Extraction and structure-function properties of lentil protein, and its applications as an egg replacer in baked goods

by

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Abstract

Consumption of lentils relates to several health benefits. Lentils are also a good protein source that is waiting for exploitation of their full potential. This is largely due to the unknown relationship between its molecular structure and functionalities and the lack of knowledge of the impact of the extraction and environmental conditions on those properties. Therefore, the objective of this thesis research was to achieve a more complete knowledge of the lentil proteins and use this knowledge in the potential development of value-added food products.

The first goals were to develop a protein extraction protocol to prepare a lentil protein isolate or concentrate, and to understand lentil protein functionalities. The optimized conditions for protein extraction were determined to be pH 9.0 with a solid/solvent ratio of 1:10 (w:v). SE-HPLC and SDS-PAGE results showed that globulins were the major proteins in the protein concentrate. Environmental pH influenced protein solubility and surface charge, and subsequently the gelling and foaming properties. Excellent foaming capacity was identified for lentil proteins, which motived us to study their air-water interfacial behavior.

Once the lentil legumin-like protein fraction was isolated, its structure and functional properties were characterized in detail. This protein was capable of forming long-life foams at pH 5.0 and 7.0. The latter were especially stable because the combination of the α-helix secondary structure, medium molecular size, and balance between solubility/hydrophobicity contributed to building strong protein networks at the interface. At pH 5.0, the protein formed a dense and thick network composed of randomly aggregated protein particles at the interface. At pH 3.0, the unordered structure increased intra-protein

flexibility, producing a less compact interface that reduces the elasticity modulus with time and reduced foam resistance against collapse. Polysaccharides were then added to the lentil legumin-like protein system to investigate the feasibility of further improving foaming properties. When the protein was mixed with guar gum, xanthan gum, and pectin, foam stability (*FS*) was remarkably increased at pH 5.0. This stability was closely related to the adsorption of aggregates on to the interface to form strong interfacial networks, avoiding coalescence and coarsening of the foams. Aggregates also plugged the junctions of the Plateau borders, thus slowed down the drainage by a jamming effect. Similarly, the polysaccharides improved the *FS* at pH 3.0, in which associative interactions dominated and a coacervation phenomenon was observed. The coacervates may have stabilized the foams against collapse because they increased the foam viscosity and formed a coacervate network that might have a gel-like network behavior. At pH 7.0, the *FS* of the mixtures was poor, reducing the mean life of the original foam. Thermodynamic incompatibility phenomena at pH 7.0 produced a phase separation of polymers in the bulk and at the interface. Phase separation induced a disruption of this interfacial membrane making it weaker and easier to break, thus reducing *FS*. This phenomenon might be a result of a partial displacement of the protein by the polysaccharides through an orogenic mechanism.

Finally, the feasibility of using lentil protein to partially replace egg white in angel food cake and completely replace egg/milk protein in muffin was evaluated in terms of physical properties and sensory analysis. Using lentil protein did not change the final volume of the cakes, but it contributed to holding the crumb structure after baking by the formation of an entangled network structure that did not affect the dough network formation. Sensory evaluation by a consumer panel showed that the use of lentil protein increased compressive stress, chewiness, and density of the cakes. Yet, angel food cake

formulation with 50% replacement had a “nutty”flavor and a higher moistness that was appreciated by the consumers. In fact, lentil protein showed a strong water holding capacity that reduced the baking loss in the cakes. Additionally, a 100% replacement of egg and milk protein by lentil protein produced a muffin that was preferred by the consumers.

With this thesis, a deeper understanding of lentil protein functionalities was gained, in relation to molecular structures as impacted by environmental conditions (pH and presence of polysaccharides), which established a good foundation for applying this knowledge in developing lentil protein as a food ingredient and product applications.

Preface

The research contained in this thesis was led by Dr. Lingyun Chen as the principle investigator. The research was financially supported by the Natural Sciences and Engineering Research Council of Canada (NSERC), Alberta Crop Industry Development Fund Ltd. (ACIDF), Alberta Innovates BioSolutions (AI Bio) and Saskatchewan Pulse Growers. Lingyun Chen thanks the NSERC-Canada Research Chairs Program for its financial support. Marcela Jarpa-Parra thanks CONICYT-Chile for the support given through the scholarship Becas Chile.

This thesis contains original work done by Marcela Jarpa-Parra under the supervision of Drs. Lingyun Chen and Feral Temelli, and has been written according to the guidelines for a paper format thesis of the Faculty of Graduate Studies and Research at the University of Alberta. It is comprised of seven chapters: Chapter 1 provides a general introduction and the objectives of the thesis. Chapter 2 is a literature review concerning the lentil protein chemistry, structure, functionalities and food applications. I was responsible for writing the first drafts of these chapters and Dr. Feral Temelli contributed to chapter revisions and preparations.

Chapter 3 has been published as "Optimization of lentil protein extraction and the influence of process pH on protein structure and functionality" in *LWT-Food Science and Technology* and Chapter 4 has been published as "Impact of pH on molecular structure and surface properties of lentil legumin-like protein and its application as foam stabilizer" in *Colloids and Surfaces B: Biointerfaces*. I was responsible for the literature search relevant to the above studies, designing and performing the laboratory experiments, data analysis and writing the first drafts. Drs. Lingyun Chen and Feral Temelli contributed to discussion of experimental design, data interpretations, manuscript revisions and submission. Dr. Fatemeh Bamdad provided substantial assistance in conducting SE-HPLC analysis and data interpretation. Dr. Yi Xiang Wang provided support in preparing manuscripts.

Chapter 5 describes the “Stability mechanisms of lentil legumin-like protein and polysaccharide foams”. I was responsible for designing and performing laboratory experiments, data analysis and writing the first draft of the chapter. Chapter 6 refer to the “Effect of lentil protein as egg replacer on the rheology, microstructure and quality of angel food cake and muffin”. I was responsible for collecting the relevant literature, designing and conducting experiments, data analysis and writing the first draft of the manuscript. Drs. Lingyun Chen and Feral Temelli contributed to data interpretations and chapter revisions. Dr. Wendy Wismer was involved with the design and interpretation of sensory analysis. Leah Wong, Weijuan Huang, and Dr. Ewelina Eckhart provided generous assistance in performing sensory analysis. Dr. Jay Han and Zhigang Tian provided support in extracting and preparing food grade lentil protein concentrate. The sensory analysis in Chapter 6, received research ethics approval from the University of Alberta Research Ethics Board, Project Name “Total or partial replacement of egg white protein with pulse protein in angel food cake and muffins”, Study ID: Pro00053903, February 20, 2015.

Call to Me and I will answer you,
and will tell you great and hidden things
that you have not known.

Jeremiah 33:3 (ESV)

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List of Abbreviations and Symbols

ACE	Angiotensin I-converting enzyme
ANCs	Anti-nutritional compounds
ANS	1-anilinonaphthalene-8-sulphonate
BL	Weight loss during baking
CD	Circular dichroism spectroscopy
CLSM	Confocal laser scanning microscopy
CV	Coefficients of variation
DE	Degree of esterification
DPPH	2,2-diphenyl-1-picrylhydrazyl
E	Dilatational modulus
EAI	Emulsifying activity index
ESI	Emulsifying stability index
FC	Foaming capacity
FI	Fluorescence intensity
FRAP	Ferric reducing antioxidant power
FS	Foam stability
FTIR	Fourier transform infrared spectroscopy
G'	Storage modulus
G"	Loss modulus
HM	High methoxyl pectin
HPMC	Hydroxypropylmethylcellulose
JAR	Just-about-right scale
K	Consistency coefficient
k_{adsp}	Adsorption rate constant
k_{diff}	Diffusion rate constant
k_{reag}	Rearrangement rate constant
LM	Low methoxyl pectin
MGC	Minimum gelling concentration
n	Flow behavior index
NPFs	Novel protein foods
OAC	Oil-absorption capacity
ORAC	Oxygen radical absorbance capacity
RSM	Response surface methodology
S_0	Surface hydrophobicity index
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE-HPLC	Size exclusion high performance liquid chromatography
SEM	Scanning electron microscope
t	Time

TPA	Texture profile analysis
WAC	Water-absorption capacity
WPI	Whey protein isolate
α	Particle radius
ΔE^*	Total color difference
ε	Permittivity
η	Dispersion viscosity
η_{app}	Apparent viscosity
κ	Debye length
λ	Decay constant
π	Surface pressure
σ	Shear stress
$\dot{\gamma}$	Shear rate

CHAPTER 1

Introduction and Thesis Objectives

1. 1 Introduction

Proteins have always been part of the human diet to ensure sustainable living. Modern food production greatly relies on proteins of animal origin to provide energy, nutrients, and functionality to accomplish that. However, for economic, environmental, and sustainability reasons, concerns have been raised in the last two decades to warn about the need to change the human diet toward diets containing less animal protein and more plant protein [1]. Thus, the market is changing, e.g., U.S. per capita meat consumption has been declining since 2007, while sales of plant protein-based meat alternatives increased by 8% between 2010 and 2012 [2]. Also, vegetarians and vegans represent about 4% of the U.S. population, but 47% of consumers eat at least one vegetarian meal a week. People are concerned about the high cost of meat and seafood, genetically modified organisms (GMOs) and preservatives, and the environmental impact of animal protein-based food production [2]. Yet, this is a big challenge for the agri-food industry for both technological and consumer acceptance reasons [1, 3]. Thus, food research in plant proteins from cereals and legumes has been very productive and the search for novel protein foods (NPFs) has led to an increase in knowledge and development of numerous food products from soybean, pea, and oilseeds among others. In this field, the study of lentil proteins, structure, functionality, utilization for food product development and consumer acceptability is introduced in this thesis research.

1.1.2 Pulses and lentil production in Canada

The production of field crops in Canada for the 2014-15 crop year is estimated at 76 million tonnes (Mt), about 3% above the five-year (2008-12) average [4]. Among those, the supply of pulses (peas, lentils, beans, and chickpeas) and special crops are projected to reach 6.1 Mt by the end of 2015, while exports are forecast to stretch to 5.2 Mt. According to the Food and Agriculture Organization (FAO), "the term 'pulses' is limited to crops harvested solely for dry grain, thereby excluding crops harvested green for food (green peas, green beans, etc.), which are classified as vegetable crops. Also excluded are those crops used mainly for oil extraction (e.g. soybean) [5]". The total lentil production is likely to be 1.8 Mt and exports are expected to be 1.6 Mt, leaving about 200,000 t for domestic use in 2015 [4]. The domestic use is mostly for food as whole grain, feed waste, dockage, and seed use. Considering that the protein content in lentil is around 25% on average, this means a high amount of under-utilized proteins are available for use by the Canadian industry and to add more value to the lentil crop. Thus, it is necessary to develop processing capacity for lentil in Canada in order to generate economic returns on crop components and maximize value addition by exporting higher value ingredients/products rather than seeds. Lentil protein may be used to replace existing proteins and to produce new products to meet consumer demand for non-animal protein and plant-based protein products with high nutritional value. Also, considering that lentil protein is rich in some essential amino acids, which are deficient in cereals, it is an excellent complement to cereal-based products to form foods with added health benefits.

1.1.3 Chemistry of pulses and lentils

The composition of pulses depends on several genetic and environmental conditions. They usually contain a large amount of carbohydrates, such as starch and oligosaccharides of the raffinose family, ranging from 24 to 68%, and large amounts of crude fiber, ranging from 1.2–13.5% with cellulose and hemicellulose as the major components [6]. They are also a good source of minerals, vitamins, and important micronutrients [6-9]. However, it contains only marginal amounts of isoflavones that have been associated with the prevention of prostate cancer, improved immunity, cardiovascular health, and prevention of bone loss associated with aging [6]. Pulses also vary based on their total phenolic contents and antioxidant activities. Lentils have the highest phenolic, flavonoid and condensed tannin contents (6.56 mg gallic acid equivalents g^{-1}, 1.30 and 5.97 mg catechin equivalents g^{-1}, respectively). Pulses with the highest total phenolic content such as lentil, exert the highest antioxidant capacity measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging, ferric reducing antioxidant power (FRAP), and the oxygen radical absorbance capacity (ORAC) methods [9].

Pulses also contain an appreciable amount of protein ranging from 15 to 45%, depending on the species. The crude protein values reflect the amount of proteins plus a mixture of nitrogenous compounds, such as free amino acids, amines, purine and pyrimidine bases, and nucleic acids [7]. The proteins of pulses are mainly storage proteins. Nutritionally, these storage proteins are relatively low in sulfur-containing amino acids, such as methionine and cysteine. However, they are a good source of lysine and arginine, which are important for use in balancing the deficiency in cereal-based diets. Lentil is high in protein (24.3% to 30.2%) and especially rich in lysine and leucine [11, 12].

In addition, pulses are a rich source of energy with very low glycemic index values, which may help to regulate blood sugar and lipid levels in both diabetic and healthy individuals [6]. It may also reduce the risk of overweight or obesity possibly by improving satiety and lowering energy intake [6]. The nutritional characteristics of lentil have been associated with cholesterol and lipid lowering effects in humans, along with reducing the incidence of colon cancer and type-2 diabetes [10]. Also, addition of pulse components, such as starch and protein, may impart unique functional properties to food products by modifying their texture [13].

A downside of pulses might be the presence of anti-nutritional compounds (ANCs) and allergens that may reduce their potential health benefits [6]. Some ANCs found in pulses include lectins (carbohydrate-binding proteins), protease inhibitors, and the angiotensin I-converting enzyme (ACE) inhibitor. However, in many instances the effects of ANCs may be diminished after cooking [6]. The presence of allergens in pulses is also a growing concern, because some of them seem to be an important cause of IgE-mediated hypersensitivity in the Mediterranean region and India [14]. Protease inhibitors found in lentils have been characterized as members of the Bowman-Birk family. Lectin is found in high amount in lentils but it can be completely removed after a treatment of 72 h fermentation at 42 ˚C. Yet, lectins from lentil are non-toxic [9]. The content of α-amylase inhibitors differs greatly among legumes, with the highest amounts found in dry beans. In lentils, α-amylase inhibitor activity was undetected [9]. However, the presence of these ANCs in lentil may have a positive effect too. For example, they may have potential use as antihypertensive agents [12] because of their relatively good ACE inhibitory activity or they may be used as protein markers of cancer, as in the case of lectins [9].

Interest in lentil protein has grown lately due to its high nutritional value, good Leu/Ile and Leu/Lys ratios (1.24–1.98 and 1.08–2.03, respectively) [11], high digestibility (~83%) [15], and its potential use in food product applications due to its good functional properties, such as solubility, emulsifying and foaming capacity [11, 15-17].

1.2. Scope of the Study

Lentil is a health-promoting food that is a good source of several nutrients, such as proteins. However, lentil proteins are not utilized to the same extent as other pulse proteins, such as pea protein or oil seed proteins, such as soybean [19] because of some gaps in our knowledge. Therefore, the overall aim of this thesis research is to deepen and expand our knowledge that is critical to address this gap and facilitate wider applications of lentil proteins.

In order to achieve this goal, this work aimed to provide an optimized protein extraction protocol based on alkaline method that guarantees both maximum protein content and yield of the extract along with a characterization of lentil protein molecular structure and functionalities. After that outstanding foaming properties were identified, and with the purpose of getting a deeper understanding, the main protein fraction was isolated and its structural properties, including molecular weight, hydrodynamic size, surface charge, hydrophobicity, and conformation were studied in relation to its air-water interfacial behavior at different pH values. This was supplemented by the exploration of the impact of polysaccharides on its foaming and surface properties and this knowledge was utilized to produce bakery products that were assessed by sensory and physical methods.

Therefore, a deeper understanding of lentil protein functionalities in relation to pH, its structure and interactions with other biopolymers was achieved, which established a good foundation for applying this knowledge in developing food products.

1.3 Hypotheses and Objectives

The research presented in the following chapters was based on the following hypotheses:

1. By selecting appropriate processing conditions, an alkaline extraction protocol can be established in order to guarantee the maximum yield and purity of lentil protein.
2. Legumin-like protein (13S) as the major globulin protein in lentil plays an important role in determining lentil protein functionality.
3. Addition of polysaccharides can improve lentil protein functionality by modulating protein-polysaccharide interactions.
4. Lentil protein can be used as a foaming ingredient in bakery products.

1.3.1 Thesis objectives

The objective of this thesis research was to achieve a more complete knowledge of the lentil proteins and to use this knowledge in the potential development of value-added food products incorporating lentil proteins.

1.3.1.1 Specific objectives

The specific objectives were:

1) To develop a protein extraction protocol based on alkaline method to prepare a lentil protein isolate or concentrate (Chapter 3),
2) To characterize lentil protein molecular structure and functionality (Chapter 3),

3) To separate and purify lentil legumin-like protein and study the effects of pH on foaming and surface properties in relation to protein molecular structures (Chapter 4),
4) To investigate the foaming properties of lentil legumin-like protein-polysaccharide mixtures, and explore the impact of different polysaccharides on their foaming and surface properties (Chapter 5), and
5) To assess lentil protein as an alternative ingredient to replace egg protein in bakery products and evaluate its effect on their rheological, physical, and sensory properties (Chapter 6).

CHAPTER 2

Literature Review

2.1 Introduction

Mature pulse seeds are normally high in protein because throughout their development, pulse seeds accumulate protein [10]. These proteins are principally storage proteins that are classified as albumins, globulins, and glutelins according to their solubility behavior, and the salt-soluble globulins are the main fraction present in pulses [19]. Based on their sedimentation coefficient, two types of globulin usually prevail in pulses, vicilin or legumin, which sediments at 7S or 11S, respectively. Albumins that are soluble in water account for 10–20% of the total protein, while glutelins, soluble in dilute acid and base solutions, account for 10–20% of the total protein found in the pulses [20]. Besides the water-soluble storage proteins, there are other proteins that constitute part of the defensive mechanism of the seed, mainly enzymes, enzyme inhibitors, such as angiotensin I-converting enzyme (ACE) inhibitor, and lectins, which are considered as antinutritional factors for the human diet [10]. As for other proteins, the molecular structure of the pulse proteins impact the physicochemical properties of the various protein constituents, e.g. the albumin fraction is characterized by a low to medium molecular weight and a hydrophilic surface that renders the proteins water soluble, whereas the globulins that are multi-subunit molecules of high molecular weight, have a relatively hydrophobic surface that limits their solubility in aqueous media [19].

2.2 Lentil Protein Chemistry and Structure

Lentil (*Lens culinaris*) is a leguminous plant high in fibre and low in fat. Like most legumes, lentil is a rich source of protein, having between 20.6-31.4% proteins [11]. Most of these are storage proteins located in the cotyledon, containing a low percentage of sulfur-containing amino acids.

Lentil proteins are comprised of around 16% albumins, 70% globulins, 11% glutelins, and 3% prolamins [21]. Albumins, glutelins, and prolamins have a molecular weight of about 20 kDa, 17 to 46 kDa, and 16 to 64 kDa, respectively, and they are comprised of approximately 13, 4, and 10 polypeptides each. Globulins contain both legumin and vicilin-like proteins. The first group, also called 11S proteins, consists of six polypeptide pairs that interact non-covalently and have a molecular weight (*Mw*) of 320 to 380 kDa. Each of these polypeptide pairs is comprised of an acidic subunit of about 40 kDa and a basic subunit of about 20 kDa, linked by a single disulfide bond [20]. The second group of proteins is referred to as 7S proteins and are generally isolated from seed extracts as trimers of glycosylated subunits with a *Mw* of 50 to 60 kDa [22]. The isoelectric points of lentil proteins vary from pH 3.0 to 5.5, at which about 20% of the protein still remains in solution [23]. All protein fractions are glycosylated, particularly the vicilins, containing about 2.8% carbohydrate [11, 12, 24].

2.3 Lentil Protein Extraction

Lentils and other pulses are traditionally consumed whole and mainly prepared as salads and soups [25]. Thus, novel applications need to be identified to increase its use in the food industry. Fiber, starch, and protein concentrates or isolates can be extracted and

separated from lentil and other pulses [18], and then, they can be utilized as ingredients in the preparation of diverse food products.

Extraction of pulse proteins may be relatively easy using wet processes, as they are highly soluble under alkaline and acidic conditions. They are commonly extracted by alkaline solubilisation, first dispersing the pulse flour in water at pH 8.0 to 10.0, followed by stirring of the dispersion. After that, the insoluble material is removed by centrifugation and proteins are recovered by adjusting the supernatant pH to a value around 4.5, where proteins are precipitated iso-electrically. The final concentrate or isolated protein is then dried using a spray-, drum- or freeze-drying method [26].

Most of the previous studies have focused on lentil proteins extracted with diluted sodium hydroxide [23] under a single pH condition that varies from one study to another. For example, Joshi et al. [27] produced lentil protein isolate by alkaline extraction at pH 8.0, room temperature and 1:10 solid-to-solvent ratio, and compared the physicochemical characteristics of the isolated proteins obtained by the three methods of drying (freeze drying, spray drying, and vacuum drying). Similarly, Boye et al. [18] extracted lentil protein using isoelectric precipitation or ultrafiltration after extraction at pH 9.0 and 25 °C, using 1:10 solid-to-solvent ratio from red and green lentils and compared their yields and protein contents. Their concentrates had a protein content between 78.2 to 88.6% with a yield of 50.3 to 62.8% based on the protein content of the starting dehulled flour. On the contrary, Alsohamy et al. [16] extracted lentil protein using seven different pH values (6.0, 7.0, 8.0, 9.0, 10.0, 11.0, and 12.0) and three different methods of protein recovery (isoelectric precipitation, ammonium sulfate precipitation, and alcohol precipitation) at room temperature and 5:100 solid-to-solvent ratio. The optimum pH of extraction was 12.0 with ammonium sulfate and alcohol precipitation giving the highest protein recovery (93

and 100%, respectively). Lee et al. [26] performed a comprehensive investigation of extraction conditions. They determined the best parameters of protein extraction of green and red lentils, considering five pH values (distilled water and pH 8.0, 8.5, 9.0 and 9.5) and four temperatures (22, 30, 35, and 40 °C), while keeping the solid-to-solvent ratio constant (1:10) and analyzed the results according to protein content and percentage of starch damage. Upon evaluation of all extraction conditions, pH 9.0 at 30 °C was chosen as the optimum extraction condition for green lentil (56.6% of protein), while pH 8.5 at 35 °C was chosen for red lentil (59.3% of protein). Regarding the structure of the protein, most of the studies just determined the type of protein fraction present based on *Mw* determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

In conclusion, protein extraction conditions vary from one study to another without providing a full justification for it. Also, there is a lack of systematic studies showing how extraction parameters impact lentil protein yield and purity, and how the extraction conditions influence the lentil protein conformational structure.

2.4 Lentil Protein Functionality

Depending on the method of extraction and the conditions used to isolate or concentrate the proteins, their functionality may vary due to their compositional and physicochemical characteristics and the magnitude to which these characteristics are affected [13]. United States trading rules establish protein contents at a minimum of 70% and 90% for protein concentrates and isolates, respectively, on a dry-matter basis. Generally, the studies related to protein functionality evaluate their gelling, emulsifying, foaming, solubility, and water- and oil-absorption capacity. However, most of the studies assess the functional properties only at one pH, which is generally neutral pH.

Protein solubility is one of the important functional properties as most of the other functional properties depend on it [27]. Thus, the solubility profile at different pH levels may serve as a useful indicator of the performance of the protein in food systems, and also of the extent of protein denaturation caused by the extraction method. According to Boye et al. [18] regardless of the extraction process, lentil protein shows a minimum solubility at the pH range of 4.0 to 6.0, and two regions of maximum solubility at pH levels lower or higher than this range. Similar results were obtained by Ma et al. [28] and Bora [17]. Other authors like Joshi et al. [27] and Can Karaca et al. [29] only determined the solubility at neutral pH.

Water-absorption capacity (WAC) and oil-absorption capacity (OAC) are the terms often used to refer to the amount of water and oil that can be absorbed per gram of sample, respectively. WAC values constitute useful indices of the ability of the protein to prevent fluid leakage from a product during food storage or processing [13, 19]. WAC values for lentil protein isolates or concentrates range between 0.4 and 4.0 g g^{-1} [17, 18, 26, 27], whereas, OAC usually ranges from 1.1 to 2.6 g g^{-1} [17, 18] depending on the lentil variety and the extraction conditions. All these studies were carried out at a single value of pH that varied from one study to another.

The emulsifying ability of a protein is its ability to help in the preparation of an emulsion with oil droplets of a relatively small size by forming a film around them while they are dispersed in an aqueous medium, thereby preventing droplet aggregation that might lead to phase separation [29]. Emulsifying properties of proteins are normally described by two parameters: emulsifying activity index (EAI) and emulsifying stability index (ESI). EAI is the maximum surface area created per unit of protein and it is usually determined by applying turbidimetry to highly diluted emulsion samples. ESI measures the

ability of the emulsion to hold its structure over a defined time period and it is determined by monitoring the decrease in turbidity with time of a diluted emulsion during short-term storage [19]. Studies related to emulsifying properties of lentil protein show great variability depending on the conditions of testing and protein extraction. In general, lentil protein emulsifying properties are similar to those of other pulses, with values ranging from 5 to 45 m^2 g^{-1} [18, 27, 29] for EAI and 19 to 245 min for ESI. Most of these studies were done at pH 7.0, with the exception of Joshi et al. [27] who tested both, EAI and ESI in the pH range of 3.0 to 7.0. They found that lentil protein isolate has a higher EAI compared to whey protein isolate, but lower ESI than sodium caseinate and whey protein isolates. They did not consider different conditions of protein extraction. On the contrary, Lee et al. [26] tested emulsifying properties of proteins extracted at different pH levels, while Can Karaca et al. [29] compared proteins recovered by isoelectric precipitation and salt extraction, though both were tested only at neutral pH.

Foams are formed when proteins diffuse and adsorb to air–water interface reducing the surface tension, while partially or totally unfolding and forming an interfacial film around the air bubbles, which helps to prevent the foam from collapsing immediately after formation and, sometimes, during storage [18]. The foaming properties of proteins are usually expressed in terms of the foaming capacity index (*FC*), which represents the relative increase in the volume of a protein solution, resulting from air incorporation and the foam stability index (*FS*), which measures the ability of the system to retain the air in the form of bubbles during aging [19, 21, 24]. This functionality is not extensively studied and overall, the results are difficult to compare since they differ in the method of calculation. Boye et al. [18] compared foaming properties of lentil protein at pH 7.0 with those of chickpea and pea recovered by isoelectric precipitation and ultrafiltration. The

method of extraction had no impact on the *FC* of the lentil protein, but it made a difference on the *FS*, with lentil protein performing fairly well compared to the other pulses. Bora [17] determined the *FC* and *FS* of lentil protein at several pH values, but his results are not comparable to those of Boye et al. [18]. There were also significant differences between the values at different pH levels, but neither a correlation was found nor was any explanation offered. Lee et al. [26] also measured *FC* and *FS* at pH 7.0 for lentil protein extracted at different pH values, but once again the results are not comparable to those of other authors.

Protein gelation normally occurs when proteins form a three-dimensional network after heating to a temperature higher than the protein denaturation temperature followed by subsequent cooling. Gelling capacity is usually expressed by the minimum gelling concentration (MGC), which may be defined as the minimum protein concentration required for a self-supporting gel to form. The lower the MGC, the better is the ability to form gels. Pulse protein gel formation is frequently favored by hydrophobic interactions and, in some cases, covalent disulfide bridges may also contribute to the network development. Similar to the foaming properties, there are only a few studies related to the gelation of lentil protein. Joshi et al. [27] studied the effect of different drying methods after protein extraction on gelation properties at pH 7.0. MGC values ranged from 11 to 14% w/v of protein. Whereas Boye et al. [18] found MGC values from 8-12% w/v of protein, depending on the extraction method, at the same pH. MGC for lentil protein was similar to that of chickpea and pea proteins.

Even though some studies have addressed the effect of different conditions of extraction or testing on lentil protein functionality, those studies are still lacking in making a deeper connection between these conditions and their influence on the lentil protein structure and the subsequent functional properties of these extracts. Since environmental

conditions, protein treatment, physicochemical and structural properties impact protein functional properties, more fundamental research that addresses this gap is critical to facilitate wider applications of lentil proteins. Also, some of these functional properties have received less attention than others, as in the case of foaming properties. Thus, more research is also needed to increase the knowledge in this field, considering that a large segment in the food market, such as bakery, confectionery, and dairy desserts, is comprised of products for which foaming is essential.

2.4.1 Foam properties of proteins

Foam is a dispersed system where gas bubbles are trapped in a continuous liquid phase, but it will eventually break down because it is thermodynamically unstable. Proteins are used to provide kinetic stability in foams because of their surface activity, which helps foam formation and stabilizes the foam system [30]. Foaming properties are often characterized by foaming capacity, foam stability, and interfacial rheology, and they are widely applied in the food industry to provide unique structures and textures to products like aerated chocolate, soufflés, mousses, ice cream, cappuccino, bread, cakes, etc. [31].

Foaming capacity is defined as the capacity of the liquid phase to trap gas, and foam stability is the ability to retain the gas for a certain period of time, while interfacial rheology provides information on the adsorption behavior and interactions of molecules at the liquid/gas interface by measuring the viscoelastic response of the interface to shearing forces. The foaming properties of proteins depend on many intrinsic factors, including size, structure, hydrophobicity, surface potential, and environmental and processing factors, i.e., pH, temperature, and protein concentration [30]. Complete characterization of foams should include determination of how macroscopic properties, such as foaming capacity and

stability, are affected by foam microstructures and interfacial interactions at the molecular level. Also, since food systems are complex and frequently formed by a mixture of different ingredients, it is important to study the effect of adding other components such as polysaccharides into the protein foam system.

In the literature, there are several methods to determine the foaming capacity of a protein, but generally they involve measuring the volume or weight of foam obtained from a known volume and concentration of a protein dispersion before and after foam generation using a graduated cylinder or by conductimetry. The volume of foam can be expressed as the percent volume increase, the fold of volume increase, or the percentage of overrun among others [32, 33]. Instability in protein foams is most likely dictated by several mechanisms acting simultaneously: drainage of liquid from and through the foam layer due to gravity, coalescence of bubbles due to instability of foam film, and Ostwald ripening, which is the diffusion of gas from small to larger bubbles or to the atmosphere, because the pressure in small bubbles is greater than that in larger ones. Methods capable of measuring these processes are frequently used, such as conductivity or fluid drainage rate [34].

As mentioned previously in this section, there are some studies where *FC* and *FS* of lentil protein have been determined, though they are not totally comparable because of the diverse methodologies utilized [17, 18, 26]. Also, those studies focus on determining *FC* and *FS* values under different treatment conditions and comparing them with other proteins, but provide no fundamental reasons for lentil protein foaming behavior.

2.4.2 Surface properties

2.4.2.1 Surface tension

As amphiphilic molecules, proteins are capable of locating themselves at the interface of an air-water system, reducing the surface tension, which facilitates foam formation and stabilizes the foam system by forming a film around the bubbles, whose physicochemical characteristics will determine the stability of the foam [35].

Surface tension of liquids is due to the cohesive nature of its molecules at the surface that allows them to resist an external force. Proteins are able to reduce surface tension because they diffuse to the air-water interface and form a layer or film by adsorbing and re-orienting themselves. This surface activity, as is called, is related to its physical, chemical and conformational characteristics, which are affected by extrinsic factors such as pH, ionic strength, temperature, etc. Thus, generally, increasing the bulk concentration of a protein will speed the decay of surface tension because of the higher number of molecules that transport and adsorb to the interface [10]. Also, as diffusion is inversely proportional to the radius of a particle, smaller molecules will move and adsorb faster to the interface compared to the larger molecules. Diffusion is also inversely related to the viscosity of the medium [35-37]. Frequently, the reduction of surface tension is described by a surface tension vs time curve divided into four regions; an induction region (short times), a rapid fall region, a mesoequilibrium region, and an equilibrium region (at long time periods) [39]. These regions are related to the protein kinetics at the interface when it is forming: (i) Diffusion of protein from bulk to the interface and (ii) adsorption at the interface often accompanied by structural rearrangement [40, 41]. Dynamics of both processes can be observed through shear and dilatational rheology.

Three main studies reported the surface activity of lentil protein. Karaca et al. [29], investigated the interfacial tension at neutral pH in a mixture of flaxseed oil and lentil protein obtained using two methods of extraction. Also, Joshi et al. [27] studied the interfacial tension behavior of lentil protein in a mixture with olive oil at pH 7.0, though they also investigated the effect of protein concentration. Then, they studied the impact of pH, ionic strength, heat treatment, and presence of a reducing agent, dithiothreitol (DTT) in solutions of 10 mg/mL. Finally, Avramenko et al. [42] examined the interfacial tension in mixtures of lentil protein and flax oil as a function of the degree of hydrolysis at pH 7.8. As can be inferred, these studies have been connected to the emulsifying properties. There are no studies where surface activity of lentil protein had been linked to its foaming properties.

2.4.2.2 Shear and dilatational rheology and foaming kinetics

Extrinsic factors such as pH and ionic strength affect the physical, chemical, and conformational properties of the proteins, which in turn modulate their interfacial behavior (adsorption, structure, mechanical properties, etc.) [43]. Therefore, interfacial shear rheology parameters, such as storage modulus (G') and loss modulus (G"), are good tools to elucidate the interfacial composition and protein-protein interactions, such as non-covalent intermolecular interactions and covalent disulfide cross-linking, which help proteins to form a continuous viscoelastic film around the air bubbles [36, 40]. The kinetics of adsorption of proteins at the air-water interface can be monitored through the time evolution of surface tension and surface dilatational properties [43].

Some of the findings related to these parameters indicate that proteins with different levels of exposed hydrophobicity and net charge may adsorb to the air–water interface at different rates [41]. Besides that, molecular flexibility and susceptibility to conformational changes affect diffusion of the protein molecules to the interface, as well as their solubility

and hydrodynamic diameter [37]. Also, pH and therefore protein aggregation would affect foam stability, depending on the size of aggregates. They can act by destabilizing the foam or as steric stabilizers in the interfacial films [41]. In addition, foaming capacity and stability might be affected by protein diffusion and adsorption, conformational changes at the interface, rate of surface tension decay, and dilatational elasticity in different ways, depending on the specific protein under study [41].

Foaming kinetics is the result of the protein adsorption dynamics at the air-water interface, which is closely related to the structure and characteristics of the proteins [36]. As described before, there are two main processes occurring when interface is forming: diffusion and adsorption. In the former process, the rate of diffusion to the interface is dictated by the protein concentration in the bulk and by the size of the protein molecules [37, 41]. Then, in the following adsorption stage at the interface, there is some kind of an energy barrier whose magnitude is related to the physico-chemical character of the protein, e.g., the exposure of hydrophobic groups [41]. So, every protein molecule reaching the interface will become adsorbed at a different rate, depending on the balance of the interactions between the periphery of the protein and the interface. A conformational reorganization might follow, characterized by the unfolding of the protein that allows further exposure of protein hydrophobic sites to the air phase [38, 43]. This ‘surface denaturation’ is enhanced with time and space to spread out at the interface but is restricted by kinetic limitations. After this, the development of intermolecular cross-linking, strengthening of the monolayer, and probably some local phase separation would lead to a structural consolidation where a concentrated glass-like or gel-like layer of densely packed protein molecules is established. These processes influence the long-term mechanical properties, as demonstrated through time-dependent surface rheological measurements [40].

Several studies have demonstrated the relationship between the protein kinetics at the interface, protein structure, and surface properties. Martin et al. [44] studied the ability of β-casein, β-lactoglobulin, ovalbumin, and soy glycinin to form a network at the air/water interface and their suitability as foaming agents and foam stabilizers, and showed that adsorption and unfolding rate are the most important factors for foam formation and that creation of a rigid network at the interface might help to stabilize the foam. They found that protein molecular size, protein structure, and hydrophobicity seem to influence the adsorption rate, e.g. difference in adsorption behavior between proteins with more or less the same molecular size, such as glycinin and ovalbumin is probably due to a difference in molecular functional properties, such as the exposed level of hydrophobicity. They also observed that β-casein is likely to adsorb faster at the interface because disordered, small, and flexible proteins are more surface active than ordered, rigid, and larger proteins [44]. In addition, the differences in the ability to produce a foam between the various protein solutions were related to the differences in the adsorption rate observed and to the structure of the protein, e.g. the lack of foaming capacity observed for ovalbumin was explained by its high molecular weight and rigid structure that slowed down its adsorption and unfolding at the interface within the time scale of the foam experiment. Zhang et al. [45] compared the adsorption behavior of β-lactoglobulin, α-lactalbumin, and β-casein and confirmed the findings of Martin et al. [44] related to β-casein, but also showed that the structure as defined by pH, does appear to alter both the foamability and adsorption of β-lactoglobulin and α-lactalbumin at the interface. α-Lactalbumin was preferentially adsorbed at the interface compared to β-lactoglobulin at low pH because of the molten globular state of α-lactalbumin and the rigid structure of β-lactoglobulin [45]. Martin et al. [46] followed the

change in conformation during adsorption of β-casein, α-lactoglobulin, and glycinin at the air/water interface. For α-lactoglobulin and ovalbumin, they detected structural changes in the secondary protein structure from β-sheet to random coil upon adsorption. For β-casein, no conformational changes on the secondary folding level were detected upon adsorption or as a function of time, whereas for glycinin prepared at pH 3.0 infrared reflection-adsorption spectrometry showed formation of intermolecular β-sheet structures and just about after adsorption, the creation of a strong network. Furthermore, in foams prepared from the glycinin solution at pH 3.0, large aggregates were found. The absence of intermolecular β-sheet structures in glycinin adsorbed at pH 6.7 seemed to explain the less strong network or the interfacial gel formed under this condition, which was confirmed by rheological experiments. Based on the comparison of the conformational changes in adsorbed layers of these proteins, it can be concluded that each protein behaves differently, depending on its flexibility and internal structure [46]. No studies on the relationship between lentil protein structure and its surface properties have been found.

2.4.3 Improving the foam properties of proteins

Destabilization of foams occurs due to simultaneous drainage, bubble coalescence, and coarsening [34]. As a general rule, proteins are good foaming agents because they strongly adsorb to the gas–water interface due to their hydrophobic regions formed by the hydrophobic amino acid residues. Furthermore, due to interactions between the adsorbed molecules, they give structural coherence to the interfacial layer. Additionally, proteins may produce steric and electrostatic stabilization. Any factor acting against these elements will modify protein surface activity, and in turn, foaming capacity and stability. Such factors include unfolding due to heat and addition of detergents or other chemical agents

among others [47]. Then, foaming capacity and stability can be improved by physical, chemical, and enzymatic treatments, as shown by several studies, including those previously cited. As additional examples, depending on the pH value, enzymatic hydrolysis of protein might have a positive effect on foam capacity and stability because it can increase the exposure of hydrophobic residues, which would increase the interfacial adsorption [43]. Thermal treatments may improve significantly the stability and foaming capacity of some proteins over a range of temperatures [48]. Acid treatment that cause conformational changes and dissociation enhanced the foam formation capacity and stabilization of amaranth and soybean proteins [33]. Polysaccharide addition causes different effects on foaming properties depending on the polysaccharide chemical structure, bulk viscosity, the relative concentration, and protein-polysaccharide interactions in solution and at interface [38].

2.4.3.1 Protein-polysaccharide interactions

When a polysaccharide and a protein are mixed together in a solution, one of two main possibilities can be observed. For dilute solutions, the protein and the polysaccharide are co-soluble and the mixture is stable [49]. If polymer concentrations increase, a two phase separation phenomena can be observed, depending on the affinity between the different biopolymers and the solvent. Thermodynamic incompatibility or segregative phase separation, is generally observed when there is a net repulsion between the two biopolymers, i.e. both protein and polysaccharide carrying a similar net charge. Thus, solvent-protein or solvent-polysaccharide interactions are favored to the detriment of protein-polysaccharide and solvent-solvent interactions. As a result, the system separates into two phases, each being enriched with one of the two biopolymers [49, 50]. However, reversible complexes have been also observed between anionic polysaccharides and anionic

proteins, which are formed by the interactions between anionic reactive sites of polysaccharide and small cationic reactive sites of protein that result in the formation of anionic soluble “protein-polysaccharide” aggregates [51]. Also, near the pI of protein, when it is carrying nearly zero overall charge and the polysaccharide is negatively charged, weak reversible complexes can be formed as well, mainly due to hydrogen bonding or hydrophobic interactions [49].

