A Thesis for the Degree of Master of Science

Preparation of amylosucrase treated potato starch complexed with butyric acid and its structural characteristics

아밀로수크레이스 처리한 감자전분-부티르산의 복합체 형성과 그 구조적 특성

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농학석사학위논문

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Preparation of amylosucrase treated starch complexed with butyric acid and its structural properties

by

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ABSTRACT

Potato starch was modified by using amylosucrase (AS) from *Neisseria polysaccharea* for 3 and 24 h and complexed with butyric acid. Branch-chain length distribution, thermal properties, crystallinity, and digestibility were changed by the AS-treatment. The proportion of A chains (DP $\leq$ 12) decreased, while those for both B2 chains (DP 25-36) and B3 chains (DP 25-36) increased with increasing reaction time. Thermal properties of AS-treated starch such as the onset, peak and conclusion temperatures of gelatinization, and melting enthalpy increased with enzyme reaction time. However the complex of AS treated starch had the lower melting temperature than that of their control due to the bulky carboxylic group of butyric acid. The carboxylic group induced disruption of amylopectin double helices, resulting in the decrease of the level of crystalline order. Therefore, the X-ray pattern of AS-BAs complex displayed V type pattern with a peak of gentle slope, and the R value of 1022/995 cm^{-1} in FTIR showed that AS-Bs were less ordered that ASctrs. In previous studies, there were many reports that amylose could form a complex with a fatty acid, which is with a longer chain length than caprylic acid. To date, there has been minimal research regarding

amylopectin-fatty acid inclusion complex. Butyric acid has a very low ability of forming a complex with raw starch. Thus, this study hypothesize the possibility that amylopectin-short chain fatty acid inclusion complex could be formed by controlling of the branch chain length of amylopectin. The starch-butyric acid could support the prevention and therapy for inflammatory bowel disease like Crohn's disease. Therefore starches complexed with butyric acid could provide functional food ingredients that could enhance the health.

Keywords: amylosucrase, potato starch, inclusion complex, butyric acid

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ABBREVIATIONS

AS: amylosucrase

RDS: rapidly digestible starch

SDS: slowly digestible starch

RS: resistant starch

DP: degree of polymerization

BA: butyric acid

Raw ctr: raw starch incubated at 90℃ for 24 h without butyric acid

AS3: AS treated potato starch for 3 h

AS3ctr: AS3 incubated at 90℃ for 24 h without butyric acid

AS24: AS treated starch for 24 h

AS24ctr: AS24 incubated at 90℃ for 24 h without butyric acid

ASctrs: both AS3ctr and AS24ctr

Raw-BA: raw starch complexed with butyric acid

AS3-BA: AS3 complexed with butyric acid

AS24-BA: AS24 complexed with butyric acid

AS-BAs : both AS3-BA and AS24-BA

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Introduction

Starch is the main form of storage carbohydrate in plants. It has two types of biopolymers. Amylose is a linear glucose polymer with α-(1,4) linkage. Amylopectin is a branched glucose polymer with α-(1,4) and α-(1,6) linkages (Sajilata et al., 2006)

Based on the rate and extent of starch digestion in vitro, starch can be classified into three fractions: rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistance starch (RS) (Englyst et al., 1992). RDS increases the level of blood glucose and insulin rapidly, whereas SDS is digested slowly throughout the small intestine and provides a prolonged release of glucose. It is helpful in preventing diabetes and cardiovascular disease (Brennan, 2005). RS is an indigestible starch in the small intestine, but can be fermented in the large intestine. It plays a role as a dietary fiber and gets a lot of interest in relation to physiological role and health effect (Shu et al., 2009).

Amylose has capability to form complex with non-polar compounds. The structure is usually induced by presence of a suitable guest molecule such as

iodine, linear alcohols, and fatty acids or flavors, forming a crystalline structure of V type (Biais et al., 2006; Buleon et al., 2007). This results in a left-handed single helix including a hydrophobic interior to binding site with a high affinity for the ligands. Therefore, the guest molecules are usually hosted into the helical cavity. This structure provides certain forces requires to keep a stable helix conformation (Putseys et al., 2010). Therefore, it required heat energy to dissociate the complex, and the polymorphs of amylose inclusion complexes can be classified according to the required heat energy for dissociation, type Ⅰ and type Ⅱ (Karkalas et al., 1995). Type Ⅰ complex has a low melting temperature with the formation of individual helical segments which are randomly and less-ordered oriented. On the other hand, type Ⅱ complex has a higher melting temperature with semi-crystalline structure.

In the previous studies, amylose chain length, amylose content and fatty acid chain length are related to the crystal thickness and level of organization of amylose-fatty acid complexes (Godet, Bizot, et al., 1995). The extent of complexation between amylose and the ligand increases with longer amylose chain length (Gelders et al., 2005), and amylose contents (Chang et al., 2013). In the case of longer amylose chain length or higher amylose content, the more stable complexes are formed. Regarding the effect of lipid on

complexation, the heat stability of the complex increases with increasing fatty acid chain length (Tufvesson & Eliasson, 2000). Especially, saturated fatty acids with chain length from C12 to C18 result in stable complexes.

So far, inclusion complexes of amylopectin have been less studied than those of amylose. Due to steric hinderance and short branched chain length, amylopectin has a limited ability to form complexes (Chang et al., 2013; Copeland et al., 2009).

Amylosucrase (EC 2. 4. 1. 4., AS) from *Neiserria polysaccharea* elongates the non-reducing ends of amylose and amylopectin. It catalyzes the reaction to produce α-(1, 4) glucans using sucrose, while releasing fructose (Rondeau-Mouro et al., 2004). It uses the glucose obtained by splitting sucrose to elongate glucan (Potocki-Veronese et al., 2005). Amylosucrase-treated starches increase SDS fraction in comparison to normal starches due to crystallites formed by elongated branch-chains of amylopectin (Shin et al., 2010).

