Enzyme-Resistant Starch. I. Characterization and Evaluation by Enzymatic, Thermoanalytical, and Microscopic Methods[1]

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ABSTRACT

Formation of enzyme-resistant starch (RS) during autoclaving and cooling was investigated in starches from wheat, maize, potatoes, peas, waxy maize, and amylomaize. Amylose content and yield of RS were positively correlated. The highest yield (21.3%) was obtained from amylomaize VII starch (70% amylose). Formation of RS in amylomaize VII starch was affected by the starch/water ratio, autoclaving temperature, and number of autoclaving-cooling cycles. The number of cycles exerted the most pronounced effect on RS; increasing the number of cycles up to 20 raised the RS level to over 40%. Differential scanning calorimetry thermograms of amylomaize VII preparations and isolated RS exhibited an endothermic transition over a similar temperature range (120–165°C), which could apparently be attributed to the melting of amylose crystallites. With increasing levels of RS in amylomaize VII starch preparations, a linear increase of melting enthalpies of amylomaize VII starch was recorded. Melting enthalpies of RS indicated changes in the quality of RS with increasing yields of RS. Furthermore, the thermoanalytical data suggested that amylose-lipid complexes were not involved in the formation of RS. Structural differences between heat-moisture and enzyme-treated amylomaize VII preparations, as illustrated by scanning electron microscopy, could be related to different melting enthalpies determined by differential scanning calorimetry.

A series of recent studies demonstrated that a portion of starch in starch-based foodstuffs escapes digestion in the small intestine of man, albeit to various degrees and for different reasons (Anderson et al 1981, Asp et al 1986, Englyst and Cummings 1985, Englyst et al 1987, Stephen et al 1983). Factors that were shown to affect starch digestion in foods include degree of gelatinization, granule particle size, amylose/amylopectin ratio, starch-protein interactions, amylose-lipid complexes, and percentage of retrograded starch (Holm et al 1987). Englyst and Cummings (1987) proposed a classification of starch based on its digestibility. Their scheme distinguishes among readily digestible starch in freshly cooked foods, partially resistant starch such as raw potato and banana starch, and resistant starch (RS), which is formed as a result of food processing. The amount of RS in cooked or baked foods such as bread, pasta, cereals, legumes, and potatoes is small; it reaches levels up to 3% (Englyst et al 1982, 1983).

Interest in RS results from its inclusion in the insoluble dietary fraction when applying enzymatic-gravimetric methods such as the AOAC method (AOAC 1985). Studies conducted to evaluate the nutritional properties of RS (Asp et al 1986, Bjorck et al 1987, Englyst and Cummings 1985, Englyst and MacFarlane 1986, Wyatt and Horn 1988) show that RS in processed foods resists not only amylolytic hydrolysis in vitro but also in the human small intestine. However, once RS reaches the colon it is fermented with the formation of volatile fatty acids. In addition to physiological studies, physico-chemical investigations have been carried out to explain generation of RS (Berry 1986, Englyst and MacFarlane 1986, Ring et al 1988, Sievert et al 1987, Siljestrom and Asp 1985). Accordingly, the current theory of RS formation can be summarized under the term "starch retrogradation" or "starch recrystallization." Upon cooling of gelatinized starch the dispersed starch molecules reassociate spontaneously. A portion of the starch crystallites formed resists enzymatic attack but can be fully or partially solubilized in 2*M* KOH or dimethylsulfoxide and subsequently rendered accessible to amylolytic enzymes. Investigations of RS by enzymatic assays (Berry 1986, Bjorck et al 1987, Englyst et al 1982, Ring et al 1988), iodine-binding capacity (Matsunaga and Kainuma 1986, Ring et al 1988), differential scanning calorimetry (Sievert et al 1987), X-ray diffraction (Berry et al 1988), and column permeation chromatography (Berry et al 1988, Matsunaga and Kainuma 1986) indicate that mainly recrystallized amylose is involved in formation of RS.

The objective of this study was to characterize RS by enzymatic, thermoanalytic, and microscopic methods in relation to process conditions that affect its formation.

