A Thesis for the Degree of Master of Science

In vitro digestion of the amylosucrase treated waxy corn starches fitted to 1st-order kinetics

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농학석사학위논문

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위 원 장 서 진 호 인

부위원장 문 태 화 인

위 원 장 판 식 인

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In vitro digestion of the amylosucrase treated waxy corn starches fitted to 1st-order kinetics

by

Kim, Ha Ram

Advisor: Tae Wha Moon, Professor

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Department of Agricultural Biotechnology

Seoul National University

ABSTRACT

Englyst et al. classified the starch into three fractions of rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS), according to the time of digestion. This classification has been argued because the *in vitro* digestion of starch can be described by a single rate constant k of a first order reaction. This study aimed to ascertain individual existence of RDS and SDS fractions in a single starch source. Waxy corn starch was modified by amylosucrase (AS) to obtain starch samples with increased SDS contents determined by Englyst method. Hydrolysis curves were obtained for native and AS-treated starches and then fitted to the logarithm of slope (LOS) plot, which can reflect the change of k during first order reaction. LOS plots for AS-treated starches revealed a discontinuity, demonstrating that digestion proceeded in two separated phases. It provided the evidence of a fraction that is digested more rapidly than the remainder, or RDS and SDS as a structural feature. Rate constants for digestion of AS-treated starches could be categorized into 2 groups and named k_{RDS} and k_{SDS}, respectively. The values of the k_{RDS} and k_{SDS}, and the contents of RDS and SDS estimated by LOS plot method of AS-treated starches were affected by the amount of AS employed. This intimated that highly extended branch chains would favor the formation of the more slowly

digestible form of RDS and SDS. It seemed that the LOS plot approach would be a better investigative tool for classification of starch fractions by its digestibility. The structural characteristics AS-treated starches were examined before and after removal of RDS and SDS fractions. The changes in branch chain length distribution implied the branch chains with certain DP contribute to the organization of each fraction. Undigested RS was composed of rather short chains with DP 13-24. After removal of RDS and SDS fractions, chains of DP ≥ 37 and DP ≥ 25 decreased, respectively. The AS-treated starches displayed B type X-ray pattern, and the relative crystallinity increased with the amount of AS, and also according to the degree of hydrolysis of RDS and SDS fractions. In conclusion, the branch chain length distributions of AP determined the primarily generated crystallite organization of AS-treated starches, causing the diversity of digestion property. The different values of k_{RDS} and k_{SDS} among starch samples reflected the different structures of RDS and SDS.

Keywords: slowly digestible starch, amylosucrase, digestibility, branch chain length distribution, first order equation

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INTRODUCTION

Carbohydrates are one of the important energy-providing macro-nutrients in food, and starch is the main source of digestible carbohydrate in the human diet. The glucose generated from starch digestion plays an important role in energy metabolism. Public awareness of health and diet has increased considerably. Obesity and related metabolic diseases such as diabetes and cardiovascular disease have been related to an increase in the consumption of refined carbohydrates (Zhang et al., 2008b). It is generally known that foods containing a similar amount of starch can induce different postprandial blood glucose level and insulin responses after consumption (Patel et al., 2014). In this regard, the concept of glycemic index (GI) was introduced to classify foods on the basis of their postprandial blood glucose response (Jenkins et al., 1981). The GI is defined as the postprandial incremental glycemia area after a meal, expressed as the percentage of the corresponding area after an equi-carbohydrate portion of a reference food (glucose or white bread).

Englyst et al. (1992) introduced a classification system to describe the starch digestion property with reference to specific time frame. The fraction digested within first 20 min is defined as rapidly digestible starch (RDS), the fraction digested between 20 and 120 min is slowly digestible starch (SDS),

and the undigested part after 120 min represents resistant starch (RS). This system implies that starch granules contain individual fractions that have different enzyme susceptibility and has been widely quoted in numerous studies. Previous studies have shown that GIs of food products are positively correlated with the amount of RDS (Englyst et al., 1999; Englyst et al., 2003), whereas the SDS may be more desirable for healthy food products. The principal health effect of SDS is a slow and prolonged postprandial glucose release profile (Seal et al., 2003). Thus SDS may be beneficial in food products that can provide a more consistent source of exogenous glucose to the body. Also, SDS can have implications for physical and mental performance, satiety, and diabetes management (Lehmann & Robin, 2007a; Wolf et al., 1999). Therefore, improving food quality with a higher amount of SDS is becoming an interest for food industry (Zhang et al., 2006b).

However, some researchers argue that assigning labels of RDS and SDS to the certain time frames of a digestibility curve does not give an accurate description of the enzyme process of starch hydrolysis. For example, for cooked or gelatinized starch, regardless of the 'Englyst' classification, digestibility data proceed as a first-order reaction described by a single rate constant (Dhital et al., 2010; Goñi et al., 1997), i.e. all digestible fractions have the same intrinsic reactivity. In this context, the terms of 'RDS' and

'SDS' are considered as not proper for describing the digestion behavior of starch granules.

Meanwhile, a research group recently introduced an improved first-order kinetic model for the analysis of starch hydrolysis using a 'logarithm of slope' (LOS) plot (Butterworth et al., 2012; Patel et al., 2014). This analysis allows an estimation of several digestion kinetic parameters: the rate constant, k, is represented by the negative slope of the linear plot, and the total starch digested, C_∞, can be calculated from the y-axis intercept. These studies showed that digestion of native granular starch does NOT follow a single first-order reaction. The digestion process is described by two separate first-order reactions that differ in their rate constant. Therefore, the LOS plot approach would be a useful investigative tool for accurate determination of RDS and SDS starch fractions, if present, from discontinuities in the linear plot (Patel et al., 2014).

According to previous studies (Zhang et al., 2008a; Zhang et al., 2008b; Zhang et al., 2006b), the content of SDS has a parabolic relationship with the weight ratio of short chains (DP < 13) to long chains (DP ≥ 13) of amylopectin (AP) in a variety of maize mutant samples. This suggestion was supported by the results of plentiful studies (Casarrubias-Castillo et al., 2012; Miao et al., 2014; Shin et al., 2010). In addition, the molecular structural

features of AP such as molecular weight, dimension or size, density, degree of branching, and distribution of short chains influence the starch hydrolysis (Goesaert et al., 2010; Miao et al., 2011; Naguleswaran et al., 2014).

A few studies employed amylosucrase (EC 2.4.1.4., AS) from *Neisseria polysaccharea* to produce starches with extended AP branch chains (Kim et al., 2013; Kim et al., 2014; Shin et al., 2010). AS is a glucosyltransferase which produces an insoluble α-1,4-linked glucan polymer by consumption of sucrose releasing fructose. This reaction uses the energy generated by splitting sucrose to synthesize glucan polymer. When the starch exists as a glucose acceptor, elongation of the glucosyl units occurs at non-reducing ends of external chains (Buttcher et al., 1997; Potocki de Montalk et al., 2000; Rolland-Sabaté et al., 2004). Shin et al. (2010), who conducted modification of several starches with AS, reported a noticeable increase of SDS content by approximately 25% in waxy starches. In other study, SDS increased as the reaction time increased from 1 to 6 h during 45 h AS treatment on waxy corn starch (Kim et al., 2014). On the basis of the suggestion that the slow digestion property of the starch material could be manipulated according to its AP fine structure, waxy corn starch was modified with AS at various levels to obtain starch samples with different SDS contents determined by Englyst method. Digestibility of AS-treated

starch samples was examined and fitted to first order equation for LOS plotting.

The objectives of this study were to ascertain individual existence of RDS and SDS fractions in a single starch source and to compare the structural characteristics of RDS and SDS fraction in recrystallized starch. It is expected that this study would contribute to a better understanding of the relationship between starch molecular structure and digestion property.

MATERIALS AND METHODS

1. Materials

Waxy corn starch was obtained from Ingredion (Westchester, IL, USA). Amylosucrase (AS) from *Neisseria polysaccharea* was provided by Food Microbiology and Biotechnology Laboratory of KyungHee University. α-Amylase from porcine pancreatin (type VI-B, A3176) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and its activity stated by the supplier was 30 U/mg solid. Amyloglucosidase (AMG 300L, activity 300 AGU/mL), isoamylase (activity 1,000U) and GOD-POD assay kit were from Novozymes (Bagsvaerd, Denmark), Megazyme (Bray, Ireland), and Embiel Co. (Gunpo, Korea), respectively.

2. Methods

2.1. Enzyme assay of AS activity

The gene of AS from *Neisseria polysaccharea* was cloned and expressed in *Escherichia coli* BL21 (DE3). Purification of the enzyme was carried out with affinity chromatography using nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen, Hombrechtikon, Switzerland) as described in a previous study

(Jung et al., 2009). Activity of AS was determined based on the method of van der Veen et al. (2004) with slight modification. An aliquot of diluted AS (0.05 mL) was mixed into the solution composed of 0.1 mL of 4% sucrose, 0.1 mL of 1% glycogen, and 0.25 mL of 0.1 mM sodium citrate buffer (pH 7.0). The amount of released fructose was quantified after incubation of the mixture in a water bath at 30°C and 80 rpm for 10 min. One unit (U) of AS was defined as the amount of enzyme that catalyzes the production of 1 μM of fructose per min by consumption of sucrose (Potocki de Montalk et al., 2000).

2.2. Preparation of AS-treated starches

Starch was dispersed in 100 mM sodium acetate buffer (pH 7.0) with 100 mM sucrose to make a 2% suspension (w/v) of final volume of 150 mL. The suspension was boiled with vortex mixing for 30 min and then cooled to 30°C. AS was added to the starch suspension (2,500 U, 5,000 U, 10,000 U, and 20,000 U/30 mL-starch suspension) and incubated for 6 h in a water bath at 30°C and 80 rpm. The samples were named according to the relative amount of AS added (AS1, AS2, AS4 and AS8, respectively).

Three volumes of ethanol were added to stop the enzyme reaction, and the AS-treated starch was precipitated by centrifugation (10,000 xg, 10 min).

The pellet was washed with distilled water 3 times, freeze-dried, ground and passed through a 100-mesh sieve to be used as a sample. Control was prepared according to the same procedure without addition of AS.

