Extraction, extrusion processing, and functional behavior of plant proteins

by

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Abstract

Due to environmental, health, social, and ethical concerns associated with animal-based protein production, there is a growing demand for alternative protein sources. Plant-based proteins offer advantages such as cost-effectiveness, environmental sustainability, and nutritional value. In addition to widely utilized proteins such as soy protein, pea protein, or wheat gluten, sorghum proteins are gaining interest due to their gluten-free nature and unique functional features. The goal of this study is to extract proteins from grain sorghums and investigate their physicochemical properties and functionalities, as well as exploring physicochemical and functional changes of plant proteins during extrusion texturization. The specific objectives were to: 1) investigate the impact of genotype and fermentation on the physicochemical and functional properties of sorghum kafirins; 2) develop an efficient method for sorghum protein concentrate extraction, optimize its extraction process and analyze their physicochemical and functional properties; 3) evaluate the effects of raw material formulation and extrusion processes on the properties and structural conformation of plant-based proteins (including soy protein, pea protein, and wheat gluten).

The extracted kafirins had protein content ranging from 75 to 85%. Protein molecular weights were consistent across different sorghum types but altered after fermentation, likely due to high-temperature fermentation conditions. Kafirin properties, including structure, color, and surface hydrophobicity, varied among sorghum types, with fermentation showing minimal effects. Kafirins from distiller's grains (DGs) demonstrated lower solubility but varied emulsifying properties, water holding capacity (WHC), and oil holding capacity (OHC). Black sorghum DG kafirin had slightly lower in vitro protein digestibility (around 74%) compared to other sorghum DGs (75-79%), possibly due to higher residue tannin content.

A novel process was developed for extracting sorghum protein concentrate with 85% protein content and approximately 95% protein recovery rate. Sorghum proteins extracted from flour exhibited higher α-helix and random coil structures, total sulfhydryl content, WHC, OHC, and in vitro digestibility compared to proteins from sorghum gluten meals, likely due to the different procedures. The sorghum protein concentrate also displayed higher crude fat content, α-helix and random coil structures, surface hydrophobicity, and OHC compared to commercial plant proteins (soy protein, pea protein, and wheat gluten), attributed to differences in amino acid compositions and protein structures.

Texturized proteins with varying cold-swelling protein (CS) proportions experienced decreased α-helix and increased β-sheet content post-extrusion, indicating significant conformational changes. Disulfide bonds were identified as primary forces forming fibrous structure integrity. After extrusion, free amino and sulfhydryl groups, along with surface hydrophobicity, experienced a notable decline. Protein digestibility, WHC, and OHC displayed fluctuating patterns with varying CS ratios, whereas emulsifying, foaming, and gel-formation properties deteriorated in texturized proteins.

In conclusion, different pretreatments and extraction methods influence sorghum protein properties and functionalities. Extrusion processes significantly alter protein structures and properties. Therefore, the processing method plays an important role in addition to the type of protein source in determining protein functionalities and practical applicability across various plant sources.

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Chapter 1 - Overview of sorghum and sorghum proteins

Abstract

Sorghum is the fifth largest cereal crop in the world and is famous for its excellent agronomic traits such as drought resistance and heat tolerance. It is also a valuable source of nutrients and bioactive compounds in the human diet. Sorghum is rich in protein and has attracted extensive research in recent years aimed at developing effective methods for extracting sorghum protein. Sorghum proteins have unique properties and functionalities. However, when it comes to practical applications, certain modifications are often required to make the protein fit for a specific purpose. In addition, sorghum protein has a wide range of applications in both food and non-food fields. This article aims to provide a basic understanding of sorghum protein and its diverse applications.

Keywords: sorghum, sorghum protein, extraction method, applications.

1.1 Introduction:

As the global population continues to grow significantly, the demand for daily food supply has risen substantially. The issue of food adequacy has attracted widespread attention from researchers in recent years. Protein, being a vital macronutrient, is particularly worthy of consideration (Sim et al., 2021). Traditionally, animal protein has been the primary source of protein supply, with people meeting their daily protein needs through the consumption of meats such as poultry, pigs, and cattle. However, the raising cycle of these livestock is longer and consumes more land and energy, making it a comparatively inefficient approach (Calicioglu et al., 2019). Consequently, there is a growing change towards exploring non-animal protein sources, including plant-based proteins, insect-based proteins, or cellular agriculture. These alternatives not only offer cost advantages but also have shorter growth cycles and similar nutritional content compared to animal meat (McClements, 2020).

