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Carbohydrate Polymers 57 (2004) 177–190

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

Waxy maize starch subpopulations with different gelatinization temperatures

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Accepted 19 April 2004

Abstract

The objective of this research was to obtain further evidence that starch preparations contain subpopulations of granules with properties that differ from those of other subpopulations. Waxy maize starch was used in this investigation. Nongelatinized waxy maize starch granules (both chemically modified and unmodified) were recovered after suspensions were heated to different temperatures between the initial and final gelatinization temperatures under conditions that minimized leaching of starch polymer molecules. Nongelatinized starch granules that were recovered after heating to 74 °C had a lower normalized concentration of A chains (DP 6-12) and became less substituted upon hydroxypropylation compared to granules recovered after heating to 70 °C and unfractionated granules. Granules of hydroxypropylated starch that were recovered after heating to higher temperatures in the gelatinization temperature range were less substituted (had lower MS values) compared to granules recovered after heating to lower temperatures and unfractionated granules. Results confirm that subpopulations with different physical and chemical properties exist in waxy maize starch. It is hypothesized that granules which gelatinize at higher temperatures are more crystalline due to a higher percentage of amylopectin A chains.

Keywords: Waxy maize starch; Starch granule subpopulations; Starch gelatinization; Starch branch chain length; Hydroxypropylstarch

1. Introduction

It is widely held that all populations of starch granules are heterogeneous. It is known that granules from different cultivars differ and believed that those from a single seed, tuber, or other organ of a single plant differ from each other, but there is little direct proof of heterogeneity. It has been reported that there are at least three subpopulations of normal maize starch granules that appear to be products of developmental regulation (Jones, Srienc, & Roessler, 1992; Wasiluk, Fulcher, Jones, & Gegenbach, 1994). One type of heterogeneity is that evidenced by as granules of different sizes (see, for example, Stapley & BeMiller, 2003 and references therein) and shapes (Jane, Kasemsuwan, Leas, Zobel, & Robyt, 1994) from a single source. Individual granules from a single source exhibit different behaviors (see, for example Jones et al., 1992; Morrison & Gadan, 1987; Wasiluk, Fulcher, Jones & Gegenbach, 1994). A] simple example is that individual granules lose birefringence in water over a short temperature range of the order of 1 °C, whereas total gelatinization of starch occurs over a much wider range of temperature of the order of at least 10 °C (Evans & Haisman, 1982). And granules differ in the degree of structural integrity after gelatinization (see, for example Obanni & BeMiller, 1995, 1996). It can be assumed that differences in gelatinization temperature and structural integrity are due to differences in granule organization.

There are various levels of molecular organization within any granule that range from nonorganized areas to supermolecular structures (Gallant, Bouchet and Baldwin, 1997; Gidley & Bociek, 1985; Muhrbeck, 1991; Oostergetel & van Bruggen, 1993; Morgan, Furneaux & Larsen, 1995). With such a range of physical structures in a natural product composed primarily of two polymolecular and polydisperse polymers, it is predictable that variation would exist; but the full extent of heterogeneity, the biological reason for it, and the impact heterogeneity has on the use of starch as a food or feed or on modification reactions remains to be determined.

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doi:10.1016/j.carbpol.2004.04.017

In this research, waxy maize starch granules were separated into different subpopulations according to their gelatinization temperatures. The fine structures and reactivities of each subpopulation were investigated. The objective was to determine if subpopulations of maize starch granules with different gelatinization temperature ranges have physical and chemical properties that are different from those of other subpopulations.

2. Experimental

2.1. Materials

Commercial waxy maize starch was provided by Tate and Lyle North America/A.E. Staley Mfg. Co. (Decatur, IL, USA). Sodium sulfate was purchased from Aldrich Chemical Co. (Milwaukee, WI). Propylene oxide was purchased from EM Science (Gibbstown, NJ). Sodium chloride was obtained from VWR International (West Chester, PA). α-Amylase from *Bacillus* species (EC 3.2.1.1) with an activity of 51 units/mg[1] solid and amyloglucosidase (EC 3.2.1.3) from *Aspergillus niger* with an activity of 6100 units/ml[2] were purchased from Sigma Chemical Co. (St Louis, MO).

2.2. Hydroxypropylation

Waxy maize starch was hydroxypropylated by the method of Shi and BeMiller (2002) Sodium sulfate was added to the reaction system to protect starch granules from swelling and gelatinization.

2.3. Fractionation of granules by gelatinization temperature

Waxy maize starch (2 g) was suspended in 100 ml of 40:60 ethylene glycol (EG)–water (v/v). The suspension was heated to different temperatures within the gelatinization temperature range as determined by differential scanning calorimetry (DSC, described below) for 1 h in a water bath with constant gentle shaking. Swollen, gelatinized granules were further damaged by application of mechanical shearing force produced with a magnetic stir bar (5 min). The mixture was then centrifuged at low speed. After centrifugation, the supernatant was decanted, and the top layer of lower-density, gelatinized granules was scraped off. The centrifugation was repeated three times using 40% aqueous EG to resuspend the granules. Recovered, non-gelatinized starch granules were washed three times with 40% acetone to remove residual ethylene glycol and air dried.

2.4. Leaching

Highly substituted hydroxypropylated waxy maize starch (MS 0.18) and a fraction of it recovered at 58 °C were used to determine leaching. A 2% (dwb) suspension of each starch in 40% ethylene glycol was held at 58 °C for 80 min. Leaching of starch molecules was measured by reading the absorbance of the triiodide–starch complex of the supernatant at 560 nm.

2.5. Differential scanning calorimetry (DSC)

For DSC analysis, a Perkin–Elmer DSC 7 analyzer equipped with a thermal-analysis data station (Perkin–Elmer Corp., Norwalk, CT) was used. The samples were prepared with a water to starch ratio of 2:1. Samples were heated from 30 to 110 °C at a rate of 10 °C/min. Gelatinized samples were stored for seven days at 4 °C to determine starch gel retrogradation as described by White, Abbas, and Johnson (1992). Thermal transitions for gelatinization were characterized by T_o (onset temperature), T_p, T_c (conclusion temperature), and ΔH (enthalpy of gelatinization). Melting of retrograded starch was characterized by T_R, and ΔH_R (enthalpy of retrogradation). All enthalpy calculations were based on dry-starch weight. Reported results (Table 1) are averages of at least two replicates for each sample.

To determine T'_g (Table 4), samples were prepared with a water to starch ratio of 2:1 w/w, heated to 100 °C at a rate of 10 °C/min for gelatinization, cooled to −40 °C with He, and then reheated to 45 °C at a rate of 2 °C/min.

2.6. Granule-size distribution

Granule-size distributions of unmodified waxy maize starches were determined by the procedure described by Stapley and BeMiller (2003). Starch was suspended in 95% ethanol to aid in spreading the granules into a monolayer on a microscope slide. From 439 to 840 granules of each sample were analyzed using a Leitz Laborlux 12 POL microscope at 160× magnification. Scion Image (Scion Corporation) software was used to measure the major axis of each granule.

2.7. Isolation of small starch granules

Small unmodified waxy maize starch granules were isolated using the sedimentation procedure described by Stapley and BeMiller (2003) A 250-ml buret was filled with distilled water. A slurry of waxy maize starch (0.4 g in 20 ml water) was added to the top of the column. After 2 h, nonsedimented granules were collected and air-dried.

2.8. Enzyme-catalyzed hydrolysis

Hydrolysis of unmodified waxy maize starch granules with α-amylase was performed by the procedure of

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[1] One unit liberates reducing power equivalent to 1.0 mg of maltose from starch in 3 min at pH 6.9 and 20 °C.

[2] One unit liberates 1.0 mg of glucose from starch in 3 min at pH 4.5 and 55 °C.

