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The structure of four waxy starches related to gelatinization and retrogradation*

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ABSTRACT

Starches from two waxy rices, a waxy maize, and a waxy barley showed onset temperatures (T_o) of gelatinization in excess of water of 349.2, 340.5, 339.1, and 331.1 K, respectively, as determined by differential scanning calorimetry. The T_o values, as well as peak (T_p) and final (T_c) transition temperatures, decreased in the same order as their X-ray crystallinity. Annealing the starches increased T_o and T_p, but the differences remained the same. After lintnerization in 2.2M HCl at 30°, the acid-resistant fractions from the four starches gave the same d.s.c. thermograms and the same constitutive molecular dextrins before and after debranching, as determined by high-performance anion-exchange chromatography with pulsed amperometric detection. Gels (50% water) of the four starches all had the same, but reduced, T_o values after retrogradation. These results indicate that the molecular structure of the microcrystalline region is the same in the four granular starches, and that T_o is controlled indirectly by the surrounding amorphous regions. The retrogradation of the four waxy starches appeared to be directly proportional to the mole fraction of unit chains with d.p. 14–24 and inversely proportional to the mole fraction of unit chains with d.p. 6–9.

INTRODUCTION

The temperature at which starch gelatinizes[1–4] generally correlates[5] with the degree (quantity) of starch crystallinity. However, the underlying cause has been attributed[6,7] indirectly to an increase in the glass-transition temperature (T_g) of the amorphous phase of the granule, which precedes the melting temperature of the crystallites. Crystallite perfection (quality) also has been proposed[8] to explain differences in gelatinization temperature.

Recrystallization or retrogradation of amylopectin (AP) in gelatinized starch is dependent on AP structure in an unknown fashion. The order of the retrogradation of APs from potato, tapioca, and kuzu starches has been related[9] to average chain length. Cereal amylopectins retrograde[10,11] slower than pea, potato, and canna amylopectins, and this difference has also been attributed to shorter average chains in cereal AP. However, even among cereal amylopectins[11], corn AP retrogrades faster than barley AP, which retrogrades faster than wheat AP.

Recently, Koizumi and Hizukuri[12] used high performance anion-exchange chro-

* Dedicated to Professor David Manners.

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matography with pulsed amperometric detection (h.p.a.e.c.–p.a.d.) to separate and quantitate individual unit chains of AP up to d.p. 55. They demonstrated differences in unit-chain distributions among seven APs. We now report the characterization of four waxy starches by h.p.a.e.c.–p.a.d. and by conventional structural methods, and the examination of their gelatinization and retrogradation properties by differential scanning calorimetry.

EXPERIMENTAL

Materials. — Cultivars RD4 and IR29 of milled waxy rice (WR1 and WR2) were obtained from the Pathum Thami Rice Research Center (Thanyaburi, Thailand) and the International Rice Research Institute (Philippines), respectively. Hull-less waxy barley (WB), cultivar Wanubet, was obtained from the Montana Agricultural Experimental station (Bozeman, MT). Starch was isolated from waxy rice[13] and barley[14], using dilute sodium hydroxide. Waxy maize (WM) starch was provided by A. E. Staley Manufacture Co., Inc. (Decatur, IL). The moisture contents of the starches were: WR1, 11.5; WR2, 10.8; WM, 11.0; and WB, 11.0%.

Amylopectin (AP) was isolated by dissolving starch granules in aq. 90% methyl sulfoxide followed by precipitation of AP with ethanol[15]. The precipitated material was collected by centrifugation, washed three times with ethanol and once with acetone, and vacuum-dried overnight in a desiccator containing $CaCl_2$.

Pullulanase (EC 3.2.1.41) from *Enterobacter aerogenes* and isoamylase (EC 3.2.1.68) from *Pseudomonas amyloderamosa* were obtained from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). Crystalline sweet-potato beta-amylase (EC 3.2.1.2), type 1-B, was obtained from Sigma Chemical Co. (St. Louis, MO).

Maltose monohydrate (grade HHH) was obtained from Hayashibara Biochemical Lab, Inc. Malto-oligosaccharides of d.p. 3 to 7 (pure grade) were gifts from Dr. T. Nakakuki, (Nihon Shokuhin Kako Co., Ltd., Tokyo), while those of d.p. 8 to 15 were purchased from Nakano Vinegar Co., Ltd. (Tokyo).

Lintnerization. — Starch granules (3 g) were steeped in 90 mL of 2.2M HCl at 30° in several containers. The sedimented granules were resuspended every other day by gentle shaking[16]. At specific times up to 45 days, the sediment in a container was collected by centrifugation, washed six times with water to a final pH 6.5, and dried in a desiccator at room temperature to ~8% moisture content. Solubilized carbohydrate was measured in the original supernatant solution by the phenol–H_2SO_4 reagent[17].