On the contrary, attractive interactions between protein and polysaccharide can lead to a separation phenomenon called the associative phase separation or coacervation [49, 50]. Coacervation is a liquid-liquid phase transition, whereas precipitation is a liquid-solid phase separation [50]. This occurs when the interactions between the two biopolymers are favored and the formation of soluble and/or insoluble protein-polysaccharide complexes occurs [50]. The soluble macromolecular complexes interact to form electrically neutralized aggregates that ultimately sediment to form the coacervate phase containing both biopolymers [50, 52]. The two coexisting phases are a rich solvent phase with very small amounts of biopolymers and a rich complexed biopolymer phase forming the coacervate [49, 50]. The contribution of electrostatic interactions is predominant in these mixtures of opposite charges, e.g. positively charged proteins (pH< pI) and negatively charged polysaccharides. Hydrogen bonding and hydrophobic interactions play a secondary role for the stability of the protein-polysaccharide complexes [51]. The magnitude of these interactions depends on the solution parameters such as pH, ionic strength, and temperature. At fixed conditions, type of protein/polysaccharide, molecular weight, charge density, and hydrophobicity of the bio-polymers also play a significant role [51]. Next, a brief description of the polysaccharides included in this thesis research is given and their structures are shown in Figure 2.1.

Guar gum belongs to the non-ionic galactomannans, a group of plant reserve polysaccharides that are obtained from the seeds of guar plant (*Cyamopsis tetragonoloba*). They are composed of a β-1,4-D-mannopyranose backbone with α-D-galactopyranosyl residues as single unit side chains attached at C-6. Its galactose to mannose ratio is 1.0:1.6 [53].

Xanthan gum is a high molecular weight polysaccharide produced by the microorganism, *Xanthamonas campestris*. The primary structure consists of a cellulosic backbone of β-1,4-D-glucose units, with a trisaccharide side chain on alternate glucose residues composed of two mannose units separated by a glucuronic acid with various amounts of acetyl and pyruvate substituents. The carboxyl groups on the side chains render the gum molecules anionic, with an average charge density of 0.25 mol negative charge/mol of monosaccharide [54].

Pectins are linear polysaccharides consisting mainly of a linear chain of α-1,4-D-galacturonic acid units with carboxylic groups bearing the negative charge. The charge density of pectins depends on the number of methyl ester groups attached to the carboxylic groups. The degree of esterification (DE) of the two commercially important pectins is typically 70% and 35% for high methoxyl (HM) pectin and low methoxyl (LM) pectin, respectively. This correlates with a charge density of 0.3 and 0.65 mol/mol, respectively, for HM and LM pectins [55]. The pectin used in this study is LM pectin.

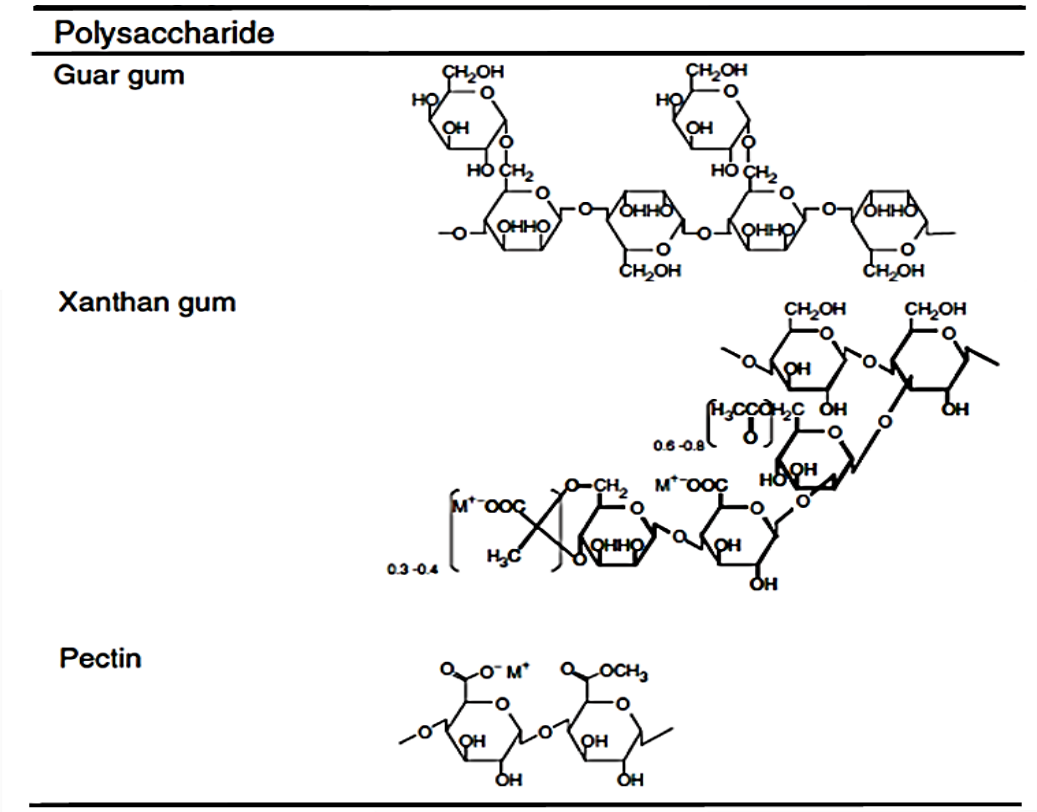

Figure 2.1 Structures of selected polysaccharides

2.4.3.2 Protein-polysaccharide mixtures in foams

The overall structure of food products containing protein-polysaccharide mixtures depends not only on the functional properties of the individual ingredients, but also on the nature and strength of the protein–polysaccharide interactions. These interactions have implications for interfacial characteristics and for the formation and stability of the foams [47]. Formation and stabilisation of foams are to a large extent determined by the compounds present at the interface. The presence of proteins empowers these properties by a combination of electrostatic and steric mechanisms. Using polysaccharides would also increase the stability of foams compared to foams stabilized by protein alone. The ability of some polysaccharides to increase the viscosity of the continuous phase improves the foam stability by reducing coalescence and foam drainage. The polysaccharides would retard

phase separation and gravity-induced creaming, enhancing the long-term stability, by controlling the rheology and network structure of the continuous phase [49]. Alternatively, the presence of polysaccharide molecules at the interface, through their interaction with adsorbed protein molecules, may enhance foam stability by preventing the close approach and interaction of air bubbles [19].

There are several studies related to the impact of protein-polysaccharide interactions on the dynamics of protein adsorption at the interface and the effect on foam capacity [55-58]. Also, the main contributions of the polysaccharides to the interfacial characteristics of the adsorbed protein films and their influence on foam stability have been reported [59-64]. Most researchers, with the exception of Miquelim et al. [56], Sadahira et al. [57], Van den Berg et al. [58], and Ruíz-Henestrosa et al. [59], studied the influence of the protein-polysaccharide interactions just at one pH value, normally below pI. In one of the most complete studies, Ruíz-Henestrosa et al. [59] analyzed the effect of sucrose on dynamic surface pressure and surface dilatational properties and foam (foam capacity and foam stability) characteristics of soy globulins (7S and 11S) at pH 7.0 and 5.0. They observed the dynamics of adsorption of soy globulins, as well as the effect of sucrose in the aqueous phase on the unfolding of the protein and compared the rate of adsorption of these proteins by varying the pH and ionic strength in the presence/absence of sucrose. They linked the dynamics of adsorption, surface dilatational properties and some molecular features of the proteins with the foaming capacity. They also suggested that the increased interfacial adsorption and its combined effects with interfacial protein interactions can explain the higher stability of the foam.

Except for the brief mention by Ruíz-Henestrosa et al. [59], the above mentioned studies do not describe the impact of polysaccharide on the molecular structure of the

protein at the foam interface and its relationship with the improved/diminished foaming properties with the required depth to understand the underlying mechanisms. Other studies on foams related to this topic reported the effect of adding xanthan gum to a native/heat denatured soy protein solution on foam stability [60]. Also, a probable change in the conformation of the protein at pH 3.0 and 7.0 affected complex formation by hydrophobic interactions in a mixture of egg white and hydroxypropylmethylcellulose (HPMC) [58]. Carp et al. [61] suggested that the character of soy protein–xanthan gum interfacial interactions may be different from those in bulk solutions due to an altered conformation of the protein at the interface, thus improving foam stability. None of these studies employed specific techniques, such as circular dichroism (CD) spectroscopy or Fourier transform infrared spectroscopy (FTIR) or others, to confirm the changes in the molecular conformation of the proteins. In addition, no reports were found about the impact of polysaccharide addition on the rheology and foaming properties of lentil protein-polysaccharide foams.

2.5 Lentil Protein and Food Applications

Modern food production greatly relies on animal protein to provide energy, nutrients, and functionality to ensure sustainable living. Yet, the market is changing and during the last decade the per capita meat consumption has been declining, while sales of plant protein-based products have increased due to consumers' economic, environmental, and sustainability apprehensions [1]. Additionally, as a result of several health benefits attributed to pulses, a change in the attitude and perception toward them has created a renewed interest toward its intake, which has increased the annual per capita consumption of pulses [62, 63]. Also, there is a low incidence of allergic reactions attributed to pulses

compared to other legumes, such as soybean [14]. Undoubtedly, this is leading the food scientists to incorporate pulse-derived ingredients into food formulations [3]. Because of their diverse range of functionalities, pulse-derived proteins can be incorporated into novel food products, such as milk substitute, curd-like products, meat products, extruded products, and baked goods [21]. However, this is a big challenge for the food industry for both technological and consumer acceptance reasons [1, 3].

As already explained in Chapter 1 (section 1.2), lentil is an excellent source of several nutritional factors and its consumption is associated with several positive effects on human health [9-11]. In addition, lentil protein has high nutritional value, good Leu/Ile and Leu/Lys ratios (1.24–1.98 and 1.08–2.03, respectively) and high digestibility (~83%) [21]. Since lentil proteins have a high lysine content, they can also satisfy nutritional requirements when incorporated into cereal-based products or combined with cereal proteins that contain adequate amounts of sulfur containing amino acids [62]. So far, lentil flour and lentil protein alone or in combination with other pulse proteins, have been incorporated with more or less success in tofu-like product, imitation milk, meatballs, and bread, among others. According to Swanson [64] lentil protein isolate produced a milk of intermediate quality equivalent to milk prepared from soy protein isolates. Cai et al. [65] prepared a curd (tofu-like product) from lentil protein that was of inferior quality compared to that of soy and pea proteins. Bamdad et al. [66] produced an edible film from lentil protein and found that it was comparable with other edible films, with good mechanical properties and water vapor permeability, as well as good solubility. Serdaroğlu et al. [67] formulated meatballs with lentil flour as an extender, resulting in greater cooking yield, fat retention and moisture retention values than those with chickpea flour. A salad dressing

was also supplemented with raw and thermally treated lentil flour, i.e., from ground roasted seeds, roasted flours, pre-cooked, ground and freeze-dried seeds; pre-cooked, ground and spray-dried seeds [28]. They measured the rheological, physical stability, microstructural and sensory properties and found that the thermally-processed pulse flours may be suitable as value-added ingredients in salad dressing applications. Finally, in the bakery field, Aider et al. [63] substituted wheat flour in bread with lentil protein. They found that the substitution produced a bread of intermediate quality with lower volume than the control and a greener color. Depending on the level of supplementation, it had similar or higher hardness than the control bread. Similarly, De la Hera et al. [68] substituted wheat flour with lentil flour to produce a layer cake and a sponge cake and studied its effect on the characteristics of the batters and of the final products. The addition of lentil flour changed the density of both cakes and reduced layer-cake volume, symmetry index, cohesiveness, and springiness. In sponge cakes, no clear trends were observed in volume or symmetry index, but the total substitution of wheat flour with lentil flour resulted in harder and less cohesive cakes, as it also increased hardness in layer cake [63]. Except for the study of Ma et al. [28], there is no other more comprehensive study that includes a lentil-based product. In terms of studying structure, texture, appearance, colour, and sensory analysis, there are no studies focusing on a product where lentil protein has been utilized as the principal source of protein.

2.5.1 Role of proteins in bakery products

There are two main proteins used in baked goods: gluten and egg proteins [69]. Its contribution to the final quality of the product will vary from one product to another. For example, gluten is the most important protein in bread and it contributes to the dough

strength, creating an elastic, extensible matrix that ensures entrapment of large amounts of carbon dioxide produced by yeast fermentation [69]. Whereas in cakes, full development of gluten into a continuous viscoelastic structure does not occur. The formulations and mixing procedures for cake do not allow gluten development, since it is diluted with eggs, fat and sugar and, therefore, gluten is less concentrated than in bread dough. It is widely accepted that in cakes, gluten serves as a viscosity enhancing water binder [70]. On the contrary, egg proteins have several functions in cakes. They provide structure when they coagulate at baking temperature, they emulsify fats because of the natural emulsifiers contained in egg yolk, which help produce smooth batters and contribute to volume and texture, and they also act as leavening agents as a result of their foaming capacity that allows them to incorporate air bubbles, which expand when heated. In addition, they contribute to cake colour, aroma, and moistness [70, 69]. Depending on the type of cake, either whole egg or only egg white is used [62, 63, 71].

Regardless of these important functions, the current trend is to reduce the egg content or to replace eggs in these products in order to decrease the long-term production cost and also satisfy consumers with specific dietary restrictions or needs [72]. However, the total or partial replacement of egg proteins with other proteins, such as plant proteins convey some issues, especially because of these excellent emulsifying, foaming, and gelation properties that define the final quality of the product [72].

There are several studies where egg protein has been totally or partially substituted with other proteins. Due to the excellent foaming and heat-induced gelation properties, whey protein isolate (WPI) has been widely used for this purpose in different types of cakes [30, 72-75]. Without other additives in the formulation, WPI can be used up to 100% to substitute egg protein [31, 73]. However, the control cake significantly outperformed the

cake formulated with the WPI in volume and also in terms of the other sensory attributes evaluated. Utilization of other additives, such as emulsifiers, i.e. sodium stearoyl-2-lactylate (SSL) [72] and hydrocolloids, i.e. hydroxypropylmethylcellulose (HPMC), methyl cellulose, xanthan and guar gum [72-74], resulted in cakes with similar characteristics to the control with respect to appearance, texture, and sensory properties. However, the level of replacement had to be reduced to 50%.

Since the use of WPI may not be an option for vegan or vegetarian consumers and because of the attributed health benefits, some studies have been conducted to substitute egg proteins with plant proteins, such as soy protein isolate and white lupine protein [76-79]. Soy protein incorporation produced denser cakes, low in volume as well as crumbly, which, according to Brewer et al. [74] might be controlled with other functional additives. However, flavor was the primary determinant of acceptability and cakes containing soy protein had an unacceptable after taste/off flavor [74, 75]. Lupine protein resulted in a decrease in cake hardness and moisture content when 25 and 50% of eggs were substituted. As well, cake volume was reduced and sometimes undesirable collapse of the cakes was observed. This problem was resolved by the combined use of emulsifiers (mono- and diglycerides) and xanthan gum that improved the internal structure of crumb, reducing shrinkage and increasing the cake height [76, 77].

Hence, much work remains to be done when targeting the substitution of eggs in cake formulations. It appears to be an interesting objective, especially for the people with specific dietary needs or restrictions (vegans, vegetarians, hypercholesterolemic, etc.) to utilize lentil protein as an egg replacer.

2.5.2 Quality assessment of cakes

An important step in developing cakes or other food products is the assessment of their quality. The main attributes of a cake are structure, texture, moistness, color, volume and flavor [78, 69]. There are a variety of cake products with a broad range of formulations that will change their attributes, making them unique [79]. They can be classified in two basic categories: foam and shortened. The former style (angel food, sponge, chiffon) depend on the foaming and aerating properties of eggs for their structure and volume The shortened-style cake (pound cake, yellow cake, chocolate cake, etc.) has a crumb structure derived from a fat-liquid emulsion that is created during batter processing [78]. Undoubtedly, cake style will impact its final quality.

2.5.2.1 Physical properties and texture analysis

The main methods used to evaluate the properties of cake products are summarized below [79]:

- Volume, often using seed displacement, laser sensor, or simply a caliper to measure the characteristic dimensions.
- Color, the preferred measurement technique is to employ a colorimeter with 45°/0° measuring geometry.
- Crumb structure, which may be assessed visually or by using objective image analysis.
- Moisture content is calculated from the loss of weight that occurs when a known weight of sample is heated at a certain temperature for a specific period of time. There are several standard methods available.

- Texture of baked products can be evaluated by a variety of tests. Although the test to apply depends on the information being sought and what bakery product is being assessed, they can be arranged in two broad groups: mechanical (Texture Profile Analysis) and sensory.

i. Texture profile analysis (TPA)

One of the most popular methods to study the textural changes in bakery products has been the TPA double-compression test based on the controlled compression of a sample through a given distance and measurement of the force required to achieve a set percentage compression of the total sample height [79]. This test is designed to mimic the bite of a sample and the result is reflected in a TPA graph from which texture parameters, such as firmness, chewiness, springiness, and cohesiveness, can be calculated [80].

ii. Sensory analysis

Sensory analysis involves the measurement, analysis, and evaluation of the sensory properties of foods and other materials using human subjects [81]. Sensory analysis of bakery products generally includes flavor, texture, and color. However, each product has its characteristic sensory profile with specific attributes [82]. Cakes are normally evaluated for appearance, color, taste, crumb texture, and overall acceptability [83, 84].

Proper evaluation of the attributes is critical to quantify sensorial responses in order to utilize statistics that will provide a rational basis for decision making about the products under investigation. The 9-point hedonic scale is one of the most popular scales and is often utilized to assess consumer acceptability. It is simple to describe, easy to use, and provides reliable and valid results. It has proven to be effective in assessing the extent of liking [81]. In addition, hedonic judgment can be combined with intensity using the just-right or just-

about-right (JAR) scales. This scale measures the consumer's reaction to a specific attribute giving direct information about it, which can be diagnostic or clarifying if the overall product appeal is deficient. When combined with hedonic judgments, the potential impact of being off from the just-right point can be estimated by using "penalty analysis" [85]. The approach is simple, consisting of (i) calculating the mean hedonic scores from the acceptability scales for groups above, below, and at the just-right category, (ii) comparing the mean of the above-JAR and below-JAR groups with the JAR group, and (iii) plotting the resulting difference (called "mean drop") in a scatter plot of the mean drop versus the percentage of the total consumer panel in each category. In this plot, any point that shows simultaneously a large mean drop and percentage of consumers indicates an attribute that can be modified to improve the product [85].

Any change in the formulation, ingredient, mixing method, etc., of a cake will impact the quality of the final product. A partial or total replacement of the egg protein will certainly modify its quality attributes. Therefore, all studies related to egg protein substitution should consider the evaluation of some or all of the properties that distinguish a cake. As already mentioned in the cases reported in section 2.5.1, replacement of egg protein by WPI, soy protein, or other proteins, will usually act at the expense of cake quality: reducing the volume, making it denser as well as crumbly, and affecting the sensory properties, such as color, taste, and geneal appearance [31, 79-84]. Use of baking additives, i.e. HPMC, SSL, methyl cellulose, xanthan and guar gum, mono- and diglycerides, etc., may produce cakes with similar quality attributes to those of egg protein-based cakes [79-82, 84].

Therefore, it is important to determine not only the suitability of lentil protein to function as a total or partial egg protein replacer in cake formulation, but also how the final quality of the cake is affected, as well as, the underlying mechanisms for these changes.

CHAPTER 3

Optimization of Lentil Protein Extraction and the Influence of Process pH on Protein Structure and Functionality[1]

3.1 Introduction

Lentil (*Lens culinaris*) is a leguminous plant high in fibre and low in fat. Like most legumes, lentil is a rich source of protein [11] with a protein content between 20.6-31.4 g/100 g. According to Osborne's classification, lentil proteins are mainly globulins (~ 50 g/100 g) consisting of legumin and vicilin-like proteins with a molecular mass of about 60 kDa and 50-80 kDa, respectively [12]. Usually, preparation of lentil protein concentrates involves alkaline extraction followed by an isoelectric precipitation step. Although there are several protocols described in the literature on alkaline extraction of lentil protein, they differ in pH, varying from 7.2 to 11.0, solid-to-solvent ratio, from 1:5 to 1:20, and temperature, from 25ºC to 40ºC, and there is a lack of systematic studies showing how extraction parameters impact lentil protein yield and purity. The limited work includes study of the effect of temperature and pH on lentil starch and protein yields by Lee et al. [26], and investigation of the impact of pH on the purity of isolates by Alsohaimy et al. [16]. On the other hand, great progress has been made recently to reveal the good functionality of lentil proteins such as solubility and emulsifying, foaming and gelation capacities [15-17]. Nevertheless, these studies have focused on lentil proteins extracted under one condition and evaluated their functionality at neutral pH. Studies are limited regarding the influence of extraction conditions on lentil protein structure and subsequent functional properties of these extracts.

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Since protein functional properties are dictated to a large extent by protein's physicochemical and structural properties as influenced by the treatment and environmental conditions [86], more fundamental research that addresses this gap is critical to facilitate wider applications of lentil proteins.

Therefore, this study aims to optimize the lentil protein extraction process using response surface methodology (RSM) to maximize both protein content and yield of the extract. The extracts, obtained at different pH treatments, were characterized for composition and structure using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), size exclusion high performance liquid chromatography (SE-HPLC) and Fourier transform infrared spectroscopy (FTIR). Functional properties were systematically evaluated at the mildly acidic and neutral pHs representative of most food processing operations. The impact of lentil protein extraction and process pH on molecular structure is discussed.

3.2 Materials and Methods

3.2.1 Raw materials and sample preparation

Large green lentils produced in Saskatchewan, Canada of mixed varieties (Greenland and Sovereign) were purchased from a local supermarket (Edmonton, AB, Canada). The whole grains (including the hull) were ground into fine flour using a Retsch centrifugal grinding mill with screen aperture size of 0.5 mm (ZM 200, Retsch, Inc., Newtown, PA, USA). The flour was packed in plastic bags, sealed, and stored at 4 ^{o}C until use. Canola oil used for protein functionality evaluation was purchased from a local grocery store (No name®, Superstore, Edmonton, AB, Canada). Unstained standard protein molecule marker for SDS-PAGE (Molecular weight of 10-250 kDa) was purchased from Bio-Rad

(Richmond, CA, USA) and standard molecular markers for HPLC analysis (thyroglobulin, 670 kDa; ferritin, 440 kDa; BSA, 67 kDa; ovalbumin, 43 kDa; cytochrome C, 13.6 kDa and aprotinin, 6.5 kDa) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were reagent-grade.

3.2.2 Experimental design for protein extraction and statistical analysis

Lentil protein was extracted according to Lee et al. (2007). Defatted and non-defatted lentil flour (100 g) was mixed with distilled water and the pH was adjusted to the desired value using 0.1 M NaOH. After extraction, the insoluble solids were removed by a centrifuge (8,500 × g for 15 min at 23 ^{o}C). The supernatant was adjusted to pH 4.2 with 0.1 M HCl, left overnight at 4 ^{o}C to precipitate the protein and then the mixture was centrifuged (1,590 x g for 30 min) to precipitate the extract. The protein precipitate was then freeze dried, and the dry powder was stored in plastic bags at 4 ^{o}C until further analysis. Two sets of extraction experiments were performed to assess the effects of different variables.

In the first set of screening experiments, the effects of four variables, X_1 (extraction temperature, 22 and 40 ^{o}C), X_2 (pH, 9.0 and 11.0), X_3 (solid/solvent ratio, 1:20 and 1:10 w:v), and X_4 (extraction time, 60 and 120 min), at two levels (Table A1-1 in Appendix A1) on lentil protein extraction were investigated in order to determine the most important factors in the extraction process. Thus, the candidate variables could be reduced and the subsequent experiments were more efficient, requiring fewer tests [87]. The maximum temperature was kept at 40 ^{o}C to avoid solution thickening due to starch swelling. The ranges for the other process variables were defined based on a review of the literature [16, 18]. The responses evaluated included protein content, defined as the percentage of protein

in the extracted dry powders, and yield, defined as the mass (g) of extract obtained from 100 g of lentil flour. This experimental design was a two-level factorial (2^k) design with 3 centre points, including 19 experimental runs and 16 treatments (Table A1-2 in Appendix A1).

The significance of each factor in the screening test was determined by the standardized Pareto chart (Figure A1.1 in Appendix A1). The length of each bar is proportional to the impact of each corresponding effect on the responses. Any bar extending beyond the vertical line, which represent the selected significance level (5%), is statistically significant. The pH and solid/solvent ratio were the most significant factors that impacted yield and protein content, respectively (p<0.05). The interaction effects of pH-temperature and solid/solvent ratio-time were also significant for protein content (p<0.05). However, an analysis of the interaction plots (not shown) revealed that significance was almost exclusively due to pH or solid/solvent ratio variation. Then, temperature and time did not play a significant role. This result was supported by the ANOVA analysis (data not shown). Protein content of the extracted samples ranged between 79.34 and 83.55 g of protein/100 g of extract (Table A1-2 in Appendix A1), which is satisfactory for application purposes, and the yield varied from 13.41 to 16.03 g of protein extract/100 g of lentil flour. Extraction of lentil protein using RSM was not reported previously; however, studies for other plant proteins indicated that both pH and solid/solvent ratio were important factors impacting protein content and/or yield [88]. Therefore, only pH and solid/solvent ratio were selected as variables for the next phase of optimization. As defatted and non-defatted lentil flours produced similar results, only the latter was utilized in the optimization stage, in order to reduce processing steps.

In the second set, the independent variables that were identified as significant in the first set were further optimized by RSM and the desirability method in an effort to maximize both protein content and yield of the extract samples, using a central composite design with four axial points and six central points augmented by two points. This enabled a better understanding of how pH and solid/solvent ratio impact protein content and yield. Analysis of variance, regression analysis, contour plot, and optimization were conducted using Design-Expert® 8.0.5 (Stat Ease, Inc., Minneapolis, MN, USA, 2010). Contour plots obtained from RSM help to understand the response when two important factors are varied simultaneously, making the selection of the values simpler following the desirability approach. The responses in terms of protein content (Y_1) and yield (Y_2) were analysed by RSM to fit the following statistical model:

$$Y_i = b_0 + \sum_{n=1}^{2} b_n X_n + \sum_{n=1}^{2} b_{nn} X_n^2 + \sum_{n<m}^{k} b_{nm} X_n X_m \qquad \text{eq. (3.1)}$$

where b_0 is the value of the fixed response at the central point of the experiment, which is the point (0,0,0); b_n; b_{nn} and b_{nm} are the linear, quadratic and cross product coefficients, respectively, and X_n, X_m are the input variables.

3.2.3 Characterization of lentil proteins

The protein content of the extracts was determined using a nitrogen analyzer (FP-428, Leco Corporation, St. Joseph, MI, USA) calibrated with analytical reagent-grade EDTA and a protein conversion factor of 6.25.

The weight-average molecular weight (M_w) of the lentil protein was determined by size exclusion high performance liquid chromatography (SE-HPLC) (Agilent series 1100, Palo Alto, CA, USA) equipped with a BioSuite™ 450, 8 µm HR-SEC column (7.8x300 mm;

Waters Corporation, Milford, MA, USA) at 25 ± 0.5ºC. The elution buffer contained 0.05 M Na_2HPO_4/NaH_2PO_4 and 0.1 M NaCl and its flow rate was 0.5 mL/min. Sample solution (50 µL, 0.5% w/v) was injected into the HPLC system and the protein elution was monitored at the UV wavelength of 280 nm. Standard molecular markers were used to calculate M_w of the distribution of samples. A calibration curve was obtained from the *log* M_w of the markers and their respective elution times (R^2= 0.95).

SDS gel electrophoresis was performed after isoelectric precipitation to determine the molecular weight of lentil protein subunits using an AmershamTM ECLTM Gel Box (GE Healthcare Bio-Sciences Corp., Pittsburgh, PA, USA). Protein samples (1.5 mg/mL) were mixed with loading buffer (0.5 mol Tris-HCl, pH 6.8, 4 g/100 mL SDS, 20 mL/100 mL glycerol, 0.5 mL/100 mL 2-mercaptoethanol, and 0.02 g/100 mL bromophenol blue w/v) and heated at 95 °C for 5 min. After cooling, samples were loaded on a pre-cast gel (4–20% gradient) and subjected to electrophoresis at a constant voltage of 120V. After electrophoresis, the gels were stained with 0.1 g/100 mL Coomassie Brilliant Blue-R-250 in water, methanol, and acetic acid (4:5:1, v:v) for 30 min and destained with water, methanol, and acetic acid (4:5:1, v:v).

The electrophoretic mobility of the extracted lentil protein samples under pH 2.0-11.0 (adjusted using 0.1 M HCl or NaOH) at 22 ºC was measured by laser Doppler velocimetry using a Zetasizer NanoS (model ZEN1600, Malvern Instruments Ltd, Malvern, UK). Electrophoretic mobility (i.e., velocity of a particle within an electric field) was related to the zeta potential (ς) using the Henry equation

$$U_E = \frac{2\varepsilon \times \xi \times f(\kappa\alpha)}{3\eta} \qquad \text{eq. (3.2)}$$

where η is the dispersion viscosity, ε is the permittivity, and $f(\kappa\alpha)$ is a function related to the ratio of particle radius (α) and the Debye length (κ).

The infrared spectra were recorded at room temperature (22 ^{o}C) using a Nicolet 6700 spectrometer (Thermo Scientific, Madison, WI, USA). The spectrometer was continuously supplied with nitrogen. Lentil protein extracts (2 g/100 mL) were dissolved in D_2O solution. To ensure complete H/D exchange, samples were prepared 2 days before and kept at 4 ^{o}C prior to infrared measurements. Samples were placed between two CaF_2 windows separated by 25 mm polyethylene terephthalate film spacer for FTIR measurement and infrared spectra were recorded at 20 ^{o}C. To study the amide I region of the protein, Fourier self-deconvolutions were performed using the software provided with the spectrometer. Band narrowing was achieved with a full width at half maximum of 25 cm^{-1} and with a resolution enhancement factor of 2.5 cm^{-1}.

For amino acid analysis, the extracted lentil protein was hydrolyzed under vacuum in 4 M methanesulphonic acid with 0.2 g/100 mL tryptamine according to the method of Simpson et al. [89] with slight modifications. Glass sample tubes (6 × 50 mm) were used in the reaction vial assembly, which were then placed in the Work Station (Waters, Milford, MA, USA). After treating as suggested in the Work Station manual, where the contents were hydrolyzed at 115 °C for 24 h, the pH was adjusted to neutral with 3.5 M NaOH. Amino acid analysis was performed using the Waters ACCQ-Tag method. The high-performance liquid chromatography (HPLC) system (Agilent series 1100, Palo Alto, CA, USA) consisted of an auto sampler and a binary pump, a control system with a column heater maintained at 37 °C, and a UV detector set at a wavelength of 254 nm. A reversed-phase AccQ-Tag 150 × 3.9 mm C18 column with a three-eluent gradient solvent system

(AccQ-Tag eluent, acetonitrile, and water) was used at a flow rate of 1.5 mL/min. Data acquisition was controlled by ChemStation software.

3.2.4 Evaluation of functional properties

In order to achieve a deeper understanding of the pH impact on the structure and functionality of the protein, the functional properties were determined for extracts obtained at pH 8.0, 9.0, and 10.0, at varying environmental pH conditions (3.0, 5.0, and 7.0).

3.2.4.1 Solubility

Protein solubility was evaluated using the method of Wang et al. [90] with slight modifications. A sample of 50 mg was suspended in 10 mL distilled water and the pH of the suspension was adjusted to 2-12 using 0.1 M HCl or NaOH solutions. The suspensions were continuously stirred for 1 h at 22 ºC and centrifuged at 8,000 × *g* for 15 min. The amount of protein in the freeze dried supernatant was determined with the nitrogen analyzer (CN-628, Leco Corporation, St. Joseph, MI, USA). Solubility was expressed as the percentage of the protein in the supernatant compared to the total protein in the extract added into the suspension. The solubility profile was obtained by plotting the average protein solubility (%) against pH values.

3.2.4.2 Gelling properties

Appropriate amounts of protein extracts were weighed and added into test tubes containing 5 mL of deionized water adjusted to pH 3.0, 5.0, or 7.0 using 0.1 M NaOH or 0.1 M HCl solutions to make suspensions with protein concentrations ranging from 2 to 20 g/100 mL. After hydration overnight, the dispersions were heated at 90 ºC in sealed tubes for 30 min, cooled immediately under tap water, and then stored at 4 ºC overnight. The minimum gelling concentration (MGC) was estimated as the concentration below which no

self-supporting gel was formed after storage at 4 °C when the tubes were turned upside down. The formed gels were placed on an Instron 5967 universal testing machine equipped with a 50 N load cell (Instron Corp., Norwood, MA, USA), and their texture profiles were tested under a double compression cycle at a crosshead speed of 1 mm min^{-1} until a deformation of 40% of initial height was reached. The compressive stress, which represents the force required to attain a given deformation per unit of area, was computed (software Blue Hill ver.2, Instron Corp., Norwood, MA, USA). Compressive stress was calculated as the compressive force (N) over the cross-sectional area of the gel (cm^2) [91]. The morphological observation of the gels was carried out with a Phillips XL-30 scanning electron microscope (FEI Company, Hillsboro, OR, USA) at an accelerating voltage of 80 kV. The samples were frozen in liquid nitrogen, freeze-dried, and the dry samples were coated with gold before observation.

3.2.4.3 Foaming properties

Foaming properties were determined according to Wang et al. [90] with slight modifications. Protein extract samples (0.5 g/100 mL) were dispersed in 20 mL of deionized water adjusted to pH 3.0, 5.0, or 7.0 using either 0.5 M NaOH or 0.5 M HCl solution. The solution was mixed for 2 min with a homogenizer (PowerGen 1000, Fisher Scientific, Fairlawn, NJ, USA) at speed six. Volumes were recorded before and after homogenization using a graduated cylinder. The foaming capacity (FC) was calculated as:

$$FC(\%) = \frac{V_{f1} - V_{f0}}{V_{f0}} \times 100 \qquad \text{eq. (3.3)}$$

where V_{f0} and V_{f1} represent the volume of the protein solution and the formed foams after homogenization, respectively. Foam stability (FS) was determined as:

$$FS(\%) = \frac{V_{f2}}{V_{f1}} \times 100 \qquad \text{eq. (3.4)}$$

where V_{f2} is the volume of foam that remained after standing for 0.5 h at 22 °C expressed as a percentage of the initial foam volume.

3.2.5 Statistical analysis

Extractions were done according to RSM, characterisation tests were performed in triplicate, and the means ± standard deviations were reported. Data collected were subjected to analysis of variance at 5% confidence level. When significant effects of treatments were found by ANOVA analysis, Tukey's multiple range test was carried out for multiple comparison of the means. All statistical analyses were carried out using Statgraphics Centurion software package, version XVI (StatPoint Technologies Inc., Warrenton, VA, USA).

3.3 Results and Discussion

3.3.1 Screening experiment and optimization of the lentil protein extraction conditions

The significance of each factor in the screening test was determined by the standardized Pareto chart (Figure A1.1 in Appendix A1) and supported by the ANOVA analysis (data not shown). The pH and solid/solvent ratio were the most significant factors that impacted yield and protein content, respectively (p<0.05), therefore, they were selected as variables for the next phase of optimization.

Then, protein extraction from lentil flour was performed following 12 combinations of the two independent variables (pH and solid/solvent ratio) at fixed time and temperature (60 min and 22 ºC). Short extraction time and ambient temperature were selected since the responses did not differ significantly using longer time and higher temperature during the screening experiments. Table 3.1 shows the response variables Y_1, Y_2 (observed responses) and $Y\hat{}_1$ and $Y\hat{}_2$ (predicted responses based on the model) obtained for each of the

treatments. Results reveal that the experimental protein content varied from 70.28 to 85.67g /100 g, and the yield varied from 12.35 to 16.50 g of protein extract from 100 g of lentil flour. The obtained protein content and yield values are comparable to those reported for other pulses [16, 88, 92].

The regression equations obtained for protein content Y_1 and yield Y_2 were as follows:

$$Y_1 = 62.80 + 468.3x_2 - 0.30{x_1}^2 \quad \text{eq. (3.3)}$$

$$Y_2 = -3.90 + 2.12x_1 \quad \text{eq. (3.4)}$$

where x_1 is pH and x_2 is solid/solvent ratio.

Table 3.1 Central composite design for independent variables X1 (pH) and X2 (solid/solvent (g/mL) ratio), coded variables, and the responses of protein content and yield.

Run	Coded variables		Uncoded variables		Protein content g protein/100 g extract		Yield g extract/ 100 g flour	
	X_1	X_2	x_1 pH	x_2 Solid/solvent ratio	Experimental Y_1	Predicted $Y^\wedge_1$	Experimental Y_2	Predicted $Y^\wedge_2$
1	1	1	9.0	1:10	83.46	86.33	14.40	15.18
2	0	0	8.0	1:13	84.13	82.06	12.63	13.06
3	-1	-1	7.0	1:20	78.17	73.12	12.55	10.94
4	1	-1	9.0	1:20	70.47	66.52	15.42	15.18
5	0	0	8.0	1:13	83.91	82.06	14.59	13.06
6	-1	1	7.0	1:10	85.30	93.48	12.89	10.94
7	0	0	8.0	1:13	83.35	82.06	13.93	13.06
8	0	1.41	8.0	1:09	85.67	94.19	14.17	13.06
9	0	0	8.0	1:13	84.50	82.06	14.27	13.06
10	0	0	8.0	1:13	84.64	82.06	13.88	13.06
11	0	0	8.0	1:13	83.22	82.06	13.98	13.06
12	1.41	0	9.4	1:13	80.50	74.76	15.49	16.03
13	-1.41	0	6.6	1:13	84.30	88.20	12.35	10.09
14	0	-1.41	8.0	1:25	70.28	63.33	13.78	13.06
16	3.5	0.6	11.5	1:11	73.62	68.87	16.50	18.41
17	0.5	-0.06	8.5	1:15	75.32	74.91	14.00	14.12

The predicted values (Table 3.1) of protein content and yield were calculated using the regression models and compared with experimental values. The regression coefficients (R^2)

were 0.81 and 0.93 for protein content and yield, respectively, indicating a reasonable fit of the models to the experimental data [88, 93]. The coefficients of variation (CV) for protein content and yield were 0.72% and 4.60%, respectively. As a general rule, a model can be considered reasonably reproducible if its CV is not greater than 10% [93]. ANOVA analysis revealed that the linear term of solid/solvent ratio and quadratic term of pH had a significant effect (p<0.05) on protein content, while only pH had a significant impact on protein yield (Tables A1-3 and A1-4 in Appendix A1). The lack of fit term that appears in the ANOVA tables is an indicator of how well the model predicts experimental response values. Since they were not significant (p>0.05), the models found were validated.

A lower protein content was observed when samples were extracted at high pH levels (Figure 3.1 A), as a higher amount of starch was introduced into the concentrate. Starch granules, already damaged by the milling process, are susceptible to alkali conditions, which increases their solubility at high pH values. Then, when the pH of the extract solution is decreased to the isoelectric point of proteins, some of the solubilized starch is precipitated along with the protein [94]. On the other hand, the yield increased with increasing pH at solid/solvent ratio level between 1:20 and 1:10 (Figure 3.1 B). This is explained by the higher solubility of lentil protein at high pH as well as the recovery of higher amounts of starch. It was reported that the high alkali concentration helps to break down the hydrogen bonds and to dissociate hydrogen from carbonyl and sulphate groups [95]. The increased surface charge on protein molecules then leads to an enhanced solubility in the solvent system. The conditions for reaching the maximum protein content and yield values are represented by the darkest zones in Figures 3.1 A and B. As both protein content and yield are important for protein extraction, optimum extraction conditions to simultaneously maximize yield and protein content were estimated by the

desirability approach. The desirability approach is a method that assigns a "score" between 0 and 1 to a set of responses and chooses factor settings that maximize the score for the optimization of multiple responses. The ideal optimum value is d=1 while an acceptable value for d can be between 0.6 and 0.8 [93]. The highest d value of 0.729 was obtained at a solid/solvent ratio of 1:10 (w:v) and pH of 9.0, which were identified as the optimum conditions for lentil protein extraction in this study. This processing condition is in agreement with that optimized for red and green lentils by Boye et al. [21]; however, validation of these optimal conditions is required.