Table 1
DSC parameters for gelatinization and retrogradation of whole populations and fractions with different gelatinization temperature ranges

Starch	Fraction[a]	T_o[b] (°C)	T_p (°C)	T_c (°C)	ΔH (J/G)	T_{oR} (°C)	T_{cR} (°C)	ΔH_R (J/G)
Waxy maize starch	Whole	65.3 ± 0.1[c]	71.2 ± 0	77.6 ± 0.2	14.7 ± 0.2	38.5 ± 0	61.6 ± 0.2	8.1 ± 0.2
	70 °C	69.3 ± 0.1	72.4 ± 0.2	77.4 ± 0.1	14.3 ± 0.2	41.0 ± 0.2	62.2 ± 0.3	8.6 ± 0.2
	72 °C	70.9 ± 0.2	73.5 ± 0.2	78.0 ± 0.2	14.0 ± 0.1	43.9 ± 0.1	62.7 ± 0.9	9.2 ± 0.5
	74 °C	73.4 ± 0.1	75.8 ± 0	80.0 ± 0.1	14.2 ± 0.1	42.7 ± 1.2	62.7 ± 0.7	9.1 ± 0.2
HPWMS 1 (MS 0.125)	Whole	56.7 ± 0.9	65.4 ± 0.8	72.7 ± 1.1	10.9 ± 0.1			
	59 °C	59.5 ± 0.3	64.1 ± 0.3	71.2 ± 0.7	11.0 ± 0.6			
	62 °C	59.8 ± 0.2	65.7 ± 0.1	73.7 ± 0.6	9.1 ± 0.5			
HPWMS 2 (MS 0.114)	Whole	54.9 ± 0.1	65.3 ± 0.2	73.3 ± 0.6	10.4 ± 1.1			
	59 °C	60.7 ± 0.4	65.2 ± 0.6	72.2 ± 0.8	10.9 ± 0			
	62 °C	61.2 ± 0.3	65.5 ± 0.7	71.4 ± 0.6	9.8 ± 0.3			
HPWMS 3 (MS 0.179)	Whole	51.6 ± 0.7	62.4 ± 0.1	71.2 ± 0.2	9.0 ± 0.4			
	56 °C	56.6 ± 0.4	61.6 ± 0.4	70.0 ± 0.8	10.3 ± 0.5			
	58 °C	57.8 ± 0.1	63.3 ± 0	71.4 ± 0.1	9.1 ± 0.2			
	60 °C	58.3 ± 0.2	62.6 ± 0.1	68.7 ± 0.1	10.4 ± 0.1			
HPWMS 4 (MS 0.176)	Whole	52.6 ± 0.1	61.8 ± 0	69.8 ± 0.1	10.0 ± 0.6			
	56 °C	56.5 ± 0.1	61.6 ± 0.1	69.4 ± 1.0	9.8 ± 0.4			
	58 °C	57.7 ± 0.2	62.7 ± 0	70.4 ± 0.2	10.4 ± 0			
	60 °C	58.1 ± 0	62.3 ± 0.4	68.8 ± 0.6	10.2 ± 0.2			

[a] Whole, whole starch population before fractionation; temperature, temperature to which the whole population of granules was heated in 40% EG before recovering the nongelatinized granules.

[b] T_o, gelatinization onset temperature; T_p, gelatinization peak temperature; T_c, gelatinization end temperature; ΔH, enthalpy of gelatinization; T_{oR}, retrogradation onset temperature; ΔH_R, enthalpy of retrogradation.

[c] Mean values ± standard deviation of at least two measurements.

Knutson, Cluskey, and Dintzis, (1982). Starch granules (0.300 g) were suspended in 15 ml of 20 mM sodium phosphate buffer (pH 6.0) containing 0.2% NaN_3 and tempered at 37 °C for 10 min. α-Amylase was immediately added to give a starch:α-amylase ratio of 1 mg/3 units.[1] The starch–enzyme mixture was incubated at 37 °C with continuous mild shaking. A 1.0-ml aliquot was removed at specific time intervals and centrifuged 5 min at 3500 rpm. After deactivation of the enzyme (by boiling the supernatant solution for 10 min), the supernatant was analyzed for reducing sugar by the Somogyi-Nelson method (Somogyi, 1952; Wood, 1994), using maltose as the standard. Percent hydrolysis was expressed as milligrams of maltose released per 100 mg of dry starch.

Partially digested granules were prepared under identical conditions by incubation with the α-amylase for 2 h, at which point 14–20% of the starch was solubilized. Granular residues were collected by centrifugation, washed with 75% ethanol to deactivate the enzyme, washed twice with water to remove soluble carbohydrate, and air dried. Partially digested granules were then treated with α-amylase for up to 21.8 h using the conditions described above for the second-step hydrolysis. Controls (without enzyme) were also prepared.

Hydrolysis of unmodified waxy maize starch granules by amyloglucosidase was done for 10 h at 37 °C by following the procedure of Kimura and Robyt (1995). Amyloglucosidase (starch:enzyme ratio of 1 mg/3 units[2]) was added to a starch granule suspension (2.0% w/v) prepared in 0.1 M acetate buffer (pH 4.5) containing 0.2% NaN_3. Aliquots were removed periodically and centrifuged 5 min at 3500 rpm. After deactivation of the enzyme (by boiling the supernatant solution for 10 min), the supernatant was analyzed for reducing sugar by the Somogyi-Nelson method (Somogyi, 1952; Wood, 1994) using D-glucose as the standard. Percent hydrolysis (mg glucose released per 100 mg of dry starch) was calculated.

2.9. Branch-chain-length distribution of amylopectin

Branch-chain-length distribution of amylopectin was determined following the procedure described by Jane and Chen (1992) and Wong and Jane (1997, 2002). Starch was debranched using isoamylase, and the branch chain-length distributions were determined using a high-performance anion-exchange chromatograph equipped with an enzyme (amyloglucosidase)-column reactor that converts all maltooligosaccharides into glucose so that all fractions have identical response factors, making the analysis quantitative, and a pulsed amperometric detector (Dionex, Sunnyvale, CA) (HPAEC-ENZ-PAD). A CarboPac PA1 anion-exchange column (250×4 mm^2) and a CarboPac PA1 guard column ($25 \times 3\ ^2$) were used for sample separation. Results reported (Table 2) are an average of at least two replicates of each sample. Chains were categorized as A, B1, B2, and longer chains according to the protocol of Hanashiro, Abe and Hizukuri (1996).

Table 2
Relative branch chain-length (CL) distributions

Starch	Fraction[a]	Peak DP[b]		Maximum DP	Average CL	% Distribution[c]			
		I[d]	II[e]			DP 6-12[f]	DP 13-24[g]	DP 25-36[h]	DP ≥ 37
Waxy starch	Whole	14	49	71	24.78	17.9	46.2	15.6	20.3
	70 °C	14	49	72	25.80	16.75	44.9	15.5	22.8
	72 °C	14	46	72	25.81	16.6	45.2	15.6	22.6
	74 °C	14	50	72	25.86	16.3	45.2	15.7	22.8
HPWMS 1	Whole	12	N/A	30	14.53	38.3	58.1	3.6	0
	59 °C	12	N/A	30	14.34	38.4	59.0	2.6	0
	62 °C	12	N/A	30	14.48	37.7	59.4	2.95	0
HPWMS 3	Whole	12	N/A	27	13.78	43.5	55.0	1.4	0
	56 °C	12	N/A	28	13.62	43.5	55.6	0.9	0
	58 °C	12	N/A	29	13.70	43.0	55.8	1.2	0
	60 °C	12	N/A	28	13.90	42.6	55.4	1.9	0

[a] See Table 1 for an explanation of starch fractions.
[b] DP, degree of polymerization.
[c] The results reported are averages of at least two replicates for each sample. Grouping of DP follows the protocol of Hanashiro, Abe, and Hizukuri (1996).
[d] Peak I consisted of A1 (DP 6-12), B1 (DP 13-24), and some B2 (DP 25-36) chains. The most abundant chain length is given in the column.
[e] Peak II consisted of B2, B3, and longer chains. The most abundant chain length is given in the column.
[f] A chains.
[g] B1 chains.
[h] B2 chains.

2.10. Molecular weight distribution of amylopectin by HPSEC-MALLS-RI

The weight-average molecular weight (M_W) and z-average radius (R_z) of gyration of amylopectin were determined using HPSEC (HP1050 series, isocratic pump) equipped with multiangle laser light scattering (MALLS, model Dawn-F, Wyatt Technology Co., Santa Babara, CA) and refractive index (RI) detectors (HP1047A) following the procedure of Yoo and Jane (2002). In the procedure, a Shodex OHpak KB-G guard column and KB-806 and KB-804 analytical columns (Shodex Denko, Tokyo, Japan) were used for the separation of amylopectin and amylose.

2.11. Molar substitution (MS)

The molar substitution of hydroxypropylated starch was determined using ^{1}H-NMR spectroscopy at 300 MHz (QE 300 spectrometer, General Electric Co., Fairfield, CT, USA) following the procedure of Stapley and BeMiller (2003). Results reported (Table 3) are means of at least two determinations.

