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Molecular order and structure in enzyme-resistant retrograded starch

M.J. Gidley[a]*, D. Cooke[a], A.H. Darke[a], R.A. Hoffmann[a], A.L. Russell[a] & P. Greenwell[b]

[a]*Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford MK44 1LQ, UK*
[b]*Flour Milling and Baking Research Association, Chorleywood, Rickmansworth, Herts WD3 5SH, UK*

Molecular features underlying the resistance to amylolytic hydrolysis in cooked and cooled gels of wheat, amylomaize V and amylomaize VII starches have been investigated using a combination of physicochemical techniques. X-ray diffraction and ^{13}C CP/MAS NMR spectroscopy indicate levels of crystalline and double helical order to be 25–30% and 60–70%, respectively, in enzyme-resistant retrograded starches. The width of features in diffraction patterns and NMR spectra indicate smaller and/or less perfectly arranged B-type double helical aggregates than found in native potato or amylomaize VII starch. Differential scanning calorimetry in excess water shows a broad endothermic transition from below 100 to *c.* 170°C which is interpreted in terms of double helix melting. Consistent with a broad melting endotherm, (linear) chain lengths present in enzyme-resistant starches cover a range of degree of polymerisation (DP) from less than 10 to *c.* 100 as determined by high performance anion exchange chromatography (HPAEC). This dispersion of chain lengths coincides with the range expected from previous studies for double helices (minimum required DP of 10) with no major intervening amorphous regions (maximum DP ~100). HPAEC analysis also shows a periodicity in chain length for DP multiples of 6 above DP18 for all three enzyme-resistant retrograded starches. A model is proposed to account for this observation based on restricted enzyme access to potential substrates arranged in double helical aggregates. In general, enzyme-resistant retrograded starch reflects features both of aggregated/gelled amylose (high double helix content; low crystallinity, DP range from junction zones of DP 10–100), and the consequence of enzyme action on such a structure (periodicity of six units from accessibility of enzyme to aggregated substrate).

INTRODUCTION

Starches in many foodstuffs escape complete digestion by pancreatic α-amylase in the human small intestine (Englyst & Cummings, 1985, 1986; Annison & Topping, 1994). Various categories of such resistant starch have been proposed (Englyst & Cummings, 1987), of which retrograded amylose is considered to be the most resistant to digestion. In attempts to mimic *in vivo* digestion resistance, *in vitro* amylolytic digestion of retrograded starches from various sources and with a range of process histories has been studied (Berry, 1986; Ring *et al.*, 1988; Russell *et al.*, 1989; Siljeström *et al.*, 1989). The main techniques which have been used to study amylolysis-resistant retrograded starches (commonly termed enzyme-resistant starch) are size exclusion chromatography (Russell *et al.*, 1989; Berry *et al.*, 1988; Leloup *et al.*, 1992; Eerlingen *et al.*, 1993*b*), differential scanning calorimetry (Sievert & Pomeranz, 1989, 1990; Gruchala & Pomeranz, 1993; Sievert & Würsch, 1993) and X-ray diffraction (Siljeström *et al.*, 1989; Berry *et al.*, 1988; Eerlingen *et al.*, 1993*a*, 1993*b*; Cairns *et al.*, 1990; Sievert *et al.*, 1991). The combination of a B-type X-ray diffraction pattern and an endothermic event in the range 130–170°C is characteristic (Eberstein *et al.*, 1980) of retrograded amylose. Molecular analysis of enzyme-resistant retrograded starch has shown that it is composed of essentially linear (1 → 4)-α-D-glucan chains (Russell *et al.*, 1989; Siljeström *et al.*, 1989) having a range of chain lengths. Depending on source and preparation conditions, number average degrees of polymerisation (DP_n) ranging from *c.* 30 to 65 have been reported (Russell *et al.*, 1989; Berry *et al.*, 1988; Leloup *et al.*, 1992), in reasonable agreement with chain lengths from enzyme-resistant retrograded amylose (Eerlingen *et al.*, 1993*b*; Jane & Robyt, 1984). In confirmation of the validity of enzyme-resistant retrograded starch as a model for *in vivo* digestion-resistant retrograded starch, amylose of DP_n35 with a differential scanning (DSC) endotherm at calorimetry at

*Author to whom correspondence should be addressed.

150°C and a B-type X-ray diffraction pattern has been isolated as a major resistant fraction at the end of the human small intestine (Faisant *et al.*, 1993*b*), along with incompletely digested (but potentially fully digestible) oligo and polysaccharides (Faisant *et al.*, 1993*a*, 1993*b*).

