A Study on the Functional Properties of Four Different Types of Plant-based Protein

A collaboration between Singapore Institute of Technology/Massey University and Big Idea Ventures

Wang Yi Jin (SIT/Massey)

Lim Wensheng (SIT/Massey)

Matthew Zhao (BIV)

3rd - 28th August 2020

Contents

1 Introduction

Recently, there has been an increased demand for plant protein as an ingredient in the food industry because of their relatively low cost and sustainability as compared to animal proteins (Peng, Kong, Chen, Zhang, Yang & Hua, 2016). Many proteins are from common food allergens such as soy, milk, egg or wheat. Hence, plant protein from pea and rice are good alternatives due to its lower allergenic potential. The aim of this project was to evaluate the functional properties of brown rice protein, pea protein, faba bean protein and pumpkin protein.

1.1 Functional Properties of Food Protein

Protein has several functional properties which can be classified into four main categories; hydration, interfacial, aggregation and gelation properties. The hydration properties of the protein are dependent on the interaction with water and it will have an effect on wettability, swelling, adhesion, dispersibility, solubility, rheological property, water holding capacity and absorption. Surface tension, emulsification and foaming capacity are examples of interfacial properties, aggregation and gelation properties are dependent on the interactions between a protein-protein molecule (Galazka, Dickinson & Ledward, 2000). Other interesting functional properties of food protein also includes flavour binding and dough formation. These functionalities greatly impact the sensorial properties of food.

1.2 Protein Hydration

The ability of the protein to interact with water affects the functional properties such as dispersibility, wettability, solubility, thickening/viscosity, water-holding capacity etc. For low and intermediate moisture food, such as bakery and comminuted meat products to be acceptable in terms of sensory characteristics, the ability for protein to bind water is important. Part of the hydration capacity is related to its amino acid composition; a protein with more charged residues has a larger hydration capacity (Damodaran, 1996).

Hydration ability is the lowest at the isoelectric pH of the protein; shifting the pH away from the isoelectric point allows the net charge and repulsive forces to be increased and more water

will be bound. As observed in most proteins at pH 9-10, the water binding capacity is at the greatest largely attributed from the ionization of sulfhydryl and tyrosine residues. Beyond pH 10, the water binding capacity is reduced because of the absence of positively charged amino groups of lysyl resides (Damodaran, 1996).

Increasing temperature decreases the water binding capacity of proteins because there is a reduction in both hydrogen bonding and hydration of ionic groups. A denatured protein exposes some of the previously buried hydrophobic groups and therefore, it is able to bind 10% more water than the native protein. However, if aggregation occurs due to denaturation, the protein-protein interactions will result in a decrease in water binding capacity (Damodaran, 1996).

1.2.1 Solubility

Solubility is an important aspect in food application as it is needed for the protein to perform various functionalities such as thickening, foaming, emulsifying, and gelling. Hydrophobic and ionic interactions influence the solubility characteristics of protein in two different ways. The solubility of protein decreases when there are more hydrophobic interactions involved, whereas solubility increases when there are more ionic interactions involved (Damodaran, 1996).

pH has a direct effect on the solubility of protein as protein carries a net positive charge when pH is below the isoelectric point and a net negative charge when pH is above the isoelectric point. Protein solubilization is promoted by electrostatic repulsion and when charged residues are hydrated. Similar to protein hydration characteristics, solubility is the minimum at the isoelectric pH of proteins and it is primarily caused by aggregation and precipitation via hydrophobic interactions. Sufficient electrostatic repulsion is needed to prevent this from occurring. Only certain proteins (e.g. lactoglobulin and bovine serum albumin), that has a large proportion of surface hydrophilic residues to surface non-polar groups, are highly soluble at their isoelectric pH. Hence, the protein will be soluble at the isoelectric pH only when the protein-protein hydrophobic interactions are less than the hydrophilicity and hydration repulsion forces from the charged residues. At alkaline pH of 8 to 9, most proteins are highly soluble. Therefore, protein extraction is carried out at this pH for plant sources such as soybean flour. Isoelectric precipitation at pH 4.5 to 4.8 can be carried out to recover the protein (Damodaran, 1996).

In general, the solubility of protein increases with temperature (0 – 40°C) at constant pH and ionic strength. Some highly hydrophobic proteins exhibit a negative relationship with temperature, for example, casein and some cereal proteins. At above 40°C, the occurrence of decreased solubility is primarily caused by denaturation (protein unfolding), exposure of the non-polar group, aggregation and precipitation (Damodaran, 1996).

1.2.2 Viscosity

In a system with high protein concentration, the viscosity coefficient tends to increase with shear rate and this means it exhibits a pseudoplastic or shear thinning behaviour. The behaviour is a result of protein molecules aligning their major axes in the direction of flow. If the protein remains oriented after shearing or flow is stopped, the solution will not be able to regain its original viscosity rapidly. Such behaviour is seen in fibrous protein such as gelatin and actomyosin. On the other hand, the globular protein is able to regain its viscosity when the flow is stopped. Such behaviour is seen in soy and whey proteins, and these solutions exhibit thixotropic behaviour (Damodaran, 1996).

1.2.3 Interfacial Properties

A protein molecule is amphiphilic and it tends to move to an air-water interface or an oil-water interface to stabilize the system. Protein-stabilized foams and emulsions are stronger than those prepared using low molecular weight surfactants. The highly viscoelastic film formed at the interface is stable against mechanical shocks and therefore, proteins have a vast application in foam and emulsion type food products. All proteins are amphiphilic but they have very different surface-active properties and is largely due to the differences in protein conformation. These include how stable or flexible the polypeptide chain is, the ability to adapt to environmental changes and the proportion of hydrophilic and hydrophobic groups on the surface of the protein (Damodaran, 1996).

1.2.4 Gelation

Protein gelation occurs when protein transforms from a “sol” state to a “gel-like” state under the presence of enzymes, heat or divalent cations. During heating, the protein undergoes

denaturation and the protein in a sol state is transformed into a “progel” state – a viscous liquid state where a certain degree of protein polymerization has occurred. Secondly, when the protein has unfolded, hydrogen bonding and hydrophobic groups are exposed and facilitates the formation of a protein network (Damodaran, 1996).

1.2.5 Protein Denaturation

Protein in its native state is the net result of several attractive and repulsive interactions emanating from various intramolecular forces and interactions between protein groups with solvent. The environment in which the protein is subjected to has a large impact on the native structure. For example, ionic strength, pH and temperature. Denaturation occurs when the secondary, tertiary and quaternary structures of the protein are altered and without cleavage of backbone peptide bonds, which resulted in a loss of ordered structure, in most instances. Denaturation can be both desirable and undesirable in food. For example, denaturation can cause insolubility and loss of some functional properties of food proteins. In the case of a desirable effect, thermal denaturation of trypsin inhibitors found in legumes significantly enhances its bioavailability and digestibility. In general, proteins that are partially denatured have better digestibility and its foaming and emulsifying properties are better than in the native state. Heat-induced gelation of food proteins also requires thermal denaturation as a prerequisite (Damodaran, 1996).

2 Materials and Methods

2.1 Materials

All the plant proteins were sourced commercially and requested from several companies. Brown rice protein (Oryzatein®, 84% protein) was obtained from Axiom Foods, Inc. Pea protein (VITESSENCE™ 1803, 80% protein), faba bean protein (VITESSENCE™ Pulse CT 3602, 60% protein) were obtained from Ingredion Singapore Pte Ltd. and pumpkin protein (60% protein) was obtained from Kündig Group. The specifications of all the proteins samples are presented in Appendix A. All the other chemicals used in this project were of analytical grade and purchased from Sigma Aldrich, St Louis, MO, USA, unless otherwise indicated.

2.2 Sample Preparation and Process Conditions

2.2.1 Pressure Treatment of Plant Proteins

10% (w/w) slurry was prepared by dissolving the protein powder into deionised water and stirred using the magnetic stirrer (400rpm) for 30 minutes at room temperature. The pH of the solution (pH 3, 5, 9) was adjusted using either 1M HCl or NaOH. Thereafter, the solution was packed into Polyamide/Polyethylene (PA/PE) pouch for HPP treatment (200, 400 and 600MPa for 10 minutes) using the high pressure processor (Hiperbaric 300i). After which, the samples were stored chilled (~4°C) overnight and 0.02% (w/w) sodium azide was added the following day.

