Damaged starch characterisation by ultracentrifugation

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Abstract—The relative molecular size distributions of a selection of starches (waxy maize, pea and maize) that had received differing amounts of damage from ball milling (as quantified by susceptibility to α-amylase) were compared using analytical ultracentrifugation. Starch samples were solubilised in 90% dimethyl sulfoxide, and relative size distributions were determined in terms of the apparent distribution of sedimentation coefficients $g^*(s)$ versus $s_{20,w}$. For comparison purposes, the sedimentation coefficients were normalised to standard conditions of density and viscosity of water at 20 °C, and measurements were made with a standard starch loading concentration of 8 mg/mL. The modal molecular size of the native unmilled α-glucans were found to be ∼50S, 51S and 79S for the waxy maize, pea and maize amylopectin molecules, respectively, whilst the pea and maize amylose modal molecular sizes were ∼14S and ∼12S, respectively. As the amount of damaged starch increased, the amylopectin molecules were eventually fragmented, and several components appeared, with the smallest fractions approaching the sedimentation coefficient values of amylose. For the waxy maize starch, the 50S material (amylopectin) was gradually converted to 14S, and the degradation process included the appearance of 24S material. For the pea starch, the situation was more complicated than the waxy maize due to the presence of amylose. As the amylopectin molecules (51S) were depolymerised by damage within this starch, low-molecular-weight fragments added to the proportion of the amylose fraction (14S)—although tending towards the high-molecular-weight region of this fraction. As normal maize starch was progressively damaged, a greater number of fragments appeared to be generated compared to the other two starches. Here, the 79S amylopectin peak (native starch) was gradually converted into 61 and 46S material and eventually to 11S material with a molecular size comparable to amylose. Amylose did not appear to be degraded, implying that all the damage was focused on the amylopectin fraction in all three cases. Specific differences in the damage profiles for the pea and maize starches may reflect the effect of lipid-complexed amylose in the maize starch.
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1. Introduction

An understanding of how the damaged starch content of flours affects functionality has been a very important requirement of the baking industry for many decades. The damaged starch fraction promotes both water absorption and endogenous β-amylase hydrolysis, which generates maltose. For traditional baking processes the maltose is consequently used by yeast to ferment to carbon dioxide (and cause loaves to rise). In view of the importance of damaged starch to the baking industry, many methods have been developed to quantify its presence. These have been reviewed and discussed in detail elsewhere.[1–16] Susceptibility to α-amylase hydrolysis is a convenient way to determine damaged starch. Unlike native starch granules, which are semicrystalline, insoluble and as a consequence inaccessible to α-amylase, damaged starch is amorphous, soluble and readily hydrolysed. Quantification of damaged starch by α-amylase hydrolysis is very convenient, although microscopy is required to understand damage at the level of the whole granule. At the other length scale, chromatographic procedures are required to understand the molecular basis of starch modification.[10,17–27] Due to

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its susceptibility to α-amylase, damaged (or amorphous) starch is much more readily digested in the gut than native starch.[28]

Using high-performance liquid chromatography (HPLC) or other chromatographic procedures (as mentioned above), it is possible to determine the molecular size distribution of native starch amylose and amylopectin molecules and to profile their products of hydrolysis. Due to inherent difficulties with molecular 'cut-offs', however, it is difficult to make a profile of starch before and after damage using a single technique (or with a single chromatographic column). However, using analytical ultracentrifugation with modern instrumentation, data capture and analysis procedures, this becomes a practical proposition.[29–31] Whilst this analytical technique cannot replace rapid laboratory assessments of damaged starch in flour processing factories, it does have the potential to complement HPLC-type approaches in terms of molecular characterisation of native and damaged starches. Furthermore, no separation media or membranes (as required by field-flow fractionation-based separation methods), with necessary assumptions concerning inertness, are required.