Differential scanning calorimetry (d.s.c.). — D.s.c. studies were performed with a Perkin–Elmer DCS-2 (Norwalk, CT) equipped with an FTS Systems Flexi-cooler and temperature controller (FTS systems, Inc., Stone Ridge, NY). The instrument was calibrated with indium. Samples (~3 mg of starch) at three starch-to-water ratios, 1:1, 1:2, and 1:3 (w/w), were prepared[18] and heated at 10 K/min from 280 to 400 K. A second aluminum pan, containing an appropriate amount of aluminum to balance the heat capacity of the sample, was used as a reference. Data were collected and analyzed using DARES (Data Acquisition, Retention and Examination System for Differential Scann-

ing Calorimetry, v. 1.4, Industrial Technology Research Institute, Cambridge, U.K., 1987). Parameters of an endotherm peak were determined as described by Lund[1]. The values of T_o and T_c, onset and final transition temperatures, respectively, were determined by the intercept of the extrapolated baseline and the leading and trailing edges of a peak. T_p was the peak temperature of the endotherm. Enthalpy (ΔH) was determined by measuring the area of the d.s.c. endotherm. All d.s.c. experiments were replicated at least twice.

Annealing of starch was done by heating (10 K/min) a mixture of starch and water (1:3 w/w) in a d.s.c. pan from 300 K to a specific temperature and holding it thereat for 2 to 48 h. The d.s.c. pan was then rapidly cooled (320 K/min) to 280 K and scanned from 280 to 400 K.

After a mixture of starch and water (1:1, 1:2, or 1:3, w/w) had been heated in a d.s.c. pan, the starch gel was stored at 4° for 1 day, then at room temperature (23 ±1°) for periods of 1–4 weeks. The extent of retrogradation of the starch gel was estimated from the enthalpy of melting of recrystallized starch measured by d.s.c.

Starch gels in the d.s.c. pan also were held at −20° for 22 h, and thawed for 2 h at room temperature; and then the cycle was repeated[19]. After a given number of freeze–thaw cycles, the sample was scanned by d.s.c. from 280 to 400 K, in order to evaluate the extent of retrogradation.

X-Ray diffraction. — X-Ray diffraction patterns of native and lintnerized starches were recorded on a Philips X-ray diffractometer, using Cu-K_α radiation at 35 kV and 20 mA, a theta compensating slit, and a diffracted beam monochromator. Relative crystallinity was estimated from the ratio of the area of peaks to the total area of a diffractogram[20].

Debranching amylopectin and lintnerized starch. — Debranching of AP and lintnerized starch was done as described by Tester and Morrison[8] with slight modification. Amylopectin (6.00 mg, dry basis) was weighed on a microbalance and dissolved in 2.99 mL of acetate buffer (pH 3.8, 10mM) by boiling briefly. The solution was cooled to room temperature, and 10 μL of isoamylase (590 units) was added. The mixture was incubated at 30° for 24 h, then boiled for 10 min, cooled, and centrifuged; the supernatant solution was used for further analysis. Debranching of lintnerized starch was done in the same way except that, in addition to isoamylase, two units of pullulanase were added and the reaction mixture was incubated at 35°.

Structural methods. — The reducing value of debranched glucan was determined by the Somogyi–Nelson alkaline-copper procedure[21,22] using maltose as standard. The heating step was extended[23] to 30 min so that the molar reducing power was independent of chain length. The number-average degree of polymerization (d.p.) was calculated as follows: 2 × weight of carbohydrate (μg)/reducing sugar (as μg of maltose). Beta-amylolysis of AP was done according to a modification[24] of Whelan's procedure[25].

Chain-length distribution was determined by h.p.a.e.c.–p.a.d. (Dionex Corp., Sunnyvale, CA). A Dionex Carbopac PA1 column (4 × 250 mm) was used with a Carbopac PA Guard column (3 × 25 mm). Pulse potentials (volts) and duration (s) on the p.a.d. were as follows: E_1 0.05 (t_1 0), E_1 0.05 (t_2 0.50), E_3 0.6 (t_3 0.51), E_4 0.6 (t_4 0.59), E_5

-0.6 (t_5 0.60), E_6 -0.60 (t_6 0.65). The sample-injection loop size was 20 μL. Results were recorded on a Chromatopac CR 601 digital integrator (Shimadzu, Kyoto, Japan).

Eluents for the h.p.a.e.c. system were prepared[26] as described previously. Eluent *A* was 150mM NaOH, and eluent *B* was 150mM NaOH containing 500mM sodium acetate. The gradient program[12] was as follows: 40% of eluent *B* at 0 min, 50% at 2 min, 60% at 10 min, and 80% at 40 min. All separations were carried out at ambient temperature with a flow rate of 1 mL/min.

To construct a standard response curve, maltose and malto-oligosaccharides of d.p. 3 to 15 were vacuum-dried at 70° for 24 h. After being cooled in the drying pistol over anhydrous calcium sulfate, standards (~ 0.4 mg) were weighed on a microbalance and dissolved in 5 mL of H_2O with 18-megaohm resistance. The standard solutions were diluted with 5 to 10 volumes of H_2O and filtered through a syringe-filter before injection. Retention time and the detector response (area/μmole of sample) of pure maltose were obtained directly, whereas the detector response for maltotriose was obtained after correcting for the presence of maltose. The detector responses for d.p. 4 to 15 were obtained in the same fashion by correcting for oligomer impurities in a sample.

The concentrations of debranched amylopectin and lintnerized starch injected were 0.5 and 0.25 mg/mL, respectively.

