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# Effects of processing high amylose maize starches under controlled conditions on structural organisation and amylase digestibility

A. Htoon [a,b], A.K. Shrestha [a,c], B.M. Flanagan [c], A. Lopez-Rubio [d], A.R. Bird [a,e], E.P. Gilbert [d], M.J. Gidley [c,*]

[a] Commonwealth Scientific and Industrial Research Organisation, Food Futures National Research Flagship, Riverside Corporate Park, North Ryde, NSW, Australia
[b] Food Science Australia, Riverside Corporate Park, North Ryde, NSW, Australia
[c] Centre for Nutrition and Food Sciences, University of Queensland, Hartley Teakle Building, St. Lucia, Brisbane, Qld 4072, Australia
[d] Bragg Institute, Australian Nuclear Science and Technology Organisation, PMB 1, Menai, NSW 2234, Australia
[e] CSIRO Human Nutrition, Kintore Avenue, Adelaide, SA 5000, Australia

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ABSTRACT

The amylase digestibility of high-amylose maize starches has been compared before and after thermo-mechanical processing. Starches were analysed for enzyme-resistant starch yield, apparent amylose content, crystallinity (X-ray diffraction), and molecular order (NMR and FTIR), both before and after treatment with α-amylase. All samples had significant (>10%) enzyme-resistant starch levels irrespective of the type and extent of thermal or enzymic processing. Molecular or crystalline order was not a pre-requisite for enzyme resistance. Near-amorphous forms of high amylose maize starches are likely to undergo recrystallisation during the enzyme-digestion process. The mechanism of enzyme resistance of granular high-amylose starches is found to be qualitatively different to that for processed high-amylose starches. For all samples, measured levels of enzyme resistance are due to the interruption of a slow digestion process, rather than the presence of completely indigestible material.



## 1. Introduction

In plants, starch is synthesized in the form of water-insoluble semi-crystalline granules with a complex architecture which is specific to each particular plant. Unlike other dietary polysaccharides, starches contain only α-glucosidic linkages and, are potentially digestible by the amylolytic enzymes secreted by the human digestive tract (Englyst & Hudson, 1996). Various studies, however, have shown that structural conformation and other factors can influence the rate and extent of starch, *in vitro* and *in vivo*, and subsequent absorption in humans and animals (Botham et al., 1997; Cairns, Botham, Morris, & Ring, 1996; Englyst, Kingman, & Cummings, 1992; Faisant, Champ, Colonna, & Buleon, 1993b; Faisant et al., 1993a, 1995; Gidley et al., 1995). The sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals has been termed 'resistant starch (RS)' (Asp, 1992; Englyst & Cummings, 1990). RS plays important physiological roles and has the potential to improve human health and lower the risk of many diet-related diseases. RS is defined as the fraction of starch not digested in the small intestine that enters the colon where it is fermented by bacteria to short chain fatty acids (SCFA) and gases (Topping & Clifton, 2001). The SCFA, particularly butyrate, have been implicated in promoting good colonic health and preventing the incidence of colo-rectal cancer (Champ, 2004; Jacobasch, Schmiedl, Kruschewski, & Schmehl, 1999).

RS is classified into four major groups, namely RSI, RSII, RSIII, and RSIV (Topping et al., 2003). RSI arises from physically inaccessible starches, *e.g.*, within plant tissue structures, RSII is due to the condensed form and partial crystallinity of native (uncooked) starch granules, RSIII is derived from recrystallised (retrograded) starches, typically after food processing, and RSIV is from chemical modification of starches that inhibit amylase digestion. RSIII or retrograded starch is the form of resistant starch most often used as an ingredient in a range of foods and is therefore of both commercial and nutritional interest.

The molecular organisation of polymers within starch granules (sources of RSI and RSII) has been reviewed (Tester, Karkalas, & Qi, 2004). Many food processing methods reduce or eliminate RSI and RSII but have the potential to generate RSIII, particularly if high amylose starches are used. The currently accepted mechanism for the resistance of RSIII to amylase digestion is that linear amylose segments align themselves after gelatinization into condensed structures based on double helices (amylose retrogradation) that render the α-1,4 glucosidic linkages inaccessible to amylase. Generally speaking, RS III has been found to contain short and linear chains of thermally stable α-1,4 glucans of about

* Corresponding author. Tel.: +61 7 3365 2145; fax: +61 7 3365 1177.
*E-mail address:* m.gidley@uq.edu.au (M.J. Gidley).

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10–100 chain lengths; significant double helix content and moderate crystallinity (mostly B-type) (Cairns et al., 1996; Eerlingen, Deceuninck, & Delcour, 1993b; Faisant et al., 1993a, 1993b, 1995; Gidley et al., 1995; Shamai, Shimoni, & Bianco-Peled, 2004). There are also a few reports of retrograded amylopectin contributing towards type III RS (Chung, Lim, & Lim, 2006; Eerlingen, Jacobs, & Delcour, 1994; Russell, Berry, & Greenwell, 1989), an issue that needs further investigation. Several studies have shown that high amylose starches and products made from them have high RS contents measured *in vivo* which correlate with enzyme-resistant values determined *in vitro* (Akerberg, Liljeberg, & Bjorck, 1998; Thompson, 2005; Leeman, Karlsson, Eliasson, & Bjorck, 2006).

Extrusion cooking is a common food processing method for foods such as breakfast cereals and pasta products. Extrusion of starch in the presence of sufficient water triggers a number of physico-chemical and functionality changes in starch granules, such as the loss of granular structure associated with melting of crystallites and underlying helices, and the generation of an amorphous structure. This structure may later reacquire ordered helical or crystalline order (=retrogradation) and become resistant to digestion by human α-amylase. Various investigators have reported that retrogradation following extrusion cooking results in formation of enzyme-resistant starch (Eerlingen, Crombez, & Delcour, 1993a; Faraj, Vasanthan, & Hoover, 2004; Kim, Tanhehco, & Ng, 2006; Unlu & Faller, 1998; Vasanthan & Bhatty, 1998). In these studies, the acquisition of double-helical or crystalline order was considered to be critical for the observed amylase resistance. The inability of a starch double helix to fit into the active site of α-amylase is a plausible logic for the ability of retrograded structures to avoid enzymatic hydrolysis.

