecular structure when using an order-of-magnitude higher MA concentration than in our previous MA + TGA experiments (Zhong et al., 2021). Thus, a major purpose of the present study is to explore the consequences of different BE concentrations on the structure and properties of maize starch granules.

2. Materials and methods

2.1. Starch and enzyme sources

Normal maize starch (NMS) (Commercial Clinton 106) was kindly provided by Archer Daniels Midland (ADM, Decatur, IL). Maltogenic α-amylase (MA) (Maltogenase, 41,530 U/mL) and branching enzyme (BE) (E.C.2.4.1.18, GH57) were kindly supplied by Novozymes, Bagsværd, Denmark. Isoamylase (E-ISAMY, 200 U/mL) and pullulanase (E-PULBL, 700 U/mL) were purchased from Megazyme (Ireland). Potato amylose (A0512) and other chemical reagents were from Sigma-Aldrich (USA).

2.2. Reducing ends produced by branching enzyme

Potato amylose (20 mg/mL) was solubilized in 1 M NaOH and incubated overnight at room temperature. Then the pH was adjusted to 7 with 2 M HCl and diluted to 5 concentrations from 0.05 to 0.3 mg/mL in 0.05 M phosphate buffer (pH = 7). Samples were pre-heated to 60 °C for 10 min, and then 1 μg/mL BE was added for 20 min until the reaction was stopped by heating at 99 °C for 15 min, while the corresponding blank samples was heated to 99 °C directly after BE addition. Next, debranching was performed by adding 1 μL of isoamylase (Megazyme) and 1 μL of pullulanase (Megazyme) and incubating at 40 °C for 120 min. Produced reducing ends were quantified using the BCA protocol (Hizukuri, Takeda, Yasuda, & Suzuki, 1981; Park & Johnson, 1949). Maltose was used as a standard and each analysis was repeated 6 times. One unit of branching activity is defined as 1 μmol of α-1,6-glucosidic linkages synthesized per minute by using amylose (0.15 mg/mL) as a substrate, i.e., the difference of the amounts of reducing ends in amylose before and after debranching.

2.3. Sequential modification of raw corn starch by maltogenic α-amylase and branching enzyme

Raw, native starch granules (10%, w/w) were treated with MA (520 U/g) in 50 mM acetate buffer (pH 5.5) containing 5 mM $CaCl_2$ at 60 °C while stirring for 3 h. The reaction was stopped by adjusting the pH of the solution to 11 (2 M NaOH) and incubated for 10 min. The inactivation of MA was tested by comparing the amount of maltose in MA treated samples before and after inactivating MA activity (incubated 3h) by HPAEC-PAD, and we secured that the amount of maltose was not changed significantly upon MA inactivation. Following adjustment of the pH back to 5.5 (2 M HCL), BE was added and the reaction mixture incubated at 60 °C for 20 h. The BE reaction was terminated as described above. The remaining salts were removed by washing the granules in MilliQ water 3 times, followed by lyophilization. The experimental setup is summarized in Table 1. Three control samples were used: 1). NMS only; 2). NMS only in acetate buffer for 20 h (BUF); 3). NMS treated with BE for 20 h (BE).

Table 1
The experiment design of dual MA and BE treatment (n = 3).

Sample code	Acetate buffer	MA (3h) U/g	BE (20 h) U/g
NMS	No	0	0
BUF	Yes	0	0
BE	Yes	0	20×10^{-5}
MA	Yes	520	0
MABE1	Yes	520	1×10^{-5}
MABE2	Yes	520	4×10^{-5}
MABE3	Yes	520	20×10^{-5}

Abbreviations: NMS, normal maize starch; BUF, NMS only in acetate buffer for 20 h; BE, branching enzyme; MA, maltogenic α-amylase.

2.4. Yield and syneresis

Yield was defined as the dry weight of non-soluble substrate relative to the original dry weight. The syneresis of starch gels was determined as follows: A starch suspension (6%, w/v in water) was heated to 90 °C while stirred for 30 min, cooled down to room temperature and stored at 4 °C for 72 h. Syneresis of samples, treated as listed in Table 1, was measured as the amount of water in % released after centrifugation at 8000 g for 20 min.

2.5. Size-exclusion chromatography (SEC)

Both native and debranched samples (1 mg/mL) were analyzed by using a size exclusion chromatography triple detection array (SEC-TDA, Viscotek, Malvern, UK) system, equipped with tandem GS-520HQ/GS-320HQ Shodex columns attached to a TDA302 detector array (Song et al., 2019). 100 μL of sample was injected and eluted in ammonium formate (10 mM) at a flow rate of 0.3 mL/min. The column temperature was 60 °C. The OmniSec Software 4.7 (Malvern Instrument, Ltd, UK) was used for data analysis. Pure maize amylose and amylopectin from maize before and after debranching were used as standards. Starches were gelatinized at 99 °C for 1 h and then debranched by 2 μL isoamylase and 2 μL pullulanase at 40 °C for 3h.

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

Gelatinized and debranched starch (5 mg/mL) was injected onto a CarboPac PA-200 column attached to an HPAEC-PAD (Dionex, Sunnyvale, CA, USA) system (Song et al., 2019). Peak integration and detector response were performed and the chain lengths distribution was calculated (Blennow, Bay-Smidt, Wischmann, Olsen & Møller 1998).

2.7. Wide angle X-ray scattering (WAXS)

The crystalline allomorphs and crystallinity were measured by adjusting the relative humidity of starch granular samples to 90% in a chamber for 48 h, followed by analysis using a SAXSLab instrument (JJ-X-ray, Copenhagen, Denmark) equipped with a 100 XL + microfocus sealed X-ray tube (Cu-$K_\alpha$radiation, Rigaku, The Woodlands Texas, USA) and a 2D 300 K Pilatus detector (Dectris Ltd, Baden, Switzerland). Hydrated samples were sealed between 5 and 7 μm mica films *in vacuo*. Standard reduction software (SAXSGUI) was used to average and correct the two-dimensional scattering data. The radially averaged intensity I is given as a function of the scattering angle 2θ in the angular range of 5°–30° using a wavelength of 0.1542 nm (Zhong et al., 2021). Relative crystallinity was calculated as described before (Brückner, 2000; Goldstein et al., 2017).

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

One-dimensional ^{1}H NMR spectra of starch samples were acquired using a 600 MHz NMR spectrometer (Bruker Avance III, Bruker Biospin, Rheinstetten, Germany) at the Department of Food Science (University of Copenhagen). The details of the samples preparation and NMR spectra acquisition are described elsewhere (Zhong et al., 2021). The degree of glucan branching of starch samples was estimated using areas of signals representing anomeric protons (δ 5.35–5.45 α1,4; δ 4.95–5.00 α1,6; δ 5.20–5.24 α-anomeric reducing end protons; δ 4.64–4.68 β-anomeric reducing end protons). Areas of signals representing anomeric protons were detected and calculated using SigMa software (Khakimov, Mobaraki, Trimigno, Aru & Engelsen 2020).

