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Polymorphism of resistant starch type III

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Abstract

Starch fraction, which is resistant to enzymatic digestion, is produced during retrogradation. This fraction, termed resistant starch type III (RSIII), has health benefits such as pre-biotic effects, improving lipid and cholesterol metabolism, and reducing the risk of colon cancer. Since RSIII preserves its nutritional functionality during cooking processes, it may be used as a food ingredient. This research is part of a project that explores the relation between the structural properties and prebiotic behavior of RSIII. A procedure was developed for the production of different RSIII polymorphs from the same plant source, and applied to three different native starches. For all three types (high amylose corn starch, wheat starch, cornflour), the polymorph structure was determined by the crystallization temperature. Retrogradation at 40 °C lead to the formation of B-type polymorph, whereas incubation at 95 °C, produced a mixture of A- and V-type polymorphs. Differential scanning calorimetry measurements showed no measurable differences in the melting temperatures between the polymorphs, and exhibit an endothermic transition over the range of $T_m = 140–170$ °C.

Keywords: Resistant starch type III; Retrograded starch; Differential scanning calorimetry; X-ray diffraction; Crystalline polymorphs

1. Introduction

Starch is an important energy source in the human's diet. It consists of two D-glucan polymers: Amylose, which is primarily linear, and amylopectin, which is highly branched. In the plant, the starch is packed in very dense paracrystalline granules (Sivak & Preiss, 1998; Zobel, 1988). Various studies have demonstrated that a part of the dietary starch, called resistant starch (RS), escapes enzymatic digestion in the small intestine (Sievert & Pomeranz, 1989). The microbial flora in the colon, however, may ferment a fraction of this RS. RS activities are similar to these of dietary fibers, including pre-biotic effect on colon microflora, altering lipid metabolism, improving cholesterol metabolism, and reducing the risk of ulcerative colitis and colon cancer. Since RS is not digested in the small intestine it also reduces the glycemic index of the food (Hoebler, Karinthi, Chiron, Champ, & Barry, 1999).

RS is classified into four types (Eerlingen & Delcour, 1995) according to the mechanism that prevents its enzymatic digestion. From these four types, RS type III (RSIII) seems to be particularly interesting. Since it preserves its nutritional functionality during cooking processes, it may be used as a food ingredient. RSIII is produced in two steps: Gelatinization, which is a disruption of the granular structure by heating with excess of water (Farhat et al., 2001) and Retrogradation, a slow recrystallization of the starch molecules upon cooling or dehydration (Sivak & Preiss, 1998). The resistant fraction may be then isolated using amylolytic enzymes such as pancreatic α-amylase (Alonso, Calixto, & Delcour, 1998) or Termamyl—heat stable α-amylase (Thompson, 2000). It has been shown that the later approach leads to formation of very thermally stable RSIII, and to yields up to 40% (Sivak & Preiss, 1998).

Long before the nutritional benefits of RSIII were realized, Katz and Derksen (1933) observed that the recrystallization conditions determine the crystal polymorph structure. They reported that retrogradation of starch gels at room temperature results in the formation of crystallites that have an X-ray diffraction (XRD) pattern identifying them as B-type structure. Starch gels that were retrograded at 70 °C have shown an A-type pattern, regardless of the crystalline patterns of the native starch from which they were prepared (Katz & Derksen, 1933). In

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1940, Bear and French confirmed these results (Bear & French, 1941). In a later work Eerlingen, Crombez, and Delcour (1993) have shown that retrogradation of gelatinized wheat starch at 0 and 68 °C was fast and yield a B-type polymorph of RSIII, whereas crystallization at 100 °C was slower and lead to formation of an A-type RSIII (Eerlingen et al., 1993). Other studies have shown that both A- and B-type polymorphs could be crystallized from short debranched glucan chains. As an example, Williamson et al. (1992) prepared A- and B-type polymorph from short amylose chains with an average DP of 20 (Williamson et al., 1992).

