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Temperature effect on retrogradation rate and crystalline structure of amylose[1]

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Commercial potato amylose was used to study temperature effects on the retrogradation of amylose solutions (3.5 mg/ml). The retrogradation rate decreased as incubation temperature increased (5 to 45°C). The degree of retrogradation within 24 h decreased from 58.8 to 7.1% as incubation temperature increased from 5 to 45°C. In the amylose solution, different-sized molecular subfractions retrograded at different rates. After incubating at 5°C for 100 days, the majority of the amylose molecules retrograded and precipitated from the solution; at 45°C, only amylose of the small-molecular subfraction (number average, DP_n = 110; weight average, DP_w = 150) retrograded and precipitated. Entanglement of molecules was observed in size exclusion chromatograms. The morphology of retrograded amylose observed by using a scanning electron microscope differed with the retrogradation temperature. The chain length of amylose crystalline segments, prepared by hydrolysis of retrograded amylose, showed a narrow distribution (polydispersity from 1.21 to 1.67). The chain lengths of resistant segments increased DP_n from 39 to 52 and DP_w from 47 to 72 for α-amylolysis and DP_n from 34 to 40 and DP_w from 48 to 67 for 16% sulfuric acid hydrolysis, when incubation temperature increased from 5 to 45°C. © 1997 Elsevier Science Ltd

INTRODUCTION

Amylose is essentially a linear molecule with few branches. Like other linear polymers, amylose molecules have a strong tendency to retrograde (Miles *et al.*, 1984). In an aqueous solution, amylose molecules can rapidly associate to build up molecular aggregates that soon exceed colloidal dimensions and precipitate or form a gel (Gidley, 1989; Gidley and Bulpin, 1989; Colonna *et al.*, 1992). The retrogradation tendency of amylose depends on amylose molecular size and concentration, temperature, pH, and the presence of other chemicals in the solution (Whistler, 1953; Suzuki *et al.*, 1985). It is well known that amylose with DP 80 to 100 has the highest retrogradation tendency (Gidley *et al.*, 1986; Pfannemuller, 1986; Gidley and Bulpin, 1989).

Retrograded amylose is a mixture of crystalline and amorphous regions and shows a B-type X-ray pattern (Kitamura *et al.*, 1984; Leloup *et al.*, 1992; Cairns *et al.*, 1995). The crystalline region is resistant to acidic and amylolytic hydrolysis. The relative amount of the crystalline region in retrograded amylose differs with the retrogradation conditions and can be as high as 65% of the total carbohydrate (Jane and Robyt, 1984; Leloup *et al.*, 1992; Cairns *et al.*, 1995). The retrograded amylose crystallites exist as double helices (Wu and Sarko, 1978a, b; Imberty and Perez, 1988), similar to the crystalline structure of native granular starch (Kainuma and French, 1972; Yamaguchi *et al.*, 1979; Oostergetel and van Bruggen, 1993).

The double helical structure of the retrograded starch has been proposed as a requisite for branch formation during starch biosynthesis (Borovsky *et al.*, 1976, 1979). The reaction of branch-chain formation catalyzed by branching enzymes is temperature dependent (Borovsky *et al.*, 1975; Takeda *et al.*, 1993). Thus, temperature affects not only branching enzyme activities but also substrate conformation. At an elevated temperature, it has been proposed that the double helix formation rate is slower, and longer chains are required to maintain a

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stable double helical arrangement (Robyt, 1984). Suzuki *et al.* (1987) have reported that an increase in incubation temperature (0 to 30°C) of a soluble starch solution results in an increase in the thermal transition temperature of the retrograded starch.

This study investigated how temperature affects retrogradation rate and retrograded amylose structure. A diluted amylose solution, in which amylose molecules were highly mobile for reassociating, was used as a model system for studying the effects on amylose double helix formation.

EXPERIMENTAL

Materials

Potato amylose (Type III) and porcine pancreatic α-amylase (Type I-A, DFP treated) were purchased from Sigma Chemical Co. (St Louis). Pullulan molecular weight standards (Shodex Standard P-82 kit, Showa Denko KK, Tokyo) were purchased from Millipore Waters (Milford, MA). The molecular weights (and polydispersities) of the pullulan standards were: 853 000 (1.14); 380 000 (1.12); 186 000 (1.13); 100 000 (1.10); 48 000 (1.09); 23 700 (1.07); 12 200 (1.06); and 5800 (1.07). Maltotetradecaose, maltoheptaose and maltose were also used as molecular weight standards. Maltotetradecaose was purchased from Nakano Vinegar Co. (Aichi, Japan); and maltoheptaose and maltose were from Aldrich Chemical Co. (Milwaukee, WI). Other chemicals were reagent grade and were used without further treatment.