In this study, two high amylose maize starches have been subjected to each of two model extrusion conditions in a capillary rheometer. Products have been characterised by X-ray diffraction, solid state $^{13}C$ NMR, infrared spectroscopy and apparent amylose analysis both before and after digestion with α-amylase. Characterisation of these samples by X-ray scattering and electron microscopy has been reported elsewhere (Lopez-Rubio, Htoon, & Gilbert, 2007). The results suggest that high amylose starches can have relatively high levels of enzyme resistance even after processing to a near-amorphous state.

## 2. Materials and methods

### 2.1. Materials

High-amylose maize starches, Hylon VII (National Starch and Chemicals Pty Ltd., Seven Hills, Sydney, Australia) and Gelose 80 (Penfords Australia, Lane Cove, Sydney, Australia) with approximately 70% and 80% amylose contents, respectively, were used as raw materials. The initial moisture content of starches was determined by oven drying at 135 °C for 2 h, and the amount of water required to give 35% or 50% w/v calculated. The calculated amount of water was added to starches and mixed in a kitchen mixer (Kenwood) for 5 min. The resulting dough was kept at 4 °C overnight in plastic sealed bags for moisture equilibration to 35% or 50% w/v prior to extrusion.

### 2.2. Extrusion by capillary rheometer

Extrusion has been chosen as a model process to study the formation of enzyme-resistant retrograded starch. In order to carry out processing under well-defined conditions, a bench top capillary rheometer (Rosand RH2000 Rheometers, Bohlin Instruments Ltd., England) was used. The capillary rheometer can deliver controlled shear and stress over a wide temperature range, with conditions monitored by a comprehensive data acquisition and analysis package, providing a high level of control over extrusion conditions. The rheometer was configured into two different settings to achieve different processing conditions (Table 1). Low shear processing was designated 'mild' (M) and high shear processing was designated 'extreme' (E). Both Gelose (G) and Hylon (H) samples were processed by each method to give GM, GE, HM, and HE samples.

### 2.3. In-vitro digestion and RS Isolation

The processed samples were ground in a mortar and pestle, passed through a 500 μM particle size sieve and a portion was dried at 135 °C for 2 h to determine the moisture content. Granular or finely ground processed sample (80 g) was mixed with artificial saliva [250 U of α-amylase (Sigma) at pH 7.0 in 1.2 L]. After 15–20 s, the mixture was incubated with acidified (0.02 M HCl) pepsin (1 mg/mL; Sigma) at 37 °C for 30 min. The solution was adjusted to pH 6.0 (NaOH) and the samples were treated with pancreatin (2 mg)/amyloglucosidase (AMG, 28 U: Sigma) enzyme mixture for 18 h at 37 °C in 0.2 M acetate buffer at pH 6.0 in a shaking water bath. Samples were inactivated by adding an equal volume of 95% ethanol and centrifuged (2000*g*, 10 min). The supernatant was discarded and the residue was washed twice, first with 0.2 M acetate buffer (pH 6.0) followed by water and then freeze-dried. A schematic diagram for high amylose starch processing is given in Fig. 1. The *in vitro* (enzyme-) RS residues are denoted by RS following the code denoting raw material and process conditions such as GRRS, GMRS, GERS, HRRS, HMRS and HERS (see Fig. 1).

The yield of enzyme-resistant starch was determined by subjecting accurately-weighed quantities of samples (ca. 500 mg) from the *in vitro* digestion process described above. The starch content of freeze-dried products was determined by dissolving in dimethyl sulphoxide, followed by complete hydrolysis to glucose using thermostable α-amylase and amyloglucosidase, and enzymatic determination of glucose (Megazyme, Ireland).

### 2.4. Apparent amylose content

The apparent amylose content of raw and processed starches was determined by an iodine colorimetric method as described by Hoover and Ratnayake (2005). Sample (20 ± 0.1 mg) in a tube was dispersed with 8 mL 90% DMSO and vortexed for 2 min. A series of potato amylose (A0512, Sigma) and maize amylopectin (S9679, Sigma) mixtures at various concentrations was prepared (to construct a standard curve) and treated in the same way as samples. The tubes were heated in a water bath at 80 °C for 15 min with mixing, cooled to room temperature and diluted to 25 mL. One millilitre of diluted solution was mixed with 40 mL distilled-deionized water and 5 mL iodine reagent (0.0025 M $I_2$/0.0065 M KI) in a 50 mL volumetric flask and volume was adjusted.

**Table 1**
Extrusion parameters for high amylose starches processed in a capillary rheometer

| Processing conditions | Moisture (%) | Temperature (°C) | Shear rate ($s^{-1}$) | Die setting |
|---|---|---|---|---|
| Low shear, high moisture and low temperature ('mild') | 50 | 100 | 150 | 1 mm diameter, 8 mm length |
| High shear, low moisture and high temperature ('extreme') | 35 | 140 | 750 | 1 mm diameter, 8 mm length |

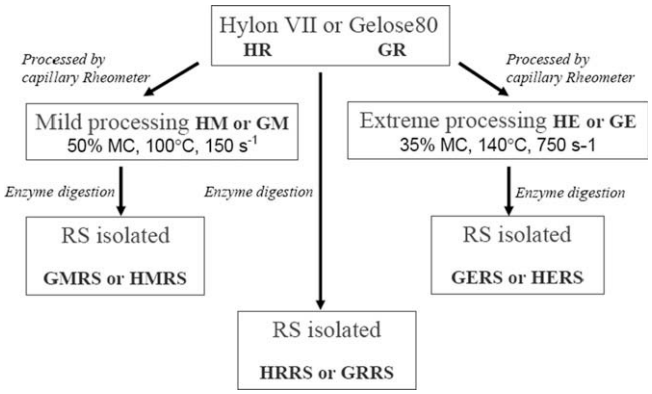


**Fig. 1.** Schematic diagram of RS isolation from high amylose starches; where, H (Hylon), G (Gelose), R (Raw), M (Mild processed), E (Extreme processed), and RS (isolated Resistant Starch fraction).

