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Hydrodynamic radii of solubilized high amylose native and modified starches by pulsed field gradient NMR diffusion measurements

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ABSTRACT

Over the last decade, there has been an increase in the application of pulsed field gradient nuclear magnetic resonance (PFG NMR) to characterize food materials. In this work, PFG NMR was used to examine the impact of chemical modification on properties of solubilised high amylose starches obtained from heated (121 °C for 15 min) starch suspensions (0.5 % w/w). The starches examined were high amylose maize starch (HAMS) and high amylose maize starch chemically modified with acetate (HAMSA), propionate (HAMSP) and butyrate (HAMSB) at degree of substitution (DS) of 0.2. The hydrothermal treatment solubilized 3.36 to 4.96 % of the starch in all samples, corresponding to concentrations of 10.36 to 10.91 μM on glucose equivalents basis. The hydrodynamic radii of the starches were 30 Å (HAMS), 45 Å (HAMSB), 60-70 Å (HAMSA & HAMSP). Contrary to expectations, the radii did not increase with the molecular weight of the fatty acid attached to the starch. Rather, the trend in the observed hydrodynamic radii was related to the physical organization of the starch molecules in higher DS granular dried starch (DS = 0.32 to 0.40) as observed by others using X-ray diffraction and small angle x-ray scattering, suggesting the preservation of the nano-structure of starch molecules upon solubilization. Upon storage (7 days at 25 °C), there was retrogradation of HAMS but not of modified HAMS. Given the correlation between retrogradation and food spoilage, the inhibition of retrogradation in the soluble fraction of modified HAMS may increase the stability of starch containing foods.

Keywords

high amylose maize starch, hydrodynamic radii, starch modification, NMR, diffusion
1. INTRODUCTION

Starch is the largest contributor to energy intake in human diets and a major energy component of animal feed. Heat processing of starch suspensions in water above the gelatinization temperature result in the formation of a starch gel. This process is due to the starch granule first swelling and then bursting, resulting in the irreversible leaching of amylose and sometimes amylopectin (Funami, Kataoka, Omoto, Goto, Asai, & Nishinari, 2005). Factors such as the type of starch, concentration, time – temperature of treatment and lipid content affect the degree of starch solubilization (Lii & Tsai, 2000; Shamekh, Forssell, & Poutanen, 1994). Retrogradation or recrystallisation of the leached fraction is a time-dependent and complex process which affects food stability, taste and texture, contributing to staling or undesirable firming of starch-containing food products upon storage (Zeleznak & Hoseney, 1986).

High amylose maize starch (HAMS) and modified high amylose maize starch (mHAMS) chemically esterified to carry acetate, propionate or butyrate entities on a proportion of free hydroxyl groups are postulated to improve gastrointestinal health by delivering health-promoting short chain fatty acids (SCFA) to host gut (Annison, Illman, & Topping, 2003; Clarke, Bird, Topping, & Cobiac, 2007). Structural characterization of these starches through X-ray diffraction (XRD) and small angle X-ray scattering (SAXS) suggested that there was more extensive remodeling of the nanostructure upon modification with acetyl compared with the longer chain butyryl group (Lopez-Rubio, Clarke, Ben, Topping, & Gilbert, 2009). It is believed that longer chain modifications lie parallel to starch molecules within starch granules whereas
the shorter chain modifications that are unable to interact with existing starch structure cause
defects within the organization of the starch granule leading to greater structural distortion.

