University of Alberta

Antioxidant Peptides and Biodegradable Films Derived from Barley Proteins

by

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Abstract

Barley protein derived antioxidant peptides and biodegradable /edible films have been successfully prepared. Alcalase hydrolyzed barley glutelin demonstrated significantly higher antioxidant capacity than those treated by flavourzyme in radical scavenging capacity ($O_2^{\cdot -}$/$OH^{\cdot}$), Fe^{2+}-chelating effect and reducing power assays. The alcalase hydrolysates (AH) was separated using ultra-filtration and reversed-phase chromatography, and assessment of the fractions indicated that the molecular size, hydrophobicity and amino acid composition of AH all contributed to their activity. Final peptides sequences were identified using LC-MS/MS. Hydrolyzed barley glutelin is a potential source of antioxidant peptides for food and nutraceutical applications. The other barley protein application—biofilms is also included in the research work. Barley protein films were prepared by thermo-pressing using glycerol as a plasticizer. The combined effects of heating temperature and the amount of plasticizer interacted to determine protein conformation subsequently the properties of the film matrix. The film barrier and mechanical properties were investigated; moreover, the changing of protein structures during forming process also been characterized. I*n vitro* degradation experiments demonstrated that barley films were resistant in gastric conditions, but can be completely degraded by intestinal enzymes. The prepared barley films have potential for the development as delivery systems for gastric-sensitive bioactive compounds to the intestine for release.

***Keywords*:** barley protein; glutelin; enzymatic hydrolysis; antioxidant activities; protein film conformation; functionalities

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This is the fourth year since I came to Canada. I have met lots of nice people and had memorable experience in this country. I would like to appreciate my parents and my professor; they gave me the great chance studying my master project and thinking my future life. I am also grateful to all the friends or even some strangers who helped me when I had hard time here. I have learned lots lots of things not only about professional knowledge. Graduation is another beginning. I will try to live my life steadfastly and happily for both myself and all the people who I love.

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ANS	1-anilinonaphthalene-8-sulfonic acid
AH	glutelin hydrolyzed by alcalase
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
DH	degree of Hydrolysis
DPPH	1,1-diphenyl-2-picrylhydrazyl
DSC	differential scanning calorimetry
E	elongation at break
EDTA	ethylenediaminetetraacetic acid
EM	elastic modulus
FTIR	fourier transform infrared spectroscopy
FH	glutelin hydrolyzed by flavourzyme
GI	gastro-intestinal
HPLC	high performance liquid chromatography
kDa	kilo Dolton
Mw	molecule weight
OD	optical density
RH	relative humidity
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	scanning electron microscope
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
T_g	glass transition temperature
TS	tensile strength
WVP	water vapor permeability

Chapter 1 Introduction and Literature Review

1.1 Barley

1.1.1 Barley Overview

Barley (*Hordeum vulgare L.*), an ancient grain, has been domesticated since 8000 B.C. Because it can survive harsh natural environment, European Union, Russia and Canada rank first three in the world in barley production (Abdellatif, Mila, Steve, & Girma, 2007; Ceccarelli, Grando, & Van, 1995). Alberta produces more than five million tons of barley per year, that is over half of the total amount of barley grown in Canada (Alberta Barley Commission, 2010). Previously, barley has significant roles in livestock feeding and beverage brewing, but less than 20% barley production is for human food or other bioproducts (Jadhav, 1998). Recently there is growing attention on nutritional value of barley for human consumption due to its high content of dietary β-glucan (Pommet, Redl, Guilbert, & Morel, 2005). This soluble dietary fiber componenet of barley is known to reduce blood cholesterol and glycemic index (Miura et al, 1996; Jean-Louis, 2009). Moreover, the tocopherols and tocotrienols, which are found in barley and extracted with its oil, show a high nutritive value, including inhibition of lipid peroxidation in biological membranes, reduction of serum low density lipoprotein (LDL) cholesterol in human and protection against heart disease, etc (Vivekananthan et al., 2003). However, there remains a need for further research into the barley proteins which compose 8-15% dry weight of barley grain

(Andersson, Andersson, & Aman, 2005). A better understanding of the physical nature of barley proteins can lead to their manipulation to obtain value-added processing and applications for human consumption.

1.1.2 Barley Grain Protein

Protein Classification

Generally, barley grain is constituted of husk, embryo, scutellum, starchy endosperm and aleurone layer. Major proteins are stored in embryo and endosperm (Doll, 1983). They can be classified into four types according to different solubility (Kapp & Bamforth, 2002; Celus, Brijs, & Delcour, 2006) as albumin, globulin, hordein and glutelin (**Table 1.1**). Hordein (35–45%) and glutelin (25–35%) are major barley endosperm proteins. Cytoplasmic proteins including albumins and globulins, which compose 3-4% and 10-20% of barley protein, respectively are enriched in barley bran and germ (L ẑztity, 1984). The hordein can be extracted with alcohol. The remaining flour can be treated by alkali solution to extract glutelin. It has been reported that it is impossible to prepare a glutelin fraction totally free of contamination from hordein.

Table 1.1 Barley Grain Protein Classifications by Solubility

Protein type	Definition
Albumins	Water soluble, coagulated when heat
Globulins	Water insoluble, soluble in dilute salt buffer (0.5M NaCl)
Hordeins	Soluble in 70% ethanol
Glutelins	Soluble in dilute acid (0.5 M acetic acid) or alkali (0.5M NaOH) buffer

Table 1.2 Five Groups of Barley Hordeins

Name	Molecular weight	Description
albumin and globulin	< 1 kDa	LMW
A Hordein	< 15 kDa	
γ Hordein	< 20 kDa	sulphur rich
B Hordein	35-46 kDa	sulphur rich
C Hordein	55-75 kDa	sulphur poor
D Hordein	> 100 kDa	HMW

Hordeins are divided into four groups based on their electrophoretic mobilities and amino acid compositions. The B (30-50 kDa, sulfur-rich) and C (55-80 kDa, sulfur-poor) hordeins take up 70–80% and 10–20% of the hordein fraction, respectively; and the D (80-90 kDa) and A (15 kDa) hordeins take up less than

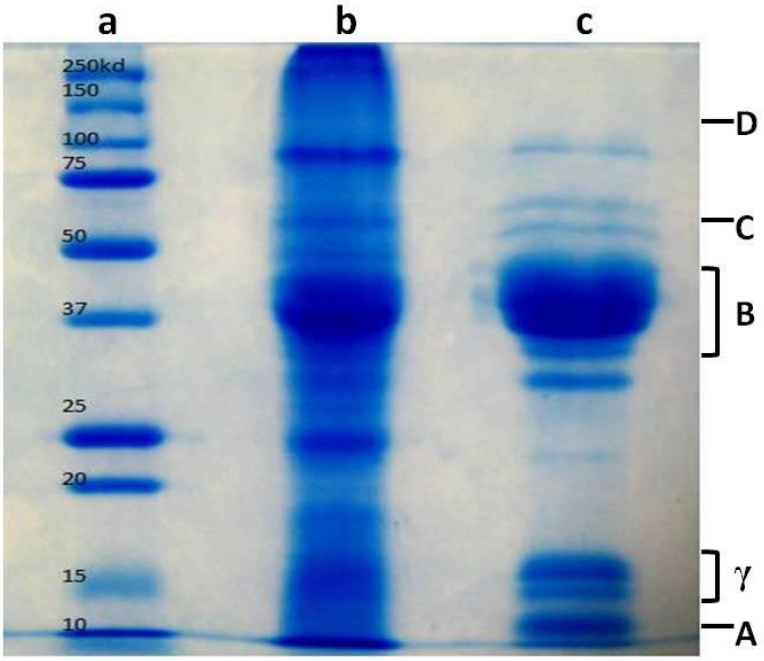

Figure 1.1 SDS-PAGE of prolamin and glutelin of barley. a is marker, b is glutelin, and c is prolamin. A, γ, B, C and D-hordeins are located as labelled.

5% of the total hordein fraction. C and some B hordeins appear as monomers, while most B and D hordeins are linked by inter-chain disulfide bridges (**Table 1.2 and Figure 1.1**). A hordein, which has average molecular weight around 15 kDa, is the smallest polypeptides. It may be alcohol-soluble albumins or globulins or breakdown products of larger hordeins rather than true hordeins (Celus, Brijs, & Delcour, 2006). In general, the barley albumin, globulin and glutelin fractions have not been investigated as extensively as the hordein fraction, thus information about their subunits is limited.

Table 1.3 Amino Acid Composition of Barley Protein Fractions (mol%)

Amino Acid name	Albumin	Globulin	Prolamin		Glutelin
			Insoluble in water	Soluble in water	
Ala	9.3	7.9	2.6	6.6	10.0
Asp	10.6	10.5	1.8	5.2	11.5
Cys	6.5	5.6	2.4	/	/
Glx	19.2	14.5	35.0	30.2	21.0
Gly	10.8	12.7	3.1	10.1	12.5
Ile	3.8	3.0	5.3	3.2	6.0
Leu	6.8	8.4	7.8	8.0	10.9
Lys	3.4	7.8	0.6	1.5	0.8
Met	2.5	1.6	1.2	1.6	1.9
Phe	3.6	4.4	6.8	4.9	5.3
Pro	6.1	7.3	20.1	13.8	2.1
Ser	6.7	7.9	5.8	7.1	9.7
Tyr	4.4	3.0	3.3	3.1	1.1
Val+Thr	6.4	5.5	4.3	4.8	7.2

Glx includes Gln and Glu

Amino Acid Composition and Protein Confirmation

According to Linko (1989) (**Table 1.3**), barley protein is an incomplete protein which has a shortage of lysine (Lys), threonine (Thr), and methionine (Met), especially in prolamin and glutelin. On the other hand, barley proteins are

generally rich of proline (Pro), glycine (Gly) and glutamine (Gln). Generally, the high content of no-polar and polar but non-charged amino acids, such as proline (Pro), methionine (Met) and tryptophan (Trp) relates to low solubility of hordeins in water. For each protein fractions, the amino acid composition is different **(Table 1.4)**. For example, hordein contains excessive high amount of Pro and Glu; whereas glutelin is lack of cysteine (Cys).

Table 1.4 Amino Acid Composition of Barley Hordeins (mol%)

Amino Acid name	γ hordein	B hordein	C hordein	D hordein
Ala	2.1	2.6	1.5	3.2
Arg	1.8	2.6	0.9	1.6
Asx	2.4	0.7	1.5	1.5
Cys	3.5	2.9	/	1.5
Glx	28.0	30.0	37.0	28.0
Gly	3.1	2.9	0.6	15.7
His	1.4	1.5	0.6	3.0
Ile	3.8	4.4	3.4	0.7
Leu	7.0	8.0	8.6	4.1
Lys	1.8	0.7	0.9	1.2
Met	1.8	1.1	/	0.4
Phe	5.6	4.7	7.7	1.3
Pro	16.8	19.4	29.1	10.5
Ser	5.6	4.7	2.5	10.5
Thr	3.1	2.2	1.2	7.3
Trp	0.7	0.7	0.6	1.2
Tyr	2.1	2.6	1.8	4.2
Val	7.3	6.2	0.3	4.1

*Asx includes Asp and Asn; Glx includes Glu and Gln

Intensive study on C hordein has already revealed that it has more ordered conformations with proline (Pro) and glutamine (Gln) taking more than 50% of its total amino acid composition. It has a molecular mass range of 52-54 kDa consisting almost entirely of repeats based on the octapeptide motif Pro-Gln-Gln-Pro-Phe-Pro-Gln-Gln and has demonstrated conformational transitions between poly-L-proline II-like and βI/III turn structures. The regular molecular structures

form a spiral super secondary structure. Research to identify conformation of other barley proteins is lacking currently.

1.1.3 Current Research of Barley Protein

Unlike other cereals, such as rice and wheat, barley is not a major crop for human consumption. Till now, high-value application of barley protein is still very limited Thus, development of value-added processing and application of barley protein will benefit barley producers and processors.

One of main problems that limit barley protein applications is the their low solubility as barley hordein and glutelin are soluble in water only in the presence of high concentrations of acid or alkali, or anionic detergents due to its high proportion of nonpolar amino acid residues and high surface hydrophobicity (Linko, 1989). Research has been conducted to improve barley protein solubility by enzymatic hydrolysis or deamidation (Bamdad, Wu, & Chen, 2011; Zhao, Tian, & Chen, 2010). More recently, it had been reported that barley prolamin has the potential of being used as emulsifying and foaming reagents by deamidation. The prolamin underwent structural changes at a low deamidation level ($\leq$ 4.7%), which led to dissociation of the protein aggregates and a great increase of protein solubility and protein surface hydrophobicity, consequently significantly improved emulsifying and foaming properties (Zhao, Tian, & Chen, 2010). Another research found that enzymatic degradation of barley hordein can produce hydrolysates with antioxidant properties which could be used in food and nutraceuticals applications (Bamdad, Wu, & Chen, 2010). In addition, barley

hordeins, glutelin and their mixture can be applied as microcapsule coating materials for protection and delivery of food ingredients and nutraceuticals, such as fish oil and beta-carotene (Wang, Tian, & Chen, 2011). In spite of significant progress above, barley proteins are still less studied and developed as value-added products compared with other crop proteins. As the third major barley producer in the world, development of new applications from barley and economically usage of barley proteins are important for Canada‘s agricultural industry.

1.2 Antioxidant Peptides

1.2.1 Damage of Oxidation and Free Radicals

Table 1.5 Reactive Species and Their Radicals

Reactive Species	Radicals
Reactive oxygen species (ROS)	Superoxide, $O_2^{\cdot -}$
	Hydroxyl, $OH^{\cdot}$
	Hydroperoxyl, $HO_2^{\cdot}$
	Lipid peroxyl, $LO_2^{\cdot}$
	Lipid alkoxyl, $LO^{\cdot}$
Reactive chlorine species (RCS)	Atomic chlorine, $Cl^{\cdot}$
Reactive nitrogen species (RNS)	Nitric oxide, $NO^{\cdot}$
	Nitrogen dioxide, $NO_2^{\cdot}$

Oxidation is a very natural process that happens during normal cellular metabolism. For food industry, unexpected oxidative reactions in foods products can affect food quality, such as off-flavor and rancidity. The main targets of the reactions that lead to foods deterioration are lipids and proteins. Lipid and protein pro-oxidation typically involves unsatisfied free radicals which are always generated from reactive species (**Table 1.5**) (Halliwell, 2001). During this process,

lipid and protein form hydroperoxides, and interact with other compounds (Elias, Kellerby, & Decker, 2008), which leads to loss of functionality.

There are intense studies recently to reveal the relationship between cells oxidation and a variety of diseases, such as cardiovascular diseases, diabetes (from protein oxidation), and even cancers (from oxidative DNA damage) (Hazen & Heinecke, 1997; Feig & Loeb, 1993). A large amount of evidences showed that oxygen and nitrogen species (ROS and RNS) can spur the mutation of cells, therefore lead to cellar dysfunction and diseases (Davies, 2005; Butterfield, 2002).

Intrinsic antioxidant systems in human metabolism can modulate the antioxidant/pro-oxidant balance to provide a favorable environment. Such a favorable balance is more difficult to maintain in food systems because processing operations increase the oxidative load by introducing oxygen, removing or destroying natural antioxidants, and promoting pro-oxidative factors (Rajapakse et al., 2005). Therefore, the strategies to enhance the oxidative stability of both biological and food systems are demanding and important for public health.

1.2.2 Developments of Antioxidants

Antioxidants have been used for centuries to delay oxidative deterioration of food consequently to prevent food quality and flavors. As a broad definition of an antioxidant, it is considered any substance that, in present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate (Halliwell & Gutteridge, 1999). In foods, antioxidants

have also been defined as ‘substances that in small amount are capable to defend or greatly reduce the oxidation of easily oxidizable matters, such as lipids and protein (Chipault, 1962).

Nowadays, hundreds of compounds have been reported as antioxidants. Synthetic food antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl-gallate are considered more powerful compared with natural antioxidants extracted from dietary plants. However, the use of synthetic antioxidants is under increasing regulatory scrutiny because of their unnatural molecular structures that may turn out to be hazardous to human health (Life Sciences Research Office, 1994). In addition, dietary antioxidants may be absorbed into human body with their extra beneficial effects. Vitamin C, and α-tocopherol have been mostly used dietary antioxidants (Traber, 1997; Rice-Evans, 2000). Other samples are catechins and quercetin; when they are absorbed into body and reach the concentrations at 0.1-1 μM in plasma, they can delay the process of lipid peroxidation in liposomes and low-density lipoproteins (LDLs) (Donovan et al, 1999; Manach et al, 1998). The demand of “natural” antioxidants in food and health applications has been increased, so new antioxidants from natural sources are required.

1.2.3 Peptides as Antioxidants

Antioxidant proteins found naturally in foods can increase the oxidative stability that is an added tool for food manufacturers to enhance food quality. Compared to other natural antioxidants, antioxidative peptides or protein

hydrolysates are relative new and competitive in terms of safety, nutrition and multifunction. In addition, unique amphipathicity of most antioxidative peptides can prohibit oxidation in both aqueous and lipoid systems. These specialities make antioxidative peptides more attractive in food industry.

Mechanisms

Recently, a great number of researches have indicated that proteins can inhibit oxidations in biological and food systems through multiple pathways, such as restraining reactive species, scavenging free radicals, binding transition metals et al (Moure, Dominguez, & Parajo, 2006). In real foods and biological systems, antioxidant actions are complex that may involve a variety of mechanisms. The most common antioxidative mechanisms of peptides can be summarized into five types which are scavenging free radicals, chelating prooxidative transition metals that catalyze the generation of reactive oxygen species, reducing hydroperoxides, inactivating reactive species, changing the physical properties of surrounding to separate reactive species and inhibiting oxidative enzymes (Frankel, 1998; Krinsky; 1992; Levine et al, 1996; Neuzil, Gebicki, & Stocher, 1993).

The most reactive amino acids are reported to possess either sulfur-containing, nucleophilic side chains or aromatic side groups, such as cysteine (Cys), tryptophan (Trp), Methionine (Met), and phenylalanine (Phe), as listed in **Table 1.6**. In addition, peptides with more hydrophobic amino acids have been reported to be related to antioxidative properties (Chen, Muramoto & Yamauchi, 1995;

Rajapakse, Mendis, Jung, Je & Kim, 2005; Xia, Bamdad, Ganzle, & Chen, 2011). On the other hand, the physical structures and amino acid

Table 1.6 Typical Antioxidant Mechanisms of Selected Amino Acids and their Formed Products. (adapted from Davies & Dean, 1997; Mendis, Rajapakse, & Kim, 2005; Wade & Tucker, 1998)

Amino Acid	Mechanisms	Formed Product
Cysteine (Cys)	(1)Abstracting hydrogen from SH group and dimerizing radicals (2)Abstracting hydrogen from SH group, then reacting with O_2 and isomerising	(1)Cystine (2) Oxy acids
Histidine (His)	Attacking $HO^{\cdot}$ or oxidizing (decomposing) its imidazole ring	2-oxo-histidine
Leucine (Leu)	Attacking $RCO^{\cdot}$ of susceptible fatty acids	Leu-NH_2
Methionine (Met)	Various reacting including radical and non-radical	Methionine sulfoxide
Phenylalanine (Phe)	(1)Attacking $HO^{\cdot}$ or one-electron oxidizing of aromatic ring (reacting with nitrogen species) (2)Dimerizing	(1)*o*-, *m*- tyrosine (2)Dimers of hydroxylated aromatic amino acids
Tryptophan (Trp)	Attacking $HO^{\cdot}$ or one-electron oxidizing ring	*N*-formylkynurenine, kynurenine,5-hydroxytrytophan,7-hydroxytryptophan
Tyrosine(Tyr)	(1)Attacking $HO^{\cdot}$ or one-electron oxidizing of aromatic ring (2)Attacking $HO^{\cdot}$ or one-electron oxidizing of tyrosine then dimerizing radicals HOCl (3) Chlorinating of tyrosine (4)Reacting with nitrogen species (5)When $O_2^{\cdot -}$ presents, forming tyrosine phenoxyl radical	(1)3,4-dihydroxy-phenylalanine (2)Di-tyrosine (3)3-chlorotyrosine (4)3-nitrotyrosine (5)Tyrosine hydroperoxides and subsequent materials

sequences of peptides also largely play roles in their antioxidant systems. Recently, a few specific active sequences have been identified that contributed to the potent antioxidant activity of peptides. In casein, dipeptide (Glu-Leu) possesses the highest radical scavenging activity; meanwhile, when Tyr, Tyr-Phe

and Tyr-Phe-Try present at the N-terminus, peptides lost their activity (Suetsuna, Ukeda, & Ochi, 2000).

Enzymatic hydrolysis

The total antioxidant activity of proteins can be improved by disrupting their tertiary structures. Due to mild process and controllable conditions, hydrolysis reaction is the most promising technique to modify proteins and produce proteinaceous antioxidant – peptides. During enzymatic treatment, molecular weight of proteins is reduced and the solubility of proteins increases. Moreover, the cleavage of peptide bonds results in an increased concentration of functional residues, free amino and carboxyl groups, therefore the antioxidant capacity of peptides is substantially higher compared to intact protein (Mannheim & Chetyan, 1992; Kristinsson & Rasco, 2000). A typical protein hydrolysate is a mixture of protein domains, peptides and free amino acids (Chang, Wu, & Chiang, 2007). The hydrolysis degree and amino acid compositions have been reported to determine the antioxidant activity of hydrolysates (Kong & Xiong, 2006).

