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An NMR spectroscopic characterisation of the enzyme-resistant residue from α-amylolysis of an amylose gel

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The mobility and conformation of amylose in a gel form and its α-amylase resistant residue were probed through NMR relaxation experiments and high resolution ^{13}C-CP/MAS NMR. The gel contained a double helical, rigid fraction and a more mobile amorphous fraction. Treatment of the gel with α-amylase resulted in the hydrolysis of part of the amorphous fraction, with further ordering of the remainder, to form a product, the amylose chains of which were essentially fully ordered and rigid as assessed by NMR.

INTRODUCTION

There is emerging evidence that a fraction of starch in our diet escapes digestion and absorption in the small intestine (Englyst *et al.*, 1992). Most of the starch that we eat has been processed by a heat/moisture treatment which disrupts the native granular structure. If the material is cooled, the solubilised and partially solubilised starch polysaccharides reassociate or retrograde. Retrograded starch is partially resistant to hydrolysis by mammalian amylolytic enzymes *in vitro*. While retrograded amylopectin is capable of being digested by the enzyme to low molecular weight products, retrograded amylose is substantially resistant (Jane & Robyt 1984; Ring *et al.*, 1988; Leloup *et al.*, 1992*a*), typical values for the resistance of a retrograded gel being in the region of 70%. The resistant product is a linear dextrin with an average degree of polymerisation in the range 20–70 (Jane & Robyt 1984; Ring *et al.*, 1988; Russell *et al.*, 1989; Siljeström *et al.*, 1989; Eerlingen *et al.*, 1993*a*), its value depending upon sample preparation, the conditions of the *in vitro* hydrolysis, and the type of average determined. More recently, it has been shown that *in vitro*, and *in vivo* resistant products escaping digestion in the small intestine, are largely comparable (Faisant *et al.*, 1993*a*, 1993*b*; Botham *et al.*, 1994). *In vivo* the major resistant fraction is a linear dextrin, with small amounts of starch oligosaccharides and a high molecular weight fraction also escaping digestion (Faisant *et al.*, 1993*a*).

While it has been shown that the porosity of substrates can restrict access of amylolytic enzymes and increase the effective 'resistance' of otherwise digestible material (Colonna *et al.*, 1992; Leloup *et al.*, 1992*a*), the molecular basis for the formation of α-amylase-resistant starch remains elusive. The observation that resistant starch residues once dissolved are susceptible to amylolysis shows that it is the physical state of starch which confers resistance. The commonest techniques used to characterise physical structure are, most directly, X-ray diffraction (Eerlingen *et al.*, 1993*a*, 1993*b*) which probes long-range crystalline order and, relatively indirectly, differential scanning calorimetry (Gruchala & Pomeranz, 1993) which measures heat flow during the melting and dissolution of ordered structures. In characterising starch structure, both the ordering of chains into double helices and the packing of helices into extensive three-dimensional crystallites must be considered (Gidley & Bociek, 1985). The strands of the retrograded amylose gel network consist of assemblies of chains (Miles *et al.*, 1985; Leloup *et al.*, 1992*b*). For relatively dilute gels (2–10%w/w) the network strands are accessible to the enyme, any resistance to amylolysis resulting from the organisation of amylose in the strand (Leloup *et al.*, 1990). Amylose gels give a weak X-ray diffraction pattern of the B-type and it is tempting to propose that this crystallinity is the origin of resistance. The level of crystallinity, determined by X-ray diffraction, of amylose gels is dependent on the conditions of storage (Eerlingen *et al.*, 1993*b*) and concentration (Cairns *et al.*, 1994), and reaches 35% for an 11%w/w gel which has been stored for 1 week at room temperature. The

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amylose gel is substantially resistant to amylolysis with the product having a crystallinity of 34–61% (Cairns *et al.*, 1994), therefore, crystallinity alone is not a sufficient explanation of resistance.

