University of Alberta

Improvement of functionality of barley protein by deamidation

by

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Dedication

This thesis is dedicated to my beloved mother and father.

Abstract

In this study, the deamidation is involved to modify the structure of barley proteins in terms of prolamin and glutelin in order to improve the functional properties of protein. A wide range of deamidation degrees (0.1% to 45%) were prepared using alkaline method. The results suggested that the optimal deamidation degree of barley prolamin is around 2.4-4.7%, where the solubility, emulsifying and foaming properties of prolamin were significantly improved at both acidic and neutral pHs. The optimal deamidation degree for glutelin is around 2.2 to 5.6%, where deamidated glutelin demonstrated markedly improved solubility at both acidic and neutral pHs. Glutelin performed strong tendency to form aggregates with spherical shape and very large molecular weight. These aggregates are important in stabilizing the emulsions at a broad range of deamidation degree (5.6-43%). These results suggest that barley protein would be an excellent candidate to develop as an emulsifying and foaming ingredient.

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I would like to dedicate this thesis to my parents Baosheng Zhao and Aiping Lu.

Table of Content

List of Tables

List of Figures

List of Abbreviations

ANS	1-anilinonaphthalene-8-sulfonic acid
CD	Circular Dichroism
DD	Deamidation Degree
DH	Degree of Hydrolysis
DTT	Dithiothreitol
EAI	Emulsion Activity Index
EC	Emulsion Capacity
ESI	Emulsion Stability Index
ES	Emulsion Stability
FC	Foaming Capacity
FI	Fluorescence intensity
FS	Foaming Stability
FTIR	Fourier transform infrared spectroscopy
Gln	Glutamine
IEP	Isoelectric Point
kDa	Kilo Dolton
Mw	Molecule Weight
OPA	o-phthaldialdehyde
Phe	Phenylalanine
Pro	Proline
SDS-PAGE	Sodium dodecyl sulphate gel electrophoresis
SEC	Size Exclusion Chromatography
TGM	Transglutaminase

Chapter 1 Introduction and literature review

1.1 Overview of barley

Barley cultivation. Barley is the fourth most important cereal in the world in terms of annual production, after wheat, rice and corn [1]. The geographical distribution of barley cultivation reveals a significant imbalance (Table 1-1) [2]. Europe and Canada are the two major contributors, whereas barley cultivation in the other continents is very limited [3]. Alberta barley producers grow more than five million tons of barley per year, which is over half of the total amount of barley grown in the country [4]. Arid and drought-inflicted climate as well as the salinity-prone soil conditions in Europe and Canada are suitable for barley cultivation [5].

Table 1-1. World barley production by country 2008-2010 (Million Tons) [4]

Country	2008	2009	2010
EU-27	57,461	65,554	61,462
Russia	15,650	23,100	18,000
Canada	10,984	11,781	9,200
Australia	7,191	7,000	7,800
Ukraine	6,000	12,600	12,000
Turkey	6,000	5,600	6,000
Others	9,282	7,827	8,832
United States	4,575	5,230	4,951
World Total	133,001	153,938	147,245

Application of barley. Unlike major cereal food staples such as wheat and rice, barley is listed in the category of "coarse grains", which means that a large proportion of barley is used as feed grain [6]. Another major application of barley is in the malting industry. Recently, however, barley has attracted increasing attention as a human food since it is enriched with dietary fiber, particularly beta-glucan which has been shown to control diabetes, to reduce cholesterol and to decrease the risk of colorectal cancer and coronary heart disease [7]. Unlike barley starch and beta-glucan, which were studied and applied widely, barley protein was neglected.

Structure of barley grain. Figure 1-1 shows the structure of cultivated barley grains. Most cultivated barleys are covered by a caryopsis and husks, which consist of two membranous sheets that enclose the caryopsis completely. In some countries, huskless barley is also grown. The caryopsis covers the aleurone layer, endosperm and embryo. The embryo and the endosperm store carbohydrates (mainly starch) and proteins that support the initial growth of the germinating embryo [8]. The main component in endosperm is starchy endosperm, which is a storage tissue surrounded by a non-starch cell layer called aleurone. Endosperm cell walls are composed of a cellulose skeleton impregnated with soluble and insoluble arabinoxylans and β-glucans. The husk constitutes 7% to

25% of the grain, while the embryo and endosperm constitute 3% and 77% of the grain respectively [9].

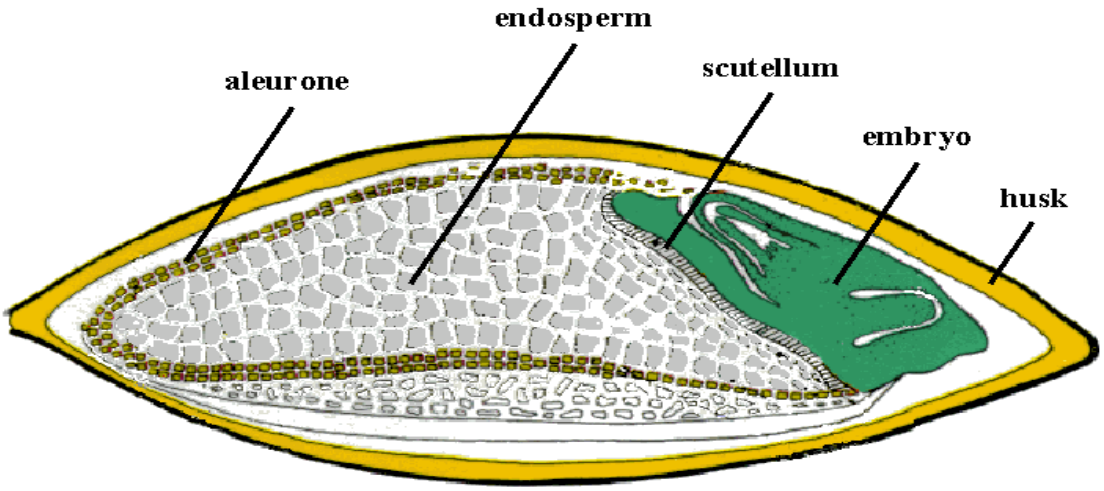

Figure 1-1. the structure of barley grain [10].

1.2 Barley protein

1.2.1 B, C, D, and γ hordein

Barley protein can be classified into B, C, D, γ hordein and small molecule proteins (albumin and globulin) according to electrophoresis mobility [11]. The amino acids composition of into B, C, D, and γ hordein was listed in Table 1-2 [12]. The highest content of amino acids is glutamate and glutamine (30%), followed by proline. Furthermore, the content of no-polar or polar but non-

charged amino acids, such as threonine, methionine and tyrosine, are very high; this may result in low solubility in water.

Table 1-2. Amino acid composition (mol %) of D-, C-, B- and γ- hordein [12-13]

Amino Acid	D	C	B	γ
Alanine	3.2	1.5	2.6	2.1
Arginine	1.6	0.9	2.6	1.8
Aspartic acid[a]	1.5	1.5	0.7	2.4
Cystine	1.5	0	2.9	3.5
Glutamic acid[b]	28.0	37.0	30.0	28.0
Glycine	15.7	0.6	2.9	3.1
Histidine	3.0	0.6	1.5	1.4
Isoleucine	0.7	3.4	4.4	3.8
Leucine	4.1	8.6	8.0	7.0
Lysine	1.2	0.9	0.7	1.8
Methionine	0.4	0.0	1.1	1.8
Phenylalanine	1.3	7.7	4.7	5.6
Proline	10.5	29.1	19.4	16.8
Serine	10.5	2.5	4.7	5.6
Threonine	7.3	1.2	2.2	3.1
Trptophanian	1.2	0.6	0.7	0.7
Tyrosine	4.2	1.8	2.6	2.1
Valine	4.1	0.3	6.2	7.3

[a] Includes Asparagine
[b] Includes Glutamine

B and γ hordeins are sulfur-rich fractions while C hordein is sulfur-poor. D hordein is the highest molecular weight fraction (Figure 1-2). B and C are the two major fractions accounting for 70-80% and 10-20% of prolamin, respectively, while D and γ make up less than 5% of prolamin [14]. Among the hordein groups, the C hordein has been intensively studied. The C hordein has a mixed structure and consists of a repeated octapeptide: Pro-Gln-Gln-Pro-Phe-Pro-Gln-Gln. The conformation of the octapeptide can alternate between poly-L-proline II-like and

BetaI/III turn structures; this process can be triggered by the temperature and solvents [15]. A negative correlation was founded, between protein content and malting quality [16]. B- and D- hordein form aggregates which is linked by thiol groups; it may adversely affect the extraction of starch during the malting procedure [17].

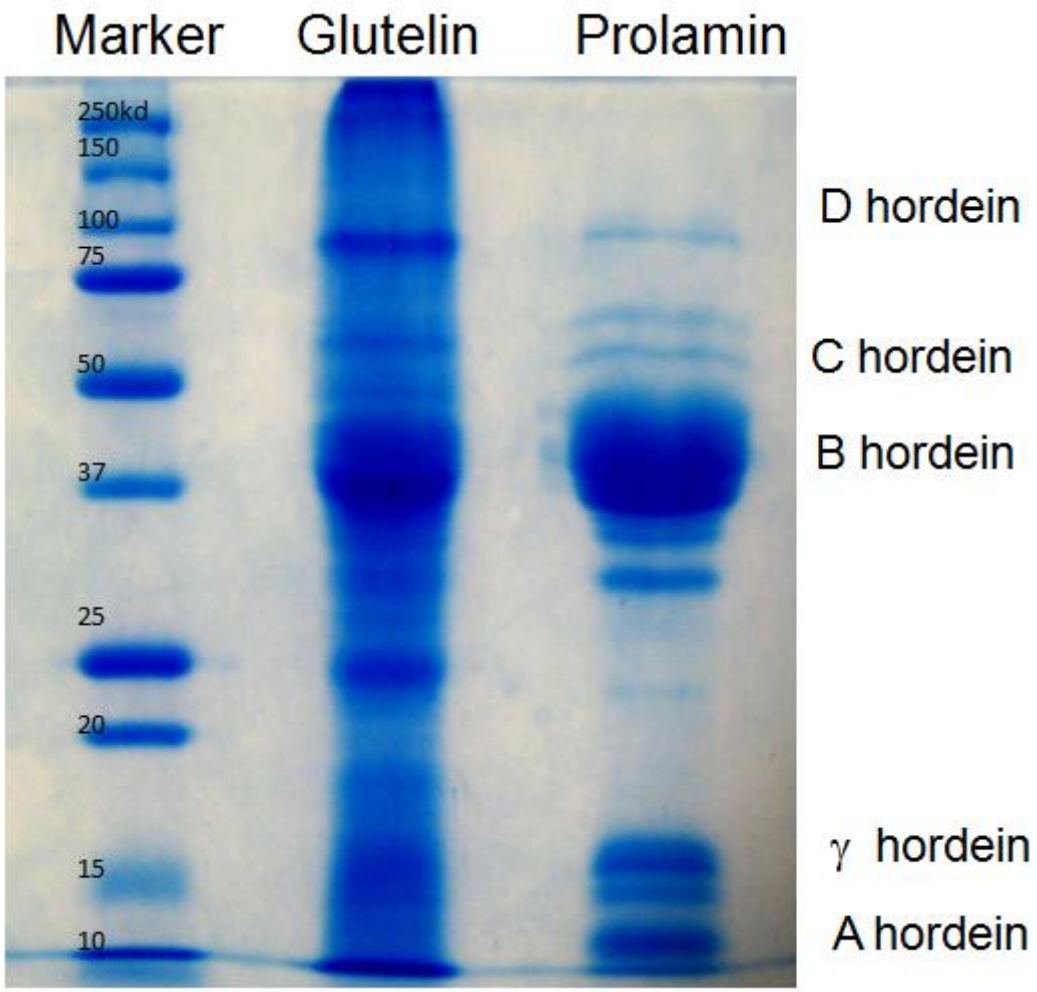

Figure 1-2. SDS-PAGE patterns of barley protein

1.2.2 Albumin, globulin, prolamin and glutelin

According to the Osborne method, barley protein can be classified into albumin, globulin, prolamin and glutelin. Barley albumin and globulin, soluble in water and dilute salt solutions respectively, only constitute about 15-30% of barley protein [18-19]. Prolamin, the alcohol-soluble protein, is the main endosperm storage protein (~45%) [20-21]. Glutelin, an alkali-soluble protein, possesses 40-45% total protein. Glutelin contain B and D hordein and other proteins that have not been extracted in the previous fraction (albumins, globulins, prolaminms) [22-24]. In fact the process of extracting and separating of glutelin can influence the composition of glutelin [25]. Due to the high content of prolamin and glutelin in barley grain, this thesis focuses on these two proteins.

1.2.3 solubility of barley protein

To some extent, the most critical criterion governing the usefulness and functional application of food proteins is protein solubility - the interaction between protein and water. This interaction may affect applications that depend on water sorption, such as, dough formation, gelation, emulsifying and foaming properties [26]. The solubility of food proteins depends on inherent properties such as the tertiary and

quaternary structures of protein, size, composition, surface charge as well as environmental conditions.

The solubility of barley protein was studied. Solubility values were higher in aqueous solution below pH2 and above pH 11(solubility = 70%). Minimum solubility (about 5%) was observed around pH6, which is close to the protein isoelectric point [27]. The effect of NaCl on the solubility of barley protein was also investigated. NaCl could decrease the protein solubility at all pH values [11]. Furthermore, the decreasing solubility of barley protein was more significant in acdic solution than in alkali solution. This could be explained by the fact that at low pH value, carboxyl groups in protein are protonated and protein is positive charged. The negatively charged Cl^- in NaCl may interact with positively charged proteins, resulting in lower repulsion force, higher hydrophobic interactions and lower solubility [28]. The authors conclude that the increased ionic strength is adverse to protein solubility.

At present, plant proteins are more and more widely used in food applications due to their functional properties, including solubility, water and oil holding capacity, emulsifying and foaming properties and other functionalities [11]. The performance of functional properties is closely related to the protein amino acid compositions and confirmations as well as to their interactions with other components in food systems [29]. Compared with other functionalities, protein emulsifying and foaming properties drew the most attention because numerous

food products are related to them; these include ice cream, yogurt, salad dressing, coffee whitener and so forth.

My preliminary research indicated that barley protein has strong emulsifying and foaming properties. Thus, this study will focus on the emulsifying and foaming properties of barley prolamin and glutelin.

1.3 Emulsion properties of proteins

Emulsions, dispersed immiscible droplets within another liquid, are stabilized by surface active compounds, such as proteins, lecithin and monoglyceride [30]. Emulsion plays an important role in the food system since it is responsible for controlling the physical properties, flavor and stability of food products. Moreover, proper emulsifiers could reduce the amount of fat in a product without adversely affecting the product's texture and sensory properties [31]. Many foods, such as comminuted meat field, coffee whiteners, milk type beverages and mayonnaise, are emulsions [32]. Emulsion capacity and emulsion stability are used to characterize emulsifying properties.

Emulsion capacity (EC) was defined as the amount of oil emulsified by a certain unit of sample at the point of emulsion collapse [33-34]. The measurement was carried out by analyzing the maximum amount of oil that the mixture would

emulsify without losing its emulsion characteristics. EC depends on the ability of the protein to decrease the surface tension at the oil/water interface and to form the adsorption films around oil droplets [34].

Emulsion stability (ES) is the ability of the emulsion to keep stable and without separation under conditions of processing and storage (Figure 1-3). ES depends to a large extent on interparticle forces, which can drive aggregation formation and determine the phase behavior of emulsions [35].

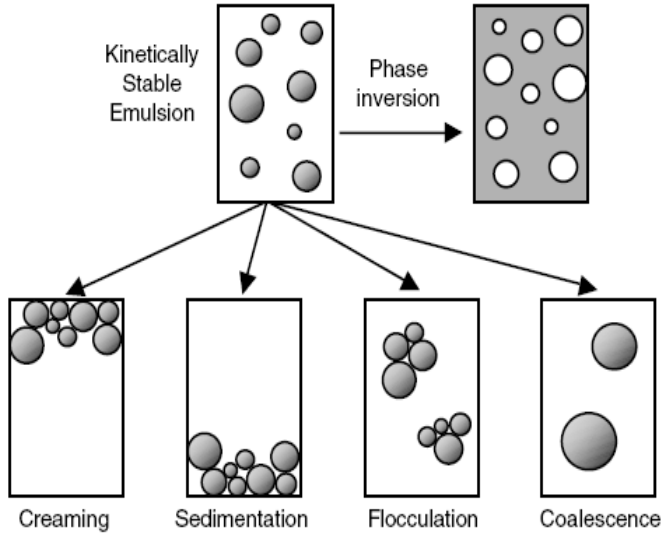

Figure 1-3. Physical mechanism of unstable emulsion [36]

1.3.1 The mechanism of emulsion formation

Emulsion systems are thermodynamically unstable, whereas, in the presence of emulsifiers (such as proteins) this system is stable for a reasonable period. Most of the proteins are amphiphilic molecules, which allow protein to adsorb into the water-oil interface to reduce interfacial tension and form thick layers which may prevent the coalescence or flocculation of droplets [36]. This procedure is thermodynamically favorable due to the hydrophobic residues on the protein surface. These residues drive the protein to move towards the oil surface, after which the protein structure is rearranged at the interface [37]. The adsorbed protein structure takes a state that lies between the native state and fully denatured state and is sometimes referred to as the molten globule state. Nevertheless, in spite of the alternation of the adsorbed protein conformation, most of the adsorbed proteins still maintain a well organized structure [38]. The adsorbed protein is capable of interacting with the protein in a solution depending on the chemical environment. The protein at the interface can also interact with itself [39].

1.3.2 Impact of protein structure on emulsion properties

The factors influencing the emulsion properties of protein products are the inner characteristics of protein and their environmental conditions. The function and

characteristics of the protein structure include interfacial tension, interfacial rheology, electrostatic interaction, surface hydrophobicity and molecular conformation and flexibility.

