

EFFECTS OF AMYLOSE CONTENT AND CHEMICALLY CROSS-LINKING STARCH ON
IN-VITRO DIGESTIBILITY AND EXTRUSION OF STARCH

by

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B. Tech., Universiti Sains Malaysia, 2003
M.S., Universiti Putra Malaysia, 2006

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College of Agriculture

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Manhattan, Kansas

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Abstract

This study focused on *in-vitro* digestibility and pasting properties of cross-linked (CL) starch with different amylose contents and different cross-linking levels, as well as physicochemical properties of extruded wheat flours with different amylose contents and functionality of amylopectin and cross-linking in improving the textural and physical properties of oat flour extrudates. Starch was CL by phosphorylation using a mixture of sodium trimetaphosphate and sodium tripolyphosphate at the ratio of 99:1 under alkaline condition. The digestibility of highly CL maize starches with different amylose contents was determined by Englyst, Available Carbohydrate Dietary Fiber and Association of Official Analytical Chemists (AOAC) Method 991.43 methods. CL waxy and normal maize starch granules swelled much more at higher temperatures, resulting in significantly lower total dietary fiber content than high-amylose maize starch. The alkali treatment study on low levels of cross-linking in starch affected the ratios of total bound phosphate esters which changed the degree of starch swelling, crystallinity and pasting properties of the CL starch. The study on the mechanism of the digestive enzymes and the extent of digestion during the incubation of CL wheat starch in AOAC Method 2009.01 showed progressive digestion after 16 h of incubation, which may not reflect *in-vivo* response in human. Extrusion of normal and waxy wheat flours resulted in the breakdown of starch and an increase in the insoluble protein, which affected the textural and structural properties of extrudate. High energy input played a major role in radial expansion of normal hard wheat extrudate, whereas higher amylopectin in soft waxy wheat flour was a dominant factor in determining the radial expansion when compared to normal soft wheat. Inclusion of soft waxy wheat in the oat flour formulations at 18% moisture content improved the textural and structural properties of extrudates. Low cross-linking level of CL waxy maize starch in oat flour formulation increased the void fraction and reduced the breaking strength of extrudates, whereas higher cross-linking levels of CL starches improved the resistant starch level on oat flour formulation but had very poor structural and textural properties. The study offers a good insight on the properties and digestibility of CL starch, as well as using low levels of CL starch to improve textural properties of nutritional extruded products. In addition, study on the extrusion of wheat flours with different amylose and protein contents provides knowledge on the influence of chemical compositions and energy input on the physico-chemical properties of extrudates.

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Dedication

To Mama and Baba for your unconditional love.....

Introduction

This dissertation consists of chapters that were written for specified journals. Hence, the required journal format was followed for each manuscript, with references following the text and figures in the requisite format for the respective journal. The basis of the research is in how cross-linking levels of starch and amylose/amylopectin ratio in starch and flour may affect the *in-vitro* digestion, pasting properties and extrusion.

Cross-linking of starch, is a well-known starch modification method (Paschall, 1964) that eliminate the shortcomings of native starch during prolonged heating and extreme processing conditions (e.g., high acidity, high shear) (Wurzburg, 2006). The detrimental effects of harsh food processing on starch can be minimized or prevented by introducing cross-linking groups in the starch molecules by binding the neighboring anhydrous glucose units in the amorphous regions. Cross-linking suppressed the swelling of starch granules due to strongly associated bonds, thus developing a short and non-cohesive paste texture and improved viscosity (Xie et al., 2005; Wurzburg, 2006). In chapter 2, low level of cross-linked (CL) waxy maize starch was subjected to alkali treatment to test on the stability and changes on the pasting properties of CL starch.

Most commercial CL starches have a low degree of phosphorylation and can be digested by α -amylase (Ostergard et al., 1988). CL starches produced with a combination of 12% sodium trimetaphosphate (STMP) and (sodium tripolyphosphate) STPP provide a significantly higher degree of substitution (0.4% bound phosphorus), relatively higher total dietary fiber content (58–76%), and more restricted swelling than native starches (Woo and Seib, 2002). Hence, CL starch at a higher cross-linking level is also known as resistant starch type 4 (RS4). Woo and Seib (2002) did extensive studies on preparation and *in-vitro* digestibility of high cross-linking levels of CL starches. We further study the effects of incubation temperatures in *in-vitro* methods (Englyst Method, AOAC Method 991.43 and Available Carbohydrate and Dietary Fiber Method) on high levels of CL maize starches with different amylose content (Chapter 1).

Several *in-vitro* methods have been suggested to quantify resistant starch (RS) in food, all of which involve enzymatic digestions on starch, removal of the digested starch and quantitation of remaining RS. The AOAC Method 2009.01 claims to determine non-starch polysaccharides, RS and non-digestible oligosaccharides content effectively in samples (McCleary et al., 2010).

However, our preliminary study shows that the AOAC Method 2009.01 gave significantly lower values for several samples including CL starch. This led us to conduct a time course study on the digestibility of commercial CL wheat starch using the AOAC Method 2009.01 to observe the digestion kinetics, specifically the enzyme mechanism and incubation time (Chapter 3).

Quantitation of RS can be carried out using direct methods or indirect methods. While direct methods determine RS in the remaining starch after removing the digested portion (Berry, 1986; Champ, 1992; Faisant et al., 1993; McCleary et al., 2002), the indirect methods measure RS by subtracting the digested starch from the total starch (Englyst et al., 1992; Tovar et al., 1990). Determination of RS is achieved by solubilizing RS and quantifying it with colorimetric methods (Berry, 1986; McCleary et al., 2002), or by isolating RS gravimetrically after ethanol precipitation (Lee et al., 1992; McCleary et al., 2010). The AOAC Method 2002.02 uses direct method that applies the solubilization of undigested portion of sample with 2M potassium hydroxide. However, this method is unable to quantify RS4 due to the insolubility of RS4 in 2M potassium hydroxide which we later resolved by developing different procedures to solubilize CL starch (Chapter 4).

Extrusion is a thermomechanical processing that involves high temperature, short time and low moisture cooking that uses mechanical energy to force out material out of the die (Riaz, 2006). Although documentation on flour or starch are available in terms of effect of extrusion processing variables (Mercier and Feillet, 1975; Paton and Spratt, 1984; Ilo et al., 1999; Ding et al., 2006), influence of moisture content (Faubion and Hosney, 1982a) and influence of minor ingredients (protein and lipid) (Faubion and Hosney, 1982b; Chanvrier et al., 2007; Arhaliass et al., 2009), no study has been done on extrusion of waxy wheat flours. We extruded normal and waxy wheat flours with different protein content at two different screw speeds and did a comprehensive study on the macro- and micro-structural changes as well as transformation of starch and protein in the extruded product (Chapter 5).

Whole cereals comprised of bran, germ and endosperm that contain vitamins, minerals, protein, lipid and fiber is a good choice of main ingredient in producing healthy expanded snacks. The most common problem encountered during extrusion of whole grain cereal or cereal with high lipids and fiber is to achieve the desired degree of expansion. Incorporation of waxy starch or flour as a minor ingredient may help better expansion (Graybosch, 1998) of less expanded product like oat flour. We studied the extrusion of soft waxy wheat flour and oat flour

blends and the textural and structural properties of the extrudates (Chapter 6). We also did a study on the RS changes, textural, micro- and macro-structural changes of extruded oat flour-CL starch formulations (Chapter 7).

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Chapter 1 - Digestibility of cross-linked maize starches differing in amylose content

1.1. Abstract

The digestibility of cross-linked (CL) maize starches with differing amylose content and the extent of digestion assayed by Englyst, Available Carbohydrate Dietary Fiber (ACDF), and AOAC 991.43 methods were investigated. CL waxy maize starch had significantly higher phosphorus content (0.36%) than normal and high-amylose (0.32%) starches. For native starches, total dietary fiber (TDF) and resistant starch (RS) increased with increasing amylose content. At 80 °C (in ACDF) and 95 °C (in AOAC 991.43), TDF content was, in order, native waxy < normal < high-amylose maize starches. CL starches had the same order, but with significantly higher TDF content due to reduced susceptibility to digestive enzymes because of cross-linking. Native and CL waxy and normal starch granules swelled much more at higher temperatures, resulting in significantly lower TDF content than high-amylose maize. Morphology of the granular starches after digestion showed less prominent degradation of starch granules in CL high-amylose starch, indicating less susceptibility to digestive enzymes.

1.2. Introduction

Starch consists of amylose and amylopectin molecules, occurs in higher plants in granular form (Pérez and Bertoft, 2010), and are commercially extracted from maize, wheat, potato, rice, tapioca, and sago (Tester et al., 2000). In humans, starch is digested predominantly by amylases in the small intestine (Tester et al., 2004). Based on the digestibility rate in humans, starch is categorized into rapidly digestible starch (RDS), slowly digestible starch (SDS), and RS (Englyst et al., 1992). RS is defined as the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals (Delcour and Eerlingen, 1996). RS is categorized into five types, RS1, RS2, RS3, RS4 and RS5. RS1 is the physically inaccessible starch normally found in whole grains; RS2 consists of ungelatinized resistant granules, and RS3 is retrograded or recrystallized starch. RS4 is chemically modified starch with additional chemical bonds formed (Sajilata et al., 2006) and RS5 is amylose-lipid complex (Brown et al., 2006).

Chemical modifications of starch have proven to affect the rate and extent of digestion in the small intestine (Wolf et al., 1999; Woo and Seib, 2002; Haub et al., 2010) based on starch source, type and degree of modification, extent of granule gelatinization, and the source of enzyme used (Filer, 1971). Most commercial cross-linked (CL) starches have a low degree of phosphorylation and can be digested by α -amylase (Ostergard et al., 1988). CL starches produced with a combination of 12% sodium trimetaphosphate (STMP) and (sodium tripolyphosphate) STPP provide a significantly higher degree of substitution (0.4% bound phosphorus), relatively higher TDF content (58–76%), and more restricted swelling than native starches (Woo and Seib, 2002). Cross-linking reduces digestibility (Woo and Seib, 2002) probably by preventing amylase molecules from entering the porous channels of starch granules (Huber and BeMiller, 2000) and interferes with the formation of the amylase enzyme and starch complex (Woo and Seib, 2002).

Several methods are available to determine RS and TDF; namely, Englyst, AOAC 985.29, AOAC 991.43, and AOAC 2002.02 methods (McCleary, 2010). Determination of RS and TDF using *in-vitro* methods is controversial because the extrinsic factor (i.e., extent of chewing, rate of orocecal transit) involved in *in-vivo* digestion was not taken into consideration in the early methods (Englyst et al., 1992). In addition, incubation time and enzyme source may affect the *in-vitro* yield of these assays (Englyst et al., 1992). Previous work by McCleary (1999) showed that increased purity and concentration of α -amylase and amyloglucosidase in AOAC 985.29 increased the TDF content in regular maize starch and high amylose maize starch. These limitations were overcome by developing an *in-vivo* digestive model in ileostomy patients that shows a high correlation of RDS with their *in-vivo* glycemic response (Englyst et al., 1992; Englyst et al., 1999). Enzymatic-gravimetric procedures using the AOAC 991.43 (2000) TDF method also show accordance with *in-vivo* values determined in ileostomy patients (Champ et al., 2003).

The available carbohydrates dietary fiber (ACDF) method was developed based on the methods of AOAC 991.43 (Lee et al., 1992) and AACC 32-07 (Prosky et al., 1988), which use digestive treatments using heat-stable α -amylase, amyloglucosidase, and protease. The test is modified by changing the α -amylase temperature from 95–100 °C (as in AOAC 991.43) to 80 °C, using acetic acid to change the pH before adding amyloglucosidase, and using a Duran bottle during incubation. The advantages of the modified method include optimum pH for

amyloglucosidase reaction and a better yield recovery. For certain samples, the ACDF method is claimed to produce results similar to the AOAC 991.43 method (Available Carbohydrates and Dietary Fibre assay procedure, Megazyme International Ireland, Ltd., 2006); however, RS2 and RS3 are less hydrolyzed in the ACDF method due to a lower temperature that resulted in higher content of RS (McCleary and Rossiter, 2006). Although digestibility of native starch had been reported (McCleary, 2007; Zhu et al., 2011; Brewer et al., 2012), digestibility of CL starches with various amylose-amylopectin ratios by different *in-vitro* methods are not well documented. Thus, the objectives of this study were to determine the digestibility of CL maize starches with different amylose contents and to compare the extent of digestion by Englyst, ACDF, and AOAC 991.43 methods.

1.3. Materials and methods

1.3.1. Materials

Waxy (Amioca), normal (Melojel), and high-amylose (HYLON VII) maize starches were obtained from National Starch LLC (Bridgewater, NJ) with amylose content of 0.5, 25, and 71%, respectively, as determined by the potentiometric iodine method (Shi et al., 1998). STMP was purchased from MP Biomedicals, LLC (Solon, CA). STPP, sodium sulfate, sodium hydroxide, and hydrochloric acid were obtained from Fisher Scientific (Pittsburgh, PA). Total Dietary Fibre assay kit (catalogue no. K-TDFR) and Available Carbohydrates and Dietary Fibre assay kit (catalogue no. K-ACHDF) were obtained from Megazyme International Ireland, Ltd. (Wicklow, Ireland). Porcine pancreatin (catalogue no. P7545), with an enzyme activity of 300 U (0.9 mg of glucose released from soluble starch in 3 min at 37 °C and pH 5.8) and amyloglucosidase (catalogue no. A7255), with an enzyme activity of 234 U (0.7 mg of glucose released from soluble starch in 3 min at 37 °C and pH 5.8), were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). All chemicals were reagent grade.

1.3.2. General methods

Phosphorus (P) content was assayed using the procedure of Smith and Caruso (1964). Moisture content was determined by using AACC Air Oven Method 44-15 (AACC 2000) at 135°C for 2 h.

1.3.3. Preparation of CL phosphorylated starch

CL waxy, normal, and high-amylose maize starches were prepared by the method from Woo and Seib (2002) using a combination of 12% STMP and STPP (99:1) as phosphorylating agents.

1.3.4. Settling volume measurement and microscopic observation

The settling volume was determined using the method of Tayal et al. (2004) with a slight modification. Starch (5g, db) was constantly stirred in 100 mL distilled water within a water bath at 37 °C for 30 min. After the slurry cooled to room temperature, it (20 g, wb) was transferred to a 100 mL graduated cylinder containing distilled water (80 g). The settling volume of the starch was taken after 24 h. The same steps were repeated with the temperature of the water bath adjusted to 80 °C and 95 °C. The slurries heated to 80 °C and 95 °C were placed on microscope slides and observed under an Olympus BX-51 microscope (Olympus, Tokyo, Japan) with a 40× objective. The native and CL starch slurries at room temperature (24 °C) were also prepared for microscopic observation as a comparison with the heated slurries.

1.3.5. Pasting properties

The pasting properties of the CL starches suspended in distilled water were determined using a Micro Visco-Amylograph (C.W. Brabender Instruments, Inc, South Hackensack, NJ). CL starch was added to distilled water to prepare a 20% suspension (dry weight basis, w/w). The heating and cooling cycles were programmed by heating the suspension from 50 °C to 90 °C (heating rate of 10 °C/min), held at 90 °C for 6 min, and cooled to 50 °C (cooling rate of 10 °C/min). The viscosity of starch suspension was expressed in Brabender Unit (BU).

1.3.6. Thermal properties

The gelatinisation temperatures and the enthalpy of native and CL starches were measured by a differential scanning calorimeter (DSC) (TA Instruments Q100, TA Instruments, New Castle, DE). The total solids content of samples was 33.3% (w/w, dry basis). After hydration for 1 h at 25 °C, 30 mg of the well-stirred sample suspensions were weighed into 40 µl aluminum crucibles and immediately hermetically sealed to prevent moisture loss. The scans were performed from 10 °C to 130 °C at a controlled constant rate of 10 °C/min. A sealed empty crucible was used as a reference, and the DSC was calibrated using indium. The gelatinisation

enthalpy (ΔH) and transition temperatures, namely the onset temperature (T_o), peak temperature (T_p), and conclusion temperature (T_c), were determined.

1.3.7. X-ray diffraction

The CL starches and their residues after digestion by Englyst, ACDF, and AOAC 991.43 methods were hydrated with distilled water to 15% moisture content by placing samples in a dessicator which the bottom part was filled with distilled water. The X-ray diffraction patterns of the hydrated starch samples were recorded on a Philips APD 3520 X-ray diffractometer (MAC Science Co. MO3XHF22, Tokyo, Japan). The CuK α radiation was set at 35 kV and 20 mA at a scanning rate of 2° 2 θ /min. The degree of crystallinity was estimated from the mathematical model of Wakelin method (Wakelin et al., 1954).

1.3.8. TDF

Two methods from AOAC Method 991.43 (2000) and ACDF were used to determine the TDF by using Megazyme (Wicklow, Ireland) kits. Both ACDF and AOAC 991.43 methods used α -amylase (activity of 10000 U/mL on soluble starch) and amyloglucosidase (3300 U/mL on soluble starch) enzymes provided from the assay kits. One U of α -amylase at pH 5.0 and 40 °C is defined as the amount of enzyme required to release one micromole of D-glucose per minute from soluble starch. One U of amyloglucosidase enzyme activity is amount of enzyme required to release 1 micromole of glucose from soluble starch per minute at 40 °C and pH 4.5. The requirements of protein and ash corrections in both methods were omitted due to the negligible amount of the compounds in starches.

1.3.9. Starch digestion profile

The RDS, SDS, and RS were determined by a modified procedure of the Englyst method (Sang and Seib, 2006).

1.3.10. Scanning electron microscopy (SEM)

The indigestible residues at the conclusion of AOAC 991.43 (2000) and ACDF methods were collected from crucibles, washed with distilled water (20 mL) three times, and centrifuged at 1500g for 10 min. The 120-min vials of the Englyst method (1992) were collected from the water bath and centrifuged at 1500g for 10 min. The supernatant was discarded, and the pellet

was washed 3 times with distilled water (20 mL) and centrifuged at 1500g for 10 min. The collected residue was stored at 4 °C for 24 h and freeze-dried for 12 h. The freeze-dried residue was gently ground using a mortar and pestle and stored in an airtight container. The CL starches were prepared as controls for comparison with the assayed CL starches.

The samples were sprinkled lightly onto a carbon double-sided adhesive tape on metal specimen stubs that were then coated with gold-palladium (60:40 ratio) under vacuum with Desk II Sputter/Etch Unit (Denton Vacuum, LLC, Moorestown, NJ). The micrographs of the samples were obtained at 1000× and 5000× magnifications with a SEM (S-3500N, Hitachi Science Systems, Ltd, Japan) at an accelerating potential of 20 kV using X-ray Detector-Link Pentafet 7021 (Oxford Instruments Microanalysis Limited, Bucks, England).

1.3.11. Statistical analysis

All data were statistically analyzed using Statistical Analysis Software (SAS) (version 9.2, SAS Institute, Cary, NC) by analysis of variance (ANOVA), and the values are expressed as means ± standard deviations from 2 replicates unless stated otherwise.

1.4. Results and discussion

1.4.1. P content

The P content of the native and CL starches are reported in Table 1-1. All the native maize starches had negligible P content (< 0.01%). The P content in native maize starches normally exist in the form of phospholipids (Morrison, 1995; Suh et al., 2004; Jiang et al., 2010). The CL maize starches showed significantly higher P content than native starches due to the addition of P from STMP and STPP during the cross-linking process. The CL waxy maize starch had relatively higher P content (0.36%) than both CL normal maize and CL high-amylose starches (0.32%). The data are in agreement with Landerito and Wang (2005), who used a slurry and oven method for cross-linking of maize starches. Both methods were reported to produce significantly higher P content in CL waxy maize compared with CL normal maize and CL high-amylose starches (Landerito and Wang, 2005). The studies of O'Brien et al. (2009) and O'Brien and Wang (2009) also showed higher P content of waxy maize starch when cross-linked using an extruder at pH 9 and 11. The researchers proposed that amylopectin could be more reactive in

the cross-linking process due to higher retained phosphate salts in the branched structure of amylopectin (O'Brien et al., 2009; O'Brien and Wang, 2009).

1.4.2. Swelling properties

Generally, the settling volume portrays the swelling properties of starch granules; i.e., higher settling volume represents higher swelling power. The settling volume was observed at 95 °C, 80 °C, and 37 °C, because these temperatures are the incubation temperatures for α -amylase enzymes in starch digestion methods of AOAC 991.43, ACDF, and Englyst methods, respectively. The settling volumes of native and CL starches (Table 1-1) were comparable at 37 °C. According to Tester and Morrison (1990), waxy and normal maize starches begin swelling at 50 °C to 55 °C; however, normal maize starch swells at a slower rate. Thus, our results suggested very little or no swelling for both native and CL granule starches at 37 °C. At 80 °C and 95 °C, both native and CL starches showed an increase in the settling volume, but the CL starches had significantly lower value than the native starches due to increased resistance against swelling as a result of stronger interactions between starch chains (Chung et al., 2004). The settling volume at 80 °C and 95 °C of both native and CL starches increased with increasing amylopectin content (waxy > normal > high amylose).

Fig. 1-1 represents the microscopic micrographs of native and CL maize starches at room temperature (24 °C), 80 °C, and 95 °C. The samples were not microscopically observed at 37 °C, mainly due to insignificant changes of settling volume at 37 °C (Table 1-1). The granule size of native starches increased drastically at 85 °C, with waxy and normal maize starches more prominently swollen than high-amylose maize starch. At 95 °C, only the high-amylose maize starch was able to retain the granule shape, whereas the waxy and normal maize showed irregular smaller pieces of swollen granules, indicating granular fermentation and solubilization. For the CL maize starches, the granule size was relatively bigger at 80 °C than those at room temperature, but the swelling of CL maize starch granules did not differ much at 95 °C compared with 80 °C, as depicted by the settling volume. All of the CL maize starches were able to retain the shape of the granules at 80 °C and 95 °C.

The present data are in agreement with Tsai et al. (1997), who demonstrated increased swelling of starch granules at higher temperatures (60 °C–95 °C). Tester and Morrison (1990) also showed a linear increase of the swelling of native waxy and normal maize starches from 50

°C to 80 °C. They found that the swelling of normal maize starch was significantly lower than waxy maize starch and suggested that higher amylose and the presence of amylose-lipid complexes in the granules can strongly restrict swelling at all temperatures. Starch swelling properties also greatly depend on the amylose and amylopectin content in the starch granules, where swelling is described as a property of amylopectin (Tester and Morrison et al., 1990). On the other hand, amylose content is inversely proportional to the degree of granular swelling (Lii et al., 1996; Shi et al., 1998), which explains the lower swelling of high-amylose maize starch. Higher amylose content is also correlated with higher rigidity (Singh, 2010); therefore, swelling will increase with higher amylopectin content and decrease with higher amylose content. The phenomenon is applicable to both native and CL starch, but cross-linking treatment further suppresses swelling due to strongly the associated bond caused by P molecules (Chung et al., 2004).

1.4.3. Pasting property

Fig. 1-2 presents the pasting curves of the CL starches. Distinguishing the pasting curves of CL samples at a lower concentration (8–10%) was difficult because of the restricted swelling of CL starches; thus, a higher concentration was used to study the pasting property of the CL (20% concentration of solids) starches. Among the CL starches, pastes from waxy maize starch had significantly higher peaks and final viscosities. During heating and holding periods, CL waxy maize starch pastes progressively increased without any breakdown, indicating stability of the pastes against shear. The pasting curve of CL normal maize starch was slightly higher than the CL high-amylose starches, and the pasting curve of the CL starches was positively correlated with settling volumes. Because the pasting properties of starch granules are closely related to swelling behavior, amylose and amylopectin contents in the starch granules play a large role in influencing the pasting curves. CL waxy maize starch consists of ~99% amylopectin, where the latter is associated with greater swelling (Tester and Morrison, 1990), as indicated in the present settling volume (Table 1-1). Both CL normal and CL high-amylose maize starches contain amylose and an amylose-lipid complex (Shi et al., 1998). The amylose-lipid complex is known to be water-insoluble and to dissociate at very high temperatures (94 °C–98 °C) (Raphaelides and Karkalas, 1988). Viscosity of starch pastes are also influenced by the synergistic effects of amylopectin chain length and amylose molecular size (Jane and Chen, 1992). Therefore, the

restriction against swelling of starch granules that caused lower pasting viscosity was more pronounced in the CL normal and high-amylose starches.

1.4.4. Gelatinization

The gelatinization transition temperatures consisting of T_o , T_p , and T_c as well as ΔH of CL maize starches before and after digestions at 37, 80, and 95 °C are presented in Table 1-2. The gelatinization of the CL waxy maize and CL normal maize starches took place between 75 °C (T_o) to 94 °C (T_c) and 72 °C (T_o) to 92 °C (T_c), respectively. The ΔH of CL waxy maize starch was relatively higher than that of CL normal maize starch. The current data of CL waxy maize starch were similar to that of Woo and Seib (2002); however, the CL normal maize starch had significantly higher gelatinization transition temperatures and ΔH than the reported value (Woo and Seib, 2002). Before digestion, endothermic peak was observed in the curves of CL waxy and normal maize starches. The residues of CL waxy and normal maize starches after digestions at 80 and 95 °C showed no endothermic values, suggesting that the starch crystallinity was already lost at both temperatures.

Gelatinization of raw CL high-amylose starch was 73 °C and extended to a higher temperature (114 °C) than CL waxy and CL normal maize starches and ΔH of 9.1 J/g, which was similar to the data in high-amylose maize starch reported by Liu et al. (2006) and Shresta et al. (2012). The endothermic peak of CL high-amylose maize starch was also broader, suggesting heterogenous structure of starch granule (Shresta et al., 2012). As opposed to other samples, residues of CL high-amylose maize starch after digestions at both 37 and 80 °C showed endothermic peaks. Interestingly, the onset and midpoint temperatures of the CL high-amylose starch digested at 80 °C shifted to higher values than before digestion and after digestion at 37 °C, which was also observed in high-amylose maize starch by Jiang et al. (2010). The significant increase was possibly due to the existence of long-chain double-helical crystallites (Jiang et al., 2010) that was resistant to the digestion at 80 °C. At higher temperature, the CL high-amylose starch showed no endothermic peak, suggesting its inability to withstand full gelatinization at 95 °C.

1.4.5. X-ray diffraction patterns

Fig. 1-3 shows changes in X-ray diffraction patterns and crystallinity for the CL starches after digestion by the Englyst, ACDF, and AOAC 991.43 methods with α -amylase incubation

temperatures of 37 °C, 80 °C, and 95 °C, respectively. Both CL waxy and CL normal maize starches showed a distinctive A-type X-ray diffraction pattern with doublet diffraction (17 and 18 2 θ) and other peaks at 15 and 23 2 θ , whereas the CL high-amylose starch displayed a B-type X-ray crystallinity with a strong diffraction at 17 2 θ and weaker diffractions at 6, 15, 19.5 and 22 2 θ .

Crystallinity of starch has been traditionally calculated by dividing the area under the crystalline peaks by the total area under the X-ray diffractogram, using an amorphous standard to separate the amorphous regions with the crystalline peaks (Wakelin et al., 1959). Improved methods to determine starch crystallinity consider not only the amorphous and double helix crystal conformations, but V-type single helix is also accounted in the quantitation. The ¹³C NMR method quantitatively analyzes amorphous, single-helix V-type and double helix conformations and proved that different source of sample has different ¹³C NMR spectral of amorphous region (Tan et al., 2007). Peak-fitting technique or crystal-defect approach applies a fitting on the amorphous and crystalline regions in the whole X-ray diffraction pattern which are able to isolate the V-type single helix form in starch. Crystal disorder or decrease in the size of the crystal is identified by the broadening of diffraction peaks (Lopez-Rubio et al., 2008).

Crystallinity of the CL maize starches after digestions was noted to decrease with the increasing temperature of incubations in the Englyst (37 °C), ACDF (80 °C), and AOAC 991.43 (95 °C) methods. CL waxy and CL normal maize starches had no crystallinity at 80 °C and all CL starches showed 0% crystallinity after digestion at 95 °C in the AOAC 991.43 method. The decrease of crystallinity after each method was more prominent in the CL waxy and CL normal maize starches than in CL high-amylose maize starch, which was also consistent with the DSC data (Table 1-2). Before digestion, the order of the crystallinity of the CL maize starches was CL waxy > CL normal > CL high amylose (Fig. 1-3) which associates higher crystallinity with higher digestion. Besides amylose content, the digestibility of starch is also believed to be controlled by the amorphous regions (Htoon et al., 2009) and the absence of pores and channels on the B-type crystallites (Dhital et al., 2010), which in this case was the CL high-amylose maize starch.

1.4.6. Starch granular morphology before and after enzyme digestion

The morphological properties of the CL maize starches and the residues after AOAC 991.43 (2000), ACDF (2009), and Englyst (1992) procedures were observed using SEM at 1000× and 5000× magnifications as shown in Fig. 1-4. The difference in the susceptibility to enzyme attack was clearly visible in all CL starches for all methods. The CL high-amylose starch granules were shown to be intact for all methods; however, high-amylose granules after 120 min of incubation at 37 °C in the Englyst method had distinctive cracks that may be caused by longer incubation time compared with the other methods at only 35 min. The CL waxy maize starch was more degraded for both AOAC 991.43 (2000) and ACDF methods. The severity of starch damage in the waxy maize starch was more prominent in the AOAC 991.43 method. Increased starch damage could be due to higher incubation temperature (95 °C) and increased granular swelling. This reasoning is strongly supported by the high settling volume of CL waxy maize starch at 95 °C (Table 1-1); therefore, the starch granules had higher surface area for the enzyme attack, resulting in a higher extent of starch surface erosion. The morphology of native maize starches digested using the Englyst method showed greater intensity of starch degradation, with the waxy maize starch being the most digested, followed by the normal and high-amylose maize starches (Brewer et al., 2012).

Both CL waxy and CL normal maize starch granules assayed from the Englyst method had “Swiss cheese” shell appearances with scattered deep holes into the granules (Fig. 1-4) as described by Robyt (2009), but the deep holes were more pronounced in the CL normal maize starch, causing some of the granules to lose granular identity. This result indicates that hydrolysis of the starch granules was more evident in the CL normal maize starch granules, which explains the significantly lower RS value (Table 1-1). The microscopic observation agrees with Kimura and Robyt (1995), who reported that high-amylose maize starch was more resistant to enzyme digestion. The modification of starch through cross-linking treatment was proven not to alter the crystalline pattern of starch granules, because cross-linking is believed to take place in the amorphous region (Hoover and Sosulski, 1986; Koo et al., 2010). The surface of type A starches (waxy and normal maize starches) has been associated with the existence of peripheral pores and channels, which allows penetration of α -amylase that resulted in an inside-out hydrolysis mechanism (Zhang et al., 2006). In comparison, the type B starches (high-amylose starch) are normally hydrolyzed progressively by amylase enzymes starting from the surface and proceeding

to the inside of the granule by exocorrosion (Gallant et al., 1997). Smooth-surfaced granules, as observed on the CL high-amylose starch granules in the present study, are also associated with suppression of enzymatic digestion (Oates, 1997).

1.4.7. Digestibility

The TDF of the samples was assayed using the AOAC 991.43 and ACDF methods to observe any significant differences in the value obtained. For the native starches, both methods yielded low TDF (< 0.2%) for waxy and normal maize starches compared with high-amylose starch (19%) (Table 2). In contrast, the CL starches had significantly higher TDF content ranging from 29% to 79%, in the order of CL waxy < CL normal < CL high amylose. The higher TDF in the CL starches indicated less susceptibility of the CL starch granules to digestive enzymes. Statistically, all samples except the native high-amylose starch showed a significantly higher amount of TDF with the ACDF method than with the AOAC 991.43.

The digestive profiles of the native and CL starches were assayed using the Englyst method to observe the digestibility of the maize starch granules at a lower temperature (37 °C). The SDS of native waxy and normal maize starches were significantly higher than that of native high-amylose starch (Table 1-1), and the RS of native high-amylose starch (~70%) was statistically higher than the other native starches. The cross-linking treatment significantly improved RS value of all starches (Table 1-1) in the order of CL high amylose > CL waxy > CL normal maize starches. The likely reason for the CL normal maize starch having the lowest RS is the significantly lower P content in CL normal maize starch than in CL waxy maize starch. Although the P content of CL high-amylose starch is similar to that of CL normal starch, the native high-amylose maize starch was more resistant to enzyme digestion than the native normal maize starch (Table 1-1).

The digestibility of starch is complex and influenced by the amylose:amylopectin ratio, amylose-lipid complexes, degree of gelatinization, percentage of retrograded starch, granular architecture, surface organization and granule particle size (Sievert and Pomeranz, 1988; Bird et al., 2009). The present study focused mainly on the effect of the amylose:amylopectin ratio on the digestibility of native and CL maize starches as noted in this and previous studies (Zhu et al., 2011). However, amylose content per se is not the reason why high amylose maize starch is more resistant to enzyme digestion. Granule architecture features, particularly surface organization

seems to be responsible for the low digestion of high-amylose maize starch granules (Shrestha et al., 2012). Because high-amylose maize starch granules do not have extensive pores, enzyme digestion has to proceed from the outside but the more molecularly organized outer regions of the granule function as an effective barrier for enzymes to access and digest the less organized interior (Shrestha et al., 2012). The digestibility of starch granules was found to be inversely correlated with the amylose content. In this study, the TDF content obtained from both methods was inversely correlated with settling volume (Table 1-1); and we believe that increased granule swelling allows α -amylase diffusion inside starch granules easier, which leads to more digestion.

In addition, shaking used in the digestion procedures causes higher inter-granular friction during movement of starch granules. The starch granules are more susceptible to shear motions with a higher degree of swelling, mostly due to granule softening (Hoover and Vasanthan, 1994). Hence, the breakdown of the CL waxy maize starch granules is easier due to a higher extent of swelling that facilitates α -amylase attack. The CL high-amylose starch granules had lower swelling which inhibited enzyme hydrolysis.

Using a lower temperature (37 °C) during incubation in the Englyst method did not cause as much swelling of starch granules at higher temperatures as in the ACDF (incubation temperature at 80 °C) and AOAC 991.43 (incubation temperature at 95 °C) methods. Less granule swelling resulted in more restriction of starch hydrolysis by digestive enzymes. This result explains the high content of SDS in the native starches, except for high-amylose starch. The cross-linking treatment increased the resistance of starch granules, thus increasing the RS content. Lack of pores in native high-amylose maize starch makes it resistant to enzyme digestion. The cross-linking treatment of high-amylose maize starch provides a stronger bond with the inclusion of P molecules in the granules, resulting in more amylolysis inhibition; hence, the RS value increased significantly. The CL starch is categorized under RS4, which is also considered dietary fiber; however, RS in CL waxy maize could be destroyed at higher temperatures, which may result in lower TDF content, as shown in the present data.

Of all methods utilized, the Englyst method is more suitable to describe digestion of food in the small intestine of humans because of the similar incubation temperatures. The present study, however, assayed raw starch, which is unlikely to describe the digestion action of cooked and processed food that is consumed by humans. Therefore, the TDF methods (AOAC 991.43 and ACDF) are more applicable to reflecting the RS content of processed food that undergoes

mechanical shear and cooking at high temperature, because the possibility of RS of CL starch being affected during food processing is reasonably high.

1.5. Conclusions

At 37 °C, the RS increased with increased amylose content for native starches. At 80 and 95 °C, the swelling of starch granules of native waxy and normal maize starches increased significantly and became more susceptible to enzyme digestion. The native high-amylose maize starch had limited swelling and higher TDF content than waxy and normal maize starches. Cross-linking bonds hindered enzyme hydrolysis and dramatically increased the RS and TDF contents of waxy and normal maize starches. For the CL high-amylose maize starch, swelling at 80 and 95 °C was low, resulting in higher TDF by both ACDF and AOAC 991.43 methods. In contrast, the CL waxy maize starch swelled more at 95 °C and yielded lower TDF as measured by the AOAC 991.43 method than with the ACDF method. Knowledge of swelling and digestion of starches with different amylose contents is useful in food processing to attain certain functionalities and nutritional properties in food products. *In-vivo* methods can be applied to further explore and understand the digestibility of CL starches.

1.6. Acknowledgements

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Table 1-1: Phosphorus content, settling volume, rapidly digestible starch (RDS), slowly digestible starch (SDS), resistant starch (RS), and total dietary fiber (TDF) of native and cross-linked maize starches with different amylose contents.¹

	Native			Cross-linked		
	Waxy	Normal	High-amylose	Waxy	Normal	High-amylose
Phosphorus (%)	0.00 ± 0.00 c	0.00 ± 0.00 c	0.00 ± 0.00 c	0.36 ± 0.01 a	0.32 ± 0.01 b	0.32 ± 0.01 b
Settling volume (mL)						
37 °C	2.00 ± 0.00 a	2.00 ± 0.00 a	2.00 ± 0.00 a	2.00 ± 0.00 a	2.00 ± 0.00 a	2.00 ± 0.00 a
80 °C	22.25 ± 0.35 a	11.45 ± 0.07 b	3.55 ± 0.07 e	4.50 ± 0.00 c	3.95 ± 0.07 d	3.00 ± 0.00 f
95 °C	25.75 ± 0.35 a	17.05 ± 0.07 b	4.30 ± 0.14 d	5.00 ± 0.00 c	4.00 ± 0.00 d	3.10 ± 0.14 e
Digestion profile ²						
RDS (%)	37.22 ± 0.82 a	29.33 ± 0.26 b	9.84 ± 0.20 c	6.37 ± 0.36 e	9.15 ± 0.39 c	7.08 ± 0.07 d
SDS (%)	62.18 ± 0.54 b	65.64 ± 1.43 a	19.76 ± 0.13 d	11.99 ± 0.37 e	29.60 ± 2.38 c	8.92 ± 0.46 f
RS (%)	0.61 ± 0.28 f	5.03 ± 1.17 e	70.40 ± 0.08 c	81.64 ± 0.73 b	61.25 ± 1.99 d	84.94 ± 0.94 a
TDF ³ (%)	0.10 ± 0.02 d	0.17 ± 0.07 d	19.32 ± 1.37 c	37.87 ± 2.82 b	65.23 ± 2.01 a	69.44 ± 2.15 a
TDF ⁴ (%)	0.03 ± 0.01 e	0.06 ± 0.01 e	18.45 ± 1.07 d	29.43 ± 1.01 c	63.24 ± 1.02 b	68.44 ± 0.73 a

¹ All values are reported on dry basis of starch. Data are means ± standard deviation. Means within rows not sharing a common letter are significantly different at $p \leq 0.05$.

² Measured by the Englyst method (Sang and Seib, 2006).

³ Measured by Available Carbohydrate and Dietary Fiber method using an assay kit from Megazyme International Ltd. (Wicklow, Ireland).

⁴ Measured by AOAC 991.43 method using an assay kit from Megazyme International Ltd. (Wicklow, Ireland).

Table 1-2: Thermal properties of cross-linked maize starches and their collected residues at the conclusion of Englyst (Sang and Seib, 2006), Available Carbohydrate and Dietary Fiber (ACDF), and AOAC 991.43 methods as measured by differential scanning calorimetry.

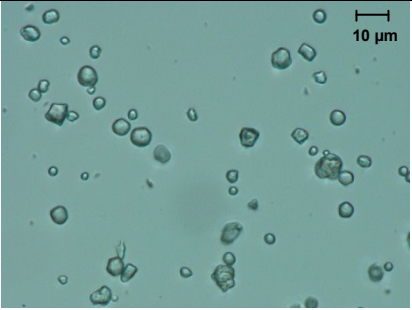
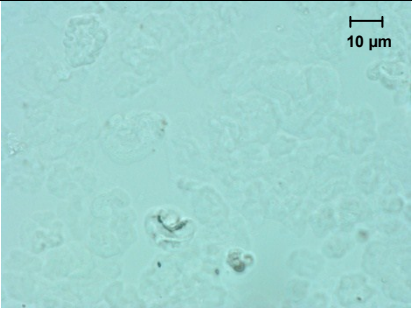
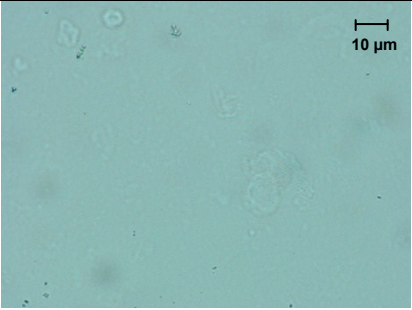

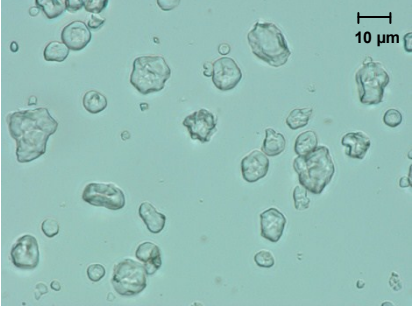
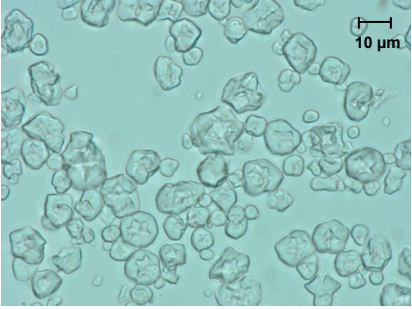


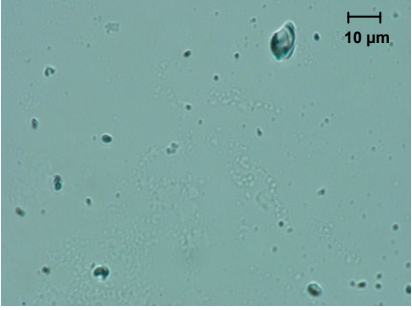
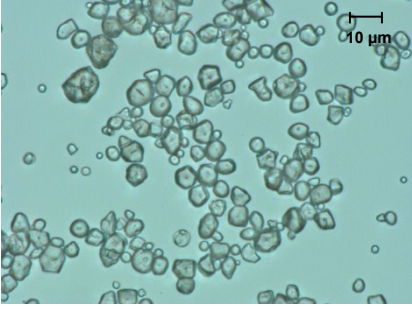


	Temperature (°C)			ΔH (J/g)
	T_o	T_p	T_c	
CLW ²	75.1 ± 1.9 a	80.2 ± 0.1 a	94.4 ± 1.3 a	17.6 ± 0.5 a
CLW after Englyst	75.6 ± 0.1 b	81.3 ± 0.1 a	94.6 ± 0.7 a	16.9 ± 0.3 a
CLW after ACDF	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b
CLW after AOAC 991.43	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b
CLN ³	72.5 ± 0.0 a	77.1 ± 0.1 a	92.7 ± 0.5 a	13.0 ± 0.0 a
CLN after Englyst	71.9 ± 0.1 a	77.9 ± 0.2 a	92.0 ± 0.1 a	12.1 ± 0.5 a
CLN after ACDF	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b
CLN after AOAC 991.43	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 a	0.0 ± 0.0 b
CLH ⁴	73.4 ± 0.0 b	86.1 ± 1.4 b	114.9 ± 0.6 ab	9.1 ± 0.5 a
CLH after Englyst	72.8 ± 0.8 b	86.9 ± 0.2 b	114.0 ± 1.8 b	9.7 ± 0.2 a
CLH after ACDF	87.4 ± 0.0 a	97.6 ± 0.1 a	116.3 ± 1.1 a	5.4 ± 0.6 b
CLH after AOAC 991.43	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c

¹ All values are reported on dry basis of starch. Data are means ± standard deviation. Starch solid was 33.3%. Means within columns not sharing a common letter are significantly different at $p \leq 0.05$.

² Cross-linked waxy maize starch.

³ Cross-linked normal maize starch.

⁴ Cross-linked high-amylose starch.

	Room temperature (24 °C)	80 °C	95 °C
Native waxy maize starch	 10 μm	 10 μm	 10 μm
CL waxy maize starch	 10 μm	 10 μm	 10 μm
Native normal maize starch	 10 μm	 10 μm	 10 μm
CL normal maize starch	 10 μm	 10 μm	 10 μm

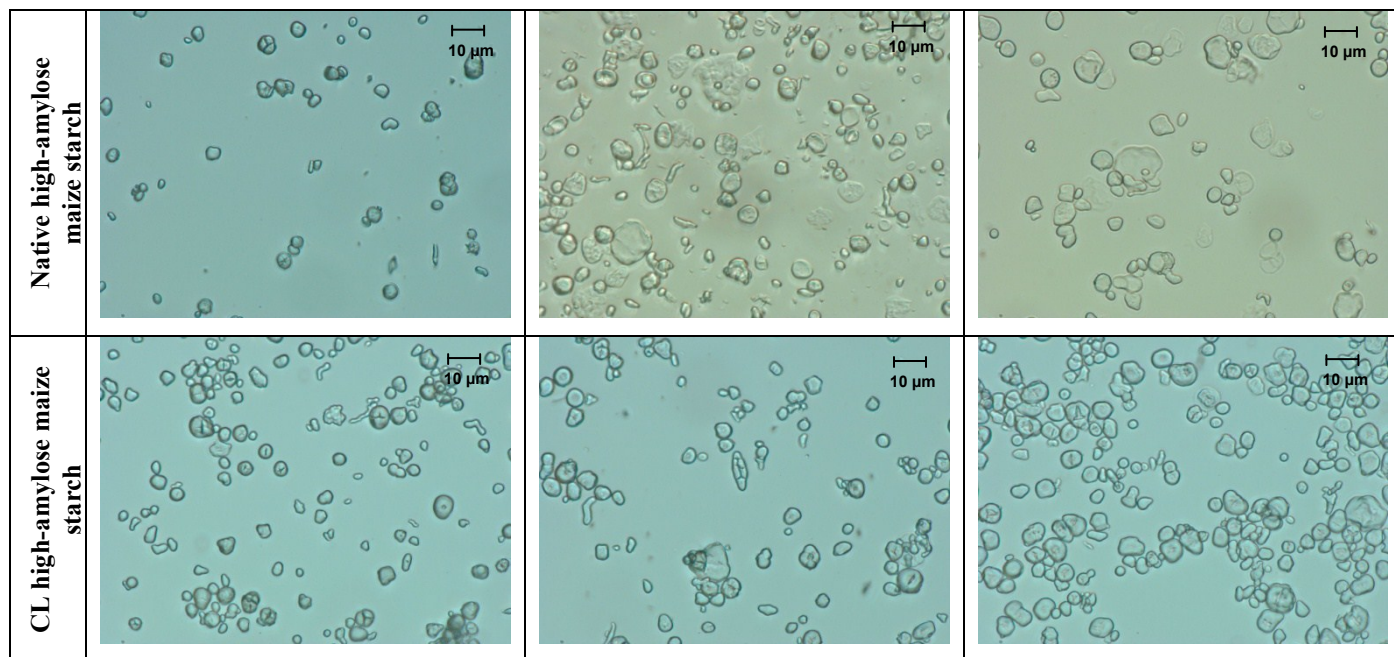


Fig. 1-1. Microscopic photos of native and cross-linked maize starches with different amylose contents at room temperature (24 °C), 80 °C, and 95 °C. The micrographs are at 40× objective.

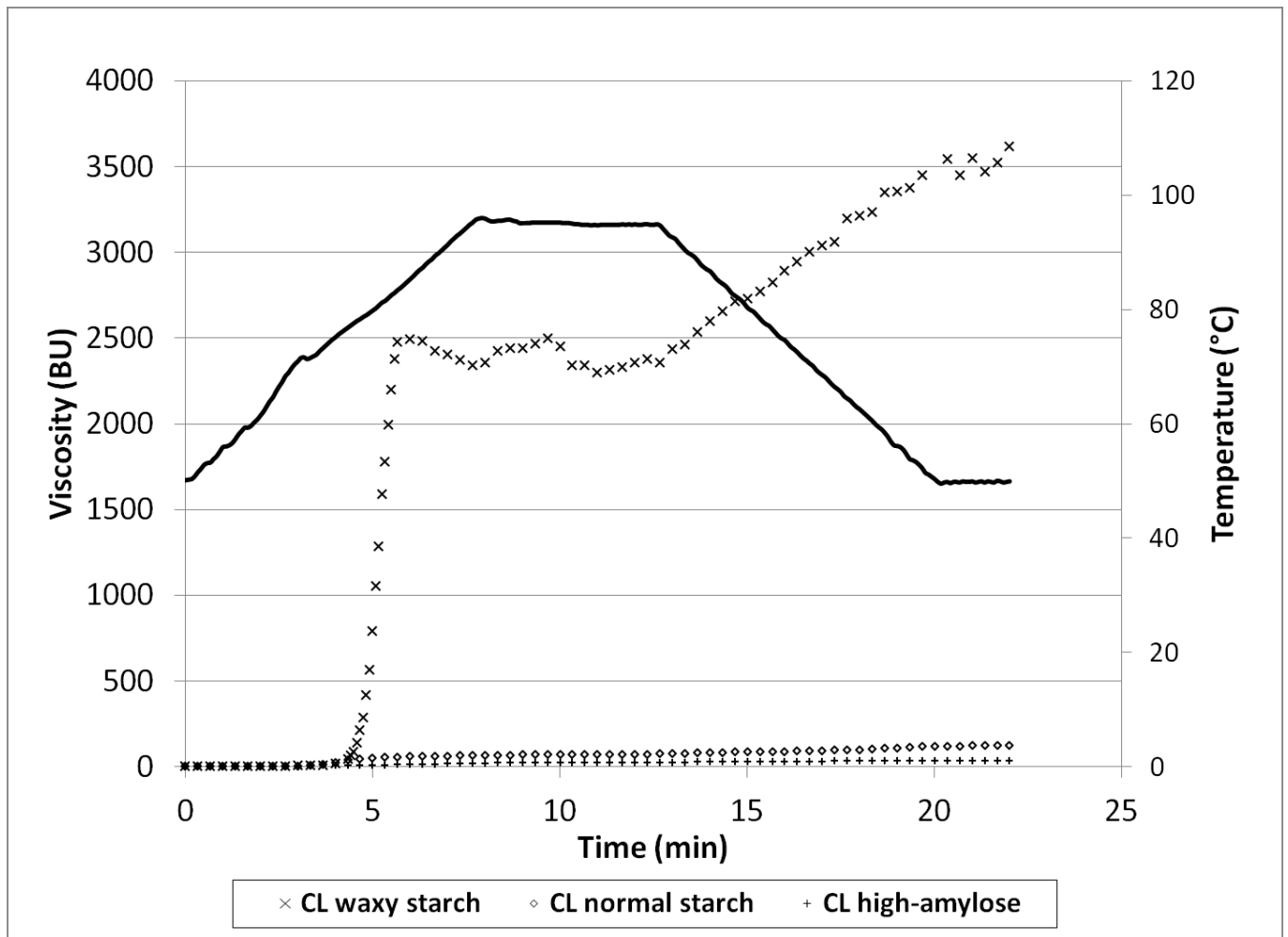


Fig. 1-2. The pasting curves of cross-linked (CL) maize starches with different amylose contents.

