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Macromolecular distribution of a mixed system on dodol ulame by Confocal Laser Staining Microscopy (CLSM)

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Abstract

The aim of this study was to learn more about the macromolecular distribution of the food matrix system in the traditional food Dodol Ulame in order to better understand the interactions between starch, fat and sugar. Microstructural stages included dye selection, staining/coloring, material mixing, and observation. The starch was stained with APTS and the fat with Nile red. The starch was stained using the double staining method with covalent labeling. The samples were analyzed with a Zeiss Inverted LSM 800 AXIO Observer equipped with an AXIO cam 503 color camera and an excitation wavelength of 665 nm and 543 nm. The results indicated that GR was polyhedral. Fat and sugar are evenly distributed in the matrix system. Some of the fat interacts with the starch granules to form a coating that prevents the starch from interacting with the sugar and inhibits the imbibition of water into the starch granules at 50 ˚C and 70 ˚C. Amylose escapes from the granules and reacts with the sugar solution at a higher temperature of 90 ˚C, while the free fat acts as a lubricant between the particles. This shows how flour, fat and sugar interact to affect the manufacturing process and the final properties of dodol ulame.

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1. Introduction

Glutinous rice flour (GR), coconut milk (CK), and palm sugar (PG) are ingredients commonly used in traditional Indonesian traditional foods, including dodol ulame. Dodol ulame is one of the traditional cuisines of Mandailing ethnic group in North Sumatra Province. Dodol ulame is interesting for its traditional and distinctive flavour and for its originality, deriving from an ancient traditional cooking process. The basic components, the raw ingredients from which dodol ulame is made, contribute to the originality of the product. Each of these basic components is molecularly dispersed and interacts with each other to provide different rheological qualities that affect the flavor and texture of dodol ulame. Both GR, CK and PG have different chemical compositions. CK is high in water and fat (1,2). Glutinous rice contains amylose and amylopectin, which are carbohydrates in the form of starch. The Amylose content in glutinous rice varies from 0 to 2% (3–6), but is 9.79% in local Mandailing varieties (2). PG has an 89.47% carbohydrate content (2), which is a reducing sugar (7).

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In the manufacture of dodol ulame, GR, CK and PG are mixed and heated for a specific time to produce a product with the desired properties. In general, starch gelatinizes when heated. The presence of CK and PG affects gelatinization. The effect of sugar on gelatinization has been studied previously. The presence of sugar increases the gelatinization temperature, maximum viscosity, breakdown viscosity, final viscosity, and setback viscosity of the starch (8–10). Sugar competes with amylose for water under water-limited conditions, which inhibits starch swelling and affects its gelatinization profile (11,12). The effect of fat on gelatinization has also been studied previously as it is related to the formation of fat-starch complexes (13). The complexity between starch and fat is believed to reduce peak viscosity and breakdown viscosity (14).

Limited studies have analyzed the interactions that occur in systems composed of GR, CK, and PG simultaneously, particularly the microstructure. Previous studies showed that GR, CK and PG formed a complex system distinct from the binary systems of GR-CK and GR-PG in terms of gelatinization profile. Both the gelatinization curve and the apparent viscosity decrease with an increasing percentage of CK and less PG in the system, which is associated with the formation of the fat-starch complex and a more dominant lubricating effect of fat in the system (2). To prove this conjecture, a deeper analysis is required, especially of the microstructure of the system.

Analysis of microstructural features can be used to gain a deeper understanding of the system in the food matrix. Microstructural visualization can provide useful information about the structure of components in an assembly system, as well as a better understanding of how changes occur in the food matrix system. Optical microscopy (OM), polarized microscopy, SEM, TEM, atomic force microscopy (AFM), and confocal laser scanning microscopy (CLSM) are some of the microscopy techniques that can be used to study the microstructure of Emulsions (15). Before TEM, SEM, and CLSM were widely used, observations of the structure of starch granules were mainly made with a polarizing microscope. A polarizing microscope is a microscope that uses polarized light to analyze the birefringence structure. Starch granules are known to have diagonally paired quadrants. The resulting color can be viewed through the compensator in a polarizing microscope (16). Microscopy techniques have been developed that can provide better microstructure images, such as CLSM

