# BARLEY (HORDEUM VULGARE L.) PROTEIN: EXTRACTION, CHEMICAL COMPOSITION AND FLAVOR INTERACTION

By

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## ABSTRACT

Barley (*Hordeum vulgare* L.) protein extracts were prepared from defatted barley flour using different techniques. Firstly, two conventional alkaline extraction methods were applied: (i) a simple alkaline extraction using 0.5M NaOH (pH 11.0) to break-open cell walls with subsequent release of the protein, and (ii) a sequential alkaline extraction method where the simple alkaline treatment was followed by an isoelectric precipitation (IEP) step. Secondly, milder enzymatic treatments to minimize protein breakdown during extraction were investigated. These included: (i) a bi-enzymatic method involving starch removal using α-amylases from *Bacillus* spp. and amyloglucosidase from *Aspergillus niger*, (ii) a tri-enzymatic sequential method using the former bi-enzymatic treatment followed by digestion with β-(1,3)/(1,4)-glucanase from *Trichoderma longibrachiatum*, and (iii) a tri-enzymatic sequential treatment combining the latter tri-enzymatic digestion followed by IEP.

Results of the study showed that both alkaline extraction methods gave similar protein recovery yields regardless of whether or not the IEP step was performed. However, the combination of IEP with alkaline method improved the protein content, increasing from 33.0% to 68.9%, which in both cases comprised mainly low molecular weight fractions. Extracts produced by the bi-enzymatic treatment had the highest protein content (49.0%) among enzymatic extractions, while those obtained by the tri-enzymatic treatment followed by an IEP step led to the highest protein recovery yield (78.3%), with 35 kDa B-hordeins being the major constitutive proteins of both extracts. Further characterization of the extracted barley proteins indicated that they exhibit pseudoplastic behavior, as revealed by functionality testing, and form stable emulsions. In addition, their highest foaming capacities (FC) and foaming stabilities (FS) were recorded at pH 3.0 and 8.0, respectively.

The protein-flavorant interactions between barley proteins and the model flavor compound vanillin (4-hydroxy-3-methoxybenzaldehyde) were assessed by two methods: (i) measurement of the proportion of unbound vanillin upon incubation with barley protein extracts at different temperatures for up to 72 hrs followed by quantification of binding affinity (number of binding sites *n*; dissociation constant $K_d$; equilibrium constant K) by Klotz plot, and (ii) characterization of the protein-vanillin complexes by fluorescence spectrophotometry analysis. The interaction

between vanillin and the barley proteins was defined as non-cooperative, as evidenced by the Klotz plot linearity. As well, increased vanillin concentrations paralleled an increase in quenching of the protein-vanillin complex fluorescence, indicating changes in the hydrophobicity sites of proteins upon their complexation with vanillin. The effects of heat- and high pressure-treatments of the proteins on their level of interaction with the flavor compound were also evaluated and compared to those of whey protein isolate (WPI) and pea protein concentrate (PPC) used as controls. Consistent with the results generated from the Klotz analysis, the lowest degree of vanillin binding for control proteins was seen with native whey protein; among barley proteins, the alkaline extract treated with high pressure showed the least interaction. Fluorescence spectroscopy analysis revealed that the interaction of vanillin was weakest with heat-treated pea protein, followed by heat-treated whey protein. Lastly, commercial WPI and PPC were incorporated into a high protein (~30%) cookies, formulated with different concentrations of vanillin. Sensory analysis of the cookies was conducted by a panel of 70 untrained panelists targeting three attributes using a 9-point hedonic scale for mean intensity scores. Cookies formulated with PPC at a flavor:protein weight ratio (WR) of 0.45 and those formulated with WPI at a WR of 0.74 received the highest scores, while the highest vanillin intensity was perceived at a WR of 0.74 for WPI and 0.59 for PPC.

## RÉSUMÉ

Des extraits protéiques d'orge (*Hordeum vulgare* L.) ont été préparés au moyen de différentes méthodes à partir de la farine d'orge dégraissée. Dans un premier lieu, deux techniques classiques d'extraction par solubilisation en milieu alcalin ont été utilisées : (i) une simple extraction alcaline dans une solution 0.5M de NaOH (pH 11.0) permettant de dégrader les enveloppes cellulaires afin de libérer les protéines dans le milieu, et (ii) une extraction alcaline séquentielle où la simple extraction alcaline est suivie d'une concentration par précipitation isoélectrique (IEP). Ensuite, nous avons procédé à une hydrolyse enzymatique ménagée de l'amidon et/ou de glucanes afin de réduire la dégradation des protéines au cours de leur extraction. Les techniques enzymatiques utilisées sont : (i) une méthode bi-enzymatique d'hydrolyse d'amidon par des α amylases de *Bacillus* spp. et de l'amyloglucosidase d'*Aspergillus niger*, (ii) une méthode tri-enzymatique séquentielle où la méthode bi-enzymatique précédente est suivie d'une hydrolyse de glucanes par une β-(1,3)/(1,4)-glucanase de *Trichoderma longibrachiatum*, et (iii) une méthode tri-enzymatique séquentielle combinant la technique précédente à l'IEP.

Les résultats de l'étude montrent que les deux méthodes d'extraction en milieu alcalin ont donné des rendements protéiques similaires indépendamment du recours à l'étape additionnelle de l'IEP. De même que les fractions protéiques des deux types d'extrait sont caractérisées par leur faible poids moléculaire. Néanmoins, l'addition de l'IEP après l'extraction alcaline a amélioré la teneur en protéines qui est passée de 33% à 68.9%. D'un autre côté, la teneur en protéines la plus élevée de 49.0% a été enregistrée dans les extraits protéiques obtenus par le traitement bi-enzymatique, alors que le rendement en extrait protéique le plus élevé a atteint 78.3% par la méthode tri-enzymatique séquentielle suivie de l'IEP. Des B-hordeins de 35 kDa de poids moléculaire sont les constituants majeurs des deux derniers types d'extrait. Une caractérisation plus poussée par des tests fonctionnels a montré que les protéines de l'orge ont un comportement pseudoplastique et forment des émulsions stables. Par ailleurs, leur capacité moussante (FC) et la stabilité de leur mousse (FS) ont atteint leurs valeurs respectives les plus élevées aux pH de 3. 0 et de 8.0.

Les interactions entre protéines de l'orge et l'agent aromatisant, la vanilline, ont été étudiées par deux moyens: (i) la mesure de la proportion libre de vanilline après mélange avec les extraits protéiques et incubation à différentes températures pour une durée maximale de 126 hrs, et la

détermination des principaux paramètres de l'affinité de liaison (nombre de sites de liaison *n,* constante de dissociation $K_d$, et constante d'équilibre K) à partir de la représentation de Klotz, et (ii) la caractérisation du complexe protéine:vanilline par spectroscopie de fluorescence. La linéarité de la représentation de Klotz obtenue révèle que la vanilline établit un type d'interaction non coopératif avec les protéines de l'orge. Aussi, l'augmentation de la concentration en vanilline qui s'est accompagnée de l'extinction de la fluorescence du complexe protéine:vanilline indique qu'un réarrangement des pôles hydrophobes des protéines a eu lieu à la suite de leur complexation avec cet agent aromatisant.

L'évaluation des effets des traitements thermiques et de haute pression sur les interactions entre les protéines de l'orge et la vanilline ont été évalués après comparaison à ceux des témoins, isolat protéique de lactosérum (WPI) et concentré protéique de petit-pois (PPC). En concordance avec les résultats obtenus de la représentation de Klotz, le degré de liaison le plus faible a été observé entre la vanilline et les protéines natives du lactosérum. Pour les protéines extraites de l'orge, celles obtenues par solubilisation en milieu alcalin et ayant subi un traitement de haute pression ont enregistré la plus faible interaction avec la vanilline. Les résultats de la spectroscopie de fluorescence montrent que la vanilline établit les plus faibles interactions avec les protéines de l'extraits de petit-pois suivi de celles de l'extrait lactosérum ayant, tous les deux, subi un traitement thermique.

Des extraits protéiniques commerciaux de WPI and PPC ont été utilisés pour enrichir en protéines (30%) des biscuits formulés avec différentes concentrations en vanilline. L'analyse sensorielle des biscuits obtenus a été réalisée par un jury de dégustation constitué de 70 panelistes non entrainés pour trois critères utilisant une échelle hédonique à 9 points et les scores moyens d'intensité ont été déterminés. Les biscuits formulés avec le PPC au ratio molaire (MR) aromate:protéine de 0.45 et ceux formulés avec le WPI au MR de 0.75 ont été les mieux notés, alors le niveau d'intensité le plus élevé a été perçu pour le WPI et le PPC pour les MR respectifs de 0.74 et de 0.59.

## CONTRIBUTION OF AUTHORS

This thesis consists of the three following chapters:

Chapter I provides a comprehensive review of the literature on the chemical, structural and functional properties of barley protein, as well as various protein extraction methods. A description of the interactions between proteins and flavors, influencing factors, and assessment techniques were reviewed.

Chapter II examines the conventional and enzymatic extraction of barley proteins, and the comparison of the protein extracts in terms of structural and functional characterization.

Chapter III assesses the binding between pea, whey, and barley proteins and vanillin using instrumental techniques, and explores the interaction sensorially in a high-protein cookie with 70 student panelists.

Connecting statements are also included to provide a succinct summary of each chapter and to introduce the next one.

Marika Houde, the author, was responsible for the experimental work and the writing of the thesis.

Dr. Salwa Karboune, the MSc student's supervisor, guided all the research and critically revised the thesis prior to its submission.

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## TABLE OF CONTENTS

## LIST OF FIGURES

### CHAPTER II



### CHAPTER III

## LIST OF TABLES

### CHAPTER II



### CHAPTER III

## LIST OF ABBREVIATIONS

| | |
|---|---|
| A-BP: | Alkaline barley protein |
| AI-BP: | Alkaline-based barley protein concentrate |
| ANOVA: | Analysis of variance |
| BE-BP: | Bi-enzymatic barley protein |
| BPI: | Barley protein isolate |
| DDT: | Dichlorodiphenyltrichloroethane |
| FTIR: | Fourier transform infrared spectroscopy |
| GC: | Gas chromatography |
| HCl: | Hydrochloric acid |
| HPLC: | High performance liquid chromatography |
| H/hrs: | Hours |
| HS-SPME: | Headspace solid phase microextraction |
| IEP: | Isoelectric precipitation |
| MW: | Molecular weight |
| MWCO: | Molecular weight cutoff |
| NaOH: | Sodium hydroxide |
| PPC: | Pea protein concentrate |
| rpm: | Revolutions per minute |
| SDS-PAGE: | Sodium dodecyl sulphate polyacrylamide |
| TE-BP: | Tri-enzymatic barley protein |
| TEI-BP: | Tri-enzymatic-based barley protein concentrate |
| Termamyl: | α-amylase *Bacillus licheniformis* |
| U: | Enzymatic units |
| WPI: | Whey protein isolate |

# INTRODUCTION

The increasing demand for health-promoting foods has fueled the development of functional protein ingredients. Furthermore, there is an ever-increasing interest in exploring new sources of plant-derived proteins, based on health, environmental, and economical rationales. Among the currently available plant-derived proteins, soy and wheat proteins remain dominant. Although the use of pulse proteins has recently grown dramatically, the demand for ancient grains has also spiked. One grain that has yet to be thoroughly investigated for its protein characteristics and functionality is barley (*Hordeum vulgare* L). Barley has been domesticated for over 10,000 years Badr et al. (2000) and is now the fourth most grown cereal globally after wheat, rice and corn (Xia et al., 2011). This crop was brought to Canada in the early 17$^{th}$ century where it has since been cultivated intensively, especially in the western region of the country (Friedt et al., 2010). With an annual production varying between 7.1 and 11.8 million tons during the last decade, Canada ranks among the most important barley producers worldwide (Statistics Canada, 2017). This production covers the national needs and a substantial part is exported mainly to China, the USA, and Japan, generating about 1.0 billion Dollars every year (Barley Council of Canada, 2017).

Barley is a multi-purpose cereal primarily utilized for animal feed (65%) and as raw material to produce malt (33%) for the brewing industry, while only 2% of the world barley supply is used directly in human consumption (Sullivan, Arendt, & Gallagher, 2013). In Canada, the figure is 80% of barley production used in livestock feeding, 15 % transformed into malt for beer-making, and a meager part of less than 5% directed for human consumption (Wang et al., 2010). The unpopularity of barley in human diet may be due, in part, to its distinct flavor being a negative acceptance factor among consumers. However, the grain gained increased popularity in recent years owing to the association of the soluble β-glucan fiber and phytochemical compounds it contains with various health benefits (Alu'datt et al. 2012; Idehen, Tang, & Sang, 2017). The approval, by the US Food and Drug Administration, of the claim establishing a relationship between β-glucan soluble fiber from barley and reduced risk of coronary heart disease (FDA, 2006) was an additional incentive that stimulated research interest on barley as a source for bioactive components (Idehen, Tang, & Sang, 2017). Although β-glucan was the most credited for therapeutic effects, it is now well established that other bioactive barley components, including minerals, vitamins, and various phytochemical molecules are also associated with health benefits (Idehen, Tang, & Sang, 2017). This was also evidenced by the observation that regular barley

consumption prevented chronic diseases, such as colonic cancer (Dongowski et al., 2002; Finn 2008), diabetes (Sullivan, Arendt, & Gallagher, 2013; Thondre, 2014), and gallstones (Hoang et al., 2011; Zhang et al., 1990). Barley contains 10-20% proteins dominated with the prolamin hordeins (52%) as the main storage proteins (87%) of the endosperm layer. Glutelin is another major storage protein of the endosperm, which represents 23% of the total barley proteins. Two other cytoplasmic proteins, albumins and globulins, represent minor storage proteins of the grain aleurone and embryo (Fontana & Buiatti, 2009; Sullivan, Arendt, & Gallagher, 2013). Barley is intensively used as a raw material in the starch industry generating large quantities of protein as a by-product that can be valorized in the food industry (Yalçın, Çelik, & İbanoğlu, 2007). Indeed, barley proteins are good candidates for value-added application as food supplements owing to their functional properties, including emulsifying capacity and stability, foaming, elasticity, cohesiveness, and water holding capacity that enhance rheological food properties (Alu'datt et al., 2014; Wang et al., 2010; Yalçın, Çelik, & İbanoğlu, 2007). Despite the limited nutritive value of barley proteins in the human diet due to their low digestibility, they constitute a good source for essential amino acids, such as threonine, valine, lysine, phenylalanine, and arginine (Sullivan, Arendt, & Gallagher, 2013). The low digestibility may, in fact, be a desirable attribute as it ensures a controlled release of bioactive compounds and exerts a protective effects on the gastrointestinal tract (Xia, Wang, & Chen, 2011). Therefore, barley proteins have a potential for application in food development with a competitive advantage compared to the trend-leading whey and soya proteins (Singh et al., 2008). This is especially timely to address the increased consumer preference for the plant-based proteins sourced from alternative whole grains, having the ability to enhance the nutritional profile and to add to the health attributes of familiar foods.

The overall objective of the study was to optimize the isolation of barley protein, characterize its structural properties, and investigate its interaction with a selected flavor by incorporation into a high-protein cookie. This was achieved by the following specific objectives:

(1) Developing an enzymatic approach for the isolation of barley proteins, comparing its efficiency with conventional extraction methods, and characterizing the structural properties of the obtained protein isolates

(2) Investigating the effects of barley and control protein systems, and their molecular forms on protein-flavor binding

(3) Formulating a cookie product containing pea protein concentrate and whey protein isolate and evaluating protein-flavor interaction with sensory analysis

There may be an opportunity to capitalize on the by-product from industrial isolation of beta-glucan from barley, where the protein-rich residue is otherwise sent for animal feed.

# CHAPTER I.

# LITERATURE REVIEW

## 1.1. Barley Proteins

### *1.1.1. Chemical and Structural Properties*

#### *1.1.1.1. Hordein*

The most abundant storage protein found in the endosperm of the barley seed is hordein, classified as a prolamin, which comprises 30-50% of the total protein. As in all seed plants, proteins are first produced as preproteins that are transported to the endomembrane site, where they are concentrated and form aggregate protein bodies. Transportation continues to the endoplasmic reticulum and Golgi apparatus during the last stages of grain filling, and is stored within vacuoles (Shewry & Casey, 1999; Steiner et al., 2011). Its amino acid composition is poor in lysine, threonine, and methionine, rendering barley an incomplete protein source, but exhibits high proportions of proline, glutamine, leucine, valine, phenylalanine and tyrosine (Steiner et al., 2011; Xia, 2012; Xia et al., 2011). Its particular amino acid content comprised of nonpolar and polar uncharged side groups (proline, methionine, and tryptophan) account for hordein's low solubility in water (Xia, 2012).

Like most prolamins, hordein is composed of a set of complex polypeptides. The five subunits can be classified based on their electrophoretic mobility and amino acid composition. α-hordeins are the smallest subunit at 15-25 kDa, and comprise mostly protease inhibitors and α-amylases (Steiner et al., 2011). It has been suggested that they may not be true hordeins, but rather metabolites of larger prolamins, or even globulins or albumins that are soluble in alcohol or salt (Celus, Brijs, & Delcour, 2006; Wang et al., 2010). Sulfur-rich β-hordeins are the largest hordein segment (32-45 kDa), representing 70-80% of hordein and can be further fractionated into β B1, β B2, and β B3 subtypes (Celus et al., 2006; Steiner et al., 2011; Wang et al., 2010). They reveal 19 amino acid residues, and a proline- and glutamine-rich central domain. A third group, C-hordeins (49-72 kDa) are low in sulfur and account for 10% of the overall hordein fractions; it has been the subject of many studies, and research work has determined that it has a structured conformation with glutamine and proline comprising 50% of its amino acid makeup (Xia, 2012). Polypeptidic chains in high molecular weight D-hordeins (>100 kDa), like in B-hordeins, are joined by disulfide bridges (Celus et al., 2006; Chanput, Theerakulkait, & Nakai, 2009; Steiner et al., 2011; C. Wang et al., 2010). The last fraction, γ-hordeins, exists as monomers with disulfide bridges, and are enriched with sulfur. The D- and γ-hordeins comprise less than 5% of total barley prolamin (Celus et al., 2006). Barley prolamin has an extended and rodlike structure (Shewry & Casey, 1999). Its

isoelectric point has been determined to be at pH 5.5 (Baxter, 1976). Makarenko and colleagues (2002) have determined that hordein contains an estimation of 52.6% poly-L-proline II helix, 19.3% β-turns and bends, 8.9% β-sheet structure, 6.9% α-helix, and 12.3% unordered conformation. Its tertiary structure has been described as having a hydrophilic core and a hydrophobic surface (Bamdad, Wu, & Chen, 2011).

#### *1.1.1.2 Glutelin*

The alkali- and detergent-soluble protein fraction, designated as glutelin, comprises 30% of barley protein, and is also located in the starchy endosperm. Due to its poor solubility in any other media, extracted glutelin is often denatured and can even degrade, rendering it difficult to perform relevant electrophoretic studies. Moreover, as the final protein fraction extracted during the sequential procedure, it is therefore not only altered by prior treatments, but contaminated by other protein fractions (Steiner et al., 2011). It has been reported that a glutelin extract cannot be exist free of hordein (Xia, 2012). However, Zhao and colleagues (2011) have determined that glutelin possesses molecular four major bands at 85-90, 55-70, 25-50 and <20 kDa, with the possibility of the band between 85-90 kDa being D-hordeins, and that the broad band at 25-50 is contaminated with B-hordein. The amino acid composition of glutelin features a high proportion of glutamine (20.2%), proline (11.2%), and glycine (8.4%), as well as an overall hydrophobic amino acids content of 35%, namely leucine, alanine and valine (J. Zhao et al., 2011). Like hordein, glutelin is low in lysine, threonine, and methionine (Xia, 2012). The isoelectric point has been determined to be in the range of pH 7.3-8.4 (El-Negoumy, Newman, & Moss, 1983). It has been suggested that hydrolyzed barley glutelin peptides may have potential as natural antioxidants due to their amino acid composition (Xia et al., 2012). The derived peptides contained hydrophobic phenylalanine and methionine, which act as radical scavengers, and glutamine, lysine, and arginine, shown to chelate and inactivate oxidative action of metal ions. Analyzing the amide I band, barley glutelin can be assigned α-helices and β-sheets, though estimations are unknown (Zhao et al., 2011).

#### *1.1.1.3. Others*

The two last protein fractions, globulins and albumins, can be extracted together using dilute salt solutions, and then partially separated by dialysis. These contain primarily metabolic proteins. The globulin fraction comprises 15% of barley protein, and is found in the embryo of the barley seed. Designated as edestin in barley, it is comprised of 4 subunits, α, β, γ, and δ which have a spherical structure (Shewry & Casey, 1999; Steiner et al., 2011). Molecular weights range between 14-58 kDa (Linko et al., 1989), and the isoelectric points of the α- and β-globulins are pI 4.0 and 4.2, respectively (El-Negoumy et al., 1983). The β-subunit contains sulfur, and does not completely precipitate upon boiling. It is highest in glycine, cysteine, and glutamine (Linko et al., 1989). Leucosin is the name given to the pure water-soluble albumin fraction in barley which is localized in the aleurone layer. It represents 11% of barley protein, and has subunits designated as α-amylase, protein Z, and lipid transfer proteins with molecular weights between 14-58 kDa (Linko et al., 1989). It is completely precipitated once subjected to boiling, with the isoelectric point situated between pH 7.8-8.7 (El-Negoumy et al., 1983; Steiner et al., 2011). A third of the amino acid composition of leucosin is glutamine, and it is very low in methionine and lysine (Linko et al., 1989).

### 1.1.2 Techno-Functional Properties

#### 1.1.2.1. Solubility

Solubility can be described as the equilibrium state between hydrophobic and hydrophilic interactions. The degree of solubility is dependent on pH, the minimum being at the isoelectric point. Other factors contributing to protein solubility are temperature and ionic strength (Bolontrade, Scilingo, & Añón, 2013). It is arguably the most important protein properties, as it is a deciding factor in terms of food system application, and influences other functional properties such as emulsification, foaming, and gelation (Soderberg, 2013; Yalçin & Çelik, 2007). As barley protein consists of numerous fractions, it is more challenging to determine the solubility than it is for a single protein. The solubility of barley is greatly dependent on the pH and the ionic strength. Barley exhibits solubility patterns similar to other plant proteins: solubility is greatest in strong acidic and alkali solutions.

Being amphoteric electrolytes, the solubility of proteins is highly dependent on the pH. A major setback that precludes wider use of barley protein has been the insolubility of hordein and glutelin in pure water. As previously mentioned, they can only be dissolved in aqueous alcohol and in dilute acid or alkali solutions, respectively. High surface hydrophobicity and a large non-polar amino acid proportion account for this characteristic (Xia, 2012). Hordein solubilizes well at pH 10 or 11, but is almost insoluble between pH 3.0 to 8.0 (Wang et al., 2010; Yalçin & Çelik, 2007). Glutelin has a minimum solubility at pH 5.0, which dramatically increases at pH 10 (Wang et al., 2010). It has been suggested that many plant proteins have the lowest solubility at their pI (Bilgi & Celik, 2004; Ma & Harwalkar, 1984; Zhao et al., 2011).

To determine the effect of salt concentration on solubility, Yalçin & Çelik (2007) examined the solubility of hordein isolate in distilled water and 0.01-0.5M NaCl, adjusting the pH with 0.5M HCl and NaOH. Solubility in distilled water was higher at pH 10 and 11, and lower at pH 4.0. In salt solutions, protein solubility was highest at pH 2.0, 10 and 11 at 0.01M NaCl, but decreased at higher NaCl concentrations under acidic and neutral conditions.

#### *1.1.2.2. Emulsification*

Emulsification relates to the grasp, adsorption, and unfolding of protein at the oil-water interface followed by the formation a viscoelastic film around an oil droplet. An emulsifier of choice will have hydrophilic and hydrophobic moieties, allowing for a quick adsorption of droplets with a consequent reduction of the interfacial tension. Emulsion stability is achieved through protein adsorption to the emulsion droplets and formation of a compact film around the droplets (Wang et al., 2010).

A study by Mohamed et al. (2007) evaluated the emulsifying properties of two barley protein isolates (BPI), both obtained using acid precipitation, with one having undergone acylation after extraction. Both BPIs showed equally high emulsifying capacity and stability. Emulsion capacity of hordein and glutelin was tested by whipping a protein-oil mixture to form an emulsion, and stability studied after centrifugal and thermal treatments (Wang et al., 2010). After centrifugation of the formed emulsion, stability was determined by measuring the volume before and after centrifugation. Samples were then heated at 80$^{o}$C for 30 minutes and cooled to 23$^{o}$C, then centrifuged again. The highest emulsion capacities and stabilities were observed at pH 3.0 and 8.0,

and decreased at pH values closer to the isoelectric point of pI 5.0. This behavior was explained by the presence of a high surface charge on protein domains required to prevent agglomeration of the suspended oil droplets. In particular, glutelin forms aggregates at the water-oil interface to enable film formation due to the proximity of high molecular weight polypeptides, thereby limiting the contact between oil droplets and, consequently, their flocculation and coalescence. Therefore, the most stable emulsion for any barley protein fraction occurs at values far from the isoelectric point. Following both treatments, glutelin displayed the highest emulsifying centrifugal stability values, and was thus the protein fraction that allowed the highest stability of the thermal emulsion. Its desirable ability to form emulsions has been partly attributed to its dual water- and oil-holding capacity. The excellent stability following heat treatment may be due to a condensed film forming around the oil droplet from gelation of the protein (Wang et al., 2010). Research by Zhao et al. (2011) has shown that the controlled de-amidation of glutelin isolated from barley dramatically improves its ability to stabilize emulsions and to solubilize at acidic and neutral pH values; this change is due to easier unfolding, and greater electrostatic repulsion between molecular chains.

#### *1.1.2.3. Foaming*

Foaming is defined as the ability of a protein to attain, adsorb, and finally unfold quickly at the air-water interface. The rate of foaming correlates to the diffusion coefficient, in which the speed of protein transport to the interface is inversely related to the protein size. During the process, proteins undergo denaturation and they subsequently expose their hydrophobic side chains to bind with the hydrophobic molecules where there is a larger denaturing interface. In addition, proteins must adjoin to and integrate the film to form the foam. It can be concluded that flexible and hydrophobic proteins are ideal foaming candidates. Foam stability is based on the resistance of the lamella, the network of interconnected films, to drain and of the air bubbles to deflate, which are dependent on the rheological and adhesive features of the interface of the film (Wang et al., 2010). It has been proposed that the isoelectric point of a protein strengthens, thickens, and stiffens films, thereby enabling them to entrap more air due to the promotion of protein-protein interactions (Wang et al., 2010) .

A study by Wang et al. (2010) examined the pH dependence of foaming capacity and stability of glutelin and hordeins at pH 3.0, 5.0 and 8.0. In this study, foaming was achieved by homogenizing

the protein solutions (0.5% w/v) at high speed, and results showed that the lowest foam capacity was attained at pH 5.0, where both proteins are the least soluble. Foam capacity was greatly enhanced for both fractions at the other pH values, as their increased solubility enhanced diffusion to the air-water interface. Hordein reached a foaming capacity of 163% at pH 8.0, while glutelin only reached a maximum of 73% at pH 3.0. Hordein's ability to adsorb to the liquid-air interface can be explained by a number of factors. It has a higher water adsorbing capacity at the air-water interface, which may be due to the surface location of the hydrophobic side chains allowing for rapid adsorption or viscoelastic-film forming around the air bubble. Alternatively, it can be due to its greater flexibility, exposing the nonpolar amino acids for improved adsorption. In contrast, for both fractions, foam stability was highest at pH 5.0 and decreased at the other pH values. In addition, glutelin demonstrated the strongest foam stability compared to hordein, reaching 95% and 55%, respectively. This research group also investigated foam stability of BPI extracted using alkaline extraction and isoelectric precipitation. The highest point of foam stability was recorded near its isoelectric point, at pH 5.0. It has been suggested that foam stability is enhanced when the protein is comprised of high molecular weight storage proteins (German, O'Neill, & Kinsella, 1985). Alternatively, partial hydrolysis of proteins which decreases stability and protein polymerization that thickens the film surrounding the air bubble (German et al., 1985; Wang et al., 2010). Yalçin et al., (2008) obtained the same results using the gas sparging method. The filtrate from 0.5% and 1.0% (w/v) BPI solutions obtained using alcohol extraction and cold precipitation was transferred into a glass-sintered column, complete with a sintered glass filter. The sample was injected with dry air for 10 seconds and the foam volume produced was immediately measured for foam capacity. Foam stability was measured as the time for the foam volume to be reduced by half. In addition, the authors noted an increase in the foam stability when the concentration of BPI solutions increased from 0.5% to 1.0% (w/v).

The effect of heat and pH denaturation on hordeins and albumins was assessed by Kapp & Bamforth (2002). Both methods of denaturation significantly increased hydrophobicity of the proteins, which has been linked to greater foam stability. Albumin demonstrated greater foam stability compared to hordein, particularly after heat treatment at 95$^{o}$C for 10 minutes, due to its flexible nature and surface elasticity. Foam stability was not affected by an acid treatment of holding the solution at pH 1.0 for 10 minutes before increasing it to 4.5.

#### *1.1.2.4. Water- and Oil- Holding Capacity*

Water and oil holding capacity in barley glutelin is particularly high and may represent an attractive property in novel food processing. Water holding capacity (WHC) is the ability of polar side chains to hold water. The highest WHC of glutelin was reported to be 4.19g/g, whereas that of hordein was about twofold lower (2.21g/g). Compared to other barley protein fractions, glutelin also shows the highest oil holding capacity (OHC), which is defined as the ability of nonpolar side chains to bind fats. These characteristics of high WHC and OHC qualify barley protein as an acceptable ingredient for foods requiring both water and oil binding. A possible explanation is the equitable distribution of polar and nonpolar amino acid residues found in glutelin; curiously, hordein, albumin, and globulin have a similar hydrophobic amino acid content as glutelin, and yet their WHC and OHC are much lower. Glutelin contains high molecular weight chains, which may form a network between the polar and nonpolar amino acids and water or oil molecules, further entrapping and binding them (Wang et al., 2010).

### 1.2. Extraction of Protein Fraction from Barley Grains

Like most other grains, barley only contains 8-13% (w/w) protein depending on the variety. Retrieving protein from ground barley flour can be performed according to numerous methodologies, mostly aqueous and solvent extraction/fractionation.

#### *1.2.1. Alkaline Extraction*

Wang et al. (2010) isolated protein fractions from pearled barley flour, in which they removed barley bran prior to treatments. The bran contains significant amounts of phytates, fibre, and phenolics, capable of binding to proteins and impeding dissociation from the matrix. Ethanol at 55-75% (v/v) was first applied to isolate hordein. Glutelin in the remaining flour was then removed following an alkaline treatment, and dispersed in the solvent at a 1:6 ratio for 2 hours at 60$^{o}$C at a constant mixing speed. An extraction efficiency of 30% using 55% ethanol at 55$^{o}$C was observed, with a protein content of 95%, while at most 45% of glutelin was extracted from the flour at pH 11.5 at 23$^{o}$C, containing 85% protein. The authors reported a 61-81% extraction efficiency with a protein content of 80-85% under conditions of pH 11.5 for 30 minutes at 23$^{o}$C for the overall protein fraction, due to the breakdown of hydrogen bonds, in parallel to the removal of hydrogen from carboxylic and hydrogen bonds, which increases the protein charge on the surface of the molecule and thereby solubility. The researchers specify that while NaOH was utilized in their

experiment, effectiveness is directly impacted by extraction factors such as time/temperature and solvent pH. A subsequent cold precipitation enhanced the separation of phenolic compounds and low molecular weight carbohydrates from hordein, allowing for improved purity. The insoluble portion thus obtained was then centrifuged at 8500 *g* for 15 minutes at 23°C. The supernatant was collected and ethanol removed by in vacuum evaporation to recover the hordein fraction.

