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Original article

Characterisation of low-digestible starch fractions isolated from amylosucrase-modified waxy corn starch

Hyeon Jeong Yoo,[1] Ha Ram Kim,[1] Seung Jun Choi,[2] Cheon-Seok Park[3] & Tae Wha Moon[1,4]*

1 Department of Agricultural Biotechnology, Seoul National University, Seoul 08826, Korea
2 Department of Food Science and Technology, Seoul National University of Science and Technology, Seoul 01811, Korea
3 Department of Food Science and Biotechnology, Institute of Life Science and Resources, Kyung Hee University, Yongin, Gyeonggi 17104, Korea
4 Center for Food and Bioconvergence, Research Institute of Agriculture and Life Sciences, Seoul National University, Seoul 08826, Korea

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Summary Amylosucrase (AS) modification of starch increases the slowly digestible (SDS) and resistant starch (RS) fractions. However, the characteristics and formation mechanism of each fraction of AS-modified starch have not been determined yet. Therefore, this study isolated SDS and/or RS from AS-modified waxy corn starches and investigated their structural characteristics. The amount of SDS+RS and RS had a positive correlation with the proportion of the medium length (13–24 of degree of polymerisation) branched chains of amylopectin. The relative crystallinity increased in the order of AS-modified starch < SDS+RS < RS, while maintaining the B-type crystalline structure. The thermal transition temperature ranges of the isolated fractions were also higher than those of undigested starches. The medium branched chains of amylopectin were presumably the clincher for the SDS and/or RS formation in AS-modified starches. The principal causes of SDS and RS formation were the chain length elongation and the subsequent retrogradation-like process, respectively.

Keywords Amylosucrase, resistant starch, slowly digestible starch, waxy corn starch.

Introduction

Starch is the main carbohydrate source in human nutrition. As mono- and di-saccharides are very important energy sources for the human body, the digestion of starch into these forms is considered to be an important metabolic mechanism.

Generally, starch has been classified into three fractions, rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS), according to the rate and extent of *in vitro* digestion (Englyst *et al.*, 1992). A RDS is digested rapidly in the small intestine, and includes fully gelatinised waxy starches. A SDS is digested completely but slowly in the small intestine and provides a sustaining effect on blood glucose level. The physiological advantages of SDS are satiety, improved glucose tolerance, diabetes management and reduction of blood lipid levels (Lehmann & Robin, 2007). RS is the starch fraction that cannot be digested by digestive enzymes but is fermented in the colon. It has hypoglycaemic and hypocholesterolaemic effects, acts as a substrate for growth of probiotic micro-organisms, and inhibits fat accumulation (Sajilata *et al.*, 2006). Starch with abundant SDS and RS fractions can be regarded as low-digestible starch (Shin *et al.*, 2007).

Because of their many health benefits, many studies have focused on the preparation and characterisation of novel starches with high proportions of SDS and RS (Shin *et al.*, 2004; Han *et al.*, 2006; Miao *et al.*, 2009; Kim *et al.*, 2014). RS has various conformations, classified as types I-V, and is mainly consisted of a crystalline region with high perfection and rigidity (Fuentes-Zaragoza *et al.*, 2011). Most commercially available RS is prepared from high-amylose starches and through retrogradation (Sajilata *et al.*, 2006; Jiang *et al.*, 2010). The preparation process of starches with a high SDS fraction is well reported, and starch samples with high SDS content have been prepared through physical (Lee & Moon, 2015) and enzymatic modifications (Shin *et al.*, 2004; Han *et al.*, 2006; Miao *et al.*, 2009). However, structural information on the SDS fraction is scarce compared with that on RS. It has been hypothesised that SDS consists of less perfect crystalline regions containing a small portion of

*Correspondent: Fax +82 2 873 5095;
e-mail: twmoon@snu.ac.kr
Hyeon Jeong Yoo and Ha Ram Kim equally contributed to this work.

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double helices and an amorphous region (Shin *et al.*, 2004). Zhang *et al.* (2008b) suggested a parabolic relationship between SDS content and the weight ratio of short chains to long chains in amylopectin.

Several studies have reported the possibility of preparing low-digestible starch products using amylosucrase (AS) (Shin *et al.*, 2010; Kim *et al.*, 2013, 2014; Zhang *et al.*, 2017) based on its glucosyltransferase activity to elongate the nonreducing ends of (1→4)-α-glucans using sucrose (De Montalk *et al.*, 1999; Rolland-Sabaté *et al.*, 2004). AS-modified starches showed increased SDS and RS contents (Shin *et al.*, 2010). It was also reported that AS reaction time had a positive correlation with the resistance to digestion of modified starches (Kim *et al.*, 2014). However, it is still unclear how the internal structure of crystallites is organised in the SDS and/or RS fractions of AS-modified starches, and their detailed structural characteristics have not been clearly determined. Therefore, the objectives of this study were to investigate the structural characteristics of the SDS and/or RS fractions isolated from AS-modified starches and to elucidate the structure-digestibility relationship of starch.

