A Thesis for the Degree of Master of Science

Structural properties and digestibility of amylopectin-palmitic acid complex formed by amylosucrase treatment on waxy corn starch

아밀로수크레이즈를 찰옥수수전분에 처리하여 형성한 아밀로펙틴-팔미트산 복합체의 소화율과 구조적 특성

February, 2014

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농학석사학위논문

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by

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ABSTRACT

Structural properties and *in vitro* digestibility of amylopectin-palmitic acid (AP-PA) complex were studied. Amylosucrase (AS) was treated on waxy corn starch (WC) for 1, 3 and 6 h to have AP chain elongated. AS-treated starch formed AP-PA complex by complexation process including heat treatment such as autoclaving of the starches at 121°C and incubation of starches at 95°C and palmitic acid mixture. AS6-PA showed the much higher ability to form complex with palmitic acid than WC-, AS1- and AS3-PA. It reflected that prolonged AS treatment time could enhance the formation of AP-PA complex. complex samples with AS-treated starches and PA (ASs-PA) had a lower relative crystallinity than their FA controls.

Both of FA controls and ASs-PA from AS-treated starches had lower *in vitro* digestibility and slower hydrolysis rate with increasing AS reaction time. AS treatment on WC reduced rapidly digestible starch (RDS) content while increased slowly digestible starch (SDS) and resistant starch (RS). The formation of complex in ASs-PA did not show significant effect on *in vitro* digestibility and maintain the low digestibility which was induced by AS treatment on WC. It might be too small amount of complexes formed in ASs-PA to affect the hydrolysis rate of AS-treated starch.

In DSC therograms, the complexes from elongated amylopectin displayed the type I peaks inducing higher T_c and ΔH than FA control while the complexes showed higher ΔH in the endothermal peak of long chain double helix. These changes of thermal properties resulted from formation of complexes were more distinct in AS6-PA than others.

Keywords: starch-lipid complex, amylopectin-palmitic acid complex, starch digestibility, waxy corn starch, amylosucrase, digestibility

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ABBREVIATIONS

AS: amylosucrase

AP: amylopectin

FA: fatty acid

PA: palmitic acid

AP-PA complex: amylopectin-palmitic complex

ASs-nf: fatty acid control samples formed with AS-treated starches and no fatty acid

ASs-PA: complex samples formed with AS-treated starches and palmitic acid

CC: complex content

RDS: rapidly digestible starch

SDS: slowly digestible starch

RS: resistant starch

DP: degree of polymerization

Short chain: the chains of DP ≤12

Medium chain: the chains of DP 13-24

Long chain: the chains of DP 25-36

Very long chain: the chains of DP ≥37

CONTENTS

List of figures

List of tables

INTRODUCTION

Starch is an important source of carbohydrate. It is consisted of amylose and amylopectin. Amylose is a linear poplymer which is made up of (1→4)-α-D-glucose units and a few (1→6)-α-D-glucose. Amylopecin is also composed of (1→4)-α-D-glucose but highly branched with (1→6)-α-D-glucose (Sajilata et al., 2006).

Amylose can form a complex with ligands such as fatty acids, iodine, monoacylglycerols, flavor compounds and hydrophobic organic polymers. When amylose exists as a signle helix, guest molecules enter the central cavities of the amylose helix during complex formation. (Costas G. Biliaderis et al., 1989; F. Tufvesson et al., 2000).

Amylose-lipid complexes decrease starch swelling capacity, solubility and granule disruption(Biliaderis and Tonogai, 1991; Lauro et al., 2000; Mira et al., 2007, Eliasson et al.,1981). Holm et al. (1983) found that amylose-lysolecithin complex was more slowly degraded by α-amylase. Besides, complex formation restricts amylose to forming double helices and recrystallization by reducing the solubility and mobility of amylose (Gudmundsson et al., 1990; Lebail et al., 2000; Z. Zhou et al., 2007). Therefore, amylose-lipid complexes have possibility of application to modification of pasting properties, to formation of slowly digestible or

resistant starch and to reduction of retrogradation (Hasjim et al., 2010; Zabar et al., 2010).

Complex formation is affected by solvent for amylose, amylose chain length, fatty acid chain length and thermal treatment. Depending on these formation conditions, mixture of starch and fatty acid forms two types of complex which have different thermal properties. Type I complex has a lower dissociation temperature than 100°C and is considered as amorphous form, whereas type II complex is organized in crystalline packing and dissociated at a higher temperature than 100°C and shows semi-crystall form (Costas G. Biliaderis et al., 1989; Lesmes et al., 2009). Type II complex, which is more heat stable than type I complex, can be obtained by heating the mixture of amylose and ligand at a higher temperature (at least 90°C) (Karkalas et al., 1995).

On the other hand, the complex is mainly formed with amylose and the ligands. Amylopectin have limited ability to form complex with ligand because of steric hindrance and short branch chain length (Heinemann et al., 2003; Hirai et al., 1994). However, the presences of amylopectin outer branches complexed with lipid although low level of complexation has been reported(Costas G. Biliaderis et al., 1991). Gudmundsson et al. (1990) reported amylopectin complexed with sodium sulfate and cetyltrimethylammonium bromide have an impact to hinder starch

retrogradation.

Amylosucrase (EC 2. 4. 1. 4., AS) catalyzes the transcosylation reaction to produce an insoluble α-1,4-glucan using sucrose, releasing fructose (Gabrielle Potocki de Montalk et al., 2000). AS especially accelerates the elongation of some external chains at their non-reducing end, in the presence of acceptor like a glucosyl unit (Rolland-Sabaté et al., 2004). AS-treatment on waxy starch increases slowly digestibility starch(SDS) and resistant starch(RS) than normal starches due to the crystallinityes formed by elongated branch chains of amylopectin (Ryu et al., 2010; Shin et al., 2010). The degree of polymerization which is induced by AS-treatment can be controlled by changing AS treatment time (Shin et al., 2010)

In the current study, AS from *Neisseria polysaccharea* was treated on waxy corn starch which consisted of mostly amylopectin for 1, 3 and 6 h. Through it, the amylopectin had elongated chain length which is favorable to form V-type complex with fatty acid (X. Zhou et al., 2013).

The objectives of the study were to prepare amylopectin-palmitic acid complexes by AS treatment on waxy corn starch and to investigate the structural properties and digestibility of amylopectin-fatty acid complex.

MATERIALS AND METHODS

1. Materials

1-1. Starch

Waxy corn starch was obtained from Samyang Genex Co. (Incheon, Korea).