The solution was vortexed and incubated for 15 min for color development at room temperature. The absorbance was measured at 600 nm. The apparent amylose content was measured from the amylopectin/amylose calibration curve.

### 2.5. Fourier transform infra-red spectroscopy (FTIR)

FTIR spectra were obtained on a Spectrum FTIR 100 series Perkin-Elmer spectrometer (UK) fitted with DTGS (deuterated triglycine sulphate) detector using the Universal attenuated total reflectance (ATR) single reflectance cell with a diamond crystal. For each spectrum, 32 scans were recorded at room temperature (about 22 °C) at a resolution of 4 $cm^{-1}$, co-added and Fourier transformed. An aliquot of sample was placed over the diamond crystal and pressed gently with a small steel cylinder until an optimum force of 100 (an arbitrary scale) was reached and scanned over the range of 1200 to 800 $cm^{-1}$. The background spectra was recorded in air (with no sample) and subtracted from the sample spectra. All the scanned spectra were baseline-corrected using the in-built programme in the Spectrum FTIR 100 system. The amplitudes of absorbance for each spectrum at 995, 1022, and 1045 $cm^{-1}$ were noted from each sample.

### 2.6. Solid-state $^{13}C$ NMR spectroscopy

Solid starch samples were analysed by $^{13}C$ NMR spectroscopy before and after processing, and after subsequent enzyme-digestion using the spectral acquisition and interpretation methodology described in Tan, Flanagan, Halley, Whittaker, and Gidley (2007). This provides a quantitative analysis of double helices, single helices, and amorphous conformational features within solid starch samples. The method gives accurate (±2%) quantification of double helix and single helix contents, but accuracy is reduced for samples with very low (<10%) total helix content as the method involves the subtraction of amorphous spectral signals from the sample spectrum, so a low helix content results in almost complete subtraction of signals, with resulting uncertainty in the residual double/single helix signal intensity.

### 2.7. X-ray diffraction (XRD)

X-ray diffraction was carried out on a Panalytical X'Pert Pro diffractometer. The instrument was equipped with a Cu long fine focus tube, programmable incident beam divergence slit and diffracted beam scatter slit (both fixed at 0.125°) and an X'celerator high speed detector. The samples were examined over the angular range of 2–40 degrees with a step size of 0.0332° and a count time of 800 s per point. Crystallinity determination was carried out using the X'Pert software. This programme automatically determines the amorphous hump of the diffraction pattern and the crystallinity can be easily calculated from the intensity ratio of the diffraction peaks ($I_{net}$) and of the sum of all intensity measured ($I_{total}$):

$$\text{Crystallinity}(\%) = 100 \times (I_{net})/I_{total}$$

## 3. Results and discussion

### 3.1. Capillary rheometer extrusion

The apparent shear viscosity (ASV) of extruded high amylose starches obtained from the capillary rheometer is in the range of 5–11 kPa s (Table 2). The values are similar to those reported previously for other starches (Alavi, Chen, & Rizvi, 2002; Singh & Smith, 1999). The ASV and the calculated specific mechanical energy (SME) (Alavi et al., 2002) of Hylon VII were slightly higher than Gelose 80 which may be due to the slightly higher amylose content. High amylose starches have been reported to produce higher shear viscosity in screw extrusion processing than their normal counterpart (Kokini, Ho, & Karwe, 1991) because of their greater proportion of linear molecular structures reinforced by hydrogen bonds which are less affected by processing (Bhattacharya & Hanna, 1987).

The SME of high amylose starches obtained from the capillary rheometer are in the range of 30–136 kJ/kg (Table 2) indicating low moisture and high shear processing ('extreme') has generated 3.5 to 4 times higher energy input than the high moisture and low shear processing ('mild'). From the SME values it is likely that the decrease in viscosity in both starches under extreme processing is due to the greater mechanical energy input. According to Wang, Chiang, Yeh, Zhao, and Kim (1989) the molecular changes occurring under our 'mild' processing could be regarded mostly as thermal diffusion conversion, with 'extreme' processing as a combination of thermal and mechanical conversion. Generally, screw shear extrusion processing (as often used in large scale processing) produces significantly higher SME and lower ASV values than die shear capillary processing. It has been reported that rice starch extruded at 100 °C, 57% moisture, 225 rpm at 5 kg/h mass flow rate generated 150 kJ/kg of SME on a twin screw extruder (Akdogan, 1996), whereas, cornmeal extruded on a twin screw ex-

**Table 2**
Apparent shear viscosity and mechanical energy data for high amylose maize starches processed by capillary rheometer

| | Hylon VII | | Gelose 80 | |
|---|---|---|---|---|
| | Mild processing(100 °C, 150 $s^{-1}$, 50% moisture) | Extreme processing (140 °C, 750 $s^{-1}$, 35% moisture) | Mild processing (100 °C, 150 $s^{-1}$, 50% moisture) | Extreme processing (140 °C, 750 $s^{-1}$, 35% moisture) |
| Apparent shear viscosity (kPa s) | 11.2 ± 1.5 | 7.3 ± 0.6 | 7.4 ± 0.2 | 5.3 ± 0.2 |
| Specific mechanical energy (kJ/kg) | 39 ± 8 | 136 ± 10 | 30 ± 2 | 122 ± 16 |

**Table 3**
Apparent amylose and resistant starch contents of raw and processed Gelose and Hylon[1]

| Samples | Amylose[2] (%) | Resistant starch[3] (%) |
|---|---|---|
| *Gelose 80* | | |
| GR | 74.8 ± 3.9 | 45.7 ± 1.3 |
| GM | 71.6 ± 4.8 | 15.6 ± 0.1 |
| GE | 67.5 ± 0.3 | 15.6 ± 0.2 |
| GMRS | 40.3 ± 0.5 | 45.2 ± 1.0 |
| GERS | 34.4 ± 0.4 | 41.2 ± 2.0 |
| GRRS | 64.4 ± 1.0 | 55.5 ± 1.4 |
| *Hylon VII* | | |
| HR | 81.1 ± 1.4 | 60.0 ± 2.3 |
| HM | 77.5 ± 0.6 | 13.8 ± 0.4 |
| HE | 71.5 ± 0.7 | 16.9 ± 0.0 |
| HMRS | 48.9 ± 0.2 | 35.1 ± 1.8 |
| HERS | 44.2 ± 0.4 | 24.8 ± 2.2 |
| HRRS | 60.3 ± 1.5 | 52.5 ± 1.2 |

