Millet Starches: Structural Characteristics and Glycemic Attributes

by

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The hypoglycemic property of millet can be exploited in developed countries for the management of type II diabetes and its complications. Understanding the attributes that confer this hypoglycemic property in millet is important in maintaining its low glycemic index following processing. This study investigated the physical and molecular characteristics of starches from pearl, proso, foxtail and finger millets, grown in Ontario, Canada, as well the effects of starch-protein-lipid interaction on millet starch hydrolysis rates and glycemic index. Proso and foxtail millet starches had similar starch characteristics but were different from finger and pearl millet starches, which were also similar. The former had more of short amylose chains with short chain segments between branch points in their branched amylose. Finger millet amylopectin crystals, melted over the widest temperature range (10.2°C), compared to pearl, foxtail and proso millet starches. The unit and internal chain profiles of the millet amylopectins

showed significant ($P < 0.05$) differences between the starches, with values of 17.9–18.1 and 11.9–12.3 calculated for average chain length (CL) and external chain length (ECL) respectively. Millet amylopectin could be structurally classified as type 2, based on the classification of amylopectins (Bertoft et al 2008). Removal of proteins, lipids or both significantly ($P < 0.05$) increased enzymatic starch hydrolysis rates and glycemic index of the millet starches, with starch-lipid interaction having more effect than starch-protein interactions. The complimentary effects of lipids and proteins on reducing starch hydrolysis rates was also observed. Millet starches complexed with palmitic, oleic, linoleic and elaidic acids to different extent. The complexing index (CI) of fatty acids with millet starches increased with increasing degree of unsaturation. Reductions in starch hydrolysis rates of the starch-fatty acid complexes depended on the amounts of the fatty acids added. Oleic acid was very effective in reducing millet starch hydrolysis rates while linoleic acid-complexed starches were much less resistant to hydrolysis. *Cis* oleic acid was more effective in reducing glycemic index than its *trans* configuration. This study showed that millet starch structural characteristics and starch-protein-lipid interactions are part of the reason why millet have low glycemic and insulinemic response.
DEDICATION

I would like to dedicate this thesis to the Almighty GOD, my wife Elizabeth Annor and my family.
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CHAPTER 1: INTRODUCTION

Millets, which are small seeded plants, are drought tolerant crops found in tropical and sub-tropical regions of the world. They grow very well in harsh environments where other crops usually fail (Abdalla et al 1998). Due to their ability to withstand drought, coupled with the production of substantial yields in a wide range of soils and climates, millets have become a staple food in many African and Asian countries (Malleshi et al 1986) where they are mostly cultivated. In North America however, millets are mainly used as bird or animal feed.

Millets are nutritionally superior to major cereals such as rice or wheat due to the presence of all the required nutrients including protein, minerals and energy (Shinoj et al 2006). For example, finger millets have about five time more calcium than wheat and about thirty more than rice. Most species of millets are richer in iron, potassium, and magnesium when compared to rice and wheat. Furthermore, the vitamin and essential amino acid contents of some of the common millet species are in most cases more than rice and wheat (Shobana et al 2013). Millets have lipid content of 1.5% to 9.3%, with unsaturated fatty acids forming the bulk of its fatty acids. The main fatty acids found in millets are palmitic, oleic and linoleic acid (Ravindran, 1991).

Millets contain about 60% starch, and just like many cereal starches, it contains between 25% to 30% amylose (Malleshi et al 1986). Millet starch granules size range from 0.8 to 25 µm, are mainly polygonal or spherical in shape with very deep
indentations due to protein bodies (Krishna and Thayumanavan 1998). Also present are numerous pores on the surface of the granules. Millets starches have not been studied as extensively as other cereal starches. Moreover, studies on millet starches over the years have focused on their physicochemical characteristics, with little information on its structural characteristics.

With recent developments of methods and instrumentation for starch research, unraveling new insights into key structural features of starches, especially their amylopectin structure, has become possible. The availability of starch debranching enzymes such as isoamylase and pullulanase, coupled with high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), has enabled researchers to study unique structural characteristics of amylopectin with increased resolution (Bertoft et al 2008; Koizumi et al 1991; Wong and Jane 1995). Detailed structural characteristics of amylopectin from different botanical sources have been reported (Hizukuri 1985; Hizukuri 1986; Inouchi et al 1987; Jane et al 1999; Koizumi et al 1991; Hanashiro et al 1996; Hanashiro et al 2005; Takeda et al 1987; Van Hung et al 2007; Bertoft and Koch 2000; Bertoft et al 2008; Kong et al 2009; Laohaphatanaleart et al 2009; Zhu et al 2011; Bertoft 2013; Kalinga et al 2013; Klucinec and Thompson 2002). No such information is available for millet starches.

One interesting property of millet is its hypoglycemic response (Shobana et al 2009), which could be exploited in main stream foods. In a study by Lakshmi and Sumathi (2002), significantly lower blood glucose levels in type II diabetes subjects were
observed when fed with finger millet-based diets compared to wheat or rice. Vijayakumar et al (2010) also reported significantly lower glycemic index and load from noodles prepared with refined wheat flour substituted with 20% millet flour. This hypoglycemic property of millets may be attributed to the interactions between starch and proteins as seen in wheat (Jenkins et al 1987) as well as with lipids. Significant decreases in starch hydrolysis rates have been reported when lipids were complexed with starches from different botanical sources (Ai et al 2013; Hasmij et al 2010; Crowe et al 2000; Kawai et al 2012; Guraya et al 1997). Understanding why millets are slowly digestible could help maintain its hypoglycemic attributes during processing.

The goal of this research was to structurally characterize millet starches and to determine whether starch-protein-lipid interactions play a role in the hypoglycemic property of millets.
CHAPTER 2: LITERATURE REVIEW

2.1 Millet

The term “millet” is broadly used to describe various small seeded grasses that are harvested for food or feed. They can be classified within the genera *Brachiaria, Digitaria, Echinochloa, Eleusine, Panicum, Paspalum, Pennisetum, Setaria* and *Sorghum* (Yang et al 2012). Several species of millets exist. These include: browntop (*Brachiaria ramose*), barnyard (*Echinochloa crus-galli*), finger (*Eleusine coaracana*), proso (*Panicum miliaceum*), kodo (*Paspalum scrobiculatum*), little (*Panicum sumatrense*), pearl (*Pennisetum glaucum*) and foxtail (*Setaria italica*) millets. Amongst these millet species, pearl millet is the most widely cultivated.

Millets are ancient crops, and have been cultivated by man for thousands of years. There is archeological evidence to show the domestication of millets in Northern China (about 5800 BC), India (about 2000 BC), Japan (about 4000 BC) and West Africa (about 2400 BC) where they eventually became staple food crops (Yang et al 2012; Liu et al 2012; Brunken et al 1977; De Wet et al 1982; Manning et al 2011). It can be argued that, the domestication of millets was successful due to their ability to tolerate conditions of drought (Brunken et al 1977).

Millets are mainly produced in Asian and African countries with India being the largest producer (FAO 2012). In Africa, Niger topped the production of millets at 108,798 tons, followed by Nigeria at 59,994 tons in 2010 (FAO 2012). In many of these
regions, millets play an important role in the food security, where they serve as major food components in various traditional foods and beverages (Chandrasekara and Shahidi 2011). Commonly eaten by the marginalized in society, millets are important ingredients in the production of bread, porridges and snack foods (Chandrasekara et al 2011).

Millets have been reported to be nutritionally superior when compared to many cereals (Saleh et al 2013; Parameswaran and Sadasivam 1994). They are good sources of proteins, carbohydrates, fiber and essential amino acids. Millets are also rich in phytochemicals and micronutrients (Singh et al 2012). Reports on the nutritional composition of millets are readily available in literature (Gopalan et al 2009; Geervani and Eggum 1989; Malleshi et al 1986; Wankhede et al 1979; Virupaksha et al 1975; Mahadevappa and Raina 1978; Barbeau and Hilu 1993; Ravindran 1991; Vadivoo et al 1998; Antony and Chandra 1999; Hemalatha et al 2007; Saleh et al 2013; Chethan and Malleshi 2007; Sriprya et al 1997; Hegde et al 2005; Kalinova and Moudry 2006; Subramanian and Viswanathan 2007; Shobona et al 2013; Chandrashekar 2010; Malleshi 2002; Malleshi 2007; Malleshi et al 2004; Malleshi et al 1996).

Health benefits associated with the consumption of millets have been documented (Shobana et al 2009). Its antioxidant (Chandrasekara & Shahidi 2011; Hegde and Chandra 2005; Subba Rao and Muralikrishna 2002; Varsha et al 2009) and antimicrobial properties (Antony et al 1998; Chethan and Malleshi 2007; Varsha et al 2009) have been reported. Phenolic extracts from millets have been reported to inhibit intestinal α-glucosidase and pancreatic α-amylase, and may play a vital role in the management of
postprandial hyperglycemia (Shobana et al 2009). It is also interesting to note the anti-ulcerative (Tovey et al 1975), cholesterol lowering (Pore and Magar 1976) and wound healing (Hegde et al 2005; Rajasekaran et al 2004) properties of millets.

2.2 Millet Starch

Starch is the major component in millet and typically ranges from 56-65% of the total seed weight (Wankhede et al 1990), though up to about 80% starch has been reported for proso millet (Casey and Lorenz 1977). Normal millet starches have amylose contents ranging from 20-32% (Beleia et al 1980; Hoover et al 1996; Kim et al 2012). Much lower amylose contents (3.3-11.4%) have been reported for some varieties of proso millet (Kim et al 2009). Choi et al (2004) reported an amylose content of 6% for waxy millet.

2.2.1 Granule Morphology

Millet starches typically have polygonal and spherical starch granules. The polygonal starch granules tend to be larger than the spherical ones. In rare cases, large spherical granules are observed (Krishna Kumari and Thayumanavan 1998). Bangoura et al (2012) observed bean-shaped granules for foxtail millet starch. Malleshi et al (1986) reported finger millet starches with rhombic shapes. Also present on the surfaces of millet starch granules are deep indentations and numerous pores. These pores, which are real features of granular morphology, are usually found on the entire granule surface (Fannon et al 1990), and sometimes more evident in some millet varieties than others (Hoover 1990; Kim et al 2009). The indentation found on the millet starch granules is believed to
be caused by compression of the granules by protein bodies in the millet endosperm (Krishna Kumari and Thayumanavan 1998).

Variations in the sizes of millet starch granules have been observed for different species. Using the Scanning electron microscope (SEM), Krishna Kumari and Thayumanavan (1998) reported starch granules sizes of 1.3-8.0 µm, 0.8-9.6 µm, 1.2-10.0 µm, 1.2-9.5 µm and 1.0-9.0 µm in diameter for proso, foxtail, barnyard, kodo and little millets, respectively. Yanez et al 1991 observed a bimodal distribution in proso millet starch granules with sizes ranging from 1.8-13 µm. Hoover et al (1990) reported starch granule sizes of 2-22 µm for three varieties of pearl millets. A mean starch granule size of 6.8-11.8 µm for 53 foxtail millet varieties was observed by Fujita et al (1996). They also observed a normal distribution curve for the starch granules with peak granular sizes ranging from 8.1-9.9 µm. Choi et al (2004) reported a granule size range of 5.0-10.0 µm for waxy millet. Kim et al (2009) after characterizing four local varieties of Korean foxtail millet, reported starch granule sizes of 4.7-25.0 µm. Granule size ranges of 10-16 µm was also have been reported for pearl millet by (Wankhede et al 1990). The differences observed in the shapes and sizes of the millet starches, according to (Wankhede et al 1990), could be due to the fact that the individual starch granules were at different stages of maturity at the time the millet seeds were harvested and starches isolated. Sizes of up to 50 µm for foxtail millet starch have also been reported (Bangoura et al 2012).
2.2.2 Starch Structures

Starch, the main form of stored energy in green plants, is composed principally of amylose and amylopectin. Amylose, which constitutes about 15-35% in most plants, is made up of α-(1-4)-linked glucose units. Making up about 65-85% of starch is amylopectin, which is a highly branched molecule with α-(1-4)-linked glucose backbone and about 5% α-(1-6)-linked branches (Perez and Bertoft, 2010). The amounts of amylose and amylopectin present in starch depend on the botanical source of the starch. In terms of molecular weight, amylopectin is larger than amylose with a molecular weight range of about $1 \times 10^7$ to $1 \times 10^9$ compare to $1 \times 10^5$ to $1 \times 10^6$ of amylose (Buleon et al at 1998). The intermediate material on the other hand is still under discussion as to whether it should be classified as amylose or amylopectin.

Starch granules consist of alternating amorphous and semi-crystalline shells known as growth rings, which are between 100 and 400 nm thick (Gallant et al., 1997). The amorphous growth rings are believed to consist of mainly amylose, while the semi-crystalline rings on the other hand consist of alternating crystalline and amorphous regions with a repeat distance of 9 to 11 nm (Cameron and Donald 1992).

al 2009; Zhu et al 2011; Bertoft 2013; Kalinga et al 2013; Klucinec and Thompson 2002). However, very little information on the structural characteristics of millet starch, can be found in literature. The few that exist are mainly on finger millet starch.

Jideani et al (1996) investigated the starch structure of tamba, a locally grown finger millet variety in Nigeria. The amylose of the millet sample showed a wide range for its weight average degree of polymerization (DP$_w$) (about 260 – 20,400) and average chain lengths of about 180 – 240 was reported. The β-amylolysis limit of the millet amylose ranged from 71- 81%, indicating the presence of considerable amount of branching in the amylose. The β-amylolysis limit of the millet amylose was higher than that of sweet potato, tapioca and kuzu amylases, but lower than that of wheat, rice and corn amylases (Takeda et al 1983 1987). The millet amylose was also reported to have about 6 glucan chains per molecule. The $\lambda_{\text{max}}$, which gives an indication of the length of glucan chains was about 659 and 650 nm the millet amylose and its respective β limit dextrins. Such high $\lambda_{\text{max}}$, values suggests that the chains between the branched points in the branched amylose of these millet starches were very long.

Jideani et al (1996) also reported an average chain length (CL) of about 20 for the millet amylopectin studied and was found to be similar to wheat, corn, waxy corn, japonica rice, but longer than that of waxy rice. A comprehensive review on the various chain categories and structures of amylopectin have been published (Bertoft 2013). The external chain length, which gives an indication of the length of the crystalline lamella of amylopectin, was calculated as between 14-15 for the millet amylopectin. The long chain
fraction of the millet amylopectin was reported to be about 4.0% by weight, and was observed to be higher than that of corn (2.4%) but lower than that of wheat (4-6%) (Hizukuri 1993). The long chain fraction of amylopectins has been reported as having considerable effects on the viscosity, retrogradation tendency and, possibly, the inclusion capacity of hydrophobic materials (Hizukuri 1989).

Madhusudhan et al (1996) also studied the structures of linear and branched fractions of Indian grown finger millet starch. The amylopectin component in this study was precipitated with concanavalin A, after which amylose was fractionated with hot 1-butanol. The starch fractions and the β-limit dextrins of the amylose component were then characterized by gel permeation chromatography after debranching with pullulanase. The $\lambda_{\text{max}}$ values reported for the amylose and amylopectin fractions of the millet starches ranged from 632 and 551 nm, suggesting shorter amylopectin chains. The β-limit dextrins of the amylose fraction was 93.7 %, suggesting it is less branched than amylose from chickpea, with a β-amylolysis limit of 79.1 %. The β- amylolysis limit reported by Madhusudhan et al (1996) is very different from the 71- 81%, reported by Jideani et al (1996). The amylopectin fraction of finger millet starch was reported as having chain length, external chain length, internal chain length and β-amylolysis value of 20.7, 12.1, 6.3 and 52.1, respectively

Gaffa et al (2004) also reported an average number degree of polymerization (DPn), chain length (CL), β-amylolysis limit, external (ECL) and internal (ICL) chain lengths of 9100, 20, 56%, 13.8 and 6.2, respectively for finger millet amylopectin. The
average chain length reported by Gaffa et al (2004) was similar to Jideani et al (1996). A- and B₁-chains of the millet amylopectin were reported to constitute about 73 % by weight of the amylopectin. The A-chains are known to form the external chains of amylopectins (Bertoft 2013). With a β-limit value of 83% reported for the amylose component of the finger millet studied by Gaffa et al (2004), there appears to be differences in the extent of branching among different varieties of finger millet. The average number of chains for the millet amylose was reported to be 4 and was less than the 6 glucose units per amylose molecule reported for another finger millet variety by Jideani et al (1996). Using the modified Park-Johnson method (Takeda 1987), the number-average degree of polymerization of the millet amylose was reported to be about 1065.

### 2.2.3 Thermal Properties

The thermal properties of millet starches have been studied using Differential Scanning Calorimetry (DSC), and using a Kofler hot stage microscope (Wankhede et al 1990; Beleia et al 1980; Yanez et al 1991). When using the DSC, starches are heated in excess water to gelatinize, and their onset (To), peak (Tp) and completion (Tc) gelatinization temperatures are respectively reported. The enthalpy of gelatinization (∆H) is determined by integrating the area under the endothermic peak produced as a result of the melting of the amylopectin crystallites in the starches. Higher melting temperatures (Tp) represent a measure of starch crystallites perfection (Tester, 1977). The enthalpy of gelatinization of the amylopectin endotherm, gives an indication of the rupturing of the links between and within the amylopectin double helices (Cooke and Gidley 1992). Significant differences in the thermal properties of millet starches has been
reported. In 1996, Fujita et al studied the thermal properties of fifty three varieties of foxtail millets cultivated in China, two of which were waxy. A peak temperature (Tp) range between 62.5-75.1 °C was observed, with enthalpies of gelatinization between 8.2 - 13.5 J/g. Hoover et al (1990) studied the thermal properties of three cultivars of pearl millets (ICTP 8203, ICMS 7703, and ICMH 356) cultivated in India and reported gelatinization temperature ranges of 61.9-70.5 °C, 60.9-67.5°C and 64.5-78.0°C for ICTP, ICMH and ICMS, respectively. Gelatinization enthalpies were 2.7, 2.5 and 3.5 for ICTP, ICMH, and ICMS, respectively. Millet starches have higher gelatinization temperatures than wheat (Hoover and Vasanthan 1994) and similar to those of rice (Hoover et al 1991).

Kim et al (2012), observed significant differences in the onset temperature (To), peak temperature (Tp), conclusion temperature (Tc), gelatinization temperature range (Tc-To) and enthalpy of gelatinization (ΔH_{gel}) of three varieties of proso millets (Andongjaerae, Danyangjaerae and Andong48ho). To ranged from 73.1-76.4 °C, Tp ranged from 78.0- 81.5 °C, and their Tc was reported to be between 79.3-86.0 °C. ΔH_{gel} ranged between 0.81 and 4.48 J/g. They suggested that the differences observed in the To of the starches may be attributed to differences in their amylose content, size, shape, distribution of starch granules and internal arrangements of starch fractions within the granules. Andong48ho was observed to have the highest gelatinization temperature and enthalpy, suggesting that the amylopectin double helices are strongly associated within the starch granule. Peak height index (PHI), which is the ratio of ΔH_{gel} to temperature of gelatinization, gives an indication of the uniformity of gelatinization. Andongjaerae and Danyangjaerae showed higher PHI values.
In an earlier report, Kim et al (2009) investigated the thermal characteristics of four Korean foxtail millets varieties and reported values of 66.4-69.9 °C, 71.0-74.2 °C, 74.1-79.5 °C and 0.219-8.219 J/g for To, Tp, Tc and ΔHgel, respectively. It can be seen that the values reported for To and Tp were lower than those reported for proso millet studied by the same authors (Kim et al 2012). Yanez et al (1991) studied the thermal characteristics of four varieties of proso millets grown in Nebraska, USA, and reported To, Tp, Tc and ΔHgel values of 67.8-69.0 °C, 72.5-73.9 °C, 80.3-81.8 °C and 3.51-3.6 J/g, respectively. Compared to maize, higher values were observed for millet. They also determined the To and Tp of the proso millet starches with Kofler hot stage microscope.

