A Thesis for the Degree of Master of Science

Retrogradation of amylosucrase treated waxy corn starch by temperature cycling: structural and digestibility properties

아밀로수크레이스 처리 후 온도 사이클링으로 노화시킨 찰 옥수수 녹말의 구조 및 소화 특성

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농학석사학위논문

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Retrogradation of amylosucrase treated waxy corn starch by temperature cycling: structural and digestibility properties

by

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ABSTRACT

In this study, waxy corn starch was treated by amylosucrase (AS), followed by retrogradation under different temperature cycles or isothermal storage. Various retrogradation conditions were used to accelerate crystallization of amylopectin chains elongated by AS: temperature cycling with a low temperature of 4 °C for 1 day and a high temperature of 20, 40, or 60 °C for 1 day for 3 or 7 cycles, and isothermal storage at 4 °C for 6 days or 14 days. The properties of AS-treated starches were determined by high-performance anion-exchange chromatography and the iodine binding capacity (IBC). The properties of retrograded starches were determined by X-ray diffractometry, differential scanning calorimetry, ^{13}C CP/MAS NMR spectroscopy, and *in vitro* digestibility.

After the AS treatment, the proportion of short chains (DP ≤ 12 and DP 13-24) decreased, whereas that of long chains (DP 25-36 and DP ≥ 37) increased. Also, IBC was similar with that of normal corn starch. Following the retrogradation for 14 days, the X-ray diffraction patterns of all AS-treated starches showed a B-type polymorph. In the control starches, compared to starches stored only at 4 °C, the starches stored under the temperature cycles exhibited lower relative crystallinity and melting enthalpy due to the melting

of unstable crystallites during storage at higher temperatures. Little difference in ordered structure content was observed. On the other hand, the AS-treated starches stored under the temperature cycles exhibited higher relative crystallinity, melting enthalpy, ordered structure content, and resistant starch content than the isothermal storage at 4 ℃. Among all starches, the AS-treated starch retrograded under 4/60 ℃ cycled condition particularly showed the most striking changes in structural and digestibility properties.

Conclusively, this study demonstrated that temperature cycling could induce reassociation of elongated chains by AS treatment and accelerate retrogradation. It also suggested that retrogradation by temperature cycling brought more changes in structural and digestibility properties of AS-treated starch than control starch.

Keywords: amylosucrase, waxy corn starch, retrogradation, temperature cycling, *in vitro* digestibility, structural properties

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ABBREVIATIONS

AM: amylose

AP: amylopectin

RDS: rapidly digestible starch

SDS: slowly digestible starch

RS: resistant starch

DP: degree of polymerization

AS: amylosucrase from *Neisseria polysaccarea*

GI: Glycemic Index

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INTRODUCTION

Starch is an important energy source in human diet. It consists of amylose (AM) and amylopectin (AP). AM has long linear chains of (1, 4)-linked α-D-glucopyranose residues, some with a few (DP>10) branches (Hizukuri et al., 1981). AP has large molecular weight and highly branched structures consisting of much shorter chains of (1, 4)-α-D-glucose residues. The branch-chains are connected by (1, 6)-α-D-glucosidic linkages (Hizukuri, 1996).

In terms of the nutrition, starch is generally classified into rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) according to the rate of glucose release and its absorption in the gastrointestinal tract (Englyst et al., 1992). RDS is digested rapidly in the mouth and the small intestine, leading to a rapid increase followed by an equally rapid drop in blood glucose level. SDS is digested slowly but completely in the small intestine and might consist of less perfect crystalline regions containing small portions of double helices and amorphous region (Shin et al., 2004). RS is not digested in the small intestine, but can be fermented in the large intestine. RS is classified into four types according to the mechanism that prevents its enzymatic digestion. Especially, RS type III is produced by two steps: gelatinization

and retrogradation (Eerlingen et al., 1993).

Jenkins et al. (1981) introduced the concept of glycemic index (GI) to classify foods on the basis of their postprandial blood glucose response. The GI is defined as the postprandial incremental glycemic area after a test meal, expressed as the percentage of the corresponding area after a carbohydrate portion of a reference food such as glucose or white bread. Both SDS and RS are correlated with a low GI (Englyst et al., 1996) and have a resistance against enzymatic digestion in the small intestine (Zhang et al., 2006). The potential health benefits of SDS are improved glucose tolerance, diabetes management, mental performance, and satiety (Lehmann & Robin, 2007). The health benefits of RS have been reported as prevention of colon cancer, hypoglycemic effects, substrate for growth of the probiotic microorganisms, reduction of gall stone formation, hypocholesterolemic effects, inhibition of fat accumulation, and increased absorption of minerals (Sajilata et al., 2006). Therefore, increasing the contents of SDS and RS in foods is an interesting subject for the food industry. To increase the fraction of low digestible starch, physical, chemical and enzymatic modifications have been employed. Among these, enzymatic modification is safer and can reduce by-products (Le et al., 2009). Physical modification is natural and safe as compared to chemical modifications (Lawal, 2005).

Amylosucrase (EC 2. 4. 1. 4., AS) from *Neiserria polysaccharea,* one of the glucoside hydrolases, has an unusual specificity for sucrose hydrolysis and synthesizes an insoluble AM-like polymer (Hehre, 1949; Potocki-Veronese et al., 2005; Potocki de Montalk et al., 2000). It elongates the non-reducing ends of AP and AM, and produces (1→4)-α-glucans using glucose from sucrose, while releasing fructose (Büttcher et al., 1997; Potocki de Montalk et al., 2000; Rolland-Sabaté et al., 2004). A previous study showed that AS-treated starch has longer branch chain length than raw starch dose (Shin et al., 2010). It could induce reassociation of chains during reaction and decrease the susceptibility to digestive enzymes, resulting in higher SDS and RS content (Kim et al., 2014). Retrogradation involving reassociation is most rapid with AM and much slower and more incomplete with AP due to the short chain length of its branches (Jenkins et al., 1998). Jane et al. (1999) and Shi and Seib (1992) reported that long chains with DP > 50 accelerated retrogradation of amylopectin, whereas the short chains (DP 6-9) retarded it. Therefore, in this study AS was used for increasing the proportion of long chains of waxy corn starch.

Retrogradation is the main category of physical modification used to achieve low-GI benefits in cooked and processed starchy foods(Hamaker, 2007). Retrogradation occurs when the starch components in gelatinized

starch reassociate in an ordered structure. Starch changes from an amorphous state to a crystalline state, and thus this retrogradation process includes crystallization. The crystallization rate and extent are mainly affected by the inherent starch properties, such as AM/AP ratio and the botanical origin of the starch and by storage conditions, such as temperature, time and moisture content (Gudmundsson, 1994; Liu & Thompson, 1998).

Crystallization occurs in three consecutive steps: nucleation (formation of critical nuclei), propagation (growth of crystals from the nuclei formed) and maturation (crystal perfection or continuing slow growth), all of which are temperature-dependent (Silverio et al., 2000). At a temperature near the starch glass transition, the nucleation rate of starch crystallization is high and the propagation rate is low, whereas the nucleation rate is low and the propagation rate is high when the temperature is close to crystal melting (Eerlingen et al., 1993).