CLSM is a type of optical microscopy that is similar in many ways to epifluorescence microscopy. CLSM works on the premise of focusing a laser beam on a specimen using an objective microscope, which subsequently excites fluorescence. Only specific fluorescent lights are detected by the dichroic mirror when a pinhole detector is present, resulting in an image (17). CLSM has several advantages, including the ability to image samples in 3D, the lack of sample preparation, good resolution, the ability to detect multiple probe fluxes, and ease of use. CLSM has limitations as it requires fluorochrome labeling (for food samples), contains solvents and processes that can damage the sample, requires special sample treatment for better images, and has a low resolution (200 nm) (17). CLSM is commonly used to monitor changes in food systems that are affected by processing factors, storage time, or absorption and digestion conditions (15) to help understand the structure of food and the interactions between the bread components (18), multiple starch granule structures (19), to visualize the structure of starch and polysaccharide composites and how they change during preparation (20). It is also used to study the interactions of food components in a mixed system, such as the interaction of starch, milk, and carrageenan (21). Therefore, CLSM is an

excellent tool for observing the distribution of and the interactions between the components that make up the food matrix.

2. Materials and Methods

The materials used in this study were GR, CK and PG, all of which were purchased from the local market in Bogor Regency (Figure 1). As a dye, 8-aminopyrine-1,3,6-trisulfonic acid trisodium salt (APTS), and Nile red from Sigma-Aldrich, NaCl, dimethyl sulfoxide (DMSO), glacial acetic acid, NaOH, aquades were used.

(a) (b) (c)

Figure 1. (a) Material to prepare dodol ulame GR; (b) CK extracted from a mature coconut; (c) PG was cut into small pieces before use.

Dodol is traditionally prepared by combining GR and CK and then heating the mixture until it forms a thick, smooth, glossy dough. Then PG was added and cooked until the dough thickened but didn't become sticky (22). Dodol ulame was prepared with modifications to facilitate the staining process and observation of microstructure. When heated to a temperature of 50-90 °C, the distribution of each component was observed. The temperature at which GR, CK and PG were gelatinized was 74.44 °C (2). The stages of microstructural analysis using CLSM in this study include dye selection, staining, material mixing, and observation.

2.1. Staining of Starch and Fat Granules

The staining procedure chosen in this study used the double staining method with covalent labeling to color the starch granules. Covalent labeling is used to ensure that one ingredient is localized with the other. The basic considerations in selecting the dye are the maxima and absolute emission spectra of each probe. Starch granules in GR were stained using APTS using the procedure described by Blennow et al. (2003) and Matignon et al. (2014) (21,23). Preparation of the APTS stock solution: 5 mg of APTS dissolved in 0.5 ml of 15% acetic acid (1.5 ml of acetic acid in 8.5 ml of distilled water). In two experiments, two (2) mg of GR were dispersed into 3 l of APTS solution and incubated at 30 °C for 15–18 hours. The starch granules were washed with 1 l of 1 M NaOH (0.4 g in 100 ml of distilled water) until the pH was 8. Furthermore, the starch granules were washed with 1 l of distilled water four times (21,23). The fat in CK is colored using Nile red. Preparation of nile redstock solution: 0.5 mg of Nile red was dissolved in 1 ml of DMSO as a solvent (stock solution). Stock solutions of Nile red were packed in light-protected containers and stored at 20°C to prevent degradation, and the required amount of solution was made on the day of the experiment. Fat staining refers

to Moreno and Bouchon (2013). A total of 4 l of stock solution was added to the sample mixture and then stirred with a stirrer for 2 minutes to homogenize the mixture (24). Mixtures with various ratios of PG are not colored. The treatment of microstructural observations with CLSM is presented in Table 1.

Table 1. Treatment of microstructural observations with Confocal Laser Scanning Microscopy (CLSM).

