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Inhibition of amylopectin retrogradation by partial beta-amylolysis

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ABSTRACT

The rate of retrogradation of amylopectin solution differs from one starch variety to another and it is thought to be due to the different length of the external chains of amylopectin. A shortening of the external chains of waxy maize and potato amylopectin was performed with beta-amylase. Partial beta-amylolysis produced a significant fraction of chains having 2–6 glucose units. A high linear correlation ($R > 0.97$) was found between the enthalpy of retrograded amylopectin measured by DSC, or percent solid measured by low frequency pulsed NMR, and average external chain length. No retrogradation appeared to occur when the external chains of both amylopectins had 11 or less glucose units on average. The inhibition of retrogradation appears to be caused primarily by the presence of very short external chains, which hinders the reassociation of the long external chains.

INTRODUCTION

The starch granule is composed of alternating crystalline and amorphous moieties. The currently accepted crystalline structure of starch granule consists of a radial arrangement of clusters of amylopectin. Each cluster contains a highly branched region (the amorphous lamella) and a domain where short-chain segments of amylopectin have formed double helices[1,2] (the crystalline lamella of the granule). These crystalline moieties are formed by the whole length of the external chains (called A chains) and part of the B chains, on which one or more A chains are attached[3,4]. The chain-length distribution, as revealed by size exclusion chromatography and high performance chromatography, gives little information on the site of the chain linking and consequently on the chain-length distribution of the external chains of amylopectin. These form the crystalline lamella of the granule and are involved in the reordering of amylopectin during retrogradation, the branching serving to initiate the crystalline arrangement[5].

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The minimum chain-length requirement for crystallisation has been found to be 10. However, in the presence of longer chains, oligomers as short as 6 glucose units can cocrystallise[6]. The retrogradation rate appears[7] to be proportional to the mole fraction of chains with dp 14–24, and inversely proportional to the mole fraction of chains with dp 6–9. Thus, the rate of retrogradation is dependent on the length of the external chains of amylopectin, the shortest being in cereals[3,8,9]. The starch granules, which have a greater proportion of longer chains (dp > 30), have the highest enthalpy and peak maximum temperature for both gelatinisation and retrogradation[10].

The retrogradation process takes place over several days, but the rate is dependent on the botanical source, hence of the external chain length[8,11]. The initial rate of retrogradation of potato amylopectin gel is almost twice as fast as those from wheat and rice. Retrogradation of starch gels can be followed by various physical methods, including rheology, differential scanning calorimetry, X-ray, low resolution pulsed proton nuclear magnetic resonance[12] and cross-relaxation[13] NMR, each technique measuring different kinetics[14]. The aim of this work was to compare the effect of beta-amylolysis on the retrogradation of an amylopectin having short external chains (waxy maize amylopectin) and an amylopectin with long external chains (potato).

EXPERIMENTAL

Materials.—Waxy maize starch was obtained from Roquette Frères (Lestrem, France). Potato amylopectin, as well as crystalline pullulanase (pullulan 6-glucanhydrolase, EC 3.2.1.41) and beta-amylase [α-(1 → 4)-glucan maltohydrolase, EC 3.2.1.2] were from Sigma, St. Louis, MO, USA. No amylose was detected in both starch samples using the colorimetric and DSC methods described by Sievert and Holm[15]. Any α-glucosidase activity (EC 3.2.1.20) contaminating beta-amylase was controlled by enzymatically measuring D-glucose present after overnight amylopectin beta-amylolysis.

beta-Amylolysis of amylopectin.—Starch (2%) was suspended in a 10 mM acetate buffer, pH 4.5, and cooked for 10 min under gentle stirring. After adjusting the temperature to 37°C, beta-amylase devoid of α-glucosidase was added and the reaction was followed by measuring the increase in reducing sugars with Nelson reagent[16] using maltose as a standard. The reaction was stopped by heating for 2 min at 100°C and then an equal volume of EtOH was added. Starch was isolated by centrifugation and dried under vacuum at 40°C. A series of amylopectins hydrolysed from 0.9 to 36% was obtained by changing the enzyme content and/or the duration of the digestion. Total starch was determined by hydrolysis with amyloglucosidase and quantification of glucose formed by the D-glucose oxidase, peroxidase reagent.

Debranching of amylopectin.—Debranching of amylopectin with pullulanase was performed essentially as follows: amylopectin (50 mg dry weight), or the calculated

amount of "shortened" amylopectin corresponding to 50 mg amylopectin, was dissolved in Me_2SO (1.5 mL) with minimum heating. The clear solution was buffered with 0.1 M Tris · acetate buffer, pH 5.5 (6 mL). The solution was incubated at 37°C and 25 μL (2 Units) pullulanase was added.