The heating of starch suspensions is an integral part of food processing and has been
extensively used to manipulate the texture of foods. The changes to starch during gelatinization
and retrogradation influence the rheological properties of starch and govern their application in
many products. Indeed there is a significant literature on the rheological properties of starches
relating to their pasting, gel-forming properties and starch retrogradation (Jacobs & Delcour,
1998; Liu & Thompson, 1998; Zavareze & Dias, 2011). Many authors have suggested that the
extent of starch solubilization is related to the starch rheological properties and the rate of
retrogradation (Bello, Waniska, Gomez, & Rooney, 1995; Lii & Tsai, 2000; Mua & Jackson,
1995). Currently, a wide array of techniques, such as turbidometry, Raman spectroscopy,
differential scanning calorimetry and oscillation rheometry are used to study the structural and
molecular behavior of starches under different conditions (Karim, Norziah, & Seow, 2000).
More recently, pulsed field gradient nuclear magnetic resonance (PFG NMR) has been used to
classify food systems such as dairy emulsions (Gabriele, Migliori, Di Sanzo, Rossi, Ruffolo,
& de Cindio, 2009), whey proteins (Colsenet, Söderman, & Mariette, 2006) and polymeric gels
(Karim, Oo, & Seow, 2007; Matsukawa, Sagae, & Mogi, 2009) with good correlation to
traditional rheological techniques. While diffusion NMR is relatively new to food science, it has
been employed successfully to study the self-diffusion of small protein molecules in solution
(Wilkins, Grimshaw, Receveur, Dobson, Jones, & Smith, 1999). This technique measures the
spontaneous and random translational motion of molecules, and this motion is highly
dependent on the size and shape of the molecule within a controlled setting. By measuring the translational self-diffusion coefficients (D) of molecules under different conditions, effective hydrodynamic radii (R_h) and association states can be determined (Yao, Howlett, & Norton, 2000).

In this study, we investigate the properties of the starch solubilized on heating suspensions in water. We examine the solubility and hydrodynamic radius (R_h) of HAMS solubilized through the process of gelatinization to deduce the consequences of acylating high amylose maize starch with acetate, propionate and butyrate and on their re-association properties. The impact of short term storage on the soluble fractions was also examined, providing insight into the relationship between solubilised starch fraction and retrogradation. This work has practical implications in that it enhances our understanding of how modification of starch can alter retrogradation processes, and consequently impact on the texture of food products.
2. METHODS

2.1 Starch samples.

HAMS is an unmodified maize starch that contains approximately 70 % amylose produced by National Starch and Chemical Company (now named Ingredion, New Jersey, US). HAMS was used as the base starch to manufacture acetylated (HAMSA), propionated (HAMSP) and butyrylated (HAMSB) starches by the company Ingredion. All four starches were supplied with moisture content (% dry basis) of 10.23 to 11.62%. The extent of acylation is expressed as a degree of substitution (DS), which can be defined as the average number of substitution groups per anhydroglucose unit in starch. DS can be calculated using the following equation:

\[ DS = \frac{[S]}{[B]} \]  

(1)

Where [S] is the concentration of the substituent in the sample and [B] is the concentration of the backbone in monomeric terms. As there are only 3 hydroxyl groups per glucose unit, the maximum DS value for starch is 3. The DS values of the HAMSA, HAMSP and HAMSB used in this study are 0.22, 0.20 and 0.20 respectively, as determined by the company Ingredion. To study batch variation, two other batches of HAMSA (DS=0.18, 0.23) and five additional batches of HAMSB (DS = 0.18 – 0.25) were obtained. These additional batches were also produced by Ingredion and all starch samples were stored under similar conditions (25 °C in sealed containers) prior to use.

2.2 NMR sample preparation.

50 mg of starch (dry basis) was suspended in D$_2$O to a concentration of 0.5 % (w/v) and centrifuged at 4000 x g for 10 min and decanted. The pellet was resuspended in 10 ml D$_2$O and
autoclaved at 121 °C for 15 min to gelatinize the starch. Samples were centrifuged at 4000 x g for 10 min and the supernatant sampled for NMR experiments. The supernatant containing solubilized starch were used for the rest of this study.

2.3 Determination of solubilized starch.

The solubility was determined in triplicate based on the method of Leach et al. (Leach, McCowen, & Schoch, 1959). Briefly, 10 ml starch dispersions (0.5% dry weight) were heated at 121°C for 15 min and then centrifuged at 20,000 g for 10 min. The supernatant was evaporated and dried in a centrifugal evaporator at 30 °C. The dry matter obtained was used to calculate % solubility. To account for the contributing mass of the fatty acids, solubilized starch was also expressed in μM. The molecular weight of starch was calculated by assuming all glucose were anhydroglucose and esterified at DS of 0.20 for all starch. The molecular weights used for anhydroglucose and esterified anhydroglucose to calculate the solubility of starch in μM glucose equivalents were 162.15, 204.18, 218.21 and 232.24 for HAMS, HAMSA, HAMSP and HAMSB respectively.

