A Thesis for the Degree of Master of Science

Structural properties and *in vitro* digestibility of amylosucrase treated waxy corn starch affected by repeated retrogradation

반복노화처리가 아밀로수크레이스 처리한 찰 옥수수 녹말의 구조 및 소화 특성에 미치는 영향

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농학석사학위논문

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Structural properties and *in vitro* digestibility of amylosucrase treated waxy corn starch affected by repeated retrogradation

by

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ABSTRACT

The structural characteristics and *in vitro* digestion properties of AS-treated waxy corn starch that underwent repeated retrogradation were investigated in this study. Various retrogradation conditions were used to accelerate crystallization of amylopectin chains elongated by AS. The retrogradation conditions were as follows: gelatinized starch gel was stored at 4 °C for 1 or 5 d to perform single-, double-, and triple-retrogradation treatment. AS control starch underwent the same procedure for comparison. High-performance anion-exchange chromatography was used to analyze the branch chain length distribution of AS-treated starches. The structural characteristics of starches after repeated retrogradation were determined by X-ray diffractometry and differential scanning calorimetry. *in vitro* digestibility was also evaluated in order to assess the effects of different crystal structures on their digestion properties. After the AS treatment, the proportion of short chains (DP 6-12) decreased, whereas that of long chains (DP 25-36 and DP ≥ 37) increased. The X-ray diffraction patterns of all AS-treated starches displayed a B-type polymorph, higher relative crystallinity (%), and greater melting enthalpy after repeated retrogradation. Regarding *in vitro* digestibility, starches treated with repeated retrogradation showed a

higher yield of RS and a lower kinetic constant. These results were more prominent in AS-treated starches with 5 d interval of repeated retrogradation. Conclusively, this study demonstrated that repeated retrogradation could induce reassociation of chains elongated by AS-treatment, thereby accelerating starch retrogradation. It is suggested that repeated retrogradation brings more changes in structural properties and digestibility of AS-treated starch than those of control starch. Also, different crystal structures induced by repeated retrogradation affect the digestion properties. These findings suggest that repeated retrogradation is one of promising technologies for developing industrial RS products in the food industry.

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

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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 polysaccharea*

RR: repeated retrogradation

SR: single-retrogradation

DR: double-retrogradation

TR: triple-retrogradation

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INTRODUCTION

Starch, the most important reserve polysaccharide, is the main source of carbohydrates in human diet. It consists of two polymers: amylose and amylopectin. Amylose (AM) is essentially long linear chains of (1→4)-linked α-D-glucopyranose residues, whereas amylopetin (AP) has a large molecular weight and a highly branched structure consisting of much shorter chains of (1→4)-α-D-glucose residues with (1→6)-α-D-glucosidic branch linkages (Sajilata et al., 2006).

For nutritional purpose, a classification system to describe starch digestion property was introduced by Englyst et al. (1992). Rapidly digestible starch (RDS) is the fraction digested within first 20 min in the mouth and the small intestine, leading to a rapid increase followed by an equally rapid drop in blood glucose level. Slowly digestible starch (SDS) is the fraction digested between 20 min and 120 min, showing slow but complete digestion in the small intestine and a prolonged postprandial glucose release profile (Seal et al., 2003). The fraction that is not digested after 120 min but can be fermented in the large intestine is referred to as resistant starch (RS). 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). The RS may be beneficial in the food products for the following reasons: RS is a good substrate for fermentation, which gives rise to an increase in short-chain fatty acid production. It is also associated with several changes in metabolism. RS intake decreases postprandial glycemic and insulinemic responses, improves body insulin sensitivity, increases satiety, and reduces fat storage (Higgins et al., 2004). Therefore, RS can be an attractive dietary target for prevention of certain common chronic diseases such as diabetes and coronary heart diseases as well as the development of weight loss diets.

However, the designation of a starch fraction as RDS, SDS, or RS based on the certain time frames of a digestibility curve does not give an accurate estimate of the enzyme process of starch hydrolysis. Instead, the rate of reaction decreases with time and a plot of the concentration of product formed versus time is logarithmic. Therefore, all digestible fractions have the same intrinsic reactivity, and the substrate decay process fits the standard first-order equation. This method can be used to investigate the kinetics of starch digestion (Goñi et al., 1997; Zhang et al., 2013), which allows an estimation of the rate constant k and the total starch digested C_{∞}.

A few of recent studies introduced the modification of gelatinized starches with recombinant amylosucrase (EC 2.4.1.4., AS) from *Neisseria polysaccharea* (Kim et al., 2013; Kim et al., 2014; Shin et al., 2010). AS

catalyzes a transglycosylation reaction to yield 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 the proportion of the branch chains with short and medium length decreases and that the proportion of long branch chains increases (Park et al., 2013; Shin et al., 2010). Accordingly, it could induce reassociation of chains during reaction and decrease the susceptibility to digestive enzymes, resulting in higher SDS and RS contents (Kim et al., 2014). Therefore, in this study, AS was used to increase the proportion of long chains of waxy corn starch on the basis of a premise that the slow digestion property of the starchy materials could be manipulated according to its AP fine structure.

When heated in water, starch granules become hydrated, swell, and transform into a paste. The granule structure is destroyed due to the melting of crystallites, followed by unwinding of double helices and breaking of hydrogen bonds. These changes are collectively referred to as starch gelatinization (Wang et al., 2015). 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, including 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) (Silverio et al., 2000). Starch retrogradation is the main means of physical modification used to achieve low-glycemic index (GI) benefits in cooked and processed starchy foods (Hamaker, 2007). The process is desirable in some applications due to the modification of the structural, mechanical, and sensory properties. Breakfast cereals, parboiled rice, dehydrated mashed potatoes, and Chinese rice vermicelli are the examples of food products manufactured by using starch retrogradation (Karim et al., 2000). It is also desirable in terms of nutritional significance, due to the slower enzymatic digestion and moderate release of glucose into the blood stream (Copeland et al., 2000, Wang et al., 2013).

Xie et al. (2014) applied starch crystallization theory to develop a method to accelerate retrogradation by introducing the term, repeated retrogradation. The effects of repeated retrogradation treatment on the structural characteristics and *in vitro* digestibility of starch samples have been investigated. The structural changes of starch samples treated with

different cycling times of repeated retrogradation significantly affect the digestibility, and repeated retrogradation treatment has been further applied in other studies to different source of starch samples (Hu et al., 2015; Hu et al., 2014). Therefore, in this study, repeated retrogradation treatment was performed in order to induce crystallization of the chains of waxy corn starch elongated by AS. Consequently, the structural characteristics as well as *in vitro* digestion properties of AS-treated starch that underwent repeated retrogradation were investigated.

MATERIALS AND METHODS

1. Materials

1-1. Starch

Waxy corn starch was obtained from Ingredion (Westchester, IL, USA).

1-2. Enzymes

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

Isoamylase (activity 1,000U) was obtained from Megazyme (Bray, Ireland). Pancreatin (P7545, activity 8 × USP/g) and amyloglucosidase (AMG 300L, activity 300 AGU/mL) were from Sigma Chemical Co. (St. Louis, MO, USA) and Novozymes (Bagsvaerd, Denmark), respectively. GOD-POD assay kit was from Embiel Co. (Gunpo, Korea).

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 shaking 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 by consumption of sucrose..

2-2. Preparation of AS-treated starch

Starch suspension (2%, w/w) was prepared by dispersing waxy corn starch in 100 mM sodium acetate buffer (pH 7.0) with 100 mM sucrose to reach the final volume of 150 mL. The 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 shaking water bath at

30 °C, 80 rpm for 24 h. The enzyme reaction was stopped by adding three-fold ethanol to the suspension, and the AS-treated starch was precipitated by centrifugation at 10,000 xg for 10 min. The pellet was washed off three times with distilled water by centrifugation at 10,000 xg for 10 min. The precipitate was freeze-dried, ground and passed through a 100-mesh sieve to be used as a sample. Control starches were prepared by undergoing the same procedure without addition of AS.

2-3. Preparation of starch samples by repeated-retrogradation

Starch suspension (20%, w/w) was fully gelatinized by heating in boiling water for 30 min and autoclaving at 121 °C for 30 min. The gelatinized starch gel was cooled to room temperature, hermetically sealed and stored at 4 °C for 1 or 5 d to perform one cycle of retrogradation treatment. The retrograded samples were heated in boiling water for 30 min and autoclaved at 121 °C for 30 min again to undergo two cycles of retrogradation treatment with the time intervals of 1 or 5 d. Under the same conditions described above, starch samples were retrograded to perform three cycles of retrogradation treatment with the time intervals of 1 or 5 d. AS control starch underwent the same procedure for comparison. The samples were named according to the

cycling times of repeated retrogradation treatments of control (C0, CS1, CD1, CT1, CS5, CD5, CT5) and AS-treated samples (A0, AS1, AD1, AT1, AS5, AD5, AT5). Each sample after retrogradation process was freeze-dried and ground to pass through a 100-mesh sieve.