Hydrolysis was monitored by the Nelson method[16]. When the hydrolysis was complete, the enzyme was inactivated at 100°C for 2 min and the solution freeze-dried. The average chain-length (CL_o) was expressed as 2 × total starch/reducing sugar (expressed as maltose) × 0.95. Prior to the chromatographic analysis the debranched amylopectin was dissolved in 0.5 N NaOH (3 mL). The chromatography was carried out on a Dionex HPIC-AS6 anion exchange column (250 × 4 mm), using a PAD II pulsed amperometric detector[17] (Dionex, Sunnyvale, CA, USA). The sample-injection loop size was 20 μL. Eluent *A* was 150 mM NaOH and eluent *B* was 150 mM NaOH containing 500 mM NaOAc. The gradient program was as follows: percent eluent *B*, 10% at 0 min, 30% at 10 min, and increase by 10% every 10 min until 80 min. All separations were carried out at room temperature with a flow rate of 1 mL/min. The PAD response factors for peaks 2–17 were taken from Murugesan et al.[18]. For longer chain lengths, the response factor for peak 17 was taken.

Physical methods.—Differential scanning calorimetry (DSC) measurements were performed with a Mettler DSC 30 instrument (Mettler, Naenikon-Uster, Switzerland) equipped with a Mettler TC11 analysis data station. In all experiments, a pan with water was used as a reference sample.

The gels were prepared as described by Krusi and Neukom[19]. A 2% starch gel was prepared by heating the suspension under gentle stirring for 15 min in a boiling bath, which was then cooled to 30°C. A sufficient starch sample was uniformly dispersed to obtain 36% dm. After degassing, the concentrated suspension was heated in a boiling bath for another 15 min. The cooled gel was incorporated into aluminium pans (Mettler ME 29990, 120 μL) with a plastic syringe and weighed accurately. The pans were sealed and stored at 20°C before measuring the melting enthalpy at 4, 12, 15, and 18 days. The DSC scan was carried out only once on each cell.

For the measurement of the gelatinisation enthalpy, a sample of ~ 30 mg dry matter of starch was weighed accurately into an aluminium pan. Then, 70 μL distilled water was added, and the sample was heated from 20 to 100°C (heating rate 2°C/min). Transition enthalpies were calculated from the area delineated by the recording trace and the baseline. DSC measurements were carried out in triplicate.

Pulsed NMR.—A Bruker-Minispec pc 20 (Bruker Rheinstetten, Germany) with an operating frequency of 20 MHz was used in the s/l-working and s/l-calculating programme modes. Measurements were performed at 4°C, in intervals up to 15 days, on triplicates of 30% w/w starch gels prepared as described above. Short Minispec test tubes (length 50 mm, diameter 10 mm) were filled with a syringe and kept in a refrigerator at 4°C.

Freeze / thaw stability (F / T).—A 2% amylopectin solution was heated at 100°C for 10 min in demineralised water with gentle mixing. Cup-screw test tubes (18 tubes) were filled with 5.0 g of the solution. The tubes were frozen in an alcohol bath at −40°C during at least 20 min, then thawed in a water bath at room temperature, and 3 tubes were centrifuged for 2 min at 570*g*. The supernatant liquid was collected and precisely weighed. The procedure was repeated several times with the remaining tubes.

Total carbohydrate in the solution and supernatant was determined by the phenol–H_2SO_4 method on 0.5 mL of fresh solution diluted in a 25-mL flask, in which a spike of alpha-amylase was added.

RESULTS AND DISCUSSION

All the products treated with beta-amylase were F/T stable to more than 5 cycles, except the waxy maize starch and potato amylopectin with a level of beta-amylolysis less than 5 and 10%, respectively.

The physical changes occurring during storage of the enzymatically treated amylopectin gels were followed by DSC and NMR. Pulsed low resolution NMR has been recently applied to follow the gelation process in different thermally reversible gelling α-glucan–water systems[12,13] (retrogradation). It is based on the principle that the signals from protons in the "solid-like" and liquid components in the system decay at significantly different rates following a 90° radio frequency pulse. This NMR technique has the advantage of being nondestructive and the evolution of a sample can be followed continuously over many days. Retrogradation measurement by DSC can be carried out at room temperature, whereas by NMR, 4°C is necessary to follow the evolution because no increase of solid content could be observed when the gels of the hydrolysed amylopectins were stored at room temperature, even after 30 days as also reported by Teo and Seow[12].