Plant-based protein is emerging as a promising substitute for animal protein. Commonly researched plant-based proteins include wheat, soybeans, or corn. Sorghum, a relatively less utilized raw material compared to other cereals, also has good application prospects. Sorghum is the fifth most-produced cereal grain in the world after corn, rice, wheat, and barley, the global annual production in 2023 is 59.92 million tons, of which the United States accounts for the highest proportion of approximately 14% (USDA, 2023). It is a C4 tropical plant with biochemical and morphological characteristics, which can improve carbon assimilation at high temperatures and therefore has good heat and drought tolerance (Paterson, 2008). Because sorghum is able to thrive in extreme conditions, it is mainly grown in semi-arid tropical and subtropical regions (J. R. N. Taylor & Emmambux, 2010). The Sorghum genus was initially established in 1794 by Moench, who named it S. bicolor. In 1971, a simplified classification was

generated and divided sorghum into 15 races (Léder, 2004). Sorghum exhibits significant genetic variability, with approximately 235,000 sorghum germplasm resources having been collected worldwide, making its classification extremely difficult (Xiong et al., 2019). Based on sorghum genotype, phenolic components, and testa color, it is broadly categorized into five types: white sorghum, yellow sorghum, red sorghum, brown sorghum, and black sorghum (Xiong et al., 2019). Sorghum pericarp color is influenced by R and Y genes, resulting in red, yellow, and white pericarp colors, while pigmented testa presence is governed by B1 and B2 genes (Dykes et al., 2005). The simultaneous expression of B1 and B2 genes leads to the production of tannins in sorghum (Rao et al., 2018).

Traditionally, the application of sorghum is mainly divided into two parts. Firstly, it serves as direct human food consumption, particularly in Africa and Asia. Secondly, it is utilized as animal feed, primarily in the United States, Mexico, Argentina, Brazil, Australia, and other countries (J. R. N. Taylor & Emmambux, 2010). Apart from the grain, sorghum stems can also be repurposed as a fiber or fuel source (Y.-H. Wang et al., 2016). In this article, we have delved into the fundamental knowledge of sorghum protein structures, extraction methods, functional properties, and potential applications. By providing a comprehensive overview of these aspects, we aim to enhance understanding and pave the way for the optimal utilization of sorghum proteins in future food industries.

1.2 Sorghum structure

The structure of sorghum is very similar to that of corn, with both horny and floury endosperm, a large, fat-rich germ, and an absence of a true husk, but sorghum grains are generally oval in shape (Taylor, 2004). The sorghum grain can be categorized into three primary sections: the bran layer (comprising the pericarp and testa), the endosperm, and the germ. Some

sorghum varieties may have a colored testa between the pericarp and endosperm (Earp et al., 2004).

The pericarp serves as the outermost layer enveloping the internal structure. In certain sorghum varieties, the pericarp may contain phenolic substances, such as phenolic acids, flavonoids, and condensed tannins (Ogbonna, 2011), imparting a bitter taste to the entire sorghum grain. Varieties with this bitter taste are often referred to as "bird-proof" due to birds generally avoiding this flavor (Léder, 2004). Additionally, these substances function to protect sorghum from insects and fungi (Taylor, 2004). The endosperm is the largest tissue in sorghum, and is rich in starch and protein components, along with some vitamins and minerals. The germ contains substantial amounts of fat and protein, also harboring fat-soluble vitamins like B complex vitamins and minerals (Slavin, 2004).

1.3 Sorghum proximate composition

The nutritional composition of sorghum is comparable to that of other grains, encompassing proteins, fats, carbohydrates, non-starch polysaccharides, as well as bioactive elements such as B vitamins and fat-soluble vitamins (D, E, and K), micronutrients, macronutrients, and non-nutrients (Tanwar et al., 2023). These constituents contribute to various health benefits, including antioxidant, anti-inflammatory, and anti-cancer effects, as well as free radical scavenging and high antioxidant activity (Chhikara et al., 2018).