### *1.2.2. Sequential Alkaline and Isoelectric Precipitation*

Mohamed et al. (2007) suggested to first defat the flour with hexane in a 4:1 ratio before extracting with dilute NaOH under room temperature conditions. The slurry is mixed for one hour, then centrifuged at 4000g for 10 minutes at 10°C and pellets re-suspended in the same weak alkali, stirred for 1 hour and centrifuged at 4000g for 10 minutes. Pooled supernatants are combined and acidified to pH 4.5 using 2M HCL, and the barley protein isolate was obtained after centrifugation at 10,000g for 20 minutes. Extraction efficiency and protein content were not reported.

This procedure was later modified by Alu'datt et al. (2012) by adding 2M NaOH to barley flour to achieve a pH of 11.0. The mixture was stirred at 23.6°C for 1 hour in a water bath, centrifuged at 10,000g for 15 minutes, and filtered through a cheese cloth. Using 0.1M HCl, the supernatant was adjusted to pH 4.6, and the precipitated proteins were centrifuged and freeze-dried. In order to isolate the globulin, glutelin-1, glutelin-2, and prolamin protein fractions, the authors used a sequential extraction procedure with four solvents: 0.5M NaCl, 0.1M NaOH, 50% glacial acetic acid, and 70 % ethanol. At each step, a meal:solvent dissolution ratio of 1:10 (w/v) was utilized for 2 hrs at room temperature. Protein content was determined to be 35.3%, and protein yield 60.15%, according to the following formula:

$$\text{Yield of Protein (\%)} = \frac{Weight\ of\ Freeze\ Dried\ Extract\ x\ Protein\ Content\ x\ 100\%}{\%Protein\ in\ Meal\ x\ Weight\ of\ Meal} \qquad \text{(Equation 1)}$$

### *1.2.3. Alcohol Extraction*

In order to produce the barley protein isolates, Yalçin and Çelik (2007) used 70% (v/v) ethyl alcohol at a solvent to flour ratio of 3:1, and added 1% (w/v) sodium metabisulphite and L-cysteine to two separate extractions, resulting in protein yields of 86.0% and 85.2%. The extraction lasted

for 2 hrs at 20ºC, after which the extracts were centrifuged at 900*g* for 30 minutes. Hordeins were precipitated from the supernatant using 1M NaCl, and recovered by centrifugation at 5000*g*.

### *1.2.4. Sequential Extraction*

The Osborne method remains a commonly accepted method for the fractionation of cereal proteins by their differential solubilities (Shewry & Casey, 1999). This procedure has been frequently applied to barley in order to isolate various fractions: water soluble proteins (albumins); salt soluble extract (globulins); alcohol soluble fraction, commonly in the presence of reducing agents (prolamins); and alkali soluble proteins (glutelins) (Celus et al., 2006; Xia, 2012). Recently, the solvents for extracting glutelin have been modified by including detergents such as sodium dodecyl sulfate (SDS) or chaotropic agents (urea), coupled with reducing agents (Wang et al., 2010).

A similar protocol suggests extracting protein twice with a solution of NaCl (5% w/v) at room temperature and isolating albumins and globulins, hordeins thrice with 55% n-propanol, and finally recovering glutelin by treatment with a mixture of SDS and urea (Celus et al., 2006). A solution containing ethanol and 2-mercaptoethanol is another means to extract hordein, while the isolation of glutelins can also be performed through reduction and alkylation by 8M urea (Wang et al., 2010). Mohamed et al. (2007) have reported extraction efficiencies of up to 94% with 2-mercaptoethanol and 0.5% SDS. The Osborne method has many advantages over other extraction procedures, such as higher versatility, rapidity, and simplicity in quantifying all the proteins found in a variety of cereals, in addition to its ability to allow for efficient protein extraction and analysis by reverse phase-HPLC (Steiner et al., 2011).

### *1.2.5. Buffer Extraction*

A buffer extraction method was developed by Finnie et al. (2002) using barley ears as the raw material. Samples were freeze-dried for 48 hours prior to extraction, and ground to a flour. Barley seed flour is dissolved in 5mM Tris (pH 7.5) containing 1mM $CaCl_2$ at 4ºC, the temperature at which the rest of the extraction steps are carried out. The slurry are stirred for 30 minutes, the insolubles are decanted by centrifugation at 11,500 *g* for 30 minutes, and the concentration of the extracted proteins are determined in the supernatant by appropriate techniques (e.g., Bradford method).

## 1.3. Flavor Binding by Protein

### *1.3.1 Types of Binding Interactions between Flavors and Proteins*

Despite its complexity, flavor is one of the food's most critical and greatly influential characteristic for consumer acceptance. Aroma is influenced by chemical reactivity of the flavor compound, the presence of light and oxygen in the food environment, as well as the components of the food matrix itself, including carbohydrates, proteins, fats, transition metals, and other products formed during processing (Weerawatanakorn et al., 2015). Chemical structures of particular aroma volatiles and the overall composition of the food can affect the transfer of flavor compounds within the matrix and their subsequent release (Seuvre et al., 2006). It is well established that certain non-volatile components, notably proteins, can significantly retain aroma compounds, which alters their release and the flavor perception during mastication (Guichard, 2006; Heng et al., 2004). Most interactions are reversible, for instance hydrophobic or hydrogen bonding, although some exceptions involving irreversible interactions do occur - in particular with aliphatic aldehydes that can covalently bind proteins (Guichard, 2006).

Many flavor-protein interactions were focused on β-lactoglobulin and soy protein. β-lactoglobulin binds and traps numerous flavor compounds, particularly ketones, aldehydes, ionones and esters, primarily through polar group interactions (Guichard, 2006). In the case of soy protein, denaturation was found to increase the binding capacity of volatiles, while proteolytic hydrolysis eliminates any binding capacity. It was inferred that the binding occurred through hydrophobic interactions, with various reports suggesting that either alcohols or aldehydes display the strongest binding capacity. Between the two classes of compounds, the interaction between aldehydes and soy proteins was shown to be partly due to covalent binding, and in part reversible binding (Plug & Haring, 1994). A few other food proteins have been studied but to a lesser extent. Unlike other whey proteins, α-lactalbumin had a poor affinity toward ketones and aldehydes. Milk's dominant protein, casein, was shown to interact with small hydrophobic compounds by trapping them inside the hydrophobic portion of its micelle. Like soy protein, the binding properties of bovine serum albumin binds in general are independent of pH and temperature conditions. It does so through poor and unspecific interactions, but has a limitless binding capacity. Specifically, BSA has an affinity for carbonyl constituents, which alter its conformation, although this affinity decreases when the protein undergoes a chemical reduction of its disulfide bridges (Guichard, 2006).

The molecular structure of certain flavor components has significant impacts on the degree of retention by proteins. Van der Waals forces are responsible for binding action of hydrocarbons, and hydrogen bonds occur between alcohols and carbonyls (Guichard, 2006). Similarly, chain length of aroma compounds is directly related to the degree of binding, chiefly through hydrophobic interactions (Heng et al., 2004).

Apart from molecular structures of either volatiles or proteins, many conditions affecting food matrices play vital roles in the degree of binding. Water content and protein structure strongly influence the degree of volatile binding. The action of binding itself may cause an alteration in conformation of the protein which can modify the flavor molecule binding ability. The effect of pH has also been investigated. It has been reported that pH can change a protein's tertiary structure, the ionic state of acid and amine groups, and its degree of reactivity (Reineccius, 2005). This has been studied using specific flavor molecules: in a 5% caseinate solution at 40ºC, the binding of butanal, hexanal and diacetyl to the protein was shown as being dependent on pH, whereas heptanone's was shown to be independent of pH (Overbosch et al., 1991). The temperature modifies the interactions between proteins and flavors. When proteins are heat denatured, this results in the dissociation and unfolding of polypeptides with a subsequent aggregation into a precipitate (Damodaran & Kinsella, 1981; Guichard, 2006). This change in protein conformation can cause a decrease in association constants, greater affinity of flavors to protein binding pockets, as well as emergence of additional binding sites on proteins. (Guichard, 2006). Through a similar mechanism, the addition of modifiers such as urea may lessen interactions, as occurs for the model system of soy proteins and 2-nonanone. Finally, the presence of salt can equally impact retention by altering the ionic force within the matrix. In the β-lactoglobulin / 2-octanone system, increasing salt concentration enhances their binding (Damodaran & Kinsella, 1981).

Flavor absorption can be defined as volatile aroma compounds being trapped onto food ingredients, such as proteins or carbohydrates. Flavor binding by adsorption to the surface of these constituents or diffusing into the interior can be of a variety of types: covalent, hydrogen, or hydrophobic interactions (Halász & Lásztity, 1991; McGorrin, 2014). The occurrence of binding depends on the availability of binding sites, which is dictated by protein structure and amino acid composition. Finally, flavor release involves liberating aroma molecules into the gaseous

environment to be perceived, as well as tasting the non-volatile components in the aqueous environment (McGorrin, 2014).

Polar compounds (e.g. alcohols) bind using hydrogen bonds, while hydrophobic interactions are frequently observed with low molecular weight nonpolar compounds. This occurs as a result of the three-dimensional configuration of proteins, resulting in a spherical structure with hydrophobic interior regions and a hydrophilic surface. The hydrophobic areas impart stabilization of the constituent, in addition to an interaction site with the nonpolar aromatic substances (Charalambous, 1981). Aldehydes and ketones bind to amino acids, and amines bind to carboxylic groups via irreversible covalent linkages, commonly seen with high molecular weight volatiles; irreversible linkages of Schiff bases can occur between carbonyl groups, found on flavor compounds, and amide side chains, such as glutamine (Hansen & Heins, 1991).

Factors in the food matrix can influence flavor-protein binding. The addition of water improves the binding of polar molecules, with no effect on nonpolar volatiles. Increasing the protein content of the product will enhance protein-protein interactions, thereby decreasing binding of volatile molecules. In solutions, pH and salt concentration can modify protein configuration, and substances that dissociate protein or break disulfide bonds result in increased binding (Halász & Lásztity, 1991). Maier and Hartmann (1977) have established the interaction between certain amino acids and volatile aroma compounds. They determined that in dry form at room temperature, lysine showed the greatest binding affinity towards ketones and aldehydes, and adsorbed to them irreversibly. Cysteine almost adsorbed as many carbonyl groups, by forming thiazolidinecarboxylic acids. Likewise, arginine, histidine, phenylalanine, tyrosine, proline, valine, leucine, and isoleucine, found in barley, also exhibited strong interactions with volatile compounds.

As discussed earlier, any flavor compound is prone to chemical changes caused by several interactions: thermal degradation, oxidative and hydrolytic reactions, and interaction with protein in food matrices, regardless of degree of volatility, neutral or acidic state, or the presence of sulfurous compounds (Weerawatanakorn et al., 2015).

#### *1.3.1.2. Interactions between Proteins and Vanillin*

Vanilla is the most commonly used flavoring constituent in the food industry, used in a range of foods including bakery, ice cream, and confectionary. The primary component, vanillin (4-hydroxy-3-methoxybenzaldahyde), is a phenolic aldehyde containing three functional groups: aldehydic, phenolic hydroxyl, and an aromatic nucleus. It has been observed that in a liquid food environment, the aldehyde group of flavor compounds binds covalently to amino groups of proteins using a Schiff base. Vanillin has been shown to interact via hydrogen bonding, hydrophobic interactions, and electrostatic interactions with amino acids and proteins. These interactions influence flavor perception during consumption, and may decrease apparent flavor intensity, though the degree is dependent on type of protein, conformational state, concentration, as well as pH and temperature of the environment (Weerawatanakorn et al., 2015).

### 1.3.2. Analysis of Flavor-Protein Interactions

#### *1.3.2.1. HS-SPME/GC*

Headspace solid-phase microextraction (HS-SPME) is a simple, sensitive, and solvent-free technique to screen volatile compounds. The headspace relates to the compounds at equilibrium between the headspace and the solution. The technique is, in part, founded on adsorption/absorption features of specific fiber coating fabric. Volatiles or semi-volatiles are released from gaseous, liquid, or solid matrices under investigation, and sorbed onto a fabric overlaid with a polymer capable of ad- or ab-sorption. The analytes are then desorbed by heating onto a gas chromatography inlet, or desorbed by solvent treatment onto a high-performance liquid chromatographic inlet. Static headspace tests have been reported to suffer from reduced sensitivity and selectivity. The partition coefficient of the volatiles or semi-volatiles between the headspace and the matrix, and between the headspace and the fiber both highly affect measurements taken by headspace solid phase microextraction (HS-SPME) , and can be influenced by factors, such as hydrophobicity, solubility, and interaction with of nonvolatile components (Jung & Ebeler, 2003). HS-SPME is recommended to be coupled with some additional analytical techniques, since it only provides limited information on the binding mechanisms, and none on the binding type (Guichard & Langourieux, 2000).

#### *1.3.2.2. Equilibrium and Non-Equilibrium Dialysis*

Equilibrium dialysis is used to monitor interactions between low affinity molecules in solution. It involves ligand binding to a constituent (e.g. protein), placed on one side of a dialysis membrane. At equilibrium, the concentration of free ligand in solution will be equivalent on either side of the membrane. Concentration will be greater in the receptor chamber, due to bound ligand, and can be analyzed for binding properties (Tromelin, Andriot, & Guichard, 2006). The main shortcoming reported with this method has been the degradation or complete loss of gaseous analytes, and the fact that it cannot differentiate between reversible and irreversible interactions (Jung & Ebeler, 2003; Tromelin et al., 2006). Nevertheless, this method is commonly used, as binding constants obtained from any study can be compared (Guichard, 2006).

#### *1.3.2.3. HPLC*

High performance liquid chromatography (HPLC) is often used for quantifying non-volatile and volatile compounds (Yang et al., 2013). A rapid and effective method for screening flavor compounds has been developed by Sostmann and Guichard (1998). A silica gel coated with stationary protein is filled into a column under vacuum and connected to an HPLC column. Binding constants can be calculated based on retention times. This test can be conducted while modifying different conditions, including pH, and various salt and flavor concentrations.

#### *1.3.2.4. Sensory Evaluation*

A common and conventional method for measuring flavor perception, sensory evaluation relies on the five senses to achieve quantitative and qualitative results, which has been implemented in testing numerous protein-flavor interactions. For example, trained panelists were invited to taste three aroma compounds, benzaldehyde, limonene, and citral in the presence of casein and whey protein, and asked for a description of how these differed from the control (Hansen & Heins, 1991). Results did not deviate far for each compound. Benzaldehyde flavor perception decreased upon interaction with casein, but no difference was noted with casein. Limonene intensity decreased when both protein concentration increased, and no change in citral perception was detected for casein or whey. The interactions with benzaldehyde and limonene could be explained via certain mechanisms: for casein, hydrophobic interactions are the primary types of binding, and whey proteins undergo cysteine-aldehyde condensation reactions as well as Schiff base formation. Another study yielded different findings. A β-lactoglobulin aqueous solution decreased methyl

ketone and eugenol odor perception, but surprisingly vanillin was unaffected, perhaps being the compound boasting the least affinity to this particular protein. Quantitatively, flavor retention rates greater than 20% were deemed perceivable by panelists who noted a significant decrease in ability to perceive odor (Guichard, 2006).

#### *1.3.2.5. Spectroscopic Methods*

While the previously mentioned methods shed insight as to the presence and degree of interactions between flavor compounds and proteins, spectroscopic methods can be used to determine the nature of these interactions. Using fluorescence spectroscopy, the shifts in wavelength ($\lambda_{max}$) and changes in intensity of the tryptophan residue can be correlated with conformational and environmental changes of the proteins (Wang & Arntfield, 2015). A decrease in fluorescence intensity occurs upon electron transfer quenching by internal or external ligands (Ghisaidoobe & Chung, 2014). By plotting the molar ratio of vanillin:protein against the change in fluorescence intensity, the association constant ($K_a$) and number of binding sites (n) can be determined (Libardi et al., 2011). It can be expected that upon complex formation between a protein and a ligand, the maximum fluorescence emission will shift, due to binding at a different receptor site or altering the protein conformation (Damodaran & Paraf, 1997).

#### *1.3.2.6. Dynamic coupled column liquid chromatography (DCCLC) and Affinity Chromatography*

DCCLC is a technique enabling the measurement of a solution saturated by the compound of interest, which coats glass beads filled in the column and is washed with water. This method has been used to study aggregate formation constants in the interaction of selected aroma molecules and β-lactoglobulin (Tromelin et al., 2006). Using DCCLC, it can be assumes that the results obtained will correlate directly to molecular interactions, but this does not account for the migration of aroma compounds in a dilute solution into the gaseous phase (Guichard & Langourieux, 2000). Affinity chromatography provides the ability to purify biomolecules depending on their chemical structure. In the case of protein-flavor analysis, this technique allows for the observation of competitive interactions between certain flavor compounds in a matrix. Together, DCCLC and affinity chromatography can help determine affinity constants for

reversible interactions (Tromelin et al., 2006).

#### *1.3.2.7. Other techniques*

With increasing evidence of the deleterious effects of protein-flavor interactions and their mechanisms, researchers have been developing other methods to quantify this phenomenon. Lubbers, Landy, and Voilley (1998) performed quantitative structure-activity relationships (QSAR), which describe the flavor-protein interaction from the perspective of the protein while contributing some information from the aroma molecule. This method has proven to be useful in determining binding sub-sites on any protein, granted sufficient information has been collected on the flavor-protein binding constants. More recently, a novel technique termed Atmospheric Pressure Chemical Ionisation-Mass Spectroscopy (APCI-MS) allows the monitoring of flavor-protein interactions in the human system. By quantifying the content of flavor freed from the nasal cavity, the influence of protein on flavor perception can be precisely demonstrated and compounds not measured are assumed to be either irreversibly bound or highly reversibly bound to proteins (Le Guen & Vreeker, 2003).

### 1.4. Determination of Binding Parameters

The quantification of binding between flavor compounds and proteins can be done experimentally or theoretically. Binding parameters include dissociation constant (k), total number of binding sites (n), enthalpy of binding (ΔH°), and entropy of binding (ΔS°). Numerous plots may be used to determine such values, some of which are described below (Klotz & Urquhart, 1949; Klotz, Walker, & Bivan, 1946).

#### *1.4.1. Scatchard Plot*

The Scatchard equation depicting the thermodynamic relationship between the binding of one ligand to multiple binding sites on a protein with the same equilibrium binding constant can be represented as:

$$\frac{v}{[L]} = Kn - Kv \qquad \text{(Equation 2)}$$

where $v$ is the number of moles of ligand (flavor) bound per mole of protein, $[L]$ is the free ligand concentration ($mol \cdot l^{-1}$), $K$ is the binding constant, $n$ is the number of binding sites (Scatchard,

1949). This can be rearranged and plotted between $\frac{v}{[L]}$ and $v$, giving a linear relationship. The slope provides the value of $-K$, and $n$ and $K$ can be determined from the y-intercept $nK$.

### *1.4.2. Klotz Plot*

The double-reciprocal form of the Scatchard plot is the Klotz plot, the most commonly used technique in binding studies (Invitrogen Corporation, 2008; Klotz et al., 1946).

$$\frac{1}{v} = \frac{1}{n} + \frac{1}{Kn[L]} \qquad \text{(Equation 3)}$$

where $v$ is the number of moles of flavor bound per mole of protein ($L$), n is defined as the number of binding sites on the protein, and $K$ is the binding constant.

Plotting $1/[L]$ vs $1/v$ causes the plot to be linear. $n$ can be determined from the y-intercept $1/n$, while the dissociation constant ($K_d$) can be calculated from the slope ($K_d/n$) and the binding equilibrium constant ($K$) can be obtained using $K=1/K_d$ (Kühn, Considine, & Singh, 2008).

### *1.4.3. Hill Plot*

As previously stated, Scatchard and Klotz plots are the most commonly used models in binding studies. Both of them assume that the protein's receptor sites are equal and independent; if they are not, the resulting plots will not be linear. The Hill plot, which accounts for this possibility, is used to determine the cooperativity between binding sites. The equation is as follows;

$$v = \frac{n}{\frac{1}{(K[L])^h} + 1} \qquad \text{(Equation 4)}$$

where $h$ is the Hill coefficient, representing the interactions between receptor sites. As is the case for other plots, the double-reciprocal form is mostly used:

$$\frac{1}{v} = \frac{1}{n(k[L])^h} + \frac{1}{n} \qquad \text{(Equation 5)}$$

The equation is plotted as $\frac{1}{v}$ against $\frac{1}{[L]}$, where $h$ can be determined using non-linear regression. If no cooperativity exists between binding sites, a linear plot will be obtained with a slope of 1.0. A slope greater than 1.0 will be indicative of positive cooperativity, while negative cooperativity will give a Hill plot with a slope less than 1.0 (Kühn et al., 2008).

### 1.5. Factors Influencing Protein-Flavor Binding

To attenuate the potential loss of flavor in high protein foods, certain approaches are being explored.

#### *1.5.1. pH of the Medium*

The pH of a protein's environment has a significant effect on its structural conformation, and can subsequently enhance or hinder ligand binding. For β-lactoglobulin, at pH values below 6.2 the entry point of the hydrophobic cavity where binding takes place is closed, and is open between pH 6.2-7.2. This may explain enhanced ligand binding at higher pH values, as well as increased specificity at lower pH values (Tromelin et al., 2006).

#### *1.5.2. Heat Treatment*

Most protein-flavor interactions have focused on native proteins, however heat treatment resulting in the denaturation of proteins is a common occurrence in food processing. As previously mentioned, the binding behavior of flavors to proteins is protein- and flavor-dependent, therefore interactions have varying outcomes. Generally, flavor binding may increase as a protein unfolds by exposing hidden hydrophobic binding sites. Conversely, interaction may decrease due to modifications of these sites, or hydrophobic protein-protein interactions upon aggregation (Kühn et al., 2008). In the case of β-lactoglobulin, Kühn et al. (2008) reported that this protein partially unfolds upon denaturation, and forms an aggregate due to hydrophobic and intra- and inter-protein disulfide bonds. The effect of heating canola protein isolate combined with aldehyde and ketone flavors was investigated using GC-SPME by Wang & Arntfield (2014, 2016). An increase in the binding interactions with increased heating time was observed with aldehydes, due to a greater occurrence of irreversible covalent interactions than reversible hydrophobic interactions. The binding trend of ketones increased and, then, decreased with heating time, suggesting that the protein-flavor interactions were substituted by the protein-protein interactions occurring from aggregation, thereby releasing the flavor compounds.

#### *1.5.3. High Pressure Treatment*

Another commonly used processing method is high pressure treatment, which is able to maintain desired quality characteristics of food. Of the few studies done on binding interactions between flavors and proteins, it has been observed that a high pressure treatment of 600 MPa for 10 minutes on whey protein concentrate (WPC) increased both the number of binding sites and dissociation

constants. However, aggregate formation resulting from the treatment restricted access to newly formed binding sites. Furthermore, after prolonged treatment of 30 minutes, the dissociation constants returned to untreated-WPC values, mitigating the effects of aggregation on the binding interactions (Liu et al., 2006). Similarly, Yang et al. (2003) treated β-lactoglobulin to 500 MPa and reported an increase in binding sites and dissociation constant, the latter indicating decreased binding affinity after high pressure treatment.

### *1.5.4. Enzymatic Hydrolysis of Proteins*

A method of preparation of barley protein hydrolysates was described by Yalçin and Çelik, (2007) using an automatic titrator to terminate hydrolysis at a desired degree of hydrolysis (DH%). The barley protein isolate is dispersed in deionized water at a protein concentration of 8.3% (w/v). Efficient condition parameters were determined to be pH 8 and at 37ºC, with the optimal enzyme to substrate ratio of 1:300 (w/w). The batch system with constant agitation and temperature has a set volume of 60 ml. The pH of the isolate is modified to 8.0-8.2 prior to hydrolysis. Enzyme is dissolved in less than 1 ml of deionized water, and added to the mixture incubating at 37ºC. The pH of the process is maintained by constant drip of 0.2 M NaOH. Upon achieving desired DH, the process is ceased by immersing the slurry in a hot water bath for 5 minutes, then cooled with cold water. The mixture can be freeze-dried and kept at 4ºC until needed.

Modifying protein side chain has been investigated as a means of reducing the binding of flavors. In this regard, Suppavorasatit, Lee, & Cadwallader (2013) investigated the effect of deamidation on soy proteins. In addition to hydrolysis, protein glutaminase releases amino acids or peptides from side chains, thereby altering the flavor profile. A sensory evaluation test was used to verify these modifications, and there was a perceived difference in taste of deamidated and control soymilk, which may be due to changes in viscosity or to mouthfeel. In addition, intensity rating for the deamidated soymilk is nearly 1.5 times greater than the control. Similarly, the best estimate odor threshold using vanillin and maltol was significantly lower for the deamidated sample, indicating that the binding affinity of the two molecules is lower than to the intact protein. A possible explanation is that the deamidation transforms the glutamine residues into glutamic acids, which decreases the presence of amide groups. The lack of bond sites hinders the covalent binding by carbonyl groups of vanillin. The binding type has been hypothesized to change from covalent to hydrophobic interactions or even hydrogen bonding and van der Waals forces. Lozano (2009)

suggested that the reduction in off-flavor binding to soy protein following deamidation is due to the decrease in asparagine and glutamine contents, as these would otherwise form Schiff bases with volatile organic compounds containing carbonyl groups.

### *1.5.5. Encapsulation of Flavors*

Encapsulation is a method of coating a solid or liquid particle, or gas cell with a thin and continuous layer, to fully protect the material held inside (Marcuzzo et al., 2010). In food applications, it is the process of protecting a flavor in a carrier as a means to prevent it from "evaporation, reaction, or migration" within a food product (Zeller, Saleeb, & Ludescher, 1998). It has been touted as a preferred method of controlling flavor loss occurring post-processing and product's shelf life, due to its ability for controlled release (Marcuzzo et al., 2010). The most widely used encapsulation manufacturing techniques are spray-drying and extrusion. Spray-drying implies entrapping flavors in a spherical sold coating of a carbohydrate matrix to reduce migration. Stability of the volatile flavor components rely heavily on the processing temperature, as they are highly heat-sensitive in addition to being difficult to encapsulate. Extrusion also aims to reduce migration through entrapment of the aroma compound in an impervious glass. Attention must be paid to ensuring the solid walls are not cracked, are of adequate thickness, and that pore production during or post processing has been minimized. Encapsulated flavors produced by both methods have been noted to experience oxidation and delayed diffusion (Zeller et al., 1998). The capsule composition can include starches, sugars, proteins, gums, and lipids (Bortnowska, 2010). Edible films are made with proteins (wheat gluten, WPI, soy) or polysaccharides (alginates, starch), due to the myriad of positive interactions within food products such as high structural and oxygen barrier properties. Certain hydrophobic elements (lipids, essential oils) may also be used in formulating encapsulation coatings as they provide a moisture barrier (Marcuzzo et al., 2010).

## CONNECTING STATEMENT I

A literature review on the isolation of barley proteins, the functional properties of proteins, and an overview of the interaction between proteins and flavors, as well as techniques to assess them, were presented in Chapter I.

Chapter II investigates the extraction of barley proteins. Multiple approaches, including conventional and enzymatic methods, are discussed in terms of protein content and recovery yield. Barley protein concentrates were characterized based on their primary, secondary and tertiary structure and compared to two control proteins, pea protein concentrate and whey protein isolate. The proteins were also exposed to heat and high pressure treatments, and the effects on structural characterization was also studied. Lastly, the functional properties (viscosity, foaming, and emulsification) of two barley protein concentrates are reported in this chapter.

# CHAPTER II

# EXTRACTION, STRUCTURAL CHARACTERIZATION AND FUNCTIONAL PROPERTIES OF BARLEY PROTEIN CONENTRATES

## 2.1. Abstract

Barley (*Hordeum vulgare* L) protein extracts were prepared from defatted barley flour using alkaline extraction and enzymatic treatments. Milder enzymatic treatments included: (i) a bi-enzymatic method involving starch removal using two α-amylases, and (ii) a tri-enzymatic sequential method using the former bi-enzymatic treatment followed by digestion with a glucanase from *Trichoderma longibrachiatum*. Results showed that the combination of alkaline extraction with isoelectric precipitation (IEP) (AI-BP) improved the protein content, which was comprised mainly of low molecular weight fractions. The bi-enzymatic treatment produced a protein concentrate with the highest protein content (49.0%), while those obtained by the tri-enzymatic treatment followed by an IEP step (TEI-BP) gave the highest protein recovery yield (78.3%). In both of the latter concentrates, 35 kDa B hordeins were the major protein fraction. Divergence between the composition of barley protein concentrates (TEI-BP; AI-BP) in secondary structure elements may be attributed to the difference in their protein profiles. Further characterization of barley protein concentrates indicated that they exhibit pseudoplastic behavior and form stable emulsions. In addition, their highest foaming capacities and foaming stabilities were obtained at pH 3.0 and 8.0, respectively.

## 2.2. Introduction

The increasing demand for health-promoting foods has fueled the development of functional protein ingredients. Furthermore, there is an ever-increasing interest in exploring new sources of plant-derived proteins, based on health, environmental, and economical rationales. Among the currently available plant-derived proteins, soy and wheat proteins remain dominant. Although the use of pulse proteins has recently grown dramatically, the demand for ancient grains has also spiked. One grain that has yet to be thoroughly investigated for its protein characteristics and functionality is barley (*Hordeum vulgare* L). Barley has been domesticated for over 10,000 years Badr, M, Sch, Rabey, Effgen, Ibrahim, et al. (2000) and is now the fourth most grown cereal globally after wheat, rice and corn (Xia, Wang, & Chen, 2011). This crop was brought to Canada in the early 17$^{th}$ century where it has since been cultivated intensively, especially in the western region of the country (Friedt et al., 2010). With an annual production varying between 7.1 and 11.8 million tons during the last decade, Canada ranks among the most important barley producers

worldwide (Statistics Canada, 2017). This production covers the national needs and a substantial part is exported mainly to China, the USA, and Japan, generating about 1.0 billion Dollars every year (Barley Council of Canada, 2017).

