EFFECT OF HIGH HYDROSTATIC PRESSURE (HHP) ON THE FUNCTIONAL PROPERTIES OF PEA PROTEIN ISOLATE (PPI)

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ABSTRACT

EFFECT OF HIGH HYDROSTATIC PRESSURE (HHP) ON THE FUNCTIONAL PROPERTIES OF PEA PROTEIN ISOLATE

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Pea proteins as plant-based proteins have attracted much interest and are preferred in the food industry due to their low allergenicity, high availability, high nutritional value, and low price in recent years. Thus, they are considered a good replacer for commonly used animal-based protein ingredients; however, better functional properties are required for the industry. The aim of this work was to investigate the influences of high hydrostatic pressure (HHP) on the functional properties of pea protein isolate (PPI). For this purpose, PPI was exposed to a pressure range of 300, 400 and 500 MPa at 25 and 50°C for 5 min. The concentration of samples was kept constant at 45% (w/v) PPI in solutions at desired pH. This process was carried out at different pH levels 3, 5 and 7 to understand the impacts of change in pH with HHP on the pea protein functionalities. WHC (water holding capacity), solubility by Lowry method, emulsion activity, viscosity, change in secondary structure analysis by Fourier transform infrared (FTIR) spectroscopy and hydration behavior by NMR Relaxometry experiments were performed on pressurized and control samples. This study showed that HHP treatments enhanced the solubility of pea protein isolate (PPI) approximately 60% at neutral pH compared to other pH values and control ($p<0.05$). WHC of PPI

significantly was reduced by HHP treatment at pH 3 compared to control and other pHs ($p<0.05$) and 500 MPa-50°C-pH 5 processing condition improved WHC of PPI significantly ($p<0.05$). Moreover, FTIR results showed that pressure treatments caused changes in the secondary structure of native PPI due to irreversible unfolding. In addition, significantly lower hydrated PPI at pH 3 was found under HHP treatment, especially at 300 MPa, compared to the control ($p<0.05$), and other processing factors affected hydration behavior of PPI insignificantly ($p>0.05$). Also, at pH 3, HHP treatments resulted in higher emulsion capability compared to control samples. Furthermore, HHP treatments caused a reduction in viscosity of PPI for all processing conditions ($p<0.05$). Therefore, HHP could be an excellent alternative to improve the functional properties of pea protein through conformational, structural, and surface change modifications, and this study can help develop new products, including pea proteins.

Keywords: PPI, HHP, functional properties, FTIR, NMR

ÖZ

YÜKSEK HİDROSTATİK BASINCIN (YHB) BEZELYE PROTEİN İZOLATININ FONKSİYONEL ÖZELLİKLERİ ÜZERİNE ETKİSİ

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Bitkisel kaynaklı bir protein olan bezelye proteinleri, son yıllarda düşük alerjenlik, yüksek bulunabilirlik, besin değeri ve düşük fiyatı nedeniyle gıda endüstrisinde büyük ilgi görmekte ve tercih edilmektedir. Bu nedenle, yaygın olarak kullanılan hayvansal bazlı protein bileşenleri yerine kullanılabilecek şeçenek olarak düşünülmüştür, ancak endüstri için daha iyi fonksiyonel özelliklere sahip olması gerekmektedir. Bu çalışmada, yüksek hidrostatik basıncın (YHB) bezelye protein izolatının fonksiyonel özelliklerine etkilerini görmek amaçlanmıştır. Bu amaçla, bezelye proteini 5 dakika süresince 25 ve 50°C sıcaklıklarda, 300, 400 ve 500 MPa basınç seviyelerine maruz bırakılmıştır ve hazırlanan örneklerin konsantrasyonu %45 olarak sabit tutulmuştur. Bu işlem, YHB ile birlikte pH değerlerindeki değişimin bezelye proteinin fonksiyonu üzerindeki etkilerini de anlamak için 3, 5 ve 7 pH seviyelerinde gerçekleştirilmiştir. Kontrol ve basınçlanan bezelye protein izolatı için; su tutma kapasitesi, Lowry yöntemi ile çözünürlük, emülsiyon aktivitesi, Vibro viskozimetre ile viskozite, Fourier Dönüşümlü Kızılötesi (FTIR) spektroskopisi ile ikincil yapıdaki değişim ve NMR Relaxometri deneyleri ve hidrasyon analizleri yapılmıştır. Bu çalışma, nötr pH'de yapılan basınç işleminin, diğer pH değerleri ve kontrol örneklerine göre bezelye protein izolatının çözünürlüğünü yaklaşık %60 oranında arttırdığını göstermiştir ($p<0.05$). Ayrıca, kontrol ve diğer pH'lere kıyasla, pH 3'te HHP işlemiyle bezelye protein izolatının su tutma kapasitesinin önemli ölçüde azaldığı görülmüştür ($p<0.05$)

ve 500 MPa-50°C-pH5 işleme koşulu, su turma kapasitesini önemli ölçüde iyileştirmiştir ($p<0.05$). FTIR verilerine göre, YHB yapıdaki geri alınamaz açılma nedeniyle proteinin ikincil yapısında değişikliğe neden olmuştur. Ek olarak, pH 3'te yapılan basınçlama, özellikle 300 MPa'da, kontrol örnekerline göre proteinin hidrasyon özelliğinin önemli derecede azalmasına neden olmuş ($p<0.05$) ve diğer proses faktörlerinin hidrasyon üzerinde çok etkili olmadığı görülmüştür ($p>0.05$). Ek olarak, pH 3'te, YHB işlemi kontrole kıyasla daha yüksek emülsiyon kapasitesine sahip protein izolatı elde edilmesini sağlamıştır. Ayrıca, YHB, tüm işleme koşulları için proteinin viskozitesinde önemli derecede azalmaya sebep olmuştur ($p<0.05$). Bu nedenle, YHB işlemi, konformasyonel, yapısal ve yüzey değişikliği modifikasyonları yoluyla bezelye proteininin fonksiyonel özelliklerini geliştirmek için mükemmel bir alternatif olabilir ve bu çalışma, bezelye protein içerikli yeni ürünlerin geliştirilmesine yardımcı olabilir.

Anahtar Kelimeler: Bezelye protein izolatı, YHB, fonksiyonel özellikler, FTIR, NMR

With love to my family…

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TABLE OF CONTENTS

ABSTRACT v

ÖZ vii

ACKNOWLEDGEMENTS x

TABLE OF CONTENTS xii

LIST OF TABLES xv

LIST OF FIGURES xvi

LIST OF ABBREVIATIONS xviii

CHAPTERS

1. INTRODUCTION 1

1.1. General Aspects of Food Proteins 1

1.1.1. Physicochemical Properties of Food Proteins 1

1.1.1.2. Influences of Extrinsic Factors on Functional Properties of Food Proteins 5

1.1.2. Sources of Plant Proteins 8

1.1.3. Industrial Applications of Plant Proteins 9

1.2. Pea Protein 10

1.2.1. Functional Properties 11

1.2.1.1. Water Holding Capacity (WHC) 12

1.2.1.2. Solubility 13

1.2.1.3. Emulsification Ability 14

1.2.1.4. Viscosity 15

1.3. High Hydrostatic Pressure (HHP) 16

1.3.1. General Information 16

1.3.2. Effects of HHP on Plant Proteins 17
1.3.3. Characterization of HHP Treated Pea Protein 20
1.3.3.1. Lowry Method.. 20
1.3.3.2. Fourier Transform Infrared (FTIR) Spectroscopy 20
1.3.3.3. Nuclear Magnetic Resonance (NMR) Relaxometry 21
Objectives 23
2. MATERIALS AND METHODS 25
2.1. Materials 25
2.2. Methods 26
2.2.1. Preparation of Samples for High Hydrostatic Pressure (HHP) Treatment 26
2.2.2. High Hydrostatic Pressure (HHP) Treatment 26
2.2.3. Characterization of Pressure Treated Pea Proteins 27
2.2.3.1. Determination of Protein Content of Pea Protein Isolate by Kjeldahl Method 27
2.2.3.2. Determination of Water Holding Capacity (WHC) 29
2.2.3.3. Determination of Solubility 29
2.2.3.4. Emulsion Activity (%) 31
2.2.3.5. Viscosity Measurements 31
2.2.3.6. Fourier Transform Infrared (FTIR) Spectroscopy Analysis 31
2.2.3.7. Hydration Behavior of Pea Protein by Nuclear Magnetic Resonance (NMR) Relaxometry 32
2.2.3.8. Statistical Analysis 32
3. RESULTS AND DISCUSSION 33
3.1. Water Holding Capacity (WHC) 33
3.2. Solubility 35
3.3. Emulsion Activity % 38

3.4. Viscosity 41
3.5. Fourier Transform Infrared (FTIR) Spectroscopy Analysis 43
3.6. Hydration Behavior of Pea Protein Isolate (PPI) by NMR relaxometry 49
CONCLUSIONS & RECOMMENDATIONS 51
REFERENCES 53
APPENDICES
A. Calibration Curve 75
B. Statistical Analyses 76

LIST OF TABLES

TABLES

Table 1.1. Bond-dissociation energy of protein-protein interactions & covalent Bond (at 0 °C) 4

Table 1.2. Functional properties of food proteins in some applications 6

Table 1.3. Various sources of food proteins 9

Table 1.4. Nine characteristic of infrared bands of peptide linkage 21

Table 2.1 The reagents for Lowry Method 30

Table 3.1. Viscosity (cP) values of HHP-treated pea protein isolate (PPI) at different pH values (3, 5 and 7) 41

Table B.1. ANOVA and Tukey's Comparison Test with 95% confidence level for determining solubility by Lowry Method 76

Table B.2. ANOVA and Tukey's Comparison Test with 95% confidence level for determining Water Holding Capacity (WHC) 79

Table B.3. ANOVA and Tukey's Comparison Test with 95% confidence level for determining Emulsion Activity 83

Table B.4. ANOVA and Tukey's Comparison Test with 95% confidence level for determining Viscosity 87

Table B.5. ANOVA and Tukey's Comparison Test with 95% confidence level for determining T_2 relaxation times by NMR Relaxometry 91

LIST OF FIGURES

FIGURES

Figure 1.1. Solubility (%) profile of untreated (25°C) and heat-treated (50–100°C) pea protein isolate at pH 3–9 14

Figure 1.2. SEM micrograph of untreated, HHP-treated and heat-treated pea protein concentrate solutions (24 g/100 g) 18

Figure 2.1. HHP equipment 27

Figure 3.1. Water holding capacity (g/g) of both control and HHP-treated pea protein isolate (PPI) at different pHs (3, 5 and 7) 33

Figure 3.3. Emulsion activity (%) of both control and HHP-treated pea protein isolate (PPI) at different pH values (3, 5 and 7) 39

Figure 3.4. FTIR graph of both control and HHP-treated pea protein isolate (PPI) at pH 3 44

Figure 3.5. Peak position and relative area of both control and HHP-treated pea protein isolate (PPI) at pH 3 44

Figure 3.6. FTIR graph of both control and HHP-treated pea protein isolate (PPI) at pH 5 46

Figure 3.7. Peak position and relative area of both control and HHP-treated pea protein isolate (PPI) at pH 5 46

Figure 3.8. FTIR graph of both control and HHP-treated pea protein isolate (PPI) at pH 7 47

Figure 3.9. Peak position and relative area of both control and HHP-treated pea protein isolate (PPI) at pH 7 48

Figure 3.10. T_2 (ms) of both control and HHP-treated pea protein isolate (PPI) at different pH values (3, 5 and 7) 49

Figure A.1. Example of a calibration curve for Lowry Method prepared by Bovine Serum Albumin for determining total soluble protein contents in both HHP-treated and control PPI samples 75

LIST OF ABBREVIATIONS

ABBREVIATIONS

HHP: High hydrostatic pressure

PPI: Pea protein isolate

WHC: Water holding capacity

FTIR: Fourier transform infrared

(O/W): oil-in-water

(W/O): water-in-oil

PL: Pulsed Light

PEF: Pulsed Electric Fields

NMR: Nuclear magnetic resonance

CPMG: Carr-Purcell-Meiboom-Gill

ANOVA: Analysis of Variance

EAI: Emulsion activity index

ESI: Emulsion stability index

EC: Emulsion capacity

BSA: Bovine serum albumin

CHAPTER I

INTRODUCTION

1.1. General Aspects of Food Proteins

1.1.1. Physicochemical Properties of Food Proteins

Food proteins bring into existence of a complex and diverse collection of biological macromolecules. Amino acids, which are building blocks of proteins, form food proteins. Generally, about 20 different amino acids are boded by peptide bonds, and they are arranged systematically. They create millions of diverse combinations with many folds or conformations, resulting in the unique structure of each protein and its function (Shang et al., 2018). For all amino acid residues, the main-chain components are identical. The carboxyl (-COOH) and amino group (-NH2) are covalently bonded to (α)-carbon that also bonds the amino acid side chain group, which is specific to each amino acid (Dietzen, 2018).

Side chain group is appropriately classified as aliphatic, acidic, basic, polar uncharged, and sulphur containing ones. Because of their specific conformational environment, the chemical properties of the amino acid side chain in protein structure may differ significantly from those of isolated protein. Hence, the occurrence of the disposition causes a change in structural, functional, and physicochemical properties of food protein (Damodaran & Paraf, 2017; Phillips et al., 1994).

The amino acid sequence designates the 3D protein structure. Various non-bonded, short, medium and long-range interactions act as a part of protein folding to a thermodynamically stable structure. All-α, all-β, α/β or α + β are classifications of protein structure and NMR and cryo-electron microscopy are some techniques used for determination of protein structure. In addition, a correctly folded protein structure reveals the biological function and also misfolding is related to different disorders.

Alteration of protein functions and interactions with other binding partners occur by means of the flexibility of protein structure (Guruprasad, 2019).

Functional behaviors of food proteins rely on their structures. Four different levels for a well-defined three-dimensional structure of proteins are primary, secondary, tertiary, and quaternary (Haque et al., 2016; Littlechild, 2013). The primary structure of a protein denotes the extended polypeptide chain. The chain length and amino acid sequence of polypeptides designate its three-dimensional structure in solution. The local spatial arrangements of a polypeptide chain as secondary structures are α- helix, and β- sheet determined by the primary structure. The occurrence of different amino acids and selection of the peptide bonds as primary linkage seem to be the intentional design by nature to control the flexibility or rigidity for polypeptides to perform lots of biological functions (Damodaran & Paraf, 2017).

With the presence of hydrogen bondings between amino and carbonyl functions of the polypeptide chain, the planarity of the peptide bond specifies a local arrangement of polypeptide backbone that is referred to by secondary structure (Sikorski, 2006). It consists of α- helices, β-pleated sheets, and random coils. α-helices can not be formed by some amino acids due to electrostatic repulsion or bulky side chains. Thus, a random-coil structure is assumed to have a minimum electrostatic free energy by the polypeptide (Onwulata & Qi, 2006a).

The parallel β-pleated sheet and the antiparallel β-pleated sheet are two sterically favorable arrangements. In fibrous protein, which has polypeptide chains organized by long strands, does not include the parallel β-pleated sheet owing to the considerable strain that is influenced by the direction of hydrogen bonds; however, in globular proteins, which has polypeptide chains folded into a spherical or globular shape, mixed β- sheets are commonly found (Phillips et al., 1994; Sikorski, 2006).

The percentages of these types of structures vary among globular proteins (Sikorski, 2006). Intramolecular and solvent interactions are minimal in the folding form of protein and the folding of the polypeptide chain of secondary structure into a compact 3D (three-dimensional) shape is referred to as tertiary structure. Disulfide bonds between cysteine residues, intramolecular hydrogen bonds, van der Waals forces, and hydrophobic interactions provide stabilization of this 3D shape (Dietzen, 2018). Proteins including more than one polypeptide chain exhibit another level of organization, which refers to the quaternary structure where the aggregation of separate polypeptide chains is observed at that structure. Hydrogen bonds and van der Waals forces play a role in the stabilization of this level. When structural changes occur, this may result in denaturation of the organized structure, which forces changes in the desired functional behavior of food proteins (Onwulata & Qi, 2006a).

By the minimization of connection with water molecules and the several apolar groups, proteins achieve a characteristic 3D structure in a physiological environment that enables the formation of a hydrophobic nucleus in the protein interior, whereas, on the surface, hydrophilic groups form intramolecular hydrogen bonds, van der Waals interactions and also hydrogen bonds with solvent molecules (Phillips et al., 1994). Protein interactions are governed by molecular forces, which are covalent bonds, ionic interactions, hydrogen bonding, hydrophobic interactions that specify the relation of the individual proteins'structure to their functionalities and also the interactions of a protein with other molecules (Haque et al., 2016; Onwulata & Qi, 2006).