1-2. Fatty acid

Palmitic acid (PA) was purchased from Sigma Chemical Co. (St. Louis, Mo, USA)

1-3. Enzymes

Pancreatin (P7545, activity 8 x USP/g) was from Sigma Chemical Co. and amyloglucosidase (AMG 300L, activity 300 AGU/mL) from Novozymes (Bagsvaerd, Denmark). Isoamylase (activity 1000U) was obtained from Megazyme(Bray, Ireland). Amylosucrase (AS) from *Neiserria polysaccharea* was provided by the Food Microbiology and Bioengineering Laboratory of Kyunghee University.

2. Methods

2-1. Enzyme assay of AS activity

The amylosucrase was purified by affinity chromatography with Ni-NTA (nickel-nitrilotriacetic acid) resin according to the method of Jung et al. (2009). Enzyme activity was determined using the method of Van der Veen et al. (2004) with a modification. The mixture of 0.1 mL of 4% sucrose, 0.1 mL of 1% glycogen, 0.25 mL of 0.1 mM sodium citrate buffer (pH 7.0) and 0.05 mL of diluted enzyme was reacted in a water bath at 30°C and 80 rpm for 10 min. The released fructose was quantified using the method of Miller (1959). One unit (U) of amylosucrase was defined as the amount of enzyme that catalyzes the release of 1 μM of fructose per min under the assay conditions.

2-2. Preparation of AS-treated starch

Starch suspension (2%, w/w) was prepared by mixing starch, 100 mM sucrose, and 100 mM sodium acetate buffer (pH 7.0) to reach the final volume of 150 mL. The starch suspension was boiled for 30 min and cooled to 30°C. Amylosucrase (10,000U/30 mL) was added to the starch suspension. After then, the starch suspension including amylosucrase was incubated in a

water bath at 30 ℃ for 1, 3 and 6 h. The enzyme reaction was stopped by adding 450 mL of ethanol to the suspension. The AS-treated starch was precipitated by centrifugation at 10,000 x g for 10 min, and the supernatant was removed. The pellet was washed with 450 mL of distilled water by centrifugation at 10,000 x g for 10 min. The pricipitate was freeze-dried, ground and passed through a 100-mesh sieve.

2-3. Preparation of amylopectin-palmitic acid complex

Amylopectin-fatty acid (AP-FA) complex prepared by following method. Starch was dispersed in distilled water (DW). The starch slurry (5%, w/v) was boiled for 30 min with vortexing and autoclaving at 121°C for 30 min to gelatinize the starch perfectly. Palmitic acid (PA) solution (10%, w/v), was prepared by dissolving 0.2g of PA in 2 mL of ethanol, added to the gelatinized starch dispersion. The mixture was continually heated in boiling water for 30 min and re-autoclaved at 121°C for 30 min. The mixture was incubated at 95°C for 24 h to promote the formation of type II complex (Fredrik Tufvesson et al., 2003). The sample was cooled to room temperature and recovered by centrifugation (10,000 x g, 20 min), washed with 50% ethanol four times and with DW two times. The final precipitates (AP-PA

complex) were freeze-dried, ground and passed through a 100-mesh sieve. Samples without the addition of PA were prepared as a control.

2-4. Determination of amylopectin branch chain distribution by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

The branch chain-length distribution of starches was determined after debranching the starch. Starch (15 mg) was dispersed in 90% dimethylsulfoxide (DMSO, 3 mL) and boiled for 20 min. Ethanol (15 mL) was added to the starch suspension and centrifuged at 10,000 ×g for 10 min to precipitate starch. DW (1.5 mL) was added to pellet and boiled for 10 min. After boiling, 1.5 mL of 50 mM sodium acetate buffer (pH 4.3) and 30 μL of isoamylase were added, and the sample was reacted in a water bath at 45°C and 50 rpm for 2 hr. To stop the reaction, samples were boiled for 10 min. Debranched sample was filtered through a 0.45 μm membrane filter and analyzed using HPAEC-PAD on a Carbo-pack PA1 anion-exchange column (4x250 mm, Dionex, Sunnyvale, CA, USA) with a pulsed amperometric detector. The sample was eluted with a gradient of 600 mM sodium acetate in 150 mM NaOH with a flow rate of 1 mL/min. The gradients of sodium acetate used were as follows : increasing from 0-20% for 0-5 min, 20-45% for 6-30 min, 45-55% for 31-60 min, 56-60% for 61-80 min, 61-65% for 81-

90 min, 66-80% for 91-95 min, and 81-100% for 96-100 min. The values of degree of polymerization (DP) were designated using a mixture of maltooligosaccharides (DP 1-7, Sigma Chemical) as standard. PeakNet software (version 5.11, Dionex) was used for calculation of peak areas. Average DP was calculated by the following equation.

$$\text{Average DP } (DP_n) = \frac{\%A_i \times DP_i}{100}$$

$$A_i = \frac{\text{peak area}}{\text{total area}} \text{ (i: raw, 1,3,6)}$$

2-5. Measuremen of complex content

Complex content (CC), percentage of complex in the starch sample, was determined based on measurenment of apparent amylose content (Kim et al., 2013). Starch-palmitic acid complex sample (20 mg) was dispersed in ethanol (0.2 mL). the starch suspecsion was mixed with 1M NaOH (1.8 mL) and heated for 10 min in boiling water. It was then cooled to room temperature. The resultant starch solution (1 mL) was diluted with DW (4 mL). The diluted solution (0.5 mL) was added to reaction solution which composed of 1M acetic acid (0.1 mL) and DW (9.2 mL). Iodine solution (0.2% I_2 and 2.0% KI) was treated to the sample solution and immediately

vortexed. After 20 min of iodine binding reaction, the absorbance (Abs) was measured at 620nm. Apparent amylose content (AAC) of control was calculated with the standard curve, that was plotted using different ratios of amylose/amylopectin mixture. Complex content (CC) was computed by following equation.

$$CC(\%) = AAC_{control} - AAC_{complex}$$

2-6. X-ray diffraction patterns

X-ray diffraction was analyzed using a powder X-ray dffractometer (Model New D8 Advance, Bruker, Karlsruhe, Germany) at 40 kV and 40 mA.

Starch sample scan was performed through 2θ range from 3° to 33° with a 0.02° step size and a count time of 2 sec. The relative crystallinity was determined by the equation below.

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

2-7. Starch digestibility

Starch digestibility was determined by the method of Brumovsky et al. (2001) with slight modification. Pancreatin (2 g) was dissolved in distilled water (24 mL) and stirred well for 10 min. It was precipitated by centrifugation at 1,500 xg for 10 min. The supernatant (20 mL) was mixed

with 0.4 mL of amyloglucosidase and 3.6 mL of DW, and incubated at 37°C for 10 min.

