University of Alberta

Emulsifying Properties of Deamidated Barley Protein Fractions

by

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Abstract

This work aims to develop value-added applications of barley protein. Our previous work demonstrated that deamidation could significantly improve solubility and emulsifying properties of barley protein. In this research, barley protein with deamidation degree of 63% was separated by ulta-filtration into three fractions carrying similar surface charge: large-sized fraction with weight-average molecular weight (M_w) exceeding 300 kDa, medium-sized fraction with M_w 20 – 300 kDa and small-sized fraction with M_w 5 – 20 kDa. The emulsifying properties of these fractions were evaluated in relation to molecular weight and hydrophobicity at different pHs. The large-sized fraction showed excellent emulsifying properties at neutral pH, but poor emulsifying stability at acidic pH. The small-sized fraction exhibited poor emulsion properties. The results indicate that the emulsifying property of the deamidated barley protein was significantly influenced by molecular weight, and the large-sized fraction can be used as potential natural emulsifier in food, cosmetic and other areas.

Key words: Barley protein, deamidation, molecular weight, emulsifying properties

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List of Abbreviation

ANS	8-anilinonaphthalene-1-sulfonic acid
CMC	critical micelle concentration
CI	creaming index
DD	deamidation degree
DLVO theory	Derjaguin-Landau-Verwey-Overbeek theory
EA	emulsion activity
EAI	emulsion acitivity index
EC	emulsion capacity
ECS	emulsion centrifuge stability
ES	emulsion stability
ESI	emulsion stability index
HLB	hydrophilic/lipophilic balance
HPLC	high-performance liquid chromatography
IEP	isoelectric point
kDa	kilo Dalton
M_w	weight-average molecular weight
SEC	size exclusion column

Chapter 1 Literature Review

1.1 General Information for Barley

Barley is ranked as the fourth important cereal in terms of production in the world [1]. The importance comes not only from its long cultivated history but also from its wide adaption to areas with extreme climate, such as high latitude regions and even deserts [2]. Based on its growing seasons, different morphologies, presence or absence of hull, and grain compositions, barley can be classified into spring or winter types, two row or six row types, hulled or hulless types, and normal, waxy or high nutrient (high amylose starch, high β-glucan, high lysine *etc.*) types [2]. The different classes of barley often present different properties and diverse physical and chemical characteristics, which lead to wide end uses [2]. For example, hulless barley is more suitable for human comsumption, while hulled barley is more favored by malting and brewing industry because its hull can contribute to beer flavor and brewing processing [2].

Barley Production. With growing and harvesting more than 50 barley varieties, Canada is one of the largest barley producers and exporters in the world [3].

Western areas, such as Alberta, Manitoba and Saskatchewan, are the major areas for barley production in Canada [3]. According to Table 1-1, Western Canada produced around 8,122 kilotons of barley in 2012, which is 11% of increase from 2011 [3]. Among these barley growing provinces, Alberta produced the most amount of barley, which is 4,607 kilotons in 2011, and 4,914 kilotons in 2012.

Table 1- 1. Barley production in Western Canada 2011-2012, adapted from [3].

Province	**Seeded area($\times 10^3$ Ha)**		**Production($\times 10^3$ Tons)**	
	2011	**2012**	**2011**	**2012**
Alberta	1442	1564	4607	4914
Saskatchewan	880	1068	2439	2639
Manitoba	138	230	261	569
Total	2460	2862	7307	8122

Barley Uses. Barley was used to be a major food source in ancient times. However, it has been replaced by wheat and rice, which present better product quality and mouth-feel [2]. According to report, a majority amount of barley (80%) is used for livestock feeding; a small amount of barley (15%) is selected for malting and brewing in beer industry; and very limited amount of barley (5%) is consumed as human food to make noodles, bread, breakfast cereals *etc.* [1, 5]. In

recent years, there is growing recommendation for increasing utilization of barley products for human consumptions, because they contain good source of dietary fiber, high content of protein, and a good source of minerals [2, 6].

1.2 Nutritional Components in Barley

Table 1- 2. Composition of hulled and hulless barley grains (dry matter basis), adapted from [7].

	Hulled		Hulless	
Components	**Average[a]**	**Range**	**Average[a]**	**Range**
Starch	58.2	57.1-59.5	63.4	60.5-65.2
Fiber	20.2	18.8-22.6	13.8	12.6-15.6
Protein[b]	13.7	12.5-15.4	14.1	12.1-16.6
Sugars[c]	3.0	2.8-3.3	2.9	2.0-4.2
Lipids	2.2	1.9-2.4	3.1	2.7-3.9
Ash	2.7	2.3-3.0	2.8	2.3-3.5

a: n=3

b: N×6.25

c: glucose, fructose, sucrose, and fructans.

The major nutritional compositions (average values) of barley kernel are showed in Table 1-2 [7]. Different types of barely grains may have different chemical compositions based on genotypes, environmental conditions *etc.* [7]. Starch, fiber, and protein account for the largest proportion of barley kernel [7]. In recent decades, there is growing research interest in barley starch, dietary fiber (β-glucan) and protein. Scientists around the world never stop from exploring commercial values for barley grains.

1.2.1 Starch

Barley contains a large proportion of starch, which is important for brewing industry. Apart from brewing industry, starch components are important factors that influence food application of barley. Barley starch is consisted of two structural types, amylose and amylopectin [7]. Amylose contents decide the end use of barley in food area. Amylose is short linear starch molecules, which is formed by α-(1,4)-D-glucose units [8]. Barley with high amylose level exhibits high expansion and low density, which can be used for snacks and ready-to-eat cereals; while barley with low amylose level shows soft and less chewy textures, which can be a good candidate for rice extender and substitute [2]. Amylopectin is the major starch type, which composes of 72% to 78% in total, in most barley species [7]. Amylopepctin is a heavily branched polysaccharides with α-(1,4)-D-glucose branched via α-(1,6)-D-glucose linkage [7]. It has been

reported that starches with high amylopectin content were digested and absorbed faster than starches that were high in amylose [9].

1.2.2 Beta-glucan

Barley contains 4 – 7% of beta-glucan [10]. Beta-glucan is a soluble dietary fiber, and it exhibits notable health benefits, including lowering blood cholesterol, reducing heart disease, controlling blood glucose and insulin levels in type-2 diabetes [2, 10]. Based on its health benefits, beta-glucan is gaining more and more popularity for scientific research in modern times. The idea of incorporating barley beta-glucan into cereal based food has also been examined [11]. It has been reported that the enrichment of barley beta-glucan in pasta and wheat bread was able to significantly lower blood glucose level [12, 13].

1.2.3 Barley Protein

The protein content in barley was commonly reported as 8 – 13% [4]. As another major by-products obtained in beer industry, protein in leftover malted barley can go up to 20% of the total waste [14]. If proteins coming from brewing industry can be efficiently recycled and reused, we will significantly enhance the commercial value of barley grains. 18 L-amino acids in total have been detected in barley proteins, as shown in Table 1-3 [7]. The high content of glutamine and asparagine residues in barley protein may cause the aggregation of protein

Table 1- 3. Amino acids composition (g/kg) of hulled and hulless barley at two types of proteins (dry matter basis), adapted from [7].

	Types 1[a]		Type2[b]	
Protein and Amino Acids	**Hulled**	**Hulless**	**Hulled**	**Hulless**
Protein(n×6.25)	13.2	14.0	11.2	11.7
Amino acids				
Alanine	0.44	0.47	0.35	0.38
Arginine	0.60	0.64	0.45	0.50
Asparagine (Aspartic acid)	0.71	0.75	0.55	0.57
Cystine	0.28	0.31	0.20	0.20
Glutamine (Glutamic acid)	2.98	3.24	2.28	2.44
Glycine	0.42	0.44	0.32	0.34
Histidine	0.26	0.28	0.20	0.22
Isoleucine	0.43	0.46	0.34	0.37
Leucine	0.79	0.84	0.67	0.71
Lysine	0.41	0.41	0.31	0.34
Methionine	0.20	0.28	0.15	0.17
Phenylalanine	0.68	0.73	0.51	0.53
Proline	1.32	1.43	0.96	0.98
Serine	0.54	0.57	0.41	0.45
Threonine	0.42	0.45	0.38	0.37
Tryptophan	0.22	0.23	0.14	0.15
Tyrosine	0.37	0.42	0.32	0.33
Valine	0.59	0.63	0.46	0.46

a: Montana Agriculture Experiment Station, unpublished data, n=8.

b: Truscott *et al.* (1998), n=6.

molecules by hydrophobic and hydrogen bonds [15]. So, barley proteins are not used often in processed food products since they have low solubility in aqueous solutions [15]. The protein quality of barley protein is relatively low, compared to meat, poultry and dairy products [7]. Quality in protein is quantified by amounts and balance between the essential and nonessential amino acids [7]. Essential amino acids refer to amino acids which cannot be synthesized by metabolic system of animals, while nonessential amino acids can be synthesized [7]. Lysine is the most limited essential amino acids in barley protein [7]. High lysine barley mutant, which contains 2 – 3% higher lysine than normal barley types has been developed and cultivated in recent years [2].

1.2.3.1 Classification of Barley Proteins

According Osborne method, cereal proteins are classified into four groups, including albumins, globulins, prolamins and glutelins [7, 16]. Albumins (3 – 5% of total barley endosperm proteins) are water soluble proteins, and globulins (10 –20% of total barley endosperm proteins) can be soluble in diluted salt solutions [7, 16]. Prolamins (also called hordeins in barley), which consist of 35 – 45% of total barley endosperm proteins, are soluble in alcohol solution; while Glutelins, which possess 35 – 45% of total barley endosperm proteins, can be soluble in alkali conditions [7, 16].

Another popular classification method, on a basis of biological function, defines barley protein into two groups - storage and nonstorage proteins [7]. The storage proteins, including hordeins, glutelins and a small amount of globulins, are located in endosperm and embryo of barley grains [7]. The nonstorage proteins, which present as enzymes or located in cell wall, will not be discussed in this thesis. Hordeins are major endosperm proteins in barley, and they have large impact on technological process and nutritional quality of barley products [7, 16]. Hordeins are complex mixtures of polypeptides. They are divided into four groups — B hordeins (sulfur rich), C hordeins (sulfur poor), D hordeins (high molecular weight) and γ hordein (sulfur rich) — on the basis of electrophoretic mobilities [7, 17]. B hordeins (35 – 46 kDa) occupy 70 – 90% of total hordein amount; C hordeins (55 – 75 kDa) and γ hordeins (< 20 kDa) account for 10 – 30% and 1 – 2% of hordein fraction, respectively [7, 16, 17]; D hordeins, which with molecular weight over 100 kDa, consist of 2 – 4% of total hordein fraction [17]. Besides the B, C, D and γ hordeins, a very low molecular weight fraction might also be separated by SDS-PAGE as well. This fraction is commonly named as A-hordein. A-hordeins, which are reported to have different amino acids compositions comparing to other hordein groups, might not belong to storage proteins [16].

1.3 Fundamental Properties of Protein

Fundamental properties of protein, including solubility, hydrophobicity and molecular size are essential parameters for understanding the basis of protein functionality [18].

1.3.1 Solubility of Protein

Solubility of portein is a thermodynamic characteristic which provides information on the equilibrium between protein-protein and protein-solvent interactions [19]. High solubility of protein is a fundamental requirement for optimum functionalities [18]. Many functionalities, including emulsification, foaming and gelation, are influenced by solubility [20]. The solubility of protein is determined by several factors, mainly including charges and hydrophobicity, folding structures of protein, and pH and ionic strength of solvent [21]. Proteins with lower hydrophobicity and higher charges, are more likely to be soluble in water [21].

1.3.2 Hydrophobicity of Protein

The tendency of non-polar substances to attach and held to each other in an aqueous phase is called hydrophobicity [22]. Hydrophobicity is one of the important parameters which can be used to predict protein’s functionality [23].

There is a correlation between the content of non-polar amino acids residues of proteins and their physical properties [24]. Hydrophobic interactions in protein molecules are essential for determining protein conformation and intervening protein-protein interactions [22]. Additionally, the number and relative size of hydrophobic regions on protein surface control the solubility of proteins and further dictate their aggregation potential under different physicochemical conditions [22]. Early research evaluated the total hydrophobicity of protein by calculating the sum of the side chain hydrophobicity [25]. However, many hydrophobic residues are embedded in the interior of protein molecules [25]. Therefore, the exposed outside hydrophobicity is more 'effective' and direct when considering protein functionalities (such as oil-holding in emulsifying properties). Several methods have been applied in scientific field for determination of hydrophobicity (such as high-performance liquid chromatography (HPLC), contact angle measurement and fluorescence probe methods) [19]. Among those methods, fluorescence probe method is the most common method to quantify protein exposed hydrophobicity [19]. Fluorescent probes, such as 1-anilinonaphthalene-8-sulfonic acid (ANS) and cis-parinaric acid (CPA), are used in probe method to test protein exposed hydrophobicity [26]. These probes will exhibit a significant increase in fluorescence when being binded to the surface hydrophobic region of proteins. The initial slope of fluorescence intensity

as a function of protein concentration is proportional to the quantum yield of fluorescence, as such that the slope of concentration-dependent quantum yield can be used to represent the ‘effective’ exposed hydrophobicity [27]. The fluorescence probe methods are relatively simple and rapid ways to quantify the exposed hydrophobicity. Protein exposed hydrophobicity, as well as charges, are major factors which influence the protein solubility [23]. Generally, proteins with high charge and low hydrophobicity can exhibit high solubility [23].

1.3.3 Molecular Weight of Protein

The molecular weight of protein or peptides may provide fundamental information on protein characteristics and functionalities. Electrophoresis and HPLC are two analytical techniques which are widely used for evaluating molecular weight of proteins. Electrophoresis is based on the mobility of charged molecules in an applied electric field [19]. In SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the relative movement of protein molecules is inversely proportional to the log of their mass [28]. If proteins of known mass are running with the unknown together, the mass of unknown proteins can be estimated [28]. Size exclusion chromatography (SEC) in HPLC is used to characterize and fractionate proteins according to size [29]. The sieving medium in SEC column is a porous gel. Protein molecules with sizes smaller than the pore diameter have more possibility to penetrate the gel and hence pass through the

column slowly; while protein molecules with sizes larger than the pore diameter are excluded from the gel and pass through the column unimpeded [29]. Depending on the passing time through the column, the weight-average molecular weight of protein can be estimated.

1.3.4 Non-nutritional Functionality of Protein in Food

Proteins exhibit a variety of functionalities, as shown in Table 1-4 [21]. Among these functionalities, emulsification, foaming and gelation are the three major important functional properties of proteins. The different functional behaviors of proteins come from the complex interactions and mutual influences among the secondary, tertiary structure, amino acids composition, amino acids sequence, and physic-chemical properties (charge, hydrophobicity, molecular weight *etc.*) of proteins themselves; or their interactions associated with the surrounding environment and other components, such as carbohydrates and lipids [20]. Generally, the cohesion, adhesion and elasticity functionality can be found in proteins coming from muscle, egg and some cereals; the emulsification can be ascribed to the proteins deriving from egg, milk; and the fat and flavor binding can be traced to the proteins coming from milk, egg, and cereals [19]. Proteins are extracted by different preparation methods, the characteristics of these proteins, including hydrophobicity and molecular weight, are basic physicochemical parameters which are essential for better understanding their functionalities and

applications [19].

Table 1- 4. Summary of protein functionalities in food systems, adapted from [21].

Functionalities	Food System
Solubility	Beverage, soups
Emulsification	Salads, ice-cream
Foaming	Whipped toppings, protein shakes
Gelation	Curds, gelatin gels
Water absorption and binding	Bakery products
Viscosity	Soups, salad dressings
Adhesion	Baked goods, pasta products, breading
Elasticity	Baked goods
Plasticity	Wheat flour dough
Flavor binding	Bakery products
Color	Roasted and fried goods
Flavor	Artificial flavors

Emulsification of Proteins. Proteins are of great importance due to their amphiphilic structure, which allows them to adsorb at oil-water interface to

decrease the interfacial tension [30]. Emulsification by proteins is widely used in food formulations, drug and nutrient delivery. [30] Cream, salad dressing and mayonnaise are typical emulsion products in food industry. [30] The advanced emulsion technologies, which allow drugs and nutrients to be coated inside of emulsion droplets, enable the agents to be delivered to the target organ in pharmaceutical applications. More information of emulsification properties will be explained later in this chapter.

Foaming of Proteins. Foam is defined as dispersed gas bubbles surrounded by continuous liquid phase [31]. Proteins exist in continuous phase and at the interface in the foam system [31]. During foaming, proteins rapidly adsorb to air/water interface thus decreasing the surface tension [31]. Once the foaming system is formed, protein will stabilize the foam system against a variety of destabilizations, such as creaming, flocculation, coalescence *etc.* [31]. Examples for food products involving foaming can be sponge cakes, whipped toppings, fudges *etc.* [20]

Gelation of Proteins. The ability of proteins to form gels and provide a network matrix to trap water under certain circumstances is an important protein functionality [20, 31]. By a range of process, one can increase the intermolecular

interaction among polypeptides [20]. Once the intermolecular interaction reaches a certain point, a continuous three-dimensional network of intertwined, partially associated polypeptides will form [20, 31]. Gelatin, protein extracted from animal and fish collagen, is the most popular gel forming material in food [20].

1.4 Deamidation

Deamidation can be defined as a chemical modification which removes ammonia from peptides or proteins via amide group hydrolysis [32]. The reaction corresponds to conversion of asparagines amide groups or glutamine amide groups to carboxylic groups [32].

$$HOOCCHNH_2CH_2CONH_2 + H_2O \rightarrow HOOCCHNH_2CH_2COOH + NH_3 \quad (1.1)$$

$$HOOCCHNH_2CH_2CH_2CONH_2 + H_2O \rightarrow HOOCCHNH_2CH_2CH_2COOH + NH_3 \quad (1.2)$$

Reactions (1.1) and (1.2) show the deamidation process for asparagines and glutamine, respectively [32]. Certain cereal, pulse and seed proteins are poor in solubility. Deamidation is a widely selected modification for improving protein's functionalities. Deamidation can be achieved both chemically and enzymatically [33]. Deamidation brings more negative charges to protein molecules by converting amid groups to carboxylic groups [15, 32]. The increased

negative charges enlarge the electrostatic repulsion among protein molecular chains, which effectively increase the solubility and emulsification functionalities of proteins [4, 15, 32]. Deamidation leads to exposure of hydrophobic region, such as nonpolar groups and sulfur rich groups, in native protein. In terms emulsion formation, the nonpolar groups contribute to hydrophobic interactions at the adsorbed protein interface, while the exposure of sulfur rich groups contribute to disulfide bond formation at the protein interface [34]. It has been reported that even small level (2-6%) of deamidation can lead to a significant improvement of protein functionalities [32]. According to Table 1-3, barley proteins have high content of glutamine (glutamic acid) and considerable amount of asparigine (aspartic acid), which makes barley proteins to be good candidates of deamidation modification.

1.4.1 Proteins Modified by Deamidation

1.4.1.1 Rice Protein

Rice proteins have rigid globular structure formed by excessive intramolecular and intermolecular disulfide bonds and hydrophobic interactions, thus they are extraordinary insoluble [19]. Rice proteins contain high proportion of glutamine and asparagine, and their side chains are responsible for promoting the aggregation of glutelin, which is another reason for poor solubility of rice proteins.

Because of the low solubility, less molecular flexibility and poor surface activity, rice protein has low level of functional properties [19]. Deamidation is considered in rice proteins to improve their solubility, as well as other functionalities [15]. The deamidated rice protein showed excellent solubility under mildly acid and neutral conditions [15]. After deamidation, most glutamine residues were converted into negatively charged glutamic acid residues, and the hydrophobic, hydrogen and some disulfide bonds in original rice proteins were broken [15]. In the meantime, the deamidated rice proteins exhibited a more open and stretched conformaiton caused by the increased electrostatic repulsions. Thus, the functionalities of rice proteins could be greatly improved.

