**Impact of reagent infiltration time on reaction patterns and pasting properties of modified
maize and wheat starches**

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Abstract

The impact of granular and molecular reaction patterns on modified starch properties was investigated as a function of the length of time allowed for reagent to infiltrate starch granules. A fluorescent reagent [5-(4,6-dichlorotriazinyl)aminofluorescein] was dispersed in aqueous normal maize or wheat starch slurries (35%, w/v) for 0, 5, 10, 30, or 60 min, after which reaction was initiated by increasing the pH to 11.5 and allowing reaction to proceed for 3 h. With increasing lengths of infiltration, the reaction became increasingly homogeneous within the granule interior (matrix) and the AM:AP reactivity ratio increased (wheat starch), as assessed by confocal laser scanning microscopy (CLSM) and size-exclusion chromatography (refractive index and fluorescence detection), respectively. A longer reagent infiltration time also led to a more inhibited (i.e., cross-linked) pasting viscosity, suggesting that both granular and/or molecular reaction patterns were altered by varied reagent infiltration times to ultimately impact modified starch properties.

Keywords: starch modification; cross-linking; fluorescent probe; derivatization; pasting property

1. Introduction

Native starch properties are improved by chemical modification, enhancing starch functionality for intended food and industrial applications. Though chemical modification of starch is industrially practiced, the impact of the granular and molecular reaction locale on the physical properties of modified starches has not yet been established.

At the granular level, microstructural features of starch granules such as pores and channels, which connect the central cavity region of granules (corn, sorghum, wheat, etc.) to the extra-granular environment, provide a direct path for reagent to access the interior regions of starch granules and facilitate reagent flow into granule matrix (Huber & BeMiller, 1997, 2000, 2001). These findings have cultivated the technical insight and capacity to directly detect reaction sites/patterns within starch granules. Reaction patterns within derivatized starch granules have been probed with anionic reagents and reagent analogs (Gray & BeMiller, 2004, 2005; Huber & BeMiller, 2001; Kim, 2009; Kim & Huber, 2013), as well as a fluorescent probe (Hong & Huber, 2015a; Kim & Huber, 2008), via confocal laser scanning microscopy (CLSM). Reaction conditions and parameters (pH, swelling inhibiting salt, and/or temperature) that promote a high degree of starch swelling generally enhance reagent penetration into granules and favor a uniform granular derivatization pattern (Gray & BeMiller, 2005). A high level of reagent (Huber & BeMiller, 2001; Kim & Huber, 2013) or an increasing hydration of granules prior to the derivatization (Hsieh & Huber, 2012) further increase reaction uniformity within starch granules. A model system approach, utilizing a fluorescent probe as a reagent, has the advantage of facilitating straightforward monitoring of not only granular, but also molecular, reaction patterns (Higley, 2005), allowing the effects of reaction conditions and parameters on starch reaction patterns to be directly assessed. On a molecular level, Hong and Huber (2015a) provided the first-

ever report of reaction kinetics for amylose (AM) and amylopectin (AP) branch chains (long, medium, short) as a function of reaction time. As reaction time increased, reagent gradually diffused from external granular surfaces into the granule matrix, altering the relative extents of reaction among AM and AP branch chains. This result suggested that starch molecular, as well as granular, reaction patterns might be altered by manipulating reaction system conditions, and that specific combinations of granular and molecular reaction patterns might have potential to produce modified starches with different physical properties.

The objective of this study was to investigate both granular and molecular reaction patterns, and their collective impact on modified starch pasting behavior, as a function of varied reagent infiltration times (i.e., length of time for reagent to penetrate starch granules before formal reaction was initiated by addition of base). While the presence of surface pores and channel structures in cereal starches has been reported to provide reagent with direct access to the granule interior (Huber & BeMiller, 1997; Kim & Huber, 2008), a direct comparison of normal maize and wheat starches has not yet been conducted to contrast similarities and differences in their granular and molecular reaction patterns under similar reaction conditions. Starch derivatives, which were produced in a model reaction system utilizing a fluorescent probe as the reagent, were assessed at the granular (CLSM) and molecular (reactivity of AM and AP branch chains) levels via intermediate pressure size-exclusion chromatography (IPSEC) equipped with both refractive index (RI) and fluorescence (FL) detection. A Rapid Visco Analyzer (RVA) was used to evaluate the pasting properties of starch derivatives. This study seeks to contribute new insight into the interrelationship between granular/molecular reaction patterns and modified starch physical properties to improve technical practices for creating functional modified starch products.

