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Kinetic studies of *in vitro* digestion of amylosucrase-modified waxy corn starches based on branch chain length distributions

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ABSTRACT

Classification of starch into rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) has been in controversy. Therefore, this study aimed to ascertain the individual existence of RDS and SDS using amylosucrase-modified starch. Enzymatic hydrolysis curves of modified waxy corn starches were obtained and fitted to a logarithm of slope (LOS) plot. LOS plots for amylosucrase-modified starches revealed a discontinuity, reflecting the change of rate constant k during digestion, such that distinguishable k s for each of the phases were defined (k_{RDS}, k_{SDS}). The LOS plot approach could be utilized as a persuasive basis for the classification of the starch fractions. The structure of amylosucrase-modified starches was characterized before and after removal of RDS and/or SDS. Branch chains with certain lengths contributed to the organization of each fraction and determined the primary crystallite configuration of amylosucrase-modified starches causing the diversity of digestion properties.

1. Introduction

Starch is the main source of digestible carbohydrate in the human diet, and the glucose generated from starch digestion plays an important role in energy metabolism. 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, Day, Butterworth, & Ellis, 2014). In this regard, Englyst, Kingman, and Cummings (1992) introduced a classification system to describe the starch digestion properties with reference to a specific time frame: rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS). This classification implies that starch granules contain individual fractions that have different enzyme susceptibility and has been widely quoted in numerous studies. However, some researchers argue that the classification into RDS, SDS, and RS is not a proper way to describe the digestion behavior of starch granules, since digestibility data proceed as a first-order reaction described by a single rate constant for cooked or gelatinized starches regardless of the 'Englyst' classification (i.e., all digestible fractions have the same intrinsic reactivity) (Dhital, Shrestha, & Gidley, 2010; Goñi, Garcia-Alonso, & Saura-Cailxto, 1997). To account for the discrepancy of the previous studies, 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, Warren, Grassby, Patel, & Ellis, 2012; Patel et al., 2014). This 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. They 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 rate constant. Therefore, the LOS plot approach would be a useful investigative tool for accurate determination of the RDS and SDS starch fractions, if present, from discontinuities in the linear plot (Patel et al., 2014).

A few studies employed amylosucrase (AS; EC 2.4.1.4.) from *Neisseria polysaccharea* to produce starches with extended amylopectin branch chains (Kim, Kim, Moon, & Choi, 2014; Kim et al., 2013; Shin, Choi, Park, & Moon, 2010). AS is a glucosyltransferase that produces an insoluble α-1,4-linked glucan polymer by the consumption of sucrose. When the starch exists as a glucose acceptor, elongation of the glucosyl units occurs at non-reducing ends of the external chains (Buttcher, Welsh, Willmitzer, &

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Kossmann, 1997; Potocki de Montalk et al., 2000; Rolland-Sabaté, Colonna, Potocki-Véronèse, Monsan, & Planchot, 2004). A noticeable increase of SDS and RS contents in waxy starches with AS modification was reported (Shin et al., 2010). The study on the increment of the SDS content of waxy corn starch through amylosucrase modification from 1 to 6 h supported the idea that the SDS content has a parabolic relationship with the weight ratio of short chains to long chains of amylopectin in maize starches (Kim et al., 2014; Zhang, Ao, & Hamaker, 2008).

The objectives of this study were to ascertain the individual existence of the RDS and SDS fractions in a single starch and to compare the structural characteristics of each fraction in amylosucrase-modified starches. On the basis of the suggestion that the slow digestion property of the starch could be manipulated according to the fine structure of amylopectin, waxy corn starch was modified with amylosucrase at various levels to obtain starch samples with different SDS contents. Digestibility of amylosucrase-modified starches was examined and discussed with regard to its first-order kinetic parameters and structural properties.

2. Materials and methods

2.1. Materials

Waxy corn starch was obtained from Ingredion (Westchester, IL, USA). α-Amylase from porcine pancreatin (type VI-B, A3176, activity 30 U/mg solid) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Amyloglucosidase (AMG 300 L, activity 300 AGU/mL), isoamylase (activity 1,000 U/mL), and a GOD-POD assay kit were obtained from Novozymes (Bagsvaerd, Denmark), Megazyme (Bray, Ireland), and Embiel Co. (Gunpo, Korea), respectively. All other reagents were of analytical grade.

2.2. Amylosucrase activity assay

The gene of AS from *Neisseria polysaccharea* was cloned and expressed in *E. coli* BL21. Its purification and activity analysis was carried out following previous studies (Jung et al., 2009; van der Veen et al., 2004). An aliquot of diluted enzyme (0.05 mL) was mixed into a solution composed of 0.1 mL 4% (w/v) sucrose, 0.1 mL 1% (w/v) glycogen, and 0.25 mL 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 (Kim et al., 2014).

2.3. Preparation of AS-modified starches

Starch was dispersed in 100 mM sodium acetate buffer (pH 7.0) with 100 mM sucrose to make a 2% (w/v) suspension. 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: AS1, AS2, AS4, and AS8, respectively. Three volumes of ethanol were added to stop the enzyme reaction, and the AS-modified starch was precipitated by centrifugation (10,000*g*, 10 min). The pellet was washed with distilled water 3 times, freeze-dried, ground, and passed through a 100-mesh sieve. The control (AS0) was prepared according to the same procedure without enzyme addition.

2.4. Measurement of degree of starch hydrolysis

The degree of hydrolysis was determined throughout the incubation period (0–720 min) following the method of Shin et al. (2004) with modification. Pancreatic α-amylase (4.51 g) was suspended in distilled water (17 mL) by magnetic stirring for 10 min. After centrifuging at 1,500*g* 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, 4 mM $CaCl_2$, made with benzoic acid saturated distilled water) 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 microtube. The tubes were removed after certain reaction times in a shaking incubator (240 rpm, 37 °C) and boiled for 10 min to terminate the hydrolysis. The glucose in the supernatant, released by the hydrolysis of starch, was measured using a GOD-POD kit after centrifugation at 5,000*g* for 10 min.

