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Structural and physical properties of granule stabilized starch obtained by branching enzyme treatment

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ABSTRACT

Chemical cross-linking of starch is an important modification used in the industry for granule stabilization. It has been demonstrated that treatment with branching enzyme (BE) can stabilize the granular structure of starch and such treatment thereby provides a potential clean alternative for chemical modification. This study demonstrates that such BE-assisted stabilization of starch granules led to partial protection from BE catalysis of both amylose (AM) and amylopectin (AP) in their native state as assessed by triiodide complexation, X-ray diffractometry (XRD) and differential scanning calorimetry (DSC). The granule stabilizing effects were inversely linked to hydration of the starch granules, which was increased by the presence of starch-phosphate esters and suppressed by extreme substrate concentration. The data support that the granule stabilization is due to the intermolecular transglycosylation occurring in the initial stages of the reaction prior to AM–AP phase separation. The enzyme activity needed to obtain granule stabilization was therefore dependent on the hydration capability of the starch used.

1. Introduction

Starch is the primary energy storage of higher plants and is an important part of the human diet. Due to its unique physical properties it is a widely used food ingredient, serving as a gelling agent, thickener or stabilizer (Mason, 2009). In its native state, starch consists of the two glucose polymers AM and AP, which by the plant have been deposited as water insoluble, highly organized, semicrystalline granules (Damager, Engelsen, Blennow, Møller, & Motawia, 2010). When heated in presence of water the starch granules swell due to water uptake of mainly AP, which thereby loses its organization. Also part of the AM leaches out and eventually the granules rupture (Srichuwong & Jane, 2007). Starch gelatinization as a fundamental characteristic makes starch a useful food ingredient. Phosphate groups covalently monoesterified to the C-6 and C-3 positions of starch are tremendously important to support starch granule plasticity, starch hydration and starch paste viscosity (Hansen et al., 2009; Hejazi et al., 2008; Viksø-Nielsen et al., 2001). The functional properties of native starches in most cases are not directly useful as ingredients. Most starches are therefore modified either physically or chemically in order to better suit diverse applications. Covalent cross-linking is a common starch modification used to obtain better granular stability and increased resistance of the starch toward acidity, heating and shearing during food processing (Chiu & Solarek, 2009). Traditionally, cross-linking is done chemically by using agents such as phosphoryl chloride, sodium trimetaphosphate, sodium tripolyphosphate, epichlorohydrin, or adipic–acetic mixed anhydride (Koo, Lee, & Lee, 2010; Mason, 2009). In recent years, there has been intense focus on cleaner technologies for modification of starch. For example, for cross-linking starch films, green alternatives using organic acids such as citric acid and malonic acid as cross-linking agents have been developed (Dastidar & Netravali, 2012; Reddy & Yang, 2012). Another green alternative using enzyme technology to obtain starch with a stabilized granular structure was introduced in a recent study (Jensen, Larsen, Bandsholm, & Blennow, 2013). Here it was shown that granule stabilization of certain starch types could be obtained by enzymatic modification using BE under specific circumstances. Stabilization herein refers to a tightly packed granular structure which was obtained after BE treatment and observed by microscopy in contrast to the control samples, which showed highly swollen "ghost structures". It was shown that potato, maize and a transgenic low-phosphate potato starch (asGWD – antisense

Abbreviations: AM, amylose; AP, amylopectin; asGWD potato starch, antisense glucan water dikinase; BE, branching enzyme; DM, dry matter; DSC, differential scanning calorimetry; GPC, gel-permeation chromatography; LD, limit dextrins; WAXS, wide angle X-ray scattering.

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Glucan Water Dikinase) could all be modified at high substrate concentration by BE, but the remaining granular structure after modification was highly dependent on the content of starch-bound phosphate. High-phosphate starches, such as potato starch, completely lost their granular structure upon treatment with heat and high activity of BE. In contrast, granules from starches with low phosphate content, such as maize, were stabilized and appeared as native granules after high activity BE treatment, while a large part of the granular organization in the control maize samples was lost. When treated at low BE activity, high-phosphate starch, as well as low-phosphate starch, lost granular structure. The observations were explained with a hypothetic model, suggesting that high BE activities were able to connect starch segments through intermolecular transglycosylation prior to gelatinization when treated at high substrate concentration and thereby keeping the main granular structure intact. A more general model for the observed molecular effects remains to be explored.

In this study, starches with different molecular and structural features, were subjected to enzyme treatments at lower starch concentrations. Physical and structural properties of these modified starches were analyzed with diverse techniques. Further evidence for the presence of extensively modified starch granules locked by a network of branch linkages formed by BE treatment is presented.