2. Materials and methods

2. 1. Starch sources and chemicals

Laboratory-isolated normal maize starch was prepared in the laboratory of J.N BeMiller (Purdue University, West Lafayette, IN) using the procedure described by Wongsagonsup, Varavinit, and BeMiller (2008). Commercial wheat starch (MidsolTM50, MGP Ingredients, Inc., Atchison, KS) consisted of approximately 99.7% A-type granule starch (volume/weight basis), as determined by particle size analysis (Geera, Nelson, Souza & Huber, 2006). The fluorescent probe, 5-(4,6-dichlorotriazinyl)aminofluorescein (DTAF), which was utilized as the starch modifying reagent, was purchased from Sigma-Aldrich Corporation (St. Louis, MO USA), while isoamylase (EC 3.2.1.68; 1000 U/ml) was acquired from Megazyme International Ireland Ltd (Co. Wicklow, Ireland) for debranching of starch chains. Other reagents and chemicals used were at minimum of analytical grade, and were obtained from Sigma-Aldrich Corporation unless indicated otherwise.

2. 2. Starch derivatization with DTAF with varied reagent infiltration times

Starch (5.0 g, db) was dispersed in deionized water (13.8 and 14.0 mL for maize and wheat starch, respectively) with stirring to maintain granules in suspension, after which 300 µL (maize) or 50 µL (wheat) of DTAF solution (10%, w/v in dimethyl sulfoxide) was added to the reaction slurry. After DTAF addition, the starch reaction slurry was stirred for varied lengths of time (0, 5, 10, 30 or 60 min) prior to adding 2 M sodium hydroxide (230 µL and 275 µL for maize and wheat starches, respectively, to achieve a reaction pH of 11.5) to formally initiate the reaction. Following pH adjustment, derivatization was allowed to proceed for 3 h at ambient temperature.

A few drops of 2 M NaOH ($\leq 100 \mu\text{L}$ in total) were added as needed to maintain the pH at 11.5 during reaction. At the conclusion of the derivatization period, the reaction was terminated by neutralizing the reaction medium with 1 M hydrochloric acid. The entire reaction slurry was transferred to a conical tube (50 mL), and centrifuged ($3000\times g$, 10 min) to recover the modified starch pellet. The starch pellet was suspended (15 min) in 85% (v/v) aqueous ethanol (40 mL) on a wrist action shaker (Model 75, Burrell Co., Pittsburgh, PA), after which it was recovered by centrifugation ($3000\times g$, 10 min). This washing procedure was repeated until the resulting supernatant became colorless (i.e., unreacted DTAF was removed). The final starch pellet was dispersed in absolute ethanol, vacuum-filtered over a Büchner funnel, and air-dried.

A reaction control (Reaction-Control) was generated for each infiltration time/reaction condition described above without the addition of reagent. An additional set of controls (Infiltration-Controls) was subjected only to the reagent infiltration step (reagent included), after which each starch was recovered immediately thereafter without undergoing the formal 3 h reaction in the presence of sodium hydroxide. All reaction controls were subjected to the same washing and recovery procedures outlined previously.

2. 3. Confocal laser scanning microscopy and light microscopy of starch granules

Granular reaction patterns for starch derivatives were visualized via confocal laser scanning microscopy (CLSM), with sample preparation conducted as described by Huber and BeMiller (2000). A CLSM system (Zeiss LSM 510, Thornwood, NY, USA) equipped with an inverted microscope was used to generate serial cross-sections ($1 \mu\text{m}$ thickness) of modified starch granules as described by Kim and Huber (2008). Excitation was achieved with an Argon laser (488 nm) operated at 30% power, and emission was regulated through a LP 505 emission

filter. During image acquisition, microscope parameters (confocal pinhole, detector gain, and amplification factor) were fixed for all starch derivatives regardless of starch type (maize or wheat) or reagent infiltration time, such that observed differences in fluorescent intensity and patterns reflected true differences in extent of reaction. A single cross-section representing the approximate geometric center of granules was utilized to assess granular reaction patterns. Acquired images were processed using Zeiss LSM Image Browser software.

Following pasting analysis (Section 5), pastes of native and DTAF-derivatized starches were visualized with a Nikon Eclipse E600 light microscope (Nikon Inc. Instrument Group, Melville, NY, USA) to compare the structural integrity of granule remnants. One drop each of starch paste and I₂/KI solution (0.2% I₂ in 2% KI, diluted 2:1 with distilled water) were placed on a glass slide, and overlaid with a glass cover slip in preparation for viewing.

2. 4. Intermediate pressure size-exclusion chromatography of debranched starch derivatives

Starch was solubilized in DMSO assisted by microwave heating, debranched with isoamylase, and prepared for intermediate size-exclusion chromatography (IPSEC) as described by Hong and Huber (2015a), except that only 25 mg of starch (db) was utilized for solubilization, and the amount of isoamylase for debranching was reduced accordingly (6.25 µL of isoamylase with 1000 U/mL of activity).

