A Thesis for the Degree of Doctor of Philosophy

Structure—Digestibility Relationship of Recrystallized Starch by Using Amylosucrase-modified Starch as a Model System

아밀로수크레이스 처리 전분 모델을 활용한 재결정 전분의 구조–소화특성 간 관계 구명

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by

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Submitted in Partial Fulfillment of the Requirement
for the Degree of Doctor of Philosophy

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ABSTRACT

Starch can be classified into three fractions of rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS), according to the time of digestion as suggested by Englyst et al. SDS and RS are generally classified as low-digestible starch, and their production and health beneficial effects have been widely studied. However, the classification of RDS and SDS has been argued because the *in vitro* digestion of starch can be described by a single rate constant k of a first order reaction. With increasing interests on carbohydrate-controlling diets because of health concerns, food industry requires a unified strategy for the production of health-functional starch. To achieve this goal, actual existence of each starch fraction should be verified primarily.

Waxy corn and waxy potato starches were modified by amylosucrase (AS) to obtain starch samples with different branch chain length distributions. Hydrolysis curves were obtained for native and AS-modified 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-modified starches revealed a discontinuity, demonstrating that digestion proceeded in two separate phases. It provided the evidence of a fraction that is digested more rapidly than the remainder, i.e., RDS and SDS, and their rate constants were

defined as 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-modified starches were affected by the amount of AS employed. The digestible properties related to RDS, SDS, and RS were investigated by both *in vitro* and *in vivo* study. This study could verify the existence of individual RDS and SDS fractions in a single starch source, and suggested the modified LOS plot approach as an alternative investigative tool for classification of starch fractions on the basis of its digestibility.

The structural characteristics of RDS, SDS, and RS were investigated by tracing the changes according to serial removal of each fraction. 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 the removal of RDS and SDS fractions, chains of DP $\geq$ 37 and DP $\geq$ 25 decreased, respectively. Certain structures found in common among RDS and SDS fractions, respectively, strongly supported the existence of individual RDS and SDS possessing specific structural characteristics of each. The AS-modified 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. The branch chain length distributions of amylopectin determined the primarily generated crystallite organization of AS-modified

starches, causing the diversity of digestion properties. Highly extended branch chains would favor the formation of more slowly digestible form of RDS and SDS. The different values of k_{RDS} and k_{SDS} among starch samples reflected the different structures of RDS and SDS.

The recrystallized starches prepared by using AS-modified waxy potato starch and amylose from potato starch by 3:1 ratio and the structures of their RDS, SDS, and RS were investigated. Co-crystallization of amylose and amylopectin occurred, where the branch chain length of amylopectin determined the length of amylose-amylopectin double helix and mobility of amylose. RDS was composed of singular amylose chains and amorphous double helices not long enough or not aligned ordered structure. Low digestible fractions, SDS and RS, were mainly composed of semi-crystalline or crystalline structure of double helices with DP 13-24. Unlike amylopectin-only environment, the amount of SDS did not show a linear increase according to the branch chain length. Certain length of amylopectin would prefer formation of SDS or RS by controlling the interaction with amylose.

In short, this study provided the knowledge on the formation of low digestible starch under various environments and detail structure of each fraction within starch. It would be utilized in the production of customized starch for specific digestive properties with health functionality.

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

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Chapter 1.
Introduction

1.1. Background

Starch is the primary storage form of carbohydrates, and is commonly found in seeds, roots, tubers, unripe fruits, stems, and leaves of plant. Starch has been the main component of the human diet. It is generally known that starch provides 70-80% of the calorie consumed by human worldwide. In addition to their nutritive value, starches and modified starches can be used to control the physical properties of many foods.

Starch and its derivatives are used for many applications in various industries such as food, paper, and textile industries. In particular, in food industry, native and modified starches are utilized as gelling, thickening, adhesion, moisture-retention, stabilizing, texturizing, and antistaling ingredients, in products like bakery products, pasta, snacks, and so on. Starch majorly contributes to the physicochemical, functional, and digestibility characteristics of those products.

Starch obtained from various sources varies in shape, size, and composition. By these different structural characteristics, starches reveal different functionalities in food product. Therefore, starch can be modified in its structure or other properties to utilize for specific purpose. In particular, in the nutritional aspects, it is important to understand the structural and physicochemical properties of starch and to control its digestibility based on

the knowledge on its properties.

Therefore, in this chapter, previous reports on general aspects of starch, properties related to structure and functionality, digestive properties of starch, and attempts to modify its digestibility were comprehensively reviewed.

1.2. General aspects of starch

1.2.1. Structure of starch

Composition

Regardless of their botanical origins, starch is basically polymers of the six-carbon sugar, D-glucose. Glucose polymerization in starch results in two types of polymers, amylose and amylopectin (Figure 1.1).

Amylose is primarily a linear molecule with α-1,4-linked glucosyl units with 500-6,000 glucose units, to have average molecular weights range of 10^5-10^6 (Roger, Tran, Lesec, & Colonna, 1996). Amylose may have up to 10 or more branches, forming predominately single chain helices. Amylose is also known to exhibit a sixfold left-handed double helical conformation.

Amylopectin is a highly branched polymer that is much larger than amylose (greater than 10^8) (Roger, Bello-Perez, & Colonna, 1999). Amylopectin is composed of α-1,4-linked glucose segments connected by α-1,6-linkeage at branch points. Amylopectin is the predominant molecule in most normal starches (~70–80%) and so is the major component, strongly influencing the physicochemical and functional characteristics of starch.

The ratio of amylose to amylopectin within a starch, their structure, and the way in which these two polymers pack themselves in the granule vary depending on the starch source, and are very important factors to affecting

the physicochemical and functional properties of starch (Fredriksson et al., 2000).

There are also minor components found in starch including lipids (up to 1% in cereal starches), proteins, phosphorus (especially in potato starch), and other minerals.

Granular structure

Amylose and amylopectin do not exist free in nature, but as components of discrete, semi-crystalline particles called starch granules. Starch granules are produced within amyloplast, in which their biosynthesis initiates at the hilum, and the granules then grow by apposition (Buléon, Colonna, Planchot, & Ball, 1998).

Though the major components of all types of starch granules are amylose and amylopectin polymers, there is great diversity in the structure and characteristics of native starch granules. Starch granules have varying characteristics according to their botanical origin, such as sizes (～1–100 μm in diameter), shapes (spherical, lenticular, polyhedral, and irregular), size distributions, and forms (simple and compound) (Tester, Karkalas, & Qi, 2004). It is known that shape and size of starch granules are responsible for the physicochemical and functional characteristics of starch.

The internal structure of starch granule is densely packed semi-crystalline in nature. The dense layer in a growth ring of repeats of alternating amorphous region and crystalline lamellae (Manners, 1989). The crystalline lamellae are made up of amylopectin double helices, which are packed in a parallel arrangement, while the amylopectin branch points are in amorphous region (Zobel, 1988). The exact location of amylose in the granule remains unclear, but is, however, considered to be interspersed between amylopectin, rather than being located in clusters, contributing mainly to the amorphous regions (Jane, Xu, Radosavljevic, & Seib, 1992).

The characteristic surface structure of starch granule has been reported according to the type of starch. Generally, starches from cereals (A-type) such as corn, sorghum, millet, large granules of wheat, rye, and barley commonly have pores on their granular surface (Fannon, Hauber, & BeMiller, 1992), while starches from potato (B-type) have a smooth surface with no pores.

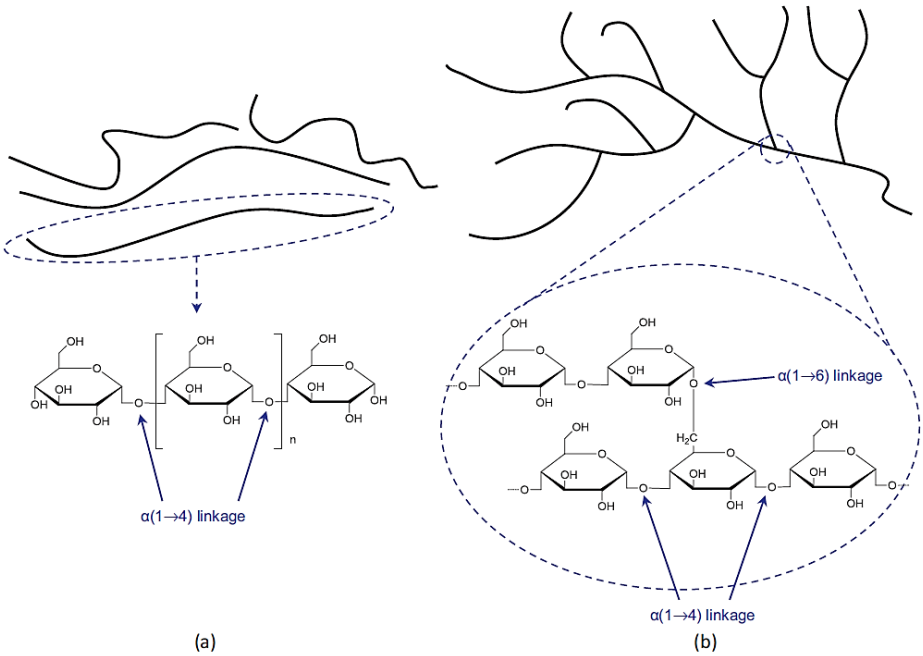


Figure 1.1. Chemical structure of (a) amylose and (b) amylopectin (Xie, Pollet, Halley, & Avérous, 2013).

1.2.2. Starch crystallinity

Wide-angle X-ray diffraction scattering studies have classified packing of amylopectin double helices of native starch into A-, B-, C-, and V-types. Native starch granules exhibit two main types of X-ray diffraction diagrams (Figure 1.2.), the A type for cereal starches and the B type for tuber and amylose-rich starches (Zobel, 1988). A-type crystalline diffraction pattern has the main diffraction doublet at 17° and 18° and peaks at 15°, 20°, and 23° 2θ. B-type crystallinity is characterized by a strong diffraction peak at 17° and weaker reflections at 5°, 15°, 22°, 24°, and 29.5°, 2θ. The C-type is shown as a combination of the A- and B-type diagrams, and is commonly found in starches from pea and various bean starches or sweet potato (Colonna, Buleon, & Mercier, 1981). The V-type can be found in starches after formation of amylose helical complexes with lipids or related compounds (Cheetham & Tao, 1998), which is recognized by a typical major peak at 19.8° and additional peaks at 7.4° and 12.9° 2θ.

Differences between the two allomorphs of A- and B-type crystals come from the relative amounts of water and the organization of the double helices in the unit cell of the crystal (Figure 1.3.). The A-type accommodates densely packed double helices, while B-type double helices are rather loosely packed. In detail, the A-type contains 4 water molecules per 12

glucose residues, while the B-type has 36 water molecules.

The degree of crystallinity of the starches can be calculated and expressed as the percent fraction of the area of the crystalline sub-diffractogram relative to the area of the total diffractogram (Nara & Komiya, 1983).

In general, a higher susceptibility of A-type crystallites with hydrolysis compared to B-type crystallites has been reported. The X-ray diffraction pattern has been related to starch digestibility since starches with B-type diffraction pattern have shown high resistant starch content, and those with A-type have presented a high level of slowly digestible starch (Zhang, Venkatachalam, & Hamaker, 2006b).

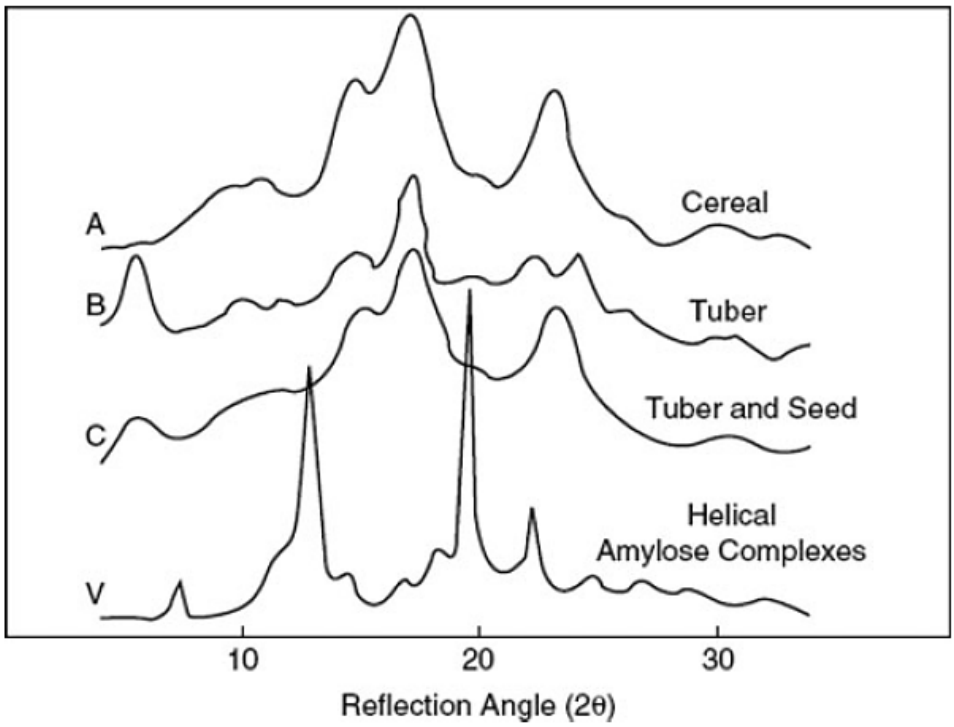


Figure 1.2. Characteristic X-ray diffraction of A, B, C, and V-type starch (Zobel, 1988).

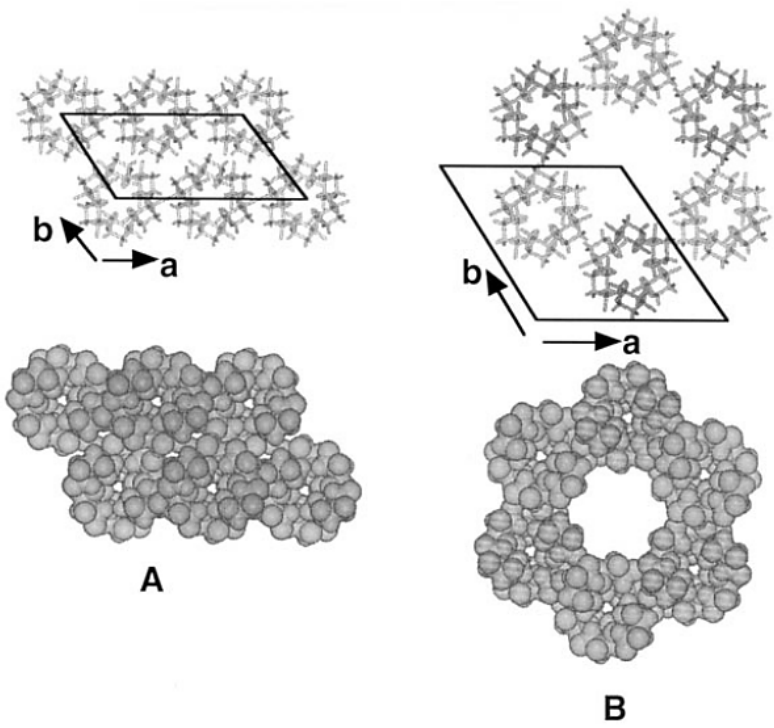


Figure 1.3. Crystalline packing of double helices in (A) A-type and (B) B-type amylose. Projection of the structure onto the (a, b) plane (Buléon et al., 1998).

1.2.3. Gelatinization, pasting, and retrogradation of starch

In its native form, a starch granule is insoluble in cold water, thus creating a suspension when mechanically dispersed in water. However, a combination of heat and water causes starch granules to undergo unique and irreversible changes called ‘gelatinization’. As the starch consumed by humans is cooked, it is important to understand the changes occurring in starch during cooking.

Starch gelatinization is the collapse (disruption) of molecular orders within the starch granule manifested by irreversible changes in properties such as granular swelling, native crystalline melting, loss of birefringence, and starch solubilization (Atwell, Hood, Lineback, Varriano-Marston, & Zobel, 1988). During gelatinization process, a starch granule absorbs a considerable amount of water and loses its semi-crystalline nature while swelling to many times its original volume. From a mechanistic perspective, heating of starch in water causes disruption of the hydrogen bonds between polymer chains, thereby weakening the granule. The point of initial gelatinization and the range over which it occurs are governed by starch concentration, method of observation, granular type, and heterogeneities within the granule population under observation.

As the heating process continues after gelatinization, the granules swell to many times their original volume, and the viscosity of the medium increases.

This phenomenon is called pasting. It involves granular swelling, exudation of molecular components from granule (amylose leaching), and eventually total disruption of the granules (Atwell et al., 1988). It should be noted that pasting is not exactly separated from gelatinization, but rather an overlapping occurrence as a continuation of gelatinization.

Starch retrogradation is a process which occurs when starch chains begin to reassociate in an ordered structure. The molecules in a gelatinized starch can undergo inter- and intra-molecular association into an ordered structure when stored under certain conditions (Atwell et al., 1988). In its initial phases, two or more starch chains may form a simple junction point which then may develop into more extensively ordered regions. Ultimately, a crystalline order appears to result in the formation of crystalline aggregates and a gelled texture.

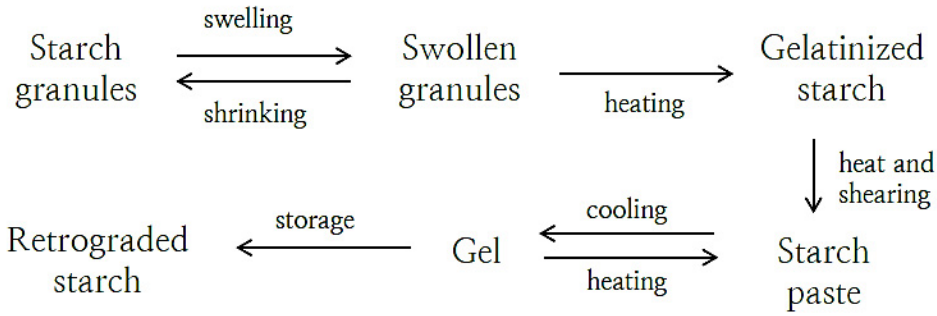


Figure 1.4. Schematic diagram for changes in starch state.

1.2.4. Recrystallization of starch

The recrystallization of starch is the changes in gelatinized/amorphous starch resulting in ordered or crystalline state from initially amorphous state. The recrystallization takes place with a certain minimum amount of water during storage at a specific temperature for a period of time. Recrystallization, or retrogradation of starch is an unavoidable phenomenon during cooling and storage of the cooked starch or starchy foods.

There are many factors influencing the recrystallization such as botanical origins, ratio of amylose to amylopectin, the presence of lipid and surfactants, fine structure of amylopectin molecule, etc. (Eliasson & Gudmundsson, 2006; Fredriksson, Silverio, Andersson, Eliasson, & Åman, 1998; Inaba, Hatanaka, Adachi, Matsumura, & Mori, 1994). Also, starch concentration, presence of other food ingredients, storage conditions including temperature, time, and water content affect the degree of recrystallization among starch molecules (Chang & Liu, 1991; Silverio, Fredriksson, Andersson, Eliasson, & Åman, 2000; Zhou, Wang, Yoo, & Lim, 2011).

The recrystallization of starch is based on a three-step mechanism: nucleation, propagation, and crystal perfection (maturation). Amylose and amylopectin generally participate in different steps during the whole recrystallization process. Amylose molecules irreversibly and rapidly

reassociate to form crystal nuclei after the first two days, considered as 'short-term' retrogradation (Fearn & Russell, 1982). Propagation and maturation occur following nucleation, and are rather associated with 'long-term' retrogradation. The crystalline region of amylopectin grows slowly around the amylose crystal nucleus and forms a perfect crystallite. To be specific, outer chains of amylopectin molecules interact with amylose nucleus to form a network, and it is referred to as co-crystallization. Nucleation and propagation are favored at the temperature near glass transition temperature (T_g) and melting temperature (T_m), respectively (Baik, Kim, Cheon, Ha, & Kim, 1997; Durrani & Donald, 1995; Silverio et al., 2000). The association of 40-70 glucose units forms the double helical amylose, whereas branch chain length of at least DP 15 is needed to cause amylopectin crystallization (Cui, 2005).

1.3. Nutritional property of starch

Recently, as people have been consuming more calories than expending, overweight, obesity, and related illnesses such as diabetes have become major public health concerns worldwide (Lehmann & Robin, 2007; Patil, 2004). Therefore, many food researchers are interested in the strategy for healthy diet. Because carbohydrate, especially starch, is the major source of human diet, food and nutritional industries have interests in slowing down the digestion rate of ingested carbohydrate sources.

1.3.1. Digestion of starch

Starch digestion and absorption consists of essentially three phases: the intraluminal phase, the brush border phase, and the glucose absorption phase. In other words, starch is ingested, then enzymatically hydrolyzed, and finally absorbed as glucose for energy metabolism in the upper gastrointestinal tract (Miao, Jiang, Cui, Zhang, & Jin, 2015). Many kinds of enzymes are involved with digestion of starch. In the gastrointestinal tract, salivary and pancreatic α-amylases break down the starch molecules into smaller oligosaccharides, such as maltose, maltotriose, and other branched oligosaccharides, which are further converted into glucose in the small intestine by the action of the maltose-glucoamylase and sucrose-isomaltase (Hoebler, Devaux, Karinthi, Belleville, & Barry, 2000; Lee et al., 2012a).

The mechanism of enzymatic hydrolysis of granular starch observed in *in vitro* approaches can be differentiated according to the type of starch and its characteristic surface structure (Figure 1.6). In the case of A-type starches, enzymatic digestion usually begins at the surface pores and interior channels, and the enzyme can easily diffuse into the interior of granule; and this side-by-side digestion gradually enlarges the channel by simultaneously digesting crystalline and amorphous regions (Zhang, Ao, & Hamaker, 2006a). The B-type starches, which have smooth surface with no pores, apparently have a surface 'barrier'. Therefore, enzyme adsorbs onto granule surface and has to break through the external surface to diffuse inside the granule (Dhital, Shrestha, & Gidley, 2010).

Moreover, the enzymatic hydrolysis of native starch granule is affected by a range of factors including granular structure (shape, size, and porosity), supramolecular structure such as organization of growth rings and degree of crystallinity, molecular structure of amylose and amylopectin, presence of non-starch materials, and so on (Ao et al., 2007; Dhital et al., 2010; Dreher, Dreher, Berry, & Fleming, 1984; Magallanes-Cruz, Flores-Silva, & Bello-Perez, 2017; Singh, Dartois, & Kaur, 2010; Zhang, Ao, & Hamaker, 2008a). Digestibility of starch has been associated with the glycemic index (GI) to assess the nutritional quality of foods. The GI has been used as an indicator

of postprandial glucose response for starch-based foods.

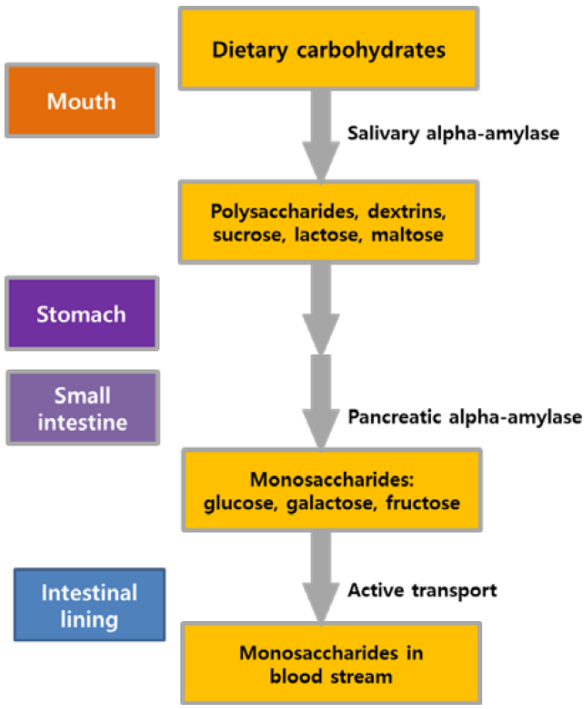


Figure 1.5. Schematic diagram for digestion of starch in human body.

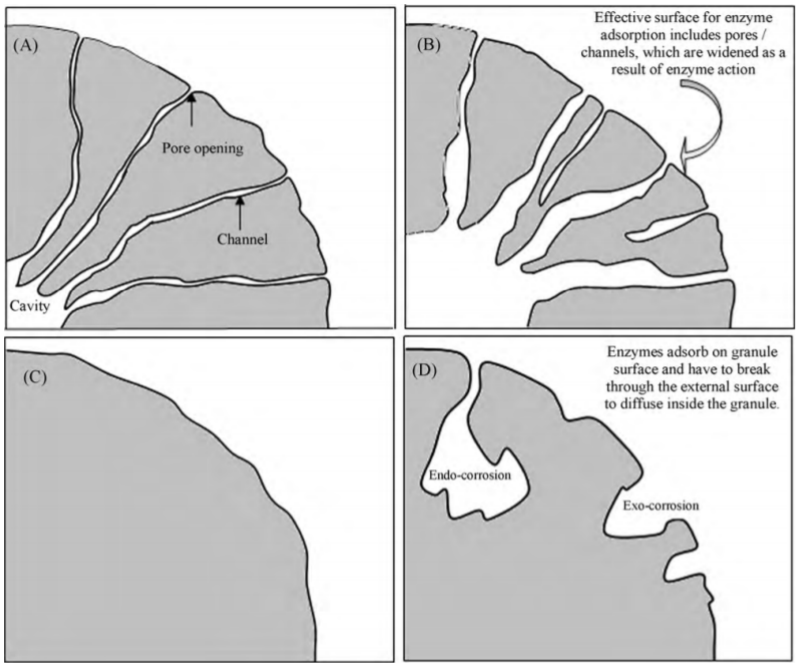


Figure 1.6. Schematic illustration of diffusion of amylase and its catalytic patterns in maize and potato starches. (A) maize starch showing pores, channels, and cavity; (B) maize starch hydrolyzed by amylase with enlarged pores, channels, and cavity; (C) potato starch lacking pores, channels, and cavity; (D) potato starch exo- and endo-corroded by amylase (Dhital et al., 2010).

1.3.2. Analysis methods of starch digestibility

The most popular tool to express the digestive property of starch *in vivo* is glycemic index (GI). The concept of GI was introduced to classify carbohydrate-based foods based on the postprandial glucose responses after consumption (Jenkins et al., 1981). GI is defined as the incremental area under the blood sugar response curve (the change in blood glucose level two hours after a meal) of 50 g available carbohydrate portion of a test food expressed as percentage of the response to the same amount of carbohydrate from a standard food (either white bread or glucose) ingested by the same subject.

The term of glycemic load (GL) has been proposed to take into account the differences in carbohydrate content among foods served for a meal (Salmeron et al., 1997). GL is calculated by multiplying the GI of a food by the amount of total dietary carbohydrate per serving, and it therefore serves as an indicator of the ability of the carbohydrate to raise blood glucose level or global dietary insulin demand. There is also a concept of extended glycemic index (EGI), proposed to measure the slow digestion of starchy foods *in vivo* on an extended time scale (He, Liu, & Zhang, 2007).

The digestibility of starch measured *in vivo* is a time-consuming and expensive process that requires many human subjects with specific attributes.

Therefore, *in vitro* tests to measure the starch digestion rate are more commonly used.

The most common tool is Englyst test. Englyst et al. (1992) suggested the analytical method to classify the starch fractions according to their digestion properties into rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS). This method imitates the physiological conditions of starch digestion, as the analyses give values in agreement with the average amount of starch escaping complete digestion and absorption in the human small intestine. Two time points of 20 min and 120 min are selected for determination of fractions. The method provides results comparable to those in *in vivo* studies and is adapted to a variety of substrates, such as minced or fresh samples. Brumovsky and Thompson (2001) suggested a modified method with reduced complexity; excluding non-starch components and considering starch only. Many recent studies also modified this method by altering type and concentration of digestive enzyme, concentration substrate, reaction time, etc. (Miao, Jiang, & Zhang, 2009a; Ovando-Martínez, Whitney, Reuhs, Doehlert, & Simsek, 2013; Shin et al., 2007).

There are also other approaches focused on measuring digestion rate of starch. An *in vitro* assay proposed by Goñi, Garcia-Alonso, and Saura-

Cailxto (1997) employs proteolytic enzymes in combination with amylases, in contrast with general assays using amylases only. They described the hydrolytic process with a mathematical first-order kinetic equation; starch hydrolysis kinetic curves can be presented as the equation $C_t = C_\infty(1 - e^{-kt})$, where C_t is the quantity of ingested starch digested at time t, C_∞ is the potentially digestible starch fraction (less than 100), k is the fractional starch digestion rate, and $\boldsymbol{t}$ is the chosen time. It allows the prediction of the percentage of total starch hydrolysis at any time with a close estimation of the GI of a food product.

A 'logarithm-of-slope (LOS)' plot was introduced recently as an improved first-order kinetic model for the analysis of starch hydrolysis (Butterworth, Warren, Grassby, Patel, & Ellis, 2012; Patel, Day, Butterworth, & Ellis, 2014). From LOS plot, the digestion process could be described by two separate first-order reactions with different rate constant values which represent digestion of RDS and SDS, respectively. This approach 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.

1.3.3. Classification of starch fractions according to nutritional value

For nutritional purposes, starch can be classified into three categories by the Englyst test (Englyst et al., 1992), depending on their rate and extent of digestion. These include rapidly digested starch (RDS), slowly digested starch (SDS), and resistant starch (RS).

RDS is rapidly and completely digested in the small intestine, and is determined by the amount of glucose released after 20 min. This is associated with elevated plasma glucose and insulin, therefore it is linked with diabetes, coronary heart disease, and with the aging process. SDS is associated with the amount of glucose released between 20 and 120 min hydrolysis, which has characteristics of complete but slow digestion in the small intestine. It has a moderate influence on the plasma glucose and insulin levels but is the most desirable form of starch from the nutritional point of view. RS is not digested in the small intestine, defined as the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals. It can be calculated by total starch minus amount of glucose released within 120 min hydrolysis.

1.4. Low-digestible starch

1.4.1. Definition of low-digestible starch

Among the three starch fractions, SDS and RS can be classified as low-digestible starch fraction. Moreover, starch with a noticeable amount of these low-digestible fractions can be called low-digestible starch.

As stated above, SDS is the starch fraction that is likely to be completely digested in the small intestine but at a slow rate. It is generally known that native A-type cereal starches have considerable amounts of SDS. There are some reports on the structural features of SDS. It has been hypothesized that SDS consists of less perfect crystalline regions containing a small portion of double helices and an amorphous region (Shin et al., 2004). In the same context, Miao et al. (2015) suggested that the structure of SDS may consist of imperfect crystallites and amylopectin with a high branching density and pattern. A parabolic relationship was reported between SDS content and the weight ratio of short chains to long chains in amylopectin (Zhang, Sofyan, & Hamaker, 2008b). Especially, the high proportion of SDS in cereal starches was correlated to a higher fraction of short chains with degree of polymerization (DP) 5-10 (Zhang et al., 2006b).

RS is not digested in the small intestine, and therefore may be fermented in the large intestine by colonic microflora like dietary fiber. Structural

features of RS have been studied comprehensively. RS is generally known to mainly consist of crystalline regions with high perfection and rigidity but also has various conformations, classified as five subcategories called RS1, RS2, RS3, RS4, and RS5 (Birt et al., 2013; Fuentes-Zaragoza et al., 2011; Wong & Louie, 2017).

Starch that is covered and protected by cell walls or other barriers from the action of the digestive enzyme, such as in whole-grain flour and unpolished rice, is classified as RS1. Starch with a high crystallinity and amylose content is RS2. For example, starches from raw bananas, potato, or high amylose maize cannot be easily reduced to degradation products that can be absorbed in the small intestine (Englyst et al., 1992).

Retrograded starch produced by cooling gelatinized starch is referred to as RS3. When the starch in a food system undergoes gelatinization and is allowed to retrograde by cooling, the reorganized and alternated orientation of starch molecules can have resistance to digestion. Retrograded starch molecules are unable to fit into the substrate binding site of amylase (Sievert & Pomeranz, 1989). RS3 consists of re-associated amylose formed during cooling of gelatinized starch, and is usually contained in moist-heat-treated foods.

The starch modified by chemical reagents and have accompanied novel

chemical bonds other than α-1,4 or α-1,6-linkages is RS4. These modifications limit the ability of the starch molecule to swell during heating, or change the structure of the molecule so it can no longer fit into the binding site of the digestive enzymes (Woo & Seib, 2002).

Finally, RS5 refers to the complex that consists of a fatty acid molecule and an amylose chain. The complexes get aggregated to form a superstructure, which were found to be resistant to enzymatic hydrolysis (Birt et al., 2013).

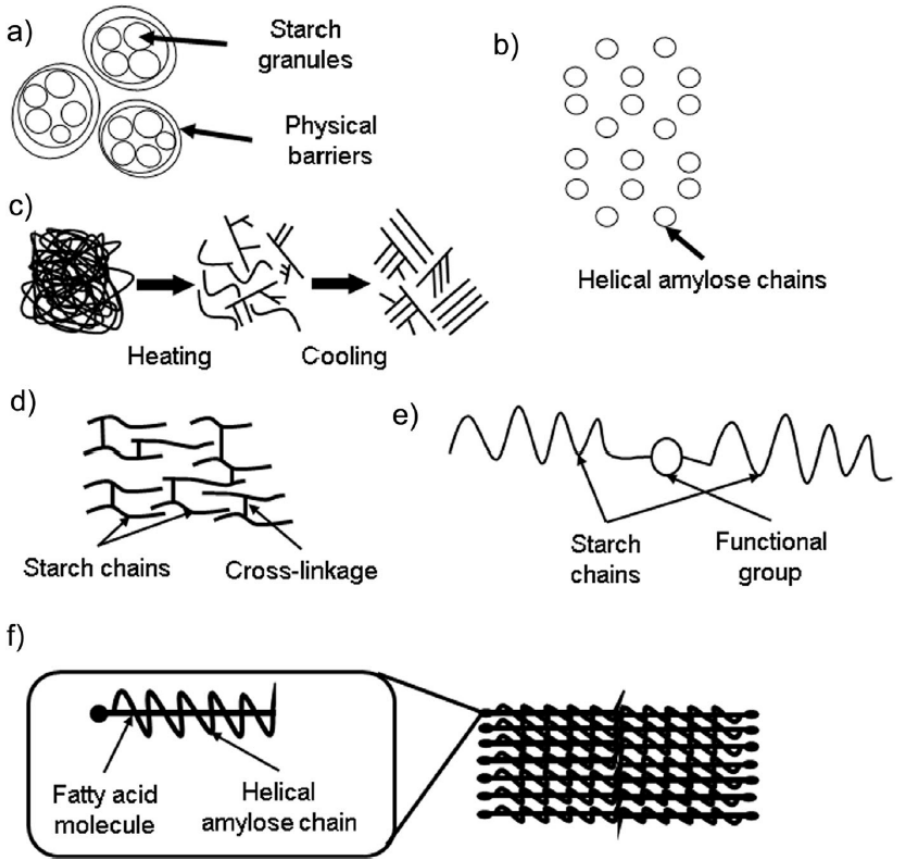


Figure 1.7. Different types of RS.

(a) RS1: the starch molecules are encapsulated by a physical barrier; (b) RS2: B-type polymorph of starch molecules. The helical amylose chains, as depicted by circles, are closely and orderly aligned, enabling the structure to resist enzyme degradation; (c) RS3: the process of retrogradation, thereby forming RS3; (d) RS4: cross-linkages and (e) starch molecules linked by a new functional group, forming RS4; (f) RS5: complex formed by an amylose chain and a fatty acid molecule, which aggregate into superstructure of RS5. (Wong & Louie, 2017).

