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Physicochemical characterization of resistant starch type V (RS5) from manggu cassava starch (*Manihot esculenta*)

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Abstract

Resistant starch has been well known to have beneficial health effects, mainly for digestive tracts. Starch isolated from cassava showed characteristics required to produce resistant starch type 5 (RS5). In this study, we modified tapioca starch through the gelatinization process with the addition of palm oil, coconut oil, stearic acid and lauric acid with 3 treatments, i.e. concentration (1) 10%, (2) 20% and (3) 30%. The result of study showed that modification of starch can decrease starch digestibility, increase levels of resistant starch and lower amylose levels. The analysis using X-Ray diffraction, strearic acid 30% had the peak at 7.5°, 22° and 24°. Peak 7.5° was a amylose-lipid complex as RS5. The profile of starch gelatinization using Rapid Visco Analyzer (RVA) showed that the addition of fatty acid can decrease the value of breakdown, setback, and peak viscosity. These results indicates modification of tapioca starch with fatty acid (stearic acid or lauric acid) can form amylose-lipid complex (RS5).

1. Introduction

Starch is a food source that has high availability and can be used as energy reserves in the form of glycogen in the human body. One type of starch that is widely used is cassava starch or tapioca. Cassava production in Indonesia reached 22 million tons in 2016 (Indonesia Ministry of Agriculture, 2016). One of the cassava varieties in Indonesia is the Manggu cassava variety which is a superior variety from Sukabumi Indonesia with a diameter of 4-5 cm (Carolina, 2009). The amount of amylose content in this cassava around 22.22% (Hartati and Hartati, 2019). Native starch has several deficiencies in physicochemical properties that can inhibit their application in industry, the modified starch techniques were used to produce resistant starch. Modification of starch can increase resistant starch that has functional properties that play a role in the processing, but also resistant to digestion (Kusnandar, 2011; Faridah, *et al.,* 2019). One type of resistant starch is a type 5 or RS5 resistant starch which is a amylose-lipid complex.

The formation of amylose-lipid complexes occurs during gelatinization and its influenced by several factors including chain length and type of fat bond (Aliasson and Krog, 1985), type of starch, water content, polymerization rate, the ratio of amylose to lipid concentration, manufacturing process, pH time and medium starches that contain high amylose will be more easily made complex (Obiro *et al.,* 2012). RS5 has stable properties for processing and resistant to hydrolysis by amylase enzymes (Jane and Robyt, 1984), limiting swelling on starch granules and being resistant to heating (Birt *et al.,* 2013). Amylose-lipid complex formations also affect the pasting properties of starch. Peak viscosity of corn starch decreased by complexing the amylose with lipid (Ai *et al.,* 2013). RS5 has been applied as bread making ingredient that produced lower glycemic index value (Hasjim *et al.,* 2010). Moreover, than that, RS5 has also been applied in bioactive nano-encapsulation production and as flavor ingredient (Obiro *et al.,* 2012).

Starch modification for complexing amylose with lipid has been exhibited by Ai *et al.* (2013) using tapioca starch with 29% amylose content which could decrease enzymatic hydrolysis from 77.5% to 65.5% with stearic acid, also with palmitic acid (66.1%), corn oil (66.4%), oleic acid (66.8%), soy lecithin (69.5%) and linoleic acid (72.0%) with 10% addition of these lipids from the dry

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weight of starch. The complex of amylose-lipid also applied in corn starch with 10% dry basis of oleic acid addition resulting in a peak temperature (Tp = 102.2±0.0ºC) and enthalpy changes (ΔH = 1.9±0.1J/g) (Ai *et al*., 2013).

High availability of resources in Indonesia, including palm oil, coconut oil, stearic acid and lauric acid and also the high amount of amylose content in Manggu cassava may be used to modify starch into amylose lipid complex.

2. Materials and methods

2.1 Materials

Manggu type cassava 9-month originating from Cikarawang, Dramaga Bogor. Palm oil and coconut oil were obtained from a supermarket in Indonesia. The oil used is olein phase, obtained from Supermarket Bogor, stearic acid triple pressed (C16:0 58.29%, C18:0 40.83%), and lauric acid (C12: 0 99.55%.

Other materials were glucose, aquadest, phenol, H_2SO_4 (Merck, Germany), KCL-HCL buffer, pepsin (Sigma P7000, USA), phosphate buffer, α-amylase (Sigma 10065, USA), KOH (Merck, Germany), HCl (Merck, Germany), amyloglucosidase (Sigma A7095, USA), NaOH (Merck, Germany), glacial acetic acid, iodine.