The physical characteristics of native and retrograded waxy maize starch and potato amylopectin are presented in Table I. The rate of retrogradation of potato amylopectin was very fast and half of the maximum of enthalpy and total solid were reached in less than a day (data not shown), whereas with waxy maize amylopectin, ~ 5 days were needed to reach half of the maximum enthalpy and 1.5 days for total solid (Figs. 1a and b). The thermograms of the native and retrograded waxy maize amylopectins are shown in Fig. 2. Two peak temperatures of melting (Tp1 57°C and Tp2 62°C) are visible even after shortening the external chains. At an early stage of retrogradation, Tp2 only was observed and progressively Tp1 appeared and finally became bigger than Tp2, confirming the findings of Inouchi et al.[20]. However these authors reported Tp1 values at around 40°C, for various corn genotypes, against 57°C for our waxy maize sample. Biliaderis et al.[21] proposed that this multiple endotherm reflects melting and retrogradation processes occurring simultaneously during heating. At a higher dilution of starch, only Tp1 appeared.

TABLE I

Characteristics of waxy maize and potato amylopectin

	Waxy maize	Potato
Native		
beta-Amylolysis limit (β_o)	57	55
Average chain length (CL)	21	33
Average external chain length (ECL_o)	14	20
Temperature of gelatinization (Tp)	70.8	69.6 (starch)
Enthalpy of gelatinisation (J/g)	13.6	9.7
Retrograded		
Temperature of melting (Tp1, Tp2)	57, 62	60.0
Enthalpy of melting after 18 days (J/g)	9.6	7.2
Percent solid after 14 days (30% gel)	12.9	10.9

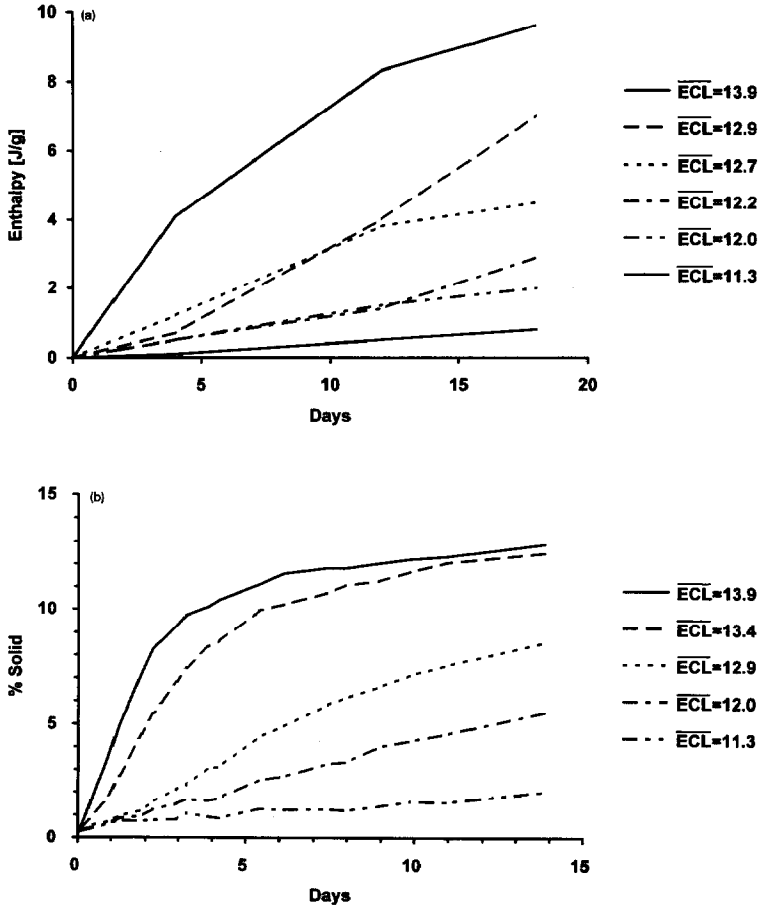

Fig. 1. Retrogradation kinetics of waxy maize starch gels: (a) enthalpy of transition measured by DSC on 36% w/w gels stored at 20°C; (b) percent solid measured by NMR on 30% w/w gels stored at 4°C.

