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Improving feed quality by proper processing of raw materials

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Improving feed quality by proper processing of raw materials

by

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A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Food Science and Technology

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ABSTRACT

Objectives of my research were 1) to understand effects of drying temperature and storage of corn kernels at an elevated humidity on starch structure and functions, and 2) to develop a method to produce cassava-based feed that resembles a commercially available corn-based feed product. Corn kernels with 32.5% moisture (db) dried at higher temperatures (80°C or 120/80°C) had higher starch digestibility than that dried at a low temperature (37°C). Starch isolated from the corn kernels dried at the higher temperatures had higher gelatinization temperature and lower gelatinization enthalpy-changes than that from corn kernels dried at the lower temperature because of partial starch gelatinization. The partially-gelatinized starch exhibited loss of Maltese-cross of the starch granules when viewed under a polarized-light microscope. The starch digestibilities of the dried-corn samples decreased after storage at 37°C and 93% relative humidity (RH) for 3 and 6 days.

Effects of storing dried corn kernels at 27°C and 85-90% (RH), resembling a tropical climate, for up to 6 months on starch structure and functions were investigated. Freshly-harvested corn samples (B816, 27.8% moisture, db) were sun-dried (SD) at 35°C or machine dried (MD) at 80°C. Storage of the dried corn kernels decreased starch digestibilities and peak viscosities of the ground corn samples. The rate of enzymatic hydrolysis of starch isolated from the sun-dried corn increased with storage, whereas that from machine-dried corn decreased with storage. Peak viscosity and gelatinization enthalpy-changes of starches isolated from both sun-dried and machine-dried corn decreased but the gelatinization temperature, pasting temperature, and percentage crystallinity of the starches increased after storing the corn. Numbers of damaged starch granules and starch granules with pinholes

increased and the molecular weights of starch and long branch-chains of amylopectin decreased with storage time of corn. The starch isolated from sun-dried corn (35°C, SD) after storage suffered greater levels of endogenous and/or microbial enzymatic hydrolysis than that from machine-dried counterpart (80°C, MD) after the same period of storage.

Cassava-based feed with slower starch digestive rate, resembling corn-based feed and improved nutritional values, was produced by blending cassava flour (CA) with other ingredients, including dietary oils (palm oil, soybean oil, and corn oil), Distiller's Dry Grains with Soluble (DDGS), or defatted-soybean meal (SBM), followed by a heat-moisture treatment (HMT). Blending dietary oils (6%, v/w) or DDGS (10%, 20%, 30%, w/w) with cassava flour followed by HMT at 100°C for 1h substantially reduced the starch digestion rate of the processed cassava flour. Dietary oils with larger proportions of unsaturated fatty acids had greater impacts on reducing the starch digestive rate of cassava flour. HMT conditions, such as heating temperature and initial moisture content of the blends, were evaluated. Cassava flour-DDGS blends had a lower starch digestive rate than cassava flour-SBM blends at equivalent protein levels.

GENERAL INTRODUCTION

Starch is an energy-reserve in plants and is stored in various plant organelles, such as roots, tubers, leaves, seeds, and fruits. Starch is mainly composed of two polysaccharides, amylopectin and amylose. Amylopectin is highly branched molecules, in which 95% of glucose monomers are linked by α -(1 \rightarrow 4) linkages and 5% are linked by α -(1 \rightarrow 6) linkages. Amylose is essentially linear molecules with few branches. Lipids and proteins are present in the starch granules in small amounts. Starch is developed in the amyloplast of plants in the form of semi-crystalline granules. Starch properties play an important role in the quality of many food and non-food products containing starch. The properties of native starches, however, may not achieve the specific functionalities needed for commercial applications. To overcome these limitations, chemical and physical modifications have been utilized to modify and improve the properties of starches. In my thesis research, I focused on understanding and modifying starch functional properties that affect the quality of animal feed.

After harvesting, shelled corn kernels are commonly dried before being processed into an animal feed. Thus, drying conditions, such as drying temperature and initial moisture content of the corn kernels, impact the processability of the dried corn and the nutritional quality of feed. In the present, freshly-harvested corn kernels were dried at three different temperatures and the changes in their starch functional properties were observed. Crops produced during the harvesting season are not used immediately. Thus, the dried corn kernels need appropriate storing before being further processed to make feed products. The effects of storage of dried corn-kernels on their starch properties, however, are not well understood. I

analyzed structures and properties of dried corn-kernels after being stored in a tropical climate storage condition and observed the changes in their starch structures and functions during 6 months of storage.

Cassava root is an excellent source of starch for food and is widely used in some countries as their main staple food. Cassava starch is relatively easy to isolate from the root because the root contains very low levels of lipids and protein. Cassava starch, however, is known to have a substantially higher starch digestive rate than corn starch. The difference in starch digestive rate between cassava starch and corn starch hinders uses of cassava to replace commonly used corn in animal feed. It is important to develop a new and simple method to improve the nutritional value of cassava flour. The objectives of my thesis research were to 1) understand effects of drying-temperature and storage of corn kernels at an elevated humidity on starch structures and functions and 2) develop a novel method to produce cassava-based feed that resembles a commonly used corn-based feed product.

DISSERTATION ORGANIZATION

First chapter of this dissertation is a review of literatures on the background knowledge relating to the research topics. Second to Forth chapters of this dissertation are organized with the format of research papers: Chapter 2 “Effects of Grain Drying Temperature on Starch Functional Properties of Corn Kernels”, Chapter 3 “Changes in Starch Structures and Functions after Storage of Dried Corn-kernels at Elevated Humidity”, and Chapter 4 “Methods to Improve Nutritional Values of Cassava Flour for Animal Feed”. These four chapters are preceded by a General Introduction and followed by a General Conclusion. A preliminary study on “Enzyme Hydrolysis Pattern of Four α -amylases” is added in the appendix.

CHAPTER 1. LITERATURE REVIEW

Starch Structures

Amylose Structure

Amylose is essentially a linear polysaccharide of D-glucose units linked by α -1 \rightarrow 4 linkages with a few branches linked by α -1 \rightarrow 6 linkages (1, 2). Amylose was initially believed to be a linear molecule because it carried only one reducing and one nonreducing terminal residue (3). Amylose was first believed to carry few branches by Kerr and Cleveland (4) because they found few nonreducing terminals per molecules of amylose. This proposed structure of amylose was later supported by findings of Kjølberg and Manners (5), Takeda et al (6), and Curá et al (7), which showed that the linkages preventing complete β -amylase degradation of amylose are α -1 \rightarrow 6 glycosidic linkages. Amylose of normal corn starch carries one α -1 \rightarrow 6 glycosidic branch for every 300 D-glucose residues (8). The molecular weight of amylose ranges from a degree of polymerization (DP) of 900 for corn starch to 2100 for potato starch (9, 10). The amylose content of normal starch is typically about 15-30%. The amylose content of starch varies with the species and mutants. Amylose is synthesized alongside amylopectin by granular-bound starch synthase (GBSS) (11). Waxy maize starch, a mutant lacking expression of GBSS activity, contains negligible amylose content (<1%) comparing with a normal maize starch (20-30%). Amylose is more concentrated at the periphery (surface) of the starch granules (12). Amylose forms a single helical structure when it is in the form of an inclusion complex with organic compounds, such as dimethyl sulfoxide (DMSO), n-butanol, and n-pentanol (13, 14). The number of glucose units per helical turn depends on the size of the complexing compound (15). The

ability of amylose to form complex and crystallize has been used to fractionate amylose from amylopectin and to modify the properties of starch (16-21). Amylose forms single helical complexes with iodine to give blue color, which is conveniently used to determine amylose content of starch (22). The amylose-iodine complex gives dark blue color, and the color intensity is related to the chain-length of amylose (23). Determinating amylose content based on iodine affinity of the whole starch (apparent amylose) inflates the real amylose content, because it includes the contribution of amylopectin or intermediate components of starch. After starch gelatinization, amylose readily retrogrades in solution. The retrogradation rate of amylose in solution depends on the chain length of amylose, pH of solution, solution temperature, and storage time (24-27).

Amylopectin Structure

Amylopectin is a branched polysaccharide of D-glucose units linked by α -1 \rightarrow 4 and contains approximately 5% branches linked by α -1 \rightarrow 6 linkages. The cluster model of amylopectin was proposed by French (28) and further defined with chain classification by Hizukuri (29) (**Figure 1**). The average branch-chain length of amylopectin varies among plant species and is related to starch functional properties, such as gelatinization temperature, paste viscosity, and turbidity of starch solution (30-34). Amylopectin with shorter average branch-chain length is found in A-type polymorphic starches, whereas amylopectin with longer average branch-chain length is found in B-type polymorphic starches (35, 36). In A-type starch, the average amylopectin branch-chain length at the periphery of a granule is shorter than those found at the inner part of the granule. In the proposed model, amylopectin consist of three types of chains, namely A-, B-, and C-chains. A-chains, which are mostly external chains, are connected to B- and C-chains at their reducing ends by α -1 \rightarrow 6 branch

linkages and carry no other branch chains. B-chains are connected to other B- or C-chains and carry A-chains or other B-chains. The C-chain is the only chain in amylopectin that carries a reducing end group (29, 37-39). After acid hydrolysis of starch, the remaining residues (Naegeli dextrans) are used to understand crystalline structures of the starch. Structures of Naegeli dextrans show that A-type starches have greater numbers of branch linkages that were preserved in the Naegeli dextrans than B-type starches. These results suggest a model that the A-type starch contains amylopectin with branch linkages scattered in both the amorphous and crystalline regions, whereas the B-type starch contains amylopectin with branch linkages present only in the amorphous regions (35)

Organization of Starch Granules

Starch is synthesized in the form of semi-crystalline granules in plants. Shapes and sizes of starch granules vary with species, mutants, maturity and growth conditions of the plant. For example, normal maize starch has spherical and polygonal granules whereas high-amylose maize mutant has a mixture of spherical and elongated granules (40). Wheat starch has a mixture of large disc-shaped (A) and small spherical granules (B). Starch granules display a birefringence pattern (Maltese-cross) when viewed under a polarized-light microscope (**Figure 2**), reflecting an ordered structure within the granule. The organic center of the Maltese-cross is the initial nucleus of the starch granule known as the hilum (41). Amylose and amylopectin molecules are aligned perpendicularly to the surface and grow in radial arrangements from the hilum to the surface of the starch granule (28, 42-43). Amylopectin molecules have clustered structures consisting of both amorphous and crystalline regions. Amylose intertwines with amylopectin molecules. The native starch double helices are stabilized by hydrogen bonds and hydrophobic interactions (44). The

native semi-crystalline structures of the starch granule are classified into A-, B-, and C-type polymorphs. A-type starches have double helices that are packed into a monoclinic lattice, whereas B-type starches have double helices that are packed in hexagonal arrangement (**Figure 3**) (45). C-type starches, such as cassava starch, have a mixture of A- and B-type unit cells. X-ray diffraction is a commonly used method to determine the crystalline packing and the degree of crystallinity in the starch granules (45, 46). The interaction between amylose and amylopectin molecules in the native starch granule preserves the integrity of the granule (47, 48).

Starch Functional Properties

Starch gelatinization and retrogradation

In its native semi-crystalline structure, the starch granule is insoluble in water at ambient temperature. In the presence of excess water, starch granules absorb water and swell to a limited extent. Increasing the temperature of the starch suspension above the gelatinization temperature of the starch causes the starch granule to irreversibly lose its crystalline structure and molecular order (49). This process is known as *gelatinization*. During gelatinization, starch molecules absorb energy (endothermic) to dissociate the native double helices into an amorphous confirmation (50). Under a polarized-light microscope, the birefringence of starch granules (Maltese-cross) disappears after gelatinization. The gelatinization of starch occurs over a temperature range (onset to conclusion) that differs for each starch variety, for example: normal maize, 64.4 to 80.4°C; waxy maize, 64.2 to 80.4°C; and normal potato, 59 to 68°C (35). The amount of water present to plasticize the starch molecule affects the gelatinization temperature. Other factors that affect the gelatinization temperature of starch

are amylopectin branch-chain length and the content of phosphate-monoester derivatives of the starch. Starch with a larger proportion of short branch-chains (DP 6-12) has a lower gelatinization temperature than that with more long branch-chains (11). Studies with starches isolated from various botanical sources have shown that gelatinization temperature is negatively correlated with the proportion of short branch chains (DP 6-12) (34) and positively correlated with that of intermediate branch-chains (DP 13-24) (51). Phosphate-monoester derivatives bound to starch molecules reduces gelatinization temperature. Charge repulsion forces between the phosphate derivatives enhance swelling of starch granules, aiding starch gelatinization (34). Gelatinization of the starch granule can be achieved non-thermally using dimethyl sulfoxide (DMSO), CaCl_2 , LiCl , and alkali solution. DMSO is capable of dissociating the starch native double helices by forming hydrogen bonds (DMSO as an acceptor) with hydroxyl groups of starch molecules. CaCl_2 (12) and LiCl (47) solutions can interact with starch and have exothermic reactions. The heat released will dissociate the native double helices of starch. Alkali, such as NaOH and KOH , can dissociate the proton of hydroxyl group of the anhydroglucose, giving negative charges to starch that repel and disrupt native double helices (52).

Gelatinized starch molecules tend to recrystallize and form double helical structures upon cooling and storage. This process is known as *retrogradation* and is commonly associated with changes in the qualities of starch products, such as bread staling and hardening of rice cakes. Retrogradation of starch molecules involves three consecutive steps: nucleation (initiation), propagation (growth), and maturation (perfection) of the starch crystallites (53), and these steps are affected by the storage temperature, moisture content, and starch structures. The nuclei of the starch crystallites develop more rapidly at a lower storage

temperature (close to the glass transition temperature), whereas propagation of the starch crystallites is greater at high storage temperature (close to the melting temperature). Amylose retrogrades faster than amylopectin because amylose is linear and has more molecular mobility to realign and to reassociate. Amylopectin with a larger proportion of short branch-chains retrogrades to lesser extents (35). Lipids in starch complex with amylose molecules and retard retrogradation (53). Phosphate-monoester in the starch molecules, such as that found in most tuber and root starches, prevent starch molecules from realigning and retrograding because of charge repulsion.

Pasting Properties

Starch pasting properties are normally determined by measuring viscosity during a programmed heating-cooling under constant stirring. Heating starch granules in excess water swells the granules and increases viscosity. Swollen starch granules are fragile and easily disrupted when shear force is applied, resulting in decreased viscosity. On cooling, the starch molecules reassociate, form a gel, and increase the viscosity. A typical starch pasting profile and pasting parameters are illustrated in **Figure 4**. The pasting parameters that are commonly reported are peak viscosity (PV), pasting temperature (PT), trough viscosity, breakdown viscosity (BD), setback viscosity (SB), and final viscosity (FV). Starches with lower amylose contents display lower breakdown and setback viscosities than starches with more amylose (54). Starches with longer amylopectin branch-chain length have higher peak viscosity (55). Waxy cereal starches typically have higher peak viscosity than their normal starch counterpart (35). Most of the tuber and root starches tend to produce high viscosity pastes that break rapidly under a moderate shearing. The maturity of starch affects its pasting

properties. Starch isolated from immature corn has higher peak viscosity and lower pasting temperature than those of mature corn (56).

Starch digestibility

In the human digestive system, starch is hydrolyzed by consecutive enzymes to produce glucose for absorption. Pancreatic α -amylase, which is secreted by the pancreas, is an endo-acting enzyme that hydrolyzes α -1 \rightarrow 4 linkages of starch molecules and produces smaller oligosaccharides and limit-dextrins. These small oligosaccharides and limit-dextrins are then further hydrolyzed by amyloglucosidase (glucoamylase), a brush border enzyme, to produce glucose before entering the small intestine where absorption occurs. Some starches that resist enzymatic digestion are transported to the large intestine and are later fermented into short-chain fatty acids. This type of starch is commonly known as resistant starch (RS) which can be further classified into five different categories (57). RS1 is physically inaccessible starch, such as that found in legumes or partly milled grain. RS2 is mainly B-type polymorphic starch that is resistant to enzymatic hydrolysis in its native granular form, such as that found in uncooked potato and high-amylose maize mutant (40). RS3 is retrograded starch which is formed upon cooling of gelatinized starch. RS4 is chemically modified starch. RS5 is a starch of amylose-lipid complex.

Amylase attacks the starch granule by adsorption of the amylase onto starch granules followed by exo-corrosion on the surface of the granule. Once the enzyme penetrates the granule, successive endo-corrosion occurs (**Figure 5**). The rate and hydrolysis pattern of each amylase hydrolysis vary with the source of the enzyme (species specific) (58-60). Svensson (61) suggests that hydrolysis of starch granules depends on the enzyme adsorption rate to the starch granule surface.

In native starch granules, starch digestibility depends on many factors, including granule size, polymorphic form, amylose content, and lipid. Native starch granule of larger size has lower starch digestibility comparing with that of smaller size starch (62). A starch granule of larger size has less surface area to volume for enzyme to adsorb to the granule. A-type starches more starch digestible than B-type starches (63). The large proportion of short-chains in the amylopectin of A-type starches makes them vulnerable to enzyme hydrolysis. B-type starches, on the other hand, contain large proportions of long branch-chains extending through multiple clusters, which prohibit rearrangements of starch molecules in the granule and maintain the integrity against enzymatic hydrolysis. A-type starches have more porous structure (approximately 0.1 μm in diameter), which increase enzyme accessibility to starch molecules (64). Confocal laser-scattering micrographs (CLSM) of A-type starches show greater numbers of open space (void-zone) in their internal structures than B-type starches (48, 65). Amylose content of starch is negatively correlated with enzyme digestibility (47, 48, 66, 67). Starches with higher amylose contents, such as Hylon-5 maize starch (50% amylose) and Hylon-7 maize starch (70% amylose), have high resistant starch content. Waxy maize starch (<1% amylose), on the other hand, has greater enzyme digestibility than their normal starch counterparts. Lipid reduces the starch digestibility by 1) forming inclusion complex with amylose or long-chains of amylopectin (68) or 2) reducing the adsorption site for the enzyme (69). The starch digestive rate has impacts on human health upon consumption of products containing starch. On the basis of the starch digestive rate, starch is classified into rapidly-digestible starch (RDS) or slowly-digestible starch (SDS) (70).

Cassava Starch

Cassava root is one important food source in tropical countries, such as Thailand, Vietnam, Indonesia, and Brazil. Cassava starch has been commonly used in both food and non-food applications. Cassava starch has low lipids and protein contents, ranging from 0.097 to 0.43% (db) and 0.009 to 0.013% (db), respectively (71-74). The amylose content of cassava starch varies from 13.6 to 23.8% (75) or 17.9 to 23.6% (72). Cassava starch is a mixture of truncated, bell-shaped, and spherical granules (**Figure 6**) (71, 74) with sizes ranging from 4 to 43 μ m for Brazilian cultivars (74) and 8 to 22 μ m for Thailand cultivars (73). Cassava starch has a C_a-type X-ray diffraction pattern with ~38% crystallinity (46). Cassava starch has a wide variety of onset gelatinization temperature, ranging from 50.7 to 57.7°C (76), 62.4 to 65.8°C (77), 53.9 to 62.1°C (72), and 65.0 to 69.0°C (78). Cassava starch is commonly known to have significantly greater starch digestibility than corn or sorghum starches (79). The main concern of using cassava product for human consumption is the presence of two cyanogenic glucosides (linamarin and lotaustralin), which accumulated in the root during plant growth (80, 81).

Starch Interactions with Other Compounds

In food products, starch is often present together with lipids and proteins. Thus, any interactions between starch and these compounds during processing impact the quality of the products. The most common interaction between starch and lipids is the formation of amylose-lipid complex. Amylose forms a single helix around the hydrocarbon chain of lipids, such as fatty acids, diglycerides, monoglycerides, and phospholipids. The fatty acid chain occupies the cavity of the single helix through hydrophobic interaction with (82). Amylose-

lipid complexes display a V-type pattern when analyzed with an X-ray diffractometer (83). The functional properties of the amylose-lipid complex are affected by the structure of the lipid. The stability of amylose-lipid complex increases with chain length of saturated lipid (84), because long-chain fatty acids form stronger hydrophobic interaction with amylose than do short-chain fatty acids. Lipids with greater degrees of unsaturation form less stable amylose-lipid complex (85). Godet et al (86) found that the amount of insoluble complexes increases with increasing chain-length of amylose. The amylose-lipid complex exists in two different structures: Type I is an amorphous amylose-lipid complex that has low melting temperature ($<100^{\circ}\text{C}$), whereas Type II is a crystalline amylose-lipid complex that has high melting temperature ($>100^{\circ}\text{C}$) (84). Amylose-lipid complex is resistant to enzymatic hydrolysis. Hasjim et al (87) proposed a method to produce starch with high enzyme-hydrolysis resistance (up to 75%) by complexing debranched high-amylose maize starch (HA7) with fatty acids.

Interaction between starch and protein is often found in wheat doughs. Starch granules appear to adhere to the protein matrix, which rapidly forms upon hydration of flour. The earlier findings by Sandstedt et al (88) show that water concentration in the dough affects the interaction (adhesion) between starch and protein. At low water concentration, it appears to have a strong interaction between protein and starch, whereas at excess water content, the starch can easily be removed from the protein matrix. Starch-protein interactions increase during dough mixing but decreases upon over mixing (89). Pomeranz et al (90) report that swollen starch granules in wheat flour, mainly large granules, interact strongly with protein matrix than do small starch granules. Increasing pH of the dough weakens the starch-protein interaction, whereas increased protein content of the dough strengthens the interaction (91).

Starch granules in pasta dough are embedded in protein matrices, which are attributed to the greater protein content of durum wheat than that of other wheat species. The protein matrix prevents the diffusion of water into the starch granule of the pasta (92) and retards the starch gelatinization during boiling (93, 94).

Modifications of Starch

Native starches have been modified chemically or physically to produce a product with desired functional properties. The following section will review current and common methods used to modify starch.

Chemical modifications of starch

Chemically-modified starches commonly found in the industry include acid-thinned starch, cross-linked starch, oxidized starch, and derivatized starches. Acid-thinned starch is produced by limited acid hydrolysis of starch. Acid-thinned starch is commonly used in paper coatings and candy manufacture. Acid-thinned starch has lower peak viscosity but higher setback viscosity than the native starch counterpart. Cross-linking of starch is probably the most important chemical modification of starch. Starch is modified by replacing the native hydrogen bonding between starch chains with stronger and more rigid covalent bonds. Starch with an appropriate degree of cross-linking has significantly lower peak viscosity and greater heat and shear stabilities than native untreated starch (95, 96). Oxidized starch is produced by oxidizing the hydroxyl groups of starch into bulky carboxyl or carbonyl groups. The oxidizing agents used in this process are hydrogen peroxide or peracetic acid (97). Similar to acid-thinned starch, oxidized starch has a lower peak viscosity than the native starch. Oxidized starch, however, has low setback viscosity because of the

steric hindrance of the bulky group in the starch chains, negative charge repulsion, and smaller molecular weight. Oxidized starch is commonly used in the paper-making industry (98). Derivatized starches are produced by substituting bulky groups into the starch to prevent retrogradation. The common derivatized starches are phosphorylated starches, acetylated starches, hydroxypropylated starches, and succinylated starches. Derivatized starch is commonly used as thickener in frozen food products because it has good freeze-thaw stability (99).

Physical modifications of starch

Annealing and heat-moisture treatment (HMT) are hydrothermal methods used to physically modify starch properties. Annealing modifies starch properties by incubating starch with excess moisture (>40% w/w) at temperatures above the glass transition temperature (T_g) but below the gelatinization temperature (T_0) (100-102). Annealing improves starch granule stability and induces perfection of starch crystallites. Annealing also increases gelatinization temperature and decreases granular swelling and amylose leaching of starch (103, 104). Jacobs et al (105, 106) show that annealing decreases the rate of enzymatic hydrolysis of the starch at the early stage of hydrolysis but increases the extent of starch hydrolysis at the later stage of hydrolysis. Annealed starch interacts stronger with added palmitic acid and forms more inclusion complexes than native starch counterparts (107). Heat-moisture treatment (HMT) modifies starch properties by incubating starch with low moisture content (<35% w/w) for a certain period (15 min to 16 h) at high temperature (84-120°C) (108). HMT increases the gelatinization temperature of starch and decreases starch swelling, amylose leaching, and rate of enzymatic hydrolysis of starch. HMT also alters the

X-ray diffraction pattern of starch from B-type pattern into a mixture of A-type and B-type pattern depending on the botanical source of the starch (109-111).

Distillers Dried Grains with Solubles (DDGS)

DDGS is a co-product obtained from the production of corn ethanol. In recent years, interests in ethanol as a biorenewable fuel have led to great increases in DDGS production. The production rate has grown by nearly 20-25% per year since 2000. In 2005, approximately 8.5 million metric tons of DDGS was produced (112). DDGS produced by conventional dry-grind method of ethanol production usually contains 28-30% crude protein, 10-13% crude fat, 4-6% ash, and 11-16% acid-detergent fiber (db) (113-115). New corn fractionation methods to produce DDGS with an improved chemical composition have been proposed, such as Quick Germ (QG), Quick Germ Quick Fiber (QGQF), and Enzymatic Milling (E-Mill) (117, 118). QG and QGQF methods remove the germ and pericarp fiber as co-products at the beginning of the dry-grind corn process. E-Mill, recovers the germ, pericarp fiber, and endosperm fiber as valuable co-products. These new methods produce DDGS with significantly higher content of crude protein (36%-59%, db), but reduces all components (119). Traditionally, DDGS has been used to fortify ruminant feed because it contains high nutritional value (120, 121). In recent years, DDGS has been incorporated into both poultry feed and marine-animal feed. Incorporation of DDGS into poultry feed increases the yellow-color density of egg yolk with no changes in feed intake and growth rate of the chickens (122, 123). DDGS with darker brown color has shown lower amino acid availability and digestibility than that with lighter color. Decreased amino acid availability and

digestibility likely resulted from the Maillard browning reaction that occurs during ethanol production (124).

Soybean Meal

Soybean meal (SBM) is an extensively used ingredient for poultry diets and is the largest source of protein in the poultry feed. Soybeans are commonly dehulled and defatted (by solvent extraction). The main reason for the popularity of SBM in the poultry diets is the unique composition of amino acids that complement the amino acid compositions in many cereal grains. SBM is an excellent source of lysine and tryptophan but is deficient in methionine and cysteine required by poultry (125). The concentration of metabolizable energy (ME) in SBM is 11-25% greater than that of other oilseed meals (126), which is attributed to the lower fiber concentration. In the early days, SBM was known to have higher protein quality than other soy product, such as soy protein isolate (SPI, 90% protein), because SBM had higher concentrations of total and digestible methionine, cysteine, and threonine (127). Recent findings, however, showed that the apparent digestibilities of amino acids in SPI fed to chicks are greater than that in SBM (128). Full-fat soybean meal is also used for poultry diets, especially for broilers and turkeys. Diets containing high levels of full-fat soybean meal must be pelleted to improve diet density and better release the nutrients (129). Chickens fed diets containing dehulled-SBM show better growth performance than chicken fed diets containing SBM with hulls (130).

Effect of storage on chemical and functional properties of crops

Storage of crops has impacts on the properties of starch, lipids, protein, and the structural interactions among them (131-135). Storage of cassava roots (136) and rice kernels (137) at 21-30°C for several months decrease starch yield. Patindol et al (137) reported decreased contents of amylose and long branch-chain of amylopectin of the isolated starch after storing rough rice at high temperature (38°C) for nine months. Changes in starch structures during storage likely attributed to enzymatic hydrolysis of starch. Storage of crops increases lipid oxidation and free fatty acid contents of the grain (138, 139). Lipids oxidation is minimal in intact grain, but more pronounced in milled flour when the native structures are destroyed (140). Chrastil (141) reported that storage of rice kernels at 40°C for 12 months increased the molecular weight of oryzenin resulting from disulfide bond formations. Storage of cereal grain changes the activity of endogenous enzymes of the grain, such as amylase, peroxidase, and phospholipase-C (142). Zhou et al (143) reported a decrease in paste peak viscosity of milled rice flour after storage at 37°C for 16 months.

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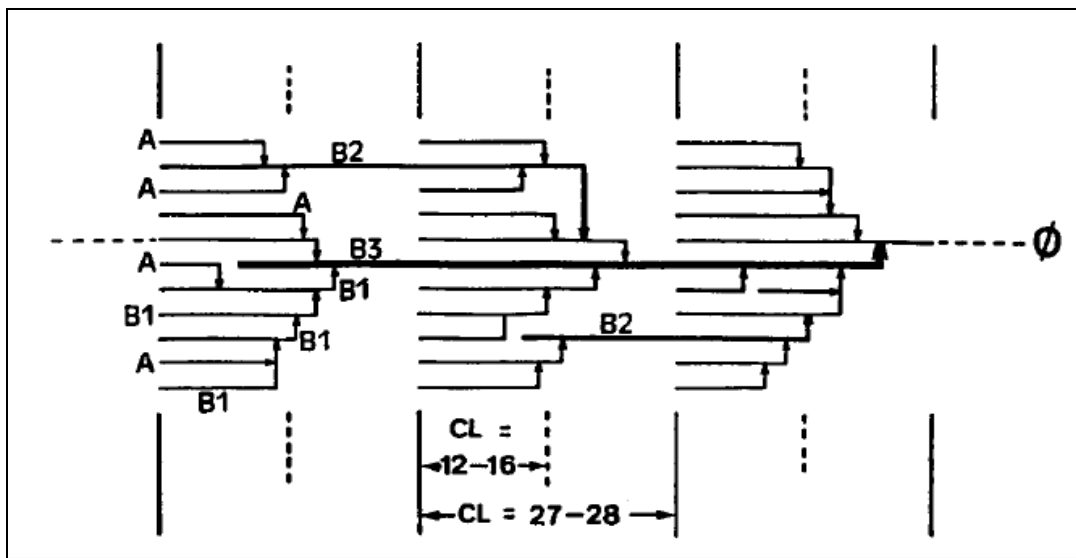


Figure 1. Proposed model of amylopectin cluster with chain classification (30).

