

*Pumpkin seed protein is a high-quality plant protein, which has all essential amino acids for the human body and can also supply essential amino acid histidine for children. When it is introduced to food products, it needs to meet some functional properties, such as solubility, emulsifying ability, foaming ability, and so on. Among them, solubility is very important because it has a great influence on other functional properties of protein. In this study, pH-shifting treatment, which is a novel method to modify protein, is applied to improve the solubility of pumpkin seed protein isolate (PSPI). PSPI treated by pH-shifting treatment was investigated at different pH values (pH 2, pH 4, pH 6, pH 8, pH 10, and pH 12), which were labeled as PSPI 2, PSPI 4, PSPI 6, PSPI 8, PSPI 10, and PSPI 12, respectively. Compared to that of control PSPI (45.6 %), only the solubility of PSPI 8 (55.5 %) showed increased ( $p < 0.05$ ) value, while the solubility of PSPI 2 (13.7 %), PSPI 4 (10.8 %), PSPI 10 (41.8 %), and PSPI 12 (13.4 %) showed decreased ( $p < 0.05$ ) value. Then the average particle size, zeta potential of the soluble protein in PSPI were analyzed, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. PSPI 2, PSPI 4, and PSPI 12 showed decreased ( $p < 0.05$ ) average particle size after the pH-shifting process. And PSPI 2, and PSPI 12 showed decreased ( $p < 0.05$ ) zeta potential. While other samples didn't show any significant difference in these two indicators. Besides, the molecular weight of the increased abundance of soluble protein bands was observed at 33 kDa and 25 kDa of PSPI 8. As the solubility of PSPI 8 increased ( $p < 0.05$ ) significantly, it might suggest the PSPI after pH-shifting treatment under pH 8 has more advantages to be used in the food industry*

*Keywords: pumpkin seed protein isolate, solubility, pH-shifting treatment, molecular weight*

# DETERMINING THE EFFECT OF PH-SHIFTING TREATMENT ON THE SOLUBILITY OF PUMPKIN SEED PROTEIN ISOLATE

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## 1. Introduction

In the past, animals were major sources of protein in Europe and the United States, such as poultry, pigs, and cattle. However, more and more people are eating plant proteins as alternatives for meat proteins. In 2016, Business Wire predicted that the vegetarian market would be twice larger than today by 2024, and plant proteins would take up nearly one-third of the entire protein market by 2054 [1]. According to the report of Worldatlas, India has the largest number of vegetarians in the world, accounting for 38 % of the Indian population [2]. The second is Taiwan, vegetarians of which occupied its 12 % population. As for Europe, Britain, Italy, Austria, Germany, Israel, about 10 % of the population are also vegetarians. In South America, 8 % of Brazilians are vegetarians. In North America, about 1.5 % of Americans are vegan, according to Wikipedia. However, Faunalytics.org showed that the US has 2 % vegetarians, and 0.5 % strict vegetarians, which is about 1.6 million people.

Nowadays, because the concept of healthy diet attracts more and more people, the number of vegetarian groups has been greatly improved. The main customers of vegetarian food have gradually developed from religious people to socially responsible people caring for environmental animals. The report showed that animal husbandry produces carbon emissions accounting for 15 % of the total global carbon emissions, more than the total carbon emissions of all global vehicles [3]. Production of carbon emissions per liter of fresh cow milk is nearly seven times that of soybean milk. At the same time, producing milk requires a lot of water resources. Studies showed that 100 liters of water are required for producing 100 ml of milk, while plant milk such as walnut milk requires only 5 liters.

The current demographics may not be accurate enough, but various statistics showed that the vegetarian population is increasing year by year, and more and more people tend to reduce meat intake. Health is a driver of choosing vegetarian milk over dairy, and growth hormone in milk may increase the risk of certain cancer, along with studies showing that

dairy products are associated with hormone-related diseases, such as acne and premature puberty [4].

Plant proteins are alternative proteins of animal proteins. According to the analysis of Technavio [1], the main ingredient for plant protein products is soy, and the soy protein-dominated market will expand to \$58 billion in 2021. However, as the market gradually matures, the second generation and the third generation of plant protein sources also continue to be updated, such as pea, lentils, and other sources of protein powder are also pushed to the market.

There are many plant protein products, such as soy milk, meat analogs. They also have the potential to be added in snacks and baby food. The sensory quality of meat analogs is close to that of meat products, but made of plant proteins. Meat analogs are successful because of their healthy figure (cholesterol-free), meat-like texture, and low cost.