It is possible that the amylose chains connecting crystallites are sufficiently rigid or inaccessible to escape amylolysis. Alternatively, X-ray measurements may only give a partial measure of the extent of ordering of amylose chains. In high resolution solid state (CP/MAS) ^{13}C-NMR experiments on amylose gels (Gidley, 1989) and starch granules (Cooke & Gidley, 1992) the extent of helicity as determined by NMR is greater than that determined by X-ray diffraction. The NMR technique is, therefore, sensitive to helix packing over a shorter distance scale. In this paper we probe both the mobility and conformation of amylose chains in a gel and its α-amylase-resistant product using NMR techniques to gain further insight into the molecular basis of resistance.

MATERIALS AND METHODS

Materials

Amylose was isolated from pea starch (Nutrio P-star 33, Grinsted Products, Suffolk, UK) by aqueous extraction at 70°C followed by purification by butan-1-ol precipitation. GPC, on TSK-GEL columns (G3000PW, G4000PW and G6000PW Toyo-Soda Ltd) calibrated with pullulan standards, indicated a weight-average degree of polymerisation, DP_w, of 1900 after appropriate correction for the relative sizes of pullulan and amylose of the same DP. A short chain amylose was prepared by hydrolysis of potato starch (Sigma, Poole, Dorset, UK) using 2.2 M HCl at 37°C for 40 days. Ion exchange chromatography followed by pulsed amperometric detection (Dionex, Sunnyvale, CA, USA) gave a DP_w of 22.

An amylose gel (7.1%w/w) was prepared from the amylose–butanol complex by evaporating the butan-1-ol at 95°C in a heated nitrogen stream and cooling to 20°C to set the gel. After ageing for 48 h, the gels were blended (25 mg/ml in 5 mM phosphate buffer, containing 0.02%w/v sodium azide, adjusted to pH 6.9) using an Ultra Turrax homogeniser at 9500 rpm for 2 min. The sample was divided into two parts: one sample for gel characterisation and one for preparation of the enzyme-resistant fraction. One fraction was treated with 10 units/ml of porcine pancreatic α-amylase (Boehringer Mannheim, Lewes, East Sussex, UK) at 37°C for 8 h. Time course studies (Sun, 1993) showed that during the first 2 h approximately 29% of the gel was hydrolysed and that at 8 h this had only increased a further 1% to 30% overall. The resistant fraction was extensively washed until no protein or oligosaccharide could be detected in the supernatant.

B-type spherulites were prepared (Ring *et al.*, 1987) by slowly cooling a 20%w/v aqueous solution of the short chain amylose from 100 to 2°C. For ^{1}H-NMR, the gel, resistant product and spherulites were dispersed in D_2O (99.99%, Sigma, UK), centrifuged and the supernatant discarded five times to replace exchangeable hydrogens with deuterium. Wet dispersions were loaded into the sample cells for spectroscopic measurements.

Methods

The ^{1}H-NMR T_2 values were determined by direct measurement of the free induction decay (FID) immediately after a 90° pulse using a Bruker MSL-100 instrument operating at 100 MHz. The 90° pulse width was 1 μs, receiver dead time 5 μs and the dwell time 4 μs (spectral width 125 kHz). The data were fitted to empirical models using the PC-based, non-linear least squares fitting package TableCurve™ (Jandel Scientific). ^{13}C-NMR spectra were obtained on a Bruker MSL-300 instrument operating at 75.46 MHz using a 7 mm diameter rotor and a double-bearing probe head at 25°C and −43°C. For cross polarisation and magic angle spinning spectra, spinning rates of 3 kHz, spin locking and ^{1}H decoupling fields of 38 kHz were employed. Other experimental parameters were as follows: acquisition time, 205 ms; recycle delay, 5 s; time domain points, 4k; contact time, 1 ms; and scans, 5500.