2.5. Determination of starch fractions using the Englyst method

Starch fractions were determined according to the degree of hydrolysis following the method of Shin et al. (2004) The amount of RDS was determined by the quantity of glucose after digestion for 10 min. SDS was the fraction digested between 10 and 240 min. The undigested fraction that remained after 240 min was defined as RS. The contents of RDS, SDS, and RS obtained were very similar to those determined using the original method of Englyst et al. (1992).

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

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

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

where C_t is the concentration of product, in other words, the degree of hydrolysis at time t. C_∞ is the corresponding concentration at the end point which is understood as the total starch digested, and k is a first order rate constant of *in vitro* digestion. Differentiation of the 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 shows a linear graph with a slope of $-k$. The y-intercept of the graph equals $\ln(C_\infty k)$; thus, the value of k can be calculated from the slope of the plot, which is referred to as a logarithm of the slope (LOS) plot (Poulsen, Ruiter, Visser, & Iversen, 2003). 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.

At the last stage of *in vitro* digestion, the ΔC was almost zero;

thus, the experimental points in that region were excluded for LOS plotting and determined as the RS region. The slope is sensitive to the change in k that occurs during a reaction, which would be revealed by the 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 the steeper slope was determined to be RDS, and the latter part was determined as SDS.

2.7. Preparation of digested starches for isolation of SDS + RS fraction or RS fraction

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

2.8. Determination of branch chain length distribution

The branch chain distribution of the starch samples was determined after debranching by isoamylase using a high-performance anion exchange chromatography system (Dionex-300, Dionex, Sunnyvale, CA, USA) with a pulsed amperometric detector according to a previous study (Kim et al., 2014). Starch samples (15 mg) were dissolved in 90% dimethyl sulfoxide (3 mL) and boiled for 30 min. Ethanol (15 mL) was added to the solution and centrifuged at 10,000*g* for 10 min. The starch pellet was resuspended with distilled water (1.5 mL) and 50 mM sodium acetate buffer (1.5 mL, pH 4.3) and boiled for 15 min mixed. Isoamylase was added to the starch suspension (200 U/g starch) and then incubated at 45 °C and 30 rpm for 2 h in a shaking water bath. The enzyme reaction was stopped by boiling for 10 min. The debranched sample was filtered through a 0.45-μm membrane filter and analyzed using high performance anion exchange chromatography with pulsed amperometric detection on a Carbo-Pack PA1 anion-exchange column (4 × 250 mm, Dionex, Sunnyvale, CA, USA) with a pulsed amperometric detector. This analysis was performed by using a gradient increase of 600 mM sodium acetate in 150 mM NaOH solution against 150 mM NaOH for sample elution as follows: 0–20% for 0–5 min, 20–45% for 5–30 min, 45–55% for 30–60 min, 55–60% for 60–80 min, 60–65% for 80–90 min, 65–80% for 90–95 min, and 80–100% for 95–100 min.

2.9. Analysis of 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 a 2θ range from 3° to 30° with a 0.02° step size and a count time of 2 s. The relative crystallinity was calculated (Nara & Komiya, 1983) using the software developed by the instrument manufacturer (EVA, 2.0).

2.10. Statistical analysis

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

3. Results and discussion

3.1. Preparation of starch samples with different branch chain length distributions

Branch chains of amylopectin were classified into four groups having chain lengths of degree of polymerization (DP) 6–12, 13–24, 25–36, and ≥37, respectively (Hanashiro, Abe, & Hizukuri, 1996). The native waxy corn starch had abundant chains of DP < 25, but a smaller proportion of longer chains (Table 1), as reported in a previous study (Zhang, Venkatachalam, & Hamaker, 2006). AS0 showed no significant difference compared with native starch because there was no enzyme addition. A dramatic change was observed after AS-modification compared with AS0. An increase in the average chain length and a decrease in the proportion of short chains (DP 6–12) were observed, as AS catalyzes the elongation of some branch chains by attaching 12 to 18 glucosyl units at non-reducing ends (Potocki de Montalk, Remaud-Simeon, Willemot, Planchot, & Monsan, 1999). Further, the degree of decrease in this range (DP 6–12) according to AS level was remarkable, and the chains with DP 13–24 had the largest proportion for all AS-modified starches, which corresponded to earlier studies (Kim et al., 2013; Kim et al., 2014; Ryu et al., 2010). The more AS that was added, the lower was the ratio value of short chains (DP < 13) to long chains (DP ≥ 13), ranging from 0.026 to 0.193 for AS8 to AS1.

3.2. Digestion profiles of AS-modified starches

The degree of digestion was measured for a reaction period of 0–360 min or more to clarify the emergence of a plateau. Fig. 1

Table 1
Branch chain length distributions of native and amylosucrase-modified starches.

Sample	Percent distribution (%)				Average DP	S/L
	DP 6–12	DP 13–24	DP 25–36	DP ≥ 37		
Native	30.22 ± 0.43^{a}	52.60 ± 0.55^{d}	12.62 ± 0.51^{e}	4.56 ± 0.45^{c}	17.87 ± 0.23^{e}	0.433^{a}
AS0	29.72 ± 0.53^{a}	52.47 ± 0.30^{d}	12.94 ± 0.47^{e}	4.87 ± 0.35^{c}	18.03 ± 0.20^{e}	0.423^{a}
AS1	16.18 ± 0.46^{b}	60.52 ± 0.43^{b}	18.31 ± 0.56^{d}	4.99 ± 0.24^{c}	19.99 ± 0.19^{d}	0.193^{b}
AS2	11.43 ± 0.13^{c}	62.52 ± 0.29^{a}	21.07 ± 0.17^{c}	4.98 ± 0.25^{c}	20.85 ± 0.13^{c}	0.122^{c}
AS4	5.47 ± 0.21^{d}	56.82 ± 1.82^{c}	31.17 ± 0.81^{b}	6.54 ± 0.82^{b}	23.36 ± 0.29^{b}	0.052^{d}
AS8	2.53 ± 0.15^{e}	46.30 ± 1.02^{e}	42.36 ± 0.33^{a}	8.80 ± 0.77^{a}	25.67 ± 0.22^{a}	0.026^{e}

The values with different superscripts in the same column are significantly different ($p < 0.05$).
DP, Degree of polymerization; S/L, ratio of short chains (DP < 13) to long chains (DP ≥ 13).
Native, native waxy corn starch; AS0, control with no enzyme addition; AS1, amylosucrase modified starch with 2,500 U amylosucrase/30 mL-starch suspension; AS2, amylosucrase 5,000 U/30 mL; AS4, amylosucrase 10,000 U/30 mL; AS8, amylosucrase 20,000 U/30 mL.

displays the enzymatic digestion profiles of native and AS-modified starches.