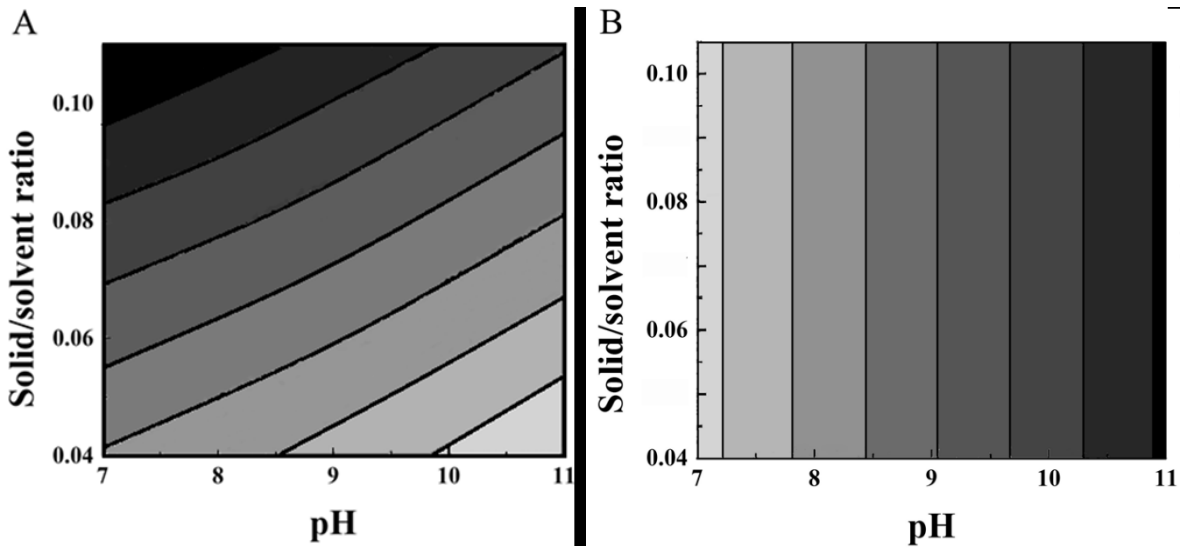


Figure 3.1 (A) Effect of pH and solid to solvent ratio on protein content of the extract. From lighter to darker color each color border correspond to 42.60, 52.22, 62.92, 73.30, 77.20, 81.88, 84.83, 86.33, and 94.19 g of protein/100 g of extract. **(B)** Effect of pH and solid to solvent ratio on yield of extract obtained from lentil flour. From lighter to darker color each color border correspond to 10.94, 12.00, 12.56, 13.10, 14.08, 15.18, 16.21, 17.36, and 18.07 g of extract/100 g of flour.

Therefore, additional extractions were conducted at pH 9.0, 1:10 solid/solvent ratio, and 22 ^{o}C for 60 min, in triplicate. The predicted protein content and yield with these parameters using the regression model were 90 g/100 g and 15.5 g of protein extract/100 g

of lentil flour, respectively. The experimental protein content of the extract and the yield obtained at the optimum conditions were 82±1 g/100 g and 15.5±0.5 g of protein extract/100 g of lentil flour, respectively. Additional analysis revealed that the sample contained 5.0±0.05 g water, 2.6±0.3 g ash, 1.6±0.4 g lipids, and 8.0±1.8 g carbohydrates per 100 g of protein extract. Under these conditions, the protein recovery was 47%.

3.3.2 Characterization of lentil protein

Since pH plays an important role in lentil protein extraction and is likely to influence protein structure and functionality, three samples extracted at 1:10 solid/solvent ratio, 22 ºC for 60 min, and pH 8.0, 9.0, and 10.0 were investigated for structure and functionality. The protein contents of these extracts obtained at pH 8.0, 9.0, and 10.0 were 83±1, 82±1, and 80±0.5 g/100 g, respectively.

Table 3.2 Amino acid composition of lentil protein extracted by alkaline method for 1 h at pH 9 and 22 ºC

Amino acids[a]	Composition % (w/w)[b]	Amino acids[a]	Composition %[b]
Asx	8.9	Val	6.6
Ser	3.1	Met	n.d
Glx	18.8	Lys	6.9
Gly	7.6	Ile	6.4
His	2.5	Leu	9.6
Arg	7.3	Phe	5.3
Thr	2.5	Trp	n.i
Ala	6.9		
Pro	5.0		
Cys	n.d		
Tyr	2.7		

''n.d.'' means not detectable, "n.i" means not included in determination.

[a] Asx represents Asn and Asp; Glx represents Gln and Glu

[b] The order of amino acids listed in the table follows the elution order of the amino acids from the reversed-phase HPLC column.

The amino acid composition of the obtained lentil protein concentrate (Table 3.2) revealed that similar to most pulse proteins, lentil protein is rich in glutamine/glutamic acid

(18.8 g/100 g protein), and lysine (6.9 g/100 g protein), but poor in sulphur containing amino acids (methionine + cystine). Lentil proteins also exhibited balanced hydrophilic and hydrophobic segments with ~40% hydrophobic residues including alanine, valine, isoleucine, leucine, phenylalanine and proline, and ~45% hydrophilic residues including glutamine/glutamic acid, asparagine/aspartic acid, lysine and arginine.

As shown in Figure 3.2 A, all samples had similar isoelectric points of pI 4.4-4.6. No significant difference (p>0.05) was observed for samples extracted at different pHs. When pH was deviated from the isoelectric point, the net charge on protein chains increased. All of the samples exhibited similar ζ charges, as the pH was gradually increased from pH 2.0 to 11.0. The highest charge reached to +30 to +40 at pH 2 and -30 to -40 at neutral and alkaline pH. The samples extracted at pH 8.0, 9.0, and 10.0 also showed similar SDS-PAGE patterns (Figure A1.2 in Appendix A1) with bands ranging in size from 12 to approximately 97 kDa. The bands in the range of 34-37 kDa and 18-23 kDa correspond to the acidic and basic subunits of the 11S fraction (legumin-like), respectively [12]. The intense band with a molecular mass of 44 kDa corresponds to subunits of vicilin-like (7S) fraction. Other bands with lower molecular mass (Mw < 15 kDa) observed in the samples are likely to be a mixture of albumin polypeptides and/or gamma-vicilin [15].

These results suggest that the protein compositions of the three extracts were similar and mainly composed of globulin proteins [92]. Limited glutelins and prolamins were extracted from lentil flour probably due to their poor solubility at the conditions applied in this study. The precipitation step at pH 4.2 after the alkaline extraction would lead to a loss of the water-soluble albumin proteins.

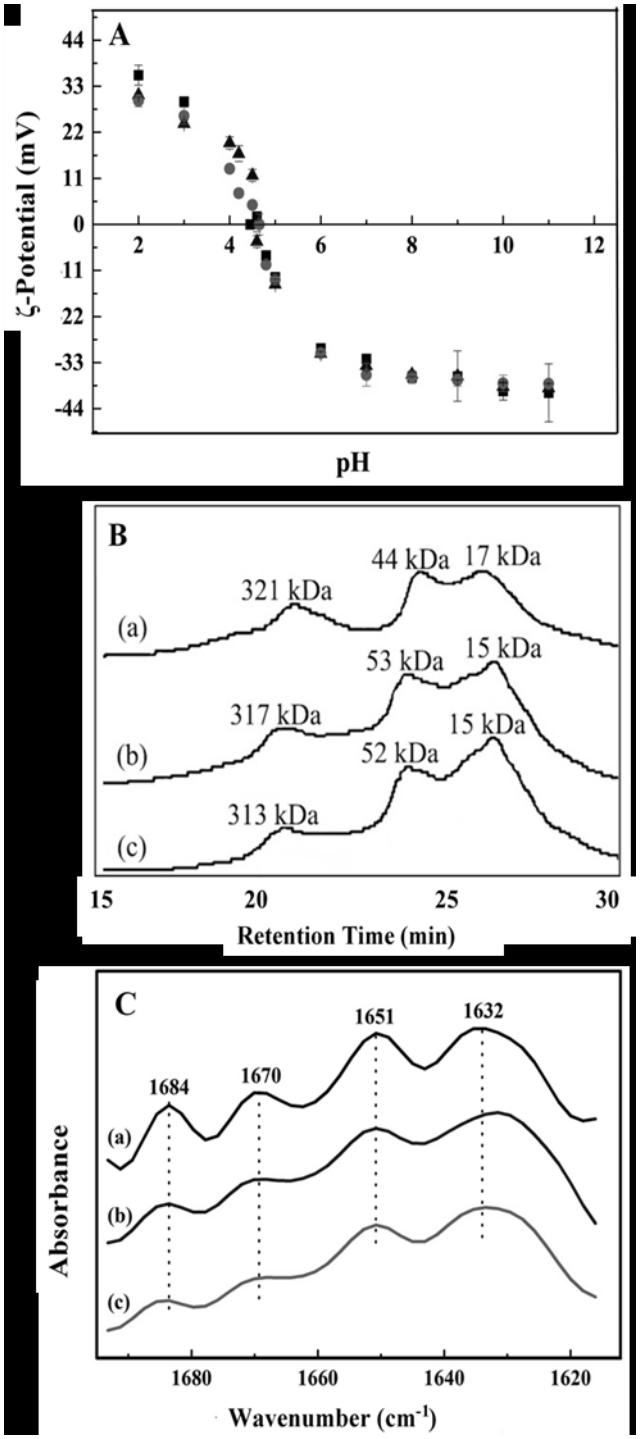


Figure 3.2 (A) ζ-Potential of lentil protein concentrate samples extracted at pH 8.0 (squares), 9.0 (triangles), and 10.0 (circles). **(B)** SE-HPLC and **(C)** Fourier-deconvoluted FTIR spectra of lentil protein concentrate samples extracted at (a) pH 8.0, (b) pH 9.0 and (c) pH 10.0.

Figure 3.2 B shows the SE-HPLC chromatograms of the samples extracted at pH 8.0, 9.0, and 10.0 under non-reducing conditions. Lentil protein shows three major peaks at around 313-321 kDa, 44-53 kDa, and 15-17 kDa, corresponding to legumins (11S), vicilins (7S) and smaller peptides [15], respectively. This result is in good agreement with that observed in the SDS-PAGE pattern. With increasing extraction pH, the peak intensity of legumin fraction decreased considerably, whereas an obvious concurrent increase of the smaller-sized peptides occurred. This suggests partial hydrolysis of legumin, resulting in the formation of small peptides when lentil protein was extracted at higher pH. Alkaline pH created a progressive dissociation/denaturation of the 11S protein to lower molecular weight subunits (7S and 2S) [96]. Since the proportion of 7S protein remained almost constant, it is likely that 11S dissociates faster than 7S at this range of pH.

Figure 3.2 C shows the FTIR spectra in the amide I region of the extracted lentil protein samples. The spectra are characterized by the presence of antiparallel β-sheets (1632 and 1684 cm^{-1}), overlapped α-helices and random coil signals (1651 cm^{-1}), and turns (1670 cm^{-1}). The bands observed at 1632 cm^{-1} were relatively more intense than those at 1651 cm^{-1}, indicative of the presence of more β-sheet structures. These results are in agreement with previous studies on lentil proteins [12, 97]. A decrease in peak intensity at 1651, 1670 and 1684 cm^{-1} was observed when the pH was raised from 8.0 to 9.0 and 10.0, suggesting a partial unfolding of the protein structure. This could also be related to disruption of hydrogen bonds and dissociation of hydrogen from carbonyl and sulphate groups at alkali conditions, which could increase protein surface charge, leading to partial protein denaturation.

3.3.3 Lentil protein functional properties

3.3.3.1 Protein solubility

Lentil protein concentrates showed minimum solubility between pH 4.0 and 5.0, with protein solubility increasing dramatically when pH deviated from the pI. Relatively high solubility was observed at pH 2.0 (above 75%) and above pH 8.0 (70-97%) (Figure 3.3), which is favorable for its potential applications. These proteins contain a significant amount of polar and hydrophilic amino acid residues such as glutamine/glutamic acid, asparagine/aspartic acid, lysine, and arginine, which contribute to favourable hydrophilic interactions between protein molecules and water, such as hydrogen bonds and electrostatic interactions. Although lentil protein is rich in hydrophobic amino acids, like many other globulin proteins, most of these hydrophobic amino acids are probably buried inside the core with hydrophilic residues located on the external surface. The protein solubility was improved at pH $\leq$3.0 and $\geq$ 6.0 for the extract obtained at pH 10.0, likely due to the partial hydrolysis as observed in SE-HPLC chromatograph at high pH, which could increase terminal residues, hence charged groups for interactions with the aqueous medium.

3.3.3.2 Gelling properties

In this study, lentil protein samples extracted at pH 8.0, 9.0, and 10.0 were tested for their gelling properties. All three protein samples possessed a denaturation temperature of 85±1 ^{o}C as determined by differential scanning calorimetry (data not shown). Gels were then prepared by heating protein solutions at 90 ^{o}C and pH 3.0, 5.0, and 7.0 for each sample. In the first step, minimum gelation concentration (MGC) was evaluated, as generally the lower the MGC values the better the gelation capacity [98]. MGC values of 8 to 10 g/100 g of protein in the extract were observed for all samples.

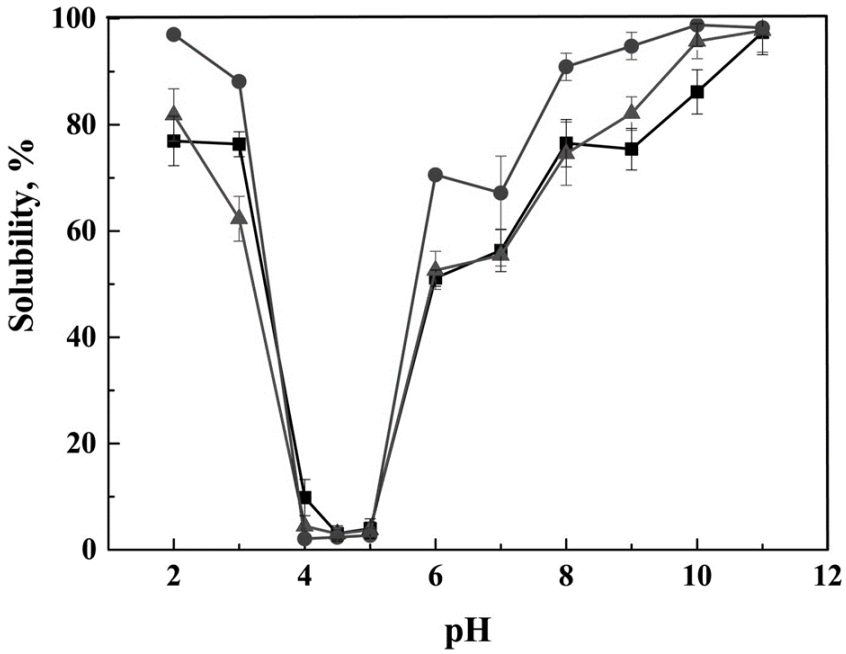


Figure 3.3 Solubility profile of lentil protein concentrates extracted at pH 8.0 (squares), 9.0 (triangles), and 10.0 (circles).

The extraction pH did not significantly affect the sample MGC values, neither did the gelation pH (p>0.05). Lentil proteins extracted in this study exhibited much lower MGC values than those reported for chickpea protein isolates and pea protein isolates (12 – 14 g/100 g) [18, 98]. A similar range of MGC values were observed for soy (9 – 11 g/100 g) and whey proteins (5 – 9 g/100g) under similar gelling conditions [99, 100]. Since gelation has an important impact on food attributes, a low MGC may be an advantage when targeting certain textural attributes at a lower concentration.

Texture profiles of the gels were then evaluated for compressive force. The gels were formed at 8, 10, and 12 g of protein/100 mL. When the concentration was higher than 12 g/100 mL, the solution became thick and a good gel could not form. Gel textural properties could not be measured if it was too soft to support itself.

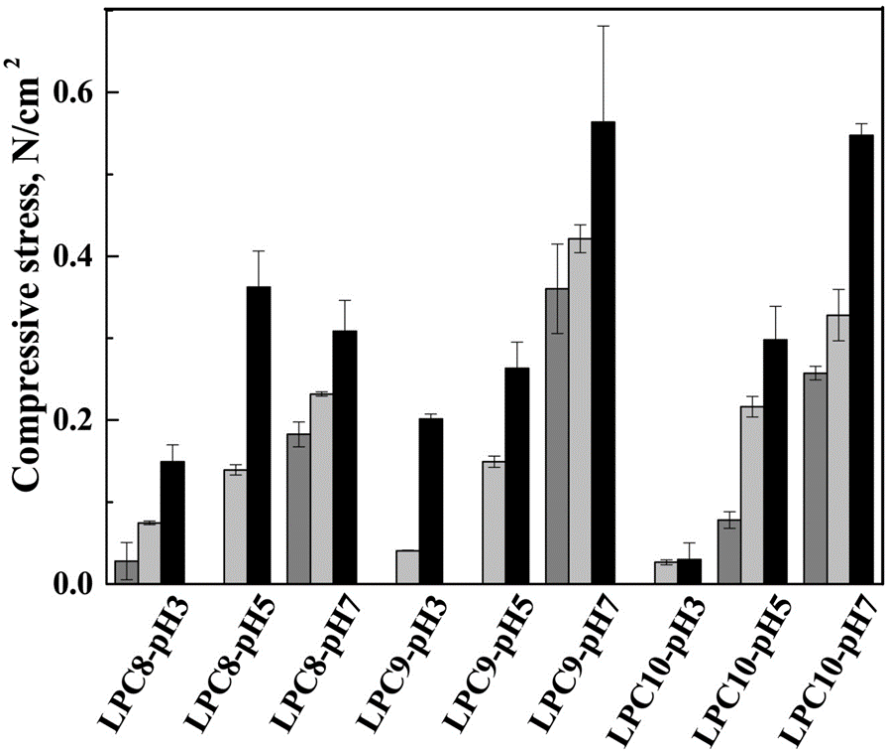


Figure 3.4 Compressive stress of the lentil protein gel as a function of protein concentration and pH of gelation (3.0, 5.0, and 7.0): (gray bar), 8 g/100 mL, (light gray bar), 10 g /100 mL, and (black bar), 12 g/100 mL. Samples were extracted at pH 8.0 (LPC8), 9.0 (LPC9), and 10.0 (LPC10).

As expected, a higher protein concentration generally resulted in significantly higher compressive stress (p<0.5) (Figure 3.4), which has been observed in other protein gels [101, 102]. Higher concentration increases the possibility of protein-protein interactions resulting in a more densely cross-linked three-dimensional network, thus contributing to increased gel strength. Gelation pH also significantly affected the gel mechanical property. Gels formed at pH 7.0 showed, in general, higher values of compressive stress. The physical properties of a gel are closely linked to its network structure, so the morphology of the gels prepared at different pH conditions using the protein extracted at pH 9.0 was observed using SEM. The gel prepared at pH 3.0 showed a network structure composed of protein strands about 1 μm thick with small pores (Figure 3.5 A), which is characteristic of a filamentous gel formed at pH values above or below protein pI. A filamentous gel was

also observed at pH 7.0, but a more dense network structure was observed, which was composed of thicker strands (≥2μm) (Figure 3.5 C). The gel prepared at pH 5.0 (Figure 3.5 B) exhibited a non-homogeneous, particulate morphology, typical of gels formed near the pI, composed of large protein aggregates. The increase in the compressive stress when gel microstructure moves from particulate to strand morphology has been observed before in whey protein gels, and it is also explained by the stiffness of the generated strand structures due to increasing number of interactions [103]. The thicker strands and the denser network structures observed in gels prepared at pH 7.0 compared to those formed at pH 3.0 can explain the dramatically enhanced gel strength obtained at neutral pH (Figure 3.4). Surface charge results indicated that the protein has a charge between -30 to -40 mV at pH 7.0, which is higher than that at pH 3.0 (~+30mV, Figure 3.2 A). Such a high charge could prevent rapid protein aggregation and allow development of hydrogen bonding and hydrophobic interactions during gel formation. Stronger gels were observed in general at pH 5.0 compared to pH 3.0 according to the compression test (Figure 3.4), which can be explained by a denser network formed at pH 5.0 that would confer more strength to the gel.

Proteins extracted at pH 9.0 and 10.0 formed gels with higher compressive stress at neutral pH than those extracted at pH 8.0 (Figure 3.4). This may be related to the partial unfolding of the protein structure and exposure of hidden active groups to facilitate protein-protein interactions during gel formation, especially at the initial stage of heating. No obvious effect of protein extraction pH on gel strength was observed when the gels were prepared at pH 3.0 and 5.0.

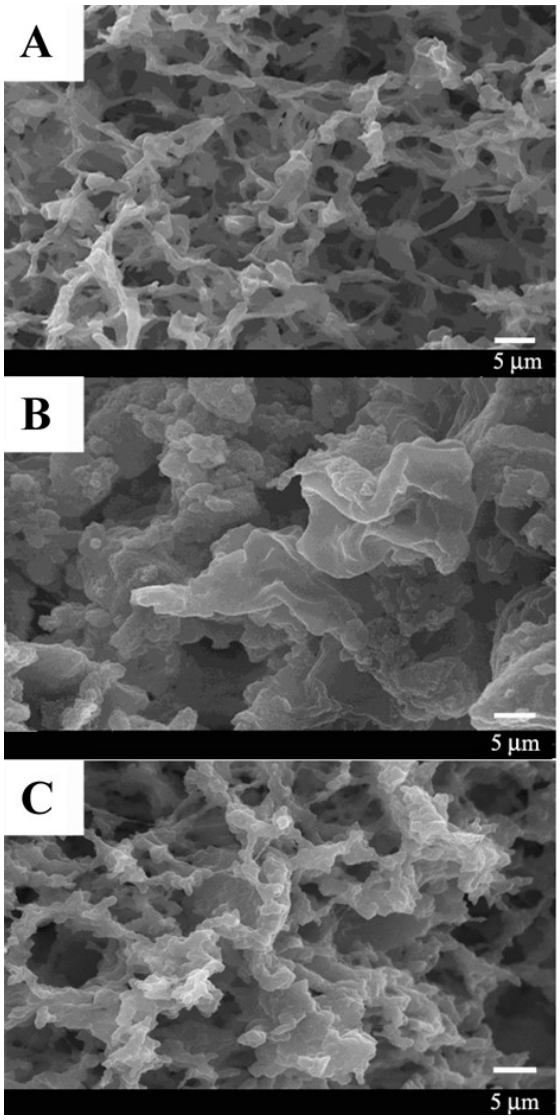


Figure 3.5 Scanning electron micrographs of lentil protein gels prepared at pH 3.0 (A), pH 5.0 (B) and pH 7.0 (C). The protein sample was extracted at pH 9.0.

3.3.3.3 Foaming properties

The pH dependence of the foaming capacities (*FC*) of the lentil protein extracted at pH 8.0, 9.0, and 10.0 are represented in Figure 3.6 A. No significant difference in *FC* values was observed between the samples extracted at different pH levels. However, significant differences occurred when the pH of solution was changed. The lowest *FC* values (550%)

were observed at pH 5.0 for all extracted lentil proteins, likely related to the lower protein solubility near the pI. When the pH of samples was decreased to 3.0, lentil protein demonstrated excellent foaming capacity of around 680%. The tests carried out in our laboratory to compare lentil protein with ovalbumin, soy protein, and whey protein under exactly the same conditions demonstrated that lentil protein could compete well with those proteins, which are widely recognized for their excellent foaming properties. It is especially interesting to notice that lentil protein possesses superior foaming capacity at mildly acidic pH compared to other plant proteins such as lupine (58%) [16], barley (70%) [90], and soy (91%) [105]. All samples demonstrated high foaming stability regardless of the protein extraction pH or the environmental pH. The *FS* values ranged from 77 to 84%, indicating more than 77% of the formed foams remained stable after standing for 30 min at room temperature. This is superior to other proteins such as barley (40-70%), chickpea, and pea (35-40%) [18, 90]. The capacity of a protein to form foam is related to its ability to reach, adsorb, and unfold rapidly at the liquid-gas interface. Also, a protein must be able to associate through protein-protein interactions and become part of the film to create a foam. The excellent foaming capacity of lentil protein is probably related to its high solubility and unique amino acid composition with balanced hydrophilic and hydrophobic segments and high surface charge. The higher solubility could make the protein chain more flexible, thus diffuse more rapidly in aqueous medium, and then be readily adsorbed at the air–water interface with hydrophilic segments oriented towards the aqueous phase and hydrophobic segments oriented towards the air. The high charge at pH 3.0 produces a large repulsion between proteins on adjacent bubble surfaces, which in turn contributes to a greater lamellar resistance [106].

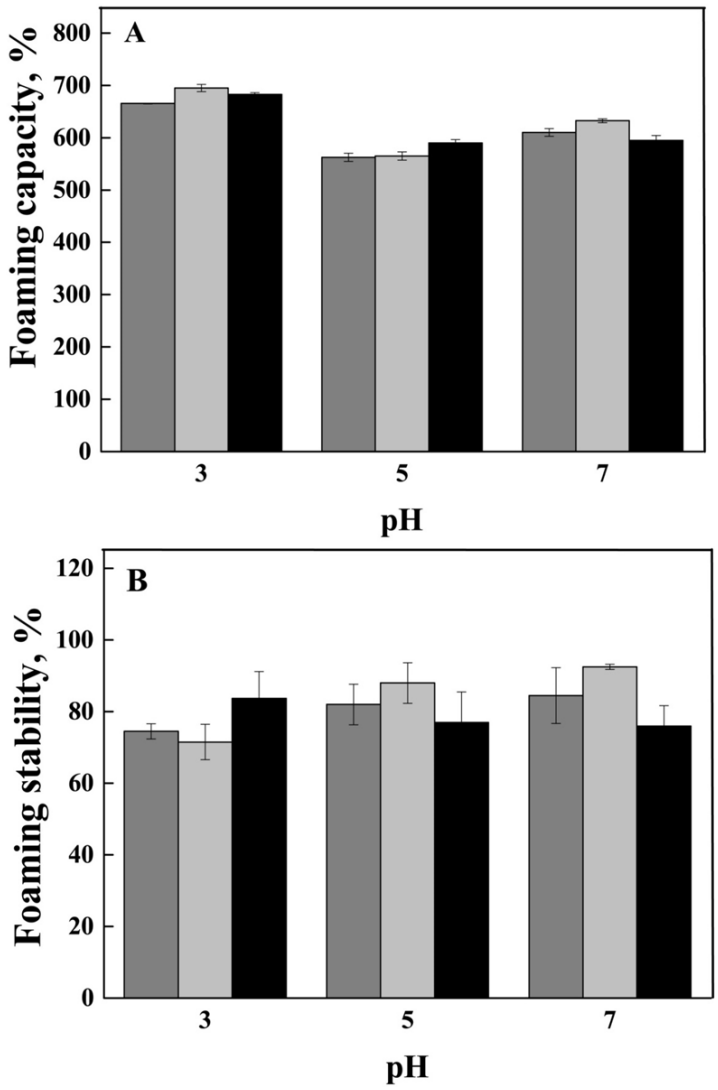


Figure 3.6 Effect of environmental pH on (A) foaming capacity (%) and (B) foaming stability of lentil protein concentrates extracted at pH 8 (gray bar), 9 (light gray bar), and 10 (black bar).

The resistance of the lamella to drain and of the bubbles to collapse is normally responsible for the overall foam stability. The stability of the foams (Figure 3.6 B) was similar in the pH range tested, which means that the interfacial film properties were not pH

dependent. Information about lentil protein molecular structure at air-water interface is limited, so further investigation is needed to better understand the molecular basis of such excellent foaming characteristics.

3.4 Conclusions

A statistical method was used to optimize the extraction of a protein concentrate from lentil seeds and the effect of molecular structure on functionality was systematically interpreted. A solid/solvent ratio of 1:10 (w:v) and pH of 9.0 were found to be optimal for the extraction process to maximize both protein yield and purity, while neither time nor temperature had a significant effect on these two parameters. SE-HPLC chromatograms and SDS-PAGE patterns showed that globulins were the major protein in the extracts. Increasing the extraction pH to 9.0 and 10.0 caused partial protein hydrolysis and unfolding as suggested by SE-HPLC and FTIR data, leading to improved protein solubility and gelling properties. Environmental pH affected protein solubility and surface charge, and subsequently their gelling and foaming properties. Excellent foaming capacity was identified for lentil proteins comparable to that of whey and egg proteins. This superior functionality may provide an opportunity for lentil protein to be used as a foaming agent of plant origin in cakes, dairy products, a variety of desserts and drinks, and especially in mildly acidic foods such as fruit-based products.

CHAPTER 4

Impact of pH on Molecular Structure and Surface Properties of Lentil Legumin-Like Protein and its Application as a Foam Stabilizer[2]

4.1 Introduction

Increasing cost of dairy-based ingredients, emerging dietary preferences (e.g., gluten-free and vegan) and consumer demand for healthier ingredients are leading the market trends towards lower cost and plant-based alternatives, which are gaining increasing market share as food ingredients and for bio-based material applications [107]. In this context, legume proteins are attracting attention because of their potential from both nutrition and health standpoint. In general, legumes are high in protein (20%-25%). The majority of storage proteins in legumes are globulins, which can be classified into two groups. Proteins in the first group have sedimentation coefficients between 10.5S and 13.0S, and are referred to as 'legumin-like' or 11S proteins. The second group has smaller sedimentation coefficients (7.0- 9.0S) and are generally isolated from seed extracts as trimers of glycosylated subunits. This group of proteins is referred to as 'vicilin-like' or 7S proteins [22]. Lentils contain 20.6-31.4% protein with legumin-like protein (~50%) as the major globulin fraction, which is comprised of a number of 6 to 19 polypeptides with a molecular weight (M_w) of 18 to 43 kDa [12].

[2] A version of this chapter has been published in *Colloids and Surface B: Biointerfaces, 132 (2015), 45-53*

Generally, it is accepted that legumin is a hexamer with a M_w of about 320 to 380 kDa, which consists of six polypeptide pairs that interact non-covalently. Each of these polypeptide pairs is comprised of an acidic subunit of about 40 kDa and a basic subunit of about 20 kDa, linked by a single disulfide bond [12]. Unlike other legumes such as soybean and pea, which have been extensively studied [108], there is limited research on the legumin-like protein of lentil except for some report on its sedimentation speed [109], immunological reactivity, composition [12] and functionalities [110, 111]. A fundamental understanding of detailed structural features of lentil legumin and its functionalities is important for its potential food applications.

In the previous study reported in Chapter 3, extraction process parameters were optimized to obtain lentil protein concentrates, and they demonstrated strong foaming capacity and stability, comparable to whey and egg protein. This superior functionality may provide an opportunity for lentil protein to be used as a foaming agent of plant origin for both food and non-food applications. In spite of its high potential, the underlying mechanism of such foaming properties of a plant protein is still unknown. As the major protein in lentil, legumin-like protein component could play an important role contributing to the foaming properties. Thus, this study aims to isolate and purify the legumin-like protein from lentil, and use it as a plant protein model to understand how protein molecular structure (molecular weight, surface charge, hydrophobicity, and conformation) impacts its surface properties (surface tension, dilatational and shear rheology), and subsequently foaming functionality. The generated knowledge may help develop strategies for modification of plant protein structures to improve their functionality for targeted applications. Most of the previous reports have focused on protein surface properties and foaming capacity at neutral pH. The previous study (Chapter 3) revealed that environmental

pH significantly influenced protein physicochemical properties and foaming functionality. Thus, the impact of environmental pH on the structure and properties of the lentil legumin-like protein was also investigated.

4.2 Materials and Methods

4.2.1 Raw materials

Large green lentil produced in Saskatchewan, Canada of mixed varieties (Greenland and Sovereign) was purchased from a local supermarket (Superstore, Edmonton, AB, Canada). The grains were ground into fine flour using a Retsch centrifugal grinding mill with screen aperture size of 0.5 mm (ZM 200, Retsch, Inc., Newtown, PA, USA). The flour was packed in plastic bags, sealed, and stored at 4 ºC until extraction. Standard protein molecule markers for SDS-PAGE (Precision Plus Protein™, M_w 10-250 kDa) and native electrophoresis (NativeMark™, Mw 20-1236 kDa) were purchased from Bio-Rad (Richmond, CA, USA) and Life Technologies (Burlington, ON, Canada), respectively. 1-Anilino-8-naphthalene-sulfonate (ANS) and the standard molecular markers for HPLC analysis (thyroglobulin, 670 kDa; ferritin, 440 kDa; BSA, 67 kDa; ovalbumin, 43 kDa; cytochrome C, 13.6 kDa and aprotinin, 6.5 kDa) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were reagent grade.

4.2.2 Lentil legumin-like protein purification

Lentil protein concentrate (85 g of protein/100 g of concentrate) was obtained as described in Chapter 3. Legumin-like protein was purified from lentil protein concentrate by rate-zonal centrifugation using a sucrose lineal density gradient according to Abe and Davies [112]. Briefly, two sucrose solutions (12 and 60 g/100 mL) were kept overnight at 4 ºC. Then, 13 mL of the 60 g/100 mL sucrose solution was placed into an Optiseal™

centrifuge tube and 13 mL of the 12 g/100 mL sucrose solution was slowly layered on top, so as not to disturb the previous one. Then, the centrifuge tubes were horizontally laid and kept at 4ºC overnight to produce the lineal sucrose gradient. The next day, the tubes were slowly returned to the vertical position and stored for two days at 4 ºC. Meanwhile, a solution of 10 mg of protein concentrate/mL at pH 8 was stirred overnight at 4 ºC. Then, 3 mL of the protein solution was placed on top of the sucrose gradient and centrifuged at 302,000 × g for 4.5 h, using an OptimaXPN-80 ultracentrifuge (Beckman Coulter Canada LP, ON, Canada). Twelve fractions of approximately 2.4 mL each were immediately collected by puncture and elution from the bottom of each tube. The collected fractions were numbered from top to bottom and analyzed by native electrophoresis and SDS-PAGE. The sedimental velocity of the fraction was also calculated, based on rate-zonal centrifugation results. The fractions identified as legumin-like protein were pooled and dialyzed for 96 h (Spectra/Por® 3 standard RCTubing, MWCO 3.5 kDa, Spectrum Laboratories, Inc.) and freeze dried. Then, all freeze dried fractions from different batches were pooled together for further testing. Purity of lentil legumin-like protein was measured using a nitrogen analyzer (FP-428, Leco Corporation, St. Joseph, MI, USA) giving a result of 81 g of protein/100 g of isolate.

4.2.3 Legumin-like protein structures and foaming properties

4.2.3.1 Protein structure characterization

Native electrophoresis and SDS-PAGE under reducing conditions were used to identify legumin-like protein and measure its molecular weight with an Amersham™ ECL™ Gel Box (GE Healthcare Bio-Sciences Corp., Pittsburgh, PA, USA) using pre-cast gel (4–20% gradient) and a constant voltage of 120V. The weight-average molecular weight (M_w) of the

legumin-like protein was determined by size-exclusion high-performance liquid chromatography (SE-HPLC) (Agilent series 1100, Palo Alto, CA, USA) equipped with a Zenix-C SEC-300 size exclusion column (3 µm, 300 A, 7.8x300 mm; Sepax Technologies, Inc., Newark, NJ, USA) at 22 ºC. The flow rate was 0.5 mL/min and the elution was monitored at 280 nm.

The electrophoretic mobility of the extracted legumin-like protein samples under pH 2.0 to 11.0 at 22 ºC was measured using a Zetasizer NanoS (model ZEN1600, Malvern Instruments Ltd., Malvern, UK).

The hydrodynamic size of the legumin-like protein was determined by dynamic light scattering (DLS) using the same Zetasizer Nano ZS particle size analyzer with a backscatter detection angle of 173°.

Solubility was evaluated as reported previously in Chapter 3 (section 3.2.4.1) and expressed as the percentage of the protein in the supernatant compared to the total protein in the extract added into the suspension. The solubility profile was obtained by plotting the average protein solubility (%) against pH values.

Fluorescence intensity (*FI*) was determined for five concentrations of lentil legumin-like protein (0.002-0.010%) and ANS was used as the fluorescence probe. The excitation/emission wavelengths were set at 390 nm and 470 nm, respectively. Net *FI* of each solution (FI_{Net}) was calculated as: (FI_{Net}) = (*FI* of protein dilution blank - *FI* of protein solution with ANS). The initial slope of FI_{Net} versus protein concentration (%, w/v) plot was calculated by linear regression analysis and used as an index (S_o) of the protein surface hydrophobicity.

The infrared spectra were recorded at 22 ºC using a Nicolet 6700 spectrometer (Thermo Scientific, Madison, WI, USA) and Fourier self-deconvolutions were performed

(FTIR) using the software provided with the spectrometer to study the amide I region of the protein. Band narrowing was achieved with a full width at half maximum of 15 cm^{-1} and with a resolution enhancement factor of 2.5 cm^{-1}.

4.2.3.2 Foam morphology

Micrographs of the foam samples were observed with a Hitachi X-650 scanning electron microscope (SEM, Hitachi, Japan) at an acceleration voltage of 6 kV. Foams were frozen in liquid nitrogen immediately after preparation and freeze-drying. Then they were sputtered with gold for 2 min before SEM observation.

4.2.3.3 Foaming properties

Protein extract samples (1 g/mL) were dispersed in 30 mL of deionized water adjusted to pH 3.0, 5.0, or 7.0 using either 0.5 M NaOH or 0.5 M HCl solutions. The solution was mixed with a homogenizer (PowerGen 1000, Fisher Scientific, Fairlawn, NJ, USA). The foaming capacity (*FC*) was calculated as: $FC = (V_{f1} - V_{f0})/V_{f0} \times 100\%$, where V_{f0} and V_{f1} represent the volume of the protein solution and the formed foams after homogenization, respectively. As an index of foam stability (FS), a "mean life" τ value was determined according to equations 4.1 - 4.3 [113]. The larger the τ value, the longer the foam will last.

$$H(t) = H(0)exp^{-\lambda t} \qquad \text{eq. (4.1)}$$

where *H(0)* is the initial foam height at time *t*=0, *H(t)* is the foam height at time t, and λ is the decay constant, which is a measure of foam decay. This exponential relationship can be converted to a linear equation by taking the natural logarithm of both sides, so that the

slope of a plot of ln($H(t)$) vs time would correspond to $-\lambda$. τ is the inverse of the decay constant of a foam system.

$$ln[H(t)] = ln[H(0)] - \lambda t \quad \text{eq. (4.2)}$$

$$\frac{d[ln(H)]}{dt} = -\lambda = \frac{1}{\tau} \quad \text{eq. (4.3)}$$

4.2.3.4 Surface properties

i. Shear rheology measurement

Surface phenomena are dependent on the bulk protein concentration [114], thus protein solutions of three different concentrations (10, 1 and 0.1 mg/mL) were prepared at pH 3.0, 5.0, and 7.0. Interfacial rheology of protein solutions at air-water interface were measured by means of a DHR3 rheometer (AT Instruments-Waters LLC, New Castle, DE, USA) using a Platinum/Iridium Du Noüy ring geometry (10 mm diameter). Strain sweep tests with strain amplitudes ranging from 0.01% to 100% were performed at 0.6283 rad/s in order to establish the linear viscoelasticity range. Then, frequency sweep measurements (0.1 to 100 rad/s) were carried out at a strain amplitude of 0.1%; a value smaller than the critical value for linear viscoelasticity. The storage modulus *G'* and loss modulus *G"* were recorded as a function of time during time sweep tests for up to 6.5 h at an oscillation frequency of 1 Hz and strain of 0.1%. The temperature of all the interfacial rheology tests was fixed at 22 ºC.

ii. Surface tension and dilatational rheology measurements

The surface tension of protein solutions was measured by the pendant drop technique with an automatic drop tensiometer (Model 400, Ramè-Hart Instrument Co., Succasunna,

NJ, USA) at 22 ºC [37]. The drop profile was fitted to the Young-Laplace capillarity equation to obtain the surface tension (γ). The dilatational rheological properties were measured with the same tensiometer at 22 ºC using an oscillation with volume amplitude of 5% at a frequency of 0.1 Hz [115]. The measured change in surface tension during a surface area change over a sequence of four sinuses was used to determine the dilatational modulus. Each sample was measured at 22 ºC in duplicate. The DROPimage Advanced software (Ramé-Hart Inc., Mountain Lakes, NJ, USA) was used for data collection and analysis. The surface dilatational modulus, *E,* is defined in equation 4 and is the change in surface tension (γ) upon a small change in surface area (A) of a constant shape [116], and the surface dilatational elastic and loss moduli *E'* and *E''* are correlated with the dilatational modulus *E* of the interface by equation 4.5.

$$E = \frac{d\gamma}{\left(dA/A\right)} = -\frac{d\pi}{\ln A} \qquad \text{eq. (4.4)}$$

$$E = \left(\gamma_0/A_0\right)(\cos\phi + i\sin\phi) = E' + iE'' \qquad \text{eq. (4.5)}$$

where, π is the surface pressure, γ_0 and A_0 are the strain and stress amplitudes, respectively, and ϕ is the phase angle between stress and strain. The ratio (γ_0 / A_0) is the absolute modulus $|E|$, a measure of dilatational resistance to deformation [116].