Several features of enzyme-resistant retrograded starch, however, remain obscure. In particular, the logical hypothesis that resistance to amylolysis is conferred by double helical crystalline states (Jane & Robyt, 1984) is difficult to reconcile with the relatively low crystallinity of enzyme-resistant retrograded starch (Berry *et al.*, 1988; Sievert *et al.*, 1991) and amylase-treated retrograded amylose (Eerlingen *et al.*, 1993*a*, 1993*b*; Cairns *et al.*, 1990). However, X-ray diffraction only detects crystallites of aggregated double helices, whereas less (perfectly) aggregated helices may also confer resistance to enzyme hydrolysis. High resolution solid state (CP/MAS) ^{13}C-NMR is a shorter distance scale probe than X-ray diffraction, giving characteristic spectra for ordered helices and non-ordered chains (Gidley & Bociek, 1985, 1988) which can be used to estimate the double helix content of solid starch samples (Gidley & Bociek, 1985). The same technique can also be used to assess whether any lipid component is present within an amylose single helix (Morrison *et al.*, 1993*b*).

It is also unclear why a certain range of chain lengths are found in enzyme-resistant retrograded starch, if many segments are derived from non-crystalline glucan regions. Size distribution measurements of chains in enzyme-resistant retrograded starch which have been reported (Russell *et al.*, 1989; Berry *et al.*, 1988; Leloup *et al.*, 1992; Eerlingen *et al.*, 1993*b*) show limited resolution and are calibrated (approximately) by secondary standards. Increased resolution and straightforward peak assignment/calibration for linear (1 → 4)-α-D-glucans can be obtained using high performance anion exchange chromatography (HPAEC) under alkaline elution conditions (Koizumi *et al.*, 1991).

We now describe studies of enzyme-resistant material from retrograded wheat, amylomaize V and amylomaize VII starches using a range of established (DSC, X-ray diffraction) and previously unused (^{13}C CP/MAS NMR, HPAEC) techniques in order to gain a better understanding of the molecular origins of enzyme resistance in retrograded starches.

MATERIALS AND METHODS

Materials

Enzyme-resistant retrograded starches were prepared as described by Russell *et al.* (1989). The samples of enzyme-resistant material from wheat and amylomaize V were starches S and Q, respectively and enzyme-resistant material from amylomaize VII was obtained as for amylomaize V (Russell *et al.*, 1989). Maltoheptaose was obtained from Boehringer, and potato and amylomaize VII (Hylon VII) starches were from National Starch. Amorphous potato starch was obtained by lyophilisation of a gelatinised paste.

Methods

DSC analyses were carried out with a Perkin-Elmer DSC7 instrument with data analysis performed using supplier's software. Samples for analysis (5–10 mg) were accurately weighed into stainless steel pressure-tolerant pans, and water added (~50 μl) such that starch concentrations were ~20% w/v. The heating rate was 10°C/min from 5 to 180°C.

X-ray diffraction patterns were obtained by using a Phillips powder diffractometer (P CW 1050/1390) mounted on a PW 1730/10 sealed-tube X-ray generator operating at the Cu-K_α wavelength (1.542 Å). Estimates of crystallinity were made by assessing the contribution of amorphous features to the total diffraction intensity over the angular range 5–35° 2Θ as described elsewhere (Chinachoti & Steinberg, 1986; Nara & Komiya, 1983).

^{13}C CP/MAS NMR spectra were obtained at 75.46 MHz on a Bruker MSL-300 instrument operating at 303 K using a double bearing probe. A single contact time of 1 ms, a spectral width of 30 kHz and line broadenings of 10–20 Hz were routinely used. Spinning rates were typically 4 kHz and ^{1}H decoupling fields of ~80 kHz (20 G) were employed. Typically 8 K time domain points were collected during a 140 ms acquisition time with a transform size of 32 K: a recycle delay of 4 s was used. For quantitative analysis of ordered/amorphous ratios (Gidley & Bociek, 1985), spectra were compared with those generated by Fourier transformation of composite free-induction decays (f.i.d.s) obtained by addition in various ratios of f.i.d.s corresponding to fully ordered and amorphous starch samples (both Fig. 5 in Gidley & Bociek 1985, 1988).

Chromatographic analysis of unit chains in enzyme-resistant retrograded starch samples was carried out using HPAEC. To 5 mg resistant starch, 50 μl 2 M KOH solution was added, mixed and diluted immediately with pure water to give a final concentration of 0.1 M KOH. The freshly prepared sample was filtered through a 0.45 μm filter (Millipore) and 200 μl of this solution was subjected to HPAEC using a Dionex DX-300 LC series HPLC system (Sunnyvale, CA, USA) equipped with a Dionex CarboPac PA-100 column (250 × 4 mm) in combination with a CarboPac PA-100 guard column, run at *c.* 20°C. Elution (1 ml/min) involved linear gradients of sodium acetate in 100 mM NaOH 175–250 mM from t = 0–20 min; 250–280 mM from t = 20–35 min; 280–305 mM from t = 35–50 min; 305–325 mM from t = 50–60 min; 325–415 mM from t = 60–130 min, followed by a 5 min wash with 500 mM NaOH. The solvents were prepared from a 50% solution of NaOH

(Fisons), filtered through a 0.45 μm filter (Millipore), degassed and stored under helium using a Dionex EDM module. The eluent was monitored using a Dionex PED-2 detector in the pulsed-amperometric (PAD) mode. A reference Ag/AgCl electrode was used with a working gold electrode with the following pulse potentials and durations: $E_1 = 0.05$ V, 500 ms; $E_2 = 0.60$ V, 80 ms; $E_3 = -0.60$ V, 50 ms. Instrument control and data collection was performed using AI-450 chromatography workstation software, release 3.31. The DP of oligomers was assigned by spiking resistant starch samples with maltoheptaose.