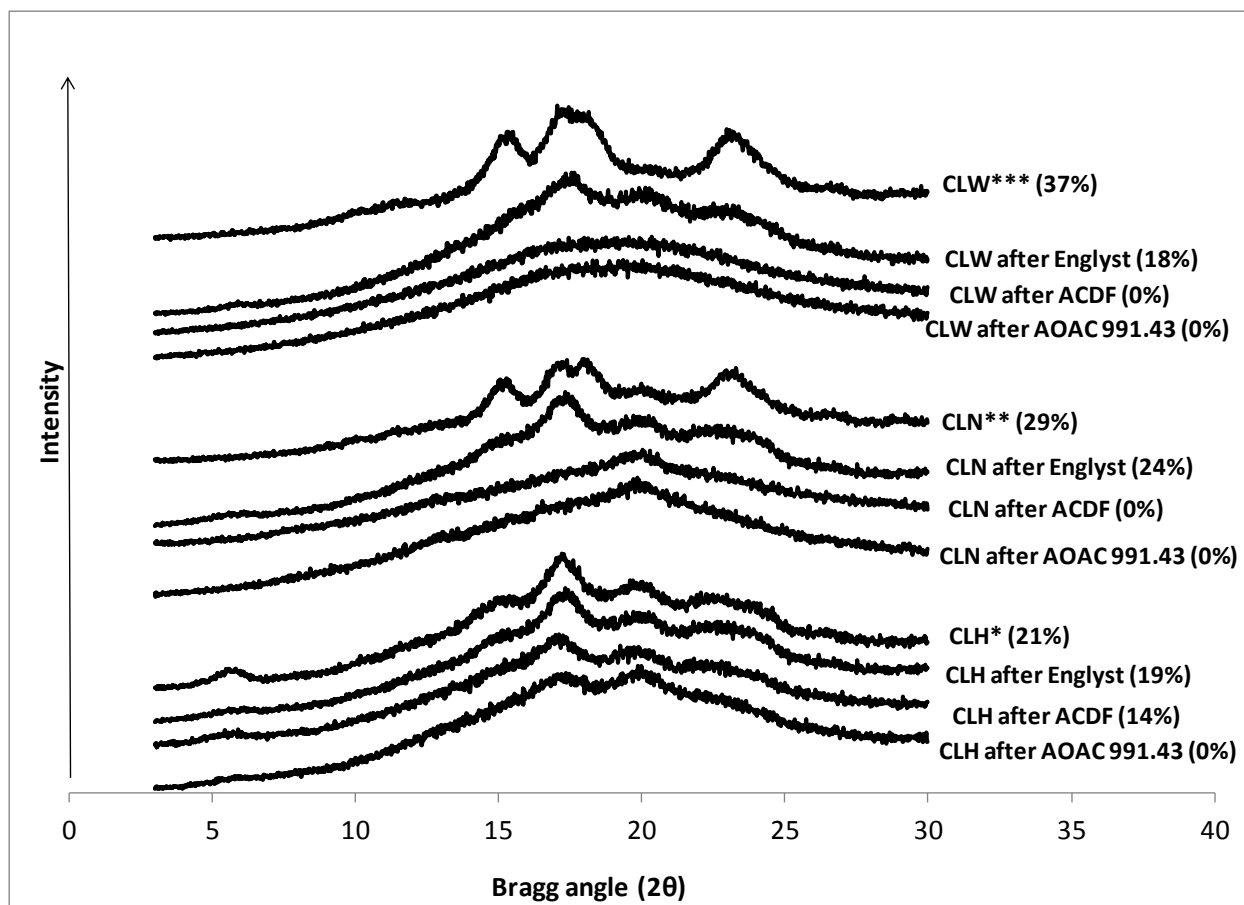
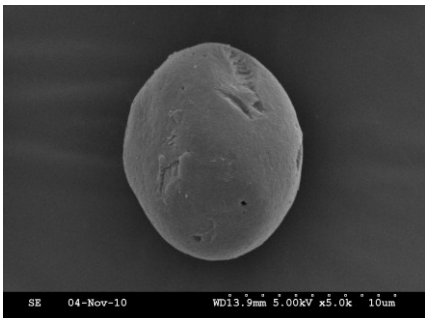
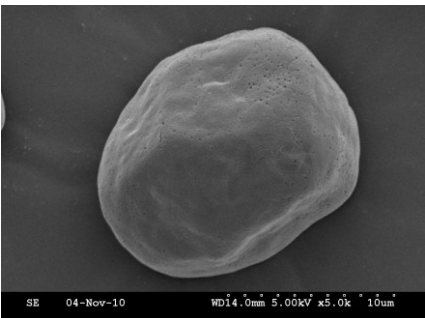
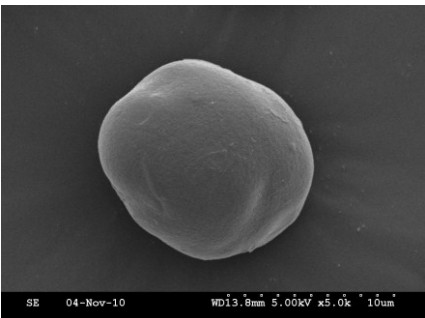
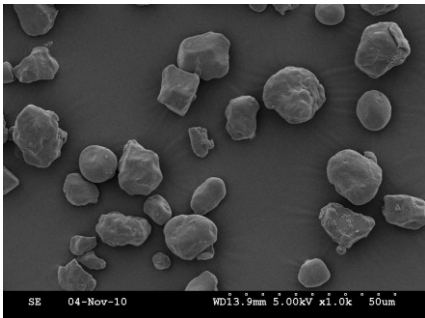
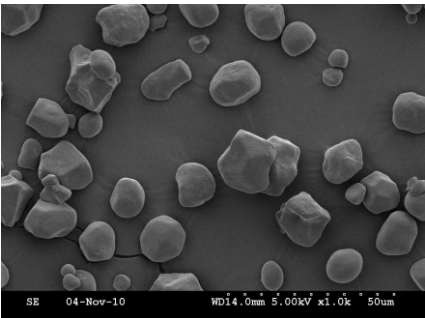
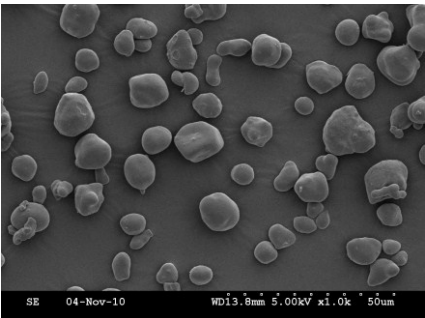
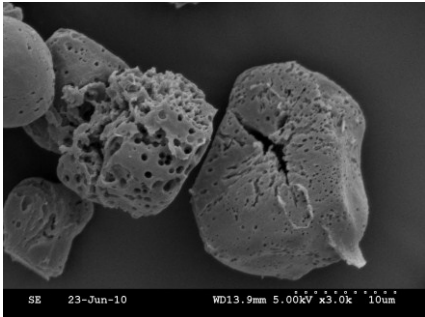


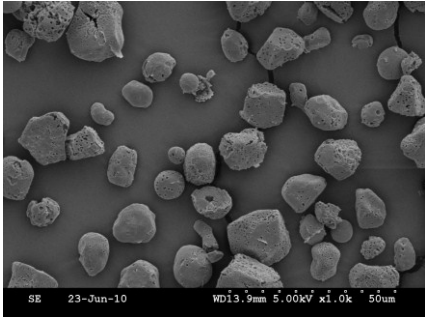
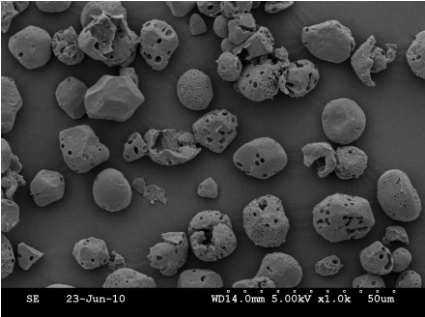
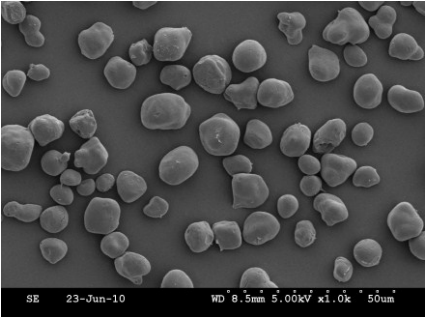


Fig. 1-3. The X-ray diffraction pattern of cross-linked maize starches with different amylose contents and the residues collected at the end of Englyst (Sang and Seib, 2006), Available Carbohydrate and Dietary Fiber (ACDF), and AOAC 991.43 methods. Crystallinity (%) is given in paratheses.

* Cross-linked high-amylose starch

** Cross-linked normal maize starch

*** Cross-linked waxy maize starch

	CL waxy	CL normal	CL high-amylose
Control starch granules (5000×)			
Control starch granules (1000×)			
Starch residues after Englyst method (5000×)			
Starch residues after Englyst method (1000×)			

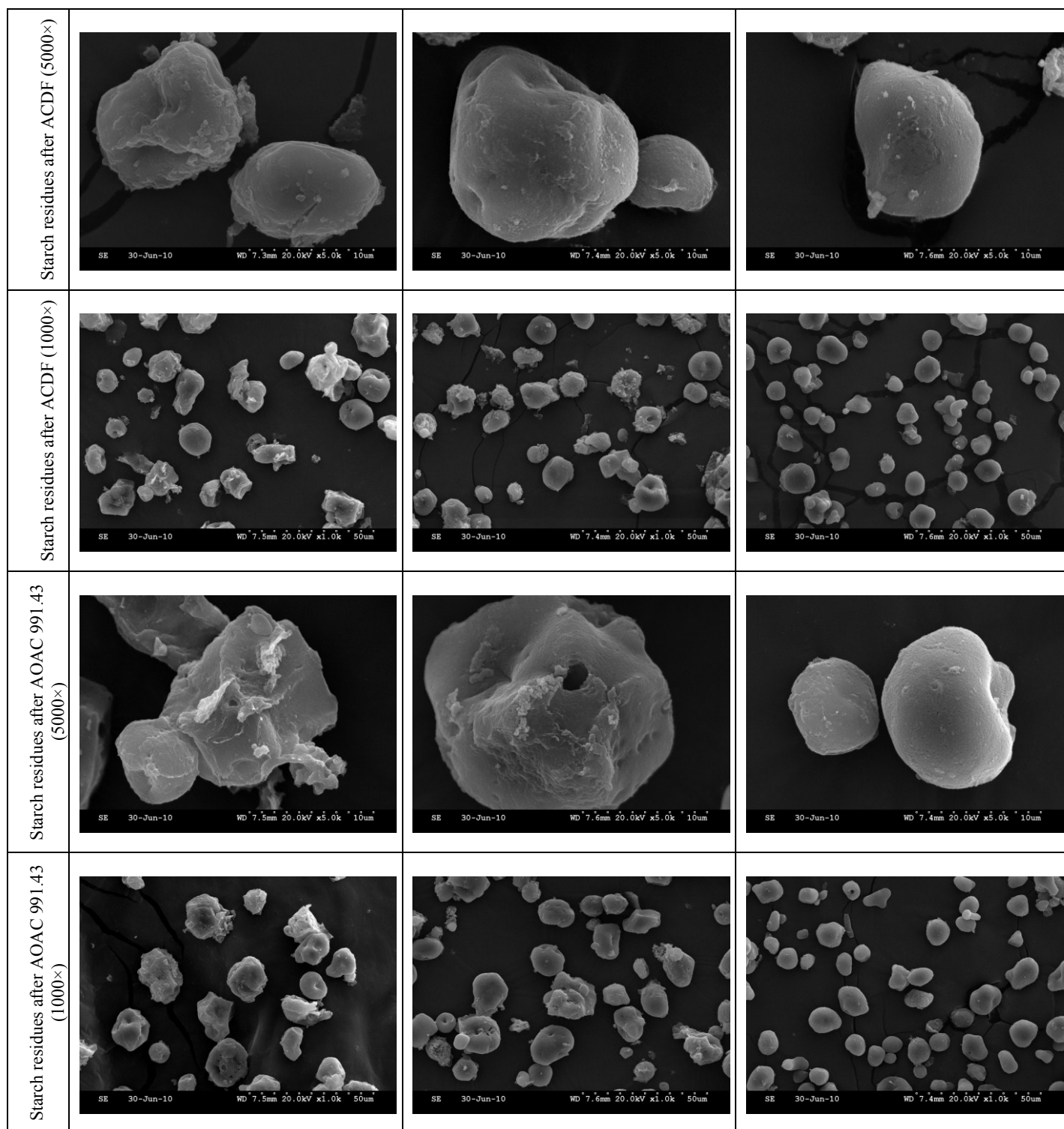


Fig. 1-4. The scanning electron micrographs of cross-linked maize starches with different amylose contents and the residues collected at the end of Englyst (Sang and Seib, 2006), Available Carbohydrate and Dietary Fiber (ACDF), and AOAC 991.43 methods. The micrographs are at 1000× and 5000× magnifications.

Chapter 2 - Structure and pasting properties of alkaline-treated cross-linked waxy maize starches

2.1. Abstract

The objectives of this study were to determine the stability of cross-linked bonds of starch with different pH values and their effects on the pasting property of waxy maize starch cross-linked by 0.05% and 3% sodium trimetaphosphate/sodium tripolyphosphate. The cross-linked waxy maize starch (CLWMS) was slurried (40%, w/w) and subjected to alkali treatments of pH 9, 10, 11, and 12 using 1.0 M sodium hydroxide at 40 °C for 4 h. The phosphorus in 3% CLWMS decreased with increasing pH and remained unchanged in 0.05% CLWMS for all pH treatments. Decreased settling volumes indicated the reduction of swelling power for the alkali-treated CLWMS at pH 11 and 12. The ³¹P spectra of 3% CLWMS at pH 12 showed decreased cyclic monostarch phosphate, monostarch monophosphate, and monostarch diphosphate, but significantly increased distarch monophosphate from 0.039 to 0.057% (46%). Crystallinity of 0.05% CLWMS and 3% CLWMS decreased after alkali treatment. Alkali treatments of cross-linked starches at various pH offer a way to manipulate the pasting and rheological properties of cross-linked starch for desired food applications.

2.2. Introduction

Phosphorylation, or cross-linking of starch, is a well-known starch modification method (Paschall, 1964). The main purpose of starch cross-linking is to eliminate the shortcomings of native starch during prolonged heating and extreme processing conditions (e.g., high acidity, high shear) (Wurzburg, 2006). These extreme conditions are common in the food industry and are normally associated with the rupturing of swollen granules and fragmentation that lead to decreased viscosity and development of the stringy and cohesive texture of native starch, especially waxy starch (Rutenberg and Solarek, 1984). The detrimental effects on starch can be minimized or prevented by introducing cross-linking groups in the starch molecules by binding the neighboring anhydrous glucose units (AGU) in the amorphous regions. Cross-linking suppressed the swelling of starch granules due to strongly associated bonds, thus developing a short and non-cohesive paste texture and improved viscosity (Xie et al., 2005; Wurzburg, 2006). The enhanced quality of starch paste texture can be achieved with a very low level of

substitutions, because even low levels of cross-linking have a marked improvement on the rheological and texture properties (Wurzburg, 2006).

Starch is chemically phosphorylated with sodium trimetaphosphate (STMP), sodium tripolyphosphate (STPP) or phosphoryl chloride to produce ester derivatives (Solarek, 1995; Woo and Seib, 2002; Sang and Seib, 2006; Sang et al., 2007). Ester derivatives produced are monostarch phosphates and/or distarch phosphates. The ratio of monostarch and distarch phosphates depends mainly on the phosphorylating agents used, concentration, reactivity, and reaction conditions (Woo and Seib, 2002; Sang and Seib, 2006; Sang et al., 2007). Monostarch phosphates are formed through the esterification of a phosphate to the hydroxyl group of one AGU, whereas distarch phosphates are produced when hydroxyl groups of two AGUs are esterified to the same phosphate group via cross-linking (Landerito and Wang, 2005). Unlike distarch phosphates, which are formed by starch reaction with polyfunctional phosphorylating agents, monostarch phosphates are produced using monofunctional reagents (Thomas and Atwell, 1999). Monostarch phosphates are produced with a higher substitution of phosphates on starch than distarch phosphates (Lim and Seib, 1993). Distarch phosphates result in improved starch texture and very low phosphorus (P) content (0.04%), whereas monostarch phosphates require significantly higher total P (0.1-0.4%) to improve appearance, hygroscopicity, transparency, swelling, and viscosity (Rutenberg & Solarek, 1984).

The pH during the starch phosphorylation process directly influences the concentration of mono- and di-esters in cross-linked starches. Early research showed that phosphate monoesters were produced using mixtures of orthophosphates at the pH of 5.0-6.5, whereas usage of STPP required pH of pH 5.0–9.0 (Kerr and Cleveland Jr., 1947). A combination of 2% STMP and 5% STPP at pH 9.5 produced cross-linked starch with better heat and shear resistance (Lim and Seib, 1993); however, Woo and Seib (2002) showed that cross-linking at pH 11.5 using a combination of 12% STMP and STPP was the optimum pH to produce resistant starch up to 0.4% total P. Understanding the structural changes of cross-linked starch caused by pH is crucial to gaining knowledge of the altered functional properties. Sang et al. (2010) reported that pH treatment on highly cross-linked starch caused a slight decrease in total dietary fiber but significantly reduced the levels of cyclic-monostarch monophosphate, monostarch diphosphate, and monostarch monophosphate at pH 12. The latter findings focused on the digestibility profile of the alkali-treated cross-linked starch, but did not reveal any alteration of pasting properties. High alkaline

treatment on cross-linked starch, particularly with a low level of phosphorylation, may have a significant effect on the pasting property. In addition, alkali treatments on cross-linked starch may have potential in manipulating the pasting property of cross-linked starch to the desired outcome; thus, the objective of this study was to determine the stability of cross-linked bonds in starch at different pH levels of aqueous sodium hydroxide and their effects on the pasting property of cross-linked waxy maize starch.

2.3. Materials and methods

2.3.1. Materials

Waxy maize starch (Amioca) was obtained from National Starch LLC (Bridgewater, NJ). STMP was purchased from MP Biomedicals, LLC (Solon, CA). STPP, sodium sulfate, sodium hydroxide, and hydrochloric acid were obtained from Fisher Scientific (Bridgewater, NJ). Thermally stable α -amylase (activity of 10000 unit (U)/mL on soluble starch) and amyloglucosidase (3300 U/mL on soluble starch) were obtained from Megazyme International Ireland Ltd. (Wicklow, Ireland). One U of α -amylase at pH 4.5 and 40 °C is defined as the amount of enzyme required to release one micromole of D-glucose per minute from soluble starch. One U of amyloglucosidase at pH 4.5 and 40 °C is defined as the amount of enzyme required to release one micromole of D-glucose per minute from soluble starch. All chemicals were reagent grade.

2.3.2. General methods

The P content was assayed using the procedure of Smith and Caruso (1964). Moisture content was obtained according to AACC method 44-15 (AACC 2000) by calculating the difference of starch weight before and after oven-drying at 130 °C for 1 h.

2.3.3. Preparation of cross-linked waxy maize starch (CLWMS)

CLWMS was prepared using the method of Woo and Seib (2002) with slight modifications. Two levels of cross-linking were prepared using 0.05% (starch basis, sb) and 3% (sb) combination of STMP and STPP (99:1) as phosphorylating agents, and these two products are labeled as 0.05% CLWMS and 3% CLWMS, respectively, throughout the paper. Preparation of 0.05% CLWMS involved mixing waxy corn starch (50.0 g, db), distilled water (70.0 ml),

STMP (0.02475 g, 0.0495%, sb), STPP (0.00025 g, 0.0005%, sb), and sodium sulfate (5 g, 10%, sb). The pH of the slurry was adjusted to 11.5 by slowly adding 1.0 M sodium hydroxide into the stirred mixture to avoid gelatinization of starch. The slurry was stirred continuously and held at 45 °C for a 3-h reaction in a water bath. The pH of the slurry was then adjusted to 6.5 by adding 1.0 M hydrochloric acid. The starch was centrifuged and washed with 150 mL distilled water seven times, dried in an oven overnight at 40 °C, and ground. The same procedure was repeated with 3% STMP/STPP to obtain a higher level of cross-linking.

2.3.4. Alkaline treatment of CLWMS

The treatment was done based on the method described by Sang et al. (2010). CLWMS (50.0 g, db) was mixed with water (70.0 ml) in five different beakers. The pH for one of the slurries was left unadjusted so it could act as the control, but the pH levels of the other slurries were adjusted to 9, 10, 11, and 12 using 1.0 M sodium hydroxide. The covered beakers containing the slurries were held at 40 °C for 4 h. After cooling to 25 °C, slurries were adjusted to 6.5 using 1.0 M hydrochloric acid. The starch was centrifuged (1500 × g, 10 min), supernatant was discarded, and starch was washed four times with distilled water, dried overnight at 40 °C, and ground.

2.3.5. Conversion of CLWMS to phosphodextrins for ³¹P nuclear magnetic resonance (³¹P NMR) spectroscopy

The treatment was based on the method described by Sang et al. (2010). CLWMS starch (1.0 g db) was weighed into a 50-mL centrifuge tube. The starch was slurried with 30 mL 2.0 mM calcium chloride at pH 8.2, and heat-stable α -amylase (100 μ l) was added. The mixture was heated in a boiling water-bath for 30 min, and 100 μ l of heat stable α -amylase was added into the mixture again. After cooling to room temperature, the pH of the mixture was adjusted to 4.5, and amyloglucosidase (200 μ l) was incorporated for 1 h to incubate at 60 °C. The mixture was adjusted to pH 7.0, centrifuged (1500 × g, 10 min), and the supernatant was freeze-dried.

2.3.6. ³¹P NMR spectra of the phosphodextrins

The freeze-dried starch digest (1.0 g, db) was dissolved in deuterium oxide (1.0 mL) containing 20 mM EDTA and 0.002% sodium azide. The pH of the solution was adjusted to 8.0 by adding 0.1 M sodium hydroxide. The proton-decoupled ³¹P NMR spectra were obtained using

a 11.75 Tesla Varian NMR System (Varian Inc., Palo Alto, CA). Using a method by Sang et al. (2007), the ^1H NMR was operated at 499.84 MHz and ^{31}P at 202.34 MHz. The ^{31}P NMR data collection was carried out at 25 °C using a delay of 6 s between pulses, with pulse width of 15.0 μs and sweep width at 12,730 Hz. The 3% CLWMS samples were run for 3 h, whereas 0.05% CLWMS samples required 24 h for ^{31}P spectra detection. The obtained spectra were processed and analyzed using Varian software VNMRJ Version 2.2C. Chemical shifts were reported in δ (ppm) from the reference signal of an 85% phosphoric acid (0.0 ppm) external standard.

2.3.7. Pasting properties of the CLWMS

Pasting properties of the CLWMS were determined using a Micro Visco-Amylograph (Brabender, Duisburg, Germany). CLWMS was added to distilled water to prepare 8% and 15% suspensions (dry weight basis, w/w) for 0.05% CLWMS and 3% CLWMS, respectively. The heating and cooling cycles were programmed by heating the suspension from 50 °C to 90 °C (heating rate of 10 °C/min), holding at 90 °C for 6 min, and cooling to 50 °C (cooling rate of 10 °C/min). The viscosity of the starch suspension was expressed in Brabender Units (BU).

2.3.8. Settling measurement of the CLWMS

Settling measurement of the CLWMS was determined by a method from Tayal et al. (2004) with slight modifications. CLWMS (5g, db) was stirred constantly in water (100 mL) and placed in water bath at 95 °C for 30 min. After the slurry cooled to 25 °C, slurry (20 g, wb) was transferred to 100 mL graduated cylinder containing water (80 g, wb). The settling volume of the CLWMS was taken after 24 h.

2.3.9. Thermal properties of the CLWMS

The gelatinisation temperatures and enthalpy of cross-linked starches were measured by a differential scanning calorimeter (DSC) (TA Instruments Q100, TA Instruments, New Castle, DE). The total solids content of samples was 33% (w/w, dry basis). After hydration for 1 h at 25 °C, 30 mg of the well-stirred sample suspensions were weighed into 40- μL aluminum crucibles and hermetically sealed to prevent moisture loss. Scans were performed from 10 °C to 130 °C at a constant rate of 10 °C/min. A sealed, empty crucible was used as a reference, and the DSC was calibrated before use with indium. The gelatinisation enthalpy (ΔH) and transition temperatures, namely the onset (T_o), peak (T_p), and conclusion (T_c) temperatures, were determined. The ΔH

was evaluated based on the area of the main endothermic peak and expressed in terms of J/g of dry starch.

2.3.10. X-ray diffraction

The alkali-treated CLWMS were hydrated with distilled water to 15% moisture content by placing samples in a hydration chamber. The X-ray diffraction patterns of the hydrated starch samples were recorded on a Philips APD 3520 X-ray diffractometer (MAC Science Co. MO3XHF22, Tokyo, Japan). The CuK α radiation was set at 35 kV and 20 mA, and a scanning rate of 2° 2 θ /min was used. The degree of crystallinity was estimated from the mathematical model of Wakelin method (Wakelin et al., 1954).

2.3.11. Statistical analysis

All data obtained were statistically analyzed using Statistical Analysis Software (SAS) (version 9.2, SAS Institute, Cary, NC) by analysis of variance (ANOVA). The means were expressed from two replicates unless stated otherwise.

2.4. Results and discussion

2.4.1. Total P content of the alkali-treated CLWMS

Before alkali treatment, P content of 0.05% CLWMS and 3% CLWMS was 0.002% and 0.101% (Table 2-1), respectively. Alkali treatment of 0.05% CLWMS at pH 9, 10, 11, and 12 showed comparable data of the P content. The 3% CLWMS under pH treatments of 9, 10, and 12 at 40 °C for 4 h showed 13–17% decrease of total P content, which is in agreement with Sang et al. (2010), who reported decreased P content at high pH. Hence, alkali treatments were able to “strip off” some P groups.

2.4.2. ³¹P NMR spectra – effects of alkaline treatment on the chemical forms of the phosphate esters on starch

Degrading large starch polymers into α , γ -dextrins by amylolytic enzymes improves their solubility in water (Sang et al., 2007; Spyros and Dais, 2009). The yielded dextrins are detectable by ³¹P NMR spectroscopy at 5–10% concentration in D₂O for acquisition of the spectra (Sang et al., 2007). The amylolytic enzymes are incapable of hydrolyzing glucosidic bonds near

phosphate groups (Kasemsuwan and Jane, 1996), so all spectral changes presented are due to alkali treatments on the CLWMS.

The ^{31}P NMR spectra of phosphate derivatives in 3% CLWMS are illustrated in Fig. 2-1, and the concentrations of each derivative are tabulated in Table 2. The ^{31}P NMR analysis was carried out on both 0.05% CLWMS and 3% CLWMS. Although 3% CLWMS was run for 24 h on the ^{31}P NMR, phosphate esters in 0.05% CLWMS were undetectable due to very low concentrations of P (0.002%). Spectra of 3% CLWMS before alkali treatment in Fig. 2-1 A has signals of cyclic-monostarch phosphate (cyclic-MSMP), monostarch monophosphate (MSMP) positioned at C3 and C6, inorganic phosphate (P_i), distarch monophosphate (DSMP), and monostarch diphosphate (MSDP). They were signaled at δ 15.4 ppm (cyclic-MSMP), δ 1-3 ppm (MSMP), δ 0.8 ppm (P_i), δ 0 to -1 ppm (DSMP) and δ -6 and -10 ppm (MSDP). The chemical shifts of the phosphate derivatives agreed with previously reported cross-linked starch data (Sang et al., 2007; Sang et al., 2010; Manoi and Rizvi, 2010). The phosphate derivatives in 3% CLWMS with alkali treatments of pH 9, 10, and 11 showed comparable changes in concentrations of phosphate esters (Table 2-2) compared with the untreated 3% CLWMS. Alkali treatment at pH 12 on 3% CLWMS caused a decrease on cyclic-MSMP, MSMP, and MSDP levels, but significantly increased the DSMP value by 20% (Table 2-2). Similar findings were observed by Sang et al. (2010), with an 18% increment of DSMP levels in cross-linked starch of 0.37% add-on P after being treated with sodium hydroxide at pH 12. At pH 12, existing MSDP or cyclic MSMP may form new cross-linked esters with a hydroxyl group on another starch, resulting in increased DSMP (Sang et al., 2010).

2.4.3. Pasting properties and settling volume

The pasting profiles of unmodified waxy maize starch and 0.05% CLWMS at 8% solids were shown in Fig. 2-2. At 8% concentration, the 3% CLWMS showed no detectable consistency by the Micro Visco-Amylograph due to the higher level of cross-linking that inhibited the starch granules from swelling. Therefore, a higher solid concentration (15%) was used for 3% CLWMS (Fig. 2-3).

It is interesting to note that 0.05% CLWMS had a much higher peak viscosity than native waxy maize starch and no significant breakdown (Fig. 2-2). Previously, waxy maize starch reacted with 0.025 to 0.050% phosphoryl chloride (starch basis) at pH 11.5 and 25 °C with 2%

sodium sulphate; had a peak consistency of 1250 -1500 BU, also much higher than unmodified waxy maize starch, which had a peak consistency of about 850 BU at 7.5% starch content (Reddy and Seib, 2000). In both cases, the cross-linked waxy maize starches had a higher consistency than unmodified waxy maize starch. At those low levels of cross-linking, starch solid content (7.5-8%) is above the close packing concentration and the cooked cross-linked starch granules are more rigid than the unmodified, resulting in higher consistency (Steeneken, 1989; Tayal et al., 2004). At 3% STMP/STPP, the starch was highly cross-linked and had a lower swelling capability. As a result, at 8% solids content, the system was still not filled by the swollen starch granules.

The settling volume of 0.05% CLWMS and 3% CLWMS (Table 2-3) was positively correlated with the pasting curves (Figs. 2-2 and 2-3). Settling volume portrays the swelling property of starch granules, where higher settling volume represents greater swelling power. The close-packing limit is dependent on the degree of swelling (settling volume) and the starch concentration (Steeneken, 1989; Tayal et al., 2004). In Table 2-3, 3% CLWMS had significantly lower settling volumes than 0.05% CLWMS due to a higher level of cross-linking that restricted starch granule swelling. The settling volumes of both 0.05% CLWMS and 3% CLWMS were not significantly affected by alkali treatment at pH 9 and 10; however, both 0.05% CLWMS and 3% CLWMS showed significantly decreased settling volumes when treated at pH 11. The decrease in settling volume explains the relatively lower viscosity of 0.05% CLWMS and 3% CLWMS treated at pH 11 (Figs. 2-2 and 2-3) compared with treatments with the lower pH values.

Both 0.05% CLWMS and 3% CLWMS pasting profiles showed a similar order of pH treatments, with notable relative viscosity differences between treatments observed in 3% CLWMS. The pasting curves of 0.05% CLWMS from pH 9 and 10 alkali treatments showed similar viscosity with the untreated 0.05% CLWMS. On the other hand, 3% CLWMS had different gelatinization peaks from pH 9 and 10 alkali treatments compared with untreated starch, but the samples treated at pH 10 had very similar viscosities to untreated samples during the cooling stage. The insignificant settling volumes of untreated, pH 9-treated and pH 10-treated 0.05% CLWMS and 3% CLWMS (Table 2-3) may explain the similar viscosities observed in the pasting curves (Figs. 2-2 and 2-3). The lower settling volumes of pH 11- and pH 12-treated CLWMS also showed correlation with the pasting curves. In addition, 3% CLWMS treated at pH 11 showed the highest P add-on content (Table 2-1), consequently reducing the swelling power.

Although 3% CLWMS treated at pH 12 showed a significant reduction of P content, the pasting curve showed lower viscosity than CLWMS treated at pH 9 and 10. This observation could be due to increased cross-linking (DSMP) content (Table 2-2), resulting in a decreased degree of granule swelling. Alkali treatments of cross-linked starch at various pH present an alternative for further manipulating the pasting and rheological properties of cross-linked starch for desired food applications.

2.4.4. Thermal properties of alkali-treated CLWMS

The gelatinization transition temperatures consisting of T_o , T_p , and T_c as well as ΔH of CLWMS and alkali-treated CLWMS are presented in Table 2-4. Gelatinization of the starch took place between 67 °C (for T_o) to 97 °C (for T_c), and an endothermic peak was observed in the DSC curves for all the samples. The T_o , T_p , T_c and ΔH of waxy maize starch was relatively higher than that reported by Woo and Seib (2002). 0.05% CLWMS and 3% CLWMS had lower gelatinization temperatures than the cross-linked waxy maize starch prepared by Woo and Seib (2002). The latter had higher level of cross-linking (0.34% P add-on) which may slow down crystal melting, resulting in higher gelatinization temperatures. Despite of the alkaline treatments, gelatinization temperatures of waxy maize starch, 0.05% CLWMS and 3% CLWMS were all very similar. ΔH was shown to markedly elevate with increment of cross-linking levels; however, all pH treatments did not affect the ΔH for both 0.05% CLWMS and 3% CLWMS. Increased ΔH of cross-linked starches may suggest an increase in order within amorphous region (Stute, 1992).

2.4.5. X-ray diffraction of alkali-treated CLWMS

Both 0.05% CLWMS and 3% CLWMS showed a distinctive A-type X-ray diffraction pattern (Fig. 2-4). The crystallinity of the untreated 0.05% CLWMS and 3% CLWMS were 34% and 35%, respectively. Just by looking at the diffractogram, it was hard to identify any changes on the crystallinity of the alkali-treated cross-linked starches. However, crystallinity was observed to decrease significantly with alkali treatments for both 0.05% CLWMS and 3% CLWMS, suggesting disorder of crystalline region in starch granules by alkali which may play a role in the significantly lower viscosity of the alkali-treated starch (Figs. 2-2 and 2-3). Although

the changes in the starch crystallinity were significant after alkali treatment, it was probably too small to be detected by the DSC (Table 2-4).

2.5. Conclusions

Treating cross-linked waxy maize starch at different pH levels was an effective way to alter its pasting properties. The level of total bound phosphate esters, cyclic-MSMP, and MSDP were reduced at high pH, resulting in changes in the degree of starch swelling, crystallinity and pasting properties of cross-linked starches. The current study also suggests that cross-linking reactions at different pH would produce products with different ratios of phosphate esters and result in different pasting properties.

2.6. Acknowledgements

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Table 2-1. Phosphorus content of cross-linked waxy maize starches (CLWMS) treated at pH 9.0, 10.0, 11.0, and 12.0 at 40 °C for 3 h.

Samples	Phosphorus content (% dry basis)	
	0.05% STMP/STPP*	3% STMP/STPP**
CLWMS	0.002 ± 0.002 a	0.101 ± 0.006 a
CLWMS treated at pH 9	0.002 ± 0.001 a	0.087 ± 0.004 b
CLWMS treated at pH 10	0.002 ± 0.002 a	0.083 ± 0.004 b
CLWMS treated at pH 11	0.002 ± 0.001 a	0.092 ± 0.003 ab
CLWMS treated at pH 12	0.002 ± 0.002 a	0.088 ± 0.005 b

Data are means ± standard deviation. Means within columns not sharing a common letter are significantly different at $p \leq 0.05$.

* Cross-linked waxy maize starch using 0.05% sodium trimetaphosphate (STMP) and sodium tripolyphosphate (STPP).

** Cross-linked waxy maize starch using 3% sodium trimetaphosphate (STMP) and sodium tripolyphosphate (STPP).

Table 2-2. Levels of different phosphate esters in cross-linked waxy maize starches (CLWMS) using 3% STMP/STPP treated at pH 9.0, 10.0, 11.0, and 12.0 at 40 °C for 3 h.

Samples	Phosphorus content (% dry basis) in the form of				
	Cyclic-MSMP ¹	MSMP ²		MSDP ³	DSMP ⁴
		C-3	C-6		
CLWMS	0.005 ± 0.001 a	0.018 ± 0.004 a	0.020 ± 0.000 a	0.004 ± 0.002 a	0.055 ± 0.004 b
CLWMS treated at pH 9	0.004 ± 0.001 a	0.017 ± 0.002 a	0.014 ± 0.002 ab	0.004 ± 0.002 a	0.049 ± 0.002 b
CLWMS treated at pH 10	0.003 ± 0.001 ab	0.013 ± 0.000 b	0.012 ± 0.003 b	0.004 ± 0.003 a	0.051 ± 0.002 b
CLWMS treated at pH 11	0.002 ± 0.001 b	0.016 ± 0.003 a	0.011 ± 0.005 b	0.004 ± 0.003 a	0.061 ± 0.005 b
CLWMS treated at pH 12	0.000 ± 0.000 b	0.012 ± 0.003 b	0.008 ± 0.002 b	0.001 ± 0.001 b	0.066 ± 0.001 a

Data are means ± standard deviation. Means within columns not sharing a common letter are significantly different at $p \leq 0.05$.

¹ Cyclic-MSMP, cyclic-monostarch phosphate.

² MSMP, monostarch monophosphate positioned at C-3 and C-6.

³ DSMP, distarch monophosphate.

⁴ MSDP, monostarch diphosphate.

Table 2-3. Settling volume of cross-linked waxy maize starches (CLWMS) treated at pH 9.0, 10.0, 11.0, and 12.0 at 40 °C for 3 h.

Samples	Settling volume (mL)	
	0.05% STMP/STPP*	3% STMP/STPP**
CLWMS	20.0 ± 0.0 a	6.2 ± 0.0 a
CLWMS treated at pH 9	20.1 ± 0.1 a	6.2 ± 0.0 a
CLWMS treated at pH 10	20.0 ± 0.0 a	6.2 ± 0.1 a
CLWMS treated at pH 11	18.0 ± 0.0 b	6.0 ± 0.0 b
CLWMS treated at pH 12	19.0 ± 0.0 ab	6.1 ± 0.1 b

Data are means ± standard deviation. Means within columns not sharing a common letter are significantly different at $p \leq 0.05$.

* Cross-linked waxy maize starch using 0.05% sodium trimetaphosphate (STMP) and sodium tripolyphosphate (STPP).

** Cross-linked waxy maize starch using 3% sodium trimetaphosphate (STMP) and sodium tripolyphosphate (STPP).

Table 2-4: Thermal properties of cross-linked waxy maize (CLWMS) starches treated at pH 9.0, 10.0, 11.0, and 12.0 at 40 °C for 3 h as determined by differential scanning calorimetry.

Samples	Temperature (°C)			ΔH (J/g)
	T_o	T_p	T_c	
<i>Waxy maize starch</i>	66.6 ± 0.1 a	76.7 ± 0.1 a	97.1 ± 0.1 a	14.7 ± 0.6 c
<u>0.05% STMP/STPP*</u>				
CLWMS	67.3 ± 0.7 a	75.8 ± 0.7 a	97.9 ± 0.3 a	17.9 ± 0.3 b
CLWMS treated at pH 12	66.2 ± 0.1 a	75.1 ± 0.1 a	97.5 ± 0.1 a	17.4 ± 0.3 b
<u>3% CLWMS**</u>				
CLWMS	68.8 ± 0.3 a	77.0 ± 0.2 a	96.4 ± 0.4 a	21.0 ± 0.9 a
CLWMS treated at pH 9	67.2 ± 0.1 c	75.1 ± 0.2 c	96.6 ± 0.4 a	21.4 ± 0.6 a
CLWMS treated at pH 10	67.5 ± 0.1 bc	75.4 ± 0.3 bc	95.1 ± 0.1 b	21.9 ± 0.2 a
CLWMS treated at pH 11	67.8 ± 0.1 b	75.9 ± 0.1 b	96.0 ± 0.5 ab	21.9 ± 0.1 a
CLWMS treated at pH 12	68.6 ± 0.1 a	77.0 ± 0.3 b	96.2 ± 0.1 a	22.3 ± 0.4 a

Data are means ± standard deviation. Means within columns not sharing a common letter are significantly different at $p \leq 0.05$.

* Cross-linked waxy maize starch using 0.05% sodium trimetaphosphate (STMP) and sodium tripolyphosphate (STPP).

** Cross-linked waxy maize starch using 3% sodium trimetaphosphate (STMP) and sodium tripolyphosphate (STPP).

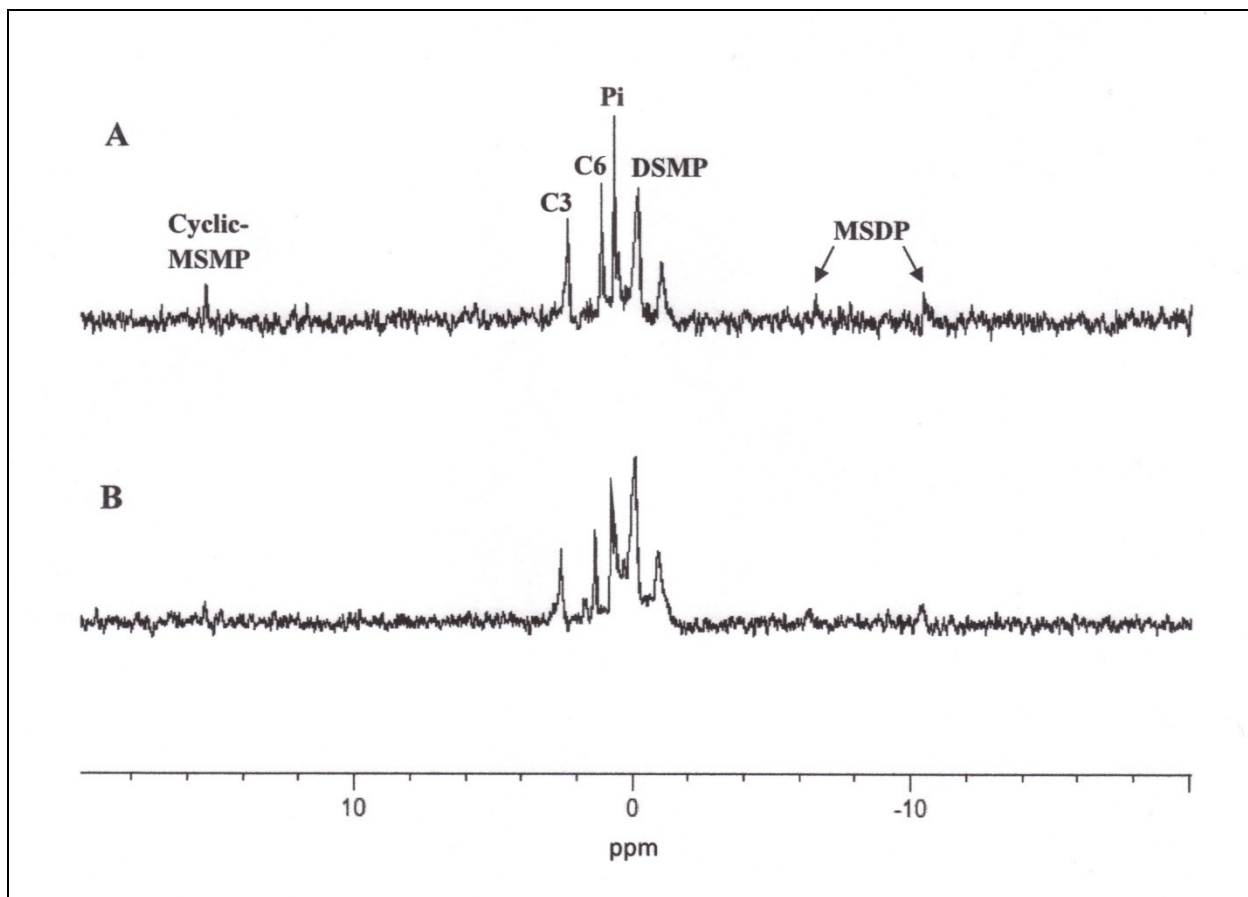


Fig. 2-1. (A) ^{31}P nuclear magnetic resonance spectra of dextrin prepared from cross-linked waxy maize starch (CLWMS) using 3% sodium trimetaphosphate/sodium tripolyphosphate (STMP/STPP), and (B) CLWMS using 3% STMP/STPP treated at pH 12 and 45 °C for 3 h. Cyclic-MSMP: cyclic-monostarch monophosphate; C3: monostarch monophosphate positioned at C3; C6: monostarch monophosphate positioned at C6; Pi: inorganic phosphorus; DSMP: distarch monophosphate; MSDP: monostarch diphosphate.

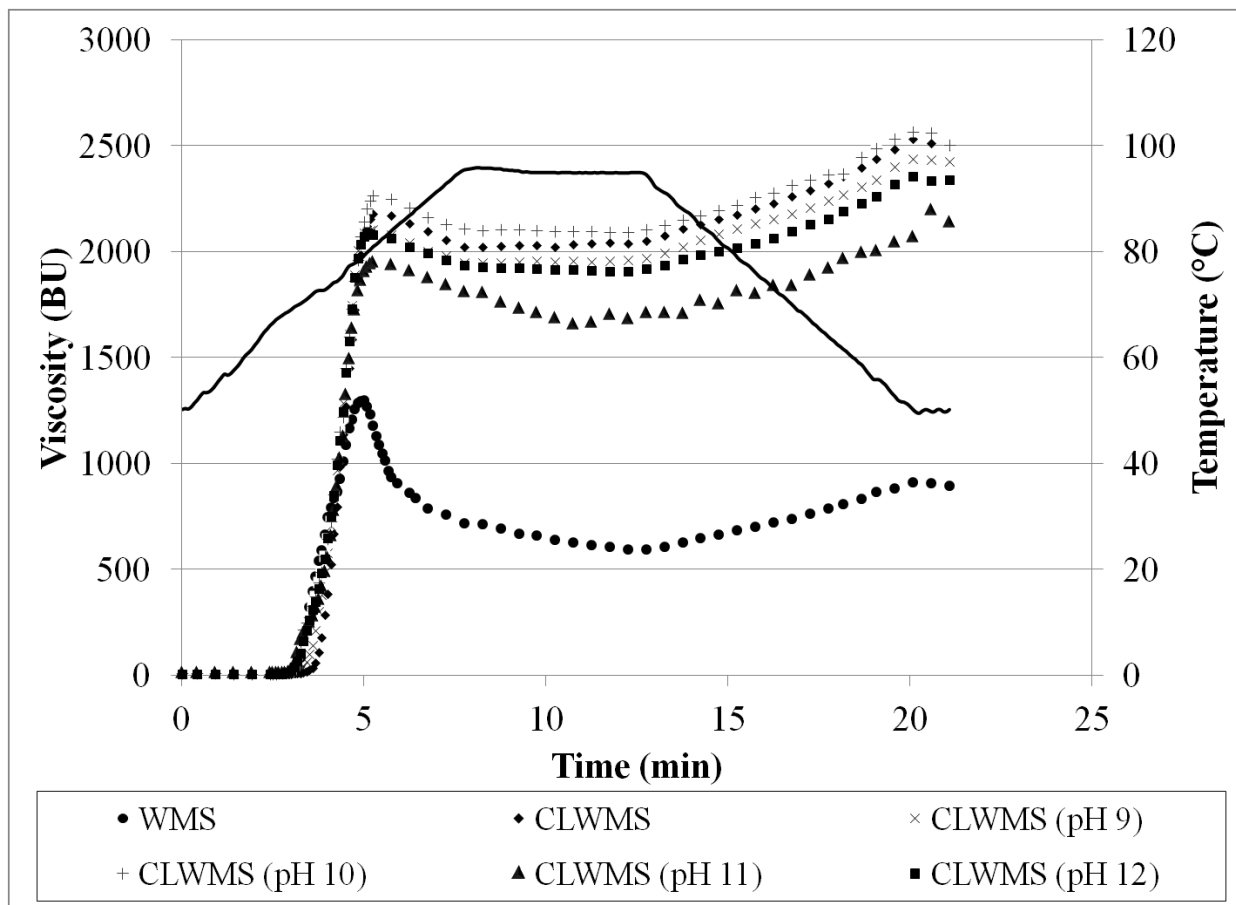


Fig. 2-2. Pasting profiles of waxy maize starch (WMS) and cross-linked waxy maize starch (CLWMS) using 0.05% STMP/STPP and the alkali-treated products. The starch content was 8% (w/w).

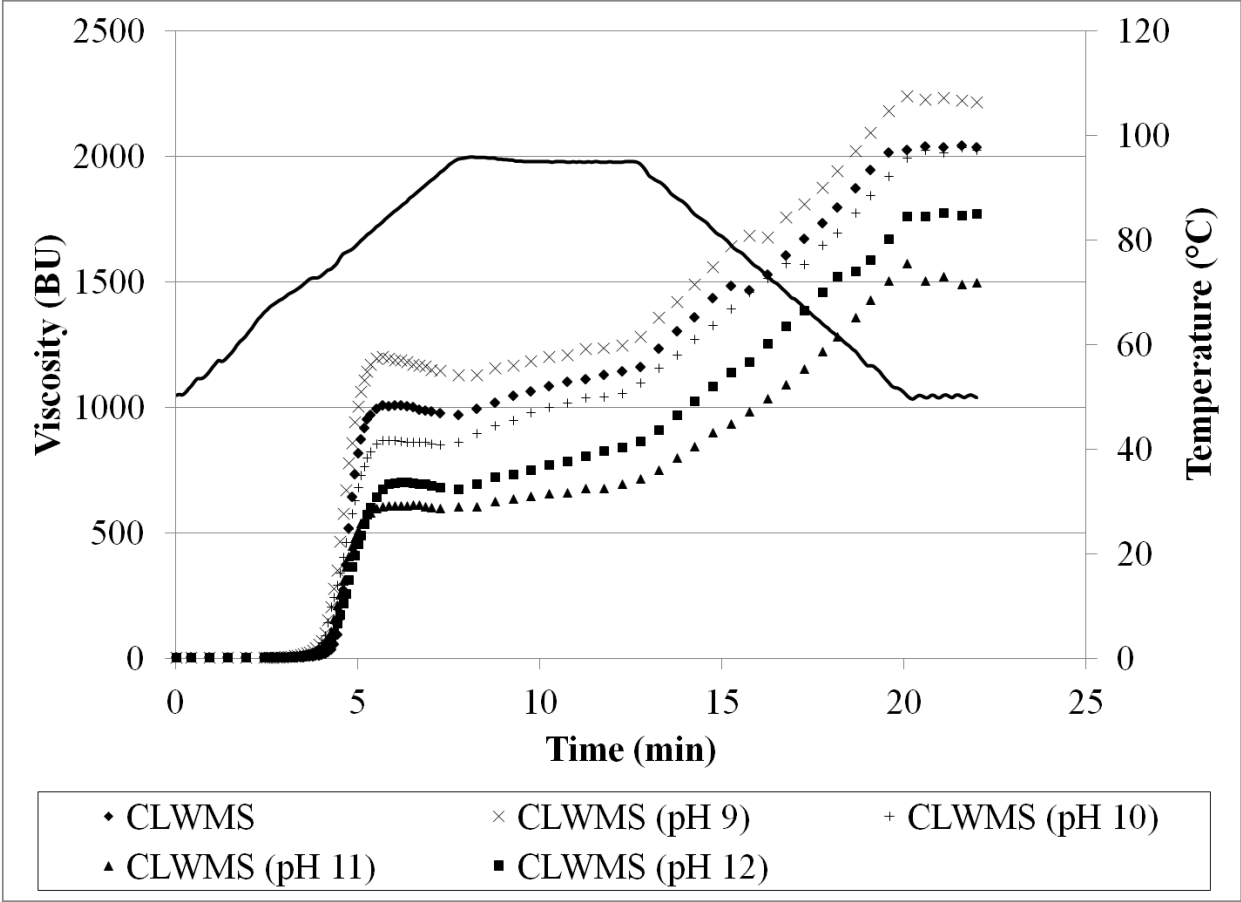


Fig. 2-3. Pasting profiles of cross-linked waxy maize starch (CLWMS) using 3% STMP/STPP and the alkali-treated products. The starch content was 15% (w/w).

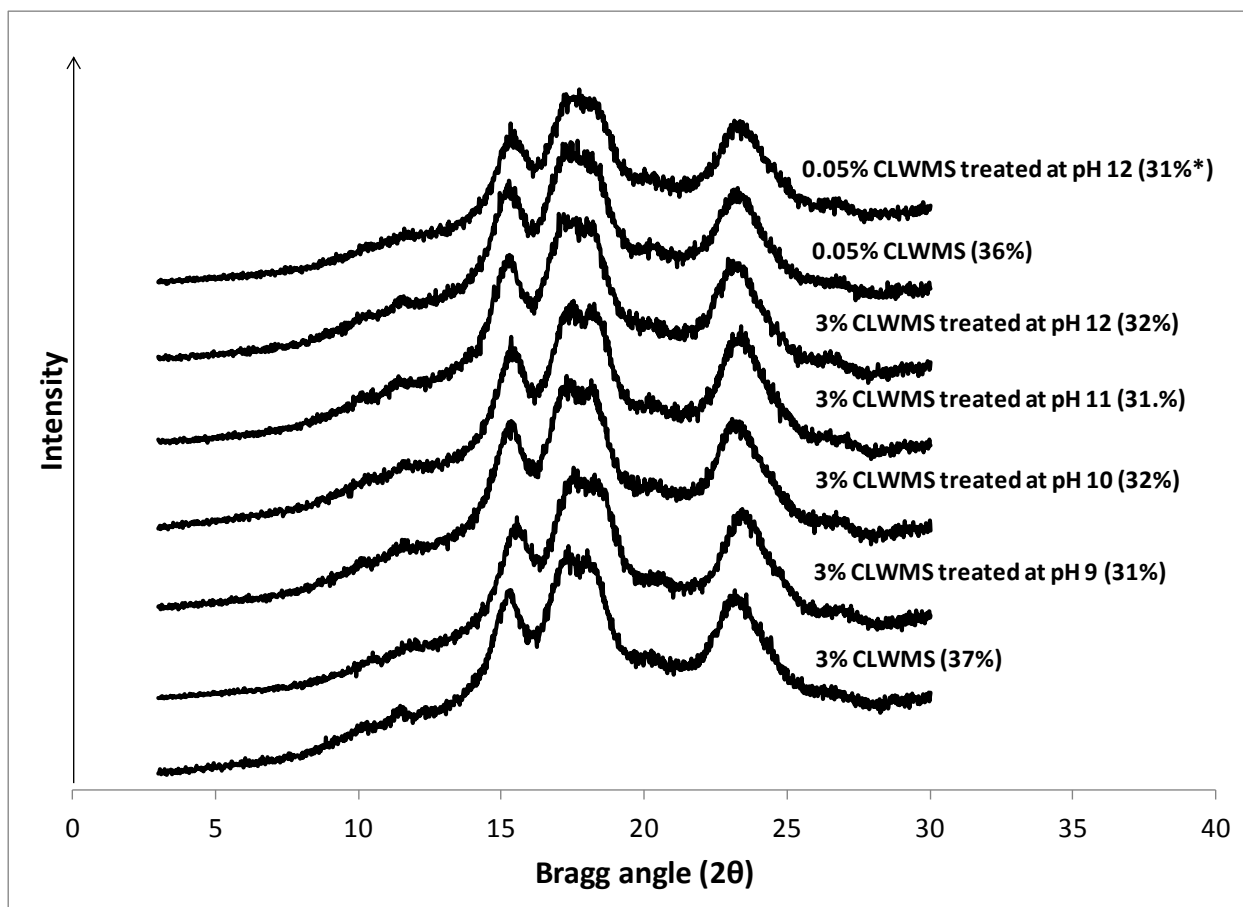


Fig. 2-4. X-ray diffraction patterns of cross-linked waxy maize starch (CLWMS) using 0.05% STMP/STPP and 3% STMP/STPP and the alkali-treated products. * Crystallinity (%) is in parantheses.

Chapter 3 - *In-vitro* enzymatic testing method and digestion mechanism of cross-linked wheat starch

3.1. Abstract

The objectives of this research were to study the *in-vitro* digestive behavior, progressive changes and the mechanism of the digestive enzymes on cross-linked (CL) wheat starch by AOAC Method 2009.01. CL wheat starch was subjected to *in-vitro* digestion using AOAC Method 2009.01 with the α -amylase/amyloglucosidase cocktail incubation time was extended to 24 h. The residue of CL wheat starch was recovered after 1, 2, 4, 6, 8, 16 and 24 h of incubation. The phosphorous content increased with incubation time and the thermal properties were comparable at all incubation times. The method yielded 25% and 16% resistant starch content after incubation for 16 h and 24 h, respectively. The morphology of CL wheat starch showed progressive surface erosion with increasing incubation time, but some starch granules remained intact and retained crystallinity, as depicted by the existence of Maltese cross under a light microscope. This study offers insight into improving the *in-vitro* digestive method so it better reflects the *in-vivo* conditions.