2.3. Digestibility of starch

2.3.1. Degree of starch hydrolysis

The degree of hydrolysis was measured at various time points throughout the incubation period (0-720 min) following the method of Shin et al. (2007) with modification. Pancreatic α-amylase (4.51g) was suspended in distilled water (17 mL) by magnetic stirring for 10 min. After centrifuging the suspension at 1,500 xg for 10 min, the supernatant (15 mL) was mixed with amyloglucosidase (0.3 mL) and distilled water (2.7 mL). The prepared enzyme 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 in 0.75 mL of 0.1 M sodium acetate buffer (pH 5.2) with one glass bead. After the sample dispersion was equilibrated in a 37°C shaking incubator (240 rpm) for 10 min, the enzyme solution (0.75 mL) was added to each sample tube. It was then incubated in a shaking incubator (240 rpm, 37°C). The tubes were removed after certain times and boiled for 10 min to terminate the hydrolysis. The glucose released by hydrolysis of starch was

obtained in the supernatant after the centrifugation at 5,000 xg for 10 min and measured using a GOD-POD kit.

2.3.2. Determination of starch fractions using the Englyst method

Starch fractions were determined according to the degree of hydrolysis. The amount of RDS was determined by the quantity of glucose after reaction for 10 min. SDS was the fraction digested between 10 and 240 min. The undigested fraction that remained after 240 min was measured as RS.

2.3.3. Determination of starch fractions according to log of slope (LOS) method

The rate constant of starch hydrolysis was estimated based on a previous study (Butterworth et al., 2012) with slight modification. In general, digestibility curves of starch can be fitted to a first-order equation (Goñi et al., 1997):

$$C_t = C_\infty\left(1 - e^{-kt}\right)$$

Differentiation of given equation gives:

$$\frac{dC}{dt} = C_\infty k e^{-kt}$$

This equation can be expressed in logarithmic form as follows:

$$\ln\left(\frac{dC}{dt}\right) = \ln(C_\infty k) - kt$$

Thus, a plot of $\ln(dC/dt)$ against t is linear graph with a slope of $-k$. The y-intercept of the graph equals $\ln(C_\infty k)$ and thus the value of k can be calculated from the slope of the plot. Poulsen et al. (2003) referred to this plot as a LOS plot, an abbreviation of 'logarithm of the slope' or 'log of slope'. The slope of a digestibility curve through several time points was determined: the slope was estimated from the fraction ΔC such as $(C_2 - C_1)/(t_2 - t_1)$, $(C_3 - C_2)/(t_3 - t_2)$, etc. and the natural logarithms plotted against the mean time, i.e., $(t_2 - t_1)/2$, $(t_3 - t_2)/2$, etc. A spread sheet was utilized to perform the calculations and regression for linear graph.

At the last stage of digestion, the ΔC revealed almost zero, thus the experimental points in that region were excluded for a LOS plot and determined as RS region. The slope is sensitive to the change of k that occurs during a reaction, which would be revealed by discontinuity in the linear plot. The intersection point of two discontinuous linear lines was the distinction point between RDS and SDS: the former region with steeper slope was considered as RDS, and the latter part was determined as SDS.

2.3.4. Preparation of digested starches for isolation of SDS+RS

fraction or RS fraction

The parallel set for determination of degree of hydrolysis was prepared and followed the same hydrolysis procedure in a 50 mL tube (with 20 times scale). The tube was incubated for certain times to hydrolyze RDS or SDS fraction determined based on the LOS plot. Soluble fractions from starch hydrolysis were removed by centrifugation at 3,000 xg for 10 min. The pellet was re-suspended in 15 mL of 0.2M phosphate buffer (pH 7.0) and treated with 0.5 mL of protease solution which contained 50 mg protease in 10 mL of the phosphate buffer to remove protein part. After the incubation in water bath (60°C) for 10 min, the undigested part of starch was precipitated by centrifugation (3,000 xg, 10 min), washed twice with distilled water, freeze-dried, ground, and passed through a 100-mesh sieve.

2.4. Determination of branch chain distribution

Starch sample (15 mg) was dissolved in 90% dimethyl sulfoxide (3 mL) and boiled for 30 min. Ethanol (15 mL) was added to the suspension and then centrifuged (10,000 xg, 10 min). The precipitated starch pellet was boiled for 15 min with distilled water (1.5 mL) and 50 mM sodium acetate buffer (1.5 mL, pH 4.3). After cooling to 45°C, isoamylase (30 μL) was added to the starch dispersion and incubated in a shaking water bath (45°C,

30 rpm). After 2 h, the dispersion was boiled for 10 min to inactivate the isoamylase.

The debranched starch solution was passed through a 45 μm syringe filter, and injected into a high-performance anion-exchange chromatography (HPAEC) system to examine branch chain distribution. The HPAEC system with a pulsed amperometric detector (PAD) was equipped with a Carbo-pak PA1 anion-exchange column (4x250 mm, Dionex, Sunnyvale, CA, USA). This analysis was performed using 150 mM NaOH for column equilibration and 600 mM sodium acetate in 150 mM NaOH for sample elution with flow rate of 1 mL/min with gradients as follows : 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.

2.5. X-ray diffraction patterns and relative crystallinity

X-ray diffraction analysis was conducted using a powder X-ray diffractometer (Model New D8 Advance, Bruker, Karlsruhe, Germany) at 40 kV and 40 mA. The sample was scanned through 2θ range from 3° to 30° with a 0.02° step size and a count time of 2 sec. The area was calculated using the software developed by the instrument manufacturer (EVA, 2.0). The relative crystallinity was calculated according to the following equation

(Nara & Komiya, 1983).

$$\text{Relative crystallinity (\%)} = \frac{A_c}{A_a + A_c} \times 100$$

A_a: area of amorphous region, A_c: area of crystalline region

2.6. Statistical analysis

All experimental data were analyzed using analysis of variance (ANOVA) and expressed as mean ± standard deviation of replicate measurement. Significant differences among mean values were compared using the Duncan's multiple range test ($p<0.05$). Statistical analysis was conducted by IBM SPSS statistics version 21.0 (IBM, NY, USA).

RESULTS AND DISCUSSION

1. Preparation of starch samples with different branch chain length

Figure 1 and Table 1 provide the branch chain length distributions of starch samples determined by HPAEC-PAD. Branch chains of AP can be classified into four groups (A, B_1, B_2, and B_3) depending on the degree of polymerization (DP), which have chain lengths of DP 6-12, 13-24, 25-36, and ≥37, respectively (Hanashiro et al., 1996), though this classification is no longer valid after AS modification. The native waxy corn starch had relatively abundant A and B_1 chains but a smaller proportion of longer chains as reported in a previous study (Zhang et al., 2006b). AS0 showed no significant difference compared with native starch because of no enzyme addition.

A dramatic change in branch chain length distribution was observed after AS treatment compared to native and AS0. Potocki de Montalk et al. (1999) informed that AS catalyzes the elongation of some branch chains of the glucose acceptor by attachment of 12 to 18 glucosyl units at non-reducing ends. Since short chains are located outside the cluster structure, they are

readily accessed by AS (Kim et al., 2014). Thus, an increase of branch chain length and a decrease in the proportion of short chains (DP 6-12) were observed, as expected. Further, the ratio of decrease in A chains was positively affected by the amount of AS.

The proportion of branch chains with DP 13-24 was the highest in all AS-treated starches, which corresponded to earlier studies (Kim et al., 2013; Kim et al., 2014; Ryu et al., 2010). The percentage of this DP range increased from approximately 52% to over 60% (AS1 and AS2) but got lowered again when the amount of AS was above 50,000 U/150 mL-suspension (AS4, AS8). The higher enzyme activity in AS4 and AS8 resulted in a decrease in this DP range (DP 13-24) but increased in the amount of DP 25-36. A previous study suggested that AS preferentially reacts at the external chains and extends them up to DP 25-36 (Kim et al., 2014). Others also reported that the amount of the B_3 chains (DP $\geq$ 37) hardly changed after AS reaction (Kim et al., 2013). In this study, observable elongation in B_3 chains occurred in the samples treated with excessive amount of AS, whereas there were no significant changes for AS1 and AS2. It seemed that these differences from the previous reports were due to the difference in the enzyme activity employed in the reaction.

Zhang et al. (2008a) demonstrated a parabolic relationship between SDS

content and the ratio of AP short chains (DP < 13) to long chains (DP ≥ 13). Thus, the ratio values of short chains to long chains were calculated for further comparison with SDS content (Table 1). Branch chain elongation and resultant decrease of short chain proportion remarkably reduced the ratio value. With more addition of AS, the lower ratio value was obtained, which ranged from 0.026 to 0.433 consequently.

Table 1. Branch chain length distributions of native and AS-treated starch samples

Sample[1)]	Percent distribution (%)				S/L[3)]
	DP[2)] 6-12	DP 13-24	DP 25-36	DP ≥ 37	
Native	30.22±0.43[a4)]	52.60±0.55[d]	12.62±0.51[e]	4.56±0.45[c]	0.433±0.009[a]
AS0	29.72±0.53[a]	52.47±0.30[d]	12.94±0.47[e]	4.87±0.35[c]	0.423±0.011[a]
AS1	16.18±0.46[b]	60.52±0.43[b]	18.31±0.56[d]	4.99±0.24[c]	0.193±0.006[b]
AS2	11.43±0.13[c]	62.52±0.29[a]	21.07±0.17[c]	4.98±0.25[c]	0.129±0.002[c]
AS4	5.47±0.21[d]	56.82±1.82[c]	31.17±0.81[b]	6.54±0.82[b]	0.058±0.002[d]
AS8	2.53±0.15[e]	46.30±1.02[e]	42.36±0.33[a]	8.80±0.77[a]	0.026±0.002[e]

[1)] Native = native waxy corn starch; AS0 = AS control without enzyme addition; AS1 = AS 2,500 U/30 mL-starch suspension; AS2 = AS 5,000 U/30 mL; AS4 = AS 10,000 U/30 mL; AS8 = AS 20,000 U/30 mL.

[2)] DP = degree of polymerization.

[3)] S/L = ratio of short chains (DP < 13) to long chains (DP ≥ 13).