2.2 Preparation and starch modification

Starch extraction can be done by the wet method based on Lingga *et al.* (1986). The process of starch modification for the formation of amylose lipid complex based on Ai *et al*. (2013). The process of modification of starch for the formation of a lipid amylose complex compound is carried out by weighing as much as 4.4 g of tapioca starch weighed into a 250 mL beaker glass, then added 10% lipid (0.4 g) or according to the concentration of lipid, and then added distillate water 12 mL, heated in a water bath with a temperature of 95°C for 8 mins with constant stirring until the starch is perfectly gelatinized. The sterilized starch sample was stored on a baking sheet of alumunium to dry on a drying oven at a temperature of 50°C for 20 hrs. The modified starch has been dried and then mashed using a High-Speed Grinder (RT-02A 150G). Modified cassava using palm oil, coconut oil, stearic acid and lauric acid with 3 treatments, i.e. concentration (1) 10%, (2) 20% and (3) 30% according to the research design (Table 1).

Table 1. Modified tapioca starch design with oil and fatty acids

Treatment	Concentration (% w/w)	Code
Control	0	K
Coconut Oil	10	MK 10
	20	MK 20
	30	MK 30
Palm Oil	10	MS 10
	20	MS 20
	30	MS 30
Stearic Acid	10	AS 10
	20	AS 20
	30	AS 30
Lauric Acid	10	AL 10
	20	AL 20
	30	AL 30

2.2.1 Resistant starch content

About 5 mg of pure glucose was diluted in 50 mL aqua and diluted (0, 10, 20, 30, 40, 50, 60 µg/mL glucose solution). Each concentration was moved into the reaction tube and 0.5 mL of phenol 5%, was added and then mixed well with a vortex. Then, 2.5 mL of concentrated H_2SO_4 was added quickly to each tube and aged for 10 mins. Each tube was aged once more for 20 mins in ambient temperature before measured in term of absorbance using spectrophotometer UV-Vis on 490 nm. The results were used as a standard curve of resistant starch content.

The sample was prepared by mixing with KCL-HCL buffer 5 mL and 0.1 mL pepsin (4000 U/10 mL buffer KCl-HCl) then incubated at 40°C for 60 mins using water bath shaker before cooled at ambient temperature. The sample was added with 4.5 mL phosphate buffer and 0.5 mL α-amylase (15.2 mg α-amylase/mL phosphate buffer) incubated in 37°C for 16 hrs using a water bath shaker, centrifuged (15 mins, 3000 rpm), then the precipitated sample was washed with 10 mL of aqua and added with 1.5 mL KOH solution 4 M before aged for 30 mins in ambient temperature. After aging, 2.75 mL of HCl 2 M, 1.5 mL of sodium acetate buffer and 40 µL amyloglucosidase was added, incubated in the water bath shaker for 45 mins in 60°C before centrifuged (15 mins 3000 rpm) then the supernatant was contained and washed with 10 mL of aquadest. The supernatant was mixed with 0.5 mL of phenol 5%, was added and then mixed well with a vortex. Then, 2.5 mL of concentrated H_2SO_4 was added quickly to each tube and aged for 10 mins. Each tube was aged once more for 20 mins in ambient temperature before measured in term of absorbance using spectrophotometer UV-Vis on 490 nm.

2.2.2 Starch digestibility

Starch sample or potato starch (0.1 g) was added 10 mL of distilled water and heated in a water bath at 90°C for 15 mins or until gelatinized. The sample was

removed and cooled under running water. The sample solution was added distilled water and phosphate buffer pH 7, incubated 37°C for 15 mins. Finally, the solution was added alpha-amylase enzyme (1 mg/mL in phosphate buffer pH 7) and incubated for 30 mins at 37° C. An aliquot (1 mL) of the sample solution was transferred to a closed test tube containing 2 mL of DNS solution and heated in boiling water for 10 mins. All samples were measured in term of absorbance using spectrophotometer UV-Vis on 520 nm. We used maltose as a standard solution for the determination of maltose content in starch samples and potato starch.