RESULTS

Characterisation by established techniques

Light microscopic observation of enzyme-resistant retrograded starch preparations showed a few residual intact granules and remnants for material obtained from amylomaize V and VII (as judged by birefringence effects) but no such structures for material obtained from wheat. It is difficult to quantify the birefringent fraction but it was judged to be minor, probably only a few per cent of the total preparation. Birefringent remnants presumably represent material not gelatinised during autoclaving (45 min at 134°C; Russell *et al.*, 1989).

DSC of enzyme-resistant retrograded starch preparations in excess water showed, for all samples, a broad endothermic transition starting at *c.* 90°C and peaking at 150–160°C. Examples are shown in Fig. 1 for enzyme-resistant retrograded starches from wheat and amylomaize VII. This response is similar to that found by some authors (Gruchala & Pomeranz, 1993; Sievert & Würsch, 1993) but starts at lower temperatures than reported by others (Sievert & Pomeranz, 1989, 1990). For such broad calorimetric events (Fig. 1), assignment of baselines is often not obvious. As well as causing a degree of uncertainty in the breadth of transitions, calculation of transition enthalpies can also be problematic. Estimates of enthalpies were obtained using a linear baseline connecting the low temperature onset and a trough reproducibly observed at ~170°C (Fig. 1). For enzyme-resistant retrograded material from wheat, amylomaize V and amylomaize VII, enthalpies of 20–25, 15–20 and 25–3 J/g were obtained. These values fall in the middle of ranges reported previously (Sievert & Pomeranz, 1989, 1990; Gruchala & Pomeranz, 1993; Sievert & Würsch, 1993).

X-ray powder diffraction patterns for enzyme-resistant retrograded starches are shown in Fig. 2 and compared with granular amylomaize VII and potato starches. For all enzyme-resistant retrograded starches, two broad maxima centred on 16–17 and 22–23° 2Θ are found in agreement with previous work on amylomaize

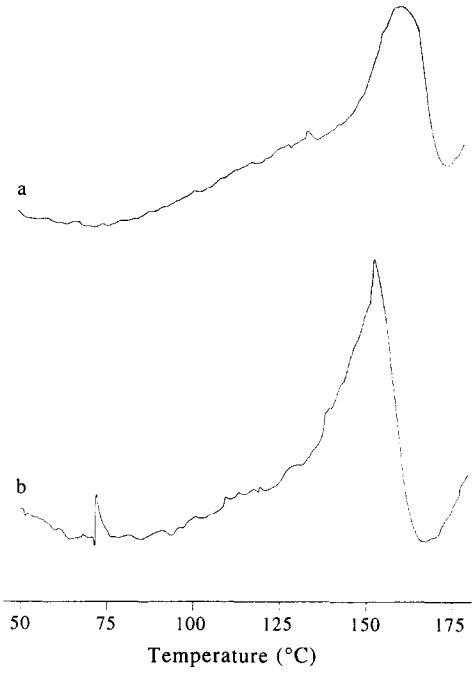

Fig. 1. DSC traces for enzyme-resistant material from: (a) retrograded wheat; and (b) amylomaize VII starches obtained by heating *c.* 20% w/v aqueous dispersions at 10°C/min.

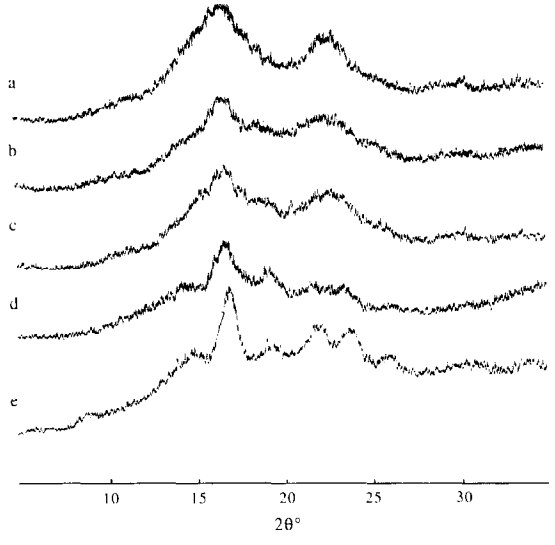

Fig. 2. X-ray powder diffraction patterns for enzyme-resistant material from retrograded: (a) wheat; (b) amylomaize V; (c) amylomaize VII starches; and native (d) amylomaize VII; and (e) potato starches.