Barley is a multi-purpose cereal primarily utilized for animal feed (65%) and as raw material to produce malt (33%) for the brewing industry, while only 2% of the world barley supply is used directly in human consumption (Sullivan, Arendt, & Gallagher, 2013). In Canada, the figure is 80% of barley production used in livestock feeding, 15 % transformed into malt for beer-making, and a meager part of less than 5% directed for human consumption (Wang et al., 2010). The unpopularity of barley in human diet may be due, in part, to its distinct flavor being a negative acceptance factor among consumers. However, the grain gained increased popularity in recent years owing to the association of the soluble β-glucan fiber and phytochemical compounds it contains with various health benefits (Alu'datt et al., 2012; Idehen, Tang, & Sang, 2017). The approval, by the US Food and Drug Administration, of the claim establishing a relationship between β-glucan soluble fiber from barley and reduced risk of coronary heart disease (FDA, 2006) was an additional incentive that stimulated research interest on barley as a source for bioactive components (Idehen, Tang, & Sang, 2017). Although β-glucan was the most credited for therapeutic effects, it is now well established that other bioactive barley components, including minerals, vitamins, and various phytochemical molecules are also associated with health benefits (Idehen, Tang, & Sang, 2017). This was also evidenced by the observation that regular barley consumption prevented chronic diseases, such as colonic cancer (Dongowski et al., 2002; Finn 2008), diabetes (Sullivan, Arendt, & Gallagher, 2013; Thondre, 2014), and gallstones (Hoang et al., 2011; Zhang et al., 1990). Barley contains 10-20% proteins dominated with the prolamin hordeins (52%) as the main storage proteins (87%) of the endosperm layer. Glutelin is another major storage protein of the endosperm, which represents 23% of the total barley proteins. Two other cytoplasmic proteins, albumins and globulins, represent minor storage proteins of the grain aleurone and embryo (Fontana & Buiatti, 2009; Sullivan, Arendt, & Gallagher, 2013). Barley is intensively used as a raw material in the starch industry generating large quantities of protein as a by-product that can be valorized in the food industry (Yalçın, Çelik, & İbanoğlu, 2007). Indeed, barley proteins are good candidates for value-added application as food supplements owing to their functional properties, including emulsifying capacity and stability, foaming, elasticity, cohesiveness, and water holding capacity that enhance rheological food properties (Alu'datt et al.,

2014; Wang et al., 2010; Yalçın, Çelik, & İbanoğlu, 2007). Despite the limited nutritive value of barley proteins in the human diet due to their low digestibility, they constitute a good source for essential amino acids, such as threonine, valine, lysine, phenylalanine, and arginine (Sullivan, Arendt, & Gallagher, 2013). The low digestibility may, in fact, be a desirable attribute as it ensures a controlled release of bioactive compounds and exerts a protective effects on the gastrointestinal tract (Xia, Wang, & Chen, 2011). Therefore, barley proteins have a potential for application in food development with a competitive advantage compared to the trend-leading whey and soya proteins (Singh et al., 2008). This is especially timely to address the increased consumer preference for the plant-based proteins sourced from alternative whole grains, having the ability to enhance the nutritional profile and to add to the health attributes of familiar foods.

The overall objective of the study was to optimize the isolation of barley proteins and characterize their structural properties. This was achieved by the following specific objectives: (1) Developing an enzymatic approach for the isolation of barley proteins and comparing its efficiency with conventional extraction methods under alkaline conditions, (2) Characterizing the primary, secondary and tertiary structural properties of two protein concentrates, obtained by using enzymatic and alkaline extractions, and (3) Assessing their functional properties.

## 2.3. Materials and Methods

### *2.3.1. Materials*

Sodium hydroxide (NaOH), hexane ($C_6H_{14}$), tris base, and sodium citrate dihydrate [$HOC(COONa)(CH_2COONa)_2{\cdot}2H_2O$] were obtained from Fisher Scientific (Fair Lawn, NJ). Hydrochloric acid was purchased from Acros (Fair Lawn, NJ). Citric acid anhydrous ($C_6H_8O_7$) was obtained from Debro (On, Ca). Potassium phosphate monobasic ($KH_2PO_4$), potassium phosphate dibasic ($K_2HPO_4$), glycerol trioleate, α-amlyase from *Bacillus* sp., α-amylase from *Bacillus licheniformis* (Termamyl™), amyloglucosidase from *Aspergillus niger*, and β-1,3,4 glucanase from *Trichoderma longibrachiatum* were purchased from Sigma Aldrich (St. Louis, MO). Deionized water (Millipore) was used in all experiments. Barley flour was manufactured by Meunerie Milanaise (Qc, Ca). SDS-PAGE broad molecular weight standard (6.5-200 kDa) was obtained from Bio-Rad (On, Ca). Pea protein concentrate (PPC) was obtained from Roquette (Nord-Pas-de-Calais, FR), and whey protein isolate (WPI) from Hilmar (Hilmar, CA).

### 2.3.2. *Preparation of defatted barley flour*

Barley flour of the 2-row variety was defatted with hexane using a solvent-to-flour ratio of 1:10 (w/v) by shaking at 200 rpm for 1h at 25ºC. The slurry was centrifuged at 8500*g* for 15 min at 4 ºC in a Beckman Avanti centrifuge, model J25-I (Beckman Coulter, CA, USA). The precipitate was then air-dried at room temperature as a thin layer and stored at 4ºC until needed.

### 2.3.3. Methods for preparation of barley protein extract

#### *2.3.3.1. Alkaline extraction*

An alkaline extraction was performed by mixing the DBF with 0.5 M NaOH (pH 11.0) at a solvent-to-flour ratio of 10:1 (w/v); e.g., 50 g DBF in 500 mL NaOH solution, for 2 h at 23 ºC. The mixture was centrifuged at 4000 *g* for 15 min and the supernatant was dialyzed for 48 h at 4 °C against Millipore water using a 2000-Da benzoylated dialysis tubing to remove low molecular weight sugars. This preparation, designated alkaline barley protein (A-BP) extract, was freeze dried to be used for further analyses.

#### *2.3.3.2. Sequential alkaline/isoelectric extraction*

The alkaline extraction was repeated as explained above (section 2.3.3.1) and followed by an isoelectric precipitation (IEP). After centrifugation of the alkaline-treated defatted flour, the pH of the supernatant was adjusted to 4.5 with 0.5 M HCl and left to precipitate overnight at 4 ºC. The pH was re-determined to confirm that the pH remained stable; the preparation was then centrifuged at 4000 *g* for 20 min, and the pellet was re-suspended in distilled water (1:1), dialyzed, and freeze-dried. This preparation was designated alkaline and IEP barley protein (AI-BP) extract.

#### *2.3.3.3. Barley protein extraction by enzymatic treatment*

To prevent excessive protein degradation during the extraction process by the alkaline methods, milder enzymatic techniques to hydrolyze barley carbohydrates (starch reserves and cell-wall glucans) and recover extracts enriched in proteins were assayed. To this end, DBF was subjected to digestion with an α-amylase (mono-enzymatic treatment), with an α-amylase followed by an amyloglucosidase (bi-enzymatic treatment), or with an α-amylase followed by an amylo-glucosidase and a β-glucanase (tri-enzymatic treatment).

#### *2.3.3.3.1. Mono and bi-enzymatic digestion approach for protein isolation*

In the mono-enzymatic treatment approach, DBF was digested with each of two α-amylases, one from *Bacillus* sp. and another, Termamyl™, from *B. licheniformis*. DBF was re-suspended in 10 mM potassium phosphate buffer (pH 6.5) at a 1:10 ratio (w/v), and either the α-amylase from *Bacillus* sp. or Termamyl™ was added to the final concentrations of 10,000U/g or 5000 U/g, respectively. The reaction mixture involving the amylase from *Bacillus* sp. was incubated at 65 ºC for 1 h, while the reaction mixture using Termamyl™ was incubated at 40 °C for 16 h. After incubation, each reaction mixture was centrifuged at 8500 *g*, and the supernatant was recovered, and freeze-dried to be stored until needed for further analyses.

In the bi-enzymatic treatment, DBF was successively digested with the α-amylase from *Bacillus* sp. followed by amyloglucosidase. Two different combinations of enzyme/flour ratios for the treatment with the α-amylase and amyloglucosidase were used. In the first combination, the α-amylase was added to a suspension (1:10) of DBF in 10 mM potassium phosphate buffer (pH 6.5) at the final ratio enzyme/flour of 5000 U/g and incubated for 1 h at 40 °C. The amyloglucosidase was then added to the reaction mixture at the ratio enzyme/flour of 330 U/g and the incubation continued at the same temperature for an additional period of 16 h. For the second combination, two aliquots of DBF suspension were digested with 1,000 U/g of α-amylase for 1h at 40 °C, then 660 U/g amyloglucosidase was added to each of them; then incubation continued at 40 °C for 4 h for one aliquot and 16 h for the other. After incubation, the reaction mixtures were centrifuged at 4000 *g* for 15 min and the supernatants were dialyzed, freeze-dried, and stored for further analyses. The resulting product was designated bi-enzymatic barley protein (BE-BP) extract.

#### *2.3.3.3.2. Tri-enzymatic digestion approach for protein isolation*

The isolation of proteins from barley upon hydrolysis of starch and the cell-wall glucan was carried out by the tri-enzymatic approach. This approach was based on the sequential digestion with the α-amylase from *Bacillus* sp. (10,000 U/g, 65 ºC, 1h, pH 6.5), the amyloglucosidase (660 U/g, 40ºC, 16 h, pH 6.5), and the β-1,3,4-glucanase (8 U/g, 37 ºC, 1 h, pH 5.0). The mixture was centrifuged at 4000 *g* for 15 min and the supernatant dialyzed and freeze-dried. This preparation was designated as the tri-enzymatic barley protein (TE-BP) extract.

#### *2.3.3.3.3. Combined tri-enzymatic approach and isoelectric precipitation*

A tri-enzymatic starch and glucan removal was carried out as described above (section 2.1.3.3.2.) and followed by the IEP, whereby the pH of the mixture was adjusted to 3.7 with 0.5M HCl and kept undisturbed overnight at 4 ºC. It was then centrifuged at 4000 g for 20 min and the pellet was recovered, dialyzed, freeze-dried, and its protein content determined. This preparation was designated tri-enzymatic and IEP barley protein (TEI-BP) extract.

### *2.3.4. Protein determination*

Nitrogen content of the barley protein extracts was determined by using a Leco TruSpec Micro CHNS (Leco Corporation, MI), following the Dumas method as described by Kirsten and Hesselius (1983). Protein content was obtained by multiplying the nitrogen content by the barley protein nitrogen conversion factor of 5.83 (Jones, 1931). The protein recovery yield (%) and the purification factor (w/w) were calculated for all extraction methods according to equations (6) and (7), respectively:

$$\text{Yield (\%)} = \frac{Protein\ content_{precipitate}}{Protein\ content_{initial\ flour}} \text{x } 100 \qquad \text{(Equation 6)}$$

Purification factor (w/w) was calculated according to the following equation:

$$\text{Purification factor} = \frac{Protein\ proportion_{initial}}{Protein\ proportion_{extract}} \qquad \text{(Equation 7)}$$

### *2.3.5. Molecular weight (MW) distribution of proteins*

The protein extracts were analyzed for molecular weight distribution by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Stacking and resolving gels contained 5.0 and 15.0% acrylamide, respectively using a 1.5-mm thick gel in a mini-gel apparatus (Bio-Rad, On, Ca). The electrophoresis was run at 120 mV using the bio-rad broad range molecular weight markers as standards. Gels were stained for 2 h in a solution of Coomassie Blue R250 (2% w/v) in a methanol:water:acetic acid (45:45:10 v/v/v), then destained overnight in a methanol:water:acetic acid (1:8:1 v/v/v) solution. Quantitative band analysis was performed with the software AlphaView SA (Alpha Innotech, San Leandro, CA).

### 2.3.6. Structural Characterization

#### *2.3.6.1. Protein primary sequence analysis*

##### *2.3.6.1.1. Tryptic Digestion*

Structural characterization of barley protein extracts was carried out by mass spectroscopy (MS/MS). Firstly, a tryptic digestion was performed by using a MassPrep liquid handling robot (Micromass, MCH, UK) according to the method of Shevchenko et al. (1996) as modified by Havlis et al. (2003). Briefly, SDS-PAGE gels were run for both AI-BP and TEI-BP extracts as described above (section 2.1.5). Four of the most abundant protein bands from each extract were collected separately in Eppendorf tubes. They were then reduced with 10 mM DDT (Dichlorodiphenyltrichloroethane) and alkylated with 55 mM iodoacetamide. Trypsin digestion began with 105 mM of modified porcine trypsin (sequencing grade, Promega, WI, USA) at 58 ºC for 1 h. The generated peptides were extracted in 1% formic acid/2% acetonitrile, followed by 1% formic acid/50% acetonitrile, and the solvent was removed by vacuum centrifugation. The dried peptide extracts were solubilized in 10 µL of 0.1% (v/v) formic acid and subjected to mass spectroscopy analysis.

##### *2.3.6.1.2. Mass spectrometry*

Mass spectrometry was performed using a Thermo Surveyor MS pump joined to a LCQ Deca XP mass spectrometer (Thermo Electron, CA, USA) connected to a nanoelectrospray ion source (Thermo Electron, CA, USA). Peptide extracts were first separated by online reverse phase nanoscale capillary liquid chromatography and analyzed by electrospray mass spectrometry. A cap trap (Michrom Bioresources, CA, USA) bound the peptide extracts at 10 µL/min, and chromatographic separation using a PicoFrit column BioBasic C18, 10 cm x 0.075 mm internal diameter (New Objective, MA, USA). A linear gradient of 2 to 50% acetonitrile and 0.1% formic acid, for 30 min, at a flow rate of 200 nL/min (obtained by flow splitting) was then performed. Xcalibur software version 1.2 in the data-dependent acquisition mode was used to collect mass spectra. Finally, following the determination in full scan mass spectra (400-2000 *m/z*), the three most intense ions underwent collision-induced dissociation. Dynamix exclusion was selected (30 s exclusion duration) and relative collisional fragmentation energy was chosen at 35%.

#### *2.3.6.1.3. Database searching*

All MS/MS samples were analyzed using Mascot software (Matrix Science, London, UK; version 2.5.1). Mascot was set up to search the ORG_HordeumVulgare_4513_20161114 database (unknown version, 2369 entries) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.100 Da and a parent ion tolerance of 0.100 Da. Carbamidomethyl of cysteine was specified in Mascot as a fixed modification. Deamination of asparagine and glutamine and oxidation of methionine were specified in Mascot as variable modifications.

#### *2.3.6.1.4. Criteria for protein identification*

Scaffold (version Scaffold_4.7.2, Proteome Software Inc., Portland, OR) was used to validate MS/MS based protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability to achieve a false discovery rate (FDR) less than 1.0% by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 95.0% probability to achieve an FDR less than 1.0% and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. The amino acid sequence of the peptides generated were determined using the FASTA format, with the most prominent accession numbers SPZ4_HORVU, Q03678_HORVU, BSZ7_HORVU.

### *2.3.6.2. Secondary structure analysis*

The secondary structures of PPC, WPI, and two barley protein concentrates (AI-BP and TEI-BP) were analyzed by Fourier transform infrared spectroscopy (FTIR). The samples were suspended in $D_2O$ at a concentration of 50 mg protein/ml. Infrared spectra were recorded using a Bio-Rad Excalibur spectrophotometer by placing 10 µl aliquots of sample in an IR cell between two $CaF_2$ windows with a 25 µm pathlength. In total, 256 scans were averaged at 4 $cm^{-1}$ resolution. Deconvolution was performed using OMNIC software (version 7.3, Nicolet, Thermo Electron Corporation) with a half-bandwith of 25 $cm^{-1}$ and an enhancement factor of 2.5. The baselines were corrected between 1600 and 1700 $cm^{-1}$. Curve fitting was performed using Origin Lab software (version 93E). Bands were assigned as specified by Kong and Yu (2007), where bands

($cm^{-1}$) at 1624 + 4.0, 1631 + 3.0 and 1637 + 3.0 are β-sheets, 1645 + 4.0 are random coils, 1653 + 4.0 are α-helices, 1663 + 4.0, 1671 + 3.0, 1683 + 2.0, and 1694 + 2.0 are β-turns.

#### *2.3.6.3. Tertiary structure analysis*

Analyses of the tertiary structure were performed using a Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon system, Edison, NJ). Excitation was between 280-300 nm at increments of 10 nm, and emission measured at 280-700 nm with a scan speed of 120 nm/min. Both excitation and emission slits were set at 1 nm and carried out at 25°C. A 0.2% (w/v) sample from each of PPC, WPI, AI-BP, and TEI-BP extracts were prepared in distilled water.

### 2.3.7. *Treatments of selected protein concentrates and isolate*

PPC (74.2% protein content), whey protein isolate (WPI, 94.9% protein content), and barley protein concentrates (AI-BP, 71.6% protein content; TEI-BP, 78.3% protein content) were subjected to a heat treatment in a water bath for 20 min at their respective denaturing temperatures of 95 °C, 78 °C, and 85 °C (Hultin, 1949; Kühn, Considine, & Singh, 2008; Wang & Arntfield, 2015). The same proteins were also subjected to high pressure treatment using a 5-L static high pressure unit (ACIP 6500/5/12VB; ACB Pressure Systems, Nantes, France). Samples (3% w/v) were prepared at in distilled water, vacuum-sealed in low density polyethylene plastic bags and treated at 600 MPa for 30 min.

### 2.3.8. *Techno-functional Properties*

#### *2.3.8.1. Viscosity Properties*

The viscosity of solutions of 1% (w/v) pea, whey, and barley proteins (AI-BP and TEI-BP) was measured at 25°C using an AR2000 controlled-stress rheometer (TA, Crawley, U.K.), equipped with a 60 mm acrylic parallel plate. The shear rate was set between 1 and 100/s. To define the flow behavior of the solutions, the Ostwald-de Waele equation was used:

$$\tau = K\left(\frac{\partial u}{\partial y}\right)^{n} \qquad \text{(Equation 8)}$$

where $\partial u/\partial y$ is shear rate ($s^{-1}$), $\tau$ is shear stress (Pa), K is the flow consistency index ($Pa.s^{n}$), and n is flow behavior index.

#### *2.3.8.2. Foaming Properties*

The method of Wang et al. (2010) was used, with slight modifications, to measure the foaming properties. Solutions of 0.5% (w/v) PPC, WPI, and barley protein concentrates (AI-BP and TEI-BP) were dispersed in deionized water. Protein solutions were foamed using a PowerGen 125 homogenizer at speed 6 for two minutes. Foam volumes were recorded before and after using a graduated cylinder. The percentage volume increase was calculated as the foaming capacity (FC):

$$FC = \frac{(V_{f-} V_i)}{V_i} x\ 100 \qquad \text{(Equation 9)}$$

where $V_f$ and $V_i$ represent the final total volume of the protein solution after homogenization and the initial total volume, respectively.

Foam stability (FS) was determined as percentage of foam that remained after the solution was left to stand for 30 minutes at room temperature:

$$FS = \frac{Foam_{final}}{Foam_{initial}} x\ 100 \qquad \text{(Equation 10)}$$

#### *2.3.8.3. Emulsification Properties*

To test the emulsifying properties, the 0.2% (w/v) solutions of PPC, WPI, and barley protein extracts (AI-BP and TEI-BP) were prepared in MilliQ water. The barley protein extracts were adjusted to the pH of 11.0 for solubility. Glycerol trioleate was added to the solutions (1.25% v/v) and emulsified using a PowerGen 125 homogenizer at 30000 rpm for two minutes. This treatment was repeated three times. Droplet size was measured with a Delsa™Nano Submicron Particle Analyzer (Beckman Coulter). Measurements were performed at 25 °C immediately after homogenization and after 2 h of incubation at 25°C. Droplet size distribution was expressed by relative volume (v/v, %) of particles of each size in sample. In addition, the extent of increase of the droplet size was estimated as the droplet diameter (nm) after incubation over the value obtained before incubation.

### 2.3.9. *Statistical analysis*

Each experiment was repeated three times and data were expressed as means ± standard deviation and analyzed using one-way analysis of variance (ANOVA) and by the student *t*-test using Microsoft (Redmond, WA) Excel software. Means were evaluated for significance by the least significant difference procedure ($p < 0.05$).

## 2.4. Results and Discussion

### *2.4.1. Selected approaches for the preparation of barley protein extracts*

Figure 2.1 illustrates the efficiency of selected approaches for the preparation of barley protein extracts in terms of recovery yield, purification factor, and protein content. The enzymatic treatments were based on the hydrolysis of starch and/or glucans surrounding the proteins into mono and oligomers. The effect of an additional isoelectric precipitation (IEP) step after the alkaline extraction or the enzymatic approaches on the purification performances was also investigated. The alkaline extraction combined with the IEP resulted in a protein recovery yield of 51.4%, protein content of 68.9% in the AI-BP extract, with a purification factor of 9.45. However, when the IEP step was omitted from the extraction procedure, a higher protein portion was recovered (57.1%), but the protein content in the A-BP extract (33.0%) and the purification factor (4.52) were significantly ($p < 0.05$) lower (Figure 2.1). These results show clearly that the addition of the IEP step as part of the alkaline extraction procedure improves the purity of the resulting protein extract. Therefore, when high purity rather than quantity of the recovered extract is the priority, performing IEP after the alkaline extraction is the method of choice. For example, this step may be especially useful if the purification procedure is aimed to obtain a protein isolate (> 90% protein). The high protein yield with alkaline extraction (pH < 11.0) was explained by the breakdown of hydrogen bonds leading to the dissociation of hydrogen ions from carbolic and sulphate groups (Shen et al., 2008). The surface charge of the proteins becomes, therefore, increasingly negative, which enhances their solubility in aqueous solutions (Wang et al., 2010). Different performances of the protein extraction from cereals were reported the literature. Under similar conditions as those used in the present study (0.015M NaOH at a 10:1 of solvent to flour for 1h at room temperature), Alu'datt, et al. (2012) recovered higher protein yield (60.15%) from barley flour, but the protein content was as low as 32.9% even when the IEP was performed. However, Mohamed et al. (2007) successfully prepared a barley protein isolate (90.5%) with a

protein recovery yield of 70% by alkaline extraction followed by IEP. From a DBF dispersion in an alkaline solution, these authors collected the supernatant and subjected the pellets to another round of alkaline extraction; the supernatants resulting from the DBF and the pellet dispersions were mixed and subjected to an IEP by acidification to pH 4.5.

In preliminary screening studies, singular enzymatic starch removal approaches using two α-amylases were investigated (data not shown). When first using 1000 U/g of α-amylase from *B. licheniformis* for 16 h at 40ºC, the recovered extract from the DBF had a protein recovery yield of 53.8% and protein content of only 12.0%. This low protein content indicates that α-amylase from *B. licheniformis* induced a limited hydrolysis of starch into mono and short oligomers that did not cross the dialysis tubing. Alternatively, the proteins may have been hydrolyzed during the extraction process due to a naturally occurring proteases in the extract.

However, the use α-amylase from *Bacillus* sp. was used at a concentration of 2000 U/g for 1 h at 65ºC gave an extract with a lower protein recovery yield of 46.8%, but its protein content was significantly higher (49.6%). Waglay, Karboune, and Khodadadi (2016) achieved a protein recovery yield of 75% from potato pulp with an enzymatic approach using 2000 U/g of α-amylase from *Bacillus* sp., followed by a digestion with 16.66 U/g of each of polygalacturonase M1 and endo-1,4-β-D galactanase. The protein recovery yield has was increased to 100% when α-amylase from *Bacillus* sp. was replaced by 1000 U/g of α-amylase from *B. licheniformis* shown to be more effective in removing starch form potato pulp (Waglay, Karboune, & Khodadadi, 2016). Therefore, that the more effective starch removal, the higher is the protein recovery yield. The lower protein recovery yields obtained in our study compared with that reported by Waglay, Karboune, and Khodadadi (2016) can be explained by the complexity of the structure of barley cell wall compared potato.

Among the investigated enzymatic approaches (mono, bi-, tri), the tri-enzymatic treatment for sequential degradation of starch and glucan resulted in the highest protein recovery yields. The latter extraction procedure, with and without IEP, yielded 78.3 and 71.6% protein extracts, respectively (Figure 2.1). Conversely, the bi-enzymatic treatment based on starch removal by amylase and amyloglucosidase enzymes resulted in the highest purification factor (6.73) compared to the other enzymatic methods, and a protein recovery yield of 25.7% (Figure 2.1).

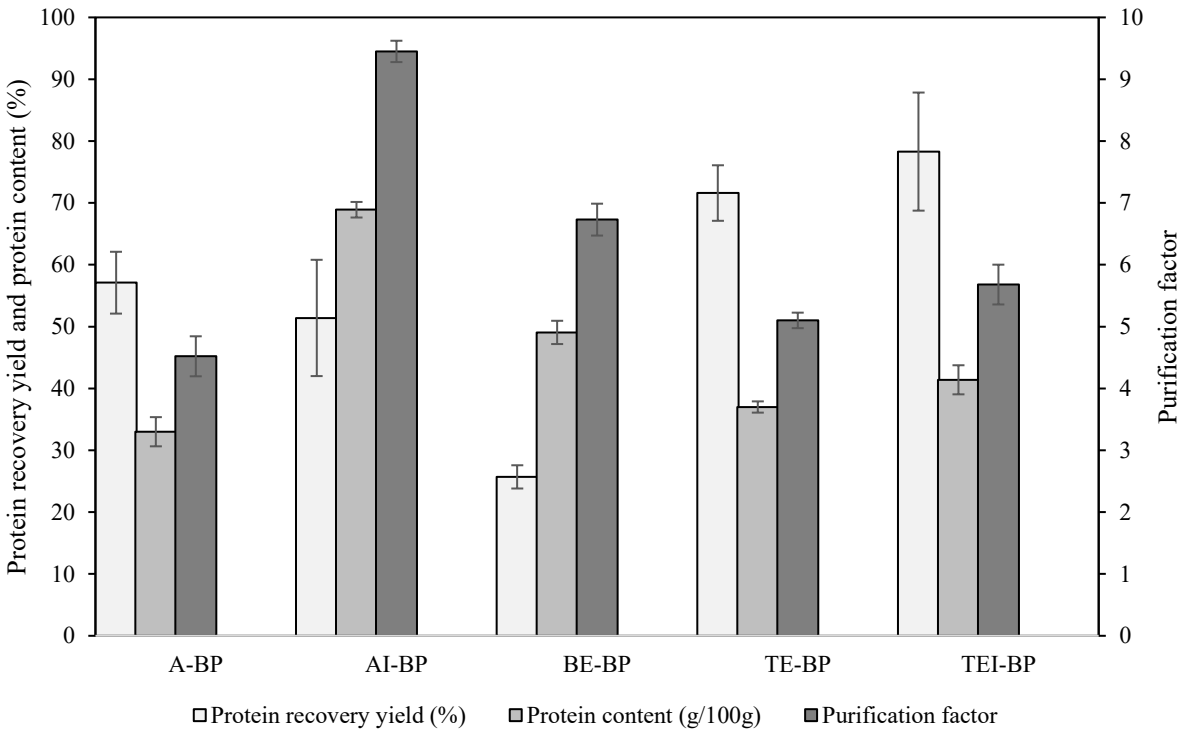


**Figure 2.1** Protein recovery yield (% ▭), protein content (% ▭) and purification factor ( ▭) of protein extractions from defatted barely flour (DBF). Alkaline extraction (A-BP) was performed with 0.5M NaOH for 2 h; bi-enzymatic treatment for starch removal (BE-BP) was performed with 10,000 U/g α-amylase from *Bacillus* sp. and 660 U/g amyloglucosidase, with an incubation time of 17 h tri-enzymatic starch and glucan removal (TE-BP) was performed with 10,000 U/g α-amylase from *Bacillus* sp. and 660 U/g amyloglucosidase, with an incubation time of 18 h, and 8 U/g β-1,3,4-glucanase for 1 h. Isoelectric precipitation (IEP) was performed by adjusting the pH to 4.5.

The bi-enzymatic treatment using both *Bacillus* sp. α-amylase and amyloglucosidase was then retained as the most efficient for protein extraction. Enzyme concentration and time of digestion variations were investigated. The bi-enzymatic treatment experiment using 10,000 U/g α-amylase and 660 U/g amyloglucosidase and an incubation time of 17 h gave an extract with the highest protein content of 49.1% and the lowest protein recovery yield of 25.7% (Figure 2.1).

The incubation time of the reaction mixture appears not to affect the performance of the treatment. After an initial incubation of DBF with α-amylase (1,000 U/g) for 1 h, further digestion of the reaction mixture with 660 U/g of amyloglucosidase for 4 h resulted in purification factor, protein recovery yield, and protein content of 6.06%, 28.8 %, and 44.2 %, respectively. These results did not differ significantly ($p > 0.05$) from those obtained when the incubation period in the presence of amyloglucosidase was extended for 16 h instead of 4 h (Figure 2.1). Conversely, the enzyme concentrations had a significant ($p < 0.05$) impact on the purification performances. A twofold reduction of enzyme concentrations (5000 U/g α-amylase and 330 U/g amyloglucosidase), resulted in a decrease in the purification factor and protein content to 4.68% and 34.1%, respectively; whereas the protein recovery yield was increased to 45.6%. It is worth mentioning that different performances were reported in the literature. For example, protein concentrates with higher protein contents were obtained by alkaline extraction from oat bran pre-treated with the cell wall degrading enzymes at different concentrations (Jodayree, Smith, & Tsopmo, 2012). According to these authors, α-amylase concentrations of 60 and 2300 U/g yielded extracts with concentrations of 70 and 72%, while treatment with amyloglucosidase at the concentrations of 8, 50, and 100 U/g produced protein concentrates with 82, 57 and 47% protein content, respectively. The higher performances in protein extraction obtained by Jodayree, Smith, and Tsopmo (2012) as compared to our results may be due to the difference in the experimental conditions (enzyme concentration, reaction temperature, pH used for alkaline extraction, nature of the raw material, centrifugation conditions). Therefore, the assignment of the discrepancies between our results and those of the latter authors to specific factors may be a mere speculation.

An approach using three enzymes to breakdown the plant cell polysaccharides and glucan polymers was investigated. As barley contains ~5% β-glucan, the addition of glucanase was required for an enhanced protein recovery yield (Jodayree, Smith, & Tsopmo, 2012). The results obtained show that the addition of glucanase without IEP has increased the protein recovery yield from 25.7% to 71.6%, while the purification factor decreased from 6.73 to 5.1

(Figure 2.1). Addition of the IEP step after the alkaline extraction has increased the protein content to 41.4% and the purification factor to 5.68. At the same time, it resulted in the highest protein recovery yield (78.3%) among all extraction methods tested.

### 2.4.2. Characterization and profiling of barley protein extracts

#### *2.4.2.1. Protein profiles of barley protein extracts*

Table 2.1 summarizes the results of the profiles of proteins extracted from barley by different techniques, as shown by SDS-PAGE analysis. The table shows the low MW (< 20 kDa) protein fraction was largely predominant in A-BP and AI-BP concentrates where it represented 70.5% and 69.5% of the total proteins, respectively. The predominance of this fraction, corresponding to globulin, indicates a severe protein degradation under the alkaline environment. Nonetheless, the extraction of high MW proteins (e.g., hordeins) in higher amounts compared to low MW proteins (e.g., globulin and albumin) by alkaline and IEP method from barley was reported previously (Bilgi & Çelik, 2004). In the latter study, the SDS-PAGE pattern revealed a major band corresponding to B-hordein between 35-55 kDa and weak band corresponding to D-hordein between 85-90 kDa; the bands corresponding to albumin (< 20 kDa) also had weak relative intensities. Conversely, our results showed that the barley proteins extracted by the alkaline method were largely dominated (~70%) by the low MW fraction of MW < 20 kDa, regardless of whether or not an IEP step was applied. In contrast, the large MW proteins (> 85 kDa) were either absent (AI-BP) or present in the small proportion of 8.1%. The bi-enzymatic approach yielded barley protein concentrates where the low MW (< 20 kDa) protein fraction represented 25.9% of the total protein fractions, and the large MW (> 85 kDa) protein fraction represented 36.2% (Table 2.1). Moreover, large MW fractions in the ranges of 55-80 kDa and > 85 kDa predominated in the TE-BP barley protein concentrate, where they represented 28.41% and 39.32%, respectively. The addition of an IEP step to the tri-enzymatic treatment enriched the extract (TEI-BP) in the 55-80 kDa protein fraction, corresponding to globulin, to reach 64.64%. It is worth mentioning that the low MW protein fraction (< 20 kDa) was smaller in BE-BP compared to alkaline extracts (A-BP and AI-BP) and absent in both TE-BP and TEI-BP extracts. As expected, these results indicate that the harsh conditions of the alkaline extraction induce partial degradation of barley proteins resulting in increased proportions of the low MW proteins.

