

## Enzyme-Resistant Starch. V. Effect of Retrogradation of Waxy Maize Starch on Enzyme Susceptibility

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### ABSTRACT

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Gelatinized waxy maize starch (100% amylopectin) was subjected to different time and temperature conditions of storage to obtain samples with differences in the extent of amylopectin retrogradation. The resulting samples showed melting endotherms with varying onset, peak, and completion temperatures, as well as varying melting enthalpies. X-ray diffraction

also showed variance in the degree of crystallinity. Increased retrogradation extents (higher melting temperatures, melting enthalpies, and higher crystallinity levels) caused reduced enzyme susceptibilities to pancreatic  $\alpha$ -amylase and amyloglucosidase at 37°C.

Results from a number of studies (Berry 1986; Berry et al 1988; Siljeström et al 1989; Sievert and Pomeranz 1989, 1990; Czuchajowska et al 1991; Eerlingen et al 1993) led to the conclusion that resistant starch type III, formed after gelatinization of starch, consists mainly of retrograded amylose. However, in most studies, storage conditions were not optimized to favor amylopectin retrogradation, or else resistant starch was determined after hydrolysis at 100°C with a heat-stable  $\alpha$ -amylase. At such a high temperature, retrograded amylopectin is expected to be melted because an endotherm at  $\sim 60^\circ\text{C}$  is observed with differential scanning calorimetry when retrograded amylopectin is heated in water.

Amylopectin consists of  $3 \cdot 10^5$ – $3 \cdot 10^6$  glucose residues (Zobel 1988). It is highly branched. Three types of chains can be distinguished. Short S chains with a degree of polymerization (DP) of 14–18 glucose residues, are organized in a cluster structure (Robin et al 1975). Inner, long L chains have a DP of 45–55. A single chain of DP  $>60$  bears the reducing end-group (Mercier 1973, Watanabe and French 1980). Polymodal chain distributions have, however, also been reported (Hizukuri 1986). Retrogradation of amylopectin includes a crystallization process of the outer short branches (DP 14–18). In contrast to what is observed with

amylose, the crystallization of amylopectin is a slow process continuing over a period of several days or weeks. Due to the limited dimensions of the chains, the stability of these crystallites is lower than that of amylose crystallites. As mentioned above, a melting endotherm at  $\sim 60^\circ\text{C}$  can be observed when retrograded amylopectin is heated, while melting temperatures of  $\sim 150^\circ\text{C}$  are found for amylose crystallites. In general, crystallization, consisting of nucleation, propagation, and maturation (slow crystal growth and crystal perfection), strongly depends upon temperature. In a partially crystalline polymer system such as the starch gel, the crystallization process can only occur within the temperature range between the glass transition temperature of the system and the melting temperature of the crystals—the rubbery state (Levine and Slade 1990). In the case of crystallization of amylopectin in a starch gel, the glass transition temperature is  $\sim -5^\circ\text{C}$  and the melting temperature of the B-type crystals is  $\sim 60^\circ\text{C}$ . Therefore, the crystallization, and thus the retrogradation of amylopectin, can be influenced by the time and temperature conditions of storage.

In this study, we investigated whether the differences in retrogradation grade, obtained by subjecting the gelatinized waxy maize starch to different time and temperature conditions of storage, caused changes in the enzyme susceptibility at 37°C.

### MATERIALS AND METHODS

#### Materials

Waxy maize starch (Meriwax, 100% amylopectin) was supplied by Amylum (Aalst, Belgium). It contained 11.5% moisture

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(method 44-15A, AACC 1983) and was used as such in the experimental work. Enzymes used for the determination of resistant starch were pancreatin (P1625, Sigma Chemical Co., St. Louis, MO) with an  $\alpha$ -amylase activity of 3 U/mg (one U is defined as the amount of enzyme converting 1 mg of potato starch into soluble carbohydrates after 5 min in water at 40°C); amyloglucosidase from *Aspergillus niger* (Novo Nordisk, Bagsvaerd, Denmark) with an activity of 300 AGU/ml of preparation (one AGU is the amount of enzyme that hydrolyzes 0.342 mg [ $1 \mu M$ ] of maltose at 25°C in an acetate buffer, pH 4.3, in 1 min). The glucose oxidase colorimetric kit for glucose determination (nr166391, GOD-PAP) was from Boehringer (Mannheim, Germany).