[1] Values are reported in 'g/100 g, as is' basis.
[2] Mean ± standard deviation (of duplicate).
[3] Mean ± standard deviation (of triplicates).

truder at 140 °C, 30% moisture, 500 rpm (845 $s^{-1}$) at 30 kg/h showed SME of 360 kJ/kg and ASV of 0.6 kPas (Chang, Martinez-Bustos, Park, & Kokini, 1999). Therefore, conditions used here in a capillary rheometer would not be expected to generate equivalent samples in a screw extruder.

### 3.2. Apparent amylose content

The apparent amylose content of all starches was measured by the iodine binding capacity of amylose-iodine inclusion complexes (colorimetric method). Table 3 shows the apparent amylose content of granular ('raw') Gelose, Hylon and their processed products. The amount of apparent amylose in Hylon (81.1%) was slightly higher than previously reported values of 66.5% (Themeier, Hollman, Neese, & Lindhauer, 2005 using AOAC method); 71% (Shi, Capitani, Trzasko, & Jeffcoat, 1998 by potentiometric iodine method); 75.5% (Jiang & Liu, 2002, methods not known); and 70% (National Starch, Bridgewater, USA). For Gelose, the resultant apparent amylose content (74.8%) is slightly lower than 80%, as given in manufacturer's product specification (Penford Starch, Australia).

Extrusion under the given set of conditions reduced the apparent amylose content of both starches (Table 3). Based on initial amylose content, there was about 5% reduction during mild processing and about 10% reduction in extreme processing for both Gelose and Hylon. Extrusion processes involve the application of heat, high pressure, and shear forces that cause gelatinization of starches and may result in molecular degradation (Vergnes, Villemaire, Colonna, & Tayeb, 1987). Amylose is a long, mostly linear glucose polymer with a typical molecular weight of about $10^5$–$10^6$ corresponding to degree of polymerization of 500–5000 (Takeda, Hizukuri, Takeda, & Suzuki, 1987). The gelatinised amylose under extrusion may undergo degradation resulting in shorter amylose molecules with a reduced iodine binding capacity, thereby lowering the overall apparent amylose content of starches. Various investigators have also reported the lowering of amylose chain length during extrusion (Unlu & Faller, 1998; Valle, Colonna, & Patria, 1996).

Apparent amylose contents of residues from mild and extreme-processed Gelose and Hylon after 18 h digestion with starch-hydrolysing enzymes, *i.e.*, GMRS, GERS, HMRS and HERS, are also shown in Table 3. All the processed starches showed a marked decrease in apparent amylose content: a decrease of 56% and 51% for mild and extreme-processed Gelose and 63% and 62% mild and extreme-processed Hylon, respectively. In a similar study, Jiang and Liu (2002) reported apparent amylose content of 70.6% and 62.7% after 2 and 48 h incubation of raw Hylon VII (75.5% apparent amylose). Our study showed a much greater reduction in apparent amylose content for extruded high-amylose starches. This could be due to the fact that the starches after extrusion are no more in a granular state. For raw starches, it is suggested that resistant fractions are likely to contain non-attacked granules with native apparent amylose contents. However, resistant fractions from processed starches are more likely to be molecularly-resistant low molecular weight amyloses, showing a reduced apparent amylose value.

### 3.3. Enzyme-resistant starch contents in extruded and spray dried samples

Enzyme-resistant starch contents of raw and processed Gelose and Hylon, and the residues of these starches after 18 h incubation with pancreatic amylase are shown in Table 3. As expected, the enzyme-resistant starch (RS) contents of Gelose and Hylon were quite high at 45.7% and 60%, respectively. A range of RS values have been reported for Hylon VII such as 54.4% by Themeier et al. (2005), 69.5% by Evan and Thompson (2004), 52.5% by Jiang and Liu (2002). No literature value was available for RS content of Gelose 80. Comparison of RS content from a particular starch with values obtained in other studies is difficult due to the different methodologies used (Goni, Garcia-Diz, Manas, & Saura-Calixto, 1996). Faisant et al. (1993b) reported three different RS values for three different methods for retrograded and complexed starch. The current method is intended to simulate starch digestion in humans and accurately predicts the RS content of foods as consumed. Only assays that gave acceptable RS values of 2.0–2.9% for reference material (Kellogg's corn flake) were accepted.

Extrusion cooking of high amylose starches significantly reduced the resistant starch content, with all processed samples having a similar RS content (13.8–16.9%). The degree of reduction in RS content compared with the granular form was higher for Hylon (77% in HM and 72% in HE, with respect to HR) compared to Gelose (66% in GM and GE, with respect to GR). Starch granules are disrupted by thermal and mechanical energy during extrusion cooking which would be expected to increase accessibility of amylases to starch polymers. Upon cooling, hydrated amylose (and amylopectin) chains may undergo a process ('retrogradation') of molecular re-association into double helices, stabilised by hydrogen bonds, and may consequently acquire resistance to enzymic digestion. However, the relatively low moisture content and rapid drying that occurs in extrusion and model processes such as described here would not be expected to result in extensive retrogradation. Other studies have shown that extrudates of high amylose starches favour formation of RS more than extrusion of low amylose flours (Faraj et al., 2004; Unlu & Faller, 1998; Vasanthan, Gaosong, Yeung, & Li, 2002). The extrudates in this study were immediately dried and ground into fine powder. It is likely that the current processing conditions did not favour the retrogradation of Gelose/Hylon and the fragmented starches remained more or less in an amorphous condition.