Slade and Levine (1987) applied polymer crystallization theory to develop a method to accelerate retrogradation termed temperature cycling. By cycling between the temperature of greatest nucleation rate and the temperature of greatest propagation rate for set periods of time, the rates of both retrogradation of concentrated starch pastes and staling of bread crumb were increased over those which occurred under optimal isothermal

conditions. Therefore, in this study, temperature cycling was done in order to induce crystallization chain of waxy corn starch elongated by AS.

There are no studies about retrogradation of AS treated waxy corn starch by temperature cycling. The objective of this study was to investigate structural and digestibility properties of temperature cycled waxy corn starch after AS treatment.

MATERIALS AND METHODS

1. Materials

1-1. Starches

Waxy corn starch and normal corn starch was obtained from Samyang Genex Corp. (Incheon, Korea).

1-2. Enzymes

Amylosucrase from *Neiserria polysaccharea* was provided by the Food Microbiology and Biotechnology Laboratory of Kyunghee University.

Isoamylase (activity 1000U) was obtained from Megazyme (Bray, Ireland). Pancreatin (P7545, activity 8 × USP/g) was from Sigma Chemical Co. (St. Louis, MO, USA) and amyloglucosidase (AMG 300L, activity 300 AGU/mL) from Novozymes (Bagsvaerd, Denmark).

2. Methods

2-1. Enzyme assay of AS activity

The AS was purified by affinity chromatography with Ni-NTA (nickel-nitrilotriacetic acid) resin according to the method of Jung et al. (2009). Enzyme activity was determined using the method of van der Veen et al. (2004) with a modification. The mixture of 0.1 mL of 4% sucrose, 0.1 mL of 1% glycogen, 0.25 mL of 0.1 mM sodium citrate buffer (pH 7.0) and 0.05 mL of AS was reacted in a water bath at 30 °C and 80 rpm for 10 min. The released fructose was quantified using the dinitrosalicylic acid method of Miller (1959). One unit (U) of AS was defined as the amount of enzyme that catalyzes the release of 1 μM of fructose per min under the assay conditions.

2-2. Preparation of AS-treated starch

Starch suspension (2%, w/w) was prepared by mixing waxy corn starch, 100 mM sucrose, and 100 mM sodium acetate buffer (pH 7.0) to reach the final volume of 150 mL. The starch suspension was boiled for 30 min and cooled in a water bath at 30 °C for 30 min. AS (20,000U/30 mL) was added to the starch suspension and incubated in a water bath at 30 °C for

24 h. The enzyme reaction was stopped by adding two-fold ethanol to the suspension. The AS-treated starch was precipitated by centrifugation at 10,000g for 10 min, and the supernatant was removed. The pellet was washed off three times with distilled water by centrifugation at 10,000g for 10 min. The precipitate was freeze-dried, ground and passed through a 100-mesh sieve. AS-control was native waxy corn starch.

2-3. Preparation of retrograded starch by temperature cycling

Starch suspension (20%, w/w) was prepared and gelatinized using an autoclave at 121 ℃ for 30 min. The gelatinized starch gels were cooled to room temperature, hermetically sealed and stored under different temperature conditions: constant temperature of 4 ℃ or cycles of 4 ℃ for 1 day and subsequent 20 ℃, 40 ℃ or 60 ℃ for 1 day, respectively. Each sample after 0, 6, and 14 days was then freeze-dried, ground and passed through a 100-mesh sieve.

2-4. Determination of branch chain length distribution by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

The branch chain length distributions of native and AS treated starch mixtures were determined by debranching the starches with isoamylase. Starch (15 mg) was dispersed in 90% DMSO (3 mL) and boiled for 30 min. Ethanol (15 mL) was added to the starch suspension to precipitate starch and centrifuged at 10,000g for 10 min. Then distilled water (1.5 mL) was added to the pellet and boiled for 15 min. After boiling, 1.5 mL of 50 mM sodium acetate buffer (pH 4.3) was added and boiled for 20 min. Isoamylase (30 μL, 1000 U/mL) was added to the starch dispersion and the sample was incubated at 45 °C and 30 rpm for 2 h in a water bath. Enzyme reaction was stopped by boiling for 10 min. Debranched sample was filtered through a 0.45 μm membrane filter and analyzed using HPAEC-PAD on a Carbo-pack PA1 anion-exchange column (4x250 mm, Dionex, Sunnyvale, CA, USA) with a pulsed amperometric detector. The sample was eluted with a gradient of 600 mM sodium acetate in 150 mM NaOH with a flow rate 1 mL/min. The gradients of sodium acetate used were as follows: increasing from 0-20 % for 0-5 min, 20-45 % for 6-30 min, 45-55 % for 31-60 min, 56-60 % for 61-80 min, 61-65 % for 81-90 min, 66-80 % for 91-95 min, and 81-100 % for 96-100 min. The values of

DP were designated using a mixture of maltooligosaccharides (DP 1-7, Sigma Chemical) as standard. PeakNet software (version 5.11, Dionex) was used for calculation of peak areas. Number-based average DP (DP_n) was determined by following equation.

$$DP_n = (\%A_i \times DP_i) / 100$$

A_i : peak area / total area (i: 1, 2, 3 …)

2-5. Determination of iodine binding capacity (IBC)

IBC was measured by the colorimetric method outlined by AACC Approved Method 61-03 (AACC, 2000). Starch (20 mg) was dispersed in absolute ethanol (0.2 mL), and then mixed with 1 M NaOH (1.8 mL) with vigorous vortexing. The starch suspension was cooked for 10 min, and cooled to room temperature. The resultant starch solution (1 mL) was diluted to 10 mL with distilled water. An aliquot (0.5 mL) of the diluted starch solution was combined with 1 M acetic acid (0.1 mL) and Lugol's solution (0.2 mL; 0.2% I_2 + 2.0% KI), and diluted again to 10 mL with distilled water, followed by holding for 20 min in the dark. The absorbance of the color-developed starch solution was measured at 620 nm. IBC of the starch sample was determined from a standard curve prepared with amylose from potato and amylopectin from maize (Sigma–

Aldrich Chemical Co., St. Louis, MO, USA).

2-6. X-ray diffraction patterns and relative crystallinity

X-ray diffraction was analyzed using a powder X-ray dffractometer (New D8 Advance, Bruker, Karlsruhe, Germany) at 40 kV and 40 mA. Starch sample scan was performed through 2θ range from 3° to 30° with a 0.02° step size and a count time of 2 sec. The relative crystallinity was determined by the following equation according to the method of (Nara & Komiya, 1983). The area was calculated using the software developed by the instrument manufacturer (EVA, 2.0).

$$\text{Relative crystallinity (\%)} = \left(\frac{\text{Crystalline area}}{\text{Total curve area}}\right) \times 100$$

2-7. Measurement of thermal properties

Thermal properties of the samples were investigated using a differential scanning calorimeter (DSC, Diamond DSC, Perkin-Elmer, Waltham, MA, USA). Each sample (10 mg) was weighed in a hermetic aluminum pan (Seiko, Tokyo, Japan), and 40 μL of distilled water was added. The sample

pan was sealed and kept at room temperature overnight for equilibrium. An empty aluminum pan was used as a reference, and indium was used for calibration. DSC scan was performed from 30 ℃ to 180 ℃ at 10 ℃/min. The onset temperature (To), the peak temperature (Tp), the conclusion temperature (Tc), and the melting enthalpy (△H) were recorded.

