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An enzymatic approach to quantify branching on the surface of starch granules by interfacial catalysis

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ABSTRACT

Enzymatically modified starch granules are useful in the food industry by endowing improved thermal properties, resistance to digestion and complexation capacity. However, it is of interest to correlate structural features on the granular surface with functional characteristics relevant to given applications. To meet this requirement, a method was developed to quantify the density of α-1,6 branch points on differently structured starch granules as based on interfacial enzyme catalysis. The branch points are attacked by pullulanase, a debranching enzyme, and the branch point density, as calculated from the kinetic attack site density ($^{kin}\Gamma_{max}$), was linked to the chain length distribution (CLD) of the released segments. The procedure involved a combination of conventional and inverse Michaelis–Menten (MM) kinetics for pullulanase degradation of native, branching enzyme- or 4-α-glucanotransferase-modified granular waxy and normal maize starch (WMS and NMS). The treatment by branching enzyme increased the branch point density for WMS from 1.7 to 3.3 nmol/g starch granules. CLD analysis indicated that 4-α-glucanotransferase catalyzed hydrolysis and/or cyclization on the surface of the granules, rather than disproportionation. The CLD data reflected the different spatial organization of amylopectin chains within WMS and NMS granules related to their different amylose contents of 0.7 and 20.7%, respectively. Scanning electron microscopy confirmed that the starch granules retained the morphology without prominent cracks or pores after pullulanase hydrolysis for the analysis of interfacial kinetics. Comparison with the corresponding gelatinized starches gave new insights into the connection between substrate structure and specificity of the two glucotransferases acting on the different starches.

1. Introduction

Starch is a widely occurring renewable plant polysaccharide that plays a major role in the food industry (Chi et al., 2021). For most applications, starch is gelatinized in heat-moisture processes (Liu et al., 2020; Zhong et al., 2022). However, focus on sustainability and energy-saving motivates use of the raw starch granules and their applications are emerging (Liu et al., 2020; Zhong et al., 2022). To confer novel functionalities and enhance its positive attributes, starch is generally subjected to functional improvements by structural engineering using enzymatic, chemical or physical treatments (Li et al., 2023; Miao & Bemiller, 2023). Clearly enzyme treatment of native starch granules represents an environmentally friendly strategy. Moreover, it is attractive because it avoids high viscosity and instability caused by retrogradation as compared to treatment of gelatinized starch (Wang, Li, Copeland, Niu, & Wang, 2015). Overall, there is currently a growing interest in the application of various transglycosylases or hydrolytic enzymes for modifying granular starches (Guo, Deng, Lu, Zou, & Cui, 2019; Miao & Bemiller, 2023; Zhong et al., 2022). Notably, maize starch granules modified by branching enzyme (Ren et al., 2020; Zhong et al., 2021) or cyclodextrin glycosyltransferase (Dura & Rosell, 2016) have shown higher resistance to digestion.

Several techniques have been used to analyze enzyme-modified starches, including high performance anion exchange chromatography

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with pulsed amperometric detection (HPAEC-PAD), size exclusion chromatography-multi-angle laser light scattering-refractive index detection (SEC-MALLS-RI) and ^{1}H NMR (Zhai, Li, Bai, Jin, & Svensson, 2022). However, these methods were developed for solubilized starch and are not suitable for direct analysis of structural changes on starch granule surfaces. Recently we introduced a procedure for kinetics analysis of the interfacial hydrolysis of α-1,4-linkages on the surface of different granular starches using the *exo*-acting glucoamylase and *endo*-acting α-amylase (Tian, Wang, Liu, et al., 2023; Tian, Wang, Zhong, et al., 2023; Wang, Tian, et al., 2023). This involved combining conventional and inverted Michaelis-Menten (MM) kinetics having substrate and enzyme, respectively, in excess, which lead to values of the density of enzyme attack sites, $^{kin}\Gamma_{max}$ (in units of mol/g), on the granules (Tian, Wang, Liu, et al., 2023; Wang, Tian, et al., 2023). The used approach was inspired by kinetics analysis of the heterogenous catalysis of cellulase depolymerization of crystalline cellulose (Kari, Andersen, Borch, & Westh, 2017).

