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Iodine binding to explore the conformational state of internal chains of amylopectin

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ABSTRACT

Previous studies have found that the proportion of long chains of amylopectin correlates to its functional and nutritional properties. As a possible explanation of this correlation, the iodine binding property of amylopectin internal chains was investigated as an indirect evidence of their ability to form helices for intra- or inter-molecular interactions. Waxy and amylose-extender waxy corn starches were hydrolyzed by β-amylase for varying periods of time to incrementally remove the external chains, and the absorbance and the wavelength of maximum absorbance of iodine binding were examined. Experimental results suggest that iodine can bind with both external and internal chains; a significant amount of absorption comes from the latter, as stepwise removal of external chains only somewhat reduced absorption. Internal amylopectin chains, thus, were concluded to likely pre-exist in helical form, as opposed to a conformational change into helices facilitating iodine binding in the absence of external chains. Such internal chain helical structures possibly drive intermolecular interactions that would explain why amylopectin with high proportion of internal chains form harder gels, create pastes less prone to shear breakdown, and are more slowly digesting.

1. Introduction

Many studies have shown that the proportion and the chain length of the long chains of amylopectin have special effects on its functional properties (Benmoussa, Moldenhauer, & Hamaker, 2007; Gudmundsson & Eliasson, 1990; Han & Hamaker, 2001; Jane & Chen, 1992; Jane, Xu, Radosavljevic, & Seib, 1992; Jane et al., 1999; Kalichevsky, Orford, & Ring, 1990; Lu, Chen, & Lii, 1997; Matalanis, Campanella, & Hamaker, 2009; Miles, Morris, Orford, & Ring, 1985; Shi & Seib, 1992; Shi & Seib, 1995; Wang, White, & Pollak, 1993; Yuan, Thompson, & Boyer, 1993). A greater extent of retrogradation of waxy starches was found to be directly proportional to the mole fraction of the unit chains of DP 14–24 by Shi and Seib (1992). In the study of Yuan et al. (1993), the changes of enthalpy of both gelatinization and retrogradation of the corn starch from the *ae wx* genotype were higher than those of the *wx* and *du wx* genotypes, which may be attributed to the greater amounts of longer chains in *ae wx* amylopectin. More long chains or longer chains in amylopectin correlated to lower RVA breakdown in rice amylopectin (Han & Hamaker, 2001), easier retrogradation and reduced digestibility in rice starch (Benmoussa et al., 2007), and greater increase in storage modulus of gels during retrogradation (Matalanis et al., 2009). The long chains might give more opportunities for interactions among chains of amylopectin, forming inter- or intra-molecular double helices, or among chains of amylopectin and amylose, thus, affecting functional properties of starch. Our recent study on waxy rice amylopectins (Shen and Hamaker,unpublished) revealed that the long chains of amylopectin, without interference from amylose, still impact the functional properties of starch, showing significantly different behaviors in DSC, rheology and gel microstructure.

Studies regarding the contribution of long chains of amylopectin to its functionality have focused on increased interactions among external chains of amylopectin (Cameron, Durrani, & Donald, 1994; Klucinec & Thompson, 2002; Klucinec & Thompson, 1999; Miles et al., 1985; Ring et al., 1987). Based on the theory of Klucinec and Thompson (1999, 2002), two types of junction zones can form during amylopectin retrogradation affecting their functionality: (i) intermolecular double helices from external chains termed as the Type 1 junction zone where external chains from two different molecules form double helices, and (ii) an intramolecular double helix could participate in a semicrystalline aggregate with double helices from a separate molecule, forming an intermolecular aggregate of double helices of external chains

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as the Type 2 junction zone. They stated that Type 1 junction zones are not likely to form because the external chains preferably form intramolecular double helices regardless of internal chain flexibility. Regarding the relationship of long chains and enhanced functionality, in this theory, longer or higher proportion of long internal chains would produce greater flexibility and encourage greater chances for the external chains or double helices to interact intermolecularly and to form junction zones.