2-4. Determination of branch chain length distribution

The branch chain length distributions of control and AS-treated starch mixtures were determined by debranching the starch with isoamylase. Starch sample (15 mg) was dissolved in 90% dimethyl sulfoxide (3 mL) and boiled for 30 min. Ethanol (15 mL) was added to the starch suspension to precipitate starch, and then the sample was centrifuged at 10,000 xg for 10 min. The precipitated starch was boiled for 15 min with distilled water (1.5 mL), and then 1.5 mL of 50 mM sodium acetate buffer (pH 4.3) was added and boiled for another 20 min. After cooling to 45°C, isoamylase (30 μL) was added to the starch dispersion, and the sample was incubated in a shaking water bath (45°C, 30 rpm) for 2 h. 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 analyzed using 150 mM NaOH for column equilibration and 600 mM sodium acetate in 150 mM NaOH for sample elution with a flow rate of 1 mL/min with gradients 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. Peak areas were calculated using PeakNet software (version 5.11, Dionex).

2-5. X-ray diffraction patterns and relative crystallinity

X-ray diffraction analysis was performed 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 and Komiya, (1983). The area was calculated using the software developed by the instrument manufacturer (EVA, 2.0).

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

2-6. Thermal properties

Thermal properties of control samples and AS-treated samples were examined 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 24 h for an equilibrium. An empty aluminum pan was used as a reference, and indium was used for calibration. DSC scan was performed from 30 °C to 170 °C with a rate of 10 °C/min. The onset temperature (T_o), the peak temperature (T_p), the conclusion temperature (T_c), and the melting enthalpy ($\triangle H$) were recorded.

2-7. Starch digestibility

2-7-1. Degree of starch hydrolysis

Starch digestibility was determined by the method of Brumovsky and Thompson (2001) with slight modification. Pancreatin (6 g) was dissolved in distilled water (72 mL) with magnetic stirring for 10 min. The suspension was precipitated by centrifugation at 1,500 xg for 10 min. After mixing the supernatant (60 mL) with amyloglucosidase (1.2 mL) and distilled water (10.8 mL), the solution was kept in a water bath at

37 °C for 10 min.

The starch sample (30 mg) was weighed into a 2 mL-microtube and suspended with sodium acetate buffer (0.75 mL, 0.1 M, pH 5.2) with one glass bead. After vortexing each microtube, the sample dispersion was equilibrated in a shaking incubator (240 rpm, 37 °C) for 10 min. Then prepared enzyme solution (0.75 mL) was added to each tube, and the starch sample was incubated in a shaking incubator (240 rpm, 37 °C). The microtubes were removed at certain times and boiled in a cooker for 10 min to stop the reaction.

The glucose released by hydrolysis of starch was obtained from the supernatant after the centrifugation at 5,000 xg for 10 min. Then the glucose content was determined by the glucose oxidase method (Karkalas, 1985) using a commercially available kit (Embiel Co., Gunpo, Korea).

2-7-2. Determination of starch fractions using the Englyst method

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

2-7-3. Determination of starch fractions using first-order equation

The digestibility curves of starch can be fitted to the standard first-order equation (Goñi et al., 1997):

$$C = 1 - e^{-kt}$$

where t is the digestion time, C is the fraction of digested starch at digestion time t, and k is the digestion rate constant($\min^{-1}$).

The equation can be expressed in logarithmic form as follows:

$$\ln(1 - C) = -kt$$

The plot of ln (1 – C) against t is linear graph with a slope of **-** k. At the late stage of digestion, the C was not significantly different in the degree of hydrolysis. Therefore, the experimental points in that region were excluded for the digestibility curves of starch using standard first-order equation and determined as RS region.

2-8. 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 determined by HPAEC-PAD are presented in Figure 1 and Table 1. In general, branch chains of AP are categorized into four groups, which are 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 relatively higher proportion of short chains such as A and B_1 chains but a lower proportion of longer chains (Zhang et al., 2006). Shin et al. (2010) reported that an increase in the chain length of AP and a decrease in the proportion of short chains were induced by AS treatment. In this study, the highest peak was shifted to the right compared with that of AS control (Figure 1). After AS treatment, the proportion of A chain (DP $\leq$ 12) decreased by 80%, while the proportions of both B_2 chain (DP 25-36) and B_3 chain (DP $\geq$ 37) increased by 87% and 79%, respectively, compared with those of control. These results were due to the short chains that are located outside the cluster structure, which are readily accessible by AS (Kim et al., 2014). Potocki de Montalk et al. (2000) reported that

AS catalyzes the elongation of branch chains of AM and AP by attaching 12 to 18 glucosyl units at non-reducing ends.

Double helices formed by the extended branch chains by AS treatment could hinder the access of hydrolytic enzymes, thus inhibit the digestion by hydrolytic enzymes. 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).

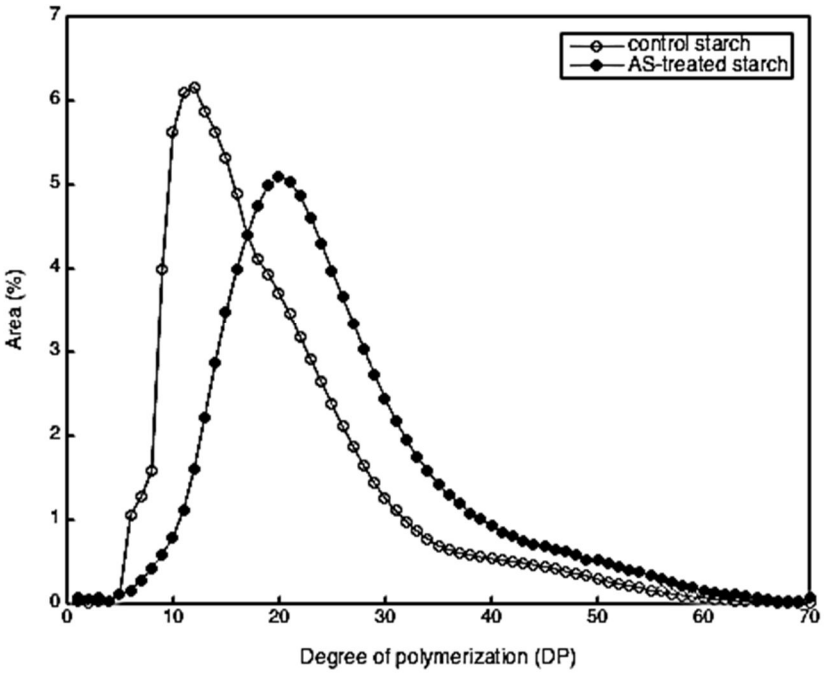


Figure 1. Branch chain length distributions of control starch (○) and AS-treated starch (●).

Table 1. Branch chain length distributions of AS-treated starches

Sample	Percent distribution (%)				
	DP[1)] ≤ 5	DP 6-12	DP 13-24	DP 25-36	DP ≥ 37
Control starch[2)]	0.22±0.04[a4),5)]	25.77±0.86[a]	50.00±0.30[a]	15.72±0.22[b]	8.28±0.45[b]
AS-treated starch[3)]	0.32±0.07[a]	4.96±0.17[b]	50.57±0.56[a]	29.35±0.34[a]	14.79±0.35[a]

[1)] DP, degree of polymerization.

[2)] Control starch denotes the starch without enzymatic treatment.

[3)] AS-treated starch denotes the starch treated 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 the same column are significantly different (p<0.05).

2. X-ray diffraction patterns and relative crystallinity

The X-ray diffraction patterns of the starches are displayed in Figure 2. The corresponding X-ray diffraction parameters and crystallinity level calculated from the diffraction peak area and total diffraction area are given in Table 3.

Control starches did not show any noticeable major peak, due to the complete gelatinization during sample preparation. With increasing retrogradation time, however, the peaks from 14° to 17° started to develop. CT1 and CT5 samples exhibited stronger peaks compared with other control samples. It is reported that retrograded normal and waxy corn starches displayed a B-type X-ray pattern after further retrogradation (Zhou and Lim, 2012). Therefore, it can be assumed that control starch could have a B-type X-ray pattern after repeated retrogradation.

The AS-treated starch, on the other hand, showed a B-type X-ray pattern with reflection intensities at values of 5.5°, 14.5°, 17°, 19.3°, 22°, and 24°, which corresponded to the previous studies (Kim et al., 2013; Kim et al, 2014; Shin et al., 2010). Branch chain elongation resulting from AS action facilitated and solidified the inter-chain association, which in turn led to a stable B-type polymorph (Ryu et al., 2010). The B-type X-ray pattern was

maintained after RR-treatment. A similar result was reported in the work of Zhou et al. (2012) where retrograded normal and waxy corn starches had a B-type configuration, which was typical for retrograded starches. It has also been reported that retrograded starch shows a B type pattern, which is induced from the regions composed of double helices in a hexagonal structure (Kim et al., 2009). Whether starch has an A or B type pattern in the nature, starch gels display a B type pattern on retrogradation (Katz, 1934). It is 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 a B type pattern. RR-treated AS starches with the time interval of 5 d showed a strong diffraction peak at 5.5°, and all RR-treated AS starches presented a strong diffraction peak at 17°. The increased intensity was attributed to double helices packing more efficiently in the crystallites (Miao et al., 2010).