MATERIALS AND METHODS

Commercially available starches were used for preparation of RS: wheat starch (Ogilvie Co., Montreal, Canada); pea starch (Nutrio P-STAR 33, Grindsted Products Inc., Kansas City, KS); potato starch, food grade (AVEBE America Inc. Hopelawn, NJ); and four maize starches: regular (PTP), waxy (Amioca), and amylomaize (V and VII) from American Maize Products Co., Hammond, IN.

Formation of RS

Heat-moisture treatment and subsequent cooling of the starch sample is a prerequisite for RS formation. Twenty grams of starch (as-is basis) were weighed into a 1,000-ml beaker and suspended in distilled water. Starch/water ratios used were 1:2, 1:3.5, and 1:10. The suspensions were autoclaved for 1 hr at 121, 134, and 148°C using a thermostatically controlled autoclave (Barnstead Co., Boston, MS). After autoclaving, the samples were allowed to cool and stored overnight in a refrigerator (4°C). These autoclaving-cooling cycles were repeated up to 20 times. The treated samples were freeze-dried. The dried material was ground on a Udy cyclone mill (Fort Collins, CO) to pass a screen with 0.5-mm diameter openings.

Determination and Isolation of RS

RS was estimated by an enzymatic-gravimetric assay. It was considered as the residue remaining after incubation of the sample with a heat-stable α-amylase (Termamyl 120 L, Novo Laboratories Inc., Danbury, CT) and amyloglucosidase (from *Aspergillus niger,* no. A-3042, Sigma Chemical Co., St. Louis, MO). This assay followed the pattern of the AOAC method for the determination of total dietary fiber (AOAC 1985). The method was modified for determination of insoluble dietary fiber. Concentrations of enzymes were adapted to analyses of starchy, basically protein-free products: 0.5-g samples were incubated with 0.2 ml of Termamyl at 100°C for 30 min (pH 6.0). Then, 0.5 ml of amyloglucosidase was added and the sample incubated at 60°C for 30 min (pH 4.5). After filtration, the insoluble residue was either dried overnight at 105°C or vacuum-dried. The material obtained was finely ground and used for further characterization.

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Differential Scanning Calorimetry (DSC)

DSC measurements were performed with a Perkin-Elmer DSC-4 instrument equipped with a 3600 thermal analysis data station and a Perkin-Elmer graphics plotter 2 (Perkin-Elmer Corp. Instrument Div., Norwalk, CT). An indium standard was used for temperature and energy calibration.

Samples of approximately 20 mg were weighed accurately into Perkin-Elmer aluminum pans (no. 0319-0218). About 40 μl of distilled water was added, and the pans were sealed and allowed to equilibrate overnight. The DSC run was performed from 20 to 180°C at the 5°C/min heating rate. A pan with inert material (Al_2O_3) and water represented the reference sample.

T_i (initial transition temperature) and T_c (completion transition temperature) of a peak in the thermogram were determined as the points at which deviations were noted from the linear portions of the scan before and after the peak, respectively. Transition enthalpies (ΔH) computed as J/g were calculated from the area under the curve described by the recorded trace and a straight baseline joining T_i and T_c. Using T_o (onset temperature) computed by the DSC instrument instead of T_i for determination of transition enthalpies led to misleading results because of the irregular shape of some peaks. T_p (peak transition temperature) was defined as the temperature at the peak maximum.

Scanning Electron Microscopy

For examination by scanning electron microscopy (SEM), dried and finely ground samples were placed on double-stick Scotch tape mounted on aluminum specimen holders. The samples were coated with a thin film of gold (30 nm) by Technics Hummer V Sputtering, and viewed and photographed in a Hitachi S570 scanning electron microscope at 20 kV. Photographs were taken at 3,000 magnification on a Polaroid type 55 film.