RESULTS AND DISCUSSION

Gelatinization temperature. — The four waxy starches were of the A-type polymorphic form[27] with three strong reflections at 2θ values of 15°, 17°, and 23° in their X-ray diffractograms (Fig. 1). WR1 was the most crystalline starch, and WB the least. The order of crystallinity of the starches also was reflected in the yields of acid-resistant residues. WR1 consistently gave the lowest conversion into solubilized material throughout hydrolysis (Fig. 2), indicating that it was the most crystalline starch. On the other hand, WB gave the highest yield of solubilized material.

The gelatinization temperatures of the four waxy starches in 75 wt% of water (Table I) increased in the same order as their X-ray crystallinity (Fig. 1). Recently, Tester and Morrison[8] found that six cultivars of waxy rice with high gelatinization temperatures (T_o 60–65°) showed somewhat greater X-ray crystallinity than six waxy rice starches with low gelatinization temperatures (T_o 49–52°). Even though there appears to be a correlation between T_o and ΔH in Table I, that correlation is not always observed[8,28–36]. In fact, we believe that T_o and ΔH are controlled by different structural features as discussed below.

Annealing the four waxy starches at 6° below T_o for either 2 or 48 h increased T_o equally for all starches (Table II), whereas there was little change in ΔH. Annealing at T_o for 2 h again increased the gelatinization temperature by 12–13°, but significantly decreased ΔH. In addition to the increase in T_o values, the peak width of d.s.c. thermograms was narrowed by annealing. For example, after annealing at 6° below T_o for 48 h, T_p and T_o became separated by ~ 2 K as opposed to ~ 5 K before annealing (Table I). If annealing perfected the A-type crystallites in the four waxy starches, why did the perfected crystals retain their relative differences in T_o and T_p values (Table II)?

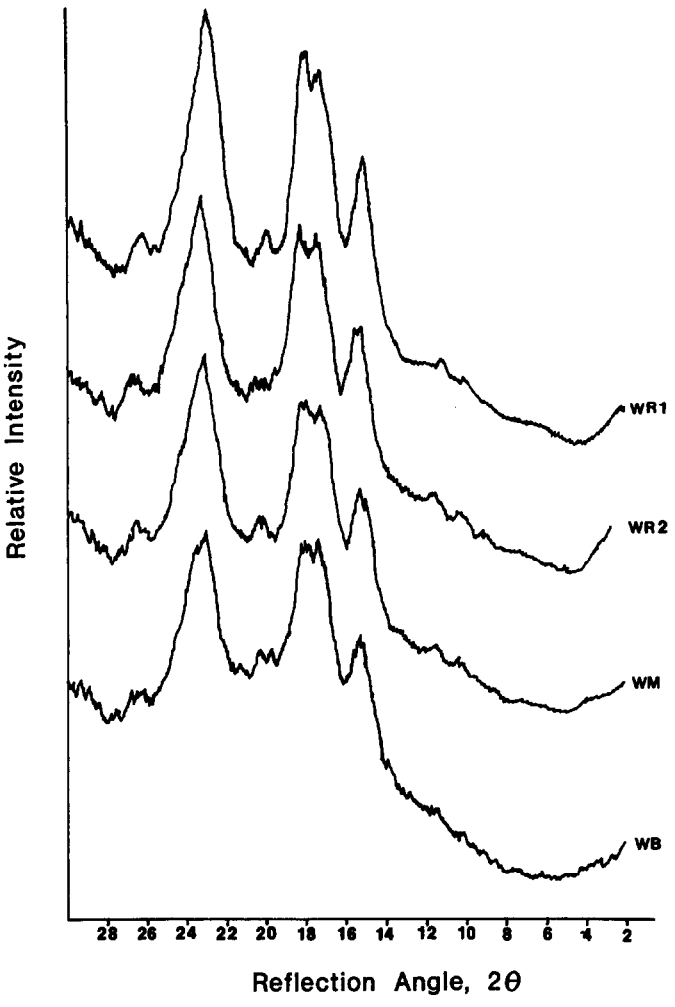

Fig. 1. X-Ray diffractograms of four waxy starches: waxy rice RD4 (WR1), waxy rice IR 29 (WR2), waxy maize (WM), and waxy barley (WB). Relative crystallinity was 57, 50, 45, and 40 for WR1, WR2, WM, and WB starches, respectively.

As the amorphous phase was removed by acid at 30°, the width of the gelatinization endotherm broadened as illustrated for lintnerized waxy-rice (IR 29) starch in Fig. 3. Similar results have been reported[2,16,37]. T_o decreased with hydrolysis time up to 7 days, whereas T_p decreased to a minimum after 2 days of linterization and then increased. The T_o values of linterized starch from the four waxy starches converged at ~315 K after digestion for 7 days. Indeed, the entire thermogram of each of the four acid-resistant residues became essentially the same after 7 days of digestion, and also after 21 days of digestion (Fig. 4). The d.s.c. thermograms after 21 days of digestion (Fig. 4) approached the true melting of crystallites since the amorphous region in a starch granule had been removed selectively by acid.

