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Enzyme digestibility of starch and methods to produce enzyme-resistant starch to improve human health

Jovin Hasjim
Iowa State University

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Enzyme digestibility of starch and methods to produce enzyme-resistant starch to improve human health

by

Jovin Hasjim

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

DOCTOR OF PHILOSOPHY

Major: Food Science and Technology

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Iowa State University
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2009

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The objectives of this study were to understand how intrinsic and extrinsic factors of o2 and QPM starches affected the enzyme digestibility of their starches, to develop novel and economical technologies to produce RS for health benefits. Enzyme digestibilities of starch present in opaque-2 maize (o2) and quality protein maize (QPM) were analyzed to evaluate the uses of the two maize mutants for food, feed, and ethanol production. The starch granules of the dry-ground o2 maize and QPM were hydrolyzed faster than those of the dry-ground wild-type (WT) maize using porcine-pancreatic α-amylase (PPA). The differences in the hydrolysis rates of these starch granules were attributed to the lower protein content of the o2-maize kernels in general and the lower amylose content of the severe o2 mutant B46o2 and QPM starches than the WT counterparts when subjected to PPA hydrolysis. The starch granules in the dry-ground maize samples were also hydrolyzed faster than the starch granule isolated from whole kernels by wet milling. This difference could be attributed to the presence of mechanically damaged starch granules and endogenous amylases in the dry-ground maize samples. All the results suggested that the starch granules in the o2 maize and QPM are desirable for feed and ethanol production.

A novel starch with high enzyme resistance was produced by complexing high-amylose maize starch VII (HA7) with fatty acid (FA) to increase the starch-lipid complex content of the HA7 granules and to enhance the enzyme resistance. The production of the HA7-FA complex involved pre-swelling and debranching HA7 starch to facilitate the starch-lipid complex formation. The resistant starch (RS) contents of the HA7-FA complex made from stearic acid and palmitic acid were up to 75% determined using AOAC Method 991.43 for
dietary fiber. The presence of the amylose-lipid complex in the HA7-FA products was confirmed using DSC thermograms and X-ray diffractograms. The increase in the enzyme resistance of the HA7-FA products was attributed to the amylose-lipid complex formation and the restriction of starch granule swelling during cooking. The restricted swelling of the starch granules reduced the accessibility of amylase to hydrolyze the starch. Bread made from 60% of the HA7-palmitic acid (PA) complex containing 34.4% RS was used for a human-feeding study. After ingesting the HA7-PA bread, the postprandial plasma-glucose and insulin responses of 20 healthy male human subjects was reduced by 45% and 57%, respectively, from that obtained after ingesting white bread as the control. The results suggested that the HA7-PA can be used for interventions of insulin resistance and metabolic syndrome including diabetes, obesity, and cardiovascular disease.

Extrusion cooking was used to enhance the RS content of normal-maize starch. Acid-modified normal-maize starch was extrusion cooked followed by hydrothermal treatment at 110°C. The RS contents of the extruded products were not significantly different from those obtained from the batch-cooked products. The RS contents of the batch-cooked and extruded products increased up to 17% and 33% determined using the AOAC Method 991.43 and Englyst’s method, respectively, after the hydrothermal treatment. The RS contents of the batch-cooked and extruded products were attributed to the presence of retrograded amylose and crystalline starch-lipid complex. Both had melting temperatures above 100°C.
GENERAL INTRODUCTION

Starch is the major reserve polysaccharide in plants, which also serves as an important energy source for food, feed, and ethanol production. Starch is found in grains, roots, tubers, stems, leaves, and fruits and present as granules with semi-crystalline structure. Starch consists of two glucans: amylopectin and amylose. Amylopectin is highly branched molecules, in which 95% of glucose monomers are linked by \(\alpha-(1\rightarrow4)\) linkages and 5% are linked by \(\alpha-(1\rightarrow6)\) linkages. Amylose is essentially linear molecules with a few branches.

Starch granules of some varieties are highly susceptible to amylase hydrolysis and are desirable for feed and ethanol production using cold fermentation. This starch can be easily converted to glucose to provide energy for animal growth and development and to provide glucose for ethanol fermentation without cooking. The enzyme hydrolysis of the starch is affected by the extrinsic and intrinsic factors of starch. The presence of protein matrix and cell wall material surrounding starch granules can prevent the accessibility of enzyme to hydrolyze the starch. The susceptibility of the starch granules are also greatly affected by their amylose content, starch polymorphism, structure of amylopectin, and the presence of amylose-lipid complex in the starch granules. Amylose negatively correlates with the susceptibility of starch to amylase hydrolysis because it intertwines with amylopectin, and holds the integrity of the starch granules. Amylopectin of the A-type starch has larger proportions of short branch-chains, which result in more open space (weak points) in the granule for amylase to penetrate and hydrolyze the starch. Thus, the A-type polymorphic starch is more digestible than the B- and C-type polymorphic starches. The presence of
amylose-lipid complex inhibits the swelling of starch granules and reduces the accessibility of enzyme to hydrolyze the starch.

Maize mutants with a greater lysine content, such as opaque-2 (o2) and quality protein maize (QPM), have been developed to improve nutritional value, especially for use as animal feed. The enzyme digestibility and the properties of the o2 and QPM starches are not well studied. This information is important to fully understand the quality of o2 maize and QPM as feed for providing energy for animal growth and development.

On the contrary, enzyme-resistant starch (RS), which cannot be hydrolyzed by digestive enzymes, is beneficial for human health for lowering postprandial plasma-glucose and insulin responses. Therefore, RS can be used for the interventions of insulin resistance and metabolic syndrome, including diabetes, obesity, and cardiovascular disease. RS is also readily fermented by gut microflora to produce short-chain fatty-acids, which can prevent the development of cancerous cells in the colon.

Many technologies have been developed to produce RS, including repeated cycles of autoclaving and heating and complete debranching of starch molecules. Those production techniques of RS, however, involve lengthy and high-energy processes and were costly, which make the RS less affordable for consumers and restrict its use as ingredient in food products. In addition, some RS’s are not heat stable and lose their enzyme resistance during cooking. With the reasons stated, it is imperative to develop simpler and more economical technologies to produce heat-stable RS. Thus the RS and its health benefits are more affordable for consumers. The objectives of this study were to understand how intrinsic and extrinsic factors of o2 and QPM starches affected the enzyme digestibility of their starches, to develop novel and economical technologies to produce RS for health benefits.
This dissertation consists of four papers. The first paper, “Kernel composition, starch structure, and enzyme digestibility of opaque-2 maize and quality protein maize,” has been accepted by the Journal of Agricultural and Food Chemistry for publication. The second paper, “Novel resistant starch from high-amylose maize starch VII – fatty acid complexes. Part1. Preparations,” and the third paper, “Novel resistant starch from high-amylose maize starch VII – fatty acid complexes. Part2. Reducing postprandial plasma-glucose and insulin,” follow the format of the Cereal Chemistry for submission to the Cereal Chemistry. The fourth paper, “Production of resistant starch by extrusion cooking of acid-modified normal-maize starch,” will be submitted to the Journal of Food Science. The four papers are preceded by a General Introduction and a Literature Review and followed by a General Conclusion, an Appendix, and Acknowledgements. The Literature Cited in the Literature Review is listed in alphabetical order of the first author’s last name.
LITERATURE REVIEW

Structure of starch granules

Starch is the major reserve polysaccharide in plants. It is synthesized in semi-crystalline granular structure in grains, roots, tubers, stems, leaves, and fruits. The morphology and the structure of the starch granules depend on the botanical sources (Jane et al 1994), the organs of the plants (Vrinten and Nakamura 2000, Li et al 2007), and the stages of development (Briones et al 1968, Li et al 2007). The diameter of the starch granules range from submicron to more than 100 microns and display many shapes, including spherical, disk, oval, polygonal, elongated, etc. (Jane et al 1994, Li et al 2007). The starch granules are water insoluble, thus they are easily isolated from the plant organs through wet milling or recovered after chemical modification or derivatization.

Normal starch consists of two glucans: amylopectin and amylose. Amylopectin is highly branched molecules with $\alpha$-(1$\rightarrow$4) glycosidic-linked short linear chains connected by $\alpha$-(1$\rightarrow$6) glycosidic linkages. Amylose is comprised of primarily linear molecules with $\alpha$-(1$\rightarrow$4) glycosidic linkages; some consist of a few branches. The branch chains of the amylopectin are packed into a semi-crystalline structure of double helices in the starch granules (Figure 1) (Jane et al 1997). Amylose is present in an amorphous form intertwined and interspersed with the amylopectin (Jane et al 1992). Amylopectin and amylose are synthesized side by side by different enzymes, which are soluble starch synthase and granular bound starch synthase, respectively (Nakamura 2002). Granular bound starch synthase is also responsible for the biosynthesis of super-long branch chains of the amylopectin, which have properties similar to amylose (Yoo and Jane 2002, Inouchi et al 2005). Amylopectin
and amylose are arranged in radial direction in the starch granules. The amylopectin crystalline structure gives a Maltese-cross patterns to the starch granule when it is observed under a polarized-light microscope (Jane et al 2003).

The amylose content of normal starch is about 15%-30%. Waxy starch is devoid of amylose (Hovenkamp-Hermelink et al 1987, Nakamura et al 1995), whereas high-amylose starch contains more than 50% amylose (Regina et al 2006, Li et al 2008). Waxy starch also shows greater crystallinity than normal starch, which is attributed to the greater amount of amylopectin in the waxy starch (Fujita et al 1998, Jane et al 1999). Consequently, high-amylose starch has less crystallinity than normal starch. The amylopectin of the waxy starch possesses a larger molecular weight than that of the normal starch, whereas that of the high-amylose starch possesses a smaller molecular weight than that of the normal starch (Yoo and Jane 2002). This is attributed to that more ADP-glucose is available for the biosynthesize of amylopectin in the waxy starch, and the pool of ADP-glucose has to be partitioned to synthesize amylose and amylopectin in the normal and high-amylose starches. The molecular size of the amylose in cereal starches is smaller than that of the amylose in tuber and root starches (Takeda et al 1987, Jane and Chen 1992).

The semi-crystalline structures of native starch granules are divided into A-, B-, and C-type polymorphs. The A-type polymorph has a monoclinic unit cell, and the B-type polymorph has a hexagonal unit cell (Figure 2) (Buléon et al 1998). The C-type polymorph has a combination of the A- and B-type polymorphs. The amylopectin of the B-type polymorphic starches has a smaller amount of short branch-chains than that of the A-type counterparts (Hizukuri 1985). The branching points of the B-type polymorphic amylopectin are mostly located in the amorphous regions, whereas the branching points of the A-type
counterpart are scattered in both amorphous and crystalline regions (Figure 1) (Jane et al 1997). The B- and some C-type starch granules are resistant to enzyme hydrolysis, whereas the A-type starch granules are easily hydrolyzed (Jane et al 2003). The A-type starch granules show more pinholes on the surface and serpentine-like channels inside the granules than the B-type starch granules (Fannon et al 1992, Huber and BeMiller 2000). The internal structure of the A-type polymorphic starch granules is more loosely-packed than that of the B-type counterparts (Figure 3) (Jane and Shen 1993, Pan and Jane 2000, Jane 2006, Jane 2007). The homogenous internal structure of the B-type polymorphic starch could be attributed to the large proportion of long branch-chains of amylopectin which spans thru several multiple clusters of amylopectins and holds the crystalline lamellar (Srichuwong and Jane 2007).

The internal structure of the starch granules is not homogenous (Jane 2007). The amylose content is greater at the periphery than that at the core of the starch granules (Jane and Shen 1993, Pan and Jane 2000). This is attributed to that amylose is synthesized more actively at the later stage of starch accumulation (Li et al 2007). Thus larger (more matured) granules contain more amylose than smaller (less matured) granules. The amylopectin molecules at the periphery also consist of a greater amount of shorter branch chains than those at the core of the granules (Jane and Shen 1993, Pan and Jane 2000). This could be attributed to the activities of different soluble starch synthases and starch branching enzymes at different stage of starch accumulation (Gao et al 1997, Fujita et al 2006).
Starch gelatinization and retrogradation

Heating starch granules with sufficient amount of water causes a irreversible change of semi-crystalline structure to amorphous structure, which is also known as gelatinization (Atwell et al 1988). Heating starch granules in water below the gelatinization temperature causes the granules to swell, and the swelling process is reversible. The gelatinization process is associated with the dissociation of the double helices in the starch granules. The gelatinization properties (gelatinization temperature and enthalpy change of gelatinization) depend on the structure of the amylopectin, the amylose content, and the phosphate-monoester derivatives of the starches (Srichuwong and Jane 2007). The amylopectin with larger population of short branch-chains (DP 6-12) shows a lower gelatinization temperature than does that with smaller amount of short branch-chains. This is attributed to that the short branch-chains do not span through one complete crystalline lamellae and cause crystalline defects in the starch granules (Jane et al 1997, Jane et al 1999). The amylose is present in amorphous form in the starch granule reducing its crystallinity and, consequently, reducing the enthalpy change of starch gelatinization. High-amylose maize starch has a gelatinization temperature above 100°C because of its long branch chains of amylopectin (Li et al 2008). Starch with high proportion of the phosphate-monoester derivatives on its amylopectin also had a low gelatinization temperature because of the repulsion force between the negative charge of the phosphate-monoester derivatives (Jane et al 1999).

Gelatinized, amorphous starch molecules tend to recrystallize in a double helical structure during storage, which is also known as starch retrogradation. The rate of starch retrogradation depends on the amylose content, the amylopectin structure, the lipid content, the storage temperature, and the moisture content of starch paste. Amylose retrogrades faster
than amylopectin because of the linear structure and the high mobility of the amylose molecules (Sievert and Wusch 1993). The dissociation temperature of the retrograded amylose is above 120°C (Sievert and Pomeranz 1989, 1990, Gruchala and Pomeranz 1993, Klucinec and Thompson 1999). The retrogradation of the amylopectin molecules requires several days of storage because of its short branch chains. The short branch-chains (DP 6-12) of the amylopectin retrograde to a lesser extent than the longer branch chains (Jane et al 1999). The very long branch-chains of the amylopectin have properties similar to amylose, which increase the rate of retrogradation. The presence of lipids and phospholipids restricts the swelling of starch granules and the dispersion of starch molecules during cooking, which keeps the starch molecules in close proximity for retrogradation (Jane et al 1999). The presence of lipids also forms complex with amylose and prevents amylose from retrogradation (Slade and Levine 1987). The repulsive force of charged moieties on starch molecules, such as phosphate-monoester derivates in tuber and root starch, can reduce the tendency for starch molecules to retrograde (Thygesen et al 2003). Introduction of short chains on starch molecules through chemical modification, such as hydroxypropylation, creates steric hindrance for starch retrogradation (Perera and Hoover 1999).

The rate of retrogradation can be modified by changing the storage temperature for nucleation, propagation, and maturation of starch crystallites (Slade and Levine 1987). The nuclei of the starch crystallites form more rapidly at a lower temperature (close to the glass transition temperature), whereas the propagation of the starch crystallites is greater at a higher temperature (close to the melting temperature). Water molecules are required as plasticizers to lower the glass transition temperature of the starch molecules and to increase the mobility of starch molecules (Zeleznak and Hoseney 1987, Jang and Pyun 1997, Liu and
Thompson 1998). The maximum degree of starch retrogradation is achieved at an intermediate moisture content (50-60%).

**Enzyme digestion of starch**

Enzyme hydrolysis of starch molecules to produce glucose is important to provide energy for plant metabolism, food, feed, and ethanol production. The starch granules are hydrolyzed at a slower rate by enzymes than the gelatinized, amorphous starch molecules. The susceptibility of the starch granules depends on the granular size, the polymorphism, the structure of the amylopectin, the amylose content, the lipid content, and the reaction pattern of the enzyme. In general, the larger starch granules are normally digested at a slower rate than the smaller starch granules because the larger granules have a smaller relative surface space for enzyme hydrolysis (Tester et al 2004). The A-type polymorphic starch granules, such as waxy amaranth starch and waxy rice starch, is more easily hydrolyzed by enzyme than the B- and some C-type polymorphic starch granules, such as potato starch, green banana starch, and high-amylose maize starch (Figure 4) (Jane et al 2003). This is attributed to that the branching points of the amylopectin in the A-type polymorphic starch are scattered in both amorphous and crystalline regions, creating weak points in the crystalline regions for enzyme hydrolysis, whereas those of the B-type counterparts are mostly located at the amorphous region, resulting in a more perfect crystalline structure (Jane et al 1997). The internal structure of the A-type polymorphic starch granules also contains more voids than that of the B-type counterparts, making it more accessible for enzyme hydrolysis (Figure 3) (Jane 2006, Jane 2007). In addition, the A-type starch granules show more pinholes on the
surface than the B-type starch granules (Fannon et al 1992), which are the result of the hydrolysis by endogenous amylase (Jane et al 2003, Li et al 2007).

The amylose content is known to be negatively correlated with the starch susceptibility to amylase hydrolysis (Knutson et al 1982, Dombrink-Kurtzman and Knutson 1997, Jane et al 2003, Jane 2006). Waxy starch granules (0-8% amylose) are more susceptible to enzyme hydrolysis than normal starch granules (~25% amylose), and the normal starch granules are more susceptible to enzyme hydrolysis than high-amylose (amylose-extender) starch granules (>50% amylose) (Fuwa et al 1977, Jane et al 2003). The starch granules in the maize floury endosperm also contain less amylose than those in the maize vitreous endosperm, thus the starch granules in the floury endosperm are more susceptible to endogenous amylases and show more pinholes on the granule surface (Dombrink-Kurtzman and Knutson 1997, Jane et al 2003). Some legume starches, such as mung bean and wrinkled pea starches, show low enzyme susceptibilities because of their high amylose contents (Banks et al 1974, Fleming and Vose 1979).

Beside the waxy maize mutant, the starch granules of brittle-1, brittle-2, shrunken-2, sugary-1, sugary-2, and opaque-2 maize mutants show greater susceptibilities to amylase hydrolysis than does the starch granules of the normal maize (Fuwa et al 1977, Perera et al 2001). This can be attributed to the alteration of the enzymes used in the starch biosynthesis upon the mutation on the maize kernel. The sugary-1 mutation produces a α-glucan with a greater number of branches (phytoglycogen) than the amylopectin in the normal maize (Wong et al 2003). The branch-chains of the phytoglycogen or sugary-amyllopectin of the sugary-1 starch also consist of a large proportion of DP <12. The sugary-2 maize starch granules contain greater amorphous regions and more voids than the normal maize, thus the
sugary-2 maize starch granules are easier to be hydrolyzed by amylase (Perera et al 2001, Jane 2006). The activity of soluble starch synthase and granular-bound starch synthase are lower in the opaque-2 maize than in the normal maize, which could results in a lower total starch and amylose contents (Joshi et al 1980).

The hydrolysis of α-amylases on the starch chains depends on their action patterns, which include multichain (random) attack and multiple-attack (Figure 5) (Robyt and French 1967, Atichokudomchai et al 2006). For example, heat-stable α-amylase from Pseudomonas fluorescens has a greater degree of multichain attack action pattern and produces dextrins with a narrower molecular-weight distribution than heat-stable α-amylase from Bacillus licheniformis (Atichokudomchai et al 2006). Porcine pancreatic α-amylase at optimal conditions of pH and temperature has a greater degree of multiple attack action pattern and produces dextrins with a greater affinity to bind with iodine than human salivary and Aspergillus oryzae α-amylases (Robyt and French 1967). The sizes of binding sites of α-amylases also vary; for example, pancreatic and salivary α-amylases have 5 glucose-unit binding site, whereas α-amylase from Bacillus subtilis has 9 glucose-unit binding site (Jane and Robyt 1984). The size of the binding site determines the size of the smallest dextrin that can be hydrolyzed by the α-amylase. In addition, some α-amylases and glucoamylases contain one non-catalytic starch-binding domain, which binds to starch granules and disentangles the double helical structure on the surface of the starch granules (Chen et al 1995, Southall et al 1999). The starch-binding domain facilitates the enzyme hydrolysis of the starch granules, but does not show significant effect on the enzyme hydrolysis on gelatinized starch.
**Resistant starch**

In the early days, starch was considered to be completely digested and absorbed in the small intestine. Later, it was found that a fraction of starch (resistant starch or RS) survived the enzyme digestion in the digestive track (Englyst and Cummings 1985, Björck et al 1986, Faisant et al 2007). RS is not absorbed in the small intestine, and is passed onto the colon, where it is fermented by the gut microflora. The fermentation of RS in the colon produces short-chain fatty acids and other organic acids (Englyst and Macfarlane 1986, Wyatt and Horn 1988, Phillips et al 1995, Jenkins et al 1998, Ferguson et al 2000), and releases hydrogen through exhalation (Anderson et al 1981). Examples of RS include raw potato starch, raw high-amylose maize starch, raw green banana starch, and retrograded amylose in bread (Englyst and Cummings 1985, Jane 2006, Jane 2007).

RS has demonstrated similar physiological benefits as dietary fibers (Asp et al 1987), and it has been proposed that RS should be included in the definition of dietary fibers (Asp 1996, DeVries et al 1999, Goodlad and Englyst 2001). Consumption of RS has shown to reduce glucose absorption and insulin secretion, which prevents insulin resistance and metabolic syndrome, including obesity, diabetes, and heart disease (DeFronzo and Ferrannini 1991, Jenkins et al 1998, Ludwig 2002). Butyrate, a product of RS fermentation in the colon can prevent the development of cancerous cell in the colon (Jenkins et al 1998, Ferguson and Harris 2000). Furthermore, the amount of RS that reaches the colon is larger than that of other dietary fibers, thus it is more important as a substrate for colonic fermentation (Cummings and Englyst 1987).

RS has been classified into four types according to the nature of the enzyme resistance and the structure of the starch (Englyst et al 1992, Eerlingen and Delcour 1995). RS type 1
(RS1) is physically inaccessible starch, which is protected by a protein matrix or cell wall material, such as that in whole grains, legumes, and pasta. RS type 2 (RS2) is native, uncooked semi-crystalline granular starch that displays the B- and some C-type polymorphs, such as uncooked potato starch, green banana starch, and high-amylose maize starch. RS type 3 (RS3) is retrograded amylose formed in cooked starchy food. RS type 4 (RS4) is chemically modified or cross-linked starch, which is less accessible for enzyme hydrolysis. In addition to the four types of RS, resistant dextrins (Brimhall 1944, Thompson and Wolfrom 1958, Ohkuma and Wakabayashi 2001, Wang et al 2001) and amylose-lipid complexes (Jane and Robyt 1984, Cui and Oates 1999, Gelders et al 2005) are also included in RS because they are resistant to enzyme hydrolysis.

RS1: Physically inaccessible starch

RS1 is entrapped in the protein matrix or cell wall material and is physically inaccessible for enzyme hydrolysis. For example, starch granules in the vitreous endosperm of cereal grains are embedded in a dense protein matrix (Figure 6). The protein matrix is relatively water-impermeable, which retards starch swelling during cooking and acts as a barrier for amylase hydrolysis (O'Dea et al 1980, Rooney and Pflugfelder 1986, Correa et al 2002). Grinding of raw grains or seeds increases the susceptibility of starch to enzyme hydrolysis because the starch granules are free from the protein matrix (O'Dea et al 1980, Jenkins et al 1988). RS1, however, is not observed in the floury endosperm of cereal grains because the protein matrix is weak and discontinuous (Figure 6) and the starch is more accessible for enzyme hydrolysis.
Starch granules in pasta are also embedded in protein matrix, which is attributed to the greater protein content of durum wheat than that of other wheat species. The protein matrix forms a barrier and prevents the diffusion of water into the center of the pasta (Cunin et al 1995). As the result, the starch granules in the center of the pasta are not fully gelatinized during boiling and are less susceptible to amylase hydrolysis than the starch in white bread (Figure 7A) (Granfeldt et al 1991, Barkeling et al 1995). The digestibility of starch in pasta increases when protein is removed using proteases (Fardet et al 1998, Zhang and Hamaker 1998).

The RS1 in legume seeds are surrounded by cell wall (Figure 8A). The rigid cell wall retards the penetration of water into the legume seeds, and thus prevents the starch granules from getting enough water to swell and to gelatinize during cooking (Figure 8B) (Kon et al 1971, Wursch et al 1986). Thus, the starch granules in the cooked whole bean are not completely gelatinized and less digestible than those in the cooked ground bean.

Although RS1 showed benefits for human health, its presence in the cereal grains is not desirable as feed for small animals such as young chicks. The entrapped starch is not available to provide energy for the growth and development of the animal. It is also not desirable in the ethanol production because it reduces the final ethanol yield. Heat processing has been used to reduce the RS1 content in the grains through protein denaturation and starch gelatinization (Nnanna and Phillips 1990). Milling of the grains increases starch digestibility by reducing the particle size, disrupting the protein matrix, and releasing the starch granules (O'Dea et al 1980, Weurding et al 2001). Other treatments, such as protease incubation and steam flaking, can also weaken the protein matrix (Zhang and Hamaker 1998, Zinn et al 2002). Endogenous proteases and amylases in the grains can be...
activated through germination, which increases the hydrolysis of both protein and starch (Nnanna and Phillips 1990, Kataria et al 1992).

**RS2: Native B-type semi-crystalline starch**

Isolated uncooked starch granules with the B- and some C-type polymorphs (RS2), such as uncooked potato starch, green banana starch, and high-amylose maize starch, are resistant to enzyme hydrolysis (Figure 4) (Jane et al 2003). This is attributed to the homogenous internal structure of the B-type polymorphic starch granules (Figure 3) (Jane 2006, Jane 2007). The branch chains of the B-type polymorphic amylopectin are also longer than those of the A-type counterpart and the branching points of the B-type polymorphic amylopectin are mostly located at the amorphous regions, which results in a more perfect crystalline structure (Figure 1) (Jane et al 1997).

The RS2, in general, loses its enzyme resistance after heat processing. Starch gelatinization during heat processing converts semi-crystalline structure (RS2) to highly digestible, amorphous structure (Knutson et al 1982). The high-amylose maize starch with an amylose content above 50%, however, has high gelatinization temperature above 100°C (Li et al 2008). This is attributed to that the *amylose-extender* mutation increases the contents of amylose and long branch-chain of amylopectin (Jane et al 1999). Thus a fraction of the native crystalline structure (RS2) remained in the high-amylose maize starch after heating at the water-boiling temperature (Li et al 2008).

The heat stability and the enzyme resistance of the granular starch (RS2) can be enhanced using mild acid hydrolysis and hydrothermal treatments (Brumovsky and Thompson 2001). Acid hydrolysis of the amorphous regions in the starch granules results in
a higher percentage of crystalline regions. Hydrothermal treatments allow the starch crystallites to rearrange to a more perfect crystalline structure. The increase in the crystallinity and the perfection of the starch crystallites results in an increase in the RS content of the granular starch.