As society develops, people's eating habits are gradually shifting from carbohydrate-based food architecture to a high-protein dietary structure. However, animal food provides protein, but also brings high oil, high cholesterol, and then promotes hypertension, hyperlipidemia, and other rich diseases. Since plant protein products can not only provide protein, but also provide good taste. Therefore, the vegetarian industry and meat substitutes will be a major trend that cannot be ignored in the future.

As one of the plant proteins, pumpkin seed protein is a high-quality protein. It can be extracted from pumpkin seed meal, which is a by-product during the oil production process. Pumpkin seed protein is composed of albumin, globulin, glutenin, and proline [5]. The essential amino acids composition of pumpkin seed protein (except His) can provide the minimum requirement according to FAO/WHO (2007) for adults, which makes it a good candidate for nutrient supplements and functional ingredients in the food industry [6].

However, when pumpkin seed protein is introduced into food products, it needs to meet some requirements of functional properties in food processing, such as solubility, water-absorbing ability, oil-absorbing ability, foaming ability and gelling ability. Among them, solubility is one of the most important functional characteristics, which affects other functional characteristics of protein, such as emulsifying property, foaming property, gel property and so on. Therefore, the relevant task is to improve the solubility of pumpkin seed protein by the method of pH-shifting treatment for its better application in food products.

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## 2. Literature review and problem statement

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The researches on the functional properties of pumpkin seed protein were mainly focused on its isolate or different protein fractions (water-soluble protein, alkali-soluble protein, salt-soluble protein), and its enzymatic hydrolysis products. The previous study showed that pumpkin seed protein had low solubility (<20 %) in acidic pH regions (pH<5), but it increased sharply when the pH was higher than 6, and reached the maximum value (70–80 %) when the pH exceeded 7 [7]. Scientists reported that the globulin in pumpkin seed protein reached the maximum solubility (91 %) when the pH was 7.69 and the NaCl concentration was 3.99 % [8]. Besides, it has been shown that the enzymatic hydrolysis products of pumpkin seed protein isolate (PSPI) were higher than the solubility of PSPI, especially when the pH was close to pI [9].

These reports provide a better understanding of the solubility of pumpkin seed protein or its fractions in different environmental conditions, like pH, ionic strength, and interface pressure. However, there is little information about the improvement of its functional properties by the methods like ultrasonic processing, heat treatment, pH-shifting, and so on. These methods are commonly applied to improve the functional properties of plant proteins. Among them, pH-shifting treatment attracts more and more interest as it is simple and convenient recently. It can unfold protein structure under an extreme acid or alkali condition and then refold in a neutral condition. The paper showed that the solubility of soy protein isolate improved slightly when the pH was altered to 10.0 [10]. It also demonstrated that the solubility of pea protein isolate was increased from 8.17 % to 54.94 % by pH-shifting treatment [11]. As shown in the paper, the solubility of hemp seed protein isolate increased from 20.6 % to 97.5 % after a combined pH 12 shift and heating treatment at 80 °C for 60 min [12]. The study showed that pH-shifting treatment can improve the solubility of peanut protein from 79.39 % to 89.87 % [13]. All these investigations showed that pH-shifting treatment is a good way to improve the solubility of plant proteins.

Since there is little information about the modification of pumpkin seed protein isolate (PSPI), pH-shifting treatment was introduced to improve the solubility and application of PSPI. Experiments on the solubility of PSPI, average particle size, zeta potential, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of soluble protein in PSPI were carried out by pH-shifting treatment at pH 2, pH 4, pH 6, pH 8, pH 10, and pH 12.

In short, as a plant protein, pumpkin seed protein isolate has high nutritive value. pH-shifting is a simple and convenient way to improve the protein solubility. pH-shifting treatment can improve the solubility of pumpkin seed protein isolate. Determining the particle size, zeta potential and abundant change of the soluble protein bands of PSPI can provide a certain theoretical basis for its further application.

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## 3. The aim and objectives of the study

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The aim of the work is to study the effect of pH-shifting treatment on the solubility of pumpkin seed protein isolate (PSPI), which might improve the functional properties of PSPI. This could make it possible for PSPI to be a good candidate for nutrient supplements and functional ingredients in food processing.