Unlike the crystalline forms of native starch, which consists mostly of amylopectin (Zobel, 1992), it is believed that RSIII contains mainly retrograded amylose (Eerlingen et al., 1993; Sievert, Czuchajowska, & Pomeranz, 1991). Therefore, its melting temperature might be expected to be similar to that of high molecular weights crystalline amylose, i.e. around 150 °C (Eerlingen et al., 1993). This property makes RSIII an appropriate candidate as a heat-stable pre-biotic food additive, which may be used in cooked or baked goods. Previous studies compared the melting behavior of crystallites made of short-chain amylose. Williamson et al. (1992) found a melting temperature of 88 °C for A-type polymorph, whereas B-type spherulites melted at 71 °C (Williamson et al., 1992). It should be noted that these temperatures are much lower than the melting temperatures of high molecular weights crystalline amylose, presumably due to the low molecular weight. The question whether the RSIII polymorphism affects its thermal behavior was never addressed before. It was reported that B-type RSIII residues exhibit an endothermic transition over temperature range of 135–170 °C (Gidley et al., 1995; Yoon & Lee, 1998). However, the melting transition of A-type polymorph was not examined. Therefore, the possibility that the differences in the melting behavior are kept for the high molecular weights crystallites still remains to be examined.

The work described in this paper is part of a project that explores the relation between the structural properties and pre-biotic behavior of the different polymorphs of RS III. Understanding the relation between RSIII polymorphism and its resistance is critical for the development of RS with improved prebiotic properties. In native starch, the enzymatic resistance is dependent mainly on the vegetal source of the starch (Gallant, Bouchet, & Baldwin, 1997). In general, cereal starches, which typically exhibit A-type patterns, were attacked more rapidly by α-amylase than potato starch, which exhibits B-type patterns, but this may be due to the granular size and the differences in the surface/volume ratio (Alonso et al., 1998; Ring, Gee, Whittam, Orford, & Johnson, 1988). Williamson et al. (1992) compared the enzymatic resistance of the different polymorphs, using A and B-type crystallites of amylose with an average DP of 20. They showed that the B-type crystallites were hydrolyzed more slowly than the A-type ones (Williamson et al., 1992). However, in order to compare the enzymatic resistance of the different polymorphs of RSIII, it is necessary to isolate both RSIII polymorphs from the same vegetal source. Therefore, the objectives of this project were first, to develop a procedure for the formation of different polymorphs of RSIII from the same vegetal source, by controlling the crystallization conditions, and using different enzymatic procedures. Second, to investigate the relations between the crystallization conditions and the thermal stability of different polymorphs of RSIII.

2. Materials and methods

2.1. Materials

RSIII samples were produced from high amylose corn starch (HACS), (National starch & chemical, Manchester, UK), wheat starch (Sigma S-5127, Rehovot, Israel) and cornflour (Galam Ltd, kibbutz Ma'anit, Israel). Enzymes used for isolation of RSIII were purchased from Sigma Chemical Co., St Louis, MO: Termamyl, i.e. a heat stable α-amylase from *Bacillus licheniformis* (A-4551), α-amylase type VI-B from porcine pancreas (A-3176), amyloglucosidase from *aspergillus niger* (A-9913), protease from *streptomyces griseus* (P-6911).

2.2. Preparation of RSIII

Starch samples (10 g) were suspended in 100 ml of miliQ water overnight and then autoclaved for 120 min at 121 °C. The samples were taken out of the autoclave and immediately transferred into constant-temperature ovens, where they were incubated at either 40 or 95 °C for 24 h.

2.3. Isolation of RSIII

RS was isolated using two methods: (1) a modification of the enzymatic-gravimetric procedure for the determination of total dietary fiber (Sievert & Pomeranz, 1989); (2) a low temperature digestion (Alonso et al., 1998).