Starch digestibility was measured using the formula as follows:

$$\text{Starch digetibility} = \frac{[\text{Maltose}]\text{sample} - [\text{Maltose}]\text{blank sample}}{[\text{Maltose}]\ \text{starch} - [\text{Maltose}]\text{blank starch}} \times 100$$

2.2.3 Amylose content

A total of 0.1 g sample was weighed into 100 mL volumetric flask in triplicate. Then, 1 mL of 95% ethanol and 9 mL of 1 N NaOH were added and boiled in waterbath for 10 mins. After cooling, distilled water was added to make the volume exactly 100 mL. A blank solution was prepared following the previous steps except taking the sample to the volumetric flask. Then, 5 mL of sample solution was transferred to an empty 100 mL volumetric flask, then 70 mL distilled water, 1 mL of glacial acetic acid, and 2 mL of iodine solution were added. The volume was adjusted to exactly 100 mL with distilled water and left to stand for 20 mins. The absorbance was measured at 620 nm after setting zero with the blank solution. The value of the absorbance was calculated into the apparent amylose content using a standard calibration curve developed from potato amylose standard.

2.2.4 Crystalization analysis using X-ray difraction

The ground sample was adjusted to have 95% moisture content and 25% w/w humidity. X-ray diffraction was operated at 600 W, 40 kV, 15 mA and CuKα1 (0.154 nm). The analysis was conducted on 5 - 30º (2θ) for 16 mins and 14 seconds with 0.026° per steps and the time per steps was 229.5 s. The degree of cristallinity was determined using Origin Pro 8 software.

2.2.7 Morphology using Scanning Electron Microscopy (SEM)

The ground sample was put onto the specimen holder which has been coated with carbon tape. The coating process was conducted using sputter coater of quorum type Q150R ES with gold and sputter current 20 mA for 60 s. Then, the coated sample was put into a chamber. Morphology of the sample was observed on the screen with 50, 100, 500, 1000, 2000 and 5000 times of magnification. The results were captured using a camera with an SE detector (secondary electron), with a working distance of 8.0 mm and EHT 16.00 kV.

3. Results and discussion

3.1 Effect of modification on resistant starch (RS) content, digestibility of starch (DS) and amylose content

Based on the results of resistant starch content in Figure 1, native had the lowest resistant starch content that is 14.80±0.34% and control of 17.07±0.52%. The levels of resistant starch to native tapioca starch were higher when compared with the resistant starch content of tapioca according to a study conducted by Pereira and Leonel (2014) that is equal from 0.56 to 1.1%. High levels of resistant starch in this research can be caused by the method of resistant starch analysis which was used not through the high heating stage, so this process allows gelatinization process on starch, and the result obtained is quite large. Resistant starch content calculated based on total dietary fiber in unheated corn starch had higher RS 41.5±1.6% compared with preheated starch at 95ºC temperature of 35.4±1.1% (Hasjim *et al.,* 2010).

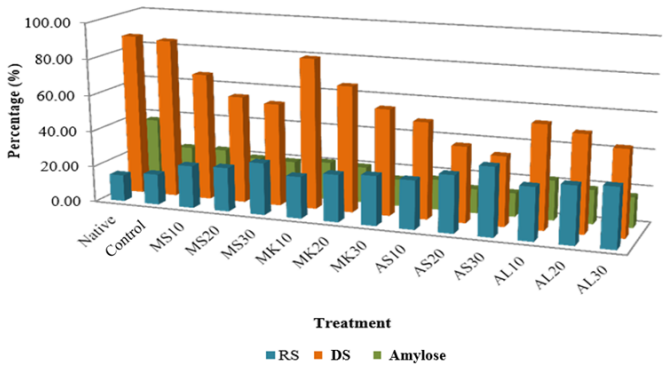

Figure 1. Resistant starch content (RS), digestibility of starch (DS) and amylose content of native and modified starch.

Resistant starch levels in the controls experienced a non-significant increase compared to native due to the retrograde process that allowed the establishment of RS3. AS30 produced the highest resistant starch content of 37.87±1.44%, whereas the starch content was resistant to the treatment of stearic acid with concentrations of 10 and 20% respectively of 26.89±0.93% and 31.61±0.73%. Modifications using palm oil (10-30%) produce resistant starch of 23.65±0.99%, 24.42±0.84% and 28.88±1.50%; MK (10-30%) respectively 23.12±0.91%, 26.18±0.79%, and 27.40±0.93%, whereas AL treatments (10-30%) were 29.12±1.04%, 31.77±2.11% and 33.01±1.01%, respectively. Multivariate analysis results show that the type of oil and fatty acids and concentrations used to cause significant differences. Hasjim *et al.* (2010) stated that there was an increase in the level of starch resistant to corn starch forming a complex compound with 10%

(bk) of palmitic acid to 39.2±1.6% which was significantly different from the control. Different resistant starch content due to free fatty acids can form the amylose lipid complex, besides the length of the chain also affects the formation of lipid amylose complexes, stearic acid has a longer carbon chain (C18: 0) compared to lauric acid (C12: 0). The amount of resistant starch content is influenced by several factors such as the type of starch used, the type and concentration of lipids, also the condition for the formation of amylose lipid complex (Lau *et al.,* 2016). The addition of rice bran oil can improve the resistant starch content in instant rice than control and result in higher slow digestion starch (Luangsangkul and Ritudomphol, 2018).