2.3 Determination of Protein Solubility

The biuret and Lowry method were used to determine the soluble protein content by using the Total Protein Kit (TP0200 and B3934) obtained from Sigma Aldrich. The kit contains protein standard (bovine serum albumin), biuret reagent and Folin and Ciocalteu's Phenol reagent.

2.3.1 Preparation of Protein Standard Curve

0.5 ml of the protein standard, containing 100 mg/ml bovine serum albumin, was pipetted into a 50 ml volumetric flask and topped up to the mark with 0.85% sodium chloride solution to make a concentration of 1000 μg/ml. This solution was further diluted to make protein concentration of 0 μg/ml, 250 μg/ml, 500 μg/ml and 750 μg/ml as indicated in Table 1. 2.2 ml of biuret reagent was added to each solution, mixed and left to stand at room temperature for 10 minutes and following that, 0.1 ml of the Folin and Ciocalteu's Phenol was added to the solution, mixed and left to stand for 30 minutes. The absorbance of the solution was read at 400 – 1000 nm using Spark® Multimode Microplate Reader (Tecan Trading AG, Switzerland) with test tube 1 as a reference. The absorbance values were plotted against protein concentration.

Table 1: Solutions used to obtain a calibration curve.

Test Tube	Diluted Protein Standard (ml)	0.85% Sodium Chloride Solution (ml)	Protein (μg/ml)
1	0	0.20	0
2	0.05	0.15	250
3	0.10	0.10	500
4	0.15	0.05	750
5	0.20	0	1000

2.3.2 Preparation of Sample for Analysis

The sample (10% w/w) was centrifuged at 20,000 × g for 30mins, 20°C and the supernatant was then filtered through a 0.45μm syringe filter (Sartorius, Minisart® Syringe Filter, Germany). The filtered supernatant was diluted with 0.85% sodium chloride to give a final protein concentration of 150 – 1000 μg/ml. 2.2 ml of biuret reagent was added to each solution, mixed and left to stand at room temperature for 10 minutes and following that, 0.1 ml of the Folin and Ciocalteu's Phenol was added to the solution, mixed and left to stand for 30 minutes. The reference used was prepared in the same way as described in 3.4.1. The absorbance of the solution was read at 735 nm using Spark® Multimode Microplate Reader (Tecan Trading AG, Switzerland). Triplicate measurements were taken for each sample.

2.4 Determination of Phase Stability

50ml of the sample was poured into a clear polystyrene tube and stored chilled (~4°C). In order to ensure homogeneity in each tube, the sample was stirred for at least one minute before pouring into the tube. Visual observation and height of sediment were taken after 24 hours and 1 week of storage. The images of the samples were taken in a benchtop photobooth with controlled lighting. Phase stability (%) is calculated using the equation below:

$$\text{Phase stability (\%)} = \frac{\text{Height of solution in tube} - \text{Height of bottom layer in tube}}{\text{Height of solution}} \times 100$$

2.5 Determination of Zeta Potential

Zeta potential of 1% (w/w) protein solutions before and after treatment were determined by electrophoretic light scattering using Horiba SZ-100 zeta potential analyser. The sample was injected into a carbon electrode cell (6mm) and a measurement of the particle electrophoretic mobility results in the calculated zeta potential. Triplicate measurements were taken for each sample using Smoluchowski model at 25°C.

2.6 Statistical Analysis

Analysis of variance was conducted with the ANOVA function of Minitab 18 Statistical Software (Minitab®, United States). Significant differences between sample means were analysed using Tukey's range test and at a significance level of 0.05 (95% confidence interval). The mean and error bar using the standard deviation of the results were presented. Different lower-case letters indicate that there is a significant difference between the mean values.

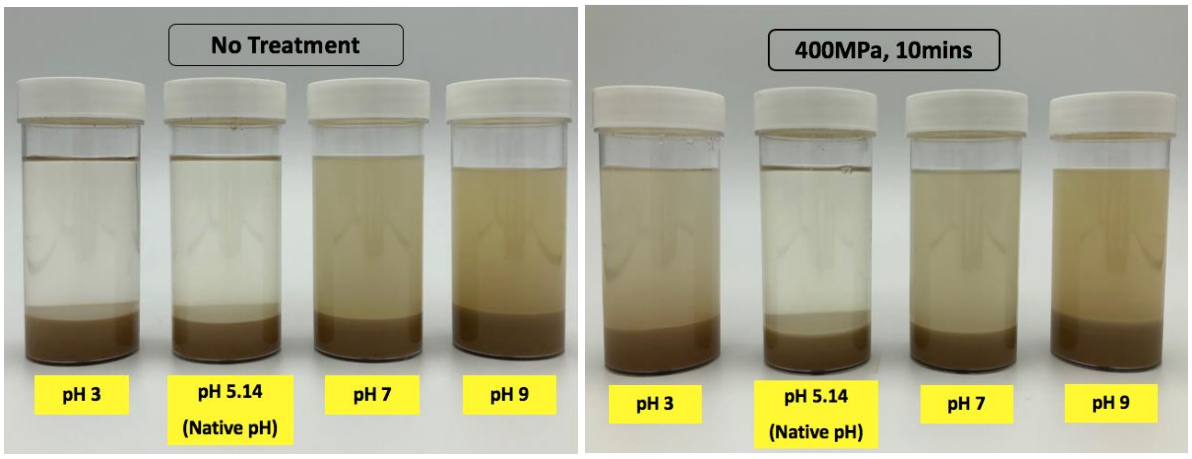
3 Results and Discussion

3.1 Screening of Plant Protein

3.1.1 Effect of pH and HPP Treatment

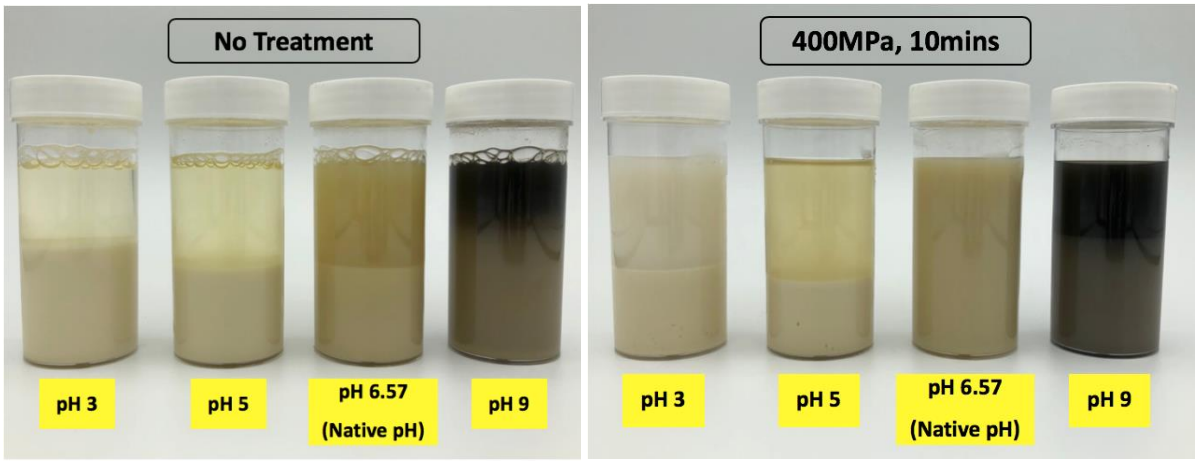
3.1.1.1 Phase Stability

The phase stability of 10% w/w brown rice protein, faba bean protein, pumpkin protein and pea protein in deionised water at varying pH are presented in Figure 1, 2, 3 and 4. Figure 1(a), 2(a), 3(a) and 4(a) were samples that did not go through any pressure treatment while Figure 1(b), 2(b), 3(b) and 4(b) were samples subjected to pressure treatment of 400MPa for 10 minutes. Generally, it could be deduced that all the plant proteins are highly insoluble in water as evidenced by the poor phase stability, sedimentation and phase separation in all the samples without treatment. Only faba bean protein at native pH resulted in a stable suspension after HPP treatment at 400MPa for 10 minutes as shown in Figure 2(b).