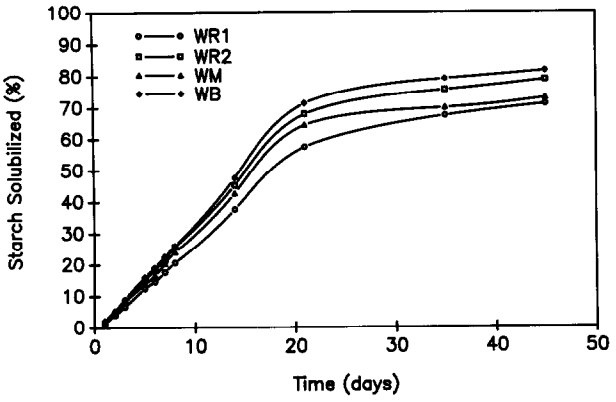

Fig. 2. Starch solubilized by steeping waxy starches in 2.2M HCl at 30 °C.

TABLE I

Gelatinization of waxy starches[a] in water (75%)

Sample	*Transition temp.*			*Enthalpy*
	T_o *(K)*	T_p *(K)*	T_c *(K)*	ΔH *(cal/g)*
WR1	349.2	354.2	360.1	4.6
WR2	340.5	346.6	353.8	3.6
WM	339.1	343.8	351.5	3.7
WB	331.1	335.7	341.2	3.4

[a] Each result is the average of three determinations. Standard deviation ±0.4 K and ±0.1 cal/g.

The structure of the crystalline residues of the four waxy starches after 21 days of acid digestion was also the same, even though the yields of the solubilized material varied from 57–71% (Fig. 2). All four acid-resistant residues comprised almost identical dextrin molecules as shown by h.p.a.e.c.–p.a.d. Upon debranching, the chain-length distributions were also identical with an average d.p. 14.5 ± 0.5. Chromatograms of one lintnerized starch before and after debranching are shown in Fig. 5. Moreover, when gels (75 wt% of water) of the lintnerized starches were prepared and retrograded at 23° for 2 weeks, the retrograded material of each of the four gels showed essentially the same thermograms with T_o 328 K, T_p 336 K, and ΔH 5.1 cal/g.

The similarities of the d.s.c. thermograms and the molecular structures of the crystalline residues from the four starches support the concept that the melting temperature (T_o) of the crystallites in native starch is controlled indirectly by the surrounding amorphous regions.

Retrogradation. — Starch pastes (50% of H_2O) in d.s.c. pans were retrograded by storing at room temperature. After 1 week, all four retrograded waxy starches gave the

TABLE II

Gelatinization of starch after annealing in water (75%)

Sample	*Annealing conditions*[a]			*Transition temp.*[b]	*Enthalpy*[b]
	Temp. (K)	*ΔT (K)*	*Time (h)*	T_o *(K)*	*ΔH (cal/g)*
WR1	–	–	–	349.2	4.6
	349	0	2	360.7 (+11.5)	1.5 (−3.1)
	343	6	2	356.3 (+ 7.1)	4.5 (−0.1)
	343	6	48	359.9 (+10.7)	4.7 (+0.1)
WR2	–	–	–	340.3	3.6
	340	0	2	353.1 (+12.9)	1.7 (−1.9)
	334	6	2	347.9 (+ 7.6)	3.7 (+0.1)
	334	6	48	352.4 (+12.1)	4.0 (+0.4)
WM	–	–	–	339.1	3.7
	339	0	2	351.6 (+12.5)	1.0 (−2.7)
	333	6	2	346.3 (+ 7.2)	3.3 (−0.4)
	333	6	48	350.7 (+11.6)	3.5 (−0.2)
WB	–	–	–	331.1	3.3
	331	0	2	344.4 (+13.3)	0.4 (−2.9)
	325	6	2	339.1 (+ 8.0)	2.7 (−0.6)
	325	6	48	343.6 (+12.5)	2.8 (−0.5)

[a] $\Delta T = T_o$ − annealing temperature. [b] The data in parentheses are differences between T_o or ΔH values after and before annealing.

same values of T_o (320.5 ±0.4 K) and T_p (330.2 ±0.6 K). After 2 weeks, transition temperatures increased to $T_o \sim 322$ K and $T_p \sim 331$ K, and after 4 weeks to $T_o \sim 324$ K and $T_p \sim 333$ K. Common transition temperatures of $T_o \sim 318$ K and $T_p \sim 326$ K also were observed when the gels (75% of water) of the four starches were subjected to seven freeze–thaw cycles. On the other hand, the ΔH values were different for the retrograded starches (Figs. 6 and 7), indicating that different quantities of crystallites had formed.

In other work by Ward[38], retrograded wheat and corn starches in 75 wt% of water gave the same melting transition temperature, whereas the native starches had T_o values separated by ~10°. Once again, there was no difference between T_o values of retrograded APs from wheat and corn starch. Kalichevsky *et al.*[11] also reported that retrograded APs from maize, wheat, and barley starch gave similar peak temperatures (326–328 K) for their melting transition, whereas AP from canna, potato, and pea showed somewhat higher temperatures (330–333 K).