It is well known that iodine can complex with amylopectin to create a purple-red color (Banks, Greenwood, & Khan, 1971; Bates, French, & Rundle, 1943; Gerard, Barron, Colonna, & Planchot, 2001; Gidley & Bulpin, 1987; John, Schmidt, & Kneifel, 1983; Rundle & French, 1943). Bates et al. (1943) and Gidley and Bulpin (1987) proposed that the iodine complex formation requires a helical conformation of chains with at least 6 D-glucose residues in one helix turn. However, there is no detailed information in the literature on the iodine binding spectrum of amylopectin with varied chain length distributions. Chauhan and Seetharaman (2013) recently did provide evidence that supports the possibility for internal chains of amylopectin to interact with iodine.

The purpose of this study was to better understand the role of the long chains of amylopectin in driving the functional properties mentioned above, which are dependent on enhanced intermolecular interaction. We hypothesized that, in addition or separate to the above theory of the two types of junction zones formed through interactions among external chains, long internal chains may themselves be involved in certain chain interactions, through intermolecular entanglements and/or intermolecular double helices perhaps with other external chains. A prerequisite to formation of internal chain double helices is that the internal long chains either exist naturally in a single helical conformation or take on such structure when gelatinized. However, this second possibility is unlikely considering the steric hinderance from the external chains and the movement constraints from the branched structures.

In the current study, β-amylolysis of normal waxy and *ae* waxy (with high proportion of long chains) corn starches was employed to produce β-dextrins with different external chain lengths. The iodine binding spectra of the generated structures were used to investigate how varied external chain lengths bind iodine, and to indicate whether the internal chains of amylopectin can bind iodine, thus indicating the existence of a turned or helical structure.

2. Materials and methods

2.1. Materials

Waxy corn starch (*wx*) and amylose extender waxy corn starch (*ae wx*) were gifts from Tate and Lyle, Inc. (Decatur, IL). β-Amylase from barley, isoamylase from *Pseudomonas* sp., and pullulanase from *Klebsiella planticola* were purchased from Megazyme International (Wicklow, Ireland). β-Amylase activity was 50,000 U/g, as determined by the manufacturer. Stock β-amylase solution was diluted with 0.02 M sodium acetate buffer (pH 6.0) to 100 U/mL and 200 U/mL. Isoamylase activity was 1000 U/mL as determined by the manufacturer. Stock isoamylase solution was diluted 2000 times with 0.02 M sodium acetate buffer (pH 4.75). Stock pullulanase solution (720 U/mL as determined by the manufacturer) was also diluted 2000 times with the sodium acetate buffer. Pullulan standards were from Polymer Laboratories Inc. (Amherst, MA). Iodine solution (0.2% I_2 and 2% KI) was prepared with 100 mM phosphate buffer (pH 7.0) and stored in a brown bottle at room temperature.

2.2. Preparation of nongranular starch

Nongranular starches were prepared from the *wx* and *ae wx* as described by Klucinec and Thompson (1998) with modifications. Granular starches (200 mg) were dispersed in 10 mL of 90% (v/v) dimethyl sulfoxide (DMSO) by heating the mixtures in a boiling water bath with constant stirring for 3 h. Following dispersion, 40 mL of ethanol were added slowly to the mixture, and the mixture was then centrifuged at 6500 g for 15 min. The supernatants were discarded and the pellets were washed by suspending them in 5 mL of ethanol followed by recentrifugation at 6500 g for 15 min. The precipitate was then dried in a vacuum desiccator.

2.3. β-Amylolysis

β-Amylolysis was carried out according to the method described by Xia and Thompson (2006) with minor modifications. Vacuum-dried nongranular waxy starch (12 mg) was dispersed in 120 μL of 90% DMSO by heating in a boiling water bath for 10 min. After heating, 880 μL of 0.02 M warm sodium acetate buffer (pH 6.0) was added and mixed with the samples. The mixture was then heated in a boiling water bath for 10 min and placed into a 50 °C water bath. To six tubes of 1 mL mixture, a 50-μL aliquot of β-amylase solution (100 U/mL) was added. Samples were held at 50 °C for 1 min, 5 min, 30 min, 1 h, 3 h, and 5 h, respectively, with constant agitation. To another two tubes of 1 mL, a 50-μL aliquot of β-amylase solution (200 U/mL) was added and held at 50 °C for 24 h with constant agitation. Blank sample was prepared by adding a 50-μL aliquot of sodium acetate buffer (0.02 M, pH 6.0) to a tube of 1 mL mixture, named as 0 min sample. After designated incubation time, all the samples were heated in a boiling water bath for 10 min to stop the reaction.