2. Materials and methods

2.1. Materials

Starches from potato, maize, pea, tapioca and wheat were obtained from KMC (Brande, Denmark). Low phosphate potato starch was isolated from a transgenic glucan water dikinase antisense suppressor line (asGWD) (Viksø-Nielsen et al., 2001).

A preparation of BE (α-1,4 → α-1,6 glycosyltransferase, E.C. 2.4.1.18) was obtained from Novozymes, Bagsværd, Denmark. The enzyme was produced by heterologous expression of a synthetic gene coding for glycogen BE from *Rhodothermus obamensis* in a genetically modified strain of *Bacillus subtilis*. The preparation was tested for absence of amylolytic activity (<0.2 CU/mL by Ceralpha method (Megazyme, Wicklow, Ireland).

α-Amylase from *Bacillus amyloliquefaciens* (EC 3.2.1.1) was obtained from Megazyme (Wicklow, Ireland). The activity of α-amylase was determined at pH 6.5 and 25 as previously described °C (Bertoft, Manelius, & Qin, 1993). β-Amylase of barley (EC 3.2.1.2, specific activity 705 U/mg) was obtained from Megazyme.

2.2. Enzymatic modification

Reaction mixtures were prepared in microfuge vials with 30% and 40% (w/v) dry matter (DM) starch, respectively. Samples of 1.00 g were thus prepared by mixing 300 mg or 400 mg DM starch, respectively, with 50 mM sodium phosphate buffer, pH 6.5, and BE at 25 °C. BE was added in volumes equalling 0, 750, 1500 and 2250 BE units per gram sample, respectively, and are here denoted control samples and samples with low, medium and high BE activity respectively. We defined one BE unit as the quantity of the enzyme that causes a decrease in absorbance at 660 nm of an AM–triiodide complex of 1%/min (pH 7.2; 60 °C). The samples were incubated in a thermomixer (Eppendorf, Hamburg, Germany) with continuous mixing (1400 rpm) for 1 h at 70 °C. Following incubation, samples were cooled to 25 °C and the enzyme was inactivated by lowering pH to 1 using 4 M HCl, incubated for 15 min at 25 °C, and neutralized to pH 6.5 using 4 M NaOH and dried at 30 °C *in vacuo*. Low substrate controls comprised 3% DM and 0, 75 or 225 BE units per sample, respectively.

Time course studies were conducted with 1.00 g samples as described above. Individual samples were prepared for each time point and the reaction was stopped at the given time as described above.

Control and high BE activity samples of maize, potato and asGWD potato starch were prepared on a larger scale (250 g reaction mixture). The reactions were carried out in beakers in a water bath and were stirred by a steel propeller on a shaft. Stirring was stopped once the reaction mixture had reached 70 °C.

2.3. Starch phosphate monoester content

Starch phosphate monoester content was determined as content of glucose 6-phosphate and glucose 3-phosphate after hydrolysis of starch and analyzed by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Dionex BioLC system; Dionex Corp., Sunnyvale, CA, USA). Starch hydrolysates with verified glucose-6-phosphate and glucose-3-phosphate contents were used as standards and injected repeatedly along with the samples (Blennow, Bay-Smidt, Wischmann, Olsen, & Møller, 1998).

2.4. Microscopy

The granular state of the starch samples was visualized by bright field light microscopy as described by Jensen et al. (2013). Fluorescence images for visualization of enzyme penetration into the starch granules were obtained after staining the samples (5 mg) with 30 μL SYPRO Ruby Protein Gel Stain (Invitrogen, California, USA) for 2 h using a fluorescence Leitz, SM-Lux-Pol, Leica Microsystems (Leitz, Wetzlar, Germany) microscope with a fluorescent filter (450 nm). Images were taken using a Leica DC300F camera and IM50 software.

2.5. Average DP of debranched starch determined by HPAEC-PAD

Samples were gelatinized and enzymatically debranched at 40 °C by 0.3 unit isoamylase (Megazyme) per 1 mg of sample. The obtained linear glucan fragments were analyzed by high-pressure anion-exchange chromatography with pulsed amperometric detection. Samples of 20 μL (100 μg of linear α-glucan) were injected on a CarboPac PA-200 column using 0.4 mL/min flow rate, 150 mM isocratic NaOH and the following NaOAc gradient profile: 0–5 min: 0–110 mM linear gradient, 5–130 min: 110–350 mM convex gradient. Single peaks between the degree of polymerization (DP) 2–80 were integrated and corrected for detector response (Blennow et al., 1998), and the average DP was calculated from the corrected values of the relative content of each chain.