Retrogradation studies on millet starches have also been reported. Afolabi et al (2012) reported retrogradation temperatures of 54.7 °C, 58.1 °C, and 62.3 °C for To, Tp and Tc, respectively for finger millet. The enthalpy of retrogradation was 1.62 J/g.

2.2.4 Pasting Properties

The pasting properties of starch are primarily changes that occur when starch is heated in excess water in the presence or absence of shear. These changes include swelling of starch granules, leaching of molecular materials from the granules and eventual disruption of the granules especially when shear is applied (Tester and Morrison 1990). Using the Brabender viscoamylograph, Krishna, Kumari and Thayumanavan (1998) determined the pasting properties of proso, foxtail, barnyard, kodo and little millets. Significant differences were observed in the gelatinization temperature, peak
viscosity, hot paste viscosity, cold paste viscosity, break down and set back. Gelatinization temperature was the highest (84.9 °C) for barnyard millet, while proso millet starch had the lowest (75.8 °C). A significant positive correlation between amylose content and temperature of gelatinization was also observed. Peak viscosity of the millet starches ranged from 375 Brabender units (BU) for barnyard to 520 BU for proso millet starch. The highest cooked paste viscosity was observed for barnyard millet starch, followed by kodo millet starch. For cold paste viscosity, values of 850 BU, 900 BU, 980 BU, 1050 BU and 1165 BU were observed for proso, foxtail, barnyard, kodo and little millet starches, respectively.

Beleia et al (1980), in studying the pasting properties of pearl millets, reported a pasting temperature of 76.5 °C. Differences between maximum and minimum viscosity values at 95 °C was 100 BU. Larger differences were however observed between the pearl millet starches after they were held at 95 °C for 1 hour and also during the cooling cycle. Hoover et al (1996) also reported differences in the pasting properties of pearl millet varieties. They suggested viscosity differences observed at 95 °C could be due to the degree of crystallinity, extent of amylose leaching and amount of amylose lipids. While Hoover et al (1996) observed an increase in viscosity during the holding period at 95 °C, decreases in viscosity during the same period have been reported by others (Beleia et al 1980; Wankhede et al 1990; Freeman and Bocan 1973). Very high pasting temperatures, which were likened to those only exhibited by cross-linked and legume starches, were observed for the pearl millet starches used in their study.
Adebowale et al (2005) investigated the hydrothermal treatment of different varieties of finger millet starch and determined their pasting characteristics with the Rapid Visco-Analyzer (RVA). Peak viscosity, trough viscosity, breakdown, final viscosity, setback and pasting temperatures values of 386.8 rapid visco units (RVU), 153.2 RVU, 225.5 RVU, 377.2 RVU, 184.0 RVU and 64.4 °C, respectively, were reported. Abd Allah et al (1987) reported a temperature of gelatinization of 90.8 °C for pearl millet. Choi et al (2004) reported an initial temperature of gelatinization of 75.2 for waxy millet. This value was higher than that (69.0-69.3 °C) observed for waxy millet by Kim et al (1987). Bangoura et al (2012) reported gelatinization temperatures of 76.1 °C for yellow and white foxtail millet starches, with peak viscosities of 3322 centipoise (cP) and 3321 cP respectively.

2.2.5 Swelling and Solubility Characteristics

Swelling and solubility behavior of starches in aqueous solutions are studied to investigate the nature of associated bonding forces within starch granules (Wankhede et al 1990). Beleia et al (1980) observed a two-stage swelling and solubility pattern in millet starches. Swelling power of 8.0% and 8.3% at 60 °C and 12.4 % and 12.2% were reported for yellow finger millet and white finger millet starch, respectively (Bangoura et al 2012). Solubility of about 26% for both millet starches was also reported. Hoover et al (1996) studied the swelling factor and amylose leaching of pearl millet starches at temperatures between 50-95 °C. In their study, an increase in swelling factor and amylose leaching with increasing temperature for the millet starches, which was most predominant between 60 and 70 °C was observed. They also observed that the swelling factor of the pearl millet
starches were higher than those of wheat and rice. Lower swelling factor in one of the millet starches was attributed to its higher content of lipid-complexed amylose and/or the presence of more crystallites within the granule.

Afolabi et al (2012) did not observe any significant difference in the swelling factor of finger millet starch at temperatures between 85-95 °C. Adebowale et al (2005) reported a decrease in the swelling factor of finger millet starches when they were hydrothermally treated. Higher swelling factor for annealed finger millet starches versus hydrothermally treated ones was also reported. The lower swelling factor of hydrothermally treated finger millet starch, they suggested could be attributed to restriction in percolation of water within the starch matrices due to increased starch crystallinity. All the finger millet starches solubilized at different rates at different temperatures. Abd Allah et al (1987) observed higher swelling factor for pearl millet compared to yellow corn and sorghum. Malleshi et al (1986) observed a decrease in the swelling factor but an increase in the solubility of millet starches when germinated.

### 2.3 Glycemic Attributes of Millet

As far back at 1957, the importance of millets in the management of type two diabetes and its complications was established (Ramanathan and Gopalan 1957). Since then other studies have confirmed this hypoglycemic property (Kurup and Krishnamurthy, 2011; Lakshmi Kumari and Sumathi, 2002; Mani et al 1993; Shobana et al 2007; Shukla and Srivastava 2011; Urooj et al., 2006). Chhavi and Sarita (2012) baked bread from wheat flour substituted with between 30-60% of finger millet flour and
determined sensory preference and glycemic index of the bread samples. Bread baked from 30% millet substituted wheat flour was the most preferred. Breads from millet substituted wheat flour had a significantly lower area under the blood glucose response curve as compared to bread from refined wheat. The glycemic index of bread containing foxtail millet flour was 49.5, whereas that for refined wheat flour was 67.8.

Anju and Sarita (2010) reported a glycemic index of 50.8 for biscuits prepared from refined wheat flour substituted with 45% foxtail millet versus 68 for biscuits from refined wheat flour. Geetha & Easwaran (1990) showed a significant decrease in postprandial glucose after feeding type II diabetes subjects for a month with breakfast foods incorporated with 20% finger millet. Lower glycemic index was reported for the consumption of 20% finger millet substituted noodles versus that from 100% refined wheat flour noodles (Shukla and Srivastava 2011). Abdelgadir et al (2004) showed that the consumption of millet acida (porridge) followed by wheat gorasa (pancakes) resulted in significantly lower postprandial glucose and insulin responses compared to the consumption of maize acida. In a recent study, feeding genetically obese type-II diabetic mice under high-fat feeding conditions with proso millet showed improved glycemic responses. (Park et al 2008).

The reason why millet has lower glycemic response compared to other cereals is, very poorly understood. Mohan et al (2005) studied the characteristics of native and enzymatically hydrolyzed granular millet starches and observed that the molecular weight and degree of crystallinity of residues from finger millet starch hydrolyzed with an
enzyme mixture of α-amylase, β-amylase and amyloglucosidase were significantly higher than those of rice. This suggests that finger millet starch was much more resistant to enzymatic hydrolysis than rice starch, which was fully hydrolyzed. They concluded that the resistance of finger millet starch to digestive enzymes could be due its rigid starch granule architecture compared to rice. Singh and Ali (2006) after comparing the in-vitro hydrolysis of various starches by α-amylase, observed that the resistance to enzymatic hydrolysis in order of decreasing resistance was finger millet > potato > chickpea > rice > sorghum > green gram > wheat > tapioca > waxy rice > maize.

The inhibitory effects of millet phenolic compounds on α-glucosidase and pancreatic amylase has been mentioned by Shobana et al (2009). They concluded that millet seed coat phenolics were effective non-competitive inhibitors of carbohydrate-hydrolyzing enzymes. They went on to further suggest that millet seed coat extracts may delay or interfere with the absorption of dietary carbohydrates in the small intestine resulting in the suppression of postprandial glucose spikes. Chandrasekara and Shahidi (2011) later found that the insoluble bound form of phenolics attached to the cell wall materials of millet grains was the major contributor to its total phenolic content. Hydroxybenzoic acids, hydroxycinnamic acids and flavonoids were reported as the main classes of phenolic compounds in the whole millet grain. Subba Rao and Muralikrishna (2002) also reported the presence of both free and bound forms of phenolic acids in finger millets.
Fiber in millets has also been mentioned as one of the reasons for its hypoglycemic property. Soluble and insoluble fiber contents of 2.5% and 19.5%, respectively, has been reported (Shobana and Malleshi 2007). A considerable increase in soluble fiber content of finger millet was observed after decortication, though a decrease in the dietary fiber was reported. The increase in the soluble fiber content has special nutritional significance due to its physiological advantages in terms of hypoglycemic and hypocholesterolemic characteristics. The formation of resistant starch in millet during processing contributed to dietary fiber content, which complemented the health benefits of finger millet (Shobana and Malleshi 2007).

Effects of protein encapsulated granule matrix and starch–lipid complexes has been suggested (Mohan et al 2005) as part of the reason for the hypoglycemic property of millet. Protein fractions such as albumins, globulins and glutenins, glue protein bodies into a matrix surrounding starch granules, which acts as a barrier to amylases (Hamaker and Bugusu 2003). Jenkins (1987) studied the effects of starch-protein interactions on the starch digestibility of wheat and reported a decrease in glycemic response and reduced rate of reaction due to the interaction of starches with proteins.

Starch-lipid complexes, particularly lipid complexes with amylose have been reported to influence the susceptibility of starch to enzymatic degradation (Ai et al 2013; Hasmij et al 2010; Crowe et al 2000; Kawai et al 2012; Guraya et al 1997). Seneviratne and Biliaderis (1990) observed an inverse relationship between the extent of enzymatic hydrolysis of amylose-lipid complexed superstructures and the degree of organization of
helices into larger domains of ordered chains in aggregated structures. A comprehensive review on the formation, identity and physical properties of the amylose-lipid complex has been reported (Putseys et al 2010). Enzymatic hydrolysis of amylose-lipid complexes has been reported to be a two-step process. The first step involves the rapid hydrolysis of amorphous areas of the complex and then followed by a slower degradation of the amylose inclusion complex (Galloway et al 1989; Jane and Robyt 1984; Godet et al 1996). Holm et al (1983) observed a substantially reduced susceptibility of potato amylose complexed with lipids to α-amylase in vitro. However, addition of excess enzymes completely hydrolyzed the complex after 3 hours. Kawei et al (2012) observed significant reductions in enzymatic hydrolysis of potato starch when complexed with fatty acids. They went on to report that oleic and lauric acid resulted in the largest reductions. Enzymic hydrolysis rates of starch-linoleic acid complexes were not significantly lower than that of the native starch. Understanding the effects of corn oil, soy lecithin, palmitic acid, stearic acid, oleic acid, and linoleic acid on the enzymatic hydrolysis of normal corn, waxy corn, tapioca and high-amylose corn starches was studied (Ai et al 2013). Significant decreases in starch-hydrolysis rates of all the starches except waxy corn when cooked with the lipids was observed. It was also reported that lipids of different structures showed different effects on starch-hydrolysis rates of starch-lipid complexes. Guraya et al (1997) not only reported significant reductions in starch hydrolysis rates when lipids were complexed with rice starch, but also indicated that long-chain saturated emulsifiers reduced digestibility more than short-chain saturated and unsaturated emulsifiers.
2.4 Conclusions

Millets can be exploited in the management of type II diabetes and its complications due to its low glycemic property. However, the reasons for this hypoglycemic property in millets are not totally understood. Even though millet phytochemicals and fiber have been mentioned as reasons for their low digestibility, little information exist on the contribution of its starch structure and starch-protein-lipid interactions. Understanding the structural characteristic of millet starches and its glycemic attributes could shed more light on the hypoglycemic property of millets. Furthermore, studies undertaken on millet starch structural characteristics have focused on only one or two millet species and have only used size-exclusion techniques. With the advent of high-performance anion exchange chromatography (HPAEC), the fine structures of millet starches can be studied in more detail.

2.5 Objectives

1. Study the physical and molecular characteristics of millet starches

2. Investigate the unit and internal chain profile of millet amylopectin

3. Study the effects of starch-protein-lipid interactions on the in vitro starch digestibility and expected glycemic index of millet starch
4. Investigate the effects of types and amounts of fatty acids on the in vitro starch digestibility and expected glycemic index of millet starch

2.6 Significance of the Project

This research will generate information on the physical and molecular characteristics of millet starches, as well as information on the unit and internal structure of millet amylopectin. The data will improve our understanding on the fine structure of starch. It is known that millets could be useful in the management of type II diabetes due to their low insulinemic response. Understanding the structural attributes of millet starch and the details that may confer this hypoglycemic property is important, as it will allow us to design healthier food products.
2.7 References (Chapters 1 and 2)


Anju, T. and Sarita, S. 2010. Suitability of foxtail millet (Setaria italica) and barnyard millet (Echinochloa frumentacea) for development of low glycemic index biscuits. Malya J Nutr. 16:361-368.


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CHAPTER 3: PHYSICAL AND MOLECULAR CHARACTERIZATION OF MILLET STARCHES
Abstract

This study investigated the physical and molecular starch characteristics of four Canadian grown millet species – pearl, foxtail, proso and finger millet. The millet starch granules ranged from about 2.5–24 µm in size and were mainly polygonal with few spherical ones. Their amylose contents ranged from 28.6–33.9% with finger and pearl millets having much more of long amylose chains than short amylose chains compared to foxtail and proso millets. Starches also differed in the molecular structure of their branched amylose, with finger and pearl millets having longer glucan chains between branch points. The enthalpy of gelatinization of starch granules ranged from 11.2–13.2 J/g, while the enthalpy of melting of the retrograded starches ranged from 2.2–5.9 J/g. The onset temperature of gelatinization ($T_o$) of the starches ranged from 62.8–70.6 °C. Addition of iodine vapor to the granular starches showed significant ($p < 0.05$) differences in the ratio of the absorbance to scattering coefficient (K/S) values, indicating differences in the rigidity of the glucan chains present in the granules. Starches with short amylose chains possessed higher K/S values. Iodine vapor addition resulted in altered X-ray diffractogram peak intensities. The study suggested differences in the structure and granular architecture of the millet starches.

Keywords: Millet, physical characteristics, molecular characteristics, iodine, X-ray
3.1 Introduction

Millets are small seeded crops of importance in the diets of many populations in some parts of the world. They can be classified into major or minor millets. The major millets comprise pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*) and finger millet (*Eleusine coracana*) (Amadou et al 2011). The minor millets include barnyard millets (*Echinochloa colana*), kodo millet (*Paspalum scrobiculatum*), and little millet (*Panicum miliare*) (Shinoj et al 2006). Millets are believed to be amongst the first crops cultivated in Central and Eastern Asia, Russia, China, India and some parts of Africa (Amadou et al 2011). It is of particular importance to the populations in these regions, where it forms a major part of their diets (Fujita et al 1996). In North America, however, millets are mainly used as animal forage.

Millets are good sources of minerals (2.5-3.5%); especially, calcium, iron, and phosphorous (Devi et al 2011; Subbarao & Muralikrishna, 2001). They contain about 5–10% protein, 65–75% carbohydrates and 15–20% dietary fiber (Chethan and Malleshi 2007a). With about 344 mg calcium/100 g, millets are considered to have the highest calcium content relative to other cereals. Millets also contain polyphenols, phytates, tannins, and trypsin inhibitors (Thompson 1993). The dietary fiber and polyphenols present in millets are known to offer several health benefits, such as antidiabetic, antioxidant, hypocholesterolaemic, antimicrobial effects and protection from diet related chronic diseases to its regular consumers (Devi et al 2011). In addition to health benefits, millets are gluten-free (Pagano 2006), making it suitable for coeliacs.
Regular consumption of finger millet has been reported to help in the management of type II diabetes and its complications by regulating glucose homeostasis (Devi et al 2011; Tovey 1994). Some of these positive health benefits of millets, which could be exploited in North America, can be linked in part to the characteristics of their starches (Singh et al 2010). A look through literature, however, reveals the scarcity of scientific reports on the characteristics of millet starches compared to other cereals such as rice or wheat. Additionally, research on millet starches over the years has focused mainly on their physicochemical properties, and not on their molecular characteristics. Notable amongst these reports are Fujita et al (1996); Hoover et al (1996) and Malleshi et al (1986a, 1986b). For millets to be incorporated into the main stream food systems in North America, it is important their starch characteristics at the molecular level are well documented, to better explain some of their unique characteristics, such as their hypoglycemic property (Singh et al 2010). The objective of this study, therefore, was to investigate the physical and molecular characteristics of starches of millet species grown in Canada.

3.2 Materials and Methods

3.2.1 Materials

The seeds of four millet species, namely proso, foxtail, finger, and pearl, were obtained from the Agriculture Environmental Renewal Canada (AERC) Inc, Delhi, Canada. These seeds were kept at -20 °C and later analyzed in this study.
3.2.2 Methods

3.2.3 Starch Extraction

The millet seeds were milled for 2 min with a Smart grind Stainless Steel Coffee Bean Grinder, model CBG100S (Black and Decker, USA) into flour, after the seeds were frozen in liquid nitrogen. Starch was extracted from the flour samples according to the method of Waduge et al (2010) with modifications. 100 g of millet flour was stirred with 2 L sodium borate buffer (12.5 mM, pH 10, containing 0.5% SDS [w/v] and 0.5% Na$_2$S$_2$O$_5$ [w/v]) for 5 min to extract the proteins, and the residue recovered by centrifugation at 900 × g for 5 min. The extraction step was repeated again. The resulting residue was washed three times with distilled water and recovered by centrifugation (900 × g for 5 min). The residue was then suspended in distilled water and stirred overnight to further release the protein from the starch granules, after which the starch slurry was passed through four layers of cheesecloth and then through 70 μm nylon mesh. The slurry was centrifuged and the brown layer, formed on the top of the starch layer, was scraped off using a spatula. The starch was then suspended again in water and centrifuged at 1600 × g for 10 min. These steps were continued until all the brown particles were removed from the starch fraction. Starch yield was expressed as the dry weight per weight of millet flour. Starch damage was determined using the Megazyme Starch Damage Kit (Megazyme International Ltd, Bray, Ireland)
3.2.4 Starch Granule Size Distribution

Particle size distribution of starch granules was determined using the Mastersizer 2000 (Malvern Instruments Ltd, Worcestershire, UK) with a refractive index of 1.33 for the aqueous continuous phase, and a refractive index of 1.52 for the starches in the dispersed phase, and sample absorption of 0.001.

3.2.5 Starch Surface Morphology

The surface characteristics of the millet starches was viewed using the Hitachi S-570 Scanning Electron Microscope (Hitachi Scientific Instruments, Rexdale, Ontario, Canada) after starches were sputtered with 15 nm of gold dust on a stub. The working distance used was 15 mm with a voltage of 10 kV.

3.2.6 Size Distribution of Debranched Millet Starches

Duplicate starch samples (4 mg) were dissolved in 90% dimethyl sulphoxide (DMSO; 100 L) and heated in a warm water bath (80 °C) for 5 min and then stirred slowly overnight at room temperature (25°C). 750 L water (80 °C) was then added to the sample, after which 100 L of 0.01 M sodium acetate buffer (pH 5.5) was added. 1 L of isoamylase (465 U/mL) and 1 L of pullulanase M1 (925 U/mL) (Megazyme International, Bray, Ireland) were added and the mixture incubated overnight with stirring at slow speed at room temperature (25°C). At the end of the incubation, samples were placed in a boiling water bath for 5 min to inactivate the enzymes. 50 L of 5 M NaOH
was added, and the sample was diluted to 1.5 ml with deionized water. The sample (1 mL) was applied on a column (1 × 90 cm) of Sepharose CL 6B (GE Healthcare, Uppsala, Sweden), and eluted with 0.5 M NaOH at 1 mL/min. Fractions (1 mL) were analyzed for carbohydrates with the phenol–sulphuric acid reagent (Dubois et al 1956). The content of amylose and amylopectin of the samples was determined by dividing the chromatogram at the lowest point between the two peaks (Sargeant 1982). To distinguish between short and long chains of amylose, the amylose fraction was further divided into the fraction eluted at the void of the gel and the fraction eluted between this and the amylopectin chains.