4.2.3.5 Statistical analysis

All samples were prepared and tested in triplicate and results are presented as Mean ± SD. One way or two way analysis of variance (ANOVA) was carried out using Origin 9.1 software (Origin Lab Corporation, Northampton, MA, USA), and statistical differences among sample means were determined using Tukey's test at 95% confidence level.

4.4 Results and Discussion

4.4.1 Lentil legumin-like protein structures

Rate-zonal centrifugation of lentil legumin-like protein resulted in a sedimental velocity of 13S. Native electrophoresis result (Figure 4.1 A, lane N) shows a band with a molecular weight between 346 and 426 kDa. Typically, legumin hexamers consist of six subunits of approximately 60 kDa that interact non-covalently, each one comprised of an acidic subunit (α) of around 40 kDa and a basic subunit (β) of approximately 20 kDa, linked by a single disulfide bond. SDS-PAGE of lentil legumin-like protein under reducing conditions (Figure 4.1 A, lane F) shows three bands with relative molecular weights of 32, 42, and 47 kDa, respectively, corresponding to the acidic polypeptide chains. The other two bands at 18 and 20 kDa correspond to the two basic polypeptide chains. Similar legumin subunits were previously identified in lentils [12], peas, soybeans and fava beans [117]. Aydemir and Yemenicioğlu [111] identified bands between 15 and 20 kDa, 30 and 40 kDa, and 40 and 70 kDa for the crude lentil protein using SDS-PAGE, whereas, for the isoelectric precipitated extract they found intensive spots of protein between 21 to 23 kDa and around 26 kDa, and less intense spots above 30 kDa. The protein bands identified in this work for lentil legumin-like protein are more similar to the crude protein extract prepared by Aydemir and Yemenicioğlu [111]. Figure 4-1 B shows the SE-HPLC chromatogram of the lentil legumin-like protein. The sample possesses one major peak with a weight-average molecular weight of 347 kDa. This result is in good agreement with that observed in the SDS-PAGE pattern. Small peaks appearing in the chromatogram might be identified as small amounts of 7S and 3S proteins present in the sample [118].

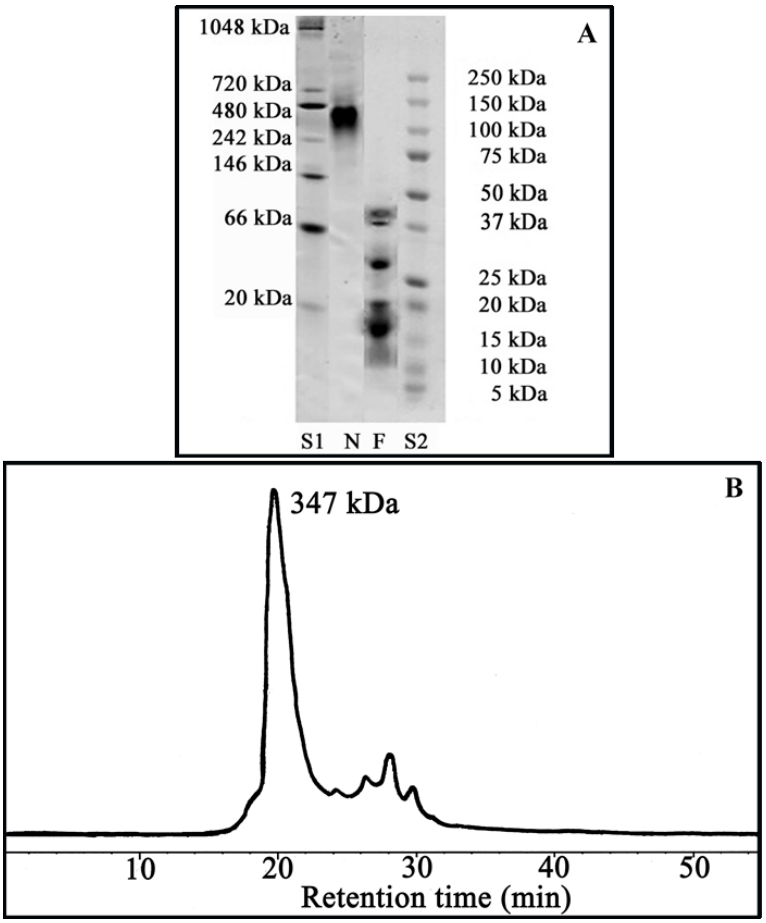


Figure 4.1 (A) Lentil legumin-like protein under (N) native gel and (F) SDS-PAGE gel conditions. S1 and S2 are protein molecular standards in native and SDS-PAGE gel, respectively. **(B)** SE-HPLC chromatogram of lentil legumin-like globulin enriched fraction.

The amino acid composition analysis (methodology and results provided in Table A2-1, Appendix A2) revealed that, similar to most legume globulin proteins, lentil legumin-like protein is rich in glutamine/glutamic acid, asparagine/aspartic acid (about 26% in total) and glycine (9%), but poor in sulfur containing amino acids (1%), methionine and cysteine. This protein exhibited balanced hydrophilic and hydrophobic segments with ~40% hydrophobic residues including alanine, valine, isoleucine, leucine, phenylalanine and

proline, and ~38% hydrophilic residues including glutamine/glutamic acid, asparagine/aspartic acid, lysine and arginine.

As shown in Figure 4.2 A, lentil legumin-like protein has an isoelectric point (*pI*) around 4.6. The highest charge reached +38 mV at pH 2.0 and -28 mV at alkaline pH from 8.0 to 11.0. The hydrodynamic size distribution of lentil legumin protein at different pH levels is shown in Figure 4.2 B. The protein sample presented monomodal size distributions at both pH values: 3.0 and 5.0, with a peak at 7 nm and 120 nm, respectively. While a bimodal distribution with two peaks at 12 and 40 nm was observed at pH 7.0. According to Ruiz-Henestrosa et al. [37], estimated size of glycinin assembled forms 3S and 11S are 6.8 and 14.6 nm, respectively. Dissociation of the 13S into 7S or 3S was observed in pea legumin, soy glycinin, and lupine legumin [96]. Thus, the hydrodynamic size observation suggests that lentil legumin-like protein was dissociated from 13S into 3S form at pH 3.0. At pH 5.0, the charge of lentil legumin-like protein is close to 0 because of the proximity to its isoelectric point. In the presence of minimum electrostatic repulsion, protein aggregation could occur, leading to increased size. At pH 7.0, the major peak represents 13S protein, while the smaller peak represents small aggregates. The presence of some aggregates at pH 7.0, but not at pH 3.0 may be related to the lower net surface charge (-25 mV compared to +35 mV). These small aggregates might not be detected during SE-HPLC analysis due to M_w exclusion limit of the column and the presence of a guard column.

As shown in Figure 4.3 A, the surface hydrophobicity was the lowest at pH 5.0 (S_0 value of 1200), but dramatically increased at pH 3.0 and 7.0 with S_0 values of 5200 and 2200, respectively.

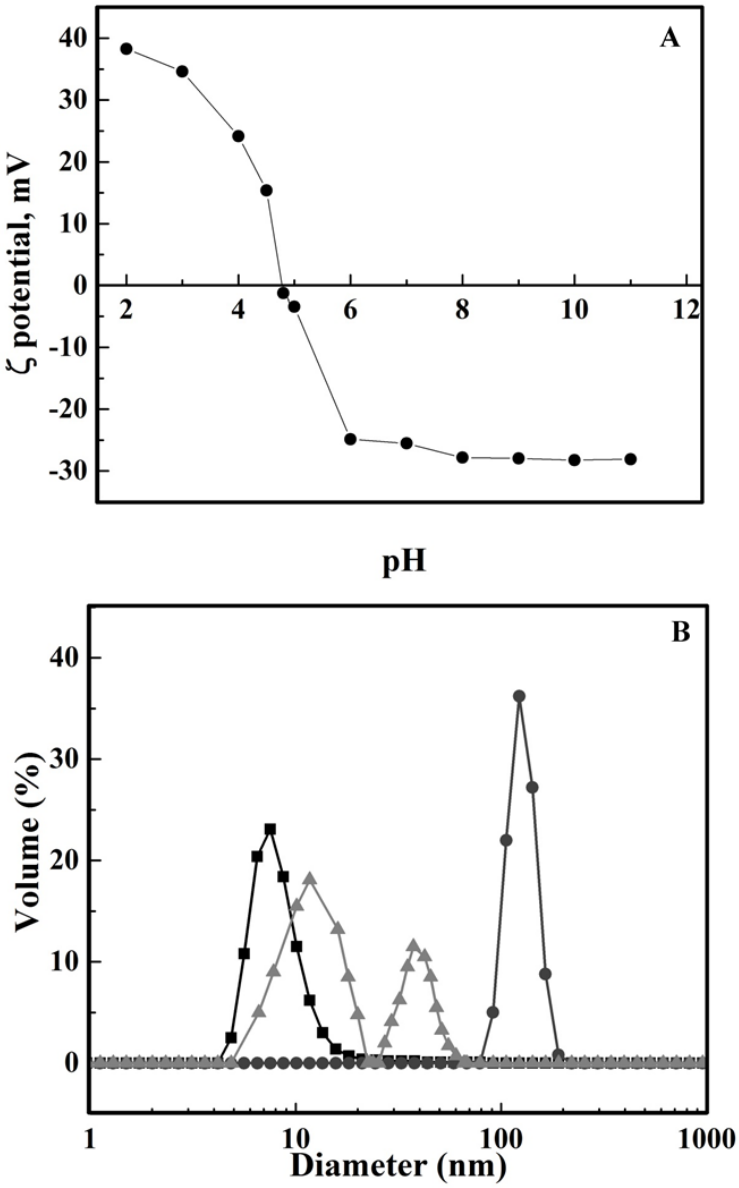


Figure 4.2 (A) ζ-Potential of lentil legumin-like protein as a function of pH. Values are reported as the mean of 3 samples ± standard error of the mean. **(B)** Molecular size of lentil legumin-like protein in solution at pH 3.0 (square), pH 5.0 (circle), and pH 7.0 (triangle).

The S_0 values determined by ANS are higher than those reported for other legumins such as pea and soybean, which had S_0 values from 94 to 2000 [29]. Higher surface hydrophobicity of lentil legumin-like protein might be attributed to a higher exposure of aromatic and aliphatic amino acid residues. Lower S_0 values were observed at pH 5.0

because protein tended to aggregate when pH was near its pI, thus hydrophobic groups become hidden inside the aggregates and less accessible to ANS. Higher S_0 value observed at pH 3.0 than that at pH 7.0 can be related to dissociation of legumin-like proteins at pH 3.0 to expose more hydrophobic regions [119]. In addition, ANS as an anionic probe could interact with positively charged sites on the proteins at low pH, thus resulting in overestimation of hydrophobicity to a certain extent [120].

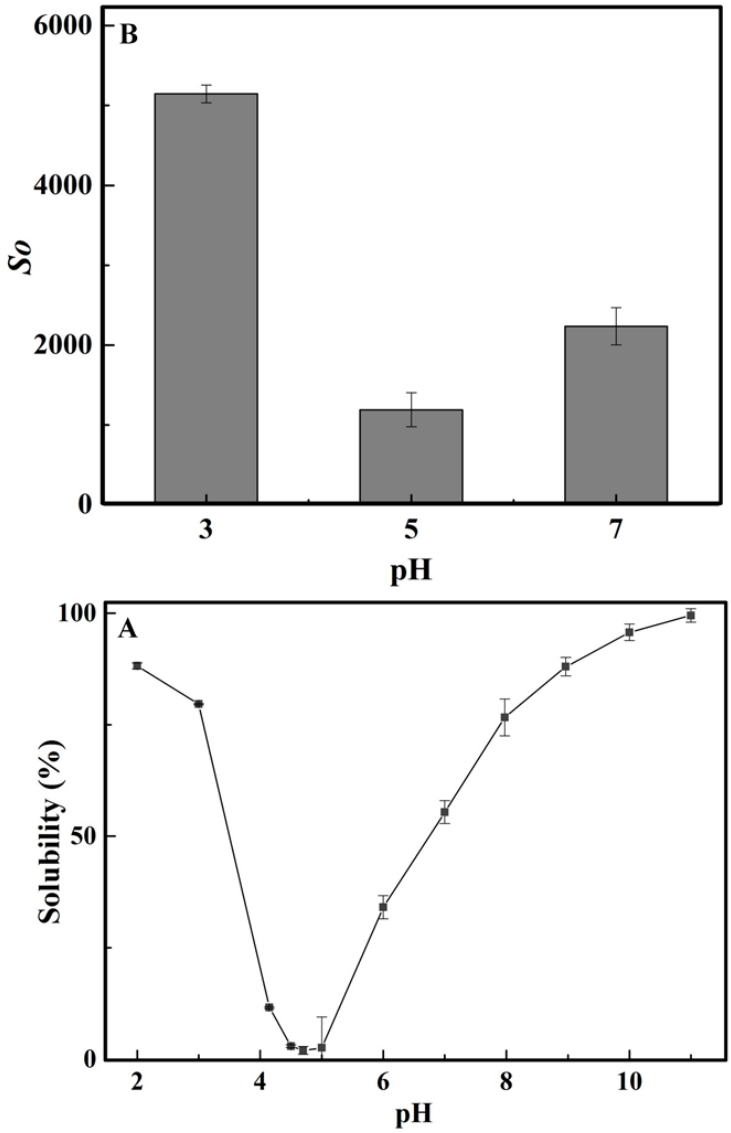


Figure 4.3 (A) Surface hydrophobicity index (S_0). **(B)** Solubility of lentil legumin-like protein as a function of pH. Values are reported as the mean of 3 samples ± standard error of the mean.

Lentil legumin-like protein showed minimum solubility around 4.7 (near pI), with protein solubility increasing dramatically when pH deviated from the pI. Relatively high solubility was observed at pH 2 (above 85%) and above pH 8 (70-95%) (Figure 4-3 B), which is favorable for its potential applications. It is interesting to notice that lentil legumin-like protein possesses both high surface hydrophobicity and solubility when pH was deviated from protein *pI*, thus it is likely that a balanced level of hydrophilic and hydrophobic amino acid residues are exposed at the protein surface.

4.4.2 Foaming properties

The foaming capacity (*FC*) as a function of pH is presented in Figure 4.4 A. The *FC* is high at all pH levels, ranging from 403 to 425%, which is better than that reported for soy and pea 11S protein in a similar pH range [37]. The *FC* value of the lentil protein extract found in the previous study was higher (Chapter 3, section 3.3.3.3), likely due to the synergistic effect of legumin-like lentil protein and the other protein components present in the lentil grain, especially vicilin-like protein [29]. Figure 4.4 A insert shows the fitting of the experimental data of natural logarithm of foam height versus time for the lentil legumin-like protein. From these plots, the mean lifetime (τ) was calculated to give values of 25.5, 50.7, and 84.1 min at pH 3.0, 5.0, and 7.0, respectively (Figure 4.4 A). A foam can decay smoothly or collapse very fast. Foams formed at pH 5.0 and 7.0, which is the common pH range of most food and non-food applications, can be classified as long-life foams, which normally show decay τ value of 50 min or higher [121]. Long-life foams are particularly useful in the food industry when products must be processed for long periods and it is important to keep the aerated structure before solidifying or gelling (e.g. mousse and ice cream).

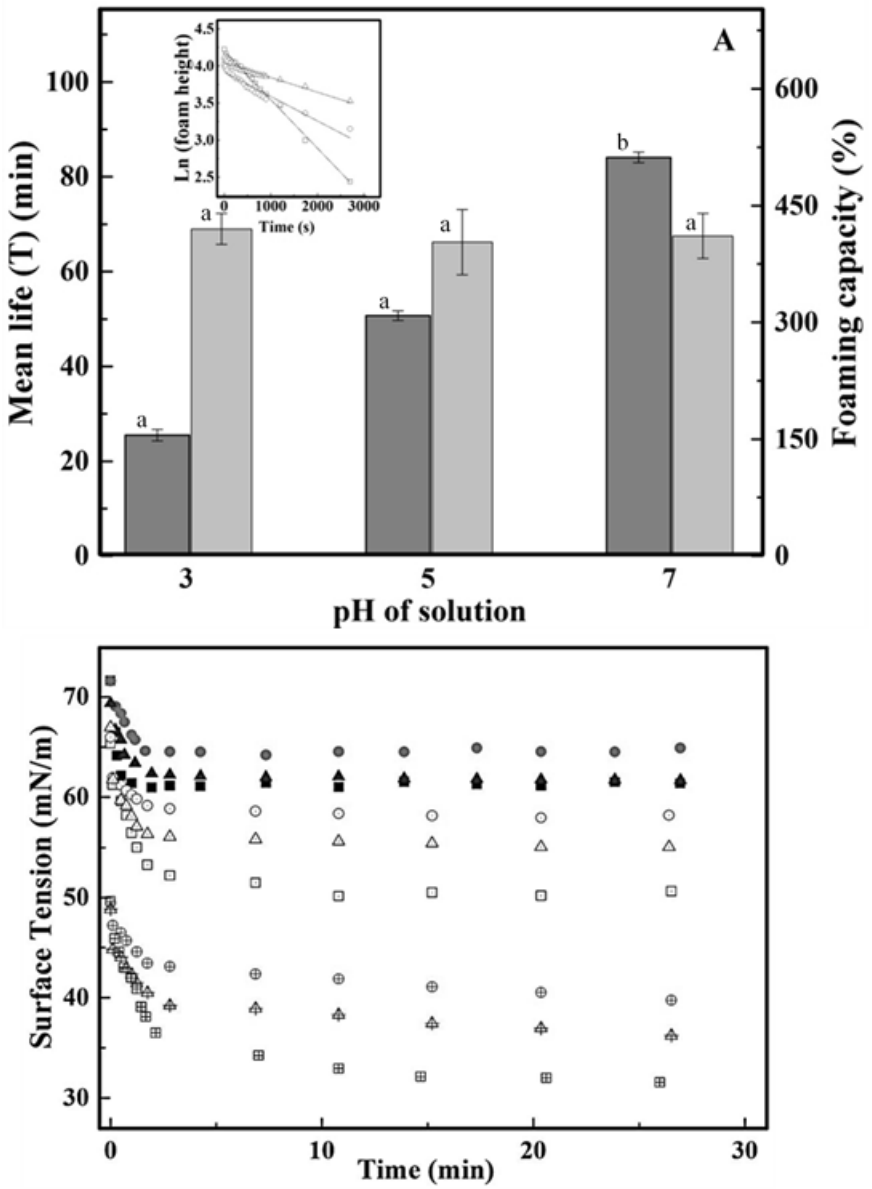


Figure 4.4 (A) Mean life (dark gray bars) and foaming capacity (light gray bars) values of foams stabilized by lentil legumin-like protein at pH 3.0, 5.0, and 7.0. The insert shows the plot of natural logarithm of foam height at pH 3.0 (squares), 5.0 (circles), and 7.0 (triangles) versus time which was used to calculate mean life. The fitting of the models (R^2) was higher than 0.92 in all cases. Bars without a common letter have a significant difference ($p < 0.05$). **(B)** Surface tension of lentil legumin-like protein versus time in solutions at pH 3.0 (square), pH 5.0 (circle), and pH 7.0 (triangle) (solid symbol: 0.1mg/mL, open symbol: 1 mg/mL, crossed symbol: 10 mg/mL)

4.4.3 Impact of protein structure on surface properties

The good foaming properties of lentil legumin-like protein observed above led to the investigation of how protein molecular structure influences surface phenomena and the subsequent foaming properties.

4.4.3.1 Surface tension

Reducing surface tension involves proteins migrating to the air-water interface and forming a layer or a film by adsorbing and re-orienting themselves. Increasing the bulk concentration until a certain concentration of protein will speed the decay of surface tension because of the higher number of molecules that transport and adsorb to the interface [39]. The decrease in the surface tension with increasing concentration of protein was also observed by Tomczynska-Mleko et al. [122] in whey protein isolate. At higher values (6–11%) the whey protein concentration did not affect the change in the surface tension, likely due to a complete coverage of the surface by proteins at that point [122]. This is also observed in lentil legumin-like protein, as shown in Figure A2.1 (Appendix A2). Critical micelle concentration (cmc) of lentil legumin-like protein is 5 mg/mL, above which no further decrease is observed in the static surface tension (Figure A2.1). In addition, the rate of surface tension decay will increase because of increased diffusion and adsorption of the molecules. As diffusion is inversely proportional to the cube root of the molecular weight, smaller molecules will move and adsorb faster to the interface compared to the larger molecules [116]. In addition, proteins with a flexible structure and higher surface hydrophobicity possess more capacity of lowering the surface tension because they can strongly adsorb at the interface [123]. As shown in Figure 4.4 B, higher protein concentration led to lower values of surface tension.

The capacity for lentil legumin-like protein to reduce surface tension follows the sequence of pH 3.0 > pH 7.0 > pH 5.0. This correlates well with previously characterized protein structures at different pH levels. Lentil legumin-like protein showed smaller size, higher solubility, and surface hydrophobicity at pH 3.0 than pH 7.0 and thus could migrate and adsorb onto the water/air interface more rapidly at acidic pH than at neutral pH. On the other hand, large size, low solubility, and surface hydrophobicity at pH 5.0 resulted in a relatively low surface activity. Lentil legumin-like protein showed a similar capacity for decreasing the surface tension when compared to β-lactoglobulin and soy glycinin at pH 7.0 [37]. A study of the pH impact on the surface tension of the whey protein isolate by Tomczynska-Mleko et al. [122] also showed a better capacity of reducing surface tension at pH 3.0 than at other pH values, but a lower surface activity at pH 7.0 than lentil legumin-like protein. Since surface tension is not only related to the electric charge of the protein but also other physical-chemical characteristics of the protein, differences between whey protein and lentil legumin-like protein are likely related to the latter.

4.4.3.2 Surface rheology

Surface shear rheological parameters are sensitive to surface composition and protein-protein interactions [40], and normally higher values of storage modulus (*G'*) indicate stronger interactions. As shown in Figure 4.5 A, higher protein concentration led to higher *G'* values ($p<0.05$) due to more intermolecular interactions and strengthening of non-covalent bonds within an adsorbed layer of proteins [40]. During the whole test period *G'* was 10 fold higher than *G"* (data for G" not shown). This result indicates that legumin-like lentil protein was able to form an elastic network at the water/air interface at all the pH levels tested, which plays an important role contributing to the foam stability as an elastic

film and can better resist deformation and also recover its natural shape when the deforming force is removed.

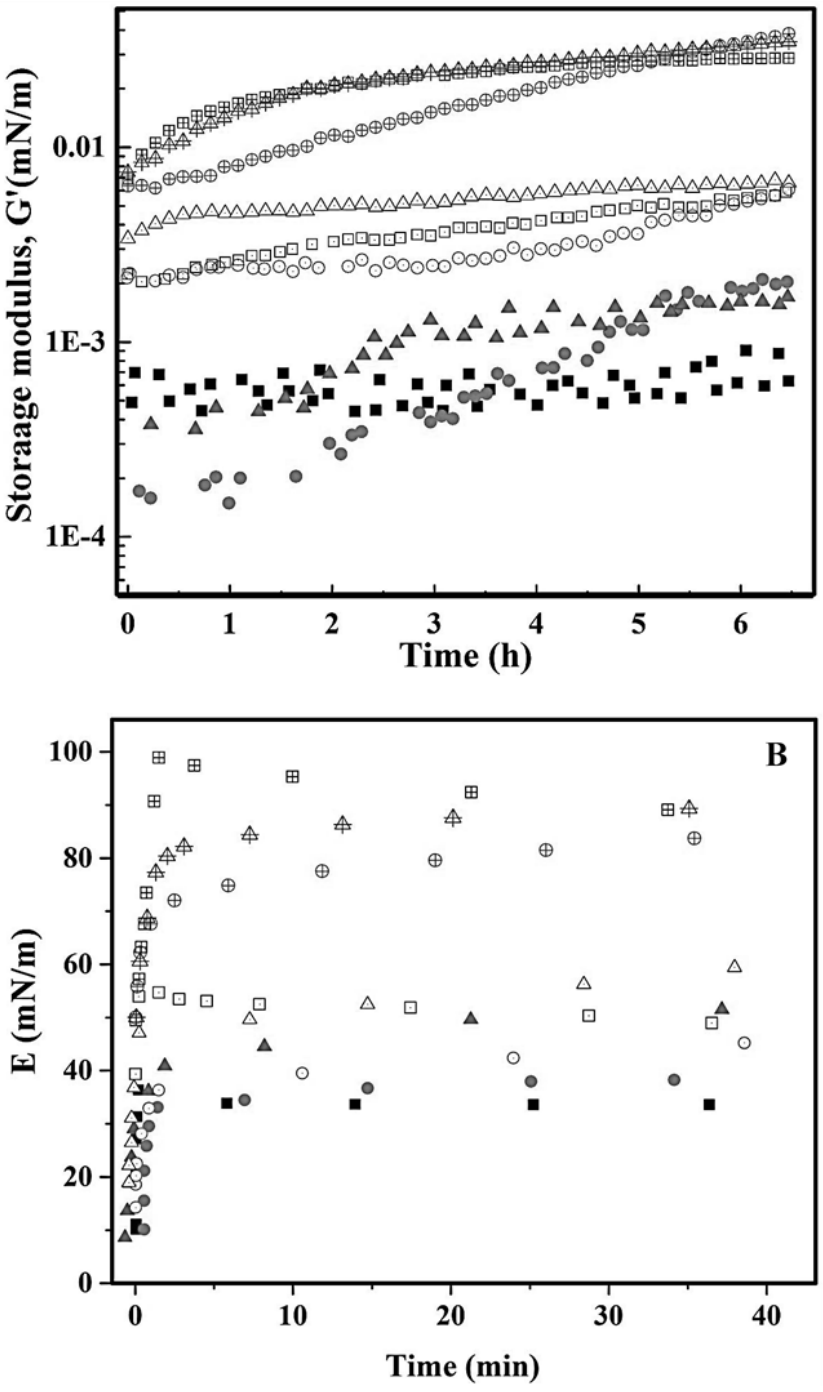


Figure 4.5 (A) Shear storage modulus (G') and **(B)** dilatational modulus (E) of lentil legumin-like protein versus time in solutions at pH 3.0 (square), pH 5.0 (circle), and pH 7.0 (triangle)(solid symbol: 0.1mg/mL, open symbol: 1 mg/mL, crossed symbol: 10 mg/mL)

The *G'* value increased more rapidly at pH 3.0 and 7.0 especially at 10 mg/mL due to the fast protein diffusion and adsorption because of the higher solubility and smaller hydrodynamic diameter. In addition, their higher surface hydrophobicity could facilitate protein adsorption at the air/water interface. A different kinetic of adsorption was observed at pH 5.0, where lag times (initial time where no change in the surface properties was observed) were indicative of slower diffusion of the protein molecules to the interface and hindered adsorption due to the low molecular flexibility and susceptibility to conformational changes due to aggregation [37]. It is noticed that the *G'* values at pH 5 still showed an increasing trend even after 6 h at all the pH levels tested. This is generally related to protein multilayer building at the interface, which might in part explain the high stability of the foam [124]. The end values of *G'* were higher at pH 5.0 and 7.0 at the protein concentrations of 10 and 0.1 mg/mL and similar at different pHs when concentration was 1.0 mg/mL.

The evolution of the surface dilatational modulus (*E*) with time is shown in Figure 4.5 B. E value is the result of the intra-protein flexibility and the aggregated inter-protein network strength [125]. It is also correlated with diffusion and adsorption of protein molecules toward the interface [116]. At pH 3.0 a faster network building generated higher values of elasticity at short time (<2 min) due to higher protein diffusion. Though the dilatational elasticity of the surface layer at pH 7.0 and 5.0 did not reach the same level as that at pH 3.0 at the initial stage, they became similar in the relatively long time period (>10 min). The decayed E value suggests that weaker elastic protein networks could be maintained at the air-water interface at pH 3.0.

4.4.3.3 Kinetic study

At surface pressure values where diffusion process is the rate-determining step of protein adsorption at the interface, a modified form of the Ward and Torday equation can be used to calculate the diffusion coefficient [37]:

$$\pi = 2C_0KT\left(\frac{Dt}{3.14}\right)^{1/2} \qquad \text{eq. (4.6)}$$

where π is the surface pressure calculated as the change in surface tension compared to that for water, C_0 is the protein concentration in solution, D is the diffusion coefficient or diffusivity, K is the Boltzmann constant, T is the absolute temperature, and t is the time. Then, a plot of π vs $t^{1/2}$ should be linear with a slope equal to the diffusion rate constant (k_{diff}). The plots for all the samples gave a straight line at the initial stage (data not shown). As shown in Table 4.1, k_{diff} is higher at higher protein concentration in the bulk phase and the value for k_{diff} decreases in the sequence pH 3.0>pH 7.0>pH 5.0 as k_{diff} increases as the hydrodynamic size decreases according to the Stokes-Einstein equation.

After the diffusion control step, the protein adsorption kinetic can be evaluated using the first-order rate equation [37, 116]:

$$ln\left[\frac{\pi_\infty - \pi_t}{\pi_\infty - \pi_0}\right] = -kt \qquad \text{eq. (4.7)}$$

where π_∞, π_t, and π_0 are the surface pressures at the steady or equilibrium state, at any time t, and at $t = 0$, respectively, and k is the first-order rate constant. The plot of ln [($\pi_\infty - \pi_t$) / ($\pi_\infty - \pi_0$)] versus t is characterized by two regions: the first one corresponding to the adsorption period (or penetration of the interface), and the second one for the

rearrangement period, each one characterized by its own constant, k_{adsp} and k_{reag} [116]. Adsorption is favored by exposition of hydrophobic residues [41], so that protein molecules with higher surface hydrophobicity, as at pH 3.0 and 7.0 (Figure 4-3 A) exhibited higher adsorption rate (k_{adsp}).

Table 4.1 Characteristic interfacial parameters for the diffusion, adsorption, and rearrangement at the air-water interface.

pH	Concentration, mg/mL	k_{diff}, (mN m^{-1} s$^{-0.5}$)^{A}	k_{adsp}x10^{-5}, (s^{-1})^{A}	k_{reag}x10^{-5}, (s^{-1})^{A}
3	0.1	0.12±0.01^{a,x}	2.01±0.6^{a,x}	8.16±0.8^{a,x}
5	0.1	0.07±0.02^{a,y}	1.06±0.1^{a,y}	10.4±0.5^{a,y}
7	0.1	0.09±0.04^{a,z}	1.99±0.5^{a,x}	12.3±0.7^{a,y}
3	1.0	0.24±0.02^{b,x}	2.80±0.2^{b,x}	9.62±0.3^{b,x}
5	1.0	0.20±0.03^{b,y}	2.23±0.3^{b,y}	15.9±0.2^{b,y}
7	1.0	0.21±0.06^{b,z}	.3.01±0.3^{b,x}	18.8±0.1^{b,y}
3	10	0.26±0.02^{c,x}	2.94±0.1^{b,x}	17.7±0.6^{c,x}
5	10	0.21±0.05^{c,y}	2.45±0.1^{b,y}	21.6±0.3^{c,y}
7	10	0.24±0.06^{c,z}	3.19±0.2^{b,x}	27.3±0.9^{c,y}

Linear regression coefficient are all higher than 0.91.
^{A}: Values are presented as mean ± SD based on three measurements.
^{a,b,c}: Indicate significant differences among samples due to concentration ($p < 0.05$).
^{x,y,z}: Indicate significant differences among samples due to pH ($p < 0.05$).

Smaller protein size (less aggregates) also contributed to protein adsorption at the interface. After the protein has been adsorbed, the rearrangement process takes precedence. According to Beverung et al. [126], in the rearrangement process, proteins continue to slowly change their conformation and build a viscoelastic interfacial film. Additional layers might be added onto the monolayer interface when conformational changes of adsorbed proteins provide a favorable environment for sublayer proteins to interact with the adsorbed molecules. As shown in Table 4.1, high k_{reag} values at pH 7.0 and pH 5.0 were observed

compared to those at pH 3.0. Considering the fact that long-life foams were obtained at pH 7.0 and 5.0 (Figure 4.4 A), the rearrangement process is likely to play a major role contributing to the formation of a stronger surface network, leading to improved foam stability. In order to have a better understanding of this system, protein molecular rearrangement at the interface was investigated using FTIR in the next step.

4.4.4 FTIR spectra of protein in solution and at the foam surface

Figure 4.6 A shows the FTIR spectra in the amide I region of the legumin-like protein prepared at pH 3.0, 5.0, and 7.0. Beta-sheet (1632 cm^{-1}) and β-turn (1660 cm^{-1}) structures are the major secondary structures for lentil legumin-like protein at all pH levels tested. This is typical for most of legumins, which indicates that about two-thirds of the amino acid residues in each of the subunits are involved in the β-sheet and turn conformations [22]. The fact that lentil legumin-like protein is rich in Asp, Asn, and Gly (Table A2-1, Appendix A2) might explain these structures, since the presence of a glycine residue close to the Asn/Asp residue provides additional flexibility in the main chain that enables the turn structures [127]. Beta-structures can be amphipathic with alternating hydrophobic and hydrophilic residues, which creates a hydrophobic and a hydrophilic segment [127]. Even when most of hydrophobic residues might be buried in the core of the globular protein, some bulky ones, such as phenylalanine and tyrosine, could stay on the surface, creating hydrophobic patches and increasing the surface hydrophobicity of the molecule [128]. When pH was decreased from 7.0 to 3.0, the portion of β-sheets (1628 to 1632 cm^{-1}) decreased to a certain extent, probably induced by charge changes on protein molecular chains.

FTIR was also applied to study the protein conformational changes when adsorbed at the interface and the spectra are shown in Figure 4.6 B. Compared to the lentil legumin-like protein solutions, a major peak at around 1651 cm^{-1} that corresponds to α-helix can be identified at pH 7.0, along with β-sheets (1634 and 1683 cm^{-1}) and β-turns (1670 cm^{-1}).

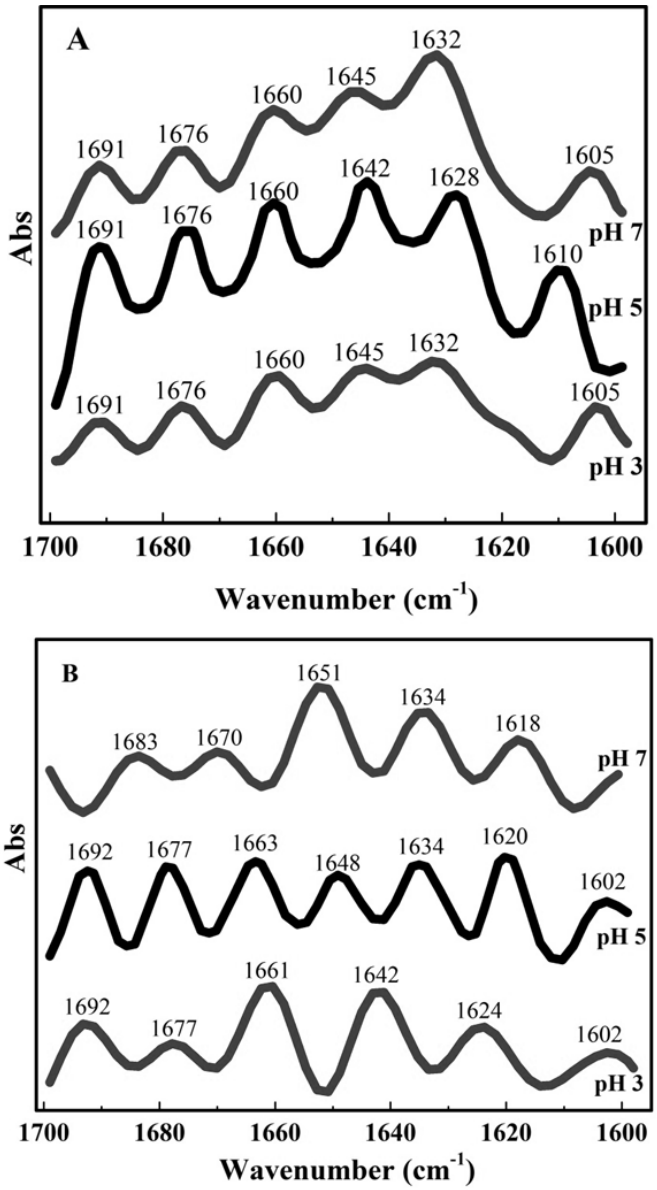


Figure 4.6 Fourier-deconvoluted FTIR spectra of lentil legumin-like protein in solution **(A)** and at the foam surface **(B)** at pH 3.0, 5.0, and 7.0

The absorption at 1618 cm^{-1} suggests formation of protein aggregates at the interface. At pH 5.0, a strong aggregation is observed (1620 cm^{-1}), as well as β-sheets (1634 cm^{-1}) and β-turns (1663 and 1677 cm^{-1}). At pH 3.0, the adsorbed protein is characterized by unordered structures (1642 cm^{-1}), β-turns (1661 and 1677 cm^{-1}), and some aggregates (1624 cm^{-1}).

Based on these spectra, it is suggested that lentil legumin-like protein conformation changed when adsorbed at the interface, depending on the environmental pH. At pH 7.0, lentil legumin-like protein formed a α-helix structure at the interface. The presence of α-helix structure could contribute to the formation of more stable interfaces and multilayer interfaces, likely due to the possible creation of more non-covalent intramolecular interactions. α-Helices could be adsorbed with their long axis parallel to the air/liquid interface, which would allow the side chains that protrude to interdigitate, so α-helices would be packaged side by side with hydrophilic side chains extending towards the aqueous phase and hydrophobic side chains penetrating into the hydrophobic part of the interface [129]. At pH 5.0, the slower protein adsorption rate together with the minimum electrostatic repulsion allowed lentil legumin-like protein to build a strong aggregated multilayer interface that helped the foam to resist collapse. The presence of β-structures could contribute to strong interfacial network building because of its rigidity [46]. These molecular structures could partially explain the high stability of lentil legumin-like protein stabilized foams at pH 5.0 and 7.0 (Figure 4.4 A). On the other hand at pH 3.0, unordered structures increased intra-protein flexibility, producing a less compact structure and relaxed interface that reduces the elasticity modulus [125]. Consequently, the foam would have a lower resistance against collapse.

Tomczynska-Mleko et al. [122] studied the effect of pH on the structure of whey protein isolate in relation to its surface activity. They observed that partial unfolding of proteins associated with a reduction in the number of α-helix structures and an increasing number of disordered structures, causes a decrease in the surface tension of the protein solutions, likely due to a more flexible protein structure that can increase the adsorption at the interface.

4.4.5 Foam morphology

Scanning electron microscopy (SEM) observation of foams prepared at pH 3.0 showed a less compact structure (insert of Figure 4.7 A) and it seems the overall foam structure collapsed after freeze drying. Foam morphology at pH 5.0 (insert of Figure 4.7 B) exhibits a particulate morphology characteristic of protein networks formed at pH near its pI. Such network is composed of randomly aggregated protein particles forming a thick and dense interfacial film. The foam prepared at pH 7.0 shows a dense and almost homogeneous network where holes left by bubbles are clearly visible (Figure 4.7 C), suggesting a strong interfacial network was formed, which could well maintain shape and structure during the freeze-drying process.

