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Physicochemical and functional characterization of pumpkin seed protein isolate†

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The increasing demand for plant-based protein sources has driven extensive research into various underutilized seeds and their protein isolates. This research addresses the gap by systematically investigating the physicochemical, functional and morphological properties of pumpkin seed protein isolate (PSPI). By establishing the potential of PSPI as a versatile plant-based protein source, this research expands the knowledge base for its application in food product development. The proximate composition (moisture, ash, fat, protein, and carbohydrate) content of PSPI was found to be 7.56%, 2.58%, 1.28%, 85.78%, and 2.8%, respectively. The dispersibility increased with an increase in pH (from 9 to 12). The mineral composition was found to be Mg (4.20), P (0.62), K (457.82), Ca (3.76), Fe (5.81), Cr (0.10), Mn (1.46) and Cu (0.21) mg/100 g. SDS-PAGE showed 10 detectable bands with molecular weights ranging from 5 to 250 kDa. All the essential and non-essential amino acids were present, indicating the presence of good quality proteins. The onset and melting point temperatures of PSPI were found to be 69.3 °C and 75.6 °C, respectively. This research advances the field of food science and technology by identifying PSPI as a promising plant-based protein source in the formulation of food, contributing to the development of nutritious and sustainable food products.

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Sustainability statement

Among the foods with maximum wastage, pumpkin stands out. Pumpkin seeds are an excellent source of several nutrients and have been exploited for their oil, leaving the other portion as waste, which is a rich source of protein. By exploring the physico-chemical and functional properties of pumpkin seed protein, a new paradigm can be established for the development of protein enriched foods or pumpkin protein based meat alternatives, which ultimately paves the way to achieve SDGs 2 and 12.

1. Introduction

Fruits and vegetables are widely grown food crops due to their nutrients and health benefits. To meet the rising demand, the production and processing of these crops have significantly expanded. However, the economic and nutritional quality of the processing industry has declined, leading to increased environmental concerns.[1] The food sector has faced significant challenges in reducing processing-related waste and in processing and disposing of waste in an environmentally responsible way. More focus has also been placed on utilizing resources to transform these by-products as well as wastes into beneficial products.[2]

Pumpkin (*Cucurbita moschata* Duch., Cucurbitaceae) is a common plant utilized in the food industry to make juices, purees, alcoholic beverages, and jams. It grows in subtropical, tropical, and warm climates worldwide.[3] Because of their high level of nutritional content and health-promoting properties, pumpkin seeds, a food oil crop, are frequently used in vegetable oil production. Despite being primarily thought of as agro-industrial waste, pumpkin seeds are a rich source of nutrients with interesting nutraceutical properties.[4,5]

A significant amount of pumpkin seed cake is formed as a byproduct of increasing pumpkin-seed oil production and is often utilized as livestock. Recently, pumpkin seed cake has attracted a lot of interest due to its high-level content of protein (60–65 percent), which makes it an attractive as well as potential protein source of plant origin. Furthermore, the main protein found in pumpkin seeds is 12S globulin, which shares a structural resemblance with globulins present in legume seeds.[6] This structural similarity suggests that pumpkin seed protein isolate

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(PSPI) may exhibit functional properties similar to those of legume seed proteins, including emulsifying, foaming, and gelling characteristics.[7] Pumpkin seed proteins are broadly utilized as food ingredients in addition to their pharmacological characteristics, which include antidiabetic, hepatoprotective, anti-cancer, and antioxidant properties.[8]

Pumpkin seeds contain several key protein fractions, including cucurbitin, 18S globulin, and albumins. The cucurbitin molecule consists of six identical subunits, each weighing 54 kDa, totaling a molecular weight of 325 kDa. Each subunit is composed of two disulfide-linked polypeptide chains, with molecular weights of approximately 22 kDa and 33 kDa.[9] Additionally, Blagrove and Lilley identified smaller quantities of 18S globulin, a dimer of 12S components.[10] Although present in lower concentrations than globulins, pumpkin seed albumins also contribute to the overall protein content. Together, globulins and albumins make up approximately 59% of the crude protein in pumpkin seeds.[4] One specific protein, 2S albumin, is formed by two disulfide-linked polypeptide chains—a smaller chain of about 4.8 kDa and a larger one of roughly 7.9 kDa—resulting in a total molecular weight of approximately 12.5 kDa.[11]

The valorization of plant proteins involves their extraction and isolation by targeting their isoelectric points. Isoelectric precipitation is a common method for protein isolation, where proteins are first solubilized in a high pH range of 8 to 10, followed by adjusting the solution to the isoelectric pH using mineral acids such as hydrochloric or sulfuric acid. Plant protein isolates with tailored functional properties have become a valuable alternative for meeting diverse industrial production needs. They are extensively utilized in food applications, serving as water retention agents, foaming agents, stabilizers, adhesives, and gelling agents.[4]

The physicochemical and heat-induced gelation properties of pumpkin seed protein isolate (PSPI) were investigated and compared to those of soybean protein isolate (SPI) and pea protein isolate (PPI). These plant proteins underwent treatments using ultrasound under either acidic or alkaline conditions (pH 3 or 11). PSPI exhibited distinct amino acid content and subunit composition compared to SPI and PPI. Rheological and textural analysis revealed that native PSPI displayed the highest gel strength among the three proteins, attributed to its high hydrophobicity and the presence of insoluble particles that enhanced the protein solution. Notably, PSPI demonstrated significant improvements in gel properties following ultrasound or alkaline treatment compared to SPI and PPI, likely due to increased surface hydrophobicity, enhanced solubility, and reduced particle size. Thus, PSPI shows superior heat-induced gelation properties over SPI and PPI, making it a promising alternative for food industry applications.[12]

This study aims to explore the physico-functional and thermal properties of pumpkin seed protein, with a focus on its potential for new product development and as a valuable source for protein supplementation. By examining these characteristics, the research seeks to contribute to a deeper understanding of pumpkin seed protein's applications in food innovation, emphasizing its functional versatility and nutritional benefits.