A starch sample (30 mg) was dispersed in a 2 mL-microtube with sodium acetate buffer (0.75 mL, 0.1 M, pH 5.2) with one glass bead. After mixing each microtube, it was equilibrated in a shaking incubator (240 rpm at 37°C) for 10 min. Then, 0.75 mL of the prepared enzyme solution was added to the tube, and the starch sample was incubated in a shaking incubator (240 rpm at 37°C) for 10 min and 240 min, respectively. The reaction was stopped by boiling for 10 min. The glucose released by the hydrolysis of starch was obtained in the supernatant after the centrifugation at 5,000 xg for 10 min. The glucose content was measured using a GOD-POD kit (BCS Co., Anyang, Korea).

Starch fractions were classified based on the rate and degree of hydrolysis. Rapidly digestible starch (RDS) was measured by the quantity of glucose after reaction for 10 min. Slowly digestible starch (SDS) was the fraction digested between 10 and 240 min. Resistant starch (RS) was the unhydrolyzed fraction after 240 min.

2-8. Enzymatic hydrolysis parttern of starches

The starch hydrolysis curve was calculated on the basis of the percentage of total glucose released. The amount glucose released was measured after

reaction for 0, 10, 20, 30, 60, 120 and 240 min. The reaction method followed the starch digestibility method except for reaction time.

2-9. Measurement of thermal properties

Thermal properties of samples were investigated using a differential scanning calorimeter (Diamond DSC, Perkin-Elmer, Waltham, MA, USA). Each sample (8 mg) was weighed in a hermetic aluminum pan (Seiko, Tokyo, Japan), and DW (24 μL) was added. The sample pan was sealed and kept at room temperature overnight for equilibrium. An empty aluminum pan was used as a reference. DSC scan was performed from 30°C to 180°C at 10°C/min.

2-10. Statistical analysis

All the experiments were done in triplicate, and the data were expressed as mean±standard deviation. Analysis of variance (ANOVA) was performed and the mean separations done by the Duncan's multiple-rage test ($p<0.05$). All the statistical analyses described above were conducted using IBM SPSS Statistics (version 21.0.0, Chicago, IL, USA).

RESULTS AND DISCUSSION

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

The branch-chain length distributions of raw and AS-treated starches are displayed in Figure 1. Additionally, the relative percentages of peak area with degree of polymerization (DP) of starches are presented in Table 1. Amylopectin branch-chains are classified into A chain (DP 6-12), B1 chain (DP 13-24), B2 chain (DP 25-36) and B3 chain (DP ≥ 37) depending on degree of polymerization (Hanashiro et al., 1996).

Waxy corn starch showed larger proportions of short chains such as A and B1 than AS-treated starches, while showing smaller proportions of medium and long chains such as B2, B3 and B4. This result agreed with a previous study regarding A-type starch such as waxy corn starch (G. Zhang et al., 2006).

The AS-treated starches showed the tendency that the proportions of short chains such as A and B1 ~~was~~ decreased and the proportions of long chain, B3 ~~was~~ increased depending on amylosucrase reaction time. This tendency in branch-chain length was also observed in a previous study (Shin et al., 2010). These results were caused by the elongation of A and B1 chains by amylosucrase. The shift of the highest peak to the right, indicating a higher

DP value, was observed with increasing amylosucrase reaction time.

After 6 h reaction of amylosucrase, the branch chain length distributions showed nonsignificant differences. G. Potocki De Montalk et al. (1999) reported that amylosucrase catalyzes the attachment of 12 to 18 glucose at non-reducing ends of amylose and amylopectin. The half-life of amylosucrase is 21 h at 30°C (Okada et al., 1974). Thus, the elongation of the branch-chain might be completed within 6 h of amylosucrase reaction.

According to Godet et al. (1995b), chain length of starch affects structural properties of amylose-lipid complexes such as melting temperature and crystallinity Therefore, in this study, amylopectin-palmitic acid complex(AP-PA) samples were prepared with raw waxy corn starch(WC) and AS-treated starches of which reaction time were 1 h(AS1), 3 h(AS3) and 6 h(AS6) except 24 h(AS24) to investigate the effects of branch chain distribution of amylopectin on structural properties and digestibility of AP-PA. The elongated branch chain by amylosucrase treatment preferably formed double helices which impeded the enzyme access. It might be associated with a reduction of digestibility by hydrolytic enzymes. The resistance starch, which has perfect crystallites, could be resulted from the increase of long branch-chains and decrease of short branch-chains in amylopectin (Shin et al., 2010). Therefore, the AS-treated starch could have higher levels of SDS and RS than raw waxy starch.

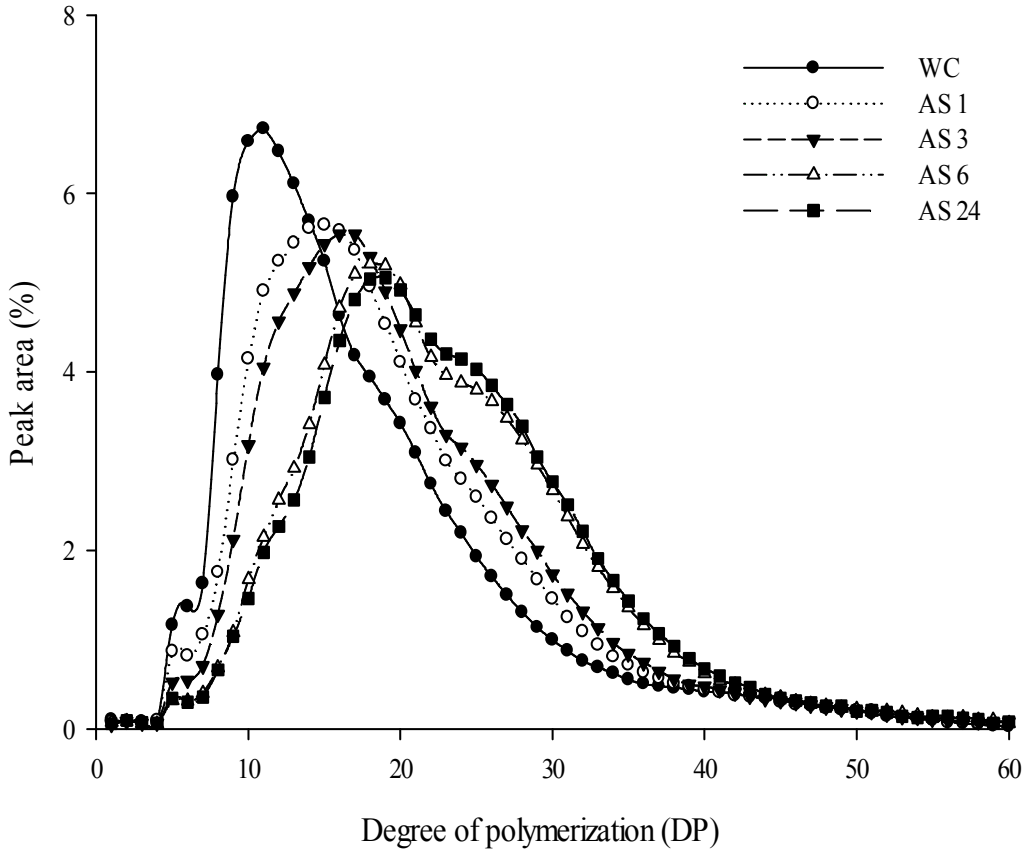

Figure 1. Branch-chain length distributions of waxy corn starch(WC) and AS-treated starches. AS1, 3, 6 and 24 are AS-treated starches for 1, 3, 6 and 24 hours.