Native starch reached a plateau at 120 min of digestion, and the observed C_∞ (maximum degree of hydrolysis) was approximately 84%. Although the plateau time for AS0 appeared quite early (15 min), its C_∞ (81.1%) was similar to that of native starch. The high digestion rate of AS0 was due to destruction of the inherent granular structure during the gelatinization process of sample preparation. The amorphous regions generated by gelatinization are easily accessed by digestive enzymes (Zhang, Venkatachalam, & Hamaker, 2006). The resistance to enzymatic hydrolysis of AS-modified starches was greatly influenced by the action of AS. The plateau time was delayed to 60, 150, 210, and 360 min as the amount of AS doubled (AS1, AS2, AS4, and AS8, respectively). The C_∞ exhibited values slightly exceeding 80% for AS1 and AS2, and was considerably decreased in AS4 and AS8 (77.02% and 67.39%, respectively). The requirement of the longer hydrolysis time of the smaller amount of digestible starch (C_∞) clearly demonstrated the slower digestion property caused by AS-modification.

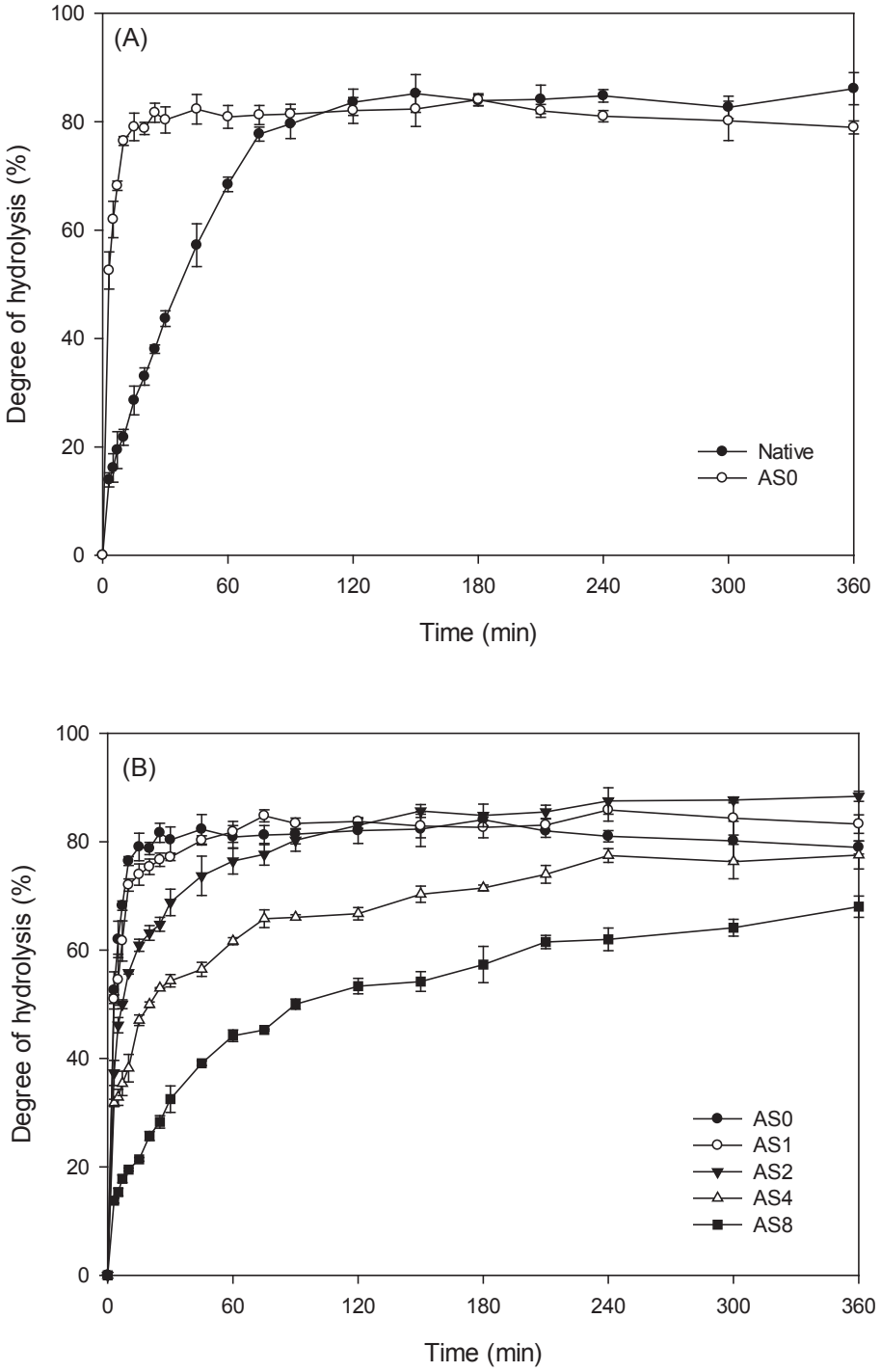

Fig. 1. Digestion profiles of amylosucrase-modified starches (0–360 min).