2. Materials and methods

2.1 Materials

Pumpkin seeds have been sourced from Punjab Agriculture University (Variety: PAU MAGZ KADU NO. 1). All the reagents and chemicals utilized for the research were of analytical reagent grade.

2.2 Methods

2.2.1 Preparation of defatted pumpkin seed meal. The pumpkin seeds underwent thorough washing and cleaning in order to separate and remove any contaminants. After that, the seeds were dried for 48 hours at 45 °C using a tray dryer. The seed kernels were separated manually. Using a lab scale grinder, the kernels were reduced to fine powder. Defatting was done using *n*-hexane (30 : 1, v/w) following the method reported by Devi *et al.*[1] with slight modifications. The defatted sample was dried at a temperature of 40 °C and kept at a storage temperature of 4 °C until further use.

2.2.2 Preparation of pumpkin seed protein isolate (PSPI). PSPI was isolated by alkali extraction with isoelectric precipitation as per the method described by Vinayashree and Vasu[8] with slight modifications. Protein dissolution was facilitated by suspending the defatted pumpkin cake in a solution of an alkali at room temperature (pH 10.00), which was adjusted using 1 mol per dm^3 NaOH (1 : 25 w/v). The slurry was filtered after being gently stirred for 30 min. By adding 1 mol dm^{-3} of HCl to bring the filtrate's pH down to 5.00, the dissolved proteins were precipitated. Centrifugation was used to separate the precipitate from the phase of liquid for 20 min at a temperature of 4 °C and 10 000 rpm. The precipitate was then freeze-dried in a lyophilizer (LABFREEZE Scientific Limited) and maintained at −80 °C for further evaluation.

2.3 Physicochemical characterization of pumpkin seed protein isolate

The PSPI has been analyzed for different characteristics as discussed below.

2.3.1 Determination of moisture, ash, protein, and mineral content in PSPI. The moisture as well as ash content of PSPI has been evaluated as per AOAC protocols.[13] The protein content in pumpkin seed protein isolate (PSPI) was measured using the Kjeldahl method following standard AOAC procedures.[13] A conversion factor of 6.25 was applied to convert nitrogen content to protein content. All measurements were done in triplicate.

Elemental analysis was determined using a PerkinElmer ELAN DRC II type inductively coupled plasma mass spectrometer, which has a quadrupole, a reaction cell for interference elimination, a mass range of 3 to 240 *m*/*z*, and a resolution of less than 1 amu (IU36, Serial Q1970307H). Standard calibration solutions made from the stock solutions (Multi-element Calibration Standard 3, PerkinElmer) were used to calibrate the device.[14]

2.3.2 Bulk density, Hausner ratio, tapped density, and compressibility index. To measure the bulk density, 25 g of PSPI was placed into a measuring cylinder, and the volume of the sample was recorded. The bulk density was then calculated as

the weight-to-volume ratio (g mL^{-1}). For the tapped bulk density, the cylinder was tapped until no further reduction in volume was observed, and this final volume was recorded. Eqn (1) and (2) were used to determine the tapped density and the Hausner ratio (HR), respectively. The Compressibility Index (CI), which reflects the powder's compressibility, was calculated using eqn (3).

$$\text{Tapped density (g mL}^{-1}) = m_f/v_f \quad (1)$$

$$\text{HR} = \frac{\rho_{\text{tapped}}}{\rho_{\text{bulk}}} \quad (2)$$

$$\text{Compressibility index} = 100(v_0 - v_f)/v_0 \quad (3)$$

where m_f, v_f, and v_0 stand for the mass of samples in a measuring cylinder following tapping, the final tapped volume, and the apparent unsettled volume, respectively.

2.3.3 Water absorption capacity (WAC) and oil absorption capacity (OAC). Water Absorption Capacity (WAC) and Oil Absorption Capacity (OAC) were determined following the method described by Sharma *et al.*[15] with slight modifications. 1 g of sample was mixed with 10 mL of distilled water or oil for 30 s and then allowed to rest at room temperature for 30 min. The samples were subsequently centrifuged at 10 000 rpm for 10 min. The amount of water or oil retained per gram of sample was calculated by measuring the volume of the supernatant.

2.3.4 Dispersibility. 3 g of PSPI were added to distilled water in a measuring cylinder, and the pH was adjusted to between 9 and 12 using diluted HCl or NaOH solution. The final volume was brought up to 30 mL with distilled water. After stirring continuously for 2 h and allowing the mixture to settle, dispersibility was calculated using eqn (4) as outlined by Sharma *et al.*[15] with slight modifications.

$$\text{Dispersibility}(\%) = \frac{\text{Total volume} - \text{settled volume}}{\text{Total volume}} \times 100 \quad (4)$$

2.3.5 Color. A colorimeter (D25 LT Hunter Associates Laboratory, USA) was used to measure the *L*, *a*, and *b* values for assessing the sample color. The *L* value represents lightness, ranging from 0 (black) to 100 (white). The *a* value indicates the red–green spectrum, with higher positive values reflecting greater redness. The *b* value corresponds to the yellow–blue spectrum, where higher positive values indicate a more intense yellow.