2-8. Solid-state ^{13}C cross-polarization and magic-angle spinning (CP/MAS) nuclear magnetic resonance (NMR) spectra

A Bruker AVANCE 400 WB (Bruker, Karlsruhe, Germany) equipped with CP-MAS accessories was used for ^{13}C CP/MAS NMR analysis. Cross-polarization (CP), magic angle sample spinning (MAS), and high power decoupling conditions were used to observe NMR spectra (single scan). The acquisition time was 35 ms, time domain points, 2.2 k, and line broadening 10 Hz. The samples were spun at a rate of 5 kHz at room temperature in a 4-mm rotor with a spectral width of 3.1 kHz. Spectra were referenced to the high-field resonance of adamantine (29.5 ppm).

The ordered (double-helical) to amorphous ratio was obtained by comparison between spectra of sample and amorphous starches (Gidley, 1985). Resonance peaks in the regions 60-64, 68-77, 82-85 ppm are

characteristic for amorphous material, while the signal at 94-105 ppm is characteristic for ordered (double-helix) structure (Gidley & Bociek, 1985). The data were processed and calculated of integrated peak areas using the processing tools included in the Topspin 1.3 software (Bruker, Karlsruhe, Germany).

2-9. Starch digestibility

Starch digestibility was determined by the method of (Brumovsky & Thompson, 2001) with slight modification. Pancreatin (6 g) was dissolved in distilled water (72 mL) and stirred well for 10 min. It was precipitated by centrifugation at 1,500g for 10 min. A 60 mL aliquot of supernatant was mixed with 1.2 mL of amyloglucosidase and 10.8 mL of distilled water, and incubated at 37 °C for 10 min.

A starch sample (30 mg) was dispersed in a 2 mL-microtube with sodium acetate buffer (0.75 mL, 0.1 M, pH 5.2) with one glass bead. After mixing each microtube, it was equilibrated in a shaking incubator (240 rpm, 37 °C) for 10 min. Then, 0.75 mL of the prepared enzyme solution was added to the tube, and the starch sample was incubated in a shaking incubator (240 rpm, 37 °C). A microtube was removed each time at 10, 20, 30, 60, 120, and 240 min and boiled in a cooker for 10 min to stop

reaction.

The glucose released under hydrolysis of starch was obtained in supernatant after the centrifugation at 5,000g for 10 min. The glucose content was determined by the glucose oxidase method (Karkalas, 1985) using a commercially available kit (Embiel Co., Gunpo, Korea). To measure the content of released glucose, 11-fold diluted supernatant (0.1 mL) was added to a 2 mL-microtube containing 1.5 mL of glucose oxidase and peroxidase reagent. The microtube was incubated in a water bath at 37 °C for 20 min. The absorbance of the sample was then read at 505nm.

Starch fractions were classified based on the rate and degree of hydrolysis. RDS was measured by the quantity of glucose after reaction for 10 min. SDS was the fraction digested between 10 and 240 min. RS was the unhydrolyzed fraction after 240 min.

2-10. Statistical analysis

All the experiments were done in triplicate, and data were expressed as mean±standard deviation. Analysis of variance (ANOVA) was conducted and the mean separations done by the Duncan's multiple range test at a significance level of 0.05. All the statistical analyses described above were

conducted using PASW statistic 18 (SPSS corp., Chicago, IL, USA).

RESULTS AND DISCUSSION

1. Branch chain length distributions of AS-treated starches

The branch chain length distributions and the relative percentages of peak area with DP of starches are shown in Figure 1 and Table 1, respectively. In general, AP branch chains are categorized into A chain (DP 6-12), B_1 chain (DP 13-24), B_2 chain (DP 25-36) and B_3 chain (DP $\geq$ 37) depending on degree of polymerization (Hanashiro et al., 1996). The waxy corn starch, one of A type starches, has a larger proportion of short chain such as A chain but smaller proportions of longer chains (Zhang et al., 2006). Shin et al. (2010) reported that AS treatment induced an increase in the chain length of AP and decrease in the proportion of short chains. Figure 1 showed highest peak was shifted to right compared with that of AS control. After the AS treatment, the proportion of short A chain (DP $\leq$ 12) and B_1 chain (DP 13-24) of AS-treated starch decreased. On the other hand, the proportions of long B_2 chain (DP 25-36) and B_3 chain (DP $\geq$ 37) increased compared to those of control. These results were due to the elongation of external chains by AS. Potocki de Montalk et al. (2000) reported that AS elongates external chains of AM and AP by catalyzing

the attachment of 18 glucosyl units at non-reducing ends.

The extended branch chains by AS treatment favored the formation of double helices which hinder enzyme access and it might be accelerated by retrogradation. The increased proportion of long chains and the decreased proportion of short chains could lead to the formation of more perfect crystallites resulting in the resistance to starch-digestive enzymes (Shin et al., 2010).

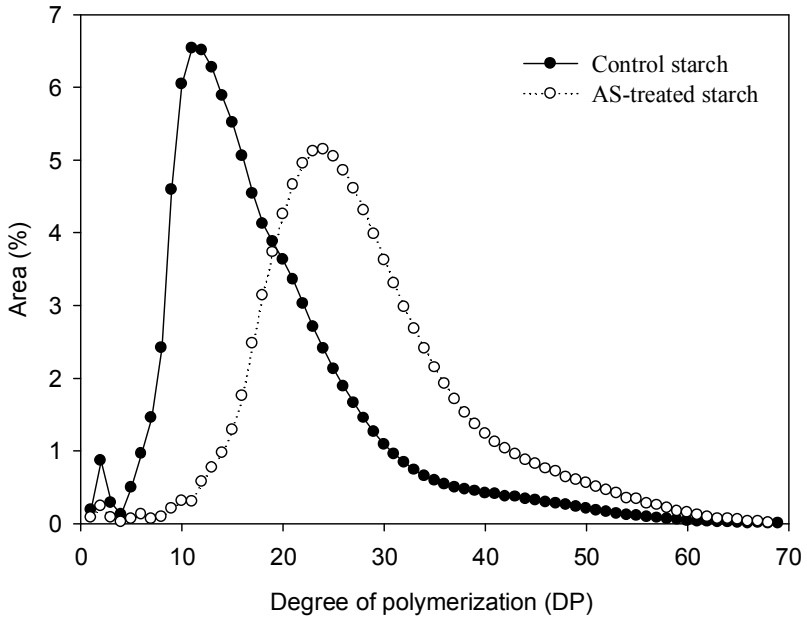


Figure 1 Branch chain length distributions of control starch (●) and AS-treated starch (○).

Table 1 Branch chain length distributions of AS-treated starches

Samples	Percent distribution (%)				
	DP[1)] ≤ 5	DP 6-12	DP 13-24	DP 25-36	DP ≥ 37
Control starch[2)]	1.5±0.6[a,4),5)]	28.4±4.0[a]	50.4±2.9[a]	13.8±1.3[b]	6.0±0.8[b]
AS-treated starch[3)]	0.8±0.2[b]	1.7±0.4[b]	38.2±2.4[b]	41.8±2.2[a]	17.4±1.0[a]

[1)] DP, degree of polymerization.

[2)] Control starch with no enzymatic treatment.

[3)] AS-treated starches with 20000 U/30 mL for 24 h

[4)] Data are expressed as average value and standard deviation.

[5)] The values with different superscripts in a same column are significantly different (p<0.05).