1.4.1.2 Wheat Proteins

Wheat protein exhibits low solubility and poor foaming, emulsifying and gelling properties [35]. Wheat gluten gives elasticity to dough, but these proteins have limited other applications. The groups of monomeric and polymeric storage protein are named as gliadins and glutenins, respectively. To be specifically, gliadin is rich in glutamate and glutamine, while glutenin is high in proline and glycine [35]. Deamidation is believed to be an excellent modification for increasing solubility for wheat gluten, since wheat gluten contains a high content of glutamine residues. It has been proved that even a low level of deamidation may cause significant improvement of protein functionalities [35]. Deamidated

wheat gluten has an amphiphilic nature and still maintains its long-chain structures. Based on these characteristics, deamidated wheat gluten showed good emulsifying property.

1.4.1.3 Zein

Maize ranks as one of the top cereals based on production in the world [36]. The starch and oil extracted from maize seeds have been widely used in food industry. However, the maize protein, namely, zein, is not utilized well due to its poor functionalities [36]. According to reports, zein consists of high content of nonpolar amino acids, including leucine, proline and alanine. Almost all of the β- and γ-carboxyl groups of glutamic and aspartic acids are amidated in the form of glutamine (21%) and asparagine (5%), which facilitate the aggregation of zein by engaging in hydrogen bonding with their polar terminal amino groups [36]. All these characteristics lead to the low solubility of zein in water. The solubility and emulsifying properties of zein can be efficiently enhanced by deamidaiton, thereby, an extensive usage of deamidated zein in food industry can be expected [36].

1.5 Emulsion

Emulsion is a mixed, heterogeneous system which contains two (or more)

immiscible liquid phases, with one phase dispersed in the other as droplets [37]. Simple emulsion system can be classified into oil-in-water (O/W) system and water-in-oil (W/O) system [37]. Besides, various types of multiple emulsions, such as oil-in-water-in-oil (O/W/O) or water-in-oil-in-water (W/O/W), have also been introduced in some systems [38].

In food industry, emulsification can be achieved by a high speed mixer, a colloid mill, or a high pressure homogenizer [39]. During emulsification, vigorous mechanical energy is required to break down large deformable drops [39]. At the same time, a large amount of new liquid interface is created [39]. Thermodynamically, the mechanical energy required for making emulsion can be calculated as [39]:

$$\Delta G = \gamma \Delta A \qquad (1.\ 2)$$

ΔG: The required energy (free energy change) (J)

ΔA: The increase in the total interfacial area (m^2)

γ: Interfacial tension (N/m)

However, the actual required amount of mechanical energy in practice can be much larger than this calculated ΔG [39]. Because the small droplets have highly

curved surfaces, if we want to break the larger droplets into small ones, the rapid disruptive mechanical energy needs to exceed the interfacial force that holding larger droplets together [39]. In order to disrupt a droplet with radius of R, the required external pressure gradient should be [39]:

$$\frac{\Delta p}{R} = \frac{2\gamma}{R^2} \qquad (1.3)$$

Δp : Laplace pressure, $\Delta p = 2\gamma/R$ (Pa/m)

So, the input mechanical force needs to develop a very high level of local pressure gradient during emulsification [39]. The local gradient pressure can be generated from: intense laminar flow including shear and extensional deformation; or inertial effects including turbulence and cavitation [39].

1.5.1 The Physical Chemistry of Emulsions

In emulsion systems, emulsifier molecules on the surface suffer different quantity of van der waal forces from molecules in bulk liquid [40]. Thus, surface free energy is accumulated at interface. In Gibbs model, the surface free energy dG^{σ} consists of three parts: surface entropy $S^{\sigma}dT$, surface energy $Ad\gamma$, and composition $\sum n_i^{\sigma}\ d\mu_i^{\sigma}$ [40, 41]. Gibbs – Duhem equation is given below [41]:

$$dG^{\sigma} = -S^{\sigma}dT + Ad\gamma + \sum n_i^{\sigma}\ d\mu_i^{\sigma} \qquad (1.4)$$

S^{σ}: Surface entropy

A : Surface area

γ: Surface tension (surface energy per unit area)

$\sum n_i^{\sigma}\ d\mu_i^{\sigma}$: The number of moles of emulsifier component i with chemical potential of μ_i^{σ}

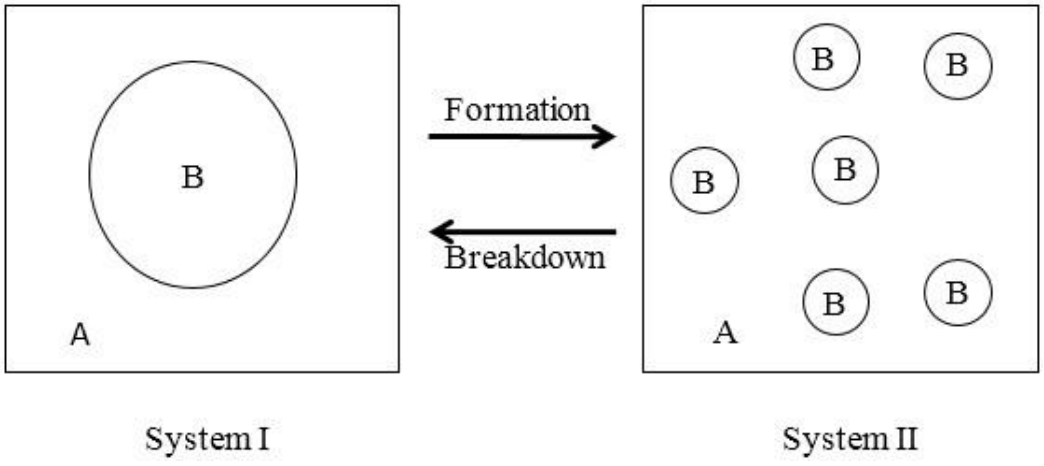


Figure 1- 1. Emulsion formation and breakdown. Source is adapted from [41].

Consider a system with two different phases – phase A and phase B, as shown in Fig. 1-1 [41]. The surface area in system I and system II is A_I and A_{II}, respectively. By converting the system I to system II, we will largely increase the surface area ($A_{II} >> A_I$). The change in free energy during emulsion formation comes from two components: a surface energy term, which can be expressed by

$\Delta A\gamma$ (positive value); and an entropy change term, which is equal to $T\Delta S$ [41]. According to Eq. 1.5, the free energy for the transition can be expressed as [41]:

$$\Delta G^{form} = \Delta A\gamma - T\Delta S^{conf} \quad (1.5)$$

$\Delta A = A_{II} - A_I$;

$\Delta S^{conf} = S_{II} - S_I$;

If ΔG^{form} is negative, the transition from system I to system II will be thermodynamically favorable; if ΔG^{form} is positive, the transition from system I to system II will be thermodynamically unfavorable. Because emulsion formation is accompanied by production of great number of small droplets, the change for surface energy is positive [41]. For most emulsion systems, $\Delta A\gamma \gg T\Delta S^{conf}$, then ΔG^{form} is positive [41]. This means emulsion formation is nonspontaneous and the emulsion system is thermodynamically unstable [41]. In order to completing emulsification, the input of mechanical energy, as well as the addition of surface-active agents (emulsifiers) are required [42].

1.5.2 Destabilization Mechanisms of Emulsions

Emulsion systems generally tend to change from the small droplets form to two bulk phase form, since the later form is more energetically favorable [43]. Four major destabilization mechanisms — creaming/sedimentation, Ostwald ripening,

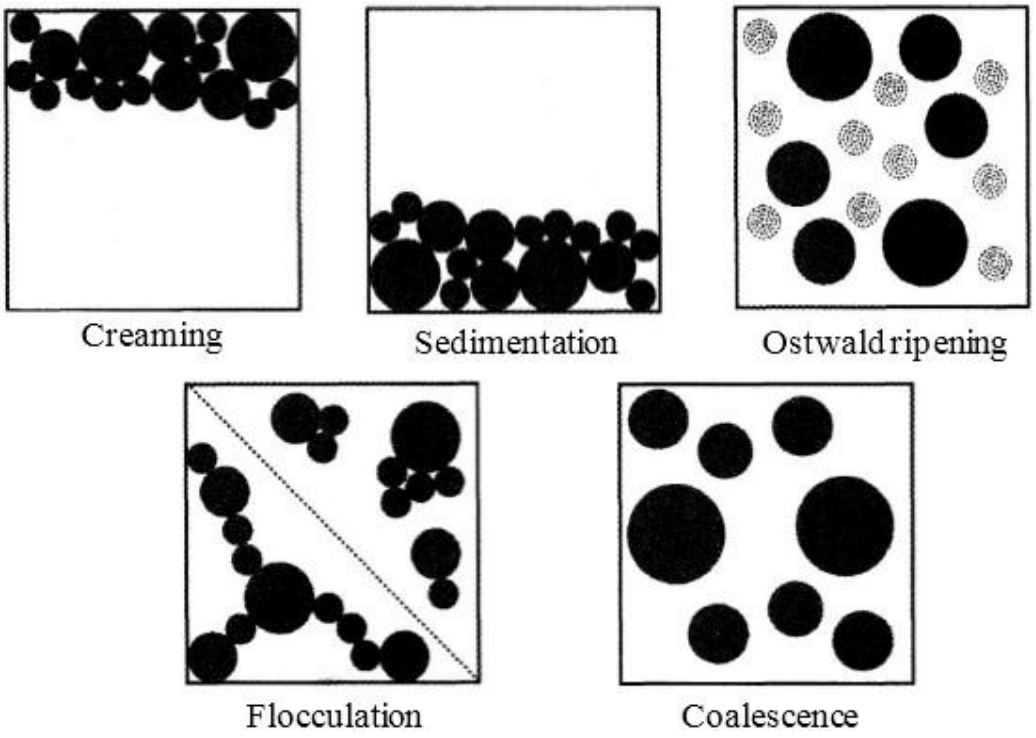


Figure 1- 2. Emulsion destabilization process. Source is adapted from [43].

flocculation/aggregation and coalescence — are involved in emulsion system [44].

1.5.2.1 Creaming and Sedimentation

Metastable fresh emulsion is a polydispersed system animated by Brownian motion [43]. Creaming is also called gravitational separation [45]. Creaming occurs when dispersed phase and aqueous phase have different densities [43, 45]. If the dispersed phase has lower density under the influence of gravity, droplets in emulsion will tend to move upward, which is called creaming [45]; if the dispersed phase has higher density, droplets will tend to move downward, which

is called sedimentation [45]. Since the density of oil is lower than the density of water in normal case, so O/W emulsions are more likely to suffer creaming, while W/O emulsions are more likely to experience sedimentation [45]. Creaming does not involve rapture of close droplets, it is a simple separation process whereby the original emulsion is separated into two emulsions, the top creaming layer is richer in dispersed phase, while the bottom layer contains negligible amount of dispersed phase unless the dispersed phase is in small (< 1 μm) and stable emulsions [46].

1.5.2.2 Ostwald Ripening

Ostwald ripening corresponds to the process, in which large droplets grow at the sacrifice of small ones due to transportation of dispersed phase from small droplets to large droplets [43, 45]. Compare to small droplets, large droplets are more energetically favorable. Ostwald ripening is governed by the trend of interfacial energy reduction, and it is a slow process that eventually causes disappearance of small droplets [43, 47].

1.5.2.3 Flocculation

Flocculation is also referred to aggregation. It refers to a process when two or more droplets held together [43, 45]. Flocculation can lead to two types of network systems, one is a system condensed with discrete aggregates called flocs, which is compact flocculation, another is a system filled with a single expanded

network structure, which is open flocculation [43]. Flocculation happens due to one of two distinct mechanisms: bridging or depletion flocculation [48].

Bridging Flocculation. Polymers could either directly adsorb to the interface or attach to the already adsorbed emulsifier layers, hence they can promote flocculation by forming bridges [45]. At a low emulsifier concentration, bridging flocculation may occur when the emulsifier is not sufficient for fully covering the oil-water interface, the emulsifier may attach some of their hydrophobic regions on one droplets, while other hydrophobic regions on another droplets [45, 47]. Bridging flocculation may occur when the concentration of emulsifier is high enough to be present in the continuous phase [45]. For example, when an emulsifier molecule is weakly attached to a droplet, its polar segments can desorb and attach to another neighboring droplet [45]. Bridging flocculation may also occur when a polymer in the continuous phase has electrical charges which are opposite to the charges of the dispersed droplets [45].

Depletion Flocculation. When nonadsorbing colloidal polymers or their micelles are present in aqueous phase, they tend to be excluded from the narrow region between two droplets due to osmotic effects [48]. This phenomenon causes an attractive force between two neighboring emulsion droplets. This attractive force

increases with increasing concentration of colloidal polymers [48]. Once the attractive force is large sufficiently to overcome the repulsive force between two droplets, depletion flocculation occurs [48]. A variety of biopolymers or emulsifiers, including low molecular weight surfactants (Tween 20), polysaccharides (modified starch), and proteins (whey), are capable of causing depletion flocculation when their concentration is sufficiently high [48].

1.5.2.4 Coalescence

Coalescence refers to a process which two or more emulsion droplets flow together to form a new larger droplet due to film rupture [43, 45]. The rate of coalescence relies on droplets contact rates and surfactant properties [43]. In O/W emulsions, coalescence will finally cause the formation of oil layer on top of emulsion systems [45].

Among these destabilization processes, creaming and Ostwald ripening does not require close contact of two droplets, while flocculation and coalescence need encounter of two droplets [43]. There is a complex set of relationship among all these destabilization processes [43]. Creaming, in addition to Brownian motion, increases the rate of droplets approach to each other [43]. This will eventually influence the rate of flocculation or coalescence [43]. Ostwald ripening is affected by the distance of nearby droplets, since the dispersed phase is transported

between them [43]. Hence, Ostwald ripening rate can be accelerated when emulsions are either encounter creaming or flocculation [43].

1.5.3 Interactions in Emulsion Systems

The interactions within emulsion systems are responsible for most of important physicochemical properties of emulsions [49]. If we assume there are two types of interactions in emulsion systems, one attractive and one repulsive, the overall stability of emulsions will depend on the balance between attractive and repulsive interactions [49]. If the attractive interaction is dominant in whole system, emulsion droplets will tend to aggregate or coalescence [49]. If the repulsive interaction is dominant, emulsion droplets will tend to remain as individuals, which means the emulsion system will remain stable [49]. Generally, Van der Waals attraction, electrostatic repulsion and steric repulsion are the three major interactions in emulsion systems [41]. Among these interactions, electrostatic repulsion and steric repulsion are the two main stabilization mechanisms for emulsions [47].

1.5.3.1 Van der Waals Attraction

Van der Waals force can be defined as the universal attractive force among atoms and molecules [51]. Intermolecular van der Waal force acts on all kinds of molecules in emulsions, which leads to colloidal van der Waal interaction between

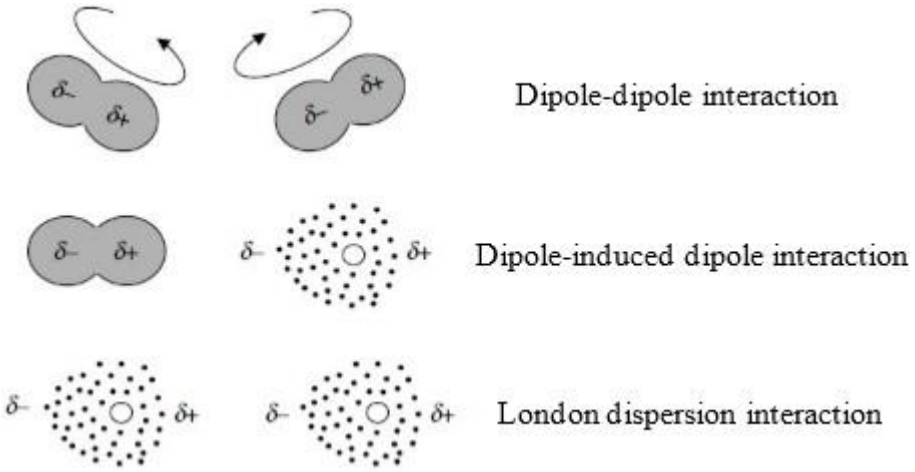


Figure 1- 3. Schematic representation of van der Waals interactions, adapted from [50]

emulsion droplets [49]. There are three types of van der Waals attraction: (I) dipole-dipole interaction; (II) dipole-induced dipole interaction; (III) induced dipole-induced dipole interaction (London dispersion interaction) [41]. The dipole-dipole interaction originates from interaction between two permanent dipoles that are constantly rotating [50]. Though each individual rotating dipole is zero charge in total, a weak attractive force can be generated between different dipoles due to the movement of one dipole causes some correlation of the nearby dipole [50]. The dipole-induced dipole interaction arises from the interaction between a permanent dipole and a neighboring molecule [50]. The permanent dipole can induce an alteration in the distribution of electrons in the neighboring molecule, and thereby forming an induced dipole [50]. Then attractive interaction

will happen between the permanent dipole and the induced dipole [50]. London dispersion interaction originates from the interaction between two neighboring molecules [50]. In molecules, the electrons are constantly moving around the nucleus, which lead to an uneven distribution of negatively charged electrons and positively charged nucleus [50]. As a result, an instantaneous dipole, which can also induce a dipole in a nearby molecule, is formed [50]. Eventually, the instantaneous attractive interaction between the two molecules is generated [50].

The van der Waal interaction between two spheric droplets, with radii a_1 and a_2, can be expressed as [51]:

$$V_A = -\frac{A_{131}}{6h}\frac{a_1 a_2}{a_1+a_2} \quad (1.6)$$

V_A: Van der Waal force between two spherical droplets;

A_{131}: Hamaker constant for medium;

h: Distance between two droplets (h << a)

If $a_1 = a_2 = R$, van der Waal force can be calculated as [41]:

$$V_A = -\frac{AR}{12h} \quad (1.7)$$

With decreasing of h (close approach of emulsion droplets), V_A will increase

dramatically. For the droplets made from similar materials (emulsion systems), medium 1 interacting across medium 2, the Hamaker constant can be calculated as [51]:

$$A_{131} = (\sqrt{A_{11}} - \sqrt{A_{22}})^2 \quad (1.8)$$

Thus, we can conclude that the van der Waal interaction between two spherical emulsion droplets is always attractive (positive Hamaker constant) [51].

Hamaker constant can roughly represent the strength of van der Waals interactions between macroscopic bodies. For any materials, the Hamaker constant can be calculated by [41]:

$$A = \pi^2 C \rho_1 \rho_2 \quad (1.9)$$

C: Coefficient in particle-particle pair interaction;

ρ (1 or 2): The number of atoms or molecules per unit volume;

The Hamaker constant can varied from 3×10^{-21} to 10×10^{-21} J in most food emulsions [51].

1.5.3.2 Electrostatic Repulsion

Electrostatic force, as well as steric force, is major repulsive force for stabilizing

emulsion systems. The droplets in emulsion system are normally stabilized by the same kinds of emulsifiers, which exhibit the same charge [49]. The electrostatic

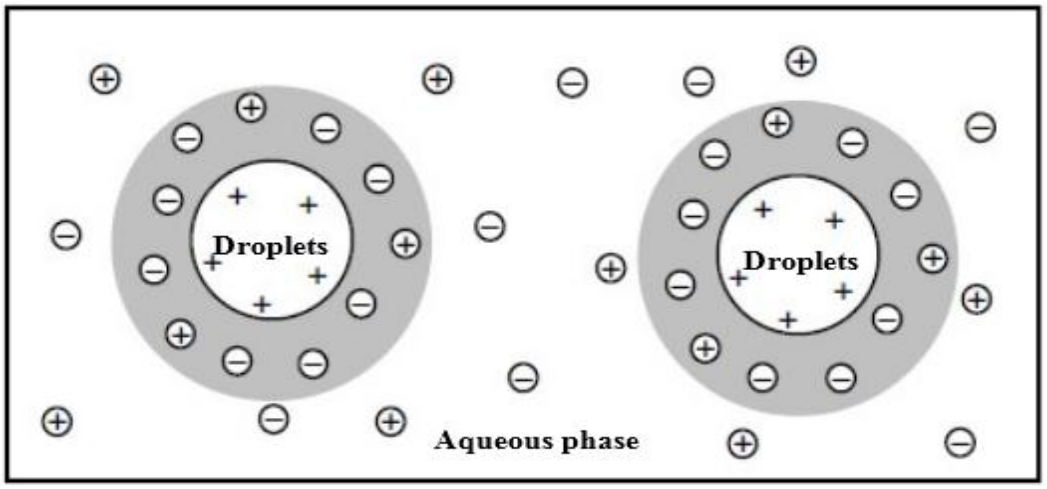


Figure 1- 4. Emulsion droplets surrounded by a layer of counterions, adapted from [49].

repulsion is based on the repulsive force that is generated between two approaching droplets with similar charges [47]. If the repulsive force is sufficiently strong, the droplets will repel each other from close contact [47]. The electrostatic interaction in emulsion system depends both on droplets electrical charges and aqueous ionic concentrations [49]. In emulsions, the isolated droplets with electrical charges are neutralized by a layer of counterions, as shown in Fig. 1-4 [49]. If the ionic strength is high, the electrostatic repulsion will decrease.

