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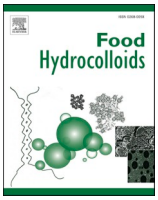

Insights into high hydrostatic pressure pre-treatment generating a more efficient catalytic mode of maltogenic α-amylase: Effect of multi-level structure on retrogradation properties of maize starch

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ABSTRACT

Maltogenic α-amylase (MA) is an enzyme used to retard starch retrogradation in the baking industry. However, sometimes it exhibits low efficiency which may restrict the enzymatic application. This study investigates the effects of using High hydrostatic pressure (HHP) as a pre-treatment to promote MA catalysis. The results showed that HHP resulted in more indentations and fissures of the starch granules promoting the susceptibility of MA. While MA treatment alone introduced short malto-oligosaccharide chains with degree of polymerization (DP) 1–5, the combined HHP-MA treatment exhibited a higher proportion of DP 1–9, but a lower proportion of DP 13–24. Furthermore, when increasing the pressure, the ratio of α-1,6 to α-1,4 glycosidic bonds increased in the MA-treated starch. MA modification on the HHP-pretreated starch showed increasing gelatinization temperature and enthalpy, while it had a reverse impact on long-term retrogradation enthalpy due to the shortening of the outer chains of amylopectin. LF-NMR was applied to analyze the water mobility and compartmentalization in starch gel systems after 1- and 7-days storage. Compared with single MA-treated system, HHP-MA treated starches showed no significant changes in the value of the transverse relaxation times, but less amount of bulk water was detected due to higher amounts of soluble small oligosaccharides. In conclusion, HHP pre-treatment combined with MA could improve the water holding properties of retrograded starch gels and that HHP prior to MA catalysis provides an efficient alternative method to modify starch-based food products with anti-stalling properties.

1. Introduction

Starch is an important energy source in human diets, and it has wide and fundamental applications in the food industry. However, starch-based foods usually face the challenge of staling during the processing and storage, which result in quality loss and firmness increase. In some cases, this undesirable change is caused by aggregation and reassociation of the starch component in food. Therefore, modification for starch is necessary to retard the retrogradation and improve the quality of starchy food. In the past decades, enzymatic starch modification has been applied as a clean and safe strategy for providing fewer by-products and specific for substrates (Zhong, Xu et al., 2022). Enzyme technology has many applications in the baking industry because carbohydrate-active enzymes specifically react with carbohydrate components, such as starch, in complex food systems. Maltogenic α-amylase (MA, EC 3.2.1.133) are added to starch-based foods, such as baking products, to retain moisture more efficiently and to increase softness, freshness, and shelf life. It is a member of glycoside hydrolase family 13, and it can accomplish exo-hydrolysis by hydrolyzing α-1,4 glycosidic bonds from the non-reducing ends to form the maltose. It can

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also catalyze an endo-action on amylase and amylopectin to form the malto-oligosaccharides to reduce the chain length of amylopectin and form less clusters (Wang, Bai et al. 2022).

The major limitation to use MA extensively is attributed to lower efficiency and prohibitive cost. Granule starch is in a highly ordered and compact manner, and it is difficult for the enzymes to penetrate and get access to the substrate. For this reason, it is necessary to do some pre-treatments to gelatinize and promote enzymatic modification of granular starch. There exist two strategies to increase the susceptibility of native granular starch to enzyme modification: 1) pre-swelling of the starch granule to a medium state between native starch and totally fragmentized starch pastes and 2) making porous starch (Zhong, Xu et al., 2022). The second strategy usually use hydrolases to get low yield of targeted starch, which is inefficient and economically unattractive. In order to disrupt the internal structure while maintaining the granule morphology, so-called non-crystalline granular starch (NCGS) has been prepared by several various methods, such as heating in hot ethanol (Zhong, Herburger et al., 2022), alcoholic-alkaline treatment (Majzoobi & Farahnaky, 2021), spray drying (Dries et al., 2017), cross-linking (Liu, Zhang, Shen, Hu, & Li, 2010) or combinations thereof. Among these methods, heating in hot ethanol is a popular, but energy-demanding method, by which the native granular starch is heated in aqueous ethanol up to 60 °C, and subsequently high temperatures (140°C–180 °C) are needed to remove the ethanol by washing or evaporation. Alcoholic-alkaline treatment is the most widely used method, but it is not suitable for industrial mass production (Majzoobi & Farahnaky, 2021).

High hydrostatic pressure (HHP) gradually obtains an increasing interest from the industry due to the advantages of high efficiency, simple operation, and non-by-products of chemical reagents, which are very attractive attributes for subsequent large-scale industrial production in the modified starch industry. In comparison the compression energy of 1 L of water at 400 MPa is 19.2 kJ, as compared to 20.9 kJ for heating 1 L of water from 20 to 25 °C. For large scale operations in the food industry, HHP is environmental-friendly and energy-saving processing technology (Rastogi, Raghavarao et al., 2007). In our previous, HHP is more controllable and milder processing than heating (Liu, Wang, Liao, & Shen, 2020). It has been noted that the porous structure can be produced by HHP treatment, making specific surface area in rice starch (Wang, Xue, Yousaf, Hu, & Shen, 2020). Moreover (Pei-Ling, Qing, Qun, Xiao-Song, & Ji-Hong, 2012), has reported that the use of HHP potentially enable to produce NCGS to enhance the efficiency of enzymes. According to Guo et al. (Guo, Zeng et al., 2015) the swelling power of lotus seed starch samples increased with increasing pressure. Swelling was mainly caused by amylopectin, and the amylopectin remained stabilized to promote granule swelling of starch after HHP treatment (Castro, Alexandre, Saraiva, & Pintado, 2020). Many researchers have focused on the ability of HHP treatment to enhance enzyme catalysis, and it has been reported that HHP can be utilized to improve α-amylase and β-amylase catalysis in the production of sugar syrups and alcoholic products (Eisenmenger & Reyes-De-Corcuera, 2009). However, there is limited information about the potential and mechanism of HHP as a pre-treatment to promote the MA catalysis of granular maize starch. Compared with single MA modification, granular maize starch after HHP pre-treatment may have unique characteristics to influence MA hydrolysis on maize starch, thus dual HHP and MA modification will be effective for starch modification, resulting in better anti-aging properties and longer shelf-life for starch-based bakery food products.

To test this hypothesis, normal maize starch was selected due to its wide application in starch industry. Furthermore, the effect of single HHP, single MA and dual HHP-MA modification on the structural properties including morphology, molecular structure and ordered structure, and the resultant effect of these structure changes on retrogradation properties including thermal, pasting properties and water migration of starch were evaluated. To our knowledge, this is the first systematic investigation on the study of possible role of HHP pre-treatment in catalysis mode of MA from the perspective of supramolecular structure and retrogradation properties. The results will provide insights for the application of the HHP as a pre-treatment before catalysis to extend the shelf life and keep the freshness of starch-based products.

