Changes in the Molecular Structure of Potato Starch Conferred Digestion Resistance by Fatty Acid Addition and Heat Treatment

Shiori OKI¹, Chika SATO², Natsuki KUBOTA², Keita NAKASHIMA³, Kyoko ISHIKAWA¹,², Yoshinobu AKIYAMA¹,²,†

¹Graduate School of Bioresource Science, Akita Prefectural University, 241-438 Kaidobatanishi, Nakano, Shimoshinjo, Akita city Akita 010-0195, Japan
²Faculty of Bioresource Science, Akita Prefectural University, 241-438 Kaidobatanishi, Nakano, Shimoshinjo, Akita city Akita 010-0195, Japan
³House Foods Corporation, 1-4 Takanodai, Yotsukaido, Chiba 284-0033, Japan

X-ray diffraction (XRD) was used to analyse potato starch with a 20% moisture content that was made digestion-resistant by the addition of palmitic acid and heat treatment at temperatures in the range of 120–160°C. This starch was also analysed for the quantity of fatty acids able to form starch complexes and in vitro digestion test. Changes in molecular structure that accompany starch–fatty acid complex formation were then investigated based on the results from these analyses. XRD analysis confirmed that fatty acid addition caused emergence of new peaks in the region of 2θ=13, 19°. There was a strong positive correlation between the 2 new peaks and the internal free fatty acid content, which also increased at higher heat treatment temperatures. This positive correlation suggested these peaks are attributable to structures from the starch–fatty acid complex.

Keywords: resistant starch, internal free fatty acid, starch–fatty acid complex, X-ray diffraction

1. Introduction

As the prevalence of diabetes mellitus continues to rise, this lifestyle disease has recently become a serious health issue in Japan. Focusing on controlling postprandial blood glucose, the authors are conducting studies aimed at developing starches that can suppress postprandial elevation of blood glucose. Many digestion-resistant starches currently on the market, such as digestion-resistant dextrins and cross-linked starches, are created by chemical modification. These starches must be displayed as food additives on food labelling, and there is a need for new digestion-resistant starches that are more acceptable to consumers and not categorized as food additives. Mori et al. [1] reported that forming a starch–fatty acid complex by the simple food processing treatment of adding fatty acids (palmitic acid and linoleic acid) and heat treatment introduces fatty acids into the helical structure of starch and can confer digestion resistance to potato starch. Nakashima et al. [2] also reported that adding palmitic acid and linoleic acid, as well as lauric acid, myristic acid, stearic acid, and oleic acid also conferred digestion resistance to potato starch, and the quantity of fatty acids capable of forming a complex with starch (hereinafter referred to as “internal free fatty acids”) contributed to reduced digestibility. Nevertheless, the detailed mechanism behind formation of this starch–fatty acid complex, which is regarded as the reason digestion resistance is conferred to starch, has yet to be elucidated.

Powder X-ray diffraction (XRD) is one method of analysing physical changes to starch based on its crystal structure. Starches are broadly separated into A-, B-, and C-types based on the crystal structural pattern observed in X-ray diffraction charts obtained by XRD analysis. Cereal starches, such as rice and wheat starch, are categorized as A-type starches. When these starches are subjected to heat–moisture treatment by heating above the gelatinization temperature under conditions of constant moisture content, their double–helical structure (A-, B-, and C-type) changes to a single–helical structure (V-type) [3]. V-type amylose that has a single helical structure is known to form complexes with iodine, fatty acids, and other compounds [4]. Since rice starch, an A-type starch, contains a large quantity of lipids [5],

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when Shih et al. [6] subjected rice to heat–moisture treatment, they reported the formation of amyllose–lipid complexes and observed a peak in the region of $2\theta=20^\circ$. Meanwhile, potato starch, which is categorized as a rhizome starch, is a B-type starch. When subjected to heat–moisture treatment, B-type starches change into C-type starches, which have an X-ray diffraction pattern between that of A- and B-type starches [7]. Wang et al. reported that effects of fatty acid chain length on properties of potato starch–fatty acid complexes under high moisture content at 63°C [8], however there are no reports concerning potato starch–fatty acid complexes forming at low moisture content and over 120°C. Potato starch, a B–type starch, contains a small quantity of lipids compared to cereal starches, and amylose–lipid complex formation, which is confirmed to occur after heat–moisture treatment of A-type starches, has not been reported to occur with B–type starches.

One objective of this study was to determine by X–ray diffraction the changes in molecular structure that arise from subjecting starch to a digestion resistance treatment consisting of the addition of free fatty acids and heat treatment. A further objective of this study was to quantify the amount of internal fatty acid, which is considered able to bond with starch during starch–fatty acid complex formation and thereby confer digestion resistance to starch, and investigate the relationship between changes in molecular structure and internal free fatty acid content to elucidate the mechanism by which the addition of fatty acids confers digestion resistance to starch.