Butyric acid is a kind of short fatty acid with 4 carbons skeleton produced by the microflora fermentation in colon. (Kumar et al., 2002; Smith et al., 1998) Previous studies have shown butyric acid has various physiological functions associated with bowel disease (Chapman et al., 1994) Patients suffering from inflammatory bowel disease as well as colon cancer typically are deficient in butyric acid producing bacteria and have a low level of butyric acid in the colon (Chapman et al., 1994). In the experimental animal studies, mice suffering from gut inflammation have shown that their symptoms are improved after applying butyric acid as part of their diet. (Furusawa et al., 2013) It has been shown shows that colonocyte utilizes butyric acid as a preferred energy source for the metabolism. Thus, butyric acid has potential for use in prevention and treatment of gut inflammation and colorectal cancer.

The objectives of the present study were: (1) to investigate the formation of amylosucrase treated potato starch inclusion complexes with a short chain fatty acid and (2) to evaluate the effect of the starch chain length on the formation of AS-treated starch-fatty acid inclusion complexes.

MATERIALS AND METHODS

1. Materials

1-1. Starch2-1. Enzyme assay of AS activity

Potato starch (KMC, Brande, Denmark) used, which contained 20.0% moisture, 0.10% protein, and 0.27% lipid. Sucrose was purchased from Junsei Chemical (Tokyo, Japan).

1-2. Enzymes

Pancreatin (P7545, activity 8 x USP/g) was from Sigma Chemical Co. (St. Louis, MO, USA) 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 (20,000U/30 mL) was added to the starch suspension. After then, the starch suspension including amylosucrase was incubated in a

water bath at 30 °C for 3 and 24 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 xg for 10 min, and the supernatant was removed. The pellet was washed with 450 mL of distilled water by centrifugation at 10,000 xg for 10 min. The precipitate was freeze-dried, ground and passed through a 100-mesh sieve. AS-control was prepared according to the same method for the AS-treated starch preparation without the amylosucrase addition.

2-3. Preparation of starch complexed with butyric acid

Starch (1 g) was dispersed in 20 mL distilled water (5%, w/v), and then preheated for 10 min with vigorous vortexing. The starch suspension was autoclaved at 121℃ for 30 min. Butyric acid (0.5 mL; 5.4 mM/g starch) dissolved in distilled water (10 mL) was added to the starch suspension at 90 ℃. After vortexing for 30 S, the suspension was incubated in a water bath at 90℃ and 80 rpm for 24 h.

2-4. Preparation of debranched starch

Starch (15 mg) was dispersed in 90% DMSO (3 mL) and boiled for 30 min. Ethanol (15 mL) was added to the starch suspension to precipitate starch and centrifuged at 10,000 xg for 10 min. Then distilled water (1.5 mL) was added to the pellet and boiled for 15 min. After boiling, 1.5 mL of 50 mM sodium acetate buffer (pH 4.3) was added and boiled for 20 min. Isoamylase (30 μL, 1000 U/mL, Megazyme) was added to the starch dispersion, and the sample was incubated at 45°C, 30 rpm for 2 h in a water bath. Enzyme reaction was stopped by boiling for 10 min.

2-5. 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 by debranching the starch with isoamylase. 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 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. The average DP was calculated using the following equation:

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

Ai: peak area / total area (i: 1,2,3….)

2-6. Determination of apparent amylose content (AAC) and complex content

The measurement of the apparent amylose content (AAC) was based on a colorimetric method described by AACC Approved Method 61-03 (AACC, 2000). Starch (20 mg) was dispersed in absolute ethanol (0.2mL), and then mixed with 1 M NaOH (1.8 mL) with vigorous vortexing. The starch suspension was heated for 10 min in a boiling water bath and cooled to room temperature. The resultant starch solution (1 mL) was diluted to 10 mL with

distilled water. An aliquot (0.5 mL) of the diluted starch solution was combined with 1 M acetic acid (0.1 mL) and Lugol's solution (0.2 mL; 0.2 % I_2 + 2.0 % KI), and diluted again to 10 mL with distilled water, followed by holding for 20 min in the dark. The absorbance of the color-developed starch solution was measured at 620 nm.

Complex content was deduced from the AAC value of control to that of complex. The complex content was evaluated using the following equation:

$$\text{Complex content (\%)} = \text{AAC for control} - \text{AAC for complex}$$

2-7. Measurement of thermal properties

Thermal properties of the samples were investigated using a differential scanning calorimeter (Diamond DSC, Perkin-Elmer, Waltham, MA, USA). Each sample (10 mg) was weighed in a hermetic aluminum pan (Seiko, Tokyo, Japan), and 40 μL of distilled water 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.

2-8. X-ray diffraction patterns and relative crystallinity

The freeze-dried samples were equilibrated over saturated solution in a closed box at room temperature for at least 48 h before analysis. X-ray diffraction was analyzed using a powder X-ray diffractometer (Model New D8 Advance, Bruker, Karlsruhe, Germany) at 40 kV and 40 mA. Starch sample scan was performed through 2θ range from 5° to 35° with a 0.02° step size and a count time of 2 sec. The area was calculated using a software developed by the instrument manufacturer (EVA, 2.0). The relative crystallinity was determined by the equation below.

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

2-9. Fourier transform infrared (FT-IR) spectroscopy

FT-IR spectra were measured with a FT-IR spectrometer (Nicolet 6700, Thermo Scientific Corp., Waltham, MA, USA) using OMNIC software. The absorbance spectra, obtained at resolution 8 cm^{-1} in the range of 800-1200 cm^{-1}, were 32 scans on average. Each spectrum was recorded against an empty state as background, and the spectra were baseline-corrected. The ordered extent was observed using ZnSe windows.

2-10. Starch digestibility

Starch digestibility was determined by the method of Brumovsky and Thompson (2001) with slight modification. Pancreatin (1.5 g) was dissolved in distilled water (18 mL) and stirred well for 10 min. It was precipitated by centrifugation at 1,500 xg for 10 min. A 15 mL aliquot of supernatant was mixed with 0.3 mL of amyloglucosidase and 2.7 mL of distilled water, 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 under hydrolysis of starch was obtained in 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. RDS was measured by the quantity of glucose after reaction for 10 min. SDS was the fraction digested between 10 and 240 min. RS was the unhydrolysed fraction after 240 min.

2-11. Statistical analysis

All the experiments were done in triplicate, and data were expressed as mean ± standard deviation. Analysis of variance (ANOVA) was conducted and the mean separations were done by the Tukey's HSD test ($p<0.05$). All the statistical analyses described above were conducted using SPSS (version 18, Chicago, IL, USA).