2. Materials and methods

2.1. Materials

Normal maize starch (Cstar Gel 03401) was provided by Cerestar Company (Belgium). Maltogenic α-amylase (MA) was kindly provided by Novozymes (Bagsvaerd, Denmark). Debranching isoamylase (200 U/mL) were provided by Megazyme (Ireland). Pancreatin (Cat. No. P7545) and amyloglucosidase (Cat. No. A7095) were supplied by Sigma (St. Louis, MO, USA). D-Glucose Assay Kit was bought from Megazyme corporation (Co. Wicklow, A98 YV29, IRELAND). All other chemicals and reagents were of analytical grade.

2.2. Preparation of HHP-treated starches

The normal maize starch suspension (20% w/w) was prepared for HHP treatment. Before the pressurization, the slurries were sealed and vacuumed in polyethylene bottles. In order to get the partially gelatinized normal maize starch with around 50% and 75% gelatinization degrees, the applied pressure was held at 500 MPa, and the pressurization time was set to 5 and 15 min, respectively. At the end of the constant pressure phase, the depressurization was applied instantaneously (less than 2 s).

2.3. Enzymatically modified starches

Native and HHP-treated maize starch (3 g) were suspended in 30 mL sodium acetate buffer (pH = 5.5), and kept in an incubator at 50 °C for 10 min. Then 52U/g MA was added and incubated at 50 °C for 3 h in a shaking incubator with 150 rpm. The enzymatical treatment was terminated by adding 1 M NaOH to adjust pH to approximately 11.0, and then use 1 M HCl to adjust the pH back to about 5.0. Subsequently the enzymatically modified starch granules were collected by centrifugation at 4500*g* for 5 min and was washed with milliQ water three times. Finally, the samples were freeze-dried for 48 h and stored in a desiccator. Control samples were native maize starch (NMS) and native maize starch treated by buffer (NMS-buffer).

2.4. Granule morphology (Scanning electron microscopy)

The morphologies of native and modified maize starch granules were measured by a scanning electron microscope (SEM) (SU8010, Hitachi, Japanese). Samples were sprinkled on double-sided adhesive tape mounted on an aluminum stub and then coated with gold before imaging.

2.5. Chain length distribution (High-performance anion exchange chromatography)

Starch samples (5 mg/mL) were gelatinized at 99 °C for 1 h. After cooling to room temperature, 2 μl isoamylase was added to each sample, and then kept at 40 °C for 3 h. Following centrifugation, 40 μl supernatant was injected onto a CarboPac PA-200 column attached to a high-performance anion exchange chromatography (HPAEC-PAD) (Dionex, Sunnyvale, CA, USA) system. The chain length distribution was calculated following the method reported by Blennow et al. (Blennow, Bay-Smidt et al., 1998).

2.6. Branching degree (Proton nuclear magnetic resonance spectroscopy)

5 mg starch samples were dissolved in 1 mL deuterium oxide, and heated at 99 °C for 2 h. Upon cooling down, samples were freeze-dried. Before measurement, samples were re-dissolved in 90% deuterium oxide mixed with 10% heavy water, and reheated at 100 °C for 30 min. The ratio of α-1,6 to α-1,4-glycosidic bonds was detected using one-dimensional proton (^{1}H) nuclear magnetic resonance (-NMR) spectroscopy. Proton NMR spectra were acquired on 500 MHz NMR spectrometers (Bruker Avance III, Bruker Biospin, Rheinstetten, Germany) and analyzed using SigMa software (Khakimov, Mobaraki et al., 2020).

2.7. Short-range ordered structure (Infrared spectroscopy)

The short-range ordered structure was explored by a Fourier-transform infrared (FT-IR) spectrometer (IS50, Thermo Fisher Scientific, Hudson, USA), equipped with an attenuated total reflectance (ATR) single reflectance germanium crystal, using the method by Liu et al. (Liu et al., 2020) with a minor modification. The OMNIC (Thermo Fisher Scientific, USA) software was used to adjust baseline and calculate the ratio of 1047/1022 cm^{-1} after deconvolution.

2.8. Long-range ordered structure (Wide-angle X-ray scattering)

Before wide-angle X-ray scattering (WAXS) measurements, samples were stored in a sodium chloride humidity chamber (90% relative air humidity) for 3 days and then measured on a Nano-inXider instrument from Xenocs (Grenoble. France) using a Cu Kα source with a 1.54 Å wavelength and a 2D 300 K Pilatus detector (Dectris Ltd. Baden. Switzerland). The relative crystallinity of the starch samples was calculated by MATLAB trapez integration to determine the amorphous component by drawing a smooth curve following the method of Frost et al. (Frost, Kaminski, Kirwan, Lascaris, & Shanks, 2009).

2.9. Thermal properties (Differential scanning calorimetry)

A differential scanning calorimeter (DSC, 200 F3, Netzsch, Germany) was used to determine the gelatinization and retrogradation properties. 3 mg (20%, w/w) starch suspension was sealed in a standard aluminum pan and equilibrated overnight at room temperature. The scanning of samples was performed from 20 to 120 °C at 10 °C/min heating rate. The gelatinization onset temperatures (T_o), peak temperatures (T_p), conclusion temperatures (T_c), and enthalpy changes (ΔH_g) of samples were calculated using the STARe evaluation software (Version 15.0 Mettler Toledo, Schwerzenbach, Switzerland). After gelatinization, the sample pans were placed in a plastic valve bag and stored at 4 °C for 7 days, and rescanned using the same heating procedure.

2.10. Pasting properties (Viscometry)

The pasting properties of samples were investigated by a Rapid Visco Analyzer (RVA, Newport Scientific, Australia). The starch samples (10% w/v) were prepared and then stirred for 30 s, then measured using ICC Standard Method No.162.

2.11. Water migration (Proton nuclear magnetic resonance relaxometry)

Proton nuclear magnetic resonance (NMR) relaxometry also called low field NMR (LF-NMR) measurements of samples were performed at 0.47 T magnetic field strength using a MQR Spectro-P spectrometer (Oxford Instruments, Oxfordshire, UK), controlled by the Oxford Instruments NMR Application Developer software. Starch samples (3 g) were added into 27 mL distilled water to mix using magnetic stirrer (700 rpm) and then heat in water bath at 90 °C, until the paste cannot be stirred. After heating, the samples were totally transferred into 18-mm tubes to cool down to 25 °C. Carr-Purcell-Meiboom-Gill (CPMG) sequences were used to investigate spin-spin relaxation times with the parameters a receiver gain of 5, number of echoes of 10000, 180-degree pulse length of 10.43 μs, 90-degree pulse length of 5.26 μs, 16 scans with recycle delay. After measurement, the sample tubes were sealed and stored at 4 °C for 1 and 7 days, and retested by CPMG sequences. The LF-NMR data was analyzed by both distributed and discrete exponential fitting - combining the two methods serves to validate the realism of the results from the labile and multiparametric distributed fitting. Distributed exponential fitting was performed using Inverse Laplace Transform (Contin function) in Origin 2020 (OriginLab, Massachusetts, USA) to recover the distribution of transverse T_2 relaxation times. A total of 256 logarithmically distributed relaxation times from 0.1 to 2000 ms were fitted simultaneously. Discrete exponential fitting was obtained using in-house MATLAB (R2019a, MathWorks, MA, USA) scripts (Pedersen, Bro, & Engelsen, 2002) designed for fitting the relaxation curves to a sum of exponential decays according to Equation (1):

$$I_N(t) = \sum_{i=1}^{N} M_{0,i} \bullet exp\left(\frac{-t}{T_{2,i}}\right) + B \quad (1)$$