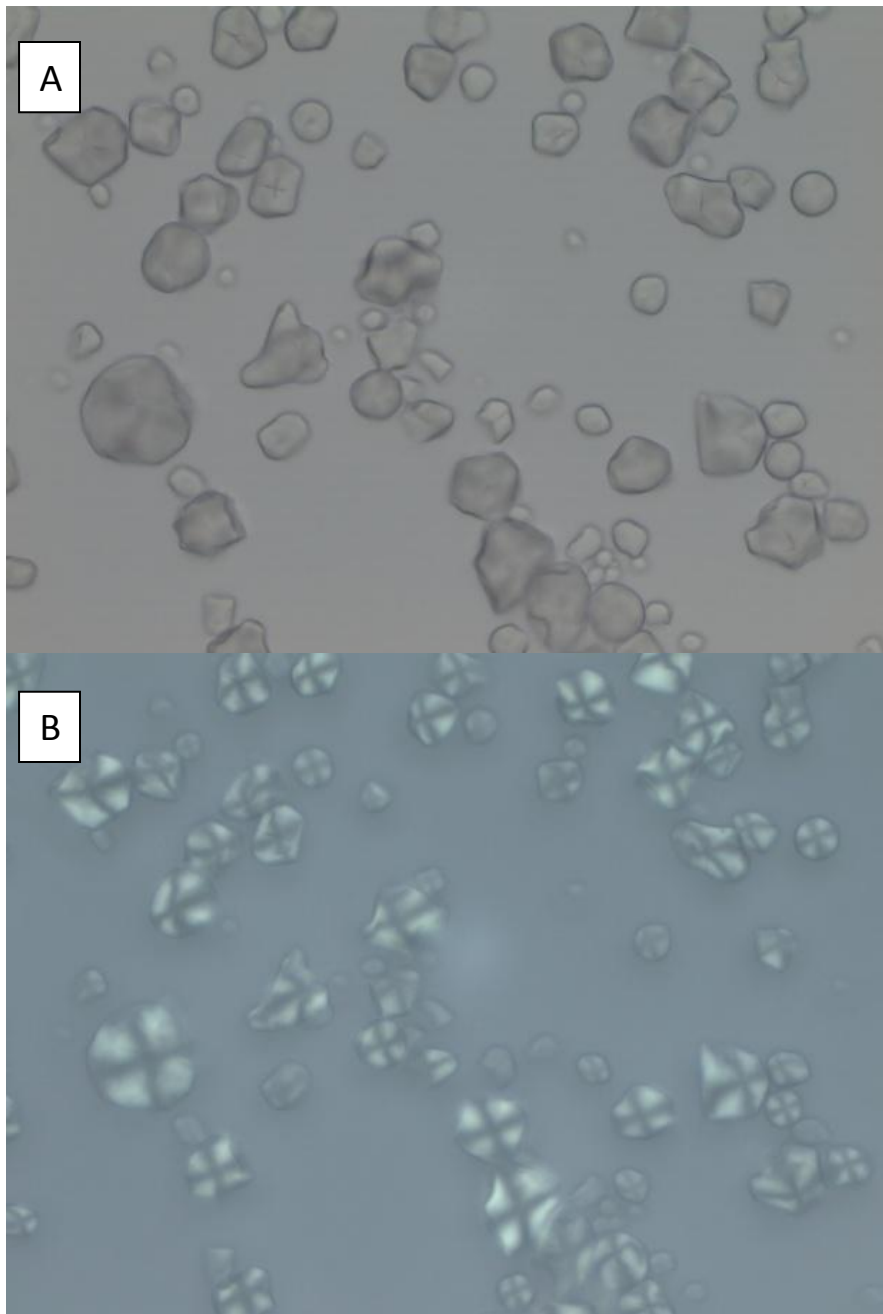


Figure 2. Light micrograph (100X) (A) and polarized-light micrograph (B) of normal maize starch showing a typical birefringence of starch commonly known as “Maltese-cross”.

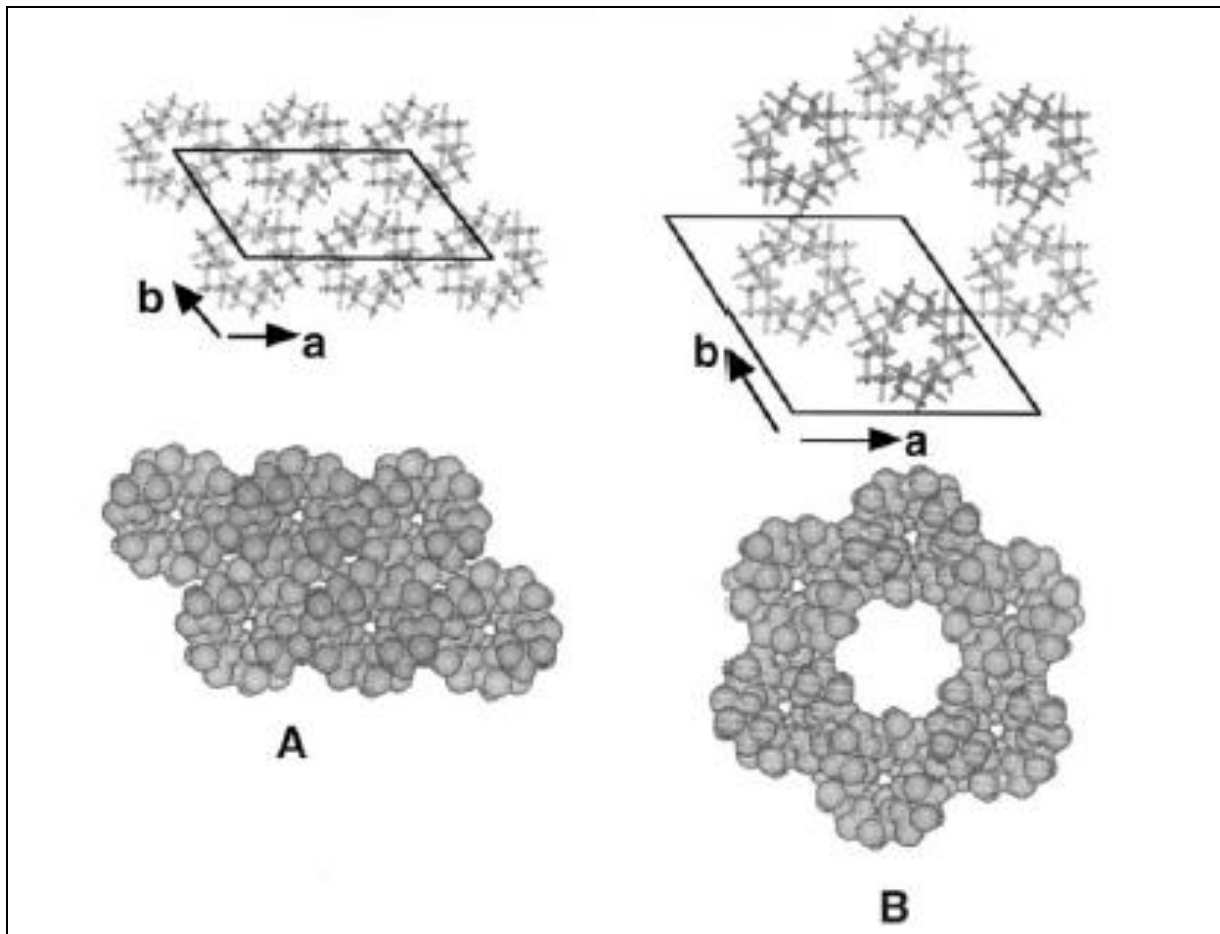


Figure 3. Double helices packing of amylopectin in A) A-type starches, and B) B-type starches (44).

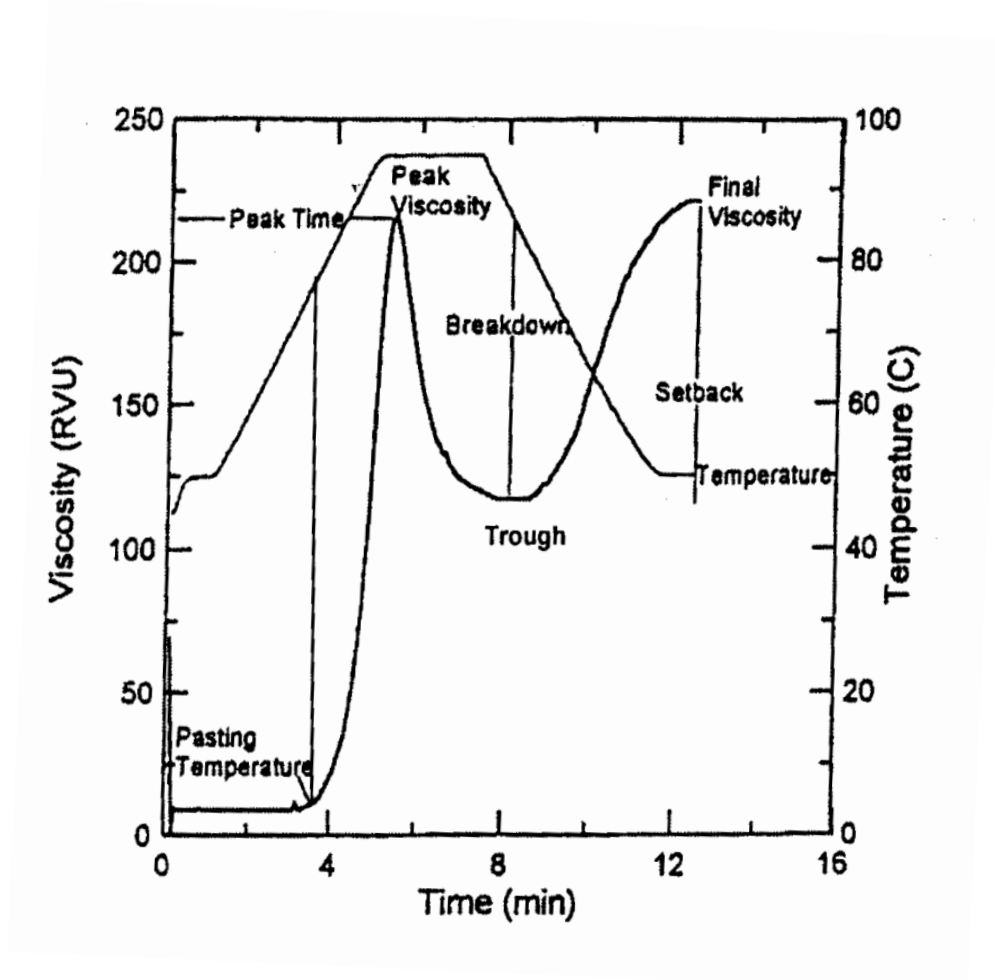


Figure 4. Typical starch pasting profiles and pasting parameters measured using Rapid Visco Analyzer (RVA) (144).

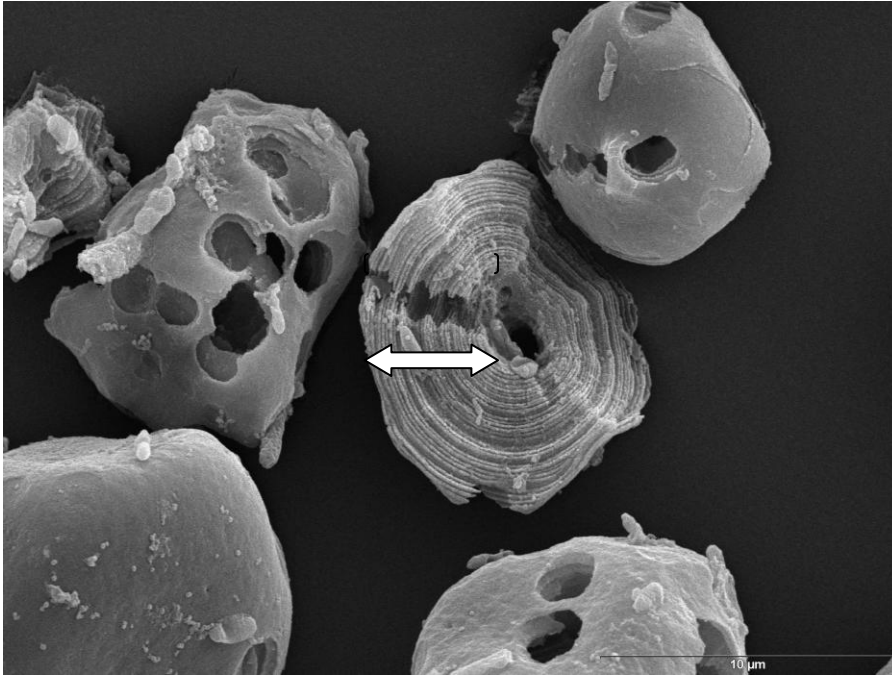


Figure 5. Scanning electron micrograph of amylase hydrolyzed starch (normal maize) residues illustrating the successive endo-corrosion mechanism of enzyme hydrolysis

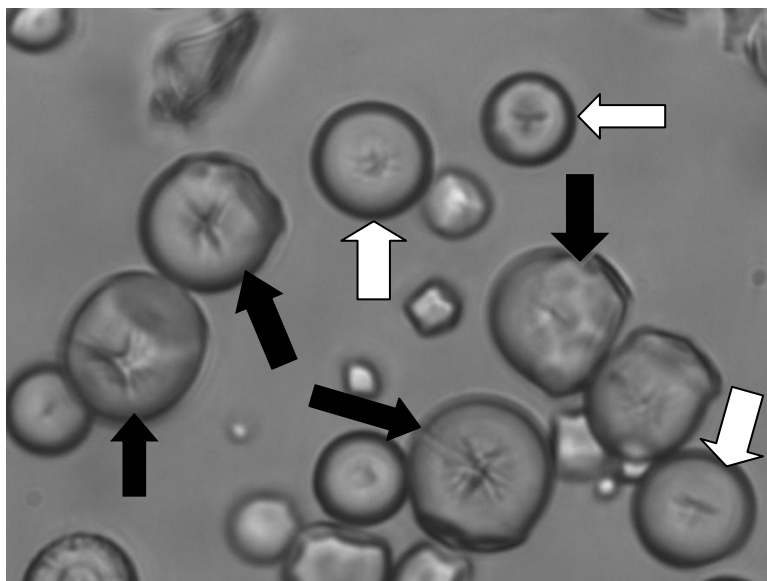


Figure 6. Light micrograph of cassava starch showing some typical bell-shaped (black arrow) and spherical (white arrow) granules.

CHAPTER 2. EFFECTS OF GRAIN DRYING TEMPERATURE ON STARCH FUNCTIONAL PROPERTIES OF CORN KERNELS

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ABSTRACT

The objective of this study was to understand effects of drying temperature on starch functional properties of corn kernels. Corn kernels of a maize hybrid line (B73xMo17, 32.5% moisture, db) were dried at three different drying temperatures (37, 80, 120/80°C). The corn kernels dried at higher temperatures (80 and 120/80°C) showed higher starch digestibility (78.8 and 81.5% after 8 h hydrolysis for 80 and 120/80°C, respectively) than that dried at a lower temperature (37°C) (65.7%). The polarized-light micrographs of starch isolated from corn kernels dried at higher temperatures showed some starch granules that lost their birefringence (Maltese-cross). Starch isolated from corn kernels dried at higher temperatures (80 and 120/80°C) showed higher gelatinization temperature and lower gelatinization enthalpy-changes. Starch digestibility of the dried-corn samples decreased after storage at 37°C and 93% relative humidity (RH) for 3 and 6 days.

INTRODUCTION

After harvesting, shelled maize kernels are commonly dried at high temperature to reduce the moisture content before storage. Effects of high-temperature drying on maize qualities,

such as starch yield, starch paste property, and resistance to mechanical forces have been reported (1-3). It is a common practice in some countries to harvest maize at high moisture contents (30-35%). Maize kernels with high moisture contents are susceptible to starch gelatinization when they are exposed to high-temperature drying. After gelatinization, starch loses its native crystalline structure and becomes more susceptible to enzymatic hydrolysis. Starch gelatinization is an endothermic reaction and can be determined using differential scanning calorimetry (DSC) (4). An increase in drying temperature of corn kernels results in higher onset starch gelatinization temperature and lower gelatinization enthalpy change (5, 6). Gelatinized starch molecules tend to retrograde and form double-helical crystallites during storage (7). Starch retrogradation rate depends on the storage conditions (temperature and relative humidity), amylose content, amylopectin structure and lipid content of starch (8-10). Retrograded starch is highly resistant to enzymatic hydrolysis and correlated with quality changes, such as bread staling and hardening of rice cakes. High-temperature drying of corn kernels decreases protein yield and protein nutritive value of the kernels (11, 12). High-temperature drying, however, does not change the fatty acid composition of maize kernels (13). The objective of this study was to understand effects of grain drying temperature on starch functional properties of corn kernels. This study will provide useful information to select a proper drying temperature for grain processing.

MATERIALS AND METHODS

Materials

A maize hybrid used in this study (B73xMo17) was grown in 2008, in Ames, IA, and provided by Dr. Michael Blanco of USDA-ARS. Freshly harvested maize kernels (32.5%

moisture, db) were dried at three different temperatures. Sample A was dried at 80°C for 5 hours using a flow-through air convection oven (VWR HAFO series Mod.1675, Westchester, PA). Sample B was dried at 120°C for 30 minutes to reduce the moisture content to approximately 20-21% followed by drying at 80°C for 3 hours using the same oven as used for sample A. These drying temperatures were used to mimic the actual drying conditions in animal feed production. Sample C was dried at 37°C for approximately 4 days using a convection oven (Napco Mod. 320). The moisture content of each maize sample after drying was analyzed using a gravimetric method. The dried-corn samples were ground using a Cyclone Mill (UDY Corporation, CO, USA) equipped with a screen with 0.5 mm openings. This grinding step was repeated twice to ensure homogeneous fine particle size. Porcine pancreatic α -amylase (PPA) and glucoamylase (GA) from *Aspergillus niger* were purchased from Sigma Aldrich Corporation (St. Louis, MO).

Starch digestibility

Starch digestibility of each dried corn sample was analyzed following the method of *Hasjim et al (14)* with modifications. Ground corn samples (<0.5mm particle size), each containing 200 mg of starch (db), were suspended in a phosphate buffer solution (20 ml, 0.02M, pH 6.9, containing 0.25 mM CaCl₂) and equilibrated at 40°C for 1 h. One ml of a PPA solution (200 units) was added to the suspension. Enzyme hydrolysis was carried out at 40°C, and aliquots (0.2 ml) of the hydrolysate were withdrawn at different time intervals. The aliquots were centrifuged at 6059 g for 5 minutes, and the supernatant was collected and diluted with a sodium acetate buffer solution (0.02 M, pH 4.5). Glucoamylase (10 units) was added to the supernatant, and the hydrolysis was carried out at 50°C for 2 h. The glucose

content of the hydrolysate was determined using Glucose assay kits (GOPOD assay).

Percentage starch hydrolysis (%) was calculated using the following equation:

$$\text{Percentage starch hydrolysis(\%)} = \frac{\text{Glucose content} \times 0.9}{\text{Initial starch weight (dry basis)}}$$

Storage of ground corn dried at different temperatures

Corn kernels dried at three different temperatures (80, 120/80, and 37°C) were ground (<0.5 mm particle size) and stored in chambers of 93 or 100% relative humidity (RH) at 37°C. An aqueous solution of saturated potassium nitrate (KNO₃) was used to provide 93% RH, and water was used to provide 100% RH. These storage conditions were chosen to mimic the storage conditions in the tropical climates.

Starch isolation

Dried corn kernels (50 g) were steeped in an aqueous solution of 0.45% sodium metabisulfite in a refrigerator for overnight. Germs of steeped corn-kernels were removed. The degermed-corn samples were ground using a commercial blender (Osterizer-14Speeds Blender, US) for 5 min. The slurry was filtered through a nylon screen with a pore size of 53 µm and washed with excess water. The residue was ground again with additional distilled water until no more starch was released. Starch was collected by centrifugation, re-suspended in an aqueous solution of 0.1 M NaCl containing 10% toluene, and stirred for 1 h to remove protein. This treatment was repeated until the toluene layer became clear and contained no protein. The purified starch was washed twice with distilled water, rinsed twice with 100% ethanol, dried at 37°C for 48 h, and kept in a sealed container before use (15).

Thermal properties of isolated starch

Thermal properties of the starch isolated from dried corn-kernels were analyzed using a differential scanning calorimeter (DSC) (Diamond DSC, Perkin-Elmer, Norwalk, CT) (16). Each isolated starch samples (2 mg, db) was precisely weighed in an aluminum pan, and deionized water (6µl) was added. The mixture was sealed and allowed to equilibrate at the room temperature for 1 hour. The sample was heated from 10°C to 110°C at a rate of 10°C/minute. An empty pan was used as the reference, and indium was used as a reference standard.

Light microscopy

Starch isolated from corn kernels dried at different temperatures was sprinkled on glass slides (75mm x 25mm), and a glycerol-water (50:50) solution was used as a medium. Starch samples were observed using a Nikon Labophot Series Microscope (Frank. E. Fryer. Co, Edina, MN) equipped with a digital camera (Infinity-3, Lumenera Corporation, Minneapolis, MN). Digital images were captured using imaging software (Infinity ANALYZE version 4.5.0)

RESULTS AND DISCUSSION

Drying regimes and the moisture contents of the dried samples are summarized in **Table 1**. Starch digestibility of ground maize samples dried at different temperatures are shown in **Figure 1**. The sample dried at 120/80°C showed the greatest rate of starch hydrolysis, whereas the sample dried at 37°C showed the least. **Figure 1** showed that after 8 h

hydrolysis, sample B reached 81.5% starch hydrolysis followed by sample A at 78.8% and sample C at 65.7% (**Figure 1**). After 24 h hydrolysis, all the samples were completely or close to be completely hydrolyzed (99.8-100%), indicating no significant effect of drying temperature on resistant starch formation.

Thermal properties of the starch isolated from the dried maize samples (A, B and C) are summarized in **Table 2**. Starch isolated from sample B, which was dried at 120/80°C, had the lowest enthalpy change (11.56 J/g) followed by that from sample A (12.14 J/g) and sample C (14.21 J/g). The differences in gelatinization enthalpy-changes were attributed to partial gelatinization of starch in samples A and B (17, 18). Starch partial gelatinization caused disassociation of some of the starch native double helices. Thus, the starch required less energy to be fully gelatinized during DSC thermal scanning. Starch isolated from samples A and B had higher onset gelatinization temperatures (T_o) than that from sample C. The increase in the onset gelatinization temperature of starch after high-temperature drying has been reported in the literature (19). The gelatinization of starch in the samples A and B occurred on those less-stable double helices of the starch molecules, which readily gelatinized during heating. Thus, the double helices of the starch samples remaining after drying were more stable and displayed higher onset gelatinization temperatures (20). Partial gelatinization of starches in samples A and B resulted greater rate of starch digestibility shown in **Figure 1** (21)

Partial gelatinization of starch was reflected by loss of birefringence (Maltese cross) of isolated starch granules when viewed under a polarized-light microscope. The bright-field and polarized-light micrographs showed that starch isolated from sample B suffered a greater degree of starch partial gelatinization than starch isolated from samples A and C (**Figure 2**).

Sample B, dried at 120/80°C, had more starch granules without birefringence than the sample A, dried at 80°C. Sample C, on the other hand, had intact starch granules that exhibit clear birefringence. Guler et al reported that heating the wheat dough at 65 and 85°C during pasta preparation caused some starch granules to lose their birefringence (22).

Starch digestibilities of the samples decreased after storage at 37°C and 93% RH for 3 and 6 days (**Table 3**). Exposure of starch to high humidity at 37°C during storage likely caused gelatinized-starch molecules to retrograde and crystalline starch to anneal. The decreases in starch hydrolysis rate were more significant during the first 4 h hydrolysis. For example, after 6 days of storage, sample A showed 3.7, 5.2, and 4.0% reduction in starch digestibility after 1 h, 2 h, and 3 h of hydrolysis, respectively, comparing with 1.4% after 8 h hydrolysis (**Table 3**). The samples stored at 100% RH showed severe mold growth after 1-2 days of storage. Samples A and B showed earlier signs of mold growth than sample C. All samples stored at 93%RH showed mold growth with the same trend after 7-8 days of storage. These results suggested that drying at high temperatures decreased microbial stability of the samples during storage at 37°C and high relative humidity.

CONCLUSION

Grain drying temperature of corn kernels affected the starch functional properties of the dried products. Drying corn kernels with high moisture contents (32.5%, db) at the high temperatures (80 and 120/80°C) resulted in partial gelatinization of starch. The partially gelatinized starch showed loss of Maltese-cross when viewed under a polarized-light microscope. The partially gelatinized starch was responsible for the greater rate of starch

digestibility, the high starch onset-gelatinization temperature, and the lower gelatinization enthalpy-change of samples dried at the higher temperature. All dried corn samples showed decreased starch digestibility after storage at 37°C and 93% RH for 3 and 6 days.