**Table 2.1.** Molecular weight distribution as a percentage of total proteins extracted by different extraction techniques.

| Extraction technique | >85 kDa | 80-55 kDa | 54-30 kDa | <20kDa |
|---|---|---|---|---|
| A-BP[a] | 8.1 | 9.9 | 11.4 | 70.5 |
| AI-BP[b] | - | 5.09 | 25.4 | 69.5 |
| BE-BP[c] | 36.2 | 16.5 | 20.8 | 25.9 |
| TE-BP[d] | 28.41 | 39.32 | 32.26 | - |
| TEI-BP[e] | 25.8 | 64.64 | 9.57 | - |

[a]A-BP, alkaline barley protein extract,
[b]AI-BP, alkaline extraction with IEP barley protein extract,
[c]BE-BP, barley bi-enzymatic enzymatic extraction; starch removal with treatment of the supernatant obtained by centrifugation of defatted barley slurries with α-amylase and amyloglucosidase
[d]TE-BP, tri-enzymatic barley protein extract; starch and glucan removal with treatment of the supernatant obtained by centrifugation of defatted barley slurries with α-amylase, amyloglucosidase and glucanase
[e]TEI-BP, tri-enzymatic and isoelectric precipitation (IEP) barley protein extract; starch and glucan removal with α-amylase, amyloglucosidase, and glucanase treatment of a suspension of pellet obtained by centrifugation of the supernatant of defatted barley slurries, which had been subjected to isoelectric precipitation.
- Absence

#### *2.4.2.2. Identification of proteins by MS/MS*

Major bands in SDS-PAGE pattern of AI-BP and TEI-BP concentrates were further characterized by peptide mapping with a tryptic digestion followed by mass spectrometry peptide analysis. The bands corresponding to the MW of 22, 40, 44, and 61 kDa of the AI-BP concentrate, and those corresponding the MW of 34, 62, 67, and 105 kDa of the TEI-BP concentrate were selected for peptide mapping. The molecular weights and amino acid sequences of the peptides generated by the trypsin hydrolysis are reported in supplementary materials. Peptide mapping identified embryo globulin (Q03678; MW 72.251 kDa) and B3 hordein (I6SJ26; MW 35.424 kDa) in AI-BP concentrate (Table 2.2), while B3 hordein, globulin, and D hordein (Q40054; MW 75.102 kDa) were identified in TEI-BP concentrate (Table 2.3). The protein fraction corresponding to globulin, which was detected in AI-BP and TEI-BP concentrates, showed different homologies with the reference globulin (Q03678). The protein fraction of AI-BP had a slightly higher homology (43%) with the reference globulin than that extracted in TEI-BP concentrate (40%). This suggests that the protein fraction corresponding to globulin may have been partially hydrolyzed during the enzymatic extraction. On the other hand, B3 hordein in AI-BP concentrate and D hordein in TEI-BP concentrate presented the low percent identities of 13% and 1.5% with the reference B3 hordein (I6SJ26) and D hordein (Q40054), respectively. Again, it can be anticipated that no such partial degradation appeared to have occurred for B3 hordein isolated from the AI-BP, whose MW, as determined by SDS-PAGE (40 kDa), or D hordein (MW 105 kDa) were higher than those of the reference proteins (35.424 and 75.105 kDa, respectively) (Tables 2.2 and 2.3). Additionally, B1 hordein of 34 kDa MW was detected only in AI-BP concentrate (Table 2.2). These results corroborate those of the SDS-PAGE analysis showing that the proteins fraction of 30-54 kDa MW, corresponding to B hordeins, represented 25.4% of the total proteins in the AI-BP concentrate (Table 2.2). In addition to the above-discussed major barley proteins extracted from DBF with different techniques, other proteins were identified by peptide mapping. The most prominent of these was a storage proteins of the endosperm albumin, serpin Z4 (43.277 kDa MW). The highest homology (66%) was observed between serpin Z4 and an intact protein detected in AI-BP concentrate. An intact protein, homologous to serpin Z4 (54% homology), was also identified in TEI-BP concentrate by peptide mapping. Other minor proteins playing different roles in barley grains were identified in this study. For example, protein fractions in AI-BP and TEI-BP concentrates were characterized to share homologies of 29% and 19% respectively, with the

**Table 2.2.** Mass spectroscopy characterization of peptides generated by tryptic digestion of four major bands isolated from SDS-PAGE of barley protein concentrate obtained by alkaline extraction and isoelectric precipitation (AI-BP).

| Molecular weight (kDa) of the major SDS-PAGE bands | | | |
|---|---|---|---|
| 22[a] | 40[b] | 44[c] | 61[d] |
| (Number of peptides) Sequence (Actual MW in Da) | | | |
| (2) [e](R)TPDYVEEAR(Q) (1078.49) | (5) (R)ILPFGIDTR(V) (1030.58) | (2) (M)ATTLATDVR(L) (946.5) | (1) (R)DDQQQHGRHEQEEEQGR(G) (2104.89) |
| (2) (K)TQQAGHAIQSR(A) (1195.61) | (4) (R)ILPFGIDTRVGV(-)[f] (1285.74) | (3) (R)LSIAHQTR(F) (924.52) | (1) (R)HGEGEREEER(G) (1226.53) |
| (1) (K)TqqGHAIQSR(A) (1197.59) | (1) (R)TLPmmCSVNVPFYR(I) (1745.79) | (1) (R)LSIAHqTR(F) (925.5) | (2) (R)RPYVFGPR(S) (990.54) |
| (1) (K)TqQAGHAIqSR(A) (1197.61) | (1) (K)VFLQQQCSPVAmSQR(I) (1793.85) | (1) (R)SAISSNPER(A) (959.47) | (2) (R)IIQSDHGFVR(A) (1170.61) |
| (1) (R)AGHTAGAGAGAGTRASS(Q) (1398.66) | (1) (K)VFLQQQCSPVAmSQR(I) (1793.86) | (10) (R)DQLVAILGDGGAGDAK(E) (1498.76) | (2) (R)ALRPFDQVSR(L) (1187.64) |
| (1) (R)GQmGEQmKGmLQEK(A) (1641.71) | | (2) (R)DqLVAILGDGGAGDAK(E) (1499.76) | (2) (R)VAImEVNPR(A) (1043.54) |
| (1) (R)AEETRAGHTAGAGAGAGTR(A) (1739.84) | | (1) (K)ELNALAEqVVQFVLANESSTGGPR(I) (2529.27) | (1) (R)VAImEVnPR(A) (1044.53) |
| (3) (R)GSYVQVQHGGQYGAGQQQHGR(G) (2241.04) | | (1) (K)AKTQSVDFQHK(T) (1287.66) | (4) (R)AFVVPGFTDADGVGYVAQGEGVLTVIEnGEKR(S) (3294.64) |
| | | (1) (K)AKTQSVDFqHK(T) (1288.64) | (3) (K)EGDVIVAPAGSImHLANTDGR(R) (2138.04) |
| | | (1) (K)AKTqSVDFqHK(T) (1289.64) | (2) (K)EGDVIVAPAGSImHLANTDGRR(K) (2294.14) |
| | | (1) (K)TqSVDFQHK(T) (1089.51) | (1) (K)ILHTISVPGK(F) (1063.63) |
| | | (2) (K)TQSVDFQHK(T) (1088.52) | (2) (K)FQFLSVKPLLASLSK(R) (1676.99) |
| | | (1) (K)TQSVDFqHK(T) (1089.51) | (1) (K)TSDERLER(L) (1004.49) |
| | | (1) (K)TLEAVGQVnSWVEQVTTGLIK(Q) (2272.2) | (2) (R)ASEEQLR(E) (831.41) |
| | | (1) (K)TLEAVGqVNSWVEqVTTGLIK(Q) (2273.16) | (3) (R)EAAEGGQGHR(W) (1010.5) |
| | | (8) (K)QILPPGSVDNTTK(L) (1368.72) | (1) (R)DTFNLLEQRPK(I) (1359.72) |
| | | (5) (K)qILPPGSVDNTTK(L) (1369.74) | (1) (R)LYEADAR(S) (836.4) |
| | | (2) (K)GAWDQKFDESNTK(C) (1524.69) | (5) (R)SFHALANQDVR(V) (1256.62) |
| | | (1) (K)FDESNTK (C) (839.37) | (1) (R)RGSESESEEEEEQQR(Y) (1807.76) |
| | | (4) (K)KQYISSSDNLK(V) (1281.66) | (1) (R)RGSESESEEEEEQQRYETVR(A) (2456.08) |
| | | (3) (K)KQYISSSDnLK(V) (1282.65) | (1) (R)GSESESEEEEEQQR(Y) (1651.64) |
| | | (2) (K)KqYISSSDNLK(V) (1282.64) | (1) (R)GSESESEEEEEQQRYETVR(A) (2299.97) |
| | | (1) (K)KqYISSSDnLK(V) (1283.64) | (2) (K)LGSPAQELTFGRPAR(E) (1598.85) |
| | | (1) (K)QYISSSDnLK(V) (1154.54) | (2) (R)EVQEVFR(A) (905.56) |
| | | (7) (K)QYISSSDNLK(V) (1153.56) | (2) (R)AQDQDEGFVAGPEQQSR(E) (1860.82) |
| | | (2) (K)VLKLPYAK(G) (930.59) | (1) (R)AQDQDEGFVAGPEqQSR(E) (1861.82) |
| | | (2) (K)RQFSmYILLPGAQDGLWSLAK(R) (2409.25) | |
| | | (1) (R)QFSmYILLPGAQDGLWSLAK(R) (2253.15) | |
| | | (5) (K)RLSTEPEFIENHIPK(Q) (1808.94) | |
| | | (4) (K)RLSTEPEFIEnHIPK(Q) (1809.93) | |
| | | (7) (R)LSTEPEFIENHIPK(Q) (1652.84) | |
| | | (6) (R)LSTEPEFIEnHIPK(Q) (1653.83) | |
| | | (2) (K)QTVEVGRFqLPK(F) (1401.76) | |
| | | (11) (K)FKISYQFEASSLLR(A) (1687.86) | |
| | | (2) (K)FKISYqFEASSLLR(A) (1688.89) | |
| | | (8) (K)ISYQFEASSLLR(A) (1412.73) | |
| | | (6) (K)ISYqFEASSLLR(A) (1413.72) | |
| | | (13) (R)ALGLQLPFSEEADLSEmVDSSQGLEISHVFHK(S) (3528.7) | |
| | | (1) (R)ALGLQLPFSEEADLSEmVDSSqGLEISHVFHK(S) (3529.78) | |
| | | (1) (K)SFVEVNEEGTEAGAATVAmGVAmSmPLK(V) (1915.54) | |

[a] Homologous to oleosin (Accession number Q43769, MW 18,493.7 Da); [b] Homologous to hordein B3 (Accession number I6SJ26, MW 35,423.5 Da); [c] Homologous to serpin Z4 (Accession number SPZ4, MW 43,277.0 Da); [d] Homologous to globulin (Accession number Q03678, MW 72,250.9 Da); [e] Residues between parenthesis are the cleavage sites of trypsin (N-terminal of the peptide) and the residue before the cleavage site of the enzyme (C-terminal of the peptide); [f] Gap of indeterminate length ; Lowercase symbols designate amino acid residues that may vary in this position

**Table 2.3.** Mass spectroscopy characterization of peptides generated by tryptic digestion of four major bands isolated from SDS-PAGE of barley protein concentrate obtained by tri-enzymatic extraction with IEP (TEI-BP)

| Molecular weight (kDa) of the major SDS-PAGE bands | | | |
|---|---|---|---|
| 34[a] | 62[b] | 67[c] | 105[d] |
| | (Number of peptides) Sequence (Actual mass) | | |
| (2) [e](R)ILPFGIDTR(V) (1030.58) | (1) (R)LSIAHQTR(F) (924.52) | (1) (R)RGEGERDEEQGDSR(R) (1618.69) | (1) (R)QYEQQTEVPSK(g) (1335.63) |
| | (1) (R)SAISSNPER(A) (959.47) | (1) (R)GEGERDEEQGDSR(R) (1462.6) | (1) (R)QYEqQTEVPSK(g) (1336.62) |
| | (6) (R)DQLVAILGDGGAGDAK(E) (1498.76) | (1) (R)RPYVFGPR(S) (990.54) | |
| | (1) (K)TQSVDFQHK(T) (1088.52) | (2) (R)IIQSDHGFVR(A) (1170.61) | |
| | (1) (K)TqSVDFQHK(T) (1089.51) | (2) (R)ALRPFDQVSR(L) (1187.64) | |
| | (1) (K)TLEAVGQVnSWVEQVTTGLIK(Q) (2272.2) | (1) (R)ALRPFDqVSR(L) (1188.62) | |
| | (10) (K)QILPPGSVDNTTK(L) (1368.72) | (3) (R)VAImEVNPR(A) (1043.54) | |
| | (1) (K)LILGNALYFK(G) (1150.68) | (1) (R)VAIMEVNPR(A) (1027.55) | |
| | (1) (K)GAWDQKFDESNTK(C) (1524.69) | (5) (R)AFVVPGFTDADGVGYVAQGEGVLTVIEnGEKR(S) (1647.82) | |
| | (2) (K)KQYISSSDNLK(V) (1281.66) | (1) (R)AFVVPGFTDADGVGYVAqGEGVLTVIENGEKR(S) (3294.65) | |
| | (1) (K)QYISSSDnLK(V) (1154.54) | (8) (K)EGDVIVAPAGSImHLANTDGR(R) (2138.05) | |
| | (2) (K)QYISSSDNLK(V) (1153.56) | (1) (K)ILHTISVPGK(F) (1063.63) | |
| | (2) (K)VLKLPYAK(G) (930.59) | (2) (K)ILHTISVPGKFQFLSVKPLLASLSK(R) (2722.62) | |
| | (4) (K)RLSTEPEFIENHIPK(Q) (1808.94) | (2) (K)FQFLSVKPLLASLSK(R) (1676.99) | |
| | (2) (K)RLSTEPEFIEnHIPK(Q) (1809.93) | (1) (K)TSDERLER(L) (1004.49) | |
| | (4) (R)LSTEPEFIENHIPK(Q) (1652.84) | (1) (R)LFNQRqGQEK(T) (1247.67) | |
| | (2) (R)LSTEPEFIEnHIPK(Q) (1653.83) | (2) (R)ASEEQLR(E) (831.41) | |
| | (1) (K)QTVEVGRFqLPK(F) (1401.76) | (3) (R)WPLPPFR(G) (911.5) | |
| | (5) (K)ISYQFEASSLLR(A) (1412.73) | (2) (R)GDSRDTFNLLEQRPK(I) (1774.89) | |
| | (7) (R)ALGLQLPFSEEADLSEmVDSSQGLEISHVFHK(S) (3528.7) | (2) (R)DTFNLLEQRPK(I) (1359.72) | |
| | (1) (R)ALGLqLPFSEEADLSEmVDSSqGLEISHVFHK(S) (1765.37) | (1) (R)DTFNLLEqRPK(I) (1360.7) | |
| | (1) (K)SFVEVNEEGTEAGAATVAmGVAmSmPLK(V) (1915.54) | (3) (R)LYEADAR(S) (836.4) | |
| | | (2) (R)SFHALANQDVR(V) (1256.62) | |
| | | (1) (R)SFHALAnQDVR(V) (1257.61) | |
| | | (1) (R)RRGSESESEEEEEQQR(Y) (1963.85) | |
| | | (3) (R)RGSESESEEEEEQQRYETVR(A) (2456.08) | |
| | | (2) (R)GSESESEEEEEQQRYETVR(A) (2299.97) | |
| | | (2) (K)LGSPAQELTFGRPAR€ (1598.85) | |
| | | (3) (R)EVQEVFR(A) (905.56) | |
| | | (1) (R)EVqEVFR(A) (906.44) | |
| | | (3) (R)AQDQDEGFVAGPEQQSR(E) (1860.82) | |

[a] Homologous to B3 hordein (Assession number I6SJ26, MW 35,423.5 Da); [b] Homologous to serpin Z4 (Assession number SPZ4, MW 43,277 Da); [c] Homologous to globulin (Assession number Q03678, MW 72,250.9 Da); [d] Homologous to D-hordein (Assession number Q40054, MW 75,102 Da); [e] Residues between parenthesis are the cleavage sites of trypsin (N-terminal of the peptide) and the residue before the cleavage site of the enzyme (C-terminal of the peptide); [f] Gap of indeterminate length ; Lowercase symbols designate amino acid residues that may vary in this position.

calcium-binding oil-body surface proteins caleosin (Q6UFY6; 28.188 kDa MW). Moreover, the polypeptides of < 20 kDa MW, shown to constitute 69.5% of the AI-BP concentrate (Table 2.2), were characterized to comprise a variety of heat shock proteins, alpha-amylase trypsin inhibitors, and oleosins of smaller MW (data not shown).

Overall, the above findings are in good agreement with those reported previously in the literature. Chmelik et al. (2002) identified a different 30-kDa B3 hordein (P06471) in barley protein obtained by alkaline extraction. Kaspar-Schoenefeld et al. (2016) identified different types of hordein [B3 hordein (P06471), γ-hordein 1 (35_69), and γ-hordein 3 (P80198)], and serpin Z4 (35_14790) in proteins extracted from barley caryopses in a buffer solution of tetracetic acid (TCA) and 2-mercaptoethanol in acetone. Analysis of fractions of pepsin-hydrolysates of oat bran proteins revealed that many peptides were generated from 12S globulin (Bobalova, Salplachta, & Chmelik, 2008). The accession numbers of B3 hordein and serpin used in the present study differ from those reported by other authors, which may be due to the difference in the barley variety used in each study and/or to the genetic polymorphism of barley depending on the geographical origin (Tang, Ding, & Hu, 2002).

### 2.4.3. *Structural and techno-functional properties*

The secondary and tertiary structures, as well as functional properties of the barley protein concentrates (AI-BP and TE-BP) were investigated along with PPC and WPI, as standards.

#### *2.4.3.1. Secondary structures and their stability*

The results of the characterization of the secondary structures of the PPC, WPI, and the barley protein concentrates (AI-BP and TEI-BP) in the native, heat-denatured and high pressure-treated states by FTIR. Figure 2.2 shows the variations in the proportions of structural elements obtained from the amide I region (1600-1700 $cm^{-1}$), stemming from the peptide's C=O group stretch vibration considered to be the region where the main protein peaks are located (Withana-Gamage et al., 2011; Xin et al., 2011). Native PPC has been characterized to be composed of 29% α-helix (Figure 2.2) as seen with an amide I band peak at 1650 $cm^{-1}$, and a peak at 1636 $cm^{-1}$ showing 27% intramolecular β-sheets (Belton et al., 1997). The proportions of β-turns (24%) and intermolecular β-sheets (20%) in native PPC are in agreement with those reported previously for field pea protein

(Shevkani et al., 2015). The results of FTIR analysis showed that the native WPI was composed of 10% intramolecular β-sheets, 4% β-turns, and 86% random coils (Figure 2.2). These results differ from those reported by Haque et al. (2015) who showed that WPI was composed of 43% β-sheets, 34% β-turns, and 23% α-helix. The discrepancy between our results and those reported by Haque et al. (2015) could be due to prior processing of the protein. In the native state, the AI-BP concentrate was mostly comprised of β-turns (47%) and α-helices (25%), while intra- and inter-molecular β-sheets represented 17% and 11%, respectively. TEI-BP concentrate showed a large peak at 1652 $cm^{-1}$, indicating that it is comprised mainly of α-helices (93%), with 4% random coils and 3% intramolecular β-sheets (Figure 2.2). Divergence between the composition of barley protein concentrates in secondary structure elements may be attributed to the difference in protein profiles. TEI-BP concentrate contained a higher proportion of globulins and D hordeins, and a lesser amount of B hordeins and serpins than AI-BP concentrate.

Upon heat denaturation, the secondary structure composition of PPC changed to 8% β-turns, 7% intermolecular β-sheets, and 85% random coils (Figure 2.2). A study conducted on soy protein isolate (SPI) under conditions similar to those adopted herein (80ºC for 5 min), showed that the proportion of β-sheets decreased from 28% to 26%, while the proportions of β-turns and α-helices increased by 1% and 5%, respectively (Wang et al., 2014). The heat-induced self-rearrangement from β-sheet structures to β-turns and α-helices may be due to the unfolding of the soy protein subunit glycinin, with subsequent exposure of the hydrophobic groups to the polar environment allowing them to interact and form aggregates. As β-sheets are located in the interior of a protein, their proportion would be decreased as a result of protein aggregation (Long et al., 2015; Wang et al., 2014). The secondary structure conformation of heat denatured WPI consisted of 14% α-helix, 45% intramolecular β-sheets, and 41% β-turns, while no α-helix was found in the native WPI, whose secondary structure consisted of β-sheets (10%), β-turns (4 %), and random coils (86%) (Figure 2.2). These results are in line with those reported by O'Loughlin et al. (2015) who showed that WPI denaturation by heat treatment (90 °C for 5 min) induced an increase in the α-helix proportion and a loss in random coils. These authors also showed that random coils may be increased or decreased by thermal denaturation of WPI depending on its chemical composition, where β-lactoglobulin appears to play the major role. In the case of PPC, an increase in α-helices and in random coils accompanied by a decrease in intramolecular β-sheets was observed.

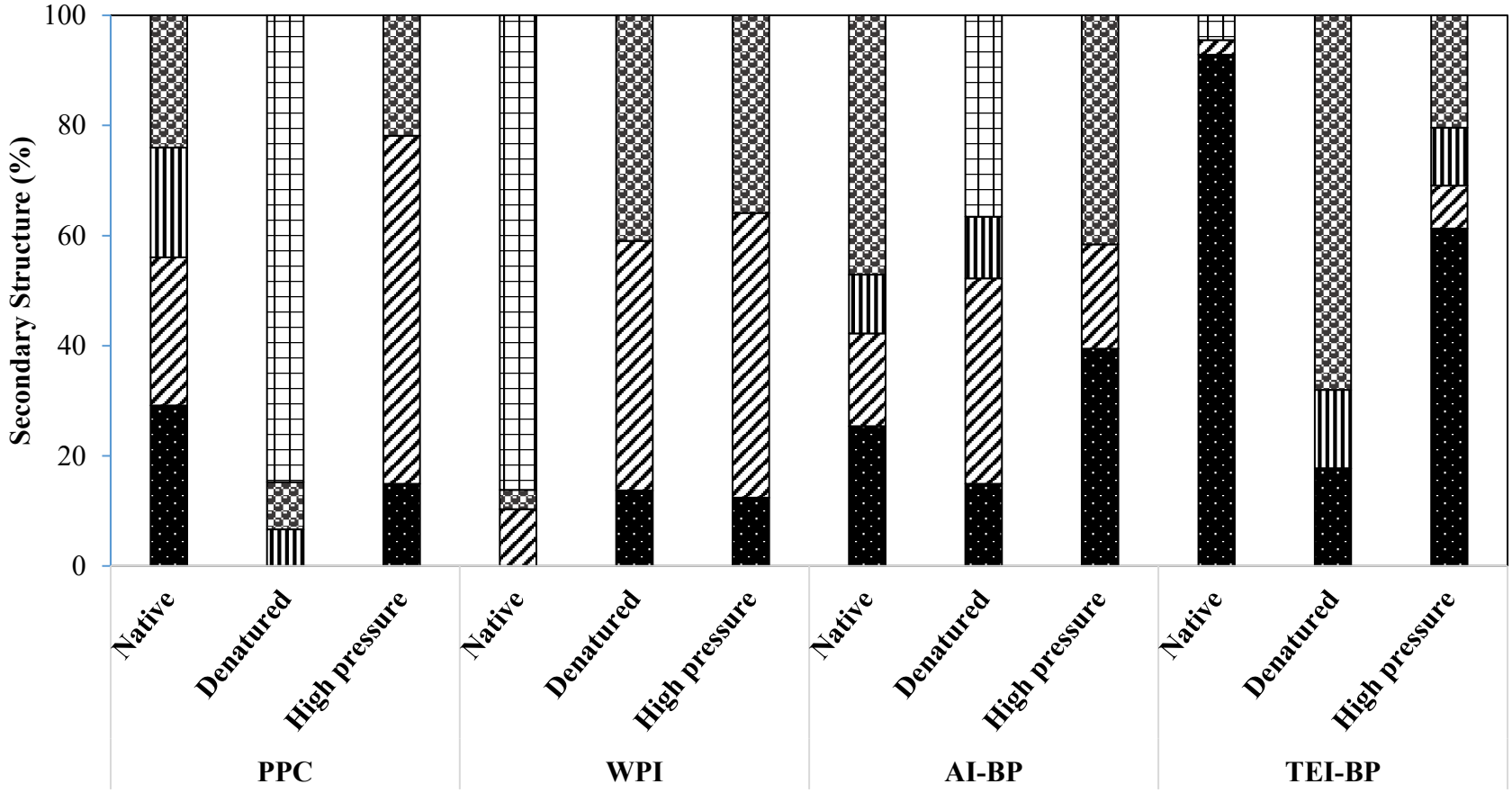


**Figure 2.2.** Proportions of secondary structures of pea protein concentrate (PPC), whey protein isolate (WPI), alkaline extraction barley protein concentrate (AI-BP), and tri-enzymatic starch and glucan removal barley protein concentrate (TEI-BP) in the native, denatured, and high pressure-treated states, obtained using Fourier transform infrared spectroscopy: $\alpha$-helix (▪), intramolecular $\beta$ sheet (▨), intermolecular $\beta$ sheet (▥), $\beta$-turn (▩), random coils (卄).

The barley protein concentrates had a remarkably different secondary structure composition. Thermal denaturation of AI-BP concentrate resulted in a greater share of intramolecular β-sheets and random coils (37% each), and in a decrease in α-helices (15%) and intermolecular β-sheets (11%) proportions. The proportion of α-helix in TEI-BP concentrate was decreased by heat treatment from 93% to 17%, while β-turns and intermolecular β-sheet, absent in native TEI-BP, were found at the proportions of 68% and 14%, respectively in the denatured form of the protein concentrate.

PPC exposed to high pressure showed peaks at 1651, 1637, 1637, and 1667 $cm^{-1}$, as determined by FTIR analysis (data not shown), corresponding to 15% α-helix, 63% intramolecular β-sheets and 22% β-turns (Figure 2.2). High pressure treatments reduce the number of noncovalent bonds, partially unfolding the protein and allowing for the SH-groups of cysteine to become available for interactions that cause structural changes (Janssen et al., 2017). This is in agreement with a previous report on the increase of β-turns in lentil proteins upon high pressure treatment, which may be due to the stabilizing effect of SH-groups on β-turns (Tiwari, Gowen, & McKenna, 2011; Trivedi, Laurence, & Siahaan, 2009). The application of high pressure treatment (600 MPa) to SPI resulted in a steady unfolding of the protein, as revealed by an increase in the band intensity located in the amide I region (Ma & Tang, 2009). Figure 2.2 shows that the secondary structure of WPI after exposure to high pressure (600 MPa for 30 min) was composed of 12% α-helix, 52% intramolecular β-sheets and 36% β-turns. Alvarez (2004) observed two major peaks after 30 min after exposure of WPI to 400 MPa pressure: the most significant corresponded to antiparallel β-sheets, and the smaller one to β-turns. The increase in β-sheet proportion is due to parallel β-sheets rearranging, and the unraveling of the tertiary structure, exposing the β-sheets to the surface. In the present study, an increase in α-helix proportion was also observed upon pressure treatment (Figure 2.2). This was explained by the conversion of native β-sheets of β-lactoglobulin to non-native α-helices under high pressure (Tromelin, Andriot, & Guichard, 2006). Contrary to WPI, different conformational change patterns in the structural composition of AI-BP were caused by thermal denaturation and high pressure treatment. The secondary structure composition of the high pressure-treated AI-BP resembled that of its native state, with β-turns, α-helices, and intramolecular β-sheets representing 41%, 39%, and 19%, respectively (Figure 2.2). The predominance of α-helices in the high pressure-treated TEI-BP concentrate (61%) resembled that

of the native state, while β-turns, intermolecular β-sheets (20%) and intramolecular β-sheets comprised 20, 11 and 8% of the secondary structure. (Figure 2.2).

The secondary structure composition of barley protein concentrates was reported to be variable depending on the type of barley and the extraction methods. For example, hulless barley protein concentrate obtained by the addition of cellulose and heat mixing was composed of 45% β-sheets, 20% α-helix, and 35% β-turns (Jia & Wang, 2015). Jiang et al. (2015) reported that native oat protein extract is comprised of 29% α-helix, 27% random coil, 22% intramolecular β-sheets, and 3% intermolecular β-sheets. The effect of heat and high pressure treatments on barley protein extracts have not been extensively investigated. Changes in secondary structure of wheat roots after heat treatment was studied by Zhao et al. (2011) and found that heat denaturation decreased the α-helix from 41% to 23% and increased β-turns from 18% to 27%, and random coil from 25% to 55%, while no change occurred for the β-sheets element, which remained the same (16%). High pressure treatment at 650 MPa for 15 min also denatured basmati rice proteins, but produced opposite effects, as it resulted in a reduction in β-turns, and an increase in α-helix and β-sheets (Ahmed, 2009).

#### *2.4.3.2. Tertiary structures and their stability*

Fluorescence spectroscopy analysis was used to characterize the tertiary structure of the proteins barley protein concentrates, as well as the quenching of tryptophan. The fluorescence intensities obtained refer to the quantum yield, or to the ratio of the amount of fluorescent emitted photons to the amount of absorbed photons. These values are correlated to the presence of two aromatic amino acids: tryptophan and tyrosine, as these residues have suitable fluorescent properties that can be used to monitor changes in protein folding. The intensities depend on the polarity of the environment; in a hydrophobic environment, the amino acid residue is buried within the core of the protein and emits more photon, resulting in a high fluorescence intensity. When the environment is more hydrophilic, more photons are absorbed, and the residues emit low fluorescence intensities (Anonymous, 2007). The maximum fluorescence intensity fluorophores depends on their microenvironment; for tryptophan it is in the range of 308-355 nm (Vivian & Callis). The changes in fluorescence intensity of PPC, WPI, and both barley protein concentrates (AI-BP, and TEI-BP), in their native, thermally denatured, and high pressure-treated states were investigated (Figure 2.3). The wavelength of maximum fluorescence intensity varied from 332-

350 nm, as shown in Table 2.4. The change in fluorescence intensity for PPC varied depending on heat and high pressure treatments (Figure 2.3A). The high-pressured samples had the highest intensity (8370 a.u) of all proteins analyzed (Table 2.4), surpassing the native sample by 35.9%. These results suggest that either the tryptophan residues are aggregated or the microenvironment became less polar because of the loss of water molecules during the application of high pressure treatment. The intensity of heat denatured samples decreased by 2.6% compared with that of native state (6160 versus 6000 a.u) (Figure 2.3A, Table 2.4), suggesting that the aqueous environment may have permeated the tryptophan microenvironment after heat treatment. At an excitation wavelength of 290 nm, the $\lambda_{max}$ of 345 nm, characteristic of tryptophan, was recorded for native PPC. Upon heat denaturation and high pressure treatment of this protein, a red shift in $\lambda_{max}$ 348 nm to 346 nm, respectively, was observed (Table 2.4). This shift reveals greater uncovering of the tryptophan residue of the protein and its exposure to the aqueous environment, as was also noted by other authors (Yin et al., 2008).