As the AS-modified starches were recrystallized after gelatinization (Kim et al., 2014), their digestion property could be accounted for based on the following mechanism, which is similar to retrogradation. During the gelatinization of starch, the original crystalline structures of amylopectin get disintegrated, and the polysaccharide chains form a random configuration (Singh, Kaur, & McCarthy, 2007). Then, re-association among them is induced by longer branch chains forming strong, stable, and long double helices, thereby producing a superior crystalline structure. On the other hand, the short or weak double helices formed by short chains would produce an imperfect crystalline structure (Jane et al., 1999; Srichuwong, Sunarti, Mishima, Isono, & Hisamatsu, 2005). Retrogradation of long linear chains is one of the mechanisms for the slow digestion property of starches after gelatinization (Zhang et al., 2008). Thus, in the present study, the AS-modified amylopectin possessing a large amount of long chains and a small amount of short chains could lead to the formation of crystallites with a higher level of perfection than the unmodified one (Kim et al., 2014). Further, the elongation of branch chains by AS would permit the formation of ordered crystallites that inherently had been hindered by α-1,6-linked branch points in waxy type starches (Ryu et al., 2010; Shin et al., 2010). Since the crystalline regions possess low susceptibility to enzymatic hydrolysis (Zhang, Venkatachalam, & Hamaker, 2006), the elongation of branch chains and their accelerated re-association into double helices might improve the slow digestion property of AS-modified starches. To interpret these changes in digestibility in terms of branch chain length and crystallinity, structure analysis was conducted on digested starch residues.

3.3. 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, exhibiting abundant SDS (Lehmann & Robin, 2007). RDS content was the highest in the AS0, decreasing with the amount of employed AS. No change was observed in the SDS content of AS8 compared to AS4, although there was a remarkable reduction of RDS. RS increased with an increase in the amount of AS, which was especially high in AS8 (39.54%). The SDS level showed significant differences ($p < 0.05$) among samples. With an increase in the AS amount, AS-modified starches consequently showed a decrease in the ratio of short chains to long chains, as well as an increase in SDS content, supporting a previous report (Zhang et al., 2008). Thus, the desired amount of SDS could be produced by modifying the branch chain length of amylopectin.

Table 2
RDS, SDS, and RS contents of native and amylosucrase-modified starches determined using the Englyst assay method.

Sample	RDS (%)	SDS (%)	RS (%)
Native	21.77 ± 1.46^{e}	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}

The values with different superscripts in the same column are significantly different ($p < 0.05$).
RDS, rapidly digestible starch; SDS, slowly digestible starch; RS, resistant starch.
Native, native waxy corn starch; AS0, control with no enzyme addition; AS1, amylosucrase modified starch with 2,500 U amylosucrase/30 mL-starch suspension; AS2, amylosucrase 5,000 U/30 mL; AS4, amylosucrase 10,000 U/30 mL; AS8, amylosucrase 20,000 U/30 mL.

3.4. Determination of starch fractions based on LOS plot

LOS plots of native waxy corn starch and AS0 displayed a single line (Fig. 2A and B), supported by a high determination coefficient ($r^2 = 0.945$ and $r^2 = 0.957$, respectively). These results strongly indicated that these starches were hydrolyzed at the same rate over the entire digestion period and did not consist of distinct structures with different digestibility. The C_∞ calculated according to the obtained LOS linear equation closely agreed with the experimentally measured amount of totally digested starch. The k of native starch was lower than that of AS0. Considering that hydrolysis of starch predominantly occurs in the amorphous regions (Gallant, Bouchet, Buleon, & Perez, 1992), the rapid and singular digestion rate of AS0 can be understood by its extremely high amount of amorphous region, classified into RDS by Englyst hypothesis (Zhang, Venkatachalam, & Hamaker, 2006). The particular structure of native A-type starch could account for the rather slow and simultaneous digestion of native starch (Benmoussa, Suhendra, Aboubacar, & Hamaker, 2004). It is initiated by the migration and attachment of amylolytic enzymes to channels penetrating from the surface into the granular interior (Fannon, Hauber, & BeMiller, 1992). Due to the tight linkage between adjacent amorphous and crystalline layers, the two regions are concurrently hydrolyzed, leading to a constant slow digestion profile (Zhang, Ao, & Hamaker, 2006).

A LOS plot of the digestibility curve of AS1 revealed a discontinuity around 15 min of digestion (Fig. 2D). It demonstrated that AS1 consisted of distinct fractions having different digestion rates, and that these distinct fractions were identified with considerably different rate constants ($k = 0.254$ min^{-1} for the rapidly digested fraction and $k = 0.030$ min^{-1} for the slowly digested fraction). The final C_∞ calculated from the LOS plot (85.14%) agreed very well with the measured data (83.59%). Meanwhile, there was also a slight possibility to consider the LOS plot of AS1 as a single equation (Fig. 2C). However, it was not acceptable due to its too low determination coefficient ($r^2 = 0.686$) and the great gap between C_∞ calculated from a single linear regression (44.21%) and experimentally measured value (83.59%).

The LOS plots of AS2 and AS4 also revealed two distinct lines (Fig. 2E and F), and thus provided the evidence of fractions those were digested more rapidly (RDS) or more slowly (SDS), respectively. The LOS plot of AS8 (Fig. 2G) was better described by a single linear graph rather than two linear graphs (Fig. 2H, $C_\infty = 91.02\%$). Therefore, the presence of separate rapidly digested and slowly digested components in AS8 was not conceded. The rate constant ($k = 0.011$ min^{-1}) of AS8 was similar to the k values of the slower phase of other AS-modified starches.