1.5.3.3 Steric Repulsion

Steric force is produced by nonionic emulsifiers or polymers [41]. For example,

protein polymers can adsorb to the surface of emulsion droplets and extend into

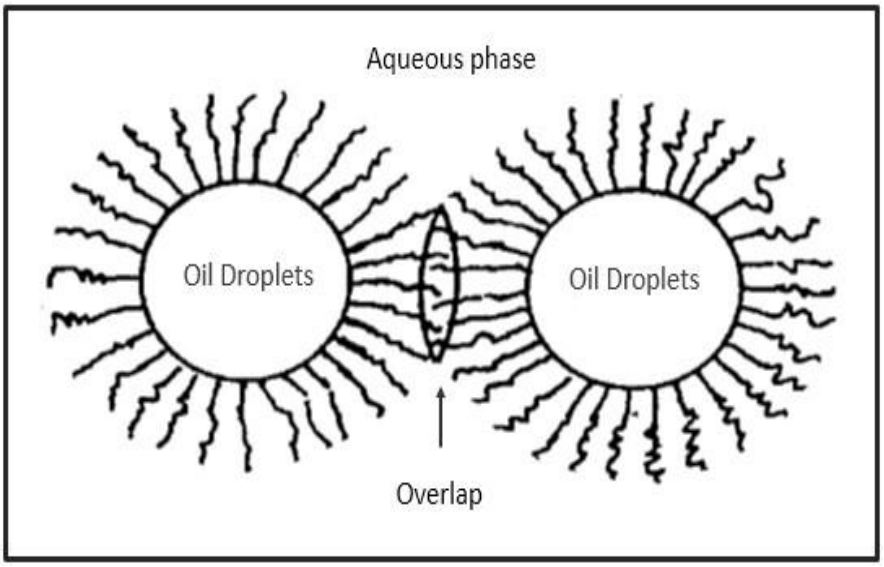


Figure 1- 5. Schematic representation of steric repulsion, adapted from [52].

the aqueous phase, building up a volume restriction or a physical barrier for close contact of droplets [47]. When protein coated droplets approach to each other, their interfacial layers overlap at close proximation, which results in repulsive force [49]. At such close droplets distance, steric interactions are strongly repulsive, which protects emulsion droplets from aggregation or coalescence [49]. However, the steric force has very short range, and it decreases rapidly when the distance between two droplets is larger than the sum of their apparent radii (drop plus associated layers). Steric repulsion strongly depends on the characteristics of emulsifier layers, including thickness, packing, rheology, and molecular interactions *etc.* [49].

1.5.3.4 Hydrophobic Interaction

The hydrophobic interaction occurs when emulsion interface has significant amount of hydrophobic area, or the emulsifiers have hydrophobic groups exposed to aqueous solutions [49, 51]. It arises either by insufficient emulsifier coverage at emulsion interface or by heat-induced emulsifier denaturation [49]. Hydrophobic interaction (long range), which can be surprisingly large, plays a very important role in stabilizing protein molecular structure, as well as the emulsion stability.

1.5.3.5 Hydration Interaction

Hydration interactions (short range) are also named as hydrophilic interactions. Different from attractive hydrophobic interactions, hydration interactions lead to repulsive force [49]. Hydration interactions arise from the bonding of water molecules to dipolar and ionic groups [49]. When the two droplets approach to each other to very close proximation, the bonds between the water molecules and dipolar or ionic groups become disrupted, which lead to repulsive force [49].

1.5.4 DLVO Theory

DLVO theory names after the scientists Deryaguim, Landau, Verwey, and Overbeek, who were responsible for developing this theory. DLVO theory suggests that the stability of emulsions against creaming, Ostwald ripening,

flocculation and coalescence is achieved by combined effect of attractive force and repulsive force [43]. The most important forces for emulsion system would be van der Waals attractive and electrostatic repulsive forces [43]. The total potential energy of interaction can be obtained by summation the van der Waals and electrostatic interactions [43, 53]. At small separations, the total energy is dominated by the van der Waals attraction, where it forms a deep attractive force well named as primary minimum [43, 53]. At a larger separations, van der Waals

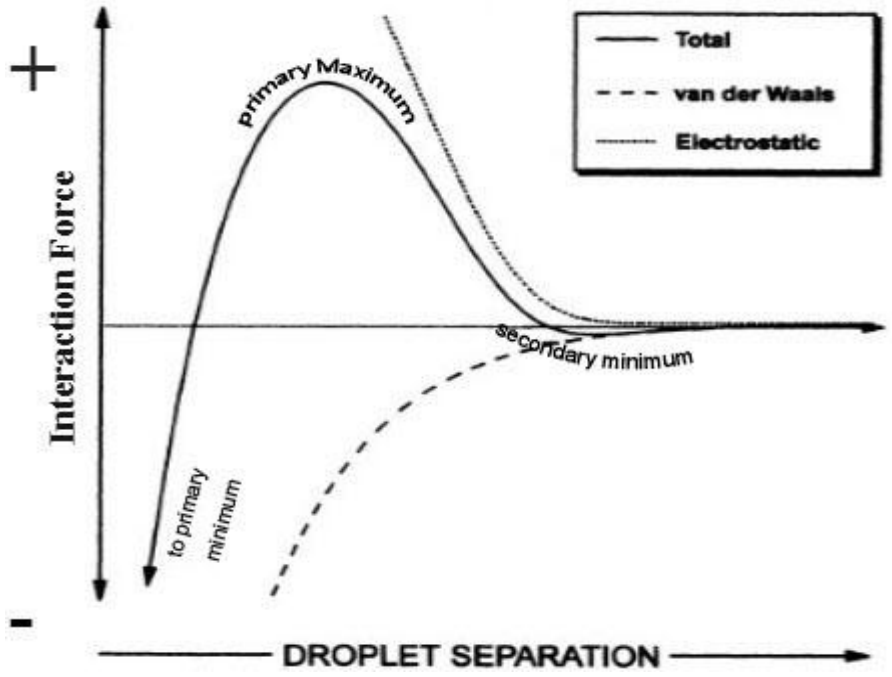


Figure 1- 6. Schematic total interaction force between van der Waals and electrostatic interactions, adapted from [43].

attraction becomes decreased, the energy curve rises to the primary maximum, where the electrostatic repulsive force dominates [43, 53]. At a very larger

distance, the curve decreases again, crossing the axis to from a second attractive force well - secondary minimum, where van der Waals force is dominant again [43, 53]. This theory reveals that the total interaction energy is always attractive for both small and very large separations, and there is a medium distance range in which the repulsive force dominates [43, 53]. The shape of this energy curve is very important for understanding the chemical behavior and stability of emulsion systems [43, 53].

1.5.5 Emulsifiers

Emulsifiers are typical surface active molecules. They are a group of amphiphilic compounds which anchor themselves to some extent at the interface, where they are able to form a protective membrane that prevents droplets from coalescence [54, 55]. In order to minimize the interfacial tension between two immiscible phases, emulsifiers can adapt an orientation at the interface, with their hydrophilic head group extended into the aqueous phase and their lipophilic tail into organic phase [54]. Effective emulsifiers are considered to be capable of: (I) rapidly decreasing interfacial tension during emulsification by quickly adsorbing at oil-water interface; (II) binding strongly at the interface once adsorption happens; (III) effectively protecting emulsion droplets against diverse destabilizations, including flocculation and coalescence [39]. The protection happens in the early stage of emulsion by dynamic surface tension effects (the

Gibbs-Marangoni mechanism), or in the later storage stage of emulsion by repulsive colloidal interactions (electrostatic, steric interactions) [39].

1.5.5.1 Classification of Emulsifiers

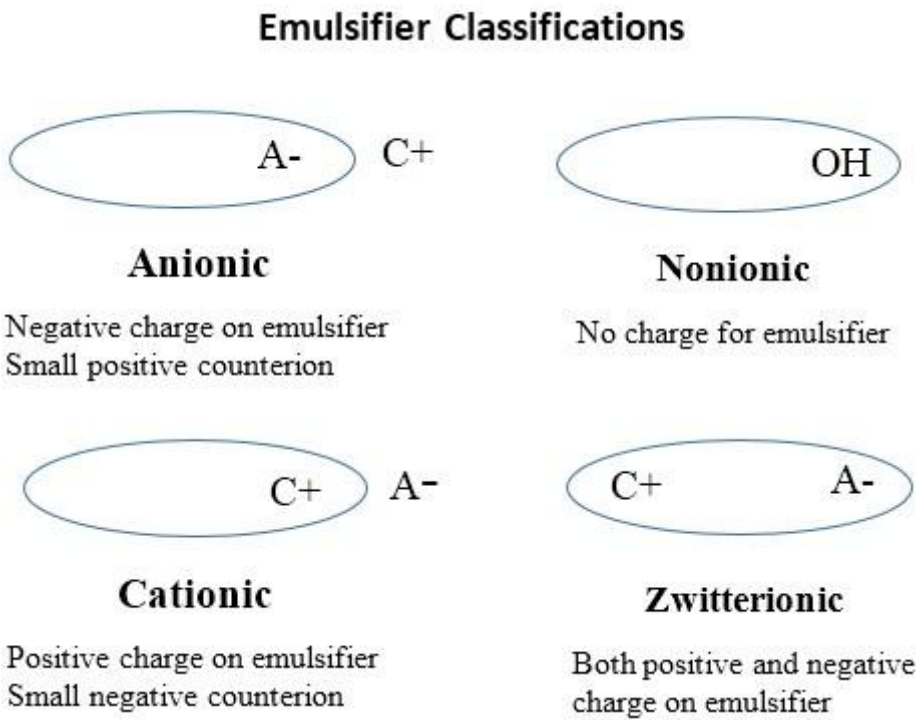


Figure 1- 7. Structures of anionic, nonionic, cationic, and zwitterionic emulsifiers, adapted from [56].

Emulsifiers can be classified into anionic emulsifiers, nonionic emulsifiers, cationic emulsifiers, and zwitterionic emulsifiers based on their dissociation in water, as shown in Fig. 1-7 [55, 56]. Anionic emulsifiers exist in water as an amphiphic anion, and a small cation courterion. Anionic emulsifiers occupy around 50% of emulsifier production in the world market [55]. The most common

products of anionic emulsifiers in the market include detergents, soaps, foaming agent and wetting agent [55]. Nonionic surfactants, which have no charge for hydrophilic part, do not ionized in water [55]. Nonionic emulsifiers account for about 45% of all surfactant industrial production [55]. They are mainly used in cleaning detergents [57]. Cationic emulsifiers can be dissociated in aqueous phase into an amphiphilic cation and a small anion courterion [55]. They are greatly used in corrosion inhibition [55]. Zwitterionic emulsifiers contain both anionic and cationic dissociations, which means they exhibit both negative and positive charges [55, 56]. Phospholipids belong to one of popular Zwitterionic emulsifier products [55].

1.5.5.2 Critical Micelle Concentration for Emulsifiers

In diluted solutions, with constant temperature, the emulsifier 'surface excess' concentration can be calculated as (Gibbs' isotherm) [40, 47]:

$$\Gamma = -\frac{1}{RT}\frac{d\gamma}{d\ln C} \qquad (1.10)$$

Γ: Concentration of the adsorbed emulsifiers on the interface (concentration per unit area)

R: Gas constant

T: Absolute temperature

C: Bulk emulsifier concentration

In case of emulsion system with extra added electrolyte, the right hand side of Gibbs' isotherm should multiply by the factor 2 [40]. Most emulsifiers have

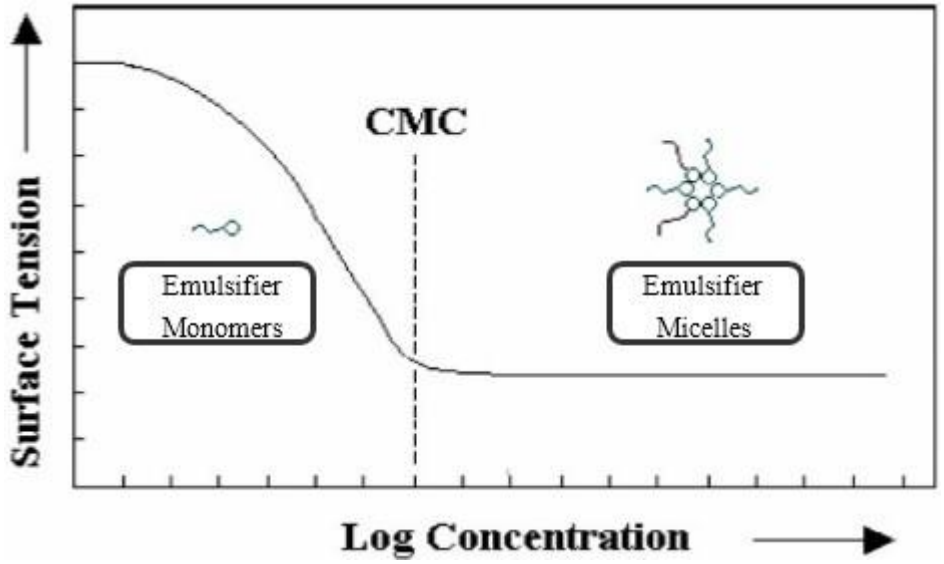


Figure 1- 8. Schematic representation of micellization of emulsifiers, adapted from [47].

ability to form micelles in aqueous phase [47]. Micelles are defined as emulsifier aggregates [47]. The specific concentration, which micelles are observed in aqueous solution, is called critical micelle concentration (CMC) [47]. Below CMC, emulsifiers present as monomers in aqueous solution and the interfacial tension can be calculated following Gibbs' isotherm [47]. Above CMC, the free emulsifier concentration in aqueous phase becomes constant, since more additional emulsifiers are about to aggregating to form micelles. In this case, the

interfacial tension becomes constant as well [47].

1.5.5.3 Food Emulsifiers

Food emulsifiers are present in most fat and oil involving processed foods. They have at least two important characteristics: surface-active and edibility [58]. The most well-known food emulsifiers used in food industry are phospholipids, lecithins, polysaccharides, proteins, *etc.* [56]. Most food emulsifiers are made from food themselves. For example, casein from milk protein and phospholipids from eggs (or soy) are well-known emulsifiers [56].

1.5.6 Protein Emulsifiers

Proteins, which consist of mixtures of ionic, non-ionic, polar and non-polar regions, are very complex amphipathic molecules [59]. This characteristic enables protein to be surface-active [59]. During emulsification, protein molecules rapidly adsorb to the interface of newly formed droplets [59]. This protein layer generated at the liquid-liquid interface prevents emulsion system from various mechanisms of destabilization [59]. In a protein stabilized emulsion system, if pH value of emulsion system is above its isoelectric point, protein will act as anionic emulsifiers; while if pH value of emulsion system is below its isoelectric point, protein will act as cationic emulsifier [56]. Proteins are very efficient emulsifiers. Compared with polysaccharide-based emulsifiers, proteins exhibit higher binding

affinities, surface activities and lower saturation surface loads (1 – 2 mg/m^2). These characteristics mean a smaller quantity of protein emulsifier can be used to produce and stabilize emulsions [39].

1.5.6.1 Protein Conformation

In aqueous solution, the protein polypeptide chain is often presented in different levels of 'folded' state, depending on different pH, ionic strength, and temperature, *et al.* [60]. Different from carbohydrates, lipids and soluble polymers, protein has four fundamental structures. The primary structure of protein is the basic amino acids sequence which makes up the whole protein molecule structures [60]. The secondary structure refers to the local conformation of the polypeptides backbone in elements, including α-helix, β-sheet, random coil and turn [60]. The tertiary structure of protein is highly structured domains, which are made up by different arrangements of secondary structures [60]. The quaternary structure corresponds to assembly of three dimensional forms of polypeptides [60]. Protein contains different kinds of amino acids residues based on their complex structures, and they exhibit a stable preferred conformation in solution [61]. The 'native protein' is a result of a delicate balance between all attractive and repulsive forces (electrostatic force, van der Waal force, steric force, ion-paring, hydrogen bonding and hydrophobic force) to achieve the easiest accessible and free energy minimal status [62]. The three steps for protein to form a protective film at the

interface can be simplified as: (I) movement from aqueous phase to interface; (II) adsorption; and (III) unfolding and rearrangement [63]. During emulsification, proteins adapt another conformation at oil-water interface [61]. This conformation largely depends on the distribution of hydrophobic amino acid side chains within the protein structures [61]. In theory, the hydrophobic amino region favors the oil phase, while the hydrophilic region prefers the water phase [61]. The stabilization of emulsions by adsorbed protein is by electrostatic and steric repulsion [44]. The electrostatic stabilization comes from electrical charges of the droplet surface, while the steric stabilization comes from polymeric (steric) barrier of the droplet surface [44].

1.5.6.2 Protein Layer Adsorbed at Interface

After protein adsorbing to the interface, the properties of this interface layer are controlled by its equilibrium structure and conformation, and the magnitude of intermolecular interaction at the formed interfacial film [64]. The properties of this absorbed layer play an important role in manipulating the physicochemical properties of emulsions, including their stability and their correlations with taste, texture, and digestions [62]. Different proteins form different structures at the interface [62]. Globular proteins, including β-lactoglobulin and α-lactalbumin, can form a thin dense interface, thereby stabilizing emulsion droplets mainly by electrostatic repulsion; flexible proteins (caseins), which form thick disperse

interface, stabilizing emulsion mainly by steric and electrostatic repulsions [62]. The re-arrangement of protein interface after adsorption may enable hydrophobic region of globular protein to interact with each other, thus globular proteins tend to form denser interface than flexible proteins [62].

Protein 'Folding' and 'Unfolding' Mechanism. It is well known that proteins adopt different structures from their native conformations when they adsorb from aqueous phase into the interface [62]. In aqueous solution, the protein conformation is governed by the balance between the energy penalty correlated with hydrophobic region surrounded by water molecules and the configuration entropy gain correlated with an open structure [62]. During adsorbing at the interface, the protein conformation is controlled by the balance between the energy penalty associated with moving hydrophobic side chains from oil phase to water phase and the configuration entropy gain [62]. The 'unfolding' mechanism of protein interface is associated with every possible molecular forces in protein. Upon homogenization or stirring, the gain in configuration entropy is correlated with the hydrogen bonding and hydrophobic interactions that are disrupted by shear force [62]; meanwhile, the level of protein re-arrangement is possibly influenced by the gain in free energy originating from the transfer of hydrophobic side chains to the oil phase [62]. Several techniques, such as Fourier transform

infrared spectroscopy (FTIR), are used to measure the protein folding at the interface [62] .

Molten Globule State of Protein. The concept of interfacial molten globule state has been developed to describe the structure of protein at interface [62]. Supporting by several experiment evidence (the protein at interface loss all of absorption bands near UV wavelength), proteins are proposed to adopt a 'molten globule' state at interface in which they lose most of their tertiary structures and adopt a conformation which is rich in secondary structures [62]. Proteins prefer to form α-helix rich and β-sheet rich conformation after adsorbing to interface [62]. After adsorption, proteins need to adopt their conformation in a restricted 2D space. This restricted space forces proteins to adopt in their most compact conformation — α-helix and β-sheet. So, a majority of protein formed interface have high level of α-helix and β-sheet [62].