Preparative ultracentrifugation has in the past been used to separate amylose and amylopectin from within dissolved starch,[32] although recent methodology has focused on rate-zonal methods.[33] Analytical ultracentrifugation, and in particular the sedimentation velocity technique, offers the possibility to quantify the amylose and amylopectin components in terms of their relative amounts and molecular sizes (in terms of the sedimentation coefficient) without prior fractionation.[32] Early studies have used the Schlieren optical system where estimates for the amylose/amylopectin ratio were made by assessing the areas under the Schlieren (refractive index gradient) boundaries assigned for amylose (slower sedimenting) and amylopectin (faster sedimenting) components. The appearance of a new generation of analytical instrument, the Beckman XL-I in 1996[34] with full on-line data capture and concomitant advances in analysis software, offers the possibility of estimating molecular size distributions in terms of the sedimentation coefficient distribution.[35]

Although the sedimentation coefficient is not an absolute marker for size (it depends also on conformation), it is still very useful when absolute molecular weight estimates are not possible. Good examples are for the seed globulins and ribosomes. Sedimentation coefficients can be converted into estimates for molecular weights if either assumptions concerning shape or other information, such as the translational diffusion coefficient, is available. Using this approach, Lelievre et al.,[36] for example, reported that according to sedimentation coefficient data, the weight-average molecular weight of wheat amylopectin dissolved in dimethyl sulfoxide (DMSO) was $\sim 10^7$ g mol^{-1} irrespective of source. The

same group characterised β-limit dextrin using the same approach.[37] Millard et al.[38] dissolved waxy maize starch in 90% DMSO and estimated the weight-average molecular weight for amylopectin, which they reported as 593×10^6. Fronimos[39] reported a molecular weight for waxy-amylopectin in 90% DMSO of $\sim 50 \times 10^6$, which is more in common with Lelievre et al.[36] data. In terms of representing the molecular integrity of starches, and in particular the changes caused by processing or other sources of damage, it is perhaps more convenient to represent sizes in terms of the sedimentation coefficient distribution.

The objective of this particular study was to utilise the analytical ultracentrifuge to characterise and quantify the molecular composition of starches from different sources that had been physically damaged, to different extents, by ball milling. These starches have been characterised previously by a range of chemical and physical methodologies (where the amount of damaged starch was determined by α-amylase hydrolysis).[23] The data generated from this ultracentrifuge study provide the basis of comparison with these earlier physicochemical data and a potential tool to understand more fully the effects of mechanical damage on starch. These data also provide a potentially novel insight into the effects of attrition on starch structure and properties.

2. Materials and methods

2.1. Materials

The starches utilised for this study were waxy maize, pea and maize that had been subjected to different amounts of damage by ball milling using a Pascall mill (Crawley, Sussex) fitted with porcelain pots (10 cm diameter by 15 cm height) each containing a large number of porcelain balls of varying diameter (16×19 mm, 36×13 mm and 86×10 mm). These have been described in detail elsewhere,[23] although some compositional data are presented in Table 1.

2.2. Preparation of starch solutions

Starch samples (ca. 80 mg) were weighed into 10-mL screw-capped tubes and solubilised in 10 mL of 90% (v/v) DMSO.[40] The concentration of dissolved starches were determined according to Dreywood,[41] and then solutions were adjusted to 8.0 mg mL^{-1}.

2.3. Sedimentation velocity using the analytical ultracentrifuge

Sedimentation velocity experiments were performed using a Beckman Instruments (Palo Alto, CA) Optima XL-I analytical ultracentrifuge. Dissolved starch samples

Table 1. Composition of starches utilised in this study[23]

Starch/milling time (h)	AP[a] (%)	FAM[b] (%)	LAM[c] (%)	Damage[d] (%)	AP[a,e] (%)	Low MWt AP[e,f] (%)	SAP[h] SAM[i] (modal)	
Waxy maize								
0	99.2	0.8	0	2.2	100	nd[g]	50	—
1				23.8		nd[g]		
3				54.8		nd[g]		
8				85.3		47		
48				96.7		Amorphous		
Pea								
0	63.8	36.2	0	0.2	83	nd[g]	51	14
1				13.0		5		
3				34.0		4		
8				71.7		3		
48				94.2		Amorphous		
Maize								
0	73.8	21.7	4.5	0.7	84	nd[g]	79	12
1				14.9		46		
3				36.9		43		
8				68.1		>50		
48				91.2		Amorphous		