Tasks to be solved in the research process:

- to determine the solubility of PSPI;
- to determine the average particle size of soluble protein in PSPI;
- to determine the zeta potential of soluble protein in PSPI;
- to determine the abundant change of the soluble protein bands of PSPI by SDS-PAGE.

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## 4. Materials and methods

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In this study, pumpkin seeds were bought from Alibaba in China. All reagents used in this experiment were of analytical grade. Pumpkin seed protein isolate (PSPI) was obtained from defatted pumpkin seed meal by alkali solution and acid precipitation. Defatted pumpkin seed meal was suspended in deionized water and the pH of the suspension was adjusted to 10.5.

After the extraction process of 1 h, the pumpkin seed meal suspension was centrifuged at  $4,000\times g$  for 15 min. The extracts were acidified to pH 4.5 after filtered. By centrifugation for 20 min at  $4,000\times g$ , the protein precipitates were obtained and then freeze-dried for further pH-shifting treatment.

The pH-shifting treatment of PSPI was carried out as follows. The pH of native PSPI suspension was adjusted to 2, 4, 6, 8, 10, and 12 by 1 mol/L HCl or 1 mol/L NaOH solutions, respectively. All pH-shifting treatments were conducted for 1 h and then adjusted to neutral pH. The mixture was dialyzed and then freeze-dried. In this study, PSPI 2, PSPI 4, PSPI 6, PSPI 8, PSPI 10, and PSPI 12 were used to represent the samples treated by pH-shifting treatment for 1 h under pH 2, pH 4, pH 6, pH 8, pH 10 and pH 12, respectively. Except for pH-shifting treatment, the control PSPI was achieved using identical steps with PSPI 2, PSPI 4, PSPI 6, PSPI 8, PSPI 10, and PSPI 12. All prepared samples were stored at 4 °C for further investigation.

The soluble protein was determined according to the Bradford assay. The PSPI dispersion (1.0 mg/mL, w/v, pH 7.0), which had been stirred for 1 h at room temperature, was prepared and then centrifuged at  $4000\times g$  for 20 min. Protein solubility of PSPI flour was calculated as the proportion of soluble protein content and the total protein content.

The particle size distribution and zeta potential of soluble protein in PSPI were determined by the Zetasizer Nano-ZS90 (Malvern Instrument Ltd., UK) following the previously described method with minor modification [13]. The PSPI (1.0 mg/mL, w/v, pH 7.0) dispersion was stirred for 1 h at 25 °C and then centrifuged ( $10000\times g$ ) for 15 min to get the supernatant. 1 ml of the supernatant was added into the quartz cuvette to determine the zeta potential and particle size distribution, respectively. All measurements were performed three times at room temperature.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of soluble protein in PSPI was performed following the reported method with minor modification [14]. The PSPI dispersion (2 mg/mL) was prepared and then centrifuged to obtain the supernatant. 80  $\mu$ L of supernatant and 20  $\mu$ L of SDS-PAGE sample loading buffer (5 $\times$ ) were mixed and boiled at 100 °C for 5 min, and then cooled to room temperature. Electrophoresis was conducted with 5 % stacking gel and 12 % separating gel at 80 V for 35 min, and then at 120 V for about 40 min. Proteins were observed by Coomassie Brilliant Blue R-250 staining.

All measurements were performed three times. Analysis of variance was adopted to determine the significant difference among independent variables ( $p < 0.05$ ). SPSS software (version 26.0, SPSS Inc., Chicago, IL, USA) was adopted for data analysis.

## 5. Results of studying the effect of pH-shifting treatment on the solubility of pumpkin seed protein isolate

### 5.1. Results of determining the solubility of pumpkin seed protein isolate

Solubility is an important functional property of protein, which has a great impact on the gel-forming ability and emulsifying ability [15]. The results of the solubility of control pumpkin seed protein isolate (PSPI) and pH-shifting treated PSPI samples are shown in Fig. 1. The solubility of control PSPI and

pH-shifting treated samples (PSPI 2, PSPI 4, PSPI 6, PSPI 8, PSPI 10, and PSPI 12) was determined in the present study. The solubility of the control PSPI was 45.6 %. Compared with that of the control, the solubility of PSPI 2, PSPI 4, PSPI 10 and PSPI 12 was significantly declined to 13.7 %, 10.8 %, 41.8 %, 13.4 % ( $p < 0.05$ ), respectively. This might be due to that some polar groups (such as carboxyl and amide groups) were buried after pH-shifting treatment at pH 2, pH 4, pH 10, and pH 12, as a result, the interaction ability with water was decreased [16]. It has been reported that the solubility of peanut protein isolate also showed decreased ( $p < 0.05$ ) value after pH-shifting treatment at pH 2 and pH 12 [13].