Electrostatic interaction. Electrostatic interaction determines emulsion properties since the droplets have the same electrical charge in the same system; therefore, the electrostatic interaction is repulsive. Hence, it plays a major role in preventing the coalescence and aggregation of droplets. The electrostatic interaction in the emulsion is influenced by the droplet surface and ionic composition in the surrounding aqueous medium [40-41]. pH value may change the surface charge, and hence may affect emulsion properties. For example, when the pH value is near the isoelectric point, the protein has low repulsion force, resulting in emulsion droplet aggregation and low emulsion stability. At higher or lower pH value, the protein will be charged, leading to strong repulsive force between emulsion droplets and higher emulsion stability [42]. In addition, ionic strength is another important parameter for electrostatic interaction. Certain ionic compounds can decrease the repulsive force between droplets by reducing the thickness of the electrical double layer at the oil/water interface; hence the emulsion droplet will tend to aggregate, leading to low emulsion stability. By contrast, increasing ionic concentration may increase the amount of loading proteins, leading to higher emulsion capacity, presumably because the

electrostatic repulsions between adsorbed and non adsorbed proteins was reduced [43-44].

Surface hydrophobicity. Emulsifying capacity correlates to the surface hydrophobicity in many proteins, such as soy, canola and sunflower proteins [45-46]. The surface hydrophobicity affects the equilibrium between protein-solvent interactions (hydrophilic) and protein-protein interactions (hydrophobic) and hence emulsion properties. Surface hydrophobicity is influenced by environmental conditions, such as pH value, ionic strength and temperature. For instance, at the isoelectric point, the protein-protein interactions are so strong that the hydrophobic groups are buried inside; thus the interaction between protein residues and the oil phase to form the emulsion is prevented [42]. Hence, unstable emulsion is usually found at the IEP. Ionic strength can alter the surface hydrophobicity by influencing the structural arrangement of water molecules. Therefore, the ionic strength can influence emulsion capacity [47]. High temperature is another important factor since the high temperature may enhance hydrophobic interaction and therefore affect the emulsion capacity [48].

Molecular conformation and flexibility. Protein can exist in a number of different conformations, which allow such macromolecules to adopt a conformation at the lowest free energy under the prevailing environmental conditions [49]. Generally, a helical conformation is preferable. This could be

related to the fact that the helix allows a molecule to maximize the number of energetically favorable inter-molecular and intra-molecular interactions, while minimizing the number of energetically unfavorable ones [50]. Protein flexibility is another important factor in determining the emulsion properties. A flexible molecule allows the protein to change the structure as it adsorb at the water/oil and water/air interface to form a stable film. Thus the flexible protein is preferable for emulsion properties. In fact, according to current understanding, both the conformation and flexibility are highly dependent on the environmental conditions; this is particularly important in understanding and predicting the behavior of many ingredients in food emulsions [51-53]. For example, increasing the temperature could lead to partial unfold of protein which can increase the amount of surface load protein. As a result, emulsion capacity and emulsion stability will be increased [54].

Interfacial tension. Interfacial tension is the imbalance of molecular interactions between molecules adsorbed at the interface. It can affect the droplet size of the emulsion and emulsion stability [52]. Protein can increase the system stability by minimizing the thermodynamically unfavorable contact between the two molecules (water and oil) and hence decreasing the interfacial tension.

Interfacial rheology. Interfacial rheology influences the formation, stability and texture of emulsion [55]. In general, most of the proteins adsorbed at the interface tend to form a high interfacial viscous or elastic membrane by extensive

intermingling or cross-linking; thus the emulsion may remain stable for a long time [56].

1.3.3 Emulsion properties of barley protein

According to previous research in our lab, barley protein has high emulsifying capacities but it need to be dissolved at pH 11 before emulsion formation [57]. This procedure limited the usage of barley protein in the industry. The high emulsifying capacity of barley protein is also reported by other authors. Mohamed et al. reported that, compared with defatted and low fat soy flours, the emulsifying capacity of barley protein is 4-7 times higher, whereas the emulsifying stability is similar to others [58]. In addition, Bilgi and Celik reported a positive correlation between the solubility and emulsifying properties (EC and ES) of barley proteins [11].The effect of pH on emulsifying properties was also studied. The lowest EC and ES were at pH6, which is close to the isoelectric pH of barley proteins; whereas the emulsifying properties improved at pH values below and above pH6 [11]. This could be because at the isoelectric point, the protein-protein interaction is increased, leading to low surface charge and low solubility. Increased pH results in higher solubility. The high solubility will increase the protein concentration at the interface, and thus enhance the formation of interfacial films and improve emulsion properties.

1.4 Foaming properties of proteins

The most common examples of food foams are whipped cream, ice cream, aerated desserts and mousses. In these foams, which are thermodynamically unstable, gas is maintained as a distinct dispersed phase in a liquid matrix. Foaming capacity and foaming stability are used to characterize foaming properties [59].

Foaming capacity FC is determined by the speed of the protein adsorption and protein rearrangement at the air-water interface to reduce the surface tension [60]. Generally, the foaming capacity of protein increases with an increase in the protein concentration in solution. Once the interface is saturated by proteins, the foaming capacity is at a maximum. This property corresponds to protein surface activity.

Foaming stability FS is another important property for food foam since it can affect the quality of the product. Foam stability reflects a balance between force within the films and the other forces. Drainage, gravity effects, hydrostatic phenomena, Van der Waals and electric attractive force are destabilizing [61].

1.4. 1 The mechanism of foam formation

The diffusion of proteins from solution to the interface is thermodynamically favorable because the conformational and hydration energy of the protein may be lost at the interface [62]. Hydrophobic segments tend to adsorb at the air water interface easily with change or loss in tertiary structures. In the initial stage, the adsorption rate is diffusion controlled. In the second stage, the protein penetrates and creates space in the existing films, and rearranges its structure at the surface. This procedure is important in determining the reaction rate [63]. In the penetration stage or rearrangement stage, the protein may subsequently unfold. Therefore, a protein with a solid structure is not ideal to form foam. But the protein can be modified into a desirable structure with higher molecule flexibility, and hence it can penetrate into the surface easily to form a stable film [64]. After diffusion and successful adsorption, only limited unfolding of proteins occurs at the interfacial film. This is governed by factors including thermodynamic conditions, protein size, type, flexibility and the relative concentration. Few or several segments of the molecule may adsorb at the interface. A research results indicated that alpha helix and beta sheet balance was an optimum structure of unfolded BSA [65]. They confirmed that a completely disordered molecule was not optimum for adsorption and disordered molecules did not occupy the maximum area. On the other hand, protein with the partly unfolded structure is

important to interfacial behavior and properties. Thus, retention of an ordered structure is important for interfacial behavior and foam properties [66].

1.4.2 Impact of protein structure on foaming properties

Table 1-3. Molecular characteristics and their functions in foam formation [67-68]

Molecular Characteristics	Function in foam formation
Solubility	Facilitates rapid diffusion to interface
Size	Affects the interaction in the surface and film formation
Amphipathic	Provides unbalanced distribution of charged and ploar residues for improved interfacial interactions
Flexibility	Facilitates phase behavior and unfolding at interface
Electrostatic interaction	Affects protein-protein interaction in the film and charge repulsion between neighboring bubbles
Retention of structure	Enhances overlap and segmental interactions in films
Surface hydrophobicity	Favors adsorption at the water/air interface and facilitate secondary interactions of the proteins

As mentioned before, the surface active properties are related to whether the protein can diffuse to, adsorb, unfold and rearrange at the interface. Thus, several molecular characteristics become important (Table 1-3). Among all of them, solubility is one of the most crucial factors for rapid film formation, since the

soluble protein can rapidly diffuse to the new interface to reduce interfacial tension [67].

1.4.3 Foaming properties of barley protein

Kapp and Bamforth compared the foaming capacity of barley prolamin and barley albumin [69]. The foaming capacity of prolamin is lower than FC of albumin and this difference became exaggerated through heat denaturation. They also found a positive correlation between surface hydrophobicity and foam stability in both cases. The foaming capacity of barley proteins was 12% less than FC of soy protein isolates [58]. They also compared the effect of acetylation and crosslinking on the foaming properties. The results indicated that acid-precipitated and acetylated barley protein had a similar foaming capacity to FC of native barley protein whereas the foaming stability of native barley protein is higher than FS of acid-precipitated and acetylated barley protein. This is probably because the barley protein isolates, the least soluble one, showed the lowest surface tension and was the most effective in lowering the surface tension; therefore, its foaming stability was higher than FS of others. By contrast, the acid-precipated and acetylated barley protein showed higher solubility (50 fold and 10 fold higher respectively), and were the least effective in lowering the surface tension and hence leading lower foaming stability [58].

In summary, several studies demonstrated the excellent foaming and emulsifying properties of barley proteins, and revealed that the intrinsic properties of proteins and extrinsic conditions could affect their functional properties. However, due to the low solubility of barley proteins in neutral condition, the application is very limited. Thus it is necessary to find an optimal modification method to change the protein structure in order to enhance their solubility and improve their emulsifying and foaming properties.

1.5. Modification to improve the functionalities of barley protein

The application of most plant proteins, including barley proteins, wheat proteins and zein was limited by their low solubility [70- 74]. In the last 20 years, the deliberate modification of food proteins to improve their functionalities has become increasingly popular. In fact, researchers have already achieved significant success in this field. The functional properties of proteins including solubility were dramatically increased [70-74]. Modification of protein usually refers to physical, chemical or enzymatic treatments which change its conformation and structure, and consequently alter its physicochemical and functional properties [75]. Numerous researches, including enzymatic modification and chemical modification, were carried out to increase the solubility.

1.5.1 Hydrolysis

Most of the commercial enzymatic modifications involve protein hydrolysis. The main purpose is to increase functional properties and solubility and to remove undesirable flavors. The degree of hydrolysis is important. For example, a low degree of protein hydrolysis (DH) can improve the foaming property of soy protein [76-77]. This phenomenon was also observed on sunflower protein and other proteins; it could be attributed to a higher molecular flexibility of proteins after partial enzymatic hydrolysis [76-79].

Moreover, peptides of smaller size tend to penetrate into the interface without structural change. As a consequence, the foam property could be increased. Moreover, the limited hydrolysis also improved the dilatational elasticity and viscoelasticity of the film, leading to a higher foaming property [78]. However, excessive hydrolysis, resulted in formation of peptides with small molecular weight, rarely formed stable and thick films, leading to reduced foaming property [80]. In order to have a high foaming property, high foam overrun and strong viscoelastic films are necessary, but in general they are contradictory. To achieve a good overrun, the protein should be small and flexible. To form strong viscoelastic films, the protein should be large and able to interact at the air water interface. Thus, the appropriate degree of hydrolysis is important to maintain the balance of the required characteristic of molecules leading to good foaming properties.

1.5.2 Disulfide bond crosslinking

Proteins can react with each other or with polypeptide chains by covalent bond to form inter-molecular crosslinkings and intra-molecular crosslinkings, respectively [81]. Crosslinking can affect the protein conformation, flexibility and functional properties. Disulfide bond crosslinking is the most common type of covalent crosslinking; it is carried out by oxidative coupling between two cysteine residues. An example in food systems is the gel formed by proteins, such as those of milk, eggs and soybeans, during heating treatment [82]. Disulfide interchange reactions during the mixing of wheat flour and water also result in developing the protein network which is necessary for bread making [83].

1.5.3 Maillard interaction

Maillard reaction has been used widely in the food industry to produce attractively colored and flavored product. The maillard reaction is a complex cascade chemical reaction. The reactive carbonyl groups in sugar and the nucleophilic amino groups in amino acid interact when heated or in presence of enzymes to form a complex mixture of molecules [84]. The product of protein crosslinking through maillard interaction in food systems has been well established. However, the precise chemical structure is still obscure [85]. It is

believed that some of these products could be formed through the condensation of lysine residues with arginine residues and reducing sugars. However, the exact mechanism remains unclear [86]. More research is needed in this area.

1.5.4 Succinylation

Succinylation may change the basic groups to the carboxyl group and lead to a high net negative charge. The protein dissociates into their subunits and thus the conformation may alter. Reversibility of this type of modification can be achieved by acylating with unsaturated dicarboxylic anhydrides. Succinylation could increase the protein solubility by increasing the net charge, and hence, could induce higher emulsification and foaming properties [87]. Succinylating egg yolk has already been used in mayonnaise and salad dressing [88]. The change in soy protein conformation, amino acid composition and surface hydrophobicity during succinylation was determined by Achouri and Zhang [89]. The results suggested that the surface hydrophobicity decreased dramatically as the succinylation level increased, probably due to the higher frequency of charged groups at the surface. As a result, the emulsifying capacity decreased at lower levels of succinylation. However, when the succinylation level was greater than 30%, the emulsifying property increased noticeably. It is probable that at the highest succinylation level, the surface hydrophobicity is very high, due to the inner hydrophobic groups exposed at the surface. Moreover, as the author mentioned, the higher solubility,

lower molecular weight and higher molecular flexibility may contribute to the high emulsion property as well [90].

1.5.5 Deamidation

As compared to the modification methods described above, deamidation is a more suitable and more attractive modification for barley protein, since it is rich in amidated glutamic and aspartic acid residues [91].

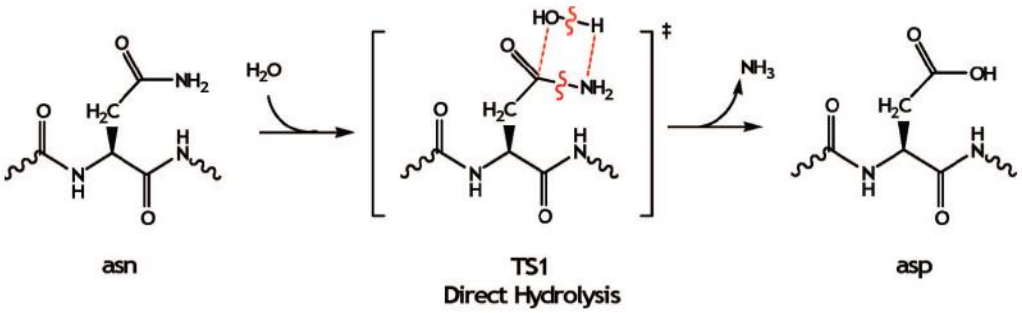

Figure 1- 4. Deamidation procedure:direct hydrolysis from ASN to ASP[91]

Deamidation can be achieved through the application of enzyme and chemical (mild acid and alkali) treatment. During deamidation, amid groups in the protein are converted into carboxyl groups (Figure 1-4). Consequently, asparagine and glutamine residues can be transferred to aspartate and glutamate residues [92]. Thus, the protein is partially unfolded and hydrophobic groups of the protein are exposed [94-95]. In this way, the surface charge and electrostatic repulsion can be

increased, and the protein molecular size and hydrogen bonding can be reduced; hence cereal protein solubility can be significantly enhanced [93].

Deamidation has been demonstrated to be an effective method of improving the functionalities of many plant proteins. The key point is controlling the degree of deamidation. Deamidation increases the solubility of protein. However, a deamidation degree that is extremely high could result in decreasing protein functionalities. For example, the emulsion properties of deamidated wheat gluten increased initially but decreased at high deamidation degree [96]. This may be because an excessive increase in the net charge will prevent the formation of an elastic film at the oil water interface through weakened protein-protein interactions. Moreover, the globular structure may be lost and optimum size of peptides might be decreased by hydrolysis during deamidation. Therefore, the protein layer around the oil droplets is thinner leading to less stable emulsion [96]. The emulsifying properties of deamidated rice endosperm proteins were significantly higher than unmodified rice protein [97]. This might be attributed to the high solubility and partial unfolding of the deamidated protein. However, the sample with high deamidation degree showed lower emulsifying properties. Therefore, the researchers concluded that it is important to make a balance between deamidation and peptide bond cleavage to improve emulsifying properties [97]. Foaming capacity of oat protein could be enhanced significantly by deamidation [98]. Because the deamidation could increase the protein solubility and decrease the molecular size; it could facilitate the diffusion of the

protein to the interface to form a stable film. However, the foaming stability decreased because excessive surface charge could reduce protein interactions and prevent formation of an elastic film at the air water interface [98].

1.6 Review summary and research objective

Barley protein has the potential of being used as emulsifying/foaming reagents in foods due to the relatively high surface hydrophobicity of the protein molecules, and their good capacity to form a coating at water/air and water/oil surface to stabilize the emulsions and foams.

According to preliminary research results, as compared to other commercially available proteins such as zein, soy, whey and gluten, barley protein shows high emulsion and foaming properties. However, the solubility of barley protein is relatively low, which limits its application in industry. Therefore, it is necessary to modify the structure of barley proteins to improve their solubility but maintain the high emulsion and foaming properties.

Owing to a high content of glutamine, barley protein is a suitable candidate for deamidation, targeting improved functionalities. However, research on barley protein deamidation is limited to few publications on antioxidant property [99-

100]. The impacts of deamidation on the structure and functional properties of barley protein are unclear.

The aim of this study is to test the hypothesis that the solubility, foaming and emulsifying properties of barley protein will be increased when it is subjected to deamidation. The protein structure transition at low deamidation level and its functionality change as a consequence of such structure transition were emphasized.

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Chapter 2 Effect of deamidation on the molecular structures and functional properties of barley prolamin

2.1 Introduction

Deamidation is one of the most commonly utilized chemical modifications to improve solubility and other functional properties of food proteins [1]. In cereals, deamidation is a particularly important modification since up to one-third of their total amino acid content is glutamine (Gln) [2-3]. The conversion of the amide groups on glutamine side chains into acid groups is believed to improve cereal protein solubility as a high content of glutamine residues may cause the aggregation of the protein molecules via hydrogen bonding. Deamidation may also partially unfold the protein and indirectly lead to protein hydrolysis by cleavage of the peptide bond [4]. These changes improve the functional properties of wheat, corn, rice and soy proteins, making them useful for the food and pharmaceutical industries [4-11]. However, excessive molecular charges on proteins and peptide bond cleavage could affect the protein structure and cause undesirable functional properties that reduce their utility [4]. Therefore, optimization of the DD is crucial to

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achieve better functionalities. Additionally, since deamidation rates as low as 2-6% can enhance the functional properties of proteins [5], changes in protein structure (molecular weight, conformation and surface charge and hydrophobicity) may require accurate and fine adjustment to enable appropriate and optimal structure modification. In general, systematic research of protein molecular structure and subsequent functionality as a function DD values is limited. More specifically, information about protein structure transition at low deadmidation levels and subsequent functionality is lacking.