2.9. Scanning electron microscopy

The morphology and topography of the starch granular samples were analyzed after fixing and sputter-coating granules with gold and subsequent imaging using field emission scanning electron microscopy (FE-SEM) (FEI Quanta 200) (Zhong et al., 2021).

2.10. In vitro digestion

In vitro digestion of raw starch and retrograded starch (99 °C heating for 1 h followed by storage at 4 °C for 1 day) were analyzed using a modified Englyst protocol (Englyst, Englyst, Hudson, Cole, & Cummings, 1999; Zhang et al., 2011). Starch (100 mg dw) and water (5 mL) were mixed in a 50 mL polypropylene centrifuge tube containing five glass beads. After vortexing for 5 min, 10 mL sodium acetate buffer (0.1 M, pH 5.2) was added. The dispersion was equilibrated in a shaking water bath at 37 °C for 30 min, followed by adding 18.75 mg pancreatin and 13.4 μL amyloglucosidase in 2.5 mL 0.1 M sodium acetate buffer. Aliquots (0.1 mL) were taken after 20 and 120 min. The reaction was stopped by adding 1 mL 95% ethanol. After centrifugation of the dispersion at 5000 g for 10 min, the glucose content was determined using the Megazyme GOPOD kit (K-GLUC, Megazyme). The amount of starch digested between 0 and 20 min was defined as rapidly digested starch (RDS), and the starch digested between 20 and 120 min as 'slowly digested starch' (SDS). The remaining residues were defined as 'resistant starch' (RS).

2.11. Pasting- and dynamic gelling properties

The pasting behavior of 8% (w/v) starch pastes was measured using a Rapid Visco Analyzer (RVA, Newport Scientific, Australia) and ICC Standard Method No. 162. Starch gels prepared from the RVA were used for rheological analysis by a Discovery HR-3 Rheometer (TA Instruments) at 25 °C immediately. The remaining gels were stored at 4 °C for 7 days and analyzed as described above. The following parameters were calculated: storage modulus (G′), loss modulus (G″), loss tangent (G'/G″, tan δ), and the modulus of complex viscosity (η*).

2.12. Statistical analyses

WAXS and ^{1}H NMR analyses were performed once for each sample. All other experiments were performed at least in duplicates. When appropriate, statistically significant differences were analyzed using one-way analyses of variance (ANOVA) followed by Duncan's test ($p < 0.05$) using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Reducing ends produced by BE

The hydrolytic activity of BE was analyzed by incubating BE-treated amylose with and without addition of isoamylase and pullulanase (debranching enzymes). The α-1,4 → α-1,6 transglucosylation activity of BE was analyzed by comparing the amounts of reducing ends of amylose incubated with BE before and after debranching (Fig. 1). BE showed optimum α-1,4 → α-1,6 transglucosylation activity at 0.15 mg/mL amylose concentration, while the activity decreased at higher amylose substrate concentrations. Hence, the activity of BE was 0.001 U/mL, according to the definition described in section 2.2.

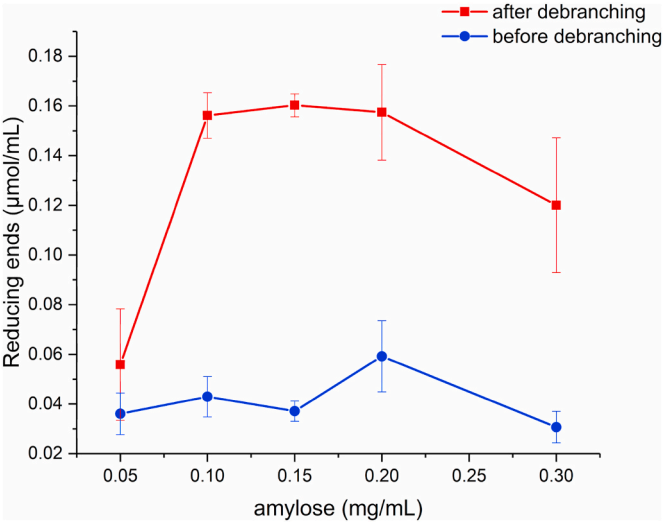

Fig. 1. The branching and hydrolytic activity of branching enzyme (BE). The activities are quantified by following the increase in reducing ends over time. Maize amylose standard was incubated with BE (1 μg/mL) in phosphate buffer with pH 7.0 at 60 °C. The samples were debranched by isoamylase and pullulanase.

Table 2
Yield and syneresis when offering starch gels to various enzyme preparations.

Sample code	Yield (%)	Syneresis (%)
NMS	100.0 ± 0.0^a	35.2 ± 0.5^e
BUF	90.7 ± 0.7^b	36.3 ± 0.5^d
BE	90.6 ± 0.3^b	36.5 ± 1.2^d
MA	69.9 ± 0.4^d	37.8 ± 1.3^d
MABE1	67.8 ± 0.1^e	40.8 ± 0.5^c
MABE2	69.5 ± 0.3^d	52.6 ± 1.5^a
MABE3	72.2 ± 0.2^c	47.2 ± 0.3^b

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

3.2. Yield and syneresis

Starch granules incubated in buffer (BUF) had a 90% yield of insoluble material due to the loss of solids during the washing process. Incubating starch with BE resulted in a similar yield as compared with BUF (Table 2). As expected, MA incubation significantly ($p < 0.05$) reduced the yield to ~70%. Post-treatment of MA-treated samples with a low dosage of BE (MABE1) slightly further decreased the yield; interestingly, the yield slightly increased linearly when the BE concentration was increased to medium (MABE2) and high (MABE3). This indicates that the soluble oligosaccharides and polysaccharides produced by MA were re-grafted onto starch molecules by BE.

BUF, BE, and MA treatments showed negligible effect on the syneresis of the starch gels. However, unexpectedly, we found that BE significantly increased the syneresis level of starch gels when pre-treated with MA, especially in the medium MA dosage group (MABE2) (Table 2). During syneresis, water is expressed from the starch network (retrogradation), i.e. syneresis indicates the tendency of starch preparations to retrograde (Karim, Norziah, & Seow, 2000). As shown before