1.4.2. Beneficial physiological effects of low digestible starch

Consumers and food industry are interested in foods with low GI, which have low postprandial glycemic response and high indigestible carbohydrate content. As a representative example, consumption of low-GI starchy food induces a reduction in plasma triacylglycerols, phospholipids levels, and epididymal adipocyte volume as well as a tendency toward lower plasma insulin levels (Lerer-Metzger et al., 1996). Therefore, production and characterization of SDS and RS have been widely studied due to the findings of their beneficial health effects.

The ultimate benefit of SDS is the control of blood glucose level. The benefits of SDS have been explained by its low glycemic index, which is related to the reduction of the risk of diverse chronic degenerative diseases such as type 2 diabetes, overweight, and obesity (Jenkins et al., 2002). A significant reduction of potential risk for metabolic syndrome was also observed in the study comparing SDS and RDS diet (Ells, Seal, Kettlitz, Bal, & Mathers, 2005). Moreover, SDS intake causes a decrease in the rate of gastric emptying, and therefore reduces food intake and prolongs satiety by controlling incretin hormone secretion (Strader & Woods, 2005). Also, a relationship between SDS and mental performance was reported (Benton & Nabb, 2003). The aforementioned advantages of SDS imply that SDS-rich

foods may provide wide health benefits.

The nutraceutical importance of RS is attributed to its fermentation in large intestine as well as the role as a low-GI ingredient. Therefore, replacement of digestible carbohydrate by RS for improvement works effectively in postprandial glycemic control (Wong & Louie, 2017).

Moreover, the microbial metabolites resulting from RS fermentation, such as short chain fatty acids like acetate, propionate, and butyrate, have physiological and metabolic impacts on human health; e.g. stabilization of blood glucose levels and suppression of cholesterol synthesis in the liver (Haralampu, 2000). It has been recently proposed that gut microbiotas are a key component in the mediation of the metabolic benefits of RS: RS can lead to a healthy gut microbiota, by acting as a substrate for the selective growth of probiotic bacteria such as lactobacilli, bifidobacteria, and streptococci (Keenan et al., 2015). RS consumption exerts hypoglycemic and hypocholesterolemic effects, and also prevents colon cancer, reduces gall stone formation and fat accumulation, and increases mineral absorption (Sajilata, Singhal, & Kulkarni, 2006).

1.4.3. Attempts to produce low digestible starch

Native starch granules can be modified for a wide range of applications and functionality. For starch products with high SDS and/or RS, structural modification of granular starch or starch molecules has been achieved using physical, enzymatic, chemical, and genetic methods or a combination of them.

Chemical modification

The most common chemical modification processes are acid treatment, cross-linking, oxidation, and substitution including esterification and etherification. These attempts change properties of starch by introduction of functional groups within the starch molecule. The effect of the type of chemical treatment and/or the degree of modification on the extent of starch digestion has been comprehensively compared by Wolf, Bauer, and Fahey (1999). Cross-linked starches that contain high amount of RS were produced by treating starches with sodium trimetaphosphate and sodium tripolyphosphate, where the level of RS increases over 75% (Woo & Seib, 2002). Acid treatment such as citric acid is effective for increasing SDS and RS formation, giving a dramatic increase of SDS or RS (Shin et al., 2007; Xie & Liu, 2004).

Physical modification

Physical modification provides structural changes without the covalent linkages (Eliasson & Gudmundsson, 2006), and includes hydrothermal treatment, recrystallization, polymer-entrapment, extrusion, and so on. Hydrothermal treatments such as heat moisture treatment (HMT) and annealing have been extensively used to increase the SDS and RS fractions in starches, without destroying the granular structure and birefringence. The digestibility of sweet potato starch was lowered, and especially the SDS content could be doubled compared to the raw starch (Shin, Kim, Ha, Lee, & Moon, 2005). Hydrothermal treatment effectively worked on starches from various sources, such as rice, corn, pea and lentil, oat, waxy potato, and so on (Anderson, Guraya, James, & Salvaggio, 2002; Chung, Liu, & Hoover, 2009; Lee, Kim, Choi, & Moon, 2012b; Ovando-Martínez et al., 2013). Zhang, Hu, Xu, Jin, and Tian (2011) employed temperature-cycled retrogradation to obtain 51.62% of SDS from waxy rice starch by the similar mechanism of producing RS3.

Enzymatic modification

Controlled enzymatic treatment of starch is an alternative approach to change the starch supramolecular structure and thereby to achieve

appropriate digestibility and glycemic response. Debranching enzymes such as pullulanase and isoamylase hydrolyze α-1-6 -linkages of amylopectin to produce linear chains which are then recrystallized to increase SDS or RS fractions (Miao et al., 2009a; Shin et al., 2004). Modification of maize starch with β-amylase and transglucosidase increase the number of short chains and alpha-1,6 linkages, which leads to slower digestion (Miao et al., 2014a). Ao et al. (2007) reported that β-amylase, transglucosidase, and maltogenic α-amylase reduced the digestion of starch with an accompanying increase of SDS content. Tapioca starch modified by combined treatment of branching enzyme and maltogenic amylase showed increased amylolytical-resistance (Le et al., 2009). Amylosucrase treatment on starch (Kim et al., 2013; Kim, Kim, Moon, & Choi, 2014; Kim, Kim, Choi, Park, & Moon, 2016a; Ryu et al., 2010; Shin, Choi, Park, & Moon, 2010) was recently employed to increase its resistance to digestion, by re-organization of starch molecules via chain length elongation.

Genetic modification

Genetic modification of starch biosynthesis involves developing a strategy to generate new cultivars with desired functionality through extensive breeding and characterization of the resulting varieties. Genetically

controlled factors that affect the type of starch produced include starch structure, starch content, interacting cell components, and starch granule architecture.

1.4.4. Amylosucrase-modified starch and its applications

A few studies employed amylosucrase (EC 2.4.1.4., AS) to produce starches with low digestibility (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. AS catalyzes the elongation of some branch chains by attaching 12 to 18 glucosyl units at non-reducing ends (de Montalk, Remaud-Simeon, Willemot, Planchot, & Monsan, 1999). AS can be obtained from various strains such as *Neisseria polysaccharea*, *Deinococcus geothermalis*, *Deinococcus radioduransm*, *Alteromonas macleodii*, and so on, and the characteristics of the enzyme from each strain has been studied (Moulis, André, & Remaud-Simeon, 2016).

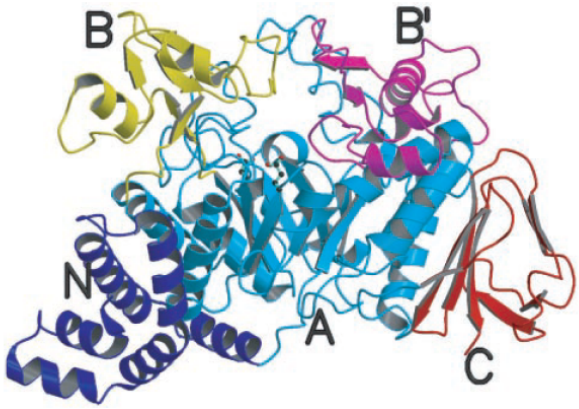
When the starch exists as a glucose acceptor, elongation of the glucosyl units occurs at non-reducing ends of external chains (Büttcher, Welsh, Willmitzer, & Kossmann, 1997; de Montalk et al., 2000; Rolland-Sabaté, Colonna, Potocki-Véronèse, Monsan, & Planchot, 2004). Shin et al. (2010), who conducted 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 of

AS treatment on waxy corn starch (Kim et al., 2014). The low digestion property of AS-modified starch has been confirmed through both *in vitro* and *in vivo* (Kim et al., 2016a).

Attempts to strengthen the low-digestion property of AS-modified starch have been conducted. Dual modification using AS and branching enzyme (Jo et al., 2016), and HMT treatment following AS-modification (Kim, Kim, Choi, Park, & Moon, 2016b) were reported. Park, Kim, Kim, and Moon (2013) encapsulated AS-modified starch using alginate to provide physical barrier against digestive enzyme attack.

Moreover, AS-modification enables the formation of lipid inclusion complexes with waxy corn starch (Kim, Kim, Choi, Park, & Moon, 2017), suggesting its potential to act as a prospective delivery system of fatty acids or other hydrophobic materials. It also could be utilized as a food ingredient that requires a low-glycemic response.

(A)



(B)

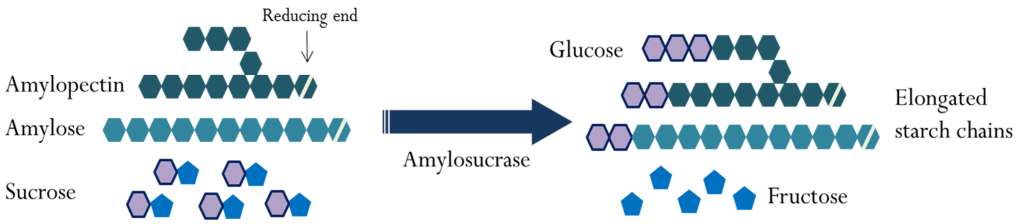


Figure 1.8. (A) Three-dimensional structure of amylosucrase (Skov et al., 2001) and (B) schematic diagram for the elongation of starch chains by amylosucrase reaction.

1.5. Research objectives

On the basis of the nutritional classification of starch into RDS, SDS, and RS fractions, formation and physiological effects of each fraction have been studied widely. However, at the same time, there was also skeptical opinion on the classification of starch digestible fractions. As interests in diets controlling carbohydrate consumption have been rising recently, a unified strategy for the production of health-functional starch is required in the food industry. To achieve this goal, the actual existence of each starch fraction should be verified primarily.

Moreover, it is important to have knowledge on certain structures that assign low digestible properties to starch for effective production of low digestible starches. Though there have been numerous studies producing starches containing low digestible fractions, profound and specific understanding on the structure of respective fraction is lacking.

Furthermore, it has been hard to trace the specific effect of a certain factor on the digestibility, since many previous studies employed various starches from different botanical source and with different physicochemical characteristics. The fact that starchy foods are cooked before consumption, and therefore the gelatinization and resultant retrogradation of starch are unavoidable phenomena is also a problem. An approach using recrystallized

starch may provide useful information and accurate investigation under controlled experimental environment. On the basis of the former statements, this study employed recrystallized starch prepared by using amylosucrase-modified starch as a model system, to control every condition except the branch chain length of amylopectin as the primary factor.

The first objective of this thesis was to suggest an analytical method that can verify and define the RDS, SDS, and RS fractions accurately. To achieve this goal, amylosucrase-modified starches with different structures were prepared and their digestion properties were analyzed based on 1^{st}-order kinetics. The second objective was to investigate the structural characteristics of each digestible fraction based on the degree of polymerization of glucose unit in amylopectin branch chains, using amylosucrase-modified waxy starch. The third objective was to elucidate the interaction between two starch component molecules and the formation of low digestible starch fractions accorded to it, via recrystallization of AS-modified waxy starch and amylose. A comprehensive understanding on the relationship between molecular structure and digestibility of starch would be provided through this study.

Chapter 2. Modified LOS plot method for *in vitro* digestibility analysis using amylosucrase-modified waxy starches with different branch chain length distributions

* Part of this chapter was published in *Food Hydrocolloids* (H.R. Kim et al., 2017, Kinetic studies of in vitro digestion of amylosucrase-modified waxy corn starches based on branch chain length distributions, 65, 46-56)

2.1. 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 are intimately related to an increase in the consumption of refined carbohydrates (Zhang, Sofyan, & Hamaker, 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). 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 a 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 is defined as resistant starch (RS). This system implies that a particular starch granule contains individual fractions that have different enzyme susceptibility, and their study 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, Englyst, Hudson, Cole, & Cummings, 1999; Englyst, Vinoy, Englyst, & Lang, 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 have implications for physical and mental performance, satiety, and diabetes management (Lehmann & Robin, 2007; Wolf et al., 1999). Therefore, improving food quality with a higher amount of SDS is of interest to 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's 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 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 in a variety of maize mutant samples. This suggestion was supported by the results of plentiful studies (Casarrubias-Castillo, Hamaker,

Rodriguez-Ambriz, & Bello-Pérez, 2012; Miao et al., 2014b; Shin et al., 2010). In addition, the molecular structural features of amylopectin such as molecular weight, dimension or size, density, degree of branching, and distribution of short chains influence the starch hydrolysis (Goesaert, Bijttebier, & Delcour, 2010; Miao, Zhang, Mu, & Jiang, 2011; Naguleswaran, Vasanthan, Hoover, & Bressler, 2014).

A few studies employed amylosucrase (AS; EC 2.4.1.4) 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 the 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, Welsh, Willmitzer, & Kossmann, 1997; de Montalk et al., 2000; Rolland-Sabaté et al., 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 AS 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, 2008b).

The objectives of this study were to develop an alternative method to analyze *in vitro* digestibility of starch based on rate term, and thereby to ascertain the individual existence of RDS and SDS fractions in a single kind of particular starch. On the basis of the suggestion that the slow digestion property of the starch material could be manipulated according to its amylopectin fine structure, waxy corn starch and waxy potato starch were modified with AS at various levels to obtain starch samples with different SDS contents. The digestibility of AS-modified starch samples were examined and discussed with regard to their first-order kinetic parameters, based on comprehensive comparison between conventional and newly-developed analysis method in this study.

2.2. Materials and methods

2.2.1. Materials

Waxy corn starch and waxy potato starch were obtained from Ingredion (Westchester, IL, USA) and Avebe (Veendam, Netherland), respectively. 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 300L, 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.

2.2.2. Enzyme assay of AS activity

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 of 4% (w/v) sucrose, 0.1 mL of 1% (w/v) 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 shaking 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).

2.2.3. 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 shaking water bath at 30°C and 80 rpm. The samples were named according to the type of starch and relative amount of AS. For example, AS-modified waxy corn starches were presented as WCAS1, WCAS2, WCAS4, and WCAS8, and AS-modified waxy potato starches were labeled with WP. 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 samples (WCAS0 and WPAS0) were prepared according to the same procedure without enzyme addition.

2.2.4. 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. 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 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.

2.2.5. Determination of *in vitro* digestibility

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 (4.51g) 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.

2.2.6. Determination of starch fractions using the Englyst method

Starch fractions were determined according to the degree of hydrolysis as described by Shin et al. (2004), which is a generally accepted modified method of original Englyst's standard. 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).

2.2.7. Determination of starch fractions according to the modified 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):

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

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

$$\boldsymbol{\frac{dC}{dt} = C_\infty k e^{-kt}} \qquad \textbf{(2)}$$

This equation can be expressed in logarithmic form as follows:

$$\boldsymbol{\ln\left(\frac{dC}{dt}\right) = \ln(C_\infty\, k) - kt} \qquad \textbf{(3)}$$

Thus, a plot of $\ln(dC/dt)$ against t shows a linear graph with a sloe 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: Therefore, C_{int} (C_t at t=t_{int}) was the amount of RDS where t_{int} is the intersection time of two linear plots. SDS was calculated by the subtraction of the amount of RDS from total amount of digestible starch.

2.2.8. 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 4 sec. The relative crystallinity was calculated (Nara & Komiya, 1983) using the software developed by the instrument manufacturer (EVA, 2.0).

2.2.9. Postprandial blood glucose response in mice

Male CD1 mice (ICR) of 9-week old were employed and housed in an approved laboratory animal facility for a 7-day adaptation period. Blood glucose level and body weight of each mouse were measured after 16 h of fasting, and mice were grouped to have similar average blood glucose level and body weight among all groups. Eight mice were adopted for each treatment group, and each group was fed 10% glucose solution or 10% AS-modified waxy corn starch suspension in distilled water (w/v) via an oral zonde needle by 10 mL per kg body weight. Blood samples were taken from the tail vein of each mouse at 0, 15, 30, 60, 90, 120, 150, 180, and 240 min after consumption of the starch suspension or glucose solution. Blood serum glucose levels were measured using an Accu-Chek® (Performa, Roche Diagnostics GmbH, Germany). All institutional and national guidelines for the care and use of laboratory animals were followed (IACUC approval

number SNU-170921-1).

2.2.10. Statistical analysis

Analysis of variance using Duncan's multiple range test ($p<0.05$) and Pearson's correlation analysis was conducted using IBM SPSS statistics version 21.0 (IBM, Armonk, NY, USA) to compare the significant differences among mean values of the experimental data.

2.3. Results and discussion

2.3.1. Preparation of starch samples with different branch chain length

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, 2006b). Proving the general knowledge that potato starch has rather long amylopectin chains, the native waxy potato starch had a smaller amount of short chains with DP 6-12 and a larger amount of long chains of DP≥25 compared with waxy corn starch. Particularly, its proportion of very long chains (DP≥37) was twice of that of waxy corn starch.

AS0 starches of both starches showed no significant difference (p>0.05) compared with their native starch because there was no enzyme addition. Dramatic changes were observed after AS-modification compared with AS0. Significant increases in the average chain length and decreases in the proportion of short chains (DP 6-12) of 50-90% were observed, by the action of AS that catalyzes the elongation of some branch chains by attaching 12 to 18 glucosyl units at their non-reducing ends (de Montalk et al., 1999). The

chains with DP 13-24 had the largest proportion for most of AS-modified starches, which corresponded to earlier studies (Kim et al., 2013; Kim et al., 2014; Ryu et al., 2010). The more AS 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 waxy corn starch, and from 0.022 to 0.149 for waxy potato starch.

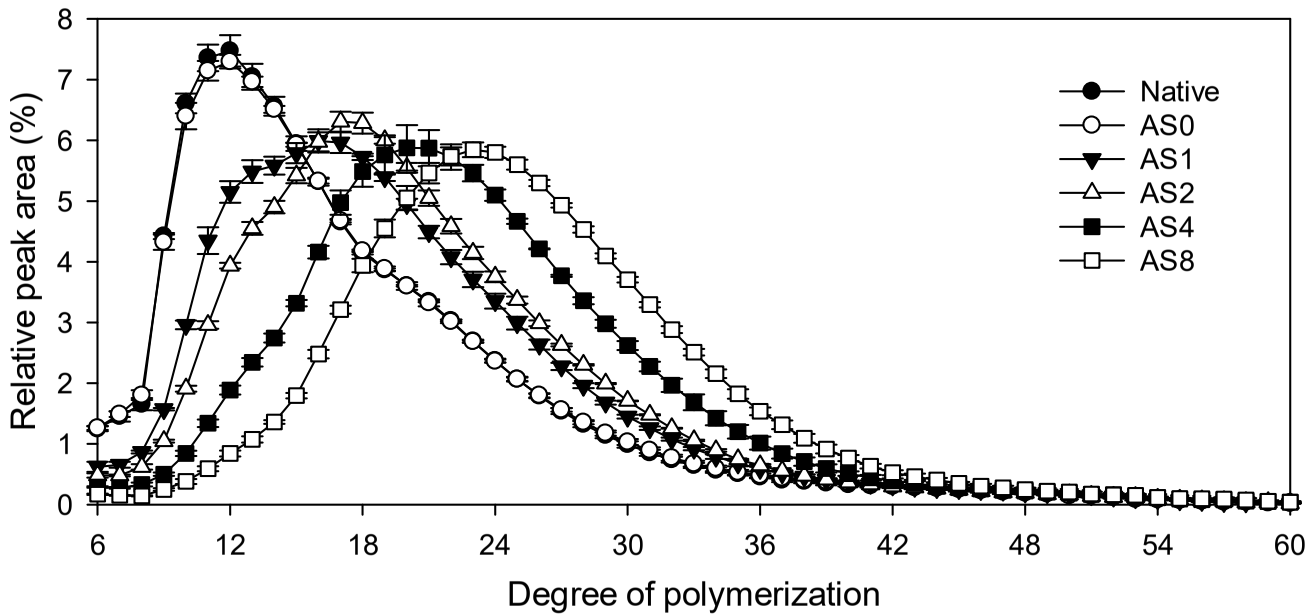
Table 2.1. Branch chain length distributions of native and amylosucrase-modified starch samples

Sample		Percent distribution (%)				S/L	Average DP
		DP 6-12	DP 13-24	DP 25-36	DP ≥ 37		
WC	Native	30.22±0.43^{a}	52.60±0.55^{d}	12.62±0.51^{e}	4.56±0.45^{c}	0.433±0.009^{a}	17.87±0.23^{e}
	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}	18.03±0.20^{e}
	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}	19.99±0.19^{d}
	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}	20.85±0.13^{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}	23.36±0.29^{b}
	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}	25.67±0.22^{a}
WP	Native	22.58±0.19^{a}	52.85±2.52^{c}	15.66±1.38^{e}	8.84±0.95^{b}	0.292±0.003^{a}	20.59±0.41^{e}
	AS0	22.25±0.46^{a}	52.77±0.10^{bc}	15.63±0.05^{e}	9.31±0.66^{b}	0.286±0.008^{b}	20.44±0.23^{e}
	AS1	12.99±0.00^{b}	58.06±0.62^{a}	20.48±0.44^{d}	8.46±0.18^{b}	0.149±0.000^{c}	21.80±0.09^{d}

AS2	6.84±0.06^{c}	55.40±0.27^{ab}	28.83±0.02^{c}	8.92±0.35^{b}	0.073±0.001^{d}	23.76±0.11^{c}
AS4	4.80±0.10^{d}	51.05±1.37^{c}	34.37±0.87^{b}	9.76±0.57^{b}	0.050±0.001^{e}	24.97±0.29^{b}
AS8	2.11±0.01^{e}	35.96±0.04^{d}	44.67±0.11^{a}	17.26±0.13^{a}	0.022±0.000^{f}	28.40±0.05^{a}

The values with different superscripts in the same column of same sample are significantly different (p<0.05). WC, waxy corn starch; WP, waxy potato starch; Native, native starch; AS0, control with no enzyme addition; AS1, starch modified 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; DP, degree of polymerization; S/L, ratio of short chains (DP < 13) to long chains (DP ≥ 13).

(A)


Relative peak area (%)
Degree of polymerization
Native
AS0
AS1
AS2
AS4
AS8
0
1
2
3
4
5
6
7
8
6
12
18
24
30
36
42
48
54
60

(B)

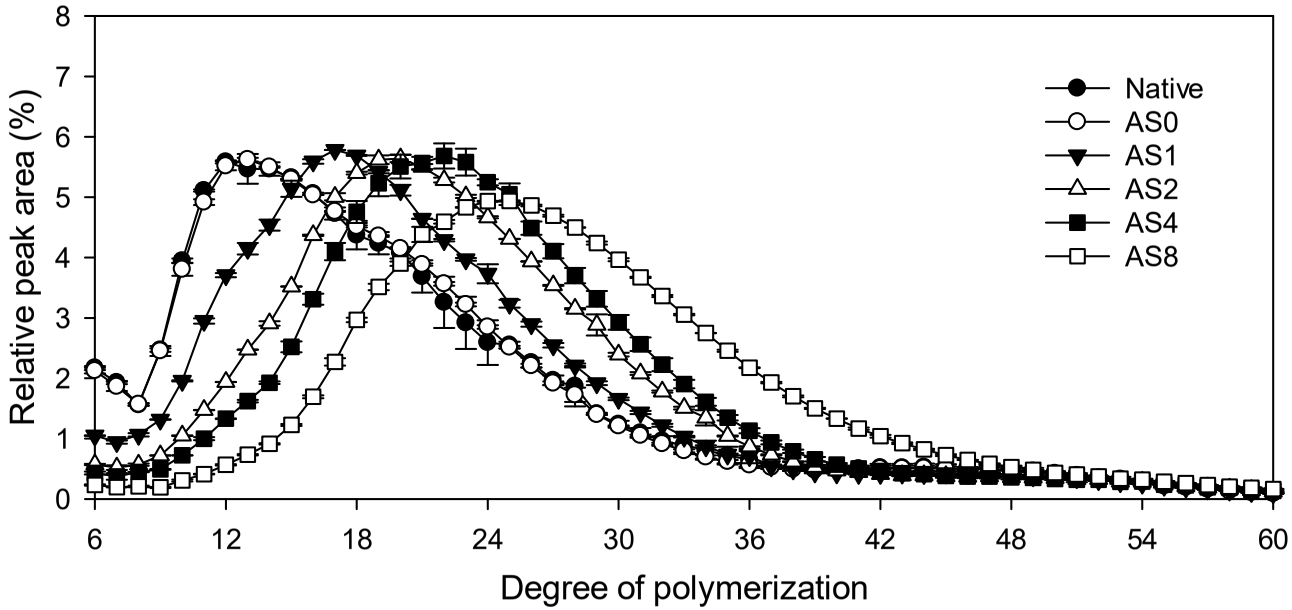


Figure 2.1. Branch chain length distribution of amylosucrase-modified (A) waxy corn starches and (B) waxy potato starches. Native, native waxy corn starch; AS0, control without enzyme addition; AS1, starch modified with 2,500 U amylosucrase/30 mL-starch suspension; AS2, amylosucrase 5,000 U/30 mL; AS4, amylosucrase 10,000 U/30 mL; AS8, amylosucrase 20,000 U/30 mL.

2.3.2. Digestion pattern 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. Figure 2.2 displays the enzymatic digestion profiles of native and AS-modified starches.

In the case of waxy corn starch, the 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 WCAS0 appeared quite early (15 min), its C_∞ (81.1%) was similar to that of native starch. WPAS0 also revealed plateau time of 15 min with maximum hydrolysis degree of 84.4%. The high digestibility of AS0 starches was due to destruction of the inherent granular structure by the gelatinization process during sample preparation. The amorphous regions generated by gelatinization are easily accessed by digestive enzymes (Zhang et al., 2006b). Meanwhile, native waxy potato starch did not reach plateau throughout the hydrolysis period of 360 min. The degree of hydrolysis of the starch till 360 min was quite low, showing only 28.6%. This result supports the high resistance of waxy potato starch against digestive enzyme, as native granular potato starch is known as a representative RS type 2.

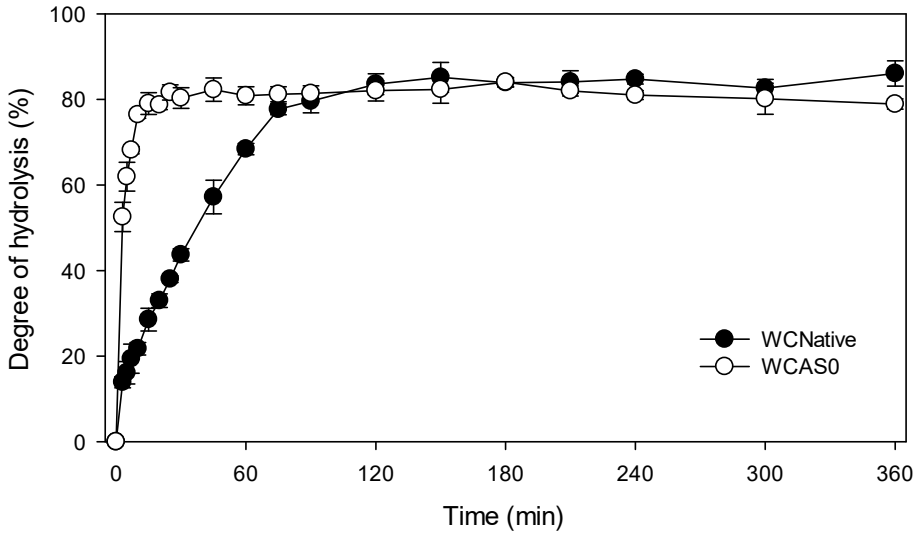
The resistance to enzymatic hydrolysis of AS-modified starches was greatly influenced by the action of AS. The plateau time was delayed to 60,

150, 210, and 360 min as the amount of AS doubled in waxy corn starch (WCAS1, WCAS2, WCAS4, and WCAS8, respectively). The C_∞ exhibited values slightly exceeding 80% for WCAS1 and WCAS2, and then considerably decreased in WCAS4 and WCAS8 (77.0% and 68.4%, respectively). Similar trends were also observed in waxy potato starches according to AS-modification: plateau times were recorded as 30, 150, 210, and 300 min, and C_∞ gradually decreased from 81.9% to 63.1%, for WPAS1-WPAS8. The requirement of the longer hydrolysis time for a similar or smaller amount of digestible starch (C_∞) clearly demonstrated the slower digestion property caused by AS-modification.

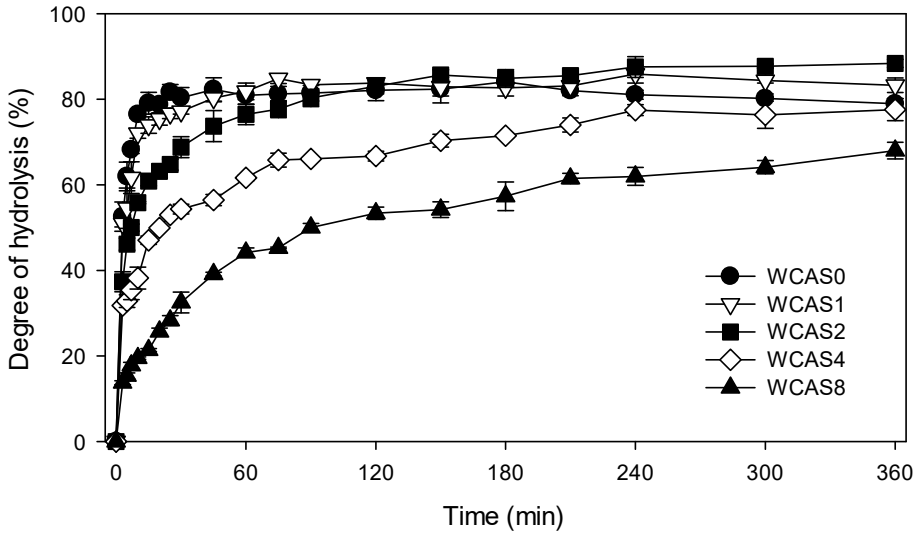
As the AS-modified starches were recrystallized after gelatinization, their digestion property could be accounted for based on the following mechanism, which is similar to retrogradation. During the gelatinization of starch, the original crystalline structures of amylopectin get disintegrated, and the 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., 1999b; 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., 2008b). 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 have low susceptibility to enzymatic hydrolysis (Zhang et al., 2006b), the elongation of branch chains and their accelerated re-association into double helices might improve the slow digestion property of AS-modified starches.

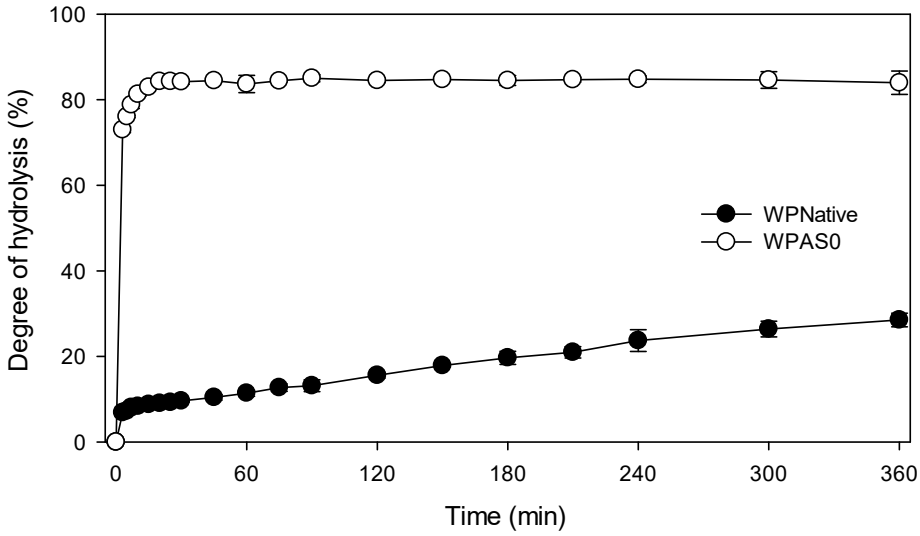
(A)


Degree of hydrolysis (%)
Time (min)
WCNative
WCAS0

(B)


Degree of hydrolysis (%)
Time (min)
WCAS0
WCAS1
WCAS2
WCAS4
WCAS8

(C)



(D)

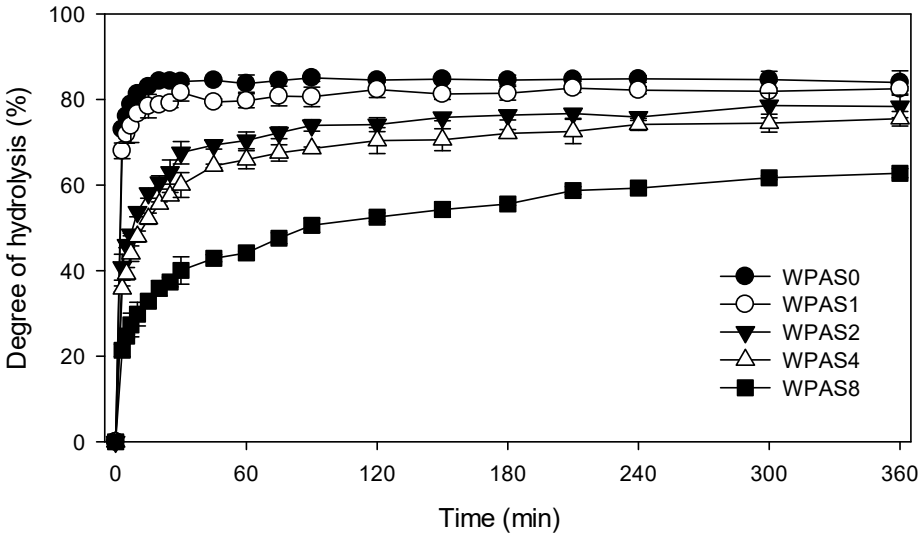


Figure 2.2. Digestion profiles of AS-modified (A), (B) waxy corn and (C), (D) waxy potato starches. Native, native waxy corn starch. AS0, control without enzyme; AS1, starch modified with 2,500 U amylosucrase/30 mL-starch suspension; AS2, amylosucrase 5,000 U/30 mL; AS4, amylosucrase 10,000 U/30 mL; AS8, amylosucrase 20,000 U/30 mL.

2.3.3. Determination of starch fractions using the traditional Englyst's method

The *in vitro* digestibility of starch samples measured using the Englyst method is presented in Table 2.2. Native waxy corn starch showed a typical digestion property of A-type granular starch, exhibiting abundant SDS (Lehmann & Robin, 2007). Native waxy potato starch, which is a representative RS, had a considerably large amount of RS (76.26%) and very small RDS (8.36%).

RDS content was the highest in the AS0 in both starches, significantly decreasing with the amount of employed AS. 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., 2008b). Thus, the desired amount of SDS could be produced by modifying the branch chain length of amylopectin. AS8 starches had no significant difference (p>0.05) in SDS amount compared to their respective AS4 starches, while steep increases in RS were observed in both waxy corn and waxy potato starches, achieving approximately 40%. When treated with the same level of AS, waxy corn starch always had a more amount of SDS and waxy potato starch had more RS fractions, which was in consistency with a previous report (Shin et al., 2010).

Table 2.2. RDS, SDS, and RS contents of native and amylosucrase-modified starches determined using the Englyst assay method

Sample		RDS (%)	SDS (%)	RS (%)
WC	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}
WP	Native	8.36±0.80^{f}	15.38±1.85^{c}	76.26±2.54^{a}
	AS0	81.33±0.52^{a}	3.49±0.33^{e}	15.18±0.79^{d}
	AS1	73.86±2.21^{b}	9.24±2.76^{d}	16.90±0.54^{d}
	AS2	53.69±1.09^{c}	22.20±1.88^{b}	24.11±0.80^{c}
	AS4	47.96±1.29^{d}	26.21±1.73^{a}	25.84±1.40^{c}
	AS8	29.82±2.76^{e}	29.46±2.96^{a}	40.72±0.22^{b}

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 starch; AS0, control with no enzyme addition; AS1, starch modified 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.

2.3.4. LOS plotting of digestibility curve using modified method

LOS plots of native waxy corn starch and WCAS0 displayed a single line (Figure 2.3A and 2.3B), 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 waxy corn starch was lower than that of WCAS0 (by 10-fold). Considering that hydrolysis of starch predominantly occurs in the amorphous regions (Gallant, Bouchet, Buleon, & Perez, 1992), the rapid and singular digestion rate of WCAS0 can be understood by its extremely high amount of amorphous region, classified into RDS by Englyst hypothesis (Zhang et al., 2006b). 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 et al., 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 et al., 2006a).