Type	Medium paste	GR (starch) - APTS	CK(Fat) – *Nile red*	PG	Temp (°C)	Observed suspension
Single	NaCl	X	-	-	50	Paste
		X	-	-	70	Paste
		X	-	-	90	Paste
Binary (GR- CK, GR-PG suspension)	PG solution	X	-	-	50	Paste
		X	-	-	70	Paste
		X	-	-	90	Paste
	CK	X	-	-	50	Paste
		X	-	-	70	Paste
		X	-		90	Paste
Ternary (GR-CK-PG suspension with ratio 1.0:2.0:1.0)	CK	X	X	-	50	Paste
					70	Paste
					90	Paste

2.2. Mixing Material

GR, CK, and PG were combined in a ratio of 1.0:2.0:1.0. An equal volume of the 2 ml mixture was then heated. The heating method involved gentle stirring on a hotplate with a beaker of water and a thermometer to control the temperature. The temperature was raised at a rate of 5 °C per minute from 50 °C to 90 °C. The mixture was held in place by a sliding concave that had been warmed to 50 °C, which was then covered with a coverslip. After preparation, the combination was monitored at room temperature.

2.3. Observation

The samples were analyzed with a Zeiss Inverted LSM 800 AXIO observer equipped with an AXIO cam 503 color camera. Excitation wavelengths of 665 nm and 543 nm were used. The spectra-filtering technology allows the system to work in precise wavelength intervals and maximize the reception wavelength zone. APTS detects light with an excitation wavelength of 488 nm and an emission wavelength of 500–530 nm (21,22). In a neon laser with a pinhole of 90 nm, Nile Red exhibits an excitation wavelength of 543 nm and an emission wavelength of 598 nm. To decrease noise, each line is scanned three times and averaged during image acquisition.

3. Results and Discussion

3.1. Glutinous Rice Flour Microstructure

The microstructure of glutinous rice starch observed by CLSM is shown in Figure 2. The microstructure of glutinous rice has a polyhedral structure. Starch is made up of granules of varied shapes and sizes, depending on the source, such as round, oval, transcuted elliptical, and polygonal (4,25,26). The glutinous rice starch from Mizoram, India, also has a polyhedral structure (3). Glutinous rice starch has a polyhedral structure with a smooth surface and does

not shatter (27). In this study, individuals and groups/aggregates of starch granules were detected, confirming the existence of amylose. Compared to individuals, aggregates and elongated granules had the highest amylose content (27).

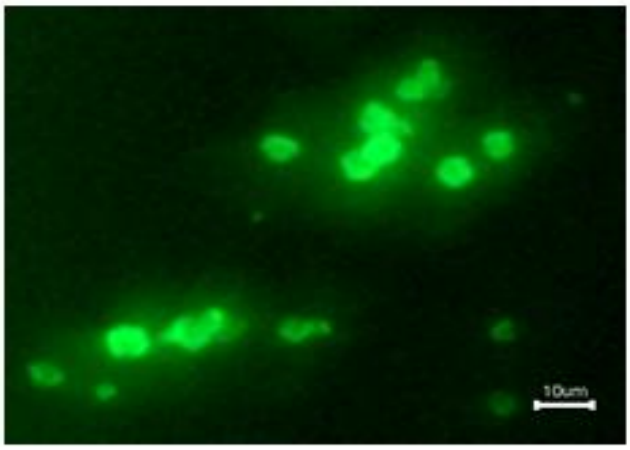
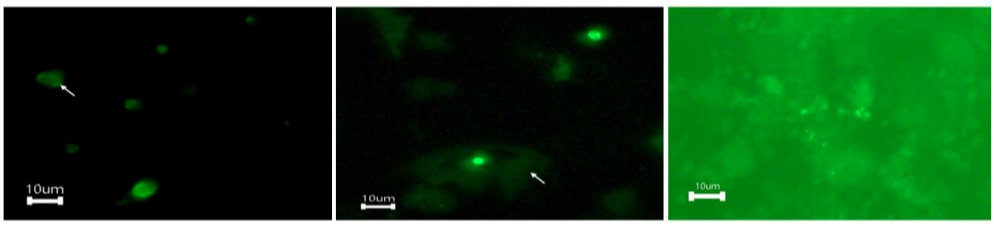


Figure 2. Microstructure of glutinous rice starch was observed using CLSM.