Here, we adopt the kinetics analysis of heterogenous catalysis to enumerate α-1,6-linked branch points hydrolyzed by *Bacillus licheniformis* pullulanase (*Bl*Pul) (Abdel-Naby, Osman, & Abdel-Fattah, 2011) on the surface of granules of waxy and normal maize starch (WMS and NMS). This new method was validated using the same WMS and NWS granular starches, which were pretreated by either branching enzyme from *Rhodothermus obamensis* (*Ro*BE; EC 2.4.1.18; glucoside hydrolase family 13, GH13) that catalyzes the introduction of new α-1,6 linked branch chains (Tetlow & Emes, 2014) or by 4-α-glucanotransferase from *Thermoproteus uzoniensis* (TuαGT; EC 2.4.1.25; GH77), which is able to catalyze four reactions on starch, namely hydrolysis, coupling, cyclization and disproportionation (Wang et al., 2020). The disproportionation reaction is particularly attractive as it delivers elongated exterior branch chains in amylopectin by transfer of short fragments from amylose to non-reducing chain ends via new α-1,4-linkages (Wang et al., 2020). In the current study, surprisingly it was found that TuαGT did not elongate the chains on the surface of granular starches, but rather catalyzed hydrolysis and/or cyclization. Despite this, the contrasting effects of *Ro*BE and TuαGT on solubilized and granular starches provide novel insights into modification of starch granules using glucanotransferases.

2. Material and methods

2.1. Materials

Waxy maize starch (WMS) was a kind gift of Cargill, USA, and normal maize starch (NMS) of Archer Daniels Midland (ADM, Decatur, IL). Pullulanase M2 from *Bacillus licheniformis* (*Bl*Pul, E-PULBL, 900 U/mL) was purchased from Megazyme Co. Ltd (Wicklow, Ireland). Branching enzyme from *Rhodothermus obamensis* (*Ro*BE, 5.98 U/mg) was a kind gift of Novozymes, Denmark. *Thermoproteus uzoniensis* 4-α-glucanotransferase (TuαGT, 542 U/mg) was produced as described (Wang et al., 2020).

2.2. Modification of granular starch

Starch (6%, w/v), washed twice with MilliQ water and once with reaction buffer (20 mM sodium citrate, pH 6.0), was suspended in reaction buffer and modified by either 1.0 U *Ro*BE or 32.5 U TuαGT per 1 g starch (50 °C, 20 h). As a control, starch was incubated with reaction buffer (50 °C, 20 h). Reactions were terminated by addition of Na_2CO_3 (final concentration: 0.3 M) followed by centrifugation (10,000 *g*, 5 min) after 10 min of incubation. The unmodified (control) and modified starch granules were washed with MilliQ water and freeze-dried.

2.3. Modification of gelatinized starch

Starch (6%, w/v) was washed as above and suspended in reaction buffer, gelatinized (99 °C, 30 min), cooled and modified by either 1.0 U *Ro*BE (60 °C, 20 h) or 32.5 U TuαGT (70 °C, 20 h) per 1 g starch. As a control, gelatinized starch was incubated with reaction buffer (60 °C, 20 h). Reactions were terminated by heating (100 °C, 30 min). The unmodified and modified starch were precipitated by three volumes of ethanol, centrifuged (10,000 *g*, 5 min), kept overnight at −80 °C, and freeze-dried.

2.4. Chain length distribution (CLD)

Granular starch (50 mg/mL, w/v), resuspended in 50 mM sodium acetate pH 5.5, was debranched by 50 nM (final concentration) *Bl*Pul (25 °C, 30 min), followed by centrifugation (10000 *g*, 5 min). Gelatinized starch (5 mg/mL, w/v) was suspended in 50 mM sodium acetate pH 5.5, gelatinized again (99 °C, 30 min), debranched by 50 nM (final concentration) *Bl*Pul (42 °C, 2 h) and centrifuged (10000 *g*, 5 min). The supernatants were analyzed by HPAEC-PAD to determine the CLD as described (Christensen et al., 2022).

2.5. Determination of attack site density ($^{kin}\Gamma_{max}$) on the starch granule surface

The attack site density, $^{kin}\Gamma_{max}$, was determined for *Bl*Pul by a combination of conventional and inverse MM kinetics, adopting procedures applied for enzymatic hydrolysis of solid polysaccharide substrates as discussed in more detail elsewhere (Andersen, Kari, Borch, & Westh, 2018; Kari et al., 2017, 2018; Tian, Wang, Liu, et al., 2023; Tian, Wang, Zhong, et al., 2023; Wang, Tian, et al., 2023). Briefly, in conventional MM analysis starch granules (15–150 mg/mL, 135 μL) were pre-incubated (25 °C, 15 min, 1100 rpm), added *Bl*Pul (15 μL, final 62.5 nM) and incubated (25 °C, 1100 rpm). For inverse MM analysis, *Bl*Pul (0.3–625 nM) was added to starch granules (20 mg/mL) and after 30 min, which is within the linear range of hydrolysis (data not shown), aliquots (100 μL) were transferred to new tubes, mixed with 20 μL 1.8 M Na_2CO_3 to terminate the reaction, and centrifuged (10000 *g*, 5 min). The concentration of reducing sugar in the supernatants was determined using the PAHBAH assay with glucose as standard (Lever, Powell, Killip, & Small, 1973).

