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Influence of incubation temperature and time on resistant starch type III formation from autoclaved and acid-hydrolysed cassava starch

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Abstract

Raw cassava starch, having 74.94 and 0.44 g/100 g resistant starch type II and III (RS II and RS III), respectively, was autoclaved at 121 °C in water, 1, 10 or 100 mmol/L lactic acid. The formation of RS III was evaluated in relation to variable incubation temperature (−20 to 100 °C), incubation time (6–48 h) and autoclaving time (15–90 min). Negligible to low quantities of RS III (0.59–2.42 g/100 g) were formed from autoclaved starch suspended in 100 mmol/L lactic acid, whereas intermediate to high quantities (2.68–9.97 g/100 g) were formed from autoclaved starch suspended in water, 1 or 10 mmol/L lactic acid, except for treatments with water or 10 mmol/L lactic acid incubated at 100 °C for 6 h (1.74 g/100 g). Autoclaving times corresponding to maximum RS III contents were 15 and 45 min for water and 10 mmol/L lactic acid, respectively. Whereas, the RS III fractions from cassava starch suspended in water had melt transitions between 158 and 175 °C with low endothermic enthalpies (0.2–1.6 J/g), the thermal transitions of the acid treated samples were indistinct.

Keywords: Cassava starch; Resistant starch type II; Resistant starch type III; Differential scanning calorimetry

1. Introduction

Cassava is principally exploited as subsistence food and animal feed in the tropical belt and developed countries, respectively, though its importance as a source of industrial starch is rising in countries with economies in transition (Moorthy, 2002). The commercial value of cassava starch can be improved further by modifying its functional and nutritional characteristics into speciality secondary products. One such product is resistant starch (RS), an indigestible starch fraction that resists enzymatic hydrolysis in the small intestine and instead passes on to the colon where it is fermented by the bacterial flora. The fermentation products, comprising short chain fatty acids, rectal gases and biomass, may be partially absorbed to provide energy to the mucosal cells or support growth and metabolism of the colonic flora, whereas the undigested material is excreted in the stool. Nutritional studies have shown that RS improves lipid and cholesterol metabolism, increases faecal bulk, reduces the risk of ulcerative colitis, colorectal cancer, coronary heart disease, constipation and Type II diabetes and binds toxins, bile acids and carcinogens (Haralampu, 2000; Morita et al., 2005; Xue, Newman, & Newman, 1996).

Starch digestibility in the gastro-intestinal tract is highly variable, ranging from rapidly digestible through slowly digestible to indigestible RS. The rapidly and slowly digestible fractions are digested and absorbed in the small intestine, generally within 120 min of consumption of food, whereas RS is fermented in the colon (Englyst, Kingman, & Cummings, 1992). The nature of RS in foods is variable and is classified on the basis of its botanical source and processing. It occurs as physically inaccessible starch (RS I), resistant starch granules (RS II), retrograded starch (RS III) and chemically modified starch (RS IV). Despite these delineations, the extent of homogeneity within each

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type of RS remains unclear and caution is called for when differentiating the types (Thompson, 2000). The RS I fraction is physically inaccessible to digestion by entrapment in a non-digestible matrix whereas RS II is native dehydrated granular starch packed in a compact molecular structure that limits the accessibility of amylases. The RS I and II fractions occur in whole cereal grains and root crops, respectively, and have insignificant nutritional value because of their thermal instability. The RS III fraction is formed by recrystallisation of amylose polymers, subsequent to gelatinisation, into enzyme-resistant double helices stabilised by hydrogen bonds and is characterised by a high thermal stability (Englyst et al., 1992; Haralampu, 2000; Thompson, 2000). Resistant starch type IV is formed by chemical modification of starch.