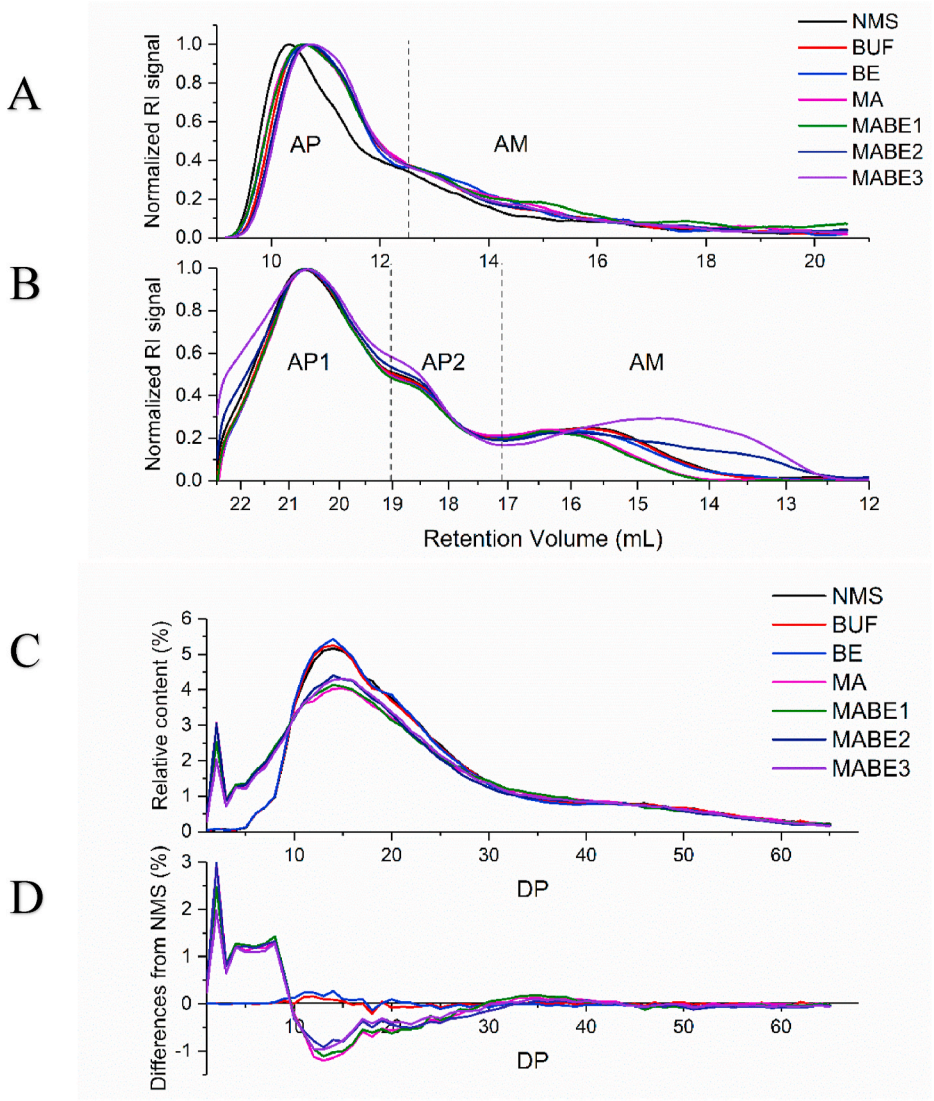

Fig. 2. Molecular structure of A) native starches and B) debranched starches analyzed by SEC, C) Chain length distribution of amylopectin analyzed by HPAEC, D) different chain length distributions of amylopectin samples (NMS set as control). Abbreviations: BE, branching enzyme; BUF, buffer; MA, maltogenic α-amylase; NMS, normal maize starch.

(Ao et al., 2007; Le et al., 2009; Lee et al., 2008), BE decreases retrogradation in gelatinized starch systems by creating branches in amylose/amylopectin. Therefore, our data suggest that BE exhibits different modification patterns when acting on granular starch or MA pre-treated starch.

3.3. Molecular size distribution

Chain length distribution (CLD) profiles derived from SEC data of raw (not debranched) starches revealed 2 main components that can be referred to as amylopectin and amylose (Fig. 2A). Buffer incubation of starch granules and treatments by MA alone or BE alone had negligible effects on amylopectin degradation. Interestingly, we recently found that starch granules treated with MA at relatively low concentrations significantly hydrolysed amylopectin molecules (Zhong et al., 2021). In contrast, at high MA concentration (10-fold higher) as used in our study, we surprisingly found low hydrolytic activity. Therefore, we suggest that this effect might be due to high hydrolytic activity of MA at low concentrations (Zhong et al., 2021) and higher transglucosylation activity at high MA concentrations as documented by the high ratio of α-1,6 to α-1,4 linkages found for the MA-treated samples. The CLD profiles of debranched samples (Fig. 2B) exhibited three main, but overlapping, components: short amylopectin (DP 6–36), long amylopectin (DP 37–100) and amylose chains (DP > 100), as previously reported (Zhong et al., 2020). As expected, buffer alone and BE alone had no effect on CLD profiles of debranched samples, and MA treatment resulted in the hydrolysis of preferably amylose chains, which is consistent with the effect of lower MA dosage on granular starch (Zhong et al., 2021). The MABE1 treatment profile was similar to the MA only treatment, indicating that BE did not alter the molecular structure when used at a low concentration. However, increasing the BE dosage surprisingly increased the amount of amylose and the amylose peak moved to a position at a lower retention volume, i.e. corresponding to a higher molecular weight. This demonstrates that, despite the well documented α-1,4 → α-1,6 transferase activity of BE, high BE dosage can increase both the relative amount of amylose chains and the amylose chain lengths rich in α-1,4 bonds.

3.4. Amylopectin chain lengths distribution

As demonstrated by HPAEC-PAD analyses, both MA and MABE treatments significantly affected the CLD of amylopectin (Fig. 2C and D).

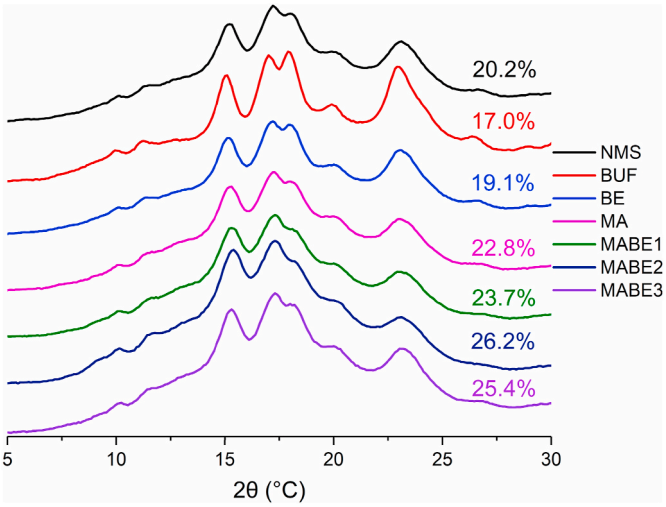

Fig. 3. WAXS spectra. Abbreviations: BE, branching enzyme; BUF, buffer; MA, maltogenic α-amylase; NMS, normal maize starch.