Materials and methods

Materials

Waxy corn starch was obtained from Samyang Genex Corp. (Incheon, Korea). The gene of *Neisseria polysaccharea* encoding AS was cloned and expressed in *E. coli*, and AS was purified following the method of Jung *et al.* (2009). One unit (U) of AS corresponds to the amount of enzyme that catalyses the production of 1 μM of fructose per min in the assay condition (Kim *et al.*, 2014). All other chemicals were of analytical reagent grade.

AS-modified starch production

Waxy corn starch was modified with AS according to a previous study (Kim *et al.*, 2014). Briefly, 3 g of starch and 5.13 g of sucrose were suspended in 100 mM sodium acetate buffer (pH 7.0). It was boiled with vortex mixing for 30 min and then cooled to 30 °C. AS (10 000 U per 30 mL starch suspension) was added to the starch suspension, to adjust the final volume of 150 mL. After incubating for 1, 3, 6, 9, 15 or 24 h in a shaking water bath at 30 °C and 80 rpm, the enzyme reaction was terminated by adding three volumes of ethanol to the suspension. After centrifugation at 10 000 ***g*** for 10 min, the precipitated AS-modified starch was recovered and washed three times using distilled water. The pellet was freeze-dried, ground, and passed through a 100-mesh sieve. A control sample was prepared by incubation for 24 h using the same method without the enzyme addition.

Analysis of reducing sugar concentration after AS modification

After the enzyme reaction, 2 mL of each suspension was boiled for 10 min, cooled to room temperature, and centrifuged at 7000 ***g*** for 20 min. Then, 500 μL of supernatant was diluted to be mixed with 500 μL of 3,5-dinitrosalicylic acid solution, boiled for 5 min, and cooled on ice for 20 min. The absorbance of each sample was measured at 575 nm (Miller, 1959).

Determination and isolation of starch fractions

The starch fractions were measured according to the method of Englyst *et al.* (1992), with a slight modification (Shin *et al.*, 2004). Pancreatin (2 g, P7545, activity 8 × USP g^{-1}; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in distilled water (24 mL) with stirring for 10 min and then was precipitated by centrifugation at 1500 ***g*** for 10 min. The supernatant (20 mL) was mixed with amyloglucosidase (0.4 mL, AMG300L, activity 300 AGU mL^{-1}; Novozymes, Bagsværd, Denmark) and distilled water (3.6 mL) and incubated at 37 °C for 10 min.

A starch sample (30 mg) was dispersed in a 2-mL microtube containing sodium acetate buffer (0.75 mL, 0.1 M, pH 5.2) with one glass bead and was equilibrated in a shaking incubator (37 °C, 240 rpm) for 10 min. Then, the prepared enzyme solution (0.75 mL) was added to each microtube, and the starch sample was incubated in a shaking incubator (37 °C, 240 rpm) for 10 or 240 min. The enzymatic reaction was terminated by boiling for 10 min, and the glucose released under hydrolysis was measured using a glucose oxidase-peroxidase kit (BCS, Anyang, Korea). The RDS was measured as the quantity of glucose after reaction for 10 min. SDS was the fraction digested between 10 and 240 min. RS was the unhydrolysed fraction after 240 min (Shin *et al.*, 2004).

A parallel set was prepared, and the enzyme reaction was terminated by adding three volumes of ethanol to the suspension at 10 and 240 min. After centrifugation at 10 000 ***g*** for 10 min, the recovered precipitates were washed with distilled water, and then the denatured protein layer was scrapped out repeatedly. The pellet was freeze-dried, ground and passed through a 100-mesh sieve. The recovered parts after 10 and 240 min of enzymatic hydrolysis were regarded as the SDS+RS and RS fractions, respectively.