**RS3: Retrograded amylose**

Gelatinized and amorphous starch molecules tend to retrograde into a double helical structure during storage. The orderly aligned crystallites of the retrograded amylose (RS3) are enzyme resistant and have a melting temperature above 120°C, thus they remain in food products after heat processing (Sievert and Pomeranz 1989, 1990, Gruchala and Pomeranz 1993, Klucinec and Thompson 1999). Starch with large amounts of amylose and/or long branch chains of amylopectin, such as legume and high-amylose cereal starches, has been used to produce RS3 (Sievert and Pomeranz 1989, 1990, Vasanthan and Bhaty 1998). The long-branch chains of amylopectin have properties similar to amylose, which increase the apparent amylose content of the starch. On the contrary, the short-branch chains of amylopectin form double helices that are not long enough to produce stable crystallites.

Extrusion cooking has been used recently to produce RS3 from flours and starches (Vasanthan et al 2002, Faraj et al 2004, Agustiniano-Osornio et al 2005). Extrusion is commonly used in food industry to make breakfast cereals, snack foods, and many other similar food products. The RS content of the extruded foods depends on the screw speed, extrusion temperature, and moisture content. The optimum RS3 content is achieved when the starch is cooked by extrusion at low screw speed (higher residence time), which allows
the amylose chains to retrograde (Agustiniano-Osornio et al. 2005). The fresh extrudate, however, usually contains RS3 below 10% because it is highly amorphous.

The RS3 content in starch can be optimized by altering the conditions for nucleation and propagation of amylose retrogradation (Slade and Levine 1987, Jang and Pyun 1997). Repeated cycles of autoclaving and cooling of starch have been used to optimize the nucleation and propagation of retrograded amylose crystallites (RS3) (Sievert and Pomeranz 1989).

Enzyme debranching and mild acid hydrolysis of starch can increase the linear chains for greater amylose retrogradation (Vasanthan and Bhatt 1998, Guraya et al. 2001). Debranching enzyme, such as isoamylase and pullulanase, hydrolyzes α-D (1→6) glycosidic branching of amylopectin and produces linear molecules. Acid hydrolyzes the amorphous regions of the starch granules, where branching points of amylopectin are mostly located, producing linear chains.

RS4: Chemically modified starch

Esterification of starch using acetic anhydride, propylene oxide, or octenyl succinic anhydride has been used to increase the resistance of starch granules to enzyme hydrolysis (RS4) (Chung et al. 2008b, He et al. 2008). The hydrophobic moieties, such as acetyl group, hydroxypropyl group, and octenyl group, increase the water insolubility of the starch granules and decrease the accessibility of enzyme to hydrolyze starch molecules. The hydrophobic moieties also prevent the starch molecules from fitting to the enzyme binding site and being hydrolyzed.
Crosslinking of starch granules using sodium trimetaphosphate (Woo and Seib 2002, Sang and Seib 2006), phosphorus oxychloride (Han and BeMiller 2007), or citric acid (Unlu and Faller 1998, Xie and Liu 2004) also increases the RS4 content of starch. Crosslinked starch molecules have reduced flexibility to fit into the enzyme binding site (Shin et al 2004). In addition, crosslinking holds the starch molecules together because it limits the swelling of starch granules and prevents the dispersion of starch molecules during cooking (Shin et al 2003).

**Resistant dextrins**

Resistant dextrins have linkages that are not hydrolyzed by amylases, including \( \alpha-D (1 \rightarrow 2) \), \( \beta-D (1 \rightarrow 6) \), and \( \beta-D (1 \rightarrow 2) \) linkages. These unique linkages in the resistant dextrins are produced by heating starch granules in a dry condition at a high temperature up to 200°C (pyrolysis) either alone or in the presence of a small amount of acid catalyst (Brimhall 1944, Thompson and Wolfrom 1958, Wang et al 2001). Starch is hydrolyzed at the beginning of the reaction until most of the moisture is consumed. As water molecules become scarce, the reverse reactions take place, which are transglycosylation and repolymerization of the starch. The resulting products are randomly-branched molecules, which have a low ability to retrograde and a great water solubility. A commercial resistant dextrins have been produced by pyrolysis with subsequent controlled amylase treatment (Ohkuma and Wakabayashi 2001). These highly branched, low molecular-weight resistant dextrins are highly water soluble and produce low viscosity in solution, which are highly suitable for uses in beverage products.
**Amylose-lipid complexes**

The linear molecules of amylose spontaneously form single helical complex with lipids, such as diglycerides, monoglycerides, phospholipids, fatty acids, and other molecules with hydrophobic moieties. The hydrocarbon chain of the lipid occupies the central cavity of the helix through hydrophobic interaction with the amylose molecules (French and Murphy 1977). The amylose-lipid complex shows a V-type polymorphism. The number of glucose units per helical turn depends on the size of the complexing compound. When amylose is complexed with butyl alcohol, tert-butyl alcohol, and 1-haphthol, the number of glucose units per turn is six, seven, and eight, respectively (Jane and Robyt 1984).

The melting temperature of amylose-lipid complex also increases with the length of fatty acid chains, but it decreases with the degree of unsaturation of fatty acid chains (Raphaelides and Karkalas 1988, Tufvesson et al 2003b, a). Longer fatty-acids form a stronger hydrophobic interaction with amylose than do the shorter fatty-acids. Unsaturated fatty acids have bent structure, and thus they form less perfect complex with amylose. Furthermore, the complexes of unsaturated fatty acids with cis configuration have lower melting point than those of unsaturated fatty acids with trans configuration (Raphaelides and Karkalas 1988). This is attributed to that the cis double-bonds give rise to a kink structure, whereas the trans double-bonds have an almost linear structure.

The amylose-lipid complex also exists in two forms (Biliaderis and Galloway 1989, Biliaderis and Seneviratne 1990, Tufvesson et al 2003a). Amorphous amylose-lipid complex (form I) has lower dissociation temperature (< 100°C) than the crystalline complex (form II) (> 100°C). The amorphous complex can be converted to the crystalline complex by
incubating at temperatures above the dissociation temperature of the amorphous complex, and below the dissociation temperature of the crystalline complex.

Amylose-lipid complex is resistant to amylase hydrolysis (Jane and Robyt 1984). The amylose-lipid complex also entangles with amylopectin molecules in the starch granules, which restricts the starch swelling during heating in excess water and reduces the enzyme accessibility to hydrolyze starch molecules (Morrison et al 1993, Cui and Oates 1999). Furthermore, the crystalline amylose-lipid complex (form II) is more resistant to amylase hydrolysis than the amorphous amylose-lipid complex (form I) (Seneviratne and Biliaderis 1991). The collapsed helical conformation of starch prevents its binding to amylases (Figure 9) (Jane and Robyt 1984, Kitahara et al 1996, Gelders et al 2005).

Health benefits of resistant starch

*Reduction of glycemic index and prevention of hyperinsulinemia*

Glycemic index (GI) is originally developed for diabetic patients to avoid highly digestible starchy foods that cause a rapid increase in postprandial blood glucose response (Jenkins et al 1981, Jenkins et al 2002). The GI of a food is defined as the percentage of the total area under the curve (AUC) of the postprandial blood glucose response within 2 hours after the ingestion of a defined amount of a sample food (usually 50 g carbohydrates) compared with the AUC after the ingestion of a reference food with the same amount of carbohydrates. The reference food is usually glucose solution or white bread. White wheat bread, cooked-potato food, and breakfast cereals usually have a high GI because the carbohydrates are easily digested and absorbed in the small intestine (Figure 7A) (Björck et al 2007). RS (RS1-4, resistant dextrins, and amylose lipid complex) is slowly or not
digestible and dilutes the amount of the digestible carbohydrates in food, and thus show a low GI (Figure 7A) (Douglass 1975, Horwitz and Slowie 1975, Jenkins et al 1998, Ohkuma and Wakabayashi 2001, Chung et al 2008a, Chung et al 2008b).

For the general public, a low-GI food is also perceived as a healthy food choice. Ingesting a high-GI food increases the blood glucose concentration rapidly to above physiological range (a hyperglycemic state). Severe hyperglycemia is usually associated with excessive thirst (polydipsia), excessive passage of urine (polyuria), and weight loss (Nathan et al 2006). At the hyperglycemic state, a large amount of insulin is secreted by the pancreatic beta-cells (hyperinsulinemia) to increase glucose uptake from the blood stream to the muscle and the liver, and thus to lower the blood glucose level to the normal range (Figures 7A and B) (O'Dea et al 1980, Jenkins et al 1988, Ludwig et al 1999). A hypoglycemic state may follow the hyperglycemic state when the persistent high level of blood insulin maintained and reduced the blood glucose levels to below the physiological range. Hypoglycemia is associated with symptoms, such as tremulousness, palpitations, tachycardia, sweating, hunger, anxiety, nausea, drowsiness, dizziness, blurred vision, etc (Weinger et al 1995, Cryer 1999). Severe hypoglycemia may lead to seizure, coma, and death. The hypoglycemia-induced hunger also increases the consumption of more high-GI foods and repeats the hyper- and hypo-glycemic cycle. Severe episodes of hyper- and hypoglycemia are common acute complications in patients of insulin-dependent diabetes mellitus (Cryer et al 1989, Polonsky et al 1992, Weinger et al 1995). The incident of hyper- and hypo-glycemia cycle can be avoided by ingesting low-GI foods containing RS.
Prevention of insulin resistance and metabolic syndrome

Repeated incidents of hyperinsulinemia from frequent consumption of high-GI foods may also reduce the sensitivity of the body to respond to the action of insulin (insulin resistance) (Byrnes et al 1995). As the result, the pancreatic beta-cells augment the secretion of insulin to overcome the insulin resistance, which, in turn, repeats the state of hyperinsulinemia. The cycle of insulin resistance and hyperinsulinemia has been associated with metabolic syndrome, including non-insulin-dependent diabetes, obesity, hypertension, lipid abnormalities, and atherosclerotic cardiovascular disease (DeFronzo and Ferrannini 1991, Ludwig 2002). Hence low-GI foods containing RS can be used to improve the insulin sensitivity and to prevent the metabolic syndrome (Higgins 2004).

RS can also be used to intervene obesity by reducing the total energy intake, which leads to less fat deposited in the adipose tissue (de Deckere et al 1995, Ranhotra et al 1996, Ludwig et al 1999). Because RS is not readily available for energy production, the body utilizes fat in the adipose tissue for energy. In addition, high-RS foods prevent the hypoglycemia-induced hunger and promote satiety as they stay longer in the digestive system, thus they reduce the total food intake (Raben et al 1994, Brand-Miller et al 2002).

Decreases in the serum triglyceride and cholesterol concentrations are also observed with the ingestion of high-RS diets, which can be used to reduce the risk of cardiovascular disease (de Deckere et al 1993, Mathe et al 1993, Ranhotra et al 1997, Lerer-Metzger et al 2007). The hyperinsulinemia-induced hypoglycemia suppresses glucose oxidation and increases the release of free fatty acids to the blood stream similar to a fasting state; this can be prevented by consuming high-RS foods (Felber et al 1987). The reduction in the insulin secretion with the consumption of high-RS foods also decreases the triacylglycerol and cholesterol
syntheses in the liver. In addition, RS lowers serum cholesterol by promoting the excretion of fecal bile acids through the complex formation with amylose (Abadie et al 1994, Levrat et al 1996).

Colon microflora fermentation and prevention of colon cancer


The increase in the production of short-chain fatty acids in the colon can be attributed to that the fermentation of RS alters of the microbial population in the colon. An increase in the numbers of Lactobacillus and Bifidobacterium species and a decrease in Enterobacterium species were observed with ingestion of RS (Kleessen et al 1997, Silvi et al 1999). In addition, the RS3 shows a greater increase in the numbers of Lactobacillus and Streptococcus in the cecum than does the RS2, thus more propionate is produced in the colon with the ingestion of the RS3 (Kleessen et al 1997). Similarly, raw potato starch produces more butyrate than the raw high-amylose maize starch, which could be attributed to the difference in starch structure and amount of fermentable starch (Ferguson et al 2000). An increase in the fecal Bifidobacterium populations was reported when human subjects were supplemented with resistant dextrins (Fastinger et al 2008). RS also plays a protective role in the colon by
creating an environment that suppresses the growth of pathogenic organisms (Kleessen et al 1997, Silvi et al 1999).

In addition, RS prevents colon carcinogenesis by diluting and promoting excretion of potential carcinogens. Fecal bulk increased by RS reduces the contact between the carcinogens and the colonocytes, hence its reduces the absorption of the carcinogens (Phillips et al 1995, Hylla et al 1998, Jenkins et al 1998). RS also promotes the secretion of potential carcinogens out of the body through the complex formation with amylose (Abadie et al 1994).

**Literature cited**


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Fig. 1. Proposed models of branching patterns in starches with (a) A-type and (b) B-type polymorphic crystalline structure (Jane et al 1997). A indicates amorphous regions and C indicates crystalline regions.
Fig. 2. Crystalline packing of double helices in starches with (A) A-type and (B) B-type polymorphic crystalline structure (Buléon et al 1998).
Fig. 3. Internal structures of (A) sugary-2 maize starch, (B) waxy maize starch, (C) normal maize starch, (D) potato starch, (E) high-amylose maize VII starch, and (F) banana starch studied using confocal laser-light scanning microscopy (Jane 2006).
Fig. 4. Enzyme digestibility of native starch granules with different crystalline polymorphisms indicated by letters above the bars (Jane et al. 2003).
Fig. 5. Proposed action patterns of α-amylases (Robyt and French 1967).
Fig. 6. Scanning electron micrographs of maize vitreous and floury endosperms. 2-mercaptoethanol treatment is used to remove protein bodies, and pancreatic α-amylase and amyloglucosidase treatment is used to remove starch granules. Bars indicate 10 μm.
Fig. 7. Mean incremental (A) blood glucose responses and (B) insulin responses after ingestion of (—) bread, (---) spaghetti, (---) thin linguine, (---) thin linguine with egg, and (….) thick linguine (Granfeldt et al 1991).
Fig. 8. Optical micrographs of (A) raw white kidney bean starch granules in the cells (phase contrast), and (B) gelatinized white kidney bean starch trapped in cooked cells (Wursch et al 1986).
Fig. 9. Proposed crystalline-lamellar structure of amylose-V complex (Jane and Robyt 1984). Arrows indicated the weak points of the lamella for amylase hydrolysis.
CHAPTER 1. KERNEL COMPOSITION, STARCH STRUCTURE, AND ENZYME DIGESTIBILITY OF opaque-2 MAIZE AND QUALITY PROTEIN MAIZE

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ABSTRACT

Objectives of this study were to understand how opaque-2 (o2) mutation and quality protein maize (QPM) affect maize kernel composition and starch structure, property, and enzyme digestibility. Kernels of o2 maize contained less protein (9.6-12.5%) than those of the wild-type (WT) counterparts (12.7-13.3%). Kernels of a severe o2-mutant B46o2 also contained less starch (66.9%) than those of B46wt (73.0%). B46o2 and QPM starches contained less amylose (28.0% and 26.0%, respectively) than others (31.9% - 33.7%). The B46o2 starch also consisted of amylopectin with fewest branch chains of DP 13-24. Thus the B46o2 starch was the most susceptible to porcine pancreatic α-amylase (PPA) hydrolysis. Starches of the dry-ground o2 maize and QPM were hydrolyzed faster than that of the dry-ground WT maize, resulting from the reduced protein content of the o2-maize kernels and the reduced
amylose content of the B46o2 and QPM starch. Starch in the dry-ground maize sample was hydrolyzed faster by PPA (85-91%) than was the isolated starch (62-71%), which could be attributed to the presence of mechanically damaged starch granules and endogenous amylases in the dry-ground maize samples. These results showed that o2 maize and QPM had highly digestible starch and could be desirable for feed and ethanol production.

**KEYWORDS:** opaque-2 maize, quality protein maize, dry-ground maize, starch digestibility, physicochemical properties

**INTRODUCTION**

Maize (*Zea mays* L.) is an important crop for food, feed, and fuel applications. Zein is the major protein of maize; however, it is deficient in essential amino acids, tryptophan and lysine. Lysine deficiency in humans and animals causes symptoms of growth impairment, anemia, hypoproteinemia, fatty liver, etc. (1, 2).

A mutant called opaque-2 (o2) contains an increased amount of lysine and has been used to improve the protein quality of maize (3). The o2-maize mutants are known to contain less α-, β-, γ-, and δ-zein, which results in increases in the contents of other proteins, including globulin and albumin (3-5). The lysine content of o2 maize can be up to twice of that of normal maize. Despite the improvement in the amino acid composition and nutrition value, most o2-maize lines are not well adapted because of their low grain yields, soft and chalky endosperm, and high susceptibility to kernel breakage and to pest and mold damages (6).

Quality protein maize (QPM) was developed using modifier genes to produce a vitreous endosperm while maintaining the nutritional advantages of o2 maize (6). The vitreous
endosperm, containing an elevated level of $\gamma$-zein, increases the resistance of $o2$ maize to diseases and insect damages, increases the grain yield, and improves grain appearance (7).

The physicochemical properties of starch of $o2$ maize and QPM, however, are not well understood. It has been reported that the activities of soluble starch synthase in $o2$ maize are terminated earlier than the normal counterpart, resulting in a decrease in starch accumulation (8). The activity of granular-bound starch synthase in $o2$ maize is also lower than that in the normal counterpart. Starch granules in the $o2$-maize endosperm are synthesized less vigorously than in the normal-maize counterpart. Thus they are packed more loosely, giving $o2$-maize kernels an opaque appearance (9). Starches of single- and double-mutant $o2$-maize kernels have been shown to be digested more rapidly by $\alpha$-amylases than that of the normal maize (10).

The objectives of this study were to understand how the $o2$ mutation and QPM affect the kernel composition, the starch physicochemical properties, and the starch digestibility. This information is needed for future breeding and selecting quality $o2$ maize and QPM for food, feed, and ethanol production. In this study, we analyzed kernel compositions, starch structures and properties, and starch digestibility of dry-ground wild-type (WT) maize, $o2$ maize, and QPM. The enzyme digestibility of starch in the dry-ground maize sample was also compared with that of the isolated starch obtained from wet milling of the whole kernels. Two sets of $o2$ maize inbred lines, B46o2 (severe phenotype) and M14o2 (mild phenotype), their near-isogenic WT counterparts, B46wt and M14wt, and a QPM sample were selected for this study. The set of near-isogenic lines in the B46 genetic background was selected because the B46o2 mutant exhibits an extreme phenotype, including the highest lysine content, the lowest kernel density, and the lowest warm germination percentage among eight
genetic lines studied by Jia et al. (11). The other set of near-isogenic lines in the M14 genetic background was selected for its mild phenotype among the eight genetic lines (11).

MATERIALS AND METHODS

Materials. Five maize inbred lines, B46wt, B46o2, M14wt, M14o2, and a germplasm source known to be a QPM inbred, used in this study were grown at the Iowa State University Agronomy Farm (Boone, IA) in 2004. A single ear of each genotype was produced by self-pollination. The ears were dried and shelled by hand. The dry-ground maize was prepared by grinding whole kernels using a Cyclone Mill (UDY corp., Fort Collins, CO) to pass through a 0.5-mm screen.

Porcine pancreatic \( \alpha \)-amylase (PPA), maltohexaose, and maltoheptaose were purchased from Sigma Chemical Co. (St. Louis, MO). Glucoamylase (GA) from Aspergillus niger, D-Glucose assay (GOPOD) kits, and Total Starch assay kits were purchased from Megazyme International Ireland Ltd. (Co. Wicklow, Ireland). Crystalline \textit{Pseudomonas} isoamylase was purchased from Hayashibara Shoji, Inc. (Okoyama, Japan), which had a specific activity of about 66,000 units/mg protein.

Starch Isolation. Starch was isolated using a wet-milling method (12). Samples of maize whole kernels and dry-ground maize (50 g) were steeped in a sodium metabisulfite solution (150 mL, 0.45% w/v) overnight in a refrigerator before blending. For the whole kernels, pericarps and germs were manually removed before the endosperms were blended. The starch isolated from the steeped endosperms was designated as “isolated starch”.

Kernel Composition. The nitrogen content of the dry-ground maize was determined using a macro-Kjeldahl method (13). The protein content was calculated by multiplying the
nitrogen content with 6.25. Lipids were extracted from the dry-ground maize using hexanes in a Soxhlet extractor for 24 h and quantified following AACC Method 30-25 (14). The starch content of the dry-ground maize was determined using Megazyme Total Starch assay kit following AACC Method 76-13 (14). The protein, lipid, and starch contents were determined in duplicate.

**Starch Granule Morphology.** The morphology of starch granules isolated from whole kernels (isolated starch) and that of starch granules isolated from the dry-ground maize were examined using a scanning electron microscope (SEM) (JEOL JSM-5800LV, Tokyo, Japan) at the Bessey Microscopy Facility, Iowa State University (Ames, IA), following the method of Jane et al. (15).

**Starch Digestibility.** Starch (100 mg, dry basis, db) or a dry-ground maize sample containing 100 mg of starch (db) was suspended in deionized water (5.0 mL) and equilibrated in a water bath at 37°C with agitation for 10 min. PPA (125 units) in a phosphate buffer solution (0.10 M, pH 6.9, 5.0 mL) containing calcium chloride (0.25 mM) and sodium azide (0.04% w/v) was added to the starch or the dry-ground maize suspension. The mixture was vortexed and incubated in the same water bath for 48 h. Aliquots (0.7 mL) were removed from the hydrolysate after 0, 3, 6, 24, and 48 h of incubation. The reaction was stopped by adjusting the pH to 4.0 using a HCl solution (1.0 M, 20 μL) and then adjusted to pH 6.0 using a NaOH solution (1.0 M). The aliquots were centrifuged at 6,600g for 15 min. The supernatants (0.10 mL) containing soluble dextrin were further digested at 50°C for 1 h with GA (0.25 units) in an acetate buffer solution (200 mM, pH 4.5, 0.90 mL) containing sodium azide (0.02% w/v). The glucose content of the supernatant after GA
hydrolysis was quantified using a D-Glucose assay (GOPOD) kits. The starch digestibility was determined in triplicate. The enzyme digestibility was calculated as:

\[
\text{Starch digestibility} = \frac{\text{amount of glucose produced}}{\text{initial dry weight of starch}} \times 0.9
\]

**Amylose Content of Starch.**

**Iodine Potentiometric Titration.** The amylose content of starch was determined on the basis of the iodine affinity of defatted starch. The analysis was done in triplicate using an automatic potentiometric titrator (702 SM Tirino, Metrohm, Herisau, Switzerland) (16). Starch was defatted using an aqueous methanol solution (85% v/v) in a Soxhlet extractor for 24 h.

**Gel Permeation Chromatography (GPC).** The molecular-weight distribution of starch molecules was analyzed using GPC (16), and the amylose content was calculated on the basis of molecular weight, that is, the second peak in the GPC profile. Starch (15 mg) was wetted with water (0.15 mL) and then mixed with dimethyl sulfoxide (1.35 mL). The starch dispersion was heated in a boiling water bath with stirring for 1 h and then stirred at room temperature for an additional 16 h. Starch was precipitated using 4 volumes of absolute ethanol, centrifuged, and redispersed in water (5 mL) in a boiling water bath with stirring for 30 min. The starch dispersion was filtered through a nylon membrane of 5.0-μm pore size and then injected into a GPC column (2.6 cm i.d. × 80 cm) packed with Sepharose CL-2B gel (Pharmacia, Inc., Piscataway, NJ). The column was eluted with an aqueous solution containing 25 mM sodium chloride and 1 mM sodium hydroxide in an ascending direction. Fractions (4.8 mL) of the eluent were collected. The total carbohydrate content (CHO) was determined using phenol and sulfuric acid (17). The blue value (BV) was obtained using
iodine/potassium iodide solution (18). The colors developed from CHO and BV analyses of each fraction were quantified using a microplate reader (ELx808, Bio-Tek Instruments, Inc., Winooski, VT) at 490 and 630 nm, respectively. The analysis was done in duplicate.

**Solubility and Swelling Power.** The solubility and the swelling power were analyzed by heating an aqueous suspension of starch (1% w/v, db, 5.0 mL) or dry-ground maize containing the same amount of starch in a shaker water bath at 80°C and 120 rpm for 40 min (19). The analysis was done in duplicate.

**Branch-Chain Length Distribution of Amylopectin.** Amylopectin was fractionated from amylose using n-butanol and was then debranched using isoamylase (20). The debranched chains were labeled with 8-amino-1,3,6-pyrenetrisulphonic acid (APTS), and the branch chain-length distribution was analyzed using capillary electrophoresis (P/ACE MDQ, Beckman Courter, Fullerton, CA) (21). Maltohexaose and maltoheptaose were used as reference standards. The analysis was done in duplicate.

**Starch crystallinity.** The X-ray diffraction pattern of the starch and the percentage of crystallinity were determined using a D-500 diffractometer (Siemens, Madison, WI) (22). 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.

**Thermal properties of starch.** The thermal properties of native isolated starch and retrograded starch were analyzed using a differential scanning calorimeter (DSC-7, Perkin-Elmer, Norwalk, CT) (16). The starch sample was heated from 10 to 110°C at a rate of 10°C/min. An empty pan was used as the reference pan, and indium was used as a reference standard. The gelatinized-starch sample was kept in a refrigerator at 4°C for 7 days and reanalyzed to characterize the retrograded starch. The analysis was done in triplicate.
Retrogradation (%) = \frac{\Delta H \text{ of the melting of retrograded starch}}{\Delta H \text{ of starch gelatinization}} \times 100\% \\

**Statistical Analysis.** Mean values of the kernel compositions 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**

**Kernel Composition.** Starch, protein, and lipid contents of kernels of the different maize lines are shown in Table 1. Both \( o2 \) mutants contained less protein than their WT counterparts. The severe \( o2 \) mutant B46\( o2 \) contained less starch than B46\( wt \). The apparent larger lipid content of the B46\( o2 \) maize kernels could be a result of the decreases in both protein and starch contents of the kernels.

**Starch Granule Morphology.** SEM images of starch granules isolated from dry-ground maize and isolated from whole kernels (isolated starch) by wet milling are shown in Figures 1 and 2, respectively. The starch granules isolated from the dry-ground WT maize and QPM (Figure 1A,C,E) showed more mechanical damage (debris and broken granules) than those isolated from the dry-ground \( o2 \) maize (Figure 1B,D). Hila of some starch granules isolated from the dry-ground WT maize and QPM were exposed to the surface. The exposed hila were indicated with arrows (Figure 1A,C,D,E).

Starch granules isolated by wet milling of steeped whole kernels (Figure 2), however, did not show mechanical damage as observed with those isolated from the dry-ground maize (Figure 1). The B46\( wt \) starch showed more polygonal-shaped granules (Figure 2A) than the
B46o2 starch (Figure 2B), and the B46o2 starch had more spherical granules. In contrast, the M14WT and the M14o2 starches showed similar starch granule morphology (Figure 2C,D, respectively), which was attributed to the mild mutation of M14o2. The QPM starch had some irregular-shaped granules, which were larger than starch granules of other maize lines (Figure 2E). Some WT starch granules showed dimplelike indentations (marked with asterisks), which were not observed in the o2 and the QPM starch granules. The B46o2 and the QPM starch granules showed significantly more pinholes (marked with arrows) than the other maize starch granules.