Based on the results of the analysis, decreased digestibility of starch oil and fatty acid starch concentration ranged from 18.97% to 50.99%. Starch without modification had the highest starch digestibility value of 89.42±1.16%, while the lowest starch digestibility is owned by 30% stearate acid-modified starch (AS30) of 38.42±1.52% which was significantly different from native and control. Modification with stearic acid at other concentrations, i.e. 10% and 20% had starch digestibility 51.98±0.37% and 41.90±0.60% significantly lower than native and control, respectively. The modified starch digestibility value by using lauric acid at concentrations of 10, 20 and 30% was also significantly lower than that of native and control, respectively 57.10±0.18%, 53.63±0.52% and 47.56±1.11%. Modification using palm oil produces starch digestibility of 70.44±0.21%; 59.40±0.39% and 56.90±0.17%, while modification with coconut oil resulted in starch digestibility of 83.30±0.44%, 69.73±1.04% and 58.77±0.93%, respectively. These results indicate that the process of starch modification with palm oil, coconut oil, stearic acid and lauric acid can significantly decrease starch digestibility. Fatty acid lower starch digestibility is greater than the treatment using oils. The decrease in starch digestibility also correlates with increased concentrations of oil or fatty acids added to the modification process. Ai *et al.* (2013) states that there is a decrease in enzymatic hydrolysis using PPA (porcine pancreatic α-amylase) in tapioca starch to 66.4% in addition to 10% corn oil, 72.0% in 10% lauric acid addition, and 65.5% in acid addition stearate 10% after incubation for 120 min. Farooq *et al.* (2017) also state that non-waxy rice with palm oil complexed have significantly increased the resistant starch content and decreased the rapidly digestible starch. The amylose-palm oil complex can block the enzymes to penetrate inside the starch granules that produce a form of a single helical V-amylose structure and cause a high enthalpy change. Decreased starch digestibility also occurred in the millet that the content of rapidly digestible starch will be decreased with the addition of fatty acids. Besides that, polyunsaturated fatty acids have a low ability compared to monounsaturated fatty acids at the rate of starch digestion (Kawai *et al.,* 2012; Annor *et al.,* 2015).

The results of amylose content analysis are shown in Figure 1. Amylose content with the addition of oil and fatty acid resulted in lower amylose content than control. The lowest levels of amylose were found in AS30, which was 13.11±0.70%, which was significantly different from amylose content in native starch and treatment control. The amylose content obtained at the treatment controls was 22.84±1.55%, the results showed a significant decrease in the amylose levels in the native, due to the amylose leaching during the gelatinization process, allowing the formation of complex associations that could not form the complex with iodine in the retrogradation process (Wang *et al.,* 2015). Decreased amylose levels in the treatment of adding oils and fatty acids are due in large part to amylose forming complex compounds with fatty acids so that the amylose available to form complex compounds with iodine is reduced. The results of the amylose content in Figure 1 show that the more oils or fatty acids added will decrease the amylose content which may form the compound of the complex with iodine. Bhatnagar and Hanna (1997) stated that iodine binding capacity at control was 3.68% and there was a decrease in lipid addition in the extrusion process such as stearic acid (2.18%) and coconut oil (2.62%). Decreased iodine binding capacity (IBC) or amylose ability to form complex compounds with iodine indicates the increased formation of lipid amylose complexes. The treatment of fatty acids will reduce the amount of amylose because fatty acids more easily form complex compounds with amylose (Tufvesson *et al.,* 2003).