In contrast, incubation with buffer and treatment with BE only had neglectable effects, supporting our data from yield analyses and SEC above. As expected from its catalytic specificity, incubation of starch granules with MA increased the amount of chains with DP 2–9, while amylopectin chains with DP 10–29 strongly decreased (Fig. 2D). Interestingly, incubation with BE slightly decreased chains with DP 2–9, while chains with DP 10–29 increased, suggesting that BE elongates short amylopectin chains with DP 2–9 to longer amylopectin chains with DP 10–29. In contrast, incubation with transglucosidase (TGA) increased DP 2–9 chains and reduced the amount of DP 10–29 chains (Zhong et al., 2021), indicating different mechanism of BE and TGA when modifying amylopectin molecules, even though the predominant activity of both BE and TGA is transglucosylation.

3.5. Crystalline structure

WAXS patterns revealed that incubation with buffer and treatment with MA or BE alone had no significant effect on the crystalline allomorph (Fig. 3). Strong diffraction peaks (2θ) occurred around 15° and 23°, and an unresolved doublet at 2θ 17° and 18°, demonstrating an A-type crystalline allomorph (Cheetham & Tao, 1998). However, BE post-treatment (MABE) displayed a tendency to alter the doublet peak to single a peak, i.e., BE post-treatment induced a weaker peak at 2θ 18° and a stronger peak at 2θ 17°, showing that BE affected the crystalline allomorph to some degree. The presence of a large peak at 17° is a typical feature of the B-type allomorph (Cheetham et al., 1998). Generally, the transformation from A-type allomorph to C- and B-type allomorphs occurs when the amylose content increases (Cheetham et al., 1998), which is consistent with an increased amylose content upon BE post-treatment as monitored by SEC (Fig. 2B). Therefore, our results suggest that BE post-treatment altered the A-type allomorph to B-type allomorph by increasing the amount of amylose chains.

Moreover, the buffer-incubated starch granules showed a slightly lower crystallinity than native starch granules, which is possibly due to the non-enzymatic hydrolysis of amylopectin molecules (Fig. 2A), corresponding to our previous study (Zhong et al., 2021). BUF treatment can be regarded as a type of annealing physical modification, which requires the presence of moisture and a temperature that is higher than the glass transition temperature and lower than the gelatinization temperature (Schmiele, Sampaio, Gomes, & Clerici, 2019). It has been reported that annealing results in the formation of pores on the surface of starch granules (Nakazawa & Wang, 2003; O'Brien & Wang, 2008), reflecting the hydrolytic effect of annealing on starch granules. Treating starch with BE caused a slight increase in crystallinity when compared with BUF, implying that BE treatment only slightly modified the crystalline structure of NMS. MA treatment increased the crystallinity (Fig. 3). This effect might be due to MA's transglucosylation activity as was suggested based on the SEC data (Fig. 2B). Furthermore, we observed that MA, supplied at a 10-fold lower concentration, reduced

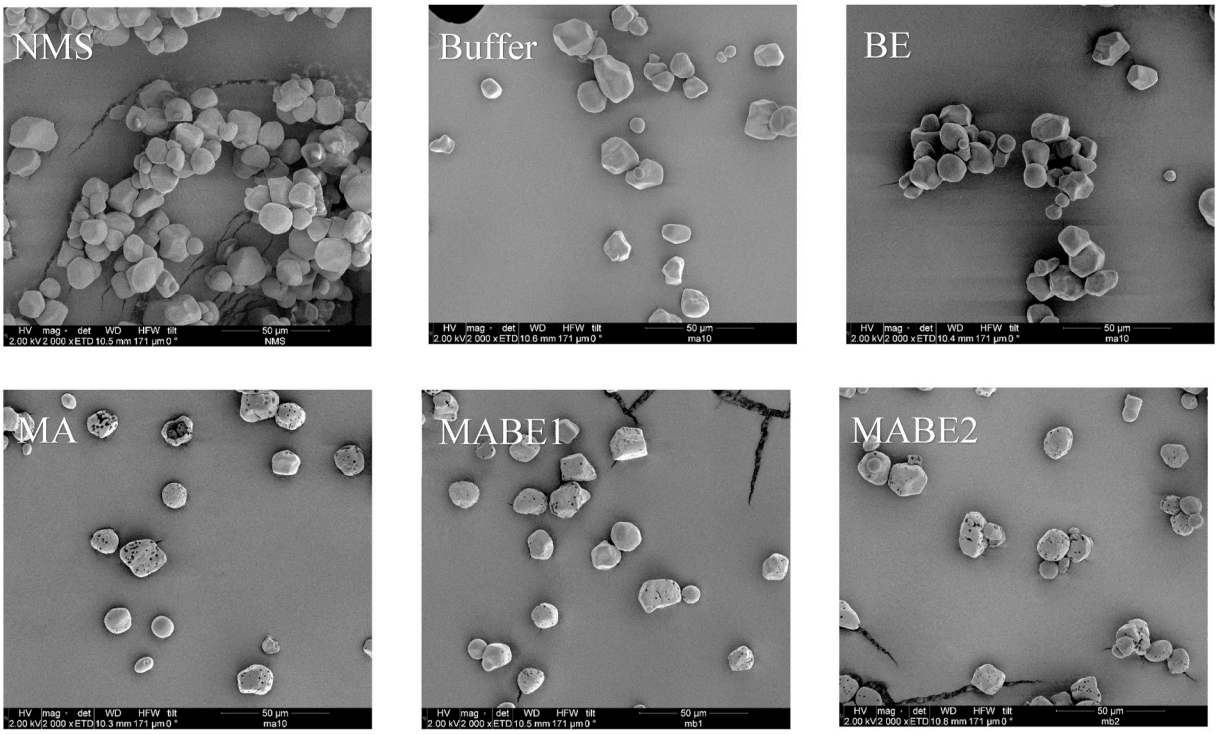

Fig. 4. SEM micrographs showing the morphology of starch granules after different enzymatic treatments. Abbreviations: BE, branching enzyme; BUF, buffer; MA, maltogenic α-amylase; NMS, normal maize starch.

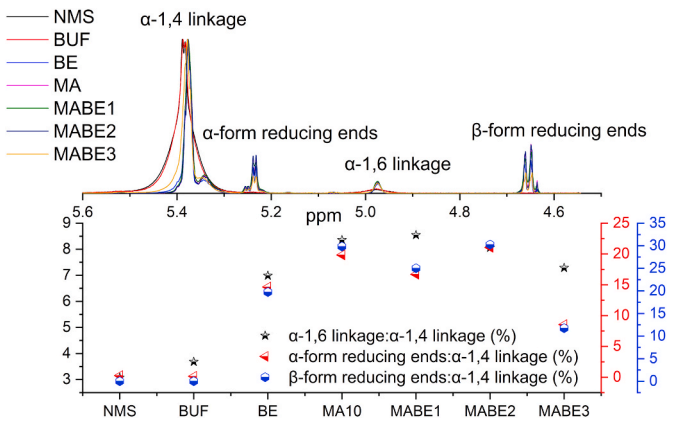

Fig. 5. ^{1}H NMR spectra. Abbreviations: BE, branching enzyme; BUF, buffer; MA, maltogenic α-amylase; NMS, normal maize starch.

the crystallinity of starch. This effect is possibly directly related to its α-(1 → 4)-hydrolytic activity (Zhong et al., 2021) and consistent with our molecular structure data, i.e., MA showed high hydrolytic activity towards amylopectin at low dosage but its molecular size was restored to some degree at high BE-dosage treatment. BE post-treatment of MA-treated granules significantly increased their crystallinity, especially in MABE2 and MABE3 samples. Interestingly, MABE3 also caused a higher yield when compared with MA, implying strong transglucosylation effect of BE when supplied at high concentrations. It has been documented that the amylose content in native starches was negatively correlated with the relative crystallinity (Cheetham et al., 1998); however, our results suggest that BE can produce a novel starch type with a B-type like allomorph and a high crystallinity.