2. Iodine binding capacity (IBC)

AM forms single-helical complexes with iodine to give a blue color (Conde-Petit et al., 1998). Long branch chains of AP, like AM, bind iodine to form a single helical complex during colorimetric procedure. Consequently developing a blue color inflates the iodine binding capacity and the apparent AM content of the starch (Jane et al., 1999). Because of this, IBC of starches was examined and the results are shown in Table 2. IBC of normal corn starch was similar with that of AS-treated starch, despite the starch containing no AM. This result indicated elongated branch chains of AP by AS treatment formed a single helical complex like AM.

Table 2 Iodine binding capacity of AS-treated starches

Samples	Iodine binding capacity (%)
Control starch[1)]	0.4±0.3[b,3),4)]
AS-treated starch[2)]	26.2±0.6[a]
Normal corn starch	25.8±0.6[a]

[1)] Control starch with no enzymatic treatment.

[2)] AS-treated starches with 20000 U/30 mL for 24 h

[3)] Data are expressed as average value and standard deviation.

[4)] The values with different superscripts in a same column are significantly different (p<0.05).

3. X-ray diffraction patterns and relative crystallinity

X-ray diffraction patterns and relative crystallinities of the starches are shown in Figure 2 and Table 3, respectively. The raw waxy corn starch showed major peaks at 15°, 17°, 18°, and 23°, presenting a typical A type pattern (Hizukuri et al., 1980). No pattern of was not shown for control starch due to gelatinization during sample preparation. With increasing retrogradation time, the peaks from 10° to 14° were started to develop. The peak of 4/60 °C, 14 d sample exhibited stronger than other samples. It was possible that retrograded control starch could have a B-type X-ray pattern after further retrogradation. Zhou and Lim (2012) reported that retrograded normal and waxy corn starches had B-type configuration, which was typical for retrograded starches. The AS-treated starch showed a B-type X-ray pattern with the peaks at 5.5°, 14.5°, 17°, 19.3°, 22°, and 24°. Branch chain elongation resulting from AS action facilitated and solidified the inter-chain association, which in turn led to stable B-type polymorph (Ryu et al., 2010). Generally, B-type crystallites have a cluster with higher DP as compared to A-type amorphous lamellae (Gérard et al., 2000).

The peak intensity of control starch decreased with cycling at high temperatures, because unstable crystallites formed under temperature cycling

could be melted. When starch was temperature cycled, the crystallites were formed weakly than those of starch stored at 4 °C. The peak intensity of AS-treated starch increased with increasing cycled temperature. As retrogradation of AS-treated starch was progressed, the peaks were observed 20° and 22°, a general peak shown on retrograded starch. Kim et al. (2009) reported that retrograded starch showed a B type pattern, which was induced from the regions composed of double helices in a hexagonal structure. Whether starch has an A or B type pattern in the nature, starch gels formed a B type pattern on retrogradation (Katz, 1934). It has been known that short chains of AP induce an A type pattern, and long chains of AP are involved in the formation of a B type pattern (Pohu et al., 2004). Long branched chains in the AS-treated starches could act like long AM (Rolland-Sabaté et al., 2004). These AM-like long chains could contribute to the formation of B type pattern.

Differences in relative crystallinity probably result from differences in 1) crystal size, 2) AP chain length, 3) extent of interaction between double helices and 4) orientation of the double helices within the crystalline domains (Gunaratne & Hoover, 2002). The relative crystallinity of all samples gradually increased with increasing retrogradation time. The 0 d sample of control starch exhibited the lowest relative crystallinity due to the gelatinization during the sample preparation. In control starches, compared

with other samples, the 4 °C sample showed higher relative crystallinity. Zhou and Lim (2012) reported that the starch gels stored under the isothermal condition had slightly higher relative crystallinity than those stored at cycled temperatures.

Especially, the 4/60 °C, 14d sample of AS-treated starch showed a much higher relative crystallinity compared with the other samples, indicating that its crystalline structure was more densely packed than that of the other samples due to enhanced retrogradation. This result suggested that the temperature cycling at 4/60 °C promoted crystallization of extended chains of AP by AS treatment than isothermal 4 °C storage did.

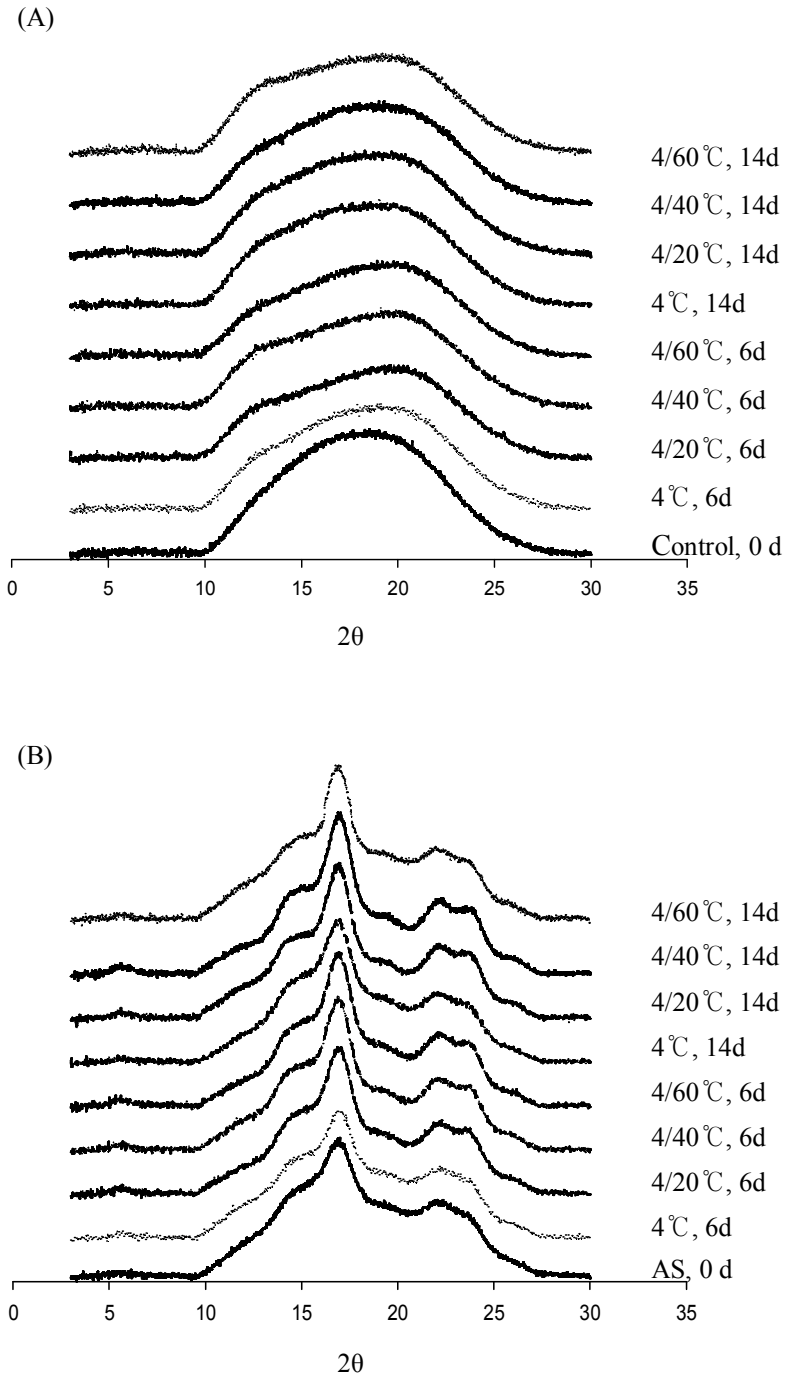


Figure 2 X-ray diffraction patterns of starches. (A): control starch, (B): AS-treated starch

Table 3 Relative crystallinity of starches.