The largest proportion of carbohydrates in sorghum is starch. In addition, it also contains small amounts of soluble sugars, pentosans, cellulose, and hemicellulose (Léder, 2004). While sorghum starch shares high similarities with corn starch in amylopectin, the amylose of sorghum starch may exhibit more branched, resulting in higher gelatinization temperature than corn

starch. However, the higher tannin content in sorghum can inhibit its starch digestibility to some extent (J. R. N. Taylor & Emmambux, 2010).

The protein content and composition of sorghum vary based on factors such as genotype, water availability, temperature, soil fertility, and environmental conditions. Typically ranging from 6% to 18%, with an average of 11% (Lasztity, 2017). Sorghum protein primarily consists of prolamins and glutenins, lacking the essential amino acid lysine but containing elevated levels of sulfur-containing and hydrophobic amino acids (Léder, 2004). As sorghum lacks gluten components, it is a friendly alternative for those allergic to gluten, serving as another source of grains in their diet (Tanwar et al., 2023).

Sorghum grain lipids are mainly located in the germ area and have a unique fatty acid composition, including linoleic acid (18:2), oleic acid (18:1), palmitic acid (16:0), linolenic acid (18:3), and stearic acid (18:0) (Serrano et al., 2023). It is rich in linoleic acid (49%), oleic acid (31%), and palmitic acid (14%) (Léder, 2004). At the same time, the soluble fiber in sorghum also plays important roles in regulating blood sugar levels, lowering cholesterol, and promoting satiety (Khalid et al., 2022).

Furthermore, sorghum grains are abundant in B vitamins such as thiamine and riboflavin, carotenoids, tocopherols, essential minerals (phosphorus, magnesium, iron, copper), and trace minerals (zinc, calcium, potassium), all contributing significantly to human health (Tanwar et al., 2023). In addition, it is particularly noteworthy that sorghum is rich in unique antioxidant components, including polyphenols, tannins, flavonoids, stilbenes, phytosterols, and polyeicosanols (Przybylska-Balcerek et al., 2019).

1.4 Sorghum protein

As a valuable cereal crop, the proteins in sorghum are critical. Based on the principle of Osborne, proteins in sorghum can be categorized into albumins (water-soluble), globulins (salt-soluble), kafirins (prolamins, aqueous alcohol-soluble), crosslinked kafirins (aqueous alcohol + reducing agent-soluble), and glutelins (detergent + reducing agent + alkaline pH-soluble) (Jambunathan et al., 1975). Prolamins in sorghum, also known as karfirin, make up a significant portion of sorghum proteins, making up approximately 70% of total storage protein and have high levels of proline and glutamine (B. R. Hamaker et al., 1995). Therefore, the proteins in sorghum can be also divided into only two types: prolamin and non-prolamin (B. Hamaker & Bugusu, 2003).

1.4.1 Kafirin classification and structure

Kafirins can generally be divided into four classes: α-, β-, γ- or δ-kafirins, and their classification is based on the different molecular weights, amino acid composition, and solubility of proteins (Belton et al., 2006). Sorghum endosperm contains about 66%-84% α-kafirins, 8%-13% β-kafirins, 9% to 12% γ-kafirins, and very low level of δ-kafirins (Lasztity, 2017).

The α-kafirins are the most abundant class, the molecular weight of them are about 22-26 kDa. And they can be divided into two groups of polypeptides of 23 kDa and 25 kDa (Belton et al., 2006). These proteins are rich in nonpolar amino acids, mainly in the form of monomers and oligomers. These proteins are not extensively cross-linked and primarily form intramolecular disulfide bonds (Labuschagne, 2018).

The β-kafirins have the molecular weight of about 18 kDa, which have the great abundance of sulfur-containing amino acids (methionine and cysteine) (de Mesa-Stonestreet et

al., 2010). But β-kafirin is a minor component of the protein body and is absent in some sorghum lines (Duodu et al., 2003).

The γ-kafirins have the molecular weight of about 20 kDa, and have the great abundance of proline, cysteine, and histidine. Both β- and γ-kafirins can form intermolecular and intramolecular disulfide bonds and are highly cross-linked (de Mesa-Stonestreet et al., 2010). The γ-kafirins are very unique in sorghum proteins, when they act as reduced subunits, they are soluble in water, but when in native state they are insoluble, which may because they stay in stable polymers (Belton et al., 2006).