**Starch Digestibility.** Digestibility of starch in the dry-ground maize and the isolated starch using PPA is shown in Figure 3A,B, respectively. In the first 3 h of the PPA hydrolysis, the rates of starch digestibility of the dry-ground WT-maize samples were slightly higher than those of the dry-ground o2-maize and QPM samples (Figure 3A, inset). After 6 h of hydrolysis, the dry-ground o2-maize and QPM samples displayed substantially greater starch hydrolysis than did the dry-ground WT-maize samples (Figure 3A).

For enzyme hydrolysis of isolated-starch samples, the severe o2 mutant B46o2 starch showed the greatest rate of hydrolysis (Figure 3B). Other isolated-starch samples showed similar digestibility rates. At the end of 48-h PPA hydrolysis, the mild o2 mutant M14o2 starch showed the second largest hydrolysis. All dry-ground maize samples displayed greater starch digestibility than the isolated-starch samples.

**Amylose Content of Starch.** Amylose contents of the isolated-starch samples determined using iodine potentiometric titration and using GPC are shown in Table 2. GPC profiles of the isolated-starch samples are shown in Figure 4. The first peak of the GPC profile was amylopectin, and the second peak was amylose, which developed darker blue
color when stained with iodine. The amylose contents determined using GPC were similar to those determined using iodine potentiometric titration. The B46o2 and the QPM starches had the lowest amylose contents.

**Solubility and Swelling Power.** The solubility and the swelling power of the dry-ground and the isolated-starch samples are shown in Table 3. The dry-ground o2 had greater solubility and swelling power than the dry-ground WT-maize and QPM samples. For isolated-starch samples, the B46o2 and the QPM starches had the lowest solubility and swelling power.

**Branch-Chain Length of Amylopectin.** The branch-chain length distributions of debranched amylopectin are shown in Figure 5 and are summarized in Table 4. The percentages of branch chains of DP 6-12 were similar for all five amylopectin samples. The B46wt and B46o2 amylopectin exhibited the least percentages of branch chains of DP 13-24, whereas the M14wt and M14o2 amylopectin exhibited the largest. The average DPs of amylopectins of all maize lines were around 21.

**Starch Crystallinity.** All starches showed the A-type X-ray diffraction pattern (Figure 6). The degrees of crystallinity were similar for all of the isolated-starch samples.

**Thermal Properties of Starch.** Thermal properties of the isolated-starch samples are shown in Table 5. The B46wt and B46o2 starches had slightly lower gelatinization temperatures and melting temperatures of retrograded starch than the M14wt and M14o2 starches.
DISCUSSION

Kernels of B46o2 and M14o2 contained less protein than their WT counterparts (Table 1), which could be attributed to the suppression of zein biosynthesis in the o2-maize mutants. The solubility and the swelling power of the dry-ground o2-maize samples were greater than those of the WT counterparts (Table 3), whereas the solubility and swelling power of the isolated o2 starches were smaller than those of their respective WT counterparts (Table 3). On the basis of these results, the greater solubility and swelling power of the dry-ground o2 maize were attributed to the larger albumin and globulin contents of the o2 mutants. Albumin and globulin are more water soluble and swell more than zein (23).

The surface of some WT starch granules had dimplelike indentations (Figure 2A,C), which were results of spherical-shaped protein bodies present between closely packed starch granules during kernel development (24). After the protein bodies were removed, the indentations remained on the surface of starch granules. The smooth surface of the o2 and QPM starch granules, without the dimplelike indentations (Figure 2B,D), suggested that either starch granules were not tightly packed in the kernel or protein bodies were absent in o2-maize kernels. The large concentrations of zein present in the protein matrices of the WT and QPM kernels functioned like cement and bound starch granules together tightly (25). During dry grinding, the protein matrices were very hard to break apart; thus, the starch granules yielded to the force of grinding and were broken, some with hila exposed (Figure 1A,C,E). This was similar to the broken starch granules observed in the dry-milled flour of hard wheat (25). The dry-ground o2-maize samples, however, contained fewer mechanically damaged starch granules (Figure 1B,D) than the dry-ground WT-maize and QPM samples.
These results suggested that the weak protein matrices in the o2 kernels were broken apart easily during dry grinding and resulted in less damage to starch granules.

The substantial reduction in the starch content of B46o2-maize kernels (Table 1) might result from early termination of the starch biosynthesis in the o2 mutant (8). The spherical granules of the isolated B46o2 starch (Figure 2B) resembled starch isolated from premature kernels during maize kernel development (26). The WT starches displayed more polygonal-shaped granules (Figure 2A,C), which were the results of starch granule deformation when they were vigorously growing and competing for limited space in the endosperm. The B46o2 starch contained less amylose than did the B46wt starch (Table 2), which also resembled premature maize starch (26). It is known that amylose is more actively synthesized at the later stage of starch biosynthesis (26-28) and the amylose content of starch is negatively correlated with starch susceptibility to amylase hydrolysis (27, 29). The large number of pinholes on the surface of the B46o2 starch granules (Figure 2B), resulting from endogenous amylase hydrolysis (26, 29), and the greater starch digestibility of the B46o2 maize (Figure 3A,B) could be attributed to its lower amylose content. The large numbers of pinholes on the surface of the B46o2 and QPM starch granules also resulted in low swelling power and solubility of the two starches (30) (Table 3).

The B46wt and B46o2 starches displayed slightly lower gelatinization temperatures than the M14wt and M14o2 starches (Table 5), which correlated to fewer branch chains of DP 13-24 in the amylopectins of B46wt and B46o2 starches (Table 4). It has been proposed that a decrease in branch chains of DP 13-24 causes defects in the amylopectin crystalline structure and results in a lower gelatinization temperature (31). The defects in the crystalline structure
of starch granules also increased the susceptibility of the B46o2 starch to PPA hydrolysis (Figure 3B).

The QPM starch granules contained the lowest amylose content (Table 2) and showed the largest number of pinholes on the granule surface (Figure 2E). The isolated QPM starch, however, displayed less PPA hydrolysis than the isolated B46o2 starch. This could be related to its large starch-granule size, which had smaller relative surface space for enzymes to hydrolyze (32). It is known that starch granules with less amylose contents and smaller granular sizes are more quickly digested (29).

The fact that the dry-ground WT-maize samples were more susceptible to PPA hydrolysis during the first 3 h of digestion (Figure 3A, inset) could be a consequence of the greater proportions of mechanically damaged starch granules in those samples (32). After 6 h of PPA hydrolysis, the starches of the dry-ground o2 maize, however, were hydrolyzed to greater extents than those of the WT counterparts. These observations were likely the results of highly digestible B46o2 starch (Figure 3B) and the lower protein contents of the o2 kernels (Table 1). Protein, surrounding starch granules, acts as a barrier for enzyme hydrolysis. The starch digestibility of the dry-ground QPM sample reached to a similar level as that of the dry-ground B46o2 sample after 48 h of PPA hydrolysis, which could relate to the low amylose content of the QPM starch (Table 2).

The percentage starch hydrolysis of the dry-ground maize (85%-91%) was larger than that of the isolated starch (62%-71%) after 48 h of enzyme hydrolysis using the same ratio of PPA to starch (Figure 3A,B, respectively). These could be attributed to the presence of mechanically damaged starch granules in the dry-ground maize samples (Figure 1), which were not observed in the isolated-starch samples (Figure 2). Furthermore, endogenous
amylases present in the dry-ground maize, which were removed from the isolated-starch samples, could also enhance the hydrolysis of the starch granules in the dry-ground maize (33).

In conclusion, mechanically damaged starch granules in the dry-ground WT maize were more susceptible to PPA hydrolysis at the first 3 h of digestion. After 6 h of digestion, starches in the dry-ground o2-maize and QPM samples were hydrolyzed faster than those in the dry-ground WT-maize samples because of reduced protein contents of the o2 mutants and more digestible B46o2 and QPM starches. The dry-ground maize also displayed larger starch digestibility than did the isolated starch, which was attributed to the presence of mechanically damaged starch granules and endogenous amylases in the dry-ground maize samples. The o2 and QPM starches in dry-ground maize and as isolated starch were more readily digested by enzymes to provide energy for animal growth and to produce glucose for alcohol fermentation. Thus, the o2 maize and QPM showed potential uses for feed and ethanol production.

LITERATURE CITED


Table 1. Kernel Compositions of WT, o2, and QPM Maize (db)\textsuperscript{a}

<table>
<thead>
<tr>
<th>lines</th>
<th>starch\textsuperscript{b} (%)</th>
<th>protein\textsuperscript{c} (%)</th>
<th>lipid\textsuperscript{d} (%)</th>
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</thead>
<tbody>
<tr>
<td>B46wt</td>
<td>73.0 ( \pm ) 0.4\textsuperscript{b}</td>
<td>13.3 ( \pm ) 0.0\textsuperscript{d}</td>
<td>4.3 ( \pm ) 0.2\textsuperscript{a}</td>
</tr>
<tr>
<td>B46o2</td>
<td>66.9 ( \pm ) 1.4\textsuperscript{a}</td>
<td>12.5 ( \pm ) 0.0\textsuperscript{bc}</td>
<td>5.1 ( \pm ) 0.1\textsuperscript{b}</td>
</tr>
<tr>
<td>M14wt</td>
<td>71.1 ( \pm ) 0.8\textsuperscript{b}</td>
<td>12.7 ( \pm ) 0.0\textsuperscript{c}</td>
<td>4.5 ( \pm ) 0.1\textsuperscript{a}</td>
</tr>
<tr>
<td>M14o2</td>
<td>74.1 ( \pm ) 0.8\textsuperscript{b}</td>
<td>9.6 ( \pm ) 0.0\textsuperscript{a}</td>
<td>4.2 ( \pm ) 0.2\textsuperscript{a}</td>
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<tr>
<td>QPM</td>
<td>72.4 ( \pm ) 1.3\textsuperscript{b}</td>
<td>12.1 ( \pm ) 0.3\textsuperscript{b}</td>
<td>4.2 ( \pm ) 0.1\textsuperscript{a}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Values with the same letter in the same column are not significantly different at \( p < 0.05 \).

\textsuperscript{b}Starch contents were determined using Megazyme Total Starch assay kit following AACC Method 76-13 (14).

\textsuperscript{c}Protein contents were determined using macro Kjeldhal method (13).

\textsuperscript{d}Lipid contents were determined using AACC Method 30-25 (14).
Table 2. Amylose Contents of Isolated WT, o2, and QPM Starches

<table>
<thead>
<tr>
<th>lines</th>
<th>amylose content (%)</th>
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<tr>
<td></td>
<td>iodine</td>
<td>GPC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>potentiometric</td>
<td>titration</td>
<td></td>
</tr>
<tr>
<td>B46wt</td>
<td>33.7 ± 0.8</td>
<td>32.5 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>B46o2</td>
<td>28.0 ± 2.7</td>
<td>28.9 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>M14wt</td>
<td>32.9 ± 1.8</td>
<td>31.8 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>M14o2</td>
<td>31.9 ± 0.2</td>
<td>31.0 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>QPM</td>
<td>26.0 ± 2.0</td>
<td>28.5 ± 0.8</td>
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</tr>
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Table 3. Solubility and Swelling Power of Dry-Ground WT, o2, and QPM Maize and the Isolated Starches from Whole Kernels by Wet Milling$^a$

<table>
<thead>
<tr>
<th>lines</th>
<th>dry-ground maize</th>
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<th>wet-milled starch</th>
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<tbody>
<tr>
<td></td>
<td>solubility (%)</td>
<td>swelling power (g/g)</td>
<td>solubility (%)</td>
<td>swelling power (g/g)</td>
</tr>
<tr>
<td>B46wt</td>
<td>10.3 ± 0.7b</td>
<td>13.4 ± 0.5ab</td>
<td>22.0 ± 1.5b</td>
<td>15.4 ± 0.1bc</td>
</tr>
<tr>
<td>B46o2</td>
<td>12.7 ± 0.2c</td>
<td>16.0 ± 0.7b</td>
<td>16.7 ± 0.5a</td>
<td>14.2 ± 0.2b</td>
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<td>M14wt</td>
<td>9.6 ± 0.5b</td>
<td>13.7 ± 1.0ab</td>
<td>20.6 ± 0.2ab</td>
<td>16.2 ± 0.2c</td>
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<tr>
<td>M14o2</td>
<td>14.2 ± 0.5c</td>
<td>16.7 ± 0.1b</td>
<td>20.2 ± 1.0ab</td>
<td>15.5 ± 0.5c</td>
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<tr>
<td>QPM</td>
<td>5.2 ± 0.2a</td>
<td>12.9 ± 0.7a</td>
<td>17.1 ± 1.6a</td>
<td>13.0 ± 0.4a</td>
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</table>

$^a$Values with the same letter in the same column are not significantly different at $p < 0.05$. 
Table 4. Branch-Chain Length Distribution of Amylopectins of WT, o2, and QPM Starches Analyzed Using Fluorophore-Assisted Capillary Electrophoresis

<table>
<thead>
<tr>
<th>lines</th>
<th>Branch-chain length distribution</th>
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<tr>
<td></td>
<td>DP 6-12 (%)</td>
<td>DP 13-24 (%)</td>
<td>DP 25-36 (%)</td>
<td>DP &gt; 36 (%)</td>
<td>average DP</td>
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<tr>
<td>B46wt</td>
<td>11.5 ± 0.0</td>
<td>41.7 ± 0.3</td>
<td>17.1 ± 0.6</td>
<td>29.7 ± 0.8</td>
<td>21.2 ± 0.1</td>
<td></td>
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<tr>
<td>B46o2</td>
<td>11.2 ± 0.1</td>
<td>41.3 ± 0.7</td>
<td>15.9 ± 0.5</td>
<td>31.6 ± 1.1</td>
<td>21.5 ± 0.2</td>
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<tr>
<td>M14wt</td>
<td>11.9 ± 0.3</td>
<td>43.2 ± 1.2</td>
<td>15.9 ± 0.1</td>
<td>29.0 ± 1.4</td>
<td>20.9 ± 0.3</td>
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<tr>
<td>M14o2</td>
<td>11.6 ± 0.3</td>
<td>43.0 ± 0.1</td>
<td>15.2 ± 1.2</td>
<td>30.2 ± 0.9</td>
<td>21.1 ± 0.1</td>
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<tr>
<td>QPM</td>
<td>11.4 ± 0.2</td>
<td>42.3 ± 0.6</td>
<td>16.1 ± 0.0</td>
<td>30.2 ± 0.7</td>
<td>21.2 ± 0.2</td>
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Table 5. Thermal Properties of Isolated WT, o2, and QPM Starches

<table>
<thead>
<tr>
<th>lines</th>
<th>starch gelatinization</th>
<th>melting of retrograded starch</th>
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<tr>
<td></td>
<td>$T_o^a$</td>
<td>$T_p$</td>
<td>$T_c$</td>
</tr>
<tr>
<td></td>
<td>(°C)</td>
<td>(°C)</td>
<td>(°C)</td>
</tr>
<tr>
<td>B46wt</td>
<td>65.9</td>
<td>70.3</td>
<td>75.0</td>
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<tr>
<td>B46o2</td>
<td>65.7</td>
<td>71.8</td>
<td>77.5</td>
</tr>
<tr>
<td>M14wt</td>
<td>65.9</td>
<td>72.6</td>
<td>78.5</td>
</tr>
<tr>
<td>M14o2</td>
<td>67.4</td>
<td>72.3</td>
<td>78.6</td>
</tr>
<tr>
<td>QPM</td>
<td>66.0</td>
<td>70.8</td>
<td>76.1</td>
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$^aT_o$ = onset temperature, $T_p$ = peak temperature, $T_c$ = conclusion temperature, and $\Delta H$ = enthalpy change.
Fig. 1. Scanning electron micrographs (1500× magnification) of starch granules isolated from dry-ground maize of (A) B46wt, (B) B46o2, (C) M14wt, (D) M14o2, and (E) QPM. Arrows mark broken starch granules with exposed hila.
Fig 2. Scanning electron micrographs (1500× magnification) of starch granules isolated by wet-milling from whole kernels of (A) B46wt, (B) B46o2, (C) M14wt, (D) M14o2, and (E) QPM. Numbers in parentheses are the percentages of granules with pinholes. Arrows mark starch granules with pinholes, and asterisks mark starch granules with dimplelike indentations.
Fig. 3. (A) Starch digestibilities of dry-ground WT, o2, and QPM maize and (B) that of isolated starches from whole kernels by wet milling. The inset in A shows a magnified plot of the first 4 h of starch digestibilities of the dry-ground maize samples. PPA was used for the digestibility studies.
Fig. 4. Molecular-weight distribution of isolated (A) B46wt, (B) B46o2, (C) M14wt, (D) M14o2, and (E) QPM starches using Sepharose-CL-2B gel permeation chromatography. 

CHO (○) and BV (●). The first peak is designated to amylopectin and the second peak is designated to amylose.
Fig. 5. Branch-chain length distributions of amylopectins of (A) B46wt, (B) B46o2, (C) M14wt, (D) M14o2, and (E) QPM starches using fluorophore-assisted capillary electrophoresis.
Fig. 6. X-ray diffraction of isolated WT, o2, and QPM starches. Numbers in parentheses are the percentage of crystallinity.
CHAPTER 2. NOVEL RESISTANT STARCH FROM HIGH-AMYLOSE
MAIZE STARCH VII – FATTY ACID COMPLEXES. PART 1.

PREPARATIONS

A paper to be submitted to Cereal Chemistry

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Department of Food Science and Human Nutrition, Iowa State University, Ames, IA 50011.

ABSTRACT

The objectives of this study were to produce a novel resistant starch by complexing high-
amylose maize starch VII (HA7) with fatty acid (FA) and to understand the mechanism of
amylose-lipid in increasing enzyme resistance of starch. The production of the HA7-FA
complex involved heating HA7 starch granules and debranching to enhance the formation of
the starch-lipid complex. The resistant starch (RS) content of the HA7-FA complex was up
to 75% determined using AOAC Method 991.43 for dietary fiber. The amylose-lipid
complex in the HA7-FA products was confirmed using DSC thermograms and X-ray
diffractograms. The HA7-FA complex samples prepared at 80°C were still in granular
shape, whereas those heated at higher temperature were agglomerated, and some lost their
granular structure. Defatting of the HA7-FA products reduced their RS contents by 6.5-
31.7%. The resistant residues of the HA7-FA products prepared at 80°C were still in granular shape after enzyme hydrolysis at boiling-water temperature, whereas the resistant residues of the native HA7 and the HA7 control showed partially hydrolyzed granules and dispersed materials. The amylopectin in the native HA7 was hydrolyzed by amylase to a greater extent than that in the HA7-FA complex samples prepared at 80°C. The increase in the heat stability and the enzyme resistance of the HA7-FA products was attributed to the amylose-lipid complex, which restricted the swelling of the starch granules during cooking and reduced the accessibility of amylase to hydrolyze starch.

Key words: resistant starch, high-amylose maize starch VII, fatty acids, amylose-lipid complex, enzyme debranching

INTRODUCTION

Resistant starch (RS) is starch or a fraction of starch that is not hydrolyzed by enzymes in the digestive track, but it is fermented by the gut microflora in the colon (Englyst and Cummings 1985, Englyst and Macfarlane 1986, Wyatt and Horn 1988). RS has shown to lower postprandial plasma-glucose and insulin concentrations. This is beneficial for interventions of insulin resistance and metabolic syndrome, including obesity, diabetes, and heart disease (DeFronzo and Ferrannini 1991, Jenkins et al 1998, Ludwig 2002). The fermentation of RS in the colon produces short-chain fatty acids including butyrate, which can prevent the development of colon cancer (Jenkins et al 1998, Ferguson and Harris 2000).
Native high-amylose maize starch VII (HA7), a B-type polymorphic starch with amylose content about 70%, is highly resistant to amylase hydrolysis (RS type 2) (Brumovsky and Thompson 2001, Jane 2006). The RS content of the HA7 starch, however, decreases when the starch is heated at 100°C in excess water. This is attributed to the partial gelatinization of the HA7 starch granules (Haralampu 2000). Treatments on the HA7, such as partial acid-hydrolysis followed by a hydrothermal treatment, has been used to enhance the heat stability and enzyme resistance of the HA7 starch granules (Brumovsky and Thompson 2001).

The objective of this study was to produce a novel RS by complexing HA7 starch with fatty acids (FA). Amylose-lipid complex is resistant to enzyme hydrolysis (Jane and Robyt 1984, Kitahara et al 1996, Gelders et al 2005). Amylose-lipid complex also restricts the swelling of starch granules during cooking, which reduces the accessibility of the enzyme to hydrolyze the starch molecules (Tester and Morrison 1990, Cui and Oates 1999). The physicochemical properties of this novel RS were also analyzed and discussed.

**MATERIALS AND METHODS**

**Materials**

High amylose maize starch VII (HA7) was a gift of Cargill (Hammond, IN). Palmitic acid (PA, Cat. No. W283207), stearic acid (SA, Cat. No. W303518), heat-stable α-amylase from *Bacillus licheniformis* (Cat. No. A3403), protease (Cat. No. P3910), glucoamylase from *Aspergillus Niger* (Cat. No. A9913), pullulanase from *Bacillus acidopullulyticus* (PUL, Cat. No. P2986), tris(hydroxymethyl)aminomethane (Cat. No. T1503), 2-morpholinoethanesulfonic acid (Cat. No. M3671), celite (Cat. No. C8656), D-glucose (Cat.
No. G8270), and amylose (Cat. No. A0512) were purchased from Sigma-Aldrich Corp. (St. Louis, MO) and used as received. Technical grade isoamylase (ISO) from *Pseudomonas amylofera* was a gift of Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan), which had specific activity of about 62,000 U/mL protein.

**Complex formation**

A suspension of HA7 (10% w/w) was heated at various temperatures: 80°C (30 min), 100°C (30 min), and 100°C (30 min) followed by autoclaving (121°C, 15 min). Each of the heat-treated HA7 samples was cooled to 25°C, adjusted to pH 3.5 or 5.0, and incubated with ISO (250U/g starch, dry starch basis, dsb) or PUL (5U/g starch, dsb), respectively, at 60°C for 24 hr with agitation. One unit ISO is defined as the amount of enzyme which hydrolyzes waxy maize starch, liberating reducing sugar equivalent to 1.0 µmole glucose per min at pH 3.5 and 60°C. One unit PUL is defined as the amount of enzyme which hydrolyzes pullulan, liberating reducing sugar equivalent to 1.0 µmole glucose per min at pH 5.0 and 40°C. The ISO- and PUL-treated-HA7 suspensions were heated back to the same temperature as the first heating treatments (80°C or 100°C) and mixed with FA (10%, w/w, dsb) for 30 min to form HA7-FA complex. The HA7-FA complex was recovered by centrifugation, washed with distilled water and then 50% ethanol, dried in a convection oven at 50°C to a moisture content below 12% (w/w), and ground using a Cyclone Mill (Udy Corp., Ft. Collins, CO) to pass through a 0.5-mm screen.

The HA7 controls were heated at the same temperatures as the HA7-FA complex samples, but without the debranching and the complex formation with FA. To understand the impact of each treatment to the enzyme resistance of the HA7 starch, the heat-treated
HA7 was also subjected to the ISO or PUL debranching reaction alone, and the heat-treated HA7 without the ISO and PUL debranching was subjected to the complex formation with FA. Native HA7, the HA7 control, and the HA7-FA complex samples were defatted using a methanol solution (85%) in a Soxhlet extractor for 24 hr and dried at 37°C for 24 hr. The defatted HA7 samples were used to analyze the effect of the FA-complex formation on the enzyme resistance of the HA7 starch.

Abbreviations were used to describe the treatments of the HA7 samples; for example, HA7(80°C) +PUL+SA represents that HA7 was heated at 80°C for 30 min, subjected to PUL debranching reaction at pH 5.0 and 60°C for 24 hr, and then complexed with SA at 80°C for additional 30 min. The abbreviations (100°C) and (AU) stand for heating at 100°C for 30 min and heating at 100°C for 30 min followed by autoclaving at 121°C for 15 min, respectively.

**Solubility and swelling power of starch**

The solubility and swelling power of the HA7 starch after the heating treatment was analyzed using the method of Sriruchwong et al. (2005). HA7 starch suspensions (1% w/v, 5 mL) in tared 50-mL centrifuge tubes were heated at various temperatures: 25°C (30 min), 80°C (30 min), 100°C (30 min), and 100°C (30 min) followed by autoclaving (121°C, 15 min). Each suspension after the heating treatment was centrifuged at 2000 × g for 15 minutes. The supernatant was collected and the swollen granules were weighed. The total soluble carbohydrate content (CHO) in the supernatant was determined using phenol and sulfuric acid (Dubois et al 1956). The soluble amylose content in the supernatant was determined using a blue value test (BV) with an iodine/potassium iodide solution (Juliano
1971). The colors developed from the CHO and BV tests were quantified using a microplate reader (ELX808, Bio-Tek Instruments, Inc., Winooski, VT) at 490 and 630 nm, respectively. Glucose and amylose were used to generate standard curves for CHO and BV, respectively.

\[
\% \text{ Total soluble carbohydrate} = \frac{\text{dry weight of soluble carbohydrate}}{\text{dry weight of starch}} \times 100
\]

\[
\% \text{ Soluble amylose} = \frac{\text{dry weight of soluble amylose}}{\text{dry weight of starch}} \times 100
\]

\[
\text{Swelling power (g/g)} = \frac{\text{weight of swollen granule} \times 100}{\text{dry weight of starch} \times (100 - \% \text{ total soluble carbohydrate})}
\]

**Molecular-weight distribution of starch**

The molecular-weight distribution of starch molecules was analyzed using gel permeation chromatography (GPC) (Song and Jane 2000). A starch sample (6 mg) was wetted with water (0.06 mL) and then mixed with DMSO (0.54 mL). The starch dispersion was heated in a boiling-water bath with stir for 1 hr and then stirred at 25°C for additional 16 hr. Starch was precipitated using 4 volumes of absolute ethanol, centrifuged, collected, and redispersed in water (2 mL) in a boiling-water bath with stir for 30 min. The starch dispersion was filtered through a nylon membrane with 5.0-micron pore size and injected into a GPC column (1.5 cm i.d. x 50 cm) packed with Sepharose CL-2B (Pharmacia, Inc., Piscataway, NJ). The column was eluted with an aqueous solution containing sodium chloride (25 mM) and sodium hydroxide (1 mM), in a descending direction. Fractions (1.66 mL) of the eluent were collected and analyzed using the CHO and BV tests similar to those used in the analysis of starch solubility (Section 2.3).
Starch morphology

The morphology of the native and processed HA7 samples was analyzed using scanning electron microscopy (SEM) (Jane et al 1994). The starch sample was spread on a silver tape mounted on a brass disk, and then coated with gold/palladium (60/40) using Polaron E5100 sputter coater (Polaron Equipment Ltd., Watford, UK). The starch images were captured at a magnification of 1500X using a scanning electron microscope (JSM-5800LV, JEOL, Tokyo, Japan) at Bessey Microscopy Facility, Iowa State University, Ames, IA.