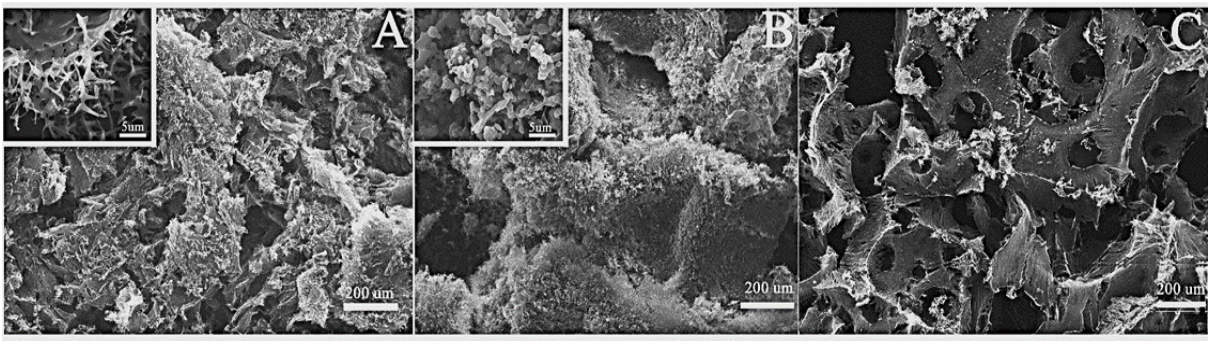


Figure 4.7 SEM micrographs of foams produced from lentil legumin-like protein at (A) pH 3.0, (B) pH 5.0, and (C) pH 7.0.

4.5 Conclusions

This study revealed the high foaming capacity of lentil legumin-like protein and long-life foams were obtained at pH 5.0 and 7.0. Evaluation of the foaming kinetics and protein conformation suggested that the foaming stability of lentil legumin-like protein was dependent on the conformation of the protein at the air-water interface that strongly affected adsorption and re-organization of the protein layer at the interface. Foams prepared at pH 7.0 showed dense and strong networks at the interface, where combination of the α-helix secondary structure, medium hydrodynamic molecular size, and balance between solubility/hydrophobicity all contributed to building of strong protein networks at the interface. At pH 5.0, the protein formed dense and thick interface network composed of randomly aggregated protein particles. At pH 3.0, the smaller hydrodynamic size and high surface hydrophobicity led to the formation of an initially elastic surface layer. However, the unordered structure increased intra-protein flexibility producing a less compact structure and relaxed interface that reduces elasticity modulus with time. Consequently, the foam would have a lower resistance against collapse. This research also provides support information for potential use of lentil protein as a foaming ingredient in food and non-food products where long-life foams are required.

CHAPTER 5

Stability Mechanisms of Lentil Legumin-Like Protein and Polysaccharide Foams

5.1 Introduction

Formation and stabilization of foams are to a large extent determined by the properties of the compounds they contain at the interface. The presence of proteins empowers these properties by a combination of its surface activity and electrostatic and steric mechanisms [34]. Protein foam properties can be improved by the addition of polysaccharides, and the foam stability depends on the interactions between these two kinds of biopolymers and the ability of their mixture to retard gravity-induced drainage by controlling the rheology and network structure of the continuous phase [49, 130]. Complex coacervation, miscibility, and segregation are the possible phenomena that arise in aqueous solutions of protein and polysaccharide mixtures [130]. If the pH of the medium is reduced to below the isoelectric point (p*I*) of the protein, complex coacervation occurs as a result of net electrostatic attractive interactions between the biopolymers carrying opposite charges and implies the separation of two phases, one rich in the complexed biopolymers and the other phase depleted in both. Above the isoelectric point, because of the repulsive electrostatic interactions and different affinities towards the solvent, thermodynamic incompatibility between the polymers occurs. Above a critical concentration and/or at high ionic strength, protein and polysaccharide may segregate into different phases. However, if the concentration is diluted, they could co-exist in a single phase (miscibility) in domains in which they mutually exclude one another [49].

As mentioned before in Chapter 1 (section 1.1.3), lentil is a rich source of nutritional factors, including protein [10, 8], whose consumption has been associated with several health benefits [10]. In the previous study reported in Chapter 4, it was found that lentil legumin-like protein has an isoelectric point (*pI*) around 4.6 with balanced hydrophilic (~38%) and hydrophobic (~40%) residues. It also possesses both high surface hydrophobicity and solubility when pH was deviated from its *pI*. Thus, it demonstrated excellent foaming capacity and stability, being capable of forming long-life foams with a mean lifetime of 51 and 84 min at pH 5.0 and 7.0, respectively.

The polysaccharides selected for this study are guar gum, xanthan gum, and pectin. Some characteristics of these polysaccharides have been previously described in Chapter 2 (section 2.4.3.1). In addition, it is important to highlight other characteristics related to their structure and the possible interactions they can participate in.

The galactose to mannose ratio of guar gum (1:2) leads to galactose-rich and galactose-poor regions [53]. The less soluble galactose-poor regions can associate causing guar gum to form large intramolecular complexes that give guar gum its rheological properties [131]. It can swell and dissolve readily in cold water, therefore, it is widely used in the food industry as a thickening, water holding or stabilizing agent. For uncharged polysaccharides, such as guar gum, the primary physical interactions are hydrogen bonds that allow them to bind with the surrounding water molecules when they are dispersed in solution, but they may also form intra- or intermolecular hydrogen bonds with polar functional groups [132]. Xanthan gum is a stiff high molecular weight polysaccharide (2×10^6 to 5×10^7 Da) and can form highly viscous solutions. Pectin (2×10^4 to 4×10^5 Da) is a commonly used branched ionic polysaccharide, with neutral “hairy regions” attached.

The purpose of the present study was to systematically investigate how the presence of different polysaccharides may impact the conformation, surface properties of the lentil legumin-like protein, and its capacity to stabilize foams under different environmental pH conditions. It is hypothesized that a synergistic effect could be achieved by modulating protein-polysaccharide interactions for improved foam stability.

5.2 Materials and Methods

5.2.1 Raw materials

Lentil legumin-like protein extract (83% w/w d.b.) was obtained by rate-zonal centrifugation using a sucrose lineal density gradient as described in Chapter 4 (section 4.2.2). Guar and xanthan gums, Rhodamine B, and Calcofluor White (Fluorescent brightener 28) were obtained from Sigma-Aldrich Canada Co. (Ontario, ON, Canada). The LM Pectin (*Mw* of 2×10^4 -4×10^5 Da, as specified by the supplier) was provided by MP Biomedicals, LLC (Solon, OH, USA). All other chemicals were reagent-grade.

5.2.2 Preparation of the protein-polysaccharide mixtures

Pectin, guar or xanthan gum (1 mg/mL) solution was mixed with the lentil legumin-like protein (10 mg/mL) solution by magnetic stirring. Mixtures were prepared at three different pH levels (3.0, 5.0, and 7.0) adjusted using 0.1 M HCl or NaOH.

5.2.3 Electrophoretic mobility

The electrophoretic mobility of the protein-polysaccharide mixtures under pH 2.0 to 11.0 (adjusted using 0.1 M HCl or NaOH) at 22ºC was measured by laser Doppler velocimetry using a Zetasizer NanoS (model ZEN1600, Malvern Instruments Ltd., Malvern, UK) as described in Chapter 3 (section 3.2.3).

5.2.4 Foam properties

Foaming capacity was determined as in Chapter 3 (section 3.2.4.3). Briefly, 30 mL of protein-polysaccharide mixtures were mixed for 2 min with a homogenizer (PowerGen 1000, Fisher Scientific, Fairlawn, NJ, USA) at speed six. The foaming capacity (FC) was calculated as: FC(%) = $(V_{f1} - V_{f0})/V_{f0} \times 100$, where V_{f0} and V_{f1} represent the volume of the protein-polysaccharide mixture and the formed foams after homogenization, respectively.

As an index of foam stability (FS), a "mean life" τ value was determined as in Chapter 4 (section 4.2.3.3). Foam drainage was also calculated by measuring the volume of the drained liquid from the foams in a graduated cylinder and expressed as the liquid fraction (ε) in the foam (volume of liquid / volume of foam) [133].

5.2.5 Surface properties

5.2.5.1 Surface tension of protein-polysaccharide solutions

The surface tension of the protein-polysaccharide mixtures was measured at 22 °C with a DHR3 rheometer (AT Instruments-Waters LLC, New Castle, DE, USA) using a Platinum/Iridium Du Noüy ring (diameter of 10 mm). The ring was immersed in the mixture solution and gradually raised above the surface. The maximum value of the force at the detachment of the ring from the surface of the solution was recorded for surface tension calculation [123]. Three independent samples of each protein-polysaccharide mixture were measured.

5.2.5.2 Shear rheology measurement of protein-polysaccharides foams

The viscoelastic properties of the protein-polysaccharide foams were measured as a function of time. Samples were analyzed by means of a DHR3 rheometer (AT Instruments-Waters LLC, New Castle, DE, USA) fitted with a cone and plate attachment with a

diameter of 35 mm and angle of 2°. Strain sweep tests with strain amplitudes ranging from 0.01% to 100% were performed at 0.6283 rad/s in order to establish the linear viscoelastic range. Based on these results, frequency sweep measurements (0.1 to 100 rad/s) were carried out at a strain amplitude of 1%; a value smaller than the critical value for linear viscoelasticity. Oscillatory measurements were performed in the linear region at a frequency of 0.6283 rad/s and strain of 1%, and the storage modulus *G'*, loss modulus *G''*, and *tan (δ) = G''/G'* were recorded. In addition, shear rate-shear stress and shear rate-apparent viscosity data were collected as shear rate was increased linearly between 1 and 100 s^{-1} over a total run time of 5 min. During the analysis, the sample was kept at 22 °C and all measurements were done in triplicate using independently prepared samples.

5.2.6 Protein conformation in foams with polysaccharide

Infrared spectra of foams were recorded at 22 ºC using a Nicolet 6700 spectrometer (Thermo Scientific, Madison, WI, USA) and Fourier self-deconvolutions were performed (FTIR) using the software provided with the spectrometer to study the amide I region of the protein. Band narrowing was achieved with a full width at half maximum of 10 cm^{-1} and with a resolution enhancement factor of 1.0 cm^{-1}.

5.2.7 Microstructure of protein-polysaccharide mixtures and their foams

Images of the protein-polysaccharide mixtures were taken with a Zeiss Axio Imager M1 fluorescence microscope with a 10× objective (Carl Zeiss Microscopy, Jena, Germany) using the filter set 10 (excitation:450-490 nm, emission: 515-565 nm). Rhodamine B was used for labeling of lentil legumin-like protein and calcofluor white was used for labeling of the polysaccharides. After adding the fluorescence dye, the protein and the polysaccharides were separately stirred for 12 h at 22 °C, dialyzed against distilled water in

the dark, and freeze-dried. Protein-polysaccharide suspensions were prepared in darkness as described previously in section 5.2.2.

The microscopic structure of the protein-polysaccharide foams were examined using a laser scanning confocal microscope Zeiss LSM710 (Carl Zeiss Microscopy, Jena, Germany) with a 20× objective. Foams were prepared immediately before measurement, from protein-polysaccharide mixtures dyed and mixed as for fluorescence microscopy. Foam samples were loaded on the microscope slides and the fluorescent images were analyzed at the wavelengths of 420 and 516 nm for Calcofluor and Rhodamine B, respectively. Images were processed with the ZEN 2009 LE software (Carl Zeiss AG, Oberkochen, Germany).

5.2.8 Statistical analysis

All samples were prepared and tested in triplicate and results are presented as mean ± standard deviation. One way or two way analysis of variance (ANOVA) was carried out using Origin 9.1 software (Origin Lab Corporation, Northampton, MA, USA), and statistical differences among sample means were determined using Tukey's test at 95% confidence level.

5.3 Results and Discussion

5.3.1 Foaming properties

Destabilization of protein foams occurs due to simultaneous drainage, bubble coalescence, and coarsening [34]. Polysaccharide addition might impact stability depending on their interactions with protein, both at the interface vicinity and in the bulk, which are also influenced by the polysaccharide chemical structure, bulk viscosity, and their relative concentration in solution among others [38]. Thus, guar gum, xanthan gum and pectin were

selected to study their combined effect with the lentil legumin-like protein to stabilize foams, representing non-ionic and anionic polysaccharides with different molecular weights. All the samples demonstrated a good foaming capacity (*FC*) as shown in Figure 5.1 A with *FC* values in the range of 358 to 478%. Under all the tested pH conditions, the *FC* values of the mixtures were close to those measured for lentil legumin-like protein alone, suggesting that the selected polysaccharides did not contribute to further improvement of the foaming capacity of the lentil legumin-like protein. However, the polysaccharides had a significant effect on the foam stability (*FS*), depending on the pH as shown in Figure 5.1 B.

At pH 3.0, guar gum, pectin, and xanthan gum increased the mean life of lentil legumin-like protein foam from 26 to 29, 42, and 87 min, respectively. At this pH, coacervates can be formed between lentil legumin-like protein and anionic polysaccharide such as xanthan and pectin due to the attractive interactions between the anionic groups on the polysaccharide chains ($-COO^-$) and the cationic groups on the protein chains ($-NH_3^+$) [134, 135]. Although guar gum is considered to be a non-ionic polysaccharide, it exhibits a small negative charge (-0.40 ± 0.23) mV at pH 3. Therefore, opposite charges between lentil legumin-like protein and guar gum could allow development of weak electrostatic interactions. Furthermore, complexes may be formed by hydrophobic interactions between guar gum and lentil legumin-like protein that shows a strong surface hydrophobicity at this pH (Chapter 4, section 4.4.2).

This kind of interaction has been previously observed between whey protein and guar gum and also in other proteins and non-ionic polysaccharides such as egg white and hydroxypropylmethylcellulose (HPMC) mixtures [58, 136].

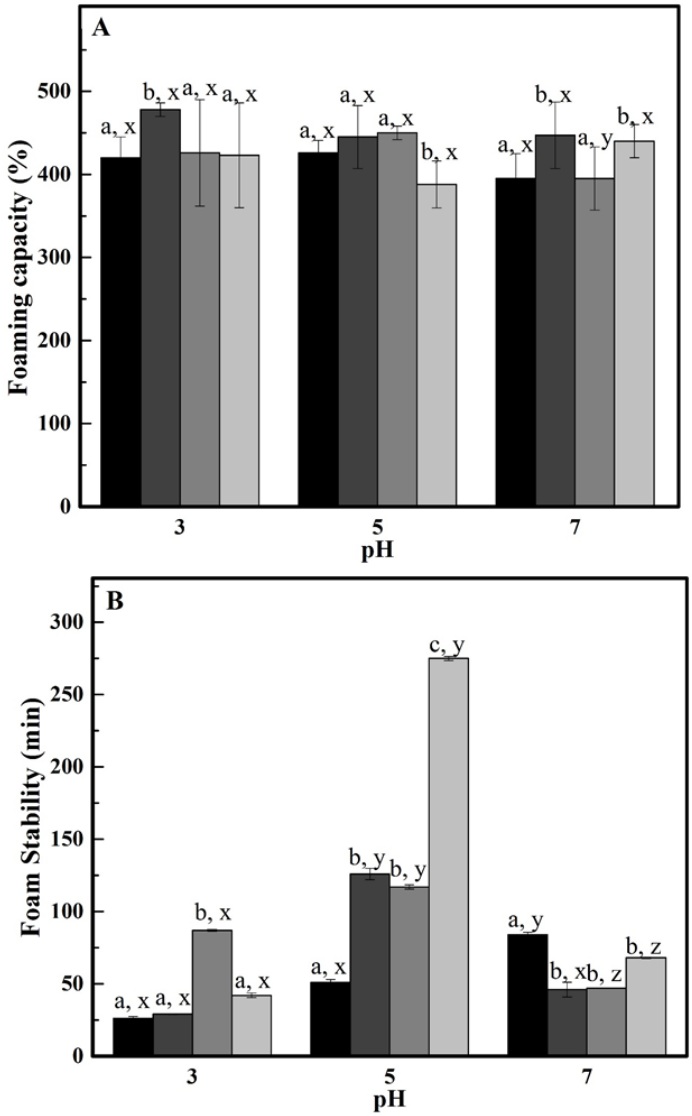
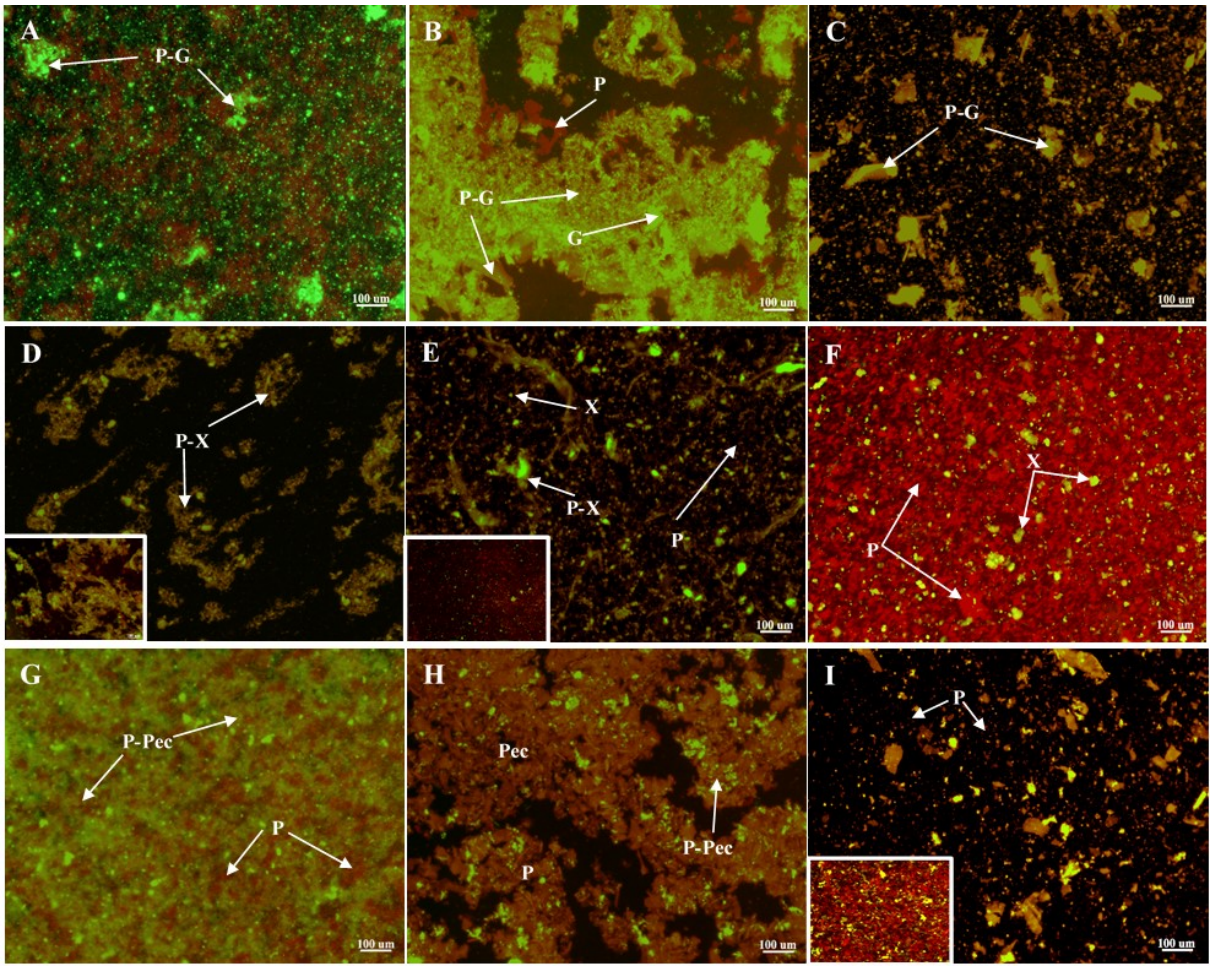


Figure 5.1 (A) Foaming capacity and (**B)** foam stability of lentil legumin-like protein-polysaccharide mixtures as a function of pH. (Black bar: lentil legumin-like protein, dark gray bar: with guar gum, gray bar: with xanthan gum, light gray bar: with pectin). Bars without a common letter have a significant difference ($p < 0.05$).
[a,b,c]: Significant difference between samples at fixed pH. [x,y,z]: significant differences between pH value for the same sample.

Fluorescent micrographs of the mixtures at pH 3.0 are shown in Figures 5.2 A, D, and G with protein visualized as red and polysaccharide as green/yellow. For the protein-guar gum mixture, polysaccharides and protein remained co-soluble. Yet, some larger

aggregated structures are observed (indicated with arrows in the figure), suggesting primary coacervation of the system [136]. A higher negatively charged polysaccharide is expected to have a higher degree of inter-biopolymer interaction with the protein, which may lead to the formation of primary soluble macromolecular complexes, then to form insoluble



complexes that ultimately sediment to form the coacervate phase containing both biopolymers [49].

Figure 5.2 Fluorescent micrographs of lentil legumin-like protein mixed with guar gum **(A, B, C)**, xanthan gum **(D, E, F)**, and pectin **(G, H, I)** at pH 3.0 (first column), pH 5.0 (second column), and pH 7.0 (third column). P: Lentil legumin-like protein; G: Guar gum; P-G: Lentil legumin-like protein and guar gum; Pec: Pectin; P-Pec: Lentil legumin-like protein and pectin; X: xanthan gum; P-X: Lentil legumin-like protein and xanthan gum. Protein is in red and polysaccharides in green/yellow. Inserts show the protein-polysaccharide system after 5 – 10 min.

Mixture with pectin (Figure 5.2 G) shows two main domains: one composed of just lentil legumin-like protein and another one with protein-pectin aggregates, both remaining loosely compacted. This could represent the macromolecular complex stage during coacervate formation [57]. However, in the protein-xanthan gum mixture, the micrograph (Figure 5.2 D) shows well defined coacervates, already separating from the solvent phase to form the coacervated phase (insert in Figure 5.2 D). Also the structure of the protein-polysaccharide complexes relates to the stiffness of the polysaccharides. The more rigid the polysaccharide, the larger the coacervates that will be formed with well-defined structures. Xanthan gum has the highest rigidity, followed by pectin and guar gum [52, 136].

Previous studies on β-lactoglobulin in the presence of polysaccharides showed similar results [52, 136] with xanthan and pectin forming complexes that were denser and more compact in structure than guar gum, the latter remained mostly in a co-soluble state. The presence of coacervates could stabilize foam interfaces against disproportionation as a result of the formation of electrostatically cross-linked interfacial networks [137, 56].

The small coacervates could also act as Pickering particles that are able to stabilize foams by the formation of a coherent structural barrier of adsorbed particles at the surface of dispersed bubbles [138, 139]. Also, coalescence is avoided by electrostatic or steric repulsions between the coacervate stabilized air bubbles [137]. Because of its higher charge density and more rigid structure, xanthan gum might produce larger complexes that would build a thicker interfacial layer [140], hence a more stable foam.

At pH 5.0, a strong synergy in the foam stability occurred and the stability of the foams dramatically increased by the presence of polysaccharides with the mean life value increasing from 51 min to 126, 117, and 275 min, with guar, xanthan, and pectin, respectively (Figure 5.1 B). Near the protein isoelectric point (*pI*), protein-polysaccharide

aggregation could occur via hydrophobic interactions, as well as some electrostatic interactions due to positively charged “patches” on the protein surface. Turgeon et al. [52] reported that protein-polysaccharide aggregates increased the viscosity of the interfacial layer and they could form strong, thick, and dense viscoelastic networks at the interface that exhibited low gas permeability and improved foam stabilization properties. Thus, the synergistic effect could be related to the adsorption of lentil legumin-like protein-polysaccharide aggregates at the interfacial layer to form stronger networks at the interface and their effect on the viscoelastic behavior of the interfacial layer and the bulk foam [141]. These aggregates might also be able to plug the junctions of the Plateau borders (liquid-carrying channels formed where three lamellae meet), so that the drainage of the liquid would slow down [142], further contributing to foam stabilization. This speculation is supported by the results of fluorescence microscopy. Fluorescent micrographs of the guar gum and pectin mixtures with protein at pH 5.0 (Figures 5.2 B and H) show the formation of aggregates, which exhibited different morphology from that of protein alone (Figure A3.2, Appendix A3) for both size and shape. The protein-xanthan mixture presented a different structure (Figure 5.2 E) with the aggregates breaking apart into smaller structures, which might indicate that segregative phase separation is starting. At the same pH and polysaccharide concentration, phase separation behavior in protein-polysaccharide systems is highly dominated by polysaccharide charge density [55]. The higher segregation rate for xanthan gum could be related to its higher charge density [143].

At pH 7.0, none of the polysaccharides improved the foam stability, instead a significant decrease compared to the control was observed (Figure 5.1 B). The mixtures of proteins and polysaccharides at pH 7.0 are governed by segregative phenomena due to the repulsion produced by electrostatic repulsive interactions and different solvent affinities

[144, 145]. Thus, the system undergoes liquid-liquid separation at molecular scale, but the system remains as one phase at macroscopic scale. If the incompatibility is greater, it may lead to polymer concentration in two different phases [144, 50] forming a water-in-water emulsion system with "droplets", rich in polysaccharide surrounded by a continuous phase rich in protein, or vice versa, depending on the ratio of the biopolymers [143]. In Figures 5.2 C, F, and I, the protein-polysaccharide systems at pH 7.0 clearly showed the phase separation process with the identification of characteristic droplet-like domains. In Figure 5.2 C, guar gum separation from lentil legumin-like protein was not complete and some protein-polysaccharide aggregates still remained. Whereas for pectin, the phase separation was almost complete after 4 h (insert in Figure 2I) and a protein-rich phase was formed by that time. For the mixture with xanthan gum, an "emulsion-like" structure of spherical aggregates of polysaccharides in a protein-rich continuous phase was formed, almost immediately after mixing (Figure 5.2 F) [146]. The differences in the phase separation behavior are likely due to the differences in the charge density of the polysaccharides [55]. Such phase separation creates enriched protein or polysaccharide regions at the interface as segregated patches dispersed, thus breaking the continuity of the protein film around the bubbles [147].

5.3.2 Surface and viscoelastic properties

The effect of polysaccharides on the viscoelastic and surface properties of the mixture was also studied. The addition of the polysaccharide did not significantly impact the surface tension of the mixtures (data not shown). As shown in Figures 5.3 A, B, and C, the apparent viscosity (η_{app}) of all foams stabilized with either protein alone or protein-polysaccharide mixtures exhibited shear thinning behavior. Shear thinning has been

observed in other protein-based foams such as milk foams and egg white foams with or without the addition of polysaccharides [145-147]. Shear thinning phenomenon in foam is mainly caused by the disruption of the foam structure either by the alignment of the protein chains in the direction of the shear force, or by mechanical damage during the measurement [151]. All foams at pH 3.0 and 5.0 exhibited a higher apparent viscosity than those at pH 7.0 at any shear rate due to the formation of coacervates and aggregates, respectively. A slightly higher apparent viscosity was observed at pH 5.0 than 3.0 probably due to the larger size of the aggregates formed near the protein pI. In general, the addition of guar gum, xanthan gum, and pectin to the protein produced higher values of η_{app} than protein alone at low shear rates at both pH 3.0 and 5.0 (Figures 5.3 A and B). Low shear rate viscosity can be related to long-term stability in foams because typical shear rates experienced by materials under gravity-induced drainage vary from 0.01/s to 1/s [152, 115]. Whereas at pH 7.0, the addition of polysaccharides decreased the foam η_{app} value at lower shear rates to lower values than the original foam with protein alone (Figure 5.3 C), which might be related to the observed segregation phenomenon at this pH value (Figure 5.2). Indeed, at this pH, the η_{app} value of protein-polysaccharide foams was lower at any shear rate than that of the protein foam.

The foam viscosity correlated well with the stability of the foams. The higher viscosity at lower shear rate of lentil legumin-like protein-polysaccharides at pH 3.0 and 5.0 could favor the immobilization of the lamellar water surrounding the gas bubbles, improving the stability of the foam against drainage, coalescence, and coarsening [153, 154]. This phenomenon was also observed for egg white-pectin coacervates at acidic pH when the protein was at a higher concentration than the polysaccharide. Under such conditions, the concentration of the non-complexed protein increased and protein was responsible for film

formation around the bubbles. Then, these proteins act as anchor for the complexes to build a secondary layer, which contributed to the formation of a more stable film, inhibiting the bubble coalescence [153, 155].

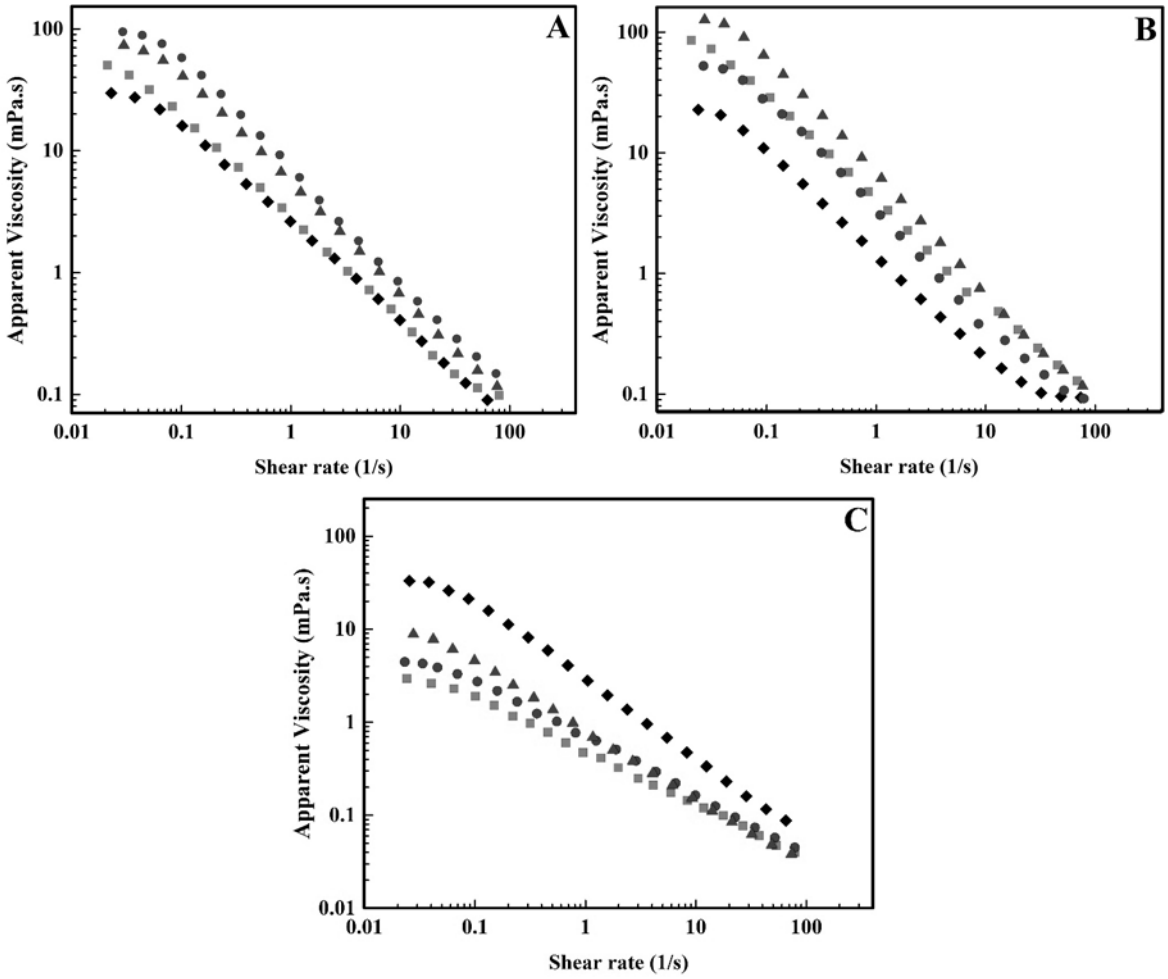


Figure 5.3 Apparent viscosity of lentil legumin-like protein-polysaccharide foams as a function of shear rate at **(A)** pH 3.0, **(B)** pH 5.0, and **(C)** pH 7.0 (lentil protein: diamonds, with guar gum: squares, with xanthan gum: circles, with pectin: triangles).

Also, the soluble complexes migrate to the lamella, increasing the viscosity by forming gel-like structures when they get connected, providing some rigid and thick bridges between the bubbles, and thus the liquid drainage was slower when compared to the foam

formed only with the protein [155]. A similar explanation can be applied to the lentil legumin-like protein systems in the presence of polysaccharides in this study.

Viscoelastic moduli can help to understand some of the underlying phenomena of foam stability as *G'* is dependent on bubble diameter (*D*) and liquid fraction in the foam (ε) [156]:

$$G' \propto \left(\gamma/_D\right) g(\varepsilon) \qquad \text{eq. (5.4)}$$

where g(ε) is a decreasing function of only ε. During drainage, if the bubbles get bigger, this will lead to faster drainage, implying a drier foam and then even faster coarsening. Thus, *G'* will continuously decrease as the foams coarsen and collapse [156]. Figures 5.4 A, B, and C show the aging behavior of the foams represented by the storage modulus (*G'*) as a function of time. Concerning the loss modulus *G''*, similar qualitative features were observed, thus results were not shown. At pH 3.0 and 5.0 (Figures 5.4 A and B), all lentil legumin-like protein-polysaccharide foams showed a higher *G'* value than the protein foam. At pH 7.0, only the foam containing xanthan gum had a higher G' at t = 0 compared to that of the protein, but the G' decreased rapidly and showed a lower value than that of the foam stabilized by protein alone after 15 min. Previous studies have revealed that G' was strongly related to the cohesion within and between the complexes present in adsorbed layers. Thus, the higher *G'* values support the statement that strong, cohesive, and thicker interfacial layers were created by adsorbed complexes at pH 3.0 and 5.0 [134, 140, 157]. If the polymers do not have a tendency to form complexes, as at pH 7.0, they cannot form thick aggregated layers nor show strong cohesion within the adsorbed layer [140]. This evolution of *G'* with time is sensitive to surface composition and structure changes and

normally higher values of storage modulus (*G'*) indicate stronger interactions in the interfacial networks [40].

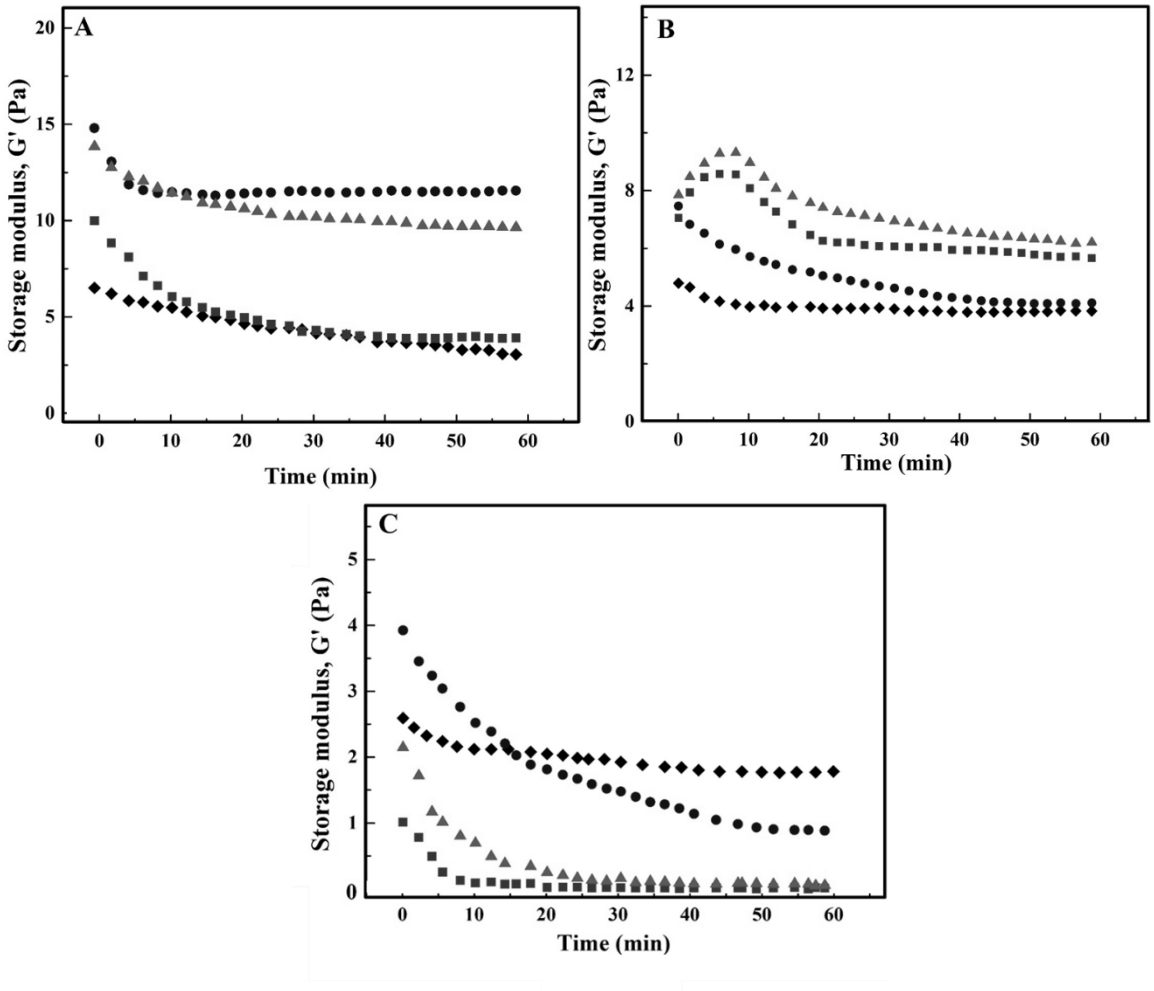


Figure 5.4 Storage modulus of lentil legumin-like protein-polysaccharide foams as a function of time at **(A)** pH 3.0, **(B)** pH 5.0, and **(C)** pH 7.0 (lentil protein: diamonds, with guar gum: squares, with xanthan gum: circles, with pectin: triangles).

It can also give information about the jamming effect produced when the liquid in a foam is confined to a narrow Plateau border, due to, for example, the presence of protein-polysaccharide complexes [150]. Under this jamming effect, the foam will change from exhibiting a liquid-like behavior towards a solid-like behavior with the *G'* value increasing with time [156]. The fluid jamming is not permanent, the foam will finally drain as a consequence of the foam coarsening with the decay of the *G'* with time [156]. This jamming effect is clearly seen in the foams containing guar and pectin at pH 5.0 (Figure 5.4 B), where a continuous increase of *G'* is observed in the first 10 min. This phenomenon can be related to the formation of large aggregates (Figures 5.2 B and H) that were confined to the Plateau borders in the lamellae networks, slowing down the drainage of the liquid fraction (Figure 5.5 B), by reducing the Plateau border cross section [133, 156]. As time passed (after around 15 min) coarsening relaxed the confinement and the fluid jamming, then *G'* decreased and yielding occurred as the gas diffusion and coarsening continued because the films do not block gas diffusion [133, 156]. Such jamming effect significantly reduced the foam drainage as shown in Figure 5.5 B, subsequently contributing to the excellent foam stability at pH 5.0 [150], especially for the lentil legumin-like protein-pectin foam. Additionally, in this foam, the low net surface charge of the aggregates regulated by the charge density of the pectin, may allow greater cohesion between aggregates within the adsorbed layer, which, along with their size could have contributed to building of a thicker interfacial layer [140]. Hence, greatly improved foam stability was observed with the highest *FS* value. The jamming effect was not observed at pH 3.0 where coacervates existed (Figure 5.4 A). The initial *G'* values of protein-polysaccharide foams as well as their liquid fractions at pH 3.0 (Figure 5.5 A), are higher than those at other pH values. A possible explanation could be that the initial liquid fraction and *G'* were the result of the

formation of electrostatically cross-linked gel-like interfacial structure promoted by the presence of coacervates that acted against coarsening and coalescence. Such a gel-like layer at the interface also inhibited foam drainage by holding the water in its structure [158]. This increase in *G'* due to the formation of electrostatic complexes and the gel-like structure at the interface that stabilized the foams was previously observed in the β-lactoglobulin-pectin and the polyelectrolyte-surfactant systems [134, 158].

