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Study of rice-starch structure by dynamic light scattering in aqueous solution

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Abstract

The expansion of various rice starches in solution was studied using dynamic light scattering (DLS) in order to provide information on their microstructure. Hydrodynamic diameters of starch molecules were found to change with solvent conditions. The component starches in the rice varieties studied had similar sizes in pure water (between 125 and 235 nm), but showed different expansion behaviour with changes in salt concentration, with addition of urea (which disrupts hydrogen bonding) and with the addition of 1-butanol (which reduces solvent polarity). Some starches showed a gradually increasing size with increasing [NaCl], while others showed an initial steep increase followed by more gradual behaviour. The size distributions from DLS indicated two (and possibly three) components at about 100 and 1000 nm. The larger component was largely responsible for the expansion with added salt, an effect which is ascribed to the greater ability of larger chains to expand. Chain length distributions (CLD) of debranched starch from the same samples were examined by capillary electrophoresis (CE) for correlation with the expansion behaviour obtained by DLS. The ratio of shorter to longer chains partly correlated with the classes of expansion behaviour. The data suggest that the expansion behaviour is sensitive to the branching structure (connectivity) as well as to the distribution of the lengths of the branches.

Keywords: Starch; Dynamic light scattering; Molecular weight distribution; Capillary electrophoresis; Rice; Starch structure; Chain length distribution

1. Introduction

Rice starches of similar amylose content can have markedly different textural properties (Ong & Blanshard, 1995), suggesting that amylopectin and amylose *structure* may be a more important determinant of physical processing properties than the simple ratio of the two starch components. Examples of structural information are average molecular weights and molecular weight distributions of both the whole amylopectin (or amylose) and its individual branches, the average size and size distribution of the whole amylopectin, and branch density distribution. Textural properties may depend on different degrees on all of these structural characteristics. These structural characteristics also control the volume occupied by a starch molecule, and the change in expansion caused by changes in a solution property. The importance of knowing more than just a simple average of a single property is illustrated by the fact that the hydrodynamic volume of two starch molecules with the same total molecular weight, but with differing distributions of branches, will almost always be different. Knowledge about the dependence of hydrodynamic volume (including its variation with solvent) on molecular architecture will also aid in relating molecular architecture and textural properties.

Techniques to explore the dimensions of starch molecules in solution include light scattering, both static (SLS) and dynamic (DLS, also known as photon correlation spectroscopy, PCS). With SLS, weight-average molecular weight and root-mean-squared radius of gyration ($\langle s^2 \rangle^{1/2}$) can be obtained, while DLS provides the hydrodynamic radius (R_h) from measurement of the diffusion coefficient (Bloomfield, 2000), and (under restrictions discussed

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below) information of the distributions of the hydrodynamic radii, i.e. size distributions. These techniques provide some information on the dimensions of starch molecules in solution, giving a measure of their average swollen density, but provide no direct information on microstructure (Bello-Perez, Roger, Colonna, & Paredes-Lopez, 1998; Ring, L'Anson, & Morris, 1985). Complementary information may be achieved by examining starch structure by SLS and DLS after a separation technique, for example, size exclusion chromatography (Radosta, Haberer, & Vorwerg, 2001) or field flow fractionation (van Bruijnsvoort, Wahlund, Nilsson, & Kok, 2001).

Changing the properties of the solvent may lead to changes in the swollen macromolecule dimensions, which will be governed by solvent-induced changes in the free energies of solvation of each branch, constrained by branch connectivity. Salt–starch–water interactions in the gelatinisation of starch in different salt solutions have previously been studied by differential scanning calorimetry and differences were attributed to structure-forming and structure-breaking properties of various salts (Jane, 1993), but the effect of solvent properties on the dimensions of gelatinised starch was not examined.

The purpose of this study is to investigate the relationship between chain length distributions (CLD) of debranched starch and the average size distributions of the branched starch. The CLD of debranched starch was analysed by capillary electrophoresis (CE) (Morell, Samuel, & O'Shea, 1998; O'Shea, Samuel, Konik, & Morell, 1998). The size and size distributions of the branched starch were determined by dynamic light scattering. The objective is to see if the debranched chain length distributions are sufficient to explain the observed expansion behaviour, or if the expansion behaviour is determined by additional structural parameters of the starch, such as distributions of the branch connectivity.

2. Materials and methods

2.1. Rice varieties

Rice, *Oryza sativa*, was grown in the 2001/2002 and the 2002/2003 seasons in a temperature-controlled glasshouse at the Yanco Agricultural Institute, Yanco, NSW, Australia. All rices were either commercial varieties or advanced and stable breeding lines from the Australian Rice Improvement Program. These were Tarra 140 (waxy, but measured 6.5% amylose, 2002), YRW 4 (waxy, measured as 8.3% amylose, 2003), Shimizu-Mochi (waxy, measured as 9.0% amylose, 2003), YRM 44 (19.1% amylose, 2002), Amaroo (20.0% in 2002, 21.0% in 2003), Millin (19.5% in 2002, 21.0% in 2003), Vialone Nano (21.9% in 2002, 24.4% in 2003) and I-Geo-Tze (IGT) (29.3% in 2003). All rice varieties used in this study were *japonica*, apart from Tarra 140 and IGT, which are *indica* types. The amylose content was determined by a standard iodine-binding assay (Juliano et al., 1981). Starch was extracted and purified from these rices using protease (Sigma) and soxhlet extraction (Chiou, Martin, & Fitzgerald, 2002).

2.2. Preparation of samples for DLS

Starch (5.0 mg) was gelatinised by adding sodium hydroxide (Aldrich, 0.25 M, 1.0 g) and heating in a closed container (10 min, 130 °C). Filtered deionised water (MilliQ, Millipore) was then added until the solution weighed 5.0 g. Ion-exchange resin (3.0 g, washed Amberlite MB-3) was then added and the solution was incubated (30 min, 60 °C), filtered on a tightly packed glass wool column, and the filtrate adjusted to 10.00 g with filtered deionised water. The heating to 130 °C here and elsewhere is to re-solubilise the pellet (hot water alone is not enough to do this, so sodium hydroxide and high temperature are required). The samples in this procedure were dried as described in order to control the mass (20 mg) used in the measurements. Different starches have different ratios of soluble components, and this standardises the measurement of the pellet.