[a] Amylopectin fraction of total α-glucan from iodine binding.
[b] Free amylase.
[c] Lipid-complexed amylase.
[d] Determined according to the α-amylase susceptibility method of Karkalas et al.[10]
[e] Analytical ultracentrifuge.
[f] Where amylose is subtracted from the low-molecular-weight fraction.
[g] Not discernable.
[h] Sedimentation coefficient of amylopectin.
[i] Sedimentation coefficient of amylase.

or 90% DMSO (0.38 mL) were injected into the solution or reference solvent channels, respectively, of the 12-mm optical path length cells. The cells were loaded into an 8-hole titanium rotor and placed into the centrifuge. Samples were centrifuged at 25,000 rpm at a temperature of 20.0 °C. Concentration profiles in the ultracentrifuge cell were registered using the Rayleigh interference optical system. Standard Fourier transform software was used to convert the fringe profiles into plots of fringe displacement, 'j' (relative to the meniscus) versus radial position, 'r'.[42] Data were analysed using the ls-$g^*(s)$ method with SEDFIT[43] and a curve-fitting module 'Multi Gaussian' applying the algorithm PROFIT (Quantum Soft, Switzerland).

The software used employed a change in the concentration distribution in the ultracentrifuge cell as a function of radial position and time to generate an apparent distribution of sedimentation coefficients. The method is based on numerical solution of the classical Lamm[44] equation for sedimentation and diffusive transport, a solution rendered possible with the on-line data capture facility of the new-generation analytical ultracentrifuge. The methodology was developed by Schuck[43] and Dam and Schuck,[45] and its adaptation to the study of polysaccharides was recently considered by Harding.[42] The form of the distribution was presented in terms of $g^*(s)$ versus $s_{20,w}$. For comparison purposes, the sedimentation coefficients were normalised to standard conditions of density and viscosity of water at 20 °C in the conventional way[46] to yield $s_{20,w}$. Usually, corrections are made for non-ideality effects by measuring $s_{20,w}$ at a variety of concentrations and extrapolating to zero concentration. For comparative studies like these, this is impractical, and hence all measurements were performed at a standard starch loading concentration of 8 mg/mL.

The difference between $s_{20,w}$ measured at a finite concentration and the zero-concentration value can be significant.[42] The star in $g^*(s)$ indicates that it is an *apparent* distribution meaning the sedimentation coefficients have not been corrected for non-ideality, and diffusion effects have not been taken into account.[47] However, since starch polysaccharides are very slow to diffuse, this contribution is likely to be small.