The δ-kafirins have the molecular weight of about 13 kDa and are rich in methionine. However, δ-kafirins can not be identified when detected by SDS-PAGE at the protein level (Belton et al., 2006), which just make up 1% of total storage protein (Watterson et al., 1993).

Since the interest of prolamins in sorghum has recently increased now, several methods have been investigated for extracting kafirins from different sorghum source like sorghum flour or bran for food and other uses (J. R. N. Taylor et al., 2006).

1.4.2 Non-kafirin proteins

Albumin and globulin, crucial components involved in metabolic processes, are predominantly present in the germ of the sorghum kernel (J. R. N. Taylor & Schüssler, 1986). These proteins exhibit an average protein content ranging from approximately 10% to 30%. Their molecular weights, as revealed by SDS-PAGE, span from 14 kDa to 67 kDa (J. R. N. Taylor & Schüssler, 1986). Notably, the amino acid profile of albumin and globulin differs from prolamin, showing higher lysine content (Bean et al., 2011).

In sorghum, glutelin serves as a structural element within the matrix, positioned both peripherally and internally in the endosperm (Bean et al., 2011). This protein plays a vital role in

connecting protein bodies to starch granules, making it the second most important component of the endosperm. Reports indicate that glutelin constitutes 25% to 50% of the total protein content, with molecular weights ranging from 20 to 67 kDa according to SDS-PAGE (Van Der Walt et al., 1984).

1.5 Sorghum protein extraction methods

The study of the chemistry, structures and functionality of sorghum proteins could lead to new food uses. Therefore, to achieve this purpose, more and more methods are developed for sorghum proteins extraction in recent years, such as extraction using wet-milling methods (de Mesa-Stonestreet et al., 2010) or extraction using chemical solvents (Espinosa-Ramírez & Serna-Saldívar, 2016a).

1.5.1 Wet milling method

Wet milling is a traditional method to separate the components of grain, such as germ, bran, fiber, starch, and protein. Nowadays, there are not many sorghum separations based on wet-milling process. Rooney and Serna-Saldivar describe a commercial wet-milling process for grain sorghum (de Mesa-Stonestreet et al., 2010).

First, sorghum grains undergo a soaking process in water to toughen the bran and soften the endosperm, making it easier to separate the components. To aid in this separation, chemical reagents or enzymes, such as sulfur dioxide, sodium metabisulfite, sodium bisulfite, or sodium bisulfite, can be introduced into the soaking water at an effective concentration ranging from 0.05% to 0.30% (R. A. Buffo et al., 1997; Xie & Seib, 2002; Serna-Saldívar & Mezo-Villanueva, 2003). These chemicals serve to dissolve the protein matrix that envelops the starch granules within the endosperm. Additionally, lactic acid (at concentrations of 0.40% to 1.4% [w/w]) may be incorporated to enhance protein solubilization. Enzymes such as fiber-degrading enzymes,

cell wall-degrading enzymes, and proteases can also be employed for this purpose (Mohen o-Pérez et al., 1999; F. C. Wang et al., 2000; Mezo-Villanueva & Serna-Saldívar, 2004; Chew-Guevara et al., 2016). Furthermore, sodium hypochlorite and KOH can also be added to bleach the sorghum kernels, facilitating the extraction of the white starch component (Pérez Sira & Lares Amaiz, 2004).

Optimal conditions for the soaking step can be achieved by adjusting the temperature and holding time, typically ranging from 48 to 55 °C for 24 to 48 hours. In laboratory wet milling process, the standard ratio of grain to soaking water is 1:2, while in commercial wet milling, it is 1:5 (R. A. Buffo et al., 1997; F. C. Wang et al., 2000; Xie & Seib, 2002; Serna-Saldívar & Mezo-Villanueva, 2003). The predominant starting material for wet milling experiments is whole grain sorghum. However, it's noteworthy that most wet milling experiments on sorghum kernels have predominantly concentrated on starch recovery, with minimal attention given to protein recovery.

1.5.2 Sequential extraction

The traditional technique of plant protein fractionation, based on Osborne's work, involves using different solvents (Van Der Walt et al., 1984). These solvents divided the proteins into four parts separately including albumin, globulin, prolamin, and glutelin. This sequential extraction process can be employed to isolate protein fractions from sorghum, enabling the analysis of various protein components to understand their distribution and unique properties.