Disulphide Bonds at Interface. Eric Dickinson brought out an idea that proteins can form a strong film at the emulsion interface by forming intermolecular disulphide linkages [65]. As we mentioned previously, the adsorption of protein to oil-water interface leads to the exposure of protein residues, which are buried inside of the native structures. Consequently, the embedded –SH groups are

exposed to outside [65]. This will trigger: (I) oxidation of two free –SH residues to form a disulphide linkage; or (II) SH-SS interchange reaction to form disulphide bonds [65]. Thus, a gradual strengthening of the protein adsorbed interface is achieved by protein polymerization through disulphide bonds [65].

Molecular Structure and Surface Properties. There is relationship between protein molecular structure and surface properties. Three representative molecular structures of proteins are studied: globular structure (lysozyme), rod-like structure (bovine serum albumin), flexible coil structure (β-casein) [66, 67]. Globular and rod-like structures are rigid conformations, while flexible coil structures are highly dynamic conformations [67]. It was demonstrated that the flexible molecular structures (β-casein) could cause rapid initial adsorption to the interface during emulsification, followed by rearrangement of the protein polymer molecules due to their relatively high mobility. The flexible protein molecules predominantly form the 'loop-tail-train' structure with their non-polar and polar regions protruding into the oil and aqueous phase (loops and tails), respectively, while with their neutral regions remaining in interface (trains) [66, 67]. In contrast, the globular molecular structure adsorbs slowly to the interface, with their non-polar regions facing towards the oil phase, while their polar regions remaining in water phase [66, 67]. After that, they build up an additional

multilayer around the monolayer when the concentration of proteins is high enough [66]. Once adsorbed, globular proteins often experience ‘unfold’, which results in an enhancement of interfacial structures supported by strong chemical bonds [67]. While the rod-like molecular structures behave in an intermediate pattern, they form an interface similar to globular molecules, but they will behave like flexible coil structure when heat is involved in the system [66]. Consequently, the globular proteins form thin and compact interface, while the flexible coil proteins form open and thick interface, though the rearrangement of protein molecules is slow for globular proteins, while fast for flexible proteins [67, 68].

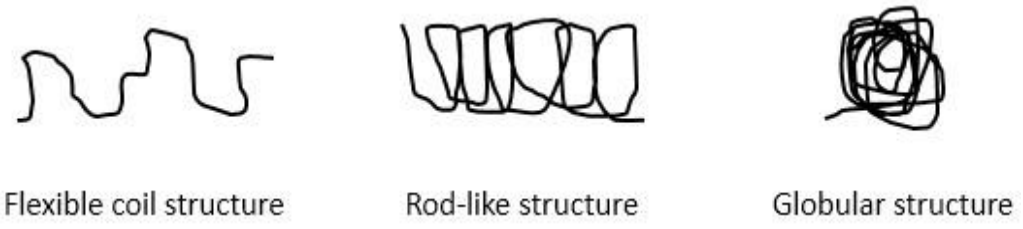


Figure 1- 9. Three representative structures of proteins. Source is adapted from [67]

The rheological behavior of the protein interface is also closely related with molecular structure [66]. Globular and rod-like molecules have more ordered secondary structures, such as α-helix, thus they are more likely to form cross-linking and chain entanglement than flexible coil molecules [50]. The great

enhancement of crosslinking and entanglement exhibits a large resistance to shear and dilation [50]. So, the globular and rod-like proteins tend to form a higher visco-elastic film at the interface, while the flexible proteins tend to from a liquid-like film at the interface [50, 66]. This will in turn relate to emulsion stability.

1.5.6.3 Examples for Emulsifying Proteins

Milk (casein) and Whey Proteins. Milk and whey proteins have been the subject of emulsion study for decades. Casein has high level of proline peptides, which enable it to be relatively hydrophobic and thermal stable. Casein is capable of forming a compact aggregated film by hydrophobic interactions at the interface, so the emulsion made from casein is very stable. Whey proteins can be good candidates for emulsifying agents, however, they are not thermal stable. β-Lactoglobulin and α-lactalbumin are two major whey proteins that are studied for emulsifying properties. β-Lactoglobulin, which has molecular weight around 18kDa, shows a great proportion of ordered structures consisting of β-sheet (43%-50%) in secondary and tertiary structures; while α-lactalbumin, which has molecular weight around 14kDa, exhibits a low level of ordered structures consisting of only 30% α-helix and 9% β-sheet [19]. The main disadvantages for using whey as emulsifiers are their susceptibility to heat denaturation and their limited process selection for avoiding any possible heat

involved technology to produce emulsions [19].

Soy Proteins. The β-conglycinin in soy proteins shows good emulsifying properties. Several studies have successfully produced stable emulsions using soy proteins as emulsifiers. The dextran molecules that conjugated to soy proteins were proved to greatly increase the hydrophilicity and steric repulsion of the emulsion droplets, thereby stabilizing the emulsion against heat treatment, changes in pH, increase in ionic strength, and storing for long time [19]. It has also been demonstrated that polysaccharide-soy protein conjugates can effectively improve the emulsifying properties by successfully reducing the emulsion droplets size and protecting emulsion against creaming [19].

1.5.7 Factors Influence Emulsion Stability

1.5.7.1 Volume Fraction (∅)

The concentration of dispersed phase is normally expressed by the ratio of the volume of the droplets to the volume of the whole emulsion, which is also called 'volume fraction' (∅) [67]. The volume fraction of emulsion not only influences the types of emulsion that could be produced (O/W or W/O), but also affects droplets disrupture and coalescence phenomena in emulsion system [67]. Hence, volume fraction is an important parameter for emulsion evaluation.

1.5.7.2 Droplet Size

In emulsion research, the evaluation of droplet size as a function of time period is a major parameter to investigate the stability of emulsions because several instability phenomena can affect or be affected by the droplet size [69]. For example, coalescence and Ostwald ripening can promote generating larger size droplets, and creaming is affected by the size of droplets [69]. So, the droplet size measurement is valuable for emulsion research. Several techniques have been widely used to measure droplet size, such as microscopy, light scattering, and ultrasonic methods *etc.* [69].

In terms of food industry, the primary goal of emulsion processing is to produce uniform, physically stable products. Generally, decreasing the mean size of emulsion droplets and narrowing their size distribution can increase product stability. However, smaller emulsion droplets will refract light differently, thereby causing a lighter color and greater opacity in products. The smaller emulsion droplets may also influence the texture and flavor release of products. So, it is important to weigh balance between the benefit of developing more stable emulsions by reducing droplet size and the impact of small size to color, texture and flavor of emulsions [50].

1.5.7.3 Electrophoresis and Zeta-potential

The charge of emulsifiers and the surface charge of emulsions are important parameters for controlling emulsion stability. If we assume the emulsifiers are proteins. When protein stabilized emulsion is subject to an electric field, the droplets with charge will migrate towards the oppositely charged electrode [70]. The velocity of the droplets is measured and expressed as mobility [71]:

$$U_E = \nu/E \qquad (1.\ 11)$$

U_E: Mobility;

ν: Velocity of droplets;

E: The strength of applied field.

By measuring the electrophoretic mobility of the emulsion droplets, zeta-potential can be determined by using Henry Equation [70, 72]:

$$U_E = \frac{2\varepsilon z f(Ka)}{3\eta} \qquad (1.\ 12)$$

ε : dielectric constant;

z :zeta-potential;

f(Ka): Henrry's function (1.5 for aqueous solution; 1 for non-polar solvent);

η : viscosity.

The measurement of zeta-potential is normally used to predict the stability of colloidal systems. For example, when the zeta-potential of emulsion is high (either largely negative or largely positive), the emulsion droplets will strongly repel each other, and the emulsion system will be highly stable.

1.5.7.4 pH

PH and Solubility. The relationship for pH and solubility is usually a U-shaped curve for pure proteins [73]. The minimum solubility occurs when the pH reaches to the protein's isoelectric point. Proteins will exhibit high solubility when the pH is far away from their isoelectric point [73].

PH and Surface Charge. The charge of emulsifiers, as well as the resultant emulsion interface charge, is closely related to pH of the environmental solutions [61]. When the pH is approaching to the isoelectric point of emulsifiers, the charge of emulsifier becomes zero [61]. This phenomena decreases the electrostatic repulsion, thus promoting the flocculation and coalescence of emulsion droplets. A stable emulsion needs to carry a sufficiently high droplets surface charge to protect the emulsion droplets from close contact [61].

1.5.7.5 Ionic Strength

Some proteins (albumins) can be found soluble in pure water in vitro, but proteins

cannot be found in pure water in vivo. The ionic strength is approximately 0.15M NaCl in most biological systems [73]. The solubility of some proteins, such as globulins and some albumins, increases before the concentration of neutral salts in aqueous phase increasing to a certain level, after this certain maximum salt concentration, the solubility of protein starts declining [73]. Neutral salt with concentrations around 0.1 – 1M may increase protein solubility depending on the salt, pH, temperature and protein types [73]. The salt ions can be attracted by oppositely charged residue of proteins and form a double layer around it, which reduces the electrostatic interactions between protein molecules, thereby increasing the protein solubility [73]. When the ionic concentration is higher than 1*M*, most of the water molecules are tightly bound to the salt ions, which largely decrease the interactions between protein molecules and water molecules [73]. The interaction between protein and protein (surface hydrophobic interaction) becomes stronger, and the proteins start to associate or aggregate [73].

In terms of protein stabilized emulsions, the ionic strength influences the emulsion systems either by impacting the protein solubility or by impacting the effective charge on emulsion droplets. The strong ionic strength reduces the effective emulsion interface charge, which consequently decreases the strength of electrostatic repulsion. At the meantime, the ionic strength may also modify the

conformation of the adsorbed protein interface, thus impacting the steric repulsion [61].

Protein Molecular Weight. The functionalities of protein are governed by their molecular characteristics, such as molecular weight and molecular structures [67]. Several researches have suggested that the peptides molecular weight may correlate with their emulsifying functionality. Jeon *et al.* fractionated cod frame protein hydrolysates (30 kDa, 10 kDa, 5 kDa, and 3 kDa) based on molecular weight, and they reported that 10 kDa fraction showed the best emulsifying properties over all pH ranges, followed by 30 kDa fraction, which also showed excellent emulsifying properties [74]. Wang *et al.* produced four peptide fractions (100 kDa, 50 kDa, 20 kDa, and permeate) from wheat gluten, and they claimed that 50 kDa gluten fraction tends to have higher emulsion activity index among pH 2 and pH 10 [75]. Agboola *et al.* highlighted the importance of high molecular weight whey peptides for emulsion stability, they found that the increased concentration of high molecular weight peptides at the interface can lead to improved emulsion stability [76]. Other researches have reported that peptide fractions with chain length over than twenty amino acids tended to have good emulsifying properties [74].

1.5.7.6 Thermal Treatment

Temperature has profound influence on protein solubility and denaturation. Generally, the protein solubility is increasing before the temperature reaching to 40 – 50 °C [73]. When the temperature increases further, the non-covalent bonds involved in stabilizing the secondary and tertiary structure disrupt [73]. Hydrophobic groups buried inside of these secondary and tertiary structures become exposed. In order to reducing the contact between hydrophobic groups and water molecules, the hydrophobic interactions between the hydrophobic groups themselves happen dramatically. Thus, protein heat-induced denaturation occurs [73].

Thermal stability is important for designing and operating the manufacture process used in protein stabilized emulsions. Measuring the heating stability of emulsion products may help us to thoroughly understand the influence of temperatures that emulsion experienced during processing and consumption. Protein can be denatured by heating. Based on the denaturation of emulsifiers, heating emulsion can cause the formation of aggregation or gels, which is unfavorable in emulsion systems [61].

1.5.7.7 Rheology of Emulsions

Rheology is a property that describing the relationship between the applied force and the resulting flow or deformation [77]. From a fundamental aspect, emulsion rheology can provide detailed information for emulsion droplet aggregation and strengths of interdroplet interactions, thus predicting the shelf life (stability) of emulsion products; from an industrial processing aspect, the rheological properties are important for designing a processing operation which depends on how the products behave when they under a shear flow, passing through a heat-exchanger, flowing within pipes, or pumping into a packages; from a commercial aspect, the rheological parameters are useful for texture or sensory description, such as creaminess, thickness, smoothness, and spreadability *et al.* [77, 78]. The rheology measurements are widely used to provide insightful information about the structure organization and droplets interactions in emulsion systems [77]. For instance, the viscosity versus shear rate measurement can be used to interpret the strength of droplet interactions within emulsions [77]. The viscosity of emulsions can be expressed by:

$$\eta = \mathrm{f}(\eta_0, \phi, \mathrm{d}, \mathrm{A}) \qquad (1.\ 13)$$

η: Viscosity;

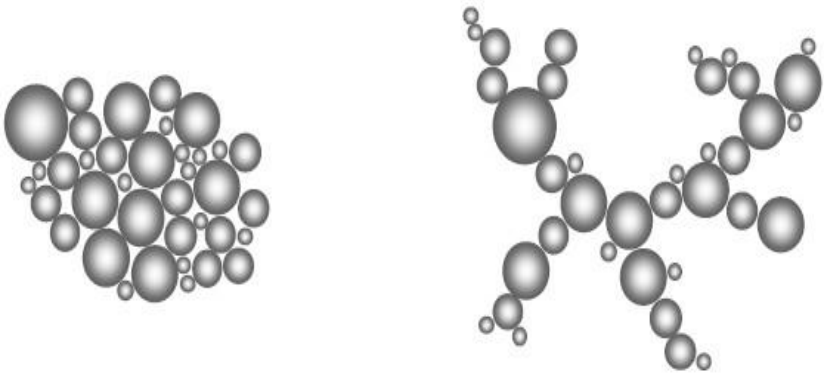
η_0:Viscosity of continuous phase;

ϕ: Phase volume fraction;

d: Droplet size;

A: State of aggregation.

To be specifically, if the viscosity for continuous phase is settled, the total emulsion viscosity increases along with the increased dispersed phase volume fraction, as well as increased aggregations [68]. However, the viscosity of emulsion reduces with growing droplet size [68].



Compact aggregates Open aggregates

Figure 1- 10. Schematic representation of compact and open aggregates. Source is adapted from [68].

The effects of droplet size and aggregation are normal primary parameters for investigating concentrated emulsion systems ($0.05 < \emptyset < 0.49$), since the emulsion

droplets in this system interact appreciably with each other through hydrodynamic interactions and droplets collisions [77]. When the size of emulsion is produced within a similar range, rheology can be used to reflect aggregation of emulsions. The interactions between droplets determine whether the aggregates are relatively open or compact, as showed in Fig. 1-10 [68]. Open aggregates entrain large amounts of continuous phase [68]. Open aggregates are formed when the attractive interactions between the emulsion droplets are strong; while compact aggregates are developed when the attractive interactions are weak [68]. So emulsion systems with open aggregates normally exhibit higher viscosity than the systems with compact ones [68]. The aggregation state of emulsions depends on the shear rate, and shear history (for thixotropic system) [68]. At low shear rate, the hydrodynamic force is not large enough to break the bonds holding droplets together, so the aggregates behave like particles with fixed sizes and shapes, hence the emulsion may have a constant viscosity [68, 79] Along with the increased shear rate, the hydrodynamic force is large enough to break down the aggregates into small droplets, thereby decreasing the emulsion viscosity [68, 77]. Thus, concentrated emulsions usually exhibit a shear thinning (non-Newtonian) behavior [68].

1.5.8 Parameters Used to Evaluate Emulsions

1.5.8.1 Emulsion Capacity, Emulsion Stability & Emulsion Activity

Emulsifying property is an important functionality for many food proteins. It is commonly discussed in terms of emulsion capacity (EC), emulsion stability (ES), and emulsion activity (EA) [80].

Emulsion Capacity. The emulsion capacity (EC) is defined as the maximum amount of oil that can be emulsified by a standard amount of protein under specific circumstances [80]. The emulsion capacity of proteins is associated with the interfacial area that can be coated by the available proteins [80]. The EC is expressed as the volume of oil per unit weight of emulsifiers (proteins) [81].

Emulsion Stability. Emulsions, which undergo various destabilizations, including creaming, Ostwald ripening, flocculation and coalescence, are thermodynamically unstable. ES is normally measured by the amount (or rate) of oil (or cream) separating from the emulsion through a certain period of time at specified temperature and gravitational field [80]. The emulsion stability is related to the constancy of the protein interface. It is usually evaluated by emulsion centrifuge stability (ECS) or creaming index (CI) [80].

Emulsion Activity. The emulsion activity is associated with the ability of proteins to adsorb at and stabilize the oil-water interface. It is usually expressed by emulsion activity index (EAI) and emulsion stability index (ESI) [80]. The EAI is determined by a turbidimetric method to measure the absorbance of light by diluted emulsion at wavelength of 500 nm [21, 80]. EAI can be presented by the interfacial area of per unit weight of protein [21, 80]. ESI is examined as the change of EAI based on a specific time period [80].

1.5.8.2 HLB

Table 1- 5. A summary for HLB ranges of surfactants and their applications. Source is adapted from [82].

HLB values	Utilizations
4-6	W/O emulsifiers
7-9	Wetting agents
8-18	O/W emulsifiers
13-15	Detergents
15-18	Solubilizes

The term ‘HLB’ stands for hydrophilic/lipophilic balance [58]. It has been used to calculate the balanced polarity of emulsifier molecules and predict the emulsion

types [37, 59]. Theoretically, high HLB values can be related to high water dispensability [54]. If emulsifiers were dispersed into the continuous phase, high HLB (8-18) emulsifiers are suitable for producing and stabilizing O/W emulsion, while low HLB (4-6) emulsifiers are suitable for preparing W/O emulsion [54]. The utilization for different surfactants with different HLB values is summarized in the table 1-5. The HLB values can be calculated by [82]:

$$\mathrm{HLB} = 7 + \sum(\text{hydrophilic group numbers}) - \sum(\text{hydrophobic numbers}) \quad (1.13)$$

However, HLB has been proven less useful for food emulsion systems, which exhibit very complicated nature [58]. Other properties, such as the balanced structures of emulsifier molecules and the capacity of emulsifiers to create and stabilize emulsion, are proven to be more essential [58].

1.5.9 Application of Emulsions

Emulsion plays an important role in human life. The earliest known emulsion products are milk and dairy products, such as butter, mayonnaise and cheese. In recent decades, emulsions are widely used in cosmetic and pharmaceutical products in order to increase the adsorption of certain nutrients. Other applications of emulsion products, including paints and dry-cleaning detergents, have also

Table 1- 6. Emulsion application in industry, adapted from [41].

Industrial systems	Applications
Food emulsions	Mayonnaise, salad creams, deserts, beverages *et al.*
Personal care & Cosmetic products	Hand-creams, lotions, hair-sprays, sunscreens
Pharmaceuticals	Anesthetics of O/W emulsions, lipid emulsions
Paints	Emulsions of alkyd resins, latex emulsions
Dry-cleaning	Dry cleaning oil with water droplets emulsified inside

been studied. The industrial products consisting of emulsions are shown in Table 1-6 [41]. Since a considerable number of emulsion applications were developed or will be possibly applied to the above-described utilizations, providing an insightful understanding for emulsion formation and stabilization is of great scientific and practical importance.

1.6 Hyphothesis and Objectives

1.6.1 Background

As one of the most important crops in the world, barley is widely cultivated in western Canada [3]. In order to develop value-added opportunities of barley grains for human consumption and increase revenue return for barley producer and processor, one of the nutrient compounds in barley — protein, was investigated in this research.

Barely protein exhibits low solubility in water, which leads to its limited application in food and non-food area. To achieve better solubility, as well as other improved functionalities, protein modification is required. In this research, deamidation was chosen to modify the barley protein, and the corresponding enhanced emulsifying functionality was evaluated.