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Table 1. Drying process and moisture contents of the corn kernels used in samples A (80°C), B (120/80°C) and C (37°C)

Sample ID	Initial Moisture (%)	First Dryer		Second Dryer	
		Temperature (°C)	Moisture Out (%)	Temperature (°C)	Moisture Out (%)
A (80°)	32.5 ± 0.8	80	12.1	-	
B (120°/80°)	32.5 ± 0.8	120	21.3	80	11.2
C (37°C)	32.5 ± 0.8	37	10.9	-	

Table 2. Thermal properties of starches isolated from corn kernels dried at A (80°C), B (120/80°C) and C (37°C)

Sample	Thermal Properties			
	To (°C)	Tp (°C)	Tc (°C)	ΔH (J/g)
A (80°)	64.61 ± 0.09	69.70 ± 0.14	74.40 ± 0.13	12.14 ± 0.07
B (120°/80°)	65.19 ± 0.12	72.36 ± 0.36	74.94 ± 0.02	11.56 ± 0.10
C (37°C)	60.62 ± 0.12	67.76 ± 0.04	73.59 ± 0.56	14.21 ± 0.19

Table 3. Starch digestibility of finely-ground corn samples dried at different temperatures (A, B and C) stored at 37 °C and 93 % relative humidity for 0, 3 and 6 days

Hydrolysis Time (h)	Percentage Hydrolysis (%)								
	A			B			C		
	0d	3d	6d	0d	3d	6d	0d	3d	6d
1	47.3 ± 0.6	45.2 ± 1.3	43.8 ± 2.7	51.5 ± 0.6	47.0 ± 0.8	47.4 ± 0.5	30.6 ± 0.5	28.2 ± 2.3	26.9 ± 1.4
2	57.4 ± 2.3	53.5 ± 1.1	52.1 ± 0.5	60.4 ± 0.8	58.5 ± 2.1	58.8 ± 0.8	38.2 ± 1.7	37.6 ± 0.2	36.4 ± 0.8
3	64.5 ± 1.1	61.0 ± 2.1	60.4 ± 3.1	68.0 ± 2.3	67.0 ± 1.4	66.5 ± 2.1	46.2 ± 0.3	43.4 ± 0.7	43.2 ± 0.7
4	67.9 ± 0.2	64.7 ± 1.2	64.8 ± 0.5	70.0 ± 1.8	69.6 ± 1.7	69.4 ± 0.4	49.6 ± 0.6	48.8 ± 0.5	49.0 ± 1.5
6	73.3 ± 2.4	72.6 ± 3.1	72.1 ± 0.7	75.4 ± 0.4	75.9 ± 1.6	75.6 ± 0.4	58.3 ± 0.8	58.5 ± 0.3	58.1 ± 0.4
8	78.8 ± 2.7	77.1 ± 1.8	77.4 ± 1.5	81.5 ± 1.5	80.3 ± 2.1	80.3 ± 0.8	65.7 ± 1.3	66.8 ± 2.7	65.1 ± 1.5
24	99.8 ± 0.6	98.4 ± 1.6	99.9 ± 2.3	100.4 ± 2.3	100.1 ± 2.1	99.2 ± 3.8	99.9 ± 2.0	99.8 ± 1.2	98.3 ± 2.1

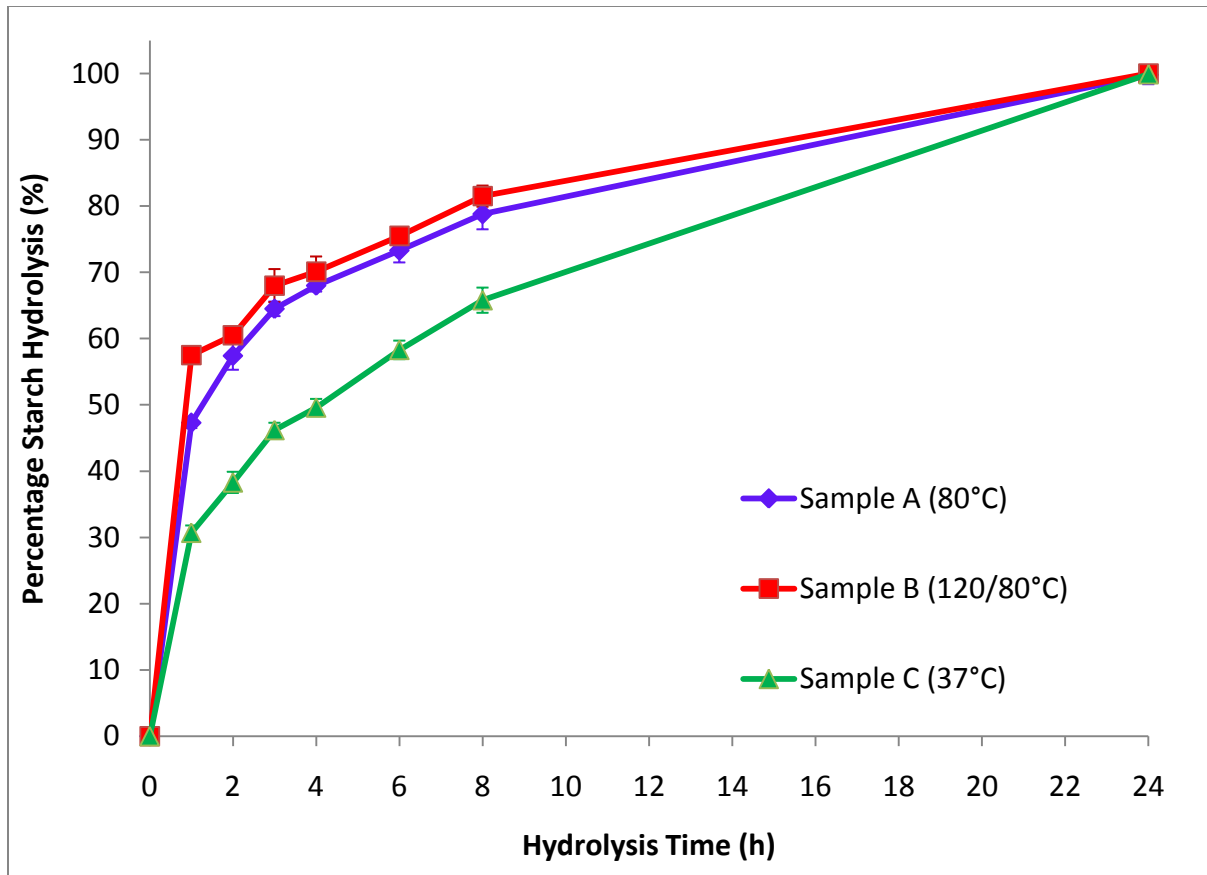


Figure 1. Starch digestibility of finely-ground corn samples dried at different temperatures measured as on the basis of glucose production over starch content on dry weight basis

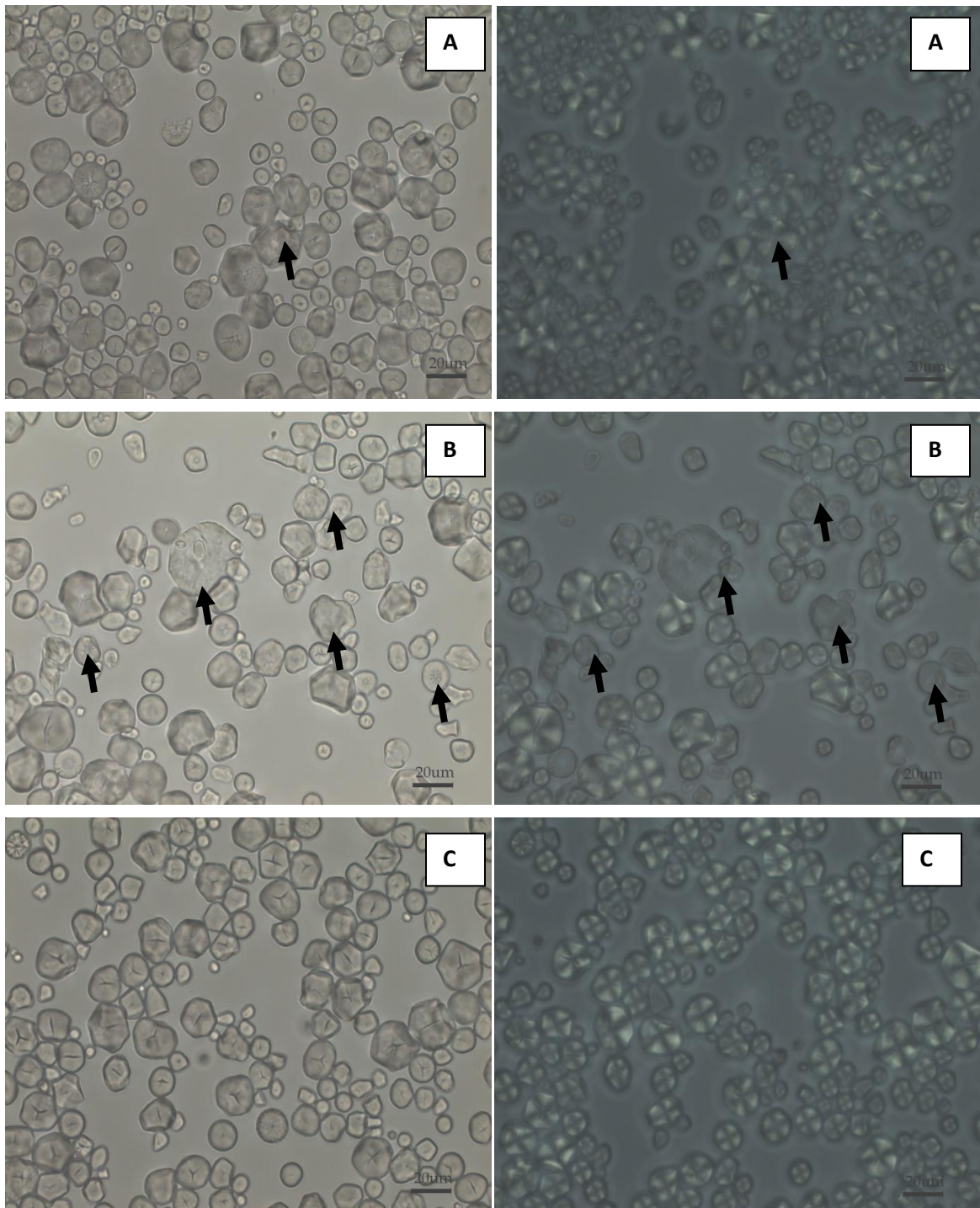


Figure 2. Bright-field and polarized-light micrographs (400X) of starch isolated from maize dried at A (80°C), B (120/80°C), and C (37°C)

Arrow indicates partially-gelatinized starch granule

CHAPTER 3. CHANGES IN STARCH STRUCTURES AND FUNCTIONS AFTER STORAGE OF DRIED CORN KERNELS AT AN ELEVATED HUMIDITY

A paper to be submitted to the Journal of Agricultural and Food Chemistry

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ABSTRACT

The objective of this study was to understand effects of storing dried corn kernels at 27°C and 85-90% relative humidity for up to 6 months on starch structures and functions. Corn kernels were sun dried at 35°C or machine dried at 80°C prior to storage. Starch digestibility and peak viscosity of both ground sun-dried and machine-dried corn samples decreased after storage. The rate of enzymatic hydrolysis of starch isolated from the sun-dried samples increased but that from the machine-dried samples decreased with the storage time of the corn. The gelatinization temperature, pasting temperature, and percentage crystallinity of the isolated starch increased but the gelatinization enthalpy-change and peak viscosity of the starch decreased with storage time of the corn. Numbers of damaged starch granules and

starch granules with pinholes increased and the molecular weight of starch and long branch-chains of amylopectin decreased with storage time.

INTRODUCTION

Storage of dried crops is a common practice in food and agriculture industries. Changes in chemical structures and functional properties of the crops after storage impact their processability and qualities of products made from them. Extensive storage is known to reduce starch yield during wet milling because of starch degradation and interactions between starch and other components of the grain (1). Storage increases lipid oxidation and free fatty acid contents of the grain (2, 3). The free fatty acids can form helical complex with amylose or long branch-chains of amylopectin and alter the physical and nutritional properties of the grain (4, 5). Storage of crops also decreases grain protein solubility and digestibility (6, 7). *Chrastil et al* (8, 9) reported that storage increased the molecular weight of the protein in rice grain through disulfide bond formation. Storage of crops changes the activity and properties of endogenous enzymes present in the grain, such as amylases, proteases, and phosphatase (10).

Starch is widely used in food and non-food applications. Thus, changes in starch functional properties significantly impact the quality of products made from the starch (11). *Patindol et al* (12) reported that storage of rough rice at controlled temperatures of 4, 21, and 38°C up to nine months affected starch pasting and thermal properties and decreased long branch-chains of amylopectin. Effects of storage of dried corn kernels at elevated humidity on starch structures and functional properties are not well understood. The objective of this

study was to understand effects of storage of dried corn-kernels at 27°C and 85-90% relative humidity (RH) on starch structures and functions. In the present study, shelled corn kernels (B-816, 27.8% moisture, db) were sun-dried at 35°C (SD) or machine-dried at 80°C (MD).

MATERIALS AND METHODS

Materials

Shelled corn kernels (B-816, 27.8% moisture, db) were dried using sun-drying (35°C) and machine-drying methods (80°C) until they reached the moisture contents of 12.5-13.1% (db). Dried corn kernels were stored at 27°C and 85-90%RH for up to 6 months. Each of the stored corn-samples was ground using a commercial blender (Panasonic MX-J1G, Indonesia), and sieved using a sieve with 1.0 mm openings. The ground samples were kept in a sealed container at -15°C prior to analyses. The dried-corn samples without storage (0 month) were used as the control. B-816 is a tropical maize variety that was grown and provided by Charoen Pokphand Indonesia. The drying and storage of this corn were done in Indonesia, and coarsely ground corn samples were sent by express mail to Iowa State University for characterization of corn and isolated starch. Porcine pancreatic α -amylase (PPA) and amyloglucosidase from *Aspergillus niger* were purchased from Sigma Aldrich Corporation (St. Louis, MO). Glucose assay kits (GOPOD format, Catalog: K-GLUC) were purchased from Megazyme International (Wicklow, Ireland).

Starch isolation

Dried corn samples (50 g) were steeped in an aqueous solution of 0.45% sodium metabisulfite in a refrigerator overnight. The steeped sample was ground using a commercial blender (Osterizer-14Speeds Blender, US) for 5 min, and the process was repeated three times. The ground sample was filtered through a nylon screen with a pore size of 53 μm and washed with excess water. The residue was ground again with additional distilled water until no more starch was released. Starch was collected by centrifugation, re-suspended in an aqueous solution of 0.1 M NaCl containing 10% toluene, and stirred for 1 h to remove protein. This treatment was repeated until the toluene layer became clear and contained no protein. The purified starch was washed twice with distilled water, rinsed twice with 100% ethanol, dried at 37°C for 48 h, and kept in a sealed container until used (13).

Starch digestibility

Starch digestibilities of ground corn samples and isolated starch samples were analyzed following the method of *Hasjim et al (14)* with modifications. Isolated starch (200 mg, db) or finely-ground corn (<0.5mm particle size) containing 200 mg of starch (db) was suspended in a phosphate buffer solution (20 ml, 0.02 M, pH 6.9, containing 0.25 mM CaCl_2) and equilibrated at 40°C for 1 h. An aliquot of each sample was withdrawn before adding PPA and analyzed for the initial soluble-sugar content. One ml of PPA solution (200 units/g of starch) was added to the suspension. Enzyme hydrolysis of starch was carried out at 40°C, and aliquots (0.2 ml) of the hydrolysate were withdrawn at different time intervals. The aliquots were centrifuged at 6059 g for 5 minutes, and the supernatant was collected and diluted with a sodium acetate buffer solution (0.02 M, pH 4.5). Amyloglucosidase (10 units) was added to the supernatant, and the hydrolysis was carried out at 50°C for 2 h. The

glucose content of the hydrolysate was determined using Glucose assay kits (GOPOD format). The analysis was done in duplicate. Percentage starch hydrolysis (%) was calculated using the following equation:

$$\text{Percentage starch hydrolysis(\%)} = \frac{\text{Glucose content} \times 0.9}{\text{Initial starch weight (dry basis)}}$$

Pasting properties of starch

Pasting properties of ground corn samples and isolated starch samples were analyzed using a Rapid Visco-Analyzer (RVA-4, Newport Scientific, Sidney, Australia) (15). Isolated starch (2.24 g, db) or finely-ground corn (<0.5 mm particle size) containing 2.24 g of starch (db) was suspended in distilled water in an RVA canister making up the total weight to 28 g. The starch suspension was premixed at 960 rpm for 10 seconds, equilibrated at 50°C for 1 min, heated to 95°C at a rate of 6.0°C/min, maintained at 95°C for 5.5 minutes, and then cooled down to 50°C at a rate of 6.0°C/min. A constant paddle rotating speed (160 rpm) was used after the premixing step and through the analyses. The analysis was done in duplicate.

Thermal properties of starch

Thermal properties of the isolated starch samples were analyzed using a differential scanning calorimeter (DSC) (Diamond DSC, Perkin-Elmer, Norwalk, CT) (16). Each starch sample (2 mg, db) was precisely weighed in an aluminum pan, and deionized water (6 µl) was added. The mixture was sealed and allowed to equilibrate at the room temperature for 1 h. The sample was heated from 10 to 110°C at a rate of 10°C/minute. An empty pan was

used as the reference, and indium was used as a reference standard. The analysis was done in triplicate

Morphology of starch granules

Morphology of isolated starch granules was studied following the method of Jane et al (17). Starch was suspended in absolute methanol, and a drop of the suspension was placed on silver tape, sticky side down, attached to a brass disk and sputter coated with gold/palladium (60/40). The mounted specimens were captured at a magnification of 1500X using a scanning electron microscope (SEM, JEOL model, JSM-5800LV Tokyo, Japan) at the Microscopy and Nanoimaging Facility (Bessey Hall, Iowa State University, Ames, IA).

Starch Crystallinity

The X-ray diffraction patterns and percentages crystallinity of isolated starch samples were studied using X-ray diffractometry (18). Samples were equilibrated in a chamber of 100% relative humidity at room temperature (22-24°C) for 24 h. The X-ray diffraction patterns of starches were obtained with copper K_{α} radiation using a diffractometer (D-500, Siemens, Madison, WI) at Town Engineering X-Ray Facility (Iowa State University, Ames, IA). The diffractometer was operated at 27 mA and 50 kV. The scanning region of the two-theta angle (2θ) was from 4 to 40° at 0.05° step size with a count time of 2 seconds.

Molecular-weight distributions of starch

Molecular-weight distributions of isolated starch samples were determined using gel permeation chromatography (GPC) (16). Starch was dispersed in 90% DMSO by heating and

stirring the starch suspension in a boiling-water bath for 1 h and followed by stirring at room temperature (22-24°C) overnight to make a 1% (w/v) dispersion. The dispersed starch was recovered by precipitation with ethanol (5X, v/v) and centrifuged at 6059 g for 30 minutes. The collected sample was then re-dispersed in hot deionized water to make a 0.3% dispersion by heating in the boiling-water bath for 30 minutes. After cooling to room temperature, the starch dispersion was injected into a GPC column (1.5 cm i.d. x 50 cm) packed with Sepharose CL-2B gel (Pharmacia, Inc., Piscataway, NJ). Eluent of each fraction (1.66 ml) was collected, and the total carbohydrate content (CHO) of the eluent was analyzed using a phenol-sulfuric acid method (19), and the blue value (BV) was analyzed by mixing the eluent with an iodine/potassium iodide solution at 1:1 (v/v) (20). The colors developed from the CHO and BV analyses were quantified using an Ultra Microplate Reader (EL_x808, Bio-Tek Instruments, Inc., Winooski, VT) at 490 and 630 nm, respectively. The analysis was done in duplicate.

Branch-chain length distribution of amylopectin

Amylopectin branch-chain length distribution of the isolated starch sample was analyzed following the method of *Morell et al* (21). Amylopectin was separated from amylose by complexing and precipitating the amylose molecule with n-butanol (22). The isolated amylopectin was debranched using isoamylase (Megazyme, Ireland) at 40°C for 16 h, dried, and derivatized with 8-amino-1, 3, 6-pyrenetrisulphonic acid (APTS). The branch-chain length distribution was analyzed using capillary electrophoresis (P/ACE) (MDQ, Beckman Courter, Fullerton, CA). The analysis was done in duplicate.

Statistical Analysis

Mean values of the starch digestibility, pasting and thermal properties of starch, molecular weight distribution of starch, and branch-chain length distribution of amylopectin were analyzed using analysis of variance (ANOVA) with the General Linear Model procedure in SAS version 9.1 (SAS Institute, Inc., Cary, NC). Differences were evaluated by t-test using Tukey's adjustment. The significance level was set at p -value < 0.05 .

RESULTS AND DISCUSSION

Starch digestibility of ground corn samples (<0.5 mm particle size) after 0 to 6 months of storage are shown in **Table 1**. The concentration of soluble sugars of the sun-dried corn samples (0 h, before adding PPA) increased from 2.5% on 0 month to 4.4% after 6 months of storage (**Table 1**). In contrast, the concentration of soluble sugars of the machine-dried corn samples showed no clear trend of changes after storage. The increasing concentrations of soluble sugars of the sun-dried corn after storage were attributed to starch hydrolysis during storage. It is plausible that either endogenous amylases or amylases of contaminating microorganisms present in the sun-dried corn hydrolyze the starch during storage. Most of the amylases could be inactivated during machine drying at 80°C , and, thus, there was no trend of increases in soluble sugar content with storage of machine-dried corn samples. Both the sun-dried and machine-dried corn samples showed decreases in starch digestibility after storage (**Table 1**). The pasting properties of the ground corn samples (<0.5 mm particle size) after different periods of storage are shown in **Figures 1**. The peak viscosities (PV) of the corn samples gradually decreased between 0 and 6 months of storage, from 164.0 to 107.3

RVU and 168.3 to 113.3 RVU for sun-dried and machine-dried corn, respectively. These results agreed with that reported by Zhou et al on decreases in the peak viscosity of rice flour after storage (23).

Enzymatic hydrolysis rates of starches that were isolated from the dried-corn samples after storage, however, showed different trends from those of ground corn samples (**Table 2**). The starches isolated from sun-dried corn samples showed increases in the rate of starch hydrolysis from 77.2 (0 month) to 84.9% (6 month) after 8 h hydrolysis. In contrast, the starches isolated from the machine-dried samples showed decreases in the rate of hydrolysis from 79.1 (0 month) to 75.6% (6 month) after 8 h hydrolysis (**Table 2**). Similar to the ground corn samples (**Table 1**), the concentration of soluble sugars of the sun-dried starch samples (0 h, before adding PPA) increased from 2.4 to 5.1% after 0 to 6 months of storage, whereas, that of the machine-dried counterparts showed little differences (**Table 2**). The starch isolated from machine-dried corn sample without storage showed a slightly greater initial rate of enzyme hydrolysis (e.g. 47.5% at 2 h hydrolysis) than that of the sun-dried counterpart (44.1% at 2 h hydrolysis) (**Table 2**). This difference could be a result of heating the corn kernels at 80°C during machine drying, which caused partial gelatinization of starch. Gelatinized starch is known to be more easily digestible (24). After storage, the gelatinized starch could be retrograded and became resistant to enzyme hydrolysis (**Table 2**). More discussion will follow.

Thermal properties of the starch isolated from sun-dried and machine-dried corn samples after different periods of storage are shown in **Table 3**. The onset gelatinization temperature (T_o) of the starch increased after 0 to 6 month storage (68.74 to 71.21°C and 68.76 to 71.95°C for sun-dried and machine-dried starch samples, respectively). The starch isolated

from machine-dried corn without storage showed lower gelatinization enthalpy-changes (12.28 J/g) than that from the sun-dried counterpart (13.21 J/g). This result confirmed that drying corn kernels with 27.8% moisture content at 80°C caused partial gelatinization of starch. The gelatinization enthalpy-changes (ΔH) of starches from both sun-dried and machine-dried samples decreased with storage. The decreases in the gelatinization enthalpy-change after 6 months of storage were greater for the starch isolated from sun-dried samples (2.67 J/g) than that from the machine-dried counterparts (1.57 J/g) (calculated from data in **Table 3**).

The X-ray diffraction patterns and percentages crystallinity of the starch samples isolated from sun-dried and machine-dried corn after storage are shown in **Figures 2**. All the starch samples showed typical A-type diffraction patterns with major 2θ peaks at 15°, 17°, 18°, and 23° (25). Starch isolated from the sun-dried corn without storage showed a higher percentage crystallinity (28.6%) than that from the machine-dried samples (27.4%). The lower percentage crystallinity of the machine-dried starch sample further supported the observation of partial-gelatinization of starch in the machine-dried corn. The percentage crystallinity of the starch increased with storage from 28.6 (0 month) to 31.9% (6 month) and 27.4 (0 month) to 28.4% (6 month) for the starch isolated from sun-dried and machine-dried samples, respectively. The increase in the percentage crystallinity of the starch suggested improved quality and increased size of crystallites of the starch after storage at 27°C and 85-90% RH (26), which differed from decreased amounts of double helices in the starch reflected by decreased gelatinization enthalpy-changes (**Table 3**). Differences in crystallinity changes between the starch samples of sun-dried and machine-dried corn will be discussed later.

Pasting properties of the starches isolated from sun-dried and machine-dried corn samples after storage are shown in **Figures 3A and B** and summarized in **Table 4**. The pasting temperature (PT) of starch isolated from dried corn stored for 0 to 6 months increased from 72.5 to 75.6 °C and 73.6 to 75.4°C for the sun-dried and machine-dried corn, respectively. The starch from sun-dried corn after 0 and 2 months of storage showed a peak-viscosity range of 158.2-160.9 RVU, whereas that after 3 to 6 months of storage showed remarkable decreases in the peak viscosity to 128.5-132.5 RVU (**Figure 3A** and **Table 4**). The peak viscosity of the machine-dried starch samples gradually decreased, from 154.6 to 133.8 RVU after 0 to 6 month storage (**Figure 3B**). The decreases in the peak viscosity of the ground corn after storage (**Figures 1A and B**) were mostly related to decreases in the peak viscosity of the starch. The decreases in the peak viscosity of the ground corn samples after storage (56.7 and 55.0 RVU for sun-dried and machine-dried corn, respectively) were more pronounced than that of the isolated starch samples (25.7 and 20.8 RVU for starches of sun-dried and machine-dried corn, respectively), indicating that non-starch components of the grain, such as free fatty acids from lipids, which complex with starch, and disulfide bond formation of protein, also contributed to the reduced peak viscosity of the ground corn after storage.

The morphology of starch granules isolated from dried corn after storage using SEM is shown in **Figure 4**. The SEM images show increases in the number of damaged starch granules (broken granules and debris) after storage of both the sun-dried and machine-dried corn. The number of damaged starch granules was larger in the starch from sun-dried corn than that from machine-dried corn (**Figure 4**). Starches isolated from machine-dried corn showed fewer starch granules with pinholes on the surface than that from sun-dried corn after

the same period of storage (**Figure 4**). The difference was attributed to less starch-hydrolyzing enzyme activities in machine-dried corn, resulting from high-temperature drying (80°C). The larger number of damaged starch granules in the starch from sun-dried corn coincided with its greater rate of enzymatic hydrolysis (**Table 2**). Damaged starch granules had more internal structures exposed, which were more susceptible to enzymatic hydrolysis (14). The presence of damaged-starch granules also decreased the peak viscosity of the isolated starch (**Table 4**) (27).

Molecular-weight distributions of the isolated starches analyzed using GPC displayed two peaks, representing amylopectin (first peak) and amylose (second peak) (**Figure 5**). The proportion of the second peak of starches isolated from sun-dried and machine-dried corn increased from 28.9 to 35.7% and 28.2 to 31.7%, respectively, after 0 to 6 month storage (**Table 5**). The increases in the proportion of the second peak were attributed to the hydrolysis of amylopectin molecules. The partially-hydrolyzed amylopectin molecules had smaller molecular weights, which were co-eluted with amylose and increased the size of the second peak. The hydrolysis of the amylopectin molecules was more pronounced in the starch isolated from sun-dried corn after storage than that from the machine-dried counterparts. These results confirmed that the activity of starch-hydrolyzing enzyme was reduced after machine drying at 80°C. The co-elution of the partially-hydrolyzed amylopectin molecules with amylose in the chromatogram was evident by decreases in the ratio of blue value (BV) to total-carbohydrate content (CHO) of the second peak, from 4.94 to 3.88 and 3.84 to 3.61 (**Table 5**) for the sun-dried and machine-dried starch samples, respectively, after 0 to 6 month storage of dried corn kernels. The reduction in the BV of the peak resulted from the presence of partially-hydrolyzed amylopectin molecules that were

highly branched and gave less blue-color when complexed with iodine. Furthermore, the enzymatic hydrolysis of starch molecules in the corn samples during storage reduced the structural integrity of the starch granules and resulted in a greater number of damaged starch granules after storage (**Figure 4**). The reduced molecular weight of starch after storage of corn resulted in lower viscosities as shown in **Figures 1 and 3**.

The amylopectin branch-chain length distributions of the starch isolated from the dried-corn samples after storage are shown in **Figure 6** and summarized in **Table 6**. The amylopectin branch-chain length of the starch sample without storage showed a typical bimodal distribution, consisting of a large proportion of short A- and B1-chains (DP 6-24) (15, 28). The branch-chain length distribution profiles of starch isolated from sun-dried corn showed obvious increases in DP 22 to 32 and decreases in the long branch-chains (DP 40-60) after 3 month storage (**Figures 6A-6E**). After 6 months of storage of sun-dried corn, the percentage of long branch-chains (DP>36) of amylopectin decreased from 12.1 to 8.8% and the percentage of intermediate branch-chains (DP 25-36) increased from 9.8 to 11.9%. Starch isolated from the machine-dried corn samples did not show any obvious changes in branch-chain length distribution before 4 months of storage (**Figures 6F-6J**). The decrease in the percentage of long branch-chains was more pronounced in the starch isolated from sun-dried corn (12.1 to 8.8% after 6 months of storage) than that from the machine-dried counterparts (12.7 to 10.7%) (**Table 6**). The results of decreases in long branch-chains of amylopectin after storage were in agreement with the findings of Patindol et al (12). The changes in amylopectin branch-chain length distributions of isolated starch samples were attributed to the enzymatic hydrolysis of starch that took place during storage as previously suggested (29, 30). Alpha-amylase attacked the amorphous region of amylopectin, which consists of long

branch-chains, and hydrolyzed the long branch-chains to intermediate chain-length.

Reduction in the molecular weight of starch has been reported to accelerate crystallization of starch and increase the resistant starch content (31). The reduced proportion of long branch-chains of amylopectin and smaller starch molecules in sun-dried corn after storage facilitated crystallization of starch. Thus, the percentage crystallinity of the sun-dried starch increased from 28.6 to 31.9% after 6 months of storage, which was substantially greater than the machine-dried counterparts (27.4 to 28.4%) (**Figure 3**). Tester and Morrison reported that hydrolysis of amylopectin by both enzymatic and chemical treatments decreased the starch swelling power (32, 33). Accordingly, the enzymatic hydrolysis of starch molecules after storage of corn (**Table 6**) resulted in lower peak viscosity of the isolated starch (**Figure 3** and **Table 4**) and ground corn samples (**Figure 1**).

CONCLUSION

Storage of sun-dried and machine-dried corn kernels at 27°C and 85-90% relative humidity for up to 6 months altered structures and functions of their starch. Starch hydrolysis rate and peak viscosity of the ground corn decreased with storage. The decreases in the peak viscosity of the ground corn were partially attributed to the decreases in the peak viscosity of the starch. The gelatinization temperature, pasting temperature, and percentage crystallinity of the isolated starch increased with the storage of the corn. Numbers of damaged starch granules and starch granules with pinholes increased and the molecular weight of starch and percentage of long branch-chains of amylopectin decreased with storage of corn, indicating starch hydrolysis taking place during the storage of corn. Starch isolated from sun-dried corn

after storage displayed greater levels of enzymatic hydrolysis than that from the machine-dried samples, suggesting more amylase activities remaining in the corn samples after sun-drying at 35°C than machine-drying at 80°C.