The signal intensity $I_N(t)$ is given as a sum of mono-exponentials (N) each described by a signal amplitude $M_{0,i}$, a time constant $T_{2,i}$, and a baseline offset. t is the acquisition time. For more details on this approach see (Hansen et al., 2009).

2.12. In-vitro digestibility

Starch samples (100 mg) were suspended in 7.5 mL acetate buffer (pH 5.5) and then incubated at 40 °C for 20 min. The reaction was started by adding 5 mL enzyme solution containing pancreatin (5 × 103 USP/g) and amyloglucosidase (66 U/g) into suspension while stirring at 250 rpm. Aliquots (0.1 mL) were collected at 20 and 120 min and the reactions were terminated by adding 1 mL of ethanol (95%). The reaction mixtures were centrifuged at 20,000 rpm for 5 min and the released glucose in the supernatant was measured by the GOPOD kit (Megazyme, Ireland). The amount of rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) was determined by using the method of Englyst et al. (Englyst, Kingman et al., 1992).

2.13. Statistical analysis

Except for WAXS measurement, each experiment was performed at least in duplicate. Origin 2020 was used to draw plots. Significant differences ($p < 0.05$) among different samples were evaluated using ANOVA and Duncan's test using SPSS 25.0 software (IBM Corporation, Armonk, NY, USA).

3. Results and discussion

3.1. Morphology

The measured SEM morphologies display significant differences among the native and modified maize starches (Fig. 1). As shown in Fig. 1, native maize starch (NMS) exhibits an irregular polygonal and spherical shape with a smooth surface. Some holes, indentations and fissures that appear on the granule surface of maize starch after HHP treatment, indicate that HHP treatment squeeze the starch granule to become rough and bumpy. The HHP-treated (500 MPa-5 min) maize starch displayed a typical doughnut-shaped due to the fact that HHP tend to induce an endo-corrosion (Liu et al., 2020). In some cases, it would appear that the HHP treatment squeeze large amounts of water into the granule through the holes which result in the collapse and disruption of starch leaving only the starch granule envelope. The modification using MA for 3 h on NMS, led to the formation of visible pores and channels. MA treatment can hydrolyze the granules from the surface with exo-attack, followed by penetration to the inside of the

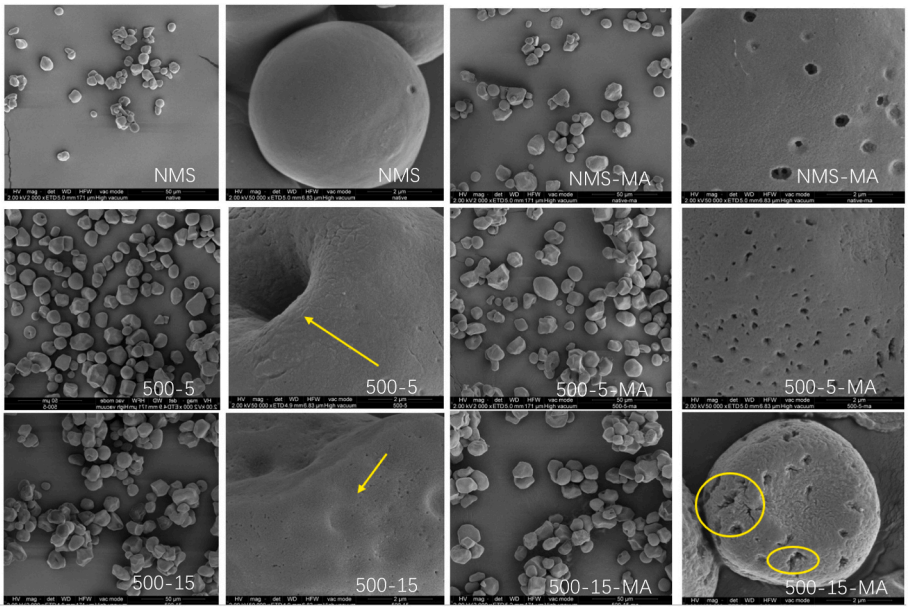

Fig. 1. The microscopic images (× 2000 and × 50000) of native and modified maize starches (NMS: native maize starch; NMS-buffer: native maize starch modified by buffer; NMS-MA: native maize starch modified by maltogenic α-amylase; 500–5: native maize starch treated by 500 MPa for 5 min; 500-5-MA: native maize starch treated by 500 MPa for 5 min combined with maltogenic α-amylase modification; 500–15: native maize starch treated by 500 MPa for 15 min; 500-15-MA: native maize starch treated by 500 MPa for 15 min combined with maltogenic α-amylase modification).

granule to generate pores. This is in accordance with the previous study of normal maize starch hydrolyzed by MA (Li, Kong, & Ai, 2022). However, for HHP pre-treated maize starches, the already formed "outside-in" tunnels is directly promoted by the attack from MA, resulting in more and larger pores and cracks, while the overall granule structure remain intact. Therefore, the combined effect of HHP and MA treatment made larger changes to the surface morphology compared with the single HHP or MA treatment. Latip et al. (Hj.Latip et al., 2021) found that the α-amylase treatment produced the largest pore size and ratio of cavity-to-granule diameters of porous starches compared to that of other enzymes. Based on the morphology of HHP-MA treated maize starch, it would appear that this combined treatment has great potential as porous food material for carrying flavors and aromas.