The previous work of our lab investigated the deamidation on functional properties of barley glutelin and hordein [4, 83]. A broad range of deamidation levels of glutelin (~ 1 – 43%) and hordein (~ 0.7 – 40%) were prepared, and their emulsifying and foaming properties were systematically examined [4, 83]. The optimal functionalities were achieved at the deamidated range between 2.2 – 5.6% and 2.4 – 4.7% for barley glutelin and hordein, respectively [4, 83]. It was also

shown that even a small level of deamidation could significantly improve the solubility of barley protein, and thereby further improving emulsifying functionality [4, 83]. Thus, the previous research proved that barley glutelin and hordein could be used as excellent candidates for emulsifying ingredients. It was also found that large molecular weight fraction of deamidated protein may closely relate to high emulsion stability.

1.6.2 Hypothesis

It is hypothesized that there is positive relationship between the molecular weight of deamidated barley protein and its emulsifying properties, and fractions with larger molecular weight could better stabilize emulsion system. Therefore, in this research, deamidated barely protein was separated by ultrafiltration into fractions of different molecular weight. The effect of molecular weight on emulsifying properties of deamidated barley protein was systematically investigated. If the hypothesis can be proved through this research, new processing can be developed to concentrate most effective deamidated barley protein fraction as a new natural emulsion stabilizer for food, cosmetic and other applications. In this way, additional revenue return can be generated to benefit barley producers.

1.6.3 Objectives

1.6.3.1 The Long-term Goal

Modify barley protein to develop novel plant based emulsifier for food, cosmetic and other applications.

1.6.3.2 The Short-term Goal

1. Separate deamidated barley protein based on molecular weight using membrane filtration technique

2. Investigate physic-chemical properties (charge and hydrophobicity) of each fraction sample.

3. Study the effect of molecular weight of deamidated barley protein on emulsifying properties at different conditions (oil-to-water ratio, pH and temperature).

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Chapter 2 Emulsifying Properties of Deamidated Barley Protein Fractions

2.1 Introduction

Barley is ranked as the fourth most important cereal in terms of production in the world [1]. With more than 50 barley varieties being grown and harvested, Canada is one of the largest barley producers and exporters in the world [2]. The majority of cultivated barley (80%) is used for livestock feeding; while only a small amount of barley (15%) is utilized for malting and brewing in the beer industry; and a very limited amount of barley (5%) is consumed as human food to make noodles, bread, and breakfast cereals [1, 3]. In order to increase commercial value of barley grains and generate more revenue return to barley producer and processor, a growing research interest has been focused on value-added barley products, such as dietary fibers (β-glucan) and protein. Scientists around the world never stop their efforts to explore and improve the commercial uses of barley grains.

Barley protein is one of major by-products obtained in the beer industry. Barley protein contains high levels of hydrophobic amino acids. In addition, the high

content of glutamine and asparagine residues makes barley protein very prone to aggregation by hydrophobic and hydrogen bonds [4]. As a result, barley protein is only soluble in alcohol, urea, or alkali solutions, which leads to limited application of barley protein as food ingredients [4, 5].

Deamidation is a widely selected modification for improving protein's functionalities. It can be defined as a chemical modification which removes ammonia from amide side chains of peptides or proteins via hydrolysis [6]. The reaction leads to conversion of asparagine and glutamine amide groups to carboxylic groups [6]. The relevant reactions are:

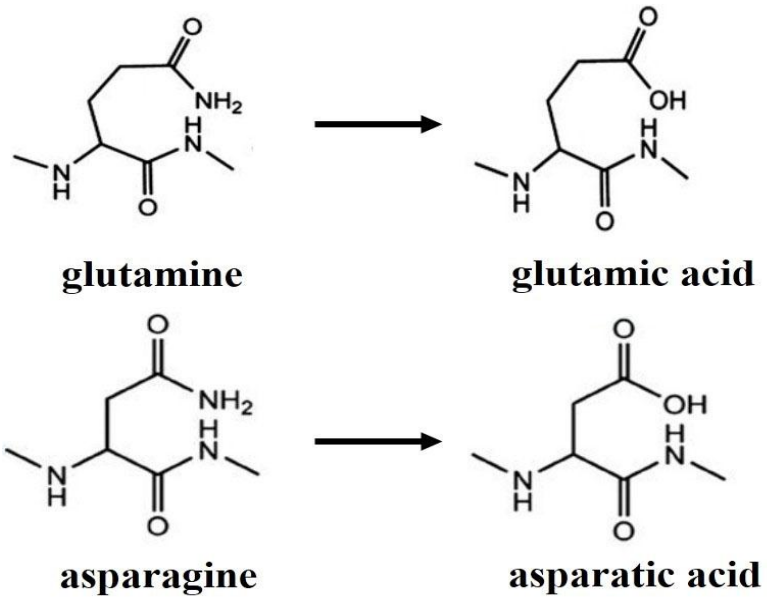


Figure 2- 1. Deamidation of asparagine and glutamine, adapted from [7].

Fig. 2-1 shows the deamidation reaction of asparagine and glutamine, respectively [7]. Deamidation brings more negative charges to protein molecules by converting amide groups to carboxylic groups [4, 6]. The increased negative charges intensify the electrostatic repulsions among protein molecular chains, which effectively increase the solubility and emulsifying functionalities of proteins [4-6]. The enhanced repulsive force leads to partial unfolding of the protein chains, which results in more exposure of hydrophobic regions. In emulsion formation, the increased hydrophobic groups will positively contribute to hydrophobic interactions at the protein adsorbed interface. In addition, higher exposure of sulfur rich groups due to partial unfolding will contribute to disulfide bond formation which improves the cohesiveness and strength of the protein layer at the interface [8]. It had been reported that even small levels (2 – 6%) of deamidation could lead to a significant improvement in protein functionalities [6]. Based on the high content of glutamine (glutamic acid) and considerable amount of asparagine (aspartic acid) in barley protein, deamidation modification was selected in this research to improve the emulsifying properties of barley protein.

The emulsifying properties of barley proteins with different deamidation degree (DD) were evaluated in research previously conducted in our groups. It had been shown that the optimal functionalities were achieved at the deamidation

range between 2.2 – 5.6% and 2.4 – 4.7% for barley glutelin and hordein, respectively [5, 9]. The author also demonstrated that deamidation led to significant increase in negative charge of molecules due to amide side chain conversion combined with protein hydrolysis (peptide bond cleavage) [5]. Thus, during deamidation, barley proteins have been converted from their native structure to protein fractions or peptides of smaller molecular weight with different physico-chemical properties (charge, molecular weight and hydrophobicity). The relationship between molecular weight and emulsifying properties of deamidated barley protein was studied in this research. Previous research suggested that the molecular weight of peptides may correlate with their emulsifying functionality. Jeon *et al.* fractionated cod frame protein hydrolysates based on molecular weight (30 kDa, 10 kDa, 5 kDa, and 3 kDa) and reported that 10 kDa fraction showed the best emulsifying properties over all pH ranges, followed by 30 kDa fraction, which also showed excellent emulsifying properties [10]. Wang *et al.* produced four peptide fractions (100k Da, 50 kDa, 20 kDa, and < 20 kDa) from wheat gluten. They concluded that 50 kDa fraction showed a higher emulsion activity index in the pH range from 2 to 10 [11]. Agboola *et al*. highlighted the importance of high molecular weight whey peptides for emulsion stability. They found that the increased concentration of high molecular weight peptides at the interface could lead to improved emulsion

stability [12]. Other researchers have also reported that peptides fractions with chain length over twenty amino acids exhibited better emulsifying properties as compared to short peptides [10].

The objective of this research is to examine the correlation between molecular weight and emulsifying properties of deamidated barley proteins. In order to obtain fractions with similar charges and different molecular weights, the deamidated barley protein, at 63% DD, is to be fractionated using ultrafiltration membranes (with 10 kDa, 50 kDa and 300 kDa molecular weight cut-off membranes). The corresponding molecular structures and emulsifying properties of fractions with different molecular weight are investigated.

2.2 Materials and Methods

2.2.1 Materials

Regular barley grains (Metcalfe) were provided by Alberta Agricultural and Rural Development, Lacombe, Alberta. Canola oil, used for experiments was purchased from a local supermarket (Edmonton, AB, Canada). Ammonia Assay Kit for deamidation degree determination, 1-anilinonphathalene-8-sulfonic acid (ANS) for hydrophobicity analysis and standard molecular weight markers for HPLC

analysis were ordered from Sigma-Aldrich, Canada. All other chemicals were of analytical grade.

2.2.2 Barley Protein Preparation

Barley protein isolates were extracted from barley grains using alkaline extraction, based on the methods reported by Wang *et al.* [13]. Pearled barley flour was dispersed in alkaline solution (pH 9 – 11.5 adjusted with 0.5 *M* NaOH) and constantly stirred for 1.5 h at room temperature. After extraction, the supernatant was collected by centrifuge (8,500 × *rpm* for 15 min), followed by adjusting the pH to 5.0 (by 0.5 *M* HCl). Protein was then precipitated from the supernatant and obtained by centrifugation at 8,500 *rpm* for 15 min at room temperature. All isolated proteins were freeze-dried and stored at 4°C before further use [13].

2.2.3 Deamidation

Barley protein, obtained from barley protein isolates described above, was deamidated by acid methods. Barley protein isolates were suspended in 0.2 *M* HCl solution (2%, w/v). Then, the protein suspension was heated up to 90°C and kept at this temperature for 4 h under constant stirring. After the reaction, the sample suspension was cooled down to room temperature and neutralized using 0.5 *M* NaCl. The degree of deamidation (DD) was calculated as the ratio of ammonia released from the sample to that total ammonia released from completely

deamidated protein using Ammonia Assay Kit. The complete deamidation was conducted by stirring barley protein isolates in 2 *M* HCl (2%, w/v) for 4h. According to the result, the DD of the deamidated barley protein used in this thesis was 63%.

2.2.4 Molecular Weight Distribution Profile

Molecular weight distributions of deamidated barley proteins were determined by size exclusion column (SEC) (Superdex 20010/300 GL, Amersham Boisciences, NJ) using high-performance liquid chromatography (HPLC) system (1200 Series, Agilent Technologies, US). During the operation, 50 μL of sample solution was injected into the HPLC system. The protein sample was chromatographed at room temperature using 0.1 *M* phosphate buffer containing 0.1 *M* sodium chloride as mobile phase at a flow rate of 0.5 ml/min, and detected using the UV/Vis detector at 280 nm. Standard molecular weight markers were measured by HPLC, and the standard curve for molecular weight was obtained to calculate the weight-average molecular weight of deamidated proteins.

2.2.5 Preparation of Deamidated Barley Protein Fractions

The different molecular weight fractions were obtained by membrane filtration using a series of ultrafiltration membranes (Pall Corporation, US) connected to a pump (Masterflex console drive, Cole-Parmer Instrument Company, Canada). The

deamidated barley protein solution was allowed to pass through molecular weight cut-off (MWCO) membrane of 300 kDa (OS300T12), 50 kDa (OS050T12) and 10 kDa (OS010T12). The fractions (> 300, 10 – 50 and ~ 10 kDa) were collected and freeze-dried. After preparation, the molecular weight of each fraction was verified by HPLC to ensure the successful separation.

2.2.6 Zeta-potential

Zeta potential of three protein fractions at different pHs (3 – 10) was measured by monitoring the direction and velocity of protein molecule movement under an applied electric field using Zetasizer Nano-ZS (Model ZEN3600, Malvern Instruments Ltd., Malvern, UK). All the measurements were performed in triplicates and the average values were reported.

2.2.7 Surface Hydrophobicity of Protein

The surface hydrophobicity of protein was determined using fluorescence probe method. 8-Anilinonaphthalene-1-sulfonic acid (ANS) was selected as the fluorescence probe according to the method of Kato and Nakai [14]. The sample solutions were diluted in phosphate buffer (pH 7) at room temperature to reach different concentrations (0.005 – 0.02%, w/v). 20μl of ANS (8 *mM*) were added to the diluted sample solutions, and the fluorescence intensity (FI) was determined at 390 nm (excitation) and 470 nm (emission) by Spectrometer (SpectraMax M3

Multi-Mode Microplate Reader, Molecular Devices, US). The surface hydrophobicity was reported as the initial slope of fluorescence intensity versus sample concentration (mg/ml) calculated by linear regression analysis.

2.2.8 Emulsification Properties

Emulsificaiton properties were determined at pH 3, 5 (0.2 *M* citrate buffer) and 7 (0.2 *M* phosphate buffer) in buffers according to the method of Zhao *et al.* [5, 9]. Each protein fraction was dispersed in 20 ml and 30 ml buffer solutions, followed by addition of 20 ml and 10 ml canola oil to prepare emulsions with 1:1 and 1:3 oil to aqueous phase ratio, respectively. Then, the mixtures were homogenized for 2 min using a high speed homogenizer (PowerGen Model 1000 Homogenizer, Fisher Scientific) at the speed of 30,000 *rpm* to prepare emulsion. Emulsion samples were centrifuged for 5 min at 1500 g. The emulsion centrifuge stability (ECS) was calculated as follows: $\mathrm{ECS}\% = V_{e1}/V_{e0}$, where, V_{e1} is the volume of emulsion layer remaining after centrifuge, while V_{e0} is the total volume of emulsion before centrifugation. To study the thermal stability, emulsion samples were heated in 80°C water bath for 30 min, followed by centrifugation for 5 min at 1500 g after they cool down. The emulsion thermal stability was calculated as: $\mathrm{ETS}\% = V_{e2}/V_{e0}$, where, V_{e2} is the volume of emulsion layer remaining after centrifuge of heated emulsion samples. The released oil volume (ml) after centrifuge was also recorded.

2.2.9 Micrograph of the Emulsion

The diluted (30 times dilution) emulsion samples were observed under 40×microscope (Primo Star microscope, ZEISS, Germany). The microscope was equipped with a CCD camera (AxioCam ERc5S, ZEISS, Germany) for image capture.

2.2.10 Statistical Analysis

All experiments were conducted in triplicates. Results were reported in figures as mean ± standard deviation. The error bars in figure stands for standard deviations. Originpro 8 software (OriginLab Corporation, Northampton, MA, USA) was used to analysize data. Statistical evaluation was performed by analysis of variance (ANOVA) using SAS statistical software (SAS Institute, Inc., Cary, NC). The level of significance used was $p < 0.05$.

2.3 Results and Discussion

2.3.1 Molecular Weight Distribution

The weight-average molecular weight (M_w) distribution of the deamidated barley protein was examined by size exclusion column (SEC) in high-performance liquid chromatography (HPLC). According to Fig. 2-2, deamidated barley protein

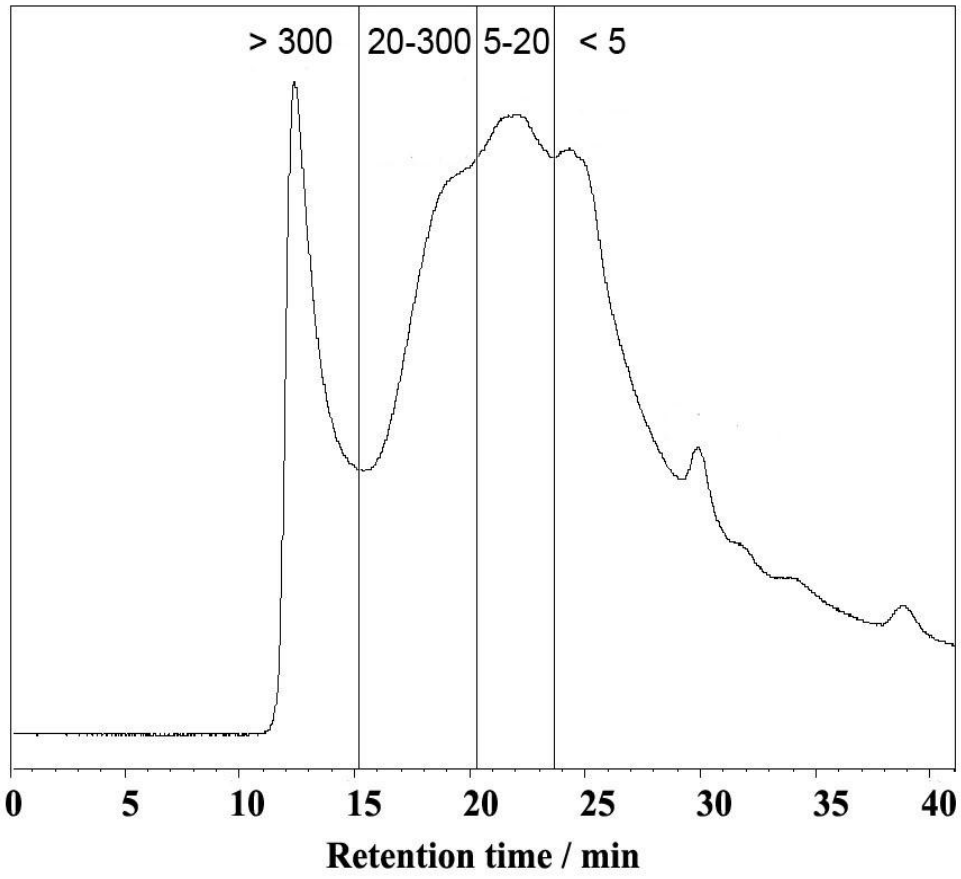


Figure 2- 2. SEC-HPLC chromatograms of deamidated barley protein (63%DD), the Mw values showed in this graph are in the unit of kDa.

contains 5 major peaks, which corresponded to M_w exceeding 300 kDa, M_w between 20 and 300 kDa, M_w between 5 and 20 kDa, and M_w lower than 5 kDa (two peaks), respectively. The peak for large-sized fraction (M_w > 300 kDa) was sharp. This peak could be attributed to aggregates of large soluble polypeptides after deamidation [5, 9]. In previous research, a narrow and sharp peak assigned to large M_w (> 600 kDa) was reported by Zhao *et al.* [5, 9]. The

authors reported that a significant increase of aggregates (large polypeptides) band could be observed at relatively high deamidation degree (DD) at a sacrifice of small peptide band [5, 9]. To be specific, the elution volume area of aggregate band could be greatly enhanced up to 43% of DD for deamidated barley glutelin [5]. As a result, the observation of sharp peak at large-sized fraction (M_w > 300 kDa) for 63% (DD) deamidated barley protein was in agreement with previous result [5, 9]. The fractions with M_w range between 20 – 300 kDa and 5 – 20 kDa could be assigned to deamidated barley protein peptides. The two peaks which showed M_w in the range of lower than 5 kDa were believed to be small peptides caused by protein hydrolysis during deamidation, since their M_w were much lower than barley protein subunits.