3. Results

3.1. General considerations

Sedimentation coefficient $g^*(s)$ versus $s_{20,w}$ distribution plots for starch samples exposed to varying degrees of ball-milling are shown in Figures 1–3 for waxy maize, pea and maize starches, respectively. In these plots, the
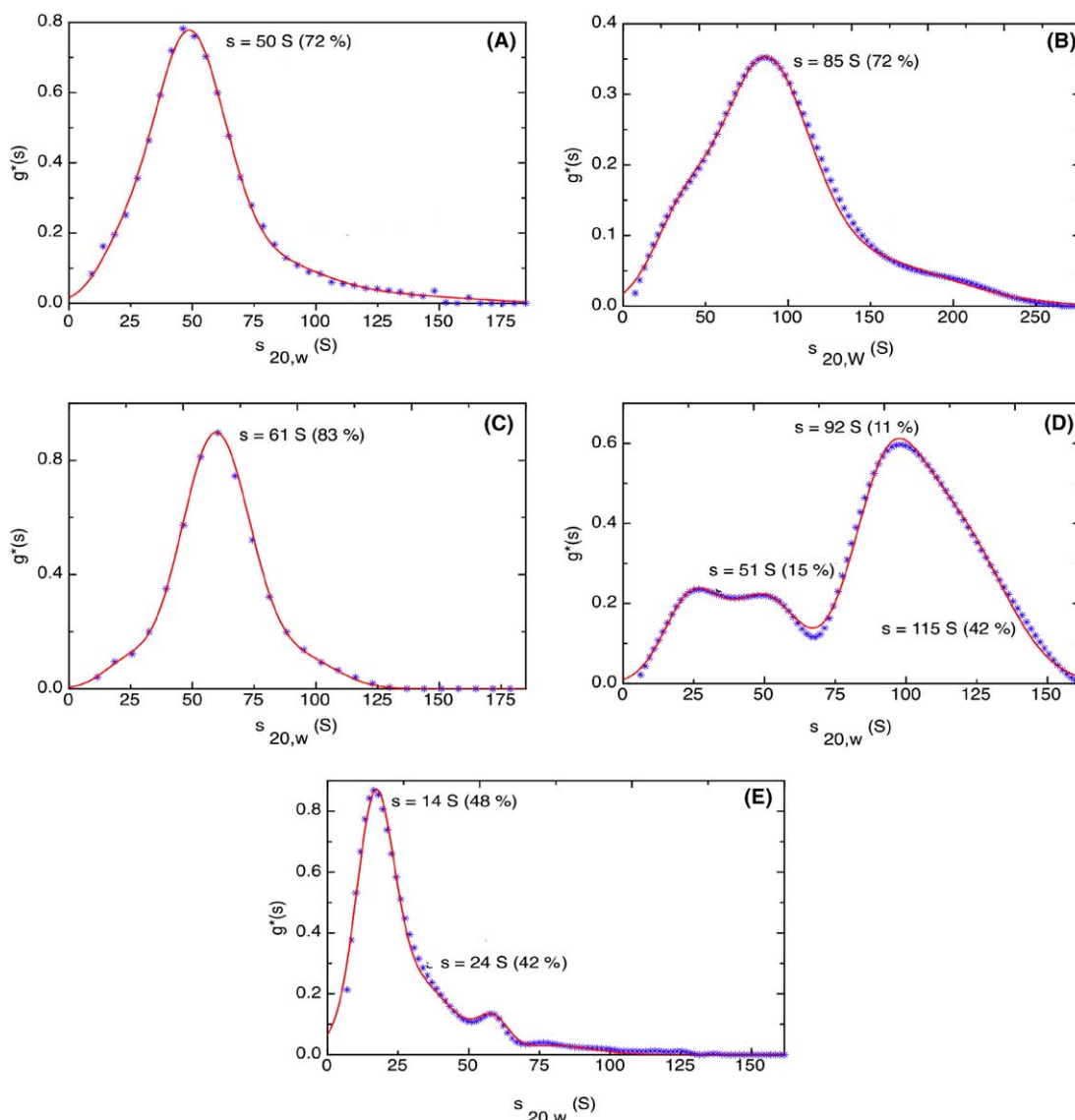
Figure 1. Sedimentation coefficient distribution $g^*(s)$ versus $s_{20,w}$ profiles for waxy maize starch: (A) 2.2% damage to crystalline starch, (B) 23.8% damage, (C) 54.8% damage, (D) 85.3% damage and (E) 96.7% damage.

distribution data have been subjected to multi-Gaussian, non-linear least squares analysis to identify essential features, which are reported as the main sedimenting species and their approximate relative weight concentration as a percentage of the total amount of sedimenting material.

3.2. Waxy maize starches

For the waxy maize starches, as the amount of damaged starch increased from the native starch content of 2.2% (enzymatic method, Table 1), a reduction in the sedimentation co-efficient of amylopectin was apparent from the ultracentrifuge distribution (Fig. 1). Interestingly, at least to a content of 54.8% starch damage (enzymatic assay), the ultracentrifuge detected reason-