2.4 NMR spectroscopy and translational diffusion measurements by PFG NMR.

All NMR spectra were acquired on a Bruker Avance II 800 MHz spectrometer equipped with a cryoprobe and a single gradient (G_z). For the measurement of translational self-diffusion coefficients (D), a stimulated echo sequence with shaped bipolar gradient pulses for the encoding and decoding of diffusion was used. Water suppression was achieved by WATER suppression by GrAdient Tailored Excitation (WATERGATE) (Piotto, Saudek, & Sklenar, 1992).
For each measurement, a series of 16 diffusion-weighted one-dimensional spectra was recorded in a two-dimensional manner using sine-shaped gradient pulses of 3.5 ms duration (effective duration for diffusion encoding, $\delta = 4.456$ ms, after taking into account the shape of the gradient pulse used), a separation ($\Delta$) of 100.0 ms, and amplitude of the field $G_z$ ranging from 2.8 to 53.2 G cm$^{-1}$. The strength of the $B_0$ field gradient used for measuring $D$ was calibrated by measuring the $D$ of residual HDO in a 100 % D$_2$O sample at 25 °C with the same pulse sequence but with the $^1$H carrier frequency placed 2 ppm away from the H$_2$O resonance. A diffusion coefficient of $1.90 \times 10^{-9}$ m$^2$ s$^{-1}$ for residual HDO was used in the back calculation of $G_z$ (Callaghan, Le Gros, & Pinder, 1983).

### 2.5 Translational diffusion coefficients measured by PFG NMR.

For molecular self-diffusion, the attenuation of an NMR signal in the presence of a bipolar pulse pair gradient as used in the present study, is given by

$$I = I_0 \exp \left( -\gamma^2 g^2 D \delta^2 (\Delta - \delta/3 - \tau/2) \right)$$

(2)

Where $\gamma$ is the gyromagnetic ratio and $g$, $\delta$ and $\Delta$ are the amplitude, effective duration and separation of the single pair of gradient pulses, respectively. $\tau$ (s) is the delay between the end of the first lobe of gradient and the 180° radio frequency pulse in the bipolar encoding/decoding segment. All spectra were processed using TOPSPIN (Version 3, Bruker BioSpin). For diffusion weighted spectra, an exponential window function with 3 Hz line broadening was applied before the Fourier transformation (FT) and baseline corrections were performed after the FT. Translational $D$ were obtained by fitting peak intensities to Eq.2 using the relaxation $T1/T2$ module in TOPSPIN.
2.6 Internal standard for diffusion measurements.

As the ratio of solvent radius to that of solute is more than 0.01 in this work, microviscosity differences are present (Boeré & Kidd, 1983). In order to avoid the complications arising from these phenomenon and variations in sample conditions, small molecules such as dioxane were used as internal standards (Chen, Wu, & Johnson, 1995; Jones, Wilkins, Smith, & Dobson, 1997). This internal standard may be considered as an internal radius standard for calculating the effective hydrodynamic radius of starch, $R_h^{\text{Starch}}$

$$R_h^{\text{Starch}} = R_h^{\text{Ref}} \times \frac{D_{\text{Ref}}}{D_{\text{Starch}}} \quad (3)$$

$R_h^{\text{Ref}}$ for dioxane is obtained from published sources (Chen, Wu, & Johnson, 1995; Jones, Wilkins, Smith, & Dobson, 1997), while both D values are experimentally-derived to solve for $R_h^{\text{Starch}}$. This approach was employed in comparing HAMS and mHAMS by PFG NMR diffusion measurements in order to avoid the complexities arising from variations in solution viscosity and temperature.