For each sample, 10 μL was taken to determine the total carbohydrate content by phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and 40 μL was taken to determine the reducing capacity by Somogyi–Nelson method. The degree of β-amylolysis was calculated by the ratio of the reducing capacity based on glucose content over total carbohydrate content. Each sample was measured in duplicates and the average value was taken and used in the subsequent calculations.

2.4. Debranching

The β-dextrin samples were completely debranched by the method described by Klucinec and Thompson (2002) with modification. β-Dextrin samples (0.5 mL/tube) were mixed with 1.5 mL of ethanol, held at 4 °C for 30 min, and then centrifuged (270 g) at room temperature for 10 min. Precipitates were washed with 500 μL ethanol at room temperature and centrifuged at 1020 g for 10 min. The washing procedure was repeated. Precipitates were dried in a vacuum desiccator.

Dried precipitates were pooled (about 3 mg) to mix with 50 μL of 90% DMSO and heated in a boiling water bath for 10 min. While mixtures were still hot, 350 μL of 0.02 M, pH 4.75, warm sodium acetate buffer was added. Mixtures were then heated in a boiling water bath for 10 min and cooled to 37 °C. To each cooled mixture, 40 μL of diluted isoamylase solution was added. Samples were held at 37 °C for 24 h with constant agitation, and then heated in a boiling water bath for 10 min to stop the reaction. To the isoamylase-debranched samples, 40 μL of diluted pullulanase solution was added to ensure complete debranching. Samples were held at 37 °C for 24 h, and then heated in a boiling water bath for 10 min.

A 100-μL aliquot of each completely debranched sample was dried in a vacuum evaporator (SpeedVac SC 100H, Savant). The dried samples were dissolved in 0.5 mL of 90% DMSO followed by heating for 10 min.

Size-exclusion chromatography was conducted using a HPSEC-RI system equipped with a Waters 1515 isocratic pump, a Waters

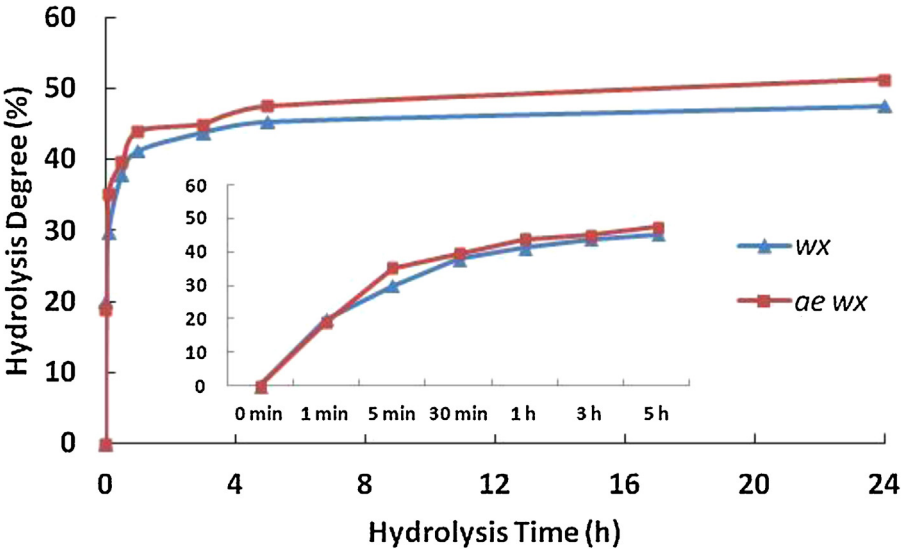

Fig. 1. β-Amylolysis degree of *wx* and *ae wx*.

2414 refractive index detector (Waters, Milford, MA), two Zorbax PSM 60-S columns connected in series (Quantum Analytics Inc., Foster City, CA), and a 20-μL sample loop. The column and detector temperatures were set at 35 and 40 °C, respectively. Samples were injected and chromatographed at a flow rate of 0.5 mL/min using DMSO as the mobile phase. Pullulan molecular weight standards were used for column packing calibration.