Conventional MM kinetics (substrate in excess) were analyzed according to eq. (1), where S_0^{mass} is substrate mass load and $K_{1/2}$ the mass load at substrate half-saturation. Non-linear regression analyses of the data returned values of V_{max} (in M·s^{-1}) and $K_{1/2}$ (in g·L^{-1}).

$$v_0 = \frac{V_{max} \cdot S_0^{mass}}{K_{1/2} + S_0^{mass}} \quad (1)$$

Inverse MM kinetics (enzyme in excess) were analyzed according to eq. (2), where E_0 is enzyme concentration and K_M the enzyme concentration at enzyme half-saturation. Nonlinear regression analysis of data led to $^{inv}V_{max}$ (in g·L^{-1}·s^{-1}) and K_M (in M).

$$v_0 = \frac{^{inv}V_{max} \cdot E_0}{K_M + E_0} \quad (2)$$

The $^{kin}\Gamma_{max}$ was determined from V_{max} (eq. (1)) and $^{inv}V_{max}$ (eq. (2)) using eq. (3) as previously described (Kari et al., 2017).

$$\frac{\frac{^{inv}V_{max}}{S_0^{mass}}}{\frac{V_{max}}{E_0}} = {}^{kin}\Gamma_{max} \quad (3)$$

For validation of quasi-steady state assumption (QSSA) (Kari et al., 2017) see Supplementary material.

2.6. Scanning electron microscopy (SEM)

Starch granules (20 mg/mL) were suspended in reaction buffer, treated with 625 nM *Bl*Pul (25 °C, 30 min, 1100 rpm) and the reactions were terminated by addition of Na_2CO_3 (final concentration: 0.3 M) and centrifuged (10,000 *g*, 5 min) after 10 min. The starch granules were washed with MilliQ water and freeze-dried. For imaging, all starch

granules were mounted on carbon tapes on aluminum SEM stubs and sputter-coated with 6 nm gold under a Leica EM ACE200 gold coater (Leica Microsystems, Wetzlar, Germany). Both overall and detailed morphology of granular starch samples were visualized using field emission scanning electron microscopy (FE-SEM) using an FEI Quanta 200 microscope at 3500 × and 15,000 × magnification, respectively, as previously described (Tian, Wang, Liu, et al., 2023).

3. Results and discussion

3.1. CLD of starches before and after enzymatic modification

Treatment of starch by either *Ro*BE or TuαGT led to an increase in the number of branch chains and longer branch chains, respectively (Table 1). It is noteworthy that the enzyme modifications of both granular and gelatinized starches were carried out using the same enzyme concentration and starch loads. Importantly, the precise substrate concentration represented by accessible branch points (α-1,6 glucosidic bonds) on the starch granule surface was not accurately known.

The *Ro*BE-modified granular WMS exhibited 2.5-fold higher and 9.5-fold lower proportion of A-chains (DP < 12) and B_1-chains, respectively, compared to native WMS granules (Table 1, Fig. 1A). On the other hand, in *Ro*BE-modified granular NMS A-chains only increased 1.9-fold while B_1-chains decreased 10.7-fold compared to the native NMS (Table 1, Fig. 1B). Thus *Ro*BE-catalyzed transfer of maltooligosaccharide chains yielded new branches in amylopectin, leading to a shorter average length of the branch chains released from the granules by *Bl*Pul. By contrast, modification of gelatinized WMS using *Ro*BE increased the proportion of A-chains by only 1.3-fold, and reduced the proportion of B_1-(DP 13–24) and B_3-chains (DP > 37) by 1.1-fold (Table 1, Fig. 1C). Similarly, *Ro*BE-modified gelatinized NMS exhibited 1.2-fold increase in A-chains and 1.1−1.2-fold decrease in B_1- and B_3-chains compared to gelatinized unmodified NMS (Table 1, Fig. 1D). Consequently, *Ro*BE demonstrated highly efficient catalytic activity in introducing new branch chains onto the surface of starch granules as shown by the *Bl*Pul CLD analysis confirming the *Ro*BE-mediated enrichment of short chains on the granular surface.

The TuαGT modification of granular WMS (Fig. 1A) resulted in a 1.2-fold increase in A-chains and decreased B_1, B_2- and B_3-chains by 1.1-, 1.1- and 1.8-fold, respectively, compared to native WMS. This resulted in a slight overall decrease in DP_{Ave} (Table 1). A similar trend was seen by TuαGT modification of NMS granules, where 1.3-fold higher and 1.2-fold lower proportions, respectively of A- and B_1-chains were obtained relative to native NMS granules (Fig. 1B; Table 1). The CLD patterns for the corresponding native and modified gelatinized starches differed, as in case of WMS, both A- and B_1-chains slightly decreased by 1.1-fold, while B_2- and B_3-chains increased by 1.2- and 1.1-fold, respectively. NMS exhibited a similar pattern, although its B_1-chain content remained unchanged (Table 1). The CLD data supported the role of TuαGT catalyzing disproportionation between glucan chains, resulting in less short and more long chains. Notably, among the four different reactions catalyzed by TuαGT, only hydrolysis and cyclization will lead to an overall decrease in average chain length as found for the surface of modified granular starches (Table 1). Therefore, TuαGT apparently mainly catalyzed hydrolysis and/or cyclization of branch chains on the starch granules, but catalyzed disproportionation of the branch chains in gelatinized starches (Table 1).