3.6. Morphology

As expected, NMS, BUF, and BE-treated starch granules showed a smooth surface (Fig. 4). This indicates low hydrolytic effect of BE. In contrast, MA formed extensive pores in granules (Fig. 4). BE post-treatments did not further affect the pore size of granules, which demonstrates negligible effect on starch morphology and corresponds to our yield data.

3.7. Degree of branching

Four peaks in the region of 4.6–5.6 ppm were displayed in the ^{1}H NMR spectra (Fig. 5A). MA alone strongly increased the relative ratio of α-1,6 to α-1,4 linkages (Fig. 5B), which supposedly is an effect of the hydrolysis of α-1,4 linkages. BE alone had a similar effect on increasing the relative ratio of α-1,6 to α-1,4 linkages (Fig. 5B), underlining that BE exhibited α-1,4 → α-1,6 transglucosylation activity when acting on starch granules. In our previous study (Zhong et al., 2021), we found that pores and internal channels were required for the efficient modification by TGA and BE. However, granules treated with BE alone did not show pores at the surface (Fig. 4) suggesting that BE alone is only capable to modify the surface of the granules. As expected, treatment with MABE1 slightly increased the ratio of α-1,6 to α-1,4 linkages when compared with MA treatment only, implying that MA pretreatment facilitates the formation of α-1,6 glucans at low BE concentrations. Interestingly, higher BE concentrations as used in the MABE2 and MABE3 treatments displayed lower α-1,6 to α-1,4 ratios than in the MABE1 group. A similar effect of BE on linkage composition when offered at high concentrations was found for partly gelatinized and highly concentrated starch preparations (Jensen, Larsen, et al., 2013; Jensen, Zhu, et al., 2013). Our results show that BE produced a different transglucosylation pattern in solid or semi-solid starch systems when compared with gelatinized starch systems, where the ratio of α-1,6 to α-1,4 linkages is increased as expected (Ao et al., 2007; Le et al., 2009; Lee et al., 2008). The peak signals at 5.23 and 4.65 ppm reflect the α- and β-anomeric reducing end protons, respectively (Bai, Shi, Herrera, & Prakash, 2011). The intensity of these two signals is associated with the hydrolysis level of starch granules (Zhong et al., 2021). We found that MA increased the intensities of these two signals as expected (i.e. increased starch hydrolysis), while BE post-treatments decreased the intensities of these signals as an effect of possible transglucosylation activity. This effect is supported by the higher recovery found for the starch products modified by BE post-treatment.

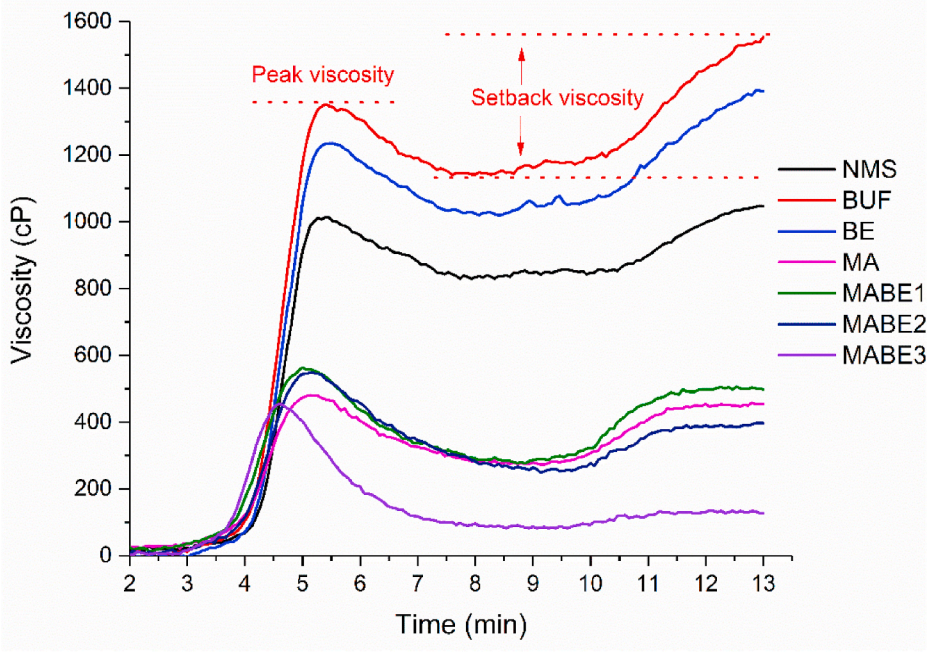

Fig. 6. Pasting properties analyzed by RVA.

Table 3
In vitro digestion of native and retrograded starches.

Sample code	Raw starch			Retrograded starch		
	RDS (%)	SDS (%)	RS (%)	RDS (%)	SDS (%)	RS (%)
NMS	26.6 ± 0.5^{e}	58.2 ± 0.7^{a}	15.3 ± 1.0^{e}	75.6 ± 1.3^{a}	13.1 ± 0.7^{c}	11.3 ± 0.6^{f}
BUF	30.1 ± 1.2^{d}	45.3 ± 2.3^{b}	24.6 ± 1.4^{bc}	76.9 ± 0.3^{a}	13.7 ± 1.2^{c}	9.4 ± 1.5^{g}
BE	29.8 ± 0.3^{d}	47.1 ± 1.3^{b}	23.1 ± 1.0^{c}	70.3 ± 0.5^{c}	11.5 ± 0.6^{d}	18.2 ± 0.1^{b}
MA	39.3 ± 0.5^{a}	42.6 ± 0.3^{c}	18.2 ± 0.8^{d}	70.8 ± 1.6^{c}	9.4 ± 2.2^{e}	19.7 ± 0.6^{a}
MABE1	37.7 ± 0.4^{b}	41.2 ± 0.8^{d}	30.0 ± 0.8^{a}	74.4 ± 1.6^{b}	11.4 ± 1.4^{d}	14.1 ± 0.2^{d}
MABE2	35.8 ± 0.5^{c}	38.9 ± 0.1^{e}	25.2 ± 0.4^{b}	68.1 ± 1.2^{d}	18.5 ± 1.0^{b}	13.4 ± 0.2^{e}
MABE3	36.1 ± 0.1^{c}	41.4 ± 1.9^{d}	22.4 ± 1.9^{c}	62.9 ± 0.8^{e}	20.7 ± 1.2^{a}	16.4 ± 0.3^{c}

