Enzyme-Resistant Starch. II. Differential Scanning Calorimetry Studies on Heat-Treated Starches and Enzyme-Resistant Starch Residues

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ABSTRACT

Enzymatic assay of heat-treated starches from amylomaize VII, regular maize, wheat, peas, and potatoes indicated that enzyme-resistant starch (RS) was present in all treated starches. The RS residues exhibited an endothermic transition at ~155°C in the differential scanning calorimetry (DSC) thermogram, which apparently was due to melting of recrystallized amylose. However, with the exception of amylomaize starch, amylose crystallites could not be detected directly in treated starches by the DSC technique. Only after degradable starch structures were removed and enzyme-resistant amylose crystallites were isolated and concentrated, could their dissociation be recorded. RS residues from amylomaize VII, regular maize, and wheat starch showed an additional small endothermic transition between 41 and 67°C. Thermoanalytical characteristics of this transition were investigated using RS residues from amylomaize VII starch preparations. A DSC run of vacuum-dried RS residues with and without added water led to formation of the peak at 54°C. After cooling the residues, an exothermic transition occurred, i.e., the transformation was thermoreversible. Reheating and cooling of vacuum-dried RS in the calorimeter revealed that even temperatures up to 180°C did not completely destroy the structures responsible for peak formation. The role of starch, proteins, and lipids in generating the 54°C transition was investigated. The findings suggested that uncomplexed lipid components induced formation of this endotherm in some RS residues. Protein derived from amylolytic enzymes used in the enzymatic assay also seemed to play a role in generation of the endotherm.

Considerable interest is currently focused on enzyme-resistant starch (RS) in processed foods due to its analytical and possible nutritional significance in the dietary fiber concept (Asp et al 1988, Englyst et al 1987). Certain food processing techniques that involve retrogradation of starch were shown to result in formation of RS (Englyst et al 1983, Siljeström and Asp 1985). The resistance of retrograded starch to hydrolysis by amylolytic enzymes has long been recognized. Katz (1934) described the presence of an "amylocoagulose" in annealed starch gels; it was not digested by malt extract, was slowly hydrolyzed by hot acid solutions, but was soluble in potassium hydroxide. There is now corroborative evidence that retrograded amylose contributes substantially or even entirely, to formation of RS (Berry et al 1988, Björck et al 1987, Englyst and Macfarlane 1986, Matsukura et al 1983, Ring et al 1988, Sievert and Pomeranz 1989). Differential scanning calorimetry (DSC) thermograms of amylose and amylomaize starch samples (Biliaderis et al 1985; Eberstein et al 1980; Ring et al 1987, 1988; Russell et al 1987) and RS isolated from bread, pasta, and retrograded amylomaize starch gels (Sievert et al 1987 and 1988, Sievert and Pomeranz 1989) exhibited an endothermic transition at ~155°C that could be attributed to the melting of crystallized amylose. Significantly higher RS contents were found in cooked pasta than in bread, which probably was due to different degrees of starch gelatinization resulting from different processing conditions. More water was available to gelatinize starch during pasta cooking than during bread baking. Consequently, more amylose could recrystallize in pasta, resulting in higher amounts of RS. Russell et al (1989) reported that crude preparations of RS contain protein derived from enzymes used for isolation of RS and lipid. None of these components, however, were found to be essential for resistance to amylolysis.

The objective of this study was to determine and interpret DSC patterns of heat-treated maize, wheat, pea, and potato starches and of isolated RS fractions.