2.6. Amylose (AM) content

AM content was determined by triiodide colorimetry (Wickramasinghe, Blennow, & Noda, 2009).

2.7. Iodine vapor exposure

The ability of iodine to complex starch chains in granules was determined by equilibrating starch samples at room temperature in a desiccator (25 cm diameter and 2.0 cm deep) containing saturated K_2SO_4 solution (300 mL). Samples were weighed and equilibrated to 0.97 water activity. Sodium azide (1%, w/w) was added to the K_2SO_4 saturated salt solution to prevent microbial growth. To determine iodine binding, a thin layer of the equilibrated starch sample (0.2 g) was spread in a plastic dish and exposed for 24 h at room temperature to iodine vapour generated from 2 g of iodine crystals placed in the desiccator containing the starch samples. The

Table 1
Selected structural properties of the investigated starches.

	Amylose (%)	Phosphate (nmol/mg)	Average DP	Gelatinization temperature[a] (°C)
Potato	22	22.0	25.9	61.3
Tapioca	26	4.5	24.8	65.3
Pea	36	2.8	24.4	70.2
asGWD potato	36[b]	2.3[b]	25.4	64.1
Maize	30	<1	25.2	73.7
Wheat	33	<1	23.9	72.1

[a] Determined by DSC, average of duplicates.
[b] Data obtained from Kozlov, Blennow, Krivandin & Yuryev, 2007.

desiccator was covered with aluminum foil to avoid exposure to light.

2.8. Wide angle X-ray diffraction (WAXS)

The hydrated starches with and without iodine exposure were packed tightly into a round aluminum holder. X-ray diffractograms were obtained with a Rigaku X-ray diffractometer (Rigaku-Denki, Co., Tokyo, Japan) with operating conditions of target voltage 40 kV, current 44 mA, scanning range 3–35°, scan speed 2.00°/min, step time 0.95, divergence slit width 0.5°, scatter slit width 0.5°, sampling width 0.03° and receiving slit width 0.3 mm.

2.9. DSC

Samples were analyzed in the range from 30 °C to 100 °C at a scanning rate of 10 °C per min using a Perkin Elmer Diamond DSC (Perkin Elmer, Massachusetts, USA). The samples were analyzed in slurries of 2 mg sample and 8 μL 10 mM NaCl in triplicates. Perkin Elmer Pyris 7.0 software was used to determine the parameters onset temperature (T_O), peak temperature (T_P), conclusion temperature (T_C) and enthalpy change (ΔH) (Carciofi et al., 2011).

2.10. Preparation and analysis of limit dextrins (LD) of clusters

LDs of clusters were produced by a modified method of Bertoft, Koch, and Åman (2012). Whole starch samples (70 mg) were dissolved in 90% DMSO (700 μL) overnight and diluted with boiling water (5.6 mL). The temperature was adjusted to 25 °C and α-amylase diluted in NaOAC buffer (0.01 M, pH 6.5), was added to a final carbohydrate concentration of 10 mg/mL and enzyme concentration of 0.09 U/mL. Under these conditions the reaction rate was slow at 120 min as determined by preliminary time course experiment followed by gel permeation chromatography on sepharose CL 6B as described previously (Kong, Corke, & Bertoft, 2009). In order to have comparable data all reactions were therefore stopped after 120 min by addition of 140 μL NaOH (5 M) and left for 2 h at room temperature. The clusters were precipitated by addition of 5 vol of methanol, incubation for 1 h and centrifugation at 4000 × *g* for 10 min. The methanol washing was repeated once and subsequently the clusters were dried at room temperature. The clusters were resuspended in H_2O to a concentration of 36 mg/mL, diluted to 27 mg/mL with NaOAC buffer (0.01 M, pH 6.0) and β-amylase (2 μL) was added. The reaction mixture was incubated at 40 °C for 3 h, and the reaction terminated by boiling for 10 min. Salt and maltose were removed from the β-LD of clusters using two PD-10 desalting columns (GE Healthcare Life Sciences, NJ, USA) coupled in tandem. The size distribution of β-LD of clusters was analyzed by gel-permeation chromatography as described above.

