University of Alberta

Extraction of Triticale Distillers Grain Proteins for Adhesive Development

by

A. M. Nandika Priyantha Bandara

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To my dearest mother and father,

for their endless love and support

ABSTRACT

Triticale (×*Triticosecale Wittmackk*) is being actively explored as a feedstock for bioethanol production in Canada. As the main co-product of bioethanol processing, triticale distillers grain contains 20-43% protein (dry basis), and mainly used as animal feed. The purpose of this study was to find new uses of triticale protein as adhesive. Proteins from triticale distillers wet grains (DWG) and distillers dried grains with solubles (DDGS) were extracted by five methods: pH shifting method, 60% ethanol, alkaline ethanol, glacial acetic acid, and enzyme-aided extraction. Extraction with alkaline ethanol and glacial acetic acid gave higher protein contents (~61–65%) than those obtained from other extraction methods (~23–24%); however, enzyme-aided extraction using Protex 6L yielded 75–82% protein at a content of 43–57%. To prepare adhesives, proteins were modified by NaOH, urea and glutaraldehyde; effects of modifications on protein structure were analyzed by FTIR, and their adhesion properties were measured by automated bonding evaluation system (ABES II). The highest ($p<0.05$) adhesion strength was observed in acetic acid extracted proteins; glutaraldehyde modification acetic acid extracted proteins increased the adhesion strength from 2.56, 0.84, 1.11 MPa to 3.86, 2.03, 2.60 MPa for dry, wet and soaked adhesion strength, respectively. Increases in α-helical conformation and molecular weight were observed for glutaraldehyde modified proteins. Development of adhesive from triticale proteins might represent new uses of triticale distillers grain.

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LIST OF ABBREVIATIONS

DDGS:	Distillers dried grains with solubles
DWG:	Distillers wet grains
DWGad:	Air dried distillers wet grains
DWGfd:	Freeze dried distillers wet grains
DDGSae:	Distillers dried grains with solubles, alkaline ethanol extracted proteins
DWGae:	Distillers wet grains, alkaline ethanol extracted proteins
DDGSaa:	Distillers dried grains with solubles, acetic acid extracted proteins
DWGaa:	Distillers wet grains, acetic acid extracted proteins
P6L:	Distillers dried grains with soluble proteins, extracted with Protex6L enzyme
TDGP:	Triticale distillers grain protein
SDS-PAGE:	Sodium dodecyl sulphate poly acrylamide gel electropherosis
FTIR:	Fourier Transform Infrared Spectroscopy
ADF:	Acid detergent Fibre
NDF:	Neutral detergent fiber
MW:	Molecular weight
HMW:	High molecular weight
KDa:	Kilodalton
MPa:	Mega Pascal

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Overview of triticale

Triticale (×*Triticosecale Wittmackk*) is a minor crop grown in most part of the world. It is the first man-made cereal developed by hybridization of female parent wheat (*Triticum* ssp.) and male parent rye (*Secale* ssp.) (Oettler 2005; Mergoum et al 2009). The primary objective of developing triticale was to combine the yield potential and food value of wheat with the hardiness and environmental adaptability of rye (Ruskin 1989; Ammar et al 2004; Mergoum et al 2009; Wrigley et al 2010). Triticale is now a commercial crop grown in a variety of climatic conditions.

1.1.1. Background and history of triticale

The first ever triticale hybrid was developed in 1875 by A. Stephen Wilson in Scotland (Ammar et al 2004; Chapman et al 2005). But it was a sterile hybrid due to the problem associated with pollen functionality (Ammar et al 2004; Wrigley et al 2010); therefore, attention was given to the cytology and meiotic characteristics to improve the crop performance (Oettler 2005). A stable and reproductively functional triticale variety or the first ever true triticale variety was developed in 1888 by a Germen scientist named Rimpau (Ammar et al 2004; McGoverin et al 2011). Triticale hybridization program was initiated by Muntzing in Sweden and by Oehler in Germany in 1930s and triticale breeding

program was initiated in USSR, Hungary, Switzerland, and Sweden in 1940s (Oettler 2005). The first commercial hexaploid triticale breeding program was started in 1950s in Spain, Hungary and Canada (Ammar et al 2004; Oettler 2005). “Triticale No. 57” and “Triticale No. 64” are the first commercially available triticale cultivars released by Hungarian triticale breeding program in 1968 made by intercrossing hexaploid and octoploid triticale varieties (Ammar et al 2004). Poland (Arseniuk and Oleksiak 2004) and France (Ruskin 1989) started their own triticale breeding programs in 1960s while Brazil, Portugal (Ruskin 1989) and Australia (Mergoum and Macpherson 2004) began their work in 1970s. Initiating a triticale breeding program in International Maize and Wheat Center (CIMMYT) is a key milestone in triticale breeding programs as it led an essential role in triticale crop development (Ammar et al 2004; Mergoum et al 2004).

Canadian triticale breeding program was started in 1950s at the University of Manitoba. The first commercial hexaploid triticale variety named “Rosner”, which is a spring triticale variety, was released from the same university in 1969 (Ammar et al 2004; Chapman et al 2005). “Welsh” and “Carman” were the other spring triticale varieties released by Canadian triticale breeding program at University of Manitoba during the early stages of development. During the same time, winter triticale varieties such as “OAC Wintri”, “OAC Trillium”, and “OAC Decade” were released from winter triticale breeding program established at Ontario Agricultural College (OAC) in Guelph, Canada. Later, several triticale varieties were developed in Canada and

around the world with different quality attributes that make them suitable for a variety of environmental and soil conditions (Ammar et al 2004; Mergoum et al 2009).

1.1.2. Production status in Canada

In addition to the conventional breeding method, the adoption of new breeding technologies has increased its cultivation around the world at different climatic conditions (Mergoum et al 2009).

Table 1.1 – World triticale production volumes (metric tons) and harvested area (ha) in 2009. Adapted from Chapman et al (2005) and FAOSTAT (2010),

Country	Production volume (Metric tons)	Area harvested (ha)
Poland	5,234,000	1,465,000
Germany	2,514,390	401,081
France	2,015,600	355,506
Belarus	1,788,310	516,589
Australia	545,000	350,000
Russia	508,460	187,000
Lithuania	426,000	136,100
Hungary	360,719	125,365
China	350,000	206,000
Sweden	255,400	53,700
Denmark	233,100	44,400
Check republic	222,711	52,950
Spain	140,600	61,100
Brazil	122,495	65,525
United Kingdome	66,000	16,000
Switzerland	56,295	9,279
Belgium	45,117	6,192
Portugal	33,000	20,000
Canada*	500,000	110,000

* based on the data from Chapman et al (2005)

Currently, the major triticale growing countries in the world are Poland, Germany, Canada, China, Australia, Mexico, Belgium, and Switzerland (Kent and Evers 1994; Mergoum et al 2004). Table 1.1 shows the world triticale production volumes and area harvested in selected major triticale producers.

World triticale production has increased over the years at a steady manner. World production and the total area harvested are illustrated in figures 1.1. According to the 2009 Food and Agriculture Organization data, Poland is the leading triticale producer in the world, followed by Germany, France, Belarus, Australia and Russia based on production volumes (FAOSTAT 2010).

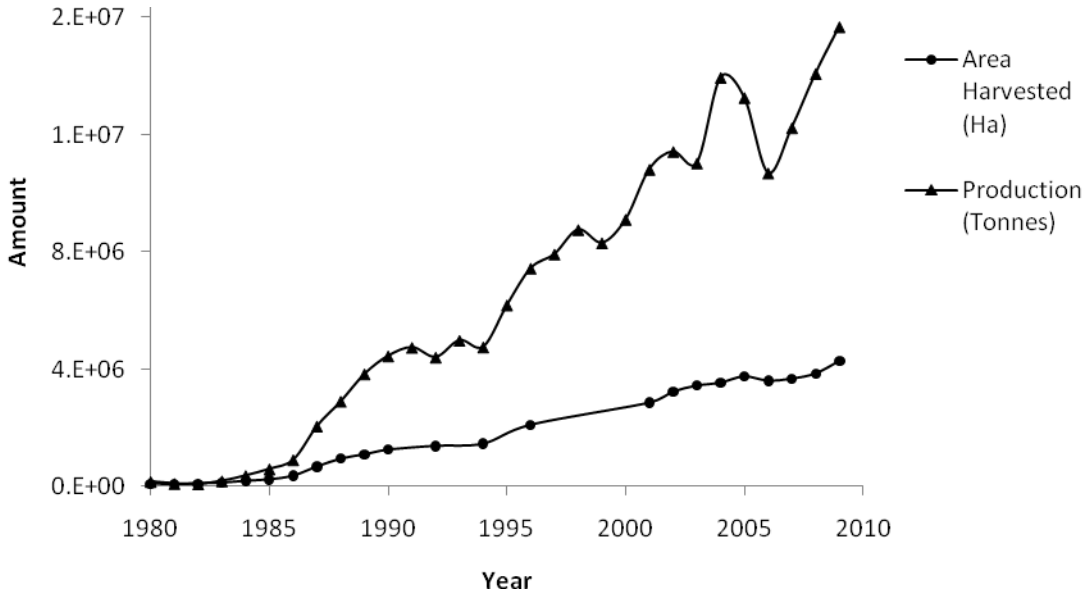

Figure 1.1 – World triticale cultivation area and production quantity since the 1980s (adapted from FAOSTAT 2010).

Even though Poland started their triticale program in 1960s, and released their first commercial variety (winter variety “Lasko” and spring variety “Jago”) in 1988, they experienced a rapid growth in triticale production over the past two decades (Arseniuk and Oleksiak 2004). The highest yield per land area was reported in Belgium, followed by Switzerland (FAOSTAT 2010; McGoverin et al 2011).

Canadian triticale production increased steadily since its first introduction to the country. Cultivation area has increased from 17,000 ha in 1996 to 110,000-120,000 ha by 2003 (Chapman et al 2005; Briggs 2007). Western Canada is the largest triticale producer, where Alberta accounts for 80% of total Canadian triticale production (Chapman et al 2005). Both winter and spring varieties grown in Canadian prairies are used mainly for forage applications, and as feed grains for pig, poultry and cattle (Salmon 2004; Briggs 2007). Most of the spring varieties prefer brown soil zones in Alberta, Saskatchewan, and Manitoba whereas winter varieties prefer brown and black soil zones, which provide agronomic advantage to triticale over the other crops (Ammar et al 2004; Salmon 2004).

1.1.3. Composition of triticale grain

Triticale grain composition can vary with the growing environment, climatic conditions, soil conditions and the triticale variety (Chapman et al 2005). Table 1.2 illustrates the chemical composition of winter and spring triticale varieties grown in Canada. According to a study done by Salmon et al

(2002), chemical composition of Canadian triticale varieties does not vary greatly among each other.

Table 1.2 – Chemical composition of Western Canadian triticale varieties. Adapted from Salmon et al (2002)

Chemical	Winter variety		Spring variety			
composition	*Pika*	*Bobcat*	*AC Certa*	*AC Ultima*	*Pronghorn*	*AC Vista*
Moisture	7.9	7.1	7.0	7.7	8.5	7.3
Ash	1.95	2.00	1.96	2.07	1.78	1.83
Protein	13.1	12.9	11.6	12.7	11.6	12.2
Lipid	1.65	1.78	1.71	1.72	1.66	1.65
Beta-glucan	0.65	0.64	0.79	0.54	0.50	0.62
Starch	54.7	55.36	54.0	57.3	59.4	58.1
Pentosan	4.84	3.78	4.46	4.81	5.44	4.17
SDF			3.68	3.61	2.10	3.38
ISF			14.53	12.08	12.97	11.18
TDF			18.21	15.69	15.07	14.56

(Mean %, w/w dry matter basis, except for moisture content)

SDF: Soluble dietary ficre, ISF: Insoluble dietary fibre, TDF: Total dietary fibre

The starch content of the triticale varieties grown in western Canada is close or higher than those of wheat (Salmon, Temelli, Spence. 2002; Ammar et al 2004). Around 66–73% starch content was reported for triticale and the amylose content was 12.8–35.1% out of the total starch (Leon et al 1996; Ciftci et al 2003; Pejin et al 2009). The amylose content can vary greatly among the triticale varieties (Mohammadkhani et al 1999; Martin et al 2008; Dennett et al

2009). High amylose content is beneficial in human food application as a low glycemic food. The content of triticale non-starch polysaccharides such as arabinoxylans is equal to those of wheat (O'Brien 1999). Table 1.3 represents the chemical composition of rye, wheat and triticale.

Table 1.3 – Chemical composition of triticale, wheat and rye grains. Adapted from Chapman et al (2005).

	Triticale	Wheat	Rye
Protein	10.3 – 15.6	9.3 – 16.8	13.0 – 14.3
Starch	57 – 65	61 - 66	54.5
Crude fibre	3.1 – 4.5	2.8 – 3.9	2.6
Free sugars	3.7 – 5.2	2.6 – 3.0	5.0
Ash	1.4 - 2.0	1.3 – 2.0	2.1

(% grain weight, dry weight basis)

1.1.3.1 Triticale protein

Triticale has lower protein content compared to Canadian Western Red Spring (CWRS) wheat, but higher than that of barley, oat, rye and corn (Chapman et al 2005). Depending on the variety and growing conditions, the total crude protein content of triticale can vary from 9% to 20% (Johnson and Eason 1988; Heger and Eggum 1991; Boros 1999; Erekul and Köhn 2006; Kara and Uysal 2009; Pejin et al 2009). Although early triticale varieties contain higher protein contents than those of the bread wheat varieties, the protein content of newly developed triticale cultivars is comparatively lower due to the increased starch content (Stallknecht et al 1996). In contrast to the reduction in

protein content, lysine content increases in low protein varieties. Triticale protein is considered to have higher biological value due to higher lysine content, which is a deficient amino acid in most of the other cereal grains (Kies and Fox 1970; Villegas et al 1970; Stallknecht et al 1996).

Protein composition in triticale can vary with different varieties. Several analytical techniques, such as SDS-PAGE and Acid – Polyacrylamide gel electrophoresis (A-PAGE) (Tohver et al 2000; Makarska et al 2008; Salmanowicz and Nowak 2009), Lab-on-a-Chip method (Jonnala et al 2010), capillary zone electrophoresis (Salmanowicz and Nowak 2009), and confocal laser scanning microscopy, (Naguleswaran et al 2011) were used to characterize triticale protein composition. High proportions of albumins and globulins were observed in some triticale varieties using size exclusion chromatography (Jonnala et al 2010; Naeem et al 2002). Gliadins and secalins, monomeric proteins with intra-disulfide bonds, account for more than 50% of storage proteins in triticale (Siriamornpun et al 2004). Other several polymeric proteins stabilized by inter-chain disulfide bonds such as high molecular weight glutenin, low molecular weight glutenin, and high molecular weight secalins were reported (Siriamornpun et al 2004; Salmanowicz and Nowak 2009).

Electrophoretic characterization of triticale protein using lab-on-a-chip method has revealed the presence of two high molecular weight (HMW) proteins named HMW glutenin sub-units, which are comparable to HMW glutenin in wheat, and HMW secalins, which are comparable to secalins in rye (Jonnala et al 2010). In another study, SDS-PAGE analysis showed that the

glutenin pattern in most triticale cultivars is closely related to that of rye (Tohver et al 2005) where some varieties showed similar glutenin patterns to wheat. In a recent study, the distribution of proteins in triticale starch granules has been studied using confocal laser scanning microscopy and proteins were observed in both granular surface and internal channels of triticale starch (Naguleswaran et al 2011).

1.1.4. Potentials and new trends in triticale utilization

Since its first introduction to the world, triticale has been screened rigorously for various applications including food, feed and other industrial uses (McGoverin et al 2011). Due to the agronomic advantages of triticale, researchers focused on evaluating its baking potentials as an alternative cereal (Naeem et al 2002). Even though there are limited food applications in some countries such as Canada, India, and USA, triticale is not used as a major raw material for the baking industry around the world (Stallknecht et al 1996; Salmon et al 2004). Soft texture of triticale flour is the main issue in using triticale for baking purposes (Li et al 2006). Dough properties such as increased stickiness, inferior rheological properties and low strength in gluten network were observed in triticale dough compared to the wheat dough (Naeem et al 2002; Tohver et al 2005; Martinek et al 2008). Low water absorption, low dough strength due to poor gluten network, shorter dough development times and less tolerance to mixing were also reported in triticale dough mixtures in studies done using farinograph, alveograph, and mixograph (Lorenz et al 1972; Macri et al

1986; Rakowska and Haber 1991). Other than baking potential, triticale flour has been evaluated for tortilla chip production (Serna-Saldivar et al 2004). 100% triticale flour resulted in defective tortilla chips, whereas 50% blend with wheat flour provided acceptable performance. Crackers (Perez et al 2003) and high fiber extruded snacks (Onwulata et al 2000) were also developed using triticale flour. Triticale has the potential to be used for food consumption alone or in combination with wheat flour (Pena 2004).

Currently, triticale is primarily used as an animal feed in the livestock industry, either as grain, forage, silage, hay and straw or as a combination of those (Stallknecht et al 1996; Salmon et al 2004). Potential use of triticale as poultry feed has been well studied in the past (Johnson and Eason 1988; Boros 1999; Ciftci et al 2003; Jondreville et al 2007; Pourreza et al 2007; Zarghi and Golian 2009). A higher content of phosphorous in triticale compared to the other feed grains provides a distinct advantage to use triticale in poultry diet as they reduce the requirement for phosphorous supplementation. Furthermore, the presence of phytase enzyme in triticale provides additional advantage in poultry feed applications as it reduces the cost of phytase enzyme, which is added in feed formulation for releasing bound nutrients, specifically minerals such as iron (Jondreville et al 2007). Although it contains a high content of lysine (Brand et al 1995), increased proportions of triticale in swine diet have reduced the feed intake of pigs. Apart from these applications, triticale has been successfully used in ruminant feed formulations such as goat, sheep and cow diets (Emile et al 2007; Fulkerson et al 2007; Lema et al 2007; Vatandoost et al 2007).

Producing bioethanol is the most recent trend in triticale application (McGoverin et al 2011). The presence of an autoamylolytic enzyme complex, which converts starch into fermentable sugars (Pejin et al 2009), provides a competitive advantage to triticale over the other feedstock in bioethanol production (Eudes 2006) due to low external enzyme requirement (McGoverin et al 2011). Cost efficiency of bioethanol production from winter cereals has been studied by several researches (Rosenberger et al 2002; Boehmel et al 2008; Deverell et al 2009). According to the results of Rosenberger et al (2002), triticale showed higher cost efficiency in bioethanol production compared to rye and wheat. Other than starch, lignocellulosic biomass such as triticale straw and hay were also used in bioethanol production studies (Chen et al 2007). In a study about combustion quality of whole crop as a solid fuel source, triticale was reported as the best feedstock due to low NOx and SO_2 emission compared with rye and wheat (Lewandowski and Kauter 2003). Increasing demand for an alternative source of biofuel has encouraged the utilization of triticale due to its low food value (McGoverin et al 2011).