Resistant starch type III is of particular interest as an ingredient in the food industry because of its physical and nutritional functionality and processing stability (Haralampu, 2000; Thompson, 2000). Several methods have been proposed for the formation of RS III and the yields depend on the physical state of the food, its water and lipid contents, amylose to amylopectin ratio, degree of amylose polymerisation, nature of double-helical polymorphs, the presence of added ingredients and process and storage conditions (Eerlingen, Crombez, & Delcour, 1993a; Eerlingen, Deceuninck, & Delcour, 1993b; Eerlingen & Delcour, 1995; Englyst et al., 1992; Thompson, 2000). Furthermore, quantification of RS III as an independent entity from dietary fibre has made it possible to grade foods on the basis of their RS contents (Gõni, García-Diz, Maňas, & Saura-Calixto, 1996).

Qualitative differences between native starch and RS are evaluated by differential scanning calorimetry (DSC) and X-ray diffraction. Whereas DSC measures thermal transitions of polymers, which reflect differences in associative bonding between the molecules, X-ray diffractograms allow the classification of starch into A-, B-, or C-patterns, which are associated with cereals, roots and tuber crops and legumes, respectively (Englyst et al., 1992). Starch granules exhibiting B- or C-patterns are generally more resistant to amylolytic digestion than those with A-patterns, though the process of RS formation may change the nature of the diffractograms (Eerlingen et al., 1993a; Shamai, Bianco-Peled, & Shimoni, 2003).

The objective of this study was to determine the quantities of RS III that can be formed from cassava starch by varying the processing conditions, and type and concentration of solvent. Thermal transitions of the native cassava starch and RS III were measured using a DSC.

2. Materials and methods

2.1. Cassava starch extraction and composition

Cassava (*Manihot esculenta* Gaerth), purchased in Machakos district in Kenya, was peeled and grated in a laboratory-size cassava grater (KIRDI, Nairobi, Kenya) before sieving through a filter cloth. The starch milk was allowed to sediment for 2 h before draining the filtrate. The starch-cake was sun-dried over a period of 2 days before determining its proximate composition using standard AACC methods (1984). A Luci 100 colour difference meter (Dr. Bruno Lange GmbH, Berlin, Germany) was used to determine whiteness of the starch.

Starch content was determined by adding 100 mg sample to 5 mL of 8 mol/L hydrochloric acid followed by 20 mL dimethyl sulphoxide. The Erlenmeyer flask was closed with Parafilm M (Pechiney Plastic Packaging, IL, USA) and incubated at 60 °C for 30 min. It was then cooled to 25 °C before adding 50 mL distilled water and adjusting the pH to between 5 and 7 with 5 mol/L sodium hydroxide. The contents were transferred to a 100 mL volumetric flask, filled to mark with distilled water and filtered before measuring starch content using a Boehringer Mannheim/R-Biopharm starch kit (R-Biopharm, Darmstadt, Germany). Rapidly and slowly digestible starch fractions and RS II and RS III in the raw cassava starch were determined by enzymatic-colorimetric methods (Englyst et al., 1992). Starch digestion index was defined as the ratio of rapidly digestible starch to total starch, and expressed as a percentage.

Amylose content was analysed using the method described by Freitas, Paula, Feitosa, Rocha, and Sierakowski (2004). Cassava starch was weighed (200 mg) and solubilised with 2 mL of 1 mol/L sodium hydroxide and 4 mL distilled water. The test-tubes were capped and heated for 30 min in a water-bath at 95 °C with occasional mixing. The solution (0.1 mL) was added to 5 mL of 0.5 % trichloroacetic acid and mixed before adding 0.05 mL of 0.01 mol/L iodine–potassium iodide solution (1.27 g I_2/L + 3 g KI/L) and further mixed. The blue colour was read at 620 nm after 30 min at 25 ± 1 °C against water using a Helios spectrophotometer (Thermo Electron Corporation, Cambridge, Britain). A standard curve was obtained from duplicate determinations using 0–100% amylose from potatoes (Fluka Chemie GmbH, Buchs, Switzerland), the difference being made up with amylopectin from potato starch. A regression function was used to determine amylose content in the cassava starch.