3.2.7 Size Distribution of Millet Starch Components and their B-Limit Dextrins

Duplicate starch samples (8 mg) were dissolved in 90% DMSO (200 L) and heated in warm water (80 °C) for 5 min and then at room temperature (25°C) overnight. The samples were then diluted with warm water (800 L). The size-distribution of the starch components was chromatographed on a Sepharose CL-2B column (1.6 × 32 cm) (Pharmacia, Uppsala, Sweden). A dissolved starch solution of 400 L was eluted through the column with 0.01 M NaOH at a rate of 0.5 mL/min. Fractions of 1 mL were collected, and the carbohydrate content was determined by the phenol-sulfuric acid reagent (Dubois et al 1956). The wavelength maxima (λ_max) of the glucan-iodine complex were determined with the WPA Spectrawave S800 Diode Array Spectrophotometer (Biochrom Ltd., Cambridge, United Kingdom) after the addition of 0.1 mL 0.01 M I₂/0.1 M KI solution prior to the neutralization of the collected fractions with 1 mL of 0.01 M HCl. β-
Limit dextrins (β-LD) of the millet starches were prepared according to the method reported by Bertoft (2004). Starch (8 mg) was dissolved in 90% DMSO (200 μL) overnight, after which 2 μL of β-amylase (4 U/mg) was added at pH 6 (0.01M NaOAc buffer, 100 μL). The resulting mixture was again stirred overnight and at room temperature (25°C) and diluted to 1 L with water, after which 400 μL of the mixture was applied to the Sepharose CL-2B column and analyzed.

3.2.8 Thermal Properties

Gelatinization and retrogradation parameters of millet starches were measured using a TA Instruments, Q1000 differential scanning calorimeter (DSC) (New Castle, DE, USA) equipped with a thermal analysis data station and data recording software (TA Instruments, Universal Analysis 2000, DE, USA). The DSC was earlier calibrated with indium. Starch dispersions in water (1:3) were equilibrated for 3 h at room temperature and scanned between 20–120 °C at a rate of 5 °C/min. After samples were scanned, they were kept at 4 °C for 7 days and then re-scanned using the same temperature and heating profiles. An empty aluminum pan was used as a reference. The transition temperatures reported are the onset (T_o), peak (T_p) and conclusion (T_c) temperatures. The enthalpy (ΔH) of gelatinization and melting of retrograded starch was estimated by integrating the area between the thermogram and a base line under the peak and was expressed as J/g of dry starch. Determinations were done in duplicate.
3.2.9 Exposure of Starches to Iodine Vapor

The millet starches were equilibrated in duplicates to 0.97 water activities ($a_w$) in desiccators containing a saturated solution of $\text{K}_2\text{SO}_4$ at room temperature. The equilibrated starches (0.2 g) were spread over a plastic pan and exposed to iodine vapor generated from 2 g of iodine crystals placed in a desiccator for 24 h at room temperature (Saibene et al 2008).

3.2.10 K/S Absorbance Spectra

The K/S (absorption coefficient/scattering coefficient) value of iodine exposed starches were evaluated by using CM 3500-d spectrophotometer (Konica Minolta Sensing Inc., Mahwah, NJ, USA) equipped with Spectra Magic NX CM-S 100 software. K/S is defined as $(1-R)^2/2R$, where R is the reflectance of the sample expressed as a fraction between 0 and 1 (Billmeyer & Saltzman 1981). Measurements were taken over a wavelength range from 400 to 700 nm with granular starch spread over an 8 mm sample cell. Starch not exposed to iodine was used as the reference. All determinations were done in duplicates.

3.2.11 Wide-Angle X-Ray Diffraction Analysis

Wide-angle X-ray scattering (WAXS) diffractograms were obtained for the starches in duplicates, packed tightly into a 1×1 inch depression on a quartz plate, using the radiation produced by a copper ($\text{K}_{\alpha 1} = 0.154$ nm) X-ray tube in the Rigaku X-ray diffractometer (Rigaku-Denki, Co., Tokyo, Japan). The diffractometer was set to the
following operating conditions: target voltage 40 kV, current 40 mA, scan speed 1°/min, sampling width 0.02°, divergence slit width 0.5°, scatter slit width 0.5°, receiving slit width 0.3 nm, and scanning range 3–35° 2θ. The diffractograms obtained were smoothed with the Jade software (version 6.5, Material Data Inc., California, USA) and normalized to equal total scattering in 3–35° 2θ range. Relative crystallinity (RC) was calculated according to the curve fitting procedure described by Lopez-Rubio et al (2008) using Igor Pro software (version 6.0.5.0, Wave Metrics Inc., Oregon, USA).

3.2.12 Statistical Analysis

Statistical analysis was performed using SPSS 16.0 with one way ANOVA at an alpha level of 0.05. Results are the averages of independent experiments.

3.3 Results and Discussion

3.3.1 Millet Starch Granules

The dry-weight yield of starch extracted from the millet flour samples ranged from 63.4% for finger millet to 93.7% for proso millet (Table 3.1). Pearl millet and foxtail millets had similar starch yields of 70.4% and 69.1%, respectively. The damaged starch contents of the millet samples ranged from 0.8% to 1.2% (Table 3.1).
Starch granule sizes ranged from 2.5 to 24 µm (Fig. 3.1). Foxtail millet starch granules were the largest amongst the samples, followed by pearl millet with granules ranging from about 3.5 µm to about 23 µm. Proso and finger millet had smaller granules with similar size distributions, and a peak size of about 5.7 µm. In contrast, the peak size of pearl and foxtail millet starch granules was about 7.6 µm.

Millet starch granules were mainly polygonal in shape with a few spherical granules (Fig. 3.2). The polygonal granules were generally larger than the spherical ones. It was, however, noted that finger millet did not have any spherical starch granules. The starch granules of the millet samples also showed indentations on their surfaces, especially on the large polygonal granules.

3.3.2 Molecular size distributions/characteristics of millet starches

The amylose content of the millet starches was determined by analyzing the chain profile of the debranched starches with gel-permeation chromatography on Sepharose CL-6B (Fig. 3.3). Apparent amylose contents were 28.6%, 33.9%, 32.4% and 32.5% for foxtail, proso, and finger and pearl millet starches, respectively (Table 3.2). Gel-permeation chromatograms revealed differences not only in the amylose content, but also in the relative amount of long chains (LCAM) and short chains (SCAM) of the amylose (Fig. 3.3). Table 3.2 shows the relative amounts and ratios of LCAM:SCAM of the samples. Finger and pearl millets had higher amounts of the long amylose chains resulting in high
ratios of 1.27 and 0.95, respectively. In contrast, foxtail and proso millets had more of the short amylose chains with consequent low ratios (0.60 and 0.63, respectively).

The molecular size distributions and the $\lambda_{\text{max}}$-values of the whole millet starches are shown in Fig. 3.4. All samples possessed a bimodal size distribution, with large size glucan molecules, corresponding to amylopectin, eluting in the void and smaller sized molecules, representing amylose, eluting after the void (Sargeant 1982). The $\lambda_{\text{max}}$ of the amylopectin fractions ranged from 550-590 nm. In these fractions, proso millet recorded lower $\lambda_{\text{max}}$ than the other samples. A sharp increase in the $\lambda_{\text{max}}$ to >600 nm marked the elution of the amylose component. The $\lambda_{\text{max}}$ of the amylose fraction was similar in all samples.

The molecular size distributions and the $\lambda_{\text{max}}$-values of the $\beta$-LDs of the millet starches are also shown in Fig. 3.4. The huge maltose peak stemmed from the removal of the linear amylose component and the external chains of the branched starch polymers (Takeda et al 1990). The $\beta$-LDs of all samples recorded lower $\lambda_{\text{max}}$-values (all below 600 nm) compared to their respective native starches. The $\lambda_{\text{max}}$-values of pearl and finger millets were generally higher, ranging from 560 to 589 nm, compared to 553–572 nm and 551–575 nm for foxtail and proso millets, respectively. For the amylopectin fractions, the $\lambda_{\text{max}}$-values tended to be slightly higher for finger and pearl millets (~556–576 nm) compared to foxtail and proso millets (~551–567 nm). The $\lambda_{\text{max}}$ of the apparent branched amylose fractions was similar or only slightly higher than for the amylopectin fraction in
all samples. Notably, however, the lower molecular weight molecules in fraction numbers > 40 were higher in finger and pearl millets than in foxtail and proso millets.

### 3.2.3 Physical Structure and Properties of Millet Starches

The iodine binding properties of the granular millet starches were also analyzed by exposing them to iodine vapor. Fig. 3.5 shows the K/S spectra of the millet starches over the range 400 to 700 nm. The absorbance maximum for all samples was approximately 550 nm. There were, however, significant differences in the K/S intensities. While finger millet recorded the lowest K/S intensities, proso and foxtail millets had the highest K/S values and pearl millet was intermediate.

X-ray diffractograms of the millet starches with and without iodine exposure is shown in Fig. 3.6. The millet starches possessed the typical A-type X-ray diffraction pattern with characteristic peaks at 15° and 23° and a doublet at 17° and 18° 2θ. The addition of iodine to the starches did not change the X-ray patterns, but resulted in differences in the peak intensities. An increase in the intensity at 20° 2θ, due to the single helical complex formed with iodine, was observed in all samples. In addition, a decrease in the intensity of the peak at 15° and the doublet at 17° and 18° 2θ was observed in all samples. This decrease was more pronounced in foxtail and proso millets than in finger and pearl millets. The peak at 23° 2θ remained unchanged after iodine addition to foxtail and proso millets, which was in contrast to finger and pearl millets that showed a marked
reduction in the peak intensity. The exposure of the millet starches to iodine vapor resulted in a significant increase in their relative crystallinity from 27–30% to 38–41% after iodine exposure (Table 3.3).

The gelatinization properties of the millet starch granules are shown in Fig. 3.7. The onset temperature \( T_o \) increased in the following order; pearl (62.8°C) > finger (63.9°C) > foxtail (66.7°C) > proso millet (68.4°C). Peak temperature \( T_p \) followed the same order and was between 67.9 and 72.2°C. The conclusion temperature \( T_c \) was above 72°C in all samples. Even though proso and foxtail millet recorded similar temperature melting ranges for their amylopectin component (7.6°C), their melting temperatures and enthalpies were significantly different: proso millet had a \( \Delta H \) of 13.1 J/g while foxtail millet had a \( \Delta H \) of 11.8 J/g. The \( \Delta H \) values of the other two samples were 12.3 J/g (pearl millet) and 13.2 J/g (finger millet).

The DSC parameters of the retrograded starches are shown in Fig. 8. The thermal properties of melting of the retrograded starches were generally typical for cereal starches, but \( T_o \) of finger millet was lower (38.8°C) than for the other millets. Finger millet also possessed a higher \( \Delta H \) of retrogradation (5.88 J/g) than the other millets. For the other samples \( \Delta H \) ranged from 2.18 J/g to 4.02 J/g.
3.4 Discussion

Starch yield largely depends on the treatment methods used for its extraction. In this study, the alkaline extraction method was used, and resulted in starch yields between 63.4 and 93.7%. Han and Hamaker (2002) reported that the alkaline method resulted in higher yield and purity. John et al (1999) reported a reduction in the yield of starch from samples pre-treated with potassium metabisulphite. They attributed this observation to a mild oxidative degradation during the pretreatment process, due to the cleavage of glucosidic linkages by sulphite. Ji et al (2004) reported that sedimentation as a starch isolation method resulted in higher starch yield compared to centrifugation. Starch damage is also an important factor to consider when extracting starch, because of its many effects on the properties of the starch (Morrison et al 1994). Damage to starch stems mainly from the milling of the seeds into flour. Morrison et al (1994) reported that both the enthalpy and the temperature of gelatinization decreases with increased starch damage. It is thus important to use procedures that result in very low amount of damaged starch. In this study, low starch damage values of 0.8–1.2% were recorded.

The morphological characteristics of the starch granules analyzed in this study (Fig. 3.1 and 3.2) were consistent with what has been reported in literature (Malleshi et al 1986b; Krishna and Thayumanavan 1998). The granule sizes of the samples were, however, larger than that reported by Krishna and Thayumanavan (1998). Using the scanning electron microscope, they reported that the size of the starch granules ranged from about 0.8 µm to 10 µm in proso, foxtail, barnyard, and kodo millets. Fujita et al (1996), after studying the particle size distribution of several varieties of foxtail millets,
observed an average granule size of 6.8 to 11.8 µm, with each granule size population showing a normal distribution curve. A bimodal distribution was reported for proso millet by Krishna and Thayumanavan (1998) but was not confirmed in this study. Pearl millet has been reported to have exclusively spherical starch granules (Malleshi et al 1986b); however, the pearl millet used in this study was found to have both spherical and polygonal granules. The indentations found on the millet starch granules are believed to be caused by protein bodies in the millet endosperm (Krishna and Thayumanavan 1998).

The results of this study showed that the millet starches differed in their molecular characteristics. The amylose content was higher than reported by Fujita et al (1996) for 18 millet varieties. This was possibly due to the fact that Fujita et al (1996) determined the amylose content by the amperometric titration method, as opposed to the gel permeation method used in this study.

The internal chains of the millet amylopectin and branched amylose were long enough to bind with iodine (Fig. 3.4). Shen et al (2013) reported the binding of amylopectin internal chains with iodine when the β-limit dextrins of waxy and amylose extender waxy corn starches were exposed to iodine. They suggested the pre-existence of amylopectin internal chains in helical forms that facilitated their binding to iodine in the absence of external chains. Chauhan and Seetharaman (2013) also concluded that longer internal amylopectin chains are helical and complex with iodine. The ability of the internal chains of the branched amylose fraction of the millet starches to bind iodine could therefore suggest that they also pre-exist in helical conformation. Interestingly, the
\( \lambda_{\text{max}} \)-values of the \( \beta \)-limit dextrins of the amylose fraction indicated differences in the internal chain segments. The higher \( \lambda_{\text{max}} \)-values of finger and pearl millets branched amylose suggested longer internal chains compared to foxtail and proso millets as is visualized in Fig. 3. 9.

The gelatinization parameters of the millet starches (Fig. 3.7) compared well with results reported in literature. After studying the thermal properties of three pearl millet varieties, Hoover et al (1996) reported \( T_o \), \( T_p \) and \( T_c \) ranges of 60.9°C–64.5°C, 67.5°C–75.0°C and 60.5°C–78.0°C, respectively. Those of waxy millet were reported to be 65.3°C, 76.4°C and 81.6°C, respectively (Choi et al 2004). The higher temperatures of gelatinization of foxtail and proso millet suggest a more perfect crystalline organization than in pearl and finger millets. The enthalpies of gelatinization of foxtail and pearl millets were, however, lower than in proso and finger millets, which means the latter had more and longer double helices formed by the external chains of the amylopectin component (Qi et al 2003). Further, foxtail and proso millets starch granules had higher \( T_o \), \( T_p \) and \( T_c \) compared to finger and pearl millets (Fig. 3.7). Based on the observation by Vamadevan et al (2013) that the internal structure of amylopectin correlates with \( T_o \), it is likely that the two groups of millet starches possess differences in their amylopectin internal structure, which influences the stability of the crystalline component of the starch granules. Thus, it appeared that not only the amylose component, but also the amylopectin component exhibited differences in molecular structure. These variations also appeared to affect the melting enthalpies of the retrograded starches (Fig. 3.8), which were also higher in finger and proso millets.
The decrease in the peak intensities at 15° 2θ and the 17° and 18° 2θ doublets observed in the X-ray diffractograms of samples after iodine exposure (Fig. 3.6) was not unique to the millet starches used in this study. Similar decreases in peak intensity in normal maize starch and even the disappearance of some peaks in high-amylose maize starch after their exposure to iodine have been observed earlier (Cheetham and Tao 1998). All samples had similar relative crystallinity without iodine, and upon the addition of iodine, significant increases were observed (Table 3.3). The relative crystallinity herein reported was calculated by dividing the area under crystalline peaks by the total area under the diffractogram. Hence, it represents a combination of the A- and V-type crystallites.

Despite the overall increase in relative crystallinity upon exposure to iodine, the X-ray diffractograms of proso and foxtail millets possessed similar changes in the diffraction patterns, while pearl and finger millets showed changes of a different kind (Fig. 3.6). Similarly, the K/S spectra of proso and foxtail millets were different from that of pearl and finger millets (Fig. 3.5). Interestingly, these observations were also reflected in the molecular characteristics of amylose in the samples (Fig. 3.3). Foxtail and proso millets had more short chains in their amylose component and, hence, a lower ratio of LCAM:SCAM compared to finger and pearl millets (Table 3.2).

The amylopectin of finger millet melted over the widest temperature range (Tc–To = 10.2°C), followed by pearl millet (Fig. 3.7). In contrast, foxtail and proso millets showed similar and narrower melting temperature ranges (Tc–To = 7.6°C). The melting
temperature range \((T_c-T_0)\) gives an indication of the homogeneity and quality of the amylopectin crystals. A narrow melting range indicates amylopectin crystals with more homogeneous quality and uniform stability, and vice versa (Ratnayake et al 2001). Thus, the amylopectin crystals of proso and foxtail millet starches appear to be more homogeneous, compared to pearl and finger millet starches. The higher enthalpy of melting of the retrograded finger millet starch compared to proso, foxtail and pearl millets could be due to a large variation in stability of its recrystallized amylopectin (Ratnayake et al 2001). It is, however, not clearly understood why the enthalpy of melting of the retrograded starches and gelatinization did not follow the same trend as with their temperatures of gelatinization and other molecular parameters reported in this study.

Furthermore, the intensity of the K/S value, which is defined as the ratio between the absorbance and the scattering coefficients, suggested differences in the flexibilities of the glucan chains inside the starch granules (Saibene et al 2008). The lower K/S intensities of finger and pearl millet starches suggested that the population of glucan polymers available to form inclusion complexes with iodine in these samples was considerably lower than in foxtail and proso millets. Apparently, however, the more flexible polymers in the starch granules in foxtail and proso millets did not render the crystalline structure unstable. On the contrary, they were more stable than granules in finger and pearl millets because they gelatinized at higher temperature (Fig. 3.7). Finally, Fig. 3.6 shows that the millet starches gave rise to different changes in their crystallinity patterns upon interaction with iodine vapor. Thus, the decrease in the intensity of the
double peak at 15° and 17° 2θ was more pronounced in finger and pearl millets than in foxtail and proso millets. On the other hand, the peak at 23° 2θ decreased only in foxtail and proso millets and not in the other millets. While this result also points to differences in the granular architecture, the actual underlying structural phenomena behind the shifts in the peak intensities remain unknown.

3.5 Conclusions

This study showed significant differences in the amylose structure and granular architecture of the millet starches. Foxtail and proso starches were found to have comparable properties, which were different from that of finger and pearl millets. Foxtail and proso millets starch granules possessed higher K/S intensities upon iodine complexation, and lower gelatinization temperatures with lower temperature ranges, compared to finger and pearl millets. The millet starches showed differences in their branched amylose structures as well: Foxtail and proso millets had more of shorter amylose chains, whereas pearl and finger millets had longer internal glucan chains between the branches in the branched component of their amylose.