1.2.4 Antioxidant Peptides Derived from Food Proteins and their Applications

There are many antioxidant peptides that have been identified from different food protein sources. These studies demonstrated the ability of protein hydrolysates to inhibit lipid oxidants and scavenging free radicals in foods. The peptides derived from casein (Diaz et al, 2003), blood plasma (Faraji, et al, 1991), soy (Pena-Ramos & Xiong, 2003), egg yolk (Sakanaka & Tachibana, 2006) and

whey protein (Shantha, Crum, & Decker, 1994) have been shown activity to inhibit lipid oxidations in meat products. Decker and Crum (1991) also reported that carnosine can inhibit myoglobin oxidation consequently reduce the discoloration of muscle foods. Peptides have also been used for inhibiting oxidation or free radicals in other food systems. For example, peptides from potato protein have shown effective activity to inhibit autoxidation of oil-in-water emulsions (Cheng, Chen, & Xiong, 2010).

1.2.5 Potential Application of Barley Glutelin Hydrolysates

In previous research, Bamdad (2011) et al, found that barley hordein hydrolysates possess potential antioxidant activity, especially in free radicals (such as DPPH and superoxide) scavenging. In addition, several factors, such as degree of hydrolysis and enzyme types, can impact the activity of the hydrolysates. Glutelin, which is characterized by high proportions of glutamine (20.2%), proline (11.2%) and glycine (8.4%) (L âszty, 1984), is the other major barley grain protein (35-45% of the total storage protein). Barley glutelin is enriched in hydrophobic amino acids (around 35%), with the highest levels corresponding to Pro, Leu, Ala and Val which has been reported to contribute to antioxidative properties (Chen, Muramoto, & Yamauchi, 1995; Rajapakse et al, 2005; Elias, Kellerby, & Decker, 2008). It was anticipated that peptides enriched with hydrophobic amino acids could be released by enzymatic hydrolysis to exert antioxidant functions. On the other hand, hydrolysis can increase the solubility of barley glutelin, and subsequent improve their functionality in food systems.

However, there is little information regarding the hydrolysis of barley glutelin and the antioxidant activities of the generated peptides.

1.3 Protein-based films

1.3.1 The important roles of protein-based materials

In the past century, numerous synthetic polymer-based films have been developed to protect and preserve food, pharmaceuticals and other products. They can prevent products from surrounding environment, such as moisture, oxygen, light et al; therefore extend their shelf life and keep their quality. However, packaging based on synthetic polymers from petrochemical components such as

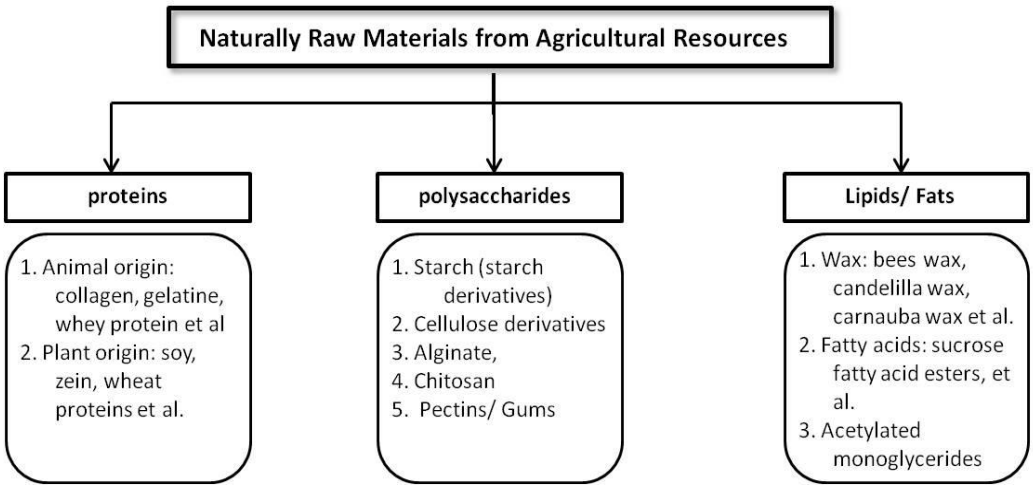

Figure 1.2 Raw materials from agricultural resources have been used to form biodegradable or edible packaging films and coatings.

polyolefin, polyesters and polyamides are not biodegradable and generate toxic gases during burning. Besides, their price also likely increased because petroleum is not infinite resource. In addition, health and safety issues also limit the

utilization of the synthetic packing materials as edible coatings or supporters. Eventually, biodegradable, renewable and edible food packing materials are intensively required in the future.

At present, some natural materials from agricultural resources have been studied with the purpose to replace the synthetic ones. Generally, these agricultural raw materials can be classified into three categories: proteins, polysaccharides and lipids (**Figure 1.2**) (Tharanathan, 2003; Zhang & Mittal, 2010). These biomolecules bright a surge of new types of packaging materials into various industry applications. Among these biopolymers, proteins are the major constituent, which have been used as edible packaging materials for a long time due to their excellent physical and chemical properties.

Compared with other raw materials, proteins are based on 20 amino acids. Their various functional side groups give each protein unique character, such as isoelectric point and spatial structure. In addition, their secondary, tertiary and quaternary structures are based on different types of molecular interactions, such as covalent bonding, hydrogen bonding, electrostatic and hydrophobic interactions. Furthermore, tertiary protein structure can be globular, fibrous or random shape. When these domains interact with each other into quaternary structure, proteins possess unique structure or bioactivity. Finally, the secondary, tertiary and quaternary structures of proteins can be modified by various physical or chemical processing. During modifications, protein interactions can change and rebuild then result in various film properties. Therefore, protein-based films have become one main focus of packaging material research.

1.3.2 Preparation of Protein-based Film

Processing

Generally, there are two process pathways used for forming protein-based biodegradable and edible films: the wet (casting) and the dry (compression molding and extrusion) process.

Solvent casting

Films and coatings based on proteins are formed by evaporation of a protein solution. In the most cases, protein are dissolved into water, the solubility can be enhanced by heating or adjusting pH value. However, for water insoluble proteins, such as corn zein and wheat gluten, ethanol solutions are required (Krochta, 2002). A plasticizer or a surfactant is also added. After heating, blending and degassing, the protein solution is casted on product surface or directly evaporated to form a film. For forming coatings on the surface of food products, it involves four steps: dipping, spraying, panning or enrobing the food. These coating methods have been applied to fresh products, such as vegetables and fruits, for keeping moisture and providing integrity. Another widely used application is gelatin soft capsules casting from aqueous solutions in the pharmaceutical industry.

Compression molding

Thermoplastic polymers are macromolecules where lateral cohesion is only ensured by low-energy interactions sensitive to plasticizers and temperature changes (Oudet, 1994). Proteins could be considered as partially thermoplastic

polymers that could be changed in a reversible way from a rigid state to a soft state through a temperature increase or plasticizer addition (Cuq, Gontard, & Guilbert, 1997). Thus, thermoplastic processing is applied to modify the physical and chemical structures of proteins in a film. In present of a plasticizer, the combination of high temperatures, high pressures and low moisture within a short time can cause transformation of protein-plasticizer mixtures from the glass state into viscoelastic melt state. The glass transition behavior of proteins depends on type and density of intermolecular interactions. When the molding temperature is above the glass transition temperature of protein, denaturation of protein results in promoted protein-protein and protein-plasticizer cross-linking via hydrogen bonding, hydrophobic and even covalent interactions (Pol et al, 2002).; meanwhile, the solubility of films reduces (Sothornvit et al, 2003). After cooling, compact protein films are formed with rearranged protein interactions. The main advantage of compression molded processing is that the mechanical and barrier properties of films can be adjusted in a large range by changing plasticizers and temperatures. So far, many research have been done on corn zein (di Gioia &Guilbert, 1999), soy protein (Cunningham et al, 2000), myofibrillar protein (Cuq et al, 1997) and wheat gluten (pommer et al, 2005) based thermoplastic films. The applications of the protein films depend on their solubility. The water insoluble films can be used as wraps or covers to keep moisture content of foods. On the other hand, partially soluble films can deliver food ingredients or capsulate drugs to achieve controlled release.

Extrusion

Recently, extrusion processing is one of the most widely applied techniques in packaging industry. Most plastics are produced in this way, such as low density polyethylene (LDPE) films. Due to the thermoplastic behavior of proteins, they can be converted into films by adapting conventional extrusion method. Generally, this process includes heating, cooling, feeding, conveying, compressing, shearing, reacting, mixing, melting, blending, amorphousizing, cooking, and shaping (Hernandez-Izquierdo & Krochta, 2008) (**Figure. 1.3**). It is a heating and pressure

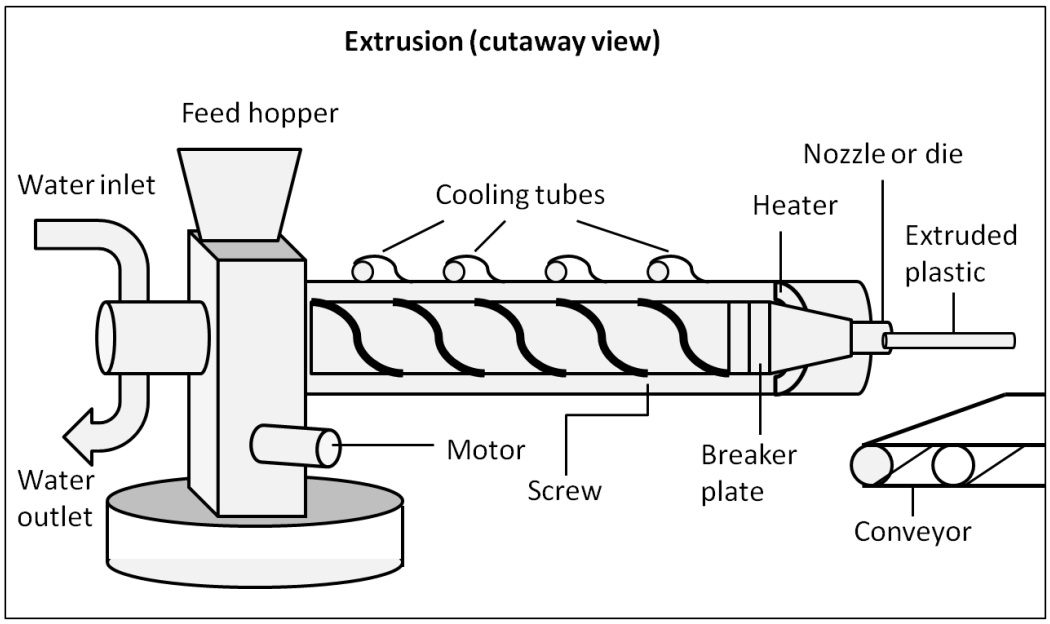

Figure 1.3 Extrusion system (cutaway view) for produce extruded protein based plastic.

required processing; however, compared to casting, it avoids the steps of adding and removing solvent. In addition, extrusion can continuously produce large amount of plastic protein materials, which is more feasible for industry production. The color, texture, density and other functional properties of films can be

controlled when adjust the parameters such as temperatures, residence time, torque, mechanical energy input, pressure at the die and degree of screw fill (Hauck & Huber, 1989).

These extruded proteins films are heat-sealable. Therefore, Hernandez-Izquierdo (2007) indicated that whey protein-based sheet could be made into pouches to carry dry milk powders or other dry foods. In spite of high potential, research to develop protein-based packaging by extrusion process is still limited.

Common process-related factors of film formation

During film-making processes, several factors play the key roles which greatly impact the functional properties of the films through changing the molecular interactions or protein structures. Most studied have been focused on these factors with the purpose to achieve improved protein film structures and properties. As individual protein and processing have their own parameters, optimizing various combinations of the factors is the biggest challenge to commercialize the protein-based films.

Plasticizers

A plasticizer is essential to prepare a good film. Normally, protein films and coatings are brittle and stiff because of extensive interactions among molecular chains, such as hydrogen bonds, electrostatic forces and hydrophobic bonds. Plasticizers enter between proteins chains, physicochemically associate with protein chains and increase free volumes and chain mobility, therefore the three-

dimensional structures of the materials are modified (Gennadios, 2005). Thus addition of plasticizer into polymeric materials results in a reduction of protein chains interactions, a lowering of protein glass transition temperature and an improvement of films flexibility (Swin et al, 2004). At the same time, the film elongation (stretchiness) and permeability increases and the strength decreases. Plasticizers, which are generally accepted for protein-based films, include water, glycerol, propylene glycol, sorbitol, sucrose, polyethylene glycol, fatty acids, lipids, monoglycerides and other liquid organic compounds (Wang et al, 2004; Wang, Crofts, & Pauda, 2003). Glycerol is the most often used plasticizer in 3%-50% w/w concentrations for protein films. The presence of hydrophilic glycerol also affect the moisture content by attracting water from surrounding and impact the properties of the films (Gennadios et al, 1993).

There are a large amount of studies to investigate the influences of plasticizer type and amount on the molecular reactions of protein films. It has been reported that increasing amount of glycerol from 0% to 33% (w/w) improved extensibility of wheat gluten film but reduced elasticity and water vapor barrier properties (Gontrd, Guilbert, & Cuq, 1993). Pommet et al (2005) found that increased plasticizer (octanoic acid) led to protein aggregations involving sulfhydryl/disulfide interchanging. For soy protein based films, addition of hydrophobic plasticizers can extensively enhance moisture barrier property (Rhim et al, 2000).

Temperature

Beside plasticizer content, the structure mechanism can be controlled by processing temperature. Protein structures are sensitive to changes in temperature. During a heating processing, protein molecules transform from a glassy to a rubbery state; meanwhile, the molecules become disorder and chains mobility increase. Heating treatment can promote protein network cross-linking via disulfide and hydrophobic bonds (Hernandez-Izquierdo & Krochta, 2008, Sabato et al, 2001). On the other hand, too high temperature can induce protein degeneration, therefore weaker protein network structures of the films.

DSC (differential scanning calorimetry) is a widely used technique to characterize the thermal transitions of a polymer. The glass transition temperature (Tg), melting, crystallization, thermal denaturation, aggregation, and protein degradation can be detected by DSC. For example, after thermal denaturation, soy protein film showed a single T_g instead of the two characteristic T_g values (Zhang et al, 2001). In addition, Fitzsimons et al (2007) found the existence of an exothermic peak that reflects the slow formation of intermolecular bonds (aggregation) in a thermal gelation of whey proteins.

Other additives

For some cases, cross-linking agents or enzymes are also employed in a filming forming processing. The aim is to improve moisture resistance, cohesion, mechanical strength and barrier properties. The enzymes, such as transglutaminases and peroxidases are normally used to achieve this purpose (Motoki et al, 1987). For example, egg protein network treated by transglutaminases showed significantly reduced water vapor permeability (WVP)

(Lim, Mine, & Tung, 1998). Chemical crosslinking reagent (such as formaldehyde) had been added in wheat gluten films to increase covalent binding between protein molecules, leading to higher tensile property and lower elasticity (Micard, Belamri, Morel, & Guilbert, 2000). However, adding covalent cross-linking agents in food-grade films is still highly questionable.

1.3.3 Films Properties

Mechanical properties

One of most important applications of a packaging film is to maintain the structural integrity and improve mechanical handling. Improving mechanical properties of protein-based films is important for them to replace synthetic polymer films. These properties include tensile strength (TS), elongation (E), and elastic modulus (EM).

Tensile properties are important mechanical characteristics of films that indicate the ability to maintain the film integrity under the stress occurring during food processing, handling, and storage of packaged materials (Rhim & Lee 2004). Tensile strength (TS) represents the maximum stress developed in a film during tensile testing (Gennadios and others 1994). Elongation is described as a degree to which film can stretch before breaking (Krochta, 2002). This parameter shows the flexibility and extensibility of films. Film toughness is approximated by a combination of TS and E. Elastic modulus (EM) indicates film stiffness as determined by ratio of pulling force/area to degree-of-film-stretch (Krochta, 2002).

The mechanical properties of materials are largely associated with distribution

Table 1.7 Mechanical Properties of Protein-based Films and Synthetic films

Film formulation	Forming method	TS (MPa)	E (%)	Reference
LDPE	Extrusion	9-17	500	Briston (1986)
HDPE	/	17-35	300	Briston (1986)
PP	/	42	300	Briston (1986)
Polyester	/	175	70-100	Briston (1986)
PVC	/	93	30	Lacroix & Cooksey (2005)
40% Gly-WPI	Thermal-compression	8	85	Sothornvit et al (2007)
50% Gly-WPI	Thermal-compression	4	94	Sothornvit et al (2007)
49% Gly-WPI	Extrusion	4.2	121	Hernandez-Izquierdo (2007)
25% Gly-SPI	Thermal-compression	13	17	Stuchell & Krochta (1994)
pH 11 SPI	Casting	3.1	187.3	Gennadios et al (1993)
Oleic acid-CZ	Extrusion	4.2	96.3	Wang &Padua (2002)
35% Gly-FMP	Casting	17	23	Cuq et al (1995)
20% Gly-WG	Casting	1.6-23	1-350	Lens et al (2003)
pH 3 WG	Casting	0.9	260	Gennadios et al (1993)

*Abbreviations: LDPE=low density polyethylene, HDPE= high density polyethylene, PP=polypropylene, PVC=polyvinyl chloride, Gly=glycerol, WPI=whey protein isolate, SPI=soy protein isolate CZ=corn zein, FMP=fish myofibrillar protein, WG=wheat gluten.

and concentration of inter- and intramolecular interactions. In a film, the density and cohesion of spatial protein network structures also result in various mechanical performances. Normally, network stabilization relies on covalent bonds or other high energy bonds (Farnum, Stanley, & Gray, 1976). On the other hand, when most inter-protein interactions are low energy or density, films are easily distended. That is also the reason most thermal compressed films show higher TS and lower E values, compared to solvent casted films. During a heating-cooling process, protein structures unfold, side chains move and react, and then more stable interactions form. In addition, protein nature, plasticizer, temperature and other factors all impact the mechanical properties of films by changing the molecular interactions of protein networks. As shown in **Table 1.7**,

generally, protein films have lower TS and E value than synthetic polymer films; however, their mechanical properties are adequate to be used as packaging products, such as casings, coating and pouches.

Permeability properties

Another main interest in edible films could be their potential of barrier properties. As a general requirement for food or drug packaging, films need to provide appropriate permeability against moisture, oxygen, aroma, oil and color with a resulting in maintaining quality and shelf life. Permeability, a steady-state property, is defined by Krochta (2002) as

$$\text{Permeability} = \frac{\left(\begin{matrix}rate\ of\ permeation\ through\\ film\ in\ amount\ per\ time\end{matrix}\right)(film\ thickness)}{\left(\begin{matrix}permeant\ concentration\ or\ partial\\ pressure\ difference\ across\ film\end{matrix}\right)(film\ area)}$$

Where the concentration or partial pressure difference is between the phases adjacent to the two sides of the film.

One of the biggest challenges of using proteins film as food or drug packaging is to optimize the film formulation (e.g. type and amount of protein and plasticizer), and forming conditions to achieve the desired barrier properties. The barrier properties of protein materials are dependent on network type and density in protein films, and more particularly on the distribution of nonpolar and polar amino acids (Guilbert & Graille, 1995). If protein composition and network structural organization allow some groups to be free in films, the films may permit interactions with passing molecules. In general, most free hydrophilic groups of proteins favor water vapor transportation, rather than hydrophobic gas transportation (such as oxygen and aroma).

The permeability of protein-based films can be influenced by many factors, such as the polar character of plasticizers and proteins, the humidity of testing condition, film thickness et al. According to previous studies, protein-based films have impressive gas barrier properties when they are not moist, especially against O_2. For instance, O_2 permeability of wheat gluten film was 800 times lower than low-density polyethylene and 2 times lower than polyamide (Bakker, 1986; Gontard et al, 1996). However, compared to edible waxes and most synthetic films, protein-based films have relatively high water vapor permeability (WVP) values. The low WVP values can be achived by using hydrophobic proteins (such as corn zein and wheat gluten), plasticizing with polar plasticizers, heating to increase the cross-linking degree (compression molding), and adding some hydrophobic materials (such as oil) (Krochta, 2002; Sothornvit et al 2003; Liu, Kerry, & Kerry, 2006). There are a few researches on aroma and oil permeability of protein films. Miller and Krochta (1998) reported that whey protein films showed higher citrus aroma barrier property than vinylidene chloride copolymer (co-VDC) film under same conditions. In addition, whey protein also showed potential for food service industry as oil barrier warp or box materials (Chan, 2000).