Determination of branched chain length distribution

Branched chain length distribution of starches was measured after debranching as described in the previous research (Shin *et al.*, 2010). Briefly, completely dissolved starch samples (15 mg starch in 3.0 mL of

25 mM, pH 4.3 sodium acetate buffer) were incubated after isoamylase addition (30 μL, 1000 U mL^{-1}; Megazyme, Bray, Ireland) at 45 °C and 30 rpm for 2 h in a 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 analysed using high performance anion-exchange chromatography on a Carbo-Pack PA1 anion-exchange column (4 × 250 mm; Dionex, Sunnyvale, CA, USA) with a pulsed amperometric detector. This analysis was performed using a gradient increase of 600 mM sodium acetate in 150 mM NaOH solution against 150 mM NaOH for sample elution. Degree of polymerisation (DP) values from 1 to 7 was designated using a mixture of maltooligosaccharides (DP 1-7; Sigma).

Measurement of thermal transition properties

Thermal transition properties such as the gelatinisation onset (T_o), melting (T_m) and conclusion (T_c) temperatures, the melting temperature range ($T_r = T_c – T_o$), and the melting enthalpy (ΔH) were investigated using a differential scanning calorimeter (Diamond DSC; Perkin-Elmer, Waltham, MA, USA). Each sample (10 mg) was weighed in a hermetic pan, and distilled water (40 μL) was added. The sample pan was sealed and kept at room temperature overnight for equilibrium. The sample pan was heated from 30 to 160 °C at 10 °C min^{-1}. An empty pan was used as a reference.

X-ray diffraction patterns and relative crystallinity

X-ray diffraction analysis was performed using a powder X-ray diffractometer (D5005; Bruker, Karlsruhe, Germany) at 40 kV and 40 mA, with Cu K_α radiation of 0.154 nm wavelength. A starch sample scan was performed through a 2θ range from 3 to 30° at a step time of 4 s. The relative crystallinity was calculated using the Origin 7.5 program (Microbial, Northampton, MA, USA). The crystallinity was determined using the equation below.

$$\text{Degree of crystallinity (\%)} = \left(\frac{\text{Area of the peaks}}{\text{Total curve area}}\right) \times 100$$

Statistical analysis

All experimental data were analysed using analysis of variance and expressed as the mean ± standard deviation of triplicate samples. Significant differences among mean values were compared using Duncan's multiple range test ($P < 0.05$). Statistical analysis was conducted with IBM SPSS statistics version 21.0 (IBM, Armonk, NY, USA).

Results and discussion

Structural characterisation of AS-modified starches

Amylosucrase changed the structures of the starch molecules, and the degree of changes determined by the enzyme reaction time was in agreement with our previous findings (Shin *et al.*, 2010; Kim *et al.*, 2014). In detail, the concentration of released fructose increased rapidly during the initial 6 h of the enzyme reaction and increased little during an additional 18-h reaction (Table 1). These results revealed that the attachment of glucosyl units at nonreducing ends occurred intensively in the early stage (initial 6 h) of the enzyme reaction and then slowed. Changes in the branched chain length distribution (Table 2) showed a decrease in short chains (DP ≤ 12) but an increase in both medium (DP 13–24) and long chains (DP 25–36) with reaction time, as previously reported (Shin *et al.*, 2010; Kim *et al.*, 2014).

The control showed no peak in the X-ray diffractogram due to gelatinisation process (data not shown), whereas the AS modification increased the B-type X-ray peak intensity and relative crystallinity in a reaction time-dependent manner (Table 3). Thermal parameters of T_m, T_c, T_r and ΔH also gradually increased as AS reaction time increased (Table 4). In sum, the elongation of branch chains induced the formation of double helices, which contributed to the crystalline structure and resulted in stronger or more stable crystallites with more heterogeneous composition (Barichello *et al.*, 1990; Larsson & Eliasson, 1991; Lopez-Rubio *et al.*, 2008; Shin *et al.*, 2010) in AS-modified starch.

In vitro digestibility of AS-modified starches

The *in vitro* digestibility of AS-modified starches as a function of reaction time is presented in Fig. 1. Overall patterns of the changes in starch fractions showed consistency with a previous report (Kim *et al.*, 2014). In

Table 1 Concentration of fructose released during amylosucrase modification of waxy corn starch

Modification time (h)	Released fructose (mM)
Control	12.6 ± 0.1^{f}
1	15.8 ± 0.3^{e}
3	24.2 ± 0.6^{d}
6	33.2 ± 0.2^{c}
9	33.3 ± 0.4^{c}
15	38.1 ± 0.1^{a}
24	37.2 ± 0.0^{b}

The values having different superscripts are significantly different ($P < 0.05$) based on Duncan's multiple range test.

