Understanding the Effect of Non-starch Grain Components on the Amylolysis of Starch in Whole Grains

by

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ABSTRACT

Bioethanol industry is fast growing as it is a clean substitute of fossil-fuel. Starch is a clean, cheap & non-toxic source for bioethanol production. Efficient conversion of starch to fermentable sugars is important. The objective of this research was to investigate the effect of non-starch grain components on the amylolysis of starches isolated from triticale, wheat, corn and barley grains. Dry-milling and non-starch components of flours may impact the enzymatic hydrolysis of starch to glucose. The particle size distributions of ground flours from whole grain triticale, barley, wheat and corn were evaluated and the effects of pre-washing with water, hexane, 100% ethanol or 50% ethanol on flour composition and the amylolysis of starch were studied. Grinding grain to pass through a 0.5 mm sieve effectively released starch granules from endosperm cells. Pre-washing with water or 50% ethanol decreased the protein, phytic acid and total phenolics contents of flours and, except for corn flour, increased starch content. Pre-washing with water reduced the β-glucan content of barley flour by 98%. Pre-washing with hexane or 100% ethanol removed about 80 to 97% of the lipid from the flours. Each of the pre-washings was associated with a significant change, positive or negative, in the extent of α-amylolysis for one or more of the flours. The degree of α-amylolysis in the unwashed flours ranged from 22.4-26.1%, and from 21.6-28.1% in pre-washed flours. Pre-washing of flours increased the degree of hydrolysis achieved with a sequential α-amylase/amyloglucosidase treatment, with values ranging from 61.4-72.8% in pre-washed flours compared to 56.2-57.8% in unwashed flours. The highest degrees of hydrolysis were achieved with 50% ethanol pre-washing at 72.4 and 72.8% for triticale and barley flours, respectively. The degree of α-amylase/amyloglucosidase hydrolysis

obtained for isolated starches ranged from 83.7-93.0%. This study clearly demonstrated that the partial removal of non-starch components from whole grain flours by solvent pre-washing enhanced the degree of amylolysis of starch.

The presence of phenolics in cereal grain is thought to influence starch amylolysis during liquefaction and saccharification of whole grain flours. To understand amylolysis systems and inhibition mechanisms, the composition and concentration of phenolic acids in whole grain flours of triticale, wheat, barley and corn were analyzed by HPLC. The total phenolic acid contents, representing the sum of 11 phenolic acids in each of the four grains, were 1171, 1732, 1599 and 2331 μg/g, respectively, with more than 76% found in the bound form. Ferulic, coumaric and protocatechuic acids were the major phenolic acids in triticale and wheat. Gallic acid also was rich in triticale. Ferulic, coumaric, hydroxybenzoic, gallic acids and catechinhydrate were predominant in barley. In corn, ferulic, coumaric, gallic, catechinhydrate, naringin, and syringic acids were abundant. Based on these profiles, pure phenolic acids were added individually and collectively to isolated starches at amounts either equivalent to or three times those in the whole grains. The degree of starch hydrolysis with α-amylase and amyloglucosidase decreased up to 8% when individual phenolic acids were added. The decreases were more pronounced when phenolic acids were added collectively (4-5% with α-amylase and 9-13% with sequential α-amylase and amyloglucosidase). Study of a phenolic acid-starch-enzyme model system indicated that a phenolic acid-enzyme interaction was the dominant contributor to the interference, but a phenolic acid-starch/dextrin interaction also played a significant role. Heating augmented the interaction between phenolic acids and the enzymes and starch/dextrin. Phenolic acids thus can contribute to the resistance

of starch to enzymatic hydrolysis and/or the loss of enzyme activity during starch amylolysis.

The effect of phenolic acids on starch amylolysis in their bound or “native” form as exists in the bran and also in the free form. Three different fiber concentrates (FC) were used in this study; one contained bound phenolic acids (FC1), one contained free phenolic acids (FC2), and the third had no phenolic acids (FC3). The degree of starch amylolysis of triticale, wheat, corn and barley flours were most significantly decreased ($p< 0.05$) by adding FC2, and next by FC1, and least by FC3.(19, 9.1 and 4.8%, respectively, for liquefaction with alpha-amylase and (14.25, 4.6 and 2.7%, respectively for liquefaction and saccharification with amyloglucosidase).

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2nd	Second
FAO	Food and Agriculture Organization
MT	Million Tonnes
DP	Degree of polymerization
PA	Phenolic acids
Da	Dalton
KDa	Kilo Dalton
AM	Amylose
AP	Amylopectin
db	Dry basis
T_g	Glass transition temperature
SGAPs	Starch granule associated proteins
h	Hour
DNS	Dinitrosalicylic acid
KV	Kilo volt
DH	Degree of hydrolysis
AACC	American Association of Cereal Chemists
AOAC	The Association of Analytical Communities
GAE	Gallic acid equivalent
GLM	General Liner Model
SAS	Statistical Analysis System
SEM	Scanning Electron Micrograph

KNU/g	Kilo Novo Units/Gram
HPLC	High Performance Liquid Chromatography
SSF	Simultaneous saccharification fermentation
InsP	Inositol phosphate
ND	Not detectable
FC	Fibre concentrate
TAA	Thermostable alpha amylase
AMG	Amyloglucosidase

Chapter 1

Introduction and objectives

1.1 Introduction

Bioethanol is currently the most widely used liquid biofuel in the world. In the year 2000, global ethanol production was ~19 billion L, and production almost quadrupled over the past decade approaching 76 billion L by 2008 (MRA, 2008). In North America cereal grains are the major starchy raw-material for ethanol production in the bio-ethanol industry due to their abundance and relatively lower cost. Bioethanol produced from energy crops or agriculture crops such as sugarcane or cereal grains is called "a first generation" biofuel (Gomez, et al., 2008). Ethanol production from grain involves the following steps: a) the amylase hydrolysis of starch from grain flour into yeast fermentable sugars such as glucose, maltose and maltotriose; b) yeast fermentation of the sugars to ethanol; c) ethanol distillation and then dehydration. Efficient and quantitative conversion of starch into yeast fermentable sugars is a crucial step for fermentation and impacts the final ethanol yield. One of the challenges in streamlining starch hydrolysis is overcoming the negative effects of non-starch grains components, such as phenolics, protein, lipid, pentosan, phytic acid, and beta-glucan (Chethan et al., 2008; Faraj, 2004; Funke and Melzig 2005; Gibson and Strauss, 1992; Kikunaga et al., 1991; Lauro et.al., 2000; Nyman and Bjorck, 1989; Rohn et al., 2002; Shobana et al. 2009; Sreerama et al., 2010). The contents, structure, and confounding interactions of these components differ amongst grains and are very complex. Since ethanol industry is increasingly using low grade cereal grains, these challenges need to be understood and

mitigated if pure starch is replaced by low grade whole grain flours, a cheaper alternative, as the raw material of choice for industrial ethanol production.

Despite extensive studies documented on the effect of non-starch grain components on starch hydrolysis (amylolysis) in the literature, a substantial research gap still exists regarding how different phenolic acids present in cereal grains, individually and in combination in their "bound" and "free" states, affect starch liquefaction and saccharification. This study thus focused towards understanding how phenolics from various botanical origins, such as triticale, wheat, barley and corn, can influence starch susceptibility towards amylolysis.

1.2 Hypotheses and objectives:

It was hypothesized that complete or partial removal of non-starch grain components by pre-washing of whole grain flours with appropriate solvents would improve the efficiency of starch hydrolysis by amylases, and thus would decrease the cost of ethanol production. Non-starch components can be partially or completely solubilized and removed by different solvents depending on grain source, composition and component structure, physical and chemical interactions, and washing conditions. The strategic selection of appropriate solvents to optimize both the hydrolysis of starch and the extraction of valuable grain components is anticipated to make grain starch hydrolysis for ethanol production even more industrially viable. Phenolic acids in whole grain flours, either individually or in combination, would significantly mitigate starch liquefaction and saccharification. The negative effect of phenolic acids on starch liquefaction and saccharification would be dependent on whether they exist in free or bound forms.

The specific objectives were:

Chapter 3

a) To determine the effect of pre-washing using different solvents on the composition of whole grain flours, since washing can remove components that interfere with starch hydrolysis.

b) To evaluate the degree of amylolysis of starch by α-amylase or a combination of "α-amylase + amyloglucosidase" in different grain flours, with and without solvent pre-washings.

c) To correlate solvent washing, subsequent changes of chemical composition in flour, and the extent of starch hydrolysis by different amylase enzymes.

Chapter 4

a) To isolate starch from the whole grain flours of triticale, wheat, barley and corn.

b) To determine the content of individual phenolic acid, in their free and bound forms, present in the whole grain flours.

c) To investigate the effect of phenolic acids on amylolysis, added individually or collectively, at the equivalent levels as they would exist in the whole grain flour.

d) To understand the effect of temperature on the interactions between phenolic acids, enzymes and starch.

Chapter 5

To establish a protocol to isolate from whole grains a dietary fiber concentrate with bound phenolics. To investigate the effect of "dietary fiber bound phenolics", in their native form as they would exist in whole grains, on starch liquefaction and saccharification.

Chapter 2

Literature review

2.1 Cereals

Cereal grains are produced in greater quantities worldwide than any other crop for human consumption and as feed for livestock. The total world production of all cereal grains is 2,778 million MT per annum (FAOSTAT, 2013). Wheat, rice and corn are the most important grains in the human diet with a production of 713.2, 745.7 and 1016.7 million MT, respectively (FAOSTAT, 2013). Whereas minor grains include oats, barley, triticale, sorghum and millet with a total production weight of 23.8, 144.8, 14.6, 61.4, and 29.9 million MT per annum, respectively (FAOSTAT, 2013; Hillman et al., 2001). In Canada, most of the grains are produced in the Prairie provinces, Alberta, Saskatchewan, and Manitoba, where the average annual cereal production is 66.1 million MT (Canadian Wheat Board, 2011; FAOSTAT, 2013).

Cereal grains produce a one seeded fruit called caryopsis which is commonly called kernel or grain. Whole grains are composed of endosperm, germ and bran. The endosperm alone make about 75-80% of the grain weight, whereas the contribution of the germ and bran to the total weight may vary among different grains and varieties. The Whole Grain Council defines whole grains as products made from whole grains that contain all the essential parts and naturally occurring nutrients of the entire grain seed (Whole Grains Council, 2004). Whole grains such as barley (*Hordeum vulgare* L.) and oat (*Avena sativa L.*) like all other cereal grains, contain carbohydrates, proteins, lipids, minerals, vitamins, dietary fibers and other minor components (Evers and Millart, 2002). Furthermore, whole grains also contain unique phytochemicals. For instance, various

classes of phenolic compounds in grains include derivatives of benzoic and cinnamic acids, anthocyanidins, quinones, flavonols, chalcones, flavones, flavanones, tocopherols, tocotrienols, oryzanols and amino phenolic compounds (Lloyd et al., 2000; Shahidi and Naczk, 1995; Thompson. 1994). These phytochemicals play important structural and defense roles in the grain. Ferulic acid and other phenolic acids protect wheat kernels by providing both physical and chemical barriers (Arnason et al., 1992; Hahn et al., 1983).

2.1.1 Triticale

Triticale (X *Triticosecale wittmack*) is a relatively new crop species which was derived from combining the genomes of wheat (*Triticum*) and rye (*Secale*) (Qualset and Guedes-Pinto, 1996). Triticale has the potential to serve as a substitute for common feed grains like wheat or even as a partial substitute for common protein sources such as soybean meal (Varughese et al., 1996). The first triticale was produced from hexaploid bread wheat and diploid rye, but was a disappointment as the resultant progeny were unstable, variable and sterile (Stoskopf et al., 1993). In 1937, chromosome doubling was discovered, followed by effective embryo culture techniques in 1940 which allowed the reliable production of fertile hexaploid triticale could be produced (Stoskopf, 1985). The modern hexaploid triticale now come in both spring and winter types, and are widely cultivated due to their superior tolerance to abiotic stresses and their ability to be productive with low input systems (Royo et al. 1995; Varughese, 1996). In Canada, the Prairies are the main area of production for triticale, where both spring and winter types are grown primarily as forage, silage or pasture. In Alberta, winter triticale is grown extensively to extend grazing periods during the fall or even seeded in the spring to provide pasture in dry land areas. Triticale's high whole plant dry matter production

along with its excellent digestibility has allowed farms to use newer spring cultivars as a conserved fodder (Salmon et al. 1996).

Triticale production on a global scale accounts for approximately 5.5 million tonnes (MT) per year or 1% of wheat production (Schlegel, 1996). Canada produces approximately 66,000 hectares of triticale (FAO, 2010). The low production of triticale in Canada is due to more production of wheat and corn which are used for various food and industrial applications. The chemical composition of triticale grain is closer to that of wheat than rye. Table 2.1 shows a general composition of triticale as compared with wheat and rye. Triticale has high starch content (53-57%) which makes it a good candidate for the bioethanol industry (FAO, 2004)

2.1.2 Barley

Barley (*Hordeum vulgare*) is an ancient crop which belongs to the family *Poaceae*, the tribe *Triticeae* and the genus *Hordeum* (Nilan and Ullrich, 1993; Newman and Newman, 2008) and it can be grown under a wide range of environmental conditions. Barley is classified into spring or winter, two-row or six-row and hulled or hulless types. In general, the genotypes of barley grain are based on amylose content (normal, waxy, and high amylose), lysine, β-glucan and proanthocyanidin contents (Baik and Ullrich, 2008). Two-rowed barley is preferably used for malting/brewing and food processing due to its uniform kernel size, whereas the six-rowed barley is used mainly as livestock feed (Bhatty, 1993).

Barley is the fourth largest grain crop after wheat, rice, and corn, with an annual production of 144.3 MT (FAO, 2014). The top ten barley producing countries are Russia,

Table 2.1 Approximate composition of triticale, wheat and rye grains (dry basis)

Cereal	Protein (%)	Starch (%)	Crude fibre (%)	Ether extract (%)	Free sugars (%)	Ash (%)
Spring triticale	10.3-15.6	57-65	3.1-4.5	1.5-2.4	3.7-5.2	1.4-2.0
Winter triticale	10.2-13.5	53-63	2.3-3.0	1.1-1.9	4.3-7.6	1.8-2.9
Spring wheat	9.3-16.8	61-66	2.8-3.9	1.9-2.2	2.6-3.0	1.3-2.0
Spring rye	13.0-14.3	54.5	2.6	1.8	5.0	2.1

(FAO, 2004)

Ukraine, France, Germany, Canada, Spain, Australia, United Kingdom, Turkey and United States of America (FAO, 2014). In Canada, the barley production was 7.1 MT in 2014 (Figure 2.1) (FAO, 2014). Of the total world barley production, 50% is used for animal feed, 30% for beer, whiskey and syrup making, 10% for seed and 10% for food (McKenna, 2006). Interest in incorporating barley in human diet is increasing because of its high nutritional value (Newman and Newman, 2008). The health benefits of barley include lowering blood cholesterol (low density lipoprotein-LDL), and lowering both the glycemic index and body mass index, which in turn can then decrease the risk of heart disease and type-2 diabetes. The beneficial effects of barley are due to the presence of several bioactive compounds such as β-glucan, tocopherols and tocotrienols in the grain (Vasanthan et al., 2004; Baik and Ullrich, 2008).

Barley grows well outside the corn-belt area where there is demand for ethanol, and this makes barley a potential feedstock for ethanol in these regions (Kim et al, 2008; Sohn et al, 2007). Corn is not a common feedstock for fuel ethanol production in Europe; therefore, barley has been successfully used to replace corn (Sohn et al., 2007). Furthermore, the production of DDGS (Distillers Dried Grains with Solubles) with a high protein content as a byproduct improves the feasibility of barley as a feedstock for potential fuel alcohol production (Ingledew et al, 1995).

Barley composition is influenced by both environmental and genetic conditions as well as the interaction between them (Andersson et al, 1999). The chemical compositions of hulled and hull-less barley grains are shown in Table 2.2 (Xue et al. 1997; Andersson et al, 1999; Griffey et al., 2009). Starch is the major component of barley grains with a

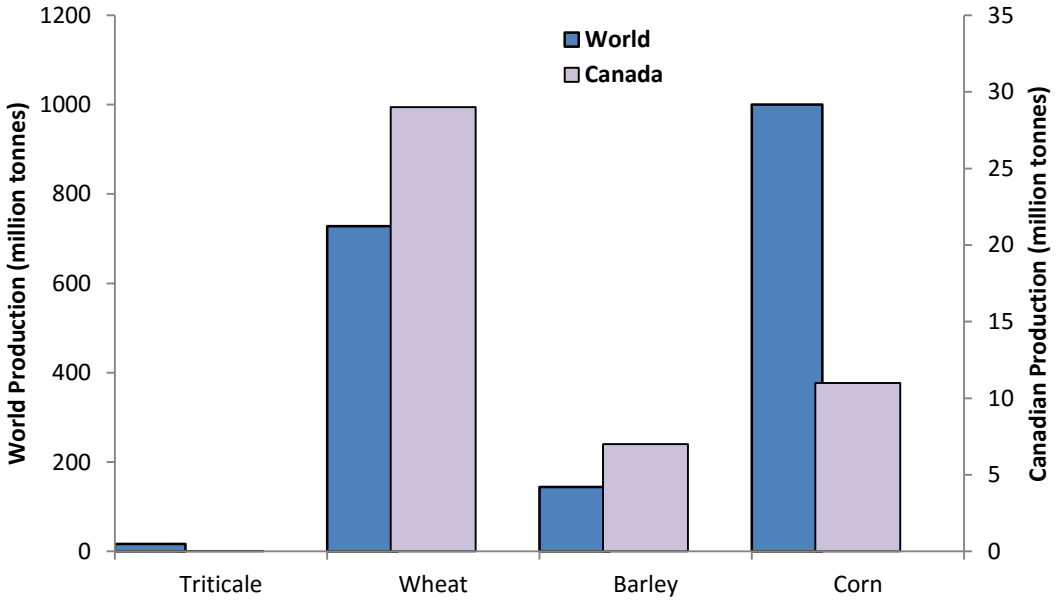


Figure 2.1 Canadian and Total world production of triticale, wheat, barley and corn in 2014 (FAO, 2014)

content of ~ 65% (Song and Jane, 2000). Non-starch carbohydrates in barley contribute the major portion of the total dietary fibre such as cellulose, lignin, and β-glucan (Temelli, 1997).

2.1.3 Wheat

Wheat (*Triticum aestivum* L.) is one of the most important and commonly grown food grains in the world, and a major crop in Canada. About 17% of the global crop acreage is occupied by wheat, which is used to feed about 40% of the world's population and to provide 20% of the caloric and protein requirements in human nutrition (Gupta et al., 2005). In 2001, wheat was the number one crop in Canada which was grown on more acres than any other crop, followed by barley (*Hordeum vulgare*), alfalfa (*Medicago sativa*), canola (*Brassica napus*) and soybeans (*Glycine max*). Most of the spring wheat in Canada is grown in the Prairie provinces of Alberta, Saskatchewan, and Manitoba in which they accounted for 99% of the acres produced in 2001 (Statistics Canada, 2001). In addition, because of its high quality and protein content, Canadian spring wheat commands a high export demand (Curtis, 2002). Following USA, Canada is the 2nd largest wheat and wheat flour producing and exporting nation in the world, accounting for 18% of the world exports.

Wheat is the third largest food crop grown in the world, next to corn with rice being second. In general, wheat is widely used in daily life to make flour for breads, cookies, cakes, noodles and pasta due to its unique elastic protein complex (Iqbal et al., 2007). In addition, wheat is used to produce vinegar, alcoholic beverages and biofuel through fermentation. In 2014, the world wheat production was about 728 MT. Canada produced about 29 MT of wheat on 8.26 million hectares in particular in Alberta,

Table 2.2 Average chemical composition of hulled and hulless barley grains

Components	Hulled barley (% Dry Basis)			Hulless barley (% Dry Basis)		
Starch	56.45	52.1-63.8	53.7	61.45	23.9-64.4	59.7
Protein	8.95	8.7-10.5	15.9	9.04	11.3-18.1	16.5
Lipid	2.29	2.2-3.5	2.1-3.1	2.43	2.9-6.2	-
B-glucan	4.17	2.8-6.9	5.2	4.34	4.1-8.0	5.6
Ash	2.23	2.3-2.6	2.8	1.81	2.0-2.3	2.1
Reference	Griffey et al. (2009)	Andersson et al. (1999)	Xue et al. (1997)	Griffey et al. (2009)	Andersson et al. (1999)	Xue et al. (1997)

Saskatchewan, and Manitoba (Fig 2.1). Wheat yield in Canada was slightly lower than that of the world average yield, 30946 Hg/Ha versu 32893 Hg/Ha, respectively (FAO, 2014).

As shown in Table 2.1, wheat grains consist mainly of starch (61-66%), protein (9.3-16.8 %), crude fibre (2.8-3.9 %), lipids (1.9-2.2 %), free sugars (2,6-3.0 %), and ash (1.3-2.0 %) (FAO, 2004). The high starch content of wheat grain makes it a potential feedstock in bioethanol production. In North America, cereal grains are the predominant ethanol feedstock. Ethanol operations in USA exclusively operate almost on corn feedstock. Corn is not consistently available in western Canada, resulting in the use of either wheat or barley (Sosulski and Sosulski, 1994). In 2008, Western Canada produced 20 MT of non-durum wheat and about 6.6% of this crop was directed to bioethanol production (CWB, 2009). In 2009, Canadian wheat based ethanol production was 487 million L (CRFA 2009).

2.1.4 Corn

Corn (*Zea mays* L.) is one of the big three major cereal crops grown in the world and belongs to the grass family (Gramineae). Corn kernels are used as feedstock in food, feed and ethanol industries. Corn kernel consists of the pericarp, endosperm, and germ (Farnham et al., 2003). The endosperm constitutes 82-84% of the kernel and consists mainly of starch (86-89% dry basis) and protein (7-9%). The germ represents about 11.5% of the kernel and it stores nutrients and hormones, which are mobilized by enzymes during the initial stages of germination. Corn germs are also good source of oil and protein, which constitute about 33% and 18%, respectively (Logan et al. 2001, and Watson, 2003).

Corn is the most widely grown grain crop in the Americas with 361 MT grown annually in the United States alone in 2014 (FAO, 2014). The ten major corn producing countries are USA, China, Brazil, Mexico, Argentina, Indonesia, India, France, South Africa and Ukraine, while Canada is ranked 11th with a production of 11.5 MT (Figure, 2.1) (FAO 2014). In the USA, the main raw material for ethanol production is corn and in 2007 more than 61 MT of corn were used for ethanol production, representing 19% of the US corn crop (Urbanchuk, 2006). The food prices are significantly affected by the use of corn for bioethanol production. About 80% of the food prices in the US are mainly a result of the cost of production, transportation, and marketing. Increasing energy costs affect food prices at all levels, but especially in transportation. Bioethanol production also has an indirect effect on other food crop prices. Use of corn for bioethanol production increases the demand for corn, and therefore increases corn prices. This results in farm acreages turning from other food crops to corn production, and in turn reduces the supply of the other food crops and increases their prices (McNew and Griffith 2005).

The chemical composition of corn is shown in Table 2.3 (Cortez and Wild-Altamirano, 1972; Wallace et al., 2005). The main component of corn grain is starch with a content of 66.0 – 75.9%, followed by protein (5.2 – 13.7%), and oil (2.2 – 5.7%).

2.2 Starch

2.2.1 General

Starch is considered to be the one of the abundant biomass resource in the world, and is the primary storage carbohydrate in higher plants (Whistler, 1984; Jane, 1995). Starch, as the energy-reserving compound, is produced by green plants and can be found in different parts of the plant, including seeds, leaves, fruits, stems, tubers and roots.

Table 2.3 Chemical composition of corn

Component	% Dry Basis
Starch	66 – 75.9
Crude fibre	0.8 – 2.9
Protein	5.2 – 13.7
Oil	2.2 – 5.7
Ash	1.2 – 2.9
%Moisture	9.6 – 15

(Cortez and Wild-Altamirano, 1972 and Wallace et al., 2005)

The primary botanical sources of starch listed in the order of commercial importance are: corn, wheat, potato, rice and tapioca (Gordon, 1999). These starches have received attention on their structure and functional properties due to their utilization in both food and non-food industries. Starches are used extensively in food and non-food applications such as thickening agents, gel forming agents, colloidal stabilizers, binders, water-retention agents, and coating and/or glazing agents (Pomeranz, 1991). Starch has been used in the manufacture of paper, textiles, cosmetics, pharmaceuticals, agrochemicals, detergents, and bioethanol (Murthy, et al., 2011).

Many reviews on different aspects of starch are available including reviews on physico-chemical characteristics (Parker and Ring, 2001; Sajilata et al., 2006; Copeland et al., 2009; Hoover et al., 2010), factors affecting digestibility (Wiseman, 2006; Copeland et al., 2009; Dona et al., 2010), nutritive value (Tapsell, 2004; Svihus et al., 2005) and health (Nugent, 2005; Lehmann and Robin, 2007; Fuentes- Zaragoza et al., 2010). The relative low cost and renewability of starch contribute to its extensive utilization within food and non-food industries (Whistler, 1984; Jane, 1995). Native starches hold natural physical defects, and poor functional properties, such as poor shear and heat resistance, high tendency toward retrogradation, poor solubility, all of which can limit their utilization in both existing and emerging industrial applications (Ratnayake et al., 2001; Hoover and Ratnayake, 2002; Singh et al., 2007). To overcome these natural limitations and expand its utilization, native starches are physically (e.g., pregelatinized, heat-moisture treated, pulverized) and/or chemically (e.g., cross-linked, stabilized, oxidized) modified. As a result, the majority of native starch used as a food ingredient is

first chemically modified to improve and extend its physical properties in accordance with the intended application (Hoover and Sosulski, 1991; Alexander, 1992).

2.2.2 Starch granule composition and structure

Starch is present in the form of granules within cell walls and embedded in the protein matrix in the endosperm of cereal grain and cotyledons of legume seeds. The size and shape of starch granules is representative of plant species and maturity (Deshpande and Damodaran, 1990). The most common granule shape is oval, however round, spherical, elliptical, kidney-shaped, and unusual shaped granules have also been reported (Hoover and Sosulski, 1991; Deshpande and Damodaran, 1990). Wheat, barley and triticale contain two types of granules: lenticular large (A-) granules with a diameter >10μm up to 40μm and round small (B-) granules with a diameter <10μm down to 1μm. 77% of triticale starch granules are over 10μm. Corn has polygonal shaped granules with a diameter range from 2-30μm. However, in high-amylose corn starch some of the granules are elongated or filmentous (Liu, 2005; Makowska, et al., 2014; Vamadevan and Bertoft, 2014).

Starch granules are composed of two distinct homopolymers of α-D-glucose, amylose (AM) and amylopectin (AP), both of which account approximately 98-99% of the starch granule on a dry basis. AM is predominantly a linear-chain polymer, with up to 6,000 glucose units linked together by α-(1-4) glycosidic bonds (Figure 2.2a). The molecular weight of amylose molecules is in the range of 10^5-10^6 Da with a degree of polymerization (DP) between 700-5000 anhydro-glucose units (Greenwood, 1970; Takeda et al., 1989; Hizukuri et al., 1997). Starch granules in cereals may contain AM in a free form, attached with AP branch points, or in a complex form attached with lipids.

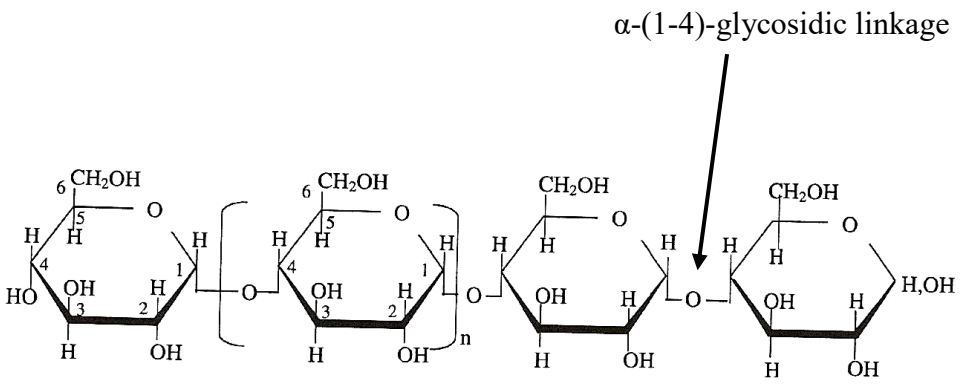


Figure 2.2a Structure of amylose molecule (Adapted with minor modification from Tester et al., 2004) with permission of Elsevier Ltd.