However, according to Chao et al. (2013), a decrease of almost 70% in intensity was observed upon application of 600 MPa high pressure to a 1% (w/w) commercial pea protein isolate (PPI) compared with a control under atmospheric pressure (0.1 MPa). A similar red shift from 372 nm to 375 nm was seen after exposure of PPI to 600 MPa for 5 min, the difference in values with the present study being attributed to the raw material preparation and reduced exposure time. The increase in fluorescence intensity after application of 600 MPa to kidney bean protein isolate was also reported by Yin et al. (2008) who also demonstrated that this treatment caused a red shift of $\lambda_{max}$ from 332 nm to 335 nm. The $\lambda_{max}$ for high pressure treated PPC in the present study was at a shorter wavelength, indicating that the protein used retained its native folded structure more than those reported in the other studies. For example, an increase in intensity following heat treatment of PPI at 100ºC for 30 min was observed by Chao et al. (2013). Such discrepancies in reported results can essentially be attributed to the different nature of the protein extract samples used in each study. In particular, Chao et al. (2013) used commercial pea protein with a protein content 8% greater than that used in our study, and the method used for protein extraction was not clearly described by the authors. However, the differences in the solvents used, the holding time for high pressure treatment, and denaturing temperatures may indeed account for the different results obtained in each study. In our study, the $\lambda_{max}$ with WPI decreased from 335 nm to 332 nm and 331 nm for heat- and high pressure-treated samples, respectively (Table 2.4).

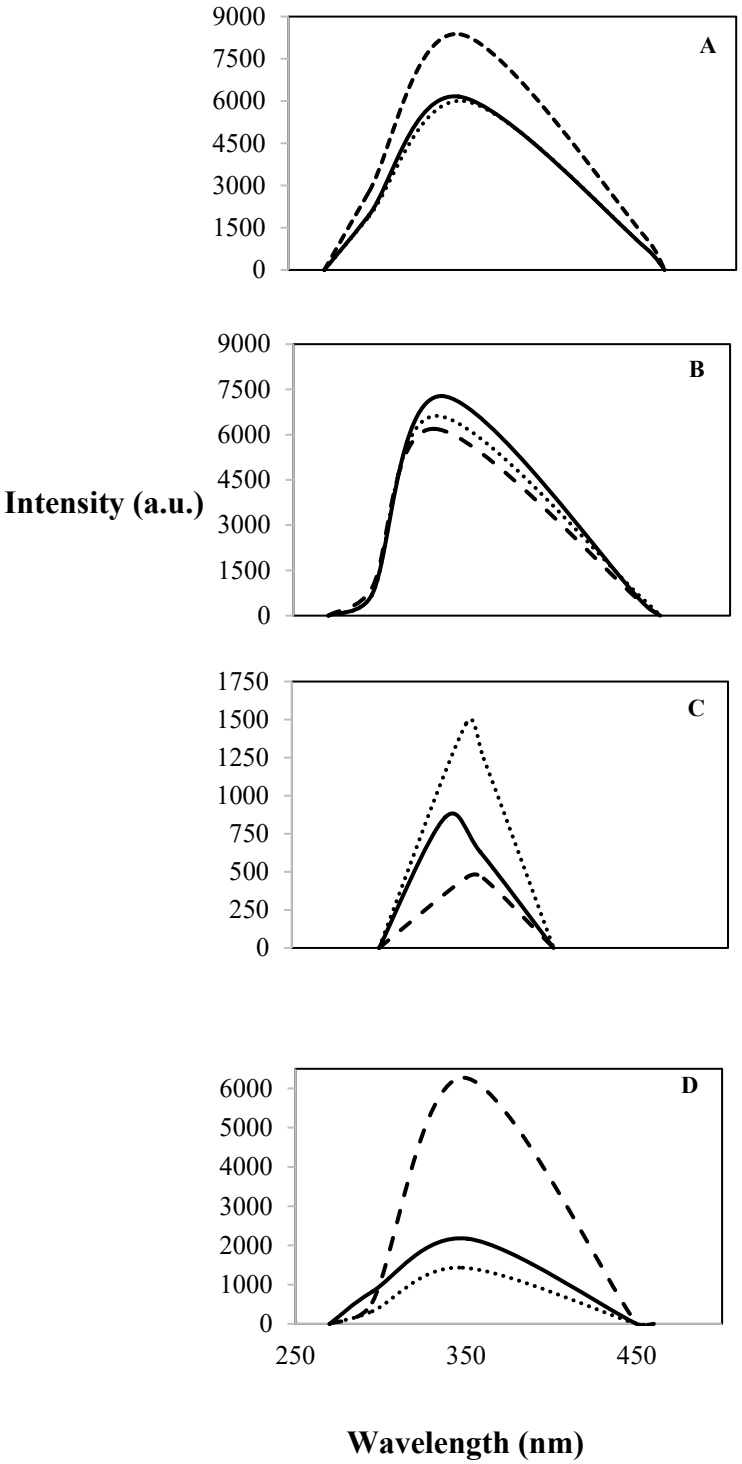


**Figure 2.3.** Fluorescence intensities of PPC (A), WPI (B), AI-BP concentrate (C), and TEI-BP concentrate (D) in the native ( ——), heat-treated ( ····· ), and high pressure-treated ( - - - - - ) states.

The maximum intensity for native WPI was 7280 a.u. (Figure 2.3B). The intensity decreased to 6622 a.u and 6192 a.u for the heat-denatured and high pressure-treated samples, respectively (Figure 2.3B, Table 2.4). The decrease in intensity was unexpected, as heat treatment is known to enhance exposure of the tryptophan residue with a consequent increase in fluorescence (Arriaga, 2011; Chao et al., 2013); yet, such decrease in intensity was occasionally reported (Qi & Onwulata, 2011). According to Miwa et al. (2013) (Miwa, Yokoyama, Nio, & Sonomoto, 2013), heat treatment at 85 °C for 30 min has doubled the fluorescence intensity of WPI in an aqueous solution of 1.5% (w/v). Shifts in $\lambda_{max}$ obtained from the present study are in accordance with those of Arriaga (2011), who determined that a maximum fluorescence was observed at 336 nm for native whey protein, which increased to 342 nm when the sample was heat-treated.

Liu et al. (2005) observed an increase of 120% in fluorescence intensity with a shift of $\lambda_{max}$ from to 335 nm to 339 nm in a whey protein concentrate exposed to 600 MPa high pressure treatment for 30 min. The temperature of 50 ºC used in the latter study during high pressure treatment may have had a compounding effect, resulting in an upward shift in $\lambda_{max}$.

The results on the behavior of AI-BP concentrate in response to heat and pressure treatments are summarized in Figure 2.3C and Table 2.4. It can be seen from these results that the AI-BP recorded the lowest intensity values, which ranged between 462 nm and 1476 nm. For the whole range of wavelengths studied, opposite effects were observed upon exposure of AI-BP to high pressure or thermal denaturation, with high pressure treatment causing a decrease in the fluorescence intensity, which has, on the contrary, increased upon heat treatment. Compared with native samples, the maximum fluorescence intensity was indeed reduced by 46.3% in high pressure-treated of AI-BP samples, and increased by 71.6% in thermally denatured ones (Table 2.4).

Additionally, heat and high pressure treatments caused a shift in $\lambda_{max}$ from 338 nm to 350 nm (Table 2.4). The low fluorescence intensity of the three AI-BP samples compared with the other protein concentrate samples may indicate excessive protein-protein interactions that has occurred in a manner to hide the tryptophan residues, or penetration of the aqueous environment in the microenvironment (Chao et al., 2013). Opposite results were recorded for TEI-BP concentrate showing the greatest change in fluorescence intensity between the native and high pressure-treated samples, with the latter almost doubling in intensity (increase of 189%) (Figure 2.3D). A smaller, yet noticeable decrease in intensity (34%), was seen with the thermally denatured sample. The

**Table 2.4** Changes in maximum fluorescence (FI) intensities and the corresponding shifts of barley protein concentrates, prepared in this study, and commercial PPC and WPI (standards), as a response to heat and high pressure (HP) treatments.

| Protein extract | State | Maximum FI (a.u.) | FI Variation (%)[1] | Maximum wavelength (nm) |
|---|---|---|---|---|
| PPC[a] | Native | 6160 | | 345 |
| | Heat-treated | 6000 | -2.6 | 348 |
| | HP-treated | 8370 | 35.9 | 346 |
| WPI[b] | Native | 7280 | | 335 |
| | Heat-treated | 6622 | -9.0 | 332 |
| | HP-treated | 6192 | 85.1 | 331 |
| AI-BP[c] | Native | 860 | | 338 |
| | Heat-treated | 1476 | 71.6 | 350 |
| | HP-treated | 462 | -46.3 | 350 |
| TEI-BP[d] | Native | 2170 | | 351 |
| | Heat-treated | 1432 | -34,0 | 348 |
| | HP-treated | 6272 | 189,0 | 349 |

Excitation wavelength was 290 nm.
[a]PPC, pea protein concentrate
[b]WPI, whey protein isolate
[c]AI-BP, alkaline extraction with IEP barley protein concentrate
[d]TEI-BP, barley tri-enzymatic treatment with IEP concentrate

native protein had a $\lambda_{max}$ at 351 nm, which then decreased to 348 nm and 349 nm after thermal denaturation and high pressure treatment, respectively (Table 2.4).

#### 2.4.3.3. Techno-functional Properties

##### *2.4.3.3.1. Viscosity*

The viscosity of each concentrate and isolate was determined at 25ºC. The sheer stress increased linearly with increasing sheer rate for PPC and AI-BP concentrates (Figure 2.4), suggesting a Newtonian behavior wherein the viscosity is independent of the rate of shear (RheoSense Inc, 2017). The viscosities of TEI-BP concentrate and WPI decreased with the sheer rate, suggesting a shear thinning behavior. The protein samples showed similar relative viscosities between are similar at low shear rates. Sharma et al. (2011) hypothesized that the high viscosity seen for PPC may be due to the formation of an interfacial layer of adsorbed protein at the interface of the sample. The flow behavior index ($n$) was determined. At a value of $n = 1$, the flow is considered Newtonian, at $n < 1$ it is pseudoplastic, and at $n > 1$ it is a less common dilatant fluid. PPC and WPI samples had flow behavior indices (n) close to one (0.9), revealing their Newtonian behavior. A native faba bean protein solution (10%, w/v) was considered to have a pseudoplastic behavior, since its flow behavior index was determined to be 0.535. However, a chickpea protein solution (2%, w/v) was reported to have a Newtonian behavior with a flow behavior index of 1.0 (Liu & Hung, 1998; Prahl & Schwenke, 1986). Consistent with our results, Vardhanabhuti and Foegeding (1999) found that native WPI exhibits Newtonian behavior. Both barley protein concentrates exhibited flow behavior indices $n<1$ (0.65), demonstrating their pseudoplastic behavior (Table 2.5). This may also be due to the fact that the weak interactions between the particles of these concentrates, which facilitates their disruption under low shear rates as was suggested by Yin, Zhang, and Yao (2015) for pea protein isolates. This also explains the sheer thinning behavior of barley protein, which is in agreement with the findings of Xu, Carson, and Kim (2015), who reported that the flow behavior of wheat protein isolate in aqueous solution (25%, w/v) had a flow behavior index of 0.8. Comparable low viscosity behaviors were obtained for all the protein samples studied analyzed in this study (Table 2.5). Average viscosities of AI-BP and TEI-BP concentrates (4000 and 2000 mPa·s) were higher than those of WPI (1000 mPa·s) and lower than those PPC (5000 mPa·s). Values of the viscosities obtained for both barley samples are more than half those obtained for amaranth protein extract (Condés et al., 2012). Under the same shear rate

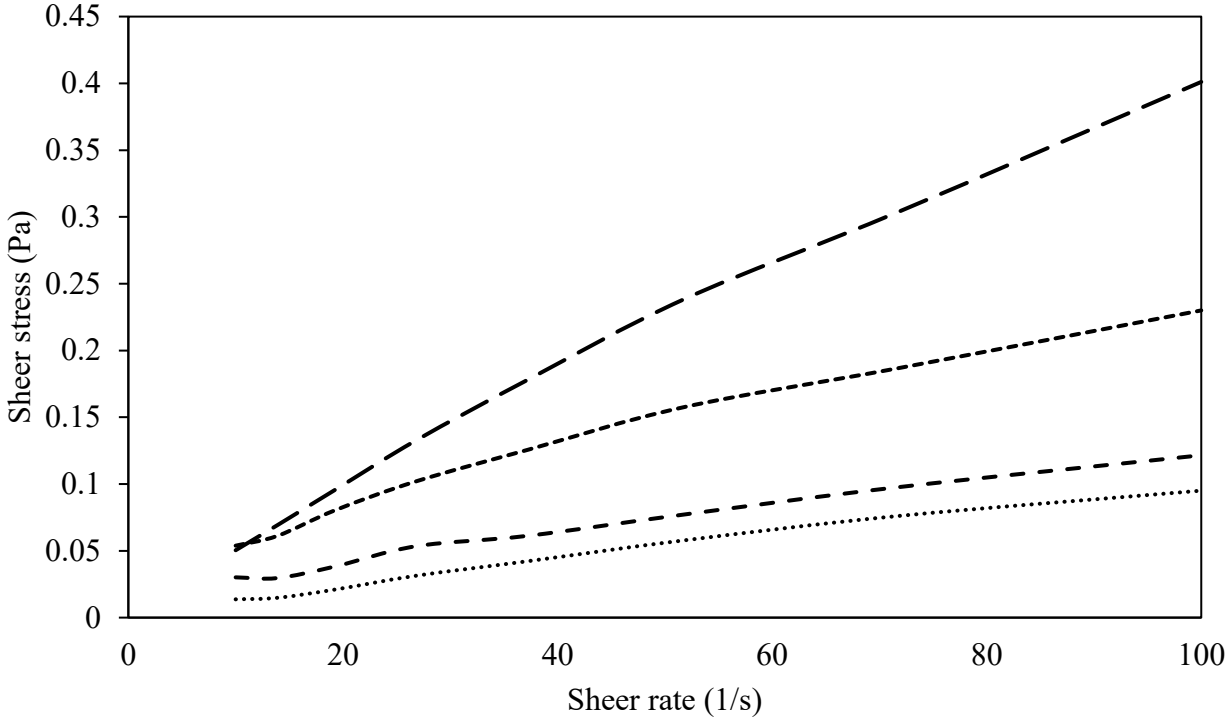


**Figure 2.4**. Changes in sheer stress as a function of sheer rate at 25°C for solutions containing 1% protein. Chart equations were $y = 0.0065x^{0.9029}$ for PPC[a] (— —), $y = 0.0015x^{0.9076}$ for WPI[b] (·····), $y=0.0117x^{0.6505}$ for AI-BP[c] ( - - - - - ), and $y = 0.0060x^{0.6505}$ for TEI-BP[d] ( — — ).

[a]PPC, pea protein concentrate
[b]WPI, whey protein isolate
[c]AI-BP, alkaline extraction and IEP barley protein concentrate
[d]TEI-BP, tri-enzymatic and IEP barley protein concentrate.

**Table 2.5.** Flow behavior indices and average viscosities of protein samples.

| Sample | Flow behavior index (n) | Viscosity[e] (*mPa·s*) |
|---|---|---|
| PPC[a] | 0.9029 | 5000 |
| WPI[b] | 0.9076 | 1000 |
| AI-BP[c] | 0.6505 | 4000 |
| TEI-BP[d] | 0.6505 | 2000 |

[a]PPC, pea protein concentrate.

[b]WPI, whey protein isolate

[c]AI-BP, alkaline extraction with IEP barley protein concentrate

[d]TEI-BP, tri-enzymatic treatment with IEP barley protein concentrate

[e]The viscosity of solutions of 1% (w/v) pea, whey, and barley proteins was measured at 25 °C using a controlled-stress rheometer using a cone-and-plate geometry. The shear rate was set between 1 and 100 $s^{-1}$.

conditions, the viscosities decreased for all protein concentrate samples, except for WPI whose viscosity remained unchanged. The decrease in viscosity may be explained by the degradation of aggregates present in solution, due to a degree of insolubility in water, which was demonstrated to prevent the formation of an established network (Condés et al., 2012).

#### *2.4.3.3.2. Foaming properties*

Foaming capacity and stability of barley protein concentrates, in addition to PPC and WPI used as standards were determined at pH values of 3.0, 5.0, and 8.0. The impact of pH on foaming capacity (FC) is shown in Figure 2.5. The highest FC values was determined at pH 3.0 for all protein extracts. As the pH increases, FC values fluctuate in different ways among the protein extracts (Figure 2.5A). PPC exhibited the highest FC values of 88.7%, 83.3%, and 71.3% at pH 3.0, 5.0, and 8.0 respectively, indicating that the FC of this protein concentrate is relatively independent of the pH within the range studied. Likewise, the FC of AI-BP concentrate did not vary significantly ($p > 0.05$) within the same pH range, as it decreased only slightly from 88.7% at pH 3.0 to 75.3% at pH 8.0 (Figure 2.5A). However, the pH greatly affected the foaming capacity of the other protein concentrates in different ways. TEI-BP concentrate displayed similar behavior to the pH variations as did WPI. They both had the lowest FC values (28.7% and 47.3%, respectively) at pH 5, and the highest FC values (70% and 63.3%, respectively) at pH 3.0. In addition, their FC values at pH 8.0 (58.7% and 62.0%, respectively) were close to those they recorded at pH 3.0 (Figure 2.5A).

After 30 min standing at room temperature, the foam stabilities (FS) of protein concentrates at different pH values varied greatly (Figure 2.5B). The foam produced by PPC was the most stable at all the pH values studied (> 95%), and the lowest FS was recorded for TEI-BP concentrate at pH 5.0. However, the FS properties of the latter concentrate were significantly improved at pH 3.0 and 8.0 and their FS values reached 78% and 91.4%, respectively (Figure 2.5B). The FS of AI-BP did not vary greatly with the pH; it was 64% at pH 3.0 and increased to 75% at pH values of 5.0 and 8.0. A different pattern was noted for the WPI whose FS reached 98% at pH 5 and declined sharply to 74.4% and 56% at pH values of 3.0 and 8.0, respectively.

Adebiyi and Aluko (2011) observed a higher foam volume for field pea protein isolate (FPI) at pH 9.0, and slightly lower volumes at pH 4.0 and 7.0. Also, the greatest foam stability of this protein isolate was observed at pH 9.0 and the poorest stability at pH 4.0. Increased electric charge density with increased alkalinity enhances electrostatic repulsions, thereby preventing rapid coalescence

of foam particles. These results were in accordance with those reported by Shevkani et al. (2015) showing that the highest FC of the FPI was obtained at pH 2.0 and pH 9.0, and the lowest FC at pH 4.0. According to these authors, the foam stability of this protein isolate as function of the pH varied in the same way as in our case. Marinova et al. (2009) demonstrated that WPI displayed the highest foaming capacity at its isoelectric point, which is around pH 4.0, and the least at pH 3.0 and pH 7.0. This was explained by the changes in structure of the adsorption layer depending on the pH. However, Zayas (1997) observed an increase in foam volume of WPI from pH 4.0 to pH 7.0, and attributed this variation was attributed to the modification of properties caused by the WPI preparation methods used. Moreover, Yalçın, Çelik, and İbanoğlu (2007) reported high foam formation of 1.0% (w/v) ethyl-alcohol-extracted barley protein isolates at pH 2.0 and pH 8.0. This can be due to the higher absolute net electric charge causing greater protein flexibility, thereby facilitating movement to the air-water interface and the encapsulation of air molecules. The lowest foaming capacity was also observed at pH 6.0, close to the isoelectric point, due to protein-protein interactions. This also correlates well with minimal water solubility of the protein, which is the primary condition required for foaming. Although Yalçın, Çelik, and İbanoğlu (2007) used gas sparging method, low foaming capacity at pH 5.0-6.0 and enhanced volumes at extreme pH values were consistent with our results.

The foaming capacity of barley protein fractions was found to be greater at pH 3.0 and pH 8.0 due to their higher water solubility and ease to migrate to the air-water interface (Wang et al., 2010). Such findings were also observed for TEI-BP concentrate in our study, but not for AI-BP concentrate (Figure 2.5A). It was also suggested that the foam of the barley protein fraction is most stable at pH 5.0, close to its isoelectric point, due to the neutral net charge stabilizing the air trapped in the protein film. This agrees with our results for AI-BP concentrate, but contrasts with those observed for TEI-BP concentrate (Figure 2.5). A possible explanation is the difference between the protein profiles and, hence, between the isoelectric points of the two protein concentrates. A further explanation can be provided by the greater proportion of higher molecular weight fractions in the BPI shown to possess high foam strength and stability (Wang et al., 2010).

#### *2.4.3.3.3. Emulsifying properties*

Emulsifying abilities were assessed by measuring the droplet size distribution and stability of emulsions. The results of droplet size distribution in the protein emulsions are summarized in

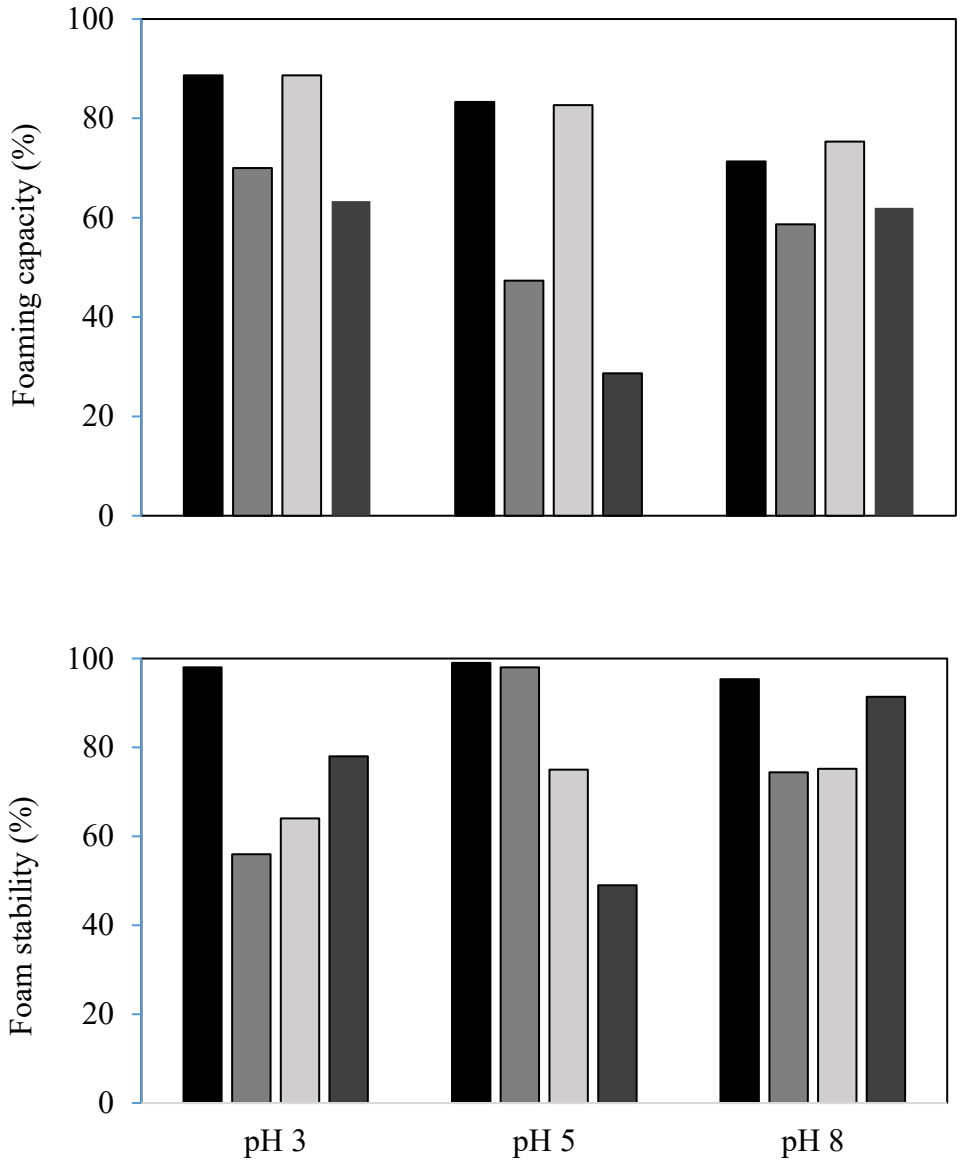


**Figure 2.5.** Effect of pH on foaming capacity (A) and stability (B) of 0.5% (w/v) (PPC[a] ■, WPI[b] ■, AI-BP[c] ■, and TEI-BP [d] ■) solutions at 25°C.

[a]PPC, pea protein concentrate
[b]WPI, whey protein isolate
[c]AI-BP, alkaline extraction with IEP barley protein concentrate
[d]TEI-BP, tri-enzymatic treatment with IEP barley protein concentrate

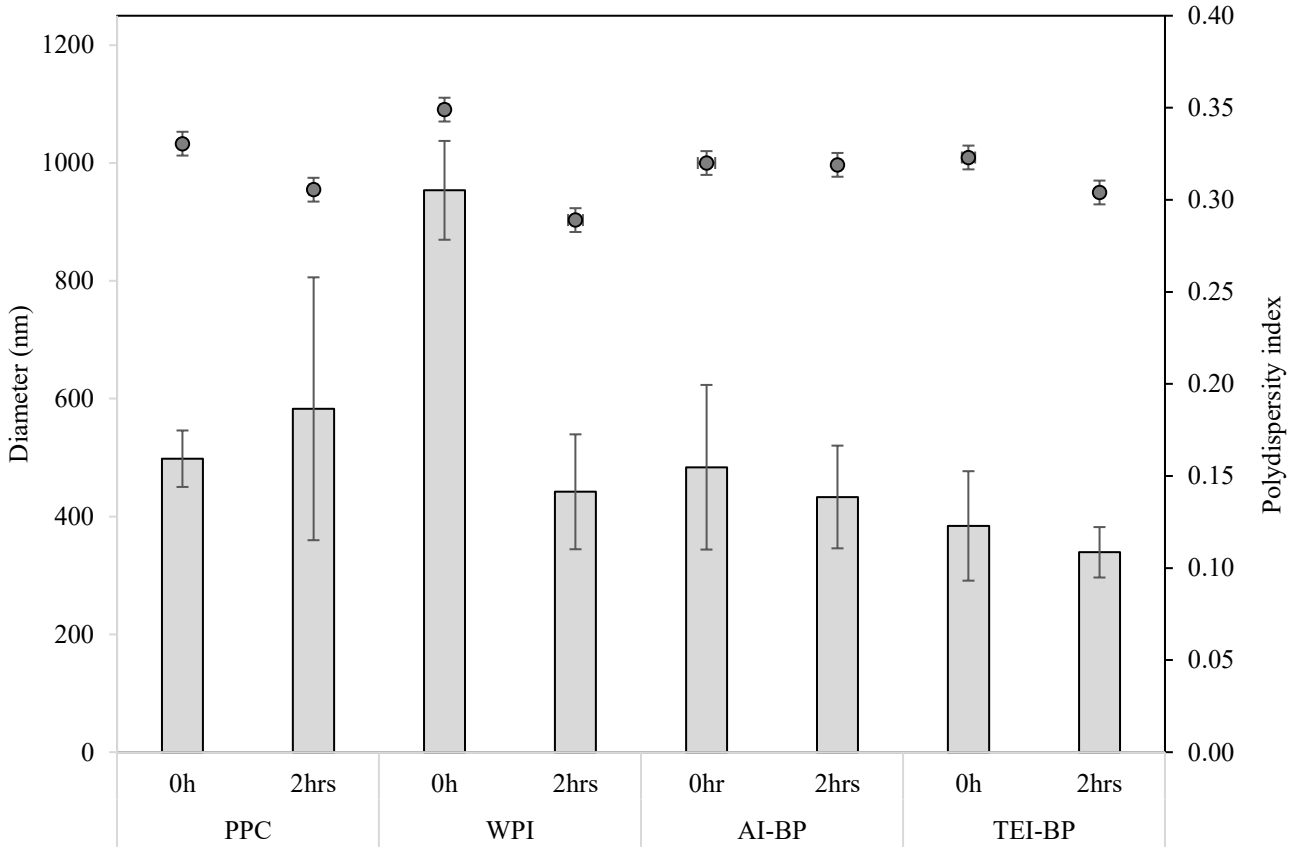


**Figure 2.6.** Droplet size ( ) and polydispersity indices ( ) of protein/glycerol trioleate emulsions, immediately after preparation and 2 h later. PPC, pea protein concentrate; WPI, whey protein isolate; AI-BP, alkaline extraction with IEP barley protein concentrate; TEI-BP, tri-enzymatic treatment with IEP barley protein concentrate

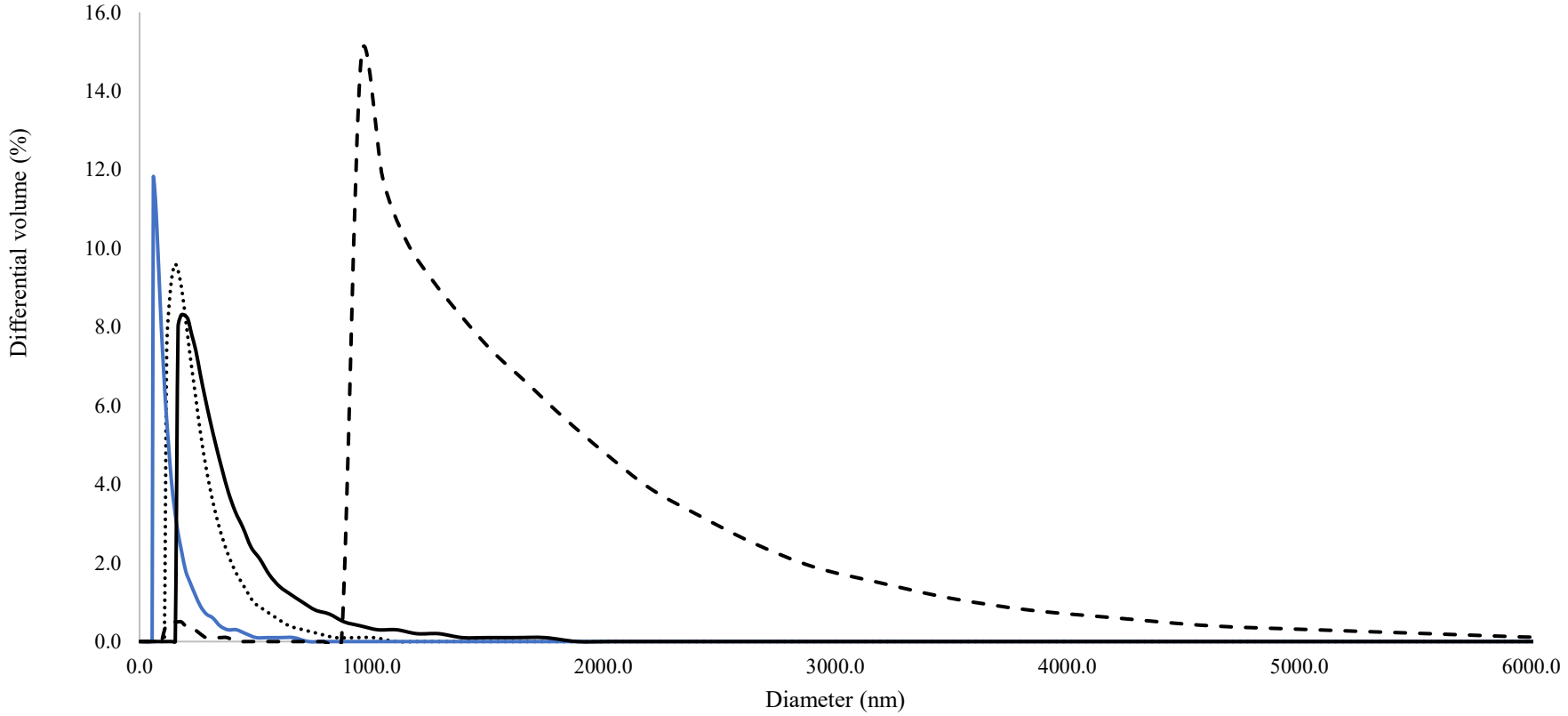


**Figure 2.7.** Particle size distribution for emulsions prepared with PPC – – –, WPI ——, AI-BP ·····, and TEI-BP —— as emulsifiers. PPC, pea protein concentrate; WPI, whey protein isolate; AI-BP, alkaline extraction with IEP barley protein concentrate; TEI-BP, tri-enzymatic treatment with IEP barley protein concentrate.