Table 2 Branched chain length distribution of amylopectin of amylosucrase-modified waxy corn starch and its amylolytic digested starch fractions

Starch fraction	Modification time (h)	Per cent distribution (%)					
		DP ≤ 5	DP 6–12	DP 13–24	DP 25–36	DP ≥ 37	Average DP
RDS+SDS+RS	Control	$^{z}1.7 \pm 0.1^{a}$	$^{x}33.3 \pm 2.3^{a}$	$^{z}46.6 \pm 2.4^{c}$	$^{x}13.2 \pm 1.0^{d}$	$^{x}5.2 \pm 1.2^{a}$	$^{x}21.4 \pm 0.0^{d}$
	1	$^{y}1.2 \pm 0.1^{b}$	$^{y}24.3 \pm 2.9^{b}$	$^{z}52.5 \pm 1.4^{b}$	$^{x}16.4 \pm 1.8^{c}$	$^{x}5.6 \pm 0.5^{a}$	$^{x}23.1 \pm 0.5^{c}$
	3	$^{y}0.8 \pm 0.1^{c}$	$^{z}16.6 \pm 2.6^{c}$	$^{z}55.9 \pm 1.4^{a}$	$^{x}20.5 \pm 1.5^{b}$	$^{x}6.2 \pm 0.3^{a}$	$^{x}25.0 \pm 0.5^{b}$
	6	$^{z}0.6 \pm 0.0^{c}$	$^{x}12.9 \pm 2.5^{cd}$	$^{z}57.7 \pm 1.2^{a}$	$^{x}23.2 \pm 1.8^{ab}$	$^{x}5.6 \pm 0.5^{a}$	$^{x}25.7 \pm 0.4^{ab}$
	9	$^{z}0.8 \pm 0.3^{c}$	$^{x}13.2 \pm 3.0^{cd}$	$^{z}56.8 \pm 0.9^{a}$	$^{x}23.3 \pm 1.8^{ab}$	$^{x}5.9 \pm 0.6^{a}$	$^{x}25.7 \pm 1.0^{ab}$
	24	$^{z}0.6 \pm 0.0^{c}$	$^{x}10.1 \pm 1.9^{d}$	$^{y}57.1 \pm 0.3^{a}$	$^{x}26.0 \pm 1.8^{a}$	$^{x}6.2 \pm 0.1^{a}$	$^{x}26.7 \pm 0.6^{a}$
SDS+RS	Control	$^{x}6.0 \pm 0.1^{a}$	$^{x}34.9 \pm 3.5^{a}$	$^{y}54.2 \pm 2.7^{c}$	$^{z}4.9 \pm 0.9^{f}$	$^{y}0.0 \pm 0.0^{e}$	$^{z}14.3 \pm 0.5^{f}$
	1	$^{x}3.7 \pm 0.7^{b}$	$^{x}30.1 \pm 0.2^{b}$	$^{y}58.0 \pm 2.2^{ab}$	$^{y}6.7 \pm 0.9^{e}$	$^{y}1.5 \pm 0.4^{d}$	$^{y}15.8 \pm 0.4^{e}$
	3	$^{x}2.6 \pm 0.2^{c}$	$^{y}18.5 \pm 0.2^{c}$	$^{y}59.7 \pm 0.6^{ab}$	$^{y}15.3 \pm 0.1^{d}$	$^{y}3.9 \pm 0.1^{b}$	$^{y}18.8 \pm 0.0^{d}$
	6	$^{x}3.5 \pm 0.1^{b}$	$^{x}13.3 \pm 1.4^{d}$	$^{y}60.7 \pm 1.3^{a}$	$^{y}19.1 \pm 0.2^{c}$	$^{y}3.4 \pm 0.0^{c}$	$^{y}19.5 \pm 0.2^{c}$
	9	$^{y}2.4 \pm 0.0^{cd}$	$^{x}10.3 \pm 0.1^{e}$	$^{y}59.5 \pm 0.2^{ab}$	$^{x}23.5 \pm 0.1^{b}$	$^{y}4.3 \pm 0.0^{a}$	$^{y}20.9 \pm 0.0^{b}$
	24	$^{y}1.9 \pm 0.1^{d}$	$^{x}8.2 \pm 0.0^{e}$	$^{y}56.8 \pm 0.3^{bc}$	$^{x}27.7 \pm 0.1^{a}$	$^{y}1.3 \pm 0.1^{d}$	$^{y}22.0 \pm 0.1^{a}$
RS	Control	$^{y}3.1 \pm 0.1^{a}$	$^{y}25.6 \pm 1.0^{a}$	$^{x}62.6 \pm 0.7^{c}$	$^{y}8.4 \pm 0.2^{c}$	$^{y}0.3 \pm 0.1^{c}$	$^{y}16.1 \pm 0.2^{e}$
	1	$^{x}3.3 \pm 0.1^{a}$	$^{y}23.5 \pm 0.8^{b}$	$^{x}67.4 \pm 2.1^{b}$	$^{y}5.6 \pm 1.2^{d}$	$^{z}0.1 \pm 0.2^{d}$	$^{y}15.8 \pm 0.1^{e}$
	3	$^{x}2.7 \pm 0.2^{b}$	$^{x}22.2 \pm 0.9^{c}$	$^{x}64.8 \pm 1.2^{c}$	$^{z}8.7 \pm 0.1^{c}$	$^{z}1.7 \pm 0.0^{a}$	$^{z}17.0 \pm 0.1^{d}$
	6	$^{y}2.7 \pm 0.1^{b}$	$^{x}14.8 \pm 0.4^{d}$	$^{x}71.8 \pm 0.7^{a}$	$^{z}10.1 \pm 0.2^{c}$	$^{z}0.5 \pm 0.0^{c}$	$^{z}17.5 \pm 0.0^{c}$
	9	$^{x}2.8 \pm 0.1^{b}$	$^{x}12.1 \pm 0.6^{e}$	$^{x}71.6 \pm 1.9^{a}$	$^{y}12.9 \pm 2.4^{b}$	$^{z}0.4 \pm 0.1^{c}$	$^{z}18.2 \pm 0.4^{b}$
	24	$^{x}2.4 \pm 0.0^{c}$	$^{x}10.0 \pm 0.1^{f}$	$^{x}68.5 \pm 0.3^{b}$	$^{y}17.8 \pm 0.1^{a}$	$^{y}1.3 \pm 0.1^{b}$	$^{z}19.3 \pm 0.0^{a}$