VII (Sievert *et al.*, 1991; Eerlingen *et al.*, 1993*a*) and characteristic of a B-type diffraction pattern as also described for enzyme-resistant amylose (Eerlingen *et al.*, 1993*b*) and wheat starch (Siljeström *et al.*, 1989; Berry *et al.*, 1988). B-type diffraction patterns with increased resolution of features have been obtained from granular amylomaize VII and potato starch (Zobel, 1988). Estimates of amorphous to crystalline ratios can be obtained from powder diffraction patterns by

apportioning intensity between a broad amorphous 'halo' and peaks due to crystalline material. Using this method, percentage crystallinities for granular amylomaize VII and potato starches of *c.* 17 and 25%, respectively, were obtained in agreement with previous reports (Nara & Komiya, 1983; Zobel, 1988; Cooke & Gidley, 1992). By the same method, percentage crystallinities for enzyme-resistant retrograded starch from wheat, amylomaize V and amylomaize VII were estimated to be *c.* 30, 25, and 30%, respectively. Estimated errors on these determinations are ±2% for granular starches and ±3–4% for the broader patterns from enzyme-resistant retrograded starches.

^{13}C CP/MAS NMR spectroscopy

High resolution solid state ^{13}C-NMR spectra of enzyme-resistant retrograded starches obtained using cross-polarisation, magic angle spinning and high power dipolar decoupling (CP/MAS) are shown in Fig. 3 together with comparative spectra from granular and amorphous potato starches. Intensity in the regions 90–110, 67–90 and 58–67 ppm is assigned to C-1, C-2, 3, 4, 5 and C-6 sites, respectively (Gidley & Bociek, 1985).

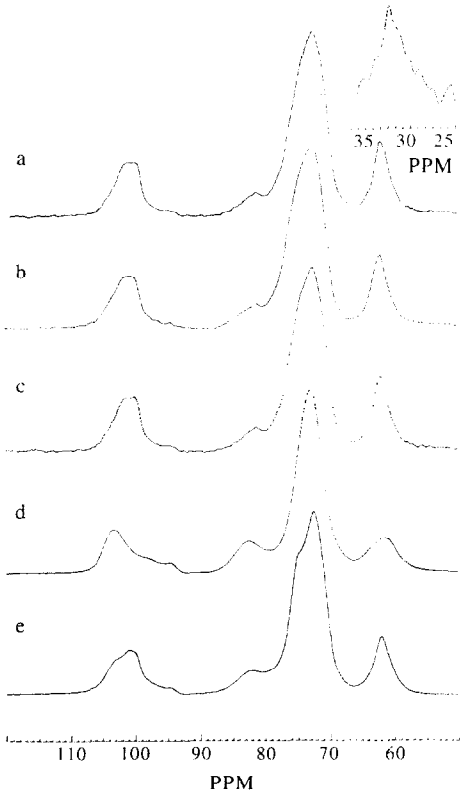

Fig. 3. ^{13}C CP/MAS NMR spectra for enzyme-resistant material from retrograded: (a) wheat; (b) amylomaize V; (c) amylomaize VII starches; and for (d) amorphous; and (e) native potato starches. The 25–35 ppm insert shows the lipid methylene region of the spectrum for enzyme-resistant material from retrograded wheat starch with a vertical expansion of X10 compared with other spectra.

The lack of sharp signals is consistent with the relatively low level of crystallinity in the samples (Gidley & Bociek, 1988; Gidley, 1989). It has previously been shown that ^{13}C CP/MAS NMR spectra for granular starches can be interpreted as a composite of intensity features from ordered (double helical) and non-ordered (amorphous single chain) material (Gidley & Bociek, 1985). This is based primarily on chemical shift and lineshape differences for C-1 and C-4 sites in double helical and amorphous states. In particular, amorphous material (e.g. Fig. 3d) displays a C-1 resonance with a maximum at 103–104 ppm and a characteristic envelope of intensity between 93 and 106 ppm (Gidley & Bociek, 1985), whereas B-type double helical conformations give rise to intensity only between 99 and 102 ppm with a doublet structure for highly organised arrays of helices (Gidley & Bociek, 1985, 1988; Gidley, 1989). For C-4 sites, double helical conformations give rise to chemical shifts within the envelope of intensity from 68 to 78 ppm which also includes C-2, 3, 5 intensity. C-4 sites in amorphous material give a broad signal ranging from below 80 to 87 ppm (Fig. 3d). Intensity in this region in starch spectra is, therefore, assigned to amorphous material although partition of the total C-4 signal between double helical and amorphous states is difficult due to overlap with signals from C-2, 3 and 5. Based on matching observed spectra with composite spectra obtained from various combinations of signals from model amorphous and double helical material, granular potato starch (Fig. 3e) was shown to have an amorphous to double helix ratio of 50:50 (Gidley & Bociek, 1985). For each of the enzyme-resistant retrograded starch samples (Fig. 3a–c), relative C-1 intensity is higher in the range 99–102 and lower in the ranges 93–99 and 102–106 ppm. This indicates a higher double helical content than for granular potato starch. From spectral matching, the double helix to amorphous ratio for all three resistant retrograded starches is estimated to be 60–70:40–30 with material from wheat and amylomaize VII lying towards the top of the double helical content range and material from amylomaize V having a double helical content lower in the range.