Samples		Relative crystallinity (%)
Control starches		
0 d		16.0±0.1[f, 1), 2)]
6 d	4 °C	17.0±0.2[bcd]
	4/20 °C	16.8±0.2[cd]
	4/40 °C	16.6±0.3[de]
	4/60 °C	16.3±0.2[ef]
14 d	4 °C	18.2±0.3[a]
	4/20 °C	17.3±0.4[b]
	4/40 °C	17.3±0.3[b]
	4/60 °C	17.2±0.4[bc]
AS-treated starches		
0 d		34.2±0.3[e]
6 d	4 °C	35.1±0.4[d]
	4/20 °C	35.6±0.4[cd]
	4/40 °C	35.8±0.4[c]
	4/60 °C	36.4±0.3[b]
14 d	4 °C	36.7±0.2[b]
	4/20 °C	36.6±0.3[b]
	4/40 °C	36.7±0.3[b]
	4/60 °C	37.7±0.2[a]

[1)] Data are expressed as average value with standard deviation.

[2)] The values with different superscripts in a same column are significantly different ($p<0.05$).

4. Thermal properties

The thermal properties of control starches and AS-treated starches retrograded by temperature cycling are shown in Table 4 and Table 5. The peak temperature (T_p) indicates structural stability. The onset temperature (T_o) and conclusion temperature (T_c) are related with melting of the weakest crystallites and strongest crystallites, respectively (Barichello et al., 1990; Biliaderis et al., 1980). The variations in ΔH represent differences in bonding forces between the double helices in the AP crystallites, resulting in different alignment of hydrogen bonds within starch molecules (McPherson & Jane, 1999).

The first peaks indicate the melting of AP crystallites formed by aggregation between adjacent double helices during retrogradation and the second peak means the melting of the complex within elongated long linear chains (Hoover et al., 1996). Control starches showed only the first peak, indicating retrograded AP crystallites melted at relatively low temperature. In general, starch retrogradation occurs in two stages. AM retrogradation is considered to be a rapid process completed within 48 h, whereas AP retrogradation may continue for weeks (Miles et al., 1985). Retrograded AP melts in the approximate temperature range from 40 to 70 °C, whereas

retrograded AM melts in a higher temperature range, from 120 to 170 °C (Sievert & Pomeranz, 1990). The AS-treated starches showed the second peak at around 130 °C and 150 °C. During retrogradation, the elongated chains of AP in the AS-treated starch behaved like long linear AM chains. The 0 d sample of control starch did not exhibit any peaks due to the gelatinization during the sample preparation. In case of control starches, ΔH of all samples increased with increasing retrogradation time. Also, T_o and T_p increased slightly, and T_c had no significant differences. Compared to the isothermal storage at 4 °C, the temperature cycled starches resulted in higher $T_{o,}$ indicating the AP crystallites formed by the temperature cycling were more uniform and more thermally stable than those formed by the isothermal storage. These results were in agreement with previous studies (Baik et al., 1997; Park et al., 2009; Zhou et al., 2010). This could possibly be regarded as an annealing effect, where the growth of more stable crystallites can occur at the expense of the less stable ones (Silverio et al., 2000). The temperature cycled starches showed lower ΔH than that of isothermal storage at 4 °C. It is probable that the imperfect crystallites formed at 4 °C were melted during storage at higher temperatures (20 °C, 40 °C, and 60 °C), which lowered the overall number of crystallites. Similar to this study, Silverio et al. (2000) reported that a decreased melting enthalpy was observed with waxy maize starch after a temperature cycling of 6 and 40 °C. This result suggested that

cycled temperature storage appeared to induce the formation of more perfect and stable crystallites.

In case of AS-treated starches, crystallites formed at 4 °C were not melted by temperature cycling. It could be explained though T_o of native AS-treated starch was 78 °C (data not shown), which was higher than cycled temperature condition (20 °C, 40 °C, and 60 °C). There was no significant difference in thermal transition parameters between 4/20 °C and 4/40 °C. The ΔH increased as the retrogradation time increased, indicating continuous propagation of starch recrystallization. After retrogradation for 14 days, the 4/60 °C sample had higher ΔH than the 4 °C sample. It is in accordance with the results of the relative crystallinity (Table 3). It might result from the crystallites further perfected under a propagation temperature of 60 °C. Consequently, cycled temperature storage induced recrystallization of chains, resulting in the formation of double helices of elongated chains of AP.

Table 4 DSC parameters of control starches.

Samples		Peak Ⅰ				Peak Ⅱ			
		T_o (°C)[1)]	T_p (°C)	T_c (°C)	ΔH (J/g)	T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)
0 d		N.D.[2)]						N.D.	
6 d	4 °C	40.0±0.8[d,3)]	48.5±0.6[e]	65.9±0.7[ab]	2.6±0.4[e]				
	4/20 °C	41.3±0.4[d]	49.2±0.3[e]	66.7±0.5[ab]	2.4±0.2[e]			N.D.	
	4/40 °C	43.4±1.0[c]	52.0±0.8[d]	66.9±0.7[ab]	2.4±0.1[e]				
	4/60 °C	46.2±0.2[b]	56.7±0.5[b]	67.1±0.5[ab]	2.1±0.1[f]				
14 d	4 °C	41.2±0.9[d]	49.7±0.5[e]	66.2±1.0[b]	3.7±0.1[a]				
	4/20 °C	43.5±0.9[c]	51.9±0.4[d]	66.5±0.7[ab]	2.8±0.1[d]			N.D.	
	4/40 °C	43.4±0.5[b]	54.1±0.4[c]	67.2±0.1[ab]	2.9±0.1[c]				
	4/60 °C	49.3±0.3[a]	57.9±0.3[a]	67.4±0.5[a]	3.3±0.1[b]				

[1)] T_o, T_p, T_c and ΔH indicate the onset, peak and conclusion temperature, and enthalpy change of melting of melting, respectively.

2) Not detected.

3) The values with different superscripts in a same column are significantly different ($p<0.05$).

Table 5 DSC parameters of AS-treated starches.