AS, amylosucrase; RDS, rapidly digestible starch; SDS, slowly digestible starch; RS, resistant starch; DP, degree of polymerisation.
The values having different superscripts (a, b, c, d, e and f) within a column of a given starch fraction are significantly different ($P < 0.05$). Within a column of the same per cent distribution, the values for the same modification time at different starch fractions which have different superscripts (x, y and z) are significantly different ($P < 0.05$) based on Duncan's multiple range test.

Table 3 Relative crystallinity of amylosucrase-modified waxy corn starch and its amylolytically digested starch fractions

Modification time (h)	Relative crystallinity (%)		
	AS-modified starch (RDS+SDS+RS)	SDS+RS	RS
Control	$^{z}12.2 \pm 0.1^{f}$	$^{y}20.7 \pm 0.2^{d}$	$^{x}23.3 \pm 0.2^{e}$
1	$^{z}14.1 \pm 0.3^{e}$	$^{y}22.6 \pm 0.3^{c}$	$^{x}25.5 \pm 0.3^{d}$
3	$^{z}15.8 \pm 0.5^{d}$	$^{y}22.8 \pm 0.0^{c}$	$^{x}26.8 \pm 0.1^{c}$
6	$^{z}21.2 \pm 0.6^{c}$	$^{y}25.5 \pm 0.4^{b}$	$^{x}28.0 \pm 0.6^{b}$
9	$^{z}23.7 \pm 0.3^{b}$	$^{y}25.8 \pm 0.7^{b}$	$^{x}28.3 \pm 0.3^{b}$
24	$^{y}26.5 \pm 0.1^{a}$	$^{y}27.0 \pm 0.3^{a}$	$^{x}29.3 \pm 0.6^{a}$

AS, amylosucrase; RDS, rapidly digestible starch; SDS, slowly digestible starch; RS, resistant starch.
The values having different superscripts (a, b, c, d, e and f) in each column are significantly different ($P < 0.05$). The values having different superscripts (x, y and z) in each raw are significantly different ($P < 0.05$) based on Duncan's multiple range test.

detail, there were drastic changes in RDS, SDS and RS contents during the initial 6-h modification, similar to the changes in the concentration of fructose released during enzyme reaction. Compared with the control, SDS and RS increased from 5.9 to 43.7% and from 13.4 to 26.1% after 24-h modification, respectively, while the RDS content gradually dropped from 80.7 to 31.2%.

In this study, starches underwent gelatinisation before the AS treatment and the original crystalline structures of native starch were fully disintegrated. Then, during AS treatment, elongation of branch chains occurred. The reassociation between the elongated chains resulted in the formation of the double helical structures, leading to the creation of crystalline structures. This reorganisation process looked similar to the 'retrogradation' of fully gelatinised starch, although it could not be completely the same (24 h is not enough time for complete retrogradation of waxy starch). It is known that the chains of DP 13–30 majorly contribute to the degree of retrogradation, and the associations of these chains form crystallites, which can act as the anchor points for the long chains of DP > 30 (Zhang *et al.*, 2008a,b). The elongated branched chains of AS-modified starches increased the relative content of these chains (DP ≥ 13), improving the crystalline structure and resulted in reduced accessibility of digestive enzymes.