2.5. Determination of chain length

The above β-dextrins were divided into two batches. The first batch was submitted to a complete debranching. The second batch of β-dextrins was hydrolyzed again by β-amylase (180 U/mL) for 24 h into a β-LD and the β-amylase re-hydrolysis degree was determined by calculating the ratio of the reducing capacity after re-hydrolysis based on glucose content over total carbohydrate content.

The average chain length (CL) was determined by calculating the ratio of the total carbohydrate concentration to the concentration of reducing ends of debranched β-dextrin samples from the above first batch. The average external chain length (ECL) and the average internal chain length (ICL) were calculated as follows (Yun & Matheson, 1993):

$$\mathrm{ECL} = (\mathrm{CL} \times \text{Degree of } \beta\text{-amylase re-hydrolysis from the above second batch}) + 2$$

$$\mathrm{ICL} = \mathrm{CL} - \mathrm{ECL} - 1$$

2.6. Iodine binding

For each β-dextrin sample, one aliquot of 400 μL was taken, to which 10 μL of iodine solution (0.2% I_2, 2% KI) was added, and the tube was wrapped with aluminum foil. All the samples were held at room temperature for 0, 30 and 60 min after the addition of iodine solution and then wavelength scanning from 400 to 800 nm was performed with a DU800 UV/Vis spectrophotometer (Beckman Coulter, Inc.). The maximum absorbance and the corresponding wavelength (λ_{max}) were recorded. Each sample was measured in duplicates and the average value was taken.

3. Results

3.1. Change in absorbance and λ_{max} of iodine binding during β-amylolysis of amylopectin

The time-course of the hydrolysis of *wx* and *ae wx* corn starches with β-amylase is shown in Fig. 1. *Wx* starch amylopectin showed little overall reduction in iodine binding with increasing β-amylolysis, while *ae wx* starch iodine binding reduced substantially with initial β-amylolysis (Fig. 2). At the initial 1 min of *wx* starch hydrolysis, when the degree of β-amylolysis was about 20%, there was slight lowering in absorbance, which followed by an increase when the hydrolysis reached 30% at 5 min. Notably, *ae wx* starch leveled off at approximately the same absorbance level as *wx* starch (an absorbance value of 1) at around 1 h when the hydrolysis corresponded to ~40%.

Somewhat different trends were seen for λ_{max} values over the 24 h β-amylolysis period of *wx* and *ae wx* starches (Fig. 3). For *wx*, λ_{max} shifted slightly downward from around 555 to 540 nm in the first 5 min of hydrolysis (30% β-amylolysis, Fig. 1). In the case of *ae wx*, there were two notable λ_{max} shifts: (i) about 30 nm decrease from around 590 nm to 560 nm when hydrolyzed for 1 h (~44% β-amylolysis), and (ii) about 15 nm decrease reaching around 545 nm between 1 and 5 h (44–48% hydrolysis).

λ_{max} of *ae wx* was greater than that of *wx* when hydrolyzed for less than 30 min. After 5 h of hydrolysis, λ_{max} values were both close to 540 nm.

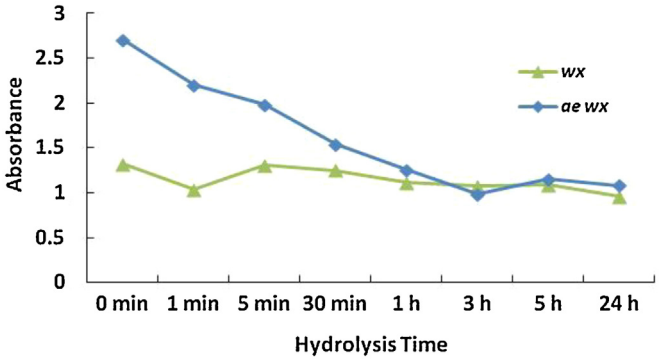

Fig. 2. Absorbance of β-dextrins of *wx* and *ae wx* binding with iodine.

Table 1
Chain length distributions of *wx* and *ae wx* β-dextrins.

