EFFECTS OF THERMAL AND HIGH PRESSURE TREATMENTS ON STRUCTURAL AND FUNCTIONAL PROPERTIES OF PEA SEED (*PISUM SATIVUM* L.) PROTEINS AND ENZYMATIC PROTEIN HYDROLYSATES

BY

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ABSTRACT

The effects of heat or high pressure treatment on the physicochemical and functional properties of pea proteins were evaluated by measuring polypeptide composition, hydrophobicity, solubility, gelation, emulsification, foaming, water-holding capacity and oil-holding capacity. Heat processing (≥ 70 °C) and high pressure treatment (≥ 200 MPa) led to significant increase (from 1.41 to 2.42) in hydrophobicity of native pea proteins. Native gel electrophoresis showed that the content of 11S protein decreased (increased aggregation) as intensity of pressure treatment was increased. In contrast the 7S protein was resistant to pressure-induced protein aggregation. The solubility and emulsifying capacity of pea proteins processed under higher pressure or heat at neutral pH had slight decreases probably due to the formation of aggregates. High pressure treatment of pea proteins led to reductions in the amount of protease required to produce renin-inhibitory peptides.

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DEDICATION

I wish to dedicate this thesis to

My great parents, Heju Chao and Yunru Zhang

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CHAPTER ONE - INTRODUCTION

The utilization of proteins has received much attention in past decades and it is hardly in doubt that larger supplies will be needed in the future to satisfy growing demand (Johnson & Brekke, 1983). Legumes such as peas, beans and peanuts, have been used as sources of protein in the diet of people from many cultures for centuries. Moreover, industry has been turning towards plants as a preferred alternative of protein ingredients to animal-based sources (e.g., milk proteins, chicken proteins, etc), mainly due to the abundant plant resources but also because of increased consumer concerns over the safety of animal-derived products and nutritional considerations to reduce fat consumption. Religious concerns are also an important factor contributing to the increased popularity of plant-based food products because certain religions prohibit consumption of specific animal products. For example, Muslims and Jews are prohibited from consuming pork products while Hindus do not consume cow (beef) products. The food industry's continuing increased interest in plant-derived food ingredients such as those from pea seeds serves to promote the study of plant protein utilization in the formulation of new food products.

As pea plant is less sensitive and can tolerate low temperatures for germination and growth, the cultivation of peas provides a good cool-season alternative for regions that are not suitable for growing soybeans because of their climate conditions (Miller, Fischer, & Marqusee, 2002). Pea is one of the world's oldest crops because records indicate it was grown in the Middle East approximately 9000 years ago. At present, it is being grown in 84 countries including Canada, China, Russia and the United States.

Canada is the leading country for pea production, with an annual production that accounts for 30% of the world's pea stock in 2007-2008 (Agriculture and Agri-Food Canada, 2011). Green and yellow peas are primarily produced in Canada, and only small quantities of maple, marrowfat and Austrian peas are produced (Pulse Canada, 2011).

The composition of pea proteins, like other legume proteins can be classified into three main classes as follows:

1) globulins (legumins, vicilins and convicilins), which are the main storage proteins of the plant seed;

2) Albumins, which consist of molecules that have a functional role in the seed;

3) "Insoluble" proteins, which are rarely studied.

In addition, the pea proteins are characterized by high lysine content, which is deficient in many other proteins of plant origin including cereals; therefore, pea proteins play a central role in food protein supply and human nutrition of many countries (Tomoskozi, Finance, & Aranyi, 2002).

In order to develop plant protein usage as food ingredients, their physicochemical and functional properties must be evaluated. Although pea proteins are nutritionally excellent, their functional behavior in food system does not seem good enough to meet the expectations of the food industry.

1.1 OBJECTIVES OF STUDY

The objectives of the study were to:

- Determine the effects of high pressure and thermal treatments on functional and physicochemical properties of pea proteins

- Determine effects of high pressure and thermal treatments on yield and in vitro bioactive properties of enzymatic pea protein hydrolysates

1.2 HYPOTHESES

- Pea protein functional properties such as emulsification, foaming and gelation can be improved through high pressure or thermal treatment.
- High pressure or thermal treatment will enhance enzymatic digestion of pea proteins to produce higher yields of peptides and increased anti-hypertensive activity of the peptides

CHAPTER TWO - LITERATURE REVIEW

2.1 PEA SEEDS AND PROTEINS

In the food industry, proteins represent an important class of active ingredients because they possess a range of dynamic functional properties (Phillips, Horn, & Smith, 1995). These functional properties reflect complex interactions between the composition, structure, conformation, and other physicochemical properties of the protein (Kinsella & Shetty, 1981). Studies have shown that emulsion and foam formations are dependent on protein levels in pea flours based on the fact that the protein-deficient flours exhibited poor functional properties (Aluko et al., 2009). Therefore, it is important to determine the functional, physiochemical, and nutritional properties of high protein pea products such as pea protein isolates in order to broaden utilization in the food industry.

2.2 FUNCTIONAL PROPERTIES OF PEA PROTEINS

Pea protein isolate has been shown to perform many functions desirable for food ingredients in processed foods (Sumner, Nielsen, & Youngs, 1981). A previous work showed that after adding pea protein concentrate to extend ground beef in preparation of hamburgers, the beef patties were softer, tenderer and required less force to compress than all–beef patties; the pea-modified cooked hamburger also presented less fat-retention than regular beef patties (Vaisey, Tassos, Mcdonald, & Youngs, 1975). One study conducted in early 80s showed that after spaghetti was fortified with pea proteins, the products were observed to have reduced raw noodle strength and cooking time with higher cooking losses. However, the sensory evaluation of color, flavor and texture of pea protein fortified noodles compared favorably with control noodles. This study also

showed that precooking the pea proteins would improve the dough strength and flavor of the spaghetti (Sumner & Nielson, 1981). The study of pea protein utilization has not progressed much since these two decades; the reason probably is that functional properties of pea proteins have not changed much through the seed production cycles. Thus, the use of different processing treatments that could improve functional properties of pea proteins needs to be studied.

Many studies have been conducted to evaluate the functional properties of pea protein isolates. Proteins with low solubility indices in aqueous environments have limited functional properties and food uses. Studies showed that pea protein solubility was between 82% (Butt & Batool, 2010) to 87% (Nielsen, Sumner, & Whalley, 1980), which is comparable to values reported for soybean proteins. The ability of proteins to aid the formation and stabilization of food emulsions is critical for many applications in chopped, comminuted meats, cake batters, coffee whiteners, milks, mayonnaise, salad dressing, and frozen desserts (Kinsella, 1979). The ratio of the height of emulsion layer to the height of liquid layer, which was noted to calculate emulsion activity, was shown to be between 38% (Nielsen, Sumner, & Whalley, 1980) to 45.5% (Butt & Batool, 2010). The emulsifying stability was shown to be between 43.19% (Butt & Batool, 2010) and 100% (Aluko, Mofolasayo, & Watts, 2009). The capacity of proteins to form stable foams with gas by forming impervious protein films is an important property in cakes, soufflés, whipped toppings, fudges, etc (Kinsella, 1979). The percentage volume of pea proteins induced by whipping, which would express the foaming capacity, was observed between 78% (Butt & Batool, 2010) and 143% (Sumner, Nielsen, & Youngs, 1981), while the foaming stability was between 79% (Butt & Batool, 2010) and 98% (Fernandez-Quintela,

Macarulla, Del Barrio, & Martinez, 1997). The ability of proteins to form gels and provide a structural matrix for holding water, flavors, sugars, and food ingredients is useful in food applications, and in new product development, and it provides an added dimension to protein functionality (Kinsella, 1979). Least gelation concentration (LGC), which indicates the gelation ability of pea proteins, is important for its application in comminuted sausage products and many other oriental textured foods. LGC of pea proteins has been reported to be about 18% (Butt & Batool, 2010; Fernandez-Quintela, Macarulla, Del Barrio, & Martinez, 1997). The capacity to retain moisture or lipids is extremely important in a variety of food products, especially meat and baked products (Kinsella, 1979). Good water-holding or oil-holding capacity could not only improve the texture but also the flavor. Pea proteins were shown to have about 152% (Butt & Batool, 2010) to 170% (Fernandez-Quintela, Macarulla, Del Barrio, & Martinez, 1997) for water-holding capacity, and 140% (Butt & Batool, 2010) to 120% (Fernandez-Quintela, Macarulla, Del Barrio, & Martinez, 1997) for oil-holding capacity.

From previous studies, pea protein isolates showed good functional properties, but still has not fulfilled the food industry expectation for large-scale utilization in product formulation. These functional properties can be improved by many methods, such as thermal treatment, high-pressure treatment, and enzymatic hydrolysis.

2.3 HIGH PRESSURE TREATMENT OF PROTEINS

High-pressure treatment is increasingly being used in food industry. High-pressure exposure in excess of 100 MPa is being used to inactivate microorganisms as well as to induce texture changes in foods without thermal degradation. This treatment could offer the chance of producing food of high quality, greater safety and increased

shelf life. Generally, four levels of structure could be distinguished in protein. During pressurization, the structure of proteins would be rearranged but covalent bonds are almost unaffected and hence the primary structure will remain intact (Hayashi, Balny, Cheftel, & Heremans, 1991; Hayashi, Kawamura, Nakasa, & Okinaka, 1989). Meanwhile, secondary structure changes occur and lead to irreversible denaturation, which is due to the rupture of hydrogen bonds at high pressures (Hama & Suzuki, 1972). The tertiary and higher structures of proteins formed through non-covalent bonds such as hydrogen bond, electrostatic interaction, hydrophobic interaction, etc., are more susceptible under high pressure treatments, which can modify the physiochemical properties of proteins (Hendrickx, Denys, Indrawati, Ludikhuyze, Van den Broeck, & Weemaes, 1998).

Previous studies have reported that different structural behavior of soybean protein fractions has been observed after high pressure treatment. For example, 200-600 MPa pressure treatment caused the molecular dissociation and structural unfolding of soybean 7S and 11S fractions (Puppo et al., 2004). Research also observed that 7S dissociated into partially or totally denatured monomers after 400 MPa high pressure treatment, while 11S became aggregated (Molina et al., 2002). This might be the reason why most of the high pressure treatments have always used 200, 400 and 600 MPa, since proteins are denatured at these levels.

In the past decades, there have been many studies on the influence of high pressure treatments on functionality of various proteins. For example, high pressure treatment could decrease the solubility of proteins, but this effect was not very significant (Apichartsrangkoon, 2003; Lim, Swanson, & Clark, 2008; Torrezan, Tham, Bell, Frazier, & Cristianini, 2007). And the improvement of emulsifying activity of soybean, lupin

proteins, etc. through high pressure treatments has been confirmed by many studies (Kajiyama, Isobe, Uemura, & Noguchi, 1995; Molina, Papadopoulou, & Ledward, 2001; Torrezan, Tham, Bell, Frazier, & Cristianini, 2007); high pressure treatments can also improve foaming stability of proteins. Ibanoglu et al. (2001) and Lim et al. (2008) reported that high pressure treatments can induce unfolding of whey proteins (WP) leading to the increase in protein flexibility (Ibanoglu & Karatas, 2001; Lim, Swanson, & Clark, 2008). Besides, Liu et al. (2005) have reported that hydrophobicity of WP was increased after 10 min high pressure treatment due to changes in protein conformation that increased flexibility (Liu, Powers, Swanson, Hill, & Clark, 2005). However, there is very limited data available on the effect of high pressure treatment on functionality and physicochemical properties of pea proteins.

2.3 THERMAL TREATMENT OF FOOD PROTEINS

In the food industry, thermal processing is usually carried out to build or enhance texture, flavor, digestibility, microbiological safety, and inactivate toxins (Boye, Ma, Ismail, Harwalkar, & Kalab, 1997). In molecular terms, during thermal processing, the polypeptides chain has increased "thermal motion". The thermal treatment would destroy the delicately balanced intra-molecular forces and drastically reorganize all levels of protein structure (Davis & Williams, 1998). Hydrophobic groups that had been inwardly-oriented are turned outwards, exposing them to unfavorable interaction with water. The extent of protein unfolding would increase with increase in extent of heating (Boye, Ma, Ismail, Harwalkar, & Kalab, 1997). The protein would have lost almost all secondary and tertiary structures when the temperature exceeds 80°C, and would adopt a configuration

that approaches a fully unfolded, random coil conformation (Privalov, Tiktopulo, Venyaminov, Griko, Makhatadze, & Khechinashvili, 1989). As a general method to modify the functionality and physicochemical properties of proteins, studies on the heat-induced modifications of protein functionality have been limited mainly to milk proteins, egg proteins, soy proteins and proteins from Phaseolus legumes. And these studies found that thermal treatment can induce changes in secondary and tertiary structures of proteins as well as formation of disulfide bonds, thereby modifying functionality of proteins especially solubility, emulsifying property and foaming properties.

The improvement of functionality of proteins through mild heat-treatment has been confirmed by many studies. For example, the dry-heating process was proved to improve simultaneously the foam capacity and the emulsifying activity through partial protein unfolding and increased surface hydrophobicity, without impairing protein solubility and the stability of protein-stabilized emulsions and foams (Kato, Ibrahim, Watanabe, Honma, & Kobayashi, 1989; Talansier, Loisel, Dellavalle, Desrumaux, Lechevalier, & Legrand, 2009). Nicorescu et al. (2010) found that dry heating had a positive influence both on foam ability and foam stability of egg white protein, but had a negative effect on those of whey protein isolate. Heat treatment (50–100°C) of sodium caseinate near the isoelectric point for 5 min resulted in enhanced emulsifying ability and capacity (Jahaniaval, Kakuda, Abraham, & Marcone, 2000). Heat treatment led to extensive denaturation, decreased free sulfhydryl (SH) contents and concomitant increases in surface hydrophobicity of proteins isolated from kidney, red and mungbeans (Tang, Sun, & Yin, 2009). The protein solubility and emulsifying activity index (EAI) were also significantly improved by the heat treatment. However, to date, scanty

information is available concerning the influences of heat treatment on the functional properties of pea proteins.

2.4 ENZYMATIC HYDROLYSIS OF PROTEINS

As discussed before, the potential utilization of pea proteins is linked to their functional properties and nutrition value. These functional properties and nutrition value can be improved not only by thermal or high pressure treatment, but also by enzymatic hydrolysis, yielding products which are better suited to compete with soy protein or other, relatively expensive proteins such as egg-white (Sijtsma, Tezera, Hustinx, & Vereijken, 1998). Previous work has shown that pea proteins were highly available for use but difficult to digest completely (Gatel, 1994). Thus, protein hydrolysis could improve this situation. Compared to chemical hydrolysis, enzymatic methods are mild and enhance retention of nutritional quality of the original protein (Parrado, Millan, Hernandezpinzon, Bautista, & Machado, 1993). The application of enzymatic hydrolysis to modify proteins might produce wider range of usage in food applications. Not only could enzyme hydrolysis improve the functional properties of proteins (Balti, Bougatef, El-Hadj Ali, Zekri, Barkia, & Nasri, 2010), but also it could generate bioactive peptides with various activities, such as ACE inhibition (Kim, Rybalkin, Pi, Wang, Zhang, Munzel, et al., 2001), free radical scavenging or renin inhibition (Udenigwe et al., 2009; Girgih et al., 2011). Pownall et al. (2010) reported that after enzymatic hydrolysis, pea seed peptide fractions had ability to scavenge free radicals, chelate metals and inhibit linoleic acid oxidation. Another study confirmed that the catalytic specificity of proteolytic enzymes plays a major role in determining the functional, nutritional, and bitterness properties of pea protein hydrolysates (Humiski & Aluko, 2007). The research also concluded that

enzymatic pea protein hydrolysates could be used as potential ingredients to formulate functional foods and nutraceutical products (Pownall et al., 2010). Thus, it is essential to evaluate the nutritional quality of enzyme-treated pea proteins.

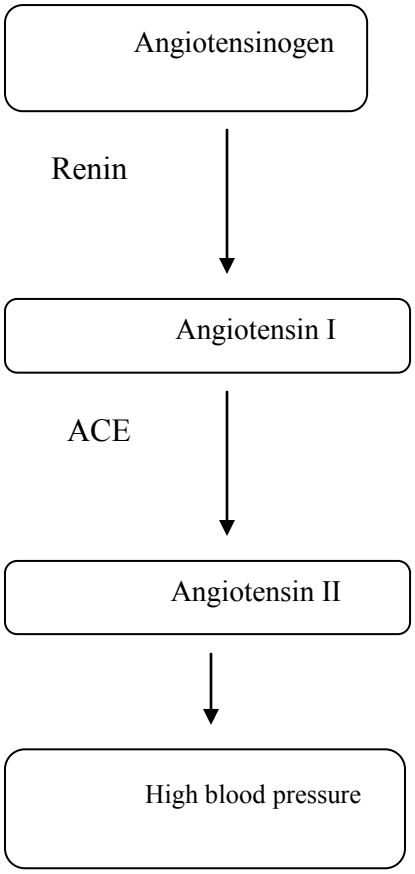
Enzymatic digests from several proteins have been shown to have the potential to prevent long-term human diseases. After enzymatic hydrolysis of proteins, bioactive peptides would be released. Numbers of food sources have been considered as sources of bioactive peptides. Animal proteins, including casein, whey, egg white, marine proteins and meat muscle proteins, have been shown to possess efficient bioactivity. Typical plant proteins which could produce bioactive peptides are soybean, pea, oat, hempseed, canola and flaxseed (Udenigwe & Aluko, 2012). After oral consumption, peptides are released from proteins as they pass through the human gastrointestinal system. Small peptides are transported through brush border membrane by peptide transporters and are actually absorbed faster than free amino acids.

Structures of peptides are closely related to the bioactivity of peptides. Take anti-hypertensive activity for example; it has been recognized that ACE-inhibitory peptides possess a characteristic pattern different from that of inactive peptide molecules (Yamamoto, Akino & Takano, 1994). It has been expected that the structure of long-term peptide conformation adopted in the specific environment of the binding site will influence binding to ACE (Fitzgerald & Meisel, 2000). Moreover, the conformation changes of proteins at the C-terminal position of an ACE-inhibitory peptide could significantly influence its interaction with the enzyme (Recio & Belloque, 2004).

In humans, the renin-angiotensin system (RAS) is responsible for regulating blood pressure and fluid homeostasis (Balti, Nedjar-Arroume, Adje, Guillochon, & Nasri,

2010), with angiotensin I-converting enzyme (ACE) and renin as the two key enzymes. ACE is a membrane-bound dipeptidylcarboxypeptidase that catalyzes conversion of inactive angiotensin I to the active form, angiotensin II by removing a dipeptide from the carboxyl-terminal (Li & Aluko, 2010). Thus, ACE inhibition could prevent excessive increase in blood pressure. Renin is an enzyme responsible for converting angiotensinogen to angiotensin I by cleaving angiotensinogen between its 10^{th} and 11^{th} amino acids (Udenigwe, Li, & Aluko, 2012). The Renin-angiotensin system is shown in Figure 1.

Figure 1. The renin-angiotensin system



Since 1980s, numerous articles have been published discussing the effects and the mechanism of ACE inhibitors. It has been reported that the acute and long-term blood pressure reduction was correlated both with the pretreatment levels and the decreases in plasma angiotensin (Rasmussen et al., 1985). The inhibition of angiotensin II formation could explain the acute hypertension-reducing action of some medications (Rasmussen et al., 1985). A large number of bioactive peptides from natural food sources have been reported to have ACE-inhibitory activities. Mung bean protein subjected to 2 hrs of alcalase hydrolysis showed good ACE-inhibitory activity (Li, Wan, Le, & Shi, 2006). Peanut flour subjected to 6 hrs of alcalase hydrolysis also has been reported to have ACE-inhibitory activity of up to 45% (Guang & Phillips, 2009b). Corn gluten meal has shown ACE inhibition bioactivity after 5 hrs of alcalase hydrolysis (Yang, Tao, Liu, & Liu, 2007). Moreover, the ACE-inhibitory activity of enzymatic hydrolysates from soybean, wheat and rice has also been reported (Guang & Phillips, 2009a). In 2010, purified pea protein hydrolysate was reported with high ACE-inhibitory activity (Li & Aluko, 2010).