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

Sample	Percent distribution (%)					Average DP
	DP[1] ≤5	DP 6-12(A)	DP 13-24(B1)	DP 25-36(B2)	DP ≥37(B3)	
WC[2]	1.6 ±0.2[a 4)5)]	32.5 ±0.4[a]	47.5 ±0.1[c]	12.6 ±0.2[d]	5.9 ±0.1[c]	17.8 ±0.0[d]
AS1[3]	1.1 ±0.2[b]	20.6 ±0.8[b]	52.9 ±1.0[ab]	18.6 ±1.2[c]	6.7 ±0.6[bc]	20.0 ±0.4[c]
AS3	0.8 ±0.2[bc]	15.2 ±1.1[c]	53.5 ±0.7[a]	22.9 ±1.0[b]	7.6 ±0.7[ab]	21.4 ±0.4[b]
AS6	0.5 ±0.1[c]	7.2 ±1.5[d]	51.1 ±1.8[b]	32.8 ±1.5[a]	8.4 ±1.6[a]	23.8 ±0.5[a]
AS24	0.6 ±0.26[c]	7.3 ±1.1[d]	51.0 ±1.1[b]	32.6 ±1.0[a]	8.5 ±0.7[a]	23.8 ±0.1[a]

[1] DP; degree of polymerization

[2] WC; raw waxy corn starch with no enzyme treatment

[3] AS1, 3, 6, 24; AS-treated starches with enzyme treatment for 1 h, 3 h, 6 h, and 24 h

[4] Data are expressed as average value and standard deviation.

[5] The value with different lowercase superscripts in a same column are significantly different (p<0.05).

2. Complex content

Amylose has ability to make a complex with iodine when it exists as a single helix while the long branch chains of amylopectin also bind iodine like amylose (Yasui et al., 2009). After amylose complexed with lipid added, the amount of amylose-iodine complex is reduced depending on the degree of amylose-lipid complex formation (G.A. et al., 1964; Liu et al., 1997).

Thus, the formation of amylopectin-palmitic acid (AP-PA) complex would reduce the iodine binding capacity of starch by changing ~~of~~ long branch chains from a single helix form to a complex with fatty acid. The complex content (CC) of AP-PA complex samples formed with waxy corn starch or AS-treated starches and palmitic aicd were determined by the method of Kim et al. (2013) with a slight modification.

The AS6-PA showed the highest CC value, 2.01%. The CC values for ~~of~~ WC-PA, AS1-PA and AS3-PA were 0.00, 0.21 and 0.01 %, respectively. ~~It~~ These results indicated that the starch treated with AS for 6 h had the higher ability to form an AP-PA complex than raw waxy starch and the starches treated with AS for 1 h and 3 h.

Longer amylose chains can complex more lipid molecules. On the other hand, if the amylose chains are shorter than DP 30 to complex with a lipid,

they have low ability to form amylose-lipid complexes (Gelders et al., 2004; Gode et al., 1995).

Therefore, a high proportion of long branch chains including B2 (DP 25-36) and B3 (DP≥37) in the AS6 would contribute to more complex with palimic acid compared with WC, AS1 and AS3. For AS1-PA and AS3-PA, similar CC values were exhibited, and it might be caused by the same levels of the B3 chains of the starches treated with AS for 1 h and 3 h. Waxy corn starch did not complex with PA. Godet et al. (1995a) reported that the reasons why short branch chains of amylopectin is hard to form inclusion compounds might be related to single helical conformation, branching flexibility and intromolecular crowding.

Table 2. Complex content of amylopectin-palmitic acid complex samples

Sample	Complex content
WC-PA[1)]	0.00 ± 0.05 [c 2) 3)]
AS1-PA	0.21 ± 0.03 [b]
AS3-PA	0.01 ± 0.08 [bc]
AS6-PA	2.01 ± 0.20 [a]

[1)] -PA; starches underwent complexation with palmitic acid

[2)] Data are expressed as average value and standard deviation

[3)] The value with different lowercase superscripts in a same column are significantly different (p<0.05).

3. X-ray diffraction patterns and relative crystallinity

The X-ray diffraction patterns of the FA control samples, which had the complexation process without palmitic acid and amylopectin-palmitic acid(AP-PA) complex samples are presented in Figure 2 and Figure 3, respectively. Their relative crystallinity is presented in Table 3. The relative crystallinity of WC-PA could not be measured because of free palmitc aicd peaks. Waxy corn starch has an A-type pattern which exhibits major peaks at 15°, 17°, 18° and 23° (Hizukuri et al., 1980; Shin et al., 2010). However, for waxy corn starch FA control (WC-nf), no peaks were shown in X-ray diffraction. It indicated that WC-nf was mainly composed of amorphous region. This result was in agreement with a preivous study reporting that the AS control formed with waxy corn starch and kept at 30°C for 24 h without enzyme addition did not show any peaks (Shin et al., 2010). Cooke et al. (1992) found that waxy corn starch after gelatinization easily lost crystalline structure compared with normal corn starch. Thus, it indicated that the crystallinity of waxy corn starch was distrupted during heat treatment including boiling and autoclaving to create AP-PA complex

The FA controls of AS-treated starch, AS-treated starches complexed without fatty acids(ASs-nf), showed a weak B-type X-ray parttern with main

peaks at 5.6°, 15.3°, 16.9°, 22° and 23°. The peak intensity of the AS-treated starches increased with longer reaction time. Therefore, it could mean that the AS treatement on waxy corn starch caused the change of the X-ray parttern from A-type to B-type. The AS-treated amylopectin has been reported to have a B-type crystalline partten and the amylpectin with long brach chain performs like long amylose(Rolland-Sabaté et al., 2004). Besides, the B-type pattern is also found in the retrogradated starches due to the aggregation of the longer chains (Hizukuri et al., 1980; Pohu et al., 2004)

For complex samples, the WC-PA displayed peaks at 4.9°, 7.4°, 12.3°, 20.5°, 21.5° and 24° indicating free palmitic acid peaks (Bhosale et al., 2010; Exarhopoulos et al., 2012). It implied that free fatty acid was not washed out completely in the WC-PA in spite of repeating the washing process 4 times, while the free fatty acids in the other complex samples were removed by the process as the samples showed no free fatty acid peaks.