3. Results and discussion

3.1. Isolation of higher-gelatinization-temperature subpopulations of granules

Waxy maize starch was used because leaching from it is minimal, as it contains no amylose. Furthermore, a 40% ethylene glycol (EG) solution was used to make granule suspensions to minimize changes due to leaching. (EG is nonvolatile, eliminating changes in concentration with time. Preliminary work indicated that leaching from waxy maize starch granules in 40% EG was insignificant. See later.) When waxy maize starch (both modified and unmodified) suspensions in 40% EG solution were heated to temperatures within the gelatinization range, some granules completely gelatinized while other granules still showed a clear ‘Maltese Cross’ under polarized light, indicating retention of crystalline order (Fig. 1, bottom). This suggested that there were different subpopulations of starch granules that gelatinized at different temperatures, which is not

Table 3
Molar of substitution (MS) values of hydroxypropylated waxy maize starch (HPWMS) granules

Starch	Fraction[a]	MS
HPWMS 1	Whole	0.125
	59 °C	0.116
	62 °C	0.110
HPWMS 2	Whole	0.114
	59 °C	0.101
	62 °C	0.100
HPWMS 4	Whole	0.179
	56 °C	0.162
	58 °C	0.155
	60 °C	0.149
HPWMS 4	Whole	0.176
	56 °C	0.163
	58 °C	0.156
	60 °C	0.148

[a] See Table 1 for an explanation for starch fractions.

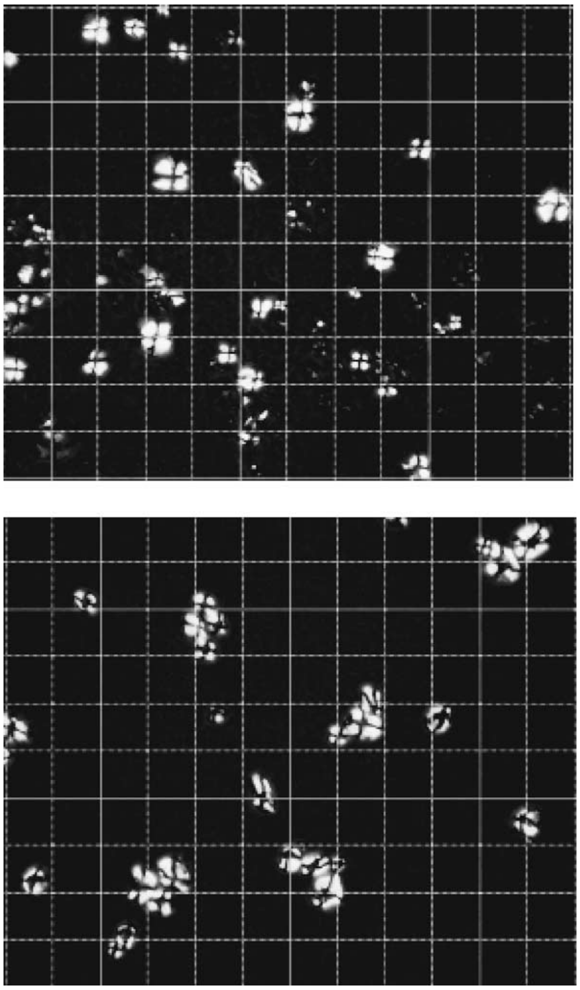

Fig. 1. Polarized-light micrographs of unmodified waxy maize starch: top, granules after holding at 70 °C for 1 h in 40% ethylene glycol; bottom, granules recovered by centrifugation.

surprising since, as already mentioned, individual granules gelatinize in a narrow temperature range (~1 °C) while an entire population will gelatinize over a range of at least 10 °C (Evans & Haisman, 1982). Centrifugation by the described procedure successfully removed all gelatinized starch granules and recovered most nongelatinized starch granules (Fig. 1, bottom). The yield of nongelatinized starch granules recovered decreased as the heating temperature increased. For unmodified waxy maize starch, yields of recovered nongelatinized starch granules at 70, 72 and 74 °C were approximately 65, 40 and 20%, respectively.

3.2. DSC

Thermal stabilities of subpopulations fractionated at different temperatures were verified by gelatinization properties determined by DSC. Compared with the whole population of starch granules before fractionation, gelatinization thermograms of recovered, nongelatinized starch granules shifted to higher temperatures and had narrowed ranges (Fig. 2). The results of DSC analysis of unmodified waxy starch and four hydroxypropylated waxy starches and their subpopulations fractionated at different temperatures are summarized in Table 1. Retrogradation of hydroxypropylated (HP) waxy maize starch was not significant enough to give consistent measurement under the experimental conditions used in this study; therefore only retrogradation data for unmodified waxy maize starch is given.

As previously reported by Kim and Eliasson (1993) and Seow and Theramalar (1993), hydroxypropylation effected significant decreases in both gelatinization temperatures (including T_o, T_p and T_c) and ΔH associated with gelatinization. Over the range of MS studied, the extent of decrease in gelatinization temperatures (including T_o, T_p and T_c) became more pronounced as the level of substitution increased. The T_o of two unfractionated HP waxy maize starches with MS ~ 0.2 was in the range 51.6–52.6 °C and of two HP waxy maize starches with MS ~ 0.1 in the range 54.9–56.7 °C, as compared to unmodified waxy maize starch which had a T_o of 65.3 °C. A similar trend was also observed in T_p and T_c as a function of MS. However, ΔH was not significantly affected by increasing MS. In contrast, Kim and Eliasson (1993) reported that ΔH decreased as MS increased from 0.045 to 0.17 for hydroxypropylated potato starch.

Compared with the whole population of starch granules before fractionation, recovered nongelatinized starch granules had higher T_o. Nongelatinized granules recovered at higher temperatures had higher T_o than did nongelatinized granules recovered at lower temperatures. For both unmodified and HP waxy maize starch, small changes (slight increase or decrease) in T_p and T_c were found after fractionation. Removal of granules with lower gelatinization temperatures had no effect on ΔH.

For unmodified waxy maize starches, the starch granules with higher gelatinization temperatures formed retrograded gels that required a higher temperature (T_{oR}) and more energy (ΔH_R) to melt as compared to the retrograded gel formed from a paste of the whole starch population. T_{oR} and ΔH_R for the whole starch population were 38.5 °C and 8.1 J/g, whereas T_{oR} and ΔH_R for pastes made from higher T_g fractions fell in the ranges 41.0–42.7 °C and 8.6–9.2 J/g (Table 1).

3.3. Leaching of starch molecules

When a suspension of starch granules in water is heated, the granules, well and leaching of starch polymer molecules occurs. Hydroxypropylated molecules leach more easily. Leaching is also more pronounced from granules containing amylose (Shi & BeMiller, 2002). We did not want the granules to be changed during the heating process, so leaching of starch molecules from starch granules had to be minimized.

In this research, 40% EG was used to minimize leaching. Leaching of starch molecules from hydroxypropylated

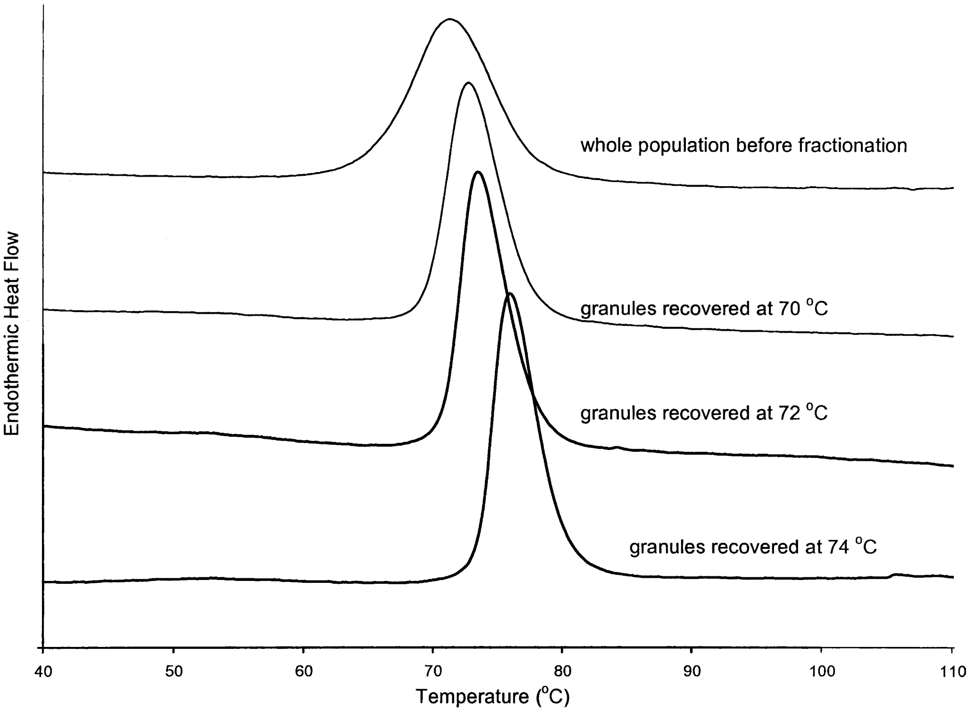

Fig. 2. DSC gelatinization thermograms of unmodified waxy maize starch and its fractions recovered at different temperatures.

starch granules (MS 0.176) before and after fractionation at 58 °C in 40% EG was examined (Fig. 3). When a suspension was heated to 58 °C, which is within the gelatinization temperature range, considerable dissolution of starch molecules occurred. After recovering the nongelatinized granules and reheating them to 58 °C, no significant leaching of starch molecules was observed. The stability of the recovered granules indicates that leaching/dissolution occurred only from gelatinized granules.