2. Materials and Methods

2.1 Materials

Potato starch and palmitic acid (Wako Pure Chemical Industries Inc., Osaka, Japan) were used. Porcine pancreas $\alpha$-amylase (EC 3.2.1.1) and amyloglucosidase (EC 3.2.1.3) (Megazyme Inc., Bray, Ireland) were used for enzymatic starch hydrolysis. For methyl esterification of palmitic acid, 12% (w/w) boron trichloride–methanol (Sigma–Aldrich Inc., St. Louis, MO, USA) was used.

2.2 Sample preparation

Potato starch with 15% moisture content was adjusted to 20% moisture content (in wet basis), and palmitic acid was added to 5% (w/w in wet basis) in starch and mixed. The fatty acid 5% added sample and a raw starch sample adjusted to 20% moisture content were each placed in stainless–steel airtight containers and heated in an oil bath until the internal sample temperature reached a target temperature in the range of 120–160°C. Sample temperature was measured with a thermocouple inserted in the sample container. After reaching the target temperature, the samples were rapidly cooled in ice water, then milled by a powder mill (Y–308B, Osaka Chemical co., ltd. Osaka Japan), and sieved between 75 and 250 $\mu$m. The sample with palmitic acid was used as the palmitic acid 5% added sample. The sample with no palmitic acid added was subjected to the same heating method and used as the heated sample.

2.3 Measurement of starch digestion rate by enzymatic hydrolysis

Sample digestibility was measured using the method described by Nakashima et al. [2]. After adding 15 mL of 0.1 M maleate sodium buffer solution (pH 6.0) containing 0.003 M of CaCl$_2$ to 75 mg of sample, the mixture was heated at 100°C for 60 minutes. After cooling to 37°C, samples were incubated after adding 0.069 U of porcine pancreas $\alpha$-amylase (37°C, 90 min, 100 turn per minute). During incubation, 0.4 mL of the sample was collected with a microtube at 15, 30, 60 and 90 minutes, and immediately heated at 100°C for 5 minutes to deactivate the $\alpha$-amylase. Samples were then centrifuged (11,752×g, 5 min) and 0.1 mL of supernatant was collected. After adding 0.37 mL of 0.4 M sodium acetate buffer solution (pH 4.5) and 0.1 U of amyloglucosidase to the supernatant, the mixture was shaken (60°C, 45 min, 85 turn per minute). Glucose was then quantified by the glucose oxidase/peroxidase method. Measurements were performed 3 times for each sample with palmitic acid added and for each sample with no fatty acid added (heated sample).

2.4 Calculation of estimated glycaemic index

Estimated glycaemic index (EGI) was calculated using the method described by Goni et al. [9]. The relative digestion rate at arbitrary time $(H_i)$ was calculated using eq. (1) by comparing the sample digestion rate to the digestion rate of a white bread standard food. In the equation below, $H_{90}$ is the digestion rate after 90 minutes of enzymatic hydrolysis.

$$Hi=100 \times \left(\frac{\text{sample } H_{90}}{\text{white bread } H_{90}}\right) \quad (1)$$

EGI was calculated using eq. (2) proposed by Goni et al. [9].

$$\text{EGI}=39.21 + 0.803 \text{ Hi} \quad (2)$$
2.5 Determination of the quantity of internal free fatty acid

Internal free fatty acids (IFFA) were quantified using the method described by Nakashima et al. [2]. Using Soxhlet extraction, samples underwent reflux extraction for 17 hours at 85°C with t-butyl methyl ether to extract the outer fats. After removing outer fats, residual samples underwent reflux extraction 3 times for 3 hours at 75°C with 85% methanol to extract the internal fatty acids. Solvent was then removed from the extraction liquid, and the resulting liquid was brought to a fixed volume with n-hexane. This liquid then underwent methyl esterification using 12% (w/w) boron trichloride–methanol, and the IFFA content was determined using a gas chromatograph (GC–2010, Shimadzu Co., Kyoto, Japan). This analysis was performed 3 times for each sample.

2.6 X-ray diffraction (XRD) analysis

The X-ray diffractometer used was an UltraX 18 (Rigaku Denki Co., Ltd., Tokyo, Japan). X-ray diffraction charts were obtained under conditions of a Cu–Kα X-ray source, 50 kV tube voltage, 27 mA tube current, and 2θ = 5.0–40.0° diffraction angle. X-ray diffraction analysis was performed on the heated sample (with no fatty acid added), the fatty acid 5% added sample, and the residual sample after removing palmitic acid attached to the starch surface by Soxhlet extraction. Peak intensities were calculated with RINT 2000/PC soft (Rigaku Denki Co., Ltd. Tokyo, Japan). Analysis was performed 3 times for each sample.