3.2. Molecular structure

The branch chain length distributions of native and modified maize starch samples were measured by HPAEC and the results are presented in Fig. 2 and Table 1. Compared with NMS, adding MA introduce short chains with DP 1–5 going from 0.22% to 1.30%, whereas the proportion of longer chains with DP 13-24 decreased. This suggest that MA can hydrolyze the α-1,4 glycosidic bonds with exo-action state to shorten the amylopectin external chains and the enzyme action sites might be very close to the branch points of amylopectin. As a result of the steric barrier of amylopectin, it takes a long time for the inner chain of amylopectin to bind to the active site of MA. Therefore, only exo-hydrolysis occurred in NMS-MA starches. Zhai et al. (Zhai, Li et al., 2022) demonstrated that MA-treated wheat starch display a similar pattern with an increasing amount of short chains with DP < 5. For the HHP-MA treated maize

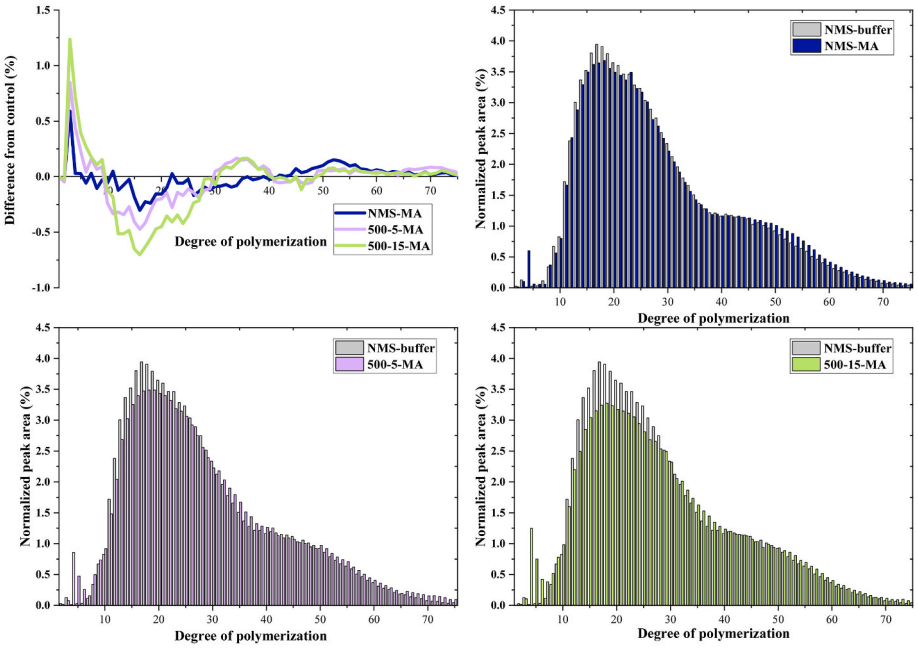

Fig. 2. The branch chain length distribution of native and modified maize starches.

Table 1
The multi-scale structure parameters of native and modified maize starch.

Sample name	Branch-chain length distribution (%)					α-1,6/α-1,4	RC	$R_{1047/1022}$
	DP 1-5	DP 6-12	DP 13-24	DP 25-36	DP > 36			
NMS	0.22 ± 0.00^{a}	8.6 ± 0.1^{a}	43.5 ± 0.6^{a}	25.1 ± 0.0^{a}	22.7 ± 0.3^{a}	0.026 ± 0.001^{a}	28.57%	0.761 ± 0.052^{a}
NMS-buffer	0.23 ± 0.01^{a}	8.5 ± 0.0^{a}	43.9 ± 0.9^{a}	25.2 ± 0.2^{a}	22.9 ± 0.2^{a}	0.027 ± 0.000^{a}	28.65%	0.678 ± 0.011^{a}
500–5	0.26 ± 0.08^{a}	8.5 ± 0.2^{a}	39.5 ± 1.2^{bc}	24.9 ± 0.6^{a}	26.7 ± 2.1^{a}	0.027 ± 0.001^{a}	18.03%	0.436 ± 0.079^{b}
500–15	0.22 ± 0.00^{a}	8.4 ± 0.2^{a}	40.0 ± 1.3^{b}	24.9 ± 0.7^{a}	26.0 ± 2.0^{a}	0.026 ± 0.001^{a}	16.70%	0.291 ± 0.036^{c}
NMS-MA	1.30 ± 0.09^{b}	8.5 ± 0.1^{a}	40.2 ± 1.7^{b}	24.6 ± 0.6^{a}	25.1 ± 1.2^{a}	0.031 ± 0.001^{b}	22.23%	0.545 ± 0.057^{d}
500-5-MA	1.56 ± 0.17^{c}	8.8 ± 0.3^{a}	37.7 ± 1.0^{bc}	24.9 ± 0.4^{a}	26.7 ± 1.2^{a}	0.035 ± 0.000^{c}	20.84%	0.475 ± 0.025^{bd}
500-15-MA	2.50 ± 0.06^{d}	9.1 ± 0.0^{b}	37.3 ± 0.3^{c}	25.1 ± 0.0^{a}	24.4 ± 1.3^{a}	0.048 ± 0.001^{d}	19.62%	0.533 ± 0.057^{d}

Values in the same column of the same category with the different letters differ significantly ($p < 0.05$).

starches the chains with DP 1–9 increased significantly, and the highest proportion of DP 1–9 was found for the 500-15-MA treated maize starch. HHP pretreatment itself had only a slight impact on the chain length distribution of maize starch. However, it was found that it can disrupt the crystal structure to form a state between native semi-crystalline structure and paste. The less-crystalline granular starch generated by HHP promoted the endo-action of MA. Therefore, in HHP-pretreated maize starch, MA was not only conducive to produce short chains by attacking the side chains with exo-action, but also acted as an endo-activity enzyme to attack the longer chain segments between branch points (Tetlow & Bertoft, 2020). When the applied pressure duration increased from 5 min to 15 min, a rise in relative contents was observed for branches DP 1–5 and DP 6–12, from 1.56 to 2.50% and from 8.8% to 9.1%, respectively. In addition, DP 13-24 decreased to 37.7% and 37.0% at 500-5-MA and 500-15-MA starch, which implies that the action of MA is mainly on medium-length chains, producing a short-clustered molecular structure. In addition, as shown in Fig. 2, an increase in chains with DP 30–40 indicated that HHP pre-treatment could promote MA to possess multimolecular catalytic properties, transferring the shorter chains to longer chains.

^{1}H NMR spectroscopy can be used for quantitative analysis of the degree of branching (Gidley, 1985). The ratio of α-1,6 to α-1,4-glycosidic bonds in NMS was determined to be 2.6% (Table 1). Buffer and single HHP treatment showed to have no significant effect on the changes of glycosidic bonds. According to the branching degree measured by HPAEC and the peak area ratio of α-1,6-glycosidic bonds, the addition of MA in NMS showed a slight increase on the proportion of α-1,6-glycosidic bond, indicating MA can cleave the α-1,4-glucan chain. As previously stated, MA treatment was responsible for the increasing ratio due to degradation of the external chains (Zhong, Keeratiburana et al., 2021). In contrast to NMS-MA, samples modified by the combined HHP-MA treatment significantly increased the ratio of α-1,6 to α-1,4 linkages. This increase can be an effect of both the hydrolysis of the α-1,4 linkages, and the reaction of transfer α-1,4 to α-1,6 linkages of MA (Zheng, Zhang, Li, Zhu, & Zhu, 2022). Overall, HHP is an efficient pre-treatment for enhancing the susceptibility of MA, which can lower the concentration of MA and shorten the incubation time.