Thermal properties of starch

The thermal properties of the native and processed HA7 samples were analyzed using a differential scanning calorimeter (DSC-7, Perkin-Elmer, Norwalk, CT). Each starch sample was precisely weighed to approximately 5 mg (dsb), mixed with 15 μL of deionized water, and sealed in a stainless-steel pan (Perkin-Elmer, Norwalk, CT). After allowing the starch sample in the stainless-steel pan to equilibrate at 25°C for 1 hr, it was heated from 10°C to 150°C at a rate of 10°C/min (first scan), cooled down to 10°C at a rate of 40°C/min, and reheated to 150°C at a rate of 10°C/min (rescan). The rescan was carried out to analyze the dissociation of the amylose-lipid complex that was formed spontaneously during rapid cooling. An empty stainless steel pan was used as the reference, and indium was used for calibration.

Starch crystallinity

The X-ray diffraction patterns of the native and processed HA7 samples were analyzed using a D-500 diffractometer (Siemens, Madison, WI) (Yoo and Jane 2002). The
differential 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.

**Resistant starch content**

The RS content was analyzed using an enzymatic-gravimetric method for dietary fiber, AOAC Method 991.43 (Horwithz 2003) with modification (Li et al. 2008). For the molecular-weight distribution of the resistant residue after enzyme hydrolysis following the AOAC Method 991.43, the resistant residue was collected by filtering through a Büchner funnel with a layer of filter paper (Whatman Paper No. 1). For the morphology of the resistant residue, the enzyme digestate was centrifuged at 8,281 × g for 20 minutes, and the resistant residue was suspended in absolute ethanol until needed.

**Statistical analysis**

Analysis of variance (ANOVA) was analyzed using the general linear model procedure in SAS version 9.1 (SAS Institute, Inc., Cary, NC) to determine the effect of the different treatments (heating temperatures, debranching reactions of starch, and complex formation of starch with FA’s) on the RS content. The RS content after a treatment was averaged over all the RS contents regardless of the other treatments. The significance level was established at \( p < 0.01 \) and the multiple comparisons were evaluated using Tukey’s Honest Significant Difference test.
RESULTS AND DISCUSSION

Swelling and solubility of HA7 starch

The swelling power, the total soluble carbohydrate, and the soluble amylose contents of the HA7 starch after the heating treatments at various temperatures (at 25°C, at 80°C, at 100°C, and at 100°C followed by autoclaving) are shown in Table 1. The HA7 samples suspended at 25°C and heated at 80°C had less swelling and less soluble carbohydrate than the HA7 samples heated at 100°C and heated at 100°C followed by autoclaving. The soluble amylose contents of the HA7 samples suspended at 25°C and heated at 80°C were also much lower than those of the HA7 sample heated at 100°C and heated at 100°C followed by autoclaving. In addition, the yield of the resulting RS products might be smaller when the HA7 starch was processed at a higher temperature because of the loss in the form of the soluble carbohydrate during the processing.

Molecular-weight distribution of starch

The molecular-weight distribution of the native HA7 showed a two peaks; the 1st peak (Fraction #14 to #21) consisted of amylopectin and the 2nd peak (Fraction #22 to #56) consisted of amylose and intermediate component (Figure 1) (Li et al 2008). All the HA7 samples debranched using either ISO or PUL showed reduced amylopectin contents than the native HA7 and the HA7 controls (Figure 2). The HA7(80°C)+ISO and HA7(80°C)+PUL samples (Figures 2B and C, respectively) retained a greater amount of amylopectin than the HA7 samples debranched after being heated at 100°C and at 100°C followed by autoclaving. These differences were attributed to that the lesser swelling of the HA7 starch granules
heated at 80°C (Table 1) reduced the accessibility of ISO or PUL to debranch amylopectin in the HA7 granules. The HA7(AU)+ISO and the HA7(AU)+PUL samples (Figures 2H and I, respectively) had the smallest amylopectin contents. These samples had the greatest swelling and the most soluble carbohydrate (Table 1), and thus their amylopectin was the most susceptible to ISO and PUL hydrolysis. The ratio of BV to CHO of the 2nd peak of the ISO-debranched HA7 samples (Figures 2B, E, and H) was greater than that of the PUL-debranched HA7 samples (Figures 2C, F, and I), suggesting that the debranching of the HA7 starch using ISO produced more linear molecules than that using PUL.

**Starch morphology**

The native HA7 starch granules showed spherical, rod, and filamentous granules (Figure 3). The HA7 samples heated at 80°C (Figures 4A-C) still retained the individual granular structure. Those heated at 100°C (Figures 4D-F) showed starch-granule agglomerates, and those heated at 100°C followed by autoclaving (Figures 4G-I) had lost their granular structure. Heating at temperatures above 80°C caused more amylose to leach out of the granules (Table 1), and the leached-out amylose held the starch granules together. The HA7(80°C)+PUL+PA and HA7(80°C)+PUL+SA samples (Figures 4B and C, respectively) had a smoother surface than the HA7 control (80°C) (Figure 4A) sample, which is attributed to that the amylose-FA complex restricted granule swelling during the processing. In addition, the granular HA7-FA complex processed at 80°C was more easily recovered after processing than the highly agglomerated HA7-FA complex prepared at 100°C and at 100°C followed by autoclaving.

**Thermal properties**
The thermal properties of the native HA7 and the HA7 samples after various treatments (heating temperatures, debranching reactions of starch, and complex formation of starch with FA’s) are shown in Table 2, and the thermograms are shown in Figure 5. The native HA7 displayed a very broad thermal transition from 70.4°C to 125.3°C with two peaks at 75.3°C and 98.4°C (Figure 5A). The first peak at 75.3°C was associated with the gelatinization of the amylopectin crystalline structure, the second peak at 98.2°C to 101.6°C was associated with the dissociation of the amylose-lipid complex, and the tail up to 125.7°C was associated with the melting of heat-stable amylose double helices (Jane et al 1999, Brumovsky and Thompson 2001, Li et al 2008).

The HA7 samples heated at 80°C lost the first peak because they had been partially gelatinized during the processing. The HA7(80°C)+PA and the HA7(80°C)+SA samples showed similar DSC profiles as the HA7 control (80°C). The HA7(80°C)+PUL sample had higher onset temperature and greater enthalpy change than the HA7 control (80°C), which could be attributed to a stronger double helical structure of the PUL-debranched amylopectin. The amylopectin of the HA7 starch is known for having longer branch chains than that of the normal or waxy maize (Jane et al 1999). Debranching of the HA7 amylopectins increased the freedom of the long branch-chains, and thus it could retrograde more efficiently. The lesser enthalpy changes of the HA7(80°C)+PUL+PA and HA7(80°C)+PUL+SA samples when compared with the HA7(80°C)+PUL sample could be a result of the FA-complexes preventing starch from retrograding (Slade and Levine 1987). The enthalpy changes of the HA7(80°C)+PA and HA7(80°C)+SA samples were smaller than those of the HA7(80°C)+PUL+PA and HA7(80°C)+PUL+SA samples because PUL-debranching of the HA7 amylopectin increased the freedom of the branch-chains of the amylopectin to form
complex with FA. The enthalpy change of the HA7(80°C)+PUL+SA sample was slightly greater than that of the HA7(80°C)+PUL+PA sample, which could be attributed that SA formed a stronger hydrophobic interaction with starch molecules than did PA (Tufvesson et al 2003). All the samples of HA7 starch complexed with PA and SA also showed a large peak at ~62°C and ~70.5°C, which was the melting temperature of the fatty acid crystallites.

Rescanning of the HA7 samples immediately after the first scan was carried out to analyze the dissociation of amylose-lipid complex that is formed spontaneously during rapid cooling. The dissociation temperature of amylose-lipid complex during the rescan of the native HA7 and the HA7 control (80°C) samples (Table 2 and Figure 5B) were flat and broad (64.1-108.0°C), which could be attributed to the presence of a heterogenous amylose-lipid complex (Klucinec and Thompson 1999). The endotherm of the rescan of the HA7-FA complex samples prepared at 80°C showed a peak with a shoulder or two peaks at 82.7-89.3°C and 101.6-103.6°C, which was associated with the melting of amorphous (form I) and crystalline amylose-lipid complex (form II), respectively (Biliaderis and Galloway 1989, Biliaderis and Seneviratne 1990). The HA7(80°C)+SA and the HA7(80°C)+PUL+SA samples had higher dissociation temperature of the amylose-lipid complex than did the HA7(80°C)+PA and the HA7(80°C)+PUL+PA samples because SA formed a stronger hydrophobic interaction with starch molecules than did PA (Tufvesson et al 2003). In addition, the melting of the retrograded amylose was not observed in any samples during the rescan.
Starch crystallinity

The X-ray diffraction patterns of the HA7 samples treated at 80°C, at 100°C, and at 100°C followed by autoclaving are shown in Figures 6A, B, and C, respectively. All the native HA7, HA7 control, and HA7+PUL samples showed the B-type polymorphic pattern (Kasemsuwan et al 1995, Jane et al 1999). All the HA7 control and HA7+PUL samples had weaker peak intensities than the native HA7 starch because they had been partially gelatinized. All the HA7-SA samples prepared at 100°C and at 100°C followed by autoclaving showed a mixture of the B- and V-type polymorphic patterns (Figures 6B and C). The V-type polymorphic pattern, however, was less noticeable in the HA7-SA complex sample prepared at 80°C (Figure 6A). The HA7-SA complex sample prepared at 80°C had less swelling (Table 1) and less debranched amylopectin (Figure 2) than did those prepared at higher temperature, thus the amylose-lipid complex in the HA7-SA complex sample prepared at 80°C was not enough to be detected using X-ray diffraction method. All the HA7-SA complex samples also showed sharp peaks (2θ = 6.5°, 21.5°, and 24.0°) associated with crystalline SA.

Resistant starch content

The RS contents of the HA7 control and the HA7 starch after various treatments (heating temperatures, debranching reactions of starch, and complex formation of starch with FA’s) are shown in Table 3. The RS contents were significantly greater for the processed HA7 samples prepared at 80°C than those prepared at 100°C and at 100°C followed by autoclaving (Figure 7A). The differences were partly attributed to a lower degree of starch gelatinization at 80°C than at higher temperature (Haralampu 2000). The HA7 samples
heated at 80°C also had less soluble amylose than did those heated at 100°C and at 100°C followed by autoclaving (Table 1), thus the FA-complexes were formed inside the granules of the HA7 samples prepared at 80°C. The HA7-FA complex samples heated at temperatures below 80°C, however, had lower RS content (data not shown) because starch granules were not adequately swollen to be hydrolyzed by debranching enzymes and to form complex with FA.

Debranching of the HA7 amylopectin using ISO or PUL also significantly increased the RS contents of the HA7 starch (Table 3, Figure 7B) because it increased the freedom of amylopectin branch-chains to retrograde and to form complex with lipid. The debranching of the HA7 starch using ISO resulted in a larger RS content than that using PUL, which could be attributed to the greater amount of linear chains in the ISO-debranched HA7 samples compared with the PUL-debranched HA7 samples (Figure 2).

The treatment of the HA7 starch with FA’s significantly increased the RS contents of the HA7 starch (Table 3, Figure 7C). This was attributed to the formation of the amylose-lipid complex, which is resistant to enzyme hydrolysis (Jane and Roby 1984, Kitahara et al 1996, Gelders et al 2005). In addition, the amylose-lipid complex restricts the swelling of the starch granules during cooking, thus decreasing the accessibility of the enzyme to hydrolyze the starch (Tester and Morrison 1990, Cui and Oates 1999). PA and SA were chosen for this study because they are straight-chain molecules and are more effective for amylose-helical complex formation than unsaturated and short-chain FA’s. Unsaturated FA’s have bent structure because of the cis double bonds, and short-chain FA’s are more water soluble, and thus they are less effective for amylose-helical complex formation (Tufvesson et al 2003). The HA7-SA complex samples had greater RS contents than the HA7-PA complex.
counterparts. This could be attributed to that SA formed a stronger hydrophobic interaction with starch molecules than did PA, reflecting from the greater enthalpy change of the HA7-SA complex sample during the first scan and the higher onset melting point during the rescan than those of the HA7-PA complex counterparts (Table 2 and Figure 5).

The combined treatments of HA7 with debranching enzyme and FA gave much greater RS contents than either treatment alone. The largest RS content (75%) was achieved when HA7 starch was heated at 80°C, debranched with ISO, and complexed with SA. This is attributed to that the debranching reaction enhanced the formation of amylose-lipid complex in the HA7 samples and the amylose-lipid complex reduced the swelling of the HA7 starch granules during cooking and reduced the susceptibility of the HA7 starch to amylase hydrolysis.

To confirm the effect of the FA-complex formation on the RS content, the native HA7, the HA7 control (80°C), the HA7(80°C)+PUL+PA, and the HA7(80°C)+PUL+SA samples were defatted and then analyzed for their RS contents. The RS content of the HA7(80°C)+PUL+PA and HA7(80°C)+PUL+SA samples decreased to a greater extent (20.7-31.7%) than did the native HA7 and HA7 control (80°C) sample (6.5-12.8%) after defatting. This showed that there were more amylose-lipid complexes in the HA7-FA complex samples than the native HA7 and HA7 control (80°C) sample. The RS content of the defatted HA7(80°C)+PUL+SA sample was greater than that of the defatted HA7(80°C)+PUL+PA. This suggested that SA was not easily removed by methanol extraction because it formed a stronger hydrophobic interaction with starch molecules than PA.
Morphology of resistant residues

The resistant residues of the native HA7 and the HA7 control (80°C) sample after enzyme hydrolysis following the AOAC Method 991.43 (Figures 8B and D, respectively) showed different morphology from their non-digested counterparts. The spherical granules of the resistant residues of the native HA7 and the HA7 control (80°C) sample became half-shelled shape, whereas the filamentous granules remained intact. The half-shelled granules suggested that the hila of the spherical granules were mostly hydrolyzed by amylase, but the periphery of the granules remained intact. It is known that the molecules at the hilum are more loosely packed and are more susceptible to amylase hydrolysis than those at the periphery (Baker et al 2001, Jane 2007). The enzyme-resistant periphery of HA7 granule was attributed to that amylose was more concentrated at the periphery than in the hilum, intertwined with amylopectin, and held the molecules together (Jane 2007). Dispersed materials were also observed surrounding the partially hydrolyzed starch granules.

In contrast, the resistant residues of the HA7(80°C)+PUL+PA and the HA7(80°C)+PUL+SA samples (Figures 8F and J, respectively) were slightly swollen, but remained intact. Fewer half-shelled granules were observed in the resistant residues of the HA7(80°C)+PUL+PA sample than in the resistant residues of the native HA7 and the HA7 control (80°C), and even fewer was observed in the resistant residue of the HA7(80°C)+PUL+SA sample. The resistant residues of the HA7(80°C)+PUL+PA and the HA7(80°C)+PUL+SA samples had smooth surface, and no dispersed materials were observed. These results confirmed that the amylose-lipid complex restricted granule swelling and reduced the accessibility of amylases to hydrolyze molecules at the hilum (Morrison et al 1993, Cui and Oates 1999).
The morphology of the resistant residue of the defatted HA7(80°C)+PUL+PA sample was similar to that of the resistant residues of the native HA7 starch and the HA7 control (80°C) sample. The resistant residue of the defatted HA7(80°C)+PUL+SA sample was less hydrolyzed and less dispersed than the resistant residue of the defatted HA7(80°C)+PUL+PA sample. These results agreed with the reduced RS contents of the defatted HA7-FA complex samples compared with their non-defatted counterparts (Table 4). Defatting removed most of the lipids in the HA7 samples including those that were already forming complexes. The defatted samples, thus, were not protected by the amylose-lipid complex and were digested in the similar fashion as the native HA7 and HA7 control (80°C) samples.

**Molecular-weight distribution of resistant residues**

The amylopectin of the resistant residues of the HA7 control (80°C), HA7(80°C)+PUL+PA, and HA7(80°C)+PUL+SA samples was reduced after the enzyme hydrolysis following the AOAC Method 991.43 when compared with their non-digested counterparts (Figure 9). The amylopectin fraction of the resistant residue of the native HA7 starch was almost undetectable (Figure 9B). The amylopectin fractions of the resistant residues of the HA7(80°C)+PUL+PA and the HA7(80°C)+PUL+SA samples (Figures 9D and F, respectively) were still larger than that of the resistant residue of the native HA7 starch. Furthermore, the small-molecule fraction in the resistant residue of the native HA7 starch had a smaller ratio of BV to CHO than that in the non-digested native HA7 starch. This indicated a higher concentration of small branched-molecules resulting from random-fasion α-amylase hydrolysis. The ratio of BV to CHO of the small-molecule fractions of the resistant residues of the HA7(80°C)+PUL+PA and the HA7(80°C)+PUL+SA samples
was similar to their non-digested counterparts, which suggested that the amylose-lipid complex protected amylopectins from amylase hydrolysis.

**CONCLUSION**

The RS content of the HA7 starch increased when the HA7 starch was debranched and complexed with FA. Heating of the HA7 starch was used to swell the starch granules, which enhanced the debranching reactions and complex formation with FA. The debranching enzyme hydrolyzed HA7 amylopectin and increased the freedom of the branch chains to retrograde and to complex with FA. The HA7 starch was still in granular shape when it was processed at 80°C, whereas that processed at higher temperature was agglomerated and some lost its granular structure. DSC diffractograms showed that the native HA7 starch contained amylopectin crystalline structure, amylose-lipid complex, and amylose double helices. The amount of amylose-lipid complex in the HA7 samples was greater after the treatment with FA, which was also confirmed by their X-ray diffractograms.

The largest RS content (75%) was achieved when HA7 starch was heated at 80°C, debranched with ISO, and complexed with SA. Heating the HA7 starch at 80°C swelled the HA7 starch with less starch gelatinization, lower soluble total carbohydrate, and lower soluble amylose than the heating of HA7 starch at temperature higher than 80°C. Using ISO to debranch the HA7 starch produced more linear molecules than using PUL, and yielded greater RS contents. SA is more hydrophobic than is PA, thus it formed a stronger hydrophobic interaction with starch molecules when forming amylose-lipid complex. Defatting of HA7-FA complex samples using methanol solution removed lipids and reduced
the amylose-lipid complex, thus resulted in reduced the RS contents. The amylopectin of the
HA7-FA complex samples prepared at 80°C was less susceptible to amylase hydrolysis than
that of the native HA7. The resistant residues of the HA7-FA complex samples prepared at
80°C remained intact granular shape, whereas those of the native HA7 and the HA7 control
(80°C) showed partially hydrolyzed starch granules with dispersed materials. The amylose-
lipid complex restricted swelling of HA7 starch granules during cooking, and decreased the
accessibility of amylase to hydrolyze starch in the granules.

LITERATURE CITED


Biliaderis, C. G. and Seneviratne, H. D. 1990. On the supramolecular structure and

starch by partial acid hydrolysis and hydrothermal treatments of high-amylose maize


Table 1. Swelling of high-amylose corn starch VII and total soluble carbohydrate and soluble amylose contents during heating at various temperatures.

<table>
<thead>
<tr>
<th>Heating temperature</th>
<th>Swelling (g/g)</th>
<th>Soluble materials Total (g/g)</th>
<th>Amylose carbohydrate (%)</th>
<th>Amylose (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°C</td>
<td>2.8 ± 0.4</td>
<td>3.3 ± 0.3</td>
<td>1.9 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>80°C</td>
<td>5.2 ± 0.3</td>
<td>7.2 ± 1.8</td>
<td>4.4 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>100°C</td>
<td>8.4 ± 0.4</td>
<td>27.6 ± 3.6</td>
<td>25.4 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>100°C followed by autoclaving</td>
<td>8.9 ± 0.2</td>
<td>34.6 ± 1.4</td>
<td>33.5 ± 0.5</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Thermal properties of high-amylose maize starch VII samples after various treatments

<table>
<thead>
<tr>
<th>Treatments(^1)</th>
<th>Fist Scan</th>
<th>Rescan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(T_o)</td>
<td>(T_p1)</td>
</tr>
<tr>
<td>Native HA7</td>
<td>70.4 ±0.3</td>
<td>75.3 ±0.4</td>
</tr>
<tr>
<td>HA7 control (80(^\circ)C)</td>
<td>84.1 ±0.3</td>
<td>-</td>
</tr>
<tr>
<td>HA7(80(^\circ)C) +PA</td>
<td>79.2 ±0.4</td>
<td>-</td>
</tr>
<tr>
<td>HA7(80(^\circ)C) +SA</td>
<td>83.9 ±0.6</td>
<td>-</td>
</tr>
<tr>
<td>HA7(80(^\circ)C) +PUL</td>
<td>84.3 ±1.8</td>
<td>-</td>
</tr>
<tr>
<td>HA7(80(^\circ)C) +PUL+PA</td>
<td>75.7 ±1.2</td>
<td>-</td>
</tr>
<tr>
<td>HA7(80(^\circ)C) +PUL+SA</td>
<td>78.9 ±0.2</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^1\)HA7 = high-amylose maize starch VII, PA = complexing with palmitic acid, SA = complexing with stearic acid, and PUL = debranching with pullulanase. All processed samples were prepared at 80\(^\circ\)C.
Table 3. Resistant starch contents of high-amylose maize starch VII samples after various treatments.

<table>
<thead>
<tr>
<th>Treatments¹</th>
<th>RS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heating at 80°C</td>
</tr>
<tr>
<td>HA7 control</td>
<td>36.7 ± 0.2</td>
</tr>
<tr>
<td>HA7+PA</td>
<td>58.3 ± 1.7</td>
</tr>
<tr>
<td>HA7+SA</td>
<td>59.8 ± 2.8</td>
</tr>
<tr>
<td>HA7+ISO</td>
<td>57.8 ± 0.1</td>
</tr>
<tr>
<td>HA7+PUL</td>
<td>46.0 ± 1.6</td>
</tr>
<tr>
<td>HA7+ISO+PA</td>
<td>74.3 ± 2.4</td>
</tr>
<tr>
<td>HA7+PUL+PA</td>
<td>69.9 ± 2.2</td>
</tr>
<tr>
<td>HA7+ISO+SA</td>
<td>74.8 ± 1.5</td>
</tr>
<tr>
<td>HA7+PUL+SA</td>
<td>71.6 ± 0.3</td>
</tr>
</tbody>
</table>

¹HA7 = high-amylose maize starch VII, PA = complexing with palmitic acid, SA = complexing with stearic acid, ISO = debranching with isoamylase, and PUL = debranching with pullulanase.
Table 4. Resistant starch contents of high-amylose maize starch VII-fatty acid complex samples before and after defatting.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>RS (%)</th>
<th>Before defatting</th>
<th>After defatting</th>
<th>Difference defatting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native HA7</td>
<td>41.5 ± 1.6</td>
<td>28.7 ± 1.5</td>
<td>12.8</td>
<td></td>
</tr>
<tr>
<td>HA7 control (80°C)</td>
<td>36.7 ± 0.2</td>
<td>30.2 ± 1.3</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>HA7(80°C)+PUL+PA</td>
<td>69.9 ± 2.2</td>
<td>38.2 ± 0.7</td>
<td>31.7</td>
<td></td>
</tr>
<tr>
<td>HA7(80°C)+PUL+SA</td>
<td>71.6 ± 0.3</td>
<td>50.9 ± 0.4</td>
<td>20.7</td>
<td></td>
</tr>
</tbody>
</table>

1HA7 = high-amylose maize starch VII, PUL = debranching with pullulanase, PA = complexing with palmitic acid, and SA = complexing with stearic acid.
Fig. 1. Gel permeation chromatogram of native high-amylose maize starch VII starch.
Fig. 2. Gel permeation chromatograms of high-amyllose maize starch VII before and after debranching. (A) HA7 control (80°C), (B) HA7(80°C)+ISO, (C) HA7(80°C)+PUL, (D) HA7 control (100°C), (E) HA7(100°C)+ISO, (F) HA7(100°C)+PUL, (G) HA7 control (AU), (H) HA7(AU)+ISO, and (I) HA7(AU)+PUL. (○) Total carbohydrate and (●) blue value.
Fig. 3. Scanning electron micrograph of native high-amylose maize starch VII.
Fig. 4. Scanning electron micrographs of high-amylose maize starch VII processed at various conditions. (A) HA7 control (80°C), (B) HA7(80°C)+PUL+PA, (C) HA7(80°C)+PUL+SA, (D) HA7 control (100°C), (E) HA7(100°C)+PUL+PA, (F) HA7(100°C)+PUL+SA, (G) HA7 control (AU), (H) HA7(AU)+PUL+PA, and (I) HA7(AU)+PUL+SA.
Fig. 5. DSC profiles of high-amylose maize starch VII after various treatments. HA7 = high-amylose maize starch VII, PA = complexing with palmitic acid, SA = complexing with stearic acid, and PUL = debranching with pullulanase. All processed samples were prepared at 80°C.
Fig. 6. X-ray diffractograms of high-amylose maize starch VII processed at (A) 80°C, (B) 100°C, and (C) 100°C followed by autoclaving. HA7 = high-amylose maize starch VII, PA = complexing with palmitic acid, SA = complexing with stearic acid, and PUL = debranching with pullulanase.
Fig. 7. Effects of (A) heating temperatures, (B) debranching reaction of starch, and (C) complex formation of starch with fatty acids on the resistant starch contents. The resistant starch content was pooled together from other treatments that were not being analyzed. Values with the different letter in the same chart are significantly different at $p < 0.01$. 
Fig. 8. Granule morphology of the resistant residues of high-amylose maize starch VII samples. (A) Native HA7, (B) residue of native HA7, (C) HA7 control (80°C), (D) residue of HA7 control (80°C), (E) HA7(80°C)+PUL+PA, (F) residue of HA7(80°C)+PUL+PA, (G) defatted HA7(80°C)+PUL+PA, (H) residue of defatted HA7(80°C)+PUL+PA, (I) HA7(80°C)+PUL+SA, (J) residue of HA7(80°C)+PUL+SA, (K) defatted HA7(80°C)+PUL+SA, and (L) residue of defatted
Fig. 9. Gel permeation chromatograms of resistant residues of high-amylose maize starch VII samples. (A) Native HA7, (B) residue of native HA7, (C) HA7(80°C)+PUL+PA, (D) residue of HA7(80°C)+PUL+PA, (E) HA7(80°C)+PUL+SA, and (F) residue of HA7(80°C)+PUL+SA.
CHAPTER 3. NOVEL RESISTANT STARCH FROM HIGH-AMYLOSE MAIZE STARCH VII – FATTY ACID COMPLEXES. PART 2.
REDUCING POSTPRANDIAL PLASMA-GLUCOSE AND INSULIN

A paper submitted to Cereal Chemistry

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ABSTRACT

A novel resistant starch (RS) previously was produced by introducing a starch-lipid complex into high-amylose maize starch VII granules (HA7-palmitic acid (PA) complex). The objective of this study was to examine the potential health benefits of the novel RS on reducing postprandial plasma-glucose and insulin responses. The postprandial plasma-glucose and insulin responses of 20 healthy male human subjects were analyzed after ingesting breads made of 60% HA7-PA complex (RS bread) and white bread on separate days for comparison. The RS bread contained 34.4% RS (determined using AOAC Method 991.43) and showed reduced postprandial plasma-glucose and insulin concentrations (45% and 57%, respectively) when compared with the white bread control. The postprandial plasma-insulin response also declined faster after ingesting the RS bread than the white
The reduction in the postprandial plasma-insulin response was greater in the subjects with greater body mass index (BMI). The results suggest that the HA7-PA complex can be used for the interventions of insulin resistance and metabolic syndrome including diabetes, obesity, and cardiovascular disease.