Haikerwal & Mathieson (1971) explored different extraction procedures for sorghum kernel proteins in their study. The method utilized "classical" solvent extraction based on Osborne solubility, involving multiple steps to extract protein fractions, yielded 8% albumin, 20% globulin, 30% prolamin, and 12% soluble glutelin in sorghum flour, with approximately

30% of proteins remaining challenging to extract. Another extraction technique was used for 41 low-tannin sorghum types, involving 1.25 M NaCl to separate albumin, globulin, and low molecular weight nitrogen, 60% (v/v) tert-butanol plus 0.05% (w/v) DTT to extract prolamin, and the resulting portions were identified as glutelin. Amylase was added to purify the glutelin by removing starch from the proteins. On average, all sorghum types showed a 23.0 ± 2.7% salt-soluble protein fraction, 48.0 ± 5.0% prolamin, and 24.7 ± 4.3% glutelin, achieving an average protein recovery rate of approximately 96% (Van Der Walt et al., 1984).

In another study by J. R. N. Taylor & Schüssler (1986), sequential extraction was employed to isolate protein fractions from three components (endosperm, germ, and pericarp) of two low-tannin sorghum grains. The majority of proteins were found in the endosperm, mainly in the form of prolamin and glutelin. In the germ, proteins consisted mainly of albumin, globulin, and some low molecular weight nitrogen. Extracting proteins from the pericarp under the same conditions proved challenging, suggesting an association of these proteins with cell walls.

1.5.3 Ethanol extraction

To effectively extract kafirin from sorghum, aqueous ethanol at elevated temperature or aqueous tert-butanol at ambient temperature is consistently utilized, applying the solubility preference of prolamin. Emmambux and Taylor (2003) outlined a method for sorghum protein extraction, particularly kafirin, using ethanol. In this approach, sorghum flours were mixed with 70% ethanol, 0.35% NaOH (w/v), and 0.5% sodium metabisulfite (w/v) at 70 ℃ for 1 hour. Subsequently, acid was employed to adjust the pH of the supernatant to 5.0, precipitating the proteins. While kafirin can also be extracted from sorghum bran using this method, the efficiency is comparatively lower than that from whole sorghum flours, resulting in a protein recovery rate

of 15.9–26.7%. This discrepancy may be attributed to the lower prolamin content in sorghum bran (da Silva & Taylor, 2004).

Y. Wang et al. (2009) modified this method slightly for extracting proteins from sorghum dried distiller's grains (DDGS). They diluted the supernatant to 40% using distilled water before adding acid to obtain the protein sediment. The protein purity reached 94.88%, with an extraction rate of 56.8%. Another method employed by Y. Wang et al. (2009) involved using 70% ethanol under acidic conditions (adjusting pH to 2.0 using 20% (v/v) HCl) and adding sodium sulfite as a reducing agent to extract sorghum proteins from DDGS. The protein content obtained from this method was 42.32%, with an extraction rate of 24.2%. The efficiency of this method is relatively lower, indicating that the alkaline condition is effective in breaking down disulfide bonds and aiding in the exposure of kafirins from the protein matrix.

1.5.4 Acetic acid extraction

Due to its relatively low dielectric constant, glacial acetic acid possesses the ability to dissolve hydrophobic proteins, making it an effective solvent for extracting sorghum proteins. J. Taylor et al. (2005a) conducted experiments using various extractants, including ethanol, isopropanol, and glacial acetic acid, to extract sorghum proteins and assess their efficiency under different conditions. The study presented the yield, kafirin recovery rate, and protein purity of extracted proteins using different solvents and conditions. The conclusion highlighted that presoaking sorghum flours for 16 hours with 0.5% (w/w) sodium metabisulfite at 25°C, followed by stirring in glacial acetic acid for 1 hour and adjusting the pH to 5 with NaOH, resulted in a final protein yield of 59.3%, kafirin recovery rates ranging from 81.2% to 87.2%, and a protein purity of 92.9% (after defatting). Wang et al. (2009) made modifications to this method and successfully extracted sorghum proteins from sorghum DDGS. The protein content was

determined using the nitrogen combustion method with a nitrogen conversion factor of 6.25. The extracted proteins exhibited remarkably high protein purity (98.94%) and yield (44.1%). The soaking step played a crucial role in this method, reducing the disulfide bonds of the sorghum matrix protein, facilitating the release of kafirin for a more effective reaction with acetic acid (J. Taylor & Taylor, 2018).