Samples were withdrawn from the ovens and suspended in 500 ml phosphate buffer (pH 6.0, 55.6 mM). The samples were filtered trough a 1 mm strainer and the filtrates were placed in a constant-temperature water bath under mild shaking. Then, an enzyme was added in order to digest the amorphous phase. For the enzymatic-gravimetric procedure of total dietary fibers (TDF), Termamyl (2 mg Termamyl/10 g starch) was added and the mixture was shaken for 30 min at 95 °C. For the low-temperature procedure, α-amylase (4 g α-amylase/10 g starch) was added and the mixture was shaken for 16 h at 37 °C. Following the enzymatic digestion, the samples were cooled to room temperature, pH was adjusted to 4.5 using a 2% phosphoric acid solution, Amyloglucosidase (10 ml/10 g starch) was added, and the samples were incubated for 30 min at 60 °C.

The samples were centrifuged (10 min, 1500 × *g*), and the residues were re-suspended in 500 ml of phosphate buffer (pH 7.5, 90.9 mM). Protease was added (10 ml of solution, containing 16 mg of protease in 100 ml of buffer pH 7.5/10 g of starch), and the residues were incubated for 4 h at 42 °C. Three more cycles of centrifugation (10 min, 1000 × *g*) and washing with miliQ water were applied to each sample. Finally, samples were lyophilized overnight.

2.4. Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) measurements were performed using a Perkin Elmer DSC7. Data analysis was performed using supplier's software (Perkin Elmer Corp., Norwalk, Connecticut). An indium standard was used for calibration. Samples (4–5 mg of RSIII and two times the RS weight of miliQ water) were placed in Perkin Elmer stainless steel pressure-tolerant pans (No. 0319-0029). The pans were sealed and allowed to equilibrate overnight at ambient temperature. An empty pan served as the reference. The DSC run was performed from 25 to 200 °C at 10 °C/min heating rate. All the DSC results were evaluated from the mean of three separate determinations for each sample. Rescanning the heated RSIII residues immediately after the first scan showed no residual peaks.

2.5. X-ray diffraction

Wide-angle X-ray powder diffraction analysis was performed using a Philips PW3710 diffractometer and Pw3020 goniometer (Philips, MBLE, Brussels, Belgium) operating at 10 kV and 10 mA with Cu Kα radiation ($\lambda = 0.154$ nm). Dried starch samples were packed in an aluminum frame. Diffractograms of RSIII samples were acquired at an angular range of 2θ from 5 to 30° with step size 0.05°. Counting time was 4 s on each step.

3. Results and discussion

3.1. Effect of the retrogradation temperature on the XRD of RSIII

RS type III is believed to consist mainly of retrograded amylose. Thus, HACS that contains 70% amylose was first chosen for the preparation of RSIII. XRD was used to follow the structural changes of starch during the formation of RSIII. The diffraction pattern obtained from native HACS is shown in Fig. 1. Although this starch is classified as a B-type starch (Zobel, 1988), the diffraction pattern exhibits, besides the B-type profile, an additional peak at $2\theta = 19.9°$ (marked as an arrow in Fig. 1). A peak in this location is characteristic to V-type polymorph. Similar combination of B- and V-type polymorphs in HACS was mentioned before by Sievert et al. (1991).

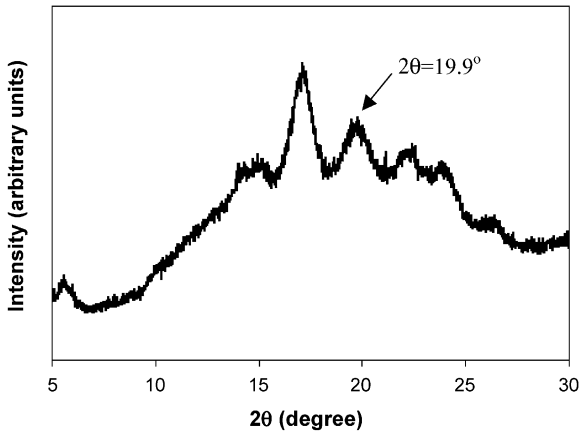

Fig. 1. X-ray diffraction pattern of native HACS. The characteristic peak of a V-type polymorph is indicated by an arrow.