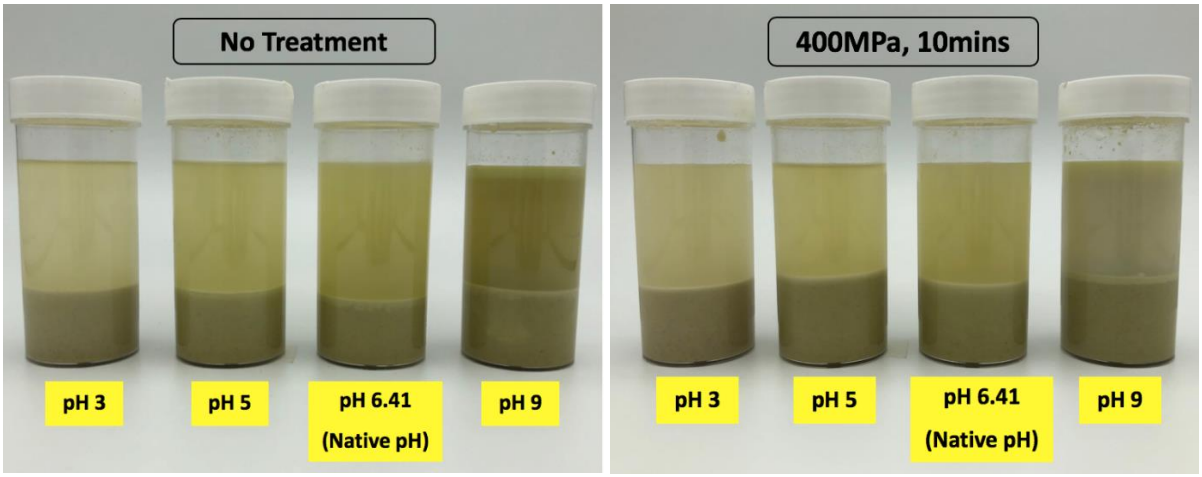
(a) (b)

Figure 1: Phase stability of (a) untreated and (b) pressure treated, 400MPa, 10 minutes 10% w/w brown rice protein at different pH.



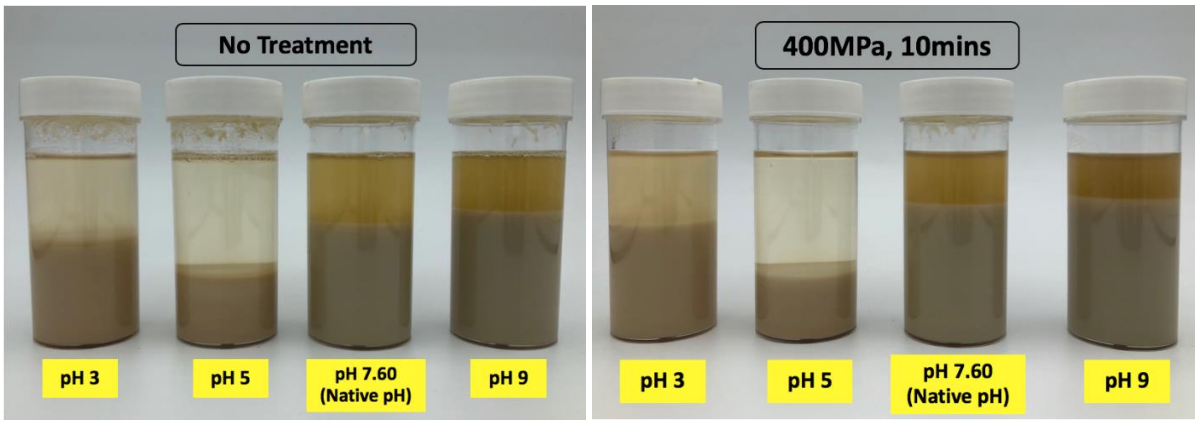
(a) (b)

Figure 2: Phase stability of (a) untreated and (b) pressure treated, 400MPa, 10 minutes 10% w/w faba bean protein at different pH.



(a) (b)

Figure 3: Phase stability of (a) untreated and (b) pressure treated, 400MPa, 10 minutes 10% w/w pumpkin protein at different pH.



(a) (b)

Figure 4: Phase stability of (a) untreated and (b) pressure treated, 400MPa, 10 minutes 10% w/w pea protein at different pH.

3.1.1.2 Solubility

Measuring the solubility of protein is one of the methods used to understand protein denaturation and aggregation (He et al., 2016). The effect of HPP treatment (400MPa, 10mins) on the solubility of brown rice, faba bean, pumpkin and pea protein are presented in Figure 5, 6, 7 and 8. The highest solubility was seen at the alkaline pH (pH 9) for all the plant proteins. It is typical for protein to have the highest solubility at alkaline pH because above the isoelectric pH, protein carries a net negative charge. Electrostatic repulsion and hydration of charged

residues promote solubilisation of the proteins. Usually, solubility is the minimum at the isoelectric pH (which is typically between 4.5-4.8) because of the lack of electrostatic repulsion, which causes aggregation and precipitation through hydrophobic interactions (Damodaran, 1996).

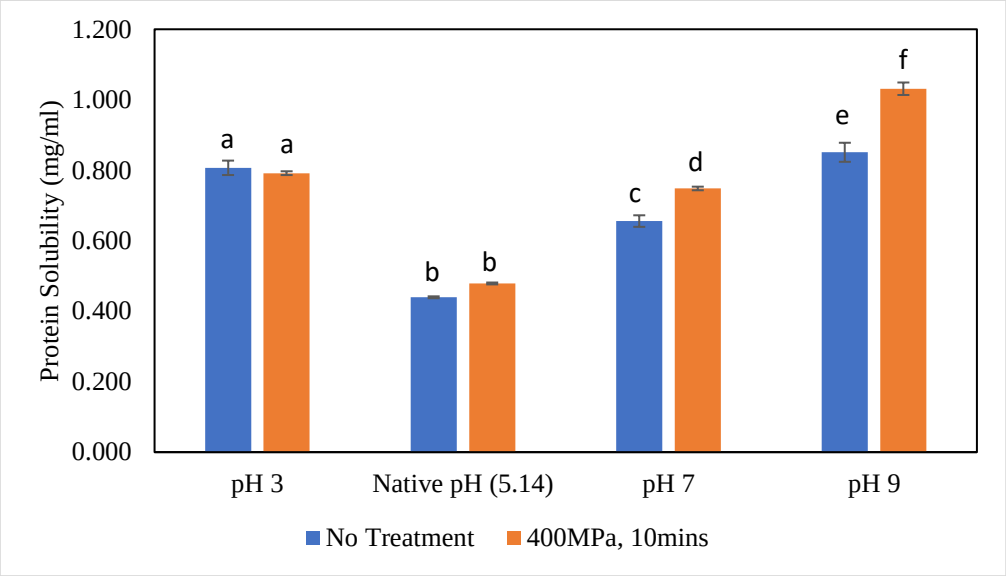


Figure 5: Effect of pressure treatment, 400MPa, 10mins and no treatment on 10% w/w brown rice protein solubility at different pH. Columns with different letters indicate significant differences ($P < 0.05$).

The application of rice protein in the food industry is limited due to its poor solubility (Li et al., 2018). Glutelin, the major fraction of rice protein, are extensively aggregated, disulphide bonded, and glycosylated and therefore it is difficult to solubilize (Hoogenkamp et al., 2017). As seen from Figure 5, the solubility of rice protein is low and the application of pressure treatment only slightly increased the solubility at pH 7 and pH 9.

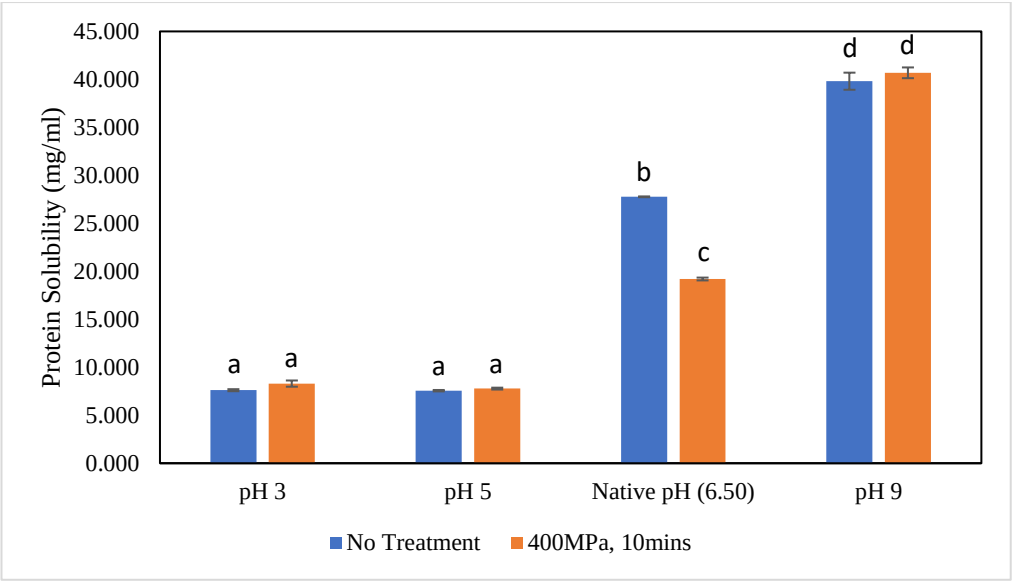


Figure 6: Effect of pressure treatment, 400MPa, 10mins and no treatment on 10% w/w faba bean protein solubility at different pH. Columns with different letters indicate significant differences ($P < 0.05$).