Faraj et al. (2004) also reported that extrusion followed by drying and grinding of barley flours decreases RS content, especially at low moisture/very low or high temperature. Other extrusion studies also showed lower or similar RS values for, *e.g.*, barley flour (Ostergard, Bjorck, & Vainiopaa, 1989), rice and amaranth starch (Parchure & Kulkarni, 1997), or wheat flour (Siljerstrom et al., 1986). Other studies involving extrusion at high moisture and, particularly, after promotion of retrogradation by appropriate storage conditions, have shown increases in the RS content of starch. Kim et al. (2006) reported an 11 times increase in RS content of wheat pastry flour when extruded at higher feed moisture and stored at 4 °C for 7–14 days. Other investigators have also reported higher yields of RS (up to 38%) when high amylose corn starch was ex-

truded at high moisture content (Sievert & Pomeranz, 1989; Unlu & Faller, 1998).

### 3.4. Resistant starch contents in enzyme-hydrolysed residues

The resistant residues from enzyme-treated raw and processed Gelose and Hylon were subjected to a second round of enzyme-digestion to determine if the RS values obtained after 18 h were due to a limit being reached in digestion. The results (Table 3) showed significant RS contents for all samples (GRRS, GMRS, GERS, HRRS, HMRS, and HERS), with values between 25% and 55%. This shows that, for all samples, 18 h of enzyme-digestion does not lead to a fully enzyme-resistant residue. The implication is that, physiologically, it is likely that resistant starch entering the colon is not completely resistant to enzyme-digestion in the conditions of the small intestine, but that the kinetics of digestion are sufficiently slow that gut transport leads to passage into the colon before sufficient time has elapsed for digestion in the small intestine. In other words, the time required for complete hydrolysis exceeds upper gut transit time. This suggestion is consistent with studies in human ileostomates showing that transit time in the upper gut and starch excretion at the terminal ileum are inversely related (Silvester, Englyst, & Cummings, 1995). For both Hylon and Gelose starches, the raw form has the highest 'second round' enzyme resistance (55%, 52%, Table 3), and the extreme-processed samples has the lowest (25%, 41%, Table 2). This suggests that the extent of structural disorganisation is a determinant of enzyme digestibility, even after 2 × 18 h treatments. Jiang and Liu (2002) also reported a kinetic effect on the degree of hydrolysis of 13%, 16%, and 20% for Hylon VII after incubation with pancreatic amylase for 5, 25, and 48 h. They also reported that raw Hylon yielding 52.5% RS generates a residue of 52.9 and 55.7% RS when subsequently incubated with enzymes for 2 and 48 h, respectively. Compared to Hylon, residues from digested Gelose contained slightly higher RS than raw Gelose.

### 3.5. Molecular and crystalline order before and after enzyme-digestion

#### 3.5.1. NMR spectroscopy and X-ray diffraction

Fig. 2a shows $^{13}$C NMR solid state spectra for starch samples before and after digestion. Relative intensities of bands are analysed quantitatively as a combination of double helices (B-type), single helices (V-type) and amorphous chains (Tan et al., 2007) with results shown in Table 3. Quantitative analysis involves the subtraction of a standard amorphous starch spectrum from the test spectrum until there is no residual intensity at 84 ppm (a region of the spectrum with intensity due solely to amorphous conformations). Example non-amorphous spectra obtained on subtraction are shown in Fig. 2b. The main intensities for C-1 (95–105 ppm) is centred on 100–101 ppm as expected for B-type double helices, with a lesser intensity due to V-type helices with characteristic intensities at 103 and 81 ppm. The relatively unresolved spectral

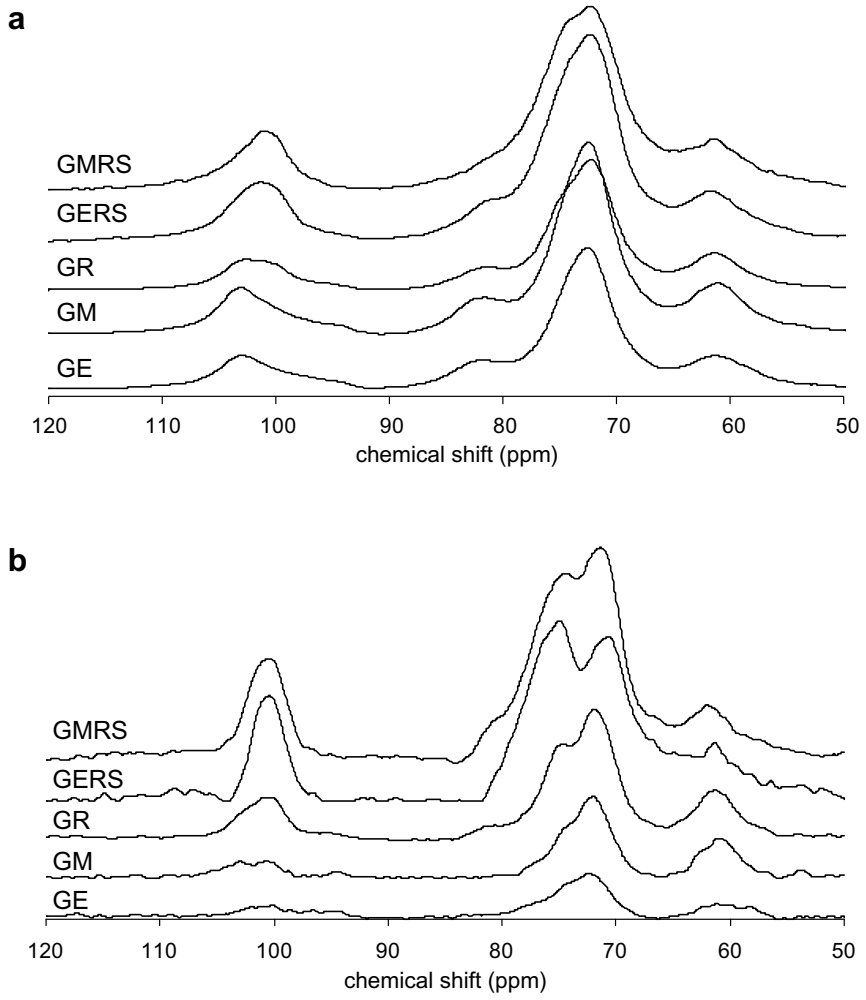


**Fig. 2.** (a) $^{13}$C solid state NMR spectra for Gelose-based samples, (b) $^{13}$C solid state NMR spectra for Gelose-based samples after subtraction of amorphous component (Tan et al, 2007).