1.5.5 Detergent extraction

To facilitate the protein extraction process, a certain degree of protein structure destruction is required. Therefore, amphiphilic molecules such as detergents, including sodium dodecyl sulfate (SDS) or urea, can function as effective reagents for protein extraction. Park & Bean (2003) utilized SDS along with a reducing agent to extract sorghum proteins from sorghum flours. They optimized the extraction conditions for increased efficiency, employing a 12.5 mM sodium borate buffer at pH 10.0, containing 1% SDS and 2% β-mercaptoethanol to solubilize the total protein. Furthermore, they used 60% tert-butyl alcohol or 70% ethanol to precipitate non-kafirin components from the total protein extract. Their findings demonstrated that the choice between tert-butyl alcohol and ethanol did not significantly impact the final protein content, but ethanol was considered a safer option for food applications. Physical treatments can also be incorporated to facilitate the extraction process. Zhao et al. (2008) employed a similar method with the addition of sonication to extract sorghum proteins from both mashed and non-mashed sorghum flours. Sonication was found to extract more polymeric proteins from the samples, as it is believed to possess the capability to break down covalent bonds during energy application, resulting in lower molecular weight proteins. Another study was conducted by Li et al. (2018) used a urea-cysteine based system, employing 8M urea and 8 wt% cysteine in an alkaline condition to successfully extract proteins from sorghum DDGS. Urea was employed to enhance

protein solubility, while simultaneously, cysteine and the alkaline condition were utilized to disrupt disulfide bonds and hydrophobic interactions within the sorghum proteins.

1.5.6 Enzymatic extraction

The enzyme amyloglucosidase is employed to hydrolyze starch in sorghum flours, a process referred to as liquefaction in some studies on corn or rice. This enzyme acts on the α-1,4 glycosidic bonds found in amylose and amylopectin, effectively eliminating the starch. This transformation leads to a flour with a higher protein content and enhances the yield of sorghum proteins during the extraction process (Paraman et al., 2006). In a method developed by Castro-Jácome et al. (2020), amyloglucosidase was applied to hydrolyze starch in sorghum flour. Subsequently, 70% ethyl alcohol (containing 0.35% w/v sodium metabisulfite) was used to extract the kafirin from the residues gotten above. The optimized pretreatment conditions included employing 0.086% amyloglucosidase per substrate for 83 minutes, resulting in 4.12% ± 0.02% of kafirin (based on total protein) with a purity of 74.2% ± 0.47%. The α-amylase can also be employed to assist in starch hydrolysis in sorghum extraction process to degrade the bonds of starch and protein matrix. Wulandari et al. (2023) isolated proteins from sorghum flours using only amylase, achieving the highest protein content (58.39%) with an enzyme addition of about 1.35% and a hydrolysis time of 2.7 hours. The article's equation predicts that by increasing amylase to 2.63% and reducing the time to 2 hours, the protein content could potentially reach 90%.

1.6 Sorghum protein functional properties

1.6.1 Water and oil holding capacity

The water holding capacity (WHC) and oil holding capacity (OHC) values indicate the amount of water or oil that protein samples can retain. These values serve as crucial indicators

for assessing the potential applications of proteins based on specific purposes, such as evaluating syneresis in yogurt or enhancing juiciness in plant-based meats (Ma et al., 2022). Additionally, during food preparation, the interaction of protein with water or oil can deeply affect the food texture and flavor (Zayas, 1997). Amoura et al. (2020) reported water holding capacity (WHC) and oil holding capacity (OHC) of sorghum proteins varied between 1.82 to 2.20 g water /g protein and 1.34 to 1.41 g oil /g protein, respectively. Their findings revealed that the wet milling process enhanced the ability of kafirins to bind both water and oil. In a study by Espinosa-Ramírez et al. (2017), extracted sorghum proteins exhibited WHC ranging from 1.85 to 2.82 g water /g protein. Additionally, they observed that the wet milling process facilitated water absorption, while the addition of protease did not significantly impact WHC. The trends in OHC were similar, with kafirins ranging from 1.57 to 2.56 g oil /g protein. Espinosa-Ramírez & Serna-Saldívar (2016) investigated the WHC and OHC of kafirin extracts, reporting values in the range of 1.99 to 2.96 g water/g protein and 2.04 to 2.76 g oil/g protein, respectively. They observed no significant differences in WHC and OHC when kafirins were extracted from whole or decorticated sorghums. However, variations in WHC and OHC were noted among different sorghum genotypes. High-tannin sorghum kafirin extracts exhibited the highest water and oil retention, while white waxy sorghum kafirin extracts displayed the lowest values.