As seen from Figure 6, the application of pressure treatment on faba bean protein significantly decreased the solubility at native pH. The decrease in solubility may be due to the formation of protein aggregates as HPP denatures the protein, exposing its hydrophobic residues and hence promotes protein-protein interactions. Despite a decreased in protein solubility, the result from the phase stability test (Figure 2(b)) showed that HPP treatment had a positive effect on the phase stability of faba bean protein at native pH. The network formation resulted from protein-protein interactions might have helped to entrap water and therefore, a stable suspension was observed.

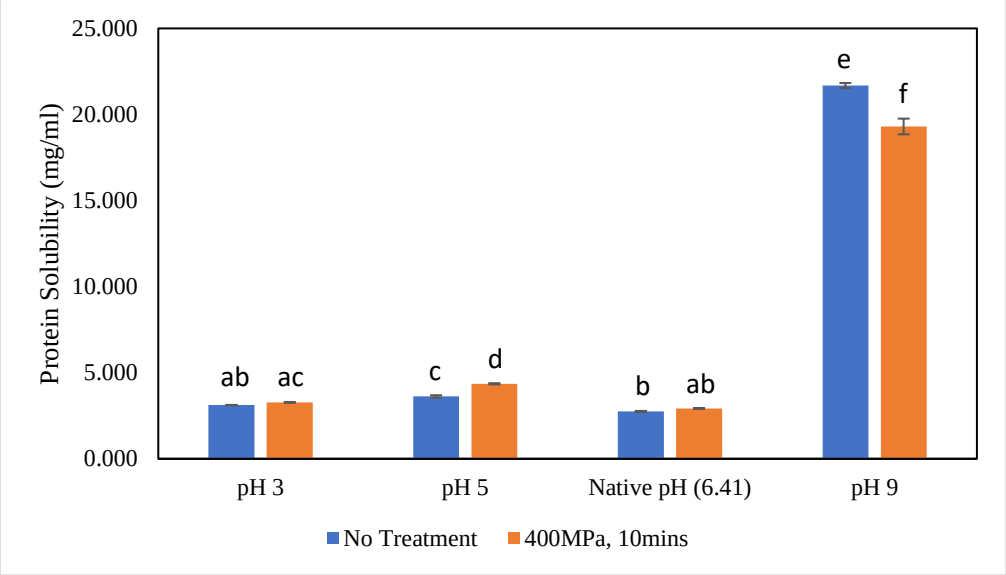


Figure 7: Effect of pressure treatment, 400MPa, 10mins and no treatment on 10% w/w pumpkin protein solubility at different pH. Columns with different letters indicate significant differences ($P < 0.05$).

The solubility of pumpkin protein was generally low at pH 3, 5 and native pH. However, high solubility was observed at pH 9. One study has also reported that solubility of pumpkin seed protein is low in the acidic pH (pH < 5) and increases drastically when pH is above 6 (Rezig et al., 2013).

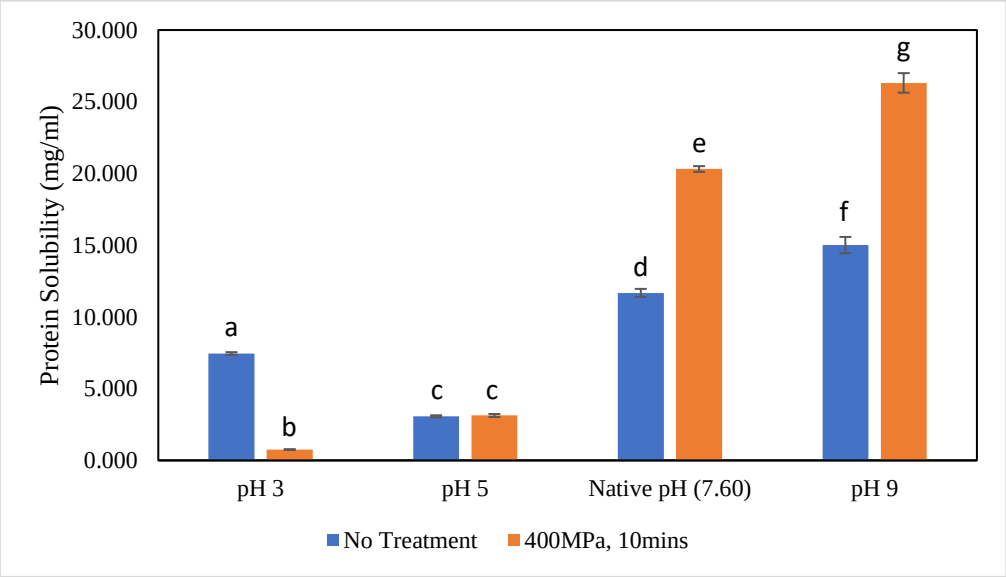


Figure 8: Effect of pressure treatment, 400MPa, 10mins and no treatment on 10% w/w pea protein solubility at different pH. Columns with different letters indicate significant differences ($P < 0.05$).

Pressure treatment on pea protein had an effect on the solubility at native pH (7.60) and pH 9 whereby its solubility increased significantly. This is in contrast with a study conducted by Chao et al. (2018) on isolated yellow field pea protein isolate subjected to pressure treatment of (200, 400, 600MPa for 5 minutes). It was reported that at pH 7-9, pea protein subjected to 400MPa has significantly lower values when compared to the untreated sample.

Based on the solubility results, untreated faba bean protein has the highest solubility at all the pH. The effect of HPP treatment on the individual plant protein was also very different due to their difference in structure and characteristics. Pea protein was shown to have the largest change in solubility after HPP treatment while brown rice protein has the least changes. As pressure treatment modifies the structure of the protein, pea protein is more susceptible to the effect of pressure as evidenced by the large change in functionality (solubility).

3.1.1.3 Zeta Potential

The zeta potential of brown rice, faba bean, pumpkin and pea proteins, with and without pressure treatment were measured to further understand the unfolding and aggregation of the proteins after HPP treatment, the results are presented in Figure 9, 10, 11 and 12 respectively. A decrease in zeta potential after pressure treatment reduces the surface charge and may induce the formation of protein aggregates. Brown rice protein at pH 7, pH 9, faba bean protein at pH 5, pumpkin protein at pH 5, native pH and pea protein at native pH, showed a decrease in zeta potential after pressure treatment. Brown rice protein at native pH, faba bean protein at pH 3, pH 5, pumpkin protein at pH 9 showed an increase in surface charge after pressure treatment. An increase in zeta potential after HPP treatment represents an increase in the surface charge, which could result in higher inter-molecular electrostatic repulsions, inhibit further aggregation and improve the stability of the protein dispersion (He et al., 2016).