3.6 Acknowledgements

We acknowledge Dr. Anand of Agriculture and Agri-Food Canada, Delhi Research Farm Delhi, Ontario, for providing the millet samples for the study. This research was funded by a grant from the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA).
### 3.7 Tables and Figures

Table 3.1: Starch Yield and Starch Damage of Millet Starches

<table>
<thead>
<tr>
<th>Sample</th>
<th>Starch yield (%)</th>
<th>Starch damage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foxtail</td>
<td>69.1</td>
<td>0.9 ± 0.03</td>
</tr>
<tr>
<td>Proso</td>
<td>93.7</td>
<td>0.9 ± 0.02</td>
</tr>
<tr>
<td>Finger</td>
<td>63.4</td>
<td>1.2 ± 0.01</td>
</tr>
<tr>
<td>Pearl</td>
<td>70.4</td>
<td>0.8 ± 0.01</td>
</tr>
</tbody>
</table>
Table 3.2: Amylose Content and Composition of Millet Starches

<table>
<thead>
<tr>
<th>Index</th>
<th>Foxtail</th>
<th>Proso</th>
<th>Finger</th>
<th>Pearl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylose (%)</td>
<td>28.6b</td>
<td>33.9a</td>
<td>32.4a</td>
<td>32.5a</td>
</tr>
<tr>
<td>$\text{LC}<em>\text{AM}$/$\text{SC}</em>\text{AM}$</td>
<td>0.60a</td>
<td>0.63a</td>
<td>1.27c</td>
<td>0.95b</td>
</tr>
<tr>
<td>$\text{LC}_\text{AM}$ (%)</td>
<td>10.6a</td>
<td>13.1b</td>
<td>18.1c</td>
<td>15.8d</td>
</tr>
<tr>
<td>$\text{SC}_\text{AM}$ (%)</td>
<td>17.9c</td>
<td>20.8d</td>
<td>14.3a</td>
<td>16.7b</td>
</tr>
</tbody>
</table>

*Values with the same letters are not significantly different within rows*

$\text{LC}_\text{AM}$ = long chain amylose; $\text{SC}_\text{AM}$ = short chain amylose
Table 3.3: Relative Crystallinity of Native and Iodine Vapor Exposed Millet Starches\textsuperscript{2}

<table>
<thead>
<tr>
<th>Sample</th>
<th>Crystallinity (%)</th>
<th>Native starch</th>
<th>Iodine exposed starch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foxtail</td>
<td>29a</td>
<td>40a</td>
<td></td>
</tr>
<tr>
<td>Proso</td>
<td>27a</td>
<td>41a</td>
<td></td>
</tr>
<tr>
<td>Finger</td>
<td>30a</td>
<td>38a</td>
<td></td>
</tr>
<tr>
<td>Pearl</td>
<td>28a</td>
<td>39a</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{2}Values with the same alphabets are not significantly different within columns.
Figure 3.1: Particle Size Distribution of Millet Starches
Figure 3.2: Scanning Electron Micrographs of Millet Starches

(a = Foxtail; b = Proso; c = Finger; d = Pearl)
Figure 3.3: Sepharose CL-6B Gel-Permeation Chromatogram of Debranched Millet Starches (LC\textsubscript{AM} = Long Chain Amylose; SC\textsubscript{AM} = Short Chain Amylose; AMP = Amylopectin Chains)
Figure 3.4: Sepharose CL-2B Gel-Permeation Chromatograms of Whole Millet Starches and their β-LD. Lines Represent Carbohydrate Content and the Symbols Represent the $\lambda_{\text{max}}$-Values.
Figure 3.5: K/S Spectra of Millet Starches. K/S is the ratio of absorption and scattering coefficients of the starches.
Figure 3.6: X-Ray Diffractogram of Millet Starches with (- - -) and without (—) Iodine Exposure
Figure 3.7: Gelatinization Parameters of Millet Starches ($T_o$ = Onset Temperature; $T_p$ = Peak Temperature; $T_c$ = Conclusion Temperature; $\Delta H$ = Enthalpy of Gelatinization. Values with the Same Letters Are Not Significantly Different)
Figure 3.8: Melting Parameters of Retrograded Millet Starches ($T_o =$ Onset Temperature; $T_p =$ Peak Temperature; $T_c =$ Conclusion Temperature; $\Delta H =$ Enthalpy of Melting of Retrograded Starch. Values with the Same Letters are Not Significantly Different)
Figure 3.9: Proposed Branched Amylose Structure of (a) Proso and Foxtail Millets and (b) Finger and Pearl Millets. Internal Chain Segments between Branch Points (Dotted Lines) are Longer in Finger and Pearl Millets.
3.8 References


CHAPTER 4: UNIT AND INTERNAL CHAIN PROFILE OF MILLET AMYLOPECTIN
Abstract
The unit chain compositions of debranched foxtail, proso, pearl and finger millet amylopectins and their \( \varphi,\beta \)-limit dextrins were analyzed by high performance anion-exchange chromatography. The \( \varphi,\beta \)-limit dextrins reflected amylopectin internal chain profiles. The amylopectins had average chain lengths (CL) ranging from 17.94–18.12. The ranges of external chain length (ECL), internal chain length (ICL) and total internal chain length (TICL) of the millet amylopectins were 11.85–12.33, 4.75–5.09 and 11.64–12.28, respectively. The relative molar concentration of B-chains in the amylopectins was close to 50\% in all samples. Significant differences were, however, observed in the proportions of very short "fingerprint" B-chains (B\(_{\text{fp}}\), DP 3–7) and the major group of short B-chains: foxtail and proso millets possessed high amount of B\(_{\text{fp}}\)-chains, whereas finger and pearl millets had higher amount of BS\(_{\text{major}}\)-chains, suggesting possible differences in the fine structure of the clusters and building blocks of the amylopectins. Millet amylopectin can be classified structurally as type 2.

Keywords: Millet, amylopectin, \( \varphi,\beta \)-limit dextrins, internal structure, external structure
4.1 Introduction

The confirmation of amylopectin as the major and highly branched component of starch by Meyer and coworkers in 1940 initiated research into how the chains of the molecule are organized. Peat et al (1952) defined A-chains as chains that do not carry other chains, whereas B-chains carry other chains. Hizukuri (1986) suggested that the A-chains are the shortest chains, whereas somewhat longer chains with a degree of polymerization (DP) of 20–24 are short B-chains, which he called B1-chains. Much longer chains with DP >36 were divided into B2-chains (DP 42-48) and B3-chains (DP 69-75). The actual arrangement of these chains into the branched amylopectin macromolecule is still a matter of debate, however. Two major models of the structure are discussed today, namely the cluster model and the two-directional building block backbone model (Bertoft 2013).

The unit chain distribution of the amylopectin molecule can be studied by specifically hydrolyzing the α-(1,6)-linkages in the molecule with the debranching enzymes isoamylase and pullulanase, and analyzing the size distribution of the chains with high-performance anion-exchange chromatography (HPAEC). The chains can then be grouped into short (DP <36) and long chains (DP >36) (Bertoft 2004). The chains of amylopectin can also be classified as external and internal. While the external chains are found in the crystalline parts of the amylopectin molecule, the internal chains are generally considered to be in the amorphous part where most of the branches are also found (Pérez and Bertoft 2010). To isolate the internal chains of amylopectin, the
external chains are removed by exo-acting enzymes. The most commonly used is β-amylase, which attacks the glucan chains from their non-reducing ends, producing maltose. Since β-amylase cannot by-pass a branch point in the amylopectin molecule, the resulting β-limit dextrin represents the internal part of the amylopectin molecule with the branches (Manners 1989). Another exo-acting enzyme is phosphorylase a from rabbit muscle, which produces φ-limit dextrins. The successive use of phosphorylase a and β-amylase results in the production of φ,β-limit dextrins (φ,β-LDs), where all A-chains and the external segments of the B-chains appear as maltosyl and glucosyl stubs, respectively (Bertoft 1989). The φ,β-LD allows the average chain lengths and relative molar amounts of different chain categories of the internal chains of amylopectin (which are the B-chains) to be estimated. According to Bertoft et al (2008), the short internal B-chains of amylopectin can be divided into “fingerprint” B-chains (Bfp) with DP ≤7, and a major group of short chains (BSmajor) with DP ~8–25. Long B-chains (BL) are divided into B2-chains (DP 26–50) and B3-chains (DP >50), apparently corresponding to the respective groups found in the original amylopectin. From the internal chain profile it is possible to calculate the total internal chain length (TICL), defined as the average length of the B-chains without their external chains (Bertoft 1991). A comparison of the average chain length (CL) of the amylopectin and the limit dextrin also enables an estimation of the average external chain length (ECL), defined as the length of the segments of the chains that extend from the outermost branch to the non-reducing end of the chains, and the average internal chain length (ICL), defined as the length of the chain segment between two branches in the amylopectin molecule (Manners 1989). Previous studies on unit chain profiles (Hizukuri 1985; Hizukuri 1986; Inouchi et al 1987; Jane et al 1999;
Koizumi et al 1991; Hanashiro et al 1996; Hanashiro et al 2005; Takeda et al 1987; Van Hung et al 2007) as well as the internal structure (Bertoft and Koch 2000; Bertoft et al 2008; Kong et al 2009; Laohaphatanaleart et al 2009; Zhu et al 2011; Bertoft 2013; Shi and Seib 1995; Kalinga et al 2013; Klucinec and Thompson 2002) of amylopectins from different botanical sources have been reported. Based on the finding of Bertoft et al (2008), amylopectins from various starches were grouped into four structural types with respect to their internal chain profile.

In this study, the unit chain composition of amylopectins from four different millets, namely foxtail, proso, finger, and pearl millets, are reported. Millets are small-grained cereals and are one of the most important drought-resistant cereal crops. Compared to other cereals, millets are much more resistant to diseases and pests; they mature faster, and give greater yields (Devi et al 2011). In places where millets are cultivated, they provide food security for the population (Pradhan et al 2010). In North America and many European countries, millet has not been recognized as a single important food commodity, though its use in multigrain and gluten-free products has been mentioned (Saleh et al 2013). Nutritionally, millets compare very well with majors cereals such as rice and wheat (Parameswaran and Sadasivam 1994). Just like many other cereals, the main component of millets is starch (about 58-70%) (Abdalla et al 1998) and it possesses many characteristics similar to those of other cereal starches. However, the structural characteristics of the amylopectin component of millet starches have not been documented previously.
4.2: Materials and Methods

4.2.1 Materials

Seeds of proso, foxtail, finger, and pearl millets were obtained from Agriculture Environmental Renewal Canada (AERC) Inc, Delhi, Canada. These seeds were kept at -20 °C and later used for the study.

4.2.2 Methods

4.2.3 Starch Extraction

Millet starch was extracted from their flour samples according to the method reported previously (Annor et al 2013).

4.2.4 Amylopectin Fractionation

The fractionation of amylopectin was done according to the method reported by Klucinec and Thompson (1998) with some modifications. Granular starch (2 g) was dispersed in 90% dimethyl sulfoxide (DMSO) (40 mL) in a water bath (90 °C), and stirred constantly with a magnetic stirrer for 3 h under nitrogen gas. The clear starch solution was then cooled slowly to 65 °C and 4 volumes (160 mL) of ethanol was added drop-wise using a peristaltic pump to precipitate the starch. The starch-ethanol mixture was cooled to room temperature (25 °C) and centrifuged at 8000 x g for 10 min at 4 °C. The supernatant was discarded and the precipitated starch was washed once again with...
ethanol to obtain non-granular starch. The non-granular starch was dissolved in 90 % DMSO (56 mL) at 90 °C under nitrogen for another 3 h with constant stirring. A mixture of, 1-butanol (23.5 mL) and isoamyl alcohol (23.5 mL) and water (324 mL) being stirred vigorously was added drop-wise to the dissolved starch, after it had been cooled to 85 °C. After the addition of the 1-butanol, isoamyl alcohol and water mixture, the entire mixture was cooled to about 28 °C for 20 h. The entire mixture was then centrifuged at 22,360 x g at 4 °C for 30 min. The supernatant, which was the amylopectin fraction, was decanted carefully and reduced in volume to about 50 mL by rotary evaporation at 50 °C. The 1-butanol–isoamyl alcohol–water mixture was added again to the amylopectin solution to precipitate any remaining amylose, after which the volume of the amylopectin supernatant was once again reduced to about 50 mL. Three volumes of methanol was then added to the amylopectin solution and left overnight at room temperature to be precipitated. The precipitated amylopectin was obtained after centrifugation at 22,360 x g at 4 °C for 20 min. The amylopectin was re-dissolved in hot water (20 mL), re-precipitated with 3 volumes of ethanol for 1 h at room temperature and collected by centrifugation. The precipitated amylopectin was dissolved in hot water (20 mL) and then freeze dried.

4.2.5 Analysis of Amylopectin Purity by Gel-Permeation Chromatography

The purity of the amylopectin samples was analyzed after debranching of the sample by gel-permeation chromatography on Sepharose CL-6B according to the procedure previously reported (Laohaphatanaleart et al 2009). The total carbohydrate
content of the various test tube fractions was determined with the phenol-sulphuric acid method (Dubois et al 1956). The column was calibrated with both linear and branched dextrins of known DP as previously described (Bertoft & Spoof 1989).

4.2.6 Production of φ,β-limit Dextrins

The φ,β-limit dextrins were produced according to the method of Bertoft (2004) with modifications as reported by Kalinga et al (2013). It involved removal of the external chains of the amylopectin by the successive use of phosphorylase α and β-amylase.

4.2.7 Analysis of Unit Chain Distributions of Amylopectins and their φ,β-limit Dextrins

Millet amylopectins or their φ,β-limit dextrins (2.0 mg) were dissolved in 90% DMSO (50 µL) with gentle stirring overnight. The solution was diluted by adding warm water (400 µL) (80 °C) after which 0.01 M sodium acetate buffer (50 µL) (pH, 5.5) was added. Isoamylase (1 µL) and pullulanase M1 (1 µL) (Megazyme International Ireland, Bray, Ireland) were added to the mixture, which then was stirred overnight at room temperature. After debranching, the enzymes were inactivated by boiling for 5 min, the volume adjusted to obtain a final concentration of 1 mg/mL, the sample filtered through a 0.45 µm nylon filter. The filtered sample (25 µL) was injected into the Dionex ICS 3000 HPAEC system (Dionex Corporation, Sunnyvale, CA, USA) equipped with a pulsed amperometric detector (PAD), CarboPac PA-100 ion-exchange column (4 x 250 mm) and
a similar guard column (4 x 50 mm). The samples were then eluted with a flow rate of 1 mL/min. The eluents used were A (150 mM sodium hydroxide) and B (150 mM sodium hydroxide containing 500 mM sodium acetate). An elution gradient was made by mixing eluent B into eluent A as follows: 0–9 min, 15–36% B; 9–18 min, 36–45% B; 18–110 min, 45–100% B; 100–112 min, 100–15% B; 112–130 min, 15% B was used. The system was stabilized by elution at 15% B for 60 min between runs. The areas under the chromatograms were corrected to carbohydrate concentration using the method of Koch et al (1998).

4.2.8 Statistical Analysis

Statistical analysis was performed using SPSS 16.0 with one way ANOVA at an alpha level of 0.05.

4.3 Results and Discussion

The purity of the amylopectin samples isolated from the millet starches was analyzed by gel-permeation chromatography on Sepharose CL-6B after debranching. All preparations showed a purity of about 96%, indicating the presence of small amounts of amylose. The amylose found in the fractionated amylopectin may be attributed to real amylose chains or very long chains of amylopectin. Such very long chains, also called “super long chains”, have been reported in some amylopectins (Inouchi et al 2005; Takeda 1987; Laohaphatanaleart et al 2009).
A detailed analysis of the unit chain profile of the amylopectins was performed with high-performance anion-exchange chromatography (HPAEC) and is shown in Fig. 4.1. Interestingly, millet amylopectin samples possessed two different types of profiles, with foxtail and proso millets being similar to each other, whereas finger and pearl millets were similar to each other and different from foxtail and proso. The groove position, marking the division between the short and long chains, also differed between the samples. For proso and foxtail millet amylopectins, the division was at DP 35 or 36, whereas the division for finger and pearl was at DP 36 and 38, respectively. Also the peak-DP of the long chains of proso and foxtail millet amylopectins was slightly lower at DP 43 than that of pearl and finger millets at DP 44.

The internal chain distributions of the millet amylopectins obtained from their φ,β-LDs are shown in Fig. 4.2. Foxtail and proso millets were observed to have similar internal chain profiles, and they were different from finger and pearl millets. The divisions of the various chain categories of finger and pearl millets were generally at higher DP than for foxtail and proso millets. The peak-DP of the various B-chain categories of foxtail and proso millet starches was exactly the same (Fig. 4.2). Finger and pearl millet starches on the other hand had slightly different peak-DPs and were also higher than those of foxtail and pearl millet starches.

Table 4.1 shows the average chain lengths of various chain categories and segments of the millet amylopectins. The average chain length (CL) ranged from 17.9 to 18.1, which was similar to that of other cereal starches (Bertoft et al 2008). This was
expected because cereal starch granules, which have A-type X-ray allomorph pattern, are known to have amylopectins with shorter CL compared to root and tuber amylopectins, which have longer chains and B-type granules (Hizukuri 1985). The short and long chains of pearl millet were significantly ($p < 0.05$) longer than in the other samples. The $\phi,\beta$-limit value of the millet amylopectins ranged from 57.7 for pearl to 59.8 for proso millet and were comparable to ranges of amylopectins from other plants reported in the literature (Bertoft 2008). The $\phi,\beta$-limit value reflects generally the length of the external chains, which was comparatively shorter in finger and pearl millets (ECL 11.9 and 12.0, respectively) compared to foxtail and proso millets (ECL 12.3 and 12.3, respectively). Since the external chain segments participate in the crystalline lamellae of the starch granules, foxtail and proso millets may have a thicker lamella than the rest of the samples. Generally, ECL of the millet amylopectins was similar to the amylopectins of many other cereal starches and lower than that of the B-type starches (Bertoft et al 2008, Zhu et al 2011).

The TICL of the millet amylopectins was generally lower than that of other amylopectins (Bertoft et al 2008). Zhu et al (2011) reported a mean TICL of 14.0 for eleven Chinese sweet potato genotypes. Laohaphatanaleart et al (2009) reported TICL of 12.4–13.8 for three rice varieties and 13.1–15.7 for two cassava varieties. The average internal chain length (ICL) of proso and foxtail millets was shorter than that of pearl and finger millets, suggesting that proso and foxtail millets have a more tightly branched structure in their amorphous lamellae. This was also suggested from the clearly shorter
internal length of the BS- and BL-chains in the ϕ,β-LDs of foxtail and proso millets (Table 4.1).

The relative molar amounts of various chain categories of the millet amylopectins are shown in Table 4.2. The molar amount of the A-chains ranged from 49.4% to 50.7%, with that of pearl millet being significantly lower than the rest of the samples. The $A_{fp}$-chains with DP 6–8 are unique to amylopectins from different sources (Koizumi et al 1991). They were previously supposed to be situated outside the clusters of chains in the amylopectin, and therefore only the rest of the A-chains were considered to be "clustered A-chains" (Bertoft et al 2008). Later it was found that the $A_{fp}$-chains are part of the clusters after all and cannot be distinguished from other A-chains on this basis (Bertoft et al 2012). However, the $A_{fp}$-chains are known to be too short to effectively participate in the crystallites of the starch granules (Gidley and Bulpin 1987). Therefore, only the rest of the A-chains more likely are involved in the crystallites and are here designated "crystalline A-chains" ($A_{crystal}$) rather than "clustered A-chains". Finger millet amylopectin had the least molar amounts of $A_{fp}$-chains (7.05%) while pearl millet had the most (7.20%), but these differences were very small and probably of minor importance. All the samples, except pearl millet, also showed similar molar amounts of the $A_{crystal}$-chains. Overall, however, it was noted that the relative amount of the $A_{crystal}$-chains obtained in this study was much lower than that found for many other amylopectin samples reported by Bertoft et al (2008), with the exceptions for Andean yam bean, waxy maize and amylose-free potato starch.
Within the B-chain categories, pearl and finger millets tended to have somewhat more of the short B-chains (BS) and less of the long B-chains (BL) compared to the other samples (Table 3.2). The most significant differences were, however, found among the sub-categories of the BS-chains. Thus, B<sub>fp</sub>-chains were more abundant in foxtail and proso millets (20.5 and 21.3%, respectively) than in finger and pearl millets (19.4 and 19.5%, respectively). The B<sub>fp</sub>-chains have been suggested to participate in tightly branched building blocks in clusters (Bertoft and Koch 2000). In the case of the BS<sub>major</sub>-chains, finger and pearl millets contained more (23.1 and 25.0%, respectively) than foxtail (21.6%) and proso (20.5%) millets. BS<sub>major</sub>-chains are involved in the interconnection of two or three building blocks (Bertoft et al 2012).