	0 min	1 min	5 min	30 min	1 h	5 h	24 h
wx β-dextrins							
CL[a]	21.6 ± 2.2	18.4 ± 1.6	14.0 ± 1.4	13.6 ± 1.7	12.2 ± 1.2	10.5 ± 1.0	9.5 ± 0.9
ECL	13.2	8.3	5.2	4.0	3.3	2.6	2.2
ICL	7.4	9.1	7.8	8.6	7.9	6.9	6.3
ae wx β-dextrins							
CL[a]	27.9 ± 2.3	21.7 ± 1.6	17.5 ± 1.5	16.0 ± 1.4	14.9 ± 1.6	13.9 ± 1.2	12.8 ± 1.2
ECL	17.1	9.6	5.8	4.2	4.1	3.0	2.3
ICL	9.8	11.1	10.8	10.9	9.8	9.9	9.5

[a] Values are reported as mean ± standard deviation of triplicates of total carbohydrate and reducing capacity analysis from duplicates of debranching.

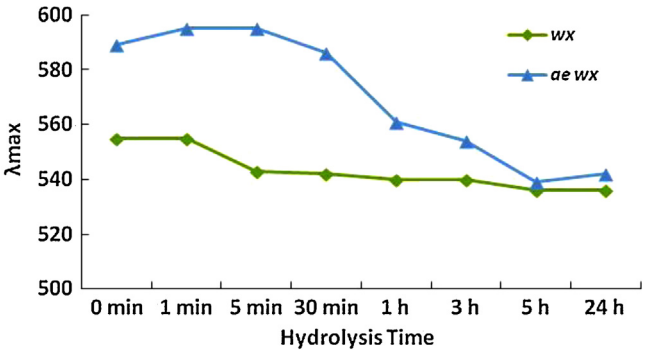

Fig. 3. λ_{max} of β-dextrins of *wx* and *ae wx* binding with iodine.

3.2. Chain length distributions of β-dextrins of wx and ae wx

Chain length distributions of *wx* and *ae wx* β-dextrins produced through increasing time of β-amylolysis are shown in Table 1. Partially hydrolyzed β-dextrins at different times were debranched and analyzed by HPSEC (Figs. 4 and 5). Over the entire β-amylolysis period to the final formation of the β-limit dextrin (β-LD), CL and ECL of the β-dextrins decreased for both *wx* and *ae wx* samples, whereas ICL as expected remained constant. For *wx* β-dextrins, CL changed from 21.6 to 9.5, ECL from 13.2 to 2.2, while ICL was around 8. For *ae wx* β-dextrins, CL decreased from 27.9 to 12.8, ECL from 17.1 to 2.3, and ICL was around 10 (Table 1).

Largest changes in chain length distribution for both *wx* and *ae wx* occurred during the initial 5 min of hydrolysis (Figs. 4 and 5). During this time, mainly peaks of DP 3 and 4 emerged and chains of DP 10–20 decreased. Later, the chain residues with DP 4 diminished, apparently reduced into DP 2, until β-LDs were formed after 24 h hydrolysis.

A comparison of *wx* with *ae wx* at the same degree of β-amylolysis (Table 1) showed that CL, ECL and ICL of the *ae wx* β-dextrins were greater than those of *wx*. After 24 h β-amylolysis, each possessed similar ECL, representing the typical residues of the external chains of β-LDs.

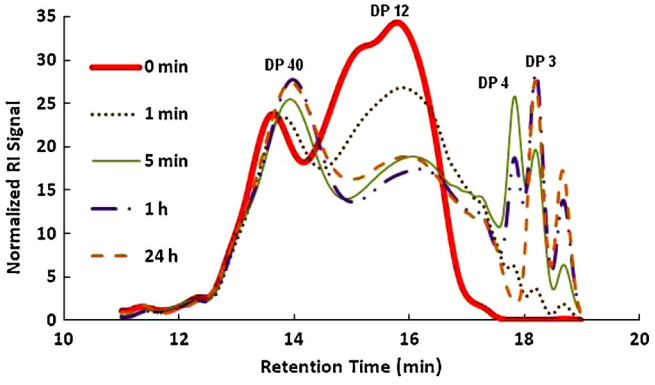

Fig. 4. Time course of β-amylolysis of *wx* followed by debranching.