Debranched starch solution was injected onto an IPSEC system (Waters Corp., Milford, MA, USA), equipped with a 1525 binary HPLC pump array, a Rheodyne 7723i manual sample injector with a 200 µL sample loop, and 2410 refractive index (RI, operated at 30 °C) and 2475 fluorescence (FL) detectors. Starch chains were eluted (0.4 mL/min) with deionized water containing 0.02% sodium azide (w/v) as a mobile phase through two successive Tricorn (10/300)

columns packed with 30 cm of Superdex-G75 (M_r : $3 \times 10^3 \sim 7 \times 10^4$) and Superdex-G30 (M_r : $\sim 1 \times 10^4$) prep grade gels, respectively (Amersham Biosciences, Piscataway, NJ). DP_w for debranched starch chain fractions [AM and AP long (AP-LC), medium (AP-MC), and short (AP-SC)] were estimated using pullulan standards (Shodex Standard P-82, JM Science, Inc, NY) as described by Kim and Huber (2010b). The series of pullulan standards consisted of M_w 47300, 22800, 11800, 5900, (Shodex Standard P-82, JM Science, Inc, NY) and 342 (Pullulan Standard 320, Fluka-Sigma-Aldrich, Buchs, Switzerland). Void and total volumes for the two columns operated in series were determined with blue dextran (Pharmacia, M_r 2×10^6) and glucose (180g/mol), respectively. M_w was determined from a plot of $\log M_w$ versus partition coefficients (K_{av}) for pullulan standards, while DP_w was calculated by dividing the obtained M_w by 162 (M_w of an anhydroglucose unit, g/mol).

The relative extent of reaction for AM or AP branch chains was expressed on a unit carbohydrate basis, termed ‘fluorescence density’ (FD). FD was calculated by dividing the FL peak area of a given chain fraction by that of its corresponding refractive index signal (see equation below).

$$\text{Fluorescence Density (FD)} = \frac{\text{Peak area of fluorescence (FL)}}{\text{Peak area of refractive index (RI)}}$$

FL peak areas for Infiltration-Control starches (0, 5, 10, 30, or 60 min reagent infiltration time) were subtracted from those of their respective starch derivatives to compensate for FL intensity incorporated onto starch chains over the course of the various reagent infiltration periods, though the contribution of FL intensity by Infiltration-Control starches was extremely small relative to that of their respective formally modified starches.

2. 5. Rapid Visco Analyzer pasting properties

Pasting properties of starch derivatives (and reaction controls) were examined with the Rapid Visco Analyzer (RVA, Newport Scientific, NSW, Australia) utilizing parameters described by Batey, Curtin and Moore (1997). Starch (1.96 g, db) was weighed into an RVA canister followed by the addition of deionized water to achieve a final net weight (starch + water) of 28.0 g. The starch suspension was subjected to a heating profile at a constant paddle speed (160 rpm) as follows: an initial hold at 60 °C (2 min), heating to 95 °C (5.83°C/min), a hold at 95 °C (4 min), cooling to 50 °C (11.25 °C/min), and a final hold at 50 °C (4 min). RVA attributes such as peak, trough, breakdown, final, and setback viscosities, as well times to pasting and peak viscosity, were determined for each generated pasting curve.

2. 6. Statistical design and analysis

All modification reactions representing each reagent infiltration time (0, 5, 10, 30, and 60 min) were replicated twice. IPSEC and RVA analysis was conducted once for each replicate, and averaged to yield a mean value of the replicates. Analysis of Variance (ANOVA) was used to examine statistical significance ($\alpha = 0.05$) among starch derivatives with different reagent infiltration times, while a least significant difference (LSD) test was used to differentiate the mean values ($p < 0.05$). The Statistical Analysis System (SAS, SAS Institute, Cary, NC, USA) was used for all statistical computations.

3. Results and discussion

3. 1. Granular reaction patterns of starch derivatives

Granular reaction patterns for normal maize (Fig. 1) and wheat (Fig. 2) starches subjected to varied lengths of reagent infiltration (0, 5, 10, 30, or 60 min) were visualized by CLSM. Reactions patterns for both maize and wheat starches differed across reagent infiltration times, indicating that the locale of reaction could be altered by varying the length of reagent infiltration, even though the formal reaction period (3 h) was held constant. Overall, an increasing length of infiltration appeared to enhance the homogeneity of granular reaction patterns.

For maize starch, when reaction was formally initiated immediately after reagent addition (Fig. 1A), external surfaces of granules, including those of channels and cavities, were primarily reacted (Fig. 1F), similar to reaction patterns observed for fast-reacting phosphoryl chloride (Huber & BeMiller, 2001). The kinetics reported for the reaction of DTAF with starch suggest that DTAF is also a relatively fast-reacting reagent, favoring reaction at granule surfaces (Hong & Huber, 2015a). The greatest apparent change in granular reaction patterns for maize starch occurred between infiltration times of 0 and 5 min (Fig. 1A and B), as channels and cavity surfaces became more extensively reacted within this timeframe. With increasing lengths of infiltration time (Fig. 1B-E), reagent diffused laterally from channels and from internal cavities outward into the granule matrix, confirming the general modes of reagent flow reported by Huber & BeMiller (1997, 2000). The reaction band at the external surfaces of granules also became broadened as the length of infiltration increased, allowing for diffusion of reagent from external granule surfaces inward into the matrix; however, this reagent path appeared to be slow and inhibited compared to that via channels and cavities. While reaction uniformity appeared greatest for reagent infiltration times of 30-60 min (Fig. 1D-E), reaction was most intense in regions adjacent to external granule surfaces, channels, and cavities, with the least reacted regions just inside from external granule surfaces, as previously noted by Huber and BeMiller (2001).