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Table 1. Starch digestibility of finely-ground (<0.5mm particle size) sun-dried and machine-dried corn after 0 to 6 months of storage at 27°C and 85-90%RH

Sample	Storage (months)	Percentage starch hydrolysis (%)*						
		0 h	1 h	2 h	4 h	6 h	8 h	24 h
B-816 SD ¹	0	2.5 ± 0.4 ^a	45.8 ± 0.4 ^a	52.0 ± 1.1 ^a	63.8 ± 3.7 ^a	71.4 ± 2.4 ^a	85.4 ± 1.7 ^a	97.4 ± 1.5 ^a
	2	2.3 ± 0.6 ^{ab}	39.7 ± 2.5 ^b	47.8 ± 1.0 ^{ab}	62.7 ± 0.1 ^a	67.4 ± 2.5 ^a	78.2 ± 0.9 ^b	90.1 ± 2.6 ^b
	3	3.4 ± 0.3 ^{bc}	38.1 ± 0.8 ^b	44.0 ± 1.9 ^b	58.4 ± 0.2 ^{ab}	60.2 ± 0.5 ^b	65.9 ± 0.7 ^c	75.6 ± 0.6 ^c
	4	3.9 ± 0.5 ^{cd}	35.7 ± 1.8 ^{bc}	43.6 ± 2.3 ^b	56.6 ± 0.1 ^b	59.9 ± 0.9 ^b	65.3 ± 0.9 ^c	74.3 ± 2.6 ^c
	6	4.4 ± 0.3 ^d	33.8 ± 1.2 ^c	43.9 ± 2.8 ^b	55.8 ± 1.1 ^b	57.9 ± 1.3 ^b	63.5 ± 1.1 ^c	74.1 ± 0.9 ^c
Least Significant Differences (LSD)		0.92	4.84	5.73	5.45	5.16	3.32	5.34
B-816 MD ²	0	1.8 ± 0.5 ^a	43.7 ± 0.2 ^a	52.4 ± 0.9 ^a	62.1 ± 0.3 ^a	72.3 ± 1.8 ^a	80.6 ± 1.4 ^a	94.9 ± 0.8 ^a
	2	1.6 ± 0.8 ^a	38.0 ± 2.1 ^b	48.9 ± 1.9 ^{ab}	57.3 ± 1.1 ^b	65.6 ± 0.9 ^b	72.3 ± 1.3 ^b	82.8 ± 1.3 ^b
	3	2.4 ± 0.6 ^a	36.8 ± 0.7 ^b	43.4 ± 0.3 ^{bc}	55.4 ± 0.2 ^b	58.8 ± 0.8 ^c	66.1 ± 0.5 ^c	77.3 ± 2.1 ^c
	4	1.8 ± 1.3 ^a	35.1 ± 0.6 ^b	41.9 ± 0.9 ^c	49.7 ± 1.9 ^c	56.8 ± 2.9 ^c	65.8 ± 0.8 ^c	76.4 ± 1.9 ^c
	6	2.1 ± 1.2 ^a	35.2 ± 1.7 ^b	41.3 ± 2.1 ^c	49.4 ± 1.1 ^c	56.4 ± 1.8 ^c	61.9 ± 0.3 ^d	76.9 ± 0.4 ^c
Least Significant Differences (LSD)		3.50	3.72	5.94	3.32	4.65	3.55	5.36

¹SD: Sun-dried

²MD: Machine-dried

*Percentage starch hydrolysis is measured on the basis of glucose production after hydrolysis divided by the initial starch weight in dry basis.

Data in the same column with different letter superscripts are significantly different ($P < 0.05$)

Table 2. Enzymatic hydrolysis of starch isolated from sun-dried and machine-dried corn after 0 to 6 months of storage at 27°C and 85-90%RH

Sample	Storage (months)	Percentage starch hydrolysis (%)*						
		0 h	1 h	2 h	4 h	6 h	8 h	24 h
B-816SD ¹	0	2.4 ± 0.2 ^a	32.6 ± 1.1 ^a	44.1 ± 1.6 ^a	60.3 ± 1.4 ^a	70.7 ± 1.3 ^a	77.2 ± 1.3 ^a	99.3 ± 1.5 ^a
	2	3.3 ± 0.6 ^{ab}	34.6 ± 1.3 ^a	45.6 ± 1.3 ^a	60.1 ± 1.8 ^a	71.7 ± 1.5 ^{ab}	78.9 ± 0.9 ^{ab}	96.0 ± 1.6 ^a
	3	2.8 ± 0.6 ^{cb}	37.4 ± 0.8 ^{ab}	48.1 ± 1.8 ^{ab}	61.1 ± 1.3 ^a	72.5 ± 1.6 ^{ab}	80.7 ± 1.3 ^{bc}	98.0 ± 1.7 ^a
	4	4.6 ± 0.2 ^c	39.4 ± 1.1 ^{bc}	51.4 ± 0.6 ^b	64.0 ± 1.4 ^a	73.5 ± 0.9 ^{ab}	83.1 ± 1.2 ^{cd}	97.1 ± 1.8 ^a
	6	5.1 ± 0.3 ^c	40.4 ± 0.7 ^c	52.4 ± 1.2 ^b	64.9 ± 2.1 ^a	74.4 ± 1.1 ^{ab}	84.9 ± 1.9 ^d	98.5 ± 1.1 ^a
Least Significant Differences		1.48	3.94	4.95	5.53	4.14	3.76	5.68
B-816MD ²	0	0.8 ± 0.3 ^a	34.2 ± 0.6 ^a	47.5 ± 0.5 ^a	63.4 ± 1.6 ^a	73.1 ± 1.3 ^a	79.1 ± 1.4 ^a	96.9 ± 0.9 ^a
	2	0.6 ± 0.1 ^a	33.4 ± 0.6 ^a	45.0 ± 0.8 ^{ab}	61.0 ± 0.3 ^a	70.7 ± 0.4 ^{ab}	78.4 ± 1.3 ^a	94.3 ± 1.3 ^a
	3	1.1 ± 0.3 ^a	34.2 ± 0.7 ^a	44.3 ± 1.4 ^{ab}	62.6 ± 0.9 ^a	72.4 ± 1.3 ^{ab}	79.5 ± 1.9 ^a	96.7 ± 0.4 ^a
	4	0.9 ± 0.2 ^a	33.2 ± 1.2 ^a	43.3 ± 1.3 ^b	58.6 ± 1.1 ^b	69.7 ± 0.9 ^{ab}	76.6 ± 1.4 ^a	93.2 ± 1.3 ^a
	6	1.2 ± 0.4 ^a	32.5 ± 0.8 ^a	44.6 ± 0.9 ^{ab}	58.8 ± 0.8 ^b	69.5 ± 1.1 ^b	75.6 ± 1.5 ^a	94.4 ± 1.5 ^a
Least Significant Differences		1.01	2.73	3.81	3.60	4.06	4.41	2.53

¹SD: Sun-dried

²MD: Machine-dried

*Percentage starch hydrolysis is measured on the basis of glucose production after hydrolysis divided by the initial starch weight in dry basis.

Data in the same column with different letter superscripts are significantly different ($P < 0.05$)

Table 3. Thermal properties of starch isolated from sun-dried and machine-dried corn after 0 to 6 months of storage at 27°C and 85-90%RH

Sample	Storage (months)	To (°C)	Tp (°C)	Tc (°C)	ΔH (J/g)
B-816SD ¹	0	68.74 ± 0.21 ^a	74.59 ± 0.12 ^a	80.71 ± 0.28 ^a	13.21 ± 0.42 ^a
	2	70.45 ± 0.17 ^b	75.71 ± 0.34 ^b	81.97 ± 0.28 ^{bc}	13.62 ± 0.23 ^a
	3	71.25 ± 0.13 ^c	75.31 ± 0.26 ^{ab}	82.42 ± 0.13 ^c	11.37 ± 0.33 ^b
	4	71.32 ± 0.22 ^c	76.02 ± 0.35 ^b	81.92 ± 0.33 ^{bc}	10.91 ± 0.22 ^b
	6	71.21 ± 0.33 ^c	75.88 ± 0.12 ^b	81.33 ± 0.42 ^{ab}	10.54 ± 0.28 ^b
Least Significant Differences (LSD)		0.69	0.86	0.90	0.95
B-816MD ²	0	68.76 ± 0.02 ^a	74.82 ± 0.12 ^a	80.31 ± 0.06 ^a	12.28 ± 0.22 ^a
	2	69.35 ± 0.19 ^{ab}	75.22 ± 0.24 ^b	80.85 ± 0.17 ^{ab}	12.06 ± 0.03 ^{ab}
	3	70.48 ± 0.08 ^b	76.02 ± 0.15 ^c	81.49 ± 0.22 ^{bc}	11.58 ± 0.17 ^b
	4	71.68 ± 0.06 ^c	75.75 ± 0.22 ^c	82.11 ± 0.36 ^c	10.85 ± 0.24 ^c
	6	71.95 ± 0.19 ^c	75.88 ± 0.24 ^c	82.42 ± 0.53 ^c	10.71 ± 0.38 ^c
Least Significant Differences (LSD)		1.04	0.55	1.00	0.61

¹SD: Sun-dried

²MD: Machine-dried

Data in the same column with different letter superscripts are significantly different ($P < 0.05$)

Table 4. Pasting properties of starch isolated from sun-dried and machine-dried corn after 0 to 6 months of storage at 27°C and 85-90%RH

Sample	Storage (months)	Pasting Temperature (°C)	Peak Viscosity (RVU)	Break Down (RVU)	Setback (RVU)	Final Viscosity (RVU)
B-816SD ¹	0	72.5 ± 0.4 ^a	158.2 ± 2.3 ^a	60.1 ± 1.3 ^a	84.7 ± 0.4 ^a	183.2 ± 2.2 ^a
	2	74.1 ± 0.1 ^b	160.9 ± 0.5 ^a	58.9 ± 0.3 ^a	86.0 ± 0.2 ^b	184.2 ± 1.3 ^a
	3	75.1 ± 0.4 ^{bc}	128.5 ± 0.3 ^b	30.7 ± 0.5 ^b	78.9 ± 0.5 ^c	177.9 ± 0.8 ^b
	4	74.8 ± 0.2 ^{bc}	131.3 ± 1.4 ^b	27.6 ± 1.3 ^b	78.6 ± 0.1 ^c	177.5 ± 1.3 ^b
	6	75.6 ± 0.5 ^c	132.5 ± 0.3 ^b	28.4 ± 1.8 ^b	79.2 ± 0.4 ^c	178.4 ± 1.1 ^b
Least Significant Differences (LSD)		1.28	4.24	3.89	1.27	4.78
B-816MD ²	0	73.6 ± 0.4 ^a	154.6 ± 1.3 ^a	59.3 ± 1.6 ^a	117.3 ± 1.4 ^a	212.4 ± 1.8 ^a
	2	74.5 ± 0.2 ^a	156.5 ± 1.2 ^a	60.2 ± 1.8 ^a	112.4 ± 1.7 ^b	211.3 ± 1.3 ^a
	3	74.1 ± 0.5 ^a	144.8 ± 0.9 ^b	42.3 ± 0.9 ^b	92.8 ± 1.1 ^c	193.8 ± 0.9 ^b
	4	74.7 ± 0.6 ^{ab}	136.8 ± 1.0 ^b	38.7 ± 2.9 ^b	78.1 ± 1.4 ^d	173.8 ± 0.9 ^c
	6	75.4 ± 0.7 ^b	133.8 ± 1.2 ^b	39.9 ± 1.4 ^b	76.3 ± 0.9 ^d	170.4 ± 1.9 ^c
Least Significant Differences (LSD)		1.47	4.06	4.79	4.38	5.29

¹SD: Sun-dried

²MD: Machine-dried

*The sample was kept under constant stirring at 160 rpm during analysis

Data in the same column with different letter superscripts are significantly different ($P < 0.05$)

Table 5. Molecular weight distribution of starch isolated from sun-dried and machine-dried corn after 0 to 6 months of storage at 27°C and 85-90%RH measured using gel permeation chromatography (GPC)

Sample	Storage (months)	Amylopectin /first peak (%)	Amylose /second peak (%)	BV: CHO of 2 nd peak*
B-816 SD ¹	0	71.1 ± 0.4 ^a	28.9 ± 0.4 ^a	4.94 ± 0.01 ^a
	2	69.4 ± 0.4 ^b	30.6 ± 0.4 ^b	4.40 ± 0.04 ^b
	3	67.5 ± 0.0 ^c	32.5 ± 0.0 ^c	4.21 ± 0.02 ^c
	4	64.4 ± 0.4 ^d	35.6 ± 0.4 ^d	4.12 ± 0.02 ^c
	6	64.3 ± 0.3 ^d	35.7 ± 0.3 ^d	3.88 ± 0.03 ^d
Least Significant Differences (LSD)		1.23	1.23	0.09
B-816MD ²	0	71.8 ± 0.2 ^a	28.2 ± 0.2 ^a	3.84 ± 0.01 ^{ab}
	2	70.8 ± 0.1 ^b	29.1 ± 0.1 ^b	3.86 ± 0.01 ^a
	3	69.9 ± 0.3 ^{bc}	30.1 ± 0.3 ^{bc}	3.82 ± 0.04 ^{ab}
	4	69.3 ± 0.4 ^c	30.6 ± 0.4 ^c	3.74 ± 0.03 ^b
	6	68.2 ± 0.1 ^d	31.7 ± 0.1 ^d	3.61 ± 0.05 ^c
Least Significant Differences (LSD)		0.89	0.89	0.11

*Blue value to total carbohydrate ratio of amylose peak calculated using normalized amylopectin peak

¹SD: Sun-dried

²MD: Machine-dried

Data in the same column with different letter superscripts are significantly different ($P < 0.05$)

Table 6. Amylopectin branch-chain length distribution of starch isolated from sun-dried and machine-dried corn samples after 0 to 6 months of storage at 27°C and 85-90%RH

Sample	Storage (months)	Branch-chain length distribution (%)			
		6-12	13-24	25-36	>36
B-816SD ¹	0	25.4 ± 0.2 ^a	52.8 ± 0.1 ^{ab}	9.8 ± 0.1 ^a	12.1 ± 0.4 ^a
	2	24.7 ± 0.4 ^b	53.1 ± 0.7 ^a	9.6 ± 0.5 ^a	11.7 ± 0.1 ^a
	3	25.8 ± 0.2 ^b	51.3 ± 0.8 ^{ab}	10.4 ± 0.1 ^a	10.4 ± 0.5 ^b
	4	26.3 ± 0.2 ^{bc}	51.2 ± 0.3 ^b	10.3 ± 0.3 ^a	9.8 ± 0.3 ^{bc}
	6	27.3 ± 0.1 ^c	51.4 ± 0.4 ^{ab}	11.9 ± 0.4 ^b	8.8 ± 0.1 ^c
Least Significant Differences (LSD)		0.95	1.76	1.17	1.17
B-816MD ²	0	24.8 ± 0.2 ^{abc}	52.8 ± 0.2 ^a	9.6 ± 0.1 ^a	12.7 ± 0.2 ^a
	2	24.6 ± 0.4 ^{ab}	52.7 ± 0.3 ^a	9.9 ± 0.5 ^a	12.6 ± 0.4 ^a
	3	24.2 ± 0.4 ^a	53.1 ± 0.4 ^a	9.7 ± 0.1 ^a	11.8 ± 0.2 ^{ab}
	4	25.7 ± 0.5 ^{bc}	52.1 ± 0.6 ^a	10.6 ± 0.3 ^a	10.8 ± 0.4 ^b
	6	25.9 ± 0.1 ^c	52.3 ± 0.5 ^a	10.5 ± 0.4 ^a	10.7 ± 0.3 ^b
Least Significant Differences (LSD)		1.28	1.37	1.16	1.13

*Maximum detectable chain length – DP 75-76

¹SD: Sun-dried

²MD: Machine-dried

Data in the same column with different letter superscripts are significantly different ($P < 0.05$)

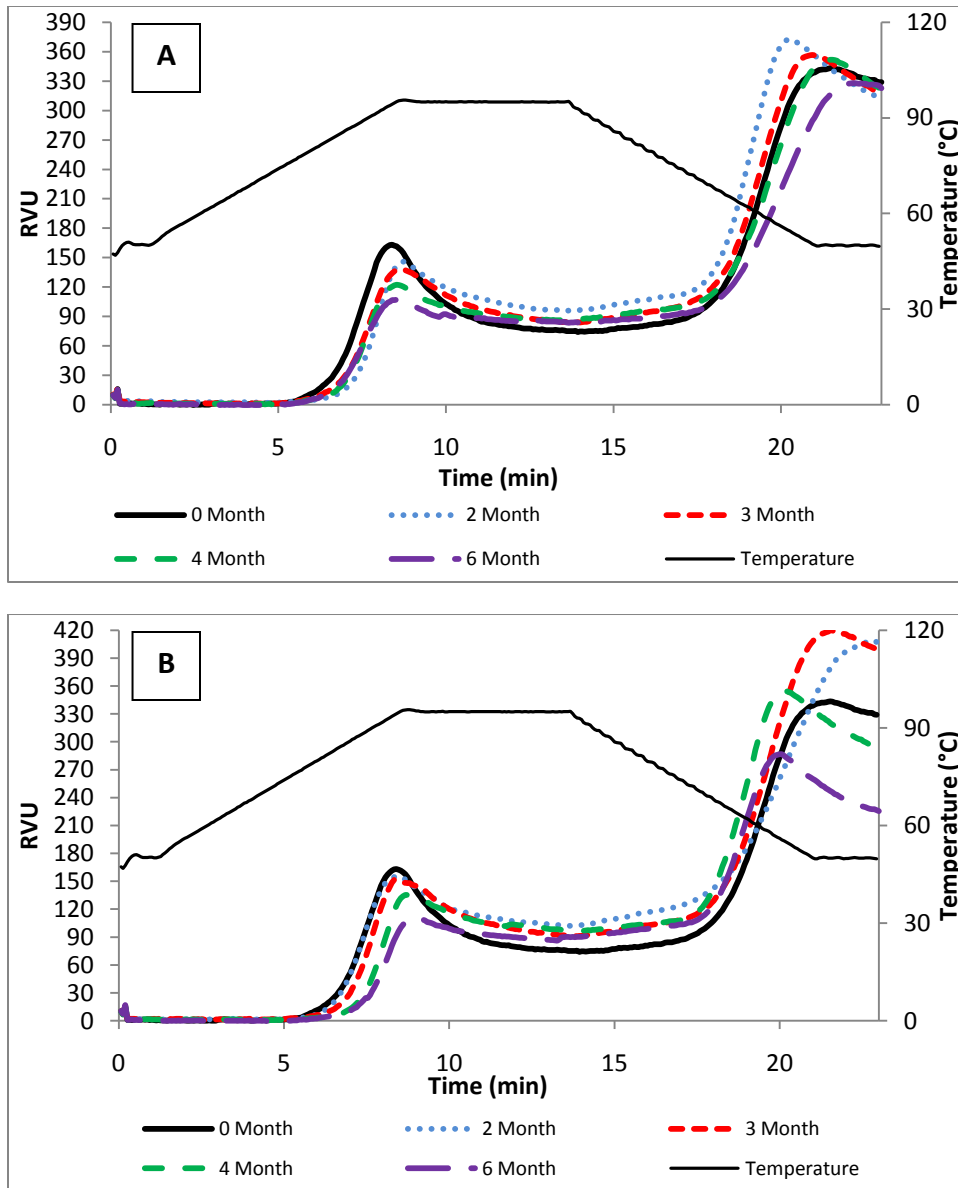


Figure 1. Pasting profiles of finely-ground (<0.5mm particle size) **A)** sun-dried and **B)** machine-dried corn after 0 to 6 months of storage at 27°C and 85-90%RH

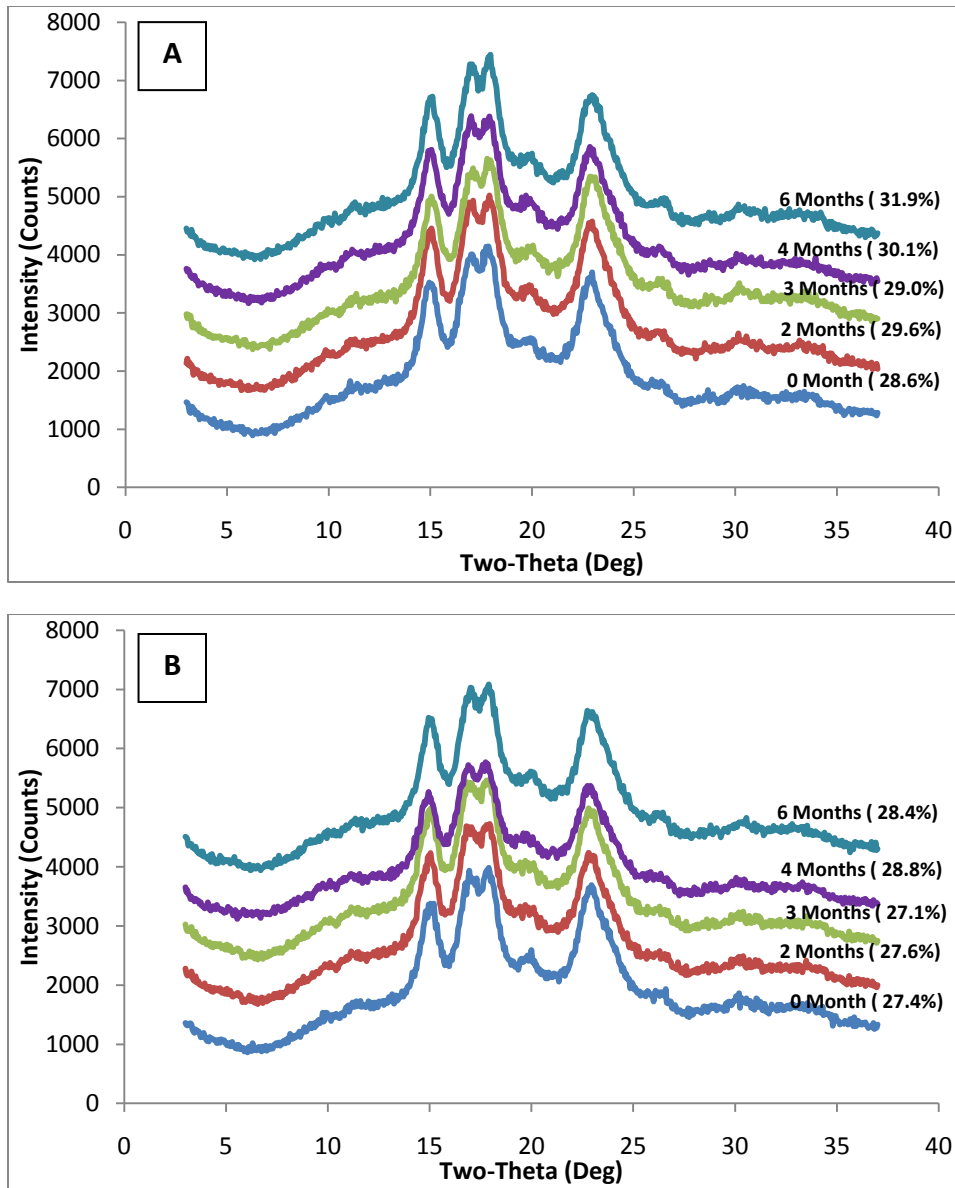


Figure 2. X-ray diffraction pattern of starches isolated from **A)** sun-dried and **B)** machine-dried corn after 0 to 6 months of storage at 27°C and 85-90%RH

*Number in parentheses showed the percentage crystallinity of the corresponding isolated starch samples

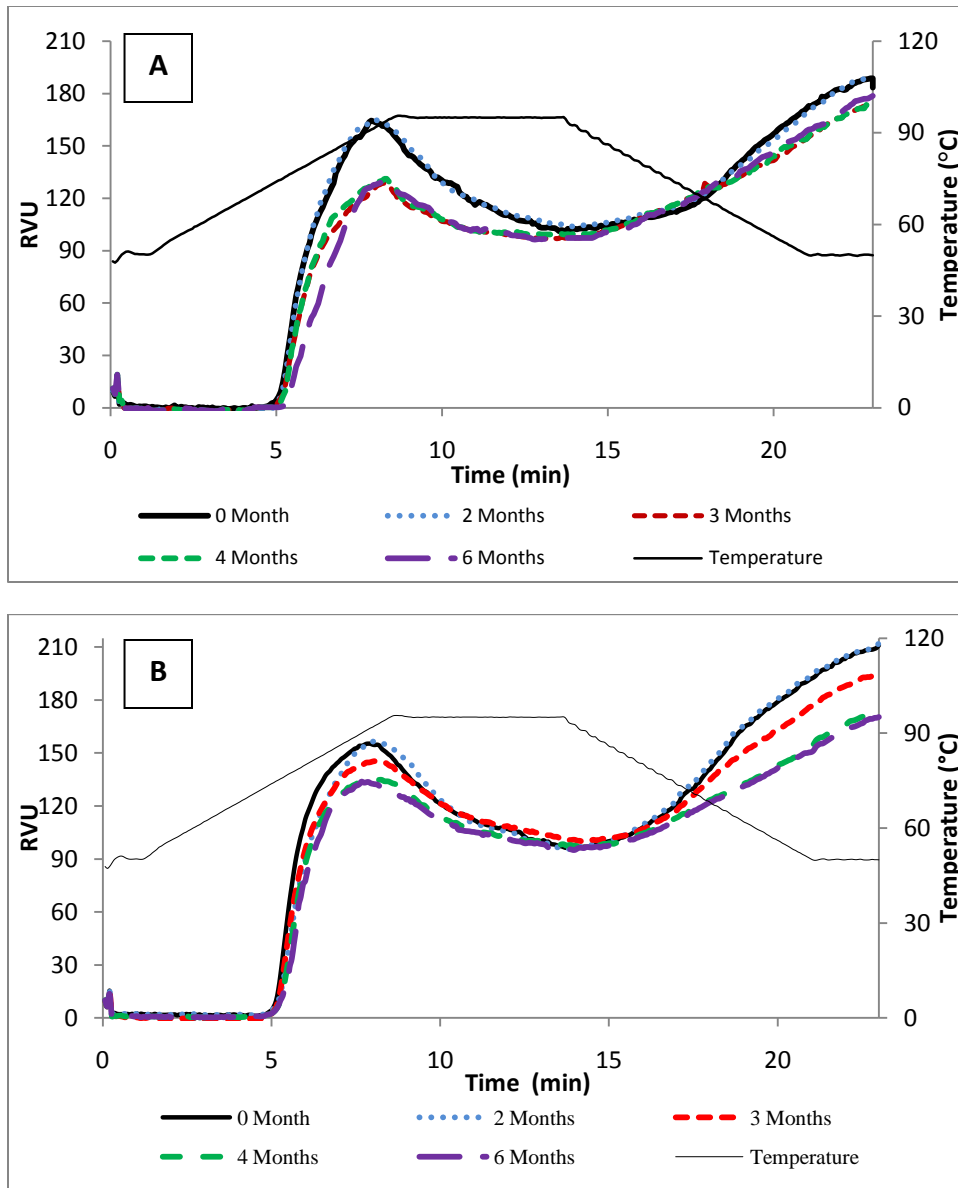


Figure 3. Pasting profiles of isolated starch from **A)** sun-dried and **B)** machine-dried corn after 0 to 6 months of storage at 27°C and 85-90%RH

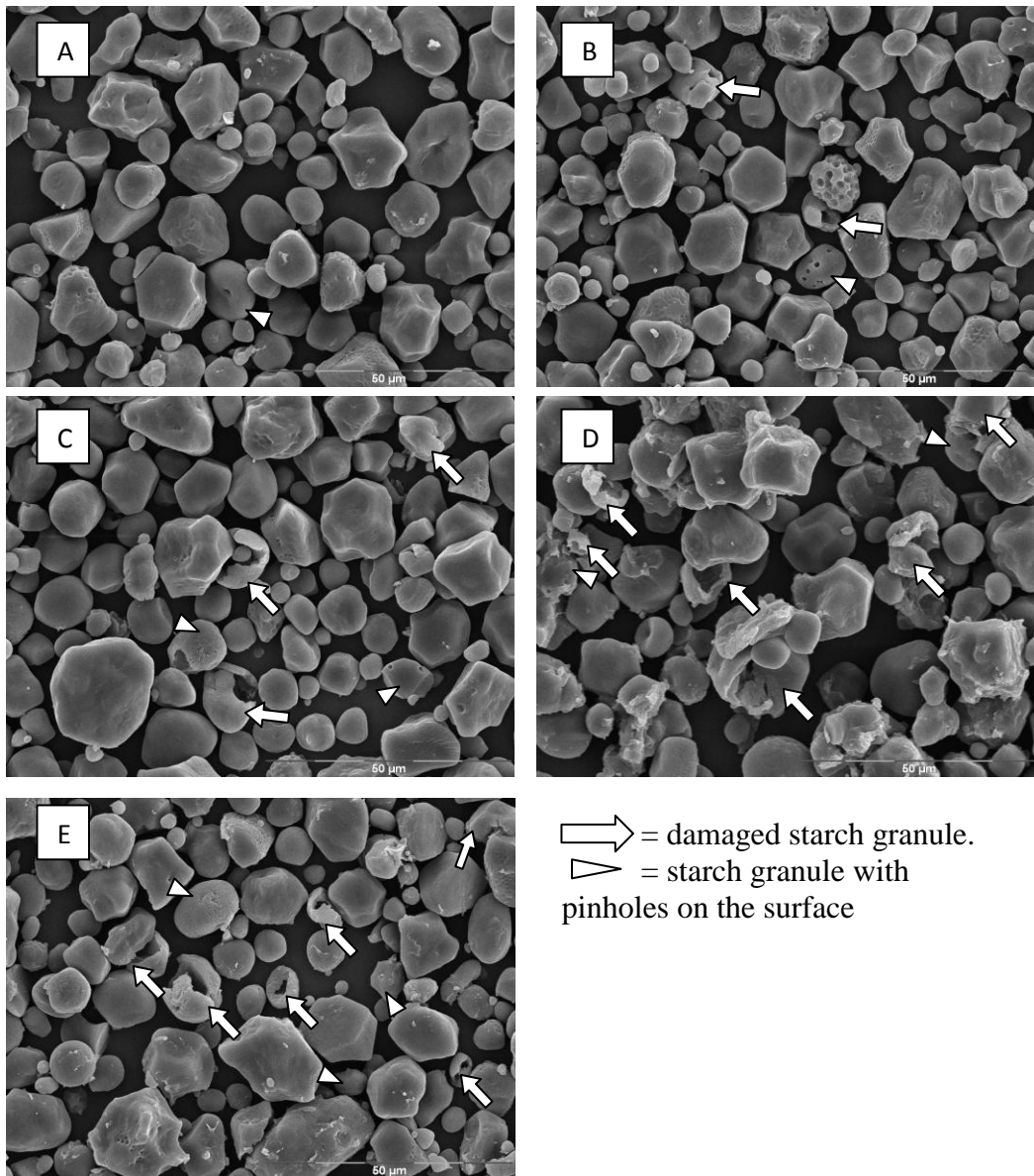


Figure 4. Scanning electron micrographs (1500X) of isolated starches. **A-E**, starch isolated from sun-dried corn after storage for **A)** 0, **B)** 2, **C)** 3, **D)** 4, and **E)** 6 months. **F-J**, starch isolated from machine-dried corn after storage for **F)** 0, **G)** 2, **H)** 3, **I)** 4, and **J)** 6 months at 27°C and 85-90%RH.