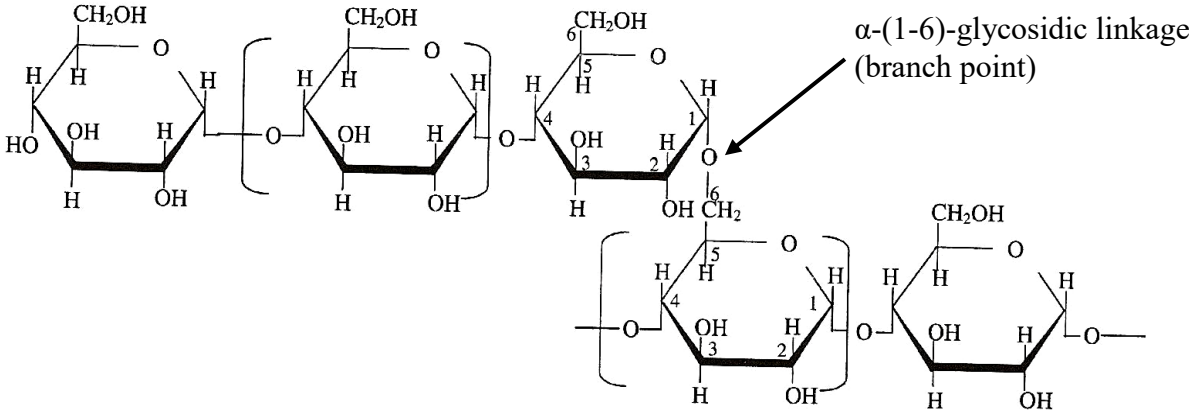


Figure 2.2b Structure of amylopectin molecule (Adapted with minor modification from Tester et al., 2004) with permission of Elsevier Ltd.

These lipids are mostly phospholipids (Ao and Jane, 2007). AP (Figure 2.2b) is a larger and highly-branched molecule compared to amylose, with an average of million glucose units linked together by α-D-(1-4) and α-D-(1-6) chains (Hizukuri et al., 1997 and Parker and Ring, 2001), a molecular weight of 10^7 to 10^9 Da, and an average chain length of 17-31 anhydro-glucose units, depending on the starch botanical origin (You et al., 1999, You and Izydorczyk, 2002, Yoo and Jane, 2002). Table 2.4 shows the differences in the basic characteristics between these two polysaccharides.

Depending on the ratio of amylose and amylopectin, starches are generally classified into 3 types: waxy (essentially devoid of amylose), normal (20-35% amylose), or high amylose (>40% amylose) (Nakamura et al., 1995; Tester et al., 2004), although this classification varies wit starch botanical origin. Wheat starch, for example, is subdivided into four categories: waxy (0-3% amylose), partial waxy (16-23% amylose), normal (25-28% amylose), and high amylose (30-37% amylose) (Nakamura et al., 1995; Graybosch, 1998; Bertolini et al., 2003; Geera et al., 2006a; Van Hung et al., 2006; Van Hung et al., 2008)..

Starch granules are semi-crystalline in nature and consist of alternating semi-crystalline and amorphous growth rings (Figure 2.4a) (French, 1984; Gallant et al., 1997; Buleon et al., 1998; Vandeputte and Delcour, 2004; Vamadevan and Bertoft, 2014). Visualization of growth ring structures by scanning electron microscopy (SEM) is enhanced via partial hydrolysis of starch molecules within granules, particularly in the amorphous regions, with either acid or α-amylase (French, 1984; Li et al., 2004; Li et al., 2006). The number and thickness of growth rings vary depending on the botanical origin

Table 2.4 Basic characteristics of amylose and amylopectin

Properties	Amylose	Amylopectin
Branch linkage (%)	0.2-0.7	4.0-5.5
Degree of polymerization	700-5000	10^4-10^5
Molecular weight (Daltons)	10^5-10^6	10^7-10^9
Average chain length	100-550	17-31
Iodine affinity (g/100g)	19-20.5	0-1.2
Blue value	1.2-1.6	0-0.2
B-Amylolisis limit (%)	70-95	55-60
Reference	Hizukuri et al. (1981, 1983, 1997, 2006), You et al. (1999), You and Izydorczyk (2002), Yoo and Jane (2002)	

of the starch (French, 1984). The semi-crystalline growth rings are suggested to possess a thickness ranging from 120 to 400 nm (French, 1984; Dang and Copeland, 2003), and become increasingly thicker, as AM content increases (Yuryev et al., 2004). It is commonly accepted that the semi-crystalline and amorphous growth rings are both comprised of continuous bundles of the crystalline and amorphous lamellae of the amylopectin, referred to as 'blocklets', as proposed by Gallant et al. (1997) (Figure 2.3b). The presence and ordered orientation of blocklets within the semi-crystalline and amorphous growth rings of various starch granules (corn, potato, pea, rice, wheat) have been visualized via atomic force microscopy (AFM) (Baker et al., 2001; Szymoska and Kork, 2003; Dang and Copeland, 2003; Ridout et al., 2006). A blocklet may possess varying dimensions (but a relatively similar spherical shape) across the various botanical sources of starch, with maximum lengths of about ~30 nm for corn starch (Baker et al., 2001), 130-250 nm for pea starch (Ridout et al., 2006), ~50 nm for potato starch (Szymoska and Kork, 2003), and 80-120 nm for wheat starch (Tester et al., 2004). There is no apparent relationship between blocklet and granule size (Tang et al., 2006).

Furthermore, blocklets comprising the semi-crystalline growth rings of starch granules are themselves believed to be composed of stacks of alternating crystalline (5-6 nm) and amorphous (2-5 nm) lamellae (Figure 2.3c) (Vandeputte and Delcour, 2004). A total lamellar repeat distance of approximately 9 nm, including both a crystalline and amorphous lamella, is commonly accepted for all starch types (Jenkins et al., 1993). The amorphous lamellae correspond to the branch point regions of amylopectin, while the crystalline lamellae are represented by the amylopectin chain clusters (Figure 2.3d)

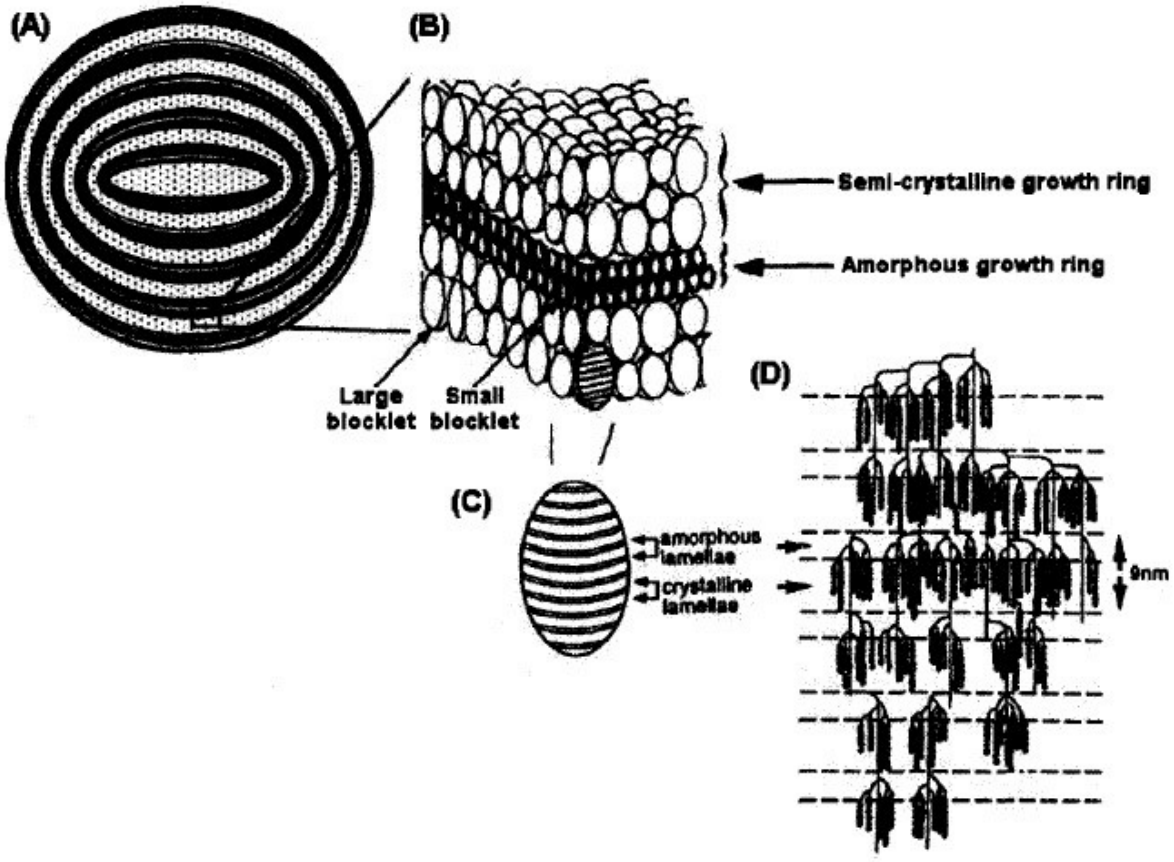


Figure 2.3: Schematic representation of a starch granule: (A) semi-crystalline and amorphous growth rings within the starch granules, (B) a stack of large and small blocklets, (C) crystalline and amorphous lamellae in blocklets, (D) aligned double helices (comprising amylopectin side chains) within a crystalline lamella and amylopectin branch points within an amorphous lamella. (Adapted with minor modification from Gallant et al., 1997; Vandeputte and Delcour, 2004) with permission of Elsevier Ltd.

(Vandeputte and Delcour, 2004). These amylopectin clusters are formed by double-helical interactions of adjacent amylopectin branch chains. It is the external A (DP 12-16) and Bl (DP 20-24) short chains of amylopectin that participate in these double helices (Tester et al., 2004).

Although the main structural features of amylopectin have been known for over 50 years, knowledge on the mode of interconnection of the different chains remains poor. During the 1970's, different molecular structures models for amylopectin were proposed. Among these, the so-called "cluster" model, has emerged as the most accepted, although there are some variations, and it is not yet clear whether it applies to all amylopectins, irrespective of the starch source (Manners, 1989). In this model, the unit chains of amylopectin were suggested to be organized into clusters (Nikuni, 1978 and French, 1972). Furthermore, Hizukuri (1986) suggested that two clusters of short chains (A and BI) are interconnected by B2-chains (DP 40-50), whereas B3-chains (DP 70-75) span over three clusters. Later, Hanashiro et al. (1996) compared the amylopectin chain length distributions from several starch sources and found a periodicity at intervals of DP 12. Chains with DP 6-12 were suggested to represent the A-chains, whereas the rest of the short chains were B1 (DP 13-24) and B2 (DP 25-36) and long chains (DP>37) were classified as B2 or B3.

Researchers have also investigated a more detailed classification of amylopectin chains. B1-chains obtained by α-amylolysis, (Bertoft and Koch, 2000) were subdivided into B1a and B1b. The cluster-based "tree" model proposed by Hizukuri (1986) classified the B-chains according to the number of clusters they participate in. The major feature of

this model is the interconnection of the clusters by the long B-chains (Bertoft, 2004). The tree model is shown in Figure 2.4

The chains of amylopectin can also be distinguished as external and internal. External chains are those between the non-reducing end group and the outermost branch point, whilst internal chains represent the segments of the B-chains between the branches, excluding the branch point residues (Bertoft, 2004). Accordingly, A-chains are external, whereas B-chains are composed of one external and one internal segment (Bertoft et al., 2008). For practical reasons, the segment at the reducing end of the molecule is also considered an internal chain (Bertoft, 2004). External chains build up the crystalline lamellae with double helical structures, and the internal chains are mainly found among the clusters of branches in the amorphous lamellae (Bertoft, 2007). For A-type starch, branch points are scattered in both crystalline and amorphous regions; while for B-type starch, most branches are clustered in the amorphous region (Jane et al., 1997). Amorphous lamellae provide chains with a certain flexibility to move around and chains can bend in directions even perpendicular to that of the double helices (O'Sullivan and Pérez, 1999).

The "backbone" model was suggested by M. Richter more than three decades ago, but first published by Babor et al. (1968). Based on the position of the long B-chains and the direction of external chains, the model was modified by Robin et al. (1975), and later further modified by Bertoft (2004, 2007, 2013), as shown in Figure 2.5. In the backbone model, the clustered chains of amylopectin are in one direction, with their external parts forming the crystalline lamella, and the long B-chains are found in a perpendicular direction. The unit chain composition is identical to that of the tree model however, the

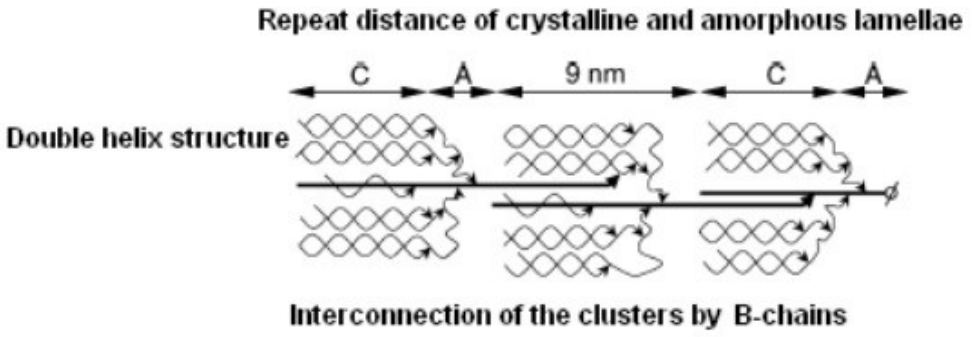


Figure 2.4 Tree model of amylopectin fine structure: clusters were interconnected by long B-chains which span over the crystalline and amorphous lamellae. C: Crystalline lamellae; A: A morphous lamellae. (Adapted with minor modification from Bertoft, 2004) with permission of Elsevier Ltd.

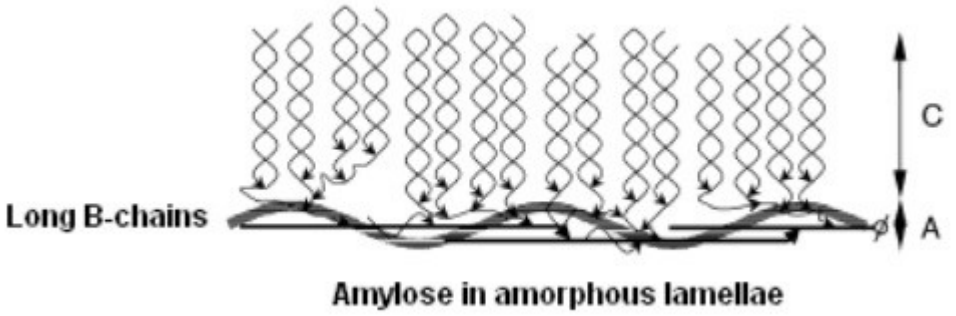


Figure 2.5 Backbone model of amylopectin fine structure: the entire long B-chains are in amorphous lamella and do not constitute part of the clusters.Amylose is shown in thick wave line. C: Crystalline lamellae; A: Amorphous lamellae. (Adapted with minor modification from Bertoft, 2004) with permission of Elsevier Ltd.

entire long B-chains are found in the amorphous lamella, forming the backbone for the whole structure. Another feature of this model is that it provides a possibility to accommodate previously reported extra-long A-chains (DP > 35) in certain amylopectin structures (Bertoft, 2004, 2007, 2008, 2013). The shortest (DP 6-8) and the extra-long A-chains, together with the long B-chains, are all found within the amorphous areas of the starch granules and do not constitute part of the clusters. The rest of the A-and B-chains are probably clustered with their external part largely involved in the crystalline lamellae (Bertoft, 2004). The backbone model may permit more flexibility to the amylopectin molecule taking into account movement of the more independent backbone chains.

Oostergetel and van Bruggen (1993) proposed a three-dimensional super-helical structure for the amylopectin clusters, which has been adapted to both the tree model and the backbone model as shown in Figure 2.6. The super-helix has a diameter of 18 nm and a central cavity with a diameter about 8 nm. In the tree model, the super-helix is a cooperative structure build-up of several individual amylopectin molecules. The double helices are lined up close together to form the left-handed crystalline lamella (Waigh et al., 1999). The directions of the clustered chains and of the individual amylopectin molecules follow that of the super-helix axis. In the backbone model, the entire super-helix is built of a single amylopectin molecule, where the amorphous lamella are built up of a true backbone formed by the long chains of the amylopectin. The direction of the clustered chains is still similar to the super-helical axis, but the direction of the amylopectin molecule follows the turns of the super-helix (Bertoft, 2004).

There are several advantages of the backbone model with regards to the amylopectin structure. The backbone model provides greater chain flexibility for the long

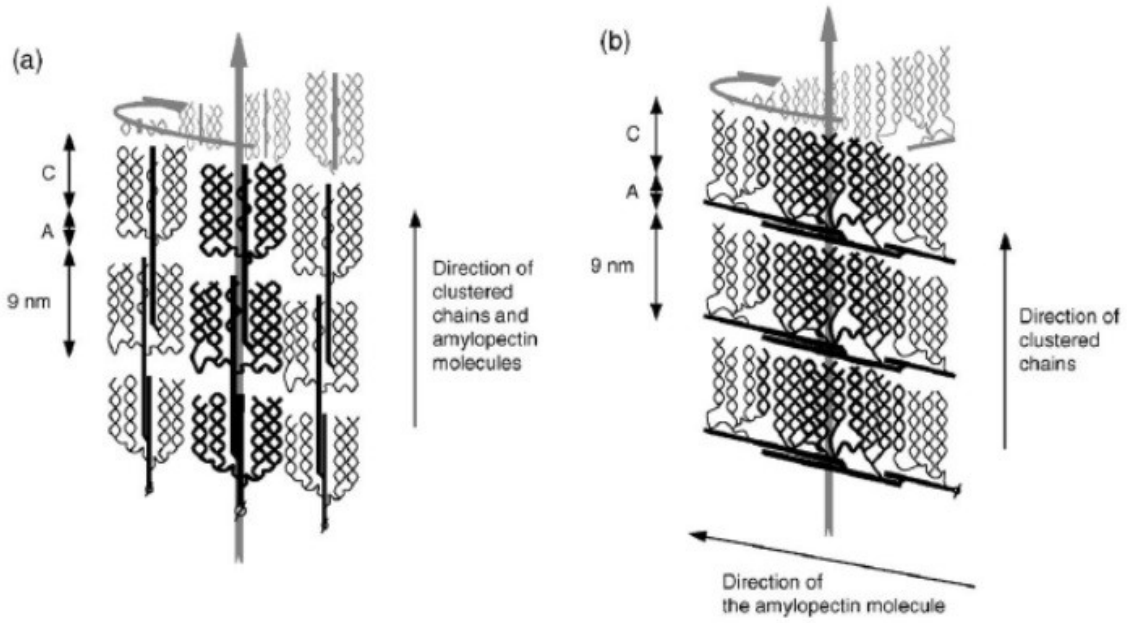


Figure 2.6 Super-helix model of amylopectin. The axis and the turns are indicated by grey arrows. (a) The super-helix structure based on the treemodel. The super-helix is a cooperative structure build-up of several individual amylopectin molecules. The directions of the clustered chains and of the individual amylopectin molecules follow that of the super-helix axis.(b) The super-helix structure based on the backbone model. The entire super-helix is built of a single amylopectin molecule. The direction of the clustered chains is similar to the super-helical axis, but the direction of the amylopectin molecule follows the turns of the super-helix.(Adapted from Bertoft, 2004 with minor modification) with permission of Elsevier Ltd.

B-chains. It offers a location for amylose to exist in normal starch, as it can be located in the amorphous lamella together with the long chains of amylopectin. Additionally, because the synthesis of the short chains is separated in space from that of the long chains, the backbone model offers an explanation on how only one chain, out of several possible chains in a cluster, is selected to continue to grow into a long, intercluster chain (Bertoft, 2004).

Double-helices of amylopectin can be arranged in A-, B-, and C- type crystalline polymorphs, as shown in Figure 2.7. The double helices of A-type polymorphs are packed into a monoclinic lattice with a low water content, whereas those of B-type polymorphs have a more open structure in a hexagonal assembly with a hydrated core (Buleon et al. 1998). The C-type crystallinity are mixtures of A- and B-type polymorphs. Type A crystallinity is present in cereal starches, type B in tubers and high amylose starches; and type C in legume starches. The extent of crystallinity in starch granules is influenced by four major factors: the amount of double helices that are organized into a crystalline array, crystallite size, amylose content, and moisture content.

2.2.2.1 Non-carbohydrate components

The moisture content of starch granules ranges between 10-15% (w/w) in cereal and 14-18% (w/w) for root and tuber starches (Tester et al., 2004). Within starch granules, lipids are present in low levels (0.6-1.2% dry basis) especially those of cereal starches, and are known as either surface or internal types, based on their location within the granule (Morrison and Coventry, 1985; Morrison, 1988). Surface lipids consist of residues of different lipid components such as tri-, di- and monoacylglycerols, phospholipids, and free fatty acids. These lipid components are

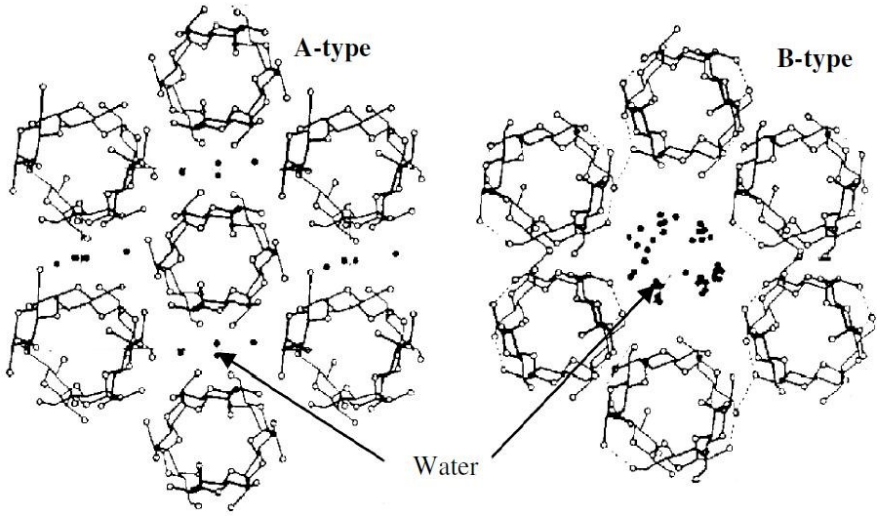


Figure 2.7 The arrangement of the double helices in starch crystalline structure into A-type and B-type polymorphs (reprinted with minor modification from Tester et al. 2004) with permission of Elsevier Ltd.

originated primarily from the endosperm, aleurone, and germ regions of the kernel (Morrison, 1988), and may be distributed on the surfaces of starch granules in either free or protein-bound form (Greenblatt et al., 1995). The major phospholipid located at both external granule and channel surfaces of waxy and normal corn starch granules is lysophosphatidylcholine, with palmitic or linoleic acid as the acyl group (Lee and BeMiller, 2008). On the other hand, internal lipids consist of both lysophospholipids (monoacyl lipids) and free fatty acids. Wheat, barley, rye, and triticale starches contain mostly lysophopholipids (e.g., lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylserine, lysophosphatidylinositol, lysophosphatidylglycerol), and variable amounts of free fatty acids. Other cereal starches such as corn contain much higher proportions of free fatty acids than lysophospholipids (Morrison, 1988, 1995). Both types of internal lipids form complexes with amylose molecules within starch granules causing an effect on starch swelling and gelatinization properties (Morrison et al., 1993; Morrison, 1995).

Starch proteins, also named as starch granule-associated proteins, are present in purified starch granules at levels ranging from 0.2-0.6% (db) for cereal starches to less than 0.05% (db) for potato starch (Baldwin, 2001; Debet and Gidley, 2006). Surface or internal types of proteins occur in starch granules based on their relative locale within the starch granule (Baldwin, 2001). Surface proteins have a low molecular weight, ranging from 5 to 30 kDa (Darlington et al., 2000; Yoshino et al., 2005). Zein and friabilin are the predominant surface proteins found in corn and wheat starches, respectively (Greenblatt et al., 1995; Oda and Schofield, 1997; Mu-Forster and Wasserman, 1998), and are remnants of endosperm proteins on granules after starch isolation. In contrast, internal

proteins with a molecular weight higher than 40 kDa are embedded within the granule matrix, or are bound to the surfaces of channel structures (Mu-Foster et al., 1996; Rahman et al., 1995; Mu et al., 1998). The best-known internal protein is GBSSI (60 kDa), which is primarily observed within the concentric rings of starch granules (Han and Hamaker, 2002). These internal proteins are residues of enzymes involved in starch biosynthesis and granule formation. More recently, proteins were detected at the surfaces of internal channel structures within sorghum, corn, and wheat starch granules (Han et al., 2005). Brittle-1 protein (40 kDa), amyloplast membrane protein, and actin (42 kDa) are proposed to be the primary proteins present at the surfaces of granule channels (Han et al., 2005; Benmoussa et al., 2005). The presence of actin within granule channels has led to the hypothesis that channels may provide a framework and/or a possible mode of substrate delivery (e.g., microtubules) for developing granules during starch biosynthesis. Furthermore, the presence or absence of SGAPs has been suggested to impact starch granule properties and also reactivity to derivatizing reagents (Han et al., 2002a; 2002b; Han and BeMiller, 2008).

Starches also contain small amounts (< 0.4%, db) of minerals (calcium, magnesium, phosphorus, potassium, and sodium) (Appelqvist and Debet, 1997; Tester et al., 2004), of which phosphorus (< 0.1%, db.) appears to be of primary functional importance in relation to starch properties, such as granule swelling, starch paste viscosity and clarity, gelatinization, and retrogradation (Noda et al., 2007). Phosphorus within starch granules is present in one of three major forms: starch phosphate monoester, phospholipids, or inorganic phosphate, the proportions and contents of which vary

depending on the botanical origin of the starch (Kasemsuwan and Jane, 1996; Blennow et al., 1998).

2.2.3 Starch gelatinization and retrogradation

2.2.3.1 Gelatinization

Starch granules are partially insoluble in water. In the presence of excess water and heat, starch granules undergo an order-disorder phase transition that leads to their solublization in water. This process has been termed gelatinization, which involves granule hydration, swelling, loss of birefringence, crystallites melting, uncoiling or dissociation of the double helices and the leaching of amylose and some amylopectin chains (Appelqvist and Debet, 1997). In the initial stages of gelatinization, the granules will start absorbing water and swell, and the molecular order of the granule structure disappears. This is manifested by loss of birefringence as observed under polarized light. The crystals then start to melt. Glass transition of the amorphous regions, at the glass transition temperature (T_g) is an important property of partially crystalline materials such as starch granules. The amorphous regions are transformed from a rigid glassy to a mobile rubbery phase when passing T_g during heating (Jacobs and Delcour, 1998) (Figure 2.8). The softening of the amorphous regions is required before melting of crystallites can take place (Slade and Levine, 1987). Therefore, T_g always precedes gelatinization.