Excessive activity of renin will cause the over production of angiotensin I, which can lead to excessive levels of angiotensin II. Thus, inhibition of the renin-catalyzed conversion of angiotensinogen to angiotensin I can reduce angiotensin II production. Although not very much research has explored the production and activity of food protein-derived peptide renin inhibitors, it has been shown that peptides are able to inhibit the active site of renin as revealed by a structural study (Sarma, Rao, Sarma, Nagaraj, & Dutta, 2002). However, renin-inhibitory activity is not related to ACE-inhibitory activity,

due to more open conformation of ACE, which makes the active site easily accessible to inhibitors unlike that of renin (Yuan, Wu, Aluko, & Ye, 2006).

The isolation of antihypertensive peptides was first reported in the early 70s. And ACE inhibitors were not reported until 1971 when the ACE-inhibiting antihypertensive peptides from snake (*Bothrops Jararaca*) venom were isolated (Ondetti, Williams, Sabo, Pluscec, Weaver & Kocy, 1971). And since then, ACE-inhibitory peptides have been studied abundantly from numerous animal and plant proteins. Till date, many ACE-inhibiting peptides have been isolated, purified and identified. Research works have showed that di- and tri-peptides are absorbed quickly and could be transported to target organs without being decomposed (Daniel, 2004; Matsui, Tamaya, Osajima, Matsumo & Kawasaki, 2002; Webb, 1990). Previous studies showed that pea protein isolate (PPI) could be hydrolyzed to small peptides which has inhibitory activities against ACE and renin. It was observed that the following three di-peptides exhibited ACE- and renin-inhibitory activities; Ile-Tyr, Lys-Ser and Glu-Phe, respectively with ACE-inhibitory activity of 72.26, 75.34, and 54.74% and renin-inhibitory activity of 38.68, 23.61, and 29.24% (Li & Aluko, 2010).

Many chronic diseases are caused by oxidative stress, and there are bioactive peptides which have been proven to be active antioxidants. There is an enormous list of plant and animal food protein sources for generating anti-oxidant peptides. The antioxidant properties of peptides include scavenging of reactive oxygen species or free radicals and inhibition of reactive oxygen species induced oxidation of biological macromolecules (Udenigwe & Aluko, 2012). Besides, the sulfhydryl functional group in

peptides, such as cysteine residues, may also be anti-oxidant peptides because this amino acid is used by cells for glutathione synthesis (Meisel, 2005; Udenigwe & Aluko, 2012).

Bioactive peptides could also lower the total cholesterol and lipid contents in blood. Studies have detected hypocholesterolemic and hypolipidemic activities of peptides from soybean protein (Nagaoka, Miwa, Eto, Kuzuya, Hori & Yamamoto, 1999), buckwheat proteins (Kayashita, Shimaoka, Makajoh, Yamazaki & Kato, 1997), etc. Peptides Val-Val-Try-Pro has been observed to exhibit lipase-inhibitory effects by the evidence of significantly decreasing postprandial triglycerides level in human subjects (Kagawa, Matsutaka, Fukuhama, Watanabe & Fujino, 1998). However, the exact mechanisms have not been clarified.

Many peptides display immunomdulatory activities. Peptides derived from casein could stimulate phagocytosis of red blood cell and protect mice from infection (Migliore-Samour & Jolles, 1989). Peptides Pro-Gly-Pro-Iso-Pro-Asn and Leu-Leu-Tyr have been shown to promote antibody formation and accelerate phagocytosis (Migiore-Samour & Jolles, 1988; Jolles & Migiore-Samour, 1986). Hydrolysed α-lactalbumin has been reported to increase mice immune response by modulating B lymphocyte and T helper cell activities (Bounous & Kongshavn, 1985). Some immunomodulatory peptides also show anti-cancer activity. Pea protein hydrolysates have been recently reported to reduce the secretion of the proinflammaroty cytokines including tumor necrosis factor-α and interleukin-6 by 35% and 80% respectively (Ndiaye, Vuong, Duarte, Aluko & Matar, 2012).

Peptides possess multiple functions have also been reported form some food sources. One Peptide (Thr-Thr-Met-Pro-Leu-Try) from milk showed both anti-

hypertensive and immunomodulatory activities (Meisel, 2004). It also has been reported that peptides (Gly-Phe-His-Iso, Asp-Phe-His-Iso-Asn-Gly, Phe-His-Gly and Gly-Leu-Ser-Asp-Gly-Glu-Trp-Gln) from beef sarcoplasmic protein had anti-hypertensive, anti-cancer and anti-microbial properties (Jang, Kang & Lee, 2008). It is very valuable to measure if one peptide have more than one function. This is because chronic diseases are always complicated and inter-related; therefore, multifunctional peptides could enhance the efficiency while reducing therapy duration, as well as the suffering of patients.

CHAPTER THREE - MATERIALS AND METHODS

3.1 PROTEIN MATERIAL

Commercial PPI was a gift from Nutri-Pea Limited (Portage la Prairie, Manitoba) and was produced using alkaline extraction followed by isoelectric pH precipitation. Details of processing of the yellow field pea seed ingredients are proprietary (Nickel, 1981). In general the process involves a similar procedure to the one described by Sosulski and McCurdy (1987). Briefly, the pea seeds are dehulled and ground into flour, which is passed through a screen to separate the coarse fiber particles. The flow-through flour is then extracted with an acidic solution, centrifuged, and the supernatant used for isoelectric protein precipitation. The product is finally spray-dried to give a protein concentrate that contains 80% protein content.

3.1.1 High pressure treatment of PPI

The pea protein (2.5g) was solubilized in 250 ml of 0.05 M phosphate buffer pH 7.0, with stirring at 4^{o}C overnight before being subjected to 0, 200, 400, and 600 MPa pressure treatments. All treated samples were freeze-dried before use and protein content determined by the modified Lowry method (Markwell et al., 1978). In the results, 0 MPa pressure treatments, as the control for the pressure treated samples, was labeled as Control 1.

3.1.2 Thermal treatment of PPI

A 5% aqueous slurry of PPI was heated to and held at 50^{o}C, 70^{o}C, 80^{o}C, 90^{o}C and 100^{o}C for 30 minutes, cooled and freeze-dried before use. In the results, 24^{o}C treated

samples, as the control for the thermal treated samples, was labeled as Control 2. Protein content was determined using the modified Lowry method (Markwell et al., 1978).

3.2 DETERMINATION OF PHYSICOCHEMICAL PROPERTIES OF PPI

3.2.1 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For non-reducing SDS-PAGE, the samples were prepared by mixing PPI with Tris-HCl buffer solution (pH 8.0) containing 10% SDS and 0.01% bromophenol blue. The sample tubes were placed in boiling water for 5 min, cooled to room temperature, and then centrifuged at 10000x*g* for 20 min. An aliquot (1 μL) of the supernatant was loaded onto the 10-15% gradient gel. Reduced samples were prepared by adding 5% (v/v) 2-mercaptoethanol (ME) to the supernatant from the 10% SDS samples. Then, all samples were loaded onto the gel. Polypeptide separation and staining were carried out using the Phastsystem Separation and Development electrophoresis unit (GE Healthsciences, Montreal).

3.2.2 Intrinsic Fluorescence

The fluorescence measurements were determined at 24^{o}C using a Jasco FP-6300 spectrofluorimeter (JASCO, Tokyo, Japan). Tryptophan was used to determine structural changes of PPI because the emission signals for tyrosine and phenylalanine were weak and insensitive. Sodium phosphate buffer (0.01M) solutions were prepared at pH values of 3.0, 5.0, 7.0 and 9.0. Each sample was dissolved in the buffer and then diluted until the protein concentration is 20 μg/ml. The intrinsic fluorescence of each protein solution then was measured. The excitation wavelength was fixed at 295 nm and the fluorescence emission spectra were recorded between 300 and 500 nm.

3.2.3 Surface Hydrophobicity

Surface hydrophobicity was determined by the method by Kato and Nakai (1980) with some modifications. Protein slurries were prepared in 0.01 M phosphate buffer (pH 7.0). The slurries were shaken for 1 hr using Eppendorf Thermomixer R, and then centrifuged at 10000x*g* for 20 min. After protein determination (Markwell et al., 1978), the supernatant was serially diluted to final concentrations of 0.0025 to 0.025 % (w/v) in 0.01 M phosphate buffer (pH 7.0). An aliquot (0.02 ml) of 8.0 mM in 0.01 M phosphate buffer, pH 7.0 containing 8-anilino-1-naphthalene sulfonic acid (ANS) was added to 4 ml of each dilution and fluorescence intensity (FI) of the mixture was measured with Fluorescence-spectrophotometer (JASCO FP-6300) at excitation and emission of 390 and 470 nm, respectively. The initial slope of the FI versus hydrolysate concentration plot was used as an index of surface hydrophobicity.

3.3 DETERMINATION OF FUNCTIONAL PROPERTIES OF PPI

3.3.1 Protein solubility

A 1% (w/v) protein aqueous slurry was prepared at different pH values (pH 3.0 to 9.0). All of slurries were shaken on an Eppendorf Thermomixer R at room temperature for 1 hr. After centrifugation at 10,000x*g* for 20 min, modified Lowry method (Markwell et al., 1978) was used to determine the protein content of each supernatant. The percentage protein solubility was expressed as: (protein content of each supernatant/total protein content in samples)*100. All determinations were done in triplicates.

3.3.2 Emulsion properties

Three different protein concentrations of 10, 25, and 50 mg/ml, were used based on a previous study on pea flours (Aluko et al., 2009). Protein slurries with different protein concentrations were prepared in 5 ml of 0.1 M phosphate buffer pH 3.0, 5.0 and 7.0 followed by addition of 1 ml of canola oil. Then the mixture was homogenized at 20,000 rpm for 1 min, and repeated after a 5-second pause using a non-fomin shaft attached to a Polytron PT 3100 homogenizer (Kinematica AG, Lucerne, Switzerland). The mean diameter of emulsion droplets was determined in a Mastersizer 2000 (Malvern Instruments Ltd., Malvern, U.K.) with distilled water as dispersant. Each sample was analyzed in triplicate.

3.3.3 Foaming properties

Slurries containing different protein concentrations were prepared in 5 ml of 0.1 M phosphate buffer pH 3.0, 5.0 and 7.0. Protein concentration were prepared at 10, 25, 50 mg/ml according to previous studies on pea flours (Aluko et al., 2009). After homogenizing at 15,000 rpm for 1 min using a 20 mm foaming shaft on the Polytron PT 3100 homogenizer, foaming capacity was determined as the increased volume of each slurry. The foam overrun was calculated as foam volume (ml)*100%/ slurry volume (ml)

3.3.4 Gelation

Protein solutions (5 ml) were made at concentrations of 8 to 26% (w/v). All samples were heated at 95^{o}C in water bath for 1 hr, and then cooled to room temperature for 2 hr. After storage at 4^{o}C overnight, the concentration at which the gel did not fall out when tube was inverted was taken as the least gelation concentration (LGC).

3.3.5 Water holding capacity (WHC)

Each protein sample (0.01g) was weighed (W_0) into 2 ml pre-weighed centrifuge tubes and then weighed as W_1. Distilled water (1.8 ml) was added to each sample followed by shaking in Thermomixer R for 5 min. Then the mixtures were centrifuged at 7000x*g* for 15 min. The supernatant were then removed and the tubes were weighed as W_2.

Water holding capacity was calculated as: WHC (g water/g protein)= $(W_2-W_1)/W_0$

3.3.6 Oil holding capacity (OHC)

Each protein sample (0.01g) was weighed (F_0) into 2 ml pre-weighed centrifuge tubes, then weighed as F_1. Canola oil (1.8 ml) was added to each sample followed by shaking in the Eppendorf Thermomixer for 5 min. Then the mixtures were centrifuged at 7000x*g* for 15 min. The supernatant was then removed and the tubes were weighed as F_2.

Oil holding capacity was calculated as: OHC=$(F_2-F_1)/F_0$

3.4 DETERMINATION OF ACE- AND RENIN-INHIBITORY PROPERTIES OF ENZYMATIC PEA PROTEIN HYDROLYSATES

3.4.1 Hydrolysis of PPI

Slurries of PPI containing 5% (w/v) protein content were prepared with distilled water and adjusted to pH 9.0 using 2 M NaOH. The slurries were heated to 50^{o}C followed by addition of alcalase (1, 2, 3, or 4% by weight of protein in the PPI). After 4 hr of hydrolysis, the reaction beaker was immersed in boiling water for 15 min, cooled to room temperature and adjusted to pH 4.0. The pH 4.0 slurries were centrifuged at 10000x*g* for 30 min and the supernatant collected and freeze-dried. The protein

concentration of the hydrolysates was determined by modified Lowry method (Markwell et al., 1978).

The peptide yield was calculated as follows:

Yield (%) = (amount of protein in the hydrolysate/ amount of protein in the PPI used for hydrolysis) * 100

3.4.2 Molecular weight distribution

Molecular weight distribution of PPI was determined by using a GE AKTA system, with Superdex Peptide12 10/300 GL 10×300 mm column (GE Healthcare, Montreal, PQ). Peptide elution was carried out at 25^0C using the buffer (50 mM phosphate containing 0.15 M NaCl, pH 7.0) at a flow rate of 0.5 ml/min; eluted peptides were detected at 215 nm. The column was calibrated with standard proteins that were prepared according to the instructions provided with the Superdex Peptide 10/300 GL high performance column. The final standard solution contained: 1 mg/ml Cytochrome C, 2 mg/ml Aprotinin, 0.1 mg/ml Vitamin B_{12} and 7.8 mg/ml Glycine. The molecular weight (MW) of peptides in samples was calculated from a linear plot of log MW versus elution volume of standard proteins.

3.4.3 Determination of ACE-inhibitory activity of peptides

The ACE-inhibitory activity was determined by the method of Holmquist et al. (1979). N- (3-[2-furyl]acryloyl)- phenylalanylglycyl-glycine (FAPGG) was used as the substrate at a concentration of 0.5 mM dissolved in 50 mM, pH 7.5 Tris-HCl buffer that contained 0.3 mM NaCl. A 1 ml aliquot of this solution was mixed with 20 µl of ACE (1 U/ml; final activity of 20 mU) and 200 µl of peptide solutions in the 50 mM Tris-HCl buffer and absorbance readings taken at 345 nm. Cleavage of the Phe-Gly peptide bond

results in a decrease in absorbance, and this was recorded every 2 min at room temperature. For the control, Tris-HCl was used instead of peptide solution.

The ACE inhibition was calculated as

ACE inhibition (%) = (Absorbance of control – Absorbance of sample)/ Absorbance of control x100

3.4.4 Renin-inhibitory activity

Renin inhibition assay was done according to the instructions provided with the Renin Inhibitor Screening Assay Kit (Cayman Chemicals, Ann Arbor, MI). The renin assay buffer was diluted 10 times with MilliQ water to give a final assay buffer of 50 mM Tris-HCl, pH 8.0 containing 100 mM NaCl. Renin enzyme solution was diluted 20 times before use. The reaction solutions were added as follows. Before reaction: 20 µl of substrate, 160 µl of assay buffer and 10 µl of MilliQ water were added to the background well, while 20 µl of substrate, 150 µl of assay buffer and 10 µl of water were added to the control well. Lastly, 20 µl of substrate, 150 µl of assay buffer and 10 µl of peptide fractions were added to the corresponding inhibitor wells. To initiate the reaction, 10 µl of renin was added to control and inhibitor wells. The total reaction solution volume was 200 µl. The microplate was incubated for 10 min at 37°C in a thermostated Spectra Max Gemini Fluorescence Microplate Reader spectrofluorimeter (Molecular Devices Sunnyvale, CA). The spectrofluorimeter was set at excitation and emission wavelengths of 340 and 490 nm, respectively and the fluorescence intensity was measured.

The Renin inhibitory activity was calculated as:

Renin inhibition (%) = (FI of control – FI of Inhibitor)/FI of control x 100

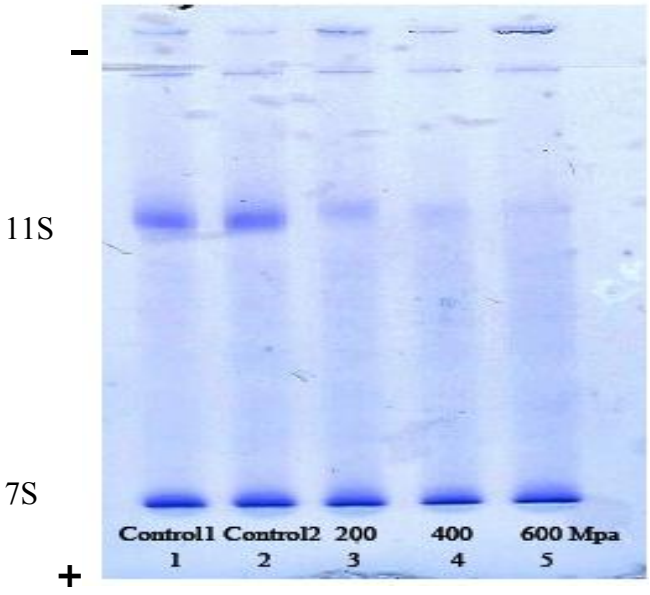
CHAPTER FOUR - RESULTS AND DISCUSSION

4.1 PHYSICOCHEMICAL PROPERTIES OF PPI

4.1.1 Polyacrylamide Gel Electrophoresis (PAGE)

The PAGE separations are shown in Figures 2-5. The native-PAGE separation is shown in Fig. 2 with lanes 1 and 2 representing untreated PPI having the same pattern and the presence of two main bands: 11S and 7S proteins. However, upon high pressure treatment (200 MPa), the quantities of 11S fraction decreased and some large protein aggregates were formed by 11S which can be seen at the top of lane 3. Under higher pressure treatments (400 and 600 MPa), 11S fraction almost disappeared. As clearly seen from lane 3, 4 and 5, high pressure treatment had no effect on the 7S subunit of PPI. Puppo et al. (2004) reported similar results about native PAGE of the legumin and vicilin of soybean proteins. However, overall, the high pressure treatment caused the conversion of PPI polypeptides into heavier aggregates as evident in the disappearance of the 11S at 200, 400 and 600 MPa (Fig. 2). The 11S protein, which is also called legumin is a hexamer with 6 subunits while the 7S protein is called vicilin and is a trimer with 3 subunits. The reason why 11S is more susceptible than 7S to pressure-induced aggregation is mainly because the 7S protein complex is held together by covalent disulfide bonds that are quite stable, while the 11S proteins are formed mainly by non-covalent bonds. High pressure treatment could break the non-covalent bonds, which causes the hexameric structure of 11S to fall apart, unfold and enhance aggregation of the unfolded protein molecules into high molecular weight polymers.

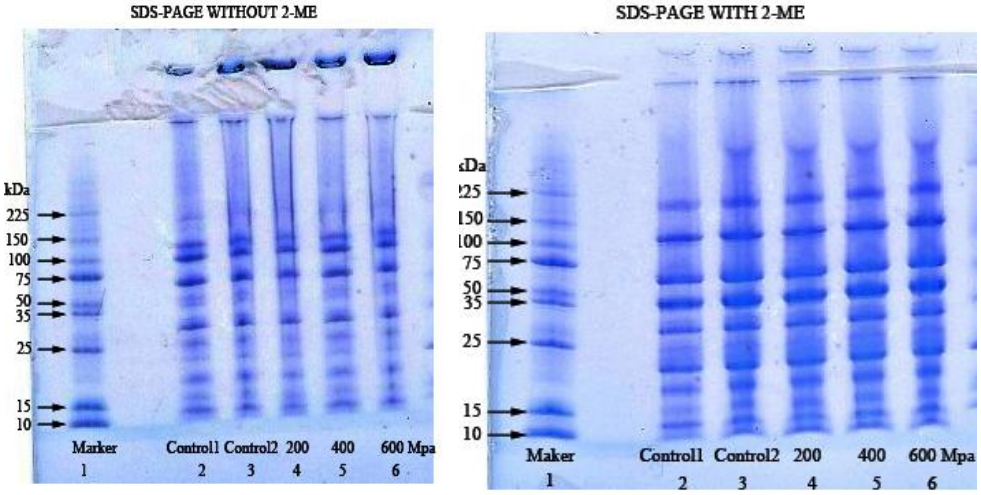
Figure 2. The native polyacrylamide gel electrophoresis (PAGE) pattern of untreated pea protein isolates (PPI) and PPI subjected to different high pressure treatments.