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

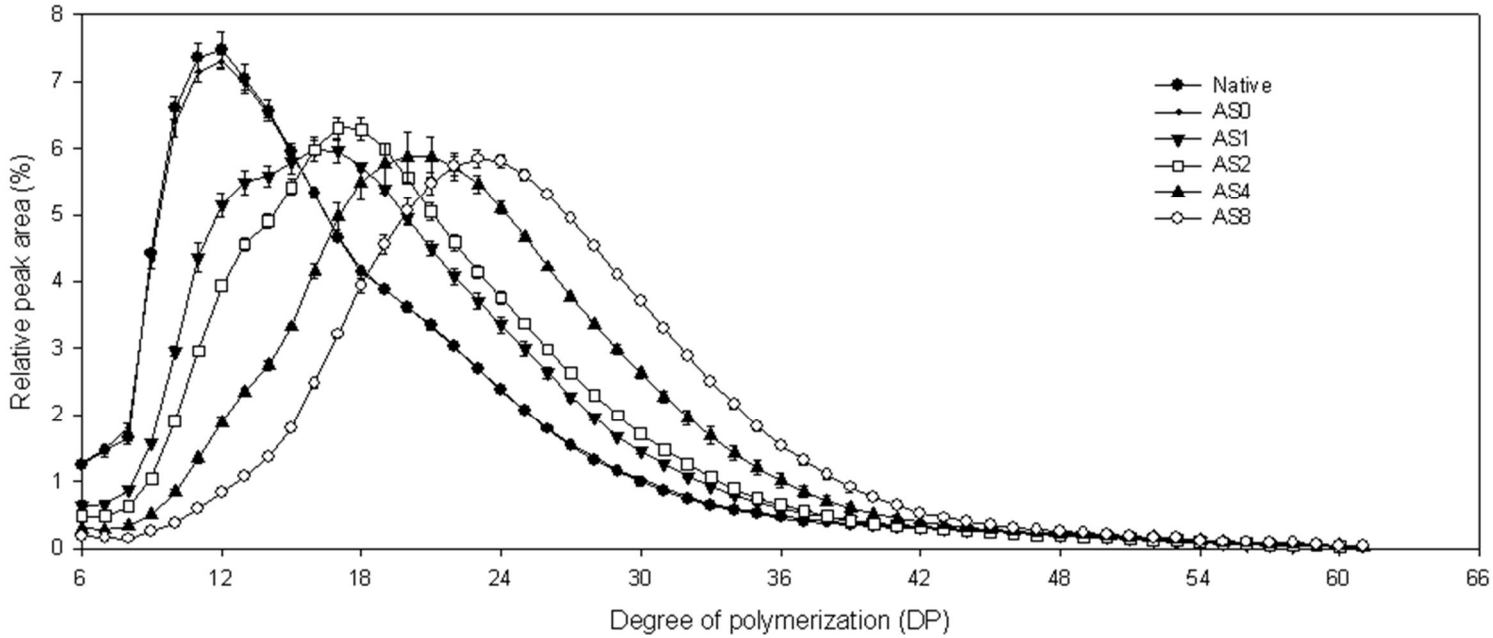


Figure 1. Comparison of the branch chain length distributions revealed in starch samples. Native = native waxy corn starch; AS0 = AS control without enzyme addition; AS1 = AS 2,500 U/30 mL-starch suspension; AS2 = AS 5,000 U/30 mL; AS4 = AS 10,000 U/30 mL; AS8 = AS 20,000 U/30 mL.

2. Determination of starch fractions using the Englyst method

The *in vitro* digestibility of starch samples measured using the Englyst method is presented in Table 2. Native waxy corn starch showed a typical digestion property of A type granular starch which exhibited abundant SDS (Lehmann & Robin, 2007b). AS0 contained the highest RDS content, because the gelatinization process during the sample preparation destroyed the inherent granular structure. The amorphous regions generated by gelatinization are easier to be accessed by digestive enzymes (Zhang et al., 2006b). After the AS treatment, the content of RDS decreased with the amount of AS employed in the reaction.

The SDS level showed significant differences among samples, ranging from 4.58 % to 40.54 %. Zhang et al. (2008a) demonstrated a significant correlation between SDS content and AP short chains or long chains, indicating a parabolic relationship between the SDS content and the weight ratio of AP short chains to long chains. With increase of the AS amount, AS-treated starches consequently showed a decrease in ratio of short chains to long chains as well as an increase of SDS content. Thus, it was found that intended amount of SDS could be produced by modifying AP branch chain length. Exceptional result of native starch should be interpreted separately,

because it retains original granule state by contrast with AS-treated starches (including AS0).

RS displayed an increment with amount of AS, especially for AS8 (39.54%). No change was observed in the SDS content of AS8 compared to AS4 although there was a remarkable reduction of RDS, and it could be explained by the increase in RS.

Table 2. Contents of RDS, SDS, and RS[1)] of starch samples determined using the Englyst method

Sample[2)]	RDS (%)	SDS (%)	RS (%)
Native	21.77±1.46[e3)]	63.01±1.03[a]	15.22±1.15[de]
AS0	76.45±0.81[a]	4.58±0.99[e]	18.98±1.05[bc]
AS1	72.01±1.13[b]	13.87±1.22[d]	14.12±2.08[e]
AS2	48.28±1.52[c]	34.46±3.13[c]	17.26±2.14[cd]
AS4	36.43±0.92[d]	42.48±0.38[b]	21.09±1.26[b]
AS8	19.92±1.22[e]	40.54±1.04[b]	39.54±0.57[a]

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

[2)] Native = native waxy corn starch; AS0 = AS control without enzyme addition; AS1 = AS 2,500 U/30 mL-starch suspension; AS2 = AS 5,000 U/30 mL; AS4 = AS 10,000 U/30 m; AS8 = AS 20,000 U/30 mL.

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

3. Digestion pattern of AS-treated starches

Degree of digestion was measured for a reaction period of 0-360 min. AS4 and AS8 were analyzed further until 480 min or 720 min to clarify the emergence of a plateau. Figure 2 displays the enzymatic digestion profiles of native and AS-treated starches, and more specific data are described in Table 3.

Native starch reached a plateau at 120 min of digestion and the observed C_∞ (maximum degree of hydrolysis) was approximately 84 % (average of the degree of hydrolysis values after 120 min). AS0 was mainly composed of the amorphous regions because it lost its native granular structure and semicrystallnity during sample preparation as described earlier. Thus the time of equilibrium, which indicates the time of "no more change occurs" in degree of hydrolysis, appeared quite early (15 min). The value of C_∞ (81.09 %) was similar to that of native starch.

The resistance to enzymatic hydrolysis of AS-treated starches was greatly influenced by the action of AS. The time of equilibrium was delayed to 60, 150, 210 and 360 min as the amount of AS doubled (AS1, AS2, AS4 and AS8, respectively). The measured C_∞ exhibited the values slightly exceeding 80 % for AS1 and AS2, and dramatically decreased in AS4 and

AS8 (77.02% and 68.39%, respectively). Requirement of the longer time to hydrolyze the smaller amount of digestible starch (C_∞) demonstrated the slower digestion property caused by AS treatment.

The AS-treated starches were recrystallized after gelatinization, and their digestion property could be accounted for based on the mechanism stated below. Zhang et al. (2008a) suggested that retrogradation of long linear chains is one of the mechanisms for the slow digestion property of starches after gelatinization. During the gelatinization of starch, the original AP crystalline structures get disintergrated, and the polysaccharide chains take up a random configuration (Singh et al., 2007). Longer branch chains contribute to retrogradation by formation of strong, stable and long double helices and thereby would produce superior crystalline structure as compared to short chains. On the other hand, the short or weak double helices formed by short chains would produce imperfect crystalline structures (Jane et al., 1999; Srichuwong et al., 2005). Thus, in the present study, the modified AP structure of AS-treated starches, that is, the increased proportion of long chains and the decreased proportion of short chains, could lead the formation of relatively perfect crystallites (Kim et al., 2014). Further, the elongation of branch chains by AS would permit the formation of ordered crystallites which may be hindered by alpha-1,6-linkaged branch points in waxy type

starches (Ryu et al., 2010; Shin et al., 2010). The crystalline regions possess decreased susceptibility to enzymatic hydrolysis (Zhang et al., 2006b). Therefore, the elongation of branch chain length and its accelerated re-association into double helices might improve the slow digestion property of AS-treated starches. To interpret this changes in digestibility in terms of branch chain length and crystallinity, structure analysis was conducted on digested starch residues.