The kinetic parameters of starch samples estimated by the LOS plot are summarized in Table 3. In addition, the contents of RDS, SDS, and RS estimated using the parameters from the LOS plot are shown in Table 4. 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-modified starches (AS1-AS4) possessed k s that were significantly different from each other. The rapidly digestible fraction, characterized by a higher k value, could have a structure that 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, Warren, & Ellis, 2011; Dhital et al., 2010). The rate constants obtained for AS-modified starches could be categorized into two groups based on the k values of AS0 and native, representing RDS and SDS, respectively (Englyst et al., 1992; Miao, Zhang, & Jiang, 2009). Because AS0 showed the rapid digestion property, the group of k close to that of AS0 was defined as k_{RDS}; for the same reason, k values at or near that of native starch were

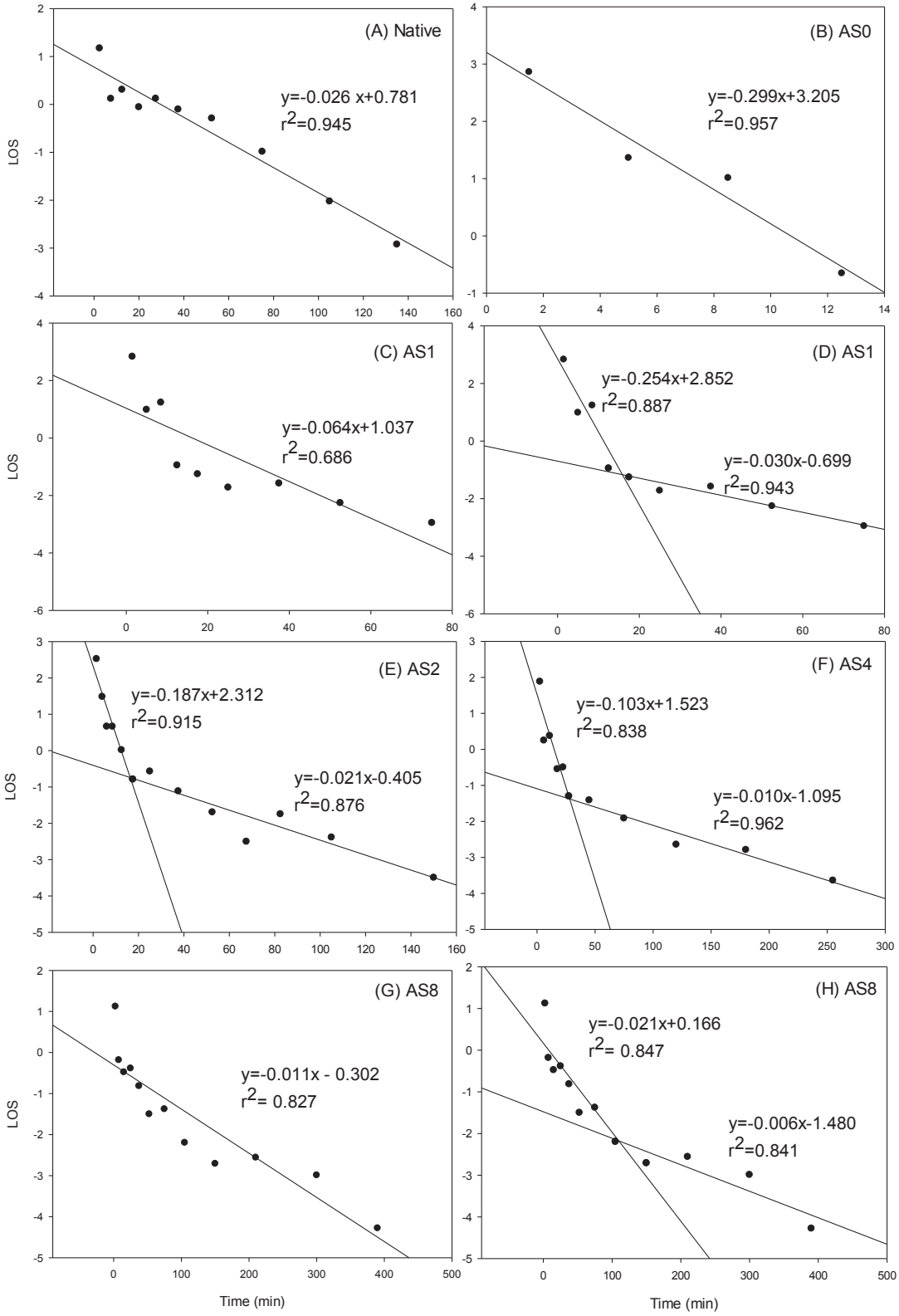

Fig. 2. LOS plots of native (A), AS0 (B), AS1 (C and D), AS2 (E), AS4 (F), and AS8 (G and H).

grouped into k_{SDS}. These two groups were clearly distinguished by approximately a 10-fold difference.

Digestion kinetic parameters of AS-modified starches implied the distinction between the rapid and slow phases in hydrolysis, suggesting the existence of RDS and SDS as a structural feature. That is, after hydrolysis of the rapidly digestible fraction (RDS), different organization of starch molecules with different enzyme susceptibility (SDS) was revealed. 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

Table 3
Enzymatic hydrolysis kinetic parameters of native and amylosucrase-modified starches estimated from the LOS plot.

Sample	k_{RDS} (min^{-1})	k_{SDS} (min^{-1})	Time of intersect (min)	C_∞ (%, calculated)	Time of plateau (min)	C_∞ (%, measured)
Native	N.D.	0.026	N.D.	83.05	120	84.34
AS0	0.299	N.D.	N.D.	82.36	15	81.09
AS1	0.254	0.030	15.9	85.14	60	83.59
AS2	0.187	0.021	16.3	86.66	150	86.61
AS4	0.103	0.010	28.2	77.26	210	77.02
AS8	N.D.	0.011	N.D.	68.46	360	68.39

k, digestion rate constant; C_∞, total starch digested; RDS, rapidly digestible starch; SDS, slowly digestible starch; N.D., not detected.
Native, native waxy corn starch; AS0, control with no enzyme addition; AS1, amylosucrase modified starch with 2,500 U amylosucrase/30 mL-starch suspension; AS2, amylosucrase 5,000 U/30 mL; AS4, amylosucrase 10,000 U/30 mL; AS8, amylosucrase 20,000 U/30 mL.

α-glucan chains. The decline of k_{RDS} and k_{SDS} values as the amount of AS increased suggested that the structural features of RDS and SDS might be dissimilar among samples.