1.2 Bioethanol industry

During the 20^{th} century, world energy consumption has increased by 17 fold, mainly due to the rapid growth in world population and industrialization, where fossil fuels provide most of the energy requirement in the world (Demirbas 2007). These conventional energy sources also provide other products such as fine chemicals, pharmaceuticals, detergents, synthetic fibers,

pesticides, fertilizers, and other petroleum based chemicals (Demirbas 2007; Naik et al 2010). Growing concerns on sustainability, environment impact, non-renewability, economic aspects and energy security has prompted to alternative energy sources to meet the future energy demands (Kamm et al 2006). Production of biofuels from renewable biomasses such as starch, lignocellulosic biomasses, plant and animal oils, and other non-conventional biomasses has gained the interest during the past three decades mainly because of their distinctive advantages such as renewability, low environment impact, and domestic energy security (Demirbas 2007; Naik et al 2010). In 2010, 13 billion gallons of bioethanol have produced in United States (RFA 2011) alone, and the amount is continuously increasing. Brazil is the leading producer of bioethanol, closely followed by USA where these two countries accounting for more than 80% of world bioethanol production (Berg 2004). Sugar cane in Brazil and corn in USA are the main feedstock for bioethanol production. Several other sources such as wheat, sorghum, tuber crops, and other minor crops are being used for bioethanol production (Kim and Dale 2004). In Canadian bioethanol production, wheat is extensively used as the main feedstock, but recently formed Canadian Triticale Biorefinery Initiative aims to develop triticale as the main feedstock for bioethanol production (CTBI 2011). Crops with fermentable sugars (sugar cane, sugar beets, and molasses) and starch based materials were used as the feedstock for first generation bioethanol production where lignocellulosic materials were used as the second generation raw materials (Cardona and Sánchez 2007; Sanchez and Cardona 2008). Most of the current bioethanol production plants

are based on first generation bioethanol technology using starch-based feedstock for bioethanol production (Cardona and Sánchez 2007).

1.2.1 Bioethanol production methods

Dry-milling and wet-milling are two main technologies used in the first generation starch-based bioethanol plants (Monceaux and Kuehner 2009). In 2006, approximately 82% of the ethanol plants operated in USA was based on dry-milling technology and the remaining 18% used wet-milling technology (Nichols and Bothast 2008). Figure 1.2 illustrates the basic production flow and differences between dry milling and wet milling ethanol production technologies.

Apart from the steeping, degermination, and gluten separation, the other processing steps are similar in both wet milling and dry milling ethanol production. In dry milling ethanol production, saccharification and fermentation is carried out simultaneously but are done separately in wet milling (Nichols and Bothast 2008). Wet milling ethanol plants provide valuble co-products such as germ oil, gluten meal, and gluten feeds (Johnson et al 2003) where dry milling ethanol plants provide basically ethanol and distillers dried grains with solubles (Kelsall and Lyons 2003). However, due to the lower capital and operating expenses associated with dry milling ethanol production, it has become the main technology used in the first generation bioethanol plants (Kelsall and Lyons 2003; Nichols and Bothast 2008).

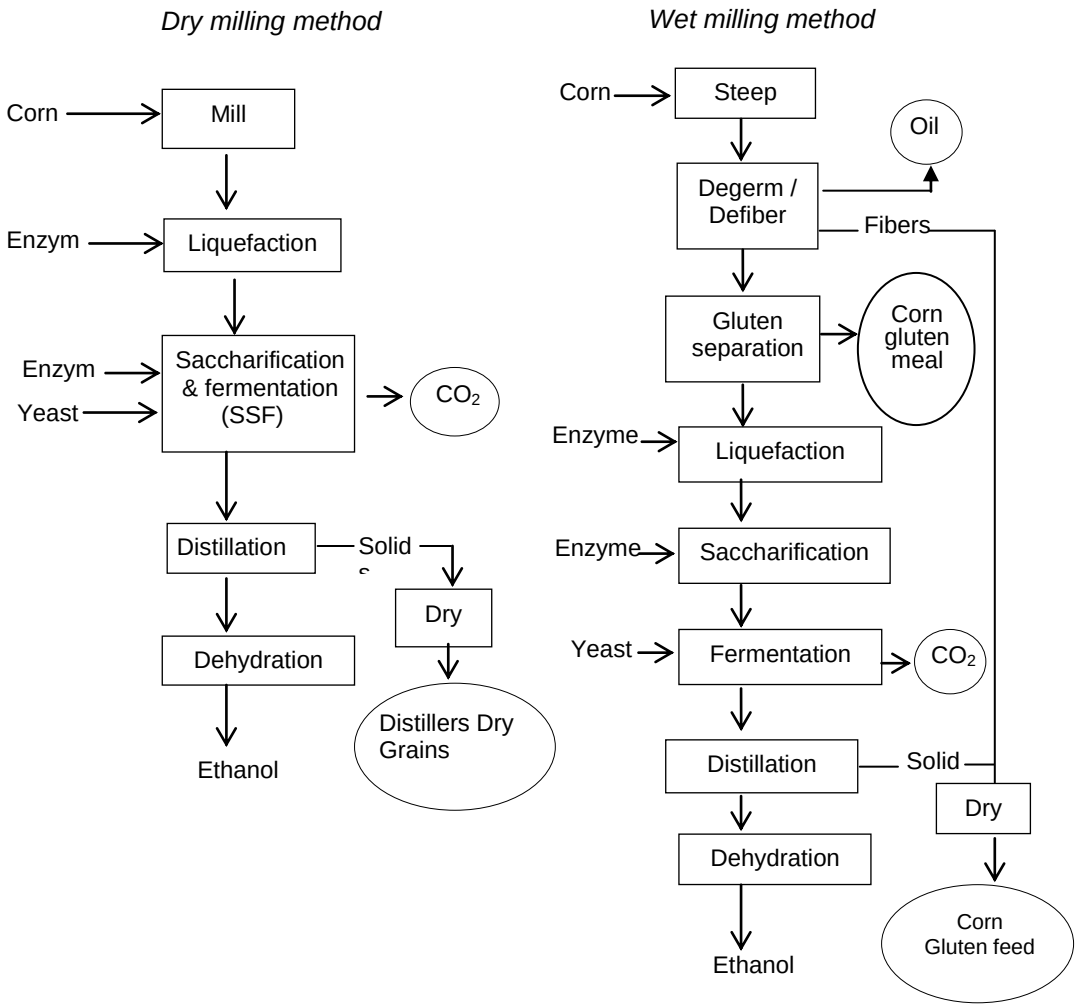

Figure 1.2 – Dry milling and wet milling ethanol production process steps Reproduced from Nichols and Bothast (2008).

Detailed processing steps involved in dry-milling and wet-milling bioethanol production have been comprehensively reviewed by several authors (Mojovic et al 2006; Balat et al 2008; Nichols and Bothast 2008; Mojović et al 2009; Monceaux, D.A., Kuehner, D. 2009). Starch gelatinization step is used in both technologies where starch mash is heated up to 120 ^{o}C using jet cookers (hydro heaters) for 1.5 to 2 hr (Serna-Saldivar 2010). Since this is an energy

intensive step, researchers are aimed to develop new technology to ferment raw starch without the gelatinization step (Wang et al 2005; Wang et al 2007; Gibreel et al 2008; Wang et al 2009).

Using of cereal for biofuel production causes issues such as food versus fuel debate, increased food price, environmental impact, carbon balance, and the sustainability that associated with the first generation biofuels. These uncertainties have motivated the researchers to focus on developing economical and sustainable technologies for the second generation bioethanol technology (Fernando et al 2006; Naik et al 2010).

1.2.2 Utilization of triticale in bioethanol industry

Not a traditional food crop, high grain yield in harsh environmental conditions, low fertilizer requirement, adaptability to different soil and climatic conditions provide distinctive advantages to triticale over the other feedstock used in ethanol production (Oettler 2005; Kučerová 2007; Pejin et al 2009). These advantages encouraged scientists to explore the potential of triticale in bioethanol production (Sosulski et al 1997; Wang et al 1997; Wang et al 1998; Wang et al 1999; Kučerová 2007; Pejin et al 2009; McLeod et al 2010; Obuchowski et al 2010). Current Canadian bioethanol plants are based on wheat as their main feedstock as corn is not a major crop in Canada. Therefore, alternative crops such as rye and triticale have gained interest in bioethanol production (Wang et al 1999). Research has been focused on evaluating the

potential of triticale to replace wheat as primary feedstock in the bioethanol industry (CTBI 2011).

So far, several studies have evaluated the potential of using triticale as bioethanol feedstock instead of wheat. Under normal gravity fermentation (20-24 g of dissolved solids / 100 g), the fermentation efficiency of triticale was reported to be 90.2 – 91.4% compared to 90 -95% in wheat (Wang et al 1997). In another study, triticale bioethanol production with very high gravity fermentation (VHG, above 300 g of dissolved solids / 100 g) showed a fermentation efficiency of 90 – 93% with the ethanol yield of 417 – 435 L/ton (dry basis). Lower mash viscosity in triticale compared with wheat and corn mashes was considered as an advantage in handling process (Wang et al 1998). Grain pearling has increased the ethanol yield up to 482 L/ton (dry basis) in very high gravity fermentation with the fermentation efficiency of 94.3% (Wang et al 1999). In a comparison between wheat, triticale, barley, and oat grown in Canadian prairies for their potential in bioethanol production, triticale ranked as the second highest in ethanol yield whereas wheat produced the highest value (McLeod et al 2010). In another comparison of four wheat varieties and four triticale varieties, autoamylolytic quotient (AAQ: measurement of enzyme system) of triticale was reported between 94.24 – 99.55% compared to 62.15 – 81.46 % AAQ in wheat. Ethanol yields obtained from this triticale equals to those of wheat even without adding technical enzymes for starch conversion (Pejin et al 2009). All these research findings provide important insights into

development of new technologies to use triticale as a valuble and low cost feedstock for fuel ethanol production in the world.

1.3. Distillers grain

World bioethanol production has increased from 17 billion liters in 2000 to 46 billion liters in 2009 (Balat and Balat 2009) while production in United States increased from 6.5 billion liters in 1999 to 39 billion liters 2009 (RFA 2011). Even though there are advantages of using lignocellulosic biomass for ethanol production, most of the current ethanol production plants heavily depend on sugary or starchy feedstock (Rausch and Belyea 2006; Balat and Balat 2009). Distillers grain (DG) is the major co-product from biofuel plants using the dry-milling ethanol processing technology, whereas gluten meal and gluten feed are the major co-products of ethanol plants based on wet milling technology (Rausch and Belyea 2006; Liu 2011). Approximately 82% of the ethanol plants operated in USA was based on dry-milling ethanol production technology while remaining 18% used wet milling technology in 2006 (Nichols and Bothast 2008). Therefore, growth in bioethanol production will eventually increase the DG production in the world (Liu 2011).

1.3.1. Composition of distillers grain

Despite the controversy of food vs. fuel debate on grain based (starch based) bioethanol production (Searchinger et al 2008), DG production has increased over the years due to increased ethanol production. United States

bioethanol industry alone has produced 10 million metric tons of DDGS in 2006 (Xu et al 2007), and increased up to 30 million metric tons in 2010 (RFA 2011). Compared to the original feedstock, the nutrient content of DDGS such as oil, proteins, and minerals, is about three times higher due to the removal of starch by converting into ethanol and CO_2 during ethanol production. This makes DDGS an ideal animal feed (Liu 2011). However, the huge variation in the nutrient composition and the quality of nutrients are the main limiting factors for utilizing DDGS in animal feed industry (Belyea et al 2004; Liu 2008). DDGS composition (Liu 2011), applications of DDGS in poultry nutrition (Świątkiewicz and Koreleski 2008), swine feeds (Stein and Shurson 2009), and in beef cattle feeds (Klopfenstein et al 2007) have been reviewed by several authors.

A summary of proximate compositions of DDGS samples obtained from different ethanol plants and sources is shown in Table 1.4. Total dry matter content of distillers grain vary from 87 to 92% (Cromwell et al 1993; Spiehs et al 2002). Cromwell et al (1993) reported the average proximate composition of crude proteins, fat, acid detergent fiber (ADF), and neutral detergent fiber (NDF) are 26.0-31.7%, 9.1-14.1%, 3.7-8.1%, 11.4-20.8%, and 33.1-43.9%, respectively in DDGS samples obtained from two different beverage ethanol plants and seven fuel ethanol plants. Spiehs et al (2002) analyzed the composition of DDGS from ten ethanol plants over 3 years. They found that the ash content was significantly different from different studies; however, the contents of total dry matter, protein and oil were not significantly different.

In a study about composition of DDGS, Belyea et al (2004) reported a residual starch content of 4.7-5.9% whereas other components vary within the values range reported by Cromwell et al (1993) and Spiehs et al (2002). In a recent study, Liu (2008) reported the average values of 27.4, 11.7, 4.4, and 4.9% for protein, oil, ash, and starch respectively on dry matter basis. The protein content reported in the study of Liu (2008) was much lower than the values reported in above mentioned studies, possibly because of using the conversion factor of 5.7 instead of 6.25 to convert nitrogen values into respective protein values. Chemical composition of distillers grain varied with the types of raw materials, processing methods, fermentation parameters, and analytical methods used (Rausch and Belyea 2006; Liu 2011).

Table 1.4 – Proximate composition of DDGS from different ethanol plants, production years and sources. Adapted from Liu (2011).

	Range			
	Cromwell et al (1993)	Spiehs et al (2002) [a]	Belyea et al (2006) [b]	Liu (2008)
No. of data points	9	10	5	6
Dry matter	87.1−92.7	87.2−90.2	N.A.	N.A.
Protein	26.0−31.7	28.7−31.6	30.8−33.3	25.8−29.1
Oil	9.1−14.1	10.2−11.4	10.9−12.6	11.0−12.2
Ash	3.7−8.1	5.2−6.7	4.3−5.0	4.0−4.9
Starch	N.A.	N.A.	4.7−5.9	3.2−5.7
Total carbohydrate	N.A.	N.A.	N.A.	55.7−57.9
Crude fiber	N.A.	8.3−9.7	9.6−10.6	N.A.
Acid detergent fiber	11.4−20.8	13.8−18.5	15.4−19.3	N.A.
Neutral detergent fiber	33.1−43.9	36.7−49.1	N.A.	N.A.

[a] Range values for means of 10 samples from different locations, [b] Range values for means of 5 samples from different years. N.A. – not analyzed

Amino acid composition is an important factor that determines the quality of distillers grain, especially in the animal feed industry. Several studies were focused on analyzing amino acid content of distillers grain proteins (Cromwell et al 1993; Spiehs et al 2002; Batal and Dale 2006; Kim et al 2008). Amino acid profile (% on dry matter basis) reported in three studies are shown in Table 1.5.

Table 1.5 – Amino acid composition (% on dry matter basis) of DDGS from different sources. Adapted from Liu (2011)

	Range		
	Cromwell et al (1993)	Spiehs et al (2002) [a]	Han and Liu (2010)
No. of data points	9	10	3
Essential amino acids			
Arg	0.95−1.33	1.11−2.17	1.16−1.40
His	0.65−0.93	0.72−0.82	0.82−1.01
Ile	1.06−1.26	1.05−1.17	0.91−1.25
Leu	3.05−4.40	3.51−3.81	3.18−3.91
Lys	0.48−0.97	0.72−1.02	0.88−1.15
Met	0.49−0.61	0.49−0.69	0.65−0.76
Phe	1.39−1.91	1.41−1.57	1.37−1.76
Thr	0.99−1.28	1.07−1.21	1.06−1.26
Trp	0.18−0.25	0.21−0.27	
Val	1.30−1.64	1.43−1.56	1.40−1.80
Nonessential amino acids			
Ala	N.A.	N.A.	1.86−2.27
Asp	N.A.	N.A.	1.77−2.16
Cys	0.49−0.66	N.A.	0.53−0.60
Glu	N.A.	N.A.	4.94−6.01
Gly	N.A.	N.A.	1.11−1.31
Ser	N.A.	N.A.	1.32−1.58
Tyr	N.A.	N.A.	0.87−1.29

[a] Range values for means of 10 samples from different locations
N.A. – not analyzed

Lysine is considerd as the most important amino acid among the others, due to its vital role in animal nutrition. However, lysine content showed huge variation among the samples tested (Cromwell et al 1993; Spiehs et al 2002). The lysine content was reported as 0.48-0.97% on dry basis with a coefficient of variation (CV) of 18.71% (Cromwell et al 1993), as 0.72-1.02% (Spiehs et al 2002) and 0.88-1.15% with CV values of 17.3% and 13.63%, respectively (on dry mater basis) (Han and Liu 2010). Variation in the lysine contents was mainly attributed to the heat damages occurred during bioethanol production and drying of distillers grain (Liu 2011). A correlation between the color of distillers grain and lysine content was observed in several studies (Cromwell et al 1993). Lysine content tends to decrease with increasing the colour of DDGS and vice versa. Similar correlation was observed in Hunter *L*, *a*, and *b* scores for colour of DDGS and lysine content (Cromwell et al 1993). As a result of variable amino acid composition, DDGS is considerd as an incomplete animal feed component despite its high protein content (Liu 2011). Arginine, histidine and methionine also exhibited a high coefficient of variation in all three studies (Cromwell et al 1993; Spiehs et al 2002).

1.3.1.1 Triticale distillers grain composition

Due to the agronomic and yield advantages, triticale is actively explored in Western Canada as a feedstock for bioethanol production (Sosulski et al 1997; Wang et al 1997; Wang et al 1998; Wang et al 1999). Therefore, potential of utilizing co-products of triticale bioethanol, particularly triticale distillers dried

grains with solubles (TDDGS) has been studied by several researchers (Mustafa et al 2000; Greter et al 2008; McKeown et al 2010; Oba et al 2010; Wierenga et al 2010). A better knowledge on composition is important in developing new technologies to utilize TDDGS for animal feed industry as well as for the other applications. Table 1.6 summarizes the proximate composition of triticale DDGS reported from three different studies.

Table 1.6 – Proximate composition of triticale dried distillers grain with solubles

	McKeown et al 2010	Wierenga et al 2010	Gibreel et al 2011
Dry matter	89.3	N.A.	N.A.
Ash	N.A.	N.A.	4.7 - 5.5
Moisture	N.A.	N.A.	2.9 - 4.1
Crude protein	30.7	36.7	45.3 - 39.0
Crude fat	5.4	N.A.	3.5 - 5.5
Crude fiber	N.A.	N.A.	5.1 - 3.6
ADF	13.7	11.4	N.A.
NDF	29.6	32.6	N.A.
Starch	N.A.	5.9	N.A.

N.A. – not analyzed

Dry matter content of triticale DDGS was reported to vary from 89 to 90% (McKeown et al 2010). Highly variable crude protein content was observed in triticale DDGS in several studies. Crude protein content of triticale DDGS was reported as 30.7 (McKeown et al 2010), 36.7 (Wierenga,2010) and 39.7% (Mustafa et al 2000). In a most recent study, TDDGS protein content was reported to be in the range of 39.0 – 45.3% depending on the triticale variety and

fermentation method (Gibreel et al 2011). Crude fat, ADF and NDF values were reported to be similar with slight variation in all studies (McKeown et al 2010). In comparison with wheat and corn DDGS, TDDGS contain higher protein content which make them suitable for animal feed industry (Mustafa et al 2000; Gibreel et al 2011).

Amino acid composition is a critical parameter which needs to be considered when using DDGS as an animal feed. Biological value of a feed is determined as the percentage of available lysine over the total lysine content (Hackler and Stillings 1967), therefore nutritional value of DDGS is mainly depending on lysine content (Gibreel et al 2011). According to a study done by Greter et al (2008) lysine content of triticale and corn DDGS were reported as 2.08% and 1.86% respectively. Similar results were observed in another study where higher lysine content was reported in pronghorn triticale variety over wheat and corn varieties under very high gravity fermentation (Gibreel et al 2011). The presence of high lysine content provides an additional advantage to TDDGS over the other distillers grain due to the improved biological value.