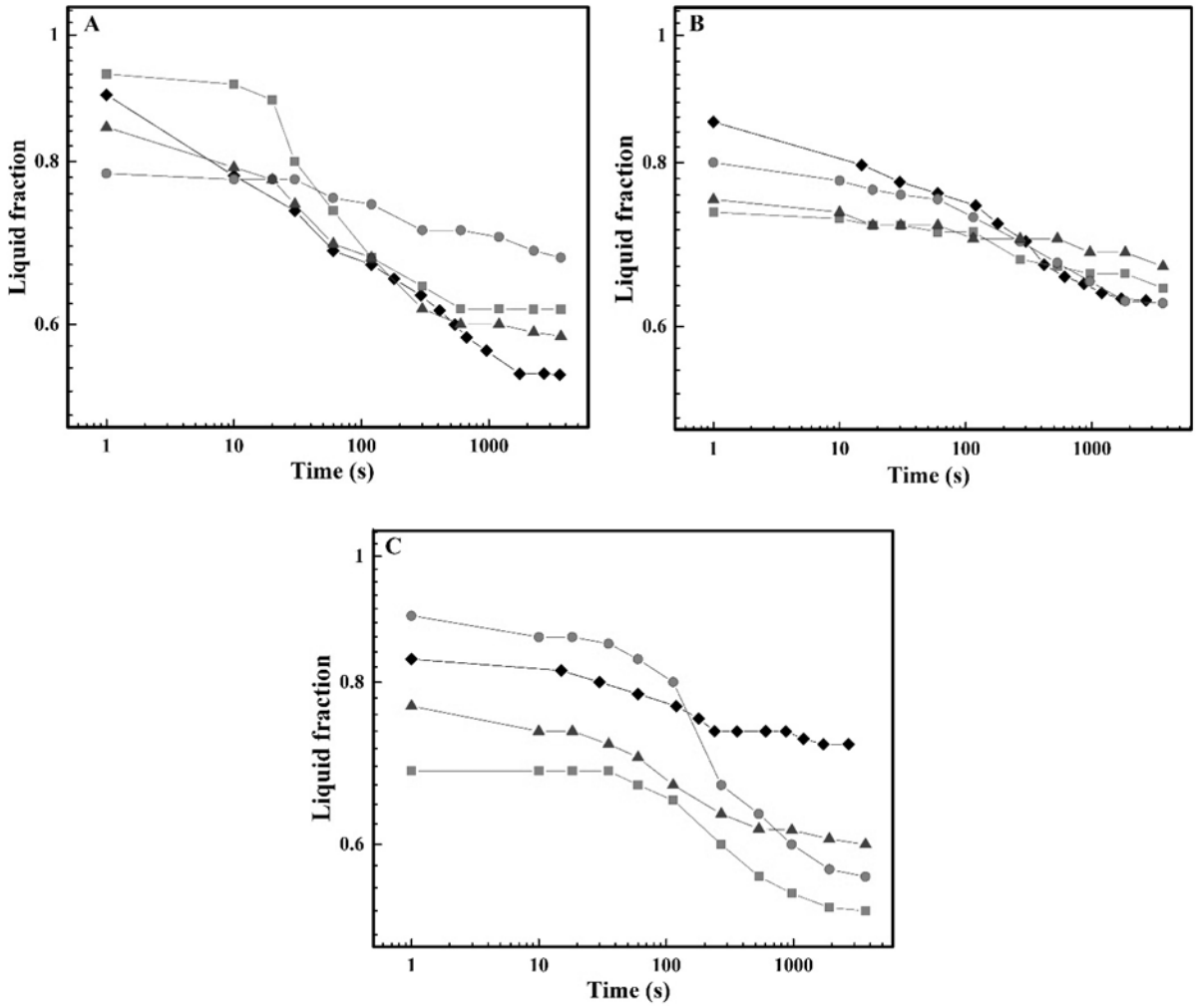


Figure 5.5 Liquid fraction in the foams of lentil legumin-like protein-polysaccharide as a function of time at **(A)** pH 3.0, **(B)** pH 5.0, and **(C)** pH 7.0 (lentil protein: diamonds, with guar gum: squares, with xanthan gum: circles, with pectin: triangles).

At pH 7.0, although the initial liquid fraction was held for a longer time (~90 sec) than at other pHs (Figure 5.5 C), lower initial values of *G'* for the system with guar and pectin, and faster and more marked decay rate of *G'* for the system with xanthan and pectin (Figure 5.4 C) were observed. Previous studies on segregative phase separation at the interfaces have demonstrated that phase separation could occur if there is an effective repulsive interaction between the polymers. This phase separation might occur not because of direct thermodynamic incompatibility between the components, but because that would trigger a preferential interaction of one of the polymers with water [159]. Specifically, in this study, the thermodynamic incompatibility between lentil legumin-like protein and polysaccharide might allow polysaccharides to hold more water in the lamellae, leading to slow drainage during the first stage of foam life. Also, the loss of the previously existing network connectivity might explain the low *G'* likely due to the lack of interactions between the phase-separated regions. There are some studies [145, 153] demonstrating the improvement of foam stability under thermodynamic incompatibility conditions in the presence of polysaccharides. Such studies, generally attribute this to a "concentration effect" on proteins at the interface produced by the phase separation. On the contrary, in this study and some other studies [60, 154] under the similar conditions, foam stability was reduced in the presence of polysaccharides at pH 7.0 probably due to different biopolymer structures and interactions.

5.3.3 Foam structure

5.3.3.1 FTIR spectra of protein at the foam surface

The previous study presented in Chapter 4 revealed that at pH 3.0, the adsorbed lentil legumin-like protein was characterized by unordered structure (1642 cm^{-1}), β-turns (1661

and 1677 cm^{-1}), and some aggregates (1624 cm^{-1}). At pH 5.0, a strong aggregation was observed (1620 cm^{-1}), as well as β-sheets (1634 cm^{-1}) and β-turns (1663 and 1677 cm^{-1}). Whereas at pH 7.0, a major peak at around 1651 cm^{-1} corresponding to α-helix could be identified, along with β-sheets (1634 and 1683 cm^{-1}) and β-turns (1670 cm^{-1}).

The Fourier-deconvoluted FTIR spectra of lentil legumin-like proteins adsorbed at the foam surface in the presence of polysaccharides are shown in Figure 5.6. Based on these spectra, it is suggested that lentil legumin-like protein underwent different conformational changes at the foam interface, in the presence of the polysaccharides depending on the environmental pH. At pH 3.0 (Figure 5.6 A), lentil legumin-like protein formed α-helix structure at the interface (1652 cm^{-1}) in the presence of polysaccharides. The α-helix conformation is energetically favorable when strong electrostatic interactions between polymers are present, contributing to the stability of the complex [160]. Beta-sheets (1635 and 1684 cm^{-1}) and β-turns (1669 cm^{-1}) were also identified. The absorption at 1618-1620 cm^{-1} suggests formation of protein aggregates at the interface for protein alone and with polysaccharides. Presence of aggregated structures in coacervates has been observed by Wang et al. [141] in β-lactoglobulin-pectin coacervates. At pH 5.0 (Figure 5.6 B), α-helix structures (1652-1656 cm^{-1}) were more prominent in the protein-polysaccharide foams than in the protein foam. This molecular configuration might have contributed to the better stability of protein-polysaccharide foams than at other pH values (Figure 5.1 B).

Alpha-helices may be better packaged side by side, since they are adsorbed with their long axis parallel to the air/liquid interface, which would allow the side chains that protrude to interdigitate creating more non-covalent intramolecular interactions leading to improved surface networks [129].

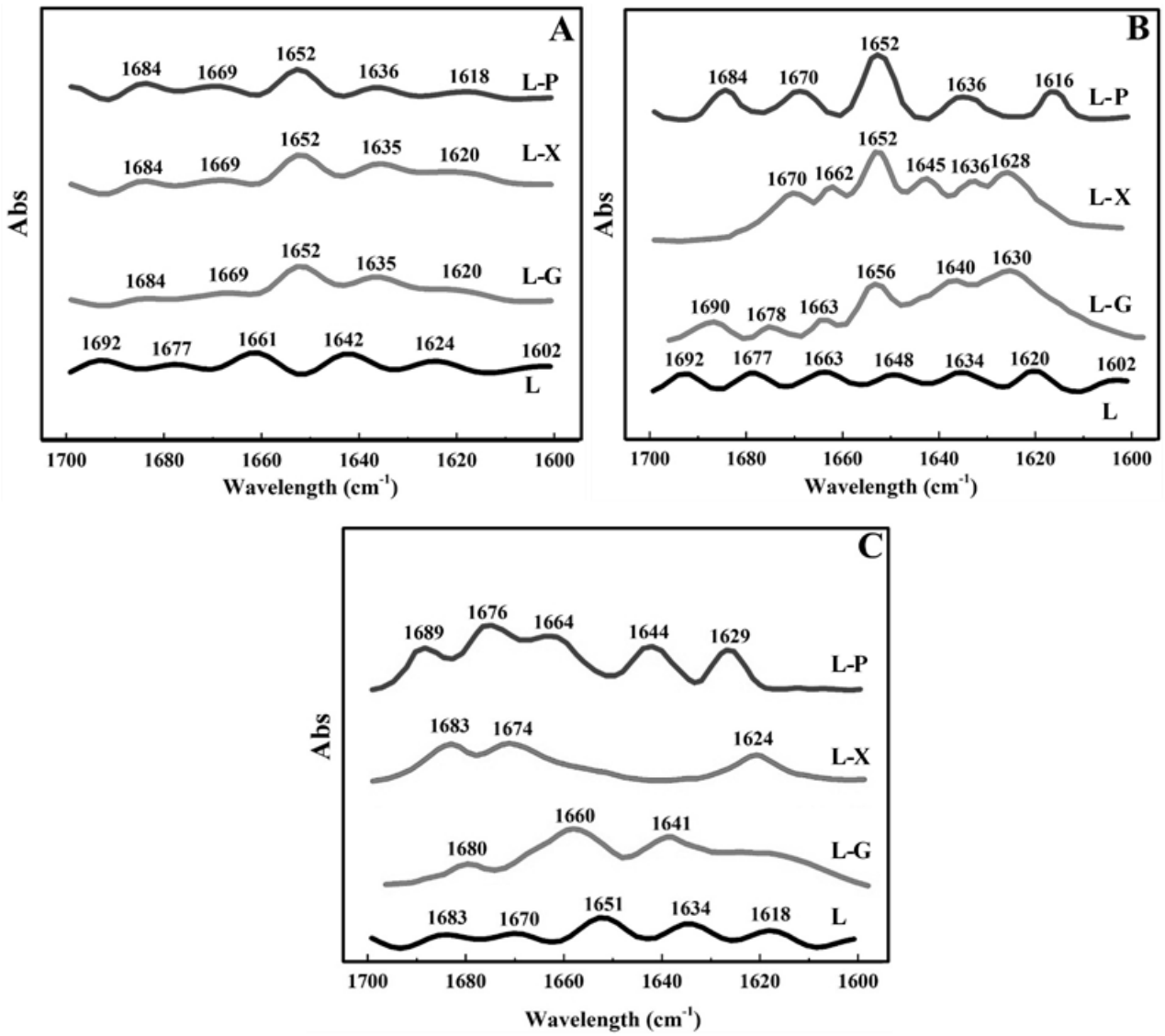


Figure 5.6 Fourier-deconvoluted FTIR spectra of lentil legumin-like protein-polysaccharide foams at the foam surface. **(A)** pH 3.0, **(B)** pH 5.0, and **(C)** pH 7.0 (lentil protein: L, with guar gum: L-G, with xanthan gum: L-X, with pectin: L-P).

The molecular structure of the protein at the foam interface suffered a remarkable change at pH 7.0 (Figure 5.6 C) that could partially explain the low stability of lentil legumin-like protein-polysaccharide foams. The protein lost its α-helix structure (1651 cm^{-1}) in the presence of polysaccharides and the protein in foams containing pectin and guar gum gained unordered structures (1640 cm^{-1}). Such changes might partially explain the lower resistance of the protein-polysaccharide foams against collapse because of the

increased intra-protein flexibility produced by unordered structures that led to a less compact structure and relaxed interface [125].

5.3.3.2 Foam microstructure by CLSM

CLSM was applied to visualize the distribution of protein (red) and polysaccharide (cyan) in the foams by staining these two components with fluorescence dyes as shown in Figure 5.7. The images confirm both the occurrence of segregative phase separation at pH 7.0 and the formation of coacervates at pH 3.0, especially in the presence of xanthan and pectin. Also, it was possible to observe the protein film around the bubbles and how its structure was changed by the presence of the polysaccharides at different pH values. At pH 3.0 (Figures 5.7 B, C, D), the surface membrane of the bubbles appeared smooth and homogeneous. The coacervates are visible in the lamellae of all the foams (marked by circles in the micrographs). Such coacervate structure and the gel-like interfacial structure could explain the increased viscosity of the foam in section 5.3.2 [133, 156]. Interestingly, the presence of the coacervates did not alter the smoothness of the protein film. Instead, they seem anchored to the protein membrane and connected with each other, building a stronger network, inhibiting the bubbles coarsening and coalescence. At pH 5.0 (Figures 5.7 F, G, H), aggregates also appeared in the lamellae of all foams. Some of the aggregates are on the surface of the bubbles, adding thickness to the bubble membrane, while others remained in the Plateau borders.

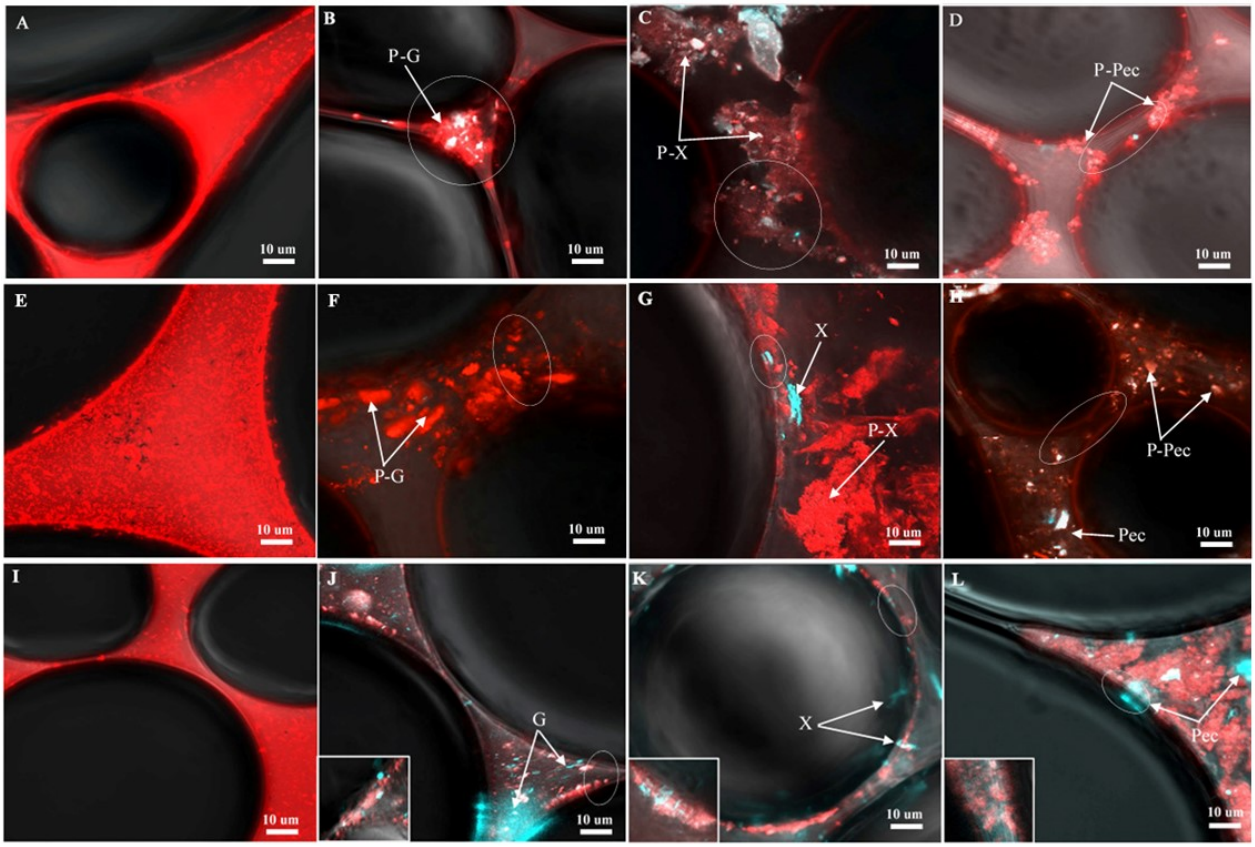


Figure 5.7 CLSM images of the lentil legumin-like protein-polysaccharides foams at pH 3.0. **(A, B, C, D)**, 5.0 **(E, F, G, H)**, and 7.0 **(I, J, K, L)**: lentil legumin-like protein, (1st column): with guar, (2nd column): with xanthan (3d column), and with pectin (4th column). Protein is in red and polysaccharides in cyan. Inserts show the protein-polysaccharide system using 40× oil immersion objective.

The latter would likely be responsible for the high viscosity determined in the protein-guar gum and protein-pectin foam (Figure 5.3 B) [133, 156]. The jamming effect of aggregates can be also confirmed in these micrographs (Figures 5.7 F and H) as delineated by the encircled zones, where the coacervates in the Plateau borders are confining the liquid in between. The presence of the aggregates again did not interrupt the smoothness of the protein membrane in the lamellae of pectin and guar containing foams, which might have influenced the stability of those foams. While for the protein-xanthan foam, it seems that the film was broken in some points compromising the interfacial network integrity (Figure 5.7 G). This may suggest an incipient phase separation occurring at this pH in the presence

of xanthan gum due to its higher charge density (Figure 5.2 E). At pH 7.0, interfacial films of all protein-polysaccharide foams seem fragmented. A close look at the inserts shows that a phase separation was occurring at the interfacial layer and this caused a disruption of the protein film around the bubbles, which explained the poor foam stability at this pH. The protein seems partially displaced through the formation of islands of both polymers with the protein being compressed into regions of increasing thickness. This gradual displacement of the protein by the polysaccharides can be explained by the orogenic mechanism [47] in which there is increasing concentration of the other component at the interface until the protein network breaks and proteins are displaced. Such orogenic mechanism was confirmed in protein-surfactant and protein-protein foams as reported by Murray [47].

5.4 Conclusions

Protein-polysaccharide interactions due to pH variation had a significant impact on the foam properties stabilized by the lentil legumin-like protein-polysaccharide mixtures. The foam life time value at pH 5.0 was dramatically increased from 51 min to 126, 117, and 275 min when guar, xanthan, and pectin were added into the protein system, respectively. The proximity to *pI* favored formation of protein-polysaccharide aggregates that could be adsorbed onto the interfacial layer to form strong interfacial networks, avoiding coalescence, and coarsening of the foams. In addition, such aggregates plugged the junctions of the Plateau borders and slowed down the drainage by a jamming effect. All these contributed to the greatly improved foam stability. The addition of polysaccharides also significantly improved foam stability at pH 3.0, with the protein-xanthan gum system being the best (mean lifetime of 87 min). At this pH, the associative interactions dominated

and a coacervation phenomenon was observed. The presence of these coacervates may have stabilized the foams against collapse due to the formation of an electrostatically cross-linked gel-like interfacial network. Also, lentil legumin-like protein underwent a favorable conformation change at the foam interface, in the presence of the polysaccharides at pH 3.0 and 5.0. Alpha-helix structures were formed in the coacervates and aggregates that might have contributed to the better stability of the protein-polysaccharide foams.

At pH 7.0, the mean life of the foams was reduced in the presence of all selected polysaccharides. Phase separation induced a disruption of the protein layer around the bubbles making it weaker and easier to break, which have a negative impact on the foam stability. This disturbance in the protein film might be a result of partial displacement of the protein by the polysaccharides through an orogenic process. In addition, proteins lost their α-helix structure, while gaining unordered structures, leading to interfacial networks with reduced resistance against collapse.

Improvement of mean life of lentil legumin-like protein foams at mild and acidic pH using guar, xanthan, and pectin creates opportunities for potential applications in food products in this range of pH.

CHAPTER 6

Effect of Lentil Protein as Egg Replacer on the Rheology, Microstructure, and Quality of Angel Food Cake and Muffin

6.1 Introduction

The previous studies of this thesis research have demonstrated good foaming capacity for lentil protein (Chapter 3 – 5). The foaming stability was especially strong that allowed the formation of long-life foams (84 min) at neutral pH. Also, good emulsifying capacity has been demonstrated by Joshi et al. [27]. Both functionalities are key during processing and structure formation of baked goods as they contribute to the aeration and stability of the batter [161, 162, 84]. Thus, lentil protein has good potential for use as a new functional ingredient in bakery products, replacing animal-based proteins such as egg white and dairy proteins. Incorporation of lentil protein in cereal-based bakery products may lead to new food products with improved nutritional value as pulse proteins are complementary to cereal proteins in essential amino acids, with the latter being high in sulfur amino acids, but limiting in lysine [62]. Also, this allows new food products to target the vegan/vegetarian population. Even partial replacement of animal proteins could be interesting for the industry because it allows long-term cost savings [73]. So far, lentil flour and lentil protein alone or in combination with other pulse proteins, have been incorporated with more or less success in tofu-like product, imitation milk, meatballs and bread [62-64, 66]. To the best of our knowledge, there are very limited literature reports regarding the utilization of lentil protein in other bakery products like cakes and muffins.

Angel food cake is a foam produced by the combination of a few ingredients, i.e. egg white, sugar, and flour. Thus, it is the most common food product utilized to test the

foaming capacity of other protein ingredients [31]. On the other hand, muffin, which is classified as a quick bread [163], is a fat-in-water emulsion/foam composed of an egg, sugar, water, and fat mixture in which flour particles are dispersed. Both products are highly appreciated by consumers due to their good taste and texture [164]. The objective of this study was to investigate the feasibility of using lentil protein to replace egg white and milk protein in angel food cake and muffin. It is also aimed to develop "high protein" foods with increased protein content in muffins. The structural characteristics of the product batters and of the baked goods were studied through rheometry, microscopy, texture analysis, as well as the consumer sensory analysis. The storage stability of the products was also evaluated using textural profile analysis (TPA) and confocal microscopy.

6.2. Materials and Methods

6.2.1 Raw materials

Cake and pastry flour (No name®, Real Canadian Superstore, Edmonton, AB, Canada), icing sugar (Rogers sugar Ltd., Taber, AB, Canada), canola oil (No name®, Real Canadian Superstore, Edmonton, AB, Canada), cocoa powder (Hershey's Canada Inc., Mississauga, ON, Canada), milk (2%, Lucerne Foods, Division of Canada Safeway Ltd., Winnipeg, MB, Canada), baking powder (Fleischmann's, ACH Food Companies Inc., Mississauga, ON, Canada), fresh eggs (Lucerne Foods, Division of Canada Safeway Ltd., Winnipeg, MB, Canada), corn starch (No name®, Real Canadian Superstore, Edmonton, AB, Canada), and salt (Windsor, Mississauga, ON, Canada) were purchased from a local supermarket. Lentil protein concentrate (LPC) containing 78.7% (d.m.), was extracted from dehulled lentil flour in the Food Processing Development Centre (Leduc, AB, Canada) according to the protocol established in Chapter 3. Egg white protein (80% protein content,

92% dry matter, code: P-19-J #428) was kindly supplied by Henningsen Food (Omaha, NE, USA).

6.2.2 Preparation of protein solutions

Lentil protein concentrate was dispersed in water while stirring to form a protein solution of 5 % (w/v) and 10 % (w/v) for muffin and angel food cake applications, respectively. Egg white protein solution (10 % (w/v) was prepared by dispersing the egg white protein in water while stirring. The protein solutions were stored at 4˚C overnight. Egg white protein solution to be used in the angel food cake was kept at room temperature for 1 h before use to achieve its full performance as a foaming agent [165].

6.2.3 Product preparation

6.2.3.1 Angel food cakes

Three kinds of angel food cakes were prepared, including control (AC-0-LPC) with egg white protein, and two with 50% (AC-50-LPC) and 75% (AC-75-LPC) replacement of egg white protein with lentil protein concentrate. The ingredients used in the formulations are shown in Table 6.1. Egg white protein solution was whipped for 30 sec at speed setting 5 (1200 rpm) with a hand mixer (Oster 2500, Inspire 240-Watt, 5-Speed, Sunbeam Products, Inc., Boca Raton, FL, USA) equipped with a wire whisk attachment. A third of the sugar was added and whipping was resumed for 1.5 min. A dry blended and sifted mixture, containing the rest of the ingredients was incorporated into the foam in small increments and manually folded into the batter. When lentil protein was used, it was separately whipped for 2 min and then carefully mixed with the foam formed with the egg white protein and a third of the sugar. Then, the rest of the ingredients were added as

previously described. Angel food cake batters were then transferred into non-stick baking pans (3.175 cm height x 7 cm diameter) and baked at 150 °C for 35 min.

Table 6.1 Angel food cake and muffin formulations.

Ingredients	Muffin			Angel food cake		
	M-0-LPC (wt %)	M-100-LPC (wt %)	M-100H-LPC (wt %)	AC-0-LPC (wt %)	AC-50-LPC (wt %)	AC-75-LPC (wt %)
Flour	20.0	20.0	16.0	16.0	16.0	16.0
Sugar	26.0	26.0	26.0	28.0	28.0	28.0
Lentil protein solution[a]	—	33.0	33.0		26.0	34.6
Egg white protein solution[b]	—	—	—	52.0	26.0	17.4
Lentil protein powder	—	—	14.0	—	—	—
Egg	11.0	—	—	—	—	—
Milk	22.0	—	—	—	—	—
Cocoa powder	4.7	4.7	4.7	—	—	—
Canola oil	16.0	16.0	16.0	—	—	—
Baking powder	0.3	0.3	0.3	—	—	—
Corn starch	—	—	—	4.0	4.0	4.0

[a]Solution concentration: 5% (w/v)
[b]Solution concentration: 10% (w/v)

6.2.3.2 Muffins

Three kinds of muffins were prepared, including a control (M-0-LPC) that was prepared with egg and milk, and two lentil protein-based muffins to totally replace the egg and milk proteins. The first lentil protein-based muffin (M-100-LPC) contained 33% (w/w) lentil protein solution, equivalent to the total protein contained in milk and egg in the control. The second formulation (M-100H-LPC) had 44% protein solution, which would allow this muffin product to qualify for a high protein content claim, according to the Canadian Food Inspection Agency (CFIA) [166]. The muffin ingredients are also shown in Table 6.1. Fresh egg and milk or lentil protein solution were first mixed with oil for 2 min at room temperature using a hand mixer (Oster 2500, Inspire 240-Watt, 5-Speed, Sunbeam Products, Inc., Boca Raton, FL, USA) at the maximum speed (1200 rpm). The rest of the

ingredients were then incorporated under constant mixing at medium speed (600 rpm) for 5 min. Muffin batters were then transferred into a non-stick baking pan (3.175 cm height x 7 cm diameter) and baked at 175 °C for 30 min.

After baking, the muffins and cakes were left to cool at room temperature for 1 h. Then, they were wrapped and stored in a closed plastic container at room temperature for up to 72 h. Sensory evaluation was conducted within 24 h after cooling. For this purpose, the muffins and angel food cakes were transferred into coded plastic bags. Four muffins and angel food cakes from the same batter were used for physical measurements and texture evaluation after 24, 48, and 72 h of storage in order to study product staling.

6.2.4 Batter specific gravity and rheological measurements

Batter specific gravity was calculated by dividing the weight of a certain volume of batter by the weight of the same volume of water [72]. Steady shear properties of product batters were obtained at constant temperature (22 °C) using a DHR3 rheometer (AT Instruments-Waters LLC, Delaware, USA) fitted with a cone and plate geometry (2°, 40 mm diameter). Fresh samples were prepared for the experiments. The gap between the cone and plate was 2 mm for angel food batters and 1 mm for muffin batters to ensure that air bubbles were not deformed before the measurement. Shear rate-shear stress, and shear rate-apparent viscosity data were collected as shear rate was increased linearly between 1 and 100 s^{-1} over a total run time of 5 min. The data for shear rate-shear stress were fitted to the Ostwald de Waele model following the power-law equation frequently used for baked products [162, 167]:

$$\sigma = K \cdot \dot{\gamma}^{n} \qquad \text{eq. (6.1)}$$

where, σ is shear stress (Pa), $\dot{\gamma}$ is shear rate (s^{-1}), K is the consistency coefficient ($Pa \cdot s^n$), and n is the flow behavior index. Parameter calculation was conducted using TRIOS software (v.3.1.0.3538, TA Instruments-Waters LLC, New Castle, DE, USA).

6.2.5 Physical properties of baked products

Angel food cake and muffin physical attributes such as height, baking loss, crumb features, and texture of the crumb were tested. Height of 4 samples for each type of product was measured after they were cut vertically through the center. Baking loss (BL) was determined by weighing the products 1 h after baking and using the following equation [72]:

$$BL(\%) = [(B - C)/IW] \times 100 \quad \text{eq. (6.2)}$$

where BL is the weight loss during baking, B is the weight of the batter before baking, C is the weight of the product after baking and IW is the initial water content of the batter. The moisture content was determined by drying triplicate batter samples in an air oven at 105ºC, according to the air oven gravimetric method AACC 44-15A.26 (AACC International, MN, USA) [168].

The color of the crumb was measured using a chromatometer (Minolta CR-410, Konica Minolta Sensing, Inc, Sakai, Osaka, Japan). Color was measured in triplicate using pieces (6 cm width x 6 cm length) from the center region of the products, and the results are expressed as an average of the measurement of L^*, a^*, and b^* values of the CIELAB system. The L^* value features the whiteness and ranges from 0 (for black) to 100 (for white), a^* ranges from -100 (for redness) to +100 (for greenness) and b^* ranges from -100 (for blueness) to +100 (for yellowness) [162, 168]. The total color difference (ΔE^*)

between the control muffin and the muffins with lentil protein was calculated as follows [169]:

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2\ ]^{1/2} \quad \text{eq. (6.3)}$$

The values used to determine if the total color difference was visually obvious were the following: $\Delta E^* < 1$, color differences are not obvious to the human eye; $1 < \Delta E^* < 3$, color differences are not appreciated by the human eye; and $\Delta E^* > 3$ color differences are obvious to the human eye [169].

Crumb characterizations include the calculation of the number of cells per mm^2 and the mean area per air cell. Images of three slices for each type of product (5 mm thick) were observed and photos were taken with a stereo microscope (Stereo Discovery.V8, Carl Zeiss Microscopy, Toronto, ON, Canada) equipped with a camera (AxioCam MRc5 (D), Carl Zeiss Microscopy, Toronto, ON, Canada). Image analysis was performed using the ImageJ software (Rasband WS., U.S. National Institutes of Health, Bethesda, MD, USA).

Crumb texture was determined by an Instron 5967 universal testing machine equipped with a 50 N load cell (Instron Corp., Norwood, MA, USA), and the texture profiles were tested under a double compression cycle at a crosshead speed of 1 $mm \cdot s^{-1}$ until a deformation of 50% and 60% of initial height was reached for muffins and angel food cakes, respectively. The samples were sliced in 30 mm height × 20 mm width × 20 mm length pieces, after the crust had been removed for the texture profile analysis. Firmness, cohesiveness, chewiness, and springiness were calculated from the force–time diagram (software Blue Hill ver.2, Instron Corp., Norwood, MA, USA). Triplicate samples from each product were analyzed.

6.2.6 Sensory evaluations

Sensory evaluation was conducted to assess the acceptability of appearance, texture, moistness, chewiness, flavor, density, and overall acceptability by a hedonic 9-point scale (1=dislike extremely, 9=like extremely) [84]. The sensory evaluation of angel food cakes was performed by 54 untrained panelists over 18 years of age from staff, undergraduate and graduate students of the University of Alberta (Edmonton, AB, Canada). Three half cake samples coded with random 3 digits of each type of angel food cake were served in a randomized order at room temperature. The panelists were asked to rinse their mouth with water between sample evaluations. The cakes were considered acceptable if their mean scores for overall acceptance were above 5 (neither like nor dislike). The participants also ranked the samples in order of preference, "1" being the most preferred, and "3" being the least. Evaluations were done in individual booths in a standard sensory panel room under white light. The sensory evaluation of muffins was conducted in a similar manner as angel food cakes, but with 70 volunteer panelists.

In addition to the hedonic judgment, a just-about-right (JAR) scale of five points was used to judge the intensity of the following attributes: chewiness, moistness, and density. The just-about-right scale contained verbal anchors ranging from "not at all" (=1) to "much too" (=5), with 3 equal to "just-about-right". This scale measures the consumer's reaction to a specific attribute giving direct information about it, which can be descriptive if the overall product appeal is deficient. When combined with hedonic judgments, the potential impact of being off from the just-right point can be estimated using "penalty analysis" [85]. The approach is simple, consisting of (i) calculating the mean hedonic scores from the acceptability scales for the groups above, below, and at the just-right category, (ii)

comparing the mean of the above-JAR and below-JAR groups with the JAR group, and (iii) plotting the resulting difference (called “mean drop”) in a scatter plot of the mean drop versus the percentage of the total consumer panel in each category. In this plot, any point that shows simultaneously a large mean drop and high percentage of consumers indicates an attribute that can be modified to improve the product [85].

6.2.7 Storage stability

The storage stability of the products was evaluated by monitoring the moisture loss and changes in the texture profile and microstructure after 24, 48, and 72 h of storage in a cabinet at room temperature in order to study product staling [169]. The moisture content was determined using the same method as for baking loss. The crumb texture profile was evaluated using the Instron 5967 universal testing machine by the same method as in section 6.2.5.

Angel food cake and muffin microstructural changes were also observed by Confocal Laser Scanning Microscopy (CLSM) (Zeiss LSM710, Carl Zeiss Microscopy, Jena, Germany). Briefly, a small flat slice (5 mm height × 20 mm width × 30 mm length) was cut from inside the products and placed on a slide. To simultaneously visualize the protein, lipid, and polysaccharides, a multiple fluorescent labelling technique was applied [170]. Rhodamine B was used to label proteins when excited at 605 nm, Nile Red was used to label lipids when excited at 488 nm, Fluorescein iso-thiocyanate (FITC) was used to label starch granules when excited at 515 nm. Labeling of muffins and angel food cakes was done in darkness. Images were processed with the ZEN 2009 LE software (Carl Zeiss AG, Oberkochen, Germany).

6.2.8 Statistical analysis

Samples for physical properties were prepared and tested in triplicate, while for the consumer panel, samples of each treatment were taken from one large batch. A one-way ANOVA of the instrumental parameters was performed to assess the effect of lentil protein incorporation on the physical or sensory characteristics of the angel food cakes and muffins. Least significant differences were calculated by the Tukey test ($p< 0.05$). For the preference ranking, the Friedman's test was used to determine the significant differences. Origin® software (Origin Lab, v.9.2, 2015) was employed for the statistical analysis.

6.3 Results and Discussion

Egg replacers currently available for bakery products are considered as important long-term cost savings [73]. In preliminary trials, the amount of lentil protein to be considered for use in muffins and angel food cakes was screened. Only the protein concentrations that produced similar products to the control with egg and dairy proteins were considered for further testing. For angel food cake, 50 and 75% replacement was applied, whereas in muffins a 100% replacement and more were achievable [166].

6.3.1 Batter specific gravity and rheological properties

To obtain a desirable final product structure, e.g. cakes with high volumes, batters must have low specific gravity and a viscosity value that helps air retention without hindering air incorporation [171]. Batters with very high or low viscosity produce bakery goods with low volume and different texture [171, 172]. Specific gravity value reflects the retention of air and leavening gases by batter, such that a low specific gravity indicates that more air has been incorporated into the batter, which will help to increase its volume during the baking

step [164]. When batter specific gravity increases, storage and loss moduli of batter decrease and so does the final volume of the product [173].

Table 6.2 shows the specific gravity of angel food cake batters of different formulations. The batter of the control angel food cake (AC-0-LPC) had the lowest specific gravity (0.33 g/L), while this value increased significantly with the increasing amount of lentil protein in the formulation ($p<0.05$), indicating less air being incorporated into the batter [174]. Lentil protein possesses good foaming capacity due to the balanced hydrophilic and hydrophobic segments (38 and 40%, respectively), suitable size and high surface hydrophobicity. Nevertheless, egg white protein is still superior in terms of air bubble incorporation and retention in the angel food cake batters. Egg white protein consists of ten different proteins ranging in *Mw* from about 10 to 76 kDa that contain from 2 to 34.7 half-cystine residues per mole [175]. The foaming capacity, which impacts directly the specific gravity of the batter, is mainly associated to globulins and ovalbumin because of their lower amounts of disulfide groups compared to the other egg white proteins [176]. The reduced number of disulfide groups allows the proteins to be partially denatured during the foaming process so that the protein molecules can be adsorbed at the air-water interface and change their conformation, exposing the sulfur containing groups and allowing them to form disulfide bridges with neighboring cysteinyl residues. This latter phenomenon contributes to the formation of a strong interfacial film that helps to retain air in the foam [176, 177]. In addition, the presence of ovomucin gives the solution an adequate viscosity for air retention [176].

Both the control batter and the lentil protein-based batters showed shear-thinning behavior as demonstrated in Figure A4.1 A (Appendix A4) with the control batter reaching the lowest values of apparent viscosity at the higher shear rates, as a result of the lower

specific gravity of the batter. The shear-thinning behavior is caused by the alignment of batter microstructure with the flow direction as the shear rate increases, and consequently the apparent viscosity decreases [162].

Commonly, bakery product batters show shear-thinning behavior [161, 168] and their rheological behavior is frequently represented by the Ostwald-De Waele model [162, 164, 178]. Table 6.2 shows the constants for the model. Flow behavior index (n) was 0.26 for the control batter and 0.17 for both AC-50-LPC and AC-75-LPC batters.

Table 6.2 Rheological and physical properties of angel food cake and muffin batters.

Samples	Flow parameters		Specific gravity	Moisture content
	K (Pa·s)	n	(g/L)	(g/100g)
Angel cakes				
AC-0-LPC	8.74^{a} (0.73)	0.26^{a} (0.02)	0.33^{a} (0.01)	51.5^{a} (2.53)
AC-50-LPC	19.74^{b} (1.41)	0.17^{b} (0.02)	0.37^{b} (0.00)	47.9^{ac} (0.74)
AC-75-LPC	20.25^{b} (0.13)	0.17^{b} (0.02)	0.47^{c} (0.01)	44.7^{bc} (0.29)
Muffins				
M-0-LPC	2.42^{a} (0.07)	0.58^{a} (0.02)	1.14^{a} (0.05)	32.2^{a} (0.30)
M-100-LPC	22.74^{b} (2.61)	0.38^{b} (0.01)	1.15^{a} (0.09)	33.4^{a} (1.95)
M-100H-LPC	71.43^{c} (3.03)	0.29^{c} (0.01)	1.10^{a} (0.04)	36.5^{a} (0.55)

Values in parentheses are standard deviations based on triplicate measurements using independently prepared samples.
$^{(a\text{-}c)}$Means in the same column for each product without a common superscript letter have a significant difference ($p < 0.05$).

When the magnitude of $n < 1$ the fluid is shear-thinning in nature [167], in which the molecular chains undergo gradual rearrangement with the shear rate, resulting in the power law behavior [167]. Low shear rates may disturb the structure to a certain degree, but the chains will still remain entangled. As the shear rate increases, disruption can occur at a faster rate than the chains can re-entangle. The decreased level of molecular entanglement results in a lower viscosity of the liquid, allowing the liquid to flow with less resistance

[179]. The increase in the apparent viscosity of lentil protein-based batters compared to the egg white-based batter suggests a more entangled structure [180, 181] in the angel food cake batters containing lentil protein. The increase in the apparent viscosity of lentil protein-based batters could also be attributed to a higher water holding capacity (*WHC*) of the lentil protein that reduces the amount of free water available to facilitate the movement of particles in the batters [71]. Lentil protein has a much higher *WHC* (close to 4.0 mL/g) [18] compared to that of egg white protein (1.68 mL/g) [182]. Also, the higher viscosity of the lentil protein-based batters might have contributed to their higher specific gravity [164].