The relative crystallinity of control starches ranged from 15.50% to 20.20% owing to the complete gelatinization prior to the enzyme reaction. The relative crystallinity of AS-treated starches gradually increased from 33.06% to 40.40% with RR-treatment, showing the highest crystallinity in AT5 sample. It could be speculated that elongated branch chains formed by AS-

treatment led to the formation of double helices, which induced formation of crystallites during enzyme reaction, and therefore increased the relative crystallinity of AS-treated starches. Regarding SR (single retrogradation)-treated samples of control and AS-treated starches (CS1 to CS5, and AS1 to AS5), the relative crystallinity increased by 8.9% and 12%, respectively. During the increased retrogradation time from 1 d to 5 d, more ordered structures within the crystalline domains could be formed. Also, some loose molecular structures could have been arranged and led to the formation of more imperfect crystallites both in the crystalline and amorphous regions, resulting in an increase in relative crystallinity (Hu et al., 2014). Relative crystallinity of DR (double retrogradation) and TR (triple retrogradation)-treated control and AS-treated samples also increased. With regard to control starches CD5 and CT5, relative crystallinity increased by 11% and 18% respectively compared with CD1 and CT1. This gradual percentage rise in control starches with repeating cycle is correlated to the retrogradation time, therefore similar results were also found in DR and TR-treated samples. In AS-treated starches AD5 and AT5, the relative crystallinity increased by 12% compared with AD1 and AT1. Relative crystallinity is associated with 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). Therefore, higher relative

crystallinity due to RR-treatment indicated that its crystalline structure was more densely packed than that of the other samples

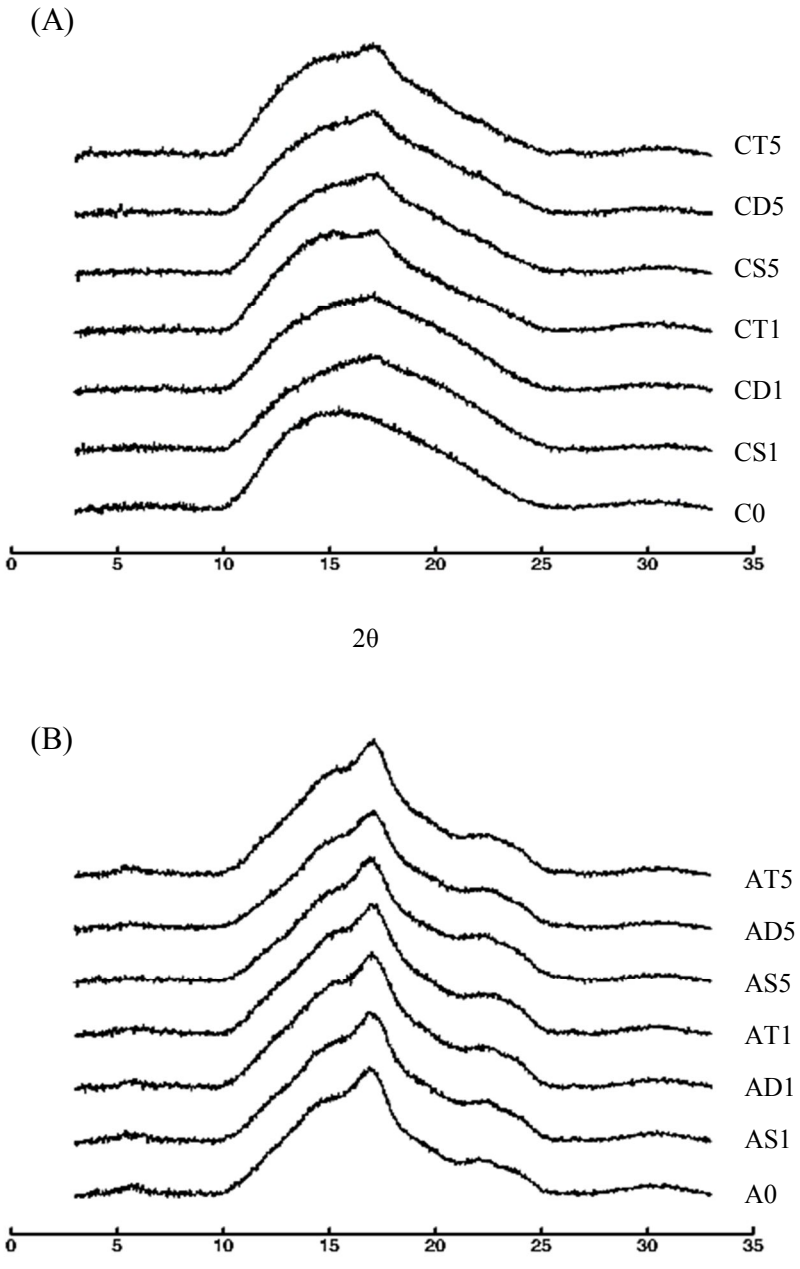


Figure 2. X-ray diffraction patterns of starches. (A): control starch, (B): AS-treated starch

Table 2. Relative crystallinity of starches

Sample	Relative crystallinity (%)
Control starches	
C0	15.86±0.23[c 1), 2)]
CS1	15.66±0.57[c]
CD1	15.50±0.43[c]
CT1	17.13±0.11[b]
CS5	17.06±0.11[b]
CD5	17.16±0.15[b]
CT5	20.20±0.26[a]
AS-treated starches	
A0	33.06±0.11[f]
AS1	35.00±0.00[e]
AD1	35.26±0.05[d]
AT1	36.06±0.11[c]
AS5	39.36±0.11[b]
AD5	39.33±0.15[b]
AT5	40.40±0.17[a]

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

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

3. Thermal properties

Thermal properties of control starches and AS-treated starches subjected to repeated retrogradation were determined using DSC. The onset temperature (T_o), peak temperature (T_p), conclusion temperature (T_c), and melting enthalpy (ΔH) are shown in Table 4 and Table 5. T_p represents structural stability. T_o and T_c are associated with melting of the weakest crystallites and strongest crystallites, respectively (Barichello et al., 1990; Biliaderis et al., 1980). ΔH reflects the overall crystallinity and is an indicator of the loss of molecular order within the granule (Hoove and Vasanthan, 1994).

The first peak indicates the melting of AP crystallites formed by aggregation between adjacent double helices during retrogradation, and the second peak indicates the melting of AP double helices of AS-treated starch within elongated long linear chains (Hoover et al., 1996).

The endothermic peak of C0 starch did not appear due to the disruption of double helices by complete gelatinization during the sample preparation. Other control starches only showed the first peak. T_o increased slightly with the retrogradation treatment, however, T_p, and T_c had no significant differences ($p > 0.05$). Also, ΔH value of SR-treated control samples

increased with increasing retrogradation time, suggesting that the repeated retrogradation treatment could result in the formation of more perfect and stable crystallites. A similar trend of ΔH values was also observed in DR and TR-treated starch samples, showing higher values in all 5 d-retrogradated starch samples. In general, starch retrogradation occurs in two stages. AM retrogradation is considered to be a rapid process completed within 48 h, whereas AP retrogradation continues 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).

Compared with control starches, all AS-treated starches displayed two distinctive peaks with increased thermal transition parameters (T_o, T_p, and T_c). In the first peak, T_p and gelatinization enthalpy increased with repeated retrogradation. Whereas in the second peak, T_p and gelatinization enthalpy significantly increased in CS1, CD1, and CT1 samples. During retrogradation process, the elongated chains of AP in the AS-treated starch behaved like long linear AM chains. Also, the enthalpy (ΔH) gradually increased with retrogradation time because of the formation of new double helices by the AS treatment. The enthalpy connected to melting of imperfect crystals is based on amylopectin, which contributes to both crystal packing and helix melting enthalpies (Lopez-Rubio et al., 2008). Also, the ΔH

increased as the retrogradation time increased, indicating continuous propagation of starch recrystallization. After repeated retrogradation with the intervals of 5 d, the samples had higher ΔH than the samples retrogradated with the invervals of 1 d. It is in accordance with the results of the relative crystallinity (Table 3). The lowest melting enthalpy (3.76 J/g) and relative crystallinity (35.0%) of AS1 sample indicated that amorphous regions and a small portion of imperfect crystallites were formed by SR treatment and that the double helical structures in the starch molecules were substantially disrupted. This was in accordance with the result of RR-treated waxy potato starch samples (Xie et al., 2014). The AT5 sample had the highest ΔH value of 7.15 J/g, suggesting that the re-association of starch molecules forms a stronger matrix or network, in which crystalline regions and more perfect crystallites exist. Therefore, repeated retrogradation induced recrystallization of chains, resulting in the formation of double helices of elongated chains of AP.