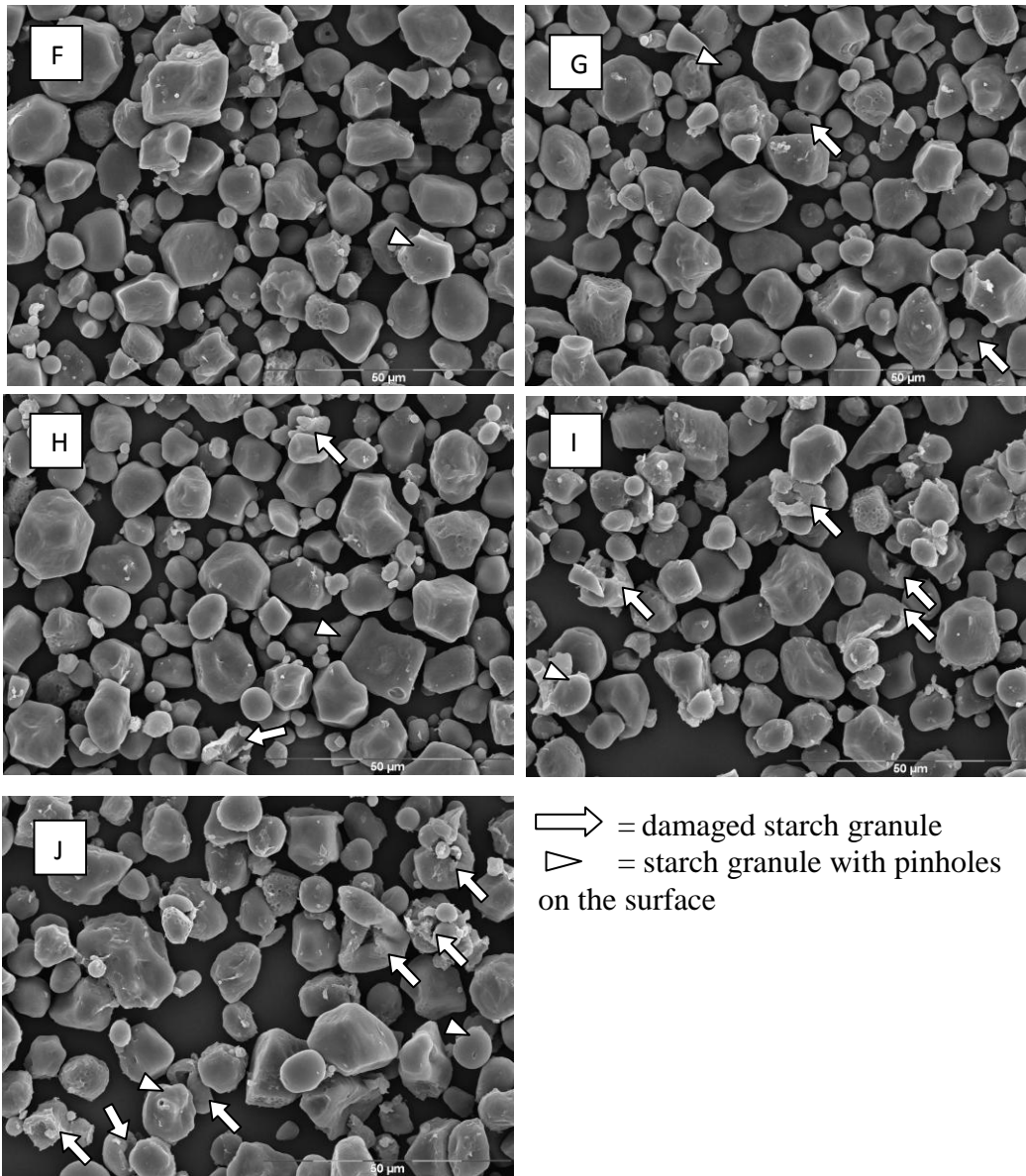


Figure 4. Continued

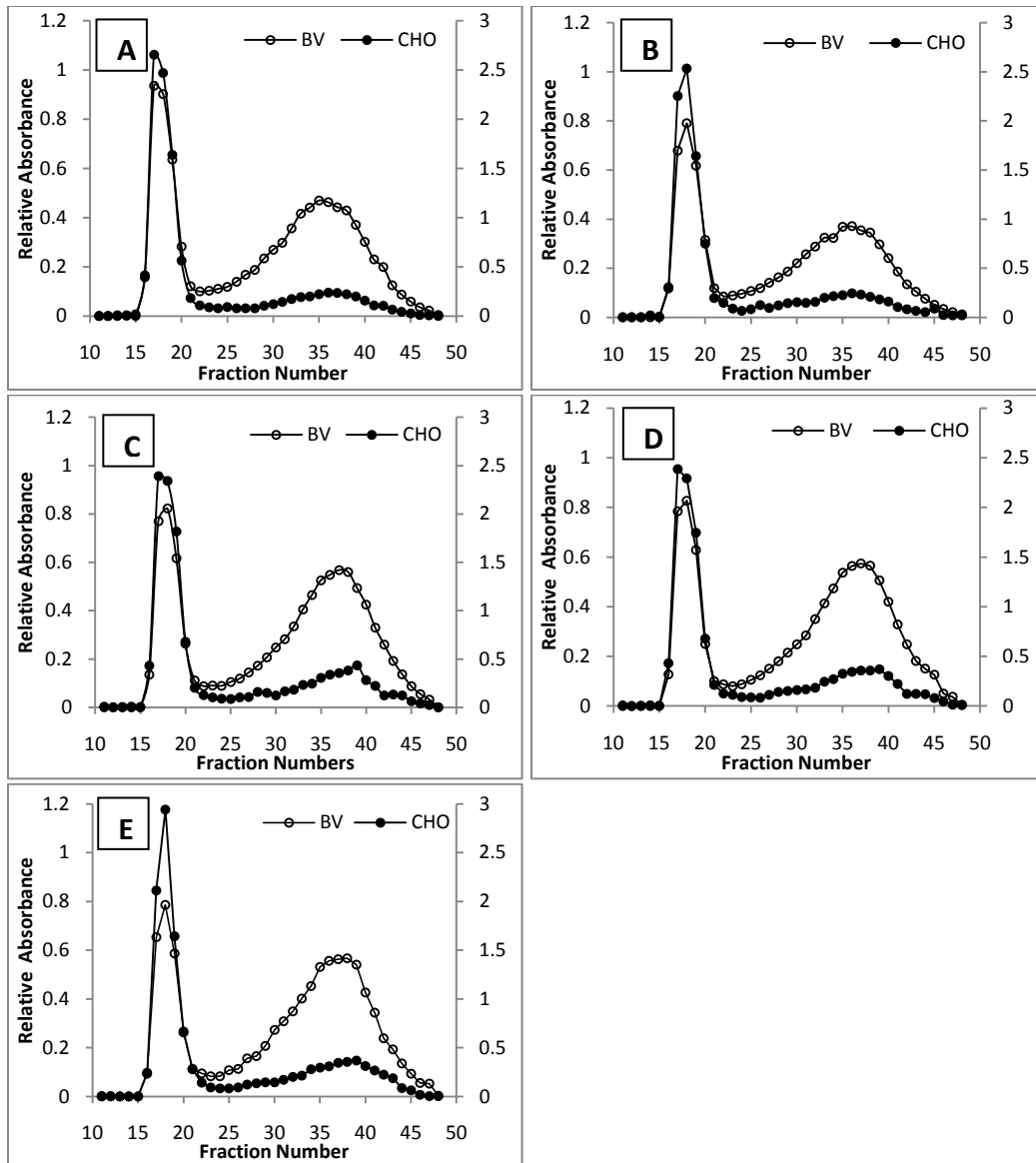


Figure 5. GPC profiles of isolated starches. **A-E**, starch isolated from sun-dried corn after storage for **A)** 0, **B)** 2, **C)** 3, **D)** 4, and **E)** 6 months. **F-J**, starch isolated from machine-dried corn after storage for **F)** 0, **G)** 2, **H)** 3, **I)** 4, and **J)** 6 months at 27°C and 85-90%RH.

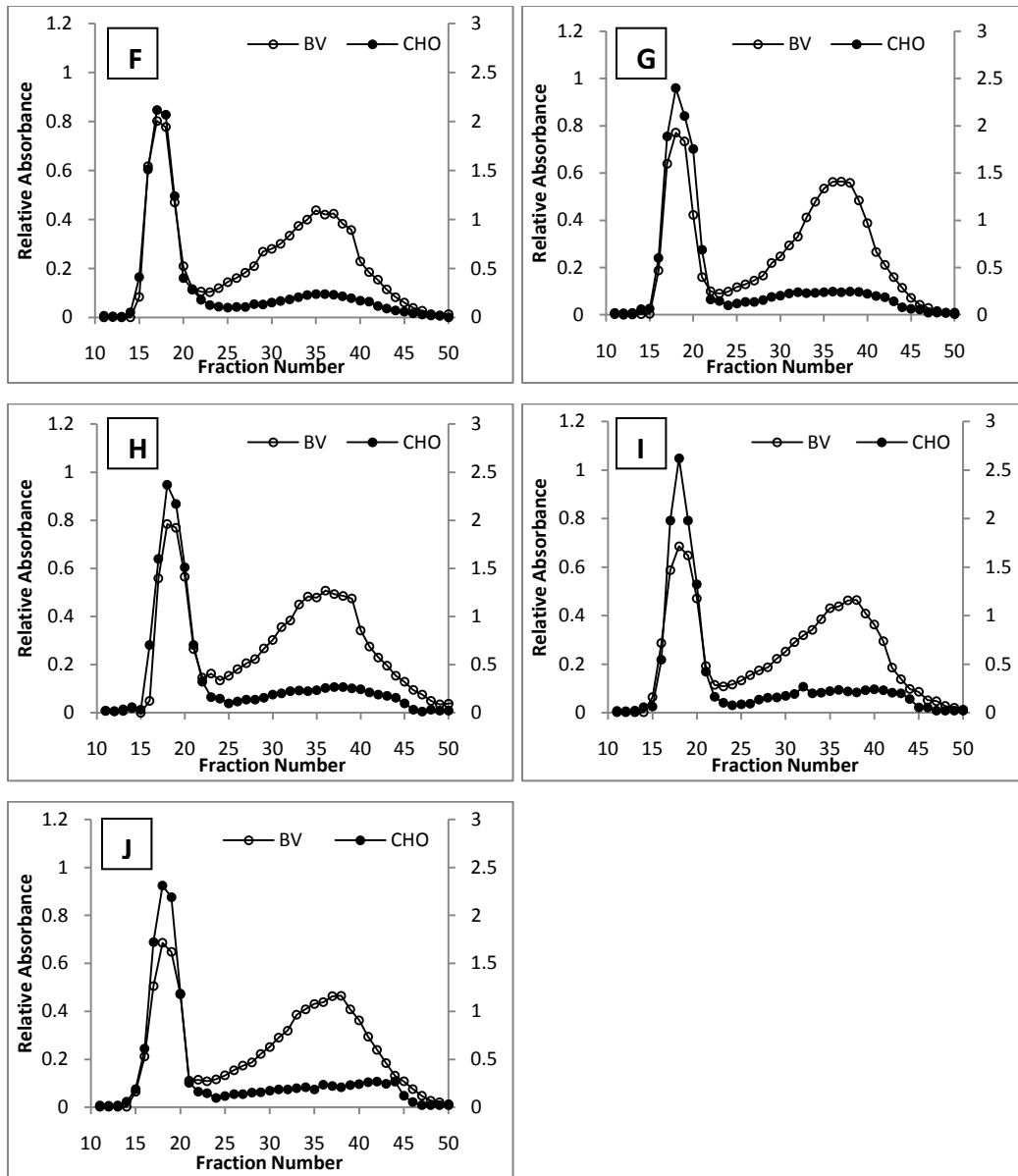


Figure 5. Continued

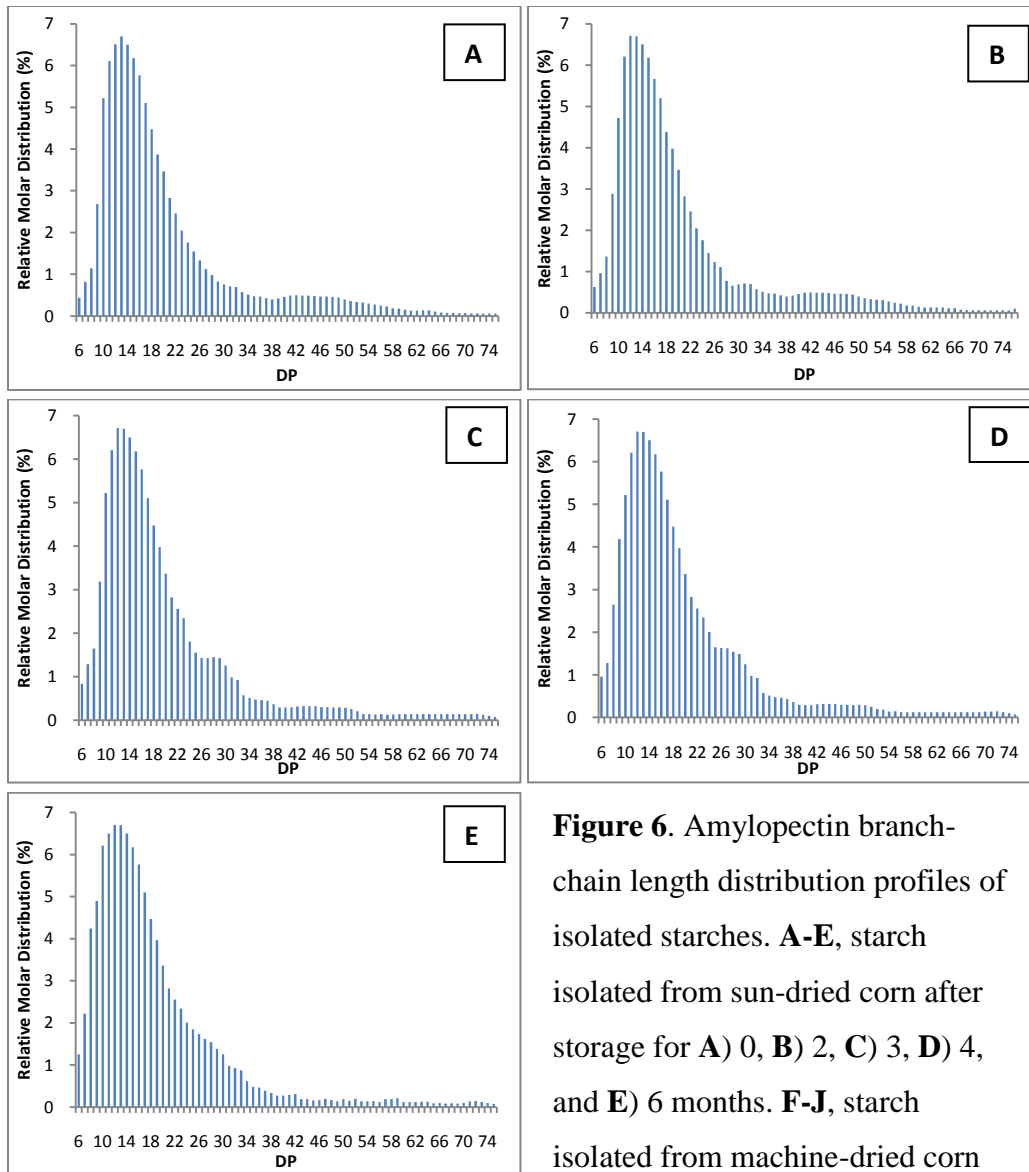


Figure 6. Amylopectin branch-chain length distribution profiles of isolated starches. **A-E**, starch isolated from sun-dried corn after storage for **A)** 0, **B)** 2, **C)** 3, **D)** 4, and **E)** 6 months. **F-J**, starch isolated from machine-dried corn after storage for **F)** 0, **G)** 2, **H)** 3, **I)** 4, and **J)** 6 months at 27°C and 85-90%RH.

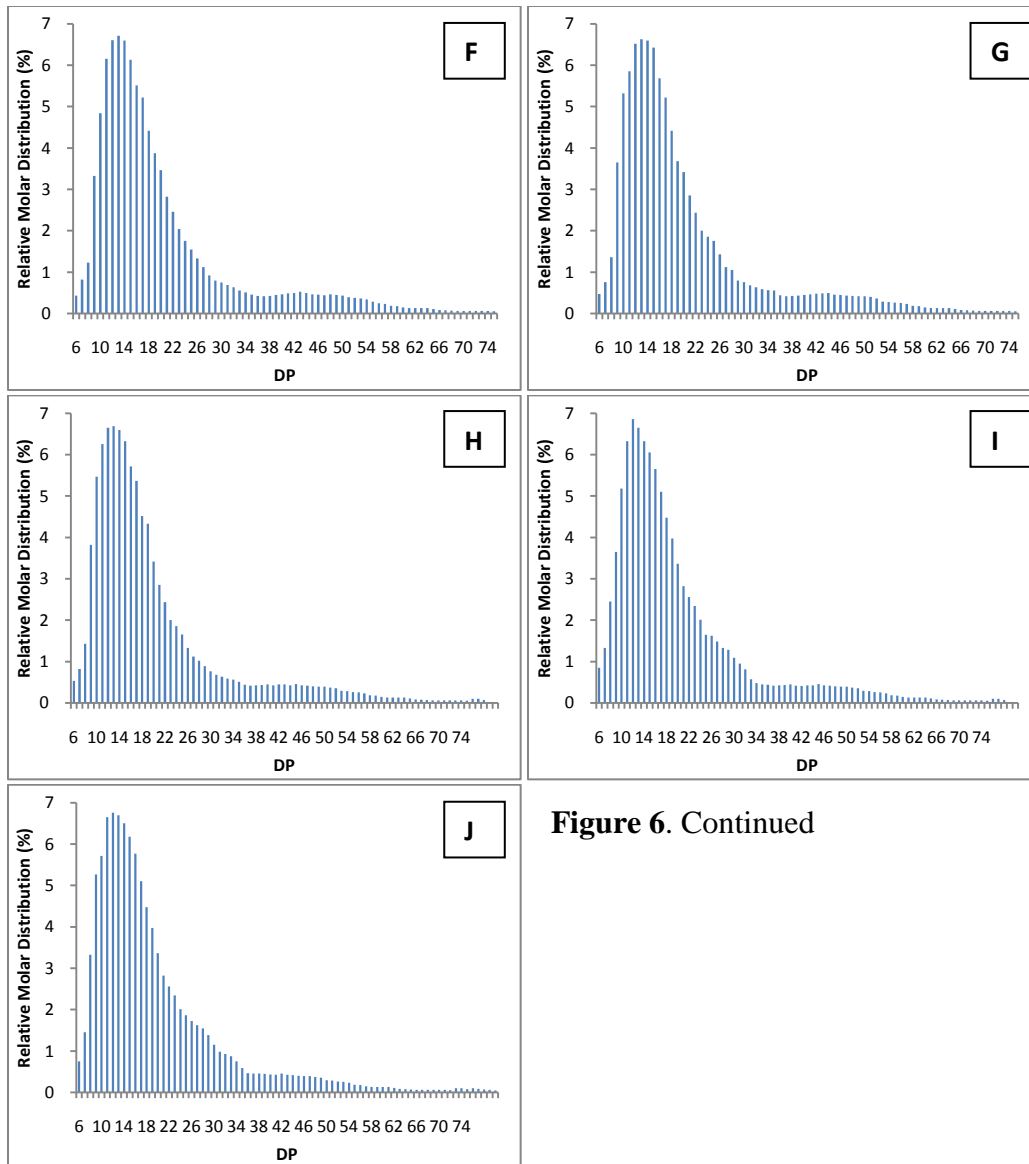


Figure 6. Continued

CHAPTER 4. METHODS TO IMPROVE NUTRITIONAL VALUES OF CASSAVA FLOUR FOR ANIMAL FEED

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ABSTRACT

The long-term objective of this study was to produce cassava-based feed that has a similar starch digestive rate and nutritional value to corn-based feed. Dietary oils (palm oil, soybean oil, and corn oil), Distiller's Dry Grain with Solubles (DDGS), and defatted soybean-meal (SBM) were used to modify the starch properties of cassava flour. Addition of 6% (v/w) dietary oils to the cassava flour followed by a heat-moisture treatment (HMT) at 100°C for 1 h reduced starch digestive rate of the cassava flour from 73.4 to 62.8, 61.6, and 56.4% after 8 h hydrolysis for palm oil, soybean oil, and corn oil, respectively. The Distiller's Dry Grain with Solubles (DDGS) substitution followed by HMT at 100°C for 1 h reduced the starch digestive rate of the cassava flour from 80.3 to 78.3, 74.1, and 72.0% after 8 h hydrolysis for 10, 20, and 30% (w/w) DDGS substitution, respectively. The cassava flour treated with defatted soybean meal (SBM), however, showed no substantial decrease in starch digestive rate. Increases in initial moisture content (IMC) of the cassava flour-blends from 18.1-20.7% to 26.1-28.0% prior to HMT at 100°C increased the starch digestive rate of the cassava flour-blends. Increase in heating temperature of HMT from 100 to 120°C for

cassava-flour blends with 11.9-12.4% moisture resulted in less reduction in the starch digestive rate of the cassava flour.

INTRODUCTION

Cassava flour obtained by grinding dried cassava root is one of the major sources for food in Southeast Asia and Africa countries. Cassava flour contains a relatively high starch content (65-70%, db), but low in lipids (0.1-0.3%, db) and protein (1-3%, db) comparing with maize and sorghum (1). Starch of cassava flour has a greater digestive rate than that of ground maize and sorghum (2). Starch with a greater digestive rate can be converted to glucose quickly and elevates plasma glucose level when other nutrients are not yet absorbed. The rapid elevation in plasma glucose level hinders amino acids absorption in the small intestine (3). The unabsorbed amino acids in the small intestine may trigger *dieresis*, a mechanism of eliminating excess electrolytes, body nitrogen, and toxin by increasing water consumption and flushing these compounds from the body (4). *Weurding et al* reported that chickens fed diets containing highly digestible starch (tapioca starch) had less body weight gain and feed conversion ratio (FCR) than chickens fed with diet containing slowly digestible starch (pea starch) (5). The fast production of glucose from starch may also cause an osmotic pressure change and contribute to diarrhea symptoms.

Dietary oils (palm, corn, and soybean oil) and Distiller's Dry Grain with Solubles (DDGS) have been used in the feed industry to improve feed quality (6-8). DDGS is a co-product of ethanol fermentation using dry-grind corn. DDGS is composed of nonfermentable components of the original grain, namely protein, lipids, and fiber. This product is relatively

inexpensive because of a large supply in the market. In 2006, approximately 10 million metric tons of DDGS was produced (9). Many applications of this byproduct, including bioplastics (10) and human food additives (11), have been developed in addition to feed supplement.

Soybean meal is commonly used in animal feed since early 1940's (12). The main reason for the use of soybean meal in animal feed is its unique amino acid compositions that complement the deficiencies in amino acids, such as lysine and tryptophan, in cereal grains (13, 14). This excellent source of protein, however, has deficiencies in mineral content, such as calcium and phosphorus. Raw soybean meal contains anti-nutritional factors, such as trypsin and chymotrypsin inhibitor, that decrease protein digestion and absorption (15). A proper heating treatment is necessary to inactivate these anti-nutritional factors prior to incorporating soybean meal into an animal diet (16, 17). The percentage of soybean meal incorporated in the animal diet depends on the nutritional requirement and the growth stage of animal (18, 19).

Heat moisture treatment (HMT) is a common physical method used to modify starch properties (20, 21). It involves incubation of the starch at 30% (db) moisture, and high temperatures (84-120°C) (22). In this study, we added dietary oils (palm oil, soybean oil, and corn oil), DDGS, or defatted soybean meal (SBM) to cassava flour followed by heat-moisture treatment (HMT) to reduce the starch digestive rate. The objective of this study was to produce cassava-based feed that has a nutritional value similar to corn-based feed.

MATERIALS AND METHODS

Materials

Dried cassava chip and coarsely-ground maize (B-16) used in this study were obtained from Charoen Pokphand (Jakarta, Indonesia). Two batches of dried cassava chip were received in 2008 and 2009. The 2008 crop of dried cassava chip was used in the study with dietary oils, and the 2009 crop was used for processing with DDGS and SBM. Dried cassava chip was ground using a commercial coffee grinder (Krupps, Shelton, CT) to produce cassava flour (CA). Dietary oils (palm oil (PO), soybean oil (SO), corn oil (CO)) were obtained from The Center for Crops Utilization Research (CCUR, Ames, IA). The fatty acid compositions and free fatty-acid contents of the dietary oils used in this study are listed in **Table 1**. DDGS (10.3% lipids and 22.1% protein, db) was obtained from Dr. Charles Hurburgh of Grain Quality Laboratory (GQL, Ames, IA). SBM (1.7% lipids and 47.5% protein, db) was provided by Charoen Pokphand (Jakarta, Indonesia). Porcine pancreatic α -amylase (PPA) and glucoamylase (GA) from *Aspergillus niger* were purchased from Sigma Aldrich Corporation (St. Louis, MO). Glucose assay kits (GOPOD format, Catalog: K-GLUC) were purchased from Megazyme International (Wicklow, Ireland).

Dietary oil compositions

Fatty acid compositions of the dietary oils were analyzed following the method of Hammond (23). Oil aliquots (2 ml) were converted to fatty acid methyl esters (FAME) by reacting the oil with 1 M sodium methoxide at 40°C for 1 h. The FAME were analyzed by gas chromatography using a Hewlett-Packard 5890 Series II chromatograph with a flame ionization detector and split/splitless injector. The GC column used in this study was 15 m with a 0.25 mm x 0.2 μ m film of SP-2230 (Supelco, Bellefonte, PA). Temperatures used

during chromatographic analysis were 230°C for injector temperature, 230°C for detector temperature, and the oven temperature increased from 150 to 180°C at 5°C/min with no holding time. The rates of carrier and auxiliary gas (He) were set at 5.4 and 19.4 ml/min, respectively. The hydrogen flow was at 13.9 ml/min, and air flow at 42.6 ml/min. The FAME composition was expressed as uncorrected relative area percentages of the detector output. The free fatty acid contents (FFA) of dietary oils used in this study were determined using AOAC method 940.28 (24).

Sample preparations:

Cassava flour-oil blends: Cassava flour (10 g, 11.16% moisture content, db) was mixed with 6% (v/w) of each of the dietary oils. The mixture was blended thoroughly in a flat-bottom container using a stirrer in a water-bath at 50°C. Each mixture was transferred into a sealed container and heated at 100°C using a convection oven (Isotemp@Oven Model 655F, Fisher Scientific, Chicago) for 1 h. The same method was used to prepare mixtures of isolated cassava starch (CAS, moisture content 6.71%, db) and dietary oils.

Cassava flour-DDGS blends: Cassava flour (100 g, 13.2% moisture content db) was thoroughly mixed with DDGS at different proportions (10, 20, and 30% of total weight, w/w) in a zip-lock plastic bag. The moisture content of the mixture was adjusted by spraying a predetermined amount of distilled water to the sample, mixed, and equilibrated overnight. Three different initial moisture contents (IMC) were used (12.4, 18.1, and 26.1%, db). Each mixture was transferred into a sealed container and heated at three different temperatures (80, 100, and 120°C) using the same convection oven for 1 h.

Cassava flour-SBM-oil blends: Cassava flour (100 g, 13.2% moisture content, db) was thoroughly mixed with SBM at different proportions (5, 10, 15, and 30% of total weight, w/w) in a zip-lock plastic bag. Approximately 10g of the cassava flour-SBM mixture (moisture content 10.4%) was blended thoroughly in a flat-bottom container with corn oil at different proportions (1.5, 3, and 6% v/w of cassava flour). Three different initial moisture contents (IMC) were used (13.7, 20.7, and 28.0%, db). Each mixture was heated at 100°C using the same convection oven for 1 h.

Particle size distribution of samples

Each of the samples was sieved using U.S.A Standard Testing Sieves (Fisher Scientific, U.S) with sieves numbers: 30 (0.6 mm), 50 (0.3 mm), 140 (0.1 mm), and 270 (0.05 mm). The series of sieve were shaken using Ro-Tap Sieves Shaker (CE Tyler Corporation, U.S) for 10 minutes. Particle-size distributions of the samples were determined using a gravimetric method.