As the fourth most widely cultivated cereal in the world after wheat, rice and corn [1], barley is gaining increasing popularity as a part of the human diet because of the recent health claim made about its β-glucan [13]. This soluble dietary fibre component of barley is known to reduce both blood cholesterol [14] and the glycemic index [15]. Additionally, barley represents a potential abundant, affordable source of plant proteins. The overall barley grain protein content is 8 to 13% depending on the variety [16]. Prolamin, barley prolamin extracted with alcoholic media, comprises approximately 35–55% of the total barley grain protein, and is the main storage protein for barley [17]. Barley prolamins 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 (70–80% and 10–20% of the prolamin

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fraction, respectively) and the D (80-90 kDa) and A (15 kDa) hordeins (less than 5% of the total prolamin fraction). The A hordeins, are likely alcohol-soluble albumins or globulins, or breakdown products of larger prolamins rather than true prolamins. C and some B hordeins appear as monomers, while most B and D hordeins are linked by inter-chain disulfide bridges [18]. Prolamin is rich in hydrophobic amino acids (40%), with the highest levels corresponding to proline, leucine, and valine [19]. This amino acid profile results in high protein surface hydrophobicity, which favors rapid adsorption at the hydrophobic interface and then form a viscoelastic film to stabilize foams and emulsions [19]. However, these features also result in a marked reduction in water solubility and a tendency of protein aggregation. Both of these changes hinder their functional application, since protein water solubility is critical to impart other desired and necessary properties such as emulsifying and foaming functionalities [20]. Since prolamin also has a very high Gln content [19], it is a suitable candidate for evaluate deamidation for improving protein solubility. However, research on prolamin deamidation is limited [21-22].

The objective of this research was to investigate the structure, solubility, foaming and emulsifying properties of barley prolamin when it is subjected to deamidation. The protein structure transition at low deadmidation level and its functionality change as a consequence of such structure transition was emphasized.

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2.2 Materials and Methods

2.2.1 Materials

Regular barley grains (Falcon) were kindly provided by Dr. James Helm, Alberta Agricultural and Rural Development, Lacombe, Alberta. Protein content was 13.2% as determined by combustion with a nitrogen analyzer (Leco Corporation, St. Joseph, MI) calibrated with analytical reagent grade EDTA and a calculation factor of 6.25. Canola oil used for the emulsification study was purchased from a local supermarket (Edmonton, AB, Canada). Unstained standard protein molecule marker for SDS-Page was purchased from Bio-RAD (Richmond, CA, USA). Ammonia Assay Kit, o-phthaldialdehyde (OPA) reagent, 1-anilinonaphthalene-8-sulfonic acid (ANS) and standard molecular markers for HPLC analysis (BSA, 67 kDa; ovalbumin, 43 kDa; lactoglobulin, 35 kDa; cytochrome C, 13.6 kDa; aprotinin, 6.5 kDa and vitamin $B_{12,}$ 1.4 kDa) were purchased from Sigma-Aldrich, Canada. All other chemicals were of reagent grade

2.2.2 Extraction of barley prolamin

Barley prolamin was extracted according to our previous work [19]. Briefly, after pearling and milling processing, barley endosperm flour was dispersed

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in the 55% ethanol solution at a solvent to flour ratio of 6:1 with stirring for 2 h at 60^{o}C. After extraction, the solid part was removed by centrifuge (Beckman Coulter Avanti J-E Centrifuge System, US) at 23 ℃ at 8500 × g for 15 min. The prolamin fraction was isolated from the supernatant by cold precipitation at 4 ^{o}C overnight. The isolated prolamin was lyophilized and the dry powders were stored in plastic bags at 4 ^{o}C before further processing.

2.2.3 Preparation of deamidated prolamin of different DD

Deamidated prolamin was prepared using a slightly modified version of method by Yong et al. [23]. A 5% (w/v) prolamin was suspended in 70% (v/v) ethanol with 0.5 M NaOH solution at 40 and 60 ^{o}C, respectively. Samples were withdrawn at different time intervals and neutralized using 0.5 M HCl before dialysis against deionized water and then freeze dried.

2.2.4 Determination of the DD value

The degree of deamidation (DD) was determined by measurement of the released ammonia after deamidation by using Ammonia Assay Kit according to the manufacturer's instruction. DD was calculated as the ratio of ammonia

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generated in the modified sample to that of the completely deamidated protein. Complete deamidation was achieved by refluxing the sample with 2 M HCl for 2 h.

2.2.5 Electrophoretic mobilities

The electrophoretic mobilities of the deamidated prolamin samples in different pH buffers (pH 3 and 5: 0.2 M acetate buffer, pH 7: 0.2 M phosphate buffer) were measured by laser Doppler velocimetry using a Zetasizer NanoS (model ZEN1600, Malvern Instruments Ltd, UK). Electrophoretic mobility (i.e., velocity of a particle within an electric field) was related to the zeta potential (ς) using the Henry equation [24]

$$U_{\mathrm{E}} = \frac{2\varepsilon \times \zeta \times f(\kappa\alpha)}{3\eta}$$

Where η is the dispesion viscosity, ε is ther permittivity, and f ($\kappa\alpha$) is a function related to the ratio of particle radius (α) and the Debye length (κ). The results are reported as the average of at least three measurements. Typical standard deviations were less than $\pm$ 3mV. The same buffers were used in the following studies.

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2.2.6 Determination of the degree of hydrolysis (DH)

Degree of hydrolysis was assayed directly by quantification of cleaved peptide bonds as assessed by the o-phthaldialdehyde (OPA) method [25]. The o-phthaldialdehyde (OPA) reagent was prepared by dissolving 7.62 g disodium tetraborate decahydrate and 200 mg SDS in 150 mL deionized water, followed by addition of 160 mg OPA dissolved in 4 mL ethanol and 176 mg, 99% dithiothreitol (DTT). The volume of the mixture was adjusted to 200 mL by deionized water. OPA reagent (3 ml) was then mixed with 400 uL deamidated prolamin samples (10 mg/mL) and the mixture solution was measured using a spectrophotometer (Jenway 6505 UV/Vis Spectrophotometers, UK) at 340 nm. The standard solution was prepared by dissolving 10 mg serine into 100 mL deionized water.

2.2.7 Surface hydrophobicity

Surface hydrophobicity of the deamidated prolamins in sodium phosphate buffer (pH 7) was determined using a fluorescence probe, 1-anilinonaphthalene-8-sulfonic acid (ANS), according to the method of Kato and Nakai [26]. Fluorescence intensity (FI) was measured at wavelengths of

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390 nm (excitation) and 470 nm (emission) using a fluorospectrometer (FP-6300, Jasco, Tokyo, Japan). The surface hydrophobicity degree (S_o) was calculated by linear regression analysis using the slope of the straight line obtained by plotting the FI as a function of the protein concentration.

2.2.8 Electrophoresis

Sodium dodecyl sulphate (SDS) gel electrophoresis was performed to evaluate prolamin subunit molecular weight change after deamidation using a vertical mini-gel system (Mini-PROTEIN Tetra Cell, BIO-RAD, USA). Deamidated prolamin samples were mixed with the loading buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS (w/v), 20% glycerol (v/v), 0.05% 2-Mercaptoethanol (v/v) and 1% bromophenol blue (w/v)) and then heated at 100 ^{o}C for 5 min. After cooling, 18 μL samples (5 mg/mL) was loaded on 5% stacking gel and 12% separating gel and subjected to electrophoresis at a constant voltage of 80V. After electrophoresis, the gels were stained with 0.1% Coomassie Brilliant Blue-R-250 in water-methanol-acetic acid (4:5:1) for 30 min and destained with water-methanol-acetic acid (4:5:1).

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2.2.9 Size Exclusion Chromatography (SEC)

SEC chromatography was performed using a HPLC system (Varian ProStar, US) combined with a size exclusion column (Superdex^{TM} 200 10/300 GL, Amersham Biosciences, US). 50 mM phosphate buffer containing 150 mM sodium chloride was used as a mobile phase at a flow rate of 0.4 mL/min at 25 ± 0.5 ^{o}C. 50 μL sample solution was injected into HPLC system and the protein was monitored at the UV wavelength of 280 nm. Standard molecular markers were used to calculate the weight-average molecular weight (M_w) of the deamidated prolamins.

2.2.10 FTIR spectroscopy

Protein conformation was studied with a Fourier transform infrared (FTIR) spectroscopy (Varian FTS-7000, US) in the wavenumber range from 400 to 4000 cm^{-1} during 128 scans, with 4 cm^{-1} resolution. 5% deamidated prolamin samples were dissolved in D_2O solution. To ensure complete H/D exchange, samples were prepared 2 days before and kept at 4 ℃ prior to infrared measurements. Samples were placed between two CaF_2 windows separated by 25-μm polyethylene terephthalate film spacer. To study the amide I region

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of the protein, Fourier self-deconvolutions were performed using the software provided with the spectrometer. Band narrowing was achieved with a full width at half maximum of 20 cm^{-1} and with a resolution enhancement factor of 2.0 cm^{-1}.

2.2.11 pH solubility profile

Deamidated prolamins (125 mg) were dispersed in 25 ml pH 3, 5 and 7 buffers. The dispersions were mixed for 1 h at room temperature using magnetic stirrer before centrifuging at 3000 × g for 20 min at 4^{o}C. The supernatants were filtered through a Whatman No. 1 filter paper to obtain clear filtrates. The soluble supernatant was used for protein content determination according to the method of Bradford et al [27]. Solubility is expressed as the percentage of the protein content in the supernatant to the amount of the total protein in the solution.

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2.2.12 Foaming properties

Foaming capacity and stability (FC and FS) were determined according to Ahmedna et al [28] with a slight modification. Protein samples (0.5% w/v) were dispersed in 50 mL buffer at pH 3, 5 and 7. The solution was mixed for 2 min with a homogenizer (PowerGen 1000, Fisher Scientific, Fairlawn, NJ, US) at speed "three" for 2 minutes. Volumes were recorded before and after homogenization. The percentage volume increase was calculated according to the following equation: Foaming capacity (FC) = $(Vf_2 - Vf_1) / Vf_1 \times 100$, where Vf_1 and Vf_2 represent the volume of the protein solution and the formed foams before and after homogenization. FS was determined as the volume of foam that remained after 0.5 h at room temperature expressed as a percentage of the initial foam volume: FS = $Vf_2' / Vf_2 \times 100$.

2.2.13 Emulsion properties

Emulsifying centrifugation and thermal stability (ECS and ETS) were determined according to Yasumatsu et al. with a slight modification [29]. Protein samples (0.5% w/v) were dispersed in 50 mL pH 3, 5 and 7 buffers, followed by addition of 50 mL canola oil. The mixture was homogenized

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(PowerGen 1000, Fisher Scientific, Fairlawn, NJ, US) at speed "three" for 2 minutes to form an emulsion. The emulsion was then transferred to centrifuge tubes and centrifuged at 1500 × g for 5 min at room temperature (23^{o}C). ECS was calculated by measuring the volume of emulsion formed Ve_1 and total volume *Vet* occupied in the tubes and recorded as EC = Ve_1 / *Vet* × 100. The emulsion samples were then heated to 80 ℃ in a water bath for 30 min and cooled to room temperature. Upon cooling, these tubes were centrifuged at 1500 × g for 5 min at room temperature. The volume of the remaining emulsified fraction Ve_2 was recorded. The ETS was calculated according the equation: ES = (Ve_2 / *Vet*) × 100.

Statistical analysis

All experiments were performed at least in triplicate. Error bars on graphs represent standard deviations obtained from the statistical model. Statistical comparisons were made by analysis of variance (ANOVA). The level of significance used was $p < 0.05$.

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2.3 Results and Discussion

2.3.1 Alkaline deamidation of prolamin

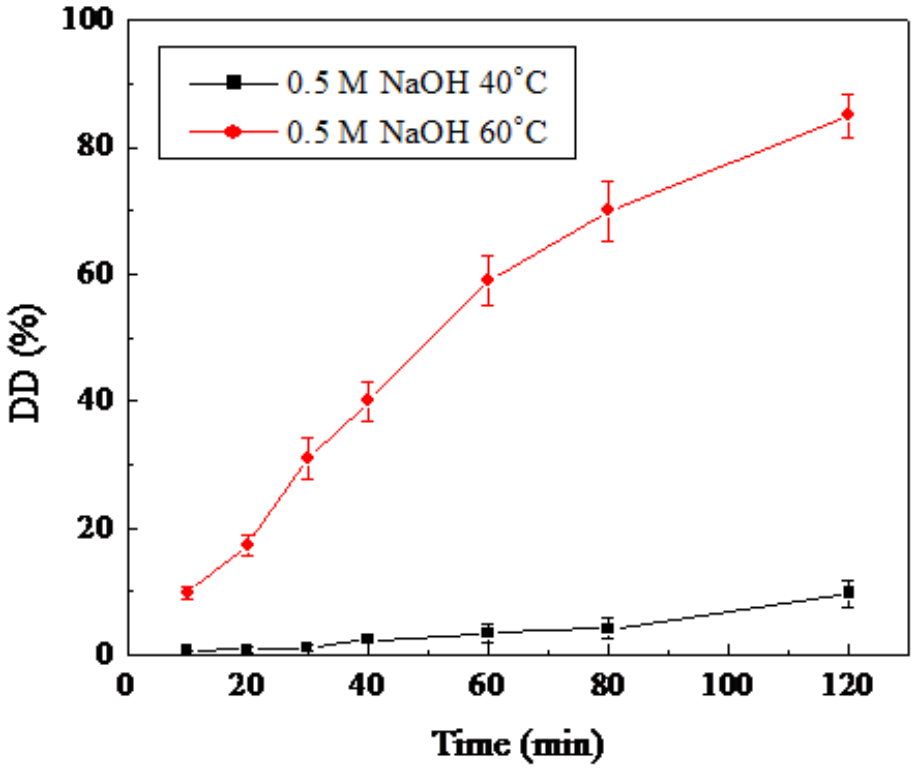

Figure 2-1. Time-dependent increase of DD value of prolamin induced by alkaline reaction.

The protein content of isolated barley protein fractions was 93%. Preliminary data showed that alkaline method was more efficient to prepare deamidated prolamin than acidic method. This is in agreement with previous report for

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zein [4]. Thus alkaline method was selected in this research to prepare deamidated prolamins. In order to study the effect of deamidation on prolamin structures and properties, samples of a relatively broad range of DD values were prepared. Those within limited DD range (DD ≤ 10%) were emphasized. Thus, reactions were conducted in 70% (v/v) ethanol solution with 0.5 M NaOH at two selected temperatures (40 and 60 ^{o}C) based on preliminary data.

Figure 2-1 shows the DD obtained as a function of the reaction time. At 40 ^{o}C, DD value reached 9.8% after 2 h. Prolonged time did not result in further increase of the DD value. Increasing temperature to 60 ^{o}C significantly enhanced reaction rate ($p < 0.05$) and DD reached more than 80% within 2 h. Samples that possessed DD values in the range of 0.7-9.8%(0.7,1.2,2.4,4.7 and 9.8%) and 17-40%(17,31 and 40%), obtained at 40 and 60 ^{o}C, respectively, were selected for further study.

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2.3.2 Characterization of deamidated prolamins

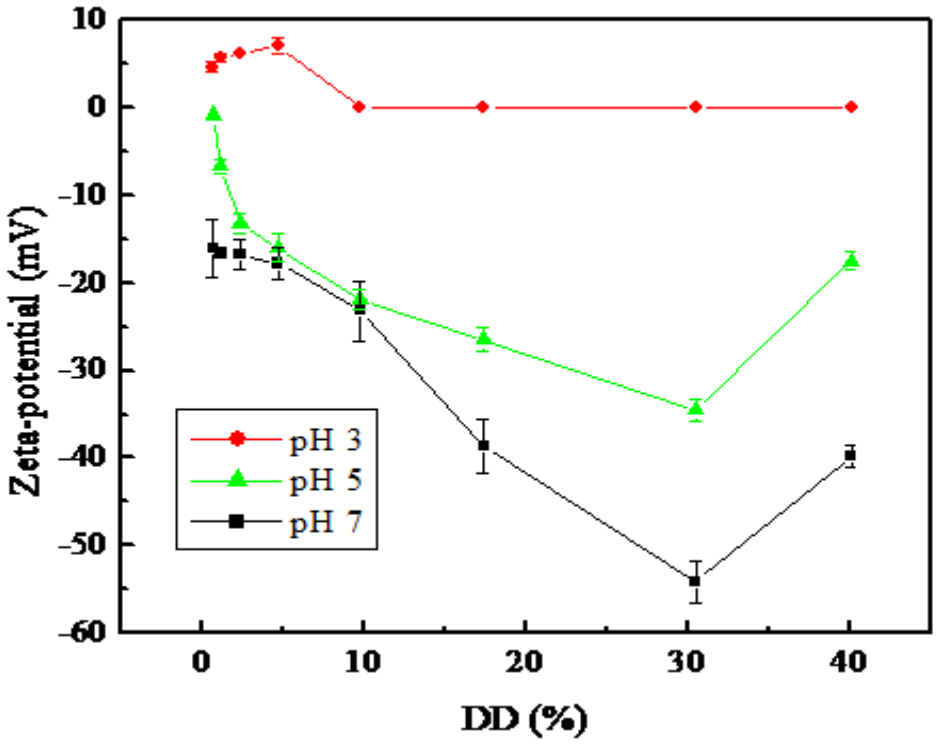

Figure 2-2. Electrophoretic mobilities of the deamidated prolamins at different pH as a function of DD value.

The zeta-potential of the prolamin samples in different pH buffers are expressed as a function of their DD value (Figure 2-2). Limited surface charge (- 5 mV) was observed for prolamin with a DD of 0.7% at pH 5. This value, however, increased greatly to - 33 mV at DD 31%, and then decreased to -17 mV at DD of 40%. The surface charge of the deamidated prolamin changed in the same way at pH 7, but the zeta-potential was generally higher than those at

pH 5, especially at relatively low or high DD range. On the other hand, the protein molecule surfaces were slightly positively charged (+ 5 mV) at pH 3 when the DD was 0.7%. With increasing of the DD value to 4.7%, prolamin surface charge decreased to near zero. These data suggest that the isoelectric point (IEP) of prolamin (without modification) is between pH 3 and 5. As the DD value increased, its IEP shifted to pH 3 due to an increase in the amount of carboxyl group on protein side chains. This is in agreement with research obtained from other proteins where their IEP normally shift to acidic pH after deamidation [5]. The introduction of the carboxyl group on protein side chains as a result of deamidation also explained the increase of the protein surface charge with DD until 31% at pH 5 and 7. The decrease of zeta-potential at a DD of 40% in both pH 5 and 7 buffers was unexpected. This may be attributed to cleavage of the small peptides with high density of charge due to significant hydrolysis. These small peptides were lost during dialysis, even though very low molecular weight cut-off (1 kDa) dialysis tubing was used in this research.