Preparation of retrograded amylose

Potato amylose (2.0 g) was wetted with methanol (10 ml) before dispersing in 500 ml water. The aqueous mixture was mechanically stirred at 85–90°C for 6 h to disperse amylose and to evaporate the methanol and some residual butanol present in the commercial amylose. The solution was then filtered through Whatman No. 4 filter paper to remove insoluble substances. The final concentration of the amylose solution, measured by using the phenol–sulfuric acid method (Dubois *et al.*, 1956), was 3.5 mg/ml. The amylose solution was dispensed into glass vials and autoclaved at 125°C for 30 min to sterilize it. The solution was then incubated at 5, 15, 25, 35 and 45°C for extended periods to facilitate retrogradation.

Preparation of amylose crystallites

Amylose crystallites were obtained by hydrolyzing the retrograded amylose with porcine pancreatic α-amylase at 37°C for 48 h or with 16% sulfuric acid at 25°C for 30 days (manually shaken each day) (Jane and Robyt, 1984). The crystallites resistant to enzyme or acid hydrolysis were washed with deionized water and isolated by centrifugation at 6700 *g* for 15 min (JA-21 Beckman Instruments, Fullerton, CA).

Molecular weight of potato amylose

Potato amylose molecular weight was analyzed by using high-performance size-exclusion chromatography (HPSEC) with three sequentially connected columns of TSK-GEL (G6000PWXL, G4000PWXL and G3000PWXL) (300 × 7.8 mm) with a PWXL Guardcolumn (40 × 6 mm) (Tosohaas, Montgomeryville, PA) at 80°C and a 0.6 ml/min flow rate. The separation was detected by using a HP 1047A Refractive Index Detector (Hewlett Packard, Wilmington, DE) at 50°C. Deionized water from a Milli-Q water system (Millipore Co., Bedford, MA), filtered through a 0.2 μm nylon membrane, degassed, and pressurized by using helium sparge in an Ultra-Ware Integrated HPLC Mobile Phase Handling System (Vineland, NJ), was used as the mobile phase. An HP 1050 Series Pump equipped with a 20 μl sample injection loop was used to deliver the mobile phase. Chromatographic data were collected and processed by using an NEC computer with Maxima 820 Gel Permeation Chromatographic Software (Millipore, Waters Chromatography Div., Milford, MA). A narrow-standard calibration procedure (Malawer, 1995) was performed by using pullulan and malto-oligosaccharide as standards. A cubic molecular weight calibration curve was obtained by using the log molecular weight versus the retention time of the standard. The slice interval of the chromatogram was set at 10 s and the molecular weight distribution of the interval was assumed to be monodispersed (Malawer, 1995). The number and weight average molecular weight (M_n and M_w) and polydispersity (D) were calculated by following their definition (Malawer, 1995):

$$M_n = \frac{\sum A_i}{\sum (A_i/M_i)} \quad M_w = \frac{\sum (A_i \times M_i)}{\sum A_i} \quad D = \frac{M_w}{M_n}$$

In the equations, A_i is the area of slice i and M_i is the molecular weight of slice i (Malawer, 1995). The molecular size of the amylose is reported as the pullulan-equivalent degree of polymerization (DP) calculated as molecular weight divided by 162. Before HPSEC analysis, potato amylose samples (2 mg/ml) were dissolved in 90% dimethyl sulfoxide (DMSO) by heating in a water bath at 100°C for 20 min, stirred at room temperature overnight, and filtered through a 0.45 μm nylon membrane.

Rate of amylose retrogradation

As retrogradation proceeded, the rate of amylose retrogradation was determined by measuring the decrease of

amylose concentration (total carbohydrate) in the supernatant by using the phenol–sulfuric acid method (Dubois *et al.*, 1956). The retrograded amylose was removed from the supernatant solution by centrifugation at 11,500 *g* for 10 min (Model 59A Microcentrifuge, Fisher Scientific, Springfield, NJ).