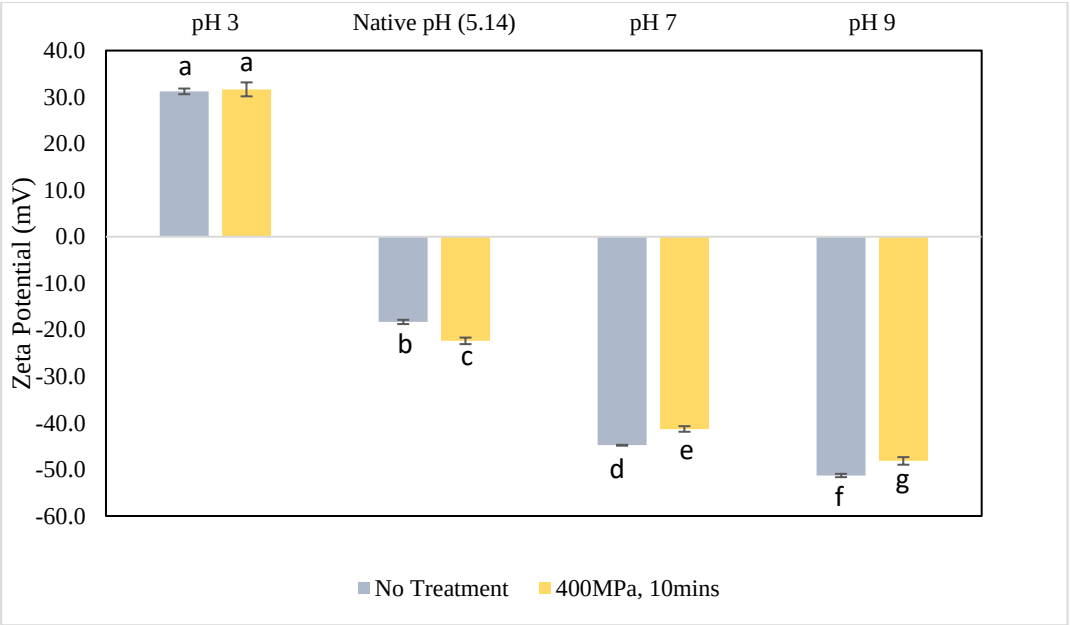


Figure 9: Effect of pressure treatment, 400MPa, 10mins and no treatment on zeta potential of 1% w/w brown rice protein at different pH. Columns with different letters indicate significant differences ($P < 0.05$).

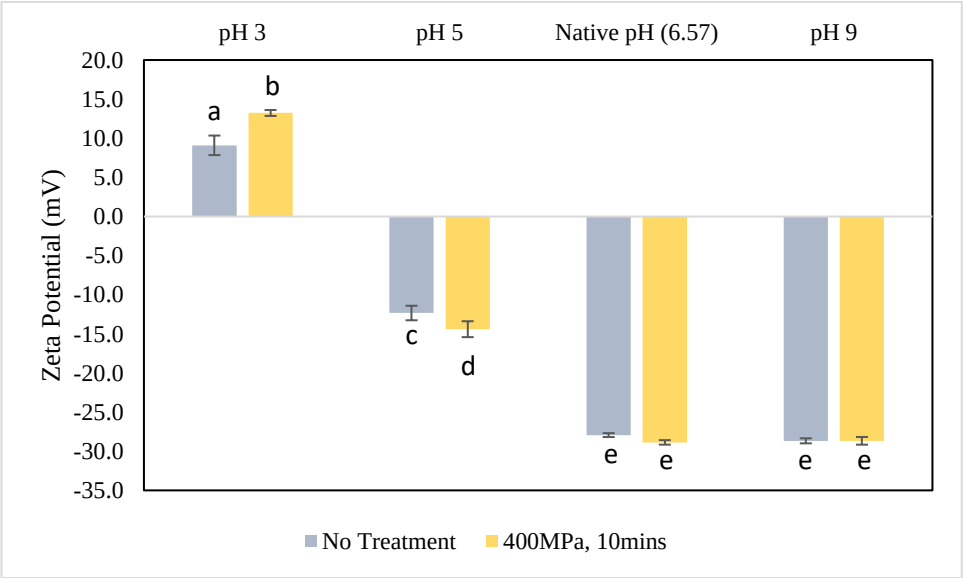


Figure 10: Effect of pressure treatment, 400MPa, 10mins and no treatment on zeta potential of 1% w/w faba bean protein at different pH. Columns with different letters indicate significant differences ($P < 0.05$).

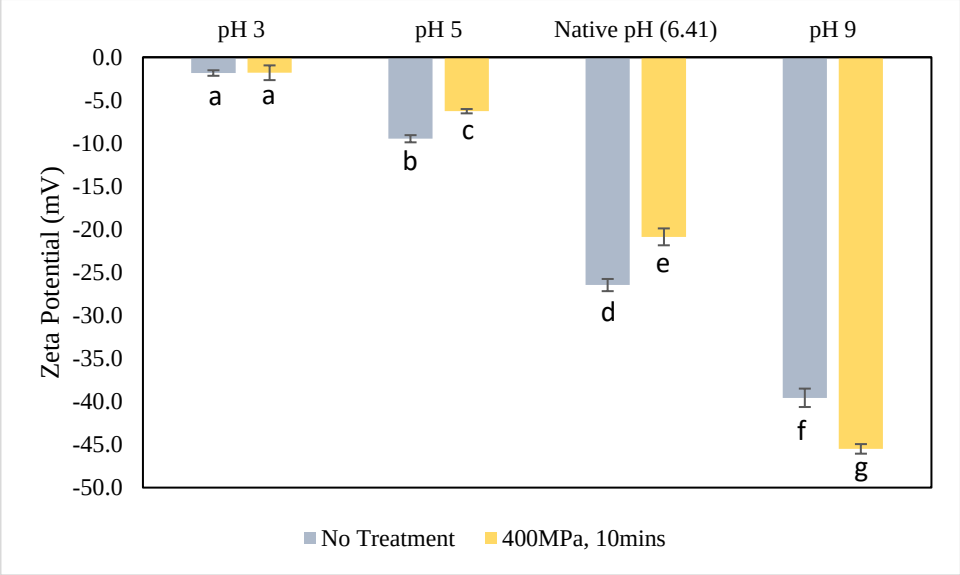


Figure 11: Effect of pressure treatment, 400MPa, 10mins and no treatment on zeta potential of 1% w/w pumpkin protein at different pH. Columns with different letters indicate significant differences ($P < 0.05$).

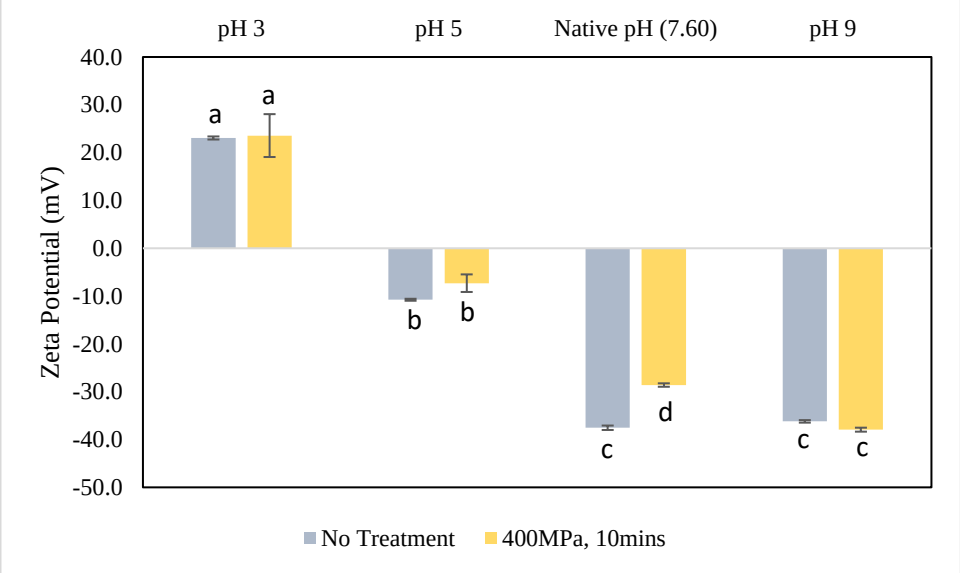


Figure 12: Effect of pressure treatment, 400MPa, 10mins and no treatment on zeta potential of 1% w/w pea protein at different pH. Columns with different letters indicate significant differences ($P < 0.05$).

3.1.2 Effect of Varying Pressure

3.1.2.1 Phase Stability

The effect of different levels of pressure treatment on the phase stability of brown rice, faba bean, pumpkin and pea protein at native pH are presented in Figure 13, 14, 15 and 16 respectively. Only one significant finding can be seen in Figure 14 for faba bean protein at native pH, HPP treatment at 400MPa and 600MPa for 10 minutes as it resulted in a stable suspension.