This study focuses on the three deamidated protein fractions based on different molecular weights, which are > 300, 10 – 50, and ~ 10 kDa. The three target fractions were separated from the 63% (DD) deamidated barley protein using ultrafiltration membrane with M_w cut offs of 300, 50 and 10 kDa. Their corresponding M_w distribution after separation was verified by SEC-HPLC. The SEC chromatograms of each deamidated protein fraction in phosphate buffer (pH 7) were depicted in Fig. 2-3. The large-sized polypeptide fraction exhibited M_w exceeding 300 kDa; the medium-sized polypeptide fragment showed

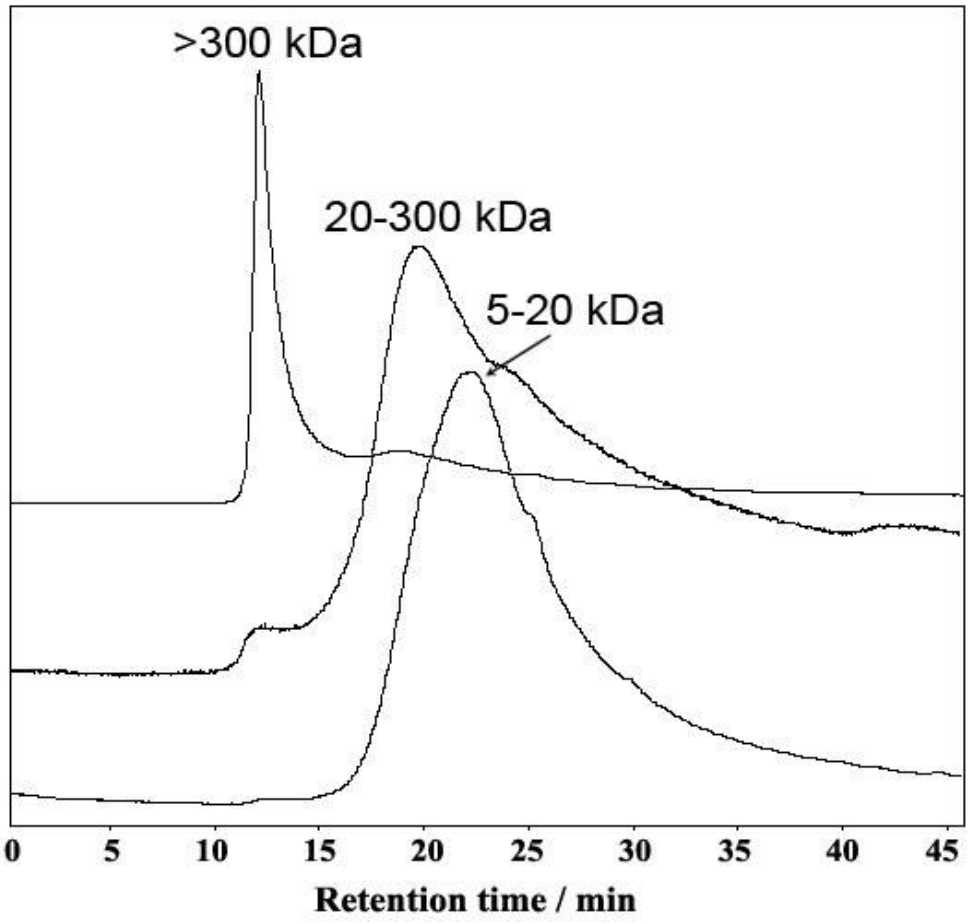


Figure 2- 3. SEC-HPLC chromatograms of different molecular weight fractions.

M_w between 20 and 300 kDa; while the small-sized polypeptide fraction displayed M_w between 5 and 20 kDa. According to Fig. 2-3, each fraction exhibited a single major band, which proved that the membrane fractionation process was successful. These three deamidated barley protein fractions were used for further analysis in this research.

2.3.2 Zeta-potential

The surface charge of deamidated protein fractions dependent on the pH of aqueous phase is shown in Fig. 2-4. The zeta-potential of protein fractions was highly positive (~ +40 mV) at pH 3, and it became less positive with increasing pH until it decreased to zero at pH ~ 4.2, which suggested that the isoelectric point (IEP) of the deamidation barley protein was around pH 4 – 5. Then, the zeta-potential became increasingly negative (reached to ~ -45 mV) when the pH was further increased to 6. These results indicated that the surface charge of protein was positive at acidic pH and negative at basic pH. The positive charge at pH lower than IEP was caused by protonation of the nitrogen in the amino groups

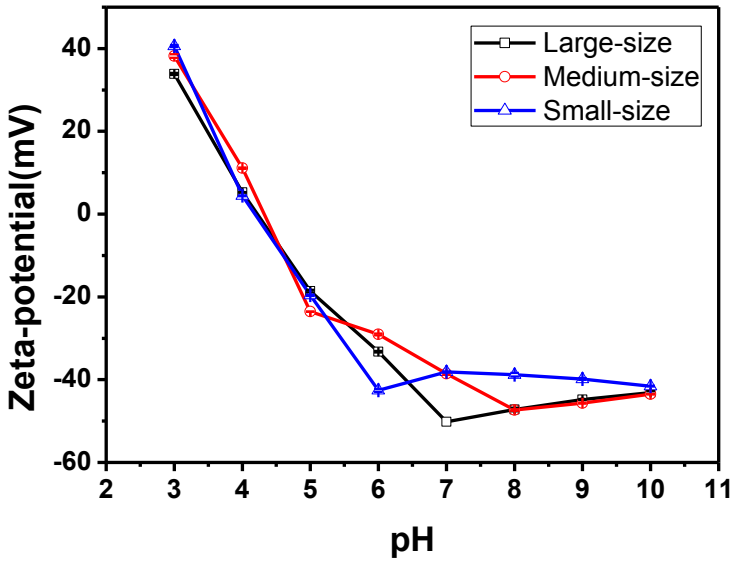


Figure 2- 4. Electrophoretic mobilities of deamidated barley protein fractions as a function of pH. The values in this figure are average of triplicates.

of the proteins, which changes amino groups from neutral ($–NH_2$) to be positively charged ($–NH_3^+$), while the negative charge at pH higher than IEP was arisen from the changes of carboxyl group from neutral (-COOH) to be negatively charged ($-COO^-$) [15]. The highly positive or negative charges at pHs far away from IEP (3 or 7, respectively) facilitated dispersion of protein molecules in aqueous phase, which may further improve emulsion stability by increasing the electrostatic repulsion between emulsion droplets. It has been reported that the IEP of untreated barley protein is around pH 6, which means the surface charge of barley protein at pH 5 should be positive [9]. However, the zeta-potential of each deamidation fraction switched to around -20 mV at pH 5, which indicated the shift of IEP to more acidic pH caused by increasing amount of carboxyl group in protein molecular chains [5, 9, 16]. According to Fig. 2-4, it could be concluded that all deamidated barley protein fractions have almost the same surface charges under similar pH condition. Thus, the protein fractions with different molecular weights (from small to large) and the same surface charge were obtained. Due to the deamidation modification, the physic-chemical properties of barley protein change as follows: change of molecular weight, increase of surface charge, and exposure of hydrophobic regions. As a consequence, protein emulsifying functionality can be improved potentially. In this research, we target to study the relationship between molecular weight and emulsifying properties of deamidated

barley proteins.

2.3.3 Surface Hydrophobicity

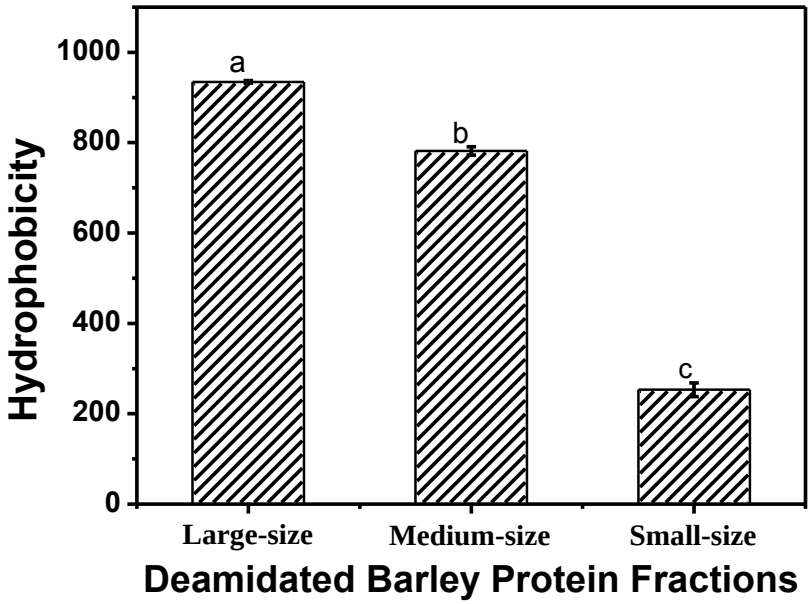


Figure 2- 5. Hydrophobicity of deamidated barley protein fractions at pH 7. The values in this figure are average of triplicates. Bars with different lowercase letters are significantly ($p < 0.05$) different.

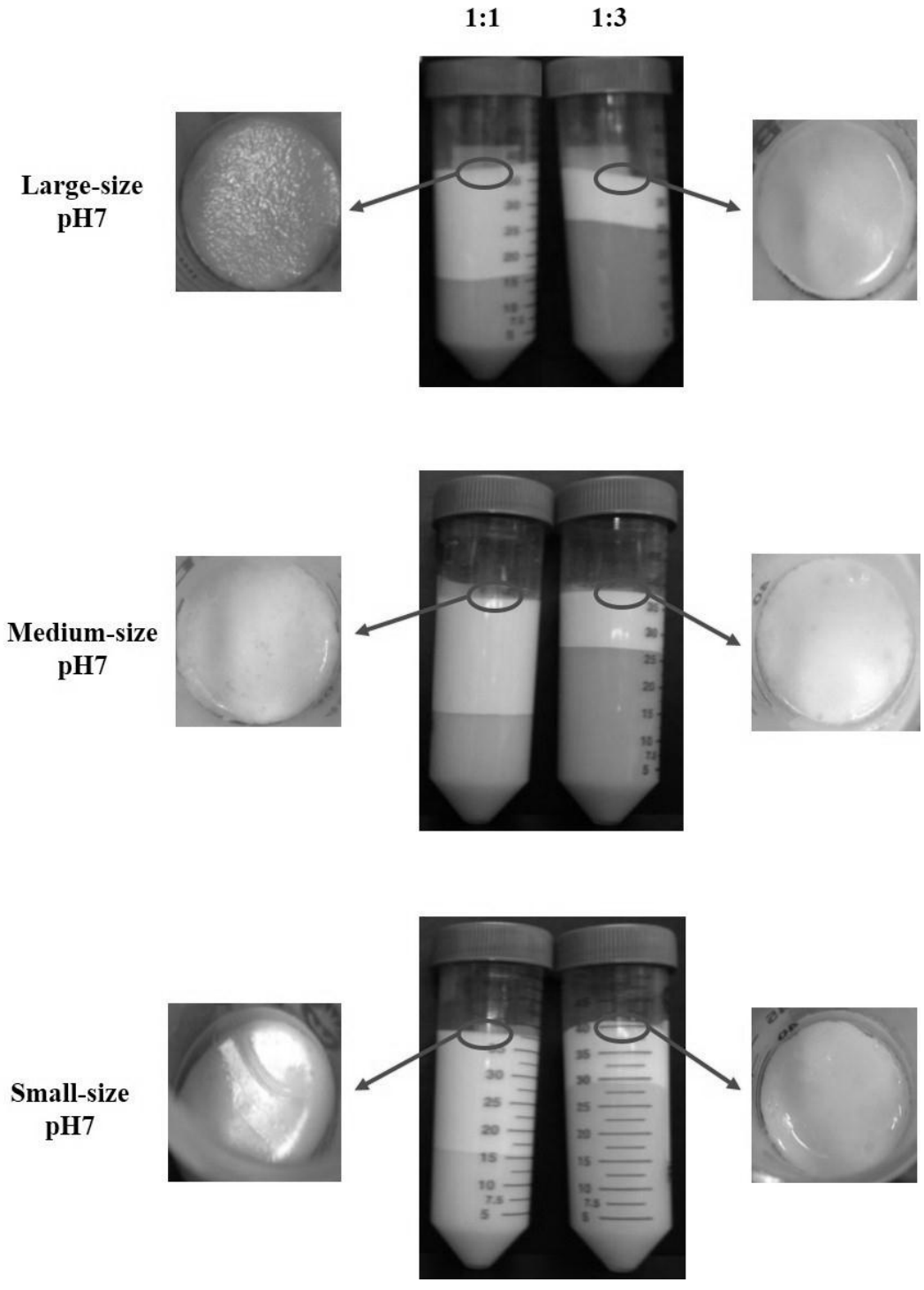
Surface hydrophobicity (S_o) is an indicator of the amount of hydrophobic region on the protein in contact with the polar aqueous phase [11]. It is also an index of protein's potential for intermolecular interactions, and consequently its functionality [11]. Higher surface hydrophobicity can enhance interactions between hydrophobic regions of protein and non-polar oil molecules, which

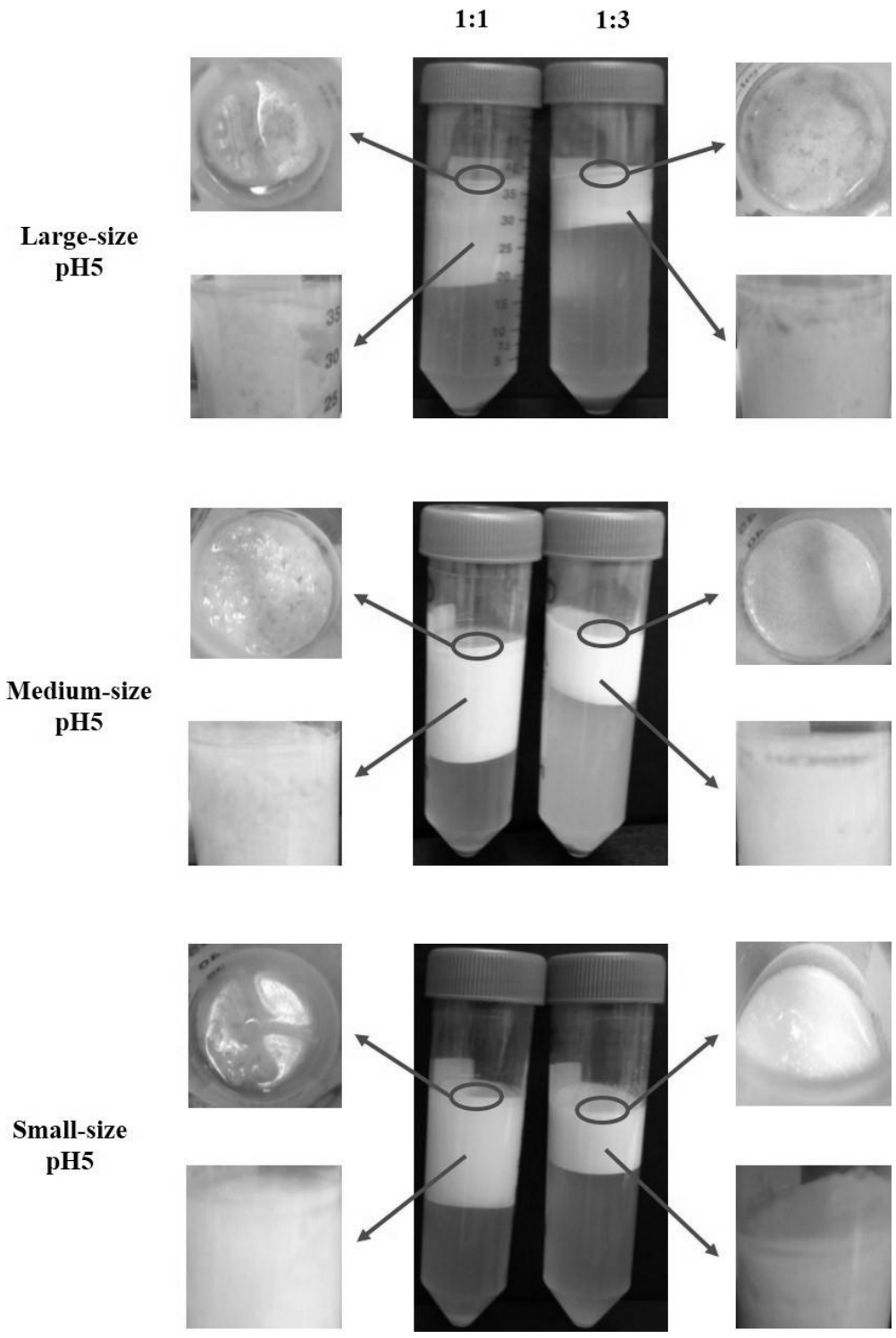
facilitate emulsion formation. The surface hydrophobicity for each deamidated barley protein fraction was showed in Fig. 2-5. According to Fig. 2-5, the highest surface hydrophobicity (S_o = 935) was observed for the large-sized fraction. At smaller M_w range, surface hydrophobicity reduced progressively to 782 and 253 for medium-sized and small-sized fractions, respectively. Barley protein was characterized by high levels of non-polar amino acids, such as proline, leucine, alanine and valine [5]. As a consequence of deamidation, some non-polar amino acid residues buried inside of native barley protein were exposed due to unfolding and cleavage of protein molecular chains [16]. Thus, the surface hydrophobicity of deamidated barley protein is relatively high comparing to other protein emulsifiers, such as whey protein (S_o ~ 43 or 97) and soy proteins (S_o ~ 17) [17-19]. Large-sized fraction is likely to have longer and more complicated molecular chains and structures than small-sized fraction. Thus, it may contain more non-polar regions, which results in higher surface hydrophobicity. The surface hydrophobicity can also be correlated with the structure of protein fractions. Bamdad (2013) reported that large molecular weight fractions possessed higher surface hydrophobicity in barley protein hydrolysates due to high content of protein tertiary structure [20]. The surface hydrophobicity can be an important factor influencing the emulsifying functionalities. The high surface hydrophobicity allows a better molecular anchorage at the oil-water

interface, which might better stabilize emulsion.

2.3.4 Emulsion Centrifuge Stability and Emulsion Thermal Stability

It has been reported that deamidation can lead to significant improvement of emulsifying properties due to the charge increment and the exposure of hydrophobic residues [5, 9, 16]. The emulsion centrifuge stability (ECS) was evaluated through morphology of the top layer (in terms of integrity and the released oil layer) and the percentage of the remaining emulsion layer. Fig. 2-6 presents the images for emulsion centrifuge stability of barley protein fractions at different oil volume fractions (Ø) and pH conditions. The oil volume fraction was normally chosen among 20 – 30% in other oil in water (O/W) emulsion studies [12]. Ø = 25% (1:3 oil-to-water ratio) and Ø = 50% (1:1 oil-to-water ratio) were selected in this research to evaluate emulsion with normal and high oil content using deamidated barley proteins. According to Fig. 2-6, deamidated barley protein was able to produce much more volume of emulsion layer at high oil volume fraction (Ø = 50%). This could be related to the relatively high hydrophobicity of deamidated protein fractions, which enabled it to have strong ability to bind non-polar oil at emulsion interface. The emulsion properties affected by protein molecular weight and environmental pH were systematically evaluated. At pH 7, the large-sized and medium-sized fractions tended to have