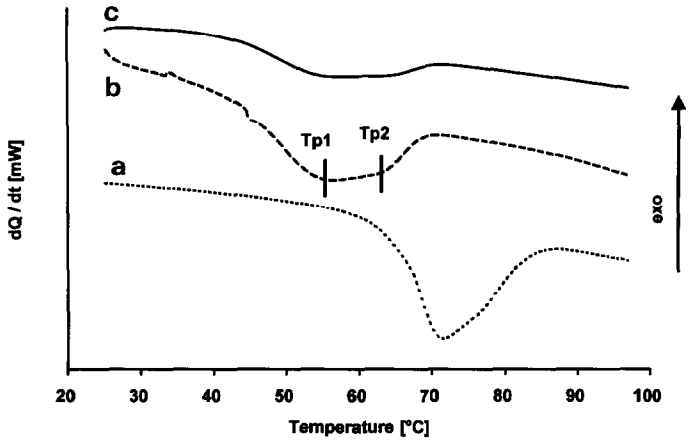

Fig. 2. DSC thermograms of waxy maize starch gels: (a) native; (b) 15 days retrograded; (c) 15 days retrograded of 12.6% hydrolysed amylopectin with beta-amylase.

Shortening of the external chains of both amylopectins reduced the rate of retrogradation, but the endothermic transition temperatures did not change by shortening the outer chain as shown in Fig. 2 for retrograded native and 12.6% waxy maize amylopectin. Other work[7,8] has shown that different retrograded cereal starches give the same melting transition of amylopectin – with some exceptions like maize mutants[21] – whereas the native starches can have gelatinisation temperatures separated by ca. 10°C (ref 10). Similarly, the solid content measured by NMR increased much faster in the gels formed by native amylopectin, and reached a plateau after 6 days of gel storage at 4°C (Fig. 1 b). So, the more the amylopectin was hydrolysed, the lower was the retrogradation measured by DSC and NMR.

The average external chain length (ECL) of the enzyme treated amylopectins can be calculated by the following equation: $ECL = ECL_o - \beta/\beta_o(ECL_o - 2) = (\beta_o - \beta)CL + 2$ in which β is the extent of beta-amylolysis and β_o is the beta-amylolysis limit. The calculated average external chain lengths ($ECL_o = \beta_o\ CL + 2$) of amylopectin from waxy maize and potato amylopectins presented in Table I are in agreement with the values calculated by Inouchi et al.[4], Hizukuri[9], and Banks et al.[22].

These results, plotted in Figs. 3 and 4, show very high correlation ($R > 0.97$) between melting enthalpy (DSC) or percent solid (NMR), and external chain length for both amylopectins. The intercept of the regression lines, on the chain length axis, occurs at 11.3 and 10.7 glucose units, respectively, for waxy maize and 10.3 and 10.8 glucose units for potato amylopectin. Same values were reached when data from other storage times were plotted. So, no molecular ordering and aggregation would occur when the average external chain length is below ~ 11–12 units in both amylopectins.

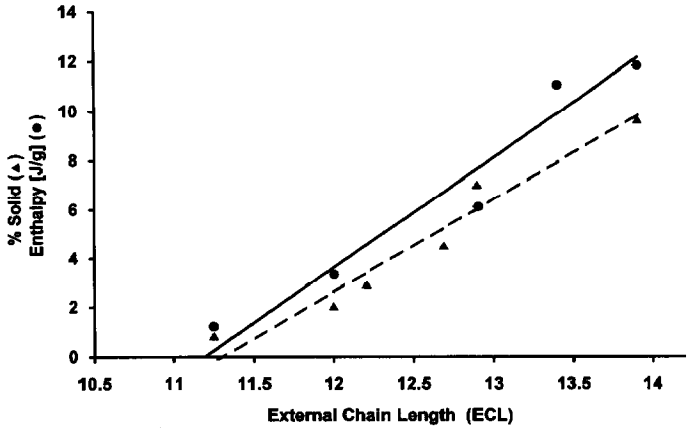

Fig. 3. Transition enthalpy (●, DSC, 12 days, 20°C), and percent solid (▲, NMR, 8 days, 4°C) as function of average external chain length of waxy maize starch gel (36% dm).