RESULTS AND DISCUSSION

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

Figure 1 shows the branch chain length distributions of starches before and after AS treatment. The relative percentages of peak area of degree of polymerization (DP) groups is 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 and longer chain (DP≥37) depending on the degree of polymerization (Hanashiro et al., 1996).

This result showed the difference in branch-chain length distribution between raw and AS-treated starches. After the AS treatment, the portion of short A chains of all AS-treated starches decreased. On the other hand, the proportions of long B1, B2 and B3 chains increased compared with those of raw starches. In addition, the average DP of starches remarkably increased from 16.05 to 23.75. These results were due to the elongation of external chains by amylosucrase. Shin et al. (2010) reported that amylosucrase treatment resulted in the increase in the branch chain length of the amylopectin and the decrease in the proportion of short chains by the

elongation of external chains at non-reducing end of an acceptor molecule by amylosucrase. Therefore, compared with Raw, the distribution curves of AS treated starches shifted to higher DP with increasing reaction time, and the proportion of low DP fraction decreased in this study.

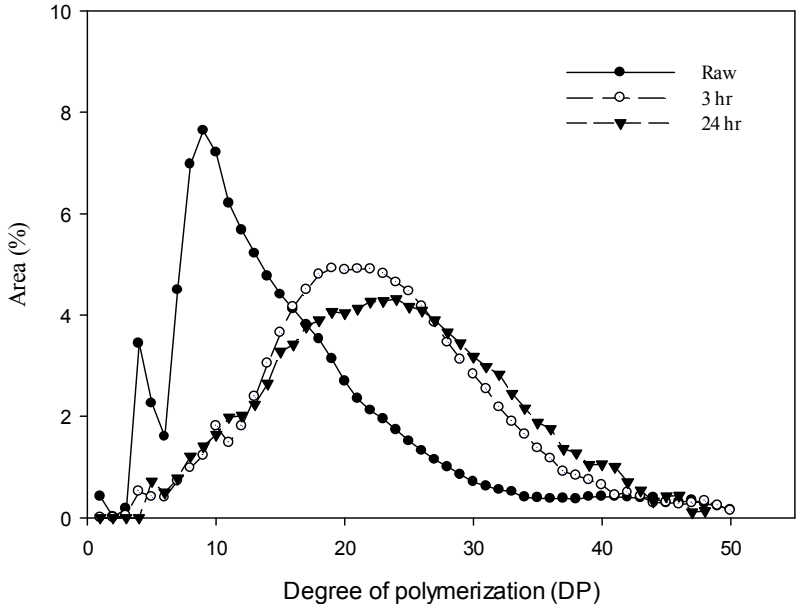

Figure 1. Branch-chain length distributions of AS-treated starches

Table 1. Percent branch-chain length distributions of AS-treated starches

Samples	Percent distribution (%)					Average DP
	DP[1] ≤ 5	DP 6-12 (A)	DP 13-24 (B_1)	DP 25-36 (B_2)	DP ≥ 37 (B_3)	
Raw	5.51±0.81[a, 3), 4)]	39.44±1.14[a]	38.88±0.92[c]	11.61±1.96[b]	4.57±0.71[b]	16.05±0.52[c]
AS3	1.29±0.32[b]	9.32±0.82[b]	49.76±2.19[a]	34.46±1.65[a]	5.16±1.91[b]	22.59±0.34[b]
AS24	1.02±0.32[b]	8.30±1.30[b]	44.17±2.48[b]	36.48±0.03[a]	8.42±0.56[a]	23.75±0.35[a]

[1)] DP, degree of polymerization.

[2)] Data are expressed as average value and standard deviation of three replications.

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

2. Apparent amylose and complex contents

The apparent amylose content of starches and the value of complex contents are shown in Table 2. This result shows that the effect of AAC on the extent of starches complexed with butyric acid.

For determination of AAC, colorimetric spectra via amylose-iodine complex formation were used and defined the amount of amylose in starches (raw, As 3, AS 24). Iodine is able to penetrate the granule and form a complex with lipid free amylose and long linear branches of amylopectin. The AAC value of AS treated starches was significantly higher that of raw resulting in the higher blue value of iodine - amylose complex of AS treated starches that of raw starch. Thus, the AAC value increased as the chain length of starch increased. This result represents the increase of the binding-site where the amylose or amylopectin-iodine complexation can occur, and the binding-site also depends on the elongation of chain length that was dependent upon AS treatment time.

On the other hand, the content of complex also increased with increasing chain length. It indicates that the ability to form complexes with butyric acid increases as the chain length increases. In previous studies, there were some reports that butyric acid rarely form a complex with potato starch. However, butyric acid had the ability to form a complex with starch having extended

chain length, even though it had very low ability of complex formation with raw starch in this study.

Table 2. Apparent amylose and complex contents

	AAC	Complex content (%)
Raw	28.19±330.28[f]	
Raw ctr	54.81 ±0.42[a]	
Raw-BA	45.15±0.85[b]	9.66±0.78[c]
AS 3	30.23±0.49[e]	
AS 3 ctr	29.78±0.68[e]	
AS 3-BA	6.84±0.28[i]	22.94±0.61[b]
AS 24	36.89±0.29[d]	
AS 24 ctr	39.18±0.88[c]	
AS 24-BA	9.87±0.50[h]	29.31±0.47[a]

[1)] Data are expressed as average value and standard deviation of three replications.

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

4. Thermal properties

The thermal properties of complex control (ctr) and complexed starches (-BA) were measured by differential scanning calrorimetry (DSC) and are displayed in Table 3. Changes in Onset (T_o), melting peak (T_p), conclusion (T_c) temperatures, and melting enthalpy (ΔH) of endotherms have been shown to reflect the crystallinity and structure of starches. Generally, T_p corresponds the perfection of crystals, T_o and T_c are associated with melting of the weakest crystallites and the strongest crystallites, respectively (Barichello et al., 1990; Biliaderis et al., 1980). The extent from T_o to T_c ($T_c - T_o$; ΔT) is related to the degree of heterogeneous of the structure (Wongsagonsup et al., 2008). ΔH values are used to measure the needed energy to dissociate helical structure of complexes or disrupt the double helices of long chain length in crystallites (Singh et al., 2006).