1.3.2. Utilization of distillers grain

Increase in bioethanol production generates increased amounts of DDGS, the main byproduct of dry-milling ethanol production. Under normal operating conditions of a bioethanol plant, income generated from ethanol is only sufficient enough to cover the overhead expenses of the plant; the income generated from DDGS sales would provide profits for the bioethanol industry

(Bonnardeaux 2007). Currently, DDGS is mainly used as a feed ingredient in animal feed industry, especially in non-ruminant diets (Rausch and Belyea 2006; Bonnardeaux 2007). DDGS has higher protein content compared to unfermented feedstock due to starch removal during fermentation, thus considerd as a protein source in feed ingredients (Klopfenstein et al 1978). Certain unidentified growth factors present in DDGS encouraged the researchers to use them in poultry feed formulations. However, later studies revealed that these unidentified growth factors are vitamins synthesized during fermentation and trace minerals released from their bound forms (Lumpkins et al 2005). Extensive research was carried out in evaluating potential of DDGS in ruminant diets (Klopfenstein et al 1978; Firkins et al 1985; Ham et al 1994; Al-Suwaiegh et al 2002; Klopfenstein et al 2007), swine diets (Cromwell et al 1993; Stein et al 2006; Whitney et al 2006; Pedersen et al 2007; Stein and Shurson 2009) and in aquaculture (D Webster et al 1992; Tidwell et al 1993; Chevanan et al 2009).

Current researches in triticale DDGS are mainly focused on the animal feed industry. Dry matter digestability of TDDGS showed a similar pattern to wheat and rye DDGS which make them suitable for ruminant nutrition (Mustafa et al 2000). Greter et al (2008) replaced the corn DDGS in lactating dairy cows with triticale DDGS with no negative impact on milk production. Instead, they observed a higher fat concentration and yield in cow milk fed with triticale DDGS. Triticale DDGS showed the ability to replace barley grain or canola meal in lamb diets up to 20% without reducing dry matter intake, average daily gain, and carcass quality of the lamb (McKeown et al 2010). In another study,

researchers replaced up to 10% of broiler diets with TDDGS without any adverse effects on performance and breast muscle yield (Oryschak et al 2010).

Several researchers are working on developing technologies to use DDGS in human food applications due to its high protein content (Bonnardeaux 2007). Several studies aimed to evaluate the baking potential of DDGS in different foods such as bread and cookies (Rasco et al 1987; Rasco et al 1990a; Rasco et al 1990b; Abbott et al 1991; Brochetti et al 1991). Production of pasta and spaghetti enriched with DDGS are the other areas of interest in human food applications (Wu et al 1987). However, undesirable taste and the odor generated from DDGS are the key limiting factors in utilizing them in human food applications (Wu et al 1987; Bonnardeau 2007).

Industrial application is another area of interest in utilizing DDGS (Wu et al 1987; Bonnardeaux 2007). Fractionation of valuble compounds such as oil (Singh and Cheryan 1998; Cheryan 2002), protein (Wolf and Lawton 1997; Cookman and Glatz 2009; Wang et al 2009) and fiber (Srinivasan et al 2005) has been evaluated as the potential value additions to DDGS. Apart from that, DDGS has been used in developing biomaterials such as phenolic resins (Tatara et al 2009) and DDGS filled plastics (Otieno et al 2006).

1.3.3. Protein extraction from distillers grain

Because of its high protein content, extracting proteins from distillers grain has become a subject of interest (Anderson and Lamsal 2011). Fractionation of corn distillers grain protein was the first ever study carried out

on DDGS protein extraction (Wu et al 1981). A low total extraction yield (14%) was reported in this study with extraction solutions of water, sodium chloride, 70% ethanol, and 70% ethanol with DTT; however, a extraction yield of 30% was reported with a mixture of borate, SDS and DTT in the same study (Wu et al 1981). A extraction yield of 3.2 – 6.6% was reported in another study using a solvent containing 0.1 M NaOH, 0.1% DTT, and 0.5% SDS (Wolf and Lawton 1997). Low protein content was the main issue related with this extraction method (Wolf and Lawton 1997; Xu et al 2007).

Extraction of zein from DDGS using 70% ethanol and 0.25% sodium sulfite solution at pH 2.0 resulted in a extraction yield of 44% at a protein content of 90% (Xu et al 2007). In another study, 40% of protein and 50% of the total lysine content were extracted using an alkaline solution of 1 M NaOH at 70 ^{o}C. However, protein content of the extract was not measured in this study (Bals et al 2009). Alkali ethanol solution showed better extraction yield than alkaline solutions alone from DDGS (Rosentrater et al 2006). Cookman and Glatz (2009) reported that the protein content of corn DDGS was reduced from 36% to 8% when a mixture of 45% ethanol and 55% 1 M NaOH (v/v) was used for protein extraction, but details on protein content of extract was not reported. In a recent study, an extraction yield of 56.8% with a protein content of 94.88 % was reported for sorghum DDGS using the same alkaline ethanol solution (45% ethanol and 55% 1 M NaOH, v/v) (Wang et al 2009), which was much higher than other reported results. This may be due to the differences in the protein composition between sorghum and corn DDGS, and in processing conditions.

1.4. Wood adhesive industry

Adhesive industry is one of the fast growing industries in the world. Total world usage of formulated adhesives in 2009 was recorded as 16.6 billion pounds (worth of US $ 20.6 billion). The market value of adhesives went down due to economic crisis in 2009, however it is expected to grow in a rate of 4.5% up to 2014 (KNGInc 2010). In 1998, North America alone consumed 1.78 billion pounds of adhesives (Sellers 2001) and this has increased up to 3.98 billion pounds in 2009 (KNGInc 2010). Table 1.7 represents the volumes and values of world adhesive industry by regions according to the multiclient study on "The global adhesive industry 2009-2014".

Urea formaldehyde (UF), phenol formaldehyde (PF), melamine formaldehyde (MF), melamine urea formaldehyde (MUF), and aqueous polymer isocyanates (API) are considered as the leading products in wood adhesive industry (Rowell 2005; Ji-you 2008; Gu and Cai 2010). These thermosetting resins are widely used over the other adhesives due to their unique advantages such as low cost, excellent thermal properties, high adhesion strength, and easy processing and handling (Pizzi 1994a; Pizzi 1994b; Rowell 2005; Gu and Cai 2010).

Table 1.7 – Current status and future projections of global adhesive consumption by region (in billions). Reproduced from a market report on "The global adhesives industry 2009 - 2014" (KNGInc 2010)

	Year						
	2008		2009		2014		AGR
Region	lb	$	lb	$	lb	$	09 - 14
Asia-Pacific	6.6	7.1	6.7	7.0	9.1	9.8	6.5
Europe	5.4	7.3	4.9	6.7	5.7	7.9	3
North America	4.4	6.3	4.0	5.7	4.5	6.5	3
South/Central America	0.9	1.0	0.8	1.0	1.0	1.2	5
Rest of the world	0.2	0.2	0.2	0.2	0.3	0.3	4
Total	17.5	21.9	16.6	20.6	20.6	25.7	4.5

AGR – Annual growth rate

UF resins are developed by polymeric condensation of aldehydes (formaldehyde in most cases) with compounds contain aminic or amidic group (Pizzi 1994b; Rowell 2005). Because of the unique properties such as water solubility which facilitate easy application to wood surface, hardness, nonflammability, and absence of colour, UFs became the most widely used thermosetting adhesive in wood industry (Pizzi 1994b; Dunky 1998; Rowell 2005). Phenol formaldehyde resins are developed through polycondensation of phenolic compounds with formaldehyde. However, unlike the other products derived from polycondensation, PF can be existed in different isomer forms as both ortho and para positions of phenols can react with formaldehyde (Pizzi 1994a). Both UF and PF are used in the exterior grade wood product developments where they are exposed to extremely harsh climatic conditions (Pizzi et al 1981; Rowell 2005). MF and MUF are also used for exterior and

semi exterior applications due to their high adhesion strength, durability, and more specifically because of the excellent water resistance compared with UF and PF. Generally, MF resins are expensive and urea is added to form MUF to reduce the cost (Pizzi 1994c; Rowell 2005).

Even though these synthetic polymers are being widely used, increasing petroleum price, environmental concerns over the formaldehyde emission and non-renewability have raised questions on the future use of them in the wood industry (Pizzi et al 1981; Mo et al 2004a; Gandini 2008). Therefore, research is being emerged in developing biodegradable adhesives from renewable polymer resources (Lambuth 1994b; Kumar et al 2002; Raquez et al 2010). Polysaccharides (such as cellulose, chitin, and starch), lignin, suberin, vegetable oils (canola oil, corn oil etc), tannins, furans, and proteins are some of the renewable polymers that have the potential to be used in wood adhesive industry (Gandini 2008). Among these polymers, proteins has extensively studied for their potential in wood adhesive preparation (Lambuth 1994b; Huang and Sun 2000a; Huang and Sun 2000b; Zhong et al 2001; Kumar et al 2002; Liu and Li 2002; Liu and Li 2004; Mo et al 2004; Wang et al 2007; Li et al 2009; Khosravi et al 2010; Lei et al 2010; Raquez et al 2010; Khosravi et al 2011).

1.4.1. Protein based adhesives

Increased concerns over the environmental issues related with synthetic non-biodegradable polymers such as formaldehyde emission and consumer demand for environmental friendly bio-based adhesives are the key driving

forces behind increased research and developments on biodegradable polymers (Dewar 2007). There are several renewable materials such as soybean meal, canola meal, brewers spent grain, and distillers grain derived from agricultural and processing industries that contain high amount of proteins (Lambuth 1994a; Lambuth 1994a; Kolster et al 1997; Kumar et al 2002; Anderson and Lamsal 2011). Protein is considerd to be an excellent biodegradable polymer that can be effectively utilized in developing biomaterials, such as adhesives, plastics, films, coatings, fibers, and composites (Lambuth 1994a; Lambuth 1994a; Kolster et al 1997; Kumar et al 2002; Barone and Schmidt 2006). Proteins were used as adhesives to bond materials from ancient times (Lambuth 1994a). Animal blood, milk proteins, fish skin extracts have been used as glue from early civilization (Lambuth 1994a). Bovine serum albumin is still using as one of the major components in wood adhesive. However, plant proteins have not been extensively studied for adhesive preparations until recent past (Kumar et al 2002; Barone and Schmidt 2006).

1.4.1.1 Adhesion mechanisms

Several theories have been proposed to explain the possible mechanisms of adhesion (Schultz and Nardin 2003). Mechanical interlocking was the first theory introduced to explain the adhesion mechanism. According to this theory, interlocking (mechanical keying) of adhesive molecules with cavities and pores of solid surfaces is the basis of adhesion (Borroff et al 1951; Schultz and Nardin 2003). Electronic theory of adhesion suggests that, adhesion is caused by

formation of double electron layer on the surface due to the electrostatic attraction between the electron band structures of adhesive and the solid surface (Deryaguin 1957). According to the weak boundary layer (WBL) theory of Bikerman (1968), cohesive strength of WBL is the main factor that determines the adhesion strength. As per the adsorption (thermodynamic) theory of adhesion, interatomic and intermolecular forces established at the interface provide the means of adhesion. Interfacial forces are created (van der Waals and Lewis acid base interactions) based on the thermodynamic properties such as surface free energy of the adhesive and the surface (Sharpe 1963). Adsorption theory is one of the most widely accepted adhesion mechanisms in adhesion science (Schultz and Nardin 2003). According to the diffusion theory, which introduced by Voyutskii (1963), creating an interface via diffusion of macromolecules across the wood and adhesive or within the adhesive is belived to be another mechanism of adhesion. This mutual diffusion will help polymers in attaching with themselves (auto-adhesion) or with other surfaces. There is another theory about the adhesion mechanisms involving chemical bonds. According to the chemical bonding theory, the chemical bonds formed at the adhesive – material surface are directly contributed to the adhesion strength. Reactivity of the substrate and adhesive is a critical factor in chemical bonding. Chemical interactions such as covalent bonds are referred as the primary force that contributes to higher bond strength, where secondary force interactions (eg: van der Waals, hydrogen bonds) have lower bond strength (Pape and Plueddemann 1991; Schultz and Nardin 2003).

1.4.1.2 Factors affecting protein based wood adhesive performance

Formation of an adhesive bond between the adhesive and the substrate is a complex process. Several internal and external factors can affect the formation and performance of adhesive – substrate interactions (Kumar et al 2002). Particle size of any adhesive is an important factor in determining the adhesion strength. Generally, 3000 – 6000 cm^2/g specific surface area is considerd to be a satisfactory range for adhesive particle size (Lambuth 2001). Surface characteristic of substrate is another important factor affecting the bond strength. Surfaces with either very high roughness, or too smooth surfaces would create adhesive failure due to lack of sites/pores for adhesive penetration into wood surface in order to make weak boundary layer (Mackay 1998). Interlocking by adhesive penetration and molecular attraction forces (van der Waals and hydrogen bonds) are the main methods of bond formation (Kumar et al 2002). Adhesion strength of soft maple wood was reported as 280 N, while lower values were reported for hard woods (walnut – 135 N) and soft woods (yellow pine – 0 N, poplar – 71 N) with smooth surfaces (Kalapathy et al 1995).

Viscosity of the adhesive directly affects to the adhesive behaviour such as flow and spreading properties; based on the application method and desired use, an optimum viscosity can vary from 500 to 75000 cP (Lambuth 1977). High absorbing materials such as paper, soft board, and dried wood aggregates require a viscosity of 500 – 5000 cP, whereas wood laminates require (both hot press and cold press) a high viscosity ranging from 5000 – 25000 cP. In no clamp cold

press technology, a viscosity of 8000 – 20000 cP is desirable for adhesives to gain acceptable performances (Kumar et al 2002). In a study to test the effect of pH on adhesive properties (Kalapathy et al 1996), increasing pH resulted in lower viscosity which adversely affected on the adhesion strength. Structure of the protein is a critical factor in protein based adhesives. Dispersion of proteins in the water is necessary to increase the availability of polar and apolar groups to interact with wood surface during the adhesion. In the native state of protein, most of the polar and apolar functional groups are unavailable due to internal bonding and protein folding (Lambuth 1977; Lambuth 1994b; Kumar et al 2002). Processing parameters such as press conditions, pre-pressing drying time, and protein concentration in the glue also have an impact on adhesion strength (Zhong et al 2001). Increase in pressing time (1 to 10 minutes), pressure (0.4 – 4.0 MPa) and temperature (25 to 100 ^{o}C) has resulted in higher adhesion strength (Hettiarachchy et al 1995; Kalapathy et al 1995; Kalapathy et al 1996; Zhong et al 2001).

1.4.2. Plant protein based adhesives

Protein based adhesives were used as gluing materials from ancient times. However, in the early stages, research attention was focused on animal protein sources such as blood, milk proteins, fish skin extract and bovine serum albumin (Barone and Schmidt 2006). Plant proteins have not been extensively studied for adhesive preparations until recent past (Barone and Schmidt 2006). Soy protein is the most widely studied plant protein source for adhesive

preparations (Lambuth 1977; Lambuth 1994b; Hettiarachchy et al 1995; Kalapathy et al 1996; Sun and Bian 1999; Huang and Sun 2000b; Hojilla-Evangelista 2002; Kumar et al 2002; Liu and Li 2002; Li et al 2009). Wheat gluten is another plant protein studied widely for wood adhesive applications with different modifications (D'Amico et al 2010; Khosravi et al 2010; Lagrain et al 2010; Lei et al 2010; Nordqvist et al 2010). Apart from these two main proteins, *Jatropha curcas* proteins (Hamarneh et al 2010) and rice bran proteins (Pan et al 2005) were tested for adhesive properties.

1.4.2.1 Soy protein adhesives

Despite having low oil content (~ 20% on dry matter basis) compared with other oil seeds such as canola (~ 45 % oil content on dry matter basis), 52 % of the world oil production is coming from soybean (Kumar et al 2002). One metric ton of soy oil production generates 4.5 metric tons of soybean meal with an approximate protein content of ~ 44 % (on dry matter basis) (Kumar et al 2002). Soy proteins mainly composed of albumins and globulins, where globulins make the major fraction of soy protein isolate (Kinsella 1979; Wool and Sun 2005). Globulins consist of glycinin (molecular weight of 320 – 360 KDa) and conglycinin (molecular weight of 150 – 190 KDa). Six acidic-basic subunits are coupled together by disulfide bonds to make glycinin while conglycinin has only three subunits (Kumar et al 2002; Nordqvist et al 2010).

Protein denaturation by alkaline agents (such as NaOH) (Hettiarachchy et al 1995; Kalapathy et al 1995; Kalapathy et al 1996; Kalapathy et al 1997;

Khosravi et al 2010; Khosravi et al 2011), urea (Sun and Bian 1999; Huang and Sun 2000a), guanidine hydrochloride (Huang and Sun 2000a), sodium dodecyl sulphate (Huang and Sun 2000b), sodium dodecyl benzene sulfonate (Huang and Sun 2000b), and enzymes (Hettiarachchy et al 1995; Kalapathy et al 1995; Kalapathy et al 1996) are the methods used to modify the adhesive properties of soy protein. Alkali modification showed increasing adhesion strength as well as the water resistance properties at increasing pHs from 8 to 12; the highest adhesion strength was reported at pH 12. The dry adhesion strength of alkali modified soy protein was reported to range from 0.75 MPa to 1.97 MPa (Hettiarachchy et al 1995). In two other studies, adhesion strength of alkaline modified soy proteins was reported as 3.0 - 6.5 MPa (Sun and Bian 1999), and 5.0 – 5.7 MPa (Mo et al 1999). In a recent study, alkali modifications of wheat gluten and soy protein were reported to increase their adhesion strength to the level of the European standard EN 204 for wood adhesives (10, 8, 2 MPa for dry, soaked and wet adhesion strength, respectively) (Nordqvist et al 2010).

Urea is a strong protein-denaturing agent that has been used in soy protein modification to improve adhesion strength. Huang and Sun (2000a) reported the dry adhesion strength of urea modified soy proteins varied from 4.2 to 5.9 MPa, or from 3.7 MPa to 5.5 MPa depending on the type of wood surface used (Mo et al 2004). The wet adhesion strength and soaked adhesion strength ranged from 0.4 MPa to 2.5 MPa and from 2.1 MPa to 4.9 MPa, respectively (Huang and Sun 2000a).

Cross-linking was used to stabilize protein structure in aqueous solutions via disulfide bond formation. In a study on glutaraldehyde aided cross-linking of soy proteins, the adhesion strength was reported as 6.81 MPa, 3.04 MPa, and 6.27 MPa for dry, wet and soaked adhesion strength respectively (Wang et al 2007). Chemical modifications such as esterification (Wang et al 2006) and grafting with different functional groups (Liu and Li 2002; Liu and Li 2004) were also used to improve the adhesion strength. The optimum adhesion strength after 10 hr esterification reaction time were reported as 5.73, 2.16, and 5.67 MPa for dry, wet and soaked strength respectively (Wang et al 2006). Grafting of dopamine into soy protein isolate has improved the water resistance and adhesion strength, however it was observed that amount of dopamine grafted has a correlation with adhesion strength (Liu and Li 2002). SDS (0.5% and 1%) and sodium dodecylbenzene sulfonate (SDBS) modifications were reported to increase the adhesion strength of soy protein isolate (Huang and Sun 2000b).

1.4.2.2 Wheat gluten protein adhesives

Wheat is the second largest cereal crop, second only to corn in the world based on production volumes (FAOSTAT 2010). It is an unique cereal among the others, due to its protein composition and functionalities which enable dough formation (Lagrain et al 2010). Monomeric gliadins with molecular weight of 30 – 60 KDa and polymeric glutenin with a diverse molecular weight ranging from 80 KDa to 250 KDa (can be increased up to 1000 KDa) are the two main components of gluten proteins (Veraverbeke and Delcour 2002; Lagrain et al

2010). Isoelectric pH of wheat gluten is 7.0, therefore it is not dispersed in distilled water (Khosravi et al 2011). Gluten is a co-product of wheat wet milling operations carried out either for starch separation or for bioethanol production (Khosravi et al 2011).