A LOS plot of the digestibility curve of WCAS1 revealed a discontinuity around 15 min of digestion (Figure 2.3C). It demonstrated that WCAS1 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 estimated C_∞ (85.14%) calculated from the LOS plot, which is the sum of two C_∞s from two distinct linear graphs, agreed very well with the measured data (83.59%). Meanwhile, there was also a slight possibility to consider the LOS plot of WCAS1 as a single equation (Figure 2.4). However, it was not acceptable due to its lower determination coefficient (r^2=0.686) and the great gap between estimated C_∞ calculated from a single linear regression (44.21%) and experimentally measured value (83.59%).

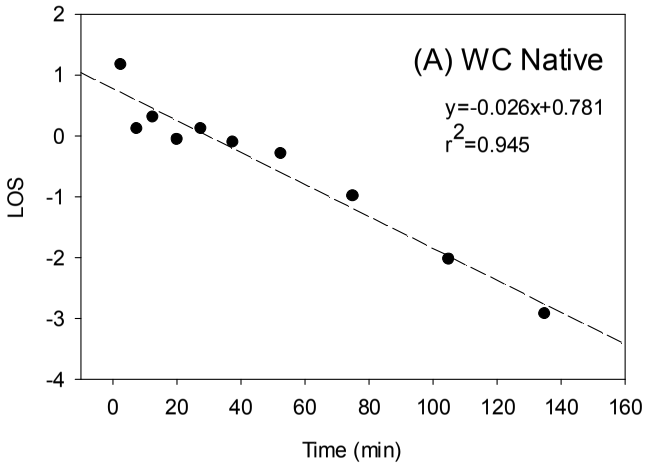
The LOS plots of WCAS2 and WCAS4 also revealed two distinct lines (Figure 2.3D and 2.3E), and thus provided the evidence of fractions those were digested more rapidly (RDS) or more slowly (SDS), respectively. The LOS plot of WCAS8 was better described by a single linear graph (Figure 2.3F) rather than two linear graphs. Therefore, the presence of separate

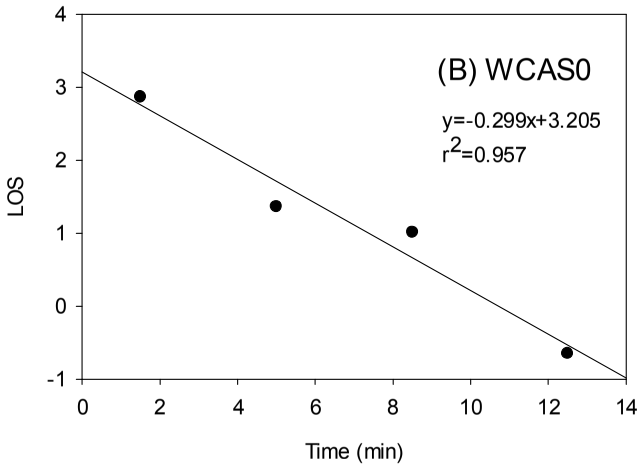
rapidly digested and slowly digested components in WCAS8 was not conceded. The rate constant (k=0.021 min^{-1}) of WCAS8 was similar to the k values of the slower phase of other AS-modified starches.

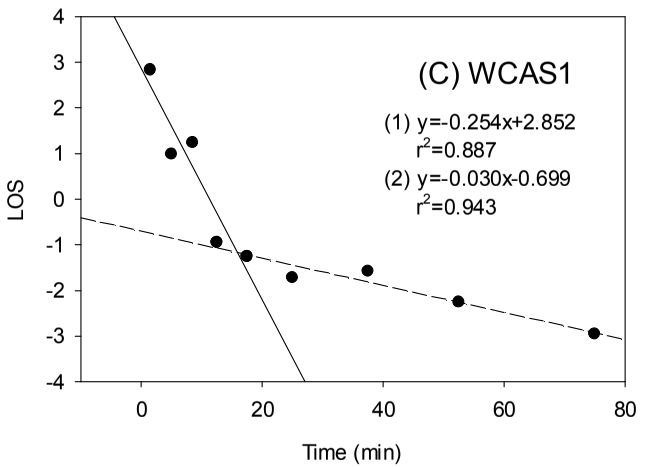
Native waxy potato starch had extraordinary digestive profile (Figure 2.2) and LOS plot graph (Figure 2.5A) among all samples. Initially, digestion starts with the attachment of amylolytic enzymes on the surface of granules, known as 'adsorption' step. Rapid and raging digestion with high rate constant (k=0.247 min^{-1}) was observed with rather short duration of about 12 min, which would be involved with surface-oriented digestion. A waxy potato starch granule does not have any pores on its surface, therefore its digestion process can be understood as a 'layer-by-layer' mechanism (Dhital et al., 2010). Therefore, this later digestion phase is a quite time-consuming step with an extremely low rate constant (k=0.002 min^{-1}), which is a 10-10^2 folds lower value compared with all rate constant values of other samples. Though the actual C_∞ was not observed, the estimated C_∞ of waxy potato starch was also very low (47.7%). As aforementioned in Englyst's fractionation result, these characteristics (low rate constant and low amount of digestible starch) reflect property as RS of native waxy potato starch.

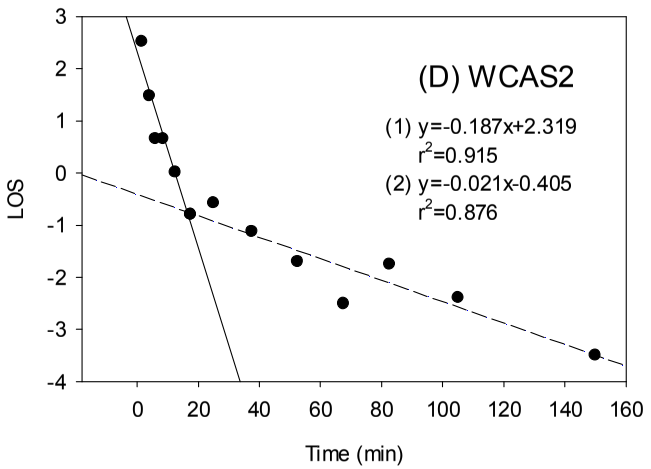
AS-modified waxy potato starches revealed similar tendency in their LOS plot graphs with waxy corn starches of their respective corresponding

treatment level. WPAS0 had a singular digestion rate graph with the high rate constant (k=0.367 min^{-1}), and this showed consistency with the digestive pattern of WCAS0, reflecting the digestive property of amorphous structure as RDS. LOS plots of WCAS1, WPAS2 and WPAS4 revealed distinctive two linear expressions: first phase with slope of k=0.302, 0.179, and 0.152 min^{-1} and second phase with slope of k=0.071, 0.026, and 0.020 min^{-1}, respectively. The high determination coefficient and close correspondence of measured C_∞ and estimated C_∞ strongly supported the idea of two digestive phases caused by two distinct fractions. WPAS8 also displayed a similar graph with WCAS8, a singular line with low k value (k=0.016) and low C_∞ close to 65%. However, estimated C_∞ and measured C_∞ showed disagreement in WPAS0 and WPAS1.


(A) WC Native
y=-0.026x+0.781
r2=0.945
LOS
Time (min)


(B) WCAS0
y=-0.299x+3.205
r2=0.957
LOS
Time (min)


(C) WCAS1
(1) y=-0.254x+2.852
r2=0.887
(2) y=-0.030x-0.699
r2=0.943
LOS
Time (min)


(D) WCAS2
(1) y=-0.187x+2.319
r2=0.915
(2) y=-0.021x-0.405
r2=0.876
LOS
Time (min)

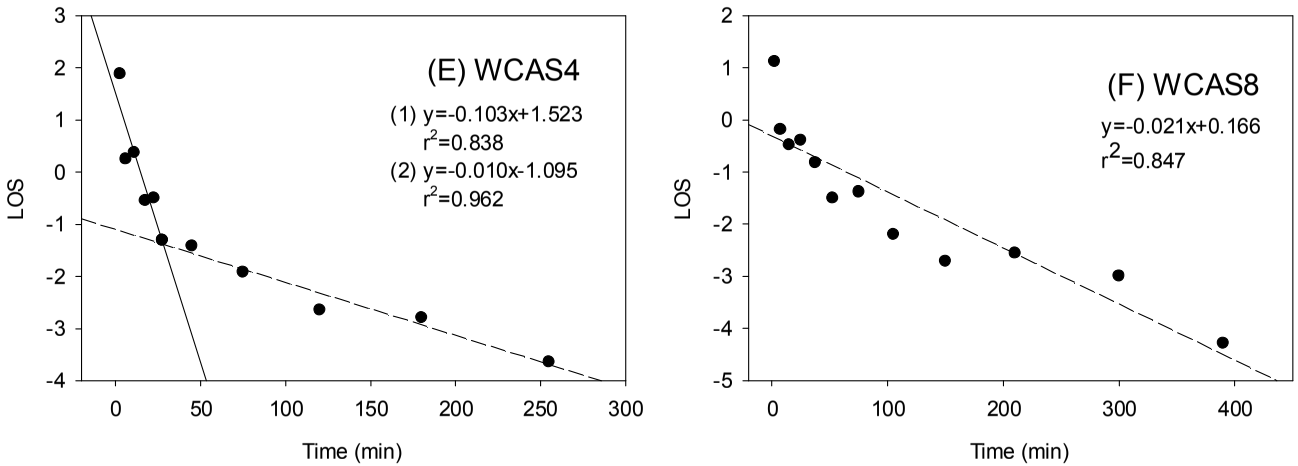


Figure 2.3. LOS plot of amylosucrase-modified waxy corn starches. WC, waxy corn starch; Native, native waxy corn starch; AS0, control without enzyme addition; AS1, starch modified with 2,500 U amylosucrase/30 mL-starch suspension; AS2, amylosucrase 5,000 U/30 mL; AS4, amylosucrase 10,000 U/30 mL; AS8, amylosucrase 20,000 U/30 mL. Solid line indicates rapid digestion phase, and broken line indicates slow digestion phase.

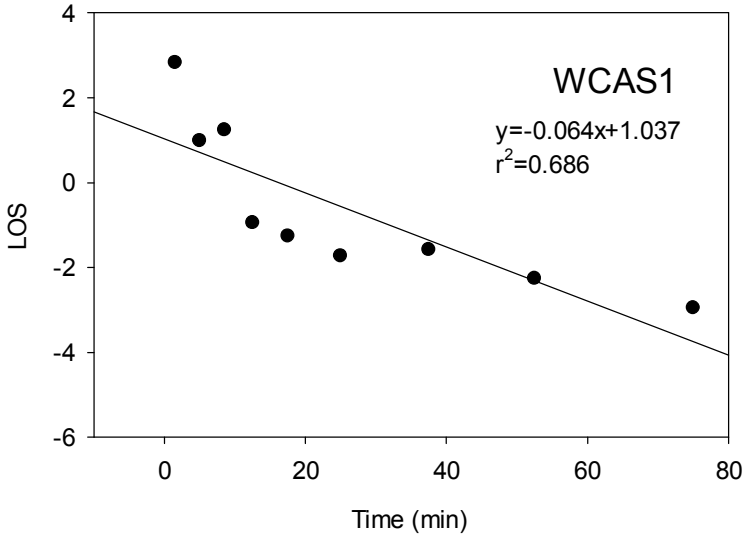
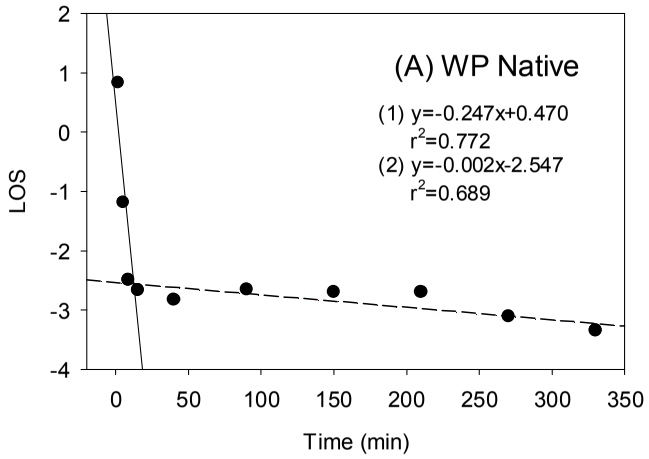
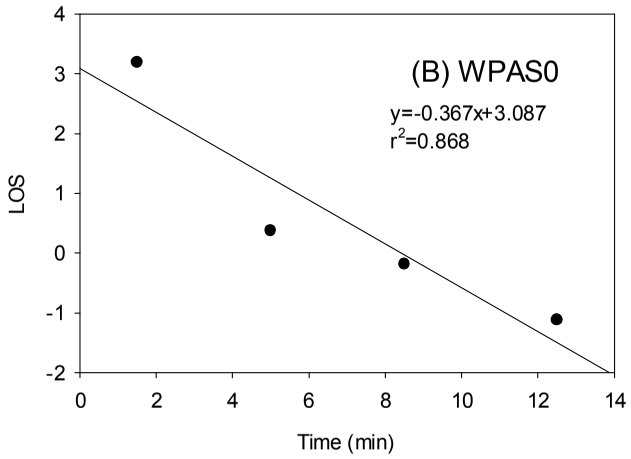
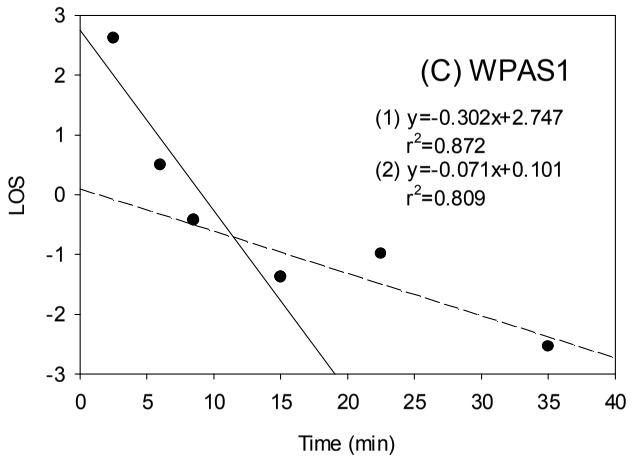
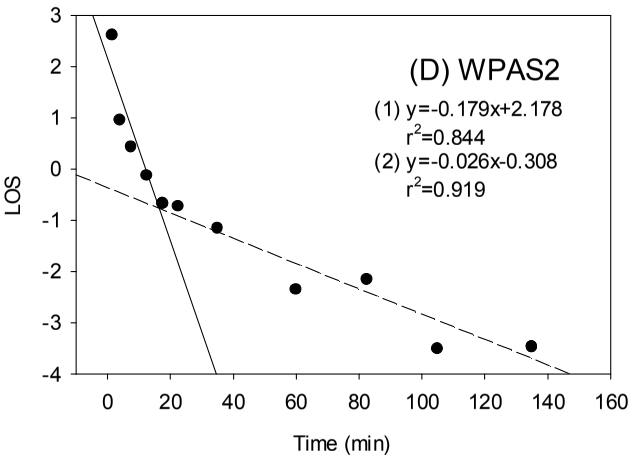


Figure 2.4. Representative example of wrong LOS plot regression (WCAS1, waxy corn starch modified with 2,500 U amylosucrase/30mL-starch suspension). The estimated C_∞ from the singular equation (44.2%) was very different from experimentally measured value (83.59%).


(A) WP Native
(1) y=-0.247x+0.470
r2=0.772
(2) y=-0.002x-2.547
r2=0.689
LOS
Time (min)


(B) WPAS0
y=-0.367x+3.087
r2=0.868
LOS
Time (min)


(C) WPAS1
(1) y=-0.302x+2.747
r2=0.872
(2) y=-0.071x+0.101
r2=0.809
LOS
Time (min)


(D) WPAS2
(1) y=-0.179x+2.178
r2=0.844
(2) y=-0.026x-0.308
r2=0.919
LOS
Time (min)

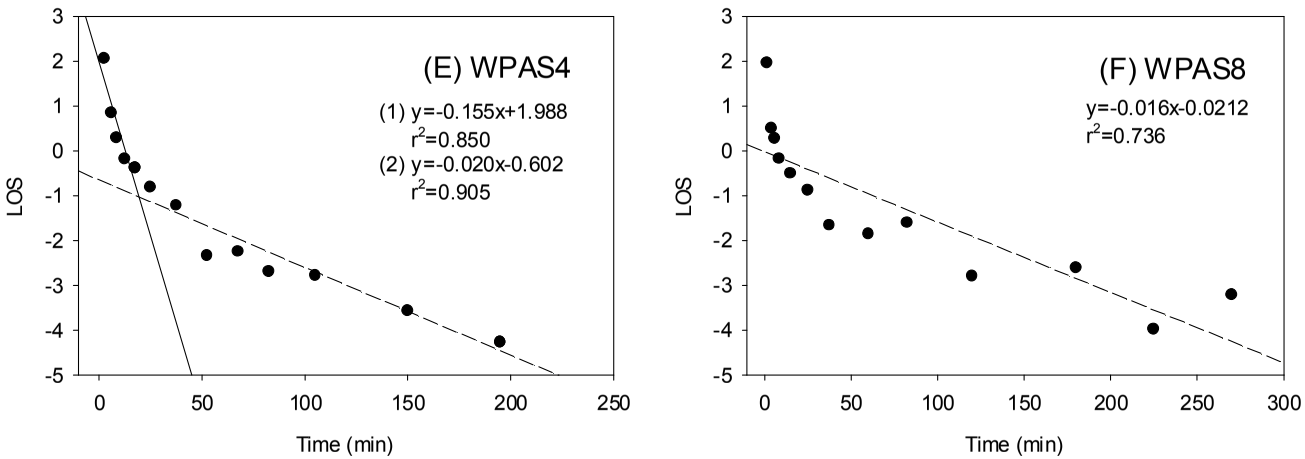


Figure 2.5. LOS plot of amylosucrase-modified waxy potato starches. WP, waxy potato starch; Native, native starch; AS0, control without enzyme; AS1, starch modified with 2,500 U amylosucrase/30 mL-starch suspension; AS2, amylosucrase 5,000 U/30 mL; AS4, amylosucrase 10,000 U/30 mL; AS8, amylosucrase 20,000 U/30 mL. Solid line indicates rapid digestion phase, and broken line indicates slow digestion phase.

The kinetic parameters of starch samples estimated by the LOS plot are summarized in Table 2.3. Digestion processes of native waxy corn starch, WCAS0, WCAS8, WPAS0, and WPAS8 were described by a single rate constant, which presented high ks for AS0 starches but low ks for the others. Other AS-modified waxy corn starches (AS1-AS4) possessed 2 ks that were obviously 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 starches employed in this study could be categorized into two groups based on the k values of AS0 starches and native waxy corn starch, representing RDS and SDS, respectively (Englyst et al., 1992; Miao et al., 2009b). Because AS0 starches showed the rapid digestion property, the group of k close to that of WCAS0 and WPAS0 was defined as k_{RDS}; for the same reason, k values at or near that of native waxy corn starch were 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 within a particular starch as a structural feature. That is, after dominant 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 starches, which are highly modified with AS, were 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. Changes in k and C_∞ according to the degree of modification were observed in this study, as explained by that abundant long branch chains induce formation of crystallites with highly ordered structure, resulting in resistance against digestion. The decline of k_{RDS} and k_{SDS} values with the increasing amount of AS suggested that the detail structure of RDS and SDS might have small dissimilarity among samples. As an implication of the suggestion, 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 (15.9-28.2 min for WCAS1 to WCAS4, and 11.4-19.2 min for WPAS1 to WPAS4, respectively). In other words, the smaller amount of RDS (Table 2.3) required more time to be digested.

C_t throughout the whole digestion range (0-360 min) were calculated (equation 4) using the k and C_∞ obtained via LOS plotting and compared

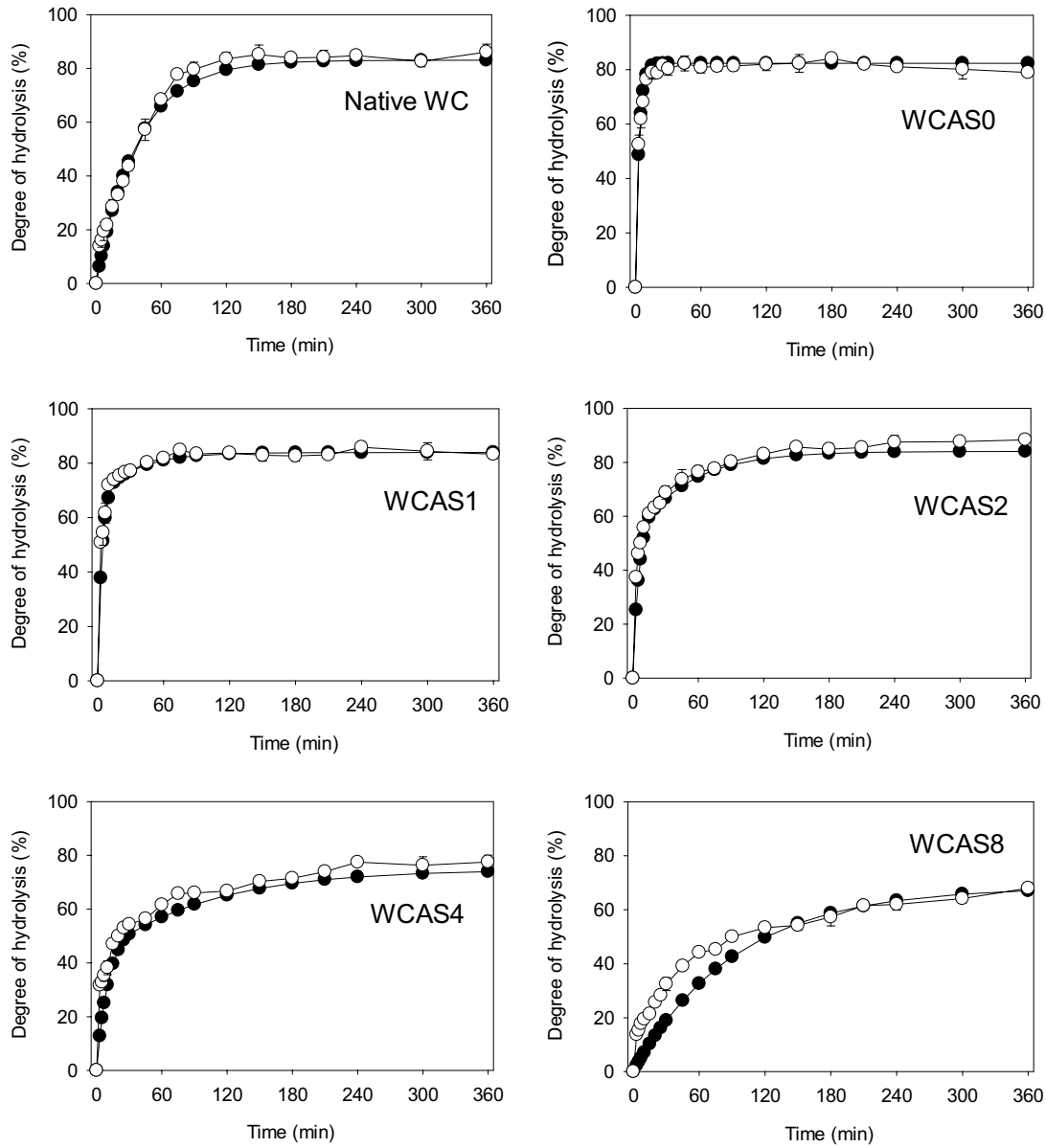
with the actual degree of digestion (Figure 2.6). The very close match of two curves derived from measurement and estimation could confirm the reliability of the modified LOS plot method used in this study.

Table 2.3. *In vitro* digestion parameters obtained from modified LOS plot analysis

Sample		k_{RDS} (min^{-1})	k_{SDS} (min^{-1})	t_{int} (min)	Time of plateau (min)	Measured C_∞ (%)	Estimated C_∞ (%)
WC	Native	N.D.	0.026	N.D.	120	84.34	83.05
	AS0	0.299	N.D.	N.D.	15	81.09	82.36
	AS1	0.254	0.030	15.9	60	83.59	85.14
	AS2	0.187	0.020	16.3	150	86.61	86.66
	AS4	0.103	0.010	28.2	210	77.02	77.26
	AS8	N.D.	0.011	N.D.	360	68.39	68.46
WP	Native	0.247	0.002	12.3	N.D.	N.D.	47.73
	AS0	0.367	N.D.	N.D.	15	84.36	59.74
	AS1	0.302	0.071	11.4	30	81.85	67.24
	AS2	0.179	0.026	16.3	150	76.94	77.59
	AS4	0.152	0.020	19.2	210	74.16	72.19
	AS8	N.D.	0.016	N.D.	300	63.07	66.19

WC, waxy corn starch; WP, waxy potato starch; Native, native starch; AS0, control without enzyme; AS1, starch modified 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; k_{RDS}, rate constant for the RDS phase of starch hydrolysis; k_{SDS}, rate constant for the SDS phase of starch hydrolysis; C_∞, the maximum degree of hydrolysis where estimated C_∞ was the sum of two C_∞s from two linear equation; t_{int}, the time when the two linear graphs intersect; time of plateau, the time when the degree of hydrolysis reached plateau revealing no more significant changes; N.D., not detected.

(A)


Native WC
WCAS0
WCAS1
WCAS2
WCAS4
WCAS8
Degree of hydrolysis (%)
Time (min)
0
20
40
60
80
100
0
60
120
180
240
300
360

(B)

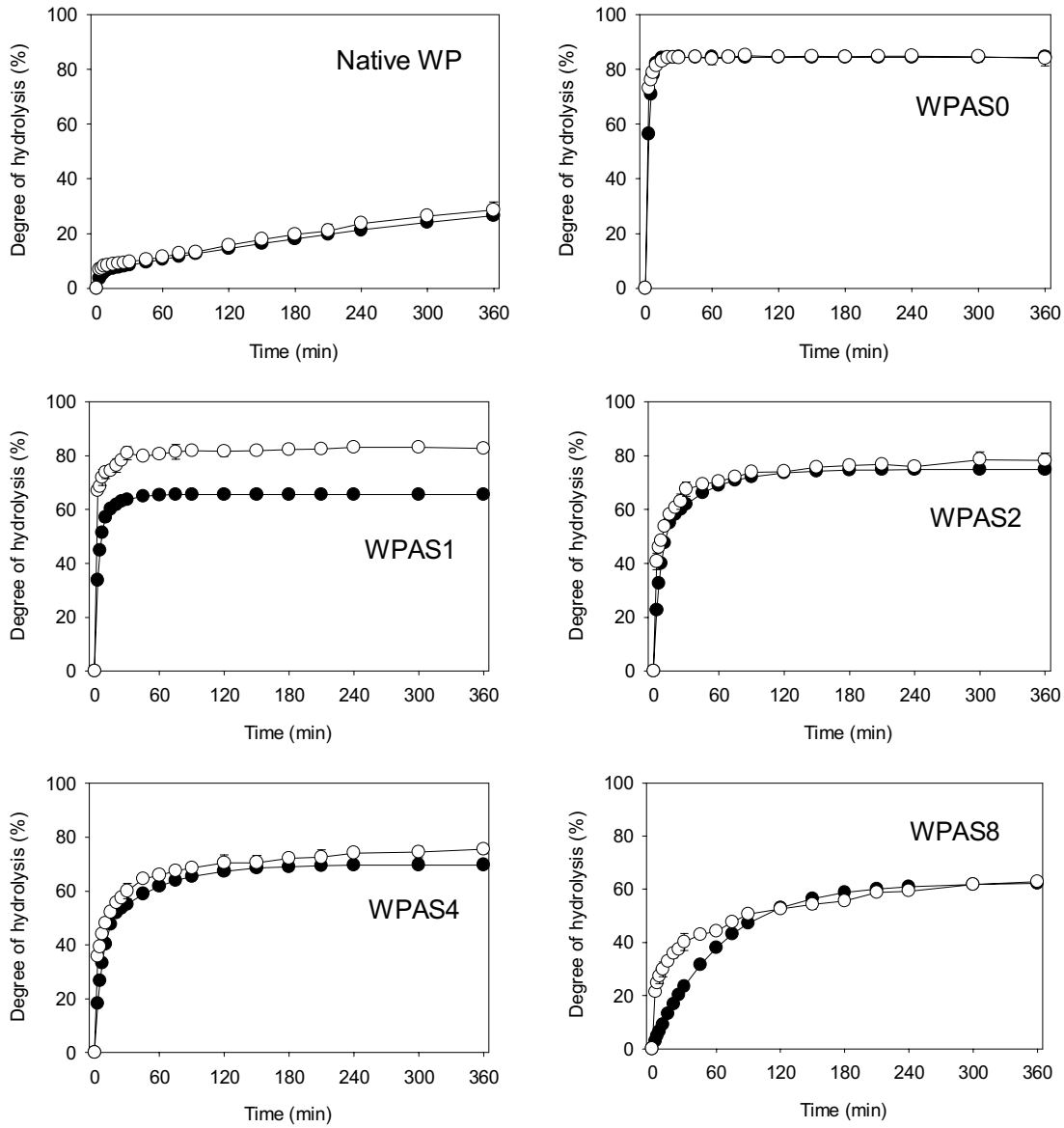


Figure 2.6. Comparison of experimental data (open circle) and estimations based on modified LOS plot (closed circle) of amylosucrase-modified (A) waxy corn starches and (B) waxy potato starches. WC, waxy corn starch; WP, waxy potato starch; Native, native starch; AS0, control without enzyme; AS1, starch modified 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.

2.3.5. Determination of starch fractions based on modified LOS plot method

The contents of RDS, SDS, and RS estimated using the parameters from the LOS plot are shown in Table 2.4. 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. With consideration of other parameters from LOS plot, it was found that more time was required for the hydrolysis of a smaller amount of RDS, with lowered digestion rate constant. In other words, the AS treatment and resultant elongation of branch chains decreased the amount of RDS fraction and induced a more slowly digestible form of 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 of both starches, minor changes in the amount of SDS were observed in contrast to the increases in the RS contents. It 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 starches 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 modified LOS method was

not consistent with that determined by the method of Englyst. The method suggested by Englyst et al. (1992) based on a specific time frame is widely accepted in the food industry as it is quite simple and is applicable to a variety of samples from complex food materials to pure starch. This classification method is considered to be easier and a more general tool for 'intuitive comparison' of digestion properties of different starches, which can be advantageous when investigating starch hydrolysis. However, it does not deal with the factor of rate, which corresponds to the terms of 'rapid' or 'slowly' digestible starch. Therefore, the LOS plot approach utilized in this study would be a reasonable investigative tool for accurate determination of RDS and SDS (Patel et al., 2014) as well as RS.

Table 2.4. Contents of RDS, SDS, and RS of starch samples estimated using the LOS plot

Sample		RDS (%)	SDS (%)	RS (%)
WC	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
WP	Native	8.24	41.63	50.13
	AS0	84.36	N.D.	15.64
	AS1	74.04	7.81	18.15
	AS2	58.72	18.22	23.06
	AS4	55.00	19.16	25.84
	AS8	N.D.	62.36	37.64

WC, waxy corn starch; WP, waxy potato starch; Native, native starch; AS0, control without enzyme; AS1, starch modified 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 where the amount of RS was calculated based on the measured C_∞; N.D., not detected.

2.3.6. Versatility of the modified LOS plot method on starches with various digestive property

Application of LOS plot method for the analysis of *in vitro* digestibility of starch was introduced in the study of Butterworth et al. (2012) for the first time by using purified native starches. The study observed that some native granular starches exhibited two digestive phase with a distinctive rate constant for each. However, in spite of such an obvious observation, the authors did not support the concept of RDS and SDS. As the current study aimed to verify the validity of classification by Englyst et al. (1992), the respective existence of RDS and SDS, the reaction conditions for *in vitro* digestion of starch such as concentration of starch and enzyme were set based on the method of Englyst et al. (1992). Therefore, this study would be a meaningful combination of the two methods, which supports the concept of digestible fraction classification via kinetics approach.

The LOS plot method has also been used to analyze homogenized food materials (Butterworth et al. (2012) using the data adopted from Goñi et al. (1997)), 24 h-retrogadated starches (Patel et al., 2014), and hydrothermally-processed chickpea and durum wheat flours (Edwards, Warren, Milligan, Butterworth, & Ellis, 2014) by the same research group. Recently, the digestion property of starchy food product such as pasta was also examined using the same method (Zou, Sissons, Gidley, Gilbert, & Warren, 2015).

However, investigation of *in vitro* digestive properties of modified starch, especially with low digestibility, using the first-order kinetics-based analysis has never been attempted. Therefore, this is the first case of applying LOS method to the investigation of low-digestible starch produced by modifying molecular structure.

Moreover, some flaws were found in the previous studies. Firstly, the previous studies did not measure the degree of digestion after certain time, and just estimated the expected amount of total digestible starch (C_∞) without verifying the accuracy of their estimation by comparing with actual data. In this regard, the originally suggested LOS plot method applied uniform time standard (60 min for gelatinized flours and 120 min for other cases) for regression into linear expression. However, the regression based on specific time standard can induce lack or surplus of information, which can act as bias. Some over- or underestimated C_∞ values not corresponding to their digestibility profile graphs were found in their studies. Starches with various digestibility understandably exhibit digestible durations varied among starch samples as presented in Table 2.3, and therefore this study employed the actual digestible part only to accomplish precise fitting. The RS related term which has not been mentioned in the previous studies could be defined. In this study, the time of plateau was defined as the cut-off point for RS region.

Moreover, the digestion mode of two distinct parts (rapid digestion and slow digestion) seemed quite misunderstood by the authors; in the previous study using retrograded starch (Edwards et al., 2014), the estimation of C_t for the case of two distinct graphs was suggested as follows:

$$\boldsymbol{C_t = \begin{cases} C_{\infty 1}\left(1 - e^{-k_1 t}\right) & if\ t \le t_{int} \\ C_{\infty 1} + C_{\infty 2}\left(1 - e^{-k_1 (t - t_{int})}\right) & if\ \ t \ge t_{int} \end{cases}} \quad (4)$$

where t_{int} is the intersection time of two linear plots. $C_{\infty 1}$ and $C_{\infty 2}$ corresnpond to $C_{\infty RDS}$ and $C_{\infty SDS}$ defined in this study, respectively. The premise for this equation to be valid is that completely distinctive two digestion steps occur before and after t_{int} consecutively: hydrolysis of SDS fraction occurs after complete extinction of RDS fraction. However, it does not seem that persuasive. Basically, starch molecules are composed of glucose, and the structure of RDS and SDS is determined by the way of organizing starch chains linked by α-1,4- or α-1,6-linkages. Since the starch hydrolysis enzyme (majorly α-amylase) acts on α-1,4-linkage, it is hard to say that there is no possibility of exposure of SDS structure to the hydrolysis enzyme during RDS digestive phase, unless RDS has the conformation of perfectly covering and protecting SDS and every part of RDS get hydrolyzed exactly at the same rate and by the same extent.

Instead, it is more acceptable that RDS digestive phase tentatively

includes hydrolysis of SDS fraction. In other words, the hydrolysis of RDS and SDS occurs rather simultaneously, where the digestion of RDS occurs dominantly firstly and that of SDS appears after exhaustion of RDS. It allows the consideration that hydrolysis of SDS occurs moderately over whole digestion period. Therefore, total C_∞ can be obtained by simple sum of two C_∞s. Taking the statements above into consideration, the equation (1) could be expressed as follows:

$$C_t = \begin{cases} C_{\infty SDS}\left(1 - e^{-k_{SDS}t}\right) + C_{\infty RDS}\left(1 - e^{-k_{RDS}t}\right) & if\ t \le t_{int} \\ C_{\infty RDS} + C_{\infty SDS}\left(1 - e^{-k_{SDS}t}\right) & if\ t \ge t_{int} \end{cases} \quad (5)$$

where $C_{\infty RDS}$ and $C_{\infty SDS}$ are the C_∞ referred to as RDS and SDS phase, respectively. A singular LOS plot corresponds simply to equation (3).