It is clearly known that different attractive and repulsive forces, caused by various intramolecular forces and the interactions of the different groups with the surrounding solvent water generate protein native structure that is the most stable thermodynamically and in the lowest energy state (Table 1.1) (Ustunol, 2014a). Also, the results from various studies about the thermodynamics of protein denaturation confirmed that the van der Waals interactions of polar groups and hydrogen bonds provide a considerable contribution to the enthalpy change for protein folding (Pace et al., 2004). Hence, denaturation causes changes in the secondary and higher-order

structural configuration of proteins to comprise the interruption of these non-covalent forces causes unfolding (Onwulata & Qi, 2006; Haque et al., 2016). However, there is no change in protein primary structure because of the change in the original environment of the native protein (Ustunol, 2014a).

Table 1.1. Bond-dissociation energy of protein-protein interactions & covalent bond (at 0 °C) (Cheftel et al., 1992)

	Bonding Type	**Bond-dissociation Energy [kJ/mol] (per bond)**
Non-covalent interaction	Wan-der Waals repulsion	0.5-5
	Hydrophobic interactions	4-12
	Hydrogen bonds	8-40
	Electrostatic interactions	40-80
Covalent Interaction	Disulfide bonds	268
	Peptide bonds	305

Enzymatic reactions, pH, ionic strength changes, and heating or high-pressure treatments can be reasons for changing protein structure and further denaturation (Kristo & Corredig, 2014; Haque et al., 2016). Basically, first extension of protein occurs and chain-to-chain interaction may arise through interactions of hydrogen, ionic, hydrophobic, and covalent bondings (Martins et al., 2018). For instance, under an extreme pH environment, having the proteins' higher net negative or positive charge density and strong intramolecular electrostatic repulsions cause the swelling and unfolding of the protein (Ustunol, 2014b). Thus, denaturation of proteins may be desirable when it improves the functional properties of food proteins, depending on the extent of denaturation and modification. For this reason, it should be controlled and mechanisms of processes need to be understood.

1.1.1.2. Influences of Extrinsic Factors on Functional Properties of Food Proteins

Protein functional properties can be described briefly throughout storage, cooking and consumption. Some physicochemical properties influence protein behavior in food systems. Examples of such functional properties comprise solubility, water and fat holding capacity, emulsifying capabilities and foaming (Kinsella, 1976; Boye et al., 2010; Chang et al., 2015) (Table 1.2).

Functional properties of proteins show complicated interactions between structure, conformation, composition and physicochemical properties by the relation of other food components and the environment (Kinsella, 1976; Mirmoghtadaie et al., 2016. The main characteristics are molecular weight, size, shape, amino acid composition, structure, charge distribution, flexibility, and hydrophobicity important intrinsic factors that could affect proteins' functionality (Fernández-Quintela et al., 1997; González-Pérez & Arellano, 2009). Besides these intrinsic factors, the source of protein, composition, the preparation method, thermal history and the processing conditions, like temperature, pressure, pH, ionic strength, other ingredients, are critical for the variation in functionality (Lu et al., 2020).

Table 1.2. Functional properties of food proteins in some applications (Barać et al., 2015)

Techno-functional Property	Mode of action	Food system
Solubility	Protein Solvation	Beverages
Water absorption	Hydrogen bonding of water	Meats, sausages, breads, cakes
Viscosity	Thickening; water binding	Soups, gravies
Gelation	Protein matrix formation and setting	Meats, curds, cheese
Cohesion-Adhesion	Protein act as adhesive material	Meats, sausages, pasta
Elasticity	Hydrophobic binding in gluten; Disulfide links in gels	Meats, bakery
Emulsification	Formation and stabilization of fat emulsions	Sausages, soups, cakes
Fat absorption	Binding of free fat	Meats, sausages, doughnuts
Flavor-binding	Adsorption; Entrapment; Release	Bakery
Foaming	Form stable film to entrap gas	Whipped toppings, chiffon desserts

It was seen in some studies that applied temperature changes the functional properties of food proteins, significantly affecting the protein structure in the processes. Heat treatment above the denaturation temperature usually induces partial unfolding and subsequent aggregation of protein (Peng et al., 2016).

For solubility, for example, it was observed that owing to the coagulation of the egg white protein, minimum solubility was seen at 60°C (Gomes & Pelegrine, 2012). In addition, soy protein is known to be more heat stable than animal proteins. The study on soy protein products showed that for 25, 50, and 75°C processing, small solubility changes were observed for most of the isolates, and they maintained solubilities at 75°C however, protein solubility increased at 50°C compared to 25°C for some of the isolates (Lee et al., 2003). Another example showed that, in general, after ultrasound treatment, increasing temperatures between 40 and 50°C enhanced the solubilities (Jambrak et al., 2009). In addition, the study about the effects of increasing

temperature (from 20 to 80°C) on whey protein dispersions' viscosity with temperature was attributed to the aggregation of whey protein molecules (Benoit et al., 2013).

Also, changing of protein conformation influences the water holding capacity due to various types of processing especially heating. For instance, for sausage batters, myosin and actomyosin interactions were affected by the temperature that they decreased the availability of polar groups because of binding between protein molecules, which resulted in less water holding capacity of sausage batters (Zayas, 1997).

Changing pH is a vital factor since it strongly affects the functional properties of food proteins. The net charge on the protein is zero at the isoelectric point that differs according to the source of the proteins, so important variations in the functionality of proteins are seen at different pH values (Kinsella, 1976; Haque et al., 2016; Soderberg, 2013).

Among protein functions, solubility is sensitive to the change of pH, so it should be under control to be applicable in food systems. Therefore, increasing solubility is expected as the pH of the samples moves away from the isoelectric point of the protein and better functional properties could be observed (Wolf, 1970; Juandoo, 1980; Soderberg, 2013). For instance, the study on the behavior of whey protein dispersions at different pH values showed that at acidic pH, due to greater net positive charge on protein molecules, higher degree of hydration of proteins resulted in a greater affinity for water molecules. Also, at pH 6, the lowest viscosity comes from repulsive forces predominated at lower shear rates and lower interactions between protein aggregates (Carr et al., 2003; Dissanayake et al., 2013). In addition, most of the food proteins show poor emulsifying at pH around the isoelectric point because of the low solubility and charge neutralization (Ustunol, 2014a).

High Hydrostatic Pressure (HHP) is another factor that may modify food components functionality by disrupting hydrophobic and electrostatic interactions and thus, the new bonds forming can occur that resulting in aggregation and precipitation (Qin et

al., 2013; Mirmoghtadaie et al., 2016). For instance, studies on walnut and sweet potato proteins with various HHP treatments indicated that the unfolding degree of proteins was triggered by a disordered structure resulting from rising pressure, especially above 350 MPa, which caused important results for their techno-functional properties (Queirós et al., 2018). Another study showed that exposure pressures 200 and 400 MPa on red kidney bean (Phaseolus vulgaris L.) protein isolate resulted in a significant increase in surface hydrophobicity and at 400 MPa or higher pressures enhanced protein solubility of red kidney bean protein isolate (Yin et al., 2008). Also, Yang, Li, Zhu and Zhang (2009), who investigated functional properties of egg white treated by HHP range of 0-600 MPa and time range of 0-20 min showed an increase in the viscosity and the surface tension.

A variation in functionality of food proteins can arise from intrinsic factors, different environmental factors and exposured conditions. In order to reach enhanced properties of food proteins, manipulation of protein structure through processing, the interaction of the protein with other components in foods, and alteration of environmental conditions are some applicable ways.

1.1.2. Sources of Plant Proteins

The population continues to grow and the sustainability of food production becomes one of the most relevant issues. It is clear that the overwhelming majority of food raw materials and proteins have an important part of the human body and growing population (Lasztity & Abonyi, 2009). Food proteins are obtained from various sources that are mostly derived from animal or plant origins (Day, 2015; Martins et al., 2018; Shang et al., 2018; Loveday, 2019).

Presently, consumption of food proteins from animals is ~ 35 % and plants is ~ 65% (Haque et al., 2016). This trend is expected since animal proteins are expensive with regard to market price, the requirement of land and environmental effects. Also, plant proteins are versatile, having better health-oriented composition and economic option compared to animal proteins (González-Pérez & Arellano, 2009; Day, 2015; Saldanha

Do Carmo et al., 2016; Balandrán-Quintana et al., 2019). For these reasons, there is a growing interest in the application of plant proteins in terms of those from legumes, like pea, chickpea, soybeans for food formulations (Table 1.3).

Table 1.3. Various sources of food proteins (Haque et al., 2016)

Source	Protein
Animal	Muscle proteins (myofibrillar, sarcoplasmic, stroma proteins)
	Blood proteins (hemoglobin, plasma protein)
	Connective tissue proteins (collagen, plastin)
	Milk proteins (whey, casein, lactoferrin)
	Egg proteins (egg albumin, yolks)
Botanical	Cereal proteins (gluten, zein, barley, oats, rice proteins)
	Legume and pulse proteins (protein isolates from peas, soybeans, lupines, lentils)
	Oil seeds proteins (protein isolates from rapeseed, canola, cottonseed, peanut, chiaseed, flaxseed)
	Tuber proteins (potato protein)
Microbial	Fungal proteins (mycoproteins)

Plant proteins are broadly categorized in terms of their solubility, prosthetic group, shape regulatory properties, and biological activities. Firstly, they were classified according to solubility that are albumins, globulins, glutenins, and prolamines (Fukushima, 1991; Shewry et al., 1995; Onwulata & Qi, 2006b). For example, albumins are the most water-soluble globular protein, whereas globulins are insoluble in water but soluble in a dilute salt solution. Legumes include predominantly albumins and globulins (Gueguen, 1983; Loveday, 2019).

1.1.3. Industrial Applications of Plant Proteins

In recent years, consumers tend to prefer healthy, quick, and eco-friendly foods, so the food industry has focused on protein-based structures according to consumer demands, and it is looking for less expensive proteins for usage in the production of modern

convenience foods. Although, even if the protein market is dominated by wheat, soy proteins like using in the processed meat industry for unique functionalities in food processing, during last years, other plant proteins such as some legume and seed proteins are introduced to the industry for applications as raw materials like isolates (Tömösközi et al., 2001; Tarté, 2009; Martins et al., 2018).

Currently, many researchers work on other potential plant proteins by relying on their functionality, processing, and industrial application. For example, canola proteins were found to be applicable ingredients in the beverage industry (Ustunol, 2014b). Another example is that pea proteins were shown as a good candidate for foam stabilization in aqueous food products (Saldanha Do Carmo et al., 2016).

1.2. Pea Protein

Pulses comprise peas (Pisum sativum L.) are from the pods of the legume plants that contain around 20-30 % protein, more than 50% starch, and low in fat relying on genotype and ecophysiological factors (Fernández-Quintela et al., 1997; Toews & Wang, 2013; Peng et al., 2016).

Pea protein has a well-balanced amino acid composition, and it is rich in lysine whereas limiting in tryptophan and the sulphur-containing amino acids (González-Pérez & Arellano, 2009; Jiang et al., 2017; Lu et al., 2020; Peng et al., 2016; Reinkensmeier et al., 2015).

The main storage proteins of pea are globulin (~55 - 65 %), albumin (~18 - 25%) and the remainings are prolamin and glutelin. Globulin can be further fall into two main types, which are 11S legumin or glycinin and 7S vicilin or β- and ϒ- conglycinin based on sedimentation coefficients (S) (Loveday, 2019; Lu et al., 2020). The important point for these types is that content, composition and structure are main differences between legumin and vicilin where their association–dissociation properties and surface structures are the most critical parameters for contributions of the functionality of pea protein and legumin content is dominated compared to vicilin (Lu et al., 2020).

Commercial pea protein ingredients are in three forms that are pea flour, pea protein concentrate and pea protein isolate which is obtained by wet processing applying alkali or acid solubilization, after the processing of isoelectric precipitation or an ultrafiltration that generates higher protein content ~85-95% (Day, 2015).

Recently some emerging ingredients come from pea, and especially proteins have attracted much interest in the food industry. They has been preferred in a variety of food production areas like cereal and bakery as gluten-free goods, snack bars, pasta, meal replacement beverages, baby food formulations, vegetarian and vegan products and seafood products (Han et al., 2010; Reinkensmeier et al., 2015).

Previous studies showed that pea protein isolate (PPI) could be applicable as desirable ingredients in food formulations. For instance, Vaisey et al. (1975) reported that pea protein can be added to ground beef to produce beef patties. They showed that patties containing pea protein were softer and tenderer than beef-soy and control patties (100% beef). In addition, Sumner et al. (1981) indicated that pea protein fortified spaghetti resulted in the reduction of raw noodle strength and cooking time; however, it caused higher cooking losses.

The popularity of pea protein in the food industry to develop innovative products are due to high nutritional value, availability, low cost and good functional properties in food applications.

1.2.1. Functional Properties

Having acceptance as an end product in the food industry, it is now clear that nutritional value, sensory characteristics, and functional properties of proteins are essential points (Soderberg, 2013). As mentioned in the study, functional properties of proteins are influenced by processed conditions like pressure treatments, extraction methods, high moisture extrusion cooking, or environmental factors like pH, ionic strength (Tang & Sun, 2011; Soderberg, 2013; Toews & Wang, 2013). Therefore, even if soy protein is extensively studied legume protein in terms of functional

properties among other plant proteins, pea protein is a good alternative for innovative food ingredient formulations in the food industry, and it is important to find new methods to improve its functionalities (Burger & Zhang, 2019).

1.2.1.1. Water Holding Capacity (WHC)

Water Holding Capacity (WHC) of proteins can be described as the ability of a protein structure to keep its own water or added water during application of forces or centrifugation from being released from the three-dimensional structure of the protein (Zayas & Zayas, 1997; Haque et al., 2016). In other words, it is the amount of water retained per gram of protein material (Kiosseoglou & Paraskevopoulou, 2011; Lam et al., 2018).

One of the hydration properties that play a crucial role in the mouthfeel is finished products' texture and flavor retention (Stone, Avarmenko, et al., 2015). In addition, storage stability, functional properties, and quality of eating also relate to water holding. For instance, high water holding capacity is desired for baked products, resulting in a reduction of moisture loss and maintaining freshness (Ge et al., 2020).

The amino acid composition and protein conformation of protein are important for the determination of water holding capacity. The charged, amide and hydroxyl groups, backbone peptide groups and nonpolar residues of amino acids are binded by water molecules and for each one, the ability to bind water is different. For example, highly charged proteins show higher electrostatic attraction toward water (González-Pérez & Arellano, 2009; Lam et al., 2018).

The concentration of protein, pH, ionic strength, temperature, presence of other components like salts, length and rate of process treatments and conditions also impact the water holding capacity of pea proteins. For instance, the water holding capacity of pea protein is affected by extraction methods according to the study that extracted by salt had a higher WHC (2 g/g) than extracted by micellar precipitation method which was 1.1 g/g (Stone, Karalash, et al., 2015). In addition, in most cases, by increasing of

temperature from 21 to 70°C for 10 min resulted in a substantial increase of water holding capacity (WHC) of legume proteins which is due to temperature effects of side chains and polar groups allowed to more excellent hydrogen bonding (Sosulski & Mccurdy, 1987).

1.2.1.2. Solubility

Solubility could be described as the existence of an equilibrium between protein-protein and protein–solvent interactions (Kiosseoglou & Paraskevopoulou, 2011; Soderberg, 2013). Solubility is very important for food systems since it is necessary for other functional properties of proteins like emulsification, gelling, and foaming in food applications (Tarté, 2009; Qin et al., 2013).

Some factors that influence the solubility of pea proteins are pea genotype, protein extraction method, pH, ionic strength and also processing history such as temperature, the pressure of protein products has a significant impact on this property (Barać et al., 2015; Reinkensmeier et al., 2015; Ge et al., 2020).

For instance, pea proteins show a U-shape of pH-solubility dependence means that major pea proteins are globulins tend to show minimum solubility at pH environments around the protein's isoelectric point in which electrostatic repulsion and ionic hydration of molecules reach a minimum and solubility is high above or below the isoelectric point (J. I. Boye et al., 2010; Barać et al., 2015). Additionally, according to Chao & Aluko (2018), the solubility profile of pea protein isolate changing with pH and temperature was shown in Figure 1.1. Moreover, another study about heat treatment on pea protein isolate showed that at 95 °C, no significant decrease in solubility was observed (Peng et al., 2016).