The ASs-PA showed a B-type pattern like the ASs-nf which had peaks at 5.6°, 15.3°, 16.9°, 22° and 23°, but a lower relative crystallinity than their FA control. It implied that the crystallinity decreased because of the formation of AP-PA complexes. Although the formation of AP-PA complexes were verified in the CC result (Table 2), the reason why the AP-PA complex did not show a V-type X-ray pattern was supposedly that the AP-PA complex do

not have sufficient crystallinity to be reflected as a peak in X-ray diffraction. Cheetham et al. (1998) reported that complexation of iodine with waxy corn starch decreases intensity of the X-ray pattern than before indodination becaurse iodine molcules could disrupt some of the existing double helical structure by incorporation into crystalline regions. Besides, the complex with V-type peaks around 7.5°, 13° and 20° are results from type II complex which form semi-crystalline while type I complex form amorphous region (Buléon et al., 1998). Therefore, AS-treated starches were considered to form mainly type I complexes with palmitic acid decreasing the relative crystallinity.

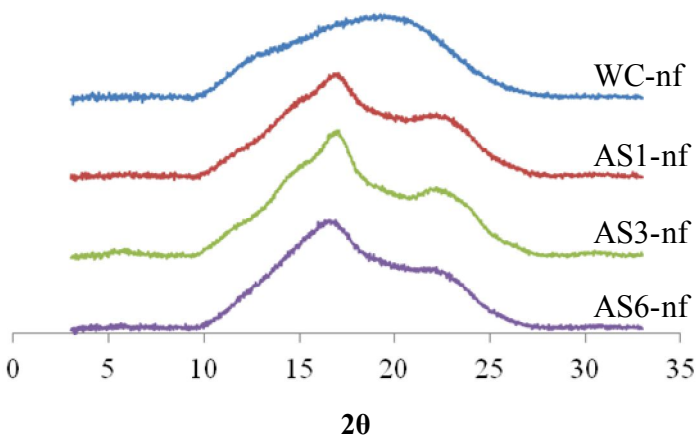

Figure 2. X-ray diffraction patterns of fatty acid controls

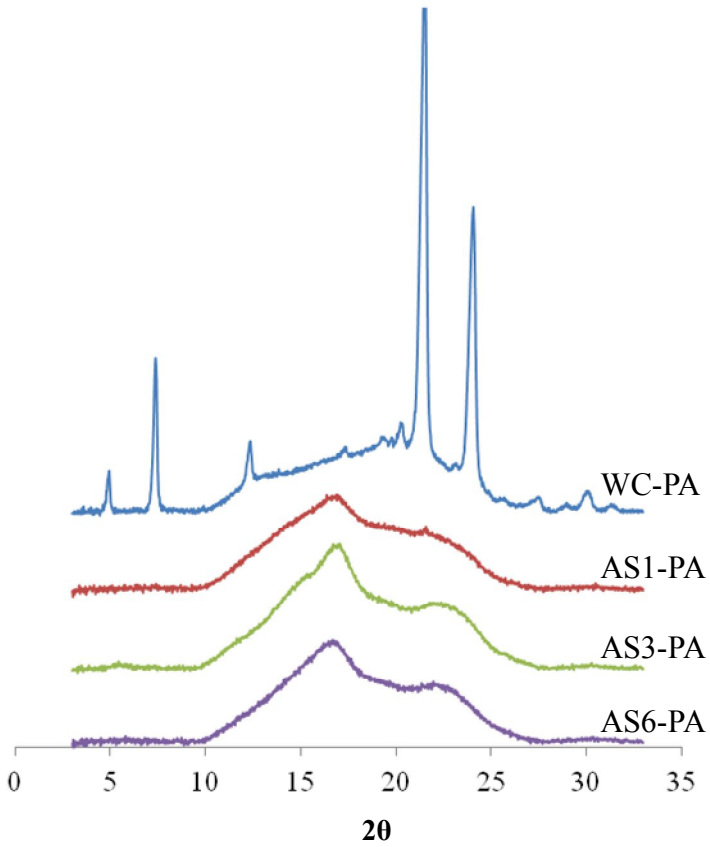

Figure 3. X-ray diffraction patterns of amylopectin -palmitic acid complex samples.

Table 3. Relative crystallinity of fatty acid controls and amylopectin-palmitic acid complex samples.

FA control	Relative crystallinity		Complex
WC-nf[1)]	21.1 ± 0.6[f 3),4)]	N.A.[5)]	WC-PA[2)]
AS1-nf	25.2 ± 1.2[d]	22.2 ± 0.6[ef]	AS1-PA
AS3-nf	33.7 ± 1.0[a]	31.2 ± 0.5[b]	AS3-PA
AS6-nf	28.7 ± 1.3[c]	23.7 ± 1.0[de]	AS6-PA

[1)] -nf, FA control; starches underwent complexation without fatty acid

[2)] -PA; starches underwent complexation with palmitic acid

[3)] Data are expressed as average value and standard deviation.

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

[5)] N.A.; not available data

4. Starch digestibility

The *in vitro* digestibility of FA control and complex samples were shown in Table 4. Starch is classified into three fractions, RDS, SDS and RS by Englyst procedure (Englyst et al, 1992). For FA control, the WC-nf had the highest RDS contents, 77.0%. It was caused by the gelatinization process which induced disruption of the semicrystalline structure of raw starch granules. Gelatinized starches have a higher RDS content than raw starches (Cousin et al., 1996; G. Zhang et al., 2006).

The contents of RDS of the AS1-nf, AS3-nf and AS6-nf were 77.0%, 67.4%, 60.0% and 48.6%, respectively. This result showed the tendency that RDS was decreasing with increasing reaction time. On the contrary, the SDS contents of of ASs-nf were risen from 16.4% to 27.7% and the RS contents of ASs-nf were also increased from 16.3% to 23.7% with increasing AS reaction time. This tendency of *in vitro* digestibility of AS-treated starches was agreed with the previous study(Shin et al., 2010). SDS is affected by amylopectin structure and chain length. Long chain of amylopectin associated with high content of SDS(Genyi Zhang et al., 2008a; Genyi Zhang et al., 2008b). The lower *in vitro* digestibility of the ASs-nf samples than that of WC-nf could be explained with branch-chain length distribution.

After AS treatment, AS-treated starches have longer branch chains than before(Rolland-Sabaté et al., 2004; Shin et al., 2010). The longer branch chains favored to be crystallized causing higher melting temperature as it was shown in DSC result Table 3 (Jane et al., 1999b; Shin et al., 2010). The starches with a higher crystallinity have reported that show resistance to hydrolysis enzyme because reduce the accessibility of the enzyme to reaction point in the starch. (Cai et al., 2010; Eerlingen et al., 1993; B. Zhang et al., 2012).