1:1
1:3
Large-size
pH7
Medium-size
pH7
Small-size
pH7


1:1
1:3
Large-size
pH5
Medium-size
pH5
Small-size
pH5

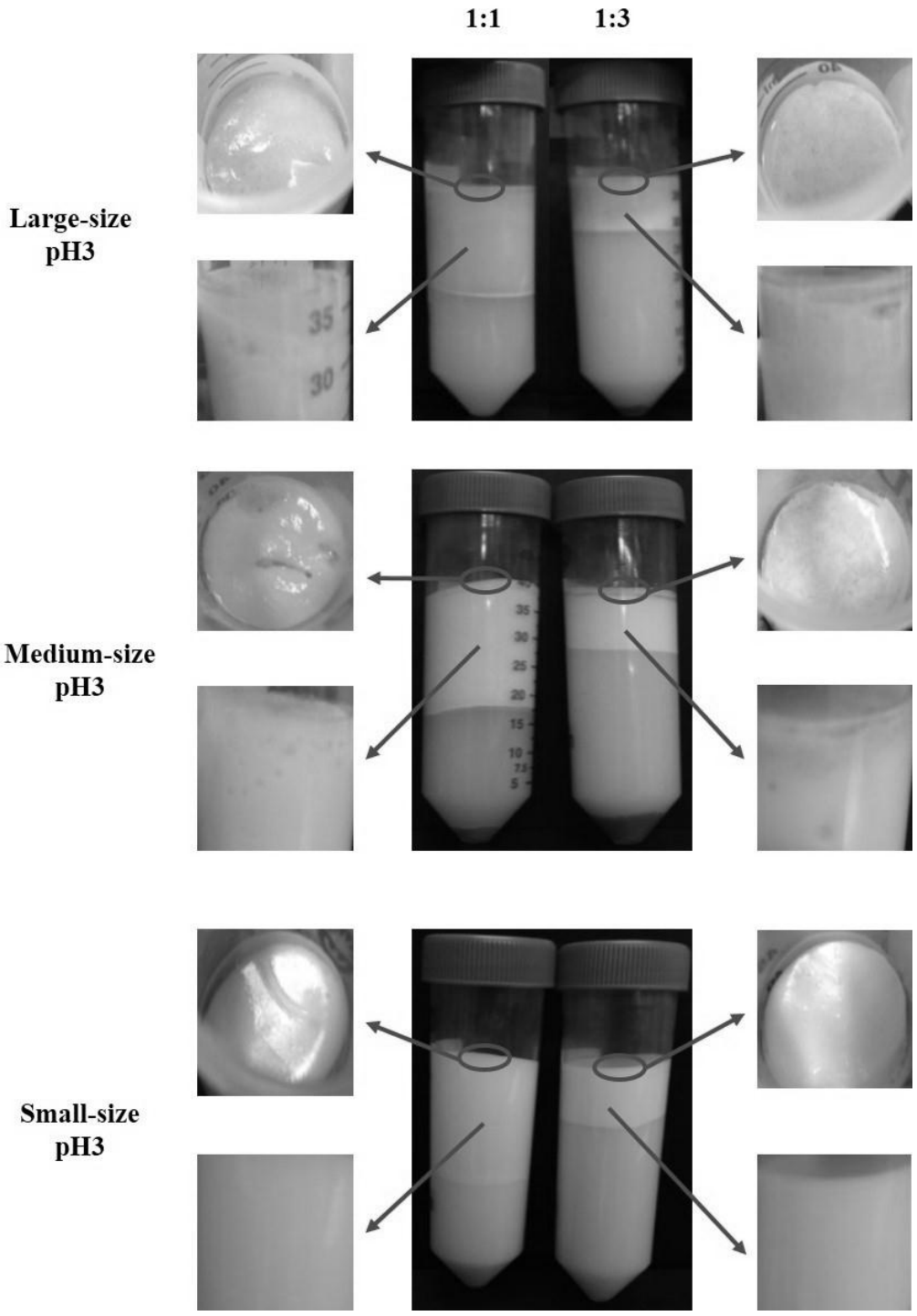


Figure 2- 6. Photos of emulsions after centrifugation when prepared at different pHs and oil-to-water ratios

more capacity to bind oil under the tested centrifuge acceleration, since there was no oil release for emulsions using these two fractions after centrifuge, while oil layer could be observed in emulsions using small-sized fraction. Oil observation revealed that the samll size fraction could not successfully stabilize oil droplets under 1500 *g* centrifuge acceleration at pH 7. It was claimed that large-sized and medium-sized fractions have higher hydrophobicity than small-sized fraction at pH 7. High hydrophobicity improved interaction between oil and non-polar region of protein, which was favorable for emulsion formation and stabilization. In addition, the relatively large polypeptides allowed the formation of steric proteic network that covers oil droplets. When pH decreased to 5 (near IEP), the emulsifying properties greatly decreased since protein fractions had low solubility due to reduced charge. According to the observation, emulsions stabilized by large-sized fraction showed the most significant changes after centrifuge, with the top of emulsion system being full of oil and large emulsion flocs loosely dispersed in aqueous phase. Comparing to large-sized fraction, medium-sized fraction showed better ECS for stabilizing emulsions at pH 5, which remained their milky color in emulsion layer, though some small holes in cream layer of the centrifuged emulsions were observed. Emulsions using small-sized fraction had the least changes at pH 5 comparing with their performance at pH 7. At pH 3, the emulsifying ability for deamidated barley protein fractions was still low. Obvious

oil release could still be observed in emulsions formed by large-sized fraction, and a thin layer of oil could be observed in both emulsion systems made by medium-sized and small-sized fractions. The small-sized fraction, with high solubility among all pHs, produced emulsion with similar morphology between pH range from 3 to 7. Thus, it could be concluded that the environmental pH could significantly influence the emulsions stabilized by large-sized fraction, but pH conditions barely affected emulsions stabilized by small-sized fraction. This may be caused by large-sized fraction having lower solubility than small-sized fraction at acidic conditions, thus they cannot have sufficient emulsifier concentration to fully cover the oil droplet interface, leading to poor emulsifying properties.

Fig. 2-7 shows oil release of different emulsion systems after centrifuge under pH 3, 5 and 7. High oil-to-water ratio (1:1) led to more oil release. Emulsion system containing 50% oil fraction showed higher oil release than emulsions containing 25% oil fraction, as shown in Fig. 2-7. There was no oil layer observed for large-sized and medium-sized fractions stabilized emulsions while 2 ml and 0.9 ml oil layer was observed at the top for small-sized fraction stabilized emulsions at 1:1 and 1:3 oil-to-water ratio, respectively. The fraction of large-sized and medium-sized had strong surface hydrophobicity as shown

previously, the high amount of exposed hydrophobic patches on the protein molecular chains facilitated their binding to hydrophobic oil molecules at the

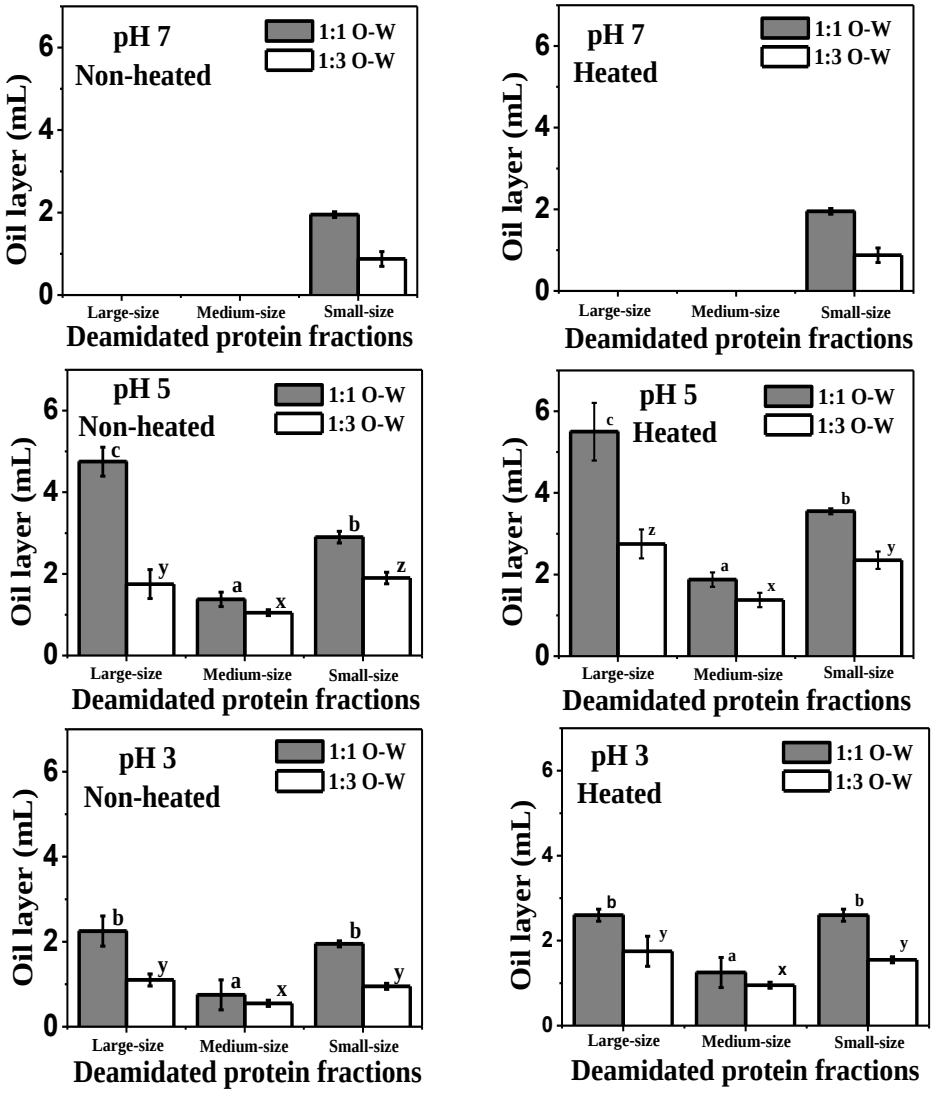


Figure 2- 7. Oil layer (ml) for each centrifuged emulsion systems at different pHs (pH 3, 5 and 7). The values in this figure are average of triplicates. Bars with different lowercase letters are significantly ($p < 0.05$) different.

emulsion interface, and then the protein molecules could aggregate by hydrophobic interaction to form strong films around the oil droplets [5, 9]. As a result, the large and medium size deamidated fractions tended to form more stable emulsion under certain centrifuge acceleration at pH 7. At pH 3 and 5, all the emulsions showed oil release after centrifugation. At pH 5, emulsions stabilized by large-sized fraction released the most amount of oil, which was up to 4.75 ml oil at the top for ∅ = 50%, and 1.75 ml for ∅ = 25%. While emulsions stabilized by medium-sized fraction only released 1.4 ml for ∅ = 50% and 1 ml for ∅ = 25%. The amount of oil release slightly increased (3ml for ∅ = 50%, 2 ml for ∅ = 25%) for small-sized fraction stabilized emulsions at pH 5, but it decreased back when pH lowered to 3. At the same time, the emulsion sample of large-sized fraction still kept large amount of oil release at pH 3, while medium-sized fraction stabilized emulsion maintained low level of oil release. After heating treatment, the oil release of emulsion systems did not have significant difference compared to the non-heating emulsions, which means the deamidated barley protein fractions could form thermal stable emulsion systems. In conclusion, large-sized fraction had good emulsifying property at pH 7, but it was vulnerable to acidic pHs; medium-sized fraction stabilized emulsion did not have oil release at pH 7, and it had the lowest oil release at both pH 3 and 5. Small-sized fraction showed the least changes of emulsifying properties when pH switched from 3 to 7.

ECS and ETS were calculated and presented in Fig. 2-8. For non-heating emulsions prepared at the same oil-to-water ratio, all the protein fractions showed similar ECS at pH 7. The ECS at pH 7 was slightly higher than the ECS at pH 3

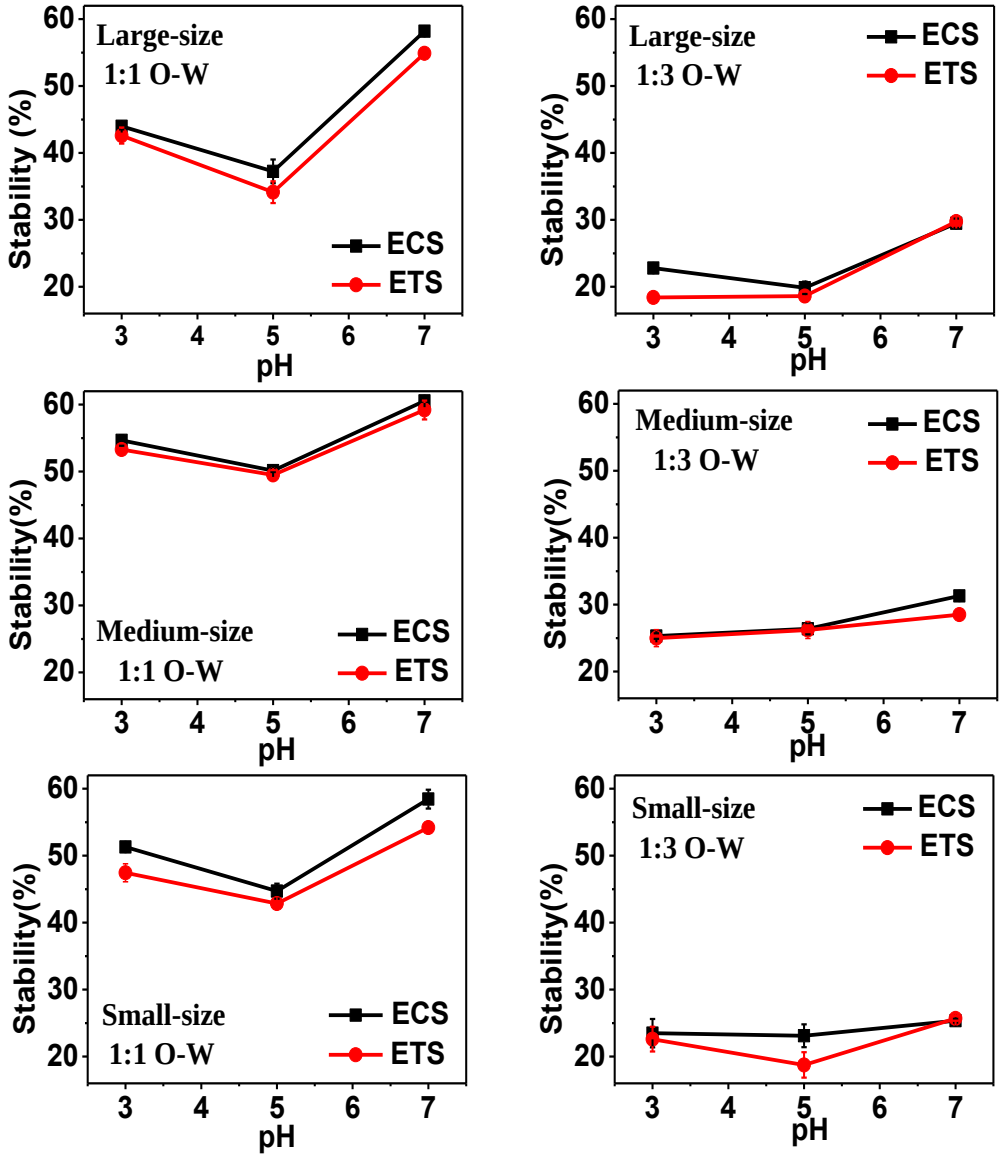
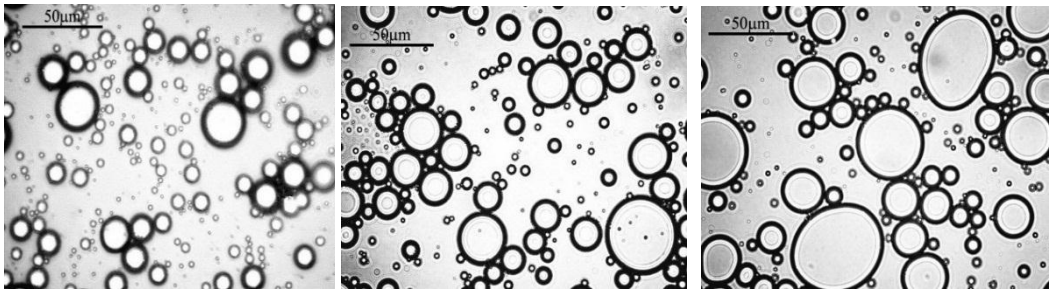


Figure 2- 8. Emulsifying stability of deamidated barley protein fractions as a function of pH. The values in this figure are average of triplicates.

and 5, this was probably due to the enhanced electrostatic repulsion in emulsions caused by high charge of protein fractions, which prevents the adjacent emulsion droplets from close contact [5, 9]. The emulsion stabilized by large-sized fraction (1:1 oil-to-water ratio) tended to have less ECS at pH 3 and 5, which was 44% and 37%, respectively. While emulsion stabilized by medium-sized fraction (1:1 oil-to-water ratio) exhibited 55% ECS at pH 3 and 50% ECS at pH 5, and emulsion stabilized by small-sized fraction (1:1 oil-to-water ratio) showed 51% ECS at pH 3 and 45% ECS at pH 3. Emulsions using 1:3 oil-to-water ratio showed significantly lower ECS than those prepared at 1:1 ratio. At 1:3 oil-to-water ratio, the amount of oil that can be emulsified was low, as compared to 1:1 ratio. As a consequence, the low ECS was observed. The deamidated protein fractions with relatively high hydrophobicity, exhibited stronger oil binding ability, thus, they were able to have higher ECS when oil-to-water ratio increased to 1:1. The systems of 1:3 oil-to-water ratio and 1:1 oil-to-water ratio showed similar trend for ECS. The changes of ECS for large-sized fraction at different pHs were in agreement with our previous discussion that large-sized fraction was vulnerable to acidic conditions when stabilizing emulsion. Similar ECS and ETS values were shown for non-heating and heating emulsion samples at different pHs, which indicates that emulsions stabilized by deamidated protein (barley) fractions have excellent thermal stability. This may be attributed to

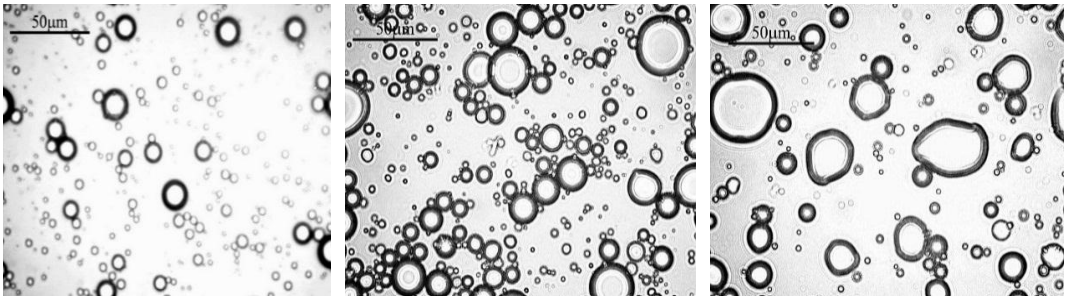
further gelation of the deamidated protein at the oil-water interface during thermal treatment [5, 9].

2.3.5 Optical Micrographs



0 h 108 h 120 h

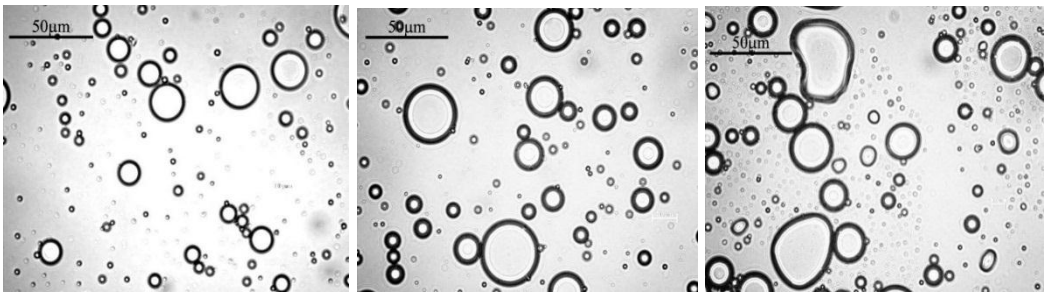
(a)



0 108 h 120 h

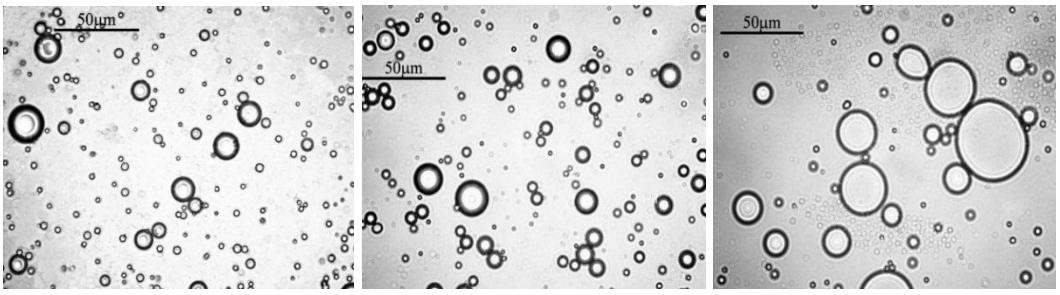
(b)

Figure 2- 9. (a) Image of optical microscope for emulsion (1:1 oil-to-water ratio) made from large-sized fraction at pH 7. (b) Image of optical microscope for emulsion (1:3 oil-to-water ratio) made from large-sized fraction at pH 7.