To verify the points in dispute stated above, the digestibility data obtained in this study was also fitted through original LOS plot method employing 0-120 min range. As the digestion of RDS occurred at very initial stage of whole digestion period, it was always included within 120 min. Therefore, RDS-related terms ($C_{\infty RDS}$ or k_{RDS}) from the two methods showed consistency. Moreover, C_∞ and k_{SDS} also showed agreement in the case that the time of plateau of the starch did not largely differ from 120 min (Figure 2.7A). This agreement between the parameters from the two methods surely makes sense since both methods employ the same principle (pseudo-

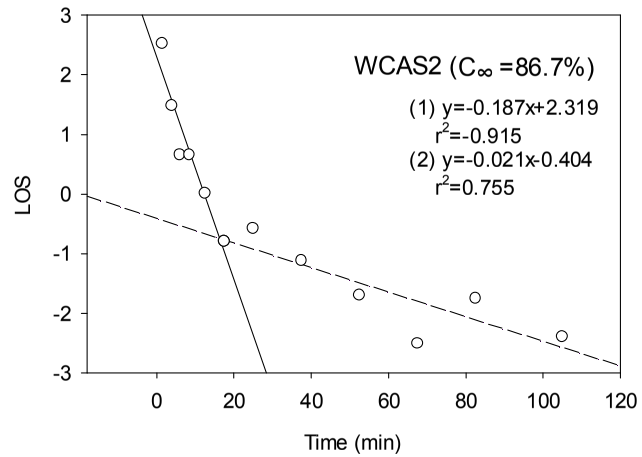
1st order kinetics) and equation. However, some cases revealing a big difference between the two methods for the same starch were also found. AS0 starches, which had a short digestion time and reached RS regions within 15 min (Figure 2.7B), showed a quite low determination coefficient of single linear expression and the estimated C_∞ had a big difference from experimental data. There was also a possibility of the emergence of two linear graphs, distinct from the LOS plots obtained via modified method. In this case, the error was due to that data points of degree of digestibility, which did not significantly (p>0.05) increase were put into consideration for regression. AS8 starches and native waxy potato starch, which had very prolonged digestion duration (over 300 min) showed rather a low determination coefficient in SDS phase and the estimated C_∞ not similar to experimental data (Figure 2.7C-E). The lack of sufficient information on later part of digestion led to the wrong prediction.

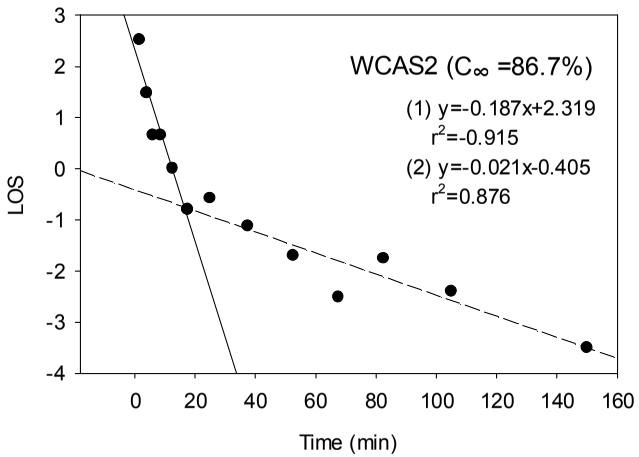
Figure 2.8 is the representative example of the comparison between the reliability of the two methods. The estimated digestion profile of starch samples stated above revealed less similarity with experimental digestive curve, compared to the estimation using the modified method.

Conclusively, LOS plotting and regression based on specific time standard could not accurately conjecture digestion property of some starches. On the other hand, the requirement for customized setting of curve-fitting period for

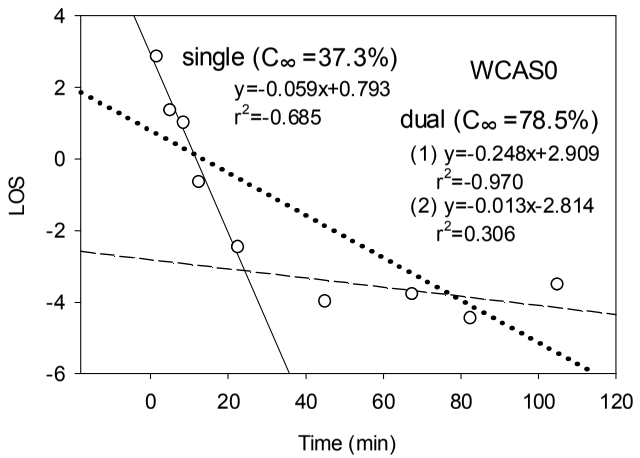
respective samples was verified. Especially, starches with various digestion properties, including low-digestible starch, accurate estimation was possible by using the modified LOS plot method. Therefore, the modified method suggested in this study would possibly be applied to any starches and utilized as a universal and effective tool for analyzing digestibility of starch.

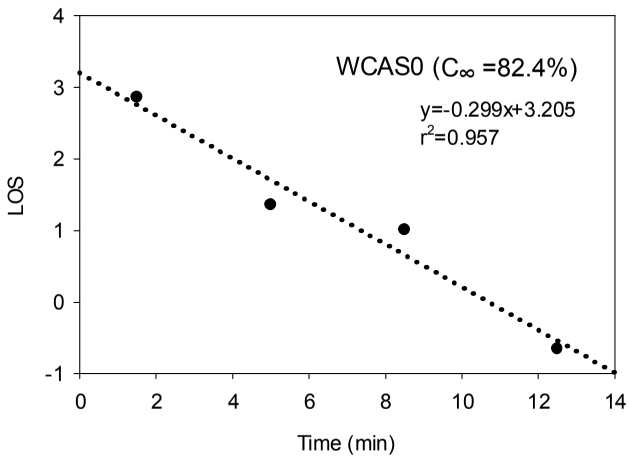
(A)


WCAS2 (C∞ =86.7%)
(1) y=-0.187x+2.319
r2=-0.915
(2) y=-0.021x-0.404
r2=0.755
LOS
Time (min)

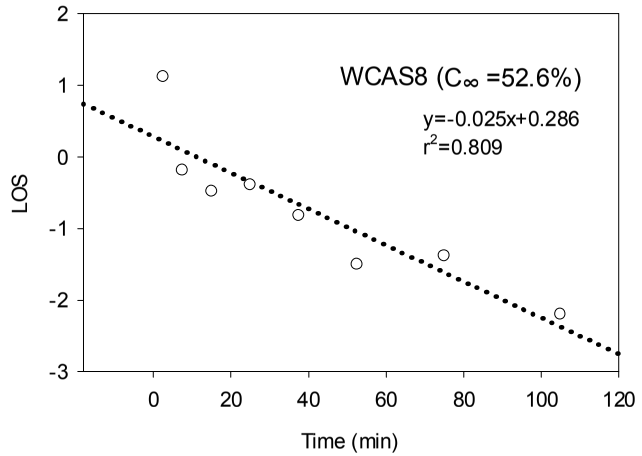

WCAS2 (C∞ =86.7%)
(1) y=-0.187x+2.319
r2=-0.915
(2) y=-0.021x-0.405
r2=0.876
LOS
Time (min)

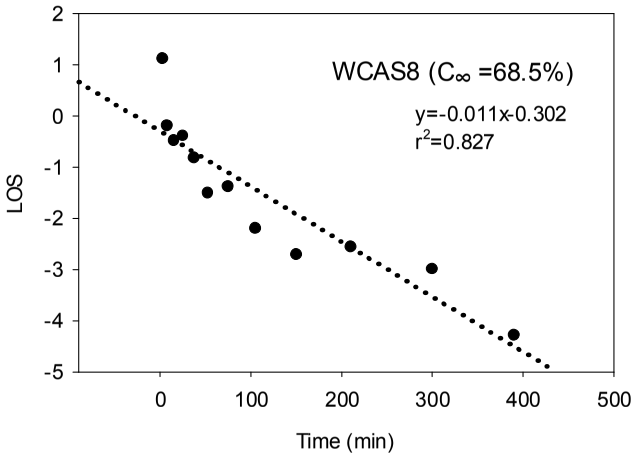
(B)


single (C∞ =37.3%)
y=-0.059x+0.793
r2=-0.685
WCAS0
dual (C∞ =78.5%)
(1) y=-0.248x+2.909
r2=-0.970
(2) y=-0.013x-2.814
r2=0.306
LOS
Time (min)

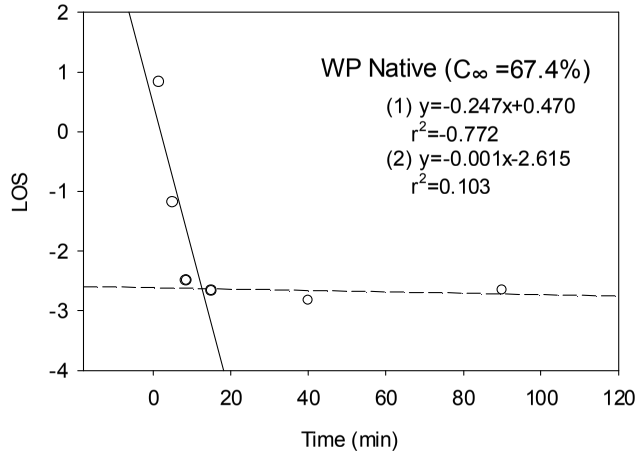

WCAS0 (C∞ =82.4%)
y=-0.299x+3.205
r2=0.957
LOS
Time (min)

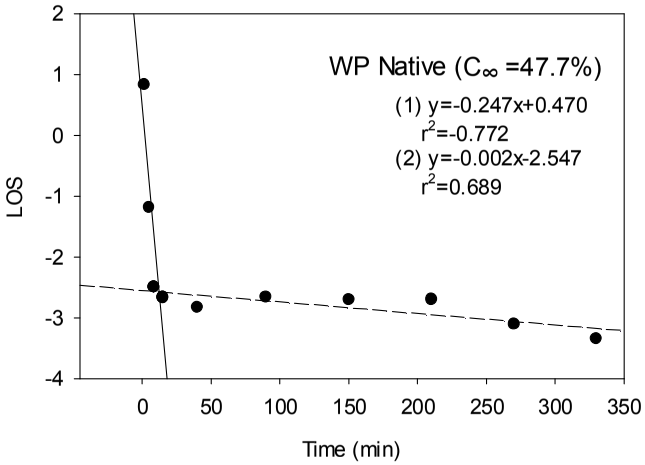
(C)


WCAS8 (C∞ =52.6%)
y=-0.025x+0.286
r2=0.809
LOS
Time (min)


WCAS8 (C∞ =68.5%)
y=-0.011x-0.302
r2=0.827
LOS
Time (min)

(D)


WP Native (C∞ =67.4%)
(1) y=-0.247x+0.470
r2=-0.772
(2) y=-0.001x-2.615
r2=0.103
LOS
Time (min)


WP Native (C∞ =47.7%)
(1) y=-0.247x+0.470
r2=-0.772
(2) y=-0.002x-2.547
r2=0.689
LOS
Time (min)

(E)

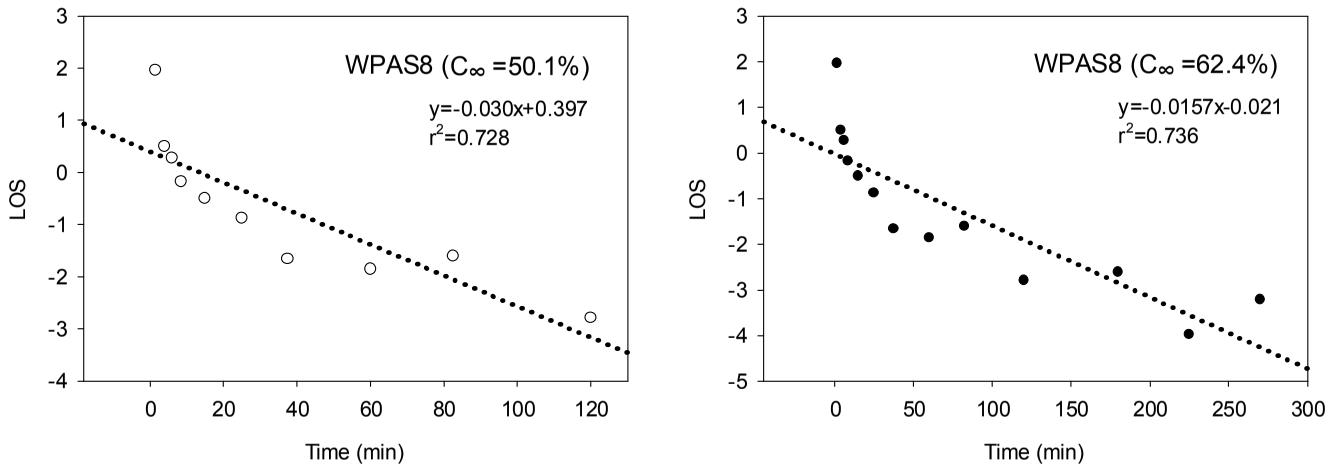


Figure 2.7. Comparison of the LOS plot graphs of original and its modification methods. LOS plot graphs using 120 min range as originally suggested were marked with open circle (left), and LOS plot graphs using modified method employing only digestible phase were marked with closed circle (right). (A), a representative example of the similarity between the two methods on the same sample; (B)-(E), representative examples of the dissimilarity between the two methods on the same sample. Solid line and broken line of two linear regressions indicate rapid and slow digestion phases, respectively. Dotted line indicates singular linear regression. The determination coefficient and estimated C_∞ from each regression are presented.

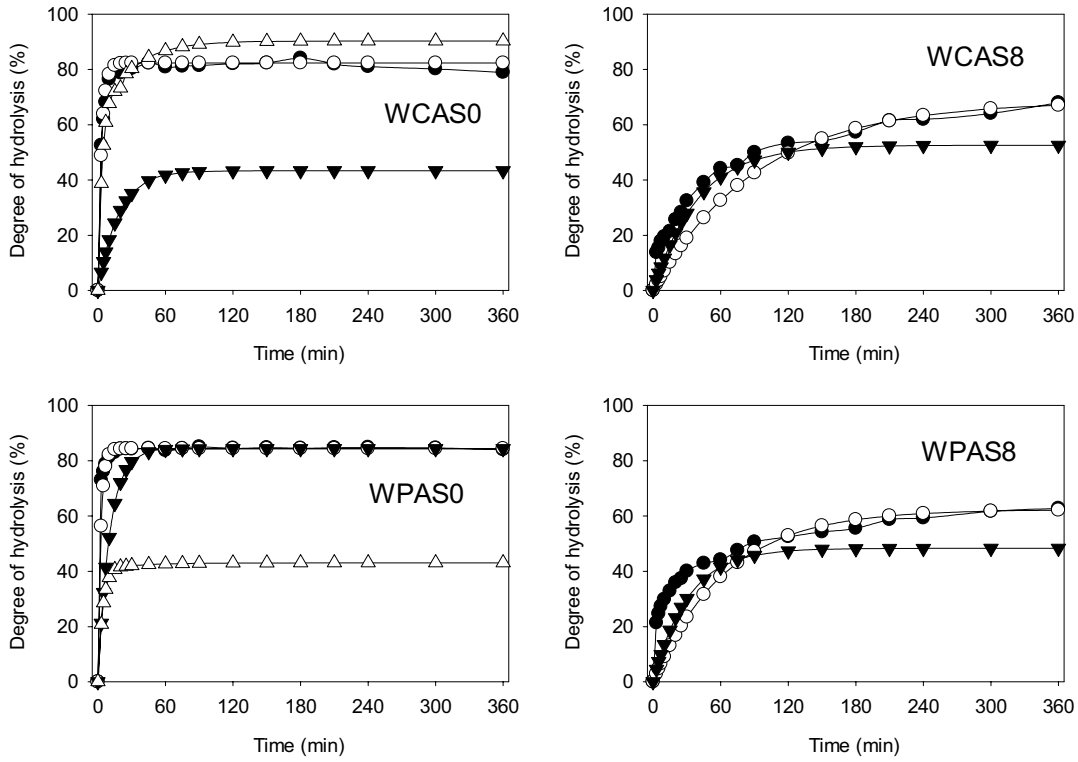


Figure 2.8. Comparison of experimental data (open circle) and estimations based on modified LOS plot (closed circle) to estimations based on originally suggested LOS plot method (open or closed triangle).

2.3.7. Relationship between *in vitro* and *in vivo* digestibility

The changes in blood glucose level in mice after consumption of AS-modified starches were examined using waxy corn starch samples, comparing with glucose. Glucose caused a sudden increase in postprandial blood glucose level for the first 15 min after consumption (168.4 mg/dL), then a steep decrease to approximately 150 mg/dL until 120 min. WCAS0 exhibited delayed peak time (the time revealed the highest blood glucose level increment) to 30 min and a slow rate of decrease in blood glucose level compared with glucose. This phenomenon occurs because stepwise hydrolysis is required to convert the starch into smaller compounds, whereas glucose can be readily absorbed into the blood. These small differences aside, the overall postprandial blood glucose changing pattern in mice of WCAS0 was similar to that of glucose, strongly supporting its digestive property as RDS.

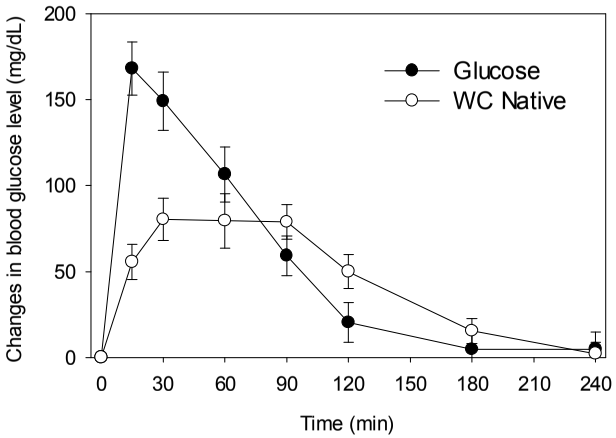
AS-modified starches revealed pronounced low digestibility compared to glucose or WCAS0. Representatively, their peak value was significantly (p<0.05) lower than that of glucose (168.4 mg/dL). The maximum increase in glucose levels throughout the measurement range for WCAS0-WCAS2 was 141.3, 102.8, and 91.6 mg/dL, respectively, showing a decrease with an increased degree of starch chain elongation. WCAS4 and WCAS8 had

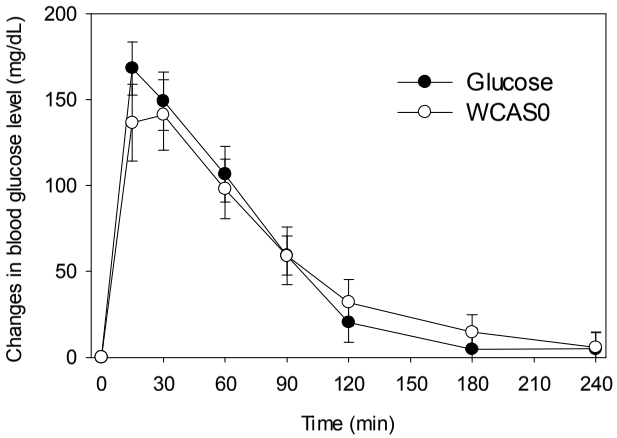
similar peak values of 59.3 and 60.5 mg/dL, respectively, which were significantly lower than even that of native starch (80.4 mg/dL).

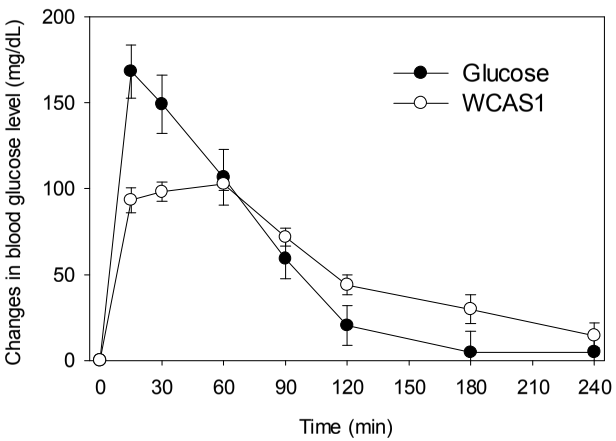
Moreover, the shapes of the graphs of AS-modified starches over the measurement range resembled that of native waxy corn starch, which is a representative SDS, rather than glucose or WCAS0 (RDS). In detail, in contrast to the triangular shape of the graph for glucose due to the steep increase and decrease of blood glucose level, the modified starches and native starch revealed the graph of a trapezoidal shape with a rather moderate increase up to the plateau which continues for 60-120 min followed by a slow and gradual decrease of glucose level. Therefore, AS-modification of starch allowed a sustained blood glucose level enabling a continuous supply of blood glucose throughout the digestive process. The considerable amounts of RS in WCAS4 and WCAS8 (22.7% and 31.6%, respectively) were reflected as noticeably decreased area under curve (60.5% and 73.1%, respectively). This showed consistency with the previous understanding that AUC is related to the amount of RS. A previous study reported that highly AS-modified adlay starch revealed a similar *in vivo* profile to conventional RS (Novelose 240), indicating the dominant RS characteristics of highly AS-modified starch (Kim et al., 2016a).

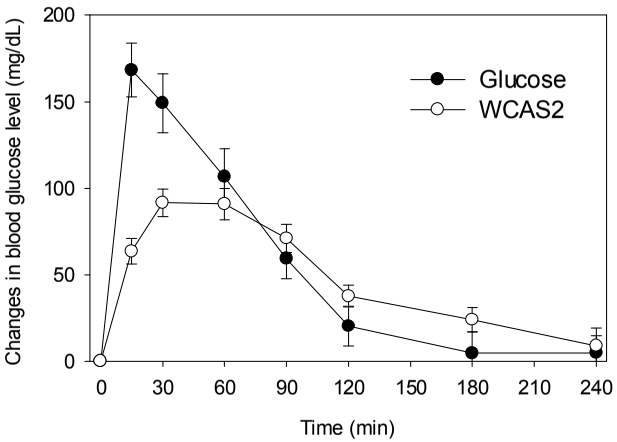
The relationship among the *in vivo* and *in vitro* digestion parameters was

determined. As this study deduced *in vitro* digestion parameters related to hydrolysis rate of RDS and SDS, the rate-related parameters were comprehensively compared with *in vivo* result. It is well known that the amounts of RDS and SDS have a relationship with peak value and average blood glucose level during whole duration (positively and negatively correlated, respectively). Based on the linear relationship between *in vitro* parameters, e.g. the amount of RDS and k_{RDS} (r=0.968, p<0.01), a correlation between the rate-terms and blood glucose level could be expected. As expected, the *in vitro* k_{RDS} and k_{SDS} showed significantly positive correlations with peak value and average blood glucose level (k_{RDS}, r=0.965 and r=0.987; k_{SDS}, r=0.979 and r=0.981, respectively for each, p<0.05). Therefore, it could be concluded that the blood glucose level after consumption of starch could be determined by the rate of digestion as well as the amount of each digestible fraction. The k_{SDS} also showed a relationship with the rate of the decrease in blood glucose level (r=0.982, p<0.05). The property of SDS that prolonged glucose supply with moderate increase and decrease in blood glucose level was directly supported by this study.


Changes in blood glucose level (mg/dL)
Time (min)
Glucose
WC Native
0
50
100
150
200
0
30
60
90
120
150
180
210
240


Changes in blood glucose level (mg/dL)
Time (min)
Glucose
WCAS0
0
50
100
150
200
0
30
60
90
120
150
180
210
240


Changes in blood glucose level (mg/dL)
Time (min)
Glucose
WCAS1
0
50
100
150
200
0
30
60
90
120
150
180
210
240


Changes in blood glucose level (mg/dL)
Time (min)
Glucose
WCAS2
0
50
100
150
200
0
30
60
90
120
150
180
210
240

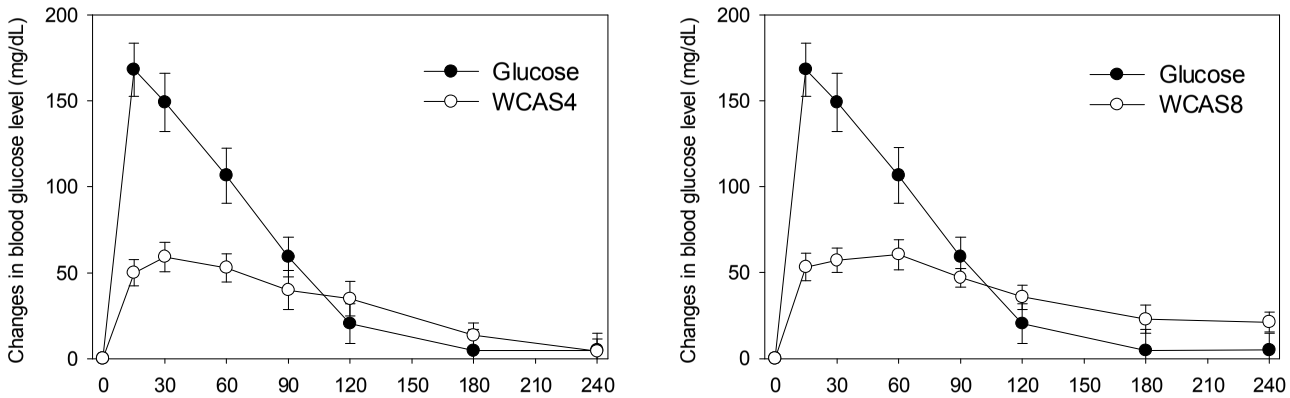


Figure 2.9. Changes in blood glucose level in mice after consumption of amylosucrase-modified starch samples.

2.4. Conclusions

In this study, a modified LOS plot method was suggested, and its accuracy and versatility over various starches could be confirmed via comparison with experimental data and estimated curves. The LOS plots with a singular linear plot proved the existence of RDS only or SDS only in some starches. At the same time, emergence of two distinctive linear plots in their LOS plots for other starches proved the co-existence of respective RDS and SDS in a particular starch. Not only the amount of each starch fractions but also the rate constants for each fraction could be obtained using the modified LOS plot method, where the correlation between rate terms and changes in blood glucose level in mice were examined.

Considering the clear categorization based on the unique digits of k_{RDS} and k_{SDS}, the specific structural features corresponding to respective RDS and SDS were expected. Moreover, minor changes within k_{RDS} or k_{SDS} themselves implied the determinant role of branch chain length distribution for organization of the structure of digestible fractions. Therefore, a follow-up study was required to establish the relationship between digestion property and branch chain length.

Since physiological changes after consumption is a time-based phenomenon, conventional Englysts' method (classification based on time

frame) has been favorable to describe postprandial effect. However, some positive correlation could be derived between *in vitro* digestion parameters from modified LOS plot analysis and *in vivo* postprandial blood glucose level changes. Precise and repetitive further researches are required to develop crucial factors to forecast the physiological changes based on *in vitro* digestion results.

Conclusively, this study confirmed that the concept of RDS, SDS, and RS suggested by Englyst et al (1992) is valid, and therefore could set forth a counterargument on the negative opinion against the classification of RDS and SDS. It would support the significance of the studies on health beneficial effects of SDS. Moreover, modified LOS plot method suggested in this study could complement the previous classification standards. This can be employed as a useful tool for the fractionation of starch into RDS, SDS, and RS.

Chapter 3.
Structure-digestibility relationship of amylosucrase-modified waxy corn starch: structure and formation mechanism of each starch fraction

* Part of this chapter was published in *Food Hydrocolloids* (H.R. Kim et al., 2017, Kinetic studies of in vitro digestion of amylosucrase-modified waxy corn starches based on branch chain length distributions, 65, 46-56) and *International Journal of Food Science and Technology* (H.J. Yoo & H.R. Kim et al., 2018, Characterization of low-digestible starch fractions isolated from amylosucrase-modified waxy corn starch, 53, 557-563)

3.1. Introduction

Starch is the main carbohydrate source in human nutrition. Since 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. In general, 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, Kingman, & Cummings, 1992). RDS is digested rapidly in the small intestine and includes fully gelatinized waxy starches. 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 a 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 hypoglycemic and hypocholesterolemic effects, acts as a substrate for the growth of probiotic microorganisms, and inhibits fat accumulation (Sajilata, Singhal, & Kulkarni, 2006). Starch with abundant SDS and RS fractions can be regarded as low digestible starch (Shin et al., 2007).

Because of their substantial health benefits, many studies have focused

on the preparation and characterization of novel starches with high proportions of SDS and RS (Kim et al., 2014; Miao et al., 2009a; Shin et al., 2004; Zhang et al., 2006b). 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 (Jiang, Lio, Blanco, Campbell, & Jane, 2010; Sajilata et al., 2006). 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, 2015a) and enzymatic modifications (Miao et al., 2009a; Shin et al., 2004; Zhang et al., 2006b). However, structural information on the SDS fraction is scarce compared with that on RS. It has been hypothesized that SDS consists of less perfect crystalline regions containing a small portion of 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) (Kim et al., 2013; Kim et al., 2014; Shin et al., 2010; Zhang et al., 2017) based on its glucosyltransferase activity to elongate the non-reducing ends of (1→4)-α-glucans using sucrose (De

Montalk, Remaud-Simeon, Willemot, Planchot, & Monsan, 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 organized 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 the present 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.

3.2. Materials and methods

3.2.1. Materials

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

3.2.2. Preparation of AS-modified starches

Waxy corn starch was dispersed in 100 mM sodium acetate buffer (pH 7.0) with 100 mM sucrose to make a 2% (w/v) suspension. It was boiled with vortex mixing for 30 min to completely gelatinize the starch and then cooled to 30°C. AS (5,000 U, 10,000 U, and 20,000 U/30 mL starch suspension; labeled as AS2, AS4, and AS8, respectively) was added to the starch suspension, and the volume was finally adjusted to 150 mL. After incubating for 6 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.

3.2.3. Determination and isolation of starch fractions

Pancreatic α-amylase (4.51 g) was suspended in distilled water (17 mL) by magnetic stirring for 10 min. After centrifuging at 1500 ×*g* for 10 min, the supernatant (15 mL) was mixed with amyloglucosidase (0.3 mL) and distilled water (2.7 mL). A starch sample (30 mg) was dispersed in a 2 mL microtube containing 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 storing in a shaking incubator (240 rpm, 37°C) for 10 min, the prepared enzyme solution (0.75 mL) was added to each microtube and incubated in a shaking incubator (240 rpm, 37°C). A microtube was removed at certain times and boiled for 10 min to stop the reaction. The supernatant was collected after centrifugation at 5,000 ×*g* for 10 min (4°C), and the amount of glucose released by hydrolysis of starch was analyzed using a GOD-POD kit. The time of plateau when the degree of digestion did not increase significantly (p>0.05) was defined for the standard of RS. The digestibility data during actual digestible phase were put into the equation of 1^{st}-order kinetics, and then expressed as log of slope (LOS) plots. The

intersection point of two discontinuous linear lines in their respective LOS plot graph was the distinction point between RDS and SDS.

The parallel set for determining the degree of hydrolysis was prepared and incubated for certain times to hydrolyze the RDS and/or SDS fractions. 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.

3.2.4. Determination of branch chain length distribution

Branched chain length distribution of starches was measured after debranching. The completely dissolved starch samples (15 mg starch in 3.0 mL of 25 mM, pH 4.3 sodium acetate buffer) was incubated at 45°C and 30 rpm for 2 h in a water bath, after isoamylase addition (1,000 U/mL, Megazyme, Bray, Ireland) by the amount of 200 U/g-starch. 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 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. Degree of polymerization (DP) values were designated using a mixture of maltooligosaccharides (DP 1-7, Sigma).

3.2.5. X-ray diffraction patterns and relative crystallinity

X-ray diffraction analysis was performed using a powder X-ray diffractometer (New D8 Advance, Bruker, Karlsruhe, Germany) at 40 kV and 40 mA, with Cu K_a radiation of 0.154 nm wavelength. A starch sample scan was conducted through a 2θ range from 3 to 30° at a step time of 2 sec. The relative crystallinity was calculated via the software (EVA, 2.0) developed by the instrument manufacturer, according to the following equation (Nara & Komiya, 1983).

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

3.2.6. Statistical analysis

All experimental data were analyzed 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).

3.3. Results and discussion

3.3.1. Structural characteristics of AS-modified starches

The native waxy corn starch had abundant chains of DP<25, but a smaller proportion of longer chains (Table 3.1), as reported in a previous study (Zhang et al., 2006b). A dramatic change was observed in AS-modified starches compared with unmodified one. Significant increases in the average chain length and considerable decreases in the proportion of short chains (DP 6-12) were observed, due to the action of AS that catalyzes the elongation of some branch chains by attaching 12 to 18 glucosyl units at their non-reducing ends (de Montalk et al., 1999). The chains with DP 13-24 had the largest proportion for AS-modified starches, which corresponded to earlier studies (Kim et al., 2013; Kim et al., 2014; Ryu et al., 2010). With increasing amount of added AS, the proportion of short chains (DP<13) and longer chains (DP≥13) greatly decreased and increased, respectively.

The crystalline packing arrangements of the AS-modified starch samples 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 3.2) with major peaks at 15°, 17°, 18°, and 23° 2θ (Hizukuri et al., 1980) and the relative crystallinity of 44.8%. The crystalline structure of the native starch was changed after AS treatment, revealing peaks at 5.5°, 14.5°, 17°, 19.3°, 22°, and 24° (Figure 3.2). Therefore, it was recognized that the crystalline structure of the native starches reorganized into dim B type polymorph after AS reaction, which corresponded to the previous reports (Kim et al., 2013; Kim et al., 2014; Shin et al., 2010). Retrograded or reorganized starch reveal B-type crystallinity resulting from the aggregation of long linear chains (Pohu, Planchot, Putaux, Colonna, & Buleon, 2004). Miao et al. (2009a) reported that the relative crystallinity is influenced by average amylopectin chain length, crystal size, amylopectin content, and extent of interaction between double helices. In this study, the amylopectin chain length was the primary factor that determined the extent of interaction between double helices. The chain length determined the amount of double helix and their packing and orientation. According to the previous report, 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-modified starches were more raised and sharpened, and peak intensity increased as the amount of AS increased. The relative crystallinity of AS-modified starches

also gradually increased according to the amount of AS.

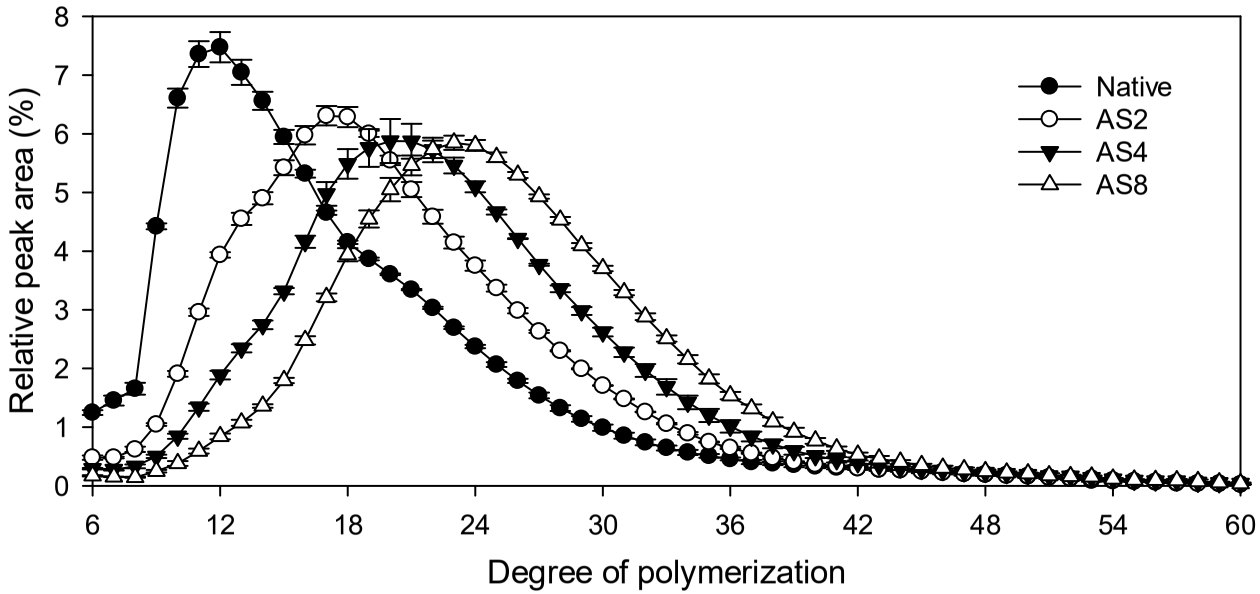


Figure 3.1. Branch chain length distribution of amylosucrase-modified waxy corn starches. Native, native waxy corn starch; AS2, starch modified with 5,000 U amylosucrase/30 mL-starch suspension; AS4, amylosucrase 10,000 U/30 mL; AS8, amylosucrase 20,000 U/30 mL.