The four waxy starches have different gelatinization temperatures (Table I); yet, after retrogradation, their melting temperatures were the same. The retrograded crystallites melted 12–27 K below the crystallites in the native starches. This change in melting behavior reflects the dynamic plasticization of water on the amorphous phase during the melting of crystallites in granular starch[3,4]. On the other hand, during retrogradation, fully hydrated starch recrystallizes in the B-type polymorphic form[15,39], which contains 36 water molecules in one unit cell[40] as opposed to 4 water molecules in A-type starch[41]. Recently, Whittam *et al.*[42] produced highly crystalline A- and B-type

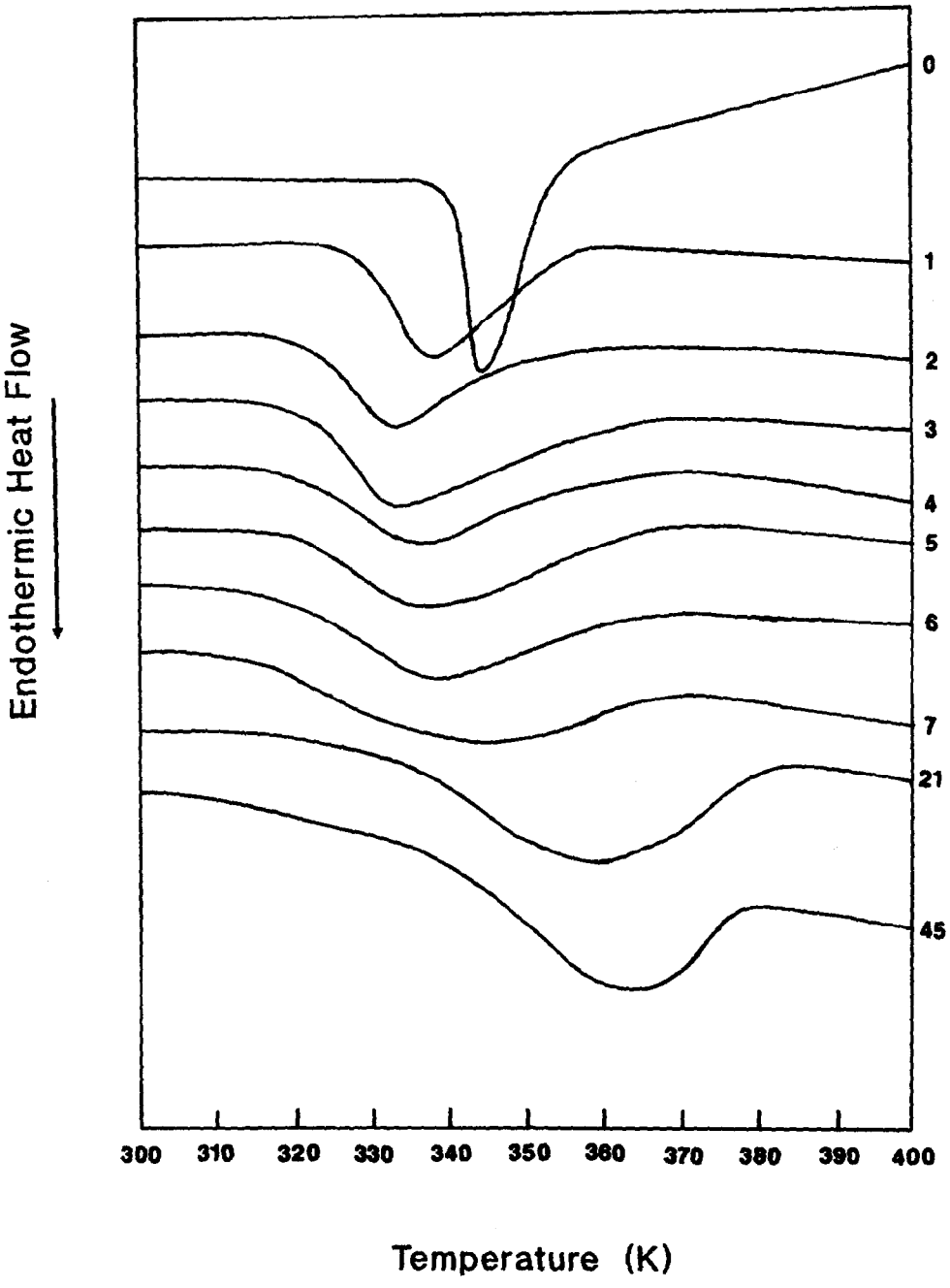

Fig. 3. D.s.c. thermograms of lintnerized waxy rice (IR29) starch. The number next to each curve gives the duration of lintnerization in days.