1.3.4 Applications of Protein-based Biodegradable and Edible Films

A packaging film can protect food or drug product against oxygen intrusion, flavor loss, oil exudation or moisture migration. The integrity and appearance of products also can be improved by packaging films or coatings. The protein-based

biodegradable and edible packaging materials are both eco- and user- friendly that is extensively required by food, agriculture and pharmacy industries. Patently,

Table 1.8 The Potential Uses for Protein-Based Films and Coatings

Uses	Edible	Biodegradable
Food covers, wraps and separation layers	×	×
Food casings, pouches, bags and labels	×	×
Food coatings	×	
Food ingredient microcapsules	×	
Drug coatings, capsules and microcapsules	×	
Disposable food service items (plates, cutlery, cups, containers, boxes, etc)		×
Trash bags (garden, restaurant)		×
Water-soluble bags / microcapsules for fertilizer, pesticides, etc.		×
Agricultural mulches		×
Paper coatings		×
Loose-fill packaging		×
Disposable medical products (gloves, gowns, etc.)		×
Disposable personal care napkins, sanitary pads, diapers, etc		×

*Adapted from Krochta (2002)

protein-based films can be used as fresh produce coating, snack pouches, fried foods wraps, flavors capsules, drug tables et al. (**Table 1.8**). In addition, protein-based biodegradable materials also are formed into plant or weedicide containers. When the container disintegrates, the release protein can provide fertilizer to benefit plant growth. New protein-based materials are targeted for replacing current synthetic material applications. Compared to a large amount of researches

on protein films, there are limited applications have been brought into industries. Though a bit higher cost and lower efficiency, bio-packaging materials are still strongly recommended in future.

1.3.5 Potential application of barley protein for film applications

Hordein and glutelin are the major barley storage proteins. Similar to wheat gluten, barley proteins, especially hordein show good cohesive and elastic properties (Payne & Corfirld, 1979), thus are interesting candidates for film formation. Additionally, these proteins are known to be hydrophobic, and according to the Goldman–Engelman–Steitz (GES) scale (Engelman, Steitz, & Goldman, 1986) their hydrophobic amino acid content is around one third of the total amino acid content, with the highest level corresponding to Leu, Val, Phe and Tyr. This hydrophobic nature may provide barley protein materials a good barrier capacity compared with many other natural materials. Moreover, barley proteins exhibit relatively low digestibility which may limit their nutritive value (Gaylord, Barrows, & Rawles, 2008), however, materials with such characteristic could be interesting for protection and controlled release of bioactive compounds in the gastro-intestinal (GI) tract. In spite of great potential as natural biodegradable films for both food and pharmaceutical applications, research on barley protein films has never been reported.

1.4 Conclusion and Research Objective

Base on the literature review, barley proteins show potential to be converted into either antioxidant peptides or biodegradable films. However research in these

areas is limited. The goal of this research is to develop value-added processing and applications of barley protein as antioxidant peptides and biodegradable films. The specific objectives are as follows:

1. Antioxidant peptides from barley glutelin
   (1) Optimize the barley glutelin hydrolysis to generate antioxidant peptides
   (2) Evaluate antioxidant capacity using five different assays (DPPH/, superoxide/hydroxyl radical scavenging activity, reducing power smf Fe^{2+}-chelating effect)
   (3) Understand the relationships between peptide structures (molecular weight, hydrophobicity, amino acid composition and sequence) and their antioxidant properties.
2. Biodegradable barley protein-based film
   (1) Form the barley protein films by compression molding technology using glycerol as a plasticizer.
   (2) Correlate the film molecular structures (analyzed by FTIR, DSC and SDS-PAGE methods) and their mechanical/barrier properties.
   (3) Conduct *in vitro* degradation and cytotoxicity essay to study the potential of a barley protein film as a drug control-released system

The core piece of these two works are to find out the relationships between molecular structures of barley proteins and their functionalities, therefore to produce new methods to develop barley protein based products. The success of these works can benefit local barley farmers, Canadian agricultural sectors and industries in food and pharmaceutical areas.

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Chapter 2 Fractionation and characterization of antioxidant peptides derived from barley glutelin by enzymatic hydrolysis

2.1 Introduction

Barley (*Hordeum vulgare* L.) is the fourth largest cereal crop in the world both in terms of quantity produced and in area of cultivation. Canadian barley is mainly used for livestock feed. It is important to explore value-added applications of barley and its components to assist development of the Canadian agricultural economy. Protein is the second most abundant component (8-13%) in barley grain after starch (Wang, Tian, Chen, Temelli, Liu & Wang, 2010). Barley proteins are regarded as contaminants by the brewing industry as some of them have undesirable qualities. These proteins are precipitated out in the spent grains and mainly used as animal feed. Recently the use of barley protein in food and nonfood applications is also gaining interest as an emulsifying agent (Zhao, Tian & Chen, 2010) and a microencapsulation material (Wang, Tian & Chen, 2011). Glutelin, a major fraction of barley protein (35-45% of the total storage protein) (L âsztity, 1984), is characterized by high proportions of glutamine / glutamic acid (20.2%), proline (11.2%) and glycine (8.4%) (Wang, Tian, Chen, Temelli, Liu & Wang, 2010). Additionally, barley glutelin is enriched in hydrophobic amino acids (around 35%), with the highest levels corresponding to Pro, Leu, Ala and Val (Bamdad, Wu & Chen, 2011).

Natural antioxidants have attracted increasing interest because they are generally recognized as safe. Alpha-tocopherol (Liebler, Baker & Kaysen, 1990) and carotenoids (Huang & Frankel, 1997), as well as polyphenol compounds (Urizzi et al., 1999) are natural antioxidants. Recently, there has been mounting evidence that protein and peptides are potentially excellent food additive antioxidants. They inhibit oxidation through multiple pathways including scavenging free radicals, chelating prooxidative transition metal ions, reducing hydroperoxides and inactivating reactive oxygen species (Hook, Burton, Yasothornsrikul, Hastings & Deftos, 2001). In addition, unique amphipathicity of most antioxidative peptides allow them to prohibit oxidation in both aqueous and lipoid systems (Wu, Chen & Shiau, 2003). These special properties make antioxidative peptides very applicable to the food industry. A protein's antioxidative activity is limited by its tertiary structure, since many peptides and amino acids with antioxidative potential are buried within the protein core inaccessible to pro-oxidants. Enzymatic hydrolysis is an effective method to release these antioxidative peptides from protein molecules. Antioxidative activity has been identified in several food protein hydrolysates, including those derived from whey protein (Pena-Ramos, Xiong & Arteaga, 2004), fish protein (Dong, Zeng, Wang, Liu, Zhao & Yang, 2008), egg yolk (Park, Jung, Nam, Shahidi & Kim, 2001), porcine haemoglobin (Chang, Wu & Chiang, 2007), zein protein (Kong & Xiong, 2006), chickpea protein (Li, Jiang, Zhang, Mu & Liu, 2008) and wheat gluten (Kong, Zhou & Hua, 2008) . The antioxidant activities of peptides are closely related to their amino acid constituents and their sequences. Several

amino acids, such as His, Tyr, Met, Lys, Trp and Phe are generally accepted as antioxidants in spite of their pro-oxidative effects in some cases (Jung, Kim & Kim, 1995; Pihlanto, 2006). Furthermore, peptides with more hydrophobic amino acids have been reported to be related to antioxidative properties (Chen, Muramoto & Yamauchi, 1995; Rajapakse, Mendis, Jung, Je & Kim, 2005). There is little information regarding the hydrolysis of barley glutelin and the antioxidant activities of the generated peptides.

Barley glutelin peptides possess high hydrophobic amino acid content, and it was anticipated that they could be released by enzymatic hydrolysis to exert antioxidant functions. Barley glutelin is soluble in water only in the presence of high concentrations of acid or alkali, or anionic detergents due to its high proportion of nonpolar amino acid residues and high surface hydrophobicity (Wilson, Shewry, Faulks & Miflin, 1981). It was expected that enzymatic hydrolysis of barley glutelin can remarkably improve its solubility in neutral pHs and therefore extend its potential over a broader range of food and non-food applications. In this study, barley glutelin was hydrolyzed by alcalase and flavourzyme. The impacts of peptide bond cleavage on glutelin molecular structure, including surface hydrophobicity and molecular weight, and their subsequent antioxidant activities, were investigated. The antioxidant potential of barley glutelin hydrolysates was studied using the scavenging effect on DPPH/superoxide/hydroxyl radicals, the reducing power, and iron chelating activity. Furthermore, amino acid composition and sequence of the peptides were evaluated to determine their relationship with the antioxidant activity.

2.2 Materials and methods

2.2.1. Materials

Regular barley grains (cv. Falcon) were kindly provided by Dr. James Helm, Alberta Agricultural and Rural Development, Lacombe, Alberta, Canada. Barley glutelin was extracted according to our previous work (Wang et al, 2010). After pearling and milling, the pearled grain flour was treated with ethanol solution (60%, v/v) to isolate the hordein fraction, and then the residue was treated with alkaline solution (pH 11.5) to extract glutelin. The protein content of isolated glutelin was determined by combustion with a nitrogen analyzer (Leco Corporation, St. Joseph, MI, USA) calibrated with analytical reagent grade EDTA. A factor of 5.83 was used to convert the nitrogen to protein. Alcalase (endoproteinase from *Bacillus licheniformis*, 3.0L) was obtained from Novozymes China Inc. Flavourzyme (from *Aspergillus oryzae,* 500L), 2-deoxy-D-ribose (DR), ethylenediaminetetraacetic acid (EDTA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), glutathione (GSH), 2,4,6 trinitrobenzene sulfonic acid (TNBS), butylated hydroxytoluene (BHT), 1-anilino-8-naphthalene-sulfonate (ANS), hydrogen peroxide (30%) and the standard molecular markers for HPLC analysis (thyroglobulin, 670 kDa; ferritin, 440 kDa; BSA, 67 kDa; ovalbumin, 43 kDa; cytochrome C, 13.6 kDa; aprotinin, 6.5 kDa and vitamin B_{12}, 1.4 kDa) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium ferricyanide, 3-(2-pyridyl)-5,6-bis(4-phenyl-sulphonic acid)-1,2,4-triazine (ferrozine), trichloroacetic acid (TCA), 2-Thiobarbituric acid (TBA), pyrocatechol violet,

pyrogallol, and L-ascorbic acid were obtained from Fischer Scientific (Edmonton, AB, Canada). All other chemicals were of analytical grade.

2.2.2. Enzymatic Hydrolysis of Barley Glutelin

Glutelin was hydrolyzed by alcalase (AH) and flavourzyme (FH) at an optimized enzyme/substrate ratio according to preliminary experiments for each enzyme. Glutelin (2.0 g) was firstly dispersed in 100 ml deionized water using a homogenizer (PowerGen-1000, Fisher Scientific, Fairlawn, NJ, USA). After pH and temperature adjustments, proteases were added to the protein suspensions to initiate hydrolysis. Alcalase hydrolysis was conducted at the enzyme/substrate ratio of 0.12 AU/g protein, at pH 8.0 and 50 ˚C. Flavourzyme hydrolysis was performed at the enzyme/substrate ratio of 40 LAPU/g protein, at pH 7.0 and 50 ˚C. The pH value of the hydrolysis mixture was readjusted and optimized every 10 min during hydrolysis with 0.5M NaOH and 0.5M HCl. Hydrolysis was continued for 4 h and the hydrolysate samples were taken out at different time intervals (0.5, 1, 1.5, 2, 3 and 4 hours). At the end of the hydrolysis period, the pH was brought to 7.0. All the hydrolysate solutions were then heated at 95 ˚C for 5 min to inactivate the enzyme and centrifuged at 5,000 $\times g$ at 23 ˚C for 10 min (Beckman Coulter Avanti J-E Centrifuge System, CA, USA) to separate the soluble hydrolysates from the non-soluble substances. The soluble hydrolysates were lyophilized and stored at 4 ˚C until analyzed. Protein content of the hydrolysates was determined by the nitrogen analyzer using the same method as indicated in 2.1.

2.2.3. Degree of Hydrolysis (DH)

The DH of the hydrolysates was determined using the TNBS method (Adler-Nissen, 1979) with modifications. Hydrolysate samples (0.25 ml, 0.8 g/l) were pipetted into test tubes containing 2.0 ml of sodium phosphate buffer (0.2 M, pH 8.2) then 2.0 ml of TNBS reagent (0.01%) was added, followed by mixing and incubation at 50 ˚C for 60 min in a covered water bath (to avoid exposure to light). At the end of incubation, the reaction was terminated by addition of 4.0 ml HCl (0.1 M) to each tube. The solutions were cooled to 23 ˚C for 30 minutes, and the absorbance was measured at 340 nm with a UV-visible spectrophotometer (model V-530, Jasco, CA, USA). The total number of amino groups was determined in a sample completely hydrolyzed with 6N HCl at 110 ˚C for 24 h. L-Leucine (0-2.5×10^{-3} M) was used to construct a standard curve. The free amino content in barley glutelin hydrolysate samples were expressed as Leu amino acid equivalents, based on the equation of the Leu standard curve generated. The DH values were calculated using the following formula:

$$\text{DH}\ (\%) = (h/h_{tot}) \times 100 \qquad (1)$$

where h is the number of peptide bonds broken during hydrolysis expressed as mmol/g of protein and h_{tot} is the total amount of peptide bonds in the protein substrate determined from the amino acid composition. For barley glutelin, h_{tot} was 7.93 mmol/g of protein.

2.2.4. Characterizations of the Hydrolysates

Surface hydrophobicity (H_0) of barley glutelin hydrolysates was determined using the a polar fluorescent dye, ANS (Kato & Nakai, 1980). Samples were

prepared as 1% (w/v) protein solutions followed by five further dilutions in phosphate buffer to obtain a final concentration ranging from 0.0025-0.0375% (w/v). Twenty μL ANS solution (8.0×10^{-3} M in 0.l M phosphate buffer, pH 7.4) was added to 4 mL sample. Relative fluorescence intensity was measured within 5-15 min after mixing using the Jasco FP-6300 spectrofluorometer (Tokyo, Japan). Excitation and emission wavelengths were set at 390 nm and 510 nm, respectively. The initial slope of the relative fluorescence intensity versus protein concentration plot was calculated by linear regression analysis and used as an index of the protein surface hydrophobicity (H_0).

The average molecular weight (M_w) of the glutelin hydrolysates was determined by high-performance size exclusion chromatography (SE-HPLC) (Agilent series 1100, California, US) equipped with a Biosuite^{TM} 125/5 μm HR-SEC column (7.8 × 300 mm; Waters Corporation, Massachusetts, USA) at 25 ±0.5 ˚C. The elution buffer contained 0.05M Na_2HPO_4/NaH_2PO_4 and 0.1M NaCl and its flow rate was 0.5 ml/min. Sample solution (20 μl) was injected into the HPLC system and the protein elution was monitored at the UV wavelength of 280 nm. Standard molecular markers were used to calculate M_w of the hydrolysates. A calibration curve was made from the log M_w of the markers and their respective elution times ($R^2 = 0.99$).

For amino acid analysis, the extracted barley glutelin and the prepared peptides were hydrolyzed under vacuum in 4 M methanesulfonic acid with 0.2% (w/v) tryptamine according to the method of Simpson, Neuberger and Liu (1976) with slight modifications. Glass sample tubes (6×50 mm) were used in the reaction vial

assembly, which were then placed in the Work Station (Waters, Milford, MA, USA). After treating as suggested in the Work Station manual, where the contents were hydrolyzed at 115 ˚C for 24 h, the pH was adjusted to neutral with 3.5 M NaOH. Amino acid analysis was performed using the Waters ACCQ-Tag method. The high-performance liquid chromatography (HPLC) system (Agilent series 1100, Palo Alto, CA, USA) consisted of an autosampler and a binary pump, a control system with a column heater maintained at 37 ˚C, and a UV detector set at a wavelength of 254 nm. A reversed-phase AccQ.Tag 150 × 3.9 mm C18 column with a three-eluent gradient solvent system (AccQ.Tag eluent, acetonitrile, and water) at a flow rate of 1.5 ml/min. Data acquisition was controlled by ChemStation software.

2.2.5. Antioxidant Properties

2.2.5.1. DPPH˙ scavenging activity

The scavenging effect of barley glutelin hydrolysates on DPPH free radical was measured according to the method of Tang, He, Dai, Xiong, Xie & Chen, 2010. Aliquots of samples (1.0 g/l) were mixed 1:1 (v/v) with 0.1×10^{-3} M DPPH in anhydrous ethanol. The mixture was shaken and left for 30 min at room temperature protected from light. BHT at concentrations of 0.01 and 0.1 g/l were used as positive controls. The reduction of DPPH free radicals was determined by measuring the absorbance at 517 nm with the UV-visible spectrophotometer (model V-530, Jasco, CA, USA). The ability of the hydrolysates to scavenge the DPPH free radicals was calculated according to the following equation:

$$\%\text{DPPH free radical scavenging} = 1 - (A_{s1}/A_{c1}) \times 100 \qquad (2)$$

where A_{s1} and A_{c1} represent the absorbencies of the sample and the control (deionized water instead of hydrolysates), respectively.

2.2.5.2. Superoxide radical ($O_2^{\cdot -}$) scavenging activity.

The superoxide radical scavenging activity was estimated at 25 ˚C by spectrophotometrically monitoring the inhibition of pyrogallol autoxidation (Li et al., 2008). This assay is dependent on the reducing activity of the test compound by an $O_2^{\cdot -}$ dependent reaction, which releases chromphoric products. Eighty µl of glutelin hydrolysates at 2.0 g/l was mixed with 80 µl of 0.05 M Tris–HCl buffer (pH 8.3) in a 96-well microplate followed by the addition of 40 µl of 1.5 mM pyrogallol in 10^{-3} M HCl. The rate of $O_2^{\cdot -}$-induced polymerization of pyrogallol (ΔA_s/min) was measured as an increase in absorbance at 320 nm for 5 min at 23 ˚C. BHT at concentrations of 0.01 and 0.1 g/l was applied as positive control and Tris–HCl buffer was used instead of hydrolysates in blank experiments (ΔA_c/min). The $O_2^{\cdot -}$scavenging activity of hydrolysates was calculated using the following equation:

The $O_2^{\cdot -}$scavenging activity= $[(\Delta A_c/\ min) - (\Delta A_s\ /min)]/\ (\Delta A_c/\ min) \times 100$ (3)

2.2.5.3. Hydroxyl radical ($OH^{\cdot}$) scavenging activity.

The hydroxyl radical scavenging assay was carried out using the method described by de Avelar, Magalhaes, Silva, Souza, Leitao and Hermes-Lima (2004) after minor modifications. Barley glutelin hydrolysate (1.0 g/l, 250 µl), EDTA (2.4×10^{-3} M, 42 µl) and $FeCl_3$ (0.5×10^{-4} M, 400 µl) were dissolved in phosphate buffer (pH 7.4) and mixed thoroughly. Ascorbic acid (0.1 M, 1 µl), DR (0.2 M, 14 µl) and H_2O_2 (0.01%, 142 µl) were added in and the mixture was

incubated at 37 ˚C for 60 min. Then the mixture was boiled for 15 min with TBA (10.0 g/l, 1ml) and TCA (28.0 g/l, 1ml). Afterwards, the mixture absorbance was measured at 532 nm (A_{s2}) with the UV-visible spectrophotometer. Phosphate buffer (250 µl) was used as blank control. Hydroxyl radical scavenging ability was evaluated as the inhibition rate of 2-deoxy-D-ribose oxidation by hydroxyl radicals. BHT (0.01 and 0.1 g/l) was used as the positive control. Results were determined using the following equation:

$$\text{Hydroxyl (OH) Scavenging Activity (\%)} = (A_{s2} - A_{c2}) / A_{c2} \times 100\% \quad (4)$$

Where A_{s2} and A_{c2} represent the absorbance of the sample and the control (phosphate buffer instead of hydrolysates), respectively.

2.2.5.4. Reducing power.

The reducing power of barley glutelin hydrolysates was measured according to Oyaizu's method (1986). One ml of hydrolysate (2.0 g/l) was added to a solution containing 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50 ˚C for 20 min. Then, 2.5 ml of 10% trichloroacetic acid (TCA) was added to stop the reaction. After centrifugation at 5,000 × *g*, 10 min at 23 ˚C, the supernatant was collected and 2.5 ml was diluted with 2.5 ml deionized water and 0.5 ml of 1 g/l $FeCl_3$ in a test tube. After a 10 min reaction, the absorbance of the resulting solution was measured at 700 nm. The blank contained everything except the sample. An increased absorbance of the mixture indicated the increased reducing power. L-ascorbic acid (0.01 and 0.1 g/l) was used as a positive control.

2.2.5.5 Fe^{2+}-chelating activity.

The ferrous (Fe^{2+}) chelating activity of barley protein hydrolysates was measured as Kong et al. described (1990). Briefly, 0.5 ml hydrolysate samples (1.0 g/l) were mixed with 1.0 ml of $FeCl_2$ (0.2×10^{-4} M) and 1 ml of ferrozine (0.5×10^{-3} M), the mixture was vortexed and kept at room temperature for 15 minutes prior to measure the absorbance at 562 nm (A_{s3}). Deionized water was used as blank (A_{c3}) and EDTA (0.01 and 0.1 g/l) was used as a positive control. The chelating ability was calculated as follows:

$$\text{Chelating Ability (\%)} = (A_{c3}-A_{s3})/A_{c3} \times 100\% \quad (5)$$

Where A_{s3} and A_{c3} represent the absorbance of the sample and the control (deionized water instead of hydrolysates), respectively.