ably unimodal distributions (70–80% of the total material) with the indication of the presence of a second component at the leading shoulder of the Gaussian peak and the appearance of a shoulder on the trailing (low-molecular-weight) edge for the 23.8 and 54.8% damage levels. The sedimentation coefficient of the main amylopectin component was 50S, 85S and 61S for 2.2% (Fig. 1a) 23.8% (Fig. 1b) and 54.8% (Fig. 1c) damage, respectively. Increasing the damage to 85.3% yielded a rather different $g^*(s)$ distribution where it can be seen from Figure 1d that there were four principal components present. These represented a peak at 51S (~15% of the total), 24S (12%, not tagged in Fig. 1d), 92S (11%) and 115S (42%). When the starch became almost amorphous, (96.7% damage level, Fig. 1e), the sedimentation coefficient was found to have dropped considerably
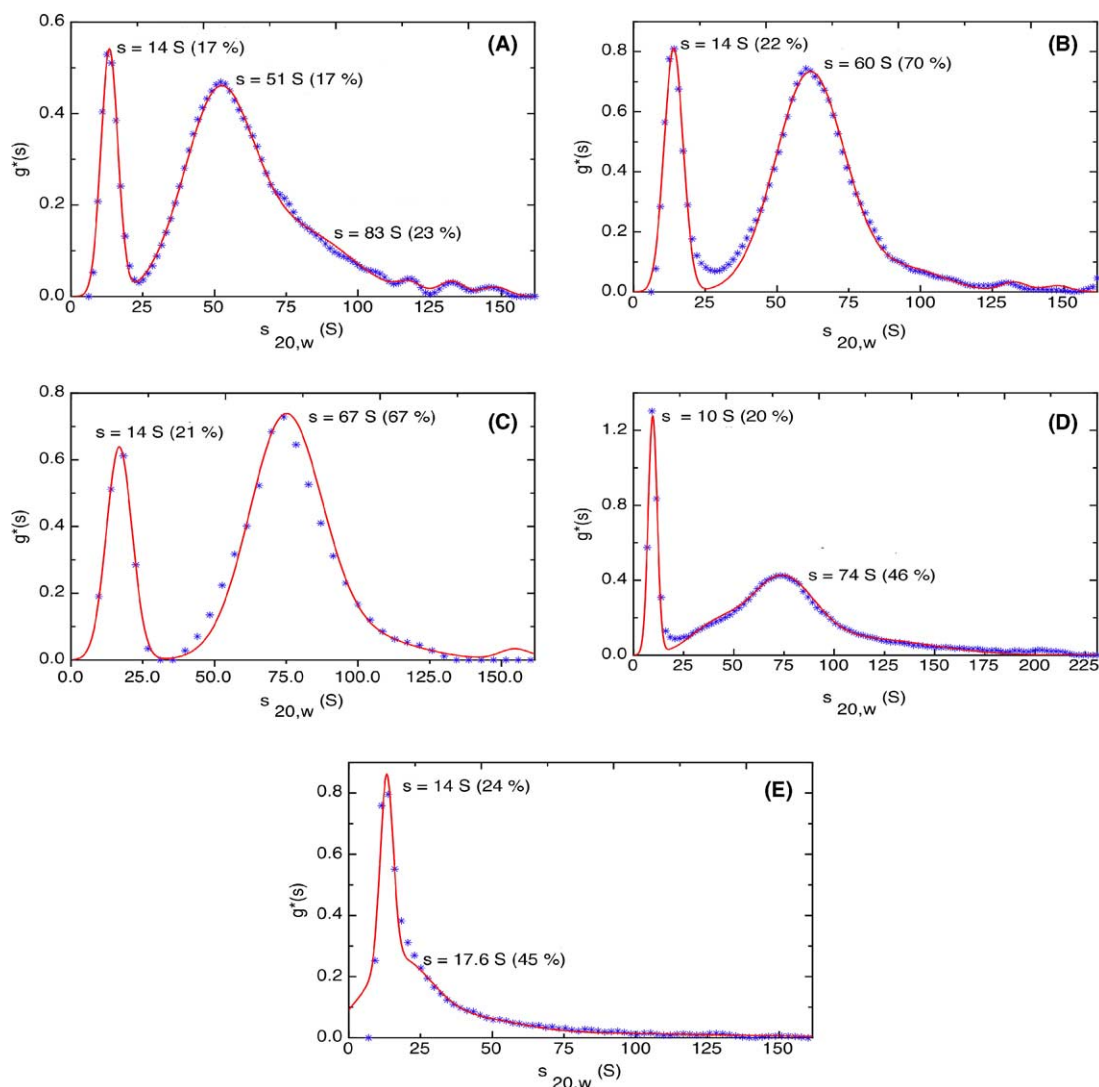
Figure 2. Sedimentation coefficient distribution $g^*(s)$ versus $s_{20,w}$ profiles for pea starch: (A) 0.2% damage to crystalline starch, (B) 13.0% damage, (C) 34.0% damage, (D) 71.7% damage and (E) 94.2% damage.