As mentioned before, RSIII was produced by two steps: gelatinization and retrogradation. Part of the gelatinized samples was incubated for 24 h at 95 °C. The resulting RSIII will be referred to in the following as HACS HT (HT stands for high temperature). The rest of the samples were retrograded for 24 h at 40 °C. The resulting RSIII will be referred to in the following as HACS LT (LT stands for low temperature). After incubation for 24 h, the resistant fraction (RSIII) was isolated via enzymatic digestion. Two types of procedures were compared: digestion with porcine pancreas α-amylase at low temperature, and digestion with Termamyl, a *B. licheniformis* heat stable α-amylase, at high temperature. The resulting RSIII will be referred to in the following as the 'amy' version and the 'ter' version, respectively.

A comparison between diffraction patterns of the four versions of RSIII obtained from HACS is presented in Fig. 2. As can be seen, diffraction patterns of the RSIII samples

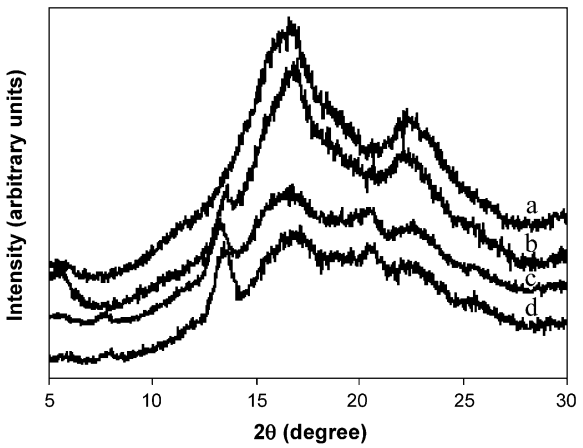

Fig. 2. X-ray diffraction pattern of RSIII produced from HACS. (a) HACS LT-ter: RSIII residues that were incubated at low temperature and were digested with termamyl. (b) HACS LT-amy: RSIII residues that were incubated at low temperature and were digested with α-amylase. (c) HACS HT-ter: RSIII residues that were incubated at high temperature and were digested with termamyl. (d) HACS HT-amy: RSIII residues that were incubated at high temperature and were digested with α-amylase.

differ from that of the native HACS, as the peaks at $2\theta = 15$ and 24° disappeared. Furthermore, it can be clearly seen that different incubation temperature has led to the formation of two different diffraction patterns. Retrogradation at low temperature (LT) have led to the formation of B-type pattern as indicated by the pronounced peaks at $2\theta = 17$ and 23° (Sievert et al., 1991; Yoon & Lee, 1998). The diffraction patterns from samples that were retrograded at high temperature show peaks at $2\theta = 8$, 13.5 and 20°, which are typical to V-type structure. However, the shoulder at $2\theta = 15°$ and the peak at $2\theta = 26°$ indicate that A-type crystals co-exist in these samples. (Yoon & Lee, 1998; Zobel, 1988)