219 For wheat starch, the reaction pattern for a reagent infiltration time of 0 min (Fig. 2A) was
220 likewise generally confined to external surfaces of granules, though a minority of granules
221 exhibited some reaction within the granule matrix. Some very fine channel structures were
222 reacted within wheat starch granules (even at an infiltration time of 0 min – especially in granules
223 that exhibited reaction within the granule matrix), though a relatively lesser number of channels
224 was directly observed within wheat compared to maize starch granules (Fig. 1F). Channels within
225 wheat starch granules were generally more difficult to discern, as they are narrower and more
226 densely packed with protein than those of maize starch (Kim & Huber, 2008). Channels became
227 more visible within wheat starch granules as reagent infiltration time was extended to 5 and 10
228 min (Fig. 2B and C) or longer (Fig. 2D and E). While most granules exhibited reaction within the
229 granule matrix at an infiltration time of 10 min, reagent did not appear to extend all the way into
230 internal cavities, which appeared as dark voids at the centers of granules with this length of
231 infiltration (Fig. 2C). At infiltration times of 30 and 60 min (Fig. 2D and E), reaction was
232 visualized within granule concentric rings, and also extended into granule cavity regions (denoted
233 by concentrated fluorescence at granule centers), most likely via the route of channels (Fig. 2F).

234 **Fig. 1.**

235 **Fig. 2**

3. 2. Molecular reaction patterns of starch derivatives

3. 2. 1. IPSEC debranched chain length profiles

Both RI and FL signals of debranched starch derivatives generated by varying reagent infiltration time (0, 5, 10, 30, or 60 min) are shown in Fig 3 (A: maize, B: wheat). Estimated RI chain-length distributions for AP-LC ($DP_w > 33$; $DP_w > 36$), AP-MC ($14 < DP_w \leq 33$; $13 < DP_w \leq 36$), and AP-SC ($DP_w \leq 14$; $DP_w \leq 13$) of maize and wheat starches, respectively, were in general agreement with those reported by Hanashiro, Abe and Hizukuri (1996). Starch derivatives were deemed to be fully debranched based on virtually identical peak distribution patterns observed for both starch derivatives and Reaction-Controls (no DTAF reagent added).

In debranched chain profiles of starch derivatives, there was a tendency for the boundary between the AM and AP-LC FL peaks, as well as the apex of the AP-LC FL peak, to shift upstream in the chromatogram relative to their respective defined RI peak boundaries (Fig. 3). Thus, some of the FL intensity comprising the shifted AP-LC FL peak was attributed to AM based on defined RI peak boundaries. In our prior study (Hong & Huber, 2015b), a distinct FL peak isolated by ion exchange chromatography that eluted in the border region between AM and AP-LC RI peaks was determined to be a highly reactive long chain fraction known as "intermediate material" (IM). Klucinec and Thompson (1998) reported IM material to possess a branch chain fraction similar to AP, excepting a population of very long chains. The reactivity of IM chains was reported to be more akin to that of AP rather than AM (Hong & Huber, 2015b). A more detailed report defining the noted reactivity and granular locale of starch IM will be discussed elsewhere (Hong et al., in preparation). However, in the ensuing discussion, it is important to acknowledge that some FL intensity attributed to RI-defined AM and AP-LC peak

regions also encompassed IM. Thus, calculations depicting extents of reaction for individual starch chain fractions reflect only approximate values.

Regardless of infiltration time, FL intensity was not equally distributed across starch chain fractions for either normal maize or wheat starches (Fig. 3A and B). The greatest differences in FL intensity amongst AM and AP branch chains were generally observed between the 0 and 5 min infiltration times (especially for wheat starch; Fig. 3B), with smaller relative differences observed between infiltration times over the range of 5-60 min. However, most differences in FL intensity observed across reagent infiltration times were noted amongst AM and AP-LC fractions. For maize starch, short infiltration times (0-5 min) appeared to favor reaction of the lowest molecular weight AM chains (Fig. 3A), with reaction shifting to higher molecular weight AM chains at longer lengths of reagent infiltration (10-60 min). It is plausible that the low molecular weight AM chains are located closer to granule surfaces (including those of channels and cavities), whereas the higher molecular weight traditional AM chains are located further into the granule matrix (based on enhanced reactivity with longer lengths of reagent infiltration). Roger and Colonna (1996) likewise reported that low molecular weight AM (8×10^4 - 1.4×10^5 g·mol⁻¹) was predominantly leached (70-90% of the total) from maize starch granules compared to high molecular weight AM (2 - 6×10^5 g·mol⁻¹) at temperatures below 85 °C [might also suggest a location at or near granule surfaces (including surfaces of channels and cavities)] for lower molecular weight AM). This finding coincides with the observation of Glaring, Koch and Blennow (2006), who suggested AM to be more concentrated in the centers of granules (adjacent to cavities) based on the staining of starch reducing ends with a fluorescent probe.