Amylose-fatty acid complexes exist in two thermally distinct forms, namely type Ⅰ and type Ⅱ, depending on the dissociation of helical amylose complexes (Godet, Bizot, et al., 1995; Karkalas et al., 1995; Snape et al., 1998; Tufvesson & Eliasson, 2000). Type Ⅰ forms have lower dissociation temperatures, a random distribution of aggregated helical amylose chains, and are considered to be amorphous region. On the other hand, type Ⅱ forms have a typical crystalline organization of amylose complexes that require

higher temperature to dissociation, and they are responsible for the distinctive V type XRD pattern. The various forms of amylose complexes are generated depending on the conditions employed during complexation: temperature, ligand type and reaction time (H.D. Seneviratne & C.G. Biliaderis, 1991).

4-1. Control samples

Ctr starches that were incubated at 90℃ for 24 h displayed a single endothermic peak for retrogradation. When AS reaction time increased from 0 to 24 h, all of T_o, T_p, T_c, and ΔH increased and the melting temperature range ($T_o - T_c$) broadened gradually. This thermal effect could be described with the melting of starch crystallites that were due to packing of the double helices formed between elongated amylopectin chains. The melting process of crystallites involves the uncoiling and melting of the branch chains of amylopectin that are packed as double helix. Starches with longer amylopectin branch chains and higher crystallinity display higher transition temperature (T_o, T_p, T_c), and increased ΔH values (Singh et al., 2006). Shin et al. (2010) also had demonstrated the increases in the transition temperature and enthalpy of AS treated ctr starches, resulting from the longer interchain associations of elongated chains in amylopectin and amylose. The obtained

result in this study was entirely consistent with above mentioned previous reports.

The T_p and ΔH of ASctrs exhibited significantly higher than those of Raw ctr. Higher values in T_p and ΔH are influenced by length of double helices, and represent the degree of ordered crystalline region and structural stability (Hoover & Manuel, 1996; Shin et al., 2010). Therefore, based on this result, it could be inferred that the crystallites of ASctrs samples in this study were more stable than those of Raw ctr. The variability in endotherm peak of ASctrs would be drawn from elongated chains, which facilitate the formation of double helices causing higher values in thermal parameters (T_p and ΔH). The crystallites consisting of elongated chains of amylopectin and amylose provided the variability in ΔT values as well as the higher transition temperature. The association of elongated chains of AS treated starch seemed to contribute the heterogeneity of crystalline region, following the formation of crystallites varying in size and perfection.

4-2. Complex samples

In contrast to Ctr samples, melting endotherms of complexes with BA involved dissociation peak, which was separate from the starch gelatinization, indicating helical complex formation. This finding also showed a considerably different thermal behavior between AS treated and un-treated starch complexes.

Raw-BA exhibited a broad endotherm at about 135℃ (peakⅢ), which was mainly attributed to the melting of amylose complexes. Previous studies demonstrated that endotherms at about 100℃ were attributed to the phase transition of amylose-lipid complexes, whereas endothermic peaks above 145℃ corresponded to melting of retrograded or associated amylose crystallites (Eliasson & Krog, 1985). Thus, it could be considered that the endotherm of peakⅢ was indicative as a melting or dissociation of amylose-BA complex, which corresponded to the melting of type Ⅱ complex. However the amount of type Ⅱ complex crystals might be very small in reference to the ΔH value of peakⅢ.

In striking contrast to raw-BA, both AS3-BA and AS24-BA showed multiple endothermic transition below temperature of 100℃. These bimodal

melting profiles could suggest the presence of crystalline structures with different thermal stability (Biliaderis et al., 1985). It could be interpreted that the first (peak Ⅰ) corresponded to melting of complex formed by elongated amylopectin, whereas the second (peak Ⅱ) endotherms were related to crystallites formed by amylopectin not turning into a complex.

AS-BAs gave rise to the first peak (peak Ⅰ) at about 66℃, even though ASctrs didn't at 66℃, suggesting that the crystallites of peak Ⅰ were formed by some structural reorganization caused by BA addition. Furthermore, The previous study reported that long branch chain amylopectin could form helical complex as like amylose (Jane et al., 1999). Therefore, thermal profile of peak Ⅰ was attributed to the dissociation of complexes formed by elongated amylopectin branch chains which are capable to act like amylose for complexation.

T_p of complex generally increases with the amylose chain length, and the higher T_p of complex reflects that the thermal stability of helical complex is greater (Eliasson & Krog, 1985; Godet, Bizot, et al., 1995; Kawai et al., 2012). From in this viewpoint, the low T_p values of AS-BAs showed that elongated amylopectin branch chains formed thermally less stable complexes with BA, compared to Raw-BA, indicating the presence of type Ⅰ

complexes which are characterized by more disordered and imperfect crystalline conformation than type Ⅱ complexes (Biliaderis et al., 1985).

ΔH values of endothermic transition are a measure of the amount of complex (Kawai et al., 2012), which is attributed to the dissociation of inclusion complexes. This results demonstrated that the compexes formed with the extent of AS24-BA>AS3-BA>Raw-BA. Furthermore, in considering of complex content values (table 2) and ΔH as a measure to quantity of the complex, AS-BAs exhibited higher values of complex content and higher enthalpy (peak Ⅰ) compared to raw-BA. In addition, it could be found that the result of ΔH values concurred with the complex content values.

On the other hand, AS-BAs showed lower values of T_p than AS-ctrs. This resulted from the carboxylic group of butyric acid. As the helical space in amylose is too narrow to accommodate bulky groups, the polar groups cannot be included in the helix (Biliaderis et al., 1985). Therefore the bulky carboxylic group located outside the helices could disrupt chain packing, and resulting in lowering the dissolution temperature due to higher steric interactions caused by the carboxylic group (Snape et al., 1998).

In case of the second peak (peak Ⅱ), it would be presumably regarded as the melting of un-complexed amylopectin with double helical structure. The transition of peak Ⅱ showed the melting temperature similar to those of

ASctrs (AS3ctr and AS24ctr) which were associated with melting of amylopectin double helices. In addition, ΔH in the second peak of AS-BAs were lower compare to ASctrs. This was probably due to the disruption of amylopectin double helices (Hoover & Manuel, 1996).