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

3.8. Pasting behavior profiles

The pasting behavior of starch preparations as analyzed by RVA showed that the control BUF sample increased the peak viscosity, while BE alone treatment recovered this viscosity (Fig. 6). More notable decreases of the peak viscosity were found for MA- and MABE-treated starches. MA disrupts the starch granular microstructure by producing surface pores, which decreases the swelling ability of granules (Keeratibura, Hansen, Soontaranon, Blennow, & Tongta, 2020ab ; Zhong et al., 2021), resulting in a lower peak viscosity (Fig. 6). As found for the granule yield and morphology, BE post-treatment (MABE) had an insignificant effect on the integrity of granules. Likewise, effects on the peak viscosity were negligible, especially for MABE1 and MABE2 treatments. Both BE alone and MA alone treatments significantly decreased the setback viscosity when compared with the NMS and BUF samples, indicating that treatment with these enzymes separately reduced the short-term retrogradation (recrystallization) of the starch upon cooling as shown by a negative correlation between setback viscosity and the relative ratio of α-1,6 to α-1,4 linkages (Ren et al., 2017). No significant decrease in setback viscosity was found for the MABE samples at either low or medium BE concentrations, while the setback viscosity was remarkably decreased at a high BE concentration (MABE3), suggesting that high BE loadings can further reduce short-term retrogradation. The MABE3 treatment combination had an insignificant effect on the relative ratio of α-1,6 to α-1,4 linkages when compared with MABE2 (Fig. 5B), and thus there might be some other factors affecting the consequences of MABE3 treatment on setback. We suggest that the increased content of long amylose chains is the main contributor to the decreased setback in the MABE3 sample, an effect previously demonstrated for rice starch (Tao, Li, Yu, Gilbert, & Li, 2019).

3.9. In vitro digestion

We studied the *in-vitro* digestion of both granular and retrograded starch (Table 3). In the raw granular system, slowly digested starch (SDS) predominated in all samples (Table 3), due to the presence of semi-crystalline structures that resist enzymatic hydrolysis. In the retrograded system, rapidly digested starch (RDS) dominated (Table 3). The digestibility of retrograded starch is mainly related to the re-association of amylose and amylopectin molecules during storage, (Tian & Sun, 2020). As mentioned above, we suggest that buffer treatment can lead to molecular annealing, an effect including the reorganization of the semi-crystalline structure of the starch granules, typically causing a higher resistance to digestibility (Schmiele et al., 2019). It should be noted that freshly-gelatinized starch can provide a relevant food nutrition model. However, the dynamics in such a system are complex and related to the molecular structure, amylose reorganization and hydrolytic kinetics of the substrates (Witt, Gidley, & Gilbert, 2010). Hence, albeit the relevance of such a system for food applications, we have chosen more stable granular and retrograded systems for our degradation tests.

We found a higher content of resistant starch (RS) in the granular BUF sample when compare with the raw starch in the granular system (Table 3), suggesting that annealing occurred. BE treatment alone produced similar amounts of RDS, SDS and RS as compared with BUF, suggesting again that BE had no notable effect on granular maize starch. Due to the hydrolytic activity of MA, pre-treatment with this enzyme decreased the resistance of granular starch against pancreatic hydrolases and amyloglucosidase, which was reflected by a significant increase of RDS and decrease of both SDS and RS. Compared with MA, BE post-treatment (MABE) mainly increased the RS content, especially at the medium concentration (MABE2), and correspondingly decreased the RDS and SDS contents. The effect of BE on transforming the starch A-type allomorph to B-type allomorph was possibly the main reason for an increased RS content, because the B-type allomorph shows a higher amylolytic resistance than the A-type allomorph (Cheetham et al., 1998).

In the retrograded starch system (Table 3), BUF also had limited effect on digestibility, which can be related to the minor changes in the molecular structure found for this sample. Treatment with MA alone and BE alone resulted in a higher RS content and lower RDS content. These enzymes increase the ratio of α-1,6 to α-1,4-linkages, which can impair the catalytic rates of the α-1,4-specific hydrolytic enzymes (Ao et al., 2007; Kittisuban, Lee, Suphantharika, & Hamaker, 2014; Le et al., 2009; Lee et al., 2008). Interestingly, the MABE samples, especially MABE3, mainly increased the levels of SDS, whereas RDS decreased slightly but the RS levels remained the same. Syneresis data showed that MABE-treated starches had a high tendency for retrogradation, which is a key factor for the resistance of starch to hydrolytic enzymes (Kim, Kim, Moon, & Choi, 2014). Hence, the recrystallization process of MABEs was more intensive than in other samples, and as an effect, these starches showed a higher resistance to pancreatin and amyloglucosidase. It has been demonstrated for the maize starch system, in freshly cooked starch preparations, that an increased amount of α-1,6 linkages was the main factor contributing to the decreased enzymatic digestion rate (Ao et al., 2007). However, we found in our retrograded starch preparations that the level of retrogradation was the main factor affecting the digestion rate. The MABE treatment decreased the RS content and RS in all the retrograded starch preparations exhibited a recrystallized B- and V-type crystalline structure. The retrogradation time was set for only 1 day, which mainly involved the rapid recrystallization of amylose molecules, while the slow recrystallization of amylopectin molecules was still expected to be ongoing (Wang, Li, Copeland, Niu, & Wang, 2015). Our SEC molecular profiling data showed that post-treatment with BE (MABE samples) resulted in higher amounts of long amylose chains (Fig. 2B). Such long chains are expected to be less prone to reorganize due to steric molecular entanglement (Chung & Liu, 2009), keeping the system in an amorphous state, decreasing the level of amylose retrogradation and the content of RS.