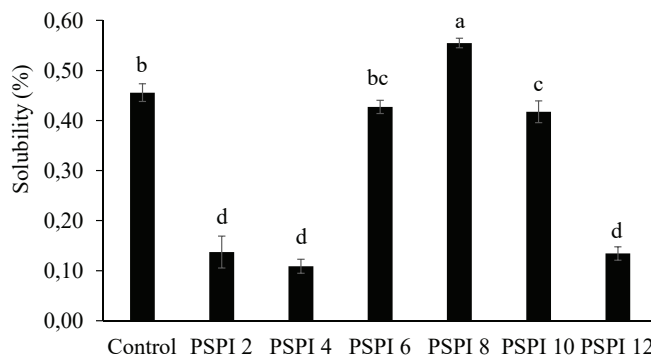


Fig. 1. Solubility of control pumpkin seed protein isolate (control PSPI) and pH-shifting treated pumpkin seed protein isolate samples (PSPI 2, PSPI 4, PSPI 6, PSPI 8, PSPI 10, and PSPI 12)

Besides, in this study, the solubility of PSPI 6 (42.7 %) didn't show any significant ( $p > 0.05$ ) difference, while the solubility of PSPI 8 increased to 55.5 % ( $p < 0.05$ ). This might be because the hydrophobic and van der Waals interactions were disrupted during the unfolding and refolding process of protein molecules [17]. Moreover, proteins might be in an unfolded state after alkaline or acidic treatment, the interactions between side chains became weaker, and conformation structure became flexible, which caused an increase in the protein-water interactions and the solubility of proteins [10].

### 5.2. Results of determining the average particle size of soluble protein in pumpkin seed protein isolate

The average particle size of soluble protein in control PSPI and pH-shifting treated PSPI samples (PSPI 2, PSPI 4, PSPI 6, PSPI 8, PSPI 10, and PSPI 12) is shown in Fig. 2.

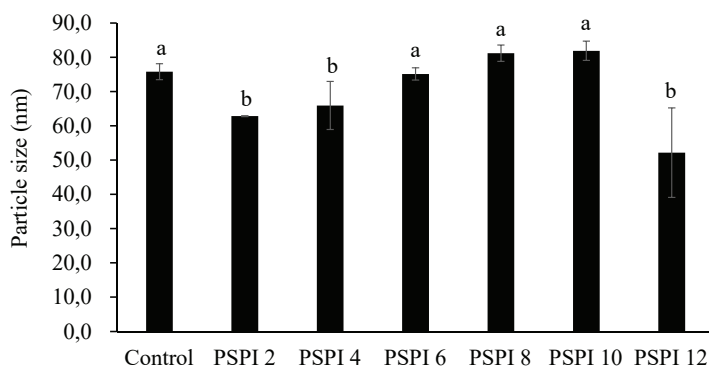


Fig. 2. Average particle size of soluble protein in control pumpkin seed protein isolate (control PSPI) and pH-shifting treated pumpkin seed protein isolate samples (PSPI 2, PSPI 4, PSPI 6, PSPI 8, PSPI 10, and PSPI 12)

Particle size is important for the solubility and emulsifying ability of protein. The average particle size of soluble protein in control PSPI was 75.79 nm. The pH-shifting process significantly reduced the average particle size of soluble protein in PSPI 2 (62.81 nm), PSPI 4 (65.92 nm) and PSPI 12 (62.17 nm), while this parameter of soluble protein in PSPI 6 (75.12 nm), PSPI 8 (81.15 nm), and PSPI 10 (81.88 nm) didn't show any significant ( $p>0.05$ ) difference. It has been suggested that the decrease of protein particle size might facilitate the improvement in emulsifying properties of protein [13]. However, the average particle size of PSPI protein including insoluble protein and soluble protein is still needed for further investigation.

**5. 3. Results of determining the zeta potential of soluble protein in pumpkin seed protein isolate**

The zeta potential of soluble protein in control PSPI and pH-shifting treated PSPI samples (PSPI 2, PSPI 4, PSPI 6, PSPI 8, PSPI 10, and PSPI 12) were is shown in Fig. 3.