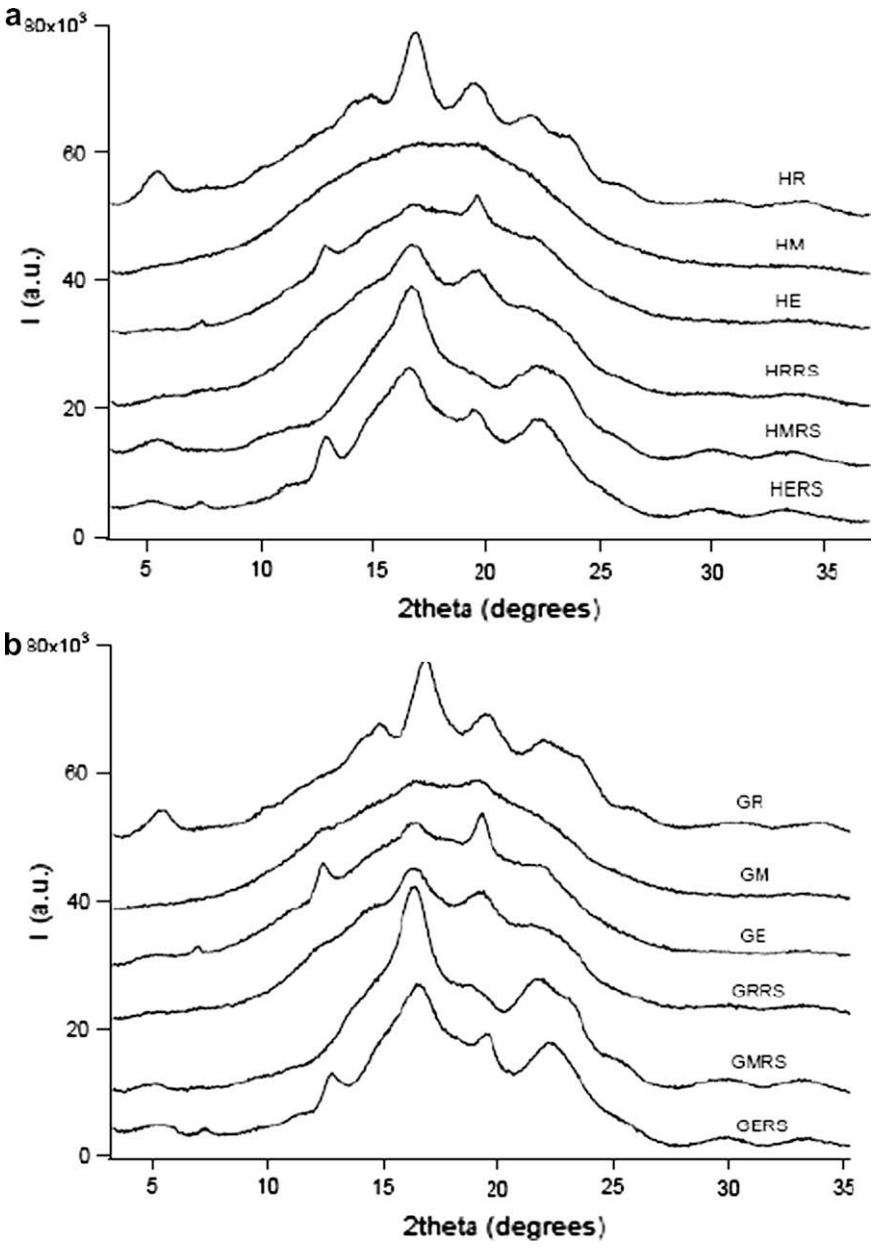


**Fig. 3.** X-ray diffractograms of (a) Hylon and (b) Gelose before and after processing and digestion. Data have been offset for clarity.

features of the non-amorphous spectrum are consistent with a relatively low level of crystalline register of double helices (Gidley & Bociek, 1985). Fig. 3 depicts X-ray diffraction traces for samples before and after processing and digestion. Major signals are consistent with mostly B-type crystallinity (peaks at around 5.5°, 15°, 17°, 22.2°, 23.7° and 26.2° $2\theta$) with some evidence for V-type peaks (*e.g.*, at $2\theta \sim 19.8°$). NMR spectroscopy and X-ray diffraction therefore show similar ordered types. However, the levels (Table 3) of crystalline order (X-ray diffraction) were consistently lower than those for helix content (NMR), suggesting that not all of the starch molecules ordered helically are arranged into crystals, as observed previously for granular starches (Gidley & Bociek, 1985). Quantitative analysis of processed and enzyme-resistant Hylon and Gelose starches (Table 3) shows that all processed samples have a low (but non-zero) level of molecular order (<13%) and crystallinity (<6%). However, levels of molecular and crystalline order are significantly higher for enzyme-resistant residues than for the processed samples from which they were derived (24–40% and 19–21%, respectively). In contrast, levels of crystalline order were low in enzyme-resistant residues from raw (granular) starch. These results show that:

i. high levels (>10%) of resistant starch can be obtained for processed high amylose maize starches with low levels of molecular and crystalline order,
ii. increases in order/crystallinity must have occurred during the enzyme-digestion process for at least some starches, as *e.g.*, there is a ten-fold increase in crystallinity for mild processed Hylon (2.1% vs. 20.3%) whereas the yield of the resistant residue was 14%.
iii. The relationship between crystallinity and enzyme resistance is different for processed samples (evidence for enhanced crystallinity) and granular starches (major decline in crystallinity levels).