^{13}C CP/MAS NMR spectroscopy can also be used to assess whether lipids in starch samples are present as inclusion complexes or free lipids (Morrison *et al.*, 1993*b*). Inclusion complexes give rise to a lipid methylene carbon resonance at 31.2 ± 0.4 ppm whereas free lipids are either too mobile to give signals under the 'solid state' acquisition conditions (Morrison *et al.*, 1993*b*) or, if more solid, give a signal at 34–35 ppm. The inset in Fig. 3a shows an expansion of the spectral region from 25 to 35 ppm for enzyme-resistant retrograded wheat starch with an intensity peak at 31.3 ppm consistent with the presence of complexed lipid as was found previously for native and acid-degraded wheat starches (Morrison *et al.*, 1993*a*). Similar signals were also found for enzyme-resistant

retrograded material from amylomaize V and VII (data not shown) consistent with the presence of lipid inclusion complexes as found in native maize starches (Morrison *et al.*, 1993*c*). Although signal responses from lipids in inclusion complexes have not yet been shown to be quantitative under the acquisition conditions used, a rough estimate of relative abundance can be made by assuming that signal responses from lipid and carbohydrate sites are equivalent. In this way, percentages of glucan present as a lipid complex in enzyme-resistant retrograded material from wheat, amylomaize V and VII were estimated to be ~5, 7 and 8%, respectively, by assuming a lipid content (based on methylene carbons) of 10% in glucan complexes (Morrison *et al.*, 1993*b*).

High performance anion exchange chromatography (HPAEC)

Previous HPAEC analyses (Koizumi *et al.*, 1991) of starch-based oligosaccharides (primarily derived from debranched amylopectin) have demonstrated resolution of individual (1 → 4)-α-D-glucan oligomers up to a degree of polymerisation (DP) of *c.* 50. As previous size exclusion chromatographic studies of enzyme-resistant retrograded starch suggested the presence of longer chains (Russell *et al.*, 1989), elution conditions were developed which allowed good separation of oligosaccharides for DP values up to at least 80. This involved changes in elution gradient through the course of each fractionation as described in the Materials and Methods section. Typical chromatograms for resistant retrograded starch from wheat, amylomaize V and amylomaize VII are shown in Fig. 4. The sharp nature of individual peaks confirms that, within the fractionated range, materials were essentially linear as significant branching leads to additional non-regular peaks (Ammeraal *et al.*, 1991) and/or loss of resolution. Peaks within chromatograms were assigned by 'spiking' of samples with maltoheptaose to establish the location of the DP7 peak followed by sequential assignment of peaks with increasing elution time to increasing DP values. Good resolution of peaks up to DP 70–80 is observed for all enzyme-resistant retrograded materials with at least partial peak resolution up to DP 90–100. No attempt has been made in this study to establish response factors for individual oligomeric species, so quantification of relative abundances across the full fractionation range cannot be attempted. However, as response factors are not expected to vary dramatically above approximately DP 15 (Koizumi *et al.*, 1991) relative intensities of similarly sized oligosaccharides can be used as a measure of relative abundance.

A striking feature of chromatograms from all three enzyme-resistant retrograded starches is a relatively consistent periodicity in intensity as a function of oligomer size. This is most marked for enzyme-resistant