Potential of wheat gluten to use as an adhesive (binder) to produce particle boards has been evaluated previously (Khosravi et al 2010; Khosravi et al 2011). 20% (w/w) protein in water and 20% (w/w) protein in alkaline solution (0.1 M NaOH) are main two dispersions used in particle board production from green and dried wood chips. Wheat gluten exhibits the acceptable properties for particle board production irrespective of the method of dispersion preparation. However, temperature used for adhesive preparation affected adhesion strength where low temperatures provided the highest adhesion strength compared to adhesives prepared at high temperature conditions (Khosravi et al 2010). Apart from temperature, methods of adhesive applications can also affect the quality of finished particle boards (Khosravi et al 2011). Modification of wheat gluten hydrolyzate using formaldehyde and glyoxal resins resulted in adhesives with strength comparable to standard specifications required for particle board production (Lei et al 2010). Wheat flour based adhesives (protein content at 12.43 ± 0.14%) prepared by dispersing in water have shown optimum adhesion strength of 5.8 MPa at curing temperature of 105 ^{o}C (D'Amico et al 2010). Current literature on wheat gluten based adhesives is very limited, as the interest on gluten based adhesives was emerged very recently.

1.5. Review summary and research objectives

Due to the increased concerns over the scarcity of petroleum based resources, energy security and environmental pollution, world bioethanol production have gained its momentum during past few decades (Demirbas 2007; Demirbas 2010). In Canadian context, wheat is the main feedstock for bioethanol production. However, utilizing wheat for bioethanol production has its own drawbacks due to being a major food crop. Bioethanol production from triticale has been extensively studied in Canada in order to use triticale as primary feedstock for bioethanol production (McGoverin et al 2011). Canadian Triticale Biorefinery Initiative (CTBI) is a recently formed institute to develop new technologies to utilize triticale as main feedstock for integrated biorefineries in Canada (CTBI 2011). Among the other provinces in Canada, Western Canada has the highest cultivation area and production volume of triticale (Salmon 2004a; Salmon et al 2004b).

Increase in bioethanol production has resulted in a great deal of distillers grain over the past decade. Main applications of distillers grain are in the animal feed industry; however, attempts are being made to explore new food and non-food applications due to its high protein content (Xu et al 2007). Triticale distillers grain contain 30.7 – 45.3% protein (Bonnardeaux 2007; Gibreel et al 2011), which is higher than those of corn and wheat distillers grain with the protein content ranging from 25.8 to 33.3% (McKeown et al 2010; Gibreel et al 2011). However, protein extraction from triticale distillers grain has not been studied, except very limited literature available regarding the protein extraction

from corn and wheat WG. There is a need to develop methods of protein extraction from triticale DDGS. Because of the higher protein content in triticale DDGS than corn and wheat DG, there is a possibility of extracting triticale proteins for industrial uses, instead of using them in low value animal feed market. One example is to develop adhesive from triticale proteins.

Wood adhesives industry is one of the largest industries especially in North America. Petroleum based resins are currently dominated in this industry (Liu 2011). However, increasing petroleum price, environmental concerns over formaldehyde emission, and non-renewability of petroleum-based adhesives have prompted scientists to look for new alternatives (Rowell 2005; Ji-you 2008). Thus, developing novel wood adhesives based on renewable polymers such as proteins has gained research interest during recent past. Soy protein and wheat gluten are the most commonly studied protein sources for adhesive preparation (Mo et al 2004; Gandini 2008). Wheat gluten showed increased adhesion strength after modification, which make them suitable for developing wood adhesives (Lambuth 1994; Hettiarachchy et al 1995; Kalapathy et al 1995; Kalapathy et al 1996; Kalapathy et al 1997; Huang and Sun 2000a; Huang and Sun 2000b; Kumar et al 2002; Khosravi et al 2010; Raquez et al 2010). Triticale contains gluten protein fraction similar to the wheat proteins (D'Amico et al 2010; Khosravi et al 2010; Lei et al 2010; Khosravi et al 2011). Therefore, extracted proteins from triticale distillers grain may have the same potential as wheat gluten to use in wood adhesive preparation. Extraction of protein from

triticale distillers grain and development of their value added applications will eventually support the Canadian bioethanol production in future.

1.5.1. Hypothesis

Triticale distillers grain contains higher amount of proteins compared with corn and wheat distillers grain. Extraction and exploring value-added potential of these proteins will help to use them on industrial applications, instead of using them in low value animal feed. Similar to wheat gluten, triticale also contains gluten protein fraction in its composition. Thus, it may be possible to improve the adhesive properties such as adhesion strength and water resistance of triticale distillers grain protein with different protein modifications.

1.5.2. The objectives of the research

- To develop methods of protein extraction from triticale distillers grain (triticale distillers wet grain, distillers died grains with solubles)
- To evaluate the potential of triticale distillers grain proteins in preparing bio-based adhesives

1.6. References

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CHAPTER 2

PROTEIN EXTRACTION FROM TRITICALE DISTILLERS GRAIN

2.1. Introduction

Increasing concerns about the scarcity of petroleum-based fuels have led to search for fuels from renewable resources (Xu et al. 2007). According to the 2011 annual report of the Renewable Fuels Association, the total renewable bioethanol production of United States in 2010 was 13.5 billion gallons per year, and is predicted to increase to 20.5 billion gallons by 2015. Corn, wheat, sorghum, and sugar cane are the major crops used in the production of bioethanol (Sosulski et al. 1997; Mojovic et al. 2006; Balat and Balat 2009). In the bioethanol industry, after distillation of ethanol (Kim et al. 2008), the resultant whole stillage is the major by-product. The whole stillage is separated into distillers solubles (liquid thin stillage) and solid distillers wet grains (DWG), and then dried to improve the shelf life. Condensed distillers solubles are added to DWG and then dried to produce distillers dried grains with solubles (DDGS) (Ganesan et al. 2006). An increasing demand for bioethanol yields a great deal of distillers grains. In 2006, the bioethanol industry in the United States generated 10 million metric tons of DDGS (Xu et al. 2007); the production of DDGS increased to 30 million metric tons in 2010 (RFA 2011). DWG and DDGS are used mainly as animal feed, where the beef, dairy, swine and poultry industries consumed 41%, 39%, 10%, and 9%, respectively, of the total distillers grain

volume in the United States, whereas only 1% is used for other applications (RFA 2011).

Triticale is being actively explored as a feedstock for bioethanol production in Western Canada. The total Canadian triticale production was over 66000 metric tons in 2010 (CANSIM 2011). Triticale, a cross between wheat (*Triticum*) and rye (*Secale*), is the first man-made cereal. The different varieties of triticale depend on the genetic composition of the parental species (Chen and Bushuk 1970). Triticale proteins contain more salt soluble proteins but fewer storage proteins than wheat and their lysine content is higher than that of wheat and rye (Ahmed and McDonald 1974; Pena 2004). Jonnala et al. (2010) reported that triticale varieties contain more albumin and globulin proteins than their parent species. As a hybrid between wheat and rye, the composition of triticale proteins shows large heterogeneity. Around 50% of triticale storage proteins consist of gliadins and secalins which are basically monomeric proteins containing intra-molecular disulfide bonds. Triticale glutenins are considered as polymeric proteins linked by interchain disulfide bonds (Siriamornpun et al. 2004; Salmanowicz and Nowak 2009). Using confocal laser scanning microscopy, triticale proteins were found in starch granular surfaces and in internal channels (Naguleswaran 2011). Triticale has the potential to be used as forage, feed grain, food uses, and for alcohol production (Sosulski et al. 1997; Wang et al. 1997; Wang et al. 1998). Dough made from triticale flour is weak due to poor quality gluten, but a triticale / wheat flour blend has been used successfully in making bread and in extruded snack production (Cluskey et al.

1979; Varughese et al. 1996; Tohver et al. 2000; Naeem et al. 2002; Perez et al. 2003; Pena 2004). Low cost of triticale starch compared with wheat and corn starches, and not having a value as a traditional food commodity are the major advantages of using triticale over wheat and corn as a fuel ethanol feedstock (Wang et al. 1998).

Development of new methods to utilize triticale ethanol by-products is essential to improve the economic value of triticale in the bioethanol industry (Wolf and Lawton 1997; Briggs 2007). Triticale DWG and DDGS contain 20–43% protein depending on the variety of triticale being used and the methods of production applied (Pena 2004; Wierenga et al. 2010; Gibreel et al. 2011). Extraction of proteins from DDGS is challenging as a consequence of protein denaturation occurred during the bioethanol production process. Previous research reported protein yields from corn DDGS ranging from 3.2 to 6.6% using a solvent containing 0.1 M NaOH, 0.1% DTT, and 0.5% SDS (Wolf and Lawton 1997), 40% using an alkaline solution at 70 ^{o}C (Bals et al. 2009), or 44.0% using 70% ethanol and 0.25% sodium sulfite solution at pH 2.0 (Xu et al. 2007). However, Wang et al. (2009) report a extraction yield of 56.8% with a protein content of 94.8 % from sorghum DDGS using a mixture of alkaline ethanol solution (ethanol/1 M NaOH, 45/55, v/v). Similar difficulties have been observed in protein extraction from brewer's spent grains due to excessive denaturation (Diptee et al. 1989; Tang et al. 2009). Research on protein extraction from triticale DDGS has not been reported in the literature. Therefore,

the objective of this research was to evaluate different methods of extracting proteins from triticale DWG and DDGS.

2.2. Materials and Methods

2.2.1. Materials and chemicals

Triticale DWG, DDGS, and triticale grains (used as the control sample) were generously provided by Pound-Maker Agventures Ltd. (Lanigan, Saskatchewan, Canada). DWG and DDGS were stored at -20 ^{o}C after receiving until use for protein extraction. All chemicals purchased were ACS grade unless otherwise noted. Sodium bisulfite ($NaHSO_3$) and sodium metabisulfite ($Na_2S_2O_5$) were purchased from Acros Organics (Morris Plains, NJ, USA). Tris-HCl 4–15% acrylamide linear gradient gel and precision plus protein molecular weight standard marker were purchased from Bio-Rad (Hercules, ON, Canada). Protex 6L enzyme was provided by Genencor International Inc. (Rochester, NY, USA). All other chemicals were purchased from Fischer Scientific (Ottawa, ON, Canada).

2.2.2. Sample preparation

DWG was either air dried at 50 ^{o}C using a cabinet air dryer for five days, or freeze dried using a Virtis Freeze Dryer (Biopharama Process Systems Ltd., Hampshire, U.K.) for five days, to study the effect of processing temperatures on protein extraction. DWG air dried samples (DWGad), DWG freeze dried samples (DWGfd), and DDGS samples were ground using a coffee grinder,

passed through a sieve (0.425 mm, Canadian Standard Sieve Series no. 40), packed in polyethylene bags, and kept at -20 ^{o}C until use.

2.2.3. Experimental design

Extraction was carried out in triplicate unless otherwise noted. Triticale grains (variety Ultima) were used as control. Chemical analyses were performed in duplicate for each replicate (a total of six determination for each sample).

2.2.4. Protein fractionation by a modified Osborne method

Samples of DWGad, DWGfd, DDGS, and triticale grains were subjected to Osborne fractionation as described by Chen and Bushuk (1970) with slight modifications. Water soluble, salt (0.5 N NaCl) soluble, ethanol (70%, ethanol/water, v/v) soluble, and propanol (50% propanol plus 1% acetic acid) soluble proteins were fractionated by subsequent extraction. A 1:10 (w/v) sample to solution ratio was used in all extractions and two subsequent extractions were carried out with one solution. Extractions were performed by stirring samples for 30 minutes at 300 rpm and 25 ^{o}C. Supernatants were collected after centrifugation at 3300 g for 15 minutes and 25 ^{o}C. Extracts were freeze-dried and analyzed for protein content.

2.2.5. Effect of pH on protein extraction

20 grams of DDGS were mixed with 200 mL of 10 mM phosphate buffer (pH 7.0), and the pH of the slurries was adjusted from 8.5 to 12.0 (at 0.5

intervals) using 1 N NaOH or 1 N HCl. The slurries were stirred (300 rpm, 25 ^{o}C) in an orbital shaker for 30 min. The supernatant was collected after centrifugation (3300 g, 15 minutes, 25 ^{o}C) and freeze dried for further analysis. The effects of raw materials on protein extraction were performed using 10 mM phosphate buffer (pH 10.0) for two subsequent extractions, supernatants were pooled for analysis.

2.2.6. Protein extraction with different ethanol concentrations

20 grams of DDGS were mixed with 20 to 90% ethanol at a 1:10 solid to liquid ratio (w/v), and stirred for 30 min (300 rpm, 25 ^{o}C) in an orbital shaker. After extraction, the supernatant was collected after centrifugation (3300 g, 15 minutes, 25 ^{o}C) and freeze dried for further analysis. Effects of raw materials on the protein extraction were performed in 60% ethanol for two subsequent extractions, supernatants were pooled for analysis.

2.2.7. Alkaline ethanol extraction of protein

Alkaline ethanol extraction of protein was performed according to Cookman and Glatz (2009) with slight modifications. DDGS (15 grams) was mixed with 150 mL of alkaline ethanol solution (45% ethanol + 55% 1 N NaOH), stirred for 2 hours (300 rpm, 25 ^{o}C), and centrifuged at 3000 g for 20 min at 25 ^{o}C to collect supernatant. For one set of experiments, the supernatant was freeze dried and analyzed for protein content; for another set of experiments, the supernatants were acidified to pH 4.6 (isoelectric pH for triticale distillers

grain proteins was determined by a series of precipitations [*data not shown*]) with 3 N HCl to precipitate the protein. Precipitates separated by centrifugation were washed with deionized water two times and freeze dried for further uses.

2.2.8. Extraction of proteins with glacial acetic acid

Extraction of proteins with glacial acetic acid was performed according to the method of Taylor et al. (2005) with slight modifications. DDGS (10 grams) was mixed with 0.5% (w/w) sodium metabisulfite, stirred for 16 hours at 100 rpm, centrifuged at 3,500 g for 15 min at 4 ^{o}C, and filtered through Whatman grade-4 filter paper. Filtrates were mixed with 50 mL of glacial acetic acid and stirred for 2 hours at 300 rpm. Supernatants were separated after centrifugation (3500 g for 15 min at 4 ^{o}C), acidified to pH 4.6 with 3 N HCl, and centrifuged again at the above conditions to obtain precipitate. Precipitate was washed with deionized water (100 mL) two times, and freeze dried.

2.2.9. Extraction of proteins with Protex 6L enzyme

Enzyme aided protein extraction from distillers grains was carried out based on the method described by Cookman and Glatz (2009) with slight modifications. 20 grams of milled samples were mixed with 300 mL of deionized water. After adjusting the temperature to 50 ^{o}C and the pH to 8.0, 1 mL of Protex 6L enzyme was added to initiate the digestion. The pH was kept constant by Titrando (Metrohm, Herisan, Switzerland) and the temperature was maintained using a circulating water bath. After 2 hours of incubation, the

temperature was increased to 80 ^{o}C and held for 10 min to deactivate the enzyme. The supernatant was separated after centrifugation (3500g, 15 minutes, 4 ^{o}C) and freeze dried for further analysis.

2.2.10. Protein content and yield calculation

The nitrogen content was determined by a Leco TrueSpec CN Elemental (carbon-nitrogen) Analyzer (St. Joseph, Michigan, USA). The protein content was calculated using a conversion factor of 5.9 (Kim et al. 2008). The extraction yield was calculated as the ratio between the amount of protein extracted and the total protein in the starting sample, and was expressed as percentage.

2.2.11. SDS-PAGE analysis of extracted proteins

Extracted proteins were prepared at a concentration of 2 mg/mL using 2% SDS containing 8 M urea, and mixed with 5% BME (50 µL of β-mercaptoethanol (BME) + 950 µL of Laemmli sample buffer) at a ratio of 1/1 (v/v). After heating at 95 ^{o}C for 5 min in an Eppendorf Thermomixer® Dry Block Heating Shaker (Eppendorf Canada, Mississauga, ON, Canada), samples were centrifuged using a Minicentrifuge (Fisher Scientific, Ottawa, ON, Canada). Supernatants (15 µL) were loaded on a Tris-HCl 4–15% linear gradient gel, and the gel was run at 200 V for 0.5h in a Mini-PROTEAN® II electrophoresis cell (Bio-Rad, Hercules, Canada). Gels were stained with Coomassie brilliant blue for 0.5 h, followed by distaining with 50% methanol and 10% acetic acid in water for 2 h. The molecular weights of the protein bands

in the gels were analyzed using AlphaEaseFC image analysis software (Alpha Innotech, San Leandro, CA, USA).

2.2.12. Statistical analysis

Analysis of variance (ANOVA) followed by Duncan's multiple range test was carried out to determine the effects of extraction methods and starting materials on the protein yield and protein content using Statistical Analysis System Software (SAS version 9.0, SAS Institute, Cary, NC). Effects of extraction method and starting material were evaluated at the 95% confidence level with three extraction replicates.

2.3. Results and Discussion

2.3.1. Protein fractionation of triticale distillers grain

Osborne fractionation was performed to determine the protein solubility of different triticale starting materials as summarized in Table 2.1. Triticale grains in the study showed a lower water, ethanol, and acetic acid soluble proteins, but a higher salt soluble protein fraction than those results of Chen and Bushuk (1970) and Ahmed and McDonald (1974). The difference may be due to the different varieties used in the two studies, and different conditions used in the Osborne fractionation. There was no difference between the fractions extracted from DWGad and DWGfd, which indicated that the solubility of triticale proteins was not affected by the methods of drying. DDGS samples displayed a different protein composition from that of unfermented triticale

grains, which indicated the effect of bioethanol processing on the protein solubility; for example, DWGad and DWGfd grains showed significantly lower ($p < 0.05$) amount of water soluble proteins than those of unfermented triticale grains, whereas DDGS had the highest water soluble protein fraction, which may be due to the addition of condensed distillers soluble to DDGS during processing. All DDGS samples showed significantly lower amount of salt soluble, ethanol soluble, and propanol soluble proteins than those of unfermented triticale grains.

Table 2.1- Protein composition of triticale distillers grain (DWGad, DWGfd) and triticale grains (raw triticale grains) fractionated based on a modified Osborne method. Data were analyzed by analysis of variance (ANOVA) followed by Duncan's multiple range test (n=3). Values in the same column with different superscripted letters are significantly different from each other ($p < 0.05$)

Sample	**Water solubles (%)**	**0.5 M NaCl solubles (%)**	**70 % ethanol solubles (%)**	**50% propanol + 1% acetic acid solubles (%)**
Triticale (Ultima)	20.84 ± 0.15^{b}	13.48 ± 0.66^{a}	20.01 ± 0.73^{a}	11.43 ± 0.01^{a}
DWGad	12.56 ± 1.48^{c}	6.29 ± 0.13^{b}	2.97 ± 0.03^{b}	2.10 ± 0.28^{b}
DWGfd	11.73 ± 0.42^{c}	5.55 ± 0.29^{bc}	3.25 ± 0.06^{b}	2.08 ± 0.11^{b}
DDGS	23.18 ± 0.21^{a}	4.82 ± 0.95^{c}	2.52 ± 0.11^{b}	1.54 ± 0.02^{c}

The total extraction yield was 32% from DDGS and 23% from DWG based on the Osborne method, compared to 65% from unfermented triticale grains. This implies that the low solubility of proteins is due to protein denaturation occurred during bioethanol processing. During bioethanol production, grains are subjected to continuous cooking using hydro-heaters at 120 ^{o}C for 60–75 min and then liquefaction at 85-90 ^{o}C for 2–3 h (Serna-Saldivar 2010), processes that may contribute to the low extractability of DDGS proteins.