Subtle FL signals were also observed for AM and AP branch chains of Infiltration-Control starches (reagent infiltration for up to 60 min, and starch was collected immediately thereafter

281 without formal initiation of the reaction) of both maize and wheat (Fig. 3). Thus, some degree of
 282 reaction of starch chains (most notably AM, AP-LC, and AP-MC) with DTAF reagent occurred
 283 at neutral pH, though the extent of reaction was very low (required addition of a second y-axis to
 284 visualize on the same graph for maize starch; Fig. 3A) relative to that of formally-reacted starch
 285 derivatives (i.e., reacted at pH 11.5).

Fig. 3

3. 2. 2. Reactivities of starch chains

Reactivities (fluorescence density, FD) for maize (Table 1) and wheat (Table 2) starch chains were quantitatively assessed by dividing the FL peak area of a given chain fraction in IPSEC debranched chain length profiles (Fig. 3) by its respective RI peak area. The relative FD proportions (%) for each chain fraction (as a percentage of the sum total of all chain FD proportions) are presented in parentheses for each infiltration time.

Total FDs (overall extent of reaction) generally trended upward for both maize and wheat starch derivatives as reagent infiltration time increased, though no accompanying statistical differences were distinguished. Total FDs for maize starch were about approximately 4-fold greater than those of wheat starch, even though approximately 6-fold more reagent was required for maize versus wheat starch to achieve the desired changes in starch pasting behavior (Section

3.3). However, the ranking of starch chains in order of decreasing FD (AP-LC > AM > AP-MC > AP-SC) was not different for the two starches. The predominant reaction of AP-LC and AM is likely explained by their location within starch granule amorphous regions, which are thought to be most accessible to reagent. Conversely, the lowest reactivities were observed for AP-MC and AP-SC, most likely due to their participation in the granule crystalline structure, making them less available for reaction (Chen, Huang, Suurs, Schols & Voragen, 2005; Kavitha & BeMiller, 1998; Manelius, Buléon, Nurmi & Bertoft, 2000; Manelius, Maaheimo, Nurmi & Bertoft, 2002; Manelius, Nurmi & Bertoft, 2000; Richardson, Nilsson, Cohen, Momcilovic, Brinkmalm & Gorton, 2003; Steeneken, Tas, Woortman, Sanders, Mijland & de Weijs, 2008).

For both maize and wheat starch derivatives, FDs for AM were approximately 1.5-3 fold greater than those of AP, regardless of reagent infiltration time (Tables 1 and 2). The strictly aqueous medium used in this study (compared to the aqueous ethanol medium of Hong and Huber (2015a, b), as well as time allotted for reagent infiltration, provided both enhanced reagent penetration into granules and greater access to starch chains within amorphous regions (e.g., AM), increasing their reactivity.

For both maize and wheat starch derivatives, FDs for AM gradually increased with longer lengths of reagent infiltration. However, the AM:AP FD ratio for maize starch remained constant as a function of reagent infiltration time (Table 1), suggesting that the relative proportions of reacted AM and AP chains was consistent as reaction progressed further into the granule matrix.

In contrast, the AM:AP FD ratio for wheat starch significantly increased with increasing lengths of infiltration time (Table 2). Thus, for wheat starch, it appeared that AM reactivity increased as the reagent penetrated further into the granule matrix (Fig. 2), implying a greater concentration of AM within interior regions of granules (as opposed to their external surfaces).

However, the increased AM reactivity of wheat starch might have been simply due to enhanced granule hydration/swelling proportional to the length of the reagent infiltration period. To reconcile these two competing hypotheses, starch granules were stirred in deionized water (as opposed to a combined system of deionized water and DTAF reagent) for 60 min, after which DTAF reagent and NaOH were immediately added, and reaction was allowed to proceed for the standard 3 h (S60). Proportional FD values for AM and AP branch chains of S60 most closely mirrored those of the 0 min reagent infiltration time (Table 2), indicating a lack of a granule hydration/swelling effect during infiltration on molecular reaction patterns. Thus, observed changes in AM reactivity over the course of reagent infiltration were most likely attributable to the granular locale of reaction as reagent penetrated further into the interior/matrix of starch granules.

Table 1.

Mean¹ fluorescence density (FD) and FD proportion of normal maize starch chains.