2.3.6 Amino acid composition. The amino acid composition was determined using high-performance liquid chromatography (HPLC). To profile the amino acids, PSPI underwent both acid hydrolysis (for all amino acids except tryptophan) and alkaline hydrolysis (specifically for tryptophan), following a method by Arte *et al.*[16] with slight modifications. For the determination of all amino acids except tryptophan, 33 mg of protein isolate was acid hydrolyzed with 0.5 M HCl at 110 °C for 24 h. The acid-hydrolyzed sample was then centrifuged at 10 000×*g* for 10 min at 4 °C, and the supernatant was neutralized. For derivatization, the sample was diluted in a 1 : 4 (v/v) ratio with borate buffer.

For tryptophan analysis, 33 mg of the protein isolate was hydrolyzed under alkaline conditions using 4 M NaOH at 110 °C for 24 h. This alkali-hydrolyzed sample was pre-oxidized with 78.1% (v/v) acetic acid and a 10% mixture of hydrogen peroxide (30%) and phenol (5 mM). Oxidation was halted by adding sodium bisulfite, followed by neutralization and centrifugation at 10 000×*g* for 10 minutes at 4 °C. The supernatant was collected for analysis.

A 1 μL aliquot of the derivatized sample was injected into an HPLC system (Shimadzu HPLC Systems, Kyoto, Japan) with a 2.1 × 100 mm column and 1.8 μm particle size. Peak areas and retention times were recorded, and the amino acid concentrations were quantified in mg mL^{-1} of protein. All analyses were conducted in triplicate to ensure accuracy.

2.3.7 Antioxidant activity of PSPI. The DPPH radical scavenging activity was determined based on a method followed by Xu *et al.*[17] with slight modifications. For each test, a 2 mL aliquot of the sample solution at the same concentration was mixed with 2 mL of DPPH solution (0.2 mmol L^{-1} in ethanol). The reaction mixture was kept in the dark at room temperature for 50 min. The absorbance of the resulting solution was measured at 517 nm using a UV-vis spectrophotometer (UV-2800AH, Shanghai, China). The radical-scavenging capacity of PSPI was evaluated by calculating the change in DPPH radical concentration using eqn (5).

$$\text{Scavenging activity}(\%) = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100 \quad (5)$$

2.3.8 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoretic patterns of PSPI were analyzed using SDS-PAGE. A 50 mg sample of PSPI was suspended in 1 mL of 2× Laemmli buffer (containing 62.5 mM Tris–HCl at pH 6.8, % SDS, 25% glycerol, and 0.01% bromophenol blue). PSPI reduction was achieved with 5% β-mercaptoethanol, followed by incubation at 50 °C for 90 min and boiling for 5 min. The sample was then centrifuged at 11 000×*g* for 20 min at 25 °C, after which the residue was discarded. For SDS-PAGE, 10 μL of the supernatant was loaded per lane on a Mini-Protean 3 system (Bio-Rad Laboratories, Hercules, USA) using a 4% stacking gel and 15% resolving gel, with electrophoresis conducted at 25 mA. The gels were stained overnight with Coomassie Brilliant Blue R-250 and then destained using a 20% methanol and 10% acetic acid solution. The stained gels were scanned, and protein molecular weights were determined using a BioRad EZ imager (Bio-Rad Laboratories, Hercules, USA). Each SDS-PAGE analysis was performed in triplicate.

2.3.9 Particle size distribution (PSD) and ζ-potential. The ζ-potential and particle size distribution (PSD) of the sample were measured using a Malvern Zetasizer Nano ZS. To prepare the dispersion, the sample solution was mixed with 0.01 M phosphate buffer (pH 7.0). ζ-Potential and PSD measurements were then taken simultaneously.

2.3.10 *In vitro* digestibility. The *in vitro* protein digestibility of the protein isolate was determined using the method followed by Chavan *et al.*[18] with slight modifications. A 250 mg sample was suspended in 15 mL of 0.1 M HCl with 1.5 mg of

pepsin and gently shaken for 1 h at 30 ± 2 °C. The resulting suspension was neutralized with 0.5 M NaOH and then treated with 4.0 mg of pancreatin in 7.5 mL of phosphate buffer (0.2 M, pH 8.0). This mixture was shaken for 24 h at 30 ± 2 °C. After digestion, the mixture was filtered through Whatman No. 1 paper, and the residue was air-dried, washed with distilled water (1 : 10, w/v), and prepared for protein determination using the Kjeldahl method following AOAC (1990) protocols. Protein digestibility was calculated using eqn (6).

$$\textit{In vitro}\ \text{protein digestibility}\ (\%) = (I - F/I) \times 100 \qquad (6)$$

where I is the protein content of the sample before digestion and F is the protein content of the sample after digestion.

2.3.11 Fourier-transform infrared spectroscopy (FTIR). The pumpkin seed protein isolate (PSPI) was ground into a fine powder using a commercial blender and used immediately. Infrared (IR) measurements were carried out over a spectral range of 600–4000 cm^{-1} with a resolution of 4 cm^{-1}, using a Jasco FT-IR-4100 spectrophotometer (Oklahoma City, OK, USA). The sample was prepared using the KBr pellet technique, with calcined potassium bromide as the matrix material. A mixture of 3 mg of PSPI and 200 mg of KBr was pressed into 15 mm dies under a pressure of 10 tons for two minutes. The spectral data were then analyzed using Origin 6.0.