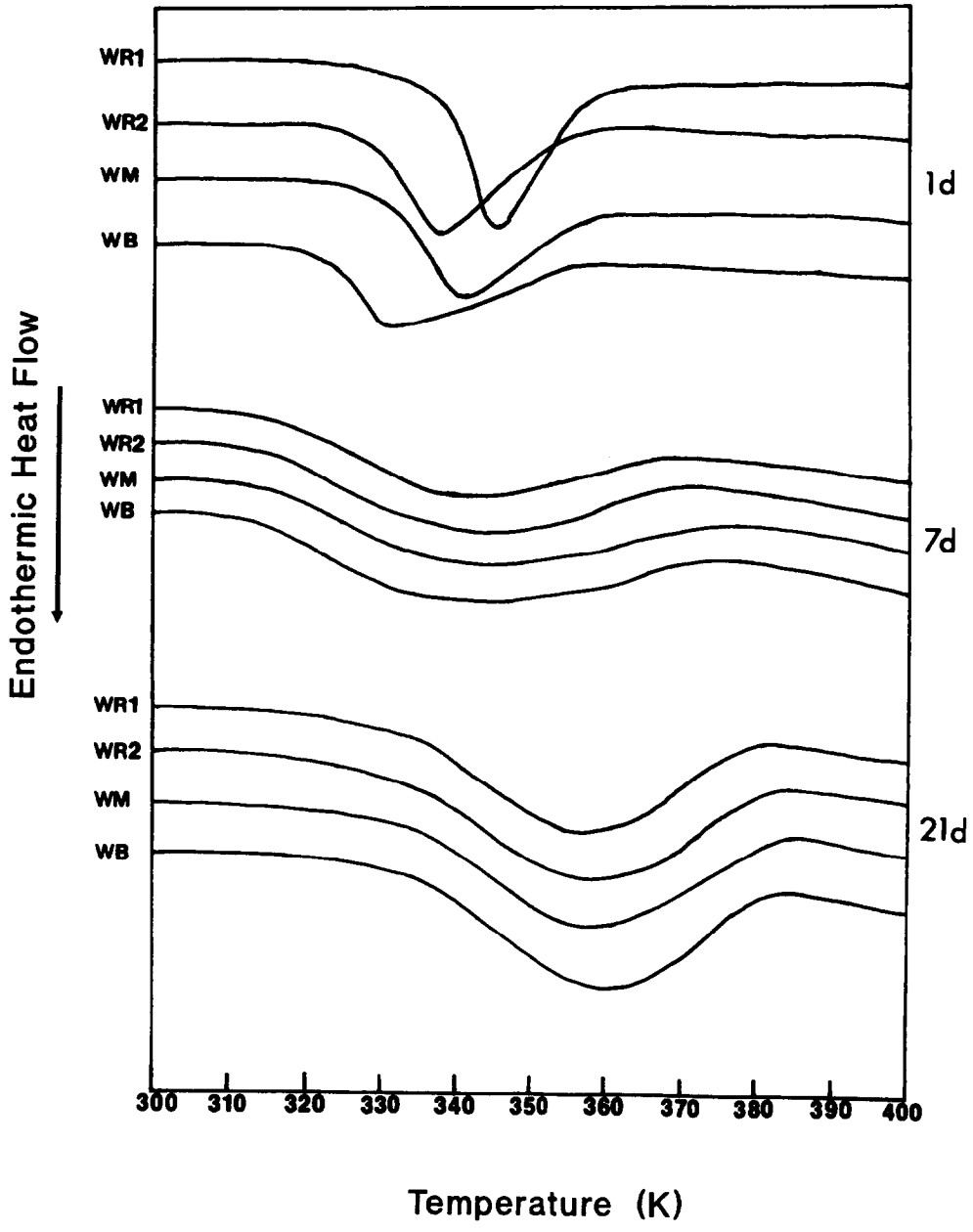

Fig. 4. D.s.c. thermograms of lintnerized waxy starches after digestion for 1, 7, and 21 days. The number next to each group of four curves gives the duration of lintnerization in days.

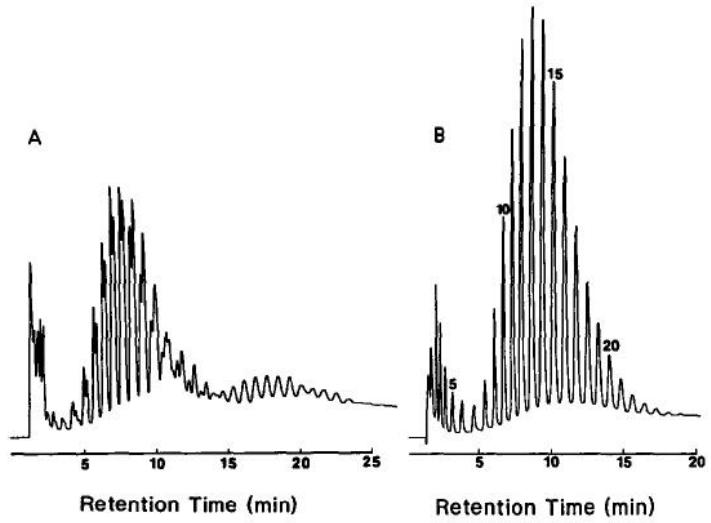

Fig. 5. Chromatograms of lintnerized waxy rice starch (WR2) before (A) and after (B) debranching, as determined by h.p.a.e.c.–p.a.d. WR2 was hydrolyzed in 2.2M HCl at 30 °C for 21 days. Numbers above peaks are chain lengths.