Table 4.3 shows selected molar ratios of the different chain categories from the millet amylopectins and their φ,β-limit dextrins. These ratios, together with the relative molar composition of the different chain categories (Table 4.2), can be used to classify the samples on the basis of their structure into one of the four types of amylopectin proposed by Bertoft et al (2008). As seen in Table 4.2, the molar amounts of B3-chains of the millet amylopectins ranged between 0.5 and 0.7. These values fit into the range of 0.7-1.0 proposed for type 2 amylopectins. Also the molar concentrations of the B2–chains (5.5–6.8) fit with the range proposed for type 2 amylopectins. Additionally, the molar concentrations of BS-, B<sub>fp</sub>- and BS<sub>major</sub>-chains, as well as the molar ratios listed in Table 4.3, all fitted very well with the ranges reported for type 2 amylopectins. Thus, there remained no doubt as to which structural type the amylopectin component of millet
starches belongs. Other amylopectins of this type are found in maize and rice (Bertoft et al. 2008).

In as much as these ratios are determining the type of amylopectin structure, they indicate important structural features of the amylopectins. The ratio of A:B-chains of the millet amylopectins was close to 1.0 (Table 4.3) indicating a similar proportion of the Staudinger and Haworth conformations in the structure (Staudinger and Husemann 1937, Haworth 1937). The ratio of BS:BL for the millet amylopectins significantly differed from each other, and it was higher in finger and pearl millets (6.1 and 7.4, respectively) than in foxtail and proso millets (5.9 and 5.6, respectively) This ratio gives an indication of the proportion of B-chains involved in the internal structure of the clusters (BS-chains) and the chains interconnecting them (BL-chains) according to the traditional cluster model of amylopectin (Hizukuri 1986). However, in the building block backbone model, the BL-chains are mainly parts of the backbone, whereas BS-chains mainly, but not entirely, are found as side-chains to the backbone and interconnect building blocks (Bertoft 2013). If it is assumed that one crystalline A-chain generally forms a double helix with the external segment of one BS-chain, as the double helix has been modeled (Imberty and Perez 1989; O'Sullivan and Perez 1999), then the ratio of A\text{crystal}:BS should be 1.0. Indeed, the ratio was close to 1.0 in all the millet amylopectins. It was, therefore, of interest to note that the ratio of A\text{crystal}:all B-chains (A\text{crystal}:B) was below 1.0, which implied that the BL-chains likely did not participate in the formation of double helices. The most significant differences between the individual millet samples were noted in the ratio of B\text{fp}:BS\text{major}. Foxtail and proso millets possessed clearly higher ratios than finger
and pearl millets. These interesting differences suggested that foxtail and proso millets on one hand, and finger and pearl millets on the other hand, possessed unique structures of their amylopectin components on the level of the building blocks and their organization within the macromolecule (Bertoft et al. 2012; Bertoft 2013). The unravelling of the details at this structural level has, however, to await a more detailed investigation of the cluster structure of the samples.

4.4 Conclusions

The amylopectins of the four millet types studied showed significant differences in their unit and internal chain profiles. For most of the structural parameters studied, foxtail and proso millets were similar to each other but different from finger and pearl millets. Significant differences were also observed in the relative amount of the Bfp- and BSmajor-chains. The differences observed in the internal chain composition of the millet amylopectins suggest that the fine structure of their clusters and building blocks is considerably different. Finally, the structure of millet amylopectin can be classified as type 2, according to the classification previously proposed by Bertoft et al. (2008).

4.5 Acknowledgements

We acknowledge Dr. Anand of Agriculture and Agri-Food Canada, Delhi Research Farm Delhi, Ontario, for providing the millet seeds for the study. This research was supported by a grant from the International Development Research Centre (IDRC) in Canada.
### 4.6 Table and Figures

Table 4.1: Average Chain Lengths of Different Chain Categories and φ,β-limit Values of Millet Amylopectin obtained by HPAEC*  

<table>
<thead>
<tr>
<th>Sample</th>
<th>Foxtail</th>
<th>Proso</th>
<th>Finger</th>
<th>Pearl</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL</td>
<td>18.0a</td>
<td>18.1b</td>
<td>17.9a</td>
<td>18.0a</td>
</tr>
<tr>
<td>SCL</td>
<td>15.5a</td>
<td>15.5a</td>
<td>15.5a</td>
<td>15.8b</td>
</tr>
<tr>
<td>LCL</td>
<td>51.0b</td>
<td>50.3a</td>
<td>51.4b</td>
<td>52.1c</td>
</tr>
<tr>
<td>ECL</td>
<td>12.3d</td>
<td>12.3d</td>
<td>11.9a</td>
<td>12.0b</td>
</tr>
<tr>
<td>ICL</td>
<td>4.8a</td>
<td>4.8a</td>
<td>5.1b</td>
<td>5.0b</td>
</tr>
<tr>
<td>TICL</td>
<td>11.6a</td>
<td>11.7ab</td>
<td>12.3c</td>
<td>11.9b</td>
</tr>
<tr>
<td>φ,β-limit value</td>
<td>59.7c</td>
<td>59.8c</td>
<td>57.7a</td>
<td>58.2b</td>
</tr>
<tr>
<td>CL&lt;sub&gt;LD&lt;/sub&gt;</td>
<td>7.3a</td>
<td>7.3a</td>
<td>7.6b</td>
<td>7.5b</td>
</tr>
<tr>
<td>BS-CL&lt;sub&gt;LD&lt;/sub&gt;</td>
<td>8.8b</td>
<td>8.5a</td>
<td>9.2c</td>
<td>9.5d</td>
</tr>
<tr>
<td>BL-CL&lt;sub&gt;LD&lt;/sub&gt;</td>
<td>35.5a</td>
<td>36.6b</td>
<td>38.1c</td>
<td>37.9c</td>
</tr>
</tbody>
</table>

CL = average chain length of amylopectin  
SCL = CL of short chains  
LCL = CL of long chains  
ECL (external chain length) = CL x (φ,β-limit value/100) + 1.5  
ICL (internal chain length) = CL - ECL – 1  
TICL (total internal chain length) = B-CL<sub>LD</sub>-1  
φ,β-limit value was calculated from the difference in CL of amylopectin and its φ,β-LD.  
CL<sub>LD</sub> = average chain length of φ,β-limit dextrin  
BS-CL<sub>LD</sub> = CL of short B-chains  
BL-CL<sub>LD</sub> = CL of long B-chains  
*Values with the same letters are not significantly (p < 0.05) different within each row
Table 4.2: Relative Molar Amounts (%) of Chain Categories in Amylopectins of Millet Starches\textsuperscript{z}

<table>
<thead>
<tr>
<th>Sample</th>
<th>Foxtail</th>
<th>Proso</th>
<th>Finger</th>
<th>Pearl</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-chains\textsuperscript{a}</td>
<td>50.7b</td>
<td>50.7b</td>
<td>50.4b</td>
<td>49.4a</td>
</tr>
<tr>
<td>Afp\textsuperscript{b}</td>
<td>7.1b</td>
<td>7.1b</td>
<td>7.1a</td>
<td>7.2c</td>
</tr>
<tr>
<td>A\textsubscript{crystal}\textsuperscript{c}</td>
<td>43.6b</td>
<td>43.6b</td>
<td>43.4b</td>
<td>42.2a</td>
</tr>
<tr>
<td>B-chains\textsuperscript{d}</td>
<td>49.3b</td>
<td>49.3b</td>
<td>49.6b</td>
<td>50.6a</td>
</tr>
<tr>
<td>BS</td>
<td>42.1ab</td>
<td>41.8a</td>
<td>42.6b</td>
<td>44.6c</td>
</tr>
<tr>
<td>BL</td>
<td>7.2b</td>
<td>7.4c</td>
<td>7.0b</td>
<td>6.0a</td>
</tr>
<tr>
<td>B\textsubscript{fp}\textsuperscript{e}</td>
<td>20.5b</td>
<td>21.3b</td>
<td>19.4a</td>
<td>19.5a</td>
</tr>
<tr>
<td>BS\textsubscript{major}\textsuperscript{f}</td>
<td>21.6b</td>
<td>20.5a</td>
<td>23.1c</td>
<td>25.0d</td>
</tr>
<tr>
<td>B\textsubscript{2}\textsuperscript{g}</td>
<td>6.7d</td>
<td>6.8c</td>
<td>6.4b</td>
<td>5.5a</td>
</tr>
<tr>
<td>B\textsubscript{3}\textsuperscript{h}</td>
<td>0.5b</td>
<td>0.7d</td>
<td>0.6c</td>
<td>0.5a</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Detected as maltose after debranching of $\varphi,\beta$-LD.

\textsuperscript{b} “Fingerprint” A-chains at DP 6-8 in the original amylopectin sample.

\textsuperscript{c} A\textsubscript{crystal} -chains calculated as all A-chains–Afp.

\textsuperscript{d} B-chains correspond to DP $\geq$3 in $\varphi,\beta$-LDs, and were divided into short (BS) and long chains (BL) at between DP 22–25 and above respectively depending on the sample.

\textsuperscript{e} “Fingerprint” B-chains at DP 3–7 in $\varphi,\beta$-LDs.

\textsuperscript{f} The major group of short B-chains at DP 8 to 22–24 depending on the sample.

\textsuperscript{g} Long chains between DP 22 or 25 and 55 depending on the sample.

\textsuperscript{h} Long chains at DP $\geq$56

\textsuperscript{z}Values with the same letters are not significantly different (p < 0.05) within each row.
Table 4.3: Selected Molar Ratios of Different Chain Categories of Millet Amylopectins and their φ,β-limit Dextrins

<table>
<thead>
<tr>
<th>Sample</th>
<th>Foxtail</th>
<th>Proso</th>
<th>Finger</th>
<th>Pearl</th>
</tr>
</thead>
<tbody>
<tr>
<td>A:B</td>
<td>1.0a</td>
<td>1.0a</td>
<td>1.0a</td>
<td>1.0a</td>
</tr>
<tr>
<td>S&lt;sub&gt;ap&lt;/sub&gt;:L&lt;sub&gt;ap&lt;/sub&gt;</td>
<td>13.4b</td>
<td>12.4a</td>
<td>13.7c</td>
<td>15.9d</td>
</tr>
<tr>
<td>BS:BL</td>
<td>5.9b</td>
<td>5.6a</td>
<td>6.1c</td>
<td>7.4d</td>
</tr>
<tr>
<td>A&lt;sub&gt;crystal&lt;/sub&gt;:BS</td>
<td>1.0a</td>
<td>1.0a</td>
<td>1.0a</td>
<td>1.0a</td>
</tr>
<tr>
<td>A&lt;sub&gt;crystal&lt;/sub&gt;:B</td>
<td>0.9b</td>
<td>0.9b</td>
<td>0.9b</td>
<td>0.8a</td>
</tr>
<tr>
<td>B&lt;sub&gt;fp&lt;/sub&gt;:BS&lt;sub&gt;major&lt;/sub&gt;</td>
<td>1.0b</td>
<td>1.0b</td>
<td>0.8a</td>
<td>0.8a</td>
</tr>
</tbody>
</table>

S<sub>ap</sub> (short amylopectin chains)

L<sub>ap</sub> (long amylopectin chains)

The rest of the abbreviations explained in Table 4.3.

Values with the same letters are not significantly different (p < 0.05) within each row.
Figure 4.1: The Unit Chain Profile of Debranched Millet Amylopectins Obtained By High Performance Anion-Exchange Chromatography (HPAEC) with Arrows and Numbers Indicating DP.
Figure 4.2: The Unit Chain Profile of Debranched ϕ,β-limit Dextrins of Millet Amylopectins Obtained by High-Performance Anion-Exchange Chromatography (HPAEC) with Arrows and Numbers Indicating DP. Short B-chains (BS) are sub-divided into “fingerprint” B-chains (Bfp), and a major group (BSmajor), whereas long B-chains (BL) are subdivided into B2- and B3-chains.
References


CHAPTER 5: IN VITRO STARCH DIGESTIBILITY AND EXPECTED GLYCEMIC INDEX OF KODO MILLET (*Paspalum scrobiculatum*) AS AFFECTED BY STARCH-PROTEIN-LIPID INTERACTIONS
Abstract

The effect of starch-protein-lipid interaction on the in vitro starch digestibility and expected glycemic index (eGI) of kodo millet flour was investigated. Debranned MF, the flour with lipid removed, protein removed or both lipid and protein removed (MF-L-P) were subjected to digestion assays. The in vitro starch digestibility and eGI of the millet samples and millet starch were compared with rice or wheat flour. Rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) of the samples were also calculated. Protease treatment and defatting resulted in significant reduction ($P < 0.05$) in protein and lipid contents of samples. Significant increases in the in vitro starch digestibility and eGI of samples was observed after removal of proteins, lipids or both. The effect of lipid removal on in vitro starch digestibility of kodo millet was found to be more significant, compared with when proteins were removed. The eGI increased from 49.4 for cooked MF to 62.5 for MF-L-P. The eGI of cooked kodo millet starch was significantly lower than that of cooked rice flour. The RS of cooked rice was the least amongst the samples. The in vitro starch digestibility and eGI of rice was significantly higher than those of MF. Processes applied to kodo millet such as decortication that results in the removal of proteins, lipids or both (especially lipids) would result in an increase in its in vitro starch digestibility and eGI. We therefore advocate for the development of acceptable products from whole millets to maintain its hypoglycemic property.

Keywords: Kodo millet, in vitro digestibility, expected glycemic index, starch
5.1 Introduction

Millet is an important crop for populations in many African and Asian countries. Because of its ability to withstand droughts, to produce substantial yields in a wide range of soils and climates where other cereal crops fail (Malleshi and Desikachar 1986), it has become the staple food in these populations. Millet is also nutritionally better, compared to other major cereals such as rice or wheat, and the presence of all the required nutrients in millet makes them suitable for the manufacture of healthful foods (Shinoj et al 2006). There are many different species of millets, including pearl millet (*Pennisetum glaucum*), which is classified as major millet. Foxtail millet (*Setaria italica*), proso millet (*Panicum miliaceum*), finger millet (*Eleusine coracana*) and kodo millet (*Paspalum scrobiculatum*), amongst others are known as minor or small millets. Millets are reported to reduce the risk of diabetes mellitus due to low glycemic and insulinemic response based on indigenous knowledge within different cultures in India. In a study by Lakshmi and Sumathi (2002), they reported that the consumption of finger millet based diets resulted in significantly lower blood glucose levels compared to wheat or rice when fed to type II diabetes subjects. This hypoglycemic property of millet starches may be attributed to many factors including the digestibility of starch, presence of molecules that binds starch and processing effects (Englyst et al 1996; Garcia-Zaragoza et al 2010).

Factors such as the interaction of starch, proteins and lipids on the functional properties of foods have been investigated (Zhang et al 2006). Zhang et al (2006) reported that a three-component interaction among starch, lipids, and protein possibly
occurs in foods, and this phenomenon has profound effects on the nutritional and functional quality of foods. Proteins, usually in the form of a matrix encapsulate starch granules, reducing the overall in vitro starch digestibility particularly in wheat (Jenkins et al 1987). High amounts of lipids are also known to confer resistance to starch towards α-amylolysis. Amylose complexed with lipids has been reported to be highly resistant to α-amylase compared to free amylose (Holm et al 1983). This observation implies that amylose-lipid complexes have the tendency to result in reduced spikes in blood glucose levels when consumed. Increased accessibility of α-amylase to starch substrates after pre-digestion with pepsin has been reported (Malouf et al 1992). With protein and fat contents up to 16% and 7%, respectively (Ravindran 1991), starch-protein-lipid interactions could play a role in explaining the hypoglycemic property of millets. A search through literature, however, reveals that little or no information exist on the role played by proteins and lipids in influencing the in vitro starch digestibility and glycemic index of millets. This study investigates the effects of starch-protein-lipid interaction on the in vitro digestibility and expected glycemic index of kodo millet.

5.2 Materials and Methods

5.2.1 Materials

Kodo millet (COCU9 variety) was provided by Malathi Ravindran from Tamilnadu Agricultural University, Coimbatore, India. The millet samples were debranned with a modified rice pearler and milled with a UDY mill (Fort Collins, CO, USA). Long-grain rice was obtained from a local supermarket in Guelph (Ontario,
Canada). Commercial strong baker’s wheat flour was obtained from ADM (Montreal, Quebec, Canada). Kodo millet starch was extracted from the debranned kodo millet flour (MF).

5.2.2 Methods

Part of the debranned MF samples were defatted (MF-L) or treated with proteases (MF-P), and parts of the defatted millet flour were again treated with proteases (MF-L-P) to remove fat, proteins or both fats and proteins, respectively.

5.2.3 Defatting

Flour samples were defatted with petroleum ether using the Soxhlet extraction to extract non-polar lipids. The extraction was done for 5 h at 30°C. After the petroleum ether extraction, the defatted samples were again treated with 3:1 (v/v) n-propanol/water in a Soxhlet apparatus for 5 h at 45°C. The defatted samples were then dried at 50°C overnight.

5.2.4 Protease Treatments

Debranned or defatted millet flour samples were treated with proteases according to the method reported by Marti et al (2011) with modifications. A protease solution (100 mL) containing 0.25 g trypsin from porcine pancreas (13,000-20,000 BAEE units/mg
protein; Sigma-Aldrich, Oakville, ON, Canada), 0.25 g of papain from *Carica papaya* (≥3 units/mg, Sigma-Aldrich) and 4 L of proteinase K (30 units/mg; Sigma-Aldrich) was prepared in 50 mM sodium acetate buffer, (pH 8) and added to 10 g of sample. After overnight incubation at room temperature under magnetic stirring (150 rpm), the suspension was centrifuged (10 min, 3000 × g). The residue was then washed three times with deionized water. The protease treatment was repeated again for 3 h, and the washing step with water was repeated. The brown protein top layer of the samples was gently removed with repeated addition of water and centrifugation till there was no brown layer. The sample was then dried at 50°C overnight.

5.2.5 Starch Extraction

Kodo millet starch was extracted from the debranned flour according to the method reported by Waduge et al (2010) with modifications. MF (100 g) was stirred with 2 L sodium borate buffer (12.5 mM, pH 10, containing 0.5% SDS [w/v] and 0.5% Na2S2O5 [w/v]) for 5 min and the residue recovered by centrifugation at 900 × g for 5 min to extract the proteins. The extraction step was repeated once again. The residue was washed three times with distilled water and recovered by centrifugation. The residue was then suspended in distilled water and stirred overnight to further release the protein from the starch granules, after which the starch slurry was passed through four layers of cheesecloth and then through 70 μm nylon mesh. The slurry was centrifuged and the brown layer, formed on the top of the starch layer, was scraped using a spatula. The starch was then suspended again in water and centrifuged in 50 mL centrifuge tubes at
1600 × g for 10 min. These steps were continued until all the brown particles were removed from the starch fraction.