2.2. Formation of RS III

Cassava starch was suspended (1 g in 10 mL) in distilled water, 1, 10 or 100 mmol/L L(+) lactic acid (Sigma–Aldrich Laborchemikalien GmbH, Seelze, Germany) and autoclaved for 1 h at 121 °C before incubation. To determine the optimum incubation temperature and time, the experiment was set-up as a completely randomised design with five levels of incubation temperature (−20, 4, 30, 60 and 100 °C) and three levels of incubation time (6, 24 or 48 h). Treatment conditions for each solvent that resulted in the highest RS III content were used in the subsequent experiment that was set-up as a completely randomised design with the independent variable having five levels of

autoclaving time (15, 30, 45, 60 and 90 min). The extent of hydrolysis of cassava starch was monitored by viscosity measurements of autoclaved starch (3 g in 30 mL distilled water). The autoclaved starch (25 g) was weighed in a Z3-DIN coaxial cylinder system. Viscosity was measured at 30 °C by applying a shear rate of 100 s^{-1} using an UM Physica Rheometer (Physica Messtechnik, Stuttgart, Germany).

2.3. Isolation of RS III

Resistant starch type III was isolated by modification of the enzymatic-gravimetric procedure for the determination of total dietary fibre (Eerlingen et al., 1993a; Shamai et al., 2003). Enzyme preparations were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Samples were removed from the incubator and suspended in 50 mL phosphate buffer (pH 6.0) at 37 °C before adding α-amylase (0.4 g/g starch, Sigma A-3176) and incubated at 37 °C for 16 h. The samples were cooled to 25 °C before adjusting the pH to 4.5 using 2 mL/100 mL phosphoric acid solution. Amyloglucosidase (1 mL, Sigma A-7095) was added and the samples incubated at 60 °C for 30 min. After enzymatic digestion, the samples were transferred into pre-weighed centrifuge tubes and centrifuged using a Biofuge Stratos centrifuge (Kendro Laboratory Products GmbH, Langenselbold, Germany) at 5000 rpm for 15 min and the residue washed twice with phosphate buffer (pH 7.5) before re-suspending in 50 mL phosphate buffer (pH 7.5). Protease (Sigma P-2143) was added (1 mL of solution containing 16 mg protease/100 mL phosphate buffer, pH 7.5) and the residue incubated at 42 °C for 4 h. The sample was centrifuged at 5000 rpm for 15 min in two cycles while washing with distilled water then dried to constant weight at 60 °C. The RS III was determined as the insoluble residue after enzymatic digestion of starch and removal of amylolytic enzymes with protease. The results were expressed as percent RS III on dry matter basis.

2.4. Differential scanning calorimetry

Differential scanning calorimetry measurements were made using a DSC121 instrument (Setaram Instrumentation, Caluire, France) equipped with an analysis data station. An indium standard was used for calibration. Samples (5 mg and 10 μL distilled water) were weighed into stainless steel crucibles and sealed with aluminium rings and stainless steel lids, and then allowed to equilibrate at 25 °C for 24 h. The samples were heated from 25 to 200 °C at 5 K/min and weighed before and after heat treatment to check for potential moisture loss. Initial transition temperature and completion transition temperature of a peak in the DSC thermograms were determined as the points where the scan deviated from linearity before and after the peak. Peak transition temperature was defined as the temperature at peak maximum. Melting transition enthalpy, ΔH (J/g), was calculated from the area under the curve described by the recording trace and the baseline joining the initial and final transition temperatures.

3. Results and discussion

3.1. Composition of isolated cassava starch

The proximate composition of the extracted cassava starch on a dry matter basis was 95.09, 0.07, 0.55, 1.10 and 0.42 g/100 g for starch, fat, ash, fibre and protein, respectively. Removal of the skin and rind and washing the tubers prior to crushing contributed significantly to an acceptable white colour indicated by a lightness value (L^*) of 95.7. The high clarity of cassava starch results from the weak associative bonds between starch molecules in the granules (Moorthy, 2002). Cassava starch had an amylose content of 25.2 g/100 g. The amylose content of starch and its degree of polymerisation significantly influences the yield of RS III, and it has been proven that amylose-rich starches give higher RS III yields than normal or waxy starches (Morita et al., 2005; Thompson, 2000; Xue et al., 1996).