2.2.6. Fractionation of Antioxidant Peptides by Ultra-filtration

Protein samples hydrolyzed for 2h with alcalase demonstrated the highest activities and were selected for further analysis. The lyophilized barley glutelin hydrolysate was dissolved in deionized water and passed through an Ultra/Diafiltration system equipped with Centramate Cassettes filtration system (T-series Omega, PALL Life Science, Ann Arbor, MI, USA) using membranes with molecular weight cutoff values of 10 and 1 kDa. The fractions with M_w distribution of > 10 kDa, 1-10 kDa and <1 kDa were collected, lyophilized and stored at 4 ˚C. The antioxidant properties of the AH fractions were evaluated using the same methods as described in 2.5. The concentration of the peptides used was 1.0 g/l for all tests.

2.2.7. Fractionation of Antioxidant Peptides by RP-HPLC

The AH fraction with M_w < 1 *k*Da was selected for further fractionation by reversed-phase high performance liquid chromatography (RP-HPLC). The lyophilized sample (100 g/l) was reconstituted in 0.1% trifluoroacetic acid (TFA) solution and injected into a HPLC system equipped with a Zorbax SB-C18 column (5 μm, 4.6 × 150 mm; Agilent, CO, USA). The gradient elution was performed at a flow rate of 1.0 ml/min with eluent A as 0.1% TFA in distilled water and eluent B as 0.1 % TFA in acetonitrile (ACN). Separation was performed using a linear gradient elution of 5 to 40% B in 30 min and the peptide peaks were monitored at UV wavelengths of 280 nm. The peaks corresponding to peptides were collected as four fractions and freeze-dried. The antioxidant properties of these fractions were evaluated using the same methods as described in 2.5. The concentration of the peptides used was 1.0 g/l for all the tests.

2.2.8. Identification of Peptides by Mass Spectroscopy (LC-MS/MS)

The most potent antioxidant fraction identified in 2.7 was analyzed by LC-MS/MS to identify the peptide sequence. The peptides were subject to LC-MS/MS analysis on a UPLC (Waters, Milford, MA) coupled with q-Tof premier mass spectrometer (Waters, Milford, MA). Five μL of the resultant peptide digests was loaded onto a nanoAcquity UPLC system with peptide trap (180μm x 20mm, Symmetry® C18 nanoAcquity™ column, Waters, Milford, MA) and a nano analytical column (75 μm × 100 mm, Atlantis™ dC18 nanoAcquity™ column, Waters, Milford, MA). Peptides were separated with a gradient of 1-65%

solvent A (acetonitrile, 0.1% formic acid) over 35 min at a flow rate of 300 μL/min. The flow entered directly into the mass spectrometer via a nanoLockspray ionization source in a positive ion mode (capillary voltage of 3.80 kV and source temperature of 100 ˚C). Spectra were recorded over the mass/charge (m/z) ranges of 100-1000 in MS mode and 50-1500 in MS/MS mode. The signal threshold to perform auto-MS/MS in the data-dependent acquisition was 20 counts/s in total ion current, and the precursor ions were isolated within a range of m/z 3.0. Instrumental control and data analysis were performed using MassLynx software (Micromass U.K. Ltd., Manchester, U.K.). Peaks Viewer 4.5 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) was used in combination with manual de novo sequencing to process the MS/MS data and to perform peptide sequencing.

2.2.9. Statistical Analysis

Each type of hydrolysate was prepared in two independent batches. The hydrolysate structure characterization and antioxidant property measurements were done in triplicate for each batch. Data are represented as the mean of two batches ± SD. For hydrolysate fractionation with ultra-filtration and reverse phase HPLC, one batch of the sample was randomly selected and the antioxidant data are the mean of three independent determinations ± SD. Statistical significance of the differences was determined by Student's t-test ($p < 0.05$).

2.3. Results and Discussion

2.3.1. Barley Glutelin Hydrolysis

The protein content of the isolated barley glutelin and the hydrolysates was 86% and 80-84% (w/w) on a dry basis, respectively. After 1h hydrolysis, more than 40%

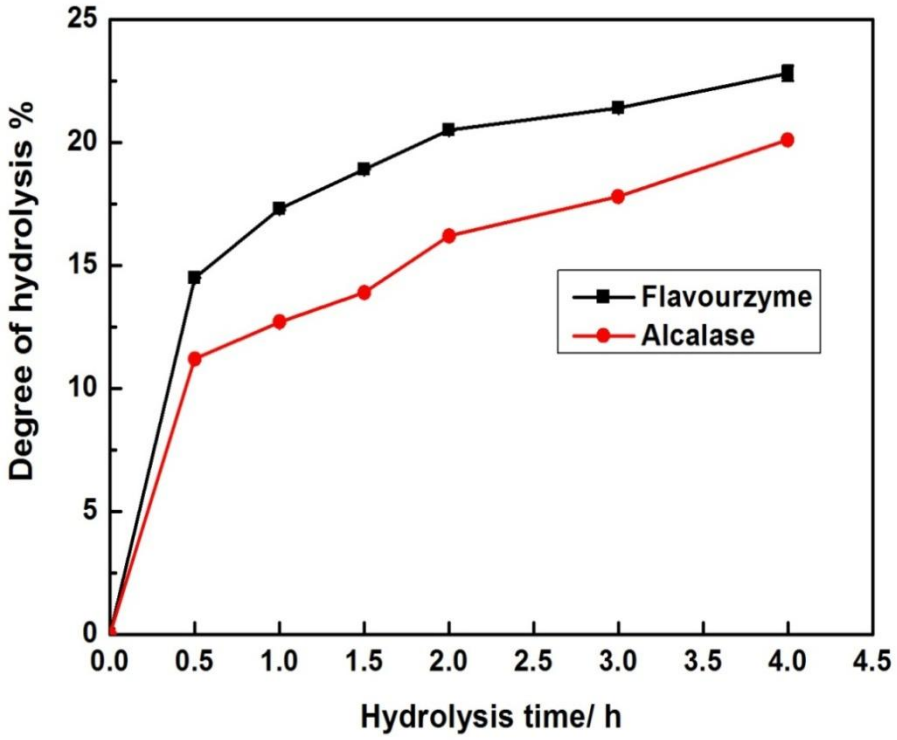


Figure 2.1 Degree of hydrolysis of barley glutelin treated by flavouzyme and alcalase

(w/w) hydrolysates were soluble at 2.0 g/l. Two hours of hydrolysis led to hydrolysates completely soluble in water at the same concentration. The antioxidant activity of the protein hydrolysates depends on the protein substrate, the specificity of the enzyme, the conditions used during proteolysis and the

degree of hydrolysis. Therefore barley glutelin hydrolysis was performed using two selected proteases, alcalase and flavourzyme, at different hydrolysis times. As shown in **Figure 2.1**, the degree of hydrolysis (DH) increased rapidly in the first 0.5 h, followed by a slower rate of increase up to 4 h, with the DH value ranging from 12.7 to 22.8%. The DH of flavouzyme hydrolysates (FHs) was higher ($P < 0.05$) than that of alcalase hydrolysates (AHs) at comparable hydrolysis times. Flavourzyme is an endo- and exopeptidase enzyme mixture, which has broad specificity to produce small-size peptides and free amino acids (Ven, Gruppen, Bont & Voragen, 2002). As an endo-protease, alcalase cleaves peptide bonds at the interior of the polypeptides chain; thus, it mainly produces small- and medium- sized peptides (Klompong, Benjakul, Kantachote, Hayes & Shahidi, 2008). Consequently, flavourzyme treatment results in hydrolysates with higher DH.

The SE-HPLC chromatograms of the FH after 0.5h of hydrolysis was characterized by two major peaks (P3 and P4) at M_w of 1.4 kDa and 0.7 kDa, respectively and a broad peak (P1) with rather high M_w (17.2 kDa), as well as several small peaks (P2) in the M_w range of 5.1-3.2 kDa (**Figure 2.2A (a)**). After 4 h hydrolysis, P1 disappeared, whereas the P2 amplitude was dramatically enhanced. For AH chromatogram after 0.5 h of hydrolysis, three major peaks were identified with M_w of 22.5 kDa (P1'), 7.8 kDa (P2') and 1.5 kDa (P3') (Fig.**2A (b)**). Meanwhile, several shoulder peaks were found between P2' and P3'.

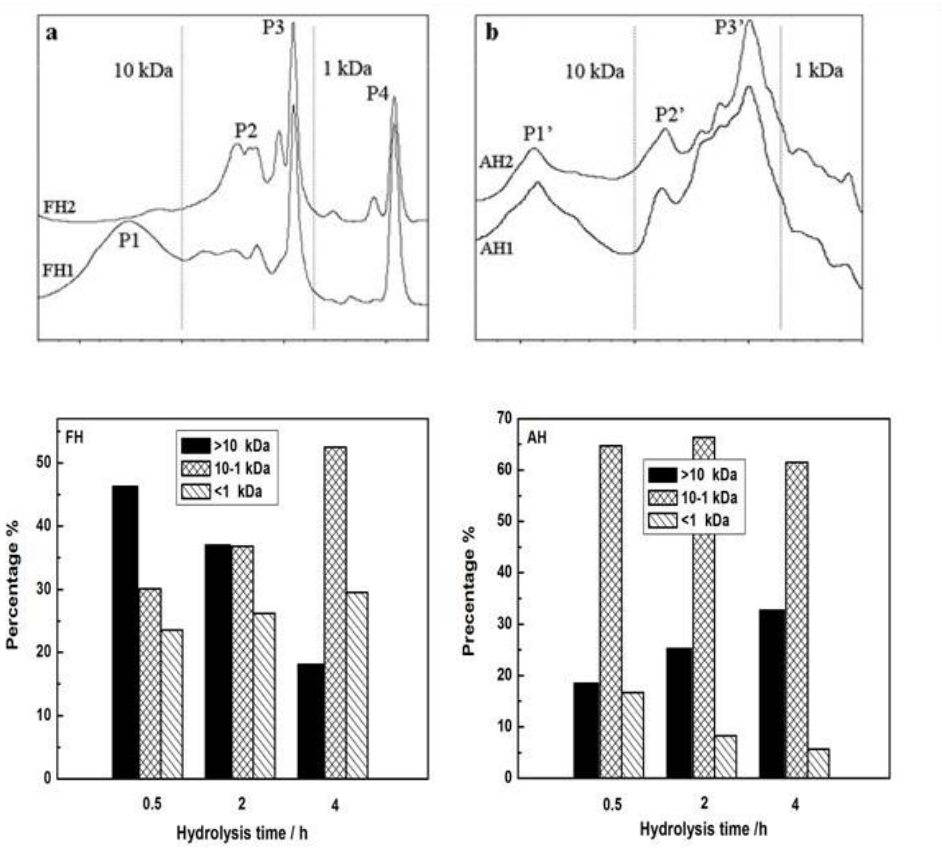


Figure 2.2 (A) SE-HPLC chromatogram of barley glutelin hydrolysates after 0.5 and 4 h of hydrolysis treated by (a) flavourzyme (FH) and (b) alcalase (AH). FH1 and FH2 are flavourzyme hydrolysates after 0.5 and 4 h of hydrolysis, respectively; AH1 and AH2 are alcalase hydrolysates after 0.5 and 4 h of hydrolysis, respectively. (B) Relative area (%) of the peptide peaks in barley glutelin hydrolysate fractions prepared with flavourzyme (FH) and alcalase (AH) after 0.5, 2 and 4 h of hydrolysis.

After 4 h hydrolysis, P1' and the shoulder peaks were significantly reduced while P3' was enhanced remarkably. The SEC-HPLC profiles were then divided into

three fractions based on their apparent M_w. Fraction I corresponds to large-sized peptide fragments (M_w > 10 kDa), fraction II to medium-sized peptide fragments (1 kDa < M_w < 10 kDa) and fraction III to mall-sized peptide fragments (M_w < 1 kDa). Fig. 2B summarizes the quantitative changes of these three fractions during hydrolysis, represented by the area of each fraction relative to the total area of the SE-HPLC chromatogram. In the case of FHs, with increasing hydrolysis time, fraction II increased significantly ($p < 0.05$) with concurrent reduction of fraction I in the chromatogram, indicating a degradation of large peptides to medium-sized peptides. Hydrolysis with alcalase resulted in the medium-sized peptides as the major fraction which depicts the endoprotease nature of the enzyme. Slight increase in the proportion of fraction I might be due to formation of some aggregates a result of protein unfolding. This result agrees with DH data that barley glutelin was more extensively hydrolyzed by flavourzyme treatment, since both medium (53%) and small-sized (28%) peptides dominated in the FHs after 4 h hydrolysis, whereas medium-size peptides (65%) prevailed in AHs with only 7% small-size peptides observed.

Due to cleavage of peptide bonds, proteolysis is accompanied by gain or loss in hydrophobicity (Liu, Kong, Xiong & Xia, 2010). As shown in **Figure 2.3**, H_0 of FH decreased sharply when the hydrolysis time was increased to 1.5 h, and then increased gradually in the next 2.5 h. The peptides that were released during the first 1.5 h may have great flexibility to expose more hydrophilic groups outward in the aqueous system (Liu et al., 2010). With a deeper hydrolysis, the released peptides may change their conformations to expose hydrophobic amino

acid residues. Enzymatic hydrolysis by alcalase was accompanied by a slight increase of H_0 in the first 1 h, and then it levelled off in the next 3 h. The average surface hydrophobicity of FHs was significantly higher than that of AHs ($p < 0.05$).

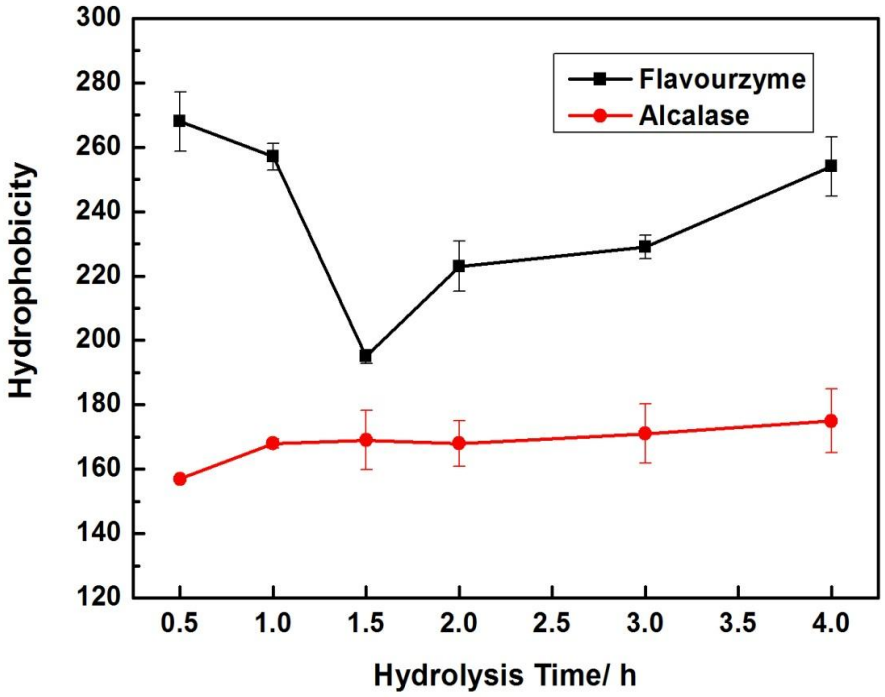


Figure 2.3 Changes in surface hydrophobicity of barley glutelin hydrolysates during hydrolysis.

2.3.2. Impacts of Hydrolysis on Antioxidant Activity of Barley Glutelin

The antioxidant properties of the hydrolysates prepared at different incubation times were evaluated based on their radical scavenging capacity

(DPPH/$O_2^{\cdot-}$/$OH^{\cdot}$), Fe^{2+}-chelating effect and reducing power. Our preliminary data indicated that the antioxidant activity of peptides was dose-dependent, thus the peptide concentrations used in each assay were optimized and the lowest effective concentrations (1.0 or 2.0 g/l) were selected. The antioxidant activity of unhydrolyzed glutelin was not tested because it made a turbid solution due to low solubility.

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. Therefore, DPPH is often used as a

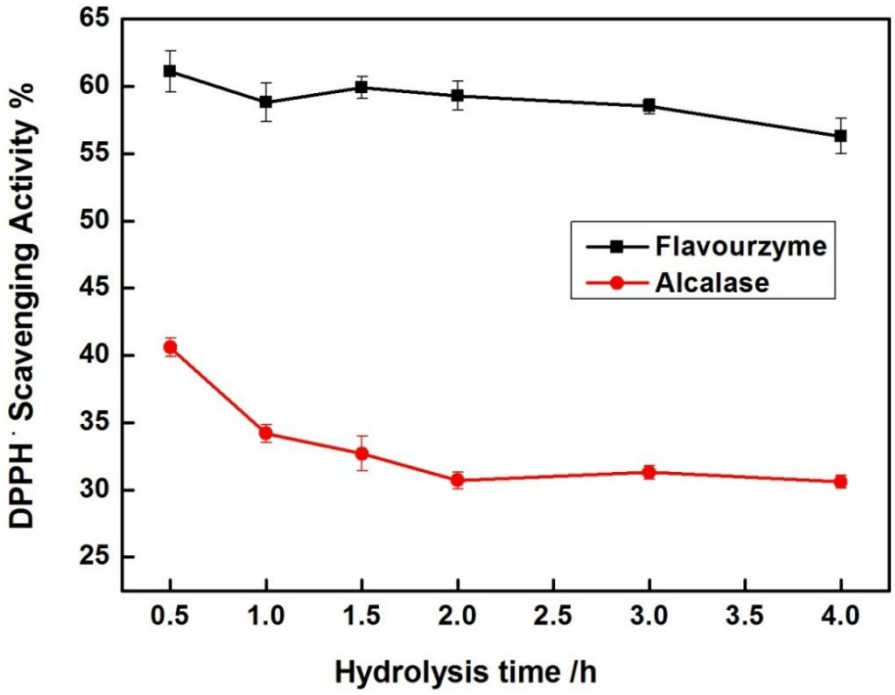


Figure 2.4 (A) DPPH radical scavenging activity (1.0 mg/ml)

substrate to evaluate the antioxidant activity of an antioxidant. As described in **Figure 2.4A**, FH showed a slight decrease in DPPH scavenging ability during the first 1 h of hydrolysis which thereafter remained almost unchanged. The same trend was also observed for AH, but the decrease was more pronounced during the first 2 h. FHs showed a much higher DPPH radical scavenging activity than AHs, probably due to their significantly higher surface hydrophobicity. It has