Table 3. DSC parameters of control starches

Sample	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)
C0	N.D.[2)]						N.D.	
CS1	41.29±0.42[a3)]	54.78±0.28[a]	65.52±5.73[b]	1.70±0.44[e]				
CD1	42.27±0.90[a]	56.49±1.86[a]	68.22±8.08[b]	2.14±0.27[e]			N.D.	
CT1	45.79±7.98[a]	55.65±1.23[a]	76.40±1.09[a]	2.73±0.15[d]				
CS5	46.27±0.97[a]	54.90±2.61[a]	64.97±3.99[b]	4.33±0.11[c]				
CD5	47.08±0.81[a]	55.98±0.20[a]	65.54±0.29[b]	5.16±0.27[b]			N.D.	
CT5	45.28±1.19[a]	54.91±0.41[a]	66.76±2.82[b]	6.35±0.11[a]				

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

[2)] Not detected.

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

Table 4. DSC parameters of AS-treated starches

Sample	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)
A0	80.63±1.49[ab2)]	89.52±0.87[d]	94.25±0.97[c]	1.21±0.17[c]	140.84±0.03[ab]	148.60±0.00[a]	153.28±1.02[a]	1.97±0.03[b]
AS1	83.62±1.40[a]	93.83±0.67[c]	109.23±1.30[b]	3.76±0.22[b]	140.53±3.04[ab]	140.97±3.00[b]	142.70±2.62[b]	0.39±0.07[c]
AD1	80.86±0.76[ab]	94.10±0.66[c]	118.30±10.47[ab]	3.97±0.44[b]	141.88±1.49[ab]	148.57±4.36[a]	154.78±3.06[a]	1.41±0.29[b]
AT1	77.09±6.56[b]	93.43±0.10[c]	97.25±1.65[c]	3.80±0.54[b]	143.35±0.17[a]	150.48±1.11[a]	154.77±0.93[a]	3.46±0.84[a]
AS5	77.87±0.24[b]	93.38±0.00[c]	117.79±6.00[ab]	5.82±0.17[ab]	141.59±1.17[ab]	149.87±0.19[a]	155.03±1.83[a]	1.52±0.13[b]
AD5	77.68±3.24[b]	97.18±0.25[a]	114.08±3.77[b]	5.35±0.29[ab]	144.01±0.87[a]	151.43±0.28[a]	155.85±0.36[a]	1.47±0.08[b]
AT5	77.84±1.48[b]	95.22±0.87[b]	124.59±0.87[a]	7.15±2.92[a]	138.27±3.81[b]	149.99±0.53[a]	154.56±1.81[a]	1.99±0.19[b]

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

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

4. Digestion pattern of RR-treated starches

The enzymatic hydrolysis pattern for control and AS-treated starch samples are displayed in Figure 3 and Figure 4, respectively. Degree of hydrolysis was measured from 0 to 360 min for control starch samples, and AS-treated starch samples were analyzed from 0 to 420 min to clarify the emergence of a plateau. Table 5 and Table 6 describe the degree of hydrolysis for control and AS-treated starch samples in detail.

As shown in Figure 3, no distinctive change was found in control starch samples. However, the time of plateau and C_∞ (maximum degree of hydrolysis) differed among control starch samples. C0, CS1, and CS5 samples reached a plateau at 10 min of digestion, whereas samples treated with repeated retrogradation exhibited a plateau after 15 min of digestion (CD1=15 min; CT1, CD5=20 min; CT5=30 min). C_∞ was obtained by measuring the average of the degree of hydrolysis values after reaching the plateau. Compared with samples treated with 1 d interval of repeated retrogradation, smaples treated with 5 d interval of repeated retrogradation showed a lower average C_∞ value of 85 % (Table 9). During the sample preparation process, the control starch samples lost its native granular

structure and semi-crystallinity, thus the time of equilibrium appeared at a quite early stage of the starch hydrolysis.

The equilibrium time for AS-treated starch samples were delayed to 240, and 300 min (240 min=AS1 and AD1; 300 min=A0, AT1, AS5, AD5, and AT5). The measured C_∞ values for A0, AS1, AT1 and AD1 were approximately 70 %. This value was decreased in AS5, AD5 and AT5 samples (67.08 %, 66.73 %, and 64.96 %, respectively). Compared with control starch samples, the AS treatment caused AS-treated starch samples to resist to the enzymatic hydrolysis.

After gelatinization process, the long linear chains undergo retrogradation, which is one of the mechanisms for the formation of slow digestion fraction of starches (Zhang et al., 2008). In other words, longer branch chains form strong, stable and long double helices, leading to a more perfect crystalline structure, whereas short or weak double helices formed by short chains produce imperfect crystalline structures (Jane et al., 1999). In this study, enzymatic modification with AS increased the proportion of long chains and decreased the proportion of short chains, rendering AS-treated starch samples to form relatively perfect crystallites.

Table 5. Degree of hydrolysis of control starch samples

Time (min)	Sample[1)]						
	C0	CS1	CD1	CT1	CS5	CD5	CT5
3	75.14±2.40$^{d2)}$	71.34±2.48^{c}	72.29±2.92^{f}	71.17±2.64^{e}	70.94±3.30^{c}	68.34±2.76^{d}	63.76±2.91^{f}
6	82.51±2.31^{c}	80.25±1.92^{b}	81.50±1.93^{e}	81.42±1.82^{d}	78.95±1.98^{b}	77.16±0.56^{c}	74.26±2.74^{e}
10	85.99±0.18^{abc}	84.53±1.77^{ab}	83.11±1.42^{de}	82.51±2.82^{cd}	83.28±3.03^{a}	82.87±2.36^{b}	81.39±2.28^{cd}
15	85.28±1.13^{bc}	84.02±1.02^{ab}	84.73±1.52^{abcde}	83.07±2.66^{bcd}	83.46±1.55^{a}	82.61±0.61^{b}	81.05±2.06^{d}
20	86.14±3.30^{abc}	85.15±2.19^{a}	82.30±0.71^{e}	84.01±0.49^{abcd}	83.36±1.63^{a}	84.76±1.74^{ab}	82.79±0.06^{bcd}
25	86.35±0.49^{abc}	86.70±1.24^{a}	83.09±1.16^{de}	85.43±0.98^{abc}	85.41±1.01^{a}	84.43±2.10^{ab}	82.49±0.58^{bcd}
30	85.82±3.33^{abc}	84.37±1.30^{ab}	83.74±2.29^{cde}	85.49±1.52^{abc}	85.34±0.79^{a}	85.66±2.58^{ab}	84.18±2.33^{abcd}
45	84.73±2.09^{bc}	86.05±1.22^{a}	84.97±1.54^{abcde}	87.67±2.57^{a}	87.75±2.28^{a}	82.92±0.67^{b}	84.20±1.63^{abcd}
60	85.53±1.72^{bc}	87.32±5.37^{a}	86.44±0.48^{abcd}	87.79±2.43^{a}	87.11±4.89^{a}	84.65±4.40^{ab}	84.89±3.41^{abcd}
90	87.90±2.06^{ab}	86.27±3.65^{a}	85.00±2.51^{abcde}	87.22±1.99^{a}	84.72±1.40^{a}	86.95±1.08^{a}	86.31±1.21^{ab}

Time (min)	Sample[1)]						
	C0	CS1	CD1	CT1	CS5	CD5	CT5
120	88.19±2.51^{ab}	86.07±4.65^{a}	86.71±5.03^{abcd}	85.68±3.16^{abc}	85.61±2.67^{a}	86.74±1.47^{a}	87.41±1.93^{a}
150	86.59±3.02^{abc}	87.42±2.41^{a}	87.56±1.34^{abc}	86.67±2.22^{ab}	85.86±2.69^{a}	86.23±1.67^{ab}	87.49±3.11^{a}
180	87.51±2.66^{ab}	86.46±3.22^{a}	88.90±1.32^{a}	85.64±1.27^{abc}	87.27±1.94^{a}	87.63±1.00^{a}	87.27±1.33^{a}
210	87.71±0.56^{ab}	88.38±0.50^{a}	88.39±1.89^{ab}	87.66±1.50^{a}	85.96±1.79^{a}	86.95±1.23^{c}	85.63±1.29^{c}
240	89.94±3.08^{a}	88.63±1.55^{a}	88.30±2.70^{ab}	87.40±3.12^{a}	86.80±1.25^{a}	86.97±1.93^{bc}	86.65±1.33^{b}
300	87.15±1.43^{ab}	87.46±1.85^{a}	84.52±0.49^{bcde}	87.35±1.56^{a}	84.68±0.83^{a}	84.27±1.43^{ab}	85.34±3.48^{ab}
360	87.67±1.22^{ab}	87.42±0.89^{a}	87.82±1.49^{ab}	87.32±1.90^{a}	85.63±0.75^{a}	85.94±0.95^{a}	85.45±0.94^{a}

[1)] C0=control starch without retrogradation treatment; CS1=single-retrogradation for 1 d; CD1=double-retrogradation with 1 d interval; CT1=triple-retrogradation with 1 d interval; CS5=single-retrogradation for 5 d; CD5=double-retrogradation with 5 d interval; CT5=triple-retrogradation with 5 d interval.