A number of analytical methods have been used to study starch gelatinization such as viscoamylography, Kofler hot-stage microscopy (Watson, 1964), pulsed NMR (Lelievre and Mitchell, 1975), differential scanning calorimetry (DSC) (Donovan, 1979), enzymic digestibility (Shiotsuba, 1983), X-ray diffraction (Zobel et al., 1988) small angle

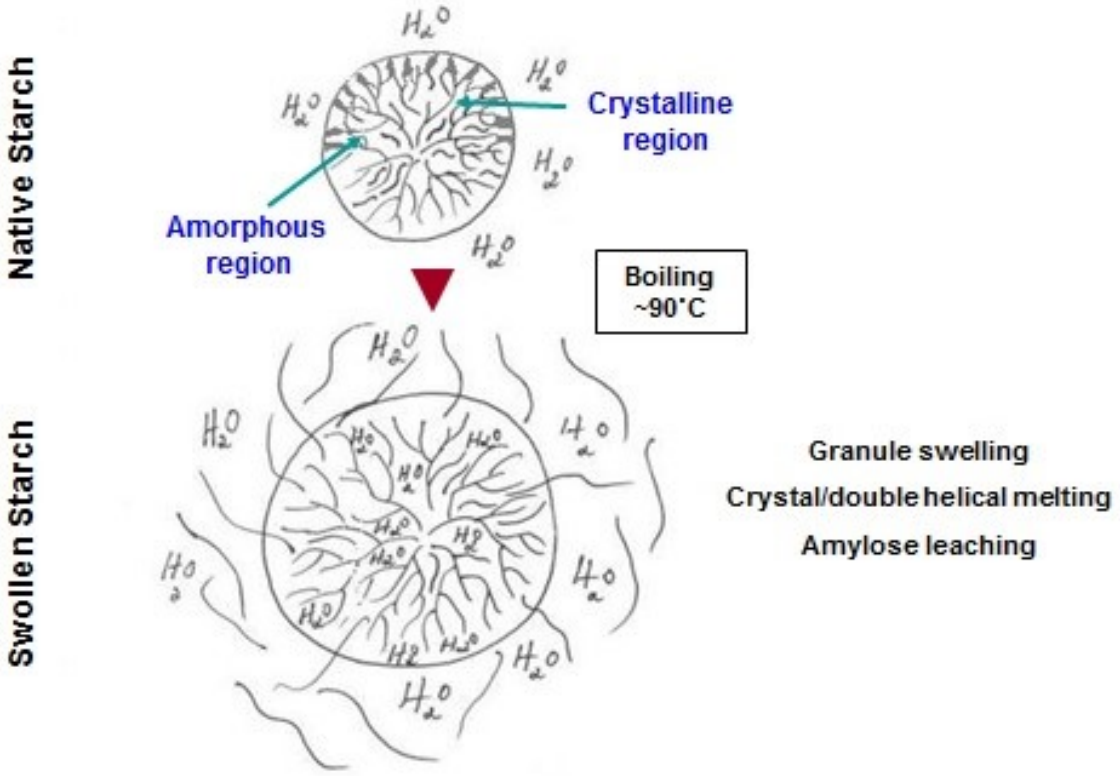


Figure 2.8 Schematic representation of starch gelatinization

X-ray scattering (Jenkins and Donald, 1998) and small angle neutron scattering (Jenkins and Donald, 1998). Differential scanning calorimetry (DSC) has been widely used to study the gelatinization parameters of starches and it measures the gelatinization transition temperatures: onset (To), mid-point or peak (Tp), conclusion or end (Tc) and gives a path to study the effect of water content on gelatinization temperature (Ratnayake, et al., 2002). The gelatinization transition temperatures are the temperatures at which the melting of starch crystals or crystallites occurs, thereby reflecting the perfection of starch crystallites (Liu, 2005). The Tc-T0 represents the gelatinization range. It has been postulated that the molecular architecture of the crystalline regions, which corresponds to the distribution of amylopectin short chains (DP 6-11) influences the DSC parameters and not the proportion of crystalline region, which corresponds to the amylose to amylopectin ratio (Noda et al., 1996). There are many factors that influence starch gelatinization such as water content, amylose/amylopectin ratio, presence of salt and pretreatments such as hydro-thermal treatments. A number of explanations for the melting behavior of starch in excess or limited amount of water have been offered. Most of these explanations do not account for the fact that gelatinization of some starches starts at the hilum and proceeds to the peripheiry of the granule (Hoseney et al., 1986). In excess water, gelatinization is a swelling driven process (Jenkins and Donald, 1998). Initially the amorphous regions swell and destabilize the amylopectin crystallites by ripping apart the edges of the crystallites. Under conditions of intermediate or limited water, a biphasic endotherm occurs (Burt and Russel, 1983; Hoseney et al., 1986). A number of suggestions and interpretations have been offered for the observed double endotherms. Russel (1987a,b) interpreted the observed double endotherms are due to

double helices associated with short-range ordering involving amylose and amylopectin followed by melting of the crystallites, while Biliaderis (1990) suggested that it could be due to recrystallization.

Generally, the waxy starches from wheat, barley, maize and potatoes show lower gelatinization temperatures than their normal and high amylose counter parts (Hoover and Ratnayake, 2002; Ratnayake, et al., 2002).

Salts can influence the gelatinization temperature of starch. Depending on the nature and concentration of the salts, they can either increase or decrease the gelatinization temperature (Gough and Pybus, 1973). When starch is heated in the presence of water, it gelatinizes with an endothermic enthalpy, but in the presence of concentrated calcium chloride (>4M), it gelatinizes with an exothermic enthalpy (Evans and Haisman, 1982). Sodium sulphate substantially increases gelatinization temperature, while sodium thiocynate (>2M) gelatinizes starch at room temperature (Evans and Haisman, 1982). Several hypotheses for the mechanisms have been proposed. These include dipole-ion interactions between starch molecules and cations and anions, the viscosity of the salt solution (Jane, 1993), the hydration energy of the salt solution (Gough and Pybus, 1973; Jane, 1993).

Thermal treatments can affect the gelatinization characteristics of starch. Annealing and heat-moisture treatments are two common hydrothermal treatments used for modifying physicochemical properties of starch. Both treatments are physical treatments that involve incubation of starch granules at certain moisture content for certain period of time at a temperature above the glass transition temperature but below the gelatinization temperature (Jacobs and Delcour, 1998; Tester and Debon, 2000). The main difference between the two treatments is that annealing is carried out in excess

water (>60%, w/w) or at intermediate moisture content (40-55%, w/w), while heat-moisture treatment is carried out a low moisture levels (<35%, w/w) (Jacobs and Delcour, 1998; Tester and Debon, 2000). Heat-moisture treatment, as for annealing, increases the gelatinization temperature but the gelatinization temperature range remains the same or broadens rather than narrowing, as observed for annealing (Hoover and Manuel, 1996; Hoover and Vasanthan, 1994).

2.2.3.2 Retrogradation

Retrogradation is the structural transformation that takes place when gelatinized starch is cooled and stored. The actual mechanism(s) has not been fully elucidated. However, it is postulated that upon cooling of the gelatinized starch paste, the amylose polymer chains begin to reassociate as double helices. The double helicals can form tightly packed three-dimensional structures that are stabilized by hydrogen bonds between the hydroxyl groups of starch chains (Eerlingen and Delcour, 1995; Haralampu, 2000; Hoover, 2001; Karim et al., 2000).

Starch retrogradation is a non-equilibrium, thermoreversible recrystallization process that is governed by a consecutive three step mechanism of nucleation (formation of double helixes of 40-70 glucose units between the ends of amylose molecules, favouring elongation), propagation (packing of double helical regions of chain folding) and maturation (Liu, 2005). The kinetics of starch retrogradation exhibit a strong temperature dependence because the nucleation rate increases exponentially with decreasing temperature down to the T_g, while propagation rate increases exponentially with increasing temperature up to the melting temperature (Silverio et al., 2000).

Crystallization can only occur in the temperature range between the T_g and the melting temperature because nucleation and propagation are liquid state events which require orientation mobility of the polymer chains (Silverio et al., 2000).

Starch retrogradation has been investigated by a number of techniques including X-ray diffraction, DSC, spectroscopic methods (NMR, Raman, FT/IR) and rheological techniques. Irrespective of the X-ray diffraction pattern in the original starch granule, retrograded starch shows mostly a B-type pattern. Retrograded amylose and amylopectin crystallites have been shown to melt at 120-170 °C (Sievert and Pomeranz, 1989), and 55-70 °C (Eerlingen and Delcour, 1995), respectively.

Starch retrogradation is influenced by the botanical source, the fine structure of amylopectin (ex: chain length and distribution), amylose:amylopectin content ratio, and molecular size and size distribution of starch (Liu, 2005). Although the retrograded starch contains both crystalline and amorphous regions (Ratnayake, et al., 2002), it is highly resistant to amylase hydrolysis. It has been reported that the amylases hydrolyze the glycosidic bonds located in the amorphous regions of the retrograded starches, leaving the crystalline double helical regions intact (Robyt, 2009). However, because the starch retrogradation is a kinetically controlled process, the alteration of time, temperature and water content during processing can produce a variety of products (Liu, 2005).

Both gelatinization and retrogradation influence starch amylolysis. Gelatinization loosens and opens up the compact structure of starch granules and thus increases the susceptibility of starch to amylolysis. In contrast, retrogradation restricts easy access of enzymes to starch.

2.2.4 Starch conversion to fermentable sugars

The starch conversion to fermentable sugars can be considered a two-step process. In the first step, raw starch is gelatinized and partially hydrolyzed to form maltodextrins with different degrees of glucose polymerization. This step is generally known as starch thinning or starch liquefaction. After this step, the dextrose equivalent - a measurement of the reducing content of a starch hydrolysate - is calculated as dextrose on a dry basis, and for the maltodextrin solution is 20 or less. If maltodextrins are the end product, the hydrolysis process stops at the point. However, if the required end product is yeast fermentable sugars (i.e., glucose), the maltodextrin solution must be further hydrolyzed in a second step known as starch saccharification. The dextrose equivalent of the final product is above 20 (Lyons, 2003).

2.2.4.1 Acid hydrolysis

Acid hydrolysis of starch proceeds in random fashion, acids cleaving both α-(1-4) and α-(1-6) glycosidic linkages at nearly the same rate shortening the chain length, thereby altering the structure and the properties of the native starch. During acid hydrolysis, the hydronium ion (H_3O^+) carries out an electrophillic attack on the oxygen atom of the α-(1-4) glycosidic bond. Then the electrons in one of the carbon- oxygen bonds move onto the oxygen atom to generate an unstable, high-energy carbocation intermediate. The carbocation intermediate is known as Lewis acid, which subsequently reacts with water resulting in the regeneration of a hydroxyl group and cleavage of the glycosidic linkage (Gao, 2008; Hoover, 2000).

Studies have shown that starch hydrolysis by acid follows a two-stage pattern. The first stage is a relatively fast hydrolysis stage which is mainly responsible for the hydrolysis of the amorphous regions of the starch granule; while, the second stage, which

is a slow hydrolysis stage, corresponds to the hydrolysis of the crystalline regions within the starch granule (Gerard et al., 2002; Hoover and Vasanthan, 1994; Jacobs et al., 1998; Jane et al., 1997; Kainuma and French, 1971; Li et al., 2001; Nakazawa and Wang, 2003; Shi and Seib, 1992; Vasanthan and Bhatty, 1996; Waduge et al., 2006).

Other reactions also occur as the hydrolysis proceeds. One such reaction is the dehydration of glucose which yields hydroxymethylfurfural. This compound may turn to levulinic and formic acids, or polymerize to form compounds believed to be intermediates in colour formation (Hebeda, 1987).

2.2.4.2 Enzymatic hydrolysis (amylolysis)

Enzyme hydrolysis of starches is not only important for the production of various industrial products such as sweeteners, syrups, ethanol, and other chemicals, but is also beneficial for understanding the starch granule structure and better control of enzymatic susceptibility. Cooking, liquefaction and saccharification are three stages in high temperature starch hydrolysis, where two main enzymes, α-amylase and glucoamylase, are used. One approach for the production of fermentable sugars is to cook the starch at 105°C with α-amylase, hold at 90 to 95°C with α-amylase, cool to 30 to 32°C and simultaneously saccharify and ferment by the addition of glucoamylase and yeast (Power, 2003) (Figure 2.9)

Amylases are enzymes that hydrolyze amylose and amylopectin. α-amylase is an endoenzyme that cleaves α-1,4 bonds in the starch chain until the chain lengths reaches 10 to 20 glucose units. The results of α-amylase hydrolysis is a mixture of dextrins from amylose and amylopectin and also large fragments that contain α-1,6 bonds. Common α-

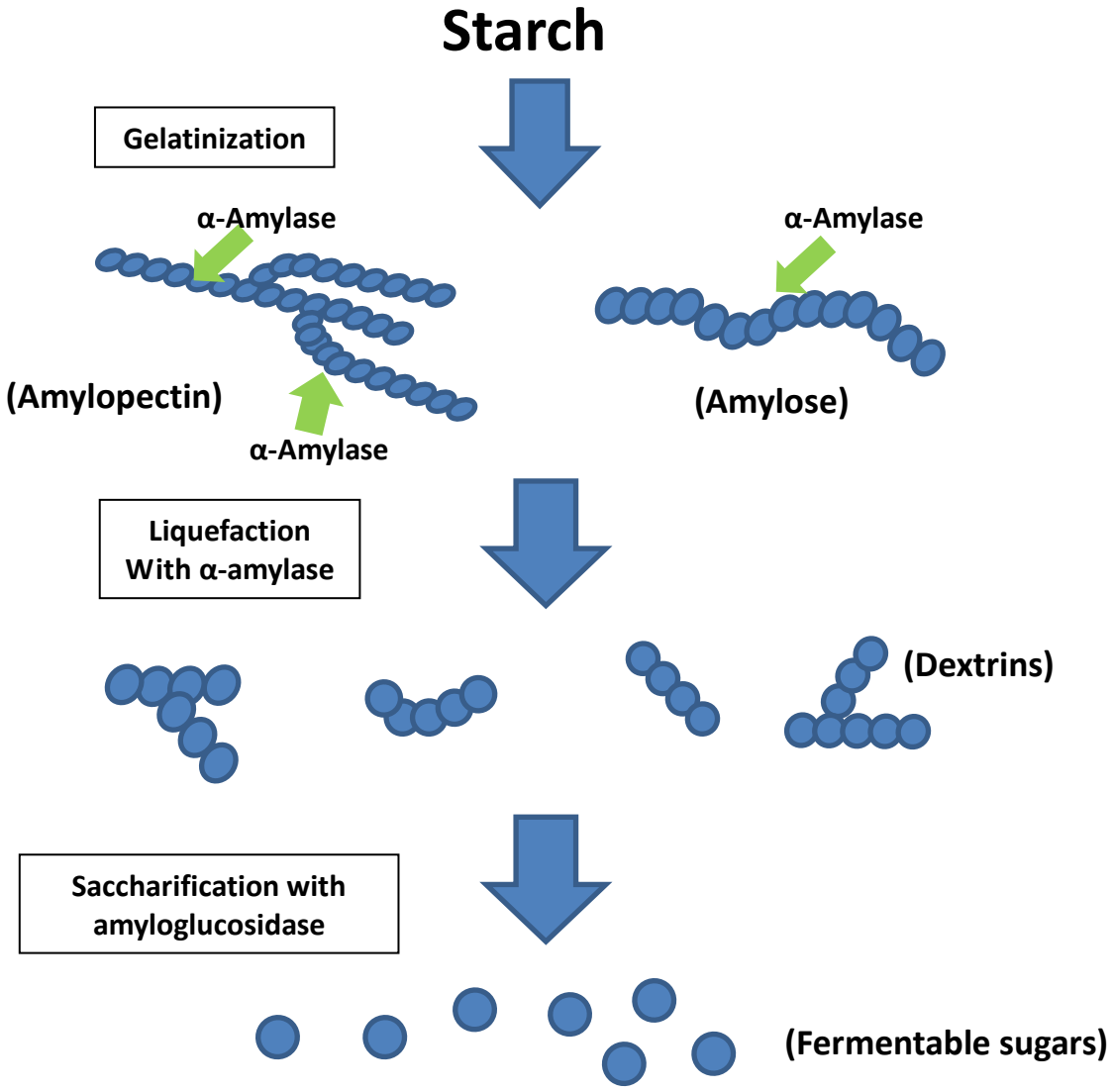


Figure 2.9 Schematic representation of a-amylase and glucoamylase action on starch

amylase is a bacterial enzyme. Beta-amylase is an exoenzyme that breaks the alternate (second) α-1,4 bond encountered as it moves along the chain from the non-reducing end and produces maltose.

Glucoamylase (or amyloglucosidase) is exoenzyme that hydrolyzes both α-1,4 and α-1,6 bonds and convert dextrins to glucose. Common glucoamylase is a fungal enzyme, and works at a lower pH and temperature than most commonly used α -amylases (Robyt, 1984; Whitaker, 1984). Amyloglucosidase and β-amylase are exoenzymes which act from the non-reducing end, producing mono and disaccharides, such as glucose by amyloglucosidase and maltose by β-amylase. Pullulanase is a debranching enzyme that can hydrolyze a α-(1,6) linkages (Bertoldo and Antranikian, 2002). Alpha (1-6) linkages also are hydrolyzed by amyloglucosidase but less rapidly. Reversion products which are oligosaccharides linked by resistant α-(1,6) linkages can be formed at high glucose concentrations (Roy and Gupta, 2003). It is more efficient to supplement amyloglucosidase with pullulanases, which rapidly hydrolyze α-(1,6) linkages and restrict reversion product formation.

In the cooking stage, starch granule structure breaks down, allowing liquefaction enzymes to react with starch granules. When corn slurry is cooked, starch granules absorb water, swell (gelatinize) and gradually lose their crystalline structure. Alpha-amylase can access starch molecules after gelatinization. Furthermore, when starch molecules are heated in excess water, water molecules become linked through hydrogen bonds to expose hydroxyl groups of amylose and amylopectin, which causes an increase in granule swelling and improves solubility. Granules continue to swell as the temperature is increased to above the gelatinization range. Corn starch granules may

swell 30 times their original volume (Singh et al., 2003). As starch granules swell, they become increasingly susceptible to shear and disintegrate. Another reason for the cooking process in dry grind ethanol is for the sterilization of the mash to minimize bacteria growth. Alpha-amylase cannot break α-(l,6) linkages and bypass the branch points in amylopectin to produce α-limit dextrins (Figure. 2.9) (Power 2003).

Enzymatic hydrolysis may be affected by starch granule size, shape and amylose content (Tester et al., 2006). The efficiency of absorption of the enzyme and the surface area accessible by enzymes critically affects kinetic parameters (Bertoff and Manelius, 1992). The hydrolysis of different starches by amylases proceeds in an initially rapid hydrolysis stage followed by a progressively slow or constant hydrolysis rate. The two-stage hydrolysis is more pronounced at higher enzyme concentrations (Bertoff and Manelius, 1992; Kimura and Robyt, 1995; Planchot et al., 1995). The hydrolysis rates at the initial stage are similar in waxy and normal maize starch, large granules of barley and wheat starches, but initial hydrolysis products differ (Bertoft et al., 2000). Waxy starches are usually hydrolysed faster than normal starches (Bertoft and Manelius, 1992; MacGregor and Balance, 1980). Among tuber and root starches, potato, yam, and taro starches are more resistant to amylases (Hoover, 2001; Valetudie et al., 1993). Most cereals (A-polymorph) starches are more readily hydrolysed than amylomaize and potato (B-polymorph) starches (Oates, 1997; Planchot et al., 1995).

The hydrolysis by a certain enzyme influenced by the diversities in composition and structural features of starches within species includes amylose-amylose and amylose – amylopectin interchain association (Vasanthan and Bhatty, 1996), amylose-lipid complex (Appelqvist and Debet, 1997; Lauro et al., 1999; Morrison, 1995), and type and

degree of crystallinity (Gerard et al., 2001). pH and temperature can affect liquefaction and saccharification processes. Each enzyme has an optimal pH (Mathewson, 1998). A change in slurry pH can affect the amino acid's ionization. When charges on the amino acids change, hydrogen bonding within the protein molecules change as does the molecular shape. The new protein shape may not be effective in attaching and interacting with the substrate. More damage among the molecules can be caused by too high a temperature. More damage increases the likelihood that the substrate will collide with the active site of the enzyme, increasing the rate of an enzyme catalyzed reaction. Above a certain temperature, the activity begins to decrease due to denaturation of the enzyme (Mathewson, 1998)

2.3 Bioethanol production

2.3.1 Bioethanol overview

Ethyl alcohol, also known as ethanol, is a colorless, flammable, volatile liquid that is widely used to produce beverages, solvents, and fuels. Ethanol is produced either synthetically through the hydration of ethylene (petrochemical) or biologically through yeast fermentation of simple carbohydrates (bioethanol) (Mills and Ecklund 1987). Bioethanol is considered the most utilized biofuel in the globe.

Ethanol can be produced from starchy crops or sugar-containing plants. Currently the feedstocks used for fuel ethanol production include corn, sugarcane, sugar beets and sorghum, but almost exclusively from corn in the United States and from sugarcane in Brazil (Gnansounou, 2009; Sanchez and Cardona, 2008). The worldwide production in 2008 was estimated 67.5 billion liters of ethanol; and USA was the leading producer (36 billion liters) followed by Brazil (22 billion liters). In 2015 the worldwide production of

bioethanol went up to 95 billion galons (Baier, et al. 2009; Renewable Fuel Association, 2016). As a result, bioethanol is considered as a bio-renewable source of energy and is anticipated to be one of the dominationg biofuels in the transport industry in the next few years (das Neves, et al., 2007; Rosillo-Calle and Walter, 2006).

In Western Canada, bioethanol is mainly produced from wheat and corn. The use of these two grains in bioethanol production may influence the availability of them for food. On the other hand, triticale and barley are less utilized cereals (Davis-Knight and Weightman, 2008; Gibreel et al., 2009; and Wang et al., 1997), and may be considered sources for bioethanol production with less impact on food markets. Triticale is especially a good candidate for ethanol production on marginal lands.

2.3.2 Current technologies of bioethanol production

2.3.2.1 First generation bioethanol

The first generation ethanol plants are divided into two types. The first ones are called sugar-based plants because they mainly utilize sugar from sugarcane and are mostly located in Brazil. The second type is called “starch-based ethanol plants” and are generally utilize grains and specially corn. These latter facilities are mostly predominant in the USA followed by other countries such as Canada, China, France, Germany and Sweden (Lennartsson, et al., 2014)

There are two major traditional industrial processes for producing bioethanol from cereal grains: “wet milling process” and “dry-grind” process. Wet milling formerly dominated as the method of ethanol production in the United States, but the dry-grind process is now the most widely used industrial method and represents >70% of the

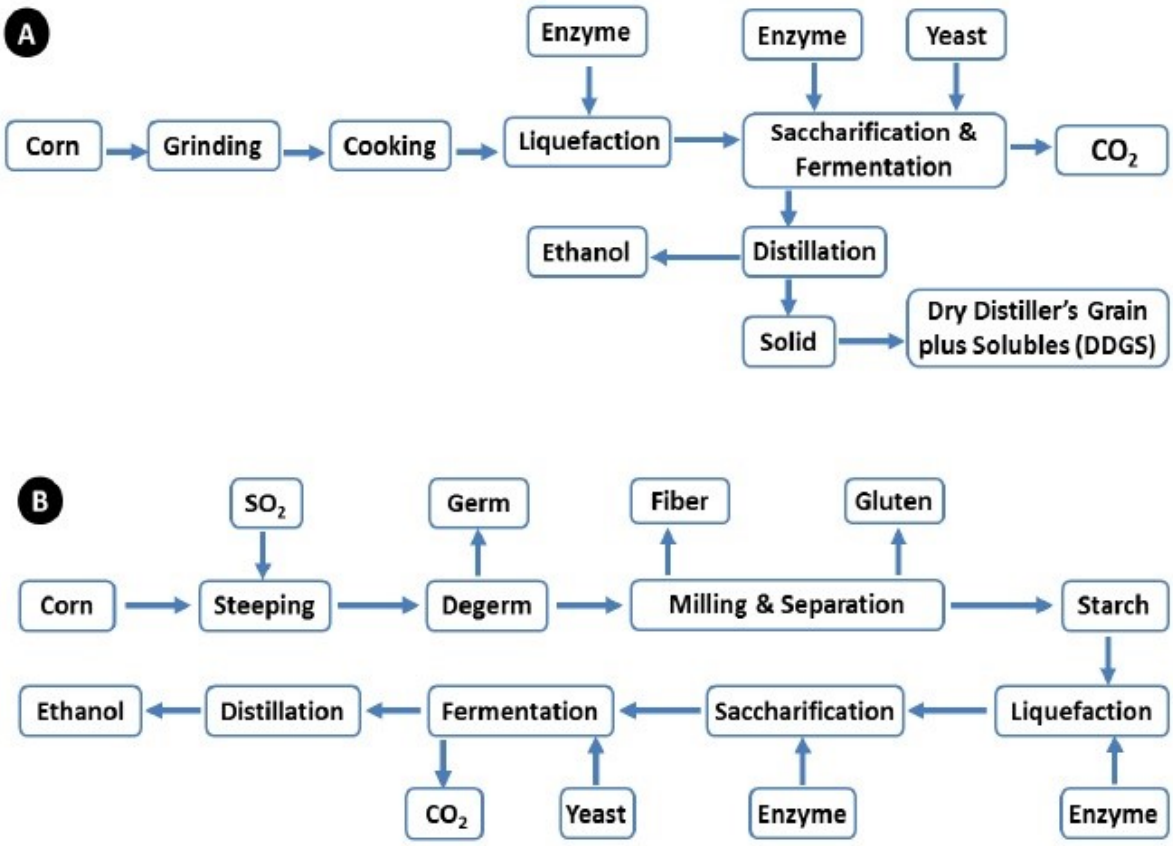


Figure 2.10 Representations of (A) dry-milling and (B) wet-milling processes involved in bioethanol production. (Adapted with minor modification from Naik. 2010) with permission of Elsevier Ltd.

ethanol processing (Moseir and Ilelej 2006; Naik, et al., 2010; Tiffany and Eidman 2005). Figure 2.10 presents the differences between dry-milling and wet-milling processes. In the dry grinding technique, dry ground grain flours are fermented straight to ethanol. The only co-product, distiller's dry grains with soluble (DDGS), is sold as animal feed. DDGS, which consists of the dried residual materials from the fermentation, contains the residual starch which escaped amylolysis hydrolysis, the non-fermentable parts of the grains and the yeast produced during the fermentation (Berg, 2004; Naguleswaran, 2013). In wet milling, corn kernels or any other whole grains are fractionated into each of their major individual components: starch, gluten, germ and fiber .This imparts two very important advantages compared to dry grind. First, the parts of the corn can each be marketed separately. So the germ is used to produce corn oil. The gluten is sold as high protein feed to the poultry industry, and the fiber is combined with liquid streams, dried and sold as a low- protein animal feed. Second, the wet mill produces a pure starch stream, which allows for the starch to be made into numerous different products. In addition to being fermented to ethanol, the starch can be modified for use in textile, paper, adhesives or food (Berg, 2004).

Starch bioconversion into fermentable sugars is generally achieved in two steps: liquefaction and saccharification. As shown in Figure 2.10, liquefaction by α-amylase in the dry-milling process is used to turn the insoluble starch into soluble dextrins. This step can be done separately, or in conjunction with cooking in a process called jet cooking process (Chen, et al., 2008). Saccharification is a process that turns the liquefied starch or dextrins into fermentable sugars which can be fermented by yeast (*Saccharomyces cervisiae*) into ethanol (Naguleswaran, 2013; Power, 2003).

Depending on the milling process, starch hydrolysis and fermentation steps are carried out in three different configurations:

1) Separate hydrolysis and fermentation (SHF): Both starch hydrolysis and fermentation steps were done separately in distinct reactors as shown in Figure 2.10b (Balat, 2009; das Neves, et al., 2007; Naguleswaran, 2013). After starch is completely hydrolyzed in the first reactor by liquefaction and saccharification process, the fermentable sugars are then transferred to a second reactor in which yeast is added to the mixture for fermentation. One of the benefits of this configuration is that there are no interactions between starch hydrolysis and sugars fermentation processes, making it more flexible with a higher ethanol yield than the other configurations (das Neves, et al., 2007). The disadvantages are the inhibition of amylase activity due to the accumulation of sugars resulting in a reduction in the ethanol yield, and longer time and higher level of energy neede to complete the production (Balat, 2009; das Neves, et al., 2007; Naguleswaran, 2013; Vinh, 2003).

2) Simultaneous Saccharification and Fermentation (SSF): This configuration is similar to the SHF configuration by having two reactors, but in the SSF configuration, both saccharification and fermentation steps are combined and carried out in the second reactor as illustrated in Figure 2.10a. Thus, the sugars produced during saccharification are simultaneously fermented by yeast. The major advantages of this configuration over SHF is that the ethanol yield is higher due to the high conversion rate, and saves time up to 25% (Balat, 2009; das Neves et al., 2007; Naguleswaran, 2013 Vinh, 2003).

3) Raw-starch Hydrolysis and Fermentation (RHF): This technique is called the "cold–cook" process, and it is different from the SHF and SSF techniques in that it uses

improved granular starch hydrolyzing enzymes (GSHE) that not need cooking at high temperature (90-95˚C) in order to convert the starches into sugars (Gibreel, et al., 2009; Gibreel et al., 2011; Reed, 2012). The whole operation of the RHF technique can be performed in one reactor under low temperature (<58˚C) conditions. First, a short incubation time is important to produce enough sugars to activate the yeast cells and multiply before introducing them into the reactor. The GSHE, such as Stargen 001™ and Stargen 002™ developed by Genencor International (USA), are a mixture of both α-amylase and amyloglucosidase that were produced from genetically engineered microorganisms. The benefits of using these enzymes are that they improve the productivity, lower the energy consumption, give higher yield, and saves on capital expenses by reducing the number of unit operations (Genencor International, 2009). Also, the co-product (DDGS) is rich in protein, and has other high value products such as tocopherols, tocotriols and fatty acids due to the low temperature used during the process (Gabreel et al., 2009; Gibreel et al., 2001; Naguleswaran, 2013).

2.3.2.2 Second generation technologies

Second generation bioethanol production utilizes lignocellulosic materials as input. Lignocellulosic biomass has been suggested as the most promising alternative to the traditional starch feedstock. Lignocellulosic feedstocks have the best well-to-wheel assessment, considering its abundance, low cost and high polysaccharides (cellulose and hemicellulose) content (Fujii et al., 2009). Intensive research and developments in the last decades on lignocellulosic materials will most likely make them important feedstocks for ethanol production in the future (Taherzadeh et al., 2007).