2.3.2. Effect of pH on protein extraction

Effects of pH and raw materials on protein extraction are shown in Figures. 2.1 and 2.2. Extraction yield was not affected by pH from 8.5 to 11; the highest extraction yield (23.28%) was observed at pH 12.0. The protein content of the extracted samples showed the same trend as that of extraction yield where the highest yield was also found at pH 12.0. Our results compare well with those of Wolf and Lawton (1997) who reported a extraction yield of 25% from corn DDGS extracted by 0.1 M NaOH containing 0.1% DTT and 0.5% SDS. They also suggested that the low extractability of DDGS proteins may be due to protein denaturation occurred during ethanol production. A similar trend in extracting denatured proteins from different protein meals such as defatted soybean meal, almond meal, jojoba meal, and corn DDGS has been reported (Wolf et al 1994; Wolf 1995). These studies emphasize the need of a higher pH in the extraction of DDGS proteins. According to Bals et al (2009), the

extraction yield increased from 3% to 25% as the NaOH concentrations increased from 0.01 N to 1 N.

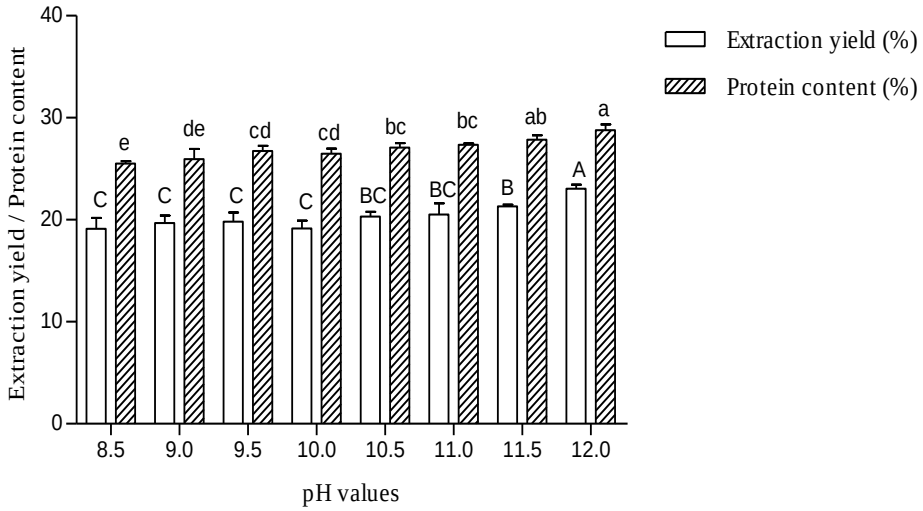

Figure 2.1 - Effect of different pH values on the extraction yield and protein content of extracts obtained from DDGS. Bars with different letters above mean values are significantly different. Upper case letters denote the differences in extraction yield whereas lower case letters represent the differences in protein content of extract.

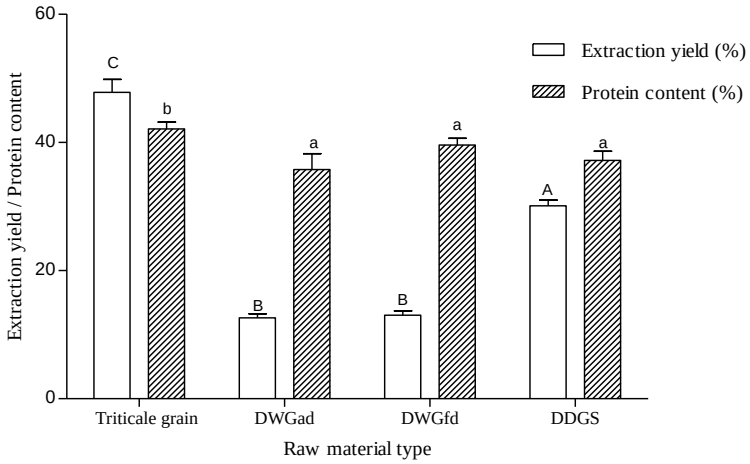

Figure 2.2 - Effect of raw materials (DWGad, DWGfd, and DDGS) on protein extraction obtained with pH shifting extraction. Bars with the different letters above the mean values are significantly different. Upper case letters denote the differences in extraction yield whereas lower case letters represent the differences in protein content of extract.

Our study showed that there was no difference in the protein contents extracted from three different distillers grain except for the triticale grains which showed significantly higher (42.09%) protein content in the extract. Extraction yield significantly varied ($p < 0.05$) where triticale grains exhibited the highest extraction yield (47.78%) and DDGS showed a significantly higher extraction yield over the other two distillers grain (Figure 2.2). The higher extraction yield in DDGS may be due to the addition of condensed distillers solubles, present proteins and/or nonprotein compounds, to the DDGS before drying, which are not present in the distillers wet grains.

2.3.3. Effect of ethanol concentrations on protein extraction

The effect of ethanol concentration on protein extraction yield and protein content of DDGS are shown in Figure 2.3. Protein extraction yield was not affected at ethanol concentrations ranging from 20% to 60%, but was significantly reduced at higher ethanol concentrations; the lowest protein extraction yield was obtained at 90% ethanol. Protein content was the highest at 60% ethanol and the lowest at 90% ethanol. DDGS proteins may be more

soluble at 60% ethanol. A recent study showed that 16% of the proteins in corn DDGS could be extracted at 60% ethanol (Cookman and Glatz 2009), compared to 29% from triticale DDGS and ~ 12% from DWGad and DWGfd in this study.

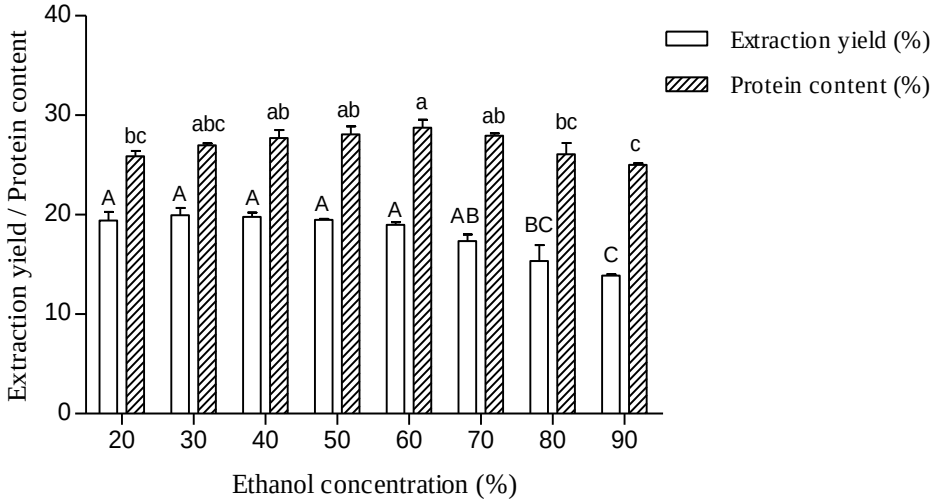

Figure 2.3 - Effect of different ethanol concentrations on protein extraction from DDGS. Bars with the different letters above the mean values are significantly different. Upper case letters denote the differences in extraction yield whereas lower case letters represent the differences in protein content of extract.

Effect of different raw materials on protein extraction at 60% ethanol is shown in Figure 2.4. The triticale grains had the highest extraction yield (42.90%) but its protein content was significantly lower than that of DWGad and DWGfd. The extraction yield was significantly higher ($p < 0.05$) from DDGS than from DWGad and DWGfd. The protein contents of DWGad and DWGfd (51.33% and 53.53%, respectively) were significantly higher ($p < 0.05$) than that of DDGS (40.33%). The presence of condensed distillers solubles may account

for the higher extraction yield in DDGS compared to DWGad and DWGfd. However, the protein content of extracted fractions can be affected by these solubles as they can also be dissolved with 60% ethanol.

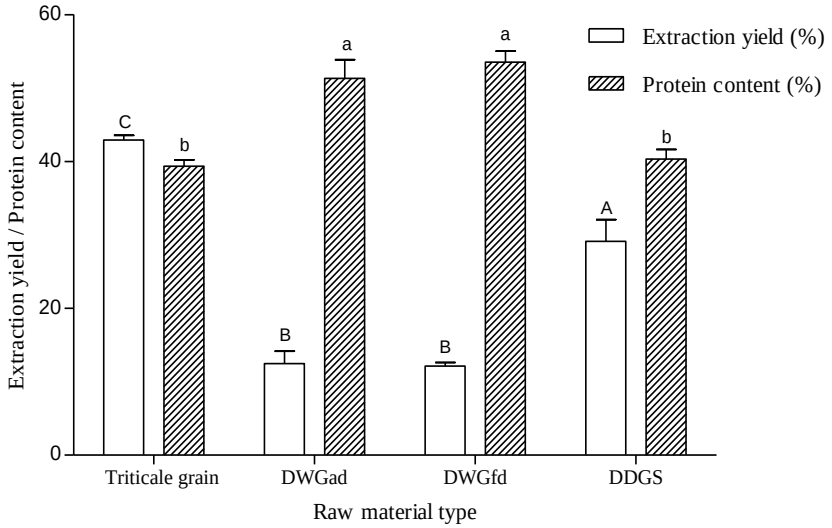

Figure 2.4 - Effect of raw materials (DWGad, DWGfd, and DDGS) on protein extraction using 60% ethanol method. Bars with the different letters above the mean values are significantly different. Upper case letters denote the differences in extraction yield whereas lower case letters represent the differences in protein content of extract.

Protein contents of the extracts obtained from DDGS were significantly lower than those of DWGad and DWGfd using both 60% ethanol extraction and pH shifting extraction methods. Bals et al (2009) reported that DDGS comprises around 20% water soluble nonprotein compounds. The effect of these water solubles may be the reason for lower protein content in pH shifting extracts

compared to 60% ethanol extracts. The study of Bals et al (2009) showed that NaOH increased the biomass solubilization which decreases the protein content of the extract.

2.3.4. Alkaline ethanol extraction of protein

The results of alkaline ethanol extraction of triticale proteins with and without isoelectric precipitation are shown in Figure 2.5.

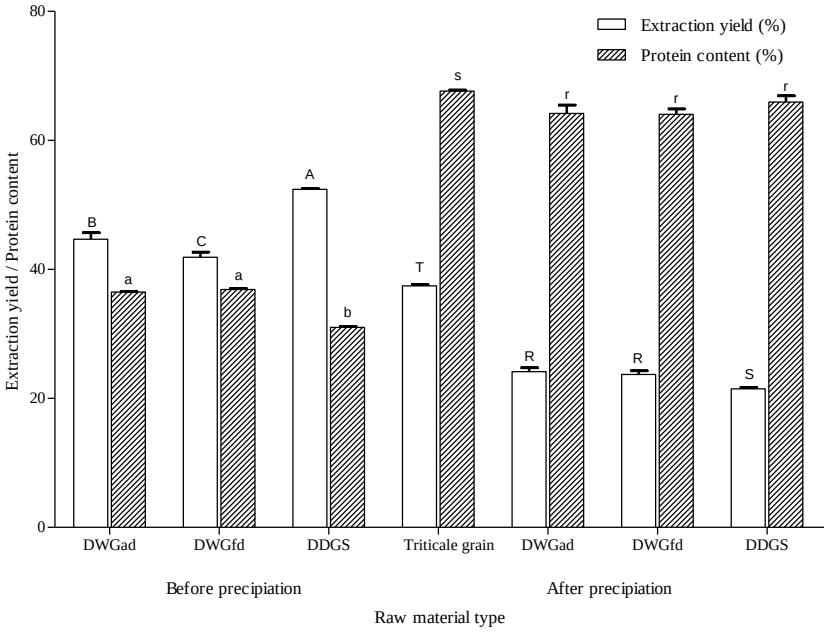

Figure 2.5 - Effect of raw materials (DWGad, DWGfd, and DDGS) on protein extraction using alkaline ethanol solution (55% 1 M NaOH and 45% ethanol) method with/out isoelectric precipitation. Bars with the different letters above the mean values are significantly different. Upper case letters denote the differences in extraction yield whereas lower case letters represent the differences in protein content of extract.

Without isoelectric precipitation, DDGS samples gave the highest ($p < 0.05$) extraction yield (52.41%), followed by DWGad (44.68%) and DWGfd (41.90%). The protein content varied from 31% to 36%, where DWGad and DWGfd extracts had significantly higher protein content (36.48% and 36.88%, respectively, $p < 0.05$) than the DDGS extract (31%). Isoelectric precipitation at pH ~ 4.5–4.7 could significantly improve the protein content from 36.48% to 64.18% for DWGad, from 36.88% to 64.05% for DWGfd, and from 31% to 65.93% for DDGS, but the extraction yields were drastically reduced. Compared to the distillers grain, triticale grains exhibited a significantly higher ($p < 0.05$) extraction yield (37.07%) and protein content (67.65%) (Table 2.2). It should be noted that not all proteins could be precipitated at the isoelectric pH. Both the protein content and yield of alkaline ethanol extracts were significantly higher than those of pH shifting and 60% ethanol extracts (Table 2.2). Alkaline ethanol is more efficient in extraction of denatured proteins from the distillers grain.

Bals et al (2009) extracted 42% of the protein from corn DDGS using the same method, but the protein content of the extract was not reported. Cookman and Glatz (2009) reported that the protein content of DDGS was reduced from ~36% to ~8% after extraction, but they didn't report the extraction yield. An extraction yield of 56.8% and a protein content of 94.88% were recently reported for sorghum DDGS using the same extraction method (Wang et al 2009); this value is much higher than values in previous studies and our results. Differences in protein composition between sorghum and triticale DDGS, as well as

differences in processing methods may be responsible for the difference in protein extraction yield and protein content in our study.

2.3.5. Extraction of proteins with glacial acetic acid

Extraction yield and protein content of extracts obtained from triticale grains, DWGad, DWGfd, and DDGS are summarized in Table 2.2. The yield and protein content of triticale grains were 28.84% and 65.05%, respectively; there was no difference in both yield and protein content of three distillers grain. Compared to the methods of ethanol and NaOH extraction, glacial acetic acid resulted in a higher protein recovery but the results were similar to those obtained in the alkaline ethanol extraction. However, our results were much lower than those of Wang et al (2009) who reported an extraction yield of 44.1% and a protein content of 98.94% from sorghum DDGS. This may be due to the variation in protein composition between triticale and sorghum and the different processing methods used in the ethanol production. Further prolonged incubation up to 16 hours under reducing conditions did not improve the extraction yield or the protein content of the extract in our study *(data not shown).*

Table 2.2 - Extraction yield and protein content of protein extracts from triticale distillers grain (DWGad, DWGfd and DDGS) and triticale grains (raw triticale grains) using different extraction methods.

Extraction method	Extraction yield (%)				Protein content (%)			
	Triticale Grain	DWGad	DWGfd	DDGS	Triticale Grain	DWGad	DWGfd	DDGS
pH shifting	47.78 ± 3.53	12.82 ± 0.74	13.46 ± 0.75	30.05 ± 2.71	42.09 ±1.79	35.75 ± 4.34	40.30 ± 1.51	37.07 ± 2.71
60% ethanol	42.90 ± 1.13	12.46 ± 2.91	12.11 ± 0.86	29.10 ± 5.16	39.37 ± 1.46	51.32 ± 4.40	53.53 ± 2.59	40.33 ± 2.24
Alkaline ethanol	37.07 ± 0.98	24.17 ± 1.03	23.12 ± 0.97	21.48 ± 0.38	67.65 ± 0.27	64.18 ± 2.29	64.05 ± 1.46	65.93 ± 1.70
Acetic acid	28.84 ± 1.71	23.60 ± 0.65	24.44 ± 0.47	23.17 ± 1.56	65.05 ± 2.02	62.05 ± 2.61	61.42 ± 5.83	64.03 ± 3.95
Protex 6L	64.12 ± 1.22	75.44 ± 0.64	75.14 ± 1.72	82.92 ± 2.05	46.07 ± 2.47	57.07 ± 0.92	55.28 ± 1.09	43.32 ± 1.90

2.3.6. Extraction of proteins with Protex 6L enzyme

Enzymes have been extensively studied to improve protein extraction (Kristinsson and Rasco 2000; Rosenthal et al 2001; Campbell and Glatz 2009; Cookman and Glatz 2009). Protex 6L was applied to improve the solubility, and therefore increase the protein extraction, of DDGS. Extraction yields were significantly improved, ~75% for DWGad and DWGfd samples and 82% for DDGS, in the presence of enzyme compared to other methods (Table 2.2). The protein content of DWGad (57.07%) and DWGfd (55.28%) was significantly higher than that of DDGS (43.32%). The extraction yield (64.12%) of triticale grains was significantly lower than the yields of all distillers grain, indicating that denatured proteins were digested more readily by Protex 6L than native proteins. Cookman and Glatz (2009) reported a reduction in protein content from ~36% to ~10% in corn DDGS after 2 hours digestion with Protex 6L. Using brewer's spent grains, alcalase enzyme solubilized 80% of the protein at a protein content of 60%. These results confirm the advantage of using peptidases to extract protein from denatured protein (Treimo et al 2008).

2.3.7. SDS-PAGE analysis of extracted proteins

SDS-PAGE analysis of Osborne fractions obtained from triticale grains are shown in Figure 2.6. In the water soluble fraction, molecules with smaller molecular weight mostly represent the enzymes present in the triticale grain, and it can be identified in the regions of 10, 15, 18, 20, 25, 40, and 60 kDa. In the salt soluble fractions, small protein bands can be observed at 10, 12, 30, and 35

kDa. Other than the smaller Mw protein bands (15, 30, and 37 kDa), bands at 45, 70, and 100 kDa, typical of gliadin proteins, were observed in the ethanol extract, similar to another study using winter triticale varieties (Tohver et al 2000). In the acetic acid extract, bands are visible at 37, 50, 55, 75, and 100 kDa. Protein bands at 50–55 kDa regions resemble high molecular weight glutenin proteins (Tohver et al 2000).

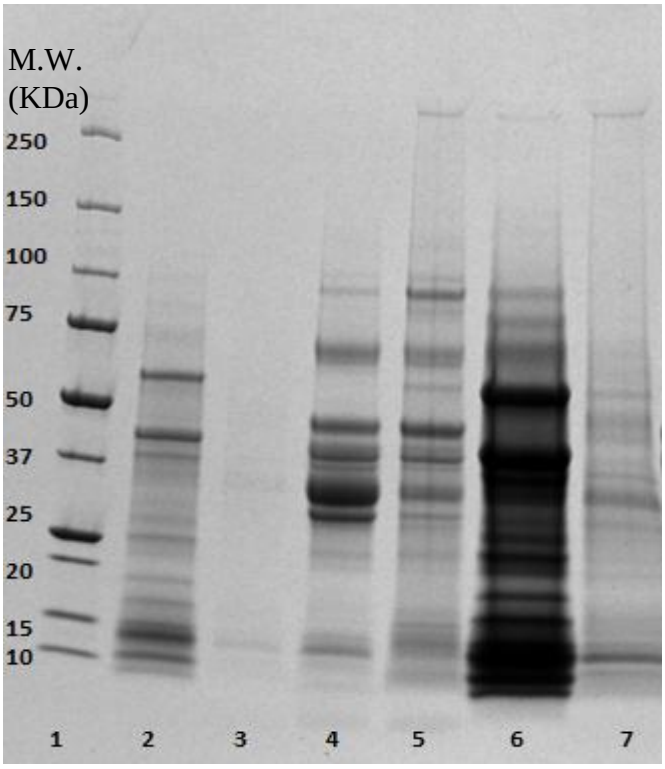

Figure 2.6 - SDS-PAGE analysis of protein extracts obtained by a modified Osborne method from triticale grains (raw triticale grains). Lane 1 represents molecular weight marker, lanes 2 to 5 represent water soluble, salt soluble, 70% ethanol soluble, and acetic acid soluble fractions, respectively. Lanes 5 and 6

represent water soluble and salt soluble fractions, respectively, with 30 μL sample.