After complexation, there were no significance changes of digestibility except WC-PA as shown in Table 4. The digestibility of amylsoe-lipid complexes is influenced by the quantity and quality of the complexes in the starch (Ai et al., 2013; Soong et al., 2013). Formation of complexes with lipid and amylose decrease RS due to competion with retorgradation of amylose(Fredrik Tufvesson et al., 2001). However, type II complex has reported that contribute somewhat to the RS content(Seneviratne et al., 1991; Fredrik Tufvesson et al., 2001). In the cuuent study, the complex samples might have no type II complex as shoewed in the X-ray pattern result(Fiqure 3). Besides, the amount of complexes in the ASs-PA samples were less than 5%. Therefore, the reason why the formation of APs-PA complex could not affect to the digestibility of AS-starch could be too small amount of complex

formed, and difficulty of fomation of type II complex in AS-treated starch.

Addition of lipid impedes starch gelatinization and invests hydrolysis enzyme resistance of the starch (Holm et al., 1983; Szczodrak et al., 1992). Hence, free fatty acid, not complexed with starch would contribute to remaining the crystallinity of WC-PA. Starch with the higher crystallinity of starch have a less proportion of RDS and more proportion of RS (Lehmann et al., 2007; Sajilata et al., 2006). Therefore, free palmitic acid in WC-PA could hinder gelatinization and caused the little decrease of RDS in WC samples.

Table 4. *in vitro* digestibility of fatty acid controls and amylopectin-palmitic acid complex samples

Sample	RDS(%)	SDS(%)	RS(%)
WC-nf[1)]	77.0 ±1.6[a 3),4)]	14.5 ±1.7 [d]	8.5 ±0.6[d]
WC-PA[2)]	72.9 ±2.8[b]	17.0 ±0.5 [cd]	10.0 ±3.1[cd]
AS1-nf	67.4 ±1.1[c]	16.4 ±4.0 [d]	16.3 ±2.9[b]
AS1-PA	70.6 ±1.1[bc]	16.8 ±3.3 [cd]	12.7 ±2.2[bcd]
AS3-nf	60.0 ±2.2[d]	25.4 ±3.6 [ab]	14.6 ±1.6[bcd]
AS3-PA	58.7 ±2.1[d]	28.3 ±1.4 [a]	12.9 ±0.7[bc]
AS6-nf	48.6 ±1.5[e]	27.7 ±4.3 [ab]	23.7 ±3.8[a]
AS6-PA	50.6 ±2.8[e]	22.3 ±4.5 [bc]	27.1 ±3.1[a]

[1)] -nf; starches being processed complexation with no fatty acid

[2)] -PA; starches being processed complexation with palmitic acid

[3)] Data are expressed as average value and standard deviation.

[4] The value with different superscripts in a same column are significantly different ($p<0.05$).

5. Enzymatic hydrolysis rate

Enzymatic hydrolysis of starch samples were calculated by amount of released glucose per starch. When AS-treated starches complexed with PA, the hydrolysis rates of them did not change significantly compared with that of FA controls. The waxy corn starch showed a slightly slower hydrolysis rate after complixation with PA. Reducing the hydrolysis rate of WC-PA could be attributed to free palmitic acid remained in WC-PA samples as shown CC result.

As it was shown in *in vitro* digestibility, AS-treated starches both with no fatty acid and palmitic aicd were presented lower enzymatic hydrolysis than WC-nf and WC-PA. It also exhibited the tendency of reducing enzymatic hydrolysis with increasing AS-treatment time as *in vitro* digestibility result showed in Table 4. Soong et al. (2013) reported the similar results that there were no significant differences of hydrolysis rate between waxy starches complexed with fatty acid and raw waxy starch because the complexes were not formed sufficiently. Because the formation of AP-PA complexes caused the decrease of double helices, which were considered as RS, AP-PA complexes could maintain the low hydrolysis rate of AS-treated starches.

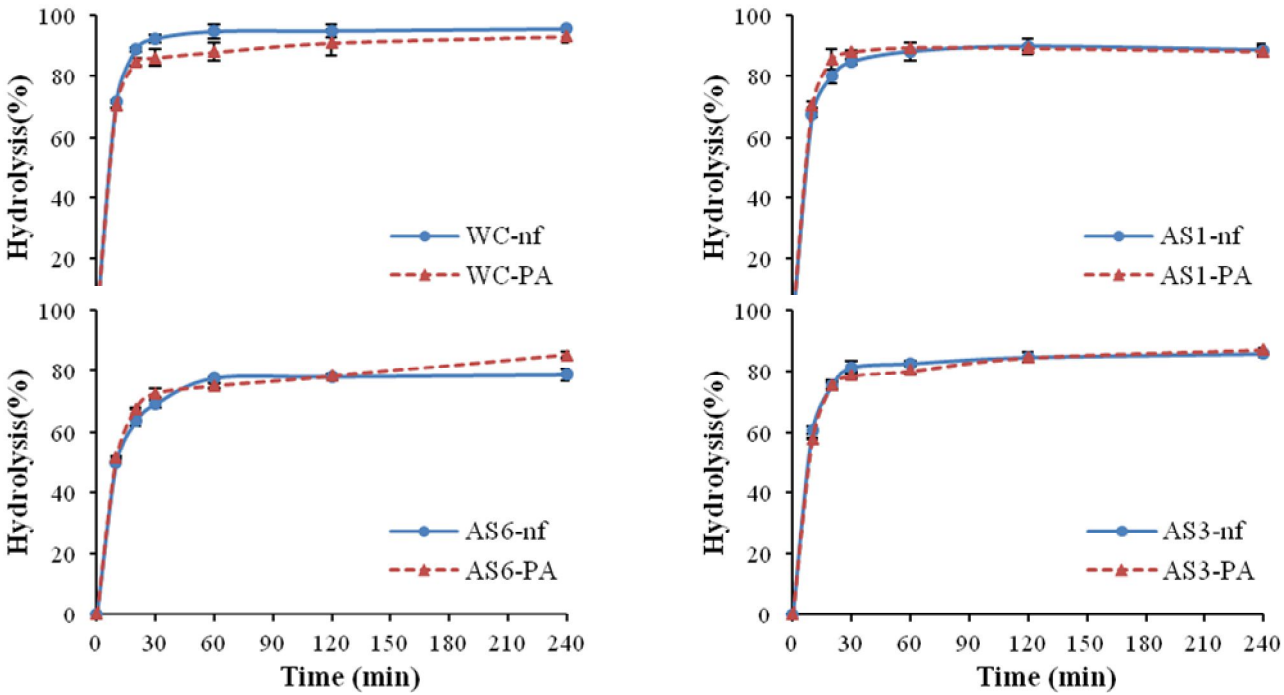

Figure 4. Enzymatic hydrolysis rate of fatty acid controls and amylopectin-palmitic acid samples formed with waxy corn starch(WC), AS-treated starches for 1 h, 3 h and 6 h (AS1, AS3 and AS6)

6. Thermal properties

Thermal properties of FA control and AP-PA samples were determined by differential scanning calorimetry (DSC). Onset (T_o), peak (T_p) and conclusion temperatures (T_c) and melting enthalpy(ΔH) were measured for the characterization of thermal properties.