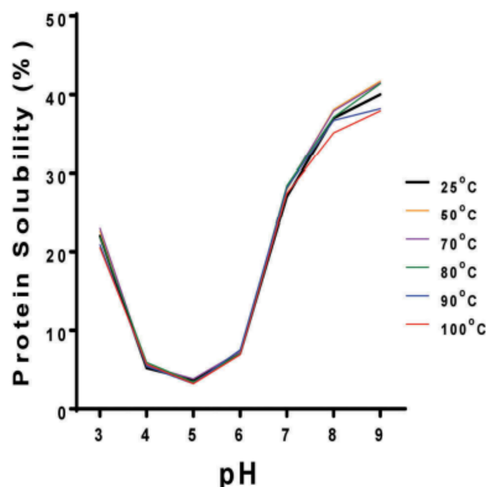

Figure 1.1. Solubility (%) profile of untreated (25°C) and heat-treated (50–100°C) pea protein isolate at pH 3–9 (Chao & Aluko, 2018)

1.2.1.3. Emulsification Ability

A system is composed of oil droplets dispersed in a watery phase is identified as an oil-in-water (O/W) emulsion, while a system composed of water droplets dispersed in an oil phase is identified as a water-in-oil (W/O) emulsion (McClements, 2009; R. S. H. Lam & Nickerson, 2013). Emulsification is an important property that protein contributes to in the development of novel foods. Many proteins are surface-active molecules preferred as emulsifiers since they facilitate the formation, enhance stability, and obtain desirable physicochemical properties in oil-in-water emulsions (McClements, 2004).

In addition, partial denaturation is generally needed for proteins to expose buried hydrophobic amino acids to the surface. By doing this, they can re-align themselves that their surface hydrophobic amino acids within the oil phase and hydrophilic amino acids within the aqueous phase (R. S. H. Lam & Nickerson, 2013). Salad dressings, dairy desserts, mayonnaise and ice cream, are some examples of protein-stabilized food emulsions in the industry (Singh & Sarkar, 2011).

Several researchers made a comparison between the emulsification ability of pea and soy protein isolates. For example, in a recent study (Aluko et al., 2009), the emulsifying capability of pea protein isolate (PPI) was found significantly higher for all concentrations (from 10 to 50 mg/ml) at pH 5 and 7. Also, Tömösközi et al. (2001) obtained that PPI had a similar emulsifying property to soy protein isolate. On the other hand, Barac et al. (2010) found that pea protein isolates demonstrated slightly lower emulsifying properties than soybean isolates under the same conditions.

The emulsifying ability of pea proteins is affected by some factors that were shown in some studies. For example, emulsion capacity is pH-dependent, and it is similar to the pH-solubility profile shown by the related study (Gharsallaoui et al., 2012). In addition, another study found that emulsifying ability can vary according to pea genotypes (Ge et al., 2020). Moreover, the study on the effects of different extraction methods on the emulsifying ability of pea protein isolates exhibited that at pH 7, extraction with ultrafiltration had a greater emulsion activity index than extraction with acid precipitation (Fuhrmeister & Meuser, 2003).

It is clear that processing parameters and environmental conditions greatly influence the emulsification properties of pea protein isolates.

1.2.1.4. Viscosity

By exposuring an applied force, viscosity is basically resistant to flow in relation to the consumer perception of liquid and semi-liquid food systems like beverages, soups, or sauces. Thus, the flow behavior is an important functional quality that should be considered during the processing and improvement of food products (Dissanayake et al., 2013).

It is affected by molecular size, shape, charge, solubility, and swelling capacity of the protein molecules and by processing conditions like temperature, concentration, pH, and ionicity (Kinsella, 1976; Juandoo, 1980). For example, according to Fleming et al. (1974), the viscosity of proteins inclines to increase with concentration. Similarly, the

study about changing viscosity of pea protein isolates with concentration showed corresponding results (Sathe & Salunkhe, 1981). Furthermore, the recent study showed that pea protein tended to create highly viscous at high concentrations and researchers pointed out that this issue can be overcome by chemical and enzymatic treatments (Lu et al., 2020).

Denaturation of proteins, for example, by heating, is also a key point that when denaturation occurs randomly, coiled molecules demonstrate higher viscosity compact folded globular molecules of the same molecular (Benoit et al., 2013; Damodaran, 2017).

1.3. High Hydrostatic Pressure (HHP)

1.3.1. General Information

High hydrostatic pressure is a non-thermal technology that has a growing interest in food processing technology. It is an effective way to decrease the level of microbial contamination without the requirement of any heat treatment and the enabling to produce foods with high sensory, nutritional quality (Erkan et al., 2010). Actually, since the late 19th century, the influences of HHP on biological materials and creatures have been investigated. For instance, in 1914, research about the relationship between HHP processing and the preservability of fruits and vegetables in microbiological aspects was conducted (Yamamoto, 2017). Then, many kinds of research and developments in terms of food and biology for HHP applications have improved until today.

For the food industry, firstly, the Japanese company was introduced fruit preserves to the market in 1990. For example, in European markets, people have preferred to consume various other HHP processed products such as fruit yogurts, fruit jellies, salads, and fruit sauces (Estrada-Girón et al., 2005). Currently, a wide range of food products like fruit juices, seafood, meat products, jams, sauces, rice and cakes were placed on shelves in markets all around the world (Trujillo, 2002; Buzrul, 2012).

It is well known that there is no requirement of active heating for the HHP process, even though passive heating by adiabatic compression and passive cooling by adiabatic decompression are needed (Yamamoto, 2017). Hence, it is a nonthermal process like Pulsed Light (PL) and Pulsed Electric Fields (PEF) (De Maria et al., 2016).

Pressure treatment is carried out between 100 and 1000 MPa and liquid or solid foods can be exposured (Erkan et al., 2010). According to the isostatic rule, the pressure is instantly and uniformly transmitted throughout a food independent of its size, shape and composition (San Martín et al., 2002). In addition, the occurrence of rising in temperature is because the adiabatic heating of foods is about 3°C per 100 MPa that relies on the composition (Bigikocin et al., 2011). Therefore, the processing equipment is composed of a pressurization system, the pressure vessel, temperature control units and product handling devices for transferring product to and from the pressure vessel (Buzrul & Alpas, 2012; San Martín et al., 2002).

In order to understand the impacts of HHP on food systems, it is necessary to take into account the combined pressure-temperature-time effect on the treated foods. In general, the operation range of temperature and time time can be within range between -20°C and 80°C and seconds to minutes, respectively (San Martín et al., 2002).

1.3.2. Effects of HHP on Plant Proteins

High hydrostatic pressure (HHP) has a wide range of applications like inactivating unwanted food enzymes, reducing the microbial population of spoilage microorganisms, and producing high-quality food products like dairy goods (Estrada-Girón et al., 2005; Trujillo, 2002). Among these applications, it plays an important role in changing various food biopolymers, such as starch gelatinization or modification of proteins (Knorr et al., 2011; Yamamoto, 2017). Moreover, low-molecular-weight compounds such as flavor compounds, vitamins, and pigments are slightly affected by the HHP process compared to thermal processes (Yordanov & Angelova, 2010).

The ways of protein modification by HHP treatment are relied on the ability to cause a volume change in the protein molecules in solution. The volume of a protein in solution comprises of its atoms’ volume and its cavities that affects interactions with the solvents (J. Yang & Powers, 2016). Therefore, HHP treatment causes compression of the protein cavities and rupturing or formation of the noncovalent interactions (Akharume et al., 2021).

Moreover, Sim et al. (2019) showed that HHP caused a network structure composed of fibrillar aggregates for pea protein concentrate samples as seen from Figure 1.2. and 550 MPa pressure treatment led to a greater extent of protein aggregation than 250 MPa.

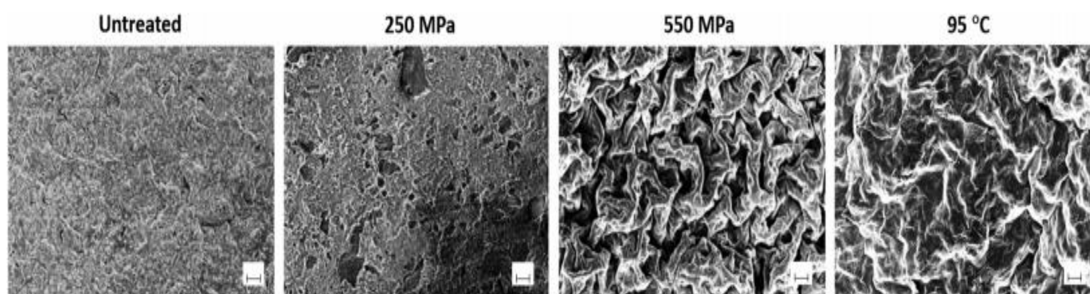

Figure 1.2. SEM micrograph of untreated, HHP-treated and heat-treated pea protein concentrate solutions (24 g/100 g) (Sim et al., 2019)

Proteins exhibit changes in their native structure under high hydrostatic pressure that may alter their functional and sensory properties (Yordanov & Angelova, 2010). It influences the non-covalent bonds, like ionic, hydrophobic, and by doing this, even though the primary structure is not affected, the secondary, tertiary, and quaternary structures may unfold and disassociation of interactive forces, especially hydrophobic bonds and electrostatic interactions occurs (Tabilo-Munizaga et al., 2014; Yin et al., 2008). This case may result in variation of functional properties of proteins.

Some studies indicate influences of high hydrostatic pressure at different levels, durations and the temperature at treatment on plant-based proteins' functionality. For

instance, the research on soy protein isolate demonstrated that for 20 min at pH 3 processing, the increase of solubility was observed over 200 MPa. In contrast, no differences were observed with higher pressures between 400 and 600 MPa (Puppo et al., 2004) and Chapleau & De Lamballerie-Anton (2003) worked on lupin proteins and they found that below 400 MPa for 10 min, solubility was not affected. Also, they stated that at 400 MPa, the pressure improved the emulsifying property of lupin proteins by decreasing droplets size, flocculation, and creaming index. Another important example is that HHP improved emulsifying activity of red kidney bean protein isolate significantly pressure between 200-400 MPa for 20 min and increasing pressure between 200-600 MPa resulted in increasing of solubility in some extent (Yin et al., 2008). In addition, Chen et al. (2019) reported that HHP reduced solubility around 3.4% cumin protein isolate at pH 3 to 9.

Although the utilization of pea protein should be more considered, there are few studies about HHP processing on pea proteins, especially from the point of functional properties. To illustrate, Chao et al., (2018) pointed out that structural changes that occurred from pressure treatments between 200 – 600 MPa for 5 min had a positive effect on emulsion formation and stability, whereas the opposite effect was observed from foaming ability. In addition, increasing concentrations (10, 25, and 50 mg/mL) resulted in high emulsifying properties with interfacial better protein packing.

It can be said that the effects of pressure treatment change with the type and concentration of protein, pH, and ionic strength of processed protein samples (Tewari, 2007).

1.3.3. Characterization of HHP Treated Pea Protein

1.3.3.1. Lowry Method

The Lowry protein assay is the most widely used and cited procedure for protein quantitation. It relies on a reaction that includes copper interacts with four nitrogen atoms of peptides to form a cuprous complex and reduce the Folin-Ciocalteu with additional steps and reagents to increase the sensitivity of detection (Shen, 2019). Interactions of the Folin-Ciocalteu reagent with the cuprous ions and the side chains of tyrosine, tryptophan, and cysteine occur to produce a water-soluble product with a blue-green color which can be detected between 650 and 750 nm (Deepachandi et al., 2020).

This method depends on the presence of readily oxidizable amino acids like tryptophan, tyrosine, cysteine so that the response can differ in view of the amino acid composition of proteins. Thus, it is important that applied protein for generating the standard curve be consistent according to experiments (Held & Hurley, 2005).

1.3.3.2. Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectroscopy allows for structural characterization of proteins and peptides and their interactions with lipid membranes (Amenabar et al., 2013; Tatulian, 2019). In the past decade, it was introduced as a useful tool to get information about protein conformation in H_2O-based solution or dried states resulting from studies of protein secondary structure and protein dynamics (Kong & Yu, 2007). In addition, The mid-infrared spectral range that is typically between 4000 to 400 cm^{-1}, which includes the vibrational frequencies of various chemical groups for all organic and inorganic compounds like in a polypeptide chain of proteins. Because they have distinct chemical composition and the vibrational frequencies, FTIR spectroscopy enables to get information on the individual functional groups of molecules (Tatulian, 2019).

Nine characteristics of absorption bands are shown in Table 1.4. It is known that protein secondary structures include α-helix, β-sheet, β- and γ-turns and FTIR spectroscopy provides spectral analysis of the composite amide I absorbance band (1700-1600 cm^{-1}) which is because of almost entirely the C=O stretch vibrations of the peptide linkages (~80%) (Kong & Yu, 2007; Tatulian, 2013). Also, the peak observed in the Amide II band (~ 1480-1575 cm^{-1}) is used to change CN stretching and NH bending (Kong & Yu, 2007; Tatulian, 2013). Also, the peak observed in Amide II band (~ 1480-1575 cm^{-1}) is used for changing of CN stretching and NH bending (Tatulian, 2013).

Table 1.4. Nine characteristic of infrared bands of peptide linkage (Kong & Yu, 2007)

Designation	Approximate frequency (cm^{-1})	Description
Amide A	3300	NH stretching
Amide B	3100	NH stretching
Amide I	1600–1690	C=O stretching
Amide II	1480–1575	CN stretching, NH bending
Amide III	1229–1301	CN stretching, NH bending
Amide IV	625–767	OCN bending
Amide V	640–800	Out-of-plane NH bending
Amide VI	537–606	Out-of-plane C=O bending
Amide VII	200	Skeletal torsion

FTIR spectroscopy is a convenient and non-destructive method that needs little sample preparation, and also it can be successfully applied under a wide range of conditions like the effect of pH and thermal treatments (Beck et al., 2017; Tatulian, 2013).

1.3.3.3. Nuclear Magnetic Resonance (NMR) Relaxometry

Nuclear magnetic resonance (NMR) relaxometry is a fast and non-destructive method that is undoubtedly an effective technique for examining water's dynamic behavior in a food system (Fan et al., 2013; Kirtil & Oztop, 2016). It provides information about the characterization of water mobility and distribution (Bertram et al., 2004).

Several interactions among the magnetic moments of the observed spins and the surrounding nuclei and electrons provide the relaxation times T_1 and T_2 that carry both structural and dynamic molecular information (Van As, 2007; Vazquez & Misra, 2019). Thus, T_1 is also described as spin-lattice or longitudinal relaxation, which is the time required for an excited self-spinning proton to achieve dynamic equilibrium after energy exchange with the surrounding lattice (Fan et al., 2013), and T_2 is also defined as spin-spin or transverse relaxation time that gives information about mobility of hydrogen molecules (Ozel et al., 2017).

For the relaxation time measurements of the sample, it can be induced with the help of a static magnetic field and applying appropriate sequences of an additional radiofrequency field (Goetz & Koehler, 2005). In addition, basically, the protons present in water molecules are exposed to an intramolecular dipolar interaction between the two proton spins within water molecules, also an intermolecular interaction with protons of neighboring water molecules. When the rotation of molecules occurs, these interactions fluctuate. If this rotational correlation time is short, like free water molecules, T_1 and T_2 are relatively long values. Furthermore, when water is close to solid surface or macromolecules, that results in decrease in the relaxation times (Van As, 2007).

The decay of the transverse magnetization, is characterized by transverse relaxation time T_2, and it is measured in the time domain through the Carr-Purcell-Meiboom-Gill (CPMG) experiments that provide information on the chemical exchange phenomenon with proton populations (Bosmans et al., 2012; Dekkers et al., 2016).

The T_2 relaxation time is more sensitive to small changes in water content and chemical exchange processes than T_1, so it is usually favored. In fact, differences in T_1 for compartments are smaller that causes an averaging effect that results in poor discrimination between water compartments (Van As, 2007; Musse et al., 2013).

From applications of NMR in protein solutions, it is revealed that transverse relaxation times in protein solutions can be explained by a mechanism of chemical exchange

between water and protein protons (Hills et al., 1989). When interactions between water and macromolecules like proteins obstruct the rotational frequency of water molecules and raise the efficiency in magnetic energy transfer, resulting in shorter relaxation times (Kirtil et al., 2014).

NMR has been used in several various protein-based studies. For instance, the study about the effects of heat treatment on β-lactoglobulin (a globular protein which constitutes about 50% of whey protein) showed that although there was no change in T_2 times for dilute solutions (1, 4, and 10 g L^{-1}) and heating temperatures (between 21 and 90°C), higher concentrations (20, 40 or 80 g L^{-1}) shortened the T_2 relaxation (Coelho et al., 2007).