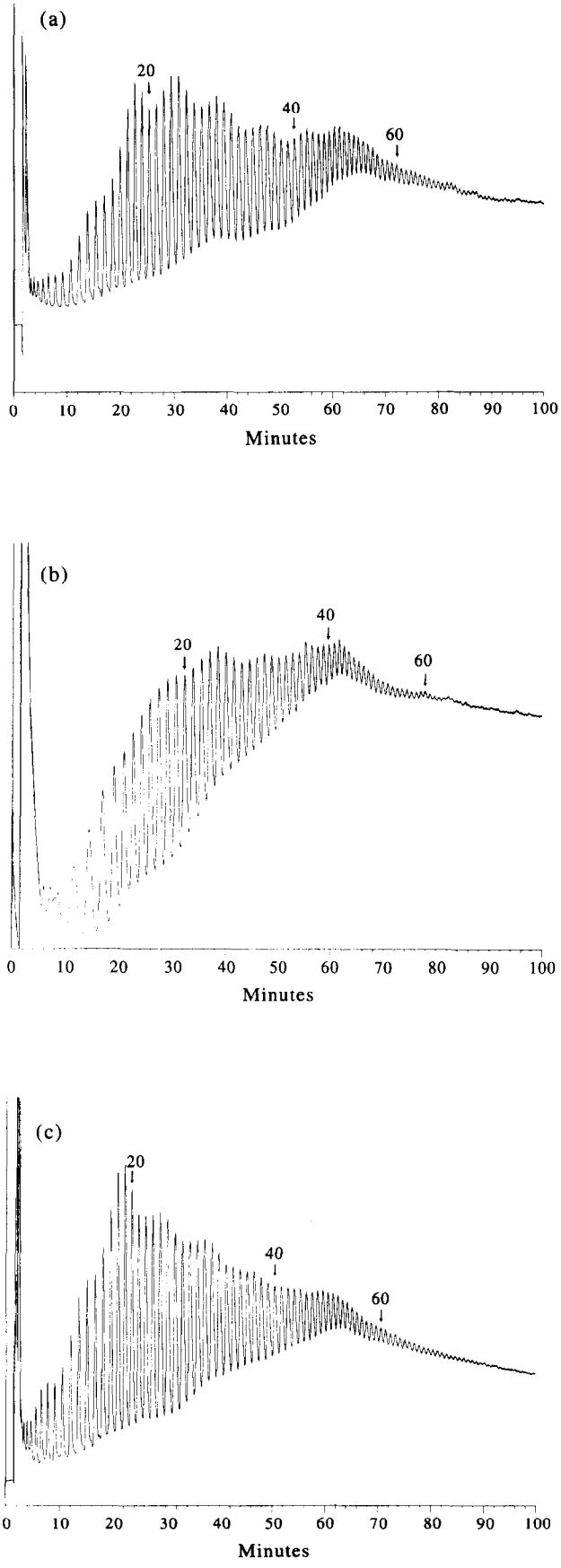

Fig. 4. HPAEC traces for enzyme-resistant material from: (a) retrograded wheat; (b) amylomaize V; and (c) amylomaize VII starches. Numbering of peaks indicates DP values.

retrograded wheat starch (Fig. 4a) where local maxima are observed for DP 18, 23–24, 29, 35–36, 42, and 47–48 with corresponding local minima intermediate between adjacent maxima. Enzyme-resistant retrograded amylomaize V starch (Fig. 4b) has local maxima at DP 19, 24, 30, 36, and 42 with troughs at intermediate positions. For enzyme-resistant retrograded amylomaize VII starch (Fig. 4c), local maxima are observed for DP 19, 24, 30 and 48 with shoulders at DP 36 and 42.

Each of the materials, therefore, exhibits a tendency to contain relatively greater amounts of chains containing approximate multiples of six residues against a background of a broad distribution of chain lengths from DP 3–5 up to *c.* DP 100.

DISCUSSION

Levels of molecular and crystalline order

Quantitative analysis of ^{13}C CP/MAS NMR spectra and X-ray powder diffraction patterns has established that the enzyme-resistant retrograded starches studied contain 60–70% double helical conformations but only *c.* 25–30% crystallinity. Therefore, more than half of the double helices in the samples are not present in domains sufficiently large and regularly arranged to diffract X-rays. Furthermore, as has been observed previously (Sievert *et al.*, 1991; Eerlingen *et al.*, 1993*a*), diffraction peaks are found to be broader and to show less structural detail than those from granular starches. It can, therefore, be inferred that crystallites within enzyme-resistant starches are smaller and/or less perfectly packed than in granular starches. Such a description is in line with model studies on amylose gels (Gidley, 1989) and enzyme-resistant material from amylose gels (Cairns *et al.*, 1990). Amylose gels show weak X-ray diffraction (Cairns *et al.*, 1990; Gidley, 1989) but a high (66–83%) level of molecular order (Gidley, 1989).

Following enzyme incubation of an amylose gel, little change in the X-ray diffraction behaviour was found (Cairns *et al.*, 1990) suggesting that network disruption by enzyme hydrolysis did not allow increased crystalline packing to occur. Studies of enzyme-resistant material from amylose at low (sub-gelling) concentrations indicated (Eerlingen *et al.*, 1993*b*) diffraction patterns sharper than for enzyme-resistant starch probably due to the reduction in network constraints at lower concentration resulting in greater lateral aggregation either before or after enzyme incubation.

The relatively high level of double helical order in enzyme-resistant retrograded starches is consistent with the proposal (Jane & Robyt, 1984) that double helices represent the primary source of resistance to enzyme hydrolysis, although the broad range of chain lengths found is not consistent with a proposed regular helix length of *c.* DP 32 (Jane & Robyt, 1984). The 30–40% of non-double helical material comprises a small (5–8% of total material) fraction of lipid complexed amylose together with less ordered material. This amorphous fraction could conceivably arise from a combination of 'fringes' at the end of helical stretches and material either trapped within aggregates of helices (contributing to broad X-ray patterns) or imperfections within otherwise helical chains. It is reasonable to assume that each of these classes of material would be resistant to amylolysis either due to their restricted size ('fringes' and imperfections in helices) or due to physical inaccessibility (trapped chains).

The overall picture of structural order in enzyme-resistant retrograded starch is, therefore, of primary structuring due to double helix formation with amorphous material either at the end of chains, in limited regions within helical chains, or trapped within aggregates, and a small amount of lipid complex. Double helices show some aggregation based on the B-type structure (Imberty & Perez, 1988) although aggregate size and/or perfection of packing are limited.