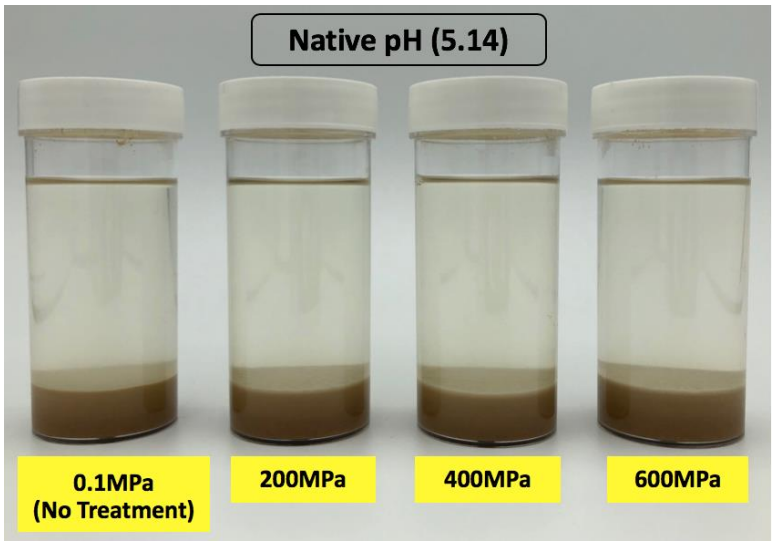


Figure 13: Effect of different levels of HPP on phase stability of 10% w/w brown rice protein at native pH = 5.14.

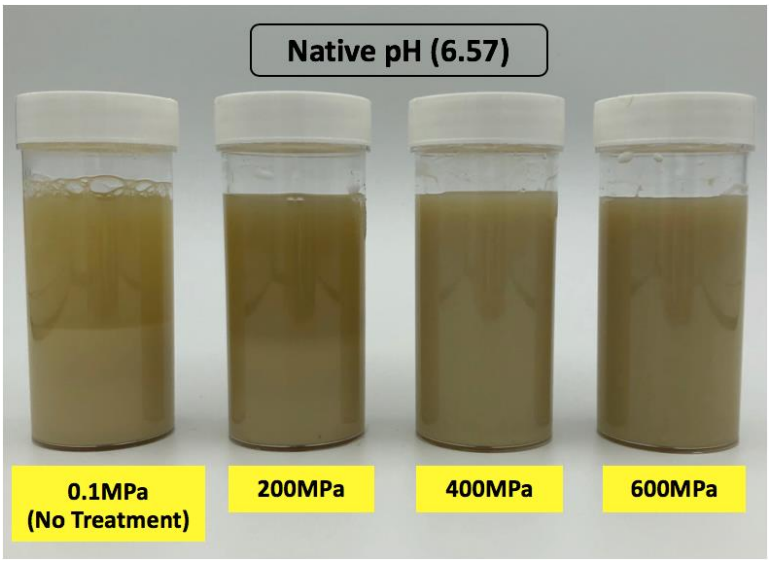


Figure 14: Effect of different levels of HPP on phase stability of 10% w/w faba bean protein at native pH = 6.57.

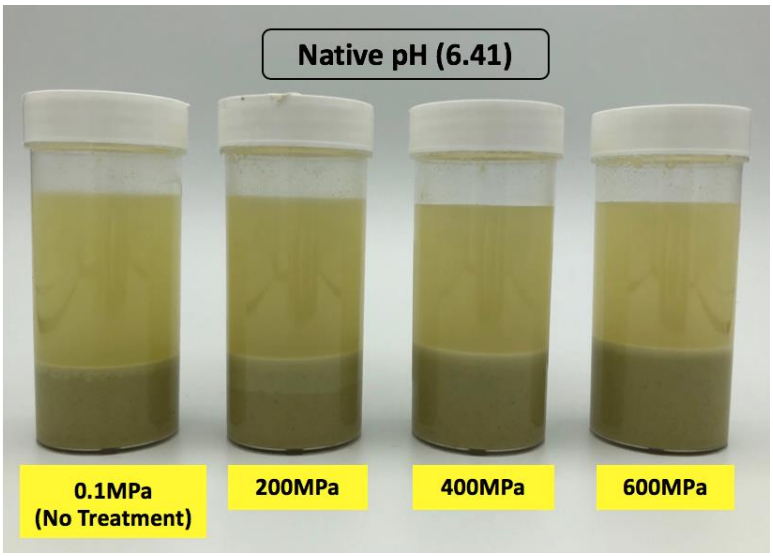


Figure 15: Effect of different levels of HPP on phase stability of 10% w/w pumpkin protein at native pH = 6.41.

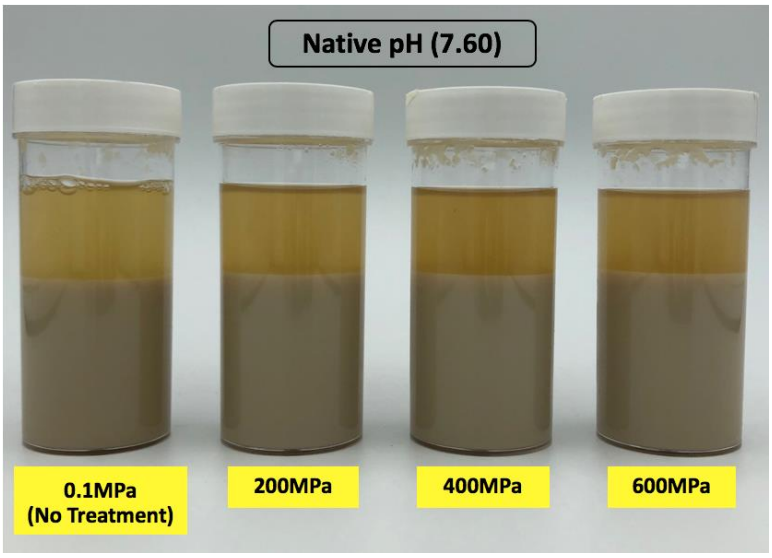


Figure 16: Effect of different levels of HPP on phase stability of 10% w/w pea protein at native pH = 7.60.

3.1.2.2 Solubility

The effect of different levels of pressure treatment on the solubility of brown rice, faba bean, pumpkin and pea protein at native pH are presented in Figure 17, 18, 19 and 20 respectively. All proteins showed a significant increase in solubility at 200MPa. This may be attributed to the partial denaturation of protein molecules, leading to unfolding and increasing the interaction between proteins and water molecules. However, when pressure condition was increased, there was a greater extent of denaturation which exposed the hydrophobic and buried intramolecular mercapto groups of proteins. Through noncovalent interactions, the protein molecules reformed macromolecular aggregates and this resulted in lower solubility (He et al., 2016). This was observed in both faba bean and pumpkin protein. Globulins, mainly legumin (11S) and vicilin (7S) are the major storage proteins in faba bean seeds. Legumin has a closely packed structure due to the hydrophobic interactions of the basic polypeptide, that is located in the interior of the molecule. After HPP treatment, the protein underwent conformational changes that exposed the aromatic amino acid residues and increases the protein surface hydrophobicity (Yang et al., 2018). Therefore, higher pressure treatment (400MPa and 600MPa) caused a greater degree of conformational change and resulted in a decrease in solubility. For samples that did not have a significant difference in solubility between the different level of pressure treatment (200, 400, 600MPa) suggests that similar soluble protein aggregates were formed (Chao et al., 2018).

The results from phase stability do not have a direct correlation with the results from solubility. For example, although faba bean protein had shown to exhibit stable suspension at 400 and 600MPa, the results from solubility showed an opposite trend; the solubility decreased at 400 and 600MPa. Till date, no study has been reported on the effect of HPP on the functionality of faba bean protein. However, it can be deduced that pressurization causes a weak gel to be formed through the crosslinking of protein molecules. The network formation helps to trap water and therefore, no separation was observed in 400 and 600MPa treated samples. Similar to faba bean protein, soy protein isolate also has 7S and 11S protein as its main fractions and is capable to form a gel at 300 MPa and above with sufficiently high concentration (Molina, 2002).