Objectives

Continuing increases growth in the popularity of plant proteins in the food industry has been quite notable. It is not surprising since consumers have an increasing tendency to attain natural, eco-friendly, and sustainable food sources. Also, it is crucial to have information about how plant protein ingredients like isolates support protein formulations since they are required for specific functions to obtain new product formulations. Therefore, it is critical to understand the effect of processing conditions on plant protein's functionality because functionality challenges with most plant proteins like pulses that limit their replacement of animal proteins. Thus, modification of proteins through alternative processing conditions is required to overcome functionality problems of plant proteins. When all of these points are taken into account, the main objective of this study is to understand the effects of high hydrostatic pressure (HHP) on the functional properties of pea protein isolate (PPI). Moreover, different conditions like pH and temperature changes were also carried out to see their effects on PPI's functionality.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Pea protein isolate (PPI) was graciously provided by Elite Naturel Organik Gıda San. ve Tic. A.Ş. (Ankara, Turkey) for this study. Protein determination of commercial protein isolate was performed by Kjeldahl analysis (% N × 6.25 for pea) according to AOAC Official Method (AOAC, 2007). Protein content was found as 85.05% (d.b.) for pea protein isolate.

So as to examine properties of both untreated and treated pea proteins, some chemicals were used for required analyses. Copper(II) sulfate pentahydrate ($CuSO_4.5H_2O$), sodium potassium tartrate tetrahydrate ($KNaC_4H_4O_6.4H_2O$), Bovine Serum Albumin (BSA), Folin-Ciocalteau's phenol reagent, sodium carbonate (Na_2CO_3), sodium hydroxide (NaOH), Hydrochloric acid (HCl), phenolphthalein ($C_{20}H_{14}O$), sulphric acid (H_2SO_4), boric acid (H_3BO_3), methyl red were bought from Sigma-Aldrich Chemical Co. (St. Louis, Missouri, USA). In addition, corn oil (Evin, Ankara, TURKEY) was purchased from the local market for usage to determine emulsification properties.

2.2. Methods

2.2.1. Preparation of Samples for High Hydrostatic Pressure (HHP) Treatment

With a concentration of 45% g/ml (w/v), pea protein samples were prepared using 1M HCl and 1M NaOH solutions to adjust desired pH levels of 3, 5, and 7. Then, HHP treatment was done for prepared doughlike samples.

2.2.2. High Hydrostatic Pressure (HHP) Treatment

High Hydrostatic Pressure (HHP) treatment was applied by using 760.0118 type pressure equipment (SITEC-Sieber Engineering AG, Zurich, Switzerland) as seen Figure 2.1. Two end closures, a tool for restraining the end closures, a hydraulic unit, a pressure pump, a temperature control device and a pressurization chamber, which has an internal diameter of 24 mm, length of 153 mm and 100 ml capacity, are parts of the HHP equipment. A built-in heating and cooling system were used to keep the inside temperature of the system constant (Huber Circulation Thermostat, Offenburg, Germany). Distilled water was used as a pressure transmitting medium that fills the vessel. For this system, the rate of increase in pressure 340 MPa/min for 400 MPa and pressure release are less than 5 seconds, so the pressurization time indicated in this study excludes the pressure increase and release times.

25 ml sterile polyethylene cryotubes (LP Italiana SPA) were filled with samples at the desired pH for high hydrostatic pressure application. They were exposed to 300, 400 and 500 MPa, at 25 and 50°C and 5 minutes. The HHP-treated samples were then freeze-dried and stored at -20 °C for further analysis. Measurements were performed in triplicate.

Figure 2.1. HHP equipment

2.2.3. Characterization of Pressure Treated Pea Proteins

2.2.3.1. Determination of Protein Content of Pea Protein Isolate by Kjeldahl Method

In order to have information about the protein content of untreated pea protein isolate, the Kjeldahl procedure was carried out according to ASTM E258 – 07 (2015) with some modifications.

Related Kjeldahl method includes three stages. Burning is the first one that each 1 g pea protein isolates were weighed and placed in the Kjeldahl tubes. Then, Kjeldahl catalyst tablet and boiling chips were added to the three tubes, and 25 ml of H_2SO_4 for each was added. So as to suppress the foam formations, an antifoam agent tablet was used, and the solution was boiled in the pre-firing device until obtaining transparency about in three hours. Then, the boiled and clarified solution in Kjeldahl tubes was cooled to room temperature.

For the distillation stage, Kjeldahl tubes and the 40% NaOH solution were placed in the distillation unit in order, and then 50 ml 4% H_3BO_3 solution and 2-3 drops of methyl red that was indicator were added Erlenmeyer flask and mixing was achieved according to the unit about in 6 minutes.

For the titration part, after adding the composed NH_3 to the H_3BO_3 solution, titration was started. For this purpose, the mixture was titrated with 0.1N HCl. When the color turned from yellow to pink, the amount of HCl consumed during the titration was noted.

The following formula was used to calculate the amount of crude protein content;

$$\text{Nitrogen (\%)} = \frac{(\text{V1} - \text{V0}) * \text{N} * 0.014}{\text{m}} * 100$$

$$\text{Protein content (\%)} = \text{Nitrogen (\%)} * \text{f}$$

Where,

V1: The consumption of HCl during titration,

V0: The consumption of HCl for blank measurement

N: The normality of the HCl used as titrant,

m: The sample weight.

f: The factor of the sample (It was taken 6.25 for pea protein isolate).

2.2.3.2. Determination of Water Holding Capacity (WHC)

The approximate water holding capacity (WHC) was determined according to the method shown by Bajaj et al. (2015) with slight modifications. At first, 5% w/v (1.25 g pea protein in 25 ml distilled water) was prepared and then Ultra Turrax T-18 (IKA, Corp., Staufen, Germany) was applied at 6,000 rpm for 5 minutes. After that, samples were put into the 25 ml cenrifuge tubes and weight of tubes with samples were noted. Then, samples were centrifugated at 4,000 rpm for 30 minutes. After centrifugation, the supernatant was carefully decanted and remaining part was weighted. Water holding capacity was calculated by using the formula given below (Bajaj et al., 2015).

$$\text{WHC}\left(\frac{\text{g H2O}}{\text{g protein sample}}\right) = \frac{\text{weight of PPI after removing supernatant} - \text{weight of PPI}}{\text{weight of PPI}}$$

2.2.3.3. Determination of Solubility

For the determination of solubility for the samples, firstly, 1% w/v (0.5 g pea protein in 50 ml distilled water) solutions were prepared. Then Ultra Turrax T-18 (IKA, Corp., Staufen, Germany) was used for mixing at 5,000 rpm for 5 minutes. After that, the samples were centrifugated at 2,500 rpm for 15 min. Finally, to calculate the solubility of the samples, Lowry method was carried out. This method basically relies on the reaction between proteins and copper ions in an alkali environment (Waterborg, 2009).

The required reagents for the method were shown in Table 2.1 below.

Table 2.1. The reagents for Lowry Method

Reagent A	2% Na_2CO_3 dissolved in 0.1 N NaOH
Reagent 1	2% $CuSO_4.5H_2O$, Copper source
Reagent 2	2% Na-K Tartarate
Lowry Reagent	Mix of Reagent A:1:2 with a ratio of 100:1:1
Folin and Ciocalteu's Phenol Reagent	Diluted 2N stock solution as a ratio of 1:1 with distilled water

Reagent A, Reagent 1 and Reagent 2 were prepared according to the composition given the reagents. For the preparation of Lowry ACR reagent; Reagent A, Reagent 2 and Reagent 1 were mixed at the ratios of 100:1:1, respectively. Then, for preparation of Folin-Phenol Reagent, 2N stock solution (commercial) was diluted as a ratio of 1:1 with distilled water. For the calibration curve, BSA (Bovine Serum Albumin) stock solution with series dilutions from 1 mg/ml to 0.03125 mg/ml was done that helps the determination of the solubility of the desired proteins.

For the experiment, after centrifugation, the supernatant was diluted with distilled water a ratio of 1:4. Then, 2.5 ml Lowry ACR reagent mixed with 0.5 ml of diluted sample and the mixture left at room temperature for 10 minutes. After that, 0.25 ml Folin Reagent were put into the tubes which vortexed by using a vortex-mixer (VM-10, Witeg Labortechnik GmbH, Germany) for minimum 8 seconds and left at room

temperature for 30 minutes. The same procedure was carried out for the blank by adding distilled water instead of a protein sample in the tube. Finally, the absorbance values of samples were read at 750 nm using Optizen POP Nano-Bio UV spectrophotometer and the calibration curve (Figure A.1) was constructed using absorbance values vs mg/ml BSA solution (y =1.685x + 0.1289).

2.2.3.4. Emulsion Activity (%)

The samples' emulsion activity was determined using the method described by Hoang (2012) with some modifications. 1% w/v (0.5 pea protein in 50 ml distilled water) solutions were prepared, then homogenised (Ultra Turrax T-18, IKA, Corp., Staufen, Germany) at 5,000 rpm for 5 min. After that, 1 ml of protein solution was mixed with 0.5 ml corn oil using the same homogenizer and homogenised at 15,000 rpm for 1.5 minutes. Then, the height of obtained emulsion samples was measured and emulsions were centrifugated at 10,000 rpm for 1 minute. Then, accepted as a non-emulsified fraction, the height of the oil fraction that remained at the top was noted. Finally, emulsion stability % was calculated by dividing the emulsified fractions by the height of the initial emulsion samples.

2.2.3.5. Viscosity Measurements

First, Pea protein isolate (PPI) was dissolved in distilled water (5% w/v), and then homogenised (Ultra Turrax T-18, IKA, Corp., Staufen, Germany) at 6,000 rpm for 5 minutes. SV-10 Vibro Viscometer was used for measurements (A&D Company, JAPAN).

2.2.3.6. Fourier Transform Infrared (FTIR) Spectroscopy Analysis

Fourier Transform Infrared (FTIR) Spectroscopy analyses were performed by using samples in powder form. The IRSpirit Spectrometer with Attenuated Total Reflectance (ATR) attachment (Shimadzu Corporation, Kyoto, Japan) was used at a resolution of 4 cm^{-1} with 32 scans in the region of 600-4000 cm^{-1}.

2.2.3.7. Hydration Behavior of Pea Protein by Nuclear Magnetic Resonance (NMR) Relaxometry

The hydration behavior of both control and treated pea protein isolates was analyzed by using NMR Relaxometry technique. For this purpose, 0.23 g pea protein powder was mixed with 0.92g distilled water (1:3) in 10 mm tubes. Then, they were analyzed by using a 0.5 T (20.34 MHz) benchtop NMR system (Spin Track, Resonance Systems GmbH, Kirchheim/Teck, Germany) to obtain T_2 (spin-spin relaxation) times for each sample by CPMG (Carr-Purcell-Meiboom-Gill) pulse sequence with an echo time of 800 ms, 500 echoes and 16 scans. Data were analysed by MATLAB (R2019b, The MathWorks Inc., Natick, MA, USA) considering mono-exponential analysis.

2.2.3.8. Statistical Analysis

Statistical analysis was carried out for sample assays in triplicates, and data were analyzed using MINITAB (Version 16.1.1, Minitab Inc., Coventry, UK). To understand the effects of each independent parameter on the functional properties, Analysis of Variance (ANOVA) was applied by using general linear model Tukey's test was performed with 95% confidence level to see the significant differences. The small letters were used to show significant differences between different HHP conditions.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Water Holding Capacity (WHC)

As mentioned in the introduction part, water holding capacity (WHC) is an important quality indicator for proteins related to the texture and freshness of food products. Thus, during processing, it is necessary to control the factors influencing the WHC of proteins. In this study, the WHC of untreated PPI was found as 4.09 g/g. This value was in the range reported by other authors for water holding capacities of PPI that were between 0.3 and 4.8 g water / g sample (Bajaj et al., 2015; Ge et al., 2020; J. I. Boye et al., 2010; Lam et al., 2018; Stone et al., 2015). Therefore, the differences may be related to cultivar or the differences in extraction methods of pea proteins.

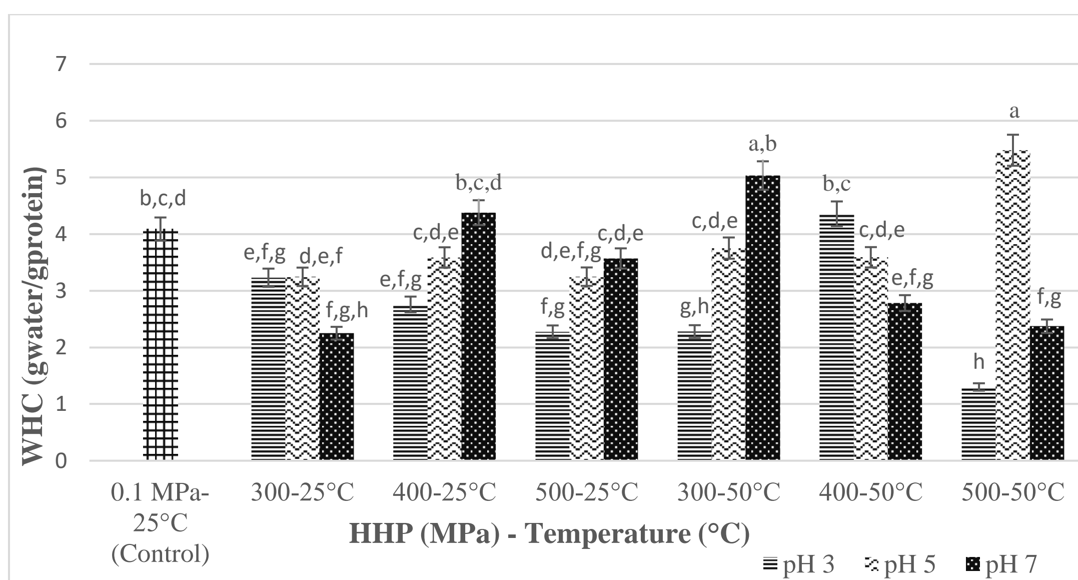

Figure 3.1. Water holding capacity (g/g) of both control and HHP-treated pea protein isolate (PPI) at different pHs (3, 5 and 7). Different small letters indicate significant differences between different HHP conditions (p<0.05)

As can be seen in Figure 3.1, all processing factors that were high hydrostatic pressure, temperature, and pH were significant ($p<0.05$) on the water holding capacity of PPI. The effect of HHP treatments was statistically analyzed alone; the pressurization at 300 and 500 MPa resulted in a significant decrease in WHC of PPIs except for 400 MPa compared to control. However, different trends were seen for pressure-temperature combined conditions. For instance, at pH 5 at room temperature, HHP resulted in no significant differences in WHC ($p>0.05$) due to pressure increase compared to control samples. Similarly, when the temperature was increased to 50°C, the proteins exposed to 300 and 400 MPa caused no significant differences in water holding capacity ($p>0.05$) compared to control, as shown in Figure 3.1. However, 500 MPa, 50°C pressure treatment yielded a maximum value of WHC (5.48 g/g) compared to the other HHP-temperature combined treatments and control samples. 500 MPa-50°C treatment at that pH could trigger the destruction of the balance of noncovalent bonds in the protein and dissociated subunits may have more binding sites that could improve WHC of PPI.

In addition, since pH 5 was close to the isoelectric point of pea protein (4.5), lower WHC of PPI was expected due to the presence of high hydrophobic interactions on the surface at pI (Haque et al., 2016; Zayas, 1997). On the other hand, no significant differences were seen in WHC at pH 5 compared to pH 3 and 7 ($p>0.05$) except for processing 300 and 500 MPa at 50°C. Thus, an increase in the WHC may be related with the high amount of polar amino acids on the surface of protein due to the dominant effect of HHP treatment other than the effect of pH.

Furthermore, HHP-treated PPI samples had significantly lower WHC ($p<0.05$) at pH 3 than pH 7 and control for almost all pressure-temperature combinations, whereas no significant differences were observed due to pressure increase ($p>0.05$). The reason for the reduction in WHC may come from the unfolding of the polypeptide chains due to the transition of the globular conformation to a random coil conformation. The availability of polar amino acid groups for binding water could have reduced. Also, WHC of protein samples decreased significantly compared to the untreated protein,

especially at 300 MPa; it had a lower value (2.25 g/g) than at 400 MPa (4.38 g/g) and 500 MPa (3.57 g/g) at pH 7 ($p<0.05$). Further rising in WHC at 400 and 500 MPa may be due to the unfolding-induced exposure of polar amino acids.

When the temperature was increased to 50°C, significantly different WHC values were obtained ($p<0.05$). For instance, at 300 MPa, PPI gained significantly better WHC (5.03 g/g) than control pea protein samples; however, an increase in pressure levels resulted in significantly lower WHC values (~ 2.5 g/g) ($p<0.05$). At that point, it could be a reason that pressure-temperature combinations resulted in extended denaturation, especially at 500 MPa, which reduced the capacity to bind water molecules due to change in protein native conformation.