RESULTS AND DISCUSSION

Formation of RS in Heat-Moisture Treated Starches

The RS content of starches that were autoclaved (1 hr at 134°C; starch/water ratio, 1:3.5 for amylomaize V and VII starches and 1:2.0 for the other starches), cooled, and dried is presented in Table I. According to recent reports (Berry 1986, Wyatt and Horn 1988), a relationship exists between the amylose content of the starches and the yields of RS. The highest yield of RS was obtained with amylomaize VII, the starch with the highest amylose fraction. Although the data suggest that only the amylose fraction is responsible for RS formation, interactions of amylose and amylopectin cannot be excluded (Ring et al 1988).

Effect of Heat-Moisture Treatment on Yield of RS

As amylomaize VII starch provided the highest yield of RS, process conditions affecting RS formation were investigated using amylomaize VII starch. The yield of RS in amylomaize VII formed as a result of heat-moisture treatment was controlled by the following factors (Fig. 1): starch/water ratio (yield of RS increased with decrease in levels of water), autoclaving temperature (even though differences between 121 and 134°C were small, higher temperatures led to a decrease in yield of RS), and number of autoclaving-cooling cycles (increasing the number of cycles increased yield of RS). Increasing the number of cycles up to 20 raised the RS level from 20 to over 40% (Fig. 2).

The results show that the conditions of heat-moisture treatment during starch gelatinization determined extent and manner of dissolution of starch polymers and subsequently association of amylose and yield of RS upon cooling. Amounts of RS in amylomaize VII gels did not change during storage (14 days at 4°C) or when different drying techniques (vacuum-drying vs. freeze-drying) were applied. Factors that influence yield of RS in starches were also described by Berry (1986) and Bjorck et al (1987).

TABLE I
Yields of Resistant Starch

Starch Type	Amylose[a] (%)	Resistant Starch[b] (%, dm)
Amylomaize VII	70	21.3 ± 0.3
Amylomaize V	53	17.8 ± 0.2
Pea starch	33	10.5 ± 0.1
Wheat starch	25	7.8 ± 0.2
Maize starch	26	7.0 ± 0.1
Potato starch	20	4.4 ± 0.1
Waxy maize	<1	2.5 ± 0.2

[a]Manufacturer's data.
[b]Values are averages of three determinations.

Characterization of RS by DSC

The DSC method, which has been used to study gelatinization as well as retrogradation of starch, was employed to characterize amylomaize starch preparations and isolated RS. DSC thermograms obtained are illustrated in Figure 3. The DSC scans on the left side constitute measurements of amylomaize VII starch, untreated (0C), and after from one to four autoclaving-cooling cycles (1C–4C). DSC scans on the right side reflect the measurements of the corresponding RS residues isolated from

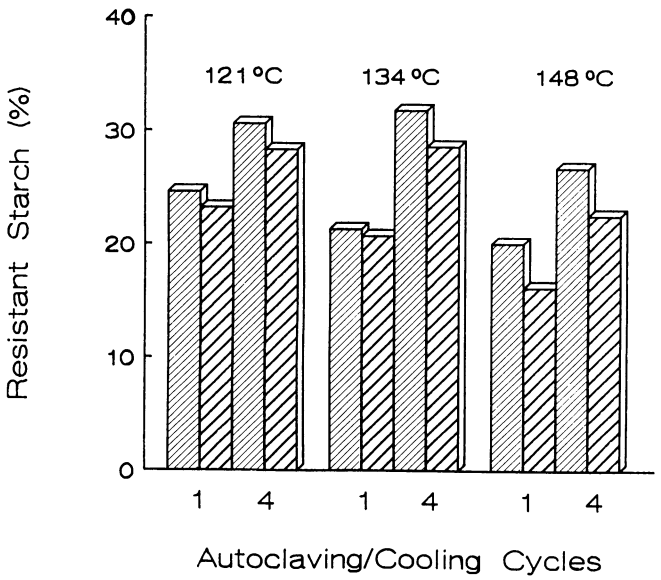

Fig. 1. Effects of starch/water ratio (1:3.5, 1:10), autoclaving temperature, and number of autoclaving-cooling cycles on yields of resistant starch from amylomaize VII starch.