2.3. Hot water soluble components

Starch (50.0 mg) and water (15.0 g, MiliQ) were mixed together and boiled in a closed container for 10 min then centrifuged (14,000*g*, 5 min). The supernatant was collected for measurement by DLS while the pellet was dried. Sodium hydroxide (0.25 M, 1.0 g) was added to a sub-sample of the dry pellet (20.0 mg) and was heated (10 min, 130 °C), then adjusted to 10.0 g with water.

2.4. Modifying solution properties

Three types of changes were made in the solvent (water): changing ionic strength, changing hydrogen bonding, and changing polarity. To change the ionic strength of the system, sodium chloride (recrystallised, Ajax) was added incrementally to increase the concentration up to saturation (6.15 M). Urea was used to break hydrogen bonds; starch (5.0 mg) was gelatinised by adding urea solution (Merck, 6 M) and heating on a hotplate in a closed container (5 min, 130 °C). The sample was then filtered and made to the appropriate concentration with filtered deionised water, with subsequent dilutions to the various concentrations, allowing the measurement of the same sample from a high concentration through to a lower concentration. To change the polarity of the solution, fresh solutions of solubilised fractions were adjusted by adding 1-butanol (BDH), up to 0.4 M (3%, w/w).

2.5. DLS measurements

DLS measurements were carried out at 25 °C with a goniometer (BI-200SM, Brookhaven) fixed at 90° and

a correlator (BI-9000AT, Brookhaven). The light source was a helium–neon laser (λ_0 = 632.8 nm, 35 mW, Spectra Physics). Samples were sonicated (Bransonic 32 bath, 30 s) before measurement. Collection time was 3 min and the effective diameter was calculated using the method of cumulants. All DLS analyses of rice samples were repeated a minimum of four times, using the same sample. Freshly made samples were tested and the results were also consistent. This consistency between multiple samples suggests that coagulation is not a factor during DLS measurements. The separated (hot water soluble and insoluble) components were also subjected to changing temperatures (from 25 to 45 °C) to observe any temperature dependence on the DLS.

The boiling is to extract hot water soluble components of starch, while the sodium hydroxide serves to completely solubilise the remaining pellet which is not hot water soluble. The ion exchange is to remove sodium ions such that there are no extra sodium ions in the system as sodium chloride is added to the system. The filtration is to remove large particulates from the system, to which DLS is very sensitive.

2.6. Capillary electrophoresis

The CLD of starches debranched by isoamylase (Batey & Curtin, 1996) was determined by the method of fluorescence-assisted carbohydrate capillary electrophoresis (CE) (O'Shea & Morell, 1996; O'Shea et al., 1998). In short, debranched starch chains (with 5 nmol of reducing ends) were labelled with 8-amino-1,3,6-pyrenetrisulfonic acid (APTS, Beckman Coulter, 5 μL, 0.2 M in 15% glacial acetic acid) as the fluorophore. This mixture was incubated with sodium cyanoborohydride solution (1 M, 37 °C, 15 h). Each run was repeated and checked for consistency.

3. Results and discussion

3.1. Angle dependence of scattering from large molecules in DLS

The internal modes of motion of branched structures have been previously studied by DLS (Gallant, Bouchet, & Baldwin, 1997), showing the angular dependence of the scattered light from starch solutions. A simple cumulants analysis can give size information at a particular angle. A dynamic Zimm plot can give the radius of the molecule (Bello-Perez et al., 1998), but only the hydrodynamic radius R_h rather than the radius of gyration $\langle s^2 \rangle^{1/2}$ as obtained by SLS. While these two quantities are related, the relation is a complex one which depends on the polymer, solvent and connectivity (Sheridan, Adolf, Lyulin, Neelov, & Davies, 2002), and thus $\langle s^2 \rangle^{1/2}$ and R_h effectively contain independent information on connectivity of (undebranched) starch. Fig. 1 shows the angle dependence of the measurement on

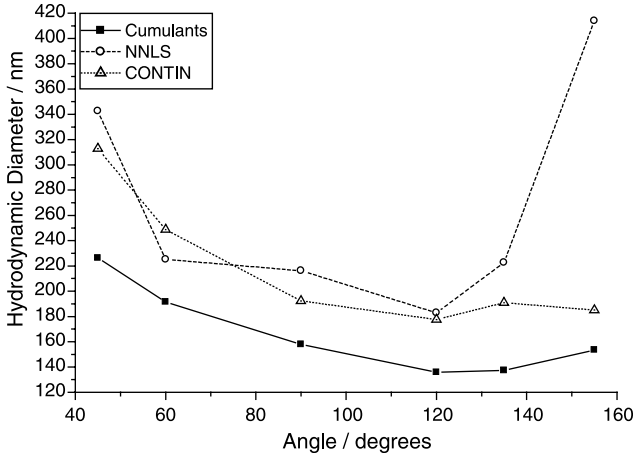

Fig. 1. Angle dependence of scattering by DLS of Tarra 140, by various fits: cumulants, non-negative-least squares: multiple pass (NNLS) and non-negative-least squares fit: regularised (CONTIN).

the hydrodynamic size of amylopectin (Tarra 140) in water by three different fits: cumulants, non-negative-least squares: multiple pass (NNLS) and non-negative-least squares: regularised (CONTIN). Of the various processing methods available (Bello-Perez et al., 1998; Cao, Sessa, Wolf, & Willett, 2000), the cumulants method was chosen for this study, as it gave a result that was least dependent on the detector angle (Fig. 1). It is possible to obtain extra data from the angular dependence of starch solutions with changing solvent behaviour, but the focus of this study is on the change in expansion of starch in solution from different sources and solvent conditions relative to one another. This is adequately achieved by comparing values obtained at a single angle, and hence DLS data were obtained only at 90°. The size range obtained here by DLS (50–350 nm) on rice starch in water is comparable to that reported for the hydrodynamic radius of amylopectin, 348 nm, for waxy maize starch as determined by analytical centrifugation (Millard, Wolf, Dintzis, & Willett, 1999).