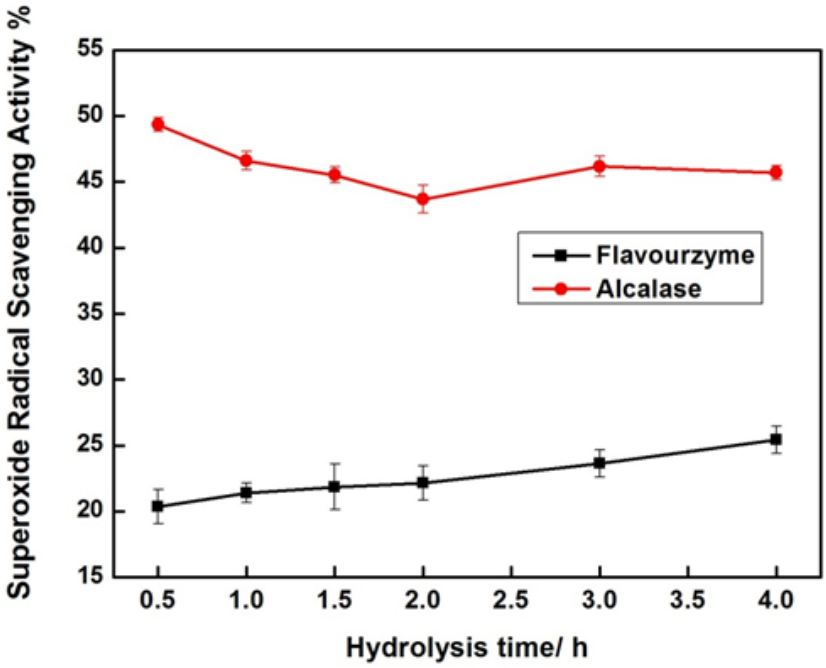


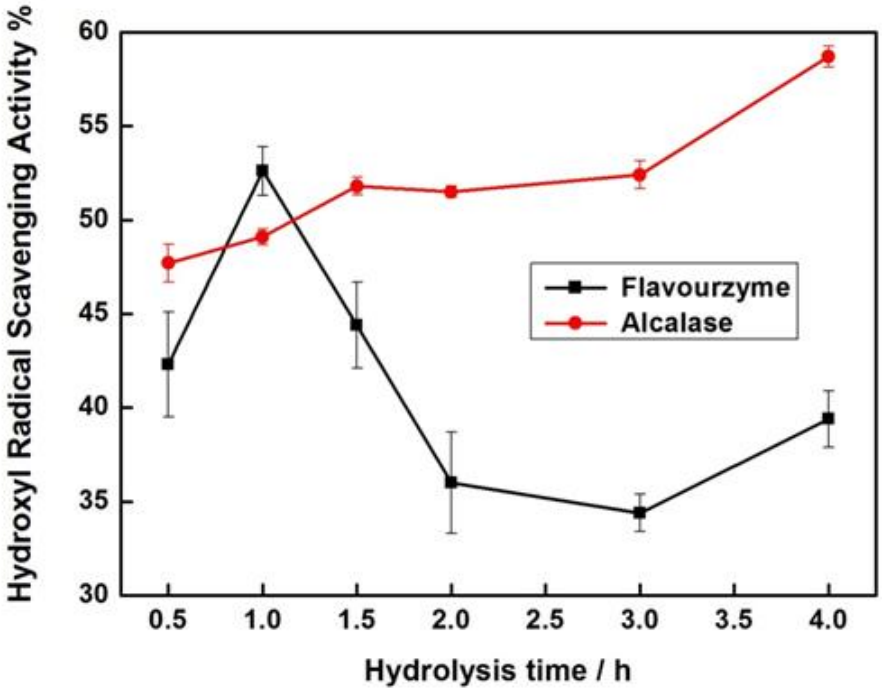
Figure 2.4 (B) superoxide radical ($O_2^{\cdot -}$) scavenging activity (2.0 mg/ml)

been reported that the high level of DPPH free radical scavenging activity of protein hydrolysates is associated with a high amount of hydrophobic amino acids or peptide (Rajapakse, Mendis, Jung, Je, & Kim 2005). FHs exhibited a moderate scavenging capacity against the DPPH radical, reaching a scavenging activity of 56-61% at the concentration of 1.0 g/l, comparable to that of whey, porcine and

chickpea protein hydrolysate (Peng, Xiong & Kong, 2009; Saiga, Tanabe & Nishimura, 2003; Xie, Huang, Xu & Jin, 2008). Superoxide anion radical ($O_2^{\cdot -}$) can produce hydrogen peroxide and hydroxyl radicals through dismutation and other types of reaction. Not only $O_2^{\cdot -}$, but also its derivatives, can cause damage to DNA and cell membranes. As demonstrated in **Figure 2.4B**, the $O_2^{\cdot -}$ scavenging of AH decreased initially (49 to 44%) in the first 2 h, and then increased slightly afterwards. Flavourzyme hydrolysis was accompanied by a slight increase of $O_2^{\cdot -}$ scavenging capacity.Despite the DPPH radical scavenging activity, in $O_2^{\cdot -}$ scavenging assay AHs exhibited superior activity compared to FHs, and the maximum scavenging value was approximately 50% for AH at the concentration of 2.0 g/l. This value is comparable to and even higher than that of many other protein hydrolysates. Zein and rice endosperm protein hydrolysates exhibited an $O_2^{\cdot -}$ scavenging ability of 11.5% at 10 g/l and < 20% at 0.5 g/l, respectively (Tang, He, Dai, Xiong, Xie & Chen, 2010; Zhang et al., 2010). Saito et al. (2003) indicated that His, Pro, and Tyr are the most important residues in radical scavenging activity of antioxidant peptides. These amino acids comprise more than 16 % of the total residues in barley glutelin (Wang, Tian, Chen, Temelli, Liu & Wang, 2010), which may explain the remarkble $O_2^{\cdot -}$ scavenging ability of barley glutelin hydrolysates. Hydroxyl radical can react with biomolecules such as amino acids, proteins, and DNA, as well as trigger lipid peroxidation (Xie et al., 2008). Therefore, removal of hydroxyl radical is probably one of the most effective defenses of a system against oxidation. In general, the inhibition effect of the AHs upon $OH^{\cdot}$ was significantly greater than that of the

FHs and it was even enhanced with prolonged hydrolysis time (47 to 58%) (**Figure 2.4C**). The $OH^{\cdot}$ scavenging capacity of FH reached a maximum at 1 h of hydrolysis (58%) at 1.0 g/l, showing potential to protect a food or living system against hydroxyl radical-induced damages.

Since compounds interfering with the catalytic activity of metal ions could affect the peroxidative process, measuring the chelating ability of the compound is important for evaluating its antioxidant activity (Saiga et al., 2003; Xie et al.,



Fihgure 2.4(C) Hydroxyl radical ($OH^{\cdot}$) scavenging activity (1.0 mg/ml),

2008). In **Figure 2.4D**, the Fe^{2+} chelating capacity of FHs was low (around 23% to 30%) and not influenced by hydrolysis time. On the other hand, Fe^{2+} chelating capacity of AH increased dramatically (53 to 88%) up to 2 h of hydrolysis, then

increased more steadily with prolonged hydrolysis time. The maximum Fe^{2+} -chelating capacity was 90% for AH after 4 h of hydrolysis at 1.0 g/l. This value is much greater than many other protein hydrolysates. Chang et al. (2007) reported a chelating ability ranging from 8 to 63% for hydrolysates derived from porcine hemoglobin at 5.0 g/l assay concentration. Corn zein and chickpea protein hydrolysates showed poor Fe^{2+}- chelating ability, even at 30-40 g/l concentration (Kong et al., 2006; Li et al., 2008). Such a high Fe^{2+} chelating capacity in AH

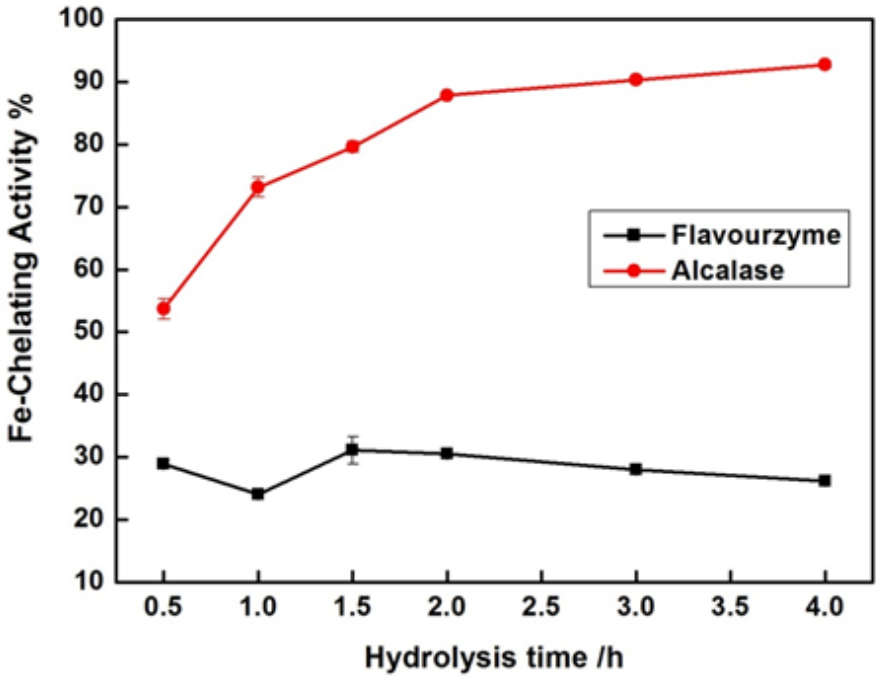


Figure 2.4(D) ferrus ion chelating activity (1.0 mg/ml)

may arise from the exposure of more acidic and basic amino acids by peptide cleavage as the carboxyl and amino groups in their side chains can bind Fe^{2+} (Zhang et al., 2010). In addition, the superior Fe^{2+} chelation ability of AHs may

contribute to their high hydroxyl radical scavenging effects due to combined effect of radical scavenging and ion chelation activity.

Free radicals form stable substances by accepting electrons and therefore the free radical chain reactions are interrupted. The reducing power assay is often used to evaluate the ability of natural antioxidants to donate an electron or hydrogen (Dorman, Peltoketo, Hiltunen & Tikkanen, 2003). As shown in **Fig. 4E**, AHs possessed significantly higher reducing power than FHs. Reducing power of AHs increased obviously (0.103 to 0.121) with increasing hydrolysis time up to

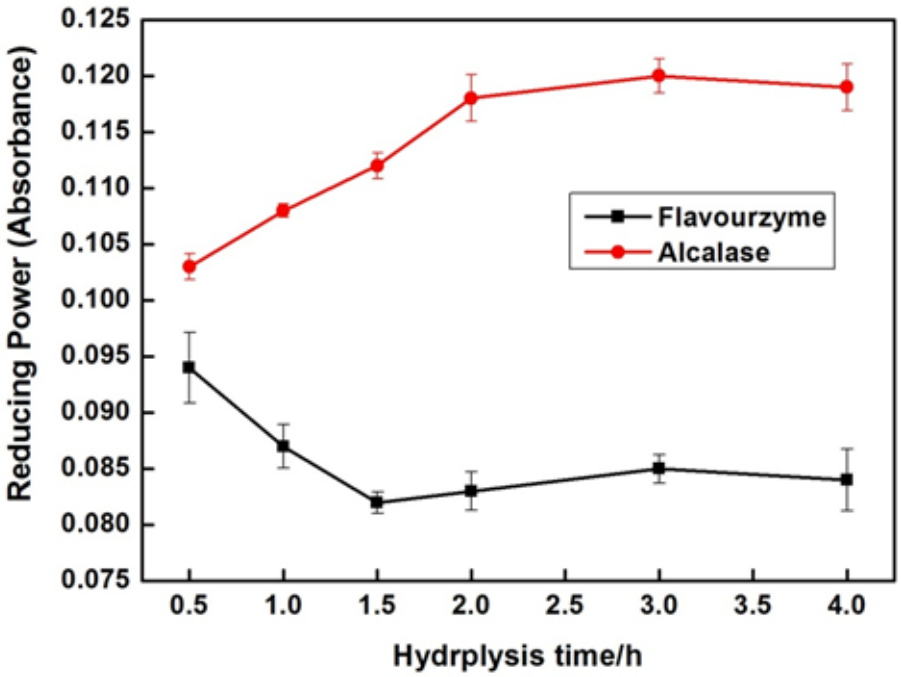


Figure 2.4(E) reducing power (2.0 mg/ml)

2h of incubation, and then leveled off during the next 2.0 h. On the contrary, reducing power of FHs was reduced significantly (0.094 to 0.083) during the first

1.5 h of hydrolysis, and then increased slightly afterwards. The increase or decrease in reducing power for AH and FH may be related to the exposure of electron-dense amino acid side chain groups, such as polar or charged moieties during hydrolysis (Bamdad, Wu & Chen, 2011). On the other hand, the phenolic and indolic groups of tyrosine and tryptophan have been reported to play important roles as hydrogen donors in a redox system (Pihlanto, 2006). At the same concentration (2.0 g/l), hydrolysates from alfalfa leaf protein and chickpea protein showed much greater reducing power with values of 0.69 and 0.2, respectively (Li et al., 2008; Xie et al., 2008).

The above results demonstrated that the type of enzyme used is a key factor in determining antioxidant activities of barley glutelin hydrolysates. AHs demonstrated significantly higher antioxidant capacity than FHs in most of the selected assays. The DPPH/$OH^{\cdot}$ radical scavenging activity, Fe^{2+} chelating ability and reducing power were related to hydrolysis time, suggesting that critical peptide size may be necessary to manifest a certain antioxidant activity. The $O_2^{\cdot}$ scavenging ability was less impacted by hydrolysis time.

2.3.3 Antioxidant Activity of Peptides from Ultra-filtration

AHs possessed higher antioxidant activities in most of the assays; therefore they were chosen as sample for analyzing antioxidant activities of the barley glutelin peptides in relation to their molecular weight. The AH sample was obtained after 2h of hydrolysis, and then separated by means of an ultra-filtration into three fractions. Fraction I corresponds to large-sized peptide fragments with M_w exceeding 10 kDa, fraction II to medium-sized peptide fragments with M_w

between 1 and 10 kDa, and fraction III to small-sized peptide fragments with M_w lower than 1 kDa. Their antioxidant activities were measured and compared to that of the hydrolysate before fractionation at 1.0 g/l (**Table 2.1A**). The large-sized peptides possessed much greater ($p < 0.05$) DPPH scavenging activity and reducing power, whereas small-sized peptides demonstrated significantly greater ($p < 0.05$) $OH^{\cdot}$ scavenging and Fe^{2+} chelating activity. In general, these optimal fractions exhibited higher antioxidant activities than the hydrolysate before

Table 2.1. Antioxidant activities of (A) barley glutelin hydrolysate (2 h of treatment) and the fractions separated by ultra-filtration (Fraction I, II and II) and (B) barley glutelin hydrolysate (M_w < 1 KDa) separated by reverse-phase column (Fraction 1, 2, 3 and 4) as well as the positive controls

Antioxidant assay	(A) Fractions separated by ultra-filtration				
	2h-AH	Fraction I (> 10 kDa)	Fraction II (10-1 kDa)	Fraction III <1 kDa	Positive control
DPPH Scavenging %	31.5 ± 0.6	61.9 ± 1.7	40.8 ± 1.0	18.3 ± 0.5	78.9 / 99.0 (BHT)
Reducing Power	0.18 ± 0.004	0.29 ± 0.009	0.065 ± 0.002	0.040 ± 0.004	0.25/0.39 (ascorbate)
$O_2^{\cdot}$ Scavenging %	18.7 ± 0.9	24.4 ± 1.1	16.2 ± 0.9	25.5 ± 0.8	32.0/47.9 (BHT)
Fe^{2+} Chelating%	86.1 ± 0.8	70.1 ± 1.8	82.5 ± 1.8	85.3 ± 1.3	38.5/98.5 (EDTA)
$OH^{\cdot}$ Scavenging %	51.1 ± 0.2	60.2 ± 1.0	63.7 ± 0.8	69.1 ± 0.9	42.4/87.2 (BHT)

Antioxidant assay	(B) Fractions separated by reverse-phase column				Positive control
	Fraction 1	Fraction 2	Fraction 3	Fraction 4	
$O_2^{\cdot}$	18.2 ± 0.9	25.6 ± 1.1	20.4 ± 0.7	21.3 ± 0.9	32.0/47.9 (BHT)
Fe^{2+}	70.1 ± 1.2	82.5 ± 0.8	85.3 ± 1.0	93.0 ± 1.1	38.5/98.5 (EDTA)
$OH^{\cdot -}$	45.9 ± 0.7	64.1 ± 0.5	65.7 ± 0.3	70.4 ± 0.5	42.4/87.2 (BHT)

* The concentration of barley glutelin hydrolysate fractions was 1.0 g/l; the concentration of BHT, ascorbate and EDTA was 0.01 and 0.1 g/l.

separation. Reducing power was dramatically improved (0.288 at 1.0 g/l) by separating large-sized peptides from barley glutelin hydrolysates, resulting in values comparable to or even greater than those from other proteins (Li et al., 2008; Xie et al., 2008). No relationship between $O_2^{\cdot}$ scavenging ability and peptide M_w could be found. The amino acid composition of these three fractions is described in **Table 2.2**. Fraction I and II had significantly higher Pro residue percentages (15.5 and 17.3%) than that of fraction III (7.7%), confirming that Pro

Table 2.2. Amino acid composition (%) of barley glutelin, and their alcalase hydrolysates separated by ultra-filtration (Fraction I, II and II) and reverse-phase column (Fraction 4).

Amino acids	Barley glutelin	Fraction I	Fraction II	Fraction III	Fraction 4
Asx	5.8	6.6	8.6	7.5	5.6
Ser	5.7	8.1	8.2	7.1	5.5
Glx	20.0	22.3	28.5	20.3	18.0
Gly	9.1	12.9	5.8	7.5	5.6
His	2.7	1.2	1.1	2.1	6.6
Arg	4.6	5.0	3.0	5.5	3.0
Thr	4.7	2.2	2.2	3.8	1.8
Ala	6.1	3.5	3.6	6.9	2.5
Pro	10.7	15.5	17.3	7.7	17.6
Cys	0.8	3.6	3.0	2.0	3.2
Tyr	3.5	2.4	2.2	2.6	2.5
Val	6.1	3.4	4.2	6.9	7.1
Met	1.5	n.d.	0.2	0.3	n.d.
Lys	3.7	4.1	2.1	3.7	1.7
Ile	3.3	3.0	3.1	4.1	6.6
Leu	8.0	3.4	4.0	8.2	9.6
Phe	3.8	3.0	3.7	3.5	9.1

*Asx represents Asn and Asp; Glx represents Gln and Glu; "n.d." means not detectable.

*The order of amino acids listed in the table follows the elution order of the amino acids from the reversed-phase HPLC chromatographic column.

*Barley glutelin amino acid composition was analyzed in our previous work (Wang, Tian, Chen, Temelli, Liu & Wang, 2010).

was less prone to cleavage by proteases and peptidases. These fractions possessed a similar amount of hydrophobic amino acid residues (Val, Leu, Ile, Met, Phe, Ala and Pro 31.8-37.6%). No significant change in the proportion of His, Tyr, Mer, Lys, Trp and Phe was observed which are generally accepted as antioxidant amino acids (Jung et al., 1995; Pihlanto, 2006). This confirms that the molecular weight of AH is critial to manifest optimum antioxidant activities. A number of studies have already shown that the antioxidant activity of peptides is dependent on their molecular weight distribution (Moure, Dominguez & Parajo, 2006; Pena-Ramos et al., 2004). The majority of previous antioxidant peptide research has shown that short peptides and amino acids are the most efficient antioxidants because their accessibility to the oxidant/antioxidant test systems is greater than that of large peptides and proteins (Hernandez-Ledesma, Davalos, Bartolome & Amigo, 2005). It is interesting that the large-sized peptide fraction was more effective in DPPH scavenging activity and reducing power in barley glutelin hydrolysates, which deserves further study. A tentative explanation could be that specific hydrophobic clusters of glutelin, with bulky and aromatic side chains, may act as hydogen donors and as direct radical scavengers (Farvin, Baron, Nielsen, Otte & Jacobsen, 2010).

2.3.4. Antioxidant Activity of Peptides Separated by a Hydrophobic Column

Peptides of <1 kDa possessed superior $O_2^{\cdot}$ and $OH^{\cdot}$ scavenging activity and Fe^{2+} chelating capacity. Hence, to further elucidate the antioxidant activity of specific peptides in this small-sized peptide fraction, the 1 kDa M_w cutoff ultra-

filtration permeate was subjected to further fractionation using a reversed-phase HPLC system. Chromatography of peptides <1 kDa produced four fractions by elution time. The $O_2^{\cdot}$ / $OH^{\cdot}$ radical scavenging activity and Fe^{2+} chelating capacity was then evaluated. The results in Table 1B demonstrated that peptides with higher hydrophobicity (fraction 4) exhibited greater $OH^{\cdot}$ radical scavenging and Fe^{2+} chelating capacity, with the maximum being 70.4% and 93.0%, respectively at 1 g/l. Still no relationship between $O_2^{\cdot}$ scavenging ability and peptide hydrophobicity could be identified. As shown in Table 2, Fration 4 had a significantly greater percentage of hydrophobic amino acid residues (52.5%) than original barley glutelin and Fration III seperated by ultra-filtration (M_w <1 kDa). This agrees with previous studies where peptides with more hydrophobic amino acids play important roles contributing to antioxidation (Chen et al., 1995, Rajapakse et al., 2005). In addition, Fraction 4 demonstrated a significantly higher His (6.6%) and Phe (9.1%) content, which have superior proton-donation ability due to their imidazole and benzyl groups, respectively (Rajapakse et al., 2005; Ren et al., 2008).

2.3.5. Amino Acid Sequence of Potential Antioxidant Peptides

Fraction 4, obtained by reversed-phase HPLC, which had relatively superior radical scavenging activity, was subsequently subjected to LC-MS/MS for peptide sequence identification. The MassLynx software identified four peptides from barley protein: Gln-Lys-Pro-Phe-Pro-Gln-Gln-Pro-Pro-Phe, Pro-Gln-Ile-Pro-Glu-Gln-Phe, Leu-Arg-Thr-Leu-Pro-Met and Ser-Val-Asn-Val-Pro-Leu. The identified peptides exhibited a high content of hydrophobic amino acid residues

such as Pro, Phe, Leu, Ile and Val, which comprise 50-67 % of the total residues. Therefore, these hydrophobic amino acids present in the sequences of barley glutelin may have significant antioxidant properties. Two peptides contained the aromatic amino acid Phe at the C-terminal end. Phe, by virtue of its aromatic ring, can act as a direct radical scavenger since it donates protons easily to electron deficient radicals and still remain stable via electron resonance over its ring (Rajapakse et al., 2005). In addition, Met was found at the C-terminal end of one peptide. Met is believed to be important in radical scavenging activity since Met is prone to oxidation to its sulfoxide (Hernandez-Ledesma et al., 2005; Ren et al., 2008). Metal-chelating amino acid residues such as Met, Glu, Gln, Lys and Arg were detected within the sequences, which have been reported to interact with metal ions through their charged groups and inactivate the prooxidant activity of metal ions (Park et al., 2001; Zhang et al., 2010). This could explain the very strong Fe^{2+}-chelating capacity of barley glutelin peptides which contributed to greater radical scavenging potential. His was not observed in the amino acid sequences despite of its high percentage in the amino acid composition of Fraction 4. It is possible that His may exist as a free amino acid to manifest antioxidant activities independently.