Key words: resistant starch, high-amylose maize starch VII, amylose-lipid complex, postprandial glucose, insulin

INTRODUCTION

Resistant starch (RS) is starch or a fraction of starch not digested and absorbed in the small intestine (Englyst and Cummings 1985). There are increasing interests in consuming RS because of its health benefits. Frequent consumption of highly digestible starchy food has shown to cause hyperglycemia and hyperinsulinemia cycle, which leads to insulin resistance and metabolic syndrome including diabetes, obesity, hypertension, and cardiovascular disease (DeFronzo and Ferrannini 1991, Ludwig 2002). Ingestion of RS reduces postprandial plasma-glucose and insulin responses (Thorne et al 1983, Byrnes et al 1995, Englyst et al 1999). Thus RS has been proposed for interventions of insulin resistance and metabolic syndrome (Higgins 2004). Lowering the postprandial plasma-insulin can also reduce the rates of cholesterogenesis, lipogenesis, and lipolysis, and thus reduce the occurrences of obesity and cardiovascular disease (de Deckere et al 1993, Mathe et al 1993, Ranhotra et al 1997).
A novel starch with up to 75% enzyme resistance has been made by introducing a starch-lipid complex into high-amylose maize starch VII (HA7) starch granules (Hasjim et al unpublished). The process involves swelling and partially gelatinizing HA7 starch granules at elevated temperature, debranching, and then complexing with lipid. The health benefits of this novel RS have not been examined. The objective of this study was to examine the effects of the novel RS on reducing postprandial plasma-glucose and insulin responses.

MATERIALS AND METHODS

Materials

High-amylose maize starch VII (HA7, AmyloGel™ 03003) was a gift of Cargill (Hammond, IN). Palmitic acid (PA, Cat. No. W283207), heat-stable $\alpha$-amylase from Bacillus licheniformis (Cat. No. A3403), protease (Cat. No. P3910), glucoamylase from Aspergillus Niger (Cat. No. A9913), tris(hydroxylmethyl)aminomethane (Cat. No. T1503), 2-morpholinoethanesulfonic acid (Cat. No. M3671), and celite (Cat. No. C8656) were purchased from Sigma-Aldrich Corp. (St. Louis, MO) and used as received. Technical grade isoamylase (ISO) from Pseudomonas amyloferansosa was a gift of Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan), which had specific activity of about 62,000 U/mL protein. Wheat flour, wheat gluten, shortening, yeast, and table salt were obtained from a local grocery store.
Resistant starch production

A HA7 suspension (10% w/w) was heated at 95°C for 1 hr with agitation, adjusted to pH 3.5 using a hydrochloric acid solution (0.5 M) at 55°C, and then incubated with ISO (500U/g starch, dry starch basis, dsb) for 12 hr with agitation to debranch the starch. PA (10% w/w, dsb) was added to the debranched HA7 suspension, and the mixture was heated at 95°C for an additional 1 hr. The resulting HA7-PA complex was recovered by centrifugation, washed with 50% ethanol, dried at 50°C to moisture content below 12%, and ground to fine powder.

Bread making

The control white bread was made of wheat flour (600 g), shortening (25 g, 52% fat), yeast (5 g), table salt (4 g), and water (330g). After allowing the bread dough to raise for 2 hr, it was baked in a conventional oven at 400°F for 25 min. The RS bread was made by substituting 75% of wheat flour (450 g) with the HA7-PA complex (398 g) and wheat gluten (78 g), and other ingredients were kept the same as those used in the control white bread. The control white bread (96% wheat flour) and the RS bread (24% wheat flour, 60% HA7+ISO+PA, and 12% wheat gluten) had the same dry proportions of starch and protein. More water (260 g) was added to the RS bread because the HA7-PA complex was harder to be mixed with water. Table 1 lists the ingredient compositions of the control white and RS breads.

Resistant residue, undigested protein, and resistant starch contents

The resistant residue content of the samples was analyzed using enzymatic-gravimetric method for dietary fiber, AOAC Method 991.43, (Horwitz 2003) with modification (Li et al
The nitrogen content of the resistant residue of wheat flour, wheat gluten, and bread samples after enzyme hydrolysis was analyzed using a macro-Kjedahl method (Jung et al 2003). The nitrogen content was converted to the protein content using a conversion factor of 5.33 (Mosse et al 1985). The RS content was calculated by following equation:

\[
\text{Resistant starch} = \text{total resistant residue} - \text{undigested protein}
\]

**Plasma-glucose and plasma-insulin concentrations**

Twenty healthy, non-smoker male human subjects were recruited for this study with age range of 19-38 years old and BMI range of 21.0-42.8 (Table 2). All subjects were not allergic or sensitive to wheat or corn, did not take any drugs, and did not have chronic illness. After an overnight fast, each subject received a slice of control white or RS bread containing 50 g of starch on a single day in a randomized crossover design with a one week washout period between treatments. Blood samples were collected from subjects every 15 min from 15 min before ingesting test bread to 120 min after ingesting test bread. The plasma-glucose concentrations were measured using a glucose oxidase analyzer (Beckman Coulter Glucose Anlyzer, Beckman Coulter Inc., Fullerton, CA) and the plasma-insulin concentrations were determined using an ultrasensitive insulin ELISA (Enzyme-Linked Immuno Sorbent Assay) kit (ALPCO diagnostics, Salem, NH). The areas under the curve (AUC’s) were calculated using the trapezoidal rule with fasting levels as the baseline; any negative area was ignored (Wolever et al 1991).
Statistical analysis

The correlations of the postprandial plasma-glucose and insulin responses to subjects’ age and BMI were analyzed using the linear regression procedure (PROC REG) in SAS (version 9.1, SAS Institute, Inc., Cary, NC). The mean values of the total AUC’s of the postprandial plasma-glucose and insulin responses were analyzed using analyses of variance (ANOVA) with the general linear model procedure (PROC GLM) in SAS. Differences were evaluated by t-test using Tukey’s adjustment. The significance level was set at $p$-value < 0.01.

RESULTS AND DISCUSSION

The total resistant residue, undigested protein, and RS contents of major ingredients (wheat flour, HA7-PA complex, and wheat gluten), white bread, and RS bread are shown in Table 3. The results showed that the wheat flour had 2.1% RS, and the HA7-PA complex consisted of 52.7% RS. The RS bread made of 60% HA7-PA complex contained 34.4% RS, which was 10 times greater than did the control white bread (3.3%). The experimental results of the RS content of the RS bread (34.4%) was similar to the calculated value (33.6%) obtained on the basis of the RS contents of the HA7-PA complex, wheat flour, and wheat gluten, thus no net loss of RS was observed during the bread baking process.

The peak maxima of the postprandial plasma-glucose and insulin responses (Figures 1 and 2, respectively) were reduced by half when the human subjects ingested the RS bread comparing with the control white bread. Even though the postprandial plasma-glucose responses at 120 min after ingesting the control white and RS breads went down to about the
same concentration, the postprandial plasma-insulin responses at 120 min after ingesting the control white bread was 6.5 times higher than that after ingesting the RS bread. The rapid reduction in the postprandial plasma-insulin response after ingesting the RS bread could reduce the incidence of developing insulin resistance (Byrnes et al 1995). The lesser increase in the postprandial plasma-insulin response after ingesting the RS bread could also reduce cholesterogenesis, lipogenesis, and lipolysis, thus can be used for interventions of obesity and cardiovascular disease (de Deckere et al 1993, Mathe et al 1993, Ranhotra et al 1997).

The total AUC’s of the postprandial plasma-glucose and insulin responses reduced significantly (45% and 57%, respectively) when subjects ingested the RS bread compared with the control white bread (Figure 3).

Despite the differences in the intensities and the AUC’s of the postprandial plasma-glucose and insulin responses with the ingestions of the control white and RS breads, the maxima of the postprandial plasma-glucose and insulin responses appeared at similar time (about 30-45 min after ingestion) for both breads (Figures 1 and 2). This suggests that the starch in the control white bread and the RS bread was digested at the same rates, but the RS bread contained less digestible starch for enzyme to convert to glucose than did the control white bread.

The average postprandial plasma-glucose response at each time point of blood collection was positively correlated with the average postprandial plasma-insulin response (Figure 4). The increase in the plasma-glucose concentration after meal (hyperglycemia) induces the secretion of insulin from the beta cells of pancreas (Berdanier 1976). Insulin, in turn, reduces the plasma-glucose concentration by regulating the body absorption of glucose from
blood to liver and muscle, where it is stored as glycogen. Excess glucose in body is also converted to fat and stored in the adipose tissue.

The total AUC’s of the postprandial plasma-glucose and insulin responses did not correlate with subjects’ age (Figures 5A and B). These results suggest that the digestion of bread and absorption of glucose in the small intestine were not affected by subjects’ age or BMI. The total AUC of the postprandial plasma-glucose response did not correlate with subjects’ body-mass index (BMI), either (Figure 5C). The total AUC of the postprandial plasma-insulin response, however, positively correlated with subjects’ BMI (Figure 5D). The results suggested that the human subjects with greater BMI might have experienced insulin resistance, and thus the beta cells of the pancreas secreted more insulin to overcome the resistance. The reduction effect of the postprandial plasma-insulin response was also more pronounced in the human subjects with greater BMI (Figure 5D). Therefore, the HA7-PA complex can be used for interventions of insulin resistance and metabolic syndrome, including diabetes, obesity, and cardiovascular disease.

CONCLUSION

The postprandial plasma-glucose and insulin responses of 20 healthy male subjects were analyzed after ingesting control white bread and RS bread on separate days. There was a significant reduction in the postprandial plasma-glucose response after the ingestion of the RS bread compared with after the ingestion of the control white bread, which led to a reduced secretion of insulin. The digestion of the bread and the glucose absorption were not affected by subjects’ age or BMI. The secretion of insulin, however, was related to subjects’
BMI, which could be attributed to the response of the pancreas to the insulin resistance developed in the subjects with greater BMI. In addition, the effect of RS bread on reducing the insulin secretion was more pronounced in the subjects with greater BMI than those with lower BMI. These results suggested that enzyme resistance of the HA7-PA complex can be used for interventions of insulin resistance and metabolic syndrome including diabetes, obesity, and cardiovascular disease.

**LITERATURE CITED**


### Table 1. Compositions of control white and RS breads.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Control White Bread</th>
<th>RS Bread</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Starch</td>
</tr>
<tr>
<td>Wheat flour (g)</td>
<td>600</td>
<td>476</td>
</tr>
<tr>
<td>HA7-PA complex (g)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Wheat Gluten (g)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Shortening (g)</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>Yeast (g)</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Table salt (g)</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Water (g)</td>
<td>330</td>
<td>0</td>
</tr>
<tr>
<td>Total weight (g)</td>
<td>964</td>
<td>476</td>
</tr>
<tr>
<td>Percentage (%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>100%</td>
<td>82%</td>
</tr>
</tbody>
</table>

<sup>a</sup>Fats, non-starch polysaccharides, vitamins, and minerals.

<sup>b</sup>Percentage of starch and protein on the dry basis of the whole ingredient.
Table 2. Age and BMI of human subjects.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Ethnicity</th>
<th>Age</th>
<th>BMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>101</td>
<td>Asian</td>
<td>29</td>
<td>27.07</td>
</tr>
<tr>
<td>102</td>
<td>Caucasian</td>
<td>29</td>
<td>26.65</td>
</tr>
<tr>
<td>103</td>
<td>Caucasian</td>
<td>24</td>
<td>24.02</td>
</tr>
<tr>
<td>104</td>
<td>Asian</td>
<td>27</td>
<td>24.08</td>
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<td>105</td>
<td>Asian</td>
<td>38</td>
<td>21.43</td>
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<tr>
<td>106</td>
<td>Caucasian</td>
<td>36</td>
<td>28.87</td>
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<tr>
<td>107</td>
<td>Caucasian</td>
<td>32</td>
<td>22.70</td>
</tr>
<tr>
<td>108</td>
<td>Asian</td>
<td>32</td>
<td>21.02</td>
</tr>
<tr>
<td>109</td>
<td>Caucasian</td>
<td>24</td>
<td>22.68</td>
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<tr>
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<td>24.26</td>
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<td>Asian</td>
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<td>207</td>
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<tr>
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<td>Caucasian</td>
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<td>31.11</td>
</tr>
<tr>
<td>209</td>
<td>Asian</td>
<td>24</td>
<td>38.42</td>
</tr>
<tr>
<td>210</td>
<td>Asian</td>
<td>21</td>
<td>26.27</td>
</tr>
</tbody>
</table>
Table 3. Total resistant residue, undigested protein, and resistant starch contents of major ingredients for bread and bread samples.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Total resistant residue&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>Undigested protein&lt;sup&gt;b&lt;/sup&gt; (%)</th>
<th>Resistant starch</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Experimental&lt;sup&gt;c&lt;/sup&gt; (%)</td>
</tr>
<tr>
<td>Wheat Flour</td>
<td>4.8 ± 0.1</td>
<td>2.7 ± 0.2</td>
<td>2.1</td>
</tr>
<tr>
<td>HA7-PA complex</td>
<td>52.7 ± 1.5</td>
<td>0</td>
<td>52.7</td>
</tr>
<tr>
<td>Wheat gluten</td>
<td>45.2 ± 1.6</td>
<td>34.2 ± 1.0</td>
<td>11.0</td>
</tr>
<tr>
<td>Control white bread&lt;sup&gt;e&lt;/sup&gt;</td>
<td>6.5 ± 0.8</td>
<td>3.2 ± 0.2</td>
<td>3.3</td>
</tr>
<tr>
<td>RS bread&lt;sup&gt;f&lt;/sup&gt;</td>
<td>37.4 ± 3.5</td>
<td>3.1 ± 0.4</td>
<td>34.4</td>
</tr>
</tbody>
</table>

<sup>a</sup>Total resistant residue was analyzed using AOAC method 991.43 for total dietary fiber (Horwithz 2003). Mean ± standard deviation from duplicates.

<sup>b</sup>Undigested protein was analyzed using macro-Kjeldahl method (Jung et al 2003) on the total resistant residue. Mean ± standard deviation from duplicates.

<sup>c</sup>Resistant starch = total resistant residue – undigested protein.

<sup>d</sup>Resistant starch was calculated on the basis of the resistant starch contents of wheat flour, HA7-PA complex, and wheat gluten.

<sup>e</sup>Control white bread contained 96% wheat flour.

<sup>f</sup>RS bread contained 24% wheat flour, 60% HA7-PA complex, and 12% wheat gluten.
Fig. 1. Average increase in postprandial plasma-glucose responses of 20 male human subjects after ingesting control white and RS breads.
Fig. 2. Average increase in postprandial plasma-insulin responses of 20 male human subjects after ingesting control white and RS breads.
Fig. 3. Average areas under the curve (AUC’s) of (A) postprandial plasma-glucose responses and (B) insulin responses of 20 male human subjects after ingesting control white bread and RS bread. The error bar shows the standard error of the average.
Fig. 4. Correlation of the average postprandial plasma-glucose and average insulin responses at each time point of blood collection of 20 male human subjects after ingestion of (♦) control white and (●) RS breads. The correlation coefficient was significant at $p < 0.01$. 

$R^2 = 0.7096$
Fig. 5. Correlation of the areas under the curve (AUC’s) of postprandial plasma-glucose and insulin responses after ingestion of (●) control white bread and (◊) RS bread to subjects’ age and BMI. (——) Trend lines of responses upon ingestion of control white bread and (---) trend lines of responses upon ingestion of RS bread. Only the correlation coefficients in plot D were significant at $p < 0.01$. 

A. Plasma-glucose

B. Plasma-insulin

C. Plasma-glucose

D. Plasma-insulin
CHAPTER 4. PRODUCTION OF RESISTANT STARCH BY EXTRUSION COOKING OF ACID-MODIFIED NORMAL-MAIZE STARCH

A paper submitted to the Journal of Food Science

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ABSTRACT:

The objective of this study was to utilize extrusion cooking and hydrothermal treatment to produce resistant starch (RS) as an economical alternative to a batch-cooking process. A hydrothermal treatment (110°C, 3 d) of batch-cooked and extruded starch samples facilitated propagation of heat-stable starch crystallites and increased the RS contents from 2.1-7.7% up to 17.4% determined using AOAC Method 991.43 for total dietary fiber. When starch samples were batch cooked and hydrothermally treated at a moisture content below 70%, acid-modified normal-maize starch (AMMS) produced a greater RS content than did native normal-maize starch (NMS). This was attributed to the partially hydrolyzed, smaller molecules in the AMMS, which had greater mobility and freedom than the larger molecules in the NMS. The RS contents of the batch-cooked and extruded AMMS products after the hydrothermal treatment were similar. A freezing treatment of the AMMS samples at -20°C prior to the hydrothermal treatment did not increase the RS content. The DSC thermograms
and the X-ray diffractograms showed that retrograded amylose and crystalline starch-lipid complex, which had melting temperatures above 100°C, were accounted for the RS contents.

Keywords: extrusion cooking, hydrothermal treatment, acid hydrolysis, resistant starch, retrograded amylose.

**Introduction**

Resistant starch (RS) is starch or a fraction of starch that is not digested by amylolytic enzymes in the digestive track (Stephen and others 1983; Englyst and Cummings 1985), but it is fermented by the gut bacteria in the colon and produces short-chain fatty acids and other organic acids (Englyst and Macfarlane 1986; Ferguson and others 2000). There are increasing interests in consuming RS because of its health benefits, including interventions of insulin resistance, obesity, diabetes, cardiovascular disease, and colon cancer (Englyst and Macfarlane 1986; DeFronzo and Ferrannini 1991; de Deckere and others 1995; Jenkins and others 1998).

Retrograded amylose or RS type 3 (RS3) is commonly found in small amounts (less than 5%) in many food products, including cooked oats, cornflakes, and bread (Englyst and Cummings 1985; Bjorck and others 1986). RS3 is heat stable, and thus it remains in the food product after cooking at the boiling-water temperature (Sievert and Pomeranz 1990; Gruchala and Pomeranz 1993). Mild acid hydrolysis and hydrothermal treatments have been used to increase the RS3 content in starch products (Vasanthan and Bhaty 1998; Shin and others 2004).
Extrusion cooking has been used recently to produce RS3 with flour and starch (Faraj and others 2004; Agustiniano-Osornio and others 2005). Extrusion cooking is commonly used in the food industry for making breakfast cereals, snack foods, and other similar food products. Its advantages over the batch-cooking process include continuous process, shorter processing time, and less energy and moisture needed (Guy 2001). The extrudate is collected as continuous strands, which is easier to handle than the batch-cooked products. The RS content of the extruded products, however, was usually low (less than 10%).

The objective of this study was to utilize the combination of mild acid hydrolysis of starch, extrusion cooking, and hydrothermal treatments as an economical alternative to the batch-cooking process in the production of RS. The enzyme resistance of the extruded starch was compared with that of the batch-cooked sample. The effect of starch molecular weight on the RS formation was also studied and discussed.

Materials and Methods

Materials

Native normal-maize starch (NMS) was obtained from Cargill (Hammond, IN). The heat-stable α-amylase from Bacillus licheniformis (Cat. No. A3403), protease from Bacillus licheniformis (Cat. No. P3910), pancreatin from porcine pancreas (Cat. No. P7545), glucoamylase from Aspergillus Niger (Cat. No. A9913 for RS content determined using AOAC Method 991.43; and Cat. No. A7095 for RS content determined using Englyst’s method), tris(hydroxymethyl)aminomethane (Cat. No. T1503), 2-morpholinoethanesulfonic acid (Cat. No. M3671), and celite (Cat. No. C8656) were purchased from Sigma Chemical
Co. (St. Louis, MO) and used as received. D-Glucose assay kits (GOPOD Format) were purchased from Megazyme International Ireland Ltd. (Co. Wicklow, Ireland).

**Acid modification**

Starch was partially hydrolyzed using a hydrochloric acid solution (1.0 M, 25% w/w) at 25°C for 40 h. The resulting acid-modified normal-maize starch (AMMS) was washed several times with distilled water until it reached a neutral pH, and then washed once with absolute ethanol before drying at 25°C. The percentage acid hydrolysis of starch was calculated on the basis of soluble sugar released to the supernatant. The soluble sugar in the supernatant was analyzed with phenol and sulfuric acid (Dubois and others 1956), and the color developed was quantified using a spectrophotometer (Beckman DU 520, Beckman Coulter, Fullerton, CA) at 490 nm. Glucose solutions at different concentrations (20, 40, 60, 80, and 100 μg/mL) were used to generate a standard curve.

\[
\% \text{ acid hydrolysis} = \frac{\text{mass of soluble dextrin}}{\text{total mass of starch}} \times 0.9 \times 100\%
\]

**Batch cooking**

Starch samples of NMS or AMMS with moisture contents of 50, 60, 67, 70, 75, 80, and 85% were sealed in containers, and heated in a boiling-water bath for 15 min with stirring until the starch gelled. The gel was then subjected to hydrothermal treatment. Batch-cooked samples processed with 67% moisture were used for comparison with extruded samples.
Extrusion cooking

The AMMS was adjusted to 40% moisture, mixed vigorously, and then allowed to equilibrate in a sealed container at 25°C overnight before extrusion. The extrusion cooking was carried out using a co-rotating extruder (Leistritz Micro-18, American Listritz Extruder Co., Somerville, NY) with a 30:1 screw length to diameter ratio and a 3.175-mm die opening. High-shear screws (with more kneading blocks) and low-shear screws (with fewer kneading blocks) were used for comparison in this study. The extruder barrel was composed of six programmable heating zones with a temperature profile from the feeder to the die: 80°C, 90°C, 100°C, 105°C, 100°C, and 45°C. The AMMS sample was fed into the extruder at a rate of about 2 kg/h. The speed of the screws was set at 100 rpm. Manual stretching of the hot extrudate at the die was applied to enhance the crystallinity of starch molecules following the method used for polyethylene terephthalate and polyethylene extrusion (Li and others 2002; Li and others 2004). Extrudates were collected as continuous strands with a moisture content of 30 - 35%, and were subjected to the same hydrothermal treatment in sealed containers as the batch-cooked samples.

Hydrothermal treatment

Samples in sealed containers were heated at 110°C for 1 or 3 d with or without a prior freezing treatment at -20°C for 8 h. The samples were then removed from the containers, dried at 110°C for an additional day, and ground subsequently using a coffee grinder and a cyclone mill (Udy Corp., Ft. Collins, CO). The ground sample was passed though a screen of 0.5-mm openings.
Average molecular weight of starch

The average molecular weights of NMS and AMMS were determined using high-performance size-exclusion chromatography equipped with multi-angle laser-light scattering and refractive-index detectors (HPSEC-MALLS-RI) (Yoo and Jane 2002). A starch sample (1 mg) was wetted with water (0.01 mL) and then mixed with dimethyl sulfoxide (0.09 mL). The starch dispersion was heated in a boiling-water bath with stirring for 1 h and then stirred at ~25°C for additional 16 h. Starch was precipitated using 4 volumes of absolute ethanol, centrifuged, redispersed in hot water (2 mL), and stirred in a boiling-water bath for 30 min. The starch dispersion was filtered though a nylon membrane of 5.0-μm pore size and then injected into a HPSEC system consisting of an isocratic pump (HP 1050, Hewlett Packard, Valley Forge, PA), a guard column (OHpak SB-G, Showa Denko K. K., Tokyo, Japan), two sequentially connected analytical columns (Shodex OHpak SB-806HQ and SB-804HQ, Showa Denko K. K., Tokyo, Japan), a multi-angle laser-light scattering detector (DAWN DSP Laser Photometer, Wyatt Tech. Co., Goleta, CA), and a refractive-index detector (Agilent 1100 series, Agilent Tech., Germany). The temperature of the column was maintained at 50°C, and the temperature of the refractive-index detector was set at 35°C. The HPSEC-MALLS-RI system was calibrated using pullulan standards (Shodex P-82, Showa Denko K.K., Tokyo, Japan). The analysis was done in duplicate.

Resistant starch content

The RS content of batch-cooked and extruded samples was analyzed using AOAC Method 991.43 for total dietary fiber (Horwithz 2003) and Englyst’s method (1992) with few modifications (Li and others 2008). For RS content determined using the Englyst’s method
(1992), the starch samples (1.00 g, dry starch basis, dsb) in an acetate buffer solution (20 mL, 0.1 M, pH 5.2) were pre-cooked in a boiling-water bath for 30 min before the analysis. The rapidly digestible starch (RDS) and slowly digestible starch (SDS) contents of the cooked starch samples were also determined after 20 and 120 min hydrolysis, respectively. The RS content determined using the AOAC Method 991.43 was done in duplicate, and the RDS, SDS, and RS contents determined using the Englyst’s method were done in triplicate.

**Thermal properties**

A precisely weighted sample (~3 mg, dsb) was mixed with deionized water (9 μL) and sealed in a stainless steel pan. The sample in the pan was allowed to equilibrate at ~25°C for 1 h. It was then heated from 10°C to 150°C with a heating rate of 10°C/min using a differential scanning calorimeter (DSC-7, Perkin-Elmer, Norwalk, CT). An empty stainless steel pan was used as the reference and indium was used for calibration. The analysis was done in duplicate.

**Starch crystallinity**

Starch samples were equilibrated in a chamber of 100% relative humidity for 24 h at ~25°C. The X-ray diffraction pattern of the starch and the percentage of crystallinity were determined using a D-500 diffractometer (Siemens, Madison, WI) (Yoo and Jane 2002). 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 sec.
Statistical analysis

Correlations of the RS content and the factors used during the cooking process and the hydrothermal treatment were analyzed using linear regression procedure (PROC REG) in SAS (version 9.1, SAS Institute, Inc., Cary, NC).

Results and Discussion

Acid modification of normal-maize starch

The hydrolysis of NMS using a hydrochloric acid solution (1.0 M) at 25°C for 40 h produced 1.62% (w/w, dsb) soluble sugar. The average molecular weight of NMS was reduced from $3.46 \times 10^7$ to $2.27 \times 10^6$ g/mol after the acid hydrolysis. The amylopectin (Fraction I) of NMS was hydrolyzed to smaller molecules (Fraction II) (Figure 1).

Resistant starch content

All batch-cooked and extruded starch products had greater RS contents than the NMS and AMMS without batch or extrusion cooking (< 2%) determined using the AOAC Method 991.43 for total dietary fiber (Table 1). The increased RS contents were the results of starch retrogradation that occurred after gelatinization of NMS and AMMS starches using either batch- or extrusion-cooking process. The RS contents of all the products further increased after the hydrothermal treatment at 110°C for 1 or 3 d because the hydrothermal treatment melted unstable crystallites and allowed the propagation of heat-stable starch crystallites (Slade and Levine 1987; Jang and Pyun 1997).
The RS contents of the batch-cooked NMS and AMMS products (BN and BA, respectively) positively correlated with the moisture contents of the starch samples during the batch cooking and subsequent hydrothermal treatment at 110°C for 1 d (Figure 2). This could be attributed to the increase in the starch retrogradation resulting from increased mobility of the starch molecules with a larger moisture content (Jang and Pyun 1997).