The SDS content was fairly constant after 6 h, showing consistency with the changes in branched chain length distribution. Therefore, the increase in SDS content was presumed to be affected mainly by the elongation of branched chains and the resultant

Table 4 Thermal transition properties of amylosucrase-modified waxy corn starch and its amylolytically digested starch fractions

Starch fraction	Modification time (h)	T_o (°C)*	T_m (°C)	T_c (°C)	T_r (°C)	ΔH (J g^{-1})
RDS+SDS+RS	Control			Not detected		
	1	$^{y}52.1 \pm 0.2^{a}$	$^{y}63.0 \pm 0.4^{d}$	$^{x}87.0 \pm 2.6^{c}$	$^{x}35.0 \pm 2.4^{c}$	$^{x}3.3 \pm 0.1^{d}$
	3	$^{y}51.8 \pm 0.9^{a}$	$^{y}66.1 \pm 1.3^{c}$	$^{y}87.3 \pm 0.3^{c}$	$^{x}35.5 \pm 0.6^{c}$	$^{x}10.1 \pm 0.0^{c}$
	6	$^{y}52.0 \pm 0.4^{a}$	$^{z}70.7 \pm 0.6^{b}$	$^{y}94.3 \pm 2.4^{b}$	$^{x}42.3 \pm 2.0^{b}$	$^{x}11.8 \pm 0.7^{b}$
	9	$^{y}51.9 \pm 0.4^{a}$	$^{y}70.6 \pm 1.2^{b}$	$^{y}101.3 \pm 0.4^{a}$	$^{x}49.5 \pm 0.7^{a}$	$^{x}11.5 \pm 1.8^{bc}$
	24	$^{y}50.4 \pm 0.6^{b}$	$^{z}79.5 \pm 0.9^{a}$	$^{x}100.9 \pm 0.6^{a}$	$^{x}50.6 \pm 0.1^{a}$	$^{x}14.8 \pm 0.3^{a}$
SDS+RS	Control	$^{x}56.8 \pm 1.9^{a}$	$^{x}73.0 \pm 0.7^{bc}$	$^{x}85.0 \pm 0.9^{c}$	$^{x}28.2 \pm 1.1^{b}$	$^{x}7.2 \pm 1.8^{b}$
	1	$^{x}55.3 \pm 2.2^{a}$	$^{x}74.0 \pm 3.2^{b}$	$^{x}89.7 \pm 1.0^{b}$	$^{x}34.4 \pm 3.2^{b}$	$^{x}3.3 \pm 1.3^{c}$
	3	$^{x}55.4 \pm 1.0^{a}$	$^{x}70.6 \pm 0.5^{c}$	$^{x}91.4 \pm 0.4^{b}$	$^{x}36.0 \pm 1.3^{b}$	$^{y}4.0 \pm 0.1^{c}$
	6	$^{y}51.9 \pm 0.6^{b}$	$^{y}73.4 \pm 0.1^{b}$	$^{x}99.7 \pm 0.9^{a}$	$^{x}47.8 \pm 1.5^{ab}$	$^{y}7.1 \pm 2.4^{b}$
	9	$^{x}54.9 \pm 0.7^{a}$	$^{x}83.1 \pm 0.7^{a}$	$^{y}101.5 \pm 2.1^{a}$	$^{y}46.6 \pm 1.4^{a}$	$^{xy}9.9 \pm 0.2^{a}$
	24	$^{x}55.0 \pm 0.1^{a}$	$^{x}84.5 \pm 0.2^{a}$	$^{x}100.3 \pm 0.6^{a}$	$^{y}45.2 \pm 0.5^{a}$	$^{y}9.1 \pm 0.3^{ab}$
RS	Control	$^{x}59.6 \pm 0.1^{a}$	$^{x}72.2 \pm 0.5^{d}$	$^{x}83.9 \pm 0.6^{e}$	$^{y}24.3 \pm 0.7^{e}$	$^{x}5.1 \pm 1.3^{bc}$
	1	$^{x}56.4 \pm 0.2^{b}$	$^{x}76.2 \pm 0.8^{bc}$	$^{x}90.4 \pm 0.6^{d}$	$^{x}34.1 \pm 0.9^{d}$	$^{x}3.1 \pm 0.8^{c}$
	3	$^{x}56.3 \pm 2.0^{b}$	$^{x}73.3 \pm 2.7^{cd}$	$^{x}90.6 \pm 1.1^{d}$	$^{x}34.2 \pm 0.9^{d}$	$^{y}3.9 \pm 0.3^{bc}$
	6	$^{x}55.5 \pm 0.6^{b}$	$^{x}79.8 \pm 1.0^{ab}$	$^{x}98.6 \pm 0.4^{c}$	$^{y}43.1 \pm 1.0^{c}$	$^{y}5.9 \pm 2.6^{b}$
	9	$^{x}55.1 \pm 0.5^{b}$	$^{x}83.2 \pm 3.8^{a}$	$^{x}105.4 \pm 0.7^{a}$	$^{x}50.3 \pm 0.2^{a}$	$^{y}8.2 \pm 0.1^{a}$
	24	$^{x}55.3 \pm 1.4^{b}$	$^{y}82.4 \pm 1.5^{a}$	$^{x}102.2 \pm 2.3^{b}$	$^{xy}47.0 \pm 3.7^{b}$	$^{y}8.8 \pm 0.5^{a}$