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

Table 6. Degree of hydrolysis of AS-treated starch samples

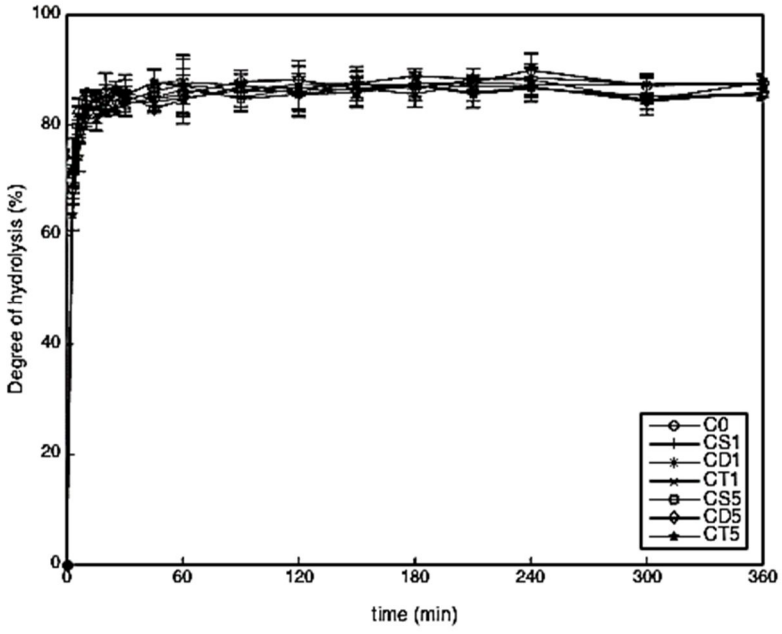
Time (min)	Sample[1)]						
	A0	AS1	AD1	AT1	AS5	AD5	AT5
3	20.27±0.54$^{n2)}$	18.03±0.58^{n}	21.09±3.66^{k}	18.52±3.01^{m}	15.15±0.81^{m}	16.89±3.20^{l}	13.50±2.48^{o}
6	24.66±0.40^{m}	21.75±1.30^{m}	23.07±4.49^{jk}	21.17±0.61^{m}	18.61±2.35^{l}	19.58±2.98^{kl}	17.10±2.03^{n}
10	27.02±2.03^{l}	25.24±0.40^{l}	25.21±2.27^{j}	24.30±0.80^{l}	20.66±2.73^{l}	21.02±2.00^{k}	19.62±0.98^{m}
15	31.08±0,40^{k}	27.03±0.68^{l}	28.53±0.75^{i}	27.16±0.97^{k}	25.60±0.64^{k}	23.42±0.54^{jk}	22.40±1.19^{l}
20	34.88±2.54^{j}	31.01±1.54^{k}	29.89±2.18^{i}	28.65±2.67^{k}	27.12±1.26^{k}	25.17±4.40^{ij}	23.94±2.51^{kl}
25	36.30±1.30^{j}	34.21±0.40^{j}	30.98±0.45^{hi}	31.64±0.82^{j}	30.80±0.92^{j}	26.07±0.72^{ij}	26.11±0.67^{jk}
30	39.98±0.69^{i}	37.55±2.69^{i}	33.36±2.16^{h}	34.10±3.18^{j}	32.45±1.13^{j}	27.39±2.99^{i}	26.87±2.01^{j}
45	44.54±1.25^{h}	42.56±0.59^{h}	42.47±0.86^{g}	39.93±1.39^{i}	36.72±0.67^{i}	34.10±0.74^{h}	30.37±0.92^{i}
60	46.43±0.59^{h}	45.57±0.28^{g}	45.57±0.09^{f}	43.52±2.56^{h}	40.93±3.51^{h}	38.57±1.10^{g}	36.06±1.82^{h}
90	51.29±1.10^{g}	49.66±1.03^{f}	50.38±0.47^{e}	48.89±1.41^{g}	45.94±2.04^{g}	43.84±1.83^{f}	43.47±1.37^{g}

Time (min)	Sample[1)]						
	A0	AS1	AD1	AT1	AS5	AD5	AT5
120	55.70±1.91^{f}	54.90±3.10^{e}	55.47±1.99^{d}	53.90±1.47^{f}	49.67±0.90^{f}	51.08±2.49^{e}	48.28±0.56^{f}
150	60.33±0.61^{e}	59.68±1.48^{d}	59.42±0.70^{c}	56.77±0.88^{e}	54.70±1.06^{e}	55.75±1.22^{d}	53.23±0.64^{e}
180	62.88±1.27^{d}	64.54±0.10^{c}	62.17±0.19^{bc}	60.84±1.01^{d}	58.00±1.62^{d}	58.69±3.80^{cd}	57.12±1.89^{d}
210	65.73±0.92^{c}	67.64±0.34^{b}	64.34±1.26^{b}	64.22±1.00^{c}	61.51±1.48^{c}	61.27±1.06^{c}	59.83±0.59^{c}
240	68.23±2.02^{b}	68.83±0.83^{ab}	67.51±1.07^{a}	66.70±0.46^{bc}	63.70±0.93^{bc}	62.50±0.70^{bc}	62.63±1.29^{b}
300	70.11±0.96^{ab}	69.44±0.97^{ab}	68.59±1.19^{a}	68.46±1.24^{ab}	66.28±0.75^{ab}	66.10±0.69^{ab}	64.33±0.77^{ab}
360	71.65±1.05^{a}	70.21±0.87^{a}	69.89±0.76^{a}	69.04±1.03^{ab}	67.26±1.13^{a}	66.94±0.84^{a}	65.27±0.90^{a}
420	72.98±1.26^{a}	70.47±0.72^{a}	70.21±0.37^{a}	69.94±1.26^{a}	67.70±0.95^{a}	67.16±2.26^{a}	65.29±0.66^{a}

[1)] A0=AS-treated starch without retrogradation treatment; AS1=single-retrogradation for 1 d; AD1=double-retrogradation with 1 d interval; AT1=triple-retrogradation with 1 d interval; AS5=single-retrogradation for 5 d; AD5=double-retrogradation with 5 d interval; AT5=triple-retrogradation with 5 d interval.

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

(A)


Degree of hydrolysis (%)
time (min)
C0
CS1
CD1
CT1
CS5
CD5
CT5

(B)

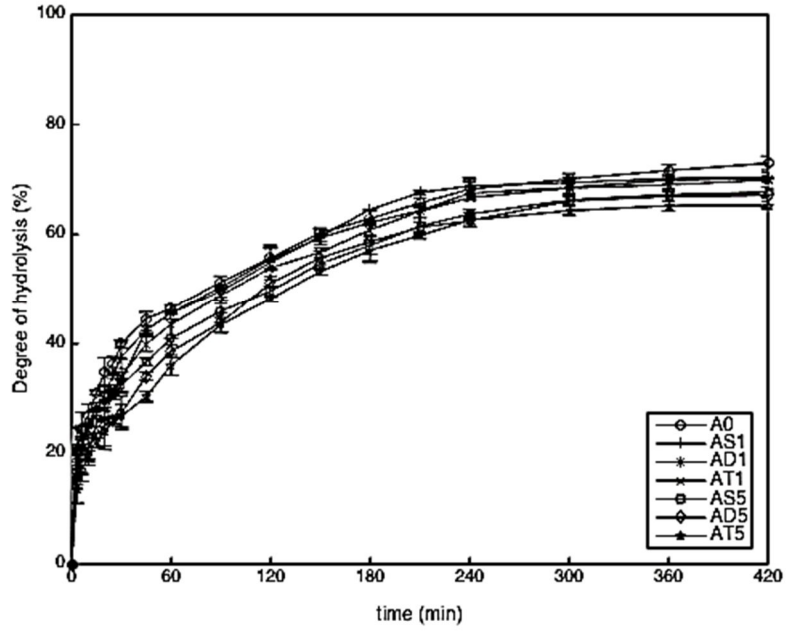

Degree of hydrolysis (%)
time (min)
A0
AS1
AD1
AT1
AS5
AD5
AT5

Figure 3. (A): Hydrolysis patterns of control starches. (B): Hydrolysis patterns of AS-treated starches.