Microsoft Excel 2010 was used for all statistical analyses and the significant test was performed by t-test.

3. Results

3.1 Effect of heating temperature on digestibility

Figure 1 shows the change in EGI of the heated sample and palmitic acid 5% added sample caused by different heating temperatures. The EGI of the palmitic acid 5% added sample was significantly lower than that of the heated sample at all heating temperatures (p<0.01). The EGI of the heated sample also increased with higher heating temperatures. This matches the damage to starch particles caused by heat-moisture treatment of rice and the accompanying increase in enzymatic hydrolysis reported by Takahashi et al. [10]. Meanwhile, the EGI of the palmitic acid 5% added sample falls with higher heating temperatures up to approximately 135°C, with a maximum decrease of 23% compared to the 120°C heating temperature. EGI then remained almost unchanged up to 150°C, but increased again from approximately 150°C.

3.2 Determination of IFFA content

The IFFA content of the palmitic acid 5% added sample is shown in Fig. 2. The IFFA content increased with higher heating temperatures to reach an IFFA content at 160°C that was approximately 6-fold the content at 120°C.

3.3 X-ray diffraction (XRD) analysis

The X-ray diffraction chart for raw sample, heated sample (140°C), palmitic acid 5% added sample (140°C),
and residual sample is shown in Fig. 3a. For the raw sample, there was a B-type X-ray pattern that is characteristic for potato starch consisting of a main peak in the region of $2\theta=17^\circ$, and 2 more peaks in the region of $2\theta=22, 24^\circ$. For the heated sample, peaks were smaller overall compared to those of the raw sample, and the 2 peaks at $2\theta=22, 24^\circ$ became a single peak as seen in the X-ray chart of A-type starches. This demonstrates that there was an increase in the amount of amorphous material that arises from partial gelatinization of starch by heat-moisture treatment [10], and shows transition from a B-type chart pattern to a C-type chart pattern [7]. For the palmitic acid 5% added sample, there was a reduction in the main peak ($2\theta=17^\circ$) that is characteristic of B-type starch, there were 3 peaks with diffraction angles in the region of $2\theta=7, 21, 24^\circ$ attributed to palmitic acid, and 2 peaks in the region of $2\theta=13, 19^\circ$ (P1 and P2, respectively). For the residual sample, the 3 peaks attributed to palmitic acid and the B-type starch main peak were absent, but P1 and P2 were present. Figure 3b shows the change in X-ray diffraction chart pattern caused by different heating temperatures for the residual sample (120–160°C). P1 and P2 intensities both increased with higher heating temperatures. Figure 4 shows the change in integrated peak intensity of the B-type X-ray chart main peak in the region of $2\theta=17^\circ$ and peaks in the region of $2\theta=13, 19^\circ$ (P1 and P2) for the palmitic acid 5% added sample. The intensity of the B-type starch main peak ($2\theta=17^\circ$) fell with higher heating temperatures, but P1 and P2 intensities increased with higher heating temperatures. When the lattice plane intervals of P1 and P2 were calculated based on Bragg’s law shown by $n\lambda=2d\sin\theta$, P1 was 0.63–0.74 nm and P2 was 0.44–0.47 nm.

### 3.4 The relationship between digestibility and P1, P2

We looked at the relationship between integrated intensity of P1 and P2 obtained by XRD and EGI for the palmitic acid 5% added sample. No relationship was found as P1 and P2 increased at higher heating temperatures, but EGI reached a lower limit at around 140°C then increased at higher heating temperatures. However, looking in more detail at EGI from 120°C to 140°C, where EGI reached a lower limit, EGI was negatively correlated with P1 (correlation coefficient of 0.62) and P2 (correlation coefficient of 0.83).

### 3.5 The relationship between IFFA content and P1, P2

The relationship between IFFA content and P1 and P2 integrated intensity for the palmitic acid 5% added sample is shown in Fig. 5. There is a strong correlation between
P1 and P2 intensity obtained by XRD and IFFA content as demonstrated by a correlation coefficient of at least 0.98 ($p<0.05$).