As shown in Figure 3A, under the non-reducing SDS-PAGE, for untreated PPI (lanes 1 and 2), there are mainly 4 bands corresponding to the molecular weight (MW) of 120, 90, 70 and 31 kDa, respectively. Besides, there are also some proteins with low molecular weights (19, 16 and 13 kDa). A protein with an apparent MW of about 90 kDa, which was present in pea and soy samples, has been reported to be lipoxygenase (Crévieu, Carré, Chagneau, Quillien, Guéguen, & Bérot, 1997). The 70 kDa protein has been considered as the subunit of convicilin, whose oligomeric structure has a molecular weight of 290 kDa (Croy, Gatehouse, Tyler, & Boulter, 1980). The 70 & 90 kDa proteins did not disappear under reducing conditions (Fig. 3B) because they have no disulfide bonds. The proteins smaller than 35 kDa including 31, 19, 16 and 13 are all subunits of vicilins which is according to previous reports (Abhyankar, Mulvihill, & Auty, 2011; Casey, Sharman, Wright, Bacon, & Guldager, 1982; Gatehouse, Lycett, Croy, & Boulter,

1982). And these bands were present as well under the reducing condition because 7S globulins are typically trimeric proteins of MW 150 to 190 kDa that lack cysteine residues and hence cannot form the disulfide bonds (Shewry, Napler, & Tatham, 1995). The 120 kDa protein may be the dimer of subunit pairs of legumin which contain "acidic" polypeptide (MW= 40 kDa) and "basic" polypeptide (MW= 20 kDa) subunits linked by a disulfide bond because this band disappeared under reducing conditions as evident from Fig. 3B (Bacon, Noel, & Wright, 1989; Gatehouse, Croy, & Boulter, 1980; Gatehouse, Lycett, Croy, & Boulter, 1982). Previous works have also showed similar polypeptide composition of untreated PPI (Ribottaet al., 2012; Shan et al., 2007; Barac et al., 2010).

Figure 3. The SDS-PAGE pattern under non-reducing (A) and reducing (B) conditions of untreated pea protein isolate (PPI) and PPI treated subjected to different high pressure treatments.

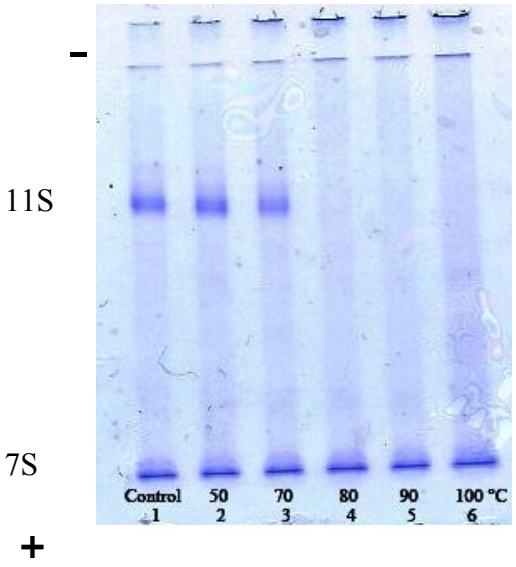


(A) (B)

The large aggregates at the top of each lane under non-reducing condition (Fig. 3A) disappeared under reducing condition (Fig. 3B), which implies that these aggregates were formed through disulfide bonds and were broken by addition of mercaptoethanol (reducing agent) to the electrophoresis buffer. After these aggregates were reduced, the native vicilin (190 kDa) appeared (Fig. 3B), which suggests the vicilin interacted with other proteins through weak disulfide bond interactions to form high MW aggregates. A new band (40 kDa) appeared and at the same time the intensity of band at 19 kDa was enhanced (Fig. 3B), which implies the legumin was reduced to its two subunits. Compared with untreated PPI, there are no changes in the composition of PPI after high pressure treatment (200, 400 and 600 MPa) under non-reducing or reducing condition, which indicates that the polymeric aggregates formed from 11S proteins during high pressure treatments were held together by non-covalent or disulfide bonds.

By observing Fig. 4, the PPI treated at 50 and 70°C shows the two main bands (7S and 11S proteins) in the native-PAGE which are same as those of the untreated PPI. But when the temperature was increased above70°C, the 11S band disappeared probably due to the denaturation of proteins thereby leading to the formation of the bigger size protein polymers. Shand et al. (2007) studied the temperature of thermal transition (T_d) using differential scanning calorimetry (DSC). Their results showed only one endothermic peak representing the denaturation of legumin and vicilin with a T_d at about 75ºC in the thermograph of commercial pea protein isolate, which is consistent with our native PAGE results (Fig. 4) that showed disappearance of the 11S at temperatures above 70^oC. Besides, Bora et al. (1994) also reported that the mixed globulins (35.7% legumin and 64.3% vicilin of pea) have a T_d between 74 to and 95 ºC.

Figure 4. The native polyacrylamide gel electrophoresis (PAGE) patterns of untreated pea protein isolate (PPI) and thermally-treated PPI

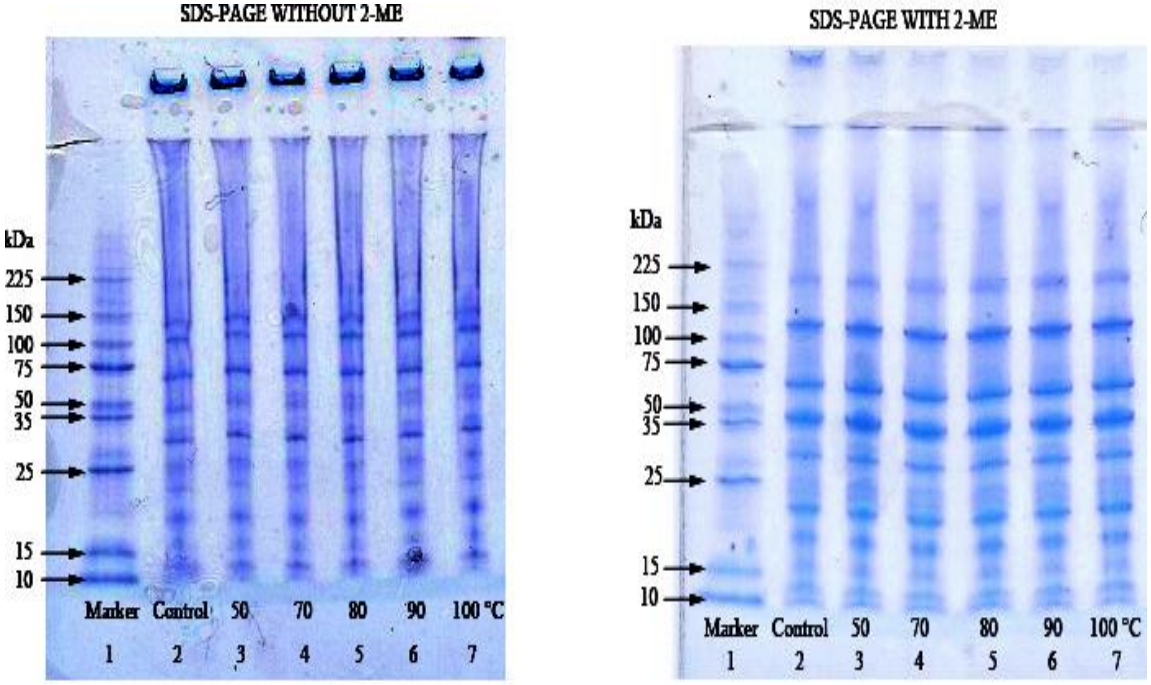


From Fig. 5A, it can be observed that under non-reducing condition, SDS-PAGE presents 4 main bands corresponding to about 120, 90, 65 and 31 kDa. There also some bands at lower MW (20 and 13 kDa). Similar to the untreated PPI, there are no differences in the composition of PPI after processing under different temperature conditions (50-100°C). In addition, quite a few of aggregates were also observed at the top of each lane, which indicates heat-induced formation of high MW protein aggregation. The thermally-treated PPI showed similar non-denaturing SDS-PAGE polypeptide composition to that of high pressure treatments, which indicates that the heat-induced aggregates were formed through non-covalent interactions. Addition of SDS disrupted the non-covalent interactions and hence similar polypeptide bands were obtained at all the heat treatment temperatures.

Under reducing condition (Fig. 5B), the lanes from 2 to 7 showed the same band, which suggests that the heat treatments caused no significant differences in polypeptide

composition. The results also mean that any heat-induced aggregate consisted of disulfide bonds, which were broken upon addition of mercaptoethanol. The thermally-treated PPI showed the same polypeptide composition under reducing condition as that of high pressure-treated PPI. The intensity of protein aggregates at the top of each lane under non-reducing conditions (Fig. 5A) were less under reducing condition (Fig. 5B), which demonstrates disulfide bonds as being involved in protein-protein interactions that formed the high MW aggregates.

Figure 5. The Sodium dodecylsulfate-polyacrylamide gel electrophoresis (PAGE) pattern under non-reducing (A) and reducing (B) conditions for untreated pea protein isolate (PPI) and thermally-treated PPI.



(A) (B)

4.2.2 Intrinsic fluorescence

The intrinsic fluorescence of each pea protein treatment is shown in Fig. 6 and 7. At pH 3.0 and 5.0, a 7 nm red shift was observed after 600 MPa treatment, which indicates changes in the polarity of tryptophan residues microenvironment of pea protein from a less polar to a more polar environment. Meanwhile, no red shift was observed for 200 and 400 MPa treated samples. This means that the 600 MPa treatment caused a more extensive protein unfolding than the 200 and 400 MPa treatments. The fluorescence intensity decreased after 600 MPa treatment, while no significant differences were observed after 200 and 400 MPa treatments; thus unfolding of protein structure was more extensive after 600 MPa treatment. This because increased interaction with the hydrolphilic environments causes quenching of tryptophan fluorescence (Schmid, 1989).

The fluorescence intensity decreased and a blue shift was observed after 50^{o}C, 70^{o}C and 80^{o}C heat treatment, which indicates decrease in the polarity of tryptophan residues microenvironment in the treated pea protein. However, an increase of fluorescence intensity was observed at 100^{o}C treatment, which might be caused by the aggregates formed under high temperature. Under aggregated conditions, the intramolecular distance between the tryptophan residues are reduced, which enhances fluorescence intensity because of increased interactions (Schmid, 1989).

Figure 6. Intrinsic fluorescence spectra of high pressure-treated pea protein isolate (PPI)

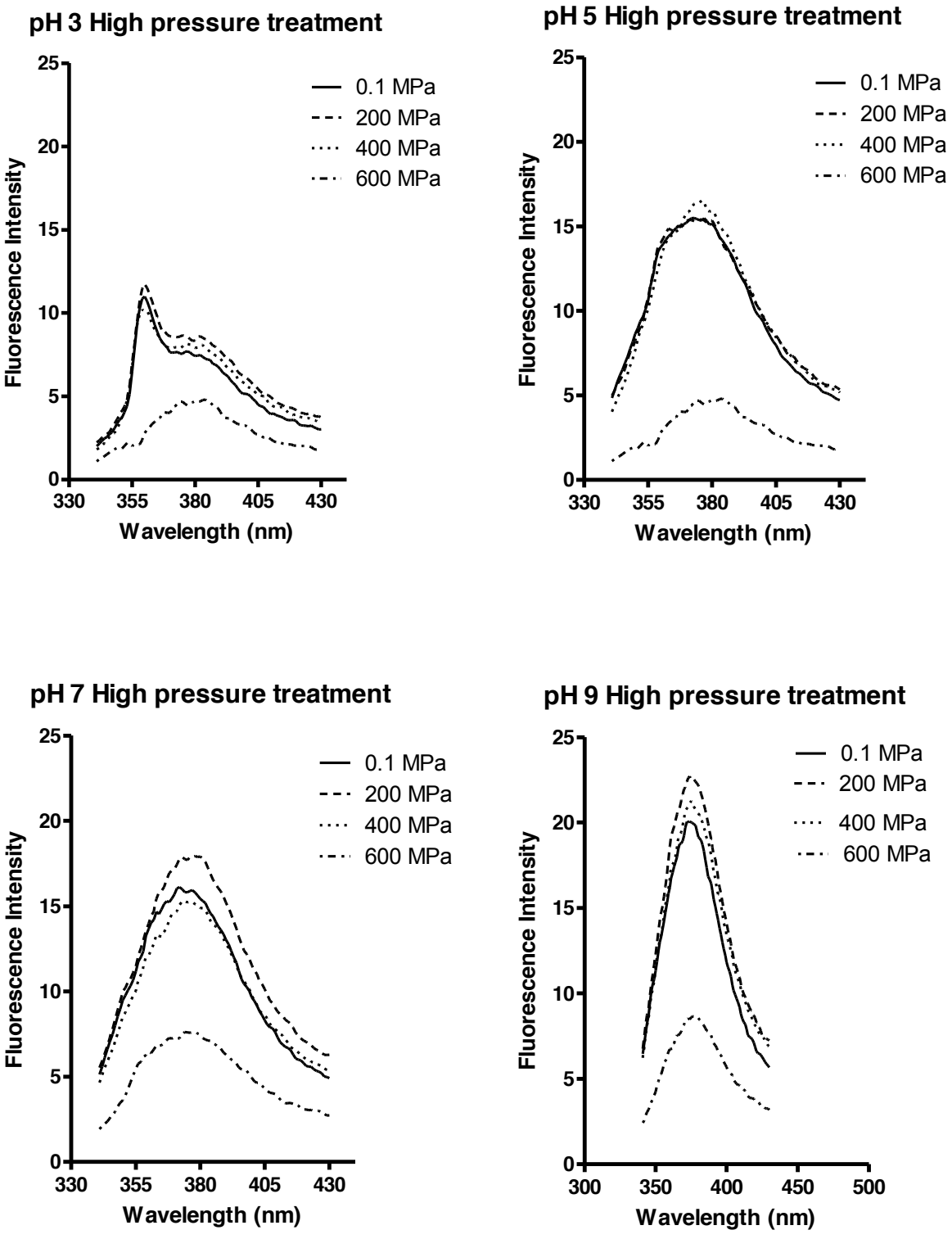
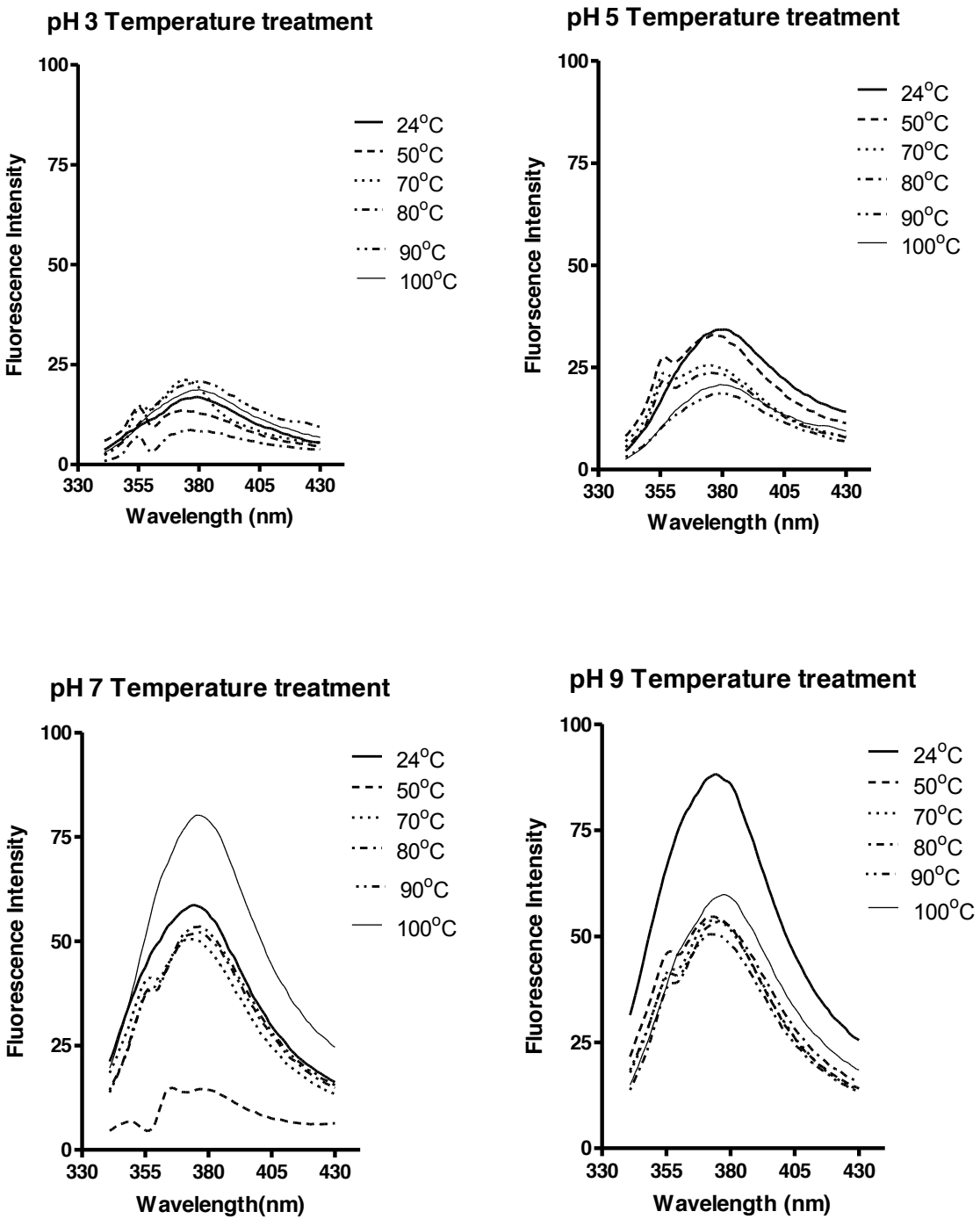


Figure 7. Intrinsic fluorescence of heat-treated pea protein isolate (PPI)



4.2.3 Surface (ANS) Hydrophobicity

The hydrophobicity of each PPI is shown in Figures 8 and 9. As shown in Fig 8, after high pressure treatment at 200, 400 and 600 MPa, the hydrophobicity of PPI was increased by 1.6, 1.4 and 1.7 fold, respectively, when compared to the untreated PPI. The increased hydrophobicity implies that the high pressure treatment resulted in unfolding of the protein's native structure thereby leading to the exposure of the hydrophobic groups buried inside of protein to the exterior of the protein molecule. A similar phenomenon was observed in the studies of soybean proteins (Puppo, Chapleau, Speroni, De Lamballerie-Anton, Michel, Anon, et al., 2004) and whey proteins (Liu, Liu, Liao, Wen, & Chen, 2004). The hydrophobicity of PPI has a negative relationship with protein solubility during pressure treatment, which can be explained by the fact that the exposed hydrophobic residues reduce protein-water interactions and lead to decreased solubility. There was a little decrease of hydrophobicity of PPI treated by 400 MPa compared with that treated by 200 MPa probably due to the aggregation of PPI that shielded the aromatic residues. But as pressure was increased to 600 MPa, rearrangement of the structural conformation of the protein aggregates may have led to increased exposure of the aromatic residues.

Heat can be used to disrupt hydrogen bonds and hydrophobic interactions. For globular proteins, the surface hydrophobicity usually increases as protein denaturation progressed. This is due to the gradual exposure of hydrophobic amino acid residues of native proteins which are buried inside of the protein molecules. As we can see from the Fig. 9, the hydrophobicity of PPI treated by 50°C had no change compared with that of untreated (native) PPI while the hydrophobicity of PPI treated by 70 and 80°C had a

significant ($p<0.05$) increase, which implies that the PPI began to denature at these temperatures. After higher temperature treatments (90 and 100°C), there is a greatly marked 2-fold increase in hydrophobicity, which implies total protein denaturation.