Starch digestibility

Starch digestibility of each sample was analyzed following the method of *Hasjim et al* (25) with modifications. Cassava flour, cassava flour-blends, or ground corn samples (containing 200 mg of starch, db) were suspended in a phosphate buffer solution (20 ml, 0.02 M, pH 6.9, containing 0.25 mM CaCl₂) and equilibrated at 40°C for 1 h. One ml of a PPA solution (200 units/g of starch) was added to the suspension. Enzyme hydrolysis was carried out at 40°C, and aliquots (0.2 ml) of the hydrolysate were withdrawn at different time intervals. The aliquots were centrifuged at 6059 g for 5 minutes, and the supernatant was

collected and diluted with a sodium acetate buffer solution (0.02 M, pH 4.5). Glucoamylase (10 units) was added to the supernatant, and the hydrolysis was carried out at 50°C for 2 h. The glucose content of the hydrolysate was determined using Glucose assay kits (GOPOD format). The analysis was done in duplicate. Percentage starch hydrolysis (%) was calculated using the following equation:

$$\text{Percentage starch hydrolysis(\%)} = \frac{\text{Glucose content} \times 0.9}{\text{Initial starch weight (dry basis)}}$$

Thermal properties of starch samples

Thermal properties of the isolated cassava starch samples (CAS) and that treated with dietary oils were analyzed using a differential scanning calorimeter (DSC) (DSC-7, Perkin-Elmer, Norwalk, CT) (26). Each starch sample (around 2 mg, db) was precisely weighed in an aluminum pan and deionized water (6µl) was added. The mixture was sealed in the aluminum pan and allowed to equilibrate at the room temperature (22-24°C) for 1 hour. The sample was heated from 10°C to 110°C at a rate of 10°C/minute, cooled down from 110°C to 10°C at a rate of 40°C/minutes, and re-heated at the same rate as the first scan. An empty pan was used as the reference and indium was used as a reference standard. The analysis was done in duplicate.

Nile Red Staining-Light Microscopy of starch samples

Stock solution of Nile red dye (500 µg/ml) in acetone was prepared and stored in a brown bottle to avoid light exposure. Approximately 10 µg of isolated cassava starch (CAS) sample and that treated with dietary oil were mixed with 500 µl of the stock Nile-red solution and

500 µl of deionized water. The mixture was allowed to equilibrate overnight at room temperature (22-24°C) with no light exposure. The mixture was then centrifuged at 6059 g for 20 minutes to remove excess dye. Starch samples were then sprinkled on a glass slide (75mm x 25mm), and glycerol-water solution (50:50) was used as a medium. Samples were observed promptly using a Nikon Labophot Series Microscope (Frank. E. Fryer. Co, Edina, MN) equipped with a digital camera (Infinity-3, Lumenera Corporation, Minneapolis, MN). Digital images were captured at 400X magnification using imaging software (Infinity ANALYZE version 4.5.0)

Solubility and swelling power of starch samples

The solubility and swelling power of starch were analyzed following the method of Srichuwong et al (27). Isolated cassava starch (CAS) and that treated with dietary oil samples were made into a suspension (1%, w/v) of 5 ml of deionized water. Each suspension was heated in a water bath at 50°C for 40 minutes with agitation. After incubation, each suspension was centrifuged at 6059 g for 20 minutes. The supernatant was collected and analyzed using phenol-sulfuric acid method (28) to determine total soluble sugar. The experiment was repeated at 60°C incubation temperature. The precipitate was weighed to determine the weight of the swollen starch. The analysis was done in duplicate. The percentage solubility and swelling power were calculated using the following equations:

$$\text{Solubility} = \frac{\text{Total weight of glucose} \times 0.9}{\text{weight of sample}} \times 100\%$$

$$\text{Swelling power} = \frac{\text{weight of swollen granules} \times 100}{\text{weight of the dry sample} \times (100 - \% \text{ solubility})}$$

Statistical Analysis

Mean values of the solubility and swelling power of starch treated with dietary oils were analyzed using analysis of variance (ANOVA) with the General Linear Model procedure in SAS version 9.1 (SAS Institute, Inc., Cary, NC). Differences were evaluated by t-test using Tukey's adjustment. The significance level was set at p -value < 0.05 .

RESULTS AND DISCUSSION

Effects of dietary oils on the starch digestive rate of cassava flour

Particle-size distributions of all the samples are shown in **Figure 1** and summarized in **Table 2**. Cassava flour consisted of a greater proportion of small particles (0.05-0.1 mm) (24.3-25.5%) than the finely-ground maize sample (0.3%). There was no change in particle size distribution observed after heat-moisture treatment (HMT) to cassava flour. The addition of dietary oils followed by HMT to the cassava flour remarkably increased the particle size of the cassava flour (**Figure 1**). Samples treated with dietary oils showed a greater proportion of large particles (0.3-0.6 mm) (49.3-60.7%) than the samples without treating with dietary oils (26.2-27.3%) (**Table 2**). The dietary-oil treatments might cause particles to adhere with each other, resulting in the greater proportion of large particles in the oil-treated cassava flour.

Starch digestive rates of cassava flour, finely-ground maize, and coarsely-ground maize comparing with oil-treated cassava flour samples are shown in **Figure 2**. HMT alone decreased starch digestive rate of the cassava flour from 76.3 to 73.4% after 8 h hydrolysis. HMT enhances interactions of starch-chains of the starch granule and improves the

crystalline structure of the starch (20). Thus, the HMT alone reduced the starch digestive rate of the sample. The addition of dietary oils to the cassava flour followed by HMT further decreased the starch digestive rate of the cassava flour samples. Dietary oils (6% v/w) added to the cassava flour decreased starch digestive rate from 73.4 to 63.8, 62.8, and 56.4% after 8 h hydrolysis for palm, soybean, and corn oil, respectively (**Figure 2**). Starch digestibility of isolated cassava starch (CAS) and maize starch (B-16) comparing with that of oil-treated cassava starch samples are shown in **Figure 3**. Similar to the cassava flour samples, addition of dietary oils to the isolated cassava starch decreased the starch digestive rate of the samples. Treating the isolated cassava starch with dietary oils decreased the starch digestive rate of the samples from 74.9 to 69.6, 66.7, and 60.1% after 8 h hydrolysis for palm, soybean, and corn oil, respectively. These results showed that dietary oil with greater degree of unsaturation had greater impact on reducing the starch digestive rates of both the cassava flour and the isolated cassava starch samples.

Solubility and swelling power of isolated cassava starch and that with dietary oils treatment are shown in **Table 3**. HMT alone increased the swelling power of the cassava starch from 4.42 to 5.32 and 4.48 to 5.01 for 50 and 60°C incubations, respectively. The addition of dietary oils to the cassava starch further increased the starch swelling power. Sample treated with soybean oil and HMT showed the greatest increase in starch swelling power (5.94 and 5.72% for 50 and 60°C incubations, respectively) comparing with other oil-treated samples. Increases in starch swelling power after addition of oils were previously reported (29). The mechanism of how dietary oils increased starch swelling power, however, was not elucidated. The solubility of cassava starch with HMT alone (CAS+HMT) was not significantly different from that of untreated cassava starch (CAS). The addition of palm oil

followed by HMT increased solubility of starch from 0.16 to 0.68% and 0.22 to 0.78% for 50°C and 60°C incubations, respectively, whereas the addition of soybean oil followed by HMT increased solubility of starch from 0.16 to 0.22% and 0.22 to 0.35% for 50 and 60°C incubations, respectively. The addition of corn oil followed by HMT, however, showed no or less impact on starch solubility of the starch samples.

Thermal properties of each of the starch samples are summarized in **Table 4**. HMT alone slightly decreased gelatinization temperatures of cassava starch samples from 65.34 to 64.63°C, 70.61 to 69.71°C, and 76.94 to 76.08°C for onset, peak, and conclusion temperatures, respectively. The addition of dietary oils to the cassava starch samples followed by HMT further decreased on the gelatinization temperatures of the samples. Decreases in onset gelatinization temperature of the HMT-treated samples were correlated with the increases in swelling power (**Table 5**) (30, 31). The gelatinization enthalpy-changes of starch samples increased after dietary oils treatment (14.89-15.46 J/g) comparing with the untreated (13.75 J/g) and HMT-treated samples (14.33 J/g) (**Table 4**). There was no melting of amylose-lipid complex peak observed after the gelatinization peak, indicating no amylose-lipid complex formed resulting from the oil treatment.

Light-micrographs of the isolated cassava starch samples with and without dietary oil treatments are shown in **Figure 4**. Nile red dye bound with the hydrophobic dietary-oil and displayed a red color when viewed under the light-microscope. Cassava starch (CAS) and cassava starch with HMT alone displayed no red color (**Figures 4A and 4B**) because of the absence of oil in the sample. After dietary-oils treatments, some granules with a dense red-color stain were observed (**Figures 4C, 4D, and 4E**). Most of the oils were found coating the starch granules on the surface. Sample treated with palm oil displayed the least number of

starch granules with oil-coating (**Figure 4C**) comparing with other dietary oils (**Figures 4D and 4E**). The coating of starch by the oils could decrease the susceptibility of starch granules to enzymatic attack (32). These results were consistent with the results that samples treated with palm oil showed the least reduction in the starch digestive rate comparing with other oils (**Figures 2 and 3**).

Effects of Distiller's Dry Grain with Solubles (DDGS) on the starch digestive rate of cassava flour

The chemical compositions (starch, protein, lipids, and ash contents) of cassava flour, cassava flour-DDGS blends, and finely-ground maize samples are given in **Table 6**.

Blending DDGS with cassava flour increased the protein and lipids contents of the cassava flour. The sample with 30% (w/w) DDGS substitution contained 3.79% lipids (db), which was similar to finely-ground maize sample (3.97%). The addition of DDGS into the cassava flour increased the protein content from 2.23 to 4.62, 7.94 and 9.07% for the blend samples containing 10, 20, and 30% DDGS, respectively.

Starch digestive rates of cassava flour and finely-ground maize (B-16) comparing with DDGS and HMT treated samples are shown in **Figure 5**. The cassava flour-DDGS blends followed by the HMT treatment showed decreases in the starch digestive rate. Similar to our previous finding, HMT alone decreased starch digestive rate of cassava flour from 83.1 to 80.3% after 8 h hydrolysis. The blending of DDGS prior to the HMT treatment further decreased the starch digestive rate from 80.3 to 78.3, 74.1, and 72.0% after 8 h hydrolysis for 10, 20, and 30% DDGS, respectively. The interactions between starch and lipids in the DDGS likely reduced the susceptibility of starch to enzyme hydrolysis as previously

suggested. From these results, we concluded that DDGS at 30% of the blends followed by HMT treatment was able to reduce the starch digestive rate of cassava flour closer to that of finely-ground maize.

Starch digestive rate of the cassava flour-30% DDGS blends with different initial moisture contents (IMC) and HMT at 100°C are shown in **Figure 6**. The increase in IMC from 12.4 to 18.1% did not show considerable changes in the starch digestive rate of the blends (72.8-73.3% after 8 h hydrolysis). Further increase in IMC of the cassava flour-30% DDGS blend to 26.1% IMC, however, caused a substantial increase in starch digestive rate (85.4%, after 8 h hydrolysis). The result indicated that starch gelatinization took place during heating of the blends with high moisture content (26.1%, db) at 100°C for 1 h. Gelatinization caused starch to lose crystallinity and became more susceptible to enzymatic hydrolysis. The increase in IMC up to 26.1% also increased the amount of undigested residue of samples from 8.7(at 12.4% IMC) to 13.7% (at 26.1% IMC), which likely resulted from retrogradation of the gelatinized starch during sample preparation.

The starch digestive rates of the cassava flour-30% DDGS blends (11.9-12.4% IMC) with and without HMT at three different temperatures (80, 100, and 120°C) are shown in **Figure 7**. The cassava flour-30% DDGS blend without HMT showed a slight reduction in starch digestive rate (82.8% after 8 h hydrolysis) comparing with the untreated cassava flour (84.3%). The cassava flour-30% DDGS blends with HMT at 80°C showed higher starch digestive rate (77.8% after 8 h hydrolysis) than the blends with HMT at 100°C (72.8%) (**Figure 7**). These results suggested that when heating at 100°C instead of 80°C, starch chains had more mobility to interact with oil in the DDGS and to further reduce the starch digestive rate. The cassava flour-30% DDGS blend with the HMT at 120°C, however, showed higher

starch digestive rate (82.6% after 8 h hydrolysis). This was a result of heating at 120°C caused partial gelatinization to the starch and increased starch susceptibility to enzyme hydrolysis. The sample treated with HMT at 120°C showed less undigested residues (3.1% reduction from the control sample) than other HMT-treated samples (7.4%-9.4%) after 24 h of hydrolysis.

Effects of defatted soybean-meal (SBM) on starch digestive rate of cassava flour.

Starch digestive rate of the cassava flour and the finely-ground maize (B-16) comparing with the cassava flour-SBM blends are shown in **Figure 8**. The cassava flour blended with SBM at different proportions followed by HMT treatment showed only slight decrease in starch digestive rate (79.1-79.8% after 8 h hydrolysis) than the untreated cassava flour (82.7%) and the cassava flour with HMT alone (80.5%) (**Figure 8**). The result showed that blending cassava flour with SBM had little impacts on starch digestive rate, which was much less than blending with DDGS at equivalent protein levels (**Figure 5**).

Addition of corn oil (CO) into the cassava flour-SBM blends further decreased the starch digestive rate (**Figure 9**). The cassava flour-15% SBM and 3% (v/w of cassava flour) added corn oil followed by HMT treatment showed lower starch digestive rate (76.7% after 8 h hydrolysis) than the untreated cassava flour (82.7%) and the cassava flour with HMT alone (80.5%). Increases in amount of corn oil addition to the cassava flour-15% SBM blends resulted in further decreases in starch digestive rate (**Figure 10**). These results further confirmed that lipids were the compound responsible for the reduction in starch digestive rate. The effect of corn oil addition, however, was substantially less than that previously found with an equivalent level of lipid addition (**Figure 1**). The SBM in the blends might

have absorbed the oil and prevent the starch of cassava flour to interact with the added corn oil, thus resulted in less reduction in the starch digestive rate of cassava flour at an equivalent lipid levels.

The effect of initial moisture content prior to HMT (IMC) on the starch digestive rate of cassava flour -15% SBM and 3% CO blends followed by HMT at 100°C are shown in **Figure 11**. An increase in IMC from 13.8 to 20.7% further decreased in starch digestive rate of the samples. The presence of additional water in the mixture plasticized the starch molecules, allowing them to interact with the oil more efficiently during treatment. Samples with IMC of 28.0%, however, showed a significant increase in starch digestive rate. Similar to our previous findings (**Figure 6**), HMT treatment of the sample with high initial moisture (28.0%) caused starch partial-gelatinization and increased the starch digestive rate of cassava flour.

CONCLUSION

The addition of dietary oils (6%, v/w) to cassava flour followed by HMT at 100°C reduced the starch digestive rate of the cassava flour. Dietary oil with greater degrees of unsaturation had greater impact on reducing starch digestive rate of the cassava flour. The DDGS substitution at 30% (w/w) followed by HMT at 100°C for 1 h reduced the starch digestive rate of the cassava flour closer to that of finely-ground maize sample (B-16). DDGS inclusion substantially increased protein and lipids contents of cassava flour blends. Increases in the initial moisture content (IMC) of the cassava flour-blends from 18.1-20.7% to 26.1-28.0% prior to HMT at 100°C increased the starch digestive rate of the cassava flour. SBM (1.7% lipids, db) substitution showed less impact on reducing the starch digestive rate

of the cassava flour than DDGS (10.3% lipids, db) at an equivalent protein levels. The results confirmed that lipids in DDGS were the compound responsible for the reduction in starch digestive rate of the cassava flour. The proposed methods required relatively simple and inexpensive processes, thus applicable for industrial application.

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Table 1. Dietary oils (palm oil, soybean oil, corn oil) compositions used in the study
Fatty Acid Content (%)^b

Fatty Acid	Palm Oil	Soybean Oil	Corn Oil
Palmitic Acid (16:0)	64.03 ± 0.47	11.57 ± 0.23	10.42 ± 0.16
Stearic Acid (18:0)	6.29 ± 0.07	4.71 ± 0.05	2.16 ± 0.03
Oleic Acid (18:1)	26.31 ± 0.27	33.53 ± 0.04	28.44 ± 0.03
Linoleic Acid (18:2)	1.39 ± 0.07	44.91 ± 0.09	55.91 ± 0.21
Linolenic Acid (18:3)	ND	4.06 ± 0.07	1.58 ± 0.03
Free Fatty acid ^a	17.80 ± 0.03*	3.51 ± 0.02*	0.16 ± 0.02*

^aFree Fatty Acid content was determined by AOAC method 940.28 (23)

^bFatty acid composition was determined following the method of Hammond (22)

*Free fatty acid content was expressed as percentage of free oleic acid present in the oil

Table 2. Particle-size distributions of cassava flour (CA), cassava flour with dietary oil addition followed by HMT*, finely-ground maize, and coarsely-ground maize

Sample	Particle-size Distribution (%)			
	>0.6 mm	0.3-0.6 mm	0.1-0.3 mm	0.05-0.1 mm
CA	11.0 ± 1.1	26.2 ± 0.8	37.2 ± 0.3	25.5 ± 0.6
CA+HMT*	10.4 ± 0.8	27.3 ± 1.0	38.1 ± 0.7	24.3 ± 1.1
CA+6%PO+HMT*	18.9 ± 0.6	56.9 ± 0.4	24.0 ± 0.9	0
CA+6%SO+HMT*	33.3 ± 1.3	49.3 ± 0.5	17.3 ± 0.4	0
CA+6%CO+HMT*	13.9 ± 0.4	60.7 ± 0.7	25.3 ± 1.1	0
Finely-ground maize	2.8 ± 0.3	57.1 ± 1.1	40.0 ± 0.8	0.3 ± 0.1
Coarsely- ground maize	54.3 ± 2.1	28.2 ± 0.9	17.3 ± 0.9	0.4 ± 0.1

*Heat moisture treatment (HMT) was conducted with 11.16-11.38% initial moisture content at 100°C temperature for 1 h

Table 3. Solubility and swelling power of isolated cassava starch (CAS) and that with dietary oils addition followed by HMT *

Sample	50°C		60°C	
	Solubility	Swelling	Solubility	Swelling
	(%)	Power	(%)	Power
CAS	0.16 ± 0.02 ^a	4.42 ± 0.29 ^a	0.23 ± 0.04 ^a	4.48 ± 0.05 ^a
CAS+HMT*	0.16 ± 0.04 ^a	5.32 ± 0.37 ^b	0.22 ± 0.01 ^a	5.01 ± 0.13 ^{ab}
CAS+6%PO+HMT*	0.68 ± 0.07 ^b	5.68 ± 0.06 ^{cd}	0.78 ± 0.08 ^b	5.60 ± 0.05 ^{cb}
CAS+6%SO+HMT*	0.22 ± 0.02 ^a	5.94 ± 0.37 ^d	0.35 ± 0.02 ^c	5.72 ± 0.38 ^c
CAS+6%CO+HMT*	0.14 ± 0.04 ^a	5.45 ± 0.06 ^{cb}	0.23 ± 0.00 ^a	5.69 ± 0.21 ^{cb}
Least Significant Differences (LSD)	0.106	0.224	0.105	0.738

*Heat moisture treatment (HMT) was conducted with 6.21-6.71% initial moisture content at 100°C temperature for 1 h

^aData in the same column with different letter superscripts are significantly different ($P < 0.05$)

Table 4. Thermal properties of isolated cassava starch (CAS) and that with dietary oils addition followed by HMT*

Sample	T _o (° C)	T _p (° C)	T _c (° C)	ΔH (J/g)
CAS	65.34 ± 0.13	70.61 ± 0.12	76.94 ± 0.27	13.75 ± 0.31
CAS+HMT*	64.63 ± 0.17	69.71 ± 0.24	76.08 ± 0.13	14.33 ± 0.25
CAS+6%PO+HMT*	64.35 ± 0.23	68.51 ± 0.14	75.00 ± 0.19	15.46 ± 0.29
CAS+6%SO+HMT*	64.31 ± 0.01	68.45 ± 0.11	75.24 ± 0.14	14.89 ± 0.05
CAS+6%CO+HMT*	63.42 ± 0.18	68.63 ± 0.06	75.07 ± 0.08	14.99 ± 0.08

*Heat moisture treatment (HMT) was conducted with 6.21-6.71% initial moisture content at 100°C temperature for 1 h

Table 5. Correlation coefficients between gelatinization temperatures and swelling power of cassava starch and that with dietary oil treatments.

Starch gelatinization temperatures	Starch swelling power	
	50°C	60°C
To	-0.759	-0.773
Tp	-0.728	-0.857
Tc	-0.940	-0.934

Table 6. Proximate analyses of cassava flour, cassava-DDGS blend samples, and finely-ground maize (B-16) samples

Samples	Starch (%)	Protein (%)	Lipid (%)	Ash (%)
CA	80.8 ± 0.5	2.23 ± 0.07	0.51 ± 0.01	2.47 ± 0.05
CA+HMT*	80.8 ± 0.5	2.35 ± 0.08	0.52 ± 0.00	2.43 ± 0.03
CA+10%DDGS+HMT*	75.3 ± 0.5	4.62 ± 0.54	1.50 ± 0.16	2.71 ± 0.00
CA+20%DDGS+HMT*	70.0 ± 0.3	7.94 ± 0.45	2.67 ± 0.08	2.83 ± 0.03
CA+30%DDGS+HMT*	64.2 ± 0.4	9.07 ± 0.41	3.79 ± 0.01	2.96 ± 0.06
Finely-ground Maize (B-16)	75.5 ± 0.6	8.07 ± 0.06	3.97 ± 0.03	1.56 ± 0.02

Percentage (%) is on flour dry basis

Heat moisture treatment (HMT) was conducted with 12.8- 13.2% initial moisture content at 100°C temperature for 1 h

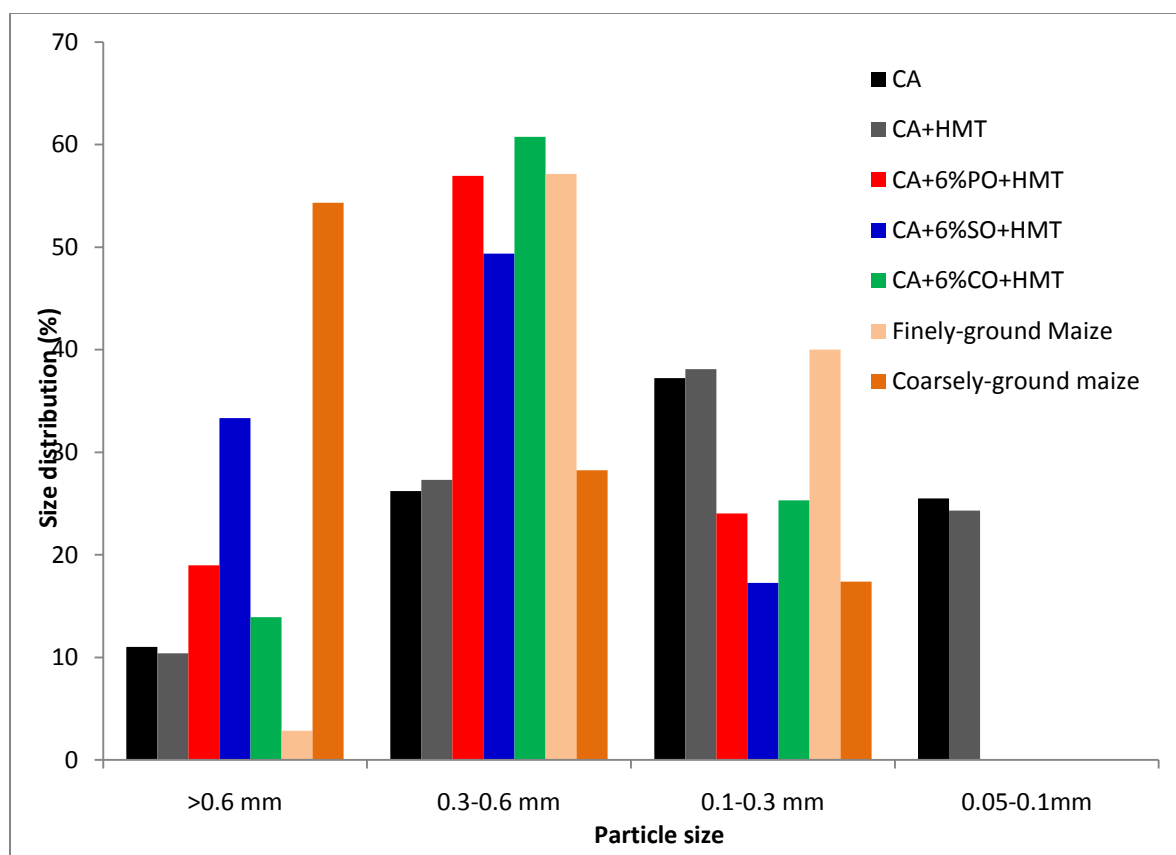


Figure 1. Particle size distribution for cassava flour (CA), finely- and coarsely-ground maize samples, and each cassava flour with dietary oil addition followed by HMT*

*Heat moisture treatment (HMT) was conducted with 11.16-11.38% initial moisture content at 100°C temperature for 1 h

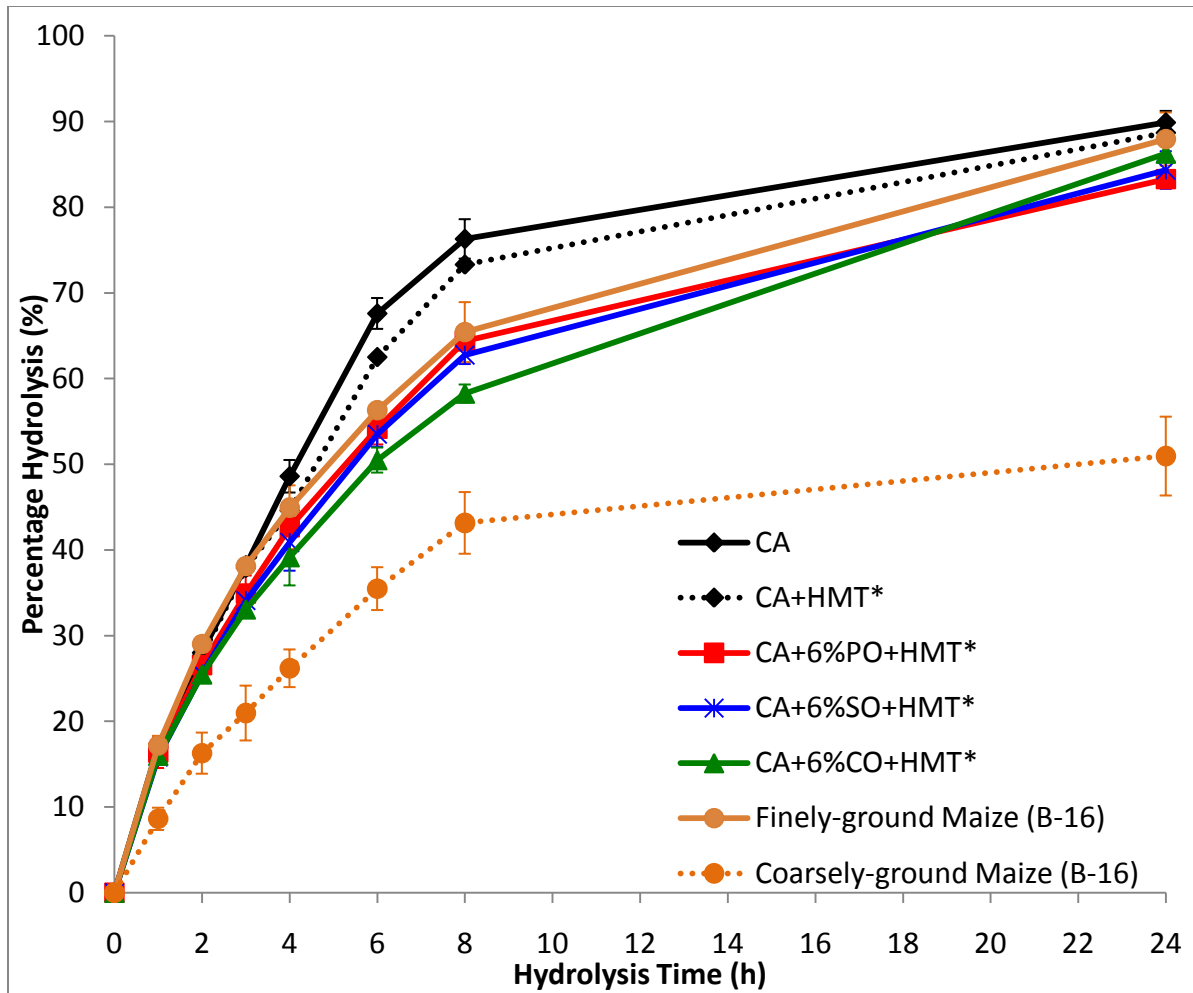


Figure 2. Starch digestive rate of cassava flour (CA), CA+HMT, CA +6% PO+ HMT, CA +6%SO+ HMT, and CA+6%CO +HMT comparing with finely-ground and coarsely-ground maize (B-16) samples. The percentage of hydrolysis is calculated on the basis of the amount of glucose produced over the starch content at dry weight basis.