Contrary to angel food cake batter, addition of lentil protein did not significantly (p>0.05) affect muffin batter specific gravity (Table 6.2), which ranged from 1.10 to 1.15. Unlike angel food cake batter in which a larger amount of air is incorporated to decrease the specific gravity, muffin batter formation relies on an emulsifying process of oil and water during which air is also incorporated into the mixture to a lesser extent. Muffin batter is a complex fat-in-water emulsion/foam, containing gas bubbles and fat as the discontinuous phase and a mixture of egg, sugar, and water as the continuous phase in which flour particles are dispersed [183]. Air is incorporated into the batter by mixing, while the leavening agent (baking powder) generates CO_2, thus the batter acquires a multiphase structure with immobile gas cells surrounded by the fat and the remainder of the ingredients dissolved or dispersed in the aqueous phase, with protein and egg lecithin acting as emulsifiers [70, 173, 184]. Obtaining similar values of specific gravity in all three kinds of muffins tested might suggest that the lentil protein has a similar capability to incorporate and retain gas in the batter during mixing [71, 72]. The muffin batters also showed the shear-thinning behavior as demonstrated in Figure A4.1 B (Appendix A4), though the difference between the samples of different formulations was more evident, for both the

flow behavior index and consistency coefficient values (Table 6.2). The higher the amount of lentil protein, the higher was the consistency index and the lower was the flow behavior index, indicating that the higher viscosity of the batter was likely due to the more entangled lentil protein structure.

6.3.2 Physical property evaluation of baked products

Lentil protein-based products demonstrated an appropriate appearance as shown in Figure A4.2 (Appendix A4). A high-quality angel food cake is produced when the matrix built on the protein foam remains stable enough to expand during heating and forms a structure in association with gelatinized starch, so that a high volume and low density cake with a fine and relatively uniform crumb structure is formed [31, 185]. Microphotographs of angel food cake sections are shown in Figure 6.1. The observation revealed differences in the internal structural features of control cakes and those with lentil protein (Figures 6.1, A, B and C) in their formulations [175] in terms of size and number of air cells. While the mean area of air cells was reduced by the presence of lentil protein, the number of air cells per unit area increased (Table 6.3) [164], thus the height of angel food cakes with lentil protein remained very close to that of the control formulations (Table 6.3). This might suggest that less air escaped from the lentil protein-based batter than the control during baking probably due to the formation of strong networks around the air cells that prevented microstructure collapse during gas expansion. The high viscosity of the batter with a more entangled structure, as reflected by a higher consistency index of the lentil protein-based angel food cake batters, might help air retention. Since air entrapment and subsequent bubble stabilization in the batter are functions mainly performed by the egg white proteins [72], the similar volume reached by the lentil protein-based angel food cakes indicates that

lentil protein can serve a similar foam stabilization function in cake products, comparable to egg white protein. Lentil protein showed superior properties to maintain cake volume compared to many other egg replacers such as whey protein isolate [175] or hydrocolloids [186] reported in the literature.

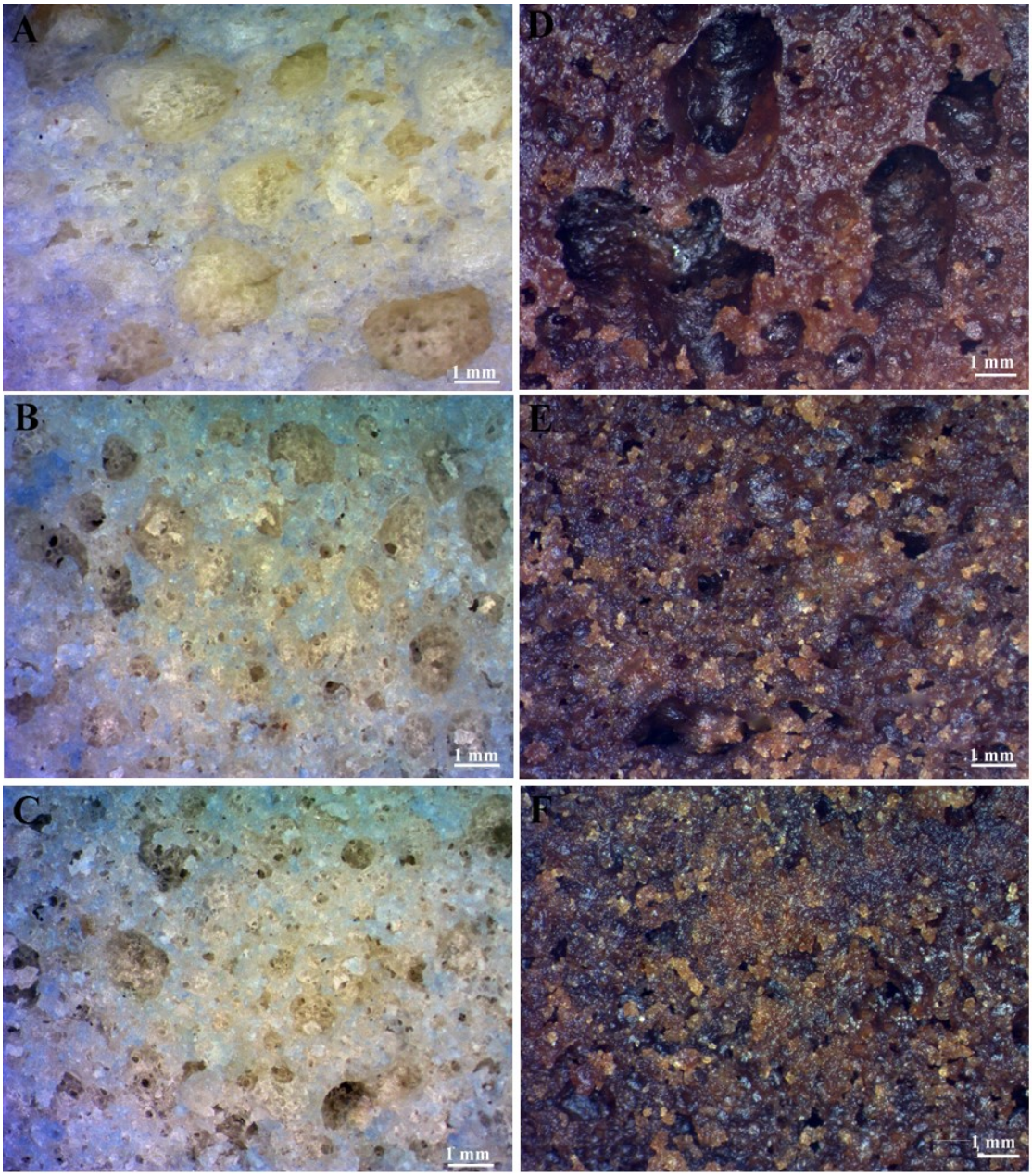


Figure 6.1 Images of angel food cake **(A, B, and C)** and muffin **(D, E, and F)** products obtained by stereo microscope for crumb characterization. **A**: AC-0-LPC, **B**: AC-50-LPC, **C**: AC-75-LPC, **D**: M-0-LPC, **E**: M-100-LPC, and **F**: M-100H-LPC.

All angel food cakes formulated by Arunepanlop et al. [175] with whey protein isolate (>25%) as an egg white replacer resulted in lower cake height than the control (100% egg white), even in the presence of other additives such as xanthan gum and hydroxypropyl methyl cellulose. Lower height was also reported by Ashwini et al.[186] when arabic gum, carrageenan, and guar gum were used in cakes to replace egg protein.

Table 6.3 Physical properties and crumb features of angel food cakes and muffins

Samples	Baking loss (%)	Height (mm)	Color				Crumb features	
			L*	a*	b*	ΔE*	Area of gas cells (mm^2)	Number of gas cell per mm^2
Angel cakes								
AC-0-LPC	37.7^{a} (0.00)	37.3^{a} (1.6)	97.0^{a} (1.7)	-1.68^{a} (0.4)	16.3^{a} (0.7)		0.31^{a} (0.87)	0.84^{a} (0.02)
AC-50-LPC	25.9^{b} (0.12)	37.0^{a} (1.0)	88.0^{b} (1.5)	0.62^{b} (0.6)	16.8^{a} (0.4)	18.9	0.21^{a} (0.50)	1.53^{b} (0.03)
AC-75-LPC	5.1^{c} (0.16)	37.0^{a} (0.5)	85.9^{b} (0.9)	2.18^{c} (0.7)	17.0^{a} (0.6)	21.2	0.16^{a} (0.87)	1.62^{b} (0.03)
Muffins								
M-0-LPC	25.5^{a} (0.04)	43.7^{a} (0.5)	57.5^{a} (3.7)	8.56^{a} (1.1)	3.84^{a} (1.7)		0.31^{a} (1.45)	1.11^{a} (0.03)
M-100-LPC	11.5^{b} (0.11)	43.0^{a} (0.8)	60.9^{a} (0.6)	3.91^{b} (0.0)	-2.74^{b} (0.3)	8.73	0.09^{b} (0.23)	1.96^{b} (0.03)
M-100H-LPC	3.5^{c} (0.06)	44.0^{a} (0.0)	61.0^{a} (3.8)	3.60^{b} (0.1)	-2.59^{b} (0.1)	8.85	0.09^{b} (0.37)	2.56^{c} (0.02)

Values in parentheses are standard deviations based on triplicate measurements using independently prepared samples.
$^{(a-c)}$Means in the same column for each product without a common superscript letter have a significant difference ($p < 0.05$).

In muffins, similar to other fat containing bakery products, air cells incorporated during mixing are released from the fat phase during the first stage of baking and they migrate to the aqueous phase. Primarily, air is entrapped by egg proteins forming mechanically strong viscoelastic films [69-71]. As the temperature rises, the viscosity of the batter decreases and the air bubbles expand resulting in the muffin rising, while the sodium aluminum sulfate and calcium acid phosphate present in the baking powder react to form CO_2 increasing the volume of the muffin. Also, some bubbles move toward the surface where some are released to the surroundings [70, 183]. Microphotographs of muffin sections are shown in Figures 6.1 D, E, and F. The muffins containing lentil protein were more dense with more air cells per unit area but the mean area of the air cells was significantly decreased (p<0.05)

compared to that of the control (Table 6.3). Though the batter and muffin formation is different from that of angel food cake, a similar result was found regarding the height of muffins, which was also not significantly impacted by the presence of lentil protein. This result indicates that lentil protein can also perform as a good emulsion and foam stabilizer in muffin products.

Baking loss is important for the weight and quality of the final baked products. In addition, it affects the freshness of the baked goods, which age earlier and become stale when too much moisture loss occurs [187]. The water retention capacity of the products is shown in Table 6.2. Angel food cakes with 50% and 75% of egg replacement had significantly lower baking loss (25.9 and 5.1 %, respectively) than the control (37.7%) (p<0.05). Similarly in muffins, the baking loss was significantly decreased from 25.5 to 3.5% when the amount of lentil protein was increased. This suggests a greater water holding capacity of lentil protein compared to egg protein. The water holding capacity of proteins is linked to the type and number of polar groups in the polypeptide chain [188, 189]. The better water binding properties observed for lentil protein may be partially related to its higher aspartic acid content, which is able to bind 4 to 7 water molecules [188]. A greater holding capacity of water in the cakes was also observed in pound cakes prepared with whey protein isolate [72].

Another quality parameter in bakery products is the color. The color is linked to the contribution of some ingredients, such as flour and egg yolk, and also to the Maillard reaction and caramelization taking place during the baking stage [69]. The interior crumb color of the angel food cakes is shown in Table 6.3. The interior of an angel food cake is known for its whiteness [178]. Both samples, AC-50-LPC and AC-75-LPC showed a significant decrease (p<0.05) in the L* value (less white) and a significant increase

(p<0.05) in the a* value (greenness) compared to the control. The color differences resulted in ΔE* values much higher than 3, indicating that both lentil protein-based cakes showed a clear color difference that was appreciable by the human eye. This was caused by the addition of a lentil protein ingredient, which exhibited a light brown color. The color of the lentil protein concentrate might be attributed to the presence of phenolic compounds bound to the protein during the extraction process, forming a protein-phenolic complex [26]. This complex, which is known to produce cross-linking and structural changes in bread dough [190], might also be partially responsible for the entangled structure and increased viscosity of the products containing lentil protein [190].

For muffins, the higher concentration of lentil protein led to a significant (p>0.05) decrease in the a* (redness) and b* (blueness) values of the final products. L* value (whiteness) increased in the lentil protein containing muffins, though it was not significantly different compared to the control (p>0.05). This also produced a color difference appreciable by the human eye (ΔE*> 3) attributed to the combined color of the lentil protein and the cocoa powder.

6.3.3 Sensory evaluation

Sensory panel results are presented in Table 6.4. For angel food cakes, appearance and texture showed a significant difference (p<0.05) between the lentil protein formulations and the control, both of which received lower scores. It is interesting that the formulation with 50% replacement obtained a slightly higher score of flavor than the control, which might be attributed to the "nutty" flavor from lentil protein according to panelist comments. Also, a higher percentage of consumers described the moistness of the AC-50-LPC product as "just-about-right" compared to the control (Figure A4.4 A, Appendix A4) due to the high

water holding capacity of the lentil protein. Texture, chewiness, and density of the angel food cake were negatively impacted by the addition of the lentil protein to a certain extent due to the high specific gravity and viscosity (consistency index) of the batter, which are directly related to the final cake quality [79, 161] because of their effect on the crumb structure.

A high specific gravity and viscosity of the batter will impact the amount of air incorporated at the mixing stage and the size of bubbles during the heating stage and it will likely produce a denser crumb [68, 172]. Indeed, the formation of an angel food cake with a denser structure when lentil protein was added has been confirmed by the morphological observation in Figure 6.1 (A, B and C). It is likely that the appearance was affected by the color of the lentil protein-based cakes, which were expected by the panelists to be as white as the control.

Table 6.4 Effect of lentil protein incorporation on sensory characteristics and preference ranking of angel food cakes and muffins

Attribute	Angel food cake			Muffin		
	AC-0-LPC	AC-50-LPC	AC-75-LPC	M-0-LPC	M-100-LPC	M-100H-LPC
Appearance	5.9^{a} (1.6)	5.1^{b} (1.7)	4.4^{b} (1.7)	6.5^{a} (1.6)	7.0^{a} (1.3)	6.9^{a} (1.4)
Flavour	5.3^{a} (1.5)	5.4^{a} (1.6)	4.8^{a} (1.5)	5.8^{a} (1.7)	6.0^{a} (1.6)	5.7^{a} (1.7)
Chocolate flavour				5.7^{a} (1.9)	6.4^{b} (1.7)	5.5^{a} (1.6)
Texture	5.4^{a} (1.9)	4.7^{a} (1.7)	4.1^{b} (1.7)	5.6^{a} (2.0)	6.3^{b} (1.7)	6.0^{ab} (1.7)
Overall acceptability	5.1^{a} (1.8)	4.8^{a} (1.7)	4.4^{a} (1.7)	5.5^{a} (1.9)	6.4^{b} (1.6)	5.7^{a} (1.6)
Preference ranking	1.7^{a} (0.8)	1.8^{a} (0.7)	3.0^{b} (0.0)	2.2^{a} (0.8)	1.6^{b} (0.7)	2.3^{a} (0.8)

Values in parentheses are standard deviations (n=54 for angel food cake, and n=70 for muffin)
$^{(a\text{-}c)}$Means in the same row for each product without a common letter differ significantly ($p < 0.05$) according to Tukey or Friedman's test.

It is interesting that the sensory panel results for muffins (Table 6.4) showed that the M-100-LPC muffin was preferred by the consumers and received higher scores for all attributes compared to the control and the high-protein muffin ($p<0.05$). No significant differences were found between the high protein muffin (M-100H LPC) and the control

(p>0.05). According to panelist comments, incorporation of the high amount of lentil protein brought a “beany” flavor to the muffin. A higher percentage of consumers described the chewiness, the density, and the moistness of the muffin as “just-about-right” compared to the control (Figure A4.4 B, Appendix A4).

Figure 6.2 A shows the mean drops in liking as a function of the proportion of consumers that checked an attribute differently from that for the ideal product for angel food cake formulations containing lentil protein (Figures A4.4 A and B, Appendix A4). The penalty analysis supported the identification guidelines for product improvement for each of the samples. In the case of angel food cake (both, AC-75-LPC and AC-50-LPC), it is suggested to reduce the density and the chewiness and increase the moistness of the samples. In the case of muffin formulations, the main sensory problems were associated with the excessive chewiness, density, and lack of moisture for sample M-100H-LPC. The latter might be related to the least baking loss experienced by the product. More bound water together with a more dense structure in this muffin may signify that the moisture is not easily released during and after the baking stage, which later is perceived by the consumers as a less moist muffin compared to the M-100-LPC sample. Finally, for muffin M-100-LPC the percentage of consumers who stated that the attributes deviated from the ideal was lower than that for M-100H-LPC and very close to the stated cut-off, in agreement with the higher overall liking score of the former sample. The main deviations from the ideal and penalties for this sample were related to the chewiness, density, and moistness, which should be adjusted in minor proportion.

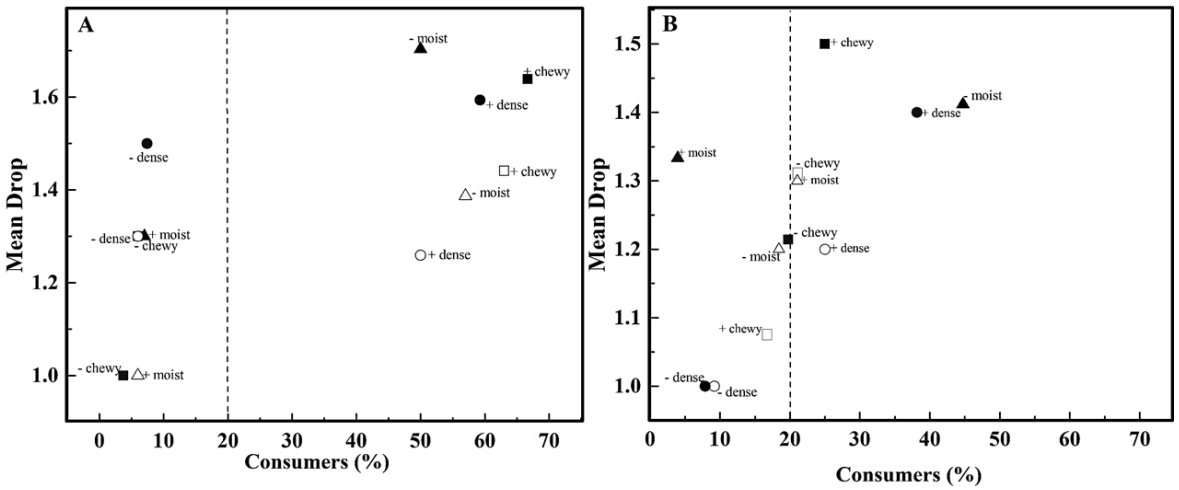


Figure 6.2 Mean drops in overall liking as a function of the percentage of consumers that checked an attribute differently than for the ideal product for angel food cakes **(A)** and muffins **(B)**. Dashed line represents the cut-off point (20%). Attributes: chewiness (square), density (circle), and moisture (triangle). Formulations: **(A)** AC-50-LPC (solid symbols) and AC-75-LPC (open symbols); **(B)** M-100-LPC (solid symbols) and M-100H-LPC (open symbols).

6.3.4 Texture profile analysis (TPA) and storage stability

The texture profile analysis was conducted for fresh products and throughout storage. The compressive stress values (Figure 6.3 A) of all fresh angel food cakes did not show a significant difference (p>0.05). All the samples, including the control showed increased compressive stress during storage with the value being doubled (control and AC-50 LPC) or tripled (AC-75 LPC) on day three. This dramatic increase in compressive stress was due to cake staling [72, 164]. Staling is a very complex phenomenon mainly related to the migration of water and the starch retrogradation during storage, which leads to product hardening [69]. Angel food cake is prone to suffer staling due to the light and airy structure of the product [69]. Its more open cell structure increases the rate of moisture diffusion from the crumb to the crust and surroundings, which also speeds up crumb drying [72, 191]. Additionally, the cakes prepared with the lentil protein presented higher chewiness values than those prepared only with egg white protein [84, 164]. This can be attributed to a reinforcement of the crumb walls surrounding the air cells due to the synergistic effect between egg white protein and lentil protein. The formation of reinforced crumb walls was

also observed in cakes containing egg white protein and whey protein or chickpea protein [62, 72].

The increased chewiness detected by the TPA was also perceived by the consumers during the sensory panel, as presented in section 6.3.3. Other parameters such as cohesiveness and springiness were not affected by the presence of lentil protein in the formulation (Figure A4.5, Appendix A4) and did not significantly change during 72 h of storage. Cohesiveness quantifies the internal resistance of food structure, while springiness gives information about the ability to recover after compression [84].

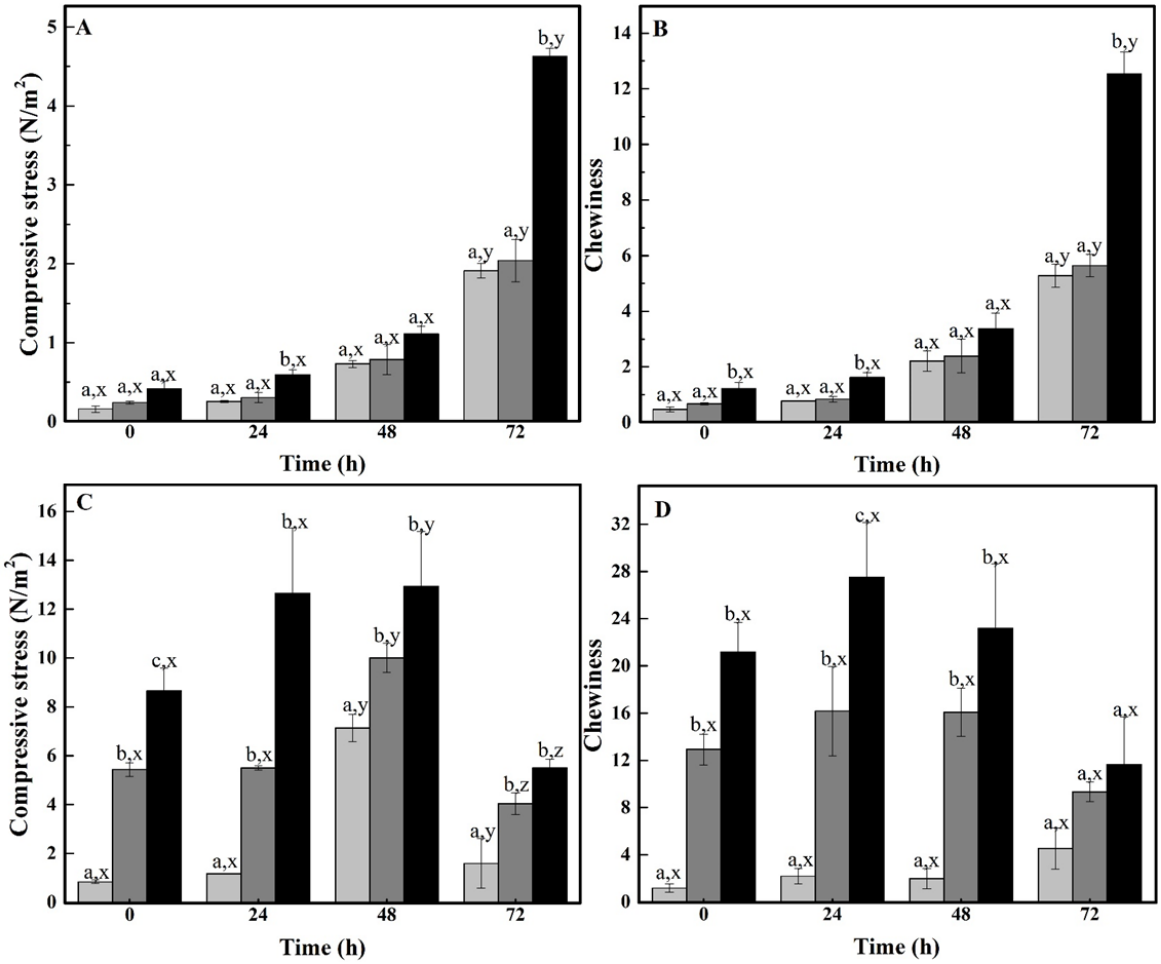


Figure 6.3 Evolution of compressive stress and chewiness with storage time for angel food cake **(A, B)** and muffin **(C, D)** products determined by TPA. For angel food cake: light gray: AC-0-LPC, gray: AC-50-LPC, black: AC-75-LPC. For muffins: light gray: M-0-LPC, gray: M-100-LPC, black: M-100H-LPC. Bars without a common letter have a significant difference ($p < 0.05$).
[a,b,c]: Significant difference between samples at a fixed time period. [x,y,z]: significant differences between days for the same sample.

According to Wilderjans et al. [192] and Moore et al. [193], cohesiveness and springiness are directly impacted by the elastic network developed by the proteins present in the dough, i.e. gluten and egg proteins. The lack of a well-developed gluten network will produce a less cohesive and elastic crumb. These results suggest that addition of lentil protein did not significantly impact gluten functionality.

The muffins with lentil protein had a significantly higher compressive stress ($p< 0.05$) over the total storage time compared to that of the control muffin. The compressive stress values decreased for all the samples at 72 h. The muffins prepared with lentil protein showed higher chewiness values than the control, which was also detected by the sensory panelists [194]. Cohesiveness (Figure A4.6 A, Appendix A4) values of lentil protein-based muffins were initially higher than the control during the first 24 h, decaying to lower values after that. The control muffin, followed the same trend, but it decayed after 48 h. In general, cohesiveness is considered as a measure of the resistance of the cake structure to compression, reflecting the development of internal bonding in a three-dimensional protein network [72]. The higher cohesiveness values of lentil protein-based muffins in the first 24 h would indicate a higher mechanical resistance, which might be associated with a strong protein network [62, 195]. It is possible that lentil protein had interacted with gluten, making a stronger network [62]. Cohesiveness is related to the internal water, which hydrates the gluten that forms the structural matrix of the dough [194]. Loss of cohesiveness during storage time is a phenomenon usually present during aging, principally produced by redistribution of the water from the gluten to the gelatinized starch and expressed as higher crumbliness and lower compressive stress of the product [164, 194, 195]. This could explain the decrease of the muffin compressive stress over storage. The

springiness value (Figure A4.6 B, Appendix A4) was not affected by the incorporation of lentil protein.

Figure 6.4 shows the changes in water content of the angel food cakes and muffins. The migration of water from the crumb to the crust and then to the surroundings reduces the water content of the bakery products [69]. It is interesting to notice that the presence of lentil protein in both angel food cakes and muffins helped to delay the water loss. This greater moisture retention capacity might also contribute to the higher values of chewiness of the products with lentil protein, making them denser to chew [164]. Doughs that contain compounds with high water holding capacity, as lentil protein, are denser and as the density increase also the firmness (compressive stress), and thus the chewiness of the cakes [196].

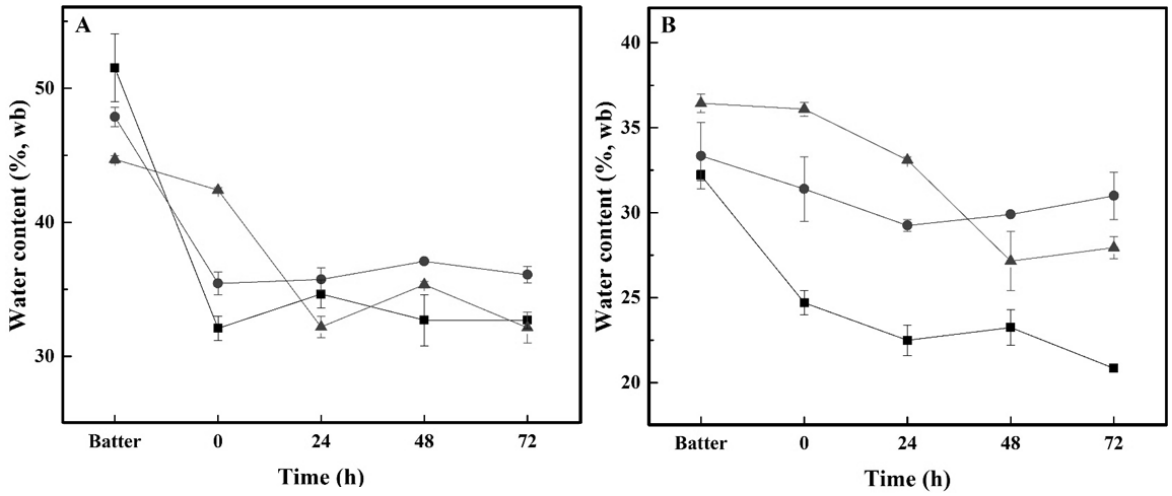


Figure 6.4 Water content as a function of time for angel food cakes **(A)** and muffins **(B)**. In angel food cakes: AC-0-LPC (squares), AC-50-LPC (circles), and AC-75-LPC (triangles). In muffins: M-0-LPC (squares), M-100-LPC (circles), and M-100H-LPC (triangles).

Overall, the storage stability of the lentil protein-based angel food cakes showed the same trend as the control, with a few significant differences during the storage time.

Actually, 50% replacement of egg white by lentil protein did not significantly change the storage stability of the angel food cake. Muffins containing lentil protein also followed the same trend as the control, though they showed significantly higher compressive stress and chewiness values during storage.

6.3.5 Confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopy (CLSM) was used to obtain images to identify different components in the angel food cakes and muffins (Figures 6.5 and 6.6, respectively) during storage. Figure 6.5 shows the microstructure of angel food cakes on day one (A, C, E) and day three (B, D, F) after preparation. All cakes present similar morphology at the microscopic level with the formation of protein (red) and starch (green) networks formed by strands and sugar (cyan) distributed within this matrix. However, some differences are apparent. The control sample (AC-0-LPC), present a loose structure on the first day of storage, while the cakes containing lentil protein have a denser and more compact structure, and the protein and starch strands are thicker than those in the control (Figures 6.5 A, C, E). The thicker protein strands were a result of a more entangled structure produced by the presence of lentil protein, as explained in section 6.3.1. The synergistic effect between the egg white and lentil protein may also help the reinforcement of the air cell wall. The denser starch strands in the cakes containing lentil protein may be related to the higher amount of water (Figure 6.4 A) in the freshly prepared samples (37% and 43% for AC-50-LPC and AC-75-LPC, respectively) due to the high water binding capacity of the lentil protein, which allowed a faster and more extensive retrogradation of the starch granules after cooling. Subsequently, denser starch strands were formed by starch aggregation linked by the amorphous regions [197]. Both phenomena, starch and protein

strand thickening would explain the higher compressive stress and chewiness of the fresh angel food cakes containing lentil protein. After 72 h of storage, the matrix structure of all angel food cakes became denser and more compact than the freshly prepared sample (Figure 6.5 B, D, and F). The protein network of the samples containing lentil protein (AC-50-LPC and AC-75-LPC) showed thicker and apparently stronger strands, while starch appeared thinner and more compact likely due to a more extensive retrogradation during storage. The latter was apparent by the decrease of the fluorescence intensity, which is caused by a reduced starch structural integrity due to retrogradation [69, 198]. Also, sugar gathered in certain parts of the foam. These changes are mainly due to a transfer of moisture from starch to gluten as the starch crystallizes and from gluten to starch during aging. The decrease in water mobility in the cakes upon staling is due to the release of water molecules from gluten into the crystalline structure of starch that develops upon staling because of the higher affinity of starch to water [69, 197]. In addition, water also moves from the crumb to the crust, reducing the amount of water available [199]. Riva et al. [199] suggested that when water mobility is high and there is a low amount of water available to sustain starch retrogradation, retrogradation may occur quickly, but in smaller extent, which might be the case for the control sample with less amount of moisture available. However, angel food cakes containing lentil protein have a higher water content, thus retrogradation was possibly slower and more extensive, leading to the higher values of compressive stress and chewiness during storage [191]. Also, crystallization of starch fractions contributed to the rigidity of the crumb structure, thus increasing compressive stress and chewiness [69].

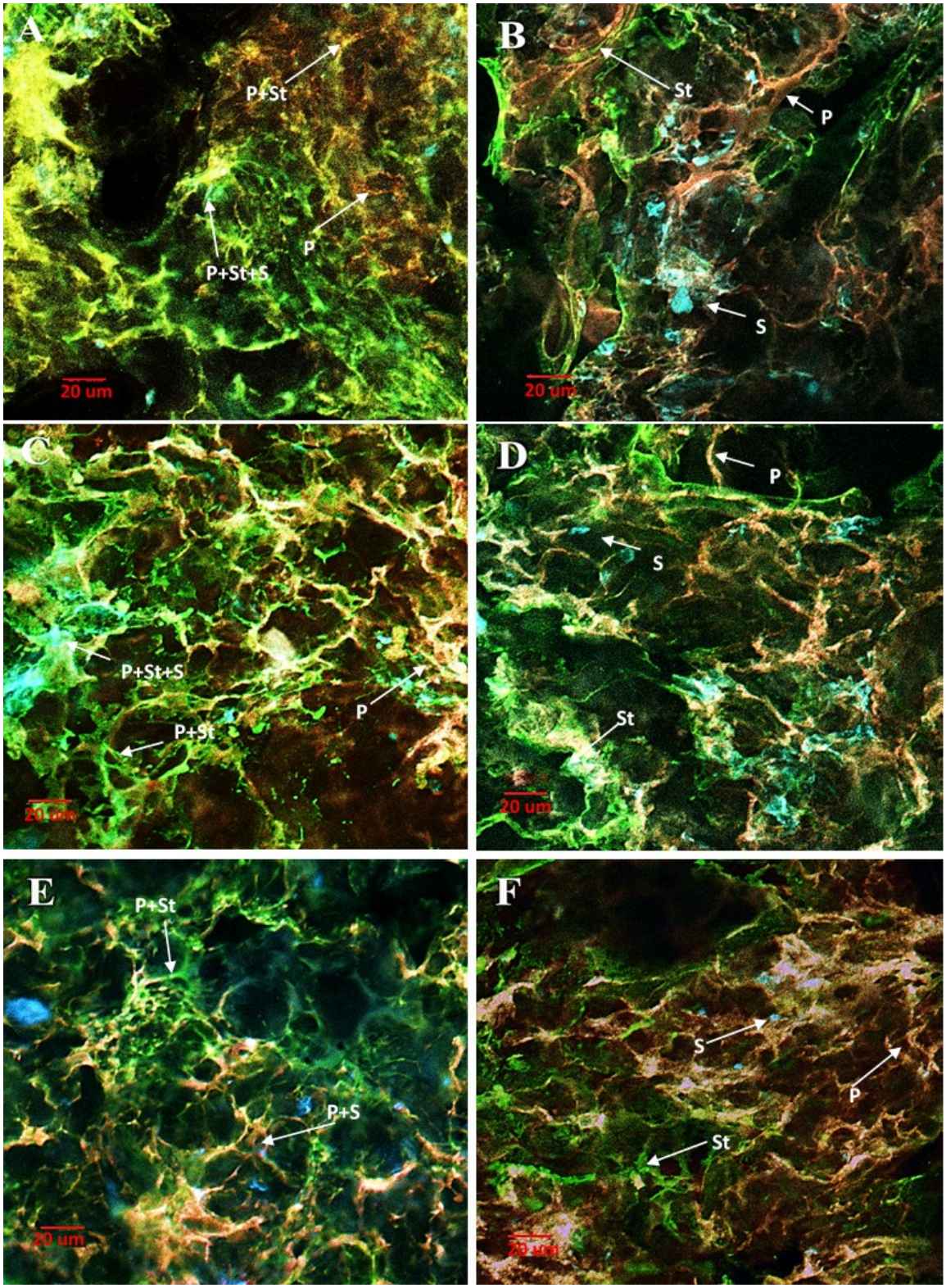


Figure 6.5 Confocal scanning laser micrographs of angel food cakes at day 1 (**A, C, E**) and 3 of storage (**B, D, F**). AC-0-LPC (**A, B**), AC-50-LPC (**C, D**), and AC-75-LPC (**E, F**).Triple labelled to show starch (St) (pale/dark green); protein (P) (red/pink); sugar (S) (cyan)

In general, muffin is considered as a solidified highly aerated emulsion, and therefore, it is a three-dimensional network [200] formed by high amounts of starch, and lower amounts of oil, and protein (Table 6.1). Confocal microscopy images of the three muffin formulations are shown in Figure 6.6. Unlike bread, muffin batter is not an optimal-mixed system, so only discontinuous protein domains are formed by gluten, egg, and milk proteins [201]. The addition of lentil protein did not impact the overall microstructure of the fresh muffins. After 24 h of storage time, the starch (green) and small oil globules (cyan) formed clusters associated with the disconnected protein-rich domains (red/pink) (Figure 6.6 A, C, and E) [201]. However, due to its higher protein content, the M-100H-LPC sample showed a matrix where both starch granules and large protein-rich areas exist [203]. It is likely that such a highly dense structure composed of starch and lentil protein matrices conferred strength to the crumb increasing the compressive stress and chewiness.

After 72 h, the protein networks in all muffins seem to be shrinking and/or losing connectivity, which explains the loss of cohesiveness of the networks, leading to reduced compressive stress, chewiness, and cohesiveness at this storage time. This could be related to the presence of higher amount of lipids in the muffins compared to the angel food cake. Figure 6.6 shows the oil globules forming larger clusters, including some that have already migrated towards the starch matrix. Lipids in cakes are known for breaking the continuity of the protein and starch structures that form the crumb [204]. Although the mechanism is still not very clear, the migration of lipids in cakes during storage has been related to softer crumbs with lower values of cohesiveness [205].

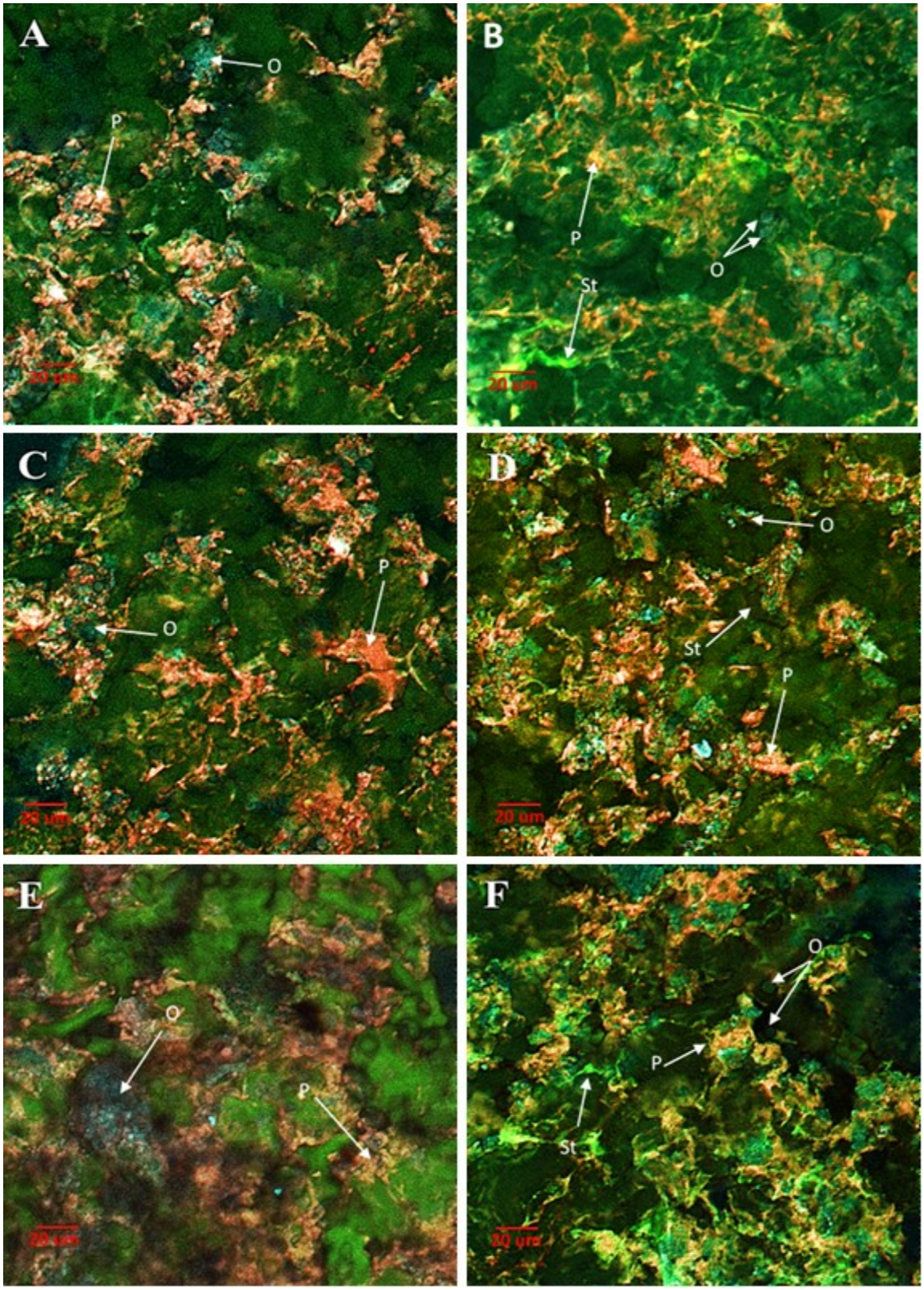


Figure 6.6 Confocal scanning laser micrographs of muffins at day 1 (**A, C, E**) and 3 of storage (**B, D, F**). M-0-LPC (**A, B**), M-100-LPC (**C, D**), and M-100H-LPC (**E, F**). Triple labelled to show starch (St) (bright/dark green); protein (P) (red); lipid (O) (cyan).