Information on the distribution of sizes was obtained by using methods to deconvolute the correlation function (e.g. CONTIN or NNLS (Bello-Perez et al., 1998; Cao et al., 2000)), as shown in Fig. 2; these distributions were essentially unchanged by varying the integration range used in the inversion over sizes where the distribution was significant. However, it is essential to be aware that it is impossible to obtain a unique distribution function from a technique, such as DLS, which does not involve physical size separation, and that it is essential to test a distribution produced by deconvoluting scattering data to see if its features are not artifacts of the deconvolution process. Fig. 2 shows (starch molecule) size distributions of three rice starch samples in two different solution environments as deconvoluted by CONTIN and by NNLS. Although there are some similarities, different distributions are obtained for the same correlation. Nevertheless, it is apparent that some features are independent of the model: all clearly show two populations,

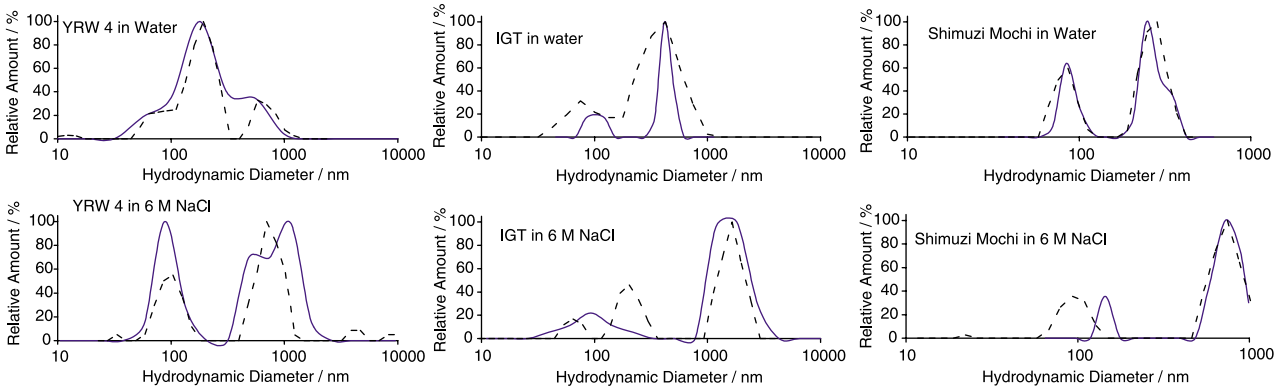

Fig. 2. Deconvoluted size distributions by two methods: CONTIN (solid line) and NNLS (broken line), for YRW 4, Shimuzi-Mochi and IGT in two different solution environments: zero salt and high salt concentration.

at about ~100 nm and ~1 μm (there is the possibility of a third population but this cannot be confirmed). These are DLS intensity distributions, not number or weight distributions, and the ratio of the areas for each population is not proportional to the ratio of the amounts of these populations.

It is likely that the larger size component comprises the so-called starch 'ghosts': portions of the gelatinised amyloplast envelope remaining after the majority of internal starch polymers have been released (Han & Hamaker, 2002). It is also apparent that much of the observed expansion with increased salt concentration can be ascribed to the larger population. This is physically reasonable: larger starch molecules will have more freedom to expand under hydration forces.

It is emphasized that these size distributions are only semi-quantitative. In order to obtain an accurate number distribution, one would need to know the refractive index (n) and measure the sample from various angles. The value of n is unknown for these starches, and moreover there will be one refractive index value for amylose and another for amylopectin, thus complicating the system. Furthermore, DLS is most sensitive to macromolecules of the largest size (Bloomfield, 2000), so the amylose and amylopectin fractions of starch may give different relative contributions to the size measurement. Obtaining a quantitative size distribution for starches therefore requires physical size separation, e.g. by hydrodynamic chromatography or one of the field flow fractionation techniques with an online DLS detector.

3.2. Separation of hot water soluble components

A simple way to separate amylose from amylopectin is on the basis of their relative solubilities in hot water (Ramesh, Ali, & Bhattacharya, 1999). Amylose is essentially linear but carries some branches (Takeda, Hizukuri, & Juliano, 1986), while amylopectin contains mainly branched clusters (Nakamura, 2002) and is of a larger molecular weight. Due to this, amylose is solubilised before the bulk of the amylopectin. While there is no guarantee that pure amylose or pure amylopectin can be obtained from this method, it can still provide some information on the relative contribution of these two components. Fig. 3 shows the comparison of the size of the unseparated original starch to those of the individual fractions. It is clear that for the waxy variety (YRW 4), there are no major differences between the three samples. For the sample containing significant amylose (IGT), it was observed that the starch suspended in the supernatant had the smallest size and starch from the pellet the largest. As DLS is biased towards larger sizes, it is not surprising that the swollen starch molecules from the unseparated sample have sizes similar to those from the pellet. No comparison of the debranched chain length distribution of the supernatant and pellet were performed, because previous studies of hot water soluble components (Mizukami, Takeda, & Hizukuri, 1999; You & Lim, 2000) show that the high molecular weight components (i.e. amylopectin) are not solubilised in hot water.

3.3. Temperature dependence

The temperature dependence of the R_h values of the separated fractions determined by DLS was obtained by

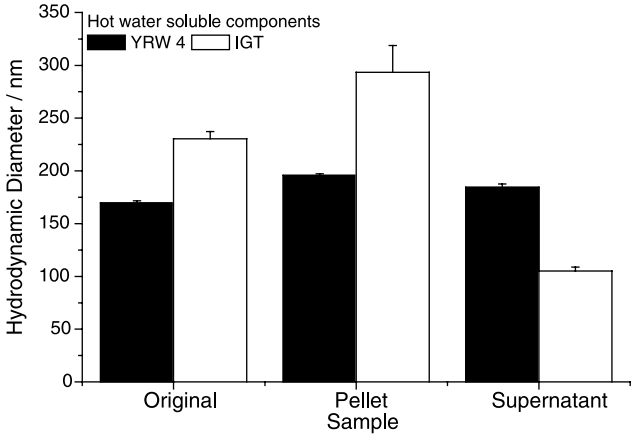

Fig. 3. Sizes of unseparated and separated hot water soluble components from waxy (YRW 4) and non-waxy (IGT) rice varieties.