Table 3.1. Branch chain length distributions of native and amylosucrase-modified starch samples

Sample	Percent distribution (%)				S/L	Average DP
	DP 6-12	DP 13-24	DP 25-36	DP ≥ 37		
Native	30.22±0.43[a]	52.60±0.55[c]	12.62±0.51[d]	4.56±0.45[c]	0.433±0.009[a]	17.87±0.23[d]
AS2	11.43±0.13[b]	62.52±0.29[a]	21.07±0.17[c]	4.98±0.25[c]	0.129±0.002[b]	20.85±0.13[c]
AS4	5.47±0.21[c]	56.82±1.82[b]	31.17±0.81[b]	6.54±0.82[b]	0.058±0.002[c]	23.36±0.29[b]
AS8	2.53±0.15[d]	46.30±1.02[d]	42.36±0.33[a]	8.80±0.77[a]	0.026±0.002[d]	25.67±0.22[a]

The values with different superscripts in the same column are significantly different (p<0.05). Native, native waxy corn starch; AS2, starch modified with 5,000 U amylosucrase/30 mL-starch suspension; AS4, amylosucrase 10,000 U/30 mL; AS8, amylosucrase 20,000 U/30 mL; DP, degree of polymerization; S/L, ratio of short chains (DP < 13) to long chains (DP ≥ 13).

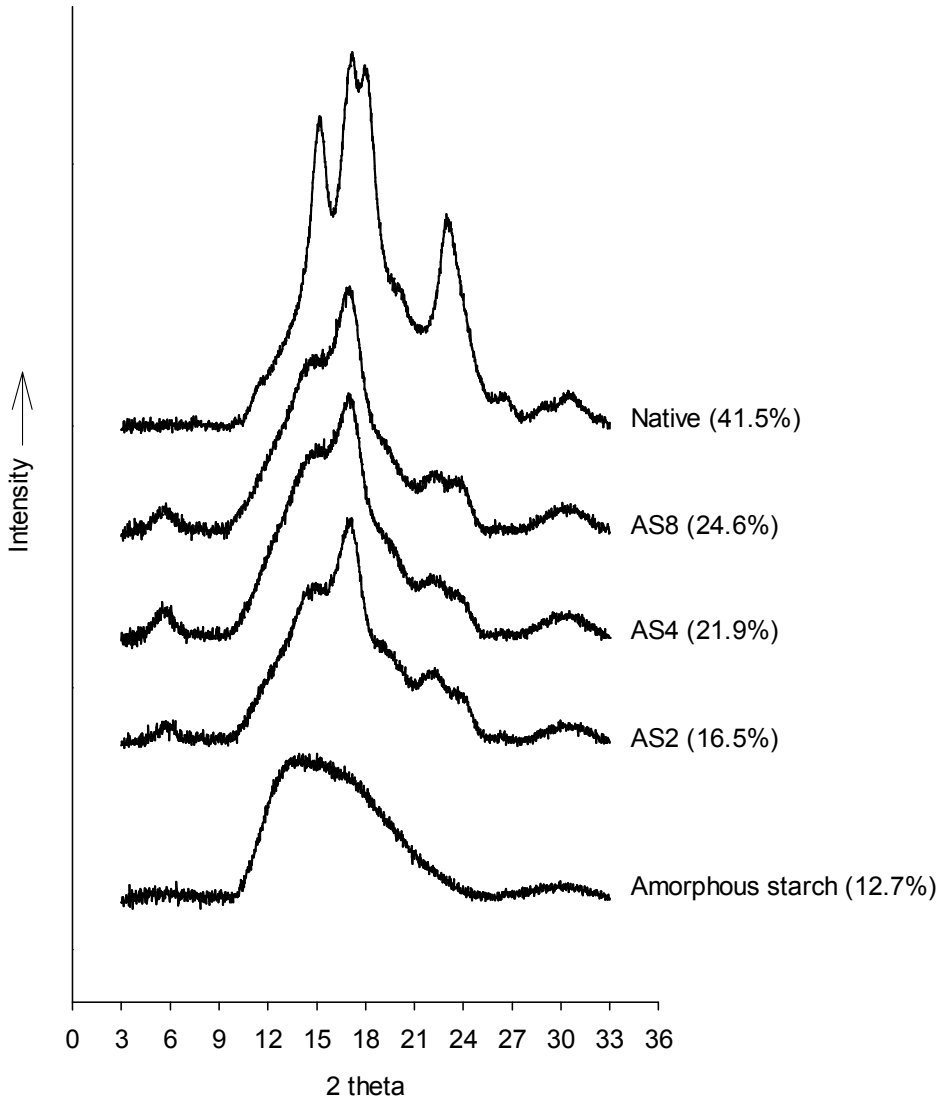


Figure 3.2. X-ray diffraction patterns of starch samples. Native, native waxy corn starch; Amorphous starch, control without enzyme addition; AS2, starch modified with 5,000 U/30 mL-starch suspension; AS4, AS 10,000 U/30 mL; AS8, AS 20,000 U/30 mL.

3.3.2. *In vitro* digestibility 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. First of all, the breakpoint for defining RS and the amount of RS were determined from the enzymatic digestion profile curves of starch samples (Figure 3.3). The degree of digestion data corresponding to digestible parts were fitted to LOS plot to obtain digestion rate constants for RDS and SDS (k_{RDS} and k_{SDS}, respectively). The kinetic parameters of starch samples estimated by the LOS plot are summarized in Table 3.2.

The native starch reached a plateau at 120 min of digestion, and the observed C_∞ (maximum degree of hydrolysis) was approximately 84%. The amount of RS was calculated by subtraction of C_∞ from 100%. The resistance to enzymatic hydrolysis of AS-modified starches was greatly influenced by the action of AS. The plateau time was shown at 150, 210, and 360 min in AS2, AS4, and AS8, respectively. AS2 exhibited the C_∞ slightly exceeding 80%, and it considerably decreased to 77.0% and 68.4% in AS4 and AS8, respectively.

LOS plots of native waxy corn starch displayed a single line with a slope of 0.026 supported by a high determination coefficient (r^2=0.945). It strongly indicated that native waxy corn starch was hydrolyzed at the same rate over

the entire digestion period and did not consist of distinct structures with different digestibility. In other words, the digestible part of native starch was not divided into RDS and SDS, but composed of only one kind of digestible fraction. The particular structure of native A-type starch could account for the rather slow and simultaneous digestion of native starch (Benmoussa et al., 2004). It is initiated by the migration and attachment of amylolytic enzymes to channels penetrating from the surface into the granular interior (Fannon et al., 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 et al., 2006a).

The LOS plots of AS2 and AS4 revealed two distinct lines, implying the existence of two fractions those were digested more rapidly (RDS) or more slowly (SDS), respectively, and these distinct fractions were identified with considerably different rate constants (Table 3.2). Their respective two rate constants from each phase were clearly distinguished by approximately a 10-fold difference. The LOS plot of WCAS8 was described by a single linear graph which corresponded to the SDS property (k=0.011 min^{-1}). It was also verified that AS8 starches, which are highly modified with AS, were composed of only SDS fractions.

Among AS-modified starches, the content of RDS decreased to a large

extent with the amount of AS whereas the proportion of SDS and RS increased. The reassociation between the elongated chains resulted in the formation of the double helical structures, leading to the creation of crystalline structures as supported by the increase in relative crystallinity (Figure 3.2). This reorganization process looked similar to the 'retrogradation' of fully gelatinized starch, though it could not be completely the same. 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; Zhang et al., 2008b). 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. In this study, starches underwent gelatinization before the AS treatment and the original crystalline structures of native starch were fully disintegrated. Then, during AS-modification process, elongation of branch chains occurred. The amorphous regions generated by gelatinization are easily accessed by digestive enzymes (Gallant et al., 1992; Zhang et al., 2006b), where RDS has amorphous structure. As the recrystallization of modified starches starts from fully amorphous state, it can be assumed that the amount and composition of RDS would differ according to how each

starch decreases the ratio of amorphous structure. Moreover, the reassociation property of each starch, such as length of double helices and their organization, would determine the resistance against digestive enzyme and the structure of SDS and RS fractions. Changes in k and C_∞ according to the degree of modification were observed in this study. Both k and C_∞ are strongly related to the increase in degree of order of the α-glucan chains (Patel et al., 2014). Therefore, 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, supporting the assumption stated above.

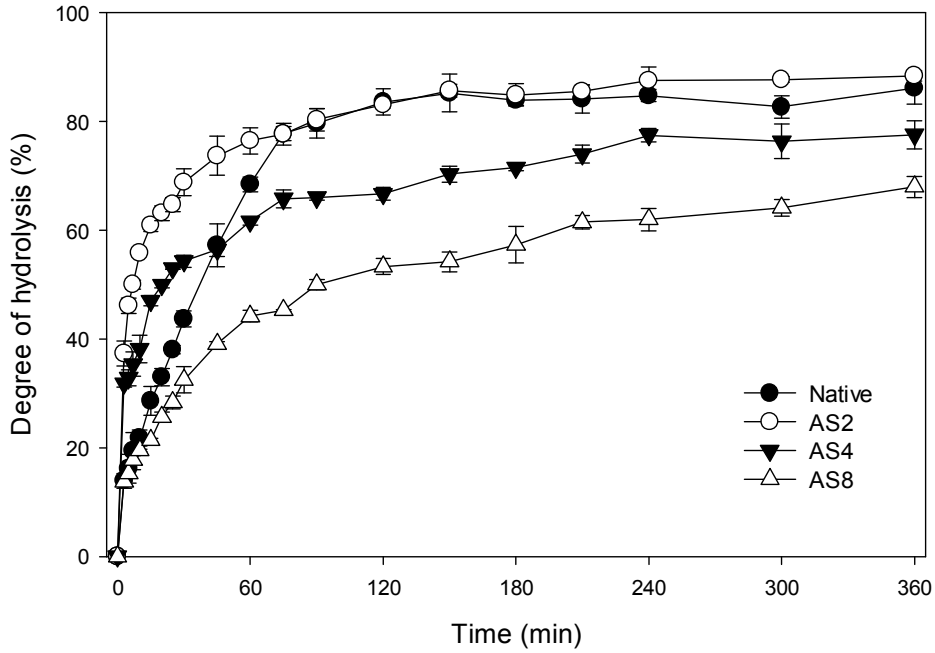


Figure 3.3. Digestion profiles of native and amylouscrase-modified waxy corn starches. Native, native waxy corn starch; AS2, starch modified with 5,000 U amylosucrase/30 mL-starch suspension; AS4, amylosucrase 10,000 U/30 mL; AS8, amylosucrase 20,000 U/30 mL

Table 3.2. *In vitro* digestibility parameters of native and amylosucrase-modified starches

Sample	RDS (%)	k_{RDS} (min^{-1})	RDS / SDS breakpoint (min)	SDS (%)	k_{SDS} (min^{-1})	SDS/RS breakpoint (min)	RS (%)
Native		N.D.		84.34	0.026	120	15.66
AS2	60.89	0.187	16.3	25.72	0.020	150	13.39
AS4	54.33	0.103	28.2	22.69	0.010	210	22.98
AS8		N.D.		68.39	0.011	360	31.61

Native, native starch; AS0, control without enzyme; AS2, starch modified with 5,000 U amylosucrase/30 mL-starch suspension; 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; k_{RDS}, rate constant for the RDS phase of starch hydrolysis; k_{SDS}, rate constant for the SDS phase of starch hydrolysis; N.D., not detected.

3.3.3. Branch chain length distributions of each fraction

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 Figure 3.4 and Figure 3.5. The branch chain length distribution of native starch was preserved in spite of hydrolysis (Figure 3.4A). It supported the no branch chain length specificity during enzyme hydrolysis in native waxy corn starch. As stated above, native granular cereal starch is hydrolyzed by a dynamic side-by-side digestion mechanism (Zhang et al., 2006a) that involves the inside-out digestion pattern. Shrestha et al. (2012) reported the similar result showing only minor change after partial digestion of normal maize starch, which is also an A-type cereal starch.

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. Meanwhile, heat-moisture treated waxy potato starch showed no significant changes in branch chain length distribution with RDS and/or SDS removal (Lee & Moon, 2015). This indicates that these two modification methods generate different structure of RDS and/or SDS each other.

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 regarding the RS from recrystallized starches (Eerlingen, Deceuninck, & Delcour, 1993; Lopez-Rubio, Flanagan, Gilbert, & Gidley, 2008). Chains with certain length commonly observed in isolated RS indicate that there are optimal-length chains to acquire the capacity to form more stable enzyme resistant structures (Lopez-rubio et al., 2008)

The dissimilarity in the DP change after hydrolysis between native and AS-modified starches could be caused by the different state and resultant different digestion patterns of the starches. The AS-modified starches lost the original granular characteristics 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 from that of native starch. The resistance of

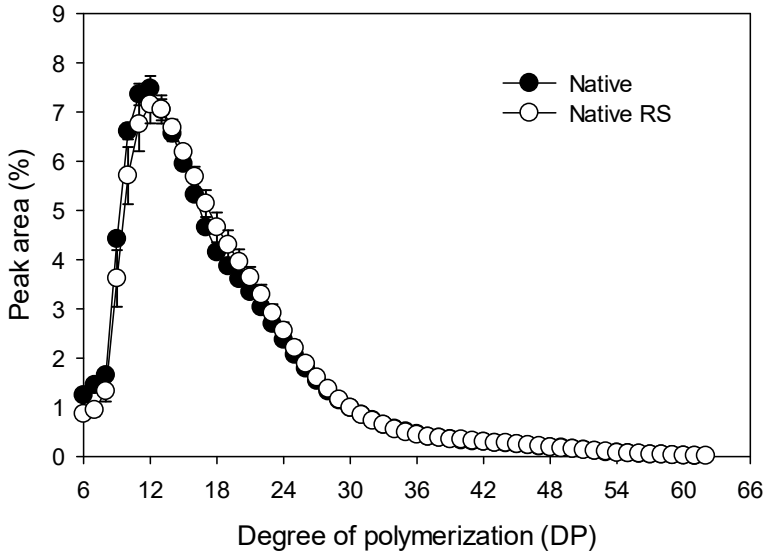
recrystallization-processed starches to digestive enzymes could be caused by the acquisition of a double-helical order (Colonna, Leloup, & Buleon, 1992). The stabilization of double helices into the crystalline structure decreases the susceptibility of starch to digestion enzymes by decreasing the effective 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).

Table 3.3. Comparison of percent distribution of branch chain length of starch samples before and after removal of RDS and/or SDS fraction

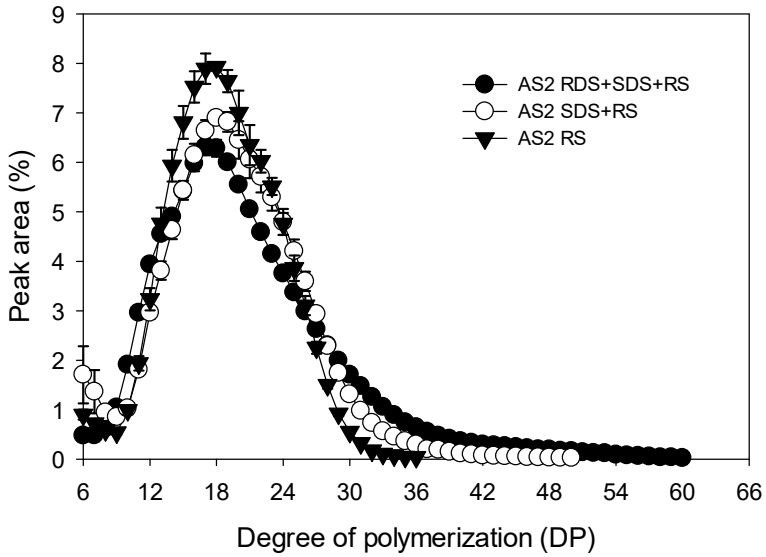
Sample		Percent distribution (%)				Average DP
		DP 6-12	DP 13-24	DP 25-36	DP ≥ 37	
Native	Whole (SDS+RS)	30.22±0.43^{a}	52.60±0.55^{f}	12.62±0.51^{g}	4.56±0.45^{c}	17.87±0.23^{i}
	RS	26.38±2.48^{b}	56.06±1.83^{e}	12.94±0.98^{g}	4.62±0.34^{c}	18.28±0.38^{h}
AS2	Whole (RDS+SDS+RS)	11.43±0.13^{c}	62.52±0.29^{d}	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	Whole (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	Whole (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}

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; DP, degree of polymerization; Native, native waxy corn starch; AS2, starch modified with 5,000 U amylosucrase/30 mL-starch suspension; AS4, amylosucrase 10,000 U/30 mL; AS8, amylosucrase 20,000 U/30 mL.

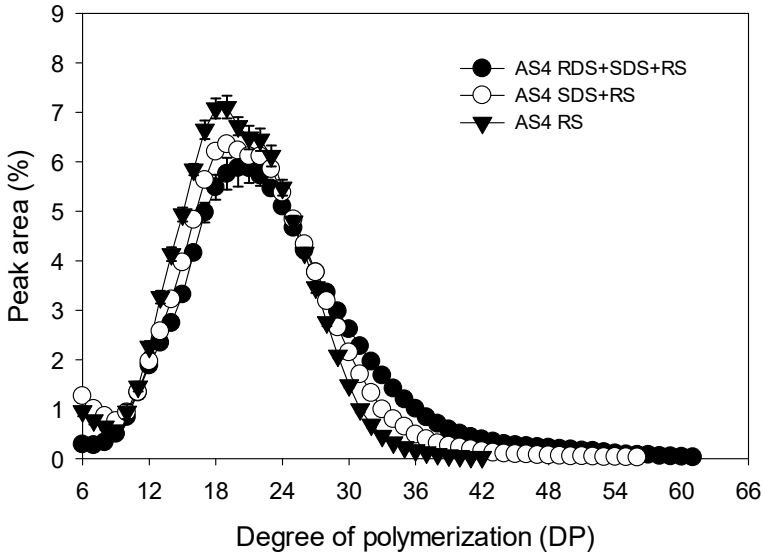
(A)


Native
Native RS
Peak area (%)
Degree of polymerization (DP)
0
1
2
3
4
5
6
7
8
9
6
12
18
24
30
36
42
48
54
60
66

(B)


AS2 RDS+SDS+RS
AS2 SDS+RS
AS2 RS
Peak area (%)
Degree of polymerization (DP)
0
1
2
3
4
5
6
7
8
9
6
12
18
24
30
36
42
48
54
60
66

(C)



(D)

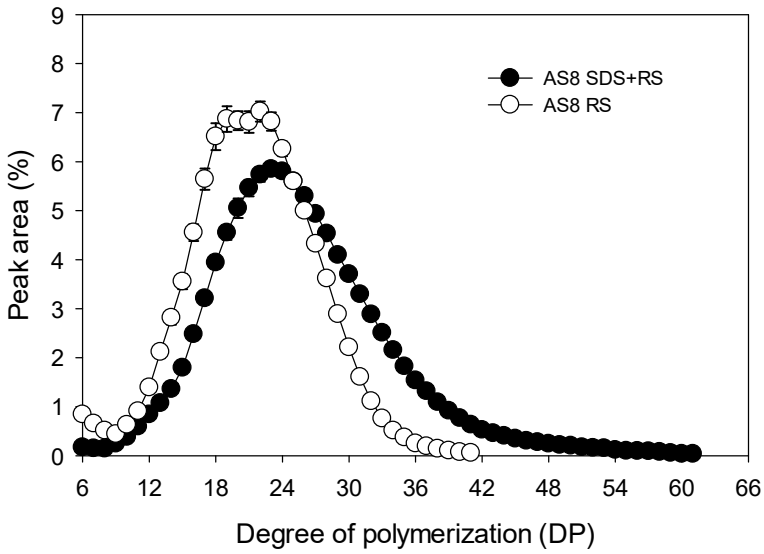
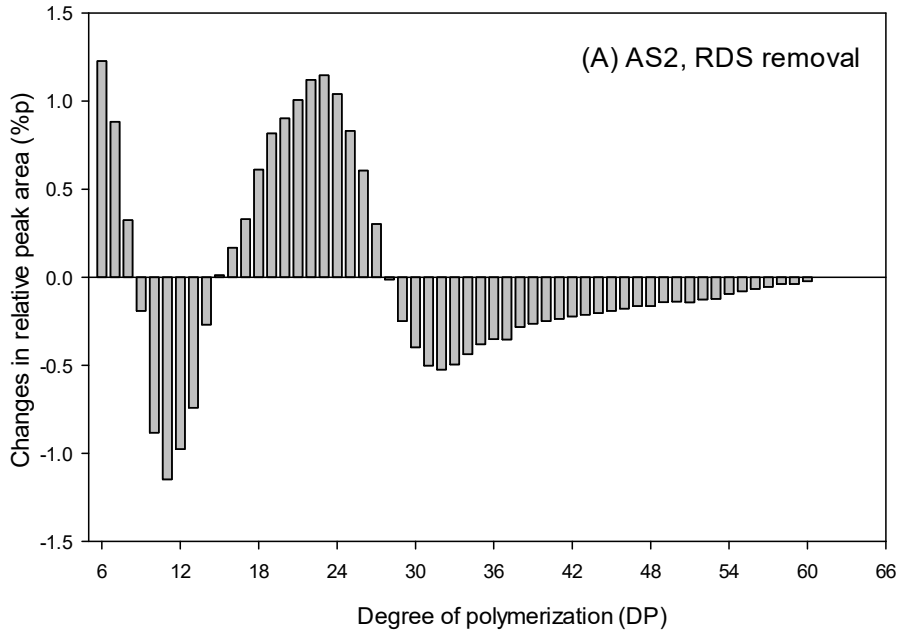
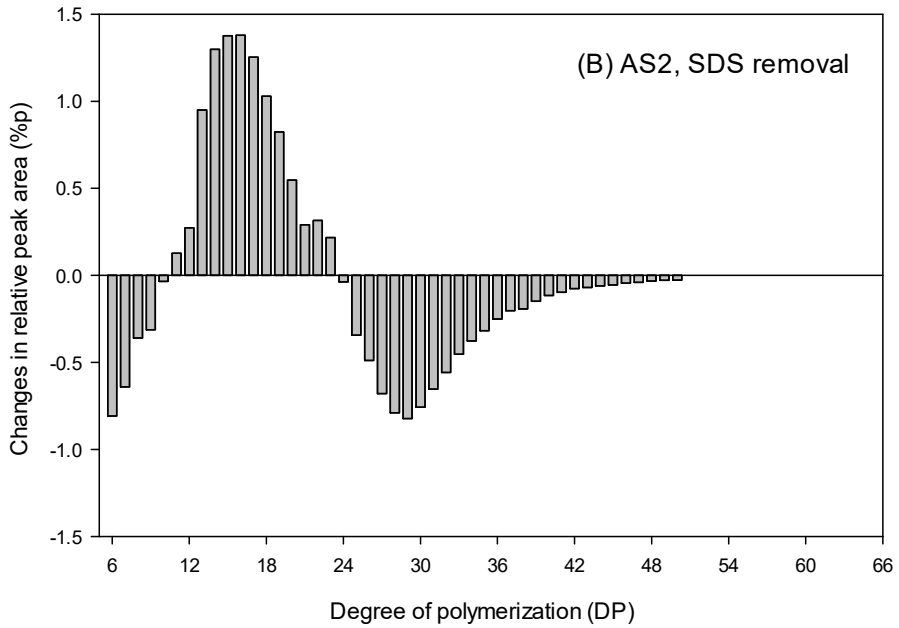


Figure 3.4. The branch chain length distributions of starch samples determined before and after removal of RDS and/or SDS fraction. (A) Native, native waxy corn starch; (B) AS2, starch modified with 5,000 U amylosucrase/30 mL-starch suspension; (C) AS4, amylosucrase 10,000 U/30 mL; (D) AS8, amylosucrase 20,000 U/30 mL.


(A) AS2, RDS removal
Changes in relative peak area (%p)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
6
12
18
24
30
36
42
48
54
60
66
Degree of polymerization (DP)


(B) AS2, SDS removal
Changes in relative peak area (%p)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
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12
18
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42
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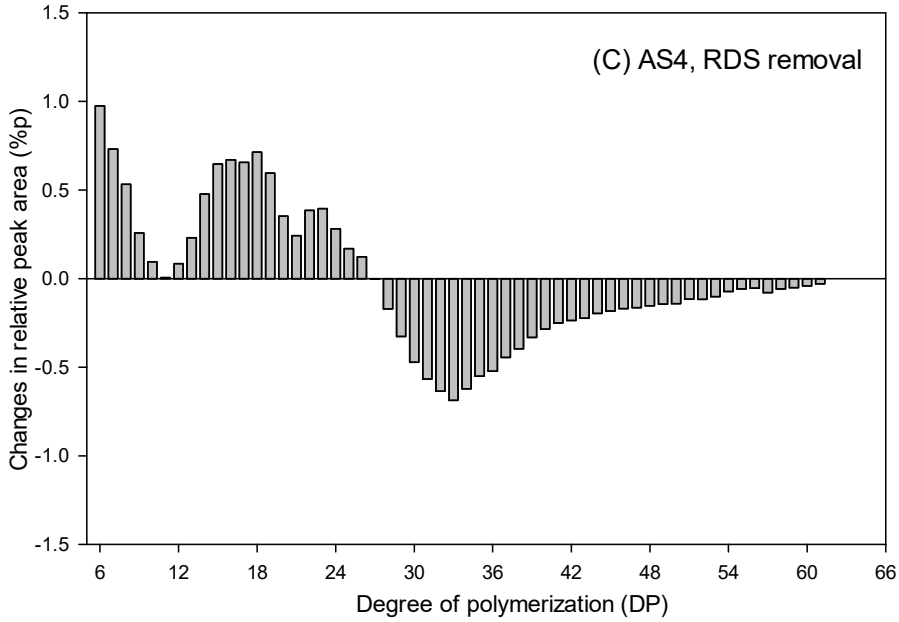
Degree of polymerization (DP)


(C) AS4, RDS removal
Changes in relative peak area (%p)
1.5
1.0
0.5
0.0
-0.5
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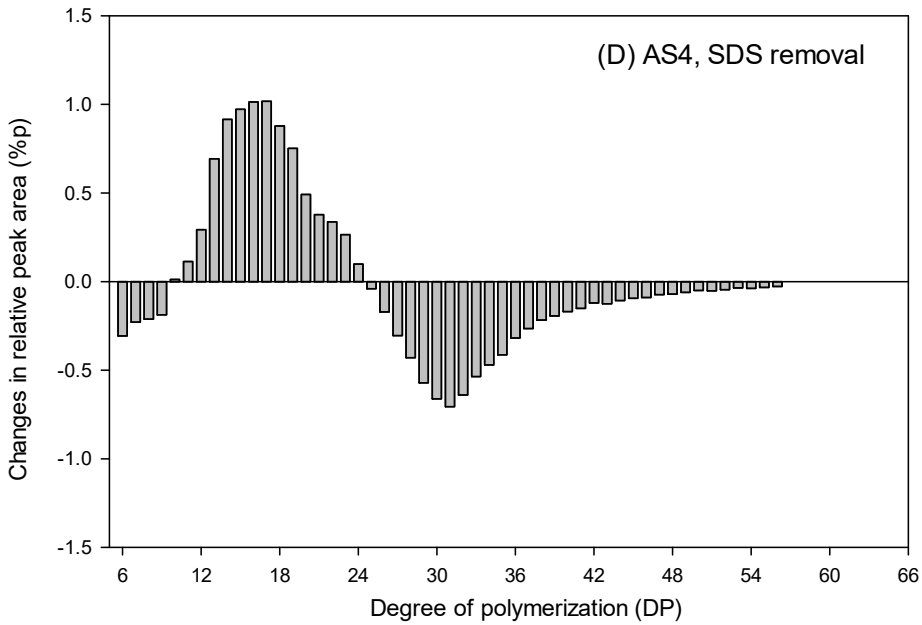
Degree of polymerization (DP)


(D) AS4, SDS removal
Changes in relative peak area (%p)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
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Degree of polymerization (DP)

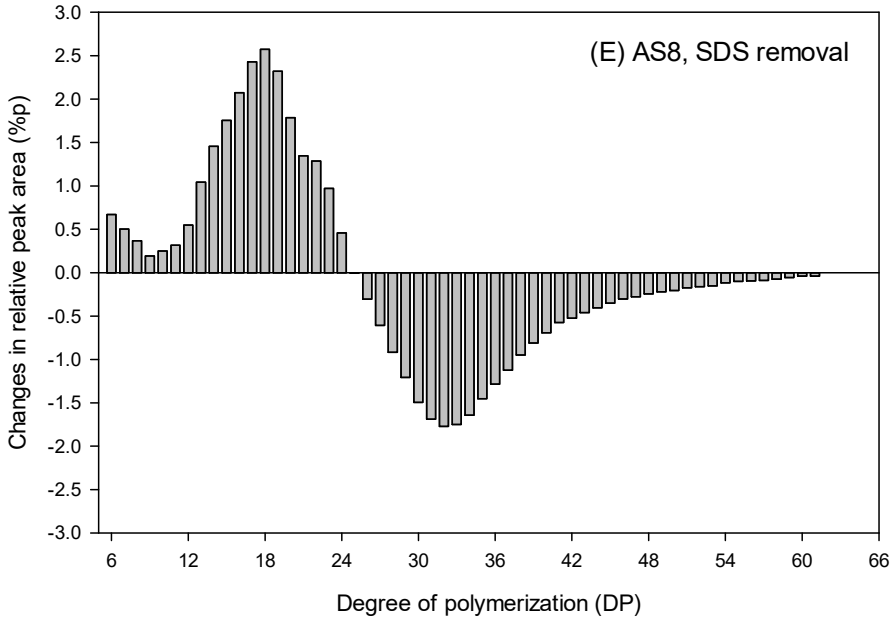


Figure 3.5. Changes in the branch chain length distributions of starch samples caused by removal of RDS and/or SDS fractions. (A), (B) AS2 = modified starch with 5,000 U amylosucrase/30 mL-starch suspension; (C), (D) AS4 = amylosucrase 10,000 U/30 mL; (E) AS8 = amylosucrase 20,000 U/30 mL. RDS = rapidly digestible starch; SDS = slowly digestible starch; RS = resistant starch.

3.3.4. Changes in crystalline properties determined by X-ray diffraction

Removal of the digestible 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 semicrystalline 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 selected regions primarily, leaving the undigested crystalline regions relatively unchanged (Shrestha et al., 2012).

After removal of RDS and/or SDS fraction, the B-type crystalline structure of the AS-modified starches was kept regardless of the degree of digestion. Shin et al. (2004) reported that SDS consisted of not only crystalline but also amorphous regions and RDS mainly composed of amorphous region. 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. Similarly, when the debranched

cassava starch was recrystallized, the crystallinities of its digested products were significantly higher than those of the undigested starches (Mutungi et al., 2011). The higher crystallinities in the digested materials coincide with higher amounts of B-type packing. AS-modified starches and their SDS+RS and RS fractions all showed increases in the relative crystallinity based on the amount of AS added. This indicated that the degree of AS treatment affected the amount of amorphous region. Therefore, the amount of RDS was gradually reduced when the degree of chain elongation was increased.

Moreover, the amount of RS and crystallinity of remaining RS fraction was high in the AS-modified starches with longer branched chains, indicating the formation of more rigid and compact RS. In general, the crystallinity of starches is attributed to average amylopectin chain length, 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., 2009b). The crystalline regions composed of long branched chains could be much more resistant to enzyme hydrolysis than crystalline regions formed with short branched chains (Zhang et al., 2006b). Therefore, 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.

Table 3.4. Relative crystallinities of native and amylosucrase-modified starches and their isolated fractions

Sample		Relative crystallinity (%)
Native	SDS+RS (whole)	41.5±1.0[b]
	RS	51.1±0.5[a]
AS2	RDS+SDS+RS (whole)	16.5±0.4[j]
	SDS+RS	19.2±1.0[g]
	RS	23.2±1.0[e]
AS4	RDS+SDS+RS (whole)	21.9±0.4[f]
	SDS+RS	25.1±0.4[d]
	RS	27.4±0.4[c]
AS8	SDS+RS (whole)	24.6±0.4[d]
	RS	27.8±0.5[c]

The values with different superscripts are significantly different (p<0.05). Native, native waxy corn starch; AS2, starch modified with 5,000 U amylosucrase/30 mL-starch suspension; 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.

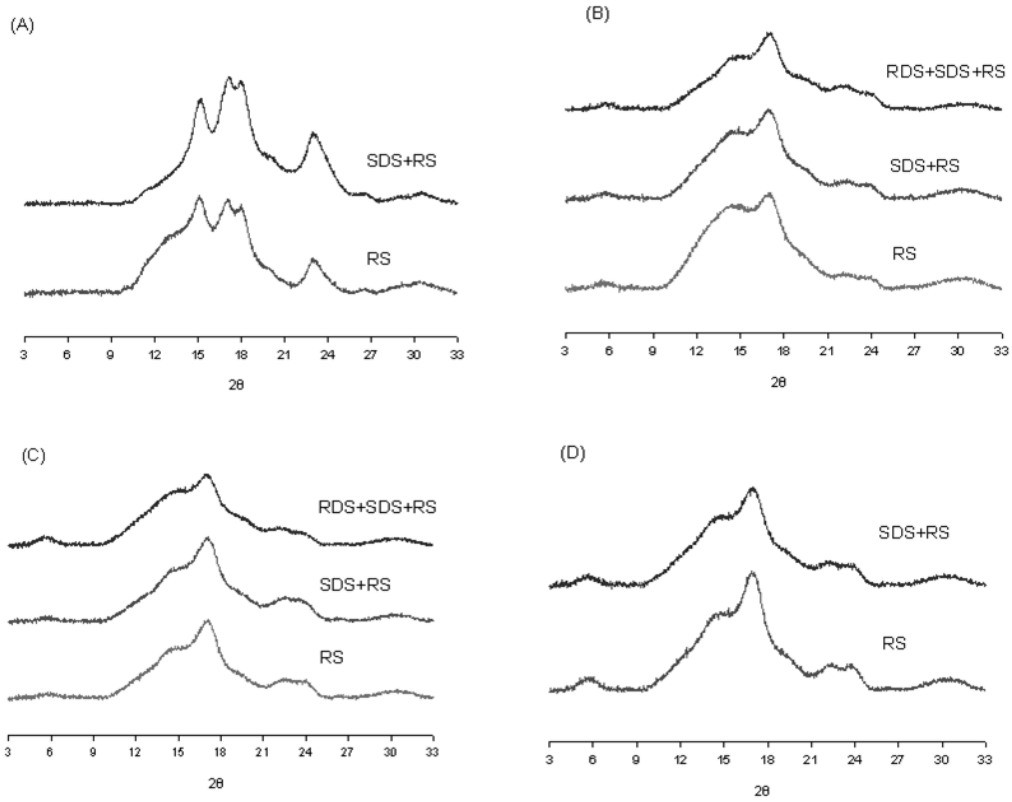


Figure 3.6. Comparison of X-ray diffraction patterns of starch samples before and after removal of RDS and/or SDS fraction. (A) Native, native waxy corn starch; (B) AS2, starch modified with 5,000 U amylosucrase/30 mL; (C) AS4, amylosucrase 10,000 U/30 mL; (D) AS8, amylosucrase 20,000 U/30 mL. RDS = rapidly digestible starch; SDS = slowly digestible starch; RS = resistant starch.