Figure 8. The relative surface (ANS) hydrophobicity (RS_0) of high pressure-treated pea protein isolate (PPI). (Untreated PPI had a So of 1.0)

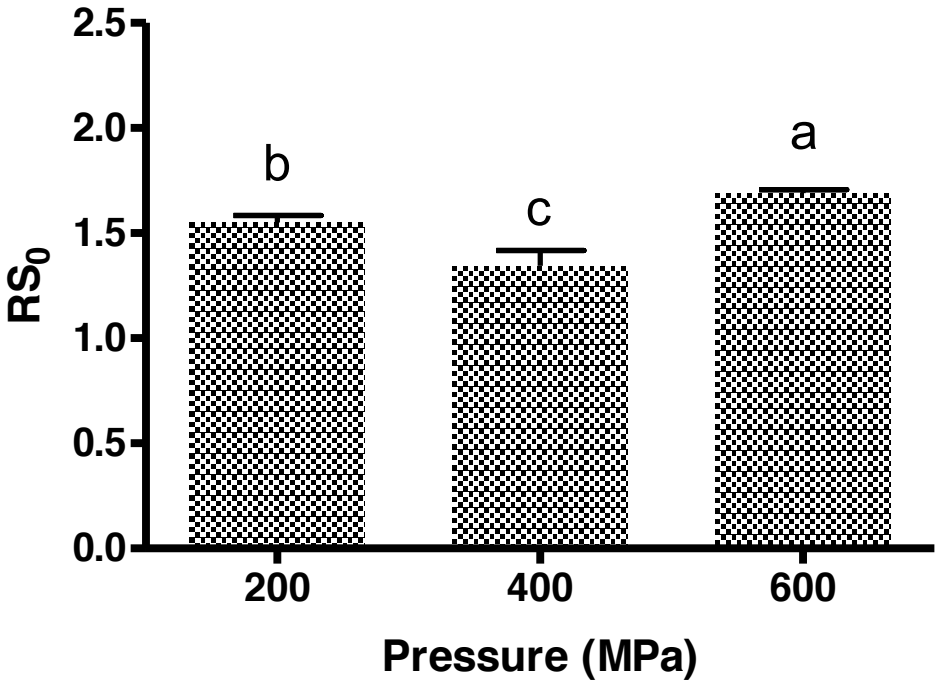
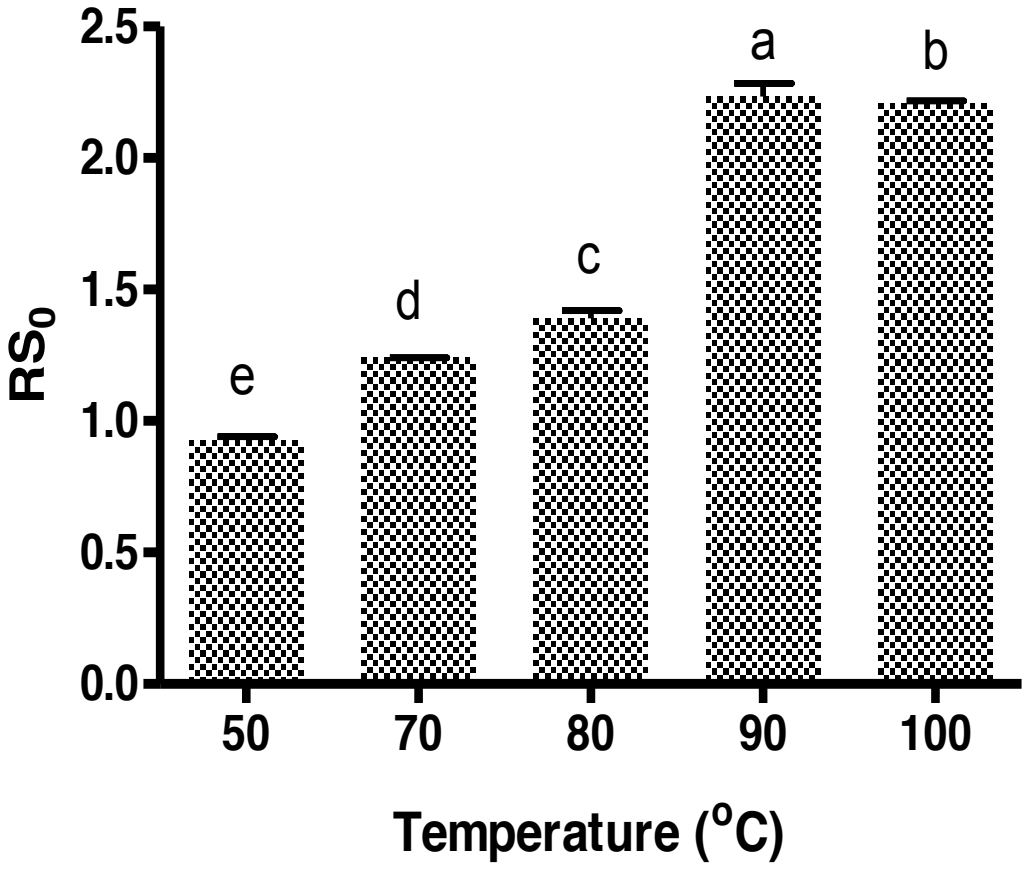


Figure 9. The relative surface (ANS) hydrophobicity (RS_0) of heat-treated pea protein isolate (PPI). (Untreated PPI had a So of 1.0).



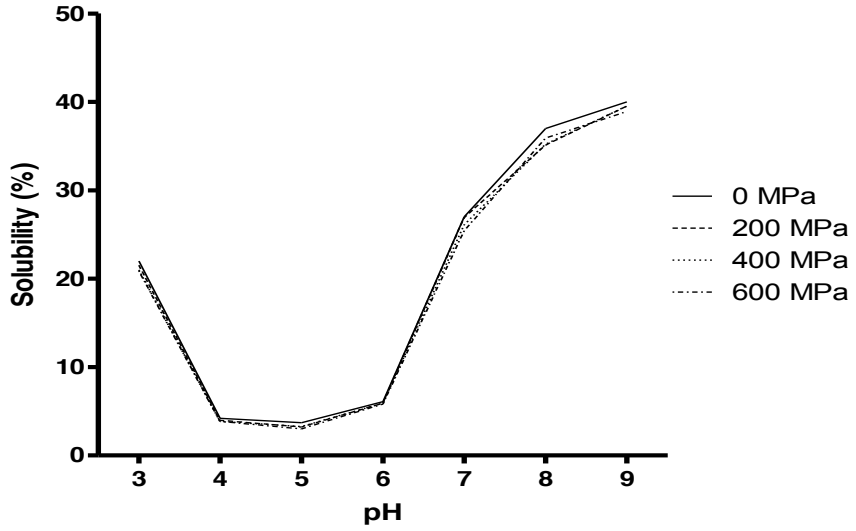
4.2 FUNCTIONAL PROPERTIES OF PPI.

4.2.1 Protein solubility

As shown in the Figures 10 and 11, the PPI at pH 3.0, 4.0 and 5.0 is almost not soluble because the net surface charge of protein almost is zero near the isoelectric point (pI). At the pI, proteins aggregate with each other strongly through van der waals interaction, hydrophobic interaction and hydrogen bonds to reduce interaction with water. With increased pH from 7 to 9 or decreased to pH 3, the solubility gradually increased. But the solubility is still relatively low (<45%), which is consistent with reports of Johnson et al. (1983), Shand et al. (2007) and Voutsinas et al. (1983) on the solubility of native PPI or commercial PPI. The increased solubility as pH values move away from the pI is due to increased net charge, which reduces protein-protein interactions but enhances protein-water interactions.

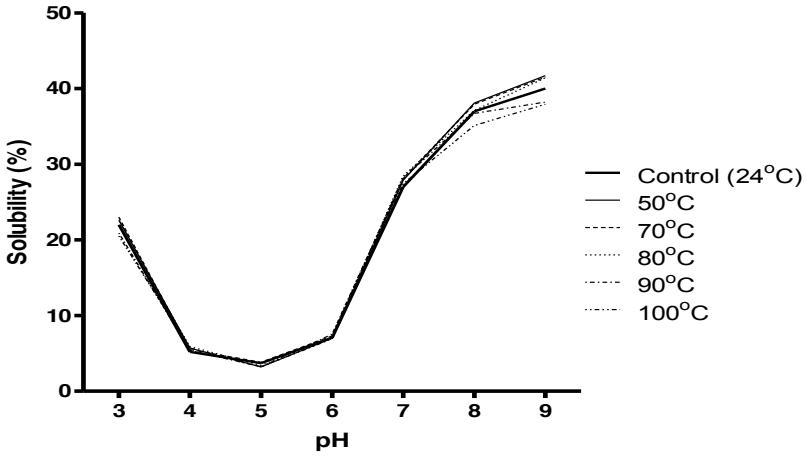
The high pressure treatment had no effect on the solubility of PPI in the pH 3-6 range. However, with increase above pH 6, high pressure treatment resulted in slight but gradual decreases in solubility, when the pressure was increased from 200 to 600 MPa. The loss of the solubility after high pressure treatment (200, 400 and 600 MPa) may be attributed to the exposing of hydrophobic and free thiol group leading to protein aggregation (Francesca E. O'Kane, Vereijken, Gruppen, & Van Boekel, 2005). A similar phenomenon was observed with soybean and lupin proteins upon high pressure (200, 400 and 600 MPa) treatments (Chapleau & de Lamballerie-Anton, 2003; Wang, Tang, Li, Yang, Li, & Ma, 2008). But Puppo (2004) obtained different results for the soybean proteins because they found an increased solubility after pressure treatments.

Figure 10. PPI solubility after high pressure treatment



The thermal treatments did not significantly affect solubility of PPI in the pH 4-6 ranges. There was a slight decrease in solubility when PPI was treated at 80°C or higher temperatures at pH >7, which is possibly due to protein aggregation. A similar result that showed decreased solubility with increased heating time has been reported by Voutsinas et al. (1983).

Figure 11. PPI solubility after heat treatment



4.2.2 Emulsifying and foaming properties

4.2.2.1 Emulsifying properties

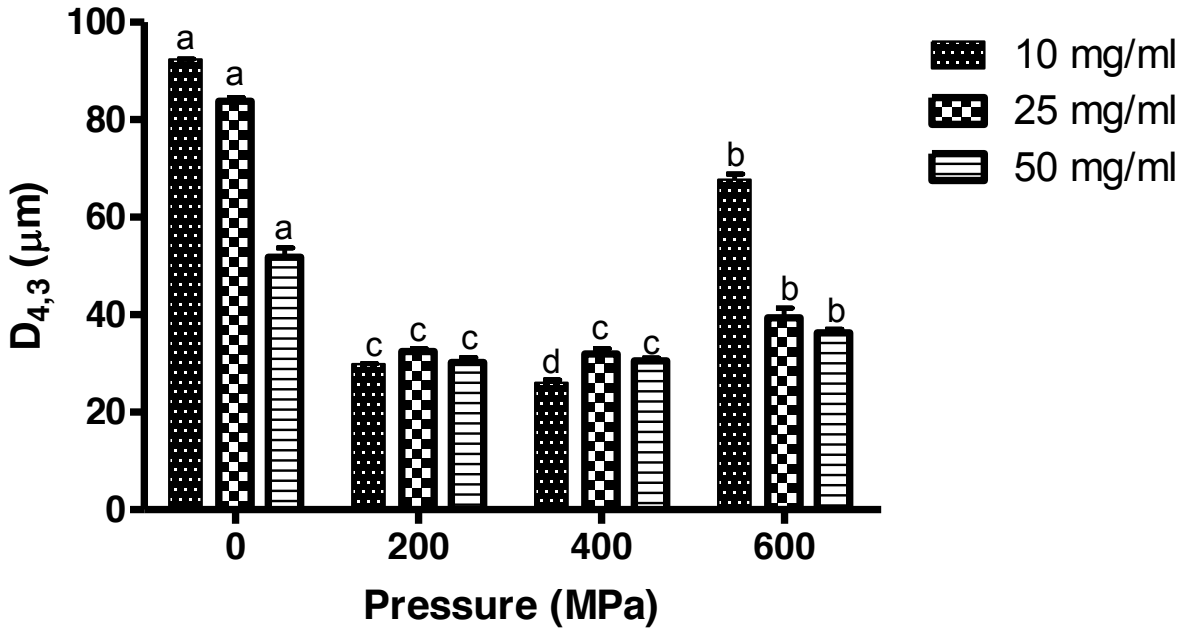
The oil droplets size ($D_{4,3}$) is a very important index to indicate the quality of emulsion. The parameter used here is volume diameter ($D_{4,3}$), which refers to the diameter of a sphere having the same volume as the particles. Monitoring the $D_{4,3}$ value will emphasize the detection of large oil particles that is characteristic of poor emulsions. A previous study (Aluko, Mofolasayo & Watts, 2009) that used a different emulsion quality parameter called the $D_{3,2,}$ which stands for the diameter of a sphere having the same surface as the particles formed by pea proteins showed values that were above 20 μm. Therefore, the index of $D_{4,3}$ was used in this study as the parameter for measuring the quality of large oil droplet-containing emulsions formed by pea proteins.

The particle sizes of emulsions prepared by PPI with high pressure or thermal treatment PPI are shown in Figures 12-17. At pH 3.0 and with increase in high pressure, there was generally a significant decrease ($p<0.05$) in oil droplet size at all protein concentrations. But the oil droplet size also differed among the high pressure treatments. This improvement of PPI emulsifying capacity may be attributed to the exposure of hydrophobic groups and formation of –S-S- leading to more adsorbed proteins at the hydrophobic oil-water interface at pH 3.0 to form the emulsion (Galazka, Dickinson, & Ledward, 1999; Vanda B. Galazka, Dickinson, & Ledward, 2000; Hayakawa, Linko, & Linko, 1996). In contrast, temperature treatments had no significant impact on the emulsifying capacity of PPI at pH 3.0.

At pH 5.0, the high pressure treatments had slight effects on the emulsifying capacity. As shown in the Fig. 12, the oil droplet size slightly decreased with increased

high pressure, but there are no differences between the emulsions processed by different high pressure treatments. Compared with the high pressure treatment, thermal treatment can markedly influence the PPI emulsion formation. As seen from Fig. 13, when the temperature is 70°C or higher, the oil droplet size greatly increased especially at the protein concentration of 25 and 50 mg/ml. The decreased emulsifying capacity of PPI treated by higher temperature (>70 °C) may be attributed to the aggregation of protein molecules (Petruccelli & Anon, 1995). The aggregation of proteins increases the diffusion time to the new interfacial surface, so the small fat oil droplets coalesce with each other and the emulsion is not stable and has a bigger droplet size (Dagorn-Scaviner, Gueguen, & Lefebvre, 1987; Phillips, 1981). Aggregated proteins also have reduced flexibility that minimizes ability to form an interfacial membrane around the oil droplets.

Figure 12. The particle size of emulsions of high pressure treated PPI at pH 3.0



At pH 7.0, the high pressure treatment slightly decreased the emulsifying capacity as shown in the Fig. 12 probably due to the formation of aggregates after the high pressure treatment. Our result is in agreement with the reports of Puppo et al. (2004) on the effect of high pressure treatment on soybean protein isolate. But Wang et al. (2008), Chapleau et al. (2003), Torrezan et al. (2007) and Molina et al. (2001) reported the reversed results on soybean protein and lupin protein. In contrast, thermal treatment higher than 90°C slightly decreased the oil droplet size which is consistent with the reports on emulsifying properties of heat denatured PPI as previously reported by Voutsinas et al. (1983).

Emulsions with three different protein concentrations (10, 25 and 50mg/ml) were prepared. Protein concentration is a very important factor influencing the formation of emulsion. Enough protein can provide a good interfacial protein layer to prevent coalescence of oil droplets and stabilize the emulsion (McClements, 2004). As shown in Fig. 12 and 13 at pH 3 and 5, increased protein concentration from 10 to 50 mg/ml decreased the particle size of emulsion (i.e. increase the emulsifying capacity) formed by PPI treated by all temperature and high pressure levels. Similar observations have been made in emulsions stabilized by whey proteins, fish gelatin and sweet potato proteins (Guo & Mu, 2011; Lizarraga, Pan, Añon, & Santiago, 2008; Surh, Decker, & McClements, 2006) studied at neutral pH. However, protein concentration does not significantly impact the formation of PPI emulsion at pH 7 with the $D_{4,3}$ of about 25 μm at all three protein concentrations despite the high pressure or thermal treatment. The results demonstrate that at neutral pH, PPI is a good emulsifier and 10 mg/ml protein concentration could provide good protein coverage at the oil/water interface. The pH

greatly impacted the formation of PPI emulsion. At pH 3, the oil droplet sizes of emulsions with protein concentration of 10 mg/ml are 90, 70, 70 and 70 μm under different pressure of 0, 200, 400, and 600 MPa, respectively. However, when pH is 5, at the same condition, the oil droplet sizes are 50, 37, 30 and 40 μm, respectively. The increased pH decreased oil droplet size because there are more proteins adsorbed to the interface at around pI leading to higher protein coverage thereby stabilizing the emulsion at pH 5. So there is a smaller oil droplet size of the emulsions at pH 5. When increased to pH 7, PPI had a better emulsifying capacity with $D_{4,3}$ of about 25-30 μm under different high pressure treatments. The results suggest that at pH 7, there are enough repulsive forces from negative surface charges at the surface of oil droplets to prevent coalescence of oil droplets into large sizes once the emulsion has been formed (Ninham, 1999; Rousseau, 2000).