3.2. Introduction

Early studies have shown that a fraction of some starches consumed by humans escape the small intestine (Anderson, Levine & Levitt, 1981; Stephen, Haddad and Phillips, 1983; Englyst and Cummings, 1985; Asp, Bjorck, Holm, Nyman & Siljestrom, 1987). The total amount of starch and products of starch degradation that resist digestion in the small intestine of healthy people is termed resistant starch (RS) (Asp, 1992). RS is further categorized into five classes: physically inaccessible starch known as RS1; granular starch known as RS2; cooked and retrograded starches known as RS3; chemically modified starches known as RS4; and amylose-lipid complex (Brown et al., 2006; Hasjim et al. 2010). The degree of starch digestibility is affected by: the structure of starch granules; the physical characteristics of food; amylose and amylopectin ratios; retrogradation of amylose, and the presence of other nutrients and antinutrients (Sharma, Yadav & Ritika, 2008; Bird et al., 2009).

Chemical modification of starch has been proven to affect the extent and rate of digestibility in the small intestine (Wolf, Bauer & Fahey, 1999), based on starch source, the type

and degree of modification, the extent of granule gelatinization and enzyme source (Filer, 1971). Cross-linked (CL) starch is one of the most highly produced and utilized chemically modified starches in the food industry (Wurzburg, 1986). The highest acceptable cross-linking levels for food starch with a 0.4% phosphorus (P) add-on is achievable by using 12% sodium trimetaphosphate (STMP) and sodium tripolyphosphate (STPP) as phosphorylating agents (Woo & Seib, 2002).

Several *in-vitro* methods have been suggested to quantify RS in food all of which involve enzymatic digestions on starch, removal of the digested starch and quantitation of remaining RS. Quantitation of RS can be carried out using direct methods or indirect methods. Whereas direct methods determine RS in the remaining starch after removing the digested portion (Berry, 1986; Champ, 1992; Faisant et al., 1993; McCleary, McNally & Rossiter, 2002), indirect methods measure RS by subtracting the digested starch from the total starch (Englyst, Kingsman & Cummings, 1992; Tovar, Bjorck & Asp, 1990). Determination of RS is achieved by solubilizing RS and quantifying with colorimetric methods (Berry, 1986; McCleary, McNally & Rossiter, 2002), or by isolating RS gravimetrically after ethanol precipitation (Lee., Prosky & DeVries, 1992; McCleary et al., 2010).

Most methods use α -amylase (Englyst, Kingsman & Cummings, 1992) or a cocktail of α -amylase and amyloglucosidase (McCleary, McNally & Rossiter, 2002; McCleary et al., 2010) to hydrolyze the RDS and SDS fractions. Enzyme cocktails are used to avoid possible inhibition of α -amylase by maltose and maltotriose (Sharma, Yadav & Ritika, 2008). Extrinsic factors (i.e., extent of chewing, rate of orocecal transit), incubation temperature, incubation time and enzyme source may affect the *in-vitro* yield of these assays (Englyst et al., 1992). Table 3-1 shows a comparison of *in-vivo* and *in-vitro* RS content using Englyst, AOAC Method 991.43, AOAC Method 2002.02 and AOAC Method 2009.01 in several samples. The RS content of each sample differed either slightly or significantly when compared with the *in-vitro* methods used. Several samples determined by the Englyst method (raw potato starch, corn flakes and raw green banana) and AOAC Method 2002.02 (raw potato starch and raw green banana) showed results consistent with the *in-vivo* data. Although AOAC Method 991.43 was reported to be consistent with ileostomy patients fed with bean and potato flakes (Schweizer, Andersson, Langkilde, Reimann and Torsdottir, 1990), the RS reported in Table 1 revealed that none of the samples except corn flakes had similar *in-vivo* compatibility with the RS determined by AOAC Method 991.43. The

significantly lower RS (determined by AOAC Method 991.43) of raw potato starch, high amylose corn starch and raw green banana was likely due to the higher incubation temperature (100 °C).

The most current *in-vitro* method, namely AOAC Method 2009.01, claims to determine non-starch polysaccharides, RS and non-digestible oligosaccharides content effectively in samples (McCleary et al., 2010). The latter method applies key features of AOAC Method 985.29, AOAC Method 991.43, AOAC Method 2001.03 and AOAC Method 2002.02 (McCleary et al., 2010). The starch digestion conditions in AOAC Method 2009.01 were similar to those used in AOAC Method 2002.02 which involve the incubation of a sample with a pancreatic α -amylase and amyloglucosidase cocktail for 16 h at 37 °C. Although the digestive enzyme concentration in AOAC Method 2009.01 was significantly higher, results produced by AOAC Method 2009.01 and AOAC Method 2002.02 were similar for most samples (McCleary, 2007). The RS content by AOAC Method 2009.1 in the samples listed in Table 3-1 showed no consistency with *in-vivo* data. Based on a comparison of *in-vitro* methods (Englyst Method, AOAC Method 991.43, AOAC Method 2009.01) on CL wheat starch, AOAC Method 2009.01 provided a significantly lower RS content (Table 3-1). AOAC Method 2002.02 was not included in the latter comparison, because CL wheat starch is not able to solubilize in 2 M potassium hydroxide for determination of glucose content using the colorimetric procedure.

The findings of RS content in CL wheat starch raise questions about to the efficiency of AOAC Method 2009.01 to quantify RS and its compatibility with *in-vivo* results. As opposed to the Englyst Method, AOAC Method 2009.01 differs in enzyme concentration, buffers, measurement method, incubation time and enzyme sources, but both methods employ the same incubation temperature (37 °C). Digestion kinetics, specifically the enzyme mechanism and incubation time in AOAC Method 2009.01, were our major interest. We predicted that at 16 h of incubation time with a relatively high amount and concentration of digestive enzyme (40 mL α -amylase (50 U/mL) and amyloglucosidase (3.4 U/mL)) would present conditions too harsh for CL wheat starch. Hence, the objectives of this research were to study the *in-vitro* digestion behavior of CL wheat starch at various time intervals, the progressive changes of CL wheat starch granules during the *in-vitro* digestion and the mechanism of the digestive enzymes during the digestion period in AOAC Method 2009.01.

3.3. Materials and Methods

3.3.1. Materials

CL wheat starch (Fibersym[®] RW) and native wheat starch (Midsol[™] 50) were obtained from MGP Ingredients, Inc[®] (Atchison, KS). The integrated total dietary fiber assay kit (catalogue no. INTDF 06/12) was purchased from Megazyme International Ireland Ltd. (Wicklow, Ireland). All chemicals were reagent grade.

3.3.2. General methods

P content was assayed using the procedure of Smith and Caruso (1964). Moisture content was obtained according to AACC method 44-15 (AACC 2000).

3.3.3. Conversion of CL wheat starch to phosphodextrins and ³¹P NMR spectra of the phosphodextrins

The treatment was based on the method described by Sang et al. (2010). Starch (1.0 g db) was weighed into a 50-mL centrifuge tube. The starch was slurried with 30 mL 2.0 mM calcium chloride at pH 8.2, and heat-stable α -amylase (100 μ l) was then added. The mixture was heated in a boiling water bath for 30 min, and 100 μ l of heat-stable α -amylase was added into the mixture again. After cooling to room temperature, the pH of the mixture was adjusted to 4.5, and amyloglucosidase (200 μ l) was incorporated for 1 h and incubated at 60 °C. The mixture was adjusted to pH 7.0, centrifuged (1500 \times g, 10 min), and the supernatant was freeze-dried.

The freeze-dried starch digest (1.0 g, db) was dissolved in deuterium oxide (1.0 mL) containing 20 mM EDTA and 0.002% sodium azide. The pH of the solution was adjusted to 8.0 by adding 0.1 M sodium hydroxide. The proton-decoupled ³¹P NMR spectra were obtained using an 11.75 Tesla Varian NMR System (Varian Inc., Palo Alto, CA). Using a method by Sang et al. (2007), the ¹H NMR was operated at 499.84 MHz and ³¹P at 202.34 MHz. The ³¹P NMR data collection was carried out at 25 °C using a delay of 6 s between pulses, with a pulse width of 15.0 μ s and a sweep width at 12,730 Hz. The samples were run for 8 h for ³¹P spectra detection. The obtained spectra were processed and analyzed using Varian software VNMRJ Version 2.2C. Chemical shifts were reported in δ (ppm) from the reference signal of an 85% phosphoric acid external standard.

3.3.4. Digestibility of CL wheat starch

Digested starch of native and CL wheat starches were determined at 0, 1, 2, 4, 6, 8, 16 and 24 h using the α -amylase and amyloglucosidase incubation procedure from AOAC Method 2009.01. To collect undigested starch for characterization, CL wheat starch was weighed to 1.0 g (db), wet with ethanol (1.0 mL), and a pancreatic α -amylase/amyloglucosidase mixture (40 mL) was added to a 250 mL glass bottle. The pancreatic α -amylase/amyloglucosidase mixture contained 50 units (U)/mL of α -amylase and 3.4 U/mL amyloglucosidase. One U of α -amylase at pH 5.8 and 37 °C was defined as the amount of enzyme required to release one micromole of D-glucose per minute from soluble starch. One U of amyloglucosidase at pH 4.5 and 40 °C was defined as the amount of enzyme required to release one micromole of D-glucose per minute from soluble starch. The bottle was capped and placed in a water bath at 37 °C with continuous (170 rpm) stirring via a stir bar. Fourteen bottles were incubated for each sample and two bottles were taken out after 1, 2, 4, 6, 8, 16 and 24 h. After cooling to 25 °C, the pH of the mixture was decreased to 2.5 to inactivate the enzyme activity with the addition 1.0 M hydrochloric acid. The mixture was held at pH 2.5 for 1 h before increasing the pH to 6.0 via the addition of 1.0 M sodium hydroxide. Subsequently, the mixture was centrifuged (1500 \times g, 10 min), supernatant decanted, pellet washed (two times) with water and centrifuged (1500 \times g, 10 min), and the pellet was then oven-dried at 37 °C for 5 h. The dried pellet (undigested portion of the CL wheat starch) was gently ground using pestle and mortar, and stored at room temperature in an air-tight container.

3.3.5. Thermal properties

The gelatinization temperatures and the enthalpy of digested CL wheat starch were measured by differential scanning calorimetry (DSC) (TA Instruments Q100, TA Instruments, New Castle, DE). The total solids content of samples was 33.3% (w/w, dry basis). After hydration for 1 h at 25 °C, 30 mg of well-stirred sample suspensions were weighed into 40 μ L aluminum pans and immediately hermetically sealed to prevent moisture loss. Scans were performed from 10 °C to 130 °C at a constant heating rate of 10 °C/min. A sealed empty pan was used as a reference and the DSC was calibrated using indium. The gelatinization enthalpy (ΔH) and transition temperatures, namely the onset temperature (T_o), peak temperature (T_p), and

conclusion temperature (T_c), were determined, based on the first-run DSC heating curves. The ΔH was evaluated

3.3.6. Microscopic observation

CL wheat starch digested after 1, 2, 4, 6, 8, 16 and 24 h of incubation were placed on microscope slides and observed under an Olympus BX-51 microscope (Olympus, Tokyo, Japan) with a 40 \times objective. CL wheat starch before digestion was also observed under a microscope for comparison with the assayed CL wheat starch.

3.3.7. Scanning electron microscopy (SEM)

The samples were sprinkled lightly onto a carbon double-sided adhesive tape on metal specimen stubs, which were then coated with gold-palladium (60:40 ratio) under vacuum with a Desk II Sputter/Etch Unit (Denton Vacuum, LLC, Moorestown, NJ). Micrographs of the samples were obtained at 1000 \times and 5000 \times magnifications by SEM (S-3500N, Hitachi Science Systems, Ltd, Japan) at an accelerating potential of 20 kV using an X-ray Detector-Link Pentafet 7021 (Oxford Instruments Microanalysis Ltd., Bucks, England).

3.3.8. Statistical analysis

All data were statistically analyzed by analysis of variance (ANOVA) using Statistical Analysis Software (SAS) (version 9.2, SAS Institute, Cary, NC) and the values are expressed as means \pm standard deviations from 2 replicates, unless stated otherwise.

3.4. Results and discussion

3.4.1. Effects of α -amylase/amyloglucosidase digestion on P content and chemical forms of the phosphate esters on starch

The P content of CL wheat starch after different incubation times with α -amylase/amyloglucosidase is reported in Table 3-2. The initial P content of CL wheat starch at 0 h was 0.36%. The P content of CL wheat starch remained constant for the first 2 h but started to increase at 4 h. The P content progressively increased with incubation time, until it reached 0.56% at 24 h, indicating the molecules with higher P density were more resistant to digestion and the regions with no or low bound P were selectively removed, thus elevating the concentration of P in CL wheat starch residue.

The ^{31}P NMR spectra of phosphate derivatives of CL wheat starch and their digestive residues after various α -amylase/amyloglucosidase incubation periods are also tabulated in Table 3-2. The effect of digestive enzymes on the CL wheat starch began after 4 h of incubation, causing an increase in the cyclic-monostarch monophosphate (cyclic-MSMP) and monostarch monophosphate (MSMP). The distarch monophosphate (DSMP) of CL wheat starch started to increase drastically after 6 h of incubation. The remaining periods of incubation showed a significant increase in MSMP and DSMP, whereas cyclic-MSMP remained constant until digestion was terminated. Results suggest that cyclic-MSMP and other phosphate esters increased due to hydrolysis of starch molecules that may not be linked to P molecules, leaving the remaining starch granules that were cross-linked to P molecules. ^{31}P NMR spectra of CL wheat starch after 16 h of digestion in Fig. 3-1 B has signals of cyclic-MSMP, MSMP positioned at C3 and C6, inorganic phosphate (Pi) and DSMP at δ 15.4 ppm, δ 1-3 ppm, δ 3.5 ppm and δ 0 to -1 ppm, respectively. The cyclic-MSMP had stronger peak detection as compared with the spectra before digestion (Fig. 3.1 A), which was consistent with the quantified cyclic-MSMP (Table 3-2).

3.4.2. Thermal properties

The gelatinization transition temperatures consisting of T_o , T_p , and T_c as well as ΔH of CL wheat starches at different times of α -amylase/amyloglucosidase incubation are reported in Table 3-3. Gelatinization of all starches took place from 72 °C (for T_o) to 94 °C (for T_c), and an endothermic peak was observed in the DSC curves of all samples. The data were slightly higher than that reported by Woo and Seib (2002) presumably due to higher P content (0.36%) in the commercial CL wheat starch in our study instead of the laboratory-prepared CL wheat starch (0.32%). T_p and T_c for digested CL wheat starch after 16 and 24 h incubation increased slightly. The ΔH for CL wheat starch at all incubation times was comparable to the ΔH before digestion, which was inconsistent with results from potato starch (Jiang & Liu, 2002) and maize starch (Shresta et al., 2012). An amylose-lipid complex peak was absent for CL wheat starch at all incubation times. Similar values for the thermal parameters imply that residual CL wheat starch residual after the α -amylase/amyloglucosidase digestion was likely to be intact and to have little or no effect on starch granule crystallinity. Native wheat starch (Colonna, Buleon & Lemarie, 1988), native barley starch (Lauro, Forssel, Suortti, Hulleman & Poutanen, 1999) and native

maize starch (Brewer, Cai & Shi, 2012) were reported to have decreased crystallinity after enzymatic digestion. The current result showed that cross-linking of wheat starch aided in the retention of starch crystallinity up to 24 h amylolysis.

3.4.3. Starch granular morphology before and after enzyme digestion

Fig. 3-2 and 3-3 represent light microscopic and SEM micrographs respectively of CL wheat starch after various α -amylase/amyloglucosidase incubation periods. The SEM results were correlated with the microscopic evidence. However, the SEM micrographs provided a better observation of the morphological changes of CL wheat starch granules from 0 h to 24 h incubation periods. At 0 h, CL wheat starch granules had a smooth surface with round shallow indentations of flower petal-like patterns, but are restricted to only some of the granules. In the first hour of digestion, mild erosion on the surface of most starch granules was observed, indicating the susceptibility of CL starch granules early in the incubation.

The progression of starch granule corrosion was observed after incubation from 1 h to 4 h. Scattered erosion on the surface of starch granules increased and deepened with time, but most starch granules still, however, remained intact, with the retention of the Maltese cross. After incubation for 6 h and 8 h, surface erosion of CL wheat starch granules intensified, causing severe damage and the disappearance of the granular identity of some starch granules. The severity of damage on CL wheat starch granules was more prominent after incubation for 16 h and 24 h, when smaller fragments of broken starch granules were evident. However, some starch granules remained intact and retained crystallinity, as depicted by the Maltese cross. This observation indicated that hydrolysis of CL wheat starch granules by an α -amylase/amyloglucosidase cocktail was progressing continuously and did not stop until the 24 h incubation was terminated.

CL wheat starch showed unequal granule degradation by digestive enzymes at all incubation times. From 1 h to 24 h incubation, some of the starch granules remained intact, whereas others had mild to extensive degradation. The same phenomenon was observed on the native wheat starch granules (Fig.3-4), although the starch damage was more prominent at 1 and 2 h of digestion. This result could be attributed to heterogeneous action of the digestive enzymes on the wheat starch granules. Starch granules are also described as unequally susceptible to enzymatic hydrolysis, with susceptibility depending on the adsorption manner of amylases on the

granule and method of isolation of the starch (Colonna, Buleon & Lemarie, 1988; Oates, 1997). Colonna, Buleon & Lemarie (1988) had a similar observation of unequal amylase digestion on native wheat starch, in which some granules had minimal pitting and a few were severely cratered. However, all large granules were completely degraded.

The mechanism of enzyme attack on starch granules is due to exo-corrosion (Shresta et al., 2012), endo-corrosion or a combination of both (Manelius, Qin, Avall, Andfolk & Bertoft, 1997; Apinan et al., 2007). The microscopic and SEM results suggest that the starch hydrolysis by amylase progressed, starting from the surface and proceeding inside of native wheat and CL wheat starch granules by exo-corrosion. In addition, the starch granules were most probably digested by the digestive enzymes using the side-by-side mechanism as observed by Zhang, Ao & Hamaker (2006) and Shresta et al. (2012) in which the enzymes digest on the amylose and amylopectin as well as the amorphous and crystalline region (Zhang, Ao & Hamaker, 2006). Unlike digested maize starches, which are reported to have holes or tunnels deepening into the interior of granules, or a “Swiss cheese” shell appearance (Robyt, 2009; Lauren, Cai & Shi, 2012), CL wheat starch granules (Fig. 3-3) and wheat starch (Fig. 3-4) had a roughened superficial surfaces and exposure of layered internal structures, which intensified with increased incubation time and caused cavities to form on some starch granules. In addition, CL wheat starch was theoretically obstructed by phosphate groups within the granule surface pores and channels, inhibiting the diffusion of amylase molecules (Thompson, Maningat, Woo & Seib, 2011).

3.4.4. Digestibility

The extent of digestibility of CL wheat starch at different incubation times (up to 24 h) was determined using the AOAC Method 2009.01. Wheat starch was also assayed using the same method as a control, because wheat starch is easily hydrolyzed by digestive enzymes (Björck et al., 1986). The amount of digested starch after each incubation period is shown in Fig. 3-5. Both starches showed rapid digestion at 1 h and the rate of digestion slowed until the end of the incubation period. The latter observation is in agreement with Bertoft and Manelius (1992), who showed two stages of starch digestion; the first being the initial rapid hydrolysis and is followed by a slower and more constant rate of hydrolysis.

For wheat starch, the amount digested was close to 70% at 1 h of incubation and 99% at 6 h, indicating a high susceptibility of wheat starch granules to hydrolysis. The SEM results (Fig. 3-4) showed the severity of starch hydrolysis at the early stage of digestion (1 h and 2 h). Our result is different from Colonna, Buleon & Lemarie (1988), who observed 74% and 91% starch hydrolysis after incubation for 10 h and 21 h, respectively. The difference could be due to enzyme source. Colonna, Buleon & Lemarie (1988) used *Bacillus subtilis* α -amylase (17.5 Phadebas Unit/ μ g protein), whereas the current study used a combination of pancreatic α -amylase (50 U/mL) and amyloglucosidase (3.4 U/mL). Pancreatic α -amylase had been suggested to be the most effective enzyme to digest native starch followed by barley, bacterial and fungal amylases (Kimura and Robyt, 1995).

When subjected to a cocktail of α -amylase and amyloglucosidase at 37 °C for 24 h, CL wheat starch was digested at a slower rate than native wheat starch and by 24 h, 84% of the CL starch was digested. The cross-linking treatment decreased the susceptibility of starch granules to digestive enzymes due to the stabilizing effect of granules with a high degree of cross-linking (Seib and Woo, 1999; Woo, Maningat & Seib, 2009), therefore causing a slower rate of digestion. The amount of digested CL wheat starch was negatively correlated with P content, but had little effect on starch crystallinity. O'Brien and Wang (2009) proposed that amylopectin could be more reactive in the cross-linking process due to higher retained phosphate salts in the branched structure of the polymer. Because amylopectin comprises of amorphous (tightly-spaced branches) and crystalline regions (parallel glucans) (Oostergetel and van Bruggen, 1989), phosphate groups may stabilize both amorphous and crystalline regions by promoting stronger interactions between the two regions. Therefore, the undigested portion of the residual starch had increased stability and was less susceptible to digestive enzymes, as depicted by the intact, yet highly roughened starch granule surfaces observed in SEM micrographs (Fig. 3-3). Increased P content with increased incubation time showed that phosphate groups were retained in the undigested residuals, reflecting the incapability of digestive enzyme to cleave bonds close to a glucose linked to a phosphate group.

Previous RS quantitation of CL wheat starch using the Englyst Method and AOAC Method 991.43 showed 83% and 76%, respectively (Woo and Seib, 2002; Yeo and Seib, 2009; Thompson, Maningat, Woo & Seib, 2011). Although the Englyst Method (incubation at 37°C) and AOAC Method 991.43 (incubation at 100 °C) use 120 min and 35 min of incubation time,

respectively, AOAC Method 2009.01 incubates samples for 16 h at 37 °C, yielding 25% RS content for CL wheat starch. The significantly lower RS content of CL wheat starch as determined by AOAC Method 2009.01 compared with the values obtained by the Englyst Method and AOAC Method 991.43 raised questions about the ability of AOAC Method 2009.01 to measure RS content accurately and consistently with *in-vivo* human conditions. At 24 h, RS content of CL wheat starch decreased further to 16%, indicating that the digestive action of the α -amylase and amyloglucosidase cocktail on CL wheat starch was still progressing. In humans, the transit time of food in the intestine takes 3-4 h (Perera, Meda & Tyler, 2010). Long-term hydrolysis may lead to an increase in substrate surface availability caused by amylase attack (Colonna, Buleon & Lemarie, 1988). Incubation time of 16 h in AOAC Method 2009.01 resulted in much lower yield of RS content as indicated in the current study, and may not reflect *in-vivo* response, which is important in the food industry.

3.5. Conclusions

CL wheat starch was assayed using the digestive enzymes and incubation conditions of AOAC Method 2009.01, and samples were collected at predetermined times. The digestion of CL wheat starch continued to increase up to 24 h. The RS value obtained at 16 h was lower than the RS content measured by the Englyst Method and AOAC Method 991.43. Microscopic and SEM results of the indigestible residues collected at predetermined times showed progressive degradation of CL wheat starch granules. However, some starch granules remained intact, which explained the comparable DSC data for CL wheat starch at all incubation times. The mechanism of enzyme attack on the CL wheat starch was exo-corrosive, as identified by the surface erosions and the non-existence of pinholes on the granules. Future studies will include assaying CL starches from different botanical sources to further understand the mechanism of enzyme attack as affected by cross-linking.

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Table 3-1. Comparison of resistant starch (RS; % total starch) content in raw potato starch, high amylose corn starch, corn flakes and raw green banana determined by *in-vitro* methods (Englyst Method, AOAC Method 991.43, AOAC Method 2002.02 and AOAC Method 2009.01) and *in-vivo* method (ileostomy model).

Source of starch	<i>In-vitro</i> RS (%)				<i>In-vivo</i> RS (%)
	Englyst	AOAC 991.43	AOAC 2002.02	AOAC 2009.01	
Raw potato starch	66.5 ^a	0.9 ^b	64.9 ^b	56.8 ^b	67.9 ^c
High amylose corn starch	71.4 ^a	25.6 ^b	50.0 ^b	49.3 ^b	43.7 ^c
Corn flakes	3.9 ^a	3.3 ^c	2.2 ^b	2.4 ^b	3.1 - 5.0 ^d
Raw green banana	54.2 ^b	7.5 ^b	51 ^b	38 ^b	55.3 ^c
Cross-linked wheat starch*	81.7	82.3	-**	23.9	-

^aEnglyst 1992, ^bMcCleary 2007, ^cLangkilde and Andersson 1995, ^dMuir and O’Dea 1992, ^eAlan and Pendlington 1999, *RS content was determined by *in-vitro* methods in Kansas State University lab, ** Unable to determine RS in Fibersym[®] RW due to insolubility of cross-linked starch in 2 M potassium hydroxide.

Table 3-2. Phosphorus content and levels of different phosphate esters of cross-linked wheat starch and their digestive residues after various α -amylase/amyloglucosidase incubation periods.

Digestion time (h)	*Total phosphorus (%)	Phosphorus content (% dry basis) in the form of			
		Cyclic-MSMP ¹	MSMP ²		DSMP ³
			C3	C6	
0	0.36 ± 0.01 d	0.000	0.078	0.054	0.228
1	0.36 ± 0.02 d	0.000	0.078	0.052	0.230
2	0.36 ± 0.00 d	0.000	0.080	0.052	0.229
4	0.38 ± 0.02 cd	0.004	0.090	0.057	0.229
6	0.40 ± 0.01 c	0.004	0.089	0.061	0.245
8	0.41 ± 0.00 c	0.004	0.099	0.062	0.246
16	0.47 ± 0.01 b	0.004	0.104	0.069	0.292
24	0.56 ± 0.00 a	0.005	0.136	0.080	0.338

Data are means ± standard deviation. Means within columns not sharing a common letter are significantly different at $p \leq 0.05$.

¹ Cyclic-MSMP, cyclic-monostarch monophosphate.

² MSMP, monostarch monophosphate positioned at C-3 and C-6.

³ DSMP, distarch monophosphate.

Table 3-3. Thermal properties of cross-linked wheat starch and their digestive residues after various α -amylase/amyloglucosidase incubation periods.

Digestion time (h)	Temperature ($^{\circ}\text{C}$)			ΔH (J/g)
	T_o	T_p	T_c	
0	72.0 \pm 0.2 a	76.2 \pm 0.2 c	89.8 \pm 0.7 bc	10.7 \pm 0.3 bc
1	72.2 \pm 0.1 a	76.1 \pm 0.1 c	90.4 \pm 0.2 c	10.8 \pm 0.2 bc
2	72.2 \pm 0.1 a	76.1 \pm 0.1 c	90.7 \pm 0.3 c	11.8 \pm 0.1 b
4	73.2 \pm 0.4 a	77.3 \pm 0.5 b	89.3 \pm 0.6 bc	11.5 \pm 0.1 b
6	72.3 \pm 0.3 a	76.6 \pm 0.3 c	91.5 \pm 0.5 b	12.2 \pm 0.1 a
8	72.3 \pm 0.1 a	77.6 \pm 0.6 b	91.7 \pm 0.5 b	12.2 \pm 0.3 a
16	73.2 \pm 0.1 a	78.3 \pm 0.3 ab	93.6 \pm 0.4 a	11.3 \pm 0.4 b
24	73.7 \pm 0.1 a	78.9 \pm 0.0 ab	93.8 \pm 0.3 a	11.6 \pm 0.4 b

Data are means \pm standard deviation. Means within columns not sharing a common letter are significantly different at $p \leq 0.05$.

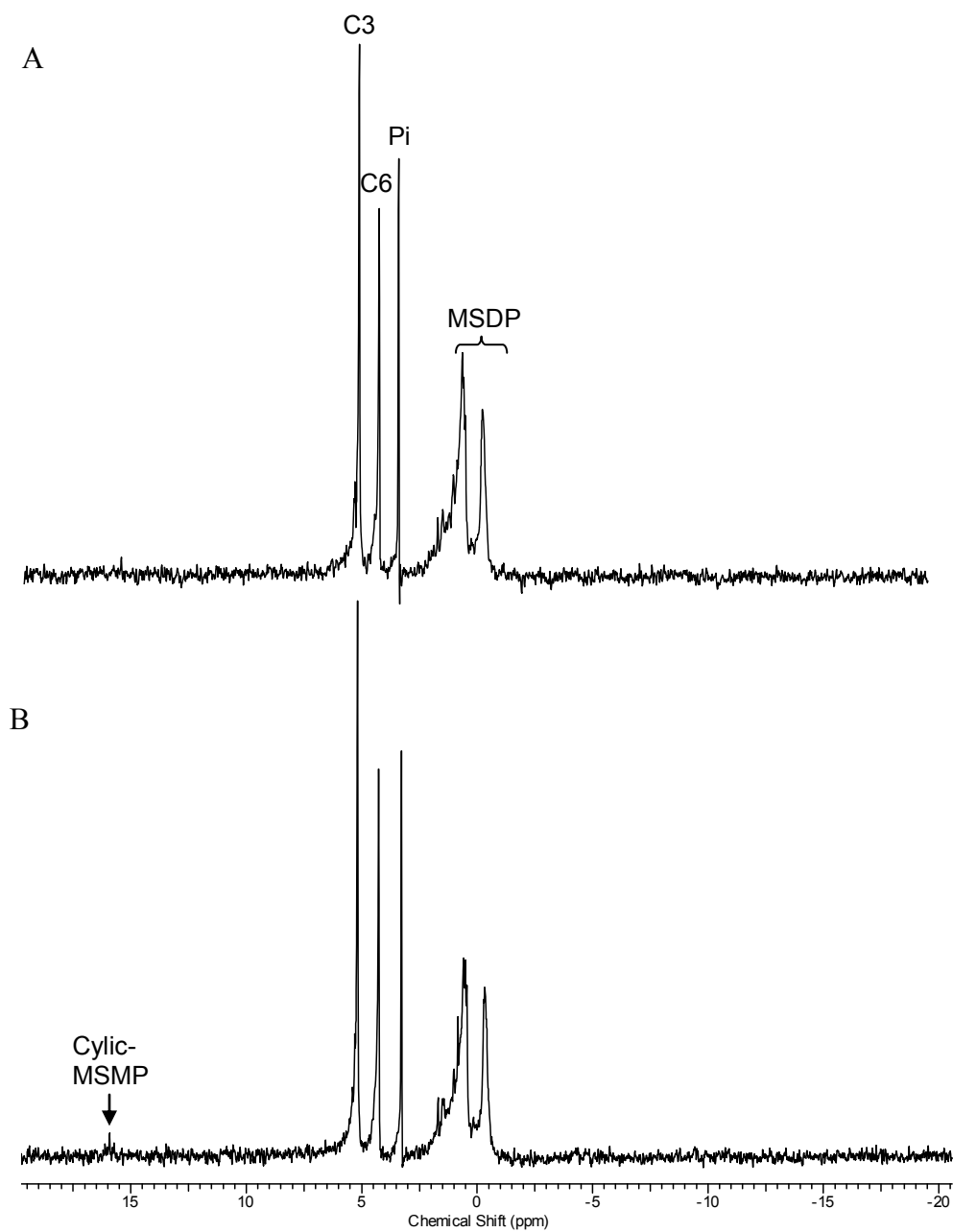
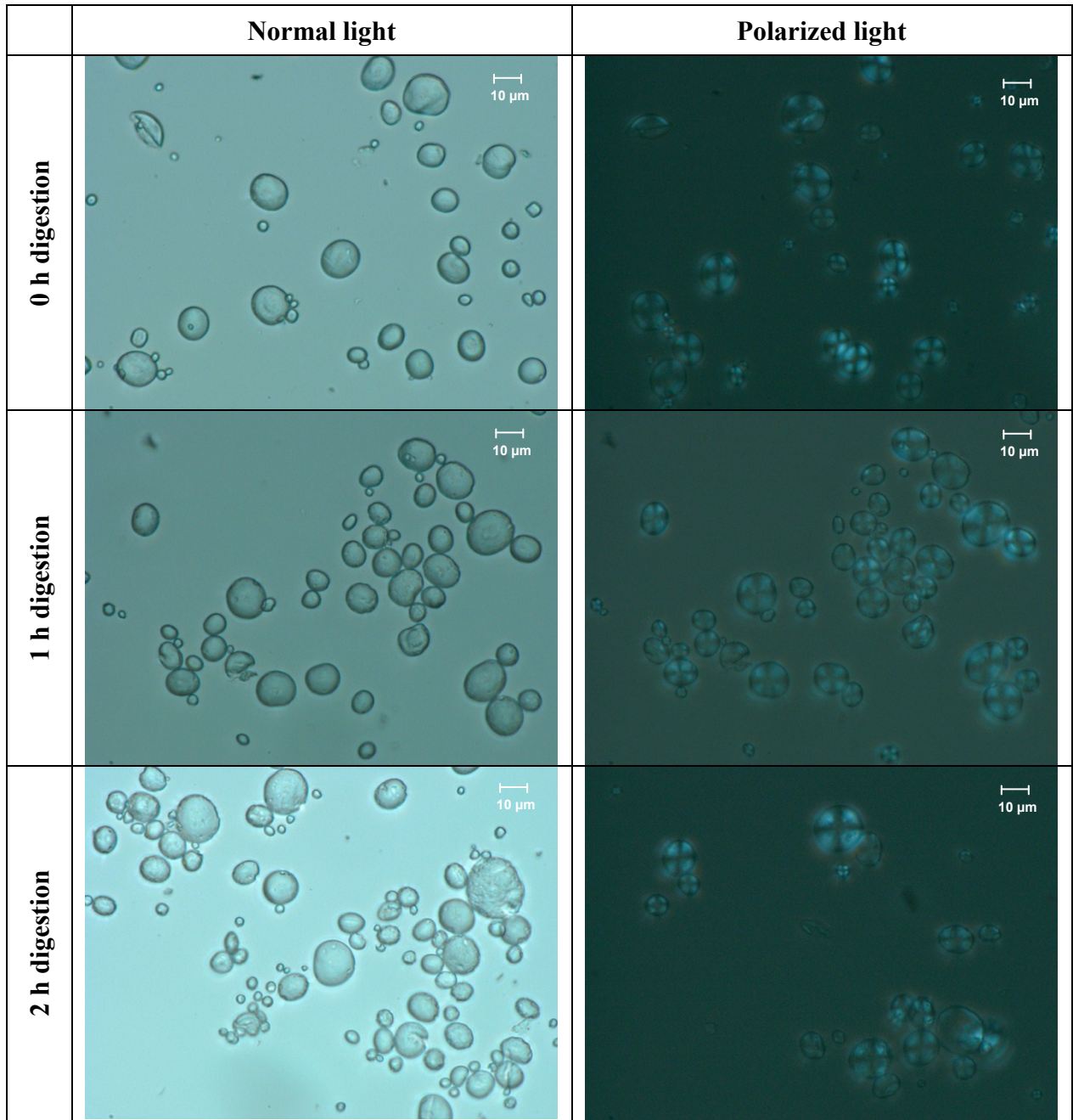
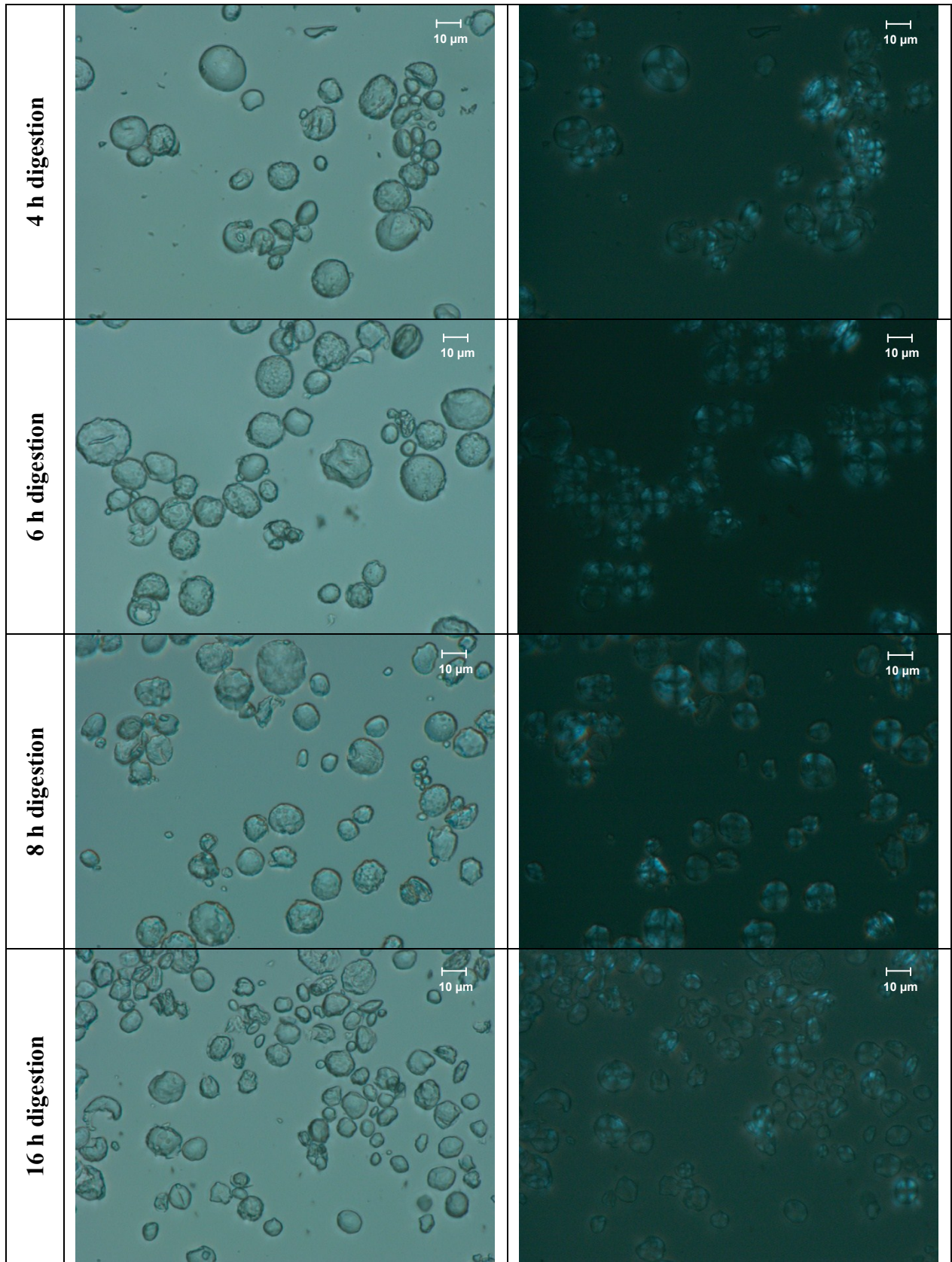


Fig. 3-1. ^{31}P nuclear magnetic resonance spectra of dextrans prepared from cross-linked wheat starch before (A) and after 16 h of α -amylase/amyloglucosidase digestion (B). Cyclic-MSMP: cyclic-monostarch monophosphate; C-3: monostarch monophosphate positioned at C3; C-6: monostarch monophosphate positioned at C6; MSDP: monostarch diphosphate; Pi: inorganic phosphorus.





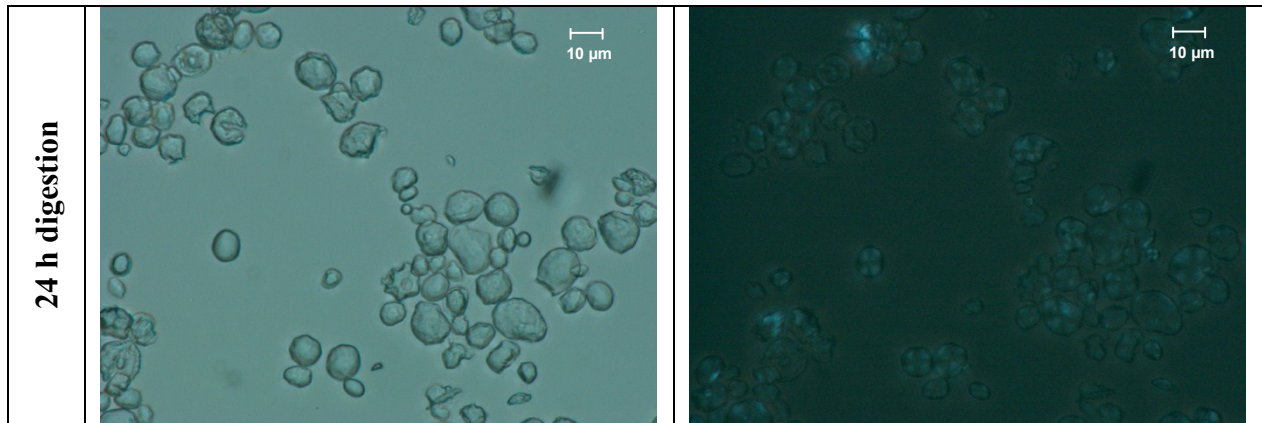
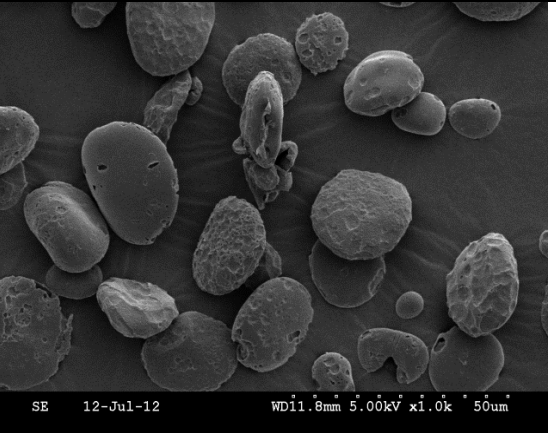
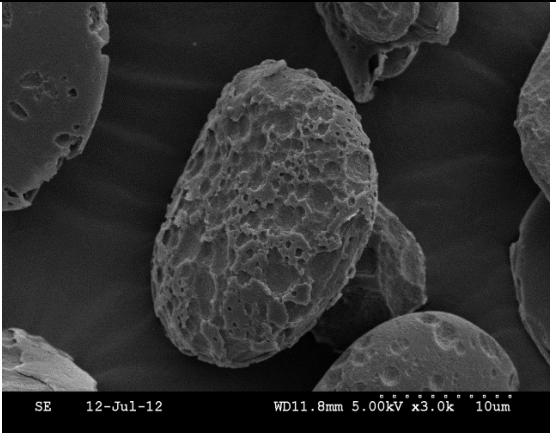
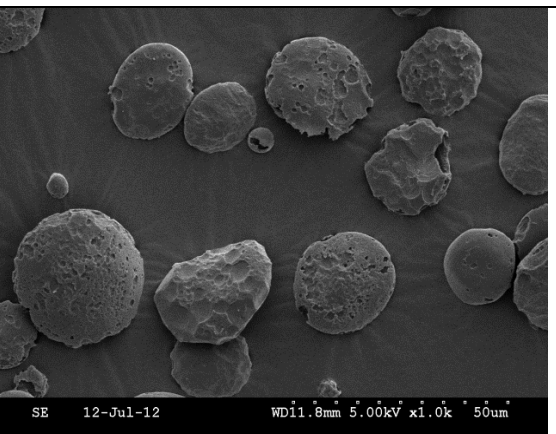
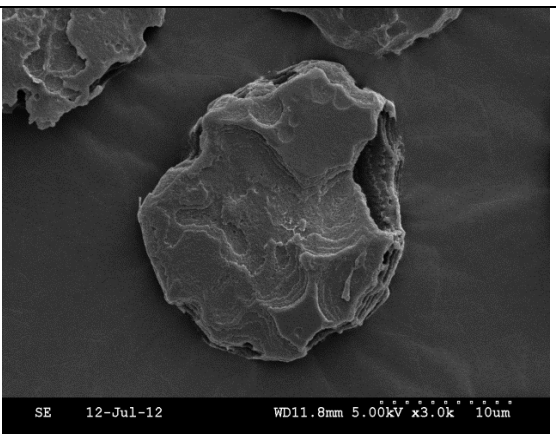
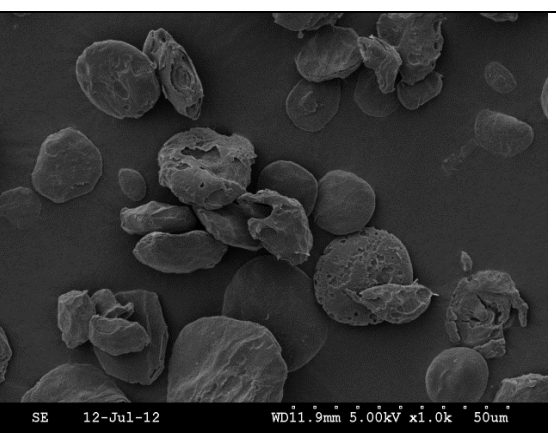
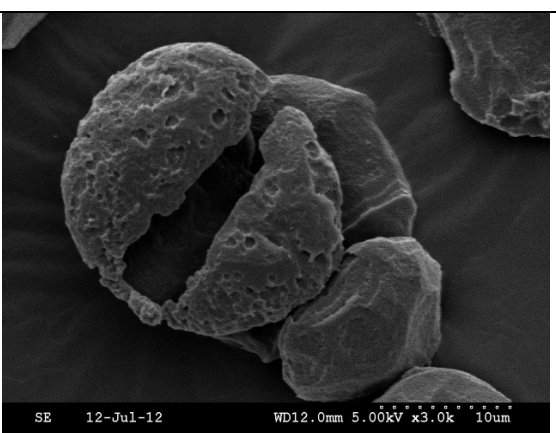


Fig. 3-2. Microscopic photos (40× objective) of cross-linked wheat starch at different incubation times of α -amylase/amyloglucosidase digestion.

	Magnification at 1000 ×	Magnification at 3000 ×
0 h digestion		
1 h digestion		
2 h digestion		

<p style="text-align: center;">4 h digestion</p>	 <p>SE 12-Jul-12 WD11.8mm 5.00kV x1.0k 50um</p>	 <p>SE 12-Jul-12 WD11.8mm 5.00kV x3.0k 10um</p>
<p style="text-align: center;">6 h digestion</p>	 <p>SE 12-Jul-12 WD11.8mm 5.00kV x1.0k 50um</p>	 <p>SE 12-Jul-12 WD11.8mm 5.00kV x3.0k 10um</p>
<p style="text-align: center;">8 h digestion</p>	 <p>SE 12-Jul-12 WD11.9mm 5.00kV x1.0k 50um</p>	 <p>SE 12-Jul-12 WD12.0mm 5.00kV x3.0k 10um</p>

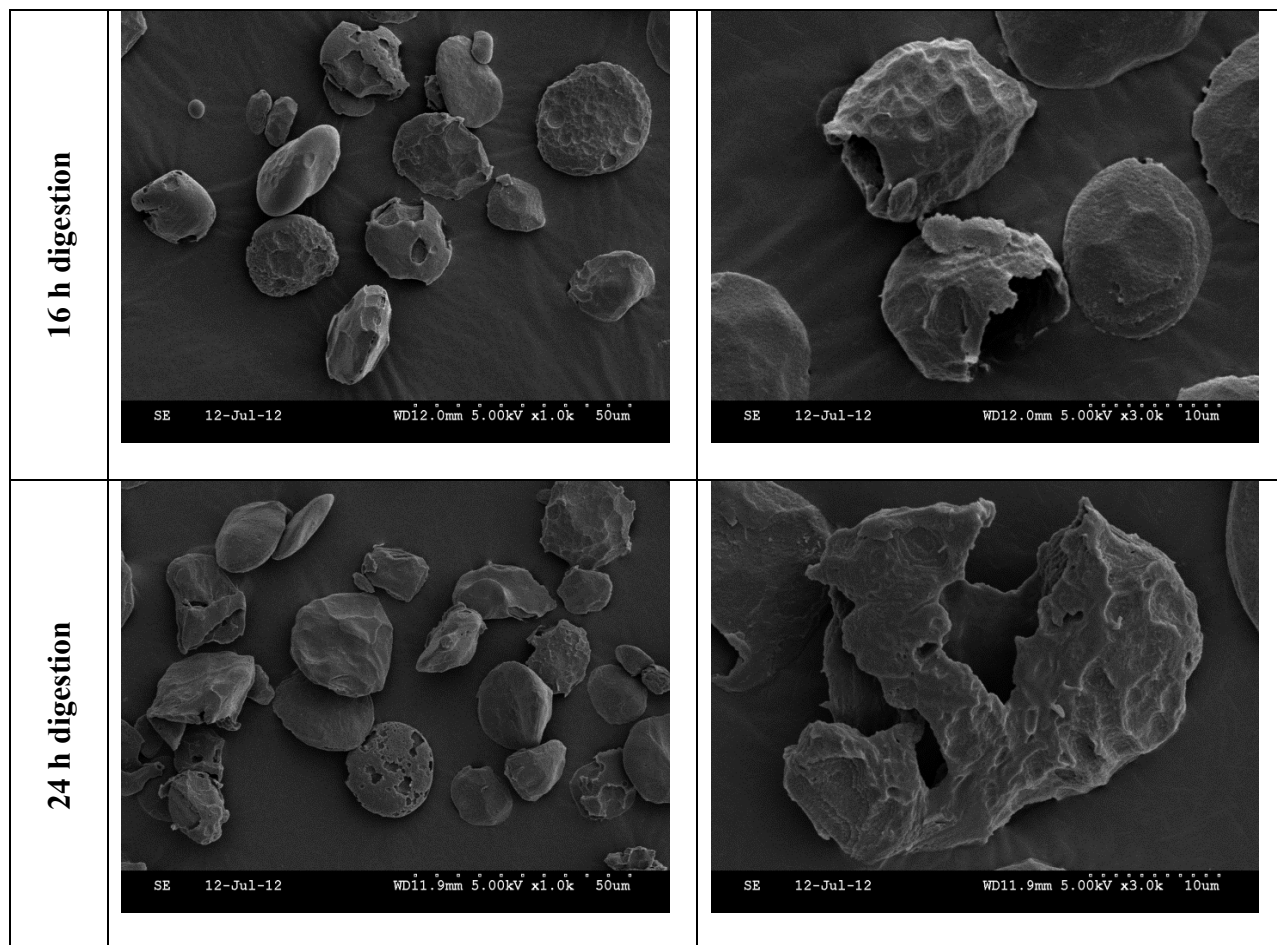


Fig. 3-3. Scanning electron micrographs (1000× and 3000× magnifications) of cross-linked wheat starch at different times of α -amylase/amyloglucosidase digestion.

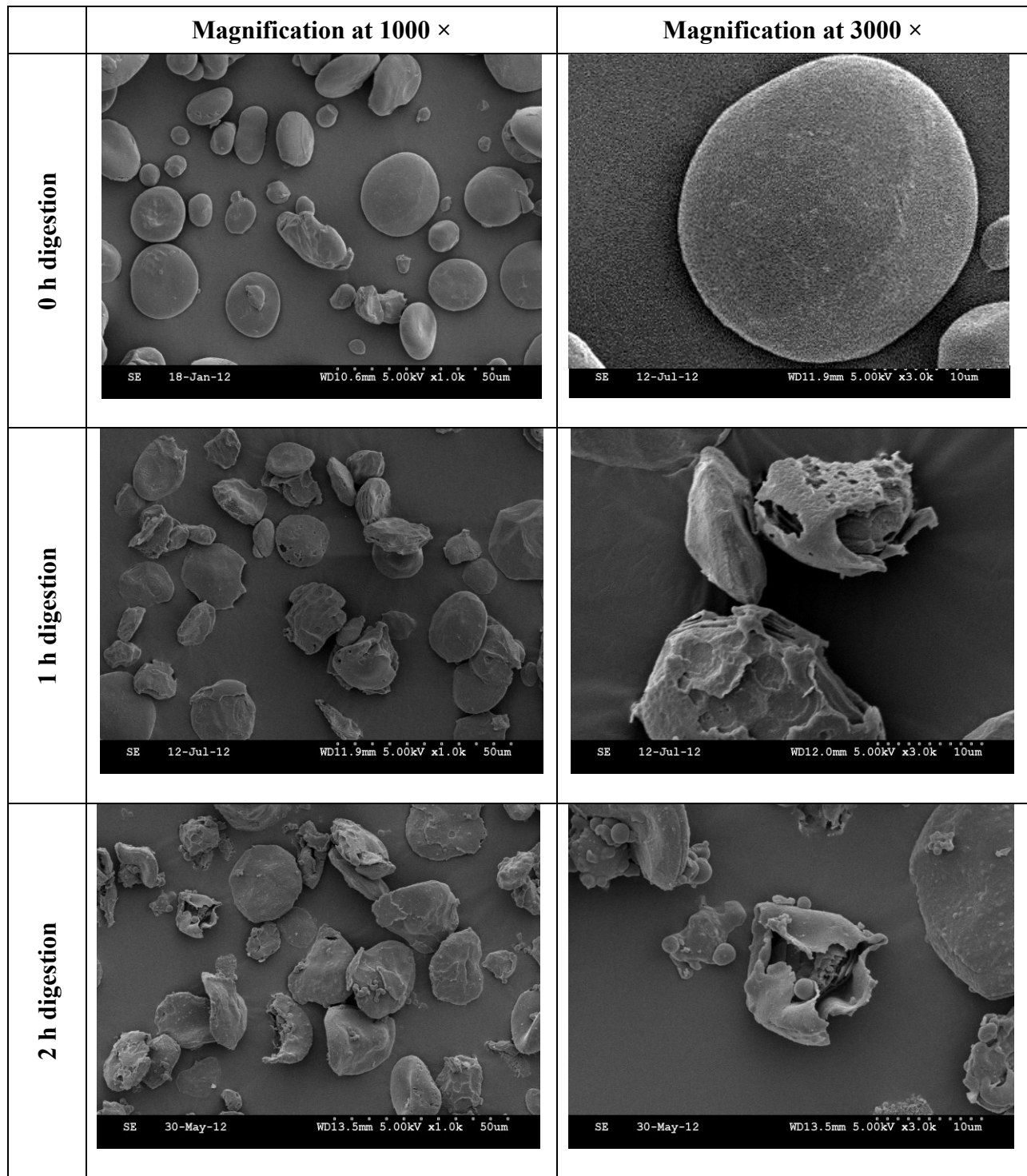


Fig. 3-4. Scanning electron micrographs (1000× and 3000× magnifications) of native wheat starch at 1 h and 2 h of amylase/amyloglucosidase digestion.