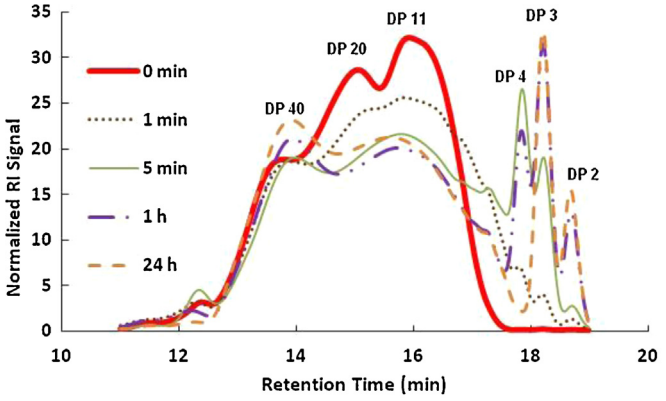

Fig. 5. Time course of β-amylolysis of *ae wx* followed by debranching.

4. Discussion

4.1. Iodine binding of wx vs. ae wx

For native *wx* starch, iodine binding with amylopectin showed similar λ_{max} as found by Davis, Skrzypek, and Khan (1994) of approximately 550 nm (Fig. 3). Although our data did not directly show the binding of iodine with internal chains in the native structure, it seems clear that iodine does bind to amylopectin internal chains albeit giving a lower absorbance of approximately 1 and lower λ_{max} values of approximately 540 nm after external chains are removed (Figs. 2 and 3). The same, but considerably more dramatic result was obtained with *ae wx*. When external chains were removed beyond their point of ability to bind iodine (<6 glucose residues), iodine binding was similar between the two starches. We interpret this data to mean that (i) *wx* amylopectin possesses lesser iodine binding to its external chains and *ae wx* significantly more due to longer ECL, and (ii) that iodine complexes with the internal chains of amylopectin while in their native (unhydrolyzed) state.

4.2. Iodine binding of external chains vs. internal chains

Generally, it has been considered that iodine binds to the external chains of amylopectin (Calabrese & Khan, 1999; Davis & Khan, 1994; Hirai, Hirai, & Ueki, 1994; Knutson, 1999). Recently, Chauhan and Seetharaman (2013) found that internal chains of amylopectin bind iodine in remaining long internal chains of lintners and already produced β-limit dextrins which provided evidence to support the backbone structural model of amylopectin. Here, we asked the question, using a different approach, of whether the internal chains are helical in the native amylopectin structure; and, if so, could

that explain why amylopectin with high proportion of internal long chains promotes intermolecular interactions to drive functional changes. To study this, the absorption profile over a stepwise time course of β-amylolysis was examined with the hypothesis that if native helical structures exist, and are not formed in the process of β-amylolysis, then an iodine binding would not abruptly change but be similar between the *wx* and *ae wx* starches when the external chains are incrementally removed; all *wx* and *ae wx* samples bound iodine (Figs. 2 and 3). This confirms that iodine complexes with internal chains as demonstrated by substantial absorbances after shortening of external chains by β-amylolysis and their concomitant loss of iodine binding ability. The finding that iodine binding of external and internal chains is different, as manifested in changes in absorbance and λ_{max}, suggests that there are potentially two types of binding between iodine and amylopectin: (i) an iodine external chain complex and (ii) an iodine internal chain complex.

4.3. Two possible scenarios of iodine internal chain binding

There are two possible scenarios regarding the iodine binding to internal chains from the current study.

In a first, and we believe more probable, scenario iodine binds initially to both external and internal chains of the native amylopectin molecule. For *ae wx*, there was a continuous drop in absorbance with increased β-amylolysis of the external chains until 1 h of hydrolysis time and a leveling off at absorbance of approximately 1. In this interpretation, this pattern of iodine binding can be explained by the fact that the longer external chains have high iodine binding, and β-amylolysis reduces binding until only internal chain binding remains at 1 h. In the case of *wx*, with shorter internal and external chains, perhaps a more compact and rigid internal structure does not allow full accommodation of iodine in the native state. During β-amylolysis, following the initial decrease in absorbance at 1 min of hydrolysis followed by increase to 5 min of hydrolysis and subsequent slow and constant decrease of absorbance to about 1 when forming β-LD, iodine could rapidly fill out the remainder of internal chains as they gain flexibility with removal of portions of external chains.