CLSM can provide the microstructure of starch granules based on the given color, which is green and can distinguish from other components, as shown in Figure 2. CLSM enables the viewing of defect-free materials in three dimensions with a remarkable optical cross-section (i.e., deep imaging of structures). In this study, the APTS probe was used to stain starch granules, resulting in green color. APTS is particularly effective and efficient for coloring starch granules (21). In this study, the GR fluorescence was not excessive, so colors were less distinct despite enhanced contrast and brightness. This is due to the low amylose content of GR, which is only 9.790 + 0.03%. (2). APTS is a dye that reacts with the decrease of amylose and amylopectin in starch granules. The area of the hilum that had more amylose than other sites stained brighter with APTS. Fluorescence increases as the amylose level of starch increases (23).

3.2. Glutinous Rice Microstructure Changes During Heating

The gelatinization process in GR starch was confirmed by changes in the starch microstructure after heating from 50°C to 90°C using CLSM. As can be seen in Figure 3 When starch granules are heated, their semi-crystalline and three-dimensional architectural structures break down, resulting in a phase change from a regular to an irregular granular structure known as gelatinization (28,29). When starch is heated, water is absorbed into the granules, causing the granules to swell severely due to increased randomness and decreased crystallinity (30).



(a) **(b)** **(c)**

Figure 3. (a) Confocal Laser Scanning Microscopy (CLSM) was used to examine changes in the microstructure of GR at temperatures of 50 °C; (b) 70 °C; and (c) 90 °C . Individual starch granules are indicated by an arrow.

The gelatinization process in the CLSM is characterized by a decrease in the brightness of the starch granules with increasing temperature. The CLSM image shows that the brightness of the starch granules starts to diminish at 50 °C, particularly on the outside of the starch granules. However, at 50 °C, some granules, especially the larger ones, still show intense fluorescence. At 70 °C, the bright fluorescence fades and expands to a deeper, black zone but still leaves a central amorphous granule region (Figure 3). The middle of the starch granule is known to include starch amylose, which is evidenced by a higher level of fluorescence intensity in the middle of the starch granule (31). The fluorescence has extended throughout the black area at 90 °C, but certain maltase crosses are still visible. The strong fluorescence gradually fades as the heating temperature rises, and the darker zone expands outwards. The ionic strength of starch granules diminishes as the temperature rises, resulting in fewer starch granules in CLSM, showing that the gelatinization process occurs gradually from the middle to the exterior.

CLSM was utilized to collect data in order to better understand how starch and fat, as well as starch and sugar, interact. The combination was heated to 50 °C, 70 °C, and 90 °C at a rate of 55 °C /min. CLSM combined with heat treatment, is possible to analyze the structure of more complex systems (19). The CK fat globules were stained with Nile red (9-diethylamino-5H benzo [α] phenoxazine-5-one). Nile red may effectively identify milk fat globules (32). The fat is represented by the red portion, while the non-fat component, such as water, is represented by the black portion. The fat in the area of observation is evenly distributed, and the larger the heating temperature, the smaller the fat droplets, which is assumed to represent the effect of heating. The viscosity of the oil and water phases in CK is dependent on the heating temperature (33). As the heating temperature rises, the viscosity lowers, allowing little fat droplets to form more easily.