SDS-PAGE results of extracts obtained by methods of alkaline ethanol extraction, reduced acetic acid extraction, and enzyme aided extraction from DWGad, DWGfd, and DDGS, and triticale grains with SDS buffer are shown in Figure 2.7. Proteins extracted from triticale grains (lane 2) appear at 10, 15, 18, 25, 36, 43, 45, 55, 62 and 70 kDa. Dennett et al (2009) reported the dominant protein band around 55 kDa regions, representing high molecular weight glutenin subunits. The authors also observed several protein bands below 40 kDa molecular weight. In our study, a smeared band near 23 kDa in the protein extracts from DWGad and DWGfd (lanes 3 and 4), and one band near 20 kDa from the DDGS extract (lane 5), represent proteins obtained using alkaline ethanol extraction. Visible bands near the end of the stacking gel in alkaline ethanol extracted proteins may represent large protein aggregates that cannot enter the running gel. Band smearing in the region of 23 kDa could be due to alkaline degradation or precipitation of proteins occurred during alkaline ethanol extractions; similar results were reported by Cookman and Glatz (2009) in SDS-PAGE analysis of alkaline ethanol extracted proteins from corn DDGS.

SDS-PAGE analysis of glacial acetic acid extracts displayed several clear bands. In DWGad (lane 6) and DWGfd (lane 7) extracts, protein bands are visible at molecular weights of 12, 16, 25, 30, 35, 43, 50, and 73 kDa, and in DDGS extracts (lane 8), bands appear close to 12, 15, 23, 25 and 50 kDa. The

DDGS extract shows two sharp bands in 23 and 25 kDa regions. No bands appear in lanes 9 and 10, which indicate extensive protein degradation occurred by the Protex 6L.

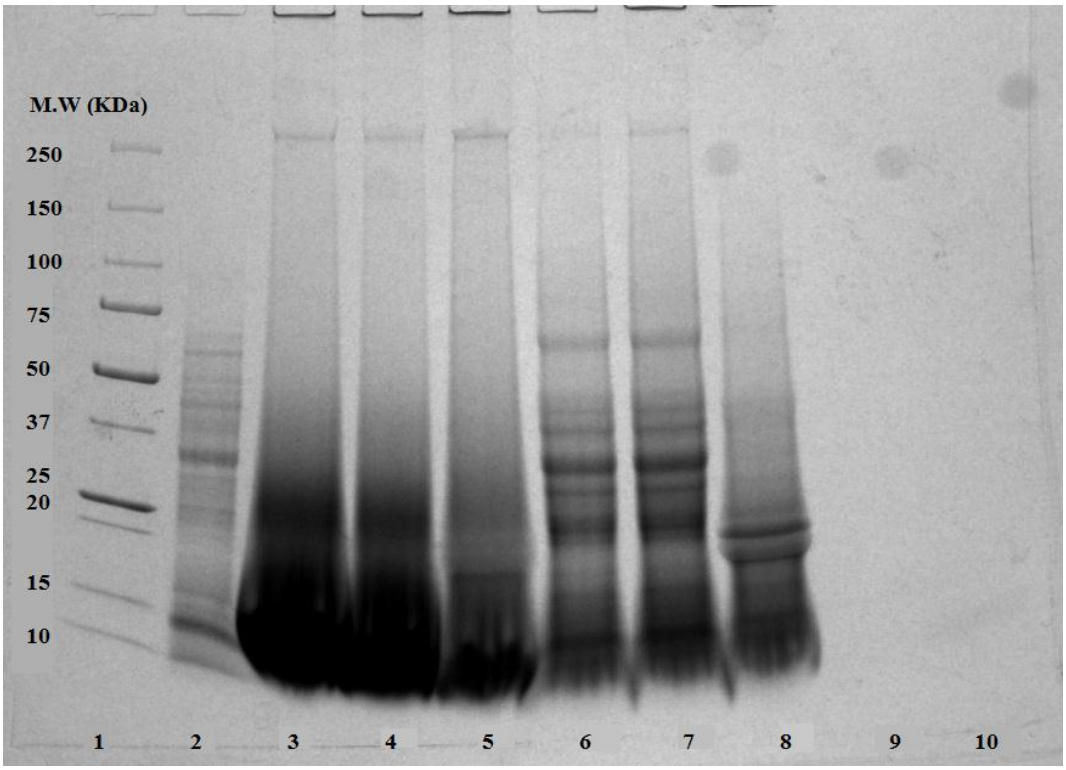

Figure 2.7 - SDS-PAGE analysis of precipitated protein obtained by three different extraction methods. Lane 1, molecular weight marker; lane 2, SDS extract of triticale grain (control); lanes 3–5, alkaline ethanol extraction; lanes 6–8, glacial acetic acid extraction; lanes 9–10, Protex 6L digested proteins. Proteins were extracted from DWGad for lanes 3, 6, and 9; DWGfd for lanes 4 and 7; DDGS for lanes 5, 8 and 10. All wells were loaded with 15 µL at a protein concentration of 2 mg/mL

2.4. Conclusion

Protein extractabilities of triticale DDGS and DWG were low based on the Osborne fractionation method. Among five methods employed to extract proteins from three triticale distillers grains, alkaline ethanol extraction and reduced acetic acid extraction showed comparable results to previous reports of protein extraction from corn DDGS. The low extractability of proteins from triticale distillers grains may be due to the heating/cooking conditions applied in bioethanol processing which denatured the proteins. One of the challenges in extraction of protein from distillers grain is improving protein content without compromising the extraction yield. Compared to the other methods tested, enzyme aided extraction resulted in a considerably high extraction yield of 75–82% and a protein content of 43–57%. Further studies are needed to improve the protein content without compromising protein yield and to determine the functional properties of the extracts.

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CHAPTER 3

ADHESIVE PROPERTIES OF TRITICALE DISTILLERS GRAIN PROTEINS

3.1. Introduction

Increasing use of triticale as the feedstock for the biofuel industry in Canada leads to increasing production of (CTBI 2011) triticale distillers grain, a co-product of triticale based bioethanol processing (Gibreel et al 2011). According to the 2011 Renewable Fuels Association Report, 98% of distillers grain produced in the United States are used as animal feed, while only 1% is used for other applications (RFA 2011). Triticale distillers grain contains a protein content of 20-43%, compared to 25-33% in corn distillers grains (Pena 2004; Wierenga et al 2010; Gibreel et al 2011; Cookman and Glatz 2009; Gibreel et al 2011; Liu 2011). Triticale contains a higher amount of water and salt soluble proteins (26-29% and 6-9%, respectively) than their parental species, wheat and rye (Chen and Bushuk 1970; Pena 2004; Tohver et al 2000; Fernández-Fígares et al 2008; Castillejo et al 2010; Jonnala et al 2010). Monomeric prolamins such as gliadins and secalins stabilized by intramolecular-disulfide bonds make 24-28% of total proteins present in triticale (Chen and Bushuk 1970; Pena 2004). Apart from monomeric proteins, 17-20% of total proteins are polymeric low molecular weight and high molecular weight glutenins, stabilized by interchain disulfide bonds (Salmanowicz and Nowak 2009).

Protein has vast potential to develop non-food uses as plastics, adhesives, and films (Lambuth 1994; Barone and Schmidt 2006). Although the wood adhesive industry is heavily dependent on petroleum based chemicals due to high performance and cheap price (Sun and Bian 1999), use of synthetic adhesives from petroleum is associated with environmental concerns due to formaldehyde emission and nonrenewability. Developing adhesives from renewable protein sources is highly encouraged because of their unique characteristics such as biodegradability, renewability, and low environmental impact (Lambuth 1994; Kumar et al 2002). Animal blood, casein and gelatin are the most commonly used animal protein sources (Lambuth 1994) while soy protein (Hettiarachchy et al 1995; Kalapathy et al 1995; Huang and Sun 2000a; Huang and Sun 2000b; Liu and Li 2002; Liu and Li 2004; Li et al 2010) and wheat gluten (Lagrain et al 2010; Nordqvist et al 2010) are the main plant proteins studied. Adhesives prepared from *Jatropha curcas* proteins (Hamarneh et al 2010) and rice bran proteins (Pan et al 2005) were also reported. Low strength and water resistance are two major challenges to overcome to make protein-based adhesives suitable for exterior applications (Kumar et al 2002). Therefore protein modifications, such as alkaline (Hettiarachchy et al 1995; Kalapathy et al 1995), urea, guanidine hydrochloride (GH), sodium dodecyl sulphate (SDS), and sodium dodecylbenzene sulphonate (SDBS) (Sun and Bian 1999; Huang and Sun 2000a; Huang and Sun 2000b) were applied to improve the adhesion strength and water resistance properties of protein-based adhesives. Esterification (Wang et al 2006), cross-linking (Wang et al 2007), and grafting

of functional groups into protein chain (Liu and Li 2002; Liu and Li 2004) were also reported. Medium density fiberboards (MDF) produced with SDS modified soy protein adhesives showed similar mechanical properties to those of commercial MDF boards (Li et al 2009). Adhesion strength of wheat gluten hydrolyzate modified with formaldehyde and glyoxal resins were comparable to the standard specifications required for particle-board production (Lei et al 2010). Comapred to soy protein and wheat glueten, triticale is not a traditional food source of proteins, which may represent its advantage. There is no report on developing adhesives from triticale distillers grain proteins. Therefore, the objective of this research is to prepare and characterize adhesive from triticale distillers grain proteins.

3.2. Materials and Methods

3.2.1. Materials

Urea, NaOH, and 25% stock glutaraldehyde solutions were purchased from Acros Organics (Morris Plains, NJ, USA). Birch wood veneer (hard wood) was purchased from Windsor Plywood (Edmonton, AB, CA). All other chemicals purchased were ACS grade unless otherwise noted.

3.2.2. Sample preparation

Triticale proteins were extracted from triticale distillers dried grain with solubles (DDGS) and distillers wet grain (DWG). Proteins were extracted with alkaline ethanol (DDGSae, DWGae), acetic acid (DDGSaa, DWGaa) solutions,

or using Protex 6 L (from DDGS only) as described in the previous chapter. All protein samples were freeze dried, and ground before stored at 4 ^{o}C until further analysis.

Hard wood veneer samples (Birch) with thickness of 0.7 mm were cut into pieces of 20 mm × 120 mm (width × length) using a sample cutting device (Adhesive Evaluation Systems, Corvallis, OR, USA), and preconditioned for seven days in a controlled environment chamber (ETS 5518, Glenside, PA, USA) at a temperature of 23 ^{o}C and at a humidity of 50% according to the requirements of ASTM (American Society for Testing and Materials) standard method D2339-98 (ASTM 2002) before using for adhesion strength measurement (Wang et al 2007).

3.2.3. Effect of various modifications and curing conditionings on adhesion strength

Effects of various methods of modifications and curing conditions on adhesive properties were studied using DWGae protein sample. Triticale protein extracts were prepared into 15% (w/v) slurries using NaOH solution at concentrations of 0, 0.25, 0.5, 1, 2, 5 mM, urea solution at concentrations of 0, 0.5, 1, 2, 4, 5 M, and glutaraldehyde solution at concentrations of 0, 20, 40, 60, 80, 100 µM, respectively, to determine the effect of concentrations on dry adhesion strength. The concentration showing the highest dry adhesion strength was selected for further study. Effects of curing temperature (80, 90, 100, 110,

120, 130 ^{o}C) and curing time (1, 2, 3, 4, 5 min) on dry adhesion strength were also studied using alkali modified (0.5 mM) DWGae samples.

3.2.4. Effect of sample preparations on adhesion strength

Five different protein samples (DDGSae, DWGae, DDGSaa, DWGaa, P6L) prepared previously were modified by 0.5 mM NaOH (stirring 300 rpm at 25 ^{o}C for 3 h), 1 M urea (stirring 300 rpm at 25 ^{o}C for 6 h), and 60 µM glutaraldehyde (stirring 300 rpm at 25 ^{o}C for 6 h), respectively, at a protein concentration of 15% (w/v) based on the established conditions above. For glutaraldehyde modified sample, 50 µL of 0.5 M NaOH solution was added to improve protein dispersion after the modification. All protein modifications were carried out in duplicate for each protein extract unless otherwise noted.

3.2.5. Measurement of dry, wet and soaked adhesion strength

About 15 µL of protein slurry was applied to each surface of two wood specimens on a 20 mm × 5 mm area. Two wood samples were placed together with a contact area of 20 mm × 5 mm and hot pressed at 110 ^{o}C, 3.5 MPa for 120 seconds using an Automated Bonding Evaluation System (ABES II, Adhesive Evaluation Systems, Corvallis, OR, USA). Dry adhesion strength (DAS) was measured immediately after the hot pressing of sample by pulling veneer pieces using the ABES II. Wet adhesion strength (WAS) and soaked adhesion strength (SAS) was measured according to the ASTM standard method D1151-00 (ASTM 2002). Hot pressed wood specimens were submerged in water

at 23 ^{o}C for 48 hr and tested immediately after removing water for wet adhesion strength (WAS) assay. To measure soaked adhesion strength, submerged samples were dried and conditioned at 25 ^{o}C and 50% humidity in a controlled environment chamber (ETS 5518, Glenside, PA, USA) for seven days prior to adhesion strength measurement. Protein modifications were performed in duplicates, and four glued specimens were prepared for each replicate for adhesion strength measurement (DAS, WAS, SAS).

3.2.6. Amino acid analysis

Amino acid analysis of protein extracts (DDGSae, DDGSaa, DWGae, DWGaa, P6L) was performed according to the method described by Sedgwick et al (1991) with slight modifications. About 50 mg of protein extracts were weighed into a screwed cap culture tubes, mixed with 3 mL of 6 M HCl solution, vortexed, purged with nitrogen and hydrolyzed at 110 ^{o}C for 24 h, and then 0.2 mL of internal standard (β-amino-n-butyric acid / ethanolamine at 25 μM/mL) and 1 mL of distilled water were added to the tubes and mixed thoroughly before centrifuge at 2500 rpm for 15 min. Supernatant (0.05 mL) obtained above was mixed with 0.2 mL saturated $K_2B_4O_7$, 0.4 mL of deionized water, and 0.05 mL of 4.29 M NaOH, filtered and analyzed by HPLC (maker, place) as described by Sedgwick et al (1991).

3.2.7. Fourier Transform Infrared Spectroscopy (FTIR)

All protein samples were freeze dried, ground with KBr into a fine powder, and made into a thin pellet prior to FTIR analysis. FTIR spectra were recorded using a Nicolet Magna 750 spectrophotometer (Nicolet Instruments, Madison, WI, USA) by cumulating 10 scans at a resolution of 8 cm^{-1} for each sample. All data were processed using OMNIC software suite (Madison, WI, USA).

3.2.8. SDS-PAGE analysis of modified proteins

Protein extracts (DDGSae, DDGSaa, DWGae, DWGaa, and P6L) with/without modifications were prepared at a concentration of 2 mg/mL using a solution containing 2% SDS in 8 M urea, and further mixed with 5% BME (50 µL of β-mercaptoethanol (BME) + 950 µL of Laemmli sample buffer) at a ratio of 1:1 (v/v). After heating at 95 ^{o}C for 5 min in Eppendorf Thermomixer® Dry Block Heating Shaker (Eppendorf Canada, Mississauga, ON, Canada), samples were centrifuged using Minicentrifuge (Fisher Scientific, Ottawa, ON, Canada) prior to sample loading. 15 µL of supernatant was loaded onto Tris-HCl 4-20% linear gradient gels, and run at 160 V for 45 min in a Mini-PROTEAN® II electrophoresis cell (Bio-Rad, Hercules, Canada). Gels were stained for 0.5 h using Coomassie brilliant blue, followed by destaining with 50% methanol and 10% acetic acid in water for 2 h. Molecular weights of protein bands in the gels were analyzed using AlphaEaseFC image analysis software (Alpha Innotech, San Leandro, CA, USA).

3.2.9. Statistical analysis

Data were analyzed using analysis of variance (ANOVA) followed by Duncan's Multiple Range (DMR) test to identify the effects of protein extractions on adhesion strengths (Dry, Wet, Soaked) using Statistical Analysis System Software (SAS version 9.0, SAS Institute, Cary, NC). Effects of protein extracts were evaluated at the 95% confidence level in duplicates.

3.3. Results and Discussion

3.3.1. Effects of concentrations of modifier on adhesion strength

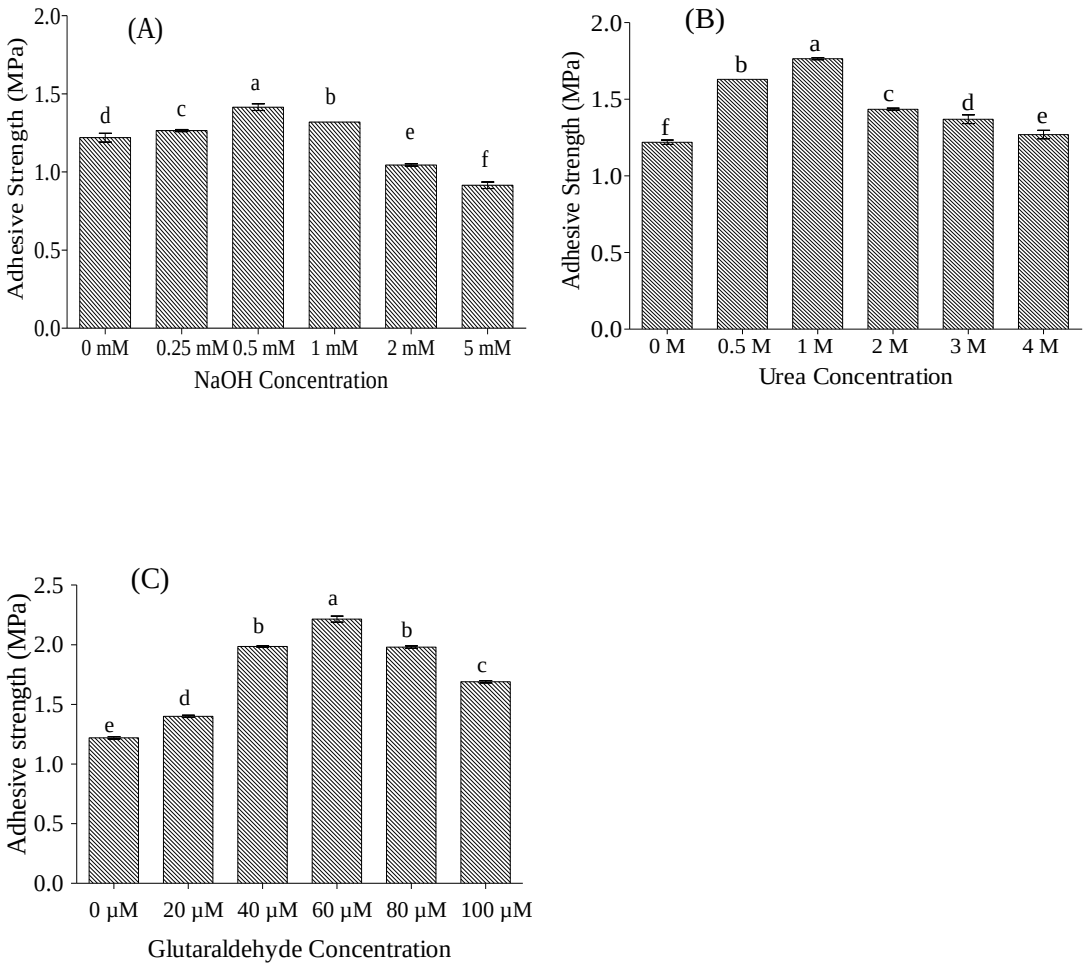

Figure 3.1 – Effects of concentrations of NaOH (A), urea (B), and glutaraldehyde (C) on dry adhesion strength of modified DWGae protein samples. Different letters on the bar represent significantly different adhesion strength ($p<0.05$).