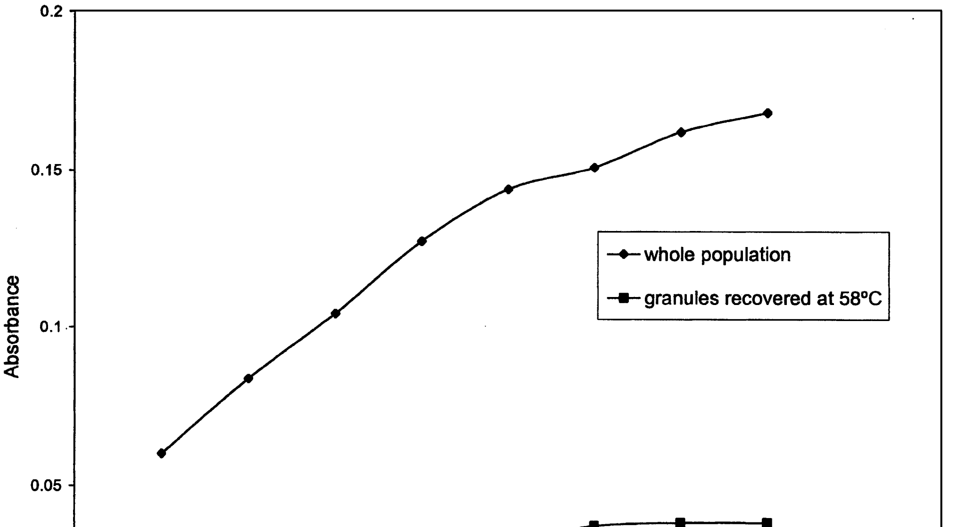

Fig. 3. Leaching in a suspension of HPWMS (MS 0.176) in 40% ethylene glycol. The x-axis is the absorbance of the supernatant treated with I_3^- reagent. Aqueous suspensions of both samples were heated at 58 °C for 80 min.

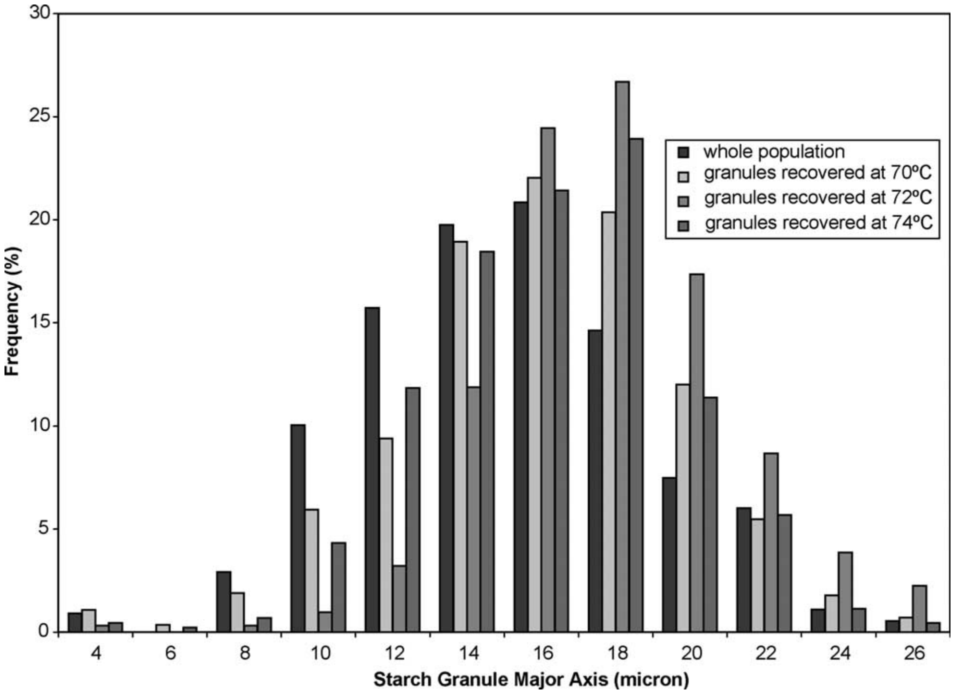

Fig. 4. Starch granule-size distribution of unmodified waxy maize starch and its fractions.

3.4. Granule-size distribution

In general, recovered starch fractions, i.e. higher T_g subpopulations, contained higher proportions of large granules (major axis from 14 to 20 μm) and a lower proportion of small granules (major axis 8–14 μm) than the whole starch population (Fig. 4). Average granule major axes for the whole population and fractions recovered at 70, 72 and 74 °C were 14.2, 15.0, 15.5, and 15.2 μm, respectively.

3.5. Gelatinization properties of small waxy maize starch granules

The increase in the proportion of large granules in the recovered fractions suggests that either (1) small granules gelatinized and were removed during the heat treatment, (2) granules underwent limited irreversible swelling during the heat treatment, or (3) larger granules were recovered preferentially by centrifugation. To distinguish between the possibilities, the gelatinization properties of smaller waxy maize starch granules were examined. Isolation of small waxy maize starch granules was done by the procedure of Stapley and BeMiller (2003). The smaller granule subpopulation had a size range of 1.96–16.78 μm with a peak at 6–8 μm, compared to the whole population of waxy maize starch granules, which had a size range of 2.5-26.43 μm, with a peak at 14–16 μm (Fig. 5). Average major axis of the isolated small granules and of the whole population were 7.92 and 14.17 μm, respectively. Compared with the whole population of waxy maize starch granules, isolated small granules had the same T_o, a higher T_p (1.7 °C higher), a higher T_c (1.6 °C higher), and the same ΔH. This result excluded the possibility that small granules were removed either during the heat treatment (hypothesis 1) or by centrifugation (hypothesis 3), indicating that granules underwent limited irreversible swelling without gelatinization during the heat treatment (hypothesis 2), a phenomenon heretofore neither observed nor thought to occur.

3.6. Branch-chain-length distribution of amylopectin

Branch-chain-length distributions of unmodified and HP waxy maize starches and their fractions as determined by the method of Wong and Jane (1997, 2002) are given in Table 2. Linear chains generated by debranching unmodified waxy maize starch with isoamylase had a bimodal distribution, with the first peak maximum at DP 14 (A chains, with a shoulder of B1 chains) and the second peak maximum at DP 46-50 (B2 chains). The maximum chain length detected by the HPAEC-ENZ-PAD system was DP 72-75. Only one peak at DP 12 was generated by debranching of HP waxy maize starches, indicating that the debranching of modified waxy maize starch by isoamylase was incomplete. This result is consistent with the report of Hood and Mercier (1978) on debranching modified (hydroxypropyl distarch phosphate) cassava starch with pullulanase and also with the finding of Kavitha and BeMiller (1998) that hydroxypropylation of potato starch amylopectin occurs primarily near branch points. The maximum DP of chains generated by debranching HP waxy maize starch detected by HPAEC-ENZ-PAD system was 30.