5. Determination of starch fractions using the Englyst method

Table 8 presents the *in vitro* digestibility of control starches measured by the Englyst method. Compared with AS-treated starches, control starches contained a higher RDS content, ranging from 81.39 % to 85.99 %. This difference was due to the complete gelatinization of control samples during the sample preparation, resulting in the disruption of the inner structure of starch granules. The result is well related to the previous study stating that the amorphous regions generated by gelatinization are easier to be accessed by digestive enzymes (Zhang et al., 2006b). As repeated retrogradation treatment proceeds, the RDS content slightly decreased, whereas no significant differences were detected in SDS and RS.

After the AS treatment, the RDS shifted to a lower value, whereas SDS and RS showed higher values compared with the control starches. Both RDS and RS covered a broad scope amongst the samples, especially the content of RDS ranged from 19.62 % to 27.02 %, and the content of RS ranged from 31.17 % to 37.49 %. The SDS content was kept fairly constant. Also, the value of RDS exhibited a slight decrease with the retrogradation time. The

most noticeable increment in the RS content was found in the samples repeatedly retrograded with the interval of 5 d (AS5, AD5, and AT5).

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). 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). Moreover, repeated retrogradation induced the reassociation of AM-like chains and formation of double helices and crystalline structures. Therefore, this result indicated that repeated retrogradation could accelerate crystal perfection.

Table 7. Contents of RDS, SDS, and RS[1)] of control starches determined using the Englyst method

Sample	RDS (%)	SDS (%)	RS (%)
C0	85.99±0.18[a2)]	3.95±3.17[a]	10.05±3.08[a]
CS1	84.53±1.76[ab]	4.09±0.81[a]	11.36±1.55[a]
CD1	83.11±1.41[ab]	5.19±4.02[a]	11.69±2.69[a]
CT1	82.50±2.82[ab]	4.89±4.10[a]	12.59±3.11[a]
CS5	83.28±3.03[ab]	3.51±1.78[a]	13.20±1.25[a]
CD5	82.87±2.36[ab]	4.09±2.10[a]	13.03±1.93[a]
CT5	81.39±2.28[b]	5.25±2.28[a]	13.35±1.33[a]

[1)] RDS = rapidly digestible starch; SDS = slowly digestible starch; RS = resistant starch.

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

Table 8. Contents of RDS, SDS, and RS[1)] of AS-treated starches determined using the Englyst method

Sample	RDS (%)	SDS (%)	RS (%)
A0	27.02±2.03[a2)]	41.20±2.62[a]	31.76±2.02[b]
AS1	23.90±3.78[ab]	44.92±3.05[a]	31.17±0.83[b]
AD1	25.21±2.27[abc]	42.29±3.13[a]	32.48±1.07[b]
AT1	24.30±0.80[abcd]	42.39±0.96[a]	33.30±0.46[b]
AS5	20.66±2.73[bcd]	43.03±1.87[a]	36.29±0.93[a]
AD5	21.02±2.00[cd]	41.47±1.33[a]	37.49±0.70[a]
AT5	19.62±0.98[d]	43.00±2.17[a]	37.36±1.29[a]

[1)] RDS = rapidly digestible starch; SDS = slowly digestible starch; RS = resistant starch.

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

6. Determination of starch fractions based on the first-order kinetics method

Enzymatic hydrolysis patterns were fitted to the plots using the first-order kinetics method (Figure 4 and Figure 5 for control starches, and AS-treated starches, respectively). When starch or starch-containing foods are digested *in vitro*, the rate of hydrolysis decreases as the digestion time is extended. Also, the concentration of product produced against the digestion time can be plotted with logarithmic scale. Therefore, digestion patterns can be further described by a single rate coefficient, as stated in the first order kinetics method (Zhang et al., 2013).

Compared with AS-treated starch samples, the determination coefficient of control starch samples were lower (R^2 as high as 0.8224 for AS1, R^2 as low as 0.5644 for AT1) due to the emergence of the time of plateau early in the hydrolysis process (Table 5). AS-treated starch samples, on the other hand, clearly showed that the digestion process follows first-order kinetics behavior, with all R^2 values above 0.9006.

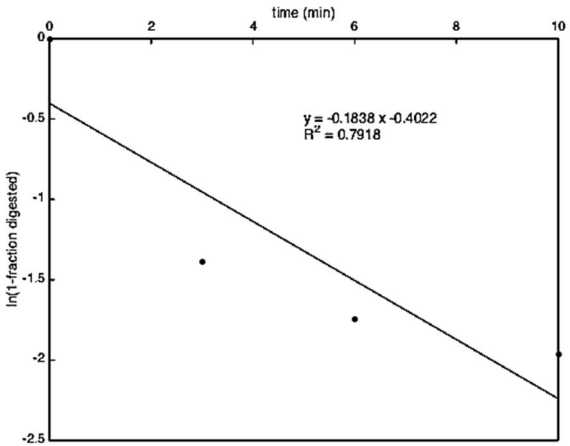
The kinetic parameters of starch samples based on the first-order kinetics method are summarized in Table 9. In order to compare the hydrolytic kinetic parameters using two different methods, the contents of starch

fractions (RDS+SDS and RS) based on the Englyst method are also presented in Table 9. The rapid digestion is characterized by a higher k value, meaning that the structure relevant to that phase is readily available to digestive enzymes. The low k value can be described by the greater difficulty that digestive enzymes experience to bind with the structural components of starch (Butterworth et al., 2011; Dhital et al., 2010). The k values for AS-treated starch samples were more than 10^2 higher than those for control starch samples. When the samples were treated with repeated retrogradation, the k values were decreased. The decrease was dramatic in the samples retrograded with longer initial storage time. During the retrogradation process, 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 (Silverio et al., 2000). It can be inferred that the samples retrogradated with 1 d interval only went through the nucleation step, whereas samples treated with 5 d interval of repeated retrogradation experienced nucleation and propagation, enabling them to resist to the enzymatic hydrolysis due to the matured and perfect crystallites.

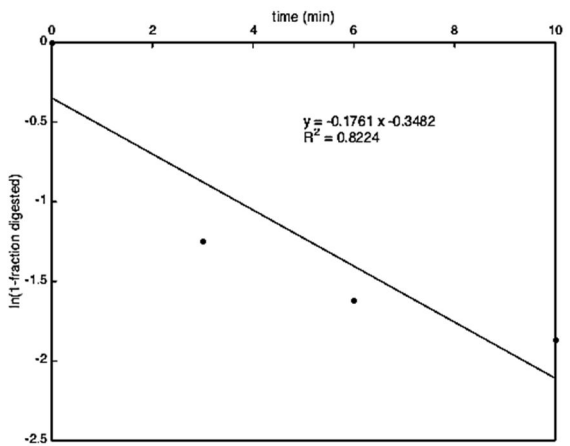
RS contents measured by using the Englyst method and the first-order kinetics method were slightly different. This difference was present because the classification system to describe the starch digestion property introduced

by Englyst et al. (1992) was based on the specific time frame, while the method originated from Goñi et al. (1997) was based on the concept that the RS was determined by using the points in the region where the C (fraction of digested starch at a certain digestion time) was not significantly different in the diegree of hydrolysis. The amount of RS fraction estimated using the first-order method increased by a large extent with the repeated retrogradation, especially in the samples treated with 5 d interval, coinciding with the result obtained by the Englyst method. When the starch is processed with thermal treatment, the starch granules are induced to swell and melt, whereupon the chains of contiguous deformed starch molecules aggregate and form sponge-like structures during retrogradation treatment (Ratnayake & Jackson, 2006). Hu et al. (2014) suggested that the morphological change during the repeated retrogradation is responsible for the difficult access of enzyme. The stated explanation was supported by the scanning electron microscopy (SEM) images where the amount of cavities decreased and more smooth regions appeared around the cavities in TR-treated starch samples. Therefore, such morphological changes may account for the lower digestion rate and higher RS yield.