3.3. Short-range ordered structure

The short-range ordered organization and structure of native and modified maize starch has been measured by ATR-FTIR and the original and deconvoluted spectra of the samples are presented in Supplementary Fig. 1. ATR-FTIR is an analytical tool which can be used to acquire the information of short-range ordered structure on the external parts with penetration depth of approx. 2 μm. In the 1200-800 cm^{-1} region, the characteristic peaks at approximately 1047 and 1022 cm^{-1} are associated to the ordered and amorphous region of starch, respectively (Lu, Ma et al., 2021). Thus, the ratio of 1047/1022 ($R_{1047/1022}$) can be used to evaluate the degree of order. Single buffer treatment had no significant effect on the order degree of starch. For highly-ordered NMS, $R_{1047/1022}$ declined to 0.545 from 0.761 when modified by MA. Accordingly, MA treatmeent led to more irregular packing of double helices. It is well-known that MA can hydrolyze starch granules by "surface pitting" mode, being able to attack the exposed structures close to the periphery of starch granules (Abedi, Sayadi et al., 2022). However, the MA modification had a reverse impact on HHP-pretreated maize starch. This phenomenon may be explained by the fact that the enzymatic hydrolysis preferentially affects the amorphous regions of HHP-pretreated starches instead of the crystalline regions, since HHP-pretreatment already enable the transition from ordered structure to a more disordered one. Higher mobility of internal chains in the amorphous region of HHP-pretreated starch is more accessible for MA to hydrolyze into maltose or malto-oligosacchrides, thus $R_{1047/1022}$ showed an obvious increase in HHP-MA treated samples compared to the HHP only treated samples. The increment of $R_{1047/1022}$ in 500–15-MA treated starch was higher than 500-5-MA treated starch. This can be explained by the effect of deepened hydrolysis of MA on the disordered chains, which contained limited helical contents in 500–15 pretreated starch.

3.4. Long-range ordered structure

The long-range ordered structure of native and modified maize starch was measured by WAXS and the spectra are shown in Fig. 3. The relative crystallinity of the samples was calculated and shown in Table 1. The native maize starch has characteristic peaks at 2θ = 15 and 23° and an unresolved doublet at 2θ = 17 and 18°, which is a typical A type allomorph (Cheetham & Tao, 1998). NMS has a relative crystallinity (RC) of 28.57%. Single buffer or HHP treatment did not change the crystallinity pattern. However, the buffer pre-treatment can increase the value of RC, suggesting that soaking incubation at pH 5.5 and at 50 °C may result in molecular reassociation which is similar to annealing (Dura & Rosell, 2016). Compared with NMS, HHP can obviously lower the intensity of diffraction peaks which was attributed to the unwinding

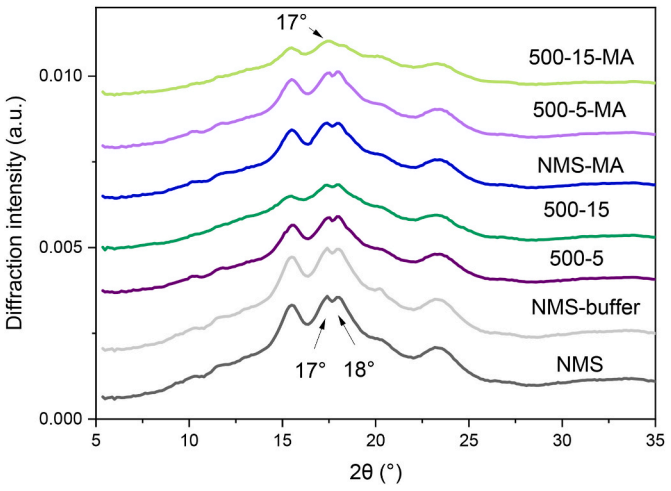

Fig. 3. The WAXS patterns of native and modified maize starches.

of double helix and disruption of crystalline structure. This result is in agreement to the results reported by Liu (Liu et al., 2020) who also demonstrated that the applied pressure result in the loss of structural organization. With the prolongation of pressurization time from 5 min to 15 min, the intensity became weaker. Moreover, RC values decreased to 18.03% and 16.70% when treated at 500 MPa for 5 min and 15 min. However, 500-15-MA modification tended to increase the intensity of peak around 17°. The strongest diffraction peak at 17° 2θ is a characteristic B-starch pattern, thus MA modification may result in forming a small part in a B-type starch structure. A similar phenomenon has also been found for a branching enzyme altering the crystal type (Zhong, Herburger et al., 2021). Compared with NMS, MA treatment reduced the value of RC slightly, and a possible reason for this result is that MA is most likely to attack the crystalline regions. This is in agreement with Zhong et al. (Zhong, Keeratiburana et al., 2021) who found that MA only modified maize starch tend to lower the RC value of starch through its α-(1 → 4)-glycosidic hydrolyzing activity. For HHP-pretreated starches, MA treatment increased the RC, which is in agreement with the $R_{1047/1022}$ results, i.e., it mainly degrade the chains in amorphous phase to decline the area of amorphous region when incubated with a short reaction time (for 3 h).

3.5. Thermal properties

The gelatinization properties as deduced by DSC of native and modified maize starch are summarized in Table 2. The onset temperature (To), peak temperature (T_P) and conclusion temperature (T_C) of native maize starch are determined to 65.94 °C, 71.23 °C and 76.78 °C, respectively, and the enthalpy of gelatinization is 28.16 J/g. In the process of endothermic transition, the water molecules enter into the amorphous area of starch, swelling them to a limited extent, and then continue to interact with crystalline region. The buffer-incubated maize starch exhibited no significant changes in To and ΔH_g. Obviously, MA treatment reduced the temperature and enthalpy of gelatinization. This result can be attributed to less content of amylopectin with a higher average chain length. MA treatment shorten the length of chain and thus reduce the content of double helices, which is consistent with the results of structural changes measured by HPAEC, ATR-FTIR and WAXS. Moreover, due to the MA hydrolysis, more pores and channels result in the absorption of water more easily than NMS. For HHP-treated maize starches, a decrease in gelatinization enthalpy (ΔH_g) demonstrate a partial gelatinization (Liu et al., 2020). By contrast, the MA treatment of the HHP-pretreated starch show an increased pattern on the gelatinization parameters. Water content plays a crucial role in starch gelatinization. It is worth noting that, HHP could enhance the susceptibility of enzyme, thus producing more polyol content (glucose, maltose, oligosaccharides, and maltodextrin) to compete with starch and delay the hydration behavior, afterwards, delaying the gelatinization. The endothermic enthalpy reflected the thermal energy associated with crystallite loss and water was a key factor to break the hydrogen bonds of the double helix (Cooke & Gidley, 1992). The higher gelatinization temperature and enthalpy in 500-5-MA and 500-15-MA samples compared with corresponding 500–5 and 500-15 samples, suggested that it took a long time for water molecules to disrupt the crystals.