2.3.12 Differential scanning calorimetry. Thermal properties were analyzed using a Differential Scanning Calorimeter (DSC 1, Mettler Toledo, Leicester, UK), calibrated for both heat flow and temperature. All measurements were performed in sealed aluminum pans, with nitrogen gas purged at a flow rate of 50 mL min^{-1}. Thermograms were analyzed using Stare software (Version 13.00, Mettler Toledo, Leicester, UK). A 3 mg sample was heated from 20 °C to 250 °C at a rate of 10 °C min^{-1} and then allowed to cool naturally to room temperature (25 °C) under a nitrogen atmosphere. The onset temperature (T_o), peak denaturation temperature (T_d), and conclusion temperature (T_e) were recorded.

2.3.13 Scanning electron microscopy (SEM). A scanning electron microscope (SEM, Model JSM-6300, JEOL; Tokyo, Japan) was used to examine the morphological structure of the PSPI. The sample was mounted onto an aluminum slide using double-sided carbon tape. It was then coated with gold using an electro-depositor (Denton Vacuum Desk II, Moorestown, NJ, USA) and observed at 1000× magnification with an accelerating voltage of 20 kV. Two different areas on each slide were analyzed.

2.3.14. Statistical analysis. Data were reported as the mean of triplicate measurements. Statistical analysis was conducted using one-way analysis of variance (ANOVA) with SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA), with a significance level set at $p \leq 0.05$ for identifying significant differences.

3. Results and discussion

3.1. Moisture, ash protein, and elemental analysis of PSPI

The moisture, ash, fat and carbohydrate contents were found to be 7.56%, 2.58%, 1.28% and 2.8%, respectively (Table 1). The protein content of PSPI was observed to be 87.78%. The results are in accordance with the outcomes attained for other oil seeds like sunflower (70%),[19] sesame seed (59%),[20] and cashew nut, (91%).[21]

Table 1 Physico-chemical, thermal, and elemental analysis of pumpkin seed protein isolate (PSPI)[a]

Parameters	Values
Moisture (%)	7.56 ± 0.02
Ash (%)	2.58 ± 0.01
Protein (%)	85.78 ± 0.05
Bulk density (g mL^{-1})	0.59 ± 0.06
Tapped density (g mL^{-1})	0.69 ± 0.03
Hausner ratio (HR)	1.16 ± 0.42
Compressibility index	4.78 ± 0.49
WAC (g g^{-1})	1.09 ± 0.07
OAC (g g^{-1})	1.17 ± 0.05
Digestibility	84.56 ± 0.07
Dispersibility	**Values**
pH 9	71 ± 0.05
pH 10	72 ± 0.03
pH 11	76 ± 0.06
pH 12	82 ± 0.03
Elemental analysis	**Values (mg/100 g)**
Magnesium (Mg)	4.20
Phosphorus (P)	0.62
Potassium (K)	457.82
Calcium (Ca)	3.76
Iron (Fe)	5.81
Chromium (Cr)	0.10
Manganese (Mn)	1.46
Copper (Cu)	0.21
Color attributes	**Values**
L^*	73.52 ± 0.30
a^*	0.37 ± 0.13
b^*	17.64 ± 0.19
Thermal properties	**Values**
T_o	69.3 ± 0.32 °C
T_p	75.6 ± 0.34 °C
T_c	104.11 ± 0.36 °C

[a] Values are expressed as mean ± SD. WAC: water absorption capacity, OAC: oil absorption capacity, T_o: onset temperature, T_p: peak temperature, and T_c: conclusion temperature.

The elemental analysis of PSPI is presented in Table 1. The PSPI's most abundant element was potassium (457.821 mg/100 g). A high potassium content boosts the body's use of iron and is advantageous for those who use diuretics to lower their blood pressure and experience excessive potassium excretion through bodily fluids. The reported iron content of 5.812 mg/100 g in pumpkin seed protein isolate fulfills approximately 35–40 percent of the RDA (Recommended Dietary Allowance), highlighting its nutritional adequacy. Also, iron has multiple uses in the body, and it aids in blood coagulation and the movement of carbon dioxide and oxygen between tissues.[22]

The concentration of magnesium in the PSPI was reported to be 4.201 mg/100 g. Mg is an essential mineral found in bone as well as teeth. It is closely linked to Ca and P and plays a role in tissue respiration, particularly in the process of oxidative phosphorylation that produces adenosine triphosphate (ATP). The intestine, kidney, and backbone need Mg to release and use parathyroid hormone.[4]

The calcium content was reported to be 3.769 mg/100 g. Both the regulation of nutrient transport through cell membranes and the alleviation of sleeplessness are assisted by calcium. Also, the concentration of manganese was reported to be 1.461 mg/100 g. Manganese is essential for all mental processes and helps carry O_2 from the lungs to the cells. It also acts as an activator for the enzyme events related to the metabolism of proteins, fats, and carbohydrates.[23]

Additionally, the concentrations of phosphorus, copper, and chromium were measured, showing relatively low values of 0.629 mg/100 g, 0.217 mg/100 g, and 0.104 mg/100 g, respectively. Phosphates can interact with more hydrogen ions; they have crucial roles as buffers that limit changes in the bodily fluids' acidity. Copper is essential for the production of red blood cells and maintains the health of nerve cells. Chromium, which is a trace element, helps to manage diabetes and blood cholesterol levels.[4]

3.2. Bulk density, tapped density, Hausner ratio, and compressibility index

As shown in Table 1, the bulk density of PSPI was found to be 0.59 ± 0.06 g mL^{-1}. This value was lower than that of sesame protein isolate (0.84 g mL^{-1}), which was reported by Sharma *et al.*,[15] but higher than that of soy isolate (0.48 g mL^{-1}) previously reported by Okezie and Bello.[24] These findings highlight the unique physical properties of PSPI, providing useful insights for formulation and industrial applications. Additionally, the tapped density of PSPI was found to be 0.69 ± 0.03 g mL^{-1}.