5.2.6 Microscopy

All samples were viewed under polarized light using the Olympus BX 60 Light Microscope (Olympus Optical, Tokyo, Japan) with a 40x magnification. Samples including those that were hydrolyzed were also viewed with the Hitachi S-570 Scanning Electron Microscope (Hitachi Scientific Instruments, Rexdale, ON, Canada) after they were sputtered with 15 nm of gold dust on a stub. The working distance used was 15 mm with a voltage of 10 kV.

5.2.7 Sample Characterization

The nitrogen contents of samples were determined using the Leco FP 528 nitrogen analyzer (Leco, St. Joseph, MI, USA). Protein contents were calculated using a factor of 5.7. The fat content of the sample was determined with the Soxhlet extraction apparatus using petroleum ether. Extraction was done for 5 h. Values were then reported in dry weight basis.
5.2.8 In Vitro Starch Digestibility and eGI

The in vitro starch digestibility on the raw and cooked samples was done using the method by Englyst et al (1992). An enzyme mixture of pancreatin from porcine pancreas (Sigma-Aldrich P-1625, activity 3x USP/g), invertase from baker’s yeast (Saccharomyces cerevisiae) (1450, Sigma-Aldrich,) and amyloglucosidase (200 U/mL p-nitrophenyl β-maltoside) (Megazyme International, Bray, Ireland) was prepared according to Englyst et al (1992). Fresh enzyme mixture was prepared for hydrolysis.

The flour samples were then weighed such that they contained 800 mg of starch on dry weight basis. For the cooked samples they were made into 10% slurry and then cooked in boiling water for 30 min with constant stirring using a magnetic stirrer at 100 rpm. Then to the samples was added 5 mL of and enzyme mixture of pancreatin from porcine pancreas (Sigma-Aldrich), invertase and amyloglucosidase after the addition of 10 mL 0.1M sodium acetate buffer (pH-5.2). The sample enzyme mixture was then incubated for 2 h with 0.1 mL aliquots taken every 20 min. The aliquots were put into 80% ethanol to stop the hydrolysis. The amount of glucose released was determined by glucose oxidase/peroxidase assay (Megazyme) and converted to starch by multiplying by 0.9. Starch hydrolyzed was reported on dry weight basis. Hydrolyzed starch was classified into rapidly digestible Starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) according to Englyst et al (1992). The calculations were as follows: RDS = glucose released at 20min x 0.9; SDS = (glucose released at 120min - glucose released at 20min) x 0.9) and RS = total starch - (RDS+SDS).
A non-linear first-order equation $C=C_\infty (1-e^{-kt})$ which was established by Goni et al. (1997) was used to describe the kinetics of hydrolysis of the samples. $C$ is the starch hydrolyzed at a time $t$; $C_\infty$ is the equilibrium concentration at the final time (120 min); $k$ is the kinetic constant and $t$ is the chosen time. The hydrolysis index (HI) was obtained by dividing the area under the hydrolysis curve (AUC) of the samples by that white bread as a reference sample as reported by Goni et al. (1997). The AUC was calculated by the equation: 

$$AUC = C_\infty (t_f-t_o) - (C_\infty/k) [1-e^{-k(t_f-t_o)}],$$

where $t_f$ is the final time and $t_o$ is the initial time. The eGI was calculated from the equation: $eGI= 8.198+0.862 (HI)$ as described by Granfeldt et al. (1992).

### 5.3.9 Total Starch

Total starch was determined by the Total starch kit from Megazyme. Total starch was reported on dry weight basis.

### 5.3.10 Statistical Analysis

Significant differences between the samples were determined by ANOVA at $P < 0.05$. Multiple range tests were done to determine which samples were different. All statistical analysis was done using Statgraphics Centurion XV, Version 15.1.02 (StatPoint, Warrenton, VA, USA).
5.4 Results and Discussion

5.4.1 Sample Characterization

The chemical composition of the samples used in the study is shown in Table 5.1. Protease treatment significantly ($P < 0.05$) reduced the protein content of the samples from 8.4% to 2.3% for the MF-P sample and to 1.9% for the MF-L-P sample. Similarly, the fat content of the sample also reduced to 0.1% after lipid extraction. The starch content was not much altered following the different treatments.

5.4.2 Microscopy

Figure 5.1 shows pictures of samples taken under polarized light. Defatting and the protease treatments did not result in the loss of birefringence of the starches. Figure 5.2 shows the SEM pictures of the various millet fractions. In figure 5.2a, millet starch granules can be seen embedded in the protein matrix in the flour. The MF samples that were subjected to protease treatments show starch granules released from the protein matrix, and with large indentations. These indentations are reported to be caused by proteins bodies (Krishna Kumari and Thayumanavan, 1998). Figure 5.3 shows the SEM pictures of the millet samples subjected to amylolytic hydrolysis without cooking. It is evident from the pictures that the mode of hydrolysis of the starch granules is inside-out, like has been reported for other starches including maize (Zhang et al 2006).
5.4.3 In Vitro Starch Digestibility of Raw Samples

The in vitro starch hydrolysis curves of the raw samples are shown in Figures 5.4. Raw rice flour had the highest hydrolysis rate compared with the different millet fractions or wheat flour samples. At 120 min of hydrolysis, about 70% of rice had been hydrolyzed compared to just about 40% for both wheat and millet flour. Zhang et al 2006 reported about 70%, 85% and about 90% percent starch hydrolyzed for rice, wheat and maize starches respectively at 120 min of hydrolyses. The relatively higher value reported for wheat might be due to varietal differences and/or amount of protein in the samples in this study, which is about 16%. The hydrolysis kinetics of raw wheat was similar to that of raw MF.

It is evident that defatting and protease treatment resulted in an increase in the rate of starch hydrolysis of kodo millet, with lipids having more effects than that observed for proteins. The influence of protein on starch digestibility has been discussed in a review by Singh et al (2010). Proteins are known to form a barrier surrounding starch granules preventing access to amylases and hence reducing starch digestibility (Rooney and Pflugfelder 1986). The reduction in the in vitro starch digestibility owing to proteins is evident in products such as pasta, in which the continuous protein matrix in the product entraps starch granules, which in turn limits the starch hydrolysis by amylases (Kim et al 2008). It has also been observed that, the removal of gluten from wheat resulted in higher absorption of glucose in the small intestine (Jenkins 1987). This observation probably explains why the RDS of the wheat flour was one of the lowest compared to the other samples.
The effects of lipid on starch digestibility can be mainly attributed to its ability to form complexes with amylose in starch (Singh et al 2010). Fatty acids in lipids form complexes rapidly with amyloses under physiological conditions contributing to the formation of resistant complexes (Seligman et al 1998). Lipid complexed amylose is more resistant to enzymatic attack as compared to pure amylose (Holm et al 1983).

The RDS, SDS and RS of the raw samples are listed in Table 5.2. MF recorded the lowest RDS (11.9%) compared to wheat (15.8%), MF-L-P (21.8%) or rice (23.3%). The removal of protein, lipids or both resulted in a significant increase in the RDS of the samples. This observation implies that defatting and/or removing the proteins from kodo millet would result in a greater spike in blood glucose response. Removal of lipids increased the RDS of the MF from 11.5% to 20.1% compared to 14.2%, when proteins were removed. This observation indicates that lipids have more effect in reducing the RDS level of MF. No definite trends in the SDS of the samples were observed. The SDS of the samples ranged from 26.6% for defatted flour to 46.3% for rice flour. The SDS of kodo millet starch was not significantly different compared to that of MF-L-P. Even though SDS of rice was the highest, it recorded the lowest RS (12.5%) amongst the samples. The RS of the samples were significantly different from each other.

5.4.4 In Vitro Starch Digestibility of Cooked Samples

Generally, the in vitro starch hydrolysis trends of the cooked samples were similar to that of the raw samples (Figure 5.5) even though higher rates of hydrolysis were
observed. Cooking results in an increased rate of hydrolysis because of starch gelatinization, thus making it more easily available for amylolysis. Figure 5.5 also indicates that removal of proteins, lipids or both resulted in increased rate of hydrolysis of cooked millet, similar to what was observed in the raw millet samples. It is also clear from the hydrolysis curves of the cooked samples that removal of lipids resulted in a higher hydrolysis of kodo millet flour compared to protein removal.

The RDS level of cooked MF-P was significantly lower than the other millet fractions, in addition to rice and wheat. The RDS levels of the cooked samples ranged from 29.2% for cooked MF to 45.7% for cooked rice, with wheat recording 32.4%. There was a general increase in the RDS of the samples that were treated to proteases, defatted or both (Table 5.3). The SDS levels of the cooked samples ranged from 15.9% for MF to 34.8% for cooked rice flour. Wheat recorded an SDS level of 30.5%. In terms of RS, rice had the least (1.6%) compared to MF with an RS level of 36.0%. Wheat and millet starch had RS levels of 11.9% and 22.0% respectively. Cooking resulted in lower RS for all of the samples. Defatting and protease treatments were found to significantly reduce the resistant starch content of MF.

5.4.5 eGI of Raw Samples

The hydrolysis kinetic constant, eGI and HI of the samples are shown in Table 5.4. The results show that defatting, protein removal or both resulted in significant increases in the eGI of MF. The eGI of the raw MF increased from 32.5 to 46.8 with
removal of both proteins and lipids. The eGI of MF-P (36.0) was significantly lower than MF-L (42.7), indicating the greater effect of lipid than protein on the starch digestibility of kodo millet. This observation is interesting, considering that many traditional processing techniques such as decortication applied to millets could result in decreases in their protein, lipids or both (especially lipids, which is mostly found in the hulls), resulting changes in the millet digestibility and increase in glucose release. The HI of the samples was calculated by comparing the area under the hydrolysis curve with that of a reference food, in this case white bread. The HI showed the same trend as with the eGI.

5.4.6 eGI of Cooked Samples

When cooked, the eGI was higher in all samples compared to the raw samples. The trends as observed in the cooked samples were similar to the raw samples. The eGI of rice was significantly higher than the millet flour samples (Table 5.5). Cooked wheat also had significantly higher eGI compared with MF. The trends observed in the HI was not different from that observed in the eGI. The removal of proteins, lipids or both increased the eGI of millet from being low (< 50) to medium (56 - 69). MF-L-P was however still less that rice flour, which was ranked as having a high eGI (> 70). The HI was also significantly increased with the removal of lipids and protein from MF. The lowest HI was observed for cooked MF, with rice flour recording the highest (77.5). The HI for wheat (58.1) was higher than that of MF but lower than MF-L-P.
5.5 Conclusion

This study suggests that the interaction between fats, proteins and starch in kodo MF plays a significant role in its in vitro digestibility and glycemic index. The removal of fats, proteins or both caused significant increases in the in vitro digestibility and eGI of the MF. The effect of lipids on the in vitro starch digestibility of the kodo millet was found to be more significant than that of proteins. It is important to note the complementary effects of proteins and lipids in reducing the glycemic index of kodo millet. This finding means that processes that result in the removal of proteins, lipids or both would subsequently result in an increase in the eGI of kodo millet flour (COCU9 variety). These findings are very important, considering that millet is mostly decorticated prior to their usage, because of the bitter taste associated with the consumption of whole millets. With most of the lipids in millets found in their hulls, this decortication would result in the significant reduction in the lipid content and hence could increase the eGI of their resulting products.

5.6: Acknowledgement:

This research project was supported by a grant from the International Development Research Centre, Canada
### 5.7 Tables and Figures

Table 5.1: Protein, Fat and Total Starch Contents of Samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein (% dry weight) (N x 5.7)</th>
<th>Fat (% dry weight)</th>
<th>Total Starch (% dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF</td>
<td>8.4c</td>
<td>1.9c</td>
<td>83.0c</td>
</tr>
<tr>
<td>MF-L</td>
<td>8.4c</td>
<td>0.1a</td>
<td>80.2b</td>
</tr>
<tr>
<td>MF-P</td>
<td>2.3b</td>
<td>1.8c</td>
<td>93.1d</td>
</tr>
<tr>
<td>MF-L-P</td>
<td>1.9b</td>
<td>0.1a</td>
<td>93.4d</td>
</tr>
<tr>
<td>Kodo millet starch</td>
<td>0.6a</td>
<td>0.2a</td>
<td>94.2d</td>
</tr>
<tr>
<td>Wheat Flour</td>
<td>15.6d</td>
<td>1.5b</td>
<td>74.6a</td>
</tr>
<tr>
<td>Rice Flour</td>
<td>8.1c</td>
<td>1.0a</td>
<td>82.0c</td>
</tr>
</tbody>
</table>

2 Values with different alphabets within a column are significantly different (P < 0.05) from each other. MF= Millet Flour; MF-L= MF without lipids; MF-P= MF without proteins and MF-L-P = MF without lipids and proteins.
Table 5.2: RDS, SDS and RS of Raw Samples

<table>
<thead>
<tr>
<th>Sample (Raw)</th>
<th>RDS (%)</th>
<th>SDS (%)</th>
<th>RS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF</td>
<td>11.5a</td>
<td>31.3b</td>
<td>40.2f</td>
</tr>
<tr>
<td>MF-L</td>
<td>20.1d</td>
<td>26.6a</td>
<td>36.2d</td>
</tr>
<tr>
<td>MF-P</td>
<td>14.2b</td>
<td>29.4bc</td>
<td>39.4f</td>
</tr>
<tr>
<td>MF-L-P</td>
<td>21.8e</td>
<td>32.8c</td>
<td>28.6b</td>
</tr>
<tr>
<td>Kodo millet starch</td>
<td>21.8e</td>
<td>33.2c</td>
<td>37.5e</td>
</tr>
<tr>
<td>Wheat flour</td>
<td>15.8c</td>
<td>27.3a</td>
<td>31.6c</td>
</tr>
<tr>
<td>Rice flour</td>
<td>23.3f</td>
<td>46.3d</td>
<td>12.5a</td>
</tr>
</tbody>
</table>

*Values with different alphabets within a column are significantly different (*P* < 0.05) from each other. RDS = rapidly digestible starch; SDS = slowly digestible starch; RS = resistant starch; MF = Millet Flour; MF-L = MF without lipids; MF-P = MF without proteins and MF-L-P = MF without lipids and proteins.
Table 5.3: RDS, SDS and RS of Cooked Samples

<table>
<thead>
<tr>
<th>Sample (Cooked)</th>
<th>RDS (%)</th>
<th>SDS (%)</th>
<th>RS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF</td>
<td>31.2b</td>
<td>15.9a</td>
<td>35.9f</td>
</tr>
<tr>
<td>MF-L</td>
<td>34.1d</td>
<td>19.0b</td>
<td>29.9e</td>
</tr>
<tr>
<td>MF-P</td>
<td>29.2a</td>
<td>24.3c</td>
<td>29.5e</td>
</tr>
<tr>
<td>MF-L-P</td>
<td>38.6e</td>
<td>25.4c</td>
<td>19.0c</td>
</tr>
<tr>
<td>Kodo millet starch</td>
<td>39.8f</td>
<td>32.4e</td>
<td>22.0d</td>
</tr>
<tr>
<td>Wheat flour</td>
<td>32.4c</td>
<td>30.5d</td>
<td>11.9b</td>
</tr>
<tr>
<td>Rice flour</td>
<td>45.7g</td>
<td>34.8f</td>
<td>1.6a</td>
</tr>
</tbody>
</table>

*Values with different alphabets within a column are significantly different (P < 0.05) from each*

RDS = rapidly digestible starch; SDS = slowly digestible starch; RS = resistant starch;
MF = Millet Flour; MF-L = MF without lipids; MF-P = MF without proteins and MF-L-P = MF without lipids and proteins.
Table 5.4: Kinetic Constant, Calculated HI and eGI of Raw Samples

<table>
<thead>
<tr>
<th>Sample (Raw)</th>
<th>Kinetic Constant ((k))</th>
<th>Calculated (HI)</th>
<th>eGI</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF</td>
<td>0.0156a</td>
<td>28.3a</td>
<td>32.5a</td>
</tr>
<tr>
<td>MF-L</td>
<td>0.0281e</td>
<td>39.7d</td>
<td>42.7d</td>
</tr>
<tr>
<td>MF-P</td>
<td>0.0197b</td>
<td>32.4b</td>
<td>36.0b</td>
</tr>
<tr>
<td>MF-L-P</td>
<td>0.0252d</td>
<td>44.8e</td>
<td>46.8e</td>
</tr>
<tr>
<td>Kodo millet starch</td>
<td>0.0243d</td>
<td>46.0e</td>
<td>47.8e</td>
</tr>
<tr>
<td>Wheat flour</td>
<td>0.0228c</td>
<td>34.1c</td>
<td>37.5c</td>
</tr>
<tr>
<td>Rice flour</td>
<td>0.0204b</td>
<td>53.33f</td>
<td>53.3f</td>
</tr>
</tbody>
</table>

Values with different alphabets within a column are significantly different \((P < 0.05)\) from each other.

HI = hydrolysis index; eGI = expected glycemic index; MF = Millet Flour; MF-L = MF without lipids; MF-P = MF without proteins and MF-L-P = MF without lipids and proteins.
Table 5.5: Kinetic Constant, Calculated HI and eGI of Cooked Samples

<table>
<thead>
<tr>
<th>Sample (Cooked)</th>
<th>Kinetic Constant $(k)$</th>
<th>Calculated HI</th>
<th>eGI</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF</td>
<td>0.0544f</td>
<td>47.9a</td>
<td>49.4a</td>
</tr>
<tr>
<td>MF-L</td>
<td>0.0515e</td>
<td>53.4c</td>
<td>54.3c</td>
</tr>
<tr>
<td>MF-P</td>
<td>0.0395b</td>
<td>50.8b</td>
<td>51.9b</td>
</tr>
<tr>
<td>MF-L-P</td>
<td>0.0462d</td>
<td>63.0e</td>
<td>62.5e</td>
</tr>
<tr>
<td>Kodo millet starch</td>
<td>0.0400bc</td>
<td>68.8f</td>
<td>67.5f</td>
</tr>
<tr>
<td>Wheat flour</td>
<td>0.0361a</td>
<td>58.1d</td>
<td>58.3d</td>
</tr>
<tr>
<td>Rice flour</td>
<td>0.0419c</td>
<td>77.5g</td>
<td>75.0g</td>
</tr>
</tbody>
</table>

Values with different alphabets within a column are significantly different $(P < 0.05)$ from each other.

HI = hydrolysis index; eGI = expected glycemic index; MF = Millet Flour; MF-L = MF without lipids; MF-P = MF without proteins and MF-L-P = MF without lipids and proteins.
Figure 5.1: Dark Field Photo Micrographs of Kodo Millet Samples (40x Magnification).

a = Millet Flour (MF); b = MF without Proteins; c = MF without Lipids; and
d = MF without Lipids and Proteins
Figure 5.2: Scanning Electron Photomicrographs of Various Millet Fraction Samples. a = Millet Flour (MF); b = MF without Proteins; c = MF without Lipids; and d = MF without Lipids and Proteins
Figure 5.3: Scanning Electron Photomicrographs of Hydrolyzed Samples. a = Millet Flour (MF); b = MF without Proteins; c = MF without Lipids; and d = MF without Lipids and Proteins
Figure 5.4: Starch Hydrolysis Kinetics of Raw Samples. MF = millet flour; –Lipids and –Proteins indicate without lipids and proteins, respectively.
Figure 5.5: Starch hydrolysis kinetics of cooked samples. MF = millet flour; –Lipids and –Proteins indicate without lipids and proteins, respectively.
5.8 References


CHAPTER 6: IN VITRO STARCH DIGESTIBILITY AND EXPECTED GLYCEMIC INDEX (eGI) OF MILLET STARCH-FATTY ACID COMPLEXES
Abstract

This study investigated whether the amounts and types of fatty acids present in millet plays any role in its hypoglycemic property. The first part of the study consisted of complexing cooked pearl, finger, proso and foxtail millets with palmitic, oleic, linoleic and elaidic acid in the amounts present in their respective millet flours. The second part involved complexing excess amount (2 mmol/g of starch) of these fatty acids to the millet starches and subjecting the complexes to in vitro starch digestibility and calculating their expected glycemic index (eGI). The complex index (CI) of the fatty acids was also determined. The CI of the fatty acids increased with increasing level of unsaturation. Significant (p<0.05) reductions in the in- vitro starch digestibility and eGI of the millet starch-fatty acid complexes was observed. Reductions in the starch hydrolysis of the samples were significantly linked to the amounts of the fatty acids added. The addition of 2.3 mg palmitic acid and 23.8 mg linoleic acid/g of proso millet starch resulted in expected glycemic index of 63.6 and 53.1, respectively. The presence of unsaturated fatty acids generally resulted in less starch being hydrolyzed. Oleic acid was the most effected fatty acid in reducing the amount of starch hydrolyzed, resulting in the decrease in the eGI of finger millet starch from 65.4 to 46.2 when added at 2 mmol/g of starch. On the other hand, linoleic acid however resulted in slightly more starch being hydrolyzed. Similarlytrans oleic acid showed to be less efficacious compared to oleic acid in a cis configuration. The amount and type of fatty acids interacting with starch plays a significant role in the hypoglycemic property of millet.