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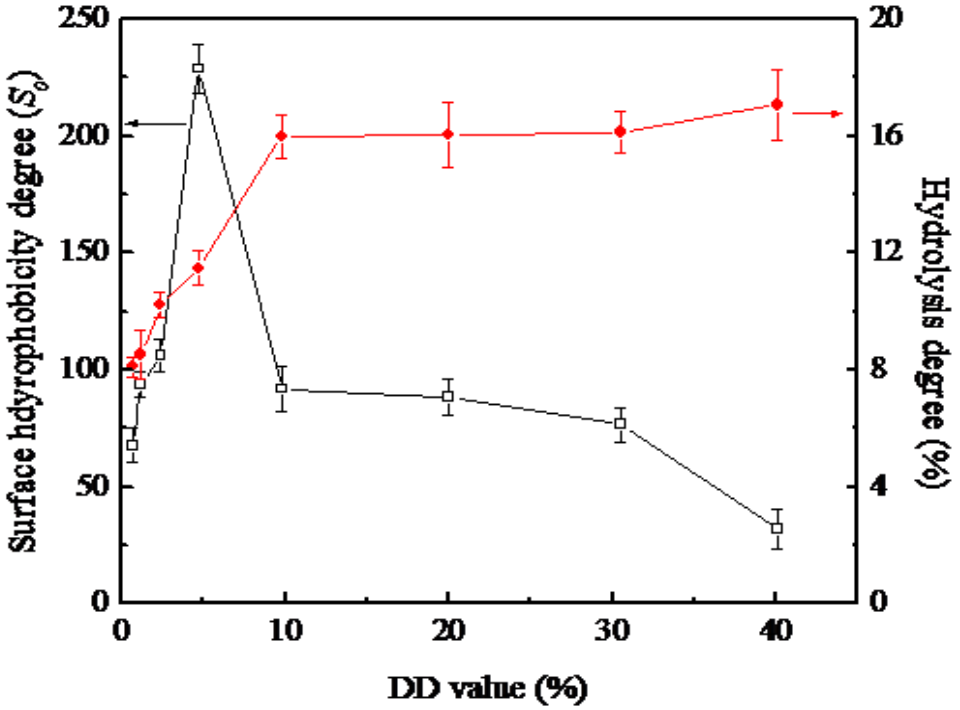

Figure 2-3. Degree of hydrolysis and surface hydrophobicity of the deamidated prolamins as a function of DD value.

The hydrolysis degree (HD) and surface hydrophobicity of the deamidated prolamin samples are shown in Figure 2-3. The HD increased linearly in proportion to the degree of deamidation until a DD of 9.8%, then the HD value leveled off. This suggests that prolamin peptide bond cleavage occurred quickly within the DD range of 0.7-9.8% and the hydrolysis rate slowed down after DD of 9.8%. S_o of the deamidated prolamin increased markedly ($p < 0.05$) with the DD increased to 4.7%, suggesting that the hydrophobic regions were progressively exposed at the molecular surface. A further increase of the

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DD value resulted in significant decrease ($p < 0.05$) of the prolamin surface hydrophobicity.

The SDS-Page patterns of the deamidated prolamins are shown in Figure 2-4. Three subunits of prolamin were identified with bands at 55-80, 30-50 and < 15 KDa corresponding to C, B and A prolamins, respectively. A weak band at 80-90 KDa corresponding to D prolamin was also observed in this work when ethanol was used as the sole extraction agent [30]. Most bands remained visible in SDS-Page pattern until a DD value of 4.7%, but the band intensity of C and B prolamins decreased gradually. After DD of 4.7%, all bands disappeared. The result indicates that partial hydrolysis occurred at DD ≤ 4.7%, whereas extensive hydrolysis took place within the DD range of 5-9.8%, resulting in formation of peptides of molecular weight less than 10 KDa.

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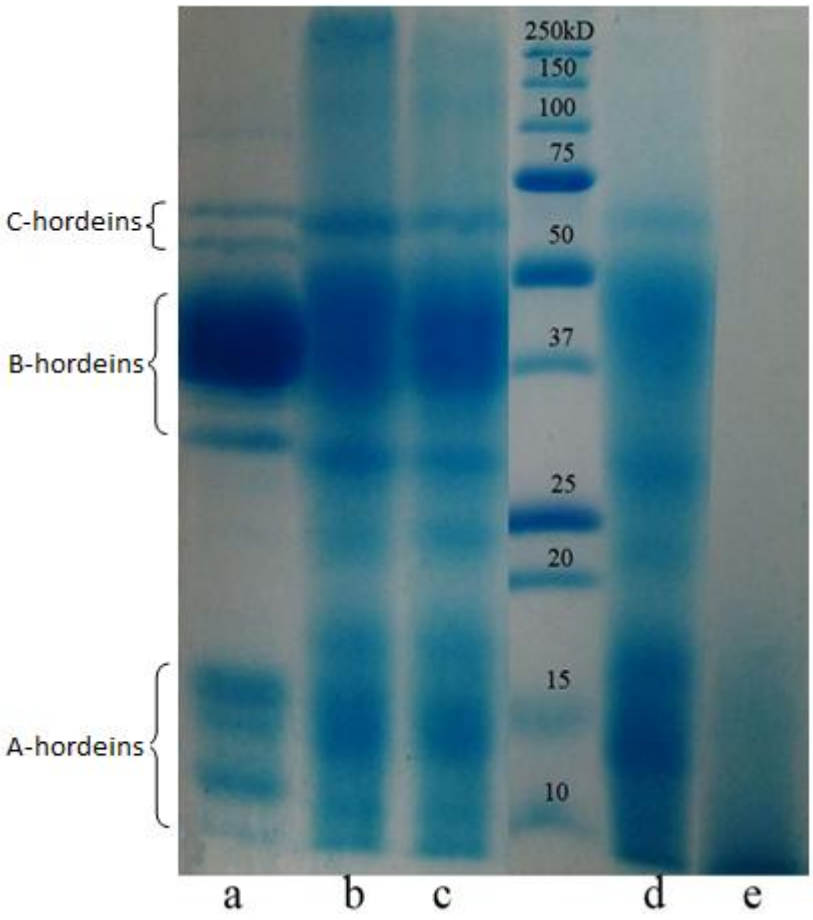

Figure 2-4. SDS-polyacrylamide gel electrophoresis of the deamidated prolamins (a: unmodified prolamin, b: DD 0.7%, c: DD 1.2%, d: DD 4.7%, e: DD 9.8%).

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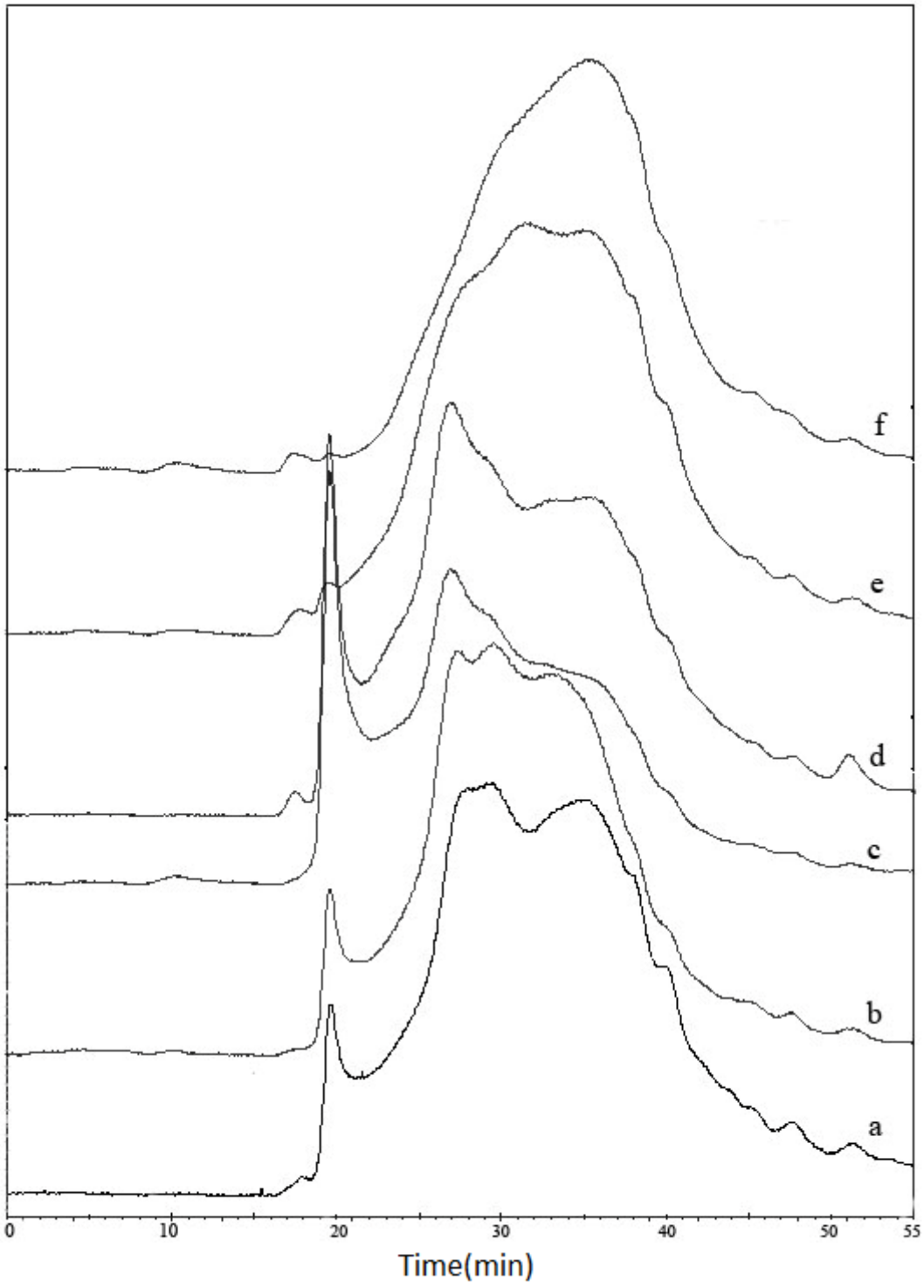

Figure 2-5. SEC-HPLC chromatograms of the deamidated prolamins (a: DD 0.7%, b: DD 1.2%, c: 2.4%, d: DD 4.7%, e: DD 9.8%, f: DD 17%).

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SEC chromatograms of the deamidated prolamins in phosphate buffer were depicted in Figure 2-5. Deamidated prolamin with the DD value of 0.7% contains two main broad peaks (Peak 1 and Peak 2) corresponding to subunits with M_w of <15 KDa and 20-67 KDa, respectively. The former can be assigned to A hordeins, whereas the latter could be B and C hordeins together. A small sharp peak (Peak 3) was also observed at 114 KDa, which could be assigned to some aggregated large peptides. This phenomenon was reported for barley proteins, where high molecular weight subunits form a backbone which binds low molecular weight subunits through disulfide bridges to form a gel-like aggregate [31]. Interestingly, increasing the DD value to 2.4-4.7% significantly altered the chromatogram patterns. Peak 2 was markedly sharpened and peak 3 amplitude was dramatically enhanced. The sharpened peak 2 corresponds to the remaining of more hydrolysis-resistant subunits, likely corresponding to C-hordeins since they are more slowly degraded than B-hordeins [31]. The increased peak 3 intensity can be attributed to an increased solubility of the large polypeptides due to an increased net negative charge by deamidation [32]. A further increase of the DD value ≥ 9.8% resulted in the dissociation of the aggregated large peptides as the peak 3 almost disappeared. Additionally, an obvious shift of the peak 2 to lower molecular weight range was observed. This indicates that the resistant subunits in prolamin started to be hydrolyzed after DD values of 4.7%. Their

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degradation may account for the extensive hydrolysis of the prolamin samples within DD range of 5-9.8%.

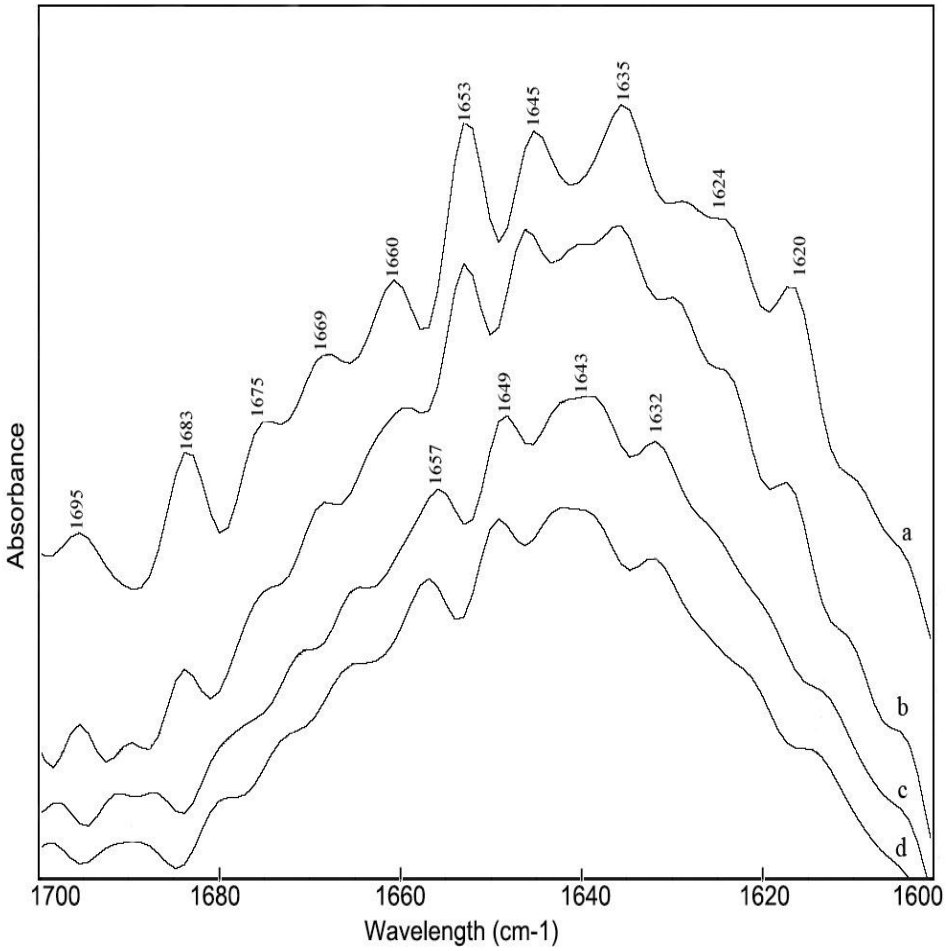

Figure 2- 6. FTIR spectra of the deamidated prolamins (a: DD 1.2%, b: DD 4.7%, c: DD 9.8%, d: DD 17%).

Fourier transform infrared spectroscopy (FTIR) is a powerful 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. The prolamin sample

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with DD of 0.7% shows several bands in the amide I region (Figure2-6), which were assigned to protein secondary structures according to previous reports [8, 33-36]: α-helices (1652 cm^{-1}), β-sheets (1617, 1635 and 1683 cm^{-1}), β-turn (1669 and 1675 cm^{-1}), and random coils (1646 cm^{-1}). The band at 1660 cm^{-1} could be mainly assigned to the carbonyl stretching of the glutamine side chain [36]. The bands at 1683 and 1617 cm^{-1} are believed to be associated with the aggregation process [33]. When the DD value increased from 2.4 to 4.7%, the intensity of the bands at both 1683 and 1617-1621 cm^{-1} decreases, suggesting disassociation of protein aggregates, probably due to increased repulsions between protein molecular chains as a resulted of increase surface charges. Marked shifts in the band positions in the wavelength range of 1623-1657 cm^{-1} were observed with a further increase of the DD value. The absorption corresponding to glutamine side chain shifted to 1656 cm^{-1}, reflecting change of intra- or inter-molecular hydrogen bonds between glutamine side chains [36]. Additionally, the α-helix band shifted to lower wavelength and the random coil band (~ 1642 cm^{-1}) intensity increased notably. This suggests that marked protein confirmation changes occurred after DD value of 4.7%, likely associated with protein partial unfolding as a result of both strong negative charge on protein molecular chains and extensive protein hydrolysis.

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A model structure has been proposed for gliadin by Friedli [37]. In this model, molecules of gliadin aggregate through hydrogen bonding to form a unit with a diameter of 32 nm with their hydrophobic groups exposed outside. These units then aggregate with each other through surface hydrophobic patches to form large aggregates, making them insoluble in water. It is proposed that prolamin may have a similar aggregation structure as gliadin owing to their similarity in amino acid composition and biological function in cereal grains. It is obvious that the IEP of the prolamin shifted from a pH of around 5 to 3 due to the introduction of additional carboxyl groups on the protein side chains as a result of deamidation. Limited deamidation (DD value $\leq$ 4.7%) can dissociate protein aggregates to a certain extent as revealed by the FTIR result. As a consequence, more hydrophobic patches on protein unit surfaces were exposed outside, thus increasing prolamin surface hydrophobicity. The dissociation of these large aggregates may have led to formation of water soluble peptide aggregates as observed in the deamidated prolamin SEC chromatograms. On the other hand, higher deamidation (DD > 5%) have led to protein unfolding and extensive hydrolysis. As a result, more polar groups on protein side chains were exposed outside, thus a decreased prolamin surface hydrophobicity was observed. In the following sections, we will evaluate the impacts of these structural changes on prolamin functional properties.

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2.3.3 Solubility

Prolamin solubility in acidic conditions (pH 3 and 5) as well as neutral pH was plotted against the DD value (Figure 2-7). Our previous research showed low solubility (< 20%) of unmodified prolamin at pH 3-7 and a significant increase of the protein solubility (approximate 50%) occurring at pH 10 [19]. After deamidation, prolamin solubility significantly increased ($p < 0.05$) at all the pHs tested.