These results ultimately confirm that a shorter average chain length reduces the retrogradation, as postulated by Kalichevsky et al.[8]. However, it is surprising that reordering of the external chains does not happen anymore when the chain length is reduced by only three glucose units in waxy maize amylopectin. Fast partition liquid chromatography, after debranching, showed that amylopectin has short chains containing a minimum of six glucose units, the most abundant having 12 glucose units. These results are translated in Fig. 5 into relative molar proportions. The chromatograms reveal that the partial beta-amylolysis generated new short chains between 2–5 glucose units and a general decrease above 11 glucose units. The same peak displacement from 13 glucose units to 11 was observed with 19.8% hydrolysed potato amylopectin, with a sharp increase in the short chains as shown

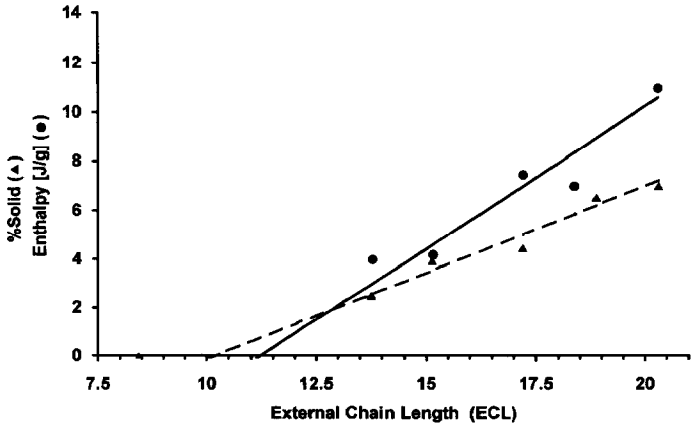

Fig. 4. Transition enthalpy (●, DSC, 15 days, 20°C) and percent solid (▲, NMR, 4 days, 4°C) as function of average external chain length of potato amylopectin gel (30% dm).

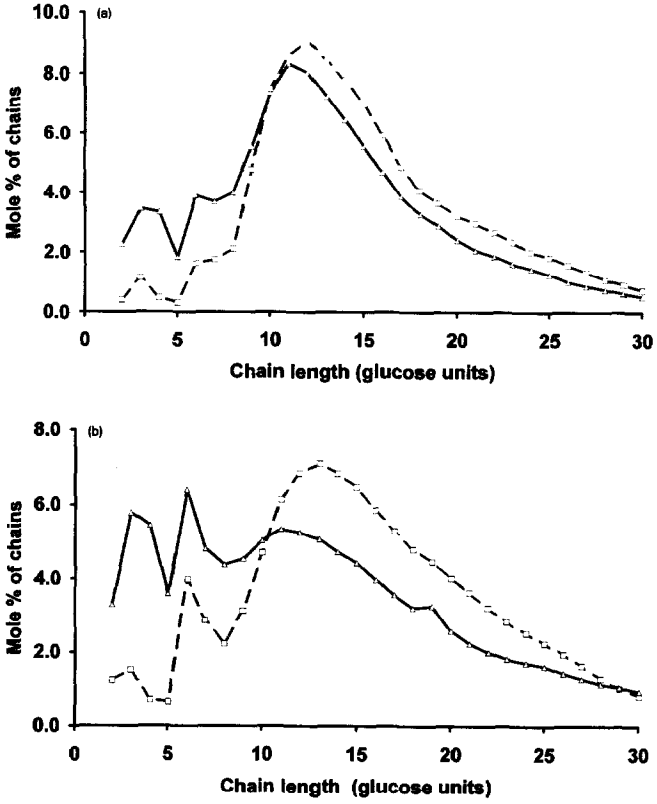

Fig. 5. Molar chain-length distributions of debranched amylopectin: (a) native waxy maize (— — —) and 12.6% beta-amylolysis (▬▬); (b) native potato (— — —) and 19.8% beta-amylolysis (▬▬).

in Table II. beta-Amylase attacks the next-to-the-last β-(1 → 4) glycosidic bond from the nonreducing end of the chains to release maltose, and it repetitively cleaves a given short chain substrate on average 4 times (8 glucose units) before dissociating[23]. The fact that a large number of small chains appears during the hydrolysis suggests that beta-amylase reduces the length of only a part of the external chains. This proposition is reinforced by the fact that the melting temper-

TABLE II

Relative molar distribution of debranched amylopectins (100% = dp 2–30).

Molar distribution	Waxy maize [a]		Potato	
	Native (%)	12.6% beta-Amylolysis (%)	Native (%)	19.8 beta-Amylolysis (%)
dp 2–5	2	11	4	22
dp 2–10	20	35	21	43

[a] Distribution profiles in Fig. 5.

atures (Tp1 and Tp2) do not change, whereas the enthalpy decreases. The chains actively involved in retrogradation of the enzymatically treated amylopectins could be further identified by studying the residue after acid hydrolysis of retrograded amylopectin as reported by Ring et al.[24]. The presence of these small "stubs" should inhibit the molecular reassociation of the longer chains as suggested by Shi and Seib[7]. This could be the reason why the reduction by an average of only three glucose units in waxy maize amylopectin is sufficient to stop the reassociation of the longer chains.

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