### Formation of the Starch Gels

Starch (500 mg) was mixed with 0.5 ml of water containing 1.7 mg of calcium propionate (preservative) and was autoclaved for 60 min at 121°C. The autoclaved starch samples were stored at different time and temperature combinations: 48 hr at room temperature; 24 hr at 6°C, followed by 48 hr at 40°C; and 24 hr at 6°C, followed by 29 days at 40°C. These time and temperature combinations were chosen from a fundamental point of view to obtain significant differences in the degree of retrogradation. At 6°C, nucleation is favored; at 40°C, propagation is promoted. Maturation is also favored at 40°C. Thus, increased retrogradation is expected with storage at 6°C and, subsequently, at 40°C, instead of storage at room temperature. Also, because amylopectin

retrogradation is a slow process, increased retrogradation is expected with storage of the starch sample for several weeks.

### Differential Scanning Calorimetry

Differential scanning calorimetry (Seiko DSC-120, Kawasaki Kanagawa, Japan) was used to determine the thermal characteristics of the native starch, the freshly gelatinized starch, and the retrograded starch samples. Indium and tin were used as standards. Measurements were performed at least in triplicate. In the analyses, starch (~5 mg) was accurately weighed in aluminum pans, and the same weight of water was added. The sample pans were analyzed (native starch) or autoclaved and stored, in the same way as outlined above, before thermal analysis. An empty pan served as the reference sample, and the starch samples were heated from 20 to 130°C at a scanning rate of 4°C/min.

### X-Ray Diffraction Analysis

X-ray diffraction analysis was performed on native starch and on freeze-dried gels (moisture contents of ~8%) with a PW 10050/25 diffractometer equipped with a proportional detector PW 1965/20 (Philips, MBL, Brussels, Belgium). The following operating conditions were used: 30 kV and 20 mA with Co radiation = 0.179 nm. Diffractograms of the samples were obtained from 3° 2- $\theta$  to 30° 2- $\theta$ .

### Enzymic Hydrolysis of the Starch

Hydrolysis was performed with a suspension of 500 mg of raw waxy maize starch and 0.5 ml of water (containing 1.7 mg of calcium propionate), as well as on freshly autoclaved (gelatinized) starch and on retrograded starches. In some instances, gel samples were disrupted by ultra-turrax mixing (4 min at 10,000 rpm) before enzymic hydrolysis. Samples were incubated at 37°C in sodium acetate buffer (20.0 ml, 0.1M, pH 5.2) containing 47 U of pancreatic amylase per mg of starch and 0.13 AGU of amyloglucosidase per mg of starch. The extent of hydrolysis was determined by measuring the production of glucose at different time intervals. To that end, 0.5 ml of the sample was added to 20.0 ml of solution containing 66% ethanol. This mixture was centrifuged for 5 min at 1,000  $\times g$ . Glucose was then determined with 100  $\mu L$  of the supernatant using the glucose test kit.

## RESULTS AND DISCUSSION

### Differential Scanning Calorimetry

Table I shows the thermal characteristics of native waxy maize starch, freshly gelatinized waxy maize starch, and retrograded waxy maize starch samples obtained after different time and temperature conditions of storage.

**Native waxy maize starch.** Native granular waxy maize starch showed three endotherms (Fig. 1) with peak temperatures ( $T_p$ ) of 69.5, 81.1, and 102.1°C, when heated in water (starch-water ratio of 1:1, w/w). The total enthalpy value was 11.5 mJ/mg of starch (dry weight). Russell (1987) also observed three endothermic transitions ( $T_p$  at 73, 90, and 103°C) when waxy maize starch was heated in the same amount of water. When higher water contents were applied, only one endotherm occurred. For a starch-water ratio of 1:1.33 (w/w), an endotherm at 69.8°C ( $T_p$ ) appeared with an enthalpy value of 14.3 mJ/mg of starch.