The microstructures of GR-CK and GR-PG in a 1:3 ratio were examined using CLSM. APTS was used to stain starch granules, but fat and sugar were not. Figure 4 shows the microstructure of GR- CK, and GR-PG at different heating temperatures, as seen by CLSM. The fluorescence produced in the GR-CK system was not very apparent, as seen in Figure 3, where the starch (green) is surrounded by whitish patches that create a circle (assumed to be fat). When the temperature was increased to 70 °C, the green fluorescence and black patches were still visible, with the green color producing a larger area than the GR-PG system at the same temperature. When fat and starch are heated to 90 °C, they take on a different structure than the GR-PG system. Starch gets trapped between the black and white areas made up of water and fat and is not evenly distributed throughout the system. The fluorescence of the GR-PG mixture produces images that make it easy to distinguish starch granules from other components. At 50 °C, the green color is a starch granule, and the black half is visible. The black element is believed to be a solution of sugar and other uncolored ingredients (water). When the temperature reaches 70 °C, the green color fades, indicating gelatinization, which occurs starch granules absorb water causing the green color around the edges to fade. A green color was found practically on all surfaces at 90 °C, which was amylose that had exuded from the starch granules and mixed with sugar and water.

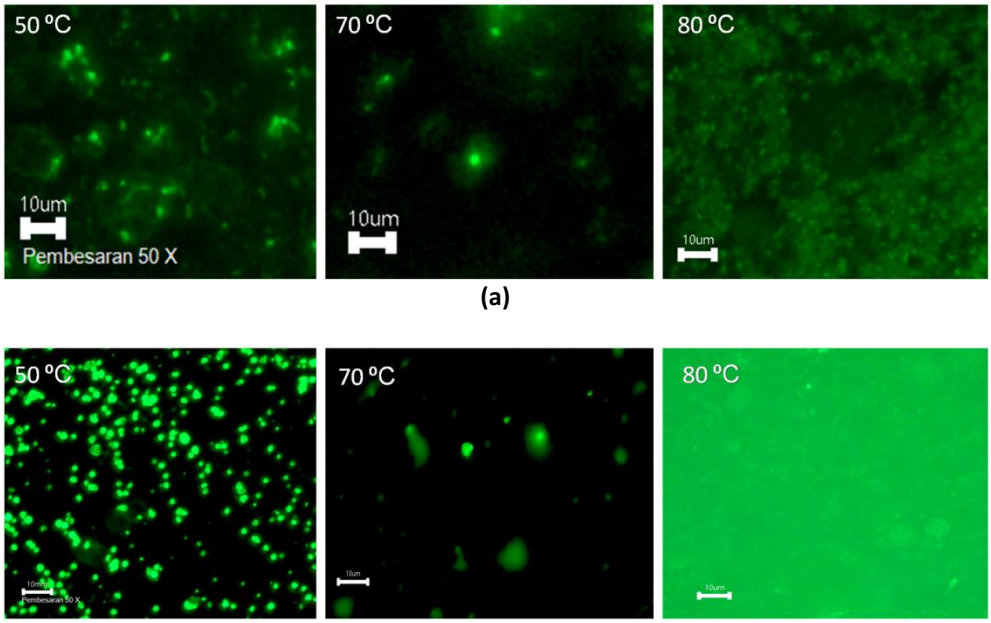
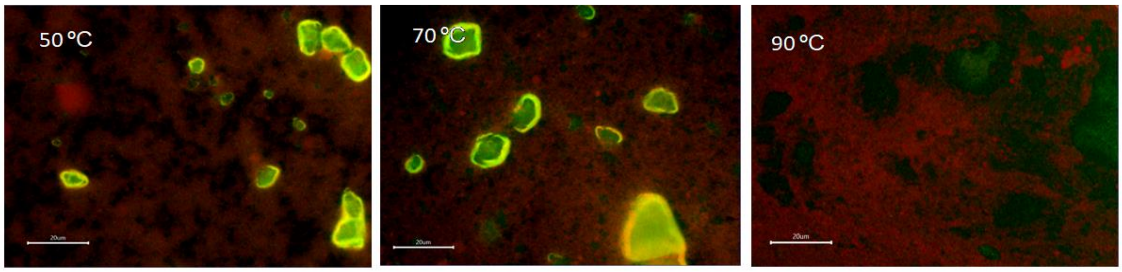


Figure 4. (a) The microstructure of the GR-CK with a ratio of 1:3 and (b) GR-PG with a ratio of 1:3 at various heating temperatures was observed using confocal laser scanning microscopy (CLSM). White arrows indicate starch granules.