Figure 2.6. The figure shows a great variation in droplet size diameter between WPI and the barley protein concentrates despite their common polydisperse blend nature based on a polydispersity index > 0.1. The pea protein emulsion showed a sharp peak and homogenous size distribution, with an average droplet size of 498.1 nm and polydispersity index of 0.33.

The wide distribution of oil droplets (Figure 2.7) indicates that PPC has low efficiency as an emulsifier. After 2 h of incubation at 25ºC, the emulsion proved stable, as the average droplet size decreased slightly from 498.1 nm to 444.7 nm, and polydispersity index decreased to 0.31 (Figure 2.6). Aluko, Mofolasayo, and Watts (2009) prepared an emulsion with 10% pea protein at pH 7.0 and recorded a droplet size of 17nm. The emulsion prepared with whey protein isolate exhibited a broader peak, with a larger droplet size diameter of 953.6 nm and a higher polydispersity index of 0.35. Droplet size was reduced to 442.0 nm, half of its original value after two hours, while polydispersity index decreased only slightly to 0.289.

In terms of emulsions made from the barley protein concentrates, TEI-BP concentrate showed the best emulsifying property, as indicated by sharp distribution peak of droplets and small diameter (384.0 nm), followed by AI-BP concentrate with droplet diameter of 483.6 nm. The polydispersity index of 0.32 was the same for both of AI-BP concentrate and TEI-BP concentrate (Figure 2.6). The difference in emulsifying properties between barley protein concentrates may relate to the difference in protein profiles, with TEI-BP concentrate containing higher proportion of globulins and D hordeins, and lesser amount of B hordeins and serpins. Following incubation, droplet size and polydispersity index remained consistent for both emulsions. Bilgi and Çelik (2004) showed an emulsifying capacity value of 40% for 0.5% barley protein concentrate at pH 8.0, despite the lower ratio of emulsifier/oil (1:1) used.

## 2.5 Conclusion

Isolation of proteins from defatted barley (*Hordeum vulgare L.*) flour was investigated using various extraction methods, which greatly influenced protein content, techno-functionality, and structural characteristics. The conventional alkaline extraction (A-BP), followed by an isoelectric precipitation (IEP) step, resulted in a recovery yield of the concentrate (AI-BP) of 51.4% with the protein content of 68.9%. Enzymatic approaches were explored as mild processes to obtain minimally degraded proteins. To this end, a tri-enzymatic approach, including the use of α-amylase from *Bacillus* sp., amyloglucosidase from *A. niger* and β-1,3,4-glucanase from *T. longibrachiatum*

followed by an IEP step produced the TEI-BP concentrate with the highest protein recovery yield of 78.3 that contained a protein content of 41.4%. The barley protein concentrates in both their native and denatured states consisted of different proportions of secondary and tertiary structures, as revealed by Fourier transform infrared spectroscopy (FTIR) and fluorescence spectroscopy analysis, respectively. In terms of functionality, the two barley concentrates had comparable emulsifying properties to those of whey protein isolate (WPI); AI-BP concentrate had enhanced foaming capacity, while TEI-BP concentrate had superior foam stability. Overall, two extraction methods yielding concentrates of differing structural characteristics and functional properties were obtained, with the potential to be incorporated into food applications while offering health benefits.

## CONNECTING STATEMENT II

The efficiency of alkaline extraction (0.05M NaOH) and tri-enzymatic extraction (α-amylase *Bacillus* sp, amyloglucosidase, and β-1,3,4-glucanase) with IEP was studied, and characterized in Chapter II.

In Chapter III, the assessment of the protein-flavor interaction between pea protein concentrate, whey protein isolate, and the two barley protein concentrates, with vanillin was investigated. The effect of heat and high pressure treatment was also determined. Two methods were used to assess the degree of binding, (i) by measuring the unbound vanillin and representing the data using the Klotz plot, (ii) through the quenching of tryptophan by fluorescence spectroscopy. A sensory study was also performed to correlate the analytical data to consumers' perceptions of vanillin, sweetness, and off-flavor in high-protein cookies.

# CHAPTER III

# ASSESSMENT OF THE INTERACTION BETWEEN VANILLIN AND BARLEY PROTEINS AND THE EFFECT OF BINDING ON SENSORY PERCEPTION IN HIGH-PROTEIN COOKIES

## 3.1 Abstract

Two methods were used to extract proteins from barley (*Hordeum vulgare* L.). A classical alkaline extraction followed by isoelectric precipitation (IEP) and an enzymatic technique using two amylases and a glucanase also followed by IEP. The protein concentrates obtained were designated AI-BP (alkaline extraction) and TEI-BP (tri-enzymatic extraction) with protein contents of 68.9% and 51.4, respectively. The interactions of these barley proteins with vanillin were assessed by two methods: (i) measurement of the proportion of unbound vanillin after incubation with the barley protein and use of Klotz plots to determine binding parameters (number of binding sites, dissociation constant, and equilibrium constant), and (ii) characterization of the protein-vanillin complexes by fluorescence spectrophotometry analysis. The interaction between vanillin and the barley proteins was found to be non-cooperative, and quenching of the protein-vanillin complex fluorescence was related to changes in hydrophobicity sites of barley proteins upon their complexation with vanillin. The effects of heat- and high pressure-treatments of the proteins on the level of their interaction with vanillin were also evaluated and compared to those of whey protein isolate (WPI) and pea protein concentrate (PPC). The lowest degree of vanillin binding for the latter proteins was seen with native whey protein. For barley proteins, the AI-BP concentrate treated with high pressure showed the least interaction. Fluorescence spectroscopy analysis revealed that the interaction of vanillin was weakest with heat-treated pea protein, followed by heat-treated whey protein. Commercial WPI and PPC were incorporated into a high protein (~25%) cookies formulated with different concentrations of vanillin. Sensory analysis showed that cookies formulated with PPC at a vanillin/protein weight ratio (V/P WR) of 0.45 and those formulated with WPI at a V/P WR of 0.74 received the highest liking rate, while the highest vanillin intensity was perceived in cookies enriched with WPI or PPC at V/P WRs of 0.74 and 0.59, respectively.

## 3.2 Introduction

Despite its complexity, flavor is one of the food's most critical and greatly influential characteristic for consumer acceptance. Aroma is influenced by chemical reactivity of the flavor compound, the presence of light and oxygen in the food environment, as well as the components of the food matrix itself, including carbohydrates, proteins, fats, transition metals, and other products formed during processing (Weerawatanakorn et al., 2015). Chemical structures of particular aroma volatiles and

the overall composition of the food can affect the transfer of flavor compounds within the matrix and their subsequent release (Seuvre et al., 2006). It is well established that certain non-volatile components, notably proteins, can retain aroma compounds, which alters their release and the flavor perception during mastication (Guichard, 2006; Heng et al., 2004). Numerous factors have significant impacts on the degree of flavor retention by proteins. In the case of the molecular structure of certain flavor components, Van der Waals forces are responsible for binding action of hydrocarbons, and hydrogen bonds occur between alcohols and carbonyl groups (Guichard, 2006). Similarly, chain length of aroma compounds is directly related to the degree of binding, chiefly through hydrophobic interactions (Heng et al., 2004).

Changes in the conformation of the protein itself may modify the interactions between proteins and flavors. When proteins are heat denatured, this results in the dissociation and unfolding of polypeptides with a subsequent aggregation into a precipitate (Damodaran & Kinsella, 1981; Guichard, 2006). This change in protein structure can cause a decrease in association constants, greater affinity of flavors to protein binding pockets, as well as emergence of additional binding sites on proteins (Guichard, 2006).

Apart from molecular structures of either volatile compounds or proteins, conditions affecting food matrices play crucial roles in the degree of binding. Water content and protein structure strongly influence the degree of volatile binding (Reineccius, 2005). Increasing the protein content of the product will enhance protein-protein interactions, thereby decreasing binding of volatile molecules (Halász & Lásztity, 1991). Moreover, the action of binding itself may cause an alteration in conformation of the protein which can modify the flavor molecule binding ability (Reineccius, 2005).

Since potential uses of proteins as functional ingredients and particularly because their interactions with flavoring components are yet to be thoroughly elucidated, the current range of their applications in the food industry remains narrow. Work on the interactions between flavors and proteins have focused on solely examining the types of binding using analytical or sensory data separately (Chobpattana et al., 2002; Damodaran & Kinsella, 1981; Tarrega et al., 2012; Wang & Arntfield, 2015a). The investigation of such interactions using both approaches together is of strong interest, as it will correlate analytical data with consumers' perception of food quality.

The overall objective of this study was to investigate the interaction between barley proteins and two control proteins with a specific flavoring agent analytically and by incorporation into a high-

protein cookie. This was achieved by the following specific objectives: (1) Investigating the effects of barley and control protein systems, and their molecular forms on protein-flavor binding, and (2) Formulating a cookie product with added pea protein concentrate (PPC) and whey protein isolate (WPI), which was then subjected to the evaluation of protein-flavor interaction by with sensory analysis.

## 3.3. Materials

### 3.3.1. *Materials*

Sodium hydroxide (NaOH), hexane ($C_6H_{14}$), Tris base, and sodium citrate dihydrate ($HOC(COONa)(CH_2COONa)_22H_2O$) were obtained from Fisher Scientific (Fair Lawn, NJ). Hydrochloric acid was purchased from Acros (Fair Lawn, NJ). Citric acid anhydrous ($C_6H_8O_{7)}$ was obtained from Debro (On, Ca). Potassium phosphate monobasic ($KH_2PO_4$), potassium phosphate dibasic ($K_2HPO_4$), vanillin, α-amlyase from *Bacillus sp.*, amyloglucosidase from *Aspergillus niger* and β-1,3,4 glucanase from *Trichoderma longibrachiatum* were purchased from Sigma Aldrich (St. Louis, MO). Deionized water (Millipore) was used in all experiments. Barley flour was manufactured by Meunerie Milanaise (Qc, Ca). SDS-Page Broad Molecular weight standard was obtained from Bio-Rad (On, Ca). Whey protein isolate (WPI) was obtained from Hilmar (Hilmar, CA), and pea protein concentrate (PPC) from Roquette (Nord-Pas-de-Calais, FR). Amicon Ultra Centrifugal Filters 0.5mL 3kDa from Sigma Aldrich (St. Louis, MO).

### 3.3.2. *Preparation of defatted barley flour*

Two-row variety barley flour underwent a defatting procedure using hexane at a solvent-to-flour ratio of 1:10 for 1hr. The slurry was centrifuged at 8,500*g* for 15 minutes at 4$^{o}$C using a Beckman Avanti Centrifuge Model J25-I. The residue was then air-dried in a thin layer at room temperature and stored at 4$^{o}$C.

### 3.3.3. Methods for preparation of barley protein (BP) concentrate

#### *3.3.3.1. Alkaline extraction / isoelectric precipitation (IEP)*

The preparation designated alkaline and isoelectric precipitate barley protein (AI-BP) was obtained by alkaline extraction followed by IEP. Defatted barley flour with 0.5 M NaOH (pH 11) at a solvent-to-flour ratio of 10:1 (w/v) for 2 h at 23 $^{o}$C. After centrifugation at 4,000*g* for 15 min, isoelectric precipitation (IEP) was carried out by adjusting the pH of the supernatant to 4.5 using

0.5 M HCl. The mixture was left to stand overnight at 4 ºC and centrifuged at 4,000*g* for 20 min. The pellet was freeze-dried, re-dissolved in distilled water (1:1), and dialyzed before determination of the protein content.

#### *3.3.3.2. Tri-enzymatic extraction/isoelectric precipitation*

The preparation designated tri-enzymatic and IEP barley protein (TEI-BP) was obtained by proteins from DBF through hydrolyzing starch and glucan carried out as the tri-enzymatic approach. This method consisted of sequential treatments with α-amylase Bacillus sp. (10,000 U/g, 65 ºC, 1 h, pH 6.5), amyloglucosidase (660 U/g, 40 ºC, 16 h, pH 6.5), and β-1,3,4-glucanase (8U/g, 37 ºC, 1 h, pH 5.0). The resulting mixture was centrifuged at 4,000g for 15 min followed by adjusting the pH of the supernatant to 3.7 using 0.5 M HCl. The mixture was left overnight at 4 ºC and centrifuged at 4,000g for 20 min. The pellet was freeze-dried, re-dissolved in distilled water (1:1), and dialyzed before determination of the protein content.

### 3.3.4. Treatments of selected protein concentrates and isolate

Heat and high pressure treatments were applied to pea protein concentrate (PPC, 74.2% protein content, w/w), whey protein isolate (WPI, 94.9% protein content w/w), and BP concentrates (AI-BP), 71.6% protein content; TEI-BP, 78.3% protein content w/w). The heat treatment for 20 min at their respective denaturing temperatures of 95ºC, 78ºC, and 85ºC, respectively was applied in water bath (Wang & Arntfield, 2015; Kühn, Considine, & Singh, 2008; Hultin, 1949)**.** The high pressure treatment was performed using a 5 L static high pressure unit (ACIP 6500/5/12VB; ACB Pressure Systems, Nantes, France). The sample concentration was 3% (w/v) in distilled water, after which they were vacuum-sealed in low density polyethylene plastic bags and treated at 600 MPa for 30 min.

### 3.3.5. Determination of protein/flavor binding parameters

#### *3.3.5.1. Spectroscopic quantification of unbound flavor*

The method of Li, et al. (2000) with some modifications was carried out for this purpose. The maximum UV wavelength for the vanillin quantification was determined to be 333 nm following UV/visible scan. Control samples containing only protein were read at 333 nm to control for any interference. Six solutions of vanillin, with a concentration varying from 0.29 to 32.86 mM, were prepared in 10 mM citrate buffer (pH 6.0). These stock vanillin solutions were added to each of

the selected protein solutions [2% (w/v)] of WPI and PPC to the final vanillin concentrations of 0.0658, 0.1314, 0.263, 0.5258, and 0.6572 mM. The vanillin-containing protein solutions were incubated at 10 °C, 25 °C, or 50 °C for varying time ranging from 0.5-72 h, until the equilibrium was reached. The time necessary to reach equilibrium was pre-determined by monitoring the amount of free vanillin over time in each of the protein/vanillin suspensions at a given temperature; when the amount of free vanillin reached its minimum for a constant period of time, equilibrium had been reached. At equilibrium, a 500-µL sample of each protein/vanillin suspension was withdrawn and ultrafiltered at 14,000*g* for 5 min, using 500 µL MWCO 3 kDa ultra centrifugal filters (Sigma-Aldrich). To improve the elution process, 250 µL of 10mM citrate buffer (6.0) was added, and centrifugation proceeded for additional 10 min. This step was repeated twice under the same conditions except that the second centrifugation round was done for 5 min instead of 10 min. The concentration of the unbound vanillin present in the filtrate was measured by spectroscopy in a Beckman Coulter system (San Ramon, USA) using the constructed standard curve of the different vanillin solution concentrations at 333 nm. The results were processed by the Klotz, Scatchard, and Hill plotting models according to Equations 11, 12 and 13, respectively, and the binding parameters were generated from the model which fits the best the experimental data:

Klotz plot:

$$\frac{1}{v} = \frac{1}{n} + \frac{1}{nK[L]} \qquad \text{(Equation 11)}$$

where $v$ is the number of moles of the flavor compound (vanillin) bound per mole of protein, $n$ is the number of binding sites on the protein, and $K$ is the binding constant. Binding parameters were obtained by plotting $1/v$ versus $1/[L]$, where $[L]$ is the concentration of unbound vanillin, $v$ is the number of moles of bound vanillin per mole of protein of which $n$ is the number of binding sites determined from the y-intercept $1/n$, the dissociation constant ($K_d$) was calculated from the slope ($1/n$K), and the binding equilibrium constant ($K$) was calculated from $K_d = 1/K$ (Kühn et al., 2008).

Scatchard plot:

$$\frac{v}{[L]} = Kn - Kv \qquad \text{(Equation 12)}$$

where $v$ is the number of moles of ligand (vanillin) bound per mole of protein, $[L]$ is the unbound ligand concentration in M, $K$ is the binding constant, and $n$ is the number of binding sites (Scatchard, 1949). This can be rearranged and plotted using $\frac{v}{[L]}$ as a linear function of $v$. The slope provides the value of -$K$, while $n$ and $K$ can be determined from the y-intercept $nK$.

Hill plot:

$$log\frac{Y}{1-Y} = h * \log[L] - h * logK_d \qquad \text{(Equation 13)}$$

The Equation 13 is plotted as $log[Y/(1-Y)]$ against $\log[L]$ where $Y$ is the fraction of saturated binding sites, $L$ is the unbound ligand (vanillin) concentration, and $K_d$ is the dissociation constant. The slope is the Hill coefficient $h$, representing the interactions between receptor sites, and which can be determined using non-linear regression. If no cooperativity exists between binding sites, a linear plot will be obtained with a slope of 1.0. A slope greater than 1.0 indicates a positive cooperativity, while negative cooperativity is indicated with a slope of less than 1.0 (Kühn et al., 2008).

#### *3.3.5.2. Spectroscopic quantification of tryptophan quenching by flavor*

Fluorescence spectroscopy was used to quantify the protein/flavor binding through the determination of the tryptophan quenching by vanillin. Analyses were performed using a Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon system, Edison, NJ). Excitation wavelength was between 250-300 nm at increments of 10 nm, and the emission was measured at 250-700 nm with a scan speed of 120 nm/min. Both excitation and emission slits were set at 1 nm and carried out at 25 $^{o}$C. Protein suspensions [0.02% (w/v)] of PPC, WPI, AI-BP, and TEI-BP at their native or denatured form were prepared in distilled water. Nine concentrations (mM) of vanillin, varying from 3.29 to 32.86 mM were prepared as stock solutions and used to spike the protein suspensions with vanillin to the final concentrations of 0.00658, 0.01314, 0.0263, 0.05258, or 0.06572 mM. Using the emission intensity values and concentrations of vanillin, a curve was plotted to determine the time necessary to reach saturation of the binding sites of the protein suspensions in their non-aggregated and aggregated forms; equilibrium was determined to be when the amount of vanillin bound to the protein reached its maximum for a constant period of time. Values were expressed as either vanillin/protein molar ratio (V/P MR), where the moles of vanillin were divided by the moles of protein, or vanillin/protein weight ratio (V/P WR) by dividing the weight of vanillin by the

weight of protein in the cookie formulation. Binding parameters including the dissociation constant ($K_d$), the association constant ($K_a$), and the number of binding sites ($n$) were also determined based on the following equation from Libardi, et al. (2011):

$$\Delta F = \frac{(F_o - F_\infty)[L]}{K_d + [L]} \quad \text{(Equation 14)}$$

where ΔF is the emission gradient after vanillin addition, $F_o$ is the emission of protein alone, $F_\infty$ is the emission of protein saturated with vanillin, $L$ is the vanillin concentration and $K_d$ is the dissociation constant. The experimental values from the fluorescence spectra were fitted to one site saturation ligand binding equation using the SigmaPlot software from SPSS Inc. (Chicago, IL) to determine $K_d$ value.

Using the following equation:

$$\frac{1}{F} = \frac{1}{F_o} + \frac{K_a}{F_o}[L]^n \quad \text{(Equation 15)}$$

where $K_a$ is the association constant. The experimental values from the fluorescence spectra were fitted to a simple power function $y = ax^b$ using SigmaPlot to determine $K_a$ and $n$ values (Libardi, et al., 2011).

### 3.3.6. Sensory Evaluation

#### *3.3.6.1. Preparation of cookies*

Cookies were prepared according to the method 10-52 of the American Association of Cereal Chemists, with some adjustments (AACC, 2000). Non-fat dry milk and ammonium chloride were omitted to observe clearly the impact of proteins on the cookie flavor, and agave syrup was added to improve the organoleptic perception of the panelists.

The preparation of the cookies began by sifting the dry ingredients of all-purpose flour (31%), WPI (30%) or PPC (30%), baking soda (0.3%), and salt (0.2%). The shortening (11.5%), agave (5%), and sugar (15%) were creamed together using a Cuisinart Smart Stick 2 Speed hand blender until fluffy and smooth texture of the mixture was obtained. The wet ingredients were then added: vanilla (4.45%; 8.9%; 13.35%; 17.8%; 22.25%; or 26.7% w/w), baking soda solution (0.8%), water when necessary, and whipped. The sifted dry blend was mixed just until it came together. Cookies of 8 grams each were weighed and shaped into a round mold. They were then baked at 190 ºC for 4-10 minutes and placed on a cooling rack.

#### *3.3.6.2. Sensory Evaluation of cookies*

The cookies were analyzed for sensory properties by 70 McGill University Food Science student volunteers. The sensory characteristics of vanilla flavor, sweetness, and off-flavor intensity, as well degree of liking were evaluated using the Compusense software (Compusense Inc., On, CA) and two scales: an intensity 9-point category hedonic scale (dislike extremely, dislike very much, dislike moderately, dislike slightly, neither like not dislike, like slightly, like moderately, like very much, like extremely) and a liking 9-point category hedonic scale (extremely weak, very weak, moderately weak, slightly weak, neither weak nor strong, slightly strong, moderately strong, very strong, extremely strong).

#### *3.3.6.3. Statistical analysis of sensory scores*

The data were analyzed using 1-way analysis of variance (ANOVA) F-test was performed, by rejecting the F-test p-value if < α 0.05 ($H_0$: $\mu_A = \mu_B$). Fisher's Least Significant Difference (LSD) was then used, by calculating the least significant difference (LSD) between means according to the following equation:

$$LSD_{A,B} = t_{\frac{0.05}{2},DFW}\sqrt{MSW(1/n_A + 1/n_B)} \qquad \text{(Equation 16)}$$

where DFW are the degrees of freedom within the group, and MSW is the mean square within, obtained from the ANOVA test, and n is the number of scores use4 to calculate the mean.

If $|\bar{y}_A - \bar{y}_B| \geq LSD_{A,B}$, the null hypothesis 05 ($H_0$: $\mu_A = \mu_B$) is rejected.

### 3.4 Results and Discussion

#### *3.4.1. Investigating binding affinity between selected proteins and vanillin*

##### *3.4.1.1 Assessment of selected binding models*

To describe the protein/vanillin binding, selected binding models, Klotz, Scatchard and Hill plots, were evaluated. The binding data were analyzed using the unbound vanillin concentrations obtained after the equilibrium binding was reached. It can be hypothesized that the quantified binding parameters are those reached at equilibrium. The Klotz plot allows not only the

determination of the binding affinity, but also the assessment of the presence of cooperativity. The Scatchard equation depicts the thermodynamic relationship between the binding of one ligand to multiple binding sites on a protein with the same equilibrium binding constant. The Scatchard and Klotz plots are the most commonly used models in binding studies. Both assume that the protein's receptor sites are equal and independent; if they are not, the resulting plots will not be linear. The Hill plot, which accounts for this possibility, is used to determine the cooperativity between binding sites.

The linear transformation of the Klotz plot (Figure 3.1A) suggests that the substrate has one type of binding site (Libardi et al., 2011). The linear transformation of the Scatchard plot is presented in Figure 3.1B. While the regression coefficient $R^2$ was favorable (0.98) for the Scatchard plot (Figure 3.1B), the $K_d$ was -0.054 M and $R_T$ value, corresponding to the total receptor concentration**,** was 1.53 mM (data not shown), deviated from those obtained with the Klotz plot. The curvilinear nature of the plots indicates either the presence of at least two groups of receptors with both high and low $K_d$ values, or a single receptor displaying cooperativity between multiple binding sites (Invitrogen Corporation, 2008). Obtaining a positive plot suggests inverse dependence of the binding constants on protein concentration, although protein-protein interactions may also be involved (Clegg and Lindup, 1984). The Hill plot gave a Hill coefficient *h* of 1.60 as determined from the slope, which is a representation of the degree of interaction (Figure 3.1C). It was not possible to obtain $K_d$, since the data did not cross the y-axis. This plot was not used for further comparison due to the low $R^2$ value of 0.78 (Figure 3.1C). Moreover, it is known that in order for the Hill equation to be appropriate for a data set, the plot obtained must be linear (data not shown), which was not the case in this study (Invitrogen Corporation, 2008).

Binding experiments with native proteins and six vanillin concentrations were performed, and analyzed using Klotz plots (data not shown). Due to limitations of the two other models, and the favorable $R^2$ values, this approach was selected for further analysis. The results showed a linear relationship for each protein, indicating a non-cooperative interaction between vanillin and all proteins. It can, therefore, be concluded that the binding of vanillin to one site of the protein does not influence the affinity of other binding sites. Similar trend was observed by Suppavorasatit, et al. (2013) who demonstrated that soy protein isolate (SPI) exhibited a non-cooperative interaction with vanillin at 25°C.

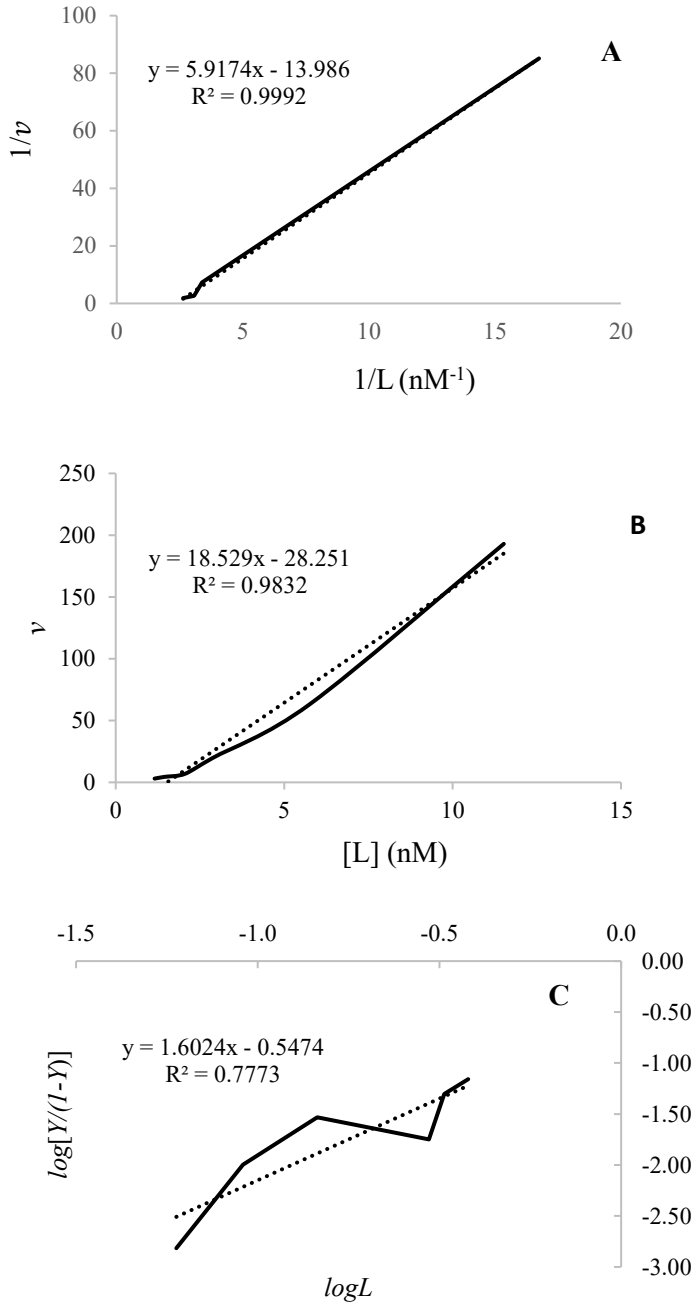


**Figure 3.1.** Klotz (A), Scatchard (B) and Hill (C) plots of selected protein concentrate, where *v* is the number of moles of bound vanillin per mole of protein, L is the unbound ligand (vanillin) concentration, and *Y* is the fraction of saturated binding sites.

### *3.4.1.2. Binding parameters of native/treated protein concentrates and isolate*

Binding parameters generated from the Klotz plots are presented in Table 3.1. For the binding equilibrium constant K, the lower its value, the weaker is the reaction, meaning that few binding interactions occur between the protein and vanillin. Conversely, the binding interactions are inversely related to the dissociation constant $K_d$, i.e., they are decreased with increasing $K_d$ values. Results of Table 3.1 show that native PPC and WPI have twice the number of binding sites compared to the barley proteins, however the K and $K_d$ are more favorable. Native barley proteins of AI-BP and TEI-BP concentrates showed both a higher incidence of binding as determined by their low $K_d$, although TEI-BP concentrate had six times lower number of binding sites. It can be hypothesized that the difference in number of binding sites is due to the protein nature of each concentrate. TEI-BP concentrate contains a higher proportion of 55-80 MW fractions, corresponding to serpin Z4, globulins, and D-hordeins, while AI-BP concentrate dominated by proteins of lower MW, including B3 hordein (unpublished data). In this regard, Marsh and Teichmann (2011) suggested that changes in conformation and surface area of an isolated protein subunit are directly correlated to its molecular weight. However, when all subunits are put together, intermolecular interactions may stabilize changes without increasing the surface area, as was observed with TEI-BP concentrate.