An additional conclusion that may be drawn from the data shown in Fig. 2 is that the enzymatic procedure did not affect the polymorph structure. Yet, a comparison between the diffraction patterns obtained from LT-amy (low temp retrogradation, digestion with pancreatic α-amylase) and LT-ter (low temp retrogradation, digestion with Termamyl) reveals a peak at $2\theta = 13.7°$ in the of LT-amy pattern, which does not exist in any other pattern. A peak in this location is distinctive to V-type polymorph (Zobel, 1988). Since it can be also seen in the diffraction pattern of the native starch (Fig. 1), it may be attributed to a residual crystalline fraction of the native starch, that did not melt during the experiment, due to the fact that HACS LT-amy is the only sample that wasn't heated beyond 60 °C (after crystallization). One more observation that can be made is that the peak at $2\theta = 5.6°$ looks sharper and more emphasized in the LT-amy pattern compared to the LT-ter pattern. It can be suggested that the slow digestion with pancreatic α-amylase results in larger ordered crystalline regions, and thus the RSIII residues exhibited somewhat sharper X-ray patterns. The results presented thus far have shown that retrogradation of molten B-type HACS at different temperatures, leads to formation of two distinguished crystalline structures, depending on the crystallization conditions. A- and B-type polymorphs were also produced from potato starch and wheat starch containing 20–25% amylose by Katz and Derksen in 1933, and later on by Eerlingen et al. in 1993. To the best of our knowledge, the present experiment is the first time were such polymorphs were produced from HACS.

In the first part of the study A- and B-type polymorphs were produced from a B-type native starch—HACS. The purpose of the second part of this work was to examine whether applying the same procedure on A-type starches leads to similar results. For this part of the study, cornflour and wheat starch were chosen. Fig. 3 shows a comparison between XRD patterns obtained from native corn starch, gelatinized corn starch that was incubated for 24 h at 95 °C (HT corn), and gelatinized corn starch that was incubated for 24 h at 40 °C (LT corn). The diffraction patterns obtained from native wheat starch, gelatinized wheat starch that was incubated for 24 h at 95 °C (HT wheat), and gelatinized wheat starch that was incubated for 24 h at 40 °C

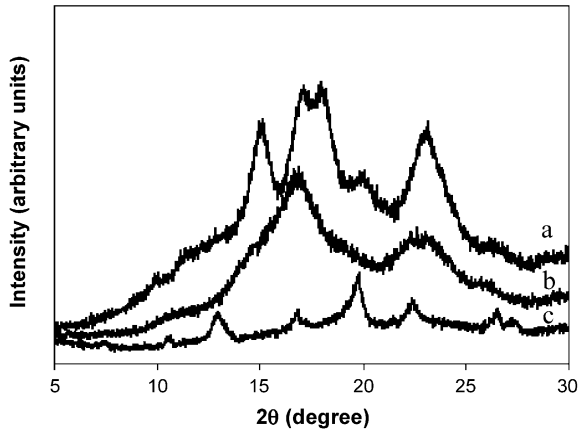

Fig. 3. X-ray diffraction of (a) native cornflour and RSIII samples produced from it at (b) Corn LT: low incubation temperature and at (c) Corn HT: high incubation temperature.

(LT wheat) are shown in Fig. 4. Since the digestion process and the enzymatic procedure did not affect the polymorph structure, at this part of the research only the 'Enzymatic-gravimetric procedure' was used for the isolation of RSIII (Sievert & Pomeranz, 1989).

As was expected, the diffraction patterns of the native cornflour and wheat starch's are typical to A-type polymorph. For both starches, low incubation temperature resulted in a formation of B-type pattern as indicated by the pronounced peaks at $2\theta = 17°$ and 23°. As with the HACS samples, retrogradation of corn starch at HT led to the formation of a mixture of V- and A-type structure, as indicated from the peak at $2\theta = 13$ and 19.8° which are characteristic for V- type and $2\theta = 16.9$ and 26.6° which are characteristic for A-type. On the other hand, wheat starch retrograded at HT resulted in a mixture of B- and A-type structure.