Zeta potential is a reflection of the net charge on the protein surface. Large absolute value of zeta potential indicates a strong electrostatic repulsion between protein molecules, which is of benefit to decrease the aggregation between proteins [18]. Compared to control PSPI (-27.10), the zeta potential of soluble protein in PSPI 4 (-24.78), PSPI 6 (-27.35), PSPI 8 (-28.10), and PSPI 10 (-29.37) didn't show any significant ( $p>0.05$ ) change. However, the zeta potential of soluble protein in PSPI 2 (-20.20) and PSPI 12 (-24.25) decreased significantly ( $P<0.05$ ), which might be because the equilibrium between positively or negatively charged groups was influenced during the pH-shifting process [19].

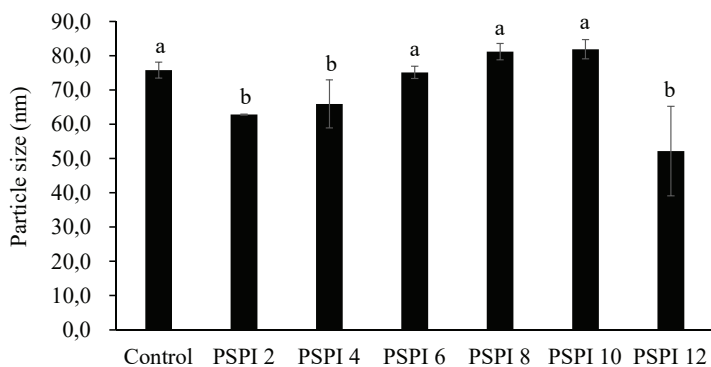


Fig. 3. Zeta potential of soluble protein in control pumpkin seed protein isolate (control PSPI) and pH-shifting treated pumpkin seed protein isolate samples (PSPI 2, PSPI 4, PSPI 6, PSPI 8, PSPI 10, and PSPI 12)

**5. 4. Results of determining the abundant change of the soluble protein bands of pumpkin seed protein isolate**

In this study, the insoluble protein in pumpkin seed protein isolate (PSPI) solution was removed by centrifugation before electrophoretic analysis, the number of protein bands is shown in Fig. 4, which can be considered to the soluble protein in PSPI. The depth of the color indicates the protein content of the bands. The deeper the color, the higher the protein abundance. In SDS-PAGE, the molecular

weight (MW) of the protein bands was determined by the protein markers shown in the first lane in Fig. 4 below.

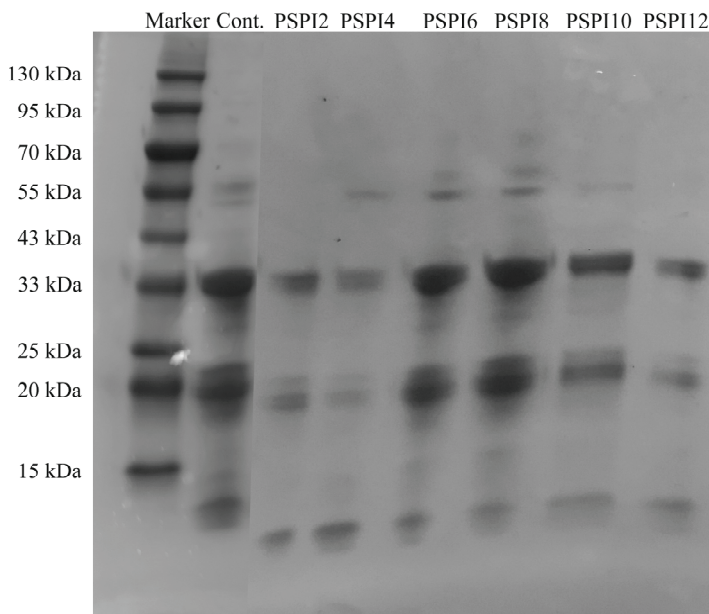


Fig. 4. Electrophoretic analysis of soluble protein in control pumpkin seed protein isolate (control PSPI) and pH-shifting treated pumpkin seed protein isolate samples (PSPI 2, PSPI 4, PSPI 6, PSPI 8, PSPI 10, and PSPI 12)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a common electrophoresis technique using a polyacrylamide gel as a supporting medium to separate proteins and oligonucleotides. The MW of protein bands of control PSPI were mainly distributed in regions around 55 kDa, 33 kDa, 25 kDa, 20 kDa, 15 kDa. Compared to control, the abundance of the protein bands of PSPI 2, PSPI 4, PSPI 10, and PSPI 12 decreased obviously and the abundance of protein bands of PSPI 8 seemed to be increased, which basically matches the solubility analysis in Fig. 1. It can be seen that pH-shifting treatment after pH 8 has an obvious improvement in the solubility of protein bands with molecular weight about 33 kDa and 25 kDa.