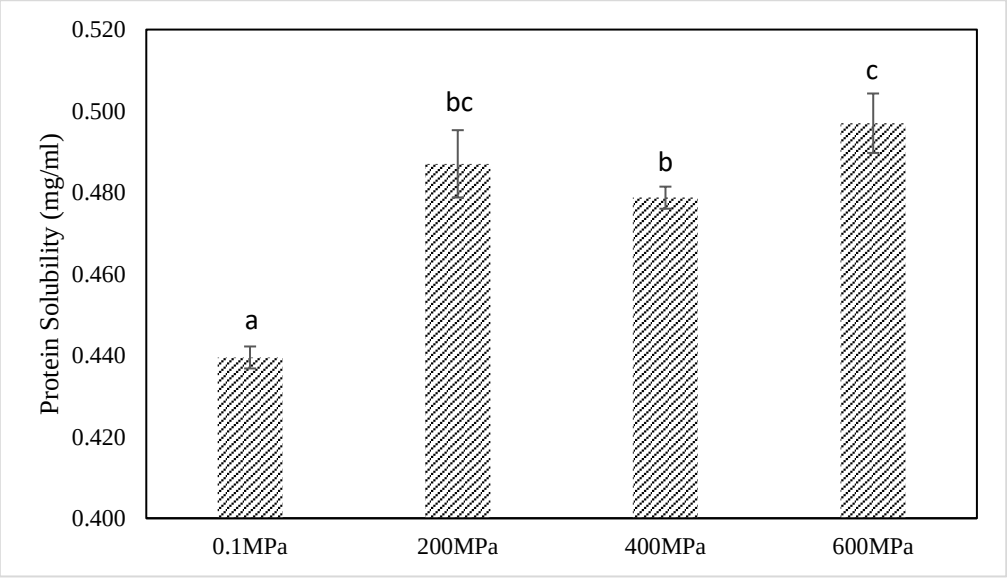


Figure 17: Effect of different levels of HPP on solubility of 10% w/w brown rice protein at native pH = 5.14. Columns with different letters indicate significant differences ($P < 0.05$).

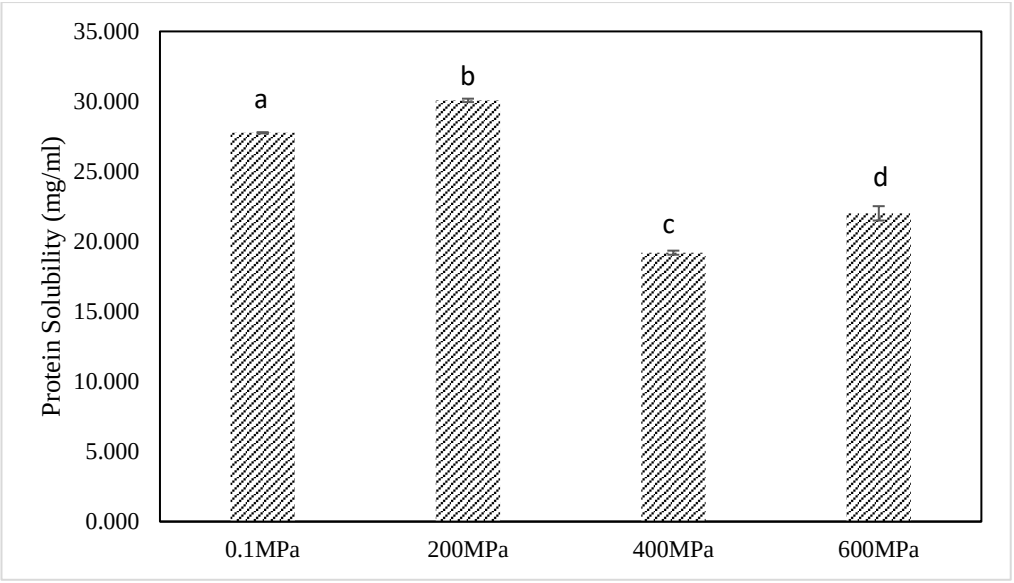


Figure 18: Effect of different levels of HPP on solubility of 10% w/w faba bean protein at native pH = 6.57. Columns with different letters indicate significant differences ($P < 0.05$).

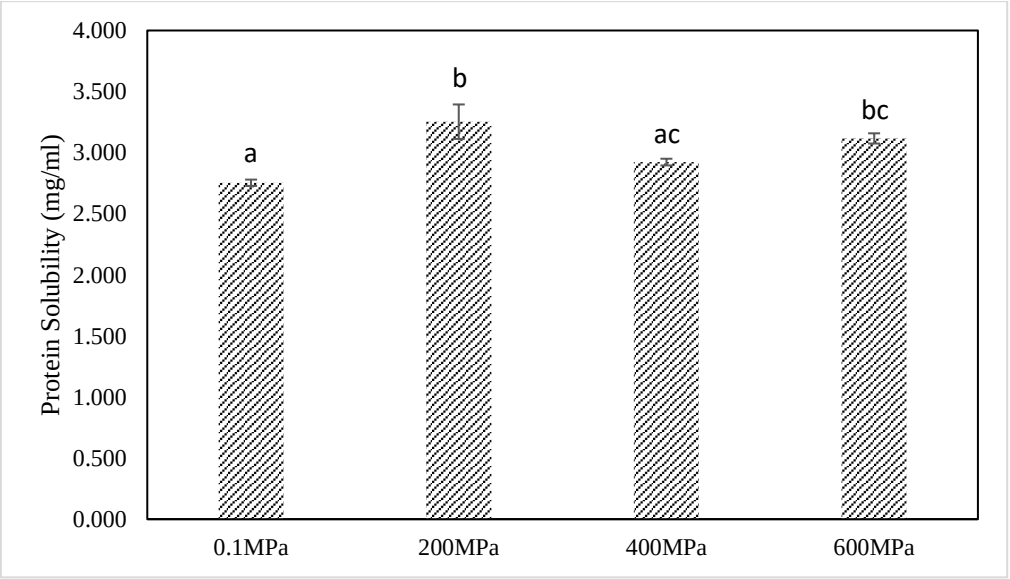


Figure 19: Effect of different levels of HPP on solubility of 10% w/w pumpkin protein at native pH = 6.41. Columns with different letters indicate significant differences ($P < 0.05$).

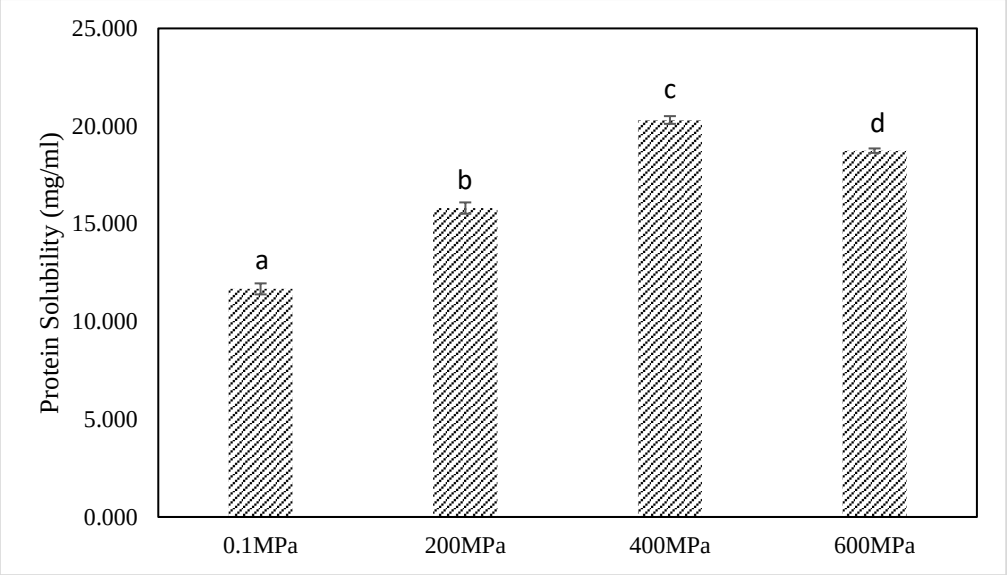


Figure 20: Effect of different levels of HPP on solubility of 10% w/w pea protein at native pH = 7.60. Columns with different letters indicate significant differences ($P < 0.05$).

3.1.2.3 Zeta Potential

The effect of different levels of pressure treatment on zeta potential of brown rice, faba bean, pumpkin and pea protein at native pH are presented in Figure 21, 22, 23 and 24 respectively and different trends were observed in each protein. Increase in HPP conditions resulted in an increase of the surface charge for brown rice protein and only slight differences were seen for faba bean protein. On the other hand, increasing the pressure treatment decreases the surface charge on brown rice protein and for pea protein, its surface charge was reduced at 400MPa.