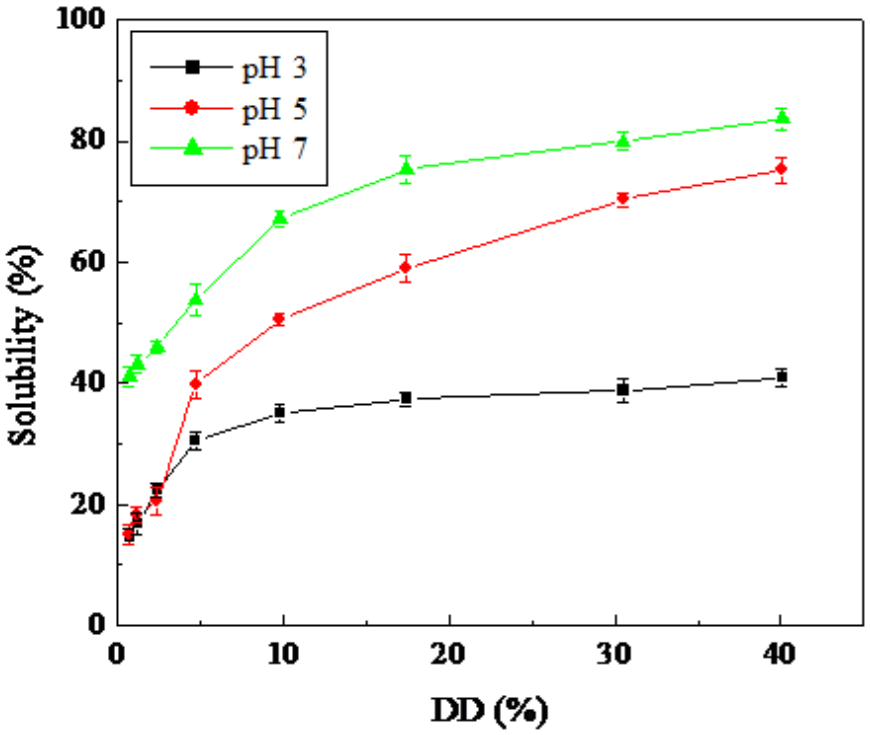

Figure 2-7. Solubility of the deamidated prolamins at different pH as a function of DD value.

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The most insightful improvement was the remarkably increased protein solubility at pH 5 from 15% to 75% with DD value increasing to 40%. Due to the high proportion of nonpolar amino acid residues and high surface hydrophobicity, prolamin is soluble in water only with the presence of alcohol, high concentrations of urea, high concentrations of alkali (pH 11), or anionic detergents, similar to other prolamin proteins [38]. The remarkable solubility at pH 5 and 7 for deamidated prolamin in this study provides a promising improvement for enabling a broader range of usage in food-processing industries. The solubility was relatively low at pH 3 due to shift of the prolamin IEP to acidic pH. The improvement in solubility within a DD range of 0.7-4.7% could be due to the dissociation of prolamin aggregates and partial protein hydrolysis. Further increased solubility after DD value of 4.7% could be attributed to protein partial unfolding and extensive hydrolysis. These structure changes led to the exposure of more charged and polar groups to the surrounding water, thus promoting protein-water interaction and an increased solubility [32].

2.3.4 Foaming and emulsifying properties

Our previous data showed rather good foaming capacity of unmodified prolamin at both pH 3 and neutral conditions (150-160%), whereas a relatively lower foaming capacity was found at pH 5 (90%). However, due to

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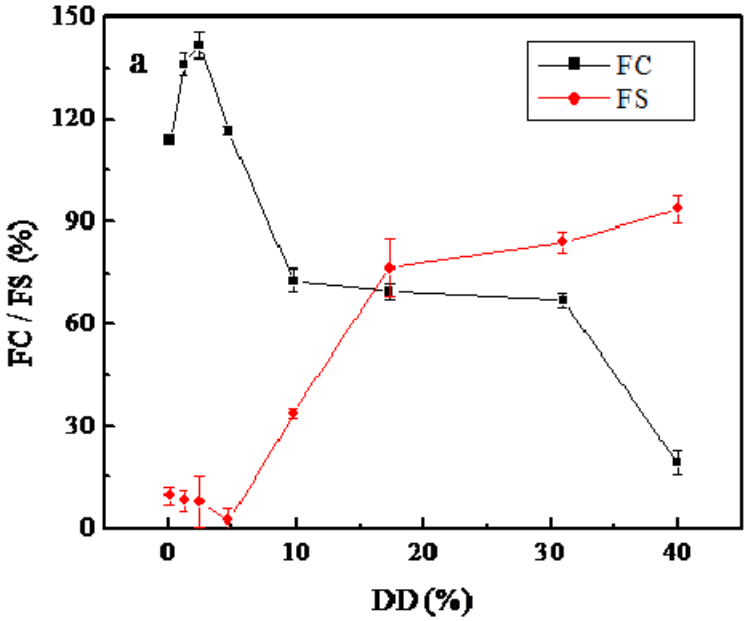
its inherent poor solubility, unmodified prolamin requires dehydration at pH 11 followed by adjusting pH back to acidic and neutral conditions to enable foaming and emulsifying functionalities. This procedure is not practical in commercial food systems. Deamidation significantly improved prolamin solubility even within a limited DD range, thus allowing functionality testing by dispersing samples at different pH buffers directly. As shown in Figure 8, with increasing of the DD value, the foaming capacity initially increased until a DD value of 2.4-4.7%, then decreased at all the pHs tested. A much more rapid decrease in FC value was observed at pH 3 and pH 5 than pH 7. The optical FC values were obtained at a narrow DD range (2.4-4.7%) where a significant improvement of foaming capacity was observed at pH 5 (145%) and pH 7 (190-200%) compared to unmodified prolamin. The optical obtained at pH 3 is on a same level as that of the unmodified sample.



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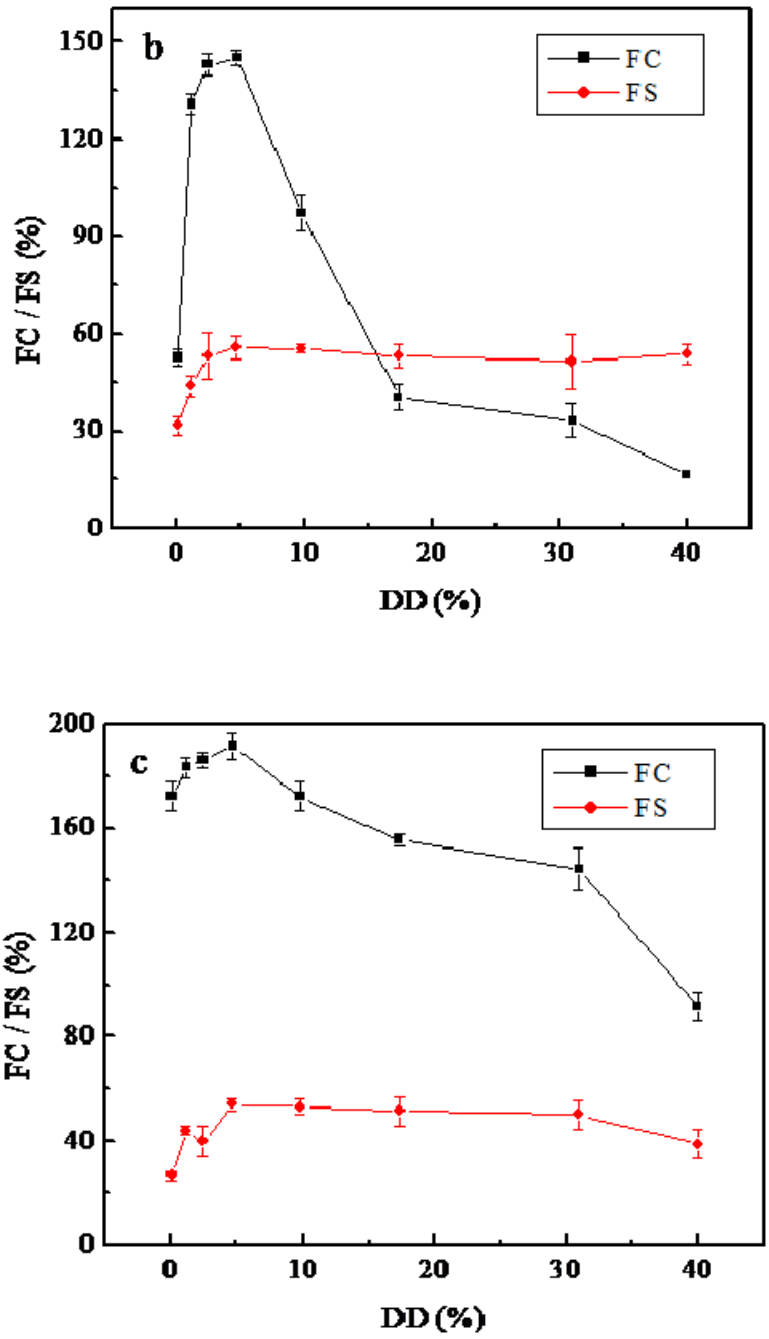

Figure 2-8. Foaming capacity and stability of the deamidated prolamins at (a) pH 3, (b) pH 5 and (c) pH 7 as a function of DD value.

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The initial increase of the FC value within the DD of 0.7-4.7% is probably due to increase of the protein solubility, enabling easily diffusion to the air/water surface. Moreover, the exposed hydrophobic side chains facilitated binding of deamidated prolamin at hydrophobic air surfaces, and then these proteins could aggregate via surface hydrophobic patches to form films around bubble surfaces. Although deamidated prolamins exhibited good solubility at a DD value > 4.7%, their surface hydrophobicity decreased with a further increasing of the DD value. This decreased protein surface hydrophobicity may be one of the major reasons accounting for the decreased FC values at DD range of 9.8-40%. The protein surface charge influenced the FC value as well. A significantly greater foaming capacity ($p < 0.05$) observed at pH 7 compared to pH 3 and 5 within the optical DD range may be related to a greater surface charge on protein molecular chains at neutral pH. This created a strong repulsion between adjacent bubbles, preventing quick foam coalescence during homogenization process. This greater surface charge could also explain the slower decrease in the rate of the FC values at pH 7 after DD value of 4.7%.

The deamidated prolamin samples possessed an increased foaming stability (20% to 50-60%) at both pH 5 and 7 when the DD value was raised from 0.7 to 4.7%, and then leveled off after DD value of 4.6%. On the other hand, low

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FS values were observed at a DD range of 0.7-4.7% at pH 3, and this value increased rapidly after DD value of 4.7%. The overall stability of a foam is related to the resistance of the lamella to drain and of the bubbles to collapse. These factors are dependent on the rheological and adhesive properties of the interfacial film surrounding the bubble [39]. Normally high molecular weight proteins exhibit greater film strength and foam stability [39]. Thus, the aggregated large peptides observed in the SEC chromatograms at the DD range of 2.4-4.7% may have contributed to the increased prolamin foaming stability at both pH 5 and 7. However, the FS values did not decrease after DD value of 4.7%. This is difficult to explain because large peptide aggregates were dissociated at a DD value ≥ 9.8% according to the SEC chromatograms. The FC values decreased significantly ($p < 0.05$) after DD value of 4.7% at both pH 5 and 7. Thus, it is deduced that less protein molecular chains have suitable molecular structures for foam forming compared to samples with DD values of 2.4-4.7%. However, once foams were prepared, the protein chains with a suitable molecular structure could form continuous and rather rigid films around bubbles. This might explain why the ES value remained almost unchanged at a DD value ≥ 9.8%. Previous literature reported that the optical foaming stability was observed near the protein IEP. Proteins can absorb better to the air/water interface at minimum electrostatic repulsion to form a rigid film against coalescence [39]. However, a very low FS value (≤ 9.8%) was observed for deamidated prolamins at a DD

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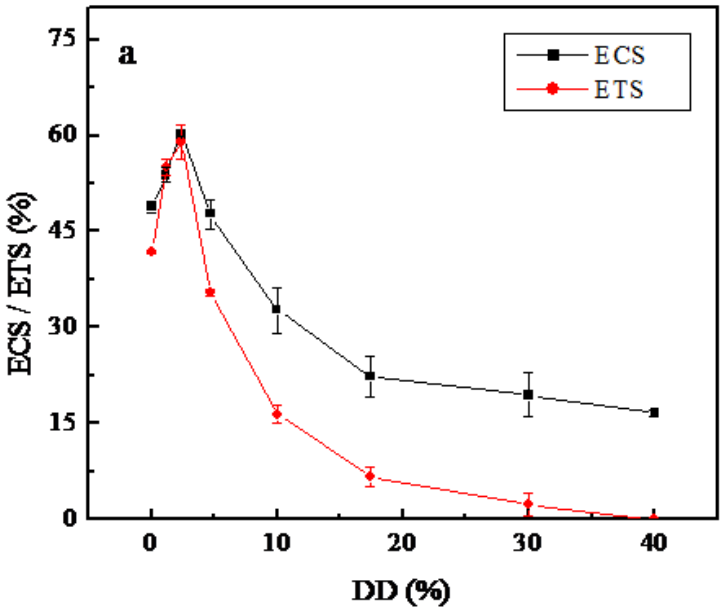
range of 0.7-4.7% when the pH was 3. It is assumed that the deamidated prolamins, including the large peptide aggregates, also formed a thick and rigid film at air/water interface at pH 3. This film may have a strong tendency to aggregate when the surface charge is low, resulting in extensive aggregation of protein films between adjacent gas bubbles, film rupture, and foam instability. Increasing of the DD value to ≥ 9.8%, large peptides were dissociated and the prolamin peptide bonds were cleaved. The hydrolyzed peptides may exhibit fewer tendencies to aggregate, forming rigid and viscoelastic films without aggregation near protein IEP. This explains a significant increase ($p < 0.05$) of the FS value observed with a DD value increasing from 9.8 to 40% at pH 3.



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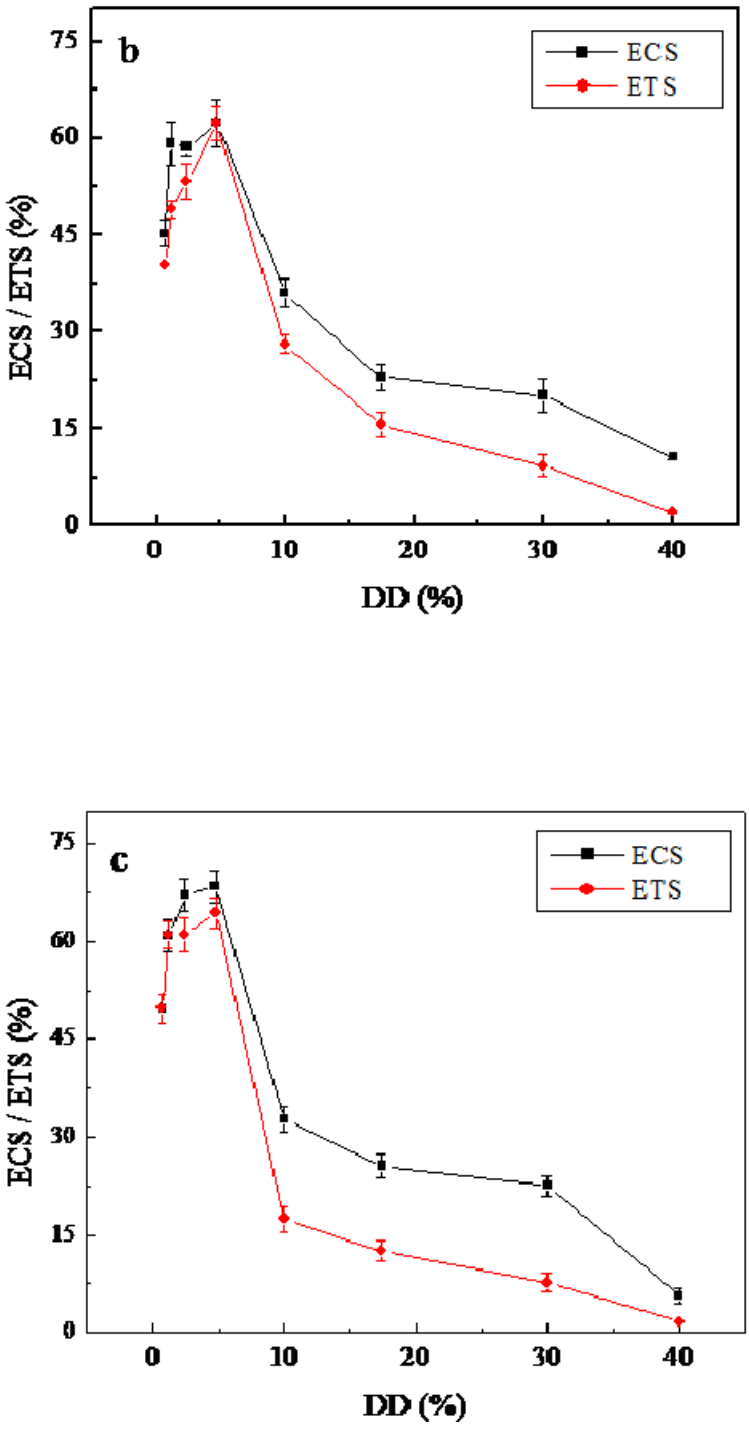

Figure 2-9. Emulsifying centrifuge and thermal stability of the deamidated prolamins at (a) pH 3, (b) pH 5 and (c) pH 7 as a function of DD value.

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Unmodified prolamin showed good emulsifying stability at both pH 3 and neutral conditions (ECS 57-61%, ETS 51%) in the authors' previous research [19], where low emulsifying property was found at pH 5 (ECS 31%, ETS 18%). These data were obtained by dehydrating unmodified prolamin at pH 11 followed by adjusting pH back to acidic and neutral conditions before evaluating their foaming property. In this study, the protein emulsifying stability was evaluated by dispersing deamidated prolamins in buffer solutions at different pH directly. As shown in Figure 2-9, an increase of the ECS value was also observed until the DD value of 2.4-4.7% at all the pHs tested. Increasing the DD value after 4.7%, the ECS value decreased rapidly. The change of the ETS values as a function of the deamidation degree followed the same trend. The initial increase of the ECS value can be attributed to increase of both protein solubility and exposed hydrophobic side chains, since protein solubility and hydrophobicity have a strong correlation with emulsifying properties [40-41]. The aggregated large peptides observed in the SEC chromatograms may also play an important role to stabilize the emulsions. Large peptides can generally form a rigid film at the oil/water interface to prevent the close contact of oil droplets, and decrease flocculation and coalescence [42-43]. A further increase of the DD value ($\geq$ 9.8%) resulted in decreased protein surface hydrophobicity as a result of protein unfolding to expose the polar side chains, dissociation of the large peptides, and extensive protein hydrolysis. All of these factors would prevent the formation of a continuous protein film at the oil–liquid interface, leading to reduced emulsion

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stability. The deamidated prolamin demonstrated an excellent capacity to stabilize the emulsion at a DD value of 2.4-4.7% as around 70% of formed emulsions remained event after heating and centrifuge processing. This favorable property is likely due to prolamin's unique molecular structure such as a strong surface hydrophobicity and a tendency to form aggregates. The excellent thermal stability may be due to further gelation of the deamidated prolamin around the oil droplets during thermal treatment to form a reinforced film.