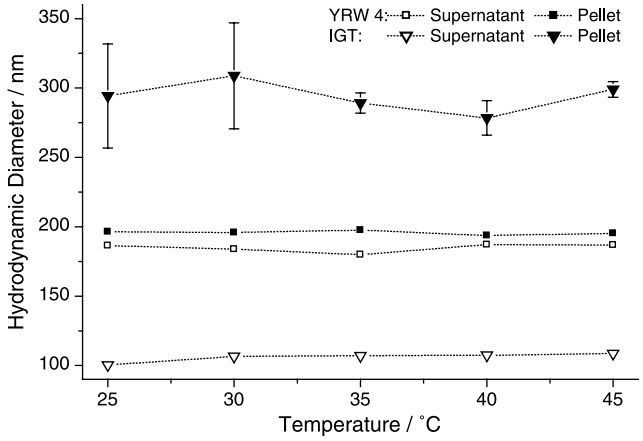

Fig. 4. Temperature effects on molecule size on separated fractions: YRW 4 supernatant, YRW 4 pellet, IGT Supernatant and IGT pellet. Error bars (shown only for IGT Pellet) are one standard deviation.

subjecting the samples to a temperature ramp from 25 to 45 °C in 5 °C increments then back to 25 °C (Fig. 4). Viscosity was corrected for the temperature change in order to maintain the accuracy of converting diffusion coefficient data (which is inferred from the DLS data) to R_h. The data show that the size of starch samples in solution had no significant temperature dependence over the range investigated.

3.4. Expansion of starches with changing salt concentrations

The hydrodynamic diameter in water of starch from all the rices examined was in the range 125–235 nm. As the salt concentration was increased, the hydrodynamic diameter for all starches increased. The system is extremely dilute (as necessitated by the technical requirements for DLS: Section 2.2) and thus it is highly unlikely that the observed changes are due to crystallisation. Fig. 5 shows the hydrodynamic sizes of the various starches against the concentration of sodium chloride. All samples were tested for stability over time and were found to be stable for at least 30 min. When kept overnight at room temperature, the samples showed different sizes as inferred from DLS; however, re-measurements after brief sonication gave a DLS diameter within the range of values obtained on the previous day. Since purified starch was used, the change in hydrodynamic diameters is due solely to the expansion or contraction of chains of amylose and amylopectin, and not attributable to a protein or lipid component.

These expansion profiles exhibit either gradual expansion in an approximately linear fashion (Tarra 140 [2002], Vialone Nano [2002], Amaroo [2002 and 2003], YRW 4 [2003] and Shimuzi-Mochi [2003]) or an initially steep expansion showing a marked inflection point between steep early expansion and then more gradual expansion at higher

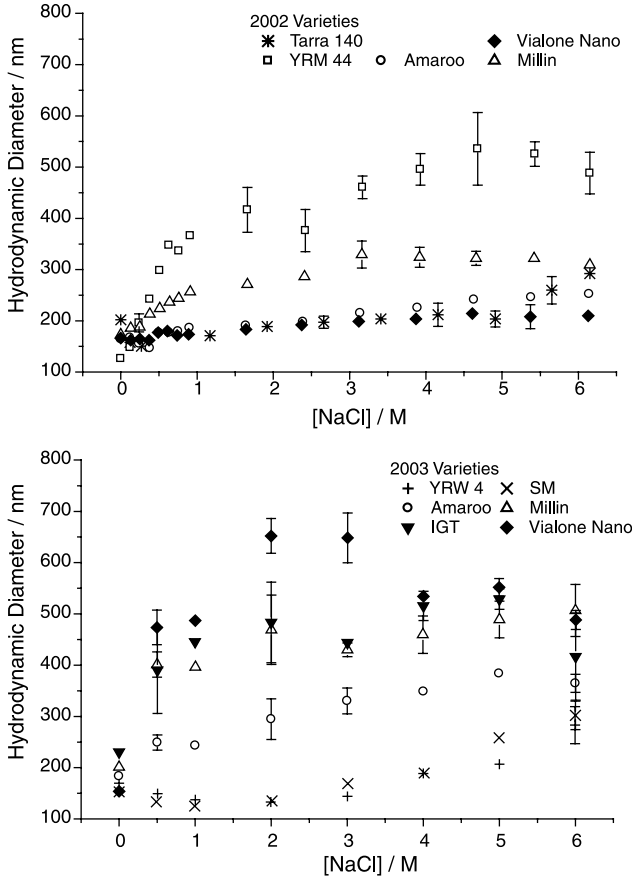

Fig. 5. Hydrodynamic diameter as a function of salt concentration of 2002 and 2003 varieties, as indicated. Error bars (shown only for some samples) are one standard deviation. 2002 varieties: * Tarra 140; □ YRM 44; ○ Amaroo; △ Millin; ◆ Vialone Nano; 2003 varieties: + YRW 4; × Shimuzi-Mochi; ○ Amaroo; △ Millin; ▼ IGT; ◆ Vialone Nano.

salt concentrations (YRM 44 [2002], Millin [2002 and 2003], Vialone Nano [2003] and IGT [2003]). The average ratios of size of the high-salt to no-salt samples for either behaviour are 1.65 and 2.63, respectively.

Artificial apparent increases in macromolecule size with salt concentration are possible if increasing salt content reduces colloidal stability and leads to coagulation. This possibility was discounted due to the stability of the DLS measurement over time, 30 min or more, at high salt concentrations and the fact that the same diameters could be recovered from the samples 24 h later after sonication, while aggregates would be expected to break up into particles of random sizes. This high degree of reproducibility was seen in all systems.