3.2. Density of BlPul attack sites ($^{kin}\Gamma_{max}$) on granular starches before and after enzymatic modification

Normally the α-1,6-/α-1,4-linkage ratio of starch is determined by ^{1}H NMR spectroscopy after complete gelatinization. However, this method is not suitable for the analysis of starch granule surfaces since these only constitute a fraction of the entire starch granule.

Table 1
Relative content of different branch chains released by *Bl*Pul from granular and gelatinized WMS and NMS before and after modification by either *Ro*BE or TuαGT.

Starch	Type of chain[a]	Native starch	*Ro*BE modified starch	TuαGT modified starch
Granular WMS	A-chain	37.4 ± 0.9[b] (100[c])	94.3 ± 3.1 (252)	44.0 ± 0.9 (118)
	B_1-chain	54.1 ± 1.1 (100)	5.7 ± 0.4 (11)	48.5 ± 1.1 (90)
	B_2-chain	7.7 ± 0.3 (100)	ND[d]	7.1 ± 0.5 (92)
	B_3-chain	0.7 ± 0.0 (100)	ND	0.4 ± 0.1 (57)
	DP_{Ave}[e]	14.9 ± 0.8 (100[f])	5.7 ± 0.4 (38)	13.1 ± 1.1 (88)
	α-1,6-/α-1,4-linkage ratio	7.2	21.1	8.3
Granular NMS	A-chain	51.0 ± 1.3 (100)	96.0 ± 2.9 (188)	65.5 ± 1.5 (128)
	B_1-chain	42.9 ± 1.1 (100)	4.0 ± 0.3 (9)	34.5 ± 0.7 (80)
	B_2-chain	5.9 ± 0.4 (100)	ND	ND
	B_3-chain	0.2 ± 0.0 (100)	ND	ND
	DP_{Ave}	12.6 ± 1.2 (100)	6.6 ± 0.5 (52)	9.5 ± 0.7 (75)
	α-1,6-/α-1,4-linkage ratio	8.7	18.0	11.8
Gelatinized WMS	A-chain	18.7 ± 0.3 (100)	25.0 ± 0.7 (134)	16.8 ± 0.4 (90)
	B_1-chain	39.0 ± 0.8 (100)	35.0 ± 0.3 (90)	35.2 ± 1.3 (90)
	B_2-chain	20.6 ± 0.6 (100)	20.6 ± 1.0 (100)	23.8 ± 1.5 (116)
	B_3-chain	21.7 ± 2.2 (100)	19.4 ± 1.0 (89)	24.2 ± 1.1 (112)
	DP_{Ave}	25.2 ± 1.8 (100)	23.9 ± 2.1 (95)	26.9 ± 1.9 (107)
Gelatinized NMS	A-chain	32.0 ± 0.2 (100)	38.7 ± 1.0 (121)	28.9 ± 1.1 (90)
	B_1-chain	41.9 ± 0.7 (100)	36.9 ± 1.1 (88)	41.3 ± 1.9 (99)
	B_2-chain	16.8 ± 0.3 (100)	16.4 ± 0.8 (98)	19.6 ± 0.5 (117)
	B_3-chain	9.2 ± 0.5 (100)	8.0 ± 0.2 (87)	10.2 ± 0.3 (111)
	DP_{Ave}	19.0 ± 0.9 (100)	17.9 ± 1.1 (94)	20.1 ± 1.4 (106)

[a] A-chain: DP 1–12, B_1-chain: DP 13–24, B_2-chain: DP 25−36, and B_3-chains: DP > 37 (Bertoft, 2017).
[b] Values are means ± standard deviation.
[c] Percentage of the relative content of chains for unmodified starch (100%) are given in parentheses.
[d] ND: not determined.
[e] Average DP.
[f] Percentage of the DP_{Ave} of chains for unmodified starch (100%) are given in parentheses.