Figure 13. The particle size of emulsions of high pressure treated PPI at pH 5.0

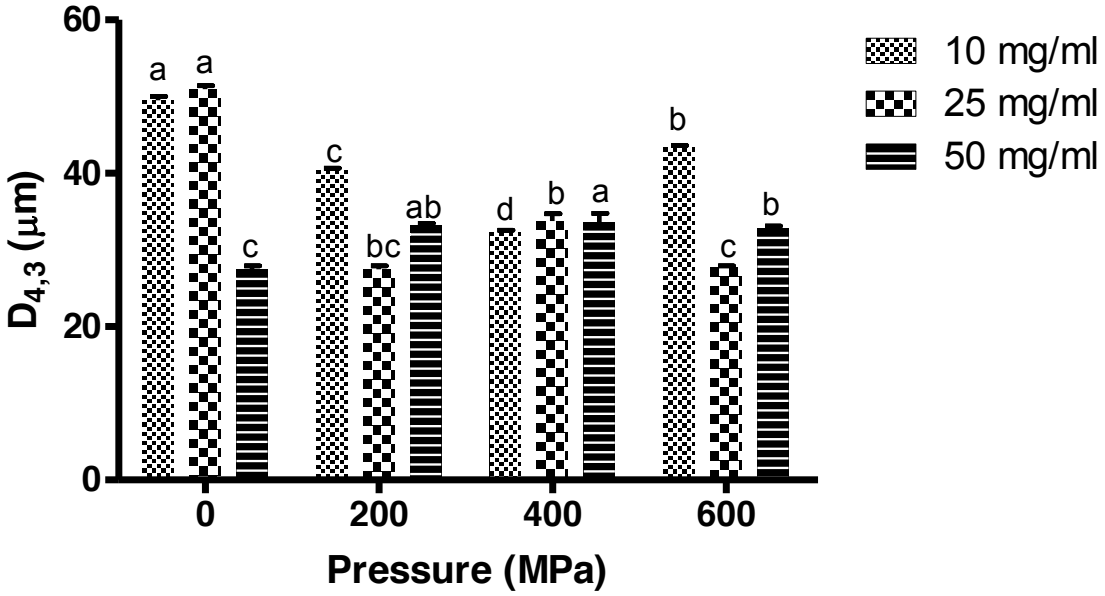


Figure 14. The particle size of emulsions of high pressure treated PPI at pH 7.0

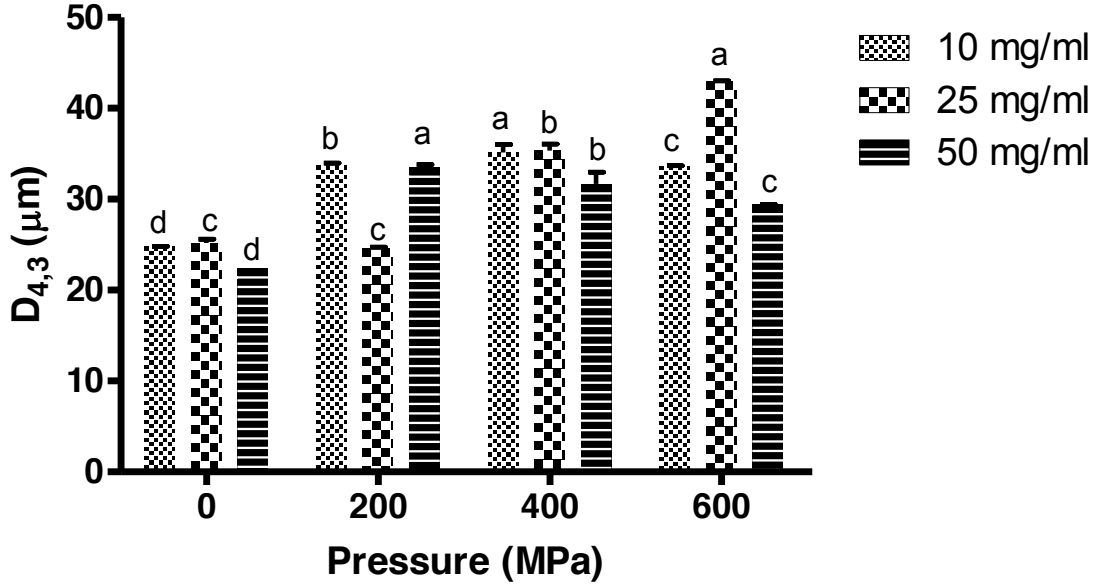


Figure 15. The particle size of emulsions of heat treated PPI at pH 3.0

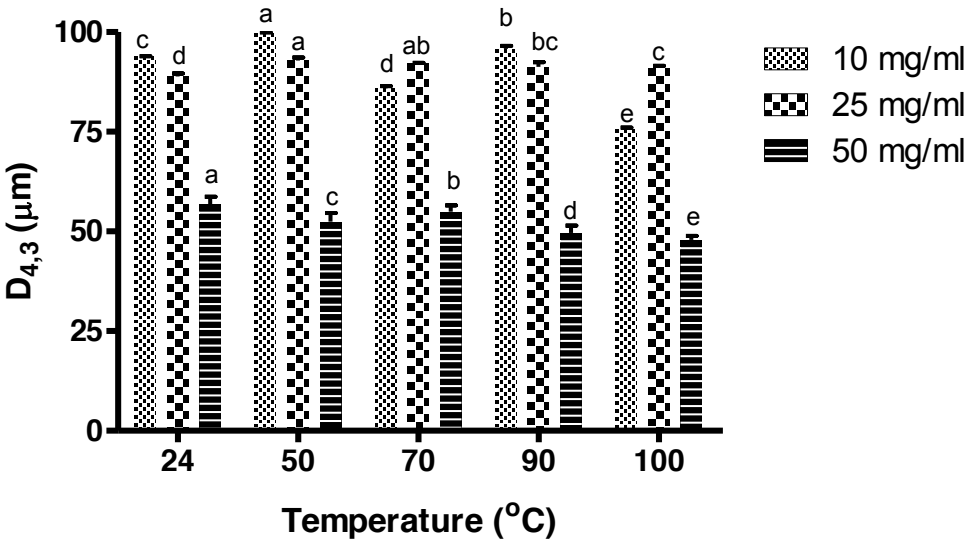


Figure 16. The particle size of emulsions of heat treated PPI at pH 5.0

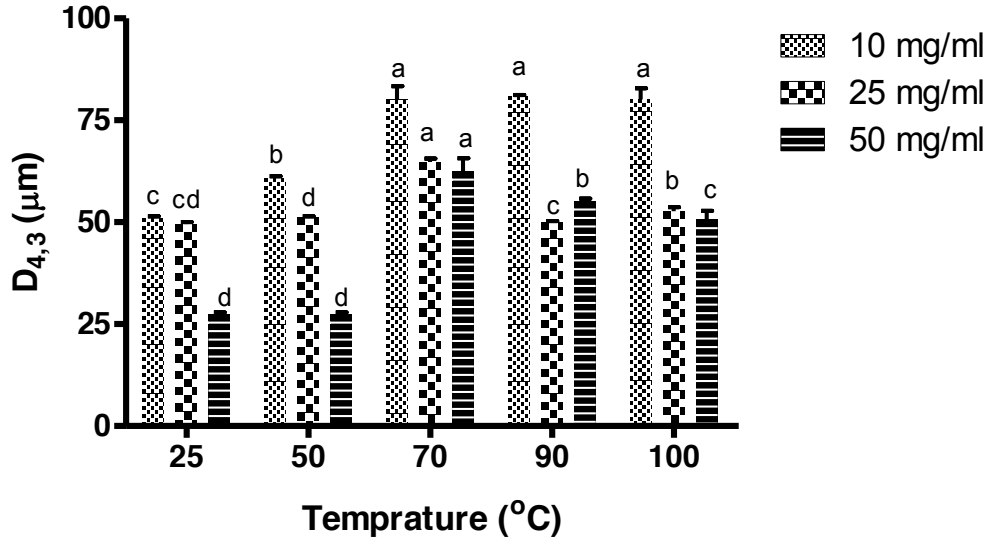
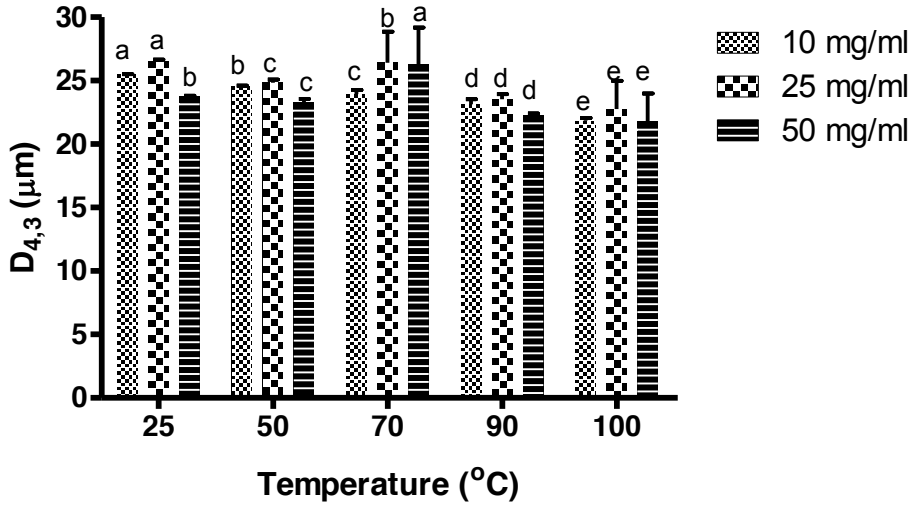


Figure 17. The particle size of emulsions of heat treated PPI at pH 7.0



Globular proteins (e.g. whey protein, soybean protein, etc) are widely used as emulsifiers in the food industry (Chen, Remondetto, & Subirade, 2006; Dickinson, 1993). Compared with other animal or plant globular proteins, the emulsifying property of the native PPI especially commercial PPI powder is not very good (Liu, Lee, & Damodaran, 1999; Sze-Tao & Sathe, 2000). There may be two key factors impacting its emulsifying capacity:

i. low solubility at a wide pH range leading to the relatively low interactions with the interface;
ii. high molecular weight and the long chains in the structure of 7S leading to high activation-energy of protein migration and unfolding at the oil-water interface.

As shown in the Figures 12 and 13, the $D_{4,3}$ of emulsion is about 25 at pH 7, so the emulsion with big oil droplets is quite unstable. Voutsinas et al. (1983) gave similar report compared with many other proteins. The creaming behavior quickly happened after the formation of emulsion such that the big oil droplets coalesce with each other and the phase separation happened after half an hour (data not shown).

As predicated in hypothesis, both high pressure and high temperature could improve the emulsifying ability of PPI.

4.2.2.2 Foaming properties

The foaming capacities are shown in Figures 18-23. As shown in Fig. 18, the high pressure treatment did not significantly impact foaming capacity at pH 3.0, 5.0 and 7.0. Increased protein concentration from 10 to 25 mg/ml also had no significant impact on the foaming capacity at pH 3.0 and 7.0; but with the further increase of protein

concentration to 50 mg/ml, there was a great increase in foaming capacity. At pH 5.0, with increasing protein concentration, there was a slight gradual increase in the foaming capacity because at around pI there is higher protein coverage to stabilize foam. Similar results were observed in the report of Akintayo et al. (1999) on pigeon pea proteins.

At pH 5.0, the foam overrun is lowest with a value between 20-45% because the proteins aggregate at pI leading to the lowest solubility which affects the diffusion of protein in air-water interface. The result of least solubility at pH 5.0 is consistent with the reports of Barac et al. (2010) on the foaming properties of pea proteins. But at pH 3.0 and 7.0, the PPI has a higher solubility so the protein can be quickly adsorbed to the air-water interface leading to better foam formation. At pH 3.0 with increased protein concentration from 10 to 50 mg/ml, the foaming capacity (overrun) of PPI treated at different temperatures (25, 50, 70, 80, 90 and 100 °C) gradually increased from 20 to ~58%. But for each protein concentration, thermal treatment had no effect on foaming capacity at pH 3.0.

At pH 5.0, the foaming capacity of PPI treated under different temperatures gradually increased from 18 to 45% with the increase of protein concentration. But the thermal treatment had no effect on foaming capacity of PPI at each protein concentration. Compared with pH 3.0, there was a little decrease in the foaming capacity at pH 5.0 due to the decreased solubility typically associated with the pI (Yatsumatsu, Sawada, & Moritaka, 1972).

At pH 7.0, the protein concentration significantly impacts the foaming capacity of PPI. With increasing protein concentration, the foaming capacity (overrun) of PPI treated by different temperature gradually increased. Temperatures ≤80°C had no significant

effect on the foaming capacity. But at higher temperatures (90 and 100^{o}C), there was a significant decrease ($p<0.05$) especially at protein concentration of 50 mg/ml in the foaming capacity because at higher temperature the PPI becomes denatured and aggregated leading the lower protein coverage at the air-water interface (Enwere & Ngoddy, 1986). The foaming stability is also quite low at pH 3.0, 5.0 and 7.0, which is in accordance with the report of Barac (2010). After 1 hr storage, the height of foam was only about 25% of initial height at pH 3.0 and 7.0 (data not shown). The low solubility is the key factor determining low foaming capacity.

From the results, high pressure treatment improved the foaming capacity of PPI, whereas, not so obvious improvement was observed for heat treatment.

Figure 18. The foam overrun of high pressure treated PPI at pH 3.0

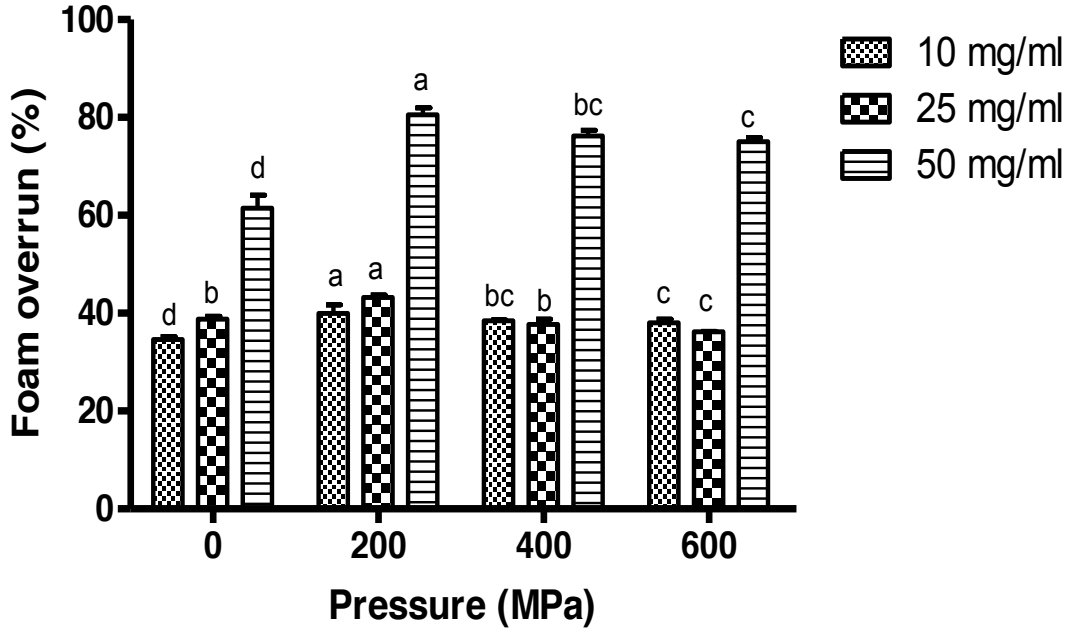


Figure 19. The foam overrun of high pressure treated PPI at pH 5.0

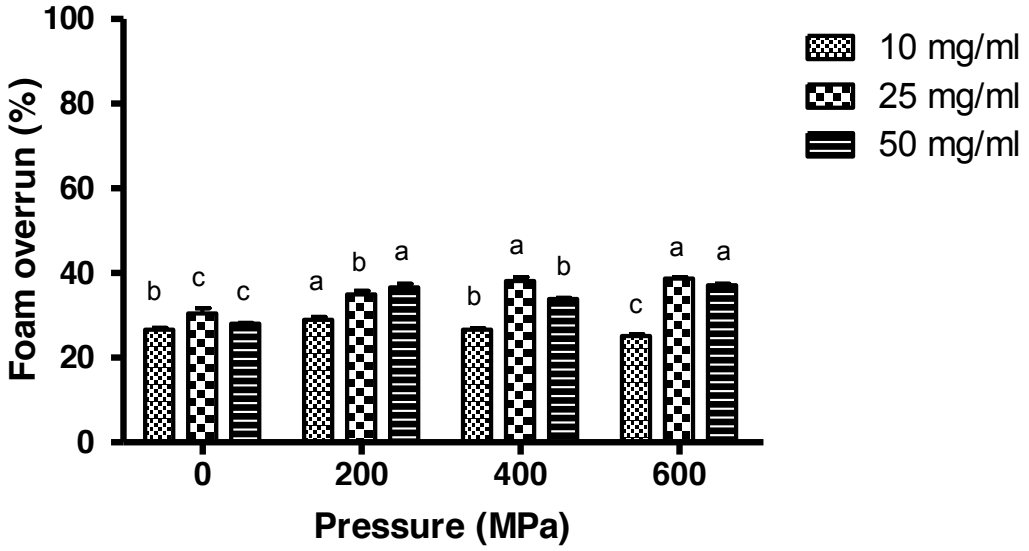


Figure 20. The foam overrun of high pressure treated PPI at pH 7.0

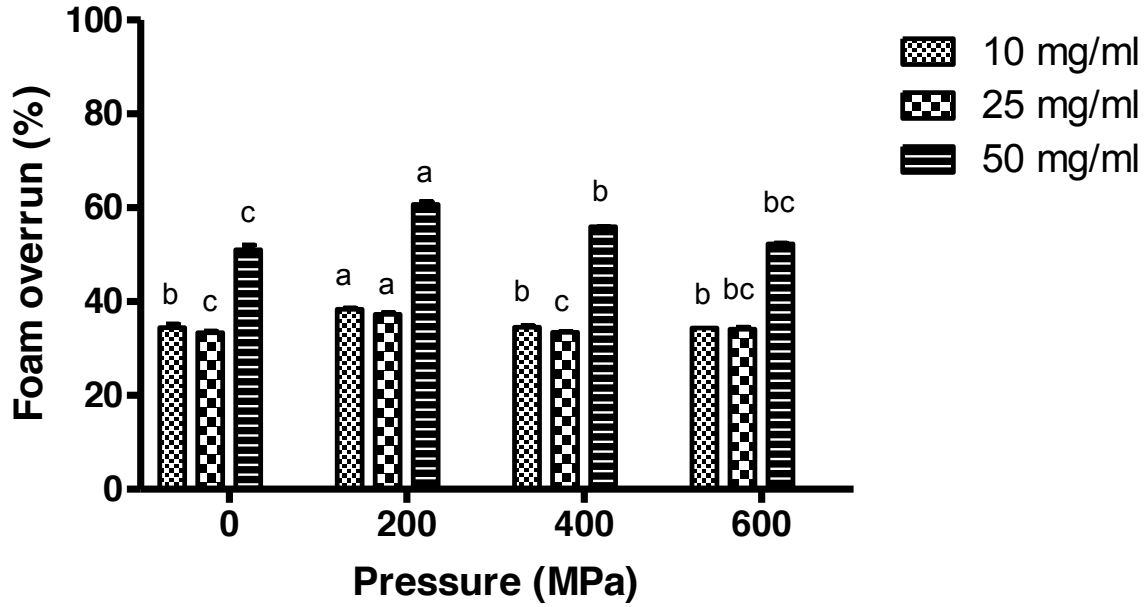


Figure 21. The foam overrun of heat treated PPI at pH 3.0

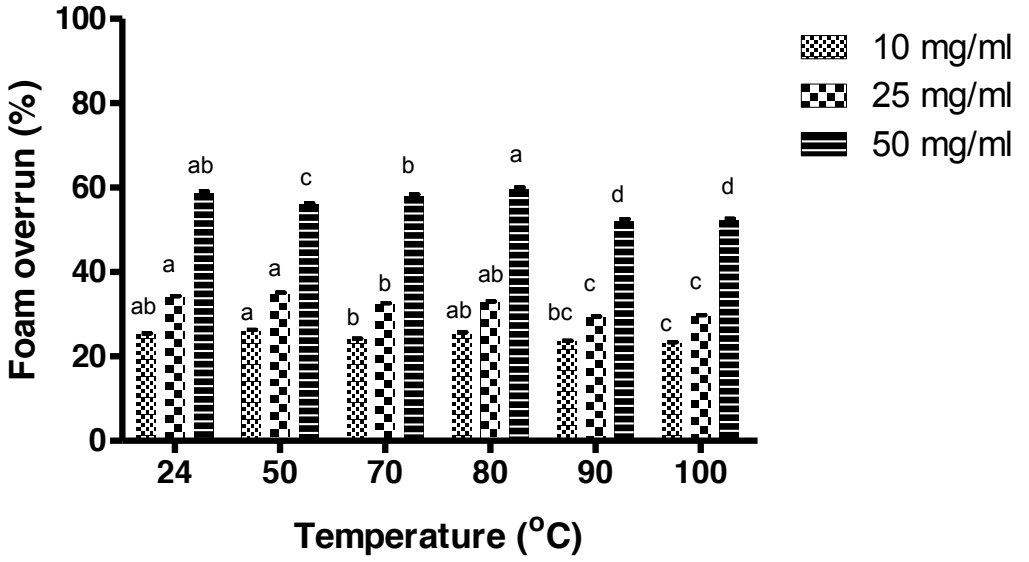


Figure 22. The foam overrun of heat treated PPI at pH 5.0

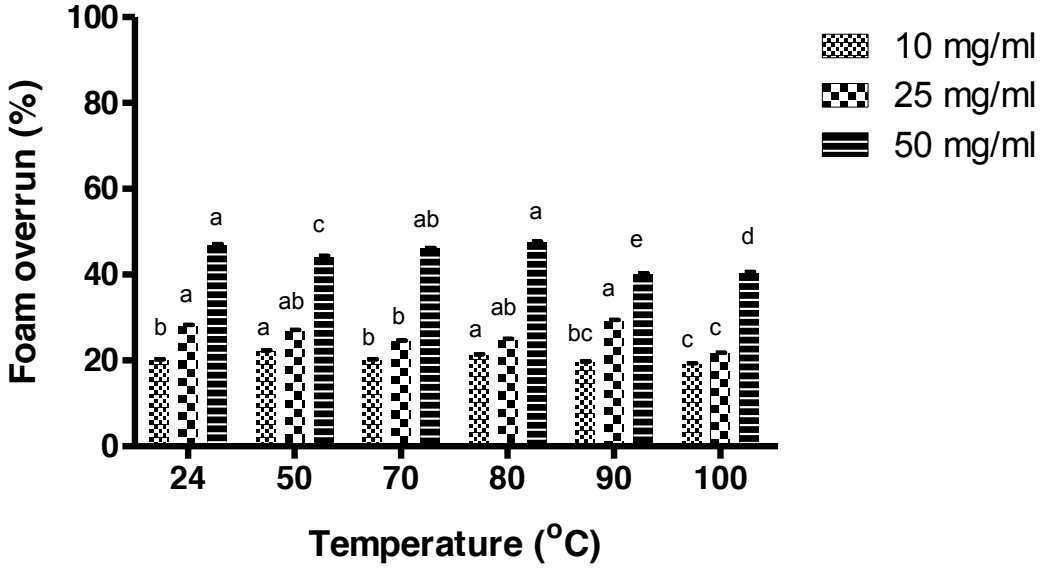
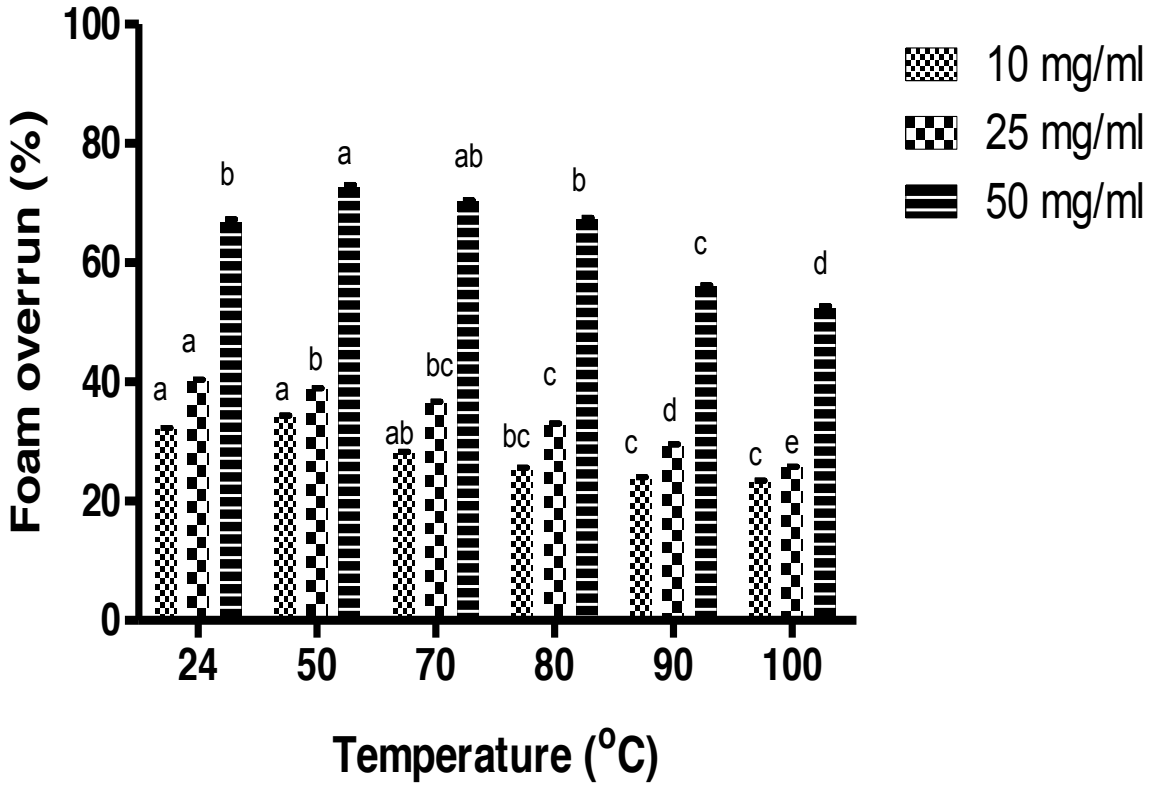