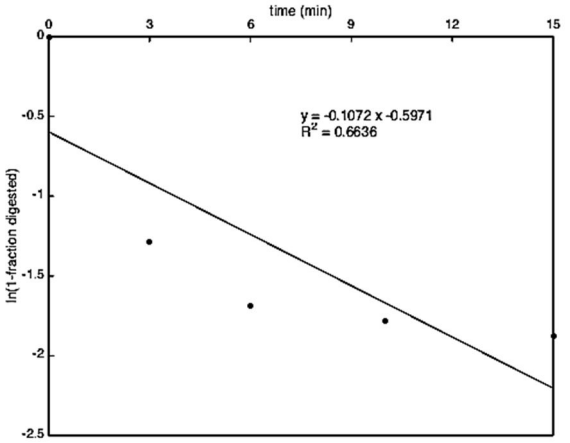
(A)


time (min)
0
2
4
6
8
10
0
-0.5
-1
-1.5
-2
-2.5
ln(1-fraction digested)
y = -0.1838 x -0.4022
R2 = 0.7918

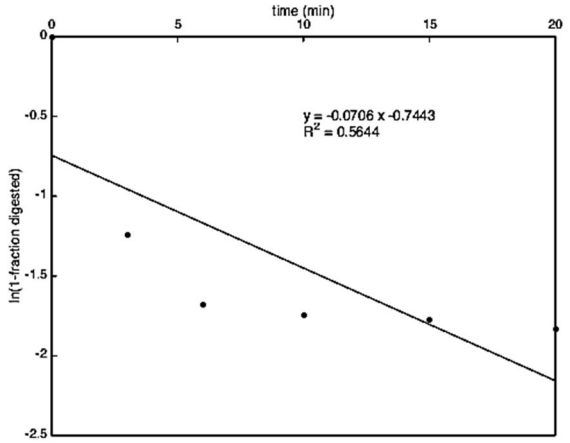
(B)


time (min)
0
2
4
6
8
10
0
-0.5
-1
-1.5
-2
-2.5
ln(1-fraction digested)
y = -0.1761 x -0.3482
R2 = 0.8224

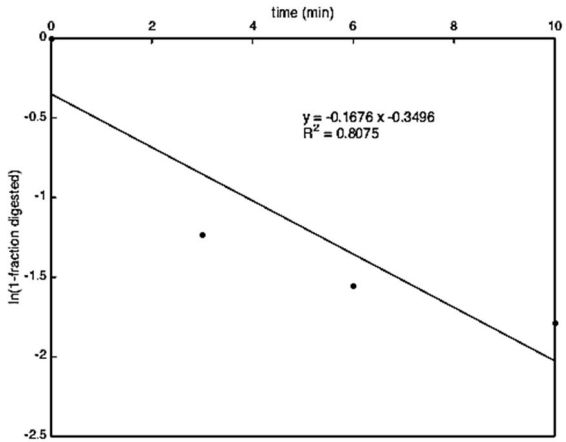
(C)


time (min)
0
3
6
9
12
15
0
-0.5
-1
-1.5
-2
-2.5
ln(1-fraction digested)
y = -0.1072 x -0.5971
R2 = 0.6636

(D)


time (min)
0
5
10
15
20
0
-0.5
-1
-1.5
-2
-2.5
ln(1-fraction digested)
y = -0.0706 x -0.7443
R2 = 0.5644

(E)


time (min)
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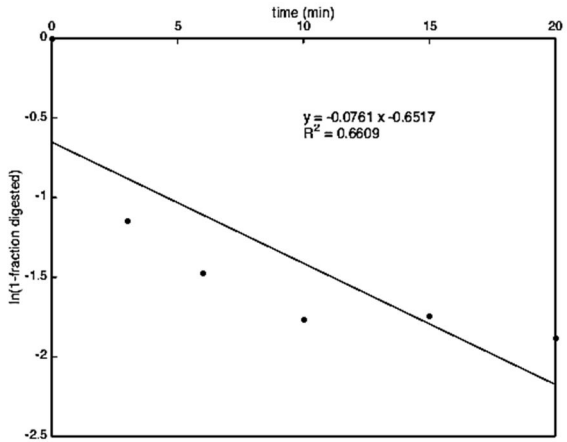
2
4
6
8
10
0
-0.5
-1
-1.5
-2
-2.5
ln(1-fraction digested)
y = -0.1676 x -0.3496
R2 = 0.8075

(F)


time (min)
0
5
10
15
20
0
-0.5
-1
-1.5
-2
-2.5
ln(1-fraction digested)
y = -0.0761 x -0.6517
R2 = 0.6609

(G)

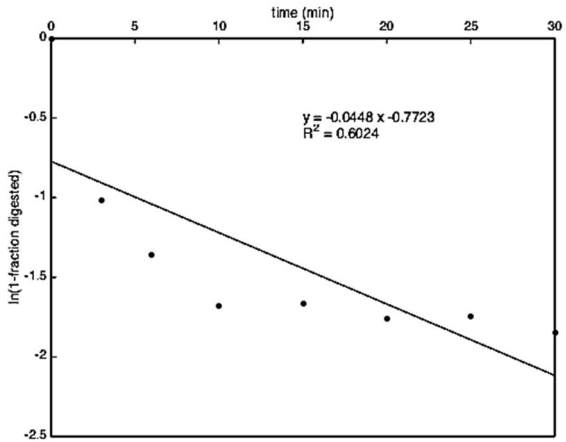
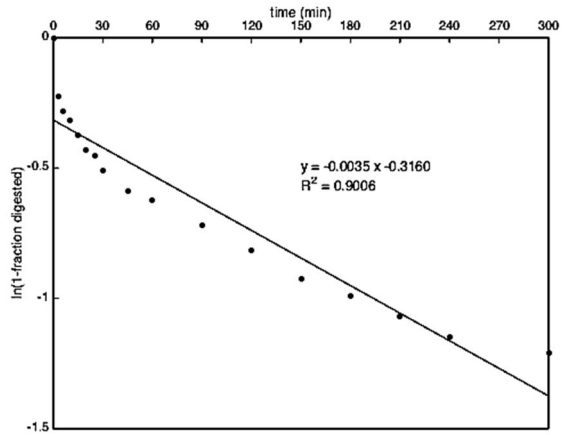
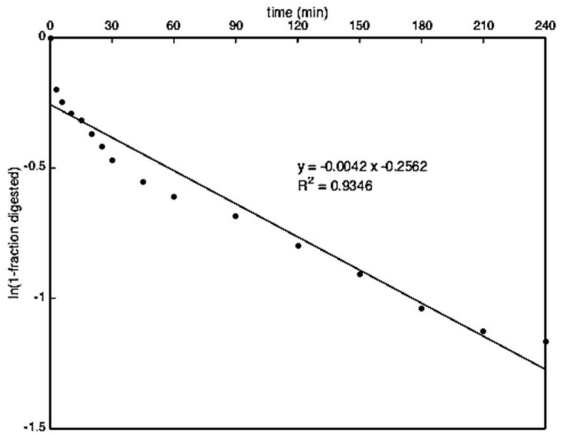

time (min)
0
5
10
15
20
25
30
0
-0.5
-1
-1.5
-2
-2.5
ln(1-fraction digested)
y = -0.0448 x -0.7723
R2 = 0.6024

Figure 4. Fitting of first-order kinetics to (A) C0=control starch with no retrogradation, (B) CS1=control starch with single retrogradation for 1 d, (C) CD1=control starch with double retrogradation for 1 d, (D) CT1=control starch with triple retrogradation for 1 d, (E) CS5=control starch with single retrogradation for 5 d, (F) CD5=control starch with double retrogradation for 5 d, and (G) CT5=control starch with triple retrogradation for 5 d.

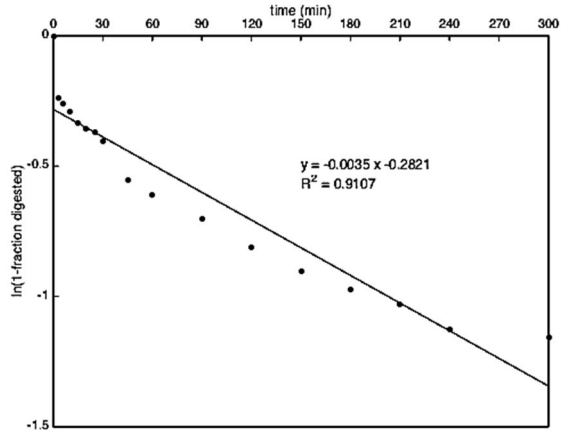
(A)


time (min)
0 30 60 90 120 150 180 210 240 270 300
0
-0.5
-1
-1.5
ln(1-fraction digested)
y = -0.0035 x -0.3160
R² = 0.9006

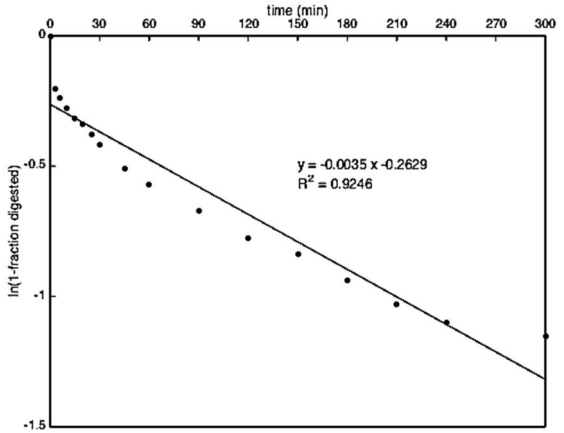
(B)


time (min)
0 30 60 90 120 150 180 210 240
0
-0.5
-1
-1.5
ln(1-fraction digested)
y = -0.0042 x -0.2562
R² = 0.9346