2.4 Conclusions

In conclusion, deamidation was an effective approach to modify barley prolamin. The prolamin structural changes as a result of deamidation greatly influenced their functionalities. Limited deamidation (≤ 4.7%) could dissociate prolamin aggregates, leading to a great increase of protein solubility and protein surface hydrophobicity. These changes occurred without dramatically altering the secondary protein structure, and therefore improved protein foaming and emulsifying properties. With a further increase of the DD value to greater than 4.7%, prolamin underwent extensive hydrolysis and a marked conformational change, leading to decreased functionalities. The optical functionalities was obtained at a narrow DD range (2.4-4.7%) where prolamin samples demonstrated significantly improved solubility, emulsifying and foaming properties at both

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acidic and neutral pHs. These results suggest that prolamin would be an excellent candidate to develop as an emulsifying and foaming ingredient. To ensure wide applications in food and cosmetic industries, research toward a better understanding and control of solubility of prolamin and its aggregates are necessary.

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Chapter 3 Effects of deamidation on structure and emulsifying property of barley glutelin

3.1 Introduction

Barley is the fourth most widely cultivated cereal in the world after wheat, rice and corn [1]. Canada is the second largest barley producer in the world with an annual production of 12 million metric tons [2]. The majority of Canadian barley crop (80%) is used as livestock feed, the remaining 15% for malting and 5% for direct human consumption. Recently, barley is gaining increasing popularity as a part of the human diet because of the recent health claim made about its β-glucan [3]. This soluble dietary fibre component of barley is known to reduce both blood cholesterol and the glycemic index [4-5]. Techniques have been developed to isolate β-glucan from barley grains as a health ingredient in food products [6]. The remaining fractions are good source of proteins, starch and lipids [7]. These compounds are awaiting research to develop their full value. Barley grain contains 8 to 13% (w/w) protein depending on the varieties [8]. Due to the high proportion of nonpolar amino acid residues and high surface hydrophobicity, these proteins are soluble in water only with the presence of alcohol, high concentrations of urea,

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high concentrations of alkali (pH 11), or anionic detergents which strongly limits their applications [9]. Additionally, barley proteins are regarded as contaminants by the brewing industry and certain particular proteins are rather undesirable compounds. Thus, they are normally precipitated out in the spent grains and used as animal feed [10]. Development of strategy to improve barley protein solubility in water is essential to expand their usages for human consumption because protein generally has to be in a solution or in a fine suspension to exert other desired functionalizes.

Deamidation is one of the most used chemical methods to alter cereal protein structures and properties by converting the glutamine (Gln) to glutamic acid (Glu) [11]. This increased electrostatic repulsion among protein molecular chains can interfere glutamine aggregation via hydrogen bonding or partially unfold protein to improve protein solubility [12]. Additionally, the deamidation process will indirectly lead to protein hydrolysis by cleavage of the peptide bond [13]. In previous work, we investigated the structure, solubility, foaming and emulsifying properties of barley prolamin, one of the major barley storage proteins (35-55% of the total storage protein), when it is subjected to deamidation [14]. The results revealed that appropriate control of deamidation degree (DD) levels is crucial to achieve better functionalities. Limited deamidation (DD 2.4-4.7%) could

significantly improve prolamin solubility and its emulsifying and foaming properties due to dissociation of prolamin aggregates. Further increasing DD value after 4.7% resulted in extensive protein hydrolysis and conformational change, leading to decreased functionalities. Similar phenomenon was observed for many other proteins such as soy, corn and rice proteins, in which, a moderate deamidation increases protein chain flexibility and expose active groups to improve their functional properties, whereas, excessive deamidation normally results in undesirable functionalities to reduce protein utility [15-17].

Glutelin is the second abundant fraction in barley storage proteins (35-45% of the total storage protein) after prolamin [18]. Its amino acid compositions are characterized by high contents of glutamine (20.2%), proline (11.2%) and glycine (8.4%). Additionally, barley glutelin is enriched in hydrophobic amino acids (around 35%), with the highest levels corresponding to proline, leucine, alanine and valine. Barley glutelin demonstrated excellent emulsifying property due to balanced ratio of polar and non-polar amino acids [19]. This structure profile enables it to adsorb to the dispersed oil droplets quickly and to reduce the interfacial tension. Additionally, glutelin may aggregates to form a strong film, which can prevent close contact between the oil droplets, and decrease flocculation and coalescence. However, due to its poor solubility, glutelin requires

dehydration at pH 11 followed by adjusting pH back to acidic and neutral conditions to enable emulsifying functionalities. This procedure is not practical in commercial food systems. To the best of the authors' knowledge, research efforts to modify molecular structure of barley glutelin to improve its functionalities in aqueous solutions are currently unavailable. The protein structures determine its functionalities. Due to differences in amino acid composition and confirmation, previous knowledge obtained for barley prolamin can not applied directly to barley glutelin.

The objective of this research was to study the feasibility to improve barley glutelin functionalities in aqueous solution by deamidation with a focus on deamidation degree on subsequent glutelin structure (molecular weight, hydrophobicity and secondary structure). The emulsifying property of the deamidated glutelin was investigated.

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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 glutelin was extracted from barley grains according to our previous work [19]. The protein content of isolated glutelin was 82% (w/w) on a dry basis as determined by combustion with a nitrogen analyzer (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. Canola oil used for the emulsification study was purchased from a local supermarket (Edmonton, AB, Canada). Unstained standard protein molecule marker for SDS-PAGE was purchased from Bio-RAD (Richmond, CA, USA). Ammonia Assay Kit, o-phthaldialdehyde (OPA) reagent, 1-anilinonaphthalene-8-sulfonic acid (ANS), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) and 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 Canada Ltd. (Oakville, ON, Canada). All other chemicals were of reagent grade.

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3.2.2 Preparation of deamidated glutelin

Glutelin (5%, w/v) were suspended in 0.1 or 0.5 M NaOH solution at 50 ^{o}C under consistent stirring. Samples were withdrawn at different time intervals (10 - 120 min). After neutralized using 0.5 M HCl, the samples solutions were dialysis against deionized water and then freeze dried. The dried samples were stored at 4^{o}C until use.

3.2.3 Characterizations of the deamidated glutelin

3.2.3.1 Determination of the degree of deamidation, hydrolysis and surface hydrophobicity

The deamidation degree (DD) was determined by measurement of the released ammonia after deamidation using an Ammonia Assay Kit according to the manufacturer's instruction. DD was calculated as the ratio of ammonia generated in the sample to that of the completely deamidated protein. Complete deamidation was achieved by refluxing glutelin with 2 M HCl for 2 h.

The hydrolysis degree (HD) of were measured by the o-phthaldialdehyde (OPA) method (24). The deamidated glutelin sample (1.25 mg/mL) was dissolved in 12.5 mM borate buffer (pH 8.5) plus 2% (w/v) SDS. This solution (50 μL) was mixed with 1mL of the reagents which was composed of 50 mL of 0.1 M borate buffer (pH9.3), 1.25 mL of 20% (w/v) SDS solution, 100 mg of N,N-dimethyl-2-mercaptoethylammonium chloride (DMMAC), and 40 mg of OPA dissolved in 1mL methanol. The mixture was allowed to stand for 2 min before measurement of the absorbance at 340 nm. The number of amino groups was determined with reference to the L-leucine standard curve (between 0.5 and 5 mM). The HD was calculated by the following equation: HD (%) = $[(\alpha - ni) / nT] \times 100$, where nT was the total number of amino groups in original glutelin after total hydrolysis with 6 M HCl for 24 h and ni was the number of amino groups in glutelin, while α was the number of free amino groups measured in the deamidated glutelin.

The surface hydrophobicity of the deamidated glutelins in sodium phosphate buffer (pH 7) was determined using a fluorescence probe, 1-anilinonaphthalene-8-sulfonic acid (ANS), according to the method of Kato and Nakai [20]. Fluorescence intensity (FI) was measured at wavelengths of 390 nm (excitation) and 470 nm (emission) using a fluorospectrometer (FP-6300, Jasco, Tokyo,

Japan). The surface hydrophobicity degree (S_o) was calculated by linear regression analysis using the slope of the straight line obtained by plotting the FI as a function of the protein concentration.

3.2.3.2 Electrophoretic mobilities

The electrophoretic mobilities of the deamidated glutelin samples in pH 3, 5 (0.2M acetate buffer) and 7 (0.2 M phosphate buffer) buffers were measured by laser Doppler velocimetry using a Zetasizer NanoS (model ZEN1600, Malvern Instruments Ltd, Malvern, UK). Electrophoretic mobility (i.e., velocity of a particle within an electric field) was related to the zeta potential (ς) using the Henry equation [21]

$$U_E = \frac{2\varepsilon \times \zeta \times f(\kappa\alpha)}{3\eta}$$

where η is the dispersion viscosity, ε is the permittivity, and f ($\kappa\alpha$) is a function related to the ratio of particle radius (α) and the Debye length (κ). The results are reported as the average of at least three measurements. The same buffers were used in the following studies.

3.2.3.3 Electrophoresis

SDS gel electrophoresis was performed to study glutelin subunit molecular weight change after deamidation using a vertical mini-gel system (Mini-PROTEIN Tetra Cell, BIO-RAD, Hercules, CA, USA). Isolated protein fractions were mixed with the loading buffer (0.125 M Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 0.5% 2-mercaptoethanol and 1% bromophenol blue (w/v) and then heated at 100^{o}C for 5 min. After cooling, 12 µL sample (3 mg/ml) was loaded on 5% stacking gel and 12% separating gel and subjected to electrophoresis at a constant voltage of 80 V. After electrophoresis, the gels were stained with 0.1% (w/v) Coomassie Brilliant Blue-R-250 in water - methanol - acetic acid (4:5:1, v:v:v) for 30 min and destained with water -methanol-acetic acid (4:5:1, v:v:v).

3.2.3.4 Measurements of size exclusion chromatography combined with a laser photometer (SEC-LP)

The SEC-LP was performed with a DAWN-HELEOS laser photometer (Wyatt Technology Co., USA) combined a Agilent 1100 HPLC system (Agilent Technologies, USA) equipped with a Biosuite^{TM} 125/5 µm HR-SEC column

(7.8×300 mm, Waters Corp., Mass., USA) and a diode array detector (Agilent Technologies, USA). Each sample was dissolved in 50 mM phosphate buffer containing 150 mM sodium chloride, which was the eluent. The flow rate was 0.5 mL/min. All the solutions were first filtered with a 0.2 μm filter (Whattman, England). Protein sample was monitored at the UV wavelength of 280 nm. Standard molecular markers were used to calculate the weight-average molecular weight (M_w) of the deamidated glutelin. Astra software equipped with the DAWN-HELEOS laser photometer was utilized for the data acquisition and the analysis of the deamidated glutelin aggregates. The weight average molecular weight (M_w) of the aggregates was determined with the Zimm fit method and a Debye plot from the light scattering signal and according to the division principle of SEC.

3.2.3.5 FTIR spectroscopy

Infrared spectra of deamidated glutelin were recorded at room temperature using a Nicolet 6700 spectrometer (Thermo Scientific, Madison, WI, USA). Nitrogen was continuously run through the spectrometer. Deamidated glutelin samples (5%, w/v) were dissolved in D_2O solution. To ensure complete H/D exchange, samples

were prepared 2 days before and kept at 4 ℃ prior to infrared measurements. Samples were placed between two CaF_2 windows separated by 25-μm polyethylene terephthalate film spacer for FTIR measurement. To study the amide I region of the protein, Fourier self-deconvolutions were performed using the software provided with the spectrometer. Band narrowing was achieved with a full width at half maximum of 20-25 cm^{-1} and with a resolution enhancement factor of 2.0-2.5 cm^{-1}.

3.2.3.6 Determination of the disulfide (SS) content

The disulfide (SS) content change during deamidation reation was determined using DTNB according to the method of Ellman [22]. For free sulfhydryl content determination, 500 uL of the 1% protein solution was mixed with 500 ul buffer containing 8 M urea, 1% SDS, and 3 mM EDTA, 0.2 M Tris-glycine, pH 8.0. After adding 20 ul of DTNB, the reaction mixture was kept at 40 ℃ for 15 min in a water bath to allow unfolding of protein, thus all -SH groups were accessible to DTNB. The solution was then shaken at room temperature for 1h. Absorbance was then measured at 412 nm and used to calculate total SH content. The blank contained everything except the sample. For determination of total sulfhydryl (SH

+ reduced SS) content, a reaction buffer was used that consisted of 8 M urea, 3 mM EDTA, 1% SDS, and 0.2 M Tris-HCl (pH 9.5), with 0.1 M Na_2SO_3 and 0.5 mM 2-nitro-5-thiosulfobenzoate ($NTSB^{2-}$) synthesized from DTNB according to the procedure of Thannhauser et al (1987). The total sulfhydryl determination was continued as described above. Free SH and total SH contents were calculated from the absorption readings using a molar absorption coefficient of 13,600 M^{-1} cm^{-1} as: $A = \varepsilon bc$, where A is the absorbance readings, ε is the molar extinction coefficient, b is the cell thickness, and c is the concentration. Disulfide content was calculated as half the difference between total SH and free SH contents using the formula SS = (TS – SH)/2, where SS is disulfide content, TS is total sulfhydryl content (free SH + reduced SS), and SH is free sulfhydryl content.

3.2.4 pH solubility profile

Deamidated glutelins (125 mg) were dispersed in 25 mL, pH 3, 5 and 7 buffers. The dispersions were mixed for 1 h at 23°C using a magnetic stirrer before centrifuging at 1,200 × g for 20 min at 4°C. The supernatants were filtered through a Whatman No. 1 filter paper to obtain clear filtrates. The protein concentration in the filtrates was determined by Bradford dye assay with bovine

serum albumin as the standard [23]. The solubility was expressed as a percentage of the total protein content of the starting sample.

3.2.5 Emulsion properties

Emulsifying property was determined according to Yasumatsu et al. (1972) with a slight modification [24]. Deamidated glutelin samples (0.5%, w/v) were dispersed in 50 mL, pH 3, 5 and 7 buffers, followed by addition of 50 mL canola oil. The mixture was homogenized at speed "six" for 2 min to form an emulsion. The emulsion was then centrifuged at 1,500 × g for 5 min. The emulsion centrifugation stability (ECS) was calculated by measuring the volume of the emulsion (Ve_1) remaining after centrifugation and before (Ve_0), and recorded as ECS% = $Ve_1 / Ve_0 \times 100$. The emulsion samples were then heated to 80 ℃ in a water bath for 30 min and cooled to 23^{o}C. Upon cooling, these tubes were centrifuged at 1,500 × g for 5 min. The volume of the remaining emulsified fraction Ve_2 was recorded. The emulsion thermal stability (ETS) was calculated according the equation: ETS% = $(Ve_2 / Ve_0) \times 100$.

Statistical analysis

All experiments were performed at least in triplicate. Error bars on graphs represent standard deviations. Statistical significances of the differences were determined by Student's t-test. The level of significance used was $p < 0.05$.

3.3 Results and Discussion

3.3.1 Preparation of glutelin of different deamidation degree (DD)

The optimization of the reaction conditions was based on preliminary data that indicated glutelin deamidation in 0.1 or 0.5 M NaOH at 50 ^{o}C was efficient to prepare samples with different degree of deamidation (DD). Samples that possessed DD values of 1.0, 2.2, 5.6 % obtained at 0.1 M NaOH at 40 ^{o}C and DD values of 15, 31, 43% obtained at 0.1 M NaOH at 40 ^{o}C (Table 3-1) were selected for the following study.

Table 3-1. Deamidated glutelins of various DD value prepared by alkaline method at different conditions

Sample	NaOH (M)	Temperature (^{o}C)	Time (min)	DD %
1	**0.1**	**50**	**40**	**1.0**
2	**0.1**	**50**	**50**	**2.2**
3	**0.1**	**50**	**80**	**5.6**
4	**0.5**	**50**	**40**	**15**
5	**0.5**	**50**	**90**	**31**
6	**0.5**	**50**	**120**	**43**

3.3.2 Characterizations of deamidated glutelin

The zeta-potential of the glutelin samples in different pH buffers is expressed as a function of their DD value (Figure 3-1). Significant increase of surface charge (near zero to -8.1) was observed for glutelin at pH 5 when DD value was raised from 1.0 to 5.6%, indicating the shifting of the isoelectric point (IEP) of glutelin to more acidic pH after deamidation [13]. Further raising the DD value after 5.6% led to gradual increase of the glutelin molecular surface negative charge due to increase in the content of carboxyl group ($-COO^{-}$) on protein molecular chains.

The increase of the zeta-potential with DD was also observed for deamidated glutelin at pH 7, but the surface charge was generally higher than those at pH 5 because more carboxyl groups were deprotonated at neutral pH. At pH 3, deamidated glutlelin molecule surface was positively charged because the carboxyl groups were protonated and the cationic amino groups ($-NH_3^+$) dominated the surface. Increasing DD value did not significantly impact the surface charge of deamidated glutelin at pH 3. According to Figure 3-1, IEP of deamidated glutelins was between pH 3 and 5 where a deamidated glutelin is electrically neutral.