3. Results and discussion

3.1. Stabilized granular structure as function of phosphate and substrate concentration

The starch types selected for BE modification in this study represented very different structural properties with regard to AM content, average degree of polymerization, and phosphate content (Table 1). The crystallinity of the native starches ranged from 25% to 35% (data not shown). These differences can be expected to be important for enzyme action and therefore generate different products following BE catalysis. A very high substrate concentration was used during the BE reaction. Hereby, not only restriction of the gelatinization process was achieved (Supplemental data 1–3, controls), but also a synchronized gelatinization was ensured as observed by microscopy (Jensen et al., 2013). As described earlier (Jensen et al., 2013), the granular structure was conserved after high activity BE modification for maize and low-phosphate potato starch while there was significant loss of granular organization in the corresponding controls. This feature was suggested to be a direct effect of intermolecular transglycosylation where chain-transfer between larger segments connects a larger fragment of either AM or AP to another molecule and releases a smaller fragment as an effect of the α-1,4 → α-1,6 action of BE. In normal potato starch such intermolecular transglycosylation, leading to granule stabilization, was likely prevented by the phosphate ester groups. These cause increased hydration and hence larger distances between chain segments and could be expected to decrease the probability of intermolecular transglycosylation by BE. An additional link between phosphate content and the substrate concentration

Table 2
The selected starch types classified according to phosphate content and DM and BE activity required for starch granule stabilization.

	Starch bound phosphate content (nmol/mg)	Substrate necessary for stabilization (% DM)	Enzyme activity needed for amylose protection
Potato	High (22.0)	>40[a]	>High[a]
Tapioca	Medium (4.5)	30/40	Medium/high
Pea	Medium (2.8)	40	High
asGWD potato	Medium (2.3)	40	High
Maize	Low (<1)	30	Medium
Wheat	Low (<1)	30	Medium
Waxy maize	Low (<1)	30	N.A.[b]

[a] No stabilization even at high BE activity.
[b] Amylose not present.