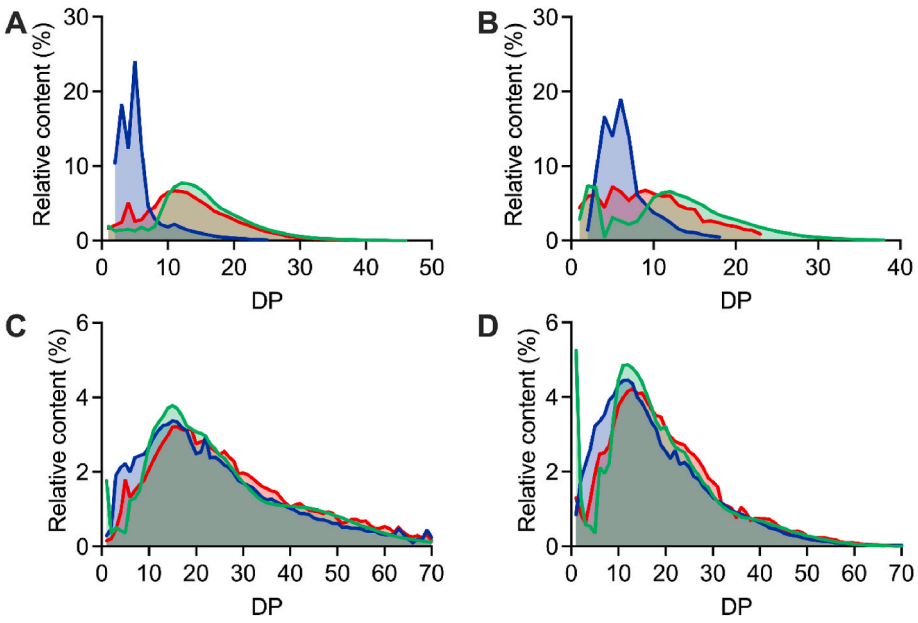

Fig. 1. Chain length distribution (CLD) of products released by *Bl*Pul from granular and gelatinized starches. (A) Granular WMS, (B) granular NMS, (C) gelatinized WMS, (D) gelatinized NMS. The granules were native (green), modified by *Ro*BE (blue) or TuαGT (red).

The cornerstone of the presented new method is the specific hydrolysis of α-1,6-linkages (branch points) on the starch granule surface by *Bl*Pul (Jung et al., 2013). This hydrolysis enables quantification of the density of branch points ($^{kin}\Gamma_{max}$) by using a combination of conventional and inverse MM kinetics implemented for heterogenous catalysis (Tian, Wang, Liu, et al., 2023; Wang, Tian, et al., 2023). The two MM equations (Section 2.5, eqs. (1) and (2)) were firstly validated under the quasi-steady-state assumption (QSSA) (Fig. S1 and Supplementary material "Validation of Quasi-Steady State Assumption (QSSA)") (Kari et al., 2017). The validity ranges of the conventional and inverse MM equations were calculated according to eqs. S1 and S2 and illustrated in Fig. S1. This confirmed that interfacial kinetics analysis for all six starch samples provided sufficient data to estimate the desired parameters (E_0, S_0^{mass}, $K_{1/2}$, K_M, $^{kin}\Gamma_{max}$). In the conventional MM approach starch in the range 20–150 mg/mL and 62.5 nM *Bl*Pul fell within the region of conventional MM, ensuring that the substrate was in excess, thus the QSSA was valid for conventional MM analysis (Kari et al., 2017; Schnell, 2014). Furthermore, for the inverse approach *Bl*Pul of 0.3–625 nM and 20 mg/mL starch were within the range of inverse MM, hence the enzyme was in excess and the QSSA was valid under inverse MM.

The attack site density, $^{kin}\Gamma_{max}$, varied significantly between the two types of starch granules. Specifically, WMS contained 1.7 nmol and NMS 0.9 nmol of α-1,6-linkages cleaved by *Bl*Pul per g of starch. This variation in $^{kin}\Gamma_{max}$ can be attributed primarily to the different amylopectin contents. Moreover, it may reflect the differences between WMS and NMS in CLD patterns and thus branch point environments, crystallinity and double helical chain contents. Notably, the $^{kin}\Gamma_{max}$ values for α-amylase acting on WMS was 0.28 nmol/g, which was 1.6-fold higher than 0.17 nmol/g observed for NMS granules (Tian, Wang, Liu, et al., 2023; Wang, Tian, et al., 2023). These previous findings are comparable with the present 1.9-fold higher attack site density for *Bl*Pul on WMS compared to NMS. Besides, kinetics analysis of glucoamylase, which removes glucose from non-reducing ends (Sauer et al., 2000), acting on six starch types showed 14.6-fold higher k_0 (molecular activity of the enzyme) on granular rice starch than on potato starch (Tatsumi & Katano, 2005). The different k_0 for different starches might stem from the density of non-reducing ends on the surface of starch granules. This suggests k_0 as an indicator for the non-reducing ends in starch, similar to the $^{kin}\Gamma_{max}$ values obtained in the present work for *Bl*Pul.