3.2. Solubility

Protein solubility is an essential functional property in various food applications. However, the low solubility of pea proteins as legume protein results in limited utilization potential in food systems since legume proteins are easily affected by different processing conditions like pH changes or extraction methods (Ge et al., 2020). Thus, changes in solubility of pea protein isolate due to HHP were investigated. The solubility results obtained from the Lowry method were presented in Figure 3.2.

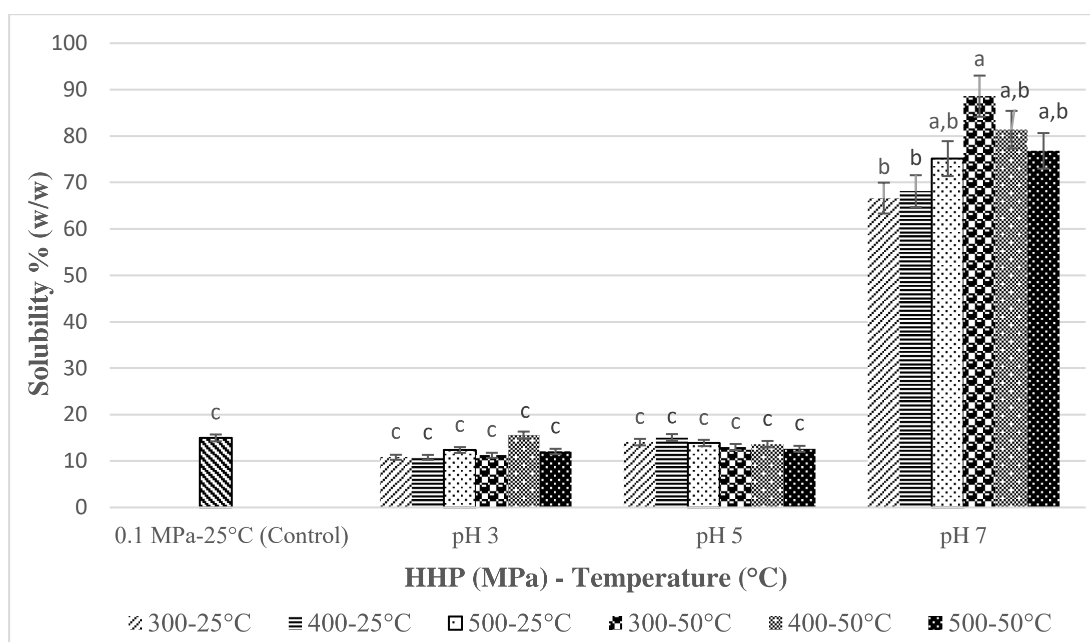

Figure 3.2. Solubility % (w/w) of both control and HHP-treated pea protein isolate (PPI) at different pH values (3, 5 and 7). Different small letters indicate significant differences between different HHP conditions ($p<0.05$)

Although no solubility differences were seen in processing between pH 3 and 5 ($p>0.05$), pH change considerably modified the solubility of PPI at pH 7. The increasing repulsion between the charged molecules could enhance the protein-solvent interactions that resulted in a significant increase in solubility of PPI ($p<0.05$). A similar observation was supported by Barac et al. (2010), who reported that six different pea varieties showed strongly pH-dependent solubility, and their solubility values were high at pH 7.0 and 8.0. Also, Chee & Ayob (2013) reported that the solubility of palm kernel cake protein by hexametaphosphate-assisted extraction showed that better solubility (87.65%) at pH 7, which was related to the presence of a lower number of hydrophobic residues at the protein surface. In addition, it is known that near the isoelectric point (pI), proteins have no net charge, so they show minimum solubility in water (Adebiyi & Aluko, 2011; Stone et al., 2015; Tang & Sun, 2010). The results agreed that at pH 5, which was around pI (4.5 for pea protein), approximately a 60% lower solubility was seen than pH 7 ($p<0.05$).

When influences of HHP treatment on the solubility of PPI were considered, no significant differences in solubility of PPI were observed alone among three different pressure levels (average value 33.95±0.14 %) ($p>0.05$); however, the solubility of PPI increased significantly compared to the control (14.97 %) ($p<0.05$) due to HHP processing. Chao et al. (2018) have pointed out that HHP treatments at 200, 400 and 600 MPa for 5 min resulted in the formation of soluble pea protein aggregates, and there were no differences in solubility due to pressure increase.

Different solubility profiles were observed at three pH levels. However, at pH 3 and 5, no significant solubility differences were obtained due to pressure or temperature increase. For instance, at pH 5, 400 MPa - 25°C (15.02 %) and at pH 3, 500 MPa – 50°C (12.05 %) showed similar solubility results compared to the control (14.97%). Therefore, it was confirmed that supplied conditions did not lead to insoluble protein aggregations at these pH levels. Similar results were reported by Li et al. (2012) where pressure treatments between 200-400 MPa, and 5-15 min at pH 3, soy protein isolate samples showed similar solubility profiles.

Apart from these results, the effects of high hydrostatic pressure application were seen at pH 7. For instance, the highest solubility value was seen for 300 MPa – 50°C (88.59 %), which was significantly higher than 300 and 400 MPa at 25°C (~ 67.39 %) ($p<0.05$). It was shown that the solubility of PPI improved due to an increase in temperature from 25 to 50°C, so significant impacts of temperature-assisted pressure treatment were inevitable. A study on the solubility change of two kinds of pea protein isolates concerning temperature changes indicated that the solubility increased with increasing temperature and below 50°C for 60 minutes of the processing; they showed lower solubility (less than 50%) (Chen et al., 2019). Moreover, thermal processing of PPI at 79-95 °C for 25 min caused a significant reduction on the solubility (Shand et al., 2007). Hence, it can be said that increasing temperature between these pressure levels at pH 7 induced the electrostatic interactions, which were the driving force for the protein-solvent association that helps solubilization.

In contrast, thermal treatments at higher levels and durations caused a reduction in solubility. In the end, in this study, the release of the denaturation step of protein causing aggregation depends on protein pH, pressure levels, and features of the protein. Also, pressure levels, treatment duration, and pressurization temperatures are critical factors for protein modifications.

3.3. Emulsion Activity

Plant proteins gain interest in emulsifying properties by means of re-aligning their surface hydrophobic amino acids within the oil phase and hydrophilic amino acids within the aqueous phase due to their amphiphilic nature (Lu et al., 2020). Properties of emulsion with proteins rely on protein characteristics like a protein source, a composition like a vicilin/legume ratio, surface hydrophilic-hydrophobic properties, processing conditions like level and duration, and environmental conditions like pH, pressure, temperature (Karaca et al., 2011).

Even though there are common methods which are the emulsion activity index (EAI), emulsion stability index (ESI), emulsion capacity (EC), or activity used to evaluate emulsion properties, they differ among researchers, and the values are reported using different units that causes difficulties in comparison (Lam et al., 2018). Therefore, in this study, emulsion activity % was determined to have information about the emulsification ability of both treated and control protein samples.

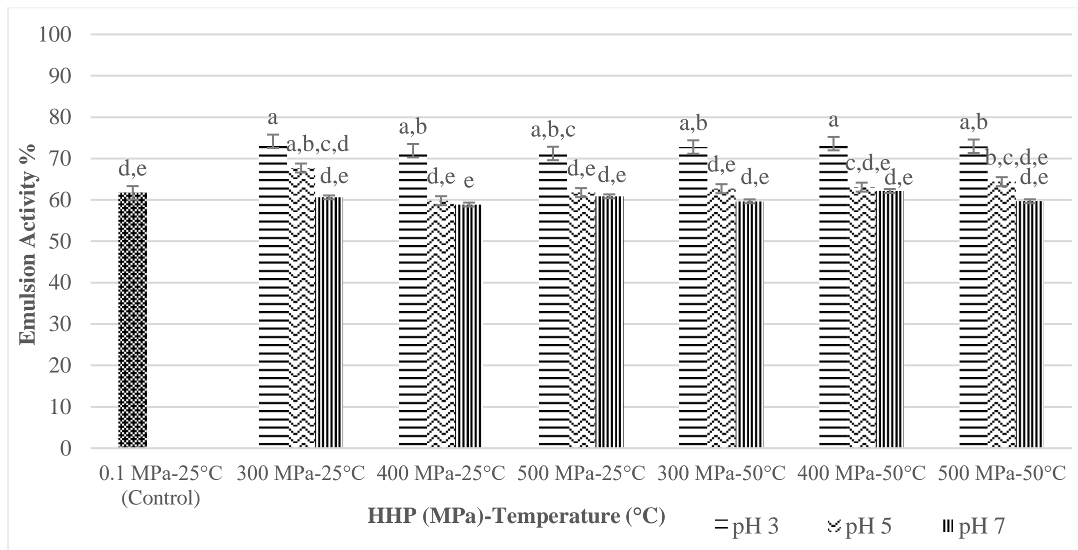

Figure 3.3. Emulsion activity (%) of both control and HHP-treated pea protein isolate (PPI) at different pH values (3, 5 and 7). Different small letters indicate significant differences between different HHP conditions ($p<0.05$)

As shown in Figure 3.3, the trends of emulsion activity % for processed PPI were given. According to the results, it can be observed that change in pH had a significant effect on emulsion activities of treated samples, especially at pH 3 ($p<0.05$). At that pH, for all pressure-temperature combinations, emulsion activity was significantly higher than pH 5 and 7 as well as control ($p<0.05$). The reason for having better emulsion activity at pH 3 than pH 7 is that pea protein isolates could form more viscoelastic interfacial films at the interface since pea globulins destrupted at pH 3 and they are dissociated to more surface-active form which is more avaliable for the adsorbtion. This observation is in agreement with Liang & Tang (2013), where the emulsification capability of pea purified globulins (vicilin and legumin) and protein isolate at pH values between 3.0 to 9.0 was better at pH 3 than at pH 7 or alkali pH. Similarly, Gharsallaoui et al. (2009) reported that PPI's emulsifying properties at pH 2.4 were better than those measured at pH 7.0 in respect to particle-size distribution and creaming stability.

Moreover, at pH 3, the lowest emulsion activity value was observed at 300 MPa - 25°C (71.22 %); still it was significantly higher than control (61.71 %) ($p<0.05$). At this point, exposure of hydrophobic groups to the interface enhanced due to the unfolding of the protein by HHP treatment, so a significant increase in emulsion activity for PPI was observed. A similar result was reported by Zhao et al. (2015), where the highest emulsion activity index was obtained at 300 MPa for 10 min on arachin, which was the major kind of protein in peanuts. Furthermore, Wang et al. (2008), confirmed that while 200 MPa led to a significant increase in emulsion activity index for soy protein isolate (SPI), the further increase of pressure levels (400 and 600 MPa) caused no change in emulsion activity index, which is in agreement with our results.

Other than these results, although the lowest emulsion activity value was observed at 400 MPa-25°C-pH 7 (58,88 %), at pH 5 and 7 (58,88%), there were no significant differences in the emulsion activity among pressure-temperature treatments at pH 5 and 7. Thus, it can be implied that HHP caused a minimal change in the structural properties of the polypeptides presents within the PPI for emulsification ability at these pHs ($p>0.05$).

Different protein modifications were observed due to HHP processing, so controversial results were seen about emulsifying properties of proteins. For instance, Chapleau and De Lamballerie-Anton (2003) emphasized that the processing at 400 MPa for 10 min enhanced emulsifying property of lupin protein (15 g/l) due to aggregation of 11S globulin and denaturation of 7S globulin and also an increase in temperature from 25 to 50°C resulted in enhanced emulsion activity for treated PPI samples.

Moreover, some authors reported that high solubility plays an essential role in having a good emulsifying property of protein samples (Deng et al., 2011; Fuhrmeister & Meuser, 2003; Gharsallaoui et al., 2012; Karaca et al., 2011; Liang & Tang, 2013; R. S. H. Lam & Nickerson, 2013). However, in our study, emulsion activity (%) values were negatively correlated with solubility ($r = -0.607$, $p<0.05$), indicating that HHP

treatment at different conditions induced protein modification by changing the hydration behavior and also the formation of small insoluble protein particles due to HHP resisted coalescence of fat globules as reported by Sumner et al. (1981), Stone et al. (2015) and Qin et al. (2013). Also, Stone et al. (2015) confirmed that higher viscosity in the continuous phase helps to inhibit coalescence.

3.4. Viscosity

As mentioned before, the viscosity of products reflects the flow behavior of substances in the liquid state and is an important parameter for food systems. Determination of viscosity index for systems is also crucial for the various types of production and design of equipment and the consumer acceptability of liquid & semisolid type foods (Mahajan & Ahluwalia, 2010; Walnofer & Horax, 2005). Thus, in this study, viscosity was used as a helpful indicator of the hydrodynamic & rheological properties of modified pea protein isolate supplied processing conditions, as seen in Table 3.1.

Table 3.1. Viscosity (cP) values of HHP-treated pea protein isolate (PPI) at different pH values (3, 5 and 7)

Pressure (MPa)-Temperature (°C)	**pH 3**	**pH 5**	**pH 7**
300 MPa-25°C	1.99 ± 0.33[b]	2.72 ± 0.71[a]	2.68 ± 0.49[a,b]
400 MPa-25°C	1.86 ± 0.29[b]	2.60 ± 0.14[a,b]	2.66 ± 0.11[a,b]
500 MPa-25°C	1.99 ± 0.14[b]	2.85 ± 0.07[a,b]	2.55 ± 0.14[b]
300 MPa-50°C	2.21 ± 0.11[b]	2.66 ± 0.02[a,b]	2.48 ± 0.21[b]
400 MPa-50°C	2.19 ± 0.27[b]	2.72 ± 0.07[a,b]	2.86 ± 0.20[a]
500 MPa-50°C	1.83 ± 0.04[b]	2.56 ± 0.07[a,b]	2.54 ± 0.15[a,b]

Different small letters indicate significant differences between different HHP conditions ($p<0.05$)

The results indicated that change in pH had a significant effect on the viscosity of samples, especially for pH 3 ($p<0.05$). Furthermore, HHP-treated PPI samples had significantly lower viscosity values than control (3.22±0,39a); the highest value was obtained at 400 MPa-50°C (2.86 cP) at pH 7. Therefore, the low viscosity of PPI at

most HHP-temperature conditions at pH 7 may probably be due to the breakdown of the existing aggregates, which were stabilized from weak interactions and modifications of protein structure as a result of pressure-temperature treatments (Peyrano et al., 2016). For instance, Li et al. (2012) have also observed a similar HHP effect on SPI (Soy Protein Isolate) (1% w/v at pH 6.8) at 300 MPa for 15 min. Herein, HHP caused considerably lower viscosity compared to that of control. Similarly, Peyrano et al. (2016) have reported that viscosity of cowpea protein isolates reduced significantly at 200, 400, and 600 MPa processing for 5 min at pH 8 and 10 compared to control.

Moreover, viscosity changed significantly at pH 3, which had the lowest value (1,83 cP) at 500 MPa-50 °C compared to other pHs and control. Therefore, the reason for higher viscosities at pH 5 and 7 may come from the different aggregation phenomena due to irreversible modification of protein structure. This result supported the concept that 7S and 11S could form the disulfide polymers due to processing conditions at pH 5 and 7, so a high amount of high-molecular-weight species may trigger increasing viscosity. In addition, Hutton and Campbell (1977) showed that the viscosity of soy isolate was higher as pH was increased from acidic to neutral pH at 4, ~25, and 90°C.

Other than these results, there were no significant differences in viscosity of pea protein isolate under different pressure-temperature combinations ($p>0.05$). Similarly, the study about the viscoelastic behavior of lupin protein (10% w/v) under HHP at 200, 400, 600 for 10 min had little or no effect on the viscoelastic properties (Chapleau & De Lamballerie-Anton, 2003). Moreover, Queirós et al. (2018) reported that HHP application at 250 MPa on soy protein dispersions showed more liquid-like behavior than control.

When the influence of temperature change was considered, our results revealed no significant differences between treated pea protein samples ($p>0.05$). However, Walnofer and Horax (2005) pointed out that the effect of heat treatment on soy protein isolate (SPI) at 50, 70, and 90°C resulted in significantly lower viscosity values.

Therefore, it can be said that level and duration of processing factors may cause various effects, and our results showed that temperature increase from 25 to 50°C was not enough to cause changes in viscosity of PPI.

In this study, the viscosity of treated pea protein isolate was positively moderate correlated with solubility ($r = 0.389$, $p<0.05$) and water holding capacity (WHC) ($r = 0.311$, $p<0.05$), so processing conditions could result in the formation of more compact aggregates that might be the reason for reduction of solubility and viscosity. Similar results were seen by Remondetto et al. (2001).