3.10. Rheological characteristics

We analyzed the rheological profiles of starch pastes stored for 1 and 7 days (Fig. 7). The parameters calculated were storage modulus (G′), loss modulus (G″), loss tangent (G'/G″, tan δ) and the modulus of complex viscosity (η*), whereas G′ indicates the strength of gels; G″ indicates the dissipated energy (viscosity) of a paste; tan δ measures how elastic (tan δ < 1) or plastic (tan δ > 1) a gel is; η* indicates the flexibility and stiffness of a material during formation (Franck, 2004, pp. 1–17; Sun, Sun, Wang, Sánchez-Soto, & Schiraldi, 2018). The rheological behavior evaluated at day 1 reflects the short-term retrogradation of a starch gel mainly affected by amylose, while the behavior at day 7

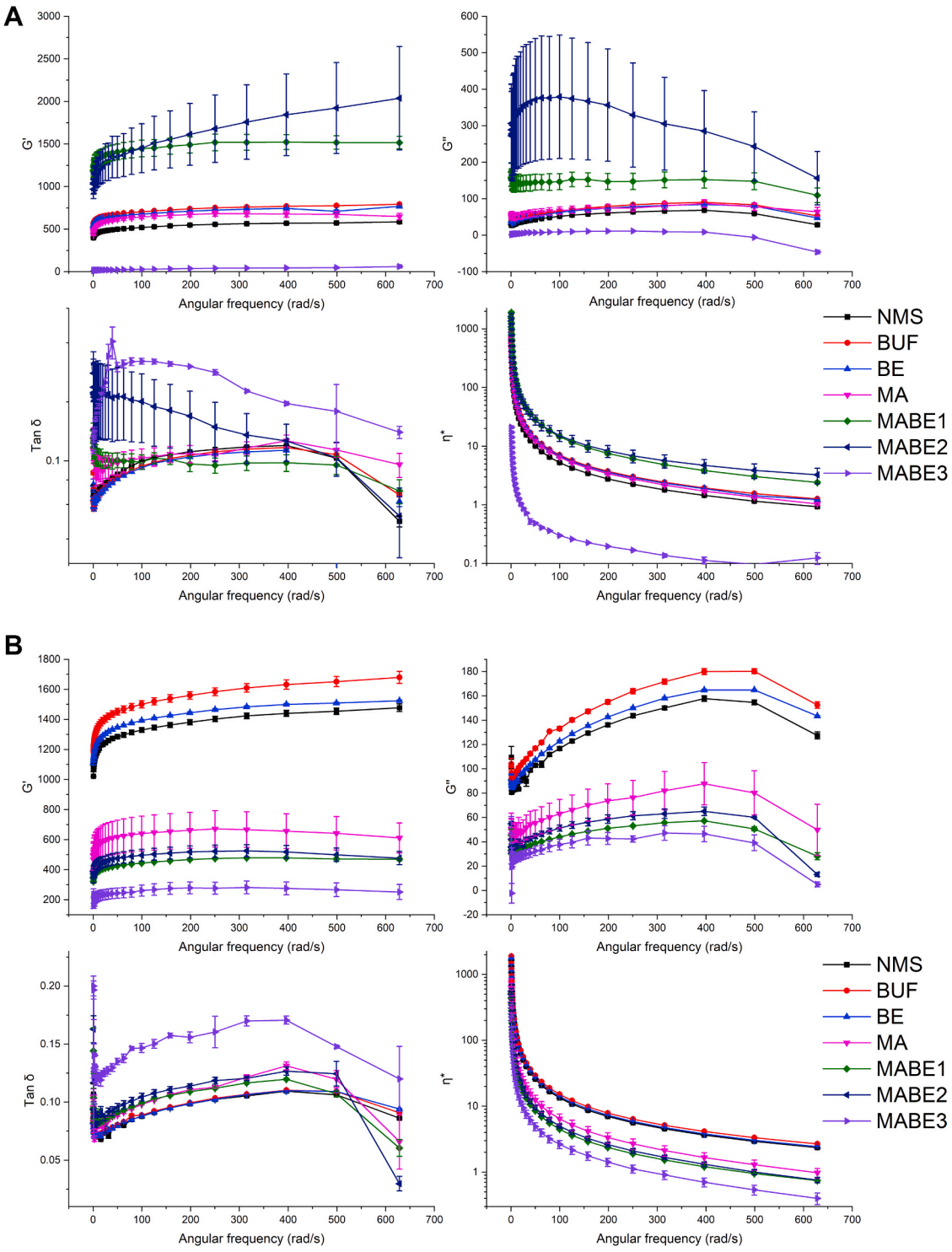

Fig. 7. Rheological characteristics of starch gels after storage for A) 1 day and B) 7 day.

reflects the long-term retrogradation reflecting re-association of both amylose and amylopectin segments.

All samples exhibited typical gel-like characteristics i.e. G′>G″ and tan δ < 1. The NMS, BUF, BE, and MA samples all showed similar values for G′, G″, tan δ, and η* at day 1 (Fig. 7A), suggesting that BE or MA treatments alone had very little effect on the short-term retrogradation of starch gels. In contrast, MABE1 and MABE2 showed significantly higher G′, G″ and η* values than NMS, whereas these values were reduced for the MABE3 sample (Fig. 7A). Thus, dual, sequential treatment (MABE) strongly affected the rheological behavior of the gels. Low and medium BE concentrations (MABE1 and 2) caused a stronger short-term retrogradation in gels, while high BE concentrations (MABE3) had reverse effects. As suggested by digestion studies, short-term retrogradation is mainly related to amylose re-association and MABE3-treated samples had more long amylose chains causing slow gelation (Clark, Gidley, Richardson, & Ross-Murphy, 1989). Interestingly, both MA and MABE1 exhibited a similar chain length distribution (Fig. 2) and degree of branching (Fig. 5). However, MABE1-treatment produced a higher degree of short-term retrogradation than MA treatment alone. This suggests that addition of BE altered the molecular structure of starch in a

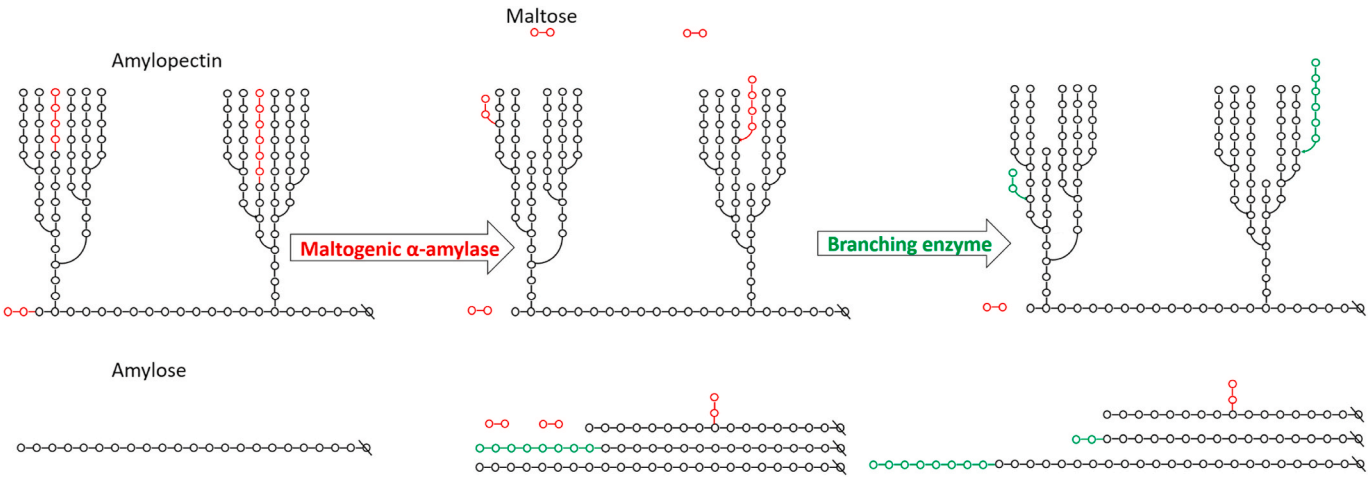

Fig. 8. Schematic catalysis pattern of sequential maltogenic α-amylase (MA) and branching enzyme (BE) treatments of granular starch.

more subtle way, for example, the positions of branching points or the internal amylopectin structure in granules, which might affect the reorganization of starch molecules. Such small variations can significantly affect the rheological behaviors of starch gels (Bertoft et al., 2016). However, further data will be required to explain such structural changes caused by MABE1 treatment.