The α-1,6-/α-1,4-linkage ratio can be calculated from the $^{kin}\Gamma_{max}$ and the values of DP_{Ave} of branch chains released by *Bl*Pul from the granular surface (Table 1) according to eq. (4).

$$\alpha\text{-}1,6\text{-}\Big/\alpha\text{-}1,4\text{-linkage ratio} = \frac{^{kin}\Gamma_{max}}{(DP_{Ave}-1)\times {}^{kin}\Gamma_{max}} \times 100 \quad (4)$$

Remarkably, the α-1,6-/α-1,4-ratio of 7.2% for the WMS granule surface was very similar to 7.0% determined for gelatinized WMS by using ^{1}H NMR spectroscopy (Chen et al., 2017). Surprisingly, the granular NMS showed an α-1,6-/α-1,4-ratio of 8.2%, which is 1.1-fold higher than of WMS, whereas a 1.4-fold lower α-1,6-/α-1,4-ratio was reported for gelatinized NMS than for WMS as determined by ^{1}H NMR (Chen et al., 2017). This discrepancy relates to NMS containing 20.7% amylose as opposed to 0.7% in WMS (Htoon et al., 2009; Tian et al., 2022). The presence of the mainly linear amylose, interspersed among amylopectin molecules in starch granules, influences the distribution of amylopectin (Bertoft, 2017). Thus, due to the very low amylose content, amylopectin is relatively evenly distributed in WMS, and a similar α-1,6-/α-1,4-ratio may be expected for the amylopectin exposed on the surface of the WMS granules as determined in the corresponding gelatinized WMS. However, in NMS granules the presence of amylose results in an uneven distribution of amylopectin on the granular surface, leading to a higher α-1,6-/α-1,4-ratio compared to in the corresponding gelatinized NMS representing the entire granule.

WMS and NMS granules modified by *Ro*BE exhibited 1.9- and 2.3-fold higher $^{kin}\Gamma_{max}$, respectively, compared to the corresponding unmodified granules (Fig. 2C, F). This increase in $^{kin}\Gamma_{max}$ aligns with *Ro*BE-catalyzed formation of new α-1,6-linkages on the granular surface, as observed for the *Ro*BE-treatment of gelatinized WMS and NMS (Table 1) and in a previous study (Ban et al., 2020). A similar effect of *Ro*BE on NMS was recently reported by using NMR for the α-1,6-/α-1,4-linkage ratio analysis on gelatinized starch after *Ro*BE-modification of NMS granules (Zhong et al., 2021). However, the NMR analysis conducted on gelatinized starch, was not suitable for direct quantification of changes in α-1,6-linkage contents resulting from surface modification.

Notably, the CLD of TuαGT-treated granular starches (Fig. 1C and D) indicated that TuαGT preferably catalyzed hydrolysis or cyclization on the granule surface, which resulted in the shortening of branch chains. Moreover, as expected, $^{kin}\Gamma_{max}$ for *Bl*Pul of TuαGT-treated WMS and NMS granules was essentially the same as for the corresponding unmodified starch granules. This indicates that shortening of branch chains