3.2. Digestible starch and RS II and RS III in raw cassava starch

The inverse relationship between the quantities of rapidly digestible starch and slowly digestible starch has been reported (Englyst et al., 1992) and was also evident in our study. The amount of digestible starch increased from 5.52 g/100 g after 20 min of in vitro enzymatic digestion to 14.19 g/100 g after 120 min and indicated that the physical form of the food hinders access of pancreatic amylase to the starch. Furthermore, the low starch digestion index (5.80 g/100 g) confirmed that most of the starch granules were undamaged and ungelatinised and therefore resistant to amylolytic attack. High starch digestion indices (approaching 100%) are associated with effective gelatinisation of the material being analysed and correlate highly with the glycaemic index (Englyst et al., 1992). The high amount of RS II in the raw cassava starch (74.94 g/100 g) is similar to values reported in the literature for root and tuber crops (Englyst et al., 1992). Resistant starch type II is a characteristic feature of raw root and tuber crops and owes its resistance to digestion by amylase to the B-type crystalline arrangement of the ungelatinised starch granules (Englyst et al., 1992).

3.3. Effect of solvent on formation of RS III

Table 1 shows the yields of RS III obtained from cassava starch suspended in distilled water, 1, 10 or 100 mmol/L lactic acid then autoclaved at 121 °C for 1 h and incubated at different temperatures and times. We considered the quantities of RS III in the raw cassava starch (0.44 g/100 g) to be negligible and did not account for them in the determination of RS III in the subsequent experiments. Preliminary studies performed using lactic

Table 1
Effect of solvent and incubation conditions on the formation of resistant starch type III from cassava starch

Incubation conditions		Resistant starch type III (g/100 g dry matter)			
Temperature (°C)	Time (h)	Water	Lactic acid (mmol/L)		
			1	10	100
−20	6	3.02	3.94	2.68	1.70
	24	3.79	4.43	5.00	1.43
	48	4.78	3.82	6.75	1.99
4	6	5.23	4.54	3.73	1.89
	24	5.97	8.01	8.08	1.39
	48	6.50	6.89	9.12	1.43
30	6	4.80	3.87	7.20	2.04
	24	5.93	5.54	8.48	2.42
	48	5.61	6.09	8.54	1.93
60	6	3.49	3.76	7.29	1.61
	24	4.96	5.71	9.89	1.65
	48	6.04	5.75	9.97	1.38
100	6	1.74	4.18	1.74	0.59
	24	9.47	8.12	4.28	0.78
	48	9.68	6.94	7.66	0.99

and citric acid concentrations exceeding 100 and 50 mmol/L, respectively, were unsuitable as these concentrations excessively acidified the starch slurries (pH < 3.0) and created unfavourable conditions for enzymatic activity in the subsequent isolation of RS III.

Irrespective of the incubation temperature or time, lower amounts of RS III were formed from cassava starch suspended in 100 mmol/L lactic acid compared to suspensions in water. When the lactic acid concentration was decreased to 10 mmol/L and further to 1 mmol/L, the RS III yields approached or exceeded those from cassava starch suspended in water (Table 1). Partial acid hydrolysis and thermal treatment improves RS III yields by inducing partial depolymerisation of amylose chains which results to increased polymer mobility for molecular rearrangement (Thompson, 2000). Excessive hydrolysis of the glycosidic bonds, as may have occurred in the 100 mmol/L lactic acid treatment, may limit amylose recrystallisation during subsequent incubation. The viscosities of the slurries gave an indication of the extent of hydrolysis. The viscosity of autoclaved cassava starch suspended in water or 1 mmol/L lactic acid was 1390 and 2140 mPa s, respectively. Both values were significantly higher ($P < 0.05$) than the viscosities of starch suspended in 100 mmol/L (20 mPa s) or 10 mmol/L lactic acid (68 mPa s).