Table 3. DSC parameters of starches complexed with butyric acid (BA) and cooked without butyric acid (ctr)

Samples	Peak Ⅰ (Type Ⅰ complex)				Peak Ⅱ				Peak Ⅲ (Type Ⅱ complex)			
	T_o (℃)[1)]	T_p (℃)	T_c (℃)	Δ H (J/g)	T_o (℃)	T_p (℃)	T_c (℃)	Δ H (J/g)	T_o (℃)	T_p (℃)	T_c (℃)	Δ H (J/g)
Raw ctr [2)]					49.56±0.18[d]	57.40±0.70[d]	68.62±0.90[d]	2.04±0.05[c, 5), 6)]				
AS3ctr		N.D.[4)]			73.88±2.99[c]	88.53±0.21[b]	101.82±0.73[b]	6.80±0.98[a]		N.D.		
AS24ctr					76.50±0.52[b]	92.74±0.03[a]	104.60±1.60[a]	6.88±0.76[a]				
Raw-BA[3)]		N.D.				N.D.			126.63±3.40	135.61±0.70	143.38±0.57	2.24±0.26
AS3-BA	56.16±1.77[a]	66.53±1.19[a]	77.86±1.26[b]	14.82±0.07[b]	81.40±1.40[a]	86.45±0.60[c]	97.32±0.79[c]	4.66±0.13[b]				
AS24-BA	53.93±0.79[b]	66.97±0.71[a]	80.24±0.06[a]	15.86±0.70[a]	81.89±0.56[a]	88.52±0.70[b]	97.34±1.04[c]	3.80±0.58[b]		N.D.		

[1)] T_o, T_p and T_c indicate the onset, peak and conclusion temperatures of melting, respectively.

[2)] Cooked starch without butyric acid at 90℃ for 24 h.

[3)] Complexed starch with butyric acid at 90℃ for 24 h.

[4)] Not detected within the temperature range.

[5)] Data are expressed as average value and standard deviation of three replications.

[6)] The values with different 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 raw and AS treated starches are shown in figure 2, and those of the starches complexed with butyric acid are displayed in figure 3.

Raw potato starch exhibited a typical B-type pattern with major peaks at 5.7°, 17.1° and 22.1° and weaker peaks at 15.1°, 19.7°, 24,1° and 26° (Hanashiro et al., 1996). AS treated starches showed similar X-ray diffraction pattern as that of raw without changes in type of X-ray diffraction pattern. Starch-butyric acid complex displayed a pattern which was a mixture of B type and V type patterns. The characteristics main peak of the V packing were observed at 19.8° (Biais et al., 2006). On the other hand, there was no peak corresponded to free recrystallized ligand indicating free butyric acid.

The crystallinity of Starch-BA complexes were higher than that of the controls (Raw ctr, ASctrs). Among the control starches, the crystallinity increased with increasing chain length. The relative crystallinity of starch was related to the amylopectin fraction and degree of disordered double helices.in starch granules (Liu et al., 2012; Vermeylen et al., 2004). The elongated chains forming double helices in ASctrs longer and greater interchain associations than those of Raw ctr (Shin et al., 2010). It was

speculated that the double helices could occur easily among elongated branch chains, and attributed to form the more perfect crystallites.

On the other hand, the crystallites of Raw-BA increased than that of Raw ctr, whereas crystallites of AS-BAs decreased than that of ASctrs. Based on the DSC data, Raw-BA complexation could cause the amylose transformation from coil form to single helical structure (Biliaderis et al., 1985). This helical structure could attribute to form more perfected conformation and thermal stable structure (Godet, Bizot, et al., 1995; Kawai et al., 2012). However, the complexation of amylopectin cause the dissociation of amylopectin double helices, resulting in the decrease of the level of crystalline order (Ma et al., 2011). Therefore, it was thought that the AS-BAs complex would have very poor crystalline structure.