As a final remark, we note that the peak at $2\theta = 5.5°$, considered as a 'finger print' for B-type structure (Yoon &

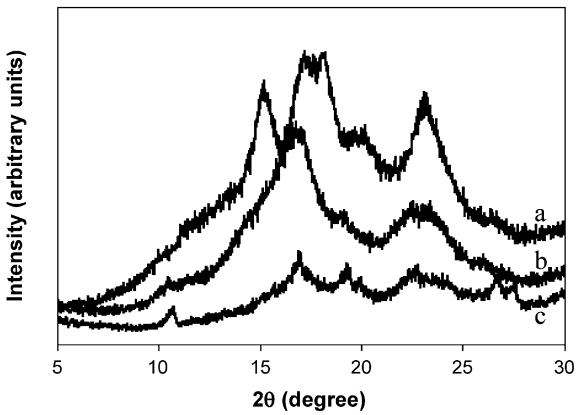

Fig. 4. X-ray diffraction of (a) native wheat starch and RSIII samples produced from it at (b) wheat LT: low incubation temperature and at (c) wheat HT: high incubation temperature.

Lee, 1998; Zobel, 1988), is not observed in the patterns of B-type RSIII produced from cornflour and wheat, although it does show in the pattern of the B-type RSIII originated from HACS (Fig. 2). It is possible that the peak at $2\theta = 5.5°$ in B-type RSIII produced from HACS arises from a crystalline fraction originated from the native starch and didn't go through melting during the experiment, therefore this peak cannot be used as indication for B-type structure in RSIII samples. In summary, it can be concluded that for all three types of starch, the retrogradation temperature influenced the crystalline structure. In all cases, and with no correlation to the amylose/amylopectin ratio or the crystalline structure of the native starch, retrogradations at LT lead to formation of a B-type structure. On the other hand, retrogradations at HT yield a mixture, which in all cases contained A-type crystals.

3.2. Effect of the retrogradation temperature on the melting temperature of RSIII

Following the formation of the different polymorphs and their characterization by XRD we studied whether the differences in the crystalline structure are also expressed in the thermal behavior. The thermal behavior of the RSIII polymorphs was studied using DSC measurements. As a mean of comparison, the gelatinization temperature of the untreated native starches was measured (data not shown). Native HACS showed a characteristic gelatinization curve with endothermic transition at a range of $T_m = 77–92$ °C and enthalpy of $\Delta H = 15.9 \pm 2.3$ (J/g dry sample). Cornflour showed a gelatinization transition at a range of $T_m = 69–70$ °C and enthalpy of $\Delta H = 13.2 \pm 0.9$ (J/g dry sample). For wheat, values of $T_m = 60–61$ °C and $\Delta H = 7.6 \pm 0.9$ (J/g dry sample) were found. These observations are in agreement with data by others (Yoon & Lee, 1998; Zobel, 1988).

The DSC thermograms of RSIII polymorphs produced from HACS are shown in Fig. 5. The transition temperatures and the corresponding enthalpies are summarized in Table 1. As can be seen, all RSIII residues exhibited an endothermic transition over a range of $T_m = 130–170$ °C, with a peak at 150–165 °C, which is characteristic to dissociation of retrograded amylose (Gidly et al., 1995; Yoon & Lee, 1998). RSIII samples which where incubated at HT exhibit another endothermic transition at a range of $T_m = 110–120$ °C. A peak in this location is distinctive to amylose–lipid complexes (V-type polymorph) (Sievert et al., 1991; Yoon & Lee, 1998). This finding is in good agreement with XRD curves in which characteristic peaks of V-type polymorph were observed (Fig. 2).

Thermal analysis by DSC suggests that digestion with Termamyl is associated with sharper transition peaks, and the phase transition begins at a higher temperature (Table 1). Furthermore, digestion with Termamyl caused a higher melting enthalpy of the 150–165 °C endotherm compare to the sample that was isolated using pancreatic α-amylase.