Adhesion strength of DWGae samples prepared at different NaOH concentrations are shown in Figure 3.1 (a). Adhesion strength was initially increased from 0 to 0.5 mM NaOH (exhibited the highest adhesion strength at 0.5 M), and started to decrease at increasing concentrations. Increasing NaCl concentrations from 0.1 M to 0.2 M was reported to decrease the adhesion strength of alkali modified soy protein due to weakening the interaction of polar groups in wood and protein (Kalapathy et al 1996). This may be due to the interference of high ion concentration with polar groups present in wood and proteins that form the bonds. Therefore, 0.5 mM NaOH solution was selected to optimize the conditions for curing. The highest adhesion strength was found at 110^{o}C curing temperature for 2 minutes (*data not shown*); thus, the curing conditions were established.

Adhesion strength of DWGae modified with different urea concentrations is shown in Figure 3.1(b). Adhesion strength was initially increased at increasing urea concentrations from 0 to 1 M, but started to decrease at further increased concentrations. The concentration of 1 M urea was selected for further study. Huang and Sun (2000) also observed similar trend in adhesion strength at increasing urea concentrations, whereas the highest strength was

reported at 3 M urea using soy protein isolate. Adhesion strength of DWGae protein sample modified with glutaraldehyde concentrations is shown in Figure 3.1(c). Adhesion strength was increased at increasing glutaraldehyde concentrations from 0 to 60 μM and started to decrease at increased concentrations. Therefore, 60 μM concentration was selected for further study. Wang et al (2007) observed similar trend in adhesion strength at increasing glutaraldehyde concentrations; however, the optimum strength was observed at 20 μM. Reduction in adhesion strength at increasing modifier concentrations may be attributed to the increased viscosity of protein dispersions and increased protein–protein interaction which results less protein-wood interactions (Wang et al 2007). Cross-linking with glutaraldehyde reinforces the network structure of protein and make them resistant to collapse in aqueous mediums (Cao et al 2000; Silva et al 2004). Thus low water solubility exist in TDGP has increased with glutaraldehyde modifications; therefore 0.5 M NaOH was added to modified proteins to improve protein dispersion.

3.3.2. Effect of protein extraction on adhesion strength of alkali modified triticale proteins

Five triticale proteins were prepared as reported previously. Effect of methods of protein extraction on adhesion strength with/without alkali modification is shown in Figure 3.2. Alkali modification enhances the adhesion strength with the exception of P6L hydrolyzate. Alkaline modifications of soy protein and wheat gluten were reported to improve the adhesive properties such

as adhesion strength and water resistance (Hettiarachchy et al 1995; Kalapathy et al 1995; Mo et al 1999; Sun and Bian 1999; Khosravi et al 2010; Nordqvist et al 2010). Alkaline modification of protein could break hydrogen bonds and unfold protein molecules, leading to exposing sulfhydryl and hydrophobic residues on protein molecules surface for bond formation with wood surface, thus increasing adhesion strength (Mo et al 2004; Hettiarachchy et al 1995). Regarding P6L hydrolyzate, as the protein molecules have already been broken into small peptides, further NaOH modification would not improve the adhesion strength.

Among the samples tested, alkali modified DDGSaa exhibited the highest strength (the dry, wet and soaked strengths were 3.22, 1.23, and 1.88 MPa, respectively), followed by DWGaa (2.97, 1.16, and 1.25 MPa, respectively). Both protein samples were extracted with acetic acid solution, and contain higher amount of gluten and gliadins compared to the other extracts, which may be the reason for better adhesion properties of acetic acid extracted proteins. Compared to the DWGaa, DDGSaa showed significantly higher adhesion strength; this may be due to the addition of condensed distillers solubles into DDGS that may contribute to the higher adhesion strength. The dry adhesion strength of alkali modified soy protein was reported to range from 0.75 MPa to 1.97 MPa, which was comparable to our results (Hettiarachchy et al. 1995). However, results from Sun's laboratory were much higher than ours, ranging from 3.0 to 6.5 MPa depending on the type of woods used (Sun and Bian 1999). In another study, dry adhesion strength at 5.0 - 5.7 MPa and wet adhesion strength at 2.25 - 2.75 MPa were observed for alkaline modified soy

proteins (Mo et al 2004). In a recent study, alkali modifications of wheat gluten and soy protein were reported to increase the adhesion strength to the level of the European Standard EN 204 for wood adhesives (Nordqvist et al 2010). Alkali modification of triticale proteins was much lower than the above standard.

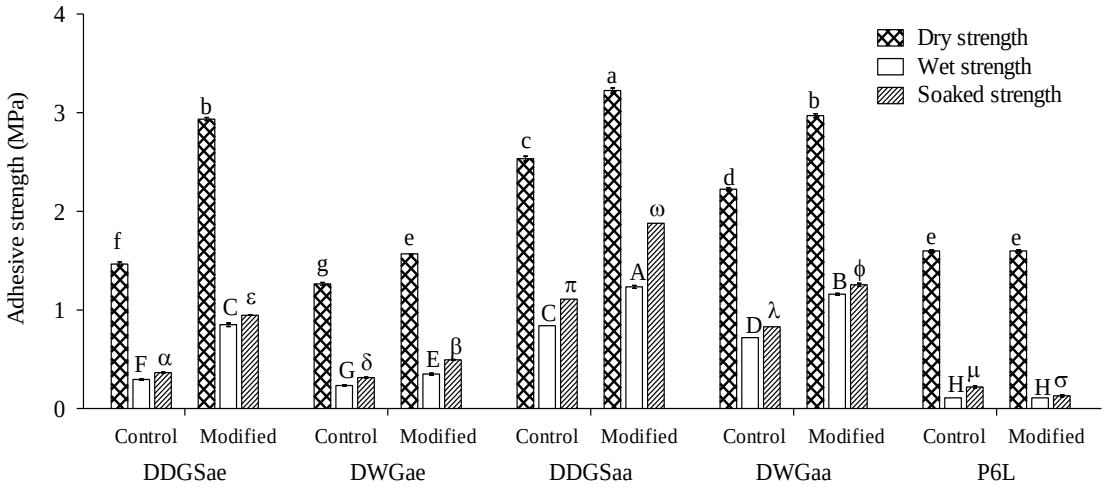

Figure 3.2 – Adhesion strength of various protein extracts with/without alkali modification. Different letters on the bar represent significantly different adhesion strength ($p<0.05$).

3.3.3. Effect of protein extraction on adhesion strength of urea modified triticale proteins

Urea modification increased the adhesion strength of all protein samples, with the exception of P6L hydrolyzate where it reduced the strength of modified proteins (Figure 3.3). The highest adhesion strength was found in DDGSaa sample (the dry, wet and soaked strengths were 2.91, 1.03, and 1.39 MPa,

respectively), followed by DWGaa samples (the dry, wet and soaked strengths were 2.78, 0.98, and 1.28 MPa, respectively). Our results suggested that proteins extracted with alkaline ethanol solutions have significantly higher adhesion strength than that of enzyme aided extract, but lower than that of acetic acid extracted proteins. Urea has been used as a protein modification agent due to its ability in denaturing protein molecules (Huang and Sun 2000a). Oxygen and hydrogen atoms present in urea have the ability to interact with hydroxyl groups in protein molecules to break hydrogen bonds, thus leading to unfold protein molecules. These unfolded proteins have better adhesion ability than folded native protein molecules (Sun and Bian 1999).

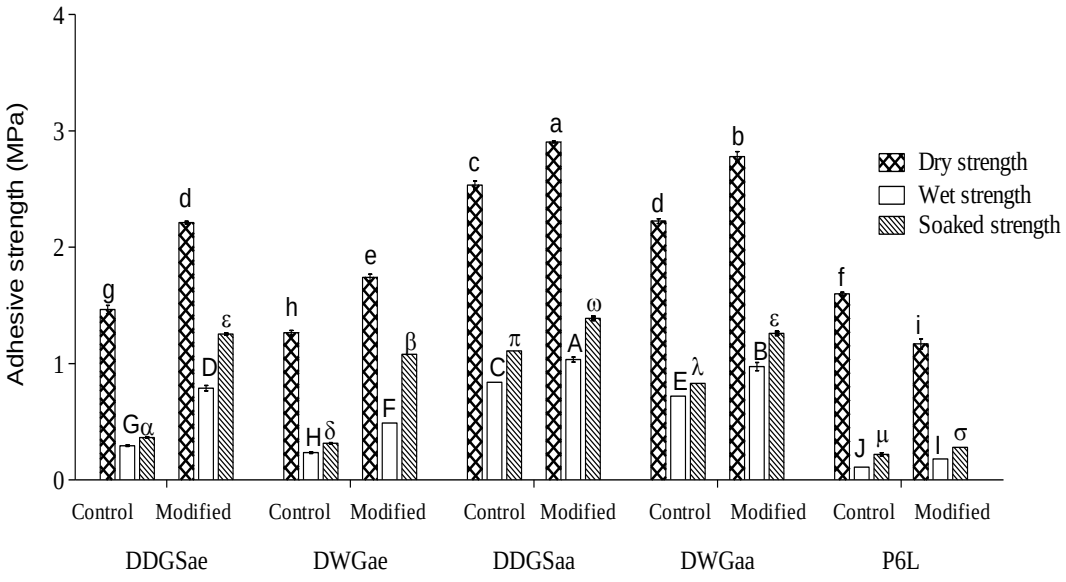

Figure 3.3 – Adhesion strength of various protein extracts with/without urea modification. Different letters on the bar represent significantly different adhesion strength ($p<0.05$).

Dry adhesion strength of urea modified soy proteins was reported to vary from 4.2 to 5.9 MPa (Huang and Sun 2000a) and from 3.1 to 6.2 MPa (Huang and Sun 2000b) depending on the wood surface used. In another study with different composition of 7S and 11S subunits, dry adhesion strength of soy proteins was reported to range from 3.7 to 5.5 MPa (Mo et al 2004). Wet adhesion strength and soaked adhesion strength were reported to vary from 0.4 to 2.5 MPa and from 2.1 to 4.9 MPa, respectively (Huang and Sun 2000a). The maximum dry adhesion strength observed for triticale distillers grain protein was 2.91 MPa (DDGSaa sample). Wet adhesion strength was drastically reduced after soaking in water for 48 h, while conditioning did not improve soaked adhesion strength. The low wet adhesion strength may be due to breaking of bonds by water molecules that penetrate into wood-glue joints during soaking, which interfere with the bond formation (Mo et al 2004).

3.3.4. Effect of protein extraction on adhesion strength of glutaraldehyde modified triticale proteins

Glutaraldehyde is used as a modification agent due to its potential of cross-linking via covalent bonds (Silva et al 2004). It has the ability to bind with amine groups of lysine, hydroxylysine, and N-terminal of polypeptide by making methylene bridges between two molecules. Other nucleophillic groups such as phenolic hydroxyl group of tyrosine, sulfhydryl group of cysteine and imidazole rings in histidine also has the ability to react with glutaraldehyde in cross-linking (Wong 1991; Lundblad 2005). Cross-linking reinforces network structure in

protein via protein polymerization, which makes protein resistant to collapse in aqueous medium (Cao et al 2000; Silva et al 2004).

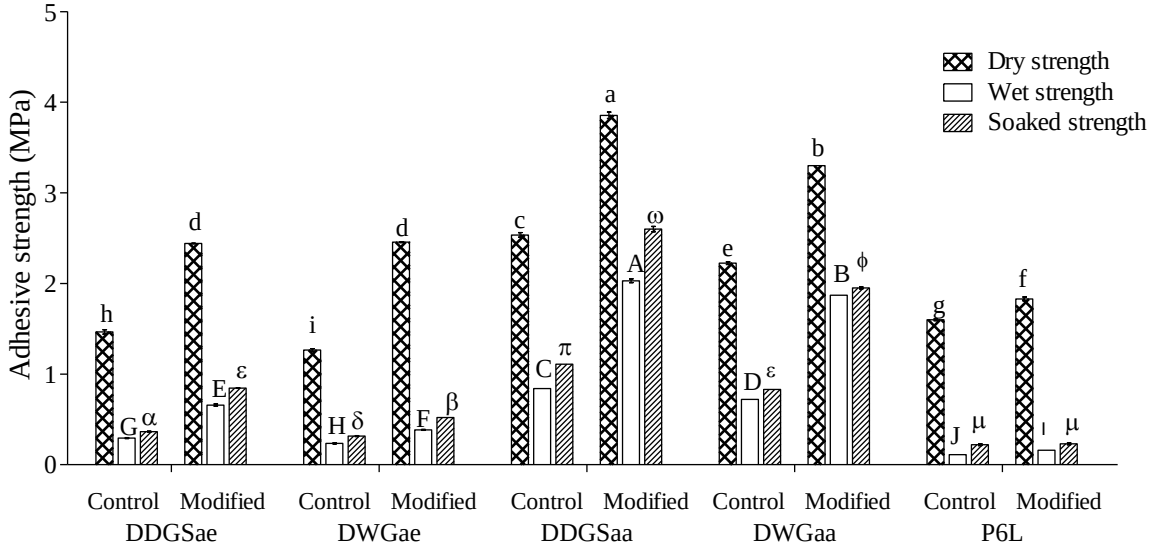

Figure 3.4 – Adhesion strength of various protein extracts with/without glutaraldehyde modification. Different letters on the bar represent significantly different adhesion strength ($p<0.05$).

Adhesion strength of various protein extracts with/without glutaraldehyde modification is shown in Figure 3.4. Similar to other two modifications, glutaraldehyde modification enhances the adhesion strength of all protein samples tested including P6L hydrolyzate. Among the protein samples tested, glutaraldehyde modified DDGSaa sample exhibited the highest adhesion strength (3.86, 2.03, and 2.60 MPa for DAS, WAS, and SAS, respectively) followed by DWGaa samples (3.30, 1.87, and 1.95 MPa for DAS, WAS, and SAS, respectively). Compared to the alkali and urea modifications,

glutaraldehyde modification led to the highest adhesion strength in dry, wet and soaked strengths ($p<0.05$) (*statistical comparison data not shown*). The formation of cross links among protein molecules may restrict the ability of water molecules to penetrate into wood-adhesive joints, thus increase the wet and soaked adhesion strength (Cao et al 2000; Silva et al 2004). Adhesion strength of glutaraldehyde modified soy proteins was reported as 6.81, 3.04, and 6.27 MPa for dry, wet and soaked adhesion strength, respectively (Wang et al 2007). These values are significantly higher than those observed in the study for triticale distillers grain proteins.

3.3.5. FTIR characterization of triticale distillers grain protein

FTIR spectra for DDGSae, DWGae, DDGSaa, DWGaa, and P6L are shown in Figures 3.5 (a) to 3.5 (e) respectively. FTIR is a widely used technique in characterization of protein secondary structure (Haris and Severcan 1999; Duodu et al 2001). Three main bands can be identified in FTIR spectra: amide I band appears in 1600 – 1700 cm^{-1} region, amide II band at 1550 – 1500 cm^{-1} and amide III around 1200 cm^{-1}, respectively (Haris and Severcan 1999; Duodu et al 2001; Grdadolnik 2002). Amide I band occurs mainly due to C=O stretching vibration of peptide bonds, while CN stretching, in-plane NH bending and CCN deformation also contributes to the amide I band (Bandekar 1992). Amide II band appears due to the combination of phase CN stretching and in-plane NH bending vibrations (Bandekar 1992; Grdadolnik 2002). C=O in-plane bending and NC stretching vibrations also have little contribution towards the amide II

band (Grdadolnik 2002). In-plane NH bending and CN stretching vibrations are the main contributors to amide III band. However, amide III band in FTIR spectra is less intensive than amide I and II bands (Haris and Severcan 1999; Grdadolnik 2002). Apart from those main bands there are some other amide modes present in FTIR spectra; such as amide IV to VII, however lower intensity of spectra made them difficult to use in protein confirmation studies (Grdadolnik 2002).

In amide I band, components appeared between spectral ranges of 1650 – 1658 cm^{-1} is characterized as α-helical structure whereas 1620 – 1640 cm-^{1}, 1670 – 1695 cm^{-1}, and 1654 – 1656 cm^{-1} represent β-sheet, antiparallel intermolecular β-sheet, and overlapped random coil, respectively. Other than that, spectral range of 1660 cm^{-1} also represents the α-helical components (Lavialle et al 1982; Jakobsen et al 1983; Bandekar 1992; Duodu et al 2001). In amide II peak, spectral range of 1545 – 1547 cm^{-1} represents the α-helical conformation whereas 1524 cm^{-1} and 1520 cm^{-1} range represents β-sheet structure and intermolecular anti-parallel β-sheet conformation, respectively (Lavialle et al 1982; Jakobsen et al 1983; Bandekar 1992; Haris and Severcan 1999; Duodu et al 2001).

Alkali modification of triticale distillers grain proteins exhibit increased β-turn and α-helix structures at 1746 cm^{-1} and 1652-1656 cm^{-1} wave length with the exeption for P6L sample where peak correspond to β-turn and α-helix structures are not visible. Urea modification of triticale proteins exhibits completely different FTIR spectra.

