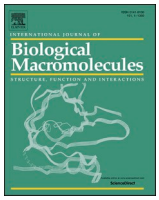
International Journal of Biological Macromolecules 283 (2024) 137421

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac



Enzymatic production and physicochemical and functional properties of sorghum protein isolates

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ARTICLE INFO

Keywords:
Sorghum protein isolation
Wet milling
Enzymatic processing
Protein functionality
Plant-based protein

ABSTRACT

Grain sorghum has emerged as a promising source for producing alternative proteins, yet current extraction methods lack efficiency. In this study, a novel enzymatic approach using α-amylase and cellulase on sorghum materials was developed to address this challenge. Comparisons were made among the proteins isolated from dry-milled sorghum flours, wet-milled sorghum gluten meals, and sorghum dried distiller's grains (DDG). Remarkably, proteins obtained from sorghum gluten meals demonstrated the highest protein purity (83–85 %) and recovery rate (92–93 %), followed by those from sorghum flour (purity 75–76 %) and DDG (purity 45–50 %). Physicochemical properties and functionalities of the isolated sorghum proteins were analyzed and compared with common commercial plant proteins (*e.g.*, soy protein isolate, pea protein isolate, and wheat gluten). Sorghum proteins exhibited higher levels of crude fat content, α-helix, and random coil structures, along with higher surface hydrophobicity and oil holding capacity (OHC) compared to the commercial plant protein isolates. Notably, proteins extracted from sorghum flours displayed slightly higher α-helix and random coil structures, total sulfhydryl content, water holding capacity (WHC), OHC, and protein digestibility compared to proteins isolated from sorghum gluten meals. Overall, this study demonstrates that enzymatic processing is feasible in producing sorghum proteins and provides insights into their basic properties and functionalities.

1. Introduction

With the increasing population and emphasis on personal nutrition [1], it is estimated that protein production for human food may need to double by 2050 [2]. Therefore, developing alternative proteins to those conventional animal proteins is crucial [3]. Currently, alternative proteins can be categorized into several types, including plant proteins, cell-cultured meat, algae proteins, and insect proteins. Plant proteins can be produced from cereals, legumes, and oilseeds [4].

Sorghum, ranked as the world's fifth-largest grain crop, offers unique functionalities and significant nutritional benefits. While predominantly utilized for animal feed in countries like the U.S. and Brazil, sorghum also serves as a staple food in regions such as Africa and Asia [5]. With the increasing prevalence of celiac disease, sorghum-based foods are emerging as a viable nutritional option for individuals with gluten intolerance. Protein, a key component of sorghum, holds immense potential as a novel plant-based protein, biomaterial for encapsulating and delivering bioactive compounds, as well as for creating edible films or emulsifiers to enhance stability in emulsions [6].

The protein content of sorghum varies from 6 % to 18 %, with an average of 11 % [7]. Kafirin (prolamin) and glutelin are the storage proteins in sorghum, accounting for approximately 90 % of the total protein. However, compared to other commonly used grain sources like soybean, pea, or wheat, the isolation of high-purity and high-yield sorghum proteins poses greater challenges. Sorghum protein extraction methods typically involve physical extraction (sonication), chemical extraction (isoelectric precipitation), and the wet milling method. Chemical extraction methods are commonly utilized, with various solvents developed for sorghum kafirin protein extraction, such as aqueous ethanol, glacial acetic acid, hydrochloric acid, or sodium hydroxide with

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https://doi.org/10.1016/j.ijbiomac.2024.137421
Received 21 June 2024; Received in revised form 25 October 2024; Accepted 6 November 2024
Available online 13 November 2024

the addition of reducing agents [8–12]. Despite these methods, protein yield has remained relatively low, ranging from only 32.0 % to 59.3 % [13], as these methods can only extract kafirin but leave the glutelin protein in the residue.

Wet milling, a common industrial process that separates components based on chemical composition like fiber, starch, and protein in cereal grains [14], results in a relatively moderate protein content in the gluten meal fraction, ranging from 44.3 % to 58.2 % [15,16], indicating the incomplete separation among components. Consequently, it is necessary to introduce chemicals or enzymes into the wet milling process to improve protein recovery.

Paraman et al. [17] used both chemical extraction method and enzymatic method to extract the proteins from rice flour. The alkaline and salt methods employed in their study resulted in proteins with protein content of 86.9 % and 87.3 %, recovering 65.9 % and 58.9 % of the total proteins, respectively. On the other side, the enzymatic method (utilizing α-amylases and cellulase) led to protein content of 85.8 % and 81.0 %, with recovery rates of 85.2 % and 86.2 %, respectively. The enzymatic approach led to a notable increase in protein recovery rate without sacrificing the purity. α-Amylases, the primary industrial enzymes, hydrolyze internal α-1,4-glycosidic linkages in starch, producing glucose, maltose and maltotriose [18]. Cellulase, another widely used industrial enzyme, degrade cellulose by hydrolyzing β-1,4-glycosidic bonds [19]. These two enzymes catalyze the hydrolysis of starch and fiber, enhancing the concentration of the protein component. In addition, combining enzymatic approach with wet milling end products could further improve protein purity and recovery.

In this study, various enzymes (α-amylases and cellulases), alone or in combination, were used to extract protein from dry milling, wet milling, and fermented sorghum materials. The objective of this research was to identify the most effective method for maximizing protein recovery from sorghum materials and characterize the physicochemical and functional properties of the isolated proteins. We also aimed to compare their properties with common commercial plant-based proteins, including soy protein isolate, pea protein isolate, and wheat gluten, and understand the influence of pretreatment and enzymatic extraction on protein characteristics.

2. Materials and methods

2.1. Materials

Red (Red NLMB) and white (White 4525) sorghum grains were provided by Nu Life Market (Scott City, KS, USA). Sorghum dried distillers' grains (DDG) were produced from solid fermentation of these two types of sorghums using commercial yeast strains (SafBrew™ DA-16) as described previously [20]. Commercial soybean protein isolates (SPI), pea protein isolates (PPI), and wheat gluten were used for comparison. Cellulase (Cellic Ctec2, 1000 BHU-2/g) was provided by Novozymes (Novozymes North America Inc., Franklinton, NC, USA). α-Amylase from *Bacillus sp.*, α-chymotrypsin from bovine pancreas (Type II, ≥40 units/mg protein), protease from *Streptomyces griseus* (Type XIV, ≥3.5 units/mg solids), and trypsin from porcine pancreas (Type IX-S, 13,000–20,000 BAEE units/mg protein) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were purchased from either Sigma-Aldrich or Fisher Scientific (Fairlawn, NJ, USA).

2.2. Pretreatments of sorghum grains

Sorghum grains were milled using a laboratory scale Ross roller mill (Model 915, Ross Machine and Mill Supply, Oklahoma City, OK, USA) to remove the bran, and fine sorghum flours were collected [21], namely fine red sorghum flour (RF) and fine white sorghum flour (WF). Additionally, sorghum DDG was ground into powder using a UYD Cyclone Sample Mill (UDY Corporation, Fort Collins, CO, USA), yielding red sorghum DDGs (RDG) and white sorghum DDGs (WDG).

Wet milling procedures were carried out following the method described by Wang & Chung [22] with some modifications. Three hundred grams of sorghum kernels were steeped in a solution containing 0.2 % (*w*/w) sulfur dioxide (3.26 g sodium bisulfite in 1000 mL distilled water is about 0.2 % SO_2) and 0.55 % (w/w) lactic acid at 50 °C for 36 h. Following steeping, the sorghum kernels were initially ground using a Waring-type blender (model CB-6, Dynamic Corp. of America, New Hartford, CT) with 2 volumes of distilled water at 12,000 *g* for 1 min. Then a Quaker city plate mill (Quaker City model 4E, Straub Co., Hatboro, PA, USA) was used for second grinding. A Ro-Tap shaker (model RX-29, W.S. Tyler Inc., Montor, OH, USA) with a standard testing sieve (U.S. No. 100, 150 μm) was then employed to remove the germ and fiber from the protein and starch slurry during a 5-min shaking process. The resulting slurry's gravity was adjusted to specific gravity (1.04) with distilled water before being pumped onto 5.08-cm × 2.44-m aluminum starch tables inclined at a slope of 1 in. at a flow rate of 50 mL/ min to separate the starch-rich and protein-rich fractions. The protein-rich fraction was further centrifuged for 10 min at 5000 *g*, with the top layer collected as sorghum gluten meal. Both red and white sorghum gluten meals (RGM and WGM) were produced, lyophilized, and grounded into fine powder. All the samples were stored at 4 °C until used. White sorghum gluten meal from pilot-scale wet-milling with 10 kg grain sorghum was also produced at the Center for Crops Utilization Research, Iowa State University (Ames, IA, USA), following the same procedures of Wang & Chung [22].

2.3. Protein isolation

Seven starting materials including sorghum flours (red & white), sorghum gluten meals from laboratory wet-milling procedures (red & white), white sorghum gluten meal from pilot scale wet-milling procedure, and sorghum DDGs (red & white) were used for protein isolation. The chemical isolation method was adapted from Paraman et al. [17] with some modifications. Two enzymatic extraction methods were employed: Method 1 solely used 2.5 % (*v/w*) α-amylase, while Method 2 employed a combination of 2.5 % (v/w) α-amylase and 2.5 % (v/w) cellulase.

In Method 1, the sorghum sample was mixed with 6 volumes of distilled water and the mixture was shaken in a water bath at 60 °C for 15 min. After the preheating process, 2.5 % (*v/w*) α-amylase was added into the slurry and the temperature was gradually raised to 70 °C then maintained for 2 h to hydrolyze the starch component. The slurry was then boiled to inactivate the enzyme and centrifuged (8000 *g*, 15 min) to collect the pellet, which was washed three times using distilled water and lyophilized.