When the moisture content of the starch samples was in a range between 70% and 85% during the batch cooking and hydrothermal treatment, the RS contents of the BN products were similar to the BA products (Figure 2). When the moisture content of the starch samples decreased to 50-70%, the BA products, however, had greater RS contents than their BN counterparts using the same batch-cooking process and hydrothermal treatment. At a moisture content of 67% and the same batch-cooking process, the BA products consistently possessed greater RS contents than their BN counterparts after the hydrothermal treatment for 1 or 3 d with or without prior freezing treatment at -20°C (Table 1). The partially hydrolyzed, smaller starch molecules in the BA samples retrograded faster than the native starch molecules in the BN samples because the smaller starch molecules had more freedom and greater mobility at a moisture content below 70% at 110°C (Zhang and Jackson 1992; Chang and Lin 2007).

The extruded AMMS products without the subsequent hydrothermal treatment produced less RS contents (2.1-2.5%) than the batch-cooked AMMS counterparts (7.7%). This difference could be attributed to that the extrusion cooking of AMMS was carried out at a lower moisture content than the batch-cooking process. After the hydrothermal treatment, the RS contents of the extruded AMMS products prepared either using low shear (LSA) screws or high shear (HSA) screws were similar to those of the batch-cooked (BA) products.
The RS contents of the batch-cooked and extruded products also increased with the duration of the hydrothermal treatment up to 3 d (Figure 3).

Stretching of synthetic linear polymers, such as polyethylene terephthalate and polyethylene, is known to increase their crystallinity (Li and others 2002; Li and others 2004). The crystallinity of the LSA sample was 14.9% and increased to 26.2% after manual stretching (LSA-MS), and that of the HSA sample was 12.2% and increased to 14.8% for HSA-MS (Table 1). The RS contents of the extruded products, however, did not increase after stretching, and some even decreased. The decrease in the RS content of the stretched extrudate could be attributed to the microporous structure formed in the extrudate during stretching (Kitamura and others 1999; Li and others 2002), which increased the surface area of the extruded products and, thus, facilitated amylase hydrolysis.

Freezing treatment at -20°C for 8 h prior to the hydrothermal treatment increased the RS contents of the BN products from 8.2% to 10.6% and from 9.5% to 13.9% after 1- and 3-d hydrothermal treatment, respectively (Table 1). Nucleation occurs more rapidly at a subfreezing temperature (Slade and Levine 1987; Jang and Pyun 1997), and thus it increased the RS contents of the BN products. The RS contents of the batch-cooked and extruded AMMS products, however, did not increase with prior freezing (Figure 4). These results could be attributed to the greater freedom and mobility of the smaller starch molecules in the AMMS, which crystallized effectively without prior freezing (Zhang and Jackson 1992; Chang and Lin 2007).

RS contents of selected samples were also analyzed using Englyst’s method (1992), and their RDS and SDS contents were determined. The RS contents of the BA, BN, LSA, and HSA products determined using the Englyst’s method (19.0-32.9%) were greater than those
of the freshly boiled NMS and the AMMS without batch or extrusion cooking (11.0-12.2%) (Table 2). These differences were attributed to the presence of the heat-stable retrograded amylose in the batch-cooked and the extruded products. The RS contents of the products, in general, increased slightly after the hydrothermal treatment.

**Starch Crystallinity**

X-ray diffractograms of the BN, BA, LSA, HSA, LSA-MS, and HSA-MS products are shown in Figure 5 and percentages crystallinity are listed in Table 1. Both NMS and AMMS samples showed the A-type X-ray diffraction pattern (Figure 5A and B, respectively). The batch-cooked and extruded products without the hydrothermal treatment had small percentages of the V- and either B- or C-type crystallinity. The BN products showed a mixture of the A- and V-type crystalline structure after 3-d hydrothermal treatment (Figure 5A), whereas the BA products showed a mixture of the C- and V-type crystalline structure (Figure 5B). The C-type crystallinity is a mixture of the A- and B-type crystalline structure. The LSA and LSA-MS products without the hydrothermal treatment had a mixture of the C- and V-type crystalline patterns (Figure 5C and E, respectively), whereas the HSA and HSA-MS products without the hydrothermal treatment had a mixture of the A- and V-type crystalline patterns (Figure 5D and F, respectively). The LSA-MS and HSA-MS products before the hydrothermal treatment had greater percentages crystallinity (Figure 5E and F) than their non-stretched counterparts (Figure 5C and D) because stretching enhanced the alignment of the starch molecules and resulted in greater crystallinity (Li and others 2002; Li and others 2004). All the extruded products after the hydrothermal treatment had a mixture of the A- and V-type crystalline patterns, which was different from the BA products (Figure
The presence of the C-type crystallites in the BA products could be attributed to the greater moisture content in the samples during the batch cooking and the hydrothermal treatment. It is known that a greater moisture-content of starch favors the B-type polymorph, whereas a limited moisture-content of starch favors the A-type polymorph (Gidley 1987). The V-type crystalline pattern of the samples indicated the presence of amylose-lipid complex in all the batch-cooked and extruded products. The percentage crystallinity of batch-cooked and extruded products increased with the length of the hydrothermal treatment (Table 1 and Figure 5), which confirmed the propagation of starch crystallites during the hydrothermal treatment (Slade and Levine 1987; Jang and Pyun 1997).

**Thermal properties**

The DSC profiles of all the batch-cooked and extruded products heated from 10°C to 150°C showed four endotherms. Endotherms I, II, III, and IV were designated for the thermal transitions that occurred from 41°C to 73°C, from 64°C to 88°C, from 84°C to 105°C, and above 102°C, respectively (Table 3). The Endotherms I and II were attributed to the melting of B- and A-type crystallites of retrograded amylopectin, respectively (Whittam and others 1990; Crochet and others 2005). The Endotherm III was attributed to the melting of the amorphous amylose-lipid complex (form I). The Endotherm IV was overlapped peaks of the melting of the crystalline amylose-lipid complex (form II) (Biliaderis and Galloway 1989) and melting of the retrograded amylose (Sievert and Pomeranz 1990; Gruchala and Pomeranz 1993).

The thermograms of BN and extruded AMMS products showed a change from the Endotherm I to the Endotherm II after hydrothermal treatments (Table 3). These results
agreed with the change from the C-type X-ray pattern to the A-type pattern after the hydrothermal treatment (Figure 5A, C, D, E, and F). The A-type polymorph was preferably produced when the starch was retrograded at a higher temperature or at a lower moisture content (Gidley 1987). The melting temperature of the retrograded starch also increases when the starch is stored at an elevated temperature, which is attributed to the formation of longer, more stable double helices (Jang and Pyun 1997). The BA products, however, did not show Endotherm II, which was consistent with its B-type crystalline component having a stronger peak at 17° 2θ (Figure 5B). This result was attributed to the higher moisture content of the BA samples during retrogradation (Gidley 1987). The presence of amylose-lipid complex in all the batch-cooked and extruded products agreed with the V-type crystalline pattern observed in the X-ray diffractograms (Figure 5).

The thermal transition peak of the Endotherm IV of the extruded products became broader after the hydrothermal treatment, and the onset temperature changed to 102°C instead of 123°C. This lower onset temperature of 102°C was attributed to the melting of crystalline amylose-lipid complex (form II), which was converted from the amorphous amylose-lipid complex (form I, Tm ~84°C in Endotherm III). The enthalpy changes of the Endotherm III of the extruded products decreased after the hydrothermal treatment, which agreed with the conversion of the amorphous amylose-lipid complex (form I, Endotherm III) to the crystalline complex (form II, Endotherm IV). The crystalline amylose-lipid complex (form II) appeared in all the batch-cooked products with or without the hydrothermal treatment. The differences between the melting temperature of Endotherm IV of the batch-cooked and extruded products could be attributed to the difference in the moisture content of starch samples during the batch- and extrusion-cooking processes (67% and 40% moisture content).
content, respectively). At the low moisture content of extrusion cooking, there was no crystalline amylose-lipid complex formation and the temperature of Endotherm IV was exclusively retrograded amylose ($T_o \sim 121 - 125^\circ C$). After the hydrothermal treatment, the crystalline amylose-lipid complex developed and appeared in the Endotherm IV with onset temperature at $103 - 107^\circ C$. On the basis of the data of the thermal properties and the enzyme resistance, we concluded that both retrograded amylose and crystalline amylose-lipid complex (form II) contributed to the RS contents (Jane and Robyt 1984; Seneviratne and Biliaderis 1991).

**Conclusion**

All the batch-cooked and extruded starch products had greater RS contents than the NMS and AMMS without batch or extrusion cooking. The RS contents of the products increased with increasing moisture content of the starch samples during the batch cooking and subsequent hydrothermal treatment at 110°C. The partially hydrolyzed, smaller starch molecules in the AMMS had more freedom and mobility to retrograde and produced greater RS content than the larger molecules in the NMS. The hydrothermal treatment at 110°C increased the RS contents of all the samples. This was attributed to the formation of heat-stable crystalline amylose-lipid complex and retrograded amylose, which was confirmed by the X-ray diffractograms and the DSC thermograms. The results suggested that the combination of mild acid hydrolysis of starch, extrusion cooking, and hydrothermal treatment can be used to increase RS content.
Acknowledgements

The authors thank Dr. Perminus Mungara for the assistance with extrusion cooking and the Department of Statistics, Iowa State University (Ames, IA), for the assistance in the statistical analysis.

References


Table 1 – Resistant starch contents (determined using AOAC Method 991.43 for total dietary fiber) and percentages of crystallinity of batch-cooked and extruded products after hydrothermal and freezing treatments.

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>Freezing treatment at -20°C (h)</th>
<th>Hydrothermal treatment at 110°C (d)</th>
<th>RSb (%)</th>
<th>Crystallinity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMS</td>
<td>0</td>
<td>1.5 ± 0.0</td>
<td>34.0</td>
<td></td>
</tr>
<tr>
<td>AMMS</td>
<td>0</td>
<td>1.7 ± 0.1</td>
<td>36.5</td>
<td></td>
</tr>
<tr>
<td>BN</td>
<td>0</td>
<td>4.5 ± 1.4</td>
<td>8.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>8.2 ± 0.8</td>
<td>20.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>9.5 ± 1.1</td>
<td>23.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>10.6 ± 1.5</td>
<td>NDc</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>13.9 ± 0.6</td>
<td>NDc</td>
<td></td>
</tr>
<tr>
<td>BA</td>
<td>0</td>
<td>7.7 ± 0.1</td>
<td>15.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>11.7 ± 1.6</td>
<td>26.3</td>
<td></td>
</tr>
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<td></td>
<td>0</td>
<td>17.4 ± 2.6</td>
<td>27.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>13.6 ± 1.5</td>
<td>NDc</td>
<td></td>
</tr>
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<td></td>
<td>8</td>
<td>15.1 ± 0.7</td>
<td>NDc</td>
<td></td>
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<tr>
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<td>14.9</td>
<td></td>
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<tr>
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<td>12.1 ± 0.7</td>
<td>36.3</td>
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<tr>
<td></td>
<td>0</td>
<td>15.5 ± 2.6</td>
<td>43.8</td>
<td></td>
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<td>8</td>
<td>9.3 ± 1.2</td>
<td>NDc</td>
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<td></td>
<td>8</td>
<td>12.9 ± 0.0</td>
<td>NDc</td>
<td></td>
</tr>
<tr>
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<td>2.5 ± 0.2</td>
<td>12.2</td>
<td></td>
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<tr>
<td></td>
<td>0</td>
<td>13.2 ± 1.6</td>
<td>28.5</td>
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<td>38.8</td>
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<td>14.5 ± 0.3</td>
<td>NDc</td>
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<td></td>
<td>8</td>
<td>15.8 ± 1.6</td>
<td>NDc</td>
<td></td>
</tr>
<tr>
<td>LSA-MS</td>
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<td>2.2 ± 0.1</td>
<td>26.2</td>
<td></td>
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<tr>
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<td>9.3 ± 3.2</td>
<td>39.5</td>
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<td>13.7 ± 1.4</td>
<td>37.1</td>
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<td>14.3 ± 1.2</td>
<td>NDc</td>
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<tr>
<td>HSA-MS</td>
<td>0</td>
<td>2.1 ± 0.1</td>
<td>14.8</td>
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<tr>
<td></td>
<td>0</td>
<td>9.3 ± 0.7</td>
<td>30.9</td>
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<td></td>
<td>0</td>
<td>12.4 ± 1.6</td>
<td>30.1</td>
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<td>9.8 ± 1.5</td>
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<td></td>
<td>8</td>
<td>14.4 ± 1.2</td>
<td>NDc</td>
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</tr>
</tbody>
</table>

aNMS = native normal-maize starch, AMMS = acid-modified normal-maize starch, BN = batch-cooked products from native normal-maize starch, BA = batch-cooked products from acid-modified normal-maize starch, LSA = acid-modified normal-maize starch products extruded using low-shear screws, HSA = acid-modified normal-maize starch products extruded using high-shear screws, and MS = manual stretching. The moisture contents of the starch samples during batch and extrusion cooking were 67% and 40%, respectively.

bResistant starch (RS) content determined using AOAC Method 991.43 for total dietary fiber. Mean ± standard deviation from duplicate.

cNot determined.
Table 2 – Rapidly digestible starch, slowly digestible starch, and resistant starch contents (determined using Englyst’s method) of batch-cooked and extruded products after hydrothermal treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>RDS</th>
<th>SDS</th>
<th>RS</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(%)</td>
<td>(%)</td>
<td>(%)</td>
</tr>
<tr>
<td>NMS</td>
<td>81.6 ± 2.3</td>
<td>7.4 ± 1.3</td>
<td>11.0 ± 1.0</td>
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<tr>
<td>AMMS</td>
<td>84.6 ± 0.3</td>
<td>3.2 ± 0.6</td>
<td>12.2 ± 1.0</td>
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<tr>
<td>BN-H0</td>
<td>62.4 ± 2.3</td>
<td>11.4 ± 4.6</td>
<td>26.2 ± 2.3</td>
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<tr>
<td>BN-H1</td>
<td>63.7 ± 1.4</td>
<td>11.8 ± 4.5</td>
<td>25.6 ± 3.8</td>
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<tr>
<td>BN-H3</td>
<td>55.5 ± 4.5</td>
<td>11.7 ± 1.6</td>
<td>32.9 ± 2.9</td>
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<td>BA-H0</td>
<td>59.1 ± 1.5</td>
<td>8.2 ± 1.7</td>
<td>32.7 ± 3.0</td>
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<td>BA-H1</td>
<td>57.2 ± 2.6</td>
<td>11.1 ± 3.3</td>
<td>31.7 ± 0.7</td>
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<td>BA-H3</td>
<td>58.2 ± 0.1</td>
<td>10.4 ± 3.5</td>
<td>31.4 ± 3.5</td>
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<td>LSA-H0</td>
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<td>7.9 ± 1.0</td>
<td>19.0 ± 0.4</td>
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<td>LSA-H1</td>
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<td>9.7 ± 0.0</td>
<td>26.6 ± 0.2</td>
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<td>LSA-H3</td>
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<tr>
<td>HSA-H3</td>
<td>62.0 ± 2.8</td>
<td>12.1 ± 1.5</td>
<td>25.9 ± 1.9</td>
</tr>
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</table>

aNMS = native normal-maize starch, AMMS = acid-modified normal-maize starch, BN = batch-cooked products from native normal-maize starch, BA = batch-cooked products from acid-modified normal-maize starch, LSA = acid-modified normal-maize starch products extruded using low-shear screws, HSA = acid-modified normal-maize starch products extruded using high-shear screws, and H# = hydrothermal treatment day.

bRapidly digestible starch (RDS) and slowly digestible starch (SDS) contents were determined using Englyst’s method (1992) after 20 and 120 min hydrolysis, respectively, and resistant starch (RS) content was the amount of starch remained after 120 min hydrolysis. Mean ± standard deviation from triplicate.
<table>
<thead>
<tr>
<th>Treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Endotherm I</th>
<th>Endotherm II</th>
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<tr>
<td></td>
<td>$T_o$ (°C)</td>
<td>$T_p$ (°C)</td>
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<tr>
<td>BN-H0</td>
<td>46.1 ± 1.5</td>
<td>57.7 ± 0.2</td>
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<tr>
<td>BN-H1</td>
<td>47.3 ± 1.1</td>
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<td>BN-H3</td>
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<td>BA-H0</td>
<td>44.7 ± 0.5</td>
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<td>43.9 ± 0.4</td>
<td>53.5 ± 0.1</td>
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<td>LSA-H0</td>
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<td>HSA-H0</td>
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<td>HSA-MS-H0</td>
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<td>43.7 ± 0.9</td>
<td>50.4 ± 1.8</td>
</tr>
<tr>
<td>HSA-M-H3</td>
<td>ND</td>
<td>ND</td>
</tr>
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</table>

<sup>a</sup>BN = batch-cooked products from native normal-maize starch, BA = batch-cooked products from acid-modified normal-maize starch, LSA = acid-modified normal-maize starch products extruded using low-shear screws, and HSA = acid-modified normal-maize starch products extruded using high-shear screws, MS = manual stretching, and H# = hydrothermal treatment day.

<sup>b</sup>$T_o$ = onset temperature, $T_p$ = peak temperature, $T_c$ = conclusion temperature, and $\Delta H$ = enthalpy change. Mean ± standard deviation from duplicate.

<sup>c</sup>ND = not detected.
<table>
<thead>
<tr>
<th>Treatment(^a)</th>
<th>Endotherm III</th>
<th>Endotherm IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(T_o)(^b) (°C)</td>
<td>(T_p) (°C)</td>
</tr>
<tr>
<td>BN-H0</td>
<td>ND(^c)</td>
<td>ND</td>
</tr>
<tr>
<td>BN-H1</td>
<td>ND</td>
<td>86.4 ± 1.4</td>
</tr>
<tr>
<td>BN-H3</td>
<td>ND</td>
<td>87.3 ± 5.2</td>
</tr>
<tr>
<td>BA-H0</td>
<td>ND</td>
<td>ND</td>
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<tr>
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<td>86.4 ± 1.4</td>
<td>90.2 ± 2.8</td>
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<tr>
<td>BA-H3</td>
<td>87.3 ± 5.2</td>
<td>89.3 ± 7.0</td>
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<tr>
<td>LSA-H0</td>
<td>84.0 ± 1.2</td>
<td>96.3 ± 1.5</td>
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<tr>
<td>LSA-H1</td>
<td>88.3 ± 4.0</td>
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<td>LSA-H3</td>
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<tr>
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<td>84.2 ± 0.2</td>
<td>96.7 ± 0.7</td>
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<tr>
<td>HSA-H1</td>
<td>91.0 ± 0.3</td>
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<tr>
<td>HSA-H3</td>
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<td>ND</td>
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<tr>
<td>LSA-MS-H0</td>
<td>87.5 ± 1.3</td>
<td>97.5 ± 0.2</td>
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<td>LSA-MS-H1</td>
<td>89.4 ± 0.5</td>
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<td>LSA-MS-H3</td>
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<td>HSA-MS-H0</td>
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<td>HSA-MS-H1</td>
<td>69.3 ± 1.0</td>
<td>90.3 ± 2.0</td>
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<tr>
<td>HSA-MS-H3</td>
<td>87.8 ± 1.1</td>
<td>92.3 ± 0.4</td>
</tr>
</tbody>
</table>

\(^a\)BN = batch-cooked products from native normal-maize starch, BA = batch-cooked products from acid-modified normal-maize starch, LSA = acid-modified normal-maize starch products extruded using low-shear screws, and HSA = acid-modified normal-maize starch products extruded using high-shear screws, MS = manual stretching, and H# = hydrothermal treatment day.

\(^b\)\(T_o\) = onset temperature, \(T_p\) = peak temperature, \(T_c\) = conclusion temperature, and \(\Delta H\) = enthalpy change. Mean ± standard deviation from duplicate.

\(^c\)ND = not detected.
Figure 1 – Molecular weight distribution of native (—) and acid-modified (––) normal-maize starch.
Figure 2 – Correlation of the resistant starch content of batch-cooked products from (●) BN and (■) BA and the moisture content during batch cooking and hydrothermal treatment at 110°C for 1 d. The resistant starch content was analyzed using AOAC Method 991.43. Error bars show the standard deviation from two measurements.
Figure 3 – Correlation of the resistant starch content of batch-cooked and extruded products ((♦) BN, (◊) BA, (●) LSA, (○) LSA-MS, (▲) HSA, and (Δ) HSA-MS) and the duration of hydrothermal treatment at 110°C without prior freezing treatment. The resistant starch content was analyzed using AOAC Method 991.43.
Figure 4 – Correlation of the resistant starch content of batch-cooked and extruded products

((•) BN, (◊) BA, (●) LSA, (○) LSA-MS, (▲) HSA, and (△) HSA-MS) and the duration of freezing treatment at -20°C followed by 1 or 3 d hydrothermal treatment at 110°C. The resistant starch content was analyzed using AOAC Method 991.43.
Figure 5 – X-ray diffraction patterns of batch-cooked and extruded samples. NMS = native normal-maize starch, AMMS = acid-modified normal-maize starch, BN = batch-cooked products from native normal-maize starch, BA = batch-cooked products from acid-modified normal-maize starch, LSA = acid-modified normal-maize starch products extruded using low-shear screws, HSA = acid-modified normal-maize starch products extruded using high-shear screws, MS = manual stretching, and H# = hydrothermal treatment day. Numbers in parentheses are the percentage of crystallinity.
GENERAL CONCLUSION

The *opaque-2* maize (*o2*) mutants had lower protein contents than the wild-type (WT) counterparts because the biosynthesis of zein was suppressed in the *o2*-maize mutants. The weak protein matrices in the *o2* kernel caused kernels to be easily broken apart during dry grinding, resulting in less mechanically damaged starch granules than did the dry-ground WT-maize and quality protein maize (QPM) samples.

Isolated starch granules obtained by wet milling of whole kernels showed no mechanical damage. The isolated starch of severe *o2* mutant B46o2 had the greatest susceptibility to porcine-pancreatic α-amylase (PPA) hydrolysis because of its low amylose content and its greater crystalline defects. Although the QPM starch had lower amylose content than the B46o2 starch, the isolated QPM starch was hydrolyzed at a slower rate by PPA because of its larger starch-granule size.

Mechanically damaged starch granules in the dry-ground WT maize were more susceptible to PPA hydrolysis at the first 3 hours of digestion. After 6 hours of digestion, starches in the dry-ground *o2*-maize samples were hydrolyzed faster than those in the dry-ground WT-maize samples because of reduced protein contents of the *o2* mutants and more digestible of *o2* starch. At the end of 48 hours of hydrolysis, the starch in the dry-ground QPM was hydrolyzed to a similar level as that in the dry-ground B46o2 maize, which could be attributed to the low amylose content of QPM starch. The dry-ground maize displayed larger starch digestibility than the isolated starch, which was attributed to the presence of mechanically damaged starch granules and endogenous amylases in the dry-ground maize.
samples. The results suggested that o2 maize and QPM are highly suitable for feed and ethanol applications.

A novel technology to produce starch with high enzyme resistance was developed by introducing amylose-lipid complex to high-amylose maize starch VII (HA7). HA7, a B-type polymorphic starch with amylose content of about 70%, contained 42% resistant starch (RS) determined using AOAC Method 991.43 for dietary fiber. The RS content of the HA7 increased up to 75% when the HA7 starch was complexed with fatty acid (FA). Pre-swelling and debranching of the HA7 starch granules was used to enhance the amylose-lipid complex formation. The greatest RS content (75%) was produced when the HA7 starch was heated at 80°C, debranched with isoamylase (ISO), and complexed with stearic acid (SA). Heating at 80°C allowed the HA7 starch granules to swell with less starch gelatinization, lower soluble total carbohydrate, and lower soluble amylose than heating at temperature above 80°C. Heating of HA7 at temperature below 80°C prior to debranching and lipid complex formation produced a lower RS content because the HA7 starch granules were not adequately swollen to react with the debranching enzyme and to complex with the FA. ISO debranching of the HA7 starch produced a greater amount of linear molecules than pullulanase (PUL) debranching of the HA7 starch, hence ISO debranching produced greater RS contents. The complex formation of the HA7 starch with SA produced greater RS contents than complex formation with PA because SA formed a stronger helical complex with starch molecules than PA. The HA7-FA complex samples processed at 80°C was still in granular shape, and those processed at higher temperature were agglomerated and some lost its granular structure.

The presence of the amylose-lipid complex in the HA7-FA complex samples was confirmed by their DSC diffractograms and X-ray diffractograms. In addition, the removal
of lipid using a methanol solution reduced the RS contents of the HA7-FA complex samples. This is attributed to the lower amount of the amylose-lipid complex in the samples after defatting. The resistant residues of the native HA7, HA7 control, and HA7-FA complex samples prepared at 80°C were collected after enzyme hydrolysis following the AOAC Method 991.43. The resistant residues of the native HA7 and the HA7 control showed dispersed materials surrounding partially hydrolyzed granules, whereas those of the HA7-FA complex were still intact granular shape. The amylopectin of the native HA7 was hydrolyzed to a greater extent by amylase than that of the HA7-FA complex samples. The results suggested that the amylose-lipid complex restricted the swelling of HA7 starch granules during cooking and protected the starch molecules from amylase hydrolysis.

A RS bread was made of the HA7-FA complex, which contained 34.4% RS determined using the AOAC Method 991.43. The postprandial plasma-glucose responses of 20 healthy male subjects were significant reduced after the ingestion of the RS bread compared with the control white bread. The reduction in the postprandial plasma-glucose responses also led to a reduced secretion of insulin by the beta-cells of pancreas. The increase in the postprandial plasma-insulin response was positively correlated with the subjects’ BMI. This could be attributed to the greater insulin secretion by the pancreatic beta-cells to overcome the insulin resistance pre-developed in the subjects with greater BMI. The reduction in the postprandial plasma-insulin response after the ingestion of the RS bread was also more pronounced in the subjects with greater BMI. Thus the HA7-FA complex can be used in the interventions to improve insulin sensitivity in human and to prevent metabolic syndrome including diabetes, obesity, and cardiovascular disease.
The other novel technology to produce RS in our study was using extrusion cooking of normal-maize starch followed by hydrothermal treatment at 110°C. The normal-maize starch was mildly hydrolyzed using a hydrochloric solution to increase the freedom and the mobility of the starch molecules so that they can retrograde effectively during the hydrothermal treatment. Freezing at -20°C of the batch-cooked and the extruded samples prior to the hydrothermal treatment did not increase the RS contents. The extrudates were also collected with manual stretching at the die while it was still hot. The degree of the crystallinity of the stretched extruded products was increased compared with the non-stretched counterparts, but their RS contents were slightly lower. This could be attributed to microporous structure formed during the manual stretching of hot extrudates, which increased the surface area of the extruded products for amylase hydrolysis. The RS content of the extruded products was up to 15.8% and 29.8% determined using the AOAC Method 991.43 and Englyst’s method, respectively, which was not significantly different from the batch-cooked counterparts. The DSC thermograms and X-ray diffractograms showed that the increase in the RS contents in the batch-cooked and extruded products after the hydrothermal treatment was attributed to the presence of retrograded amylose and crystalline amylose-lipid complex, which dissociation temperatures are above 100°C.
APPENDIX – RESISTANT FOOD STARCHES
AND METHODS RELATED THERETO (US PATENT APPLICATION)

REFERENCE TO PRIOR APPLICATIONS

The present invention claims priority to U.S. provisional patent application serial number _____.