The Hausner ratio and CI for the PSPI were found to be 1.16 ± 0.42 and 4.78 ± 0.49, respectively (Table 1). The Hausner ratio provides insight into powder flow, while the compressibility index quantifies the powder's propensity to undergo volume reduction under applied pressure, making them inversely correlated indicators of powder compressibility and flowability. A favorable (good) Hausner ratio in PSPI signifies superior flowability and reduced interparticle friction. A low compressibility index eases the transportation of food powders, prevents clump formation, and facilitates efficient mixing. The particles in a low-compressibility powder are less likely to stick together, forming agglomerates or clumps that can impede the flow. Also, manufacturers can enhance the efficiency of their processes and reduce issues related to powder handling and flow.[25]

3.3. Water absorption and oil absorption capacity (WAC and OAC)

The WAC and OAC for PSPI were found to be 1.14 ± 0.09 g g^{-1} and 1.20 ± 0.07 g g^{-1}, respectively (Table 1). The lower WAC and higher OAC values suggest the existence of more lipophilic spots in PSPI. The higher OAC can be attributed to the existence of numerous non-polar amino acids (valine, alanine, proline, leucine, tryptophan, methionine, isoleucine, glycine, and phenylalanine) that bind the fat hydrocarbon chains and result in absorption of more oil.[15]

3.4. Dispersibility

The dispersibility of PSPI was found to be dependent on pH, with measurements taken at pH levels of 9, 10, 11, and 12. The protein's ability to disperse in water reflects its reconstitution potential.[15] PSPI exhibited the highest dispersibility of 82% at pH 12, while the lowest dispersibility of 71% was recorded at pH 9 (Table 1). The increased dispersibility at higher pH levels can be attributed to the enhanced reconstitutability of the protein components. Timilsena *et al.*[26] reported similar findings for chia seed protein isolate, which demonstrated nearly complete solubility around pH 12 compared to lower pH levels.

3.5. Color

For PSPI, the L^*, a^*, and b^* values were found to be 73.52 ± 0.30, 0.37 ± 0.13, and 17.64 ± 0.19, respectively (Table 1). The yellowness of protein isolates is denoted by a positive b^* value.

3.6. Amino acid composition

PSPI was analyzed to determine the amino acid fractions as presented in Table 2. The protein isolates from pumpkin seeds

Table 2 Amino acid composition of pumpkin seed protein isolate (PSPI)

Essential amino acid	Concentration (%)	Non-essential amino acid	Concentration (%)
Leucine[a]	8.512 ± 0.12	Glycine[a]	5.912 ± 0.10
Phenylalanine[b]	7.217 ± 0.11	Cystine	5.431 ± 0.12
Arginine	3.182 ± 0.01	Alanine[a]	4.210 ± 0.03
Lysine	3.012 ± 0.08	Proline	4.126 ± 0.08
Histidine[b]	2.312 ± 0.03	Glutamic acid/glutamine	7.125 ± 0.14
Isoleucine	2.141 ± 0.06	Tyrosine[b]	3.012 ± 0.10
Methionine	7.217 ± 0.10	Aspartic acid/asparagine	8.317 ± 0.11
Valine[a]	4.021 ± 0.03	Serine	2.013 ± 0.04
Threonine	1.963 ± 0.07		
Tryptophan[b]	0.517 ± 0.01		

[a] Hydrophobic amino acid. [b] Aromatic amino acid. Values are expressed as mean ± SD.

showed a balanced amino acid composition. Among the essential amino acids, leucine (8.512%) and phenylalanine (7.217%) were found in high concentrations, respectively. Meanwhile, among non-essential amino acids, glycine (5.912%) and cystine (5.431%) were found in high concentrations. Protein antioxidant potential depends on concentration, structure, amino acid sequence, and hydrophobicity.[27] The existence of hydrophobic amino acids (glycine, valine, alanine, and leucine) in higher concentrations is responsible for PSPI's enhanced lipid solubility. Also, the protein stability is enhanced by the high concentration of hydrophobic amino acids, which compress the protein's inner core, mitigating structural disruptions and contributing to overall molecular integrity. It was also reported that aromatic amino acids, which include histidine, phenylalanine, tyrosine, and tryptophan, function as antioxidants by giving free radicals an electron and transforming them into stable molecules. The presence of histidine (2.312%) and arginine (3.182%) is justified by their capacity to donate hydrogen ions, influencing the protein's buffering capacity and potentially contributing to its functional properties.

Soy protein isolate is recognized as a complete protein, as it provides all essential amino acids in sufficient quantities. These essential amino acids include leucine, isoleucine, valine, lysine, methionine, phenylalanine, threonine, tryptophan, and histidine. Additionally, soy protein isolate contains non-essential amino acids such as alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, proline, serine, and tyrosine.[28] Similarly, pea protein isolate is also classified as a complete protein, containing essential amino acids, including leucine, isoleucine, valine, lysine, methionine, phenylalanine, threonine, tryptophan, and histidine. It also offers non-essential amino acids like alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, proline, serine, and tyrosine.[29] In contrast, rice protein isolate tends to be lower in lysine, one of the essential amino acids, when compared to soy and pea protein isolates. However, it still provides a range of essential amino acids, including leucine, isoleucine, methionine, phenylalanine, threonine, and histidine. Additionally, rice protein isolate contains non-essential amino acids such as alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, proline, and serine.[30]

Based on this information, it can be inferred that PSPI has a comparable amino acid profile to that of soy, pea, and rice protein isolates. Additionally, variability in the amino acid composition of pumpkin seeds may arise from differences in the altitude at which the plants are cultivated.[31]