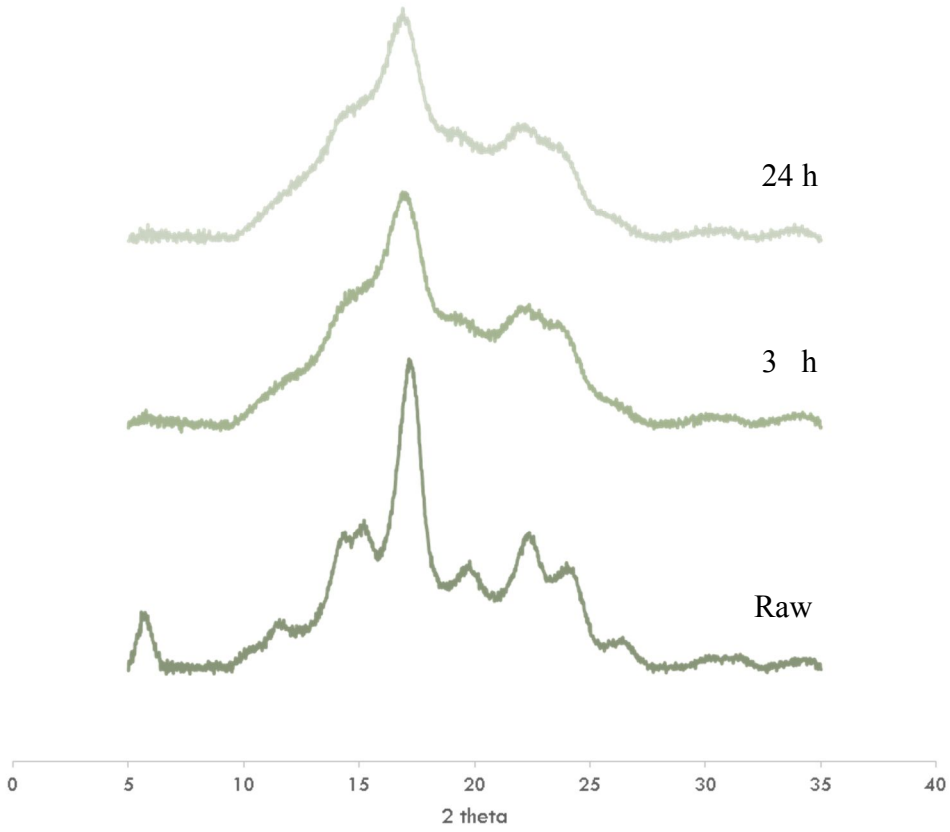

Figure 2. X-ray diffraction patterns of AS-treated starches

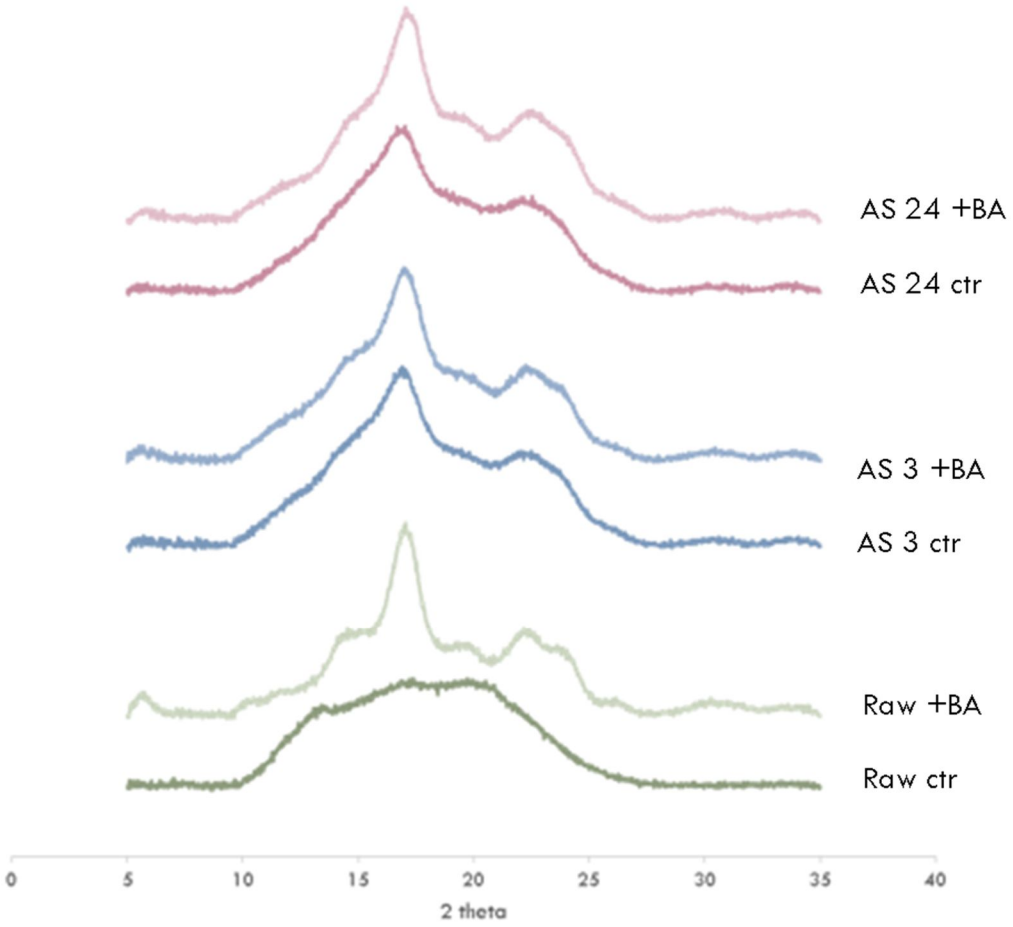

Figure 3.
X-ray diffraction patterns of starches complexed with butyric acid (BA) and cooked without butyric acid (ctr)

Table 4.
Relative crystallinity of starches complexed with butyric acid (BA) and cooked without butyric acid (ctr)

Samples	Relative crystallinity
Raw ctr [1)]	45.07±0.55[d, 3), 4)]
Raw-BA [2)]	50.10±0.46[c]
AS3ctr	52.57±0.49[b]
AS3-BA	51.93± 0.35[b]
AS24ctr	53.30± 0.17[a]
AS24-BA	52.23±0.25[b]

[1)] Cooked starch without butyric acid at 90℃ for 24 h.

[2)] Complexed starch with butyric acid at 90℃ for 24 h.

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

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

5. FT-IR spectroscopy

Fig.3 shows FTIR spectra for starch samples, and the ratios of absorbance 1022/995 cm^{-1} were presented in table 5.

In previous work, infrared spectroscopy has been widely used for investigating the changes in starch structure on a short-range molecular level. The difference between the two X-ray crystallinity patterns (A and B) is resulted from the packing double helices, defined as the long-range order, whereas FTIR is not able to tell the difference between A and B polymorphs. FTIR differences between various starches are only related to the variation in the ratio of the amounts of ordered to unordered fractions with starches (Sevenou et al., 2002). IR spectra of starch show the C-C, C-O, C-H stretching and COH bending modes in the 1300-800 cm^{-1} region of wavenumber (van Soest et al., 1995). The intensity ratios (R) of 1047/1022 and 1022/995 cm^{-1} are useful to quantify the degree of order in starch in comparisons with other starch conformation. The IR absorbance band at 1047cm^{-1} are sensitive to the amount of ordered starch or crystallites which are more organized than the amorphous region. On the other hand, the band at 1022 cm^{-1} is characteristics of amorphous region of starch, and this band is sensitive to changes of crystalline regions. The band at 995cm^{-1} represents hydrated crystalline domains related to hydrogen bonding (van Soest et al., 1995). However, the band at 1047 cm^{-1} is not sensitive to water contents,

unlike the band at 995 cm^{-1}. Varatharajan et al. (2010) have suggested that R value of 1022/995 cm^{-1} is a better indicator of changes in helical order than that of 1047/1022 cm^{-1}. Therefore, in this experiment, the R value of 995/1022 cm^{-1} seemed to represent molecular orders more adequately than 1047/1022 cm^{-1} ratio. Generally, the semi-crystalline raw starches present higher R value of 1045/1022 cm^{-1} and lower R value of $1022/995^{-1}$, whereas amorphous starches are identified with lower R value of 1045/1022 cm^{-1} and higher R value of 1022/995 cm^{-1} (Htoon et al., 2009).