3.2 Diffractogram of resistant starch

The formation of lipid amylose complexes can be observed using X-Ray diffraction at an angle of 2θ of about 7.5°, 12.7°, and 19.9° (Cheetam and Tao, 1998). The result of the diffractogram in Figure 2 shows that there is no crystalline peak in the control, MS30 and MK30. Native samples have a maximum peak at 15°, 18° and 24°, the crystals formed are crystals A. This is because the amount of amylose-lipid complex is too low, so it cannot be read by X-Ray like waxy rice samples (Farooq *et al.,* 2017). Bhatnagar and Hanna (1994) state that native starch exhibits a maximum peak at 15.1°, 16.4°, 17.5°, 18.7° and 22.6° approaching the type A crystalline pattern, whereas type B crystals have a maximum peak at 17° and type C at 15°, 17°, 23°, 31° and 38° (Marimuthu *et al.,* 2013). Based on the results of the diffractogram analysis (Figure 2), AS30 indicated a

peak at 7.5°, 11.5°, 22° and 24°. Peak at 7.5° indicates the formation of a lipid amylose complex, with AL30 having a maximum peak at 9.9°, 13.2°, 21°, 22° and 24°, but no lipid amylose complexes at 7.5°, 12.7° and 19.9° peaks. Peak 21°, 22° and 24° are due to the reflection results of pure crystals of fatty acids that do not form the association with starch as reported by Fanta *et al.* (1999) that peak at 21.4° and 23.8° increases with the increasing length of fatty acids reflecting pure crystalline from fatty acids that do not form complexes with amylose.

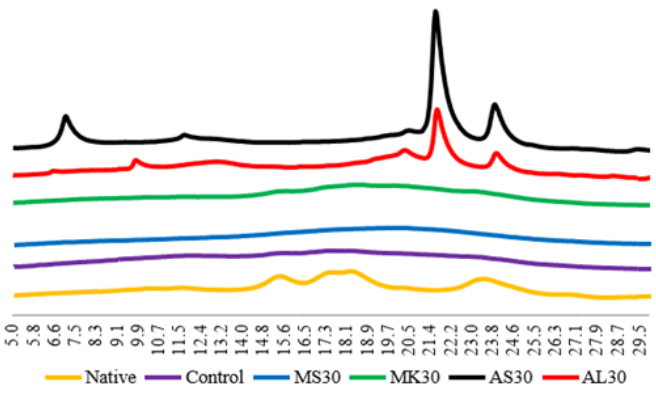

Figure 2. The result of diffractogram using X-Ray Diffraction at angle 2θ

3.3 Gelatinization of modified tapioca starch

Based on the analysis result (Figure 3), the control had the highest peak viscosity (PV) which was equal to 6319 cP. The lowest breakdown in AS30 is 153 cP, low breakdown indicates resistance to heating. The highest HPV is in control and AL30 indicates high eating quality and low cooking loss (Bhattacharya *et al.,* 1999). In addition, AS30 had the lowest setback that showed resistance to the retrograde process (Kusnandar, 2011). The controls have the fastest peak time of 4.33 mins while the AS30 has the longest peak time of 12.07 mins. Ai *et al*. (2013) states that the compound amylose lipid can increase the temperature of gelatinization, it is in accordance with the analysis results on AS30 and AL30 samples that show the temperature of gelatinization on AS30 of 90.25ºC and AL30 of 92.25ºC greater than native that is equal to 69.6ºC. The addition of oil showed different results i.e reducing gelatinization temperature by 50.25ºC at MS30 and 50.3ºC at MK30. The formation of the amylose-lipid complex in bread occurs in temperatures above 90ºC. This temperature allows the formation of well-defined crystalline structures (Lau *et al.,* 2016).

Based on Schoch and Maywald (1968), there are 4 types of starch gelatinization profiles including type A, type B, type C and type D. Type A is starch with high peak viscosity but is not resistant to heating so it is easy to decrease its viscosity. Type B has the same characteristics as type A but has a lower peak viscosity and lower decreased quantity. Type C is a starch-resistant starch, subject to limited development shown in the absence of peak viscosity and breakdown. Type D is starch with limited inflowing power is shown by a low viscosity profile. Based on the classification of native starch, control, MS30 and MK30 belong to type A, while AS30 and AL30 were C type starch.