Infiltration time (min)	Fluorescent density (FD) ² (FD Proportion, %) ³						
	AM	LC	MC	SC	AP	Total	AM/AP
0	67.12 ^{aB} (33.42 ^a)	118.89 ^{a/A} (59.21 ^a)	13.17 ^{a/D} (6.55 ^a)	1.67 ^{a/E} (0.83 ^a)	30.47 ^{aC}	39.56 ^a	2.21 ^a
5	76.99 ^{bB} (34.19 ^a)	143.64 ^{a/A} (60.01 ^a)	16.78 ^{a/D} (6.93 ^a)	2.21 ^{a/E} (0.90 ^a)	37.06 ^{aC}	46.69 ^a	2.10 ^a
10	80.34 ^{bB} (32.84 ^a)	135.23 ^{a/A} (57.54 ^a)	16.82 ^{a/D} (7.16 ^a)	2.63 ^{a/E} (1.12 ^a)	34.61 ^{aC}	45.45 ^a	2.33 ^a
30	82.11 ^{bcB} (32.84 ^a)	148.09 ^{a/A} (59.06 ^a)	17.91 ^{a/D} (7.11 ^a)	2.47 ^{a/E} (0.99 ^a)	38.38 ^{aC}	48.62 ^a	2.15 ^a
60	86.75 ^{cB} (34.69 ^a)	143.57 ^{a/A} (57.27 ^a)	17.57 ^{a/D} (6.97 ^a)	2.73 ^{a/E} (1.09 ^a)	36.57 ^{aC}	48.21 ^a	2.39 ^a

¹ Mean values of two replicate reactions for derivatives: values within a column sharing the lower-case letter are not significantly different; values within a row sharing the upper-case letter are not significantly different (p<0.05).

² Peak area of RI signal divided by peak area of FL signal in each starch chain.

³ (Chain FD/Sum of chain FD) *100

Table 2.

Mean¹ fluorescence density (FD) and FD proportion of normal wheat starch chains.

Infiltration time (min)	Fluorescent density (FD) ² (FD Proportion, %) ³						
	AM	LC	MC	SC	AP	Total	AM/AP
0	12.80 ^{aB} (24.12 ^d)	35.86 ^{a/A} (67.62 ^a)	3.99 ^{a/D} (7.54 ^{ab})	0.39 ^{a/D} (0.73 ^{ab})	8.50 ^{aC}	9.67 ^a	1.51 ^a
5	16.88 ^{ab/B} (29.48 ^c)	36.34 ^{a/A} (63.48 ^b)	3.69 ^{a/D} (6.45 ^{bc})	0.34 ^{/E} (0.60 ^{bc})	8.52 ^{aC}	10.85 ^a	1.98 ^b
10	18.59 ^{b/B} (33.41 ^b)	33.73 ^{/A} (60.57 ^c)	3.10 ^{a/DE} (5.57 ^{cd})	0.25 ^{a/E} (0.46 ^c)	7.19 ^{a/CD}	10.95 ^a	2.59 ^c
30	21.64 ^{bc/B} (32.97 ^b)	40.74 ^{a/A} (61.22 ^c)	3.60 ^{a/C} (5.31 ^{cd})	0.34 ^{a/C} (0.50 ^c)	9.16 ^{a/BC}	12.56 ^a	2.40 ^c
60	25.22 ^{c/B} (38.55 ^a)	36.77 ^{a/A} (56.12 ^d)	3.19 ^{a/D} (4.85 ^d)	0.33 ^{a/D} (0.49 ^c)	8.06 ^{aC}	12.72 ^a	3.14 ^d
S60 ⁴	12.51 ^{aB} (24.33 ^d)	34.35 ^{a/A} (66.83 ^a)	4.09 ^{a/D} (7.96 ^a)	0.46 ^{a/E} (0.90 ^a)	8.60 ^{aC}	9.67 ^a	1.46 ^a

¹ Mean values of two replicate reactions for derivatives: values within a column sharing the lower-case letter are not significantly different; values within a row sharing the upper-case letter are not significantly different (p<0.05).

² Peak area of RI signal divided by peak area of FL signal in each starch chain.

³ (Chain FD/Sum of chain FD) *100

⁴ Starch granules dispersed in water for 60 min without a reagent and proceeded 3 h reaction by adding both a reagent and NaOH.

3.3. Pasting properties of starch derivatives

Pasting profiles for native and derivatized starches of maize and wheat representing the various reagent infiltration times are presented in Fig. 4A and B, respectively, and specific RVA pasting attributes are summarized in Table 3. Pasting curves for both maize and wheat starch derivatives generally exhibited reduced viscosity profiles relative to their respective native