AS, amylosucrase; RDS, rapidly digestible starch; SDS, slowly digestible starch; RS, resistant starch.

The values having different superscripts (a, b, c, d, e and f) within a column of a given starch fraction are significantly different ($P < 0.05$). The values having different superscripts (x, y and z) within a column of the same modification time at different starch fractions are significantly different ($P < 0.05$) based on Duncan's multiple range test.

*T_o, T_m and T_c indicate the gelatinization onset, melting and conclusion temperature of crystal melting, respectively. T_r and ΔH indicate the temperature range of crystal melting (T_c–T_o) and crystal melting enthalpy, respectively.

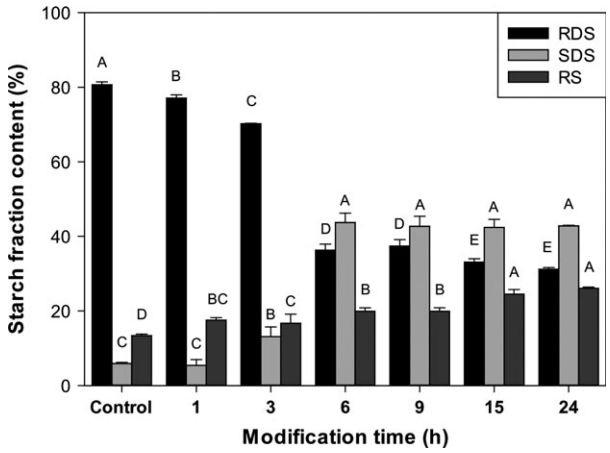

Figure 1 Digestibility of amylosucrase-modified starches. Different capital letters (A, B, C, D and E) within the same fraction are significantly different ($P < 0.05$). RDS, rapidly digestible starch; SDS, slowly digestible starch; RS, resistant starch.

double helices. However, the RS content gradually increased during the entire enzyme reaction. The formation of RS might be additionally facilitated by the promoted retrogradation-like process during enzyme reaction. The relative crystallinity also increased, possibly due to the development of crystalline regions. Recrystallisation of starch molecules during retrogradation is strongly dependent on temperature (Silverio *et al.*, 2000). In this study, the enzyme reaction temperature of 30 °C was suitable for the propagation and maturation of the interchain associations between elongated branched chains.

Branched chain length distributions of SDS+RS and RS

The branched chain length distributions of digested AS-modified starches were determined (Table 2). With increasing digestion time (sequential removal of each fraction from RDS+SDS+RS to SDS+RS, and finally RS), the proportion of long chains (DP $\geq$ 25) decreased, but those of medium chains (DP 13–24) significantly increased ($P < 0.05$). Additionally, the proportions of very short chains (DP $\leq$ 5) were higher in low-digestible starch fractions (SDS+RS or RS) than in their undigested states (RDS+SDS+RS). As AS-modified starches originally had a considerably low content of very short chains, the increment in their proportion after digestion could be explained by the degradation of medium and/or long chains. There could be two reasons for the high proportion of medium chains in spite of digestion. First, the chains buried in an internal crystalline structure would have little or no chance to be hydrolysed by digestive enzymes. In other words, because most of the medium chains

were buried in the interior of the crystalline structure, a considerable amount of medium chains remained intact despite the digestion process. Second, all chains that took part in the formation of the exterior of the crystalline structure and/or the less perfect parts of the crystalline structure, regardless of length, were rapidly and easily degraded during digestion. The remaining crystalline structure resistant to digestive enzymes was mainly composed of medium chains, either original ones or the hydrolysates of long and/or very long chains. Based on the above findings, the medium chains (DP 13–24) were the key constituents of the crystalline structures related to the SDS and RS fractions of AS-modified starch.