3.7. Antioxidant activity of PSPI

The DPPH radical scavenging assay was employed to evaluate the free radical scavenging activity of PSPI, thereby assessing its antioxidant potential. This assay indicates that the compounds being studied can act as hydrogen atom or electron donors to neutralize free radicals. Proteins can be categorized as either preventive or chain-breaking antioxidants based on their mechanisms of action. Preventive antioxidants inhibit or slow down the initiation of free radical chain reactions, while chain-breaking antioxidants interrupt the propagation of these chains and scavenge free radicals. The antioxidant activity of PSPI, represented as %DPPH radical scavenging activity, was found to be 34.1%, with an *R*-squared value of 0.993, as illustrated in ESI Fig. S1.† Several studies have shown that hydrophobic amino acids serve as antioxidants by enhancing the solubility of protein isolates in non-polar environments, which facilitates their interaction with free radicals and diminishes their reactivity.[32]

3.8. SDS-PAGE analysis

The electrophoretic pattern of PSPI is illustrated in Fig. 1. The analysis revealed ten distinct bands with molecular weights of 5 kDa, 17 kDa, 28 kDa, 36 kDa, 55 kDa, 72 kDa, 95 kDa, 130 kDa, and 250 kDa. The results also indicated a notable presence of low molecular weight proteins or smaller protein subunits, which may have formed during the defatting process due to the disruption of non-covalent bonds in the seeds. β-Mercaptoethanol (β-ME) acts as a reductant that breaks disulfide linkages between protein molecules, leading to the dissociation of aggregates into subunits.[33] Upon the addition of β-ME, the subunits in the non-reduced electrophoretic mode at 55–72 kDa disappeared, while the bands around 28 kDa became more intense (Fig. 1), indicating the presence of disulfide bonds in PSPI. Cucurbitin, a common protein in PSPI, is a hexameric globular protein comprising a β-chain of 22 kDa and an α-chain of 33 kDa, linked by disulfide bonds. The high-molecular-weight proteins (greater than 250 kDa) were likely trapped within the insoluble fiber material at pH 10. Additionally, the 2S fraction of pumpkin seed albumin consists primarily of low molecular weight (≤10 kDa) soluble proteins that remain after precipitation.[6]

3.9. Particle size distribution and zeta potential

An important metric that reflects the PSPI's functional characteristics is particle size. The particle size analysis of PSPI is shown in ESI Fig. S2.† The results show a main peak at 10 nm in the particle size distribution for the native PSPI. Isoelectric precipitation can reduce the particle size of protein isolates by causing proteins to aggregate at their isoelectric point, promoting the formation of smaller, more finely dispersed particles and enhancing solubility and functional properties in various applications. Smaller particles can improve the capability of proteins to create stable emulsions, having better reconstitution properties and increased bioavailability. Furthermore, studies have demonstrated that the enhancement of the gel characteristics of proteins was made easier by the reduction of protein particle size.[34]

Native PSPI's zeta potential is shown in ESI Fig. S3.† The ζ-potential is an important parameter for determining the net charge on complex particles and is frequently used to evaluate solution system stability. The zeta potential of native PSPI was observed to be −11.47, indicating that electrostatic repulsion causes steady dispersion, avoiding agglomeration and promoting improved colloidal stability in a variety of applications. A higher absolute ζ-potential in a solution system leads to stronger molecule repulsion and reduced aggregation.[35]

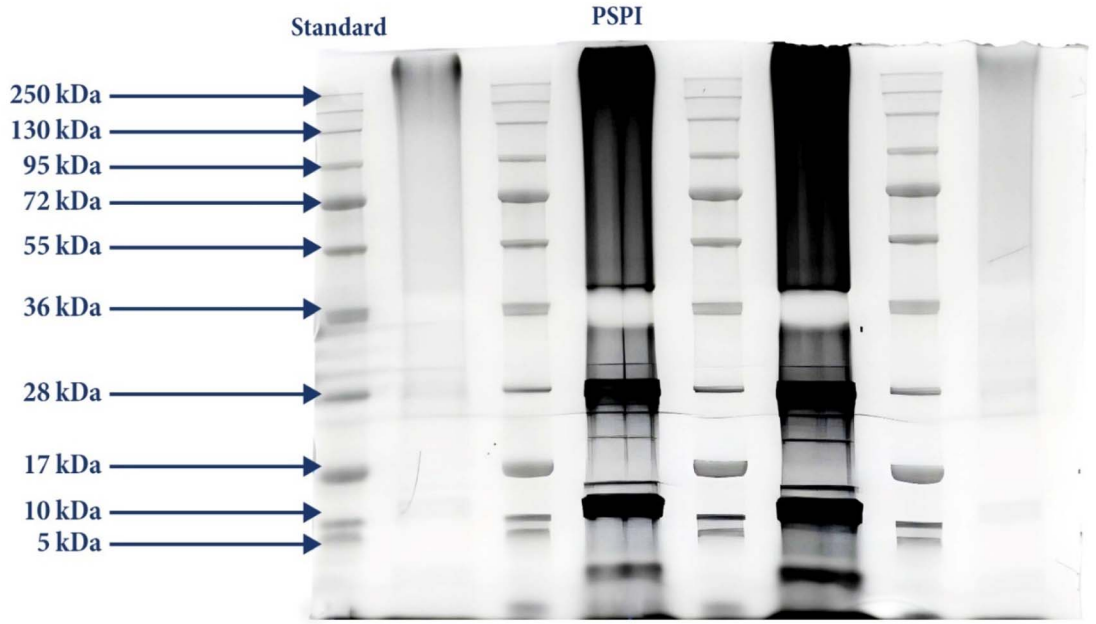

Fig. 1 SDS-PAGE profile of pumpkin seed protein isolate.