Morphology of the retrograded amylose

The morphology of retrograded amylose was studied by using a JEOL JSM-35 scanning electron microscope (JEOL, Tokyo). The lyophilized retrograded amylose gel was attached to a specimen stud and coated with 60:40 gold–palladium alloy; representative micrographs were recorded at ×10,000 magnification.

Chain length of the amylose crystallite

Chain length of the amylose crystallite, prepared by hydrolysis of retrograded amylose, was determined by using HPSEC with two sequentially connected columns of TSK-GEL (G4000PWXL and G3000PWXL) and a PWXL Guardcolumn at 50°C with a 0.5 ml/min flow rate. The amylose crystallite was solubilized in 90% DMSO and heated in a water bath at 100°C for 20 min to dissociate the crystalline structure before the analysis. Other operating conditions were the same as described for amylose molecular weight analysis.

RESULTS AND DISCUSSION

The proportion of amylose retrograding during the first 24 h decreased from 58.8 to 7.1% as the incubation temperature increased from 5 to 45°C (Table 1). The percentage of retrogradation progressing with time at different temperatures is shown in Fig. 1. The elevated incubation temperature significantly retarded the retrogradation rate of amylose solution. When incubated at 5°C, most of the solution's amylose molecules (78%) rapidly retrograded and precipitated within the first 9 days. After that, amylose continued to retrograde at a slower rate and increased up to the 85th day. When incubated at 15°C, 17.4% of amylose retrograded within the first day. On the second and third day, there was an apparent lag period in which amylose retrograded at a slower rate. After that, the retrogradation accelerated and reached 58% at the 14th day and then slowed down again.

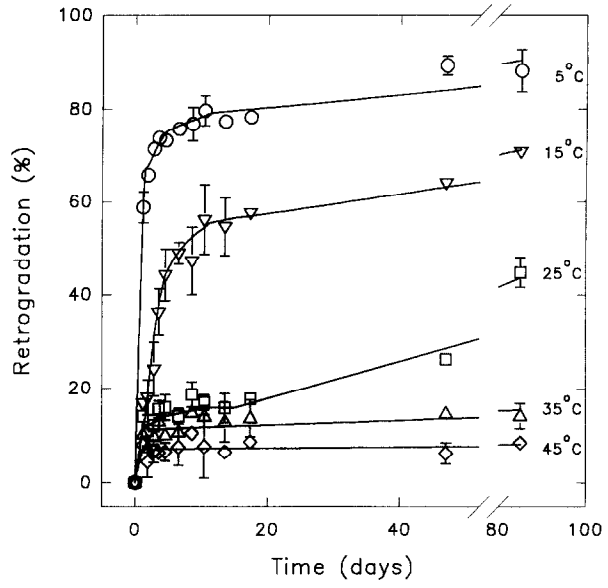

Fig. 1. The amylose solution retrogradation monitored by measuring the decrease in total carbohydrate content in the supernatant. The original amylose concentration was 3.5 mg/ml.

For the amylose solution incubated at 25°C, the retrogradation rate was much slower than at 5 and 15°C, and the retrogradation pattern differed. About 12.5% of the amylose retrograded within the first day followed by a 16 day lag period in which little additional retrogradation occurred, finally reaching 18% on the 17th day. After the lag period, amylose began to retrograde at an increased rate, reaching 45% on the 85th day. The lag period, observed at 15 and 25°C, can be attributed to the time required for nucleation (Levine and Slade, 1990). For the solutions incubated at 35 and 45°C, 9.2 and 7.1% of the amylose retrograded within the first day, respectively. After that, no significant increase in retrogradation was observed up to the 85th day.

To study the molecular weight of amylose retrograded at different temperatures during the incubation, the original amylose sample and the supernatant of the amylose solution after a 100 day incubation were subjected to HPSEC analysis. The molecular weight profiles of the amylose starting material and the amylose remaining in the supernatant after incubation at different temperatures are shown in Fig. 2. After incubating at 5 and 15°C, most amylose molecules precipitated except for a small amount at 5°C and a larger amount at 15°C. A substance shown at the void

Table 1. Retrogradation (%) of amylose solution in the first 24 h[1]

Incubation temperature (°C)	5	15	25	35	45
Retrogradation (%)[2]	58.8±3.3	17.4±0.9	12.5±0.8	9.2±1.2	7.1±0.4

[1] Means and standard deviations of duplicate samples based on three measurements for each sample.
[2] Original concentration of amylose solution was 3.5 mg/ml.