3. RESULTS AND DISCUSSION

3.1 Quantification of the soluble starch fractions.

The amount of starch solubilized on heating starch suspensions range from 3.36 to 4.96 % for all starches examined. This corresponded to similar concentrations of solubilized starch of 10.36 to 10.91 μM on a glucose equivalents basis when the mass of the esterified groups were accounted for (Table 1). The amount solubilized upon heat treatment of starch dispersions of HAMS starches is lower than that found for rice starch containing <30 % amylose (soluble up to
8 % in 90 °C water) (Lii & Tsai, 2000), large barley granules (~30 % solubilized on heating at 95°C for 30 min) (Myllrinen, Autio, Schulman, & Poutanen, 1998) and native oat starch granules containing 29 % amylose (15 % starch leached at 95°C) (Shamekh, Forssell, & Poutanen, 1994). Low solubility is a known attribute of high amylose starches. Similar observation of reduced solubility has been previously observed in high amylose pea and maize starch variants (76 % and 61 % amylose content respectively) and were found to be in the range of 5 – 10 % (Colonna & Mercier, 1985).

### 3.2 Measurement of diffusion coefficients and experimentally derived hydrodynamic radii.

Figure 1A shows the chemical structure of the four starch types and their respective ¹H NMR spectra. An example of the PFG NMR spectra for HAMSB is shown in Figure 1B. As expected, resonances from residual water (4.7 ppm) and free fatty acids were observed to rapidly decay with increasing gradient field strength. This corresponds to the greater diffusivity associated with diffusants of smaller mass. Resonances attributed to HAMS and covalently bound butyrate decayed less rapidly indicating that they diffused at a slower rate. The decay of the resonances was used to determine D and subsequently, the apparent $R_h$ values. To minimize potential variation due to changes in solution viscosities among the four starch samples, the effective $R_h$ of starch was also calculated from Eq 3 using dioxane as an internal reference molecule with an $R_h$ of 2.12 Å. All values of D and calculated apparent $R_h$ stated in this work are normalized to dioxane using Eq 3. The apparent $R_h$’s of the starches were 30 Å (HAMS), 45 Å (HAMSB), 60-70 Å (HAMSA & HAMSP) as experimentally determined using PFG NMR techniques.
Figure 2 shows the logarithmic intensities of the solubilized fractions of HAMS and acylated HAMS. To ascertain the reproducibility of the results in Figure 2, we sourced available alternate batches of starches with similar DS values, keeping in mind that it was imperative that the starches were chemically modified and stored under similar circumstances. We were able to obtain HAMSA and HAMSB that had been prepared in separate batches with similar DS values to those used above. Unfortunately, no other batch of HAMSP with a DS suitable for use in this comparative study was available. Figure 3 shows that for HAMSA and HAMSB there is no significant variation in the experimentally determined diffusion coefficients for the different batches.

The linearity of the data in Figure 2 indicates a high level of agreement with a single diffusing species described by the Stokes-Einstein equation. As the values were obtained from two independent samples on two separate occasions, it suggests negligible variations in sample viscosity and sampling environments. Various peaks from either the glucosyl or acyl groups of differing line-widths and intensities yield similar values indicating excellent internal consistency and reproducibility of the gelatinization process for these starches under these conditions.

In this work, diffusion measurements of mHAMS with shorter acyl chain modifications (HAMSA and HAMSP) show the slowest mobility while HAMSB, with the longer acyl chain modification, has a D value consistent with faster tumbling (Figure 2). Diffusion and therefore, $R_h$ of the soluble starches does not simply correlate to their mass. This is inconsistent with current expectations as one would expect that $R_h$ to increase with the molecular weight of the attached
fatty acid diffusion, since $R_n$ is largely dependent on the molecular size of the diffusant (Masaro & Zhu, 1999; Petit, Roux, Zhu, & Macdonald, 1996).  

Starch is comprised of two components, amylopectin and amylose, where amylose rather than amylopectin is leached upon gelatinization (Partridge, 1946; Tester & Morrison, 1990). The estimated and determined $R_n$ of glucose is 4.6 Å and 3.7 Å respectively (Monteiro & Hervé du Penhoat, 2001). Using Eq 3, HAMS and mHAMS possess an $R_n$ in the range of 30 - 70 Å. These values are several orders of magnitude larger than reported values of glucose monomers (Monteiro & Hervé du Penhoat, 2001). Since a similar amount of starch (on a molar basis) was leached from all starches, it is likely that the leached material from HAMS may adopt a more compacted conformation than that leached from mHAMS due to the lack of fatty acid modification. A lack of amylopectin interference also would result in a more compacted conformation (Gudmundsson, 1994).  