2.3.6. Comparison with Commercial Antioxidants

Antioxidant activities of 2h alcalase hydrolysate and hydrolysate fractions were then compared to the selected positive controls. As summarized in Table 1, the optimal DPPH scavenging activity (61.9% at 1.0 g/l) was observed for the AH fraction with M_w > 10 kDa, comparable to BHT (78.9%) at 0.01 g/l. The $O_2^{\cdot -}$ -

scavenging activities reached a maximum (48.0% at 2.0 g/l) after 0.5 h of hydrolysis by alcalase. This value was similar to BHT (47.9%) at 0.1 g/l. Therefore, barley glutelin hydrolysates can be considered as effective free radical scavengers. EDTA, a standard metal ion chelator, displayed 98.5% chelating ability at 0.1 g/l. In this test, AHs showed very strong Fe^{2+}-chelating activity with the maxium value at 93.0% at 1.0 g/l, comparable to EDTA at 0.1 g/l. AH fraction with M_w > 10 kDa showed good reducing power (0.288 at 1.0 g/l), which is comparable to ascorbic acid (0.245) at 0.01 g/l.

2.4. Conclusion

Barley glutelin can be effectively hydrolyzed by alcalase to obtain peptides with strong antioxidant activities. The activity assessment for fractions separated by ultra-filtration showed that large-sized peptides (M_w > 10 kDa) possessed greater DPPH scavenging activity and reducing power, whereas small-sized peptides (M_w < 1 kDa) were more effective in Fe^{2+} chelating activity and $OH^{\cdot}$ scavenging effect. The small-sized fraction was then separated by a RP-HPLC system and the hydrophobic fraction showed greater Fe^{2+} chelating and $OH^{\cdot}$ scavenging activity. Four peptides contributing to antioxidant activities were identified as Gln-Lys-Pro-Phe-Pro-Gln-Gln-Pro-Pro-Phe, Pro-Gln-Ile-Pro-Glu-Gln-Phe, Leu-Arg-Thr-Leu-Pro-Met and Ser-Val-Asn-Val-Pro-Leu. It is speculated that the presence of hydrophobic amino acid residues, such as Pro, Phe, Leu, Ile and Val, are important for antioxidant activities. In addition, metal-chelating amino acid residues such as Met, Glu, Gln, Lys and Arg within the

sequences of these peptides contributed to the superior Fe^{2+} chelating of the antioxidant peptides as well as their high radical scavenging potential. Although commercial synthetic or natural antioxidants are effective at lower concentartions, barley glutelin hydrolysates could be incorporated into food or cosmetic formulations in much higher proportions without significantly impacting food sensory quality potentially.

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Chapter 3 Molecular Structure, Physicochemical Characterization and in vitro Degradation of Barley Protein Films

3.1 Introduction

There is continuing interest in the production of biodegradable films based on naturally occurring biopolymers like proteins as alternatives to petroleum-based materials. Proteins from plants (wheat gluten, soy, sunflower and corn) and animals (gelatin, keratin, casein and whey) have been manufactured into films (Krochta, 2002) using casting and thermo-compression molding techniques. The advantages of latter are that it requires less processing time (2 to 3 min) and space, and starts with solvent-free protein powder. Recent research suggests that protein heat-curing creates stronger films with improved mechanical strength and barrier properties. Both heating and plasticizer play important roles in the thermal processing of plant protein-based products. Heating unfolds a protein which then facilitates the sulfhydryl-sulfide interchange and development of other protein interactions (e.g. hydrogen bonding and hydrophobic interactions) to reinforce the protein networks (Schofield, Bottomley, Timms, & Booth, 1983). The addition of plasticizing agents to protein films is often required to improve film flexibility and extensibility by reducing the intermolecular forces along polymer chains while increasing intermolecular spacing (Wang, Cao, & Zhang, 2006). Influences of plasticizers (Pommet, Redl, Guilbert, & Morel, 2005) and processing

temperatures (Cuq, Boutrot, Redl, & Lullien-Pellerin, 2000) on the properties of thermo-molded protein materials have been studied separately in previous work. However, the lack of understanding of their combined effects on molecular protein conformation has limited our ability to predict the quality of the film obtained. Most protein film research has focused on the water vapor and oxygen permeability properties of film materials and their potential to improve food quality and extend food shelf life (Krochta, 2002). More recently, there is a growing interest to use protein films to achieve site-specific or controlled release of bioactive oral pharmaceuticals/nutraceuticals (Chen, Remondetto, Rouabhia, & Subirade, 2008). However, the preliminary and integrated research necessary to evaluate the impacts of processing conditions on protein film network structures and consequently their physical and biological behavior in the gastro-intestinal (GI) tract is still limited.

Barley is the fourth most widely cultivated cereal in the world after wheat, rice and corn, and a major crop in Western Canada. Despite of its high quality due to its fertile soil, sound environmental stewardship and ideally suited climate, Canadian barley is still mainly used for livestock feed. Exploring value-added applications of barley and its components (protein, starch, lipid etc.) is important for Canadian agricultural economic development. Hordein and glutelin are the major barley storage proteins. Similar to wheat gluten, barley proteins, especially hordein show good cohesive and elastic properties, thus are interesting candidate for film formation. Additionally, these proteins are known to be hydrophobic, and according to the Goldman-Engelman-Steitz (GES) scale (Engelman, Steitz, &

Goldman, 1986) their hydrophobic amino acid content is around one third of the total amino acid content, with the highest level corresponding to Leu, Val, Phe and Tyr. This hydrophobic nature may provide barley protein materials a good barrier capacity than many other natural materials. Moreover, barley proteins exhibit relatively low digestibility which may limit their nutritive value (Gaylord, Barrows, & Rawles, 2008), however, materials with such characteristic could be interesting for protection and controlled release of bioactive compounds in the gastro-intestinal (GI) tract. In spite of great potential as natural biodegradable films for both food and pharmaceutical applications, research on barley protein films has rarely been reported (Cho & Rhee, 2009).

In this work, barley protein films were prepared by thermo-pressing using glycerol as a plasticizer. The combined effects of heating temperature and plasticizer on protein confirmation and interactions in film matrix, subsequently the film mechanical and moisture barrier properties were investigated. The film degradation and toxicity in the simulated gastrointestinal (GI) tract were evaluated through in *vitro* essays.

3.2 Materials and methods

3.2.1 Materials

Regular barley grains (Falcon) were kindly provided by Dr. James Helm, Alberta Agricultural and Rural Development, Lacombe, Alberta. Barley protein content was 13.2% (w/w, dry status) as determined by combustion with a nitrogen analyzer (FP-428, Leco Corporation, St. Joseph, MI, USA) calibrated with

analytical reagent grade EDTA (a factor of 6.25 was used to convert the nitrogen to protein). Barley protein was prepared by alkaline extraction according to our previous work (Wang et al, 2010). The protein content was 88% (w/w) as determined by the same nitrogen analyzer. Unstained standard protein molecule marker for SDS-PAGE was purchased from Bio-RAD (Richmond, CA, USA). Pepsin (from porcine gastric mucosa, 424 units/mg), pancreatin (from porcine pancreas, 200 USP units/mg), β-mercaptoethanol (β-ME), and Coomassie Brilliant Blue R-250 were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). The human colon carcinoma (Caco-2) cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). Dimethylsulfoxide (DMSO), glycerol, urea, and sodium chloride were purchased from Fisher Scientific (Markham, ON, Canada) and were used as received.

3.2.2 Preparation of Barley Protein Films

Barley protein powders with desired glycerol contents (20, 30 and 40 wt %) were mixed in a mortar and then further blended with a kitchen grinder (CBG100W, Black & Decker, USA) for 15 min. The mixtures were placed in a mold (the thickness was 0.27 ± 0.02 mm) covered with two polished stainless-steel plates and compression-molded by Carver bench top laboratory press (Model 3851, Carver Inc., IN, USA) at various temperatures (100, 120 and 140 ℃) under a pressure of 10,000 pounds for 10 min. Then the films were air-cooled to 50 ℃ under constant pressure with a cooling rate of about 3 ℃ min^{-1} before the removal from the mold, and coded as 100-20, 100-30, 100-40, 120-20, 120-30, 120-40, 140-20, 140-30 and 140-40, corresponding to the different molding temperatures

and glycerol contents. These films were kept for one week at a relative humidity (RH) of 0%, over P_2O_5 in a desiccator at room temperature before characterizations.

3.2.3 Protein Structure Characterizations

FTIR spectra of barley protein powder and films were recorded on a Nicolet 6700 spectrophotometer (Thermo Fisher Scientific Inc., MA, USA). The samples were vacuum-dried for 24 h and then placed on an attenuated total reflectance (ATR) accessory with a Ge crystal. Spectra were recorded as the average of 256 scans at 2 cm^{-1} resolution and 25 ℃, using the empty accessory as blank. During measurements the accessory compartment was flushed with dry nitrogen. For the study of the amide I region of the proteins, ATR correction and Fourier deconvolution were performed using the software provided with the spectrometer (*Omnic 8.1 software*). Fourier self-deconvolution was applied in order to narrow finer bands hidden in larger bands. Parameters used for deconvolution were: enhancement 2/bandwidth 20 cm^{-1}.

DSC analysis was carried out using a DSC Q2000 apparatus (TA Instruments, DE, USA) equipped with a refrigerated cooling system RCS90. The conditioned samples were quenched to -80 ℃ and then heated to 200 ℃ under a nitrogen atmosphere in an aluminum pan, with a heating rate of 10 ℃ per min. The glass transition temperature, T_g, was taken as the mid-point of the specific heat increment at the glass transition.

Reduced and non-reduced protein patterns of films were determined by SDS-PAGE using 5% stacking gel and 12% running gel according to the method of

Laemmli (1970). Sample solutions were 20 mM Tris-HCl-pH 6.8 including 0.1% (w/v) ground films or barley protein powder, 2% SDS and 8 M urea. In the reduced samples, 5% β-ME was also in presence to cleave disulfides bonds. The 200 μL sample solutions were shook on the vortex for 10 min and then boiled for 1 min. The mixtures were stood for 12 hours and centrifuged at 10,000 × g for 10 min. The 12 μL top layer sample was loaded onto the gel and determined at a constant current of 20 mA. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 for 1 h.

The solubility of protein films was further observed in a 0.2 M Tris-HCl (pH 9.5) buffer with 8 M urea, 3 mM EDTA and 1% SDS to determine the strength of disulfide bonds. The films which contained 0.1 g barley protein were dispersed into 15 mL buffers with a shaking plate for 30 h at room temperature.

3.2.4 Water Vapor Permeability (WVP)

WVP of protein films was tested by the modified cup method according to ASTM E96-92 standard (Mei & Zhao, 2003). Dried films were placed on the top of glass cups containing P_2O_5 powder (RH = 0%) on the bottom. O-ring lids sealed and located around the circumference of the cups' top. All the cups were in a desiccator which contained saturated sodium chloride salt solution (RH = 75%) on the bottom. The water vapor was transmitted through the film into glass cups. The weight gains of the cups were recorded and the WVP ($g\ m^{-1}\ s^{-1}\ Pa^{-1}$) of films was calculated by the following equation:

$$WVP = \Delta m \times x / (A \times \Delta t \times \Delta p) \quad (1)$$

where Δm is the weight gain (g) of glass cups during the time Δt (s), x is the

thickness of films (m), A is the exposed area of films (1.96×10^{-5} m^2), and Δp is the partial water vapor pressure (Pa). The mechanism of moisture absorption (MA) was subsequently investigated. Dried protein films were placed in a desiccator which was set relative humidity as 75% with saturated sodium chloride salt solution at room temperature. Weight change of the films with time was recorded and the absorption curves were described using a mathematical model according to Peleg's method (1988):

$$M(t) = M_0 + t / (k_1 + k_2 t) \quad (2)$$

$$1/\Delta M = k_1 / t + k_2 \quad (3)$$

where M(t) is the moisture content of protein films at time t, and M_0 is the initial moisture content. k_1 and k_2 are constants, where k_1 describes initial moisture absorption rate and k_2 indicates amount of moisture absorbed when the film reaches its equilibrium.

3.2.5 Mechanical Properties

Mechanical property testing of protein films was done using an Instron 5967 universal testing machine (Instron Corp., MA, USA) at a crosshead speed of 50 mm min^{-1} and a gauge length of 50 mm to investigate their tensile strength, percentage of elongation at the break point and Young's modulus. Five dumbbell shape samples with a dimension of 6 mm $\times$ 0.2 mm (width $\times$ thickness) were cut from each protein film. Before testing, the dried samples were allowed to rest for one week in 65% RH. The values of tensile strength, elongation at break and Young's modulus have been calculated using followed equations:

$$\sigma_s = F_s / A, \quad (4)$$

$$\varepsilon_s = l / L - 1, \quad (5)$$

$$E = \sigma / \varepsilon, \quad (6)$$

where σ_s is the tensile strength, F_s is pulling force when the sample breaks and A is the nominal area, i.e., the unreformed cross-section area of the samples. ε_s is the elongation or tensile strain at break, L is the original length and l is the final length of the sample just before it breaks. E is the Young's modulus, which is evaluated in the linear regime of the stress(σ)-strain(ε) relationship, being the slope of the σ-ε curve.

3.2.6 *In vitro* Degradation Essay

To further investigate the potential applications of barley protein films in biomaterial field, their *in vitro* degradation were tested. Films 100-40, 120-40 and 140-40 were used as examples due to their suitable mechanical properties. *In vitro* degradation of the films was carried out in the simulated gastric and intestinal fluids (SGF and SIF), respectively, at the constant temperature (37 ± 2 ˚C). Firstly, the films with a dimension of 20 mm × 15 mm (length × width) were placed into 50 mL, 0.1 M sodium phosphate buffers (pH 2.0) contained 0.1 % pepsin (SGF). After 2 h, the films were washed and placed into 50 mL, 0.1 M sodium phosphate buffers (pH 7.4) with 1.0 % pancreatin (SIF) for another 6 h. Weight change of the films with time was recorded and weight loss was calculated by the following equation:

$$\text{Weight loss} = (W_0 - W_t) / W_0 \times 100\% \quad (7)$$

where W_0 is the dry weight of original films and W_t is the dry weight of protein

films at time t. Meanwhile, the surface and cross-section of original and digested films were observed with a Hitachi X-650 scanning electron microscope (SEM, Hitachi, Japan) at an acceleration voltage of 6 kV. All samples were frozen in liquid nitrogen and snapped immediately, and freeze-dried before SEM observation. Then they were sputtered with gold for 2 min and photographed.

3.2.7 *In vitro* Cytotoxicity

In vitro cytotoxicity of original and digested protein films (2 h in SGF and then 6 h in SIF) was evaluated by MTT assay. Samples were sterilized under UV radiation for 1 h. Caco-2 cells at a density of 5.0×10^3 cells per well were seeded in the 96-well plate and incubated at 37 ℃. On the 2nd d, Caco-2 cells were exposed to original and digested protein films, and control experiment was carried out using only complete growth culture medium. After incubation for another 24 h in incubator (37 ℃, 5% CO_2), 100 L MTT solution was added to each well. After 4 h incubation at 37 ℃, 100 L DMSO was added to dissolve the formazan crystals. The dissolvable solution became homogeneous after about 15 min of shaking, and then was transferred into another 96-well plate. The optical density (OD) was measured at 570 nm with a Microplate Reader Model 550 (BIO-RAD, USA). The cell viable rate was calculated by the following equation (Tian et al, 2010):

$$\text{Viable cell (\%)} = OD_{treated}/OD_{control} \times 100 \quad (5)$$

where $OD_{treated}$ and $OD_{control}$ were obtained in the presence or absence of samples, respectively.

3.2.8 Statistical Analysis

Experimental results were represented as the mean of five batches ±SD. Statistical evaluation was carried out by analysis of variance (ANOVA) followed by multiple comparison tests using Duncan's multiple-range test at the 95% of confidence level. All the analyses were conducted using the statistical software, SAS (SAS Institute, Inc., Cary, NC), with probability of $p < 0.05$ considered significant.

3.3 Results and Discussion

3.3.1 Preparation of Barley Protein Films

Due to the high proportion of nonpolar amino acid residues and high surface hydrophobicity, barley hordein and glutelin are soluble in water only with the presence of alcohol, high concentrations of urea, high concentrations of acid or alkali, or anionic detergents (Zhao, Tian, & Chen, 2010). To avoid using of these reagents, thermo-pressing technique was used to prepare barely protein films in our research. Glycerol has a high boiling point and is protein miscible and nonvolatile, so was chosen as a plasticizer to improve film flexibility and extensibility. Our preliminary results suggested that temperature and glycerol content were the two main variables influencing barley protein film properties. The molding temperature range permitting fusion of film components together was between 100 and 140 ℃. Above the maximum molding temperature of 140 ℃, the film was very dark (protein degradation) regardless of the glycerol content. The glycerol content was varied from 20 to 40% (w/w) for each selected

temperature (100, 120 and 140 ℃), because lower than 20% and higher than 40% glycerol created films either too rigid or too soft to handle, respectively.

3.3.2 Protein Structure Characterizations

A fundamental understanding of the conformation transitions of protein and its sensitivity to processing is critical for the controlled and reproducible preparation of barley protein films. Protein unfolding and aggregation directly determine the molecular interactions, network density, and other properties. FTIR is a powerful

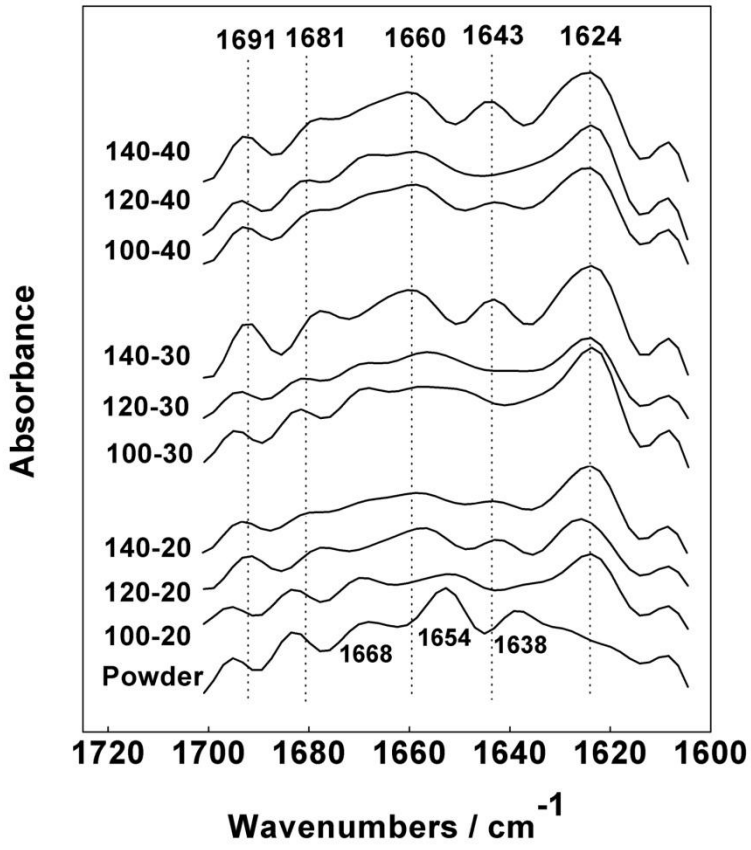

Figure 3.1 Fourier deconvoluted FTIR spectra of barley protein powder and films. The spectra are plotted in the amide I region (1600-1700 cm^{-1}) and offset along the Y axis for clarity.

tool to determine the secondary structure of a protein. Through proper fitting of the amide I band of the original FTIR spectrum of a protein, the conformation of the protein (*i.e.*, helix, sheet or turn) can be obtained. As shown in **Figure 3.1**, barley protein powder possessed six major bands in the amide I region, which have been previously assigned to protein secondary structures (Secundo & Guerrieri, 2005; Gao, et al, 2006): 1694 cm^{-1} (β-sheets/turns), 1683 cm^{-1} (β-sheets), 1668 cm^{-1} (β-turns), 1654 cm^{-1} (α-helix), 1638 cm^{-1} (β-sheets) and 1607 cm^{-1} (vibration of amino acid residues). The strong absorption at 1654 cm^{-1} indicated that α-helix dominated the secondary structures in barley protein powder. The amide I band was dramatically altered upon heating due to the gradual loss of native structures such as α-helix (1654 cm^{-1}), β-sheets (1683 and 1638 cm^{-1}) and β-turns (1668 cm^{-1}), whereas new absorptions arose at 1624 and 1643cm^{-1}. The bands at 1643 cm^{-1} were assigned to random coils. The strong absorption at 1624 cm^{-1} corresponded to intermolecular β-sheet structure which is commonly found in aggregated proteins, especially in heat-denatured proteins (Gilbert et al, 2005). These changes suggested that barley proteins were unfolded and aggregated after heat treatment, and the aggregates were mainly composed of β-sheets. When glycerol content was 20%, proteins in film networks prepared at 100 and 120 ℃ still contained native-like structures such as β-sheets or β-turns, thus the unfolded state of barley protein was not a completely unordered polypeptide chain. When temperature was raised to 140 ℃, the amide I region showed only three peaks at 1683, 1624 and 1607 cm^{-1}, indicating that barley proteins were completely