From Klotz plots, the number of binding sites (*n*) of the native PPC was determined to be 1.79 (Table 3.1). The number of binding sites of vanillin to proteins was shown to vary depending on the type of the protein and the experimental conditions of the test. For example, reported that the number of binding sites of SPI to vanillin at 25 ºC was 0.48, 2.31 at 15 °C, and 13.6 at 5 °C. Li et al. (2000) demonstrated that the numbers of binding site of soy protein and WPI to vanillin at 12 °C were 3.8 and 0.67, respectively. Similarly, the K values vary widely from a protein to another and depending on the physical state (native or denatured) of the protein. Table 3.1 shows that native barley proteins have higher K values than PPC and WPI used as standards, and that the highest K value of 10.02 $M^{-1}$ was recorded AI-BP concentrate. Highly different magnitudes of K values for vanillin binding to soy proteins of 186 x $10^4$ $M^{-1}$ at 25 ºC (Suppavorasatit et al., 2012) and 1.71 x $10^3$ $M^{-1}$ at 12 ºC (Li et al., 2000) were reported. Discrepancies in the magnitudes of K values were explained mainly by differences in the sources and methods of preparation of the protein concentrates/isolates (Suppavorasatit et al., 2012). Table 3.1 shows also that denaturation treatments affect significantly the K values. While both denaturation treatments of PPC and WPI

**Table 3.1.** The Binding Parameters Including Number of Binding Sites (n), Binding Constant (K), and Dissociation Constant ($K_d$), of Vanillin to Control (PPC[a] and WPI[b]) and Barley Protein Concentrates (AI-BP[c] and TEI-BP[d]) Generated from Klotz Plot.

| Sample | State | Binding parameters | | |
|---|---|---|---|---|
| | | n | K ($M^{-1}$) | $K_d$ (M) |
| PPC | Native | 1.79 | 0.78 | 1.28 |
| | Heat-treated[e] | 1.11 | 2.56 | 0.40 |
| | High pressure-treated[f] | 1.82 | 1.14 | 0.88 |
| WPI | Native | 1.74 | 0.29 | 3.47 |
| | Heat-treated | 0.87 | 0.76 | 1.32 |
| | High pressure-treated | 2.66 | 0.97 | 1.03 |
| AI-BP | Native | 0.66 | 10.02 | 0.1 |
| | Heat-treated | 0.21 | 3.4 | 0.29 |
| | High pressure-treated | 9.23 | 0.008 | 54.7 |
| TEI-BP | Native | 0.11 | 1.93 | 0.52 |
| | Heat-treated | 2.86 | 0.95 | 1.05 |
| | High pressure-treated | 0.68 | 3.14 | 0.31 |

[a]PPC, pea protein concentrate.
[b]WPI, whey protein isolate.
[c]AI-BP, barley protein concentrate obtained by alkaline extraction with IEP concentrate.
[d]TEI-BP, barley protein obtained by tri-enzymatic starch and glucan removal with IEP barley protein concentrate.
[e]Heat treatment of 20 min at 95 °C, 78 °C, and 85 °C for PPC, WPI, and barley proteins, respectively.
[f]A pressure of 600 MPa was applied for 30 min.

increased the corresponding K values compared with those of their native counterparts, they decreased those of AI-BP concentrate (Table 3.1). In contrast, K value of TEI-BP was decreased by thermal denaturation and increased by high-pressure treatment. The number of binding sites (*n*) was also affected by protein denaturation; with the general trend being thermal and high pressure treatments had an opposite effect on this parameter. Heat denaturation of PPC, WPI and AI-BP concentrate decreased their number of binding sites, which was, on the contrary, increased when these proteins were denatured by high pressure treatment. Conversely, heat treatment increased *n* for TEI-BP, while high pressure treatment decreased it (Table 3.1).

The dissociation constant ($K_d$) and number of binding sites of WPI and PPC were decreased upon thermal denaturation. As these respective changes in $K_d$ and *n* cancel out a reduction in binding, this suggests that none of the two treatments has affected the overall interactions with vanillin. In this regard, Wang and Arntfield (2014) observed an increase in binding of aldehydes to pea protein isolate by heat treatment at 86ºC for 30 min. Such a tendency was attributed to protein unfolding with a consequent exposure of the hydrophobic binding sites. The apparent increase in binding seen between WPI and vanillin after protein denaturation, as indicated by the increase in K values, is in agreement with the findings of Mills and Solms (1984) showing enhanced binding of aldehydes to proteins at high temperatures. In contrast, Kühn et al. (2008) did not notice a change in the degree of binding between WPI and two aldehydes upon heating a protein solution with added flavor compounds at 80ºC. However, when the flavor compounds were added prior to denaturation, a covalent interaction occurred between the aldehyde and the amino group. This inconsistency may be due to a difference in the binding mechanisms between flavor compounds of the same class, whereby some may form hydrophobic or covalent binding interactions.

Following heat-treatment, both barley protein concentrates (AI-BP and TEI-BP) had more favorable K and $K_d$ (Table 3.1). All binding parameters of the heat-treated sample either increased or decreased compared to those of their native counterparts, and the changes were more dramatic for AI-BP concentrate. For AI-BP concentrate, threefold changes were noted in each binding parameters upon heat treatment; the number of binding sites (n) decreased from 0.67 to 0.21 and K from 10.02 $M^{-1}$ to 3.4 $M^{-1}$, while $K_d$ increased from 0.1 M to 0.29 M. As for TEI-BP concentrate, the heat-treatment caused an increase in the number of binding sites by more than 25 times, with *n* being 0.11 for the native samples and 2.86 for the heat-treated ones. Moreover, the K value of

heat-treated TEI-BP concentrate samples (0.95 $M^{-1}$) was twofold lower than that of the native samples (1.93 $M^{-1}$) and, consequently, the $K_d$ value of the heat-treated samples (1.05 M) was twice as high as that recorded for their native counterparts (0.52 M) (Table 3.1). Such results demonstrate a state of the least protein-flavor interactions.

The control proteins responded differently to the high pressure treatment. While PPC's binding parameters were improved compared with those of the thermally denatured samples, WPI demonstrated the highest degree of binding, with the highest number of binding sites (2.7), K (0.97 $M^{-1}$) and $K_d$ (1.03 M) compared to all states. Kühn et al. (2008) also studied the effect of high pressure of WPI (600 MPa for 30 min at 20 ºC) on its interaction flavor compounds. These authors observed the formation of protein aggregates, and that the binding interactions were reduced with 2-nonanone and increased with *trans*-2-nonenal, while no changes occurred in the presence of 1-nonanal. Under comparable conditions (600 MPa for 32 min at 50 ºC), Yang et al. (2003) demonstrated that high pressure treatment did not alter the degree of binding between the resulting molten globule state of β-lactoglobulin and vanillin compared to the native state of the protein; however, the high pressure treatment was reported to modify the hydrophobic calyx and surface binding sites of β-lactoglobulin (Tromelin, et al., 2006). The binding of flavors to β-lactoglobulin is dependent on molecular structure of the ligand/flavor, and because of to the polar aldehyde group hindering the hydrophobic interactions, vanillin was displayed a weak interaction with β-lactoglobulin (Yang et al., 2003).

It can be seen from Table 3.1 that the high-pressure treatment had the greatest effect on AI-BP concentrate, giving the most favorable K (0.0079 $M^{-1}$) and $K_d$ (54.66 M) of all the proteins tested, meaning the least binding interaction, despite its highest number of binding sites (9.23). This treatment had the opposite effect on TEI-BP concentrate, resulting in increased binding properties, as evidenced by the higher K value of 3.14 $M^{-1}$ and the lower $K_d$ value of 0.312 M.

A reduced incidence in binding interactions upon both heat and high pressure treatments was expected, as they are known to denature proteins. Although these treatments appeared to have the same effect on the protein-flavor interactions, high pressure treatment affected the protein extracts studied herein to greater extent. This may be due to the difference in the mechanism of protein denaturation of each denaturing treatment: high pressure involves water penetrating the proteins before they unfold, while heat treatment acts essentially on nonpolar groups migrating into water (Hummer et al., 1998). An important factor in predicting covalent interactions between proteins

and aldehydes, such as vanillin, is the amount of free functional groups, particularly –SH and –$NH_2$. The lower incidence of binding with PPC and aldehydes has been attributed to less sulfur-containing amino acid residues (0.35 and 1.60 g/100g protein of Cys and Met, respectively), a weak affinity of aldehydes to –$NH_2$-containing Lys, and low level of disulfide bonds (Khattab, Arntfield, & Nyachoti, 2009; Wang & Arntfield, 2014). An opposite situation has been suggested for wheat gluten, where a high level of disulfide structure contributes to its stability and close structure, causing stearic hindrance to flavor compounds (Wang & Arntfield, 2014).

### *3.4.2. Binding study using fluorescence spectroscopy*

To evaluate the interaction between vanillin and PPC, WPI, and barley protein concentrates (AI-BP and TEI-BP), fluorescence spectroscopy was used, based on the quenching of tryptophan. While this amino acid is usually protected in the hydrophobic core of the protein, it can be exposed subsequent to conformational changes caused by denaturation or ligand binding. These molecular rearrangements will ultimately result in greater quenching of tryptophan and a decrease in fluorescence intensity. Peak emission for tryptophan is normally seen around 348 nm; however, results of Table 3.2 show that the wavelength of maximum fluorescence intensity at 25$^{o}$C varied from 332-355 nm depending on the protein source and the type of treatment. A red (higher wavelength) or blue (lower wavelength) shift in the wavelength of maximum fluorescence intensity was observed for all proteins studied upon thermal or high pressure denaturation. A second peak at 580 nm was seen in the fluorescence spectra of all samples, which did not shift following heat and high pressure treatments.

Figure 3.2 presents the spectra obtained after incubation of the native proteins with nine vanillin concentrations and measuring the fluorescence for tryptophan, and Figure 3.3 shows the corresponding spectra for these proteins after heat and high pressure treatments. Both figures show a trend of a steady decrease in the maximum intensity with increasing concentrations of vanillin for all samples. However, the most dramatic decrease in intensities was recorded when the concentration of added vanillin was changed from 3.0 mM to 6.0 mM. Thereafter, the maximum intensities continued to decrease with increased vanillin concentrations, but at significantly reduced rates compared to that observed between 3.0 and 6.0 mM of added vanillin. WPI had the highest maximum intensities in all three states (native, heated, and high-pressure treated), while

**Table 3.2.** Maximum fluorescence intensity of PPC, WPI, AI-BP and TEI-BP concentrates in the native state, and the corresponding shifts upon heat and high pressure treatments. The excitation wavelength was 290nm.

| Protein extract | Maximum wavelength for the native protein (nm) | Wavelength (nm) shift of the treated protein extracts[a] | |
|---|---|---|---|
| | | Heat-treated | High pressure-treated |
| **PPC** | 348 | 334 | 337 |
| **WPI** | 332 | 339 | 347 |
| **AI-BP** | 346 | 357 | 355 |
| **TEI-BP** | 351 | 351 | 350 |

Abbreviations are as defined in Table 3.1[a]No shift from 580nm was observed after heat and high pressure treatments

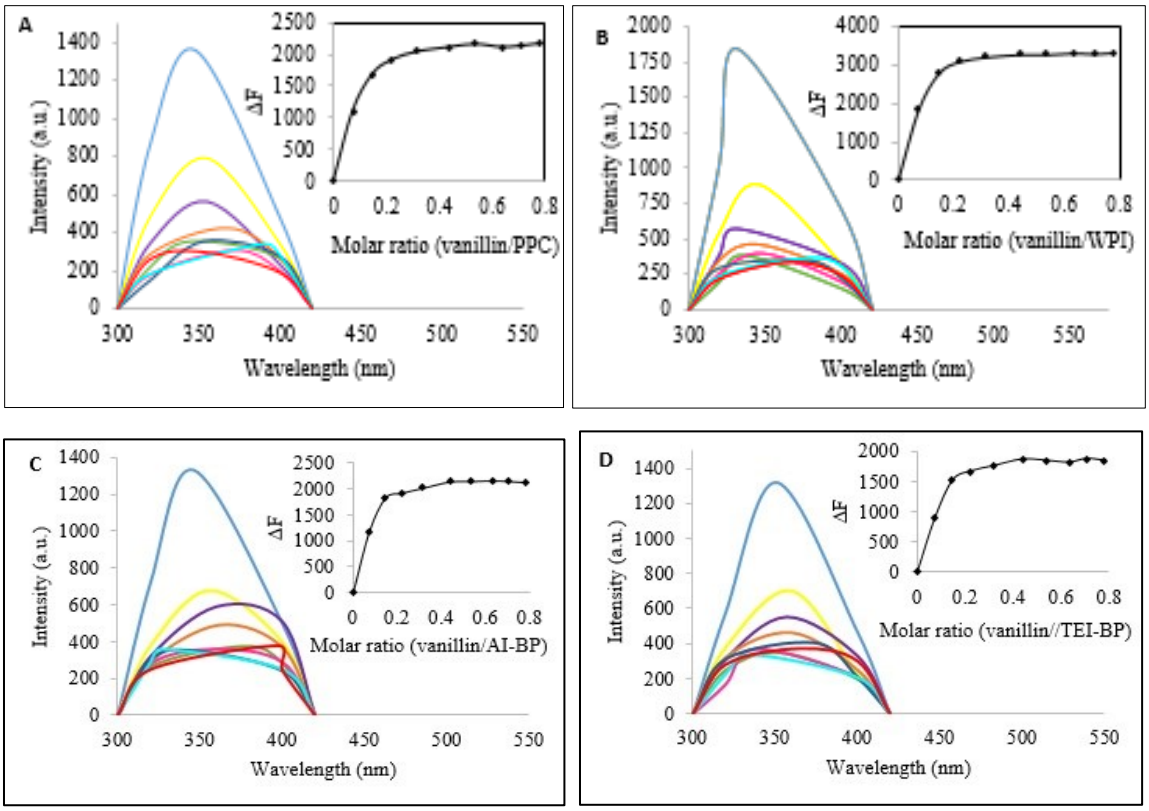


**Figure 3.2.** Interactions between vanillin and tryptophan of PPC (A), WPI (B), AI-BP concentrate (C), and TEI-BP concentrate (D) proteins in the native state, estimated by fluorescence spectroscopy at excitation of 290 nm, upon addition of vanillin at varying concentrations (mM) of 3.0 (—), 6.0 (—), 9.0 (—), 13.0 (—), 18.0 (—), 22.0 (—), 26.0 (—), 29.0 (—), 32.0 (—). Insert charts depicting the difference in emission after vanillin addition (ΔF) as function of the molar ratio are used to determine the values for saturation of the protein binding sites.

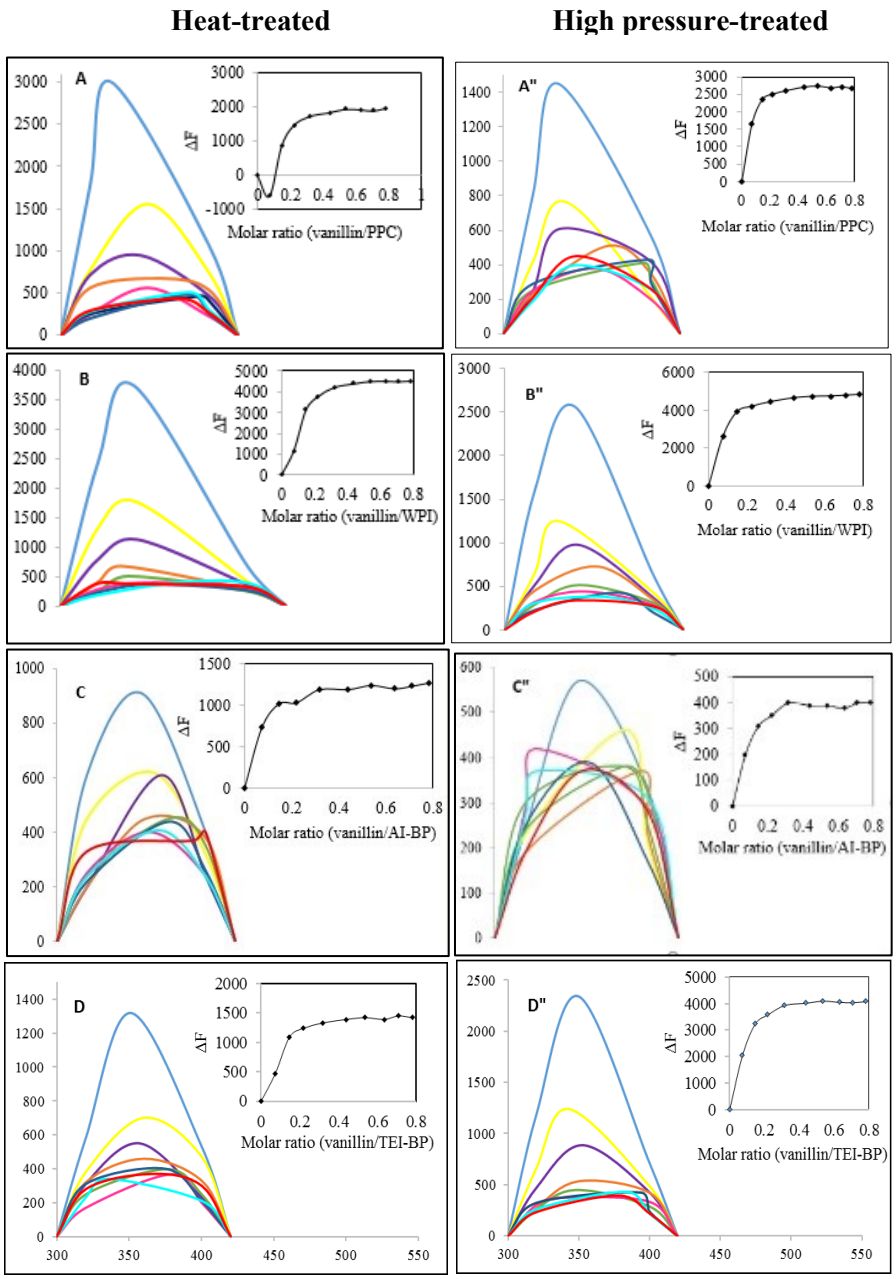


**Figure 3.3.** Effect of heat (left charts) and high pressure (right charts) treatment on the interaction between vanillin and tryptophan of PPC (A), WPI (B), AI-BP concentrate (C), and TEI-BP concentrate (D) proteins, as estimated by fluorescence spectroscopy at excitation of 290 nm, upon addition of vanillin at varying concentrations (mM) of 3.0 (—), 6.0 (—), 9.0 (—), 13.0 (—), 18.0 (—), 22.0 (—), 26.0 (—), 29.0 (—), 32.0 (—). Insert charts depicting the difference in emission after vanillin addition as function of the molar ratio are used to determine the saturation values of proteins.

the lowest maximum intensity was recorded for the alkaline extraction barley protein concentrate (AI-BP) samples (Figures 3.2 and 3.3). Among the native proteins, WPI had the highest maximum intensity of 1850 a.u, while the maximum intensities of PPC and the barley protein concentrates were similar (Figure 3.2). The greatest differences in maximum intensities are seen with the heat-treated and high pressure-treated proteins. Again, the highest maximum intensities of 3710 and 3010 a.u were recorded for the heat-treated samples of WPI (Figure 3.3B) and PPC (Figures 3.3A), respectively. Conversely, the lowest maximum intensities for the heat-treated samples were 1210 and 910 a.u recorded for TEI-BP and AI-BP concentrates, respectively (Figures 3.3C" and D"). After high pressure treatment, the maximum intensities of WPI and TEI-BP concentrate were the highest reaching 2560 and 2430 a.u, respectively (Figures 3.3B" and D").

The maximum intensity of high pressure-treated PPC (Figure 3.3A") was about twofold lower than that of high pressure-treated WPI (Figure 3.3B"), while high pressure-treated AI-BP concentrate showed the steepest decrease in fluorescence intensity and the lowest maximum intensity of all proteins, being as low as 570 a.u. Comparing the insert plots showing the saturating trend with increasing concentration of vanillin, it can be seen that WPI, and AI-BP and TEI-BP concentrates reached saturation at 0.44 molar ratio, while no change in binding interaction between vanillin and proteins was observed for PPC at a molar ratio of 0.54 (Figure 3.2). It has been suggested earlier that the inverse relationship between fluorescence intensity and vanillin concentration is due to the change in tryptophan microenvironment from non-polar to more polar. Since vanillin contains polar functional groups (aldehyde, hydroxyl, ether) and tryptophan is a hydrophobic amino acid, as flavors bind to the protein, conformational changes occur in such a way to expose the tryptophan residues that were buried in the hydrophobic core, resulting in tryptophan quenching (Damodaran & Kinsella, 1981). Another explanation for such a decrease is resonant energy transfer between the tryptophan indole ring and the flavor compound, by altering the electron density surrounding the protein and its subsequent behavior in a polar environment (Muresan et al., 2001; Liu et al., 2005). Libardi, et al. (2011) studied the change in fluorescence intensity of β-lactoglobulin with varying vanillin concentrations at pH 7.4 and obtained the same trend of decreasing fluorescence intensity with increasing vanillin concentration, as well as a similar intensity of 2500 a.u. for native β-lactoglobulin with the lowest vanillin concentration. This trend was explained by the release of water from the protein upon vanillin binding, opening the hydrophobic core, thereby enhancing the access of vanillin to tryptophan. The same pattern was seen with flavor compounds binding to

pea proteins, where it was demonstrated that the binding of aldehydes and ketones induce an unfolding effect on the protein (Wang & Arntfield, 2014). Fluorescence intensity was observed to increase following high pressure treatment (Figure 3.3). This contrasts with the results obtained by Liu et al. (2005) showing a decrease in fluorescence intensity of whey protein concentrate (WPC) subjected to 600 MPa for 0-30 min in the presence of different ketones and aldehydes. It was suggested that high pressure treatment modifies the polarity of the tryptophan microenvironment to become more polar and, hence, the binding sites of the protein more accessible (Kühn et al., 2008; Liu et al., 2006).

The results of the binding parameters for each protein are summarized in Table 3.3. The proteins had similar number of binding sites for all states, except high pressure-treated TEI-BP concentrate, which was much lower ($1.62\ 10^{-16}$). Heat-treated samples of PPC, WPI, and TEI-BP concentrate had higher numbers of binding sites than their native counterparts, with those of PPC and WPI being about threefold higher. All proteins had high association constants, suggesting a significant receptor-ligand complex interactions, and the highest were observed for the native and heat-treated WPI. The highest, and therefore most favorable from a reduced binding standpoint, $K_d$ values were obtained for the heat-treated PPC and WPI samples, followed by native TEI-BP and high pressure-treated AI-BP concentrates (Table 3.3). It can also be suggested that the control proteins (PPC and WPI) had the least interaction with vanillin, despite the higher number of their binding sites compared to both barely protein concentrates.

It should be emphasized the results for the binding parameters differed according to the analytical method used. For example, the number of binding sites (*n*) of the native proteins studied, as determined through the quenching of tryptophan (fluorescence spectroscopy), was generally lower than that obtained by using Klotz plots to determine the unbound vanillin. This reveals that sites other than tryptophan (non-specific sites) may have been involved in the protein/flavor interaction[19]. However, TEI-BP concentrate was an exception, as the value of its *n* (0.21) (Table 3.3) was twice higher that generated from Klotz plots (Table 3.1). Likewise, the binding constants were significantly greater ($p < 0.05$) for all samples when obtained through fluorescence spectroscopy, as were the dissociation constants, except for WPI (Table 3.1 versus Table 3.3). The technique of unbound vanillin showed a decrease in the value of the number of binding sites for heat-treated PPC, WPI and AI-BP concentrate compared with that of their native states (Table 3.1). On the contrary, fluorescence spectroscopy provided opposite trend showing that this

**Table 3.3.** Binding parameters of number of binding sites (n), binding constant ($K_a$), and dissociation constant ($K_d$) obtained from fluorescence spectroscopy of native, heat-treated and high pressure-treated control (PPC[a] and WPI[b]) and barley protein concentrates (AI-BP[c] and TEI-BP[d]).

| Sample | State | Binding parameters | | |
|---|---|---|---|---|
| | | n | $K_a$ | $K_d$ |
| PPC | Native | 0.21 | 2.73E+06 | 2.87 |
| | Heat-treated | 0.61 | 6.46E+05 | 17.50 |
| | High pressure | 0.15 | 5.18E+06 | 1.88 |
| WPI | Native | 0.18 | 7.03E+06 | 2.33 |
| | Heat-treated | 0.33 | 7.76E+06 | 5.72 |
| | High pressure | 0.20 | 1.32E+07 | 2.58 |
| AI-BP | Native | 0.18 | 3.02E+06 | 2.39 |
| | Heat-treated | 0.18 | 1.12E+06 | 2.33 |
| TEI-BP | Native | 0.21 | 2.10E+06 | 2.94 |
| | Heat-treated | 0.28 | 1.05E+06 | 9.50E-17 |
| | High pressure | 1.62E-16 | 3.56E+06 | 3.99E-18 |

[a]PPC, pea protein concentrate.
[b]WPI, whey protein isolate
[c]AI-BP, alkaline extraction with IEP barley protein concentrate;
[d]TEI-BP tri-enzymatic extraction with IEP barley protein concentrate.

parameter increased for the same heat-treated proteins (Table 3.3). Similar observation can be made for the binding constant (K) which was shown to be increased by thermal denaturation for PPC and WPI proteins, and decreased for the BP concentrates by when assessed by fluorescence spectroscopy (Table 3.3). However, when the Klotz plots were used, K decreased for all heat-treated proteins as compared with that of their native states with the exception of PPC (Table 3.1). The opposite trend was observed for the dissociation constant ($K_d$) determined by the two methods, as these two parameters are inversely related. Lastly, after high pressure treatment, the number of binding sites determined by both methods has increased for all treated samples, except for TEI-BP, which decreased. Likewise, both methods showed that the association constant ($K_a$) increased for PPC, WPI, and TEI-BP, and decreased for AI-BP. Similar trends for $K_d$ were shown by both techniques, in that it decreased for PPC and TEI-BP, and increased for AI-BP. A significant difference ($p< 0.05$) was observed between AI-BP and TEI-BP from each method. The differences in values obtained may reveal the degree of tryptophan availability offered by each extraction method, which would subsequently impact the binding affinity, interaction, and determined parameters between protein and ligand.

In spite of the fact that tryptophan fluoresces at 348 nm, as the protein-vanillin aggregate forms, fluorescence peaks were observed at the higher wavelength of 580 nm for native and treated protein samples (Hawe et al., 2008). Figures 4 and 5 present the results of the interactions of vanillin with aggregated proteins at 580 nm in their native and denatured forms by measuring the intensity of fluorescence emission of tryptophan. Contrary to non-aggregated proteins, stronger peak amplitudes can be seen for all proteins (Figures 3.4 and 3.5). The same trend was observed as in the non-aggregated protein-vanillin complex, depicting a decrease in the emission with increasing concentrations of vanillin. For the PPC samples, the intensity after heat treatment (346,020 a.u) was more than twice that recorded for the native (Figure 3.4) and high pressure-treated (Figure 3.5A") proteins (157,240 a.u and 163,520 a.u, respectively). From the insert plots, it can be observed that the saturation point is attained quicker when the protein is in an aggregated state, as the native protein is saturated at a molar ratio of 0.32 (Figure 3.4A), and the pressure-treated proteins at 0.44 (Figure 3.5A). The trend for WPI was slightly different, with the high pressure-treated protein showing the highest emission intensity of 83,090 a.u (Figure 3.5B"), while the heat-treated protein displayed about two fold lower intensity of 43,860 a.u (Figure 3.5B), and

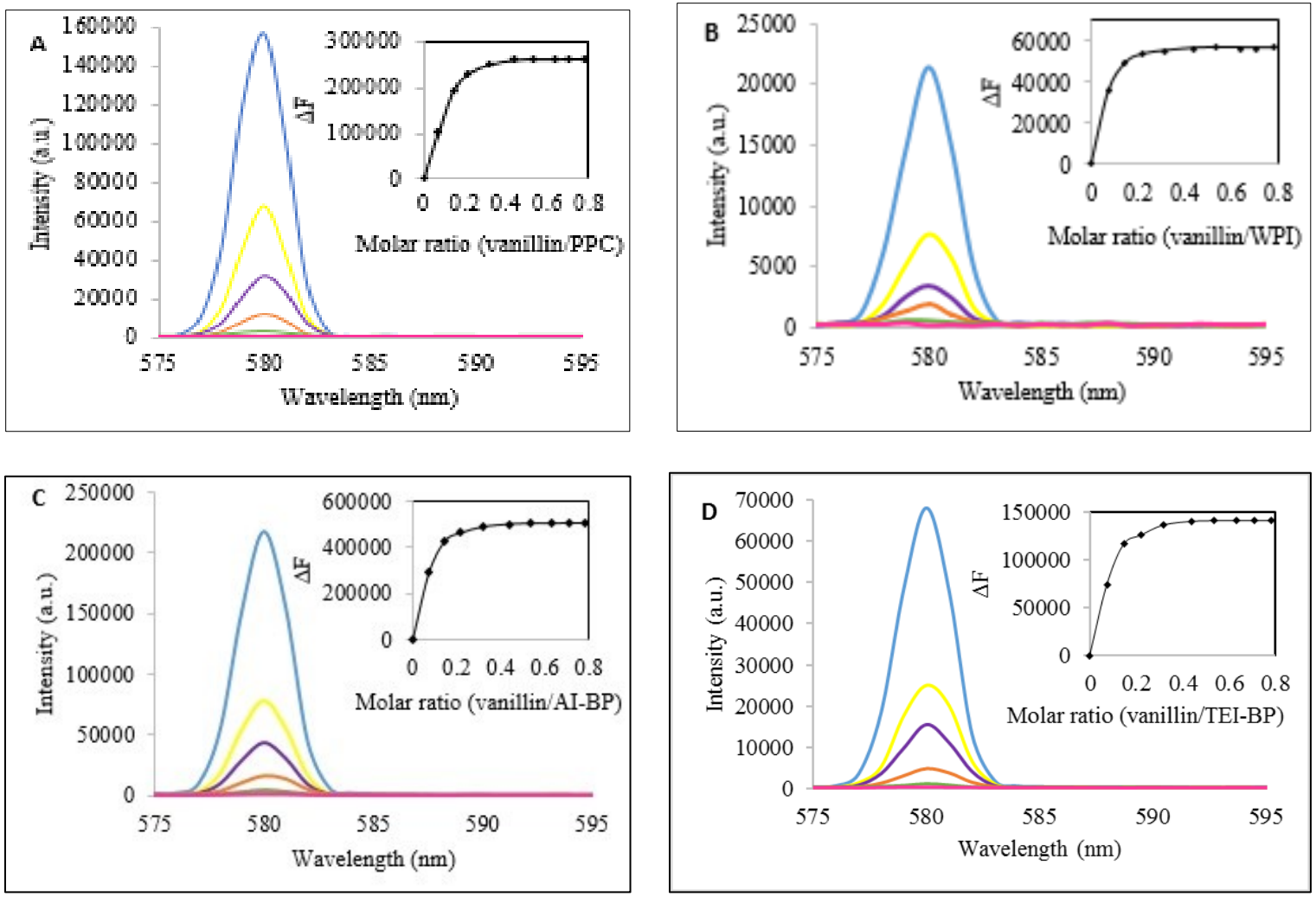


**Figure 3.4.** Interaction between vanillin and proteins aggregates of PPC (A), WPI (B), AI-BP (C), and TEI-BP concentrates (D) in the native state, as estimated by fluorescence spectroscopy at excitation of 290 nm, upon addition of vanillin at varying concentrations (mM) of 3.0 (—), 6.0 (—), 9.0 (—), 13.0 (—), 18.0 (—), 22.0 (—), 26.0 (—), 29.0 (—), 32.0 (—). Insert charts depicting the difference in emission after vanillin addition (ΔF) as function of the molar ratio are used to determine the saturation values of proteins.