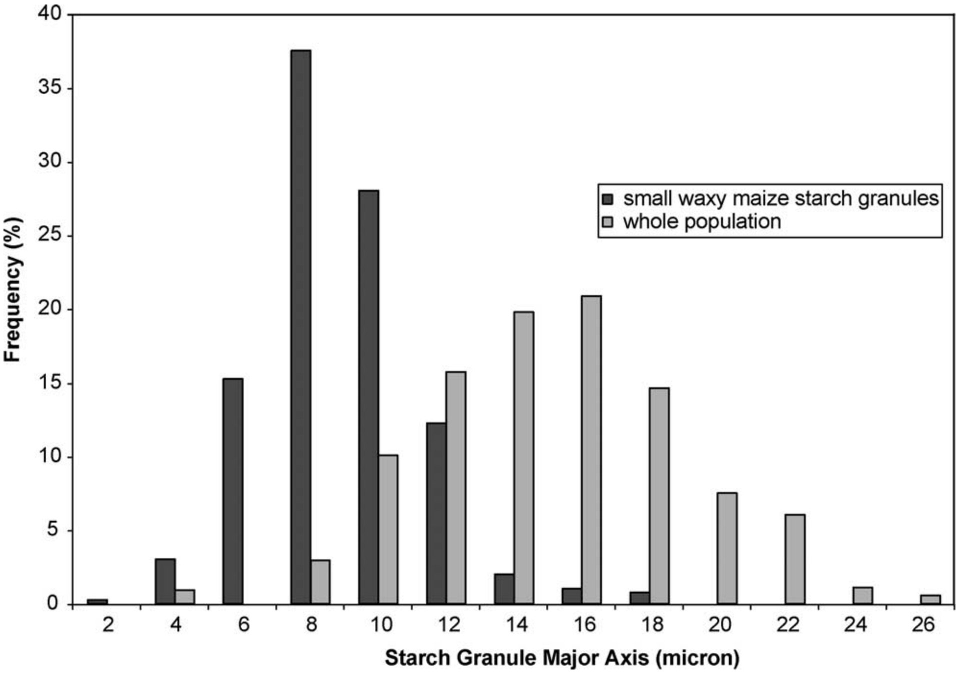

Fig. 5. Comparison of granule-size distribution of isolated small unmodified waxy maize starch granules and whole starch.

Unmodified waxy maize starch granules recovered after heating to a higher temperature in the T_g range contained a lower proportion of short A chains (DP 6-12) than did granules recovered after heating to a lower temperature or the whole granule population.

Normalized branch-chain-length distribution profiles of unmodified and modified waxy maize starches are given in Figs. 6–8. Results are expressed as means ± standard deviation of duplicate measurements, and the height of each bar at each degree of polymerization (dp) represents its relative concentration to the most populated peak (DP 14 for unmodified starches, DP 12 for modified starches, Table 2). For unmodified waxy maize starch, only chains with DP 6-24 are shown, whereas for modified starches, all

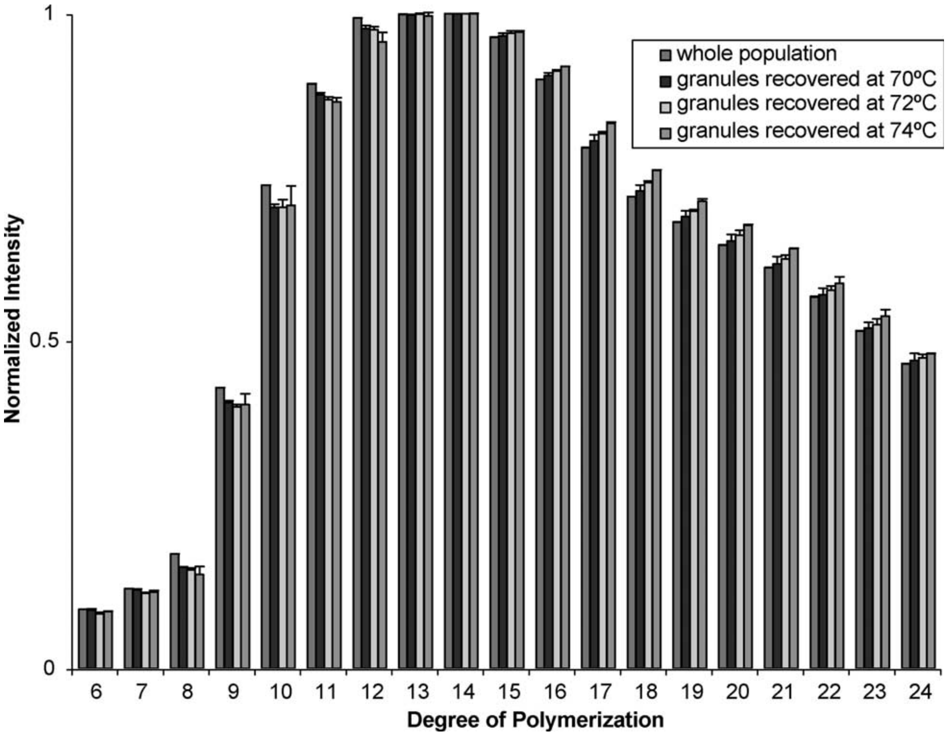

Fig. 6. Comparison of normalized branch-chain-length distributions of unmodified waxy maize amylopectin molecules before and after granule fractionation.

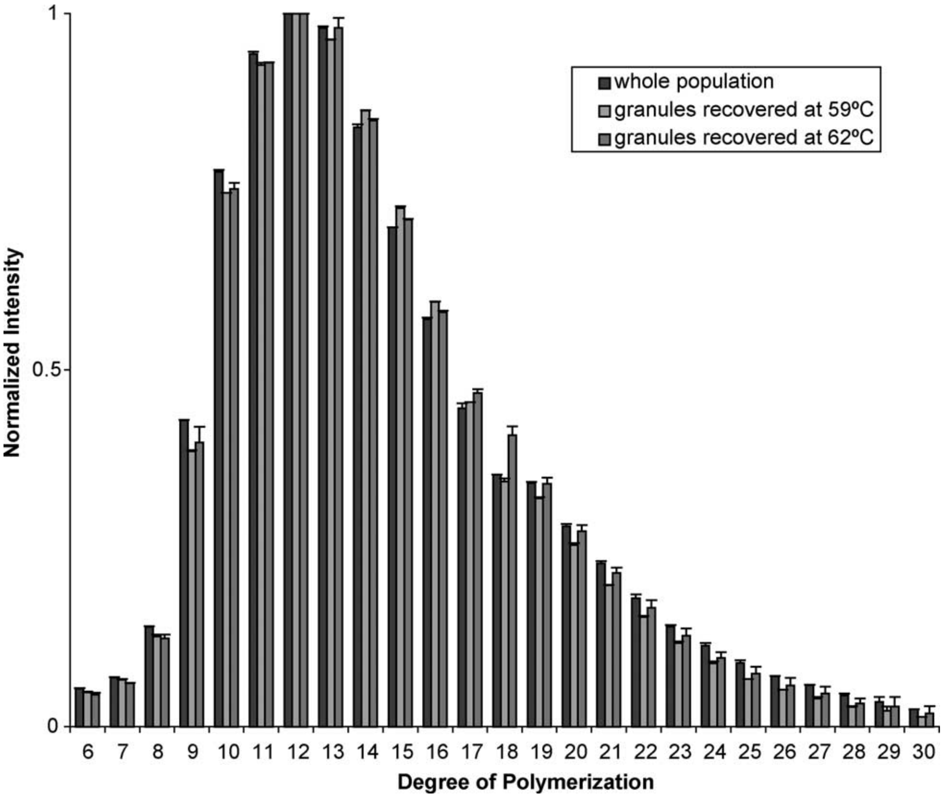

Fig. 7. Comparison of normalized branch-chain-length distributions of hydroxypropylated (MS 0.125) waxy maize amylopectin molecules before and after granule fractionation.

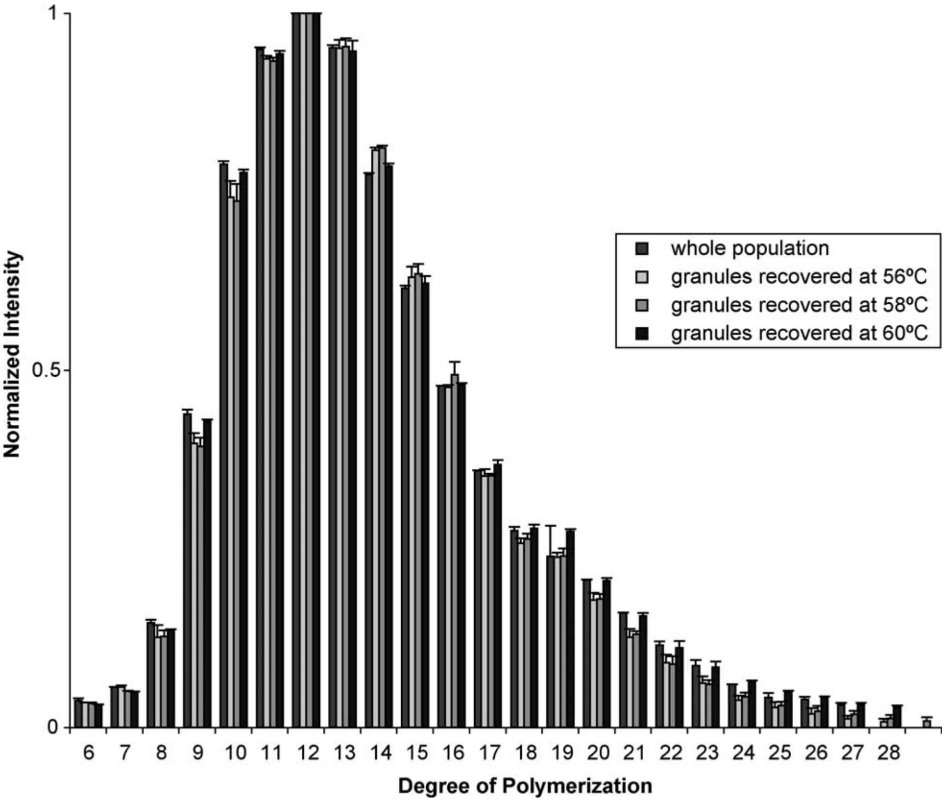

Fig. 8. Comparison of normalized branch-chain-length distributions of hydroxypropylated (MS 0.179) waxy maize amylopectin molecules before and after granule fractionation.