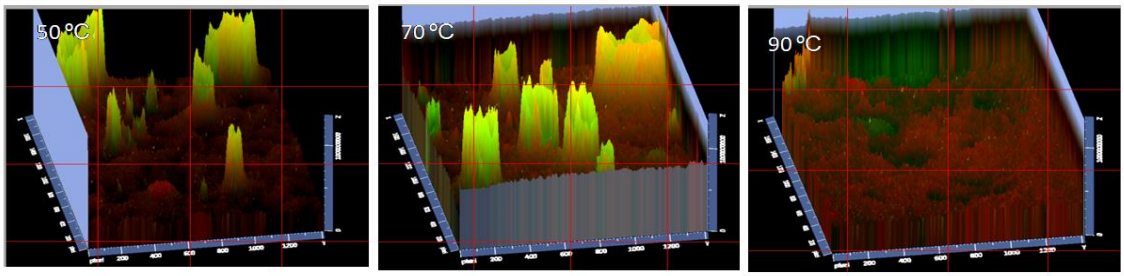
3.3. Distribution of Starch-Fat-Sugar in Dodol Ulame Mixed System

Observations were made on a complex system representing the system on dodol ulame, comprising GR, CK and PG. The double staining approach produces an image of starch with prominent contrast and clearly apparent structural details and molecular dispersion (24,34). In the double staining method, starch granules are stained using APTS, then a stock solution of Nile red with a certain concentration is added to the mixture before heating it. Figure 5 shows the microstructure of a mixed system comprising GR, CK and PG with a ratio of 1.0:2.0:1.0 observed using CLSM at various heating temperatures. The green color in the CLSM image represents the presence of starch granules, the red color represents fat and the black color represents the presence of sugar and other uncolored components in the system. Observations were made using two lasers with a wavelength of 500-530 nm for APTS, while for Nile red using a wavelength of 543 -598 nm.

The CLSM image indicates that at 50 and 70 °C, individual starch granules (green fluorescence) are surrounded by red fluorescence, which is CK fat. The presence of fat dominates the microstructure of this mixed system, which was evident by comparing red, green, and black fluorescence (considering that the amount of fat used is twice as much as the other components). The initially intense green fluorescence began to diminish and expand at a temperature of 70 °C, showing that starch had begun to expand while still being surrounded by red (fat) fluorescence. Green regions were also visible without the presence of red fluorescence, which was considered to be starch granules that had broken away and mixed with sugar. The only visible areas at 90 °C are the red fluorescent area and the green fluorescent area, which are separated by black. At this temperature, all of the starch granules should have split and the amylose should have dissolved into the sugar. Figure 5 also displays a significant number of red fluorescent spots, which are believed to contain fat that is not attached to starch granules and is therefore free fat.



(a)



(b)

Figure 5. Microstructure of the mixed system on Dodol Ulame with the ratio of GR: CK: PG 1.0:2.0:1.0 at various heating temperatures of 50 °C, 70 °C, and 90 °C were observed using confocal laser scanning microscopy (CLSM). (a) CLSM image and (b) 3D shape.

The microstructure of the mixed system is illustrated in greater detail in the 3D picture (Figure 5b). The CLSM image is reflected in Figure 3 on the horizontal Y and Z coordinates, as well as the vertical X coordinates. Starch granules (green) exhibit a higher intensity on the Z-axis at 50 °C, indicating that they are larger than fat globules (red) or sugar granules (black). These heights vary in intensity, reflecting small and large starch particles. Apart from being observable surrounding the starch granules (green), the red color (fat) is also observable on the starch granules' exterior periphery (green). The intensity of starch granules (green) was demonstrated to be higher than before at a temperature of 70 °C on the Z-axis, which represents the maximum swelling of starch granules. The red color of the starch granules can be seen clearly. There was no difference in the intensity of starch granules at 90 °C. This confirmed that the starch granules had disintegrated at 90 °C. The red area (fat) is evenly spread amongst the black and green areas as can be seen above. The green (broken starch) portions blend into the black.