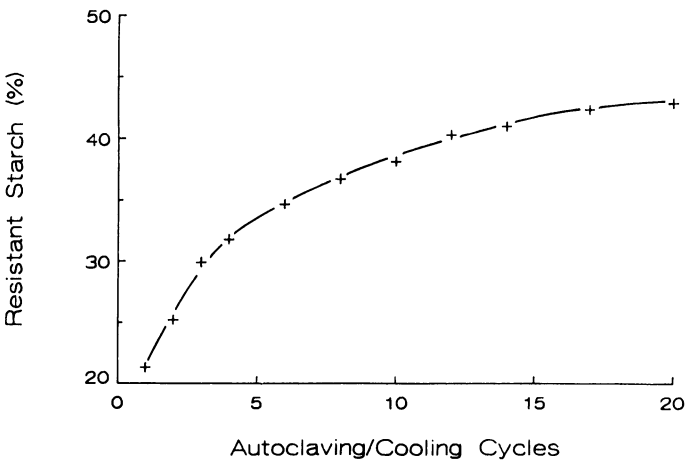

Fig. 2. Effect of repeated autoclaving/cooling cycles on yields of resistant starch from amylomaize VII starch.

untreated and treated amylomaize VII starch. All heat-moisture-treated starch preparations and RS residues exhibited an endothermic transition over a similar temperature range (120–165°C), which might be due to dissociation of crystalline amylose (Fig. 3, Table II). The findings are in good agreement with thermoanalytical data reported on retrograded starch and RS. The melting temperature of recrystalized amylose (Biliaderis et al 1985, Eberstein et al 1980, Ring et al 1987) and RS isolated from wheat starch bread (Sievert et al 1987) was estimated at ~160°C.

The thermograms of amylomaize preparations show that an increase in number of autoclaving-cooling cycles was associated with an increase in melting enthalpies. The corresponding RS residues exhibit sharper endotherms over a broader temperature range with higher melting enthalpies (Fig. 3, Table II). These features might be a result of the enzymatic treatment. Incubation with amylases removed degradable structures and thus constituted an isolation and concentration of RS present in treated amylomaize VII starch. In the temperature range of 120–165°C, no transition could be observed in native, ungelatinized starch or the corresponding oven-dried RS residue (Fig. 3). However, 15.8% of raw amylomaize VII starch was assayed as RS. Two explanations are proposed for these findings: starch fractions not hydrolyzed by enzymes may vary in their physical state and/or sample preparation such as the drying procedure affects the physical properties of starch.

TABLE II
Effects of Autoclaving-Cooling Cycles on Enzymatic (resistant starch yields) and Thermal Characteristics of Amylomaize VII Starch[a]

Number of Autoclaving-Cooling Cycles	Resistant Starch Yields (%)[b]	Transition Temperatures (T, °C)[c] and Transition Enthalpies (ΔH, J/g)[d]							
		Amylomaize VII Starch				Resistant Starch			
		T_i	T_p	T_c	ΔH	T_i	T_p	T_c	ΔH
0	15.8	nd[e]	nd	nd	nd	nd	nd	nd	nd
1	21.3	131.9	149.3	163.4	2.7	125.9	153.6	162.0	8.2
2	25.2	131.7	149.6	160.9	4.5	125.0	152.5	163.1	9.3
3	29.9	131.7	148.1	160.6	7.0	120.9	152.7	163.9	15.0
4	31.8	132.1	152.9	162.2	8.8	121.7	153.6	166.4	19.7

[a]Thermal characteristics determined by differential scanning calorimetry. Definition of parameters in text. Enthalpy values refer to oven-dried resistant starch.
[b]Values are averages of three determinations.
[c]SD <1.0°C, $n = 3$.
[d]SD <10% of the mean value, $n = 3$.
[e]None detected.