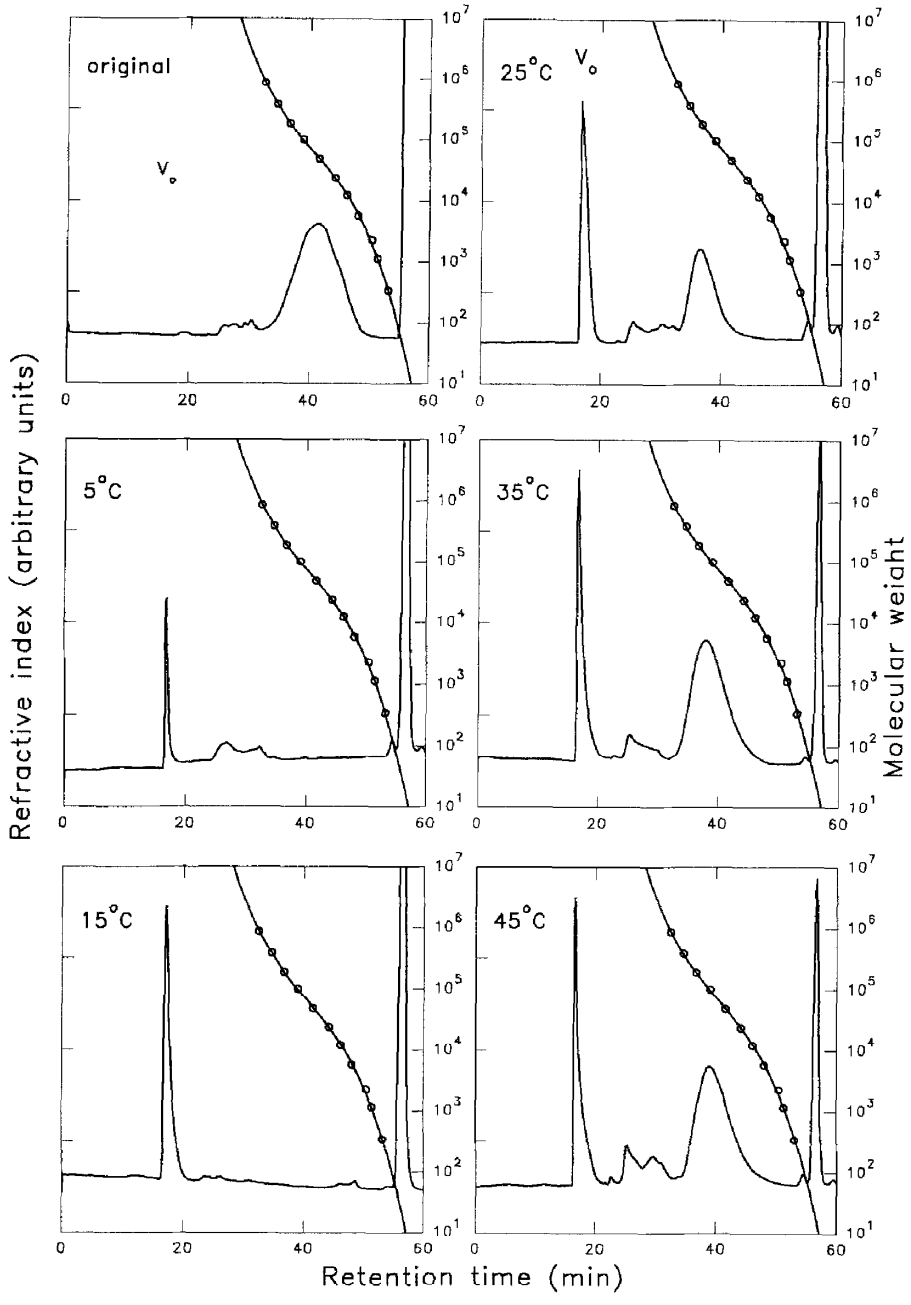

Fig. 2. The chromatogram of original amylose and retrograded amylose supernatant at different incubation temperatures. The original amylose solution with a concentration of 3.5 mg/ml was incubated at different temperatures for 100 days. The –O– is the molecular weight calibration curve of pullulan and malto-oligosaccharide standards.