RESULTS AND DISCUSSION

Figure 1 shows ^{1}H-NMR free induction decay (FID) curves for highly crystalline spherulites, an amylose gel and the enzyme-resistant products of the gel. In each case there is a clear separation of time scales. Much of the signal intensity decays within the first 50 μs and the remainder over longer time scales, i.e. approximately 1 ms. In order to quantify the contributions to the FID,

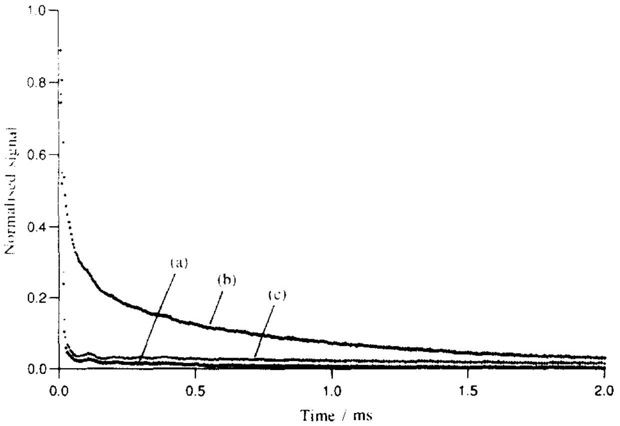

Fig. 1. Proton NMR free induction decay curves of (a) spherulites; (b) an amylose gel; and (c) resistant products of the gel.

the curves were fitted with combinations of Gaussian (fast) and exponential (slow) decay functions. Empirically, it has been found that rigid solids give rise to Gaussian contributions to the decay of the transverse magnetisation:

$$M_{yi}(t) = M_{yi}(0)\exp(-t^2/T^2_{2i}) \quad (1)$$

and the mobile fraction contributes exponential terms:

$$M_{yj}(t) = M_{yj}(0)\exp(-t/T_{2j}) \quad (2)$$

where $M_y(t)$ is the observed transverse magnetisation at time t after the pulse, $M_y(0)$ is the initial value, and T_2 is the transverse relaxation time. Subscripts i and j refer to Gaussian and exponential components, respectively.

The fractional contribution of a particular component of the decay is given by $c_{i,j} = M_{yi,j}(0)/(\Sigma_i M_{yi}(0) + \Sigma_j M_{yj}(0))$. Whilst the FIDs for the spherulites and the resistant products could be well described by the sum of a Gaussian and a single exponential, an additional exponential term of intermediate relaxation time was required to describe the data in the case of the amylose gel.

The curves can be analysed in terms of a rigid fraction with T_2 approximately 25 μs and a mobile fraction with T_2 approximately 2–5 ms (Table 1). It can be seen (Fig. 1) that the dynamics of the non-exchangeable protons in the resistant fraction are very similar to those of the spherulites and that the mobile component present in the gel is almost completely absent. Although it might be expected that in the gel there would be chains with a range of mobilities, a clear separation of timescales in the FID is observed. The predominant dynamics in the resistant product are that of a rigid solid and are very similar to that of the spherulites. The FID also provided information on the relative sizes of populations assigned as rigid and mobile in the NMR experiment. For the amylose gel these were a rigid fraction, 52%, with an average T_2 of 31 μs, and mobile fractions with T_2s of 0.2 and 2.2 ms. For the resistant products and spherulites, the mobile fraction was much reduced to 4% or less (Table 1). As the amylose gel network contained ~52% of a rigid fraction and was ~70% resistant to amylolysis with the product being essentially rigid in the NMR experiment, it follows that during the course of amylolysis there is further 'rigidification' of amylose in the network.