Method 2 followed a continuous process derived from Method 1. Following the hydrolysis with α-amylase and centrifugation (8000 *g*, 15 min), the resulting pellet was re-dispersed in three volumes of distilled water and incubated with 2.5 % (v/w) cellulase at 50 °C for 1 h. Subsequently, the slurry was boiled, centrifuged, washed for three times, and lyophilized. All the isolated proteins were stored at 4 °C until further use.

A portion of each protein was defatted using hexane at a ratio of 1:4 (protein to hexane) for three times, and the resulted defatted samples were collected and stored in the refrigerator at 4 °C.

2.4. Proximate composition analysis

The moisture, ash, and crude fat contents of non-defatted proteins and commercial plant proteins were determined using AACC method 44–15.02 [23], 08–01.01 [24], 30–10.01 [25], respectively. The crude protein content of non-defatted proteins, defatted proteins, and commercial plant proteins was determined using a FP928 LECO nitrogen analyzer (LECO Corporation, Saint Joseph, MI, USA) with a conversion factor of 6.25. Total carbohydrate content was calculated by subtracting the sum of moisture, ash, crude fat, and crude protein from 100. The

equations below represented the calculations of yield and protein recovery rate:

$$\text{Yield\%} = \frac{\text{wt of protein extract (g)}}{\text{wt of original sample (g)}} \times 100 \quad (1)$$

$$\text{Protein recovery rate\%} = \frac{\text{wt of protein in protein extract (g)}}{\text{wt of protein in original sample (g)}} \times 100 \quad (2)$$

2.5. Scanning electron microscope (SEM)

The morphology of the protein powders was analyzed using a scanning electron microscope (SEM, S-3500N, Hitachi Scientific Instruments, Mountain view, CA, USA). The protein samples were mounted on specimen stubs with colloidal silver and sputter-coated with gold-palladium. Images were then captured at magnifications of 100× and 1000×, using an acceleration voltage of 5 kV.

2.6. FTIR Spectroscopy

Fourier transform infrared (FTIR) spectra was obtained using a PerkinElmer Spectrum 400 FT-IR/NIR Spectrometer (PerkinElmer, Inc., Waltham, MA, USA) equipped with an attenuated total reflectance cell (ATR) accessory. Each sample underwent 64 scans within the range of 400–4000 cm^{-1} with an interval of 4 cm^{-1}. The secondary structures of proteins were analyzed specifically within the 1600 cm^{-1} to 1700 cm^{-1} range (amide I region) [26]. The relative areas of the amide I region were determined using OriginPro 2016 software (OriginLab, Inc., Northampton, MA, USA).

2.7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The protein profiles were determined by SDS-PAGE based on the method of Hong et al. [27] with some modifications. 150 mg proteins were suspended in 10 mL PBS buffer (pH = 6.8) containing 2 % (*w/v*) SDS and shaken at 250 rpm for 2 h. The suspensions were then centrifuged for 5 min at 8000 *g* to collect the supernatant. Both reducing and non-reducing conditions were employed for this analysis. For reducing condition, 60 μL of each sample solution was mixed with 20 μL of 4 × Laemmli buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) containing 10 % (*v*/v) β-mercaptoethanol and boiled for 5 min. After cooling for another 5 min, the mixture (10 μL) was loaded into wells of a 4–20 % Mini-Protean TGX gel (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The Precision Plus Protein Dual Color Standards (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was also loaded into the well to show the scale of reference molecular weight bands. The samples and standard were separated at 200 V in Tris-MOPS-SDS running buffer for about 38 min and the gel was transferred to the diluted Brilliant Blue R Concentrate solution (Sigma, St. Louis, MO, USA) staining for 8 min with gentle shaking. The stained gel was first washed with distilled water overnight and then washed with destaining solution (10 % *v*/v acetic acid and 30 % v/v methanol) until the background was clear. For non-reducing conditions, all procedures remained the same except for without the addition of β-mercaptoethanol.

2.8. Amino acid composition analysis

The complete amino acid profiles were analyzed following the AOAC Official Method 982.30, and the amino acid contents were presented as mg/ g protein (dry basis).

2.9. Protein solubility

The solubility of proteins was assessed following a modified method described by Liu & Hsieh [28] to better understand the covalent and noncovalent interactions contributing to the insolubility of sorghum proteins. Protein extraction was carried out using various solutions: (1) Isoelectric focus buffer (IEF): 8 M Urea and 50 mM dithiothreitol (DTT) and 2 % (*w/v*) sodium dodecyl sulfate (SDS) + 2 M thiourea + 2 % (w/v) 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) in phosphate buffer (U+D+S+T+C+P); (2) IEF w/o Urea: 50 mM DTT and 2 % (w/v) SDS + 2 M thiourea + 2 % (w/v) CHAPS in phosphate buffer (D+S+T+C+P); (3) IEF w/o DTT: 8 M Urea and 2 % (w/v) SDS + 2 M thiourea + 2 % (w/v) CHAPS in phosphate buffer (U+S+T+C+P); (4) IEF w/o Urea and DTT: 2 % (w/v) SDS + 2 M thiourea + 2 % (w/v) CHAPS in phosphate buffer (S+T+C+P); (5) IEF w/o Thiourea, SDS, and CHAPS: 8 M Urea and 50 mM DTT in phosphate buffer (U+D+P); (6) IEF w/o DTT, thiourea, SDS, and CHAPS: 8 M Urea in phosphate buffer (U+P); (7) IEF w/o Urea, thiourea, SDS, and CHAPS: 50 mM DTT in phosphate buffer (D+P); (8) PB:100 mM phosphate buffer, pH 7.5 (P).

Each sample (200 mg) was added to 10 mL of the respective solutions mentioned above. The mixture was shaken (300 rpm) for 2 h at room temperature and then centrifuged at 8000 *g* for 15 min. Protein content in the supernatant was measured using the Bradford assay. Protein solubility was calculated by comparing the protein content in the supernatant to that in the samples, expressed as a proportion.

2.10. In vitro protein digestibility

The protein digestibility was determined as described by Hsu et al. [29] with slight modifications. A 30 mL sample solution (6.25 mg protein/ mL) was prepared, and the pH was adjusted to 8.00 (± 0.02) with 1 N HCl and/or NaOH. The suspension was then shaken in a 37 °C water bath, and the pH was checked every 10 min until it stabilized. Simultaneously, the multienzyme solution was prepared with 1.6 mg trypsin, 3.1 mg α-chymotrypsin and 1.3 mg protease per 1 mL of distilled water. The pH of the enzyme solution was also adjusted and maintained to 8.00 (± 0.02) with 1 N HCl and/or NaOH. Subsequently, 3 mL of the enzyme solution was mixed with the sample solution at 37 °C and shaken for 10 min. Following the reaction, the pH immediately decreased, and the pH drop was recorded. The digestibility calculation was conducted following the equation described by Tinus et al. [30]:

$$\text{Protein digestibility\%} = 65.66 + 18.10 \times \Delta pH_{10\ min} \quad (3)$$

where ΔpH_{10min} was the change of pH in 10 min from the initial pH 8.0.

2.11. Surface hydrophobicity

Surface hydrophobicity (H_0) was determined using sodium dodecyl sulfate (SDS) binding method according to Tang et al. [31]. A 10 mg protein sample was dispersed in 40 mL of 0.1 mmol/L SDS buffer and shaken for 1 h The suspension was then dialysis using a SnakeSkin™ dialysis tubing (35 mm dry I.D.) with MW cut-off of 3.5 kDa (Thermo scientific, Rockford, IL, USA) in distilled water for 48 h. After dialysis, the solution was collected, and the volume was recorded. Subsequently, 25 mL of chloroform and 5 mL of methylene blue (24 mg/L) were added to 10 mL of the dialyzed solution and shaken for 5 min. The mixture was then centrifuged for 15 min at 2500 *g*, and the lower layer solution was determined using a double beam spectrometer (VWR UV-6300PC, Radnor, PA, USA) at 655 nm. SDS solutions with different concentrations were prepared to establish a standard curve. The surface hydrophobicity was quantified by the amount of SDS bound to the protein.

2.12. Free and total sulfhydryl content

The free and total sulfhydryl contents were determined following the method of Beveridge et al. [32]. A 75 mg sample was dispersed in 10 mL of Tris-Gly-Urea buffer (containing 0.086 mol/ L Tris, 0.09 mol/ L glycine, 0.004 mol/ L EDTA, and 8 mol/ L urea) and shaken overnight.

For free sulfhydryl content determination, 1 mL of the sample solution above was mixed with 4 mL of Tris-Gly buffer (containing 0.086 mol/ L Tris, 0.09 mol/ L glycine, and 0.004 mol/ L EDTA) and 0.05 mL of Ellman's reagent (5,5′-dithio-bis-2-nitrobenzoic acid in Tris-Gly buffer, 4 mg/mL). The mixture was then shaken (300 rpm) in the dark for 15 min and centrifuged for 8 min at 8000 *g*. The supernatant was measured at 412 nm using a UV spectrometer. Meanwhile, the Tris-Gly buffer containing Ellan's reagent was used as reagent blank, and the sample solution containing only Tris-Gly buffer was used as sample blank. The final absorbance was calculated against reagent blank and sample blank.

For total sulfhydryl content determination, 1 mL of sample solution (in Tris-Gly-Urea buffer) was mixed with 4 mL of Tris-Gly buffer and 0.05 mL of β-mercaptoethanol. The mixture was shaken for 1 h and then added 10 mL of 12 % *w/v* trichloroacetic acid (TCA). After another hour of shaking, the mixture was centrifuged for 10 min at 8000 *g* at 4 °C to remove the supernatant. The precipitate was washed with 5 mL of 12 % w/v TCA twice to get the final solid. Then, 10 mL of Tris-Gly buffer was added to the precipitate and vortexed to obtain a suspension. Next, 0.04 mL of Ellan's reagent was added into 4 mL of the suspension above, shaken in the dark for 15 min, and centrifuged for 15 min at 8000 *g*. The final absorbance was analyzed and calculated similarly to the free sulfhydryl content determination process at 412 nm. The equation used for calculating was as follows:

$$\text{SH content } (\mu\text{M SH/g}) = \frac{73.53 \times A_{412} \times \text{D}}{C} \quad (4)$$

$$\text{Disulfide bonds content } (\mu\text{M/g}) = (\text{Total SH content} - \text{Free SH content})/2 \quad (5)$$

where A_{412} was the final absorbance at 412 nm; *D* was the dilution factor (5 was used in free sulfhydryl content determination and 10 was used in total sulfhydryl content determination); C was the sample concentration (mg/ mL); 73.53 was derived from $10^6/(1.36 \times 10^4)$ where 1.36×10^4 was the molar absorptivity of Ellman' reagent and 10^6 was for conversions from the molar basis to the μM/mL basis and from mg sample to g sample.