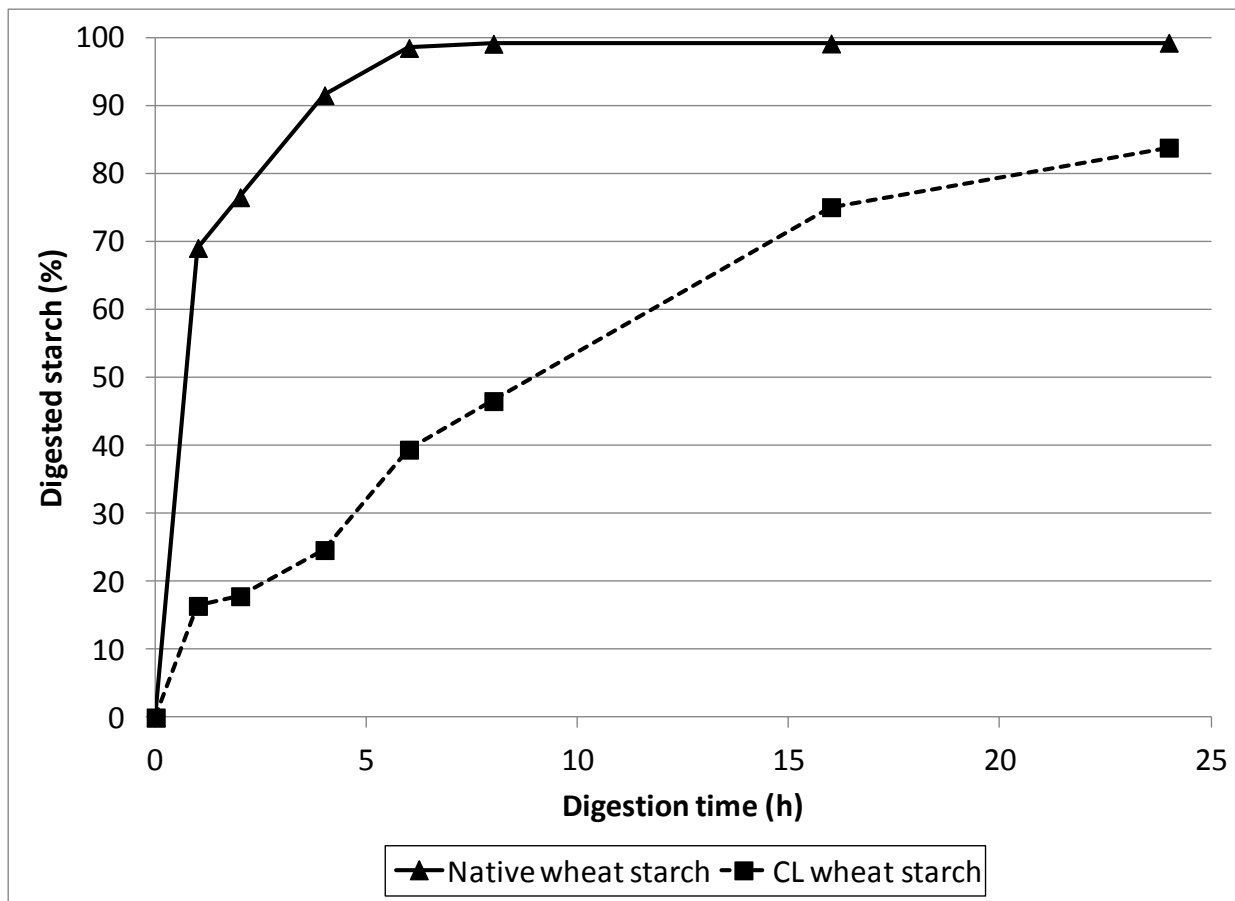


Fig. 3-5. Digested starch of cross-linked (CL) wheat starch and native wheat starch at different incubation times of α -amylase/amyloglucosidase digestion.

Chapter 4 - Assay of resistant starch content in phosphorylated cross-linked starch

4.1. Abstract

Direct assay of resistant starch (RS) in food and feed is accomplished by (i) removal of lipid, protein, and digestible starch to obtain insoluble dietary fiber and (ii) dissolution of resistant starch in the insoluble fiber followed by its quantification with specific enzymes. Cross-linked RS resists dissolution and therefore has not been assayed directly. The objective of this study was to develop a method to solubilize phosphorylated (0.4% phosphorus) cross-linked wheat starch (RS4) in the RS fraction produced after its incubation with α -amylase and amyloglucosidase for 16 h at 37 °C as directed by RS assay AOACI Method 2002.02. The residue was solubilized by conducting two back-to-back incubations with thermostable α -amylase for 30 min at 100 °C, cooling to 50 °C, then incubating with amyloglucosidase at 50 °C for 1 h. The cooling process after α -amylase incubation was done gradually by placing the mixture in a water bath at 50 °C. The degree of hydrolysis of the modified starch was determined by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (98.95%), glucose-oxidase/oxidase (95.30%), and phenol-sulfuric determination of total carbohydrate (105.22%). Analysis of the phosphorus content in the insoluble dietary fiber fraction suggested that part of the phosphorylated starch was solubilized after the 16-h α -amylase/glucoamylase digestion. Our results will provide a direct determination of cross-linked RS4 in foods.

4.2. Introduction

Initially discovered during the measurement of non-starch polysaccharides in food (Englyst, Wiggins, & Cummings, 1982), resistant starch (RS) is defined as the sum of starch and products of starch degradation that are not absorbed in the small intestine of healthy individuals (Delcour & Eerlingen, 1996). Health benefits of RS include the hypoglycaemic effect (Yamada, Hosoya, Nishimura, Tanaka, & Kajimoto et al., 2005), hypocholesterolemic effects (Han, Fukushima, Kato, Kojima, & Ohba et al., 2003), the prebiotic effect (Brown, McNaught, Ganly, Conway, & Evans et al., 1996), and improved absorption of minerals (Morais, Feste, Miller, & Lifichitz, 1996). RS is categorized into five classes: physically inaccessible starch, RS1; granular

starch, RS2; retrograded amylose, RS3; chemically modified starches, RS4; and amylose-lipid complex, RS5 (Brown, Yotsuzuka, Birkett, & Henriksson, 2006).

Chemical modification of starch may affect the rate and extent of digestibility in the small intestine (Wolf, Bauer, & Fahey, 1999) based on starch source, the type and degree of modification, and the extent of granule gelatinization (Filer, 1971). Cross-linked (CL) starch is one of the most highly produced and utilized chemically modified starches in the food industry. CL starch is used as a thickener and provides stability in acidic or hot conditions or during mechanical shearing (Wurzburg, 1986). CL starch also was shown to improve the texture and overall quality of sponge cake (Myung, Jeong, & Mal, 2001) and has been used in formulations that require pulpy texture, smoothness, flowability, low-pH storage, and high-temperature storage (Sajilata & Singhal, 2005).

Cross-linking of starch by a mixture of sodium trimetaphosphate (STMP) and sodium tripolyphosphate (STPP) by phosphorylation showed an increased amount of RS4 in the range of 5–70%. Cross-linking of wheat starch with STMP and STPP led up to up to 0.4% bound phosphorus (P) and gradually increased RS4 content to 76%. At the same time, the CL starch showed restricted swelling behavior (Woo & Seib, 2002). The possible causes of reduced digestibility of CL starch could be due to prevention of amylase molecules from entering the inside of granules via porous channels of CL starch granules (Huber & BeMiller, 2000), reduced swelling, and/or the obstructed formation of amylase and starch complex due to steric problems caused by phosphate groups on the starch backbone or by lack of chain flexibility after cross-linking of starch (Conway & Hood, 1976).

Available RS *in-vitro* methods are based on the simulation of gastrointestinal digestion of starch, followed by methods to quantify indigestible starch by chemical or gravimetric methods (Champ et al., 2003; Perera, Meda, & Tyler, 2010). Determinations of RS using *in-vitro* methods are controversial because resistance is caused principally by physical barriers to α -amylase digestion, and those barriers will withstand or succumb to *in-vitro* digestion depending on α -amylase activity, reaction conditions (pH, temperature, time, agitation), and the addition of amyloglucosidase (Kingman & Cummings, 1992; McCleary and Monaghan, 2002). These limitations were overcome by *in-vivo* digestive studies with human subjects and with ileostomy patients that validate *in-vitro* RS with *in-vivo* data (Englyst, Kingman, & Cummings, 1992) that portrays the high correlation of RDS with *in-vivo* glycemic response (Englyst, Englyst, Hudson,

Cole, & Cummings, 1999). Enzymatic-gravimetric procedures using AOAC Method 991.43 (2000) TDF method also have been reported to be consistent with *in-vivo* values determined in ileostomy patients (Champ, Langkilde, & Brouns, 2003).

Several *in-vitro* methods have been used to quantify the total RS (Englyst, Kingman, & Cummings, 1992; Goñi, García-Diza, Mañasb, & Saura-Calixto, 1996; McCleary, McNally, & Rossiter, 2002; Chung, Lim, & Lim, 2006). Many of these methods vary in terms of sample preparation, level of amylases and proteases, testing conditions (e.g., pH, temperature, time, agitation), and isolation procedure of RS. A method by Englyst, Kingman, & Cummings (1992) proposed separate quantification of RS1, RS2, and RS3. The determination of RS4 by a method developed by Englyst, Kingman, & Cummings (1992) is commonly used because of the corroboration with *in-vivo* values determined in ileostomy patients (Silvester, Englyst, & Cummings, 1995; Englyst, Kingman, Hudson, & Cummings, 1996); however, RS content was not measured directly in this method but was based on subtraction of the sum of rapidly digestible starch (RDS) and slowly digestible starch (SDS) from the total starch in a sample. Other methods (Eerlingen, Crombez, & Delcour, 1993; Faraj, Vasanthan, & Hoover, 2004), including AOAC Method 2009.01 (McCleary et al., 2010), isolate RS gravimetrically, but gravimetric separation is suitable only for analytes free of non-starch polysaccharides (Perera, Meda, & Tyler, 2010), which is not usually the case in a food system. To hydrolyze digestive starch, both AOAC Method 2009.01 and AOAC Method 2002.02 (McCleary, McNally, & Rossiter, 2002) apply the 16-h incubation at 37 °C of the α -amylase and amyloglucosidase enzyme cocktail, leaving the indigestible residue containing RS. Unlike AOAC Method 2009.01 that isolates RS by gravimetric method, AOAC Method 2002.02 determines RS by solubilizing the indigestible starch using 2M of potassium hydroxide followed by hydrolyzing the solubilized starch to glucose, which is then measured by glucose oxidase-peroxidase (GOPOD) colorimetric assay.

Available RS methods are unable to determine RS4 directly in a food system that may consist of a mixture of starch and RS4. The method by Englyst, Kingman, & Cummings (1992) is able to provide a total RS with a mixture of RS1, RS2, RS3, and RS4 or a direct measurement of RS1, RS2, and RS3. The gravimetric procedure in AOAC Method 2009.01 also measures the total RS1, RS2, RS3, and RS4, although recovery of CL wheat starch by that method is low (~25%) compared with the Englyst method (~70%). AOAC Method 2002.02 can determine only

a mixture of RS1, RS2, and RS3, and is unable to quantify RS4 due to the insolubility of RS4 in 2M potassium hydroxide. After the 16-h incubation at 37 °C of α -amylase and amyloglucosidase enzymes in AOAC Method 2002.02, the residue of CL starch left is an RS4-rich matrix that resists dissolution in 2M potassium hydroxide. A new procedure is needed to solubilize RS4 to identify the amount of RS4 in a food system. Hence, the objectives of this study were to develop procedures to solubilize CL starch (~0.4% bound P) and develop an *in-vitro* method to quantify RS4 in a food system.

4.3. Materials and Methods

4.3.1. Materials

CL wheat starch (Fibersym) and normal wheat starch (Midsol 50) were obtained from MGP Ingredients (Atchison, KS). RS assay kit (catalogue no. K-RSTAR), D-glucose assay kit (catalogue no. K-GLUC), thermostable α -amylase (activity of 3000 U/mL on soluble starch), and amyloglucosidase (3300 U/mL on soluble starch) from the total starch kit were purchased from Megazyme International Ireland Ltd. (Wicklow, Ireland). One activity unit (U) of α -amylase at pH 5.0 and 40 °C is defined as the amount of enzyme required to release one micromole of D-glucose per minute from soluble starch. One U of amyloglucosidase enzyme activity is the amount of enzyme required to release one micromole of glucose from soluble starch per minute at 40 °C and pH 4.5. All chemicals were reagent-grade.

4.3.2. General methods

The P content was assayed using the procedure of Smith and Caruso (1964). Moisture content was obtained according to AACC Method 44-15 (AACC 2000) by calculating the difference in starch weight before and after drying it at 130 °C for 1 h.

4.3.3. Solubilization and conversion of CL starch to glucose

Starch (0.1 g, db) was slurried with 100 mM sodium acetate buffer (pH 5.0; 8 mL) in a test tube. Thermostable α -amylase (20 μ L) was added, and the mixture was heated in a water bath at 100 °C for 30 min with constant stirring (magnetic stir bar). Then, another 20 μ L of thermostable α -amylase was added, and stirred an additional 30 min at 100 °C. The mixture was

cooled gradually by placing the tubes in a water bath at 50 °C for at least 15 min, after which time amyloglucosidase (40 µL) was added and the mixture incubated with constant stirring at 50 °C for 1 h. A blank sample was prepared by omitting the starch and following the same procedures.

4.3.4. Determination of glucose content

Total carbohydrate was determined using the method of Dubois, Gilles, Hamilton, Rebers, & Smith (1956). Glucose content was determined by the glucose oxidase/oxidase (GOPOD) assay procedure (McCleary & Codd, 1991) and by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Dionex Corporation, Sunnyvale, CA). Total carbohydrate and D-glucose assay were colorimetric methods, where color was measured respectively at wavelengths of 490 nm and 510 nm against blank. A sample for HPAEC-PAD was prepared by diluting the solubilized starch solution to ~20 µg/mL with deionized water and filtered through a 0.2-µm filter (Fisher Scientific, Dublin, Ireland). The sample was injected into a CarboPac PA1 column (Dionex Corporation, Sunnyvale, CA) at 25 °C. Sodium hydroxide 150 mM was employed as the eluent at a flow rate of 1 mL/min. Standard glucose solutions (10 and 20 µg/mL) were injected (10 µL) and analyzed to obtain a standard curve. Glucose released from hydrolyzed starch was quantified based on its integrated peak area relative to the area of the known glucose standard.

4.3.5. RS assay for type 4 resistant starch

The method used was based on AOAC Method 2002.02 (2011) with modifications to the starch solubilization step. Starch samples (0.1 g, db) and a combination of α -amylase (30 U/mL) and amyloglucosidase (3 U/mL) solution (4 mL) were combined in capped test tubes and placed in a shaking (200 strokes/min) water bath at 37 °C for 16 h. Then 99% ethanol (4 mL) was added to the mixture, vortexed, and centrifuged at 1500 × g for 10 min. The supernatant was decanted and 50% ethanol (8 mL) was added. The mixture was vortexed and centrifuged at 1500 × g for 10 min. After decanting the supernatant, 50% ethanol (8 mL) was again added, and the mixture was vortexed and centrifuged at 1500 × g for 10 min. The tubes were then placed upside-down to drain remaining ethanol. All decanted supernatants were combined and kept for P content analysis. The remaining pellet was the undigested RS4 and was saved for further analysis.

The next steps involved modification to the original method (AOAC Method 2002.02) to achieve solubilization of RS4. The undigested RS4 residue and 8 mL sodium acetate buffer (100 mM, pH 5) were slurried in a test tube prior to the addition of the thermostable α -amylase (20 μ L). The mixture was heated in a water bath at 100 °C for 30 min with a constant stirring, and another 20 μ L of thermostable α -amylase was added. The mixture was cooled gradually by placing the tubes in a water bath at 50 °C for at least 15 min. Subsequently, amyloglucosidase (40 μ L) was added to the mixture, which was then incubated with constant stirring at 50 °C for 1 h. The mixture was transferred to a 100-mL volumetric flask and adjusted to 100 mL using distilled water. An aliquot (1 mL) was removed, centrifuged at 1500 \times g for 10 min, then assayed for glucose using GOPOD reagent. Aliquots (0.1 mL) were transferred into glass test tubes and 3 mL of GOPOD reagent was added into each tube and incubated at 50 °C. After 20 min, the absorbance of each solution was measured at 510 nm against a blank reagent.

4.3.6. Integrated total dietary fiber

RS was determined by following the procedures for the high molecular weight dietary fiber fraction of AOAC Method 2009.01 (2011).

4.3.7. Statistical analysis

All data were statistically analyzed using Statistical Analysis Software (SAS) (version 9.2, SAS Institute, Cary, NC) by analysis of variance (ANOVA) and the values are expressed as means \pm standard deviations from 2 replicates, unless stated otherwise.

4.4. Results and Discussion

4.4.1. Solubilization of CL starch to glucose

The RS4 product was not fully solubilized using alkaline aqueous solution due to the high degree of cross-linking. The initial step in developing a direct method to determine RS content of the RS4 product was to find the right procedure to solubilize CL wheat starch. To achieve the latter, only steps 1 through 4 of the direct method of assaying for RS (AOAC Method 2002.02, Fig. 4-1) were carried out, and step 5 was replaced with multiple attempts (Fig. 4-1). The procedures that were attempted had been unsuccessful in fully solubilizing the CL wheat starch except for procedure E, which used 8M potassium hydroxide (KOH). Procedure E involved

incubation of CL wheat starch with α -amylase in boiling water for 30 min, then 2 mL 8M KOH was added and stirred continuously at room temperature for 24 h. The procedure was able to fully solubilize the CL wheat starch, as noted by the clear solution; however, the solution was yellow in color, indicating reaction of the strong alkali with starch molecules (Tomasik, 2003). Because alkaline degradation of starch may interfere with the glucose determination, the procedure using 8M KOH was deemed unsuitable.

The successful procedure to fully solubilize CL wheat starch involved incubating CL wheat starch with thermostable α -amylase for two consecutive 30-min periods at 100 °C, gradual cooling to 50 °C, then incubating with amyloglucosidase for 1 h at 50 °C. The optimum pH of both incubations was 4.5 to 5.0. Wheat starch also was solubilized using the same procedure as the control, because wheat starch is easily converted to glucose by digestive enzymes (Björck et al., 1986). Conversion of starch to glucose in the digested starch was determined by (i) phenol-sulfuric assay for total carbohydrate, (ii) HPAEC, and (iii) GOPOD.

Table 4-1 shows the total carbohydrate and glucose content of solubilized CL wheat starch and wheat starch by three different methods. Regardless of the determination method used, the glucose content of both starches was similar, indicating a similar conversion of CL wheat starch to glucose compared with the wheat starch. Based on our observation, α -amylase alone was unable to fully convert CL wheat starch to water-soluble dextrans, as depicted by a cloudy solution (Fig. 4-2 A). The α -amylase cleaves the α , 1-4 glycosidic bonds in the inner section of amylose and amylopectin to produce mono- and oligosaccharides (van der Maarel, van der Veen, Uitdehaag, Leemhuis, et al., 2002). Further incubation of CL starch with amyloglucosidase hydrolyzed dextrin to glucose because amyloglucosidase cleaves on both α , 1-4 and α , 1-6 glycosidic bonds (Pandey, Nigam, Soccol, Soccol, Singh, et al., 2000). The CL wheat starch sample had clarity similar to the blank after amyloglucosidase incubation (compare tubes on the right sides of Fig. 4-2 A and Fig. 4-2 B).

The cooling step of the α -amylase digestion from 100 °C to 50 °C should be done by placing the test tubes at ~100 °C in a 50 °C water bath for a minimum of ~15 min. It is important that the α -amylase digest be cooled only to 50 °C. If the α -amylase digestion was cool-shocked from 100 °C to 25 °C in cold water, the solution at the conclusion of amyloglucosidase incubation was cloudy (photograph not shown). The cloudy solution was centrifuged prior to digestion steps, and the supernatant was tested for glucose content, which showed only a 64%

recovery of carbohydrate, indicating incomplete solubilization of CL wheat starch. This result could be due to retrogradation or association of the α -limit dextrans during the 25 °C cooling environment, which decreased susceptibility of the α -amylase digest to glucoamylase enzyme.

4.4.2. Comparison of methods used in determination of total carbohydrate and glucose content

The total carbohydrate value was higher than the glucose content, as determined by HPAEC method and D-glucose assay (Table 4-1). The total carbohydrate method is a simple method involving usage of phenol with the presence of sulfuric acid to produce a yellow-orange solution. This method is simple, fast, cheap, and reproducible (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), but safety precautions must be taken, because the method uses concentrated sulfuric acid and 80% phenol. The D-glucose assay is also a simple method that applies glucose oxidase and peroxidase enzymes' reaction with D-glucose to produce a pink-dyed solution. Conversion of D-glucose to gluconic acid will consume oxygen. A high concentration of glucose limits the availability of oxygen for the conversion and production of hydrogen peroxide, which inhibits the activity of glucose oxidase (Houben, de Ruijter, & Brunt, 1997), hence lowering the total of glucose content quantified (Table 4-1). The HPAEC-PAD method increases the sensitivity of carbohydrate analysis and eliminates the need for derivatization (Rocklin and Pohl, 1983).

4.4.3. Development of in-vitro method for type 4 resistant starch

The CL starch is distinguished from RS1, RS2, and RS3 because it is unable to completely solubilize in 2M KOH. The unsolubilized residue can be quantified gravimetrically, but gravimetric separation is suitable only for analytes free of non-starch polysaccharides (Perera, Meda, and Tyler, 2010). Hence, a fundamental method is developed to quantify RS4 in a food system using a colorimetric procedure. The method was based on the key attributes of AOAC Method 2002.02, and its principle includes removal of digested starch to leave RS4 in the indigestible matrix, which is subsequently hydrolyzed by thermostable α -amylase and amyloglucosidase. The hydrolyzed RS4 is then diluted and measured using GOPOD colorimetric procedures.

The details of the newly developed method are illustrated in Fig. 4-3. The non-resistant starch in the sample was hydrolyzed by incubating pancreatic α -amylase and amyloglucosidase

at 37 °C for 2 h with continuous stirring. After centrifugation (1500 × g, 10 min), the RS4 matrix sediment was slurried with 100 mM sodium acetate buffer at pH 5. Then, two subsequent incubations with thermostable α -amylase at 100 °C for 30 min were carried out, followed by gradual cooling to 50 °C. Amyloglucosidase was added, and the solution was incubated for 1 h at 50 °C to hydrolyze RS4 into D-glucose. The D-glucose-rich solution was then diluted prior to GOPOD colorimetric measurement.

The RS yield of CL starch from AOAC Method 2002.02 was lower than that of Englyst method (data not shown), which was predicted due to the long incubation period (16 h) of α -amylase and amyloglucosidase. To prove this, the RS content of CL wheat starch was quantified using the newly developed method at different incubation periods (2 h, 8 h, and 16 h) during the removal of digestible starch step (Fig. 4-3). Based on Table 1, RS amount in CL wheat starch reduced significantly with increasing incubation time. The significantly lower RS may not reflect the *in-vivo* response that is important to the food industry; hence, the duration of 2 h was chosen due to the RS value similar to the RS amount assayed by the Englyst method. The RS4 content by the new developed method at 16 h was slightly lower than the RS content determined by AOAC Method 2009.01, a gravimetric subtraction method. It should be noted that in the latter case, only glucose was measured. If the total carbohydrate method were used, the RS content would be slightly higher.

4.4.4. Effect of P content after enzyme hydrolysis

The U.S. Code of Federal Regulations has approved STMP and STPP as cross-linking agents in CL starch (CFR, 2001). The cross-linking agents function to form intermolecular-level bridges between P with hydroxyl group of starch (Rutenberg and Solarek, 1984; Wurzburg, 1986). A combination of STMP and STPP with the ratio of 99:1 was proven to efficiently produce CL starch up to the 0.4% P add-on (Woo and Seib, 2002) permitted for food-grade CL starch. Determination of total RS and TDF of CL starch was reported (Woo and Seib, 2002; Sang et al., 2010), but the mechanism of digestive enzyme actions on CL starch is not well documented. The P content (Table 4-1) in the insoluble sediment of CL wheat starch after 16 h of incubation with α -amylase and amyloglucosidase (based on the procedure in the AOAC Method 2002.02) increased, whereas no P was found in the supernatant. This result suggests that the digestive enzymes are able to hydrolyze starch molecules that do not contain or are not

adjacent to a bound phosphate group to dextrans and D-glucose, leaving the starch molecules that are covalently linked to P molecules.

4.5. Conclusions

To solubilize and hydrolyze RS4, a new procedure was developed by incubating RS4 with thermostable α -amylase for 30 min at 100 °C twice, cooling gradually in a water bath at 50 °C, and incubating with amyloglucosidase at 50 °C for 1 h. The method was able to convert CL wheat starch to D-glucose similar to that of the control (wheat starch), which was determined by using total carbohydrate, HPAEC-PAD, and D-glucose assays. A new *in-vitro* method was developed based on AOAC Method 2002.02, AOAC Method 2009.01, and the developed RS4 solubilization procedure. The new method is essential to measure the RS4 content in a food system. Suggested future work includes testing the method on various food system enriched with CL starch, especially starchy food such as bakery products, pasta, and extruded products.

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Table 4-1. Resistant starch and phosphorus (P) contents of cross-linked (CL) wheat starch and wheat starch, plus total carbohydrate and glucose content of hydrolysate after α -amylase and amyloglucosidase digestion.

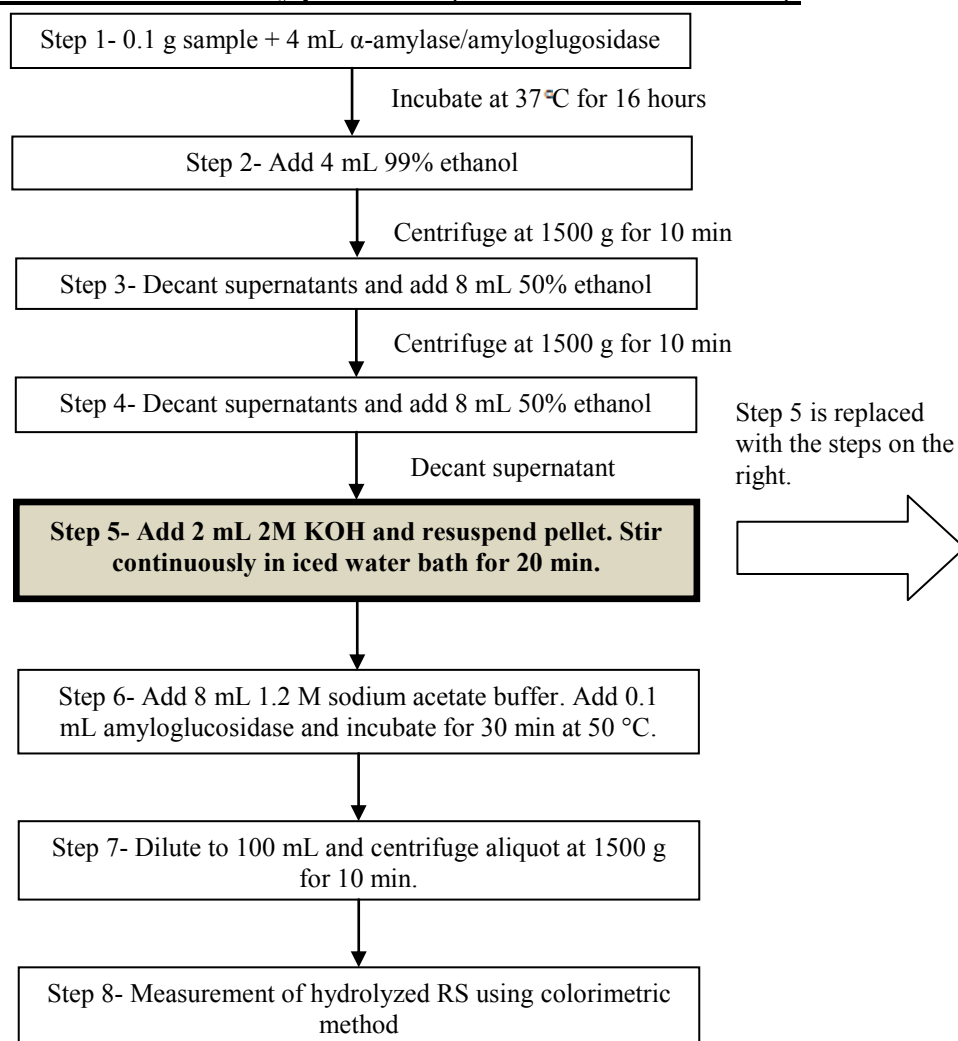
	CL wheat starch		Wheat starch
	<u>Gradual cooling</u>	<u>Shock cooling</u>	
Total carbohydrate (%)	105.22 \pm 0.54 a	-	105.62 \pm 0.62 a
<u>Glucose content (%)</u>			
HPAEC method	98.95 \pm 0.50 a	-	99.70 \pm 1.38 a
D-glucose assay	95.30 \pm 1.82 a	64.21 \pm 0.82 b	93.65 \pm 0.97 a
<u>Resistant starch (%)*</u>			
New method (2 h)	78.23 \pm 0.72 a		11.19 \pm 0.20 b
New method (8 h)	54.06 \pm 0.19 a		0.91 \pm 0.05 b
New method (16 h)	22.18 \pm 0.38 a		0.74 \pm 0.13 b
AOAC method 2009.01	23.96 \pm 1.17 a		1.48 \pm 0.69 b
<u>P content (%)</u>			
Before digestion	0.40 \pm 0.03	-	N/A
Sediment**	0.60 \pm 0.04	-	N/A
Supernatant***	0.00 \pm 0.00	-	N/A

* Resistant starch was determined by using the newly developed RS4 method with 2-h, 8-h, and 16-h incubation periods and AOAC Method 2009.01.

** P content of the RS4 residue after 16 h of incubation with α -amylase and amyloglucosidase.

*** P content in the supernatant after 16 h of incubation with α -amylase and amyloglucosidase.

The resistant starch assay procedure (AOAC Method 2002.02).



Procedures that were tried in an attempt to solubilize RS4.

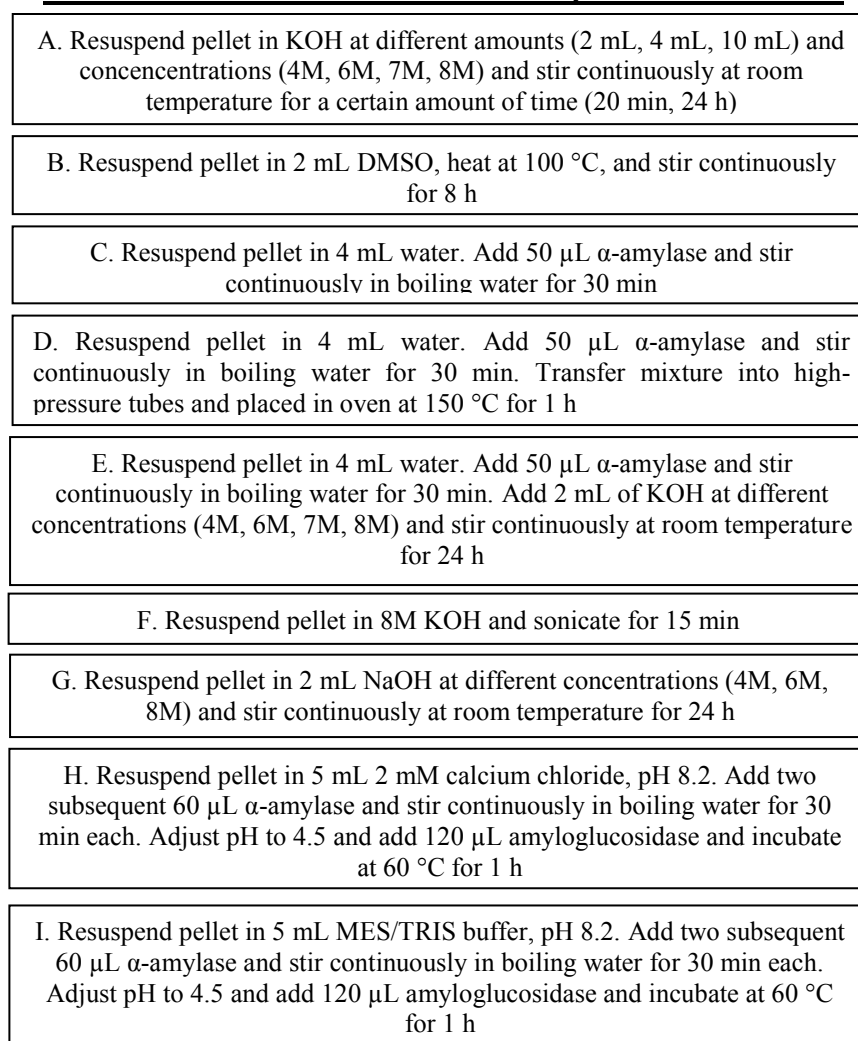


Fig. 4-1. Flow chart of direct assay for resistant starch (AOAC Method 2002.02) and the unsuccessful procedures that were tried to solubilize RS4. Steps 1–4 from the resistant starch assay procedure were followed, then step 5 was replaced with the attempted procedures to solubilize RS4. Steps 6–8 were not carried out for those attempts.

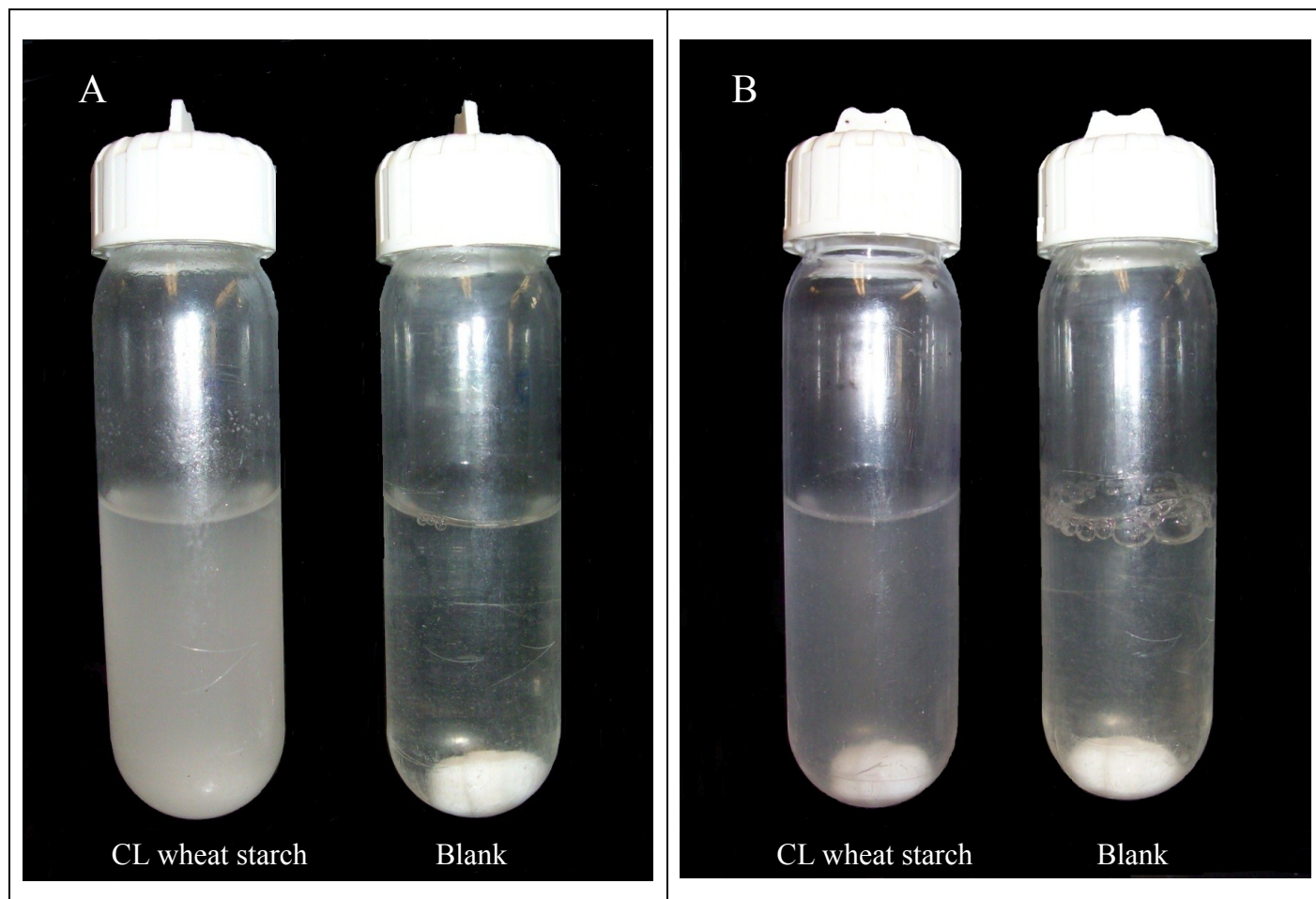


Fig. 4-2. Photograph of cross-linked (CL) wheat starch (A) after incubation with α -amylase and (B) after amyloglucosidase compared with a blank without starch. Note the cloudiness of the solution containing CL wheat starch in 2A compared with the blank.

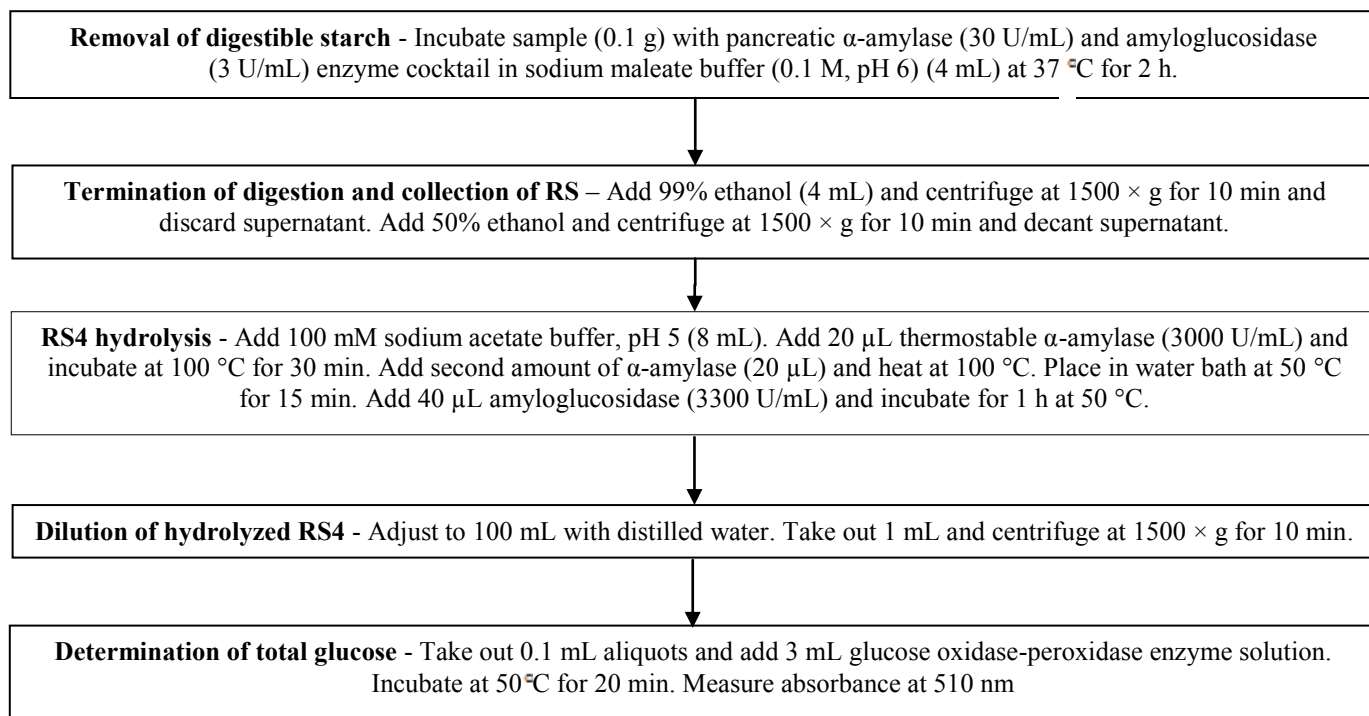


Fig.4-3. Flow chart of the new developed method to determine RS4.

Chapter 5 - Physico-chemical difference in extrudates from normal and waxy wheat flours

5.1. Abstract

The objective of this study was to compare the physicochemical properties of extruded normal and waxy wheat flours at different screw speeds. Normal (26.7% amylose) and waxy (1.4% amylose) wheat flours with different protein levels were hydrated to 18% moisture content and subjected to twin-screw extrusion at the screw speeds of 180 and 360 rpm. Higher energy input caused high radial expansion and low density of hard normal wheat extrudate, whereas higher lipid in hard waxy wheat reduced the specific mechanical energy. Soft waxy wheat extrudate had higher radial expansion than normal soft which was likely due to a better expansion and flowability of amylopectin. Extrusion resulted in the breakdown of starch and increased insoluble protein for all extrudates. After extrusion, hard waxy wheat formed relatively higher high molecular weight of insoluble protein and had excessive dextrinization of starch, causing lower radial expansion and breaking strength. The micro-structure of samples extruded with higher screw speed revealed thinner cell walls, with waxy wheat extrudates having heterogenous cell size. Different type of wheat flours offers an alternative as a functional ingredient to manipulate the physico-chemical properties of extrudates in snack or cereal applications.

5.2. Introduction

Extrusion is a thermomechanical processing which involves high temperature, short time and low moisture cooking that uses mechanical energy to force out material out of the die (Riaz, 2006). Cereal based extruded products are comprised mainly of starch. The source of starch, amylose/amylopectin ratio, and molecular weight and structure of amylose and amylopectin play a role in the expansion characteristics of the developed product (Chinnaswamy and Hanna, 1988). Starches with similar amylose and amylopectin contents from different cereals may result in different radial expansions. For example, waxy corn meal showed the best expansion from normal and high amylose corn meals (Harper and Tribelhorn, 1992), while waxy barley flour extrudates had lower expansion than the other regular barley lines (Dudgeon-Bollinger et al., 1997; Baik et al., 2004). In a comprehensive study on rice expansion conducted by

Chinnaswamy and Bhattacharya (1983, 1986), rice varieties with higher expansion had higher mean molecular weight of amylopectin. The structure of amylose and amylopectin was also noted to have differences between corn starch varieties (Banks and Greenwood, 1975; Young, 1984; Hizukuri et al., 2006).

Extrusion of starch material resulted in starch gelatinization, protein denaturation and occurrence of complexes between starch, lipids and proteins (Mercier and Feillet, 1975; Mercier et al., 1979; Colonna and Mercier, 1983; Schweizer and Reimann, 1986; Meuser et al., 1987, Bhatnagar and Hanna, 1994). Minor ingredients such as protein and lipid influence the rheological property of starch melt (Harper, 1981; Colonna, 1989). Several works had been reported on effect of oil inclusion on expansion properties of extrudates (Faubion&Hoseney, 1982b; Mohamed, 1990; Lin et al., 1997; Singh & Smith, 1997; Abu-hardan et al., 2011). These researches were conducted using different lipids at different concentrations and showed different effect on expansions properties. Addition of up to 4% corn oil showed an improved expansion of maize grits extrudates (Mohamed, 1990). Bhatnagar and Hanna (1997) did a comprehensive study on different kinds of lipid where 4% was incorporated in corn starch formulations. Addition of peanut oil, coconut oil and palm oil significantly reduced the bulk density of cornstarch extrudate as compared to the addition of myristic, stearic and behenic acids and monoglycerides which resulted in increased bulk density. Abu-hardan et al (2011) included 1-8% soybean, palm or sunflower oils into wheat starch blends and found that radial expansion increased with increasing oil content up to 5% which then reduced beyond that. In contrast, Pan et al. (1992) showed decreased expansion of rice flour extrudate with the addition of 3% lecithin and monoglycerides. The work of Faubion and Hoseney (1982) showed expansion properties were not affected by the natural lipid in wheat flour and wheat starch. However, they observed a slight decreased expansion of extrudate when 1% of free lipids from wheat flour was added. Decreased expansion of extrudate due to incorporation of lipid in extruded starch or flour could be explained by the inhomogeneous mixing of oil and other materials outside or within the extruder. This will cause separation of oil and material that causes two-phase flow. The separated oil will reduce the friction between screw and barrel surfaces due to lubrication (Yacu, 1998). In turn, shear input to the material will be reduced as well as causing a reduction in the temperature of the material (Colonna and Mercier, 1983; Lin et al., 1997) which would greatly affect starch conversion in the extruder. In addition, lipid acts as an insulating agent which coats the starch

granules and prevent water absorption. Hence, starch gelatinization will be reduced due to insufficient amount of water (Marshall et al., 1990).

Total protein is comprised of soluble protein (SP) and insoluble polymeric protein (IPP). SP consists of soluble polymeric protein (SPP) (>70 kDa), gliadin (20-70 kDa) and albumin/globulin (< 20 kDa), whereas IPP contains high molecular weight glutenin subunit (HMW-GS) (70 kDa), low molecular weight glutenin subunit (LMW-GS) (<70 kDa) and residual protein (RP). RP are the largest molecular size of glutenin that are estimated to be > 2000 kDa and may reach up to millions kDa (Huebner and Wall, 1975; Stevenson and Preston, 1996; Wahlund et al., 1996). RP is very hard to extract due to the lack of ionizable groups and lower entropy of mixing as a consequence of the very large molecular size (Southan and MacRitchie, 1999). Increased protein content has been proposed to reduce expansion of extruded products, since protein and amylose molecules have the tendency to align linearly with themselves and each other, and form amylose-protein complexes (Faubion and Hoseneay, 1982; Kim and Maga, 1987; Colonna et al., 1989; Mathew and Hanna, 1997). Cereal sources which are different in botanical origin contain different protein contents and properties. For instance, maize extrudates had higher sectional expansion than wheat extrudates (Chanvrier et al., 2007; Arhaliass et al., 2009). The capability of maize starch to expand more than wheat is mainly due to a lower level of chemically different maize proteins as compared to wheat proteins. Protein in wheat, namely gluten, is cohesive and elastic as compared to corn protein (zein) that has no stretching capabilities (Chanvrier et al., 2007). At the temperature of 60 – 70 °C during extrusion process, proteins will mostly become denatured and be insoluble, whereas starch is degraded to more soluble forms (gelatinized) at 51 – 78 °C (Huber 2001). These changes may influence the textural and structural properties of expanded extruded snack (Huber, 2001).

Waxy wheat flours have been used to extend the shelf-life of baked goods and to produce Asian wet noodle products (Graybosch, 1998). Although documentation of extrusion of wheat flour or starch are available in terms of effect of processing variables (Mercier and Feillet, 1975; Paton and Spratt, 1984; Ilo et al., 1999; Ding et al., 2006), influence of moisture content (Faubion and Hoseneay, 1982a) and influence of minor ingredients (protein and lipid) (Faubion and Hoseneay, 1982b; Chanvrier et al., 2007; Arhaliass et al., 2009), no study has been done on extrusion of waxy wheat flours. It had been documented that extruded waxy maize shows better expansion than extruded normal normal maize (Harper and Tribelhorn, 1992) and this may be

true for extruded waxy wheat. Thus, the objectives of the research were to compare the properties of extruded waxy and normal wheat flours at different screw speeds and to study the starch and protein modifications of the extruded normal and waxy wheat flours.

5.3. Materials and methods

5.3.1. Materials

Hard and soft normal wheat flours were donated from Cereal Food Company (Kansas City, KS). Hard (breed NX03Y2205) and soft waxy wheat kernels were obtained from Simons Home Store (Wahoo, NE) and WK Kellogg Institute (Battle Creek, MI), respectively. The waxy wheat kernels were milled to flour using Brabender Quadrumat Senior (Western Germany). The moisture content of wheat kernels were determined using Perten SKCS 4100 (Springfield, IL) and the amount of water needed to add was calculated to achieve tempering moisture content of 14% for soft waxy wheat and 15.5% for hard waxy wheat. The break and reduction flours were mixed together for extrusion purposes.

5.3.2 Proximate analysis

Protein of raw materials was determined via LECO TruSpec CN Carbon/Nitrogen combustion analyzer (St. Joseph, MI), while lipid and ash contents were measured according to AOAC Method 920.39 and AOAC Method 942.05, respectively. Total starch was determined by using the Megazyme International Ltd. (Wicklow, Ireland) kit.

5.3.3. Amylose content

The amylose content of sample was determined using an amylose/amylopectin assay based on the procedure of Yun and Matheson (1990) and kit was purchased from Megazyme International Ltd. (Wicklow, Ireland), with slight modifications to the centrifugation speeds from 2000 ×g to 1500 ×g.

5.3.4. Starch damage

Starch damage was determined using starch damage Megazyme International Ltd. (Wicklow, Ireland) kit and measured by the procedure of Gibson et al. (1991). The sample (100 mg) and 1.0 mL fungal α -amylase solution (50 U/mL) were vortexed for 5 sec and incubated for 10 min at 40 °C. The enzyme was inactivated by adding 8.0 mL sulphuric acid solution (0.2 %

v/v) into the mixture, stirred vigorously for 5 sec, and centrifuged at 1160 ×g for 5 min. The slurry was filtered through Whatman No 1 filter paper. Finally, an aliquot (0.1 mL) from the supernatant was collected for colorimetric determination of glucose at 510 nm against a reagent blank. Starch damage was expressed as percentage from the weight of the sample.

5.3.5. *Experimental design and extrusion processing*

A 4 × 2 factorial experimental design of four different types of wheat flour (soft normal, hard normal, soft waxy and hard waxy wheat flours) and two extruder screw speeds (180 and 360 rpm) was used. The moisture content of the blends was adjusted to 18% by spraying distilled water and mixed 1 min in a Classic Kitchen Aid mixer (St. Joseph, Michigan). The blends were kept in a fridge overnight for moisture equilibrium prior to extrusion with a Micro-18 twin screw extruder (American Leistritz Extruder Corp., Somerville, NJ). The extruder configuration parameters and the schematic of the screw configuration for the Micro-18 are in Fig. 5-1. The die used was a 2.5 mm circular die. Tests of specific mechanical energy (SME) were executed in duplicate and mechanical energy input per unit mass of extrudate was calculated as follows:

$$SME = \frac{(\tau - \tau_0) \times P_{rated} \times \frac{N}{N_{rated}}}{\dot{m}} \text{ (kJ/kg)}$$

where τ - % torque, τ_0 - no-load torque (4%), P_{rated} - rated power for the extruder (22.37 kJ/s), N - measured extruder screw speed in rpm, N_{rated} - rated extruder screw speed (336 rpm) and \dot{m} - mass flow rate in kg/s.

The extrudates were dried in an oven at 110 °C for 10 min, cooled for 5 min at room temperature and stored in plastic containers until analysis.

5.3.6. *Mechanical property*

Force–deformation data for each treatment was obtained with a Model TA-XT2i Texture Analyzer (Stable Micro Systems, Surrey, UK) equipped with a 25-kg load cell and a 38-mm-diameter test probe. Samples were compressed perpendicularly to 70% of their original height at a test speed of 5 mm/s. Thirty sample measurements were taken for each treatment. Average crushing force F_{cr} or breaking strength was calculated by using n , s and x values (Bouvier et al., 1997).

$$F_{cr} = \frac{s}{x \times n} \text{ (g)}$$

where n - number of peaks, s - integral of the curve (or area below the curve from 0% to 70% strain) and x - distance of breakage,.

5.3.7. Water absorption (WAI) and water solubility (WSI) indices

The WAI and WSI were determined by the method of Gujral and Singh (2002) on duplicates samples. Extruded samples were ground in a Waring blender (Torrington, CT) and sifted through a 250 screen sieve prior to testing. The ground sample (2.5 g) was immersed in 25 mL distilled water at room temperature, continuously stirred for 30 min, and centrifuged at 3000 $\times g$ for 10 min. The supernatant liquid was poured into a pre-weighed evaporating dish. The remaining gel was weighed, and WAI was calculated as

$$WAI = \frac{W_{\text{sediment}}}{W_{\text{dry solid}}}$$

where W_{sediment} - weight of gel and $W_{\text{dry solid}}$ - weight of dry sample.

The WSI was determined from the amount of dry solids recovered by evaporating the supernatant at 100 °C for 12 h and it was calculated as follows:

$$WSI = \frac{W_{\text{dissolved solid in supernatant}}}{W}$$

where $W_{\text{dissolved solids in supernatant}}$ - weight of dry solids from the supernatant and $W_{\text{dry solid}}$ - weight of dry sample.