3.10. *In vitro* digestibility

The *in vitro* protein digestibility of PSPI was determined to be 84.57%, respectively, using the pepsin–pancreatin enzyme systems (Table 1). The result was higher than the value of 30.50% for uncooked soybean flour reported by Ali *et al.*[36] and 35% for conophor protein concentrate reported by Gbadamosi *et al.*[37] The decrease in non-protein components, specifically polysaccharides, and the increase in protein availability for enzymatic activities may be the reason for the pumpkin seed protein isolate's (PSPI) high digestibility. The higher digestibility of the protein isolate enhances efficient nutrient absorption and promotes optimal digestion.

3.11. Fourier transform infrared spectroscopy

A chemical analysis of the functional groups present in PSPI was conducted using FTIR spectroscopy. The peak values observed in the infrared radiation range were used to identify the functional groups of active components in the sample, as illustrated in the FT-IR spectrum (Fig. 2). The FTIR spectra of PSPI were analyzed within the frequency range of 4000–600 cm^{-1}. The FTIR spectrum of PSPI revealed characteristic peaks corresponding to the amide I, amide II, and amide III groups, as well as the β-sheet conformation of proteins. The fingerprint region of proteins is identified in the range of 1633 to 1452 cm^{-1}. A broad absorption peak from 3276 to 3011 cm^{-1} corresponds to N–H and alkyl group stretching.[38] The peak between 1600 cm^{-1} and 1700 cm^{-1} is attributed to N–H stretching vibrations and reflects the secondary structure of proteins.[39] The absorption peak at 1633 cm^{-1} is significant as it corresponds to the β-sheet conformation of proteins, and it is the most intense peak observed. This peak indicates the presence of a primary amide group (amide I, –CO–NH_2), which is characterized by the stretching of C=O and C–N bonds.[40] The results suggest that the peak at 1633 cm^{-1} is crucial as it highlights the β-sheet conformation, which contributes to the functional properties of the protein. Additionally, the peak at 1525 cm^{-1} is associated with the secondary amide group (amide II, –CO–NH), which involves N–H bending and is more complex than amide I. Peaks in the range of 1300 to 1200 cm^{-1} are indicative of amide III, arising from C–N stretching and N–H deformation. The peaks at 1390, 1231, and 1160 cm^{-1} correspond to the stretching vibrations of C–N groups found in aromatic and aliphatic amines. Finally, peaks between 1069 and 652 cm^{-1} are attributed to C–O and C–H stretching.[41] These spectra align with the findings reported by Quintana *et al.*[42]

3.12. Differential scanning calorimetry

DSC reveals the structural and conformational changes in proteins. The onset and peak temperatures of the PSPI were determined using DSC, as presented in Table 1. The onset temperature (T_o) was found to be 69.3 °C. The melting point (T_m) has been linked with the maximum point (peak temperature) in the 1st endothermic peak. The outcomes demonstrate that the melting point (T_P) of isolated protein was 75.6 °C and the end temperature (T_c) was 104.11 °C, respectively. The result differs from those of previous studies on cottonseed protein isolates (CPIs) as reported by Ma *et al.*[43] Sources reported CPI onset and peak temperatures of 83.47–87.81 °C and 94.31–97.72 °C, respectively. The low PSPI onset temperature indicates that structures of the protein are more stable during heating. The wide range of temperatures observed indicates its ability to withstand various temperature conditions, suggesting inherent heat stability. This broad temperature range reflects the protein isolate's resilience, making it suitable for diverse food processing and cooking applications.[44]

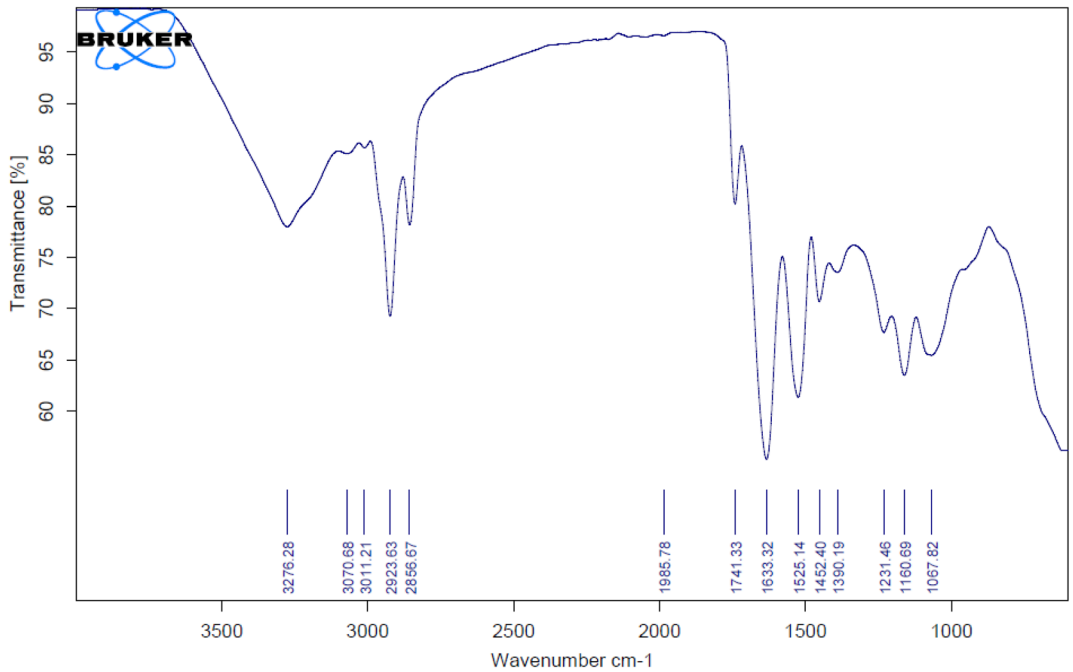

Fig. 2 FTIR spectra of pumpkin seed protein isolate.