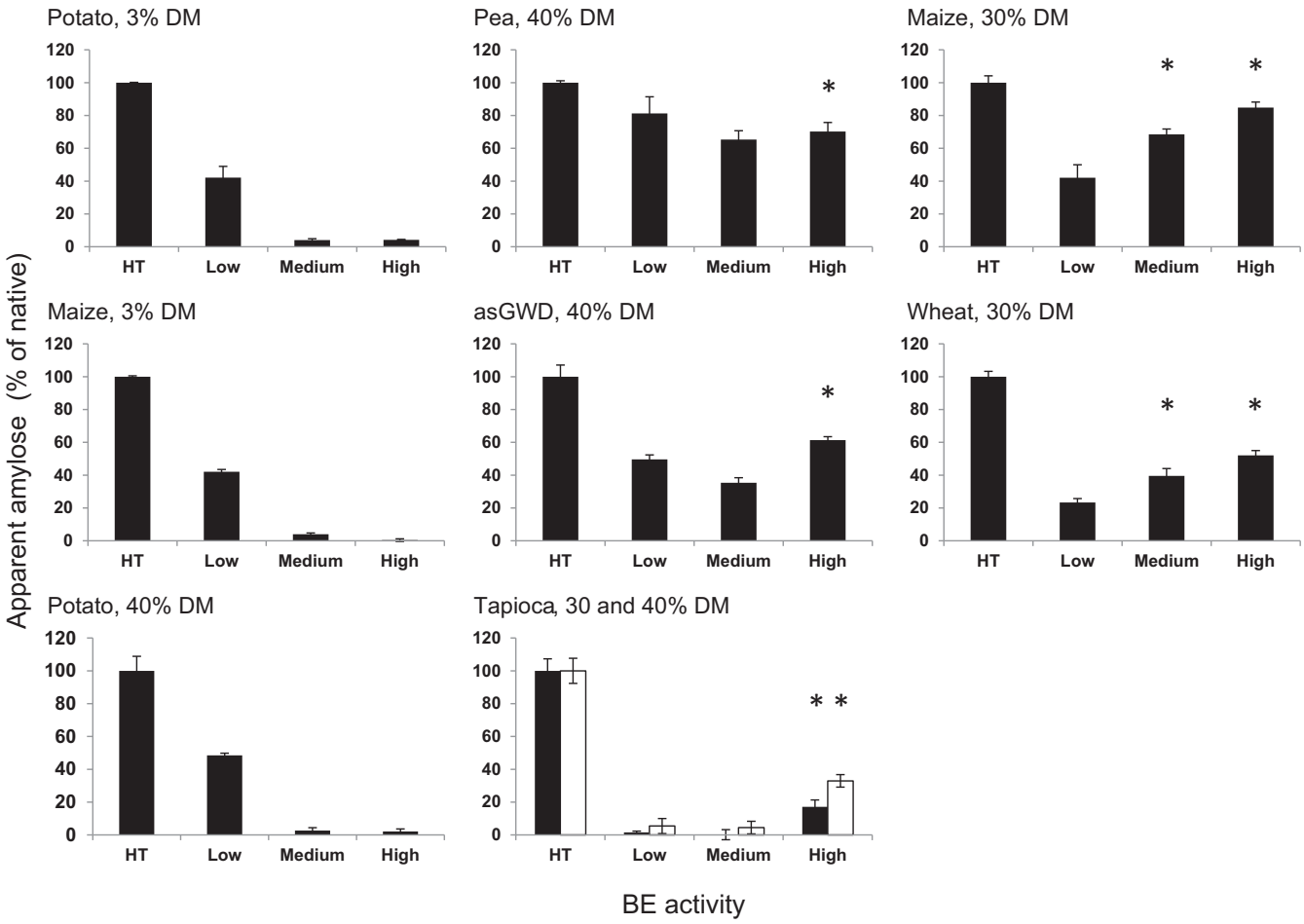

Fig. 1. Apparent AM content as a function of BE activity of different starch types treated at the substrate concentration indicated. Samples with stabilized granular structure as deduced by microscopy are indicated as *.

necessary for obtaining stabilized granular structure was found by microscopy investigations (Table 2, Supplementary material 1–3). On basis of starch phosphate content, the starches used could therefore be divided into three groups which showed different abilities to obtain stabilized granular structures as evaluated by microscopy: low-phosphate starches (<1 nmol/mg) exhibited granular stabilization at 30% DM substrate, medium-phosphate starches (2.3–4.5 nmol/mg) exhibited granular stabilization at 40% DM (with exception of tapioca starch, which showed enhanced granule stabilization), and high phosphate starch (22.0 nmol/g) remained granularly unstabilized. The disturbing effect of phosphate on granule stabilization has already been described (Jensen et al., 2013) and this study now demonstrates that even medium phosphate content (2.3–4.5 nmol/mg) can cause repulsion and prevent interlinking, but that a higher substrate concentration can compensate for the repulsion and thereby enable intermolecular transglycosylation of these starches. This supports that granule stabilization by intermolecular transglycosylation was inversely linked to starch hydration being increased by the presence of phosphate groups and suppressed by high starch DM, and both factors can influence the ability of starches to become granule stabilized by intermolecular transglycosylation. Microscopy data showing no granular structure of maize starch treated with BE at 3% DM (Supplementary data) further supports this hypothesis. It remains to be tested if even higher substrate concentrations of the potato starch may compensate for the high phosphate content, but such conditions are not readily achieved and require further development.

3.2. Apparent AM

It is expected that BE treatment would cause a decrease in apparent AM content as a result of branching of AM. Samples of all three classes of starch types having low, medium and high phosphate contents were analyzed for apparent AM content using iodide complexation in order to investigate AM branching. Interestingly, the apparent AM contents of each starch type were related to the substrate concentration at which granule stabilized structure was obtained (Fig. 1 and Table 2). At low (3% DM) starch concentration neither potato starch nor maize starch were granule stabilized under the used conditions and these samples showed decreasing AM content with higher BE activity. Even at 40% DM, potato starch showed the same trend. Higher DM could not be tested since this caused uneven distribution of BE in the reaction mixture. In the series of samples in which the starch had retained its granular structure at high BE activity, the AM content did not continuously decrease with increased enzyme concentration. Instead, the AM content decreased at the lower enzyme concentrations, but was recovered at higher BE activity. Hence, the AM content was kept high in all samples that showed granule stability, indicating that AM became protected from BE catalyzed chain transfer in these samples. The protection could possibly be due to restricted enzyme diffusion as a result of intermolecular transglycosylation between chain segments in the granule as suggested previously (Jensen et al., 2013). The high BE activity required for granule stabilization can be explained by phase-separation between AP and AM taking place during the first min after gelatinization in highly concentrated

starch systems such as starch films (Rindlav-Westling, Stading, & Gatenholm, 2002). Such separation could also be expected to take place in the present concentrated system since the DM content used allows for 2–3 times volume expansion of the granules, which is likely enough for amylose leaching and segmental chain mobility leading to at least partial phase separation. Efficient chain transfer at this initial stage after the partial gelatinization may, however, restrict phase separation between AM and AP and thereby reduce intramolecular chain branching and promote intermolecular transglycosylation and sterically block access of the enzyme to the AM further inside the granule. Too low enzyme activity would not allow for intermolecular transglycosylation before phase separation and as this process continues AM would become available for intra-molecular transglycosylation. In starches with low phosphate content, the AM became protected already at medium BE activity, whereas starches with intermediate-phosphate content (with exception of tapioca starch) required high BE activity, before AM became protected (Fig. 1). This indicates that the fairly small amount of phosphate present in these starches can prevent enzyme-assisted stabilization of the granular structures due to hydration caused by repulsion of chain segments by even small amounts of phosphate. Therefore, the granule stabilization process in the intermediate-phosphate group requires both higher substrate concentration and higher BE activity (Table 2). The greater ability of the tapioca starch to become granularly stabilized despite its moderate phosphate content suggests that factors other than phosphate are of importance, including the relative deposition of amylose and amylopectin in the starch granule. This remains to be clarified.