*3.5.2. FTIR spectroscopy*

FTIR-ATR spectroscopy has been used to further probe the changes in structure and physico-chemical properties of high-amylose starches following extrusion and enzyme-digestion. The IR spectra of "raw", "mild" and "extreme" processed and enzyme-digested Gelose 80 and Hylon VII are presented in Figs 4 and 5, respectively. Gelatinised (5% w/v starch boiled in water with stirring for 20 min) and freeze-dried regular maize starch was used as a reference for ~100% amorphous material. The spectral region of 800–1200 $cm^{-1}$ was used in this study as previous studies have suggested that bands in this 'fingerprint' region reflect changes in polymer conformation and hydration of processed starches (Bello-Perez, Ottenhof, Agama-Acevedo, & Farhat, 2005; Goodfellow & Wilson, 1990; van Soest, Tournois, de Wit, & Vliegenthart, 1995 ). These studies also showed that band intensities at ca. 1045, 1022, and 995 $cm^{-1}$ are sensitive to changes in starch conformation as inferred from, *e.g.*, X-ray diffraction (Goodfellow & Wilson, 1990; van Soest et al., 1995) or differential scanning calorimetry (Bello-Perez et al., 2005). The bands at 1047 (or 1045) and 1022 $cm^{-1}$ have been linked with order/crystallinity and amorphous regions in starch, respectively (Bello-Perez et al., 2005; Sevenou, Hill, Farhat, & Mitchell, 2002; van Soest et al., 1995). Intensity ratios of 1045/1022 and 1022/995 $cm^{-1}$ may therefore be useful as a convenient index of FTIR data in comparisons with other measures of starch conformation.

The relative intensity of FTIR bands for all samples was recorded from the baseline to peak height and the ratios for 1045/1022 and 1022/995 calculated (Table 4). This showed that the semi-crystalline raw starches and enzyme-digested residues of extrudates were generally identified with higher ratios for 1045/1022 (and lower ratios for 1022/995). Conversely, the extruded 'amorphous' starches were identified with higher ratios for 1022/995 (and lower ratios for 1045/1022). These results are in qualitative agreement with data from NMR and X-ray diffraction and resistant starch analysis that showed the crystalline nature of "raw" and "enzyme-digested" starches and the near

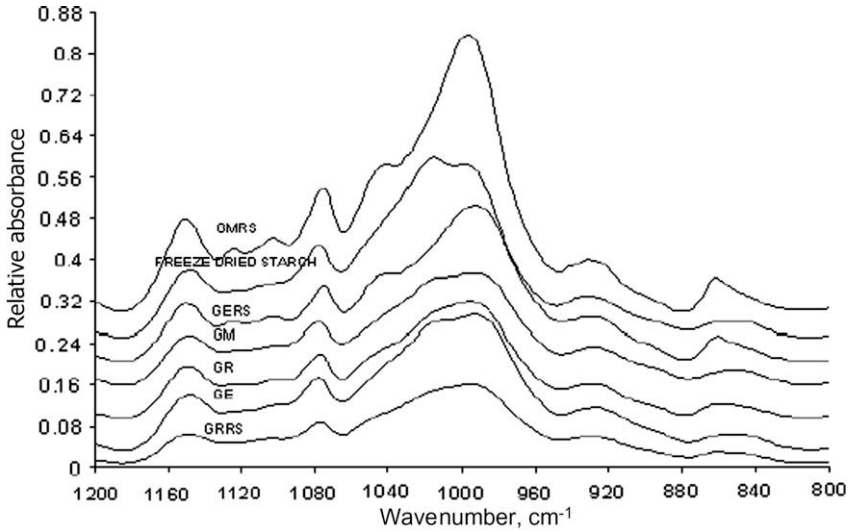


**Fig. 4.** Influence of processing and enzyme hydrolysis on Gelose 80 compared with an amorphous standard [gelatinized-freeze dried maize starch] as shown by FTIR-ATR spectra. Data have been offset for clarity.

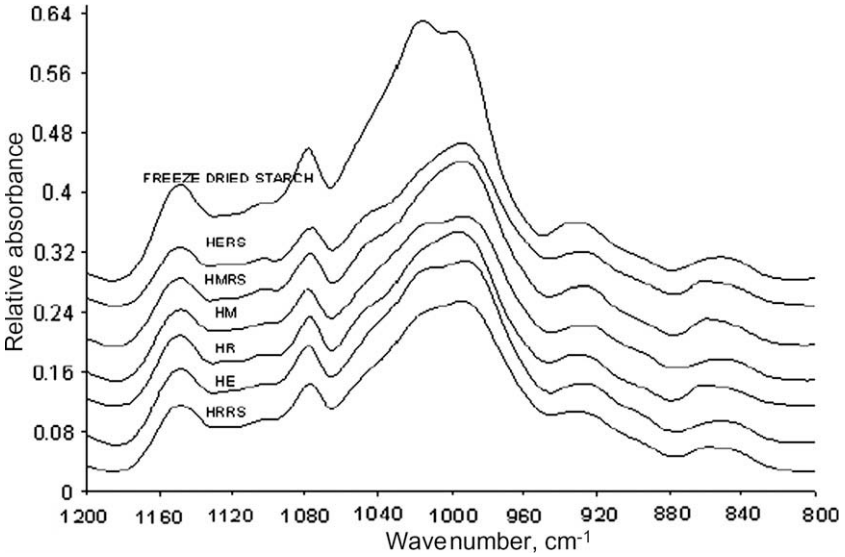


**Fig. 5.** Influence of processing and enzyme hydrolysis on Hylon VII as shown by FTIR-ATR spectra. Data have been offset for clarity.