**6. Discussion of the results of studying the solubility of pumpkin seed protein isolate**

The study of the solubility of pH-shifting treated pumpkin seed protein isolate (PSPI) samples (PSPI 2, PSPI 4, PSPI 6, PSPI 8, PSPI 10, and PSPI 12) showed that the solubility of PSPI 8 increased by 21.7 % as compared to control PSPI. The improved solubility of PSPI has great significance for its application in food products. Then the change of average particle size, zeta potential, and abundant change of soluble protein bands in PSPI were analyzed. However, according to the results, there was no significant difference found in the average particle size and zeta potential of soluble protein between control PSPI and PSPI 8. The electrophoretic analysis indicated that the molecular weight of increased abundance of PSPI 8 was around 33 kDa and 25 kDa. This range of molecular weight (25–33 kDa) was also the dominant area of protein bands in control PSPI. These results might not indicate the reason why



the solubility of PSPI 8 was changed. In the following work, it might be necessary to determine the change of free sulfhydryl groups, disulfide bonds and surface hydrophobic, because pH-shifting treatment may lead to an alteration of secondary structure or tertiary structure of protein according to the research [20]. During the unfolding process of pH-shifting, the globular protein undergoes a conformational change. Knowing the structural properties of PSPI during pH-shifting treatment might provide a certain theoretical basis for its modification in future investigation.

There are many other methods to modify protein, such as ultrasonic processing, enzymolysis method, ultra-high pressure processing technology, and so on. Different methods have different advantages and disadvantages. Protein hydrolysates are used in various sources. Although it can improve the solubility and digestibility of protein, the obtained peptides might have an unacceptable bitter flavor [21]. Besides, it needs high energy consumption, harmful chemicals, and high labor costs. In recent years, more environmentally friendly methods are provided, such as ultrasound treatment and ultra-high pressure. As a kind of physical modification methods, they have advantages of low processing cost, safety quotient, short action time and less nutritional loss. However, high power and longtime sonication of ultrasound may decrease emulsifying ability and foaming ability [22]. In our study, although pH-shifting treatment needs a lot of alkali solution and acid solution, with certain damage to the environment, it is still a simple and effective way to improve the solubility of PSPI. Our study provides a possibility to improve the solubility of PSPI by pH-shifting treatment. Besides, the multiple functional properties are important to the applied value of the plant protein. This study only improved the solubility of PSPI by pH-shifting treatment, and the effect of emulsifying ability, foaming ability and gel properties remains to be further investigated.

## 7. Conclusions

1. This study showed that the solubility of PSPI was improved after pH-shifting treatment at pH 8. The solubility of control PSPI was 45.6%, and the solubility of PSPI 8 was 55.5%. Thus, the solubility of PSPI increased by 21.7%, which is important for its application in the food industry.

2. According to the results, only the average particle size of PSPI 2 (62.81 nm), PSPI 4 (65.92 nm), PSPI 12 (62.17 nm) showed a significant difference as compared to control (75.79 nm), which showed decreased ( $p < 0.05$ ) values.

3. The results of zeta potential showed that compared to control PSPI (-27.10), only the zeta potential of soluble protein of PSPI 2 (-20.20) and PSPI 12 (-24.25) showed significant difference ( $P < 0.05$ ) and decreased values. These two indicators (particle size and zeta potential) can explain the effect of pH-shifting on the soluble protein of PSPI to some extent. However, the structural properties might also need to be done to illustrate the structural properties during pH-shifting treatment and provide a certain theoretical basis for its modification.

4. Based on the electrophoretic analysis (SDS-PAGE), the abundance of soluble protein bands of PSPI 8 was increased, which is consistent with the solubility analysis. It showed that the molecular weight of the increased abundance of soluble protein bands in PSPI 8 was around 33 kDa and 25 kDa after pH-shifting treatment.

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