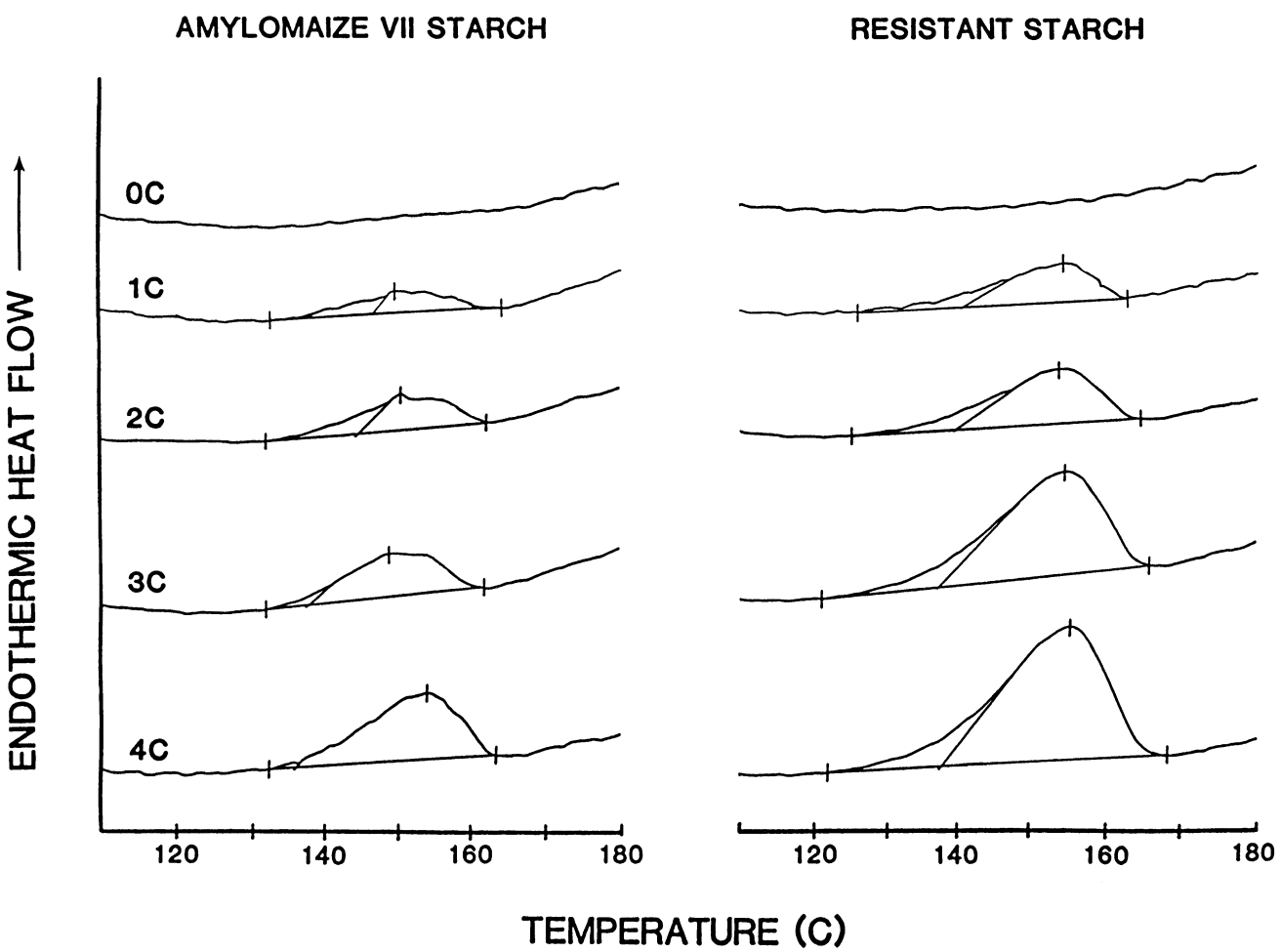

Fig. 3. Differential scanning calorimetric thermograms of amylomaize VII starch and oven-dried resistant starch. Untreated (**0C**) and after from one to four autoclaving/cooling cycles (**1C–4C**); starch/water ratio = 1:3.5, autoclaving temperature 134°C.