Samples		Peak Ⅰ				Peak Ⅱ			
		T_o (°C)[1)]	T_p (°C)	T_c (°C)	ΔH (J/g)	T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)
0 d		70.8±1.1[c,2)]	93.5±0.3[a]	101.8±1.8[d]	5.88±0.4[f]	136.2±1.6[c]	144.8± 1.4[ab]	147.3±1.2[c]	3.7±0.2[d]
6 d	4 °C	72.6±0.8[c]	93.6±1.7[a]	106.5±0.9[c]	7.0±0.5[e]	137.0±0.7[bc]	144.9±0.7[b]	147.6±0.6[bc]	5.4±0.4[c]
	4/20 °C	74.0±1.8[bc]	93.5±0.0[a]	108.4±0.4[bc]	7.0±0.5[d]	138.0±0.8[bc]	144.9±1.2[ab]	146.4±2.9[c]	5.7±0.6[c]
	4/40 °C	75.6±1.2[ab]	93.7±0.28[a]	109.3±1.5[c]	9.3±0.2[bc]	137.7±1.2[bc]	144.2±0.5[ab]	148.2±2.5[bc]	6.3±0.3[bc]
	4/60 °C	76.5±3.2[ab]	93.9±0.1[a]	113.67±0.2[a]	10.6±0.5[a]	138.6±2.1[bc]	143.3±1.5[b]	148.1±0.6[bc]	7.1±1.5[ab]
14 d	4 °C	76.0±1.4[ab]	93.1±0.5[a]	107.7±0.7[c]	8.9±0.5[cd]	137.3±0.7[bc]	144.1±0.7[ab]	148.1±0.4[bc]	6.3±0.3[bc]
	4/20 °C	76.3±1.8[ab]	93.5±0.1[a]	108.1±3.4[c]	8.7±0.7[cd]	142.6±1.5[a]	145.2±0.9[ab]	148.2±1.1[bc]	6.4±0.3[bc]
	4/40 °C	77.1±0.7[ab]	93.7±0.2[a]	112.8±0.2[ab]	9.9±0.7[ab]	141.4±1.5[a]	145.7±1.1[b]	151.1±0.5[a]	6.5±0.5[bc]
	4/60 °C	77.6±0.8[a]	93.8±0.1[a]	113.6±1.2[ab]	10.8±0.7[a]	143.0±0.8[a]	145.0± 0.9[ab]	151.8±0.4[a]	7.7±0.8[a]

[1)] T_o, T_p, T_c and ΔH indicate the onset, peak and conclusion temperature, and enthalpy change of melting of melting, respectively.

[2)] The values with different superscripts in a same column are significantly different ($p<0.05$).

5. Solid-state ^{13}C cross-polarization and magic-angle spinning (CP/MAS) nuclear magnetic resonance (NMR) spectra

The ^{13}C CP/MAS NMR spectra are shown in Figure 3. There were no striking differences in NMR spectra of control starches. In the C-1 region of AS-treated starches, where the peak was slightly shifted right to low field compared to the 0 d. These differences reflected the increase in ordered (double helix) regions of modified starches by the AS-treatment (Gidley & Bociek, 1985). The shortest A chains may disturb the formation of an ordered crystalline structure, while the longer B chains tend to form double helices which constitute crystalline region (Jane et al., 1999). By the AS-treatment, the elongated chains formed double-helical structure. Besides the temperature cycling induced reassociation of AM chains and rearrangment of starch molecules into highly ordered structure.

The proportion of ordered (double-helical) structure was represented in Table 6. In control starches, the proportion of ordered structure of the 4 °C sample slightly increased after 6 days. The AS-treated starches increased proportion of ordered structure with increasing retrogradation time. Change in the proportion of ordered structure obtained by ^{13}C CP/MAS NMR was higher than that of relative crystallinity obtained by X-ray diffraction method.

It is in accordance with the results of thermal properties (Table 4). All crystallites were not detected by X-ray diffraction method. It suggested that crystallites irregularly arrayed could build the ordered crystalline structure during retrogradation. Gidley and Bociek (1985) reported that X-ray diffraction dose not detect irregularly packed structures. This result was in agreement with a previous study (Atichokudomchai et al., 2004).

Thus, the AS-treated starch retrograded by temperature cycling contained double-helical structure and it was compact and rigid, which might cause an increase of RS in the digestion property.

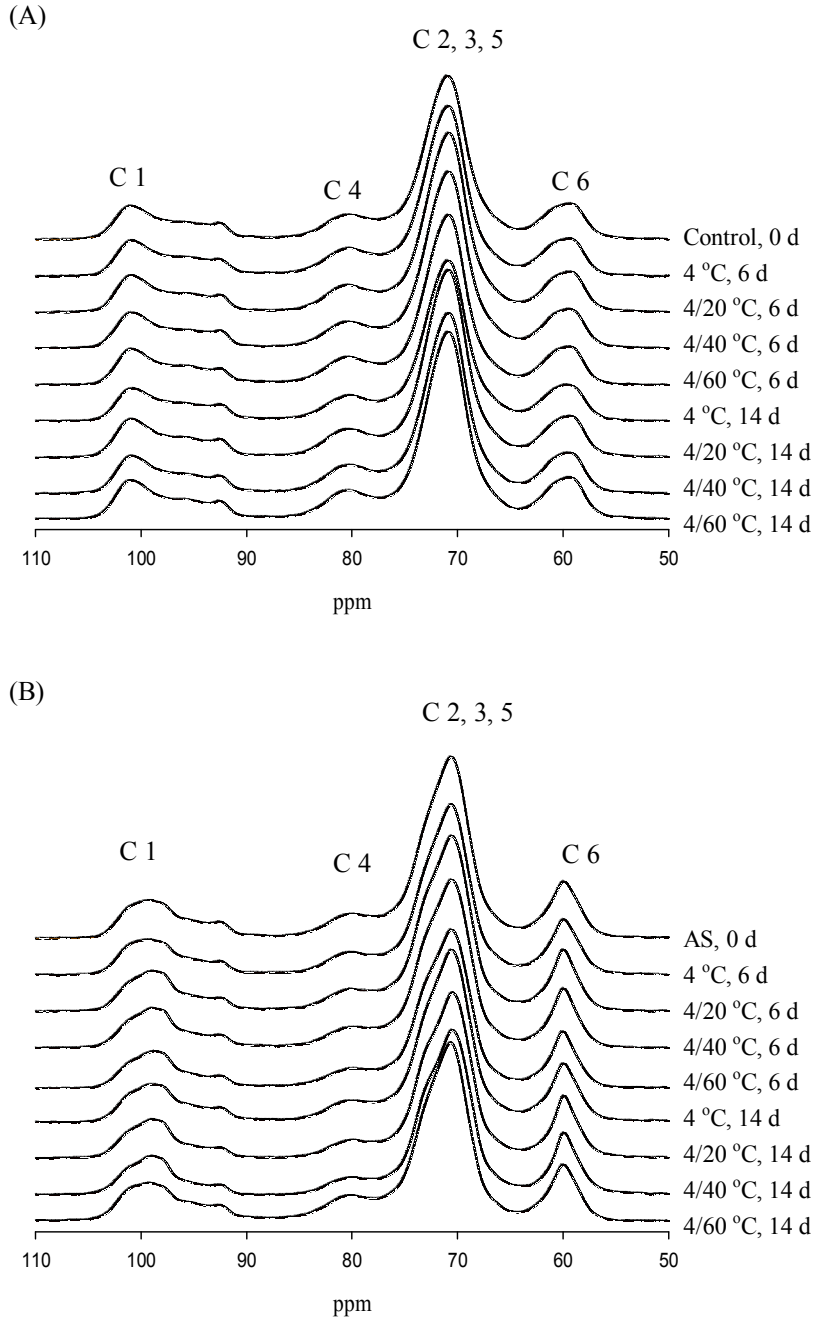


Figure 3. ^{13}C CP/MAS NMR spectra of starches. (A): control starch, (B): AS-treated starch

Table 6. The proportion of ordered structure of starches.