2.13. Water and oil holding capacity

The water and oil holding capacity of the samples was determined based on the method reported by Espinosa-Ramírez & Serna-Saldívar [9] with some modifications. For water holding capacity, a 0.25 g sample (W_0) was mixed with 7.5 mL of distilled water in a 15 mL centrifuge tube (W_2), and the resulting mixture was shaken at 300 rpm for 30 min. Subsequently, the mixture was centrifuged at 4500 *g* for 15 min to remove the supernatant. The test tube was then inverted for 5 min to drain the water residues, and the final weight was recorded (W_1). The water holding capacity was calculated as follow:

$$\text{WHC (g water/g protein)} = \frac{W_1 - W_2 - W_0}{W_0} \quad (6)$$

For oil holding capacity, the procedure was similar with method of water holding capacity. A 0.25 g sample (O_0) was mixed with 7.5 mL of soybean oil in a 15 mL centrifuge tube (O_2). The mixture was then shaken, centrifuged, and inverted to drain the oil and the final precipitate was weighted (O_1). The oil holding capacity was calculated as follow:

$$\text{OHC (g oil/g protein)} = \frac{O_1 - O_2 - O_0}{O_0} \quad (7)$$

2.14. Statistical analysis

All the experiments were conducted in duplicate at minimum, and the data were presented as mean ± standard deviation (SD). Data were analyzed using IBM SPSS Statistics software (version 27.0.1., Armonk, NY, USA). One-way analysis of variance (ANOVA) and means were compared using Tukey's test for multiple comparisons. Significant differences were considered when $P < 0.05$.

3. Results and discussion

3.1. Protein extraction and recovery

Table 1 provides the protein content of the starting materials and an overview of the treatments (*i.e.*, seven starting materials and two different enzymatic methods used for protein isolation). Notably, the protein content of sorghum gluten meals isolated through the wet milling process ranged from 36.50 % to 43.56 %, consistent with previous reports ranging between 39.02 % and 50.70 % [15,33]. Upon enzymatic treatments, the hydrolysis of starch granules and cellulose fibers facilitated the release of proteins, thereby concentrating the insoluble sorghum storage proteins (*i.e.*, kafirin and glutelin). The protein content, yield, and recovery rate of the proteins from various sorghum materials are detailed in Table 2. In this study, yield percentages represent the proportion of isolated samples weight relative to the initial material weight, while the protein recovery percentages indicate the proportion of the isolated protein weight relative to the initial protein weight.

Significant variations ($P > 0.05$) in the protein content of both non-defatted and defatted proteins were observed across different starting materials and enzyme treatments. Non-defatted proteins exhibited protein content ranging from 37.69 % to 78.98 %, while defatted proteins displayed increased protein content ranging from 40.10 % to 84.76 %. The proteins isolated from sorghum gluten meals had the highest protein content, followed by those isolated from sorghum flours and sorghum DDGs. This phenomenon was observed in both non-defatted and defatted proteins, irrespective of whether Method 1 or Method 2 was utilized. The highest protein content in non-defatted samples was noted in WGM-P (A + C), while the highest content in defatted samples was observed in the same extract. Amoura et al. [34] compared kafirin extracted from dry-milled sorghum flour and sorghum gluten meal to assess the influence of wet milling on protein extraction. They observed a higher kafirin protein content with dry milling (94.23 ± 1.43 %) in contrast to wet milling (90.07 ± 0.44 %). Notably, the protein content of the sample from sorghum flours increased significantly (approximately 20 %, $P < 0.05$) after the addition of cellulase in their isolation process. This observation suggests that the presence of fiber in the flours might significantly impact the protein isolation process, leading to increased challenges in isolating the protein in the final sample.

Protein yield varied from 9.10 % to 64.25 %. RDG (A) yielded the

Table 1
Details of enzymatic methods.

Starting materials	Protein content %	Method 1 (Add 2.5 % (*v/w*) α-amylase)	Method 2 (Add 2.5 % (v/w) α-amylase & 2.5 % (v/w) cellulase)
Fine red sorghum flour	7.28	RF (A)	RF (A + C)
Fine white sorghum flour	8.19	WF (A)	WF (A + C)
Red sorghum gluten meal	36.50	RGM (A)	RGM (A + C)
White sorghum gluten meal	43.56	WGM (A)	WGM (A + C)
White sorghum gluten meal from pilot scale plant	40.53	WGM-P (A)	WGM-P (A + C)
Red sorghum DDGs	28.42	RDG (A)	RDG (A + C)
White sorghum DDGs	26.66	WDG (A)	WDG (A + C)

Note: The protein content of samples is as-is basis.

Table 2
Protein content, yield, and recovery rate of isolated sorghum proteins.

Samples	Protein content of the extracted proteins (non-defatted) %	Protein content of the extracted proteins (defatted) %	Yield %	Protein recovery %
RF (A)	51.98 ± 0.54^{h}	54.28 ± 0.10^{j}	13.71 ± 0.09^{f}	97.91 ± 1.64^{ab}
WF (A)	56.22 ± 0.85^{g}	59.50 ± 0.28^{i}	13.21 ± 0.28^{f}	90.68 ± 0.57^{c}
RGM (A)	65.05 ± 2.33^{f}	68.52 ± 0.14^{h}	39.96 ± 0.38^{e}	71.21 ± 1.87^{e}
WGM (A)	68.27 ± 1.53^{e}	71.64 ± 0.35^{g}	45.13 ± 0.35^{d}	70.72 ± 2.14^{e}
WGM-P (A)	77.92 ± 0.33^{ab}	80.61 ± 0.14^{d}	51.37 ± 0.61^{c}	98.75 ± 1.60^{ab}
RDG (A)	37.69 ± 0.33^{k}	40.10 ± 0.11^{n}	64.25 ± 3.18^{a}	85.23 ± 4.98^{d}
WDG (A)	37.87 ± 0.15^{k}	41.03 ± 0.12^{m}	58.11 ± 1.87^{b}	82.53 ± 2.33^{d}
RF (A + C)	74.94 ± 0.08^{cd}	76.40 ± 0.48^{e}	9.10 ± 0.13^{g}	93.67 ± 1.46^{bc}
WF (A + C)	72.83 ± 0.94^{d}	74.82 ± 0.44^{f}	9.16 ± 0.31^{g}	81.47 ± 1.71^{d}
RGM (A + C)	72.84 ± 0.42^{d}	82.71 ± 0.25^{c}	46.45 ± 0.25^{d}	92.70 ± 1.04^{c}
WGM (A + C)	76.47 ± 0.02^{bc}	84.76 ± 0.37^{a}	52.37 ± 0.52^{c}	91.92 ± 0.94^{c}
WGM-P (A + C)	78.98 ± 1.99^{a}	83.49 ± 0.39^{b}	47.45 ± 1.04^{d}	92.43 ± 0.30^{c}
RDG (A + C)	42.40 ± 0.49^{j}	44.69 ± 0.34^{l}	56.47 ± 1.10^{b}	84.24 ± 0.44^{d}
WDG (A + C)	47.37 ± 0.35^{i}	49.71 ± 0.02^{k}	41.25 ± 1.91^{e}	73.27 ± 2.86^{e}

Note: Results are expressed as mean ± SD (n = 2). Different letters indicate significant differences in the same column ($P < 0.05$). The protein content of samples is as-is basis. A represents the samples are treated with α-amylase only, A + C represents the samples are treated with the combination of α-amylase and cellulase.

highest, while RF (A + C) yielded the lowest. These variations likely stem from differences in pretreatment methods. Protein recovery ranged from 70.72 % to 98.75 %, with WGM-P (A) exhibiting the highest recovery. RDG (A), despite its high yield (64.25 %), demonstrated a relatively lower protein recovery (85.23 %). This suggests that while Method 1 produced a high amount of protein from DDGs, the purity of the protein was compromised. However, kafirins extracted *via* chemical treatment from sorghum DDGS yielded protein contents of 98.94 % (using the acetic acid method) and 94.88 % (using the ethanol method) [12]; however, the protein recover rate was much lower in the previous studies, only around 24.2 % to 56.8 %.

Comparing the methods with and without the addition of cellulase, the addition of cellulase is highly beneficial to protein concentration. In most cases, the incorporation of cellulase led to an increase in protein content, indicating its potential in enhancing protein isolation efficiency. However, this improvement was not consistent across all samples, suggesting that the efficacy of cellulase is influenced by some specific characteristics of raw material, particularly the cellulose content. In the experiment conducted by Castro-Jácome et al. [35], the combined application of amyloglucosidase and ethanol for sorghum protein extraction yielded protein content of 74.2 %, accompanied by a lower recovery rate of 49.16 %. Remarkably, the enzyme significantly increased the protein purity from 68 % to 74 %, thereby improving overall extraction efficiency.

3.2. Proximate composition of sorghum proteins

The protein content, crude fat content, moisture content, and ash content of proteins from different sources are listed in Table 3. The protein contents of sorghum-based proteins were detailed in the

Table 3
Composition of isolated sorghum proteins.