As presented in Table 4, the R value of Raw ctr was 1.06, but that of Raw-BA was lower value, 0.91. It showed that the more ordered arrangement with helical structure, and it might be resulted from the formation of Raw-BA complexes corresponded to amylose fraction. As mentioned above, coil form of amylose, which are related to amorphous region, are transformed into helix form by addition of complexing agents (Biliaderis et al., 1985). This result, therefore, could be interpreted that the helical structure of complex induced the association of amorphous amylose, and contributed to more ordered organized structure compared to the coil form of amylose chains. On the other hand, R values of both ASctrs displayed 0.89 at the same time. AS-BAs exhibited 0.90 and 0.92 respectively with a slightly decrease compared to their controls. It reflected AS-BAs conformation were less ordered form compared to ASctrs. It could be interpreted that AS-BAs

related to amylopectin fraction were attributed to the dissociation of double helixes of amylopectin, or a structure formed by uncomplexed amylopectin chains (Ma et al., 2011). Furthermore, the carboxylic group of fatty acids must be located outside the helical structure, and they are expected to disrupt chain packing (Godet, Tran, et al., 1995). However, the helical structure content of starches, observed by FTIR, were not consistent with the level of crystalline packing obtained from X-ray diffractograms. This presents that not all of the starch molecules ordered helically are arranged into crystalline structure (Htoon et al., 2009).

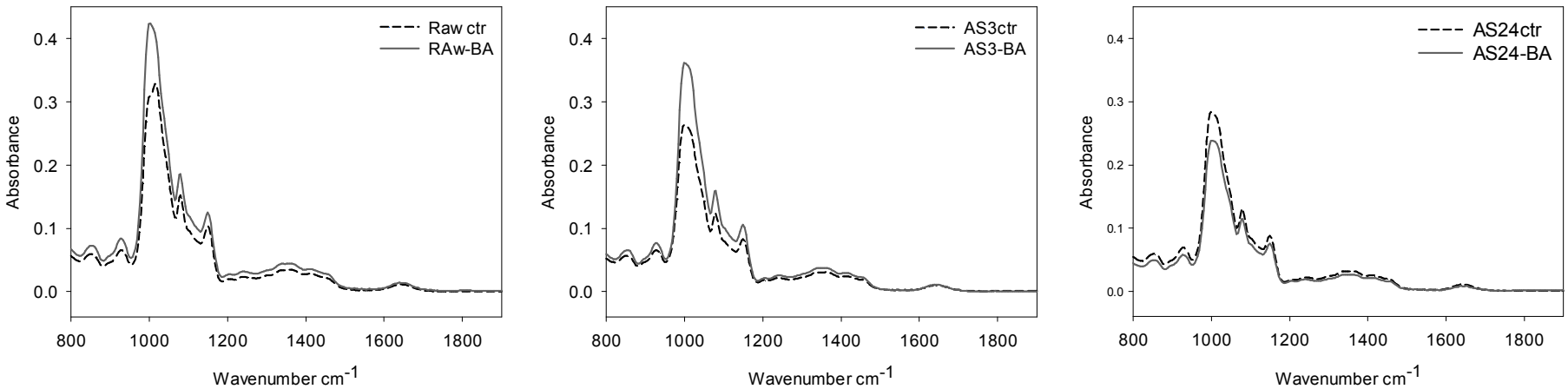

Figure 4. FT-IR spectra of starches complexed with butyric acid (BA) and cooked without butyric acid (ctr)

Table 5.
Short-range molecular orders of starches complexed with butyric acid (BA) and cooked without butyric acid (ctr) measured by fourier transform infrared spectroscopy

Samples	R (1022/995 cm^{-1}) [1)]
Raw ctr	1.06
Raw-BA	0.91
AS3ctr	0.89
AS3-BA	0.90
AS24ctr	0.89
AS24-BA	0.92

[1)] Intensity ratio

6. Starch digestibility

Table 4 show the *in vitro* digestibility of complex control (ctr) and starches complexed with butyric acid. Starch is divided into three fractions which are RDS, SDS, and RS by digestion time according to the Englyst assay (Englyst et al., 1992).

6-1. Control samples

Compared to the Raw ctr, both AS3ctr and AS24ctr, which were cooked without added BA, produced remarkably decreased RDS, but increased SDS and RS. Shin et al. (2010) also presented the digestion profile of AS treated starches, concluding that elongated chains of amylose and amylopectin allow the formation of crystallites with slow digestive property. The results obtained in presented study showed that structural changes of modified starches could affect the in *vitro* starch digestibility, in agreement with previous studies. A certain difference of digestion pattern would be due to the structural properties of the starch granules (Englyst et al., 1992; Jane et al., 1999). AS treated starches had longer chains of amylose and amylopectin than those of Raw. Longer chains to form stronger double helices result in more dense crystalline structure, which are responsible for resistant to digestibility of starches (Parada & Aguilera, 2012; Zhang et al., 2006).

6-2. Complex samples

With the addition of BA, complexes of raw and AS treated starches displayed digestion pattern with distinct differences. During complexation,

During complexation, the rearrangement caused by polar function group of butyric acid would decreases the interaction between double helical amylopectin, and promote a greater exposure of the amylopectin molecules to the action of the enzymes. This events would contribute to the hydrolysis rate of AS-BAs compared to ASctrs.

Raw-BA had a lower proportion of RDS and higher proportion of SDS and RS. It showed that the amylose-lipid complex in Raw-BA contributed to decreased enzymatic digestion rate (Kawai et al., 2012). Furthermore this trend of hydrolysis were in agreement with the melting temperature values discussed above (table 3), which showed the thermal transition of Raw-BA (135.61℃) was much higher than that of Raw ctr (57.40℃). Previous studies reported that enzymatic resistance of amylose-lipid complexes with the more crystalline structure are characterized by higher T_p value (Eliasson & Krog, 1985; H. D. Seneviratne & C. G. Biliaderis, 1991).

In case of AS-BAs, they showed that a higher proportion of RDS and lower proportion of RS compared to ASctrs. This changes could be interpreted that the bulky carboxylic group of BA influenced the accessibility

of starch digestive enzyme to starch molecules. As mentioned above, the polar group located outside helices could restrain helices packing, therefor the conformation would be more disorder, compared to AS ctrs.