volume was attributed to entangled amylose. At 25°C, about half the amylose molecules remained in the supernatant, which consisted of a large peak at the void volume and another peak of large-molecular-weight amylose (Fig. 2). At 35 and 45°C, most of the molecules remained in the supernatant; but the entangling phenomenon was obvious, as indicated by the peak at the void volume. These results indicated that following incubation, amylose molecules can be in three states: free in the supernatant, entangled and eluted at void volume, and retrograded and precipitated. The last peak of the chromatogram was the solvent peak (Fig. 2).

The number and weight average degree of polymerization (DP_n and DP_w) of the commercial potato amylose obtained by using HPSEC were 250 and 1490, respectively (Table 2), which were substantially smaller than those reported by Hizukuri and Takagi (1984). The difference in the molecular size could be caused by different starch isolation methods. The DP values also might be underestimated as a result of using the pullulan standards for molecular weights, because amylose had a slightly branched structure and a random coil conformation that resulted in a less extended molecule compared with pullulan (Hizukuri *et al.*, 1981; Hizukuri

Table 2. Molecular weights of original amylose molecules and those lost from the original chromatogram during incubation at different temperatures[1]

Temperature (°C)	Total retrogradation(%)[2]	DP_n	DP_w	Polydispersity
Original amylose	0	250±13	1490±120	5.95±1.91
Retrograded amylose				
5	88.3±4.5	234±16	1236±95	5.27±0.17
15	70.8±0.9	212±2	512±4	2.42±0.02
25	44.8±3.2	164±3	275±4	1.68±0.05
35	14.2±2.6	124±8	184±7	1.49±0.05
45	8.6±0.4	110±6	150±12	1.37±0.05

[1] Means and standard deviations of duplicate samples for total retrogradation and triplicate samples for molecular weight determination. DP_n and DP_w stand for pullulan-equivalent number and weight average degree of polymerization determined after 100 days incubation at different temperatures.
[2] After 85 days incubation.

and Takagi, 1984). Throughout this paper, the molecular weight of amylose is reported as the pullulan-equivalent molecular weight. By comparing the molecular weight profiles of the supernatant of amylose incubated at different temperatures (Fig. 2), we confirmed that retrogradation was a molecular weight dependent process.

To determine the molecular weight of the amylose that retrograded during the incubation, the chromatogram of the original amylose was subtracted by that of the supernatant after 100 day incubation to obtain a differential chromatogram. The differential chromatograms at different temperatures are shown in Fig. 3. In the differential chromatogram, the peaks that appeared above the baseline were the molecules lost during the incubation, a result of precipitation or entanglement; the peaks that appeared below the baseline represented the entangled substance which had apparent large molecular sizes in the supernatant after 100 day incubation. The DP_n, DP_w, and polydispersity of the amylose that was lost from the supernatant after 100 days of incubation at different temperatures are shown in Table 2. At 5°C, the majority of amylose molecules precipitated, making the molecular weight of the lost fraction obtained from differential chromatograms similar to that of the original amylose. At 45°C, only a small fraction of amylose molecules were lost, as indicated by the differential chromatogram, and the average molecular weight of this fraction was substantially smaller than the original molecular weight (Table 2). These results showed that amylose molecules with DP_n around 110 had a greater tendency to retrograde. This was consistent with previous reports (Gidley *et al.*, 1986; Pfannemuller, 1986; Gidley and Bulpin, 1989). The difference in the percentage retrogradation of amylose at different temperatures (Table 2) may be attributed to the different molecular weight subfractions of amylose having different critical retrogradation temperatures. Amylose of large molecular weight does not retrograde when its temperature is maintained above the critical retrogradation temperature. When the incubation temperature is maintained at 65°C and above, amylose does not retrograde (Miles *et al.*, 1985).