X-ray diffraction properties of SDS+RS and RS

During the digestion, the B-type crystalline structure of the AS-modified starches was maintained regardless of the degree of digestion (data not shown) because both SDS and/or RS fractions showed similar crystalline property with the retrograded starches which reveal a B-type crystallinity resulting from the aggregation of long linear chains (Pohu *et al*., 2004). Miao *et al*. (2009) reported that the relative crystallinity is influenced by average amylopectin chain length, crystal size, amylopectin content and extent of interaction between double helices. It has also been reported that RS is composed of crystalline regions, SDS consists not only of crystalline regions but also of amorphous regions, and RDS consists mainly of amorphous regions (Shin *et al*., 2004; Sajilata *et al*., 2006). Therefore, the relative crystallinity followed the order of AS-modified starch (RDS+SDS+RS) < SDS+RS < RS due to the removal of amorphous regions in the RDS and SDS fractions (Table 3). AS-modified starches (RDS+SDS+RS) and their SDS+RS and RS fractions all showed increases in relative crystallinity with increasing AS reaction time.

The SDS+RS fraction of the control showed an approximately 8% increase in relative crystallinity compared with its original form (RDS+SDS+RS). However, this increment after removal of the RDS fraction diminished with increasing AS reaction time, showing no significant difference ($P > 0.05$) in relative crystallinity in the 24 h-modified starch and its SDS+RS fractions. This indicated that the degree of AS modification affected the proportion of amorphous regions. In this regard, the amount of RDS of the AS-modified starch was gradually reduced from 1 to 24 h of the AS reaction.

Thermal transition properties of SDS+RS and RS

The control, which showed no melting behaviour (Table 3) due to its amorphous structure, revealed a melting peak after the removal of the RDS and/or SDS fractions. The thermal transition properties of the SDS+RS and RS fractions showed a higher melting temperature than that of the undigested AS-modified starches (RDS+SDS+RS). The stability and resistance of crystalline structures to the melting are expressed as T_m, and T_o and T_c are related to the melting of the weakest and strongest crystallites, respectively (Barichello *et al*., 1990; Larsson & Eliasson, 1991; Nakazawa & Wang, 2003). The SDS+RS fraction had the higher structural stability, causing an increment in T_m. The relatively weak crystalline structures were destroyed through the enzymatic digestion, consequently resulting in higher stability of the remaining crystallites (Singh *et al*., 2011). After removal of the RDS part mainly consisting of amorphous regions, the remaining fractions (SDS and/or RS) were composed of double helices, derived from the interactions between the branched chains giving rise to their ΔH value. The ΔH is related to the melting of imperfect amylopectin-based crystals, which contribute to both crystal packing and helix melting enthalpy (Lopez-Rubio *et al*., 2008).

The ordering and organisation levels of double helices were different according to fraction. The ΔH values were observed in the order of AS-modified starches (RDS+SDS+RS) > SDS+RS > RS. The higher ΔH value in AS-modified starches compared to those in the SDS+RS and RS fractions indicates that the AS modification caused the formation of abundant double helices that were not highly ordered. The decrease in ΔH according to the hydrolysis of each fraction might be resulted from not only the reduced amount of double helices, but also from the partial hydrolysis of packed crystallites. SDS+RS and RS both showed higher melting parameters with AS reaction time, in common with their primary state (RDS+SDS+RS). Overall, the results indicated that the interactions among the elongated branched chains of amylopectin increased the formation of SDS and RS, composed of dense crystallites.

Conclusions

Modification of waxy corn starch using AS increased the contents of medium and long branched chains and the decrease in short chains. These changes promoted and accelerated the association among amylopectin branched chains, resulting in the formation of a highly stable and more perfect crystalline structure to increase the contents of SDS and RS. Moreover, the isolated SDS and/or RS fractions showed large numbers of medium branched chains (DP 13-24) and reduced amounts of long branched chains. The medium chains with DP 13–24 could be the key constituent of the crystalline structures resistant to hydrolytic enzymes,

related to the SDS and/or RS fractions of AS-modified starch. This study also suggested that the elongation of branched chains mainly affects the formation of SDS, and that the formation of RS is additionally affected by the subsequent retrogradation-like process.

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Conflict of interest

The authors declare that there is no conflict of interest.

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