**Table 4**
Comparison of NMR, XRD and FTIR-ATR results

| Starches | NMR | | | XRD% crystallinity | FTIR 1045/1022 ratio | FTIR 1022/995 ratio |
|---|---|---|---|---|---|---|
| | % V-type polymorph | % Double helix | % Amorphous | | | |
| HR | 6 | 24 | 70 | 17.6 | 0.717 | 0.849 |
| HM | <2 | <10 | >90 | 2.1 | 0.717 | 0.904 |
| HE | <2 | <10 | >90 | 4.2 | 0.709 | 0.897 |
| HRRS | 2 | 19 | 79 | 6.6 | 0.727 | 0.885 |
| HMRS | 1 | 36 | 63 | 20.3 | 0.822 | 0.835 |
| HERS | 0 | 24 | 76 | 20.2 | 0.77 | 0.801 |
| GR | 8 | 26 | 66 | 17.4 | 0.793 | 0.921 |
| GM | 1 | 11 | 88 | 2.4 | 0.782 | 0.916 |
| GE | 0 | 10 | 90 | 5.5 | 0.766 | 0.890 |
| GRRS | 1 | 17 | 82 | 6.5 | 0.75 | 0.886 |
| GMRS | 1 | 37 | 62 | 20.7 | 0.845 | 0.706 |
| GERS | 0 | 40 | 60 | 19.3 | 0.824 | 0.689 |

amorphous nature of extruded starches. Note, however, that the FTIR spectra of extruded (and freeze-dried) high amylose maize starches differ from that of regular maize starch gelatinised by boiling in excess water and subsequently freeze-dried. This suggests a (low) level of order within extruded high amylose starches that agrees with NMR and X-ray diffraction measurements (Table 4). For each high amylose starch, the "extreme" processed starches had the lowest 1045/1022 ratio and highest 1022/995 ratio suggesting a more disordered starch conformation than "mild" processed starches. This is in apparent contradiction to X-ray data (Table 4) which showed (slightly) higher crystallinity in "extreme" processed samples; however the latter were mainly associated with V-type single helix structures, not the B-type double helix structures that dominate in native and enzyme-resistant starches.

The residues of enzyme-digested processed starches had intensity ratios associated with a more ordered form than before enzyme treatment. The intensity ratios were consistent with GMRS and HMRS being more crystalline (or less amorphous) than GERS and HERS, respectively, as also reflected by NMR and X-ray diffraction analysis. However, FTIR spectra showed little apparent change in the molecular order of "raw" starches after digestion (Table 4) which is contradictory to X-ray diffraction results that showed a marked decrease in crystallinity of 17.6–6.6% in Hylon VII and 17.4–6.5% in Gelose. We propose that, although useful for comparison between closely similar samples (*e.g.*, time courses of retrogradation), there are limits to the use of FTIR spectroscopy for quantifying molecular order in starches. This is most likely due to the complex nature of the underlying vibrations responsible for the observed bands, which makes detailed de novo assignment of bands impossible at present. In contrast, NMR and X-ray diffraction data are fully assigned, leading to a lower level of uncertainty in quantifying molecular order by these techniques. A more detailed analysis of the utility and limitations of FTIR data for analysis of starch structures will be reported elsewhere (Flanagan, Shrestha and Gidley, in preparation).

### *3.5.3. Different mechanisms are involved in enzyme-digestion of granular and processed forms of high-amylose maize starch*

Crystallinity or molecular order is often cited as a determinant of enzyme resistance for isolated starches, often designated RSII (granular starch) or RSIII ('retrograded' starch). However, this study has shown that this is not the case for high-amylose maize starches. Fig. 6 plots% crystallinity (from X-ray diffraction) against the yield of enzyme-resistant starch *in vitro* (data from Table 4). There is clearly no general relationship between the two measurements. A similar lack of correlation is seen for NMR molecular order% vs. enzyme-resistant starch content.

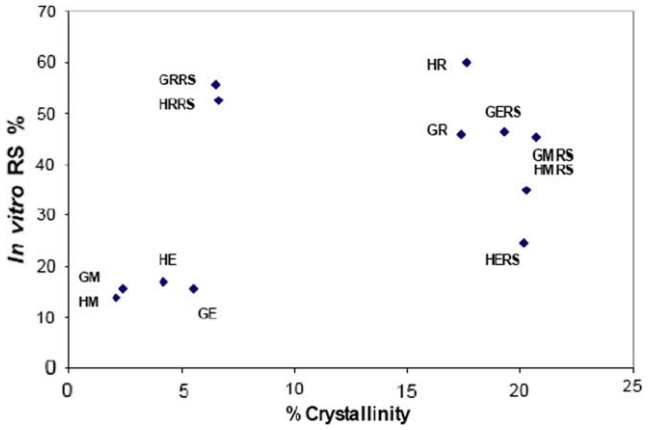


**Fig. 6.** Enzyme-resistant starch levels compared with crystallinity from X-ray diffraction for all samples studied.

Fig. 6 also illustrates a major difference between granular and processed high-amylose maize starches. Granular starches (GR/HR) lose much of their crystallinity on being subjected to one round of enzyme-digestion to yield GRRS/HRRS, but these samples themselves retain a high level of enzyme resistance to a second round of enzyme treatment (>50%). In contrast, processed starches (GE/GM/HE/HM) all show an increase in crystallinity following enzyme-digestion, and maintain a moderately high level of resistance (25–50%) on subsequent enzyme treatment. This qualitative difference in behaviour is consistent with their different categorisation as RSII and RSIII, but the present data calls into question the association of RSIII with starch that is retrograded prior to enzyme-digestion.

## 4. Conclusions

This study has identified a number of factors relevant to a greater understanding of enzyme-resistant starch:

- High-amylose starches, both raw and processed, provide significant levels ($\gg$10%) of enzyme-resistant starch, independent of the type of processing used.
- Molecular or crystalline order is not a pre-requisite for significant enzyme resistance, as near amorphous forms of high-amylose maize (such as the mild processed ones) deliver high RS contents *in vitro*.
- The comparatively high crystallinity present in enzyme-resistant fractions from processed starches suggests that molecular reorganisation is likely to take place during the enzyme-digestion process.

- The mechanism of enzyme resistance of granular high-amylose starches is not related to crystallinity and is qualitatively different to that for processed starches.
- Measured enzyme-resistant starch levels represent a single time-point in a continuing digestion process, i.e., the level of enzyme-resistant starch is under kinetic more than thermodynamic control.

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