Samples	Protein content %	Crude fat content %	Moisture content %	Ash content %
RF (A)	51.98 ± 0.54^{j}	8.38 ± 0.12^{efg}	3.74 ± 0.09^{c}	1.79 ± 0.02^{c}
WF (A)	56.22 ± 0.85^{i}	9.27 ± 0.63^{e}	0.57 ± 0.14^{g}	1.66 ± 0.05^{cd}
RGM (A)	65.05 ± 2.33^{h}	6.55 ± 0.28^{h}	0.11 ± 0.12^{g}	0.43 ± 0.00^{h}
WGM (A)	68.27 ± 1.53^{g}	6.69 ± 0.48^{h}	0.11 ± 0.04^{g}	0.59 ± 0.01^{h}
WGM-P (A)	77.92 ± 0.33^{cd}	7.61 ± 0.20^{g}	0.12 ± 0.04^{g}	0.52 ± 0.03^{h}
RDG (A)	37.69 ± 0.33^{m}	10.65 ± 0.05^{d}	0.19 ± 0.02^{g}	1.54 ± 0.03^{d}
WDG (A)	37.87 ± 0.15^{m}	12.18 ± 0.10^{c}	3.79 ± 0.58^{c}	1.79 ± 0.02^{c}
RF (A + C)	74.94 ± 0.08^{e}	7.72 ± 0.63^{g}	1.30 ± 0.14^{ef}	1.07 ± 0.02^{ef}
WF (A + C)	72.83 ± 0.94^{f}	8.09 ± 0.32^{fg}	1.81 ± 0.09^{de}	1.20 ± 0.01^{e}
RGM (A + C)	72.84 ± 0.42^{f}	15.22 ± 0.15^{a}	1.83 ± 0.11^{de}	0.50 ± 0.02^{h}
WGM (A + C)	76.47 ± 0.02^{de}	13.22 ± 0.46^{b}	2.00 ± 0.07^{d}	0.61 ± 0.01^{h}
WGM-P (A + C)	78.98 ± 1.99^{bc}	8.04 ± 0.06^{fg}	1.15 ± 0.11^{f}	0.59 ± 0.00^{h}
RDG (A + C)	42.40 ± 0.49^{l}	11.96 ± 0.88^{c}	1.39 ± 0.69^{ef}	0.99 ± 0.02^{fg}
WDG (A + C)	47.37 ± 0.35^{k}	8.89 ± 0.82^{ef}	2.27 ± 0.25^{d}	0.61 ± 0.01^{h}
SPI	86.52 ± 0.13^{a}	0.60 ± 0.07^{j}	5.64 ± 0.09^{b}	5.48 ± 0.36^{a}
PPI	80.89 ± 0.16^{bc}	1.54 ± 0.38^{i}	6.39 ± 0.12^{a}	3.95 ± 0.08^{b}
Gluten	78.47 ± 0.06^{cd}	0.44 ± 0.07^{j}	5.38 ± 0.11^{b}	0.85 ± 0.02^{g}

Note: Results are expressed as mean ± SD (n = 2). Different letters indicate significant differences in the same column ($P < 0.05$). The protein, fat, moisture, and ash content of samples are as-is basis. A represents the samples are treated with α-amylase only, A + C represents the samples are treated with the combination of α-amylase and cellulase.

preceding paragraphs, whereas commercial plant protein isolates exhibited protein content ranging from 78.47 % to 86.52 %. The crude fat content varied across different sorghum sources, ranging from 6.55 % to 15.22 %. RGM (A + C) exhibited the highest crude fat content, while RGM (A) showed the lowest. Espinosa-Ramírez & Serna-Saldívar [9] reported similarly high crude fat content in some extracted sorghum kafirins, ranging from 12.56 % to 20.25 %. In contrast, SPI, PPI, and gluten had significantly lower fat content ($P < 0.05$), averaging around 0.86 %. Notably, the moisture and ash contents of sorghum proteins ranged from 0.11 % to 3.79 % and 0.43 % to 1.79 %, respectively, all relatively lower than those in commercial plant proteins. This difference is attributed to the different drying techniques, as the sorghum proteins were lyophilized, while most commercial proteins are spray dried. Additionally, commercial samples may absorb moisture during packaging and long-term storage, leading to higher moisture content.

After defatting, four sorghum proteins isolated using the combination of α-amylase and cellulase extraction method were selected based on their high protein content. These proteins isolated from fine red sorghum flour (RF), fine white sorghum flour (WF), red sorghum gluten meal (RGM), and white sorghum gluten meal (WGM), were further characterized in comparison with three non-defatted commercial plant proteins (SPI, PPI, and Gluten).

3.3. SEM analysis

The microstructure of both sorghum proteins and commercial proteins is shown in Fig. 1. Under SEM, the isolated sorghum proteins

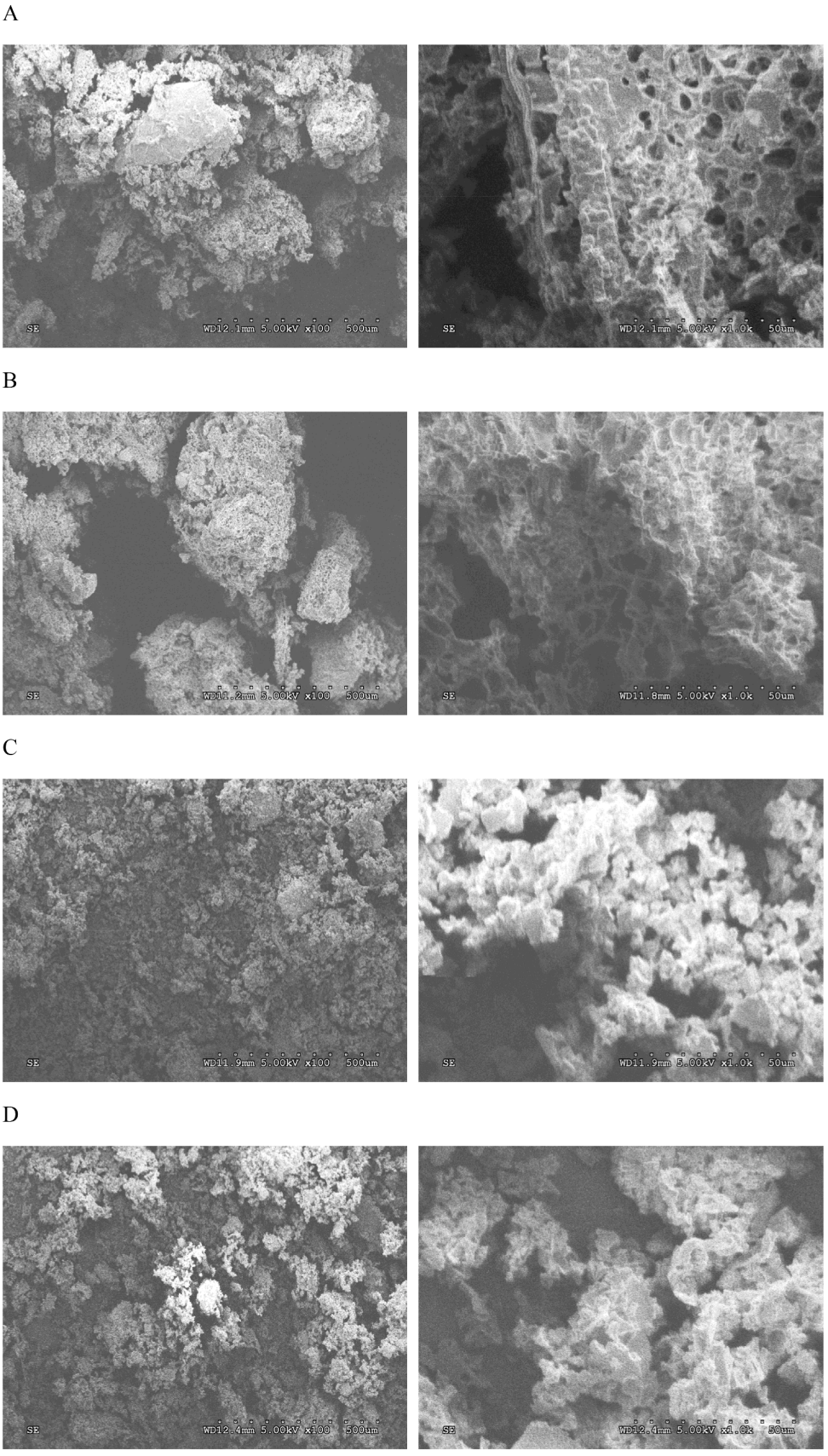
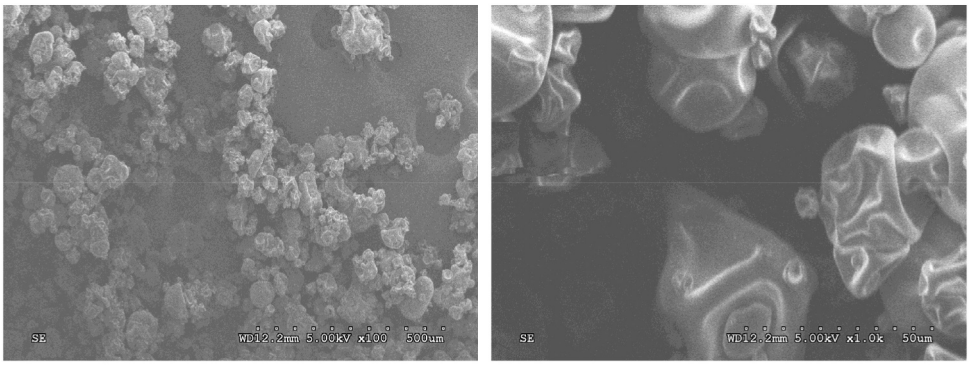


Fig. 1. SEM analysis of proteins
Note: (A) RF; (B) WF; (C) RGM; (D) WGM; (E) SPI; (F) PPI; (G) Gluten; (H) Kafirin. Samples RF (A + C), WF (A + C), RGM (A + C), and WGM (A + C) after defatting are renamed as RF, WF, RGM, and WGM, respectively. SPI means soy protein isolate and PPI means pea protein isolate. Kafirin is extracted using glacial acetic acid from white sorghum flour.