with the main component (48% of the total starch) representing 14S material, while a secondary peak remained with a higher molecular weight (24S).

From these data it is apparent that significant degradation of waxy maize amylopectin occurred at very high levels of starch damage, although, for damage below 54.8%, major degradation seemed to be absent. There was, therefore, no clear correlation between the amount of damage in the samples determined enzymatically and the distribution presented in the analytical ultracentrifuge (although the two extremes of 'native' and 'amorphous' were evident). This is not surprising perhaps, since although material accessible to α-amylase for the damaged starch enzymatic assay is amorphous and hence readily digested, its not necessarily of a particular molecular weight profile to promote hydrolysis. The α-amylase can only crudely differentiate between crystalline (arrays of double helices) and noncrystalline

α-(1→4) bonds, whilst the centrifugal method cannot discriminate between material located in amorphous and crystalline regions of native or damaged granules (as the α-glucan is all dissolved in DSMO).

3.3. Pea starches

For the pea starches investigated in this study (Fig. 2a–e and Table 1), the amylose and amylopectin fractions were discrete in the native (i.e., only 0.2% damaged material) starch, where amylose represented 17% of the α-glucan in the analytical ultracentrifuge (14S), with the remaining 83% amylopectin (51S with a higher sedimentation coefficient at the leading shoulder having an $s_{20,w}$ value of at least 83S). No intermediate peak between amylose and amylopectin was evident. The sedimentation coefficient of pea and waxy maize amylopectin molecules were similar (modal values of ~50S) at