* Heat moisture treatment (HMT) was conducted with 11.16-11.38% initial moisture content at 100°C temperature for 1 h

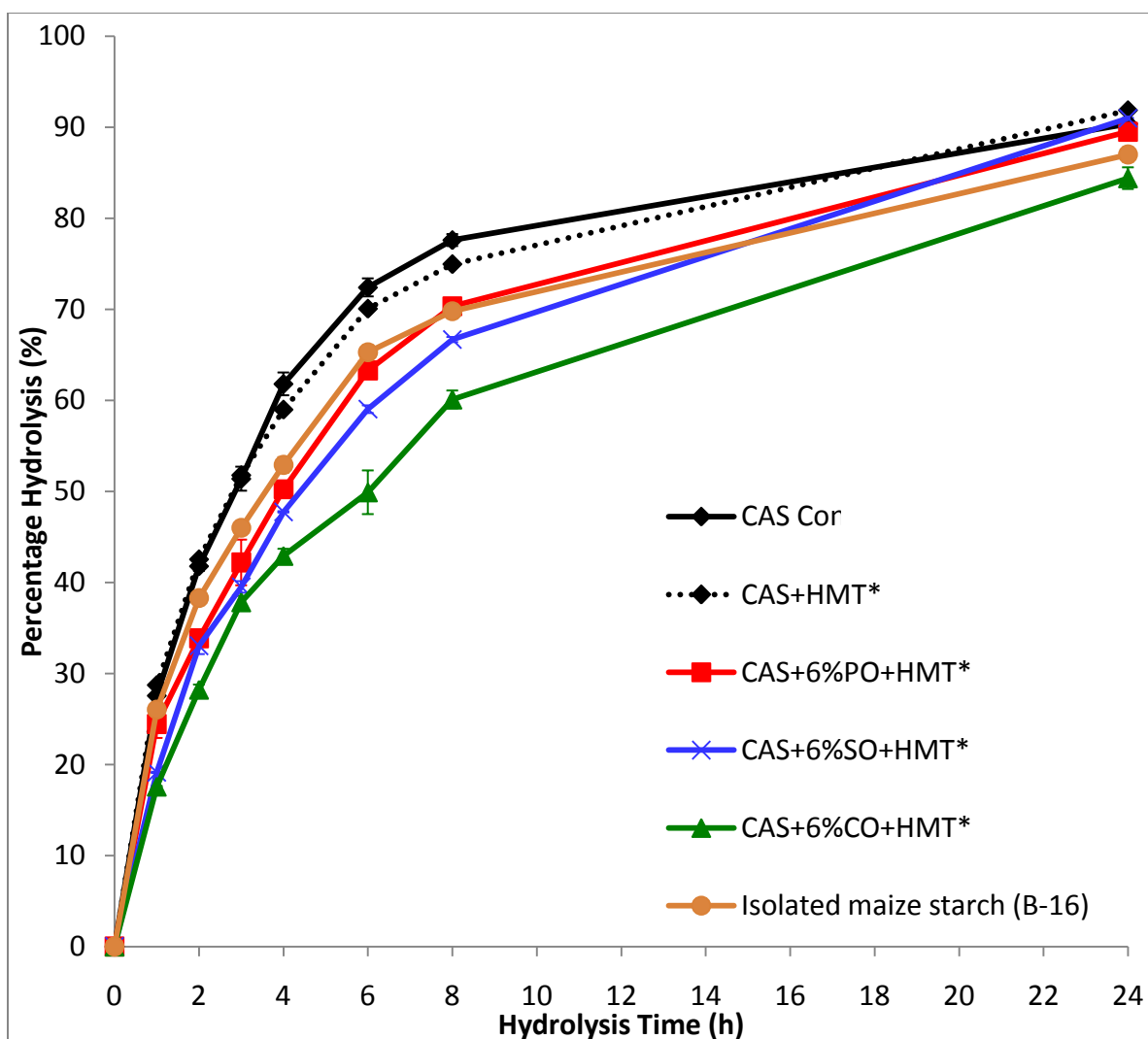


Figure 3. Starch digestive rate of isolated cassava starch (CAS), CAS+HMT, CAS +6% PO+ HMT, CAS +6%SO+ HMT, and CAS+6%CO +HMT comparing with isolated maize starch (B-16) sample. The percentage of hydrolysis is calculated on the basis of the amount of glucose produced over the starch content on dry weight basis.

Heat moisture treatment (HMT) was conducted with 6.21- 6.71% initial moisture content at 100°C temperature for 1 h

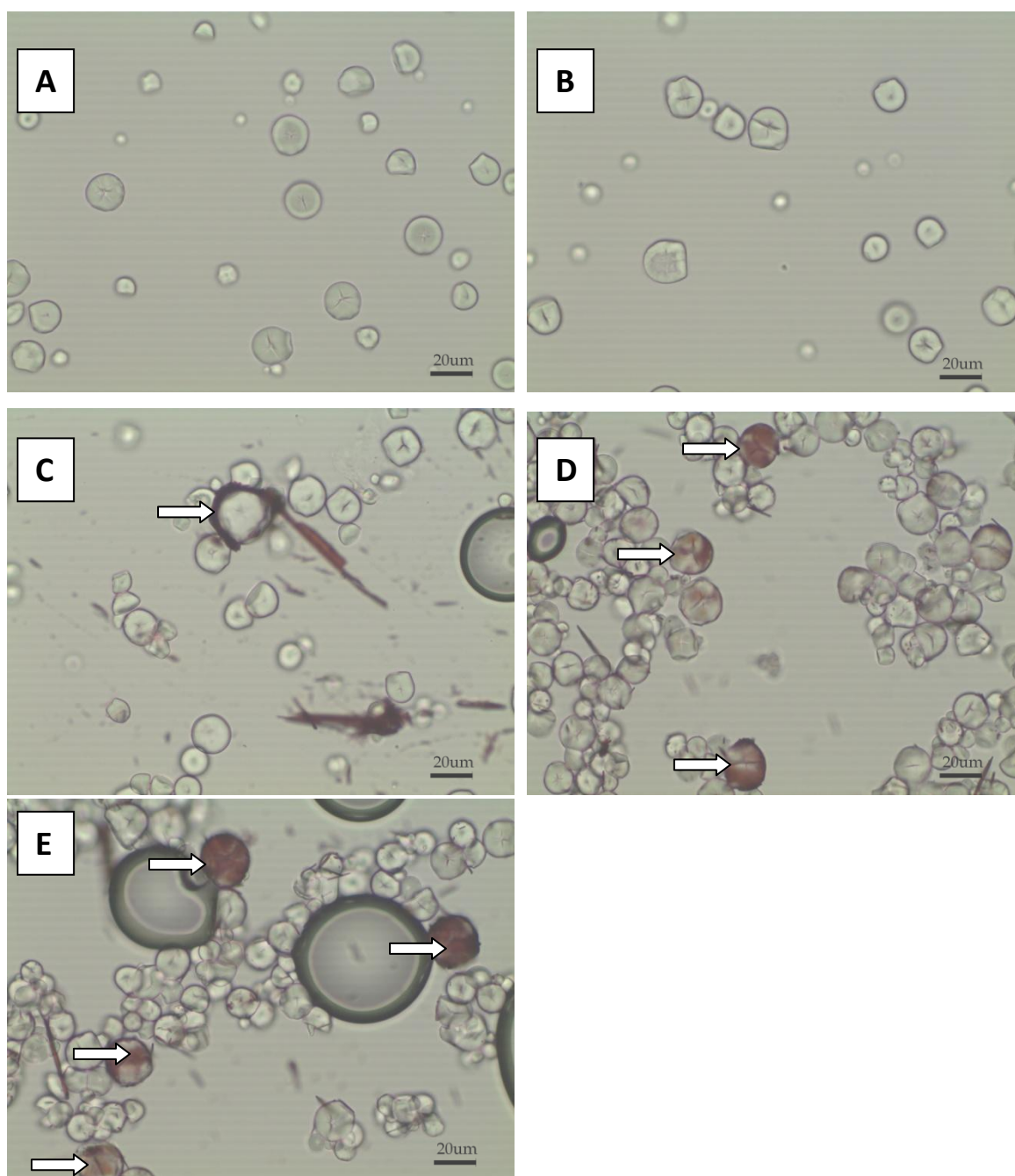


Figure 4. Light micrograph (400X) of **A)** CAS, **B)** CAS + HMT* **C)** CAS + 6%PO + HMT*, **D)** CAS + 6%SO + HMT*, **E)** CAS+ 6%CO + HMT*, with Nile red dye staining for 24 h. Arrow indicates isolated cassava starch (CAS) interacted with dietary oil, stained with Nile red dye

Heat moisture treatment (HMT) was conducted with 6.21- 6.71% initial moisture content at 100°C temperature for 1 h

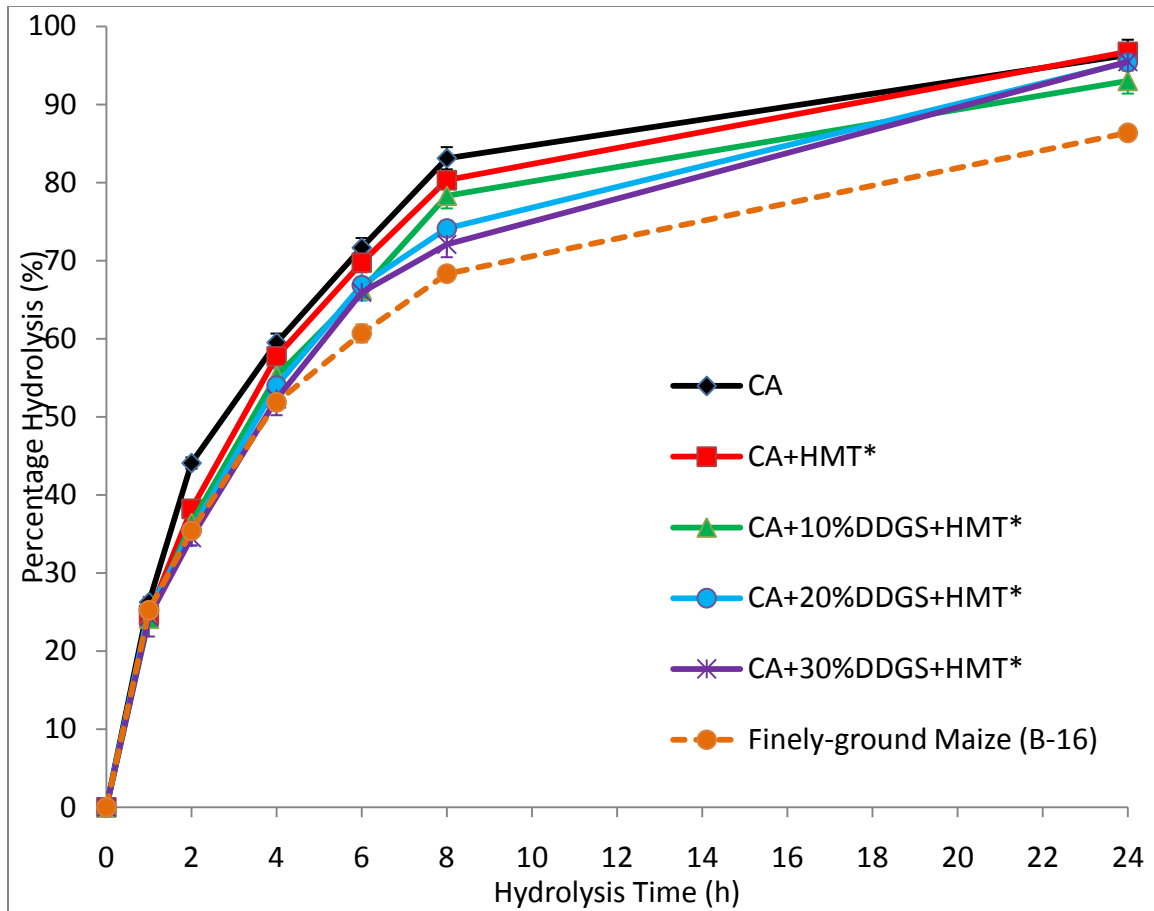


Figure 5. Starch digestive rate of cassava samples with 10%, 20%, and 30% (w/w) of DDGS substitution followed by HMT. The percentage of hydrolysis is calculated on the basis of the amount of glucose produced over the starch content on dry weight basis.

*Heat moisture treatment (HMT) was conducted with 12.8- 13.2% initial moisture content at 100°C temperature for 1 h

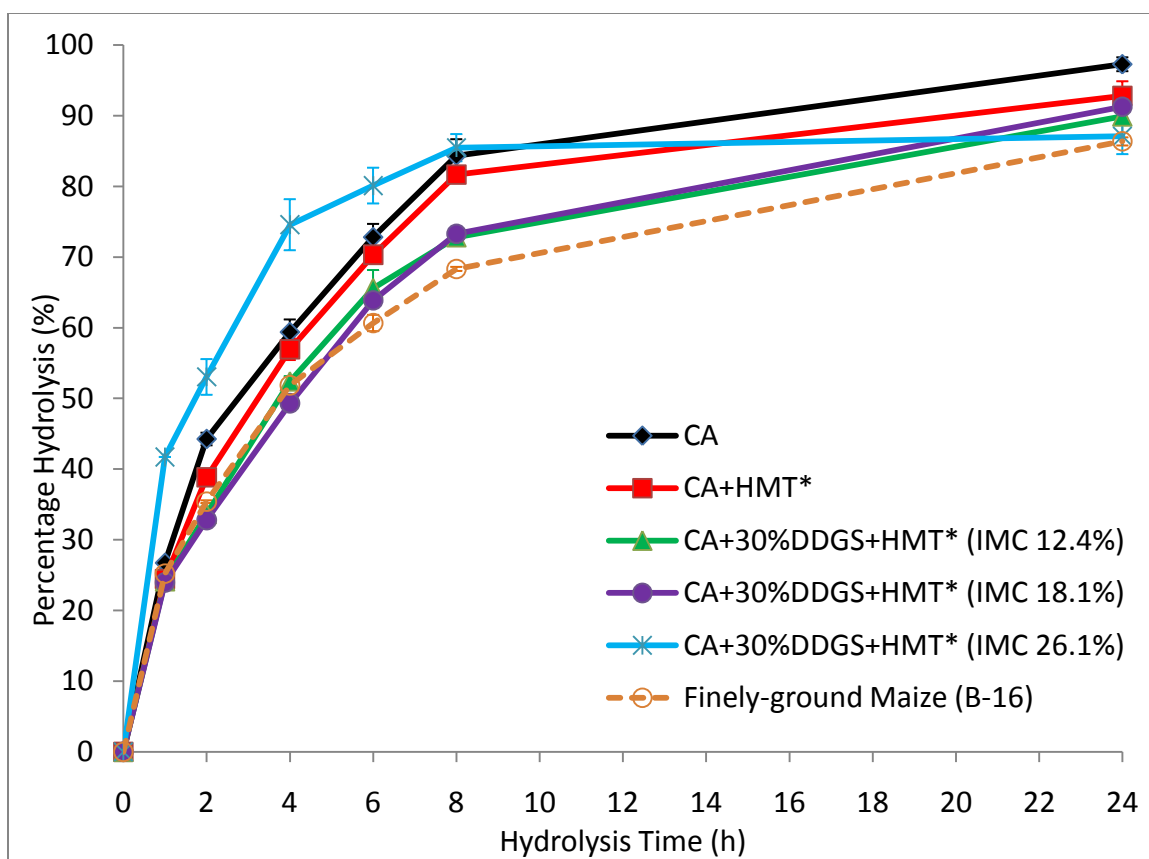


Figure 6. Starch digestive rate of cassava samples with 30% (w/w) DDGS substitution followed by HMT with different sample initial moisture content (IMC). The percentage of hydrolysis is calculated on the basis of the amount of glucose produced over the starch content on dry weight basis.

*Heat moisture treatment (HMT) was conducted at 100°C temperature for 1 h

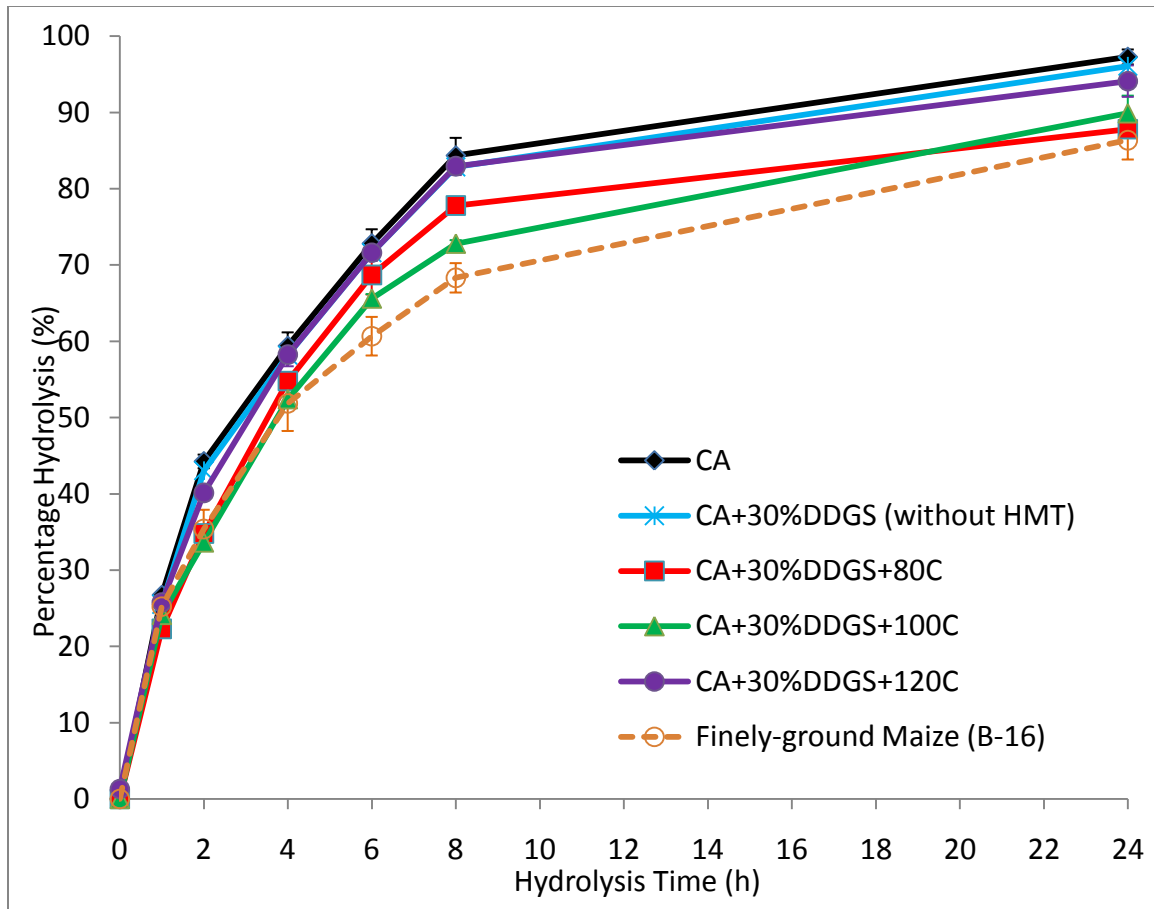


Figure 7. Starch digestive rate of cassava samples with 30% DDGS substitution (IMC= 11.9-12.4%) followed by HMT at different temperature (80°C, 100°C, 120°C). The percentage of hydrolysis is calculated on the basis of the amount of glucose produced over the starch content on dry weight basis.

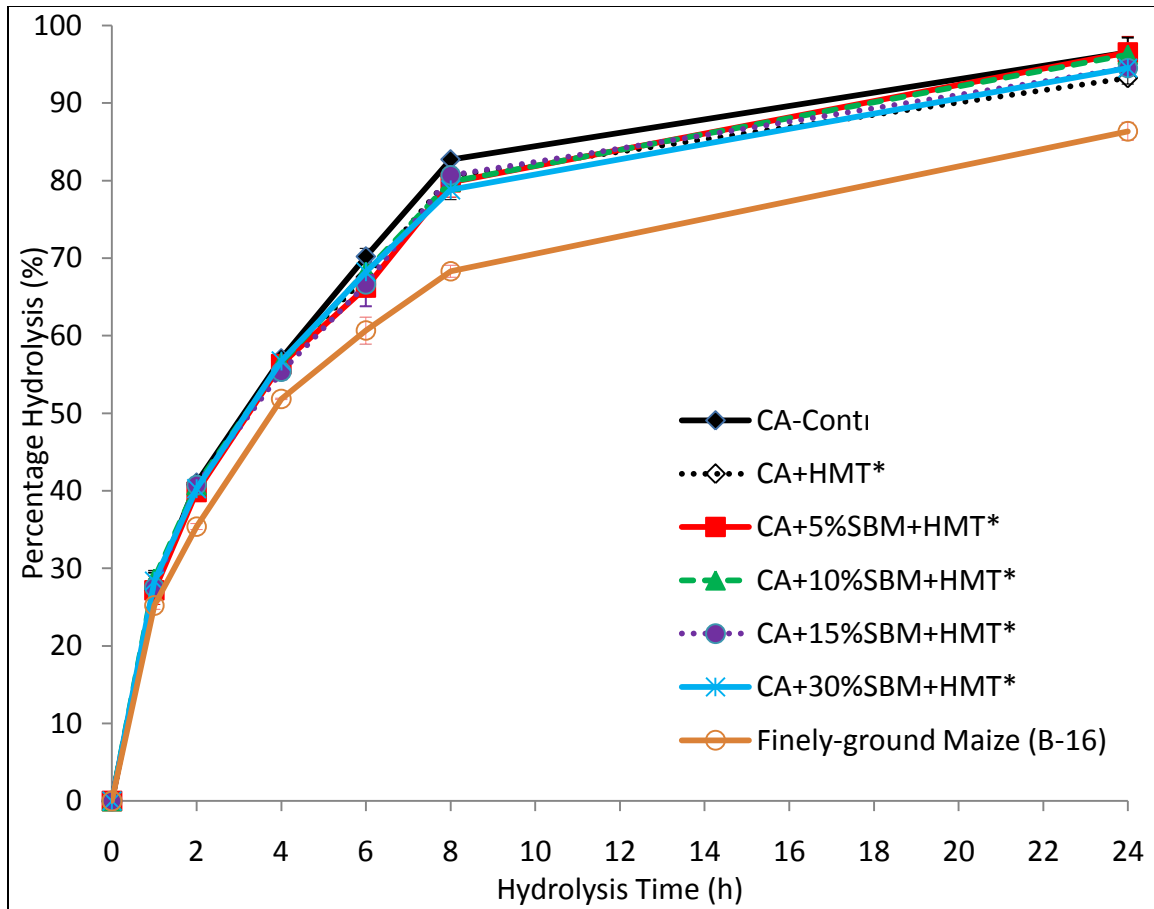


Figure 8. Starch digestive rate of cassava flour samples with 5%, 10%, 15% and 30% (w/w) SBM substitution followed by HMT. The percentage of hydrolysis is calculated on the basis of the amount of glucose produced over the starch content on dry weight basis.

*Heat moisture treatment (HMT) was conducted at 12.9-13.5% initial moisture content at 100°C temperature for 1 h

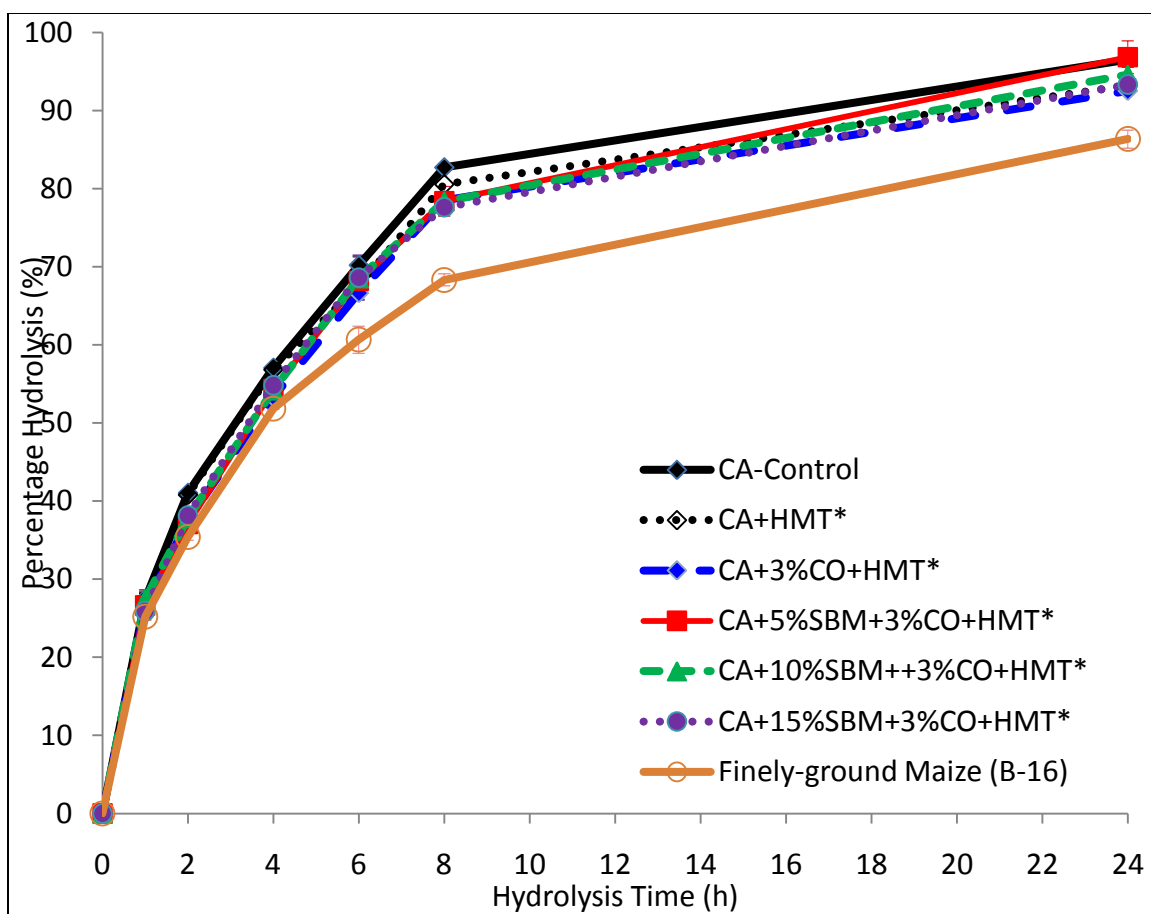


Figure 9. Starch digestive rate of cassava flour samples with 5%, 10%, and 15% (w/w) SBM substitution and 3% (v/w of cassava flour) corn oil addition followed by HMT. The percentage of hydrolysis is calculated on the basis of the amount of glucose produced over the starch content on dry weight basis.

*Heat moisture treatment (HMT) was conducted at 12.3-12.6% initial moisture content at 100°C temperature for 1 h

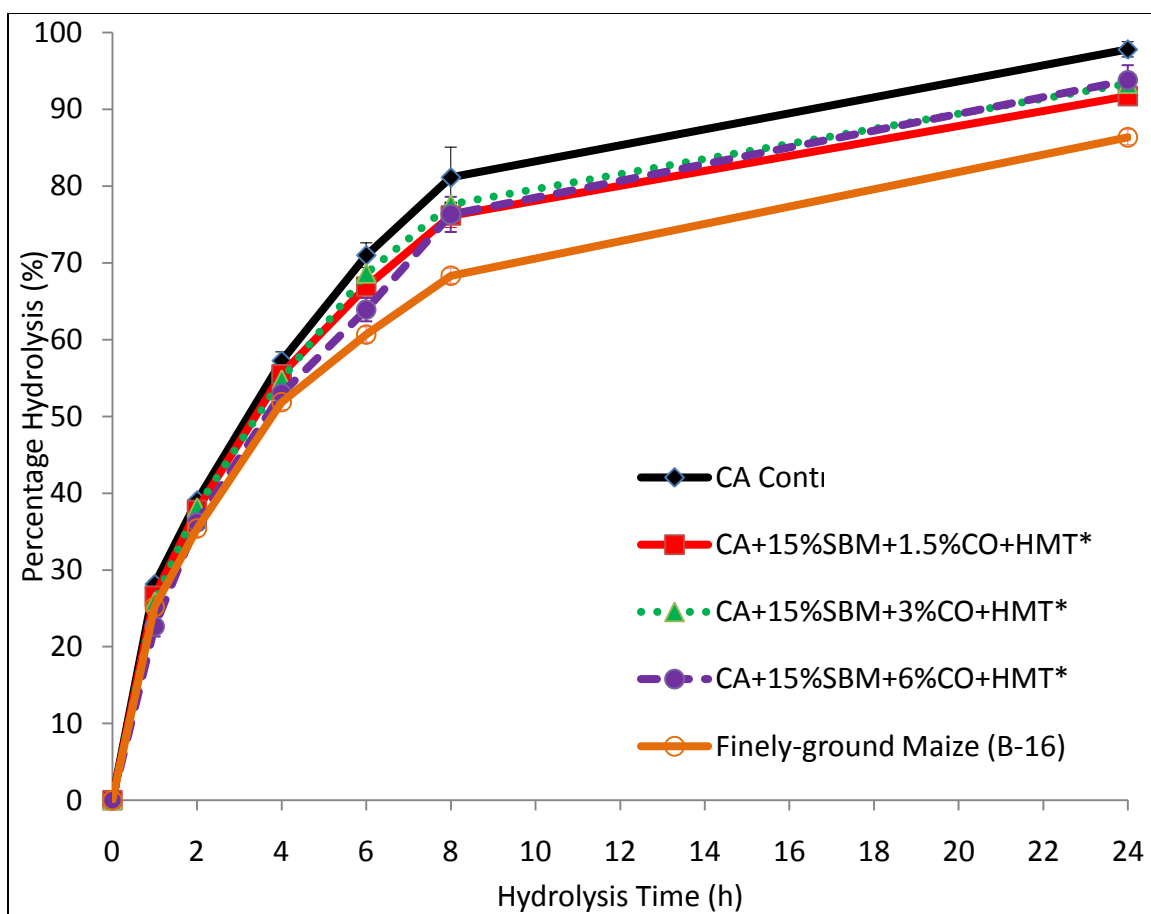


Figure 10. Starch digestive rate of cassava flour samples with 15% (w/w) SBM substitution and 1.5%, 3% and 6% (v/w of cassava flour corn oil addition followed by HMT. The percentage of hydrolysis is calculated on the basis of the amount of glucose produced over the starch content on dry weight basis.