E



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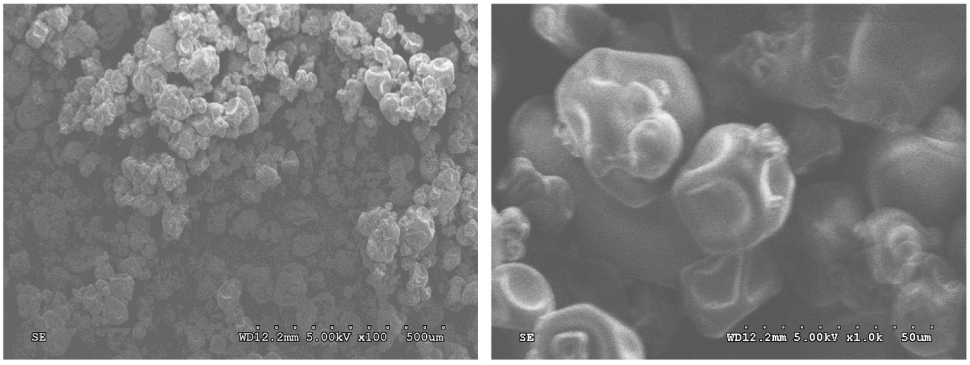

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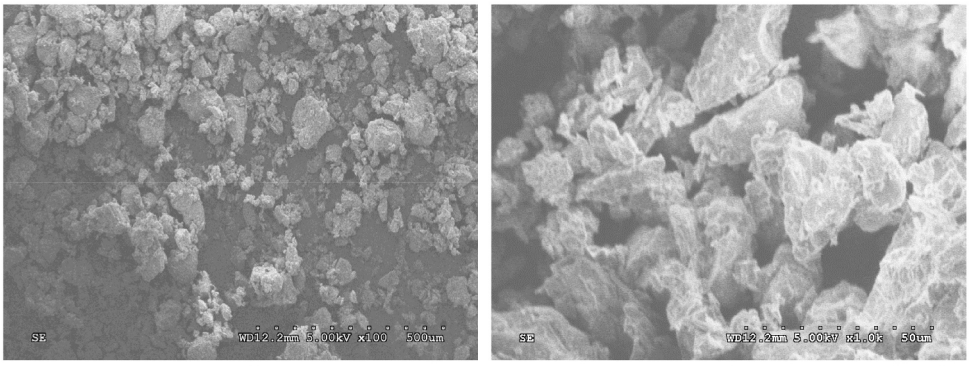

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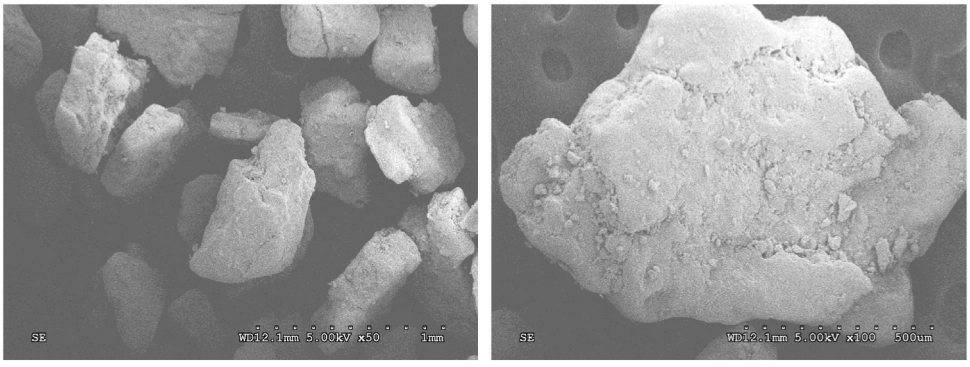

Fig. 1. (*continued*).

showed a similar microstructure, characterized by numerous holes in their protein matrix with spherical protein bodies attached. This suggests that starches were hydrolyzed by enzymes (amylase), resulting in formation of holes around the protein matrix. This structure resembles the sorghum proteins reported by Wulandari et al. [36], where amylase was used to degrade starch, leading to higher sorghum protein content. In contrast, Musigakun & Thongngam [37] observed exclusively protein bodies shapes in kafirins obtained using the ethanol extraction method, indicating a clear distinction in extracted protein types. In addition to kafirin, the current protein isolates also included non-prolamin proteins, typically acting as a coating matrix surrounding protein bodies [38]. Comparison of Fig. 1A, B with Fig. 1C, D revealed that sorghum proteins isolated from sorghum gluten meals exhibited more clumps, with the protein matrix appearing broken down into smaller particles. This could stem from the steeping step involved in the wet milling process, where sulfur dioxide cleaves disulfide bonds in proteins, reducing linkages between protein structures and forming smaller structures [39]. Additionally, Fig. 1H showed that kafirin from white sorghum, treated using a chemical method (acetic acid extraction method), displayed an uneven, blocky structure and was more separated and had larger diameters compared to enzymatically isolated proteins in this study. The acetic acid method utilized the principle of isoelectric precipitation to obtain the final kafirin, the denaturation and aggregation of kafirin potentially explaining the observed structure. This microstructure was also quite different with the structure reported in the study of Elkhalifa et al. [40], who used the ethanol method to extract kafirins, exhibited mainly spherical particles. Comparatively, the microstructures of SPI and PPI (Fig. 1E and F) exhibited similar spherical shapes with relatively smooth surfaces. In contrast, the wheat gluten in Fig. 1G displayed numerous aggregates, due to the intrinsic cohesivity and high processing temperature employed in industrial production.

3.4. FTIR

The Fourier Transform Infrared Spectroscopy (FTIR) analysis provided insights into the secondary structure distribution within the proteins. Within the infrared spectrum, secondary structure was reflected by peak distributions in the amide I band (1600–1700 cm^{-1}) [41]. The amide I band including α-helix (1630 cm^{-1}; 1648–1660 cm^{-1}; 1663–1666 cm^{-1}), β-sheet (1610–1640 cm^{-1};1670–1695 cm^{-1}), β-turns (1662–1673 cm^{-1}), and the random coil structure (1640–1650 cm^{-1}) [42]. The results, as shown in Table 4, revealed significant variability in secondary structure composition among the samples.

The α-helix content varied significantly among the samples ($P < 0.05$), with RF demonstrating the highest proportion at 31.75 %, followed by WGM at 21.63 %. Conversely, WF had the lowest α-helix content at 3.40 %. The β-sheet content ranged from 22.96 % in RF to 52.27 % in PPI, indicating notable differences in protein folding patterns among the different protein sources. The β-turn content ranged from 3.03 % in PPI to 8.59 % in WF, with significant variations observed across samples ($P > 0.05$). Random coil content, representing the least structured regions of proteins, ranged from 38.98 % to 53.01 %, with WF demonstrating the highest proportion of random coil among all samples.

Particularly, WF displayed a higher β-sheet proportion and a lower α-helix ratio compared to other sorghum-isolated proteins, possibly due to more pronounced structural folding during enzymatic treatments. The formation of intermolecular β-sheet structures during heating may promote the transition from α-helix to β-sheet, potentially leading to disulfide-linked polymer formation [38]. Comparatively, RGM and WGM exhibited similar secondary structures, with lower α-helix but relatively higher β-sheet and turns compared to proteins from sorghum flours. According to the FTIR findings from Amoura et al. [34], Elkhalifa et al. [40], and, Emmambux & Taylor [43], it is evident that the wet milling process led to an increase in β-sheet and β-turns. This observation aligns with Espinosa-Ramírez et al. [33]' s report of a predominant β-sheet structure in extracted kafirin. However, in the study by Espinosa-Ramírez & Serna-Saldívar [9], all the extracted kafirins had α: β ratios above 1, indicating a higher α-helix conformation in their protein secondary structure. This disparity may be attributed to differences in extraction methods and starting materials.

When comparing the sorghum protein isolates to commercial plant protein isolates, it is evident that sorghum proteins exhibit diverse secondary structure profiles. While sorghum proteins generally showed lower β-sheet content compared to SPI, PPI, and gluten, they exhibited comparable or higher α-helix and random coil content. These differences in secondary structure may contribute to variations in the protein structures and functional properties of sorghum proteins compared to traditional protein isolates.

Table 4
Secondary structure of proteins from FTIR.

Samples	Protein secondary structure ratio %			
	α-Helix	β-Sheet	β-Turn	Random coil
RF	31.75 ± 1.01^{a}	22.96 ± 0.62^{f}	3.98 ± 0.13^{cd}	41.49 ± 0.25^{de}
WF	3.40 ± 0.62^{d}	35.01 ± 1.40^{c}	8.59 ± 0.24^{a}	53.01 ± 1.78^{a}
RGM	16.73 ± 1.74^{c}	27.55 ± 0.23^{e}	4.44 ± 0.35^{c}	51.29 ± 1.16^{ab}
WGM	21.63 ± 3.81^{b}	26.43 ± 0.16^{e}	4.35 ± 0.47^{c}	47.59 ± 3.17^{bc}
SPI	15.13 ± 1.53^{c}	30.85 ± 0.15^{d}	6.78 ± 0.63^{b}	47.23 ± 0.75^{c}
PPI	5.72 ± 0.43^{d}	52.27 ± 1.24^{a}	3.03 ± 0.22^{d}	38.98 ± 0.58^{e}
Gluten	13.52 ± 1.32^{c}	39.17 ± 0.60^{b}	4.15 ± 0.89^{cd}	43.16 ± 1.61^{d}

Note: Results are expressed as mean ± SD (n = 2). Different letters indicate significant differences in the same column ($P < 0.05$). Samples RF (A + C), WF (A + C), RGM (A + C), and WGM (A + C) after defatting are renamed as RF, WF, RGM, and WGM, respectively. SPI means soy protein isolate and PPI means pea protein isolate.