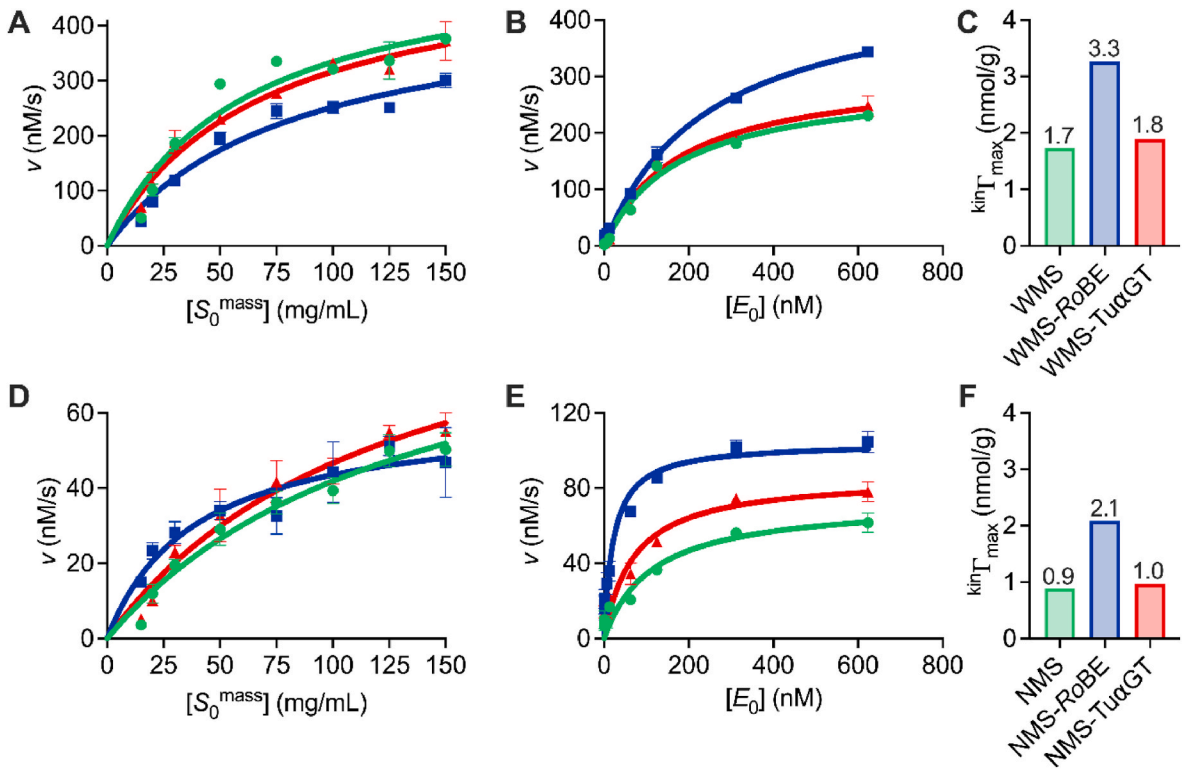

Fig. 2. Interfacial catalysis of *Bl*Pul debranching starches granules at 25 °C and pH 5.5. (A) Conventional, (B) inverse MM kinetics, and (C) $^{kin}\Gamma_{max}$ for WMS (green), WMS-*Ro*BE (blue), and WMS-TuαGT (red). (D) Conventional, (E) inverse MM kinetics, and (F) $^{kin}\Gamma_{max}$ for NMS (green), NMS-*Ro*BE (blue), and NMS-TuαGT (red).

by TuαGT did not affect the recognition of branch points by *Bl*Pul, underscoring that $^{kin}\Gamma_{max}$ is a valid parameter for determining the density of *Bl*Pul-accessible branch points on starch granule surfaces.

3.3. Appearance of starch granule surfaces after BlPul treatment

To assess the impact of *Bl*Pul treatment on the surface of different starch granules, samples subjected to 30 min of reaction in inverse MM kinetics analysis at high E_0/S_0^{mass} were examined using SEM. Prior to debranching, SEM imaging showed overall morphology (Fig. S2) and detailed surface morphology (Fig. 3) as round or irregular shaped granules with smooth surface without significant pores of native, *Ro*BE-, and TuaGT-modified granular starches (Fig. S2, Fig. 3 A–C, G–I). These results align with our previous study, indicating that the modifications caused by *Ro*BE and TuαGT did not affect the surface of the granules (Zhong et al., 2021). Importantly after *Bl*Pul hydrolysis for 30 min, the surface of the granules remained smooth without appearance of more pores or cracks (Fig. 3 D–F, J–L), supporting that the hydrolysis during the kinetic analysis primarily occurs on the starch granule surface (Fig. 3). For enzyme kinetics analysis it is assumed that $^{kin}\Gamma_{max}$ is constant throughout the reaction. While this in principle may not hold true as some substrate conversion occurs, the current set of results from interfacial kinetic analysis indicate that, the extent of substrate conversion was <0.3% in most cases although amounting to 0.5% for the highest E_0 (625 nM) and lowest S_0^{mass} (20 mg/mL). This low degree of substrate consumption indicates that the surface does not undergo significant destruction, supporting the assumption of constant $^{kin}\Gamma_{max}$ during the kinetics analysis.

4. Conclusions

In the present work, we implemented a novel approach to quantify branch points on the surfaces of WMS and NMS granules by measuring the attack site density ($^{kin}\Gamma_{max}$) for *Bl*Pul using heterogenous catalysis. This procedure involved a combination of conventional and inverse MM kinetics and was validated for *Ro*BE- and TuαGT-modified starch granules. Our results demonstrate that *Ro*BE-treatment led to the formation of shorter chains and a reduction in longer chains, as evidenced by the increased attack site density ($^{kin}\Gamma_{max}$) for *Bl*Pul and CLD analysis of the released chains. SEM confirmed that the morphology and surface appearance of the starch granules were essentially unchanged by the enzyme modifications and the pullulanase catalyzed debranching. This method serves as a valuable tool for analyzing branch structures resulting from *Ro*BE- and TuαGT-modifications of the surface of starch granules, and it can be adapted to quantify other modifications of granular starches. Additionally, the method may be applied as a tool to analyze pretreated less compact porous or cold water swollen starch granules, which are physical modifications introduced to minimize need for starch processing.

Author statement

Yu Wang designed and performed the experiments, collected the data, and drafted the manuscript.

Marie Sofie Møller and Birte Svensson developed the theoretical framework and edited the manuscript.

Yu Tian and Andreas Blennow collected the data for chain length distribution of gelatinized starches and scanning electron microscopy images.

Stefan Jarl Christensen collected the data for chain length distribution of ungelatinized starch.