Table 2
Thermal properties of native and modified maize starch stored for 0 and 7 days.

Sample name	T_O (°C)	T_P (°C)	T_C (°C)	ΔH_g (J/g)	ΔH_r (J/g)
NMS	65.94 ± 0.13^a	71.23 ± 0.12^a	76.78 ± 0.12^a	28.16 ± 0.12^a	12.52 ± 0.12^a
NMS-buffer	66.03 ± 0.04^a	71.45 ± 0.18^a	76.80 ± 0.24^a	28.58 ± 0.24^a	12.01 ± 0.23^a
500–5	65.97 ± 0.07^a	70.73 ± 0.04^b	75.84 ± 0.12^b	11.95 ± 0.13^b	9.12 ± 0.13^b
500–15	66.74 ± 0.06^b	71.74 ± 0.05^c	76.70 ± 0.13^a	7.23 ± 0.09^c	8.05 ± 0.27^c
NMS-MA	64.66 ± 0.08^c	69.39 ± 0.12^d	75.51 ± 0.10^c	25.77 ± 0.08^d	8.43 ± 0.14^c
500-5-MA	66.73 ± 0.03^b	70.90 ± 0.18^a	75.19 ± 0.06^d	18.81 ± 0.15^e	1.76 ± 0.03^d
500-15-MA	67.97 ± 0.05^d	72.25 ± 0.11^e	76.46 ± 0.14^a	12.90 ± 0.12^f	ND

Values in the same column of the same category with the different letters differ significantly ($p < 0.05$).

The retrogradation properties of starch hydrolysates after 7 days of storage at 4 °C are shown in Table 2. The retrogradation enthalpy (ΔH_r) is reduced to 8.43 J/g when NMS is treated by MA. This might be due to the shortened amylose chains and outer chains of amylopectin that retard the alignment of and generation of double helices. Short-term retrogradation is determined by amylose component, while amylopectin chains have an impact on long-term recrystallization (Li, Li, Tian, & Park, 2014). Grewal et al. (Grewal et al., 2015) showed that the extent of starch retrogradation has a positive correlation with the proportion of amylopectin chains with DP 14–24, but a negative correlation with amylopectin chains of DP 6–9. This is in good agreement with present study (See Section 3.2), where lower proportion of DP 13–24 correspond to lower ΔH_r, especially in 500–15-MA. Besides from this, shorter chains of amylopectin is unable to form stable double-helix structure, and the existence of short chains inhibited the retrogradation (Zhai, Li et al., 2022bib_zhai_et_al_2022). Based on chain length distribution presented above, a lower degree of retrogradation is observed in MA and HHP-MA treated starches. Besides that, longer internal chain segments also play an important role in retrogradation, possibly by contributing to a flexible amylopectin structure (Vamadevan & Bertoft, 2018). In HHP pre-treated starches, a more efficient MA catalysis mode occurred, possible to attack the internal longer chain segments between branch points to restrict the retrogradation.

3.6. Pasting properties

The changes of viscosity of native and modified maize starch during heating-cooling cycle are presented in Fig. 4. The peak viscosity is related to the swelling and integrity of swollen starch granules. The highest peak viscosity was observed in NMS, while a decreasing trend was observed for the 500–5 and 500-15-treated starches. This indicate that single HHP pre-treatment damage the structure organization of starch and in turn decrease the swelling ability. For both native and HHP-pretreated maize starch, the MA treatment reduce the peak viscosity which is a result from the degradation of starch chains. This effect may be explained by the cleavage of α-1,4 glycosidic bonds to smaller malto-oligosaccharides which are less resistant to shear force.

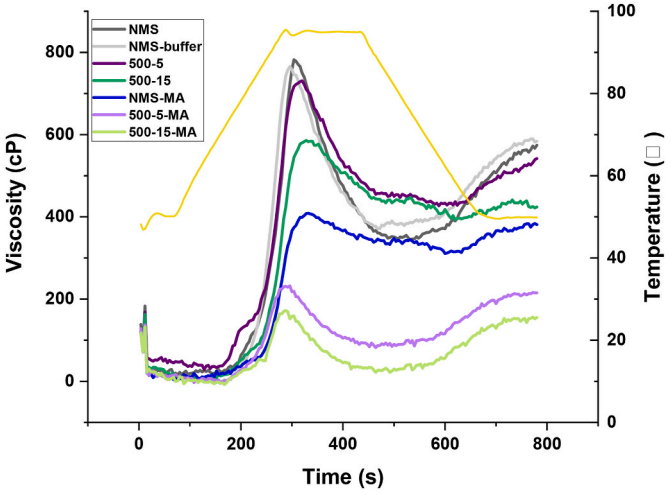

Fig. 4. Pasting properties of native and modified maize starch.

Srichuwong et al. (Srichuwong, Sunarti et al., 2005) reported that the swelling properties were mainly determined by the variation in amylopectin unit-chain length distribution, while amylose only act as a dilutant. Especially in the 500-15-MA treated starch, the pasting viscosity was quite low, indicating that the HHP pre-treatment enhance the susceptibility of MA to shorten lengths of amylose and amylopectin. When cooling down to 50 °C, viscosity begin to increase until reaching the final viscosity, due to the reassociation of the amylose molecules. Setback viscosity was calculated by subtracting peak viscosity from final viscosity, which reflect the retrogradation tendency of starch (Liu, Li, Fan, Zhang, & Zhong, 2019). This seems reasonable as the use of MA cause a significant impact on setback viscosity. In the MA treated starch, the setback viscosity was reduced, most pronounced in 500-5-MA and 500-15-MA starches which were showing lowest setback viscosity. Increasing MA concentration from 2.5 U/g to 15 U/g MA also led to approximately 48% decline in setback value, and suggesting that the short-term retrogradation of starch was inhibited (Zhai, Li et al., 2022). In this case, HHP as a pre-treatment makes sense to yield a greater ability to retard the retrogradation without increasing enzyme dosage.