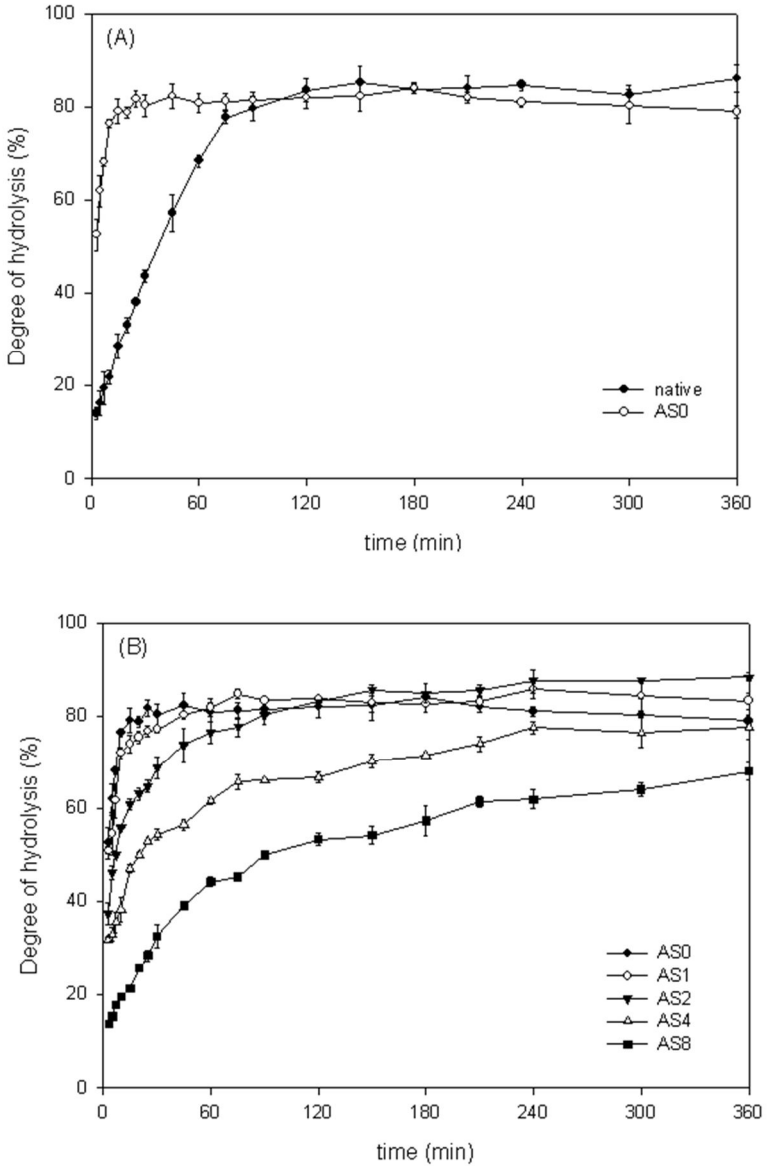


Figure 2. Digestion pattern of AS-treated starches (0-360 min). Native = native waxy corn starch; AS0 = AS control without enzyme addition; AS1 = AS 2,500 U/30 mL-starch suspension; AS2 = AS 5,000 U/30 mL; AS4 = AS 10,000 U/30 mL; AS8 = AS 20,000 U/30 mL.

Table 3. Degree of hydrolysis of starch samples

Time (min)	Sample[1)]					
	Native	AS0	AS1	AS2	AS4	AS8
3	13.92±1.30$^{a2)}$	52.56±3.43^{a}	50.94±0.79^{a}	37.33±2.32^{a}	31.79±0.67^{a}	13.76±0.45^{a}
5	16.14±2.62^{ab}	61.96±3.37^{b}	54.52±4.69^{b}	46.13±1.42^{b}	32.83±1.49^{ab}	15.34±0.71^{a}
7	19.42±3.41^{bc}	68.18±0.86^{c}	61.68±3.72^{c}	50.01±0.82^{c}	35.39±2.25^{b}	17.79±0.71^{b}
10	21.77±1.46^{c}	76.45±0.81^{d}	72.01±1.13^{d}	55.81±0.24^{d}	38.19±2.54^{c}	19.50±0.27^{bc}
15	28.58±2.66^{d}	79.05±2.54^{de}	73.95±1.95^{de}	60.89±1.12^{e}	47.04±0.97^{d}	21.39±0.34^{c}
20	33.00±1.59^{e}	78.76±1.12^{de}	75.37±1.42^{de}	63.15±1.35^{ef}	49.93±0.49^{e}	25.69±0.83^{d}
25	38.04±0.77^{f}	81.66±1.77^{ef}	76.69±1.22^{e}	64.74±1.28^{f}	52.97±0.11^{f}	28.30±1.16^{e}
30	43.69±1.44^{g}	80.33±2.41^{def}	77.17±0.64^{ef}	68.80±2.45^{g}	54.33±1.14^{fg}	32.49±2.44^{f}
45	57.22±3.94^{h}	82.30±2.72^{ef}	80.26±0.84^{fg}	73.71±3.64^{h}	56.43±1.29^{g}	39.12±0.44^{g}

Time (min)	Sample[1)]					
	Native	AS0	AS1	AS2	AS4	AS8
60	68.43±1.34^{i}	80.89±2.10^{ef}	81.83±1.94^{gh}	76.46±2.41^{hi}	61.62±0.65^{h}	44.19±1.08^{h}
75	77.71±1.30^{j}	81.23±1.76^{ef}	84.80±1.12^{hi}	77.68±2.00^{ij}	65.78±1.64^{i}	45.26±0.18^{h}
90	79.62±2.72^{jk}	81.41±1.81^{ef}	83.40±0.98^{ghi}	80.29±2.03^{jk}	66.04±0.47^{i}	50.01±0.94^{i}
120	83.59±2.43^{kl}	82.07±2.39^{ef}	83.75±0.52^{ghi}	83.05±0.57^{kl}	66.71±1.14^{i}	53.34±1.43^{j}
150	85.21±3.49^{l}	82.35±3.23^{ef}	82.91±2.17^{ghi}	85.68±1.19^{lmn}	70.30±1.51^{j}	54.19±1.82^{j}
180	83.91±0.97^{l}	84.08±1.10^{f}	82.65±1.92^{ghi}	84.87±2.09^{lm}	71.49±0.54^{jk}	58.32±3.33^{k}
210	84.12±2.63^{l}	82.01±1.19^{ef}	83.09±1.11^{ghi}	85.52±1.23^{lmn}	73.99±1.64^{kl}	61.48±1.22^{l}
240	84.78±1.15^{l}	81.02±1.05^{ef}	85.88±2.08^{i}	87.53±2.43^{mn}	77.45±1.27^{mn}	61.96±2.09^{lm}
300	82.66±2.07^{kl}	80.17±3.63^{def}	84.36±3.11^{hi}	87.70±0.53^{mn}	76.35±3.17^{lm}	64.11±1.56^{m}
360	86.10±2.96^{l}	78.95±1.19^{de}	83.27±1.71^{ghi}	88.38±0.93^{n}	77.56±2.58^{mn}	67.99±1.96^{n}

Time (min)	Sample[1)]					
	Native	AS0	AS1	AS2	AS4	AS8
420	-	-	-	-	77.42±0.69^{mn}	68.82±2.22^{n}
480	-	-	-	-	79.37±1.99^{n}	69.05±2.19^{n}
600	-	-	-	-	-	66.83±0.48^{n}
720	-	-	-	-	-	69.24±0.68^{n}

[1)] Native = native waxy corn starch; AS0 = AS control without enzyme addition; AS1 = AS 2,500 U/30 mL-starch suspension; AS2 = AS 5,000 U/30 mL; AS4 = AS 10,000 U/30 mL; AS8 = AS 20,000 U/30 mL.

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

4. Determination of starch fractions based on LOS plot

LOS plots of native waxy corn starch and AS0 displayed a single line (Figure 3A, 3B), supported by high determination coefficient (R^2=0.9449 and R^2=0.9570, respectively). It strongly indicated these starches hydrolyzed at the same rate and did not consist of distinct structures that differ in digestion rate. C_∞ calculated according to the obtained LOS linear equation closely agreed with the actually measured degree of hydrolysis values over the plateau range (Table 4). The digestibility constant for native starch was lower than that for AS0. Hydrolysis of starch predominantly occurs in the amorphous regions of the granule (Gallant et al., 1992). All digestible part is amorphous in AS0, thus rapid and singular digestion rate of AS0 can be understood by the same account for high amount of RDS measured by the Englyst test (Zhang et al., 2006b). Rather slow and simultaneous digestion of native starch is supported by the digestion mechanism of native A type starch, generally known as inside-out digestion pattern (Benmoussa et al., 2004). It is initiated by the migration of amylolytic enzymes through the channels into inside of a starch granule from surface pores (Fannon et al., 1992). Due to the tight linkage between adjacent amorphous and crystalline layers, two regions transform together throughout the digestion process though

hydrolysis of amorphous regions are favored. Therefore, digestion proceeds evenly leading to a constant slow digestion profile (Zhang et al., 2006a).

A LOS plot of the digestibility curve of AS1 revealed a discontinuity around 15 min of digestion time (Figure 3C), demonstrating that AS1 is digested in two separated phases. It allowed the identification of rapid and slow phases of hydrolysis with the considerably different rate constants (k=0.2533 min^{-1} for the more rapid phase and k=0.0296 min^{-1} for the slow phase). The final C_∞ generated by the LOS plot (85.14%) agreed very well with the measured data (83.59%). Meanwhile, there was also a little possibility to consider the LOS plot of AS1 as a single equation. However, in this case, the obtained equation was not acceptable due to the following reasons: the determination coefficient was too low (R^2=0.6855), and the C_∞ calculated from a single linear regression had a great gap with actually observed value (data not shown).

The LOS plots of AS2 and AS4 also revealed two distinct lines (Figure 3D, 3E) and thus provided evidence of a fraction that is digested more rapidly than the remainder. The time when the two phases intersect was about 16 min for AS2 and 28 min for AS4. The LOS plot of AS8 (Figure 3F) was better described by a single rate constant rather than two linear graphs. Therefore, the presence of separate rapidly digested and slowly digested

components in AS8 was not conceded. The rate constant (k=0.0108min^{-1}) was similar to the k values of the slower phase of other AS-treated starches.

The kinetic parameters of starch samples estimated by LOS plot are summarized in Table 4. Furthermore, the contents of RDS, SDS, and RS estimated using the parameters from the LOS plot are shown in Table 5. Digestion processes of native, AS0, and AS8 were described by a single rate constant, which presented a high k for AS0 but low k for the others. Other AS-treated starches (AS1-AS4) possessed two rate constants which were significantly different from each other. 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 explained 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 rate constants from AS-treated starches could be categorized into two groups with a reference to k of AS0 and native, according to the assertion that AS0 and native starch can represent the RDS and SDS, respectively (Englyst et al., 1992; Zhang & Hamaker, 2009). The group of k reflecting the first phase of rapid digestion and having values close to that of AS0 was defined as k_{RDS}, and k_{SDS} was the group including the ks of the slow digestion phase and valued at near the k of native. These groups were

clearly distinguished because k_{RDS} had the value of the tenths, whereas k_{SDS} valued from the second decimal place. Digestion kinetic parameters of AS-treated starches implied the distinction between rapid and slow phases in hydrolysis and also suggested the existence of RDS and SDS as a structural feature. That is, after hydrolysis of the rapidly digestible part (RDS), different organization of starch molecules which have different enzyme susceptibility (SDS) was revealed afterwards. It was also verified that AS8 was composed of only SDS fractions.

Patel et al. (2014) stated that both k and C_∞ are strongly related to the increase in degree of order of the α-glucan chains. Decline of the values of k_{RDS} and k_{SDS} was observed as the amount of AS increased, thus structural features of RDS and SDS might be dissimilar among samples.