The content of RDS estimated using the LOS plot decreased to a large extent with the amount of AS, agreeing with the result obtained by the method of Englyst. The time of intersection of the two lines, or the duration of the more rapid phase, was delayed according to the amount of AS employed. More time was required for the hydrolysis of a smaller amount of RDS, and the value of k_{RDS} also decreased. In other words, the AS treatment and resultant elongation of branch chains decreased the amount of RDS fraction in recrystallized starch and induced a more slowly digestible form among RDS. The increase in SDS content could also be understood along with the decrease in k_{SDS}. This implied that SDS with limited substrate availability for digestive enzyme was produced when the branch chains elongated. When comparing AS4 to AS2, a minor change in the amount of SDS was observed in contrast to the great changes in the RDS and RS contents. This suggested that the high degree of elongation of the branch chains promoted the formation of RS instead of SDS. The absence of a rapidly digestible fraction and a high amount of RS in AS8 also supported the idea that highly extended branch chains of amylopectin did not develop an easily digestible configuration during crystallization.

The amount of each fraction determined by the method of Englyst was not consistent with that determined by the LOS method. The method suggested by Englyst et al. (1992) does not deal with the factor of digestion rate, which corresponds to the terms of 'rapid' or 'slowly' digestible starch. Therefore, the LOS plot approach utilized in this study would be a more reasonable investigative tool for accurate determination of RDS and SDS (Patel et al., 2014), as well as RS.

3.5. Branch chain length distribution of AS-modified starches and their digested residues

The changes in chain length distributions of native and AS-modified starches before and after removal of the RDS and/or SDS fraction are presented in Fig. 3 and Fig. 4. Since the quantity of starch residues recovered from AS0 and AS1 was too small for further investigation, those were excluded from the structural analysis.

Table 4
RDS, SDS, and RS contents of native and amylosucrase-modified starches estimated using the LOS plot.

Sample	RDS (%)	SDS (%)	RS (%)
Native	N.D.	84.34	15.66
AS0	81.09	N.D.	18.91
AS1	73.95	9.64	16.41
AS2	60.89	25.72	13.39
AS4	54.33	22.69	22.98
AS8	N.D.	68.39	31.61

RDS, rapidly digestible starch; SDS, slowly digestible starch; RS, resistant starch; N.D., not detected.
Native, native waxy corn starch; AS0, control with no enzyme addition; AS1, amylosucrase modified starch with 2,500 U amylosucrase/30 mL-starch suspension; AS2, amylosucrase 5,000 U/30 mL; AS4, amylosucrase 10,000 U/30 mL; AS8, amylosucrase 20,000 U/30 mL.

A decrease in the DP of the detectable longest branch chain was commonly found within all AS-starches, in the order of RDS + SDS + RS > SDS + RS > RS fractions. When the RDS fraction was removed, the chains of DP ≥ 25 decreased by a significant amount. Most of the decrease was observed in the long chains with a particular DP higher than 27 or 28 (AS2 and AS4, respectively), implying that long chains contributed to the formation of the RDS fraction. The proportion of chains below DP 25 increased, but the longer chains decreased considerably. Very long chains of DP ≥ 37 were hardly detected, and the proportion of chains with DP 13–24 increased markedly as the SDS fraction was removed.

The isolated RS fractions from all of the AS-starches contained predominantly branch chains with DP 13–24. The common average DP (DP 17 or 18) of the RS obtained in the current study was in good agreement with previous results which have studied RS from recrystallized starches (Eerlingen, Deceuninck, & Delcour, 1993; Lopez-Rubio, Flanagan, Gilbert, & Gidley, 2008).

In contrast with AS-modified starches, the branch chain length distribution of native starch was preserved in spite of hydrolysis (Fig. 3A). This difference in the DP change after hydrolysis could be caused by the different digestion patterns of starch. Native granular starch is hydrolyzed by a dynamic side-by-side digestion mechanism (Zhang, Ao, & Hamaker, 2006) that involves the inside-out digestion pattern. However, the AS-modified starches, including AS0, lost the original granular properties such as surface pinhole and crystalline packing by gelatinization followed by AS addition. Recrystallization in a manner distinct from the crystalline state of granular starch caused the altered digestion pattern. The resistance of recrystallization-processed starches to digestive enzymes could be caused by the acquisition of a double-helical order (Colonna, Leloup, & Buleon, 1992). The stabilization of double helices into the crystalline structure decreases 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 the recrystallized starches (Cairns, Sun, Morris, & Ring, 1995; Gidley et al., 1995).

3.6. X-ray diffraction and relative crystallinity of AS-modified starches and their digested residues

The native waxy corn starch displayed a typical A-type crystalline pattern with a relative crystallinity of 41.5% (Table 5). The relative crystallinity of AS0 was considerably lower than that of the native starch, due to the crystalline disruption and the dissociation