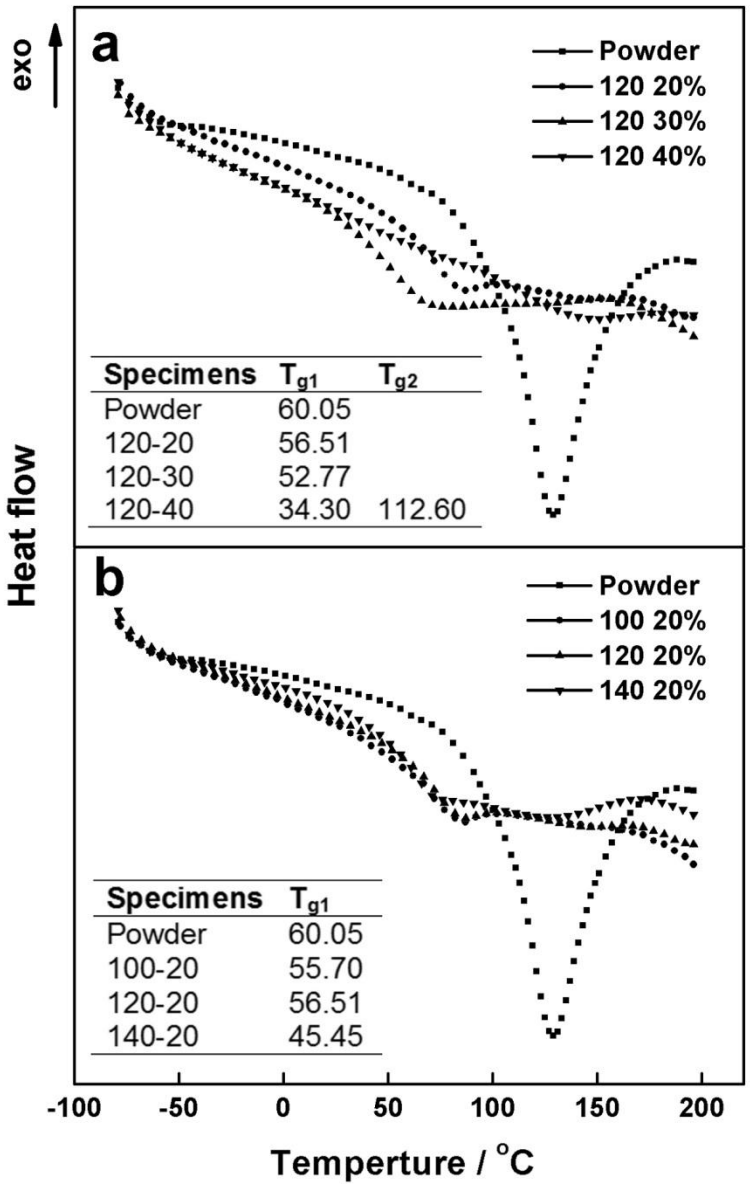

Figure 3.2 (a) DSC heat flow signal of barley protein powder and films prepared Insert table shows the glass transition temperature (T_g) of barley proteins powder and the films obtained by DSC method. at 120 ℃ with different glycerol content (20, 30, 40%); (b) DSC heat flow signal of barley protein powder and films plasticized with 20% glycerol and prepared at different temperature (100, 120, 140 ℃).

denatured. This result was similar with those reported for soy and wheat proteins where thermal processing caused protein unfolding and exposure of active side chains to promote aggregation stabilized by intermolecular interactions, and high temperature facilitates protein denaturation (Wu & Zhang, 2001; Georget & Belton, 2006). On the other hand, glycerol could reduce barley protein denaturation during thermal treatment as many native structures such as β-sheets and β-turns still remained in the spectra when increasing the glycerol content to 30 and 40%. The absorption for β-sheets shifted from 1683 to 1679-1680 cm^{-1}, whereas β-turns changed from 1668 to 1660 cm^{-1}. These shifts suggested the alternation of hydrogen bonding in protein molecular chains in the presence of glycerol. Additionally, absorption corresponding to unordered structures at 1643 cm^{-1} was clearly observed. This protein component did not form aggregates even in denatured status, probably because glycerol could prevent protein aggregation by inhibiting protein unfolding and by binding aggregation-prone regions at protein surface.

DSC spectra of protein powder and films are described in **Figure 3.2**. Barley protein powder showed a large endothermic peak at 125 ℃, corresponding to protein denaturation. This endothermic peak disappeared in protein film samples, which confirmed that the thermo-pressing treatment caused barley protein denaturation. Glass transition temperature (T_g) is an important parameter in the study of synthetic polymers and biopolymers. Generally, T_g is increased by increasing the amount of more rigid chains and bonds, bulky side groups, cross-linking between chains, and degree of crystallinity. It is expected that the

permeation of gas and vapor molecules through a film would be higher above T_g. Compared to the original protein powder, all thermo-pressing treated films possessed lower T_g values than that of untreated barley protein powder (T_g of 60.1 ℃). For films prepared at a fixed temperature, for example at 120 ℃, increasing glycerol content resulted in a decrease of the T_g and a large drop of T_g was observed (from 52.8 to 34.3 ℃) when glycerol content was raised from 30% to 40%. This was attributed to an increase of protein chain mobility and lubrication in the film matrix since the plasticizer could separate protein chains from each other, facilitating chain movement and increasing flexibility (Ghanbarzadeh et al, 2006). It was expected that a higher temperature would improve cross-linking density of the three-dimensional protein network, thus leading to increases in T_g value. However, at the fixed glycerol content of 20%, T_g did not significantly change ($p > 0.05$) when temperature was raised from 100 to 200 ℃, whereas it decreased significantly ($p < 0.05$) from 56.5 to 45.5 ℃ when temperature was increased to 140 ℃. The unfolded state of barley protein was not a completely an unordered polypeptide chain when treated at 100 and 120 ℃ with 20% glycerol, but that treated at 140 ℃ was completely denatured, as reflected by FTIR spectra. A tentative interpretation to explain the observations was that the loss of three-dimensional architecture created free end-chains, increasing free volume and decreasing cohesion, resulting in the decrease of the glass transition temperature. The T_g of polymer-plasticizer mixture also demonstrated plasticizer compatibility with biopolymer. As shown in the insert table in **Figure 3.2**, the films with 20 and 30% glycerol exhibited only one phase transition, indicating a

homogeneous mixture of protein and glycerol remained in the films. However, two glass transitions, assigned to glycerol-rich and protein-rich domains, were found for films plasticized with 40% glycerol. These two different domains were attributed to two kinds of protein chains with relatively high or low compatibility to glycerol (Chen, Zhang, & Cao, 2005). This indicated that barley protein and glycerol were not completely miscible in films plasticized with a high content of glycerol. In general, the plasticizer content had the greatest effect on the T_g of the film, where increasing plasticizer content decreased T_g significantly ($p < 0.05$).

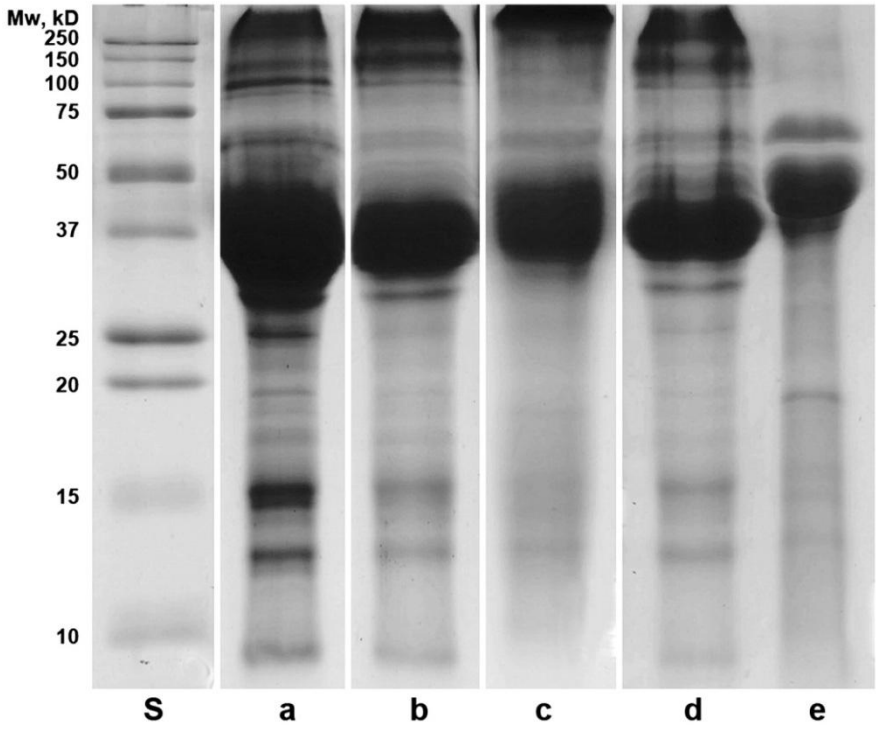

Figure 3.3 Protein patterns of barley protein power and films prepared with and without reducing agent 2-ME: (S) protein marker standard; (a) barley protein powder under reduced condition; (b) film processed at 100 ℃ with 20% glycerol under reduced condition; (c) films processed at 100 ℃ with 20% glycerol under

non-reduced condition; (d) films processed at 140 ℃ with 40% glycerol under reduced condition; (e) films processed at 140 ℃ with 40% glycerol under non-reduced condition.

Figure 3.3 shows the SDS-PAGE patterns of barley protein powder and the films under both reduced and non-reduced conditions (only film 100-20 and 140-40 were selected for demonstration, other films showed a similar SDS-PAGE pattern). Four major subunits of barley protein powder were identified with bands at 80-100, 55-80, 30-40 and <15 kDa corresponding mainly to D, C, B and A hordeins, respectively (Celus, Brijs, & Delcour, 2006). The broad band at 30-50 kDa also contained a glutelin component according to our previous work (Wang et al, 2010). The major bands with molecular weight around 30-50 kDa in films under reduced conditions shifted to higher molecular weight level while under non-reduced conditions (from 30-40 kDa to 40-50 kDa). In addition, high molecular weight components stayed in the stacking gel during SDS-polyacrylamide gel electrophoresis under non-reduced conditions. These phenomena might indicate an association of protein molecular chains during thermo-pressing treatment, probably due to the formation of both intra- and inter-disulfide bonds. The protein solubility of the films was then evaluated using a Tris-HCl (pH 9.5) buffer containing urea, EDTA and SDS (data not shown). These dissociation reagents can break hydrogen bonds, electrostatic and hydrophobic interactions between protein chains, whereas S-S bonds remain, thus the protein solubility will reflect the strength of S-S bonds developed in the film. The barley

protein powder and the films made at 100 ℃ were completely soluble regardless of the glycerol percentage. Interestingly, when processed at 120 ℃, the film with 30% glycerol was almost completely soluble, whereas those plasticized with 20% and 40% glycerol were only partially soluble. Increasing temperature to 140 ℃, all the films were partially soluble, but the solubility of the sample prepared with 30% glycerol was obviously higher than those prepared with 20% and 40% glycerol. The poor solubility of the films prepared at higher temperature could be attributed to enhanced film cross-linking via inter-molecular S-S bonds. In barley proteins, the hordein fraction is comprised of 70-80% sulfur rich B-hordein and the glutelin component also contains sulfur containing cysteine residues (Wang et al, 2010). Thiol-disulfide exchange and thiol oxidation reactions of the cysteine residues were induced during the thermo-pressing treatment, resulting in the formation of intermolecular disulfide bonds necessary to form cross-linked films (McHugh & Krochta, 1994). In this process, heating also altered the protein conformation, and the degree of protein unfolding determined the type and proportion of covalent (S-S bonds) or non-covalent (hydrophobic interactions, ionic and hydrogen bonds) interactions established between protein chains. In general, protein chains can interact more extensively, especially by disulfide bonds, when proteins are denatured. For barley protein films, a more extensive S-S bonding occurred with films prepared at a higher temperature that facilitated barley protein denaturation. The same trend has been reported in wheat gluten and egg white protein (Wang, Wei, Li, Bian, & Zhao, 2009; Plancken, Loey, & Hendrickx, 2005). In these instances, increasing glycerol from 20% to 30%

significantly weakened S-S bonds, explained by the plasticizer preventing protein intermolecular interactions by inhibiting protein unfolding and aggregation. However, the reinforcement of the S-S bonds at a glycerol content of 40% was unexpected by these authors. A decreased followed by an increased S-S bonding trend was also observed in extruded sunflower protein films plasticized by glycerol, but the authors gave no explanation (Rouilly et al, 2006). As shown in DSC result above, films plasticized with 40% glycerol were not completely

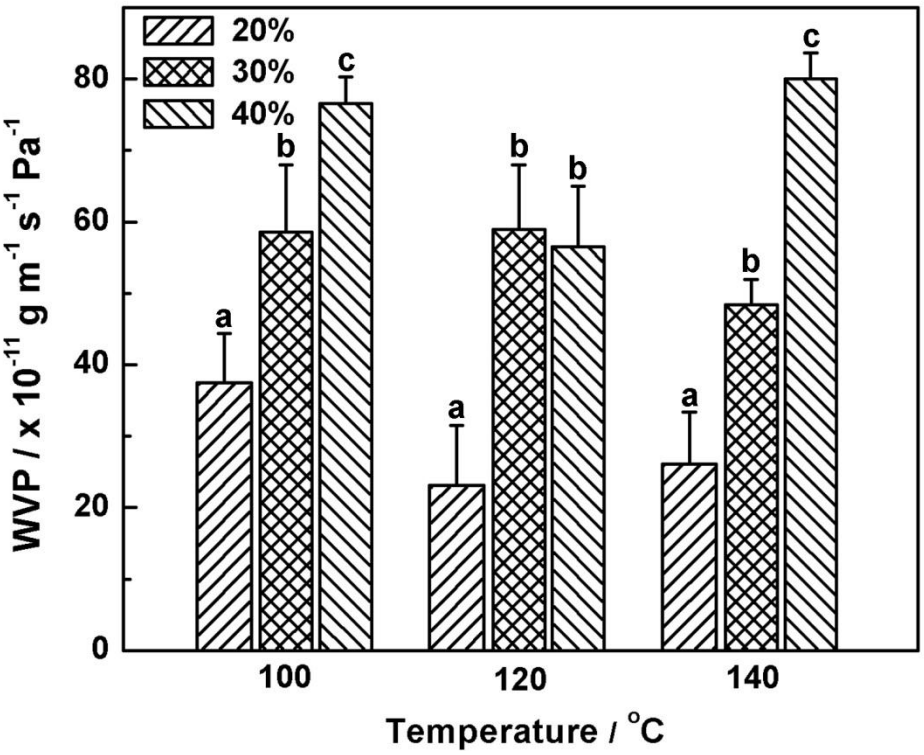

Figure 3.4 Water vapor permeability of barley protein films at 75% relative humidity. Different characters on the top of the column indicate significant difference ($p < 0.05$) due to the glycerol content.

miscible, showing both glycerol-rich and protein-rich domains. It could be

possible that this phase separation phenomenon strengthened S-S bonds in the protein-rich domains, leading to a decreased solubility of this portion of the film in the presence of the dissociation reagents. Covalent disulfide bonds are believed to play a major role in protein film formation, thus are responsible a significant part of films physical properties.

3.3.3 Water vapor permeability

Both physicochemical interactions between water and macromolecule networks on one hand, and the polymer chain relaxation with volume expansion on the other, affect water diffusion. The WVP result obtained at 75% RH is described in **Figure 3.4**. With increase of glycerol from 20% to 40%, films showed 2 to 2.5 fold higher permeability. Glycerol is an effective plasticizer with a high capacity to interact with water, which facilitates its permeation through the film. Additionally, when plasticized with a higher content of glycerol, the molecular structure becomes open and flexible enough to allow the passage of water molecules. The exception was films prepared at 120 ℃, where those plasticized with 30% and 40% glycerol showed almost the same WVP value ($p > 0.05$). This might due to the different degree of protein chain relaxation in these two networks. Films plasticized with 30% glycerol exhibited a low amount of S-S bonds compared to those plasticized with 40% glycerol as shown in the solubility test, thus the corresponding film network might have possessed a higher degree of chain relaxation. However, no clear relationship was observed between the processing temperature and the film permeability. This was different from a previous report for soy protein films where a significant reduction in film WVP

was observed as the heating temperature increased during film curing, since

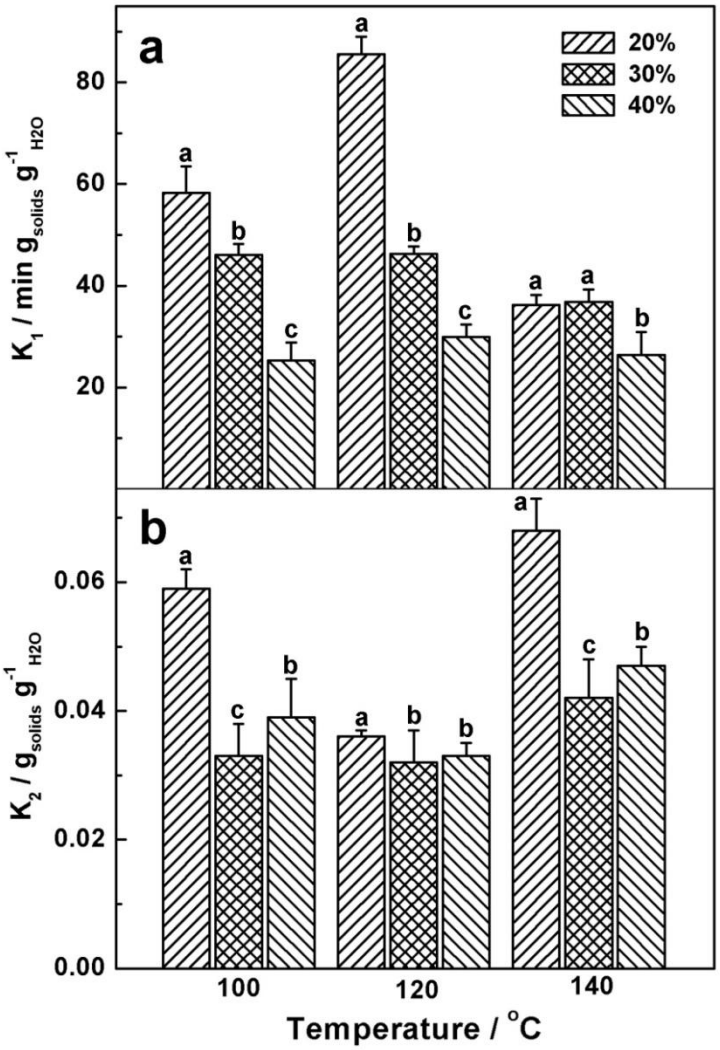

Figure 3.5 Constant values (k_1 and k_2) for moisture absorption curve equation* (Eqn (2)) of barley protein at 75% relative humidity. *All coefficients of MA curve equation were high ($r^2 > 0.99$). Different characters on the top of the column indicate significant difference ($p < 0.05$) due to the glycerol content.

thermal treatments of proteins promoted formation of intra- and intermolecular cross-links of amino acid residues (Kitabatake, Cuq, & Cheftel, 1985). This suggested that the moisture barrier property of the thermo-pressing treated barley

protein film was mainly determined by the glycerol content. Compared to other biopolymer films analyzed under similar conditions, barley protein films possessed the same range of WVP values as wheat gluten ($61.6 \pm 1.3 \times 10^{-11}$ g m^{-1} s^{-1} Pa^{-1}), zein ($11.6 \pm 1.9 \times 10^{-11}$ g m^{-1} s^{-1} Pa^{-1}) (Debeaufort, Martin-Polo, & Voilley, 1993) and cellulose (24.54×10^{-11} g m^{-1} s^{-1} Pa^{-1}) films (Park & Chinnan, 1995), while were much less permeable than gelatin films which exhibited a WVP value of $2.5 \pm 0.2 \times 10^{-9}$ g m^{-1} s^{-1} Pa^{-1} (Bae, Cha, Whiteside, & Park, 2008). For the sake of comparison, Briston reported a WVP value of 7.3-9.7 $\times 10^{-13}$ g m^{-1} s^{-1} Pa^{-1} for low density polyethylene film (25 mm thick at 38 ℃ and 90% RH) (Roy, Gennadios, Weller, & Testin, 2000). Peleg's model is an important tool to select protein-based materials for many applications. Due to different protein network structures, films possess various k_1 and k_2 values (**Figure 3.5**) which indicate the moisture absorption kinetics of films. Lower k_1 and k_2 values indicate a higher initial moisture absorption rate and larger amount of moisture absorbed until the film reach its equilibrium, respectively. At the same temperature, the films with higher glycerol content generally presented a lower k_1 value, confirming that higher glycerol content facilitated water permeation through the film ($p < 0.05$). A higher final absorption of water (lower k_2 value) occurred for the films plasticized with 30% glycerol when prepared at the same temperature. This also reflected the low cross-linking density of the films prepared with 30% glycerol where glycerol was dispersed homogenously into protein networks which maximized the loosening effect, causing the films to trap a relatively higher amount of water inside.