*Heat moisture treatment (HMT) was conducted at 12.9-13.1% initial moisture content at 100°C temperature for 1 h

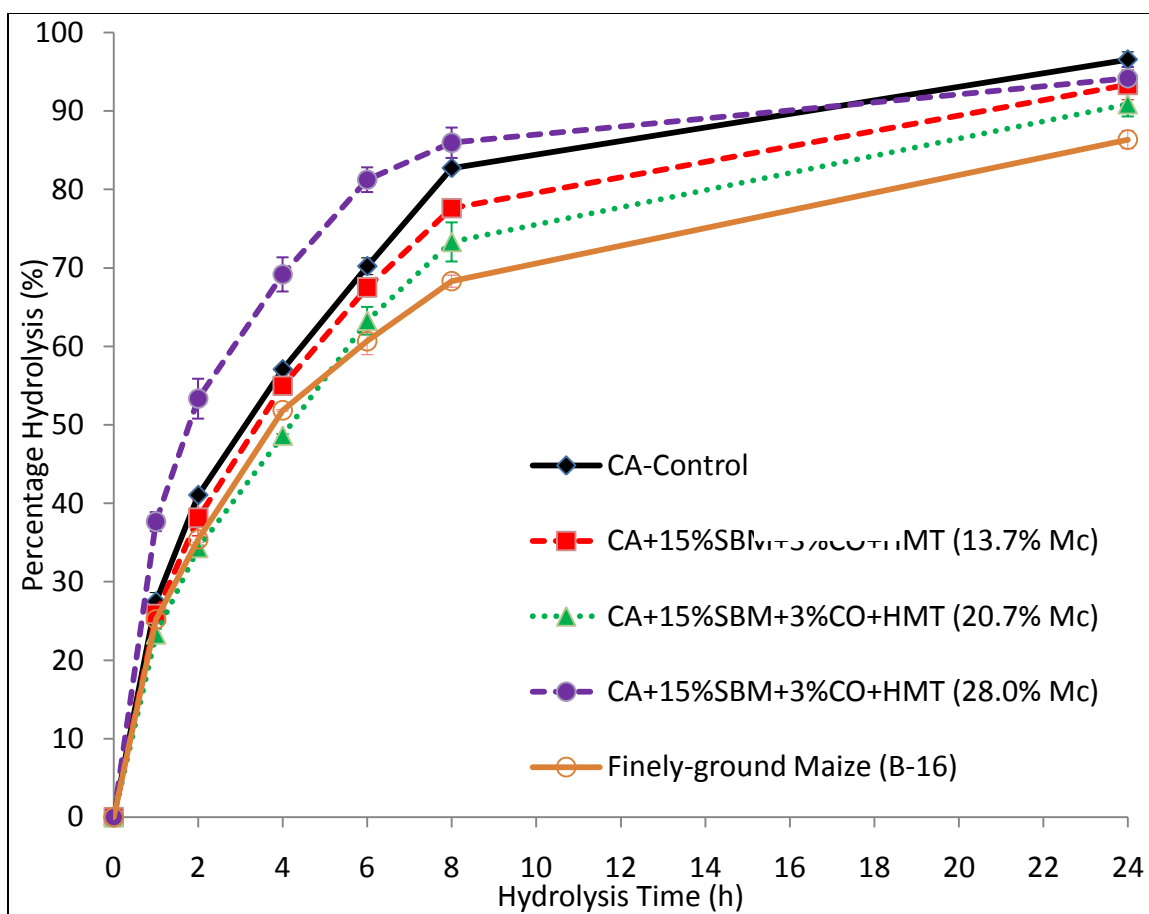


Figure 11. Starch digestive rate of cassava flour samples with 15% (w/w) SBM substitution and 3% (v/w of cassava flour) corn oil addition followed by HMT with different sample initial moisture content (IMC). The percentage of hydrolysis is calculated on the basis of the amount of glucose produced over the starch content on dry weight basis.

*Heat moisture treatment (HMT) was conducted at 100°C temperature for 1 h

GENERAL CONCLUSION

Grain drying temperature affected the starch properties of the dried products. Drying corn kernels with high moisture content (32.5%, db) at high temperatures (80 and 120/80°C) partially gelatinized their starch and increased the susceptibility to enzymatic hydrolysis. The partial-gelatinization of starch was evidenced by the loss of birefringence (Maltese-cross), the increase in starch onset-gelatinization temperature, and the decrease in gelatinization enthalpy-changes of the samples dried at higher temperatures. Storage at 37°C and 93%RH for 3 and 6 days decreased the starch digestibility of the samples. Samples dried at a higher temperature showed earlier sign of mold growth after storage than that dried at a lower temperature.

Storage of dried-corn kernels at 27°C and 85-90% for up to 6 months altered the structures and functions of their starch. Starch hydrolysis rate and peak viscosity of the ground corn samples decreased after storage. The gelatinization temperature, pasting temperature, and percentage crystallinity increased but the gelatinization enthalpy-changes and peak viscosity of the isolated starches decreased after storage of corn. Our results suggested that enzymatic hydrolysis of starch during storage were responsible to the changes in structures and functions of starch. The enzymatic hydrolysis of starch after storage was evidenced from the increases in the numbers of damaged starch granules and starch granules with pinholes and the decreases in the molecular weight of starch and percentage long-branch chains of amylopectin. The enzymatic hydrolysis of starch after storage of dried corn-kernels was more pronounced in the sun-dried samples than machine-dried samples, suggesting less

amylase activities remaining in the corn samples after machine-drying at 80°C than sun-drying at 35°C.

New methods to improve the feed quality of cassava flour were developed by introducing commonly used ingredients, such as dietary oils, Distillers Dry Grain with Soluble (DDGS), or defatted soybean-meal (SBM), into the cassava flour and followed by a heat-moisture treatment (HMT). HMT slightly reduced the starch digestive rate of the cassava flour. Addition of dietary oils increased the particle size distribution of the cassava flour samples. Addition of dietary oils (6%, v/w) followed by HMT at 100°C for 1 h substantially reduced the starch digestive rate of the cassava flour. Dietary oils with greater degree of unsaturation showed greater impact on reducing the starch digestive rate of cassava flour. DDGS at 30% of the blends reduced the starch digestive rate of the cassava flour closer to the finely-ground corn sample. DDGS inclusion increased the lipids and protein content of the cassava flour. SBM substitution showed less impact on reducing the starch digestive rate of cassava flour than DDGS at an equivalent protein levels. The results suggested that lipids were the major compound responsible for reducing the starch digestive rate of cassava flour. Increases in the initial moisture content (IMC) of the cassava flour-blends from 18.1-20.7% to 26.1-28.0% prior to HMT at 100°C increased the starch digestive rate of the cassava flour, suggesting partial-gelatinization of starch.

APPENDIX-ENZYME HYDROLYSIS PATTERN OF FOUR α -AMYLASES

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INTRODUCTION

Alpha-amylase (EC.3.2.1.1) is an enzyme that catalyzes the hydrolysis of α -1, 4 glycosidic linkages of starch, producing primarily maltose, maltotriose and maltotetraose (1). Alpha-amylase is found in the digestive tract of most vertebrate. Alpha-amylase enzyme works together with glucoamylase, a brush border enzyme, to convert the highly structured starch into glucose for absorption in small intestine. Alpha-amylase is also produced by microorganisms with unique characteristics and reaction patterns. The objective of this study was to obtain information needed to choose the appropriate α -amylase for future *in-vitro* animal feed studies. In this study we used four commercially available α -amylases (PPA, Thermamyl LC, Thermamyl SC, and D45)

MATERIALS AND METHODS

Materials

Normal maize starch was purchased from Cargill Incorporated (Minneapolis, MN). Thermamyl[®] LC (LC) and Thermamyl[®] SC (SC) are the products of Novozymes (Bagsvaerd, Denmark). Amylase-D45 (D45) was a gift from Diversa Corp (San Diego,

CA). These three alpha-amylases are thermostable. Porcine Pancreatic α -Amylase (PPA) was purchased from Sigma Aldrich Corporation (St.Louis, MO).

Amylase Assay

Amylase assay method was used to standardize the amount of enzyme units per gram of starch used in this study (2). One *Unit* is defined as the amount of enzyme required to release reducing sugar equivalent to 1 micromoles of maltose in 1 min at 40°C. One ml of enzyme solution was added to 1 ml of soluble starch substrate (10g/ L), and the mixture was incubated for three minutes at 40°C. Color reagent used in this assay was made by dissolving 100 mg of 3, 5-dinitrosalicylic acid in 20 ml of 2N sodium hydroxide and 50 ml of water containing 30 g of sodium-potassium tartrate. Two ml of color-reagent was added to stop the enzyme reaction and the mixture was heated for five minutes in a boiling water bath. The mixture was diluted and measured using a spectrophotometer at 470 nm. The determination of units of activity for each enzyme was measured by plotting the absorbance on the maltose standard curve as a reference

Starch digestibility

Normal corn starch was suspended (1% w/v) in a 0.02 M phosphate buffer with 20 ppm of Ca^{++} . Each buffer was adjusted to the optimum pH of each enzyme (pH 5.6 for Termamyl LC and SC, pH 6.0 for D45, and pH 6.9 for PPA). The starch suspension was then hydrolyzed with 200 units/g of starch of each enzyme at 40°C for 0, 1, 2, 4, 8, 24, 48 and 72 hour. The enzyme reaction was stopped by adding ethanol to denature the enzyme. The reducing sugar content of the hydrolysates was determined using the Somogyi-Nelson

method .Phenol-sulfuric method (3) was used to determine the total carbohydrate produced after 24, 48 and 72 hours.

Scanning Electron Microscopy (SEM)

Residues of starch hydrolysis were repeatedly washed with ethanol and dried at 37°C overnight. The morphology of the starch residues was analyzed by scanning electron microscopy following the method of Jane et al (4). Starch residues were re-suspended in absolute methanol, and a drop of the suspension was placed on silver tape, sticky side down, attached to a brass disk and sputter coated with gold/palladium (60/40). The mounted specimens were captured at a magnification of 1500 x using a scanning electron microscope (JEOL model, JSM-5800LV Tokyo, Japan).

Thin Layer Chromatography (TLC)

The products of amylase hydrolysis of starch were analyzed using thin layer chromatography (5). Each sample was spotted onto a 20 X 20 cm Whatman K5 TLC plate (Fisher Scientific, Chicago, IL), and the plate was irrigated 2 times with a solvent mixture of acetonitrile: ethyl acetate: 1-propanol: water (85: 20: 50: 50, by volume). The carbohydrates on the TLC plate were detected by dipping the dried plate into a methanol solution containing N-(1-naphthyl) ethylenediamine (0.3% w/v) and sulfuric acid (5% v/v), and followed by heating at 120°C for 15 min.

RESULTS AND DISCUSSION

Enzyme unit in 1 ml of solution of each α -amylase is shown in **Table 1**. SC showed the highest α -amylase activity per one ml of enzyme solution, followed by D45, LC and PPA. From the result, each enzyme was diluted at different concentrations to produce the same units of activity used for starch hydrolysis analysis. It should be noticed that the result represents each alpha-amylase activity at 40°C. Temperature of 40°C was used in this study because it is similar to the internal body temperature of most warm-blooded vertebrate, including poultry. Enzyme hydrolysis of starch using PPA, SC, LC, and D45 measured as percentages of reducing sugar are shown in **Figure 1**. PPA and SC produced greater amount of reducing sugar (39.70 % and 39.03 % after 72h hydrolysis, respectively) than LC and D45 (23.88 % and 27.63 %, respectively) (**Figure 1**). The percentages of starch hydrolysis based on glucose production are shown in **Table 2**. From the result, SC, PPA, and D45 close to completely hydrolyzed the starch after 24 h hydrolysis (92.4 %, 93.1 %, and 88.8 %, respectively), whereas LC hydrolyzed the starch at lesser extent (64.5%). The difference in starch hydrolysis pattern between that based on reducing sugar (**Figure 1**) and that on glucose production (**Table 2**) indicated the size of maltooligosaccharides produced by each of the α -amylase. From **Figure 1** and **Table 2**, LC and D45 α -amylase produced longer maltodextrins, whereas SC and PPA produced shorter maltodextrins.

The scanning electron micrographs of hydrolyzed starch residues of each enzyme are shown in **Figure 2**. The hydrolyzed residues of each enzyme were obtained after 20% starch hydrolysis based on the amount of reducing sugars that were produced. From the micrographs, PPA showed high capability to attack and to hydrolyze every starch granule. SC, LC, and D45 showed that they capable to attack some specific starch granules while leaving the other starch granule intact. From **Figure 2B**, Starch residues hydrolyzed by PPA

showed greater number of pinholes on the surface than that hydrolyzed by LC, SC, and D45. Maltooligosaccharides produced in the hydrolysates are shown in **Figure 3** and **Figure 4**. LC produced mainly G3, G4, and G5 through the hydrolysis periods. SC hydrolyzed native starch to produce hydrolysates with a large proportion of G5 residues at the initial stage of hydrolysis (24 h). SC, however, further hydrolyzed this maltooligosaccharides residues (G5) into G2 and G3 on the later stage of hydrolysis (72h) (**Figure 4**). PPA consistently produced most of its maltooligosaccharides residues as G2 and G3 through the hydrolysis periods. D45 showed significant increases in the smaller maltooligosaccharides residues (G2 and G3) after 48 h. Similar to SC, D45 initially hydrolyzed starch to produce larger maltooligosaccharides residues, and later hydrolyzed these large maltooligosaccharides residues to produce smaller maltooligosaccharides residues after 48 h hydrolysis.

CONCLUSION

Each α -amylase has its own characteristics that fit for a specific purpose. Among these enzymes, we decided to use PPA for our future *in-vitro* feed studies. PPA hydrolyzed all the available starch granules in a non-specific manner and produces more uniform maltooligosaccharides residues (G2 and G3) throughout the hydrolysis period in comparison with other α -amylase enzymes. These characteristics of PPA are appropriate for a good control and accuracy needed for *in-vitro* feed studies.

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Table 1. Enzyme units in 1 ml of solution for each α -amylase

Enzyme	Units / ml of enzyme
Thermamyl LC	920.1 \pm 62.4
Thermamyl SC	3059.9 \pm 50.4
PPA	160.5 \pm 32.5
D45	1740.2 \pm 82.1

Table 2. Percentage of starch hydrolysis measured on the basis of glucose produced on dry starch basis.

Time	Percentage of Hydrolysis (%)			
	Thermamyl LC	Thermamyl SC	PPA	D45
24 h	64.5 ± 0.6	92.39 ± 0.3	93.13 ± 1.1	88.8 ± 0.0
48 h	78.8 ± 0.7	95.00 ± 0.7	95.5 ± 3.7	97.4 ± 1.5
72 h	92.8 ± 3.1	96.32 ± 1.9	95.7 ± 1.1	99.6 ± 1.7

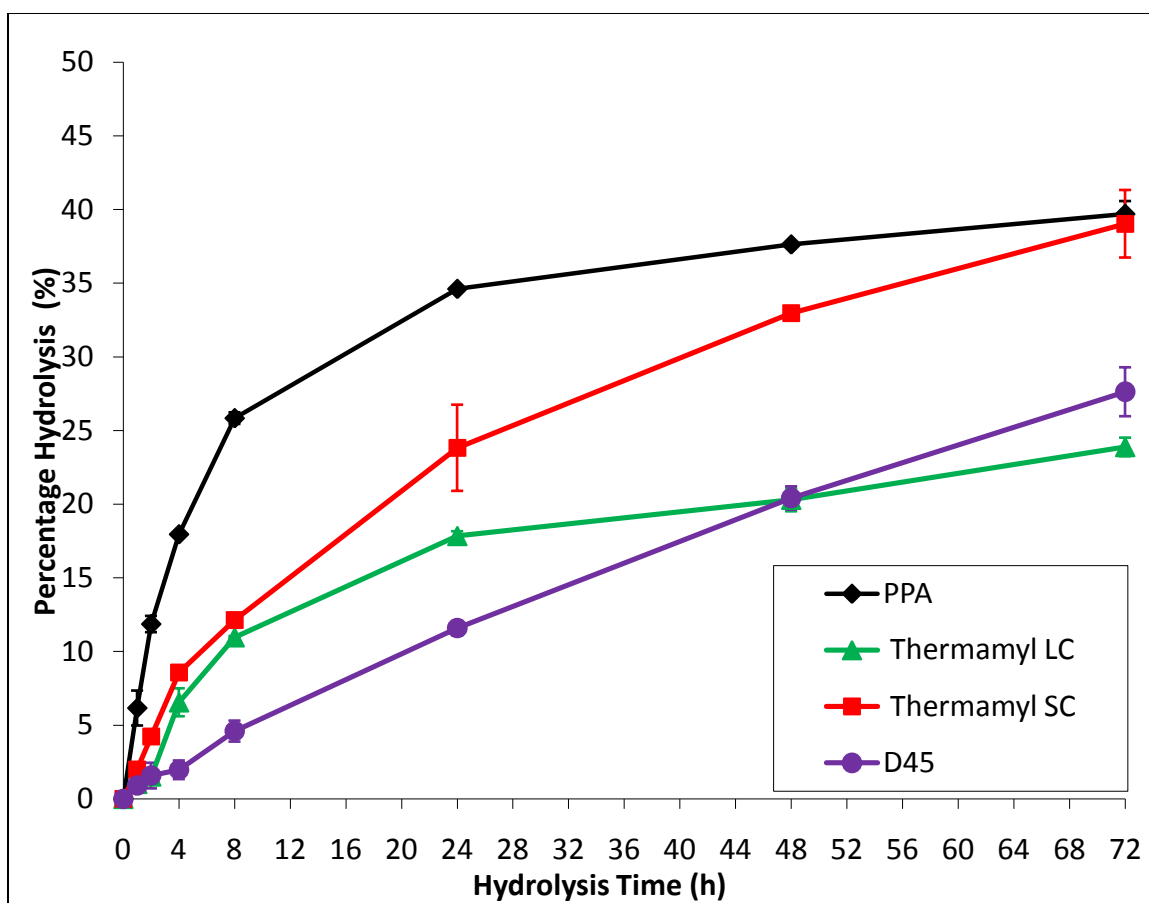


Figure 1. Starch hydrolysis pattern of normal-maize starch using four different α -amylases (PPA, Thermamyl SC, Thermamyl LC, and D45) measured as percentage of reducing sugar produced over starch content on dry basis.

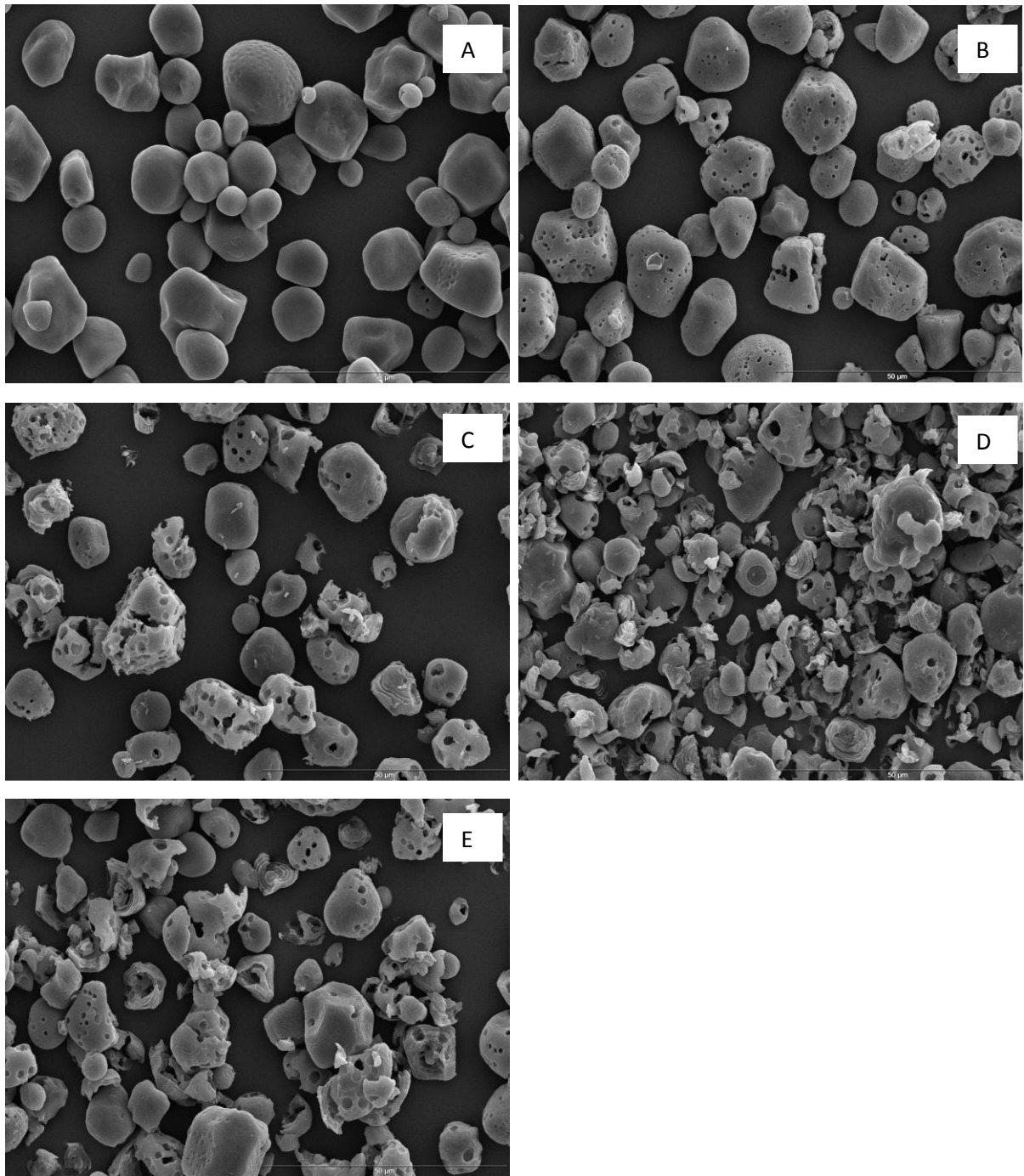


Figure 2. Scanning electron micrograph of native maize starch (A) and that hydrolyzed with PPA (B), Thermamyl LC (B), Thermamyl SC (C), and D45 (D) after 20% starch had been hydrolyzed (On the basis of reducing sugar produced)

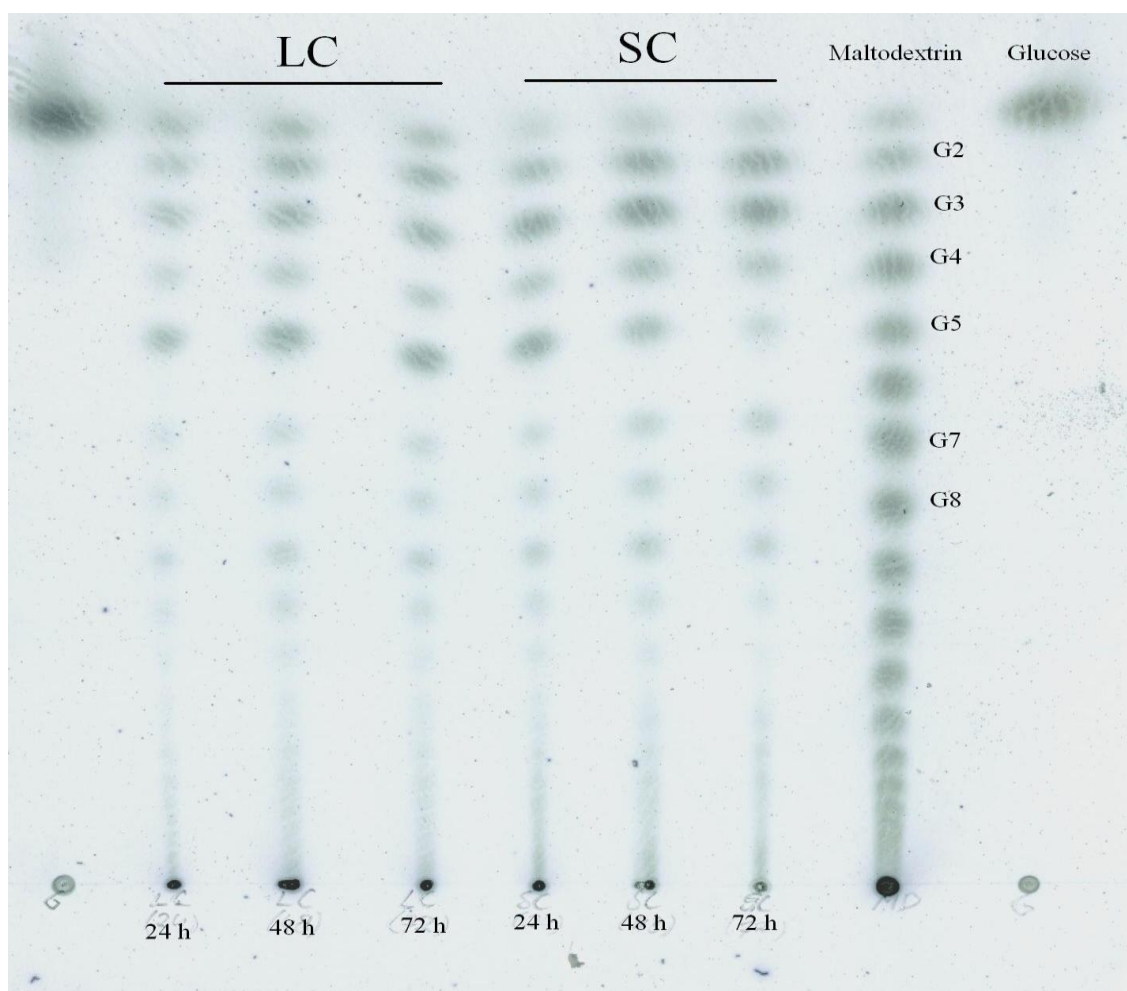


Figure 3. TLC chromatograph of normal maize starch hydrolysates produced by Thermamyl LC and Thermamyl SC hydrolysis at different time interval (24 h, 48 h, and 72h hydrolysis)

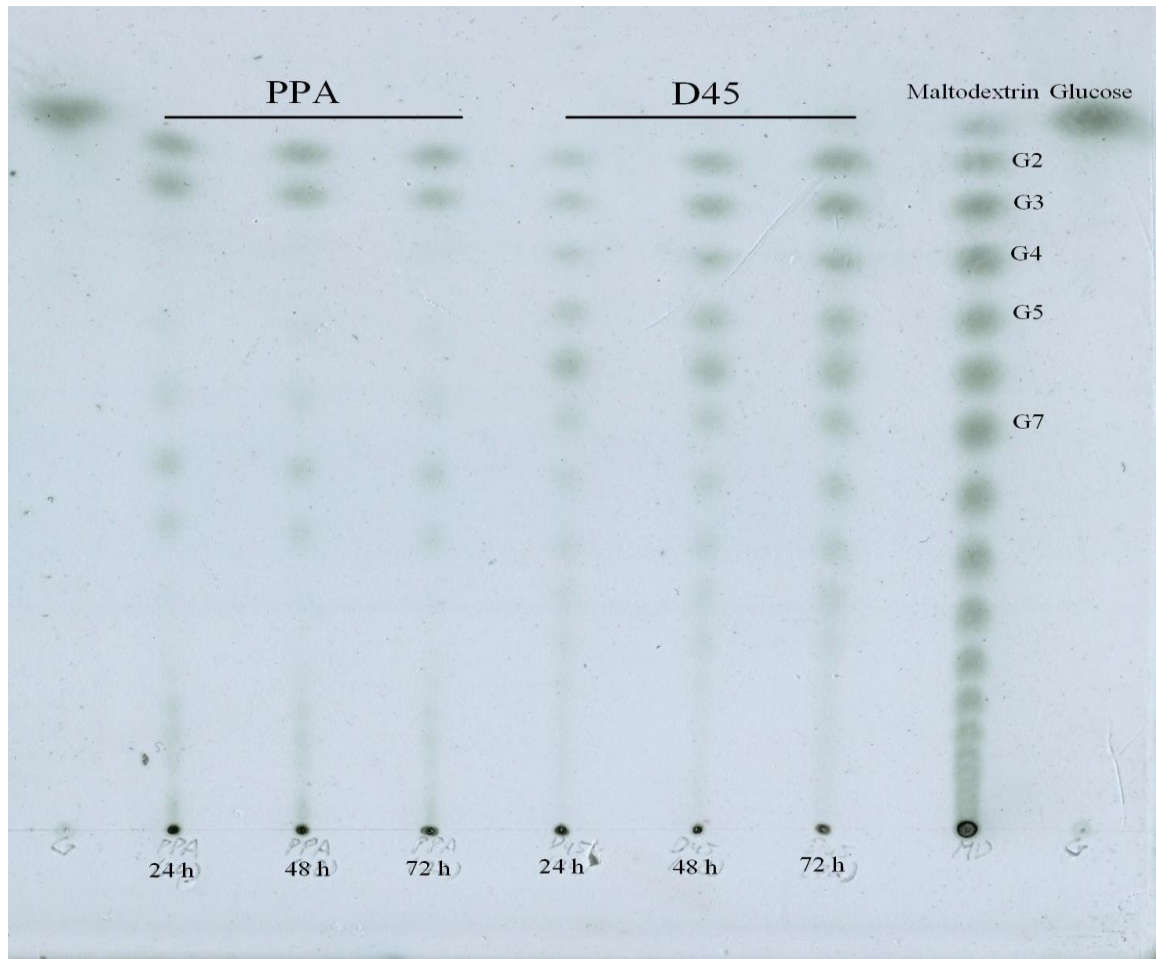


Figure 4. TLC chromatograph of normal maize starch hydrolysates produced by PPA and D45 hydrolysis at different time interval (24 h, 48 h, and 72h hydrolysis)

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