Table 1. ^{1}H-NMR T_2 and relative contribution of each component

	T_2/μs, rigid	%	T_2/ms, mobile I	%	T_2/ms, mobile II	%
Amylose gel (7.1%w/w)	31.0	51.5	0.19	19.7	2.17	28.8
Resistant products	25.4	96.0	5.18	4.0		
Spherulites	24.3	97.4	1.49	2.6		

To obtain information on the conformation of rigid and mobile components in the gel and resistant products, a ^{13}C solid state NMR experiment was performed, firstly at a temperature close to ambient to probe the conformation of the rigid fraction, and secondly at a temperature where the motion of the previously mobile fraction would be 'frozen'. Figure 2 shows the CP/MAS ^{13}C-NMR spectrum of an amylose gel measured at 25° and −43°C. The spectral features have been assigned (Gidley & Bociek, 1985, 1988; Gidley, 1989) to the different carbons of the constituent glucose rings. At 25°C, the doublet in the region 99–101 ppm has been assigned to the C-1 of the B-type crystalline polymorph of starch, the peaks in the region 69–77 ppm to C-2, -3, -4 and -5 and the peak in the region 60–64 ppm to C-6. At this temperature only the ordered conformation is detected in the CP/MAS spectra. At −43°C any amorphous material will be in a glassy state and slightly freeze-concentrated by the effect of partial freezing of water. Contributions to the spectrum from rigid amorphous carbons, therefore, appear in the CP/MAS spectrum (Fig. 2b), most noticeably in the ranges 80–83 ppm (C-4) and 96–103 ppm (C-1). The disordered material with its relatively featureless peaks, appears in the spectrum superimposed on the spectrum of the ordered material. In

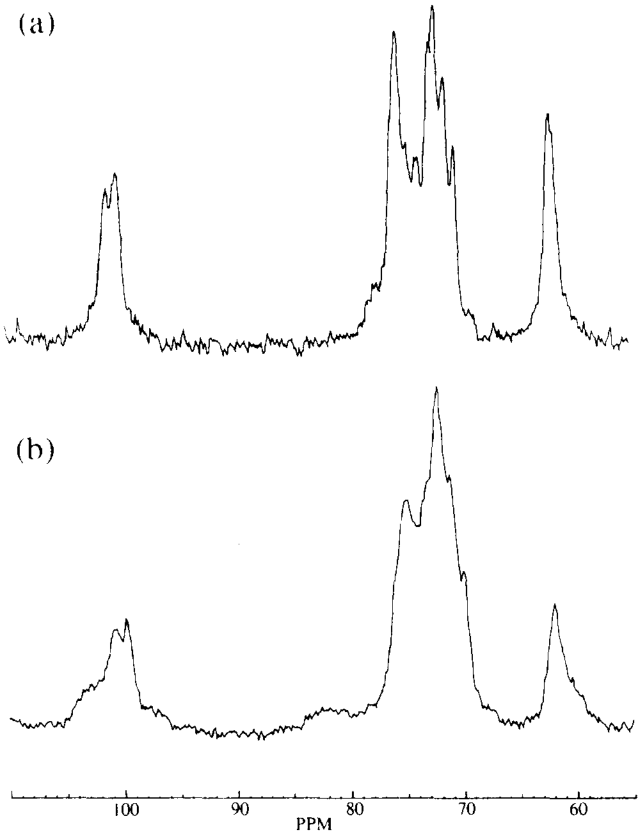

Fig. 2. The CP/MAS ^{13}C-NMR spectra of amylose gels at (a) 25°C; and (b) −43°C.