MATERIALS AND METHODS

Commercially available starches from wheat, regular maize, amylomaize VII, peas, and potatoes were used in this study. The formation of RS by autoclaving and cooling the starch material is described elsewhere (Sievert and Pomeranz 1989). The enzymatic-gravimetric procedure of determination and isolation of RS was the same as that previously described by Sievert and Pomeranz (1989). The assay followed a modified pattern of the AOAC method for the determination of dietary fiber (AOAC 1985). A 0.5-g starch sample was incubated with 0.2 ml of Termamyl 120 L, a thermostable bacterial α-amylase (Novo Laboratories, Inc., Danbury, CT), at 100°C for 30 min (pH 6.0). Then, 0.5 ml of amyloglucosidase from *Aspergillus niger* (no. A-3042, Sigma Chemical Co., St. Louis, MO) was added and the sample was incubated at 60°C for 30 min (pH 4.5). The sample was filtered through Whatman no. 41 filter paper. Washing of the residue with ethanol and acetone was omitted. For comparative studies, Termamyl was replaced by Takalite from *Bacillus licheniformis* (Miles Laboratories, Inc., Elkhart, IN). Incubation conditions were the same. For proteolytic digestion studies, protease from *Bacillus subtilis* (no. P-5380, Sigma Chemical Co., St. Louis, MO) was employed at a concentration of 0.1 unit per milligram of starch material. Protease was used for additional proteolytic treatment of vacuum-dried RS. A 0.5-g RS residue was incubated with protease at 37°C overnight in 50 ml of phosphate buffer (pH 7.5). All residues were dried under vacuum overnight at room temperature and finely ground for further characterization.

Calorimetry

DSC measurements were performed as described by Sievert and Pomeranz (1989). T_i, T_p, and T_c were defined as initial, peak, and completion temperature, respectively.

RESULTS AND DISCUSSION

DSC thermograms from autoclaved, cooled, and freeze-dried amylomaize VII, regular maize, wheat, pea, and potato starches are illustrated in Figure 1. The endothermic transitions between 42 and 72°C (Table I) most likely corresponded to melting of retrograded amylopectin (Eberstein et al 1980, Eliasson 1985, Zeleznak and Hoseney 1986). This thermal effect was not noted in amylomaize starch. The transitions, between 86 and 124°C (Table I), probably were due to disintegration of amylose-lipid complexes (Biliaderis et al 1985, Eliasson and Krog 1985, Kugimiya et al 1980). Presumably the low lipid concentration in pea and potato starch prevented the formation of thermoanalytically detectable amylose-lipid complexes (Kugimiya et al 1980,

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Lehrman and Kabat 1933, Miles et al 1985, Stute and Konieczny-Janda 1983). Treated amylomaize VII starch exhibited a transition from 132 to 163°C (Table I). This thermal effect has been attributed to melting of recrystallized amylose and was also observed in RS fractions from amylomaize VII starch preparations (Sievert and Pomeranz 1989).

Based on an enzymatic-gravimetric assay of heat-treated starches, RS values from amylomaize VII, regular maize, wheat, peas, and potatoes were 21.3, 7.0, 7.8, 10.5, and 4.4% dry matter basis, respectively. Except for amylomaize VII starch, none of the starches showed a clear transition at ~155°C (Fig. 1). However, RS residues enzymatically isolated from all five treated starches displayed prominent peaks between 120 and 177°C (Fig. 2, Table II). An increase in amylose content of the starch, generally, was associated with an increase in melting enthalpies (Table II). RS from amylomaize VII starch gave the highest and RS from potato starch the lowest enthalpy value. The findings from enzymatic and thermoanalytic measurements indicated that RS was present in all treated starches. However, the concentrations of enzyme-resistant amylose crystallites in heat-treated maize, wheat, pea, and potato starch apparently were too low to be directly recorded as a melting process by the DSC technique employed in this study; changes of enthalpies were below the sensitivity limit of the instrument. The enzymatic hydrolysis of the heat-treated starches with subsequent removal of degradable starch structures constituted a concentration and isolation of the crystallites. Consequently, the thermal energy required for melting of crystal structures could be measured by DSC.

When native, untreated amylomaize and wheat starches were incubated with Termamyl at 100°C and amyloglucosidase at 60°C, "RS residues" of 15.8 and 1.7%, respectively, were obtained. Notably, both RS residues gave small endothermic peaks in the DSC thermogram in the range from 136 to 162°C. "RS" from untreated amylomaize VII starch yielded a melting enthalpy value of 3.1 J/g, and from untreated wheat starch 2.3 J/g. Whether the structures responsible for this transition originated with crystalline amylose present in native starch or were formed during preparation of RS is yet to be determined. As reported earlier (Sievert and Pomeranz 1989), oven-dried RS residues from untreated amylomaize VII starch showed no transition at 155°C. Apparently, the severe drying procedure (16 hr at 105°C) affected physical properties of RS.