3.7. Water mobility

Water mobility and compartmentalization is a key factor contributing to the starch retrogradation properties. LF-NMR is a useful tool to measure and contrast the water distribution and migration based on the spin of the ^{1}H in water molecules. Transverse relaxation time (T_2) presents the distribution and mobility of different types of protons with different relaxation rates. In order to get a better understanding of the data, distributed exponential fitting was applied on the LF NMR data by assuming a continuous distribution of T_2 relaxation time constants. As presented in Fig. 5, three different spin-spin relaxation time constants (T_{21}, T_{22}, and T_{23}) were determined for the starches (except for NMS-MA starch) and their relative concentrations (M_{21}, M_{22}, and M_{23}) in native and modified maize starch without any storage. This indicate that three fractions of water with different mobility degrees exist in starch samples. The fast-relaxing component T_{21} may represent water molecules in close proximity to the starch polymers. The intermediate relaxation time T_{22} may represent water molecules in the hydration shells of the starch polymers, while the most abundant water component, T_{23}, may represent water molecules less bounded to the starch polymers and thus primarily affected by the presence of small soluble malto-oligosaccharides. With increasing storage time, a new water component emerges in all samples, namely T_{24}, which represent a syneresis type of almost bulk water that is released from the inside of gel to the outside. In single HHP-treated or MA-treated starches, T_{21}, T_{22} and T_{23} decreased during storage period (from 0 to 7 days), indicating that the water molecules become slightly more distributed and integrated in the starch structure. Pure MA treatment with short incubation time or low concentration is thought to be ineffective to retard the retrogradation during storage. The amylopectin may still reassociate to restrict the mobility of the water molecules, and replace part of the starch-water interaction to release bulk water. However, in HHP-MA treated starches, no significant changes occurred in the values of T_{21}, T_{22} and T_{23}, suggesting that HHP pre-treatment enhance the susceptibility of MA. Moreover, branch chain length distribution is an important factor that determines its ability to retrograde (Chang, Zheng, Zhang, & Zeng, 2021). Thus, two possible reasons could explain these phenomena: (1) MA can degrade the long amylopectin and amylose into shorter polymers, reducing the possibility of inter-chain alignment and thus is less prone to retrogradation. (2) The MA treatment can produce more sugars, replacing the water molecules to interact with starch chains, and these sugars as plasticizers retard the retrogradation compared with equal amounts of water (Smits, Kruiskamp, vanSoest, & Vliegenthart, 2003).

Discrete exponential fitting of the relaxation data was also used to reveal the state of the main water populations and their concentrations. Without any storage, samples could be adequately described by three

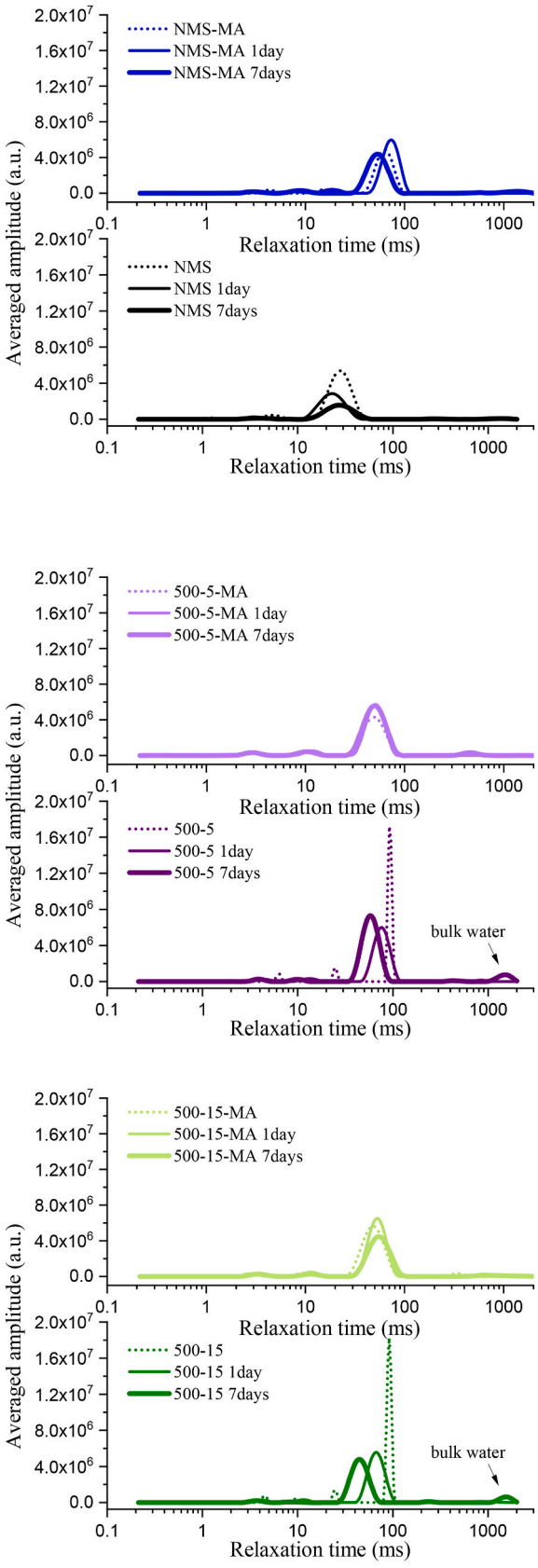

Fig. 5. LF-NMR data – Time constans distribution profiles of native and modified maize starch.

water components (T_{21}, T_{22} and T_{23}). In aged samples, they were considered as being four-component systems (T_{21}, T_{22}, T_{23} and T_{24}). The T_2 and M_2 values obtained from the discrete exponential fitting of the native and modified starch samples are presented in Supplementary Table 1. When comparing the T_2 relaxation constants obtained from discrete and distributed exponential fitting, they show a similar pattern. The combined treatment by HHP and MA was expected to yield relatively stable values of T_{21}, T_{22} and T_{23} during ageing. A significant smaller amount of "free" water (T_{24}) was found in the 500-5-MA sample compared with all the other samples. The water-binding ability promoted in the 500-5-MA gel system, in which oligosaccharides showed favorable hydrophilic properties and could absorb and hold more water (Zhao et al., 2021). However, in 500–15-MA system after 1 day, no significant change was detected in the amount of released bulk water compared with 500–15 treated starch gel, this may be due to the degraded starch granules contributing to a loosened structure untrapping the water molecules. According to the pasting results, the 500-15-MA system showed the lowest viscosity, more like a liquid rather than like a compact gel. Therefore, excessive HHP pre-treatment to promote the attack of MA marginally worked for short-term retrogradation. However, with increasing storage time, the 500-15-MA system regained the ability to restrict the generation of "free" water, as shown by a decrease of 50% in the release of "free" water. Long-term retrogradation is mainly associated with amylopectin, thus MA treatment could prevent the amylopectin from recrystallization. The produced branched oligosaccharides may hide the branches of amylopectin and thus contribute to retard the recrystallization. In conclusion proper HHP pre-treatment can increase the efficiency of MA treatment and provide optimal anti-aging function.