starches, characteristic of inhibited granule swelling and a light cross-linking effect (Xie, Liu & Cui, 2005). Hong and Huber (2015b) previously noted the likely existence of cross-linking within DTAF starch derivatives, attributable to the high reaction pH and two possible reactive sites (chlorine leaving groups) on the DTAF *s*-triazine ring (Warren, Reid & Hamalainen, 1952). To further confirm DTAF cross-linking, pastes of native and derivatized starches of maize and wheat following RVA analysis were stained (I₂/KI solution) and visualized by light microscopy (Fig. 5). For maize starch, granule remnants of DTAF-derivatized pastes with a 30 min infiltration time (Fig. 5B) retained greater structural integrity and shape after pasting than the swollen/collapsed granule remnants of native starch paste (Fig. 5A), clearly supporting a cross-linking effect. Similar evidence for wheat starch granule remnants was more subtle, though the amount of leached starch exudates from granules was less for the DTAF derivative (Fig. 5D) versus the native (Fig. 5C) starch paste, as has been previously observed for cross-linked starches (Fannon & BeMiller, 1992). In short, the observed pasting profiles of the starch derivatives and accompanying microscopic evidence of starch pastes provided further assurance that some degree of cross-linking had occurred.

Although the total FDs for both maize and wheat starches did not statistically differ across reagent infiltration times (Table 1 and 2), overall pasting viscosity (Fig. 5A and B), as well as peak viscosity values (Table 3), significantly decreased for both maize and wheat starch derivatives as reagent infiltration times increased. This suggests that pasting viscosity decreased as the reaction patterns within granules likely became more homogeneous.

Much more inhibited pasting curves were observed for wheat (Fig. 5B) compared to maize (Fig. 5A) starch derivatives, despite the fact that reagent addition levels for wheat starch were much less (0.17×) than those of maize starch. Reddy and Seib (2000) reported that 3× higher

levels of phosphoryl chloride were required for waxy maize (compared to waxy wheat) starch to effect equivalent changes in pasting behaviors. The greater viscosity inhibition observed for wheat starch derivatives at increasing lengths of reagent infiltration could be associated with the increase in the AM:AP reactivity ratio (Table 2) compared to that of maize starch, which remained constant across all reagent infiltration times (Table 1). Huber and BeMiller (2010) suggested that the effects of cross-linking on starch physical properties (e.g., swelling, gelatinization transition temperatures, etc.) are likely associated with changes to granule amorphous regions (including AM), leading to reduced mobility of these chains.

Trough and breakdown (wheat only) viscosities became gradually reduced for starch derivatives with increasing lengths of reagent infiltration (Table 3), as pasted granules became more resistant to deformation due to cross-linking (Lim & Seib, 1993). Final and setback viscosities for both maize and wheat starch derivatives were greatly reduced relative to their respective native starches, and gradually trended downward with increasing lengths of reagent infiltration. Reduced setback viscosities for starch derivatives of maize (trend only) and wheat starch derivatives likely occurred due to the substitution of starch chains with bulky, charged DTAF substituent groups, limiting intermolecular associations due to retrogradation (Kim & Huber, 2010a).

Pasting profiles of native, Reaction-Control (subjected to reaction conditions, but no reagent added), and Infiltration-Control (reagent infiltration for 60 min, after which starch was collected immediately after infiltration without formal initiation of the reaction) are provided for maize (Fig. 4C) and wheat (Fig. 4D) starches. Pasting viscosities for both Reaction-Control and Infiltration-Control starches were slightly decreased relative to those of their respective native starches, indicating that those reaction conditions imparted subtle changes to starch properties

(Sui, Shah & BeMiller, 2011). In the case of maize, a slightly reduced and delayed viscosity profile was observed for the Infiltration-Control (relative to the Reaction-Control) starch, indicating a possible slight cross-linking effect. Thus, even in the absence of alkali, some reaction likely occurred within Infiltration-Control starch granules, possibly via nucleophilic/non-starch components (e.g., starch granule-associated proteins) and some starch molecules (Sui, Huber & BeMiller, 2013, 2015). As demonstrated in Fig. 3, a minute amount of FL intensity was associated with the Infiltration-Control (60 min) sample, suggesting some reaction of starch molecules with DTAF in the absence of alkali. Sui et al. (2013, 2015) also reported that maize and wheat starches impregnated with phosphoryl chloride (POCl_3) for 3 h prior to initiating the formal reaction showed a higher incorporation of phosphorus compared to starches reacted by the traditional method (adding alkali before addition of reagent). However, this slightly enhanced reactivity did not impart any further observable cross-linking effect to the properties of these POCl_3 -modified starches. In the case of maize and wheat starches impregnated with sodium trimetaphosphate (STMP) or carboxylic acid anhydrides for 3 h prior to formal reaction, neither reactivity nor physical property benefits were observed (Sui, Huber & BeMiller, 2013, 2015). Their results are not consistent with those within our study, which observed a gradual increase in the inhibition of the pasting viscosity with increasing lengths of reagent infiltration time (up to 60 min). The collective findings of these two studies might imply that the reagent infiltration time could require optimization (i.e., a prolonged infiltration period of 3 h may be too long in some cases) and/or that the conditions or benefits of a prior reagent infiltration period are reagent-specific. Also, the possible role of starch granule associated proteins in starch modification reactions requires further investigation.