The findings indicate that the interaction occurs between starch, fat, and sugar when heated. The fat formed a layer around the surface of the starch when heated to 50 °C to 70 °C. The presence of this layer prevents amylose from interacting with sugars to form amylose-sugar complexes and prevents water molecules from entering the starch granules and binding to amylose. It confirms that the increase in gelatinization temperature is more associated with water availability for amylose in mixed systems. In the mixed system of GR, CK, and PG, the gelatinization temperature increases in the presence of CK (2). Heating at higher temperatures causes the fat layer surrounding the amylose to weaken and water is absorbed into the starch granules, causing swelling of the starch granules. Despite the fact that the starch granules swell, they are still surrounded by a coating of fat. At 90 °C, the fat-starch layer weakens and the starch granules break. The amylose comes out and combines with the

sugar solution. This phenomenon explains why the process of making dodol ulame takes a long time (more than 8 hours) because the fat in coconut milk inhibits gelatinization.

In CK, fatty acids with glycerol in the form of triacylglycerol or triglycerides are three fatty acids bonded to one glycerol. CK contains 67.5 percent triacylglycerol (35). So far, the layer formed is thought to be the result of the interaction between starch-fat. A layer forms on the starch granules as a result of hydrophobic interactions between the fatty hydrocarbon chains and the intra-helix amylose region (36). The amylose-lipid complex is made up of monoacyl fatty hydrocarbon chains that are positioned in the hydrophobic cavity of the amylose helical chain, which is made up of three amylose loops, each with six glucose residues. Amylopectin-fat complexes may form due to the GR's low amylose concentration. A helical inclusion complex requires 18–24 glucosyl residue (37). Amylopectin has a chain length of 23–44 glucosyl residues, which allows the outer branch to participate in the formation of complexes.

Under conditions of 10 grams of fat per 100 grams of amylose saturation, each monoacyl chain needed at least 18 glucosyl residues in the complex to develop (38). In the observed ratio, the CK ratio is twice as high as the GR ratio, causing the system to become saturated. Higher added fat content results in free, uncomplex fat. Free fat that does not bound to starch granules is hypothesized to act as a lubricant that lubricates between sugar and starch particles in this system. This finding confirms that at a higher percentage of CK, the interaction between starch and fat affects changes in the gelatinization profile in the mixed systems. Due to the presence of uncomplexed fat, the system becomes saturated. The uncomplexed fat lubricates the particles in the system, resulting in lower viscosity and smoother flow. The gelatinization profile changes as a result; the apparent viscosity decreases gradually and the flow index increases. The findings of this study further indicated that fat plays a role in preventing amylose from forming complexes with sugar and free fat in a mixed system of GR, CK, and PG. It acts as a lubricant, allowing the material to flow properly throughout the system.

4. Conclusions

This study shows that GR has polyhedral-shaped starch granules. Micro-structured fat and sugar are evenly distributed in the mixed system. CLSM can describe well the molecular distribution that occurs in the dodol ulame system. Some of the fat interacts with the starch granules to form a layer that prevents the starch from interacting with sugar and inhibits the imbibition of water into the starch granules at the temperatures 50 °C and 70 °C. At the temperature of 90 °C, amylose exits the granules and combines with the sugar solution, while free fat acts as a lubricant that lubricates between particles. This finding explains the interaction between starch, fat, and sugar in dodol ulame system during heating. When food components are mixed to form a dodol ulame food matrix, microstructural visualization information provides a better understanding of the changes that occur in the system. Based on the microstructural features of the material, this information is highly valuable in the design of processing methods to achieve quality and homogeneous local food items.

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Author Contributions

G.S., S., A.B.A., N.E.S. conceived and designed the experiments; G.S. and S. performed the experiments; G.S., S., A.B.A., N.E.S. analysed the data; G.S. contributed reagents/materials/analysis tools; G.S., S., A.B.A., N.E.S. wrote the paper.

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Institutional Review Board Statement

Not applicable.

Data Availability Statement

Available data are presented in the manuscript

Conflicts of Interest

The authors declare no conflict of interest.

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