3.13 Scanning electron microscopy (SEM)

Using a scanning electron microscope, the lyophilized PSPI microstructure was examined. The PSPI SEM micrographs at various magnifications are shown in Fig. 3. The micrographs obtained from SEM show clusters or aggregates of pumpkin seed protein, highlighting the tendency of proteins to form

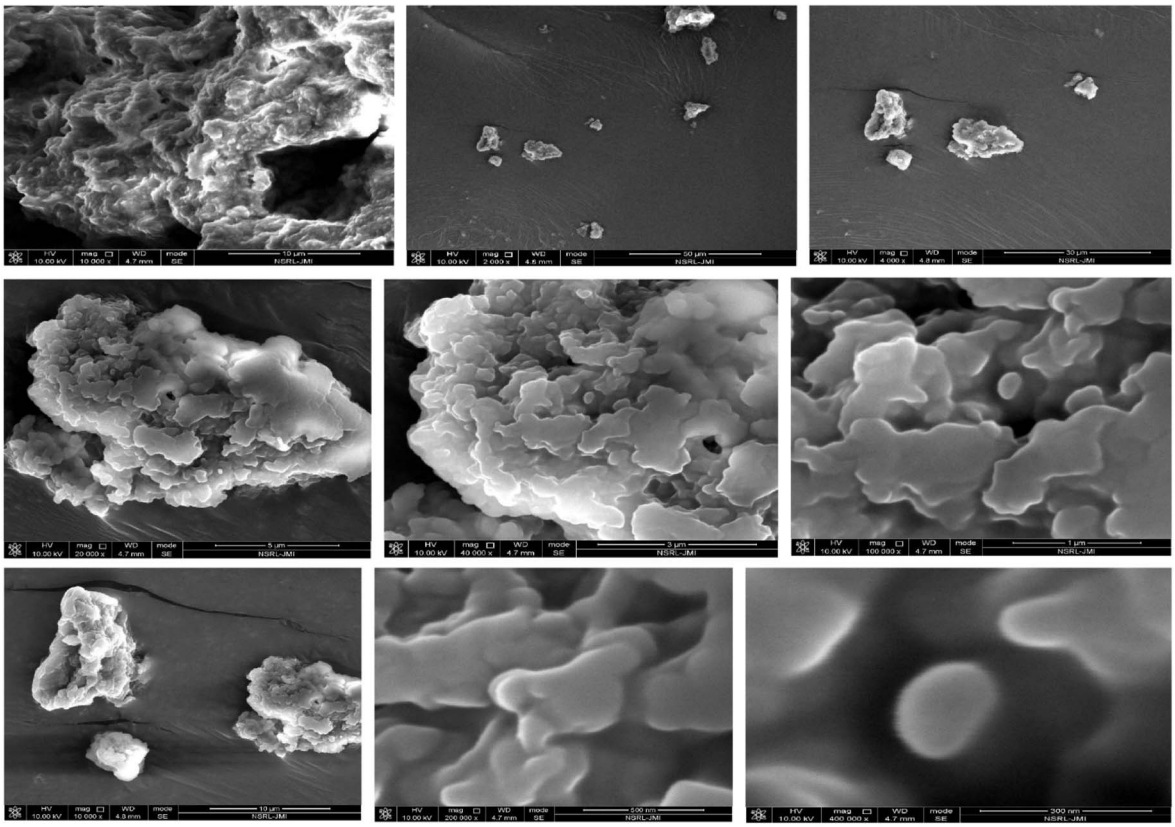

Fig. 3 Scanning electron micrographs of pumpkin seed protein isolate.

structures based on their inherent properties. The particles observed were compact with sharp edges, rough surfaces, and nonporous characteristics. SEM micrographs are important for assessing the quality and suitability of the protein for various applications such as food processing or pharmaceutical formulations, revealing information about proteins' functionality and their behaviour in different environments. Similar findings were observed by Hu *et al.*[45] for SPI (soy protein isolate) dispersion. Also, the diverse binding sites further underscore its potential for versatile interactions in various industrial applications.

4. Conclusion

This study concludes that pumpkin seed protein isolate (PSPI) is a highly prospective plant-based protein source with exceptional physicochemical, functional, and nutritional properties, as indicated by the results. Its potential as a high-quality protein alternative is demonstrated by its well-balanced amino acid profile and high protein content (85.78%). Its utility in a variety of culinary applications is further bolstered by its functional properties, which include a robust mineral composition (notably Fe, Mg, and K) and enhanced dispersibility at higher pH levels. The versatility and stability of the protein under a variety of processing conditions are underscored by the structural analysis, which reveals multiple protein bands across a wide molecular weight range. Its suitability for food processing applications is further enhanced by its thermal stability, which is characterized by onset and melting temperatures of 69.3 °C and 75.6 °C, respectively. This study's findings enhance our understanding of pumpkin seed protein characteristics and may open new opportunities for their innovative use in dietetics and health-focused food applications. Using pumpkin seed protein as a fortifying ingredient also boosts nutritional value, supplying essential amino acids, minerals, and antioxidants to create a more complete and healthful food product.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its ESI.† The corresponding author/first author can be contacted for data sharing.

Conflicts of interest

The authors declare no conflict of interest.

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