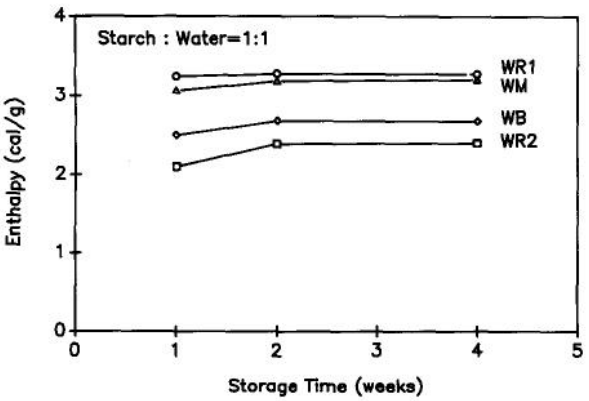

Fig. 6. Enthalpy of melting of retrograded gels prepared from waxy starches in water (50%) after storage at 23°C.

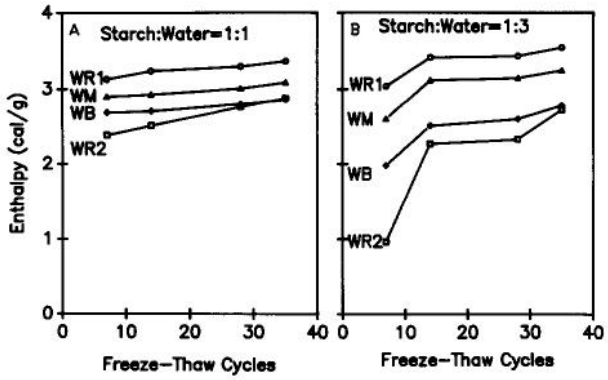

Fig. 7. Enthalpy of melting of retrograded waxy starches after freezing and thawing gels containing 50 (A) and 75% of water (B).

spherulites from a malto-oligosaccharide of d.p. ~ 15, and found that the B-type melted ~ 20 K below the A-type in the presence of >40% of water by weight. In the work reported here, the same transition temperatures (T_o, T_p) indicate that the same structure of microcrystallites developed during retrogradation of all four, cereal waxy starches.

The order of retrogradation of 50% gels of the waxy starches stored at 25° was WR1 > WM > WB > WR2 (Fig. 6). The same order was observed upon freezing and thawing of their gels (Fig. 7). Except for WR2, the order of retrogradation followed the level of X-ray crystallinity (Fig. 1) and the yield of lintnerized starch (Fig. 2). A starch of high crystallinity would be expected to retrograde readily.

To eliminate any residual effect of granular structure on retrogradation, AP was isolated from the four waxy starches. The retrogradation of the AP gels (67% of water) upon freezing and thawing is compared to that of the gelatinized starches in Fig. 8. The retrogradation of the AP gels was slower than that of the gelatinized starches, but the

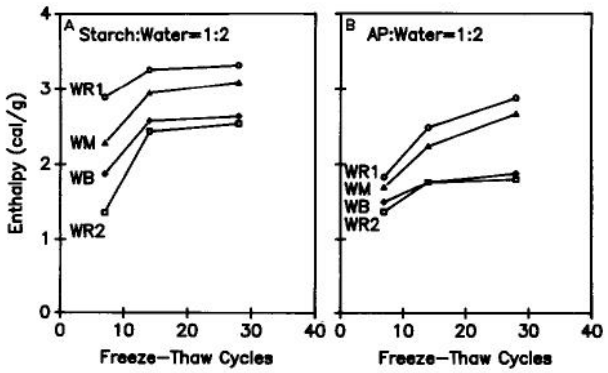

Fig. 8. Enthalpy of melting of retrograded gels (67% of water) from waxy starches (A) and amylopectins (B) after freezing and thawing.

TABLE III

Structural parameters of amylopectins

Sample	$\overline{CL}$	*Beta-amylolysis (%)*	$\overline{ECL}^a$	$\overline{ICL}^b$
WR1	19	58	13	5
WR2	21	54	13	7
WM	24	56	15	8
WB	23	58	15	7

[a] Exterior chain length, $ECL = (CL \times \text{beta-amylolysis}) + 2.0$. [b] Interior chain length, $ICL = CL - ECL - 1$.

order was identical, indicating that differences in molecular structure controlled the retrogradation of the starch gels.

Fine structure of AP. — The average chain lengths ($\overline{CL}$) of the unit chains in the four waxy starches are given in Table III. Similar structural parameters of AP from various sources have been reported in a recent review by Manners[43]. Neither the average chain length nor the external chain length explained the order of retrogradation of the starches and the differences in T_o values.

In this investigation, the four waxy starches were debranched, and the unit chains separated and quantified by h.p.a.e.c.–p.a.d. A typical chromatogram is shown in Fig. 9. Using reference malto-oligosaccharides, the molar response of the p.a.d. detector increased with chain length from 2–13. The molar responses of the components of d.p. 6

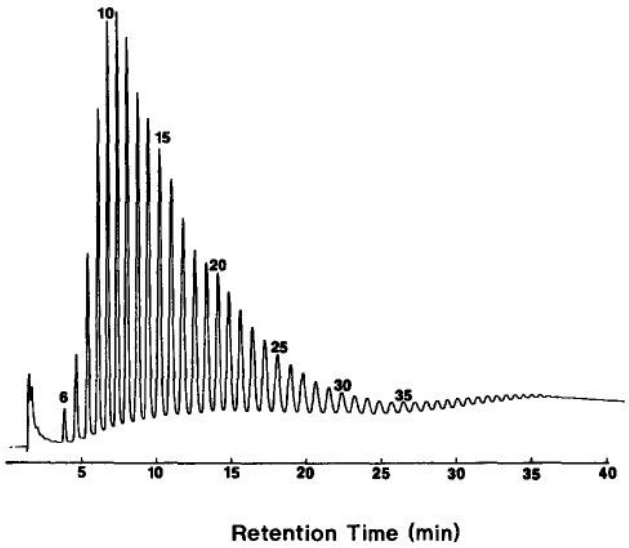

Fig. 9. Chain-length distribution of waxy rice (IR 29) starch determined by h.p.a.e.c.–p.a.d. Numbers above the peaks are chain lengths.