3.5. SDS-PAGE

The SDS-PAGE profile provided clear molecular weight information for different proteins. Fig. 2A and B showed both non-reducing and reducing conditions of the proteins. The varying intensities of the bands indicated that the solubility of SPI and PPI was significantly higher than that of other sorghum proteins and wheat gluten. The protein profiles of RF and WF were quite similar, and also, RGM and WGM exhibited the same situation. The band observed between 37 and 75 kDa likely corresponds to the dimer (45 kDa) in sorghum proteins [44] and the faint bands of high molecular weight above 75 kDa are probably kafirin trimers and tetramers. Ioerger et al. [45] demonstrated that bands around 75 kDa may also correspond to the glutelin fraction. The limited presence or low intensity of bands representing the glutelin fraction may be attributed to its insoluble nature in the extraction buffer. The report of Emmambux & Taylor [43] and Wang et al. [12] also presented the band at 46 kDa in kafirin extract.

In the profiles of proteins isolated from sorghum flours, more bands were visible in the range of 37–75 kDa compared to proteins extracted from sorghum gluten meal in both reducing and non-reducing conditions, part of the dimers were broken by the sulfur dioxide in the wet milling process. Espinosa-Ramírez et al. [33] also found that there were smaller bonds were found in the kafirins extracted from sorghum gluten meal compared to that from sorghum flours, but the bonds had much smaller molecular weight than that in present study, which may because of the effect of different extraction methods. There was a higher percentage of α-kafirin bands in kafirin from sorghum gluten meal than sorghum flours, which is similar to the situation in present study. Espinosa-Ramírez & Serna-Saldívar [9] also reported a high content of α-kafirin bands in the extracted kafirins. A faint 45 kDa band can be seen in reducing condition. Notably, after adding reducing agent, the predominant bands in the profiles changed from dimers to monomers, which indicates that the majority of dimers were disulfide crosslinked, as also found by Emmambux & Taylor [43].

The profiles of PPI and SPI were initially faint under non-reducing conditions, but became clearer upon the addition of the reducing

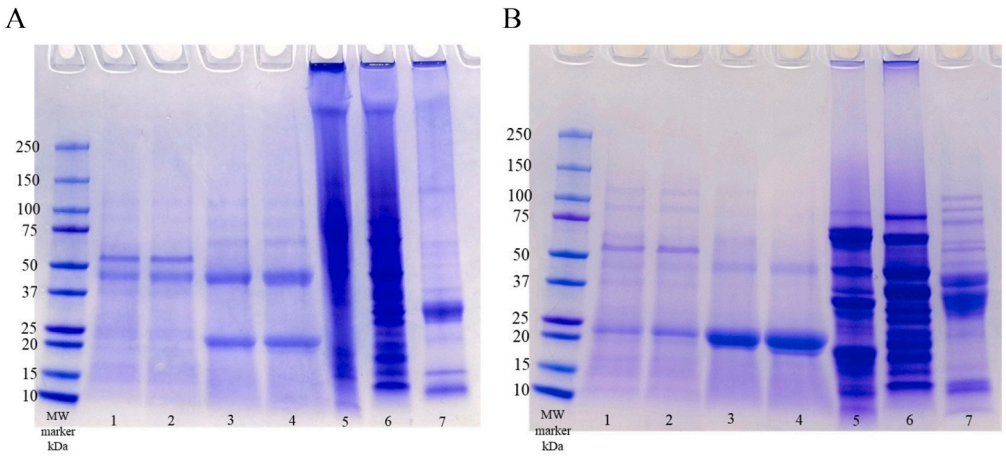

Fig. 2. SDS-PAGE profile of proteins: A. non-reducing condition; B. reducing condition.
Note: From left to right, the columns are standard; (1) RF; (2) WF; (3) RGM; (4) WGM; (5) SPI; (6) PPI; (7) Gluten. Samples RF (A + C), WF (A + C), RGM (A + C), and WGM (A + C) after defatting are renamed as RF, WF, RGM, and WGM, respectively. SPI means soy protein isolate and PPI means pea protein isolate.

agent. Under reducing conditions, the presence of three subunits of 7S globulin (α, α', and β) was observed in SPI profile, with molecular weights of 67 kDa, 71 kDa, and 50 kDa, respectively. Additionally, subunits of 11S globulin (20 kDa and 35 kDa) were identified [46]. The PPI profile also exhibited bands corresponding to convicillin, vicillin, α-subunits, and β-subunits of legumin, ranging from 70 to 100 kDa, 28 to 48 kDa, and 22 to 23 kDa, respectively [47]. Conversely, the gluten profile showed fewer bands under non-reducing conditions, possibly due to low protein solubility and the exclusion of higher molecular weight polymers from the gel. However, under reducing conditions, more bands were evident, including those indicative of high molecular weight glutenin (around 100 kDa) and the α-, β-, and γ-subunits of gliadin (30 to 50 kDa) [48].

3.6. Amino acid composition

Amino acid composition is a crucial determinant of protein quality and nutritional value, influencing various physiological processes and metabolic pathways. In this study, the amino acid profiles of different protein treatments were analyzed to assess their nutritional adequacy and potential applications in food and feed formulations, as detailed in Table 5.

Across the amino acid compositions, sorghum proteins derived from sorghum flours exhibited comparable levels of both essential and non-essential amino acids. WF exhibited slightly elevated levels of lysine, methionine, serine, and tyrosine in comparison to RF. Conversely, proteins from sorghum gluten meals displayed relatively diminished amino acid levels, particularly lysine, methionine, tryptophan, and glutamic acid, suggesting that the wet milling process led to different protein compositions, which may influence amino acid composition, resulting in

Table 5
Amino acid composition of proteins.

Amino acids (mg/ g protein)	RF	WF	RGM	WGM	SPI	PPI	Gluten	Kafirin
Essential amino acids								
Histidine	23.63 ± 0.03^{c}	22.86 ± 0.03^{d}	18.44 ± 0.02^{f}	17.49 ± 0.05^{g}	26.40 ± 0.04^{a}	25.61 ± 0.32^{b}	21.13 ± 0.02^{e}	15.16 ± 0.08^{h}
Isoleucine	43.67 ± 0.10^{ef}	43.33 ± 0.05^{f}	44.93 ± 0.16^{c}	44.35 ± 0.50^{d}	50.01 ± 0.15^{b}	51.35 ± 0.13^{a}	39.68 ± 0.12^{g}	44.01 ± 0.07^{de}
Leucine	135.96 ± 0.14^{d}	136.10 ± 0.43^{d}	149.68 ± 0.40^{b}	148.03 ± 0.05^{c}	79.47 ± 0.05^{f}	84.27 ± 0.46^{e}	69.52 ± 0.19^{g}	158.26 ± 0.34^{a}
Lysine	19.04 ± 0.04^{c}	17.90 ± 0.07^{d}	12.85 ± 0.09^{f}	12.70 ± 0.04^{f}	63.69 ± 0.09^{b}	76.04 ± 0.38^{a}	17.09 ± 0.00^{e}	7.39 ± 0.10^{g}
Methionine	17.55 ± 0.05^{c}	18.97 ± 0.15^{a}	16.41 ± 0.01^{d}	18.39 ± 0.10^{b}	12.89 ± 0.06^{f}	10.69 ± 0.06^{g}	16.28 ± 0.09^{d}	15.62 ± 0.07^{e}
Phenylalanine	54.64 ± 0.14^{c}	53.01 ± 0.06^{e}	58.52 ± 0.15^{a}	56.16 ± 0.30^{b}	53.98 ± 0.16^{d}	55.09 ± 0.35^{c}	50.65 ± 0.18^{f}	58.32 ± 0.13^{a}
Threonine	33.31 ± 0.36^{c}	33.66 ± 0.31^{c}	28.02 ± 0.09^{e}	28.64 ± 0.07^{d}	36.33 ± 0.11^{a}	35.32 ± 0.22^{b}	24.92 ± 0.04^{g}	26.01 ± 0.01^{f}
Tryptophan	9.74 ± 0.46^{bc}	10.68 ± 0.01^{b}	6.94 ± 0.60^{d}	8.58 ± 0.29^{c}	14.02 ± 1.18^{a}	10.01 ± 0.15^{b}	10.34 ± 0.66^{b}	9.31 ± 0.26^{bc}
Valine	51.91 ± 0.14^{c}	52.57 ± 0.02^{b}	50.12 ± 0.19^{e}	50.81 ± 0.32^{d}	51.15 ± 0.15^{d}	54.36 ± 0.53^{a}	41.82 ± 0.13^{g}	47.63 ± 0.02^{f}
Non-essential amino acids								
Alanine	89.07 ± 0.21^{d}	88.37 ± 0.20^{e}	97.08 ± 0.28^{b}	95.22 ± 0.21^{c}	41.72 ± 0.02^{g}	42.57 ± 0.21^{f}	25.17 ± 0.04^{h}	102.41 ± 0.05^{a}
Arginine	32.44 ± 0.36^{d}	32.85 ± 0.05^{d}	28.64 ± 0.15^{e}	28.92 ± 0.01^{e}	77.54 ± 0.11^{b}	87.71 ± 0.94^{a}	35.39 ± 0.10^{c}	21.23 ± 0.09^{f}
Aspartic Acid	64.32 ± 0.46^{d}	65.63 ± 0.54^{c}	60.89 ± 0.01^{f}	62.23 ± 0.21^{e}	114.55 ± 0.00^{b}	116.52 ± 0.19^{a}	32.11 ± 0.08^{h}	59.09 ± 0.12^{g}
Cysteine	18.86 ± 0.13^{c}	20.03 ± 0.06^{b}	14.04 ± 0.47^{e}	15.66 ± 0.19^{d}	12.03 ± 0.14^{f}	9.27 ± 0.02^{h}	22.71 ± 0.12^{a}	11.39 ± 0.19^{g}
Glutamic Acid	208.65 ± 0.31^{d}	207.32 ± 0.34^{d}	226.47 ± 0.47^{c}	226.03 ± 0.13^{c}	191.98 ± 0.44^{e}	174.03 ± 3.05^{f}	365.81 ± 0.22^{a}	239.98 ± 0.34^{b}
Glycine	29.77 ± 0.11^{d}	29.46 ± 0.05^{e}	22.89 ± 0.04^{f}	22.95 ± 0.09^{f}	40.70 ± 0.02^{a}	40.17 ± 0.08^{b}	32.99 ± 0.08^{c}	17.08 ± 0.06^{g}
Proline	82.93 ± 0.63^{c}	83.84 ± 0.19^{b}	80.73 ± 0.21^{e}	81.73 ± 0.15^{d}	51.37 ± 0.01^{f}	43.79 ± 0.47^{g}	119.55 ± 0.55^{a}	81.94 ± 0.29^{d}
Serine	40.19 ± 0.09^{b}	38.06 ± 0.13^{c}	37.43 ± 0.28^{c}	36.55 ± 0.43^{c}	45.24 ± 0.02^{a}	46.26 ± 1.81^{a}	41.38 ± 0.04^{b}	37.86 ± 0.19^{c}
Tyrosine	44.35 ± 0.16^{b}	45.34 ± 0.06^{b}	45.89 ± 0.40^{ab}	45.52 ± 1.32^{b}	36.90 ± 0.86^{c}	36.97 ± 0.81^{c}	33.43 ± 0.18^{d}	47.32 ± 0.01^{a}