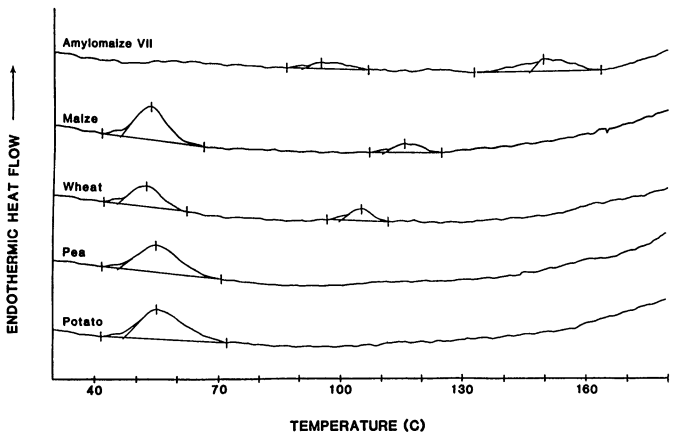

Fig. 1. Differential scanning calorimetry thermograms of autoclaved-cooled and freeze-dried starches from amylomaize VII, regular maize, wheat, pea, and potato.

Thermal effects in the range from 80 to 135°C, which are associated with disintegration of amylose-lipid complexes, could not be detected when the whole thermogram range of RS residues isolated from autoclaved and cooled starches was evaluated (Fig. 2).

In addition to the prominent peak at ~155°C, RS residues from amylomaize, regular maize, and wheat starch exhibited another small endothermic transition between 41 and 67°C (Fig. 2, Table II). Thermoanalytical characteristics of this transition were investigated using RS residues from amylomaize VII starch preparations. The enthalpy value of the peak in the RS residue isolated from native amylomaize starch was calculated to be 1.45 J/g. A DSC run of the vacuum-dried RS residue (5–6% moisture) without addition of water led to an endotherm in the same temperature range and to a slight increase of 0.27 J/g in the enthalpy value from 1.45 to 1.72 J/g (Fig. 3). When the dry-heated sample was cooled, an exothermic transition was recorded, i.e., structural reorganization took place. The peak of reorganization occurred at a lower temperature (34–44°C) than the apparently corresponding peak of disordering. Furthermore, the shape of the exotherm peak was sharper than the broad endotherm peak. Enthalpy of reorganization tended to be lower than enthalpy of disordering. The extent of thermal reversibility was examined by reheating and cooling the sample in the DSC instrument. Figure 4 illustrates that only temperatures above 100°C caused significant irreversible alterations as monitored by decreasing enthalpy values. However, the transition showed a remarkable thermal stability. Even high temperatures (100–180°C) did not destroy completely the structures responsible for formation of the transition at 54°C (T_p) under dry-heating conditions.

The reversibility of the ~54°C peak in presence of water followed a different pattern. Reheating of the sample immediately after the first run (final temperature 70°C) reduced the enthalpy value by approximately 50%. However, holding the heated RS sample in a closed pan for 24 hr at room temperature resulted in a full reformation of the peak.

As starch, proteins, and lipids (as such or their interaction) may contribute to the peak formation, subsequent investigations focused on the potential roles played by those entities.

Role of starch. The peak appeared almost in the same temperature range as melting of retrograded amylopectin. Thus, it could be concluded that amylopectin residues induced this transition. However, the fact that this peak appears in the thermogram even without addition of water contradicts the contribution of retrograded amylopectin. Thermal transitions, such as dissociation of recrystallized amylopectin or amylose, require excess of water; when freeze-dried, heat-treated starches

TABLE I
Enzymatic-Gravimetric (enzyme-resistant starch contents) and Thermoanalytical (differential scanning calorimetry) Characteristics of Heat-Treated Starches[a]

Starches	Enzyme-Resistant Starch Contents (%)[b]	Transition Temperatures T (°C)[c] and Transition Enthalpies ΔH (J/g)[d]								
		T_i	T_c	ΔH	T_i	T_c	ΔH	T_i	T_c	ΔH
Amylomaize VII	21.3	...[e]	...	...	86.4	106.5	1.2	131.9	163.4	2.7
Maize	7.0	41.5	66.1	4.8	106.3	124.2	1.2	...	...	...
Wheat	7.8	41.9	62.1	3.3	96.4	111.4	1.3	...	...	...
Pea	10.5	41.9	70.6	5.4	...	...	...	...	...	...
Potato	4.4	41.5	72.0	6.3	...	...	...	...	...	...