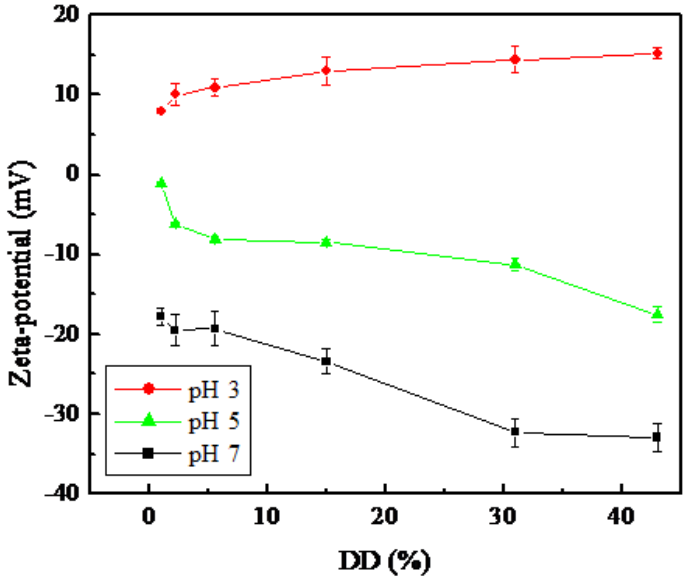

Figure 3-1. Electrophoretic mobilities of the deamidated prolamins at different pH as a function of DD value.

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The hydrolysis degree (HD) and surface hydrophobicity (S_o) of the deamidated glutelin samples are shown in Figure 3-2. Even limited deamidation resulted in extensive hydrolysis of glutelin as HD value reached 16% at DD value of 1.0%, indicating that glutelin peptide bond cleavage occurred very rapidly at initial stage of deamidation. Further increasing DD value to 43% only led to slight increase of glutelin hydrolysis degree (16 to 23%).

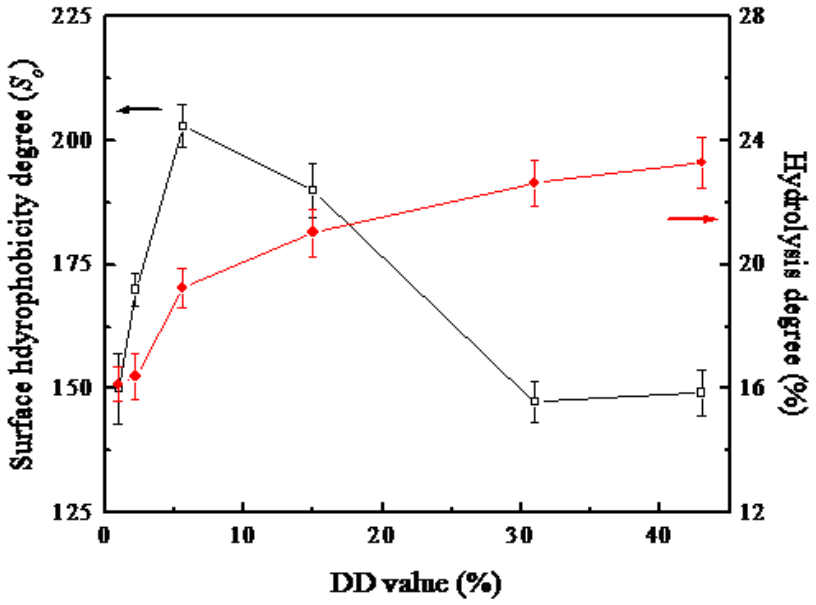

Figure 3-2. Degree of hydrolysis and surface hydrophobicity of the deamidated glutelins as a function of DD value.

Surface hydrophobicity of the deamidated glutelin increased markedly ($p < 0.05$) with the DD increasing to 5.6%, suggesting that the hydrophobic regions were

exposed outside. A further increase of the DD value resulted in significant decrease ($p < 0.05$) of the prolamin surface hydrophobicity. Barley glutelin is known to be hydrophobic with the highest level corresponding to proline, leucine, alanine and valine. Some non-polar amino acids may be buried inside the molecules. Limited deamidation reaction seems to promote the glutelin S_o increment due to hydrophobic zone exposure. As a consequence of protein unfolding and electrostatic and hydrophobic forces rearrangement, the hydrophobic regions reoriented to the surface. Whereas, higher deamidation (DD > 5.6%) could lead to protein extensive hydrolysis, resulting in shorter peptides with a higher charge density ($-NH_3^+$ and $–COO^-$ groups). Thus a decreased glutelin surface hydrophobicity was observed.

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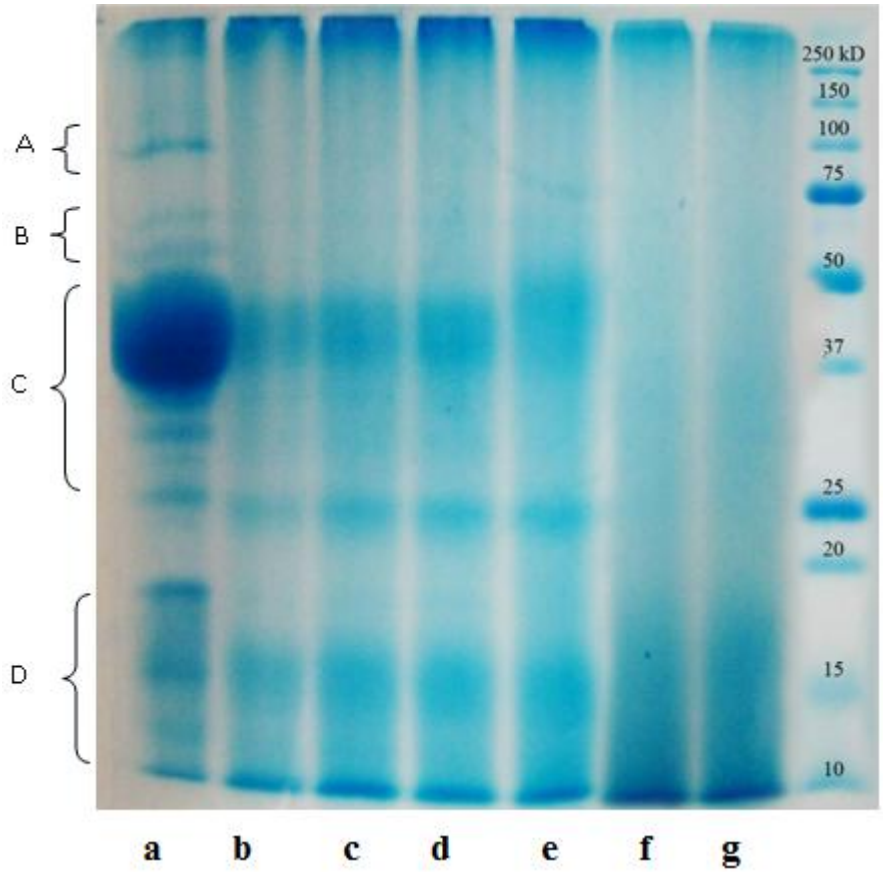

Figure 3-3. SDS-PAGE pattern of the deamidated glutelin (a: unmodified glutelin, b: DD 1.0%, c: DD 2.2%, d: DD 5.6%, e: DD 15%, f: 31% and g: 43%).

The SDS-PAGE pattern of barley glutelin (unmodified) showed 4 major bands at 85-90, 55-70, 25-50, <20 KDa, respectively (Figure 3-3). It was reported that the 85-90 KDa band could be D-hordeins, which were limited soluble in ethanol alone, but could be extracted in alkaline solution [25]. The broad band at 25-50 may contain some B hordeins, because it was not possible to prepare an

undenatured glutelin fraction free of contaminating prolamin [25]. The band of D-hordeins disappeared and the intensity of other bands significant decrease even at DD of 1%. After DD of 15%, all bands disappeared. SDS-PAGE result confirms that rapid and extensive glutelin hydrolysis occurred at initial stage of deamidation.

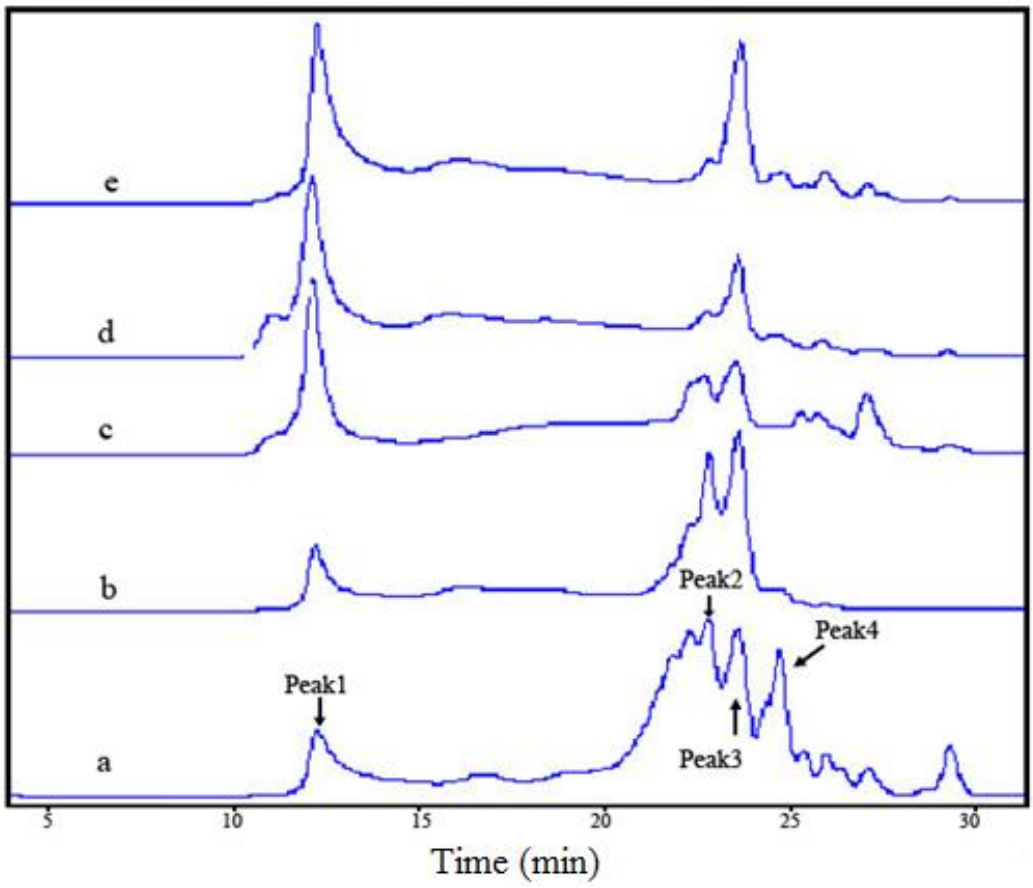

Figure 3-4. SEC-HPLC chromatograms of the deamidated glutelins (a: DD 1.0%, b: DD 2.2%, c: DD 5.6%, d: DD 15%, e: DD 43%).

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SEC chromatograms of the deamidated prolamins in phosphate buffer are depicted in Figure 3-4. Deamidated glutelin with the DD value of 1.0% contains peaks in two major elution volume areas. Peaks 2-4 appeared at the high elution volume area corresponding to M_w of 3.7 – 12 KDa, 2.5 KDa and 1.4 KDa, respectively. These peaks can be assigned to glutelin hydrolysates after deamidation as their M_w was much lower than *Mr* of glutelin subunits demonstrated in SDS-PAGE Pattern. Surprisingly, a narrow peak (peak 1) was observed at the low elution volumes, exhibited rather large M_w (>600 KDa). This peak seems to be attributed to aggregates of the deamidated glutelin. With DD increasing, hydrolysate bands intensity decreased accompanied by a significant increase in aggregate bands. The SEC data suggests that deamidated glutelin hdyrolysates have a high tendency to aggregate to form large peptides soluble in aqueous solution.

The absolute M_w and the gyration radius (R_g) for deamidated glutelin aggregates were then analyzed with a laser photometer. The LP signals corresponding to deamidated glutelin hydrolysates are very weak compared to UV signals because of their small molecular weight. The data are summarized in Table 3-2.

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Table 3-2. Results from size exclusion chromatography combined with a laser photometer (SEC-LP) method.

Sample No.	DD	SEC-LP		
		$M_{w,,a}$ (g/mol)	R_g (nm)	α
1	DD 1.0%	3.8×10^6	68.2	0.27
2	DD 2.2%	4.1×10^6	68.5	0.27
3	DD 5.6%	8.6×10^6	68.7	0.25
4	DD 15%	2.2×10^7	176.1	0.27
5	DD 43%	7.3×10^7	198.5	0.27

These aggregates exhibited very high molecular weight with M_w reaching 10^6 – 10^7. With increasing DD value from 1.0 to 43%, M_w of increased gradually from 3.8×10^6 to 7.3×10^7, meanwhile the gyration radius raised from 68 to more than 200 nm. This result indicates that crosslinking degree of deamidated glutelin hydrolysates were enhanced with DD increasing. Because the R_g is proportional to the geometrical size for linear molecules, a log-log plot of R_g versus M_w permits the extraction of information on molecular conformation [26]. Spherical molecules should yield a plot with a slope of 0.33; if the molecules are a flexible coil in a good solvent, the slope should be between 0.5 and 0.6; for a rod-like chain, the slope should be 1.0. Aggregates of the deamidated glutelin show α values varying mainly from 0.25 to 0.27, indicating a near spherical shape.

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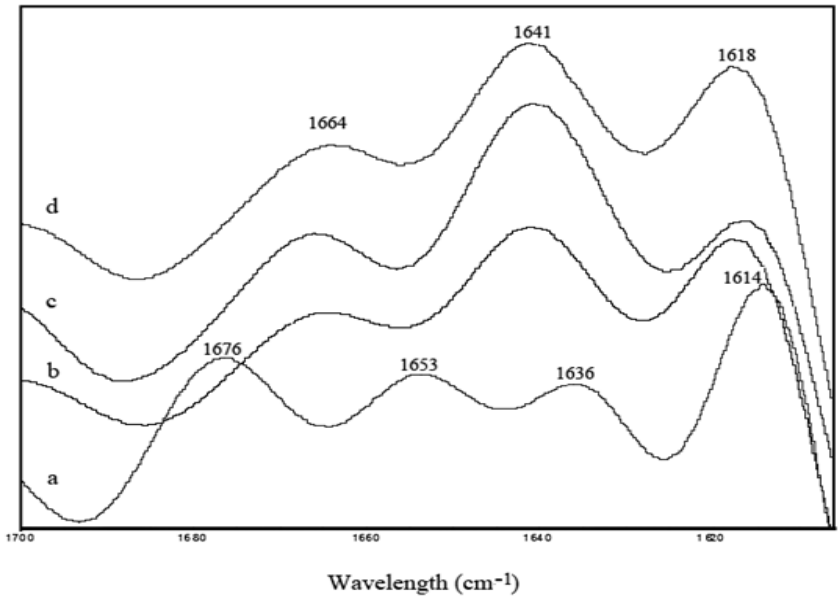


Figure 3-5. FTIR spectra of the deamidated glutelins (a: DD 1.0%, b: DD 2.2%, c: DD 15%, d: DD 43%).

In general, protein aggregation seems to be associated mainly with conformational and structural changes that expose some of the active residues. In order to have a deeper understanding of the aggregation process of deamidated glutelin, Fourier transform infrared spectroscopy (FTIR) experiment was conducted to study the glutelin secondary structure change during deamidation by analyzing the amide I band. The glutelin sample with DD of 1.0% shows several bands in the amide I region (Figure 3-5), which were assigned to protein secondary structures according to previous reports: α-helices (1653 cm^{-1}), β-sheets (1636 cm^{-1}) and β-turn (1676 cm^{-1}) [27-29]. The bands at 1614 cm^{-1} could

be a combination of intermolecular β-sheet structure (1619 cm^{-1}) and the amino acid side-chain residue vibration (1608 cm^{-1}). When the DD value was increased to 2.2%, marked shifts in the band positions were observed. The absorption corresponding to α-helices (1653 cm^{-1}) and β-sheets (1636 cm^{-1}) disappeared, whereas new absorptions arise at 1619, 1641 and 1664 cm^{-1}. The bands at 1641 cm^{-1} was assigning to random coils, whereas the bend at 1664cm^{-1} could be mainly assigned to re-organized β-turn structure. The strong absorption at 1641 cm^{-1} suggests that glutelin was denatured, likely as a result of both strong negative charge on molecular chains and extensive protein hydrolysis. The band at 1619 cm^{-1} corresponds to intermolecular β-sheet structure, formation of which appears to be a key event in the aggregation of denatured glutelin [27]. With DD increasing from 2.2 to 43%, no significant change of FTIR absorptions was observed. The FTIR result confirms that protein unfolding and aggregation occurred rapidly during initial stage of glutelin deamidation.

The change of disulfide (SS) is another factor of tendency to aggregation in a protein. In the unmodified barley glutelin, a total amount of SS group of 5.6 nmol/mg protein was obtained. As can be observed from Figure 3-6, SS content increased with DD increasing, which was more pronounced at higher DD range. When DD was 43%, SS amount was raised to 15.6 nmol/mg protein. This

indicates that some of the exposed SH groups were oxidized to form new disulfide bonds. In fact, the exposed sulfhydryl groups are rather reactive at neutral or alkaline pH and therefore can be expected to be involved in the sulfhydryl-disulfide exchange reaction during deamidation at elevated temperature [30]. This reaction has been reported to result in the formation of protein gel networks such as egg white and whey protein [31-32].

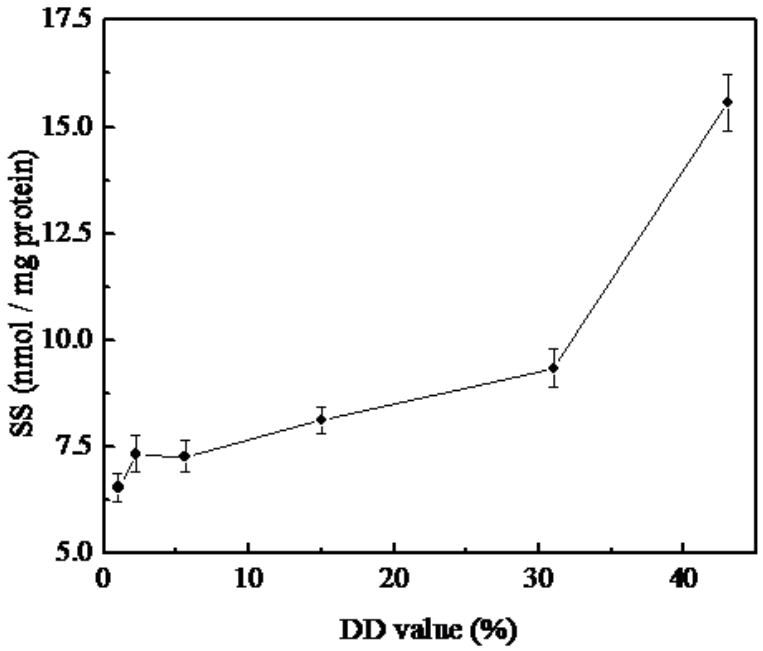


Figure 3-6. Change of disulfide bond content as a function of DD value.