In contrast to a triphasic profile, a biphasic endothermic process is generally observed when starch is gelatinized at low moisture levels. Different explanations have been proposed for this observation (Marchant and Blanshard 1978, Donovan 1979, Colonna and Mercier 1985, Russell 1987).

None of these authors dealt with the nature of a third peak ( $T_p$  at 102°C) occurring in waxy maize starch. This endotherm could not be attributed to the melting of amylose-lipid complexes, as was the case of normal maize starch, because waxy starch contains only a very small amount of both amylose and lipids. However, the third peak may be attributed to recrystallization during heating.

**Freshly gelatinized waxy maize starch.** Immediately after auto-

TABLE I  
Thermal Transition Temperatures (°C)<sup>a</sup> and Enthalpies<sup>b</sup> of Native, Freshly Gelatinized, and Retrograded Waxy Maize Starch

Starch	$T_0$	$T_p$	$T_c$	$\Delta H$
Native	60.7	69.5/81.1/102.1 <sup>c</sup>	111.1	11.5
Gelatinized	...	...	...	...
Retrograded				
48 hr at room temperature	40.7	59.7	71.9	8.2
24 hr at 6°C and 48 hr at 40°C	56.9	65.0	72.0	9.5
24 hr at 6°C and 29 days at 40°C	64.2	72.5	79.2	11.4

<sup>a</sup>  $T_0$  = onset,  $T_p$  = peak,  $T_c$  = completion.

<sup>b</sup>  $\Delta H$  in mJ/mg of starch.

<sup>c</sup> Three  $T_p$  were noticed.

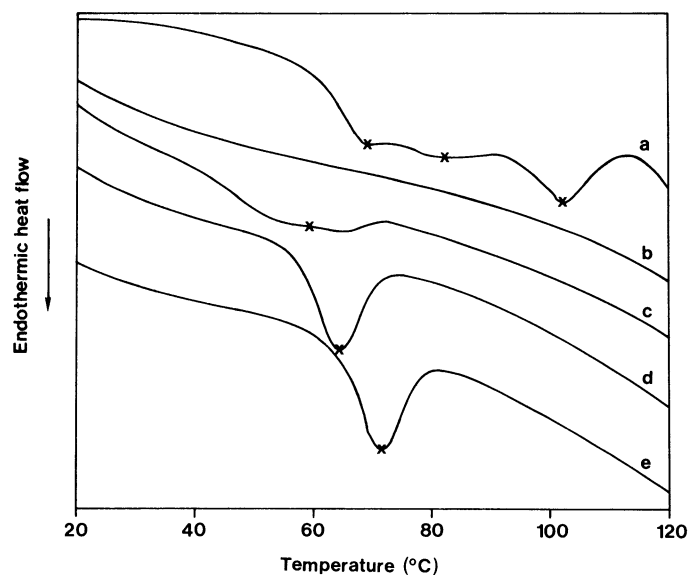
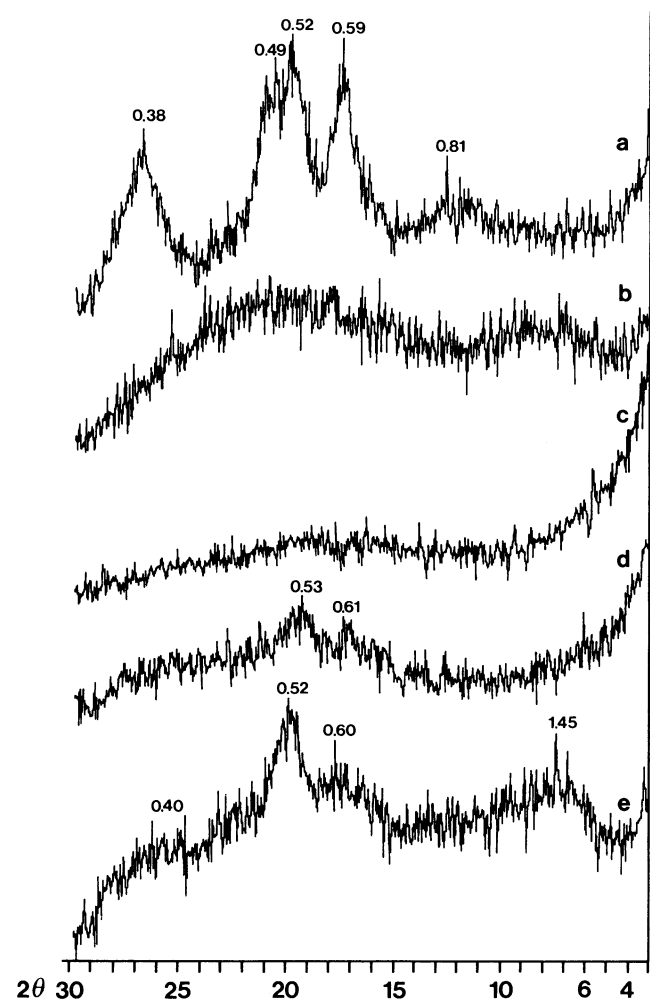