FEDERAL FUNDING STATEMENT

The present invention was not funded by federal grants or other programs.

FIELD OF THE INVENTION

[0001] The invention is in the fields of: food ingredients, particularly functional food ingredients such as digestion-resistant starches; food processing methods, particularly improved efficiencies in food starch manufacturing; and methods relating to improving health in mammals, particularly methods to reduce the negative effects of excess blood sugar, and methods to increase the positive effects of pre-biotic substances.

[0002] BACKGROUND OF THE INVENTION

[0003] Resistant starch is defined as starch that cannot be completely digested and absorbed in the small intestine. Some types of resistant starches have been shown to reduce calorie intake, moderate blood glucose levels, reduce triglyceride levels and improve colon health by increasing beneficial bacterial counts. Some resistant starches therefore also have been classified as "prebiotics," along with soluble and insoluble fibers. Although exact
modalities are unknown, some resistant starches are known to function such that little or no
digestion/absorption/fermentation commences until the molecules reach the colon. Sajilata,
et al., "Comprehensive Reviews in Food Science and Food Safety" (2006) provides a review
of resistant starch research.

[0004] In the past, commercial processing of resistant starch has been energy
intensive, requiring high heat for long periods of time, with an additional cooling or freezing
step. US Patent 5,051,271 provides an example of such processes. The resultant starches are
relatively expensive compared to other blood sugar-lowering and prebiotic foods, with some
known to lose their resistant nature when used as an ingredient in a baked item. A need
exists for improvements in processing and functionality of resistant starches.

SUMMARY OF THE INVENTION

[0005] The present processes are less time- and energy-consuming than those
in the industry. The processes do not involve severe heating and cooling/freezing cycle; the
total processing time is about two to four days, which is substantially shorter than other
technologies. Moreover, the present processes can be accomplished on readily-available and
multi-functional equipment.

[0006] The present starch material is granular and does not disperse to paste,
which makes it inexpensive compared to other resistant starches in industrial applications. If
further moisture reduction is desired, energy-saving centrifugation and/or filtration can be
used. The inventive starch material contains well-above the resistant-starch content of other
heat-stable products. Hence, they retain digestion resistance in the food products after heat
processing. This novel technology produces low-cost resistant starch.
The present invention provides modified resistant starches comprising a high-amylose starch complexed with lipid. Preferred are those resistant starches wherein the high-amylose starch is selected from the group consisting of: high-amylose cornstarch; high-amylose barley starch; high-amylose potato starch; high-amylose wheat starch; high-amylose rice starch; and high-amylose grain-derived starch. Also preferred are those modified resistant starches wherein the lipid is selected from the group consisting of: fatty acids; monoglycerides; diglycerides; and phospholipids. Most preferred are those resistant starches wherein the fatty acid is selected from the group consisting of: stearic acid; palmitic acid; myristic acid; butyric acid; oleic acid; and sodium propionate. Although also most preferred are those resistant starches wherein the high-amylose starch has been pre-treated with a debranching enzyme, and the lipid is a fatty acid, particularly wherein the debranching enzyme is selected from the group consisting of: pullulanase and isoamylase.

In particular, those modified resistant starches wherein the fatty acid is approximately 2 to 20% of the high amylose starch, when dry, and referenced by weight are provided. More preferred are those wherein the fatty acid is approximately 5 to 10%. Also provided are modified starches wherein the ratio of high amylose starch to fatty acid is from 5:1 to 50:1, preferably, those wherein the ratio is approximately 10:1 to 20:1.

Also provided are processes to produce the modified resistant starches herein, comprising: processing high amylose starch with lipid at a temperature in the range of 70°C to 90°C; and subsequently processing the starch and lipid at a temperature in the range of 40°C to 60°C. Preferred are those processes wherein the high-amylose starch is selected from the group consisting of: high-amylose cornstarch; high-amylose barley starch; high-amylose potato starch; high-amylose wheat starch; high-amylose rice starch;
and high-amylose grain-derived starch. Also preferred are those processes wherein the lipid is selected from the group consisting of: fatty acids; monoglycerides; diglycerides; and phospholipids. Preferred are those processes wherein the fatty acid is selected from the group consisting of: stearic acid; palmitic acid; myristic acid; butyric acid; oleic acid; and sodium propionate.

[0010] More preferred are those processes wherein the high-amylose starch has been pre-treated with a debranching enzyme, and the lipid is a fatty acid. Preferred are those processes wherein the pre-treating debranching enzyme is selected from the group consisting of: pullulanase and isoamylase.

[0011] Also provided are those processes wherein the fatty acid is approximately 2 to 20% of the high amylose starch, when dry, and referenced by weight, preferably wherein the fatty acid is approximately 5 to 10%.

[0012] Also provided are food compositions comprising a modified resistant starches herein.

[0013] Also provided are methods to affect the physiology of a mammal, selected from the group consisting of: decreasing plasma insulin response; decreasing plasma glucose response; increasing colonic fermentation; decreasing the risk of colon cancer; increasing digestive health; decreasing colonic pH; comprising administering the resistant starches herein, preferably in food form.

[0014] Definitions

[0015] "A" means one or more, as in "a fatty acid" means one or more types of fatty acids, and/or several molecules of fatty acid. "A resistant starch" means at least one molecule and/or at least one type of resistant starch. For instance "A modified resistant
starch comprising a resistant starch and a lipid" could mean several molecules of resistant starch and several molecules of lipids, and it can also mean several different types of resistant starches and several different types of lipids. However, the previous statement can also mean one molecule of a certain type of resistant starch, complexed with one molecule of lipid.

**BRIEF DESCRIPTION OF THE DRAWINGS**

[0016] Figure 1 is a graph of the average increase in plasma glucose responses of 20 male subjects after consuming control white bread and enzyme-resistant bread.

[0017] Figure 2 is a graph of the average increase in the plasma insulin responses of 20 male human subjects after consuming control white bread and enzyme-resistant bread.

**DETAILED DESCRIPTION**

[0018] The starches used in preparing enzyme resistant starches may be any high-amylose starch derived from any native source. Also suitable are starches derived from a plant obtained by standard breeding techniques including crossbreeding, translocation, inversion, transformation or any other method of gene or chromosome engineering to include variations thereof. In addition, starch or derived from a plant grown from induced mutations and variations of the above generic composition which may be produced by known standard methods of mutation breeding are also suitable herein.

[0019] Typical sources for the starches are cereals, tubers, roots, legumes and fruits. The source can be corn, pea, potato, sweet potato, banana, barley, wheat, rice, sago, amaranth, tapioca, arrowroot, canna, sorghum, and high amylose varieties thereof. As used
herein, the term “high amylose” is intended to include a starch containing at least about 40% amylose by weight.

[0024] The starch material useful in this invention also may include high amylose flour where the starch component of the flour contains at least 40% by weight of amylose. The term starch as used throughout this application is intended to include flour and when the high amylose content of flour is referred to throughout the application and claims, it is understood to refer to the amylose content of the starch component of the flour (e.g., 40% by weight of amylose based on the amount of starch in the flour). Such flour typically comprises protein (about 8 to 13% by weight), lipids (up to about 3% by weight) and starches (about 65 to 90% by weight) which include the specified high amylose content. Another useful high amylose starch is a substantially pure starch extracted from a plant source having an amylose extender genotype.

[0025] The resistant starch product of this invention may be used in any food or beverage product. Typical food products include, but are not limited to, baked goods such as breads, crackers, cookies, cakes, muffins, rolls, pastries and other grain-based ingredients; pasta; cereals such as ready-to-eat, puffed or expanded cereals and cereals which are cooked before eating; beverages; fried and coated foods; snacks; and cultured dairy products such as yogurts, cheeses, and sour creams.

[0026] The amount of resistant starch which can be added and used in any given food will be determined to a great extent by the amount that can be tolerated from a functional standpoint. In other words, the amount of resistant starch used generally will be as high as will be acceptable in organoleptic evaluation of the food. Generally the resistant
starch may be used in food applications at about 0.1 to 50%, by weight of the food and more particularly from about 1 to 25% by weight.

[0027] The resistant starch of this invention may also be used in a pharmaceutical or nutritional product, including but not limited to prebiotic and synbiotic compositions, diabetic foods and supplements, dietetic foods, foods to control glycemic response, and tablets and other pharmaceutical dosage forms. A prebiotic composition is a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth, activity or both of one or a limited number of bacterial species already resident in the colon. A synbiotic composition may be a yogurt, capsule or other form of introduction into the host animal, including human beings, in which prebiotics are used in combination with a live microbial food supplement. The live microbial food supplement beneficially affects the host animal by improving its intestinal microbial balance. Such live microbial food supplements may include, without limit, yeasts such as Saccharomyces, and bacteria such as the genera Bifobacterium, Bacteriodes, Clostridium, Fusobacterium, Propionibacterium, Streptococcus, Enterococcus, Lactococcus, Staphylococcus, Peptostreptococcus and Lactobacillus.

[0028] The resistant starches of the present invention can be also be used in low-fat margarine, snack dips, sour cream, mayonnaise, cream cheese and other spreads, yogurt, milkshakes, ice cream and frozen desserts. The resistant starches of the present invention are also suitable for inclusion in nutritional and dietary drinks, as well as in foods which are useful for the slow release of glucose, such as for diabetics. The resistant starch is also useful as a component of a stabilizer complex in frozen foods to control ice crystal
formation. The resistant starch of the present invention can be used in sugar-free foods as well; the amount of sugar, flour or fat in a given formulation which can be replaced with the microcrystalline starch-based product will depend in part on the formulation, the desired properties of the food and the amount of calorie and/or fat reduction or dietary fiber content desired. The product of the present invention can also be added as an extender to a formulation without reducing any of the other ingredients. The extended product has a lower calorie per volume compared with the unextended product.

[0029] For dough-based products, total amount of the flour component plus resistant starch used in the compositions of the present invention may range, for example, from about 20% by weight to about 80% by weight, preferably from about 45% by weight to about 75% by weight, based upon the weight of the dough. Unless otherwise indicated, all weight percentages are based upon the total weight of all ingredients forming the doughs or formulations of the present invention, except for inclusions such as flavor chips, nuts, raisins, and the like. Thus, "the weight of the dough" does not include the weight of inclusions.

[0030] The flour component may be replaced in whole or in part by conventional flour substitutes or bulking agents, such as polydextrose, hollocellulose, microcrystalline cellulose, mixtures thereof, and the like. Corn bran, wheat bran, oat bran, rice bran, mixtures thereof, and the like may also be substituted in whole or in part for the flour component to enhance color, or to affect texture.

[0031] Process-compatible ingredients, which can be used to modify the texture of the products produced in the present invention, include sugars such as sucrose, fructose, lactose, dextrose, galactose, maltodextrins, corn syrup solids, hydrogenated starch hydrolysates, protein hydrolysates, glucose syrup, mixtures thereof, and the like. Reducing
sugars, such as fructose, maltose, lactose, and dextrose, or mixtures of reducing sugars may be used to promote browning.

[0032] Oleaginous compositions which may be used to obtain the doughs and baked goods of the present invention may include any known shortening or fat blends or compositions useful for baking applications, and they may include conventional food-grade emulsifiers. Vegetable oils, lard, marine oils, and mixtures thereof, which are fractionated, partially hydrogenated, and/or interesterified, are exemplary of the shortenings or fats which may be used in the present invention. Edible reduced- or low-calorie, partially digestible or non-digestible fats, fat-substitutes, or synthetic fats, such as sucrose polyesters or triacyl glycerides, which are process-compatible may also be used. Preferred oleaginous compositions for use in the present invention comprise soybean oil.

[0033] In addition to the foregoing, the doughs of the invention may include other additives conventionally employed in crackers and cookies. Such additives may include, for example, milk by-products, egg or egg by-products, cocoa, vanilla or other flavorings, as well as inclusions such as nuts, raisins, coconut, flavored chips such as chocolate chips, butterscotch chips and caramel chips, and the like in conventional amounts.

[0034] The dough compositions of the present invention may contain up to about 5% by weight of a leavening system, based upon the weight of the dough.

[0035] The doughs of the present invention may include antimycotics or preservatives, such as calcium propionate, potassium sorbate, sorbic acid, and the like. Exemplary amounts may range up to about 1% by weight of the dough, to assure microbial shelf-stability.
[0036] Emulsifiers may be included in effective, emulsifying amounts in the doughs of the present invention. Exemplary emulsifiers which may be used include, mono- and di-glycerides, polyoxyethylene sorbitan fatty acid esters, lecithin, stearoyl lactylates, and mixtures thereof. Exemplary amounts of the emulsifier which may be used range up to about 3% by weight of the dough.

[0037] Production of the doughs of the present invention may be performed using conventional dough mixing techniques and equipment used in the production of cookie and cracker doughs. For example, the doughs may be sheeted, wire cut, extruded, coextruded, or rotary molded using conventional equipment. The resistant starch ingredient is preferably preblended with the flour component to obtain a substantially homogeneous mixture for mixing with the other dough ingredients.

EXAMPLES

Materials useful to make or analyze resistant starch.

[0038] The following materials were gathered for use in producing and testing resistant starch formulations and processes. High amylose cornstarch VII starch (HA7, AmyloGel™ 03003) and normal cornstarch (NC) were gifts of Cargill, Hammond, IN. Lecithin (LE, Ultralec®-P), diglycerides (DG, Enova™), and monoglycerides (MG3, Panalite® 90-03 K; MG70, Panalite® 90-70 K; MG130, Panalite® 90-130 K; the number represents the iodine value or the degree of unsaturation of monoglycerides) were gifts of ADM, Decatur, IL. The following enzymes and reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO) and used as received: sodium propionate (NaPr, Cat. No. P1880), butyric acid (BA, Cat. No. B2503), myristic acid (MA, Cat. No. M3253), palmitic acid (PA, Cat. No. P0625 and Cat. No. W283207), stearic acid (SA, Cat. No. W303518),
oleic acid (OA, Cat. No. 364525), heat-stable α-amylase from *Bacillus licheniformis* (Cat. No. A3403), protease from *Bacillus licheniformis* (Cat. No. P3910), glucoamylase from *Aspergillus Niger* (Cat. No. A9913), pullulanase from *Bacillus acidopullulyticus* (PUL, ≥400 units/mL, Cat. No. P2986), tris(hydroxylmethyl)aminomethane (Tris, Cat. No. T1503), 2-morpholinoethanesulfonic acid (MES, Cat. No. M3671), and celite (Cat. No. C8656). Technical grade isoamylase (ISO) from *Pseudomonas amylofera* (62,000 units/mL) was a gift of Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan).

**Methods used to analyze resistant starch content and protein content of food samples containing resistant starches.**

A. Enzymatic-Gravimetric Method (AOAC Method 991.43) for determining dietary fiber (resistant starch) content of a sample.

[0039] A precisely weighed starch sample (1 g, dry starch basis, dsb) was suspended in a MES-Tris buffer solution (0.05M, pH 8.2, 40 mL). Heat-stable α-amylase (500U) was added to the suspension. The mixture was incubated in a boiling-water bath with stirring for 30 minutes. The enzyme digestate was then cooled and equilibrated in a water bath at 60°C and incubated with protease (5.0 mg) at the same temperature for 30 minutes under agitation (120 rpm). The suspension was then adjusted to pH 4.4 – 4.6 by adding hydrochloric acid solution (0.561 M) and incubated with glucoamylase (300 μL) in the water bath at 60°C for 30 minutes under agitation (120 rpm). The enzyme digestate was then cooled to room temperature and filtered through a tared coarse fritted glass crucible with a layer of celite (1.0 g) on the surface as the filter aid. The collected solid residue was washed twice with 15 mL of deionized water, twice with 15 mL of 78% ethanol, twice with 15 mL of absolute ethanol, and once with 15 mL of acetone. The crucible with sample was dried in a convection oven at 110°C.
%RS = dry weight of residue/ dry weight of starch × 100

B. *Dumas Method for analyzing nitrogen contents of food samples made with resistant starches.*

The nitrogen contents of the residues of vital wheat gluten and noodle samples after enzyme treatments following the AOAC method 991.43 were detected using Dumas method (RapidN III, Elementar Americas, Inc., Mt. Laurel, NJ) following the procedure of Jung et al. (2003). The nitrogen content was converted to protein content by conversion factor of 5.33.

C. *Macro-Kjedahl Method for analyzing nitrogen contents of food samples made with resistant starches.*

The nitrogen contents of the residues of enriched bread flour, vital wheat gluten, and bread samples after enzyme treatments following the AOAC method 991.43 were analyzed using macro-Kjedahl method following the method of Jung et al. (2003). The residue from 1 g of enriched bread flour, vital wheat gluten, or bread sample was digested with 16 mL concentrated sulfuric acid in the presence of a mixture of catalyst containing CuSeO₃ and K₂SO₄ (Kjeldahl Digestion Mixture #600, Merck KGaA, Darmstadt, Germany) using a Büchi 435 Digestion Unit (Flawil, Switzerland). The distillation was carried out in an alkaline condition by adding NaOH (10N, 60mL) to the sample in a Büchi B-324 Distillation Unit (Flawil, Switzerland). The distilled ammonia was collected in a solution containing boric acid (4%) and pH indicator (0.375 g of methyl red and 0.250 g of methylene blue in 300 mL of 95% ethanol) was used to identify the end point of titration. The titration was carried out using standardized 0.1 N HCl. The nitrogen content was converted to protein content by conversion factor of 5.33.
Example 1. Preparing and analyzing novel resistant starches from HA7 and fatty acid – heating at 80°C.

[0042] An aqueous suspension of AmyloGel™ 03003 (10% w/w) ("HA7") was heated in a water bath at 80°C for 30 minutes. Fatty acid (10% w/w, dsb) was added to the suspension with stirring. The starch-fatty acid mixture was heated in the water bath at 80°C for additional 30 minutes. The mixture was then cooled to room temperature. The starch-fatty acid complex was recovered by centrifugation, washed with 50% ethanol, and dried in a convection oven at 50°C to a moisture content of below 12% (w/w). The RS contents of the resulting products are shown in Table 1.

[0043] Table 1. The resistant starch contents of Example 1.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Resistant starch (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA7 control</td>
<td>36.7 ± 0.2</td>
</tr>
<tr>
<td>HA7+10%PA</td>
<td>58.3 ± 1.7</td>
</tr>
<tr>
<td>HA7+10%SA</td>
<td>59.8 ± 2.8</td>
</tr>
</tbody>
</table>

1HA7 = AmyloGel™ 03003 (high amylose cornstarch VII), PA = palmitic acid, SA = stearic acid, and percentage = weight percentage of fatty acid, dsb.
2Resistant starch content was analyzed using AOAC Method 991.43. Mean ± standard deviation from at least 2 replicates.

Example 2. Preparing and analyzing novel resistant starches from HA7 and fatty acid – boiling only.

[0001] An aqueous suspension of AmyloGel™ 03003 (10% w/w) ("HA7") was heated in a boiling-water bath for 30 minutes. Fatty acid (2% to 10% w/w, dsb) was added to the suspension with stirring. The starch-fatty acid mixture was heated in the boiling-water bath for additional 30 minutes. The mixture was then cooled to room temperature. The starch-fatty acid complex was recovered by centrifugation, washed with 50% ethanol, and dried in a convection oven at 50°C to a moisture content of below 12% (w/w). The RS contents of the resulting products are shown in Table 2.
Table 2. The resistant starch contents of Example 2.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Resistant starch (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA7 control</td>
<td>34.7 ± 1.7</td>
</tr>
<tr>
<td>HA7+2%PA</td>
<td>35.7 ± 0.6</td>
</tr>
<tr>
<td>HA7+3%PA</td>
<td>34.2 ± 1.8</td>
</tr>
<tr>
<td>HA7+5%PA</td>
<td>36.9 ± 1.9</td>
</tr>
<tr>
<td>HA7+10%PA</td>
<td>42.0 ± 0.5</td>
</tr>
<tr>
<td>HA7+10%SA</td>
<td>44.4 ± 0.5</td>
</tr>
<tr>
<td>HA7+5%DG</td>
<td>36.8 ± 0.3</td>
</tr>
<tr>
<td>HA7+10%DG</td>
<td>37.2 ± 0.5</td>
</tr>
<tr>
<td>HA7+5%LE</td>
<td>37.4 ± 0.8</td>
</tr>
</tbody>
</table>

HA7 = AmyloGel™ 03003 (high amylose cornstarch VII), PA = palmitic acid, SA = stearic acid, DG = diglycerides, LE = lecithin, and percentage = weight percentage of fatty acid, dsb.

Resistant starch content was analyzed using AOAC Method 991.43. Mean ± standard deviation from at least 2 replicates.

Example 3. Preparing and analyzing novel resistant starches from HA7 and fatty acid – boiling/autoclave method.

An aqueous suspension of AmyloGel™ 03003 (10% w/w) was heated in a boiling-water bath for 30 minutes. Fatty acid (10% and 20% w/w, dsb) was added to the suspension with stirring. The mixture was autoclaved to 121°C for 15 minutes and heated in the boiling-water bath for additional 30 minutes. The mixture was then cooled to room temperature. The starch-fatty acid complex was recovered by centrifugation, washed with 50% ethanol, and dried in a convection oven at 50°C to a moisture content of below 12% (w/w). The RS contents of the resulting products are listed in Table 3.

Table 3. The resistant starch contents of Example 3.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Resistant starch (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA7 control</td>
<td>28.9 ± 1.8</td>
</tr>
<tr>
<td>HA7+10%PA</td>
<td>35.9 ± 1.4</td>
</tr>
<tr>
<td>HA7+20%PA</td>
<td>37.5 ± 1.7</td>
</tr>
<tr>
<td>HA7+10%SA</td>
<td>36.5 ± 2.3</td>
</tr>
</tbody>
</table>

HA7 = AmyloGel™ 03003 (high amylose cornstarch VII), PA = palmitic acid, SA = stearic acid, and percentage = weight percentage of fatty acid, dsb.
Resistant starch content was analyzed using AOAC method 991.43. Mean ± standard deviation of results obtained from at least 2 replicates.

Example 4. Preparing and analyzing novel resistant starches from HA7 and fatty acid – isoamylase treatment and heating at 80°C.

An aqueous suspension of AmyloGel™ 03003 (10% w/w) (HA7) was heated in a water bath at 80°C for 30 minutes and then cooled to room temperature. The suspension was adjusted to pH 3.5 by slowly adding hydrochloric acid solution (0.5 M). The starch suspension was incubated with isoamylase (0.4% v/w, dsb) in a water bath at 60°C for 24 hours with agitation. Fatty acid (10% w/w, dsb) was added to the isoamylase-treated-starch suspension. The starch-fatty acid mixture was heated in the water bath at 80°C for 30 minutes. The starch-fatty acid complex was then recovered by centrifugation, washed with distilled water and 50% ethanol, and dried in a convection oven at 50°C to a moisture content of below 12% (w/w). The RS contents of resulting products are listed in Table 4.

Table 4. The resistant starch contents of Example 4.

<table>
<thead>
<tr>
<th>Treatments1</th>
<th>Resistant starch2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA7 control</td>
<td>36.7 ± 0.2</td>
</tr>
<tr>
<td>HA7+0.4%ISO+10%PA</td>
<td>74.3 ± 2.4</td>
</tr>
<tr>
<td>HA7+0.4%ISO+10%SA</td>
<td>74.8 ± 1.5</td>
</tr>
</tbody>
</table>

1HA7 = high amylose cornstarch VII, ISO = isoamylase, PA = palmitic acid, SA = stearic acid, and percentage = mL/g percentage of isoamylase, dsb, and weight percentage of fatty acid, dsb.
2Resistant starch content was analyzed using AOAC Method 991.43. Mean ± standard deviation from at least 2 replicates.

Example 5. Preparing and analyzing novel resistant starches from HA7 and fatty acid -- isoamylase treatment & boiling only.

[0004] An aqueous suspension of AmyloGel™ 03003 (5% w/w) (HA7) was heated in a boiling-water bath for 30 minutes and then cooled to room temperature. The suspension was adjusted to pH 3.5 by slowly adding hydrochloric acid solution (0.5 M). The starch
suspension was incubated with isoamylase (0.4% and 0.8% v/w, dsb) in a water bath at 60°C for 24 hours with agitation. Fatty acid (10% w/w, dsb) was added to the isoamylase-treated-starch suspension. The starch-fatty acid mixture was heated in the boiling-water bath for 30 minutes. The starch-fatty acid complex was then recovered by centrifugation, washed with distilled water and 50% ethanol, and dried in a convection oven at 50°C to moisture content of below 12% (w/w). The RS contents of resulting products are listed in Table 5.

Table 5. The resistant starch contents of Example 5.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Resistant starch (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA7 control</td>
<td>34.7 ± 1.7</td>
</tr>
<tr>
<td>HA7+0.4%ISO+10%PA</td>
<td>56.6 ± 1.5</td>
</tr>
<tr>
<td>HA7+0.8%ISO+10%PA</td>
<td>55.8 ± 1.7</td>
</tr>
<tr>
<td>HA7+0.4%ISO+10%SA</td>
<td>65.6 ± 2.0</td>
</tr>
<tr>
<td>HA7+0.4%ISO+10%MG3</td>
<td>46.4 ± 3.9</td>
</tr>
<tr>
<td>HA7+0.4%ISO+10%MG70</td>
<td>48.8 ± 2.5</td>
</tr>
<tr>
<td>HA7+0.4%ISO+10%MG130</td>
<td>41.0 ± 6.2</td>
</tr>
</tbody>
</table>

1HA7 = AmyloGel™ 03003 (high amylose cornstarch VII), ISO = isoamylase, PA = palmitic acid, SA = stearic acid, MG# = monoglycerides with specific iodine value, and percentage = v/w, dsb, percentage of isoamylase and w/w, dsb, percentage of fatty acid.

2Resistant starch content was analyzed using AOAC Method 991.43. Mean ± standard deviation from at least 2 replicates.

Example 6. Preparing and analyzing novel resistant starches from HA7 and fatty acid – isoamylase treatment and boiling/autoclave method.

An aqueous suspension of AmyloGel™ 03003 (5% w/w) (HA7) was heated in a boiling-water bath for 30 minutes, autoclaved at 121°C for 15 minutes, and then cooled to room temperature. The suspension was adjusted to pH 3.5 by slowly adding hydrochloric acid solution (0.5 M). The starch suspension was incubated with isoamylase (0.4% v/w, dsb) in a water bath at 60°C for 24 hours with agitation. Fatty acid (10% w/w, dsb) was added to the isoamylase-treated-starch suspension. The starch-fatty acid mixture was heated in the boiling-water bath for 30 minutes. The starch-fatty acid complex was then recovered by
centrifugation, washed with distilled water and 50% ethanol, and dried in a convection oven at 50°C to a moisture content of below 12% (w/w). The RS contents of resulting products are listed in Table 6.