Note: Results are expressed as mean ± SD (n = 2). Different letters indicate significant differences in the same column ($P < 0.05$). Samples RF (A + C), WF (A + C), RGM (A + C), and WGM (A + C) after defatting are renamed as RF, WF, RGM, and WGM, respectively. SPI means soy protein isolate and PPI means pea protein isolate. Kafirin is extracted using glacial acetic acid from white sorghum flour.

reduced levels of specific amino acids. Notably, lysine was identified as the limiting amino acid in sorghum, with a concentration of only around 15 mg/g of protein. Notably, the amino acid content of kafirin exhibited discrepancies between sorghum proteins isolated *via* enzymatic methods, featuring relatively higher leucine, alanine, glutamic acid, and tyrosine content. This variance could be attributed to the presence of glutelin alongside kafirin in proteins obtained from enzymatic methods, a phenomenon was also observed in Li et al. [49].

PPI and SPI demonstrated higher levels of essential amino acids among the samples, exhibiting notably elevated levels of lysine and tryptophan but lower levels of leucine and methionine. Wheat gluten displayed a similar essential amino acid profile to sorghum extracts. Notably, methionine emerged as the limiting amino acid in SPI and PPI, while for sorghum protein and gluten, it was lysine. Dietary combinations of legumes with cereals can lead to a more complementary protein intake [50]. Based on polarity, the amino acids can be divided into two types: hydrophobic amino acids (non-polar) and hydrophilic amino acids (polar). Hydrophobic amino acids include alanine (Ala), valine (Val), leucine (Leu), isoleucine (Ile), proline (Pro), phenylalanine (Phe), and cysteine (Cys) [51]. Sorghum-isolated proteins demonstrated a higher content of hydrophobic amino acids such as Ala, Leu, and Pro compared to SPI and PPI, a finding corroborated by Li et al. [49]. This characteristic can contribute to their functionalities of low solubility in water and low water holding capacity, as substantiated in subsequent tests.

3.7. Protein solubility

Solubility, a key parameter shaping the functionality and versatility of proteins across industries, was thoroughly investigated in this study to discern the primary bonds governing their water insolubility. Various buffers were employed to identify the specific interactions responsible for protein insolubility. The phosphate buffer (PB) was utilized to assess the solubility of native proteins, while reagents such as urea, DTT, thiourea, SDS, and CHAPS were employed individually or in combination to target specific bonds. The urea reagent is utilized to disrupt hydrogen bonding, while DTT is employed to cleave disulfide bonds in proteins. Additionally, the combination of thiourea, SDS, and CHAPS is employed to disrupt hydrophobic interactions. Given the uncertainty regarding the predominant bonding forces in sorghum proteins, eight different buffers were formulated, each containing a distinct combination of these reagents, as detailed in Table 6.

Table 6
Extraction solvents used in solubility test.

Extracting solution	100 mM phosphate buffer, pH 7.5	8 M Urea	50 mM DTT	2 M Thiourea	2 % SDS	2 % CHAPS
Isoelectric focus buffer (IEF)	√	√	√	√	√	√
IEF w/o urea	√		√	√	√	√
IEF w/o DTT	√	√		√	√	√
IEF w/o urea and DTT	√			√	√	√
IEF w/o thiourea, SDS, and CHAPS	√	√	√			
IEF w/o DTT, thiourea, SDS, and CHAPS	√	√				
IEF w/o urea, thiourea, SDS, and CHAPS	√		√			
PB	√					

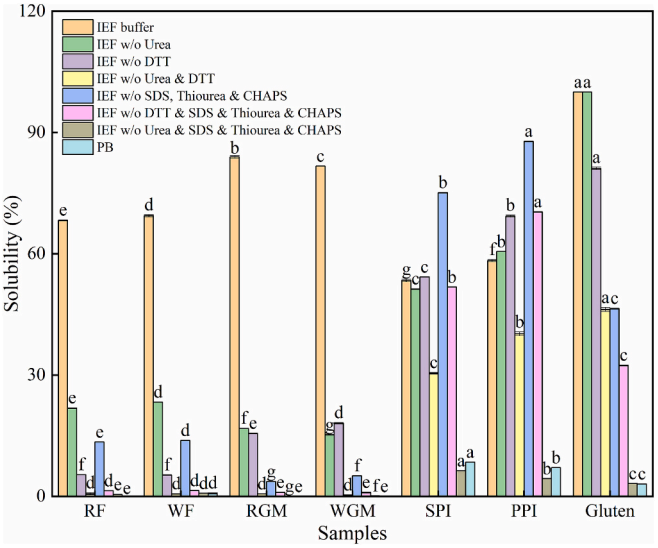

Fig. 3. Solubility results of proteins in different extraction solvents
Note: Samples RF (A + C), WF (A + C), RGM (A + C), and WGM (A + C) after defatting are renamed as RF, WF, RGM, and WGM, respectively. SPI means soy protein isolate and PPI means pea protein isolate.

The inclusion of all potential bond-breaking reagents in the isoelectric focusing (IEF) buffer theoretically allowed for the disruption of both covalent and non-covalent bonds. However, Fig. 3 revealed that, except for wheat gluten, the solubility of other proteins in the IEF buffer did not reach 100 %. This discrepancy may result in the multitude of reagents in the buffer, potentially impeding the availability of space and water molecules for protein dissolution. Sorghum proteins exhibited the highest solubility in the IEF buffer (buffer (1)), with solubility reaching 68.82 % for proteins from sorghum flours and 82.81 % for proteins from sorghum gluten meals. Buffer (2) facilitated approximately 20 % solubility, the second highest among all buffers, suggesting that the disruption of disulfide bonds and hydrophobic interactions contributed to sorghum sample dissolution. Interestingly, solubility in buffer (3) was higher for RGM and WGM compared to RF and WF, while RF and WF demonstrated higher solubility in buffer (5). This discrepancy implies that proteins from sorghum gluten meals are characterized by more hydrophobic interactions and fewer disulfide bonds than those from sorghum flours, aligning with our findings discussed in the previous section. The minimal solubility of kafirin in its native state reported by Espinosa-Ramírez & Serna-Saldívar [9] is consistent with our findings in PB (buffer (8)). Commercial proteins SPI and PPI exhibited maximum solubility in buffers (5) and (6), indicating that disulfide bonds and hydrogen bonding predominantly influenced their solubility. Similarly, gluten showed higher solubility in buffers (1) and (2), suggesting that both covalent and non-covalent bonds influence solubility, with disulfide bonds exerting a more significant influence.

3.8. In vitro protein digestibility

The *in vitro* digestibility of proteins is a critical parameter influencing their nutritional value and bioavailability. In this study, the *in vitro* protein digestibility simulated the intestinal process using the mixture of three enzymes and the digestibility was calculated by the pH change during the hydrolysis. Table 7 illustrates that PPI exhibited the highest *in vitro* digestibility, followed closely by SPI, with digestibility of 90.91 % and 88.47 %, respectively, and the result was quite similar with the results obtained from previous researches (89.08 % to 95.78 %; 81.3 % to 93 % for SPI) [52]. This suggests that PPI and SPI are readily accessible to digestive enzymes and can be efficiently broken down into smaller peptides and amino acids during simulated digestion,

Table 7
Water and oil holding capacity and protein *in vitro* digestibility.

Samples	Water holding capacity (g water/g protein)	Oil holding capacity (g oil/g protein)	*In vitro* protein digestibility %
RF	5.12 ± 0.10^{c}	5.72 ± 0.06^{b}	86.02 ± 0.38^{d}
WF	6.06 ± 0.07^{a}	6.69 ± 0.05^{a}	86.38 ± 0.13^{cd}
RGM	2.78 ± 0.03^{f}	3.47 ± 0.18^{d}	84.85 ± 0.00^{e}
WGM	3.05 ± 0.11^{e}	3.97 ± 0.28^{c}	85.30 ± 0.13^{e}
SPI	5.75 ± 0.10^{b}	1.49 ± 0.03^{e}	88.47 ± 0.26^{b}
PPI	4.80 ± 0.08^{d}	1.22 ± 0.03^{e}	90.91 ± 0.13^{a}
Gluten	1.31 ± 0.06^{g}	1.39 ± 0.04^{e}	86.57 ± 0.13^{c}

Note: Results are expressed as mean ± SD (n = 2). Different letters indicate significant differences in the same column ($P < 0.05$). Samples RF (A + C), WF (A + C), RGM (A + C), and WGM (A + C) after defatting are renamed as RF, WF, RGM, and WGM, respectively. SPI means soy protein isolate and PPI means pea protein isolate.

facilitating optimal nutrient absorption. The superior digestibility of PPI and SPI can also be attributed to their higher solubility in water, as demonstrated in the solubility test.