3.3.5. 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. 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 reducing the susceptibility to digestion (Htoon et al., 2009; Liu, Yu, Chen, & Li, 2007). In accordance with the increased crystallinity, the 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) (Figure 3.7). 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 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 the amount of double helix chain folding would be increased, but also the distance between double helices would be narrowed, resulting in a more dense crystalline structure. This hypothesis supports the existence of large amounts of RS in AS4 and AS8, possessing noticeably many long chains.

As aforementioned, 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 structure of AS-modified waxy corn starches would be formed by (1) the alignment of adjacent single helices, (2) the exposed parts of the double helix turns out of crystalline lamella, (3) non-crystalline double helical structures, and (4) 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. Further, 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 RS.

Edwards et al. (2014) stated that the rate constant is an inherent property of the enzyme, which would not change. The authors failed to clearly explain the reason for the changes in rate constant during the same reaction or depending on granule size of flour. In their studies, only the contribution of ions in reaction system as activator or inhibitor were discussed, rather than considering the structure of starch as important factor as they were not in agreement with the concept of RDS and SDS. This ambiguity could be examined by this study. If the rate of enzyme reaction differs under the totally same condition, it indicates the different characteristics of substrate. For example, the only variation in this study was branch chain length distribution. Certain structure and digestive rate constants were found in common among respect RDS and SDS fractions from different starch samples. This strongly supported the individual existence of RDS and SDS as specific structural characteristics of each. The small different structures of RDS and SDS among AS-modified starches were reflected in the minor different values within k_{RDS} and k_{SDS} groups.

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.

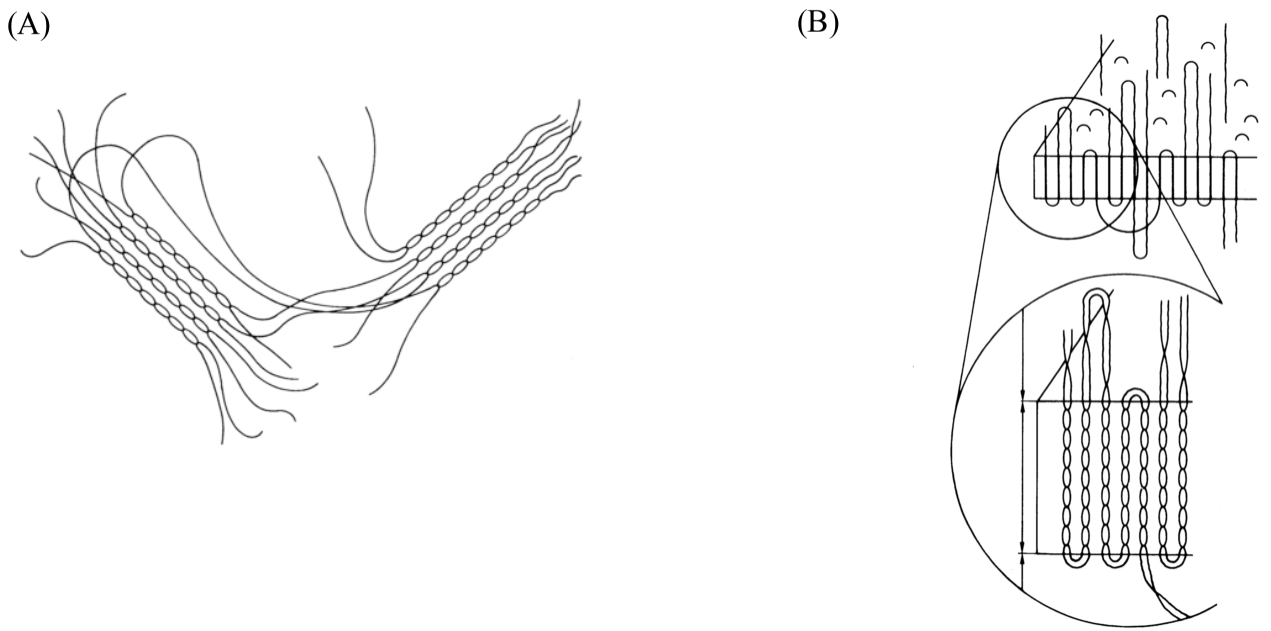


Figure 3.7. (A) Micelle model and (B) lamella model for the formation of crystalline structures in amylose solutions proposed by Eerlingen et al. (1993).

3.4. Conclusions

The branch chain length distributions and crystalline properties of amylosucrase-modified starches were characterized before and after removal of their RDS and/or SDS fractions. The structures of RDS and SDS could be estimated by comparison of the changes after their removal, and the structure of remaining RS fraction was also determined. AS-modified starches showed greatly different mode of change from native starch according to the removal of digestible fractions, due to their altered state and crystalline order.

A decrease in the length of detectable longest branch chain was observed in the order of RDS+SDS+RS > SDS+RS > RS within all AS-starches. The RDS removal caused the significant disappearance of the chains of longer than 27 or 28, implying that long chains contributed to the formation of the RDS fraction. Branch chains of DP 13-24 and DP≥25 mainly contributed to the structure of SDS. The RS fractions from all of the AS-starches contained predominantly branch chains with DP 13-24.

B-type crystalline structure of the AS-modified starches was kept regardless of the degree of digestion. Removal of the digestible fraction caused an increase in the relative crystallinity, where the crystallinity of the remaining structure was dependent on the branch chain length of their original materials.

Conclusively, the current study illustrated that different branch chain lengths of amylopectin determined the primary crystalline arrangement of recrystallized starches and, accordingly, the amounts and structures of RDS, SDS, and RS fractions. Moreover, the respective existence of RDS and SDS in the structural aspect could be strongly supported by certain structure and digestive rate constants found in common among RDS and SDS fractions respectively.

Considering that distinction between RDS and SDS was possible based on k values, and that minor structural differences among samples were reflected as differences in k_{RDS} and k_{SDS}, the modified LOS plot method utilized in this study would be useful for structural assumption of starch as well as digestibility analysis. For example, it would enable starch researchers to design and confirm a starch with specific structure.

Chapter 4.
The role of amylopectin chains length in the formation of low-digestible fraction in amylosucrase-modified waxy potato starches co-crystallized with amylose

4.1. Introduction

Starch is one of the most abundant polysaccharides in nature and is composed of two types of macromolecules, amylose and amylopectin. Amylose is primarily linear molecule with α-1,4-linked glucosyl units with 500-6,000 glucose units (Roger et al., 1996). Amylose may have up to 10 or more branches, forming predominately single chain helices. Amylopectin is a highly branched polymer that is much larger than amylose (Roger et al., 1999). Amylopectin is composed of α-1,4-linked glucose segments connected by α-1,6-linked branch points. Amylopectin is the predominant molecule in most normal starches (~70–80%) and therefore strongly influences the functionality of starch. The ratio of amylose to amylopectin within a starch, structures of amylose and amylopectin, and the way in which these two polymers organize affect the physicochemical and functional properties of starch (Fredriksson et al., 2000).

In terms of nutrition, starch is generally classified into rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) according to the rate of glucose release and the absorption of glucose in the gastrointestinal tract (Englyst, Kingman, & Cummings, 1992). RDS is digested rapidly in the mouth and the small intestine, leading to a rapid increase followed by a rapid drop in blood glucose level. SDS is digested

completely but slowly in the small intestine and is effective for improving glucose tolerance, diabetes management, mental performance, and satiety (Lehmann & Robin, 2007). RS is not digested in the small intestine, but acts as a substrate for fermentation and growth of probiotic microorganisms in the large intestine. RS can exert hypoglycemic and hypocholesterolemic effects, prevent colon cancer, reduce gall stone formation, and increase mineral absorption (Sajilata et al., 2006). Due to their health beneficial effects, increasing the SDS and/or RS contents in starch material or starchy food is a subject of interest in the food industry and physical, chemical, and enzymatic modifications have been employed to increase the fractions of SDS and/or RS in foods (Chung et al., 2009; Lee et al., 2012b; Miao et al., 2014a; Ovando-Martínez et al., 2013; Shin et al., 2010; Shin et al., 2007).

Recently, an enzyme named amylosucrase (AS; E.C. 2.4.1.4) received interest for its ability to modify molecular structure of starch and resultant lowered digestibility (Kim et al., 2014; Kim et al., 2016a; Shin et al., 2010; Zhang et al., 2018). The enzyme catalyzes a transglycosylation reaction using sucrose as a substrate while releasing fructose, thereby elongating starch chains by attaching glucose molecules to non-reducing ends of their external chains (de Montalk et al., 2000; Rolland-Sabaté, Colonna, Potocki-Veronese, Monsan, & Planchot, 2004). The adjacent elongated amylopectin

chains rapidly form double helix and crystalline structure, resulting in an increase of SDS and RS contents in AS-modified starches (Kim et al., 2014; Shin et al., 2010). However, the contribution of amylose to the molecular organization for low-digestible fraction in AS-modified starches is not well-known. Though the previous studies employing normal starches observed the changes in digestibility, they could not take amylose into account as a meaningful factor for the interpretation of the formation of SDS and RS structure (Kim et al., 2013; Ryu et al., 2010; Shin et al., 2010).

Therefore, this study examined the basic structure and digestibility of starches produced via recrystallization of amylosucrase-modified waxy starch and amylose together. The objectives of this study were to investigate the interaction between two molecules depending on amylopectin chain length, and to determine how their resultant structure affects the formation of low digestible fraction.

4.2. Materials and methods

4.2.1. Materials

Waxy potato starch was obtained from Avebe (Veendam, Netherland). Amylose from potato starch and α-amylase from porcine pancreatin (type VI-B, A3176, activity 15 U/mg solid) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Amyloglucosidase (AMG 300L, activity 300 AGU/mL), isoamylase (activity 1,000 U/mL), and a GOD-POD assay kit were from Novozymes (Bagsvaerd, Denmark), Megazyme (Bray, Ireland), and Embiel Co. (Gunpo, Korea), respectively. All other reagents were of analytical grade. 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 catalyzes the production of 1 μM of fructose per min in the assay condition (Kim et al,. 2014).

4.2.2. Preparation of the starch mixture of AS-modified waxy potato starch and amylose

Waxy potato starch was dispersed in 100 mM sodium acetate buffer (pH 7.0) with 100 mM sucrose to make a 2% (w/v) suspension. It was boiled with vortex mixing for 30 min and then cooled to 30°C. AS (5,000 U, 10,000 U,

and 20,000 U/30 mL starch suspension, labeled as AS2, AS4, and AS8, respectively) was added to the starch suspension, to adjust the final volume of 150 mL. After incubating for 6 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 (AS0) was prepared by the same method without the enzyme addition.

The AS-modified starch and amylose were mixed by the weight ratio of 3:1, and dissolved in 90% dimethyl sulfoxide (1%, w/v) via boiling for 30 min with vortex mixing to be completely solubilized. Three volumes of ethanol were added to the solution and centrifuged at 10,000 ×*g* for 10 min twice. The starch pellet was re-dissolved in distilled water (2%, w/v), and boiled for 30 min with vigorous vortexing. After autoclaving at 121°C for 30 min, it was cooled to room temperature and stored at 4°C for 48 h. The product was freeze-dried, ground, and sieved through 100-mesh sieve to be analyzed. The final starches were named as AM×ASn where n corresponds to the amount of the AS applied.

4.2.3. Determination of *in vitro* digestibility and isolation of low-digestible fractions

Pancreatic α-amylase (9 g) was suspended in distilled water (18 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). A starch sample (30 mg) was dispersed in a 2 mL microtube containing 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 storing in a shaking incubator (240 rpm, 37°C) for 10 min, the prepared enzyme solution (0.75 mL) was added to each microtube and incubated in a shaking incubator (240 rpm, 37°C). A microtube was removed at certain times and boiled for 10 min to stop the reaction. The supernatant was collected after centrifugation at 5,000 ×*g* for 10 min (4°C), and the amount of glucose released by hydrolysis of starch was analyzed using a GOD-POD kit.

The time of plateau when the degree of digestion did not increase significantly (p>0.05) was defined as the standard of RS. The digestibility data during actual digestible phase were put into the equation of 1^{st}-order kinetics, and then expressed as log of slope (LOS) plots (Butterworth et al., 2012). The intersection point of two discontinuous linear lines in their

respective LOS plot graph was the distinction point between RDS and SDS for each sample.

4.2.4. Determination of branch chain length distribution

Branched chain length distribution of starches was measured after debranching. The completely dissolved starch samples (15 mg starch in 3.0 mL of 25 mM, pH 4.3 sodium acetate buffer) was incubated at 45°C and 30 rpm for 2 h in a water bath after isoamylase addition (1,000 U/mL, Megazyme, Bray, Ireland) by the amount of 200 U/g-starch. 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 (ICS 3000 series, Dionex, Sunnyvale, CA, USA) on a Carbo-Pack PA1 anion-exchange column (4×250 mm, Dionex) equipped with an electrochemical 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. Degree of polymerization (DP) values were designated using a mixture of maltooligosaccharides (DP 1-7, Sigma).

4.2.5. X-ray diffraction and relative crystallinity

X-ray diffraction was carried out using a powder X-ray diffractometer (D8 Advance, Bruker, Karlsruhe, Germany) at 40 kV and 40 mA. The starch sample was scanned through a 2θ range from 3° to 30° with a 0.02° step size and a scanspeed of 0.5 sec/step. The relative crystallinity was calculated by the following equation (Nara & Komiya, 1983) using the software developed by the instrument manufacturer (EVA, 2.0).

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

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

4.2.6. Thermal properties

Thermal properties of the samples were analyzed using a differential scanning calorimeter (Discovery DSC, TA Instruments, New Castle, DE, USA). The starch and distilled water was weighed in a pan by the ratio of 1:3. The pan was hermetically sealed and kept at room temperature overnight for moisture equilibrium. The thermodynamic curve scan was conducted over the temperature range of 40°C to 200°C at a 10°C/min increasing rate.

4.2.7. Iodine binding property and apparent amylose content

Starch (20 mg) was first dispersed in absolute ethanol (0.2 mL), and then 1 M NaOH (1.8 mL) was added to the mixture. After boiling with vigorous vortex mixing for 10 min, the dispersion was cooled to room temperature. The cooled starch dispersion (1 mL) was diluted to 10 mL with distilled water, and 1 M acetic acid (0.1 mL) was added to an aliquot (0.5 mL) of the diluted dispersiion. It was diluted again to 10 mL with distilled water, 0.2 mL of Lugol's solution (Sigma-Aldrich) was added, and the mixture was stored for 20 min in the dark. The absorbance of the color-developed solution was scanned through the wavelength range of 400-900 nm to compare iodine binding capacity of the samples. Apparent amylose content of starch samples were calculated by comparing the absorbance of the solution at 620 nm with a calibration curve obtained from the mixtures with various ratios of amylose from potato (Sigma-Aldrich) and amylopectin in a form of waxy potato starch.

4.2.8. Statistical analysis

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)

4.3. RESULTS AND DISCUSSION

4.3.1. Production of starches with different amylopectin chain length distribution

Four samples were produced via recrystallization of AS-modified waxy potato starches and amylose, and their basic properties were examined (Table 4.1). During the preparation of AM×AS0, no elongation of branch chains occurred because there was no AS addition. It had abundant medium length branch chains of DP 13-24 with average chain length of DP 21. The branch chain length distribution of the starch samples were strongly affected by the level of AS. Steep changes in the proportion of short chains with DP 6-12 were observed in AS-modified starches, decreasing from 24.6% of AM×AS0 to 4.2% of AM×AS8. Significant increases (p<0.05) in the average chain length and long chains of DP≥25 were due to the elongation of some branch chains by AS reaction via attaching 12 to 18 glucosyl units at their non-reducing ends (de Montalk et al., 1999). The more AS that was added, the lower was the ratio value of short chains (DP<13) to long chains (DP≥13), ranging from 0.044 to 0.327 among starch samples. Since the ratio is related to the SDS content (Zhang et al., 2008a), these starches could be expected to have different digestive properties.

Because of the chain elongation, iodine binding capacity of original starch

(AM×AS0) was advanced and revealed a higher apparent amylose content. In detail, although the same amount of amylose was added, different apparent amylose content values were observed among samples. In contrast to AM×AS0 showing only the amount of added amylose (26.2%, very close to 25%), 14%p increased value was observed in AM×AS8. It supported the elongation of amylopectin chains to a high extent so that those chains contain a noticeable amount of single helical chains, which are long enough to complex with iodine molecules, and would possibly have amylose-like behavior.

All starch samples revealed B type-like X-ray diffractogram, which is a typical of recrystallized starch, as to be shown later (Figure 4.6). The relative crystallinity increased depending on the degree of elongation in amylopectin. The AS-modified waxy corn starches by different degree also showed a similar result (Kim et al., 2014). The crystalline structure is formed by the contribution of amylopectin molecules by forming double helix structure and their organization in ordered alignment. Thus, the crystalline structure of starches in this study would also be determined by the characteristics of amylopectin. During the incubation to produce the AS-modified starches, the double helices are presumably formed between elongated branch chains of amylopectin that were adjacent. The longer linear chains can easily form the

double helical structure and their aggregation could also be accelerated (Kim et al., 2016b).

Table 4.1. Structural properties of starch samples

Sample	Percent distribution (%)				S/L	Average DP	Apparent amylose content (%)	Relative crystallinity (%)
	DP 6-12	DP 13-24	DP 25-36	DP ≥ 37				
AM×AS0	24.6±2.4^{a}	46.0±2.6^{b}	18.9±0.5^{d}	10.5±0.9^{b}	0.327^{a}	20.95^{d}	26.17^{c}	15.6^{d}
AM×AS2	10.9±0.9^{b}	48.0±1.8^{b}	29.7±1.5^{c}	11.4±0.7^{b}	0.122^{b}	24.06^{c}	30.00^{b}	19.1^{c}
AM×AS4	7.5±1.2^{c}	45.7±1.0^{b}	34.2±1.6^{b}	12.6±1.0^{b}	0.081^{c}	25.33^{b}	29.57^{b}	24.8^{b}
AM×AS8	4.2±0.8^{d}	37.1±1.3^{a}	40.8±2.3^{a}	18.0±1.7^{a}	0.044^{d}	27.94^{a}	39.84^{a}	27.6^{a}

AM×AS0, mixture of amylose and amylosucrase control without enzyme; AS2, starch modified with 5,000 U amylosucrase/30 mL-starch suspension; AS4, amylosucrase 10,000 U/30 mL; AS8, amylosucrase 20,000 U/30 mL; DP, degree of polymerization; S/L, ratio of short chains (DP<13) to long chains (DP≥13). The values with different superscripts in the same column are significantly different (p<0.05).

4.3.2. *In vitro* digestibility of starch samples

As shown in Figure 4.1, noticeable differences in the enzymatic digestion profiles during 360 min were observed amongst four starch samples. A previous study explained that one of the reasons for the increases in SDS and RS fraction in AS-modified starches was the long modification time (45 h) enough for retrogradation (Kim et al., 2014). However, as this study applied the same reaction time for all samples, only the branch chain length was the primary determining factor for digestibility of different starches.

Comparing the digestibility during initial 10 min, starch with longer chains showed enhanced digestion resistance, showing the decrease in the degree of digestion values in the order of 65.7%>49.7%>41.3%>36.0%, respectively, from AM×AS0 to AM×AS8. AM×AS0 was the most vulnerable to digestion, showing the highest degrees of digestion at both initial and final stages. AM×AS0 reached a plateau after 30 min of digestion, and the observed C_∞ (maximum degree of hydrolysis) was approximately 70%. The other starches took relatively longer time to reach their respective undigestive phase. AM×AS2, AM×AS4, and AM×AS8 showed no significant (p>0.05) increase in degree of hydrolysis from 90 min, 150 min, and 150 min, respectively. The amount of RS fraction showed an increase in an amylopectin branch chain length dependent manner.

The digestion curves of starch samples were expressed in the form of LOS plots (Figure 4.2). All recrystallized starches revealed two distinct lines during their digestion, proving that their digestible part consisted of two different fractions having different affinities to digestive enzyme. The former stage with high digestion rate was classified as the digestion phase for RDS, and the latter phase with rather slow rate was corresponded to digestion of SDS. The *in vitro* digestibility parameters such as rate constants and amount of each fraction of starch samples are presented in Table 4.2.

AM×AS0 and AM×AS2 had similar duration for RDS digestion (7.9 min and 7.8 min, respectively) where the amount of RDS was much lower in AM×AS2 (65.4% and 48.9%, respectively). In other words, during similar time, the smaller amount of starch was converted into glucose under the same hydrolysis condition in AM×AS2. AM×AS4 and AM×AS8 required longer time to digest the smaller amount of RDS fraction. It indicated that not only the amount of RDS decreased, but also the structure of RDS became less vulnerable to digestion by elongation of amylopectin branch chains. This was supported by the decreased rate constant for RDS phase (k_{RDS}). A similar decrease was observed in k_{SDS} (rate constant for SDS). Edwards et al. (2014) found the differences in rate constant depending on the particle size of flour during their digestion. Patel et al. (2014) stated that k is

strongly related to the degree of order of the α-glucan chains. Therefore, differences in k_{RDS} and k_{SDS} among samples indicated the differences among RDS and SDS fractions, respectively.

It could be said that in general, under the presence of the same structure and amount of amylose, the longer amylopectin chains induce more low-digestibility of starch. However, long chains did not always contribute to the formation of SDS. An interesting changing pattern was observed in the amount of SDS fraction. In the presence of amylose, a bell-shaped pattern was revealed, and the highest SDS proportion was obtained from the AM×AS4, not in AM×AS8. SDS increased from AM×AS0 to AM×AS4 (4.6%-23.7%), and then decreased in AM×AS8 (16.4%). In the AM×AS8 starch with the longest amylopectin chain length, the formation of RS was promoted rather than that of SDS. In contrast, AS-modified waxy potato starches showed an increase in SDS proportion according to the amount of AS employed increased (data not shown). Therefore, it could be drawn that the presence of amylose plays a specific role on the formation of SDS: a specific amylopectin chain length is required to induce a proper degree of interaction with amylose for maximizing SDS content.

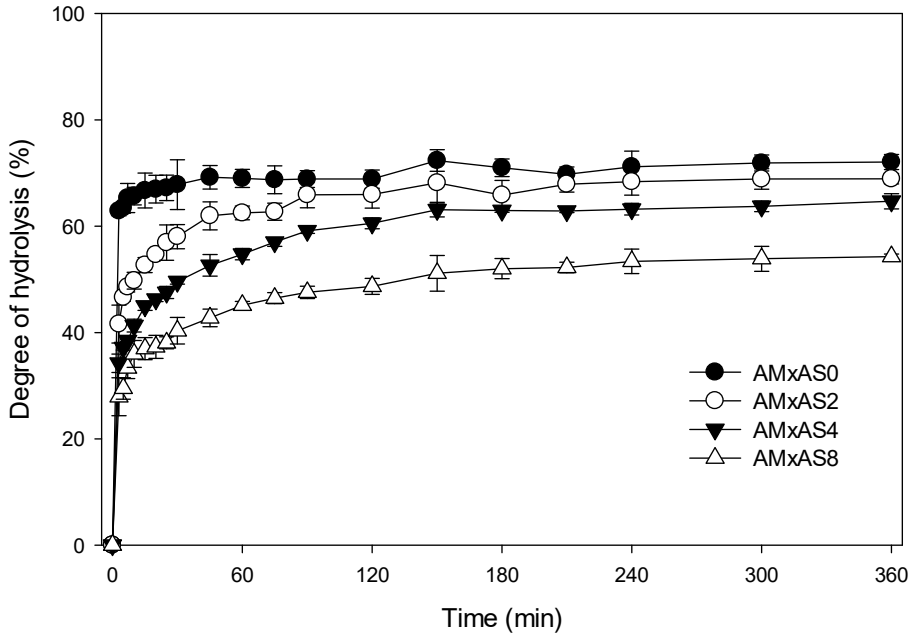
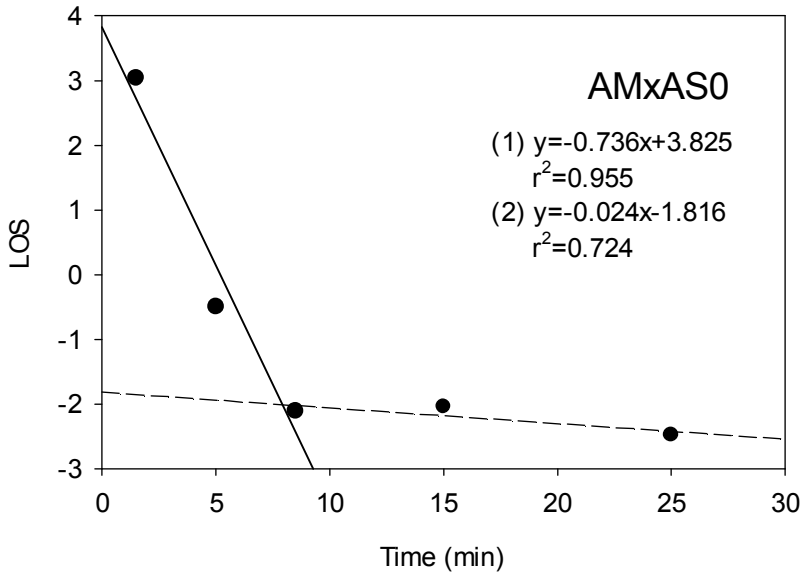
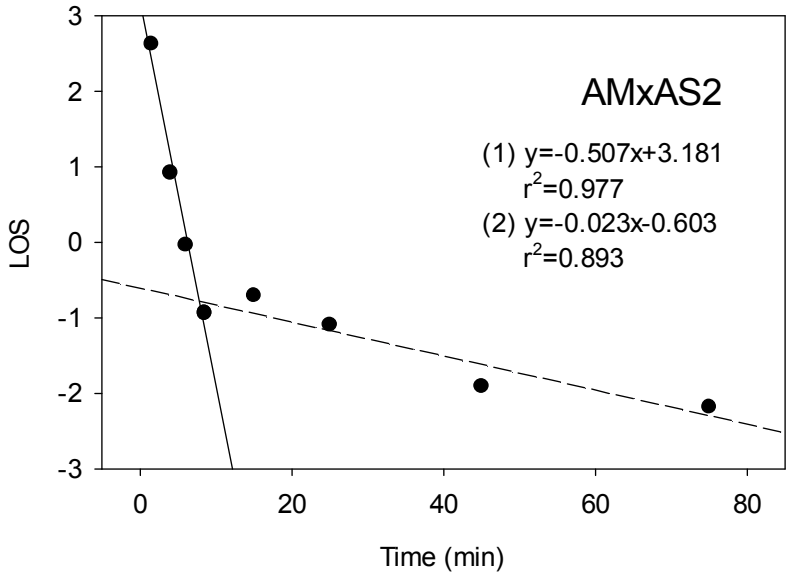


Figure 4.1. Digestion profiles of starch samples during 360 min of digestion. AM×AS0, mixture of amylose and control starch without enzyme; AS2, starch modified with 5,000 U amylosucrase/30 mL-starch suspension; AS4, amylosucrase 10,000 U/30 mL; AS8, amylosucrase 20,000 U/30 mL.

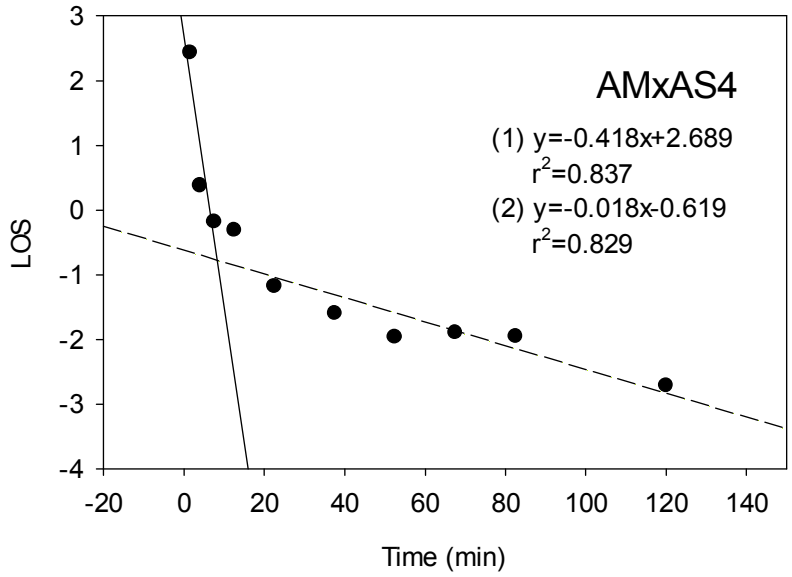
(A)


AMxAS0
(1) y=-0.736x+3.825
r2=0.955
(2) y=-0.024x-1.816
r2=0.724
LOS
Time (min)
4
3
2
1
0
-1
-2
-3
0
5
10
15
20
25
30

(B)


AMxAS2
(1) y=-0.507x+3.181
r2=0.977
(2) y=-0.023x-0.603
r2=0.893
LOS
Time (min)
3
2
1
0
-1
-2
-3
0
20
40
60
80

(C)



(D)

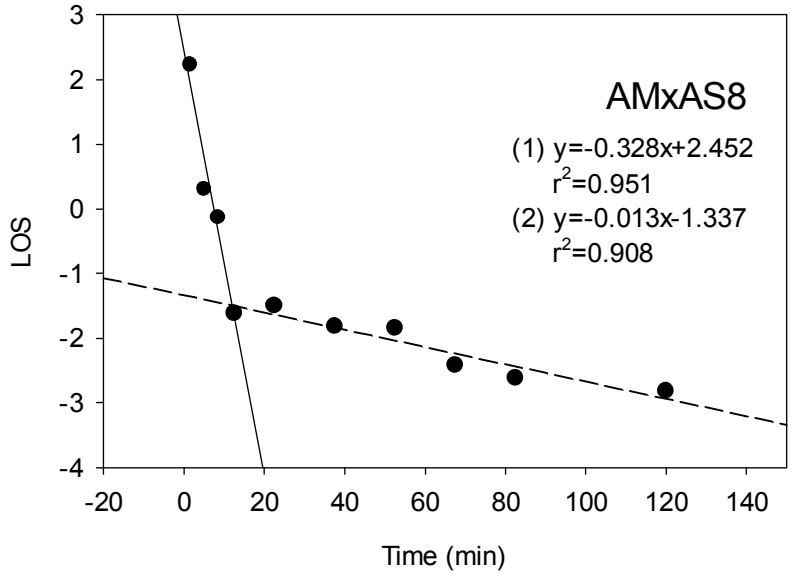


Figure 4.2. Log of slope (LOS) plots of co-crystallized starch samples. AMxAS0, mixture of amylose and control starch without enzyme; AS2, starch modified with 5,000 U amylosucrase/30 mL-starch suspension; AS4, amylosucrase 10,000 U/30 mL; AS8, amylosucrase 20,000 U/30 mL. Solid line indicates rapid digestion phase, and broken line indicates slow digestion phase.

Table 4.2. *In vitro* digestibility parameters of starch samples

Sample	RDS (%)	k_{RDS} (min^{-1})	RDS / SDS breakpoint (min)	SDS (%)	k_{SDS} (min^{-1})	SDS / RS breakpoint (min)	RS (%)
AM×AS0	65.4	0.736	7.9	4.64	0.024	30	29.9
AM×AS2	48.9	0.507	7.8	18.6	0.023	90	32.5
AM×AS4	39.7	0.418	8.3	23.7	0.018	150	36.6
AM×AS8	36.4	0.328	12.1	16.4	0.013	150	47.2

AM×AS0, mixture of amylose and control starch without enzyme; AS2, starch modified h with 5,000 U amylosucrase/ 30 mL-starch suspension; 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; k_{RDS}, rate constant for the RDS phase of starch hydrolysis; k_{SDS}, rate constant for the SDS phase of starch hydrolysis.

4.3.3. Iodine binding properties

Long linear glycosidic chains like amylose form single-helical complexes with iodine to give a blue color, whereas amylopectin appears brown color by the response to iodine addition (Conde-Petit, Nuessli, Handschin, & Escher, 1998). The development of the blue color indicates an increase in the iodine binding capacity (Jane et al., 1999). To follow the changes related to amylose, iodine binding properties of starch samples were compared before and after the removal of RDS and SDS fractions. The absorbance spectrum of 100% amylose and amylopectin (waxy potato starch) was also provided for comparison (Figure 4.3E). As all starch samples contained 25% (w/w) amylose, they revealed the typical spectrum of amylose-containing starch. AM×AS8 exhibited the spectrum closest to that of amylose, indicating its abundance of amylose-like single helical chains.

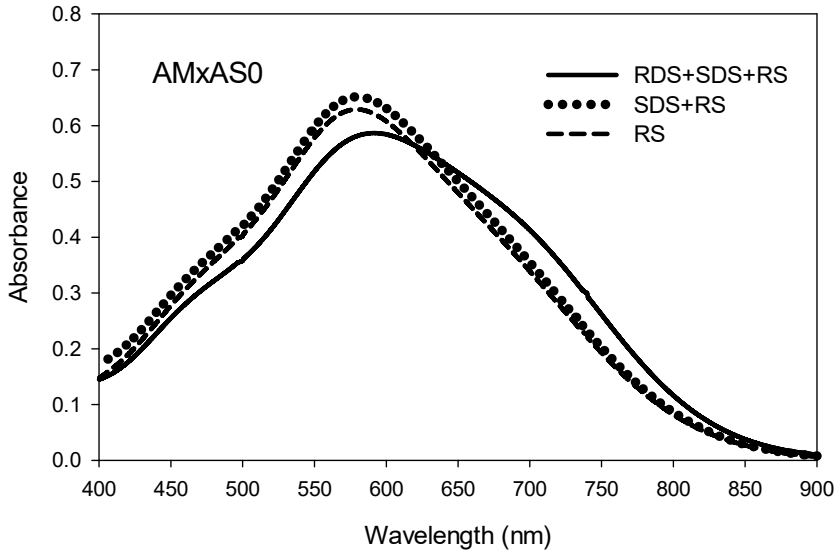
The spectrum of AM×AS0 hardly changed after the removal of its RDS and/or SDS fraction (Figure 4.3A). All three states were similar in shape of absorbance spectrum with λ_{max} at 550-600 nm range. This minor change indicates that there are enough numbers of single helix chains which can bind with iodine in its isolated SDS+RS and RS fractions. It can be easily found that the iodine-binding chains would never be originated from amylopectin, as the iodine did not bind even with the original state of

amylopectin chains in AM×AS0 (it had an apparent amylose content of only 26.17%, corresponding to the amount of amylose added). Therefore, the remaining chains after removal of RDS and/or SDS should be single amylose helices. One possible hypothesis from this result was that amylose molecules rarely contributed to the structure of RDS and SDS of AM×AS0, therefore remaining in the RS. The crystalline structure comprised of amylose double helices is very resistant to hydrolysis, and thereby considered as resistant starch (Sajilata et al., 2006). However, because the amylose molecules should have single helix conformation to bind with any ligand, this hypothesis could be neglected. The other possibility is that amylose chains still have long enough single helices to bind with iodine in spite of digestion, and therefore they could have similar iodine binding properties with their original counterpart. The DP of amylose from potato starch is reported to be 4,920 (Takeda et al., 1984), which is quite long. Long single helical chains derived from amylose remaining in SDS+RS or RS fraction would contribute to maintaining iodine binding properties.