Table 6. The resistant starch contents of Example 6.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Resistant starch (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA7 control</td>
<td>28.9 ± 1.8</td>
</tr>
<tr>
<td>HA7+0.4%ISO+10%PA</td>
<td>43.8 ± 1.3</td>
</tr>
<tr>
<td>HA7+0.4%ISO+10%SA</td>
<td>51.3 ± 0.9</td>
</tr>
</tbody>
</table>

1HA7 = high amylose cornstarch VII, ISO = isoamylase, PA = palmitic acid, SA = stearic acid, and percentage = mL/g percentage of isoamylase, dsb, and weight percentage of fatty acid, dsb.

2Resistant starch content was analyzed using AOAC Method 991.43. Mean ± standard deviation from at least 2 replicates.

Example 7. Preparing and analyzing novel resistant starches from HA7 and fatty acid – pullulanase treatment and heating at 80°C.

An aqueous suspension of AmyloGel™ 03003 (10% w/w) (HA7) was heated in a water bath at 80°C for 30 minutes and then cooled to room temperature. The suspension was adjusted to pH 5.0 by slowly adding hydrochloric acid solution (0.5 M). The starch suspension was incubated with pullulanase (0.125% to 5%, v/w, dsb) in a water bath at 60°C for 24 hours to 72 hours with agitation. Fatty acid and fatty acid salt (5% to 10% w/w, dsb) was added to the pullulanase-treated-starch suspension. The starch-fatty acid mixture was heated in the water bath at 80°C for 30 minutes. The starch-fatty acid complex was then recovered by centrifugation, washed with distilled water and 50% ethanol, and dried in a convection oven at 50°C to a moisture content of below 12% (w/w). The RS contents of resulting products are listed in Table 7.

Table 7. The resistant starch contents of Example 7.
<table>
<thead>
<tr>
<th>Treatments (^1)</th>
<th>Resistant starch(^2) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA7 control</td>
<td>36.7 ± 0.2</td>
</tr>
<tr>
<td>HA7+1.25%PUL(24hr)+10%NaPr</td>
<td>48.1 ± 2.9</td>
</tr>
<tr>
<td>HA7+1.25%PUL(24hr)+10%BA</td>
<td>44.8 ± 0.8</td>
</tr>
<tr>
<td>HA7+1.25%PUL(24hr)+10%MA</td>
<td>62.7 ± 3.0</td>
</tr>
<tr>
<td>HA7+0.25%PUL(24hr)+10%PA</td>
<td>60.9 ± 0.4</td>
</tr>
<tr>
<td>HA7+0.50%PUL(24hr)+10%PA</td>
<td>59.8 ± 0.8</td>
</tr>
<tr>
<td>HA7+1.25%PUL(24hr)+10%PA</td>
<td>69.9 ± 2.2</td>
</tr>
<tr>
<td>HA7+5%PUL(24hr)+10%PA</td>
<td>65.0 ± 0.9</td>
</tr>
<tr>
<td>HA7+0.125%PUL(72hr)+5%SA</td>
<td>72.2 ± 0.2</td>
</tr>
<tr>
<td>HA7+0.125%PUL(72hr)+10%SA</td>
<td>70.6 ± 1.1</td>
</tr>
<tr>
<td>HA7+0.25%PUL(24hr)+5%SA</td>
<td>59.4 ± 0.9</td>
</tr>
<tr>
<td>HA7+0.25%PUL(72hr)+5%SA</td>
<td>68.6 ± 1.7</td>
</tr>
<tr>
<td>HA7+0.25%PUL(24hr)+10%SA</td>
<td>61.8 ± 0.6</td>
</tr>
<tr>
<td>HA7+1.25%PUL(24hr)+10%SA</td>
<td>71.6 ± 0.3</td>
</tr>
<tr>
<td>HA7+2.5%PUL(24hr)+10%SA</td>
<td>77.0 ± 2.2</td>
</tr>
<tr>
<td>HA7+5%PUL(24hr)+10%OA</td>
<td>77.0 ± 2.1</td>
</tr>
<tr>
<td>HA7+1.25%PUL(24hr)+10%OA</td>
<td>63.2 ± 0.6</td>
</tr>
</tbody>
</table>

\(^1\)HA7 = high amylose cornstarch VII, PUL = pullulanase, NaPr = sodium propionate, BA = butyric acid, MA = myristic acid, PA = palmitic acid, SA = stearic acid, OA = oleic acid, ( ) = hours of treatment with pullulanase, and percentage = mL/g percentage of pullulanase, dsb, and weight percentage of fatty acid, dsb.

\(^2\)Resistant starch content was analyzed using AOAC Method 991.43. Mean ± standard deviation from at least 2 replicates.

**Example 8. Preparing and analyzing novel resistant starches from HA7 and fatty acid – pullulanase treatment and boiling only.**

[0006] An aqueous suspension of AmyloGel\(^\text{TM}\) 03003 (5% w/w) (HA7) was heated in a boiling-water bath for 30 minutes and then cooled to room temperature. The suspension was adjusted to pH 5.0 by slowly adding hydrochloric acid solution (0.5 M). The starch suspension was incubated with pullulanase (1.25% and 5%, v/w, dsb) in a water bath at 60\(^\circ\)C for 24 hours with agitation. Fatty acid (10% w/w, dsb) was added to the pullulanase-treated-starch suspension. The starch-fatty acid mixture was heated in the boiling-water bath for 30 minutes. The starch-fatty acid complex was then recovered by centrifugation, washed with
distilled water and 50% ethanol, and dried in a convection oven at 50\(^\circ\)C to a moisture content of below 12% (w/w). The RS contents of resulting products are listed in Table 8.

**Table 8. The resistant starch contents of Example 8.**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Resistant starch(^2) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA7 control</td>
<td>34.7 ± 1.7</td>
</tr>
<tr>
<td>HA7+1.25%PUL+10%PA</td>
<td>43.4 ± 2.8</td>
</tr>
<tr>
<td>HA7+5%PUL+10%PA</td>
<td>52.5 ± 2.6</td>
</tr>
<tr>
<td>HA7+1.25%PUL+10%SA</td>
<td>55.0 ± 0.9</td>
</tr>
</tbody>
</table>

\(1\) HA7 = AmyloGel\(^\text{TM} \) 03003 (high amylose cornstarch VII), PUL = pullulanase, PA = palmitic acid, SA = stearic acid, and percentage = mL/g percentage of pullulanase, dsb, and weight percentage of fatty acid, dsb.

\(2\) Resistant starch content was analyzed using AOAC Method 991.43. Mean ± standard deviation from at least 2 replicates.

**Example 9. Preparing and analyzing novel resistant starches from HA7 and fatty acid – pullulanase treatment and boiling/autoclave method.**

An aqueous suspension of AmyloGel\(^\text{TM} \) 03003 (5% w/w) (HA7) was heated in a boiling-water bath for 30 minutes, autoclaved at 121\(^\circ\)C for 15 minutes, and then cooled to room temperature. The suspension was adjusted to pH 5.0 by slowly adding hydrochloric acid solution (0.5 M). The starch suspension was incubated with pullulanase (1.25\%, v/w, dsb) in a water bath at 60\(^\circ\)C for 24 hours with agitation. Fatty acid (10% w/w, dsb) was added to the pullulanase-treated-starch suspension. The starch-fatty acid mixture was heated in the boiling-water bath for 30 minutes. The starch-fatty acid complex was then recovered by centrifugation, washed with distilled water and 50% ethanol, and dried in a convection oven at 50\(^\circ\)C to a moisture content of below 12% (w/w). The RS contents of resulting products are listed in Table 9.

**Table 9. The resistant starch contents of Example 9.**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Resistant starch(^2) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA7 control</td>
<td>28.9 ± 1.7</td>
</tr>
</tbody>
</table>
HA7+1.25%PUL+10%PA  
HA7+1.25%PUL+10%SA  

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Resistant starch (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native NC control</td>
<td>1.58 ± 1.32</td>
</tr>
<tr>
<td>NC+1.25%PUL+10%PA (60°C)</td>
<td>8.32</td>
</tr>
<tr>
<td>NC+1.25%PUL+10%PA (70°C)</td>
<td>6.71 ± 1.76</td>
</tr>
<tr>
<td>NC+1.25%PUL+10%PA (80°C)</td>
<td>3.80 ± 2.03</td>
</tr>
<tr>
<td>NC+1.25%PUL+10%SA (60°C)</td>
<td>2.47 ± 1.00</td>
</tr>
<tr>
<td>NC+1.25%PUL+10%SA (70°C)</td>
<td>5.37 ± 0.19</td>
</tr>
<tr>
<td>NC+1.25%PUL+10%SA (80°C)</td>
<td>7.59 ± 1.43</td>
</tr>
</tbody>
</table>

An aqueous suspension of normal cornstarch (5% w/w) was heated in a water bath at 60°C, 70°C, or 80°C for 30 minutes and then cooled to room temperature. The suspension was adjusted to pH 5.0 by slowly adding hydrochloric acid solution (0.5 M). The starch suspension was incubated with pullulanase (1.25%, v/w, dsb) in a water bath at 60°C for 24 hours with agitation. Fatty acid (10% w/w, dsb) was added to the pullulanase-treated-starch suspension. The starch-fatty acid mixture was heated in the water bath at the same temperature as the first heating (60°C, 70°C, or 80°C, respectively) for 30 minutes. The starch-fatty acid complex was then recovered by centrifugation, washed with distilled water and 50% ethanol, and dried in a convection oven at 50°C to a moisture content of below 12% (w/w). The RS contents of resulting products are listed in Table 10.
\(^1\)NC = normal cornstarch, PUL = pullulanase, PA = palmitic acid, SA = stearic acid, ( ) = processing temperature, and percentage = mL/g percentage of pullulanase, dsb, and weight percentage of fatty acid, dsb.

\(^2\)Resistant starch content was analyzed using AOAC Method 991.43. Mean ± standard deviation from at least 2 replicates.

**Example 11. Industrial scale production of resistant starches – Pilot plant**

[0001] In the pilot plant, the HA7 starch-palmitic acid complex samples were produced by heating an aqueous suspension of HA7 (10% w/w) in a steam-jacketed kettle (Model TDB/7-40, Groen, Jackson, MS) for 1 hour at 90°C - 95°C with agitation. Palmitic acid (Cat. No. W28320-7, 4% and 10% w/w, dsb) was added to the suspension in the kettle. The suspension was heated for additional 30 minutes, cooled down, centrifuged, washed with 50% ethanol, and air-dried.

[0002] The debranching-enzyme-treated-HA7-fatty acid complex samples were produced by heating an aqueous suspension of HA7 (7% w/w) in a steam-jacketed kettle for 1 hour at 80°C - 95°C with agitation and then cooled to 55°C. The suspension was adjusted to pH 3.5 and 5.0 for isoamylase and pullulanase hydrolysis, respectively. The starch suspension was treated with isoamylase (0.8%, v/w, dsb) for 12 hours in the kettle at 55°C - 60°C with agitation or pullulanase (1.25% v/w, dsb) for 24 hours at 50°C - 55°C in a hot-water-jacketed stainless steel tank (Model 70 gallon JOVC, Viatec™, Belding, MI) with agitation. Fatty acid (10% w/w, dsb) was added to the debranching-enzyme-treated-HA7 suspension. The HA7 starch-fatty acid mixture was heated back at 80°C - 95°C for additional 1 hour and stored in refrigerator overnight. The HA7 starch-fatty acid complex samples were then recovered by centrifugation, washed with 50% ethanol, and dried in a convection oven at 50°C to moisture content of below 12%. All samples prepared in the
pilot plant were ground using Magic Mill III Plus (Magic Mill, Monsey, NY). The resistant starch contents of the samples prepared in the pilot plant are listed in Table 11.

Table 11. The resistant starch contents of Example 11.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Resistant starch (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA7+4%PA^3</td>
<td>41.3 ± 2.2</td>
</tr>
<tr>
<td>HA7+10%PA^3</td>
<td>39.2 ± 1.9</td>
</tr>
<tr>
<td>HA7+0.8%ISO(12hr)+10%PA^4</td>
<td>52.7 ± 1.5</td>
</tr>
<tr>
<td>HA7+1.25%PUL(24hr)+10%PA^5</td>
<td>64.8 ± 0.3</td>
</tr>
<tr>
<td>HA7+1.25%PUL(24hr)+10%SA^5</td>
<td>66.4 ± 1.3</td>
</tr>
</tbody>
</table>

^1HA7 = high amylose cornstarch VII, ISO = isoamylase, PUL = pullulanase, PA = palmitic acid, SA = stearic acid, ( ) = hours of treatment with debranching enzyme, and percentage = mL/g percentage of pullulanase or weight percentage of fatty acid, dsb.  
^2Resistant starch content was analyzed using AOAC Method 991.43. Mean ± standard deviation from at least 2 replicates.  
^3The samples were prepared at 90-95°C and air-dried at room temperature.  
^4The sample was prepared at 90-95°C and dried in a convection oven at 50°C to a moisture content of below 12%.  
^5The sample was prepared at ~80°C and dried in a convection oven at 50°C to a moisture content of below 12%.

Example 12. Preparation and analysis of noodles made with resistant starches.

The ground starch-lipid complex was mixed with boiling water in a 1 to 1 ratio to form dough. The dough was rolled with a rolling pin to form a thin sheet, which was then cut into thin slices and air-dried. The dried noodle was kept in the refrigerator until needed. Vital wheat gluten was also incorporated in the noodle to bind the noodle together and to decrease the dispersion during boiling.

Table 12. The resistant starch contents of cooked high amylose cornstarch VII-palmitic acid complex noodles.
<table>
<thead>
<tr>
<th>Treatments</th>
<th>Initial Protein Content (%)</th>
<th>Undigested Protein (%)</th>
<th>Resistant Starch (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA7+4%PA starch</td>
<td>0</td>
<td>0</td>
<td>41.3 ± 2.2</td>
</tr>
<tr>
<td>VWG</td>
<td>100</td>
<td>7.9</td>
<td>0</td>
</tr>
<tr>
<td>HA7+4%PA noodle</td>
<td>0</td>
<td>0</td>
<td>38.7 ± 2.0</td>
</tr>
<tr>
<td>HA7+4%PA+16%VWG noodle</td>
<td>16</td>
<td>19.7</td>
<td>41.6 ± 1.2</td>
</tr>
</tbody>
</table>

1HA7 = 70% high-amylose cornstarch, PA = palmitic acid, VWG = vital wheat gluten, and percentage = weight percentage of palmitic acid, dsb, and w/w percentage of vital wheat gluten on the basis of dry HA7+4%PA starch. Noodle samples were boiled in water before enzyme treatments following the AOAC Method 991.43.
2Percentage of undigested protein from initial total protein weight analyzed using Dumas method.
3Resistant starch content (excluding the undigested protein content) was analyzed using AOAC Method 991.43. Mean ± standard deviation from at least 2 replicates.

**Example 13. Preparation and analysis of bread from resistant starches.**

[0006] For control bread, enriched bread flour (600 g) was mixed with melted soft spread (25 g, 52% fat), salt solution (4 g in 130 g water), and yeast dispersion (5 g in 100 g of warm water) using Kitchen Aid® Stand Mixer (St. Joseph, MI) with a ‘C’ dough hook at mixing speed of 2 for 3.5 minutes. The bread dough was allowed to rise in a greased pan for one hour and then in a greased loaf pan (9.5 × 5.25 × 2.625 in³) for an additional hour. The dough was baked in a conventional oven at 400°F for 25 minutes. The bread loaf was cooled at room temperature for 1 hour before storing it in a sealed zip lock bag. For enzyme-resistant bread, 75% of enriched bread flour (450 g) was substituted with resistant starch (HA7+0.8%ISO(12hr)+10%PA) (398 g), which contained 52.7% enzyme resistance, and vital wheat gluten (78 g), whereas other ingredients were kept the same. The resistant starch contents of the bread samples are listed in Table 13. Table 14 lists the composition of control white bread and enzyme-resistant bread.

[0007] Table 13. Percentages of total residue, undigested protein, and resistant starch content of bread samples.
<table>
<thead>
<tr>
<th>Samples</th>
<th>Total undigested residue (^1) (%)</th>
<th>Undigested protein (^2) (%)</th>
<th>Resistant starch (^3) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enriched Bread Flour</td>
<td>4.8 ± 0.1</td>
<td>2.7 ± 0.2</td>
<td>2.1</td>
</tr>
<tr>
<td>HA7+0.8%ISO(12hr)+10%PA starch(^4)</td>
<td>52.7 ± 1.5</td>
<td>0</td>
<td>52.7</td>
</tr>
<tr>
<td>vital wheat gluten</td>
<td>45.2 ± 1.6</td>
<td>34.2 ± 1.0</td>
<td>11.0</td>
</tr>
<tr>
<td>Control White Bread(^5)</td>
<td>6.5 ± 0.8</td>
<td>3.2 ± 0.2</td>
<td>3.3</td>
</tr>
<tr>
<td>Enzyme-Resistant Bread(^6)</td>
<td>37.4 ± 3.5</td>
<td>3.1 ± 0.4</td>
<td>34.4</td>
</tr>
</tbody>
</table>

\(^1\) Total undigested residue was analyzed using AOAC method 991.43. Mean ± standard deviation from duplicates.

\(^2\) Undigested protein was analyzed using macro-Kjeldahl method on the solid residue from the digestion according to the AOAC method 991.43.

\(^3\) Resistant starch = total undigested residue – undigested protein

\(^4\) HA7+0.8%ISO(12hr)+10%PA starch with 52.7% resistant starch content was prepared in the pilot plant as described in Example 11

\(^5\) Control white bread contained 96% enriched bread flour

\(^6\) Enzyme-resistant bread contained 24% enriched bread flour, 60% HA7+0.8%ISO(12hr)+10%PA starch, and 12% vital wheat gluten.
Table 14. The compositions of control white bread and enzyme-resistant bread.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Control White Bread</th>
<th>Enzyme-Resistant Bread</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Starch</td>
</tr>
<tr>
<td>Flour</td>
<td>600</td>
<td>476</td>
</tr>
<tr>
<td>HA7+0.8%ISO(12hr)+10%PA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vital Wheat Gluten</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Soft spread</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>Yeast</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Salt</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Water</td>
<td>330</td>
<td>0</td>
</tr>
<tr>
<td>Total weight</td>
<td>964</td>
<td>476</td>
</tr>
<tr>
<td>Percentage&lt;sup&gt;c&lt;/sup&gt;</td>
<td>100%</td>
<td>82%</td>
</tr>
</tbody>
</table>

<sup>a</sup>Resistant starch content was analyzed using AOAC Method 991.43. Mean ± standard deviation from at least 2 replicates. Resistant starch was included in the starch content.

<sup>b</sup>HA7+0.8%ISO(12hr)+10%PA starch with 52.7% resistant starch content was prepared in the pilot plant as described in Example 11.
Example 14. Analysis of plasma glucose and plasma insulin concentrations in humans who consumed foods made with the present resistant starches.

Twenty healthy male human subjects were recruited for plasma glucose and plasma insulin study. After an overnight fasting, each subject received a slice of test bread containing 50 g of starch each test day. All subjects ingested both the control white bread and enzyme-resistant bread on separate days. Blood samples were collected from the subjects every 15 minutes from 15 minutes before ingesting the test bread to 2 hours after ingesting the test bread. Plasma glucose and plasma insulin concentrations in the blood samples were analyzed by Dr. Suzanne Hendrich, Professor, and Dr. Sun-Ok Lee, Post-doctoral Research Associate, of the Department of Food Science and Human Nutrition, at Iowa State University, Ames, IA. The plasma glucose concentrations were measured using a glucose oxidase analyzer (Beckman Coulter Glucose Analyzer, Beckman Coulter Inc., Fullerton, CA) and the plasma insulin concentrations were determined using an ultrasensitive insulin ELISA (Enzyme-Linked Immuno Sorbent Assay) kit (ALPCO diagnostics, Salem, NH). Results are expressed in Figure 1 and Figure 2.

Example 15. Comparison of effects of 3 starch diets on the occurrence of pre-neoplastic lesions, Mucin Depleted Foci, induced by the chemical carcinogen azoxymethane (AOM) in F344 rats, so as to determine the ability of different starch diets to prevent or reduce colon cancer.

Five-week-old, male F344 rats were ordered as the experimental animals. They were fed with control starch diet for two weeks. Subsequently, half of the rats were injected with AOM, once weekly for two weeks. The other rats were injected with saline, once weekly for
two weeks. Control starch diet made from which contains 55% cooked normal cornstarch and other necessary rat diet nutrients, was fed during the AOM injection process. After the second AOM or saline injection, the rats were divided randomly but evenly into three diet groups. Three starch diets were fed to the groups for eight weeks. Each diet contained 55% cooked starch, together with 45% of other raw ingredients including protein, vitamin, mineral, amino acid, and fat etc that are necessary for rats’ nutritive requirements.

The control group contained cooked normal cornstarch, the second group (group 2) contained cooked high amylose cornstarch VII, and the third (group 3) contained cooked high-amylose cornstarch VII-fatty acid complex (HA7+1.25%PUL(24hr)+10%SA starch prepared in the pilot plant as described in Example 11).

After eight weeks on the three diets, the rats were killed and colons were collected as microscope observable specimens to calculate ACF and MDF number. Other endpoints were also collected such as liver weight, cecum weight, and cecum pH. The end points were compared based on carcinogen or diet groups by the means of two-way ANOVA.

Results

<table>
<thead>
<tr>
<th></th>
<th>liver weight (g)</th>
<th>cecum weight with content (g)</th>
<th>cecum weight without content (g)</th>
<th>cecum content pH</th>
<th>average MDF</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOM, control group</td>
<td>7.9±1.0</td>
<td>4.1±0.3</td>
<td>0.8±0.2</td>
<td>7.46±0.09</td>
<td>3.5 ± 1.8</td>
</tr>
<tr>
<td>AOM, group 2</td>
<td>7.8±0.7</td>
<td>16.6±3.0*</td>
<td>3.0±0.6*</td>
<td>6.60±0.58*</td>
<td>1.8 ± 1.4*</td>
</tr>
<tr>
<td>AOM, group 3</td>
<td>7.1±2.6</td>
<td>21.6±1.2*</td>
<td>2.9±0.6*</td>
<td>5.69±0.15*</td>
<td>0.3 ± 0.5*</td>
</tr>
<tr>
<td>Saline, control group</td>
<td>7.0±1.1</td>
<td>4.3±1.5</td>
<td>1.2±0.3</td>
<td>7.39±0.14</td>
<td>0</td>
</tr>
<tr>
<td>Saline, group 2</td>
<td>7.6±1.0</td>
<td>16.0±3.0$</td>
<td>2.7±0.5 $</td>
<td>6.74±0.36$</td>
<td>0</td>
</tr>
<tr>
<td>Saline, group 3</td>
<td>8.6±0.9</td>
<td>19.3±2.5$</td>
<td>3.3±0.7$</td>
<td>5.70±0.33$</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are means ± std. dev.
The two-way ANOVA results showed that:

No significant differences were observed in body weight, food consumption, or liver weight. Cecal weight was significantly elevated in the groups 2 and 3 compared with the control groups. Cecal pH was significantly decreased in the groups 2 and 3 compared with control groups. MDF was only seen with AOM and was reduced in the groups 2 and 3 compared with the control group. MDF (mucin-depleted foci) are considered as an effective biomarker of tumors and a subset of Aberrant crypt foci (ACF). ACF are the earliest, visually identifiable hyperplastic lesions considered to occur along the normal to carcinoma sequence of colorectal cancer progression in humans since their presence and numbers are highly correlated with the development of carcinomas. ACF are also used as pre-neoplastic lesions because they can be chemically induced in laboratory mammalian strains. As a subset of ACF, mucin-depleted foci (MDF) are regarded as more highly correlated to carcinoma development than ACF, although their characterization is not currently as well established as ACF. MDF can be microscopically observed after staining with alcian blue and neutral red. AOM is a carcinogen that is selective for colon, with some reports of a few small bowel/stomach tumors. Pre-neoplastic lesions could be found 8-10 weeks after AOM injection.

CLAIMS

What is claimed is:
1. A modified resistant starch comprising a high-amylose starch complexed with lipid.

2. A resistant starch of claim 1, wherein the high-amylose starch is selected from the group consisting of: high-amylose cornstarch; high-amylose barley starch; high-amylose potato starch; high-amylose wheat starch; high-amylose rice starch; and high-amylose grain-derived starch.

3. A resistant starch of claim 1, wherein the lipid is selected from the group consisting of: fatty acids; monoglycerides; diglycerides; and phospholipids.

4. A resistant starch of claim 1, wherein the lipid is a fatty acid.

5. A resistant starch of claim 4, wherein the fatty acid is selected from the group consisting of: stearic acid; palmitic acid; myristic acid; butyric acid; oleic acid; and sodium propionate.

6. A resistant starch of claim 1, wherein the high-amylose starch has been pre-treated with a debranching enzyme, and the lipid is a fatty acid.

7. A resistant starch of claim 6, wherein the debranching enzyme is selected from the group consisting of: pullulanase and isoamylase.

8. A resistant starch of claim 6, wherein the fatty acid is approximately 2 to 20% of the high amylose starch, when dry, and referenced by weight.

9. A resistant starch of claim 8, wherein the fatty acid is approximately 5 to 10%.

10. A process to produce a modified resistant starch, comprising: processing high amylose starch with lipid at a temperature in the range of 70°C to 90°C; and subsequently processing the starch and lipid at a temperature in the range of 40°C to 60°C.
11. A process of claim 10, wherein the high-amylose starch is selected from the group consisting of: high-amylose cornstarch; high-amylose barley starch; high-amylose potato starch; high-amylose wheat starch; high-amylose rice starch; and high-amylose grain-derived starch.

12. A process of claim 10, wherein the lipid is selected from the group consisting of: fatty acids; monoglycerides; diglycerides; and phospholipids.

13. A process of claim 10, wherein the lipid is a fatty acid.

14. A process of claim 10, wherein the fatty acid is selected from the group consisting of: stearic acid; palmitic acid; myristic acid; butyric acid; oleic acid; and sodium propionate.

15. A process of claim 13, wherein the high-amylose starch has been pre-treated with a debranching enzyme, and the lipid is a fatty acid.

16. A process of claim 15, wherein the debranching enzyme is selected from the group consisting of: pullulanase and isoamylase.

17. A process of claim 15, wherein the fatty acid is approximately 2 to 20% of the high amylose starch, when dry, and referenced by weight.

18. A process of claim 17, wherein the fatty acid is approximately 5 to 10%.

19. A food composition comprising a modified resistant starch of claim 1.

20. A method to affect the physiology of a mammal, selected from the group consisting of: decreasing plasma insulin response; decreasing plasma glucose response; increasing colonic fermentation; decreasing the risk of colon cancer; increasing digestive health; decreasing colonic pH; comprising administering a food composition of claim 1.
The present invention provides novel resistant starches, methods to make the resistant starches, and methods to use the resistant starches. These resistant starches may be used as an ingredient in a variety of foods to impart health benefits, such as: decreasing plasma insulin response; decreasing plasma glucose response; increasing colonic fermentation; decreasing the risk of colon cancer; increasing digestive health; decreasing colonic pH. The new methods for making the novel starches dramatically decrease the cost of producing them.
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