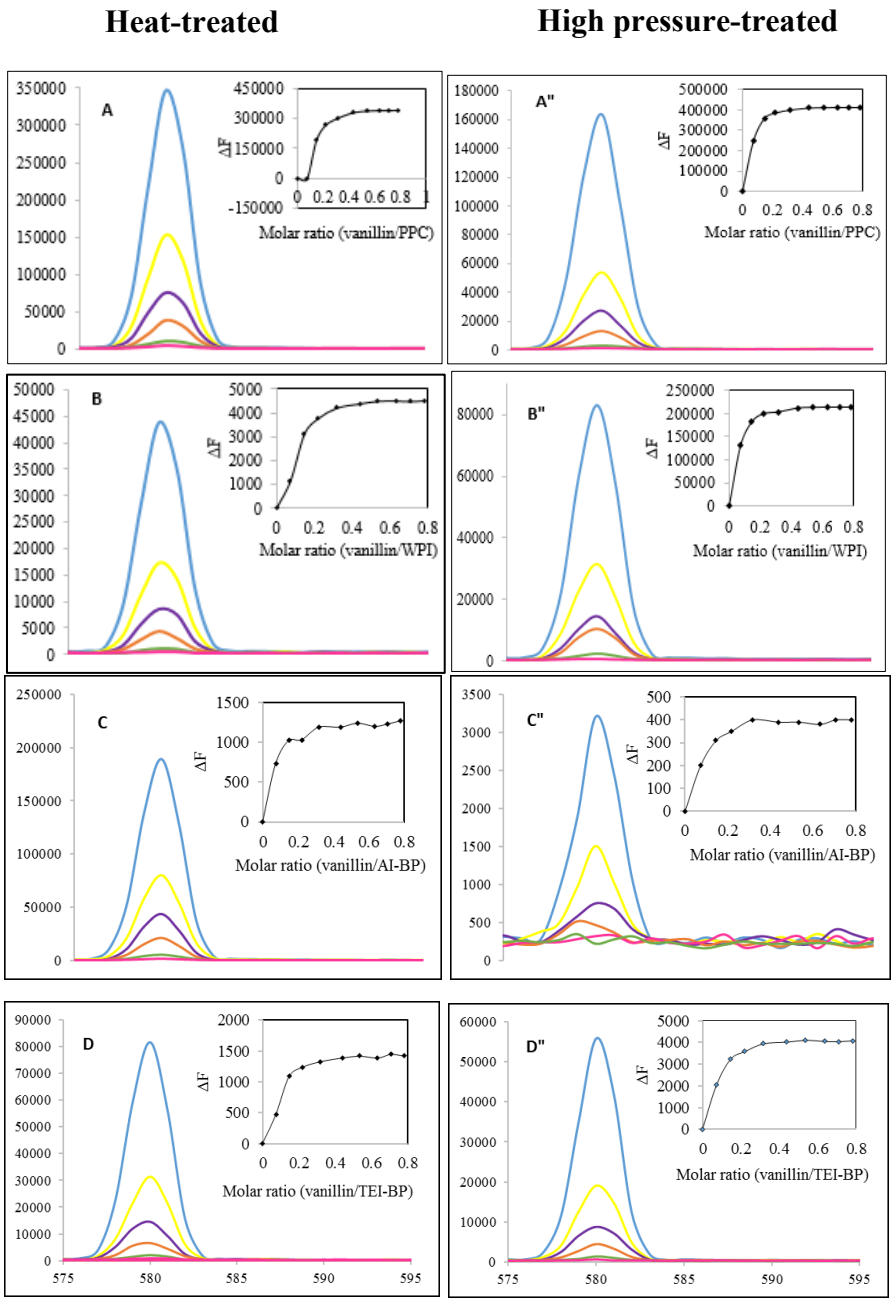


**Figure 3.5.** Effect of heat (left charts) and high pressure (right charts) treatments on the interaction between vanillin and protein aggregates of PPC (A), WPI (B), AI-BP concentrate (C), and TEI-BP concentrate (D), as estimated by fluorescence spectroscopy at excitation of 290 nm, upon addition (mM) of 3.0 (—), 6.0 (—), 9.0 (—), 13.0 (—), 18.0 (—), 22.0 (—), 26.0 (—), 29.0 (—), 32.0 (—). Insert charts depicting the difference in emission after vanillin addition (ΔF) as function of the vanillin to protein molar ratio are used to determine the saturation values of proteins.

the native state had the lowest value of 21,420 a.u (Figure 3.4B). Similarly, a change was seen in saturation point, with the native state reaching saturation first at 0.32 molar ratio (insert in Figure 3.4B), followed by 0.54 and 0.44 (inserts in Figure 3.5B) for heat-treated and high pressure-treated, respectively. Figures 3.4C and 3.5C illustrate the great variability in the spectra obtained for AI-BP concentrate depending on the treatments. The highest intensity of 217,160 a.u recorded for the native state (Figure 3.4C), was slightly decreased after heat treatment to reach 189,180 a.u (Figure 3.5C), while a dramatic decrease in the maximum intensity to reach 3210 a.u (Figure 3.5C") was induced by high pressure treatment. Accordingly, saturation was reached later and at the same molar ratio of 0.44 for the native (insert in Figure 3.4C) and heat-treated protein (insert in Figure 3.5C), while high pressure-treated AI-BP concentrate it was saturated earlier at 0.32 (insert in Figure 3.5C"). TEI-BP concentrate had lower intensity values compared to the other barley protein concentrate (AI-BP), with a peak intensity in the heat-denatured state of 81,680 a.u (Figure 3.5D) followed by a native state emission of 67,970 a.u (Figure 3.4D), and finally 55,880 a.u for the high pressure-treated protein (Figure 3.5D"). Saturation of the protein aggregates was seen at a molar ratio of 0.44 for the native and high pressure-treated proteins (inserts in Figures 3.4D and 3.5D"), while it was reached at 0.32 for heat-treated TEI-BP concentrate (insert in Figure 3.5D). Contrary to results of our study where no shift in peak wavelength was observed, (Hawe et al., 2008) noted a shift between the fluorescent dye 1-anilinonaphthalene-8-sulfonate (ANS) and β-lacamase protein indicating the exposure of hydrophobic binding sites on the protein, with a consequent stabilization of the molten globular state the protein. The decrease in emission of the protein aggregates following interaction with vanillin suggests an increase in polarity of the environment of the vanillin binding sites of the protein (Hawe et al., 2008). For AI-BP and TEI-BP concentrates, a decrease in the maximal fluorescence intensity following either heat-induced or high pressure-induced denaturation was seen. A possible explanation to this phenomenon is the steric hindrance of binding of the ligand upon protein aggregate formation (Hawe et al., 2008). Additionally, PPC and TEI-BP concentrate showed an increase in fluorescence intensity after denaturation treatments with heat or high pressure (Figure 3.5), which has been explained to be driven by the formation of large aggregates of denatured protein by these treatments (Hawe et al., 2008).

As is the case for vanillin, Nile Red dye (NRD) is weakly fluorescent in aqueous media, but fluorescence intensity increases when it binds to hydrophobic surfaces of proteins (Demeule et al., 2007). As such, protein aggregates are believed to contain more hydrophobic surfaces (Demeule

et al., 2007). Based on this property, protein aggregates are currently being applied for nanoparticle encapsulation to protect labile flavors, due to their capability to spontaneously assemble or to aggregate during processing (Livney, 2010; Ustunol, 2015).

The binding parameters of the protein aggregates were determined for each of the proteins studied and the results are summarized in Table 3.4. The proteins had similar number of binding sites for all states, although it was observed that the heat-treated PPC, and barley protein concentrates (AI-BP and TEI-BP) had a lower number of binding sites than the other states. As seen with the non-aggregates proteins, they all had high association constants, indicative of significant receptor-ligand complex interactions. The highest $K_a$ values were observed for the heat- and high pressure-treated PPC, and for the native and heat-treated AI-BP. The highest $K_d$ values were observed for the native and high pressure-treated TEI-BP concentrates (Table 3.4). In view of these data, it can also be suggested that the barley protein concentrates had the least binding interaction with vanillin, particularly true of TEI-BP concentrates, when considering its binding parameters altogether. These results differ when compared to the non-aggregated proteins (Table 3.3). In the native state of non-aggregated proteins (Table 3.3), the numbers of binding sites were lower than the proteins in an aggregated state (Table 3.4). Likewise, the association constants were lower for all samples when obtained through fluorescence spectroscopy, and the dissociation constants were greater. After heat-treatment, the number of binding sites decreased for all aggregated proteins, while the dissociation constant increased for PPC and WPI, and decreased for AI-BP and TEI-BP concentrates. The binding constant decreased for PPC, WPI, and TEI-BP concentrate, while it increased for the AI-BP concentrate.

Finally, upon high pressure treatment, a decrease in the number of binding sites was observed for the control proteins, while it increased for the barley protein concentrates when compared to the native state. Conversely, the dissociation constant increased for PPC, and decreased for WPI and both barley protein concentrates. As with the non-aggregated proteins, the differences in results may highlight the tryptophan availability within an aggregated conformation, with the protein/flavor affinity reflected by the binding parameters.

Overall, the protein aggregates behaved differently regarding their binding properties to vanillin as compared with the non-aggregated ones. No shift in maximum wavelength was observed after treatments for the aggregated proteins, suggesting that no conformational change occurred at the higher wavelength range.

**Table 3.4.** Binding Parameters of Number of Binding sites (*n*), Binding Constant ($K_a$), and Dissociation Constant ($K_d$) Obtained from Fluorescence Spectroscopy of Native, Heat-Treated and High Pressure-Treated Control (PPC[a] and WPI[b]) and Barley Protein Aggregates of AI-BP[c] and TEI-BP[d].

| Sample | State | Binding parameters | | |
|---|---|---|---|---|
| | | n | $K_a$ | $K_d$ |
| PPC | Native | 1.60E-17 | 7.99E+09 | 2.16E-16 |
| | Heat-treated | 6.07E-18 | 2.39E+10 | 7.21E-16 |
| | High pressure | 3.53E-17 | 1.21E+10 | 1.73E-16 |
| WPI | Native | 1.24E-16 | 2.30E+08 | 1.85E-16 |
| | Heat-treated | 9.06E-17 | 8.42E+08 | 1.64E-15 |
| | High pressure | 2.50E-18 | 3.43E+09 | 2.80E-16 |
| AI-BP | Native | 1.89E-16 | 2.05E+10 | 6.18E-16 |
| | Heat-treated | 8.25E-17 | 1.81E+10 | 3.11E-16 |
| | High pressure | 1.23E-16 | 8.63E+06 | 5.86E-16 |
| TEI-BP | Native | 4.28E-17 | 1.83E+09 | 1.45E-15 |
| | Heat-treated | 4.47E-18 | 8.19E+08 | 3.98E-16 |
| | High pressure | 1.56E-16 | 7.62E+08 | 1.35E-15 |

[a]PPC, pea protein concentrate. [b]WPI, whey protein isolate.

[c]AI-BP, alkaline extraction with IEP barley protein concentrate.

[d]TEI-BP tri-enzymatic extraction with IEP barley protein concentrate.

### 3.4.3. Effect of protein/flavor binding on the sensory properties

To assess the effect of protein-flavor interactions in the food product acceptance, the organoleptic properties were evaluated by sensory analysis. High protein cookies formulated with PPC and WPI and different concentrations of vanillin were used. The intensity of three main attributes (vanillin flavor, sweetness, and off-flavor) as well as their likeness were evaluated by 70 non-trained consumers. The intensity of the three attributes are shown in Figure 3.6. The 0.30 [vanillin]/[protein] (V/P) weight ratio (WR) PPC-enhanced cookie rated the highest for sweetness intensity, while vanilla flavor was most perceivable in cookies with a V/P WR of 0.59 (Figure 3.6A). Finally, cookies with 0.89 and 0.74 V/P WRs showed the highest off-flavor degree. The WPI-enriched cookies had higher overall ratings compared with those enriched with PPC (Figure 3.6B). Sweetness intensity was highest for the 0.59 V/P WR WPI-enriched cookie. The cookies with V/P WRs of 0.74 and 0.89 presented the most perceivable off-flavor, and cookies with 0.74 V/P WR had the highest intensity of vanilla flavor.

ANOVA statistical analysis provided the least significant difference (LSD) values for each sample, and the results are summarized in Table 3.5. Considering an F value >1 and $p < 0.5$, as well as the difference in mean scores between the values compared to the LSD value, samples in bold were determined to have been significantly different from each other. Results of Table 3.5 show that vanilla and off-flavor intensities were largely responsible for sample differences, and in one instance sweetness. A maximal vanilla intensity was perceived at 0.59 V/P WR PPC and 0.74 V/P WR WPI (Figure 3.6), after which it can be inferred that the panelists' palates were saturated. The consumer mean scores for vanilla intensity are consistent with those estimated analytically from the unbound vanillin concentration, and differed from those obtained using fluorescence spectroscopy. The maximal binding was reached at the highest vanillin concentration (0.89 V/P WR) for native PPC and WPI, as determined by measuring the unbound vanillin using the Klotz plots (data not shown). This contrasts with the binding parameters estimated from the aromatic amino acid quenching from fluorescence, where it was reached at 0.32 V/P MR for native PPC and WPI (Figure 3.2).

A correlation between the sensory characteristics mean scores and the liking rate are presented in Figure 3.7. Cookies formulated with PPC to enhance the protein content had a favorable liking rate for vanillin intensity, particularly those with 0.45 V/P WR, while the other cookie formulations

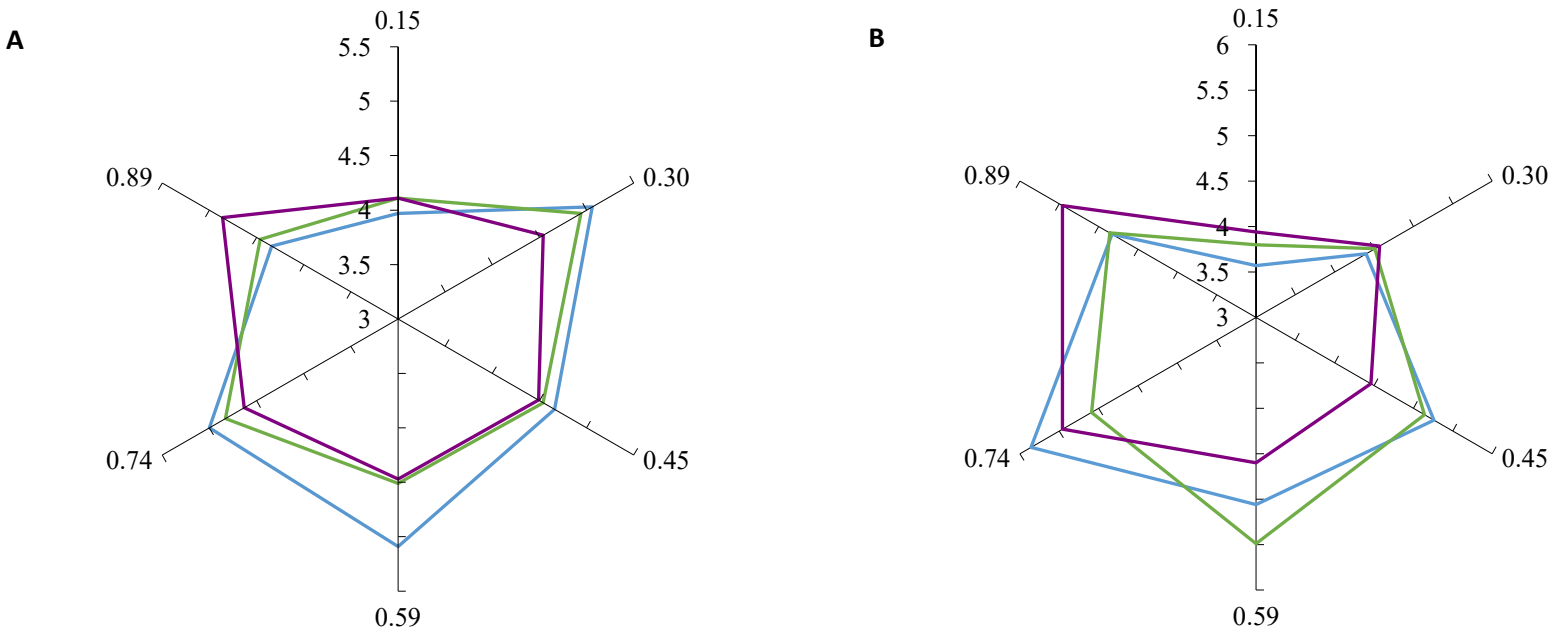


**Figure 3.6.** Mean scaling scores for sensory characteristics of vanillin intensity (—), sweetness intensity (—), and off-flavor intensity (—) for cookies formulated with PPC (A) and WPI (B), with varied [vanillin]/[protein] weight ratios (0.15; 0.30; 0.45; 0.59; 0.74; 0.89).

**Table 3.5.** Statistical interpretation of cookies formulated with pea protein concentrate (PPC), whey protein isolate (WPI), and varied vanilla concentrations.

| Protein source | V/P WR | Vanilla Intensity | | | Sweetness Intensity | | | Off-flavor Intensity | | | Liking Rate | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | F | *p* | LSD | F | *p* | LSD | F | *p* | LSD | F | *p* | LSD |
| **WPI** | 0.15-0.45 | **8.15** | **0.001** | 0.83 | 6.43 | 0.003 | 0.75 | 1.84 | 0.170 | 0.70 | 2.35 | 0.100 | 0.71 |
| | 0.59-0.89 | **5.41** | **0.007** | 0.66 | 1.60 | 0.210 | 0.71 | **5.20** | **0.008** | 0.61 | 3.10 | 0.052 | 0.56 |
| **PPC** | 0.15-0.45 | **4.48** | **0.015** | 0.73 | **3.63** | **0.032** | 0.62 | 1.10 | 0.340 | 0.63 | 1.20 | 0.310 | 0.68 |
| | 0.59-0.89 | **2.45** | 0.094 | 0.73 | 0.74 | 0.480 | 0.66 | 0.25 | 0.780 | 0.64 | 0.79 | 0.460 | 0.64 |

V/P WR, vanillin to protein weight ratio

Values in bold letter fonts are significantly different from each other ($p < 0.05$)

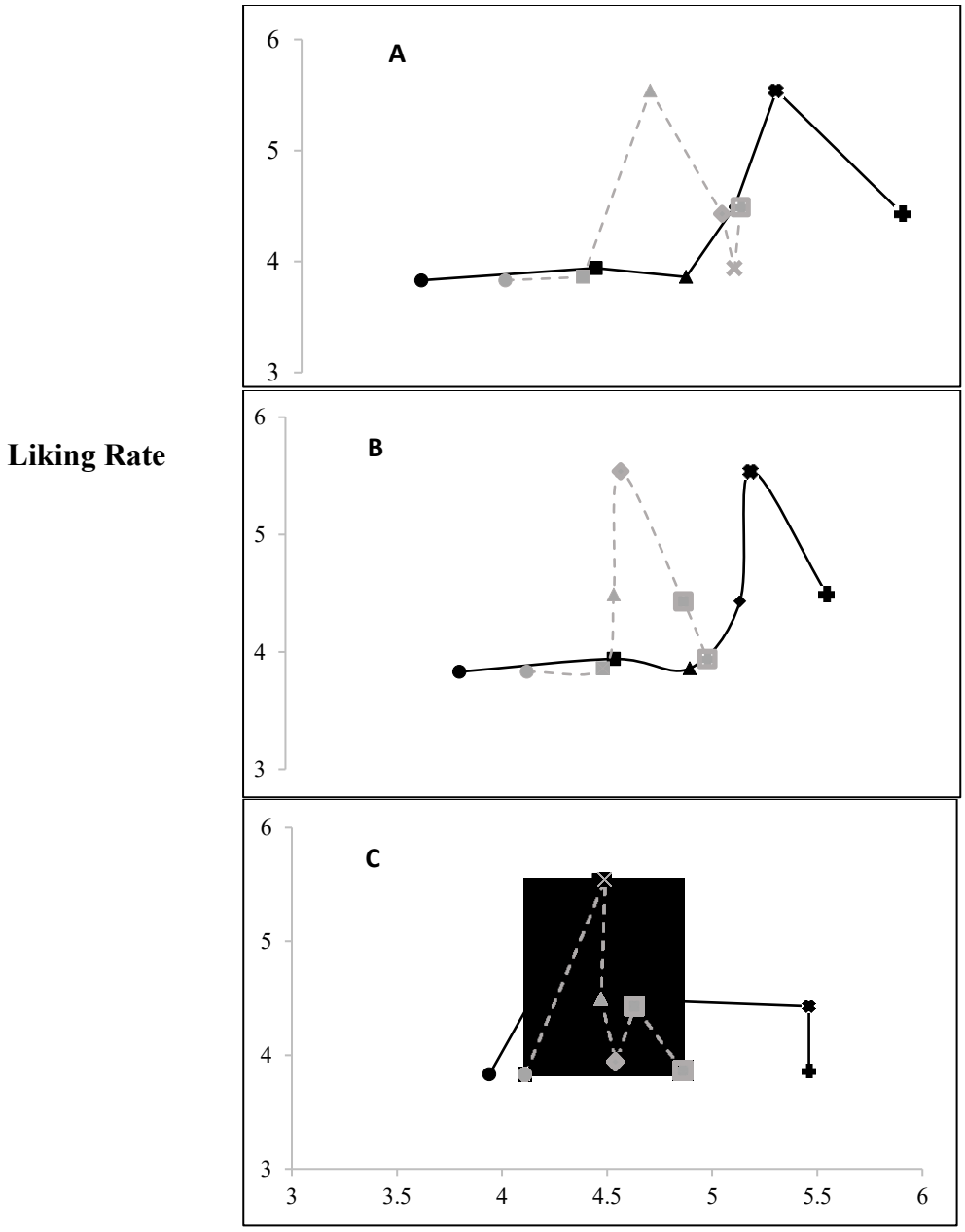


**Figure 3.7.** Liking rate as compared to the mean scores of the sensory characteristics of vanillin intensity (A), sweetness intensity (B), and off-flavor (C) for PPC-enhanced cookies (dashed line) and WPI-enhanced cookies (solid line) at [vanillin]/[protein] weight ratios of (0.15 ●; 0.30■; 0.45▲; 0.59● ; 0.74*; 0.89 ♦).

with lower and higher scores for vanillin intensity were given poorer liking rates (Figure 3.7A). For the WPI-enriched cookies, those with 0.74 V/P WR had the highest overall liking rating in terms of vanillin intensity, with the 0.89 V/P WR sample scoring the highest vanillin perception, but was liked less by the panelists (Figure 3.7A). Figure 3.7B represents the liking rate compared to the sweetness intensity. The PPC-enriched cookie that scored highest for perceived sweetness (0.89 V/P WR) had one of the lowest liking rates, compared to the 0.59 V/P WR cookie that panelists liked best. Similar results are seen with WPI-enriched cookies in Figure 3.7B as in 3.7A. Results correlate well with the findings obtained by fluorescence (Figure 3.2). As for the off-flavor intensity, the lowest overall liking rating was recorded for WPI-enriched cookies at 0.15, 0.45, and 0.74 V/P WR, and PPC-enriched cookies at 0.15, 0.59 and 0.74 V/P WR, both sets of ratings corresponding to the lowest, mid, and highest scaling score cookies (Figure 3.7C). Conversely, cookies with 0.15 V/P WR vanillin had the lowest overall liking rate for both protein adjuncts and for all three sensory attributes (Figures 3.7A, B, and C).

Overall, the degree of liking was similar between the two protein-enriched cookies, although the perception of vanilla flavor, sweetness and off-flavor was higher with in the cookies formulated with WPI. The cookies featuring PPC had the highest liking rate considering all characteristics together, despite the strong flavor of pea proteins, and for both proteins, a V/P WR of 0.74 on average had the most favorable scores for each characteristic. This contrasts with the findings of Tarrega et al. (2012) who recorded a decrease in chocolate flavor and sweetness, as well as an increase in off-flavor in a protein shake product enriched with pea protein ($\geq$25%; w/v), and to a greater degree than the same product formulated with whey protein.

### 3.5 Conclusion

Barley protein concentrates were prepared by two different techniques. The first using the conventional alkaline extraction combined with an isoelectric precipitation (IEP) yielding a barely protein concentrate with 68.9% protein content designated as AI-BP concentrate. The second is a newly developed technique in our laboratory consisting of a sequential enzymatic treatment using three different enzymes to remove starch and cell-wall glucans followed by IEP. With this technique barley protein concentrate of 41.4% protein content was obtained and designated tri-enzymatic and isoelectric barley protein (TEI-BP) concentrate. These barley protein concentrates and two commercial protein sources (WPI and PPC) were studied for their interactions with

vanillin at different concentrations. The proteins were studied either in their native and denatured (heat-treated or high pressure-treated) states. Vanillin-protein interactions were quantified by using analytical and sensory techniques. Binding parameters (number of binding sites, equilibrium constant, and dissociation constant) were generated from Klotz plots after determination of the free vanillin by UV/Vis spectroscopy. The results showed that high-pressure treatment had the greatest effect on AI-BP concentrate, resulting in the highest $K_d$ (54.66 M), followed by native WPI and native PPC, while higher degree of interaction was observed with TEI-BP concentrate (Table 1). The binding study using fluorescence spectroscopy showed a trend of direct proportionality relationship between tryptophan quenching and concentrations of vanillin for all the tested protein samples. Binding parameters were also determined, and contrary to the former analytical method, the proteins demonstrating the least interaction were the heat-treated PPC and heat-treated concentrate and the high pressure-treated AI-BP concentrate. This study also showed the effect of incorporating PPC and WPI into a high-protein cookie, formulated with varying vanillin concentrations ([vanillin]/[protein] molar ratios between 0.15-0.89). Volunteer students of McGill University at undergraduate and graduate levels (70 in number) evaluated the vanilla flavor, sweetness, and off-flavor intensities, as well as overall liking of the tested cookies, using a 9-point hedonic scale. The degree of liking was similar for the two protein-enriched cookies, although the perception of vanilla flavor, sweetness, and off-flavor was higher for the WPI-enriched cookies. These cookies with 0.45 V/P WR had the highest overall liking rating. Those enriched with PPC had a favorable liking rating, particularly those with 0.30 V/P WR. The sensory evaluation study, indicated that before the highest vanillin concentration was added, consumers' palettes reached saturation, and these results were comparable to saturation points observed by fluorescence spectroscopy tests. However, with the Klotz plots, saturation was reached at the highest vanillin concentration.

A better understanding of the structural features of barley proteins and characterization of their interactions with vanillin in food remains to be tested, as its usage in the food industry as a functional and health-promoting ingredient is anticipated to increase in the future.

# CHAPTER IV

# GENERAL SUMMARY AND CONCLUSION

Isolation of proteins from defatted barley (*Hordeum vulgare L.*) flour was investigated using various extraction methods, which greatly influenced protein content, techno-functionality, and structural characteristics. The concentrate obtained by the conventional alkaline extraction (BAE) had 33.0% protein content and 57.1% protein recovery yield; however, when this technique of extraction was followed by an isoelectric precipitation (IEP) step, recovered yield of the concentrate (AI-BP) decreased to 51.4%, but the protein content increased to 68.9%.

Two alternative enzymatic approaches were explored as mild processes to obtain minimally degraded proteins. To this end, a bi-enzymatic approach using α-amylase from *Bacillus* sp. and amyloglucosidase from *Aspergillus niger* was adopted to isolate protein by removing surrounding starches. This method produced an extract (BB-BP) with 25.7% protein and protein recovery yield of 49.1%. Finally, a tri-enzymatic approach including the addition of β-1,3,4-glucanase from *Trichoderma longibrachiatum*, to degrade the β-glucan present in barley flour (TE-BP), in addition to the former enzymes was used. The latter method followed by an IEP step to produce the TEI-BP concentrate was also investigated, and the protein contents of 71.6% and 78.3%, and protein recovery yields of 37.0% and 41.4%, respectively were obtained. The results showed clearly that the addition of the IEP step improved the purification factor for all concentrates, with BAEI concentrate having the highest, followed by TEI-BP concentrate.

The effect of heat denaturation and high pressure treatment on the structural characterization of each of the protein concentrates (AI-BP and TEI-BP) was also investigated, and compared to two control proteins of pea protein concentrate (PPC) and whey protein isolate (WPI). The barley protein concentrates in both their native and denatured states consisted of different proportions of secondary and tertiary structures, as revealed by Fourier transform infrared spectroscopy (FTIR) and fluorescence spectroscopy analysis, respectively. In terms of functionality, the two barley concentrates had comparable emulsifying properties to those of WPI; AI-BP concentrate had enhanced foaming capacity, while TEI-BP concentrate had superior foam stability.

The two barley protein concentrates (AI-BP and TEI-BP) as well as control proteins (WPI and PPC) in their native and denatured states were also incubated with different vanillin concentrations to quantify the extent of interaction with this favor compound, using analytical and sensory

techniques. Binding parameters (number of binding sites, equilibrium constant, and dissociation constant) were obtained from a Klotz plot after determination of the free vanillin by UV/Vis spectroscopy. The high-pressure treatment had the greatest effect on AI-BP concentrate, resulting in the highest $K_d$ (54.66 M), followed by native WPI and native PPC, while a higher degree of interaction was observed with TEI-BP concentrate. The binding study using fluorescence spectroscopy showed a trend of direct proportionality relationship between tryptophan quenching and concentrations of vanillin for all the tested protein samples. Binding parameters were also determined, and contrary to the first analytical method, the proteins demonstrating the least interaction were the heat-treated PPC and heat-treated WPI, followed by native TEI-BP concentrate and the high pressure-treated AI-BP concentrate.

This study also showed the effect of incorporating PPC and WPI into a high-protein cookie, formulated with varying vanillin concentrations ([vanillin]/[protein] molar ratios between 0.15-0.89). A number of 70 McGill University undergraduate and graduate volunteer students evaluated the vanillin, sweetness and off-flavor intensities, as well as overall liking of the resulting cookies, using a 9-point hedonic scale. The degree of liking was similar for the two protein-enriched cookies, although the perception of vanilla, sweetness and off-flavor was higher for the WPI-enriched cookies. The latter cookies with 0.45 MR had the highest overall liking rating. Those enriched with PPC had a favorable liking rating, particularly those with 0.30 MR. During the sensory evaluation study, indicated that before the highest vanillin concentration was added, consumers' palettes reached saturation, and these results were comparable to saturation points observed by fluorescence spectroscopy tests, while with the Klotz plot saturation was reached at the highest vanillin concentration.

The understanding of the structural characterization, functional properties and interaction of barley proteins with vanillin is expected to increase its usage in the food industry as a functional and health-promoting ingredient.

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