Chain lengths and thermal stability

The benefit of using HPAEC (Fig. 4) for the characterisation of enzyme-resistant retrograded starches is that an essentially complete description of the linear chains present in any preparation can be obtained. The main drawback of HPAEC is the difficulty of obtaining accurate quantification of relative abundances for each resolved oligomer. This arises from a combination of two effects. Firstly, response factors under the experimental conditions used need to be obtained, either by using dual detection or calibration with known concentrations of each oligomer. Secondly, the requirement for changing elution gradients through the chromatographic run (in order to effect separation across a wide DP range) results in a non-linear baseline response from the amperometric detector which is difficult to take into account.

From the results obtained (Fig. 4), a wide range of chain lengths (DP < 10 to ~100) with an approximate relative maximum at DP 20–30 and greater relative intensity for oligomers with DP of 18 and higher multiples of six can be deduced. The wide range of linear chain DP values and the maximum at DP 20–30 is in general agreement with previous studies (Russell *et al.*, 1989; Berry *et al.*, 1988; Leloup *et al.*, 1992; Eerlingen *et al.*, 1993; Jane & Robyt, 1984; Faisant *et al.*, 1993*a*, 1993*b*) using low resolution size exclusion chromatography. The upper and lower limits of DP values (~100 and 10, respectively) are consistent with their proposed origin as uninterrupted double helical lengths prior to enzyme action. From studies of purified oligomeric (1 → 4)-α-D-glucans, it has been found

(Pfannemüller, 1987; Gidley & Bulpin, 1987) that the minimum chain length required for double helix formation is 10 with B-type crystallisation being favoured for DP 13 and above. This is, therefore, proposed to be the origin for increased abundance of oligomers above DP 10 (Fig. 4). The effect of amylose chain length on phase behaviour in water has been studied using enzymically synthesised near monodisperse material (Pfannemüller *et al.*, 1971; Gidley & Bulpin, 1989). A maximum rate of aggregation in dilute (<0.1%) solution was found for DP ~80 (Pfannemüller *et al.*, 1971) which can be interpreted as being due to the upper limit of uninterrupted helix lengths as DP values of 100 and above show evidence of network formation at higher (>0.5%) concentrations (Gidley & Bulpin, 1989). If uninterrupted helix lengths have an upper limit of DP 80–100 this is a reasonable explanation for the same upper limit in enzyme-resistant starch as most helix interruptions would be expected to be enzyme-sensitive.

DSC (Fig. 1) identifies an endothermic transition for all three enzyme-resistant starches spanning from below 100 to *c.* 170°C with a maximum at *c.* 150°C. As it has been found that heating to *c.* 160°C in water is required to dissolve retrograded amylose (Gidley & Bulpin, 1989) the peak is assigned to 'melting' (unwinding) of double helical segments. The breadth of the endotherm together with the wide range of chain lengths present in enzyme-resistant starch, is consistent with temperatures required for helix melting to be increasing with helix (chain) length. Comparison with the behaviour of (most) starch granules and amylopectin gels is also consistent with melting temperatures increasing with length. For these systems, helix lengths of 12–16 residues (Ring *et al.*, 1987; Hizukuri, 1986) and melting (gelatinisation) temperatures of 55–75°C are found. This represents the shortest significantly abundant chains found in enzyme-resistant retrograded starch, and is just below the onset of the DSC endotherm.

The enthalpies associated with endotherms for heating enzyme-resistant retrograded starches in water represent a quantitative measure of ordered structure loss. For the gelatinisation of granular starches, it has been suggested that melting of double helices, and not disruption of crystalline packing, is the primary determinant of endotherm enthalpy (Cooke & Gidley, 1992). For highly ordered (and crystalline) starch fragments with melting temperatures similar to starch gelatinisation temperatures, endotherm enthalpies of *c.* 35 J/g have been obtained (Cooke & Gidley, 1992). Based on estimates of both double helix content and endotherm enthalpy for enzyme-resistant retrograded starches, extrapolated enthalpy values for fully ordered (double helical) material can be calculated. For wheat (22.5 J/g, 70% helix), and amylomaize V (17.5 J/g, 60% helix), extrapolated values of *c.* 30 J/g are found. For amylomaize VII (27.5 J/g, 70% helix) the corresponding value is 39 J/g. These values are similar to analogous extrapolated values for granular starches and experimental values for highly ordered material (Cooke & Gidley, 1992). Although both DSC and NMR quantifications for enzyme-resistant retrograded starches are less certain than for granular starches (due to broader features), the similar result obtained in this analysis suggests that enthalpies of melting/dissociation of amylose helices (in J/g) do not vary markedly with the chain length of the helix in the range encountered in granular and enzyme-resistant retrograded starches. If the helix melting enthalpy is considered to be the sum of stabilising structural features (presumably hydrogen bonds) which are present in each turn of the helix irrespective of its length, then a constant enthalpy per gram of helix would be expected, so the major factor which may lead to helix length variations would be end effects. The tentative conclusion from the current results is that end effects are not dominant in determining amylose double helix melting enthalpies.