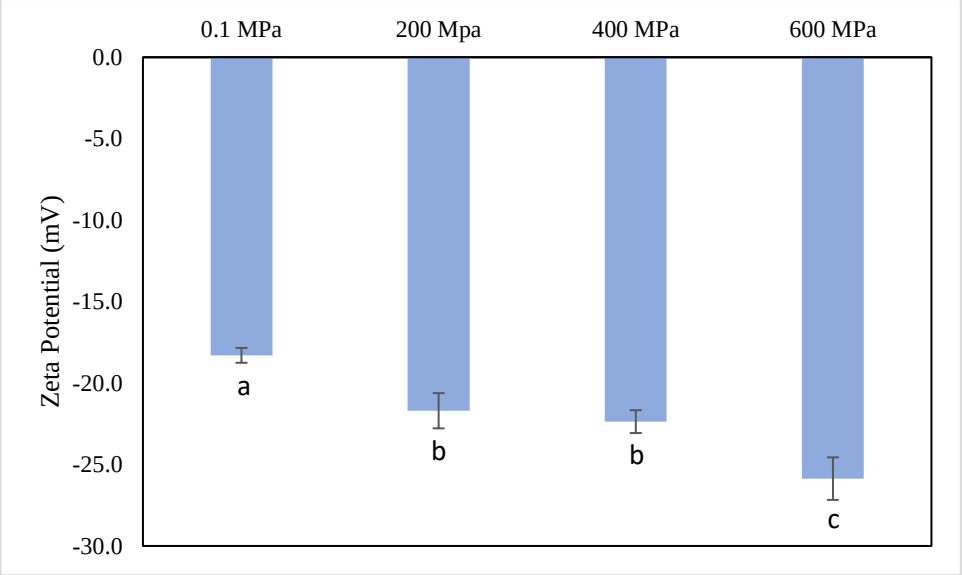


Figure 21: Effect of different levels of HPP on zeta potential of 1% w/w brown rice protein at native pH = 5.14. Columns with different letters indicate significant differences ($P < 0.05$).

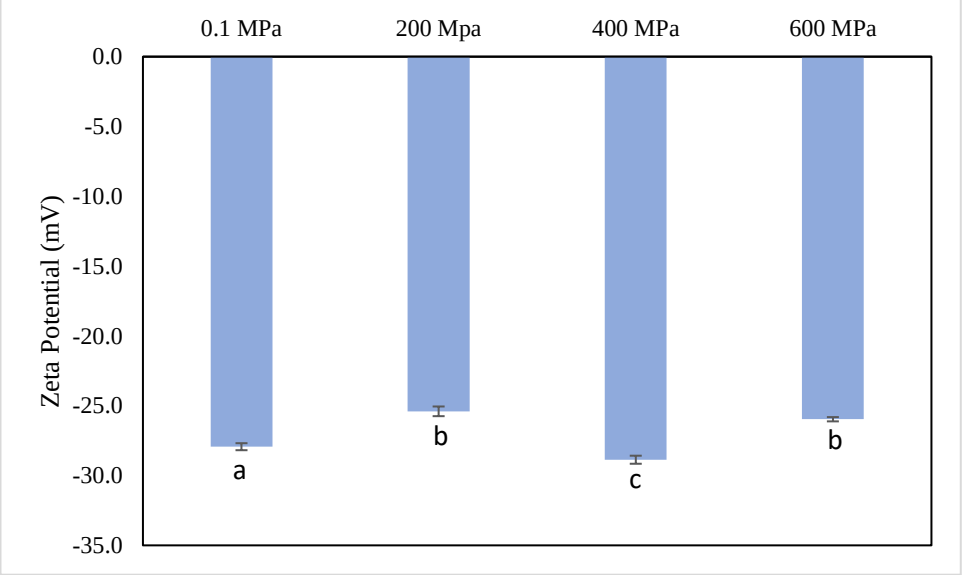


Figure 22: Effect of different levels of HPP on zeta potential of 1% w/w faba bean protein at native pH = 6.57. Columns with different letters indicate significant differences ($P < 0.05$).

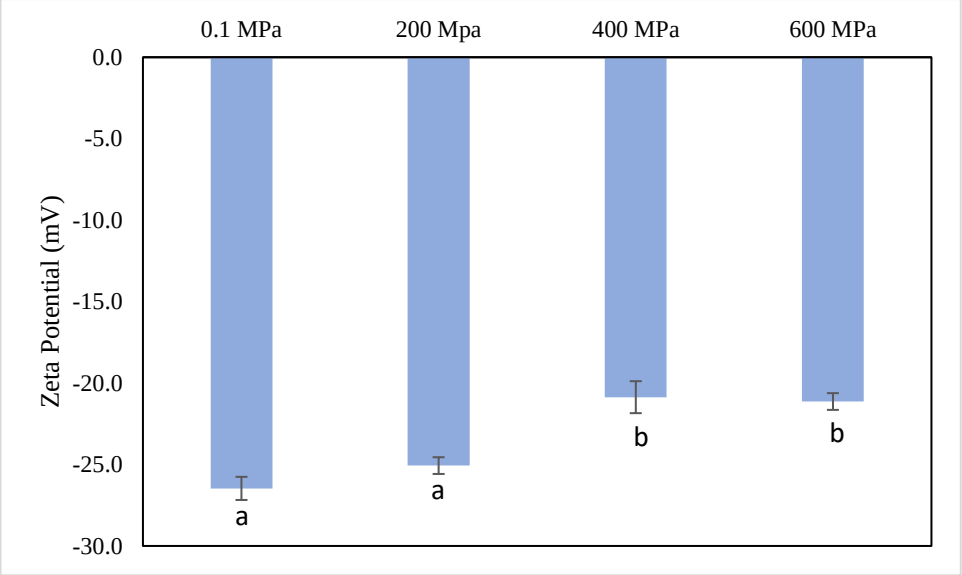


Figure 23: Effect of different levels of HPP on zeta potential of 1% w/w pumpkin protein at native pH = 6.41. Columns with different letters indicate significant differences (P < 0.05).

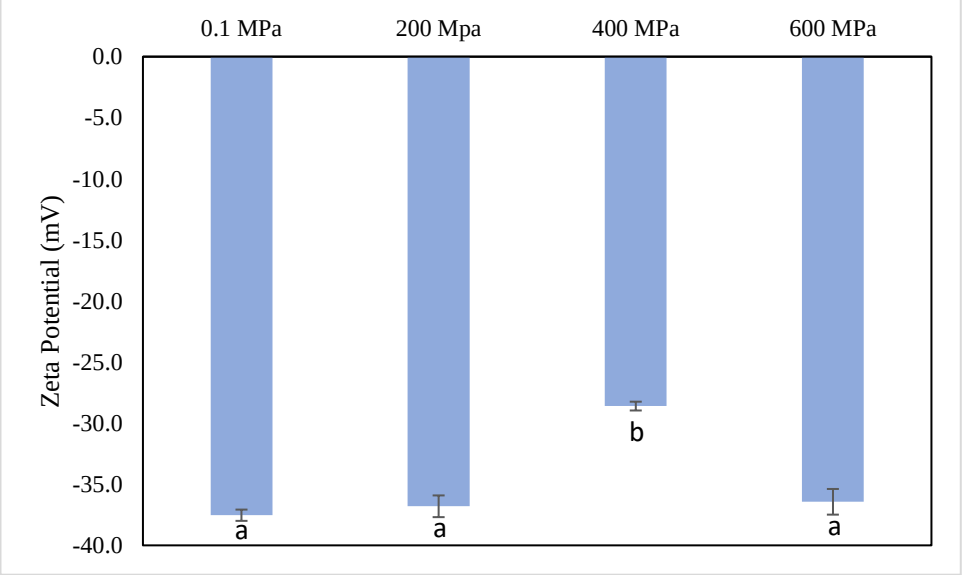


Figure 24: Effect of different levels of HPP on zeta potential of 1% w/w pea protein at native pH = 7.60. Columns with different letters indicate significant differences (P < 0.05).

4 Conclusion

The functional properties of brown rice protein, pea protein, faba bean protein and pumpkin protein were evaluated, where different types of plant proteins behave differently under pH and HPP treatment due to differences in protein conformation and other characteristics. It was found that HPP treatment of faba bean protein at 400MPa and 600MPa resulted in a stable suspension. This signifies that HPP is a potential method to modify the conformation and properties of faba bean protein. Results from solubility also showed that faba bean protein has the highest solubility at all pH compared to the other plant proteins and this may be an indicator of potential functional properties. These findings have provided useful insights to Big Idea Ventures portfolio companies.