5.3.8. Starch molecular weight distribution

The molecular weight distribution of the extrudates was determined by a gel permeation chromatography (GPC) (PL-GPC 220, Polymer Laboratories Inc., Amherst, MA) system equipped with a differential refractive index (DRI) detector and phynogel 00H-0646-KO, 00H-0644-KO and 00H-0642-KO columns (Phenomenex, Torrance, CA) connected in series. The mobile phase (dimethyl sulfoxide (DMSO) with 5 mM NaNO₃) had a flow rate of 0.8 mL/min. The column oven temperature was controlled at 80 °C. A series of dextran standards (American Polymer Standards Corporation, Mentor, OH) with different molecular weights were used to compare the retention time with the test samples for molecular weight calculation. Electronic outputs of the DRI detectors were collected by GPC software (version. 3.0, Polymer Laboratories, A Varian, Inc., Company, Amherst, MA).

For GPC analysis, 4 mg (db) of ground extrudate was dissolved in 4 mL DMSO in a boiling water bath for 24 h with constant stirring. Each solution was filtered through a 2.0- μ m filter (MILLEX AP 20 PREFILTER, Millipore, Carrigtwohill, Co., Cork, Ireland), and then the filtrate was injected into a GPC system by an autosampler. Duplicate tests were conducted for each sample and representative curves were reported.

5.3.9. Pasting profile

Pasting properties of the ground extruded samples suspended in distilled water were determined using a Micro Visco-Amylograph (C.W. Brabender® Instruments, Inc, South Hackensack, NJ). Finely ground extruded sample was added to distilled water to prepare 10% suspension (dry weight basis, w/w). The heating and cooling cycles were programmed by heating the suspension from 50 °C to 90 °C (heating rate of 10 °C/min) and held at 90 °C for 6 min and finally cooled to 50 °C (cooling rate of 10 °C/min). The viscosity of starch suspension was expressed in Brabender Unit (BU).

5.3.10. SP and IP contents

SP and IP contents in the samples were determined according to the method used by Bean et al. (1998) with slight modifications to the extraction. The sample (100 mg) was suspended in 50% propanol (1.0 mL), vortexed for 5 min and centrifuged at 10000 \times g for 5 min. This step was repeated again and the supernatants of both extractions were combined, and represent the SP extract. The remaining pellet was used to extract IPP by vortexing it with sodium dodecyl sulphate (SDS) buffer (1.0 mL) for 1 min, sonicated at 10 W for 20 sec and centrifuged at 10000 \times g for 10 min. SDS buffer consists of 0.5% and 0.05M sodium phosphate at pH 6.9. Pellet left after SDS buffer extraction was tested for RP. Another pellet after 50% propanol extractions was prepared and the total IPP was determined. RP and total IPP were determined by LECO TruSpec CN Carbon/Nitrogen combustion analyzer (St. Joseph, MI).

The extracted SP and IPP were filtered through 0.2 μ m filter (Fisher Scientific, Dublin, Ireland) and analyzed by a size exclusion high performance liquid chromatography (SEC-HPLC) (Agilent 1100 Series, Agilent Technologies, Palo Alto, CA) using Bio-Sep-SEC- 4000 column 300 x 7.80 mm (Phenomenex, Torrance, CA) that was set under the following parameters: column temperature – 30 °C, eluting solvent – acetonitrile and water containing 0.1% trifluoroacetic acid (1:1 ratio), detector – UV, detection wave – 210 nm, injection volume – 20

μL , flow rate – 0.5 mL/min, run time – 30 min. SE-HPLC analysis of SP extract provided SPP, gliadin and albumin/globulin data, whereas, HMW-GS and LMW-GS data were obtained through IPP extract.

5.3.11 Macro-structure properties

From each treatment, replicates of 30 samples were measured for bulk density (BD), piece density (PD), expansion ratio (ER) and specific length (SL). BD was measured by filling extrudates in a cylindrical steel container ($V_c = 1$ L) and recording the mass for calculation as follows:

$$BD = \frac{W_{sample}}{V_c} \text{ (g/L)}$$

where W_{sample} - mass of extrudates (g) and V_c - the volume of the steel cylinder.

PD of extrudates were calculated as shown below:

$$PD = \frac{W_{piece}}{V_{piece}} = \frac{4W_{piece}}{\pi d^2 l} \text{ (g/cm}^3\text{)}$$

where W_{piece} - mass of extrudate, V_{piece} - volume, d - diameter and l - length.

ER of extrudates were calculated as follows:

$$ER = \frac{d^2}{d_{die}^2}$$

where d - extrudate diameter and d_{die} - die diameter.

SL is calculated as follows:

$$SL = \frac{l_e}{m_e} \text{ (cm/g)}$$

where l_e - length of the extrudate and m_e - mass of the extrudate.

5.3.12. Micro-structure properties

Microstructural properties of two replicates from each treatment were analyzed using a desktop X-ray microtomography (XMT) imaging system (Model 1072, Skyscan, Aartselaar, Belgium) that was set based on Zhu et al. (2010) under the following settings: voltage - 40 kV, current – 248 μA , exposure time – 1.2 sec, rotation step - 0.9°, sample rotation – 180°, magnification – 16 \times , pixel size - 17.09 μm . Images were captured with a 12-bit, cooled CCD camera (1024 \times 1024 pixels). The total scanning time was 38 min.

The scanned images were reconstructed into 3D objects (NRecon v. 1.6.1, Skyscan, Belgium) and virtually sliced to 1000 cross-sections (CTAn 1.9.1, Skyscan, Belgium). A region of interest was set on the parameter of each cross-section for the interpolation of data. The grayscale images were converted into binary images (threshold = 15) and were analyzed in 3D to obtain void fraction, structure thickness and structure separation functions. The void fraction was calculated by subtracting the percent object volume from 100%. Structure thickness is addressed as cell wall thickness and structure separation is addressed as air cell size in this paper.

5.3.13. Statistical analysis

All data were reported as the mean \pm standard deviation (mean \pm SD). The results were analyzed by using analysis of variance (ANOVA) (SPSS version 13.0, SPSS Inc., Chicago, IL), and the Student's *t* test was used to examine the differences. Results with a corresponding probability value of $P < 0.05$ were considered to be statistically significant.

5.4. Results and discussion

5.4.1. Composition and properties of raw materials

The soft wheat flours had significantly higher total starch content than the hard wheat flours (Table 5-1). Regardless of the amylose/amylopectin ratio of wheat flours, hard normal (HN) wheat flour and hard waxy (HW) wheat flour contained significantly higher protein content than both soft normal (SN) wheat flour and soft waxy (SW) wheat flour (Table 5-1). However, HW had relatively higher protein content than HN and significantly higher lipid content, whereas protein of SN was relatively higher than that of SW. SN had relatively higher ash content than the other wheat flours. Both HW and SW had significantly higher amylopectin contents than HN and SN (Table 5-1). The latter was expected, due to existence of one, two or three granule-bound starch synthase (GBSS) null alleles in the wheat plants that reduces the amount of amylose composition in the wheat kernels (Graybosch, 1998).

Waxy wheat flours had significantly higher starch damage compared to normal wheat flours (Table 5-1). The result is expected as amylopectin has more reduced hydrogen bonding than amylose, causing amylopectin to have less resistance to mechanical damage (Bettge et al., 2000) such as in the milling process. HN and HW had significantly higher starch damage than soft wheat flours because hard wheat flours normally require higher moisture content during

tempering due to the nature of hard kernels which ensures better milling of the kernels to flours. However, higher moisture content leads to higher damage of starch molecules as water may increase the susceptibility of the crystalline structure of starch molecules towards mechanical damage (Bettge et al., 2000). The hard texture of hard wheat kernels could be due to the strong bonding between starch and protein which may have caused more starch damage.

5.4.2. Extrusion processing

The SME is closely related to the motor torque and the pressure at the die of the extruder, which greatly influences the degree of product transformation, textural characteristics, expansion and density of product (Iwe et al., 2001). Fig. 5-2 (a) shows the SME of normal and waxy wheat flours. At higher screw speed (360 rpm), SME was higher for all wheat flours except HW. The increased SME with increased screw speed phenomenon was also observed by Akdogan (1996) and Pansawat et al. (2008). The normal wheat flours had higher SME as compared to the waxy wheat flours. Normal barley flour was also observed with greater SME than waxy barley flour during extrusion of several barley flours (Baik et al., 2004). Higher amylopectin content in starch has been associated with lower viscosity during extrusion (Liu et al., 2006; Xie et al., 2009). During starch gelatinization, the branched chains of amylopectin are torn apart into short branched chains which form gel-balls comprising mostly chains from the same sub-main chain (Yu and Christie, 2005). The gel-balls have smaller size and chain lengths (4-6 glucose), resulting in much lower molecular entanglements than the linear-chained amylose (Yu and Christie, 2005). Thus, lower energy is required for these amylopectin gel-balls to move, especially with the existence of plasticizer (Yu and Christie, 2005). The latter phenomenon explains the significantly lower SME of waxy wheat flours which comprised of mainly amylopectin in the starch composition.

HN had higher SME than SN while SW showed higher SME than HW. It is common to observe higher SME for material containing higher protein content due to the poor flowability of protein. Viscosity of gluten will increase upon heating due to cross-linking of protein (Kokini et al., 1994). The significantly lower SME of HW could be explained by the relatively higher lipid composition (Table 5-1) which probably reduced the friction between the screw and barrel surfaces due to lubrication (Colonna and Mercier, 1983; Yacu, 1998). In turn, shear input to the

material and temperature will be reduced (Colonna and Mercier, 1983; Lin et al., 1997), hence lowering the SME value during extrusion process.

5.4.3. Mechanical properties

Breaking strength of normal wheat extrudates was significantly higher than the waxy wheat extrudates (Fig. 5-2 b). Extrudates with lower amylopectin content have been identified to have higher breaking strength (Mercier and Fellet, 1975; Wang, 1997) as a result of higher breakdown of amylopectin during extrusion (Harper and Tribelhorn, 1992). At the same level of moisture content, amylose molecules are more rigid and the linear structure is aligned strongly with the shear field, which is unable to efficiently breakdown and causes stronger cell walls (Kokini et al., 1992; Della Valle et al., 1996). Thus, normal wheat flours which contained significantly higher amylose (Table 5-1) had higher breaking strength than the waxy wheat flours. Protein content strongly influence the hardness of extrudate by producing rigid network that will result in increased resistance to shear and less expanded product (Chaiyakul et al., 2009). However, no significant differences were observed between hard and soft normal wheats, whereas HW had significantly lower breaking strength than SW at higher screw speed. Although HN had significantly lower ER than SN (Fig. 5-5 b), the insoluble protein in HN was higher especially at lower speed (Table 5-2), thus, resulting in similar breaking strength (Fig. 5-2 b) of extruded normal wheat flours. Increase in mechanical properties is an indicator of increased protein cross-linking after sufficient denaturing (Huang et al., 1999).

The breaking strength of all treatments was significantly lower at higher screw speed (Fig. 5-2 b). Higher SME caused excessive cross-linking which produced disrupted extrudates (Verbee and Van den Berg, 2010). Larger cells has been associated with greater expansion and weaker structure (Agbisit et al., 2007), thus implying lower breaking strength for more expanded product. However, the current result showed HW with the least ER had the lowest breaking strength. Other results in the current study suggested HW underwent dextrinization which may have caused weak cell walls, thus, lower strength was needed to break the cell walls. Further explanation for the results of HW will be discussed in depth later in the study.

5.4.4. WAI and WSI

Fig. 5-2 (c) and (d) show the water absorption index (WAI) and water solubility index (WSI) of the wheat extrudates. WAI determines the amount of water absorbed by intact starch

and functions as a gelatinization index (Anderson et al., 1969), whereas WSI measures the soluble fractions of starch and is an indicator of the amount of degraded molecular components (Kirby et al., 1988). Generally, WSI of extruded wheat flours at higher screw speed (360 rpm) was significantly higher. This was expected because at higher energy input, starch degradation would be increased. On the other hand, WAI of the extrudates was similar at both screw speeds. HW had significantly lower WAI, indicating lower levels of starch gelatinization. In addition, lower WAI had been linked to increased protein interactions (Huang et al., 1999). HW showed a prominent increase of RP after extrusion (Table 5-2), which indicated high protein cross-linking during extrusion. HW also had relatively higher lipid content which may act as a hydrophobic plasticizer. Water absorption rate was observed to decrease with higher amount of hydrophobic plasticizer (Pommet et al., 2005). WSI of HW showed relatively higher WSI values, hence a higher degree of starch degradation. High WSI has been associated with protein degradation rather than protein denaturation (Verbee and van den Berg, 2010) and high starch dextrinization (Colonna et al., 1989).

5.4.5. Starch transformations

Fig. 5-3 shows the gel permeation chromatograms of flour and extruded normal and soft waxy wheats at different screw speeds. After extrusion, extensive fragmentation characterized by the downshift of molecular weight profiles was observed in all four wheat types. Before extrusion, the HN and SN flours showed two peaks, i.e. amylose and amylopectin fractions with molecular weight between 106.3 - 107.5 and 107.3 - 109.1, respectively. The HW and SW showed one peak at a greater molecular size range of 107.5 to 109, indicating the existence of mostly amylopectin in the starch granules. After extrusion, starch greater than 108.5 was no longer detected in all wheat extrudates. The loss of this high molecular weight starch was accompanied by production of lower molecular weight fragments ranging from 103.8 to 108. The result is consistent with Colonna et al. (1984), Davidson et al. (1984) and Wen et al. (1990) who showed reduction of starch's molecular weight during extrusion cooking which is mainly due to amylopectin degradation. Higher screw speed (360 rpm) caused the peak of the curve to shift slightly to the left, indicating higher amounts of lower molecular weight starch fragments due to higher shearing input. While HN extrudates had slightly bigger molecular weights than SN extrudates, HW extrudates showed slightly smaller molecular weights than SW

extrudates where the curve of HW slightly shifted to the left. Although the differences in the molecular weights were apparently small between the HW and SW, it may suggest a higher fragmentation of HW after extrusion, as indicated by the low WAI and high WSI of HW (Fig. 5-2 c and d).

During heating and holding periods, all the viscosities of extrudate paste progressively decreased (Fig. 5-4), reflecting the instability of the paste against shear. Viscosities gradually increased during the cooling stage. All extrudates, except HW, at 360 rpm screw speed showed lower viscosities than those processed at 180 rpm which could be due to more degraded starch at higher screw speed. The HW and SW showed relatively lower viscosities with HW having prominently lower viscosities. The result could be due to more breakdown of amylopectin starch by harsh processing conditions (high temperature and shear) during extrusion (Wang, 1997). Regardless of screw speeds, HW had significantly lower viscosities than others which support the WAI and WSI results. The results suggest higher degradation of starch in HW.

5.4.6. Protein changes

The total protein, SP and IPP of wheat flour and extruded products at the screw speeds of 180 rpm and 360 rpm are tabulated in Table 5-2. Ideally, apart from the amylose content, the total protein of HN and HW should be similar. However, it was difficult to find samples that match similar protein and other compositional contents. The samples used were the best match obtained. Overall, the total protein content of samples before and after extrusion was similar, indicating there was no protein loss during extrusion. Before extrusion, SN and SW had relatively higher SPP, whereas the HN and HW had significantly higher RP. Despite the similarity of total SP and IPP of all wheat flours, SN had significantly lower HMW-GS and higher LMW-GS as compared to others. After extrusion, all the extruded samples had no SP or IPP larger than 70 kDa (soluble polymeric protein and HMW-GS). Gliadin (20-70 kDa) solubility loss could be due to the physical entrapment within the insoluble network of glutenin polymers (Redl et al., 2003). The SP content of all extruded samples decreased drastically and IPP increased significantly, with samples extruded at lower screw speed (180 rpm) having a higher amount of IPP. Loss of protein solubility is caused by the increase of density network entanglement. (Redl et al., 2003). As the size of gluten network increase, gluten polymer

becomes insoluble (Kasarda, 1999) due to an increase in covalent interactions between gluten protein (Morel et al., 2002).

From the protein perspective, the final structure of extrudate was expected to form from a complete restructuring of polymers in an oriented pattern. The first step involves the dissociation and unfolding of the protein macromolecules. The dissociated molecules will then form the final network by recombining and cross-linking through specific linkages (Redl et al., 1999). During extrusion, protein undergoes denaturation at the temperatures of 60 – 70 °C (Huber, 2001). For gluten protein in wheat, protein denaturation occurs at 55 – 75 °C where unfolding of protein molecules takes place (Schofield et al., 1983). Gluten polymerization will occur after the heating temperature reaches 60 °C (Redl et al., 1999). Added shear from extruder significantly decrease the activation energy for cross-linking, causing excessive cross-linking (Redl et al., 2003). It can be suggested that at lower screw speed, the retention time is longer, allowing more protein to denature and form insoluble protein.

During the cooking of bread dough, it has been postulated that a physical competition between starch gelatinization and protein coagulation in a continuous network occurs. If the latter prevails, starch gelatinization will be limited and there is an increase the starch dextrinization (Ding et al., 2006). During extrusion, the protein network is greater than the starch network that prevents puffing behavior (Suknark et al., 1991). Moreover, proteins compete with starch for water absorption resulting in reduction of smaller amount of water available for starch gelatinization (Suknark et al., 1991). Consequently, starch swelling is decreased due to the formation of three-dimensional protein network (Champenois et al., 1998) that reduces the extensibility of starch polymer to expand as it exits the die (Derby et al., 1975). Among the extruded products, HW at both screw speeds had significantly higher gliadin and RP, as well as lower LMW-GS. HWF with significantly higher protein content (Table 5-1), may have formed high molecular size protein networks during extrusion that restricted starch gelatinization and increases dextrinization.

5.4.7. Macro-structure of extrudate

The expansion property of an extrudate is subjected to the degree of starch gelatinization (Mercier and Feillet, 1975; Chinnaswamy and Bhattacharya, 1983; Cai and Diosady, 1993), starch breakdown (Gomez and Aguilera, 1984; Kirby et al., 1988; Cai and Diosady, 1993) and

amylose content (Chinnaswamy and Hanna, 1988). BD and PD of the extrudates were positively correlated and the ER showed inversed correlation with the densities of the extrudates (Fig. 5-5) which were in agreement with other research (Singh et al., 1996; Asare et al., 2004; Chaiyakul et al., 2009). Waxy wheat flours were expected to have higher expansion than normal wheat flours due to the nature of amylopectin molecules to expand than amylose. Other theory is soft wheat flour would expand more than hard wheat flours because of the lower protein content. However, the HN had significantly higher ER than the SN. The latter can be explained by relatively higher SME during extrusion of the HN (Fig. 5-2 a). Although SW had significantly lower SME than SN, SW had higher ER than SN. The result suggests that the amylopectin in SW may play a role in providing better expansion of extrudate. HW had the lowest ER, possibly due to the higher RP (Table 5-2) which comprised of proteins with molecule size bigger than 2000 kDa. The big protein molecules may hinder the expansion of HW, since protein may act as filler matrix in the extrudate (Riaz, 2000). The ER of all extrudates was shown to increase with higher screw speed because increased shear strains caused an increase in elastic melt stored energy, resulting in preference of radial expansion (Launay and Lisch, 1984).

5.4.8. Micro-structure

The microstructural parameters of extruded wheat samples, namely void fraction (VF), average cell size (CS) and average cell wall thickness (CWT) are presented in Table 5-3, while representative XMT image of the extruded samples is shown in Fig. 5-6. Regardless of the treatment or chemical composition, average CS of the extrudates was similar. Wheat flours extruded at 360 rpm had a lower average CWT, possibly due to increased nucleation producing more air cells and thinner cell walls. VF of waxy wheat extrudates was higher at higher screw speed. The CWT and CS distributions are provided for in-depth microstructural analysis of extrudates (Fig. 5-7). Although the average CWT varied among the samples (Table 5-3), the CWT distribution showed that the 0.1 mm thickness had the highest volume fraction for all extrudates, with samples extruded at higher screw speed having a higher fraction (25 - 35%) than at lower screw speed (17 - 25%). CS distribution displayed the extrudates processed at lower screw speed had a wider range of distribution (1 – 4 mm) as compared to extrudates at higher screw speed which had the highest peak of volumes (below 1 mm). This data was supported by the grayscale XMT image (Fig. 5-6) that showed smaller air CS in samples extruded at 360 rpm,

over that of extrudates processed at 180 rpm. However, extrusion at 360 rpm caused the CS of HN and SN to be concentrated in the range of 0.5 – 1.5 mm which were indicated by the narrow peaks, whereas the HW and SW had a wider array of air CS, reaching up to 6 mm of diameter. The latter trend suggested formation of a homogenous CS of the normal wheat extrudates and a heterogenous CS of the waxy wheat extrudates at higher screw speed.

Higher screw speed may cause nucleation to increase due to decreased pressure in the extruder (Han et al., 2002). Coalescence may also increase (Park et al., 1998) that leads to faster air cell growth and increased strain rate that favor rupture (Babin et al., 2007). HW and SW may have lower melt viscosity in the extruder as viscosity of melt was shown to decrease with increasing amylopectin (Bhattacharya and Hanna, 1987; Lai and Kokini, 1992; Babin et al., 2007; Xie et al., 2009). Short-chain branches in the amylopectin molecular structure lower the viscosity of the melt and reduce the possibility of entanglement (Harper, 1986). Hence, waxy wheat dough in the extruder might have a faster nucleation and coalescence than normal wheat, which caused heterogeneous CS in the extrudate. For normal wheat extrudates that contained higher amylose content, increased viscosity is expected, as the linear structure of amylose enhances entanglements (Harper, 1986; Della Valle et al., 1996), which also aligned strongly with the shear field (Harper and Tribelhorn, 1992). Consequently, the energy from the pressure gradient and the vaporization of moisture at the end of die are incapable of breaking most of the strongly aligned amylose linear chains (Kokini et al., 1992; Della Valle et al., 1996) and prevented the coalescence of cells. Hence, formation of CS was homogenous.

5.4.9 Effect of starch and protein changes to structure and texture of extrudates

During high shear processing of extrusion, the degradation of high molecular weight starch molecules starts with modification of supramolecular structure, followed by molecular and bond deformations (Porter and Casale, 1985). This leads to breakage of chemical bonds that change the physical and chemical properties of the polymer (Porter and Casale, 1985). With the different compositions of starch and protein, the wheat extrudates displayed different structural and textural characteristics. Although higher amylopectin has been associated with higher radial expansion (Wang, 1997), HN showed higher ER than HW. SW had better radial expansion than SN although SME of SW was much lower than that of SN. In the case of HW, higher lipid caused lower SME that may have prevented better expansion of HW. The higher mechanical

energy input resulted in higher starch gelatinization, hence, higher radial expansion. At higher screw speed, the amylose/amylopectin ratio of wheat flours also affects the micro-structure of extrudates by producing different distributions of air cell sizes in the normal and waxy wheat extrudates.

The formation of large protein network in HW, as indicated by high RP, caused limited starch gelatinization, consequently increasing starch dextrinization (Ding et al., 2006) and causing collapse of the cellular matrix under high vapor pressure at the end of die (Chinnaswamy and Hanna, 1988), and hence reducing the radial expansion. Excess dextrinization of HW was proven by the low WAI and high WSI values (Fig. 5-2), smaller molecule size distribution (Fig. 5-3), and low viscosity of extrudate (Fig. 5-4). The relatively low breaking strength (Fig. 5-2 b) of HW was also an indication of weak cellular matrix of HW due to excessive dextrinization. The amylopectin molecules in SW may have survived the excess dextrinization that occurred in the HW, due to significantly lower protein content, thus allowing significantly higher radial expansion than that of HW.

5.5. Conclusions

Starch and protein transformations after extrusion in the normal and waxy wheat flours played a large role in affecting the macro-structure and micro-structure of the extrudates. The SME had a bigger impact on the HN which leads to a higher radial expansion than HS. Although SME of SW was significantly lower than SN, SW had better radial expansion than SN which suggests the function of amylopectin was dominant in helping better expansion of extrudate. Higher screw speed contributed to increased ER and also formation of homogenous cell size for the normal wheat extrudates, and heterogenous cell size distribution for the waxy wheat extrudates. Under limited moisture content (18%), HW at both screw speeds formed greater percentages of high molecular weight insoluble proteins that may have caused limited starch gelatinization and increased starch dextrinization, leading to reduced radial expansion and breaking strength. Future work includes studying the effect of waxy wheat flour variety on extruded products.

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Table 5-1. Protein, lipid, ash, amylose, starch damage and total starch contents of normal and waxy wheat flours before extrusion.

	Normal wheat flour		Waxy wheat flour	
	Hard	Soft	Hard	Soft
Protein (%)	16.9 ± 0.0 b	13.0 ± 0.0 c	17.7 ± 0.0 a	12.8 ± 0.0 d
Lipid (%)	1.1 ± 0.0 b	1.1 ± 0.0 b	1.3 ± 0.0 a	1.1 ± 0.0 b
Ash (%)	0.4 ± 0.0 b	0.5 ± 0.0 a	0.4 ± 0.0 b	0.4 ± 0.0 b
Amylose (%)	26.7 ± 0.3 a	25.1 ± 0.6 a	1.4 ± 0.5 b	1.1 ± 0.3 b
Starch damage (%)	6.3 ± 0.1 b	4.3 ± 0.4 d	9.2 ± 0.3 a	4.9 ± 0.3 c
Total starch (%)	79.0 ± 0.0 b	82.2 ± 0.1 a	79.8 ± 0.5 b	83.1 ± 0.3 a

All values are reported on dry basis of flour. Data are means ± standard deviation. Means within columns not sharing a common letter are significantly different at $p \leq 0.05$.

Table 5-2. Total, extractable and insoluble polymeric proteins of normal and waxy wheat flours and extrudates processed at screw speeds of 180 rpm and 360 rpm.

	Hard normal wheat			Soft normal wheat			Hard waxy wheat			Soft waxy wheat		
	Flour	180 rpm	360 rpm	Flour	180 rpm	360 rpm	Flour	180 rpm	360 rpm	Flour	180 rpm	360 rpm
Protein content (%)	16.9 ± 0.0b	16.5 ± 0.3b	16.1 ± 0.1b	13.0 ± 0.1c	13.4 ± 0.0c	13.4 ± 0.1c	17.7 ± 0.2a	17.3 ± 0.2a	17.1 ± 0.1a	12.8 ± 0.3d	12.5 ± 0.0d	12.8 ± 0.1d
<u>Soluble protein*</u>	52.8 ± 0.3 c	6.4 ± 0.1 g	12.3 ± 0.2ef	55.9 ± 0.1 c	10.3 ± 0.2fg	13.3 ± 0.3ef	54.9 ± 0.2 c	14.4 ± 0.1 e	26.0 ± 0.1 d	59.0 ± 0.3 c	6.5 ± 0.1 g	12.1 ± 0.5 f
Soluble polymeric protein	12.7 ± 0.7 bc	0.0 ± 0.0 d	0.0 ± 0.0 d	14.3 ± 0.6 ab	0.0 ± 0.0 d	0.0 ± 0.0 d	11.9 ± 1.1 c	0.0 ± 0.0 d	0.0 ± 0.0 d	15.9 ± 0.4 a	0.0 ± 0.0 d	0.0 ± 0.0 d
Gliadin	36.9 ± 0.4 a	4.0 ± 0.2 ef	8.1 ± 0.2 cd	36.9 ± 0.6 a	5.4 ± 0.1 e	7.0 ± 1.1 d	36.6 ± 0.7 a	9.0 ± 0.7 c	16.6 ± 0.5 b	38.0 ± 1.5 a	2.6 ± 0.7 f	5.5 ± 0.4 e
Albumin/Globulin	3.9 ± 0.2 de	3.1 ± 0.8 e	4.5 ± 0.7 cd	4.2 ± 0.6 de	4.7 ± 0.2 bc	4.8 ± 1.0 bc	4.7 ± 0.4 bc	4.2 ± 0.8 de	8.2 ± 1.0 a	4.7 ± 0.5 bc	3.1 ± 0.4 e	5.9 ± 0.6 b
<u>Insoluble polymeric protein*</u>	47.2 ± 0.3 d	93.6 ± 0.4 a	87.8 ± 0.1 b	44.1 ± 0.1 de	89.7 ± 0.2 b	86.7 ± 0.2 b	45.1 ± 0.1 d	85.7 ± 0.4 b	74.0 ± 0.0 c	41.0 ± 0.1 e	93.5 ± 0.2 a	87.9 ± 0.2 b
HMW-GS**	31.8 ± 0.9 a	0.0 ± 0.0 e	0.0 ± 0.0 e	4.8 ± 0.5 d	0.0 ± 0.0 e	0.2 ± 0.0 e	29.2 ± 0.4 c	0.0 ± 0.0 e	0.0 ± 0.0 e	30.1 ± 0.4 b	0.0 ± 0.0 e	0.0 ± 0.0 e
LMW-GS***	11.3 ± 0.2 j	82.7 ± 0.4 b	75.9 ± 1.0 f	37.6 ± 0.8 i	80.7 ± 0.1 c	77.2 ± 0.3 e	12.1 ± 0.0 j	63.2 ± 0.4 g	57.2 ± 0.3 h	10.2 ± 0.1 k	85.5 ± 0.5 a	79.3 ± 0.5 d
Residual protein	4.8 ± 0.0 g	11.2 ± 0.4 c	11.0 ± 0.0 c	2.6 ± 0.2 h	9.0 ± 0.1 d	9.2 ± 0.1 d	4.8 ± 0.2 g	22.7 ± 0.0 a	16.6 ± 0.2 b	2.2 ± 0.1 h	7.7 ± 0.1 f	8.3 ± 0.4 e

Data are means ± standard deviation. Means within rows not sharing a common letter are significantly different at $p \leq 0.05$.

* Soluble polymeric + Insoluble polymeric protein = Total protein (100%)

** High molecular weight glutenin subunits

*** Low molecular weight glutenin subunits

Soluble polymeric protein (>70 kDa); gliadin (20-70 kDa); albumin/globulin (< 20 kDa); HMW-GS (70 kDa); LMW-GS (<70 kDa); RP (> 2000 kDa)

Table 5-3. Microstructural properties of normal and waxy wheat flour extrudates, extruded at 180 rpm and 360 rpm.

	Hard normal wheat		Soft normal wheat		Hard waxy wheat		Soft waxy wheat	
	180 rpm	360 rpm	180 rpm	360 rpm	180 rpm	360 rpm	180 rpm	360 rpm
Void fraction (%)	83.8 ± 1.2 bc	81.9 ± 0.3 cd	80.2 ± 1.5 de	76.5 ± 1.0 f	79.0 ± 0.5 ef	82.0 ± 0.7 cd	81.9 ± 2.0 cd	85.5 ± 1.2 a
Average cell size (mm)	0.2 ± 0.0 a	0.2 ± 0.0 ab	0.2 ± 0.0 a	0.2 ± 0.0 ab	0.2 ± 0.0 a	0.2 ± 0.0 ab	0.2 ± 0.0 a	0.2 ± 0.0 b
Average cell wall thickness (mm)	1.2 ± 0.2 c	0.8 ± 0.0 d	1.3 ± 0.0 bc	0.8 ± 0.1 d	1.4 ± 0.1 bc	1.3 ± 0.0 bc	1.5 ± 0.2 b	1.4 ± 0.1 b

Data are means ± standard deviation. Means within rows not sharing a common letter are significantly different at $p \leq 0.05$

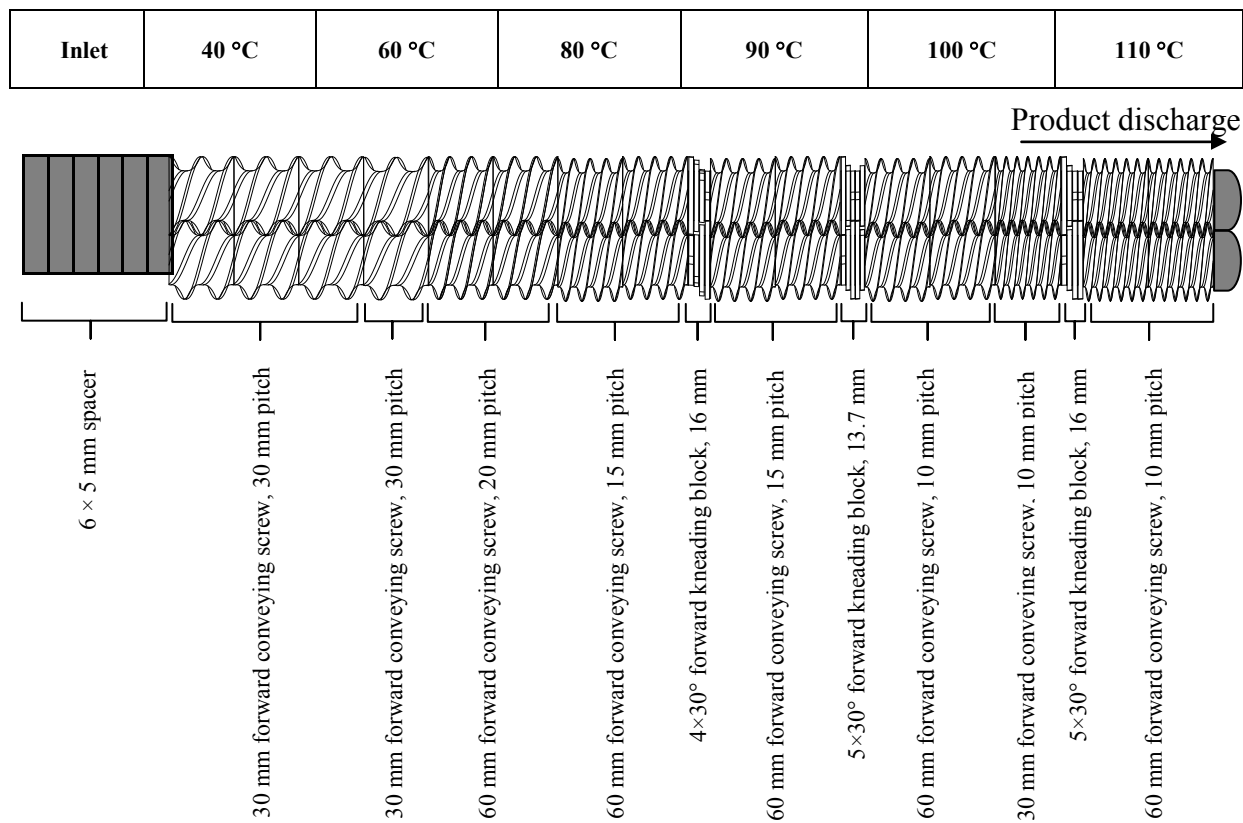


Fig. 5-1. Screw configuration and temperature profile of M-18 twin screw extruder.

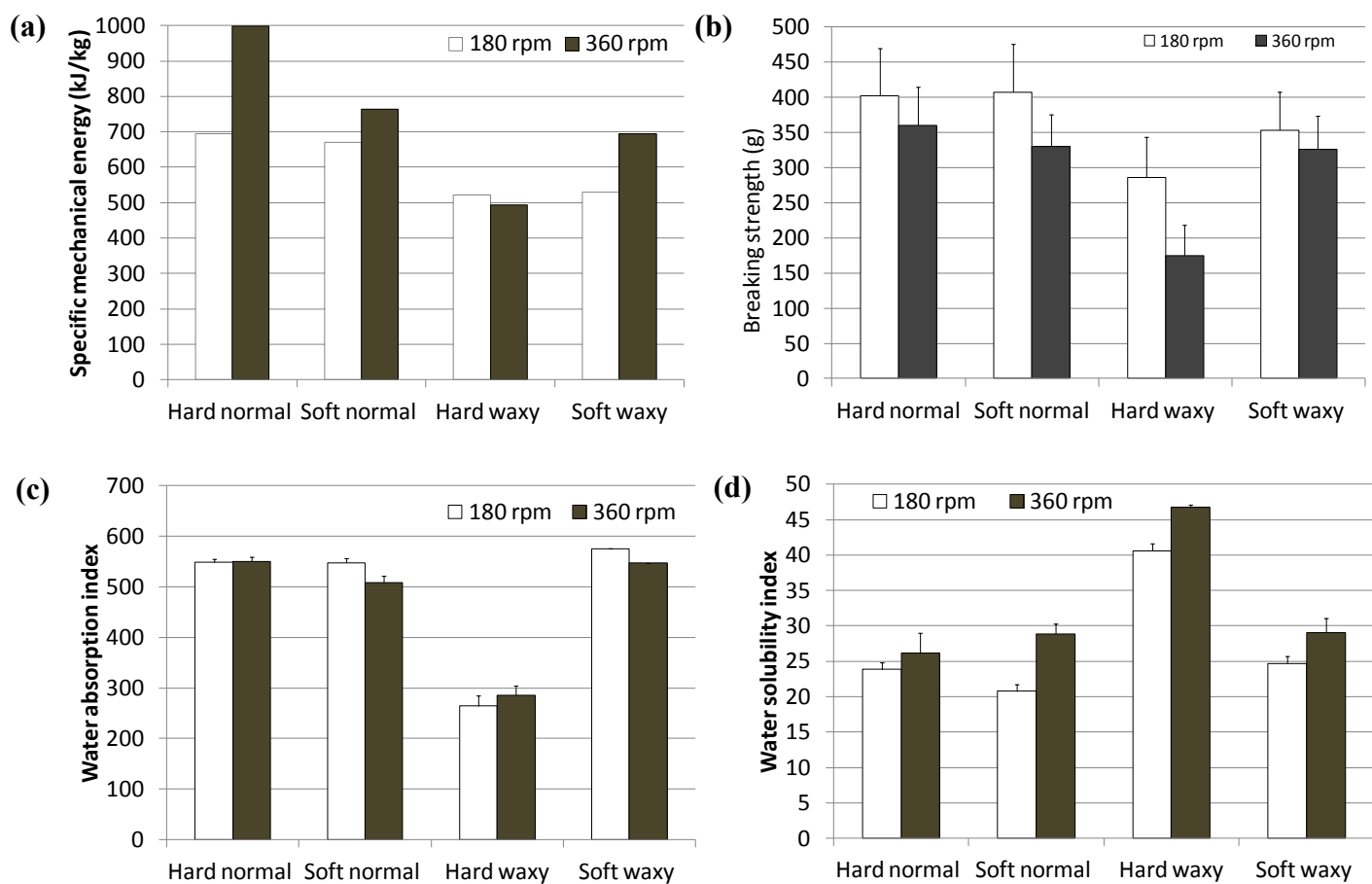


Fig. 5-2. (a) Specific mechanical energy, (b) breaking strength, (c) water absorption index and (d) water solubility index of extruded normal and waxy wheat flours at the screw speeds of 180 rpm and 360 rpm.

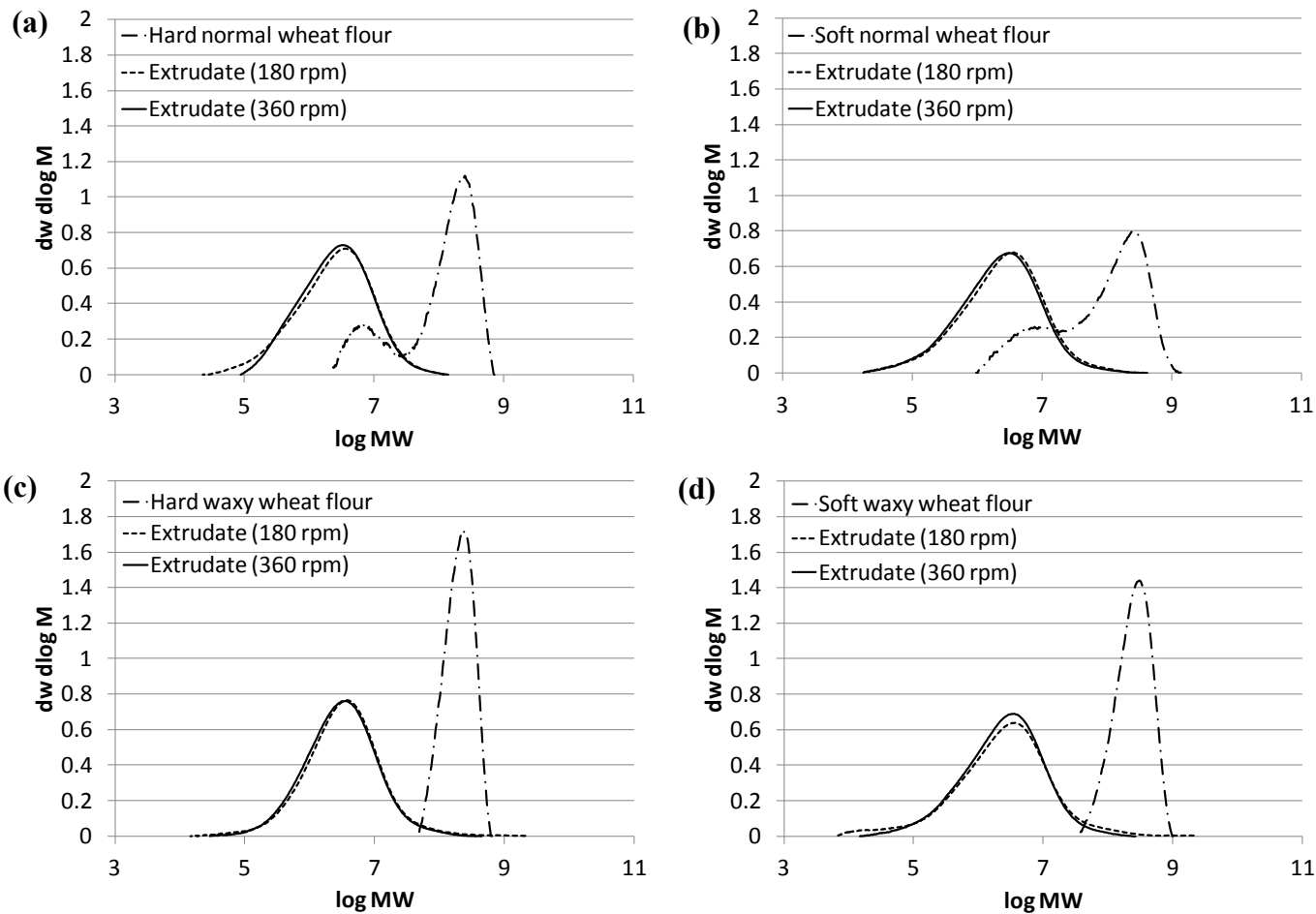


Fig. 5-3. Gel permeation chromatograms of extruded normal and waxy wheat flours at the screw speeds of 180 rpm and 360 rpm.

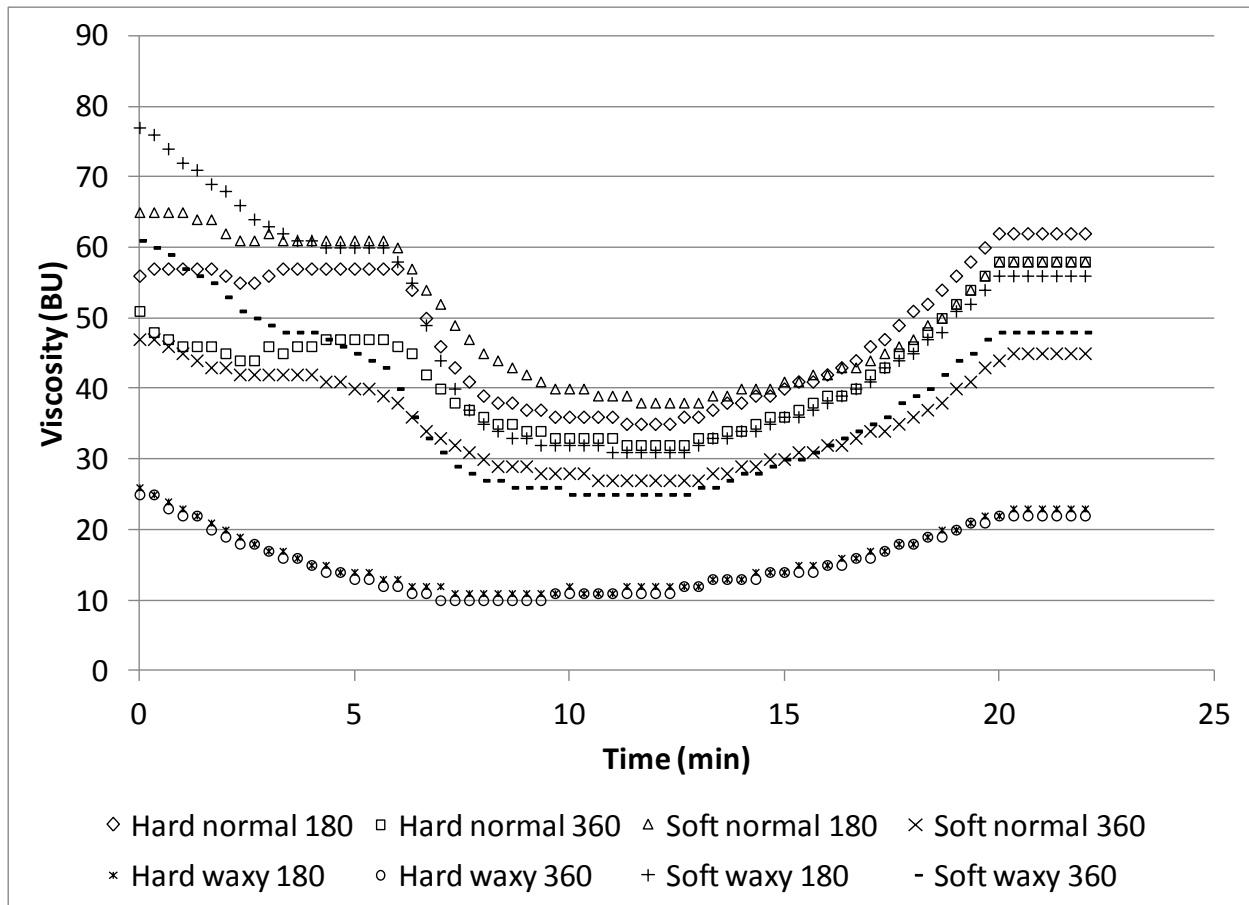


Fig. 5-4. Pasting properties of extruded normal and waxy wheat flours at the screw speeds of 180 rpm and 360 rpm.

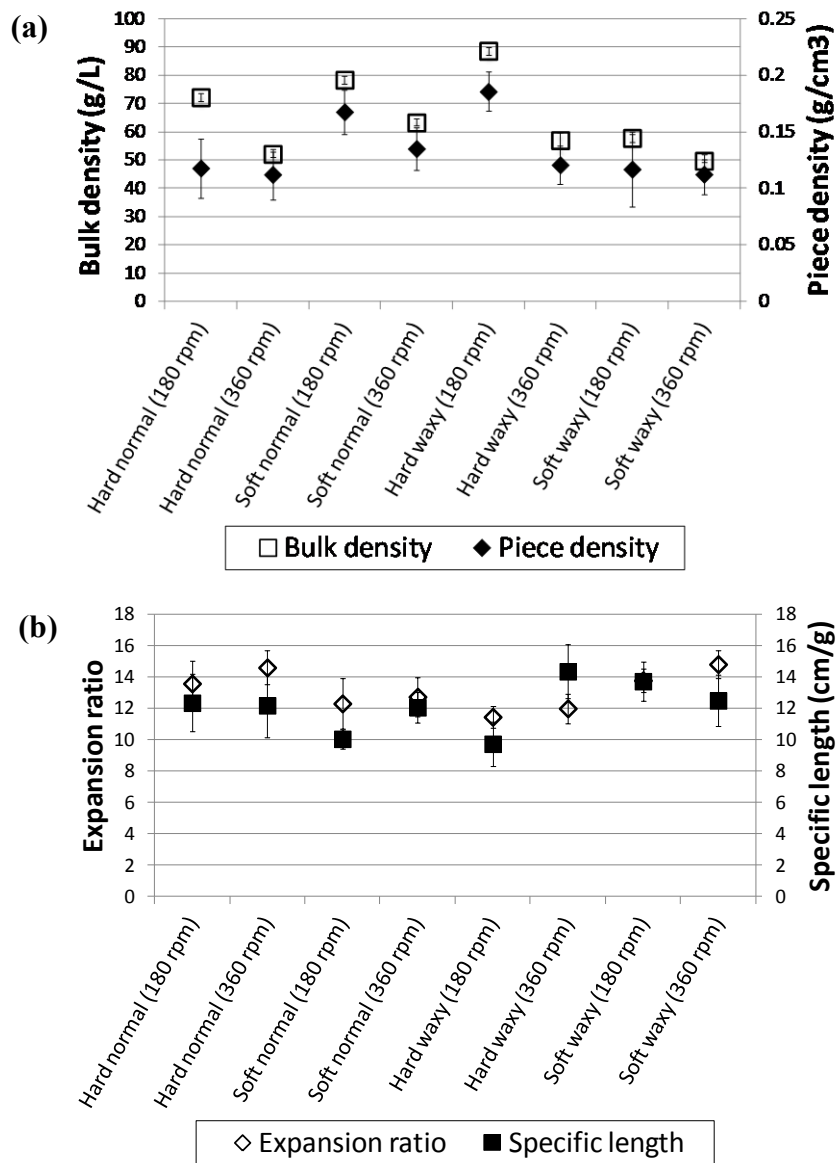
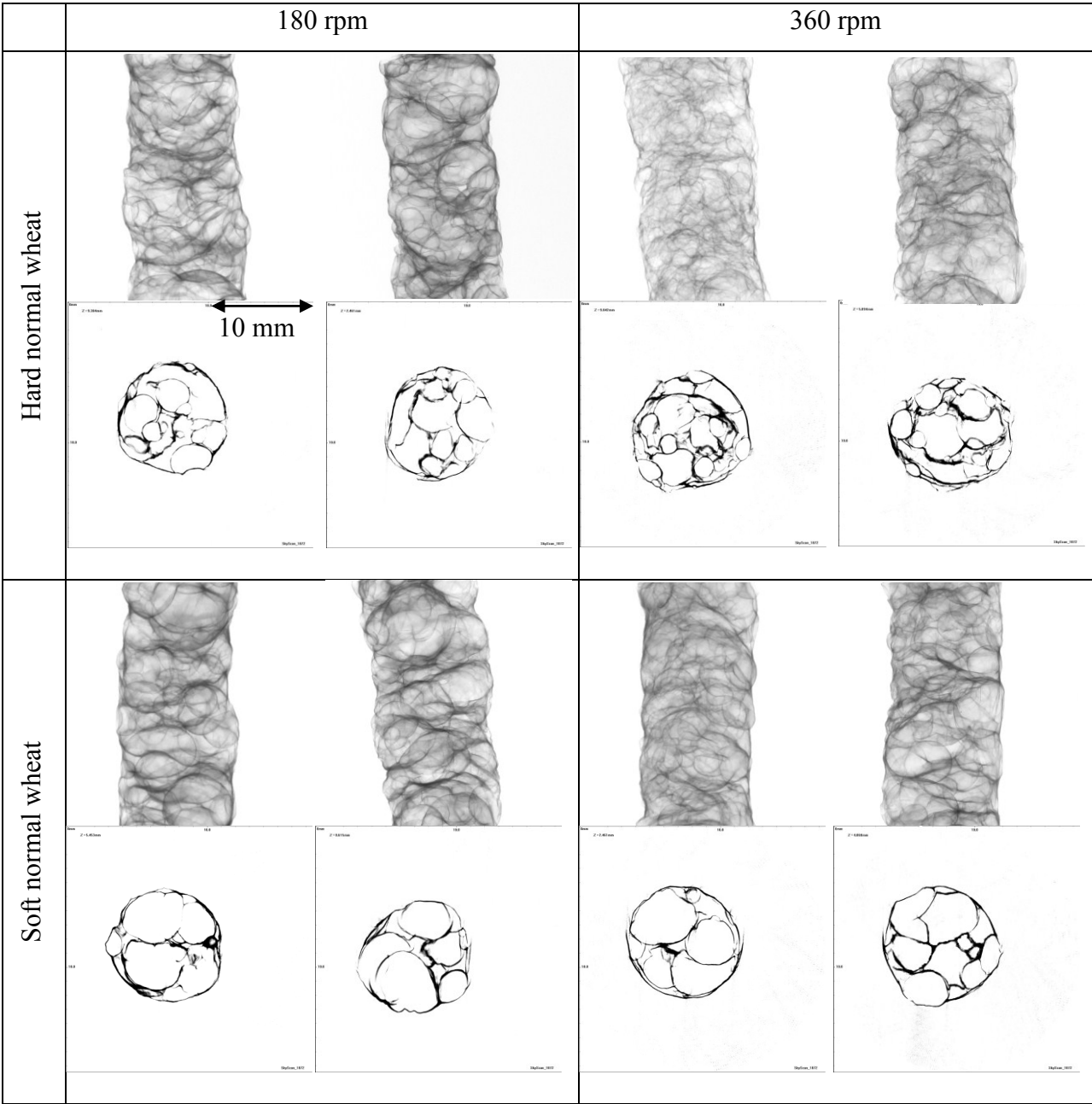


Fig. 5-5. Physical properties of extruded normal and waxy wheat flours at the screw speeds of 180 rpm and 360 rpm- (a) bulk density and piece density, (b) expansion ratio and specific length.