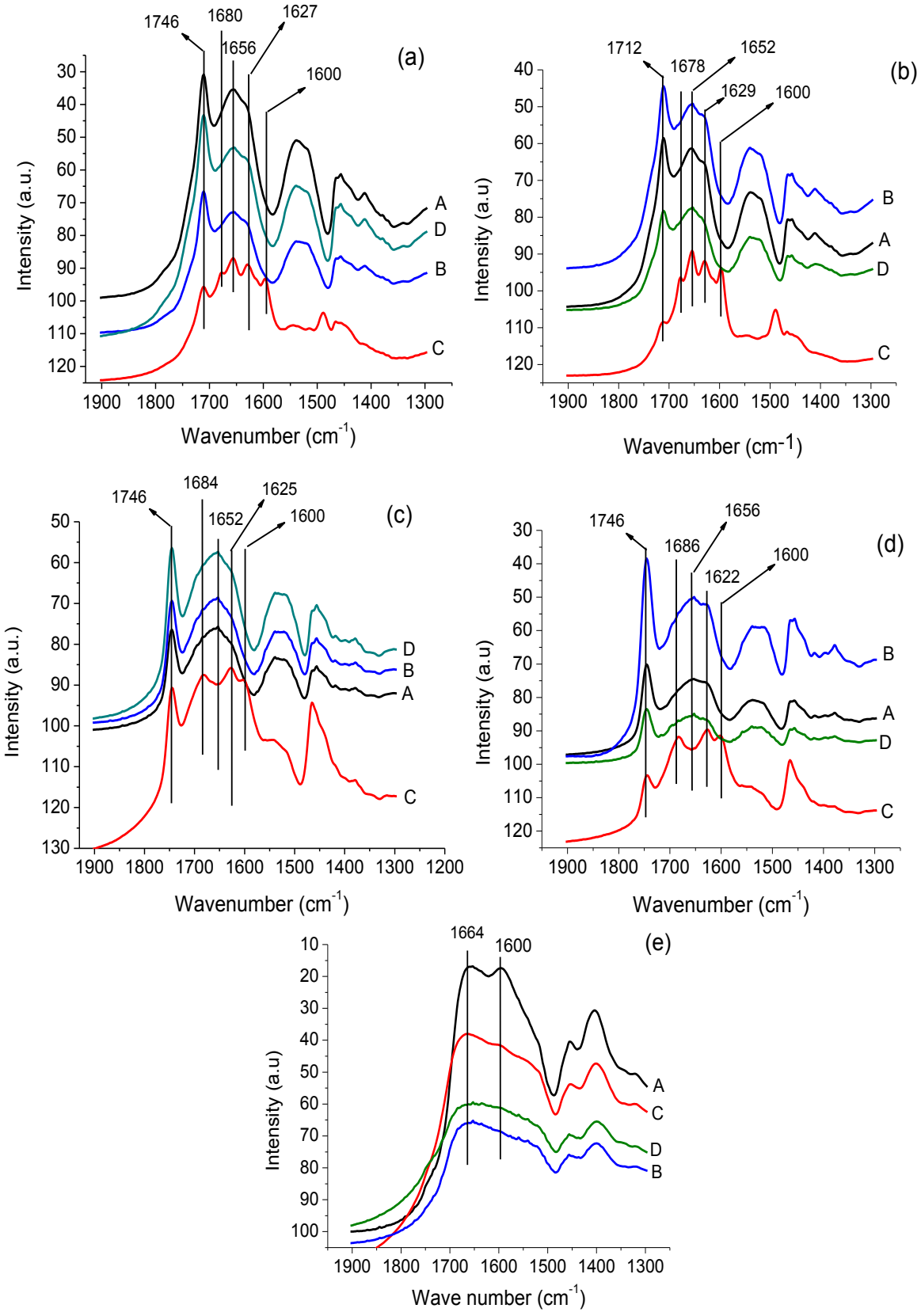
(a)
1746
1680
1656
1627
1600
A
D
B
C
Intensity (a.u.)
Wavenumber (cm-1)
(b)
1712
1678
1652
1629
1600
B
A
D
C
Intensity (a.u)
Wavenumber (cm-1)
(c)
1746
1684
1652
1625
1600
D
B
A
C
Intensity (a.u.)
Wavenumber (cm-1)
(d)
1746
1686
1656
1622
1600
B
A
D
C
Intensity (a.u)
Wavenumber (cm-1)
(e)
1664
1600
A
C
D
B
Intensity (a.u)
Wave number (cm-1)

Figure 3.5 - FTIR spectra of unmodified urea modified, alkali modified and glutaraldehyde modified (a) - DDGSae, (b) - DWGae, (c) - DDGSaa, (d) - DWGaa, (e) – P6L protein samples. A, B, C, and D denotes the FTIR spectra of unmodified, alkali modified, urea modified and glutaraldehyde modified triticale proteins respectively.

All protein samples modified with urea exhibit increased antiparallel β-sheet conformation at 1678-1686 cm^{-1} wave length (Zandomeneghi et al 2004) and decreased α-helical conformations with the exception to P6L sample. Peak arises at 1600 cm^{-1} wave length in urea modified proteins is an indication of well ordered β-sheet confirmation through intermolecular H bonding (Bandekar 1992). Ability of carbonyl groups and amine groups present in urea that can mimic proteins may be the reason for strong H bonds in urea modified proteins. However residual urea present in protein solution also has the ability to mask actual FTIR spectral pattern (Kong and Yu 2007).

With the exception for P6L protein samples, all other glutaraldehyde modified triticale proteins exhibited slight increase in α-helical conformation in FTIR spectra compared to the unmodified proteins in the range of 1652-1656 cm^{-1} wave length. Changes occurred during formation of larger molecules by cross-linking may be the reason for increases in α-helical conformation in glutaraldehyde modified proteins. FTIR spectra of P6L samples are completely different from other distillers grain protein samples due to the breakdown of polypeptides during enzymatic hydrolysis.

3.3.6. Amino acid composition of triticale distillers grain protein

Amino acid composition of triticale distillers grain protein extracts is summarized in Table 3.1. Asp, Glu and Leu are the most abundant amino acids in all protein extracts. DDGSaa showed higher percentage of Glu, Ser, Thr, Ala, Phe, and Leu than other four extracts where DWGaa contained the highest amounts of Gly, Lys and Arg. Higher levels of Asp, Val, Tyr and Ile were observed in DDGSae extract and the highest His level was detected in DWGae extracts.

A higher glutamic acid percentage was observed in acetic acid extracted proteins (11.86 and 8.20% for DDGSaa and DWGaa respectively) compared with alkaline ethanol extracted and enzyme aided extracted proteins. This could be the reason for higher adhesion strength observed in acetic acid extracted proteins (specifically in DDGSaa) as glutamic acid can create molecular attraction forces with charged groups such as carboxylic, phenolic and sulfonic acid groups in wood surfaces (Kumar et al 2002). The presence of a high proportion of His, Tyr, and Lys may also be related to increased adhesion strength in glutaraldehyde modified proteins as those amino acids can react with glutaraldehyde to form stabilized protein molecules (in aqueous solutions) via cross-linking. Reactive nucleophillic groups present in such amino acids have the ability to interact with glutaraldehyde by forming methylene bridges (Wong 1991; Lundblad 2005).

Table 3.1 – Percentage amino acid composition of triticale distillers grain protein extracts. Amino acid values are expressed in dry matter basis as a percentage and standard deviations were summarized in the table.

Amino acid	Sample				
	DDGSae	DDGSaa	DWGae	DWGaa	P6L
Asp	3.28 ± 0.40	2.58 ± 0.18	3.13 ± 0.10	2.77 ± 0.26	2.07 ± 0.79
Glu	7.84 ± 0.49	11.86 ± 0.68	5.76 ± 0.09	8.20 ± 0.12	7.59 ± 3.02
Ser	1.78 ± 0.01	2.29 ± 0.25	1.51 ± 0.09	1.69 ± 0.16	1.46 ± 0.47
His	0.73 ± 0.07	0.59 ± 0.08	0.80 ± 0.07	0.60 ± 0.11	0.48 ± 0.10
Gly	1.39 ± 0.03	1.45 ± 0.08	1.53 ± 0.07	1.74 ± 0.15	1.32 ± 0.26
Thr	0.80 ± 0.02	1.18 ± 0.06	0.78 ± 0.01	1.04 ± 0.05	0.97 ± 0.13
Arg	0.75 ± 0.04	1.72 ± 0.06	1.94 ± 0.07	2.75 ± 0.03	1.38 ± 0.10
Ala	2.83 ± 0.13	3.49 ± 0.13	2.30 ± 0.07	2.12 ± 0.00	1.90 ± 0.04
Tyr	2.47 ± 0.09	2.36 ± 0.02	2.17 ± 0.07	1.52 ± 0.02	0.67 ± 0.01
Val	2.74 ± 0.04	2.32 ± 0.08	2.50 ± 0.13	2.17 ± 0.08	1.78 ± 0.11
Phe	2.46 ± 0.01	3.00 ± 0.03	1.93 ± 0.12	1.92 ± 0.10	1.74 ± 0.06
Ile	2.26 ± 0.17	1.86 ± 0.14	1.84 ± 0.09	1.48 ± 0.05	1.29 ± 0.05
Leu	5.50 ± 0.54	6.09 ± 0.40	3.54 ± 0.12	2.94 ± 0.12	2.88 ± 0.23
Lys	0.97 ± 0.14	0.66 ± 0.05	1.54 ± 0.12	1.67 ± 0.14	0.95 ± 0.07

3.3.7. SDS-PAGE analysis of modified proteins

SDS-PAGE analysis of triticale distillers grain protein samples were shown in figure 3.6 (a to c). Similar to the results observed in chapter two, alkaline ethanol extracted protein samples (DDGSae, DWGae) do not show clear protein bands before (lanes 2, 3) and after modifications (lanes 6, 7 in Fig: 3.6 a-c). Cookman and Glatz (2009) observed similar results in SDS-PAGE of alkaline ethanol extracted corn distillers grain proteins. This may be due to interaction of

NaOH in accrylamide gel which interupt the visibility of protein bands. Alkali modification has changed the molecular weight of the protein, and it can be clearly visible in lane 8 in fig 3.6 (a).

Two intensified protein bands were appeared in alkali modified DWGaa protein sample (lane 8) at 20 KDa and 42 KDa molecular weight regions, which were also visible in urea modified and glutaraldehyde modified proteins. Urea and alkali modifications have the ability to break larger protein molecules into their respective sub units via clevage of disulfied bonds and hydrogen bonds. This may be the reason for showing intensified protein bands compared to their unmodified proteins in 20 and 42 KDa regions.

Except for the two intensified protein bands at 20 and 42 KDa [lane 8, Fig 3.6 (b)], all the other protein bands are much similar to unmodified proteins in urea modification. In all modifications, protein bands in lane 9 (DWGaa sample) exhibit a much similar pattern except for the band visible at 32 KDa molecular weight (MW) band in Fig 3.6 (c). Occurrence of this protein band may be a result of increased in MW of cross linked proteins which had smaller Mw before modification. But on the end of stacking gel, protein bands are visible compared with the unmodified proteins (Figure 3.6 c). This may be due to the inability of larger MW proteins to go through the pores in resolving gels. Wang et al (2007) reported the similar results where they observed proteins collected on the end of stacking gel. This increase in MW may have a relation with increased adhesion strength of glutaraldehude modified proteins compared with

other two modifications as large unfolded molecules can provide with more sites for binding with wood surface.

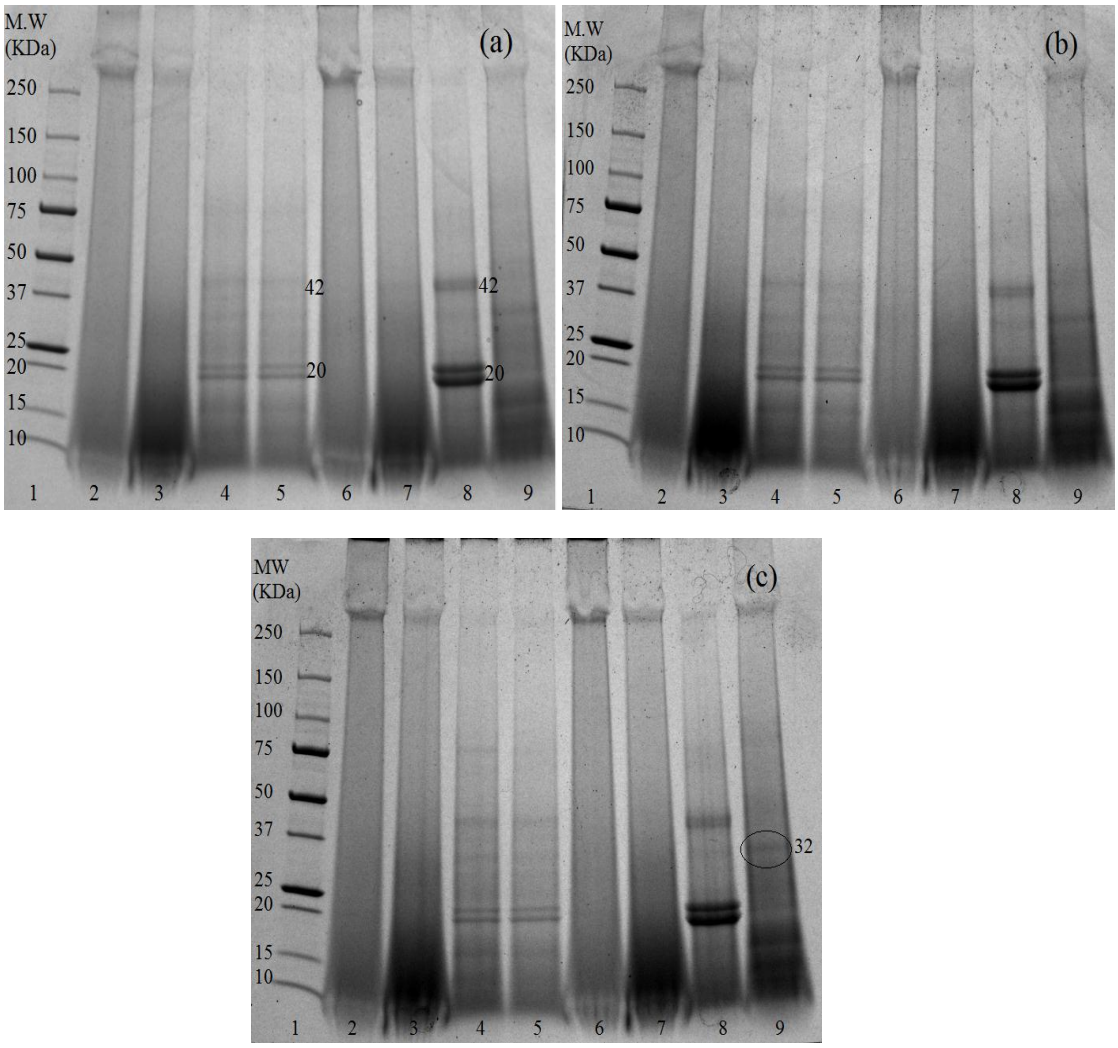

Figure 3.6 - SDS-PAGE images of modified and unmodified triticale distillers grain proteins. Lane one represent the marker protein while Lane 2 to 5 represent unmodified DDGSae, DWGae, DDGSaa, and DWGaa where lane 6 to 9 represent modified DDGSae, DWGae, DDGSaa, and DWGaa respectively. (a) – NaOH modified proteins, (b) – Urea modified proteins, (c) – Glutaraldehyde modified proteins

3.4. Conclusions and suggestions

Proteins extracted from two different triticale distillers grain (DDGS and DWG) with alkaline ethanol, acetic acid and Protex 6L enzyme were modified to improve the adhesive properties. All protein modifications significantly improved the dry, wet and soaked adhesion strength in DDGSae, DDGSaa, DWGae, DWGaa samples with an exception for P6L sample. Acetic acid extracted proteins (DDGSaa, DWGaa) exhibit higher adhesion strength than that of alkaline ethanol extracted (DDGSae, DWGae) proteins. Glutaraldehyde modification increased the adhesion strength of DDGSaa from 2.56, 0.84, 1.11 MPa to 3.86, 2.03, 2.60 MPa for dry, wet and soaked adhesion strength respectively. Even though adhesion strength was increased in a significant percentage compared with unmodified proteins, the strength values were lower than European standard (EN 204) for wood adhesives. Denaturation of triticale distillers grain proteins occurred during bioethanol production process may affect protein extraction and therefore the adhesive properties of protein extracts. Formulation of blended adhesives with other proteins with higher adhesion strength may be an alternative to use TDGPs in adhesive development. Further research is necessary to evaluate the suitability of TDGP for other industrial applications. Extracted proteins may be a valuble source to animal feed market rather than using DDGS as a feed ingredient. Further research should be carried out using distillers grain produced in raw starch ethanol fermentation as it does not have a step of heat induced gelatinization which cause the protein denaturation.

3.5. References

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CHAPTER 4

SUMMARY AND FUTURE WORK

Triticale has an important role in Canadian agriculture, especifically in bioethanol production due to their unique agronomic and yield advantages (CTBI 2011). Canadian triticale biorefinery initiative is aimed to develop triticale as a main crop platform for bioethanol production in Canada. As the main co-product generated from bioethanol processing, triticale distillers grains (DG) play an important role in generating profits for the ethanol plant (Gibreel et al 2011). Triticale DG is used mainly in animal feed industry with low revenue income (Salmon et al 2004; Oba et al 2010). Therefore, finding alternative applications for value addition to triticale DG is important to increase the economic viability of ethanol plants.

The first study in this research was focused on evaluating the protein extraction from two types of triticale distillers grains; distillers wet grains (DWG) and distillers dried grains with solubles (DDGS). In the second study, the feasibility of using triticale distillers grains proteins as bio-based wood adhesives were investigated.

Plant proteins, such as soy protein and wheat gluten have been tested for using as adhesives in the literature (Kumar et al 2002; Nordqvist et al 2010). However, due to having a food value, utilizing those proteins for adhesive development may not be feasible. Low water resistance property is the main problem associated with protein based adhesives (Kumar et al 2002; Nordqvist et

al 2010). Thus protein modifications with NaOH, urea, and glutaraldehyde were used to improve the adhesive properties and water resistance of selected protein extracts (DDGSae, DWGae, DDGSaa, DWGaa, P6L).

4.1. Summary of the first chapter

Osborne fractionation showed lower protein solubility of triticale DDGS (~ 32-33%) and DWG (~ 23-24%) compared to unfermented triticale grains (~ 65-66%). Extraction yield for DDGS and DWG was ~ 23% and 24% using acetic acid extraction, ~21% and 24% using alkaline ethanol extraction while the protein contents were 62%, 64%, 64% and 65%, respectively. Alkaline ethanol extraction and acetic acid extraction showed comparable extraction yield to previous studies on corn DDGS protein extraction. Compared to the other methods tested, enzyme aided extraction resulted in a considerably high extraction yield of 75-82% and a protein content of 43-57%. The low extractability of proteins from triticale distillers grains may be due to the protein denaturation occurred during heating/cooking conditions applied in bioethanol processing.

4.2. Summary of the second chapter

Modified triticale distillers grain proteins (DDGSaa, DWGaa, DDGSae, and DWGae) showed significantly improved ($p < 0.05$) adhesion strength except for P6L extract. Acetic acid extracted proteins showed higer adhesion strength than that of alkaline ethanol extracted triticale distillers grain proteins where the

highest adhesion strength was observed in glutaraldehyde modified proteins. Glutaraldehyde modification increased the adhesion strength of DDGSaa from 2.56, 0.84, 1.11 MPa to 3.86, 2.03, 2.60 MPa for dry, wet and soaked adhesion strength, respectively. Increases in α-helical conformation and molecular weight were observed for glutaraldehyde modified proteins using FTIR and SDS-PAGE. Compared to alkaline ethanol extracted and enzyme aided protein extract, acetic acid extracted proteins showed the highest adhesion strength in all three modifications.

However, the adhesion strength obtained for all three modifications were below the values recommended by EN 204 - European standard (10, 8, 2 MPa for dry, soaked and wet adhesion strength respectively) for wood adhesives. Loss of functionalities due to denaturation of triticale distillers grain proteins during bioethanol production process may have a negative impact on adhesive properties.

4.3. Future work

- In this study proteins were extracted from distillers grain resulted from common dry-milling bioethanol processing. Starch gelatinization step during bioethanol processing causes protein denaturation which would affect protein extraction from distillers grain. Therefore, it would be interesting to evaluate the potential of protein extraction from distillers grain generated from raw starch ethanol processing which use stargen enzymes (stargen 001, 002) instead of gelatinization step.

- In our study, hard wood (birch) was used to evaluate the adhesion strength of modified proteins. Even though adhesion strength was below the European standards (EN 204) – European standards for wood adhesives, modified triticale may have a potential to use in bonding other wood surfaces such as soft woods or in particle board production. It will be interesting to evaluate adhesion strength of modified triticale protein on other adhesive applications.
- To improve the adhesion strength and water resistance properties of triticale distillers grain protein, it can blend with another protein or synthetic polymer which has good adhesion strength.
- Grafting of synthetic polymers such as epoxy or glyoxal to improve adhesion strength and water resistance may be another area of interest.

4.4. References

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