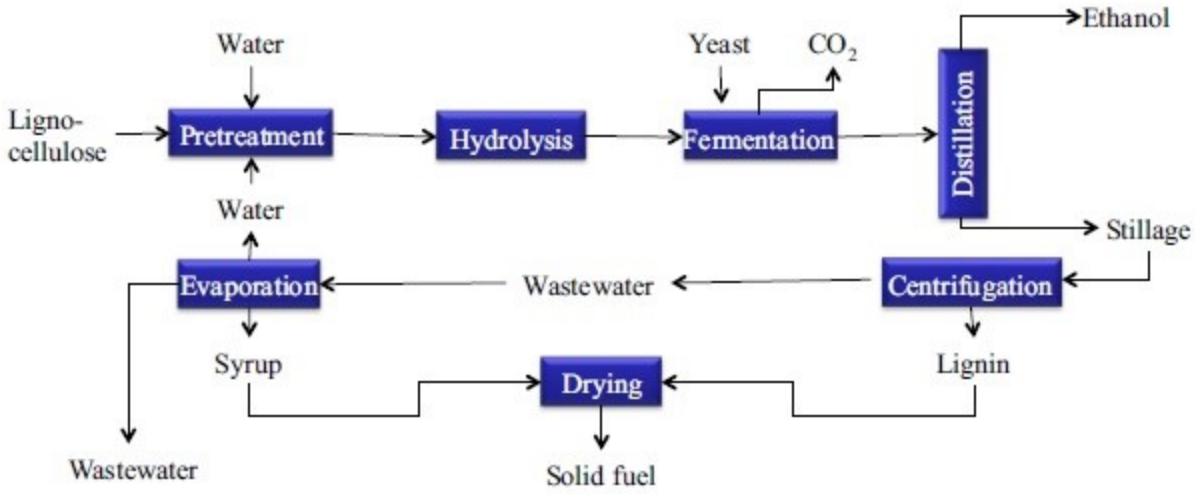


Figure 2.11 Outline for a second generation ethanol process (Adapted with minor modification from Lennartsson, et al., 2014) with permission of Elsevier Ltd.

The currently available approach for converting lignocellulosic feedstock into ethanol applies a complex and expensive multi-step process that combines thermochemical and biological methods in large, centralized processing plants. As shown in Figure 2.11, lignocellulosic conversion involves three basic steps: (1) pretreatment of raw feedstock to increase the accessibility of enzymes to the polysaccharides (cellulose and hemicellulose); (2) enzymatic hydrolysis to break down the lignocellulose constituents (polysaccharides) into a mixture of fermentable sugars; and (3) microbial fermentation, mediated by bacteria or yeast, to convert these sugars to ethanol. Making the transformation of lignocellulose to ethanol more economical and practical will require the development of molecular redesign of numerous enzymes, biochemical pathways,and full cellular systems (Lennartsson et al., 2014).

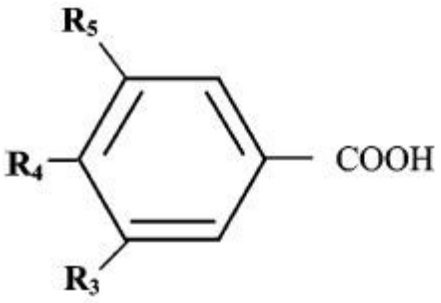
Ethanol production from lignocellulosic biomass using enzymatic hydrolysis and fermentation can be improved by: (1) development of effective pretreatment technologies that do not require expensive chemicals and/or high pressure equipment; (2) maintaining a high density of cells within the reactor to convert sugars to ethanol quickly; (3) integrating enzymatic hydrolysis of cellulose and hemicellulose with fermentation to keep sugar levels low, improving enzymatic conversion rates by minimizing the product (sugar) inhibition; (4) converting both the cellulose (glucose) and hemicellulose (xylose) to ethanol to increase the overall ethanol yield; (5) co-producing crude cellulase enzyme and/or recycle the enzymes so as to reduce enzyme costs; (6) incorporation of low temperature separation of ethanol from the reactor broth so as to keep fermentation reaction rates high, and allow recycle of enzymes without thermal destruction (Dale and Moelhman, 2005).

2.4 Phenolic compounds

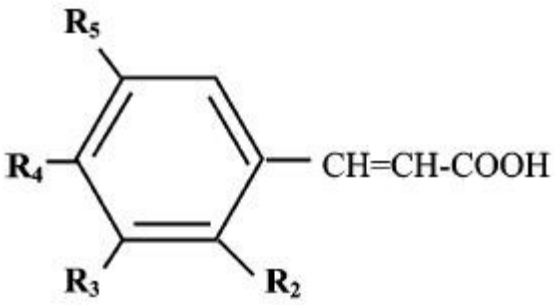
2.4.1 Chemical structure and properties

Cereal grains contain phytonutrient components such as phytates, phytoestrogens, phenols, lignins, and antioxidants. These biologically active components are generally found in the bran and germ of the grains, while the endosperm is mainly starch (Fulcher and Rooney-Duke, 2002; Slavin et al., 1999). Phenolic acids are structurally comprised of an aromatic ring to which one or more hydroxyl groups are attached and they can be categorized into two groups according to their chemical structure. These two groups are hydroxycinnamic and hydroxybenzoic acids, which generally are similer in their chemical structures but are distinguished by the numbers and positions of the hydroxyl groups on the aromatic ring (Khan and Shewry, 2009). The hydroxycinnamic group includes ferulic, *p*-coumaric, caffeic, and sinapic acids, while the hydroxybenzoic group includes gallic, vannilic, syringic, *p*-hydroxybenzoic, and protocatechuic acids (Liu, 2007; Pirjo et al., 2005) (Figure 2.12).

Phenolic acids usually exist as free acids, esterified and insoluble bound phenolics, and are concentrated in the pericarp, testa and aleurone layers. These compounds exist as glycosides linked covalently through ester and ether bonds to various sugar compounds or as complexes linked to anthocyanidins, polypeptides, amino acids, organic acids, and other phenols. The concentration of phenolic compounds in whole grains in influenced by grain type, variety, and also the part of the grain sampled (Adom and Liu, 2002; Adom et al., 2005; Clydesdale and Francis, 1976; Parker et al., 2005). The bound or insoluble phenolic acids appear to be associated with the cell walls of the grain. The most predominant phenolic acids detected in whole grains and beans are ferulic and



Benzoic acid derivatives
m-Hydroxybenzoic acid, R_3=OH
P-Hydroxybenzoic acid, R_4=OH
Protocatechuic acid, R_3=R_4=OH
Gallic acid, R_3=R_4=R_5=OH
Vanillic acid, R_3=OCH_3; R_4=OH
Syringic acid, R_3=R_5=OCH_3; R_4=OH



Cinnamic acid derivatives
O-coumaric acid, R_2=OH
m-coumaric acid, R_3=OH
P-coumaric acid, R_4=OH
Caffeic acid, R_3=R_4=OH
Ferulic acid, R_3=OCH_3; R_4=OH
Sinapic acid, R_3=R_5=OCH_3; R_4=OH

Figure 2.12 Phenolic acids of cinnamic and benzoic acid groups (reprinted with minor modification from: Pirjo et al., 2005) with permission of ACS Publications.

p-coumaric acids (Abd El Aal et al., 2001; Doherty et al., 1983; Luthria and Pastor-Corrales, 2006; Maillard and Berset, 1995; Sosulski et al., 1982). Ferulic acid can exist in the free, soluble-conjugated, and bound forms in whole grains. Studies showed that over 93% of the total content of ferulic acid in whole wheat, corn, oats, and rice grains is present in the bound form and the ratio of free, soluble-conjugated, and bound ferulic acid in wheat and corn was 0.1:1:100 (Adom and Liu, 2002).

Phenolic acids are generally known for their antioxidant properties especially ferulic acid and its conjugates (Garcia-Conesa et al., 1999). The antioxidant activity of phenolic acids is due to the free hydroxyl group attached to the benzene ring, which can donate a hydrogen atom to sequester free radicals. The resulting phenoxy radical is stabilized by rebound delocalization of charges on the benzene ring and the attached carbon-carbon double bond in conjugation with the ring (Garcia-Conesa et al., 1999; Kikuzaki et al., 2002). The electron donating methoxyl group increases the rebound stability and hence the antioxidant activity (Chen and Ho, 1997; Graf, 1992; Pekkarinen et al., 1999). In addition, the carboxylic acid group close to the carbon-carbon double bond can contribute to the stability and also provide additional attack sites for more free radicals (Graf, 1992).

2.4.2. Extraction and hydrolysis

The extraction of phenolic acids is challenging due to their structure and also their existence in multiple forms as mentioned before. A number of studies have been done to separate and identify phenolic acids. Fruits and vegetables are often cited as excellent sources of antioxidants whereas grains tend not to be mentioned due to their relatively low content and low level of antioxidant activity as reported in the literature. However,

most studies have under represented the phenolic levels of grains, since soluble phenolics are generally extracted using various aqueous solutions of methanol, ethanol and acetone (Liyana-Pathirana et al., 2006; Zhou and Yu, 2004). Recent work has shown that grains have vastly greater levels of phenolics than previously thought due to methodology improvements that measure phenolic acids in all their forms: free, esterified, and insoluble-bound. Liyana-Pathirana et al., (2006), Naczk and Shahidi (1989) evaluated free, esterified, and bound phenolics in soft and hard wheats that were fractionated into whole wheat, refined flour, and bran. The results indicated that not only the majority of phenolic compounds are present in the bran fraction, but the majority (>80%) are in bound form. The choice of methods for extracting and determining phenolic acids is affected by the different chemical properties and the conditions of extraction and hydrolysis. Soluble phenolic acids have been extracted using mixtures of methanol or ethanol, acetone, and/or water (Adom and Liu, 2002; Kim et al., 2006), cold or hot methanol (Escarpa and Gonzalez, 2000), or mixtures of methanol, water, and acetic acid (Awad et al., 2000; Lewis et al., 1998; Liyana-Pathirana et al., 2006).

The sample extract used to obtain free phenolics is then treated with acid or base to break the ester bonds attaching mainly ferulic acid (up to 95% of esterified phenolics) to sugars. Researchers have used 2M NaOH (Adom et al., 2005; Kim et al., 2006), 4M NaOH (Liyana-Pathirana et al., 2006), or multiple sequential levels from 0.1M to 4M NaOH (Parker et al., 2005) for different lengths of time, followed by neutralization or acidification with HCl. Acid hydrolysis has been also used to liberate bound phenolic acids (Hahn et al., 1983; Horvat and Senter, 1980). Acidic and alkaline hydrolyses are used to cleave the ester bond in separation and characterization of specific phenolic

compounds (Rommel and Wrolstad, 1993). The hydrolysis conditions, acid or alkaline only, or in different sequence, can significantly affect the total yield and profile of phenolic acids obtained (Kim et al., 2006). Kim et al. (2006) also found degradation of cinnamic acid derivatives, *p*-coumaric, caffeic and ferulic acids when heat was used during acid hydrolysis.

Insoluble bound phenolics in grains are freed using similar alkaline and acid hydrolysis, except this time the extractions are done on the previously extracted material. The main goal is to break ester bonds between ferulic acid and sugar structures (mostly arabinoxylan) and ether bonds between ferulic acid and lignin to free the phenolics for extraction. Because ferulic acid forms both of these types of bonds (Liyana-Pathirana et al., 2006) it may link xylan and lignin structures (Klepacka and Fornal, 2006), making it more difficult to extract. Results all agree that the majority of phenolic acids are in insoluble bound form concentrated in the bran material; this increases the importance of the particle size of the starting material to impact the final hydrolysis results. Because ferulic acid is a structural part of insoluble cell wall material, reducing the cell walls into smaller pieces would act to improve the extent of hydrolysis.

Due to the complexity of the digestion process, it is difficult to quantify the bioavailability and health benefits of bound phenolics in whole grains and bran. Bound phenolics have physiological activity, but this activity is often measured once freed from their matrix. While free phenolics and some soluble bound phenolics are absorbed in the small intestine, Hemery et al. (2010) demonstrated that reducing bran particle size by grinding correlated to an increase in *in-vitro* phenolic acid bio-accessibility from once bound phenolics.

2.5 Factors affecting starch amylolysis

2.5.1 Pre-treatment technologies

Enzymatic hydrolysis of starch can be affected by many factors. Pretreatment technologies have been developed to increase the bioconversion of starch into bioethanol including mechanical methods such as milling and decorticating (Corredor, 2006 and Perez-Carrillo et al., 2012). The effect of particle size on starch hydrolysis is mainly related to the available surface area for enzymatic action. Many studies have been conducted to investigate the kinetics of starch hydrolysis for different types of grains by different types of enzymes. Flours from four cereal grains (maize, wheat, barley and oats) have been evaluated for their *in-vitro* starch hydrolysis against the effect of different processing techniques (extrusion, cooking and grinding (0.8 and 3.0mm opening)) and the results show that the reduction of particle size increased the starch hydrolysis of the raw flours before treatment. Also, technological processing has improved the starch hydrolysis of the tested flours (Anguita et al., 2006). Wheat starch was isolated and fractionated into large (>15μm) and small granules (<10μm) by Yonemoto et al. (2007) and then were studied for their enzymatic susceptibility. They found that small granules were more susceptible to hydrolysis than large granules which was related to the larger surface area of smaller granules. Wu and Miao (2008) used wet milling for 3 h to micronize and prepare different samples of corn flour with different particle sizes from 273.6 μm to 17.5, 15.4, 14.6, 13.3 and 9.8 μm, and then liquefaction and saccharification of the flour samples were performed by using α-amylase and glucoamylase, respectively. The enzymatic hydrolysis of corn flour increases with greater wet-milling time, which is subsequently attributed to the decrease of the particle size of the starch granules. Al-

Rabadi et al. (2009) revealed that milling cereals (barley and sorghum) to a particle size ranging from ~0.1 to ~3 mm affected starch hydrolysis with porcine alpha-amylase and the rate coefficient for hydrolysis showed a decrease with increasing the size of particles.

Corredor et al (2006) investigated the effect of sorghum decortication as a pretreatment technique on ethanol production. Eliminating fibre and germ before hydrolysis allowed for greater amount of starch to be hydrolysed and ready for fermentation. In addition, some studies used protease treatment besides decortication (Perez-Carrillo and Serna-Saldivar, 2007; Perez-Carrillo et al., 2008 and Pere-Carrillo et al., 2012) on sorghum. The results showed that both sorghum decortication and protease treatments during liquefaction improved the conversion of starch into bioethanol, and they recommended using a combination of both treatments to reduce the time required for yeast to ferment starch hydrolysates.

Several studies have investigated the effect of heat treatment (cooking) and cooking time on *in vitro* starch hydrolysis. Sorghum and maize were cooked at different times and it was shown that cooking increased starch digestion by alpha-amylase, but decreased by increasing cooking time. Pressure-cooking also increased starch digestion probably through physical disruption of the protein matrix enveloping the starch (Ezeogu et al., 2005 and Ezeogu et al., 2008). Shariffa et al (2009) investigated the effect of mild heat treatment (35˚C for 24 h) against the capability of tapioca and sweet potato starches to enzymatic hydrolysis with a mixture of fungal α-amylase and glucoamylase. The degree of hydrolysis of the starches after heat-treatment showed a large increase in the hydrolysis. This could be attributed to the effect heat on the weaker areas on the starch granule allowing the enzyme to degrade the granules more expansively. It was also found

that heat treatment of starch at 100% humidity could enhance the adsorption of α-amylase. Heat-moisture treatment was used in another study at different temperatures (80, 100, 120 and 130˚C) for 16 h and a moisture content of 27% to determine the impact of these temperatures on the degree of starch hydrolysis by porcine pancreatic α-amylase. The results showed that the heat treatment changed the starch structure from B-type crystallites into A+ B-type crystallites, which affected the physicochemical properties of the starch granules at different temperatures resulting in an increase of the susceptibility of the starches towards α-amylase at 100 to 130 ˚C (Varatharajan et al., 2011). Urea was used in another study with heat treatment to investigate its influence on the amylolysis of triticale and corn starches (Li et al., 2012). Addition of urea did not significantly affect starch liquefaction and sacharrification at 30˚C. However, increasing the temperature to 50˚C for triticale and 61˚C for corn for 30 min, urea had enhanced the amylolysis of the starches by increasing both the initial hydrolysis rate and extent. Liu et al. (2015) studied the effect of heat-moisture treatment and annealing on the *in vitro* starch digestibility of buckwheat starch; they showed that the hydrolysis of heat treated samples decreased with increasing moisture level. Tawaba et al. (2015) used NaOH as a pretreatment of starch isolated from red sorghum and then studied the influence on starch hydrolysis by α- and β- amylases. The results revealed that NaOH significantly improved starch hydrolysis and the digestibility of the extracted starch was greater than that of the control flour.

Many other pretreatment technologies were demonstrated in several studies to have a better understanding of how to enhance the starch amylolysis such as supercritical-fluid extrusion (Zhan et al., 2006), electron beam irradiation (Shin and Sung, 2008) and ultrasound technology (Shewale and Pandit, 2009).

2.5.2 Non-starch components

2.5.2.1 Phenolic compounds

Phenolic compounds, which are present in the seed coats of cereal grains, are one of the most important non-starch components which affect the degree of starch hydrolysis. Polyphenols can inhibit enzyme activities such as α-amylase, α-glucosidase, β-amylase and other digestive enzymes, and this subject has been studied extensively (Funke, 2005; Lordan et al., 2013; Narita and Inouye, 2009; Rohn et al., 2002; Tawaba et al., 2015; and Worsztynowicz et al., 2014). Tannic acid in concentrations 0-3% was used to study its effect on the activity of alpha and beta amylase during germination of a low tannin sorghum variety and it was found that tannins delay starch hydrolysis by inhibiting the amylases activity (Chukwura and Muller 1982). However, Tong et al. (2014) used tannins isolated from *Eugenia jambolana* (traditional herbal tea) to investigate the effect on α-amylase activity. Results showed that using 0.125 mg of tannins on wheat flour was effective to retard enzymatic starch digestion moderately (Tong et al., 2014). The inhibitory effect of different polyphenolic compounds (luteolin, tannic acid, caffeic acid, chlorogenic acid, isochlorogenic acid, ferulic acid, gallic acid, dihydroxybenzenes, quinic acid and benzoquinone) extracted from anti-diabetic tea species on α-amylase, trypsin and lysozyme activity was investigated *in vitro*. These phenolic compounds have the ability to form quinones, which increase their reactivity towords α-amylase; they assumed that the enzyme activity decreased depending on the concentration of the compounds and the position of hydroxylic groups (Funke, 2005; and Rhon et al, 2002). Narita and Inouye (2009) studied the effect of chlorogenic acid and its components (caffeic acid, and quinic acid) from green coffee beans on the activity of two porcine

pancrease α-amylase enzymes. Both chlorogenic and caffeic acids were almost the same in inhibiting α-amylase isozymes and they were then analyzed by kinetic analysis. However, quinic acid was a poor inhibitor and it was difficult to analyze the interaction between the enzyme and the inhibitor.

Phenolic compounds from chickpea and horsegram were found in high concentrations in seed coats and cotyledon. Michaelis-Menten and Lineweaver-Burk derivations kinetic studies revealed that phenolic compounds from seed coat inhibit α-amylase activity by mixed non-competitive inhibition mechanisms for both chickpea and horse gram (Sreerama et al, 2010). Free and bound polyphenol extracts of jute leaf (*Corchorus olitorius*) were used to characterize the inhibitory action of them on α-amylase, α-glucosidase and angiotension I converting enzyme (ACE). Reversed-phase HPLC analysis revealed that chlorogenic acid (7.5 mg/mg) and isohamnetin (51.1 mg/100g) were the main free phenolic acids in the extract while caffeic acid (58.1 mg/ 100g) was the main bound phenolic acid in the extract. Both free and bound phenolic extracts inhibited α-amylase and α-glucosidase (12.5-50.0 µg/ml), and ACE (10.0-50.0 µg/ml) in a dose-dependent manner, with free extracts having significantly higher α-amylase (17.5 µg/ml) and α-glucosidase (11.4 µg/ml) and ACE (15.7 µg/ml) inhibitory activities (Oboh et al, 2012). The effect of extracted phenolic compounds on the degree of hydrolysis of isolated red sorghum starch was investigated, showing that starch hydrolysis is a log-log function of the added phenolic compounds, negatively affecting both the hydrolysis rate and the extent of maximum hydrolysis (Tawaba et al. 2015). In addition to the above research, polyphenol extracts from different plant sources (pomegranate, irish seaweed, sesame cake, cranberry bean, black chokeberry, green tea

and black tea) were used as inhibitors against α-amylase and α-glucosidase in several studies (Chiang et al, 2014; Kam et al, 2013; Lordan et al, 2013; Reshma et al, 2013; Worsztynowicz et al, 2014; and Yang and Kong, 2015). Zhu, (2015) investigated the interaction between phenolic compounds and starch, and revealed that the physicohoemical and nutritional properties of starch may be impacted by the non-covalent interactions between phenolic compounds and starch, such as amylose single helices facilitated by hydrophobic effect, or complexes with much weaker binding mostly through hydrogen bonds. The effect of these interactions on the physicochemical properties and starch hydrolysis system depends on the type and the structure of both phenolic compunds and starch as well as the method of preparing the complex.

2.5.2.2 Phytic acid

Myo-inositol hexaphosphate, commonly known as phytic acid, is widely distributed in plant seeds and grains. Information about the impact of phytic acid on starch hydrolysis is limited. Cawley and Mitchell (1968) reported that phytate suppressed α-amylase activity in sprouted wheat meal by complexing the Ca++ ions necessary for enzyme activity. Sharma et al. (1978) observed that addition of Ca++ ions up to 8 mM had no effect on the activity of wheat, maize and bacterial α-amylase. These authors also reported that at concentrations above 10 mM, Ca++ ions significantly inhibited amylase activity with or without phytate. They attributed the negative effects of phytate on amylase activity to general phytate-enzyme protein interactions, and not to complexation of Ca++ ions by phytate. The effect of dehulling of ten cultivars of beans (*Phaseolus vulgaris* L.) on phytic acid and α-amylase inhibitory activity was investigated and it has been reported that dehulling significantly increased the phytic acid content as well as the

α-amylase inhibitory activity of all beans (Deshpande et al., 1982). Bjorck and Nyman, (1987) reported that the in vitro addition of phytic acid did not significantly influence the enzyme activity. However, α-amylase and amyloclucosidase were inhibited by tannic acid and catechin. Kunckles and Betschart (1987) studied the effect of phytate and myo-inositol phosphate esters on α-amylase hydrolysis of soluble potato starch and they revealed that by using 2 mM phytate at pH 4.15 and 2 mM myo-inositol-2 monophosphate reduced starch digestion with salivary α-amylase to 8.5 and 78.3%, respectively, of the control (Knuckles and Betschart, 1987). Asghar et al. (2013) used phytic acid and different concentration of metal ions (Cu^{++}, Al^{+3}, and V^{+4}) to study their inhibitory effects on the activity of polygalacturonase. There was an increase of the inhibition rate of the enzyme activity with the increase of the concentration of phytic acid used, and also phytate-metalic complexes inhibited the enzyme activity to some extent, but significantly lower than using each of them alone

Chapter 3

Compositional changes in whole grain flours as a result of solvent washing and their effect on starch amylolysis[1]

3.1 Introduction

Cereal grains represent the major source of starchy raw material for ethanol production in the bio-fuels industry, due to their abundance and relatively low cost (Bothast and Schlicher, 2005; Molovic et al., 2009). Ethanol production from grains involves the amylase hydrolysis of starch in ground material to glucose, yeast fermentation of glucose to ethanol, and ethanol distillation and dehydration. Efficient conversion of starch into glucose is a critical determinant of process efficiency and ethanol yield. One of the challenges in obtaining efficient starch hydrolysis is to overcome the negative effects of non-starch grain components, such as protein, lipid, pentosan, phytic acid, phenolics and β-glucan. The contents, structures and interactions of these components differ among grains and are very complex. Another challenge arises from the presence of starch that is resistant to hydrolysis due to the relatively coarse particle size of whole grain flours and to the structural heterogeneity and crystalline nature of starch molecules, i.e. amylose and amylopectin, in grains. These challenges need to be understood and mitigated if grain flours are to rival pure starch as the ideal raw material for industrial ethanol production.

[1]A version of this chapter has been published as Kandil, A., Li, J., Vasanthan, T., Bressler, D. C. and Tyler, R. T. 2011. "Compositional changes in whole grain flours as a result of solvent washing and their effect on starch amylolysis" *Food Research International* 44 (2011) 167-173. I was responsible for the data collection and analysis as well as the manuscript composition. J. Li, D. C. Bressler and R. T. Tyler contributed to data collection and manuscript edits. T. Vasanthan was the supervisory author and was involved with concept formation and manuscript composition.

Research has been undertaken on hydrolysis of starch in grain flours using α-amylase and amyloglucosidase after various pretreatments, such as particle size reduction through milling (Wu and Miao, 2008), cooking at different temperatures and times (Ezeogu et al., 2008; Ezeogu et al., 2005), steam-flaking (Chuck-Hernandez et al., 2009), sonication (Shewale and Pandit, 2009), extrusion (Zhan et al., 2006), decortication (Corredor et al., 2006; Perez-Carrillo and Serna-Saldivar, 2007; Perez-Carrillo et al., 2008), protease (Perez- Carrillo and Serna-Saldivar, 2007; Wang et al., 2009), reducing agent (e.g. sodium bisulfate and 2-mercaptoethanol) (Choi et al., 2008; Ezeogu et al., 2008) and NaOH (Tawaba et al., 2015) treatments in order to improve and optimize the hydrolysis process. The influences of non-starch components on amylolysis are well documented (Singh et al., 2010; Tester et al., 2006). For example, the effects of addition of polysaccharides (Tester and Sommerville, 2003), lipid (Crowe et al., 2000; Cui and Oates, 1999; Lauro et.al., 2000), β-glucan (Faraj, 2004), phytic acid and polyphenols (Bjorck and Nyman, 1987) on the hydrolysis of pure starch have been described. However, there exist few, if any, reports on the effect of removal of non-starch grain components from whole grain flours by solvent washing on amylolysis of starch.

The non-starch components in the grain matrix interfere with the enzymatic conversion of starch to glucose during the ethanol production process. It was hypothesized that non-starch components can be partially or completely solubilized in various solvents and thereby removed from flour, the extent depending on grain source and composition, component structure and physical and chemical interactions, and extraction conditions. Efficient removal of these components should improve the efficiency of starch to glucose conversion. Furthermore, solvent washing of flours will

result in the extraction of potentially high value grain components, such as β-glucan, phenolics and lipids. The strategic selection of appropriate solvents to optimize both the hydrolysis of starch and the extraction of valuable grain components may enhance the commercial viability of ethanol production.

Barley and triticale are well adapted to Western Canadian environmental conditions and are considered economically favorable sources of carbohydrate for industrial end uses, and may be competitive alternatives to corn and wheat, the conventionally-utilized materials for ethanol production in North America and Europe. This study was carried out to improve our understanding of the impact of non-starch components in barley, triticale, wheat and corn flours on enzymatic hydrolysis of starch. The primary objectives were: 1) to examine the appearance (using SEM) and particle size distributions of whole grain triticale, barley, wheat and corn flours ground to pass a screen having an aperture size of 0.5 mm; 2) to study the effect of pre-washing using various solvents on the composition of whole grain flours; and 3) to compare the extent of starch hydrolysis by α-amylase and amyloglucosidase in whole grain flours, with and without solvent pre-washing.

3.2 Materials and methods

3.2.1 Materials

Four cereal grains were used in this study. Barley (*Hordeum vulgare* L. cv. Xena) was obtained from the Crop Development Centre, University of Saskatchewan, Saskatoon, SK, Canada. Corn (*Zea mays* L.) was supplied by Pioneer Hybrid Ltd., Chatham, ON, Canada. Canada Prairie Spring (CPS) wheat (*Triticum aestivum* L.) was provided by Alberta Agriculture and Forestry, Barrhead, AB, Canada. Triticale (x

Triticosecale cv. Pronghorn)was supplied by the Field Crop Development Centre, Alberta Agriculture and Forestry, Lacombe, AB, Canada. The grains (1 kg) were ground in a Retsch mill (Model ZM 100, Haan, Germany) using a ring sieve with an aperture size of 0.5 mm. Ground flours were stored in plastic containers at 5 °C. Liquozyme SC (α-amylase) (120 KNU/g) and Spirizyme Fuel (amyloglucosidase) (750 AGU/g) enzymes were kindly provided by Novozymes, Bagavaerd, Denmark. Pure starches were isolated from ground grain flours (0.5 kg) using the laboratory wet-extraction procedures of Mistry and Eckhoff (1992), Vasanthan and Temelli (2002) and Wolf (1965) for wheat and triticale, corn, and barley, respectively. In brief, the isolation involved preparing a dough or slurry with deionized water followed by dilute alkali washing to separate the protein-enriched fibre from the starch milk. The starch milkwas then subjected to centrifugation and water washing in order to obtain high purity starch.