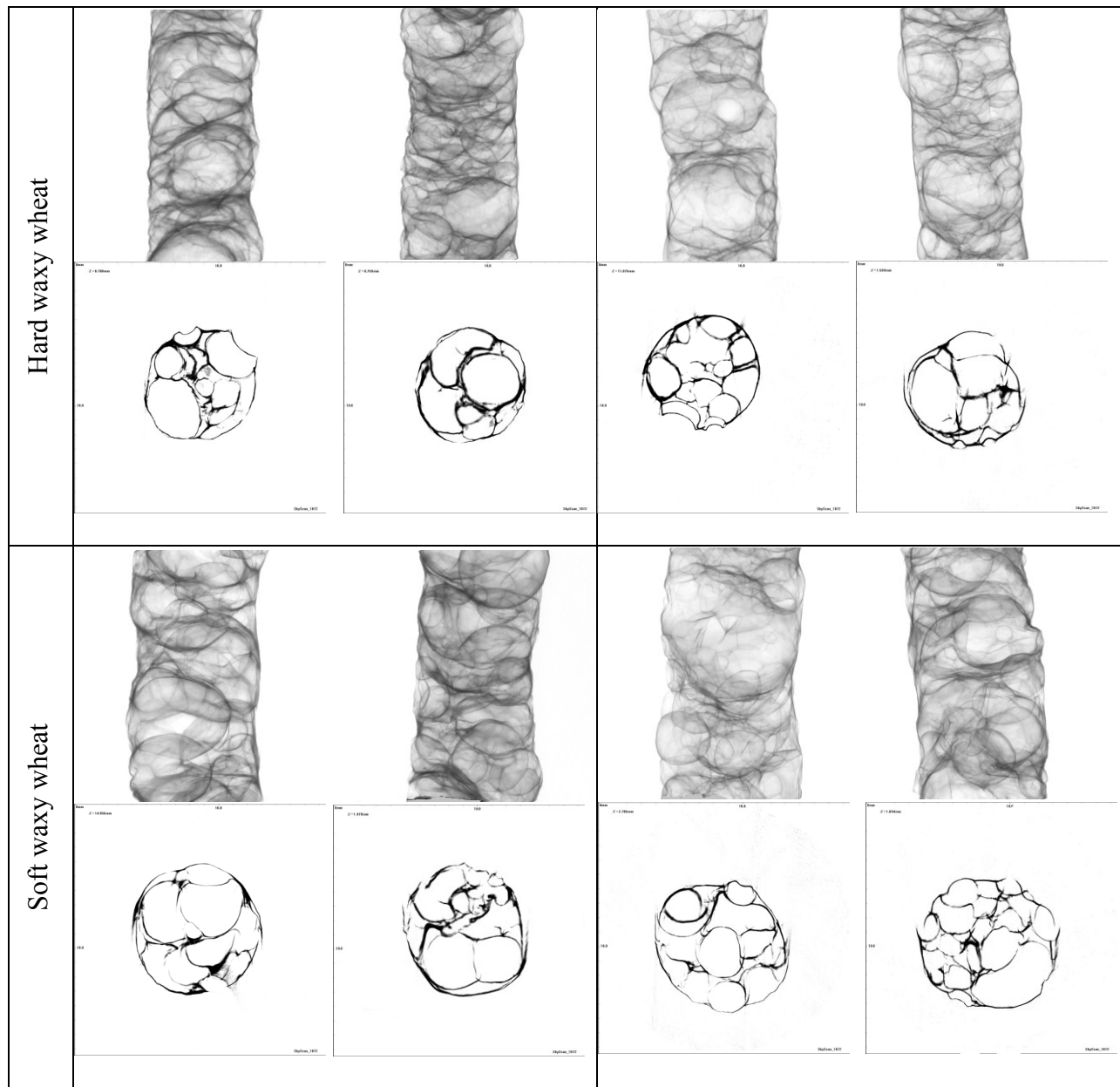


Fig. 5-6. Longitudinal (top) and radial (bottom) representative XMT images of extruded normal and waxy wheat flours at the screw speeds of 180 rpm and 360 rpm.

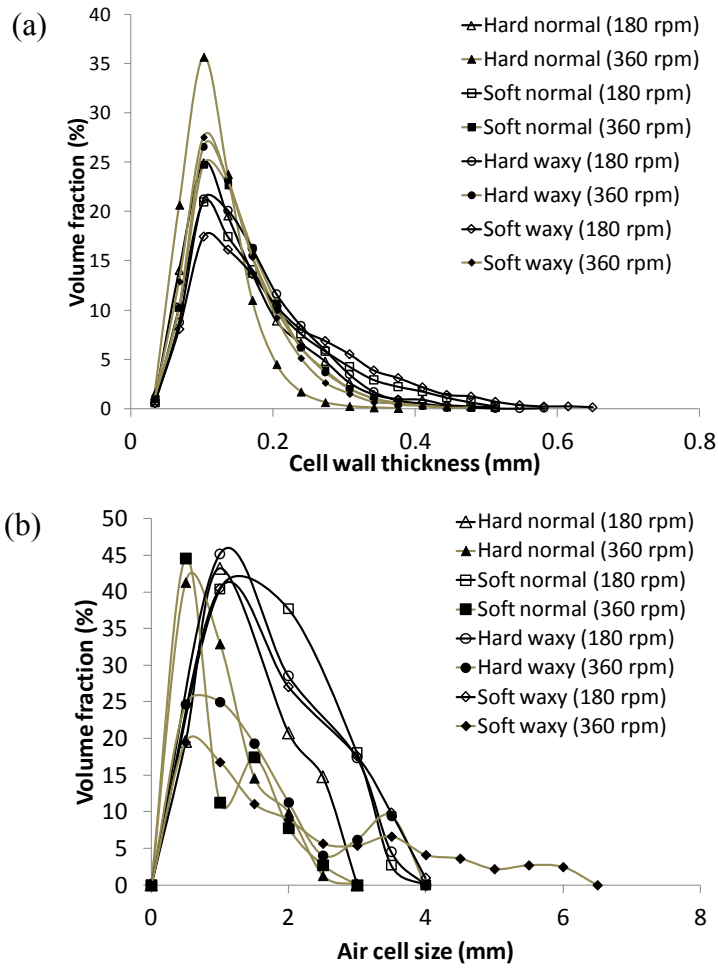


Fig. 5-7. (a) Cell wall thickness distribution and (b) air cell size distribution of extruded normal and waxy wheat flours at the screw speeds of 180 rpm and 360 rpm.

Chapter 6 - Macro- and micro-structural properties of extruded soft waxy wheat-oat flours

6.1 Abstract

The objective of this research was to study the extrusion of oat flour (OF) and soft waxy wheat (SWW) flour formulations at differing moisture contents. OF, SWW and combination of the OF and SWW (ratios of 60:40 and 50:50) were hydrated to 18, 20 and 22% moisture contents and subjected to twin-screw extrusion at the screw speeds of 400 rpm. Higher moisture content improved the expansion ratio of OF by increasing the cell wall thickness. Inclusion of SWW in the OF formulations increased the expansion ratio and reduced the breaking strength and bulk density of extrudates. At 18% moisture content, OF-SWW formulations at both ratios had the highest expansion and void fraction than OF. Production of nutritious expanded snack without compromising the extrudate's nutritional properties is possible by incorporating SWW as a functional ingredient to achieve the desired properties.

6.2. Introduction

In production of expanded snack products, the desired expansion is achievable by manipulating the amylose/amylopectin ratio in starch (Chinnaswamy and Hanna, 1988). Different ratios of amylose/amylopectin in starch produce a different outcome in terms of physical and organoleptic properties of extrudates (Chinnaswamy and Hanna, 1988). Extensive work has shown the significant effects of amylose and amylopectin on starch expansion (Mercier and Feillet, 1975; Bhattacharya and Hanna, 1987; Chinnaswamy and Hanna, 1998; Baik et al., 2004; Chanvrier et al., 2007). Waxy (~100% amylopectin content) and high amylose (50% - 70% amylose content) starches are commonly used to manipulate the amylose/amylopectin content in directly expanded extrudates (Chinnaswamy and Hanna, 1998).

Generally, extrudates with higher amylopectin content displays light characteristic with homogeneous texture and sticky external surface (Mercier and Fellet, 1975). High amylopectin content also increased expansion of extrudates that leads to puffy and crispy products with lower breaking strength (Mercier and Fellet, 1975; Wang, 1997). Expansion ratio of starch extrudates was observed to increase with increasing amylopectin with no regards to amylose content or modification of starch (Chinnaswamy, 1993). Incorporation of high amylopectin flour or starch

into a dual composition of starch-based materials have an outcome snacks with improved texture, increased crispiness, less waxy feel and high degree of mouth melt (Villagran et al., 2002). Chatel et al (2010) suggested usage of 5-15% of waxy starch from corn, rice or barley in formulation of high fiber cereal specifically whole corn, oat flour or brans for better expansion.

The drawback of using high amylopectin starch is the breakdown of amylopectin molecules by harsh processing conditions (high temperature and shear) during extrusion (Wang, 1997). Incorporation of waxy starch or flour as expanding aid may help better expansion (Graybosch, 1998) of less expanded product like oat flour. Thus, the objective of the study was to evaluate the potential of soft waxy wheat flour for production of nutritious expanded snack and to study the effect of moisture content on the extruded product.

6.3. Materials and methods

6.3.1. Materials

Soft waxy wheat kernels were obtained from Kellogg's Company (Battle Creek, MI). The waxy wheat kernels were milled to flour using Brabender Quadrumat Senior (Western Germany). The moisture content of wheat kernels were determined using Perten SKCS 4100 (Springfield, IL) and the amount of water needed to add was calculated to achieve tempering moisture content of 14%. The break and reduction flours were mixed together for extrusion purposes. Oat flour was purchased from Lortcher Agri Service (Bern, KS).

6.3.2 Proximate analysis

Protein content of raw materials was determined via LECO TruSpec CN Carbon/Nitrogen combustion analyzer (St. Joseph, MI), while lipid and ash contents were measured according to AOAC Method 920.39 and AOAC Method 942.05, respectively, by the Department of Animal Sciences, Kansas State University. Total starch was determined by using the Megazyme International Ltd. (Wicklow, Ireland) kit and β -glucan was measured by procedure from AACC Method 32-23 (AACC, 2000).

6.3.3. Experimental design and extrusion processing

A 4×3 factorial experimental design of four different types of blends, consisting of oat flour, soft waxy wheat flour and blends of soft waxy wheat-oat flours at the ratios of 40:60 and

50:50, where moisture content of blends was adjusted to 18%, 20% and 22%, were used. The moisture content of the blends was adjusted accordingly by spraying distilled water and mixed for 1 min in a Classic Kitchen Aid mixer (St. Joseph, Michigan). The blends were kept in a fridge overnight for moisture equilibrium prior to extrusion with a Micro-18 twin screw extruder (American Leistritz Extruder Corp., Somerville, NJ). The extruder configuration parameters and the schematic of the screw configuration for the Micro-18 are in Fig.6-1. The die used was a 2.5 mm circular die and screw speed was set at 400 rpm. Tests of specific mechanical energy (SME) were executed in duplicate and mechanical energy input per unit mass of extrudate was calculated as follows:

$$SME = \frac{(\tau - \tau_0) \times P_{rated} \times \frac{N}{N_{rated}}}{\dot{m}} \text{ (kJ/kg)}$$

where τ - % torque, τ_0 - no-load torque (4%), P_{rated} - rated power for the extruder (22.37 kJ/s), N - measured extruder screw speed in rpm, N_{rated} - rated extruder screw speed (336 rpm) and \dot{m} - mass flow rate in kg/s.

The extrudates were dried in an oven at 110 °C for 10 min, cooled for 5 min at room temperature and stored in plastic containers until analysis.

6.3.4. Mechanical property

Force–deformation data for each treatment was obtained with a Model TA-XT2i Texture Analyzer (Stable Micro Systems, Surrey, UK) equipped with a 25-kg load cell and a 38-mm-diameter test probe. Samples were compressed perpendicularly to 70% of their original height at a test speed of 5 mm/s. Thirty sample measurements were taken for each treatment. Average crushing force F_{cr} or breaking strength was calculated by using n , s and x values (Bouvier et al., 1997).

$$F_{cr} = \frac{s}{x \times n} \text{ (g)}$$

where n - number of peaks, s - integral of the curve (or area below the curve from 0% to 70% strain) and x - distance of breakage,.

6.3.5. Water absorption (WAI) and water solubility (WSI) indices

The WAI and WSI were determined by the method of Gujral and Singh (2002) on duplicates samples. Extruded samples were ground in a Waring blender (Torrington, CT) and sifted through a 250 screen sieve prior to testing. The ground sample (2.5 g) was immersed in 25 mL distilled water at room temperature, continuously stirred for 30 min, and centrifuged at 3000 ×g for 10 min. The supernatant liquid was poured into a pre-weighed evaporating dish. The remaining gel was weighed, and WAI was calculated as

$$WAI = \frac{W_{\text{sediment}}}{W_{\text{dry solid}}}$$

where W_{sediment} - weight of gel and $W_{\text{dry solid}}$ - weight of dry sample.

The WSI was determined from the amount of dry solids recovered by evaporating the supernatant at 100 °C for 12 h and it was calculated and calculated as follows:

$$WSI = \frac{W_{\text{dissolved solid in supernatant}}}{W}$$

where $W_{\text{dissolved solids in supernatant}}$ - weight of dry solids from the supernatant and $W_{\text{dry solid}}$ - weight of dry sample.

6.3.6. Macro-structure properties

From each treatment, replicates of 30 samples were measured for bulk density (BD), piece density (PD), expansion ratio (ER) and specific length. BD was measured by filling extrudates in a cylindrical steel container ($V_c = 1$ L) and recording the mass for calculation as follows:

$$BD = \frac{W_{\text{sample}}}{V_c} \text{ (g/L)}$$

where W_{sample} - mass of extrudates (g) and V_c - the volume of the steel cylinder.

PD of extrudates were calculated as shown below:

$$PD = \frac{W_{\text{piece}}}{V_{\text{piece}}} = \frac{4W_{\text{piece}}}{\pi d^2 l} \text{ (g/cm}^3\text{)}$$

where W_{piece} - mass of extrudate, V_{piece} - volume, d - diameter and l - length.

ER of extrudates were calculated as follows:

$$ER = \frac{d^2}{d_{die}^2}$$

where d - extrudate diameter and d_{die} - die diameter.

SL is calculated as follows:

$$SL = \frac{l_e}{m_e} \text{ (cm/g)}$$

where l_e - length of the extrudate and m_e - mass of the extrudate.

6.3.7. Micro-structure properties

Microstructural properties of two replicates from each treatment were analyzed using a desktop X-ray microtomography (XMT) imaging system (Model 1072, Skyscan, Aartselaar, Belgium) that was set under the following settings: voltage - 40 kV, current – 248 μ A, exposure time – 1.2 sec, rotation step - 0.9°, sample rotation – 180°, magnification – 16 \times , pixel size - 17.09 μ m. Images were captured with a 12-bit, cooled CCD camera (1024 \times 1024 pixels). The total scanning time was 38 min.

The scanned images were reconstructed into 3D objects (NRecon v. 1.6.1, Skyscan, Belgium) and virtually sliced to 1000 cross-sections (CTAn 1.9.1, Skyscan, Belgium). A region of interest was set on the parameter of each cross-section for interpolation of data. The grayscale images were converted into binary images (threshold – 15) and were analyzed in 3D to obtain void fraction, structure thickness and structure separation functions. The void fraction was calculated by subtracting the percent object volume from 100%. Structure thickness is addressed as cell wall thickness and structure separation is addressed as air cell size in this paper.

6.3.8. Statistical analysis

All data were reported as the mean \pm standard deviation (mean \pm SD). The results were analyzed by using analysis of variance (ANOVA) (SPSS version 13.0, SPSS Inc., Chicago, IL), and the Student's t test was used to examine the differences. Results with a corresponding probability value of $P < 0.05$ were considered to be statistically significant.

6.4. Results and discussion

6.4.1. Composition and properties of raw materials

Soft waxy wheat flour (SWW) contained significantly lower protein, ash, lipid, β -glucan and dietary fiber contents than oat flour (OF), but had relatively higher total starch content (Table 6-1). Generally, waxy wheat contains ~99% amylopectin and soft wheat contain relatively lower amount of protein than regular wheat. Wheat plants with one, two or three granule-bound starch synthase null alleles reduce the amount of amylose composition in the wheat kernels (Graybosch, 1998). OF had a better nutritional property than SWW with the higher amount of protein, dietary fiber and β -glucan. β -glucan is a soluble fiber that has some beneficial physiological properties like reducing postprandial insulin concentrations, inhibits cholesterol biosynthesis and delaying gastric emptying (Ink and Matthews, 1997). In addition, oat has significantly higher amount of lipid than other cereal which is high in unsaturated fats, including linoleic acid (Young, 1986). The SWW and OF blends will be addressed throughout the discussion as 40% SWW for the 40:60 ratio and 50% SWW for the 50:50 ratio.

6.4.2. Extrusion processing

Fig. 6-2 (a) shows the SME of OF, SWW, 40% SWW and 50% SWW with different moisture contents. SME decreased with increasing moisture content for OF, 40% SWW and 50% SWW. However, SME for SWW at 20% moisture content was significantly higher than at 18% and 22% moisture contents. At low moisture content (18%), SEM of SWW and OF was similar, but as the moisture content increased, SEM of OF reduced significantly. This suggests, at higher moisture content, the viscosity of OF melt in the extruder was lower, enabling the high amount of lipid to lubricate screw and the surface of extruder, hence lowering the SME. Oil lubrication may have caused inefficient pumping that lower the pressure at the die (Yacu et al., 1998), consequently reducing the radial expansion. Although 40% SWW at 18% moisture content showed a slightly higher SME than that of 50% SWW, SME for both treatments were insignificant at 20 and 22% moisture contents. Having higher amount of oat in the formulation, 40% SWW showed similar behavior as OF that needed more power input at lower moisture content.

6.4.3. Mechanical property

Mechanical property of extrudates is closely related to the texture of the extruded products (Maskan and Altan, 2012). The textural property of extrudates is measured by acquiring the force needed to break the sample (Sing et al., 1994). Hardness of extrudates can be determined from the maximum peak force obtained from texture analyzer (Stojceska et al., 2008). The breaking force of extruded products is showed in Fig. 6-2 (b). SWW had significantly lower breaking force than the other treatments that could be due to significantly lower protein and dietary fiber contents (Table 6-1). Generally, high protein content in material significantly increased extrudate density and breaking force (Onwulata et al., 2001; Allen et al., 2007). Protein content strongly influence the hardness of extrudate by producing rigid network that will result in increased resistance to shear and less expanded product (Chaiyakul et al., 2009). High fiber content reinforces extrudate structure and increases resistance to breakage (Rinaldi et al., 2001) as indicated by the breaking force of OF. Whether the ingredient itself contains a lot of fiber or high-fiber ingredients are incorporated into raw material, neither one usually results with decrease in the products expansion when extruded (Yanniotis et al., 2007). In addition, OF had significantly higher average cell wall thickness and smaller void fraction and average cell size (Table 6-2). High fiber extrudate normally showed thicker cell walls, smaller air cells and higher breaking strength (Yanniotis et al., 2007). The breaking force of 40% SWW and 50% SWW was lower, probably due to increased starch composition from SWW.

Moisture content had different effects on the breaking strength of the treatments. OF with 20% and 22% moisture content had higher breaking force than that at 18% moisture content. The high cell wall thickness and high void fraction (Table 6-2) of OF at 20 and 22% could be the reason of the relatively high breaking force. On the other hand, breaking force of SWW extrudate decreased over the range of moisture contents. The breaking strength was positively correlated with the ER and densities of the extrudates (Fig. 6-3). It was observed that 40% SWW had the highest breaking force at 20% moisture content. The textural property of 50% SWW had a stronger influence of SWW properties over OF, as depicted by a sharp decrease of breaking strength at 22% moisture content.

6.4.4. WAI and WSI

Fig. 6-2 (c) and (d) shows the WAI and WSI of the extrudates. Regardless of moisture content, WAI of SWW was relatively higher than that of OF, whereas 40% SWW and 50% SWW had similar WAI and WSI. In exception to SWW at higher moisture contents (20% and 22%) and OF at 18% moisture content, WAI of the extrudates was noted to be inversely correlated with WSI. Higher moisture content caused increased WAI (except OF), indicating increased gelatinization as WAI is a gelatinization index (Anderson et al., 1969). Although SWW had high WAI at 20 and 22% moisture content, the extrudates were observed to have relatively high WSI, which indicates high amount of degraded molecular components. In this case, the degraded component could be protein (Verbee and van den Berg, 2010) instead of dextrinized starch, which could also explain the low breaking strength of SWW at 20 and 22% moisture content. The observed WAI and WSI results reveal that moisture content plays a bigger role than SME in determining the characteristics of extrudates.

6.4.5. Macro-structure of extrudate

Water is crucial in gelatinization of starch, thus making it one of the major factor in affecting the ER and density of extrudates (Kokini et al., 1992; Ding et al., 2006). Fig. 6-3 showed the macro-structure properties of the extruded products. BD and PD were positively correlated; whereas ER was inversely correlated with the SL. ER was also observed to be inversely correlated with the densities. Increasing the moisture content of OF resulted in increased ER, whereas ER of SWW shifted to a lower value. SWW macro-structure properties were in collaboration with other findings that showed decreased radial expansion (Singh et al., 2007) and increased longitudinal expansion and bulk density (Launay and Lisch, 1983; Lin et al., 2000). Addition of 40% and 50% SWW in the oat formulation showed that the macro-structural properties of the extrudates were strongly influenced by the behavior of SWW extrudate, which means lower ER with increasing moisture content.

Increased moisture content in SWW may result in lower melt viscosity. Consequently, starch molecules may disentangle and align longitudinally as they flow through the screw and die, which results in lower viscoelasticity and reduced capacity to store energy. Energy storage in the melt plays a big role in influencing the extent of radial expansion, i.e., higher energy storage leads to higher radial expansion and vice versa. In addition, longitudinally aligned starch

molecules may interact and increase the intra-molecular bonding, hence reducing the ER and increasing the SL (Harper and Tribelhorn, 1992). Conversely, the soluble fiber in OF may have influenced the expansion of the extrudate at different range of moisture content. The latter was not consistent with the findings of Yao et al. (2006) that reported decreased ER of extruded oat varieties with increasing moisture content. OF was expected to have hard structure and low radial expansion due to reinforced structure of the high amount of fiber and protein. However, the soluble fiber, specifically β -glucans that is partially water-soluble (Wood, 1994) was probably more solubilized with increasing moisture that may have loosen up the high reinforcement of internal bonds between the compositions of OF melt, thus increased the ER. Insoluble fibers are more prominent in resisting expansion in extruded products (Camire and King, 1991).

6.4.6. Micro-structure

The microstructural parameters of extruded wheat samples, namely void fraction (VF), average cell size (CS) and average cell wall thickness (CWT) are presented in Table 6-2, while representative XMT image of the extruded samples is shown in Fig. 6-4. Fig. 6-5 and 6-6 showed the distribution of CS and distribution of CWT, respectively. Regardless of moisture content treatment, extruded SWW had the highest VF and CS and the smallest CWT. The CS and CWT distributions supported the results where SWW had the biggest CS distribution up to 7 mm and CWT had a narrow curve, peaking at 0.2 mm. With high composition of protein and dietary fiber, OF was not able to expand much; hence lowering the VF and CS. OF also had the highest CWT than other treatments which was in agreement with Nelson (2001) who reported smaller air cells and thicker cell walls in bran-fortified corn meal extrudates. However, incorporation of SWW in the OF formulation (40% SWW and 50% SWW) improved the overall micro-structure of the extruded products. Although the CS of 40% SWW and 50% SWW did not differ much from OF, the VF and average CWT improved significantly. The CS distribution of OF, 40% SWW and 50% SWW was concentrated at 0.1 mm. However, 40% SWW and 50% SWW had a bigger range of CS distribution that reached up to 1.4 mm in diameter. On the other hand, CWT distribution of OF had a wider array of CWT (0.1 - 1.7 mm) than other treatments, which indicates the relatively higher average CWT (Table 6-2). CWT distribution of 40% SWW and 50% SWW were in between the distributions of SWW and OF.

Effect of moisture content on the microstructural properties was obvious on all of the extruded products. While increased moisture content caused OF to have reduced VF and increased CWT, the average CS of SWW increased (Table 6-2). The significant increase in the CWT may explain the increased breaking strength over the range of moisture content (Fig. 6-2). The CS distribution of SWW showed a bigger range of CS when the moisture content was 20 and 22%, as opposed to similar CS distribution of OF at all moisture contents (Fig. 6-5). Both 40% SWW and 50% SWW showed the lowest VF at 20% moisture content (Table 6-2). The results had inverse correlation with the breaking strength (Fig. 6-2 b) which relates small air cells and thick cell walls with harder structure. CS distribution of 40% SWW and 50% SWW showed heterogeneous CS which had a wider range with increasing moisture content. 50% SWW at 22% moisture content had the biggest CS range, which was also supported by the XMT representative image (Fig. 6-4). The uneven CS of 50% SWW at 22% moisture content caused uneven support of the cellular structure, hence lowering the breaking force. CWT distribution of all treatments showed wider peak as the moisture content increased, indicating thicker CWT formation. Higher moisture content may cause reduced viscosity of melt in extruder, resulting in weak cell walls formation that may coalesce with each other as extrudate exits the die that produced thick cell walls.

6.5. Conclusions

The textural and structural properties of OF and SWW formulations were highly dependent on the composition and moisture content of the melt. Effect of moisture content was observed to be more prominent than SME on the extrudate's properties. The higher amount of protein, fiber and lipid in OF produced extrudates resulted with poor textural and structural properties. Inclusion of SWW in the oat formulation at 40% and 50% amount improved the overall textural, macro-structure and micro-structure of extrudates. Future study includes studying the effect of screw speed and temperature on the oat and SWW formulations.

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Table 6-1. Dietary fiber, protein, ash, lipid, total starch and β -glucan contents of raw soft waxy wheat flour and oat flour.

	Dietary fiber (%)	Protein (%)	Ash (%)	Lipid (%)	Total starch (%)	β -glucan (%)
Soft waxy wheat flour	0.3 \pm 0.0b	9.4 \pm 0.0b	0.4 \pm 0.0b	1.1 \pm 0.0b	76.9 \pm 0.3a	0.3 \pm 0.1b
Oat flour	7.2 \pm 0.1a	15.5 \pm 0.0a	1.8 \pm 0.0a	5.9 \pm 0.0a	57.8 \pm 0.3b	3.4 \pm 0.2a

All values are reported on dry basis of flour. Data are means \pm standard deviation. Means within columns not sharing a common letter are significantly different at $p \leq 0.05$.

Table 6-2. Microstructural properties of extruded oat flour (OF), soft waxy wheat (SWW) flour and combination of oat-soft waxy wheat flours (OF:SWW).

	Ratio	Moisture content (%)	Void fraction (%)	Average cell size (mm)	Average cell wall thickness (mm)
Oat flour	100	18	10.6 ± 0.3 g	0.2 ± 0.0 c	0.5 ± 0.0 c
		20	6.3 ± 0.3 h	0.1 ± 0.0 c	0.8 ± 0.0 b
		22	4.8 ± 0.2 h	0.2 ± 0.0 c	1.2 ± 0.2 a
Soft waxy wheat flour	100	18	90.9 ± 1.8 a	1.6 ± 0.2 b	0.2 ± 0.0 f
		20	86.7 ± 0.3 b	2.8 ± 0.4 a	0.2 ± 0.0 f
		22	85.2 ± 0.1 b	3.1 ± 0.7 a	0.3 ± 0.1 ef
OF:SWW	60:40	18	42.7 ± 0.6 c	0.3 ± 0.0 c	0.2 ± 0.1 ef
		20	10.7 ± 0.2 g	0.2 ± 0.0 c	0.6 ± 0.0 c
		22	16.2 ± 0.5 f	0.2 ± 0.0 c	0.5 ± 0.0 cd
OF:SWW	50:50	18	21.7 ± 0.2 e	0.2 ± 0.0 c	0.4 ± 0.0 de
		20	10.2 ± 0.2 g	0.2 ± 0.0 c	0.4 ± 0.1 cd
		22	31.6 ± 0.7 d	0.4 ± 0.1 c	0.4 ± 0.0 cd

Data are means ± standard deviation. Means within columns not sharing a common letter are significantly different at $p \leq 0.05$.

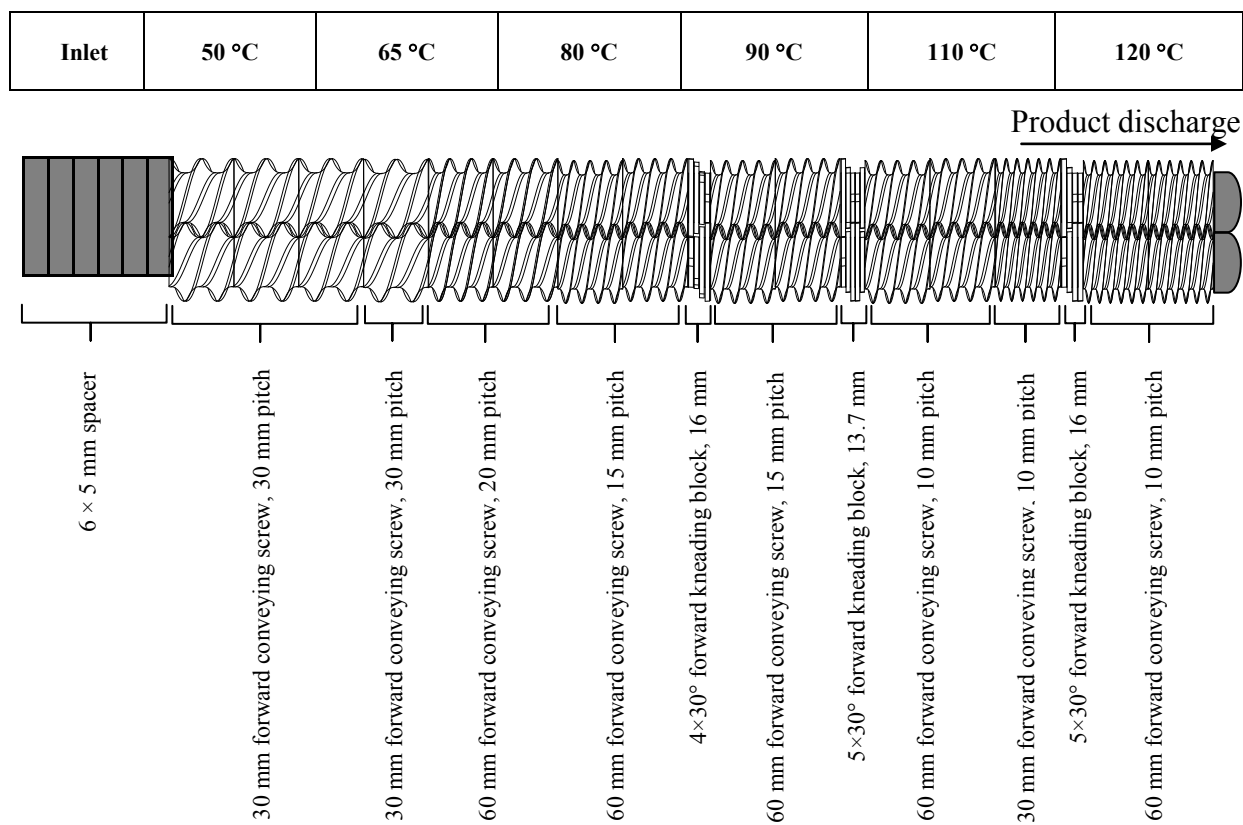


Fig. 6-1. Screw configuration and temperature profile of M-18 twin screw extruder.

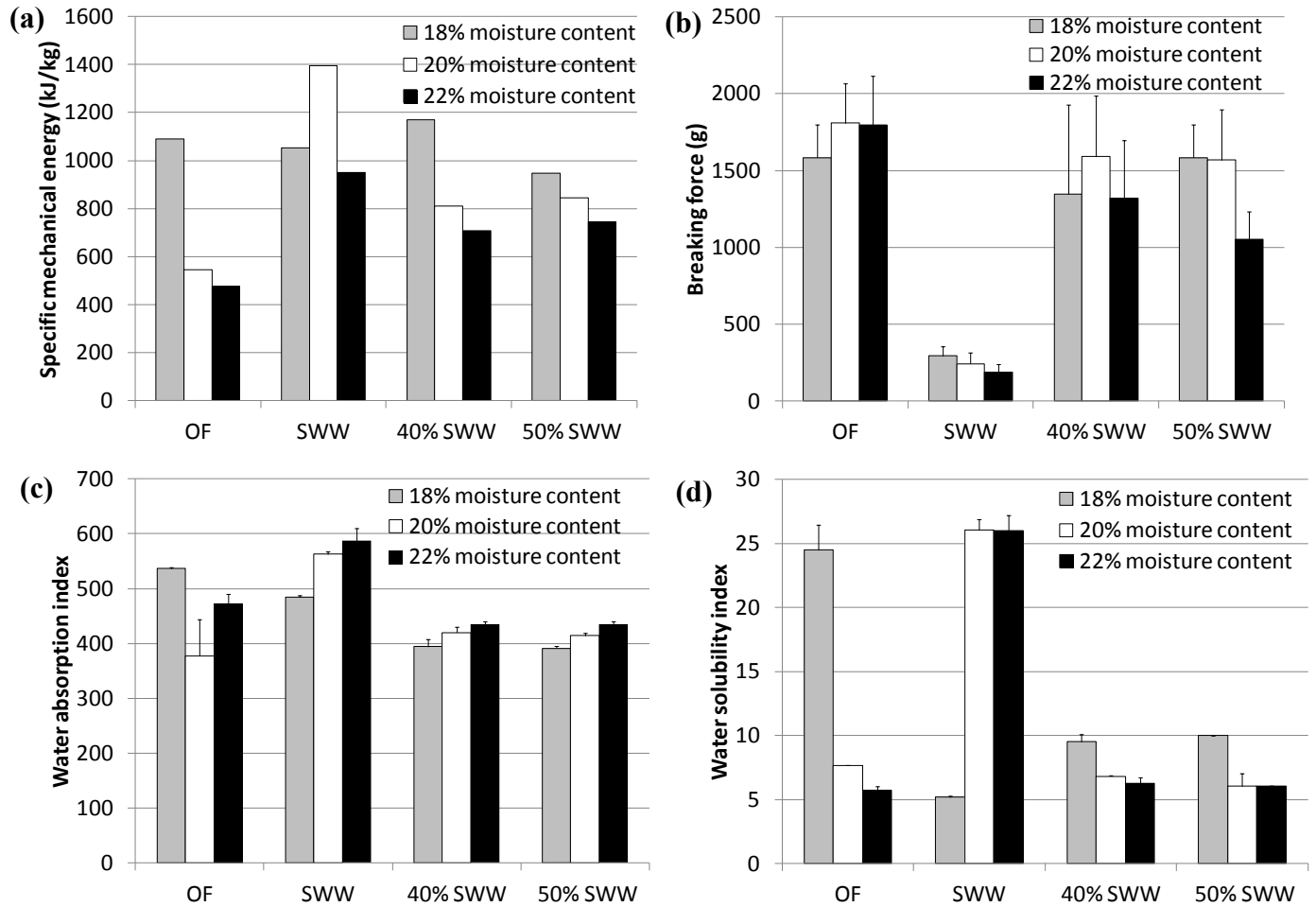


Fig. 6-2. (a) Specific mechanical energy, (b) breaking strength, (c) water absorption index and (d) water solubility index of extruded oat flour (OF), soft waxy wheat (SWW) flour and formulations of OF-SWW at the ratios of 60:40 (40% SWW) and 50:50 (50% SWW).

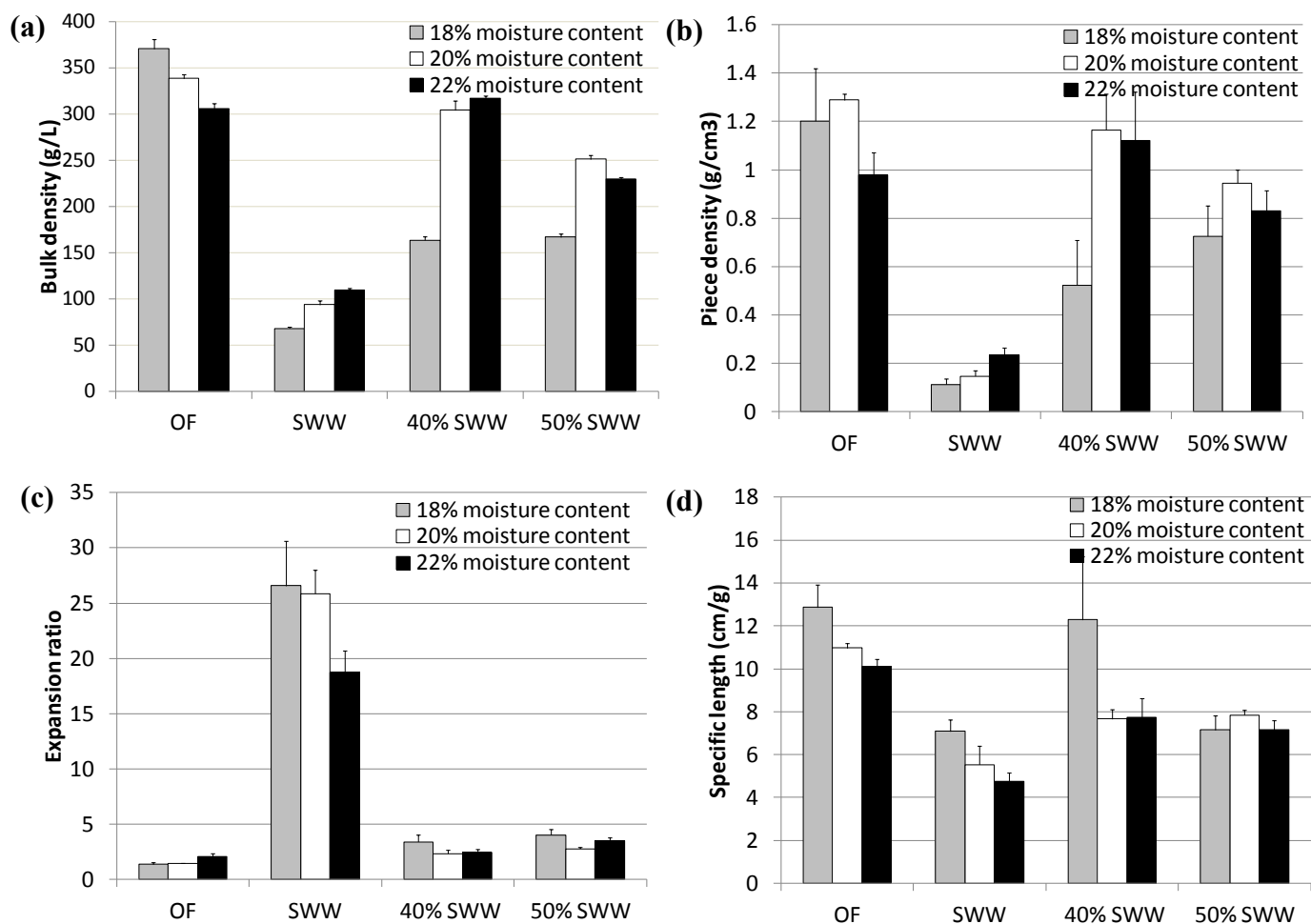
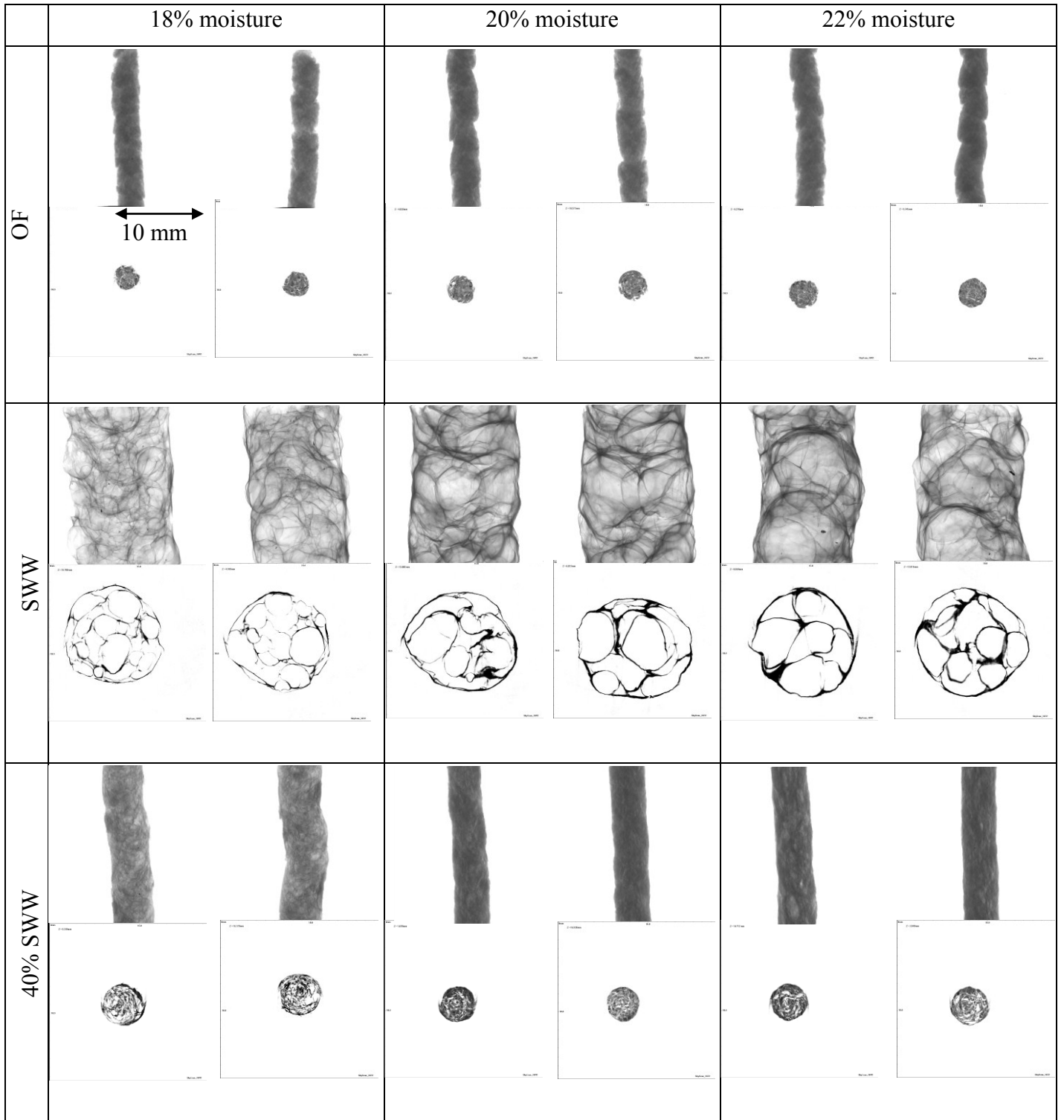


Fig. 6-3. Physical properties of extruded oat flour (OF), soft waxy wheat (SWW) flour and formulations of OF-SWW at the ratios of 60:40 (40% SWW) and 50:50 (50% SWW) (a) bulk density (BD) and (b) piece density (PD), (c) expansion ratio (ER) and (d) specific length (SL).



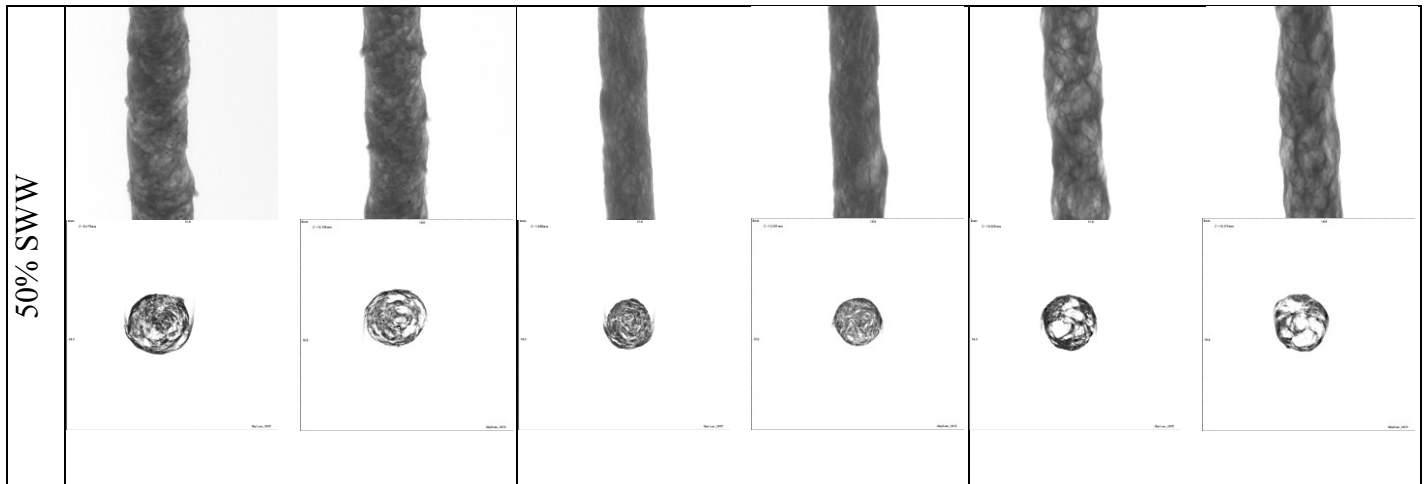


Fig. 6-4. Radial (top) and longitudinal (bottom) representative XMT images of extruded oat flour (OF), soft waxy wheat flour (SWW) and formulations of OF-SWW at the ratios of 60:40 (40% SWW) and 50:50 (50% SWW).

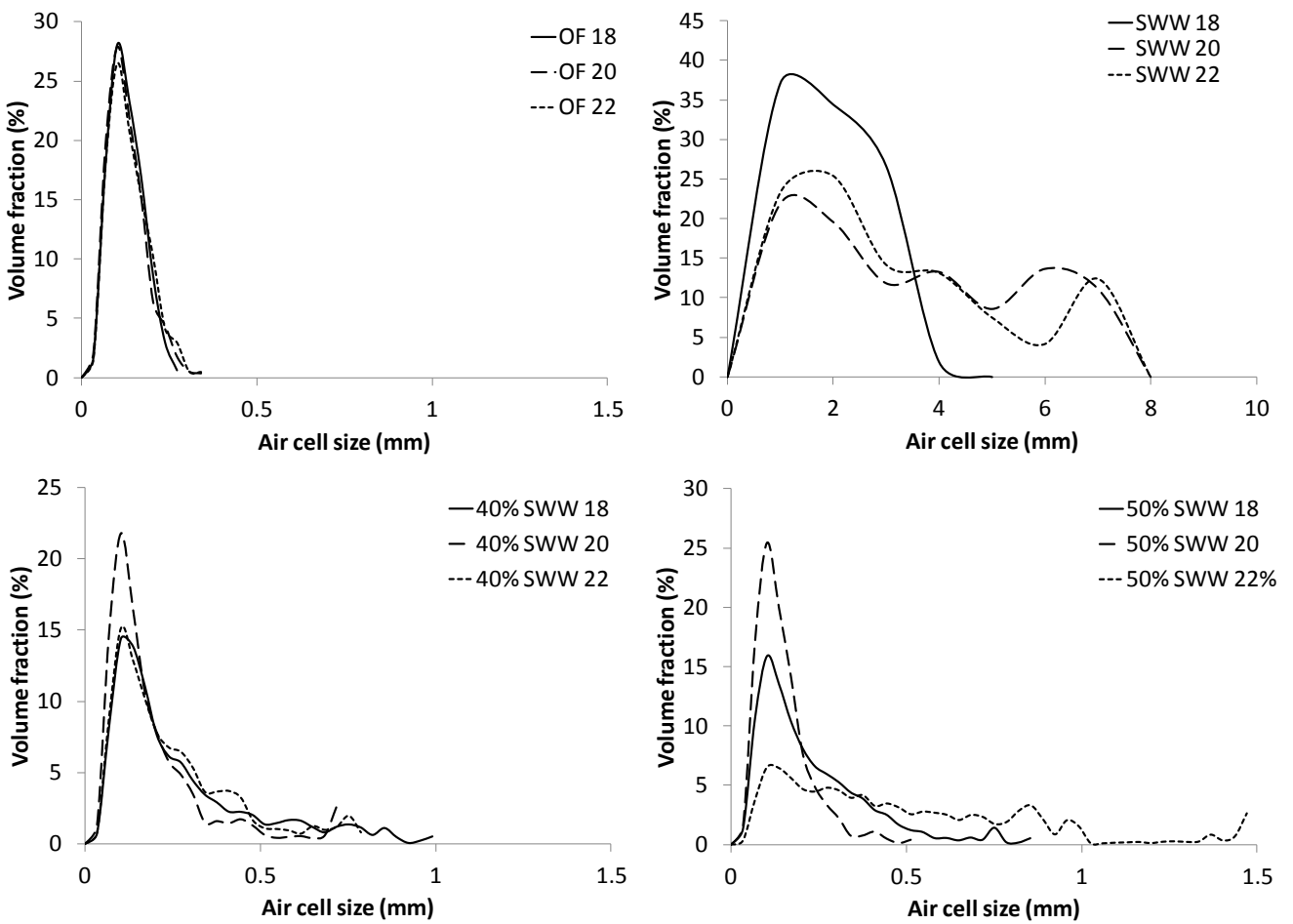


Fig.6-5. Air cell size distribution of extruded oat flour (OF), soft waxy wheat (SWW) flour and formulations of OF-SWW at the ratios of 60:40 (40% SWW) and 50:50 (50% SWW) at the moisture contents of 18, 20 and 22%.

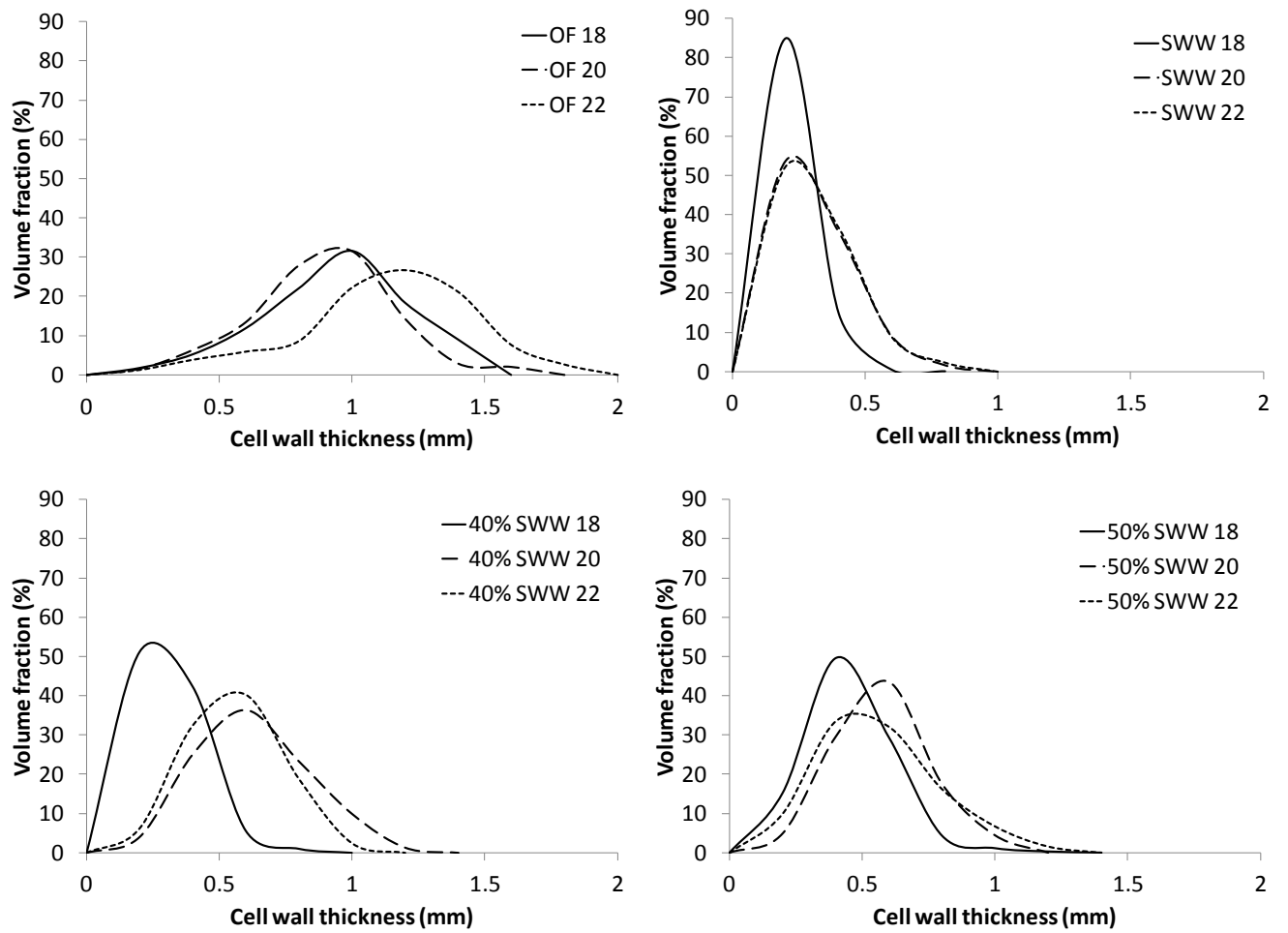


Fig. 6-6. Cell wall thickness distribution of extruded oat flour (OF), soft waxy wheat (SWW) flour and formulations of OF-SWW at the ratios of 60:40 (40% SWW) and 50:50 (50% SWW) at the moisture contents of 18, 20 and 22%.

Chapter 7 - Macro- and micro-structural properties of extruded cross-linked starch and its functionality

7.1. Abstract

The objectives of this study were to study the extrusion of cross-linked starch (CLS) at different cross-linking levels and its function as a minor ingredient in extruded oat flour (OF) formulations. CLS with different cross-linking levels and OF with 18% inclusion of the CLS were hydrated to 20% moisture content and subjected to twin-screw extrusion at the screw speeds of 350 rpm. The phosphorus content of CLS increased with increasing cross-linking levels. Low cross-linking level of CLS (0.0019% phosphorus content) in OF formulation increased the void fraction and reduced the breaking strength of extrudates. Higher cross-linking levels of CLS improved the resistant starch level on OF formulation but had very poor structural and textural properties. Varying the level of cross-linking offers an alternative a way to manipulate the structural and textural properties, as well as nutritional value of extrudates in snack or cereal applications.