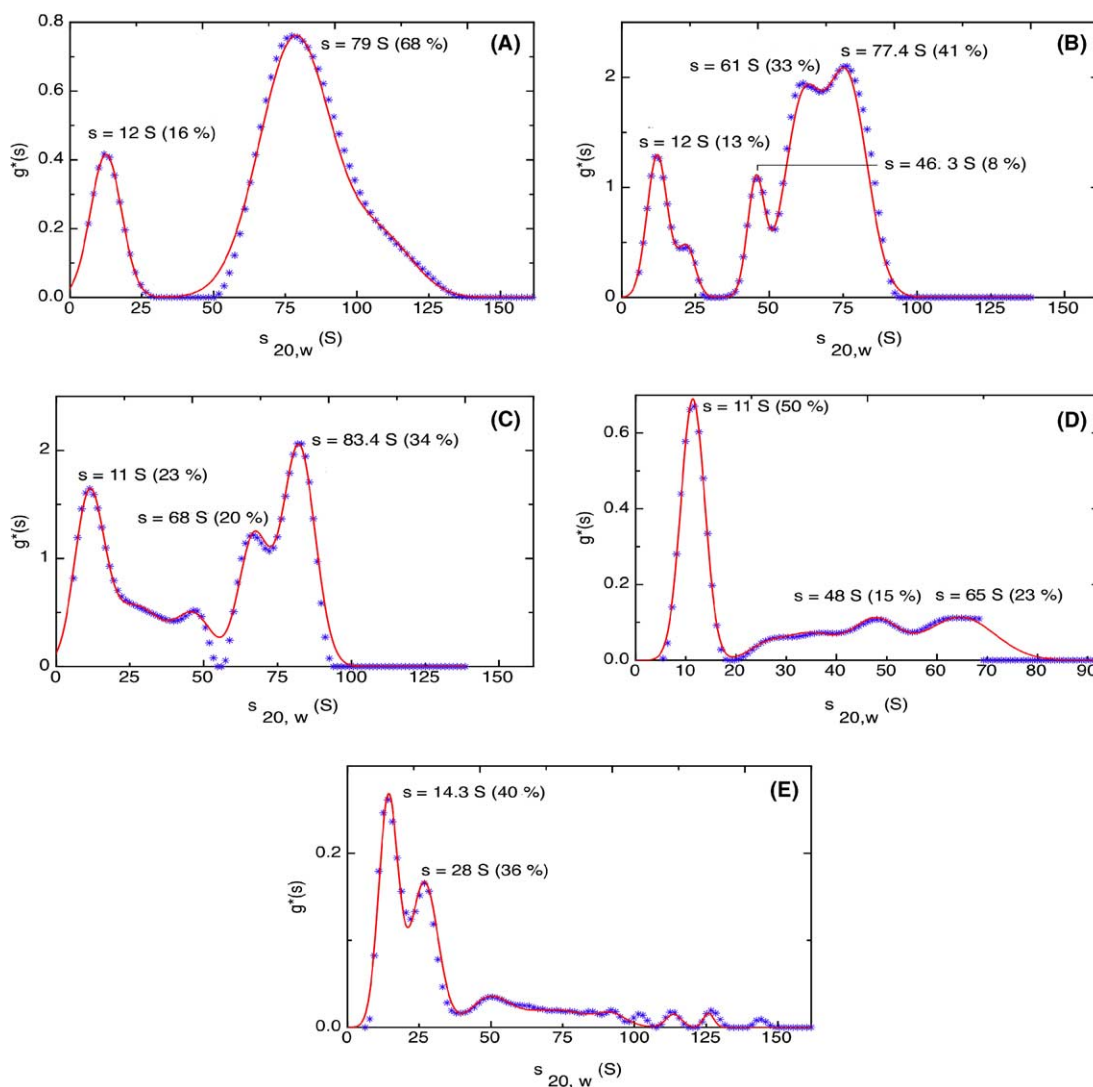
Figure 3. Sedimentation coefficient distribution $g^*(s)$ versus $s_{20,w}$ profiles for maize starch: (A) 0.7% damage to crystalline starch, (B) 14.9% damage, (C) 36.9% damage, (D) 68.1% damage and (E) 91.2% damage.

this stage. As the amount of damage increased (Fig. 2a–e), there was no significant degradation up to 34% damage, although at 71.7% damage (Fig. 2d) there was obvious depolymerisation of the amylopectin fraction, which was more marked for ∼94% damage (Fig. 2e) in common with the waxy maize. Interestingly, the amylose molecule itself (which is noncrystalline) did not seem to be modified by the damage to the crystalline starch.

In common with the waxy maize starch, for the pea starch, no clear correlation exists between the proportion of damaged material obvious in the ultracentrifuge system with the amount of material quantified by the damaged starch assay. In addition, the proportion of amylose by the analytical ultracentrifuge for the native (0.2% damage, Fig. 2a) sample represents only 17% of the total amylase, which, according to the iodine-binding method used to determine free amylose, should be higher (FAM of Table 1), representing 36.2% of the

α-glucan. This suggests that either (i) iodine-binding measures some of the amylopectin fraction as 'amylose' and overestimates it or (ii) that a discrete higher molecular weight 'amylose' fraction has a comparable molecular weight to low-molecular-weight amylopectin and sediments together in the analytical ultracentrifuge. One would expect that unbranched native amylose would show a continuous distribution of molecular weight, and so (i) appears to be the most likely explanation.