The content of RDS estimated using the LOS plot decreased by a large extent with the amount of AS, coinciding with the result obtained by the Englyst method. It was also observed that the time of intersection of two lines, or the duration time of more rapid phase, delayed with the level of AS employed. The more time was required for hydrolysis of a lower amount of RDS. In other words, the AS treatment and resultant elongation of branch chains decreased the amount of RDS fraction in recrystallized starch, and induced the more slowly digestible form of RDS.

The increase in SDS content should be understood along with the decrease in k_{SDS}. This implied that SDS with the limited substrate availability for digestive enzyme was produced when the branch chains elongated. A minor change in the amount of SDS was observed in contrast to a great decrease in RDS when comparing AS4 to AS2. It suggested that the high degree of elongation of branch chain would promote the formation of RS instead of SDS. The absence of rapid digestion phase and high amount of RS in AS8 also supported that highly extended AP branch chains did not develop the easily digestible configuration during crystallization.

The amount of each fraction determined by the Englyst method and the LOS method provided no consistency between them. The gap was striking especially for the native and AS8, which turned out to contain only SDS fraction and no RDS. The analysis method suggested by Englyst et al. does not deal with the factor of rate, which corresponds to the terms of 'rapid' or 'slowly' digestible starch. Therefore, the LOS plot approach would be a more reasonable investigative tool for accurate determination of RDS and SDS (Patel et al., 2014).

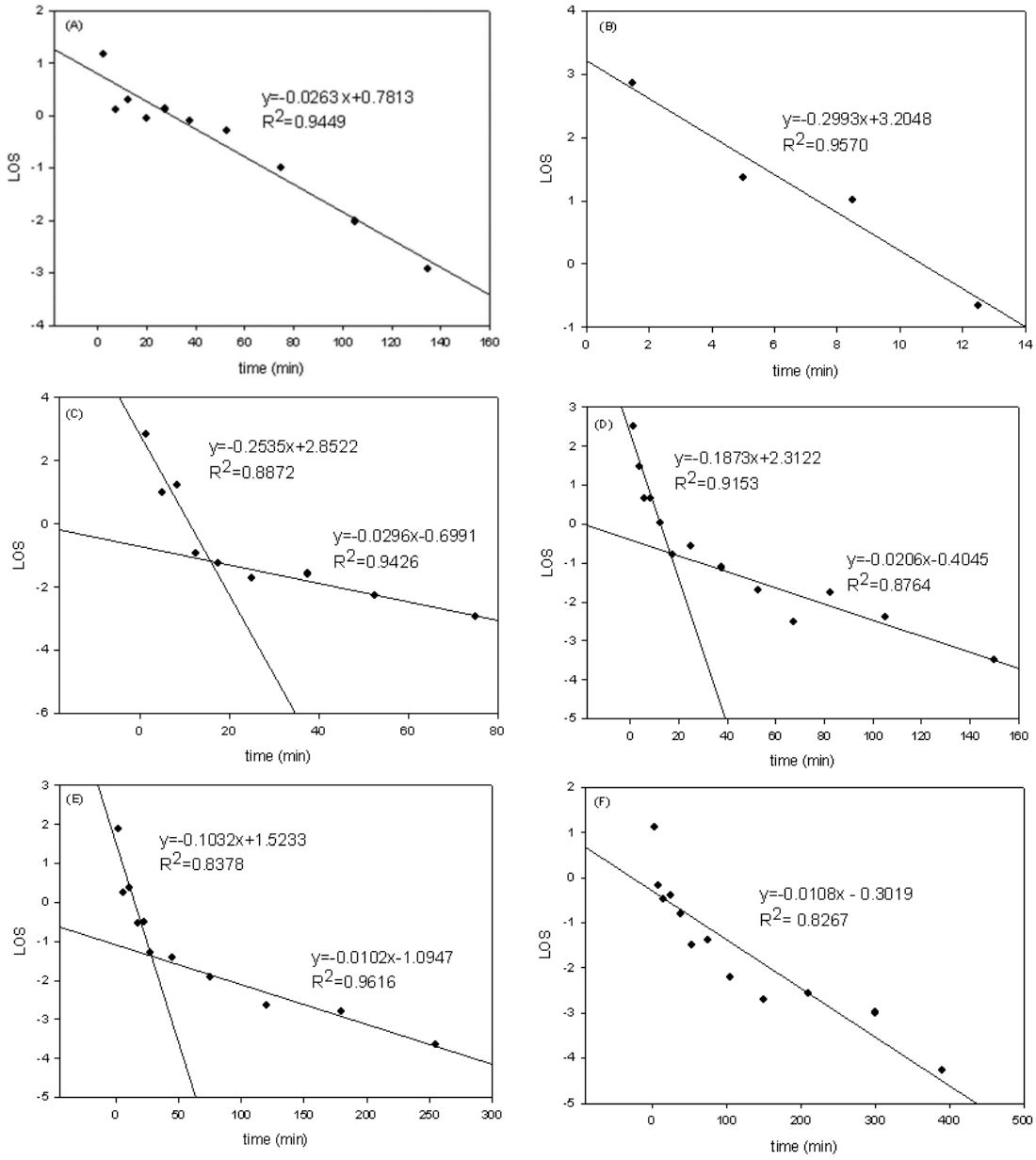


Figure 3. LOS plot of (A) native = native waxy corn starch, (B) AS0 = AS control without enzyme, (C) AS1 = AS 2,500 U/30 mL, (D) AS2 = AS 5,000 U/30 mL, (E) AS4 = AS 10,000 U/30 mL and (F) AS8 = AS 20,000 U /30mL.

Table 4. Hydrolysis kinetic parameters of starch samples estimated from LOS plot

Sample[1)]	k_{RDS}[2)] (min^{-1})	k_{SDS} (min^{-1})	Time of intersect[3)] (min)	Calculated C_∞[4)] (%)	Time of plateau[5)] (min)	Measured C_∞ (%)
Native	N.D.[6)]	0.0263	N.D.	83.05	120	84.34
AS0	0.2993	N.D.	N.D.	82.36	15	81.09
AS1	0.2535	0.0296	15.9	85.14	60	83.59
AS2	0.1873	0.0206	16.3	86.66	150	86.61
AS4	0.1032	0.0102	28.2	77.26	210	77.02
AS8	N.D.	0.0108	N.D.	68.46	360	68.39

[1)] Native = native waxy corn starch; AS0 = AS control without enzyme addition; AS1 = AS 2,500 U/30 mL-starch suspension; AS2 = AS 5,000 U/30 mL; AS4 = AS 10,000 U/30 mL; AS8 = AS 20,000 U/30 mL.

[2)] k_{RDS} = rate constant for the RDS phase of starch hydrolysis; k_{SDS} = rate constant for the SDS phase of starch hydrolysis.

[3)] Time of intersect = the time when the two linear graphs of RDS and SDS intersect.

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

[5] Time of plateau = the time when the degree of hydrolysis reached plateau, revealing no more significant changes.

[6] N.D. = not detected.

Table 5. Contents of RDS, SDS, and RS[1)] of starch samples estimated using the LOS plot

Sample[2)]	RDS (%)	SDS (%)	RS (%)	Measured C_∞[3)] (%)
Native	N.D.[4)]	84.34	15.66	84.34
AS0	81.09	N.D.	18.91	81.09
AS1	73.95	9.64	16.41	83.59
AS2	60.89	25.72	13.39	86.61
AS4	54.33	22.69	22.98	77.02
AS8	N.D.	68.39	31.61	68.39

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

[2)] Native = native waxy corn starch; AS0 = AS control without enzyme addition; AS1 = AS 2,500 U/30 mL-starch suspension; AS2 = AS 5,000 U/30 mL; AS4 = AS 10,000 U/30 mL; AS8 = AS 20,000 U/30 mL.

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

[4)] N.D.= not detected.

5. Branch chain length distribution of AS-treated starches and their digested residues

The chain length distributions of native and the AS-treated starches before and after removal of RDS or SDS fraction are presented in Figure 4 and Table 6. Changes in the amount of the polymer chains following *in vitro* digestion of AS-treated starches were displayed in Figure 5. The quantity of starch residues recovered from AS0 and AS1 was too small for a further investigation. Thus, those were excluded from structural analysis.

When the RDS fraction was removed, the fractions of DP 25-36 and DP ≥ 37 decreased by a significant level. Most of the decrease was observed in the long chains with particular DP of over 27 or 28 (AS2 and AS4, respectively), implying that long chains contributed to the formation of RDS fraction. Very long chains of DP ≥ 37 were hardly detected after hydrolysis of SDS part. DP 25 was revealed to be a breakpoint: the proportion of shorter chains below DP 25 increased, but the longer chains decreased significantly. A decrease in the DP of detectable longest branch chain was commonly found within all AS starches, in the order of RDS+SDS+RS > SDS+RS > RS fractions. The percentage of chains with DP 13-24 increased markedly as hydrolysis proceeded. Therefore, the isolated RS fracions from all AS

starches had the branch chains with DP 13-24 to a great extent. Other workers have reported that isolated RS from recrystallized starches of different origin consist of chains with average DP 19-26 (Eerlingen et al., 1993) and 13-17 (Lopez-Rubio et al., 2008). The common average DP (DP 17-18) of the RS obtained in the current study was close to the previous reports.

The isolated RS fraction from native waxy corn starch preserved the branch chain distribution of original starch granule (Figure 4A). This gap between native starch and AS-treated starches in the DP change pattern after hydrolysis could be caused by the different digestion pattern. Native granular starch is hydrolyzed by dynamic side-by-side digestion mechanism (Zhang et al., 2006a) which involves with the inside-out digestion pattern as discussed above. However, the AS-treated starches, including AS0, were gelatinized once during preparation. Therefore, the original granular properties such as surface pinhole and crystalline packing were lost. Recrystallization in the distinct manner with native crystalline state caused the altered digestion pattern. The resistance of recrystallized processed starches to digestive enzymes could be caused by the acquisition of a double-helical order (Colonna et al., 1992). The stabilization of double helices into crystalline structure lowers the susceptibility of starch to digestion enzymes by

decreasing the effective surface area and the concomitant diffusion and adsorption of the enzyme onto the solid substrate. Non-crystalline double helices and entrapped amorphous regions within imperfect crystals also induce resistance to enzymatic digestion in recrystallized starches (Cairns et al., 1995; Gidley et al., 1995). Thus, the crystalline features related to double helical order was analyzed by X-ray diffraction.