Table 6.
Contents of RDS, SDS and RS of starches complexed with butyric acid (BA) and cooked without butyric acid (ctr)

	RDS[3] (%)	SDS (%)	RS (%)
Raw ctr[1]	62.30 ±1.13[a, 4), 5)]	7.38 ±1.03[d]	30.32 ±0.95[e]
Raw-BA[2]	41.54±1.92[b]	16.91±1.25[c]	42.03±2.15[c]
AS3ctr	25.72±1.19[d]	16.31±0.42[b]	57.96±1.11[a]
AS3-BA	46.36 ±2.37[b]	18.02 ±0.89[a]	35.62 ±1.48[d]
AS24ctr	26.32 ±1.42[d]	13.08 ±0.65[c]	60.60 ±1.93[a]
AS24-BA	39.30±3.46[c]	11.88±1.16[c]	48.83±2.37[b]

[1] Cooked starch without butyric acid at 90℃ for 24 h.

[2] Complexed starch with butyric acid at 90℃ for 24 h.

[3] **RDS**: the amount of glucose released after 10 min, **SDS**: amount of glucose released between 10 and 240 min hydrolysis, and **RS**: the amount of unhydrolysed glucose after 240 min

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

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

CONCLUSION

In this study, potato starches of raw and AS treated starches complexed with butyric acid. After AS treatment, the proportion of longer branch chains increased due to the elongation of amylopectin chains. The longer chains of amylose or amylopectin showed the higher iodine binding capacity, indicating the stronger ability of complexation of starch. Thermal profile presented that the endotherms of Raw-BA was related to amylose fraction, and that of AS-BAs was related amylopectin fraction. Thermal properties of AS-BAs had the lower melting temperature than that of their control due to the bulky carboxylic group of butyric acid. The carboxylic group induced disruption of amylopectin double helices, resulting in the decrease of the level of crystalline order. Therefore, the X-ray pattern of AS-BAs complex displayed V type pattern with a peak of gentle slope, and the R value of 1022/995 cm^{-1} in FTIR showed that AS-BAS were less ordered that ASctrs. Furthermore, digestion profile showed that AS-BAs was less resistant to enzymatic hydrolysis compared to ASctrs.

This study propose the possibility that amylopectin-butyric acid inclusion could be formed by control of the branch chain length of amylopectin.

Furthermore, results obtained in this study presented that the complex with amylopectin had different structural characteristics with amylose complex. It is suggested that the starches complexed with butyric acid provide functional food ingredients containing bioactive substance. Further studies could be focus on the amylopectin complex with more various range of branch chain.

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국문 초록

Neisseria polysaccharea 유래의 amylosucrase (AS)는 수크로오스를 기질로 하여 α-(1, 4)-글루칸을 생산하는 효소로, 수크로오스의 분해로 생긴 글루코오스를 수용체 비환원성 말단에 붙여서 사슬을 연장시키는 반응을 일으킨다. 이 연구에서는 감자 전분에 3 시간, 24 시간으로 AS 처리 시간을 다르게 하여 사슬 길이를 연장시킨 후 단쇄 지방산과 복합체를 형성시키고, 복합체 함량, X-선 회절 및 상대적 결정화도, 분자배향도, 열적특성, 소화패턴을 측정하여 사슬길이를 연장시킨 전분-부티르산 복합체의 구조적 특성을 알아보았다. 그 구조적 특성을 살펴보았다.

전분 현탁액 (2% w/v)에 AS 20,000 U 를 처리하고 30°C 에서 3 시간(AS 3), 24 시간(AS 24) 동안 반응시켜 사슬 길이가 다른 전분을 얻었다. 각각의 사슬 길이를 HPAEC-PAD 를 통해 측정하여 AS 처리 시간이 증가할수록 DP ≥ 25 의 긴 사슬들이 증가함을 확인하였다. 생전분, AS 3, AS 24 전분 현탄액 (5% w/v)을 121℃에서 30 분 동안 고압솥에서 처리한 후 부티르산을

첨가하고, 90℃에서 24 시간동안 반응시켜 부티르산과 복합체를 이룬 전분을 얻었다. 겉보기 아밀로오스 함량과 복합체 형성율은 생전분, AS 3, AS 24 순으로 증가하였으며, 이는 사슬 길이가 증가할수록 복합체 형성률이 높아짐을 알 수 있었다. X-선 회절 유형에서 생전분과 AS 처리한 전분은 B 형, AS 처리한 전분의 복합체는 V 형을 나타낸 것을 통해 복합체 형성을 확인하였다. 연장된 사슬 구조를 가진 AS 처리한 전분은 열적특성에서 용융점과 용융 엔탈피 값이 생전분 보다 높았으며 상대적 결정화도, 분자배향도, 난소화성 전분(RS) 함량도 증가하였다. 한편 복합체의 열적특성에서 생전분 복합체는 아밀로오스 복합체, AS 처리한 전분복합체는 아밀로펙틴 복합체를 의미하는 흡열피크가 나타났으며, 부티르산의 카르복실기가 AS 처리한 전분-부티르산 복합체의 사슬구조 재배열 과정에 영향을 주어, 복합체의 용융점이 대조군보다 낮아졌다. 복합체의 상대적 결정화도, 분자배향도, RS 함량 변화 양상에서, 생전분 복합체는 코일형 아밀로오스가 단일나선 구조의 복합체를 형성하여 대조군보다 증가했으나, AS 처리한 전분복합체의 경우 아밀로펙틴이 이중나선 구조 대신 복합체를 형성하여 대조군 보다

감소하였다. 이 연구 결과는 AS 처리한 전분을 이용하여 복합체 형성율을 높일 뿐만 아니라 아밀로펙틴 복합체는 구조적으로 이중나선 구조가 감소하고, 카르복실기 의해 사슬구조가 정연하게 배열되는 것이 방해 받음을 보여주었다.

이전 연구에서는 카프릭산보다 사슬길이가 긴 중쇄 지방산과 아밀로스의 복합체에 관한 연구가 주로 이루어졌으며, 단쇄 지방산과 아밀로스, 아밀로펙틴의 복합체에 대한 연구는 미비한 편이다. 이 연구 결과는 AS 처리를 함으로써 전분의 사슬길이를 연장시켰을 때, 단쇄 지방산의 복합체를 형성할 수 있는 가능성을 제시하였다. 대장 건강과 관련이 있는 부티르산을 함유한 기능성 식품 개발에 활용 할 수 있을 것이다.

주요어 : 아밀로수크레이스, 감자 전분, 복합체, 부티르산,

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