Samples		Ordered[1)]	Amorphous[2)]	O/A ratio[3)]
Control starches				
0 d		47.9	52.1	0.9
6 d	4 °C	49.3	50.8	1.0
	4/20 °C	48.9	51.1	1.0
	4/40 °C	50.3	49.8	1.0
	4/60 °C	49.9	50.1	1.0
14 d	4 °C	51.3	48.7	1.1
	4/20 °C	50.0	50.0	1.0
	4/40 °C	50.4	49.6	1.0
	4/60 °C	51.1	49.0	1.0
AS-treated starches				
0 d		55.2	44.8	1.2
6 d	4 °C	56.3	43.7	1.3
	4/20 °C	56.8	43.3	1.3
	4/40 °C	57.3	42.7	1.3
	4/60 °C	57.9	42.1	1.4
14 d	4 °C	58.0	42.0	1.4
	4/20 °C	59.8	40.2	1.5
	4/40 °C	60.3	39.7	1.5
	4/60 °C	62.7	37.3	1.7

[1)] The proportion of ordered structure.

[2)] The proportion of amorphous structure.

[3)] The ratio ordered structure to amorphous structure.

6. Starch digestibility

Figure 4 shows enzymatic hydrolysis patterns of starches. The most striking result was hydrolysis pattern of the 4/60 °C sample of AS-treated starches. The 4/60 °C sample exhibited digestibility of 13.0, 14.6, 19.2, 24.2, 32.0, and 43.9% at 10, 20, 30, 60, 120, and 240 min, respectively. The digestion rate decreased under cycled temperature storage between 4 and 60 °C.

The *in vitro* digestibilities of starches are exhibited in Table 7. It is well known that in vitro digestibility of starches by α-amylase is affected by such factors as molecular associations between starch components (Dreher et al., 1984), crystalline structures (Planchot et al., 1997), and granule size (Vandeputte et al., 2003).

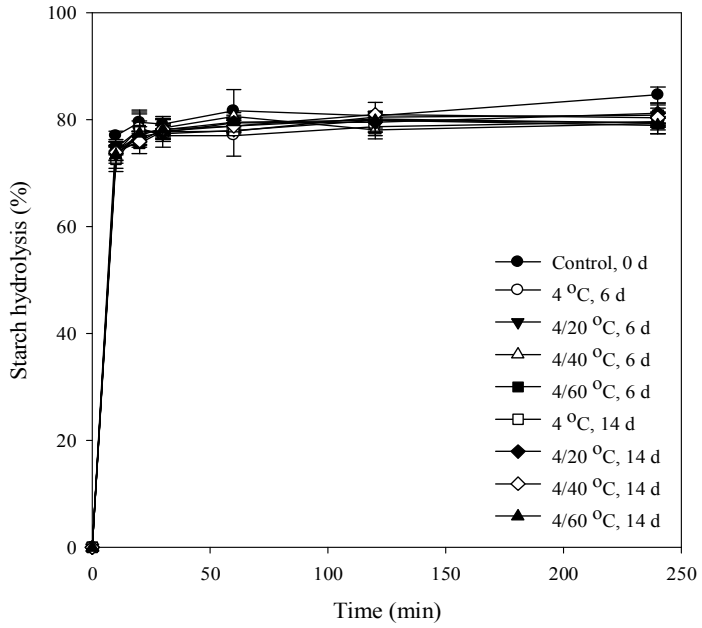
The 0d sample of control starches had the highest RDS content, because the gelatinization by autoclaving during the sample preparation destroyed the semicrystalline structure of raw starch granules (Cousin et al., 1996; Zhang et al., 2006). The amorphous regions because of gelatinization are easier to access by enzyme (Zhang et al., 2006). After retrogradation for 6 days, all starches showed an increase in the proportion of RS. Among the samples, the cycling of temperature between 4 °C and 60 °C induced the greatest

proportion of RS. Park et al. (2009) reported that temperature cycled storage induced the formation of a greater amount of RS and a much lower GI for waxy corn starch gels. The RS content is primarily related to the intensity of the crystalline matrix in retrograded starch gel (Chung et al., 2006). From 6 days to 14 days, starch digestibility of control starches did not show any significant change.

In AS-treated starches, only the 4/40 °C and the 4/60 °C samples showed significant changes. SDS decreased and RS increased whereas the RDS content was kept fairly constant as the retrogradation time increased. This could indicate that the contents of SDS were converted to proportion of RS. For AP with a high proportion of long B chains, starch retrogradation is related to its slow digestion property through a speculated anchoring effect of crystallites formed by outer A chains and some short B chains (mainly B_1 chains) on the longer chains (B_2-B_4) that comprise the main portion of SDS. This type of SDS is a physical entity that is time-dependent as longer time retrogradation will lead to the formation of RS (Shin et al., 2004; Zhang et al., 2008). Also, RS is primarily correlated to the AM content in starch (Haralampu, 2000). The elongated branch chains of AP by the AS treatment behave like linear chains of AM (Rolland-Sabaté et al., 2004). In this study, by the elongation mechanism of AS, enzymatically modified starches had longer branched chain length (Table 1). The double helices could be formed

by elongated branch chains, which could contribute to the crystalline structure in the AS-treated starch. It is possible that the crystalline conformation, including stability and perfection of the crystalline matrices, affected the resistance of the retrograded starch to enzyme digestion (Park et al., 2009). After the AS-treatment, successive treatment by temperature cycling induced the reassociation of AM-like chains and formation of double helices and crystalline structures. The crystallites formed under a propagation temperature of 60 °C were further perfected as compared with the constant temperature storage at 4 °C. Therefore, this result indicated that temperature cycling could accelerate retrogradation.

(A)


Starch hydrolysis (%)
Time (min)
Control, 0 d
4 °C, 6 d
4/20 °C, 6 d
4/40 °C, 6 d
4/60 °C, 6 d
4 °C, 14 d
4/20 °C, 14 d
4/40 °C, 14 d
4/60 °C, 14 d

(B)

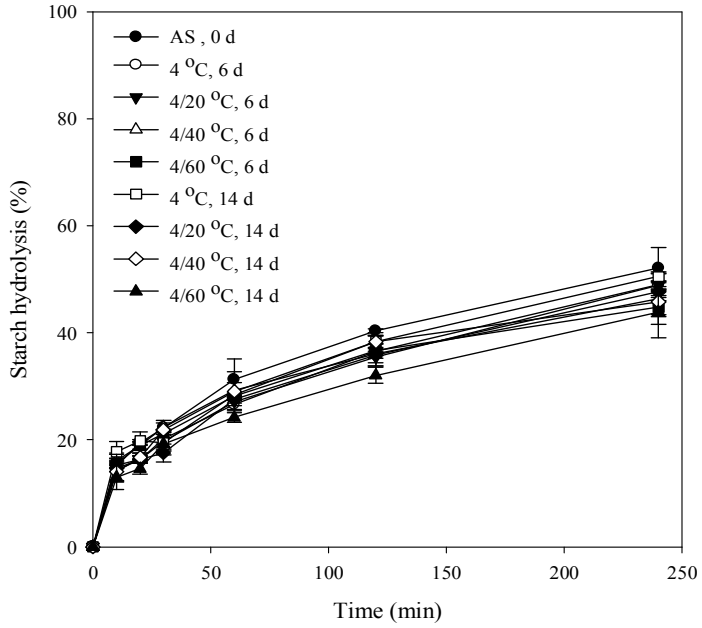

Starch hydrolysis (%)
Time (min)
AS , 0 d
4 °C, 6 d
4/20 °C, 6 d
4/40 °C, 6 d
4/60 °C, 6 d
4 °C, 14 d
4/20 °C, 14 d
4/40 °C, 14 d
4/60 °C, 14 d