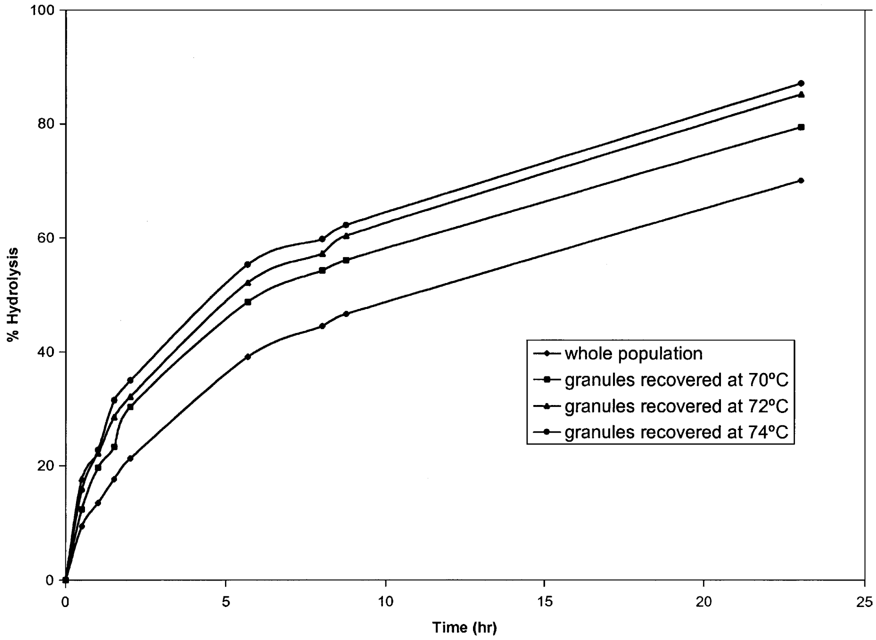

Fig. 9. α-Amylase-catalyzed hydrolysis of unmodified waxy maize starch granules as a function of incubation time. Percent hydrolysis is based on reducing power as determined by the Somogyi-Nelson method (Somogyi, 1952; Wood, 1994).

chains detected are shown. Amylopectin of granules recovered after heating to a higher temperature had a lower normalized concentration of A chains (DP 6-12) (Fig. 6, Table 2) as compared to unmodified waxy maize starch (whole population) and fractions recovered after heating to a lower temperature. For HP waxy maize starches, amylopectin of granules recovered at higher temperatures had a lower normalized concentration of chains of DP 6-11 and a greater normalized concentration of chains of DP 14-16 as compared to amylopectin of the granules of the whole population (Figs. 7 and 8).

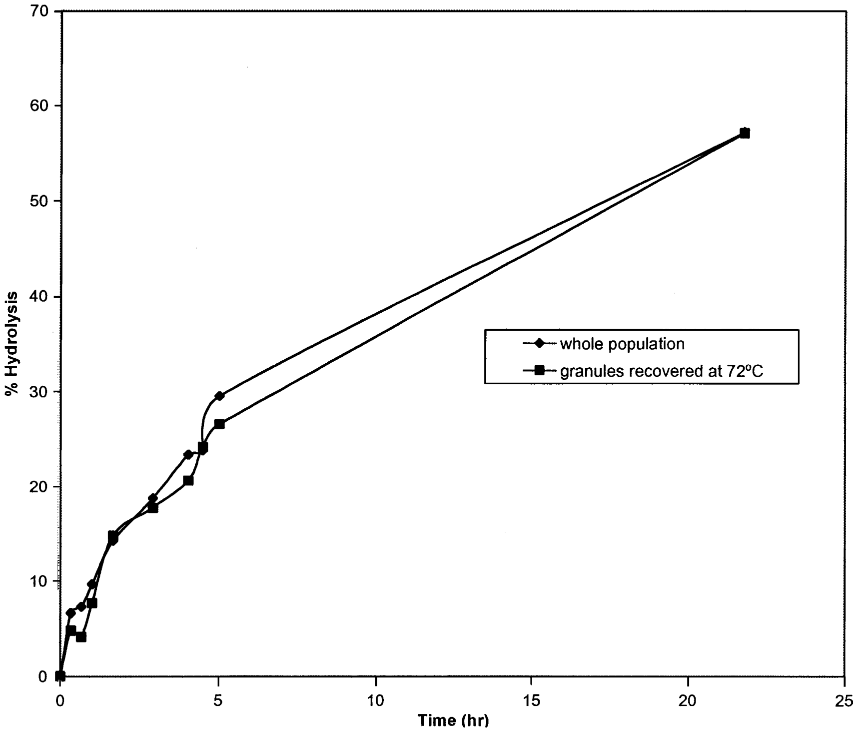

Fig. 10. α-Amylase-catalyzed hydrolysis of partially hydrolyzed (2-h incubation) unmodified waxy maize starch granules as a function of incubation time. Percent hydrolysis is based on reducing power as determined by the Somogyi-Nelson method (Somogyi, 1952; Wood, 1994).

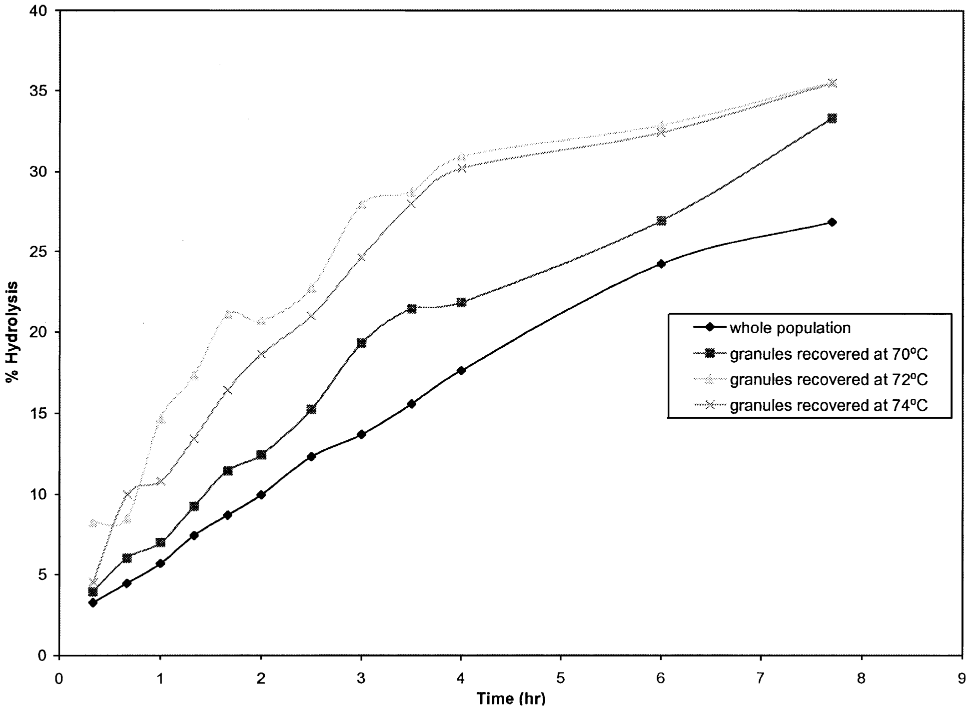

Fig. 11. Amyloglucosidase-catalyzed hydrolysis of unmodified waxy maize starch granules as a function of incubation time. Percent hydrolysis is based on reducing power as determined by the Somogyi-Nelson method (Somogyi, 1952; Wood, 1994).

3.7. Molecular weight distribution of amylopectin

No effect on M_w and R_z of the amylopectin molecules as a result of either hydroxypropylation or fractionation could be detected (Data not given).