3.4. Effect of incubation temperature and time on formation of RS III

The interaction effect of increasing incubation temperature and time significantly ($P < 0.001$) increased the yield of RS III from cassava starch suspended in water (Table 2). Contrastingly, when cassava starch was suspended in 100 mmol/L lactic acid only the incubation temperature significantly influenced ($P < 0.001$) the formation of RS III (Table 3), the highest amounts being formed at 30 °C after which the yields declined with increasing incubation temperature. When the lactic acid concentration was decreased further to 10 or 1 mmol/L, the formation of RS III was significantly affected ($P < 0.001$) by the main effects of temperature and time rather than their interaction effects (Table 3). It was also evident that further decreasing lactic acid concentration to 1 mmol/L did not substantially improve the RS III contents when compared to the 10 mmol/L treatments.

Table 2
Analysis of variance of resistant starch type III from cassava starch suspended in water[a]

Source of variation	df	SS	MSS	*F*-value
Temperature	4	32.290	8.072	27.63***
Time	2	46.832	23.416	79.38***
Temperature × time	8	47.668	5.959	20.20***
Error	15	4.425	0.295	
Total	29	131.215		

$R^2 = 0.97$.
df, degrees of freedom; SS, sum of squares; MSS, mean sum of squares.
[a] Autoclaved at 121 °C for 1 h.
*** Significant at $P < 0.001$.

Formation of RS III involves recrystallisation of amylose in a partially crystalline system in a process that is influenced by the incubation temperature and time (Eerlingen et al., 1993a; Haralampu, 2000). Polymer recrystallisation is a three-stage process that involves nucleation (formation of critical nuclei), propagation (crystal growth from the nuclei formed) and maturation (continued crystal growth and perfection). The nucleation and propagation rates determine the overall recrystallisation rate whereas the maturation rate is more temperature dependent (Eerlingen et al., 1993a). Nucleation generally proceeds rapidly when the incubation temperature is close to the glass transition temperature of starch, at about −5 °C (Chung, Woo, & Lim, 2004; Gray & Bemiller, 2003). Incubation of autoclaved cassava starch at 4 °C favoured a high nucleation rate and therefore high initial yields of RS III. Although this temperature encouraged nucleation, the yields did not improve significantly when the incubation time was extended to 48 h, probably because propagation was limited by the temperature-induced high viscosity of the medium.

Table 3
F-values of resistant starch type III from cassava starch suspended in different molarities of lactic acid[a]

Source of variation	Lactic acid concentration (mmol/L)		
	1	10	100
Temperature	9.93***	19.04***	12.63***
Time	23.70***	31.76***	ns
Temperature × time	ns	ns	ns
R^2	0.87	0.91	0.80

[a] Autoclaved at 121 °C for 1 h.
*** Significant at $P < 0.001$: ns, not significant at $P < 0.05$.

Incubation of autoclaved cassava starch below the glass transition temperature of starch (i.e. at −20 °C) or at intermediate temperature ranges (30 or 60 °C) also showed that a large proportion of RS III was formed within 6 h and the yields did not significantly increase after 24 h.

Incubation of the autoclaved cassava starch at 100 °C resulted in low RS III yields after 6 h but maximum yields were obtained after 24 h. Nucleation is limited at 100 °C because the incubation temperature largely exceeds the glass transition temperature of starch. However, propagation proceeds rapidly once a critical number of nuclei have been formed, provided the propagation temperature is close to the melting temperature of crystalline amylose (about 150 °C). The onset of propagation at 100 °C may be substrate-dependent. Whereas Eerlingen et al. (1993a) reported that RS III yields from autoclaved wheat starch incubated at 100 °C begins to rapidly increase after 2.5 h and attain maximum values after 48 h, our results showed that the yields were low after 6 h and peaked within 24 h.