The chain length distributions of the retrograded amylose crystallites that resisted acidic and amylolytic hydrolysis were examined by using HPSEC. An example of the size-exclusion chromatogram of amylose crystalline chain length, prepared by α-amylolysis of retrograded amylose at 5°C, is shown in Fig. 4. The chain lengths of the retrograded amylose crystallites prepared at different incubation temperatures showed an increasing trend as the incubation temperatures increased and a narrow polydispersity (Table 3). At lower temperatures (5, 15, 25°C), the polydispersity was not substantially changed, although the morphology and crystalline chain length of retrograded amylose differed (Table 3, Fig. 5). At higher temperatures (35, 45°C), the polydispersity and chain length were increased. At lower temperatures (5, 15, 25°C), the morphology of retrograded amylose is a gel-like block (Fig. 5). At 5°C, the structure was coarser than at 15 and 25°C and contained numerous polygonal-shaped 'nodules', which contained shorter crystallites. For this reason, the nodular structure might be the result of fast-growing crystallization. At 15 and 25°C, the structures of retrograded amylose were more homogeneous and similar to those previously reported (Leloup *et al.*, 1992). The filaments formed at 25°C were wider than those formed at 15°C. The well established structure of the retrograded amylose network formed at 15 and 25°C might be attributed to slower crystallization, which allowed the amylose molecules to align in order and to develop the network. In the amylose solutions incubated at 35 and 45°C, a small amount of fiber-like precipitate, which contained longer crystallites, formed instead of the gel-like precipitate. The fiber-like precipitate was made from a small subfraction of amylose molecules (DP_n