3.5. Fourier Transform Infrared (FTIR) Spectroscopy Analysis

FTIR analysis was used to examine the secondary structure of PPI as a consequence of HHP as seen from FTIR spectra and each peak band area. The results for HHP-treated PPI samples at pH 3, 5, and 7 were shown in figures 3.4, 3.6, and 3.8. Several chemical groups existing in amino acid residues and the peptide linkage itself resulted in plenty of vibrational bands that can be sensitive to the structural changes induced in the protein molecule under pressure-temperature treatments.

FTIR analysis was used to examine the secondary structure of PPI as a consequence of HHP, as seen from FTIR graphs and each peak band area. Also, data analysis provides information about minor variations in hydrogen bonding patterns and geometry (Kong & Yu, 2007). Amide I band is sensitive to the changes in the conformation of folding or unfolding and aggregation processes of proteins (Carbonaro et al., 2012). The amide I band (1700-1600 cm^{-1}) is mainly because of the C=O stretching vibration (Kong & Yu, 2007; Moreno et al., 2020). Thus, it is used to examine conformational changes and protein unfolding. Specifically, the major components that are α-helix; β-bands; intermolecular β-sheet aggregates; random structures were evaluated the peaks at different wavenumbers between 1650-1660 cm^{-1}; 1630-1638 cm^{-1}, 1660-1668 cm^{-1}; 1620-1630 cm^{-1}; 1640-1648 cm^{-1}, respectively by the authors (Bogahawaththa et al., 2019; Carbonaro et al., 2012). Also, Amide II

band (~1575 cm^{-1}) is used to observe the C-N stretching vibrations and N-H bending (Kong & Yu, 2007).

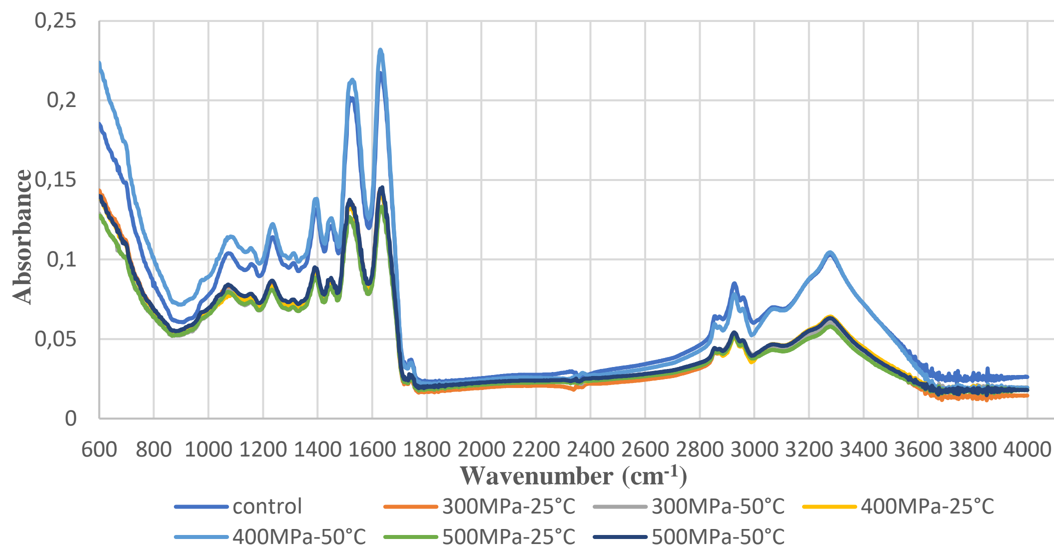

Figure 3.4. FTIR graph of both control and HHP-treated pea protein isolate (PPI) at pH 3

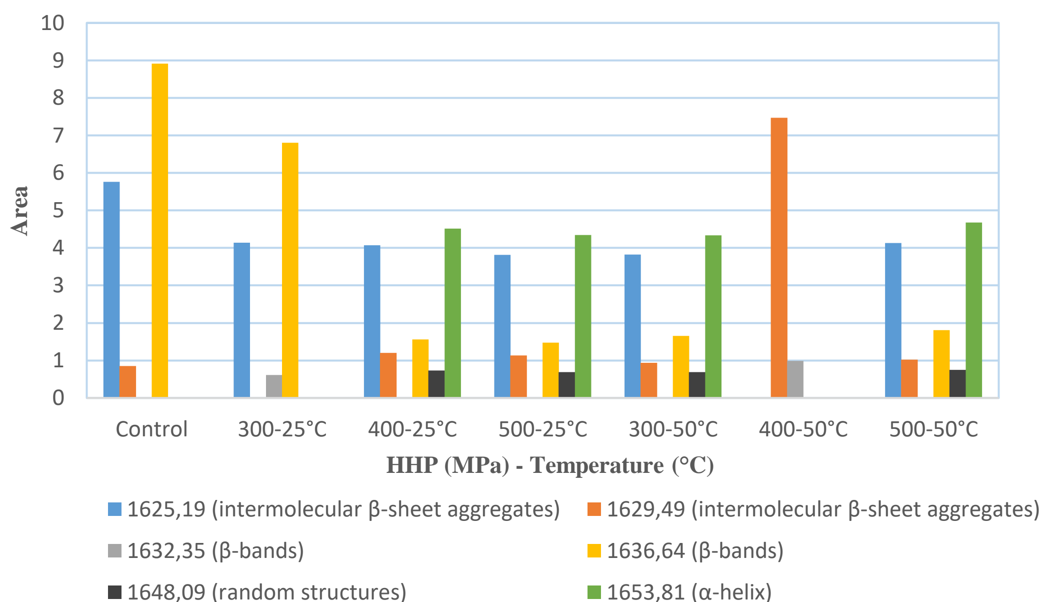

Figure 3.5. Peak position and relative area of both control and HHP-treated pea protein isolate (PPI) at pH 3

Differences in the Amide I region could be clearly observed under different HHP conditions. For all three pHs, the amide I band of most HHP treated pea protein isolate did not show the maximum at 1636.64 cm^{-1} like control samples. However, there were shifting peak bands at 1629.49, 1632.35, and 1653.81 cm^{-1} among different pressure-temperature treated PPI at various pHs (Figures 3.5, 3.7, 3.9).

The most evident modifications were mainly the disappearance of β-bands (1636.64 cm^{-1}) and shifting of intermolecular β-sheet aggregates peak bands from 1625.19 cm^{-1} to 1629.49 cm^{-1} in some cases, as can be seen from the figures. Therefore, in our results, the formation of higher intensity of intermolecular β-sheet aggregates occurred at 400 MPa-50°C-pH 3 and pH 5-500 MPa-50°C, monitored by an increase of a band at 1.625 cm^{-1} due to irreversible protein unfolding (Figures 3.5, 3.7). Similarly, Carbonaro et al. (2012) confirmed that autoclaved legume species showed the presence of the intermolecular β-sheets band in the range 1.620-1.630 cm^{-1}.

Moreover, the interesting point was that at 500 MPa-50°C-pH 5, the peak band at 1636.64 cm^{-1} disappeared, and formations of two peaks at 1629.49 cm^{-1} and more intensely at 1632.35 cm^{-1} were found. The shifting of the band to a low wavenumber like 1632.35 cm^{-1} under HHP processing might indicate the effect of hydrogen bonding, like increasing interactions with water molecules in solutions. This result also coincided with WHC results at this condition.

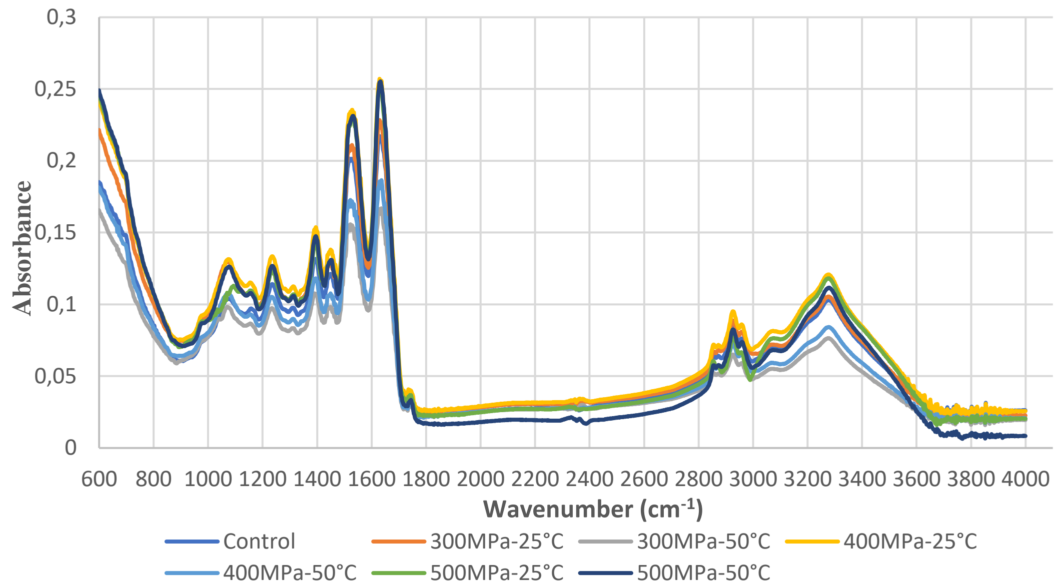

Figure 3.6. FTIR graph of both control and HHP-treated pea protein isolate (PPI) at pH 5

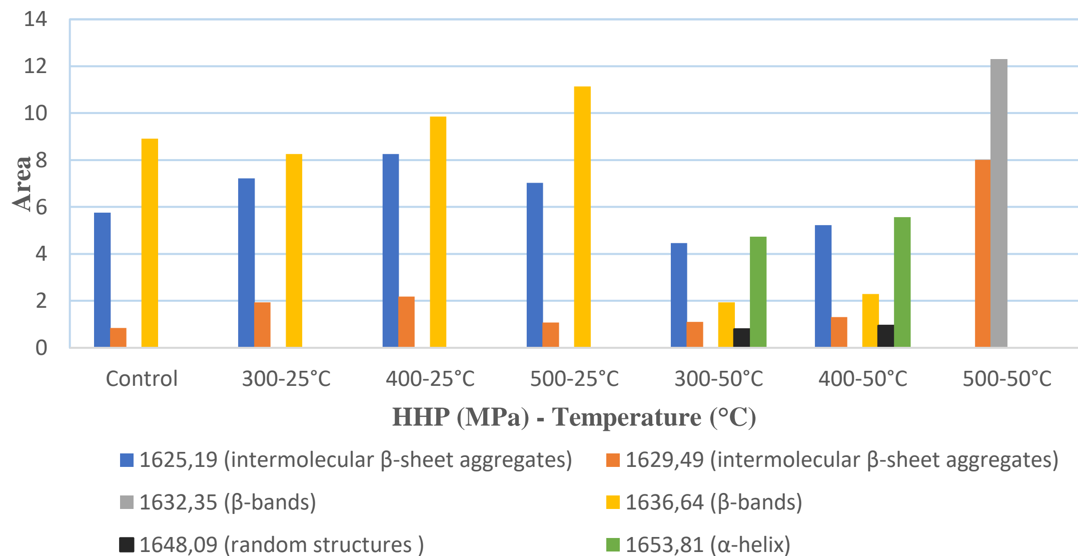

Figure 3.7. Peak position and relative area of both control and HHP-treated pea protein isolate (PPI) at pH 5

Furthermore, there were controversies about how protein solubility is affected due to intermolecular β-sheet aggregates, and they were found to be mostly water-soluble for pressure-treated PPI, according to our results. In addition, there was a formation of α-helix peak bands for pressure-treated PPI at 1653.81 cm^{-1} (Figures 3.5, 3.7 and 3.9). This observation may be related to the formation of intra- and intermolecular hydrogen bonds due to various conformational changes by the distortion of the native secondary structure (Secundo & Guerrieri, 2005). Therefore, in our case, HHP processing caused folding intermediates that can further unfold or refold and form stabilized networks by hydrogen bonds, which may prevent the forming of insoluble protein aggregates. A similar case was reported in the effects of HHP on myoglobin and on the autoclaved soybean by some authors (Carbonaro et al., 2012; Dzwolak et al., 2002).

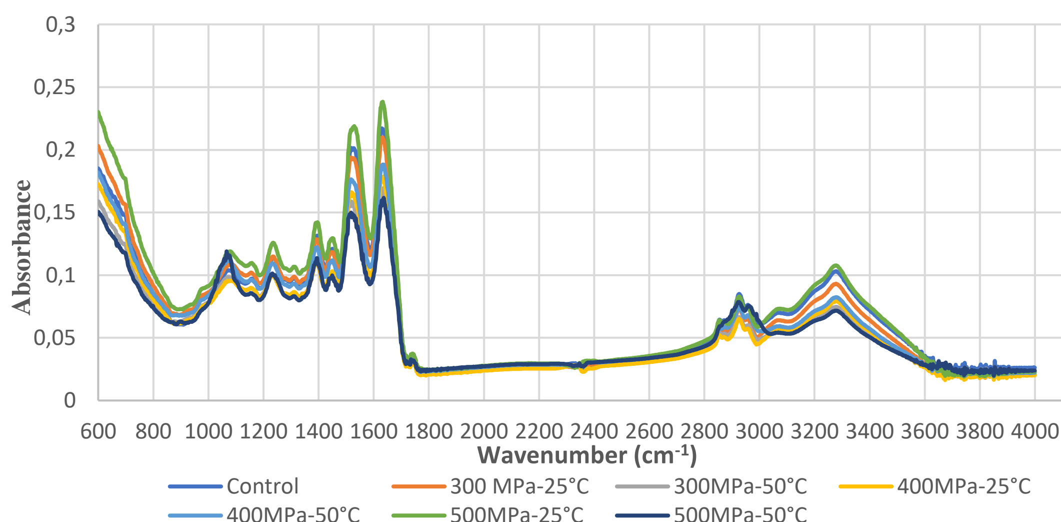

Figure 3.8. FTIR graph of both control and HHP-treated pea protein isolate (PPI) at pH 7

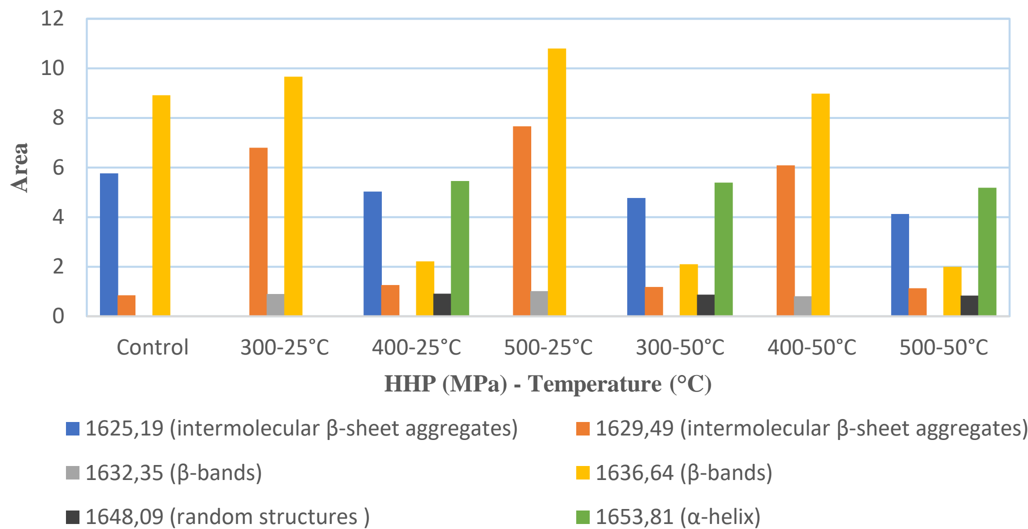

Figure 3.9. Peak position and relative area of both control and HHP-treated pea protein isolate (PPI) at pH 7

Moreover, HHP-temperature treatment caused shifting of wavenumbers mostly from 1636.64 cm^{-1} to 1629.49 cm^{-1} (intermolecular β-sheet aggregates) and 1653.81 cm^{-1} (α-helix) at pH 7. In contrast, the appearance of band peaks at 1636.64 cm^{-1} (β-bands) stayed higher intensities, especially at 300 MPa-25°C, 400 MPa-25 and 50°C compared to other pH changes and control (Figure 3.9). It shows a more rigid and folded globulin structure because β-bands have restrictions on the conformational entropy of the peptide chain for some processing conditions. This result could indicate an increase in solubility of treated PPI through the presence of soluble aggregates and partially refolding of the protein network.