When plotting the enthalpy values of amylomaize preparations versus the yields of RS, a linear positive relationship became evident (Fig. 4). Also, the enthalpy values of isolated RS increased as yield of RS increased (Fig. 5). However, this increase was found only up to the 31.8% RS level, which was achieved by four repeated autoclaving/cooling cycles. Further cycles increased RS while the melting enthalpies remained constant. The data suggest that when the number of cycles and thus yield of RS was increased, changes in the quality of RS took place. The higher melting enthalpy of RS isolated from amylomaize with 31.8% RS (four cycles) compared with the melting enthalpy of RS isolated from amylomaize with 21.3% RS (1 cycle) might be related to a higher degree of amylose polymer association in this RS fraction. The plateau of the curve in Figure 5 reflects probably a limit of crystallization of RS structures formed under the conditions applied. Performing one autoclaving-cooling cycle and extending the autoclaving time from 1 to 2 hr did not alter significantly the RS content or the melting enthalpy values of the treated amylomaize starch and the isolated RS residue. It should be noted that the investigated RS residues were oven-dried. Preliminary results showed much higher enthalpy values for vacuum-dried residues. A more detailed description of the effects of sample preparation on enthalpy values of RS will be presented elsewhere.

Rescanning the heated amylomaize preparations and RS residues immediately after the first run or after seven days of storage showed no residual or new peaks. Thus, melting of RS seems to represent an irreversible phase transition.

The thermal profiles illustrated in Figure 3 represent only a section of the DSC run performed from 20 to 180°C. The DSC curves obtained from oven-dried RS residues showed no other transition except the one from 120 to 165°C. However, vacuum-dried residues exhibited an additional small transition in the range from 40 to 60°C. It is not clear whether this thermal effect derived from retrograded amylopectin, because its association can be reversed by heating to about 70°C (Eberstein et al 1980, Eliasson 1985, Schoch 1965, Zeleznak and Hoseney 1986), or whether other factors were involved in formation of this peak. Amylose-lipid complexes that exhibit an endothermic transition at about 100°C (±20°C) (Biliaderis et al 1985, Eliasson and Krog 1985, Kugimiya et al 1980) could not be detected in RS residues. However, limitations of the DSC method to detect phase transitions below the sensitivity limit should be taken into account when evaluating the thermal profiles.

Characterization of RS by SEM

Structural differences among the heat-moisture and enzyme-treated starches were illustrated using SEM.

Raw amylomaize starch consists of granules averaging about 5 μm in diameter (Fig. 6). Amylomaize after one autoclaving-cooling cycle showed a completely different image (Fig. 7). The granular structure disappeared, and bigger, irregularly shaped particles with a continuous spongy-like, porous network were visible.

In amylomaize after four cycles this porous structure was still evident in some parts but more compact formations predominated. The higher melting enthalpy of amylomaize after four cycles compared with that after one cycle might be related to this stabilization.

In RS residues isolated from treated starch, the porous structure was no longer visible and most likely removed by the enzymic treatment (Fig. 8). In the oven-dried residue, very compact and dense formations could be observed.

The vacuum-dried residue formed an open, fluffy structure. The much higher melting enthalpy of vacuum-dried RS compared

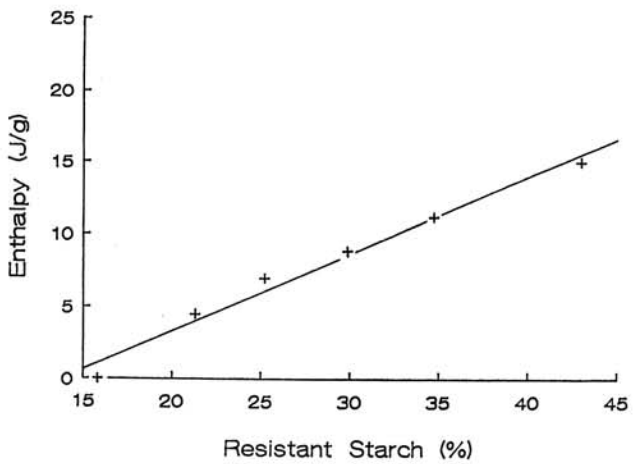

Fig 4. Plot of enthalpy values versus yields of resistant starch from amylomaize VII preparations.