All other authors contributed to the revision and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

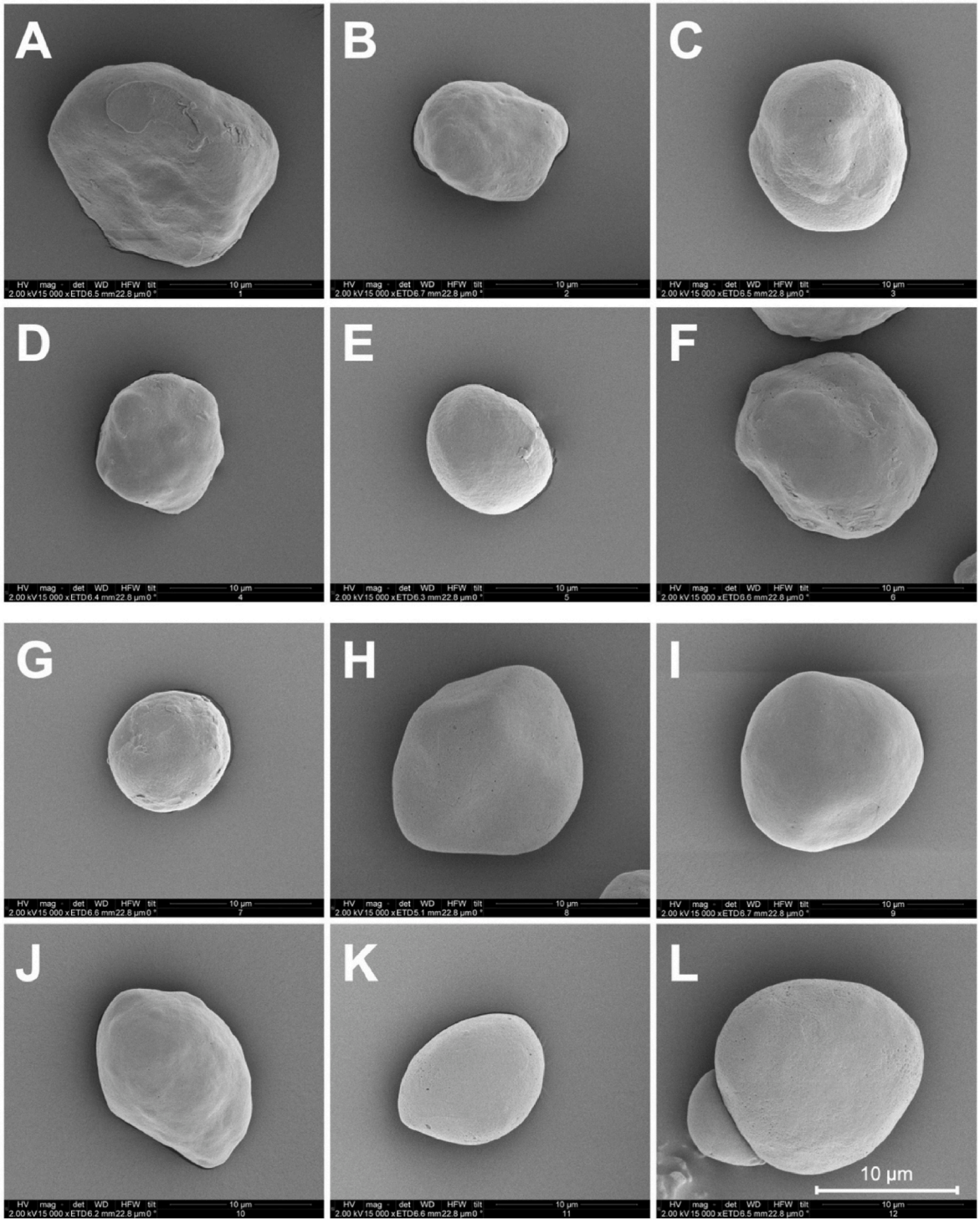

Fig. 3. SEM images of unmodified and modified starch granules before and after 30 min hydrolysis by 625 nM *Bl*Pul. Before hydrolysis (A) WMS, (B) WMS-*Ro*BE, and (C) WMS-TuαGT; after hydrolysis (D) WMS, (E) WMS-*Ro*BE, and (F) WMS-TuαGT. Before hydrolysis (G) NMS, (H) NMS-*Ro*BE, and (I) NMS-TuαGT; after hydrolysis (J) WMS (K) WMS-*Ro*BE, and (L) WMS-TuαGT. Magnification is 15,000 × .

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2023.109162.

Abbreviations

4αGT	4-α-glucanotransferase
BE	branching enzyme
*Bl*Pul	pullulanase from *Bacillus licheniformis*
CLD	chain length distribution
DP	degree of polymerization
MM	Michaelis–Menten
NMS	normal maize starch
*Ro*BE	branching enzyme from *Rhodothermus obamensis*
TuαGT	4-α-glucanotransferase from *Thermoproteus uzoniensis*
WMS	waxy maize starch

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