4. Discussion

The EGI of the palmitic acid 5% added sample was significantly lower than the heated sample at all heating temperatures ($p<0.01$). This demonstrates that palmitic acid addition and heat treatment successfully conferred digestion resistance to starch. For the palmitic acid 5% added sample, EGI decreased with higher heating temperatures in the range of 120–135°C and was at most 23% lower than the EGI at 120°C (Fig. 1). Heat–moisture treatment normally causes partial gelatinization of starch and effusion of amylose in an amorphous form. The presence of amorphous amylose together with fatty acids results in inclusion complexes formed of conjugated amylose, where the conjugated amylose is more stable than amorphous amylose and has a lower rate of enzymatic hydrolysis [11]. Consequently, though enzymatic hydrolysis increased at higher heating temperatures for the heated sample, enzymatic hydrolysis was probably suppressed in the palmitic acid 5% added sample due to starch–fatty acid complex formation arising from the addition of palmitic acid. There was almost no change in EGI between heating temperatures of 135°C and 150°C, though EGI reached a lower limit within this range, then increased starting at around 155°C. The quantity of IFFA that is presumed to form starch–fatty acid complexes with starch exceeded 4 mg per 1 g of starch on a dry weight basis at a heating temperature of around 130°C (Fig. 2). This more or less matches the result obtained by Nakashima et al. [2] who reported that adding fatty acids to starch resulted in up to 5 mg of IFFA per 1 g of starch on a dry weight basis and that IFFA contributed to reduced starch digestibility. The increase in EGI from a heating temperature of 155°C is thought to be due to damage to starch particles caused by heat–moisture treatment at high temperatures [12].

For the palmitic acid 5% added sample, the main peak of B-type starch ($M$, $2\theta=17°$) disappeared and new peaks (P1, P2) were observed in the region of $2\theta=13$, 19° from a heating temperature of around 135°C. These results indicate a change in the crystal pattern from B-type to V-type. Abraham [13] subjected cassava starch to heat–moisture treatment (18–21% moisture content, 110°C, 16 h) and confirmed peaks in the region of $2\theta=13$, 20° attributed to the amylase–fatty acid complex structure. Since these peaks occurred at almost the same diffraction angles as P1 and P2 observed in the present study, P1 and P2 are presumed to be peaks arising from the formation of starch–fatty acid complexes between the amylose of potato starch and the added palmitic acid. P1 and P2 increased in intensity with higher heating temperatures, suggesting that starch–fatty acid complex formation is driven by higher heating temperatures. When calculated based on Bragg’s law, shown by $n\lambda=2d\sin\theta$, the lattice plane interval of P1 was 0.63–0.74 nm and that of P2 was 0.44–0.47 nm. Lopez-Rubio et al. [14] reports the peaks at $2\theta=13$, 19° almost entirely consist of a single helix amylose structure, though the location of this structure on the steric structure of starch is unknown. Nevertheless, the molecular structure of starch was changed substantially by the addition of fatty acids and heat treatment, and we observed peaks attributed to a structure that resembles the structure arising from heat–moisture treatment of A-type starch. The 3 peaks in the region of $2\theta=7$, 21, 24° attributed to palmitic acid were observed on the X-ray diffraction chart of the palmitic acid 5% added sample but were absent from the residual sample after Soxhlet extraction. Meanwhile, P1 and P2, presumed to be caused by an amylose–starch complex structure, were present on the X-ray diffraction chart for the residual sample after Soxhlet extraction. This suggested that of the added palmitic acid (5% (w/w)), palmitic acid that bonded with starch to form complexes was not extracted by Soxhlet extraction by ether solvent and remained as a complex in the residual sample, and excess palmitic acid that did not form complexes was present in the starch sample in a free state. We demon-
strated that IFFA content increased at higher heating temperatures, and there was a strong positive correlation between IFFA content and P1 and P2 generation analysed by XRD. No correlation was observed between EGI and P1 and P2 generation analysed by XRD in the heating temperature range of 120–160℃. This result is due to the fall in EGI reaching a lower limit at a heating temperature of around 135℃, while P1 and P2 generation increased with higher heating temperatures. We therefore inferred that even though the formation of starch–fatty acid complexes, as evidenced by P1 and P2, is promoted by higher heating temperatures, there is an upper limit to the quantity of starch–fatty acid complexes that reduces digestibility, though further investigation of this matter is required.

The results of this study confirmed that (1) fatty acid addition and heat treatment to starch cause changes in molecular structure due to the formation of starch–fatty acid complexes and that (2) P1 and P2 are attributable to a molecular structure in starch derived from internal free fatty acids. This result supports the conjecture of Mori et al. [1] that adding fatty acids to starch confers digestion resistance by introducing fatty acids into the helical amylose structure to add a hydrophobic region. However, we were unable to obtain detailed information on the specific location of changes in molecular structure and energy needed for the formation of the starch–fatty acid complexes that arise from fatty acid addition and heat treatment of starch. We therefore intend to use XRD and differential scanning calorimetric analysis to calculate the activation energy of starch–fatty acid complex formation and a stoichiometric analysis under high moisture content to elucidate the detailed mechanism of starch–fatty acid complex formation that was identified by this study as the mechanism by which starch is conferred digestion resistance.

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