[a] Definitions of parameters in text.
[b] Values are averages of three determinations.
[c] SD < 1.5°C, $n = 3$.
[d] SD < 15% of the mean value, $n = 3$.
[e] None detected.

were scanned without additional water none of these transitions appeared. In light of those considerations, the role of ungelatinized starch in formation of the 54°C peak can be ruled out as well.

Role of proteins. The enthalpy of the peak decreased with increasing amounts of RS (Fig. 5). RS from native amylomaize starch gave the highest value (1.45 J/g); RS from amylomaize containing 43% RS provided the lowest value (0.75 J/g). The data refer to enthalpies recorded by heating the sample in DSC, in the presence of added water. Low enthalpy values throughout the RS range were monitored when Termamyl alone was used in the enzymatic assay. Replacement of Termamyl with Takalite gave similar results. Incubation of amylomaize with Termamyl and amyloglucosidase or with amyloglucosidase alone was associated with formation of the peak (Fig. 5). The "RS residue" obtained from native amylomaize constituted an exception. The peak did not appear unless incubation with Termamyl preceded incubation with amyloglucosidase. The buffer solutions employed in the enzymatic procedure did not induce peak formation, and also a DSC run with freeze-dried amyloglucosidase alone gave no thermal effect at 54°C. These findings suggested that the peak between 41 and 67°C observed in some isolated RS residues was generated by contributions of protein(enzyme)-starch interactions. Some types of amyloglucosidase were shown to have strong starch adsorption capacities (Saha and Ueda 1983, Ueda 1978). Further indication for the involvement of protein was provided by the finding that additional incubation of the vacuum-dried RS residues with protease led to complete disappearance of the 54°C transition.

As enthalpy values were determined on the basis of equal amounts of RS residues and amylose chain association increases with increasing RS content (Sievert and Pomeranz 1989), the negative relation between enthalpy values and RS content presented in Figure 5 could be due to structural differences among RS residues.

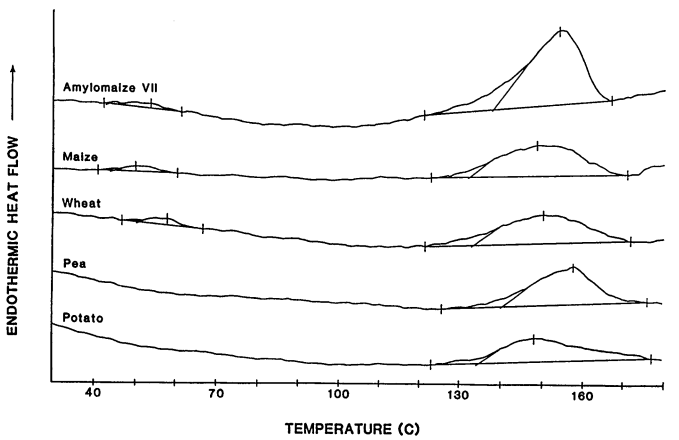

Fig. 2. Differential scanning calorimetry thermograms of enzyme-resistant starch residues isolated from autoclaved-cooled and freeze-dried starches from amylomaize VII, regular maize, wheat, pea, and potato.

It appears that the moieties involved in the transition were bound to amorphous starch structures in the residue, rather than to ordered crystalline fragments.