6.4. Conclusions

This research has demonstrated good potential to use lentil protein to replace or partially replace egg/milk proteins in bakery products. No significant change of the product volume was observed in the angel food cakes and muffins containing lentil protein compared to the control. In addition, lentil protein did not impact the dough network formation, instead, contributed to holding the crumb structure after baking by the formation of an entangled network structure. Moreover, lentil protein showed a strong water holding capacity that reduced the baking loss. On the other hand, the incorporation of lentil protein increased compressive stress, chewiness, and density of the products. Sensory tests showed that the angel food cake formulation with 50% replacement had a “nutty” flavor and a higher moisture that were appreciated by the consumers. In addition, muffin containing lentil protein in a similar percentage as egg and milk was preferred by the consumers and received higher scores for almost all the attributes. The high protein muffin showed significant differences in texture compared to the control and a “beany” flavor. A 50% replacement of egg white by lentil protein did not significantly change the storage stability of the angel food cake. Muffins containing lentil protein also followed the same trend as the control during storage, though they showed higher compressive stress and chewiness values.

CHAPTER 7

Conclusions and Recommendations

The overall goal of this Ph.D. thesis research was *"to achieve a more complete knowledge of the lentil proteins and use this in the potential development of value-added food products."*

The specific objectives targeted for achieving the overall goal and the main points of conclusion that can be drawn from the research are summarized in the following sections.

7.1. Findings and conclusions related to specific objectives 1 and 2

1) **To develop a protein extraction protocol based on the alkaline method to prepare a lentil protein isolate or concentrate.**
2) **To characterize lentil protein molecular structure and functionality.**

- Response surface methodology was used to optimize alkaline extraction of protein from lentil flour to maximize both protein content and yield. Solid/solvent ratio and pH were the significant factors that determined protein extraction efficiency. At the optimized condition of pH 9.0 and a solid/solvent ratio of 1:10 (w:v), a yield of 14.5 g of protein extract/100 g of flour was obtained with a protein content of 82 g/100 g of extract after 1 h of extraction at 22 ^{o}C. Neither time nor temperature had a significant effect on these two parameters.
- The impact of extraction pH on the molecular structures and functionality of lentil protein was investigated. Increasing the extraction pH to 10 caused partial protein hydrolysis and unfolding as suggested by size exclusion high performance liquid chromatography (SE-HPLC) and Fourier transform infrared spectroscopy analysis (FTIR), leading to improved protein solubility and gelling property. SE-HPLC

chromatograms and SDS-PAGE patterns showed that globulins were the major protein in the extracts.

- The pH influenced protein solubility and surface charge, and subsequently the gelling and foaming properties. Excellent foaming capacity was identified in lentil proteins comparable to that of whey and egg proteins. This superior functionality may provide an opportunity for lentil protein to be used as a foaming agent of plant origin in cakes, dairy products, a variety of desserts and drinks, and especially in mildly acidic foods such as fruit-based products.

7.2. Findings and conclusions related to specific objective 3

3) To separate and purify lentil legumin-like protein and study the effects of pH on foaming and surface properties in relation to protein molecular structures.

- Lentil legumin-like protein (13S) was successfully separated and purified (83% w/w) from lentil protein concentrate using rate-zonal centrifugation method, which also allowed to identify it as a 13S type protein.
- SE-HPLC chromatogram of the lentil legumin-like protein showed that its weight-average molecular weight is 347 kDa and SDS-PAGE under reducing conditions showed three bands with relative molecular weights of 32, 42, and 47 kDa, respectively, which would correspond to the acidic polypeptide chains and two basic polypeptide chains of 18 and 20 kDa.
- Molecular characterization and the surface activity study revealed that fast diffusion together with high surface hydrophobicity and solubility were responsible for lentil legumin-like protein's high foaming capacity.

- Lentil legumin-like protein was capable of forming long-life foams at both acidic and neutral pH with a mean lifetime of 51 and 84 min, respectively.
- Studies of the foaming kinetics and protein conformation suggested that the foaming stability of lentil legumin-like protein was dependent on the conformation of the protein at the air-water interface, which strongly affected adsorption and re-organization of the protein layer at the interface.
- Foams prepared at neutral pH were especially stable where the combination of the α-helix secondary structure, medium hydrodynamic molecular size, and balance between solubility/hydrophobicity all contributed to building strong protein networks at the interface.
- At pH 5.0, the protein formed dense and thick interface networks composed of randomly aggregated protein particles. Whereas at pH 3.0, the smaller hydrodynamic size and high surface hydrophobicity led to the formation of an initial elastic surface layer. However, the unordered structure increased intra-protein flexibility, producing a less compact structure and relaxed interface that reduced the elasticity modulus with time. Thus, the foam would have a lower resistance against collapse.

7.3. Findings and conclusions related to specific objective 4

4) **To investigate foaming properties of lentil legumin-like protein-polysaccharide mixtures, and explore the impact of polysaccharide on their foaming and surface properties.**

- Protein-polysaccharide interactions due to pH variation had a different impact on the foaming properties of lentil legumin-like protein-polysaccharide mixtures.

Foaming capacity (*FC*) was not significantly changed when polysaccharides were added to the solution, while foam stability (*FS*) was greatly affected.

- When lentil legumin-like protein was mixed with guar gum, xanthan gum, and pectin, *FS* was remarkably increased at pH 5.0 (approximately 2.5 to 5.5 fold). Similarly, at pH 3.0, the polysaccharides improved the *FS*, though the highest increase was around 3.5 fold. At pH 7.0 the *FS* of the mixtures was poor, reducing the mean life of the original foam.
- At pH 3.0, the associative interactions dominated and a coacervation phenomenon was observed. The presence of these coacervates may have stabilized the foams against collapse because they produced an increase in the foam viscosity and formed a coacervate network that might have a gel-like network behavior.
- At pH 5.0, protein-polysaccharide aggregates were formed. Such aggregates adsorbed to the interface to form strong and thick interfacial networks, avoiding coalescence and coarsening of the foams. Aggregates also plugged the junctions of the Plateau borders, slowing down the drainage by a jamming effect. They also dramatically increased apparent viscosity of the foams with guar gum and pectin, thus favoring the immobilization of the lamellar water surrounding the gas bubbles, improving the stability of the foam against drainage.
- Limited or segregative thermodynamic incompatibility phenomena at pH 7.0 produced a phase separation of protein and polysaccharide in the bulk and at the interfacial protein membrane according to CLSM micrographs. Phase separation induced a disruption of the protein layer around the bubbles making it weaker and easier to break, which have a negative impact on the foam stability. This

disturbance in the protein film might be a result of partial displacement of the protein by the polysaccharides through a mechanism analogous to an orogenic process.

7.4. Findings and conclusions related to specific objective 5

5) **To assess lentil protein as an alternative ingredient to replace egg protein in bakery products and its effect on their rheological, physical, and sensory properties.**

- Replacement of egg protein by lentil protein in angel food cake and muffin impacted the specific gravity and consistency index of batters, which reduced the mean area of air cells but increased the number of air cells per unit area, and thus the volume of the final product was not affected.
- Lentil protein can totally or partially substitute egg white protein in cakes producing final products with appropriate appearance, flavor, and moisture. Yet, further improvement of the formulations may be achieved by increasing the amount of water added, especially in the “high protein content” muffin.
- The substitution of egg protein by lentil protein produced denser cakes with higher values of compressive stress and chewiness than the control during storage.
- Other mechanical properties were affected in a different way, according to the type of bakery product. In angel food cakes, cohesiveness and springiness were not affected by the presence of lentil protein in the formulation, which indicated that lentil protein did not impact egg or gluten functionalities and a sufficiently strong protein network was developed. Whereas in muffins, cohesiveness was

impacted suggesting some kind of interaction between lentil protein and other ingredients that needs to be further investigated.

- Although, under the current process conditions, lentil protein did not perform with the same effectiveness as egg white foaming agent in angel food cake, in muffins it seems to perform as effective as egg/milk proteins.

7.5 Significance of this work

Rising cost of animal protein-based ingredients, growing dietary preferences (e.g., gluten-free and vegan) and consumer demand for healthier ingredients are leading market trends towards lower cost and abundant plant-based alternatives. Therefore, plant proteins are significantly gaining market as food ingredients and for bio-based material applications.

In this context, legume proteins are attracting consumers because of the nutritional and health benefits they offer. However, its incorporation into food products is still a big challenge for the food industry due to the technological and consumer acceptance issues. The findings of this research showed the excellent foaming properties of lentil protein and demonstrated the feasibility of utilizing it as an egg/milk replacer in bakery products.

To the best of our knowledge, this work is the first to address in a systematic manner the foam stability mechanism of lentil protein and its relationship with the protein structural changes at the air-water interface with and without the presence of polysaccharides.

The findings from this investigation have deepened and expanded the necessary knowledge to facilitate wider applications of lentil proteins. Hence, it may help to promote further investigation and utilization of lentil protein in the food and non-food industry. Value-added applications of lentil protein will allow the generation of additional revenues to benefit lentil producers and processors. This will also allow the development of new

health food ingredients and food products from lentil protein to benefit the food industry and consumers.

7.6 Recommendations for further work

Protein functional properties are dictated to a large extent by a protein's physicochemical and structural properties, which are influenced by the extraction method and processing conditions. This research has systematically investigated how extraction pH and environmental pH conditions impact the structure and functional properties of the lentil protein. Based on optimization of extraction pH and solid-to-solvent ratio using response surface methodology, a purity of 90% was predicted for the protein extract obtained. Yet, extractions performed under optimized conditions resulted in only 82% protein purity, likely due to the exclusion of other parameters in the model due to their low impact on the response. Also, the impact of other environmental factors that are also important for extraction and potential protein applications, such as the ionic strength and temperature were not addressed. It would be beneficial to understand the molecular structure of the lentil protein and its functional properties as impacted by such factors. As globulin proteins are soluble in salt solution, investigating ionic strength as a factor would help to determine the appropriate concentration of salt to add to the alkaline solution to maximize protein recovery and purity. In addition, further investigation on the impact of the other components present in the lentil protein concentrate is essential. As reported in section 3.3.1, the lentil protein concentrate obtained in lab-scale contained 1.6% lipids, 8% carbohydrates and 2.6% ash based on proximate composition analysis. Even though not analyzed in this research, it is also expected that the protein concentrate would contain phytic acid and phenolic compounds, which may form complexes with proteins. Therefore,

better understanding of the interactions of these components with proteins and their impact on functionality is critical. Moreover, other protein fractions beside the 13S fraction, and their contribution to lentil protein functionalities remains to be performed for full utilization of lentil protein.

This research revealed the underlying mechanism responsible for the stability of foams stabilized by lentil legumin-like protein alone, or in combination with other polysaccharides. However, it is still necessary to study the kinetics of the diffusion and adsorption of protein in lentil protein-polysaccharide systems. This should include the effect of a sequential adsorption instead of a co-adsorption of the polymers and the ratio of biopolymers mixed on the kinetics and the foaming properties. Other phenomena that may impact the foam stability should also be addressed, e.g. bubble size, interfacial elasticity, etc.

This research has demonstrated the great potential of lentil protein as a foaming ingredient. Nevertheless, it is necessary to improve the formulation and processing conditions to ensure optimum performance of the lentil protein as a foaming agent or egg replacer to achieve better final products. The challenges associated with scale up of protein extraction should not be overlooked. The purity of the protein extract obtain at pilot scale (Chapter 6) was 78.7% (d.m.), lower than that achieved at lab scale. As well, the protein recovery was 47% (Chapter 3). Therefore, further processing improvements are needed to increase protein recovery to ensure economic viability of the process. It is necessary to do a cost analysis of the lentil protein production to understand and improve its competitiveness in the global market place. Despite the demonstrated potential, further work is necessary to make the lentil protein a cost-effective functional ingredient.

Finally, this thesis research revealed the ability of lentil protein to form long-life foams at mild acidic pH without modifying the protein or using other compounds. Such a unique property in conjunction with the health and nutritional benefits may allow lentil protein to be used as a new foam stabilizer in mild acidic beverages such as fruit smoothies.

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APPENDIX A1

Table A1-1
Independent variable values of the process parameters and their corresponding levels.

Independent variable	Symbols	Level	
		-1	1
Temperature (°C)	X_1	22	40
pH	X_2	9	11
Solid/solvent ratio (w/v)	X_3	1:20	1:10
Time (min)	X_4	60	120

Table A1-2
Screening design arrangement, codified factors, variation levels, center points, and response for protein content and yield.

Run	Coded variables				Uncoded variables				Protein content,	Yield
	X_1	X_2	X_3	X_4	x_1	x_2	x_3	x_4	g protein/100 g extract	g extract/ 100 g flour
1	-1	-1	-1	-1	22	8	1:20	60	82.22	13.41
2	-1	1	1	-1	22	10	1:10	60	80.57	15.98
3	1	-1	-1	-1	40	8	1:20	60	82.41	13.95
4	0	0	0	0	31	9	1:15	90	81.07	14.07
5	-1	1	-1	-1	22	10	1:20	60	79.59	15.86
6	1	-1	1	1	40	8	1:10	120	83.09	13.82
7	-1	-1	1	1	22	8	1:10	120	82.42	13.55
8	1	1	-1	-1	40	10	1:20	60	79.77	16.03
9	0	0	0	0	31	9	1:15	90	81.86	14.74
10	1	1	1	-1	40	10	1:10	60	79.34	15.93
11	-1	1	1	1	22	10	1:10	120	80.51	15.36
12	0	0	0	0	31	9	1:15	90	81.98	14.64
13	1	-1	1	-1	40	8	1:10	60	82.51	13.71
14	1	1	-1	1	40	10	1:20	120	79.67	16.02
15	-1	-1	-1	1	22	8	1:20	120	82.72	13.77
16	-1	1	-1	1	22	10	1:20	120	80.18	15.44
17	1	-1	-1	1	40	8	1:20	120	83.55	13.61
18	1	1	1	1	40	10	1:10	120	79.38	15.2
19	-1	-1	1	-1	22	8	1:10	60	83.04	13.7

Table A1-3
Analysis of variance for response surface model for protein content.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	8.51	5	1.7	4.9	0.0238
X_1 *(pH)*	0.04	1	0.04	0.12	0.7425
X_2 *(Solid/Solvent ratio)*	2.32	1	2.32	6.7	0.0322
X_1X_2	0.027	1	0.027	0.078	0.7873
X_1^2	3.5	1	3.5	10.1	0.013
X_2^2	0.34	1	0.34	0.99	0.3489
Lack of Fit	1.36	4	0.34	0.96	0.5141

df: degrees of freedom

Table A1-4
Analysis of variance for response surface model for yield

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Block	3.53	2	1.76		
Model	13.36	3	4.45	10.53	0.0019
X_1 *(pH)*	13.11	1	13.11	31	0.0002
X_2 *(Solid/Solvent ratio)*	0.16	1	0.16	0.38	0.5526
X_1X_2	0.56	1	0.56	1.32	0.2775
Lack of Fit	1.2	6	0.2	0.26	0.9284

df: degrees of freedom

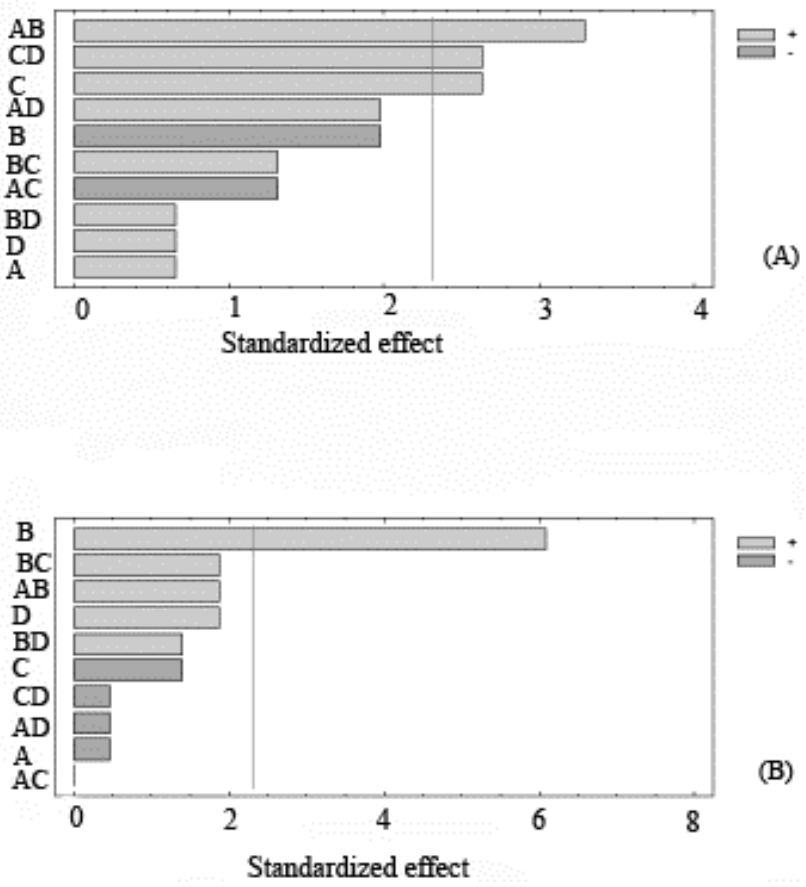

Figure A1.1 Standardized Pareto Chart of screening design for protein content **(A)** and yield **(B)**. A: temperature; B: pH; C: solid/solvent ratio; D; time. Combinations of letters represent interactions between corresponding variables. Gray bars represent an inverse correlation between variable/response.

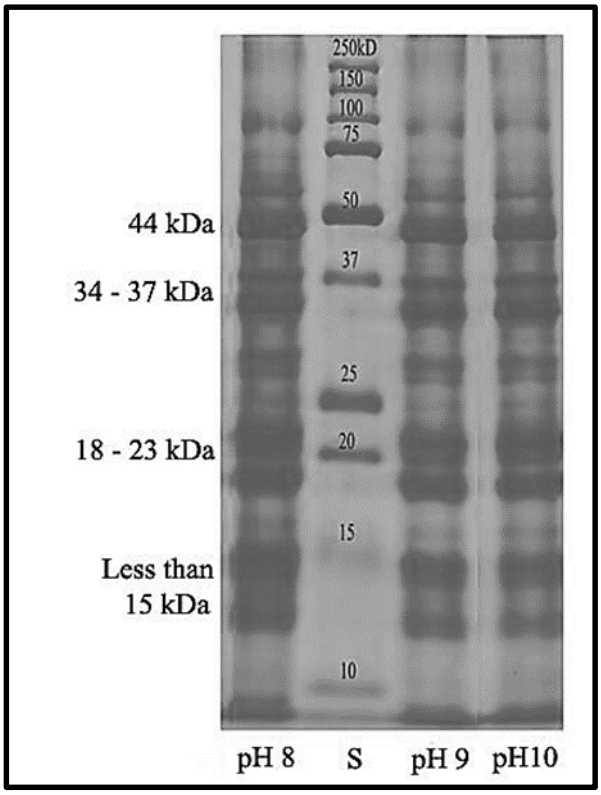

Figure A1.2 SDS–PAGE of lentil protein concentrate under reducing conditions with 2-ME: Lane 1 (left): LP extracted at pH 8.0, lane 2: MW standard, lane 3: LP extracted at pH 9.0, and lane 4: LP extracted at pH 10.0.

APPENDIX A2

A2.1. Amino acid analysis methodology and results

Amino acid analysis was performed according to Simpson, Neuberger & Liu [32]. Sample was hydrolyzed at 115 °C for 24 h, and amino acid analysis performed using a reversed-phase AccQ-Tag 150 × 3.9 mm C18 column with a three-eluent gradient solvent system (AccQ-Tag eluent, acetonitrile, and water) at a flow rate of 1.5 mL/min (Agilent series 1100, Palo Alto, CA, USA) at 37 °C, and a UV detector set at a wavelength of 254 nm. Data acquisition was controlled by ChemStation software (Agilent, Palo Alto, CA, USA).

Table A2-1
Amino acid composition of lentil legumin-like protein

Amino acid	Content, %	Amino acid	Content, %
Asx	10.4	Cys	n.d.
Ser	3.8	Tyr	2.1
Glx	15.5	Val	8.7
Gly	9	Met	1
His	3.1	Lys	6.4
Arg	6	Ile	5.9
Thr	3.6	Leu	7.7
Ala	7.6	Phe	4.7
Pro	5.2		

n.d: not determined

A2.2 Critical micellar concentration determination

Lentin legumin-like solutions were prepared in distilled water at protein concentration of 1, 2, 5, 10, 15, and 20 mg of protein/mL at pH 7.0. The surface tension of

the solutions was measured at 22 °C with a DHR3 rheometer (AT Instruments-Waters LLC, New Castle, DE, USA) using a Platinum/Iridium Du Noüy ring (diameter of 10 mm). The ring was immersed in the protein solution and gradually raised above the surface. The maximum value of the force at the detachment of the ring from the surface of the solution was recorded for surface tension calculation [25]. Each sample was subjected to three measurements.

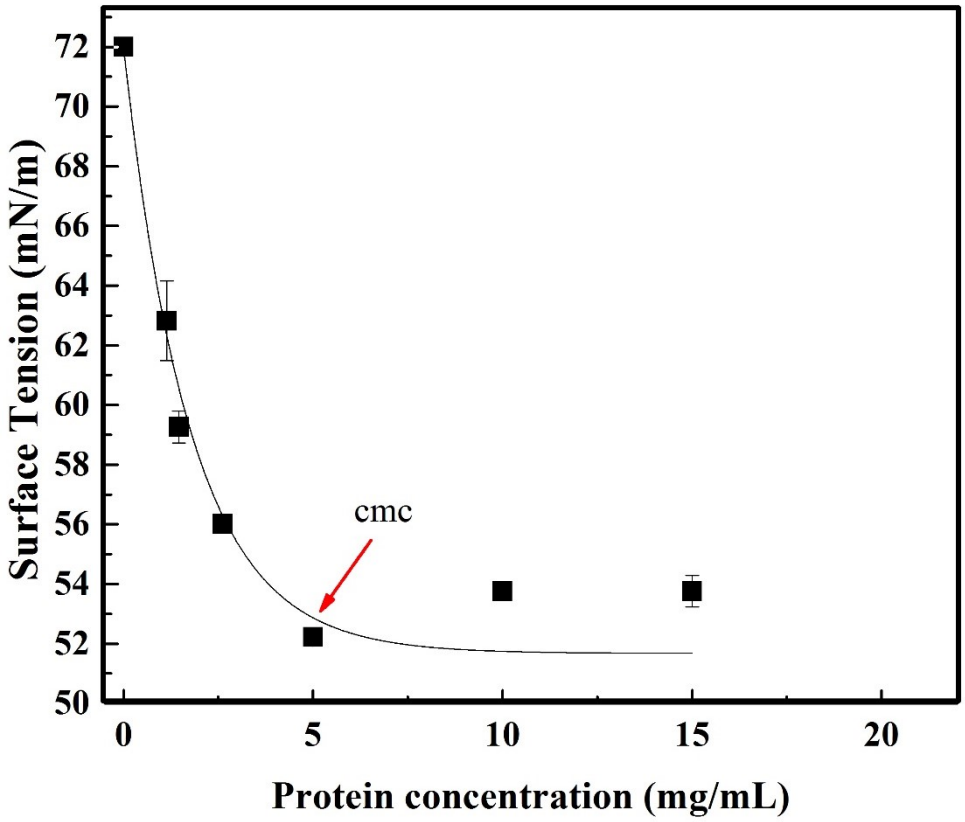

Figure A2.1 Critical micelle concentration (cmc) of lentil legumin-like protein (R^2: 0.99).

APPENDIX A3

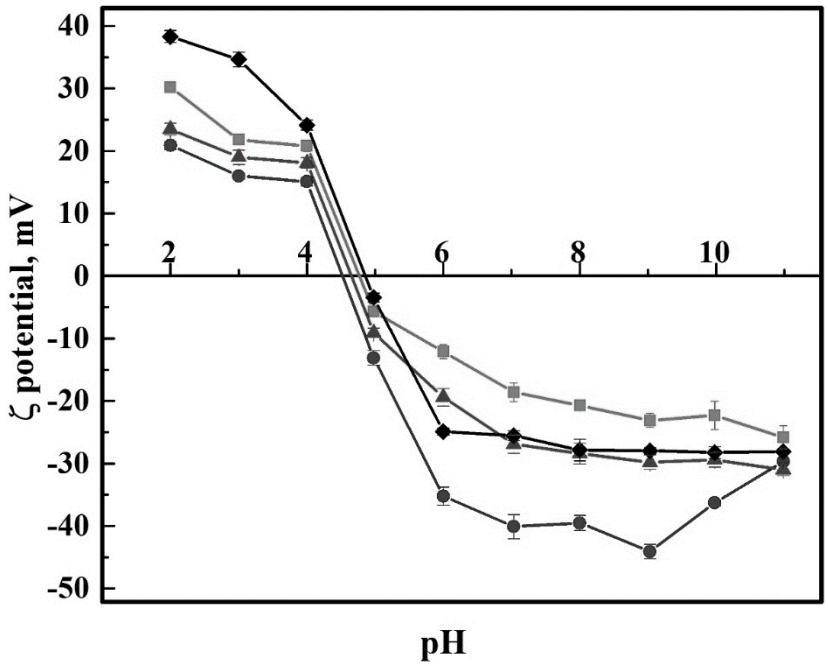

Figure A3.1 ζ-Potential of lentil legumin-like protein-polysaccharide mixtures as a function of pH. Protein (diamonds), in mixture with: guar gum (squares), xanthan gum (circles), and pectin (triangles).

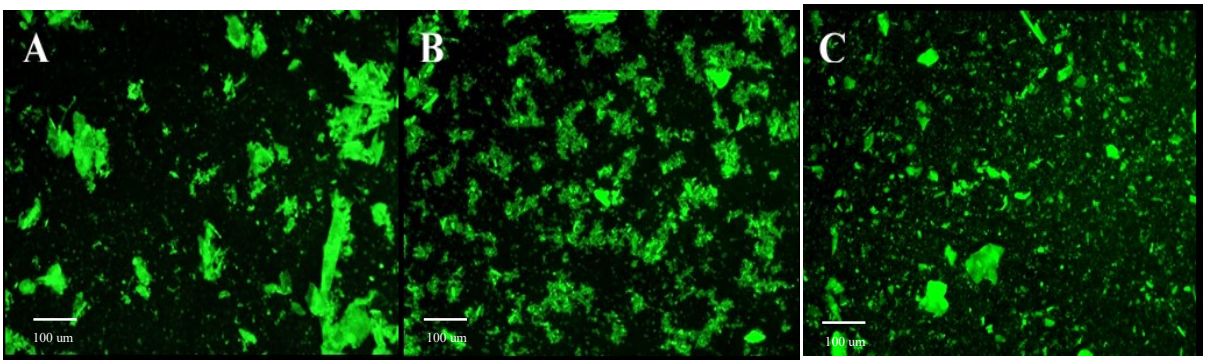

Figure A3.2 Fluorescent micrographs of lentil legumin-like protein solutions at pH 3.0 (A), 5.0 (B), and 7.0 (C)

APPENDIX A4

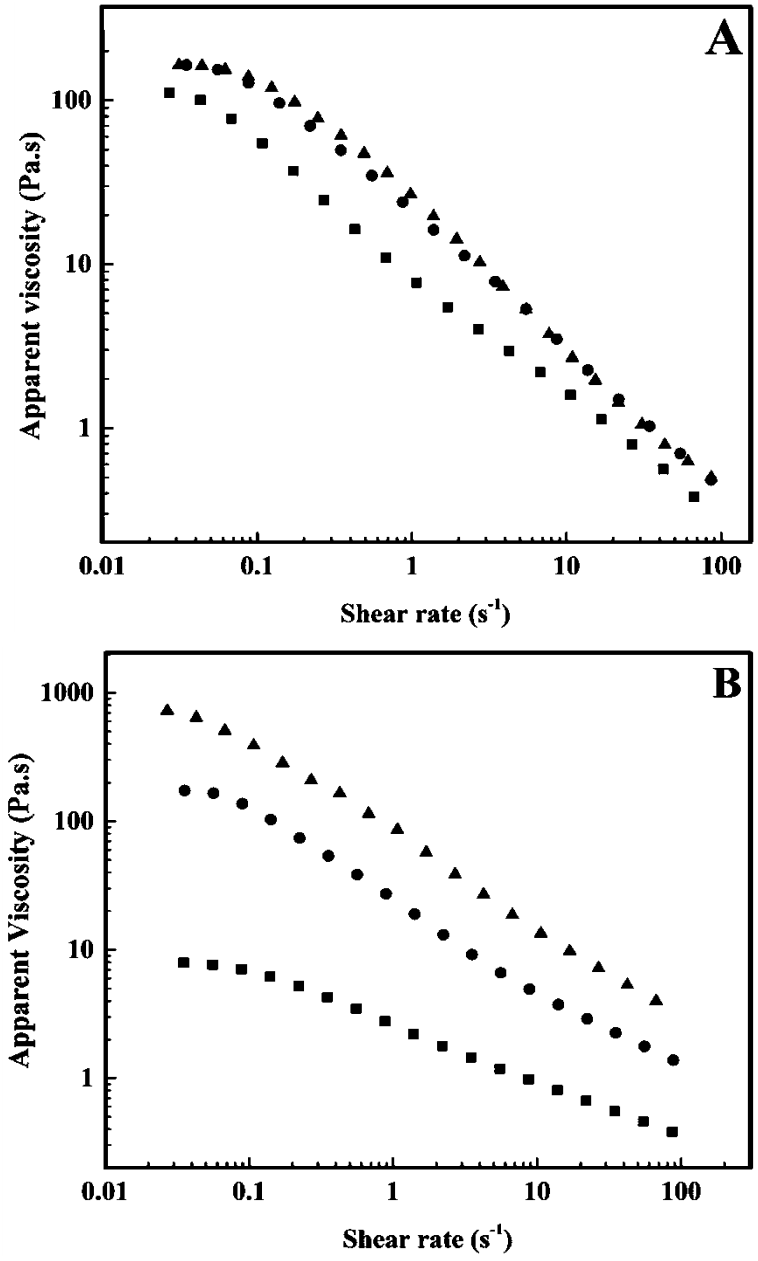

Figure A4.1 Apparent viscosity as a function of shear rate of angel food cake **(A)** and muffin **(B)**. In angel food cakes: AC-0-LPC (squares), AC-50-LPC (circles), and AC-75-LPC (triangles). In muffins: M-0-LPC (squares), M-100-LPC (circles), and M-100H-LPC (triangles).

Figure A4.2 Pictures of angel food cakes: **(A)** AC-0-LPC, **(B)** AC-50-LPC, and **(C)** AC-75-LPC.

Figure A4.3 Pictures of chocolate muffins: **(A)** M-0-LPC, **(B)** M-100-LPC, and **(C)** M-100H-LPC.

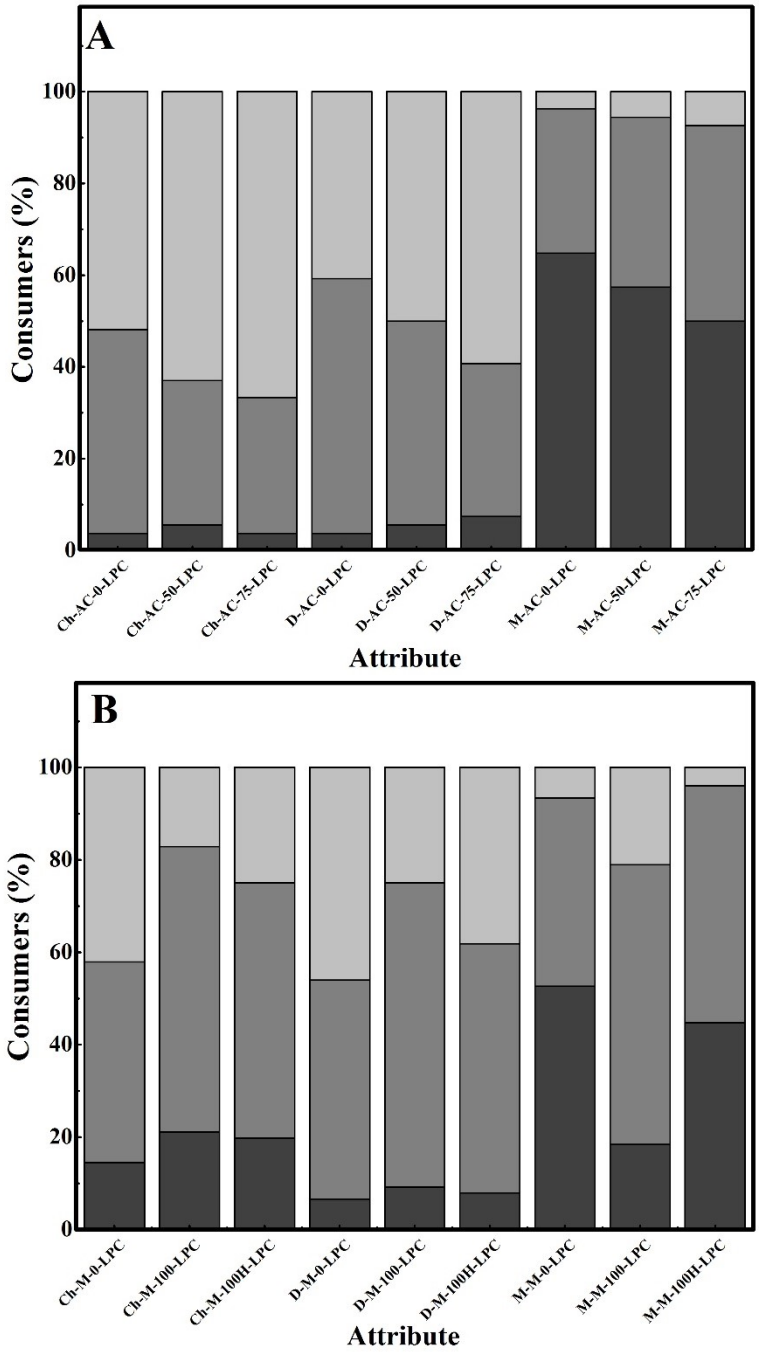

Figure A4.4 Just-about-right scale for the attributes of chewiness (Ch), density (D), and moistness (M) for the three formulations of **(A)** angel food cakes and **(B)** muffins. Dark gray: percentage of consumers that described the attribute below 3; gray: percentage of consumers that described the attribute equal to 3; light gray: percentage of consumers that described the attribute above 3.

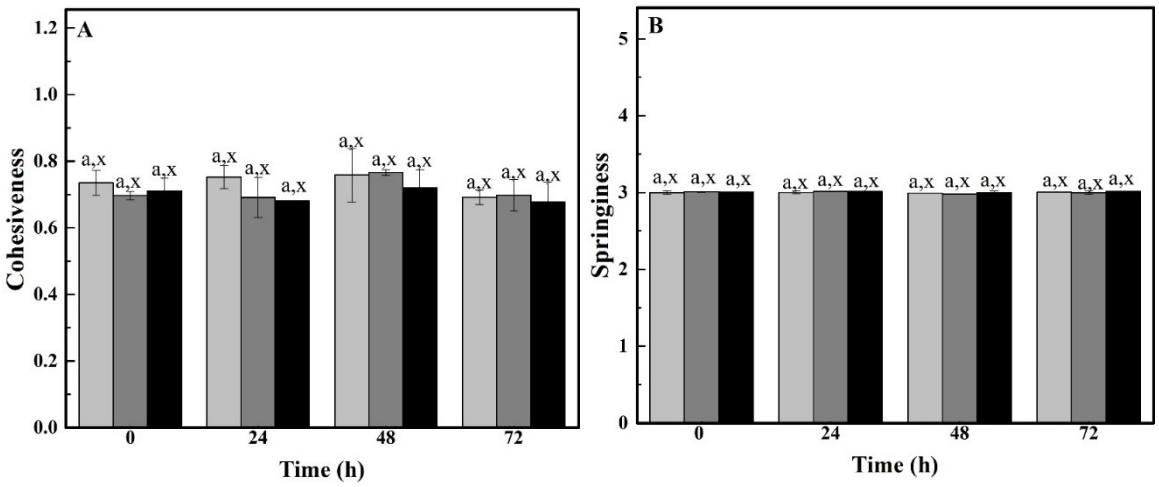

Figure A4.5 Evolution of **(A)** cohesiveness, and **(B)** springiness with storage time for angel food cake formulations. Light gray: AC-0-LPC, gray: AC-50-LPC, black: AC-75-LPC. Bars without a common top-letter have a significant difference ($p < 0.05$).
[a,b,c]: Significant difference between samples. [x,y,z]: significant differences between days

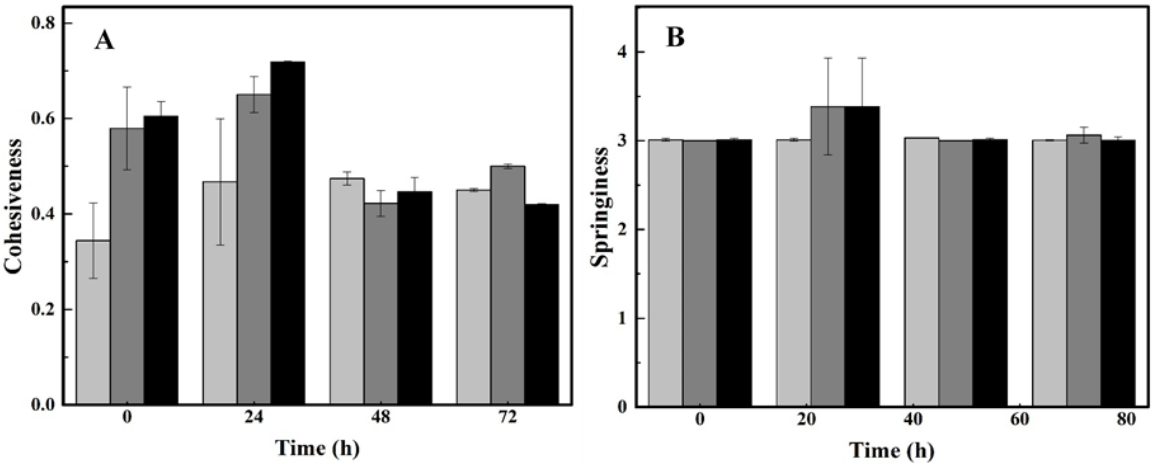

Figure A4.6 Evolution of **(A)** cohesiveness, and **(B)** springiness with storage time for muffin formulations. Light gray: M-0-LPC, gray: M-100-LPC, black: M-100H-LPC. No significant differences ($p<0.05$) were found between the samples.