Figure 23. The foam overrun of heat treated PPI at pH7



4.2.3 Gelation properties

The gelation capacity is one of the most important functional properties of globular proteins that can be used to modify food texture. Besides, protein gels can simultaneously bind water, oil, flavor, and other ingredients and stabilize them in the gel network. As shown in the Figures 24 and 25, the pea protein isolate treated by temperature or high pressure need a protein concentration higher than 20% to form very weak gel at neutral pH. Compared with soybean protein, the gelling property of pea protein is inferior. Our result is consistent with the report of O'Kane et al. (2004), and Soral-Śmietana et al. (1998) that showed poor gelling properties of pea proteins.

Figure 24. Least Gelation Concentration (LGC) of high pressure treated PPI

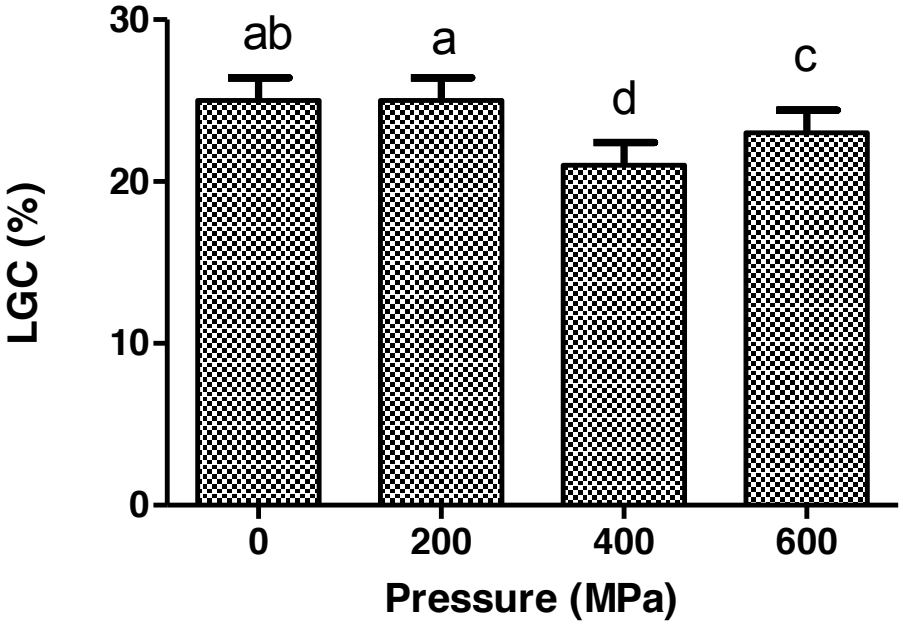
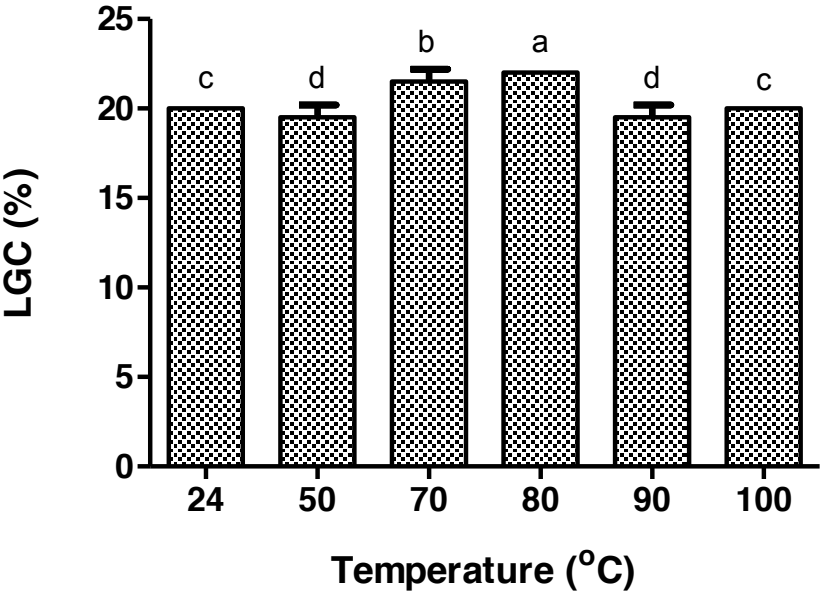


Figure 25. Least Gelation Concentration (LGC) of heat treated PPI



Sun et al. (2010) reported that PPI followed the three-step process of gelation that is generally accepted for heat-set gelation of globular proteins. This process, as described by Clark, Kavanagh, and Ross-Murphy (2001), was summarized as follows: (1) denaturation of the protein with subsequent exposure of hydrophobic groups inside of protein molecule; (2) intermolecular hydrophobic interaction or disulfide-linkage of the unfolded protein to form soluble aggregates; and (3) agglomeration of aggregates into a 3-D network that traps water molecules.

From Fig. 24, the 400 and 600 MPa treated samples exhibited slightly lower LGC values, which indicated that high pressure treatment could improve the gelation properties of pea proteins. On the contrary, as shown in Fig. 25, heat treated pea proteins showed slightly worse gelation properties at 70 and 80^{o}C when compared to the control.

O'Kane et al. (2004) observed that network formation by the 11S proteins in pea and soybean gels was mainly supported by hydrophobic interaction and hydrogen bonds, whereas disulfide bonds had minimum involvement. Mleko and Foegeding (2000) attributed the increased gel strength with increasing temperature during heating to the presence of hydrophobic interactions responsible for gelation. Therefore, it can be concluded that hydrophobic interactions and hydrogen bond are the key factors determining pea protein gelation properties.

The data showed significant less LGC for PPI with high pressure treatment, indicating high pressure treatment could improve the gelation properties of PPI. However, no significant difference was observed for heat treated PPI.

4.2.4 Water holding capacity (WHC)

The results of water holding capacities are shown in Figures 26 and 27. As shown in the Fig. 26, the WHC of pea protein treated by 0, 200 400 and 600 MPa is in the range from 0.75 to 0.83 g/g. Compared with buckwheat and soybean proteins, the PPI has quite lower WHC values (Tomotake, Shimaoka, Kayashita, Nakajoh, & Kato, 2002). This is probably due to the weak hydration of pea proteins in water leading to low WHC and solubility. There is a slight increase of WHC at 200 MPa, which could be explained by the formation of aggregates leading to holding more water inside of aggregates. However, with increased high pressure (400 and 600 MPa) WHC decreased again which could be due to some increased aggregation of pea protein molecules facilitated by the exposed hydrophobic surface. As pressure increased, the intensity of hydrophobic protein-protein interactions became greater, which causes reduced capacity for protein-water interactions.

The effect of heat on WHC of PPI is shown in Fig. 27. In general there was a significant increase of WHC via heat treatment, probably due to the formation of aggregates. This 3-D network could hold more water molecules than the native structure of PPI. The results clearly showed that after heat treatment, especially over 80^{o}C, the water holding capacity of PPI significantly increased. On the contrary, high pressure treated PPI did not exhibit significant increase compared to untreated PPI except the 200 MPa treatment. It can be concluded that the water holding capacity of PPI could be efficiently improved after high temperature and 200 MPa pressure treatment.

Figure 26. Water-holding capacity (WHC) of high pressure treated PPI

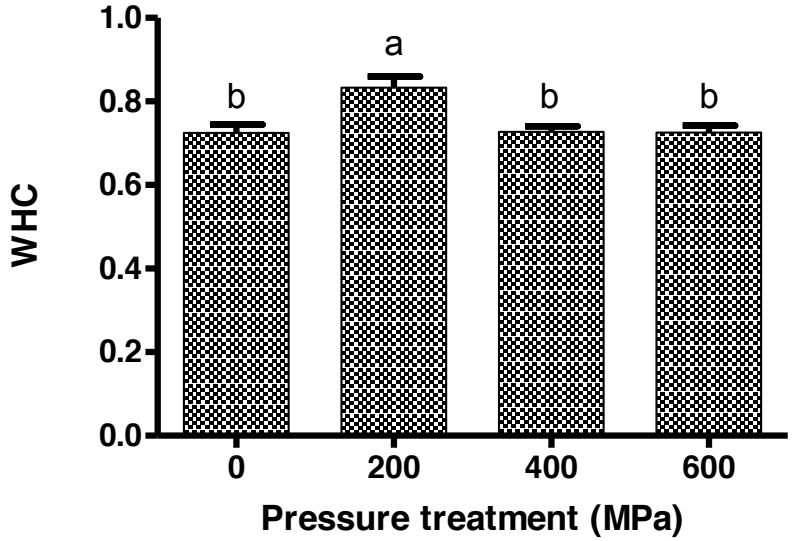
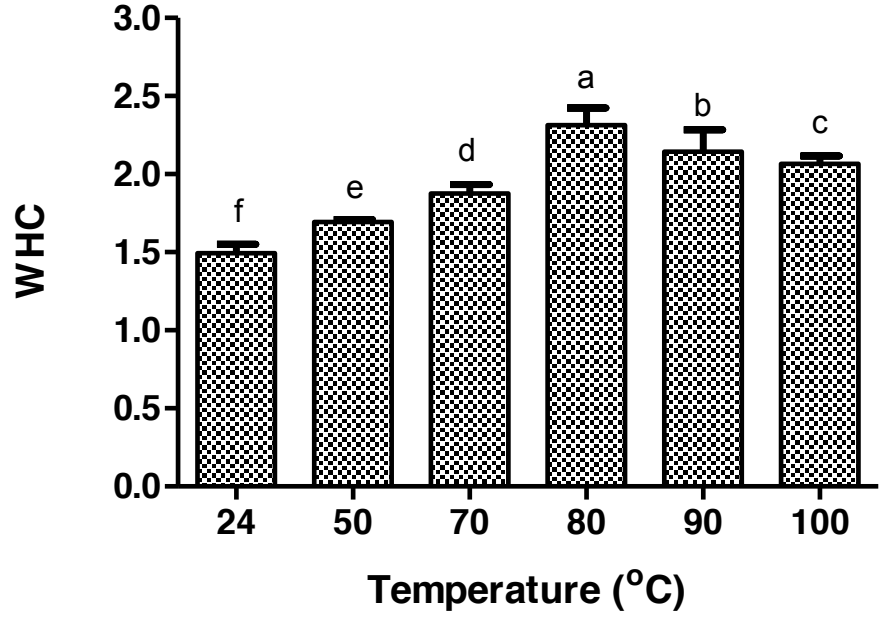


Figure 27. Water-holding capacity (WHC) of heat treated PPI



4.2.5 Oil Holding Capacity (OHC)

Results of the OHC are shown in Figures 28 and 29. The ability of proteins to bind fat depends on several parameters such as hydrophobicity, degree of denaturation, and the size and flexibility of the protein. As shown in the Fig. 28, the value of OHC of native PPI is about 2.45 g/g. The OHC of PPI was slightly improved after processing at the high pressure levels of 200 and 600 MPa. This is probably due to the partial unfolding of protein molecule leading to the exposure of hydrophobic groups buried inside of protein molecules to the exterior of protein molecules. In contrast, there is a slight decrease of OHC of PPI treated by the 400 MPa high pressure, which is probably due to the formation of aggregates mainly via hydrophobic interaction. However, the observed changes in OHC with high pressure treatments were not statistically different. As shown in Fig. 29, the values of OHC of PPI treated by 50 and 70°C are 2.9 and 3.2 g/g, respectively which are slightly higher than that of native PPI. With higher temperature treatments (80 and 90 °C), the PPI became denatured and there were many hydrophobic groups exposed to the exterior of protein molecules leading to these hydrophobic groups adsorbing more oil molecules. However, the structure of PPI treated by 100°C represents a totally denatured conformation with aggregation of protein molecules and leading to a significant decrease of OHC to 1.5 g/g. The results showed that thermal treatment was more effective than high pressure treatment as a means to improve OHC.

Similar as the conclusion got from water holding capacity, the oil holding capacity of PPI significantly increase after high than 80^{o}C temperature treatment and 200MPa pressure treatment. These results not only proved the hypotheses at the very beginning, but also provide the optimum treatment condition to food industry.

Figure 28. Oil-holding capacity (OHC) of high pressure treated PPI

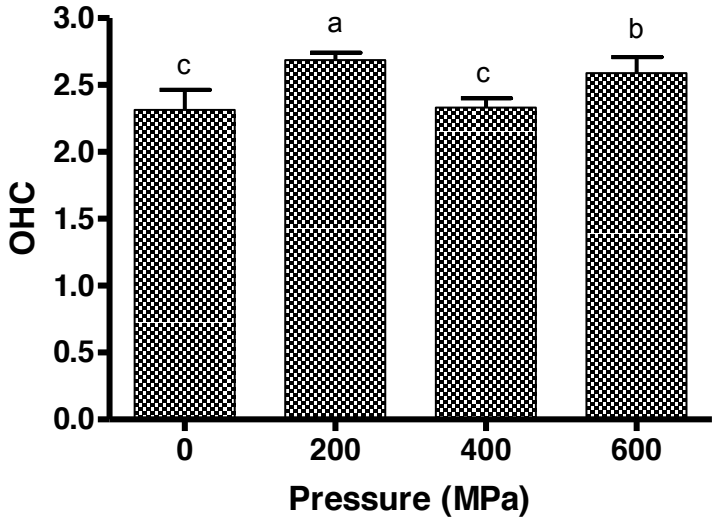
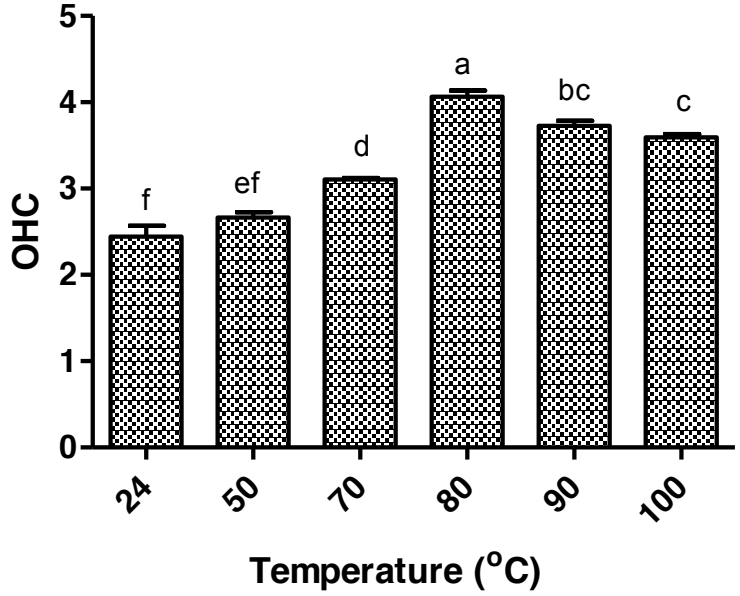


Figure 29. Oil-holding capacity (OHC) of heat treated PPI



4.3 EFFECTS OF HIGH PRESSURE AND THERMAL TREATMENTS ON ENZYMATIC HYDROLYSIS OF PEA PROTEIN ISOLATE AND FUNCTIONALITY OF RESULTANT PROTEIN HYDROLYSATES

4.3.1 Peptide yield

The peptide yields from treatment of PPI with four alcalase concentrations are shown in Fig. 30 and 31. All the PPI samples (heat or pressure treated) showed similar trend, which is gradual higher peptide yields with higher alcalase concentrations, indicating that the enzyme could hydrolyze PPI efficiently. Due to the gradually increasing peptide yield, all the four enzyme concentrations tested in this work might not be the optimum concentration.

4.3.2 Peptide identification using Fast Protein Liquid Chromatography (FPLC)

The FPLC column was used to perform peptide separation according to their molecular size. Therefore, high MW peptides will elute with shorter elution volumes while low MW peptides elute at longer volumes. The chromatogram of the standard proteins that were used to calibrate the column is shown in Fig. 32, while the chromatogram for each hydrolyzed PPI sample is shown in Figures 33-37.

The FPLC chromatograms of protein hydrolysate produced from untreated PPI and treated PPI showed peaks at similar elution volume, indicating that the peptides in each solution were similar. However, there are some differences among each peptide sample. For the protein hydrolysates from untreated PPI, as shown in Fig. 33, a peak at around elution volume 7.8 ml, which stands for peptides with MW of about 58 kDa was observed. Same peak was also observed for protein hydrolysates from the 100^{o}C treated PPI (Fig. 37). However, this 58 kDa peak was not observed for protein hydrolysates from

high pressure treated PPI (Fig. 33-36). The results indicate that the high pressure treatments were more effective in increasing susceptibility of the pea proteins to alcalase digestion. Thus, it is possible that the high pressure treatments led to greater exposure (through protein unfolding) of susceptible peptide bonds when compared to the thermal treatment. Three peaks (at elution volumes of 16.6, 17.2 and 18.7 ml) presented in the chromatogram of all samples were calculated based on the elution volume and the estimated MWs are 1856, 1555 and 998 Da, respectively.