3.4. Maize starch

For the normal maize starch (Fig. 3 and Table 1), the sedimentation coefficient of the native amylopectin fraction (79S) was greater than the waxy maize (50S) or pea starches (51S). The amylose fraction had a comparable sedimentation coefficient with the pea starch (12S for

the maize starch compared to 14S for the pea). The effect of damage on this normal starch was more complex than for the pea starches (discussed above) as a number of additional peaks caused by damage were evident from the analytical ultracentrifuge. This difference is presumably because of the presence of both free (FAM) and lipid-complexed amylose (LAM), which represents 4.5% of this α-glucan (Table 1). Although, as previously mentioned, the amylose appears to resist degradation by the milling process, the presence of the amylose (FAM or LAM) in the starch granules presumably affects the fracture mechanics of the amylopectin crystallites located nearby. Overall though, in common with the other two starches, as the amount of damage increased the amylopectin molecules were gradually fragmented by the attrition, to leave primarily amylose-like material (14S) and some fragmented amylopectin (28S material).

3.5. Dimensions

The mean diameters of the waxy maize, pea and maize starch granules were 11.8, 11.1 and 21.3 μm, respectively,[23] which might suggest that the pea starch was more susceptible to damage than the maize starches because of size.[23] In general, large granules tend to be more easily damaged by attrition.[23] In fact, the pea starch was not too dissimilar to the normal maize starch in terms of susceptibility to damage. Both of the normal starches resisted damage much more than the waxy maize starch, presumably due to the presence of the amylose.[23] Hence, the effects of granule size on the tendency of starch granules to be damaged by attrition were only part of a number of physicochemical factors that were responsible for controlling this process.

4. Discussion

The properties and molecular basis of damaged semicrystalline starch material using non-centrifuge techniques have been discussed in detail elsewhere.[10,17–21] Damaged starch represents amorphous fragments of granules and solubilised material. Whilst it has been possible to separate damaged starch fractions by liquid chromatography (LC) or high-performance liquid chromatography (HLPC) columns, the resolution of columns (typically size exclusion, SEC) favours a particular weight range and limits an overview with respect to starch composition and molecular weight distribution. The analytical ultracentrifuge also has separation limits but does provide an interesting insight into how the amylopectin molecule, which is the semicrystalline component of the starch granules, is progressively depolymerised by damage.

The starch granule comprises arrays of crystalline (registered double helices of exterior chains) and amorphous laminates of amylopectin molecules. Amylose molecules (FAM or LAM) are dispersed within this matrix. Physical attrition would be expected to initially shear fracture vulnerable bonds at the 'base' of amylopectin clusters (linked to crystallites) and thus generate low-molecular-weight material.[10,17–21] Although the analytical ultracentrifugation cannot discriminate between molecules in terms of their origin in native starch granules, it does provide an overview in terms of depolymerisation and how the different stages of damage are reflected in terms of the sedimentation coefficient (molecular weight) profile. Indeed, although a little empirical, the technique could be used to fingerprint starches in terms of their extent of physical modification.

It is not surprising that amylose resists attrition damage in that it is amorphous in starch granules. It would be interesting in future work to physically modify high amylose starches where amylose crystallites may be present[48] to see if the amylose molecules are depolymerised by damage if located in crystallites. This system is, however, complicated by the partition between FAM and LAM, which will influence the pattern of damage as discussed above.

Although modern baking methods do not require long fermentation processing, damaged starch is an important aspect of flour and starch quality because of the effects on water absorption and retention. In terms of starches, damage will also modify the rheological properties and is consequently a very important parameter to quantify and control. In this regard, the analytical ultracentrifuge is not a technique that can be used for routine quality control in the baking industry, but does provide a technique that facilitates understanding of starch modification.

5. Conclusions

This study has shown that whilst different starches respond differently to damage in terms of the fragmentation profiles for amylopectin, the amylose molecules, due to their amorphous nature, appear to resist damage using an ultracentrifuge approach. In this regard, the analytical ultracentrifuge provides a unique insight into the molecular processes associated with generating starch damage. It is evident from the data of the present study that sedimentation velocity in the analytical ultracentrifuge using modern sedimentation coefficient distribution analysis can be used to fractionate amylose and amylopectin and discriminate between starches comprising differing amounts of damage (as assessed by enzymatic assay). It may also give a more representative estimate of the amylose/amylopectin ratio compared to the more traditional methods such as the iodine-binding approach.

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