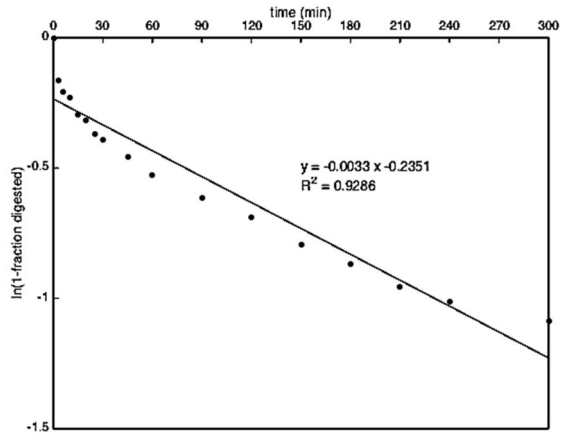
(C)


time (min)
0 30 60 90 120 150 180 210 240 270 300
0
-0.5
-1
-1.5
ln(1-fraction digested)
y = -0.0035 x -0.2821
R² = 0.9107

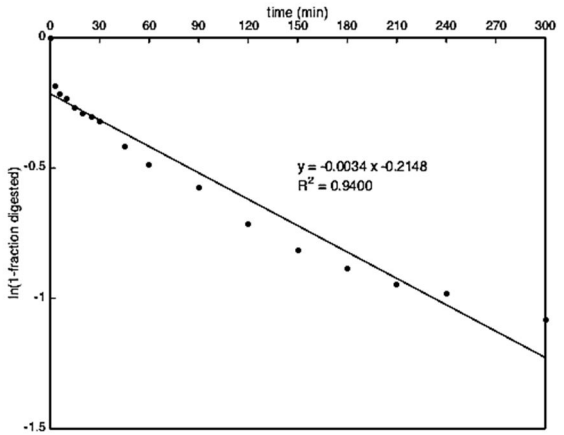
(D)


time (min)
0 30 60 90 120 150 180 210 240 270 300
0
-0.5
-1
-1.5
ln(1-fraction digested)
y = -0.0035 x -0.2629
R² = 0.9246

(E)


time (min)
0
30
60
90
120
150
180
210
240
270
300
0
-0.5
-1
-1.5
ln(1-fraction digested)
y = -0.0033 x -0.2351
R2 = 0.9286

(F)


time (min)
0
30
60
90
120
150
180
210
240
270
300
0
-0.5
-1
-1.5
ln(1-fraction digested)
y = -0.0034 x -0.2148
R2 = 0.9400

(G)

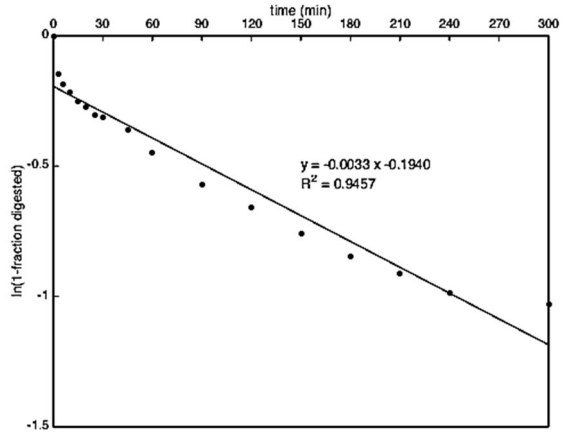

time (min)
0
30
60
90
120
150
180
210
240
270
300
0
-0.5
-1
-1.5
ln(1-fraction digested)
y = -0.0033 x -0.1940
R2 = 0.9457

Figure 5. Fitting of first-order kinetics to (A) A0=AS-treated starch with no retrogradation, (B) AS1=AS-treated starch with single retrogradation for 1 d, (C) AD1=AS-treated starch with double retrogradation for 1 d, (D) AT1=AS-treated starch with triple retrogradation for 1 d, (E) AS5=AS-treated starch with single retrogradation for 5 d, (F) AD5=AS-treated starch with double retrogradation for 5 d, and (G) AT5=AS-treated starch with triple retrogradation for 5 d.

Table 9. Hydrolysis kinetic parameters of starch samples

Sample	Englyst		First-order kinetics		
	RDS + SDS (%)[1)]	RS (%)[1)]	k (min^{-1})[2)]	RS (%)	C_∞[3)] (%)
C0	89.95	10.05	2.9×10^{-1}	13.16	86.84
CS1	88.64	11.36	2.7×10^{-1}	13.58	86.42
CD1	88.31	11.69	2.8×10^{-1}	14.10	85.90
CT1	87.41	12.59	2.8×10^{-1}	13.43	86.57
CS5	86.80	13.20	1.6×10^{-1}	14.51	85.49
CD5	86.97	13.03	2.4×10^{-1}	14.30	85.70
CT5	86.65	13.35	1.5×10^{-1}	14.10	85.90
A0	68.24	31.76	3.5×10^{-3}	28.42	71.58
AS1	68.83	31.17	4.2×10^{-3}	30.26	69.74
AD1	67.52	32.48	3.5×10^{-3}	30.95	69.05
AT1	66.70	33.30	3.5×10^{-3}	30.85	69.15
AS5	63.80	36.20	3.3×10^{-3}	32.92	67.08
AD5	62.51	37.49	3.4×10^{-3}	33.27	66.73
AT5	62.64	37.36	3.3×10^{-3}	35.04	64.96

[1] RDS = rapidly digestible starch; SDS = slowly digestible starch; RS = resistant starch.

[2] k = rate constant of starch hydrolysis.

[3] C_∞ = the maximum degree of hydrolysis.

CONCLUSION

The effects of repeated retrogradation on the structural characteristics and *in vitro* digestibility of AS-treated waxy corn starch were investigated in the current study. Elongated branch chains of AP after the AS-treatment showed higher susceptibility to the repeated retrogradation owing to AM-like properties. Storage duration also had an influence on the starch retrogradation, due to the starch nucleation as well as its prolonged propagation in the overall retrogradation process.

After repeated retrogradation, all AS-treated starches exhibited a B-type polymorph, increased relatively crystallinity, melting enthalpy, and the proportion of ordered structure. This phenomenon was due to the formation of more ordered structure, and the recrystallization between double helices formed of elongated branch chains, leading to the perfect crystal formation. AS-treated starch samples with triple-retrogradation treatment of 5 d interval, in particular, showed a much higher relative crystallinity, melting enthalpy, and the peak temperature compared with other samples. This structural change during the repeated retrogradation was responsible for the difficult access of enzyme, resulting in the higher yield of RS.

In terms of digestion properties, the concept of RDS, SDS, and RS

suggested by Englyst, is valid. However, the first-order method introduced in this study also would be an alternative tool for classifying starch into RDS, SDS, and RS, along with comparing kinetic constants of starch digestion.

With repeated retrogradation, more changes occurred in structural and digestion properties of AS-treated starches than those of control starches. These findings suggest that repeated retrogradation is one of promising technologies for developing industrial RS products in the food industry.

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국문 초록

아밀로수크레이스 (AS) 처리 후 반복 노화 과정을 거친 찰옥수수 녹말의 구조 및 소화 특성의 변화를 조사하였다. AS 처리로 연장된 아밀로펙틴 사슬의 결정화를 양적, 질적으로 가속화하기 위해 다양한 노화 조건을 사용하였다. 반복 노화 조건은 4 °C 에서 1 일 또는 5 일 저장하여 총 1 회, 2 회, 3 회 반복하였고, AS 처리하지 않은 대조구 녹말 또한 같은 조건으로 노화 처리를 하여 비교하였다. AS 처리 후 녹말의 특성은 이온크로마토그래피를 통한 사슬 길이 분포 결과로 파악하였다. 녹말의 노화 후 결정 구조 특성은 X-선 회절, 시차주사 열량계를 이용하여 알아보았고, 소화율을 측정하여 결정 구조가 소화율에 미치는 영향에 대해서도 조사하였다. AS 처리 후 짧은 사슬 분포는 감소하였고 반면에 긴 사슬 분포는 증가하였다. 반복 노화 후에 모든 AS 처리 녹말의 X-선 회절도형은 B 형을 나타내었고, 대조구 녹말과 비교하여 높은 상대적 결정화도와 용융 엔탈피값을 나타내었다. 소화율 결과에서는 높은 RS 함량과 낮은 속도 상수를 보였다. 이는 5 일 간격으로 세 반복 노화 처리한 실험구에서 더욱 큰 효과로

나타났다. 이 실험을 통해, 반복 노화는 AS 처리로 연장된 사슬의 재회합을 유도하여 노화를 가속화한다는 것을 증명하였다. 이는 대조구 녹말보다 AS 처리 녹말의 구조 및 소화 특성에 더 많은 변화를 가져왔고, 반복 노화에 의한 결정 구조의 차이는 소화율에 영향을 준다는 것 또한 확인하였다.

주요어 : 소화율, 구조적 특성, 아밀로수크레이스, 찰 옥수수 녹말, 노화

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