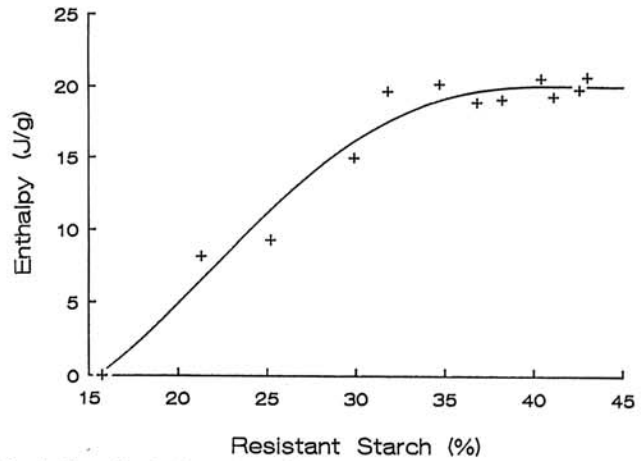

Fig. 5. Plot of enthalpy values of oven-dried resistant starch versus yields of resistant starch from amylomaize VII preparations.

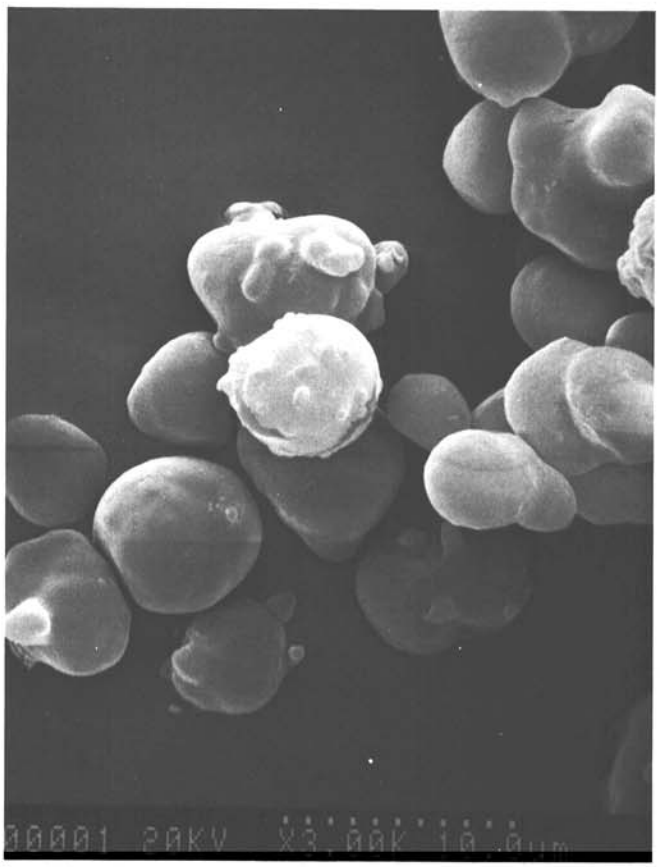

Fig. 6. Scanning electron micrograph of raw amylomaize VII starch.