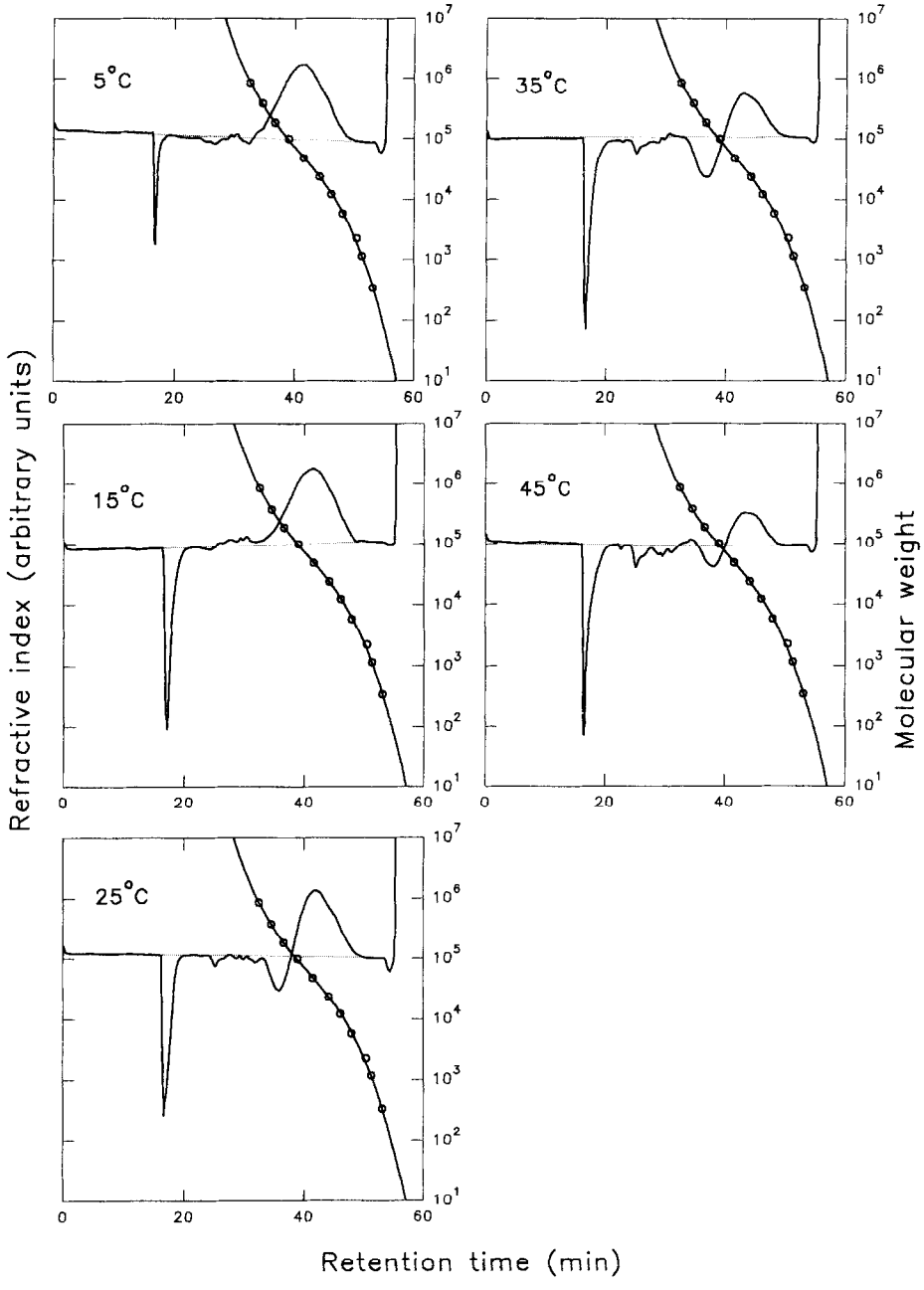

Fig. 3. The differential chromatograms of original potato amylose and retrograded supernatant at different incubation temperatures. The original amylose solution with a concentration of 3.5 mg/ml was incubated for 100 days.

Table 3. Chain length of retrograded amylose crystallite prepared at different incubation temperatures[1]

Temperature (°C)	Porcine pancreatic α-amylolysis			16% sulfuric acid hydrolysis		
	DP_n	DP_w	Polydispersity	DP_n	DP_w	Polydispersity
5	39±2	47±2	1.21±0.01	34±1	48±2	1.41±0.03
15	40±1	50±2	1.24±0.01	35±1	49±1	1.40±0.04
25	44±2	54±2	1.23±0.03	36±1	51±1	1.41±0.02
35	45±2	58±3	1.29±0.01	36±0	53±0	1.47±0.04
45	52±3	72±6	1.38±0.06	40±0	67±1	1.67±0.06

[1] Means and standard deviations of duplicate samples for total retrogradation and triplicate samples for chain length determination. Original amylose solution concentration was 3.5 mg/ml and was incubated at different temperatures for 85 days.

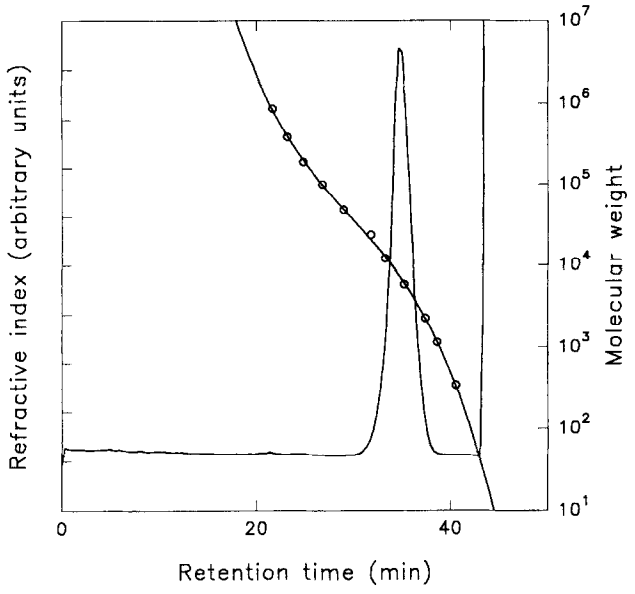

Fig. 4. The chromatogram of retrograded amylose crystallite hydrolyzed with porcine pancreatic α-amylase. The original amylose solution with a concentration of 3.5 mg/ml was incubated at 5°C for 85 days.

110 and 124), which may not be long enough to extend beyond two crystallite blocks to form a network.

At elevated temperatures, a longer chain length is required to stabilize a double helical structure. These crystalline chain length differences may illustrate the finding that the thermal transition temperature is higher for retrograded soluble starch prepared at higher temperatures (Suzuki *et al.*, 1987). In this study, the effect of temperature on the chain length of the amylose crystallites coincided with the developmental temperature effects on the branch chain length of amylopectin. Starch amylopectin developed at higher temperatures has more long branches than that developed at lower temperatures (Asaoka *et al.*, 1984, 1985; Lu *et al.*, 1996). The narrow polydispersity confirms that the retrograded amylose crystallite has a regularly helical segmental structure. These phenomena might be a result of the thermodynamic association and dissociation equilibria of amylose molecules. The chain lengths obtained from α-amylase hydrolysis were larger than those from acid hydrolysis, but the chain polydispersities were narrower. These results agree with the reports that document the difficulty for an enzyme to hydrolyze stubs shorter than the binding site of the enzyme (Jane and Robyt, 1984). The binding site of porcine pancreatic α-amylase is five glucose units (Robyt and French, 1967).