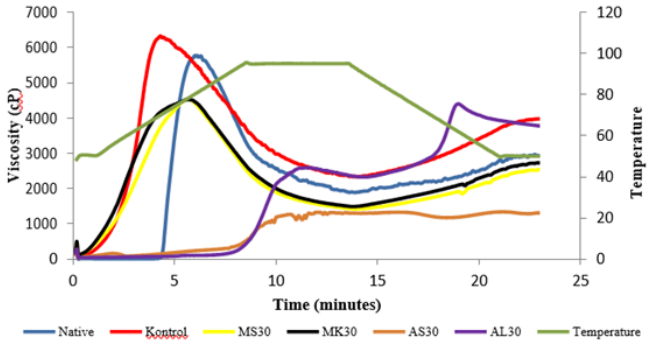

Figure 3. Profile of modified tapioca starch gelatinization using Rapid Visco Analyzer (RVA)

3.4 Modified starch morphology

The granular morphology using SEM instrument was performed on native starch, control, and some samples yielding the highest resistant starch content in each treatment i.e. MS30, MK30, AS30 and AL30. The result of observation using SEM in Figure 4A shows that

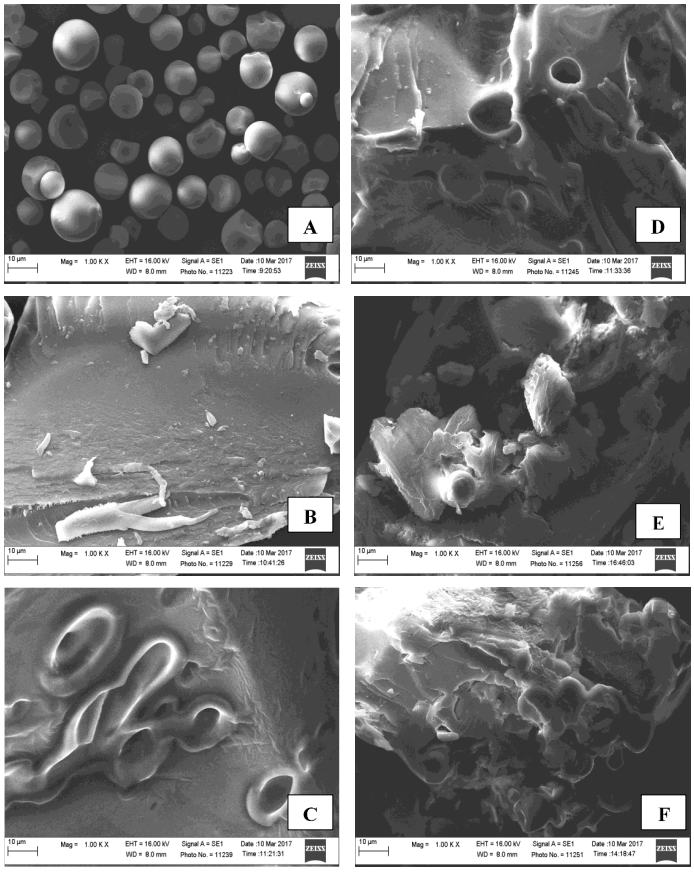

Figure 4. The morphological results of cassava starch granules interfere with native (A), control (B), 30% palm oil (C), 30% coconut oil (D), stearic acid 30% (E) and 30% lauric acid (F) at 1000x magnification

native starch had intact round granule starch. Figure 4B (control) shows the starch granules have been completely gelatinized. Figure 4C (M30), Figure 4E (AS30) and Figure 4F (AL30) indicate there were some broken starch granules, having swelling marked by increasing the size of the granule and most of the other clump-shaped granules showed that starch had undergone gelatinization process, but there are still some intact granules. The intact granules indicate an imperfect gelatinization process, due to the obstruction of water in the presence of oil or fatty acids (Gelders *et al.,* 2006).

4. Conclusion

Modification of tapioca starch through the gelatinization process with the addition of palm oil, coconut oil, strearic acid and lauric acid can decrease starch digestibility, increase levels of resistant starch and lower amylose levels. Different types of oils or fatty acids and their concentration have an effect on the increase of starch resistance and decreasing starch digestibility and amylose significantly. The analysis using X-Ray diffraction showed AS30 had a peak at 7.5°, 22° and 24°. Peak 7.5° was a lipid amylose complex whereas AL30 had a maximum peak at 21°, 22° and 24°, and other treatments did not show peak lipid amylose complexes at 7.5°, 12.7° and 19.9°. The profile of starch gelatinization using Rapid Visco Analyzer showed that the addition of fatty acid can decrease the value of breakdown, setback, and peak viscosity. AS30 had the lowest breakdown and setback of 153 cP and 135 cP and peak viscosity of 1329 cP. In addition, the addition of fatty acids was able to increase the gelatinization temperature of 90.25°C at AS30 and 92.25°C at AL30. The morphological observations of modified starch contained some intact starch granules, some of which had swelling characterized by increasing granular size.

Conflict of interest

The authors declare no conflict of interest.

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