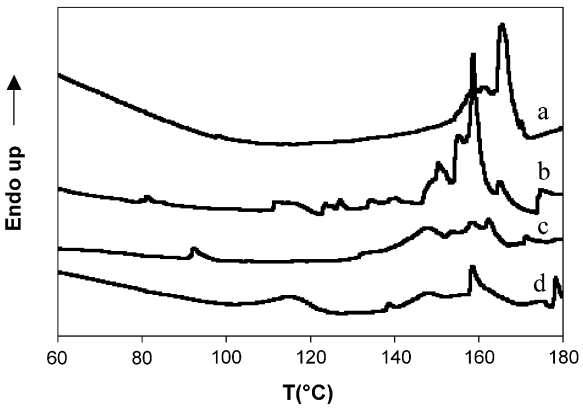

Fig. 5. DSC thermogram of RSIII produced from HACS. (a) HACS LT-ter: RSIII residues that were incubated at low temperature and were digested with termamyl. (b) HACS LT-amy: RSIII residues that were incubated at low temperature and were digested with α-amylase. (c) HACS HT-ter: RSIII residues that were incubated at high temperature and were digested with termamyl. (d) HACS HT-amy: RSIII residues that were incubated at high temperature and were digested with α-amylase.

Since it was already demonstrated that the digestion process and the enzymatic procedure do not affect the polymorph structure, it can be concluded that those differences are due to a higher degree of crystallinity. The digestion at high temperatures (with Termamyl) removed more disordered chain segments and provided RSIII residues with a higher proportion of ordered amylose fragments (Sievert et al., 1991).

The DSC thermograms of the two RSIII polymorphs produced from cornflour are shown in Fig. 6. The corresponding thermograms for wheat starch are shown in Fig. 7. The transition temperatures and the corresponding enthalpies are summarized in Table 2. All RSIII residues exhibits an endothermic transition over a range of $T_m = 140–170$ °C (Figs. 6 and 7), i.e. the transition temperature resembles to that of the RSIII produced from HACS. The cornflour samples that were incubated at HT

Table 1
DSC results for RSIII residues produced from native HACS

	HACS HT		HACS LT	
	amy	ter	amy	ter
T_{o1} (°C)	136–141	123–140	131–135	140–151
T_{c1} (°C)	167–173	170–174	164–170	169–175
T_{p1} (°C)	151–158	158–162	151–162	154–164
ΔH_1 (J/g)	17.2 ± 5.0	45.8 ± 1.5	17.7 ± 2.9	30.0 ± 1.2
T_{o2} (°C)	101–105	106–113		
T_{c2} (°C)	123–126	121–126		
T_{p2} (°C)	115–117	115–119		
ΔH_2 (J/g)	3.6 ± 0.6	2.1 ± 0.3		

Results are an average of three runs. 1,2: peak numbers; T_o : onset temperatures (°C); T_c : completion temperatures (°C); T_p : peak temperatures (°C); ΔH : transition enthalpy ± standard deviation (J/g dry sample).

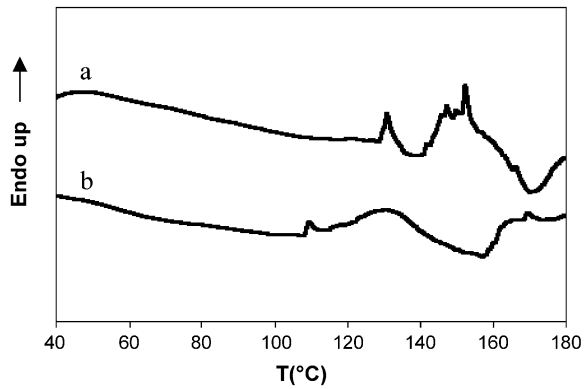

Fig. 6. DSC thermograms of RSIII samples produced from cornflour. (a) Corn LT: RSIII residues that were incubated at low temperature. (b) Corn HT: RSIII residues that were incubated at high temperature both were digested with termamyl.

(Corn HT) exhibits an additional endothermic transition at a range of $T_m = 110–120$ °C, while the sample produced from wheat (wheat HT) did not. As already mentioned, a peak in this location is distinctive to amylose–lipid complexes (V-type polymorph), which were detected by the XRD in the corn HT sample (Fig. 3), but was not seen in the diffraction pattern of the wheat HT sample (Fig. 4).