Figure 30. Peptide yields of high pressure-treated pea protein isolate digested with alcalase

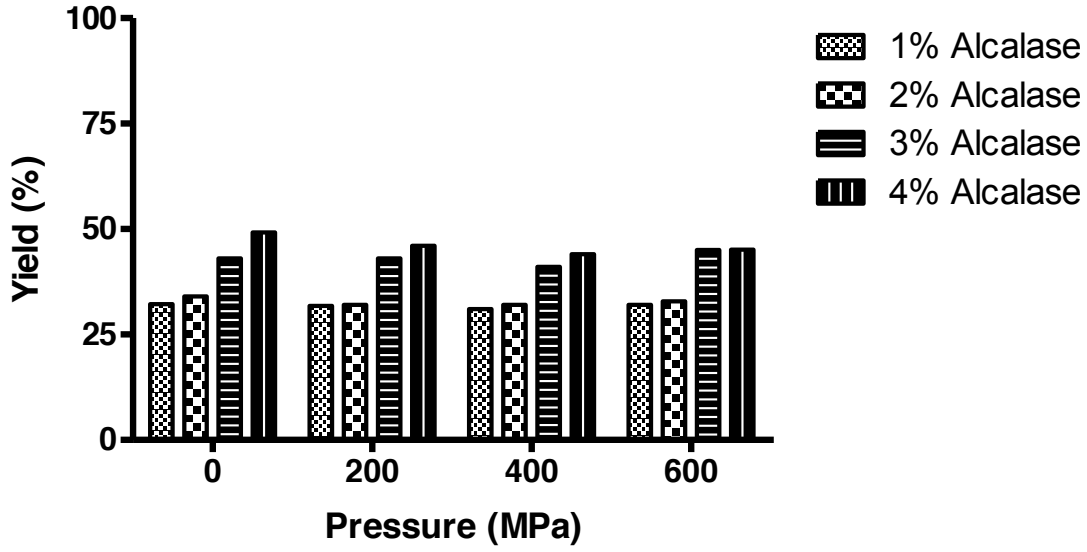


Figure 31. Peptide yields of heat-treated pea protein isolate digested with alcalase

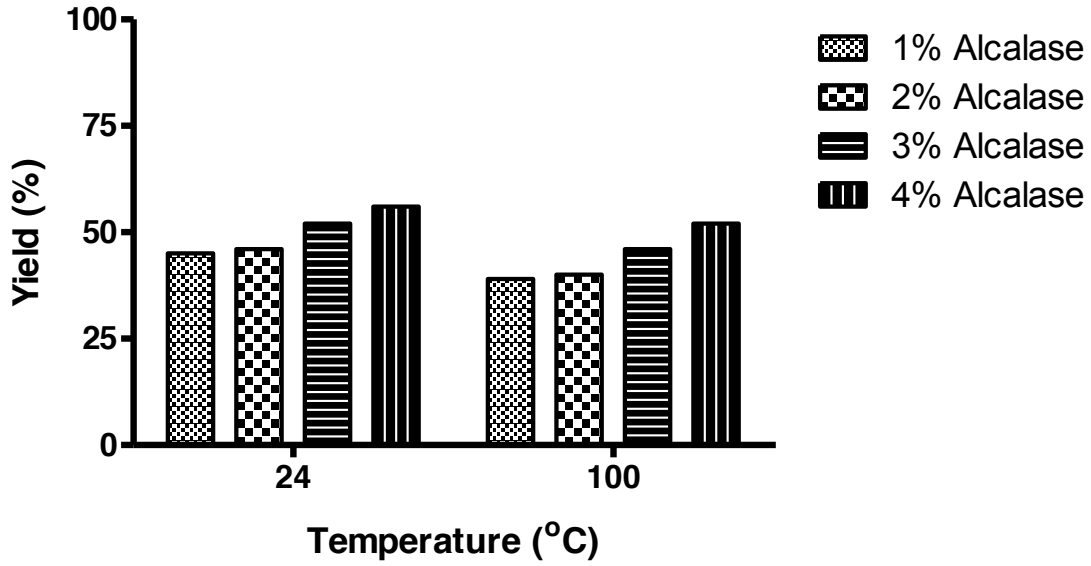


Figure 32. FPLC chromatogram of standard proteins: Cytochrome c (12,384 Da); Aprotinin (6,512 Da); Vitamin B_{12} (1,855 Da); Glycine (75 Da).

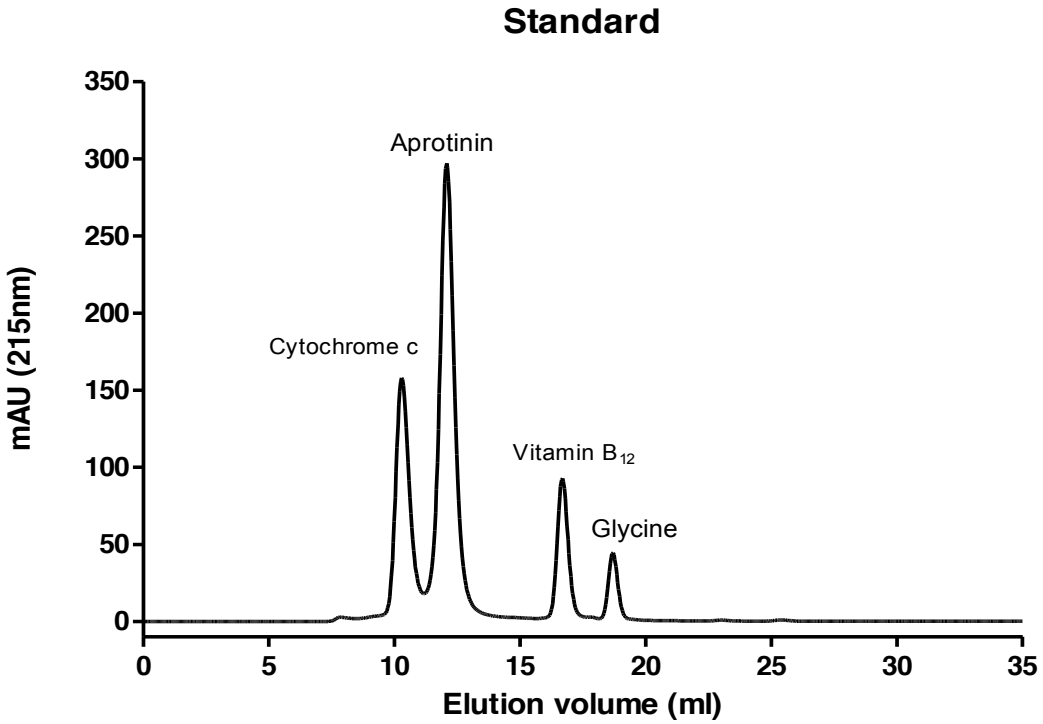


Figure 33. Chromatogram of protein hydrolysate from untreated pea protein isolate

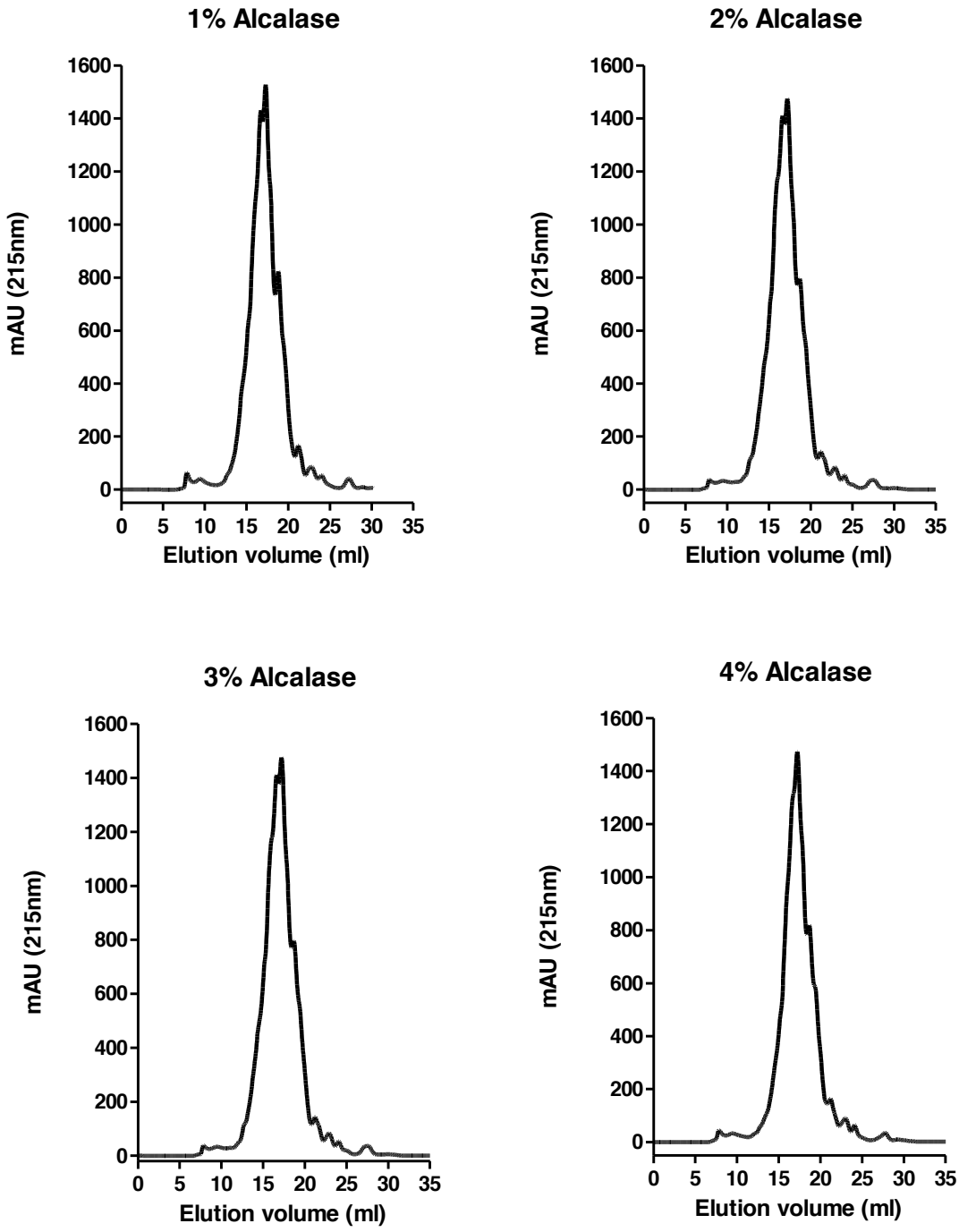


Figure 34. Chromatogram of protein hydrolysate from 200 MPa treated pea protein isolate

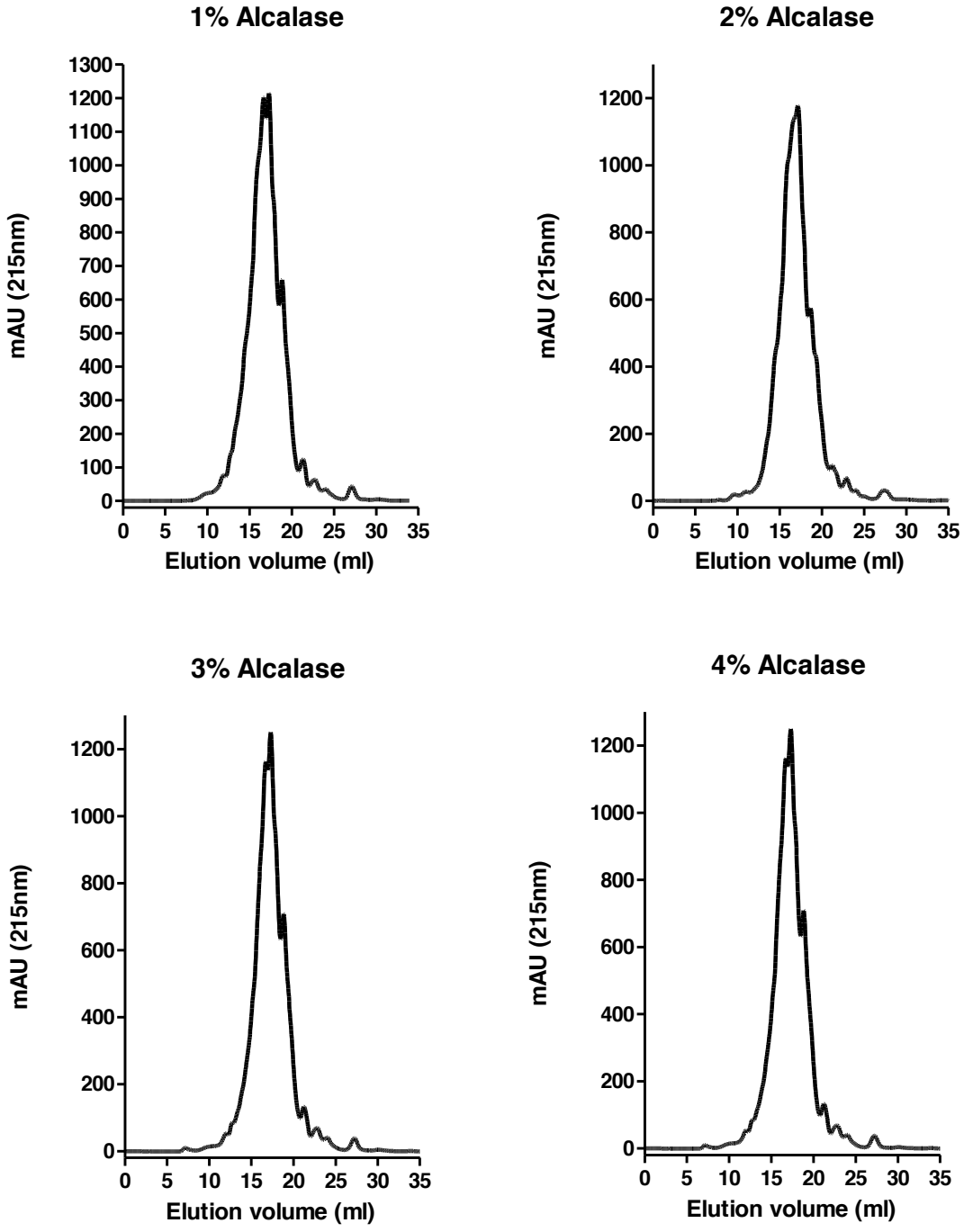


Figure 35. Chromatogram of protein hydrolysate from 400 MPa treated pea protein isolate

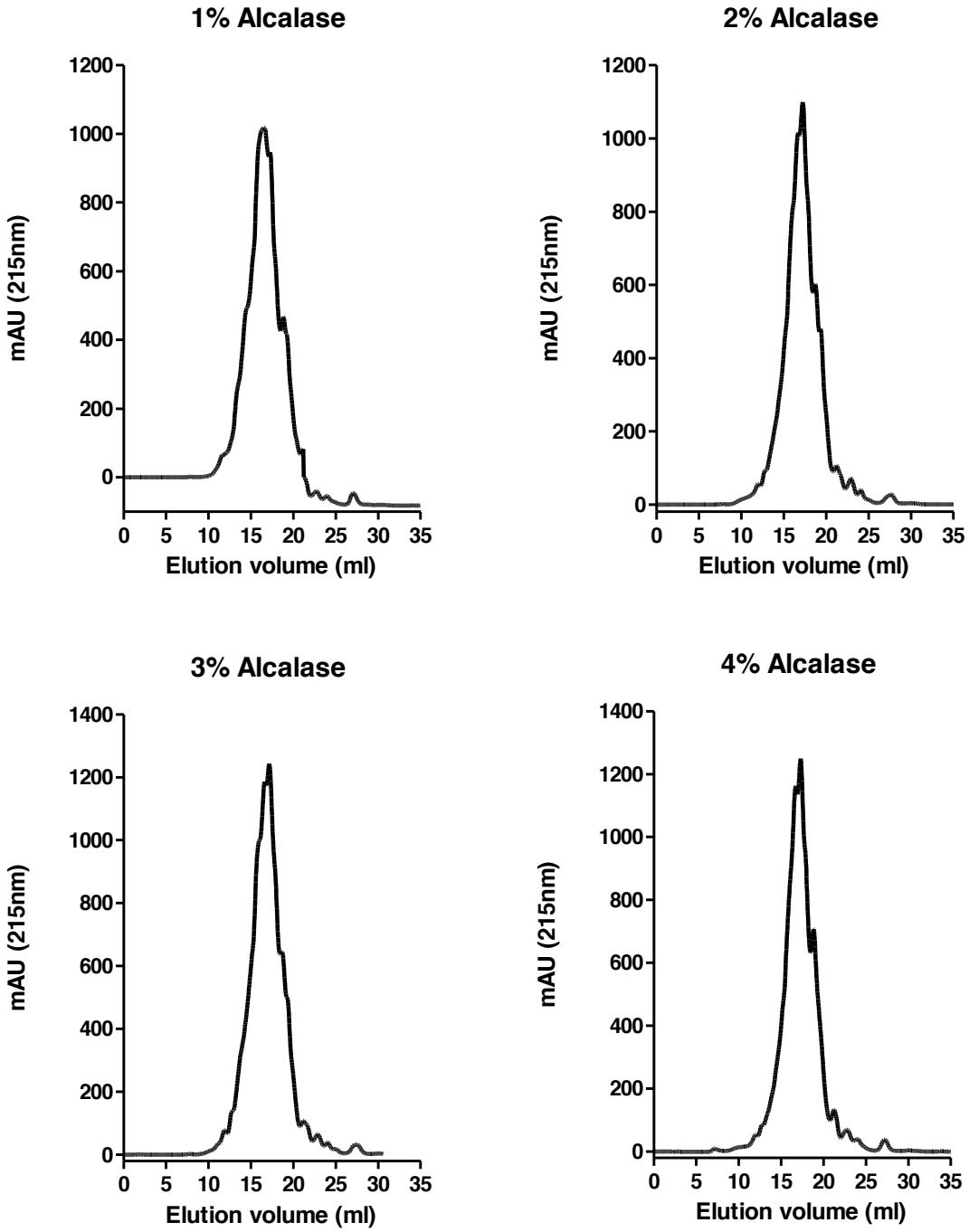


Figure 36. Chromatogram of protein hydrolysate from 600 MPa treated pea protein isolate