3.5. Effect of autoclaving time on formation of RS III

Treatments with water and 10 mmol/L lactic acid followed by incubation for 48 h at 100 and 60 °C, respectively, gave the highest RS III yields and were used to study the effect of autoclaving time on RS III formation. When water was used as a solvent, it was possible to decrease the autoclaving time to 15 min without any detrimental effect on the formation of RS III (Fig. 1). However, with 10 mmol/L lactic acid, the amount of RS III formed increased with increasing autoclaving time, attaining a maximum value at 45 min autoclaving time (Fig. 1). The RS III yields declined when the autoclaving time exceeded 45 min indicating the effects of continuing acid and thermal hydrolysis of cassava starch. These shorter autoclaving times, while giving high RS III yields (Gõni et al., 1996), are significantly better than the 1 h (Eerlingen et al., 1993a) or 2 h (Shamai et al., 2003) autoclaving times reported in earlier studies and translate to shorter process times and lower energy costs.

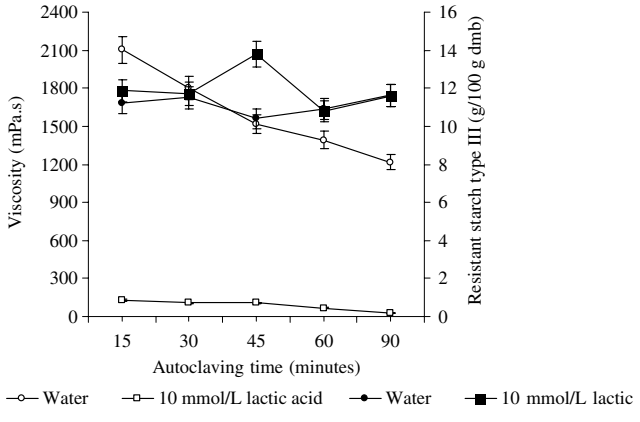

Fig. 1. Effect of autoclaving time on viscosity (open symbols) and resistant starch type III (closed symbols) formation from cassava starch. Samples were autoclaved at 121 °C. Viscosity was measured at 30 °C by applying a shear rate of 100 s^{-1}. For resistant starch type III determination, samples suspended in water were incubated at 100 °C for 48 h (circles) whereas samples suspended in 10 mmol/L lactic acid were incubated at 60 °C for 48 h (squares).

The formation of RS III is a two-stage process that involves starch hydrolysis followed by recrystallisation of amylose polymers. Whereas recrystallisation takes place during incubation, starch hydrolysis occurs during autoclaving. However, the correlation between the viscosity values, as indicators of hydrolysis, and RS III yields were low (Fig. 1). Gel permeation chromatography gives better correlations between starch hydrolysis and RS III yields (Eerlingen et al., 1993b).

Starch hydrolysis should preferably result in amylose polymers with degrees of polymerisation of 100–300 glucose units. Amylose is the principle starch fraction required for the formation of RS III because its polymers have a high degree of polymerisation that form enzyme-resistant double helices stabilised by hydrogen bonds. When the degree of polymerisation is less than 100 glucose units, the RS III yield is low because the polymers are not long enough to form enzyme-resistant crystallites. On the other hand, insufficient hydrolysis of amylose is undesirable since amylose polymers with degrees of polymerisation higher than 300 cannot effectively align to form enzyme-resistant crystallites (Eerlingen et al., 1993b). The RS III yields from amylose-rich substrates are 20–50 g/100 g (Xue et al., 1996; Morita et al., 2005). These values are almost two to five times higher than RS III yields we have reported and signify the important role of amylose in RS III formation. Contrastingly, amylopectin is not essential to the formation of RS III because its α-D-(1→6)-glucosidic linkages are hindered in movement and its chains have low degrees of polymerisation (Eerlingen, Jacobs, & Delcour, 1994).