3.8. In-vitro digestibility properties

The contents of RDS, SDS and RS in native and modified maize starches are summarized in Table 3. The NMS had 53.21%, 22.52% and 24.26% of RDS, SDS and RS contents, respectively. The HHP treatment increased the RDS and SDS content, but this is accompanied by a decrease in RS contents. This is due to the fact that the amorphous regions are favored for ready digestion, and that HHP can damage the double helices which then are much easier to digest. Deng et al. (Deng et al., 2014) has reported a similar trend in the rice starch after HHP treatment. The contents of RDS, SDS and RS in starch samples are influenced by multiple factors, including the processing method, starch source and molecular structure of starch, which could result in the increase or decrease in the starch fractions (Castro et al., 2020). The RS content was the lowest in the 500–15 treated samples than in the 500–5 treated samples. The rupture of granules produced by partial gelatinization led to the expose more enzyme-susceptible interior regions, which in turn promote the in-vitro digestion. After MA treatment, the content of SDS increased, which is in agreement with the data obtained in the previous study (Miao et al., 2014). In this study the maize starch was modified using maltogenic α-amylase for 6 h after which it displayed a significantly higher SDS content. Li et al. (Li, Li et al. 2021) ascribed the improvement in the slowly digestion pattern of pulse starch after MA treatment to the increasing content of α-1,6 glycosidic bonds. The amylolytic hydrolysis of α-1,6 linkages took place at a slower rate than that of α-1,4 linkages, which indicate that the branched oligosaccharides can be used as prebiotics to promote health and treat diseases (Miao et al., 2014). It has previously been reported that pancreatic α-amylase does not cleave linkages close to the branching point of the shortened branches, thus MA may release some starch hydrolysis products including α-limit dextrins with lower DP of amylopectin chains, influencing the extent and ratio of glucose release significantly (Korompokis, Deleu et al., 2021). Specifically, the 500-15-MA sample, showed an increase in the contents of not only SDS but also RS, suggesting that the impact of different DP chains in released starch products should be taken into account. So far, information about how the structure of dextrins affects the binding site or binding activity of the digestive enzymes is still elusive. Therefore, the use of MA to develop healthy and functional starch-based products should be continued including both in-vitro and in-vivo digestibility properties.

Table 3
The percentage contents of RDS, SDS and RS in native and modified uncooked maize starches.

Sample name	RDS (%)	SDS (%)	RS (%)
NMS	53.21 ± 0.12^{a}	22.52 ± 0.15^{a}	24.26 ± 0.17^{a}
NMS-buffer	52.22 ± 0.23^{b}	22.88 ± 0.27^{a}	24.89 ± 0.12^{b}
500–5	70.62 ± 0.23^{c}	23.37 ± 0.16^{c}	10.00 ± 0.19^{c}
500–15	77.81 ± 0.13^{d}	20.36 ± 0.11^{d}	7.82 ± 0.15^{d}
NMS-MA	48.20 ± 0.13^{e}	28.50 ± 0.11^{e}	23.30 ± 0.14^{e}
500-5-MA	56.48 ± 0.15^{f}	36.94 ± 0.22^{f}	6.57 ± 0.16^{f}
500-15-MA	58.24 ± 0.23^{g}	32.55 ± 0.21^{g}	9.21 ± 0.14^{g}

Values in the same column of the same category with the different letters differ significantly ($p < 0.05$).

To summarize, a schematic graph showing the possible mechanisms of single HHP, single MA and dual HHP-MA modification on the structural organizations of NMS was presented in Fig. 6.

4. Conclusion

HHP treatment of maize starch can be used to create a middle state between the semi-crystalline and starch paste with granular structure to enhance the susceptibility for MA treatment. HHP treatment facilitated the mobility of water molecules and ruptured the highly ordered structure. Therefore, MA treatment of maize starches can not only conducive to produce short chains by attacking the side chains with exo-action, but can also act as an endo-activity enzyme attacking the longer chain segments between branch points. Compared with pure MA treatment, the combined treatment by HHP and MA induced more pores and cracks on the surface, more drastic depolymerization of starch chains, more loss of amorphous regions and produced more malto-oligosaccharides. Furthermore, these structural variations resulted in a change of the retrogradation properties, including a decrease in the retrogradation enthalpy, a lowering of the swelling ability and setback viscosity and limited water migration into "bulk" water. Moreover, increasing applied pressure did not create extrapolative results. However, combined HHP-MA treatment can produce more slowly digestible starch, or even more resistant starch in the case of the NMS-500-15-MA starch. These results demonstrate a potential of designing starch-based products with anti-stalling properties as well as lower digestibility. In a broader perspective, the content of starch degradation fractions in the samples reminds us noticing that the effect of malto-oligosaccharides on the digestion process might be quite different from that of glucose and starch.

Author statement

Zhenyu Liu: Writing-original draft, Methodology, Formal analysis, Data analysis. Yuyue Zhong: Writing-review & editing and Conceptualization. Bekzod Khakimov: Conceptualization and Investigation. Yongxia Fu: Formal analysis. Tomasz Pawel Czaja: Data analysis. Jacob Judas Kain Kirkensgaard: Data analysis and Software. Andreas Blennow: Resources. Qun Shen: Funding acquisition, Supervision and Writing-review & editing. Søren Balling Engelsen: Funding acquisition, Supervision and Writing-review & editing.

Declaration of competing interest

The authors declare that there is no conflict of interest.

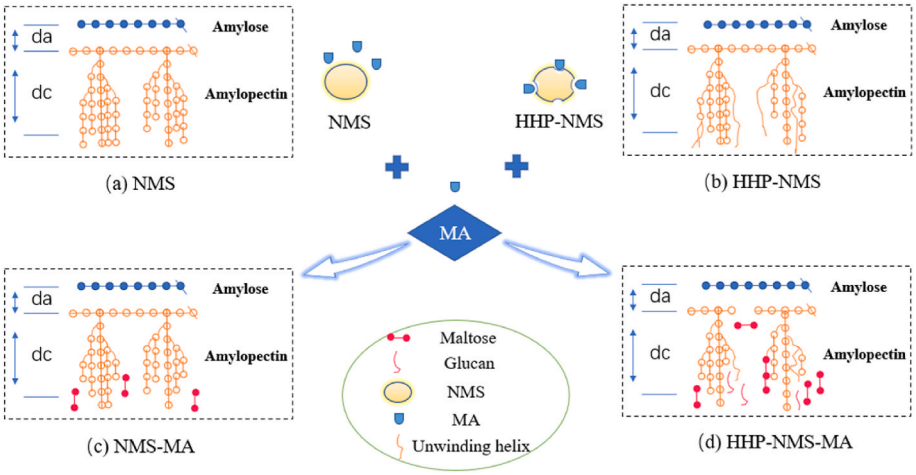

Fig. 6. The possible mechanisms of single HHP, single MA and dual HHP-MA modification on the structural organization of maize starch (da: amorphous lamellar region; dc: crystalline lamellar region; (a) NMS: native maize starch; (b) HHP-NMS: native maize starch treated by HHP treatment; (c) NMS-MA: native maize starch modified by maltogenic α-amylase; (d) HHP-NMS-MA: native maize starch treated by HHP treatment combined with maltogenic α-amylase modification).

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

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