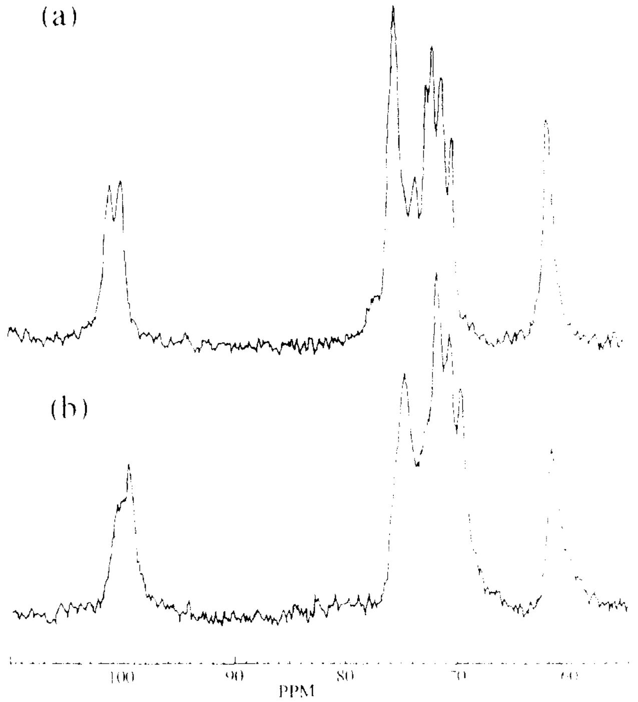

Fig. 3. The CP/MAS ^{13}C-NMR spectra of the resistant products of the amylose gel at (a) 25°C; and (b) −43°C.

contrast, the CP/MAS ^{13}C spectra of the resistant products show relatively little change on cooling from 25°C to −43°C (Fig. 3). Whilst there is a small decrease in the resolution of the peaks, none of the features characteristic of the amorphous material are present. The enzyme-resistant residue of the amylose gel is essentially fully ordered as assessed by NMR.

An estimate of the amount of amylose in an ordered double helical conformation was made by comparison of the peak areas at 25°C and −43°C. The acquisition conditions, including number of scans, were the same for both spectra. Comparison of integrals for either the C-1 region (90–107 ppm) or the full spectrum (56–107 ppm) gave an estimate of 60 ± 5% for the helix content of the amylose gel while the resistant product was essentially wholly ordered (>95%). A previous estimate (Gidley, 1989) of the double helix content of an amylose gel was 70% for a 10% w/w gel of a high molecular weight amylose. The difference observed in this study probably results from the different methods used to prepare the gel and analyse the spectra. The estimate of the fraction of amylose present in the gel in an ordered conformation, which is, therefore, rigid, is consistent with the proton NMR experiment.

From these experiments it can be concluded that for amylose gels, which are wholly accessible to mammalian α-amylase, the molecular origin of the resistance is the double helical conformation and short range packing of amylose in the B polymorph of starch. This conclusion is consistent with an earlier proposal (Jane & Robyt, 1984) and more recent related research (Gidley *et al.*, 1994). It has been observed that retrograded amylopectin also gives a B-type diffraction pattern yet is readily hydrolysed by α-amylase at 37°C (Ring *et al.*, 1988). In this case it was proposed that the temperature of incubation was sufficiently close to the melting temperature of the B polymorph in the gel for the binding of the enzyme with the substrate to cause disruption of the ordered conformation (Leloup *et al.*, 1992*a*).

The highly ordered enzyme-resistant product contrasts with that obtained by Gidley *et al.* (1994, submitted) however, this can be accounted for in terms of the different processing history of their samples. Whole starch was autoclaved at a solids content of 33% w/w and was dried and rehydrated prior to enzyme treatment. The effect of gelling at high solids content and a dehydration and rehydration step is to produce a material of low initial crystallinity which is relatively inaccessible to α-amylase (Cairns *et al.*, 1994). This leads to an enzyme-resistant residue with a relatively low crystallinity and low level of double helical order due to the presence of inaccessible amorphous material in the product.

CONCLUSIONS

From these NMR experiments it is concluded that rigid, double helical conformations of amylose of the B polymorph of starch are resistant to hydrolysis by mammalian α-amylase if the association is stable at the temperature of incubation. During amylolysis there is further ordering of amylose chains. In addition to this 'intrinsically' resistant fraction other aspects of starch and food microstructure can affect access of the enzyme to the substrate and thus influence digestibility.

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