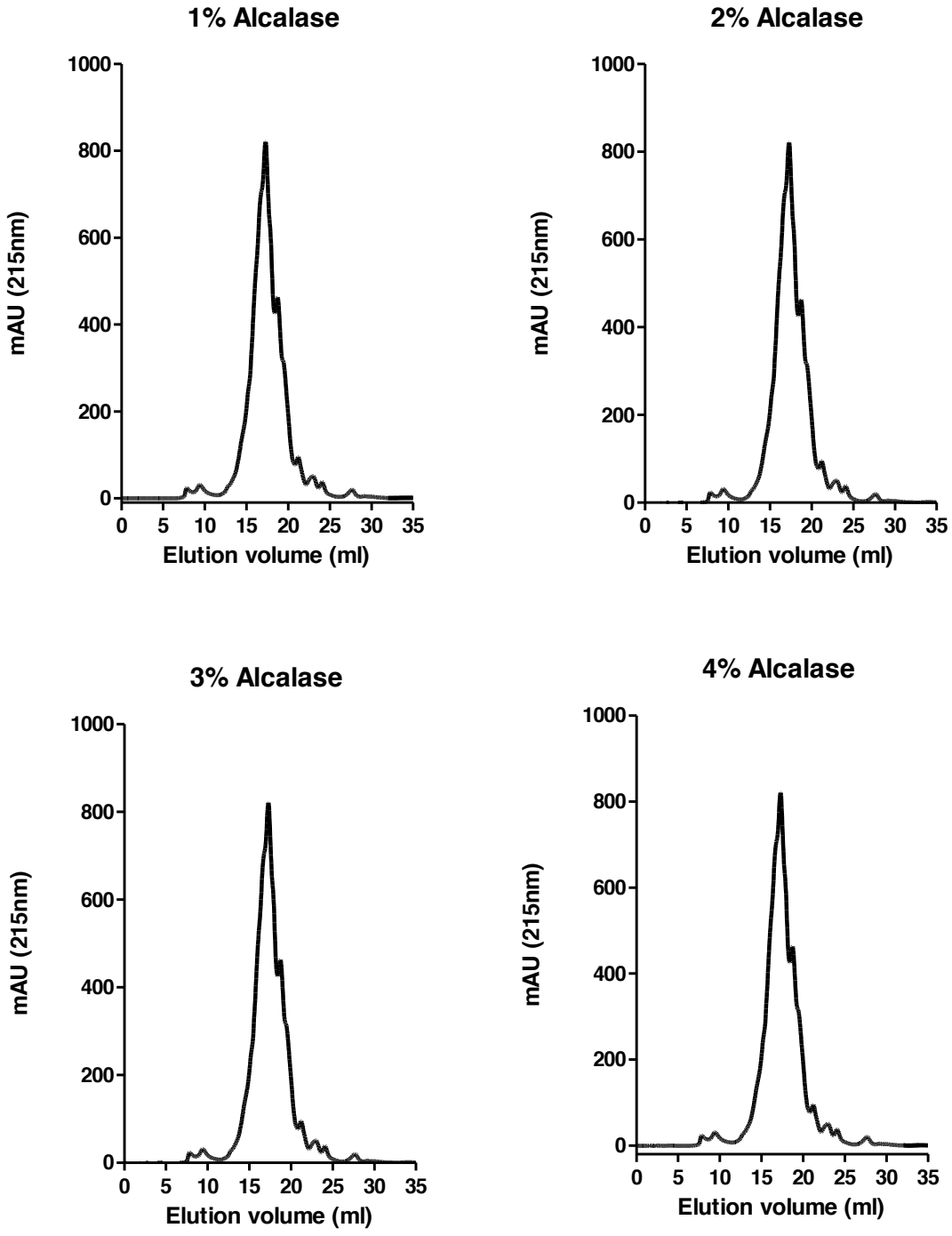
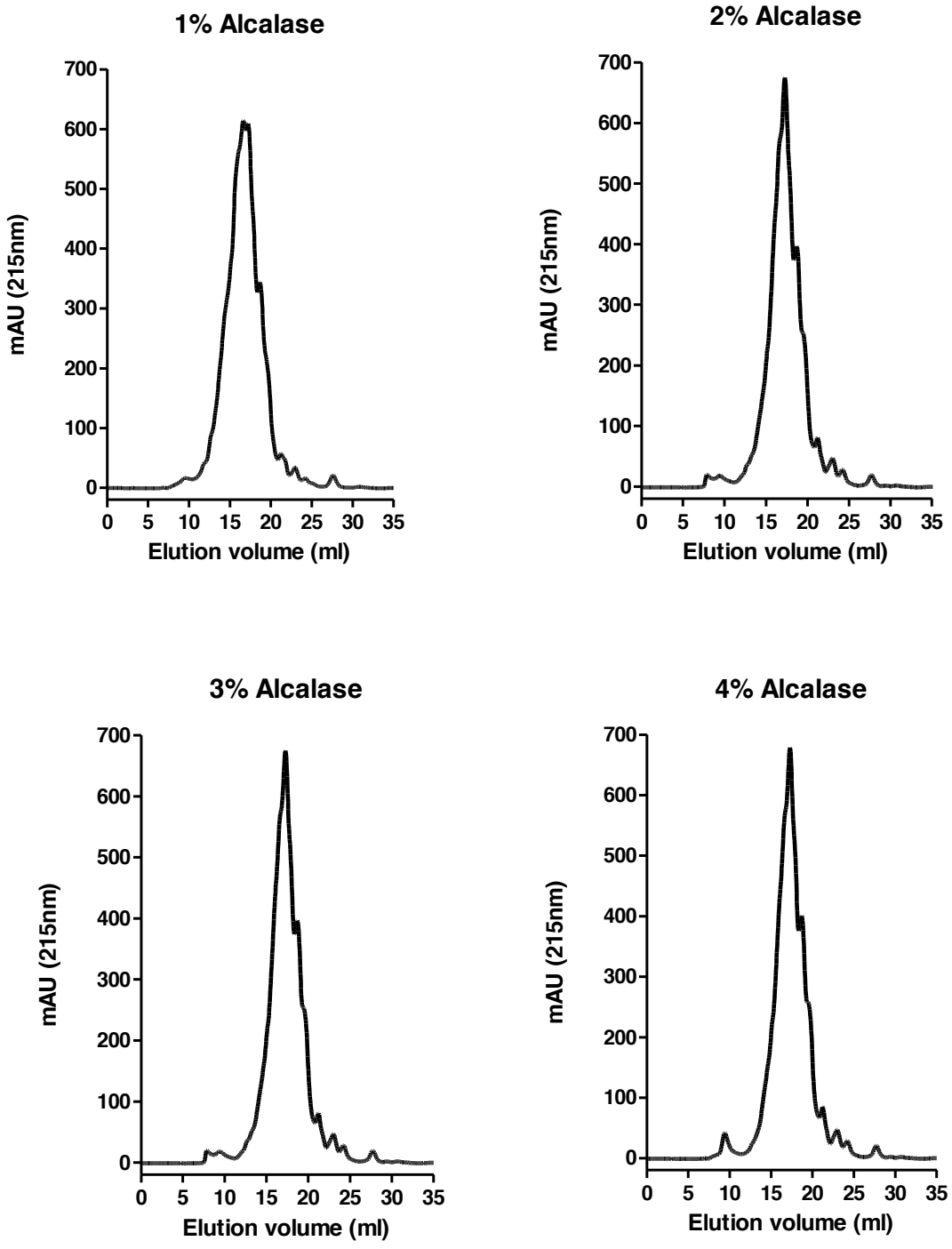


Figure 37. Chromatogram of protein hydrolysate from 100°C treated pea protein isolate



4.3.2 ACE and Renin inhibition activity

ACE and renin inhibitory activities of protein hydrolysates obtained from enzymatic (alcalase) digestion of high pressure-treated or heat-treated PPI are shown in Figures 38-41. All of the hydrolysates showed good ACE-inhibitory activity indicating pea protein is a good source of ACE-inhibitory bioactive peptides. For high pressure treatment samples, there is a little increase in ACE-inhibitory activity of the protein hydrolysates obtained from 400 and 600 MPa pressure treated PPI when compared to the untreated sample. Thus, high pressure treatment may have the potential to increase enzyme-induced liberation of ACE-inhibitory peptides from PPI. However, the inhibitory activity of the protein hydrolysates from pressure-treated samples was not as high against renin as obtained for ACE inhibition. But an increase of activity was observed for 1% and 2% alcalase hydrolysates of high pressure treated PPI when compared to the untreated PPI. Meanwhile, a significant decrease of renin-inhibitory activity was observed for 3% and 4% alcalase hydrolysates of high pressure treated PPI.

The ACE-inhibitory activity of heat treated PPI decreased significantly compared to the untreated PPI of all alcalase hydrolysates. This observed decrease might be attributed to increased aggregation of protein molecules at $100^{o}C$, which may have prevented hydrolysis of some of the critical peptide bonds that lead to release of ACE-inhibitory peptides. Meanwhile, the renin-inhibitory activity showed a decrease for the 3% and 4% alcalase hydrolysates but did not show significantly difference for the 2% and 4% alcalase hydrolysates from heat-treated PPI. Overall, the results showed that high pressure treatment was more effective than heat treatment for the purpose of alcalase-dependent release of potential antihypertensive peptides from PPI.

After high pressure treatment, it can be observed that with 1% alcalase hydrolysis, the ACE and renin inhibitory ability increased gradually by the increasing of high pressure treatment. It can be suggested that 1% alcalase is an optimum enzyme concentration for bioactive peptides generation for these treated PPI samples.

Figure 38. ACE-inhibitory activities of protein hydrolysates from alcalase digestion of high pressure treated pea protein isolate

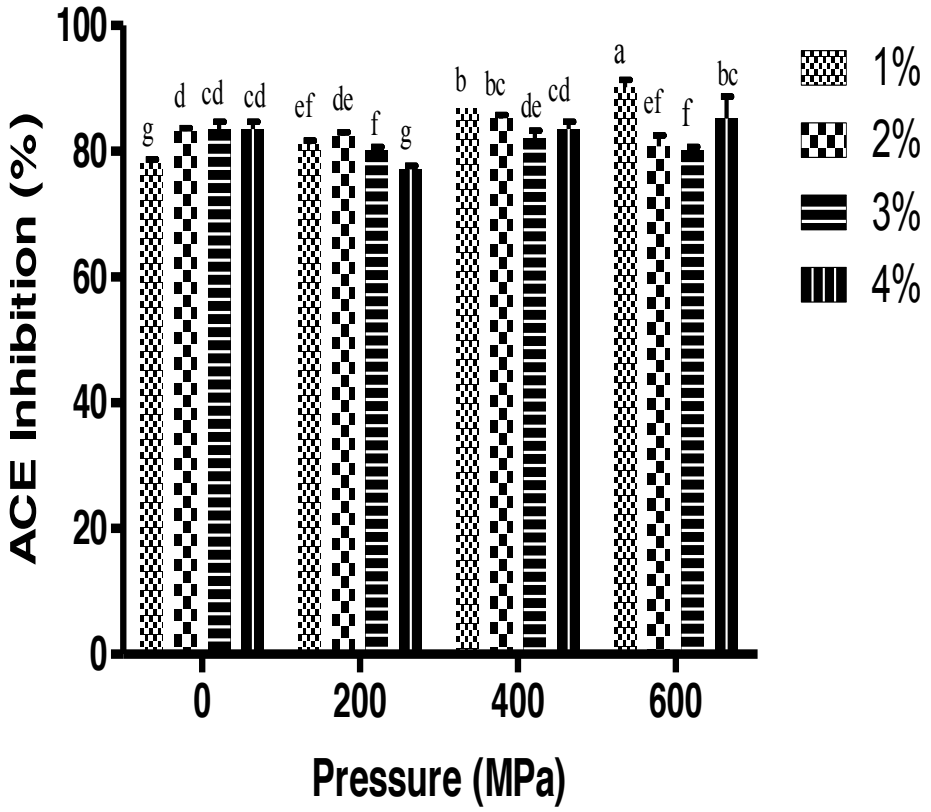


Figure 39. ACE-inhibitory activities of protein hydrolysates from alcalase digestion of heat treated pea protein hydrolysate

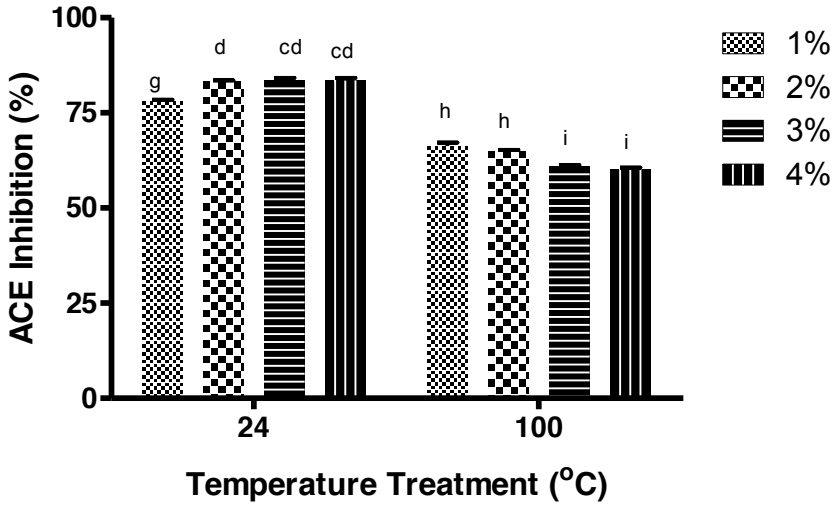


Figure 40. Renin inhibitory activities of protein hydrolysates from alcalase digestion of high pressure treated pea protein isolate

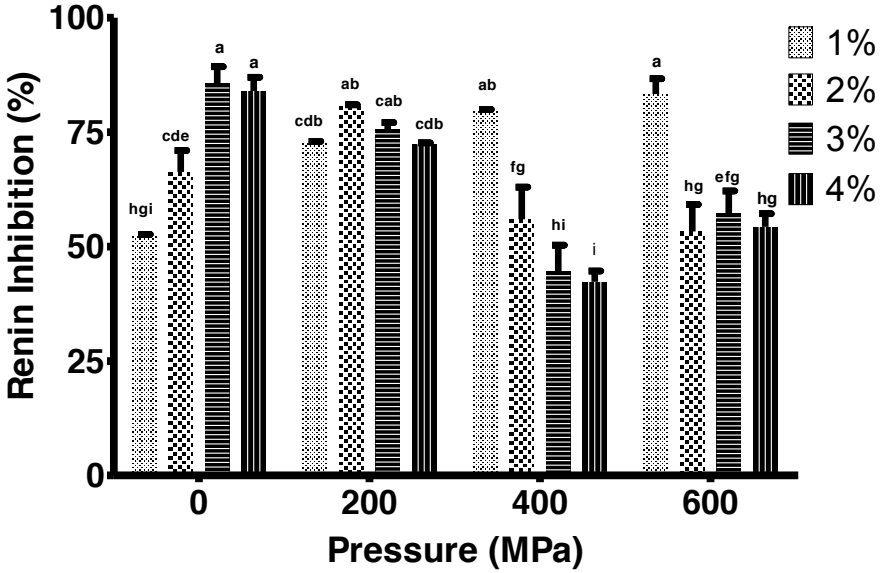
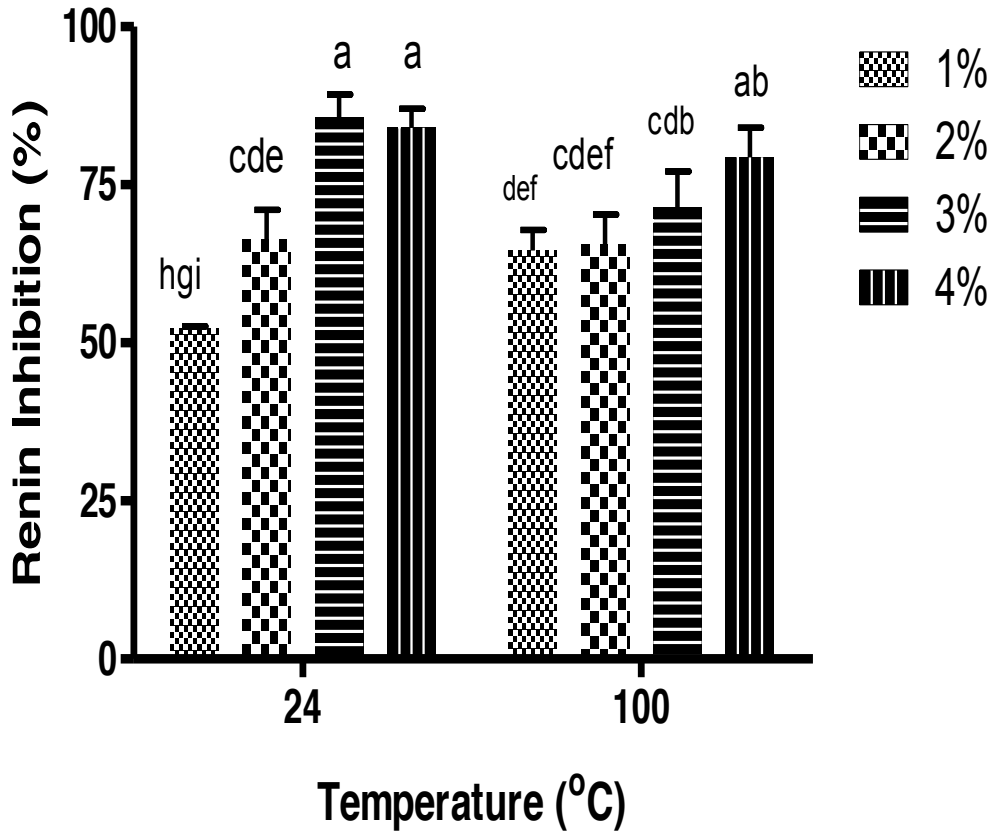


Figure 41. Renin-inhibitory activities of protein hydrolysates from alcalase digestion of heat treated pea protein isolate



CHAPTER FIVE – GENERAL DISCUSSION AND CONCLUSIONS

This study determined the effects of high pressure and thermal treatments of PPI on changes in functional and physicochemical properties as well production efficiency and activity of potential antihypertensive peptides. High pressure and temperature treatments are important tools in the food processing industry that can be used to improve functional properties of pea proteins and improve production of bioactive peptides. In this project, changes of both functional properties and physicochemical properties between treated and untreated PPI were observed. Based on data of this project, it can be demonstrated that both high pressure and heat treatment had affected tertiary and quaternary structures of protein. For high pressure treated PPI, the emulsion properties improved significantly. Furthermore, the 600 MPa treatment showed significant difference ($p<0.05$) in physicochemical properties indicating high pressure caused significant protein structure changes.

Heat treated PPI exhibited different changes and based on the study, we can conclude that protein structure of PPI underwent a sharp structural denaturation when subjected to high heat with temperatures between 70 and 80^{o}C. Physicochemical properties also showed different results between PPI treated with 50, 70 and 80^{o}C and PPI treated with 90 and 100^{o}C, which showed that treatments at higher temperatures led to more extensive changes in protein structural conformation.

As known, there are many hydrophobic groups buried inside globular protein molecules. Upon the high temperature (usually > 70 °C) or high pressure treatment, this intact protein structure would be disrupted leading to these groups exposing exterior of

molecules thereby increasing the hydrophobicity of protein molecules, which subsequently decreases the net surface charge of protein molecules (Kinsella, 1979). This can has a great impact on the functionality of globular proteins. As shown in Fig 8 and 9, the hydrophobicity of PPI is significantly increased by the thermal and high pressure treatment, which leads to the decrease of PPI solubility. At low pH (3 or 5), the high pressure treatment can improve the emulsifying capacity to some extent which may be because the increasing hydrophobicity of PPI with decreased pH leads to the more adsorbed protein concentration. However, the great increase of hydrophobicity in PPI treated under 90 °C (about 2.3 fold than that of PPI) could lead to the over-aggregation of protein thereby leading to decreased functionality. For example, the foaming capacity of PPI treated by the temperature 90 or 100 °C has a marked decrease. Besides, the increased hydrophobic interactions between denatured protein molecules induced by the high pressure improve the gelling capacity of PPI.

Based on molecular level, the hydrophobic interaction which is very strong in short range between protein molecules (Bryant & Julian, 1998), which plays a very important role in determining the functionality of proteins. It can impact the aggregation of protein molecules, the protein load to the oil-in- water interface, the magnitude of interactions between gel structural element and etc. Therefore, modifying protein structures through increasing the hydrophobicity of protein molecules is a feasible way to improve the functionality of PPI.

However, there were only small but also significantly differences between activities of protein hydrolysates produced from alcalase hydrolysis of high pressure or heat-treated PPI. All the hydrolysates from different PPI treatments showed good ACE

and renin inhibitory activities. High pressure treatment was more effective than heat treatment for the production of ACE- or renin-inhibitory peptides. This is probably because high pressure treatment is more efficient than heat treatment for unfolding proteins and exposing peptide bonds to enzyme hydrolysis.

Compared with the hypotheses at the very beginning of this project, the result showed good support to prove high pressure and heat treatment have potential effect at improving the functional properties of PPI. On the contrary, the results from anti-hypertensive properties were not very encouraging. The 100^{o}C heat treatment showed slightly decreased ACE-inhibitory peptides when compared to the untreated PPI. On the other side, peptides from PPI with 3% and 4% alcalase hydrolysis decreased the ACE and renin inhibitory activity significantly compared to the untreated PPI. However, hydrolysates from 1% and 2% alcalase concentration exhibited increased levels of renin and ACE inhibition. These phenomena could be a guide of optimum enzyme concentration, which is a quite important factor in cost saving for food industry because treatment of PPI with high pressure could enable use of lower enzyme concentrations to produce bioactive peptides.

In conclusion, PPI showed improved properties after high pressure treatment and controlled heat treatment, which demonstrated these two processing methods might increase the PPI utilization as an ingredient in the food industry.

CHAPTER SIX - FURTHER RESEARCH

From previous studies, some previously reported works have shown that functional properties of proteins would improve after high pressure or heat treatment, while some indicated insignificant improvement. Our study showed the functional properties could be improved by high pressure treatment or heat treatment. However, for deeper understanding and more convinced explanations of the underlying principle for this improvement, an in-depth study of changes in protein structure should be studied, especially the primary, secondary and tertiary structure.

Pea protein-derived peptides under normal processing conditions have been shown to possess good anti-hypertensive activity (Li & Aluko, 2010). In this project, the potential use of high pressure and heat treatments to increase the anti-hypertensive activity of pea protein-derived peptides was also observed. For a better utilization of PPI as a raw material for the production of nutraceutical ingredients, further research should be conducted to optimize the use of high pressure treatment. Since only one protease (alcalase) was used in this project, the use of other commonly available proteases such as trypsin, chymotrypsin, papain, pepsin, etc., need to be explored for generating bioactive peptides from high pressure or heat treated proteins. Also, it will be necessary to separate, purify, and identify the amino acid sequence of bioactive peptides produced from these processing conditions. Finally, testing of the protein hydrolysates in a suitable animal disease model such as the spontaneously hypertensive rat will be necessary to confirm *in vivo* potency.

CHAPTER SEVEN - REFERENCES

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