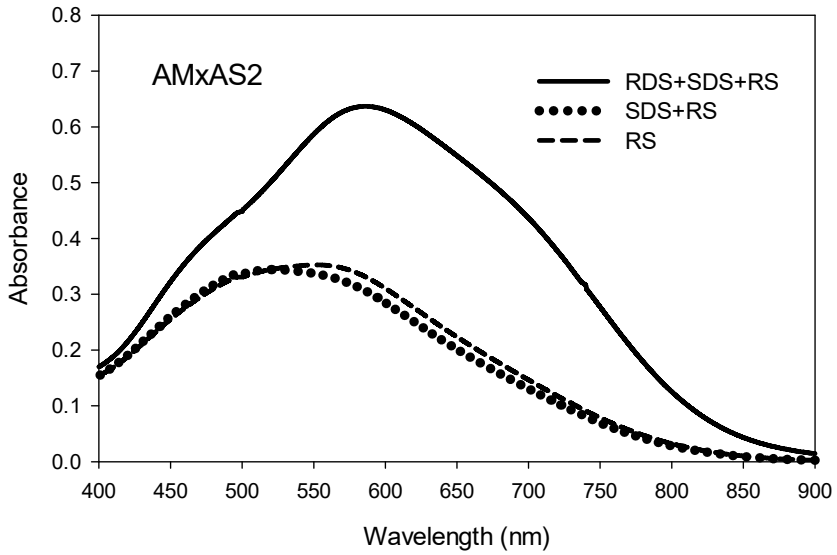
All the other starches exhibited spectra close to amylopectin rather than amylose, after the removal of RDS (Figure 4.3B-D). In detail, the absorbance noticeably decreased, and their λ_{max} values were shifted to lower wavelength. It indicated the removal of single chains that are able to incorporate iodine,

regardless of their origin, either amylose or elongated amylopectin branch chains. The SDS+RS and RS spectra from AM×AS2 were very similar to each other, whereas AM×AS4 and AM×AS8 showed additional diminution in RS spectrum after the removal of SDS fraction.

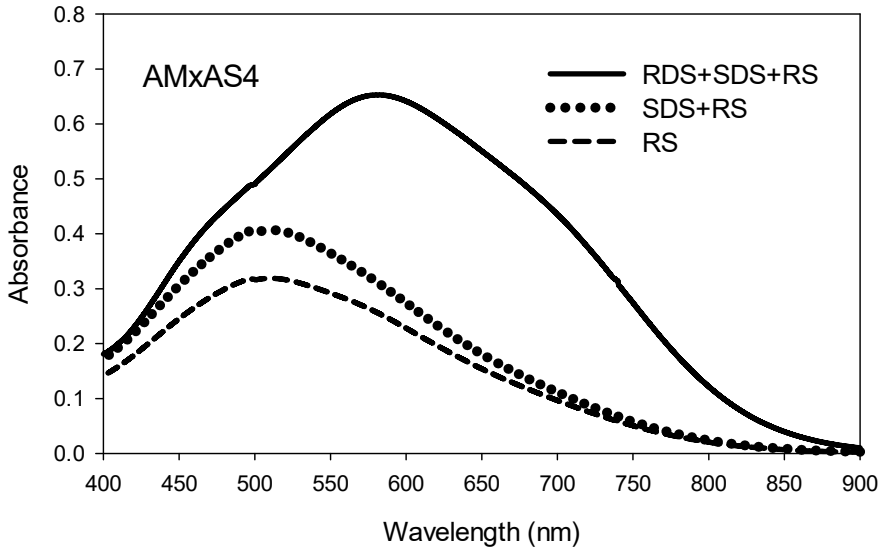
(A)


AMxAS0
RDS+SDS+RS
SDS+RS
RS
Absorbance
Wavelength (nm)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
400
450
500
550
600
650
700
750
800
850
900

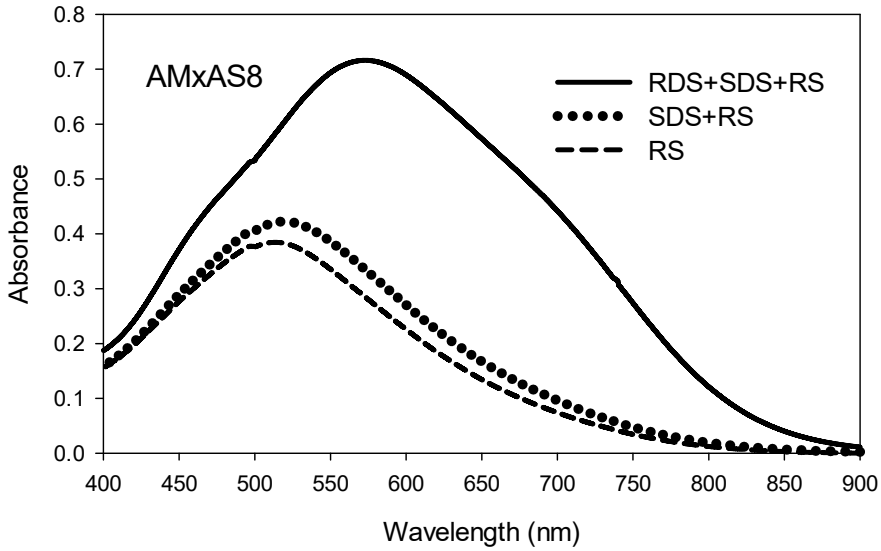
(B)


AMxAS2
RDS+SDS+RS
SDS+RS
RS
Absorbance
Wavelength (nm)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
400
450
500
550
600
650
700
750
800
850
900

(C)


AMxAS4
RDS+SDS+RS
SDS+RS
RS
Absorbance
Wavelength (nm)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
400
450
500
550
600
650
700
750
800
850
900

(D)


AMxAS8
RDS+SDS+RS
SDS+RS
RS
Absorbance
Wavelength (nm)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
400
450
500
550
600
650
700
750
800
850
900

(E)

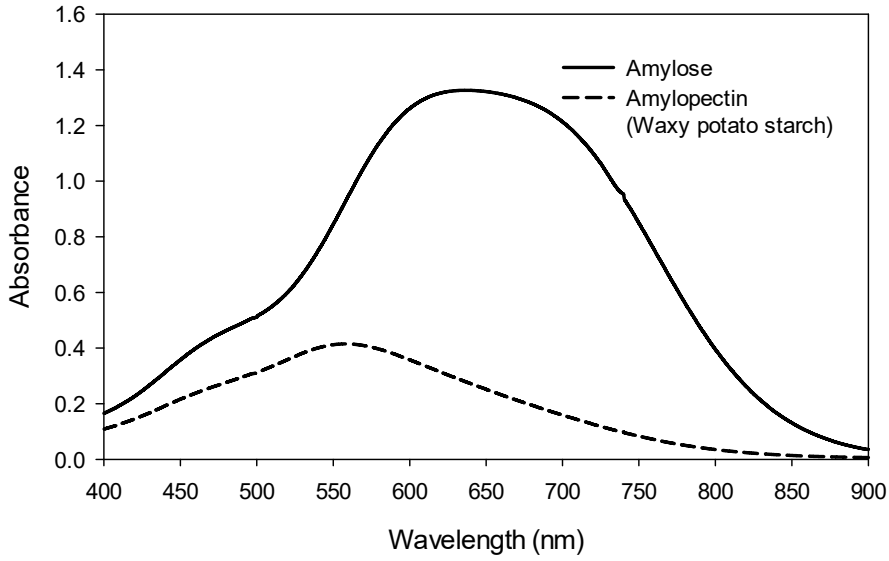


Figure 4.3. Absorbance spectrum of samples through 400-900 nm after iodine binding reaction. AM×AS0, mixture of amylose and control starch without enzyme; AS2, starch modified with 5,000 U amylosucrase/30 mL-starch suspension; AS4, amylosucrase 10,000 U/30 mL; AS8, amylosucrase 20,000 U/30 mL; RDS, rapidly digestible starch; SDS, slowly digestible starch; RS, resistant starch.

4.3.4. Changes in chain length distribution after the removal of RDS and/or SDS fraction

The chain length distributions of starch samples before and after the removal of the RDS and/or SDS fraction are presented in Table 4.3 and Figure 4.4. The detailed change in each chain is described in Figure 4.5.

With the removal of RDS, AM×AS0 exhibited a noticeable decrease in short chains and an increase in long chains. Because no chain elongation reaction occurred during digestion of RDS, it could be thought that this change was not from the loss of absolute amount of short chains. Though the long branched chains were degraded into shorter branch chains, it would not be critical enough to affect the whole proportion. Instead, it could be conjectured that the relative increase of long branched chains induced the relative decrease in the proportion of short chains. This increase of long chain seems to be the result of hydrolysis of amylose. Therefore, amylose seemed to be the main constituent of the RDS fraction of AM×AS0, as mentioned above. The removal of SDS induced a decrease of chains with DP $\leq$ 27, and increases in the rest of the chains. This change could be mainly be caused by the degradation of remaining long chains originated from amylose.

AM×AS2 showed a considerably different changing pattern according to RDS removal as compared with AM×AS0. The proportion of long chains

corresponding to DP 24-37 decreased, whereas very long chains of DP ≥ 38 hardly changed. Some short chains with DP 8-12 also decreased. The amylose in AM×AS2 would also be degraded, same as in AM×AS0, however, the contribution to the increase in long chains was not significantly observed. The exposed free amylose chains might compose amorphous structure (Jenkins & Donald, 1995) to be RDS. Regarding this phenomenon, AM×AS0 would have more and longer exposed free amylose chains compared with AM×AS2, since the length of its double helices formed between amylose and amylopectin is rather short. As a result, the severed amylose chains might be rather short in AM×AS2. The hydrolysates from amylose and long amylopectin external chains might contribute to the increase of chains with DP 13-24 and some long chains.

Hydrolysis of RDS fraction in AM×AS4 and AM×AS8 resulted in a similar change each other, where the proportion of branch chains longer than DP 22 and DP 23 decreased respectively, whereas that of the shorter chains increased. Therefore, their RDS fraction would be related to long chains, which were derived from amylose or amylopectin. Considering amylose binding properties of the undigested AM×AS4 and AM×AS8, they had enough amounts of singular helix chains. As aforementioned, they are easily attacked by digestion enzymes, and therefore the structure can be reasonably

considered as a contributor to RDS structure. The degree of change was dominant in DP 24-36, as observed in AM×AS2.

As a result of elimination of SDS fraction, AM×AS2 distinctively showed a decrease in medium-length chains with DP 16-23. Chains longer than DP 24 increased, by the result of the hydrolysis of remaining amylose or relative effect from the decrease in other chains. Considering the iodine binding property of AM×AS2 SDS+RS fraction, the existence of single helical chains with considerable length was doubtful. Therefore, the mid-length chains would positively contribute to the SDS structure of AM×AS2. In fact, its SDS+RS fraction contained chains with DP 13-24 which exceeded the half (~55%) of all chains. Decreases in short chains (DP 6-12) and long chains (DP 23-37) were observed according to the removal of SDS fraction in AM×AS4, and therefore these chains would be the major component of its SDS. The increase of medium length chains (DP 13-22 in AM×AS4) seems to be related with the hydrolysis of long chains. In case of AM×AS8, the proportions of all the long chains over DP 20 decreased, whereas the proportion of medium chains with DP 12-20 increased. SDS fraction in this starch was composed of long chains, similar to its RDS, since AM×AS8 originally had very low amount of short chains and therefore long chains should be involved in building its overall structure.

The chain length distribution of isolated RS fraction among recrystallized starch prepared using AS-modified starch (AM×AS2-AM×AS8) was quite similar in all samples. The RS fractions had an average chain length of approximately DP 23 with the highest proportion of medium chains with DP 13-24. Previous research has reported that isolated RS from recrystallized starches of different origin consist of chains with average DP 19-26 (Eerlingen et al., 1993).

In spite of hydrolysis of RDS and SDS fractions, the RS fraction of AS-modified starches had increased proportion of medium length chains compared with their undigested counterpart, instead of an increase in short 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 hydrolyzed by digestive enzymes. In other words, a considerable amount of medium chains remained intact despite the digestion process, because most of the medium chains were buried in the interior of the crystalline structure. 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 the 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. Regardless of the origin of the chains, abundance of double helices having certain length and the way of their packing seems to be related with the low-digestible fraction of AS-modified starches. By investigating the chain length distributions of SDS+RS and RS fractions, it was found that the medium chains (DP 13-24) were the key constituents of the crystalline structure related to the SDS and RS fractions of AS-modified starch.

It was clear that the chain length distribution of original starch primarily decides the low digestibility of starch. The problem was to determine the factor that decides the enzyme resistant double helices (mainly DP 13-24) to be SDS or RS. Supposing the space of same volume for all starch samples, AM×AS8 should arrange longer chains in the limited space. The highly elongated external branch chains of AM×AS8 readily form double helices with amylose chains, therefore the amylose chains has a limited degree of freedom. The double helices would be packed with high density and the tight layers of double helices prefer to be RS rather than SDS. In case of AM×AS4, the part of ordered structure would be ordered into highly digestion-resistant structure primarily, and the rest would be organized as less dense form. Amylose molecules would be involved in the formation of SDS, containing partial amorphous and partial crystalline structure, because amylose may act

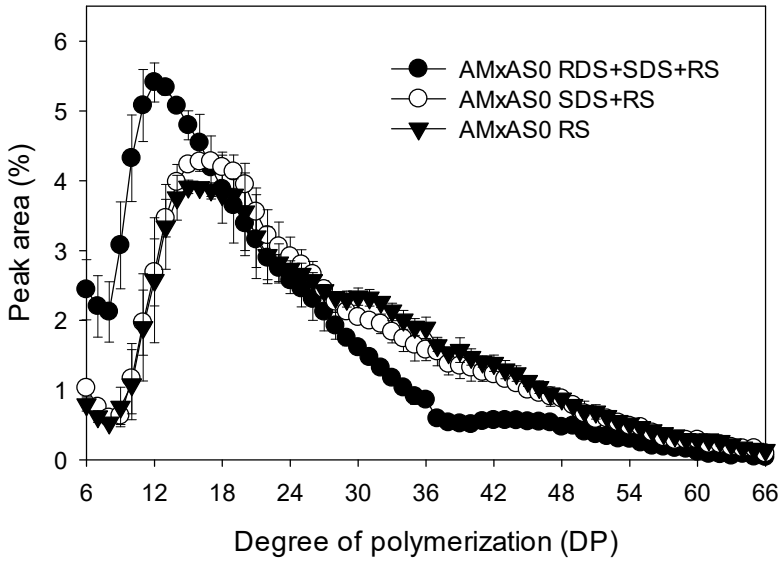
as a diluent that intrudes on the interaction of amylopectin double helices, preventing their development into perfect crystals (Jenkins & Donald, 1995). The action of amylose as a diluent would be the same for the structure formation of AM×AS0 and AM×AS2, interrupting tight packing of double helices. Though these two starches had large amounts of DP 13-24 chains in their original state, they lacked longer chains whereas space for arrangement of double helical chains would be rather sufficient. These characteristics would lead to the production of double helix layers of which the amount and the density were not enough to achieve sufficient SDS and RS compared with AM×AS4 and AM×AS8.

Table 4.3. Comparison of percent distribution of branch chain length of starch samples before and after the removal of RDS and/or SDS fraction

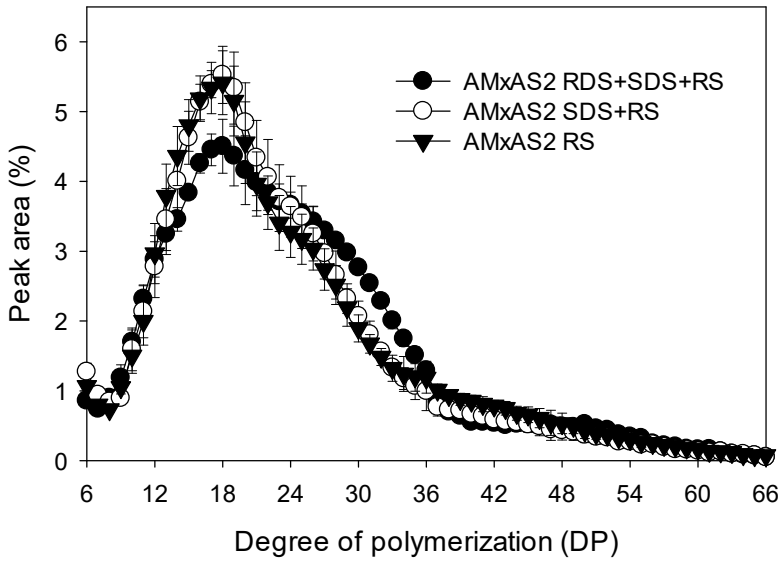
Sample		Percent distribution (%)				
		DP 6-12	DP 13-24	DP 25-36	DP ≥ 37	average DP
AM×AS0	RDS+SDS+RS	24.6±2.4^{a}	46.0±2.6^{d}	18.9±0.5^{g}	10.5±0.9^{cde}	20.9±0.1^{d}
	SDS+RS	8.8±1.4^{bcd}	45.2±2.1^{d}	25.1±0.6^{ef}	20.8±2.5^{a}	26.2±0.9^{b}
	RS	8.3±2.2b^{cd}	41.6±1.3^{e}	27.2±0.6^{def}	22.9±1.5^{a}	27.2±0.6^{a}
AM×AS2	RDS+SDS+RS	10.9±0.9^{b}	48.0±1.8^{cd}	29.7±1.5^{cd}	11.4±0.7^{cd}	24.1±0.3^{c}
	SDS+RS	10.5±1.3^{b}	54.1±2.6^{ab}	24.7±1.9^{ef}	10.7±2.7^{cde}	23.1±0.6^{c}
	RS	10.1±1.2^{bc}	52.9±3.3^{ab}	23.7±1.9^{f}	13.3±2.2^{c}	23.7±0.7^{c}
AM×AS4	RDS+SDS+RS	7.5±1.2^{cd}	45.7±1.0^{d}	34.2±1.6^{b}	12.6±1.0^{c}	25.3±0.2^{b}
	SDS+RS	9.3±1.1^{bcd}	53.1±1.3^{ab}	29.3±2.4^{cd}	8.3±1.1^{e}	23.1±0.5^{c}
	RS	7.2±1.6^{d}	55.7±2.0^{a}	27.6±3.0^{de}	9.5±0.8^{de}	23.4±0.8^{c}
AM×AS8	RDS+SDS+RS	4.2±0.8^{e}	37.1±1.3^{f}	40.8±2.3^{a}	18.0±1.7^{b}	27.9±0.4^{a}
	SDS+RS	8.5±1.5^{bcd}	48.0±0.7^{cd}	33.9±2.6^{b}	9.6±0.9^{de}	24.1±0.3^{c}
	RS	7.7±0.8^{cd}	51.4±1.8^{bc}	32.4±1.8^{bc}	8.5±0.5^{de}	23.6±0.4^{c}

AM×AS0, mixture of amylose and control without enzyme; AS2, starch modified with 5,000 U amylosucrase/30 mL-starch suspension; AS4, amylosucrase 10,000 U/30 mL; AS8, amylosucrase 20,000 U/30 mL; DP, degree of polymerization; RDS, rapidly digestible starch; SDS, slowly digestible starch; RS, resistant starch. The values with different superscripts in the same column are significantly different (p<0.05).

(A)


AMxAS0 RDS+SDS+RS
AMxAS0 SDS+RS
AMxAS0 RS
Peak area (%)
Degree of polymerization (DP)
0
1
2
3
4
5
6
6
12
18
24
30
36
42
48
54
60
66

(B)


AMxAS2 RDS+SDS+RS
AMxAS2 SDS+RS
AMxAS2 RS
Peak area (%)
Degree of polymerization (DP)
0
1
2
3
4
5
6
6
12
18
24
30
36
42
48
54
60
66

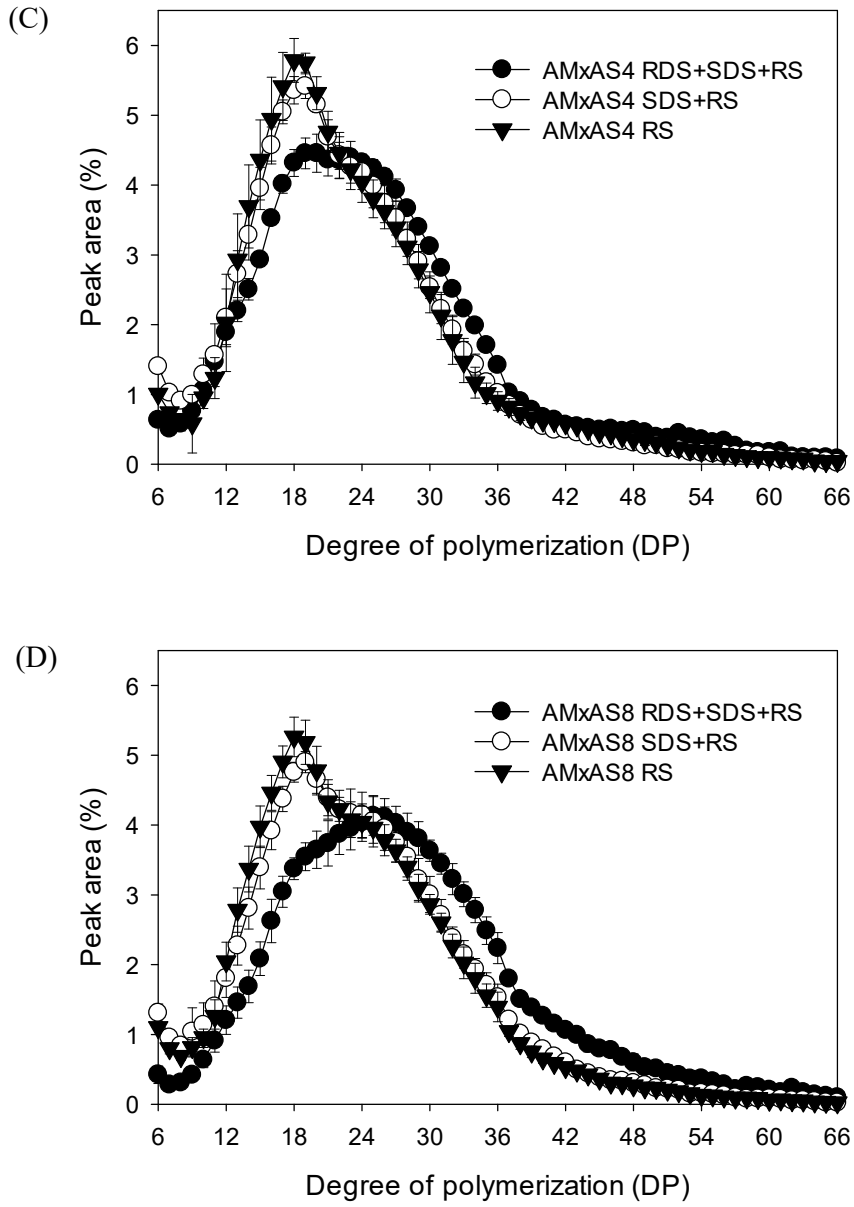
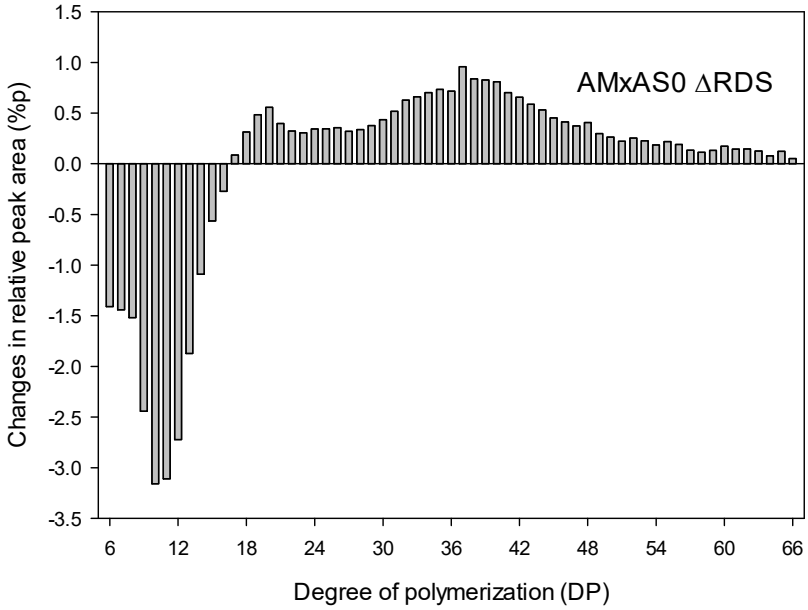
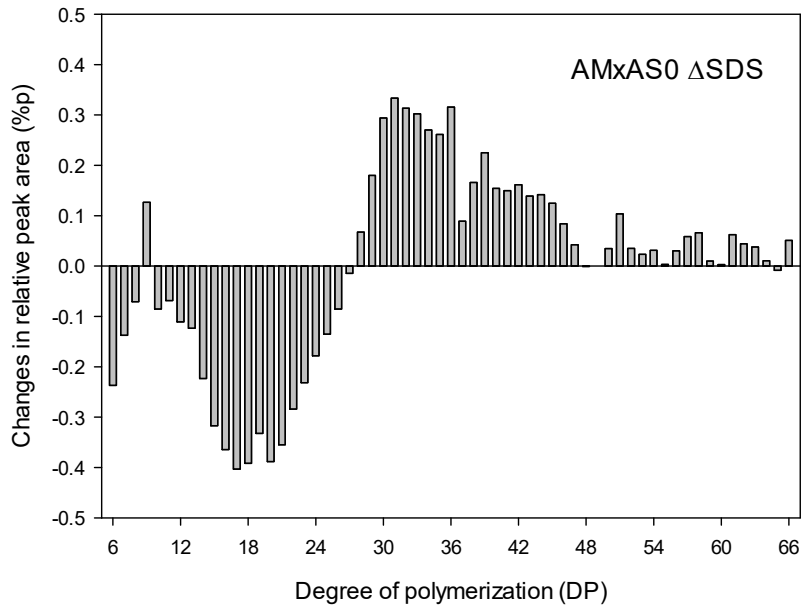


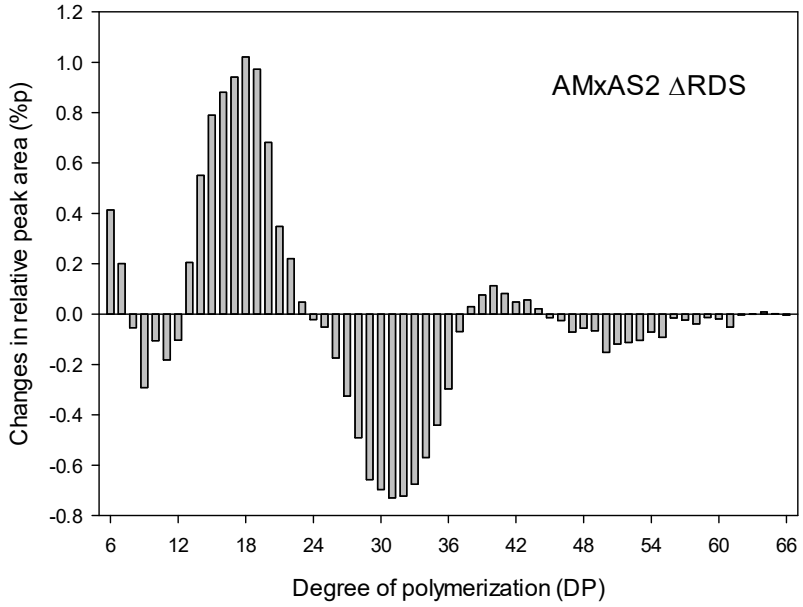
Figure 4.3. The branch chain length distributions of starch samples determined before and after the removal of RDS and/or SDS fraction. AM×AS0, mixture of amylose and control without enzyme; AS2, starch modified with 5,000 U amylosucrase/30 mL-starch suspension; AS4, amylosucrase 10,000 U/30 mL; AS8, amylosucrase 20,000 U/30 mL; RDS, rapidly digestible starch; SDS, slowly digestible starch; RS, resistant starch.

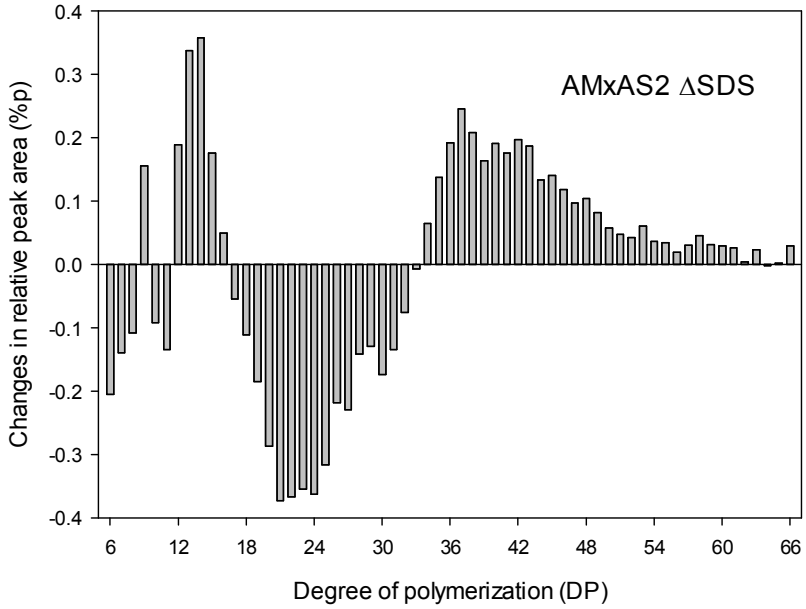
(A)


AMxAS0 ΔRDS
Changes in relative peak area (%p)
Degree of polymerization (DP)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
6
12
18
24
30
36
42
48
54
60
66

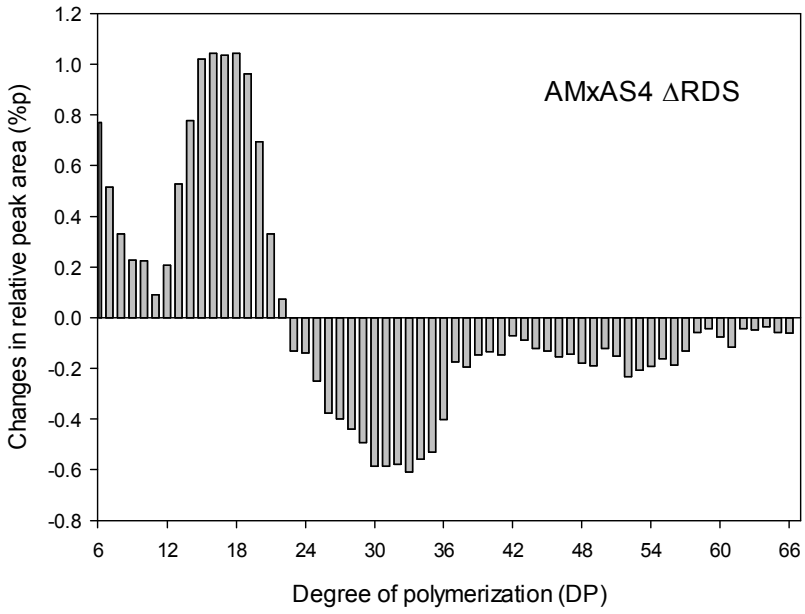

AMxAS0 ΔSDS
Changes in relative peak area (%p)
Degree of polymerization (DP)
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
-0.5
6
12
18
24
30
36
42
48
54
60
66

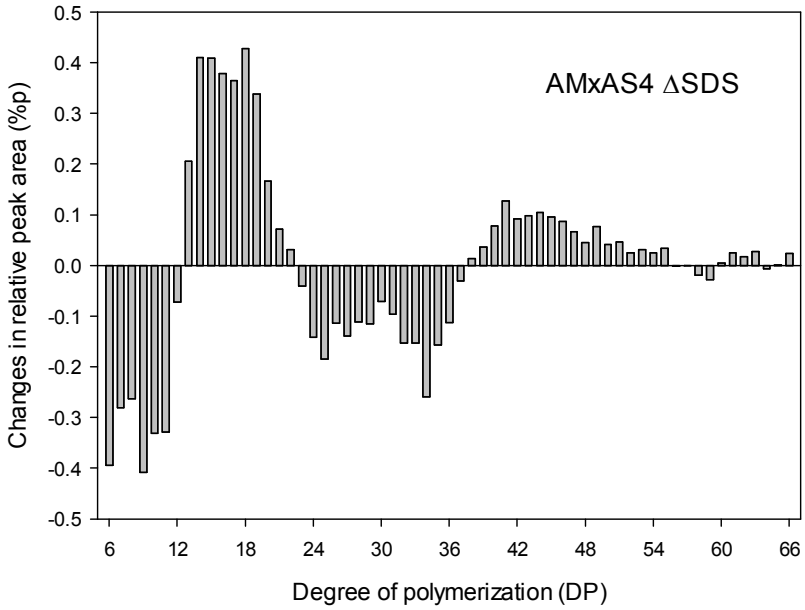
(B)


AMxAS2 ΔRDS
Changes in relative peak area (%p)
Degree of polymerization (DP)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
6
12
18
24
30
36
42
48
54
60
66


AMxAS2 ΔSDS
Changes in relative peak area (%p)
Degree of polymerization (DP)
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
6
12
18
24
30
36
42
48
54
60
66

(C)


AMxAS4 ΔRDS
Changes in relative peak area (%p)
Degree of polymerization (DP)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
6
12
18
24
30
36
42
48
54
60
66


AMxAS4 ΔSDS
Changes in relative peak area (%p)
Degree of polymerization (DP)
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
-0.5
6
12
18
24
30
36
42
48
54
60
66

(D)

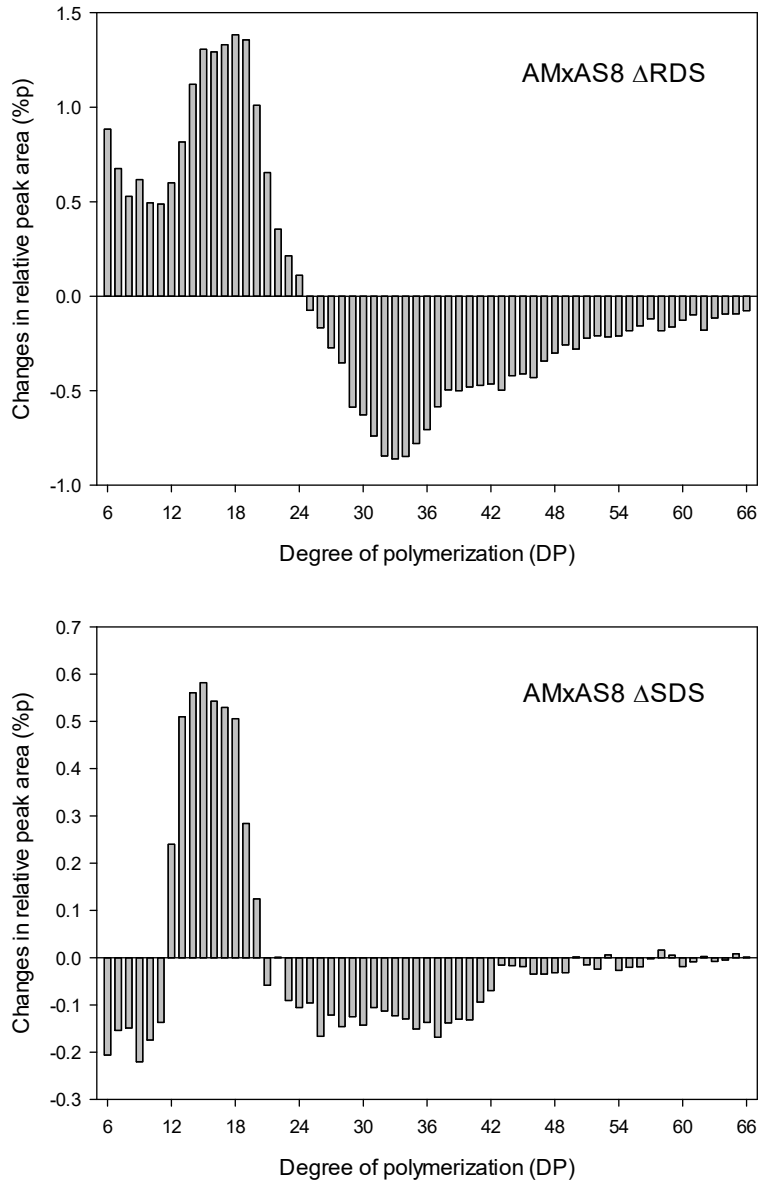


Figure 4.4. Changes in the branch chain length distributions of starch samples. AM×AS0, mixture of amylose and control without enzyme; AS2, starch modified with 5,000 U amylosucrase/30 mL-starch suspension; AS4, amylose crase 10,000 U/30 mL; AS8, amylosucrase 20,000 U/30 mL; DP, degree of polymerization; ΔRDS, removal of rapidly digestible starch; ΔSDS, removal of slowly digestible starch.