A structural model for enzyme-resistant retrograded starch

From the data obtained during this study, several aspects of the molecular and microstructural nature of enzyme-resistant retrograded starch have been determined. From X-ray diffraction, it can be deduced that only small regions of crystalline B-type packing are present, and from ^{13}C CP/MAS NMR, double helix levels of 60–70% are found. From HPAEC, a range of (linear) chains from DP 10 to DP 80–100 are observed with a significant tendency for chains to contain multiples of six units above DP 18. A general description of structures present is, therefore, of substantially double helical linear chain segments loosely arranged into aggregates with approximate B-type packing geometry, and incorporating single chain material as fringes/imperfections in helices or chains trapped within aggregates, together with some lipid inclusion complexes. Chain (helix) lengths vary widely so there is no evidence for regular helical elements of defined length (Jane & Robyt, 1984).

Each of these features is consistent with a view of amylose gelation/aggregation (Gidley, 1989; Gidley & Bulpin, 1987) which is based on interchain junction zones of a double helices occurring over a length scale of 10–100 residues with limited aggregation into B-type arrays. The tendency for enzyme-resistant retrograded starch chains to contain multiples of six units is, however, less obviously due to a feature within aggregated/retrograded amylose. In the B-type double helix structure (Imberty & Perez, 1988; Wu & Sarko, 1978) there are six residues per helix turn, three from each chain. To obtain multiples of six residues in single chains following enzyme action, helices with an even number of turns need to have been resistant.

There is no obvious reason why helices should be more resistant for even numbers of helix turns than for other helix lengths, so it is considered unlikely that the periodicity in chain length found for enzyme-resistant starch is a reflection of a periodicity of helix lengths prior to enzyme treatment. By implication, therefore, the observed periodicity is considered to arise as a result of enzyme action. One possible mechanism for this is outlined below.

For the B-type polymorph, hexagonal packing of double helices based on the unit cell illustrated in Fig. 5a occurs (Imberty & Perez, 1988; Wu & Sarko, 1978). Within individual double helices, each chain contributes three residues to each turn of the helix as illustrated in Fig. 5b. The turn of the helix above and below is composed of the complementary three residues from each chain (Fig. 5c). The same position on the double helix is, therefore, occupied by residues six units apart along the primary chain. Assuming that the primary barrier to enzyme action is a double helical conformation, the limit of enzyme hydrolysis will occur at one end of a helix. If this helix is in an environment similar to helix I in Figure 5a, the presence of adjacent helices could be envisaged to direct hydrolysis to glycosidic bonds in the vicinity of the arrow in Fig. 5a. The same argument can be made concerning enzymic hydrolysis at the other end of helical segment I. This would entail enzymic hydrolyses at equivalent geometrical positions, and, therefore, liberation of a single chain containing a multiple of six residues. For helices more peripheral in aggregate structures (e.g. II in Fig. 5a) two or three glycosidic bonds at each end of the helix may be sterically accessible. This would still lead, on average, to a multiple of six residues in liberated chains although a broader spread of other adjacent chain lengths would be expected. In the case of both helix types I and II, this proposed mechanism would be predicted to discriminate against production of chains requiring hydrolysis on opposite sides of the helix segment. Chain lengths resulting from such action (DP 21, 27, 33, 39 etc.) are indeed less abundant than those with DP of exact multiples of six.

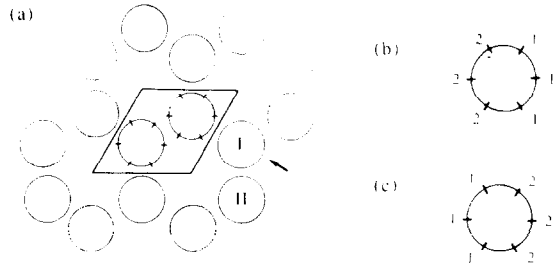

Fig. 5. (a) Schematic representation of a top view of helix packing in B-type (1 → 4)-α-D-glucan illustrating both the unit cell and relative positions of glucose residues within each double helix. Double helices I and II are referred to in the text. (b) Contribution of residues from chains 1 and 2 to one turn of a double helix. (c) Contribution of residues from chains 1 and chain 2 to the turn of a double helix directly above or below that represented in (b).

It should be stressed that the mechanism described above does not lead to all of the final enzyme-resistant starch chains as the tendency to favour exact DP multiples of six over intermediate DP is a modest one. Presumably many enzyme-resistant chains are produced from helices which do not present steric barriers to enzyme hydrolysis and would, therefore, not be expected to show any periodicity in length.

In summary, it is proposed that enzyme-resistant retrograded starch reflects features both of aggregated/gelled amylose (high double helix content, low crystallinity, DP range from junction zones of DP 10–100), and the consequence of enzyme action on such a structure (periodicity of six units from accessibility of enzyme to aggregated substrate).

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