1.6.2 Solubility

Solubility refers to the quantity of protein in a sample that dissolves in a solution under specific conditions, such as varying pH levels. The diverse solubility of proteins serves different purposes, for instance, food additives may exhibit partial solubility, complete solubility, or be entirely insoluble. Protein solubility stands as a crucial functional attribute significantly influencing the outcomes of various functional tests, providing valuable insights into their

potential applications. Factors such as protein amino acid composition, sequence, molecular weight, and conformation play pivotal roles in determining solubility values. Conversely, environmental factors, including ionic strength, solvent type, pH, temperature, and processing conditions, can also exert an influence on solubility (Zayas, 1997). Dias de Carvalho et al. (2024) reported that sorghum protein isolates exhibited the lowest solubility at pH 4.0 due to reduced electrostatic repulsion, leading to protein aggregation. And then the solubility increased at pH 9.0 and 2.0. Sorghum protein solubility ranged from 14.5 to 64.77% at pH 7, with extracts at pH 12.0 showing superior solubility, indicating the impact of extraction conditions on protein functionality. Espinosa-Ramírez & Serna-Saldívar (2016) reported that the solubility of the kafirin extracts was ranged from 0.51 to 1.00g dry matter/ 100 g initial dry matter at as-is pH. The low values indicated the hydrophobic property of kafirins. And most of the kafirins extracted from whole sorghum flours exhibited higher solubility than that from decorticated counterparts.

1.6.3 Emulsifying properties

Emulsions are defined as a dispersion of two or more immiscible liquids (like water and oil) in which one of the liquids is dispersed in the other as small droplets (0.1–100 μm) (Lam & Nickerson, 2013). Two common types of emulsions found in the food industry are water in oil (W/O) emulsions and oil in water (O/W) emulsions. A wide variety of emulsified food products are available in the market, such as beverages, milks, creams, dips, sauces, deserts, dressings, mayonnaise, margarine, and butter (Tan & McClements, 2021). Proteins are always employed as functional ingredients for the formation and stabilization of emulsion because of their amphiphilicity (Dapčević-Hadnađev et al., 2019). The emulsifying capacity (EC) and emulsifying stability (ES) are commonly used to assess the protein emulsifying properties. The

EC represents the ability of proteins to absorb both water and oil to form emulsion and the ES indicates the stability of a newly formed emulsion during a certain time (Amagliani et al., 2017). The EC values of sorghum proteins extracted from different pH conditions as reported by Dias de Carvalho et al. (2024), showed not significant differences. However, the ES value was higher for proteins extracted under pH 12 conditions compared to other extracts. According to Amoura et al. (2020), the extraction method (wet milling or dry milling methods) of kafirin seemed having a slight effect on EC (around 20 m2/ g) and ES (around 60%). Espinosa-Ramírez et al. (2017) observed that the kafirins extracted from sorghum flours the EC values were low (< 2 m2/ g) at pH 3 and 5 but increased at higher pHs, reaching a maximum at pH 9. However, the EC values of kafirin extracted from wet milling process had values < 2 m2/ g at all evaluated pHs. Besides, the protease-treated kafirin extracts all had a higher EC values compared to the regular extracts. For kafirin extracts from the study of Espinosa-Ramírez & Serna-Saldívar (2016), their EC ranged from 1.08 to 1.73 m2/ g. Among genotypes, the waxy and the red sorghum extracts presented the highest and lowest EC, respectively.