3.3.4 Mechanical Properties

Variations of mechanical properties are related to the molecular mobility and

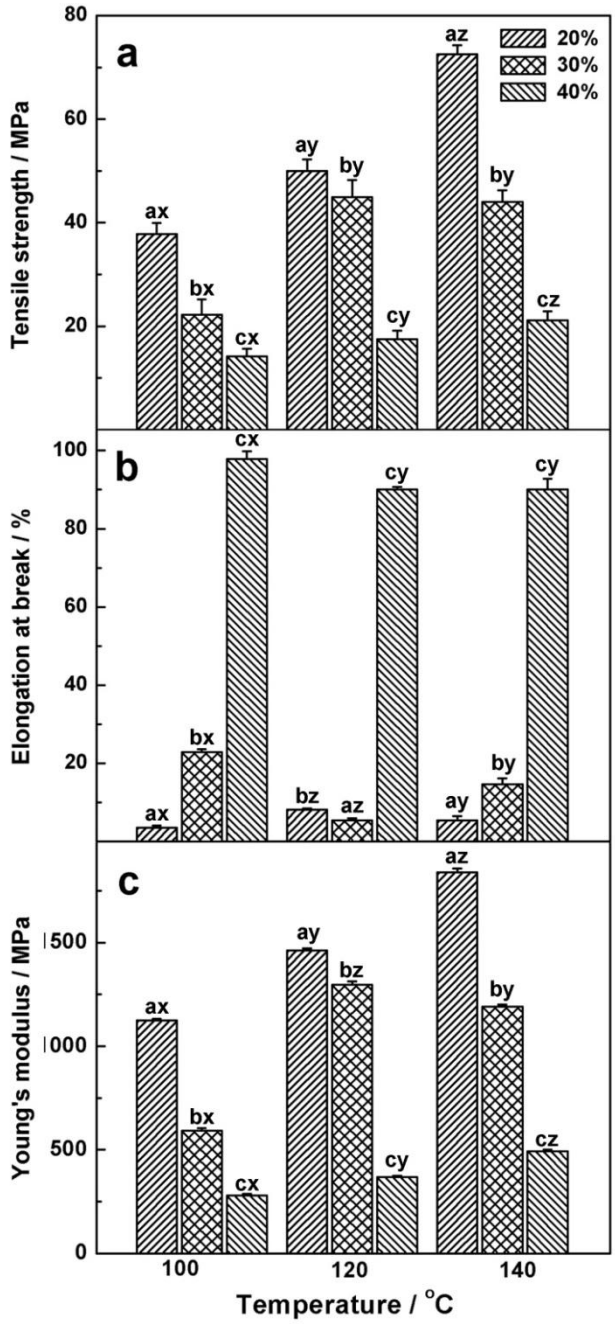

Figure 3.6 Tensile strength (a), percentage of elongation at the break point (b) and

Young's modulus (c) of barley protein films at 65% relative humidity. Different characters (a-c) on the top of the column indicate significant difference ($p < 0.05$) due to the glycerol content. Different characters (x-z) on the top of the column indicate significant difference ($p < 0.05$) due to the processing temperature.

the cross-linking density in the protein network as a result of different processing conditions. As demonstrated in **Figure 3.6**, increasing glycerol content significantly reduced the film tensile strength and Young's modulus (film stiffness), whereas it increased the film elongation percentage ($p < 0.05$). Owing to the three hydroxyl groups present in glycerol, it is expected to be extensively bonded by hydrogen bridges between protein molecules at amine, amide, carboxyl and hydroxyl sites. Additionally, being small in size, glycerol effectively increases the free volume of the system. Thus, increasing the amount of glycerol caused a decrease of tensile strength and Young's modulus, as well as an increase of elongation at "break" due to the fact that glycerol reduced the interactions between protein chains and increased the chain mobility. The impacts of temperature on barley film mechanical properties were most obvious when plasticized with a low amount of glycerol (20%). At 20% glycerol content, film tensile strength and Young's module significantly improved with temperature, increasing from 100 to 140 °C (tensile strength: 37.8 to 72.5 MPa, Young's module: 1123 to 1838 MPa). This was related to an increase in S-S bonds established at a high temperature to significantly enhance the film network cross-linking density. Other non-covalent interactions such as hydrophobic interactions and hydrogen bonds could also be developed between unfolded protein chains

during heating to reinforce the network mechanical strength. Whereas, when plasticized with 40% glycerol, the plasticizer played a more dominant role in regulating barley protein film mechanical properties, thus the impact of temperature became less dramatic (tensile strength: 14.2 to 21.1 MPa, Young's module: 279 to 493 MPa). The above results indicated that both glycerol content and thermo-pressing temperature significantly affected the mechanical properties of barley protein films. In general, barley protein films possess relatively strong mechanical properties. According to previous reports, soy protein and whey protein plasticized with 40% glycerol showed 2.6 and 8 MPa tensile strength as well as 74.5% and 85% elongation at break, respectively (Sothornvit, Olsen, McHugh, & Krochta, 2007; Cunningham, Ogale, Dawson, & Acton, 2000). Films plasticized with 40% glycerol showed very good handling properties; flexible with dramatically improved elongation as well as suitable strength, thus these films were selected for the following *in vitro* test.

3.3.5 *In Vitro* Film Degradation Essay

The degradation of the barley protein films were investigated in simulated gastric fluids (SGF) and simulated intestinal fluids (SIF) with digestive enzymes. The percentage of protein weight loss was used to determine the degree of barley protein degradation. A control experiment verified that almost no protein was released from barley protein films without digestive enzymes in pH 2.0 and 7.4 buffers, indicating that the integrity of the films was well maintained. Thus only the degradation profiles in SGF with pepsin and SIF with pancreatin were described in **Figure 3.7**. In SGF, barley protein films were degraded slowly; only

around 20% protein was released after 2 h of the test for films prepared at 120 and 140 ℃, whereas around 30% protein was released when prepared at 100 ℃. Other protein films are degraded much faster in SGF, for example, 50-60% protein was broken down after 2 h of the test in previous research (Chen, Remondetto, Rouabhia, & Subirade, 2008). When transferred into SIF, the protein degradation increased significantly and the films were steadily degraded during another 6 h of the test; 94%-99% protein was detected in the release medium after

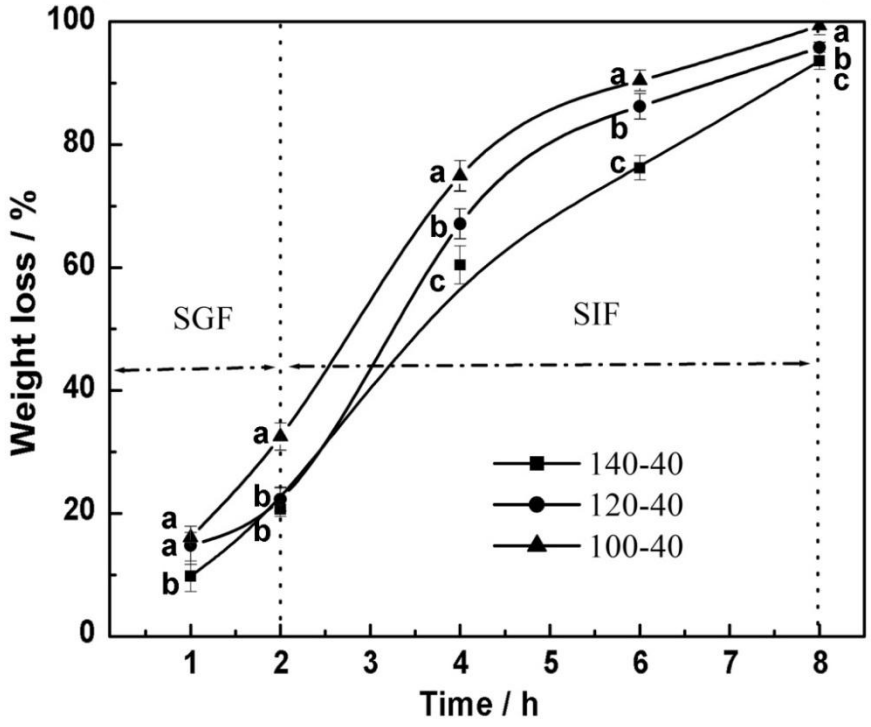

Figure 3.7 Degradation of barley protein films in SGF for 2 h, then in SIF for another 6 h. Different characters above or below the curve indicate significant difference ($p < 0.05$) due to the processing temperature.

6 h. The slow matrix degradation of barley protein films in SGF could be attributed to a high proportion of proline amino acid (~17%) residues in barley protein chains known to be pepsin resistant. In contrast, SIF barley protein networks were readily broken down by pancreatin, a mixture of several digestive enzymes containing both endo- and exo-peptidases. The films prepared at 100 and 120 ℃ had first-order degradation rates (r^2 > 0.999). On the other hand, the film prepared at 140 ℃ had a near zero-order degradation rate (r^2 = 0.991). Film degradation rates decreased significantly (p < 0.05) when the processing

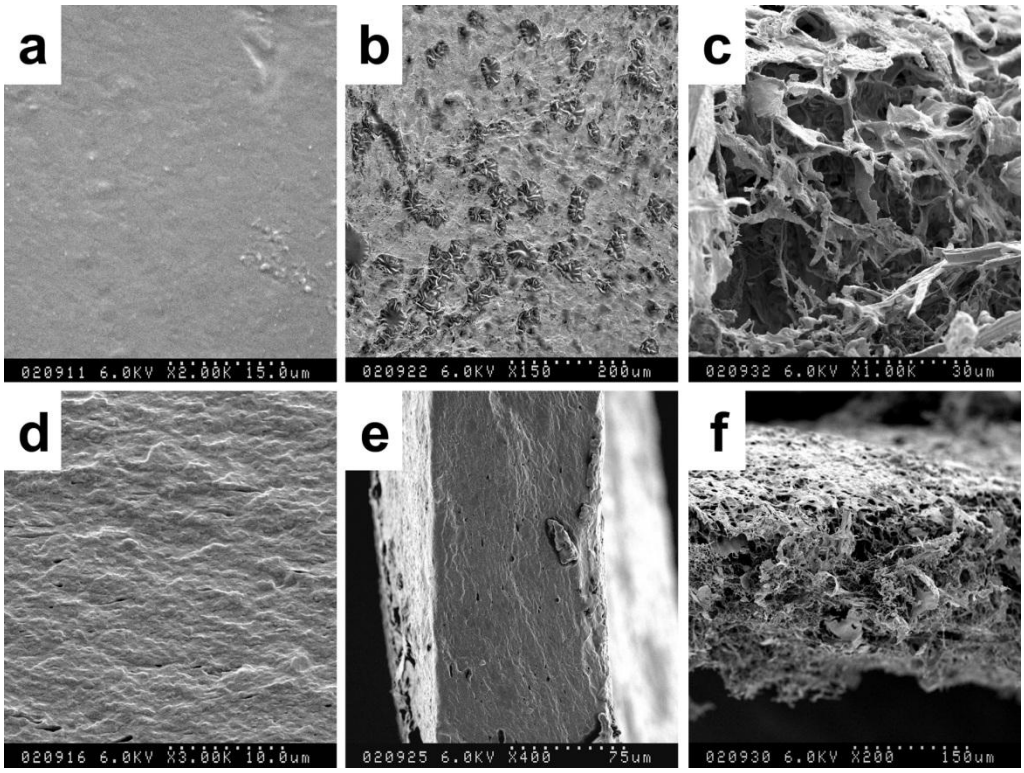

Figure 3.8 Scanning electron microscopy micrographs of surfaces (a, b, c) and cross-sections (d, e, f) of barley protein films obtained at different magnifications: (a, d) original films, (b, e) after 2 h digestion in SGF, (c, f) after 2 h in SGF and then 6 h digestion in SIF.

temperature increased from 100 to 140 ℃. This reduced degradation was caused by enhanced film cross-linking density that slowed the penetration of digestive enzymes in the film networks. The protein film surface and cross-section morphologies after incubating in SGF and SIF were then observed using a SEM. As shown in **Figure 3.8**, the barley protein film showed a very smooth surface (a) and a dense cross-section (d) without any digestion. After incubation in SGF for

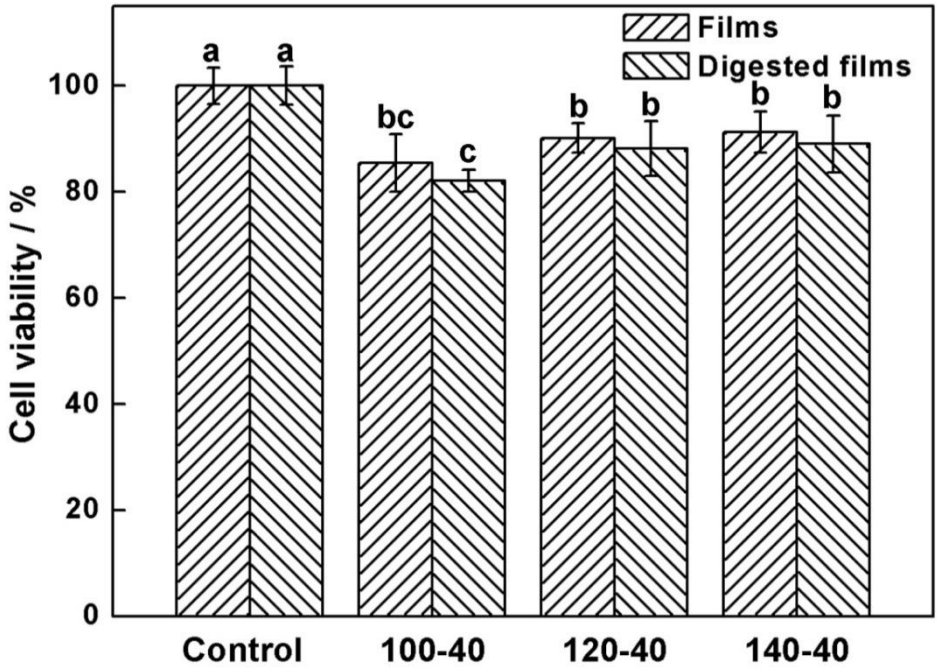

Figure 3.9 Cytotoxicity of barley protein films and their digested residues (100-40, 120-40, 140-40) relative to the nontoxic control (growth culture medium) after 24 h. Different characters on the top of the column indicate significant difference ($p < 0.05$).

2h, small pores appeared at the film surface (b) and obvious degradation was observed on the two outer layers of the film, whereas the inside of the film still possessed a dense structure (e). Such morphology indicated that barley protein film degradation probably underwent a surface erosion mechanism where degradation occurred from surface to the center. This was likely due to the hydrophobic nature of barley protein, rendering the film networks less permeable to release medium and digestive enzymes. Large pores were observed at the barley protein film surface and at the cross-section perspective (c and f) after 6h of incubation in SIF with pancreatin, confirming deep degradation occurred in the film networks. The above results suggested that barley protein films were resistant in gastric juice, whereas could be broken down completely in the intestinal juice.

3.3.6 *In Vitro* Toxicity Essay

The Caco-2 intestinal cell line is known to possess many morphological and enzymatic features typical of normal human enterocytes. To clarify the biocompatibility of barley protein films and their digested compounds, the effect of original and digested films on Caco-2 cell growth and proliferation was determined by MTT cytotoxicity assay. As shown in **Figure 3.9**, the viability of Caco-2 cells was about 85.5%, 90.1% and 91.3% for original 100-40, 120-40 and 140-40 films, respectively. After the digestion in SGF for 2h, then in SIF for 8 h, the resulted mixtures did not exhibit obvious cytotoxicity ($p > 0.05$). This indicated that these protein films had low cytotoxicity and good biocompatibility.

3.4 Conclusion

In summary, this research introduced barley protein film preparation by a

thermo-pressing technique. Both protein conformation and interactions within the film networks, and consequently the film properties, could be modulated by changing processing temperature and glycerol content. The prepared films possessed good moisture barrier and mechanical properties. The *in vitro* degradation experiments demonstrated these films were resistant in a gastric juice environment, whereas could be broken down completely in an intestinal juice environment. Such characteristics were especially relevant for developing delivery systems of gastric-sensitive bioactive compounds that could increase the likelihood of these compounds passing intact and being released at the intestine. The films showed low cytotoxicity in Caco-2 cells, demonstrated they were safe candidates for developing further edible delivery systems.

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Chapter 4 General Discussion and Conclusions

4.1 Summary of the Current Research

Base on my current research results of barley proteins, two new applications of the protein have been developed.

Antioxidant peptides

In antioxidant peptides study, barley glutelin was hydrolyzed with Alcalase and Flavourzyme. Alcalase hydrolysates (AH) demonstrated significantly higher antioxidant capacity than those treated by flavourzyme in four selected assays, radical scavenging capacity (superoxide anion/hydroxyl radicals), Fe^{2+}-chelating effect and reducing power. Ultra-filtration and reversed-phase chromatography (RP-HPLC) have been used to separate AH fractions. Assessment of the fractions indicated that the large-sized peptides (M_w > 10 kDa) possessed stronger $DPPH^{\cdot}$ scavenging activity and reducing power, whereas small-sized peptides (M_w < 1 kDa) were closely related to Fe^{2+}-chelating activity and $OH^{\cdot}$ radical scavenging effect. The hydrophobic fraction contributed more to Fe^{2+} chelating and $OH^{\cdot}$ radical scavenging activity. Four antioxidant peptides were identified using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) including: Gln-Lys-Pro-Phe-Pro-Gln-Gln-Pro-Pro-Phe, Pro-Gln-Ile-Pro-Glu-Gln-Phe, Leu-Arg-Thr-Leu-Pro-Met and Ser-Val-Asn-Val-Pro-Leu. Antioxidant activities of the AH were then compared to selected positive controls. AH had superior Fe^{2+}-chelating activity (93.0% at 1.0 mg/ml) and strong $DPPH^{\cdot}$/hydroxyl radical scavenging activity (61.9% and 70.4%, respectively at 1.0 mg/ml). Also, the reducing power was

dramatically improved (0.288 at 1.0 mg/ml) simply by separating large-sized peptides from barley glutelin hydrolysates. Thus hydrolyzed barley glutelin is a potential plant protein source of antioxidant peptides for food and nutraceutical applications.

Protein-based films

Barley protein films were prepared by a thermo-compression processing. The combined effects of heating temperatures and plasticizer amount on protein confirmation and their interactions in film network as well as the film barrier and mechanical properties were systematically investigated. Fourier transform infrared (FTIR), Differential scanning calorimetry (DSC), SDS-Page and protein solubility results indicated that heating facilitated protein unfolding, aggregation and development of covalent disulfide bonds, thus enhanced film mechanical strength. Whereas plasticizer could prevent barley protein denaturation and the protein chain interactions to certain extent that led to decreased moisture barrier capacity and tensile strength, but significantly improved film flexibility. Finally, the film which contented 40% glycerol and was molded at 120˚C has been selected as a drug controlled release system for a further degradation analyses. *In vitro* degradation experiment demonstrated these films are resistant in the gastric juice, whereas can be broken down completely in the intestinal juice. These films also showed low cytotoxicity in Caco-2 cells. Such characteristics are interesting for developing delivery systems of gastric-sensitive bioactive compounds that can increase the likelihood of these compounds passing intact into the intestine for release.

4.2 Discussion and Conclusion

Barley proteins as by-products in beer brewing industry and low-value animal feeding crop compounds have many feasibilities to achieve value-added applications. According to some previous research, Zhao et al (2010) found that low degree of deamidation significantly improved solubility, emulsifying and foaming properties of barley hordein and glutelin. Barley proteins have also been suggested to be used as microcapsules coating materials for lipophilic bioactive compounds (fish oil, β-carotene) by a pre-emulsifying process followed by a high pressure homogenization treatment (Wang, Tian, & Chen, 2011). Due to the relative hydrophobic nature and intensive interactions of barley proteins, there are more potential applications would be developed.

According to the antioxidant peptides study, the enzymatic hydrolysis of barley glutelin can produce multifunctional antioxidant peptides, acting as scavenging free radicals and chelating transitional metal ions. The antioxidant capacity of proteins can be increased at an appropriate hydrolysis degree; peptides possess stronger capacity than intact proteins. The antioxidant activity is related to unique properties contributed by their chemical composition and physical properties. As one of my further research works, understanding the relationship between peptides composition and antioxidant activity can lead to the development of new class of high effective, multifunctional, generally recognized as safe (GRAS) antioxidants for many industrial applications (Elias, Kellerby, Decker, 2008). Inhibition of lipid oxidation using barley protein peptides may by also involved in my future work.

Biodegradable and edible films from barley proteins have been developed by compress molding technique. It showed relatively high mechanical properties and moisture barrier property than other protein-based films (Bae et al, 2008; Debeaufort, Martin-Polo, & Voilley, 1993; Park, & Chinnan, 1995; Sothornvit, Olsen, McHugh, & Krochta, 2007). Barley protein-based films are very promising materials for preserving and improving food and drug quality. A point worth emphasizing is that the films are also considered as a drug or nutraceutical enteric controlled-release system. In my next step, processing factors included temperature and plasticizer will be more completely studied.

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