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Physiochemical and functional properties of *Moringa oleifera* protein obtained by isoelectric precipitation

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Abstract

The work investigated the effect of extraction pH (10 or 11) and precipitation pH (4 or 4.5) on the physicochemical and functional properties of *Moringa oleifera* protein. The physicochemical characteristics of *Moringa* leaf powder were analyzed using proximate quantification based on AOAC standard methods. The thermal, spectral, x-ray diffraction data were collected using the differential scanning calorimetric, Fourier transform infrared spectroscopy (FTIR), and wide-angle x-ray diffraction. The extraction at pH 10 and the precipitation process at pH 4.5 generated the highest protein content of 58.88%. Some of the functional properties of this pH combination were emulsion activity at 29.67%, emulsion stability at 50.45%, protein solubility at 32.36%, and water absorption capacity of 4.78 mL water/g. The thermal properties suggested the denaturation temperature of *Moringa* protein powder at 75°C, which was comparable to that previously reported plant-based protein isolate. FTIR and x-ray spectra argued the domination of amorphous protein fraction with a trace of lipid and carbohydrate. With these characteristics, protein has great potential to be used for food and drug applications.

1. Introduction

Indonesia is home to numerous medicinal plants such as ginger, turmeric, *Moringa*, lemongrass, and many other herbal plants. *Moringa oleifera*, in particular, has a high amount of protein, carbohydrates, fibres, and minerals (Mg, Ca, Na, Fe, K, P, Cu) as well as vitamins A, B, C, D and E (Bhuvaneswari *et al.*, 2014). The leaves are primarily consumed in salads or soups and are hardly processed into other food products. Having a protein content of 22-28% (w/w) of dried matter (Melo *et al.*, 2013), *Moringa* leaves can be used as a potential biomaterial for food and drug applications. Previous research has indicated the use of plant-based materials, for example, soy protein, lentil, and rapeseed protein, as microcapsules for controlled delivery of bioactive materials (Nesterenko *et al.*, 2014; Wang *et al.*, 2015; Avramenko *et al.*, 2016; Tansaz *et al.*, 2017). Therefore, it is necessary to understand the physicochemical properties of the protein fraction obtained from *M. oleifera* as a new source of biopolymer material for the controlled delivery of food nutrition.

To efficiently extract protein from plants, one needs to break down the tissues or cells of plants. The process can be achieved by either sonication or homogenization of plant solution at high frequency or pulsed followed by ionic extraction and precipitation. In this extraction process, the pH of the suspension is adjusted between 10.5 to 12 (Moayedi *et al.*, 2010). The protein solution was subjected to its isoelectric point to allow precipitation of protein fraction. A protein at its isoelectric point has a net charge of zero. Under these conditions, the electrostatic repulsion between protein molecules is reduced, and thus protein was precipitated. Most globular proteins have minimum solubility at or near their isoelectric point. Protein deposition can be induced by lowering the pH between 4 and 6 (Doran, 2013). Isoelectric precipitation can be used with other precipitation techniques using salts, organic solvents, or polymers to achieve greater yields (Novák and Havlíček, 2016). Thus far, studies on the application of isoelectric precipitation techniques for protein extraction have been conducted on soy, pea, chickpea, lentil and many other plant protein sources (Chove *et al.*, 2001; Boye *et al.*, 2010;). However, to our knowledge, none has been done for *M. oleifera* plant.

This research aimed to determine the best extraction and precipitation pH for *Moringa* protein isolation. The *Moringa* leaves were examined for their physicochemical characteristics namely moisture, ash, protein, carbohydrate, and fat content. The thermal and

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some of the functional properties of extracted protein were also analyzed. The pH variations for the extraction processes were 10 and 11, and the precipitations were pH 4 and 4.5. The treatment with the highest protein content was considered to be the best extraction method.

2. Materials and methods

2.1 Materials

The concentrate *M. oleifera* protein was prepared using hand-picked leaves collected from the Makassar region, South Sulawesi (Indonesia). BSA (12657, Lot 3245660) for protein analysis was purchased from Merck, USA. The NaOH, HCl, and the other chemicals and reagents were purchased from Sigma-Aldrich and analytical grade.

2.2 Preparation of Moringa powder

The fresh *M. oleifera* leaves were collected from Makassar region (Indonesia). The leaves were hand-picked and then sun-dried for about two days. The samples were ground and passed through a 200-mesh sieve to get an extra fine powder. The powder was treated with *n*-hexane at a 1:3 ratio (w/v) to remove the oil. The samples were air-dried and stored in a freezer at -20°C before further use. The preparation stage was following Embaby *et al.* (2018) method with modifications.

2.3 Preparation of moringa protein powder

The defatted *Moringa* protein powder was dispersed in distilled water (1:20) and stirred for 2 hrs at room temperature. Samples were adjusted to pH 10 or 11 using 1 N NaOH to allow complete solubility protein fraction and then centrifuged at 3000 rpm for 15 mins. The supernatant was separated for further isoelectric precipitation treatment. The isolation of protein was conducted using 1 N HCl at pH 4 or 4.5. The protein precipitate was separated from the solution by centrifugation at 3000 rpm for 15 mins. Samples were then neutralized to pH 7. The protein solution was dried in a freeze dryer. The preparation stage is conducted according to Tatar *et al.* (2015) method with modifications.

2.4 Proximate analysis

Proximate analysis was carried out to determine the *Moringa* powder's water, fat, and ash content following the AOAC standard method (2005). The total protein of samples was analyzed using the Lowry method (Huang *et al.*, 1976). Carbohydrate was calculated by difference. The experiments were triplicate.

2.5 Protein solubility

Moringa protein solubility was determined according to the method by Mao and Hua (2012) with modifications. An amount of 200 mg of sample was dispersed in 20 mL of deionized water. The pH of the mixture was adjusted to 7. The mixture was stirred at room temperature for 30 mins and centrifuged at 3000 rpm for 15 mins. The supernatant was separated and analyzed for its protein content using the Lowry method (Huang, Marshall, Anderson, and Charoen, 1976). The experiment was repeated three times for each preparation. The solubility of protein was calculated as follows:

$$\text{solubility (\%)} = \frac{\text{protein content in supernatant}}{\text{total protein in sample}} \times 100$$

2.6 Emulsifying properties (activity and stability)

Emulsifying activity (EA) and stability (ES) were also measured at pH 7 according to the method of Dong *et al.* (2011) and Mao and Hua (2012) with some modifications. The 500 mg of sample was added with 25 mL of de-ionised water. The pH of the mixture was set to 7. The solution was mixed with 25 mL corn oil at 15,000 rpm for 1 min and centrifuged at 3000 rpm for 5 mins. The height of the emulsion layer (H1) was recorded and compared to the height of the tube content (H2). The EA was calculated as follows:

$$\text{EA (\%)} = \frac{H1}{H2} \times 100$$

The samples were re-heated at 80˚C for 30 mins and re-centrifuged before emulsion stability (ES) analysis. The height of the remaining emulsion was measured and noted as H3. The height of the solution was recorded. All experiments were repeated three times.

$$\text{ES(\%)} = \frac{H3}{H1} \times 100$$

2.7 Water absorption capacity

Water absorption capacity was determined using the method described by Lawal and Adebowale (2004) with modifications. One g of sample (W0) was weighed into 10 mL of distilled water in the centrifuge tube. The weight of the tube plus the dry samples was recorded as W1. The solution was mixed using the vortex for 2 mins and left at room temperature (25°C for 30 mins). The samples were centrifuged at 3000 rpm for 15 mins. The water was removed and the weight of the tube plus the remaining water was recorded as W2. The water absorption capacity was determined as grams of water per grams of samples as follows:

$$WSC = \frac{W2 - W1}{W0}$$

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2.8 Fourier-transform infrared spectroscopy analysis

The FTIR spectra analysis was carried out using a Bruker Alpha FTIR spectrophotometer (Bruker Corporation, Germany). The samples were scanned within the range of 3600-600 cm^{-1} with a resolution of 4 cm^{-1}. Each sample was scanned 24 times.

2.9 Differential thermal scanning analysis

The Differential Thermal Scanning (DSC) (DSC-60 Plus, Shimadzu Corporation, Japan) was used to determine the thermal properties of *Moringa* samples. Approximately 4 mg of samples were weighed in an aluminium pan (201-52943 Al-Pan Crimping). The temperature samples were equilibrated at 25°C for 20 mins before heating up from 25 to 150°C, temperature ramp rate of 10°C/min.

2.10 Wide angle X-Ray diffraction

The diffractogram data of *Moringa* leaf and protein powder was collected using the XRD-7000 L Diffractometer (Shimadzu Co., Japan). The samples were loaded onto the X-ray measuring compartments, scanned continuously from 8˚ to 50˚ at the accelerating voltage of 40 kV and current of 30 mA. The scan speed was set at 2˚/min, and the data points were taken every 0.02˚.

2.11 Statistical analysis

The experiments were repeated in triplicate. The data were expressed as mean ± standard deviation through one-way analysis of variance (ANOVA) followed by Tukey Post Hoc test at significant differences ($p < 0.05$). The results were analyzed by IBM SPSS Statistics 26 software (IBM Australia Limited, NSW).

3. Results and discussion

3.1 Proximate analysis

Proximate analysis is a routine quantitative analysis used to determine the composition of food macromolecules. The procedure was conducted to estimate the chemical/nutritional constituents of *Moringa* leaves from the Makassar region, South Sulawesi, Indonesia. The chemical composition of fresh *Moringa* leaves and dried *Moringa* powder was analysed for their moisture content, protein, fat, carbohydrate, and mineral (Table 1). Overall, the fresh leaves contain less protein, fat, carbohydrate, and mineral than the dried powder due to the high moisture content. Moisture in fresh *Moringa* leaves was calculated at about 75% (Table 1). The sun-drying procedure allowed direct water removal from *Moringa* leaves resulting in a significant reduction of the water content of about 7.51%. The value is comparable to those findings by Sultana (2020), of about 7-8% but slightly higher than the water content reported by Melo *et al.* (2013) of about 4.09%.

The protein content of fresh *Moringa* leaves was calculated at about 6.49%. The result was almost five times lower than protein in the dried powder (28.62%). Dried *Moringa* powder has a significant amount of protein, similar to the amount of protein found in rapeseed, soybean, and microalgae estimated at 26.3, 36.4, and 37.3%, respectively (Sari *et al.*, 2013).

Table 1. Proximate analysis of fresh and dried *Moringa* leaf powder.

Parameter	Fresh *Moringa* leaf	Dried *Moringa* leaf powder
Moisture (%)	75.66±0.02	7.51±0.25
Protein (%)	6.49±0.39	28.62±0.90
Ash (%)	4.58±0.43	11.02±0.87
Fat (%)	5.06±0.08	15.24±0.39
Carbohydrate (%)	8.21±0.71	37.60±0.92

The ash content of fresh *Moringa* leaves and *Moringa* leaf powder were about 6.49 and 11.02%, respectively (Table 1). Rajput *et al.* (2017) found that ash was 4.59 and 9.53% for fresh and dried *Moringa*, respectively. Sultana (2020) reported a slightly lower amount of ash in dried *Moringa* of about 8-10%. This high ash content indicates inorganic residues in the materials, usually an indicator of the amount of minerals in food.

Dried *Moringa* leaves are estimated to have 15.24% of fat and 37.60% carbohydrate. The values were almost three times higher compared to the fresh leaves but comparable to those results summarized in Gopalakrishnan *et al.* (2016). The chemical properties of *Moringa* leave from Makassar (Indonesia) region are almost similar to the proportion of nutritional constituents reported from India and Bangladesh (Rajput *et al.*, 2017; Sultana 2020).

3.2 Properties of protein extract

3.2.1 Protein content

Previously, the proximate analysis concluded that the amount of protein found in *Moringa* leaves of 28.62% (Table 1). Next, the work utilized the isoelectric precipitation method to extract protein from dried samples. Removal of fat from the dried sample was conducted prior to the extraction process. Despite other precipitation procedures, i.e., salting-out, organic solvents, and polymer precipitation methods, the isoelectric procedure was selected due to its simplicity and routine application (Kumar *et al.*, 2003). The isoelectric precipitation utilizes the attraction between negative and positive charges of protein fraction to

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promote aggregation protein at pI of the protein. The pI for most proteins is between 4 to 7 (Novák and Havlíček, 2016). Previous studies have indicated the application of the method to extract protein from soy powder at pH 9 for extraction and precipitation at either pH 5.6 or 5.1 (Chove *et al.*, 2001). None of the studies has specifically indicated the best pH for extraction and precipitation of *Moringa* protein. Therefore, in this study, protein isoelectric separation procedures at the basic environment (pH 10 and 11) for extraction and acidic condition (pH 4 and 4.5) were utilized to select the best pH combination for protein isolation.

Table 2 shows residues of protein content at various pH combinations. The amount of protein varied from the lowest of 47.37% for the combination of pH 10 and 4 and the highest of 58.88% for pH 10 and 4.5. There was no significant difference in protein content between pH 11 and pH 10 precipitated at pH 4.5 (p<0.05) but the opposite was true for those precipitated at pH 4. In addition, The results are almost double the protein content of the *Moringa* leaf powder (28.62%). Isoelectric extraction of the yellow pea, green lentil, red lentil, desi and Kabuli chickpea resulted in 55.0, 50.3, 62.8, 53.7, and 69.1% protein, respectively (Boye *et al.*, 2010).

3.2.2 Protein Solubility

Protein solubility is the first functional property that is usually tested in protein development as a new ingredient. The solubility of this protein is related to other functional properties of proteins, especially the properties of foams, gels, and emulsions. In Table 2, protein solubility at pH 7 of samples produced from pH 10 extraction followed by pH 4.5 precipitation was the highest at 32.36%, while samples from pH 11 and 4 were the lowest at about 31.78%. Overall, there was not much difference in solubility for all treatments but for treatment pH 11 and 4 (p<0.05). However, compared to the previous study, solubility for pea, red lentils, and desi chickpea protein at pH 7 ranged from 55 to 70 % (Boye *et al.*, 2010), thus significantly higher than *Moringa* protein.

3.2.3 Emulsifying activity and stability

Protein emulsifying activity is the ability of proteins to form and stabilize emulsions. Proteins have the ability to form an absorbent surface layer that envelops oil droplets to hold oil and form an oil-in-water emulsion (Zayas, 1997). The extraction treatment at pH 10 followed by precipitation at pH 4.5, resulted in the best emulsion activity of 29.67% (Table 2). The results were 3% higher than those samples precipitated at pH 4.0 and were significantly different at p<0.05. The emulsifying activity of mucuna bean protein concentrate was found between 47-52% and improved after acetylation and succinylation of protein to about 60 and 65%, respectively (Lawal and Adebowale, 2004).

Next, heat treatment of protein has greatly affected the emulsion properties of the protein. The emulsion properties of globular proteins can be improved by heating due to the opening of the protein polypeptide chain allowing the exposure of a hydrophobic group of proteins (Zayas, 1997). In this study, the stability of the emulsion was observed after heat treatment (80˚C for 30 mins). In Table 2, the emulsion stability was between 49 to 50.45% in all samples precipitated at pH 4.5, except for a sample extracted at pH 10 and precipitated at pH 4 of 41.5% (significantly different at p<0.05). These results were comparable with the emulsifying stability of walnut protein of about 40 to 53% (Mao and Hua, 2012)

3.2.4 Water absorption analysis

The ability of the protein to absorb water plays a role in the formation of the texture of food products. Water in food plays an important role in determining the texture and mouthfeel of food. The water-binding capacity of protein depends on the composition and conformation between the protein molecules. The interaction between water and the hydrophilic groups of the protein side chains can occur through hydrogen bonds. The amount of water that a protein can hold depends on its amino acid composition, surface hydrophobicity, and processing (Zayas, 1997).

The results showed the highest water absorption capacity of *Moringa* protein was at an extraction pH of

Table 2. Protein content and functional properties of *Moringa* protein powder.

Sample	Protein (%)	Protein Solubility (%)*	Emulsion activity (%)*	Emulsion stability (%)*	Water Absorption Capacity (mL water/g protein)
pH 11 and 4.5	57.56±0.93^{a}	32.21±0.04^{a}	28.00±1.88^{a}	49.00±1.28^{a}	4.74±0.02^{a}
pH 10 and 4.5	58.88±0.93^{a}	32.36±0.02^{a}	29.67±0.94^{a}	50.45±0.64^{a}	4.78±0.06^{a}
pH 11 and 4.0	49.34±0.46^{b}	31.78±0.25^{b}	26.00±0.94^{b}	49.00±1.41^{a}	4.23±0.04^{b}
pH 10 and 4.0	47.37±0.47^{c}	32.27±0.05^{a}	26.13±0.75^{b}	41.50±2.12^{b}	4.29±0.08^{b}

Values are presented as mean±SD of triplicate measurements taken from four samples' variations. Values with different superscripts within the same column are statistically significantly difference (p < 0.05) using Tukey Post Hoc test. *Measured at pH 7.

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10 and precipitation at 4.5. The samples managed to hold 4.78 mL water/g protein. The water absorption value of *Moringa* leaf protein concentrate is above the average water absorption value for cashew protein concentrates and isolates (2.20 mL water/g protein) as reported by Ogunwolu *et al.* (2009). The high water absorption value indicates that the protein is suitable for food application.

3.2.5 Fourier-transform infrared spectroscopy analysis

Figure 1 shows the infrared spectra of *Moringa* leaf and protein powder. Generally, both samples exhibit typical amino acid/protein, carbohydrate and lipid fingerprints. Peaks were detected at 3256, 1700, 1594, 1545, 1400, and 1303 cm^{-1}, corresponding to Amide I, II, III, A and B protein fingerprints. The spectra of protein functional groups are Amide I (1600-1700 cm^{-1}), Amide II (1510-1590 cm^{-1}), Amide III (1200-1400 cm^{-1}), and Amides A and B. set at ~3300 and ~3100 cm^{-1} (Barth, 2007). The Amide I is usually centred at 1650 cm^{-1} and associated with stretching vibrations of C=O and C-N stretching, NH in-plane bend, and C-CN deformations, respectively. Amide II, which is usually centred at 1550 cm^{-1} is associated with N-H bonds, stretching of C-N bonds, C-C, C=O and N-C functional groups (Barth and Zscherp, 2002). Amine III corresponds to NH bending, CH stretching, CO in-plane bending, and CC stretching. The 3256 cm^{-1} shows NH stretching and vibration of Amine A and B corresponding to the β-sheet of the secondary structure of *Moringa* protein. This specific band position overlaps with the 0H stretching of water molecules (Mojet *et al.*, 2010). The -OH and O^- groups of tyrosine amino acid were detected in 1594 cm^{-1} and 1400 cm^{-1} for CN and COOH of proline, glutamic acid, and aspartic acid (Barth and Zscherp, 2002).

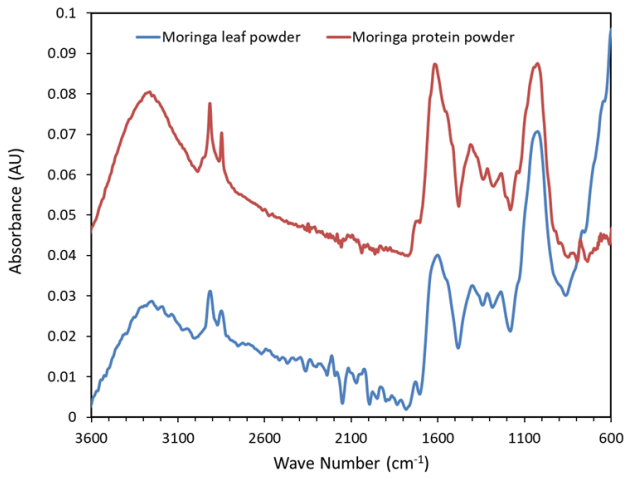

Figure 1. FTIR spectra of *Moringa* leaf and protein powder.

The absorbance at 2913 and 2846 cm^{-1} is related to the typical lipid, protein, and carbohydrate vibration. The presence of alkyl and alkenyl groups of plant cellular components such as lipid, protein, and carbohydrate were observed at 2960–2830 cm^{-1}, corresponding to CH stretch in CH_2 and CH_3. The signal band at ~2925 cm^{-1} and ~2854 cm^{-1} are the symmetric and asymmetric stretching of CH bonds of lipids (Lozano *et al.*, 2017). Carbohydrate fraction (cellulose and amylose) can be associated with signals at 1200-1000 cm^{-1} (Zimmermann *et al.*, 2017).

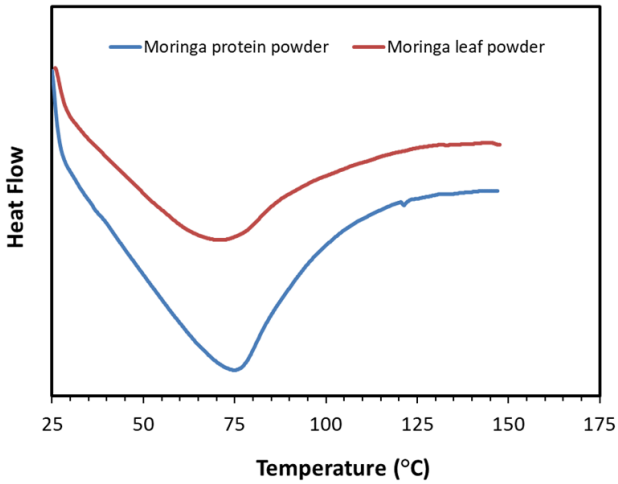

Figure 2. Denaturation temperatures of *Moringa* leaf and protein powder.

3.2.5 Differential thermal scanning analysis

The results of the thermal analysis (Figure 2) of *Moringa* leaf powder obtained mid-point thermal denaturation, which was around 71°C while protein powder was at 74°C. Meanwhile, BSA is around 60-81° C (Matsarskaia *et al.*, 2020) and soy protein is around 77 -90°C (Kitabatake *et al.*, 1990). *Moringa* denaturation temperature (powder and protein) is lower than the thermal denaturation of protein from soybeans.

3.2.6 X-ray diffraction

X-ray diffractograms (Figure 3) show the amorphicity of both *Moringa* leaf and protein powder as suggested by a broad peak at 10˚ - 35˚. The broad peak

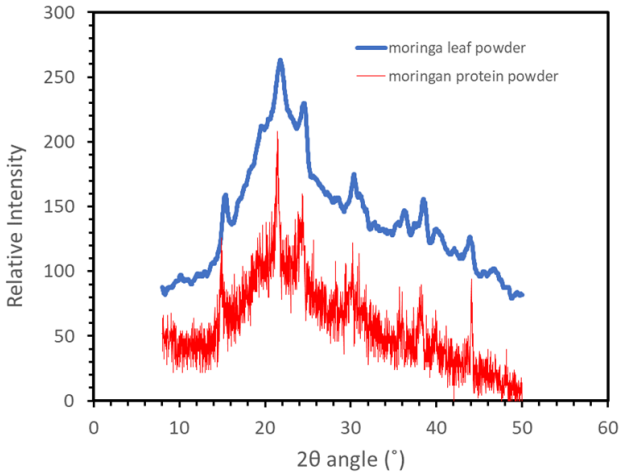

Figure 3. X-ray diffractograms for *Moringa* leaf and protein powder.

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corresponds to a typical protein fraction comparable to a previous study by Paramita and Kasapis (2018). The sharp 2θ peaks indicated the partial crystallinity of the structure at 15˚, 21˚, 25˚, 30˚, 40˚, and 44˚. The peaks at 20-21˚ in both samples were attributed to the β-sheet conformation of the protein (Paramita and Kasapis, 2018). The A-type diffraction pattern of the starch peaks at 15˚ and 25˚ (Yang *et al.*, 2019). Peak areas near ~28˚ and ~44˚ peaks are associated with the cyclic carbon and aliphatic carbon chains, respectively (Paramita *et al.*, 2015).

4. Conclusion

The best pH for protein extraction and precipitation are pH 10 and 4.5, respectively, with a protein content of about 58.88%. The functional properties (protein solubility, emulsion activity, stability, and water absorption) of protein powder from *Moringa* leaf powder at pH 10 and 4.5 are 32.36%, 29.67%, 50.45 %, and 4.78 mL of water/g sample, respectively. Compared to the existing plant-based protein concentrate, improvements in solubility and emulsifying capacity are required to advance its applications in food and pharmaceutical.

Conflict of interest

The authors declare no conflict of interest.

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