3.3. Changes in accessible linear segments

The changes in and accessibility of linear polymers following modification can be investigated by using X-ray diffractometry before and after exposure to iodine. Any long linear chain segments available to form V-helices with iodine will cause a distinct reflection at 20° 2θ. According to the diffractogram, long chains were available for iodine complexation in native maize starch (Fig. 2A) as has been reported previously (Saibene & Seetharaman, 2006). When heat-treated, an increase in the peak intensity at 20° 2θ was observed indicating an increased access of longer chains due to either chain segments of AM leaching or loosening of the granular structure during partial gelatinization allowing for increased helix formation. Interestingly, there was no difference between V-helix formation in the heat-treated sample and the sample treated at high BE activity. Therefore V-helices seem to be able to form upon iodine exposure despite the apparent tightly packed granular structure of this sample as observed by microscopy (Supplemental material 1). This agrees with the apparent AM data and maximum absorption wavelength (unpublished), which showed that there are long chains still present despite the extensively increased branching (Jensen et al., 2013). Hence, the structure of the granule stabilized BE treated maize starch was sufficiently open for iodine to enter and V-helices to form, but not open enough for the BE to act on these chains.

3.4. Crystallinity and thermal properties of granule stabilized samples

DSC and X-ray powder diffractometry were conducted to investigate if the crystalline and double helical parts of the granules were conserved in granule stabilized and destabilized samples following BE treatment. The DSC analysis showed no enthalpic transition for any of the heat or BE treated potato starches, meaning that despite the restricted water accessibility the samples were completely gelatinized (data not shown). However, maize starch samples showed distinct melting enthalpies in all samples. The melting enthalpy of the heat-treated control was decreased to approximately 50% compared to native starch demonstrating partial gelatinization and unwinding of AP double helices in the granules (Fig. 2B). In contrast to potato starch this behavior is likely an effect of the absence of phosphate and the high substrate concentration restricting gelatinization. The sample treated with low BE activity showed melting enthalpy reduction to 25%, demonstrating an expected granule destabilization of this structure. This is likely due to BE catalyzed branching in double helical parts of the granules creating structures, which similarly to glycogen, were unable to organize into crystalline structures and were thereby much more accessible for enzymatic modification (Ball et al., 1996). The granule stabilized sample treated at the highest BE activity showed a melting enthalpy of 70% of native starch and thereby higher than that of the control. This shows that the double helical part of the AP was somehow protected from unwinding and molecular separation during heat treatment, possibly due to intermolecular transglycosylation preventing swelling of the granules. These parts of the granule are likely protected from BE action resulting in increased granular stability in aqueous systems. The intermolecular transglycosylation may also prevent the introduction of branch points in the double helices and in this manner help conserve the crystalline structure. Thereby AP, just like AM, was partly conserved in its native state as an effect of the BE granule stabilization process. The data further indicates that intermolecular transglycosylation occurs mainly in the amorphous parts of the granule. From the X-ray diffraction patterns (Fig. 2A), it can be deduced that the sample treated at high BE activity showed crystallinity similar to that of the control (33% and 34%, respectively), and higher than that of the native starch (24%), indicating that a certain degree of retrogradation had taken place in both samples.

The melting temperatures of the samples were greatly affected by the different treatments (Fig. 2C). The melting temperature of the maize control was 5 °C higher than for native starch, which suggests that the part of the AP that was not gelatinized was instead annealed. When treated with BE, the melting temperature further increased up to 8 °C higher than native starch which indicates that the less perfect crystals were attacked by the BE and only highly annealed structures were left. The most granule stabilized sample, which had been treated with the highest BE activity, showed a melting temperature similar to the heat treated control, possibly reflecting that the granules were not able to further anneal due to the speculated locked, interbranched structure. The elevated melting temperature of the granule stabilized starch compared to native starch is typical for conventionally stabilized starches (Mason, 2009).