The BUF and BE samples stored for 7 days exhibited G′, G″, and η* values similar to those of NMS. MA and MABE samples showed significantly lower values for these parameters, especially in MABE3 (Fig. 7B), indicating that both MA and MABE treatments decreased the strength of gels when stored for 7 days. It has been documented that MA increases the degree of branching and thus decreases the retrogradation rate during storage (Ao et al., 2007), which produces weak gels. However, BE post-treatments further decreased the G′, G″, and η* of gels, although these treatments did not further increase the degree of branching. As mentioned above, we suggest that the low retrogradation level of these samples was associated with the presence of long amylose chains catalyzed by high levels of BE.

4. Discussion

4.1. The underlying catalysis mechanism of branching enzyme on granular starch

The effect of BE activity on reducing ends in starch molecules (Fig. 1) suggests that the predominant activity of BE on a gelatinized starch system (isolated amylose chains) was α-1,4 → α-1,6 glucan transfer, increasing the relative amounts of α-1,6 linkages. However, in a granular (solid state) starch system, BE surprisingly increased the amount of long amylose chains and, at high concentrations, did not affect the branching degree. This is an unexpected result for an α-1,4 → α-1,4 or α-1,6 → α-1,4 glucanotransferase, which is expected to decrease the amylose content and produce more short amylopectin chains as shown for a gelatinized starch system (Li et al., 2016). Similar effects have been found for high BE concentrations when acting on partly gelatinized and highly concentrated starch preparations (Jensen, Larsen, et al., 2013; Jensen, Zhu, et al., 2013). BEs require long glucan chain segments for binding and catalysis, and the binding sites for the substrate chains are distant from the active site. Moreover, BEs are capable of catalyzing both hydrolytic as well as transfer reactions (Feng et al., 2015). Hence, alternative acceptor hydroxyl groups, including water (resulting in starch hydrolysis), and OH-4 hydroxyl groups in the glucose unit (i.e. glucanotransfer) can be involved in the transfer reaction. In the solid starch granule, amylose chains are expected to be more accessible than amylopectin chains. As a result, α-1,4 → α-1,4 or reverse α-1,6 → α-1, 4 transfer reactions can be expected, which lead to the observed effects, in case that 4-position hydroxyl groups of amylose chains are available as acceptors in the granule (Hansen, Blennow, Farhat, et al., 2009; Hansen, Blennow, Pedersen, & Engelsen, 2009; Sorndech et al., 2015; Sorndech et al., 2016). These types of reactions, seemingly specific for highly concentrated reaction systems, remain to be verified. A plausible explanation is also that the BE used in our study is from family GH57 which has been demonstrated to act preferably on amylose (Marta Palomo et al., 2011), while family GH13 BEs act more equally on both amylopectin and amylose (M Palomo, Kralj, Van Der Maarel, & Dijkhuizen, 2009). Hence, rearrangement of chain segments within the amylose chain pool by GH57 BEs can result in longer amylose chains (Roussel et al., 2013).

4.2. A simplified catalysis pattern of sequential maltogenic α-amylase (MA) and branching enzyme treatments modifying molecules in granular starch

Briefly, our data suggest that MA pre-treatment predominately cleaves amylopectin chains with DP 10–29 into amylopectin chains in the DP 2–9 range, and transfers partially cleaved amylopectin chains to a new position by forming α-1, 6 linkages (Fig. 8). The data show that the crystallinity was increased by BE post-treatment, whereas the amylopectin CLD was not changed, which strongly suggests that BE possesses an α-1,6 → α-1,6 activity, i.e., BE post-treatments can for example transfer α-1,6 branches from one position to a position promoting the formation and alignment of double helices (Fig. 8). Moreover, BE increased the amount of long amylose chains and decreased the degree of branching, suggesting BE post-treatments possibly also possess α-1,4/α-1,6 → α-1,4 activities. Our data suggest that BE elongates, by glucan transfer, shorter amylose chains thereby resulting in longer amylose segments by cleaving α-1,4 or α-1,6 linkages and transfer of the cleaved segments to the short amylose acceptor chains. Such a mechanism is supported by the fact that BEs preferably use larger fragments as substrate (Roussel et al., 2013). However, the semi-crystalline structure of amylopectin and limited space in the crystalline region in granular starch possibly hinders the sequential creation of new α-1,6 linkages. The increasing yield recovery from MA to MABE3 treatments is likely an effect of BE elongating amylose chains and amylopectin side chains by transfer of MA-generated soluble hydrolytic products (e.g. short linear segments).

5. Conclusion

In this study, solid granular maize starch was treated below the gelatinization temperature by a dual, sequential catalytic approach using the enzymes maltogenic α-amylase (MA) and branching enzyme

(BE). We found that BE alters the structure and properties of starch solely as a consequence of MA pre-treatment. BE post-treatment, especially when using medium and high enzyme concentrations, increased the yield (insoluble starch), the amount of long amylose chains and the relative crystallinity of granules, and altered the crystalline type of starch by changing the crystalline allomorph from the A-type to a mixed A-, B-type. BE post-treatments had minor effects on the *in vitro* digestion of raw starch by hydrolytic enzymes, but significantly increased the slowly digested starch content of retrograded starch. The rheological characteristics of gels obtained from starch granules treated with low or medium concentrations of BE upon MA treatment and stored for 1 day promoted the short-term retrogradation. In contrast, starch modification with high BE concentration retarded the short-term retrogradation. Gels prepared from starch that were modified with MA and high doses of BE and stored for 7 days showed decreased loss moduli. In conclusion, our study provides new means to customize structural and compositional features of granular starch systems by utilizing readily available enzymes.

Funding

This study was partly supported by Archer Daniels Midland (ADM) Company, Decatur IL, and China Scholarship Council (CSC).

CRediT authorship contribution statement

Yuyue Zhong: Conceptualization, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. **Klaus Herburger:** Writing – original draft. **Jacob Judas Kain Kirkensgaard:** WAXS analysis. **Bekzod Khakimov:** NMR analysis. **Aleksander Riise Hansen:** Resources, Conceptualization, Funding acquisition, Supervision. **Andreas Blennow:** Resources, Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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