Table 6. Comparison of percent distribution of branch chain length of starch samples before and after removal of RDS or SDS[1)] fraction

Sample[2)]		Percent distribution (%)				
		DP[3)] 6-12	DP 13-24	DP 25-36	DP ≥ 37	average DP
Native	SDS+RS	30.22±0.43[a5)]	52.60±0.55[f]	12.62±0.51[g]	4.56±0.45[c]	17.87±0.23[i]
	RS[4)]	26.38±2.48[b]	56.06±1.83[e]	12.94±0.98[g]	4.62±0.34[c]	18.28±0.38[h]
AS2	RDS+SDS+RS	11.43±0.13[c]	62.52±0.29[d]	21.07±0.17[e]	4.98±0.25[c]	20.88±0.13[d]
	SDS+RS	10.67±1.30[c]	68.67±0.79[b]	19.46±0.70[f]	1.19±0.20[e]	19.72±0.25[f]
	RS	8.91±0.69[d]	78.11±0.12[a]	12.97±0.68[g]	0.00±0.00[f]	18.75±0.21[g]
AS4	RDS+SDS+RS	5.47±0.21[e]	56.82±1.82[e]	31.17±0.81[b]	6.54±0.82[b]	23.38±0.29[b]
	SDS+RS	8.15±0.44[d]	62.48±0.12[d]	26.91±0.27[d]	2.46±0.06[d]	21.37±0.10[c]
	RS	7.64±0.63[d]	70.29±1.05[b]	21.65±0.52[e]	0.43±0.05[f]	20.17±0.09[e]
AS8	SDS+RS	2.53±0.15[f]	46.30±1.02[g]	42.36±0.33[a]	8.80±0.77[a]	25.68±0.22[a]
	RS	5.38±0.12[e]	65.81±0.79[c]	28.24±0.73[c]	0.57±0.17[ef]	21.38±0.16[c]

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

[2)] Native = native waxy corn starch; AS2 = AS 5,000 U/30 mL-starch suspension; AS4 = AS 10,000 U/30 mL; AS8

= AS 20,000 U/30 mL.

[3)] DP = degree of polymerization.

[4)] RS = resistant starch.

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

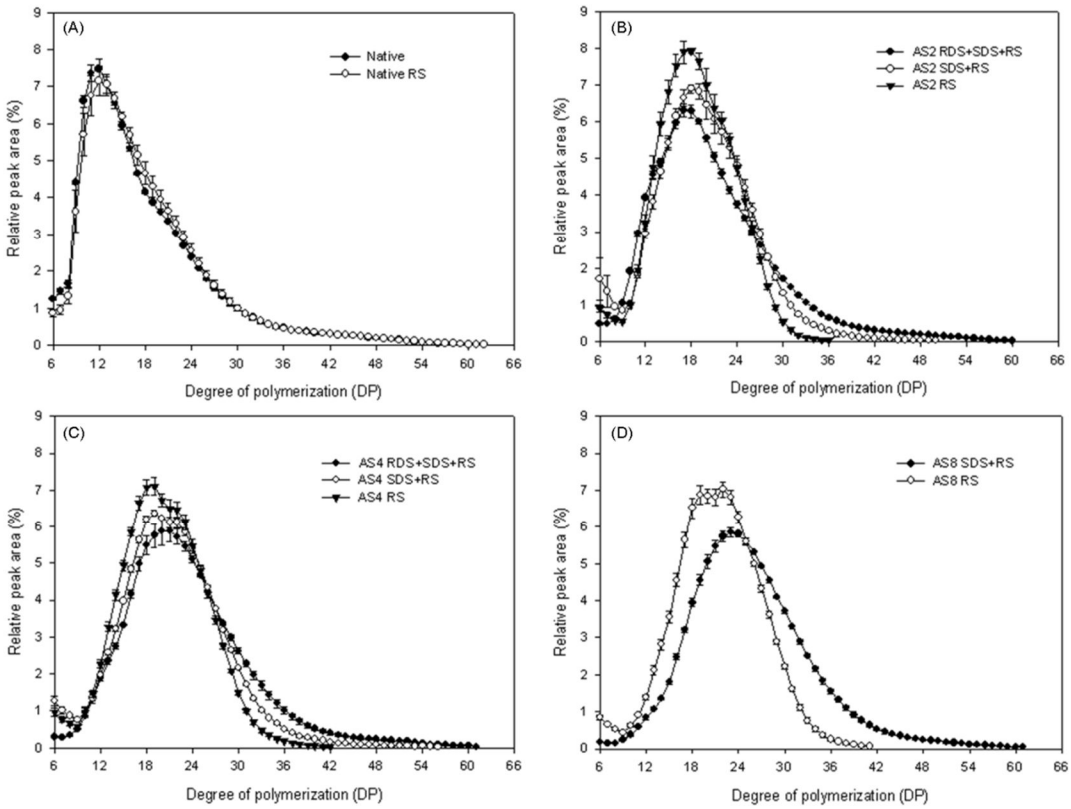


Figure 4. The branch chain length distributions of starch samples determined before and after removal of RDS or SDS fraction. (A) Native = native waxy corn starch; (B) AS2 = AS 5,000 U/30 mL-starch suspension; (C) AS4 = AS 10,000 U/30 mL; (D) AS8 = AS 20,000 U/30 mL. RDS = rapidly digestible starch; SDS = slowly digestible starch; RS = resistant starch.

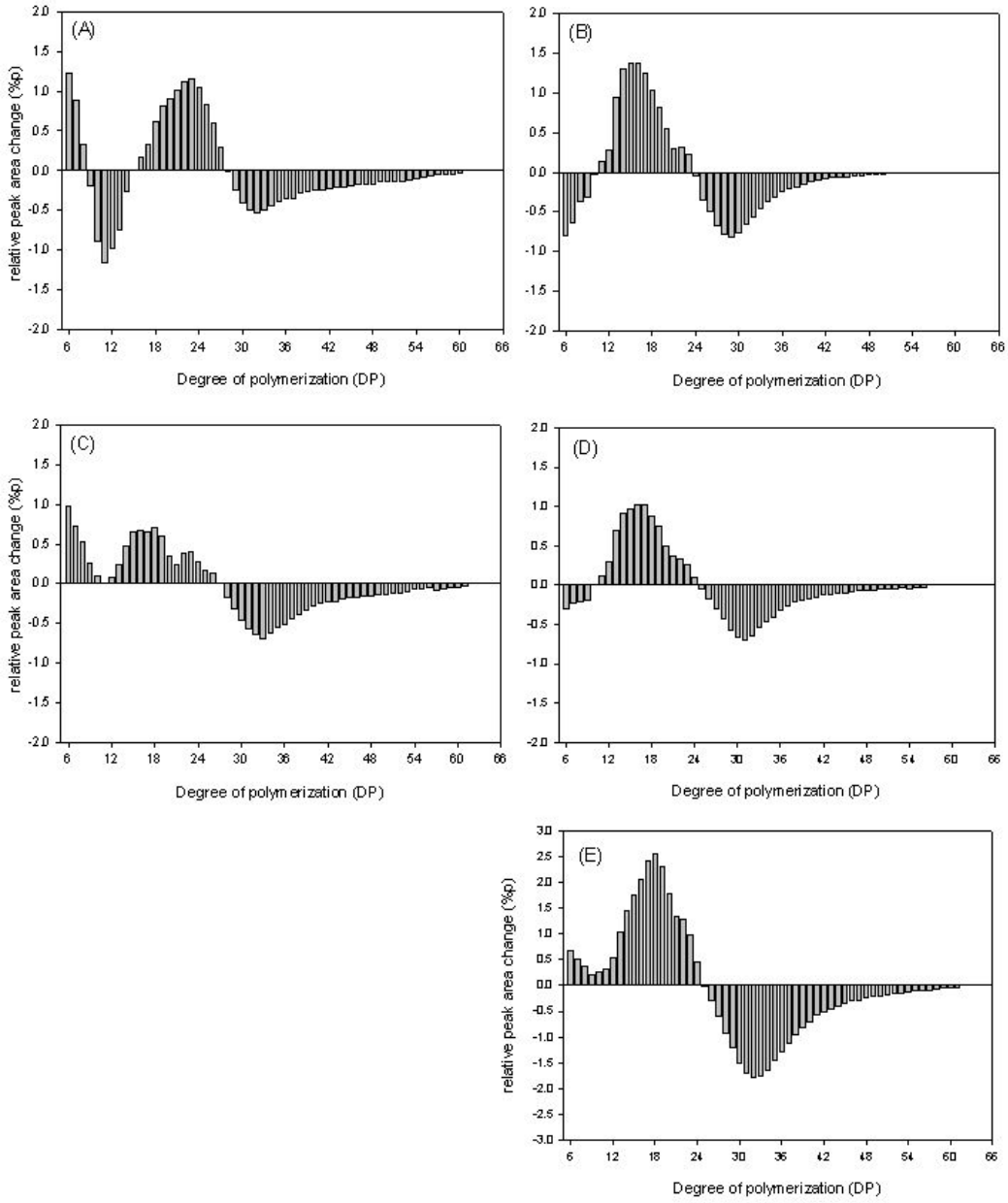


Figure 5. Changes in the branch chain length distributions of starch samples caused by removal of RDS (left) or SDS (right) fractions. (A), (B) AS2 = AS 5,000 U/30 mL-starch suspension; (C), (D) AS4 = AS 10,000 U/30 mL; (E) AS8 = AS 20,000 U/30 mL. RDS = rapidly digestible starch; SDS = slowly digestible starch; RS = resistant starch.

6. X-ray diffraction and relative crystallinity of AS-treated starches and their digested residues

The crystalline packing arrangements of the starch samples and their digested residues, which indicate isolated SDS+RS or RS fraction, were investigated with X-ray diffraction analysis.