3.5. Molecular size of β-LDs of clusters in granule stabilized and destabilized samples

BE catalyzed chain transfer is expected to mainly take place in the amorphous regions of the starch granule. A major part of the amorphous lamellae in the native granule consist of branched clusters and building blocks (Bertoft & Blennow, 2009). These clusters can be isolated by controlled α-amylase treatment. Subsequent β-amylase treatment of the clusters generates β-LDs representing trimmed clusters of importance for describing the structure of the amorphous lamellae (Bertoft, 2007). BE-treatment of starch granules is expected to lead to a slight increase in the size of the β-LDs of clusters since chains, which were previously external and degradable by β-amylase, would become internal chains after addition of new branch points. Hence, differences in the reaction catalyzed by BE depending on the phosphate content and extreme starch DM as indicated above are expected to be

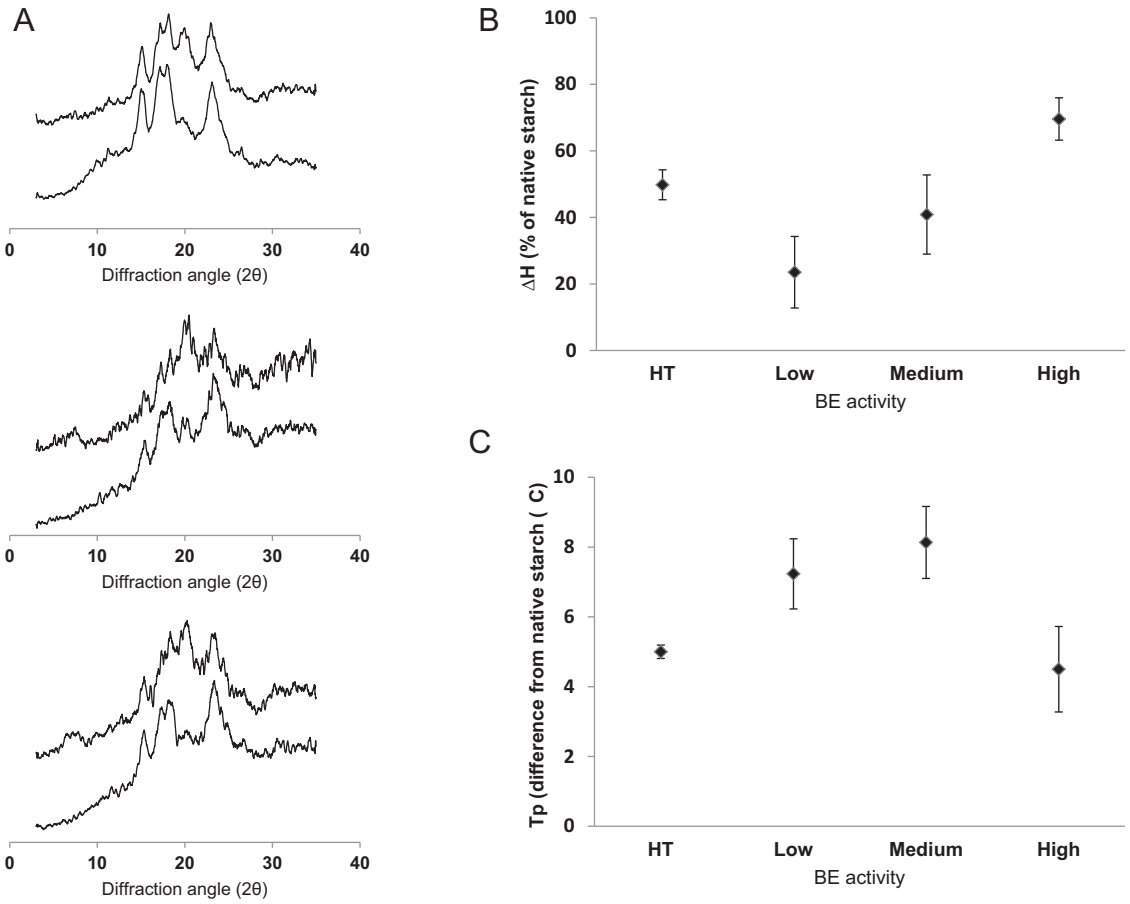

Fig. 2. Physical properties of maize starch samples. (A) Normalized WAXS diffractograms. Top panel shows native starch, middle panel shows heat treated control and bottom panel shows sample treated with high BE activity. Each sample was analyzed before and after iodine exposure, which is shown as lower and upper diffractogram within each panel, respectively. (B) Melting enthalpies of treated maize samples in percent of native maize starch as determined by DSC. (C) Difference in melting temperatures of treated maize starch samples from native maize starch as determined by DSC.