In a second and less likely scenario, iodine would initially bind only to the external chains of the native amylopectin, as held in the conventional view of iodine binding. For *wx*, after limited β-amylolysis of external chains up to 1 min of hydrolysis, less binding of iodine was found. However with continued hydrolysis, external chains were incrementally shortened and internal chains would gain more accessibility to iodine. This would result in the observed increase in absorbance at 5 min of hydrolysis and a continuation of relatively high iodine binding to the final β-LD product (Fig. 2). In this interpretation, there would be an intermediate β-amylolysis phase where iodine has the ability to bind the internal chains at the same time as it binds to parts of the external chains. The balance between the binding rate of iodine to the internal chains and the losing rate of iodine to the external chains would determine absorbance values in the phase of β-amylolysis where external chains are being shortened to the point they lose iodine binding capability. For *ae wx*, the continuous drop in absorbance could be explained by the increased time it takes to hydrolyze the comparably long external chains of *ae wx* causing a hindrance to the flexibility needed for the internal chains to bind iodine (Fig. 2). However, this explanation does not describe well the relatively flat absorbance profile of *wx* β-dextrins, as one would expect a more abrupt change in the transition of iodine binding between external and internal chains. Moreover, the necessary change in conformation of the internal chains to a helical state would require a turning of the structure that would be difficult due to steric hindrance within the structure.

4.4. Requirements of iodine internal chain binding

As a prerequisite to accommodate and provide sites for iodine binding, the internal chains of amylopectin need to: (i) be of a certain minimum chain length and (ii) be in a helical structure.

The fine structure analysis showed that the ICL of *wx* and *ae wx* were about DP 8 and 10 respectively (Table 1). This meets the basic requirement of an unbranched linear chain DP of 6 to bind iodine into one helical turn (Bates et al., 1943). For branched structures, the linear chain portion needs to be about 10 to bind iodine (Gidley & Bulpin, 1987). Indeed, as ICL represents the average chain length between branches, both shorter and longer chain segments exist, the latter especially between clusters (Kong, Corke, & Bertoft, 2009). These reports support our hypothesis that internal chains are able to form helical structures to complex iodine from the viewpoint of required chain length DP.

4.5. Relevance to starch functionality

The functional implication of internal chains of amylopectin existing in the helical conformation relates to what drives intermolecular interactions among amylopectin molecules. A growing number of correlative studies over the past 2–3 decades imply that amylopectin structures with a higher proportion of long chains form harder gels, retrograde faster to decrease digestion rate, and make pastes resistant to shear-induced breakdown, which indicates greater intermolecular interactions among these long chains. While one theory holds that such structures have more flexibility for external chain interactions (Klucinec & Thompson, 1999), the present work suggests that the long internal chains could promote such interaction as well, perhaps forming double helical structures with external chains from other amylopectin molecules. Further work is needed to investigate more directly this possibility. In a larger sense, this also brings up the point of what is the overall architecture of amylopectin, the concept of the conventional tree-like structure or the more recently suggested backbone structure (Pérez & Bertoft, 2010). Long internal chain intermolecular interactions might favor the latter over the former amylopectin structural model.

5. Conclusions

There are two types of binding involved in iodine and amylopectin interaction: (i) iodine external chain complexation and (ii) iodine internal chain complexation. The type and intensity of the iodine internal chain complexes of *wx* and *ae wx* were similar.

Two scenarios were proposed based on the results and led to interpretations on the progression of iodine binding of amylopectin during the course of β-amylase hydrolysis. In the first and most probable scenario, both external and internal linear chains of native amylopectin bind iodine. In the second and less likely scenario, only external chains of native amylopectin bind iodine, whereas their removal allows internal iodine binding. The finding that internal chains possess the ability to interact with iodine suggests that other types of interactions also are possible, such as intermolecular interactions between internal and external chains resulting in double helices.

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