3.8. Enzyme-catalyzed hydrolysis

Results of hydrolysis of unmodified waxy maize starch by α-amylase and amyloglucosidase are given in Figs. 9–11. All starch preparations gave a biphasic rate of hydrolysis, with an initial faster rate (from 0 to 2 h for α-amylase and 0–6 h for amyloglucosidase) followed by a slower rate. The explanation for this previously observed phenomena is that the enzymes preferentially attack the more amorphous regions of starch granules, with crystalline regions being hydrolyzed at a slower rate because they are less accessible (Gallant, Bouchet, Buléon, & Pérez, 1992; Vasanthan & Bhatty, 1996).

Starch granules recovered after heating to higher temperatures were more susceptible to hydrolysis by both α-amylase (Fig. 9) and amyloglucosidase (Fig. 11) than were starch granules recovered after heating to a lower temperature and the whole population. Differences in the extent of hydrolysis of the whole population as compared with the fractions were greater than the differences between the three fractions. It appears from Fig. 9 that the greatest difference in degree of hydrolysis between the whole population and the three subfractions occurs in the first 2 h. Therefore, the whole population and granules recovered after heating to 72 °C were incubated with α-amylase for 2 h; the enzyme was deactivated, and the granules were recovered. Then these granules were reincubated with α-amylase. Fig. 10 shows that, after the first 2 h, which is zero hours on this graph, the rates of hydrolysis between the whole population and the granules recovered after heating to 72 °C were essentially the same. Therefore, the initial fast stage of hydrolysis contributed most to differences in susceptibility to the enzymes, indicating that differences in digestibility are at least primarily due to variations in structures of amorphous regions.

3.9. Molar substitution of hydroxypropylated waxy maize starch fractions

Over the range of MS studied, HP waxy maize starch granules recovered after heating to higher temperatures had lower MS values than did HP waxy maize starch granules recovered after heating to lower temperatures and the whole starch population (Table 3) These results indicate that, within a population of granules modified at the same time under the same conditions, granules react to different degrees (probably at different rates). It cannot be determined from this data whether the granules in the higher-melting fraction did not gelatinize because they were less substituted or were less substituted because they had a different structure. To try to answer this question, waxy maize starch

Table 4
Molar substitution (MS) values and glass-transition temperatures of freeze-concentrated liquid phase (T'_g) of hydroxypropylated waxy maize starch fractions

Heating temperature (°C)[a]	MS[b]	(T'_g) (°C)[c]
70	0.071	−5.55
72	0.065	−5.43
74	0.054	−5.34

[a] Recovered nongelatinized granules after heating to the recorded temperature in 40% EG.

[b] Fractions hydroxypropylated under identical conditions. Averages of duplicate analyses.

[c] T'_g of frozen pastes.

was fractionated as described into fractions that were nongelatinized at 70, 72, and 74 °C and these fractions were hydroxypropylated. MS values and T'_g for the resulting products were determined. The results (Table 4) indicated a progressive decrease in MS values and an increase in T'_g as granules with lower gelatinization temperatures were removed. This provides some evidence that the granules in the higher-melting fraction did not gelatinize and became less substituted because they had a different, more crystalline structure. This seems to be in contrast to the data from digestion by enzymes which seems to indicate that the higher-melting fraction contains more regions, accessible to enzymes. Being more channelized is another possible explanation as more channelized granules are more susceptible to hydrolysis by amyloglucosidase (Gunawan, 2002).

3.10. Relationship between granule structure and DSC behavior

Gelatinization or disruption of starch crystallinity begins from the least stable crystallite within a granule and is then rapidly propagated; therefore, T_o is a measurement of perfectness of starch crystallites among all granules in a population of granules (Morrison, 1995; Tester & Morrison, 1990). Unmodified waxy maize starch granules recovered after heating to a lower temperature contained relatively less B1 chains (DP 15-24) and relatively more A chains (DP 6-13) (Fig. 6) than did granules recovered after heating to a higher temperature. It may be that the result of fewer B chains is less perfect crystallites and, thus, a lower T_o. Possible annealing could also contribute to the higher gelatinization temperatures of fractions recovered after heating to higher temperatures (Krueger, Walker, Knutson, & Inglett, 1987). However, the similar ΔH values indicates that the overall degree of crystallinity of granules within subpopulations were similar.

Introduction of hydrophilic hydroxypropyl groups is capable of disrupting inter- and intramolecular hydrogen bonds, weakening the starch granule structure, facilitating the penetration and absorption of water, and therefore increasing the initial rate of plasticization of amorphous regions and resulting in a lowering of the gelatinization temperature (Seow & Theramalar, 1993; Yeh & Li, 1996) (Table 1).

During retrogradation of starch pastes, chains reassociate to form a network structure. Short A-chains (DP 6-12 slow the retrogradation process (Shi & Seib, 1992). We have found that granules recovered after heating to a higher temperature contained proportionally less short A-chains (DP 6-12) and had higher T_{oR} and ΔH_R than granules of the whole population and fractions recovered after heating to a lower temperature (Tables 1 and 2).

3.11. Relationship between granule structure, enzyme-catalyzed hydrolysis rate, and reactivity

The degree of hydrolysis of unmodified waxy maize starch catalyzed by both α-amylase and amyloglucosidase increased in the order whole population < fraction recovered after heating to 70 °C < 72 °C fraction < 74 °C fraction (Figs. 9 and 11). A possible explanation is that starch granules were damaged during the heat treatment and fractionation. Evidence against this possibility is that granules in the fractions showed no evidence of damage (SEM observation. Data not shown). Another possible reason might be that, during heat treatment, granules underwent limited irreversible swelling, leading to structural changes, mostly in amorphous regions by which starch polymer chain organization or inter-chain associations were loosened, therefore increasing accessibility of granules to enzymes. This possibility was supported by the granule-size distribution data which showed that there were more large granules and less small granules in the three starch fractions as compared with the whole population (Fig. 4). Hoover and Vasanthan (1994) and Wang, Powell and Oates (1996) reported that annealing results in a higher susceptibility to digestion by enzymes. This may be the same phenomenon found here. However, in the earlier investigations, the almost certain leaching of starch polymers, especially amylose, during annealing can be assumed. Irreversible swelling at temperatures below the T_g, which heretofore was not thought to occur, seems to be the most plausible explanation at this time. Whatever the cause, whether irreversible swelling or something else, regions highly susceptible to enzyme-catalyzed hydrolysis seem to be generated because the differences between heated and unheated granules occur only in the first 2 h of incubation with α-amylase (Figs. 9 and 10). The change may be in the internal part of the granule, around the central cavity, since it is known that amylases act on maize starch granules from the inside out (Helbert, Schuelein, & Henrissat, 1996; Leach & Schoch, 1961; Nikuni, 1957; Nikuni & Whistler, 1957).

It is generally believed that etherification begins in amorphous regions of granules and that most reaction occurs there (Hood & Mercier, 1978; Steeneken & Smith, 1991; Yeh & Yeh, 1993) because crystalline regions are less pervious to the reagent solutions utilized in chemical modification. Subsequently, modified amorphous regions swell even more, resulting in an opening of crystalline regions to reagent solution penetration. A chains and B1 chains, which occur in double helices and are mostly located in the crystalline regions of granules, are believed to be the least chemically modified. Amorphous intercrystalline parts of B chains are probably hydroxypropylated and, thus, resistant to debranching by isoamylase. This could explain the disappearance of chains of amylopectin that span more than one crystalline cluster (B2 or longer chains) (Figs. 7 and 8).

The significant decrease of ΔH (decreased about 30%) after hydroxypropylation implies that a significant amount of reaction also occurs within crystalline regions of granules, with more reaction in the less perfect crystallites, which leads to the higher MS values in the granules with lower T_o.

4. Conclusion

Waxy maize starch granules (both native and hydroxypropylated) were separated into different subpopulations with successively higher gelatinization temperature ranges. Nongelatinized granules recovered after heating suspensions to higher temperatures, as compared to the whole population and nongelatinized granules recovered after heating to a lower temperature, were larger, had progressively higher onset temperatures of gelatinization, contained amylopectin molecules with lower normalized concentrations of A chains (DP 6-12), and became less substituted after subsequent hydroxypropylation. Previously hydroxypropylated waxy maize starch granules that were recovered after heating to a higher temperature had lower MS values and higher average chain lengths. These results indicate that subpopulations with different physical and chemical properties exist in waxy maize starch and that those granules that are more resistant to gelatinization are probably slightly more crystalline (at least probably are a little more resistant to swelling), as indicated by the fact that they become less highly substituted upon hydroxypropylation. The resistance to swelling may be the result of having a slightly higher percentage of amylopectin A chains. They, however, are more susceptible to hydrolysis catalyzed by both α-amylase and amyloglucosidase.