Starch molecules involved in crystalline alignment give rise to the peaks in X-ray diffractograms, whereas starch molecules in amorphous regions contribute to the diffused regions of the XRD patterns (Shrestha et al., 2012). The native waxy corn starch displayed a typical pattern of A type starch (Figure 7) with major peaks at 15°, 17°, 18°, and 23° 2θ (Hizukuri et al., 1980) and the relative crystallinity of 44.8 %. AS0 did not present any noticeable major peak, but a slight rise near 13°, which seemed to be an influence of annealing by 6 h incubation during sample preparation. The relative crystallinity of AS0 was considerably reduced compared to that of native starch, due to the crystalline disruption and the dissociation of double helical structure during the preparation process.

The disrupted crystalline structure was regenerated after AS treatment, revealing peaks at 5.5°, 14.5°, 17°, 19.3°, 22°, and 24° (Figure 7). Therefore, it was recognized that the crystalline structure of the waxy corn starch

changed into B type polymorph after AS reaction, which corresponded to the previous reports (Kim et al., 2013; Kim et al., 2014; Shin et al., 2010). Branch chain elongation resulting from the action of AS facilitated and solidified the inter-chain association, which in turn led to the stable B-type polymorph (Ryu et al., 2010). Therefore, B type peaks of AS-treated starches got more raised and sharpened, and peak intensity increased as the amount of AS increased. The relative crystallinity of AS-treated starches also gradually increased from A1 to A8.

The X-ray diffraction patterns and relative crystallinity of starches are shown after removal of RDS or SDS fraction in Figure 8 and Table 6. Removal of SDS fraction of native waxy corn starch caused the reduction in the intensity of the major crystalline peaks at 17°, 18°, and 23° as well as the amorphous curve (Figure 8A) and also an increase of relative crystallinity. A similar increase in crystallinity after digestion was reported in the work of Shrestha et al. (2012) for normal maize starch. Less ordered amorphous regions are more easily hydrolyzed than the ordered crystalline regions, indicating that hydrolysis by amylase predominantly occurs in the amorphous regions of the granule (Gallant et al., 1992). Native granules of waxy corn starch have a semicrystalline structure composed of alternating concentric crystalline lamellae and amorphous regions. Therefore, this

phenomenon in the native starch could be explained by that only selected regions are digested primarily, leaving the undigested crystalline regions relatively unchanged (Shrestha et al., 2012).

In AS2 and AS4, the relative crystallinity increased in the SDS+RS and RS fractions, and the increase was higher in the latter (Table 6) due to the removal of less ordered regions in RDS and SDS. In spite of no alteration in the B type diffraction pattern, the peak intensity was the highest in the isolated RS fractions among three states of samples (Figure 8B, 8C), demonstrating that the remaining regions had more densely packed and well-organized crystalline structure. AS8, which contained only SDS and RS, showed a similar change when the SDS part was removed (Figure 8D). In general, differences in crystallinity between starches are attributed to the amount of crystalline regions, size of crystal, orientation of the double helices within the crystalline domains, and extent of interaction between double helices (Miao et al., 2009). The degree of elongation seemed to be the primary factor designating the crystalline properties of AS-treated starches as well as the residual samples (SDS+RS or RS).

The AS-treated starches were produced by recrystallization, which was accompanied by the destruction of the unique surface properties and crystalline packing of native granules. This change in granular state

prohibited the enzyme diffusion into granule interior through pinholes existing at surface, a well known digestion principle of native A type starches (Huber & BeMiller, 2000). During modification of starch molecules with AS, the reconstruction of different crystallite packing and arrangement from the native starch occurred by the association of extended AP chains (Shin et al., 2010). This phenomenon might be considered similar to retrogradation; therefore digestion property of the AS-treated starch samples should be described in relation with that of retrograded starches.

The increase in crystallinity results in fewer available α-glucan chains which digestive enzymes can bind with, and thus reduces the susceptibility of retrograded starch to digestion (Htoon et al., 2009; Liu et al., 2007). In accordance with the increased crystallinity, the experimentally determined C_∞ value of AS starches decreased, meaning the increase of RS. The principal mechanism for the formation of RS in amylose solutions was proposed by Eerlingen et al. (1993) to be the aggregation of amylose helices in the crystalline B type structure over a particular region of the chain. These researchers also suggested that long amylose helices present micelle formation and chain folding (lamellar structures), corresponding to four turns of one of the amylose chains of a double helix (Figure 9). Thus, extended branch chains of AP of AS-treated starches possibly arranged into a similar

formation. The high AS level, which informs the abundant existence of long chains, accelerates the formation of double helices during recrystallization (Kim et al., 2014; Shin et al., 2010). The higher the degree of polymerization of AP is, the longer double helices would be produced. In addition, the α-1,6-linked branches of AP limit the space for arrangement of double helices. Therefore, not only the number of double helix chain folding would increase, but also the distance between double helices would be narrowed, resulting in more dense crystalline structure. These constrained conformations may restrict enzyme hydrolysis in a manner related to their proximity to the crystalline regions (Mutungi et al., 2011). This would support the high amount of RS in AS4 and AS8, possessing noticeably abundant long chains.

SDS consists of mainly imperfect crystalline regions containing small portions of double helices as well as amorphous regions (Shin et al., 2004). The part of SDS of AS-treated waxy corn starches would be formed by the alignment of adjacent single helices, exposed part of double helix turns out of crystalline lamella, non-crystalline double helical structures, and other conformations caged within imperfect region of crystals. Branch chains of DP 13-24 and DP ≥ 25 mainly contribute to these conformations, according to the chain length distribution of SDS+RS and the changes after the removal of SDS. Moreover, the part of single helix not associated into double helices

yet exposed out of micelle-conformation would be a component of RDS. Thus, long chains (DP ≥ 27 or 28) in this part disappeared as a result of RDS hydrolysis. This explanation would be a plausible reason for the high amount of short chains (DP 13-24) and lack of longer chains in undigested RS (Table 6).

Comprehensively, the branch chain length distribution of AP determined the primarily generated crystallite organization of AS-treated starches. Different crystalline arrangement caused the different structure of RDS and SDS, affecting the extent of hydrolysis. The remaining structure after hydrolysis was also affected accordingly. The different structures of RDS and SDS among AS starches were reflected in the different values of k_{RDS} and k_{SDS}.

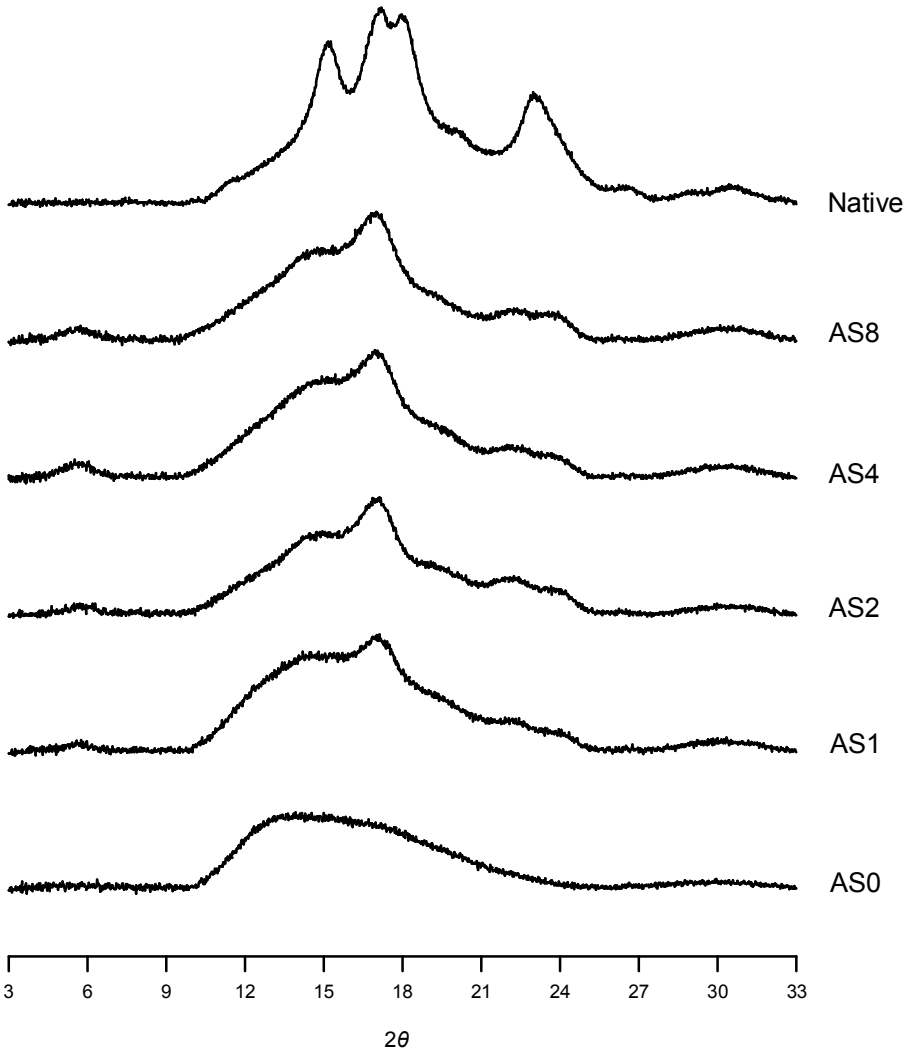


Figure 6. X-ray diffraction patterns of starch samples. Native = native waxy corn starch; AS0 = AS control without enzyme addition; AS1 = AS 2,500 U/30 mL-starch suspension; AS2 = AS 5,000 U/30 mL; AS4 = AS 10,000 U/30 mL; AS8 = AS 20,000 U/30 mL.