The T_o and T_c reflect melting of the weakest crystallites and the strongest crystallites, respectively. The T_p is considered ~~as~~ to be related to the structural stability (Barichello et al., 1990; Biliaderis et al., 1980) Biliaderis et al., 1980). ΔH reflects the overall crystallinity, representing the quality and amount of crystallites of amylopectin (F.Tester et al., 1990).

The FA controls showed two kinds of endothermic peaks. The first peak is considered as the melting peak of double helices formed by short chains of amylopectin. The second peak corresponds to the melting of double helices induced by retrogradation of amylase (Chung et al., 2003; Morrison et al., 1993). However, waxy corn starch, which was used in this study, rarely has amylose. Thus, the second peak could indicate the melting of double helices, which were consisted of long chains of amylopectin behaving like long chains of amylose.

WC-nf did not show any endothermic peak due to the disruption of

crystallinity by gelatinization during complexation process. In case of the ASs-nf, $T_{o,}$ T_{p1} and T_c gradually increased with increasing AS reaction time in the double helices peak formed by short chains. The starches with longer branch chains are favored to make interchain association (Ryu et al., 2010). Therefore, the AS-treated starches with long chain by elongation reaction show the higher crystallinity than waxy starches with less amount of long branch chains and more amount of short branch chains. Accordingly, the long chains of AS-treated starches can induce increase of gelatinization temperature (Han et al., 2006; Jane et al., 1999a). Shin et al. (2010) reported that AS-treated starches have higher T_o, T_p, T_c and enthalpy of gelatinization with increasing AS reaction time. However, in the current study, the melting enthalpy of double helix formed with short amylopectin chains decreased with AS reaction time differing from the previous study (Shin et al., 2010). It could be due to the retrogradation after autoclaving process. In the previous study, autoclaving process was not conducted on the AS-treated starches, and the autoclaving is one of the methods to make retrogradated starch (Aparicio-Saguilán et al., 2005).

During retrogradation, long chains make double helices and enhance hydrogen bonding between chains, contributing to the formation of entire crystalline region. In contrast, short chains form short or weak double helices

which would produce an inferior crystalline structure (Jane et al., 1999b; Srichuwong et al., 2005). Therefore, when the AS reaction time increased the ASs-nf had ~~the~~ lower enthalpy in double helices of short chains, and slightly higher enthalpy in double helices of long chains.

The AS3-nf showed lower T_o, T_{p1} and T_c than AS6-nf did. It might be explained by relatively higher proportions of A (DP 6-12) and B1 chain (DP 13-24) in AS3 than AS6 (Table 1). Amylopectin branch chains with DP 8-11 and DP 22-34 have negative correlations with T_c and enthalpy of retrograded starch. It indicates that starch chains with DP 8-11 or DP 22-34 can diminish retrogradation (Silverio et al., 2000). Accordingly, the T_o, T_{p1} and T_c did not show a gradual increase with AS reaction time, because A and B1 chains could interrupt starch chains to form perfect structure.

The complexation induced a new endothermic peak overlapping the double helix peak of short chains. T_c and ΔH were increased and T_{p2} appeared by the occurrence of a new peak. Generally, in normal starch, type I complex has a higher temperature range than the aggregates of amylopectin branch chains (Chang et al., 2013). However, the AS-treated starches have a high melting temperature with increasing AS-treatment time (Shin et al., 2010). Besides, the melting temperature of type I complex is lower than that of the double helix of amylose chains (Zhou et al., 2013). Therefore, the peak with two T_ps

indicates the melting peak of double helices of short chains and type I complex. This overlapped peak of type I complex was in agreement with the high amylose corn starch-lipid complex in a previous study (Ai et al., 2013). Free palmitic acid peak, which was not removed despite washing 4 times, was shown in WC-PA around 59°C as well as was found in X-ray diffraction result. It could be due to the difficulty in washing step of WC samples to be redispersed by 50% ethanol. WC-samples had a too strong association structure to be disrupted while AS-treated starch samples were easily redispersed. It could be caused by high viscosity of amylopectin. A high amount of amylopectin with little amount of amylose in waxy corn starch shows much higher peak viscosity and final viscosity than normal corn starch (Bahnassey et al., 1994; Jane et al., 1999b). WC-PA did not exhibit any complex or crystal peak indicating that WC is hard to form complex with palmitic acid and to remain crystalline after gelatinization by autoclaving treatment. On the other hand, the ASs-PA had T_{p1} and T_{p2}. T_c and enthalpy of ASs-PA in the peak of double helices consisted with short chains are higher than that of ASs-nf. The increase of enthalpy was the highest in AS6-PA, which presented the greatest ability to form complex with palmitic aicd. These differences between ASs-PA and ASs-nf might be induced by formation of type I complex. The increase of enthalpy after complexation has

been reported in the previous studies (Chang et al., 2013; Gelders et al., 2005; Zhang et al., 2004). On the contrary, in the last peak, the enthalpy of ASs-PA was maintained at a similar level or decreased, but their T_o was increased after complexation. The last peak, which appeared at above 100°C, reflects melting of double helices formed by longer branch chains of elongated amylopectin and type II complex. Type I complex is amorphous form that melts at lower temperature than 100°C, while type II complex has higher melting temperature with more ordered crystalline structure (Tufvesson et al., 2003). Thus, the decrease of enthalpy in the last peak could indicate that type II complex might not be formed between AS-treated starch and palmitic acid as X-ray diffraction results showed.

Therefore, it was suggested that the relatively weak part of double helices composed of long chains become loosened and then form type I complexes with plamitic acid instead of forming double helices. This suggestion could explain the decrease of T_o and enthalpy in the last peak of ASs-PA. Previous studies reported that formation of complexes hinders starch retrogradation by utilizing single helix to form complex with fatty acid interrupting the formation of double helices (Putseys et al., 2010; Tufvesson et al., 2001).