Among the sorghum-based samples, WF showed the highest *in vitro* digestibility, with a digestibility percentage of 86.38 %. RF, RGM, and WGM exhibited slightly lower *in vitro* digestibility compared to WF but still demonstrated respectable digestibility percentages ranging from 84.85 % to 86.02 %. The protein digestibility of extracted kafirins ranged from 86.54 % to 90.82 % [9], slightly higher than our findings. Moreover, proteins isolated from sorghum flours demonstrated relatively higher digestibility compared to those from sorghum gluten meal, possibly due to the wet milling process, which exposes the internal protein structure and potentially reveals antinutrients such as tannins [53]. Additionally, the high-temperature extraction process employed for sorghum proteins may have contributed to decreased protein digestibility, as reported by [43]. Espinosa-Ramírez et al. [33] reported higher protein digestibility for kafirin extracted from sorghum gluten meal compared to sorghum flours, indicating potential variation due to different extraction methods and sources.

Interestingly, proteins from red sorghums, both flour and gluten meal, exhibited lower digestibility compared to white sorghum. This observation may be attributed to higher levels of antinutrients, particularly tannins, in red sorghum, which can affect enzyme-protein interactions [6]. Under optimal conditions, sorghum tannins can bind and precipitate proteases and proteins significantly [54]. Wheat gluten, despite its high protein content and functionality in food processing, showed a moderate *in vitro* digestibility of 86.57 %. This suggests that gluten proteins may undergo slower digestion kinetics compared to other protein sources evaluated in this study.

3.9. Surface hydrophobicity

Surface hydrophobicity reflects the exposure of hydrophobic amino acid residues on the protein surface and can affect protein solubility and interfacial interactions [55]. In this study, we quantified surface hydrophobicity by measuring the amount of protein bound with SDS. As indicated in Table 8, WGM exhibited the highest surface hydrophobicity of 44.22 μg SDS/mg protein, followed by wheat gluten at 42.53 μg SDS/mg protein. Conversely, SPI and PPI showed the lowest surface hydrophobicity (31.62 to 32.30 μg SDS/mg protein), suggesting differences in protein surface properties among the various samples. This discrepancy could be derived from the higher proportion of hydrophobic amino acids in sorghum proteins and wheat gluten compared to SPI and PPI, as evident from the amino acid composition analysis (in Table 5). Particularly noteworthy is the higher surface hydrophobicity of proteins isolated from sorghum gluten meal compared to those from sorghum flours. This difference may be attributed to the disruption of disulfide bonds during extraction, leading to the exposure of additional hydrophobic regions on the protein surface.

Table 8
Surface hydrophobicity and sulfhydryl and disulfide bond content of proteins.

Samples	Surface hydrophobicity (μg SDS/mg protein)	Free sulfhydryl content (μmol/g protein)	Total sulfhydryl content (μmol/g protein)	Disulfide bond content (μmol/g protein)
RF	39.38 ± 0.70^{c}	0.096 ± 0.05^{d}	71.22 ± 0.00^{c}	35.56 ± 0.02^{c}
WF	36.66 ± 0.39^{d}	0.164 ± 0.05^{d}	81.71 ± 0.09^{b}	40.77 ± 0.07^{b}
RGM	41.44 ± 0.27^{b}	0.593 ± 0.08^{c}	39.29 ± 0.25^{f}	19.35 ± 0.08^{g}
WGM	44.22 ± 0.62^{a}	0.549 ± 0.12^{c}	46.79 ± 0.08^{e}	23.12 ± 0.10^{f}
SPI	31.62 ± 0.75^{e}	2.068 ± 0.04^{b}	71.51 ± 0.32^{c}	34.72 ± 0.14^{d}
PPI	32.30 ± 0.02^{e}	3.272 ± 0.00^{a}	56.72 ± 0.00^{d}	26.73 ± 0.00^{e}
Gluten	42.53 ± 0.78^{b}	0.187 ± 0.00^{d}	120.89 ± 0.09^{a}	60.35 ± 0.04^{a}

Note: Results are expressed as mean ± SD (n = 2). Different letters indicate significant differences in the same column ($P < 0.05$). Samples RF (A + C), WF (A + C), RGM (A + C), and WGM (A + C) after defatting are renamed as RF, WF, RGM, and WGM, respectively. SPI means soy protein isolate and PPI means pea protein isolate.

3.10. Free and total sulfhydryl content

Free sulfhydryl content, total sulfhydryl content, and disulfide bond content are important parameters that influence the functional properties and stability of proteins. The content of these three indicators is summarized in Table 8. Free sulfhydryl content represents the concentration of reactive thiol groups, which play a key role in protein functionality, including protein-protein interactions and protein stability. Notably, SPI and PPI exhibited the highest free sulfhydryl content at 3.072 and 2.068 μmol/g protein, respectively, indicating a greater abundance of reactive thiol groups compared to sorghum-based protein sources and wheat gluten. During the enzyme hydrolysis and deactivation process, the elevated temperature could contribute to the formation of disulfide bonds, decreasing the content of free sulfhydryl groups in sorghum proteins. The alterations of sulfhydryl groups were also reported on soy and whey, and milk proteins [56–58].

Total sulfhydryl content accounts for both free and bound sulfhydryl groups and provides insights into the overall thiol content of proteins. Gluten exhibited the highest total sulfhydryl content at 120.89 μmol/g protein, followed by SPI at 71.51 μmol/g protein. Sorghum-based protein sources showed lower total sulfhydryl content compared to SPI, PPI, and gluten, suggesting differences in thiol group concentration and protein structure among various protein sources.

Disulfide bond content reflects the presence of covalent bonds formed between cysteine residues and contributes to protein stability and structure. Gluten had the highest disulfide bond content at 60.35 μmol/g protein, followed by WF at 40.77 μmol/g protein. Sorghum-based protein sources exhibited lower disulfide bond content compared to wheat gluten, indicating differences in protein cross-linking and structure. Among the sorghum samples, proteins from sorghum gluten meal demonstrated the lowest total sulfhydryl group and disulfide bond content. This finding may be attributed to the wet milling process, which could damage disulfide bonds in proteins. Additionally, during the protein isolation process, some free sulfhydryl groups might have been lost.

3.11. Water and oil holding capacity

Table 7 displays the water and oil holding capacities of the proteins. The ability of interacting with water and oil can greatly affect the food texture and flavor during food processing [59]. Among the samples, WF

exhibited the highest WHC and OHC values at 6.06 g water/g protein and 6.69 g oil/g protein, respectively. This suggests that WF possesses superior water and oil-binding properties, which can contribute to better moisture retention and oil encapsulation in food formulations. RF also demonstrated notable WHC and OHC values, values were 5.12 g water/g protein and 5.72 g oil/g protein, respectively.

In contrast, sorghum proteins from sorghum gluten meals exhibited lower WHC and OHC values compared to that from sorghum flours. This outcome could be influenced by the presence of residual carbohydrates, which could impact the results due to incomplete hydrolysis in the samples. However, in the findings reported by Amoura et al. [34], the wet milling process led to enhanced WHC and OHC of the extracted sorghum protein, with significantly lower values obtained in both extracted sorghum proteins compared to the present study (WHC: 2.2 g water/g and 1.82 g water/g; OHC: 1.41 g oil/g and 1.34 g oil/g). Similarly, Espinosa-Ramírez et al. [33] also demonstrated higher WHC and OHC in kafirin extracted from sorghum gluten meal compared to sorghum flour, although these values were still lower than the results obtained in the present study, potentially attributed to differences in extraction methods.

Commercial plant proteins, such as SPI and PPI, displayed intermediate WHC and significantly lower OHC values compared to the proteins from sorghum flours. This may be attributed to differences in protein composition, structure, and surface properties among various protein sources. Different amino acid composition in proteins can also affect the protein functionalities [60]. Wheat gluten, while exhibiting high total sulfhydryl content and disulfide bond content, showed remarkably low WHC and OHC values, indicating limited water and oil-binding capabilities compared to sorghum-based proteins.

4. Conclusion

The utilization of both wet milling and enzymatic treatments proves beneficial for enhancing sorghum protein concentration. While amylase (2.5 % v/w) and cellulase (2.5 % v/w) individually contribute to protein isolation, their combined treatment with both enzymes demonstrates superior efficiency. This combined enzymatic approach results in sorghum proteins reaching a final protein content of 84.76 %, qualifying as an isolate, and increases protein recovery to approximately 92 % using white sorghum gluten meal.

In comparison to commercial proteins, sorghum proteins exhibit higher crude fat content, α-helix and random coil structures, greater surface hydrophobicity, OHC, and lower ash content. The physicochemical properties and functionalities of sorghum proteins from different treatments remain generally consistent. Notably, proteins isolated from sorghum flours exhibit slightly higher α-helix and random coil structures, greater total sulfhydryl content, WHC, OHC, and protein digestibility compared to proteins from sorghum gluten meals. This discrepancy is likely influenced by the wet milling procedure.

Overall, it is believed that the results from this research would be helpful for further research to produce sorghum protein concentrates/isolates and explore the diverse applications of sorghum proteins.

CRediT authorship contribution statement

Ruoshi Xiao: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Haiwei Lou:** Writing – review & editing, Investigation. **Ruijia Hu:** Writing – review & editing, Investigation. **Sang Li:** Writing – review & editing, Investigation. **Yi Zheng:** Writing – review & editing, Supervision, Resources, Investigation. **Donghai Wang:** Writing – review & editing, Supervision, Resources, Investigation. **Youjie Xu:** Writing – review & editing, Resources, Investigation. **Yixiang Xu:** Investigation, Resources, Writing – review & editing. **Yonghui Li:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Acknowledgements

This is contribution no. 24-268-J from the Kansas Agricultural Experimental Station. This work was in part supported by the Agriculture and Food Research Initiative Competitive Grant no. 2021-67021-34495 from the USDA National Institute of Food and Agriculture, and a seed grant from the Global Food Systems initiative of Kansas State University.

Data availability

Data will be made available on request.

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