0 h 60 h 72 h

(a)

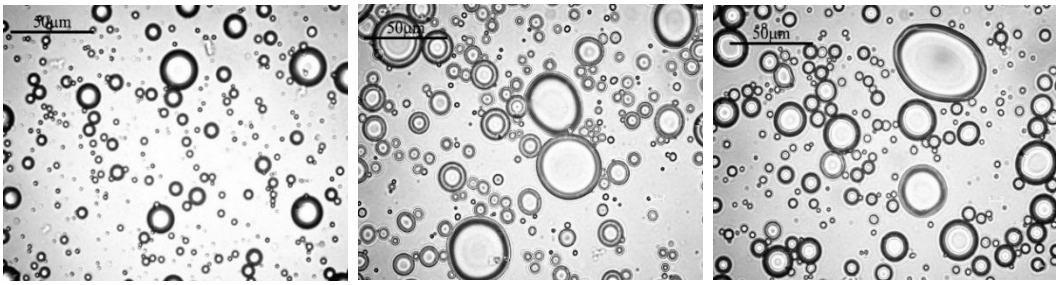


0 h 60 h 72 h

(b)

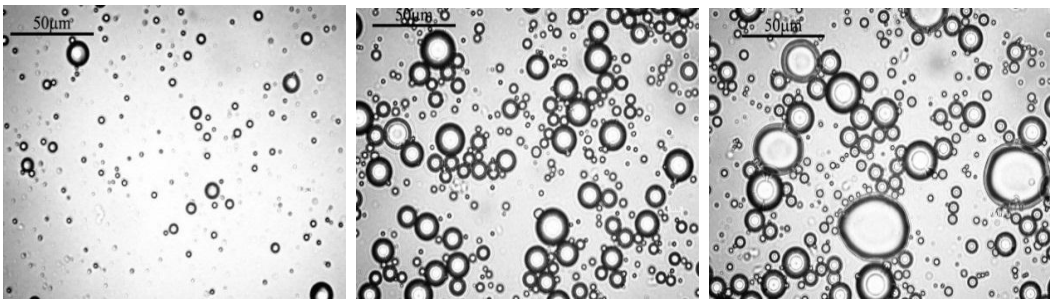
Figure 2- 10. (a) Image of optical microscope for emulsion (1:1 oil-to-water ratio) made from medium-sized fraction at pH 7. (b) Image of optical microscope for emulsion (1:3 oil-to-water ratio) made from medium-sized fraction at pH 7.

The size of emulsion droplets prepared with proteins from different fractions at pH 7 was measured by optical microscope. The image was taken at different time periods, and micrographs examples are shown in Fig. 2-9 for large-sized fraction, Fig. 2-10 for medium-sized fraction, and Fig. 2-11 for small-sized fraction. The average droplet diameter was measured and calculated using the micrographs



0 h 36 h 48

(a)



0 h 36 h 48 h

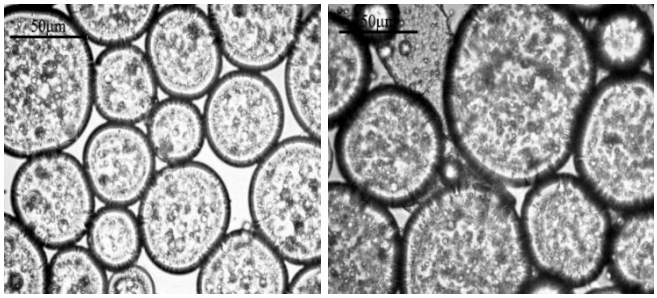
(b)

Figure 2- 11. (a) Image of optical microscope for emulsion (1:1 oil-to-water ratio) made from small-sized fraction at pH 7. (b) Image of optical microscope for emulsion (1:3 oil-to-water ratio) made from small-sized fraction at pH 7.

taken by optical microscope. For emulsions prepared using large-sized fraction as surfactant, the micrograph showed that the emulsion droplets became deformed and stretched after 120 h, so the average size measurement ended in 108 h. Examples for this emulsion sample were taken at 0 h, 108 h (the last minute for

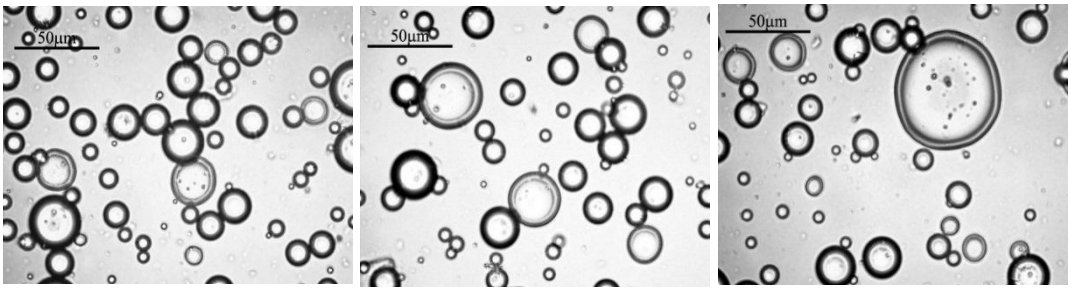
measuring size of droplets) and 120 h (the minute that size cannot be measured any more). Similarily, the sample images for emulsion made by medium-sized fraction at 0 h, 60 h, and 72 h were selected. And the sample images for small-sized fraction were chosen at 0 h, 36 h and 48 h. According to the optical microscope measurement, it was observed that the emulsion made by large-sized fraction maintained their spherical shape for 108 h, emulsion using medium-sized fraction remained their globular shape for 60 h, while emulsion stabilized by small-sized fraction kept their spherical shape for only 36 h. Hence large-sized fraction sample had ability to stabilize emulsion droplets for longer time, this observation means that emulsions formed by large-sized fraction were more resistant to coalescence. This could be attributable to fact that the large-sized protein fraction have relatively high hydrophobicity, which allowed more hydrophobic region to aggregate together to form a cohesive film around the oil droplets [5, 9]. The emulsion samples were highly unstable at pH values around IEP and at lower pH, as shown in Fig. 2-12 and Fig. 2-13. The extensive flocculation/coalescence occurred at pH 3 and 5. The poor emulsion stability at pH 5 (near IEP) could be explained by the reduction of electrostatic repulsion between emulsion droplets caused by the decrease of protein charge, while the weak emulsion stability at pH 3 could be accounted for the low solubility of protein and the denaturation of partial unfolding of protein molecules in aqueous

phase. Example images of emulsion at pH 3 and 5 were given in Fig. 2-12 and



0 h 0.5 h

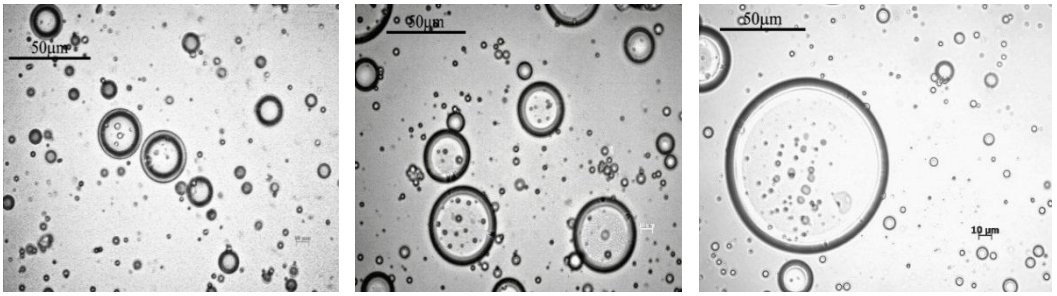
(a)



0 h 0.5 h 1 h

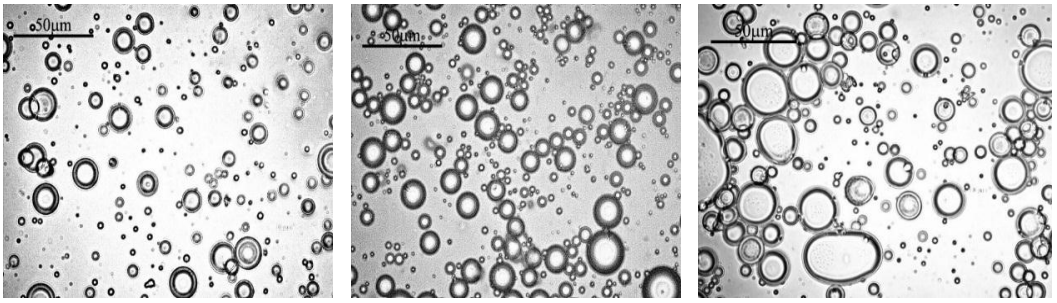
(b)

Figure 2- 12. (a) Image of optical microscope for emulsion (1:1 oil-to-water ratio) made from large-sized fraction at pH 5. (b) Image of optical microscope for emulsion (1:3 oil-to-water ratio) made from large-sized fraction at pH 5.



0 h 0.5 h 1 h

(a)



0 h 0.5 h 1 h

(b)

Figure 2- 13. (a) Image of optical microscope for emulsion (1:1 oil-to-water ratio) made from large-sized fraction at pH 3. (b) Image of optical microscope for emulsion (1:3 oil-to-water ratio) made from large-sized fraction at pH 3.

Fig. 2-13 (for large-sized fraction). The other two emulsions formed by medium-sized and small-sized fractions showed very similar images as observed for emulsions prepared using large-sized fraction at pH 3 and 5, these images were not presented in this thesis.

The measurement of emulsion droplet size provided information on the extent of

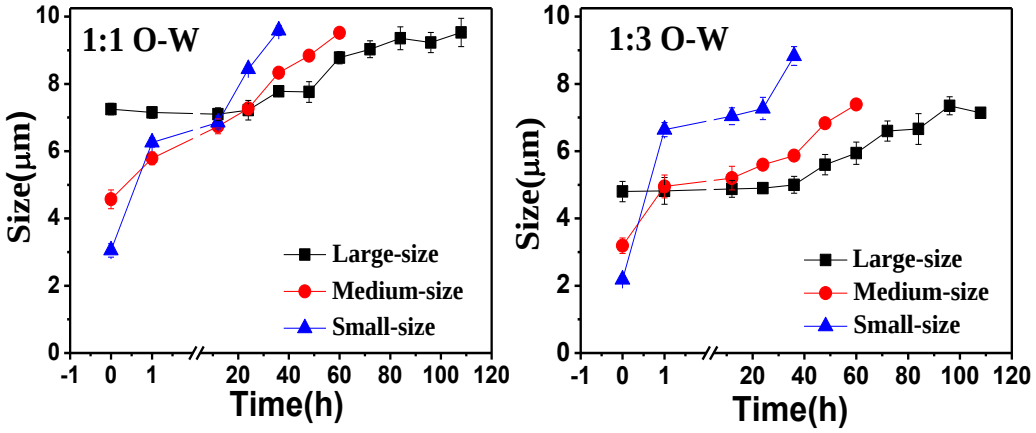


Figure 2- 14. Average diameter for emulsion made by different protein fractions at pH 7. The left figure represents emulsion using 1:1 oil-to-water ratio, while the right figure shows emulsion using 1:3 oil-to-water ratio.

flocculation/coalescence in emulsion system during different time periods [21]. For droplet size observed by microscope method, the changes of emulsion size are more related to the rate of coalescence. Though the average size for all theemulsions made by deamidated fractions had similar range (2 – 10 μm), the rate of coalescence in emulsions was different. At very beginning, the protein fraction of large size formed emulsions of relatively large size as compared to other fractions at both 1:1 and 1:3 oil-to-water ratio, and maintained over a relatively small size range (< 10 μm) for the following 108 h; The protein fraction of medium size produced emulsion droplets in the middle size range at beginning and maintained their size within 10 μm for 60 h. In contrast, the protein fraction

of small size produced smaller emulsion droplet size at early time, but the size grew fast within a short period of time (about 36 h). Thus, the rate of coalescence for emulsion made from deamidated fractions could be described as: small-sized fraction > medium-sized fraction > large-sized fraction. During emulsification, protein molecules formed an oil-water interface by rapidly adsorbing to the newly formed oil droplets, and they adapted their structure at the interface afterwards by a process called ‘unfolding’. The large-sized fraction, which was reported to have high hydrophobicity, could interact with each other to form a more cohesive film at the interface during ‘unfolding’ and further prevent close approach of emulsion droplets. Hence the protein in the large-sized fraction (aggregated large polypeptides) tended to stabilize emulsion for longer time [22]. While the small-sized fraction, with high solubility and short chain, could quickly diffuse and adsorb to the oil-water interface. As a result, small-sized fraction could form a relatively small emulsion droplets within a very short time. However, the low molecular weight fraction did not have sufficient side chains and hydrophobic region to strongly bind with each other. Therefore the emulsion stabilized by small-sized fraction showed notable coalescence in a short period of time (36 h).

2.5 Conclusions

In order to study the emulsifying functionality from the protein molecular aspect,

three fractions (large-size, medium-size and small-size), with the same charge, were produced based on different molecular weights. The large-sized fraction showed excellent emulsifying properties at neutral pH based on its high charge and hydrophobicity. The adequate charge carried by proteins ensured a successful protection of protein layer around the oil droplets by strong electrostatic repulsion. The high hydrophobicity facilitated a better anchorage of protein molecules at oil-water interface and subsequently triggered the formation of steric aggregated web around the oil droplets. Such steric web prevented adjacent oil droplets from close contact, and reduced flocculation and coalescence. However, the large-sized fraction showed poor emulsifying properties at acidic pH due to their low solubility. The medium-sized fraction showed good emulsifying properties at pH 7, again due to the high charge and sufficient hydrophobicity, but the coalescence occured faster in medium-sized fraction stabilized emulsions than in large-sized fraction stabilized emulsions. In addition, its emulsifying properties were slightly reduced when the pH decreased. The small-sized fraction proteins showed poor emulsion stability, with a large amount of oil release from centrifuged emulsion and fast coalescence within short period of time, even though the smallest emulsion droplets were formed at the beginning when emulsion was produced. Our result highlighted the importance of large molecular weight (the large aggregated polypeptides) in the centrifuge and storage stability of emulsions

formed by deamidated barley protein at neutral pH.

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Chapter 3 Conclusions and Future Work

Food emulsions play a major role in forming required structures or desirable mouth-feel characteristics in certain products, such as coffee creamer, butter, margarine, spreads, *etc.* [1]. Thus, understanding the formation, structure and physico-chemical properties of emulsion is critical for producing target emulsions with required properties and stabilities.

Barley protein is one of important by-products in brewing industry. Barley protein has limited applications due to its low solubility in water. This can be corresponding to the high content of hydrophobic amino acids within barley protein. Furthermore, it has been reported that a considerable amount of glutamine and asparagine residue makes barley protein very prone to aggregation via hydrophobic and hydrogen bonding, which becomes another reason that barley protein is insoluble in water. High level of hydrophobic amino acids allows barley protein to interact with more oil molecules during emulsion formation. In addition, a considerable amount of glutamine and asparagine residues enables deamidation modification to be selected to improve the solubility of barley protein. Therefore, barley protein can be modified by deamidation and further the deamidated barley protein can be applied as an excellent emulsifier.

3.1 Conclusions

Different molecular weight fractions were separated by ultrafiltration membranes from the deamidated (63%) barley protein, and followed by molecular weight determination using HPLC to ensure a successful separation. All the sample fractions were proven to have the same charge under the same pH condition in aqueous phase. Thus, samples with different molecular weights and same charge were developed. Different molecular weight fractions showed varied hydrophobicity. The large-sized fraction had the largest hydrophobicity, the medium-sized fraction showed middle level of hydrophobicity, and the small-sized fraction exhibited the lowest hydrophobicity. The protein in the relatively large molecular weight fractions showed better oil binding ability at pH 7 under centrifuge acceleration condition. However, pH condition largely influenced ECS and ETS for emulsions stabilized by large molecular weight fractions, especially by large-sized fraction. Protein in the small-sized fraction showed relatively weak oil binding ability at pH 7, but it remained its emulsifying properties at very similar level when pH was decreased from 7 to 5 and 3. Although the size of emulsion made from different molecular weight fractions showed similar range (2 – 10 μm), the coalescence rate were different. At pH 7, protein in the large-sized fraction formed emulsion of relatively large droplet sizes at early stage, but it tended to stabilize emulsion for a longer time than proteins in

the other fractions. While protein in the small-sized fraction formed emulsion with relatively small size at beginning, the size of emulsion droplet increased quickly within a short period of time, which means that the coalescence occurred fast in small-sized fraction stabilized emulsions.

In conclusion, the proteins in large-sized fraction showed excellent emulsifying properties at neutral pH due to its high charge and hydrophobicity. The adequate charge carried by proteins ensured a successful protection of protein layer around the oil droplets by strong electrostatic repulsion. And the high hydrophobicity facilitated a better anchorage of protein molecules at oil-water interface and subsequently triggered the formation of steric aggregated web around the oil droplets, which further prevented adjacent oil droplets from close contact, and reduced flocculation and coalescence. However, the protein in the large-sized fraction showed poor emulsifying properties at acidic pH due to their low solubility. The protein in the medium-sized fraction showed good emulsifying properties at pH 7, also due to the high charge and sufficient hydrophobicity, but the coalescence occured faster in medium-sized fraction stabilized emulsions than in large-sized fraction stabilized emulsions. In addition, its emulsifying properties were slightly reduced when the pH decreased. The protein in the small-sized fraction showed poor emulsion stability, with large amount of oil release from

centrifuged emulsion and fast coalescence within a short period of time, though the smallest emulsion droplets could be formed at the beginning when emulsion was produced. Our result highlighted the importance of large-sized fraction (the large aggregated polypeptides) in the centrifuge and storage stability of emulsions formed by deamidated barley protein at neutral pH.

3.2 Future Work

Future work for this research can follow three directions: (i) the emulsion properties of combination of different fractions; (ii) interfacial characteristics: For instance, the interfacial tension and interfacial rheology of emulsion are of great importance for providing information on emulsion formation and stability; (iii) Nano-emulsion: reducing the size of emulsion to nano-scale can be achieved by using high pressure homogenizer, and then followed by evaluation of emulsion properties under different processing and environmental conditions.

The combination of different fractions of deamidated barley protein may have potential to better stabilize emulsions for food and non-food applications. However, detailed experiments for combination of two fractions toward emulsion formation and stability will be required to perform in the future.

Interfacial region which separates oil from water phase has important influence on physic-chemical and sensory properties of food and non-food emulsions, including their formation, stability, viscosity, and flavor [2]. Hence, interfacial characteristics, such as interfacial tension, rheology, and structure are essential factors impacting emulsion properties. The change of interfacial tension as a function of time reveals how protein molecules 'adsorb' and 'unfold' at the interface; interfacial rheology gives information on elasticity of the interface; interfacial structure reveals how protein molecules interact with each other at the interface and thereby explaining the mechanism of emulsion formation and stability. Thus, interfacial tension, rheology and structure should be evaluated in future research.

Nano-emulsion subject has been gaining more and more attentions during recent decades. Preliminary experiment showed that nano-emulsion could be produced by high pressure homogenizer using deamidated barley protein as emulsifiers (data is not shown). It is known that different processing conditions, such as pressure, pass times, and oil-to-water ratio, largely influence the final quality of emulsion products. Thus, more detailed research is required to explore the optimum processing condition for producing nano-emulsion using deamidated barley proteins.

3.2.1 Non-food Applications

Cosmetics and pharmaceuticals can be example of utilizations for emulsion stabilized by deamidated barley protein in the future. In cosmetic area, the emulsifier can be used either for vehicle emulsions (coating astringents, deodorants, *etc*..) which bring target agent to intimately contact with skin, or for nonvehicle emulsions which target to maintain water-oil balance at surface of skin (cold cream, vanish cream, cream lotion *et al.*) [3]. However, further researches, such as allergy test and formulation test are required for ultimately applying our products to cosmetic fields. In terms of pharmaceuticals, poorly water-soluble drugs can be coated in emulsion droplets and administered orally to enhance the intestinal absorption [4]. It is well known that drug penetration is closely related to the size of carrier, so the pharmacological activity is greatly influenced by droplet size when using emulsions as vehicles. In order to obtaining the optimum process condition for producing nano-emulsions, further research is necessary to study the formation, stability, release behavior of nano-emulsions stabilized by deamidated barley proteins.

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