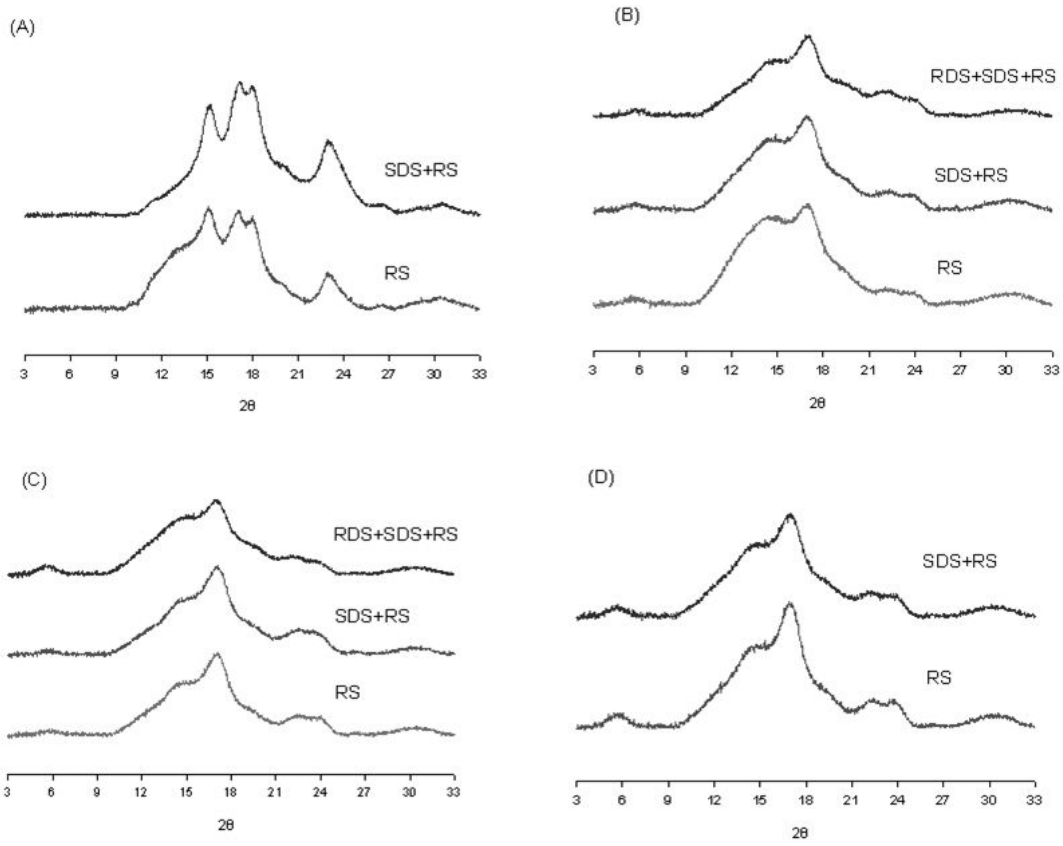


Figure 7. Comparison of X-ray diffraction patterns of starches samples before and after removal of RDS or SDS fraction. (A) Native = native waxy corn starch; (B) AS2 = AS 5,000 U/30 mL; (C) AS4 = AS 10,000 U/30 mL; (D) AS8 = AS 20,000 U/30 mL. RDS = rapidly digestible starch; SDS = slowly digestible starch; RS = resistant starch.

Table 7. Relative crystallinity of starch samples and their digested residues

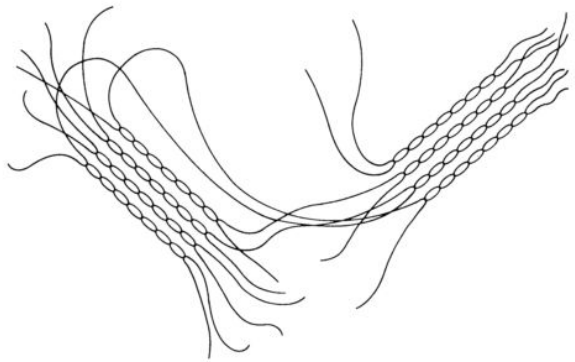
Sample[1)]		Relative crystallinity (%)
Native	SDS+RS[2)]	41.5±1.0[b3)]
	RS	51.1±0.5[a]
AS0		12.7±0.5[j]
AS1		14.6±1.0[i]
AS2	RDS+SDS+RS	16.5±0.4[j]
	SDS+RS	19.2±1.0[g]
	RS	23.2±1.0[e]
AS4	RDS+SDS+RS	21.9±0.4[f]
	SDS+RS	25.1±0.4[d]
	RS	27.4±0.4[c]
AS8	SDS+RS	24.6±0.4[d]
	RS	27.8±0.5[c]

[1)] Native = native waxy corn starch; AS0 = AS control without enzyme addition; AS1 = AS 2,500 U/30 mL-starch suspension; AS2 = AS 5,000 U/30 mL; AS4 = AS 10,000 U/30 mL; AS8 = AS 20,000 U/30 mL.

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

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

(A)



(B)

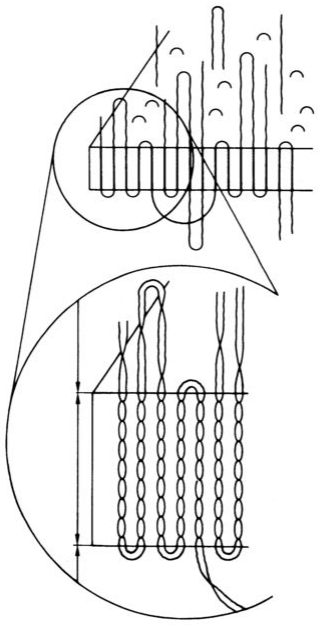


Figure 8. Micelle model (A) and lamella model (B) for the formation of crystalline structures in amylose solutions proposed by Eerlingen et al. (1993).

CONCLUSION

AS-treated starches which are known to possess abundant SDS were employed in this study to verify the existence of RDS and SDS as a structural feature. LOS plot of AS-treated starches allowed the identification of rapid and slow phases in hydrolysis, which demonstrated the presence of individual RDS and SDS fractions. Therefore, it was proved that the concept of RDS, SDS, and RS suggested by Englyst are valid, though the standard for classification described in that study was not proper. LOS plot method utilized in this study would be possibly employed as an alternative tool for fractionation of starch into RDS, SDS, and RS.

The current study illustrated that different branch chain length of AP designated the primary crystalline arrangement of recrystallized starches and accordingly determined the amount and structure of RDS and SDS. Different values of k_{RDS} and k_{SDS} observed in the starches with different AP branch chain length reflected the different structure of RDS and SDS.

The digestible fraction of highly AS-treated starch (AS8) consisted of solely SDS, thus development of industrial SDS product seemed promising. Further study requires comparison with *in vivo* test and more specific analysis of structural features.

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국문초록

Englyst 등은 전분을 소화되는 시간대를 분류 기준으로 하여 속소화성 전분 (rapidly digestible starch, RDS), 지소화성 전분 (slowly digestible starch, SDS), 난소화성 전분 (resistant starch, RS)의 세 가지 획분으로 이루어져 있다고 제안하였다. 그러나 Goñi 등이 제시한 1차 반응식을 이용한 분석법으로부터 전체 가수분해 과정에서 1개의 반응속도상수 만이 도출되므로 RDS와 SDS의 구분은 무의미하다는 주장도 제기되었다. 이 연구에서는 찰옥수수 전분에 amylosucrase (AS)를 처리하여 SDS 함량이 증가된 AS 변형 전분을 제조하였으며, 1차 반응식에 바탕을 둔 log of slope (LOS) 법을 이용하여 가수분해 유형을 분석하고 가수분해에 따른 가지사슬 길이 분포 및 X-선 회절 양상의 변화를 비교하였다. 첨가한 AS의 양이 증가할수록 (AS1-AS8) 긴 사슬의 양에 대한 짧은 사슬의 비율은 감소하였으며, Englyst법으로 측정한 SDS의 양은 증가하였다. AS 변형 전분의 시간에 따른 가수분해율은 LOS plot에서 반응속도상수 k를 기울기로 가지는 1차식 그래프로 표현되었다. 생전분 및 대조구의 가수분해 유형은 1개의 직선으로 나타나 전 구간에서 같은 속

도로 가수분해됨을 보였다. AS 변형 전분들의 가수분해 유형에서는 반응 중 k가 바뀌는 지점이 발견되었으며, 이로써 한 전분의 가소화성 부분이 서로 다른 가수분해속도를 보이는 두 종류의 구조, 즉 RDS와 SDS로 불릴 수 있는 두 개의 구조로 이루어져있음을 확인하였다. AS 변형 전분의 반응속도상수는 각각 대조구의 높은 k와 생전분의 낮은 k를 기준으로 하여 2가지 그룹으로 분류되었으며, 기준으로 사용된 전분의 소화성에 따라 이를 각각 k_{RDS}, k_{SDS} 로 명명하였다. 한편 고도로 AS 처리된 전분은 (AS8) k_{SDS}만을 보여 모든 가소화성 부분이 SDS만으로 이루어져 있음을 시사하였다. LOS법으로 추정한 각 획분의 함량은 AS 처리에 따라 RDS는 감소, SDS는 증가하였으며, 이때 k_{RDS}와 k_{SDS} 는 모두 감소하였다. 따라서 AS에 의해 사슬 길이가 길어질수록 RDS와 SDS는 덜 소화되는 구조로 형성됨을 알 수 있었다.

LOS법으로 설정한 기준점에 따라 RDS, SDS획분을 제거하였을 때 나타나는 전분의 구조 변화를 분석하였다. RS를 이루는 사슬은 대부분 DP 13-24의 비교적 짧은 길이를 나타내었으며, SDS의 가수분해 시에는 DP ≥ 25, RDS의 가수분해 후에는 DP ≥ 37 의 매우 긴 사슬이 사라진 것으로부터 각각의 사슬들이 SDS 및 RDS의 구조에

관여함을 확인하였다. AS 변형 전분은 X-선 회절에서 B형을 나타냈으며, 처리한 AS 양에 따라, 또한 RDS 및 SDS 획분이 제거됨에 따라 결정화도가 증가하였다. 결론적으로, 아밀로펙틴 가지 사슬 길이는 재결정화 전분의 일차적인 결정 특성을 결정하였으며, 이에 따라 소화 특성도 다르게 나타났다. AS 처리 전분들 간 서로 다른 k_{RDS} 및 k_{SDS}는 상이한 RDS 및 SDS의 구조 특성을 반영하였다.

주요어: 지소화성 전분, 아밀로수크레이스, 가지 사슬 길이 분포, 소화율, 1차반응식

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