3.6. Temperature of melting and enthalpy of RS III

Native cassava starch exhibited initial, peak and end gelatinisation temperatures at 59, 66 and 77 °C with an endothermic enthalpy of 3.4 J/g. Gelatinisation properties of cassava starch varies widely, with transition temperatures of 50–85 °C and endothermic enthalpies of 4.8–15.6 J/g (Defloor, Dehing, & Delcour, 1998; Freitas et al., 2004; Gunaratne & Hoover, 2002; Perez, Breene, & Bahnassey, 1998; Sajeev, Moorthy, Kailappan, & Rani, 2003). This variability of gelatinisation and enthalpy values reflect differences in degrees of crystallinity and intermolecular bonding which are in turn are influenced by genotypic and phenotypic factors. The presence of non-starch components competing for the available water with starch in cassava flour, may also contribute to delayed gelatinisation of the flour (Defloor et al., 1998).

Irrespective of the autoclaving time, RS III from cassava starch suspended in water exhibited melt transitions between 158 and 175 °C with peaks at about 164 °C (Fig. 2a). Similar melt transitions have been reported for RS III from other starchy materials (Sievert & Würsch, 1993; Shamai et al., 2003) and are characteristic to dissociation of recrystallised amylose. However, the endothermic

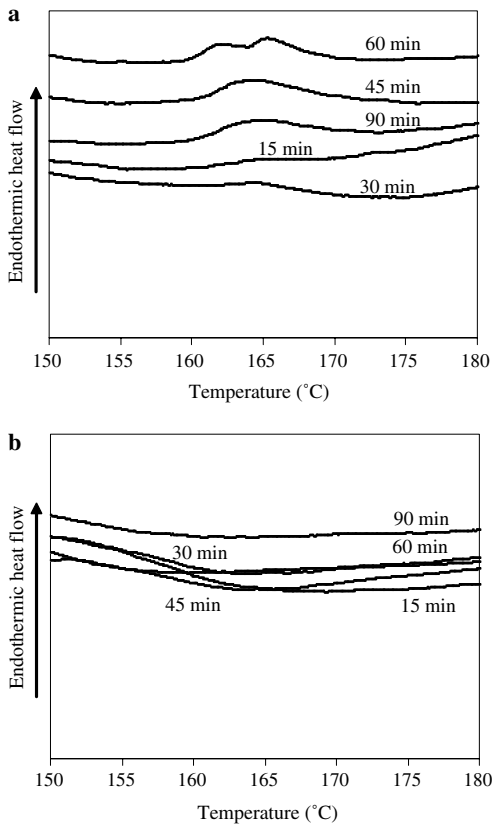

Fig. 2. Differential scanning calorimetric thermograms of resistant starch type III from (a) cassava starch suspended in water and autoclaved for 15–90 min followed by incubation at 100 °C for 48 h; (b) cassava starch suspended in 10 mmol/L lactic acid and autoclaved for 15–90 min followed by incubation at 60 °C for 48 h.

enthalpies (0.2–1.6 J/g) associated with melt transitions of RS III from cassava starch suspended in water were considerably lower when compared to the 25–30 J/g reported for RS III from cereals (Sievert & Würsch, 1993; Shamai et al., 2003). Furthermore, endothermic enthalpies associated with melting of RS III from cassava starch suspended in 10 mmol/L lactic acid were indistinct (Fig. 2b).

The melt transition of RS from the solid to the fluid state can be described as dissociation of ordered regions composed of double helical segments and double helix-to-single coil transitions (Sievert & Würsch, 1993). Based on this definition, we speculated that RS III from cassava starch had few hydrogen bond linkages between the recrystallised amylose polymers and was therefore structurally unstable and required minimal energy to dissociate. This conformational change of RS to a structurally unstable form can also be generated by repeated heating and cooling of the amylose polymer (Sievert & Würsch, 1993) or by extrusion-cooking (Chinnaswamy & Hanna, 1990; Fan, Mitchell, & Blanshard, 1996).

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