3.6. Hydration Behavior of Pea Protein Isolate (PPI) by NMR relaxometry

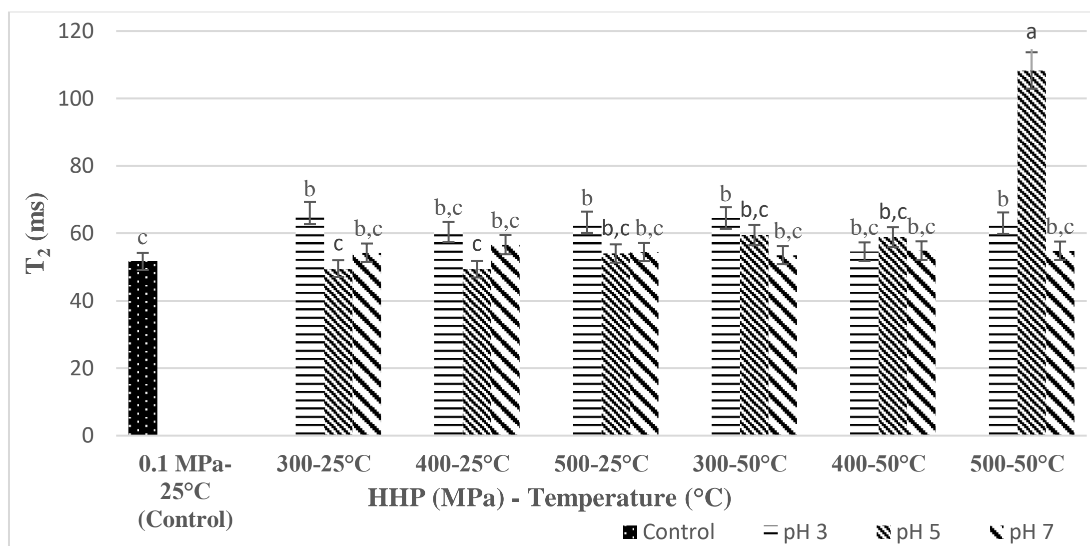

Figure 3.10. T_2 (ms) of both control and HHP-treated pea protein isolate (PPI) at different pH values (3, 5 and 7). Different small letters indicate significant differences between different HHP conditions ($p<0.05$)

It is known that protein activity is related to its hydration behavior, and H-bonding between protein surface polar groups and hydration water is a crucial factor for protein hydration (Mallamace et al., 2015). Therefore, NMR relaxometry experiments were carried out to explore the hydration behavior of HHP-treated pea protein isolate. Furthermore, T_2 relaxation times could be helpful to have information about water content, physical properties of water, and interactions with surrounding macromolecules (Kirtil & Oztop, 2016). Therefore, NMR transverse relaxation (T_2) times indicated hydration behavior due to different processing variables, as seen in Figure 3.10.

According to the statistical analysis, all factors had a significant influence on the hydration behavior of PPI ($p<0.05$). Although no significant differences were seen in T_2 times between HHP-treated samples at pH 5 ($p>0.05$), at 500 MPa-50 °C, PPI samples showed a drastic increase of T_2 relaxation time (108.31 ms) compared to

control (51.68 ms) and other HHP-treated samples ($p<0.05$). This result was attributed to the presence of a higher amount of free water content at this condition. Therefore, less hydrated PPI samples resulted in longer T_2 relaxation times (Musse et al., 2013). In addition to this, this case may arise from some relatively mobile water within interstices in the modified protein structure, thereby increasing water mobility.

When a change in pH was considered, HHP-treated PPI showed significantly longer T_2 times than control at pH 3 ($p<0.05$). Oztop et al. (2010) pointed out that conformational changes in the protein differ the accessibility of the exchangeable biopolymer protons to the solvation water. Based on this, HHP treatment increased biopolymer chain mobility that resulted in longer T_2 relaxation times at pH 3. Thus, an increase in T_2 times caused less hydrated samples at pH 3. Moreover, T_2 relaxation times did not change significantly due to increasing pressure levels and temperature at pH 3 ($p>0.05$).

Also, there were no significant differences in T_2 relaxation times between HHP-treated samples and control at pH 7 ($p>0.05$). Other than these results, temperature increase caused no significant differences in T_2 times for all conditions. Similarly, Coelho et al. (2007) have reported that heat treatment at between 21 and 90°C on β-lactoglobulin showed no change in T_2 times.

There was a moderate negative correlation between solubility and T_2 relaxation times ($r = -0.384$, $p<0.05$). Processing with HHP led to more tightly bound water, related to improved solubility of pea protein isolate samples. Also, a decrease in T_2 times can be attributed to the formation of soluble protein aggregates due to HHP processing.

CHAPTER 4

CONCLUSIONS & RECOMMENDATIONS

It can be clearly stated that the functional properties of pea protein isolate showed variations based on different processing parameters. It was seen that although pressure treatments at pH 3 and 5 did not lead to a significant change in solubility, the pressurization at pH 7 improved solubility around 60% compared to other pH values and control ($p<0.05$). Moreover, HHP at different levels did not cause a significant change in solubility at pH 3 and 5 compared to control ($p>0.05$).

Results showed some variations in the effects of HHP on water holding capacity (WHC) of PPI. HHP processing at pH 3 led to reduction of WHC significantly compared to control and pH 7 ($p<0.05$). However, HHP caused no significant differences in WHC at pH 5 compared with pH 3 and 7 for almost all conditions ($p>0.05$).

According to emulsion activity results, emulsification activity of PPI was enhanced by HHP treatments at pH 3 compared to control and other pH values ($p<0.05$), and no significant differences were observed between pH 5 and pH 7 ($p>0.05$).

Furthermore, viscosity results indicated that HHP treated PPI showed significantly lower viscosity than control at all three pH values ($p<0.05$). However, pressure increase did not lead to significant differences in viscosity of PPI at pH 3 and 5 ($p>0.05$).

In addition, HHP treatments at pH 3, 5 and 7 resulted in a change in hydrogen bonding and loss of secondary structure of PPI due to possibly irreversible protein unfolding according to FTIR analysis.

NMR Relaxometry was another analysis to get information about hydration behavior of both treated and control samples. Comparing T_2 values of control and HHP treated samples at pH 3 showed significantly slower T_2 relaxations at pH 3 ($p<0.05$) due to high water mobility due to HHP processing. Other than that, there were no significant differences in hydration behavior of HHP treated PPI due to both pressure and temperature increase ($p>0.05$) for three pHs except at 500 MPa-50°C processing. Therefore, it can be concluded that supplied conditions are critical for the alteration of hydration for pea proteins.

Based on this work, HHP treatment for all levels at pH 7 could be an advantage in enhancing the solubility and hydration behavior as well. This modification process could solve the poor solubility issue for pea proteins, and it may be an alternative for novel food applications like in beverages. Herein, in addition to these two properties, HHP processing could be applicable to improve emulsification ability at pH 3 for pea protein emulsion products and ready-to-drink beverages. When these three functional properties were considered together, HHP could help to develop novel products.

In conclusion, this study proved that the modification of pea protein could be achieved by HHP processing, leading to rupturing, denaturation, and aggregation of the protein molecules. Thus, through conformational, structural, and surface change modifications, high hydrostatic pressure (HHP) application could be a good alternative to have different functional properties of pea protein isolates (PPI).

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APPENDICES

A. Calibration Curve

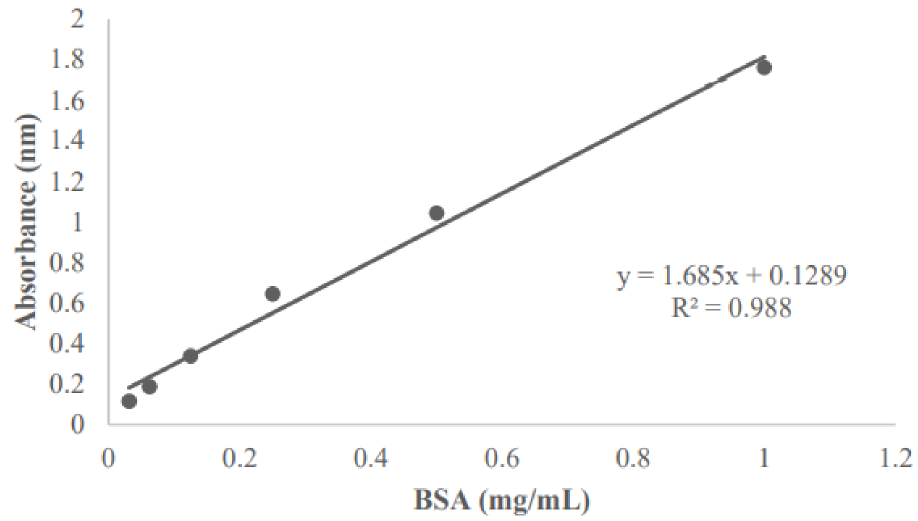

Figure A.1. Example of a calibration curve for Lowry Method prepared by Bovine Serum Albumin for determining total soluble protein contents in both HHP-treated and control PPI samples

Absorbance (at 750 nm) = 1.685 * (mg BSA/ml) + 0.1289 where R^2 = 0.988

B. Statistical Analyses

Table B.1. ANOVA and Tukey's Comparison Test with 95% confidence level for determining solubility by Lowry Method

General Linear Model: Solubility versus P-So; T-So; pH So

Factor	Type	Levels	Values
P-So	fixed	3	300,0; 400,0; 500,0
T-So	fixed	2	25; 50
pH So	fixed	3	3; 5; 7

Analysis of Variance for Solubility, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
P-So	2	0,8	0,8	0,4	0,01	0,986
T-So	1	241,1	241,1	241,1	8,46	0,006
pH So	2	47956,4	47956,4	23978,2	841,73	0,000
P-So*T-So	2	123,4	123,4	61,7	2,17	0,129
T-So*pH So	2	457,0	457,0	228,5	8,02	0,001
P-So*pH So	4	41,4	41,4	10,4	0,36	0,833
P-So*T-So*pH So	4	210,1	210,1	52,5	1,84	0,142
Error	36	1025,5	1025,5	28,5		
Total	53	50055,8				

S = 5,33731   R-Sq = 97,95%   R-Sq(adj) = 96,98%

R denotes an observation with a large standardized residual.

Grouping Information Using Tukey Method

Pressure	N	Mean	Grouping
400,0	18	34,08	A
300,0	18	34,06	A
500,0	18	33,81	A
0,1	27	14,97	B

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

pH	N	Mean	Grouping
7	18	76,118	A
5	18	13,701	B
3	18	12,130	B

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Pressure Temperature pH7	N	Mean	Grouping
300 50	3	88,591	A
400 50	3	81,370	A
500 50	3	76,820	A
500 25	3	75,145	A
400 25	3	68,142	A
300 25	3	66,639	A

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Source	DF	SS	MS	F	P
PressureTemperature	5	10,662	2,132	12,57	0,000
Error	12	2,036	0,170		
Total	17	12,698			

S = 0,4119   R-Sq = 83,96%   R-Sq(adj) = 77,28%

Grouping Information Using Tukey Method

PressureTemperature pH5	N	Mean	Grouping
40025	3	15,0212	A
30025	3	14,0716	A B
50025	3	13,8738	B
40050	3	13,6127	B C
30050	3	12,9875	B C
50050	3	12,6394	C

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Source	DF	SS	MS	F	P
PressureTemperature	5	48,997	9,799	10,14	0,001
Error	12	11,602	0,967		
Total	17	60,599			

S = 0,9833   R-Sq = 80,86%   R-Sq(adj) = 72,88%

Grouping Information Using Tukey Method

PressureTemperature pH3	N	Mean	Grouping
40050	3	15,5751	A
50025	3	12,3545	B
50050	3	12,0459	B
30050	3	11,2229	B
30025	3	10,8194	B
40025	3	10,7640	B

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Pressure

Pressure	pH 25°C	N	Mean	Grouping
500	7	3	75,145	A
400	7	3	68,142	A
300	7	3	66,639	A
400	5	3	15,021	B
300	5	3	14,072	B
500	5	3	13,874	B
500	3	3	12,355	B
300	3	3	10,819	B
400	3	3	10,764	B

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Pressure	pH 50°C	N	Mean	Grouping
300	7	3	88,591	A
400	7	3	81,370	A B
500	7	3	76,820	B
400	3	3	15,575	C
400	5	3	13,613	C
300	5	3	12,988	C
500	5	3	12,639	C
500	3	3	12,046	C
300	3	3	11,223	C

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Temperature	pH 300MPa	N	Mean	Grouping
50	7	3	88,591	A
25	7	3	66,639	B
25	5	3	14,072	C
50	5	3	12,988	C
50	3	3	11,223	C
25	3	3	10,819	C

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Temperature	pH 400MPa	N	Mean	Grouping
50	7	3	81,37	A
25	7	3	68,14	A
50	3	3	15,58	B
25	5	3	15,02	B
50	5	3	13,61	B
25	3	3	10,76	B

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Temperature	pH 500MPa	N	Mean	Grouping
50	7	3	76,820	A
25	7	3	75,145	A
25	5	3	13,494	B
50	5	3	13,264	B
25	3	3	12,355	B
50	3	3	12,046	B

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Pressure	Temperature	pH	N	Mean	Grouping
300	50	7	3	88,59	A
400	50	7	3	81,37	A B
500	50	7	3	76,82	A B
500	25	7	3	75,15	A B
400	25	7	3	68,14	B
300	25	7	3	66,64	B
400	50	3	3	15,58	C
400	25	5	3	15,02	C
300	25	5	3	14,07	C
500	25	5	3	13,87	C
400	50	5	3	13,61	C
300	50	5	3	12,99	C
500	50	5	3	12,64	C
500	25	3	3	12,35	C
500	50	3	3	12,05	C
300	50	3	3	11,22	C
300	25	3	3	10,82	C
400	25	3	3	10,76	C

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Table B.2. ANOVA and Tukey's Comparison Test with 95% confidence level for determining Water Holding Capacity (WHC)

General Linear Model: WHC versus Pressure; Temperature; pH

Factor	Type	Levels	Values
Pressure	fixed	3	300,0; 400,0; 500,0
Temperature	fixed	2	25; 50

pH	fixed	3	3; 5; 7

Analysis of Variance for WHC, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Pressure	2	2,4579	2,4579	1,2290	10,89	0,000
Temperature	1	1,2195	1,2195	1,2195	10,80	0,002
pH	2	12,1764	12,1764	6,0882	53,93	0,000
Pressure*Temperature	2	1,7002	1,7002	0,8501	7,53	0,002
Temperature*pH	2	2,8316	2,8316	1,4158	12,54	0,000
Pressure*pH	4	11,2652	11,2652	2,8163	24,95	0,000
Pressure*Temperature*pH	4	25,8314	25,8314	6,4578	57,21	0,000
Error	36	4,0638	4,0638	0,1129		
Total	53	61,5460				

S = 0,335981   R-Sq = 93,40%   R-Sq(adj) = 90,28%

Unusual Observations for WHC

Obs	WHC	Fit	SE Fit	Residual	St Resid
19	4,37805	3,58943	0,19398	0,78862	2,87 R

R denotes an observation with a large standardized residual.