3.5. Expansion of starches with changing urea concentrations

Urea is known to break hydrogen bonds between water and hydroxyl groups (Abu-Hamdiyyah, 1965), and hence can be used to probe the possible mechanisms of starch

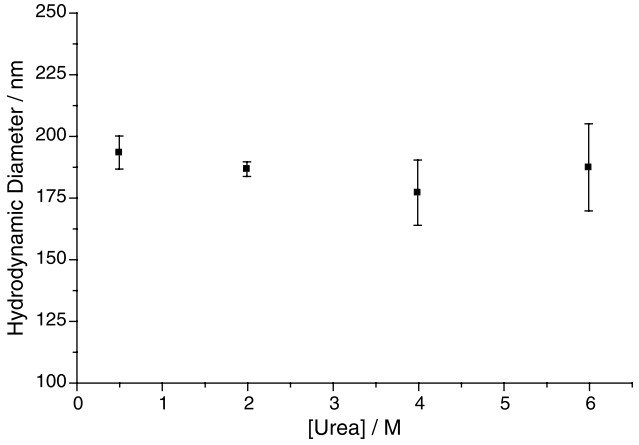

Fig. 6. Effect of urea on molecular size of Millin [2002].

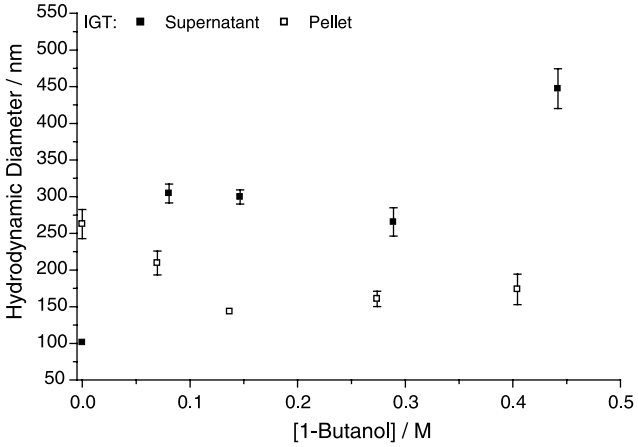

Fig. 7. Effect of 1-butanol on separated IGT. ■ supernatant; □ pellet.

expansion in solution. Urea can assist the gelatinisation and solubilisation of the starch and has been used to re-solubilise starch after fluorescent marking (O'Shea et al., 1998) or to gelatinise starches (Umemoto, Yano, Satoh, Shomura, & Nakamura, 2002). Fig. 6 shows the effect of adding urea on the hydrodynamic diameter of Millin [2002]. Millin [2002] was selected to examine the effect of urea as it was a starch which displayed a significant response to salt. The apparent size in urea, on the other hand, does not vary over the range measured and is similar to the apparent size in pure water. Although there was some variation in the profile, the magnitude of this change is not considered to be significant. Lower dilutions were not studied because attempts gave poor signal–noise ratio. It was not possible to go from low urea concentration to higher concentrations as this would require a different method for gelatinisation of the starch, introducing a new parameter.

3.6. Expansion of starches with the addition of 1-butanol

1-Butanol has been used previously to fractionate amylose from amylopectin in a solution of gelatinised, or solubilised, starch (Kamath, Bhide, & Kale, 1989; Mizukami et al., 1999; Talib, Karve, Bhide, & Kale, 1988). Amylose complexes with 1-butanol to form single-chain V-type helices (Kamath et al., 1989), which are less water-soluble. To ensure greater comparability with previous work (Kamath et al., 1989; Mizukami et al., 1999; Talib et al., 1988), hot water-soluble and insoluble starches were investigated separately. 1-Butanol content was limited to a maximum of 3.0% so that amylose would remain in solution, rather than precipitating. Fig. 7 shows the effect of 1-butanol on the molecule size of IGT supernatant and pellet. It was observed that the supernatant starch fraction started with a low hydrodynamic diameter and then expanded, while the starch fraction from the pellet initially contracted and then remained approximately the same size.

The way that these starches can expand in various solutions will depend on the starch architecture. To understand the building blocks of the amylopectin molecule, the number of chains of each particular length, the chain length distribution (CLD) is required. Rice starch contains no charged groups (compared to potato starch which contain phosphate groups), and so the changes with salt concentration are entirely a result of interaction with the water molecules.

3.7. Chain length distributions

The CLD of debranched starches describes the population of the linear components of the starch molecule once all α-1,6′ glycosidic bonds have been hydrolysed. Debranched starch chains were fluorescently marked and separated by capillary electrophoresis (CE) allowing resolution of individual degrees of polymerisation (DPs) (O'Shea et al., 1998). Absolute molecular weights are obtained because each individual molecular weight peak is resolved, and the electropherogram can be calibrated with a standard known degree of polymerisation (the hexamer of glucose in the present case). A typical electropherogram is shown in Fig. 8. The areas beneath each peak were

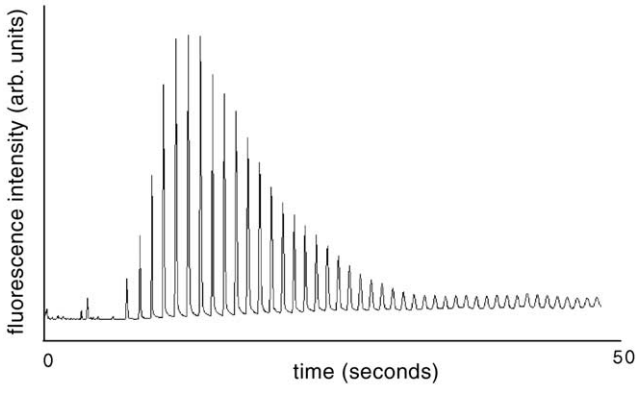

Fig. 8. Capillary electropherogram of Tarra 140.

calculated by the standard method of scaling the area with separation length and elution time (Demorest & Dubrow, 1991) to yield the chain length distribution. Size exclusion chromatography of low molecular weight starch chains gives more ambiguous information than CE due to band broadening, and moreover determining absolute molecular weights by small-angle laser light scattering detection is difficult for the lowest degrees of polymerisation of chains in the present study.

The CLDs of debranched starch are sometimes interpreted by plotting the differences in the distributions. However, such a data treatment must be treated cautiously, because it is sensitive to an arbitrary normalisation, and comparison between different data sets requires appropriate normalisation. Molecular weight distribution data can be normalised in any desired way, e.g. to unit area either under the number distribution or under the weight distribution, and one must ensure that any data interpretation is independent of the arbitrarily-chosen normalisation. As an illustration of how such a procedure can produce artifactual results, suppose one were to look at the relative amounts of short chains, degrees of polymerisation (DP) less than (say) 15, to all longer chains. The effectiveness of the fluorescent marking used in CE decreases with longer chain lengths (O'Shea et al., 1998), and hence the CE distribution for the higher molecular weight range becomes less reliable, and the relative amounts of chains of DP > 10 may be strongly sensitive to higher degrees of polymerisation that are not detected. One could also try to normalise over a fixed range of DP (say, between 6 and 25) and then subtract the results CLDs, but any conclusions so drawn are dependent on the range chosen for normalisation.

Recently, we have deduced a method for rigorously quantifying this distinction which is independent of any arbitrary normalisation (Castro, Dumas, Chiou, Fitzgerald, & Gilbert, submitted). In brief, this method uses physically reasonable mechanistic hypotheses to show that a plot of the logarithm of the number CLD (as obtained from CE) as a function of degree of polymerisation is likely to show linear behaviour over various regions. Specifically, if it is assumed that the events which lead to the CLD of debranched chains over some range of DP are only random growth and stoppage, then the number chain length distribution, or the number of chains $P(N)$ of DP N, is given by a particular case of the Schulz-Flory distribution (Clay & Gilbert, 1995; Lichti, Gilbert, & Napper, 1980; Whang, Ballard, Napper, & Gilbert, 1991)

$$P(N) = \exp\left(-\frac{k_{\text{stop}}}{k_{\text{grow}}}N\right) \quad (1)$$

where k_{stop} and k_{grow} are the rates of growth and stoppage. Because it is expected that some regions would indeed obey such kinetics in the enzymatic processes leading to starch synthesis, Eq. (1) suggests that one could plot CLDs as ln(number distribution), i.e. ln $P(N)$ as a function of N. This

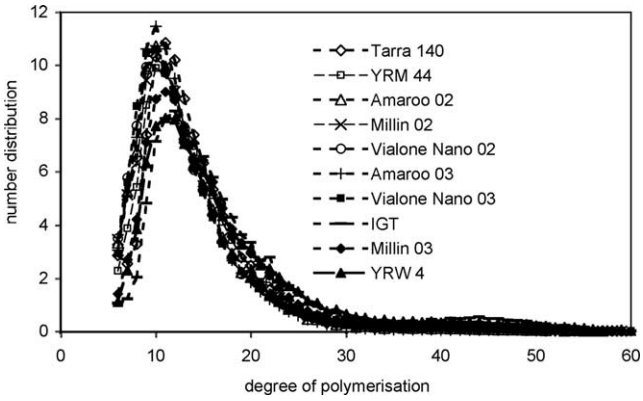

Fig. 9. Number distributions $P(N)$ (concentrations of chains of DP N) obtained by capillary electrophoresis for rice varieties as indicated.

provides a mechanistic basis for data presentation. When such plots are performed, it is found that two distinct linear regions are observed in all cereal grains to which this analysis has been applied (Castro et al., submitted): rice, maize, wheat and barley.

Fig. 9 shows the number distributions $P(N)$ as obtained by CED for all rice varieties used in the present study, and Fig. 10 shows these same data presented as ln P plots. The debranched CLD can be divided into four regions, denoted Regions 1–4, and these can be characterised by seven metrics: four slopes and three boundary values. These are: the maximum slopes of Regions 1 and 3 (s_1 and s_3, respectively), the slopes of Regions 2 and 4 (s_2 and s_4,

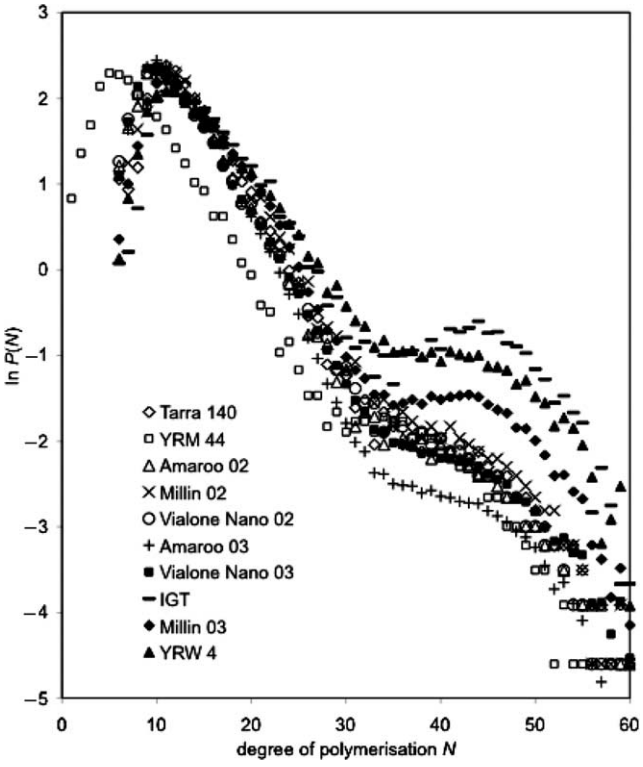

Fig. 10. Data of Fig. 9 replotted as ln P.

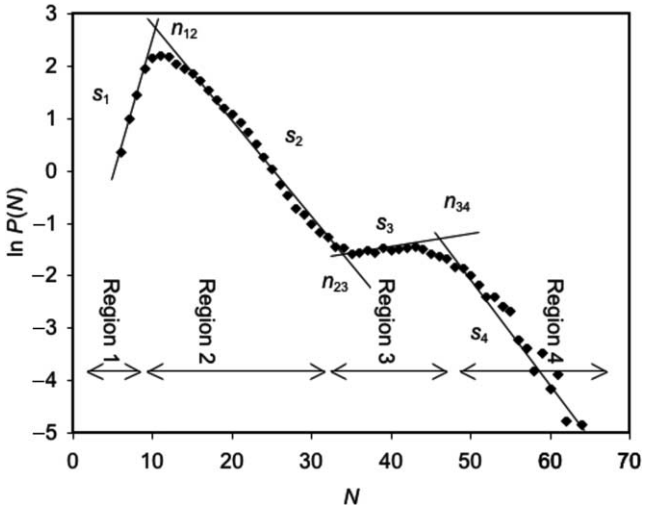

Fig. 11. The seven slope and difference metrics characterizing the ln P plot of debranched starch (the particular data are for Millin 2003).

respectively), and the degrees of polymerisation at the intersections of the tangents of neighboring Regions, $n_{1,2}$, $n_{2,3}$ and $n_{3,4}$. These seven metrics are illustrated in Fig. 11; they define the basic shape of the ln P plot. They are all independent of the normalisation of $P(N)$, i.e. multiplying $P(N)$ by an arbitrary constant does not change the value of any of these metrics. The values of the seven metrics for each variety are shown in Table 1.

This normalisation-independent representation of the MWD of debranched starch will now be used to compare differences in debranched CLDs and DLS expansion behaviour of different starches. For this purpose, the ratios of total numbers of 'short' and 'long' chains can be compared, independent of normalisation, as follows. Consider the numbers of chains between the lowest DP (6 in this case) and a DP chosen to define the boundary between 'short' and 'long' chains; this is taken here to be in Region 2. For notational simplicity, the zero-N intercept of the straight line to which the logarithm of the CLD in Region 1 is taken to be zero; this is equivalent to taking a particular normalisation, and the final result, being a ratio, will be independent of this normalisation. The straight lines describing the debranched CLD in each region are then given by:

$$\begin{aligned}
&\ln P = s_1 N && \text{for Region 1 : } N < n_{12}\\
&\ln P = s_2 N + b_2,\ b_2 = n_{12}(s_1 - s_2) && \text{for Region 2 : } n_{12} \le N < n_{23}\\
&\ln P = s_3 N + b_3,\ b_3 = b_2 + n_{23}(s_2 - s_3) && \text{for Region 3 : } n_{23} \le N < n_{34}\\
&\ln P = s_4 N + b_4,\ b_4 = b_3 + n_{34}(s_3 - s_4) && \text{for Region 4 : } N \ge n_{34}
\end{aligned} \tag{2}$$

C_{ac}, defined as the relative total number of chains between DPs a and c in a particular region, is then given by

$$\begin{aligned}
C_{ac} &= \int_a^c P(N)\mathrm{d}N\\
&= \frac{1}{s_i}(\mathrm{e}^{b_i + s_i c} - \mathrm{e}^{b_i + s_i a}), \quad \text{where in this Region ln } P = s_i N + b_i
\end{aligned} \tag{3}$$

The ratio of two areas, each calculated using Eq. (3), will then be independent of the normalisation of $P(N)$.

3.8. Effect of distributions on expansion

The full details of the expansion behaviour are governed by the complete multidimensional distribution of molecular weight of branches and the distribution of branching position. While this multidimensional description of the architecture is unknown, one may expect that varieties with short chain branches would expand less than longer chains, as the short chains are not able to occupy a large volume. For the varieties exhibiting the initially steep expansion, it is possible that the relatively unhindered stretching of segments of the long chains occurs at lower salt concentrations than expansion of the shorter chains. Fig. 12 examines this hypothesis by comparing the area ratios of chains using Eq. (3), and shows the ratio of the total number of chains with DP$\le$25 to the total number of chains with

Table 1
Metrics of the debranched CLDs for each variety, obtained from the ln P data of Fig. 10

	IGT	Tarra 140	Millin 2003	Amaroo 2002	Vialone Nano 2003	Vialone Nano 02	YRM 44	Amaroo 2003	Millin 2002	YRW4	Shimuzi
s_1	0.61	0.53	0.53	0.35	0.53	0.34	0.43	0.41	0.32	0.57	0.42
s_2	−0.16	−0.20	−0.18	−0.20	−0.19	−0.19	−0.19	−0.22	−0.19	−0.14	−0.19
s_3	0.03	−0.04	−0.01	−0.05	−0.05	−0.06	−0.02	−0.05	−0.04	−0.03	−0.1
s_4	−0.15	−0.16	−0.17	−0.12	−0.15	−0.19	−0.18	−0.25	−0.12	−0.15	−0.1
n_{12}	10.7	10.5	10.3	10.0	9.1	9.7	10.2	10.0	10.1	10.0	9.7
n_{23}	33.5	32.3	33.0	31.9	32.8	32.1	33.2	32.8	31.8	33.4	33.1
n_{34}	46.1	46.8	47.3	43.5	48.8	46.5	46.0	50.2	44.1	48.1	47.4

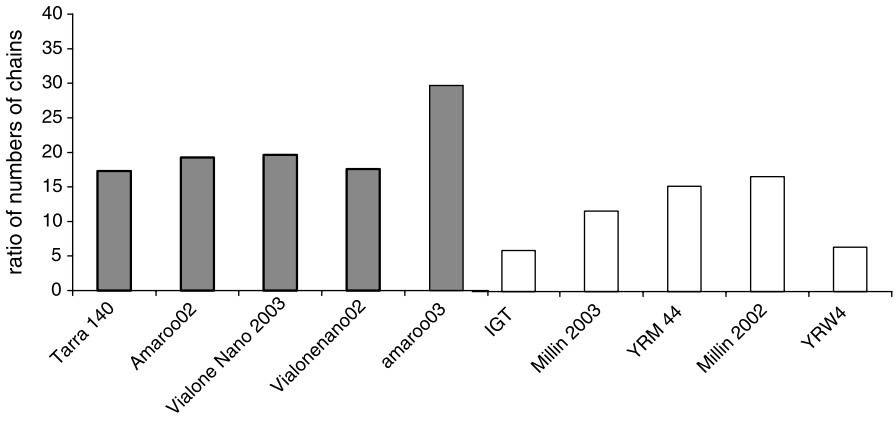

Fig. 12. Ratios of total number of chains with DP≤25 to those with DP>25, calculated using the seven metrics (values in Table 1) and Eq. (3). Grey boxes: starches showing gradual increase in size with increasing [NaCl]; white boxes: starches showing initially steep then gradual increase in size with increasing [NaCl].