425

426

427

428 **Fig. 4.**

429

Fig. 5.

Table 3.

Mean¹ pasting characteristics based on RVA profile.

Starch	Infiltration time (min)	Pasting parameters (cP)				
		Peak	Trough	Break down	Final	Setback
Maize	Native	1335	1108	227	1676	568
	0	1332 ^a	1230 ^a	102 ^a	1290 ^a	60 ^a
	5	1254 ^{ab}	1112 ^a	142 ^a	1191 ^a	79 ^a
	10	967 ^{bc}	885 ^b	82 ^a	890 ^b	5 ^a
	30	939 ^c	858 ^b	81 ^a	854 ^b	-4 ^a
	60	798 ^c	725 ^b	74 ^a	732 ^b	8 ^a
Wheat	Native	616	475	141	805	330

0	407 ^a	349 ^a	58 ^a	438 ^a	89 ^a
5	328 ^b	284 ^b	44 ^{ab}	349 ^b	65 ^{ab}
10	298 ^b	246 ^b	53 ^a	307 ^c	61 ^b
30	139 ^c	108 ^c	31 ^{bc}	155 ^d	47 ^{bc}
60	85 ^c	66 ^c	20 ^c	89 ^e	23 ^c

[†] Mean values of two replicate reactions for derivatives; values sharing a common letter within the column are not significantly different ($p < 0.05$).

4. Conclusions

This study represents the first-ever attempt to simultaneously relate granular- and molecular-level reaction patterns to the physical (pasting) properties of modified starch products. Normal maize and wheat starches were modified with a fluorescent reagent within a model reaction system under varied reaction conditions by varying the length of time for reagent to infiltrate starch granules (0, 5, 10, 20, and 60 min) prior to formal reaction.

A gradual inhibition of starch pasting properties (i.e., cross-linking effect) with increasing reagent infiltration time was concurrent with increased reaction homogeneity within the both maize and wheat starch granules. In short, a more homogeneous reaction pattern within starch granules (in the absence of significant differences in overall extent of reaction) generally appeared to provide a greater degree of viscosity inhibition during pasting. Additionally, the greater relative viscosity inhibition observed for wheat (relative to maize) starch might have been associated with the more homogeneous reaction pattern observed for wheat (relative to maize) starch subjected to the longest reagent infiltration time (60 min), further accentuating a possible impact of granular reaction patterns on modified starch properties. Aside from granular effects, the AM:AP reactivity ratio for wheat starch steadily increased as reagent infiltration time was lengthened, implying a greater reaction of AM chains as reaction progressed further into the

granule matrix. This phenomenon was less obvious for maize compared to wheat starch as AM:AP reactivity remained constant across all reaction conditions. In summary, the progressive inhibition of pasting viscosities of starch derivatives generated with increasing lengths of reagent infiltration (0-60 min) appeared to be associated with observed changes in both granular and molecular reaction patterns (most notable for wheat compared to maize starch). On a practical level, the practice of infiltrating starch granules with reagent prior to reaction has potential utility in a starch modification industrial setting, though optimization of the lengths of time for specific reagents will also require further study.

However, other factors with potential to impact on modified starch properties were not fully addressed or explained in this study. With increasing length of reagent infiltration, an increased overall extent of reaction might have existed (values trended upward, though values were not statistically differentiated), but could not be confirmed. In addition, the specific contribution of IM chains to both the granular and molecular reaction patterns, as well as modified starch properties, needs to be further investigated or defined.

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Figure captions

Fig. 1 CLSM cross-sections of maize starch granules derivatized with a fluorescent reagent following reagent infiltration times of (A) 0 min, (B) 5 min, (C) 10 min, (D) 30 min, and (E) 60 min. Reaction within channels (F) was more readily observed at higher magnifications (0 min infiltration time) (scale bars = 10 μ m; arrows indicate channels highlighted by fluorescence).

Fig. 2 CLSM cross-sections of wheat starch granules derivatized with DTAF following reagent infiltration times of 0 min (A), 5 min (B), 10 min (C), 30 min (D), and 60 min (E). Reaction within channels (F) was more readily observed at higher magnifications (30 min infiltration times) (scale bars = 10 μ m; arrows indicate channels highlighted by fluorescence).

Fig. 3 Fluorescence (FL) and refractive index (RI) profiles of debranched starch derivatives subjected to reagent infiltration times of 0, 5, 10, 30, or 60 min for normal maize (A), and wheat starch (B).

Fig. 4 RVA pasting profiles for maize (A) and wheat starch derivatives (B) according to reagent infiltration times (0, 5, 10, 30 and 60 min), and maize (C) and wheat starch controls (D). Native starch is included in both figures as a reference.

Fig. 5 Light microscopy images of starch pastes for normal maize native (A) and its derivative (B, 30 min as infiltration time), wheat native (C) and its derivative (D, 30 min).