Keywords: Millet, expected glycemic index, in vitro starch digestibility, fatty acids, complex index
6.1 Introduction

The digestibility of starch is known to vary for different foods. This variability results in different glycemic responses when various foods are consumed (Jenkins et al 1988). Based on the in vitro digestibility of starch, three different starch fractions have been defined by Englyst et al (1992). These fractions are: rapidly digestible starch (RDS), which corresponds to the amount of starch hydrolyzed after 20 min; slowly digestible starch (SDS), corresponding to the amount of starch hydrolyzed between 20 min and 120 min; and finally, resistant starch (RDS), which is the total starch of the food minus the amount of starch hydrolyzed within 120 min. The hydrolysis of starch in humans is mainly the responsibility of pancreatic amylase in the small intestines. Though salivary α-amylase also takes part in starch digestion, its effects is short-lived as it is degraded by the acidic environment of the stomach when food is swallowed (Singh et al 2010). A relationship between the rate of starchy foods digestion in vitro and their glycemic response in vivo has been established (Jenkins et al 1988; O’Dea et al 1981; and Jenkins et al 1982).

The glycemic index concept was introduced to help classify foods on the basis of the extent to which they release glucose into the blood stream when they are consumed. Glycemic index is defined as the postprandial incremental glucose increase after a test meal, expressed as the percentage of the corresponding area after an equi-carbohydrate portion of a reference food such as glucose or white bread (Singh et al 2010; Goni et al 1997; Jenkins et al 1987). Aside the nature of the starch itself (Thorne et al 1983), various factors has been reported to affect the rate of starch digestion and glycemic index of
starchy foods. Among these factors are: processing such as cooking (Bravo et al 1998); dehulling (Alonso et al 2000); popping (Capriles et al 2008), viscosity of the food matrix (Kaur & Singh 2009; Slaughter et al 2002), the amount and type of dietary fiber (Jenkins et al 1987), presence of anti-nutritional factors (Yoon et al 1983; Fariase et al 2007), the presence of proteins (Hamaker and Bugusu 2003), and the presence of lipids (Ai et al 2013; Crowe et al 2000; Kawai et al 2012; Jane and Robyt 1984).

The ability of lipids to form inclusion complexes with starch, especially with amylose, is well known. Free fatty acids (Fanta et al 1999; Tufvesson et al 2003b; Kawai et al 2012) and monoglycerides (Tufvesson and Eliasson 2000; Tufvesson et al 2003a) are examples of lipids that form helical complexes with amylose. This helical inclusion complex, whereby the guest molecules occupy the central axis of a long left-handed helix normally consist of six glucosyl residues per turn, with a distance between the turns being 0.8 nm (Raphaelides and Karkalas 1988). Conformational ordering that takes place during the formation of these complexes results in a partially crystalline amylose structure (Lui 1997). Two crystalline polymorphs of these complexes have been reported; an amorphous form (form 1) that has a temperature of dissociation below 100 °C and the crystalline form (form 2) with temperatures of dissociation above 100 °C (Tufvesson et al 2003 a, b). The dissociation temperatures of amylose lipid complexes generally increase with increases in the number of carbons in lipids, but decreases with an increase in the number of double bonds in the fatty acid chain (Eliasson and Krog 1985; Karkalas et al 1995; Raphaelides and Karkalas 1988; Tufvesson et al 2003a, b).
The susceptibility of amylose-lipid complexes to starch degrading enzymes has been studied. Seneviratne and Biliaderis (1991) reported an inverse relationship between the rate and extent of hydrolysis of amylose-lipid complexes to the degree of organization of helices into larger domains of ordered chains. The degree of organization of the amylose lipid complexes is also inversely linked to the length of both the fatty acid and the amylose chains (Godet et al 1996; Tufvesson and Eliasson 2000). Ai et al (2013) reported significant decreases in starch hydrolysis rates when corn oil, soy lecithin, palmitic acid, stearic acid, oleic acid and linoleic acids were added to corn, tapioca, and high amylose corn starch. Zhang et al (2012) also reported increases in the resistant starch fraction of high amylose starch after complexing cooked debranched high amylose maize starch with lauric acid. Hasmij et al (2010) produced a new resistant starch type 5 (RS 5) by complexing debranched high maize starch with palmitic acid. Crowe et al (2000) reported a 35% reduction in the hydrolysis when lauric, myristic, palmitic and oleic acids were complexed with amylose. Kawai et al (2012) also reported a decrease in the in vitro digestibility of potato starch when complexed with fatty acids.

Millet is a major staple crop in the arid and semi-arid regions of the world. It thrives on soils with low fertility and severe conditions such as low rainfall or intense heat. In many African and Asian countries, it is a staple diet in low income populations providing much needed calories and proteins (Sawaya, 1984). One property of millets, which can be exploited in North America, is its low glycemic and insulminemic response (Shobona et al 2009). Lakshmi Kumari and Sumathi (2002) reported significantly lower plasma glucose levels from the consumption of a millet based diet. Noodles prepared
from the substitution of 20% wheat flour with millet flour resulted in significantly lower glycemic index and load compared to that from noodles prepared from 100% wheat flour (Vijayakumar et al 2010). However, the reason why millet is slowly digestible, compared to other cereals is not clearly understood. In a recent study (Annor et al 2013), significant increases in the in vitro starch digestibility and expected glycemic index were observed when lipids were removed from debranned kodo millet flour. This study investigates whether the fatty acids present in millet play a role in their hypoglycemic property.

6.2 Materials and Methods

6.2.1 Materials

The seeds of four millet species, namely, proso millet, foxtail millet, finger millet, and pearl millet were obtained from the Agriculture Environmental Renewal Canada (AERC) Inc, Delhi, Canada. These seeds were kept at -20 °C and later used for the study.

6.2.2 Methods

6.2.3 Starch Extraction

Millet starch was extracted from their flour samples according to the method reported by Annor et al (2013). It is important to note that millet starches were defatted as part of the starch extraction procedure.
6.2.4 Sample Preparation

Millet starch (0.7 g) samples were weighed into flat bottomed flasks in duplicate and 14 mL of distilled water added (5% slurry). The samples were then cooked for 10 min in a boiling water bath, with continuous stirring. After cooking the samples, they were kept at 70 °C and palmitic acid (P0500), oleic acid (O1008), linoleic (L1376) or elaidic acid (E4637), purchased from Sigma-Aldrich, St Louis, MO, USA, were added to the samples based on levels reported in the original millet flours (Table 6.1 and 6.2). Samples were kept at 70 °C to ensure that the fatty acids melted when added to the samples. The gelatinized starch-fatty acid complex was allowed to form for 10 min with vigorous vortexing every 2 min. They were then cooled to room temperature and used immediately for subsequent analyses. This part of the study constituted part 1.

For the second part of the study, the same amount of the fatty acids (2 mmol/g of starch) was added to the cooked millet starches. A control sample, which was made up of cooked millet starch without the addition of any fatty acid was also prepared.

6.2.5 Complex Index (CI) of Gelatinized Starch-Fatty Acid Complexes

Millet starch (0.1 g) was weighed in duplicates into screw capped test tubes and 5 mL of distilled water added (2% slurry). The samples in the test tubes were cooked for 10 min in boiling water bath, with vigorous vortexing every 2 min. The cooked starch samples were kept at 70 °C and 2 mmol of palmitic, oleic, linoleic or elaidic acid per gram of starch was added to the samples. The gelatinized starch-fatty acid complex was
allowed to form for 10 min with vigorous vortexing every 2 min and cooled to room temperature. A control sample, which was made up of cooked millet starch without the addition of any fatty acid, was also prepared.

The extent of iodine complexation was determined using the procedure described by Tang and Copeland (2007). Briefly, deionized water (8.6 mL) was added to the millet starch-fatty acid complex prepared in the screw-capped test tubes and mixed thoroughly by vortexing. The suspension (200 µL) was mixed with 10 mL of distilled water and 1 mL of iodine solution (2.0% (w/w) KI and 1.3% (w/w) I$_2$) added and vortexed. The absorbance (Abs) values of the sample and a reference were measured at 690 nm. The CI was calculated according to the equation:

$$CI = 100 \times \frac{(\text{Abs of reference sample} - \text{Abs of sample})}{\text{Abs of reference sample}}$$

6.2.6 In Vitro Digestibility and Expected Glycemic Index of Starch-Fatty Acid Complexes

The in vitro starch digestibility on the raw and cooked samples was carried out using the method by Englyst et al (1992). An enzyme mixture of pancreatin from porcine pancreas (Sigma-Aldrich P-1625, activity 3x USP/g), invertase from baker’s yeast (*Saccharomyces cerevisiae*) (1450, Sigma-Aldrich,) and amyloglucosidase (200 U/mL p-nitrophenyl β-maltoside) (Megazyme International, Bray, Ireland) was prepared according to Englyst et al (1992). The mixture was prepared fresh before hydrolysis. The flour samples were then weighed such that they contained 800 mg of starch on dry weight basis. For the cooked samples they were made into 10% slurry and then cooked in boiling
water for 30 min with constant stirring using a magnetic stirrer at 100 rpm. Then to the samples was added 5 mL of and enzyme mixture of pancreatin from porcine pancreas (Sigma-Aldrich), invertase and amyloglucosidase after the addition of 10 mL 0.1M sodium acetate buffer (pH-5.2). The sample enzyme mixture was then incubated for 2 h with 0.1 mL aliquots taken every 20 min. The aliquots were put into 80% ethanol to stop the hydrolysis. The amount of glucose released was determined by glucose oxidase/peroxidase assay (Megazyme) and converted to starch by multiplying by 0.9. Starch hydrolyzed was reported on dry weight basis. Hydrolyzed starch was classified into rapidly digestible Starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) according to Englyst et al (1992). The calculations were as follows: RDS = glucose released at 20min x 0.9; SDS = (glucose released at 120min - glucose released at 20min) x 0.9) and RS = total starch - (RDS+SDS).

A non-linear first-order equation $C=C_\infty (1-e^{-kt})$ which was established by Goni et al (1997) was used to describe the kinetics of hydrolysis of the samples. $C$ is the starch hydrolyzed at a time $t$; $C_\infty$ is the equilibrium concentration at the final time (120 min); $k$ is the kinetic constant and $t$ is the chosen time. The hydrolysis index (HI) was obtained by dividing the area under the hydrolysis curve (AUC) of the samples by that white bread as a reference sample as reported by Goni et al (1997). The AUC was calculated by the equation: $\text{AUC} = C_\infty (t_f-t_i) - (C_\infty/k) [1-e^{-k(t_f-t_i)}]$, where $t_f$ is the final time and $t_i$ is the initial time. The eGI was calculated from the equation: $\text{eGI} = 8.198+0.862 (\text{HI})$ as described by Granfeldt et al (1992).
6.2.7 Total Starch

Total starch was determined by the total starch kit from Megazyme (Bray, Ireland). Total starch was reported on a dry weight basis. This was needed to calculate the expected glycemic index of the samples.

6.2.8 Statistical Analysis

All analyses were conducted at least in triplicate. Duncan’s test was used to determine significant differences between means at the level of $P < 0.05$. All statistical analysis was done with Statgraphics Centurion XV, version 15.1.02 (StatPoint, Warrenton, VA, U.S.A.)

6.3 Results and Discussion

6.3.1 Complex Forming Ability of Millet Starches with Fatty Acids

The complex index (CI), which gives an indication of the degree of starch-lipid complex formation, is shown in Fig. 6.1. The CI is based on the formation of starch-iodine complex in starch-lipid complexed samples and measures the starch that is not complexed with lipids (Gilbert and Spragg 1964; Guraya et al 1997; Kaur & Singh 2000; Tang & Copeland 2007; Kawai et al 2012). The absorbance is related to the proportion of starch that complex with iodine. Thus, the higher the CI, the more starch-lipid complexes that are formed. The millet starches showed different levels of complex formation with
different fatty acids. Pearl millet starch generally had lower CI compared to foxtail, finger or proso millet starches. Furthermore, proso and finger millet starches complexed more with palmitic (23.7% and 24.2% respectively) acid, compared to foxtail (13.3%) or pearl (14.1%) millet starches. With the exception of pearl millet starch, all the other starches formed significantly more complexes with linoleic acid. Significant increases were also observed in the CI when unsaturated fatty acids were used in all the millet starches. Furthermore, increases in CI were observed with an increase in the degree of unsaturation. This increase was significant in proso and foxtail millet starch, but not in finger and pearl millet starch. Kawai et al (2012) observed higher CI for linoleic acid (47.6%) than oleic acid (36.7%) when both fatty acids were added to gelatinized potato starch. They reported an increase in CI in the order linoleic > oleic > stearic acids. The CI values reported by Kawai et al (2012) were higher than values reported in this study. This could be due to the type of starch used. Potato starch has longer amylose (Jideani et al 1996) and amylopectin chains (Bertoft et al 2008), compared to cereal starches. The effects of amylose chain length on amylose-lipid complex formation has been reported (Gelders et al 2005; Godet et al 1993). Tang and Copeland (2007) reported maximum CI of 28.0%, 16.7% and 28.2% for palmitic, oleic and linoleic acids, respectively, when complexed with wheat starch.

Millet starches complexed to different degrees with elaidic acid, which is the trans form of oleic acid. With finger and pearl millet starches, significantly less starch-elaidic acid complex was formed compared to starch-oleic acid complex. This observation was however different for foxtail and proso millet starches. In fact with proso
and foxtail millet starch, significantly more starch-elaidic complex was formed, but for proso millets starch the amount of complex formed with elaidic and oleic was similar. Riisom et al 1984 reported mono-oleate as being more effective in complex formation than its trans counterparts. Lagendijk and Pennings (1970) had earlier suggested the opposite, because of steric hindrance of cis unsaturated lipids inside the amylose helical cavity, due to the kink in their chain. Yamada et al (1998) suggested the kink in the cis unsaturated lipids resulted in their partial inclusion in the helix cavity. This observation suggest that the complex formed between cis or trans fatty acids and starch, depends on the structural characteristics of the starch. Even though elaidic acid is not naturally present in millets, the author thought it would be interesting to investigate its effects on the starch hydrolysis rates of the millets samples used in this study, considering the fact that most commercially available refined oils which, are used for everyday cooking and food processing contain trans fats.

6.3.2 In Vitro Digestibility of Starch-Fatty Acid Complexes

This section of the study was conducted in two parts. The first part was to investigate whether the amounts of fatty acids present in the respective millet flours influenced their in vitro starch digestibility. The second part of the study involved the addition of the same level, but excess amount of fatty acids, to the cooked starches. The starch hydrolysis kinetics of cooked millet starch-fatty acid complexes added at the amounts present in the respective millets is shown in Fig. 6.2. Significant reductions in the in vitro digestion of all the cooked millet starches were observed with the addition of
fatty acids. These reductions were less in finger and pearl millet starches than in proso and foxtail. Reductions in in vitro starch hydrolysis of the millet starches also depended on the amount of fatty acid added. The more a particular fatty was acid is added, the more reduction in the starch hydrolysis of the resulting complex was observed. Kawai et al (2012) reported decreases in starch hydrolysis with increasing amounts of fatty acids added to wheat starch. For proso millet starch, linoleic acid was the most added and hence resulted in the most reduction in the starch hydrolysis of the resulting complex. In the case of finger and foxtail millet starches, the most fatty acid added was oleic acid, while it was linoleic for pearl millet.

The starch hydrolysis kinetics of cooked millet starch with excess fatty acids added is shown in Fig. 6.3. Addition of excess fatty acids, resulted in significantly more reductions in in vitro digestibility of the resulting complex, compared to the first part of the study, indicating that, the millet starch fatty acid complex formed, depended on the concentration of fatty acid present. Proso and finger millets both showed similar trends in reductions in in vitro hydrolysis of the starch-fatty acid complexes formed. The fatty acids decreased the hydrolysis of starch in the following order; oleic acid > linoleic acid > palmitic acid. Reductions in the starch hydrolysis of the millet starch-fatty acid complexes could be attributed to the restriction of starch granules swelling, as a result of the formation of amylose-lipid complexes (Hasjim et al 2010; Tester & Morrison 1990). The slower hydrolysis of the millet starch-lipid complexes can also be explained by changes in torsion (φ and ψ) angles of the glycosidic bonds when amylose changes conformation from a random coil to the helix (French and Murpny 1977). These torsion
angle changes could affect the ability of the digestive enzymes to hydrolyze the starch-lipid complexes. Addition of elaidic acid resulted in much lower millet starch hydrolysis kinetics in foxtail, finger and pearl millet starches, with very marked reductions in foxtail millet starch, though higher than oleic acid. For proso millet, the hydrolysis kinetics of elaidic seems to be slightly higher than oleic acid.

The RDS, SDS, Hydrolysis Index (HI) and kinetic constants (k) of the cooked millet starches with different amounts of fatty acids added are shown in Table 6.3. RDS of proso and foxtail millet starches were generally higher than that of finger and pearl millet starch. RDS of the millet starches decreased with addition of fatty acids. RDS of proso millet decreased from 37.2 to 33.3, 29.3, 27.0 and 29.1 with the addition of palmitic, oleic, linoleic and elaidic acids respectively (Table 6.3). The extent of RDS decrease appear to be linked to the amount and type of fatty acid added. Addition of 19.5 mg oleic acid/g starch reduced the RDS of foxtail millet from 38.5 to 23.0, compared to reductions to 34.0, 30.0 and 24.6 with the addition of palmitic, linoleic and elaidic acids respectively. With pearl millet, addition of 15.5 mg linoleic acid/g of starch reduced RDS from 35.6 to 22.5, compared to 31.2, 30.4 and 27.3 with the addition of palmitic, oleic and elaidic acid respectively. When excess amounts of fatty acids were added to the starches, the RDS of the millet starch-fatty acid complexes was in the order; no fatty acid > palmitic acid > linoleic acid > elaidic acid > oleic acid for both proso and finger millet. Thus irrespective of the type of millet starch used, addition of excess amounts of fatty acids showed a similar RDS trend. Much more decreases in the RDS were observed with the addition of excess fatty acids. Between oleic and elaidic acid, no significant
difference was observed in RDS for proso and finger millet starches. Elaidic acid resulted in an increase in RDS of foxtail millet starch and vice versa for pearl.

SDS of the samples reduced from 42.6 to 32.2, 41.5 to 29.4, 39.4 to 35.7 and 48.9 to 42.9 for proso, finger, foxtail and pearl millet starches respectively, when fatty acids amounts present in their respective flours were added. Significant reductions in SDS were observed in all millet samples when fatty acids were added. For proso and pearl millet starches, linoleic acid resulted in the lowest SDS, while the lowest SDS was reported for elaidic acid in finger and foxtail. Addition of excess amounts of fatty acids also resulted in significant differences in the SDS of samples. Both proso and finger millet starches showed a similar trend in SDS changes with the addition of excess fatty acids. SDS values for finger millet starch were generally lower than those of proso. Oleic acid resulted in the lowest amounts of SDS in both millet starches, followed by palmitic acid, linoleic acid and then elaidic acid.