3.2.2 Pre-washing using various solvents

Water, hexane, and 100% and 50% ethanol were used as solvents for pre-washing of the whole grain flours. Five grams of flour was suspended in 25 mL of solvent, shaken at ambient temperature for 1 h, and then centrifuged at 2200×g for 30 min. The residue in the centrifuge bottle was re-suspended and washed again as above, and then freeze dried (VirTis Model 50 SRC freeze dryer, SP Scientific, Warminster, PA, USA). Flours which were not solvent washed were used as controls. Each treatment was run at least in duplicate. After drying, the pre-washed flours were ground in a Braun coffee grinder (Model KSM2, Braun GmbH, Kronberg, Germany) and passed through a sieve with 0.5-mm openings. Solid loss was calculated as the difference in the dry weight of a sample before and after pre-washing.

3.2.3 Hydrolysis with α-amylase

One gram of flour (with or without pre-washing) was suspended in 10 mL of deionized water. The pH of the suspension was adjusted to 5.25 with 0.01 M HCl and then 1 μL of Liquozyme SC enzyme was added. After adjusting the volume to 20 mL, the suspension was incubated in a water bath at 85–90 °C for 30 min and then centrifuged at 2200×g for 20 min. Reducing sugars were analyzed using the DNS method described below and the degree of hydrolysis was calculated.

3.2.4 Sequential hydrolysis with α-amylase and amyloglucosidase

One gram of flour (with or without pre-washing) was suspended in 10 mL of deionized water. After adjusting the pH to 5.25 with 0.01 M HCl and addition of 1 μL of Liquozyme SC enzyme, the suspension was incubated in a water bath at 85–90 °C for 30 min and then cooled to 50 °C. The pH of the suspension was adjusted to 4.2 with 0.01 M HCl and 1 μL of Spirizyme Fuel enzyme was added. After adjusting the suspension volume to 20 mL and incubating at 60 °C for 30 min, the suspension was centrifuged at 2200×g for 20 min and the supernatant was used for determination of the degree of hydrolysis using the DNS method described below.

3.2.5 Scanning electron microscopy

Whole grain flour samples were mounted on circular aluminum studs with double-sided sticky tape, coated with gold to a thickness of 12 nm, and examined and photographed in a JEOL Model JSM 6301 FXV scanning electron microscope (JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 5 kV.

3.2.6 Particle size distribution

The particle size distributions of whole grain flours were determined using a series of Tyler sieves (32, 60, 80, 100, 200 and 325 mesh) on a sieve shaker (Model RX-812 CAN, W.S. Tyler, Mentor, OH, USA) for 10 min. Flour fractions with particle sizes of <45, 45–75, 75–150, 150–180, 180–250, 250–500 and >500 μm were collected from the sieves, weighed and expressed as weight percentages of the total flour weight.

3.2.7 Compositional analysis

Moisture and ash contents were determined according to Method 44-15A of AACC International (AACC International, 2004) and Method 923.03 of AOAC International (AOAC International, 2000), respectively. Protein content (%N×5.7) was measured with a Leco Carbon/Nitrogen determinator (TruSpec CN, Leco Corporation, St. Joseph, MI, USA). Neutral lipid was measured by the Goldfisch extraction method using petroleum ether (AACC International, 2004). Total starch, β-glucan and phytic acid contents were estimated according to the total starch assay, mixed-linkage β-glucan assay and phytic acid/total phosphorus assay procedures using kits purchased from Megazyme International Ireland, Ltd., Wicklow, Ireland. Pentosan content was determined according to the method of Hashimoto et al. (1987).

Total free phenolics were extracted by adding 2 mL of 80% methanol to 50 mg flour in a 10-mL flask, shaking the flask for 1 h, and then centrifuging at 2200×g for 15 min. The total free phenolics content of the extract was determined by the Folin–Ciocalteu method (Zhao et al., 2006). Acombination solution consisting of 1.0 mL sample extract, 2.0 mL 10% (v/v) Folin–Ciocalteu reagent, 2.0 mL 10% (v/v) sodium carbonate and 5.0 mL deionized water was prepared in a 10-mL volumetric flask and incubated at ambient temperature for 1 h. Absorbance was determined at 750 nm against

the blank. Gallic acid (0–100 μmoles/mL) was used as a standard and the total free phenolics content was expressed as gallic acid equivalents (GAE) in mg per 100 g flour (dry basis).

3.2.8 Determination of degree of starch hydrolysis

The concentration of reducing sugars in the supernatant of centrifuged samples was determined by the dinitrosalicylic acid (DNS) method (Bruner, 1964) and the degree of hydrolysis was expressed as the weight of glucose equivalents per 100 g dry starch. (See Appendix)

3.2.9 Statistical analysis

All chemical analyses and experiments were carried out in duplicate at least. One-way Analysis of variance (ANOVA) was performed using the General Linear Model (GLM) procedure of SAS Statistical Software, Version 9.1.2 (SAS Institute Inc., Cary, NC, USA, 2004). Multiple comparisons of the means were done using Tukey's test ($P<0.05$).

3.3 Results and discussion

3.3.1 Scanning electron microscopy

Whole cereal grains were ground into flours to facilitate hydration and enzyme access during hydrolysis. Figure 3.1 presents scanning electron micrographs of the flours, which were ground to pass a sieve with an aperture size of 0.5 mm. Starch granules were easily distinguishable in the micrographs. The granules appeared to be essentially free of other cellular constituents, other than a few adhering, small, irregularly shaped particles, probably pieces of protein matrix. Larger, irregularly shaped particles were visible in all flours. These likely were bran pieces or endosperm particles, i.e. starch granules

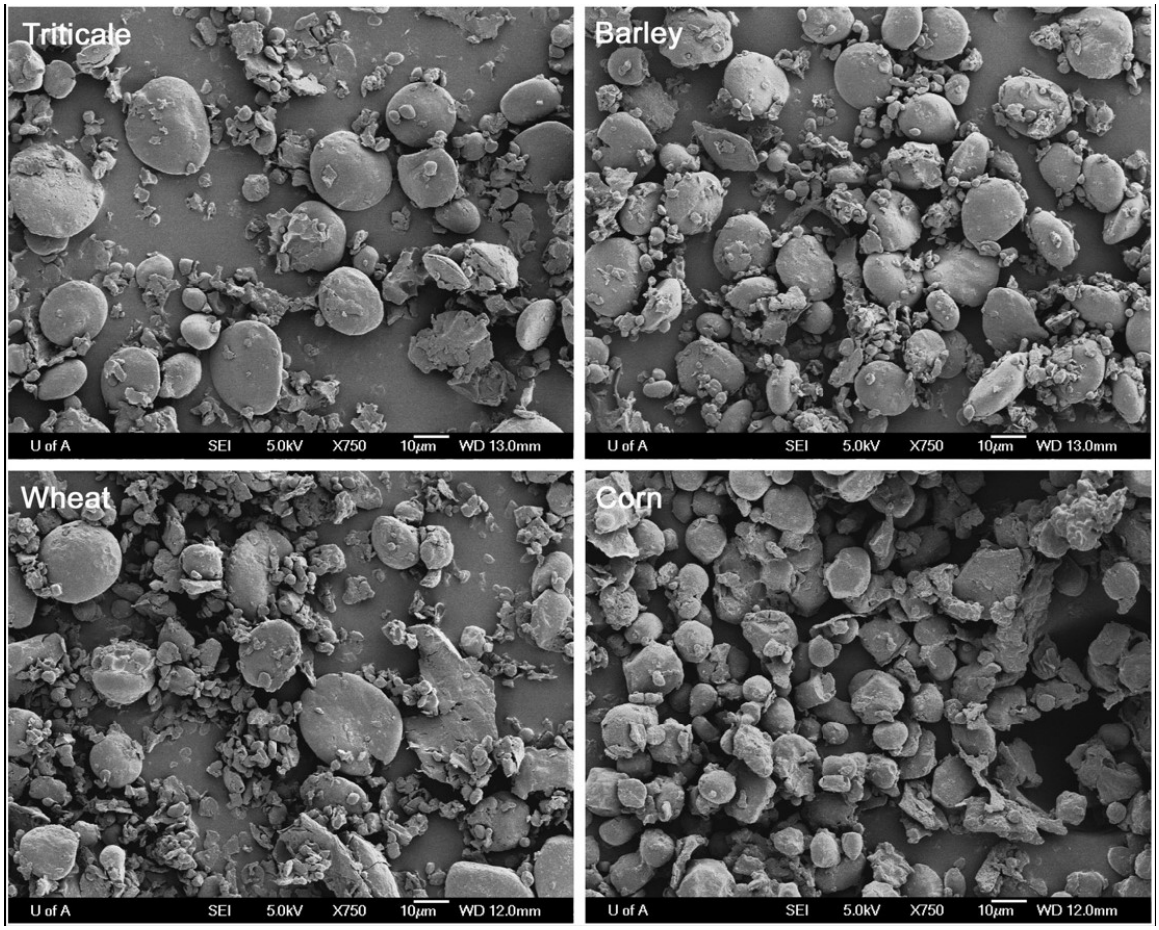


Figure 3.1 Scanning electron micrographs of grain meals

embedded in a protein matrix. Triticale, barley and wheat are known to contain two populations of starch granules, large and small. Small granules were difficult to distinguish in the three flours, as many would have been embedded in proteinaceous material as described above.

3.3.2 Particle size distributions of whole grain flours

The flours were ground to pass a screen with an aperture size of 0.5 mm. Triticale, wheat and corn flours had similar particle size distributions, whereas barley flour contained a much higher proportion of fine (<45 μM) particles (Figure. 3.2). Approximately 80% (w/w) of the particles in triticale, wheat and corn flours, and >99% of the particles in barley flour, were less than 250 μm in diameter. Al-Rabadi et al. (2009) reported that the components in particles less than 250 μm in diameter would be expected to be exposed, or completely released from broken endosperm cells, in barley and sorghum flours and the particles in this size range showed the highest *in vitro* starch digestion rates with porcine α-amylase. In the present study, scanning electron microscopy (Figure 3.1) revealed that with the milling equipment employed, grinding flours to pass a sieve with an aperture size of 0.5 mm effectively released starch granules from endosperm cells.

3.3.3 Composition of whole grain flours

Compositional analysis of the whole grain flours is presented in Table 3.1. Corn flour had the highest concentrations of starch, lipid and phytic acid, but the lowest concentrations of protein, ash, β-glucan and pentosan. Barley flour exhibited the highest concentrations of β-glucan and total phenolics. Wheat flour had the highest protein concentration. The concentrations of these non-starch components may affect the

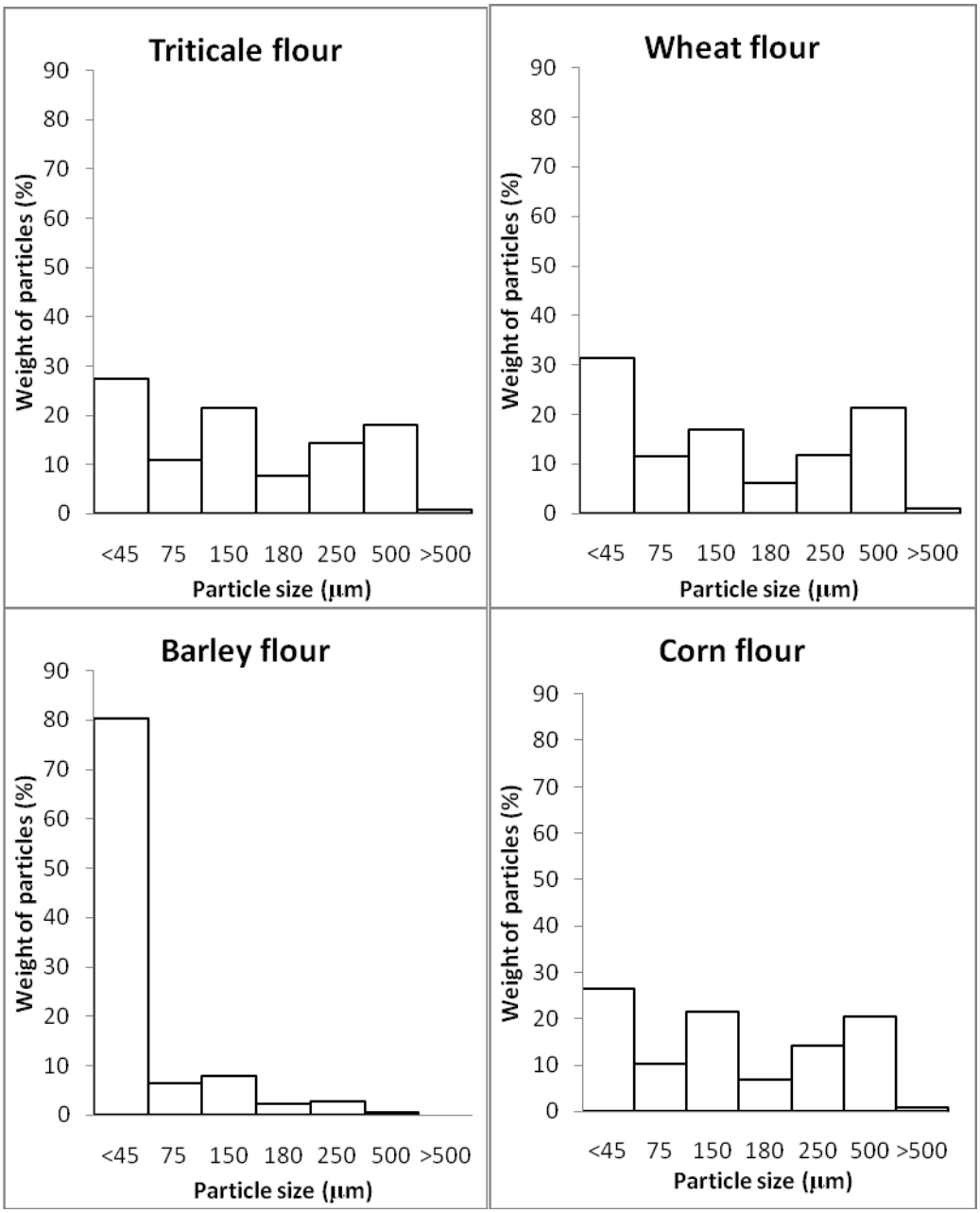


Figure 3.2 Particle size distribution of grain flours passed through 0.5 mm sieve.

Table 3.1 Composition of whole grain flours with and without solvent pre-washing (%, db).

Flour	Solvent	Starch	Protein	Lipid	Ash	β-Glucan	Phytic acid	Total phenolics (mg GAE/100	Pentosans
	Unwashed	68.87^{b}±0.08	11.73^{b}±0.02	1.27^{a}±0.18	1.95^{a}±0.02	1.17^{a}±0.04	0.62^{a}±0.09	592.16^{a}±2.91	8.47^{ab}±0.47
	Water	74.23^{a}±0.24	9.95^{c}±0.06	0.96^{a}±0.19	0.54^{c}±0.04	1.16^{a}±0.02	0.26^{c}±0.03	211.75^{d}±7.28	9.48^{a}±0.50
Triticale	**Hexane**	69.86^{b}±1.00	12.08^{a}±0.03	0.23^{b}±0.05	1.92^{a}±0.03	1.14^{a}±0.06	0.54^{ab}±0.00	537.52^{b}±1.45	8.18^{b}±0.41
	100% Ethanol	68.40^{b}±0.57	12.21^{a}±0.01	0.09^{b}±0.09	1.91^{a}±0.01	0.79^{b}±0.00	0.55^{a}±0.02	359.17^{c}±8.74	7.87^{b}±0.13
	50% Ethanol	74.39^{a}±0.63	8.06^{d}±0.13	0.95^{a} ±0.04	1.64^{b}±0.01	1.17^{a}±0.04	0.44^{b}±0.03	96.28^{e}±4.37	8.91^{ab}±0.51
	Unwashed	67.65^{b}±0.73	13.23^{a}±0.44	1.76^{a}±0.09	1.72^{a}±0.01	5.03^{a}±0.10	0.60^{a}±0.00	617.93^{a}±1.45	6.36^{b}±0.13
	Water	73.54^{a}±1.01	12.84^{a}±0.03	1.47^{b}±0.15	0.63^{c}±0.02	0.08^{c}±0.02	0.26^{d}±0.01	240.61^{d}±4.37	6.81^{ab}±0.15
Barley	**Hexane**	67.07^{bc}±0.20	13.41^{a}±0.04	0.30^{d}±0.02	1.73^{a}±0.00	5.06^{a}±0.09	0.50^{b}±0.01	526.18^{b}±5.83	6.26^{b}±0.17
	100% Ethanol	65.94^{c}±0.44	13.47^{a}±0.15	0.27^{d}±0.03	1.69^{a}±0.03	4.76^{b}±0.03	0.51^{b}±0.02	478.76^{c}±8.74	7.24^{a}±0.49
	50% Ethanol	72.79^{a}±0.33	8.39^{b}±0.39	1.22^{c}±0.11	1.42^{b}±0.04	5.05^{a}±0.04	0.46^{c}±0.01	73.60^{e}±4.37	6.60^{ab}±0.31
	Unwashed	65.52^{c}±0.14	14.80^{a}±0.25	1.08^{a}±0.27	1.78^{a}±0.04	1.20^{a}±0.04	0.69^{a}±0.00	587.0^{b}±7.28	8.69^{b}±0.21
	Water	68.29^{b}±0.50	13.93^{b}±0.05	1.01^{ab}±0.08	0.93^{d}±0.01	1.10^{b}±0.03	0.26^{e}±0.01	178.76^{d}±4.37	7.60^{cd}±0.16
Wheat	**Hexane**	66.47^{bc}±0.81	14.99^{a}±0.37	0.29^{b}±0.22	1.78^{a}±0.01	1.25^{a}±0.05	0.52^{c}±0.03	616.90^{a}±2.91	7.48^{d}±0.36
	100% Ethanol	66.87^{bc}±0.15	15.25^{a}±0.01	0.34^{b}±0.17	1.70^{b}±0.04	0.85^{c}±0.01	0.61^{b}±0.02	262.26^{c}±8.74	8.21^{bc}±0.38
	50% Ethanol	73.64^{a}±0.63	9.32^{c}±0.06	0.80^{ab}±0.23	1.60^{c}±0.00	1.23^{a}±0.01	0.46^{d}±0.04	83.91^{e}±10.20	9.64^{a}±0.15
	Unwashed	75.32^{a}±1.43	8.20^{a}±0.04	3.97^{a}±0.00	1.46^{a}±0.06	0.55^{a}±0.05	0.82^{a}±0.04	590.10^{a}±8.74	5.09^{b}±0.88
	Water	77.52^{a}±0.74	7.61^{b}±0.01	2.86^{b}±0.03	0.25^{c}±0.07	0.54^{a}±0.05	0.21^{d}±0.01	424.12^{c}±10.20	6.89^{a}±0.39
Corn	**Hexane**	75.66^{a}±1.54	8.19^{a} ±0.02	0.12^{c}±0.04	1.45^{a}±0.01	0.53^{a}±0.76	0.57^{b}±0.01	591.13^{a}±4.37	6.70^{a}±0.56
	100% Ethanol	75.62^{a}±1.31	8.51^{a}±0.40	0.35^{c}±0.38	1.42^{a}±0.00	0.22^{b}±0.04	0.61^{b}±0.02	529.27^{b}±1.45	6.84^{a}±0.22
	50% Ethanol	74.64^{a}±1.14	7.40^{b}±0.00	3.00^{b}±0.01	1.22^{b}±0.05	0.58^{a}±0.03	0.36^{c}±0.02	79.36^{d}±1.89	6.64^{a}±0.38

Each value in the table is the mean ± standard deviation of two replicates. For each flour, values in the same column with different letters are significantly different at $P < 0.05$. %Protein = %Nitrogen x 5.7. GAE = Gallic acid equivalents

efficiency and degree of starch hydrolysis in flours, and indicate the potential of recovering valuable products during solvent pre-washing of flours.

3.3.4 Effect of solvent pre-washing on the composition of flours

Washing of whole grain flours with water increased ($P<0.05$) the concentration of starch by 7.8, 8.7 and 4.2% in triticale, barley and wheat flours, respectively, but did not increase the concentration of starch in corn flour (Table 3.1). The pentosan concentration in corn flour increased ($P<0.05$) by 35.4%. In most cases, the protein, lipid, ash, β-glucan, phytic acid and total phenolics concentrations were reduced ($P<0.05$) in water-washed flours. The reduction in β-glucan concentration was particularly large with barley flour (by 98%). The total phenolics content was reduced markedly ($P<0.05$) in all flours. Overall, the effect of water-washing was to increase the concentration of starch in whole grain flours, while reducing the concentrations of most other non-starch constituents, in some cases substantially.

Hexane extracted much of the neutral lipid from the whole grain flours. Concentrations were reduced ($P<0.05$) by 82, 83, 73 and 97% in triticale, barley, wheat and corn flours, respectively. Extraction with hexane also reduced significantly ($P<0.05$) the concentration of phytic acid in barley, wheat and corn flours by 17, 25 and 30%, respectively and the total free phenolics concentration in triticale and barley flours by 9 and 15%, respectively, but had little or no effect on the concentrations of the other constituents.

The effect of washing with 100% ethanol was similar to that of washing with hexane with respect to the starch, protein, lipid, ash and phytic acid concentrations in the flours. The β-glucan and total phenolics concentrations in flours extracted with 100%

ethanol were lower ($P<0.05$) than those in hexane-extracted flours, as was the pentosan concentration in wheat flour

Washing with 50% ethanol reduced ($P<0.05$) the concentrations of protein (by 31, 37, 37 and 10%) and of total free phenolics (by 84, 88, 86 and 87%) in triticale, barley, wheat and corn flours, respectively, but had relatively little effect on the pentosan concentration, and did not reduce the β-glucan concentration, in any of the flours. The significant reductions in protein content observed with 50% ethanol washing of the flours were due to the presence of prolamins in these cereal flours, as prolamins are soluble in aqueous alcohol (Shewry and Halford, 2002). The protein concentrations were reduced to a greater extent in the Triticeae (i.e. triticale, wheat, barley) flours than in the Panicoideae (i.e. corn) flour, reflecting differences in the solubilities of prolamins in these two cereal groups (Shewry and Halford, 2002). Removal of protein from flour by solvent washing may weaken the protein matrix surrounding starch granules, which acts as a barrier to starch hydrolysis (Singh et al., 2010). The effect of washing with 50% ethanol was similar to that of water washing with respect to the starch and lipid concentrations in the flours, but washing with 50% ethanol was less effective than water washing in reducing ash and phytic acid concentrations.

Phytic acid in cereal grains exists mainly as phytate, but differs among grains with respect to its localization. Nearly 90% of the phytate in the corn kernel is located in the germ, whereas a major portion occurs in the pericarp and aleurone layers of the wheat kernel (Wu et al., 2009). A substantial proportion of the phytate was removed from all of the flours by washing with water. Phenolics in cereals consist of a range of structurally different compounds, either in free or bound form, and are mainly located in the outer

layers of the grain, although significant amounts are present in the endosperm and embryo (Naczk and Shahidi, 2006). A large proportion of the soluble phenolics is extractable with water or with aqueous polar solvents (Naczk and Shahidi, 2006). In the present study, washing with 50% ethanol reduced the total free phenolics content by 84–88% in the four flours. β-glucan is mainly located in the cell walls of the endosperm of the barley grain (Tiwari and Cummins, 2009). Water-soluble β-glucan increases the viscosity of the reaction medium, which reduces the diffusion rate of amylase enzyme molecules to starch molecules, and also is able to interact with the hydrolytic products, i.e. dextrins, from the action of α-amylase on gelatinized starch (Faraj, 2004). β-glucan in the cell wall may prevent either the passage of amylase enzyme through the cell wall or the release of starch granules from cells in flour particles, thereby impacting the rate and extent of starch hydrolysis. Of the non-starch components in cereal grains, pentosan is present in the second highest concentration, after protein, and occurs in the outer layers of the kernel. It has been reported that approximately 85–90% of the pentosan in wheat is water-unextractable (Wang et al., 2006). In this study, pentosan concentrations were affected only slightly due to its low solubility in the solvent systems employed.

3.3.5 Effect of solvent pre-washing on the loss of solids from flours

The extent of solid loss from the whole grain flours due to prewashing with various solvents is shown in Table 3.2 For triticale and wheat flours, solid losses were highest with water and 50% ethanol, and lowest with hexane, and losses with 100% ethanol were intermediate to those observed with hexane and 50% ethanol/water, whereas, for barley and corn flours, solid losses were highest with 100% and 50% ethanol

Table 3.2 Soluble solids losses resulting from solvent pre-washing (%, db).

Solvent	Triticale	Barley	Wheat	Corn
Water	15.52^{a}±0.60	3.41^{c}±0.03	12.66^{a}±0.23	5.76^{c}±0.62
Hexane	2.40^{d}±0.21	1.27^{d}±0.12	1.51^{d}±0.23	1.95^{d}±0.02
100% Ethanol	9.09^{c}±0.09	7.41^{b}±0.29	6.73^{c}±0.26	8.92^{b}±0.10
50% Ethanol	14.92^{b}±0.61	14.02^{a}±0.25	10.69^{b}±0.47	10.94^{a}±0.05

The values in the table are means ± standard deviation of three replicates. Means in the same column with different letters are significantly different at $P < 0.05$

washings. Solid losses with water extraction were lower for barley and corn flours (1.9–2.1%, respectively) than for triticale and wheat flours (3.2–4.4%). The greater losses observed for grain flours were attributed to higher levels of soluble protein, soluble β-glucan and/or soluble sugars in these flours. The relatively low solid losses seen with hexane or 100% ethanol (0.8–1.2% and 1.3–3.2%, respectively) reflect the low polarity of these solvents and their inability to extract polar (i.e. non-lipid) constituents (Table 3.1).

3.3.6 Effect of solvent pre-washing on hydrolysis of starch in whole grain flours by α-amylase and by sequential treatment with α-amylase and amyloglucosidase

As shown in Table 3.3, only slight significant ($p < 0.05$) differences in the degree of starch hydrolysis by α-amylase were observed between untreated flours (22.4–26.1%) and pre-washed flours (21.6–28.1%). Slight differences in the degree of starch hydrolysis by α-amylase were also reported by Perez-Carrillo and Serna-Saldivar (2007). It was anticipated that structural differences in the starch in the four flours and differences in the nature and concentrations of non-starch constituents, and in the nature of the associations between starch and non-starch constituents, might impact α-amylase hydrolysis. However, none of the solvent pre-washing treatments improved substantially the α-amylase hydrolysis of starch in any of the flours, suggesting that the degree of gelatinization is the dominant factor affecting starch liquefaction by α-amylase (Baks et al., 2008; Tester et al., 2004). In fact, pre-washing had a negative effect in some cases (water and 50% ethanol washing of triticale flour and barley flour, hexane washing of wheat flour). This suggests that, in some cases, solvent washing may have resulted in the concentration of certain insoluble non-starch constituents which were inhibitory for α-

Table 3.3 Degree of hydrolysis (%, db) of starch by α-amylase in unwashed and pre-washed flours.

Solvent	Triticale	Barley	Wheat	Corn
Unwashed	25.18^{a}±0.29	26.11^{b}±0.43	24.05^{b}±0.43	22.40^{b}±0.72
Water	22.40^{b}±1.02	23.74^{c}±0.29	26.42^{a}±0.29	23.43^{ab}±0.43
Hexane	24.56^{a}±0.58	28.05^{a}±0.32	21.57^{c}±0.43	21.98^{b}±0.72
100% Ethanol	23.84^{ab}±0.72	27.04^{ab}±0.58	25.60^{a}±0.58	24.77^{a}±0.87
50% Ethanol	21.88^{b}±0.87	22.45^{d}±0.36	23.53^{b}±0.87	22.60^{b}±0.14

The values in the table are means ± standard deviation of two replicates. Means in the same column with different letters are significantly different at $P < 0.05$.

amylase hydrolysis (e.g. pentosan), or the removal of certain constituents beneficial to α-amylase hydrolysis (e.g. protein, lipid, β-glucan, phytic acid, and phenolics).

Pre-washing with different solvents increased ($P<0.05$) the degree of hydrolysis of starch in whole grain flours treated sequentially with α-amylase and amyloglucosidase (Table 3.4). Pre-washing with 50% ethanol resulted in the highest increase in hydrolysis of starch in triticale, barley and wheat flours. With corn flour, the increases in the degree of hydrolysis were similar for all pre-washing treatments. The increases in degree of hydrolysis in pre-washed flours by sequential α- amylase and amyloglucosidase treatments indicate the more inhibitive effect of non-starch components on hydrolysis by α-amylase than on hydrolysis by amyloglucosidase. Even greater degrees of hydrolysis (83.7–93.0%) were observed with sequential enzyme treatment of refined starches (93.8–98.1% purity), which were largely free of non starch constituents (Table 3.5). The substantial differences observed among refined starches in the degree of hydrolysis could be due to starch structural differences.