The trend in apparent $R_n$ appears to be related to the physical organization granular dried starch where greater structural distortion to the nano-structure was found when HAMS was acetylated or propionated than when HAMS was butyrylated (Lopez-Rubio, Clarke, Ben, Topping, & Gilbert, 2009). Using X-ray diffraction and SAXS, these changes were only observed at high DS (0.32 to 0.4) and not at DS of 0.1 – 0.23. In our work, the fact that we observed a greater change in hydrodynamic radii when HAMS was butyrylated compared to when HAMS was acetylated or propionated in soluble starch at DS of 0.2, suggests that differences are obtained in hydrated structures at lower DS (0.2). It is possible that as differences are amplified
when starch is hydrated and solubilised compared to when the molecules are in a more compacted state in the dry granules. Regardless, this suggests that the nano-structure of starch molecules in the granules in the dry state is the major determination of the structure of the solubilized starch, indicating the preservation of the structure of the dry state on hydration and solubilization.

3.3 Effect of starch concentration.

Concentration did not appear to have a significant effect on $D$ although we note a slight increase at lower concentrations (Figure 4A). This increase is consistent with the knowledge that translational motion is favoured at lower solute concentrations (Chen, Wu, & Johnson, 1995; Jones, Wilkins, Smith, & Dobson, 1997; Yao, Howlett, & Norton, 2000). While the $R_h$ for HAMS and HAMSB are both significantly less than those for HAMSA and HAMSP, these values for each starch remained relatively constant at all concentrations measured (Figure 4).

Molecular interaction is often concentration dependent, as changes in concentration alter factors such as free energy and availability of binding partners (O'Shaughnessy, 1994). The independence of $D$ and $R_h$ on concentration suggests that the differences in observed mobility between the different types of starch are due to different physiochemical properties imparted by their respective modifications. In particular, the lower $R_h$ value for HAMSB relative to mHAMS is consistent with coupled butyrate moieties interacting and aligning with the glucose polymeric chains for starch both in solution, as examined here, and in the HAMSB starch granules as previously illustrated (Lopez-Rubio, Clarke, Ben, Topping, & Gilbert, 2009).
3.4 Effect of room temperature (25 °C) storage.

To investigate the impact of recrystallisation on solubilised HAMS and mHAMS, diffusion analyses were performed on seven day old starch samples at maximum concentration (Figure 4). The effect of seven day storage had minimal impact on mHAMS although it significantly decreased the D and increased the $R_h$ of HAMS. Seven days post the hydrothermal treatment; all four starches were observed to have similar $R_h$ in the range of 50 - 65 Å.

Leached starch resulting from gelatinisation is often not stable and will recrystallize in a process known as retrogradation during storage (Sobolewska-Zielinska & Fortuna, 2010). Retrogradation is a time-dependent process which is induced by factors such as starch origin, low temperature and high amylose content (Sobolewska-Zielinska & Fortuna, 2010). In the initial phase, amylose self-associates which is followed by the gelation of amylopectin (if present) which may occur over weeks (Ring, Colonna, l’Anson, Kalichevsky, Miles, Morris, et al., 1987). It is known that modification of starch may reduce or enhance this recrystallisation process, impacting food preservation (Sobolewska-Zielinska & Fortuna, 2010).

The high content of amylose in HAMS and mHAMS, coupled with the knowledge that gelatinization usually leaches amylose, suggests that the majority of leached starch material observed is amylose. Studies have shown that the presence of polar lipids such as monoglycerides may retard retrogradation (Krog & Jensen, 1970). While the exact mechanism is unknown, it is believed that such polar lipids and surfactants may complex with amylose...
altering downstream crystallisation processes (Gudmundsson, 1994). In this work, no significant changes were observed after seven days of storage for mHAMS, whereas for HAMS the $R_h$ doubled over the same time period. Esterification of HAMS with SCFA hinders amylose-amylose complex formation, altering the manner in which starch crystals may reform.