seen in altered cluster structures. Therefore samples treated at high BE activity from each phosphate-level group, high phosphate (potato), medium phosphate (asGWD potato) and low-phosphate (maize), in comparison with controls were analyzed. Firstly, it was shown that the cluster size of native starches varied greatly based on botanical origin of starch (Table 3). Such differences may reflect branching distribution and density of phosphate groups. The cluster sizes of all three modified samples on the other hand were very similar (DP = 130–131) despite the different granular integrity. The increased cluster sizes indicate that the number of branch points increased upon BE treatment regardless of starch type. The similarity in cluster sizes suggests that the products may be highly similar with regards to branching density and that only minor differences dictate the granule stabilizing effect seen for low-phosphate starch types. The very similar cluster sizes also support the suggestion that a maximal cluster size had been achieved. These data confirm previous observations on molecular structure showing very similar product structures for the different samples (Jensen et al., 2013).

Table 3
Degree of polymerization of clusters in heat treated control and BE treated samples of potato, asGWD potato and maize starches containing high, medium and low starch bound phosphate, respectively.

Starch origin[a]	Control	BE treated sample
Potato (22.0)	127	130
asGWD potato (2.3)	86	131
Maize (<1)	105	130

[a] Phosphate content in parenthesis (nmol/mg).

3.6. Initiation of the granule stabilization process

In order to obtain information on what temporal condition leads to granular stabilization and destabilization, respectively, the apparent AM content of maize starch treated at high and

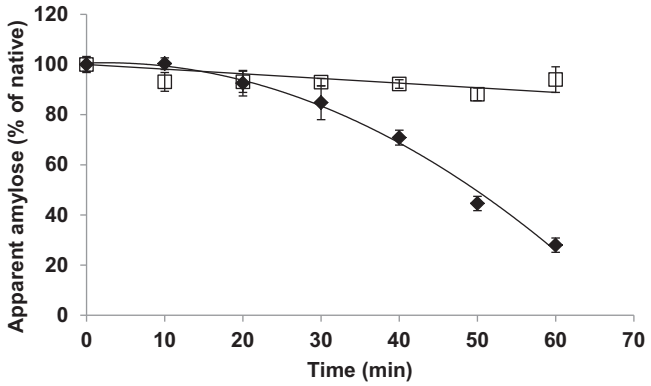

Fig. 3. Apparent AM content of maize starch as function of reaction time modified with low (♦) and high (□) BE activity, respectively.

low BE activity was determined as function of time (Fig. 3). It was shown that when the reaction was performed with high BE activity, the apparent AM content remained high throughout the 60 min of reaction. When the reaction was performed at low BE activity the apparent AM content was initially high, but then decreased after approximately 20 min of reaction. The decrease continued throughout the rest of the reaction time and the reaction did not appear to be finalized even after 60 min. This data shows that the granule stabilization obtained when using high BE activity must take place within the first 20 min. If there is not sufficient BE activity to initiate the granule stabilization process, AM will become available to BE after approximately 20 min and become branched resulting in a decrease in apparent AM. As discussed above, this may be due to molecular re-arrangement including phase separation of AM and AP (Rindlav-Westling et al., 2002) and AM re-alignment slowly taking place following heat-assisted granular opening. At low BE activity, phase separation is completed before intermolecular transglycosylation and promotes loss of granular structure and branching of individual molecules. At high BE activity, intermolecular transglycosylation can take place before phase separation is completed. The chain segments will therefore remain in the granular structure and be in close proximity promoting further intermolecular chain transfer. The data indicate that there is a susceptible phase in the first 20 min, where the granules are in a semi-native state. The chains are still distributed as in native starch, but the opened granule is, unlike native starch, susceptible to enzymatic attack by BE. The enzyme susceptibility of granule stabilized maize granules was confirmed by fluorescent protein staining. This showed that despite granule penetration of BE being heterogeneous and in most cases BE being present in the highest concentration on the surface of the granules, it did in fact also have access to the interior of the granules (Supplementary data 4).

4. Conclusions

This study demonstrated some fundamental mechanisms underlying enzymatic granule stabilization caused by intermolecular transglycosylation of starch by BE treatment. The data confirm the combined importance of starch granule hydration and subsequent AM–AP phase separation on the observed effect as controlled by natural starch-phosphate substitution and substrate concentration. The granule stabilizing effect is proposed to be a result of high chain transfer rate preceding AM–AP phase separation in the partly opened starch granule leading to protection of segments of both AM and AP in its native state. The data allows for establishing a more general protocol for conditions leading to granule stabilization and indicates that high-phosphate starches may possibly also be granule stabilized with BE under restricted conditions.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.07.071.

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