Clearly, the presence of non-starch components in the multicomponent whole grain flour matrix affects, i.e. restricts, starch hydrolysis by amyloglucosidase. The degree of restriction depends on the nature and concentration of each non-starch component and its association/interaction with starch molecules and/or hydrolytic enzymes. For example, a number of studies have reported that the protein matrix surrounding starch granules in flour blocks the access of starch hydrolysis enzymes due to disulfide bonding (Chandrashekar and Kirleis, 1988; Choi, et al., 2008; Ezeogu, et al., 2008; Hamaker et al., 1987; Oria et al., 1995; Zhang and Hamaker, 1998). Digestion of protein with protease treatment greatly improved starch amylolysis in cereal flours

Table 3.4 Degree of hydrolysis (%, db) of starch by sequential treatment with α-amylase and amyloglucosidase in unwashed and pre-washed flours.

Pre-wash solvent	Triticale	Barley	Wheat	Corn
Unwashed	56.15^{c} ± 0.87	56.73^{e}±0.55	56.42^{d}±0.46	57.83^{b}±1.28
Water	66.00^{b}±1.16	65.80^{c}±0.87	63.98^{c}±0.64	64.19^{a}±0.52
Hexane	66.93^{b}±1.02	61.38^{d}±0.46	63.32^{c}±0.29	66.07^{a}±1.08
100% Ethanol	65.80^{b}±0.58	68.65^{b}±0.52	65.59^{b}±0.87	65.94^{a}±0.67
50% Ethanol	72.38^{a}±0.61	72.80^{a}±0.84	70.09^{a}±0.81	64.35^{a}±1.16

The values in the table are means ± standard deviation of two replicates. Means in the same column with different letters are significantly different at P <0.05.

Table 3.5 Purity and degree of hydrolysis (%, db) of refined starches sequentially hydrolyzed with α-amylase and amyloglucosidase.

	Triticale	Barley	Wheat	Corn
Starch content	97.91^{a}± 0.14	93.84^{b}±0.57	98.09^{a}±0.37	93.76^{b}±0.94
Degree of hydrolysis	83.70^{c}± 0.58	92.99^{a}± 0.29	86.79^{b}± 1.45	83.70^{c}± 1.16

The values in the table are means ± standard deviation of two replicates. Means in the same row with different letters are significantly different at $P < 0.05$.

(Perez-Carrillo and Serna-Saldivar, 2007; Perez-Carrillo et al., 2008). Phenolic compounds are known to block the nucleophilic sites of degradative enzymes by binding to their amino acid side chains, and thus would inhibit the activities of amylase enzymes (Chethan et al., 2008; Rohn et al., 2002). Extraction of soluble phenolic compounds would prevent their interaction with amylase enzymes, thus improving the efficiency of starch hydrolysis. The negative effect of phytic acid on starch hydrolysis is primarily, if not totally, due to its ability to bind minerals, thereby reducing the activity of α-amylase (Isaksen, 2006). Treatment with phytase, which degrades phytic acid, largely reduced the mineral binding ability of phytic acid, thus improving starch digestibility (Isaksen, 2006). The presence of lipid inhibits starch gelatinization, and the formation of amylose-lipid complexes during heating and/or hydrolysis also impacts starch amylolysis (Lauro et al., 2000). In the present study, different solvent pre-washings removed different proportions of soluble, non-starch components, such as protein, lipid, ash, β-glucan, phytic acid and phenolics, from each flour (Table 3.1), resulting in the significant improvement of hydrolysis of starch in solvent-washed flours, at least in the case of sequential hydrolysis by α-amylase and amyloglucosidase (Table 3.4). Differences among flours in the degree of starch hydrolysis may also reflect differences in endogenous amylase activity among flours.

The non-starch components in grains are not uniformly distributed among kernel tissues (i.e. endosperm, germ and bran) and can be separated into different fractions through dry or wet milling or solvent extraction prior to starch hydrolysis. Thus, prior fractionation and separation of grain components, besides enabling recovery of valuable grain components as co-products, optimizes the starch conversion process and improves

overall conversion efficiency by increasing the concentration of starch in the raw material, and by reducing any inhibitory effects of non-starch components on starch hydrolysis.

3.4 Conclusions

Milling of triticale, barley, wheat and corn grain to pass through a sieve with an aperture size of 0.5 mm generated flours having 80–99% of particles <500 μm in diameter, and with most visible (with SEM) starch granules free of non-starch constituents. Flour composition varied with grain type, and washing with water, hexane, 50% ethanol or 100% ethanol had different effects on flour composition and soluble solid losses. The degree of hydrolysis of starch in the flours by α-amylase was similar for all flours and all washing treatments, but the degree of hydrolysis achieved by sequential treatment with α-amylase and amyloglucosidase was markedly higher in pre-washed flours, and highest in refined starches.

Chapter 4

Phenolic acids in cereal grains and their inhibitory effect on starch liquefaction and saccharification[2]

4.1 Introduction

The first generation bio-ethanol industry using cereal grains as starting material is still the most dominant entity of the bio-energy sector in North America currently. Cereal grains are rich in starch, which is converted into fermentable sugars through a common liquefaction and saccharification process by using α-amylase and amyloglucosidase. However, quantitative conversion of starch from cereal grain is a challenging step that still remains costly and suffers from low conversion efficiency due to incomplete starch hydrolysis at a commercial scale. The presence of non-starch components in grain, such as phenolic compounds, is thought to interfere with starch amylolysis during liquefaction and saccharification of whole grain flours (de Jong et al. 1987).

Cereal grains contain a variety of phenolic compounds, including phenolic acids, flavonoids, tannins, lignans and other polyphenols, which are distributed non-uniformly throughout grain tissues. They are mainly concentrated in the outer layers of the grains, such as pericarp, testa, and aleurone layers, and considerable amounts also present in the endosperm and germ of kernels (Naczk and Shahidi 2006; Dykes and Rooney 2007; Liu 2007). The phenolic compounds are diverse in their concentration, composition and structure in various grains. Phenolic acids occur in cereal grains as a major group of

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phenolic compounds in both free and bound forms, but the majority is in bound form. Ferulic acid is the most abundant phenolic acid in common cereals, representing up to 90% of total phenolic compounds, and other phenolic acids, such as vanillic, syringic, p-coumaric, caffeic, and p-hydroxybenzoic acids are present in considerably lower amounts in triticale, wheat, rye, oat and corn grains (Sosulski et al. 1982; Yu et al. 2001; Mattila et al. 2005; Dykes and Rooney 2007; Verma et al. 2009; Zuchowski et al. 2009).

Numerous references in the literature point to the inhibitory effect of phenolic compounds on enzymatic starch hydrolysis by α-amylase and amyloglucosidase, an effect which is generally attributed to the ability of polyphenols to decrease enzyme activity by binding enzymes/proteins (Thompson and Yoon 1984; de Jong et al. 1987; Rohn et al. 2002; Funke and Melzig 2005; Chethan et al. 2008; Shobana et al. 2009; Sreerama et al. 2010; Oboh et al., 2012; Kam et al., 2013; Worsztynowicz et al., 2014; Zhu 2015). However, phenolic acids with their carboxyl and hydroxyl groups are also capable of binding with starch and other polysaccharides through hydrogen bonds, chelation, or covalent bonds, forming bridges or cross-links (Gibson and Strauss 1992). Few studies have reported the contribution of the reaction of phenolic acids with starch on the inhibition of starch amylolysis, even though the interaction of tannic acid and catechin with starches (Deshpande and Salunkhe 1982) as well as the interference of gallic and chlorogenic acids with the starch-iodine reaction (Sharma et al. 1992) have been reported.

This study was designed to investigate the effects of phenolic acids either alone or in combination on the starch hydrolysis reaction from triticale, wheat, barley, and corn sources, and to determine the nature of those effects and whether they are influenced by temperature. The outcome of the research is expected to define cost efficient approaches

for quantitative starch conversion and better understand the amylolysis kinetics of starch affected by minor non-starch components.

4.2 Materials and methods

4.2.1 Materials

Four cereal grains were used in this study. Barley (*Hordeum vulgare* L. cv. Xena) was obtained from the Crop Development Centre, University of Saskatchewan, Saskatoon, SK, Canada. Corn (*Zea mays* L.) was supplied by Pioneer Hybrid Ltd., Chatham, ON, Canada. Canada Prairie Spring (CPS) wheat (*Triticum aestivum* L.) was provided by Alberta Agriculture and Forestry, Barrhead, AB, Canada. Triticale (x *Triticosecale* cv. Pronghorn) was supplied by the Field Crop Development Centre, Alberta Agriculture and Forestry, Lacombe, AB, Canada. The grains (1 kg) were ground in a Retsch mill (Model ZM 100, Haan, Germany) using a ring sieve with an aperture size of 0.5 mm. Ground flours were stored in plastic containers at 5 ºC. Liquozyme SC (α-amylase) (120 KNU/g) and Spirizyme Fuel (amyloglucosidase) (750 AGU/g) enzymes were kindly provided by Novozymes, Bagavaerd, Denmark.

HPLC grade phenolic acid standards, (p-coumaric, ferulic, p-hydroxybenzoic, vanillic, caffeic, chlorogenic, protocatechuic, gallic, catechin hydrate, naringin and syringic acids), were purchased from Sigma-Aldrich chemical Company (St. Louis, MO, USA). All other reagents used were of analytical and HPLC grade.

4.2.2 Starch isolation

4.2.2.1 Isolation from triticale and wheat flours

A dough ball washing technique was used to isolate starch from triticale and wheat flours. Stiff dough ball was prepared by mixing 100 g of flour with 60 ml of

distilled water thoroughly. The dough ball, covered with a plastic cup, was tempered at room temperature for 1-2 hr and then blended with 200 ml of distilled water at high speed in a blender. The slurry was sieved on a screen (75 μm opening). The fiber retentate on the top of screen was re-slurried with water (1:2.5w/w), further broken down with blender at high speed, and re-sieved. By repeating this step one more time, the filtrate was pooled, and centrifuged. The upper grey layer of the settlement in the centrifuge bottles was removed carefully with spatula and discarded. The bottom white starch layer was re-slurried with water and the pH was adjusted to 10 by using 0.1N NaOH. The slurry then was mixed for 30 min and centrifuged again at 3500 xg for 10 min. The starch residue was re-slurried in water (1:2.5 w/v) and neutralized by 0.1N HCl. The slurry was centrifuged at 3500 xg for 10 min. The supernatant and the upper gray layer of the residue containing mainly protein with small starch granules were discarded. The starch-rich residue in white layer was washed three more times with distilled water. The starch isolate was dried at 40 °C overnight, ground and screened through a No. 60 mesh sieve (Wolf, 1965).

4.2.2.2 Isolation from corn flour

Corn starch was isolated according to the method of Eckhoff (1992). Ground corn flour was mixed with distilled water (1:4.5 w/v) in a beaker and blended for 5 min using a lab blender (Warning, Ct. 06057, Dynamics Corp., New Hartford, NY, USA). The slurry was sieved through a 63 μm screen. The fiber residue on the screen was re-slurried with water (1:2.5 w/v) and sonicated for 30 min under continuous stirring. The slurry was then sieved (63 μm). The fiber residue was re-slurried with water (1:2 w/v) and wet milled with a polytron homogenizer (PT 2000, Kinematica AG LITTAU, Switzerland) at 30,000

rpm for 10 min. The slurry was sieved (63 μm) again. All filtrate were pooled and centrifuged at 3500 xg for 10 min. The supernatants were discarded and the crude starch residue was re-slurried with water and the pH was adjusted to 10 by using 0.1N NaOH. The slurry then mixed for 30 min and centrifuged. The starch residue was re-slurried in water (1:2.5 w/v) and neutralized. The slurry was centrifuged at 3500 xg for 10 min. The supernatant and the upper gray layer of the residue containing mainly protein with small starch granules were discarded. The starch-rich residue in white layer was washed 3 more times. The starch isolate was dried at 40 °C overnight, ground and screened through a No. 60 mesh sieve.

4.2.2.3 Isolation from barley flour

Ground barley flour was mixed with 50% ethanol (1:4.5 w/v) in a beaker and gently stirred for 30 min. The slurry was sieved through a 63 μm screen. The fiber residue on the screen was re-slurried with 50% ethanol (1:2.5 w/v) and sonicated (Sonic 300 dismembrator (90% amplitude), Systems, Corporation, Farmingdale, NY, USA) for 30 min under continuous stirring. The slurry was then sieved (63 μm) again. The fiber residue was re-slurried with 50% ethanol (1:2 w/v) and wet milled with a polytron homogenizer at 30,000 rpm (PT 2000, Kinematica AG LITTAU, Switzerland) for 10 min. The slurry was sieved (63 μm) once again. All filtrate through the sieves were pooled and centrifuged (Beckman J2-21 centrifuge, Beckman Coulter, Inc., CA, USA) at 3500 xg for 10 min. The supernatants were discarded and the crude starch residue was re-slurried with water and the pH was adjusted to 10 by 0.1N NaOH. The slurry then mixed for 30 min and then centrifuged. The starch residue was re-slurried in water (1:2.5 w/v) neutralized, and then centrifuged. The supernatant and the upper gray layer of the residue

containing mainly protein with small starch granules were discarded. The starch-rich residue in white layer was washed three more times with distilled water. The starch isolate was dried at 40 °C overnight, ground and screened through a No. 60 mesh sieve (Vasanthan and Temelli, 2002).

4.2.3 Determination of free, bound and total phenolic acids using HPLC

Free phenolics in grains were extracted by mixing 1 g of the ground flour sample with 20 mL of 50% (v/v) methanol for 1 h. After centrifugation at 4000 x g for 5 min, the supernatants were collected and used for HPLC analysis. Total phenolic acids content were extracted according to the method of (Yu et al. 2001). Ground samples (1 g) were mixed with 10 mL of 0.2 N H_2SO_4 in test tubes and heated in a boiling water bath for 1 h. Hydrolysis was terminated by cooling samples in an ice-water bath for 10 min and the pH was adjusted to 4.5 by using 0.2 N NaOH prior to the addition of 2 mL of 2.5 M aqueous sodium acetate solution containing 8% (w/v) thermo-stable α-amylase. The samples were incubated in boiling water for 1 h and then centrifuged at 4000 x g for 10 min. The supernatant was analyzed by HPLC.

HPLC analyses were performed using an Agilent HPLC system (Agilent Technologies 1200 series, Palo Alto, CA) equipped with an Agilent 717 plus autosampler coupled with a Agilent DAD (Diode Array Detector) at 360, 280 and 254 nm. Separation was performed with Zobax 300 SB-C18 (5 μm, 4.6 mm - 250 mm) column (Agilent, Palo Alto, CA, USA) at room temperature. Elution was carried out by using a gradient procedure with a mobile phase containing solvent A (0.1% acetic acid in water) and solvent B (0.1% acetic acid in methanol) as follows: 0 min, 5% B; 15 min, 20% B; 35 min, 40% B; 42 min, 65% B; 50 min, 80% B; 52 min, 5% B; 60 min, 5% B. Run time

was 60 min, the solvent flow rate was 1.0 mL/min, and the injection volume was 10 μL. Agilent "Chemstation" software version 2007 has been used for calculations of phenolic acids. The concentrations of individual phenolic compounds were calculated using standard curves. Results were expressed in micrograms per gram (Zhao et al., 2006).

4.2.4 Chemical composition

Moisture content was determined by the standard procedure of AACC (Method 44-15A (AACC International, 2004). Total starch content was estimated according to the total starch assay of Megazyme International, Ireland Ltd. (Wicklow, Ireland).

4.2.5 Starch amylolysis in the presence of individual or a mixture of phenolic acids

The effect of phenolic acids on starch amylolysis was conducted by two sets of experiments. One set of experiments was done by adding individual pure phenolic acids (in "free" form) to starch slurries at amounts either equivalent to or three times those in the whole grain flours (free and bound). The second set of experiments was done by adding a mixture of the major phenolic acids to starch slurries at amounts equivalent to those in the whole grain flours. The protocols for starch amylolysis using α-amylase or sequential α-amylase and amyloglucosidase are shown in Figure 4.1

4.2.6 Model reaction system for the interaction of phenolic acid-starch-enzyme

A model reaction system with three sets of solutions/slurries, which included pure "free" phenolic acids at the equal amount of the total phenolic acids as in whole flour, isolated starch, and/or α-amylase with and without boiling was used to study the interaction of phenolic acid-starch-enzyme. For the first set of solutions, ferulic acid or coumaric acid solution was prepared as a control. The phenolic acid concentration was equal to the level of total ferulic acid or coumaric acid in the whole grain flour. The

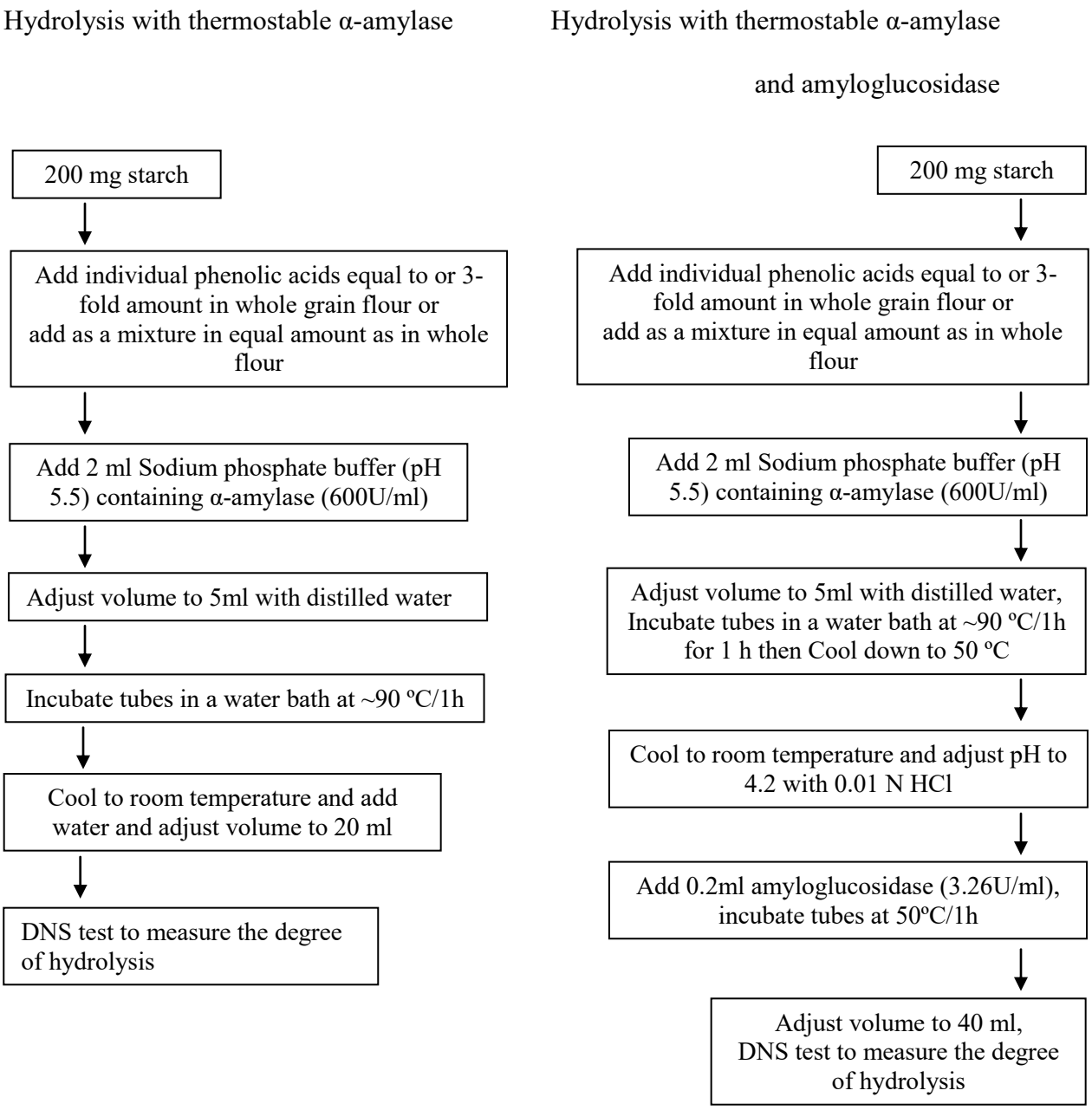

Figure 4.1 Procedure for Starch amylolysis in the presence of individual or a mixture of phenolic acids

phenolic acid solution was either heated in boiling water bath for 60 min or not heated. For the second set of solutions/slurries, isolated triticale or corn starch (4% w/v with starch purity of 97.9 and 93.8%, db, respectively) was added to the phenolic acid solution and mixed for 60 min with or without boiling. To the third set of solutions/slurries, starch (4% w/v)

of 97.9 and 93.8%, db, respectively) was added to the phenolic acid solution and mixed for 60 min with or without boiling. To the third set of solutions/slurries, starch (4% w/v) and α-amylase (150 UN) were added to phenolic acid solution with or without boiling. The contents of ferulic or coumaric acid were analyzed by HPLC according the method described by (Zhao et al. 2006).

4.2.7 Determination of degree of starch hydrolysis

The concentration of reducing sugars in the supernatant of centrifuged samples was determined by the dinitrosalicylic acid (DNS) method (Bruner, 1964) and the degree of hydrolysis was expressed as the weight of glucose equivalents per 100 g dry starch. (See Appendix)

4.2.8 Statistical analysis

All chemical analyses and experiments were carried out in duplicate at least. One-way Analysis of variance (ANOVA) was performed using the General Linear Model (GLM) procedure of SAS Statistical Software, Version 9.1.2 (SAS Institute Inc., Cary, NC, USA, 2004). Multiple comparisons of the means were done using Tukey's test ($p < 0.05$).

4.3 Results and discussion

4.3.1 Concentrations of phenolic acids in cereal grains

Table 4.1. Phenolic acids in whole grain flours (μg/g, db)

Sample		p-coumaric acid	Ferulic acid	p-hydroxybenzoic acid	Vanillic acid	Caffeic acid	Chlorogenic acid	Protocatechuic acid	Gallic acid	Syringic acid	Total phenolics
Triticale	Free	ND	20.3 ± 0.21	ND	ND	4.8 ±`0.22	ND	72.7 ± 0.86	ND	12.6 ± 0.22	110.3
	Total	258.5 ± 0.32	483.7±1.31	7.4±0.17	15.4 ± 0.14	13.9 ± 0.05	ND	199.1 ± 1.19	123.4 ± 0.79	34.1 ± 0.74	1135.5
	Bound*	258.5	463.4	7.4	15.4	9.2	ND	126.5	123.4	21.6	1025.2
Wheat	free	ND	5.4 ± 0.63	ND	ND	11.2 ± 0.4	ND	141.1 ± 0.80	ND	7.6 ±0.31	165.3
	Total	293.0 ± 0.36	771.6±0.52	9.2±0.005	19.5 ± 0.30	51.9 ± 0.47	ND	454.3 ± 1.36	66.1 ± 0.23	34.5 ± 0.19	1700.1
	Bound	293.0	766.2	9.2	19.5	40.7	ND	313.2	66.1	26.9	1534.8
Barley	free	5.0 ± 0.61	ND	ND	9.8 ± 0.13	ND	34.2 ± 0.14	29.1 ± 0.16	ND	ND	78.1
	Total	151.4 ± 1.08	132.1± 0.58	215.0 ± 0.41	49.3 ± 0.32	21.9 ± 0.02	77.1 ± 1.06	65.9 ± 0.13	158.6 ± 0.95	32.2 ± 0.37	903.5
	Bound	146.4	132.1	215.0	39.5	21.9	42.9	36.8	158.6	32.2	825.4
Corn	free	32.2 ± 0.37	37.5 ± 0.43	ND	5.2 ± 0.32	ND	17.9 ± 0.37	20.1 ±0.17	ND	21.5 ± 0.70	117.7
	Total	584.0 ± 1.0	265.5 ± 0.41	11.6 ±0.28	15.4 ± 0.73	24.4 ± 1.53	77.9 ± 0.65	47.0 ± 0.29	116.5 ± 1.04	108.4 ± 0.01	1250.7
	Bound	568.5	228.1	11.6	10.2	24.4	60.0	27.0	116.5	86.8	1133.0

Bound phenolic acids were calculated by the difference of total and free phenolic acids. ND: not detectable.

The concentrations of 11 common phenolic acids in four cereal grains quantified by HPLC are shown in Table 4.1. The majority of phenolic acids were found in bound form (90.6% in triticale, 90.5% in wheat, 76.1% in barley, and 86.5% in corn). Among individual phenolic acids determined, the main phenolic acids (content higher than 100 μg/g) were ferulic, p-coumaric, protocatechuic, and gallic acids in triticale; ferulic, protocatechuic, and p-coumaric acids in wheat; catechin hydrate, p-hydroxybenzoic, gallic, p-coumaric, and ferulic acids in barley; p-coumaric, catechin hydrate, naringin, ferulic, gallic, and syringic acids in corn. Chlorogenic acid and catechin hydrate were not detectable in triticale and wheat flour. The data indicated that the composition and concentration of phenolic acids in cereal grains vary with species. Individual and total phenolic acid contents have been reported in a wide range for various cereal grains in the literature and those values are difficult to compare due to the diversity of grain varieties, range of environmental factors, and different analytical and extraction methods used (Dykes and Rooney, 2007; Stalikas, 2007; Fernandez-Orozco et al., 2010; Menga et al., 2010).

4.3.2 Effect of individual phenolic acids on starch hydrolysis with α-amylase and with sequential α-amylase and amyloglucosidase treatments

As shown in Tables 4.2 and 4.3, the addition of individual "free" phenolic acids to starch slurries in amounts of individual phenolic acids (free and bound) equivalent to whole flours resulted in a significant decrease in the degree of hydrolysis of starch with α-amylase alone (up to 5%) and with sequential α-amylase and amyloglucosidase (up to 6%) in all four isolated starches. The degree of hydrolysis further decreased when three fold amounts of individual phenolic acids were added (up to 6% with α-amylase and up

Table 4.2 Degree of hydrolysis of starches treated by α-amylase in the presence of individual phenolic acids.

	Triticale		Wheat		Barley		Corn	
	X 1	**X 3**	**X 1**	**X 3**	**X 1**	**X 3**	**X 1**	**X 3**
Control	44.3^{a}±0.4	44.3^{a}±0.4	45.3^{a}±0.1	45.3^{a}±0.1	45.1^{a}±0.2	45.1^{a}±0.2	43.23^{a}±0.3	43.23^{a}±0.3
p-Coumaric acid	41.2^{e}±0.6	39.6^{e}±0.1	41.5^{d}±0.3	39.2^{g}±0.3	43.2^{bc}±0.3	42.0^{c}±0.0	38.2^{e}±0.4	37.4^{d}±0.6
Ferulic acid	42.1^{cd}±0.4	39.7^{e}±0.1	42.8^{c}±0.6	40.3^{ef}±0.4	43.2^{bc}±0.1	42.2^{c}±0.1	40.9^{bcd}±0.2	39.9^{bc}±0.4
p-Hydroxybenzoic acid	42.8^{c}±0.3	38.4^{f}±0.1	42.9^{c}±0.3	41.0^{e}±0.1	42.6^{c}±0.9	41.0^{d}±0.3	42.3^{ab}±0.1	39.6^{bcd}±0.8
Vanillic acid	42.7^{c}±0.2	40.7^{d}±0.5	42.7^{cd}±0.3	40.6^{e}±0.2	43.3^{bc}±0.3	41.7^{c}±0.1	41.9^{bcd}±0.4	38.0^{cd}±0.5
Caffeic acid	41.6^{de}±0.2	40.8^{d}±0.3	43.2^{bc}±1.0	42.0^{d}±0.4	43.7^{bc}±0.1	41.7^{c}±0.3	40.7^{cd}±0.6	38..0^{cd}±0.7
Chlorogenic acid	---	---	---	---	43.4^{bc}±0.3	41.8^{c}±0.2	41.0^{bcd}±0.3	38.7^{bcd}±0.7
Protocatechuic acid	41.0^{e}±0.3	39.5^{e}±0.2	42.9^{c}±0.3	39.7^{fg}±0.6	43.4^{bc}±0.1	40.7^{d}±0.2	40.4^{d}±0.4	39.7^{bcd}±0.8
Gallic acid	42.6^{c}±0.1	41.6^{c}±0.4	43.5^{bc}±0.3	42.4^{cd}±0.1	43.1^{bc}±0.3	41.0^{d}±0.3	41.7^{bcd}±0.8	40.3^{b}±1.1
Naringin	43.8^{b}±0.2	43.7^{b}±0.2	44.8^{a}±0.6	43.7^{b}±0.2	44.2^{b}±0.6	43.9^{b}±0.2	41.6^{bcd}±0.6	41.0^{b}±0.7
Syringic acid	43.4^{ab}±0.0	43.9^{b}±0.4	44.4^{ab}±0.8	43.0^{c}±0.4	43.1^{bc}±0.2	42.0^{c}±0.1	42.0^{bc}±0.3	41.0^{b}±0.2

Each value in the table is the mean ± standard deviation of two replicates. Means in the same column with different letters are significantly different ($p < 0.05$).