4. CONCLUSION

The aim of this study was to evaluate if the known biophysical characteristics of mHAMS are present in their solubilised fractions upon gelatinisation and if storage alters the $R_h$ of gelatinised soluble starch. $R_h$ of mHAMS was in the range of 50-70 Å, and was larger than that of HAMS at 30 Å. Upon storage, the solubilised mHAMS remains unaltered while HAMS retrograded to double its $R_h$. The retarded retrogradation in the solubilised mHAMS is possibly attributed to individual starch properties conferred by the polarity of the modification along starch molecules. Given the correlation between retrogradation and food spoiling, mHAMS may be protective against retrogradation, offering increased food stability in addition to its proposed health benefits.

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FIGURE LEGENDS

Figure 1: $^1$H NMR of the four starch types and PFG NMR spectra of HAMSB. (A) The structures of the four starch types, their possible chemical modification at C2, C3 and C6 and respective $^1$H NMR spectra. Resonances arising from starch glucosyl rings are labelled in blue. Fatty acids and their corresponding resonances are labelled in red with free acids denoted with (’). The same intensities are displayed in the y-dimension across all four spectra. Crossed peaks denote cropped residual water resonances. (B) Plots of $^1$H PFG NMR spectra of HAMSB shown for increasing gradient strength. Resonances from residual water and free fatty acids were observed to be rapidly decaying with an increase in field gradient strength.

Figure 2: Logarithmic intensities of the four starch types versus the strength of diffusion encoding. Logarithmic intensities of HAMS (◇◇ ◇◇), HAMSA (☐☐ ☐☐), HAMSP (∆) and HAMSB (〇) versus the strength of diffusion encoding as measured from two independent acquisitions. Error bars represent SEM.

Figure 3: Logarithmic intensities of different batches of HAMSA and HAMSB versus the strength of diffusion encoding. Logarithmic intensities of three separate HAMSA (☐☐ ☐☐) batches and six separate HAMSB (〇) batches versus the strength of diffusion encoding as measured from two independent acquisitions. Convergence of the different batches and preservation of diffusion trends across samples indicate a high level of reproducibility within sampling limits. Error bars represent SEM of the different batches used.

Figure 4: D and $R_h$ of the four starch types at different concentrations and upon storage

Experimentally determined (A) D and (B) Rh values of HAMS (◇◇ ◇◇), HAMSA (☐☐ ☐☐), HAMSP (∆) and
HAMSB (O) at different concentrations post gelatinisation and upon seven days storage as measured from two independent acquisitions. Error bars indicate SEM.
Table 1.
% Solubilized starch in gelatinized starch samples (0.5% w/v) and the calculated concentrations in glucose equivalents.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Solubility</th>
<th>µM (glucose equivalents)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAMS</td>
<td>3.28 (0.14)</td>
<td>10.36 (0.36)</td>
</tr>
<tr>
<td>HAMSA</td>
<td>4.43 (0.26)</td>
<td>10.75 (0.47)</td>
</tr>
<tr>
<td>HAMSP</td>
<td>4.68 (0.32)</td>
<td>10.91 (0.54)</td>
</tr>
<tr>
<td>HAMSB</td>
<td>4.94 (0.32)</td>
<td>10.68 (0.61)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} % Solubility was determined on a dry weight basis. Solubilized starch, expressed in µM, was derived by assuming all glucose were anhydroglucose and esterified at DS of 0.20 for all starches. The molecular weights used were 162.15, 204.18, 218.21 and 232.24 for HAMS (high amylose maize strach), HAMSA (acetylated high amylose maize starch), HAMSP (propionated high amylose maize starch) and HAMSB (butylated high amylose maize starch) respectively. In brackets are the ± SEM of three independent measurements.
Highlights

• Structure of high amylose maize starch is preserved after hydrothermal treatment

• Hydrodynamic radii of acylated high amylose maize starches are independent of mass

• Acylation retards re-association of solubilized starch on cooling and storage