Role of lipids. Two observations suggested a contribution of lipids. The investigated peak occurred only in RS residues from starches exhibiting amylose-lipid complexes (amylomaize VII, maize, and wheat starch) (Figs. 1 and 2), and thermal behavior of the transition was similar to that of lipid phase transitions. To elucidate the role of lipids, amylomaize VII starch was extracted with 1-propanol/water (3:1, v/v) under reflux. Defatted starch was autoclaved, cooled, and freeze-dried under the same conditions as nondefatted starch. Amylose-lipid complexes that were shown to be present in nondefatted heat-treated starch (Fig. 1) could not be observed in the corresponding lipid-depleted starch. A DSC examination of the RS residue from defatted raw and defatted and autoclaved-cooled amylomaize VII starch in the presence of added water gave no endotherm at 54°C. Heating without additional water gave small endotherms of 0.31 and 0.22 J/g, respectively. The involvement of lipids was further supported by the following observations. Extraction of native amylomaize starch with petroleum ether under reflux affected neither amylose-lipid complex formation nor enthalpy of the 54°C transition in the RS residue. However, when RS prepared from nondefatted native amylomaize starch was extracted with petroleum ether under reflux, the enthalpy value decreased from 1.72 to 0.23 J/g (dry heating in DSC). Cold extraction with petroleum ether reduced enthalpy of the transition to 0.57 J/g. Washing crude RS residues successively with ethanol (95%) and acetone, according to the AOAC method (AOAC 1985), resulted in loss of the transition.

In light of these findings, lipids in RS residues appeared to account for the endotherm between 41 and 67°C. Temperatures above 100°C could have caused degradation of the lipid components, resulting in decreasing enthalpy values (Fig. 4). The low transition temperatures, the appearance of the endotherm even without addition of water, the reduction of enthalpies after extraction of RS residues with petroleum ether, and the loss of this transition after treatment with ethanol and acetone suggest that lipids were present in an uncomplexed form. Melting of amylose-lipid complexes usually takes place at a high temperature range (80–135°C) (Biliaderis et al 1985 and 1986, Eliasson and Krog 1985). Also, lower molecular weight complexes such as amylodextrin-lipid complexes (Biliaderis et al 1986) and amylopectin-lipid complexes (Levine and Slade 1988) exhibit higher melting temperatures (approximately 90°C and 70°C, respectively) compared with the ~54°C endotherm observed in this study. Thus, lipid components in RS residues seemed to adhere to nonhydrolyzed starch structures. In fact, evidence for strong interactions between fatty acids and native or gelatinized starch was provided by electron spin resonance studies conducted by Biliaderis and Vaughan (1987). With regard to RS, amylase-protein seems to play a role in starch-lipid interactions. Whereas the presence of amyloglucosidase significantly affected enthalpy values of the 54°C peak, Termamyl and Takalite did not contribute

TABLE II
Enzymatic-Gravimetric (enzyme-resistant starch [RS] contents) and Thermoanalytical (differential scanning calorimetry) Characteristics of RS Fractions from Heat-Treated Starches[a]

Starches	Amylose[b]	Enzyme-Resistant Starch Contents (%)[c]	Transition Temperatures T (°C)[d] and Transition Enthalpies ΔH (J/g)[e]							
			T_i	T_p	T_c	ΔH	T_i	T_p	T_c	ΔH
Amylomaize VII	70	21.3	41.9	54.0	61.0	0.95	120.4	153.7	166.5	21.2
Maize	26	7.0	40.8	52.8	60.4	0.96	122.4	148.7	170.8	13.9
Wheat	25	7.8	46.8	58.1	66.8	0.94	121.0	150.6	171.6	12.3
Pea	33	10.5	...[f]	...	...	...	125.5	157.7	175.9	12.6
Potato	20	4.4	...	...	...	...	122.9	148.1	177.0	8.9

[a] Definitions of parameters in text.
[b] Manufacturer's data.
[c] Values are averages of three determinations.
[d] SD < 1.0°C, $n = 3$.
[e] SD < 10% of the mean value, $n = 3$.
[f] None detected.