Figure 4. Hydrolysis patterns of starches. (A): control starches with no retrogradation (●), isothermal retrogradation at 4 °C for 6 d (○), cycled retrogradation at 4/20 °C for 6 d (▼), cycled retrogradation at 4/40 °C for 6 d (▽), cycled retrogradation at 4/60 °C for 6 d (■), isothermal retrogradation at 4 °C for 14 d (□), cycled retrogradation at 4/20 °C for 14 d (◆), cycled retrogradation at 4/40 °C for 14 d (◇), and cycled retrogradation at 4/60 °C for 14 d (▲), (B): AS-treated starches with no retrogradation (●), isothermal retrogradation at 4 °C for 6 d (○), cycled retrogradation at 4/20 °C for 6 d (▼), cycled retrogradation at 4/40 °C for 6 d (▽), cycled retrogradation at 4/60 °C for 6 d (■), isothermal retrogradation at 4 °C for 14 d (□), cycled retrogradation at 4/20 °C for 14 d (◆), cycled retrogradation at 4/40 °C for 14 d (◇), and cycled retrogradation at 4/60 °C for 14 d (▲).

Table 7. Contents of RDS, SDS, and RS of starches.

Samples		RDS (%)	SDS (%)	RS (%)
Control starches				
0 d		77.0±0.8$^{a,1),2)}$	7.6±2.0^{a}	15.4±1.5^{c}
6 d	4 °C	73.8±1.4^{b}	5.9±0.9^{a}	20.4±0.6^{ab}
	4/20 °C	74.7±0.6^{ab}	4.7±2.1^{a}	20.6±2.0^{ab}
	4/40 °C	74.3±0.8^{ab}	5.0±1.2^{a}	20.7±2.0^{ab}
	4/60 °C	74.7±1.1^{ab}	4.6±1.6^{a}	20.7±1.3^{ab}
14 d	4 °C	72.5±2.3^{b}	8.25±4.0^{a}	19.2±1.9^{ab}
	4/20 °C	73.6±1.3^{b}	8.9±3.2^{a}	18.8±1.9^{bc}
	4/40 °C	73.9±2.0^{b}	6.5±1.9^{a}	19.7±1.8^{ab}
	4/60 °C	73.3±2.5^{b}	5.6±2.2^{a}	21.1±0.3^{a}
AS-treated starches				
0 d		15.1±0.4^{ab}	37.0±3.7^{a}	48.0±3.8^{c}
6 d	4 °C	15.3±1.2^{ab}	33.7±0.9^{ab}	51.0±2.1^{abc}
	4/20 °C	14.2±1.9^{b}	34.6±0.7^{ab}	51.2±2.3^{abc}
	4/40 °C	16.1±1.4^{ab}	31.0±1.9^{b}	53.6±3.3^{b}
	4/60 °C	15.6±0.9^{ab}	29.2±2.3^{b}	55.2±3.2^{ab}
14 d	4 °C	17.8±1.9^{a}	32.8±1.8^{ab}	49.5±0.9^{bc}
	4/20 °C	14.7±2.6^{ab}	32.9±0.9^{ab}	52.4±1.7^{abc}
	4/40 °C	14.0±1.8^{b}	31.9±0.9^{ab}	54.2±2.4^{ab}
	4/60 °C	13.0±2.3^{b}	30.9±2.8^{b}	56.1±4.8^{a}

[1)] Data are expressed as average value and standard deviation.

[2)] The values with different superscripts in a same column are significantly different (p<0.05).

CONCLUSION

This study showed the effects of retrogradation by temperature cycling on the structural and digestibility properties of AS-treated waxy corn starch. The long branch chains of AP elongated by AS, like AM, could bind iodine as much as normal corn starch. In case of control starches, with increasing cycled temperature, relative crystallinity and melting enthalpy decreased due to the melting of unstable crystallites formed under temperature cycling. With an increase in retrogradation time, relative crystallinity and onset temperature and melting enthalpy increased.

After retrogradation, all AS-treated starches exhibited a B-type polymorph, increased relatively crystallinity and melting enthalpy, and proportion of ordered structure. It was due to the recrystallization between double helices formed of elongated branch chains. Particularly, the 4/60 °C, 14 d sample of AS-treated starch showed a much higher relative crystallinity, melting enthalpy, and proportion of ordered structure compared with the other samples. These results suggested that temperature cycling induced formation of double helices and more perfect crystallites, leading to highly ordered crystalline structure. This structure was compact and rigid and caused an increase of RS content. Also, the change in the proportion of ordered

structure was higher than that of relative crystallinity. In the light of this result, the content of irregularly packed structures increased after retrogradation of AS-treated starch.

Overall data showed that the temperature cycling induced different retrogradation behavior compared with isothermal storage. Also, there were more changes in structural and digestibility properties of AS-treated starches than those of control starches under temperature cycling conditions. These findings suggest that temperature-cycled retrogradation is one of promising technology for producing RS in food industry.

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국문 초록

아밀로수크레이스 (AS) 처리 후 각각 다른 온도로 사이클 또는 등온 저장한 찰 옥수수 녹말의 구조 및 소화 특성의 변화를 조사하였다. AS 처리로 연장된 아밀로펙틴 사슬의 결정화를 가속화하기 위해 다양한 노화 조건을 사용하였다. 온도 사이클링은 4 °C 에서 1 일 저장하고 20, 40, 또는 60°C 에서 1 일 저장하여 3 회, 7 회 반복하였고 등온 저장은 4°C 에서 14 일 동안 보관하였다. AS 처리 녹말의 특성은 이온크로마토그래피, 아이오딘 결합력으로 파악하였다. 녹말의 노화 특성은 X-선 회절, 시차주사 열량계, 탄소-13 핵자기공명 분광, 소화율을 측정하여 알아보았다. AS 처리 후 짧은 사슬 분포는 감소하였고 반면에 긴 사슬 분포는 증가하였다. 또한 아이오딘 결합력은 일반 옥수수 녹말과 비슷한 값을 나타내었다. 14 일 노화 후에 모든 AS 처리 녹말의 X-선 회절도형은 B 형을 나타내었다. 대조군 녹말의 경우, 4 °C 에서 저장한 녹말에 비해서 온도 사이클링에서 저장한 녹말이 더 낮은 상대적 결정화도와 용융 엔탈피값을 나타내었다. 이는 높은 온도에서 저장 중에 불안정한 결정이 용융되었기 때문이다.

규칙적인 구조의 비율에서는 변화가 적었다. 반면에, AS 처리 녹말은 온도 사이클링 조건에서 상대적 결정화도, 용융 엔탈피값, 규칙적인 구조의 비율, 저항녹말 함량이 등온 저장한 녹말 보다 더욱 증가하였다. 특히, 모든 녹말 중에서 4 °C 와 60 °C 에서 사이클링하여 노화시킨 녹말이 구조 및 소화 특성에서 가장 두드러진 변화를 보였다.

이 실험을 통해, 온도 사이클링은 AS 처리로 연장된 사슬의 재회합을 유도하여 노화를 가속화한다는 것을 증명하였다. 또한 온도 사이클링에 의한 노화는 대조구 녹말 보다 AS 처리 녹말의 구조 및 소화 특성에 더 많은 변화를 가져왔음을 확인하였다.

주요어 : 아밀로수크레이스, 찰옥수수 녹말, 노화, 온도 사이클링, 소화율, 구조적 특성

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