RS increased with the addition of fatty acids to all millet starches. Like the RDS, RS seemed to be influenced by the amount of fatty acid added. For proso millet starch, 2.3 mg palmitic acid resulted in an increase in RS from 20.2 to 26.8. Addition of 7.3 mg oleic acid/g starch, 23.8 mg linoleic acid/g starch and 7.28 mg elaidic acid/g starch resulted in RS of 33.8, 40.8, and 34.2 respectively. Hence the addition of more linoleic acid to proso millet starch resulted in the most RS compared to the other fatty acids. RS of elaidic acid was similar to oleic acid when the same amounts were added to proso millet starch. For finger millet starch, the addition of 2.3 mg palmitic acid/g starch, 5.3 mg oleic acid/g starch, 2.5 mg linoleic acid/g starch and 5.3 mg elaidic acid/ g starch
resulted in increases in RS from 24.3 to 31.0, 40.1, 35.0, and 42.7 respectively. Addition of elaidic acid produced significantly more resistant starch compared to oleic acid in finger millet. Elaidic acid and linoleic acids produced the most RS in foxtail and pearl millets. Saturated amounts of the fatty acids added to the millet starches, showed oleic acid to be fatty acid, producing the most amount of RS, while linoleic acid produced the least RS for both proso and finger millet starches.

The kinetic constants ($k$), which gives an indication of the rate of hydrolysis, did not change significantly with proso millet starch when fatty acids of different amounts were added. However, when excess amounts of fatty acids were added, significant differences were observed. Addition of elaidic acid resulted in the lowest (0.025) kinetic constant, followed by oleic acid when added to proso millet starch. In the case of finger, foxtail and pearl millets starches, the addition of the different amounts of fatty acids resulted in significant differences in their kinetic constants. The reductions in the kinetic constants of the millets starches also seem to be linked to the amount and type of fatty acid added. Addition of saturated amounts of elaidic acid also resulted in the most reduction of the kinetic constant of finger millet starch.

The hydrolysis index (HI), which is used in estimating the glycemic index of foods was 70.9, 66.4, 71.0 and 68.9 for proso, finger, foxtail and pearl millet starches respectively. The addition of the different amounts of the different fatty acids used in this study resulted in significant reductions in the (HI). It was observed that the more a particular fatty acid was added to the starches, the more its effect on the HI. Addition of
excess amounts of the fatty acids resulted in significant reductions in the HI of the starches. In both proso and finger millet starches, oleic acid resulted in the most reduction in the HI. Elaidic acid resulted in significantly higher HI when compared to oleic acid. HI of linoleic acid was higher than oleic and elaidic acid. The apparent lower resistance of elaidic-millet starch complexes to enzymatic hydrolysis could be due to the fact that it might have been added at a concentration above its critical micellar concentration, resulting in self association, rather than complexing with the millet starch (Tang and Copeland 2007)

The expected glycemic index of the millet starches and their fatty acid complexes are shown in Figs. 6.4 and 6.5. Reductions in expected glycemic index of the starches were related to the amount and type of fatty acid added. For example, 2.3, 7.3, 23.8 and 7.3 mg palmitic, oleic, linoleic and elaidic acid added per gram of proso millet starch (Table 6.2), resulted in expected glycemic index being reduced from 69.3 to 63.6, 57.7, 53.1 and 57.4 (Fig. 6.5) for palmitic, oleic, linoleic and elaidic acid respectively, hence the addition of more linoleic acid, resulted in the most reduction in expected glycemic index. Addition of 2.6, 5.3, 2.5 and 5.3 mg palmitic, oleic, linoleic and elaidic acid added per gram of finger millet starch (Table 6.2) resulted in the expected glycemic index of finger millet starch reducing from 65.4 to 59.7, 54.2, 57.2 and 52.9 for palmitic, oleic, linoleic and elaidic acid respectively (Fig. 6.4), hence the addition of more oleic acid, resulted in the most reduction in expected glycemic index. Fig. 6.5 shows the expected glycemic index of the millet starches with added excess fatty acids. Oleic acid resulted in a significantly lower expected glycemic index when compared to palmitic acid, thus the
unsaturated oleic acid resulted in much less starch being hydrolyzed compared to the saturated palmitic acid. The addition of linoleic acid however, resulted in an increase in the expected glycemic index in both starches. A similar trend was observed by and Eliason and Krog (1985). This increase in the starch hydrolyzed for the millet starch fatty acid complex may be due to the lower stability of the complex (Kawai et al 2012). In studying the effects of potato amylose-fatty acid complexes on in vitro starch hydrolysis, Eliason and Krog (1985) reported that the amount of glucose release from the amylose-linoleic acid complex was similar to that of the pure amylose, which is in agreement with what was observed in this study. It was also observed that starch-oleic acid complex was much more resistant to enzymatic hydrolysis when compared to that of elaidic acid.

6.4 Conclusions

The study shows that, the fatty acid present in millet starches play a significant role in their hypoglycemic property. Not only does the amount of these fatty acids present in the millet flours matter in maintaining low digestibility of millet starches, but also the type of the fatty acids present. Generally, the unsaturated fatty acids, which form the most of the fatty acids present in millet flours, were more effective in reducing the starch hydrolysis of the millet starches when compared to palmitic acid, particularly oleic acid. The millet starch-linoleic acid complex however produced very little resistance to starch hydrolysis. Trans fatty acids, in this case elaidic acid, produced starch complexes that were much less resistant to enzymatic hydrolysis than their cis counterparts. It is therefore important to maintain the fatty acids present in millet flours when they are being processed and also to avoid processes that have the tendency to change the
configuration of the unsaturated fatty acids present in these millet flours to the *trans* form to keep its beneficially low hypoglycemic property.

6.5 Acknowledgements:

We acknowledge Dr. Anand of Agriculture and Agri-Food Canada, Delhi Research Farm, Delhi, Ontario, for providing the millet seeds for the study. This research was supported by a grant from the International Development Research Centre (IDRC) in Canada.
### 6.6 Tables and Figures

Table 6.1: Fat Content and Percent Fatty Acids of Lipids Present In Samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fat content (%)</th>
<th>Percent fatty acid of lipids present</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Palmitic acid</td>
<td>Oleic acid</td>
<td>Linoleic acid</td>
<td>*Elaidic acid</td>
</tr>
<tr>
<td>Proso</td>
<td>3.6</td>
<td>6.3</td>
<td>20.0</td>
<td>65.5</td>
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<tr>
<td>Finger</td>
<td>1.1</td>
<td>23.3</td>
<td>47.5</td>
<td>22.4</td>
<td>47.5</td>
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<tr>
<td>Foxtail</td>
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<td>8.7</td>
<td>66.3</td>
<td>22.4</td>
<td>66.3</td>
</tr>
<tr>
<td>Pearl</td>
<td>3.5</td>
<td>22.3</td>
<td>23.2</td>
<td>44.8</td>
<td>22.3</td>
</tr>
</tbody>
</table>

(Percentages of palmitic, oleic and linoleic acids based on reports by Sridhar and Lakshminarayana, 1994: Sawaya et al 1984)

*Elaidic acid amount was assumed to be the same as Oleic acid
Table 6.2: Amounts of Fatty Acids per Gram Sample Added

<table>
<thead>
<tr>
<th>Sample</th>
<th>Amount of fatty acid added (mg/g sample)</th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Palmitic acid</td>
<td>Oleic acid</td>
<td>Linoleic acid</td>
<td>*Elaidic acid</td>
</tr>
<tr>
<td>Proso</td>
<td>2.3</td>
<td>7.3</td>
<td>23.8</td>
<td>7.3</td>
</tr>
<tr>
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<td>5.3</td>
<td>2.5</td>
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<tr>
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<td>6.6</td>
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</tr>
<tr>
<td>Pearl</td>
<td>7.7</td>
<td>8.0</td>
<td>15.5</td>
<td>8.0</td>
</tr>
</tbody>
</table>

*Elaidic acid amount was assumed to be the same as Oleic acid
Table 6.3: RDS, SDS, RS, HI, and Kinetic Constant (k) of Cooked Millet Starch-Fatty Acid Complexes with Fatty Acids added at the Amounts Present in the Respective Millets\textsuperscript{z}

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fatty acid</th>
<th>RDS</th>
<th>SDS</th>
<th>RS</th>
<th>HI</th>
<th>k</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proso</td>
<td>No fatty acid</td>
<td>37.2d</td>
<td>42.6d</td>
<td>20.2a</td>
<td>70.9d</td>
<td>0.031a</td>
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<td></td>
<td>Palmitic acid</td>
<td>33.3c</td>
<td>39.9c</td>
<td>26.8b</td>
<td>64.3c</td>
<td>0.030a</td>
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<tr>
<td></td>
<td>Oleic acid</td>
<td>29.3b</td>
<td>36.9b</td>
<td>33.8c</td>
<td>57.4b</td>
<td>0.029a</td>
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<td>Linoleic acid</td>
<td>27.0a</td>
<td>32.2a</td>
<td>40.8d</td>
<td>52.1a</td>
<td>0.030a</td>
</tr>
<tr>
<td></td>
<td>Elaidic acid</td>
<td>29.1b</td>
<td>36.7b</td>
<td>34.2c</td>
<td>57.1b</td>
<td>0.029a</td>
</tr>
<tr>
<td>Finger</td>
<td>No fatty acid</td>
<td>34.2c</td>
<td>41.5e</td>
<td>24.3a</td>
<td>66.4e</td>
<td>0.030ab</td>
</tr>
<tr>
<td></td>
<td>Palmitic acid</td>
<td>30.4c</td>
<td>38.6d</td>
<td>31.0b</td>
<td>59.8d</td>
<td>0.029a</td>
</tr>
<tr>
<td></td>
<td>Oleic acid</td>
<td>28.1a</td>
<td>31.8b</td>
<td>40.1d</td>
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<td>29.4a</td>
<td>42.7e</td>
<td>51.9a</td>
<td>0.033c</td>
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<td>39.4b</td>
<td>30.6c</td>
<td>59.6b</td>
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<td>35.7a</td>
<td>39.7e</td>
<td>50.4a</td>
<td>0.026b</td>
</tr>
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<td>42.9a</td>
<td>21.5a</td>
<td>68.9d</td>
<td>0.030d</td>
</tr>
<tr>
<td></td>
<td>Palmitic acid</td>
<td>31.2c</td>
<td>44.6b</td>
<td>24.2b</td>
<td>63.5c</td>
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<td>48.0c</td>
<td>21.6a</td>
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<td>34.7c</td>
<td>49.8a</td>
<td>0.021a</td>
</tr>
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<td>27.3b</td>
<td>48.9c</td>
<td>23.8b</td>
<td>59.4b</td>
<td>0.022a</td>
</tr>
</tbody>
</table>

\textsuperscript{z} Values with different alphabets within a column are significantly different (\(P < 0.05\)) from each

HI= hydrolysis index; RDS =rapidly digestible starch; SDS = slowly digestible starch;

RS = resistant starch.
Table 6.4: RDS, SDS, RS, Hydrolysis Index, and Kinetic Constant ($k$) of Cooked Proso and Finger Millet Starch-Fatty Acid Complexes with the Same Amount of Fatty Acid (2 mmol/g of Starch) Added to their Respective Starches$^a$

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fatty acid</th>
<th>RDS</th>
<th>SDS</th>
<th>RS</th>
<th>HI</th>
<th>$k$</th>
</tr>
</thead>
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<tr>
<td>Proso</td>
<td>No fatty acid</td>
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<td>70.9e</td>
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<tr>
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<td>0.033c</td>
</tr>
<tr>
<td></td>
<td>Oleic acid</td>
<td>23.5a</td>
<td>30.6a</td>
<td>45.9d</td>
<td>46.5a</td>
<td>0.028b</td>
</tr>
<tr>
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<td>Linoleic acid</td>
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<td>35.9c</td>
<td>35.9b</td>
<td>55.5c</td>
<td>0.029b</td>
</tr>
<tr>
<td></td>
<td>Elaidic acid</td>
<td>23.8b</td>
<td>36.8c</td>
<td>39.4c</td>
<td>49.6b</td>
<td>0.025a</td>
</tr>
<tr>
<td>Finger</td>
<td>No fatty acid</td>
<td>34.2e</td>
<td>41.5d</td>
<td>24.3a</td>
<td>66.4e</td>
<td>0.030c</td>
</tr>
<tr>
<td></td>
<td>Palmitic acid</td>
<td>26.4c</td>
<td>32.0b</td>
<td>41.6c</td>
<td>51.1c</td>
<td>0.030c</td>
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<td>29.8a</td>
<td>48.2d</td>
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<td>27.5d</td>
<td>35.0c</td>
<td>37.5b</td>
<td>54.2d</td>
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<td>Elaidic acid</td>
<td>23.3b</td>
<td>36.1c</td>
<td>40.6c</td>
<td>48.6b</td>
<td>0.025a</td>
</tr>
</tbody>
</table>

$^a$ Values with different alphabets within a column are significantly different ($P < 0.05$) from each other.

HI= hydrolysis index; RDS = rapidly digestible starch; SDS = slowly digestible starch; RS = resistant starch.
Figure 6.1: Complex Index of Palmitic, Oleic, Linoleic and Elaidic Acids with Millet Starches (Values with Different Letters Are Significantly Different)
Figure 6.2: Starch Hydrolysis Kinetics of Cooked Millet Starch-Fatty Acid Complexes With Fatty Acids Added at the Amounts Present in the Respective Millets. (PA-palmitic acid, OA-oleic acid, LA-linoleic acid, EA-elaidic acid)
Figure 6.3: Starch Hydrolysis Kinetics of Cooked Proso and Finger Millet Starch-Fatty Acid Complexes with the Same Amount of Fatty Acid (2 mmol/g of Starch) Added to their Respective Starches.
Figure 6.4: Expected Glycemic Index (eGI) of Cooked Millet Starch-Fatty Acid Complexes with Fatty Acids Added at the Amounts Present in the Respective Millets. (PA-palmitic acid, OA-oleic acid, LA-linoleic acid, EA-elaidic acid)
Figure 6.5: Expected Glycemic Index (eGI) of Cooked Proso and Finger Millet Starch-Fatty Acid Complexes with the Same Amount of Fatty Acid (2 mmol/g of Starch) Added to their Respective Starches. (PA-palmitic acid, OA-oleic acid, LA-linoleic acid, EA-elaidic acid)
6.7 References


CHAPTER 7: SUMMARY AND CONCLUSIONS

The physical and molecular characteristics of starches from pearl, proso, finger and foxtail millet species, grown in Ontario, Canada were investigated. The amylose contents of these millet starches ranged from about 29-34% and differed significantly in their molecular structure. Foxtail and proso millet were observed to have more of short amylose chains. Furthermore, the branched amylose of foxtail and proso millet starches had short glucan chain between branched points. This observation was the opposite for finger and pearl millet starches. The millet starches also differed significantly (\( P < 0.05 \)) in their thermal properties. Finger and pearl millet starches had lower thermal transition temperatures, and their amylopectin crystallites melted over a wider temperature range, when compared to proso and foxtail millet starches. The determination of K/S intensities after the addition of iodine vapor to the millet starches, indicated that proso and foxtail millet starches had more flexible glucan chains, and hence their higher K/S values compared to pearl and finger millet starches. Iodine exposure of the starches suggested a negative relationship with the amount of long amylose chains of the starches, as proso and foxtail millet starches, which had more of short amylose chains, had higher K/S values. Changes in X-ray diffraction intensities of the millet starches were also observed when iodine vapor was added to the starches. While a reduction in the peak intensity at 15° 2\( \theta \) was observed for foxtail and proso, no change in intensity was observed for pearl and finger millet starches. This observation was vice versa at 23 ° 2\( \theta \). In general proso and foxtail millet starches had similar physical and molecular characteristics but were different from pearl and finger millets, which also behaved similarly.
The unit and internal chain profiles of the amylopectin of these millet starches were also investigated. Proso and foxtail millet amylopectins were observed to have similar amylopectin characteristics, as also observed in physical and molecular characteristics of their whole starches. Significant ($P < 0.05$) differences were observed in both the internal and unit chain profiles of the millet amylopectins. The average chain length of the millet amylopectins were similar to that of other cereals. The external chains of proso and foxtail millets were longer than finger and pearl millet amylopectin, suggesting that former have a thicker crystalline lamella. The molar amounts of $A_{\text{crystal}}$ chains, which are mainly responsible for the double helices in the crystalline lamella was also higher in foxtail and proso millet amylopectin when compared to finger and pearl. This observation could explain the reason why proso and foxtail millets have higher thermal transition temperatures.

Foxtail and proso millets were also observed to have shorter internal chains when compared to pearl and finger millet amylopectins. Since the internal chains are found between branched points in the amylopectin structure, this observation therefore implies that foxtail and proso millet amylopectin would have a more tightly branched structure in their amorphous lamella. This study also suggest that the double helices in these millet amylopectins were formed from one crystalline A-chain and the external segment of one short B-chain, since the ratios of $A_{\text{crystal}}:BS$ were 1.0 for all samples. The significant differences observed in $B_{fp}:BS_{\text{major}}$ suggest significant differences in the cluster and building block structure of these millet amylopectins. There also seem to be a negative relationship between $K/S$ values and average internal chain length of the millet.
amylopectins. Furthermore, finger and pearl millet starches, which had lower K/S values, recorded significantly lower amounts of “fingerprint” B-chains.

The study also showed the effects of starch-protein-lipid interactions on the starch digestibility and glycemic index of kodo millet. Starch-lipid interactions were observed to have a significant effect on the starch hydrolysis rate of millet starch. Significant increase in glycemic index was noted when debranned kodo millet flour was defatted. Removal of proteins, which encapsulate starch granules, also significantly increased starch hydrolysis rates, though its effects was not as high, compared to the removal of lipids. It is important to note that the removal of both proteins and lipids resulted in a further increase in the starch hydrolysis rates of kodo millet. This seem to suggest the complementary effects of proteins and lipids in the reduction of starch hydrolysis rates in kodo millets.

The types of fatty acids and their amounts present in the original millet flour samples were also investigated to determine their effects on millet starch digestibility. In this study, palmitic, oleic and linoleic, which are the main fatty acids present in millets were added to cooked millet starches in amounts found reported in the respective millet species to form starch-lipid complexes. Starch-fatty acid complexes from excess amounts (2 mmole/g starch) were also prepared and subjected to in vitro assays. Starch-elaidic acid complexes were prepared to determine the effect of the trans conformation of oleic acid on millet starch hydrolysis rates. Proso and foxtail millets, which had more short-amylose chains with short chains between their branch points, had slightly higher starch hydrolysis rates than pearl and finger. This observation suggests that millet starches that
have more of long amylose chains with long chain segments between branch points would likely have lower enzymatic starch hydrolysis and expected glycemic index. All the millet starches formed complexes with fatty acids, though to different extents. Oleic acid was very effective in reducing the starch hydrolysis rates of the millet starches, while linoleic acid had the least effect. Starch-elaidic acid (trans oleic acid) complex was generally much less resistant to enzyme hydrolysis than oleic acid. The extent of reduction in the hydrolysis rates by the fatty acid for finger and pearl millets was lower compared to proso and foxtail millets. This observation could be due to the relatively high amount of long amylose chains that potentially could form more stable complexes with fatty acids.

Finally, this study showed that millet starch structural characteristics and starch-protein-lipid interactions is part of the reason why millets have low glycemic and insulimemic response.

It is recommended that cluster and building block structures of the amylopectin of these millet sample is investigated to understand the fine structures of these millet amylopectins.

It would also be interesting to determine the lipid micellar concentration of millets to better understand their effects on millet starch hydrolysis rates.