Sample	Free fatty acid				Double helices of short chains, type I complex					Double helices of long chians, type II complex			
	T_o[1)] (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)	T_o (°C)	T_{p1} (°C)	T_{p2} (°C)	T_c (°C)	ΔH (J/g)	T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)
WC-nf[3)]	N.D.[2)]				N.D.					N.D.			
WC-PA[4)]	59.1 ±0.1	61.3 ±0.1	63.7 ±0.2	4.6 ±0.1	N.D.					N.D.			
AS1-nf	N.D.				49.3^{d}[4)5)] ±0.6	59.5^{e} ±0.3	N.D.	74.3^{d} ±1.4	5.6^{c} ±0.1	120.7^{cd} ±2.6	132.7^{b} ±2.6	142.8a ±4.2	1.7c ±0.3
AS1-PA	N.D.				54.8^{c} ±1.2	61.5^{de} ±0.5	83.3^{a} ±2.2	87.7^{b} ±0.5	6.2^{c} ±0.2	132.1^{b} ±1.0	132.9^{b} ±0.7	137.5^{b} ±2.1	2.4^{bc} ±1.1
AS3-nf	N.D.				55.5^{c} ±1.5	65.4^{c} ±0.6	N.D.	75.3^{d} ±2.0	4.0^{d} ±0.2	113.2^{e} ±2.5	120.1^{d} ±2.3	132.1^{c} ±1.7	2.6 ±0.5
AS3-PA	N.D.				54.4^{c} ±3.3	64.9^{cd} ±1.7	82.0^{a} ±3.4	84.0^{c} ±1.7	9.1^{a} ±1.1	118.2^{d} ±2.6	123.3^{c} ±1.4	134.5^{bc} ±3.1	3.4^{b} ±0.3
AS6-nf	N.D.				69.5^{a} ±3.5	79.2^{a} ±4.4	N.D.	88.5^{b} ±1.9	2.6^{e} ±1.0	124.0^{c} ±2.6	130.8^{b} ±1.7	143.0^{a} ±2.3	4.8^{a} ±0.5
AS6-PA	N.D.				64.3^{b} ±1.8	75.3^{b} ±0.0	82.0^{a} ±0.7	94.1^{a} ±2.0	7.8^{b} ±0.7	139.8^{a} ±0.4	140.6^{a} ±0.3	144.8^{a} ±2.9	1.8^{c} ±0.1

[1)] T_o, T_p, T_c and ΔH are onset temperature, peak temperature, conclusion temperature and enthalpy, respectively.
[2)] N.D.; not detected.
[3)] -nf; starches underwent complexation with no fatty acid.
[4)] -PA; starches underwent complexation with palmitic acid.
[5)] The values with different superscripts in a column are significantly different (p<0.05).

CONCLUSION

In the current study, palmitic acid complexed with elongated amylopectin prepared by AS treatment on waxy corn starch. AS treatment time on starch affected branch chain length distribution, thermal properties, *in vitro* digestibility and complex content. When AS6 starch underwent through a complexation process with palmitic acid, the ability to make an AP-PA complex was the highest because AS6 starch had the highest proportion of long branch chains, which can readily form complexes with fatty acids. In contrast, WC did not form complexes with palmitic acid due to highly branched chains and short chain length. Therefore, it was found that the AS treatment on waxy corn starch could enable amylopectin to form starch-fatty acid complexes despite the highly branched structure with steric hindrance. In the result, amylopectin treated with AS could be utilized as a novel starch source to form starch-fatty acid complexes.

The thermal properties indicated that formation of AS-PA complex might induce partial collapse of the relative weak structure in double helices of long chains. Hence, the complexation between AS-treated starch and palmitic acid could be a method to retard retrogradation of amylopectin by interrupting formation of double helices.

After complexation, *in vitro* digestibility which was reduced by the AS treatment was maintained. The insufficient degree of complexation affecting the hydrolysis rate resulted in no significant differences between FA controls and ASs-PA. Therefore, further studies are required to find a formation condition including the kind of fatty acid, the unit of AS treatment and the AS reaction time longer than 6 h to increase the level of complexation between AS-treated starch and fatty acids.

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국문 초록

아밀로오즈-지방질 복합체는 전분의 노화를 지연시키며, 전분 겔의 물성을 조절할 수 있고 소화효소에 대한 전분의 저항성을 증진시키는 효과로 산업적 활용 가능성이 큰 소재이다. 반면, 아밀로펙틴은 긴 선형의 아밀로오즈와는 달리, 짧은 사슬 길이와 가지구조로 인한 입체적 방해로 인하여 지방질과 복합체를 형성하기 어렵다. 이 연구에서는 이러한 복합체 형성에서의 아밀로펙틴의 한계점을 극복하기 위하여 찰옥수수 전분에 *Neisseria polysaccharea*에서 유래한 amylosucrase(AS)를 1, 3, 6시간 동안 10,000U/30mL로 처리하여 아밀로펙틴의 사슬을 연장하였다. HPAEC-PAD 결과를 통하여 AS처리 시간이 증가함에 따라 DP ≥ 25의 긴 사슬들이 증가함을 확인하였다. 이 AS처리 전분들을 팔미트산과 함께 열처리하여 아밀로펙틴-팔미트산(AP-PA) 복합체를 형성하였다.

복합체형성률을 분석한 결과, 복합체 형성률은 AS6 >AS1, AS3 > WC 순으로 나타났다. 따라서 AS 처리를 통하여 AP-PA 복합체 형성이 가능하다는 것을 확인하였으며 AS 처리 시간에 따른 사슬 길이 변화가 복합체 형성률에 영향을 미친다는 것을 알 수 있었다.

X선회절형 분석에서 AS 처리 전분-팔미트산 복합체 시료는 지방산 컨트롤과 같은 B 타입을 나타내었으며 결정성은 낮아졌다. 이를 통하여 유형 II 복합체의 형성이 잘 되지 않았다는 것을 확인하였다.

소화율 결과에서는 AP-PA 복합체의 형성이 AS처리로 인해 낮아진 아밀로펙틴의 소화율에 큰 영향을 미치지 않았다.

열적 특성 분석 결과, 복합체가 형성으로 인하여 짧은 사슬이 이룬 더블헬릭스 피크 구간에 새로운 피크가 나타났고, 긴 사슬이 이룬 더블헬릭스 피크 구간에서는 개시온도(T_o)가 높아졌다. 따라서 복합체 형성으로 인하여, 긴 사슬이 더블헬릭스 대신 팔미트산과 복합체를 형성하였다고 유추할 수 있었다.

이 실험을 통하여, AS처리 시간을 조절함으로써 아밀로펙틴과 팔미트산과의 복합체를 형성시킬 수 있음을 확인하였다. 또한 AS처리 전분에서 유래된 AP-PA 복합체가 AS처리 전분의 부분적 결정성 영역의 양과 결정성을 증가시킨다는 것을 알 수 있었다. 마지막으로 AS처리 전분의 낮은 소화율은 AP-PA 복합체 형성에 큰 영향을 받지 않고 유지됨을 확인하였다.

주요어 : 전분-지방질 복합체, 아밀로펙틴-팔미트산 복합체, 찰옥수수전분, 아밀로수크레이즈, 전분 소화율

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