Fig. 1. Differential scanning calorimetry thermograms of native waxy maize starch (a), freshly gelatinized waxy maize starch (b), gelatinized waxy maize starch stored for 48 hr at room temperature (c), gelatinized waxy maize starch stored for 24 hr at 6°C and for 48 hr at 40°C (d), gelatinized waxy maize starch stored for 24 hr at 6°C and for 29 days at 40°C (e).

clavation (gelatinization), the starch sample did not show any thermal transitions (Table I). This indicates that the gelatinization under the experimental conditions used was complete, and that retrogradation had not yet occurred.

**Retrograded waxy maize starch gels.** When the autoclaved starch samples were stored, an endothermic transition was again observed (Fig. 1). This endotherm has been attributed to the retrogradation of amylopectin. A significant increase in the onset, peak, and completion temperatures, as well as in enthalpy values, was noticed when the starch samples were stored for 48 hr at room temperature, 24 hr at 6°C followed by 48 hr at 40°C, and 24 hr at 6°C followed by 29 days at 40°C. However, the high peak and completion temperatures of two of the endotherms of native granular starch were not obtained with the time and temperature combinations used in this study. The sequential incubation at 6 and 40°C was applied to obtain extensive retrogradation in a short time by favoring the nucleation of amylopectin at a low temperature and the propagation at 40°C (Levine and Slade 1990). Also, when storing the gelatinized starch at 40°C, crystal perfection (maturation) could occur. Indeed, a drastic increase in melting temperature was observed when the samples were stored at 40°C (Table I). In the study of Russell (1987), no such increase in peak temperature was observed when gelatinized waxy starch was stored at room temperature for 1–23 days, whereas the enthalpy value increased up to ~14 mJ/mg of starch.



**Fig. 2.** X-ray diffraction patterns. Native waxy maize starch (A-type) (a). Freeze-dried starch samples include: freshly gelatinized waxy maize starch (b), gelatinized waxy maize starch stored for 48 hr at room temperature (B-type) (c), gelatinized waxy maize starch stored for 24 hr at 6°C and for 48 hr at 40°C (B-type) (d), gelatinized waxy maize starch stored for 24 hr at 6°C and for 29 days at 40°C (B-type) (e). Numbers above peaks indicate *d*-spacings in nanometers.

### X-Ray Diffraction Analysis

X-ray diffraction was performed with native starch and with freeze-dried samples of freshly gelatinized starch and retrograded gels (Fig. 2). Native waxy maize starch showed an A-type pattern, which is generally found for waxy maize starch. No significant peaks could be distinguished in freshly gelatinized starch, and gelatinized starch stored for 48 hr at room temperature. An increase in crystallinity, however, was observed when the gelatinized starch had been stored for a longer time. After storage at 6°C (24 hr), followed by 48 hr of storage at 40°C, the X-ray diffraction peaks observed resembled the B-pattern. Crystallinity increased still more when the starch gel was stored for longer periods at 40°C (Fig. 2).