In summary, retrogradation at HT led to the formation of a mixture of polymorphs A with polymorph V or B. A-type polymorph is known to have denser unit cell, and contains very few bound water molecules (Zobel, 1992). Retrogradation at LT led to the formation of polymorph B, which is less dense and includes more water molecules. Despite the differences in the polymorph density and the degree of hydration of the crystalline structure, DSC measurements showed no significant differences in the RSIII melting temperature between the two polymorphs. All six thermograms (two different polymorphs for each of the different

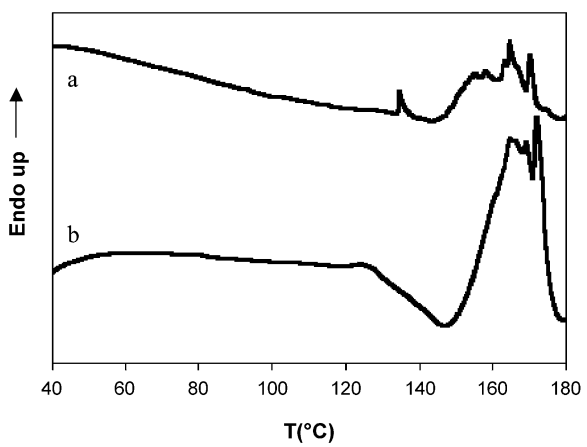

Fig. 7. DSC thermograms of RSIII samples produced from Wheat starch. (a) Wheat LT: RSIII residues that were incubated at low temperature. (b) Wheat HT: RSIII residues that were incubated at high temperature both were digested with termamyl.

Table 2
DSC results RSIII samples produced from cornflour and wheat starch

	Corn		Wheat	
	HT	LT	HT	LT
T_{o1} (°C)	104–108	138–141	146–148	143–150
T_{c1} (°C)	152–158	164–171	176–18	172–178
T_{p1} (°C)	131–139	151–157	158–172	158–165
ΔH_1 (J/g)	24.9 ± 4.4	25.5 ± 3.5	59.7 ± 4.7	35.3 ± 9.7
T_{o2} (°C)	156–158			
T_{c2} (°C)	173–178			
T_{p_2} (°C)	163–166			
ΔH_2 (J/g)	10.5 ± 0.9			

Results are an average of three runs. 1,2: peak numbers. T_o : onset temperatures (°C); T_c : completion temperatures (°C); T_p : peak temperatures (°C); ΔH : transition enthalpy ± standard deviation (J/g dry sample).

types of starch) showed endothermic transitions at 130–170 °C, which are characteristic to the dissociation of retrograded amylose. Thus, differences in the melting temperature between A- and B-type polymorphs that were observed for short-chain amylose (Whittam, Noel, & Ring, 1990; Williamson et al., 1992), do not appears in RSIII produced from native, high molecular weight starch. From examining the dependency of the thermal behavior on the enzymatic digestion, in seems that Termamyl causes higher crystallinity than amylase, namely since the digestion at HT by termamyl melts the less stabilized crystals, so the remaining fraction is more crystalline.

4. Conclusion

The main goal of this research was to produce and characterize different polymorphs of RSIII. It can be concluded that for all three types of starch used, the polymorph structure is determined by the crystallization temperature. Regardless of the chemical composition and the crystalline structures of the native starch, retrogradation at LT led to the formation of polymorph B, whereas retrogradation at HT led to the formation of mixture of polymorphs, one of which was polymorph A. In spite of the differences in the polymorph density and the degree of hydration of the crystalline structure, DSC measurements showed no measurable differences in the thermal behavior between the two polymorphs. After producing both polymorphs from the same plant, it is now possible to explore the colloid structure of the crystalline elements and the effect of RSIII crystal structure on its resistance to enzymatic and micro-flora digestion.

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