References

Evans, I. D., & Haisman, D. R. (1982). The effect of solutes on the gelatinization temperatures range of potato starch. *Starch/Stärke*, *34*, 224–231.

Gallant, D. J., Bouchet, B., & Baldwin, P. M. (1997). Microscopy of starch: evidence of a new level of granule organization. *Carbohydrate Polymers*, *32*, 177–191.

Gallant, D. J., Bouchet, B., Buléon, A., & Pérez, S. (1992). Physical characteristics of starch granules and susceptibility to enzymatic degradation. *European Journal of Clinical Nutrition*, *46*, S3–S16.

Gidley, M. J., & Bociek, S. M. (1985). Molecular organization in starches: a ^{13}C CP/MAS NMR study. *Journal of the American Chemical Society*, *107*, 7040–7044.

Gunawan, N. M (2002). *Effect of corn endosperm starch mutations on the degree of granule channelization as determined by hydrolysis with glucoamylase*. MS Thesis, Purdue University, West Lafayette, IN, USA.

Hanashiro, I., Abe, J., & Hizukuri, S. (1996). A periodic distribution of the chain length of amylopectin as revealed by high-performance anion-exchange chromatography. *Carbohydrate Research*, *283*, 151–159.

Helbert, W., Schuelein, M., & Henrissat, B. (1996). Electron microscope investigation of the diffusion of *Bacillus licheniformis* α-amylase into corn starch granules. *International Journal of Biological Macromolecules*, *19*, 165–169.

Hood, L. F., & Mercier, C. (1978). Molecular structure of unmodified and chemically modified manioc starches. *Carbohydrate Research*, *61*, 53–66.

Hoover, R., & Vasanthan, T. (1994). The effect of annealing on the physicochemical properties of wheat, oat, potato and lentil starches. *Journal of Food Biochemistry*, *17*, 303–325.

Jane, J.-L., & Chen, J.-F. (1992). Effect of amylose molecular size and amylopectin branch chain length on paste properties of starch. *Cereal Chemistry*, *69*, 60–65.

Jane, J. L., Kasemuswan, T., Leas, S., Zobel, H., & Robyt, J. F. (1994). Anthology of starch granule morphology by scanning electron microscopy. *Starch/Stärke*, *46*, 121–129.

Jones, R. J., Srienc, F., & Roessler, J. (1992). Flow cytometric determination of light scattering and absorbance properties of starch granules. *Starch/Stärke*, *44*, 243–247.

Kavitha, R., & BeMiller, J. N. (1998). Characterization of hydroxypropylated potato starch. *Carbohydrate Polymers*, *37*, 115–121.

Kim, L. F., & Eliasson, A. C. (1993). The influence of molar substitution on the thermal transition properties of hydroxypropyl potato starches. *Carbohydrate Polymers*, *22*, 31–35.

Kimura, A., & Robyt, J. F. (1995). Reaction of enzymes with starch granules: kinetics and products of the reaction with glucoamylase. *Carbohydrate Research*, *277*, 87–107.

Knutson, C. A., Cluskey, J. E., & Dintzis, F. A. (1982). Properties of amylose–iodine complexes prepared in the presence of excess iodine. *Carbohydrate Research*, *101*, 117–128.

Krueger, B. R., Walker, C. E., Knutson, C. A., & Inglett, G. E. (1987). Differential scanning calorimetry of raw and annealed starch isolated from normal and mutant maize genotypes. *Cereal Chemistry*, *64*, 187–190.

Leach, H. W., & Schoch, T. J. (1961). Structure of the starch granule. II. Action of various amylases on granular starches. *Cereal Chemistry*, *38*, 34–46.

Morgan, K. R., Furneaux, R. H., & Larsen, N. G. (1995). Solid-state NMR studies on the structure of starch granules. *Carbohydrate Research*, *276*, 387–399.

Morrison, W. R. (1995). Starch lipids and how they relate to starch granule structure and functionality. *Cereal Foods World*, *40*, 437–444.

Morrison, W. R., & Gadan, H. (1987). The amylose and lipid contents of starch granules in developing wheat endosperm. *Journal of Cereal Science*, *5*, 263–275.

Muhrbeck, P. (1991). On crystallinity in cereal and tuber starches. *Starch/Stärke*, *43*, 347–348.

Nikuni, Z. (1957). Fine structure of starch granules. Studies with electron microscope and x-ray diffractometer. *Kagaku (Tokyo)*, *27*, 283–288. Chemical Abstracts 51, 95212 (1957).

Nikuni, Z., & Whistler, R. L. (1957). Unusual structures in corn starch granules. *Journal of Biochemistry (Tokyo)*, *44*, 227–231. Chemical Abstracts 51, 67304 (1957).

Obanni, M., & BeMiller, J. N. (1995). Identification of starch from various maize endosperm mutants via ghost structures. *Cereal Chemistry*, *72*, 436–442.

Obanni, M., & BeMiller, J. N. (1996). Ghost microstructures of starch from different botanical sources. *Cereal Chemistry*, *73*, 333–337.

Oostergetel, G. T., & van Bruggen, E. F. J. (1993). The crystalline domains in potato starch granules are arranged in a helical fashion. *Carbohydrate Polymers*, *21*, 7–12.

Seow, C. C., & Theramalar, K. (1993). Internal plasticization of granular rice starch by hydroxypropylation: effects on phase transitions associated with gelatinization. *Stärke*, *45*, 85–88.

Shi, X., & BeMiller, J. N. (2002). Aqueous leaching of derivatized amylose from hydroxypropylated common corn starch granules. *Starch/Stärke*, *54*, 16–19.

Shi, Y. C., & Seib, P. A. (1992). The structure of four waxy starches related to gelatinization and retrogradation. *Carbohydrate Research*, *227*, 131–145.

Somogyi, M. A. (1952). Notes on sugar determination. *Journal of Biological Chemistry*, *195*, 19–23.

Stapley, J. A., & BeMiller, J. N. (2003). Hydroxypropylated starch: granule subpopulation reactivity. *Cereal Chemistry*, *80*, 550–552.

Steeneken, P. A. M., & Smith, E. (1991). Topochemical effects in the methylation of starch. *Carbohydrate Research*, *209*, 239–249.

Tester, R. F., & Morrison, W. R. (1990). Swelling and gelatinization of cereal starches. I. Effect of amylopectin, amylose, and lipids. *Cereal Chemistry*, *67*, 551–557.

Vasanthan, T., & Bhatty, R. S. (1996). Physicochemical properties of small- and large granule starches of waxy, regular, and high-amylose barleys. *Cereal Chemistry*, *73*, 199–207.

Wang, W. J., Powell, A. D., & Oates, C. U. (1996). Effect of annealing on the hydrolysis of sago starch granules. *Carbohydrate Polymers*, *33*, 195–202.

Wasiluk, K. R., Fulcher, R. G., Jones, R. J., & Gegenbach, B. G. (1994). Characterization of starch granules in maize using microspectrophotometry. *Starch/Stärke*, *46*, 369–373.

White, P. J., Abbas, I. R., & Johnson, L. A. (1989). Freeze-thaw stability and refrigerated storage retrogradation of starches. *Starch/Stärke*, *41*, 176–180.

Wong, K. S., & Jane, J. (1997a). Quantitative analysis of debranched amylopectin by HPAEC-PAD with a postcolumn enzyme reactor. *Journal of Liquid Chromatography Related Technologies*, *20*, 297–310.

Wong, K.-S., & Jane, J.-L. (1997b). Recent developments in starch structure analysis using high-performance anion-exchange chromatography with pulsed amperometric detection. In R. R. Townsend, & A. T. Hotchkiss (Eds.), *Techniques in glycobiology* (pp. 553–565). New York: Marcel Dekker.

Wong, K.-S., & Jane, J.-L. (2002). Starch chain length analysis by using an anion-exchange chromatography system equipped with an enzyme reactor and a PAD detector. *Journal of Chromatography Library*, *66*, 403–422. Carbohydrate Analysis by Modern Chromatography and Electrophoresis.

Wood, T. M. (1994). Enzymic conversion of cellulose into D-glucose. *Methods in Carbohydrate Chemistry*, *10*, 219–229.

Yeh, A.-I., & Li, J.-Y. (1996). Kinetics of phase transition of native, cross-linked and hydroxypropylated rice starches. *Starch/Stärke*, *48*, 17–21.

Yeh, A.-I., & Yeh, S. L. (1993). Some characteristics of hydroxypropylated and cross-linked rice starch. *Cereal Chemistry*, *70*, 569–601.