DP>25. It is apparent that there is a trend for the starches which show the initial steep size increase with increasing [NaCl] to have a larger number of small chains, consistent with the hypothesis just given. Similar dependences to those seen in Fig. 12, where the short/long boundary was chosen as $N=25$, are seen if this boundary is chosen anywhere in Region 2. However, it is plain that this dependence is only a trend: having a larger number of small chains is by no means the sole criterion for an initially steep expansion.

The inference is therefore an important one: that the expansion behaviour is governed by both the distribution of branch lengths and in some way by the connectivity of these branches.

3.9. Mechanism of starch conformation change in relation to structure

The possible thermodynamic origins of chain expansion observed in this work are either the effect of water structure or the effect of the hydrophilic domains on the starch competing for solvating water. It has been shown that starch gelatinisation in salt solutions can be attributed to structure-making/breaking effects and electrostatic interaction between salts and hydroxyl groups of the starch (Jane, 1993). With salt assisting in forming the structure of water and interactions with the hydroxyl groups, it is reasonable to assume that increasing salt concentrations enhance water–starch interactions and lead to chain expansion. The water structure formed may disrupt the starch configuration, forcing the chains apart so as not to disturb the water structure.

For those starches exhibiting an initially steep expansion with [NaCl], information on possible mechanisms can be obtained an examination of the correlation coefficients between the values of the initial slope, given in Table 2 and the various metrics, including the fractions of shorter chains obtained as above. The values of the DP defining Regions 1, 2 and 3 are not taken into account in seeking such correlations, because the differences in a given n_{ij} for different starches in the species studied here are negligible (see Table 1).

Table 2 shows that the only possibly significant correlation is with the slope of Region 1, i.e. with the variation in the smallest chains. Indeed, these chains are so short that the possibility of significant expansion is limited, and thus this apparent correlation may or may not be physically meaningful. There is a possible correlation with the DP defining the change between Regions 3 and 4, but there is no corresponding significant correlation with the total fraction of chains in Region 4 (this correlation coefficient is not given in Table 2). What is important is the general *lack* of correlation with the debranched CLDs.

Table 2
Correlation coefficient between the slope of the initial steep expansion with [NaCl] from DLS, for starches where this is observed, and various metrics of the debranched CLD, and also with the fraction of 'short' chains, as in Fig. 12

	IGT	Millin 2003	Vialone Nano 2003	YRM 44	Millin 2002	Correlation coefficient
Slope of initial expansion in DLS	293	186	310	274	87	
s_1	0.61	0.53	0.53	0.43	0.32	0.73
s_2	−0.16	−0.18	−0.19	−0.19	−0.19	0.33
s_3	0.03	−0.01	−0.05	−0.02	−0.04	0.32
s_4	−0.15	−0.17	−0.15	−0.18	−0.12	−0.51
n_{34}	46.1	47.3	48.8	46.0	44.1	0.68
Ratio of smaller chains	5.9	11.5	19.6	15.1	16.5	−0.13

This strongly suggests that *connectivity between branches* is a major effect controlling the expansion behaviour with added salt, in accord with the inferences from the area ratios given in the preceding section.

Breaking hydrogen bonds with urea should break water structure, thus producing a smaller molecule if the structure-making effects of salt are responsible for chain expansion. However, at the urea concentrations measured, there was no significant change in size of starch molecules. This supports the inference that interaction between starch and water, rather than changes in water structure, are the primary mechanism for starch expansion in this system.

The addition of 1-butanol creates amylose complexes (Kamath et al., 1989; Mizukami et al., 1999; Talib et al., 1988), where the hydrophobic chain interacts with the hydrophobic moiety of the alcohol, taking the place of lipids. It is reasonable to assume that such complexes can form with any relatively long straight chain segment capable of forming V-type helices. As amylose complexes with 1-butanol, it should create a helical structure, thus stretching out the chain. Since the supernatant sample expands with 1-butanol (Fig. 7), the supernatant must comprise largely amylose, with the pellet being largely amylopectin. As amylopectin has more short chains, they do not complex well with 1-butanol, and hence less expansion is observed. The expansion profile of the amylopectin contracts slightly before expanding, mimicking the profiles of waxy samples with salt.

It seems that the mechanism of expansion is primarily due to the hydrophilic and hydrophobic components of the starch molecules rearranging to have a favourable interaction with the solvent and generating single-chain helical domains, rather than via water structure forming and breaking. Water structure formation may be enhanced or disturbed by the additives in solution but water can also interact with the hydroxyl groups present on the starch, rearranging to accommodate the larger starch molecule. Effects due to water structure appear to play a relatively small role; however, it would be worthwhile to investigate such interactions over a wider range of starches and structure forming/breaking additives. As stated, the debranched CLD is only one of the structural characteristics which control the expansion behaviour, and it is apparent that connectivity between branches is in general a more important structural feature for this effect.

4. Conclusions

The use of DLS with changing solvent conditions, to alter ionic strength and water structure, provides a useful tool for probing starch microstructure. When the results of DLS expansion studies are coupled with accurate chain length distributions of the debranched starches, the segregation of the expansion profiles can be partially explained in terms of the relative numbers of short chains. In continuous expansion, short chains regularly expand as salt concentration is slowly increased, while in initially steep expansion, longer chains can expand and interact at a reduced salt concentration compared with shorter chains.

The microstructure of starch is very complex, with measures such as the CLD of branches and branching fraction providing only a very partial picture of this complexity. There is only some correlation between the expansion behaviour and the CLD of debranched starch, and it must therefore be the connectivity between branches which is a major controlling factor in this behaviour. The dependence of average size and size distribution on concentration of the three types of solute observed here thus provides an additional measure of the extremely complex microstructure of starches. A question of considerable interest for future investigation is whether or not this information is complementary to that provided by the temperature and frequency dependence of the viscosity of starch solutions.

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