X1 and X 3 represent the addition level of phenolic acid as equal and three fold of amount in whole grain flour.

Table 4.3 Degree of hydrolysis of starches treated by α-amylase and amyloglucosidase in the presence of individual phenolic acids.

	Triticale		Wheat		Barley		Corn	
	X 1	**X 3**	**X 1**	**X 3**	**X 1**	**X 3**	**X 1**	**X 3**
Control	86.1^{a}±0.4	86.1^{a}±0.4	87.5^{a}±0.3	87.5^{a}±0.3	90.1^{a}±0.1	90.1^{a}±1.1	82.8^{a}±0.5	82.8^{a}±0.5
p-Coumaric acid	83.3^{c}±0.1	79.3^{de}±0.1	83.8^{c}±0.1	79.5^{e}±0.6	86.1^{bc}±0.6	83.6^{cd}±0.8	79.5^{bc}±0.7	77.4^{c}±0.4
Ferulic acid	82.2^{cd}±0.4	80.0^{d}±0.4	84.0^{c}±0.6	81.8^{d}±0.3	86.5^{bc}±0.8	84.7^{bcd}±0.1	78.8^{bc}±0.4	75.8^{de}±0.2
p-Hydroxybenzoic acid	80.2^{f}±0.4	78.6^{e}±0.4	81.8^{d}±0.6	80.4^{e}±0.7	87.4^{b}±1.0	85.0^{bc}±0.6	77.9^{cd}±0.2	75.5^{de}±0.1
Vanillic acid	82.8^{cd}±0.3	81.6^{bc}±0.4	83.8^{c}±0.3	81.6^{d}±0.3	85.1^{c}±0.6	83.9^{cd}±0.4	78.3^{bc}±0.3	75.0^{e}±0.3
Caffeic acid	82.8^{cd}±0.8	81.2^{c}±0.9	84.1^{c}±0.4	81.9^{cd}±0.1	86.1^{bc}±0.7	85.0^{bc}±0.8	77.8^{cd}±0.4	75.9^{de}±0.2
Chlorogenic acid	---	---	---	---	85.1^{c}±0.6	83.6^{cd}±0.8	76.8^{d}±0.6	75.0^{e}±0.2
Protocatechuic acid	84.7^{b}±0.3	82.3^{bc}±0.2	85.8^{b}±0.2	83.3^{b}±0.3	86.3^{bc}±1.0	86.0^{b}±0.6	79.1^{bc}±0.5	77.5^{c}±0.3
Gallic acid	81.9^{de}±0.4	79.4^{de}±0.6	83.7^{c}±1.3	81.4^{d}±0.2	85.5^{c}±1.0	83.3^{d}±0.6	78.8^{bc}±0.7	76.2^{d}±0.4
Naringin	80.9^{ef}±0.6	82.5^{b}±0.6	84.0^{c}±0.5	83.3^{b}±0.3	86.0^{bc}±0.1	84.2^{cd}±0.4	78.7^{bc}±0.5	76.0^{de}±0.4
Syringic acid	81.9^{de}±0.6	80.0^{d}±0.2	84.2^{c}±0.1	82.9^{bc}±0.7	86.0^{bc}±0.3	83.6^{cd}±0.7	79.9^{b}±0.2	78.6^{b}±0.2

Each value in the table is the mean ± standard deviation of two replicates. Means in the same column with different letters are significantly different ($p < 0.05$).

X1 and X 3 represent the addition level of phenolic acid as equal and three fold of amount in whole grain flour.

to 8% with sequential α-amylase and amyloglucosidase). The study indicated that an inhibitory effect of phenolic acids on starch amylolysis occurred, however, the inhibition was limited due to the low individual phenolic acid concentration. There was no obvious trend in the effect of each phenolic acid on starch hydrolysis. In contrast, Rohn et al. 2002 reported that the inhibitory effect of phenolic acids on α-amylase activity follows the order p-benzoquinone > chlorogenic acid > gallic acid > caffeic acid > ferulic acid > quinic acid, depending on the concentration and on the number and position of hydroxyl groups of the phenolic compounds applied..

4.3.3 Effect of combination of major phenolic acids on starch hydrolysis with α-amylase and with sequential α-amylase and amyloglucosidase treatments

When a combination of major phenolic acids were added to starch slurries at amounts equivalent to those present in whole grain flours, the degree of hydrolysis of starches was significantly($p < 0.05$) decreased in all starches (4-5% with α-amylase and 9-13% with sequential α-amylase and amyloglucosidase) (Table 4.4). Compared to the starch hydrolysis with α-amylase alone, the hydrolysis with sequential α-amylase and amyloglucosidase showed a higher decrease in the degree of hydrolysis by 12.4, 15.1, 14.4 and 11.2% for triticale, wheat, barley and corn starches, respectively, indicating a combinational effect of phenolic acids on starch hydrolysis when a mixture of phenolic acids was present.

A number of studies have indicated that the inhibitory effect of phenolic compounds on α-amylase and amyloglucosidase activities is concentration dependent (Funke and Melzig, 2005; Shobana et al., 2009; Sreerama et al., 2010). The type and structure of phenolic compounds, such as the number and position of hydroxyl groups

Table 4.4 Degree of Hydrolysis (%, db) of Starches Treated with α-Amylase and Amyloglucosidase in the Absence and Presence of Phenolic Acids

Starch source	α-Amylase		α-Amylase + Amyloglucosidase	
	Starch	Starch + PAs*	Starch	Starch + PAs
Triticale	44.3[a] ± 0.83	40.2[b] ± 0.29	86.1[c] ± 1.02	75.4[d] ± 0.58
Wheat	45.3[a] ± 0.72	39.8[b] ± 0.33	87.5[c] ± 0.45	74.3[d] ± 0.87
Barley	45.1[a] ± 0.37	41.2[b] ± 0.75	90.1[c] ± 0.81	77.1[d] ± 0.15
Corn	43.2[a] ± 0.29	42.7[b] ± 0.55	82.8[c] ± 0.80	73.5[d] ± 0.52

* Phenolic acids (PAs) added to starch slurry in the equal amounts as in whole grains:
In triticale starch: p-coumaric acid, ferulic acid, protocatechuic acid, gallic acid, syringic acid.
In wheat starch: p-coumaric acid, ferulic acid, protocatechuic acid, gallic acid, naringin, syringic acid, caffeic acid.
In barley starch: p-coumaric acid, ferulic acid, protocatechuic acid, gallic acid, syringic acid, vanillic acid, chlorogenic acid.
In corn starch: p-coumaric acid, ferulic acid, protocatechuic acid, gallic acid, syringic acid, chlorogenic acid.

Means in the same row with different letters are significantly different at $p < 0.05$.

play a significant role (Rawel et al., 2002; Rohn et al., 2002; Funke and Melzig, 2005; Tadera et al., 2006). Phenolic acids may bind to the active site of enzymes or to the secondary binding site of enzyme-substrate complexes in an uncompetitive inhibition mode (Chethan et al. 2008).

4.3.4 Interaction of phenolic acids with starch and enzyme during amylolysis

Two of the major phenolic acids, coumaric acid and ferulic acid were used to study the interactions of phenolic acid with starch and/or α-amylase. As shown in Table 4.5, the change in the contents of coumaric acid and ferulic acid were minimal after heating, indicating that both phenolic acids were quite stable at boiling temperature. When α-amylase was added to the phenolic acid solution and mixed for 60 min, the contents of both coumaric and ferulic acids were slightly reduced (up to 3%) without boiling and significantly ($p< 0.05$) reduced (20-26%) with boiling, indicating that thermal treatment greatly induced the interaction of phenolic acids with the enzyme/protein. A significant reduction of phenolic acid concentration was observed in the phenolic acid–triticale starch mixture (9% in coumaric acid and 4% in ferulic acid, respectively) but not in the phenolic acid–corn starch mixture. When the mixture of phenolic acid–starch was boiled, a significant ($p< 0.05$) decrease in the phenolic acid concentration occurred in both mixtures (9-26%). When both starch and enzyme were added to the phenolic acid solution, the phenolic acid contents were further reduced by up to 7% compared to the phenolic acid solution alone and the enzyme-phenolic acid mixture. Again, boiling caused further loss of phenolic acid in the starch-enzyme-phenolic acid mixture (18-28%). Thus, the total loss of phenolic acid in the starch–enzyme–phenolic acid system with boiling was 21-32%. The study clearly indicated that an interaction occurred

Table 4.5 Phenolic acid contents in the reaction mixture of phenolic acid, starch and/or enzyme with or without boiling treatments.

Treatment	Triticale		Corn	
	Coumaric acid (µg)	Ferulic acid (µg)	Coumaric acid (µg)	Ferulic acid (µg)
Phenolic acid	191.9^{a} ±0.7	357.6^{a} ±0.4	374.5^{a} ± 0.2	172.0^{a} ±0.6
Phenolic + Boiling	192.3^{a} ±0.2	357.7^{a} ±0.3	376.8^{a} ± 0.7	178.8^{ab} ±0.7
Enzyme + Phenolic	185.2^{b} ±0.2	351.4^{b} ±0.6	364.0^{b} ±1.5	167.2^{ab} ±1.0
Enzyme + Phenolic + Boiling	142.0^{f} ±0.9	287.0^{f} ±0.8	285.8^{e} ±1.4	129.9^{d} ±0.5
Starch + Phenolic	175.1^{d} ±0.2	345.5^{d} ±1.5	374.5^{a} ±1.0	169.6^{ab} ±1.0
Starch + Phenolic + Boiling	168.5^{e} ±0.9	317.4^{e} ±0.8	341.1^{d} ±0.7	126.5^{d} ±0.9
Starch + Enzyme + Phenolic	179.9^{c} ±0.6	348.1^{c} ±0.8	352.7^{c} ±1.1	160.4^{c} ±0.9
Starch + Enzyme + Phenolic + Boiling	130.2^{g} ±0.9	284.2^{g} ±1.0	272.6^{f} ±0.4	117.6^{e} ±0.4

Each value in the table is the mean ± standard deviation of two replicates. Means in the same column with different letters are significantly different ($p < 0.05$).

between phenolic acid and enzyme and between phenolic acid and starch/dextrin. The interaction of phenolic acid with enzyme/protein generally caused more reduction of phenolic acid content (20-26%) than that with starch (9-26%), suggesting that the phenolic acid-protein reaction with enzyme is dominant. Boiling caused approximately 18-28% loss of phenolic acid in the starch–enzyme–phenolic acid mixture, playing a significant role in facilitating the interaction between phenolic acid and starch/enzyme. Both amylose and amylopectin molecules in starch granules may interact with phenolic compounds through forming inclusion complexes with amylose molecules (Beta and Corke 2004) and by binding to side chains of amylopectin and the amorphous region of starch granules, thus altering starch physicochemical properties (Zhu et al. 2008; 2009). As proposed in Figure 4.2, the phenolic acids in grain flour may bind to the starch chains increasing the resistance of starch to further enzymatic hydrolysis during starch amylolysis, especially during starch liquefaction using thermostable α-amylase. The inhibitory effect of phenolic compounds on the conversion of starch to ethanol lies mainly in the inhibition of amylase hydrolysis and not in fermentation (de Jong et al. 1987).

4.4 Conclusions

Cereal grains contain a variety of phenolic acids varying with their composition and concentration. The presence of phenolic acids in grains inhibited starch amylolysis. The inhibition of starch hydrolysis was more pronounced in the presence of a mixture of phenolic acids compared to individual phenolic acids.

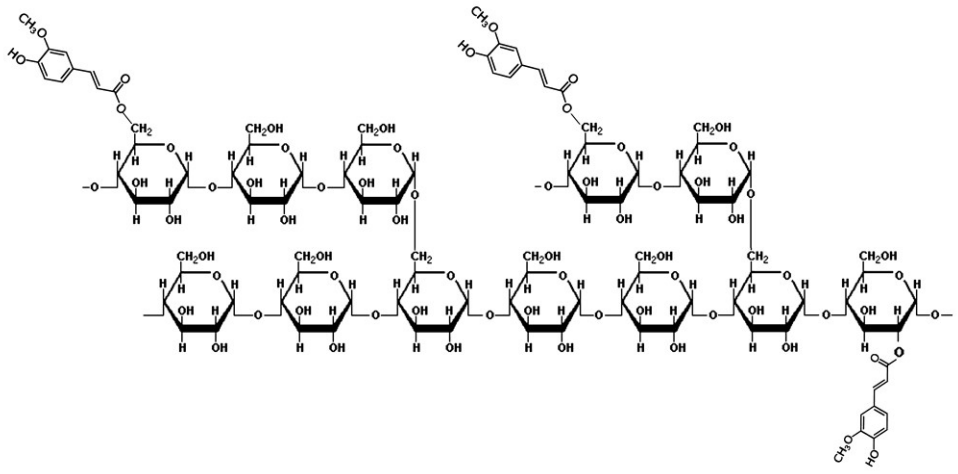

Figure 4.2 The interaction between phenolic acids with the starch chains.

Chapter 5

Native-bound phenolic acids in cereal grains inhibit starch amylolysis

5.1 Introduction

The outer bran layers and germ of cereal grains (wheat, barley, rye, oat, etc) contain a wide variety of biologically active compounds such as vitamins, antioxidants, soluble and insoluble dietary fibres, phenolic compounds, peptides, sterols, and microelements (Andreasen et al., 2001; Ragaee and Noaman, 2006; Ragaee et al., 2011; Sidhu and Kabir, 2007; Slavin, 2004; Zielinski, 2002). Phenolic compounds are those with one or more aromatic rings to which one or more hydroxyl groups are attached, and they can be categorized as phenolic acids, flavonoids, condensed tannins, coumarins and alkylresorcinols (Liu, 2004). These compounds mainly exist as glycosides linked to carbohydrates, organic acids, amines, lipids and other phenols. Phenolic acids and flavonoids are the most common phenolic compounds found in whole grains (Adom and Liu, 2002; Adom et al., 2005). Phenolic acids can be subcategorized into two major groups: hydroxybenzoic acids (vanillic, gallic, *p*-hydroxybenzoic, syringic and protocatechuic acids) and hydroxycinnamic acids (ferulic, *p*-coumaric, caffeic, and sinapic acids). They are commonly linked to cell wall components such as polysaccharides (cellulose, pentosans, beta-glucan, etc), lignin, and protein through ester bonds (Liu, 2007). Organic solvents such as methanol, ethanol, acetone and/or water mixtures are used to extract the free phenolic acids from the outer layer of the pericarp (Adom and Liu, 2002; Awad et al., 2000; Dykes and Rooney, 2007; Hahn et al., 1983; Hahn et al., 1984; Kim et al., 2006; Liyana-Pathirana et al., 2006; Mattila et al., 2005;

Sosulski et al., 1982; Subba Rao and Muralikrishna, 2002). Aqueous ethanol has been applied as the most common solvent for phenolic extraction.

Solvent extraction of bound phenolics from the bran can be effectively done through pretreatment of bran with alkali or acid, or both sequentially, in order to break covalent bonds including ester linkages between phenolic acids and cell wall polysaccharides such as arabinoxylan (Adom et al., 2005; Kim et al., 2006; Liyana-Pathirana et al, 2006; Parker et al., 2005; Verma et al., 2009). Kim et al (2006) investigated the effect of hydrolysis by acid and alkali, as well as the extraction (80% methanol) conditions on the yield and types/profile of phenolic acids from wheat bran. Approximately 90% of ferulic acid was liberated. Although the free and soluble conjugate phenolic fractions demonstrated strong antioxidant activity, the bound fraction represents the greater potential if released (Ohta et al., 1997). Liyana-Pathirana et al., (2006) utilized alkaline hydrolysis to liberate bound phenolics from wheat bran and tested them for their antioxidant activity, and showed a significantly greater contribution by the hydrolyzed bound fraction (free) than the esterified fractions. Oboh et al (2012) extracted free and bound polyphenol from jute leaf (*Corchorus olitorius*) to characterize their inhibitory action on α-amylase and α-glucosidase. The results from reversed-phase HPLC analysis revealed that chlorogenic acid (7.5 mg/mg) and isohamnetin (51.1 mg/100 g) were the main free phenolic acids in the extract, whereas caffeic acid (58.1 mg/ 100 g) was the main bound phenolic acid in the extract. Both free and bound phenolic extracts inhibited α-amylase and α-glucosidase (12.5-50.0 µg/mL) in a dose-dependent manner with free extracts having significantly higher α-amylase (17.5 µg/mL) and α-glucosidase (11.4 µg/mL) inhibitory activities.

Low grade cereal grains are commonly used as the raw material in industrial ethanol production (1st generation ethanol production), where the process demands quantitative conversion of starch to sugar through liquefaction and saccharification steps. The effect of free phenolic acids on starch liquefaction and saccharification has been studied (Chethan et al. 2008; de Jong et al. 1987; Funke and Melzig 2005; Rohn et al. 2002; Shobana et al. 2009; Sreerama et al. 2010; Thompson and Yoon 1984). However, since most of the phenolic compounds of cereal grains exist in the bran layer in a bound form, it will be important to study the effect of native bound phenolics on starch amylolysis. No such study has been reported in the literature. Therefore, the aim of the present study was to prepare fiber concentrates along with bound phenolics from triticale, wheat, barley and corn grain meals, and investigate the effect of phenolic compounds, in their native bound form as well as after liberating them into free form with acid treatment, on starch amylolysis i.e. liquefaction and saccharification.

5.2 Materials and methods

5.2.1 Materials

Four cereal grains were used in this study. Barley (*Hordeum vulgare* L. cv. Xena) was obtained from the Crop Development Centre, University of Saskatchewan, Saskatoon, SK, Canada. Corn (*Zea mays* L.) was supplied by Pioneer Hybrid Ltd., Chatham, ON, Canada. Canada Prairie Spring (CPS) wheat (*Triticum aestivum* L.) was provided by Alberta Agriculture and Food, Barrhead, AB, Canada. Triticale (x *Triticosecale* cv. Pronghorn) was supplied by the Field Crop Development Centre, Alberta Agriculture and Rural Development, Lacombe, AB, Canada. The grains (1 kg)

were ground in a Retsch mill (Model ZM 100, Haan, Germany) using a ring sieve with an aperture size of 0.5 mm. Ground flours were stored in plastic containers at 5-7ºC.

5.2.2 Chemical composition of fibre concentrates

Moisture content was determined by the standard procedure of AACC (Method 44-15A, 2004). Nitrogen content was measured by Leco® Carbon/Nitrogen determinator (TruSpec® CN, Leco Corporation St. Joseph, MI, USA) and protein content was calculated (Protien= Nitrogen x F). Total starch content was estimated according to the total starch assay of Megazyme International, Ireland Ltd. (Wicklow, Ireland). Total free phenolics was determined by the Folin-Ciocalteu method (Zhao et al., 2006).

5.2.3 Starch isolation

Isolation of starch from triticale, wheat, barley and corn grains was carried out according to the methods illustrated in Chapter 4 (section 4.2.2).

5.2.4 Preparation of fibre concentrates from cereal grains

Three different types of fibre concentrates (FC1, FC2 and FC3) were isolated from the flour/meal of triticale, wheat, barley and corn according to the protocol illustrated in Figure 5.1. Flour (200 g) of each grain was first mixed with 50% ethanol (1:3 w/v) in a beaker and vigorously stirred for 2 h before screening through a 75 micron sieve and the filtrate (ethanol wash solution) was discarded. The retentate (fibre residue) on the screen was re-slurried in 50% ethanol (1:3 w/v) and rescreened. The process was repeated two more times to ensure most starch and free phenolic acids have been removed in the ethanol wash solution. The retentate above the screen was then freeze dried (VirTis Model 50 SRC freeze dryer, SP Scientific, Warminster, PA, USA) to produce the ethanol washed fibre concentrate **(FC1)** along with most bound phenolics.

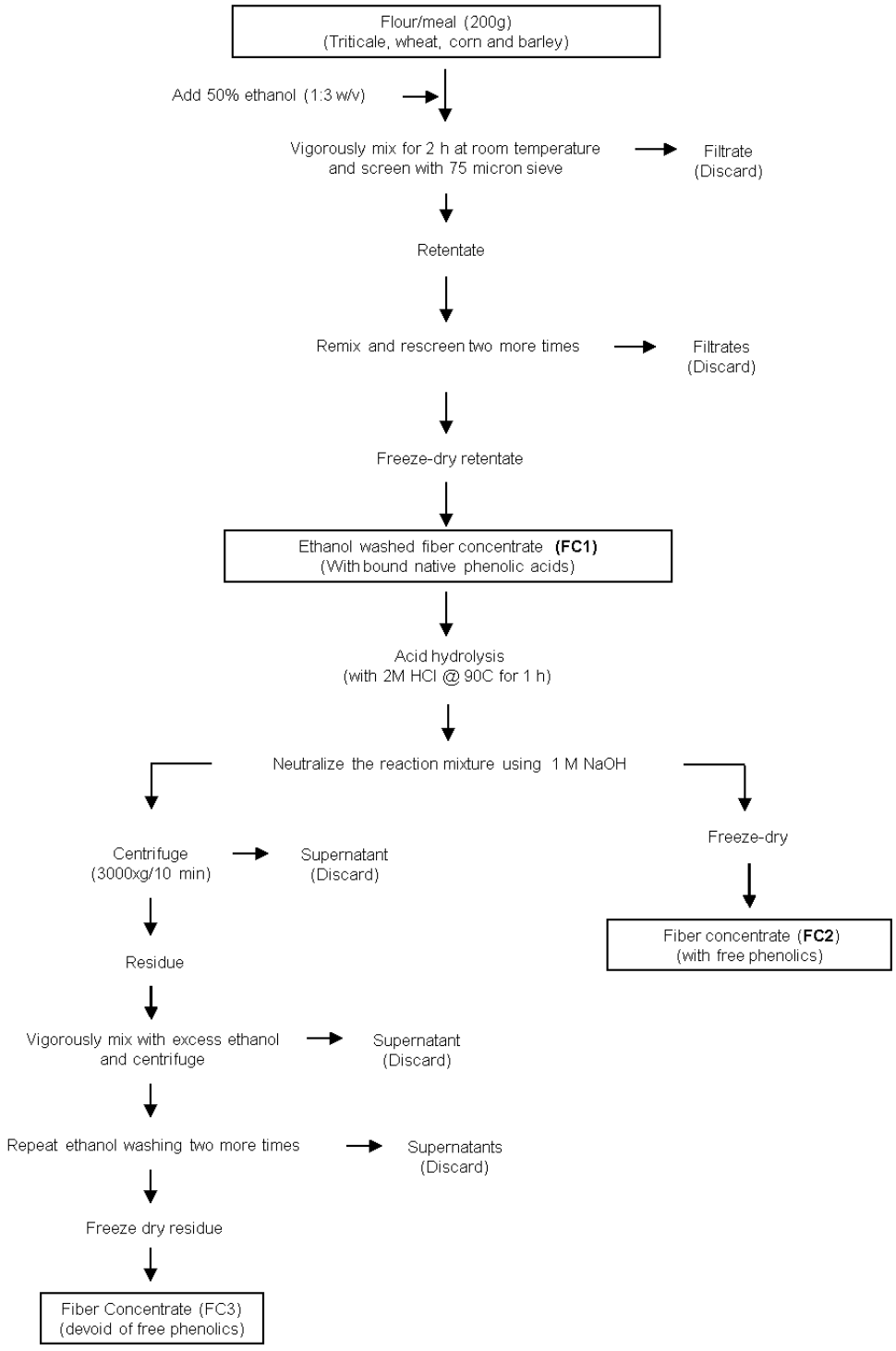

Figure.5.1 Processing of grain meals/flours into ethanol washed native fiber concentrates (FC1 -containing bound phenolics), acid treated fiber concentrate (FC2 – containing free phenolics) and acid treated and ethanol washed fiber concentrate (FC3 - devoid of phenolics)

The fibre concentrate FC1 was then acid hydrolyzed in a beaker by using 2M HCl at 90^{o}C for 1 h and then neutralized with NaOH (1M). Acid hydrolysis free both esterified as well as bound phenolics. The neutralized acid hydrolysate of FC1 was then further processed in two methods. In the first method the slurry was freeze dried "as is" to produce the fibre concentrate (**FC2)** which mostly contained acid liberated phenolic acids (i.e. free phenolics). In the second method the slurry was centrifuged (3000 x g for 10 min), supernatant discarded, and the residue was re-slurried in excess 50% ethanol (1:3 w/v) and centrifuged again (3000 x g for 10 min) to get ethanol washed residue. The process was repeated twice. The final residue was then freeze dried to produce the fibre concentrate (**FC3**) which was mostly devoid of phenolics.

5.2.5 Amylolysis of starch in the presence of fibre concentrates (FC1, FC2 and FC3)

The protocol to study the effect of fibre concentrates on starch amylolysis by α-amylase or sequential hydrolysis by α-amylase and amyloglucosidase is presented in Figure 5.2. Fibre concentrate (100 mg of FC1, FC2 or FC3) was measured in a conical flask and then mixed with appropriate amount of purified starch to adjust the total amount of starch in the mixture to 200 mg i.e. to keep the net starch content identical in all experiments. The control sample had purified starch and 100 mg of pure cellulose (Sigma-Aldrich Chemichal Company, St. Louis, MO, USA). Degree of starch hydrolysis was determined by DNS method (Bruner, 1964), where the increase in the reducing value was expressed as the weight of glucose equivalents per 100 g dry starch.

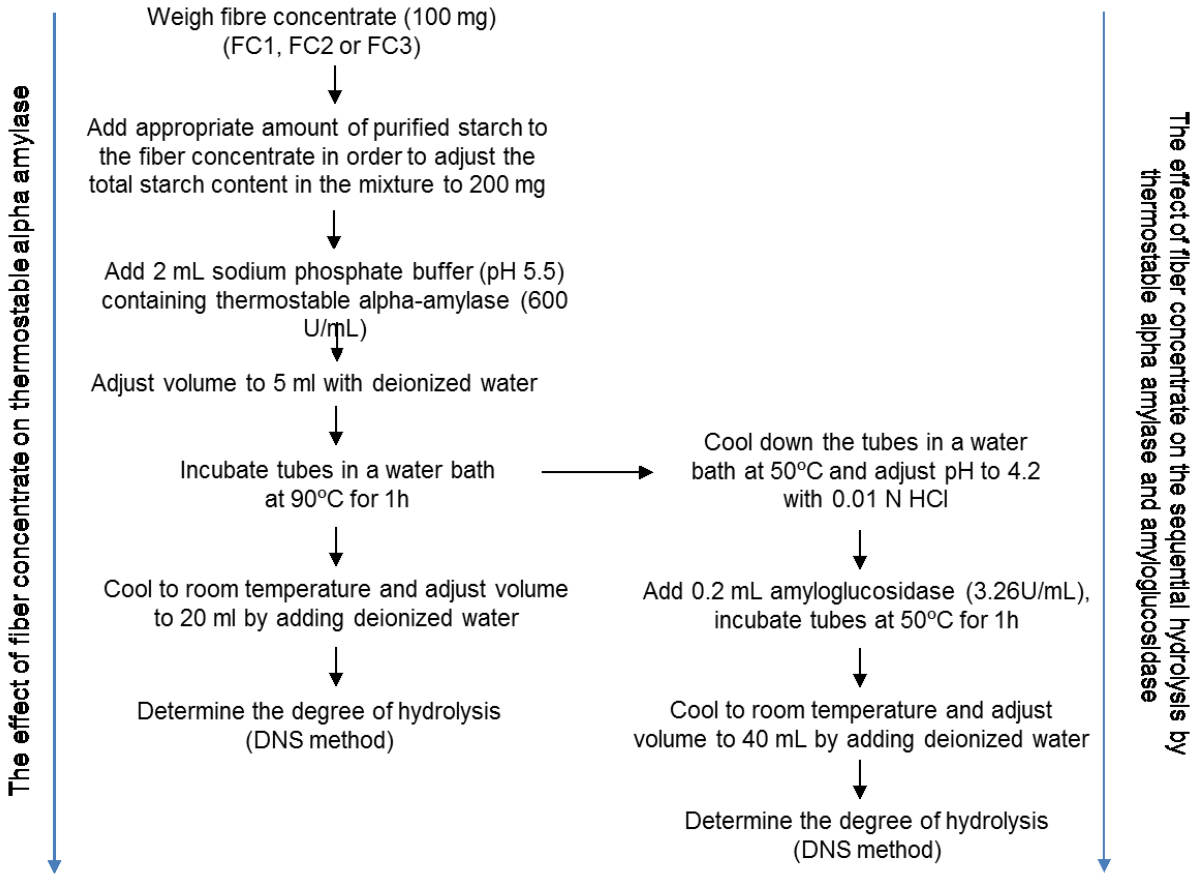

Figure 5.2: Protocol to study the effect of fibre concentrates, FC1, FC2 or FC3 on starch amylolysis

5.2.6. Statistical analysis

All chemical analyses and experiments were carried out at least in duplicate. One-way Analysis of variance (ANOVA) was performed using General Linear Model (GLM) procedure of SAS Statistical software, Version 9.1.2 (SAS institute Inc., Cary, NC, 2004). Multiple comparisons of the means were done by Turkey's test at $p < 0.05$ level.