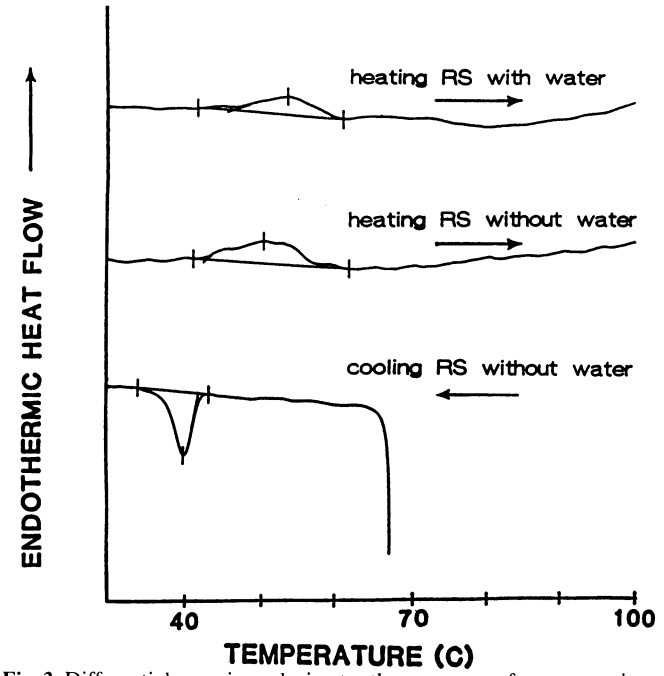

Fig. 3. Differential scanning calorimetry thermograms of enzyme-resistant starch residues isolated from native amylomaize VII starch. Heating and cooling rate: 5°C/min.

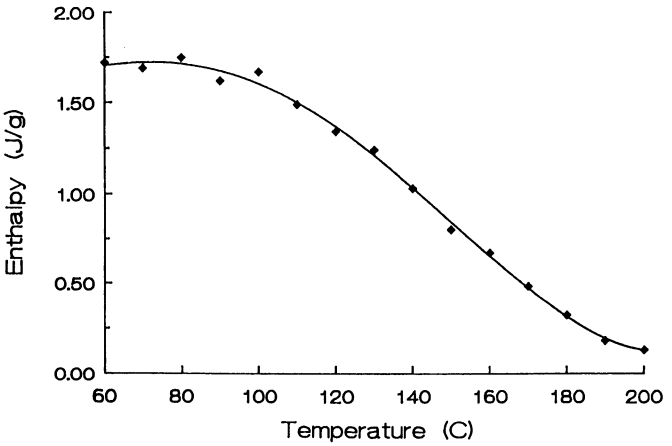

Fig. 4. Thermoreversibility of the endothermic transition at 54°C in vacuum-dried enzyme-resistant starch residue from native amylomaize VII starch. Heating at a rate of 5°C/min in the differential scanning calorimeter without addition of water.

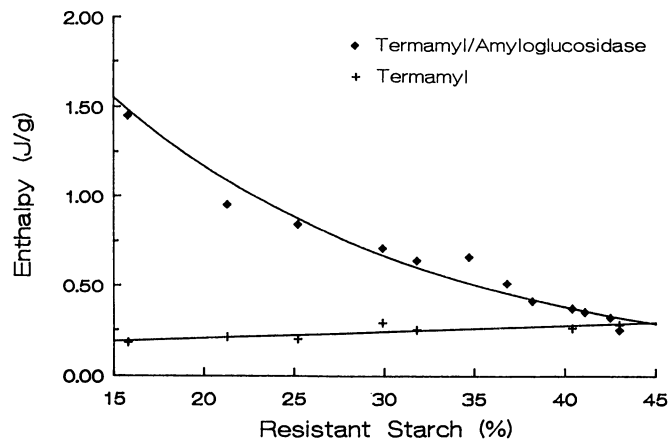

Fig. 5. Effects of enzyme-resistant starch content and different amylase applications on enthalpies of the transition at 54°C in enzyme-resistant starch residues from amylomaize VII starch preparations. Heating at a rate of 5°C/min in the differential scanning calorimeter with addition of water (starch/water ratio 1:3).

substantially to this transition. Furthermore, protease treatment of Termamyl/amyloglucosidase- or amyloglucosidase-incubated RS residues resulted in elimination of the endotherm. Thus, specific enzyme proteins might contribute to binding of lipid components to RS structures.

CONCLUSIONS

RS residues from heat-treated starches displayed endothermic transitions between 120 and 177°C that apparently were due to melting of recrystallized amylose. This provides further evidence that retrograded amylose is involved in formation of RS in heat-treated starchy materials. A small endothermic peak between 41 and 67°C observed in RS residues from amylomaize, maize, and wheat starch seemed to originate from lipid components, presumably adhering to resistant starch structures.

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