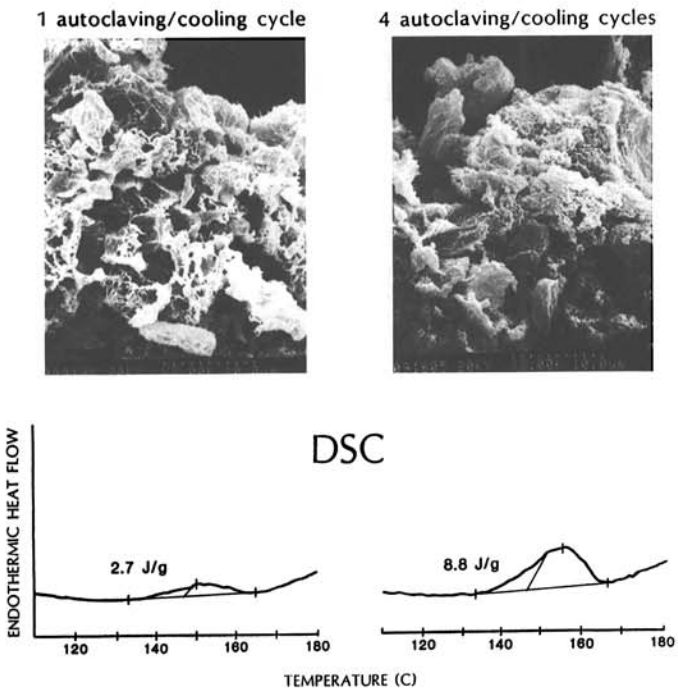

Fig. 7. Scanning electron micrographs and differential scanning calorimetric thermograms of freeze-dried amylomaize VII starch after one and four autoclaving-cooling cycles (starch/water ratio: 1:3.5, autoclaving temperature 134° C).

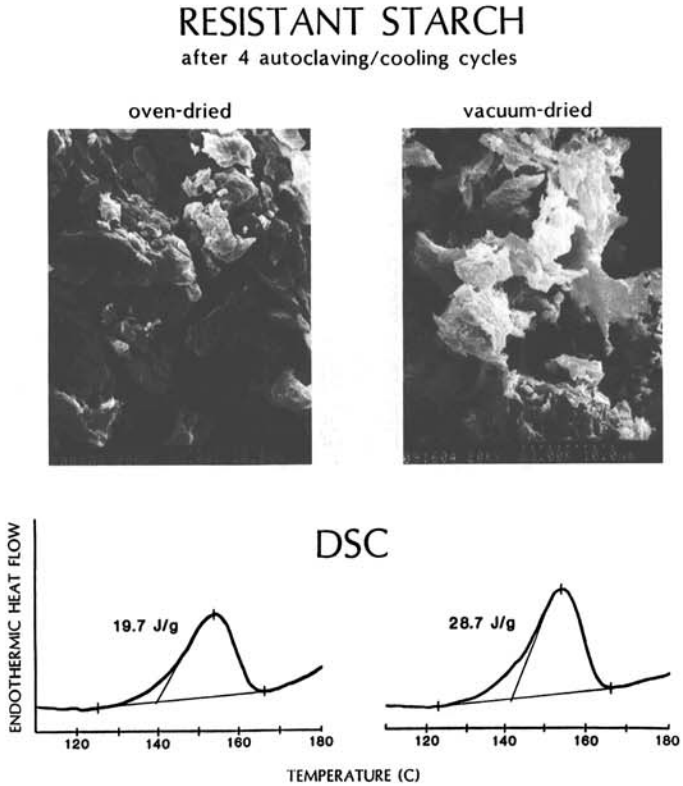

Fig. 8. Scanning electron micrographs and differential scanning calorimetric thermograms of oven- and vacuum-dried resistant starch isolated from amylomaize VII starch after four autoclaving/cooling cycles (starch/water ratio: 1:3.5, autoclaving temperature 134° C).

with the melting enthalpy of oven-dried RS (Fig. 8) might be attributed to a better hydration capacity of vacuum-dried RS and some modification of the crystalline structure during prolonged oven-drying.

CONCLUSIONS

In this study, heat-moisture treated amylomaize VII starch and isolated RS were characterized by three different techniques: an enzymatic assay, DSC, and SEM. The results provide consistent evidence that RS derives mainly from recrystallized amylose. However, RS is not a well-defined entity. Structures that are determined as RS in the enzymatic assay may vary widely in their physical properties when evaluated by DSC. To evaluate RS as recrystallized amylose and to distinguish between RS and other undigested starch structures, physical and/or chemical methods such as DSC and SEM used in this study as well as X-ray diffraction and gel permeation chromatography used by Berry et al (1988) should be employed.

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