7.2. Introduction

Consumers are demanding for tastier and healthier snacks that provide nutritious value and improves overall health. Based on the State of the Industry 2009 report, sales of healthier snacks are increasing by 8% as compared to decreasing sale of indulgent snacks. Whole cereals comprised of bran, germ and endosperm that contain vitamins, minerals, protein, lipid and fiber is a good choice of main ingredient in producing healthy expanded snacks. The most common problem encountered during extrusion of whole grain cereal or cereal with high lipids and fiber is to achieve the desired degree of expansion. High amount of fiber and protein in extruded formulation acts as dispersed phase filler, whereas lipid acts as lubricant in extruder, thus reducing expansion (Riaz, 2000).

Healthy snacks produced from extrusion of whole grain cereal such as whole white corn flour (Chatel and Chung, 2007), whole yellow corn flour (Robert and Yongsoo, 2007), whole barley flour (Dudgeon-Bollinger et al., 1997; Baik et al., 2004) and oat flour (OF) (Bindzus and Altieri, 2002) had been attempted with other minor ingredients to aid the expansion. Incorporation of healthy ingredients in snacks such as fiber (Camire and King, 1991) and

proteins (Faubion and Hosoney, 1982; Allen et al., 2007) to increase the nutritional value has also been a challenge in producing extruded expanded snacks. These ingredients inhibit expansion in extrudates that in turn affect the quality, texture and mouth feel of snack produced.

Isolated starch and/or modified starch could be used as minor ingredients to aid expansion and texture of extrudates (Huang and Rooney, 2001). Inclusion of waxy starch as a minor ingredient in the whole grain mixture has been proven to improve the expansion of extrudates. Chatel and Chung (2007) suggested incorporation of 2-10% of waxy starch by weight of the total dry cereal composition. The patent showed usage of 4% waxy maize starch in whole corn and oat cereal mixture with 16-22% total moisture content. The temperature of the extruder was operated at 160-180°C to accomplish the desired expansion (Chatel and Chung, 2007). However, waxy starch is prone to breakdown during high temperature and shear of extrusion (Mercier and Fellet, 1975; Wang, 1997).

Besides improved water-holding capacity and viscosity, resistance of waxy starch towards excessive heat and shear is also increased by chemical modification of cross-linking (Huang, 1995; Huang and Rooney, 2001). Cross-linking is achieved from low to high degree of substitution (up to 0.4% phosphorus add-on) by using cross-linking agents namely sodium trimetaphosphate (STMP), sodium tripolyphosphate (STPP), phosphoryl chloride and mixed anhydride of acetic and adipic acid (Sang and Seib, 2006). Yang et al. (1995) suggested moderate to highly cross-linked (CL) starches for high shear processes. However, excessive cross-linking results in decreased swelling capacity of the starch that produce extrudates with low expansion and non-uniform poor texture (Marotta et al., 1972). Thus, suitable degree of cross-linking and amount of CL waxy starch in formulation may result in desired degree of expansion and uniformly light texture final product (Huang and Rooney, 2001). The objective of the research is to study the extrusion of CL starch and its function to improve structural and textural properties of extruded OF.

7.3. Materials and methods

7.3.1. Materials

Waxy maize starch (Amioca) was obtained from National Starch LLC (Bridgewater, NJ) with amylose content of 0.5%, as determined by the potentiometric iodine method (Shi et al.,

1998). CL wheat starch (Fibersym) and oat flour (OF) were obtained from MGP Ingredients, Inc[®] (Atchison, Kansas) and Lortcher Agri Service (Bern, KS), respectively. STMP was purchased from MP Biomedicals, LLC (Solon, CA). STPP, sodium sulfate, sodium hydroxide, and hydrochloric acid were obtained from Fisher Scientific (Pittsburgh, PA). Total Dietary Fibre assay kit (catalogue no. K-TDFR) was obtained from Megazyme International Ireland, Ltd. (Wicklow, Ireland). Porcine pancreatin (catalogue no. P7545), with an enzyme activity of 300 U (0.9 mg of glucose released from soluble starch in 3 min at 37 °C and pH 5.8) and amyloglucosidase (catalogue no. A7255), with an enzyme activity of 234 U (0.7 mg of glucose released from soluble starch in 3 min at 37 °C and pH 5.8), were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). All chemicals were reagent grade.

7.3.2. Preparation of CL starch

CL waxy maize starch was prepared by the method from Woo and Seib (2002) using a combination STMP and STPP (99:1) at 0, 0.05, 0.1, 0.5, 1 and 2% weight of dry starch.

7.3.3. General methods

Protein of raw materials was determined via LECO TruSpec CN Carbon/Nitrogen combustion analyzer (St. Joseph, MI), while lipid and ash contents were measured according to AOAC Method 920.39 and AOAC Method 942.05, respectively. RS was determined by a modified procedure of the Englyst method (Sang and Seib, 2006). Phosphorus (P) content was assayed using the procedure of Smith and Caruso (1964). Moisture content was determined by using AACC Method 44-15 (AACC 2000) at 135 °C for 2 h. Total starch was determined by using the Megazyme International, Ltd. (Wicklow, Ireland) kit and β -glucan was measured by procedure from AACC Method 32-23 (AACC, 2000).

7.3.4. Pasting properties

The pasting properties of the CL starches suspended in distilled water were determined using a Micro Visco-Amylograph (C.W. Brabender Instruments, Inc, South Hackensack, NJ). CL starch was added to distilled water to prepare a 20% suspension (dry weight basis, w/w). The heating and cooling cycles were programmed by heating the suspension from 50 °C to 90 °C (heating rate of 10 °C/min), held at 90 °C for 6 min, and cooled to 50 °C (cooling rate of 10 °C/min). The viscosity of starch suspension was expressed in Brabender Unit (BU).

7.3.5. Thermal properties

The gelatinisation temperatures and the enthalpy of sample was measured by a differential scanning calorimeter (DSC) (TA Instruments Q100, TA Instruments, New Castle, DE). The total solids content of samples was 33.3% (w/w, dry basis). After hydration for 1 h at 25 °C, 30 mg of the well-stirred sample suspensions were weighed into 40 µl aluminum pan and immediately hermetically sealed to prevent moisture loss. The scans were performed from 10 °C to 130 °C at a controlled constant rate of 10 °C/min. A sealed empty pan was used as a reference, and the DSC was calibrated using indium. The gelatinisation enthalpy (ΔH) and transition temperatures, namely the onset temperature (T_o), peak temperature (T_p), and conclusion temperature (T_c), were determined based on the first-run DSC heating curves. The ΔH was evaluated based on the area of the main endothermic peak and expressed in terms of J/g of dry starch using the equipment's software (TA Universal Analysis, New Castle, DE).

7.3.6. Experimental design and extrusion processing

The moisture content of CL waxy starches with 0, 0.05, 0.1, 0.5, 1 and 2% STMP/STPP were adjusted to 20% by spraying distilled water and mixed 1 min in a mixer. OF and CL starch blends with the ratio of (88:18) were also prepared with 20% moisture content. The ratio was chosen because it had the best expansion based on preliminary runs of OF-CL starch blends. The CL starches used were Fibersym and CL waxy maize starches of 0, 0.05, 0.1, 0.5, 1 and 2% STMP/STPP. Fibersym was used as a replacement of 12% STMP/STPP CL waxy maize starch in OF-CL starch that could not be extruded due to clogging problems. Extrusion of Fibersym alone caused clogged extruder, hence only the OF-Fibersym was extruded. OF with 20% moisture content was extruded as control for the OF-CL starch blends. The moisture content of the blends was adjusted to 20% by spraying distilled water and mixed for 1 min in a Classic Kitchen Aid mixer (St. Joseph, Michigan). The blends were kept in a fridge overnight for moisture equilibrium prior to extrusion with a Micro-18 twin screw extruder (American Leistritz Extruder Corp., Somerville, NJ). The extruder configuration parameters and the schematic of the screw configuration for the Micro-18 are in Fig. 7-1. The die used was a 2.5 mm circular die and screw speed was set at 350 rpm. Tests of specific mechanical energy (SME) were executed in duplicate and mechanical energy input per unit mass of extrudate was calculated as follows:

$$SME = \frac{\frac{(\tau - \tau_0)}{100} \times P_{rated} \times \frac{N}{N_{rated}}}{\dot{m}} \text{ (kJ/kg)}$$

where τ - % torque, τ_0 - no-load torque (4%), P_{rated} - rated power for the extruder (22.37 kJ/s), N - measured extruder screw speed in rpm, N_{rated} - rated extruder screw speed (336 rpm) and \dot{m} - mass flow rate in kg/s.

The extrudates were dried in an oven at 110 °C for 10 min, cooled for 5 min at room temperature and stored in plastic containers until analysis.

7.3.7. Mechanical property

Force–deformation data for each treatment was obtained with a Model TA-XT2i Texture Analyzer (Stable Micro Systems, Surrey, UK) equipped with a 25-kg load cell and a 38-mm-diameter test probe. Samples were compressed perpendicularly to 70% of their original height at a test speed of 5 mm/s. Thirty sample measurements were taken for each treatment. Average crushing force F_{cr} or breaking strength was calculated by using n , s and x values (Bouvier et al., 1997).

$$F_{cr} = \frac{s}{x \times n} \text{ (g)}$$

where n - number of peaks, s - integral of the curve (or area below the curve from 0% to 70% strain) and x - distance of breakage,.

7.3.8. Water absorption (WAI) and water solubility (WSI) indices

The WAI and WSI were determined by the method of Gujral and Singh (2002) on duplicates samples. Extruded samples were ground in a Waring blender (Torrington, CT) and sifted through a 250 screen sieve prior to testing. The ground sample (2.5 g) was immersed in 25 mL distilled water at room temperature, continuously stirred for 30 min, and centrifuged at 3000 ×g for 10 min. The supernatant liquid was poured into a pre-weighed evaporating dish. The remaining gel was weighed, and WAI was calculated as

$$WAI = \frac{W_{sediment}}{W_{dry\ solid}}$$

where $W_{sediment}$ - weight of gel and $W_{dry\ solid}$ - weight of dry sample.

The WSI was determined from the amount of dry solids recovered by evaporating the supernatant at 100 °C for 12 hand it was calculated as follows:

$$WSI = \frac{W_{dissolved\ solid\ in\ supernatant}}{W}$$

where $W_{dissolved\ solids\ in\ supernatant}$ - weight of dry solids from the supernatant and $W_{dry\ solid}$ - weight of dry sample.

7.3.9. Macrostructure properties

From each treatment, replicates of 30 were measured for bulk density (BD), piece density (PD), expansion ratio (ER) and specific length. BD was measured by filling extrudates in a cylindrical steel container ($V_c = 1$ L) and recording the mass for calculation as follows:

$$BD = \frac{W_{sample}}{V_c} \text{ (g/L)}$$

where W_{sample} - mass of extrudates (g) and V_c - the volume of the steel cylinder.

PD of extrudates were calculated as shown below:

$$PD = \frac{W_{piece}}{V_{piece}} = \frac{4W_{piece}}{\pi d^2 l} \text{ (g/cm}^3\text{)}$$

where W_{piece} - mass of extrudate, V_{piece} - volume, d - diameter and l - length.

ER of extrudates were calculated as follows:

$$ER = \frac{d^2}{d_{die}^2}$$

where d - extrudate diameter and d_{die} - die diameter.

SL is calculated as follows:

$$SL = \frac{l_e}{m_e} \text{ (cm/g)}$$

where l_e - length of the extrudate and m_e - mass of the extrudate.

7.3.10. Microstructure properties

Microstructural properties of two replicates from each treatment were analyzed using a desktop X-ray microtomography (XMT) imaging system (Model 1072, Skyscan, Aartselaar, Belgium) that was set under the following settings: voltage - 40 kV, current - 248 μ A, exposure

time – 1.2 sec, rotation step - 0.9°, sample rotation – 180°, magnification – 16 ×, pixel size - 17.09 μm. Images were captured with a 12-bit, cooled CCD camera (1024 × 1024 pixels). The total scanning time was 38 min.

The scanned images were reconstructed into 3D objects (NRecon v. 1.6.1, Skyscan, Belgium) and virtually sliced to 1000 cross-sections (CTAn 1.9.1, Skyscan, Belgium). A region of interest was set on the parameter of each cross-section for interpolation of data. The grayscale images were converted into binary images (threshold – 15) and were analyzed in 3D to obtain void fraction, structure thickness and structure separation functions. The void fraction was calculated by subtracting the percent object volume from 100%. Structure thickness is addressed as cell wall thickness and structure separation is addressed as air cell size in this paper.

7.3.11. Resistant starch (RS)

The RS was determined by a modified procedure of the Englyst method (Sang and Seib, 2006).

7.3.12. Statistical analysis

All data were reported as the mean ± standard deviation (mean ± SD). The results were analyzed by using analysis of variance (ANOVA) (SPSS version 13.0, SPSS Inc., Chicago, IL), and the Student's *t* test was used to examine the differences. Results with a corresponding probability value of $P < 0.05$ were considered to be statistically significant.

7.4. Results and discussion

7.4.1. Composition of raw materials

Table 7-1 shows the protein, ash, lipid, total starch, β-glucan and total dietary fiber of OF which was compatible with that of Yao et al (2011). The high amount of protein (15.5%) and total dietary fiber (7.2%) indicated OF as a good source of raw material to produce healthy snacks. Oats has been associated with decreasing the occurrence of heart disease, obesity, type-2 diabetes and cancer (Malkki and Virtanen, 2001) due to the existence of β-glucan in the endosperm and aleurone of oats (Behall et al., 1997).

P content of CL starches is reported in Table 7-2. CL 0% had negligible P content (< 0.0001%) as it acts as control for the CL waxy maize starches which underwent the same cross-

linking procedure but without using the cross-linking agents. Hence, P content in CL 0% is the natural P existed in native maize starches in the form of phospholipids (Morrison, 1995; Suh et al., 2004). As the amount of cross-linking agents (STMP/STPP) increased from 0.05% to 2%, the P level of the CL waxy maize also elevated due to the addition of P from STMP and STPP during the cross-linking process. Ideally, CL waxy maize starch prepared with 12% STMP/STPP, a high level of CL starch, was included in the extrusion plan to evaluate the impact of higher cross-linking on extrusion. Unfortunately, the waxy nature of starch with the additional high level of cross-linking caused severe clogging of extruder. As an alternative, Fibersym, a commercial CL wheat starch was used due to the similar P level (0.38%).

The thermal properties consisting of T_o , T_p and T_c as well as ΔH of CL waxy maize starches are presented in Table 7-3. Gelatinization of the starch took place between 67 °C (for T_o) to 97 °C (for T_c) and endothermic peak was observed in the DSC curves for all the samples. There was no significant difference for all the DSC parameters tested. The similar values of the thermal parameters imply the crystallinity of the CL waxy maize starches has little or not at all affect by the cross-linking process.

Fig. 7-2 presents the pasting curves of the CL starches. Among the CL starches, pastes from CL 0% had significantly higher peaks and final viscosities. During heating and holding periods, CL waxy maize starch pastes progressively increased without any breakdown, indicating stability of the pastes against shear. As the cross-linking level increased, the viscosity of the pastes decreased which suggests increased resistance towards swelling. The settling volume of CL starches (Table 7-2) was positively correlated with the pasting curve. Settling volume shows the extent of starch molecule swelling.

7.4.2. Extrusion processing

The SME is closely related to the motor torque and the pressure at the die of the extruder, which greatly influences the degree of product transformation, textural characteristics, expansion and density of product (Iwe et al., 2001). Fig. 7-3 (a) shows the SME of extruded CL starch and OF-CL starch blends. Extrusion of CL starches had significantly higher SME than that of OF-CL starch blends. Starch was proven to have a higher requirement of SME during extrusion (Faubion and Hosoney, 1982) that could be due to finer particle size. In addition, higher lipid content of OF may lubricate screw and the surface of extruder barrel (Yacu, 1998), resulting in

slippage and reduced SME. Extrusion of CL 0% had relatively higher SME than the CL 0.05% and CL 0.1% where the same trend was observed for OF-CL starch blends. An increase of SME was observed for CL 1% and CL 2%, mostly due to the increased cross-linking of starch molecules that needed higher energy requirements to break apart the starch molecules. Higher cross-linking levels are associated with lower degree of swelling, thus lower viscosity (Fig. 7-2). Interestingly, OF-Fibersym blend had significantly lower SME than the OF-CL 2%. The fact that Fibersym had relatively higher P level than CL 2% (Table 7-2) may not contribute to the increased SME, but the higher amylopectin in CL waxy maize than Fibersym could play a role due to the fact that higher amylopectin may produce sticky flour.

7.4.3. Mechanical properties

Breaking strength of OF-CL starch is reported in Figure 7-3 (b). Breaking strength of CL starch was unable to be determined due to the very hard nature of the extrudates. Addition of 18% CL waxy maize starch caused reduced breaking strength as compared to the control. The breaking strength of the extruded OF-CL starch was similar for CL 0.05%, CL 0.1% and CL 0.5%, which was also significantly lower than CL 0%. Breaking strength gradually increased with CL 1% and CL 2%, but elevated drastically for Fibersym. The breaking strength of extrudates was positively correlated with the density and ER. Elevating the cross-linking level of waxy starch decreased the swelling capacity as indicated by the settling volume (Table 7-2) that restricted expansion, thus reducing the ER and breaking strength of extrudates. Addition of Fibersym may have caused the starch molecules in the extrudates to be more rigid due to the high cross-linking level, consequently forming stronger cell walls that required more crushing force.

7.4.4. WAI and WSI

Fig. 7-3 (c) shows the WAI and WSI of the extrudates. The CL starch extrudates were observed to have more prominent effect on the WAI and WSI, as compared to OF-CL starch extrudates. While the lower levels of CL starches showed an inverse WAI-WSI correlation with low WAI and high WSI, CL 1% and CL 2% showed similar WAI and WSI values. The latter result suggested CL starches at lower levels of cross-linking had low starch gelatinization and had relatively higher dextrinization than the CL 1% and CL 2% starch. Cross-linking may have limited gelatinization and increased the starch dextrinization effect on the lower levels of CL

starch. On the other hand, higher level of cross-linking (CL 1% and CL 2%) had stronger molecular interaction due to the increased amount of P which probably maintained the intact starch molecules. This may increased the WAI of CL 1% and CL 2%, where WAI was used to determinethe amount of water absorbed by intact starch (Anderson et al., 1969). In addition, the stronger molecular cross-linking of CL 1% and CL 2% suggest higher resistance of starch molecules towards granule tearing and dextrinization by the high shear during extrusion that explained the lower WSI which is an indicator of the amount of degraded molecular components (Kirby et al., 1988). The WSI of OF-CL starch blends followed the same trend as the CL starch extrudates with decreasing WSI value as cross-linking increased. The WAI of OF-CL starch showed comparable value, indicating similar amount of remaining intact starch and gelatinization.

7.4.5. Macrostructure of extrudate

BD and PD of CL starches and OF-CL starch blends are shown in Fig. 7-4 (a) while ER and SL are in Fig. 7-4 (b). BD and PD of CL starches and OF-CL starch showed similar increasing trends with increasing cross-linking level. BD and PD of the extrudates were positively correlated and the ER showed inversed correlation with the densities of the extrudates which was in agreement with other research (Singh et al., 1996; Asare et al., 2004; Chaiyakul et al., 2009). ER and SL of the extrudates also showed an inverse correlation. However, CL starch extrudates showed a more prominent decline of ER than OF-CL starch extrudates. The relatively low ER and high SL of OF-Fibersym was expected since it had the highest P content among other CL starches which probably caused starch molecules to bond strongly to each other, hence restricting expansion of the melt once it exited the die. Based on the physical properties of the extrudates, incorporation of 18% CL waxy maize starches in the OF blend did not improve the radial expansion and densities of extrudates.

7.4.6. Microstructure

The micro-structural properties are closely related to the textural properties of the extrudates (Babin et al., 2007; Karkle et al., 2012). The micro-structural parameters of extruded samples, namely void fraction (VF), average cell size (CS) and average cell wall thickness (CWT) are presented in Table 7-4, while representative XMT image of the extruded samples is shown in Fig. 7-5. VF and average CS of CL starch were decreasing while average CWT

increased with increasing cross-linking levels. As P content increased, starch molecules are more bonded to each other, restricting expansion and aligning perpendicularly with the direction of exiting the die, hence the lower ER and higher SL. The explanation is supported by the XMT representative images which showed very compact microstructure with very limited air cells of CL 0.05%, CL 1% and CL 2%.

Addition of 18% CL waxy maize starch in the OF formulation increased the VF and average CS, as compared to control. The average CWT of OF-CL waxy maize starches was comparable to that of control, except for OF-CL 0.05% which was significantly lower. Inclusion of 18% CL 0.05% in the OF formulation showed a significant improvement in the VF, average CS and average CWT, which had an important role of reducing the breaking strength (Fig. 7-3 b). Eventhough, OF-CL 0% had relatively higher ER, the micro-structural and the textural properties were not as good as OF-CL waxy maize starches. On the other hand, OF-Fibersym had the lowest VF and average CS as well as the highest average CWT, hence the significantly higher breaking strength. With a significantly higher P content in Fibersym, the starch molecules were expected to be tightly bonded with each other. Interactions of Fibersym with protein, lipid and starch in OF may have further restricted the expansion, or in this case, shrink the extrudate which was shown by lower ER of oat-Fibersym, in comparison to OF control (Fig. 7-4 b).

The CS and CWT distributions of the extruded products are provided in Fig. 7-6 and 7-7, respectively. Although the distribution of air CS of the CL waxy maize starches (Fig. 7-6 a) varied, all of them showed a high volume fraction at 2-3 mm cell size. However, as the cross-linking level increased, the air CS peaks were narrower, suggesting a more homogenous cell sizes. As for the OF-CL starch formulations (Fig. 7-6 b), air CS was relatively smaller (0.5-2.5 mm) as compared to CL starch (2-9 mm) with a wider range for formulations. OF-Fibersym extrudate was strictly having 0.5 mm air cell size which suggests a very limited expansion, as indicated in the XMT representative image (Fig. 7-5).

CWT distribution of CL waxy maize starch (Fig. 7-7 a) showed a variation of distribution. CL 0% had a narrow peak at 1 mm and CL 2% peaking at 4.5 mm, whereas the other CL waxy maize starches were peaking in between those two treatments. As the cross-linking increased, the CWT had a wider array. The CWT distribution of OF-CL starch (Fig. 7-7 b) was relatively lower than that of CL starch. However, all OF-CL waxy maize starch extrudates showed the highest volume fraction of CWT at 0.2-0.3 mm. OF-CL 0.05% had the

narrowest peak with the highest volume fraction at 0.2 mm, which also collaborates with the average CWT (Table 7-4). CWT distribution displayed OF-Fibersym having a wider range of distribution which peaked between 0.4 mm to 0.6 mm.

7.4.7. Resistant starch (RS)

The RS of the CL starches, OF and OF-Fibersym before and after extrusion were assayed using the Englyst method and showed in Fig. 7-8. CL starch before and after extrusion showed an increased RS with increment of cross-linking level. The cross-linking treatment of the waxy maize starch provides a stronger bond with the inclusion of P molecules in the granules, resulting in more amylolysis inhibition; hence, the RS value increased significantly. The CL starch is categorized under RS4, which is also considered dietary fiber. However, RS of all treatments decreased drastically after extrusion, as a consequence of shear and high temperature during extrusion that may have caused destruction of RS. Due to the latter effect, OF-CL waxy maize starches were not assayed, since the RS left would be mostly from OF. OF-Fibersym had a significantly higher RS (40%) than OF and still showed a good amount of RS content after extrusion (25%), indicating higher level of P may be able to retain the cross-linking effect that increased the RS content in extrudates. CL starch with high level of cross-linking had less granule swelling, resulting in more restriction of starch hydrolysis by the digestive enzymes (Woo and Seib, 2002).

7.5. Conclusions

The ER of CL waxy maize starch increased with increasing cross-linking levels but did not have an impact on the radial expansion of OF formulation. However, inclusion of 18% CL waxy maize starch at the lowest level (0.05% STMP/STPP) in the OF formulation improved the micro-structural (increased VF) and textural (reduced breaking strength) properties of extrudates. Although high cross-linking level of CL starch (Fibersym) increased the RS level on OF formulation, the high breaking strength and low expansion of the extrudate suggested it may not fulfill the palatability of consumers. Future work includes in-depth study on the effect of extrusion parameters on extrusion of low cross-linking level of CL starch with a different botanical source and amylose contents. It would also be interesting to study sensory evaluation of the extruded products.

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Table 7-1. Protein, ash, lipid, dietary fiber, total starch and β -glucan of oat flour.

	Amount (%)
Protein	15.50 \pm 0.04
Ash	1.82 \pm 0.04
Lipid	5.85 \pm 0.02
Dietary fiber	7.21 \pm 0.13
Total starch	57.78 \pm 0.33
β -glucan	3.41 \pm 0.21

Table 7-2. Phosphorus content and settling volume of waxy cross-linked (CL) waxy maize starches and Fibersym.

CL starch	Total phosphorus (%)	Settling volume (mL)
CL 0%*	0.0000 ± 0.0000 g	100.0 ± 0.0 a
CL 0.05%*	0.0019 ± 0.0001 f	83.5 ± 0.7 b
CL 0.1*	0.0043 ± 0.0000 e	61.5 ± 2.1 c
CL 0.5%*	0.0185 ± 0.0001 d	38.5 ± 0.7 d
CL 1%*	0.0322 ± 0.0001 c	36.0 ± 0.0 d
CL 2%*	0.0737 ± 0.0002 b	29.0 ± 0.0 e
Fibersym	0.3751 ± 0.0149 a	19.0 ± 0.0 f

All values are reported on dry basis of starch. Data are means ± standard deviation. Means within columns not sharing a common letter are significantly different at $p \leq 0.05$. * CL waxy maize starch prepared with sodium trimetaphosphate (STMP) and sodium tripolyphosphate (STPP) at the ratio of 99:1 between 0-2% of the starch dry weight.

Table 7-3. Thermal properties¹ of cross-linked (CL) waxy maize starches as measured by differential scanning calorimetry.

CL starch*	Temperature (°C)			ΔH (J/g)
	T_o	T_p	T_c	
CL 0%	66.6 ± 0.0 a	76.7 ± 1.1 a	97.1 ± 0.0 a	14.8 ± 0.6 a
CL 0.05%	67.3 ± 0.7 a	75.8 ± 0.7 a	97.9 ± 0.3 a	14.9 ± 0.4 a
CL 0.1	67.1 ± 0.6 a	75.8 ± 0.4 a	97.6 ± 0.7 a	15.1 ± 0.9 a
CL 0.5%	67.2 ± 0.6 a	76.2 ± 0.1 a	98.0 ± 0.0 a	15.9 ± 0.7 a
CL 1%	66.9 ± 0.2 a	75.3 ± 0.3 a	96.6 ± 1.3 a	15.9 ± 0.5 a
CL 2%	68.1 ± 0.6 a	76.2 ± 0.0 a	96.6 ± 0.4 a	15.4 ± 0.6 a

¹ All values are reported on dry basis of starch. Data are means ± standard deviation. Starch solid was 33.3%. Means within columns not sharing a common letter are significantly different at $p \leq 0.05$. * CL waxy maize starch prepared with sodium trimetaphosphate (STMP) and sodium tripolyphosphate (STPP) at the ratio of 99:1 between 0-2% of the starch dry weight.

Table 7-4. Microstructural properties of extruded cross-linked (CL) waxy maize starches and extruded oat flour (OF)-CL waxy maize starches.

	Void fraction (%)	Average cell size (mm)	Average cell wall thickness (mm)
<u>CL starch*</u>			
CL 0%	68.0 ± 2.7 b	3.4 ± 0.2 a	0.7 ± 0.0 d
CL 0.05%	46.9 ± 2.4 e	2.6 ± 0.1 b	1.2 ± 0.0 c
CL 0.1	43.1 ± 2.3 ef	2.6 ± 0.1 c	1.4 ± 0.0 b
CL 0.5%	39.5 ± 1.0 f	2.2 ± 0.2 c	1.4 ± 0.0 b
CL 1%	31.6 ± 2.2 g	1.8 ± 0.1 d	1.3 ± 0.0 b
CL 2%	3.4 ± 1.8 i	0.6 ± 0.0 fgh	3.1 ± 0.2 a
<u>OF-CL starch</u>			
OF-CL 0%	57.3 ± 4.8 d	0.4 ± 0.0 h	0.2 ± 0.0 fg
OF-CL 0.05%	73.5 ± 0.9 a	0.7 ± 0.0 efg	0.2 ± 0.0 g
OF-CL 0.1	64.5 ± 2.2 bc	0.8 ± 0.0 e	0.3 ± 0.0 fg
OF-CL 0.5%	67.8 ± 0.3 b	0.8 ± 0.0 ef	0.2 ± 0.0 fg
OF-CL 1%	62.9 ± 1.2 c	0.7 ± 0.0 efg	0.3 ± 0.0 f
OF-CL 2%	54.9 ± 0.7 d	0.6 ± 0.0 gh	0.3 ± 0.0 fg
Fibersym	11.0 ± 0.5 h	0.1 ± 0.0 i	0.4 ± 0.0 e
Control	44.9 ± 1.8 e	0.4 ± 0.0 h	0.2 ± 0.0 fg

Data are means ± standard deviation. Means within columns not sharing a common letter are significantly different at $p \leq 0.05$. * CL waxy maize starch prepared with sodium trimetaphosphate (STMP) and sodium tripolyphosphate (STPP) at the ratio of 99:1 between 0-2% of the starch dry weight.

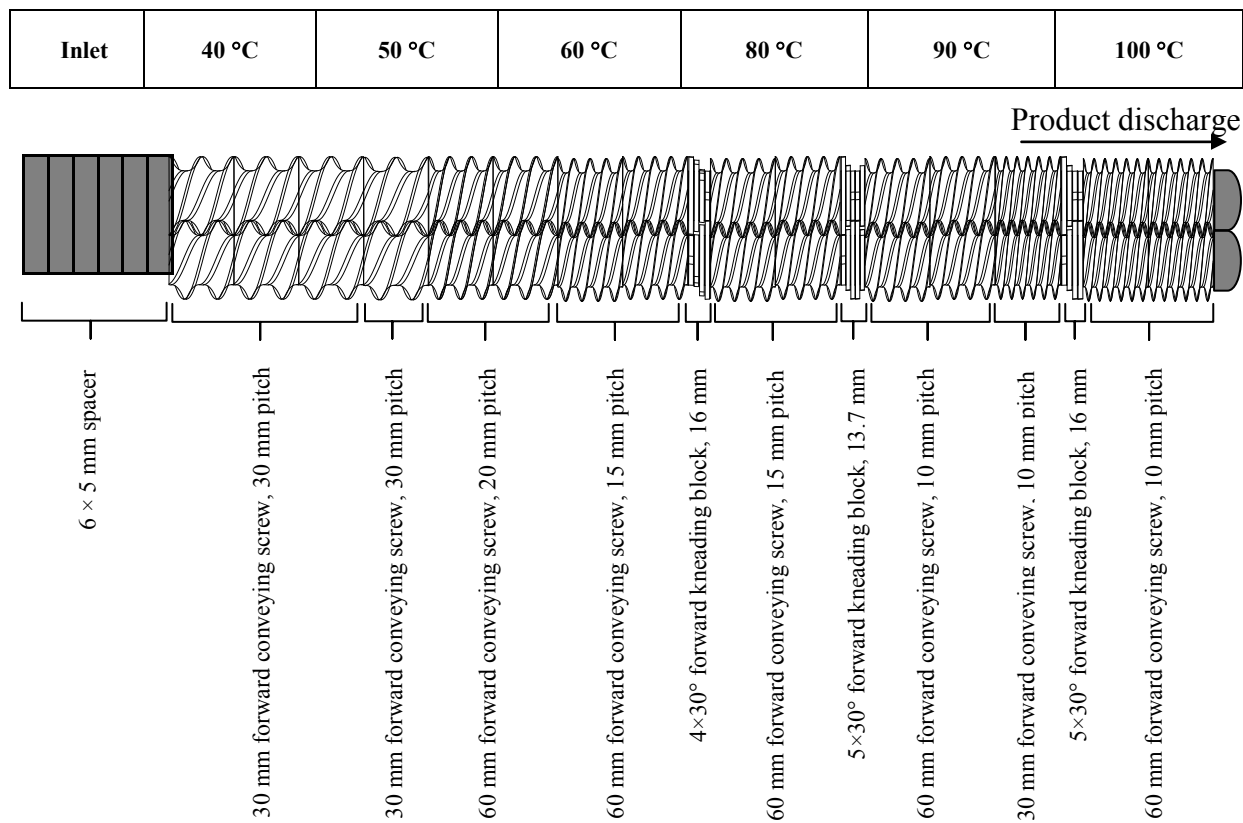


Fig. 7-1. Screw configuration and temperature profile of M-18 twin screw extruder.

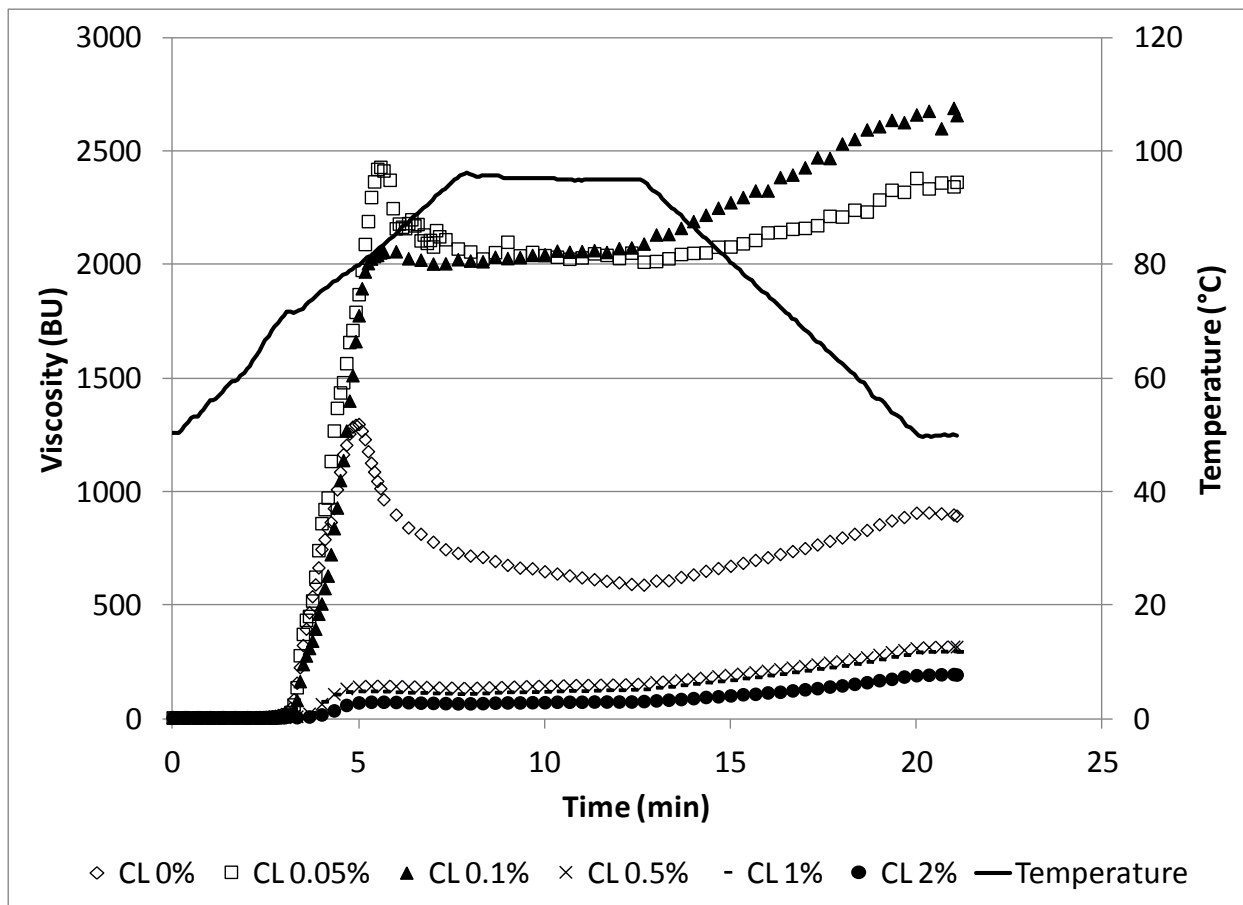


Fig. 7-2. The pasting curves of cross-linked (CL) waxy maize starches with different cross-linking levels. CL waxy maize starch was prepared with sodium trimetaphosphate (STMP) and sodium tripolyphosphate (STPP) at the ratio of 99:1 between 0-2% of the starch dry weight.

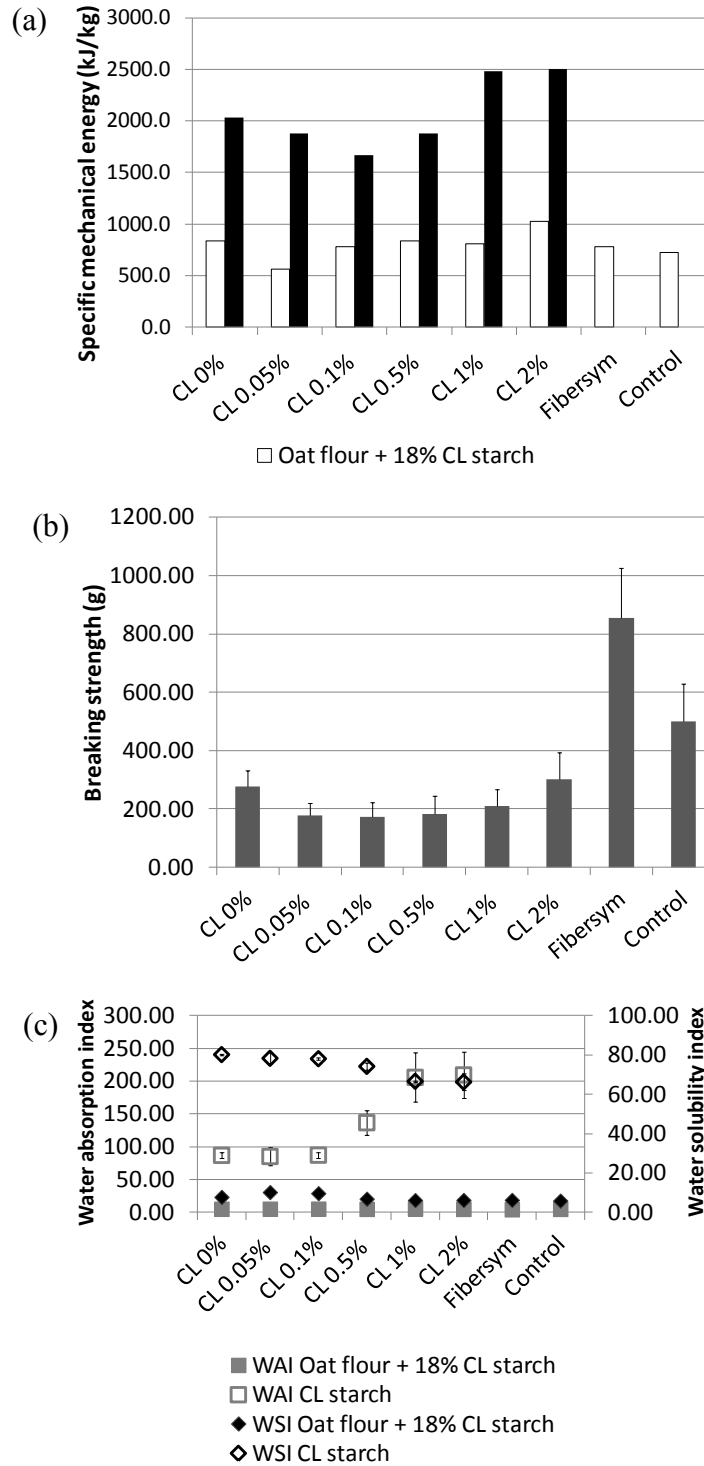


Fig. 7-3. (a) Specific mechanical energy, (b) breaking strength and (c) water absorption index (WAI) and water solubility index (WSI) of extruded cross-linked (CL) starches and extruded oat flour-CL starches. Control is extruded oat flour.

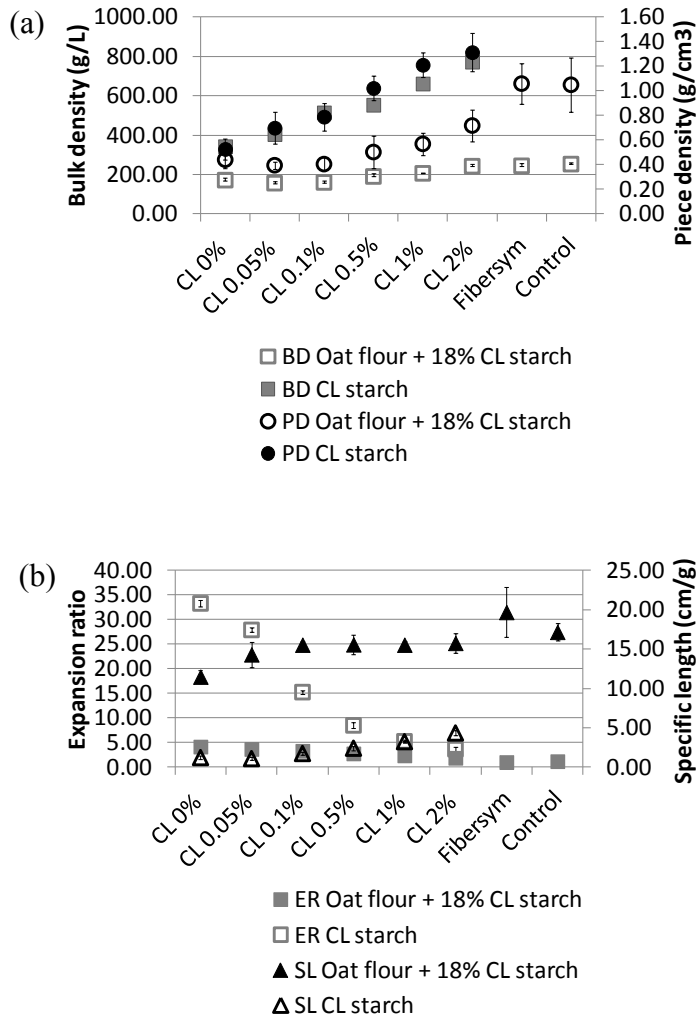
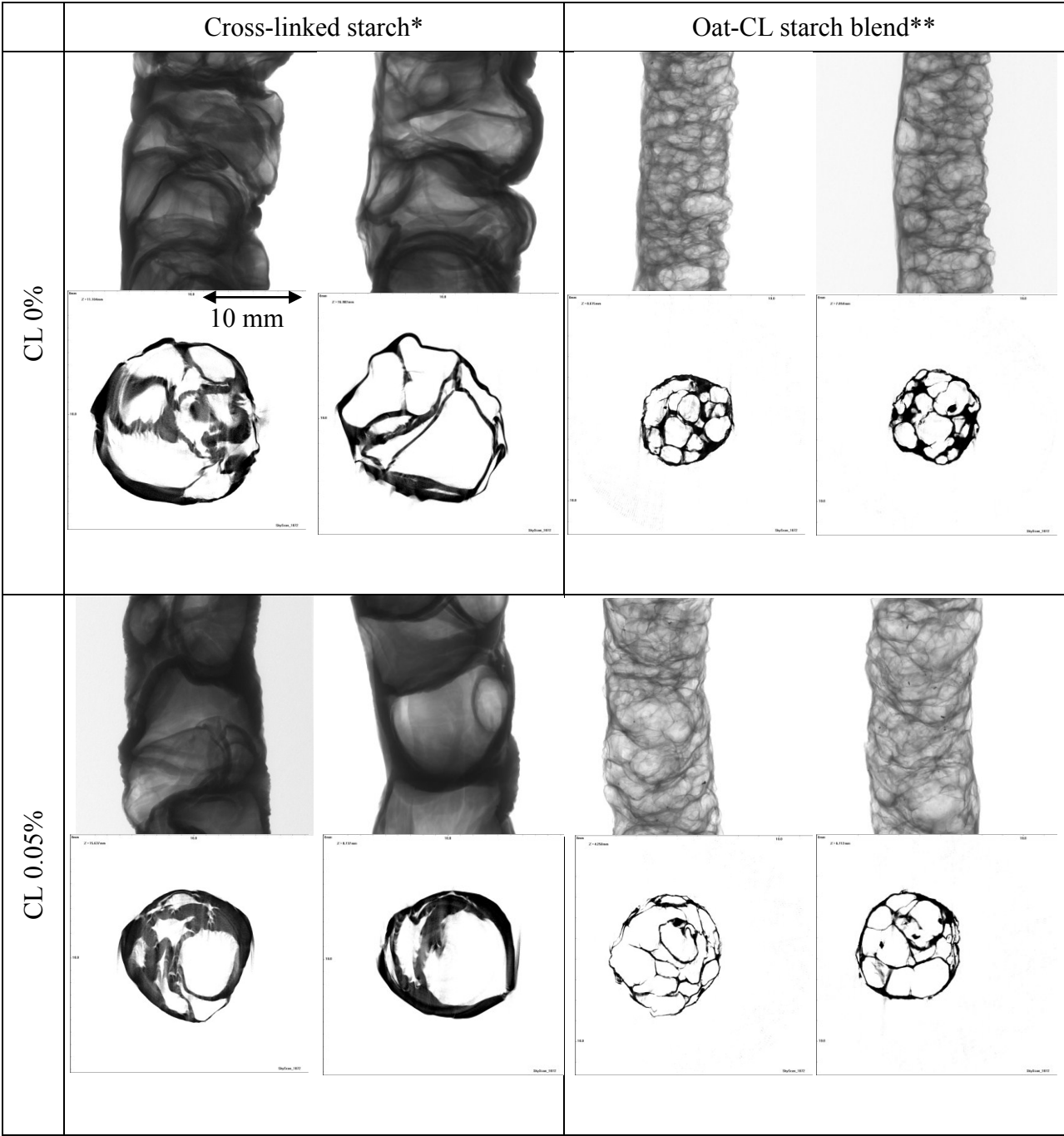
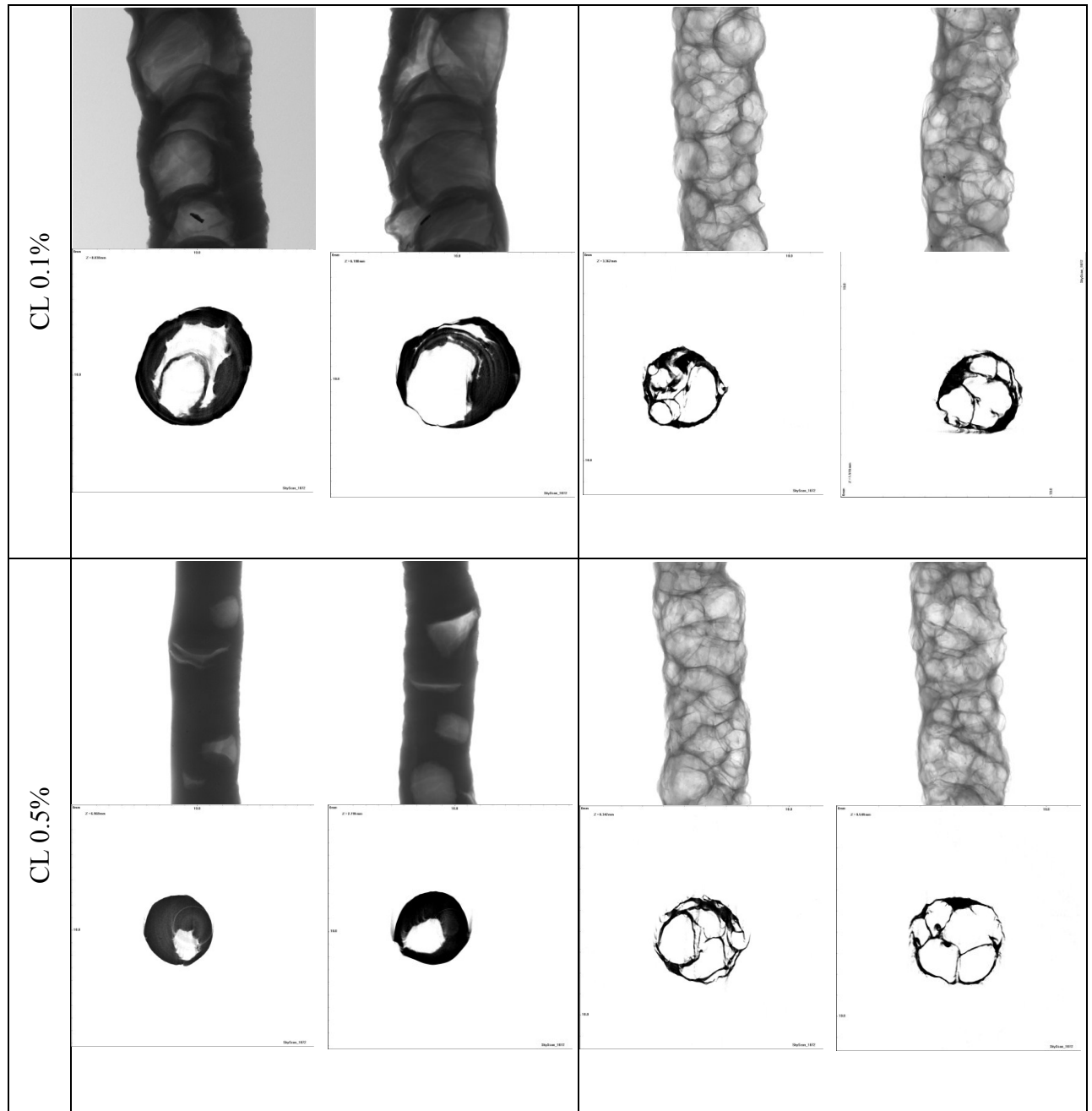
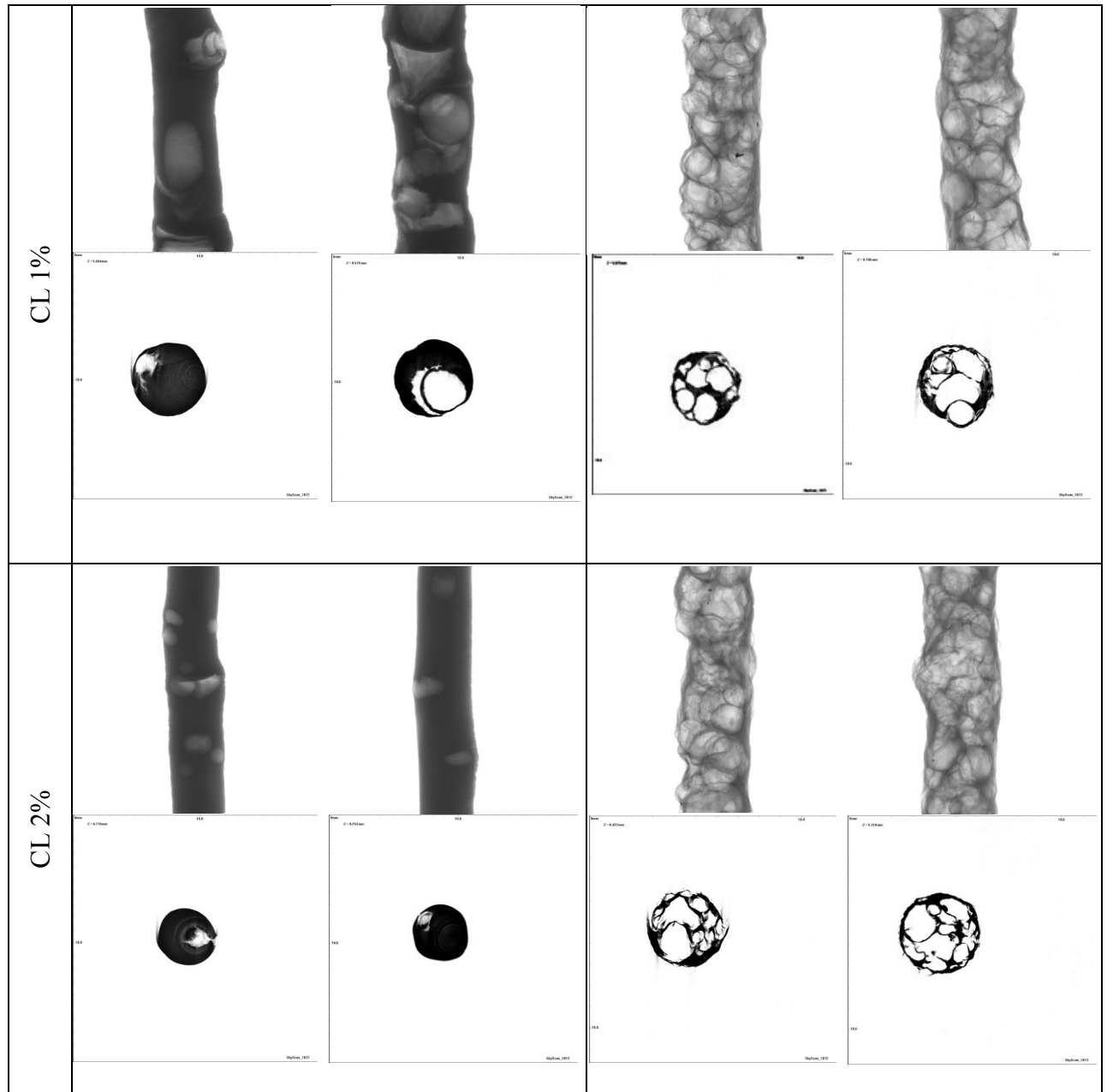


Fig. 7-4. Physical properties of extruded products- (a) bulk density (BD) and piece density (PD), (b) expansion ratio (ER) and specific length (SL) of extruded cross-linked (CL) starches and extruded oat flour-CL starches. Control is extruded oat flour.