Grouping Information Using Tukey Method

Pressure	N	Mean	Grouping
0,1	18	4,0876	A
400,0	18	3,5367	A B
300,0	18	3,2611	B
500,0	18	3,0143	B

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

pH	N	Mean	Grouping
5	18	3,8033	A
7	18	3,3587	A B
3	18	2,6502	B

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Pressure-Temperature pH5	N	Mean	Grouping
500-50	3	5,4779	A
300-50	3	3,7515	B
400-50	3	3,5894	B
400-25	3	3,5863	B

	N	Mean	Grouping
300-25	3	3,2452	B
500-25	3	3,1692	B

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

PressureTemperature pH 7	N	Mean	Grouping
30050	3	5,0315	A
40025	3	4,1474	B
50025	3	3,5685	B
40050	3	2,7820	C
50050	3	2,3731	C
30025	3	2,2499	C

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

PressureTemperature pH 3	N	Mean	Grouping
40050	3	4,3569	A
30025	3	3,0863	B
40025	3	2,7580	B
50025	3	2,2732	B
30050	3	2,2024	B
50050	3	1,2241	C

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Pressure Ph at 25°C

	N	Mean	Grouping
4007	3	4,1474	A
4005	3	3,5863	A B
5007	3	3,5685	A B
3005	3	3,2452	A B
5005	3	3,1692	B C
3003	3	3,0863	B C
4003	3	2,7580	B C
5003	3	2,2732	C
3007	3	2,2499	C

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Pressure	pH at 50˚C	N	Mean	Grouping
500	5	3	5,4779	A
300	7	3	5,0315	A B
400	3	3	4,3569	B C
300	5	3	3,7515	C D
400	5	3	3,5894	C D
400	7	3	2,7820	D E
500	7	3	2,3731	E
300	3	3	2,2024	E
500	3	3	1,2241	F

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Temperature	pH 300MPa	N	Mean	Grouping
50	7	3	5,0315	A
50	5	3	3,7515	B
25	5	3	3,2452	B
25	3	3	3,0863	B C
25	7	3	2,2499	C
50	3	3	2,2024	C

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method

Temperature	pH 400MPa	N	Mean	Grouping
50	3	3	4,3569	A
25	7	3	4,1474	A
50	5	3	3,5894	A B
25	5	3	3,5863	A B
50	7	3	2,7820	B
25	3	3	2,7580	B

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Temperature	pH at 500MPa	N	Mean	Grouping
50	5	3	5,4779	A
25	7	3	3,5685	B
25	5	3	3,1692	B C
50	7	3	2,3731	C D
25	3	3	2,2732	D
50	3	3	1,2241	E

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Pressure	Temperature	pH	N	Mean	Grouping
500	50	5	3	5,4779	A
300	50	7	3	5,0315	A B
400	50	3	3	4,3569	B C
400	25	7	3	4,1474	B C D
300	50	5	3	3,7515	C D E
400	50	5	3	3,5894	C D E
400	25	5	3	3,5863	C D E
500	25	7	3	3,5685	C D E
300	25	5	3	3,2452	D E F
500	25	5	3	3,1692	D E F G
300	25	3	3	3,0863	E F G
400	50	7	3	2,7820	E F G
400	25	3	3	2,7580	E F G
500	50	7	3	2,3731	F G
500	25	3	3	2,2732	F G
300	25	7	3	2,2499	F G H
300	50	3	3	2,2024	G H
500	50	3	3	1,2241	H

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Table B.3. ANOVA and Tukey's Comparison Test with 95% confidence level for determining Emulsion Activity

General Linear Model: Emulsion Activity versus P-Emulsion; T-Emulsion; pH-Emulsion

Factor	Type	Levels	Values
P-Emulsion	fixed	3	300,0; 400,0; 500,0
T-Emulsion	fixed	2	25; 50
pH-Emulsion	fixed	3	3; 5; 7

Analysis of Variance for Emulsion Activity, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
P-Emulsion	2	19,851	19,851	9,926	1,31	0,284
T-Emulsion	1	2,335	2,335	2,335	0,31	0,583
pH Emulsion	2	1511,181	1511,181	755,591	99,37	0,000
P-Emulsion*T-Emulsion	2	62,329	62,329	31,165	4,10	0,025
P-Emulsion*pH Emulsion	4	30,634	30,634	7,659	1,01	0,417
T-Emulsion*pH Emulsion	2	0,151	0,151	0,075	0,01	0,990
P-Emulsion*T-Emulsion*pHEmuls	4	28,525	28,525	7,131	0,94	0,453
Error	36	273,733	273,733	7,604		
Total	53	1928,739				

S = 2,75748   R-Sq = 85,81%   R-Sq(adj) = 79,11%

```
Unusual Observations for Emulsion Activity

     Emulsion
Obs  Activity      Fit  SE Fit  Residual  St Resid
 16   57,1429  61,7611  1,5920   -4,6182     -2,05 R
 25   65,3846  59,6631  1,5920    5,7215      2,54 R
 26   50,8929  59,6631  1,5920   -8,7703     -3,90 R

R denotes an observation with a large standardized residual.

Source     DF      SS    MS     F      P
Pressuree   3   205,9  68,6  2,05  0,115
Error      68  2274,8  33,5
Total      71  2480,7

S = 5,784   R-Sq = 8,30%   R-Sq(adj) = 4,26%


Grouping Information Using Tukey Method

Pressure   N    Mean  Grouping
300,0      18  66,269  A
500,0      18  65,086  A
400,0      18  64,901  A
  0,1      18  61,706  A

Means that do not share a letter are significantly different.


Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

pH Emulsion   N    Meo  Grouping
3            18  72,705  A
5            18  63,245    B
7            18  60,306      C

Means that do not share a letter are significantly different.


Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

T-Emulsion   N    Mean  Grouping
50          27  65,627  A
25          27  65,211  A

Means that do not share a letter are significantly different.


Tukey 95% Simultaneous Confidence Intervals


Grouping Information Using Tukey Method

Pressure Temperature pH5  N    Mean  Grouping
300         25           3  67,700  A
500         50           3  64,409  A B
400         50           3  63,081  A B
300         50           3  62,712  A B
```

500	25	3	61,761	B
400	25	3	59,809	B

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Pressure	Temperature pH 7	N	Mean	Grouping
400	50	3	62,144	A
500	25	3	60,868	A
300	25	3	60,608	A
500	50	3	59,675	A
300	50	3	59,663	A
400	25	3	58,876	A

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Pressure	Temperature pH3	N	Mean	Grouping
300	25	3	74,156	A
400	50	3	73,602	A
300	50	3	72,778	A
500	50	3	72,576	A
400	25	3	71,895	A
500	25	3	71,223	A

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Pressure	pH 25°C	N	Mean	Grouping
300	3	3	74,156	A
400	3	3	71,895	A
500	3	3	71,223	A
300	5	3	67,700	A B
500	5	3	61,761	B C
500	7	3	60,868	B C
300	7	3	60,608	B C
400	5	3	59,809	C
400	7	3	58,876	C

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Pressure	pH 50°C	N	Mean	Grouping
400	3	3	73,602	A

300	3	3	72,778	A
500	3	3	72,576	A
500	5	3	64,409	B
400	5	3	63,081	B
300	5	3	62,712	B
400	7	3	62,144	B
500	7	3	59,675	B
300	7	3	59,663	B

Means that do not share a letter are significantly different
Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Temperature	pH 300MPa	N	Mean	Grouping
25	3	3	74,156	A
50	3	3	72,778	A
25	5	3	67,700	A B
50	5	3	62,712	B
25	7	3	60,608	B
50	7	3	59,663	B

Grouping Information Using Tukey Method

Temperature	pH 400MPa	N	Mean	Grouping
50	3	3	73,602	A
25	3	3	71,895	A
50	5	3	63,081	B
50	7	3	62,144	B C
25	5	3	59,809	B C
25	7	3	58,876	C

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Temperature	pH 500MPa	N	Mean	Grouping
50	3	3	72,576	A
25	3	3	71,223	A B
50	5	3	64,409	B C
25	5	3	61,761	C
25	7	3	60,868	C
50	7	3	59,675	C

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Pressure	Temperature	pH	N	Mean	Grouping
300	25	3	3	74,156	A
400	50	3	3	73,602	A
300	50	3	3	72,778	A B
500	50	3	3	72,576	A B
400	25	3	3	71,895	A B

500	25	3	3	71,223	A B C
300	25	5	3	67,700	A B C D
500	50	5	3	64,409	B C D E
400	50	5	3	63,081	C D E
300	50	5	3	62,712	D E
400	50	7	3	62,144	D E
500	25	5	3	61,761	D E
500	25	7	3	60,868	D E
300	25	7	3	60,608	D E
400	25	5	3	59,809	D E
500	50	7	3	59,675	D E
300	50	7	3	59,663	D E
400	25	7	3	58,876	E

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Table B.4. ANOVA and Tukey's Comparison Test with 95% confidence level for determining Viscosity

General Linear Model: Viscosity versus P-Viscosity; pH-Viscosity; T-Viscosity

Factor	Type	Levels	Values
P-Viscosity	fixed	3	300,0; 400,0; 500,0
pH Viscosity	fixed	3	3; 5; 7
T-Viscosity	fixed	2	25; 50

Analysis of Variance for Viscosity, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
P-Viscosity	2	0,0901	0,0901	0,0451	0,32	0,729
pH Viscosity	2	5,0089	5,0089	2,5044	17,71	0,000
T-Viscosity	1	0,0043	0,0043	0,0043	0,03	0,863
P-Viscosity*T-Viscosity	2	0,3067	0,3067	0,1534	1,08	0,349
P-Viscosity*pH Viscosity	4	0,1925	0,1925	0,0481	0,34	0,849
pH Viscosity*T-Viscosity	2	0,1043	0,1043	0,0522	0,37	0,694
P-Viscosity*pHViscosity*T-Viscosity	4	0,1301	0,1301	0,0325	0,23	0,920
Error	36	5,0923	5,0923	0,1415		
Total	53	10,9293				

S = 0,376103   R-Sq = 53,41%   R-Sq(adj) = 31,40%

Unusual Observations for Viscosity

Obs	Viscosity	Fit	SE Fit	Residual	St Resid
4	2,00000	2,72333	0,21714	-0,72333	-2,36 R
22	1,78000	2,67667	0,21714	-0,89667	-2,92 R
23	2,06000	2,67667	0,21714	-0,61667	-2,01 R
24	4,19000	2,67667	0,21714	1,51333	4,93 R

R denotes an observation with a large standardized residual.

Grouping Information Using Tukey Method

Pressure	N	Mean	Grouping
0,1	18	3,2233	A
400,0	18	2,4811	B
300,0	18	2,4572	B
500,0	18	2,3850	B

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Pressure

Grouping Information Using Tukey Method

pH Viscosity	N	Mean	Grouping
5	18	2,6844	A
7	18	2,6272	A
3	18	2,0117	B

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

T-Viscosity	N	Mean	Grouping
50	27	2,4500	A
25	27	2,4322	A

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Pressure Temperature pH5	N	Mean	Grouping
50025	3	2,8467	A
30025	3	2,7233	A
40050	3	2,7167	A
30050	3	2,6600	A
40025	3	2,6000	A
50050	3	2,5600	A

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Pressure Temperature pH7	N	Mean	Grouping

40050	3	2,8567	A
30025	3	2,6767	A
40025	3	2,6633	A
50025	3	2,5467	A
50050	3	2,5400	A
30050	3	2,4800	A

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Pressure Temperature pH3	N	Mean	Grouping
30050	3	2,2133	A
40050	3	2,1933	A
30025	3	1,9900	A
50025	3	1,9867	A
40025	3	1,8567	A
50050	3	1,8300	A

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Pressure pH 25°C	N	Mean	Grouping
5005	3	2,8467	A
3005	3	2,7233	A
3007	3	2,6767	A
4007	3	2,6633	A
4005	3	2,6000	A
5007	3	2,5467	A
3003	3	1,9900	A
5003	3	1,9867	A
4003	3	1,8567	A

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Pressure	pH 50°C	N	Mean	Grouping
400	7	3	2,8567	A
400	5	3	2,7167	A
300	5	3	2,6600	A
500	5	3	2,5600	A B
500	7	3	2,5400	A B
300	7	3	2,4800	A B
300	3	3	2,2133	B
400	3	3	2,1933	B C
500	3	3	1,8300	C

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Temperature	Ph 300MPa	N	Mean	Grouping
25	5	3	2,7233	A
25	7	3	2,6767	A
50	5	3	2,6600	A
50	7	3	2,4800	A
50	3	3	2,2133	A
25	3	3	1,9900	A

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method

Temperature	pH 400MPa	N	Mean	Grouping
50	7	3	2,8567	A
50	5	3	2,7167	A
25	7	3	2,6633	A B
25	5	3	2,6000	A B
50	3	3	2,1933	B C
25	3	3	1,8567	C

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Temperature	pH 500MPa	N	Mean	Grouping
25	5	3	2,8467	A
50	5	3	2,5600	B
25	7	3	2,5467	B
50	7	3	2,5400	B
25	3	3	1,9867	C
50	3	3	1,8300	C

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Pressure	Temperature	pH	N	Mean	Grouping
400	50	7	3	2,8567	A
500	25	5	3	2,8467	A
300	25	5	3	2,7233	A
400	50	5	3	2,7167	A
300	25	7	3	2,6767	A
400	25	7	3	2,6633	A
300	50	5	3	2,6600	A
400	25	5	3	2,6000	A
500	50	5	3	2,5600	A
500	25	7	3	2,5467	A
500	50	7	3	2,5400	A
300	50	7	3	2,4800	A
300	50	3	3	2,2133	A

```
400        50         3  3  2,1933    B
300        25         3  3  1,9900    B
500        25         3  3  1,9867    B
400        25         3  3  1,8567    B
500        50         3  3  1,8300    B

Means that do not share a letter are significantly different.
```

Table B.5. ANOVA and Tukey's Comparison Test with 95% confidence level for determining T_2 relaxation times by NMR Relaxometry

General Linear Model: T2 versus P- NMR; T- NMR; pH- NMR

```
Factor  Type   Levels  Values
P- NMR  fixed       3  300,0; 400,0; 500,0
T- NMR  fixed       2  25; 50
pH NMR  fixed       3  3; 5; 7


Analysis of Variance for T2, using Adjusted SS for Tests

Source                 DF   Seq SS   Adj SS   Adj MS      F      P
P- NMR                  2  1120,77  1120,77   560,39  33,02  0,000
T- NMR                  1   684,47   684,47   684,47  40,33  0,000
pH NMR                  2   756,61   756,61   378,30  22,29  0,000
P- NMR*T-NMR            2   828,03   828,03   414,02  24,39  0,000
T- NMR*pH NMR           2  2065,01  2065,01  1032,51  60,83  0,000
P- NMR*pH NMR           4  1967,78  1967,78   491,95  28,98  0,000
P- NMR*T-NMR*pH-NMR     4  1185,70  1185,70   296,43  17,46  0,000
Error                  36  611,03   611,03    16,97
Total                  53  9219,41


S = 4,11985   R-Sq = 93,37%   R-Sq(adj) = 90,24%


Unusual Observations for T2

Obs       T2      Fit  SE Fit  Residual  St Resid
 12   41,658   49,381   2,379    -7,723     -2,30 R
 20   99,440  108,310   2,379    -8,870     -2,64 R
 21  123,480  108,310   2,379    15,170      4,51 R

R denotes an observation with a large standardized residual.


Grouping Information Using Tukey Method

Pressure   N   Mean  Grouping
500,0     18  66,33  A
300,0     18  57,90  A B
400,0     18  55,79    B
  0,1     18  51,68    B

Means that do not share a letter are significantly different.
```

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

T- NMR	N	Mean	Grouping
50	27	63,57	A
25	27	56,45	B

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

pH NMR	N	Mean	Grouping
5	18	63,27	A
3	18	61,98	A
7	18	54,76	A

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

PressureTemperature pH5	N	Mean	Grouping
50050	3	108,310	A
30050	3	59,514	B
40050	3	58,864	B
50025	3	54,030	B
30025	3	49,536	B
40025	3	49,381	B

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

PressureTemperature pH7	N	Mean	Grouping
30050	3	56,624	A
50025	3	54,884	A
50050	3	54,840	A
40025	3	54,453	A
30025	3	54,280	A
40050	3	53,507	A

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

PressureTemperature pH3	N	Mean	Grouping
30025	3	66,012	A

	N	Mean	Grouping
30050	3	64,526	A
50025	3	63,303	A
50050	3	63,057	A
40025	3	60,387	A B
40050	3	54,588	B

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Pressure pH 25°C	N	Mean	Grouping
3003	3	66,012	A
5003	3	63,303	A B
4003	3	60,387	A B C
4007	3	56,624	B C D
5007	3	54,453	B C D
3007	3	54,280	C D
5005	3	54,030	C D
3005	3	49,536	D
4005	3	49,381	D

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Pressure pH 50°C	N	Mean	Grouping
5005	3	108,310	A
3003	3	64,526	B
5003	3	63,057	B
3005	3	59,514	B
4005	3	58,864	B
4007	3	54,884	B
5007	3	54,840	B
4003	3	54,588	B
3007	3	53,507	B

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Temperature pH 300MPa	N	Mean	Grouping
253	3	66,012	A
503	3	64,526	A B
505	3	59,514	B C
257	3	54,280	C D
507	3	53,507	D
255	3	49,536	D

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Temperature pH 400MPa	N	Mean	Grouping
253	3	60,387	A
505	3	58,864	A
257	3	56,624	A B
507	3	54,884	A B
503	3	54,588	A B
255	3	49,381	B

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals

Grouping Information Using Tukey Method

Temperature pH 500MPa	N	Mean	Grouping
505	3	108,310	A
253	3	63,303	B
503	3	63,057	B
507	3	54,840	B
257	3	54,453	B
255	3	54,030	B

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Interval
Grouping Information Using Tukey Method

C34	N	Mean	Grouping
500505	3	108,310	A
300253	3	66,012	B
300503	3	64,526	B
500253	3	63,303	B
500503	3	63,057	B
400253	3	60,387	B C
300505	3	59,514	B C
400505	3	58,864	B C
400257	3	56,624	B C
400507	3	54,884	B C
500507	3	54,840	B C
400503	3	54,588	B C
500257	3	54,453	B C
300257	3	54,280	B C
500255	3	54,030	B C
300507	3	53,507	B C
300255	3	49,536	C
400255	3	49,381	C

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals