American Journal of Biochemistry and Biotechnology

Research Article

Comparative Analysis of Proteins in Two Typical Chinese Thin-Shell Walnuts

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Article history
Received: 11-07-2024
Revised: 06-11-2024
Accepted: 11-12-2024

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Abstract: This study compared functional properties and molecular characteristics of proteins from two thin-shelled walnut (*Juglans regia* L.) cultivars predominantly grown in China: Wen 185 and Lvling. Wen 185 walnut protein demonstrated significantly superior performance compared to Lvling across multiple functional parameters, including solubility, emulsifying capacity, foaming capacity, water absorption, and oil absorption. Molecular weight distribution analysis revealed that both proteins contained polypeptides ranging from 10-130 kDa; however, Wen 185 exhibited higher proportions of lower molecular weight fractions (10, 15, 20, and 48 kDa), while Lvling showed greater abundance of higher molecular weight proteins (120 kDa). The higher proportion of low molecular weight proteins in Wen 185 contributed to its enhanced solubility and surface activity. Proteomic analysis identified six proteins exhibiting significant differential expression between the two cultivars. Compared to Lvling, Wen 185 showed upregulation of three proteins enriched in β-sheet secondary structures and downregulation of three proteins with high α-helix content. Furthermore, the upregulated proteins in Wen 185 displayed higher proportions of surface net charges and solvent-accessible surface residues compared to the downregulated proteins. These structural differences correlated with the observed superior functional properties of Wen 185 protein. This study demonstrates that cultivar-dependent variations in protein molecular weight distribution, secondary structure composition, and surface charge characteristics determine the functional properties of walnut proteins. These findings provide molecular insights for selecting walnut cultivars with optimal protein functionality for food applications.

Keywords: Walnut Protein, *Juglans regia*, Protein Functionality, Molecular Weight Distribution, Secondary Structure, Proteomics, Cultivar Comparison, Emulsifying Properties

Introduction

Walnuts, also known as Juglans regia, are plants that belong to Juglans L. in the Juglandaceae family. They are tree nuts presenting a high nutritional value (Bekiroglu et al., 2022; Jahanban-Esfahlan and Amarowicz, 2018; Xu et al., 2022), being cultivated in China for thousands of years, and belonging to traditional cash crops (Elizur et al., 2020). In 2019, China presented the highest walnut harvest area (631,300 hectares) and walnut yield (252,100 tons) (the Food and Agriculture Organization (FAO) of the United Nations) (Lei et al., 2021). Worldwide, walnuts are considered one of the four major nut types (Wei et al., 2020) and are mainly composed of fat and protein. In China, these walnuts are directly consumed, and they are also used for the manufacturing of several products, including walnut oil, walnut milk, and walnut protein powder (Cindrić et al., 2018; Zhu et al., 2010). Furthermore, the fatty acids present in walnuts mainly include unsaturated fatty acids (linoleic acid) (Hayes et al., 2016), which are capable of preventing cardiovascular diseases (Wang et al., 2022; Liu et al., 2022; Bitok et al., 2017). Moreover, the yield of walnut oil increases every year (Tang et al., 2022), triggering wide research into the walnut protein. According to traditional Chinese medicine, walnut protein also has other biological functions, including invigorating the stomach, moistening the lungs, and replenishing blood. Furthermore, recent

studies demonstrated that walnut peptide exhibit several physiological functions, such as anti-aging, anti-oxidation (Ruiz-Caro et al., 2022), and blood sugar regulation (Zeng et al., 2022; Gao et al., 2020). Walnut protein contains 18 amino acids, which include 8 essential amino acids for the human body (Mao et al., 2014; Zhi et al., 2022). A previous study described walnut protein as a high-quality vegetable protein in which the essential amino acid content reaches the recommended value for adults compared to the standard FAO/ World Health Organization recommended (Lei et al., 2021). Moreover, vegetable proteins are widely accepted by consumers due to their greenness, low cost, environmental protection, renewability, and sustainability. Therefore, physical and chemical modifications of this component have attracted much attention in the research field (Wen et al., 2019). Walnut is a high-quality vegetable protein (Mao et al., 2014), whose solubility and emulsibility can be improved by ultrasonic treat and phosphorylation treat (Zhu et al., 2018; Yan and Zhou, 2021). Some studies compared walnut proteins prepared with different methods in terms of their solubility and rheological properties (Mao and Hua, 2014; Mao and Hua, 2012), and the properties were improved by adding other proteins (Chen et al., 2014), facilitating the development of vegetable protein products. However, few reports have been developed regarding different walnut proteins and their processing-related properties. The present study pays attention to elucidating the structural and functional properties of proteins in two thin-shell walnuts, Lvling (protandrous, i.e., male flowers bloom first) and Wen 185 (protogynous, i.e., female flowers bloom first) (Bernard et al., 2018). Nowadays, information technology has been applied in various fields of food systems. It is interesting that omics techniques well serve for deeply studying a component of food. Omics is a comprehensive molecular biology analysis technique. The "omics" technology, namely proteomics, genomics, transcriptomics, and metabolomics, already has a vast database of gene to protein sequences and expressions, and metabolite patterns. This study applied proteomics techniques to conduct in-depth research on walnut protein (Suwarno et al., 2023). Additionally, the link between structures and properties was explored, theoretically assisting in further studies.

Materials and Methods

Materials and Chemicals

Lvling walnut, Wen 185 walnut, and soybean oil came from a market in Baoding of Hebei Province, China; Sodium hydroxide, hydrochloric acid, PBS buffer solution, ANS solution, 1% PVPP, BPP solution, tris-saturated phenol, acetone, 8M urea +1%SDS, and protease inhibitor cocktail. All analytical reagents were provided by Beijing Solarbio Science & Technology (Beijing, China).

Sample Preparation

The walnut kernel was degreased (Kong et al., 2019) to make skim walnut powder, and alkali extraction and acid precipitation were performed (Mao and Hua, 2012). Then, the skim walnut powder was mixed with water at 1:10 (m/g:V/mL), and the pH value was adjusted to 10 using sodium hydroxide solution (1.0 mol/L), followed by 1.5 h of stirring at 55°C and 15 min of centrifugation at 4,000 r/min. After the collection of the supernatant, the pH was adjusted to 3.5 using hydrochloric acid (1.0 mol/L). The supernatant was removed after 1.5 h of stirring and 15 min of centrifugation at 4,000 r/min. Finally, the precipitates were rinsed with distilled water to become neutral, and the walnut protein powder was obtained after 24 h of vacuum freeze-drying.

Evaluation of the Properties of Walnut Protein

Solubility of Walnut Protein

For determining the solubility, 0.01g skim walnut protein powder was weighed and dispersed using 10 mL of deionized water. With the pH being adjusted to 2, 4, 6, 8, 10, and 12, respectively, the solution received 0.5 h of stirring at room temperature, followed by 15 min of centrifugation at 4,500 r/min. Finally, after the collection of the liquid supernatant, the Coomassie bright blue technique was employed to ascertain the protein Concentration (C1). The following formula was adopted for calculating the solubility (Labuckas et al, 2014):

$$S\ (\%) = \frac{(C_1 - C_0) \times V}{m} \times 100\% \quad (1)$$

The Foamability and Foam Stability of Walnut Protein

In this experiment, the samples were dispersed in 50 mL deionized water (1%, w/v) (V0), with the pH being adjusted to 2, 4, 6, 8, 10, and 12, respectively. Then the samples underwent 2 min of homogenization with a high-speed shearing machine at 12,000 rpm, followed by being moved to a 50-mL measuring cylinder to measure the Volume (V1) after 30 s. Foam stability refers to the remaining percentage of foam Volume (V2) after half an hour. Formulas below interpreted the foamability and foam stability (Wani et al., 2015):

$$F\ (\%) = \frac{V_1 - V_0}{V_0} \times 100\% \quad (2)$$

$$FS\ (\%) = \frac{V_1 - V_2}{V_1 - V_0} \times 100\% \quad (3)$$

Determination of Emulsibility and Emulsion Stability

This study also determined the emulsibility and emulsion stability. In brief, 10 mL of 1% protein solution was prepared with pH of 2, 4, 6, 8, 10, and 12. The solution added with 20 mL of soybean oil underwent 2 min of homogenization at 12,000 r/min and 20 min of centrifugation at 3000 r/min. Then, the emulsion layer and the total liquid heights were measured. The walnut protein emulsibility was calculated using the formula:

$$E\ (\%) = \frac{H_1}{H_2} \times 100 \quad (4)$$

Where:

H_1 Indicates the height of the emulsion layer in the centrifuge tube (cm)

H_2 Indicates the total height of liquid in the centrifuge tube (cm)

The above-emulsified liquid was put in a thermostatic water bath at 60°C for half an hour. After cooling to 25°C, the sample received 20 min of centrifugation at 3,000r/min for measuring the height of the emulsion layer. In this assay, the formula below interpreted the emulsion stability:

$$ES\ (\%) = \frac{H_3}{H_4} \times 100 \quad (5)$$

Where:

H_3 Indicates the height of the emulsion layer after 30 min (cm)

H_4 Indicates the initial height of the emulsion layer (cm)

Water and Oil Absorptions

Water absorption: For this assay, 0.1g skim walnut protein powder was weighted, and then added with 10 mL of deionized water to undergo 60 s of vortexing. Then, the sample received 15 min of centrifugation at 3000 r/min after 30 min of standing. Finally, the supernatant was removed to calculate the water binding capacity according to the following formula:

$$\text{Water absorption } (g/g) = \frac{M_2 - M_1}{M_0} \quad (6)$$

Where:

M_0 Denotes the mass of the sample (g)

M_1, M_2 Denote the total mass of the centrifuge tube and sample (g) and precipitation (g), respectively

Oil absorption: For the oil absorption measurements, the mixture of 5 mL of soybean oil and 0.1g skim walnut protein powder was vortexed for 60 s. After standing for 30min, the sample underwent 15 min of centrifugation at 3000 r/min, and the residual oil was absorbed using oil-absorbing paper. Finally, the formula below interpreted the oil absorption:

$$\text{Oil absorption } (g/g) = \frac{M_2 - M_1}{M_0} \quad (7)$$

Where:

M_0 Denotes the mass of the sample (g)

M_1, M_2 Denote the total mass of the centrifuge tube and sample (g) before and after oil absorption (g) (Jin et al., 2020), respectively

Evaluation of The Surface Lydrophobicity

The sample was dissolved in 10 mM PBS buffer (1%, w/w) as the mother solution, and the protein was diluted between 0.1 and 0.6 mg/mL with the same buffer, followed by the preparation of 8 mM of ANS solution in 10 mM PBS buffer, pH 7.0. Next, specific kit served for measuring the fluorescence intensity of the mixture of 4 mL of the sample and 25 μL of the ANS solution (390 nm excitation wavelength; 470 nm emission wavelength). In addition, the initial slope between fluorescence intensity and protein concentration was subjected to the linear regression analysis, yielding the hydrophobicity index (Hu et al., 2013).

Amino Acid Composition Analysis of Walnut Protein

The amino acid composition of walnut protein was ascertained following the method by Liu et al. (Liu et al., 2020). In brief, protein powder 0.5g was weighed into a closed hydrolysis tube to undergo 24 h of hydrolysis at 110 °C after added with 6 mol/L HCl (containing 0.1% phenol, v/v). After cooling, the solution was filtrated and diluted to a constant volume. Thereafter, 1 mL of the filtrate was used to concentrate and volatilize the remaining HCl on a centrifugal concentrator. The concentrated sample was then diluted 20 times, with 1 mL of the sample being analyzed via the automatic amino acid analyzer (Ding et al., 2023).

Determination of The Structure of Walnut Protein

Molecular Weight Distribution of Walnut Protein

To determine the molecular weight of walnut protein, the study conducted SDS-PAGE analysis following a previous methodology (Lamsal et al., 2007).

Sample Treatment

In this study, two walnut proteins were extracted by a lysis method (composed of 1% PVPP, BPP solution, TRIS-saturated phenol, acetone, 8M urine +1%SDS, and cocktail containing protease inhibitor). The bicinchoninic acid method served for the quantification of the protein. After, a specific amount of protein was collected from each sample to perform trypsin lysis by Filter Aided Sample Preparation (FASP) method.

Tandem Mass Tag (TMT) Labeling

For the TMT labeling, 100 μg of the peptide from each sample was collected and labeled as per the producer's protocol (Thermo Scientific). In this assay, two groups were defined, containing 3 duplicate samples in each group (6 samples in total).

First-Dimension Separation by High-pH Reversed Phase Liquid Chromatographic

The polypeptide samples were redissolved in Ultra Performance Liquid Chromatography (UPLC) loading buffer (containing 2% acetonitrile at pH = 10 adjusted with ammonia). A high-pH liquid-phase separation using a RP-C18 column was performed. Chromatographic column: ACQUITY UPLC BEH C18 Column 1.7μm, 2.1 mm × 150 mm (Waters, USA); Mobile Phase A: 2% acetonitrile (adjusted to pH = 10 with ammonia); Mobile Phase B: 80% acetonitrile (adjusted to pH = 10 with ammonia); UV detection wavelength: 214 nm; flow rate: 200 μL/min; separation time: 48 min. In this study, we collected 28 fractions by the peak pattern and time and merged them into 14 fractions. After vacuum centrifugal concentration, the fraction was dissolved by Mass Spectrometry (MS) loading buffer (composed of 2% acetonitrile and 0.1% formic acid) to perform the second-dimension analysis.

Liquid Chromatograph Mass Spectrometer / Mass Spectrometer and Data Analysis

In the LC-MS/MS analysis, the samples were separated via a reversed-phase chromatographic column: C18 column (150 μm×15 cm, Evosep). Several parameters were defined as: mobile phase A: 0.1% formic acid; mobile phase B: 100% acetonitrile and 0.1% formic acid; flow rate: 300 nL/min; and separation time: 44 min.

After, the samples were separated by chromatography, and MS was conducted by virtue of an Orbitrap Exploris 480 mass spectrometer (Thermo, USA). The MS method was set as follows: scanning range (m/z): 350-1500; collection mode: DDA; cycle time: 2 s; primary MS resolution: 60000; maximum injection time: 25 ms; fragmentation mode: HCD; secondary resolution: 15000; maximum injection time: 22 ms, fixed first mass: 110 m/z; minimum AGC target: 8e3; intensity threshold: 8.3e4; dynamic exclusion time: 30 s; reversed-phase column: C18 column (150 μm×15 cm, Evosep).

Finally, result analysis relied on the Proteome Discoverer TM Software 2.4. The raw files were submitted to the Proteome Discoverer server, and the established database was selected, followed by database search and analysis.

Statistical Analysis

Spss26.0 and Excel 2020 were used for statistical analysis of data. Origin8.6 software served for mapping; VMD software assisted in mapping the tertiary structure diagram of protein, distribution diagram of surface charges, and distribution diagram of surface residue solubility. The "IMAGE COLOR SUMMARIZER" website was selected to identify the image colors and calculate the net surface charges and soluble surface residues of proteins ratios.

Results

Differences in Lvling Walnut and Wen 185

Solubility, foamability, foam stability, emulsibility, and emulsion stability of proteins are essential properties affecting product texture (Stefanović et al., 2017). The study evaluates all the properties of two walnut proteins (Table 1). Samples treated with different pH conditions exhibited an obvious difference, indicating that they should be applied in food processing methods. In addition, the two proteins exhibited the lowest solubility at pH 6, which conformed to a previous study (Jin et al., 2020) where the isoelectric point of walnut protein was around 6. This result can explain why fermented walnut products cannot be solidified at pH 5 like in dairy products (Tan et al., 2017). As a vegetable protein, walnut protein contains a large quantity of glutelin. On these accounts, these walnut proteins had a solubility less than 0.1%, being smaller than the casein in milk products (0.8%-1.2%). Therefore, many studies focused on improving the solubility of walnut protein through physical, chemical, and biological methods (Zhu et al., 2018; Moghadam et al., 2020; Lv et al., 2019).

Table 1: Compare Physical and Chemical Properties of Wen 185 and Lvling in proteinc

pH	Solubility(×10-3g/100g)		Foamability (%)		Foam stability (%)		Emulsibility (%)		Emulsification stability (%)	
	Lvling	Wen 185	Lvling	Wen 185	Lvling	Wen 185	Lvling	Wen 185	Lvling	Wen 185
2	42.50±0.47^{A}	49.08±1.02^{B}	91.00±3.61^{a}	116.67±2.52^{b}	44.54±0.77^{A}	55.48±0.62^{B}	9.43±0.45^{A}	18.96±0.37^{B}	50.14±0.75^{A}	69.74±0.61^{B}
4	35.76±0.48^{A}	43.51±0.47^{B}	82.00±3.61^{a}	101.67±5.51^{b}	54.16±0.39^{A}	67.62±1.22^{B}	7.02±0.19^{A}	16.03±0.67^{B}	41.89±0.60^{A}	51.15±0.82^{B}
6	29.35±0.60^{A}	34.41±0.47^{B}	54.33±4.04*	69.00±5.00*	66.29±1.27^{A}	79.57±0.79^{B}	4.30±0.46^{A}	13.27±0.53^{B}	32.08±0.65^{A}	43.37±0.56^{B}
8	33.75±0.54^{A}	38.87±0.45^{B}	88.67±4.16^{a}	109.00±2.65^{b}	53.60±0.77^{A}	65.23±0.41^{B}	7.47±0.46^{A}	16.60±0.40^{B}	49.83±0.93^{A}	65.35±0.56^{B}
10	40.33±0.54^{A}	49.36±0.60^{B}	107.67±2.52^{a}	136.67±2.52^{b}	43.07±1.48^{A}	54.38±0.60^{B}	11.31±0.34^{A}	19.57±0.40^{B}	62.91±0.62^{A}	72.63±0.55^{B}
12	54.41±0.49^{A}	65.27±0.40^{B}	123.33±4.16^{a}	161.00±3.61^{b}	36.95±0.17^{A}	43.54±0.71^{B}	14.00±0.62^{A}	26.32±0.47^{B}	71.11±0.56^{A}	83.36±0.58^{B}

Note：n = 5; The varietal difference in protein physical and chemical properties, Block capitals：significant deviation（P <0.01）, Lowercase: significant deviation（P <0.05）

The emulsibility and foamability are the embodiment of the hydrophilic and oleophilic amphiphilic molecules of proteins. The results in Table 1 demonstrated that emulsibility and foamability were positively correlated with the solubility parameter. When PH of protein solution system was approximate to the pI of walnut protein, the solubility of proteins was lower, and due to a lack of surfactant, these two proteins exhibited poor foamability and emulsibility (Wani et al., 2015). Furthermore, the foaming rates of Lvling and Wen 185 were 54.33 and 69%, respectively, and the emulsibility values for them were 4.29 and 13.27%, respectively. Additionally, the emulsion stability was positively correlated with emulsibility and solubility. On the other hand, foam stability was negatively correlated with foamability and solubility. Regarding the thermodynamic analysis, the low surface tension was more conducive to foam generation since less work was required to generate the same total foam area. However, the foam stability could not be confirmed. Only when the surface membrane had certain strength can low surface tension be conducive to the foam stability. The low surface tension could induce a small pressure difference when protein solubility was smaller. Therefore, the liquid discharge speed was lower, and the liquid membrane became thin more slowly, promoting foam stability (Bolontrade et al., 2016).

It has been described that the water and oil binding capacities of proteins could significantly impact the yield, texture, and flavor of processed products. For example, changes in several biological components, including amino acid composition, protein structure, and charged groups, can occur. In this study, the differences in water and oil absorptions between Lvling and Wen 185 were evaluated (Figure 1). The water and oil binding capacities of Wen 185 were significantly higher (2.4g/g and 3.7g/g higher, respectively) compared to Lvling walnut protein. Therefore, the results suggest that Wen 185 could be a better option for obtaining walnut protein liquids and gelling products.

Additionally, surface hydrophobicity reflects the number of hydrophobic surface groups and the tertiary structure of proteins, being a relevant parameter to evaluate the strength of the intermolecular interactions. On the other hand, surface hydrophobicity remarkably affected protein stability and conformation under polar conditions, reflecting indirectly the protein conformational changes. In this study, a significantly higher surface hydrophobicity was observed in Wen 185 than in Lvling walnut protein (Figure 2) (P <0.05). This result could induce a thicker interfacial film, enhancing the film's strength and emulsibility of Wen 185 walnut protein (Ghribi et al., 2015; Wouters et al., 2016).

Comparison of the Amino Acid Composition of Walnut Proteins

Amino acid is the basic unit and the determiner of protein properties. The amino acids abundant in the two walnut proteins were glutamic acid and arginine, but these contents significantly varied among the two walnut varieties (Table 2). The glutamic acid and arginine contents were the highest (145.40 mg/g and 111.13 mg/g, respectively) in Lvling. The contents of hydrophobic amino acids in wen185 and Lvling were 40.79% and 45.53%, respectively, indicating the cause of the strong hydrophobic effect of Lvling.

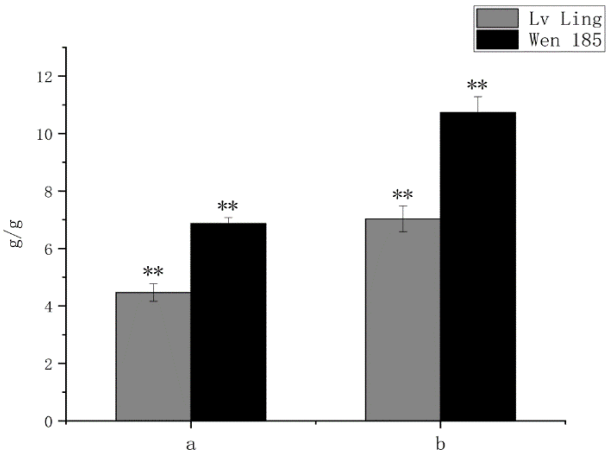

Fig. 1: Absorbent and Oil absorbency of protein in LV Ling and Wen 185
Note: a) Absorbent, b) Oil absorbency. （n=3, P <0.01）

Comparative Analysis on the Composition and Structure of Lvling and Wen 185

The difference in molecular weight has been applied to evaluate protein alterations. Therefore, in this study, these alterations in the two walnut proteins were

determined using SDS-PAGE gel electrophoresis maps (Figure 3). The results demonstrated that proteins exhibited spectral bands ranging between 10 and 130 kDa, indicating a wider molecular weight distribution of walnut proteins, with more subunit bands and relatively complex compositions. For example, the 23 and 35 kDa bands were darker and wider, corresponding to the main subunits of walnut protein. The molecular weight of the largest and smallest subunits was approximately 120 and 10 kDa, respectively.

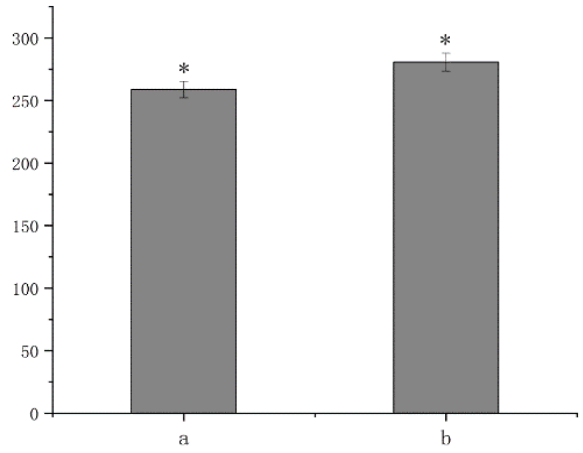

Fig. 2: The Surface Hydrophobicity Index of protein in LV Ling and Wen 185
Note: A LV Ling, b Wen 185, * significant deviation（n =3, P <0.05）

Table 2: Walnut protein amino acid composition（mg/g）

	Wen185	Lvling
Lys	29.93±0.34b	25.07±0.48a
Phe	46.76±0.68a	51.68±0.74c
Met	25.94±0.93a	28.92±0.37b
Thr	35.93±0.38b	35.76±0.16b
Ile	52.01±0.27b	54.46±0.63c
Leu	66.82±0.49b	65.18±0.75a
Val	43.39±0.82c	41.92±0.29a
Asn	87.64±0.59b	90.37±0.31c
Ser	49.97±0.15b	50.49±0.91b
Glu	142.18±0.72b	145.40±0.27c
Gly	40.41±0.79b	40.58±0.71b
Ala	42.34±0.36b	41.42±0.62ab
Cys	1.66±0.85a	3.24±0.88c
Tyr	40.25±0.24b	39.27±0.62a
His	26.61±0.19b	26.82±0.21b
Arg	102.75±0.58b	111.13±0.17c
Pre	38.43±0.63b	35.47±0.75ab

Note: $p < 0.05$

Furthermore, the content of the protein with the high molecular weight was lower, ranging between 75 and 180 kDa. this result agrees with a previous study (Zhu et al., 2018). However, the content of Wen 185 walnut protein is higher at 10, 15, 20 and 48kDa with lower molecular weight, while that of Lvling walnut protein is higher at 120kDa with higher molecular weight. The results indicate that the solubility of Lvling walnut protein was lower than Wen 185.

The proportion of Wen 185 walnut protein with high molecular weight was lower than that of Lvling walnut protein (Figure 3), which was one of the reasons why the solubility of the former is better than the latter. Additionally, a proteomic difference analysis herein engaged in understanding the significant differences in functional properties between Lvling and Wen 185 (Figure 4). Wen 185 walnut protein and Lvling walnut protein presented significant differences. To present the significant differences in expressed proteins between control groups, the proteins in control groups were expressed by FC of expression, and differential analysis was carried out on them (Tunsagool et al,. 2023). Finally, a total of 1,086 differential proteins were obtained (FC<0.83, FC>1.2, and P<0.05), including 807 up-regulated proteins (FC>1.2, and P<0.05) and 279 down-regulated proteins (FC<0.83, and P<0.05). Of all these proteins, 6 showed the most significant differences, including 3 up-regulated and 3 down-regulated proteins (Table 3). The tertiary structure diagram, surface charge distribution diagram, and surface residue solubility distribution diagram of these six proteins were examined (Figure 5). Furthermore, Figures 6a and 6b showed the ratio of net surface charge contents and the ratio of surface residues.

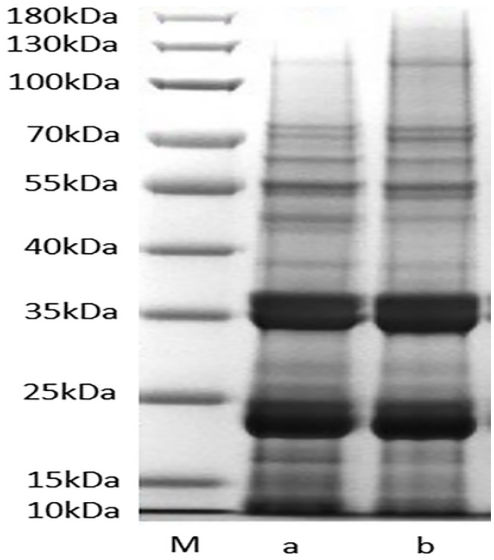

Fig. 3: SDS-PAGE of protein in LV Ling and Wen 185
Note: M Blank, a Wen 185, b, LV Ling

Table 3: The differential proteins expression

ID①	calc.pI②	FC(XJ/LL)③	P_value(XJ/LL)④	Regulate	GRAVY⑤
A0A2I4EYG9	7.85	12.33	9.0×10^{-6}	up	-0.89
A0A6P9EQ41	5.87	6.79	2.8×10^{-4}	up	-1.25
A0A6P9EX40	6.81	5.29	4.4×10^{-5}	up	-0.64
A0A2I4EB91	8.54	0.22	1.9×10^{-5}	down	0.47
A0A833TMW1	8.22	0.37	1.0×10^{-6}	down	0.08
A0A2I4E4Q9	8.88	0.38	0	down	0.52

Note: XJ: Wen 185，LL: Lvling, ①: ID in the Accession Database;②: Theoretical Isoelectric Point;③FC(XJ/LL)：Multiple，LL: Blank, ④ P_value(XJ/LL): Significant Deviation, ⑤GRAVY：Polypeptide Avergge Hydrophobicity

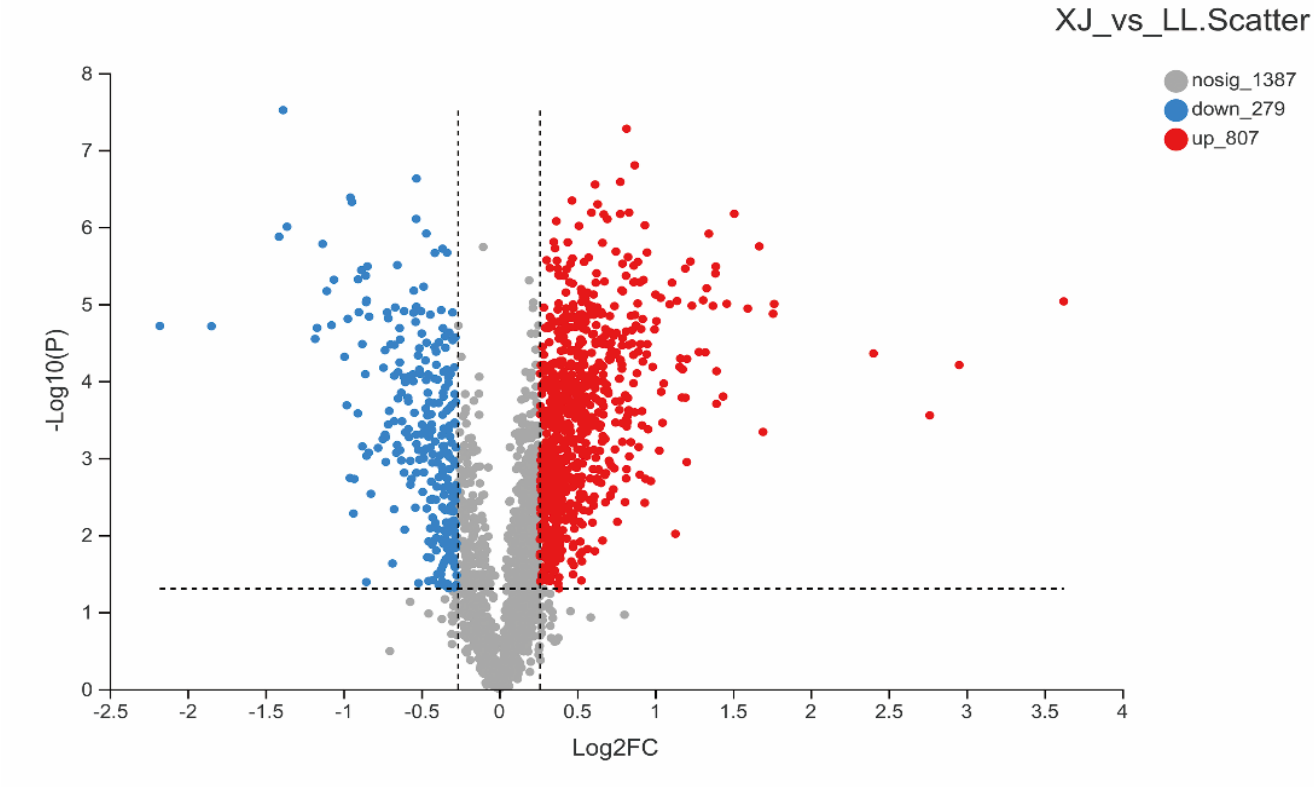

Fig. 4: Differential volcano map of protein in LV Ling and Wen 185

Note: Note: XJ represents Wen 185, and LL represents Lvling walnut protein. The abscissa denotes the protein expression differences between the two proteins, which is, the value obtained by dividing the expression amount of treated samples by the control sample. The ordinate denotes the protein expression differences (p-value). For example, a smaller p-value corresponds to a higher significant difference in the protein expression. Logarithmic processing was performed for values on the abscissa and ordinate. Each point in the figure represents a specific protein. The points on the left denote the down-regulated proteins with expression difference, and those on the right denote the up-regulated proteins with expression difference. The closer to the points on the left and right sides and those on the top, the more significant the expression difference

DOI: 10.3844/ajbbsp.2025.526.537

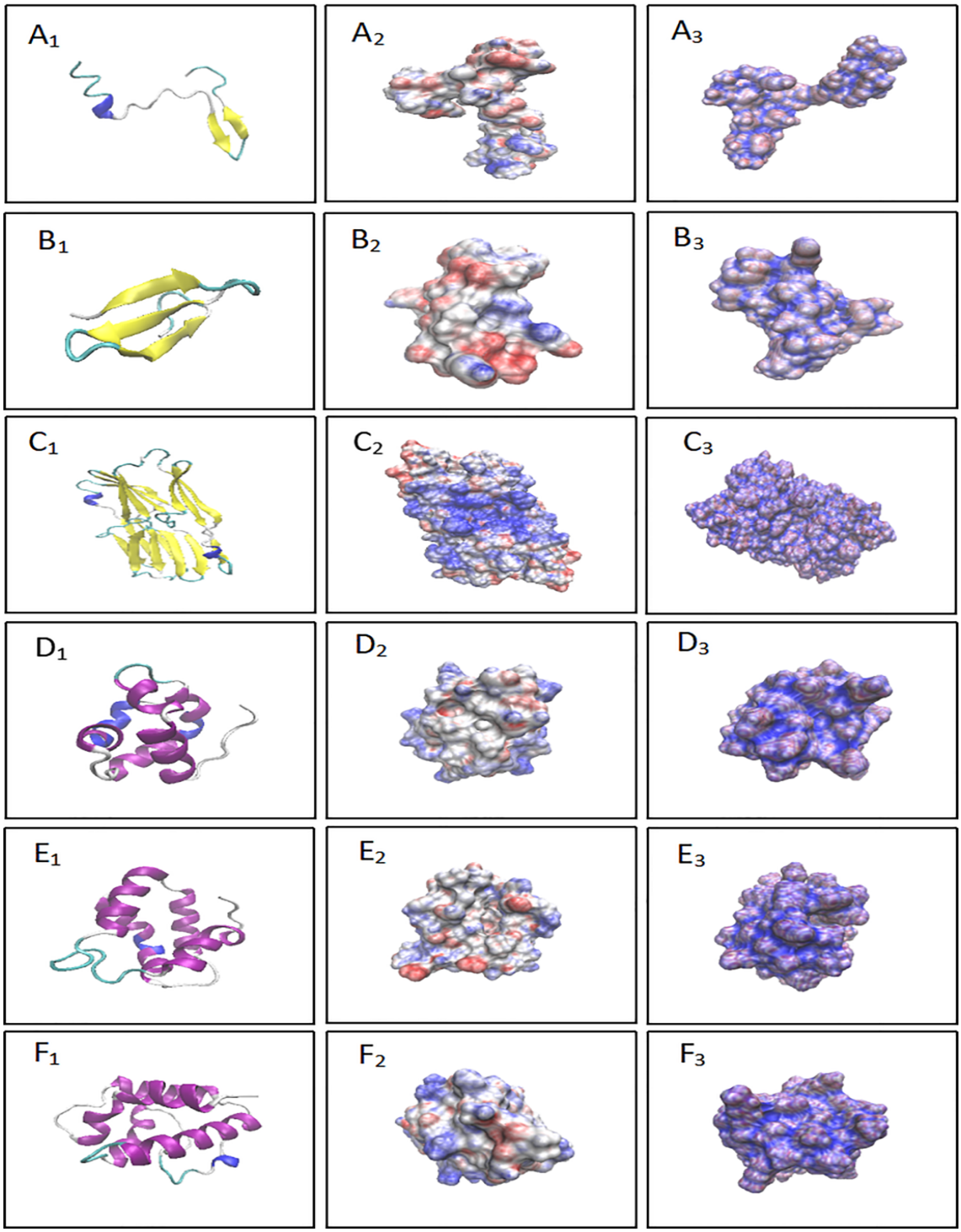

Fig. 5: 3D of six differential proteins

Note: A, B, C, D, E, and F represent differential proteins, A0A2I4EYG9, A0A6P9EQ41, A0A6P9EX40, A0A2I4EB91, A0A833TMW1, and A0A2I4E4Q9, respectively; subscripts 1, 2, and 3 represent the tertiary structure chart of the proteins (purple and blue represent two types of α-helices; yellow correspond to β-sheet; cyan characterizes β-turn; and white represents random coil), the surface charge distribution graph (blue, red, and white represent positive, negative, and no charges, respectively), and the solubility distribution of surface residues (blue and red correspond to the exposed residue and buried residue, respectively)

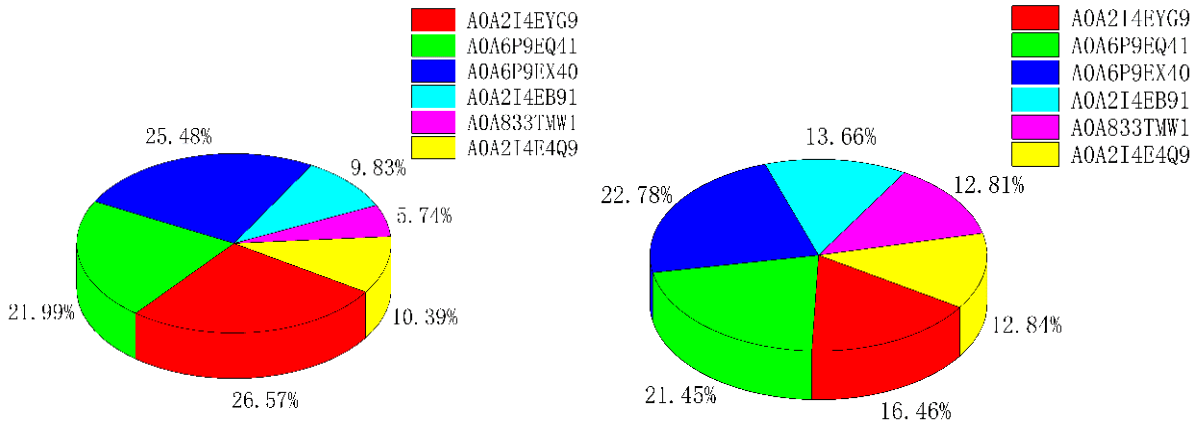

Fig. 6: The surface of net charges and protein residues solubility of six differential proteins

The content of 3 up-regulated differential proteins (A0A2I4EYG9, A0A6P9EQ41, and A0A6P9EX40) in Wen 185 were 12.33, 6.7, and 5.29 times higher, respectively, than in Lvling walnut. Furthermore, the β-sheet content in these proteins was high (Figure 5). Besides, the α-helix content was highly decreased (Figure 5). Besides, the grand average of hydropathy (GRAVY) values of these proteins were negative, indicating that they are hydrophilic proteins presenting a better hydrophilicity profile. In addition, the contents of three down-regulated differential proteins (A0A2I4EB91, A0A833TMW1, and A0A2I4E4Q9) in Wen 185 walnut were 0.22, 0.37, and 0.38 times higher, respectively, compared to Lvling walnut. These proteins presented extremely low β-sheet contents and high α-helix contents. Furthermore, the GRAVY values were positive, indicating that they are hydrophobic proteins with poor hydrophilicity. Some studies demonstrated that when the β-sheet content is much higher than the α-helix content, the protein structure becomes loose and flexible, improving the emulsibility and foamability parameters. In addition, protein unfolding can promote charged amino acid residues to be exposed to the protein surface, leading to increased charges on the protein surface and enhancing the solubility and interfacial activity (Tamm et al., 2016). For instance, when the β-sheet content increase, the α-helix content decreases and the hydrophobicity of the protein surface becomes stronger. Moreover, these increases in the hydrophobicity can help weaken the energy barrier at the air-water interface and increase its adsorption kinetics, thus enhancing the protein-water interaction (Yan and Zhou, 2021; Lam and Nickerson, 2013; Yang et al., 2018), improving the foamability and foam stability parameter (Eckert et al., 2019; Moghadam et al., 2020). Therefore, these results help to understand why the solubility, emulsibility, emulsion stability, foamability, water absorption, oil absorption, and surface hydrophobicity of Wen 185 were significantly higher compared to Lvling walnut protein. Furthermore, the 3 up-regulated differential proteins presented much higher ratio of surface net charge and surface residue solubility content versus the 3 down-regulated proteins (Figure 6). This result helps to understand better the higher solubility presented by Wen 185 compared to Lvling walnut protein. Finally, the increase in the solubility and surfactants are crucial factors for the significant differences detected in the surface activity.

Conclusion

This study analyzed two different thin-shell walnuts mainly cultivated in China, and the results demonstrated that Wen 185 was significantly better than Lvling walnut protein in several parameters, such as solubility, emulsibility, foamability, water absorption, and oil absorption. The molecular weight and proteomic analyses found that the proportion of low molecular weight in the Wen 185 was higher than in the Lvling walnut protein, Compared with Lvling walnut protein, three kinds of proteins being abundant in β-sheet structure in Wen 185 were up-regulated , while it was down-regulated three proteins which content lots of α-helix. Therefore, Wen 185 walnuts are more suitable for processing liquid walnut protein products, while Lvling walnuts are suitable for solid food processing. These differences in the biological parameters primarily resulted from the different contents and structural compositions of the differential protein caused by the variety. This article studied the protein differences in the main thin skinned walnut varieties grown in two major walnut producing areas, Xinjiang and Hebei. The sample size is relatively small, and more walnut protein difference analyses from walnut producing areas can be collected in the future to

lay a theoretical basis for selecting suitable processing materials for walnut protein products. This article combines proteomics technology to identify key proteins that affect the characteristics of walnut protein in different varieties, and deeply explores the impact of the three-dimensional structure of key proteins on functional characteristics, filling the gap in the research on the influence of protein three-dimensional structure on functional characteristics. Overall, this study indicates that exploring the biological parameters of different walnut could be highly useful in selecting the appropriate variety to obtain walnut proteins and ensure the quality of walnut protein products.

Acknowledgment

We would like to express our gratitude to MOGOEDIT for editing the English text of a draft of this manuscript.

Funding Information

National Key Research and Development Program, China (Grant 2022YFD1600402), Key research and development project of Hebei Province China, (Grant 21326304D-2), Open Foundation of Collaborative Innovation Center for Wetland Conservation and Green Development of Hebei Province (Grant No.2023hbxczx1-11)

Author's Contributions

Xu Meng: Conceptualization, methodology, investigation, resources, writing (original draft).
Tian. Yiling: Conceptualization, supervision, writing (review and editing).

Ethics

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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