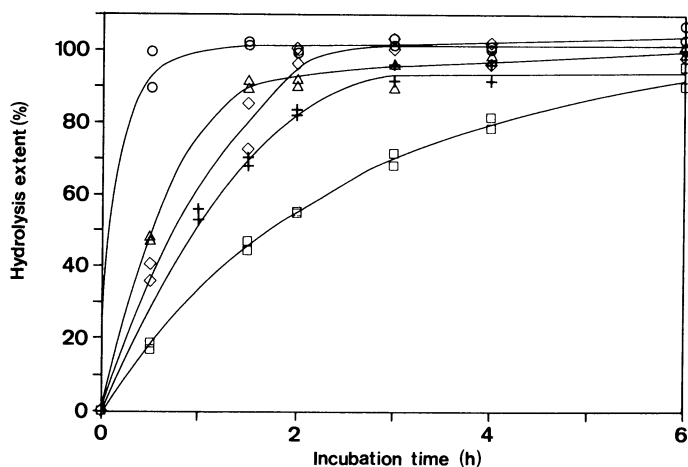
### Enzymic Hydrolysis

When the different starch samples (native waxy maize starch, freshly gelatinized waxy maize starch, and gelatinized waxy maize starch retrograded for 48 hr at room temperature, for 24 hr at 6°C followed by 48 hr at 40°C, and for 24 hr at 6°C followed by 29 days at 40°C) were subjected to enzymic digestion with pancreatin and amyloglucosidase at 37°C, significant differences were observed (Fig. 3).

**Freshly gelatinized waxy maize starch.** Freshly gelatinized waxy maize starch was hydrolyzed very readily. Complete degradation had already occurred after less than 1 hr of incubation. Because freshly gelatinized starch showed no crystallinity (Fig. 2) and no endothermic transitions (Table I), high enzymic susceptibility was expected.

**Retrograded waxy maize starch gels.** Retrograded waxy maize starch gels were more resistant than freshly gelatinized starch. Digestion rates of the waxy maize starch gels depended upon the extent of retrogradation. Indeed, the starch gel stored for 48 hr at room temperature was completely digested after 2 hr under the experimental conditions used. It showed no significant peaks on the X-ray diffraction pattern (Fig. 2), and it had an onset temperature of melting (40.7°C) that was only slightly higher than the incubation temperature of 37°C (Table I). The starch samples with increased crystallinity (X-ray diffraction, Fig. 2), increased melting temperature and higher melting enthalpy values (Table I) showed higher enzyme resistance. Almost complete degradation of waxy maize starch gel stored at 6°C followed by storage at 40°C for 48 hr was achieved after 3 hr, whereas waxy maize starch gel stored at 6°C followed by storage at 40°C for 29 days was not fully digested after 6 hr of incubation. This indicates the impact of the molecular order of the starch sample on the enzymic degradability.

LeLoup et al (1992) found that amylopectin gels, obtained after



**Fig. 3.** Enzymic hydrolysis at 37°C by pancreatic  $\alpha$ -amylase and amyloglucosidase of waxy maize starch samples: native waxy maize starch ( $\Delta$ ), freshly gelatinized waxy maize starch ( $\circ$ ), gelatinized waxy maize starch stored for 48 hr at room temperature ( $\diamond$ ), gelatinized waxy maize starch stored for 24 hr at 6°C and for 48 hr at 40°C (+), gelatinized waxy maize starch stored for 24 hr at 6°C and for 29 days at 40°C ( $\square$ ).

storage of amylopectin solutions (10–17%) at 1°C for 21 days, were totally degraded with pancreatic  $\alpha$ -amylase at 37°C within 1 hr. Differential scanning calorimetry of the gels showed that the degrees of retrogradation obtained were not as high as those in our experiments. The onset temperature of the endothermic transition was 37°C; the maximum enthalpy value obtained was 7.7 mJ/mg of starch. Thus, the results of Leloup et al (1992) are in line with our observations. Ring et al (1988) also found a decrease in pancreatic  $\alpha$ -amylase susceptibility at 37°C with increasing storage time of the starch gels (1 and 7 days at 20°C). However, the starch gels studied (potato, pea, maize, and wheat) contained both amylose and amylopectin, and the authors did not investigate whether one specific component (amylose or amylopectin), or the interaction of both, was responsible for the decrease in enzyme susceptibility.