5.3 Results and discussion

5.3.1 Chemical composition of the fibre concentrates

The chemical composition of the fibre concentrates (FC1, FC2 and FC3) isolated from triticale, wheat, corn and barley flours is presented in Table 5.1. The total dietary fiber (TDF), starch and protein contents of the fiber concentrates ranged from 55-71%, 5-19% and 6-12%, respectively. The FC3 had the lowest TDF and the lowest starch and protein contents as these components had been removed by acidic hydrolysis and subsequent ethanol washing. Palmarola-Adrados et al., (2005) reported that acid hydrolysis of wheat bran with 1% and 2% H_2SO_4 resulted in an increase in the amounts of released and hydrolyzed hemicelluloses or pentosans (i.e. dietary fiber) such as xylose and arabinose. Another study on starch-free triticale bran (SFTB) indicated that 57% arabinose which comprised the largest portion of arabinoxylan in the SFTB (about 59%) was separated and solubilized after the pretreatment of the fibrous fraction with 1.1% sulphuric acid at 120°C for 22.5 min. This could be due to the arabinoxylan structure, where the arabinose is present as side chain residues, facilitating the hydrolysis of the glucosidic bonds during the pretreatment (Garcia-Aparicio et al., 2011). The FC2 (produced through

Table 5.1: Composition of the phenolic acid-rich concentrates isolated from triticale, wheat, corn and barley flours.

Source	Fraction	Free Phenolics (GAE mg/100g)	Total Dietary Fiber (TDF) (%, db)	Starch (%, db)	Protein (%, db)
Triticale	FC1	9.99^{b}±0.35	69.3^{a}±1.7	13.94^{a}±0.66	10.15^{b}±0.17
	FC2	382.70^{a}±0.45	68.8^{b}±2.6	14.70^{b}±0.44	11.22^{b}±0.18
	FC3	7.97^{c}±0.09	63.5^{c}±1.9	6.01^{c}±0.75	8.15^{a}±0.23
Wheat	FC1	12.62^{b}±0.45	71.4^{a}±2.5	14.14^{a}±0.72	11.62^{b}±0.15
	FC2	210.36^{a}±1.09	69.8^{b}±1.8	14.51^{a}±0.75	12.34^{b}±0.26
	FC3	8.12^{c}±0.99	59.6^{c}±1.5	5.29^{b}±0.57	6.29^{a}±0.19
Corn	FC1	34.22^{b}±0.34	68.8^{a}±1.3	19.37^{a}±0.96	9.97^{c}±0.11
	FC2	675.34^{a}±0.36	67.1^{a}±1.6	18.86^{a}±0.86	8.26^{b}±0.19
	FC3	6.80^{c}±0.66	60.8^{b}±2.1	4.76^{b}±0.62	7.17^{a}±0.16
Barley	FC1	10.72^{b}±0.36	66.4^{a}±1.4	17.92^{a}±0.45	10.18^{b}±0.17
	FC2	363.43^{a}±0.21	67.9^{a}±2.4	18.12^{b}±0.45	11.29^{c}±0.15
	FC3	6.74^{c}±0.98	55.5^{b}±1.9	4.91^{c}±0.55	9.21^{a}±0.18

db, dry basis
FC1, Ethanol washed fibre concentrate
FC2, Ethanol washed and acid hydrolyzed fibre concentrate
FC3, fibre concentrate devoid of all phenolic acids
GAE, Gallic acid equivalent
Means in the same column with different letters are significantly different at $p < 0.05$.

acid hydrolysis contained the greatest quantity of free phenolics (originally existed in the bound form) followed by FC1 and FC3.Acidic and/or alkaline hydrolysis are usually used to liberate bound phenolics (i.e. break the ester bond) when extracting total phenolic compounds (Nutila et al., 2002; Kim et al., 2006), which explains the high free phenolic acids content of FC2.

5.3.2 Degree of hydrolysis of pure starches in the presence of isolated fiber concentrates from grain flours

The degree of hydrolysis/amylolysis of purified starches as affected by the addition of different fibre concentrates (FC1, FC2 and FC3) from triticale, wheat, corn and barley grains is presented in Table 5.2. The degree of hydrolysis was determined for liquefaction (i.e. after hydrolysis only with thermostable alpha-amylase) as well as for sequential liquefaction and saccharification (i.e. after sequential hydrolysis with thermostable alpha- amylase and amyloglucosidase. In general, the addition of any of the fibre concentrates (FC1, FC2 or FC3) to the purified starches significantly decreased the degree of hydrolysis of starch by alpha-amylase, and by a sequential treatment with alpha-amylase and amyloglucosidase. The addition of FC2 showed the highest reduction. This is possibly due to the liberation of bound phenolic acids from the fiber concentrates by acid hydrolysis. Free phenolics may bind to the active site of α-amylase and amyloglucosidase or to the secondary binding site of enzyme substrate complexes as non-competitive inhibitors (Funke and Melzig, 2005; Shobana et al., 2009; Sreerana et al., 2010; Tadera et al., 2006). Fractions FC1 and FC3 also showed a noticable decrease in the degree of starch hydrolysis; such changes may be due to the presence of trace amounts of free

Table 5.2 Degree of hydrolysis of purified starches in the absence and presence of fiber concentrates FC1 (with bound phenolics), FC2 (with free native phenolics) and FC3 (devoid of or contain trace amounts of phenolics)

		Degree of hydrolysis (%)	
		Liquefaction with TAA	**Liquefaction with TAA followed by saccharification with AMG**
Triticale	Starch + Cellulose	43.06^{a}±0.61	87.94^{a}±0.44
	Starch + FC1	38.33^{c}±0.17	82.40^{c}±0.15
	Starch + FC2	35.19^{d}±0.37	74.88^{d}±0.31
	Starch + FC3	40.72^{b}±0.42	85.61^{b}±0.81
Wheat	Starch + Cellulose	42.82^{a}±0.50	88.02^{a}±0.11
	Starch + FC1	38.25^{c}±0.28	84.70^{b}±0.42
	Starch + FC2	34.50^{d}±0.22	74.84^{c}±0.26
	Starch + FC3	41.33^{b}±0.39	85.28^{b}±0.88
Corn	Starch + Cellulose	42.36^{a}±0.39	91.03^{a}±0.33
	Starch + FC1	39.74^{c}±0.45	87.32^{b}±0.54
	Starch + FC2	33.50^{d}±0.19	76.11^{c}±0.98
	Starch + FC3	41.06^{b}±0.67	89.34^{ab}±0.90
Barley	Starch + Cellulose	44.61^{a}±0.22	86.37^{a}±0.89
	Starch + FC1	40.33^{c}±0.38	82.44^{b}±0.81
	Starch + FC2	36.49^{d}±0.93	75.54^{c}±0.72
	Starch + FC3	42.44^{b}±0.35	83.63^{b}±0.08

FC1, Ethanol washed fibre concentrate
FC2, Ethanol washed and acid hydrolyzed fibre concentrate
FC3, fibre concentrate devoid of all phenolic acids
TAA, Thermostable alpha amylase
AMG, Amyloglucosidase
Means in the same column with different letters are significantly different at $p < 0.05$.

phenolic acids and/or the existence of non-starch grain components in the fractions. Hemicelluloses, such as arabinoxylan and beta-glucan are two of the major non-starch carbohydrate components that are highly concentrated in the outer bran layers of grains and can also be hydrolysed and liberated during acid hydrolysis of the brans (Sasaki et al., 2000). Hemicelluloses can have two major actions when mixed with starch: a) they have the ability to absorb water, affecting the hydration of starch, thus influencing liquefaction as well as increasing the degree of starch retrogradation (molecular re-association) and the viscoelasticity of the liquefied starch mixture (Gudmundsson et al., 1991); and 2) they have the ability to form starch-pentosan complexes, and such interactions can make the starch less accessible to alpha amylase (Jankiewicz and Michniewicz, 1976; Jankiewicz and Michniewicz, 1987; Jankiewicz et al., 1979; Kim and D'Appolonia, 1977; Michniewicz et al., 1992). However, we expect that such interferences will be highly minimal due to their depolymerization by acid. In addition to pentosans and phenolic acids, several by-products can also be formed or released during the acid hydrolysis process such as furfural and 5 hydroxymethylfurfural formed by the degradation of pentoses and hexoses, respectively, as well as acetic acid. These components may also lessen starch degree of hydrolysis by amylases and also can inhibit the fermentation step during bioethanol production (Garcia-Aparicio et al., 2011; Larsson et al., 1999; Palmarola-Adrados et al., 2005).

5.4 Conclusions

Three types of fibre concentrates (FC) were prepared from triticale, wheat, barley and corn grain flours. The FC1 was rich in bound phenolic acids, FC2 had mostly free phenolic acids and FC3 contain trace amounts of phenolics. The addition of these fibre

concentrates affected the degree of starch hydrolysis with α-amylase or both α-amylase and amyloglucosidase. FC2, which mostly contained free phenolics had the greatest effect in decreasing the amylolysis of starch followed by FC1 and FC3. The study clearly indicated that both bound and free phenolics influenced the amylolysis of starch, where the greatest influence comes from free phenolics. The ethanol processing industry commonly uses crude enzyme cocktails that are not only rich in amylase, but also contain significant amounts of other enzymes that hydrolyze cell wall polysaccharides, protein, etc. These crude enzymes contain deesterases such as phenolic acid esterase (PAE) that can liberate bound phenolics into free form and thus compromise quantitative amylolysis and starch to sugar conversion efficiencies. Therefore, based on the outcome of the present study it is recommended that careful monitoring of crude enzyme cocktails for PAEs would benefit ethanol production efficiencies. Furthermore, debraning of cereal grains prior to using them in ethanol production would be beneficial because the process can eliminate bran that contains the most phenolic compounds.

CHAPTER 6

General Conclusions and Recommendations

This chapter provides a summary of the work conducted in this doctoral research, and discusses the contributions and recommendations for future research.

6.1 Conclusions

The use of renewable energy sources compared to conventional fossil fuel energy is a controversy receiving much attention in an era of increasing global energy demands. Continuing to burn fossil fuels as our major energy source, especially in light of limited fossil fuel reserves, contributes to global warming, environmental and agricultural instability, and rising ocean levels. In North America, particularly in Canada, bioethanol is produced mainly from wheat and corn. These grains represent major food sources with many food applications, thus making fuel from arable land creates a competitive market between fuel and food. The supply and demand for these two grains, corn and wheat, are influenced globally by these market trends, which may mean less availability of food corn and wheat in the developing nations.

In Canada, on the other hand, both triticale and barley are less utilized cereals (Davis-Knight and Weightman, 2008; Gibreel et al., 2009; and Wang et al., 1997), and may be considered as sources for bioethanol production with less impact on food markets. Triticale is especially a good candidate for ethanol production because it grows well on marginal lands. It is a hybrid man-made cereal grain resulting from wheat and rye and possesses wheat's nature for food production and rye's adaptive properties for difficult growing conditions. Triticale can be grown well in almost every region of Canada, and unlike wheat, it can perform well in colder and less fertile soils, sandy soils,

salty soils, acidic soils, manganese-deficient soils and drier conditions. Triticale also requires less fertilizers and herbicides, and has a greater tolerance for diseases than other crops. Indeed, it boasts a greater yield and starch content even in these unfavorable conditions (Alberta Agriculture, 2005; Gormely, 2008). These qualities make triticale the best option for dry and marginal lands of the Canadian prairies. Triticale has drawn more attention from ethanol producers over barley, since it has more starch and is hulless, making alcohol production more efficient.

Current conventional bioethanol production is increasingly using low grade cereal grains, and is a batch process which requires enzymes to hydrolyze and convert starch into dextrin and then saccharified into fermentable sugars such as glucose, maltose and maltotriose, while in parallel subject to yeast fermentation in the saccharification step. The conversion of starch into fermentable sugars is still expensive due to the extra energy used during starch gelatinization. Also, the starch conversion is not 100% efficient. This is mainly due to our lack of understanding on how the non-starch grain components interfere with the starch amylolysis. This thesis was thus motivated to better understand how different kinds of non-starch grain components, especially the phenolic compounds, affect the hydrolysis of grain starches into fermentable sugars.

In chapter 3, triticale, barley, wheat and corn grains were milled to pass through a 0.5 mm sieve, and characterized using a scanning electron microscopy (SEM) to visualize starch granules free of non-starch components. Pre-washing with water, hexane, 50% ethanol and 100% ethanol was used to determine which solvent removed the most amount of the non-starch components and how the chemical composition of each flour varied with grain type and solvent extraction system. The pre-washed flours were used in

a hydrolysis study to investigate to what extent these non-starch components affected the degree of starch hydrolysis. Although the extent of alpha-amylase hydrolysis was comparable for all flours, the results had pronounced differences when the hydrolysis was done by sequential treatment with alpha-amylase and then amyloglucosidase. Non-starch constituents were observed to have significant negative effects on the degree of hydrolysis of starch in whole grain flours, and the extraction of non-starch components from grains prior to bioconversion improved starch hydrolysis. This information represents a practical foundation of information that is relevant and could benefit the starch-based bioconversion industry.

Non-starch grain components, especially the phenolic acids, were incorporated into the amylolysis/hydrolysis experiments individually to investigate how each component in turn may impact the production of bioethanol. In chapter 4, the inhibitory effect of phenolic acids was studied with a model system within the context of industrial ethanol production to demonstrate that the interaction between phenolic acid and hydrolysis enzyme is significant. The interaction between phenolic acid and starch/dextrin substrate also significantly contributed to the inhibitory effect. Boiling enhanced the interactions in the starch–enzyme–phenolic acid system to inhibit starch conversion to sugars. In the light of this, in future pilot/industrial experiments it was proposed to avoid jet cooking of whole grain flours in the presence of thermostable alpha amylase. It was predicted this would reduce the conversion efficiency of starch into yeast fermentable sugars. From this perspective, cold starch hydrolysis and simultaneous saccharification fermentation (SSF) represents a better approach than jet-cooking and SSF. Furthermore, the findings on the interaction of phenolic acids with both the enzyme

as well as starch/dextrins offer guidance to the bioethanol industry. Judicious selection of grain varieties low in phenolics, and better process optimization choices (ie. milling, pearling, pre-treatments, jet-cooking, etc) is highly recommended in order to enhance the ability of the enzymatic system to access the greatest amount of starch while minimizing the inhibitory interactions with other grain components.

In chapter 5, fibre concentrates enriched (FC1 and FC2) or depleted (FC3) in phenolic acids were prepared from grain flours (triticale, wheat, corn and barley). The FC1 (ethanol-washed fibre concentrate) was rich in bound phenolic acids, whereas FC2 (ethanol washed and acid hydrolyzed fibre concentrate) was rich in free phenolic acids. The FC3 (ethanol washed –acid hydrolyzed – ethanol washed fibre concentrate) showed trace amounts of phenolic acids. FC3 also contained the least amount of starch and protein. The addition of the fibre concentrates to starch generally resulted in a decrease of the degree of starch hydrolysis/amylolysis in both liquefaction and saccharification processes. This was mainly due to the presence of non-starch components, such as phenolics. However, the greatest reduction of degree of hydrolysis was with the addition of FC2, indicating that bound phenolic acids in their free form may play a dominant role in the inhibition of starch hydrolysis, which may involve the action of other non-starch bran components released during the acidic pretreatment.

6.2 Contributions

This thesis research focused on studying four of the main cereal grains grown in Canada (triticale, wheat, barley and corn). These grains represent key energy sources that can be utilized in the emerging and evolving bioethanol industry. In order to enhance and accelerate the process of converting grain starches into fermentable sugars, and

minimizing processing costs at the same time, different processes were studied with the whole grain flours especially focusing on the inhibitory effects of phenolic compounds on amylolysis to better understand how to achieve the greatest yields from the raw materials.

The findings of this thesis help to fill a research gap existing in the literature and are applicable to both academic and industrial researchers alike, representing relevant contributions to the future of the bioethanol industry. The main conclusions can be summarized as follows:

- Whole grain flours contain various components besides starch that negatively affect starch amylolysis. In this study, the milling conditions were determined to achieve the best starch particle size as visualized by SEM to be mainly free from other constituents and ready for the pre washing treatments. (Chapter 3)
- Different types of solvents were used to remove non-starch components that can interfere with starch hydrolysis using a pre-washing technique. The use of these solvents was documented and ranked in their ability to remove the greatest amount of the non-starch components that interfere with starch hydrolysis (50% ethanol had the highest removal effect followed by water and 100% ethanol then hexane came the last), as shown in chapter 3.
- Phenolic acid complexes, as well as individual phenolic acids, should be removed and washed out before the conversion of starch into fermentable sugars, since they significantly decreased the starch degree of hydrolysis, as shown in chapters 4 and 5.
- Eliminating the non-starch grain components from the whole grain flours assists the conversion of grain starch to sugars. Using 50% ethanol as a pre-washing solvent, which washed out most of the non-starch grain components, increased the degree of

hydrolysis of starch by 22% for both triticale and barley, 20% for wheat and 11% for corn. This will assist the bioethanol industry to be more efficient, viable, and profitable by being able to use non-corn products, such as triticale, wheat, and barley. These non-starch extracted byproduct components such as phenolics, which function as an excellent antioxidants, could be marketed separately and add value to the process. It is noteworthy that soluble fibre β-glucan, could also be extracted and separated before using this flour source for the starch conversion into sugars. This would be especially beneficial for barley with a β-glucan content as high as 7%, and could be seen as a value added by product.

6.3 Recommendations

- Solvent prewashing and removal of non-starch grain components can be considered as an additional unit operation to improve starch conversion efficiency into yeast fermentable sugars.
- Since the phenolic compounds are mainly present in the bran layers of whole grains, it is recommended that de-branning would benefit ethanol operation.
- Since high temperature excelled the negative effect of phenolics on starch amyliolysis, cold starch hydrolysis would benefit ethanol production.
- Since free phenolics influence starch amylolysis more than bound phenolics, extra care must be given in the selection of crude enzymes for ethanol production, because any contaminant "phenolicacid-esterase" would liberate bound phenolics into their free form.

6.4 Future work

The quantitative conversion of starch into ethanol is one of the most challenging processes the bioethanol industry faces. This is especially true when using whole grain flour which contains so many other non-starch grain components that can interfere with starch hydrolysis.

Based on the studies presented in this dissertation, the following areas for future research are suggested:

- Other non-starch grain components such as phytic acid, pentosans, β-glucan, proteins, etc, in their native and enzyme hydrolysed forms can negatively influence starch amylolysis, and therefore warrant investigation.
- After pre-washing, the whole residue (starch+fibre) could also be used for the bioethanol industry. A pilot scale investigation is proposed.
- In the ethanol industry, fermentation produces a by-product known as dried distiller's grain with solubles (DDGS). This product contains both non-starch/fermentable components plus residual starch from the starch–sugar conversion process. Therefore, it is important to further understand how starch may escape from the process and precisely correlate how non-starch components and other factors affect this loss.
- This study can be extended by using different genotypes from each grain cultivated in different regions across Canada in order to more precisely understand the influence of the non-starch grain components from different grain sources. This pre-processing analysis system would allow adjusting and adapting the pre-treatments to each batch of grain, representing various genotype and regional influences, in order to optimize the starch to sugar conversion efficiency.

- While SSF-traditional technology for ethanol production dominates in North America, the new SSF-raw starch (uncooked) hydrolysis technology is getting popular among the ethanol industry. Therefore, the extension of the present study into SSH-raw starch hydrolysis would be beneficial.
- Evaluate the cost of debranning against the benefit on hydrolysis due to bran removal need to be established in the industrial prespects.

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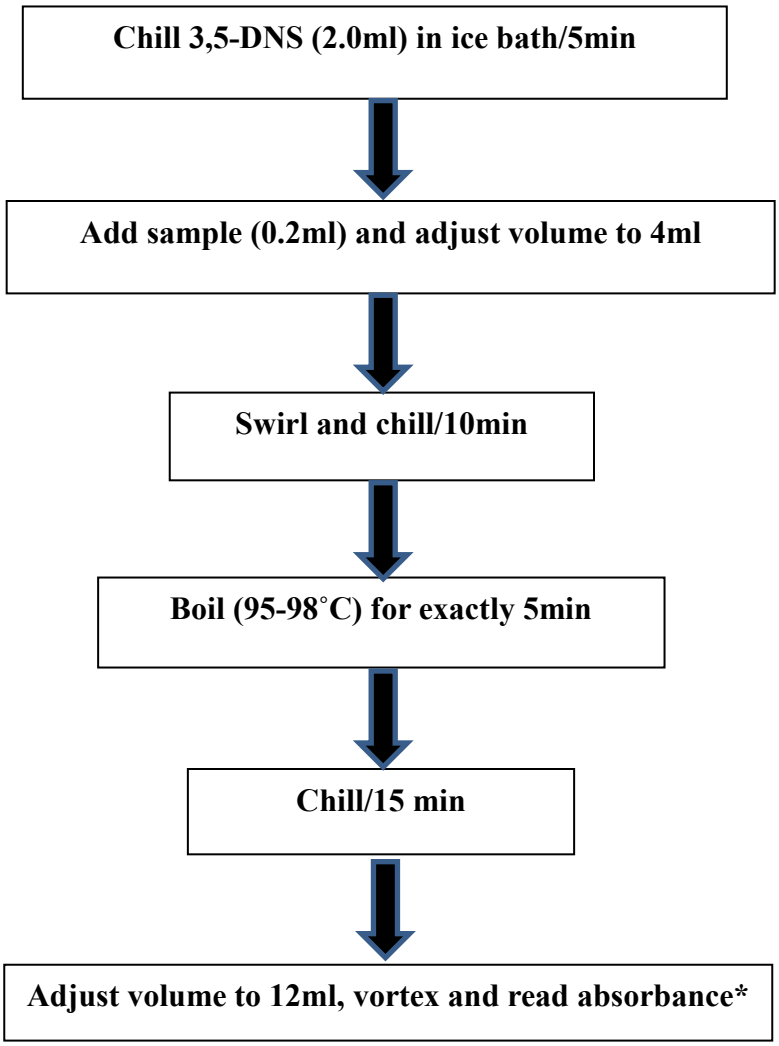
APPENDIX
Determination of reducing value using the 3, 5-dinitrosalicylic acid procedure of Bruner (1964).

Preparation of regents:

1. 20g of 3,5-dinitrosalicylic acid (3,5-DNS) was dissolved in 700ml 1.0 NaOH and made up to 1L with miliQ water. The solution was then filtered through Whatman paper (No. 2). It was kept in a dark bottle covered with aluminum foil until required for analysis.
2. Maltose standard (27.6 mM, stock solution) was prepared by dissolving 500 mg maltose into 50 ml miliQ water.

Procedure:



***The absorbance readings were taken at 540 and 590nm for the low and high concentration curves, respectively.**

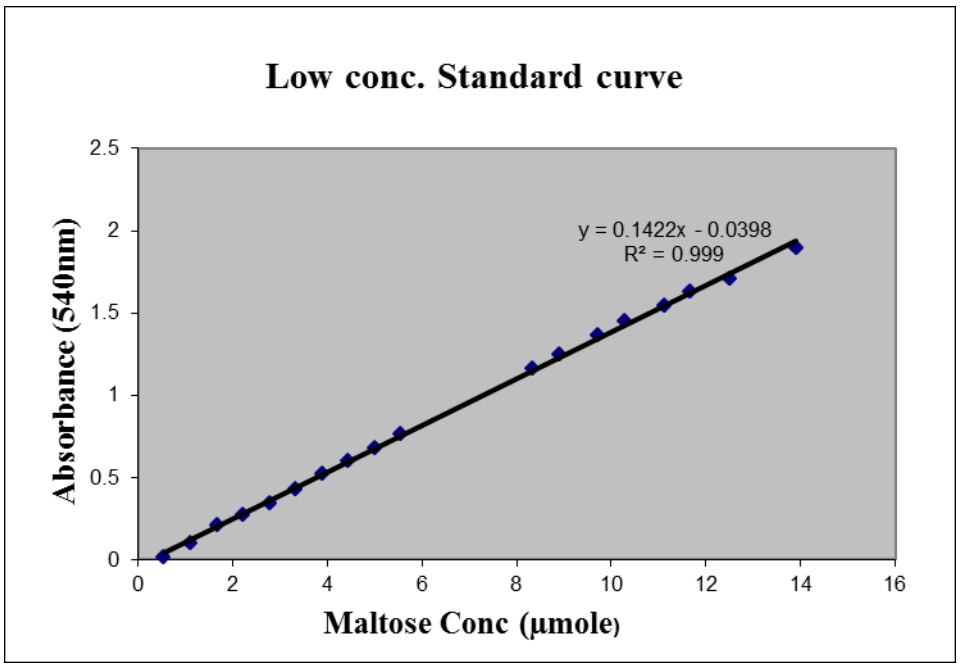
Standard curve:

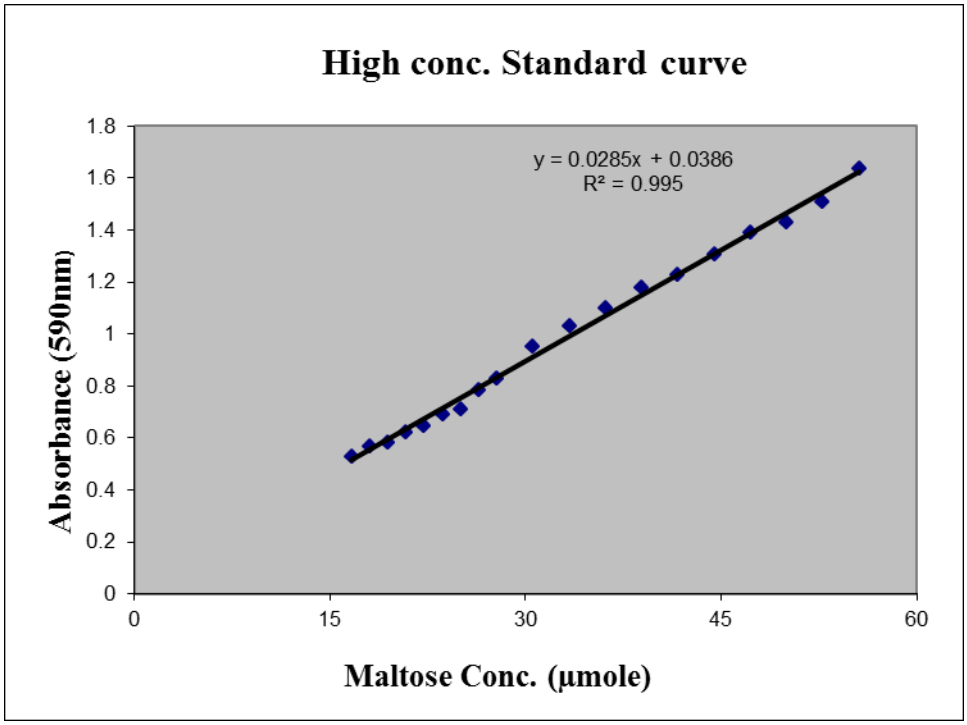
The absorbance readings are then used to create a standard curve and the linear regression line determined. Two standard curves (low and high concentration curves) were created. The standard (maltose) used for the determination of reducing sugar ranged from 0.5-14 µmoles and 12-55 µmoles.

Calculations:

The appropriate standard curve is used to determine the moles of maltose in 0.2ml aliquot. The moles of maltose are then used to calculate the total maltose formed and the degree of digestion (reducing value) using the following formula:

- µmoles of maltose in 0.2ml aliquot (at time=t) is obtained from the standard curve regression.
- Total calculated maltose = [(µmoles of maltose (at time=t) - µmoles of maltose (at time 0) x vol of hydrolysis slurry]/0.2ml
- Weight of glucose equivalents produced = Total calculated maltose (µmol) x mw glucose (g/mol)/ 1000 (µg/mg).
- Degree of hydrolysis (reducing value) at time t (%) = [(wt of glucose equivalents) x 100]/ wt of starch (db).


Low conc. Standard curve
y = 0.1422x - 0.0398
R² = 0.999
Absorbance (540nm)
Maltose Conc (µmole)
0
0.5
1
1.5
2
2.5
0
2
4
6
8
10
12
14
16


High conc. Standard curve
y = 0.0285x + 0.0386
R² = 0.995
Absorbance (590nm)
Maltose Conc. (µmole)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
0
15
30
45
60