4.3.5. X-ray diffraction and relative crystallinity of each fraction

As stated above, all starch samples and their hydrolysates revealed X-ray diffraction peaks corresponding to B-type (Figure 4.5). Regardless of botanical source, AS-modified starches were reported to have B-type crystallinity (Kim et al., 2013; Shin et al., 2010). Moreover, the method for recrystallization of AS-modified starches and amylose was retrogradation, and starches show a B-type crystalline pattern after retrogradation regardless of their original crystalline structure (Eerlingen et al., 1993; Kim, Kim, & Shin, 1997). The aggregation of longer linear chains could accelerate the formation of B-type crystalline structure (Pohu, Planchot, Putaux, Colonna, & Buleon, 2004a), and the chain elongation by AS could also contribute to it. The branches of amylopectin in AS-modified starches, which contain an amylose-like long linear structure, might reassociate with amylose or each other and organize into a crystalline structure more easily than those of unextended starches. Supporting these ideas, the starch modified with more amount of AS had higher relative crystallinity when recrystallized with amylose (Table 4.4).

Miao et al. (2009a) reported that the relative crystallinity is influenced by average amylopectin chain length, crystal size, amylose/amylopectin ratio,

and extent of interaction between double helices. The changes in crystalline peak intensity and relative crystallinity were observed after the removal of RDS and/or SDS fraction of starches.

In AM×AS0, AM×AS4, and AM×AS8, the relative crystallinity was in the order of RDS+SDS+RS<SDS+RS<RS. It was quite reasonable, because RS is mainly consisted of a crystalline region with high perfection and rigidity (Fuentes-Zaragoza et al., 2011). The undigested AM×AS0 had the diffraction peak with the lowest digestibility and unclear peaks among all samples. After digestion of RDS, its crystallinity significantly (p<0.05) increased with clearer and sharper crystalline peaks. Ensuing SDS removal also induced a significant (p<0.05) increase of the crystallinity. This result indicated that its RDS and SDS would have rather amorphous structure, and remaining crystalline structure could be revealed after removal of them. Both AM×AS4 and AM×AS8 showed significant increases in crystallinity with RDS removal, corresponding to the aforementioned hypothesis that their RDS is composed of amylose and amylose-like single amylopectin chains, and therefore amorphous. SDS+RS and RS of two starches had very similar peak intensity, with a slight increase of crystallinity. This supported the hypothesis about SDS structure that consists of less perfect crystalline regions containing a small portion of double helices and an amorphous region (Shin

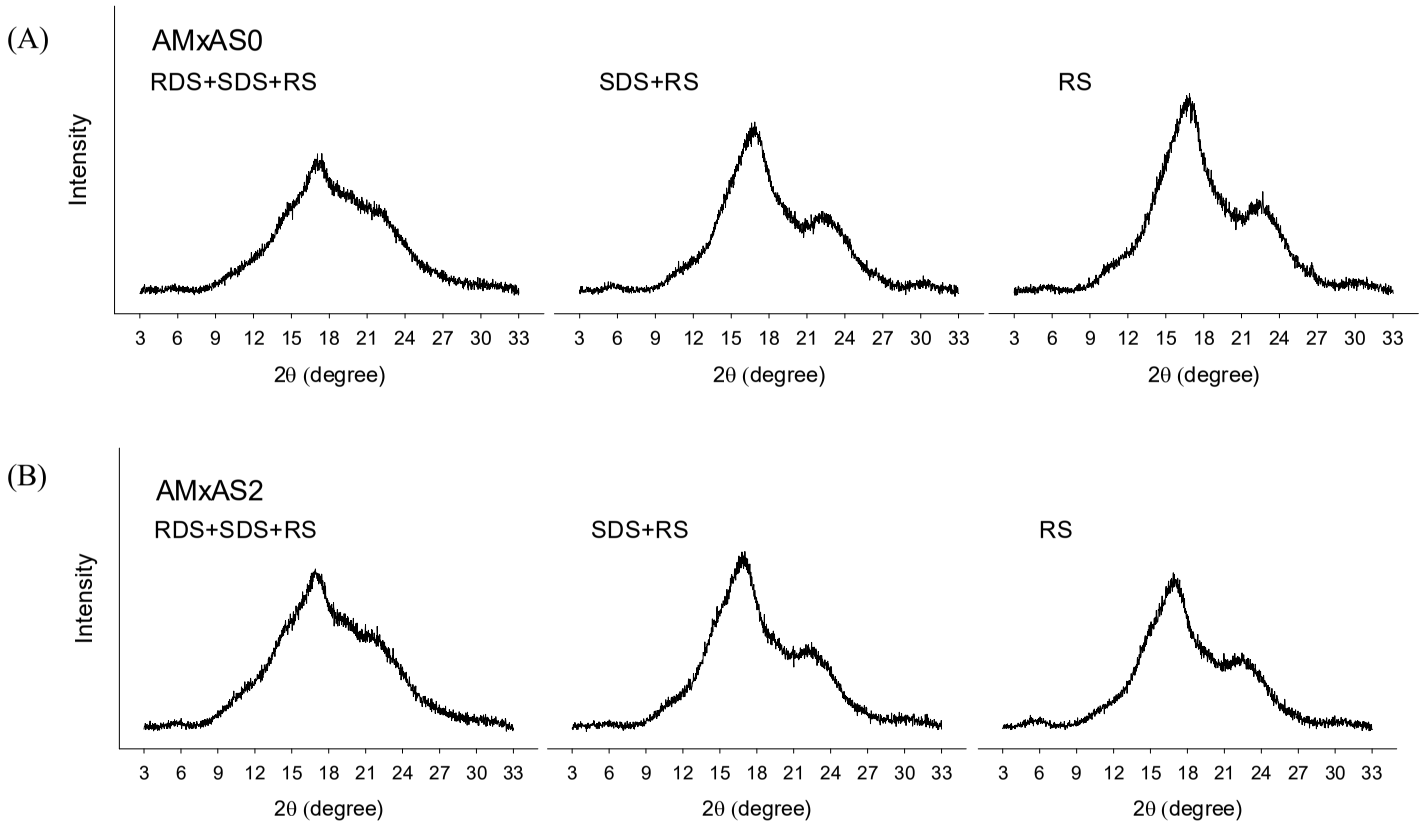
et al., 2004). The different SDS structure in AM×AS0 and AM×AS4/AS8 (amorphous and semi-crystalline) was explained by their digestibility. AM×AS0 with amorphous SDS had higher k_{SDS} where amorphous structure was more vulnerable to hydrolysis.

Meanwhile, AM×AS2 showed an unexpected changing pattern. Though the removal of RDS led to the increase in overall crystallinity, digestion of SDS resulted in the decrease in crystallinity. Considering that AM×AS0 and AM×AS2 showed a big difference in RDS amount in contrast to a small increase in RS, the arrangement of double helices in AM×AS2 would be induced toward the formation of SDS rather than RS. Therefore, its SDS would be more crystalline than AM×AS0, and the hydrolysis of SDS induced the removal of partial crystalline structure, lowering crystallinity. Moreover, in AM×AS0, crystalline structure might be induced to a certain extent between amylose chains present in its RS. However, because AM×AS2 has structural characteristics in between those of AM×AS0 and AM×AS4/AS8, which is not the most appropriate for high degree of crystallization. Therefore, RS of AM×AS2 seemed to have lower crystallinity compared with that of other starches.

Table 4.4. The relative crystallinity of samples and their respective SDS+RS and RS fractions

Sample		Relative crystallinity (%)
AM×AS0	RDS+SDS+RS	15.6 ± 0.5^{i}
	SDS+RS	25.3 ± 0.8^{f}
	RS	29.2 ± 0.8^{de}
AM×AS2	RDS+SDS+RS	19.1 ± 0.5^{h}
	SDS+RS	28.4 ± 1.0^{de}
	RS	23.0 ± 0.3^{g}
AM×AS4	RDS+SDS+RS	24.8 ± 0.8^{f}
	SDS+RS	32.0 ± 0.7^{c}
	RS	33.3 ± 0.9^{c}
AM×AS8	RDS+SDS+RS	27.6 ± 1.1^{e}
	SDS+RS	35.8 ± 1.3^{b}
	RS	37.8 ± 0.3^{a}

AM×AS0, mixture of amylose and AS control without enzyme; AS2, starch modified with 5,000 U amylosucrase/30 mL-starch suspension; 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. The values with different superscripts in the same column are significantly different ($p<0.05$).


(A)
AMxAS0
RDS+SDS+RS
SDS+RS
RS
Intensity
3 6 9 12 15 18 21 24 27 30 33
2θ (degree)
(B)
AMxAS2
RDS+SDS+RS
SDS+RS
RS
Intensity
3 6 9 12 15 18 21 24 27 30 33
2θ (degree)

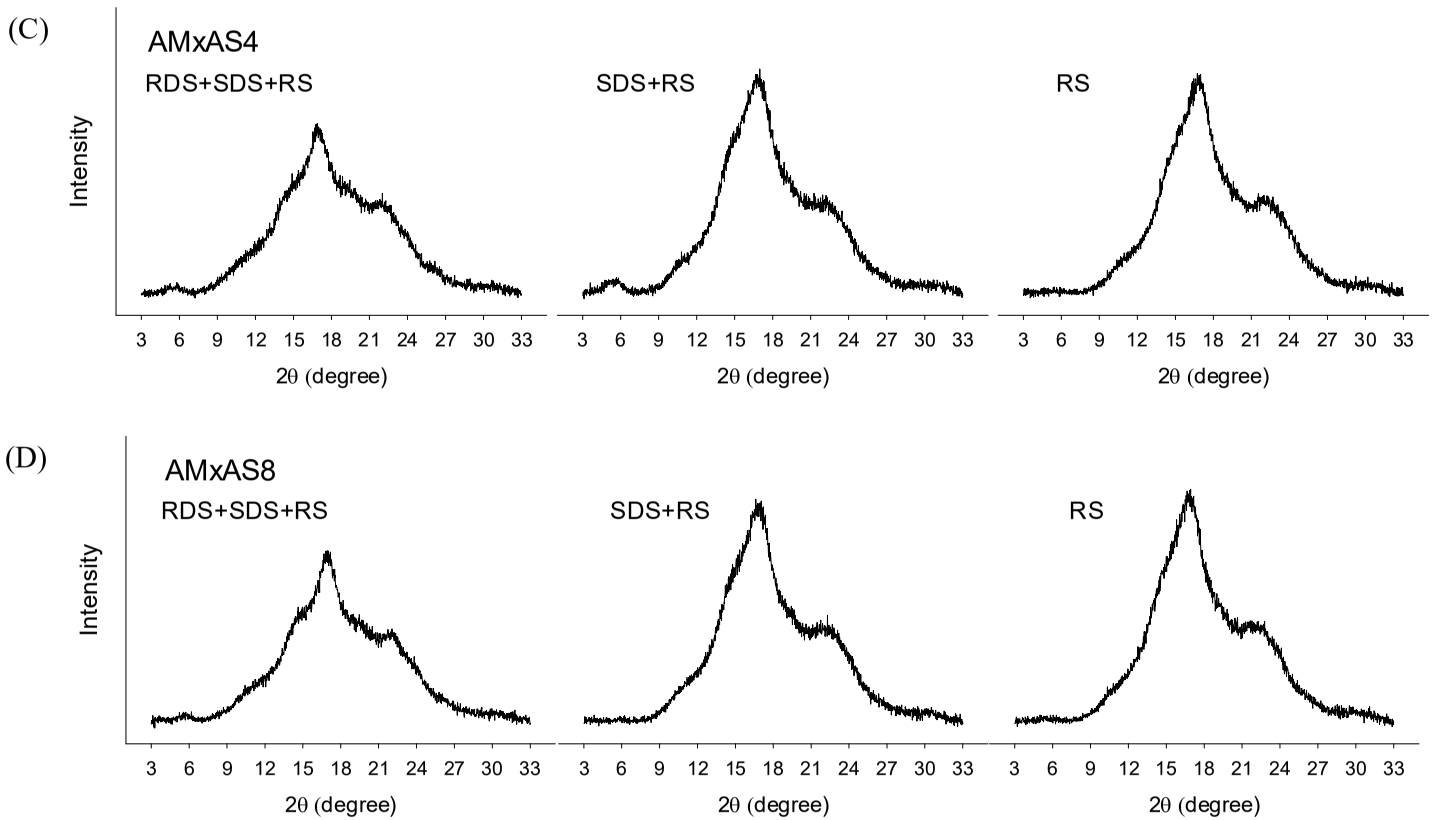


Figure 4.6.X-ray diffractograms of samples. AM×AS0, mixture of amylose and control without enzyme; AS2, starch modified with 5,000 U amylosucrase/30 mL-starch suspension; AS4, amylosucrase 10,000 U/30 mL; AS8, amylosucrase 20,000 U/30 mL; RDS, rapidly digestible starch; SDS, slowly digestible starch; RS, resistant starch.

4.3.6. Thermal properties of each fraction

DSC thermograms of starch samples and their isolated SDS+RS, RS fractions were presented in Figure 4.7. Melting temperature (T_p) were marked for each sample. The T_p indicates the quality (perfection and stability) of the crystallites, and melting temperature range is related to the degree of homogeneity (Biliaderis, 1990). The area under the peak can be considered as ΔH, where it represents the change in enthalpy caused by the melting of double helices and crystallites formed by their interaction and therefore reflects the content of double helices in a starch sample (Lopez-Rubio, Flanagan, Gilbert, & Gidley, 2008).

The starch samples in this study showed a higher melting temperature range (above 150°C) compared with previously reported melting temperatures of AS-modified waxy starches (Kim et al., 2014; Ryu et al., 2010; Shin et al., 2010). The studies reported that double helices formed with amylopectin branched chains of DP 6-24 revealed a melting peak at approximately 80°C. The melting above 140°C observed in starch is usually considered to be involved in amylose-lipid complex (Tufvesson, Wahlgren, & Eliasson, 2003). However, as all starch samples in this study were defatted during preparation, there was no possibility of the formation of lipid complex. Therefore, the presence of amylose caused the formation of a more perfect

and heat stable structure, distinct with amylopectin-only crystal. The co-crystalline structure of amylose and amylopectin requires higher melting energy than their respective crystals (Lian, Cheng, Wang, Zhu, & Wang, 2018). It has been reported that a synergistic interaction occurs between amylose and amylopectin during retrogradation, resulting in a larger endotherm (Zhou et al., 2011). Despite AS0 itself did not reveal any endothermic peak (data not shown), AM×AS0 exhibited a significant melting peak with T_p at 154.10°C. Storing at 4°C for 48 h for crystallization (retrogradation) also would enhance the degree crystallization of starch samples in this study.

Compared with undigested counterparts, SDS+RS fraction of AM×AS0 showed higher melting temperature. The non-stable and easily melting structure was discarded by the removal of RDS. Change in endothermic peak area was minor, indicating both states had a similar amount of double helices. In other words, RDS removal in AM×AS0 did not destroy double helical structure. The broadened thermogram indicated more heterogenous composition of SDS+RS than whole starch because of the random hydrolysis of starch by the digestion enzymes (α-amylase). A drastic change in chain length distribution (Table 4.2) also supported the broadening of thermogram after the RDS removal. Differences in melting temperature range of SDS+RS

and RS of AM×AS0 were not noticeable with a negligible shift in T_r and T_p.

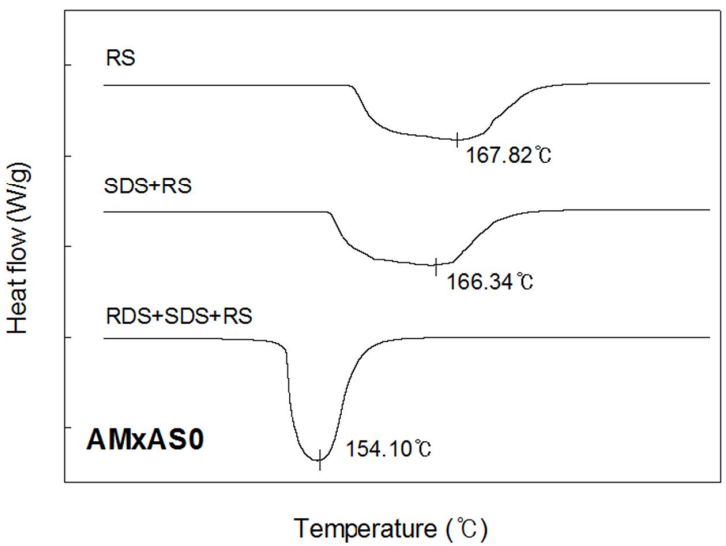
AM×AS2 had a minor change in melting temperature compared with other starches according to the hydrolysis of digestible fractions. Its melting peak area increased in the order of RDS+SDS+RS < SDS+RS < RS. With the removal of SDS, its crystallinity decreased whereas the melting peak increased. Therefore, the SDS structure had mixed structure of crystalline double helices and non-crystalline double helices indicating semi-crystalline structure.

Smaller endothermic peak was observed after the removal of RDS in both AM×AS4 and AM×AS8. Presumably, singular amylose and amylose-like chains were the major elements of their RDS structure on the basis of iodine binding properties. However, it could be found that double helices not organized into crystalline structure contributed to formation of their RDS. Because the double helices were not crystalline enough to form SDS or RS, the assumption that their RDS has amorphous structure was still valid. AM×AS4 SDS+RS had the lowest melting temperature and melting enthalpy among three states of the sample. As its SDS was thought to have rather loose packing of double helices with DP 13-24, its homogenous melting peak with earlier emergence of T_p and T_c than undigested starch was quite persuasive. Since the ΔH is related to the melting of imperfect amylopectin-

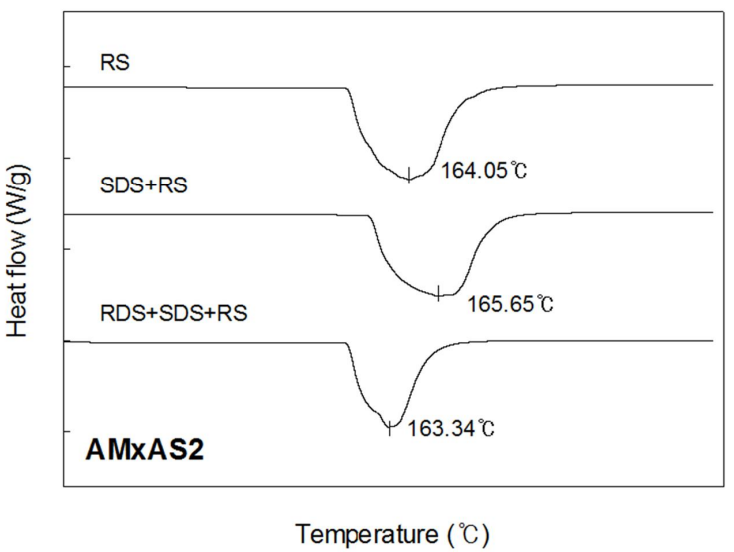
based crystals (Lopez-Rubio et al., 2008), the decrease in melting peak area according to the hydrolysis might result from not only the reduced amount of double helices, but also the partial hydrolysis of packed crystallites.

The AM×AS8 starch had rather homogenous structure with T_p at 156.54°C. This might be due to the branch chain length distribution oriented to long chains, which can be arranged into aligned structure. The melting temperature increased in the order of RDS+SDS+RS < SDS+RS < RS, whereas melting peak area decreased in the same order. It indicates that the AS modification caused the formation of abundant double helices not highly ordered.

(A)


RS
167.82℃
SDS+RS
166.34℃
RDS+SDS+RS
154.10℃
AMxAS0
Heat flow (W/g)
Temperature (℃)

(B)


RS
164.05℃
SDS+RS
165.65℃
RDS+SDS+RS
163.34℃
AMxAS2
Heat flow (W/g)
Temperature (℃)

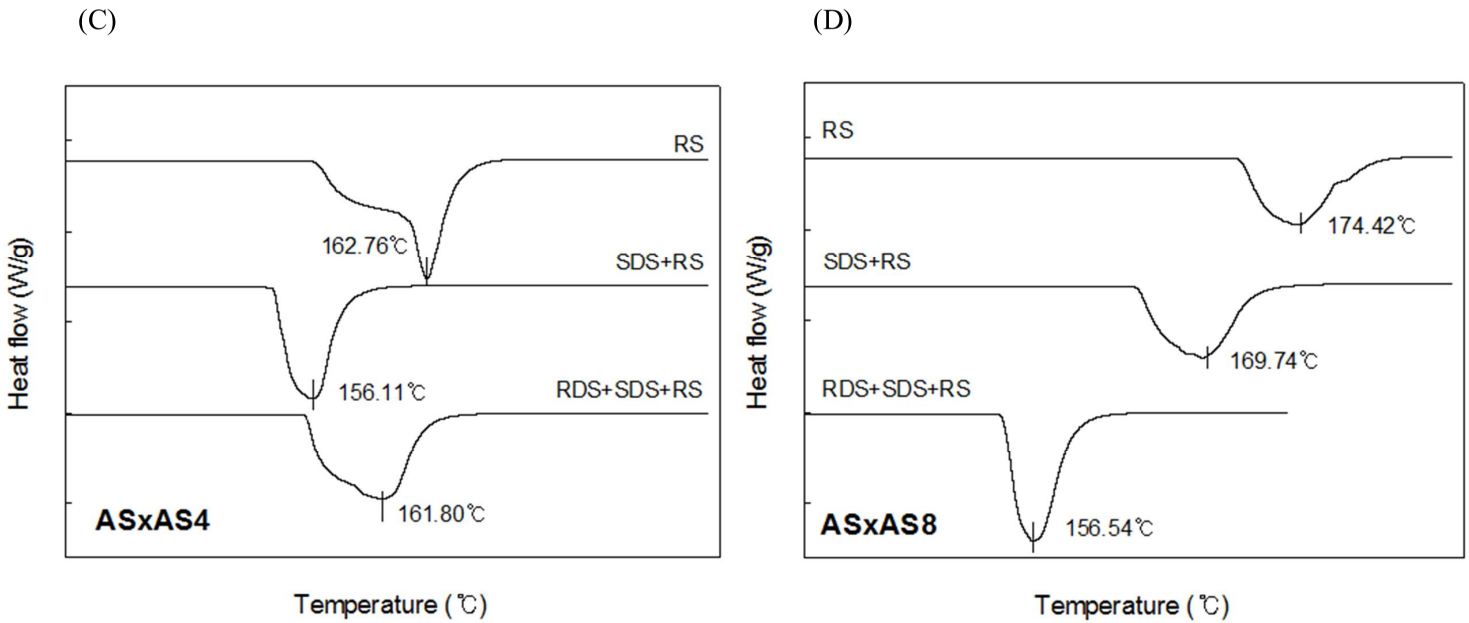


Figure 4.7. DSC thermograms of starch samples and their respective SDS+RS and RS fractions. AM×AS0, mixture of amylose and control without enzyme; AS2, starch modified with 5,000 U amylosucrase/30 mL-starch suspension; AS4, amylosucrase 10,000 U/30 mL; AS8, amylosucrase 20,000 U/30 mL; RDS, rapidly digestible starch; SDS, slowly digestible starch; RS, resistant starch. The peak temperature of each sample were marked in each thermograph.

4.3.7. Interaction between amylose and amylopectin molecules with different branch chain length distribution

In amylopectin-only systems, amylopectin branch chain length determined the crystalline structure and digestible properties of starch. The branch chain elongation resulted in reduced amount of RDS, and increases in SDS and RS in a chain length-dependent manner. As amylose molecules participated in crystallization of starch molecules in this study, the primary crystalline structure of the recrystallized starches would be different from that of amylopectin-only starches. It has been verified that waxy potato starch and amylose potato starch can exist as quite homogenous state in the ratio of 3:1 (Rindlav-Westling, Stading, & Gatenholm, 2002). The co-crystallization of two molecules, amylopectin and amylose, also has been previously confirmed in many studies (Lian et al., 2018; Rindlav-Westling et al., 2002; Zhou et al., 2011). Though DP 10 was reported as the minimum chain length required for recrystallization (Robin, Mercier, Charbonniere, & Guilbot, 1974), the short chains can participate in co-crystallization when a considerable amount of longer chains presents (Gidley & Bulpin, 1987). It indicated that short internal chains can contribute to the crystalline structure with proximate free single amylose. A single melting peak observed for the undigested recrystallized starches in this study supported the co-

crystallization, rather than respective crystals from amylose and amylopectin molecules.

There are some possible forms of interaction in the co-existing system of amylose and amylopectin as follows: Firstly, amylose can exist as free single helix with high mobility. This form of amylose generally exists over amorphous regions, connecting crystalline regions each other (Jane et al., 1992). Amylose prevents the development of amylose double helices and their crystallization, acting as a diluent (Jenkins & Donald, 1995). There are contrary reports that amylose might accelerate recrystallization between amylopectin chains. As amylose chains have much greater specific volumes owing to their linearity than amylopectin chains, water molecules become bound to amylose chains rather than amylopectin chains of the same molecular size. Reducing the available water for amylopectin to remain intact can facilitate the association of amylopectin chains (Zhou et al., 2011). Klucinec and Thompson (2002) reported that amylose at amount lower than 50% contributed to the amylopectin crystallization.

Secondly, amylose molecules can also form double helix structure by themselves. The long amylose-amylose double helix can aggregate and get organized into highly crystalline structure, which is resistant against digestion (Eerlingen et al., 1993).

Thirdly, double helices between amylopectin branched chains can be produced. Short amylopectin chains hardly associate into this form, and some studies reported that inter-chain association could only occur over DP 15 chains (Ring et al., 1987; Shi & Seib, 1992). However, long branched chains are rather easy to reassociate each other after gelatinization (Jane et al., 1999). Especially, in AS-modified waxy starches, adjacent external elongated amylopectin chains could readily form the double helical structure to form crystalline structure (Kim et al., 2014; Ryu et al., 2010; Shin et al., 2010).

Finally, the form of amylose-amylopectin double helix was supported by various studies. Schierbaum et al. (1992) proposed that the outer chains of amylopectin molecules could interact with amylose to form a network. The possibility of amylose-amylopectin double helices could be strongly supported under various concentration conditions (Zhou et al., 2011). The length of double helices formed between amylose and amylopectin external chains would be determined by the length of amylopectin chain combined together. The un-combined part of the long amylose chain would be exposed as single helix. Therefore, short amylopectin chains make short double helix, leaving abundant long singular amylose helix, which would still have mobility. On the contrary, long amylopectin chains have increased

opportunities to form double helices with amylose, producing long double helical structure. The resultant remaining single chain amylose part would be rather short and have limited mobility. In other words, the degree of freedom in amylose can also be determined by the length of amylopectin it combines with.

The double helical structures in amylose/amylopectin co-existing system can aggregate together to form crystalline regions. At the same time, disordered amorphous regions of amylose connected to the aggregated regions still remain. The chain-folded model can be used to describe the formation of crystalline regions in the retrograded starches in this study. The crystalline regions are made up of lamellae, where the chains are placed in adjacent positions, perpendicular to the surface of the lamellae, and they then fold back on themselves. The double helices will form the crystallites and the branching regions make up the folds (Farhat, Blanshard, & Mitchell, 2000).

Based on the assumption that the starch molecules should be arranged within the same volume, the structure of starches in this study could be assessed. The unextended amylopectin branched chains in AM×AS0, with rather short in their length, did not contain long single helical amylopectin chains as observed by iodine binding capacity. Instead, it would mainly have single helix amylose or amylose-containing short double helix with long

naked single chain part. Because amylose can freely act as diluent and double helices are not long enough, the degree of crystallization was moderate, resulting in high digestibility. In contrast, AM×AS8 should arrange longer chains in the limited space. Therefore, its molecular components can be (1) single helix amylopectin external chains, (2) long double helices of amylopectin themselves, and (3) double helices of amylopectin and amylose. In detail, amylose would exist as long double helix with amylopectin having short naked region, and some might as single helix. The lamella of long double helices should have many folding and resultant many layers, with cramped space in between them. Therefore, its internal structure was crystalline with many double helices, as shown in DSC, contributing to the formation of RS. Externally, single helix chains may be exposed to freely bind with iodine, composing amorphous structure. Further, loose double helices not arranged into crystalline region would also contribute to amorphous and semi-crystalline region, corresponding to RDS and SDS, respectively. As the mobility of amylose was restricted (by double helical binding and insufficient space), it could not effectively interrupt crystallization.

AM×AS2 and AM×AS4 have chain length distribution of in-between AM×AS0 and AM×AS8, and therefore might have an intermediate structure.

As they have branch chains and double helices shorter than AM×AS8, the degree of the folding of double helices and alignment of the double helical layers would not be tremendous. It indicates that, though the starches may have many double helices within their structure, they were not organized into highly crystalline structure. Instead, as stated earlier, they contributed to amorphous or semi-crystalline regions, rather than crystalline regions. This might be the reason for their lower RS amount than AM×AS8.

In summary, the chain length distribution of the original starch determined the low digestibility of starch. The length and alignment of primarily formed double helix majorly affected the low-digestible fraction, where the degree of ordered structure determined the enzyme resistant double helices with medium length (DP 13-24) would be arranged as SDS or RS. Amylose participated to constitute RDS in all starch samples. Amorphous double helices not long enough or not aligned also were component of RDS. The length of double helix between amylose and amylopectin was one of the important factors to determine the digestible properties of starch. Short amylopectin produced short double helix, therefore failed to be organized into crystalline structure and could not assign low digestibility to starch. Long amylopectin accelerated double helical formation with amylose, limiting the mobility of amylose, and therefore finally induced the increase

of RS. A certain length of amylopectin would produce proper length of double helix for semi-crystalline arrangement, maximizing the SDS amount, as supported by the higher amount of SDS in AM×AS4 than in AM×AS8.

4.4. Conclusions

This study investigated the digestive properties of the AS-modified starches according to amylopectin branch chain length in the presence of amylose. Recrystallized starches were produced by mixing AS-modified waxy potato starches with various chain length distributions and commercial amylose from potato starch with a 3:1 ratio. The interaction between amylose and amylopectin formed co-crystals affecting double helical and crystalline structures, and finally determined the digestion properties of starch samples Different proportion and respective structure of RDS, SDS, and RS of each starch sample were found via kinetics analysis.

The structural characteristics of RDS and SDS were assessed by tracing the structural changes after hydrolysis of RDS and SDS fractions, and the structure of remaining RS fraction was also investigated. The length of amylopectin was an important factor to affect the digestible properties of starch, by determining the function of amylose. RDS was composed of singular amylose chains and amorphous double helices not long enough or not aligned ordered structure. Low digestible fractions, SDS and RS, were mainly composed of semi-crystalline or crystalline structure of double helices with DP 13-24.

Through this study, the strategy to induce the formation of SDS or RS,

respectively, could be suggested. In detail, longer amylopectin chains preferred to produce RS, whereas certain length of amylopectin would produce proper length of double helix for semi-crystalline arrangement, maximizing the amount of SDS. Therefore, the production of starch with specific digestive properties seems promising.

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국문초록

Englyst 등은 전분이 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 제안하였으며, 이 중 SDS와 RS는 저소화성 전분으로 분류되어 이들의 건강적인 이점이 연구되어 왔다. 그러나 한편에서는 RDS와 SDS의 구분이 무의미하다는 주장도 제기되었다. 근래 탄수화물 조절 식이가 각광받고 있는 만큼, 건강 기능성 전분을 제조하기 위해서 식품 산업 전반에서의 공통적인 전략이 필요한 실정이다. 이를 위해서는, 각 소화 획분이 구조적으로 실재한다는 것을 뒷받침할 필요성이 대두되었다.

찰전분에 amylosucrase (AS)를 처리하여 서로 다른 가지 사슬 길이 분포를 가지는 AS 변형 전분을 제조하여 *in vitro* 상에서의 가수분해 특성을 분석하였다. AS 변형 전분의 시간에 따른 가수분해율은 1차 반응식에 바탕을 둔 log of slope (LOS) plot에서 반응속도상수 k를 기울기로 가지는 1차식 그래프로 표현되었다. 생전분 및 대조구의 가수분해 유형은 1개의 직선으로 나타나 전 구간에서 같은

속도로 가수분해됨을 보인 반면, 일부 AS 변형 전분들에서 가수분해 도중 k가 바뀌는 지점이 발견되었다. 따라서 한 전분의 가소화성 부분이 서로 다른 가수분해속도를 보이는 두 종류의 구조, 즉 RDS와 SDS로 불릴 수 있는 두 개의 구조로 이루어져 있음을 확인하였으며, 각각의 반응속도상수를 k_{RDS}, k_{SDS}로 명명하였다. 또한 쥐에서 식후 혈당 변화와 비교하여 RDS, SDS, RS의 소화 특성을 *in vitro*와 *in vivo* 모두에서 확인하였다. 따라서 이 연구를 통해 각 획분의 분류가 유효함을 증명하였으며, 속도 개념을 포괄하는 분류 방법 (변형 LOS-plot 방법)을 제시하였다.

AS 변형 찰옥수수 전분에서 RDS, SDS 획분을 제거하였을 때 나타나는 전분의 구조 변화 및 RS의 구조를 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 전분의 RDS, SDS가 공통적으로 가지는 특성이 관찰됨으로써 두 획분이 구조적으로도 실재함이 증명되었다. AS 변형 찰전분은 X-선 회절에서 B형을 나타냈으며, 처리한 AS의 수준에 따라, 또한 RDS 및 SDS 획분이 제거됨에

따라 결정화도가 증가하였다. 아밀로펙틴 가지 사슬 길이는 재결정화 전분의 일차적인 결정 특성을 결정하였으며, 이에 따라 소화 특성도 다르게 나타났다. AS 처리 전분들 간 서로 다른 RDS 및 SDS의 특성은 상이한 k_{RDS} 및 k_{SDS} 값으로 뒷받침되었다.

AS 변형 찰감자 전분과 감자 유래 아밀로스를 3:1로 혼합하여 재결정 시킨 뒤, 이들의 RDS, SDS, RS의 구조를 조사하였다. 아밀로스와 아밀로펙틴은 공동의 결정을 형성하였으며, 아밀로펙틴의 가지 사슬 길이에 따라 아밀로스-아밀로펙틴의 이중 나선의 길이 및 아밀로스의 자유도가 결정되었다. 단일 나선 형태의 아밀로스 및 정렬되지 않은 이중나선들이 RDS를 구성하였다. DP 13-24의 이중 나선들이 저소화성 획분에 기여하는 것으로 확인되었으며, 이들의 배열에 따라 SDS 및 RS 함량이 결정되었다. SDS의 함량은 찰전분 단독 환경에서와는 달리 사슬 길이에 따라 직선적인 증가를 보이지 않았으며, 아밀로펙틴의 특정한 길이의 가지 사슬이 SDS 또는 RS 각각의 생성을 유도할 수 있음을 확인하였다.

이 연구는 다양한 환경에서의 전분의 저소화성 획분의 생성 원리 및 각 획분의 구조에 대한 정보를 폭넓게 제시하였다. 이러한 결과는 저혈당 관련 건강 기능성 식품 소재로서의 전분 개발에 활

용될 수 있을 것이다.

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

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