Thus, when the extent of retrogradation increased, the enzymic susceptibility of the starch gel decreased. It is reasonable to assume that both an increase in entanglement of the molecules in the gel network (thereby reducing the accessibility of the substrate) and an increase in molecular order in the short range (double helix formation) and in the long range (crystallites formation) are responsible for the observation.

To decrease the impact of the entanglement of the molecules on enzymic susceptibility, we decided to disrupt the starch gel by ultra-turrax mixing. By doing so, we hoped to separate the impacts of entanglement in the gel network from those of molecular order. However, X-ray diffraction also revealed a decrease in crystallinity. Therefore, we were not able to evaluate the relative importance of both parameters.

Depending upon how enzyme-resistant starch is defined in vitro, retrograded amylopectin may play a role in the enzyme resistance of starch. When resistant starch is determined as the fraction of starch not digested to glucose after incubation for 2 hr at 37°C with pancreatic  $\alpha$ -amylase and amyloglucosidase (Englyst et al 1992), retrograded amylopectin can yield high levels of resistant starch for starch gels stored under specific time and temperature conditions to obtain extensive retrogradation. Indeed, a resistant starch level of 42% was measured when waxy maize starch had been stored for 24 hr at 6°C followed by 29 days at 40°C (Table II). On the other hand, when resistant starch is determined after much longer incubation times (6 hr or longer), it is obvious that resistant starch levels are very low, or even insignificant (Fig. 3). Also, when resistant starch is determined as the starch fraction surviving incubation with a heat-stable  $\alpha$ -amylase at 100°C (Sievert and Pomeranz 1989, 1990; Siljeström et al 1989), no resistant starch can be detected, because the molecular order in retrograded amylopectin would be lost at this high temperature (Table I). Thus, amylopectin would be easily degraded.

Therefore, the results of Berry (1986), who found no significant increase in resistant starch yield with increasing storage time, can be explained by the mere fact that more drastic procedures were used to determine resistant starch. Berry (1986) found only a little increase in resistant starch levels upon storage of wheat starch gels over 7 days at 4°C. However, resistant starch was determined after 16 hr of incubation at 42°C with  $\alpha$ -amylase and pullulanase.

*Native waxy maize starch.* The extent of hydrolysis of raw waxy maize starch was already 90% after 1.5 hr. Thus, although

TABLE II  
Enzyme Resistant Starch (RS)<sup>a</sup> Levels in Starch Samples

Starch	RS %
Native	8
Gelatinized	0
Retrograded	
48 hr at room temperature	5
24 hr at 6°C and 48 hr at 40°C	18
24 hr at 6°C and 29 days at 40°C	42

<sup>a</sup>Determined as the fraction of starch not digested to glucose after incubation for 2 hr at 34°C with pancreatin and amyloglucosidase.

native granular starch showed high crystallinity (Fig. 2) and a high melting enthalpy (Table I), compared with that of the retrograded starch samples, enzymic hydrolysis was not decreased in the same way. Indeed, besides crystallinity and extent of molecular organization, other factors can also limit hydrolysis. Particle size and accessibility of the substrate have been cited by Colonna et al (1992) as two such factors. For a suspension of native starch and retrograded starch gel, these factors must be different. In the case of the retrograded starch, enzymes have to diffuse into the gel network. The diffusion rate strongly depends on the gel concentration, which was rather high in our experiments (50%). The limiting step of hydrolysis of granular starch, on the other hand, is not the diffusion of the enzyme to the starch granule, but the penetration into the granule. As starch granules are, in fact, a mosaic of hard (crystalline) and soft (amorphous) material, reduced susceptibility toward  $\alpha$ -amylolysis is observed in the hard parts of the starch granules as illustrated by Gallant et al 1992 with scanning electron microscopy.

## CONCLUSIONS

Depending upon how resistant starch is defined in vitro, retrograded amylopectin may contain different levels of resistant starch. Compared with the enzyme susceptibility of native granular and freshly gelatinized waxy maize starch, enzyme susceptibility of retrograded waxy maize starch may be greatly reduced when extensive retrogradation has occurred and it is incubated with pancreatic  $\alpha$ -amylase at 37°C. Thus, influence of amylopectin (retrogradation) on enzyme resistance may be observed only when gelatinized starch has been stored in specific conditions.

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