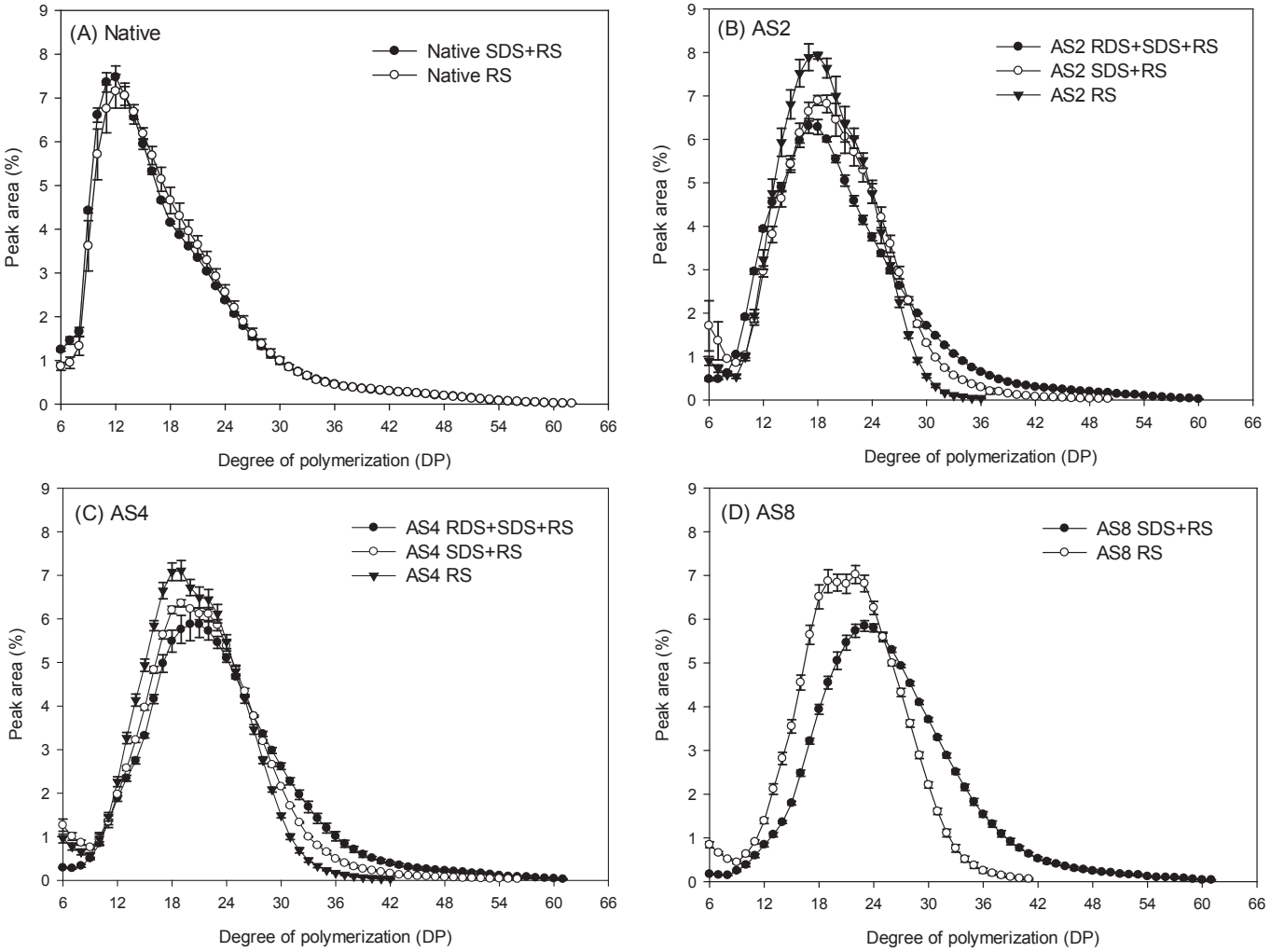

Fig. 3. The branch chain length distributions of native (A), AS2 (B), AS4 (C), and AS8 (D) determined before and after removal of the RDS and/or SDS fraction.

of the double helical structure during gelatinization before AS modification. AS0 did not present any noticeable major peak, but did show a slight rise increase near 13° (data not shown), which seemed to be due to the influence of annealing by 6 h-incubation during sample preparation. The disrupted crystalline structure was regenerated after AS modification, revealing B-type polymorph (data not shown), 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 crystallite (Ryu et al., 2010). Therefore, the peak intensity and the relative crystallinity increased as the amount of AS increased.

Removal of the SDS fraction of native starch caused an increase in the relative crystallinity. A similar increase in crystallinity after digestion was reported in a previous study of normal maize starch (Shrestha et al., 2012). Granular waxy corn starch has a semi-crystalline structure composed of alternating concentric crystalline lamellae and amorphous layers, and 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). Therefore, this phenomenon in the native starch could be explained by digestion of only selected regions primarily, leaving the undigested crystalline regions relatively unchanged (Shrestha et al., 2012).

In AS-modified starches, the relative crystallinity increased in the SDS + RS and RS fractions, indicating that the remaining parts had a more densely packed and well-organized crystalline structure. In general, the crystallinity of starches is 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-modified starches and their SDS + RS and/or RS parts.

3.7. Structure-digestibility relationship of AS-modified starch

The AS-modified starches were produced by recrystallization, which was accompanied by the destruction of the unique structure of native granules. This change prohibited enzyme diffusion into the granule interior through pinholes existing at the surface (Huber & BeMiller, 2000; Zhang, Ao, & Hamaker, 2006). During AS modification, the reconstruction of crystallite packing and arrangement which are different from those of native starch occurred by the association of elongated chains of amylopectin, which is similar to retrogradation (Shin et al., 2010). Therefore, the digestion property of the AS-modified starches should be described in relation to that of retrograded starches. The increase in crystallinity results in fewer available α-glucan chains for digestive enzymes to bind, thus