In conclusion, the retrogradation rate of a diluted amylose solution decreased as incubation temperature increased. Different molecular sized amylose subfractions had different retrogradation tendencies. Small-sized potato amylose (DP_n ~110) had a higher retrogradation tendency. The retrogradation temperature also affected

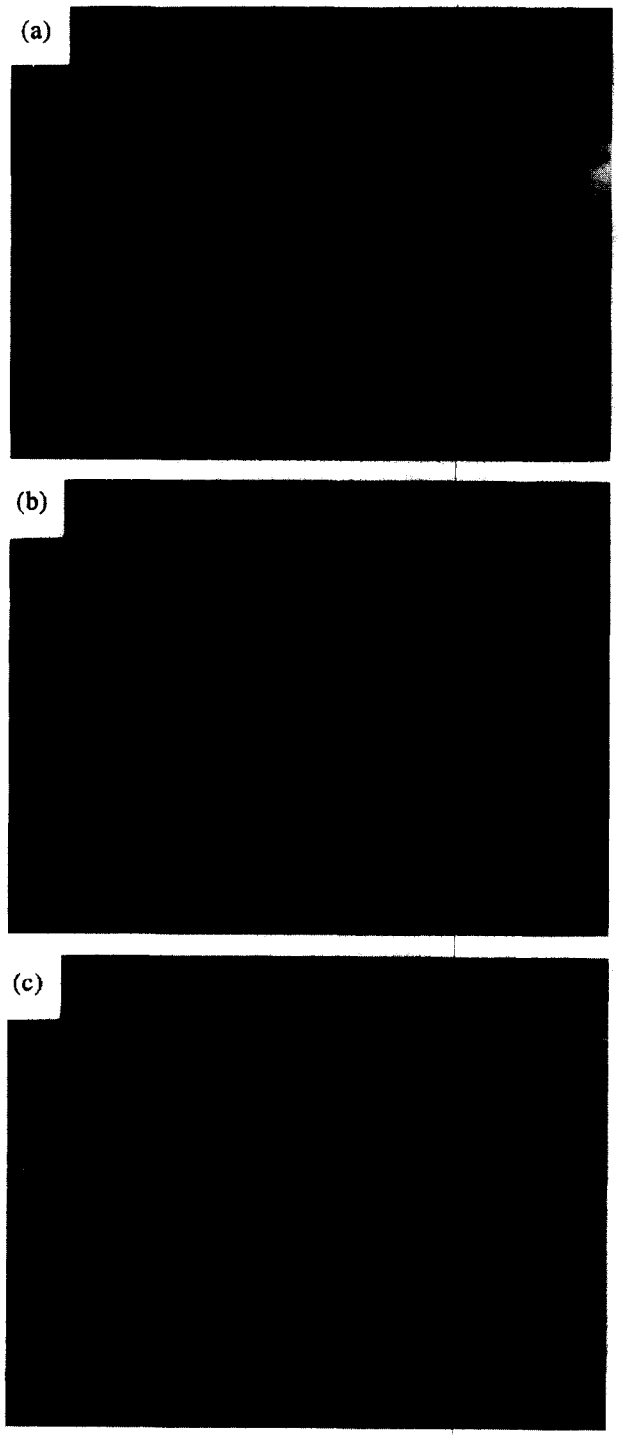

Fig. 5. The SEM photographs of potato amylose gel retrograded at different temperatures: (a) 5°C; (b) 15°C; (c) 25°C. The scale bars stand for 1 μm.

the morphology and the chain length of the crystalline region of retrograded amylose. At 5°C, retrograded amylose displayed nodular shapes; whereas, at 15 and 25°C, it displayed a well defined network. The crystalline chain length of retrograded amylose increased as the incubation temperature of the retrograded amylose increased. The retrograded amylose crystalline chains had a narrow polydispersity (1.21 to 1.67).

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