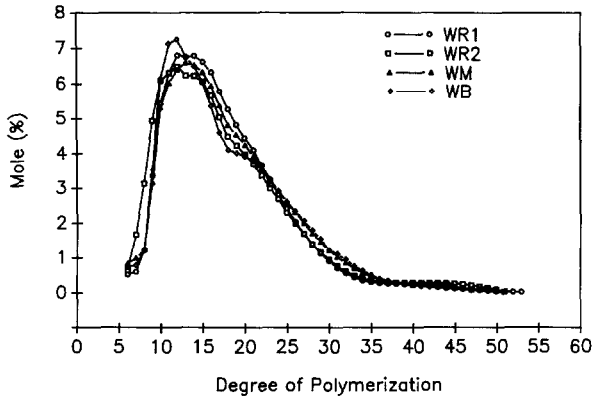

Fig. 10. Molar chain-length distributions of four amylopectins.

through d.p. 15, relative to that of d.p. 6, were 1.00, 1.06, 1.09, 1.19, 1.25, 1.33, 1.38, 1.40, 1.39, and 1.41, respectively. For d.p. > 15, the molar responses were assumed to be identical. Similar results have been reported[12] by Koizumi and Hizukuri. The standard responses were used to derive the molar distribution curves of unit chains given in Fig. 10, assuming that all chains had d.p. in the range 6–53. WR2 was unique in having a large number of short chains. WB was characterized with the highest proportion of chains having d.p. 11 and 12.

Examination of the mole fraction (%) of groups of unit chains (Table IV) revealed two factors that appeared to explain the order of retrogradation of the waxy starches. First, an increased molar proportion of the unit chains with d.p. 14–24 increased retrogradation. Structure studies[11,15,44] suggested that acid-resistant residues in retrograded gels of cereal APs had a peak d.p. ~ 16 after being debranched. Using this first factor, the order of retrogradation would be predicted to be WR1 > WM > WR2 ≈ WB. However, the data in Figs. 7 and 8 show that WR2 retrograded slower than WB.

TABLE IV

Mole fraction (%) of groups of unit chains in four amylopectins[a]

Sample	*D.p.*			
	6–9	14–24	6–24	>24
WR1	5.8	53.9	85.0	15.0
WR2	10.4	48.5	83.4	16.6
WM	6.3	51.5	82.2	17.8
WB	6.3	48.0	81.2	18.8

[a] Variation in each individual d.p. was within ±0.02%

WR2 contained almost twice the number of unit chains with d.p. 6–9 that were found in the other three waxy starches. Short unit chains with d.p. 6–9 are known[45,46] to inhibit starch retrogradation. This second factor appears to account for the discrepancy and allows the correct order of prediction WR1 > WM > WB > WR2.

Additional data to support the relationship between structure and retrogradation was found[38] on examining wheat and corn AP retrogradation. Corn AP had a higher proportion of unit chains with d.p. 14–24 and showed a greater extent of retrogradation of gels (65–75% of water) at room temperature than did wheat AP.

According to Hizukuri's revised cluster model[47] of AP, unit chains with d.p. 6–24 comprise A and B1 chains, and they are located within one cluster. The molar proportion of unit chains with d.p. 6–24 in the four waxy starches (Table IV) increased in the same order as crystallinity (Fig. 1) and T_o (Table I). The increased proportion of unit chains with d.p. 6–24 in single clusters corresponds to an increased number of branch points as well as number of chains, and results in a high molecular weight of interconnected, non-crystalline branched regions next to crystallites. The increased effective molecular weight would decrease the mobility of chain segments in the amorphous phase and restrict the swelling of the amorphous phase. As suggested by Slade and Levine[3,4], the crystalline and amorphous phases in granular starch are interdependent in their phase transition behaviors. T_g of the amorphous regions increases with an increase in the degree of crystallinity of the granules because of the rigidifying effect of the crystalline regions on the amorphous chain segments. T_o increases with increasing T_g, because melting of the microcrystallites cannot begin until the amorphous regions are mobilized at a temperature greater than T_g. While the location of T_g for granular starch is still in debate in the current literature, T_o increases with increasing degree of crystallinity of the granular starches, as shown in this study and by others[5,8].

Conclusions. — The structure of the microcrystalline regions of the four, waxy cereal starches is the same, and the temperature of melting of the crystallites in the granular starch is controlled indirectly by the surrounding amorphous regions. The retrogradation of the waxy starches appears to be directly proportional to the mole fraction of unit chains with d.p. 14–24 and inversely proportional to the mole fraction of d.p. 6–9.

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