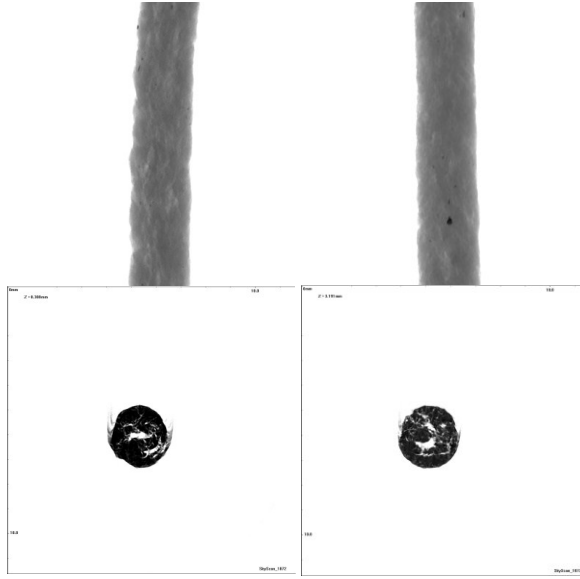
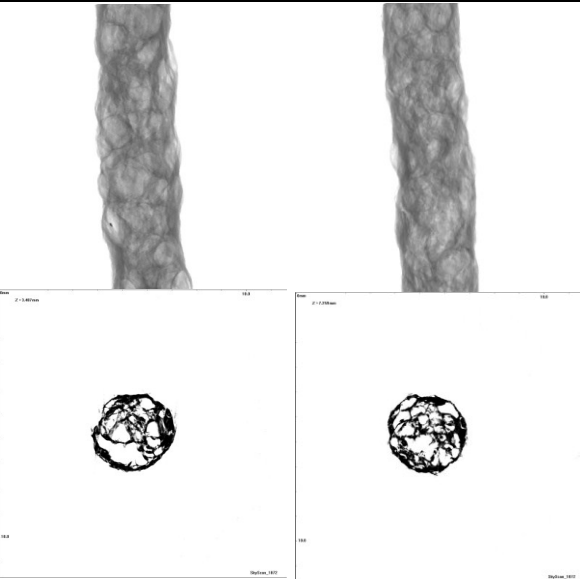
<p style="writing-mode: vertical-rl; transform: rotate(180deg);">Fibersym</p>	<p>Could not be extruded due to clogging of extruder.</p>	
<p style="writing-mode: vertical-rl; transform: rotate(180deg);">Oat flour***</p>	<p>Not applicable because only 100% oat flour was extruded to act as control.</p>	

Fig. 7-5. Radial (top) and longitudinal (bottom) representative XMT images of extruded cross-linked (CL) starches and extruded oat flour-CL starches. * Extruded CL starch; ** Extruded OF and CL starch formulations at the ratio of 82:18; *** Extruded OF is 100% which act as the control of extruded OF-CL starch formulations.

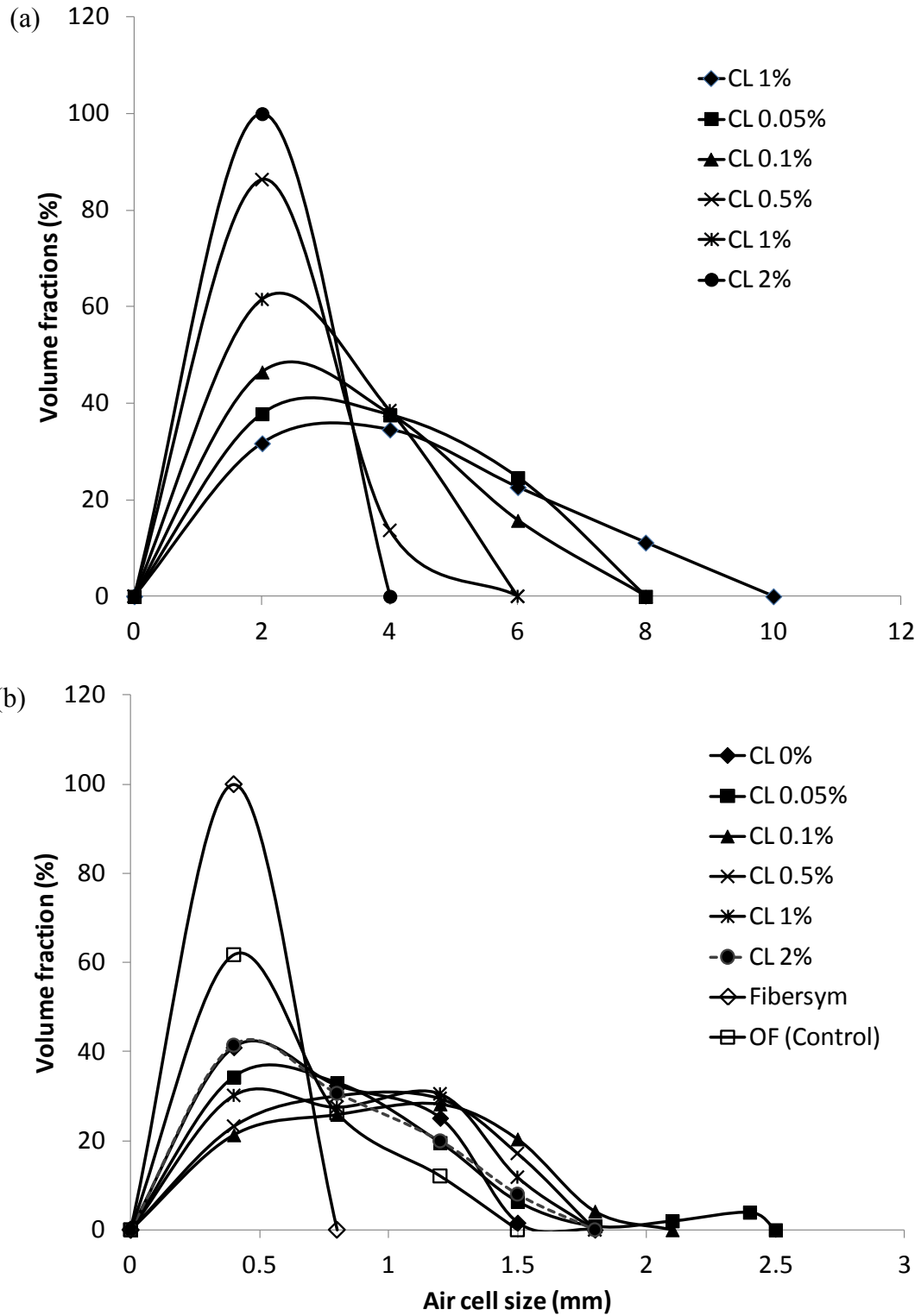


Fig. 7-6. Air cell size distribution of extruded products- (a) cross-linked (CL) starch, (b) oat flour (OF)-CL starch blends.

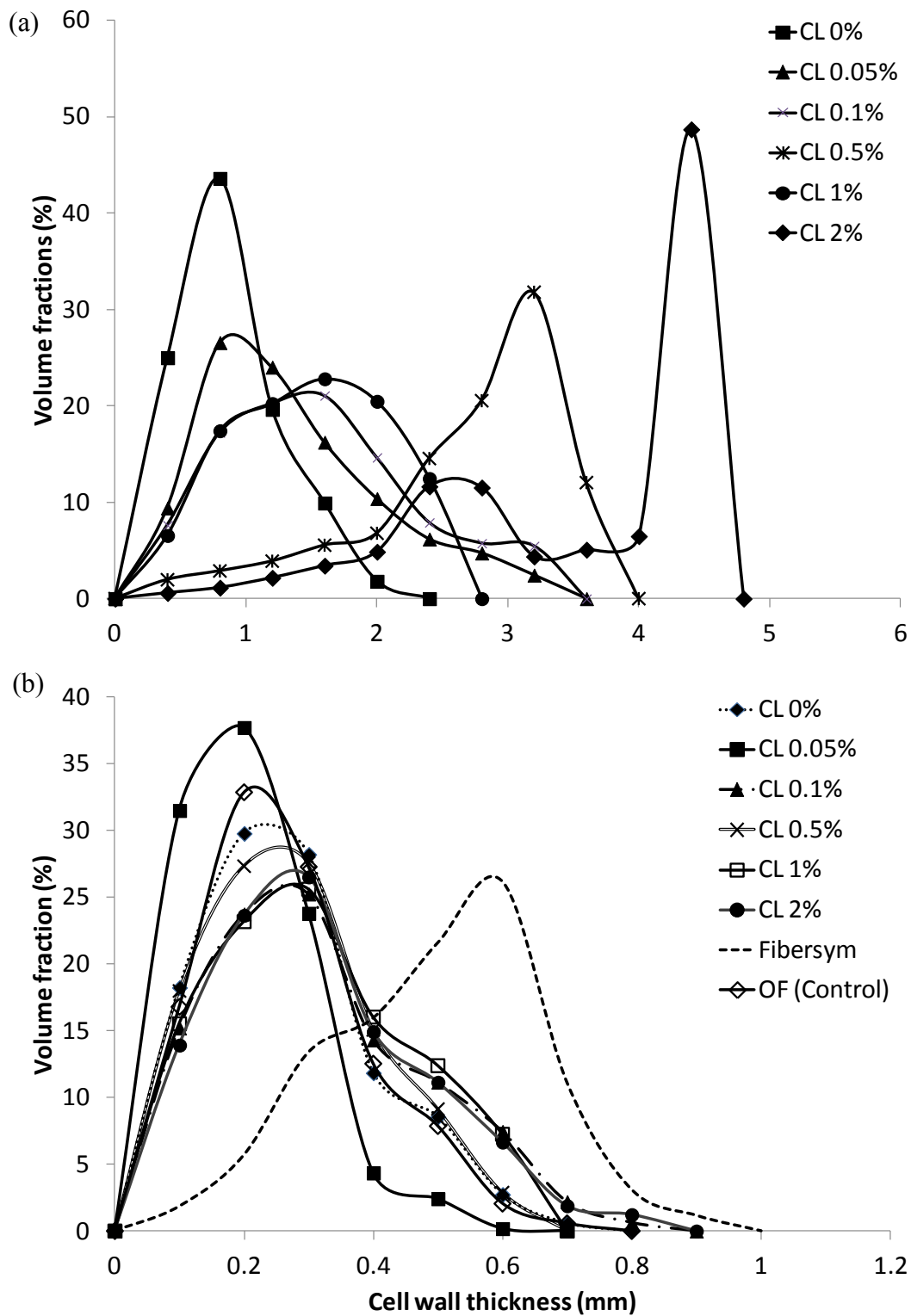


Fig. 7-7. Cell wall thickness distribution of extruded products- (a) cross-linked (CL) starch, (b) oat flour (OF)-CL starch blends.

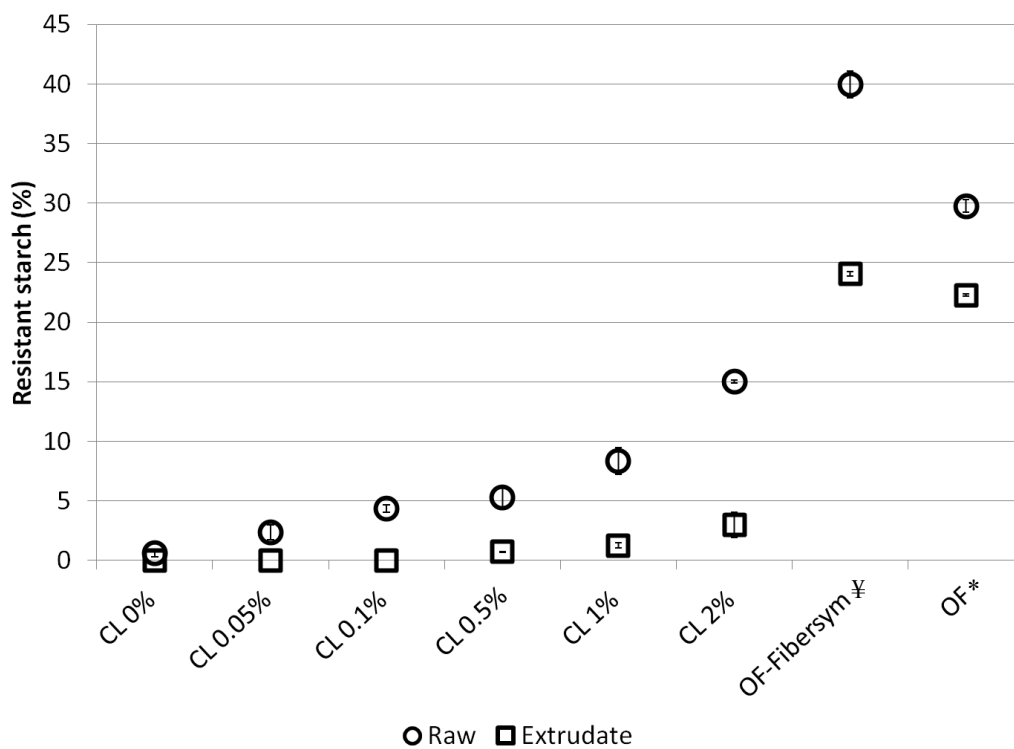


Fig. 7-8. Resistant starch content of pre-extrusion and post-extrusion of cross-linked (CL) starch. ¥ OF-Fibersym: Oat flour-Fibersym blend at the ratio of 82:18; * OF: Oat flour act as a control to compare with OF-Fibersym.

Overall conclusions

Digestibility of CL maize starch was found to elevate with increasing amylose and incubation temperature in *in-vitro* total dietary fiber and RS methods. Higher degree of swelling in CL waxy maize starch increased the susceptibility of starch granules to digestive enzymes. At higher temperature (80 and 95 °C), the swelling of starch granules of native waxy and normal maize starches increased significantly and became more susceptible to enzyme digestion. The knowledge of swelling and digestion of CL starches with different amylose contents is useful in food processing to attain certain functionalities and nutritional properties in food products

Alkali treatment on low levels of cross-linking in starch affected the ratios of total bound phosphate esters which changed the degree of starch swelling, crystallinity and pasting properties of the CL starch. The study showed that treatments at alkaline pH range offer a way to manipulate the pasting and rheological properties of CL starch.

The digestion of CL wheat starch was observed to be progressing after 16 h of incubation in the AOAC Method 2009.01 which may not reflect *in-vivo* response in human. This study offers insight into improving the *in-vitro* digestive method so it better reflects the *in-vivo* conditions, which is very important in the food industry so that it will represent the right information to the consumers on RS or total dietary fiber in food products.

CL wheat starch was able to be solubilized by using a new developed procedure that involved usage of digestive enzymes which led to an improved method to determine RS type 4 directly in food products. The developed procedure successfully solubilized RS4 by using digestive enzymes, specifically thermo-stable α -amylase and amyloglucosidase.

Extrusion resulted in the breakdown of starch and increased insoluble protein for all extrudates. High energy input plays a major role in radial expansion of normal hard wheat extrudate, whereas higher amylopectin in soft waxy wheat flour was a dominant factor in determining the radial expansion when compared to normal soft wheat. The high lipid content of hard waxy wheat caused SME to reduce, thus reducing the radial expansion of hard waxy wheat. Higher formation of high molecular weight insoluble protein in hard waxy wheat extrudate may also play a role in causing dextrinization of starch and hindering the radial expansion.

Incorporation of soft waxy wheat (40 and 50% of total weight) in oat flour formulation increased the expansion ratio and reduced the breaking strength and bulk density of extrudates.

The lowest moisture content (18%) in the oat flour-soft waxy wheat formulations produced the highest radial expansion and void fraction as compared to 20 and 22% moisture contents.

The phosphorus content of CL starched increased with increasing cross-linking levels. Low cross-linking level of CL waxy maize starch in oat flour formulation increased the void fraction and reduced the breaking strength of extrudates. Higher cross-linking levels of CL starches improved the RS level on OF formulation but had very poor structural and textural properties.

Chapter 8 – Appendix

Moisture—Air-Oven Methods (AOAC Method 44-15)

Procedure

1. Transfer 3-g portion to each of two or more tared moisture dishes. Cover and weigh dishes at once. Subtract tare weights and record weight of sample.
2. Uncover dishes and place them on shelf of oven at 103 °C; place covers under dishes. Insert shelf in oven at level of thermometer bulb. Heat for exactly 60 min after oven recovers its temperature.

Calculation

$$\% \text{ Moisture} = (A/B) \times 100$$

in which A = moisture loss in grams, B = original weight of sample.

Phosphorus content

Source: Smith, R. J., Caruso, J. 1964. Determination of phosphorus. Methods Carbohydrate Chemistry. 4, 42–46.

Reagents

- 1) Zinc acetate solution, 10% - 120 g of zinc acetate dehydrate is dissolved in 880 mL of water. The solution is filtered through Whatman No 12 if hazy.
- 2) Ammonium vanadate, 0.25% - 2.5 g of ammonium metavanadate is dissolved in 600 ml of boiling water and the solution is cooled to 60-70 °C. 20 mL of nitric acid (90 %) is added. After the solution is cooled on to room temperature, dilute it to 1 L water.
- 3) Ammonium molybdate, 5% - 50 g of ammonium molybdate tetrahydrate is dissolved in 900 mL of warm water. After the solution is cooled on to room temperature, dilute it to 1 L water.
- 4) Nitric acid, 29% - 300 mL of concentrated nitric acid (70%) is mixed with 600 mL of water.
- 5) Standard phosphorus solution, 0.1 mg of phosphorus per mL – 0.439 g of reagent grade potassium dihydrogen phosphate is dissolved in water and the solution is diluted to 1 L. Standards are prepared by mixing 0 mL (0 mg), 5 mL (0.5 mg), 10 mL (1.0 mg), 15 mL (1.5 mg), 20 mL (2.0 mg), 25 ml (2.5 mg) in 10 mL 29% nitric acid, 10 mL 0.25% ammonium vanadate and 10 mL 5% ammonium molybdate.

Procedure

- 1) Sample is weighed into dish (Normal starch or low cross-linked starch: 3 g; High cross-linked starch: 0.5 g) and add 10 mL 10% zinc acetate.
- 2) Heat on hot plate until sample is charred thoroughly.
- 3) Ignite for 2 hours at 550 °C, then cool to room temperature.
- 4) Wet residue with 3 mL of 29% nitric acid.
- 5) Evaporate completely on hot plate.
- 6) Ignite at 550 °C for 30 min and cool it to room temperature.
- 7) Residue is washed with 10 mL of 29% nitric acid and 15 mL of water.

- 8) The dish is covered with a watch glass and heated to boil for 10 min.
- 9) Solution is quantitatively transferred to 100 mL volumetric flask and filtered through Whatman No. 1 paper.
- 10) The following solutions are added in order and mixed after each addition- 10 mL 29% nitric acid, 10 mL 0.25% ammonium vanadate and 10 mL 5% ammonium molybdate.
- 11) Stand for at least 10 min and read absorbance at 460 nm.

Calculation

$$\% \text{ Phosphorus} = (P \times \text{Dilution volume} \times 100) / (\text{Aliquot volume} \times \text{sample weight in g} \times 1000)$$

In which P= phosphorus content (mg/100 mL) from calibration curve

$$\% \text{ phosphate} = \% \text{ phosphorus} \times 3.065$$

Preparation of cross-linked starch

Source: Woo, K. S., Seib, P. A. 2002. Cross-linked resistant starch: Preparation and properties. Cereal Chemistry. 79, 819–825.

Reagents

- 1) Starch
- 2) Sodium sulfate (Na_2SO_4)
- 3) Sodium trimetaphosphate (STMP)
- 4) Sodium tripolyphosphate (STPP)
- 5) 1M sodium hydroxide (NaOH)
- 6) 1M hydrochloric acid (HCl)
- 7) Distilled water

Procedure

Starch (50g, db) + Na_2SO_4 (5g) + Water (70 mL) + STMP/STPP



Adjust slurry to pH 11.5 using 1M NaOH



Hold at 45°C for 3 hours in a water bath (constant stirring)



Adjust slurry to pH 6.5 using 1M HCl



Wash 7 times



Dry at 40°C overnight



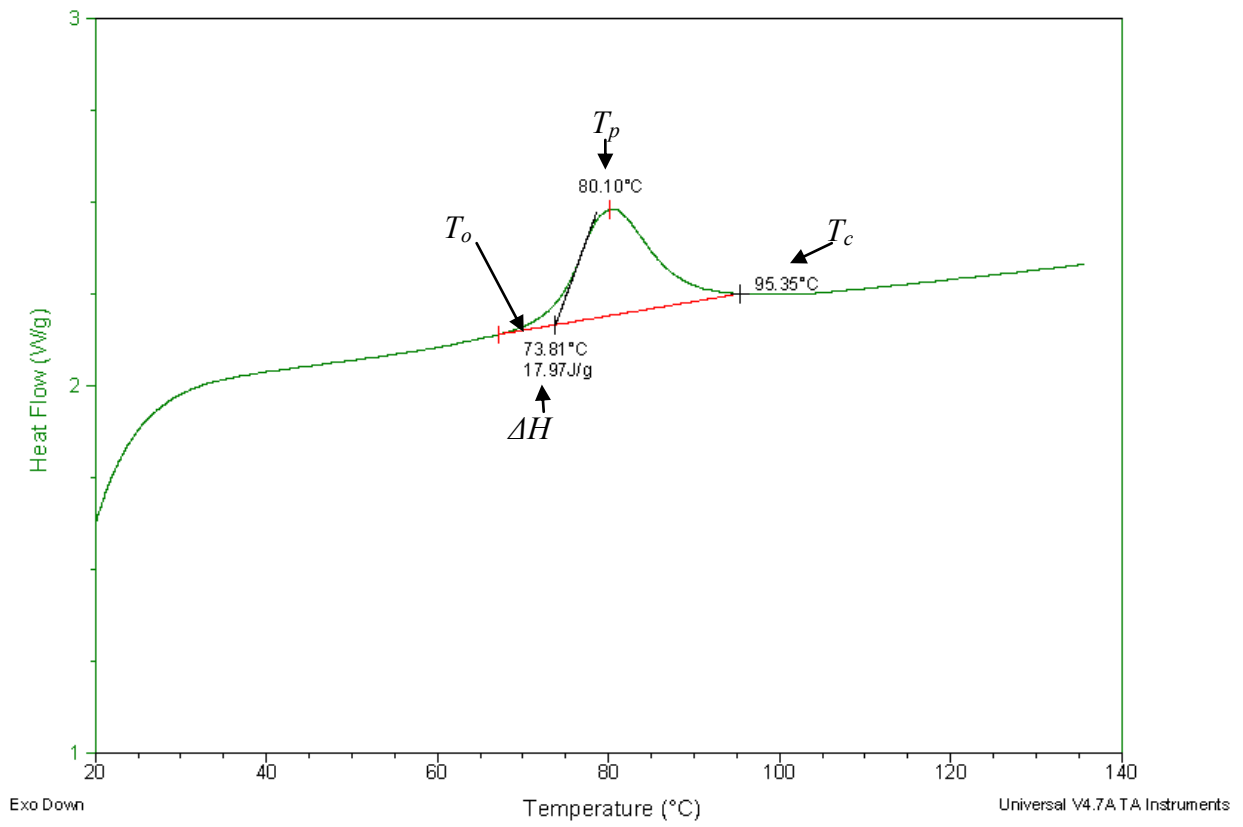
Grind to fine powder

www.researchgate.net

Photographs of settling volume cross-linked maize starches.



Differential scanning calorimeter – gelatinization transition temperature curve



Total dietary fiber method (AOAC Method 991.43)

Reagents:

- 1) Ethanol, 95% v/v.
- 2) Ethanol, 78%. Place 207 mL water into 1-L volumetric flask. Dilute to volume with 95% ethanol. Mix.
- 3) Acetone, reagent grade.
- 4) Enzymes for TDF assay (Megazyme International Ireland Limited). Store at 0-5°C.
 - a- α -Amylase, heat-stable (E-BLAAM); 3,000 Ceralpha Units/mL.
 - b- Protease (E-BSPRT); 50 mg/mL; 350 Tyrosine Units/mL.
 - c- Amyloglucosidase (E-AMGDF); 200 p -NP -maltoside Units/mL (or 3300 Units/mL on soluble starch).
- 5) Deionised water.
- 6) Celite, acid-washed, pre-ashed (Megazyme G-CEL100 or G-CEL500).
- 7) MES/TRIS buffer, 0.05 M each, pH 8.2 at 24°C. Dissolve 19.52 g 2 (N-morpholino) ethanesulfonic acid (MES) (Sigma, M 8250) and 12.2 g tris (hydroxymethyl) aminomethane (TRIS) (Sigma, T1503) in 1.7 L deionised water. Adjust pH to 8.2 with 6.0 N NaOH. Dilute to 2 L with water. It is important to adjust pH of buffer to approximately 8.3 at 20°C or approximately 8.1 at 27-28°C.
- 8) Hydrochloric acid solution, 0.561 N. Add 93.5 mL of 6 N HCl to approximately 700 mL of water in 1-L volumetric flask. Dilute to 1-L with water.

Procedure

- 1) Blanks - With each assay, run two blanks along with samples to measure any contribution from reagents to residue.
- 2) Samples
 - a- Weigh duplicate 1.000 ± 0.005 g samples accurately into 400 mL tall-form beakers.
 - b- Add 40 mL MES-TRIS blend buffer solution (pH 8.2) to each beaker. Add magnetic stirring bar to each beaker. Stir on magnetic stirrer until sample is completely dispersed in solution. (This prevents lump formation, which would make sample inaccessible to enzymes.)
- 3) Incubation with heat-stable α -amylase

- a- Add 50 μL heat-stable α -amylase solution, while stirring at low speed.
 - b- Cover each beaker with aluminium foil squares.
 - c- Place covered samples in shaking water bath at 95-100°C and incubate for 35 min with continuous agitation. Start timing once all beakers are in hot water bath.
- 4) Cool
- a- Remove all sample beakers from hot water bath and cool to 60°C. Remove foil covers. Scrape any ring around beaker and gels in bottom of beaker with spatula, if necessary.
 - b- Rinse side wall of beaker and spatula with 10 mL distilled water by using pipettor. Adjust temperature of water bath to 60°C by draining some of hot water from water bath and adding cold water.
- 5) Incubation with protease
- a- Add 100 μL protease solution to each sample.
 - b- Re-cover with aluminium foil.
 - c- Incubate in shaking water bath at 60 \pm 1°C, with continuous agitation for 30 min. Start timing when temperature of water bath reaches 60°C.
- 6) pH check
- a- Remove sample beakers from shaking water bath.
 - b- Remove covers.
 - c- Dispense 5 mL of 0.561 N HCl solution into sample while stirring.
 - d- Check pH, which should be 4.1- 4.8. Adjust pH, if necessary, with additional 5% NaOH solution or 5% HCl solution
- 7) Incubation with amyloglucosidase
- a- Add 200 μL amyloglucosidase solution while stirring on magnetic stirrer.
 - b- Replace aluminium cover.
 - c- Incubate in shaking water bath at 60°C for 30 min, with constant agitation. Start timing when temperature of water bath reaches 60°C.
- 8) Precipitation of soluble dietary fibre Add 160 mL of 95% (v/v) EtOH pre-heated to 60°C to each of the bottles detailed in point 8. Mix well on a magnetic stirrer and then remove the magnetic stirrer bar. Let the precipitate form at room temperature for 60 min.
- 9) Filtration setup

- a- Tare crucible containing Celite to nearest 0.1 mg.
 - b- Wet and redistribute bed of Celite in crucible using approximately 3 mL distilled water.
 - c- Apply suction to crucible to draw Celite onto fritted glass as even mat.
- 10) Filter enzyme mixture from Step 7 through crucible into a filtration flask.
- 11) Wash residue twice with 10 mL distilled water preheated to 70°C. Use water to rinse beaker before washing residue in crucible.
- 12) Wash residue twice with 10 mL of:
- a- 95% EtOH
 - b- Acetone
- 13) Dry crucible containing residue overnight in 103°C oven.
- 14) Cool crucible in desiccator for approximately 1 hr. Weigh crucible containing dietary fibre residue and Celite to nearest 0.1 mg. To obtain residue weight, subtract tare weight, i.e., weight of dried crucible and Celite.
- 15) Protein and ash determination.
- One residue from each type of fibre is analysed for protein, and the second residue of the duplicate is analysed for ash.

Calculation

$$\text{Dietary Fibre (\%)} = \frac{\frac{R_1 + R_2}{2} - p - A - B}{\frac{m_1 + m_2}{2}} \times 100$$

where:

R_1 = residue weight 1 from m_1 ; R_2 = residue weight 2 from m_2 ;

m_1 = sample weight 1; m_2 = sample weight 2;

A = ash weight from R_1 ; p = protein weight from R_2 ; and

B = blank

$$= \frac{BR_1 + BR_2}{2} - BP - BA$$

where:

BR = blank residue;

BP = blank protein from BR_1 ;

BA = blank ash from BR_2 .

Available Carbohydrate Method and Dietary Fiber

Reagents:

- 1) Ethanol, 95% v/v.
- 2) Ethanol, 78%. Place 207 mL water into 1-L volumetric flask. Dilute to volume with 95% ethanol. Mix.
- 3) Acetone, reagent grade.
- 4) Enzymes for TDF assay (Megazyme International Ireland Limited). Store at 0-5°C.
 - α -Amylase, heat-stable (E-BLAAM); 3,000 Ceralpha Units/mL.
 - Protease (E-BSPRT); 50 mg/mL; 350 Tyrosine Units/mL.
 - Amyloglucosidase (E-AMGDF); 200 p-NP-maltoside Units/mL (or 3300 Units/mL on soluble starch).
- 5) Deionised water.
- 6) Celite, acid-washed, pre-ashed (Megazyme G-CEL100 or G-CEL500).
- 7) MES/TRIS buffer, 0.05 M each, pH 8.2 at 24°C. Dissolve 19.52 g 2(N-morpholino) ethanesulfonic acid (MES) (Sigma, M 8250) and 12.2 g tris (hydroxymethyl) aminomethane (TRIS) (Sigma, T1503) in 1.7 L deionised water. Adjust pH to 8.2 with 6.0 N NaOH. Dilute to 2 L with water. It is important to adjust pH of buffer to approximately 8.3 at 20°C or approximately 8.1 at 27-28°C.
- 8) Hydrochloric acid solution, 0.561 N. Add 93.5 mL of 6 N HCl to approximately 700 mL of water in 1-L volumetric flask. Dilute to 1-L with water.
- 9) Sodium maleate buffer, 0.1 M, pH 6.2. Dissolve 11.6 g of maleic acid (Sigma cat. no. M-0375) in 900 mL of distilled water and adjust the pH to 6.2 with 2 M NaOH (approx 80 mL is required). Add sodium azide (0.2 g) and adjust the volume to 1 litre. Stable for > 1 year at room temperature.

Method

- 1) Blanks - With each assay, run two blanks along with samples to measure any contribution from reagents to residue.
- 2) Samples
 - a- Weigh duplicate 1.000±0.005 g samples accurately into 250 mL Duran bottles.

- b- Add 40 mL MES-TRIS blend buffer solution (pH 8.2) to each bottle. Add magnetic stirring bar to each bottle. Stir on magnetic stirrer until sample is completely dispersed in solution. (This prevents lump formation, which would make sample inaccessible to enzymes.)
- 3) Incubation with heat-stable α -amylase
- a- Add 50 μ L heat-stable α -amylase solution, while stirring at low speed.
 - b- Seal each bottle with plastic caps.
 - c- Place sealed bottles in shaking water bath at 80°C and incubate for 35 min with continuous agitation. Start timing once all beakers are in hot water bath.
- 4) Cool
- a- Remove all sample beakers from hot water bath and cool to 60°C. Remove foil covers. Scrape any ring around beaker and gels in bottom of beaker with spatula, if necessary.
 - b- Rinse side wall of beaker and spatula with 10 mL distilled water by using pipettor. Adjust temperature of water bath to 60°C by draining some of hot water from water bath and adding cold water.
- 5) Incubation with protease
- a- Add 100 μ L protease solution to each sample. Re-cover with aluminium foil. Incubate in shaking water bath at 60 \pm 1°C, with continuous agitation for 30 min. Start timing when temperature of water bath reaches 60°C.
- 6) pH check
- Remove sample beakers from shaking water bath. Remove covers. Dispense 5 mL of 0.561 N HCl solution into sample while stirring. Check pH, which should be 4.1- 4.8. Adjust pH, if necessary, with additional 5% NaOH solution or 5% HCl solution
- 7) Incubation with amyloglucosidase
- Add 200 μ L amyloglucosidase solution while stirring on magnetic stirrer. Replace aluminium cover. Incubate in shaking water bath at 60°C for 30 min, with constant agitation. Start timing when temperature of water bath reaches 60°C.
- 8) Precipitation of soluble dietary fibre Add 160 mL of 95% (v/v) EtOH pre-heated to 60°C to each of the bottles detailed in point 8. Mix well on a magnetic stirrer and then remove the magnetic stirrer bar. Let the precipitate form at room temperature for 60 min.

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Tare crucible containing Celite to nearest 0.1 mg. Wet and redistribute bed of Celite in crucible using approximately 3 mL distilled water. Apply suction to crucible to draw Celite onto fritted glass as even mat.

10) Filter enzyme mixture from Step 7 through crucible into a filtration flask.

11) Wash residue twice with 10 mL distilled water preheated to 70°C. Use water to rinse beaker before washing residue in crucible.

12) Wash residue twice with 10 mL of:

- 95% EtOH

- Acetone

13) Dry crucible containing residue overnight in 103°C oven.

14) Cool crucible in desiccator for approximately 1 hr. Weigh crucible containing dietary fibre residue and Celite to nearest 0.1 mg. To obtain residue weight, subtract tare weight, i.e., weight of dried crucible and Celite.

15) Protein and ash determination.

One residue from each type of fibre is analysed for protein, and the second residue of the duplicate is analysed for ash.

Calculation

$$\text{Dietary Fibre (\%)} = \frac{\frac{R_1 + R_2}{2} - p - A - B}{\frac{m_1 + m_2}{2}} \times 100$$

where:

R_1 = residue weight 1 from m_1 ; R_2 = residue weight 2 from m_2 ;

m_1 = sample weight 1; m_2 = sample weight 2;

A = ash weight from R_1 ; p = protein weight from R_2 ; and

B = blank

$$= \frac{BR_1 + BR_2}{2} - BP - BA$$

where:

BR = blank residue;

BP = blank protein from BR_1 ;

BA = blank ash from BR_2 .

Total Carbohydrate

Source: Dubois, M. K. A. Gilles, Hamilton, J. K., Rebers, P. A., & Smith, F. (1956).

Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, 28, 350–356.

Reagents

- 1) Sulfuric acid, reagent grade 95.5%
- 2) Phenol 80% - Add 20 g of water into 80 g of reagent grade phenol.

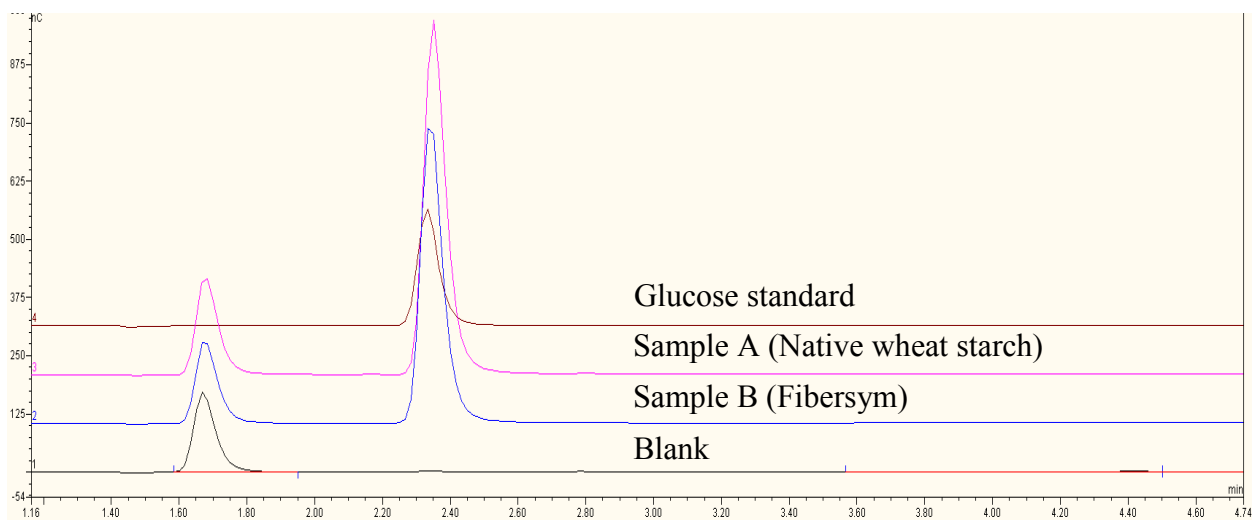
Methods

- 1) Two mL of sugar solution containing between 10 to 70 μL of sugar is pipette into a colorimetric tube.
- 2) Add 0.05 mL of 80% phenol into the tube.
- 3) Then, add 5 mL of concentrated sulfuric acid directly on the liquid surface rather than against the side of the test tube for good mixing.
- 4) Let the tubes rest for 10 min, then shake and placed for 10 to 20 min in a water bath at 30 $^{\circ}\text{C}$.
- 5) The yellow-orange color produced is measured spectrometrically at 490 nm.
- 6) Blanks are prepared by substituting distilled water for the sugar solution. The amount of sugar will be determined by reference to a standard curve previously constructed for the particular sugar under examination.

Determination of glucose by HPAEC

Standard and sample preparation

- 1) For standard stock, weigh 1 mg glucose standard and add 1 mL of deionized water into a 2 mL centrifuge tube. Vortex for 5 min to dissolve the glucose. The standard stock is diluted to 10 and 20 $\mu\text{g/mL}$.
- 2) For sample preparation, sample is prepared in concentrations within 5-35 $\mu\text{g/mL}$.



HPAEC spectra of glucose standard, diluted native wheat starch, diluted Fibersym and blank.

Integrated total dietary fiber (AOAC Method 2009.01)

Reagents:

- 1) Ethanol, 95% v/v.
- 2) Ethanol, 78%. Place 207 mL water into 1-L volumetric flask. Dilute to volume with 95% ethanol. Mix.
- 3) Acetone, reagent grade.
- 4) Stock amyloglucosidase (AMG) solution - 3300 Units/mL in 50% v/v glycerol – Solution is viscous; dispense using a positive displacement dispenser.
- 5) Pancreatic α -amylase (50 Units/mL)/AMG (3.4 Units/mL). - Immediately before use, dissolve 0.10 g of purified porcine pancreatic α -amylase (150,000 Units/g) in 290 mL of sodium maleate buffer (50 mM, pH 6.0 plus 2 mM CaCl_2 and 0.02% sodium azide) and stir for 5 min. Add 0.3 mL of amyloglucosidase
- 6) Protease (50 mg/mL; 350 Tyrosine Units/mL) in 50% v/v glycerol

Method

**Sample + maleate buffer with α -amylase & amyloglucosidase (40 mL)
Placed in shaking water bath (37°C, 16 hours)**

Add 0.75M trizma base (3 mL). Heat at 95-100°C, 20 min

Cool to 60°C, add protease (100 μL). Incubate (60°C, 30 min)

**Mix in 2M acetic acid (4 mL) & Internal standard (1 mL).
Add 95% EtOH (192 mL, preheated to 60°C) for precipitation.**

Filter

Total starch

Reagents

- 1) Sodium acetate buffer (100 mM, pH 5.0) plus calcium chloride (5 mM) - Add 5.8 mL of glacial acetic acid (1.05 g/mL) to 900 mL of distilled water. Adjust the pH to 5.0 by the addition of 1 M (4 g/100 mL) sodium hydroxide solution (approx. 30 mL is required). Add 0.74 g of calcium chloride dihydrate and dissolve.
- 2) Sodium acetate buffer (1.2 M, pH 3.8)- Add 69.6 mL of glacial acetic acid (1.05 g/mL) to 800 mL of distilled water and adjust to pH 3.8 using 4 M sodium hydroxide. Adjust the volume to 1 litre with distilled water.
- 3) Potassium hydroxide solution (2 M)- Add 112.2 g KOH to 900 mL of deionised water and dissolve by stirring. Adjust volume to 1 litre. Store in a sealed container. Stable for > 2 years at room temperature.

Procedure

- 1) Mill cereal, plant or food product to pass a 0.5 mm screen.
- 2) Add milled sample (~100 mg; weighed accurately) to a glass test tube (16 x 120 mm). Tap the tube to ensure that all of the sample drops to the bottom of the tube.
- 3) Add 0.2 mL of aqueous ethanol (80 % v/v) to wet the sample and aid dispersion. Stir the tube on a vortex mixer.
- 4) Immediately add 3 mL of thermostable α -amylase (Diluted 1:30 in Reagent 1; 100 mM sodium acetate buffer, pH 5.0). Incubate the tube in a boiling water bath for 6 min. (Stir the tube vigorously after 2, 4 and 6 min).
- 5) Place the tube in a bath at 50°C; add 0.1 mL amyloglucosidase (330 U on starch). Stir the tube on a vortex mixer and incubate at 50°C for 30 min.
- 6) Transfer the entire contents of the test tube to a 100 mL volumetric flask (with a funnel to assist transfer). Use a wash bottle to rinse the tube contents thoroughly. Adjust to volume with distilled water. Mix thoroughly. Centrifuge an aliquot of this solution at 3,000 rpm for 10 min. Use the clear, undiluted filtrate for the assay.
- 7) Transfer duplicate aliquots (0.1 mL) of the diluted solution to the bottom of glass test tubes (16 x 100 mm).

- 8) Add 3.0 mL of GOPOD Reagent to each tube (including the D-glucose controls and reagent blanks), and incubate the tubes at 50°C for 20 min.
- 9) D-Glucose controls consist of 0.1 mL of D-glucose standard solution (1 mg/mL) and 3.0 mL of GOPOD Reagent.
- 10) Blank solutions consist of 0.1 mL of water and 3.0 mL of GOPOD Reagent.
- 11) Read the absorbance for each sample, and the D-glucose control at 510 nm against the reagent blank.

Breaking strength

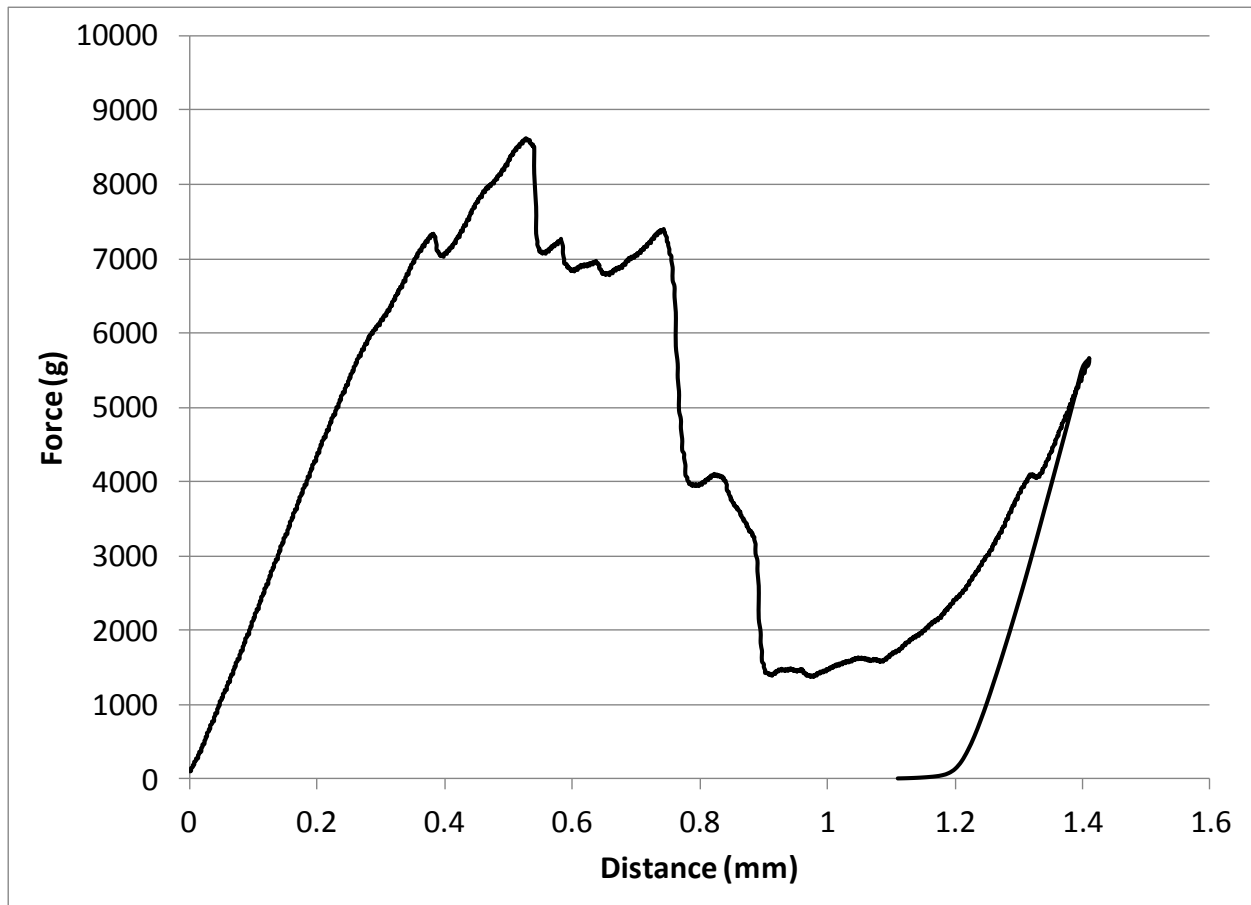
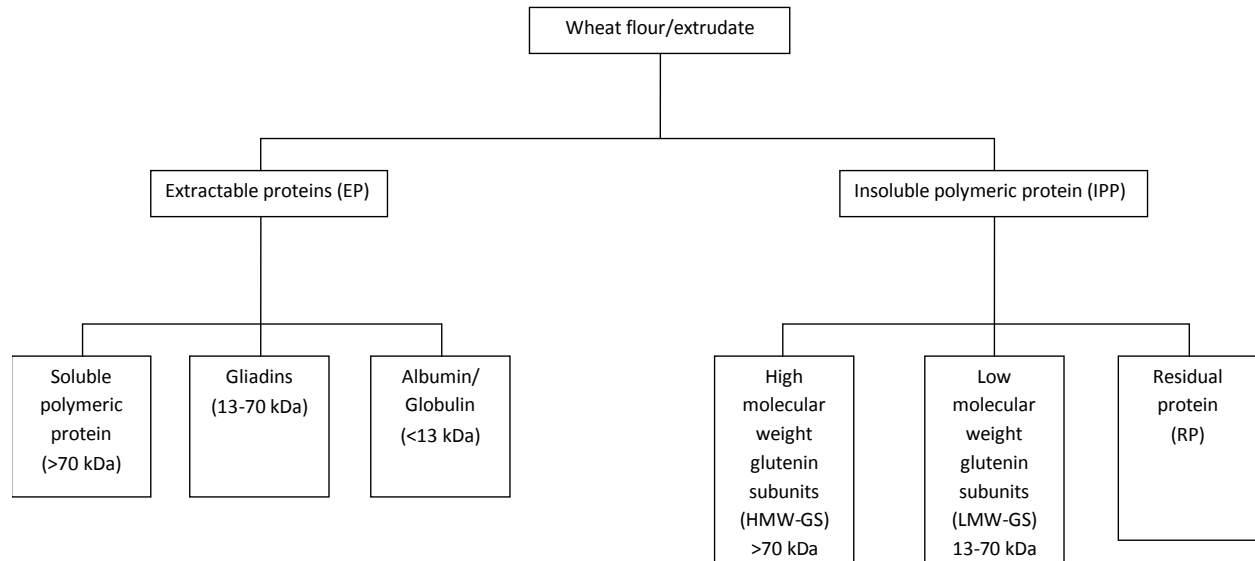


Figure shows the breaking strength (force versus distance) .

Soluble and insoluble protein content



Wheat flour/ extrudate (100mg)

Add 50% propanol (1 mL)
Vortex for 5 min
Centrifuge at 10000 g for 5 min
Repeat 2 times

Pellet (**IPP**)

Pellet 1

Add SDS buffer (1 mL)
Vortex for 1 min
Sonicate at 10W for 20 sec
Centrifuge at 10000 g for 10 min

Pellet 2

Freeze-dry

Analyze protein
content using
LECO

Supernatant

Filter through
0.45 µm filter

Analyze using SEC-HPLC

HMW-GS
(>70 kDa)

LMW-GS
(13-70 kDa)

Pellet (**RP**)

Freeze-dry

Analyze protein content
using LECO

Supernatant (**EP**)
Supernatant 1 (0.5 mL) +
Supernatant 2 (0.5 mL)

Filter through
0.45 µm filter

Analyze using SEC-HPLC

**Soluble
polymeric
protein**
(>70 kDa)

Gliadins
(13-70 kDa)

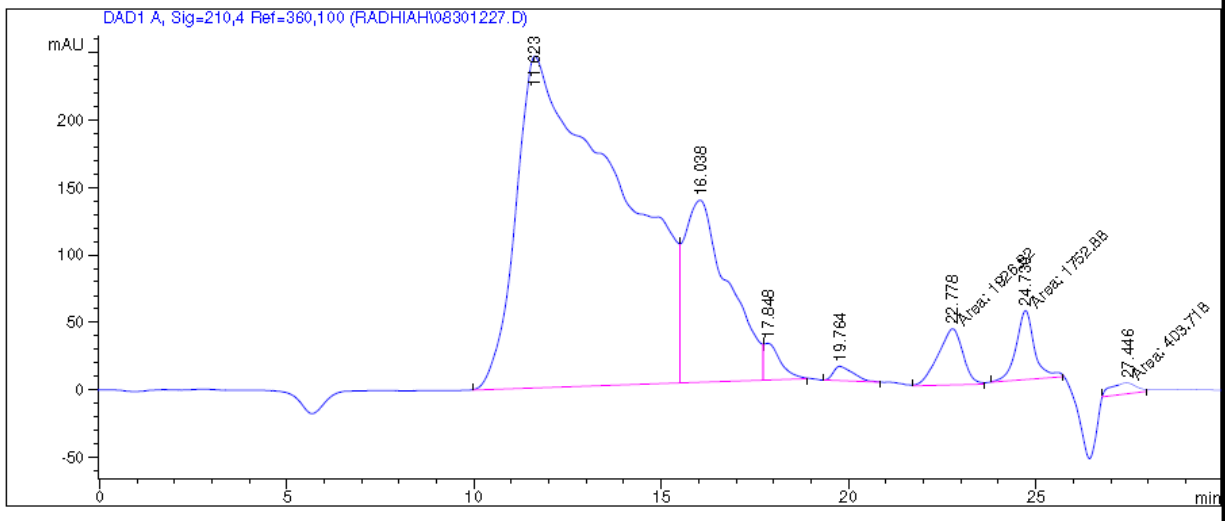
**Albumin/
Globulin**
(<13 kDa)

Total protein = EP + IPP

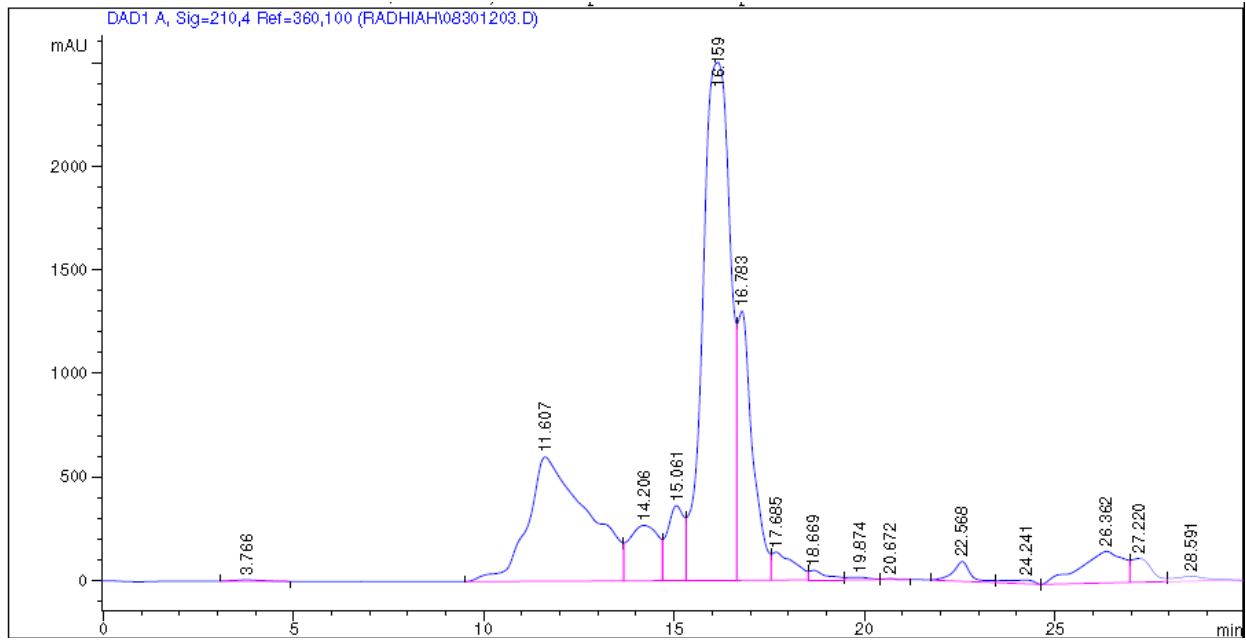
Calculations

EP = Total protein – IPP (both determined using LECO)

HMW-GS + LMW-GS = IPP – RP (RP determined using LECO)



HPLC spectra of insoluble fraction in hard normal wheat flour.



HPLC spectra of soluble fraction in hard normal wheat flour.