In the case of deamidated glutelin, such reaction resulted in formation of high molecular weight soluble aggregates instead, probably due to formation of different protein network structures. It is deduced that during deamidation

reaction barley glutelin was quickly unfolded and aggregated via intermolecular β-sheet structure. These aggregates were then stabilized through formation of disulfide bonds. In addition, heating during deamidation could promote hydrophobic interactions to promote the glutelin aggregation, originated by the exposed hydrophobic core of the unfolded protein [33- 34].

3.3.3 Comparison of deamidated glutelin with deamidated prolamin

Structures of the deamidated barley glutelin were then compared to those of prolamin investigated in our previous work. Glutelin hydrolysis was much more rapid than prolamin during deamidation as an extensive protein hydrolysis was observed even at DD value of 1.0% as demonstrated by HD, SDS-PAGE and SEC data. For the latter, extensive protein hydrolysis were only observed after DD value of around 5%. This difference was probably caused by different structures of these two barley protein fractions. Barley prolamin (1.83%, w/w) has higher amount of cysteine residue than glutelin (0.72%, w/w), its B-hordein subunit is especially rich in sulfur groups. Most B and D hordein subunits are linked by inter-chain disulfide bridges. Such greater amount of inter-chain

disulfide bonds makes prolamin more resistant to hydrolysis [25]. Additionally, C-hordein subunit consists 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 [35]. The high proline content of C-hordein makes it a challenging substrate for hydrolysis [36]. Plus, FTIR spectra indicate a rapid glutelin unfolding at the initial stage of deamidation (DD value of around 2%), whereas, no significant prolamin unfolding was observed before DD value of around 10%. Such rapid protein molecule unfolding could also promote glutelin hydrolysis during deamidation due to exposure of new residues, making additional sites susceptible to deamidation. Another major difference is that soluble glutelin aggregates with very high molecular weight were formed during deamidation due to hydrophobic interactions and disulphide-crosslinking after glutelin unfolding. Although soluble large peptides (~ 114 KDa) were also observed in SEC chromatographs of deamidated prolamin in the DD range of 2.4-4.7%, these peptides arise probably as a result of dissociation of the prolamin aggregates. With increasing DD greater than 10%, the large peptides were hydrolyzed. Whereas glutelin aggregates exhibited much higher molecular weight (10^6 – 10^7), and they are rather stable and completely soluble in aqueous solution. Actually, such phenomenon was seldom seen on other deamidated food proteins in previous literatures. The formation mechanism and kinetics of these aggregates during deamidation is still

far from clear because the published information on barley protein confirmation is very limited. However, these deamidation induced aggregation may have significance in glutelin functional properties.

3.3.4 Solubility

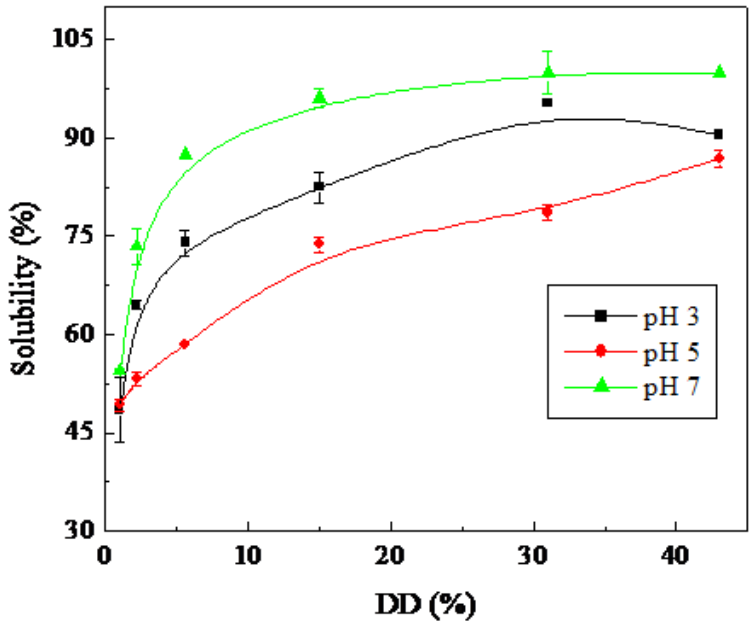


Figure 3-7. Solubility of the deamidated prolamins at different pH as a function of DD value.

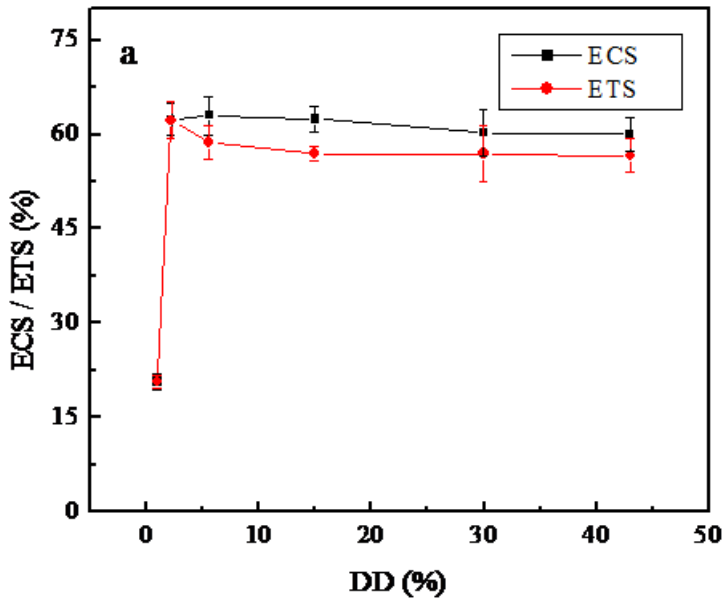
Unmodified barley glutelin showed limited solubility (< 20%) at pH 3-7. As demonstrated in Figure 3-7, even limited deamidation could significantly improve glutelin solubility as around 50% glutelin was well dissolved at both acid and

neutral pHs at DD value of 1.0%. Glutelin solubility increased rapidly when DD was raised from 1 to 15% and the solubility value reached 83, 74 and 96% at pH 3, 5 and 7, respectively. The solubility of the deamidated glutelin was the highest at pH 7, whereas lower at pH 3 and 5 when near their IEP. The improvement in solubility could be due to the protein unfolding and extensive hydrolysis. These structure changes led to the exposure of more charged and polar groups to the surrounding water, thus promoting protein-water interaction and an increased solubility [28]. The remarkably improved solubility after deamidation at both acidic and neutral pHs is expected to significantly improve glutelin functionalities in aqueous solutions to broaden its applications.

3.3.5 Emulsifying properties

Emulsions are widely used in food industry, mainly in food stuffs such as mayonnaises, sauces, creams, dairy product analogs etc. Protein emulsifying capacity and stability is related to the ability of the protein to reach, adsorb and unfold rapidly at the oil/water interface, and then to form a visoelastic film around the oil droplets. Proteins of greater flexibility and surface hydrophobicity are suitable to form emulsions [37]. Additionally, high molecular weight proteins

exhibit greater film strength and emulsion stability [38]. Barley glutelin demonstrated strong emulsifying property at both pH 3 and neutral conditions, with maximum ECS values reaching 68% and 73%, respectively, as demonstrated in our previous work. A relatively lower ECS value (35%) was found at pH 5. However, due to its inherent poor solubility, unmodified prolamin requires dehydration at pH 11 followed by adjusting pH back to acidic and neutral conditions to enable emulsifying functionalities, which is not convenient for practical applications. Deamidation significantly improved glutelin solubility even within a limited DD, thus allowing functionality testing by dispersing samples at different pH buffers directly.



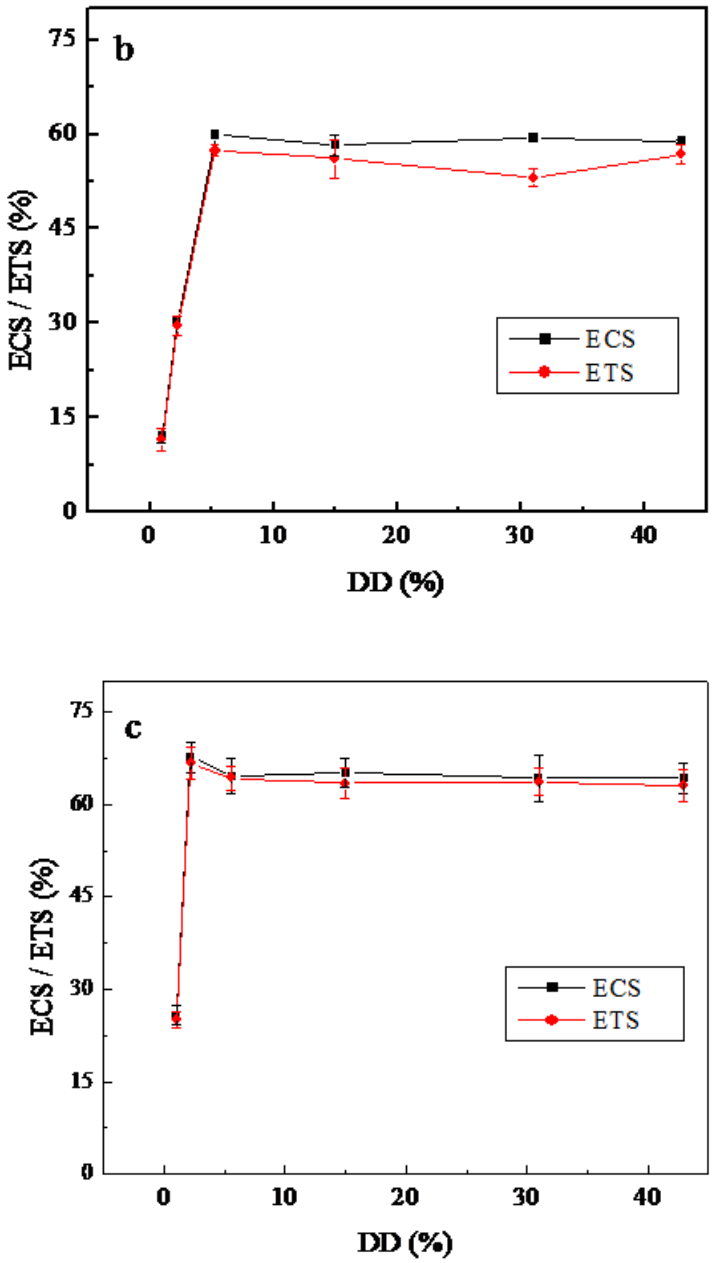


Figure 3-8. Emulsifying centrifuge and thermal stability of the deamidated glutelin at (a) pH 3, (b) pH 5 and (c) pH 7 as a function of DD value

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As shown in Figure 3-8, low ECS value (12-20%) was observed for deamidated glutelin at all the pHs tested when DD value was 1.0%. Increasing DD value from 1.0 to 5.6% greatly enhanced its emulsifying property. ECS values were raised from around 20 to 62% and 68% when DD was raised from 1.0 to 2.2% at pH 3 and 7, respectively. Although lower ECS value was observed at pH 5 (near IEP) at DD value of 2.2%, this value increased to 60% when DD was raised to 5.6%. This increase of ECS values reflects the enhanced solubility of glutelin after deamidation, enabling easy protein diffusion to the oil/water surface. Moreover, the exposed hydrophobic patches on glutelin surface facilitated their binding at hydrophobic air surfaces, and then these proteins could aggregate via surface hydrophobic patches to form films around bubble surfaces. Interestingly with further increasing of DD from 2.2-5.6 to 43%, ECS values of deamidated glutelin were almost unchanged and maintained at rather high value as more than 60% emulsion remained even after centrifugation. This phenomenon is different from those observed for many other proteins such as soy, corn and rice proteins, in which appropriate levels of deamidation improve protein emulsifying properties, however, excessive deamidation normally greatly destroy protein emulsifying capacity to reduce protein utility [15-17]. In our previous work, deamidated prolamin also demonstrated significantly improved functionalities including

emulsifying properties in aqueous solutions only at a narrow DD range (2.4-4.7%). With further increasing of DD after 4.7%, prolamin emulsifying property decreased quickly at both acid and neutral pHs. The reduced protein emulsifying property as a result of excessive deamidation is probably due to extensive protein hydrolysis during chemical deamidation reactions which would prevent the formation of a continuous protein film at the oil/water interface to stabilize the emulsions. In this work, deamidation reaction also led to extensive glutelin molecule hydrolysis, however glutelin demonstrated strong aggregation tendency during deamidation due to hydrophobic interactions and disulphide-crosslinking after glutelin unfolding. These aggregates exhibited very high molecular weight and are highly soluble and stable in aqueous solution. It is deduced that the aggregated soluble large peptides observed in the SEC-LP chromatograms play a major role to stabilize the emulsions because large peptides can generally form a rigid film at the oil/water interface to prevent the close contact of oil droplets, and decrease flocculation and coalescence [38-39]. A slightly higher ECS values were observed at pH 7 than those at pH 3 and 5 at all the DD values tested. This may be attributed to higher surface charge of deamidated glutelin when deviated from IEP which could better prevent the suspended emulsion droplets from approaching each other, thus avoid flocculation and coalescence. Similar ETS and ECS values were observed for all deamidated glutelin samples at pH 3, 5 and 7, suggesting that barley glutelin stabilized emulsions have an excellent thermal

stability which may be due to further gelation of the deamidated glutelin around the oil droplets during thermal treatment to form a reinforced film. This result indicates that deamidated glutelin can exert a strong emulsifying effect at a very broad DD range (5.6 to 43%). In this way, undesirable property changes resulted from excessive deamidation can be avoided to facilitate wide applications of deamidated barley glutelin as an emulsifying ingredient in food and non-food applications.

3.4 Conclusions

In summary, deamidation effectively improved barley glutelin solubility and emulsifying properties. Even limited deamidation (2.2%) could result in glutelin unfolding and extensively hydrolysis, which subsequently triggered glutelin aggregation based on disulphide-crosslinking and hydrophobic interactions. These aggregates exhibited very high molecular weight and are highly soluble and stable in aqueous solution which played a major role to stabilize emulsions at a broad range of DD value (5.6-43%). These results suggest that deamidated glutelin would be an excellent candidate to be developed as an emulsifying ingredient.

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Chapter 4 Summary and future work

4.1 Summary

Barley proteins constitute 8-13% in barley grains with limited application. One of the main reasons is their low solubility. Additionally, barley proteins are regarded as contaminants by the brewing industry. Thus they are normally precipitated out in the spent grains and used as animal feed. Development of strategy to improve the solubility of barley protein is essential to expand their application for human consumption because protein generally has to be in a solution or in a fine suspension to exert other desired functionalities. This study evaluated the effects of deamidation on the functionality of barley prolamin and glutelin. Samples with various deamidation degrees were prepared using the alkaline method, 0.7- 40% for prolamin and 1- 43% for glutelin. The effects of deamidation degree on protein structures, solubility, emulsifying and foaming properties were systematically investigated. Based on the results obtained in this study, my conclusions can be stated as follow:

4.1.1 Summary of deamidation of prolamin

SDS-PAGE, size exclusion chromatography (SEC) and Fourier transform infrared (FTIR) results suggested that limited deamidation ($\leq$ 4.7%) could dissociate prolamin aggregates, leading to a great increase in protein solubility and surface hydrophobicty. Increasing in the DD value above 4.7% resulted in extensive protein hydrolysis and a marked change in protein secondary structure.

The optimal functionalities were obtained at a narrow DD range (2.4-4.7%), where prolamin samples demonstrated significantly improved solubility, emulsifying and foaming properties at both acidic and neutral pHs.

These results suggest that deamidated prolamin would be an excellent candidate to be developed as an emulsifying and foaming ingredient.

4.1.2 Summary of deamidation of glutelin

The results about glutelin revealed that a rapid hydrolysis and unfolding happened at the initial stage of deamidation (DD 2.2 to 5.6%), leading to remarkably improved glutelin solubility at both acidic and neutral pHs.

Unlike prolamin, the deamidated glutelin demonstrated a strong tendency to form soluble aggregates with a spherical shape and a very large molecular weight (10^6 – 10^7 kDa), probably due to disulfide-crosslinking and hydrophobic interactions. These aggregates played a major role in stabilizing the emulsions, allowing more than 60% emulsion to remain even after centrifugation and heating treatment at a broad range in DD value (5.6-43%). In this way, undesirable property changes resulting from excessive deamidation can be avoided to facilitate wide applications of deamidated barley glutelin as an emulsifying ingredient in food and non-food applications.

4.2 Future work

Future research in this area is likely to focus on the following subjects: the detailed mechanism of emulsion and foaming properties; the reasons for the aggregation formation in glutelin and the potential for protein-polysaccharide conjugates to improve emulsifying and foaming stability.

My study found that deamidation could increase protein surface hydrophobicity, resulting in high emulsifying and foaming properties. The changes during

deamidation, such as interfacial tension, interfacial rheology and protein flexibility, may also contribute to the improvement of functionalities. Thus, experiments to evaluate the effects of deamidation degree on interfacial tension, interfacial rheology and protein flexibility should be conducted. This research could reveal the mechanism of barley protein emulsifying and foaming properties in relation to protein structure.

I also found that glutelin tends to form aggregates during deamidation which plays an important role in stabilize emulsions. According to the literature, specific amino acids sequence may lead to aggregation during protein denaturation; the ratio and the size of the aggregation are crucial for functionality. However, the information on the detailed mechanism of food protein aggregation produced by deamidation and their ability to stabilize emulsions is limited. Therefore, further research is needed in this area to reveal the mechanism of aggregation formation during deamidation.

Preliminary experiment indicated that the emulsifying and foaming properties of the deamidated barley protein can be further improved by mixing with polysaccharide (data was not shown). Thus, further research is necessary to explore the opportunity to develop a new emulsion/foam stabilizer based on deamidated barley protein-polysaccharide conjugates. Specifically, the detailed

mechanism including the interaction of protein and polysaccharide in aqueous phase and in emulsion interface is still obscure. Therefore, more research is needed in this area.