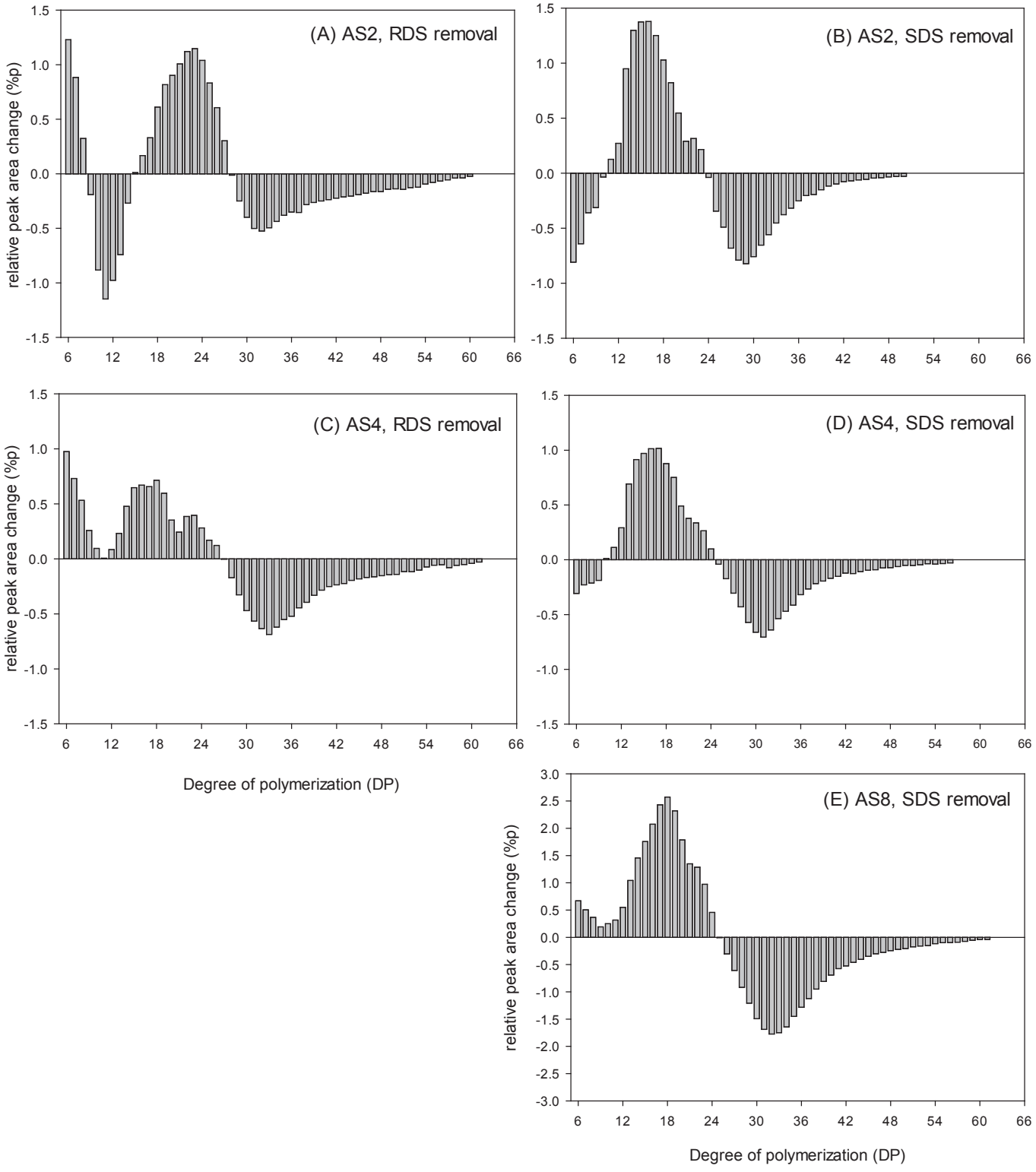

Fig. 4. Changes in the branch chain length distributions of starch samples caused by the removal of the RDS (left) or SDS (right) fraction. (A), (B) AS2; (C), (D) AS4; (E) AS8.

reducing the susceptibility to digestion (Htoon et al., 2009; Liu, Yu, Chen, & Li, 2007). In accordance with the increased crystallinity, the experimentally determined C_∞ value of the AS-starches decreased, meaning an increase in RS. The principal mechanism for the formation of RS in the amylose solution was proposed to be the formation of micelle and/or lamella-based crystalline structures by the aggregation of amylose helices in the B-type crystalline structure over a particular region of the chain (Eerlingen et al., 1993). Elongated branch chains of amylopectin of AS-modified starches were possibly arranged into a similar formation. The abundant existence of long chains accelerates the formation of double helices during recrystallization (Kim et al., 2014; Shin et al., 2010). With higher

Table 5
Relative crystallinities of native and amylosucrase-modified starches and their isolated fractions.

Sample		Relative crystallinity (%)
Native	SDS + RS	41.5 ± 1.0[b]
	RS	51.1 ± 0.5[a]
AS0	RDS + RS	12.7 ± 0.5[j]
AS1	RDS + SDS + RS	14.6 ± 1.0[i]
AS2	RDS + SDS + RS	16.5 ± 0.4[h]
	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]

The values with different superscripts in the same column are significantly different ($p < 0.05$).
Native, native waxy corn starch; AS0, control with no enzyme addition; AS1, amylosucrase modified starch with 2,500 U amylosucrase/30 mL-starch suspension; AS2, amylosucrase 5,000 U/30 mL; AS4, amylosucrase 10,000 U/30 mL; AS8, amylosucrase 20,000 U/30 mL.
RDS, rapidly digestible starch; SDS, slowly digestible starch; RS, resistant starch.

levels of AS, longer double helices would be produced from the longer amylopectin, and the α-1,6-branches of amylopectin limit the space for the arrangement of double helices. Therefore, not only would the amount of double helix chain folding be increased, but also the distance between double helices would be narrowed, resulting in a more dense crystalline structure. This hypothesis supports the large amounts of RS in AS4 and AS8, possessing noticeably many long chains.

SDS is known to consist of mainly imperfect crystalline regions containing small portions of double helices and amorphous regions (Shin et al., 2004). The SDS part of AS-modified waxy corn starches would be formed by the alignment of adjacent single helices, the exposed parts of the double helix turns out of crystalline lamella, non-crystalline double helical structures, and other conformations caged within the imperfect regions of the 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 the single helix not yet associated into double helices, and exposed out of micelle-conformation would be components of RDS. Thus, long chains (DP ≥ 27 or 28) in this part disappeared as a result of RDS hydrolysis. This explanation would also be a plausible reason for the large amount of short chains (DP 13–24) and lack of longer chains in undigested RS.

Comprehensively, the amylopectin branch chain length distribution determined the primarily generated crystallite organization of AS-modified starches. Different crystalline arrangements caused the different structures of RDS, SDS, and RS, affecting the extent of hydrolysis. The different structures of RDS and SDS among AS-modified starches were reflected in the different values of k_{RDS} and k_{SDS}.

4. Conclusions

Through this study, it was confirmed that the concept of RDS, SDS, and RS suggested by Englyst et al. (1992) is valid, though the standard for classification described by them was not appropriate. The modified LOS plot method used in this study can be employed as an alternative tool for the fractionation of starch into RDS, SDS, and RS. The current study also illustrated that different branch chain lengths of amylopectin determined the primary crystalline arrangement of recrystallized starches and, accordingly, the amounts and structures of RDS and SDS. The digestible fraction of highly AS-modified starch (AS8) consisted of solely SDS; thus, development of an industrial SDS product seems promising.

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