1.6.4 Foaming properties

Foam is a highly complex system consisting of gas (air cells), liquid (water), solids, and surfactant (an active agent on the surface, usually protein), exhibiting high viscosity, a large surface area, high surface energy, and low density (Kumar et al., 2022). The role of the surfactant is to decrease the surface tension between gas and water, facilitating the formation of smaller gas bubbles. Several methods can be employed to create foam, including gas injection into a liquid field, agitation (such as stirring, whipping, or beating), and supersaturation (dissolving gas into liquid under pressure) (Huppertz, 2010). Two key indicators used to assess protein foaming properties are foaming capacity (FC) and foaming stability (FS). FC represents

the amount of interfacial area that a protein can generate per unit weight or concentration and is influenced by polymer flexibility, charge density, and hydrophobicity. FS refers to the protein's ability to stabilize the foam (Amagliani et al., 2017). Amoura et al. (2020) reported that the extracted sorghum kafirins formed negligible foam which disappeared once the mixing process stopped, which may be due to the low solubility of kafirin in aqueous solutions. The summarized functionalities results are showed in Table 1.

1.7 Sorghum protein applications

1.7.1 As a food ingredient of gluten-free food

In recent years, more and more people will have an autoimmune disease called celiac disease (CD), when the human body with this disease consumes the grains wheat (gluten), rye (secalin) and prolamins in barley (hordein), these proteins can cause allergic reactions or harmful autoimmune reactions. CD is a chronic disease with a hereditary form for which the only cure is a lifelong abstinence from products containing gluten, secalin and hordeins. However, since gluten is found not only in food, but also in medications, vitamins, beauty products, and even postage stamps and envelope adhesives, people with celiac disease must be very cautious in their daily lives (de Mesa-Stonestreet et al., 2010).

The unique physicochemical properties of kafirin proteins, such as non-allergenicity, slow digestion by mammalian proteases, hydrophobicity, solubility in low-boiling solvents, and adoption of an elongated conformation, highlight its potential prospects for alternative sources of gluten products (Xiao et al., 2017). However, the inability of sorghum kafirins to produce elastic doughs and breads with high consumer acceptance poses challenges and drives research into the manufacture of high-quality sorghum gluten-free foods (Yousif et al., 2012). In recent years, farmers have begun producing sorghum hybrids for use in gluten-free foods for celiac patients

(Tuinstra, 2008). As a main ingredient in gluten-free products, it has become common to mix kafirin in wheat compound flour to make gluten-free bread. Other kafirin-based gluten-free products in the form of beer, biscuits, pasta, bread, waffles, etc. have entered the market. By supplementing them with other dietary nutrients, people with celiac disease and their families can have broader and healthier dietary choices (Xiao et al., 2017).

1.7.2 Edible films and coatings for food packages

Edible films and coatings are thin layers of edible materials that are applied to foods. Both technologies play an active role in improving the safety, quality and shelf life of food by preventing mechanical damage, moisture loss, gas transmission, fat migration and/or microbial spoilage of the product (Moreira et al., 2016). Edible films are food products that are molded into solid sheets and edible coatings are applied to the food product in liquid form, usually by immersing the product in a solution that then creates a structural matrix. Protein-based films/coatings are formed through the development of hydrophobicity, hydrogen bonding, and limited disulfide bonds between protein chains (A. Gennadios & C. L. Weller, 1994). Kafirin is rich in hydrophobicity and disulfide bonds, so it is called a good coating/film material, and the coating/film made of kafirin will have relatively better moisture and gas barrier properties, compared with other proteins edible film (da Silva & Taylor, 2005).

A typical kafirin film casting method involves dissolving, casting and drying a stock solution of kafirin and plasticizer on a non-stick surface to produce a film of controlled thickness (J. Taylor et al., 2005b). Solvents used to dissolve kafirin include glacial acetic acid, 70% ethanol in water, or 55% (w/w) isopropanol in water (J. Taylor et al., 2005b). Available plasticizers are typically glycerol, polyethylene glycol, lactic acid, or their mixtures. Co-

solubilization of plasticizers with kafirin can increase productivity and increase the flexibility of the polymer chains by lowering the glass transition temperature (Xiao et al., 2017).

An emerging research trend is the development of active packaging materials by incorporating bioactive compounds into kafirin films to alter the properties of the films and extend the shelf life of perishable products. Another recent research attempt was to fabricate kafirin films as scaffold matrices and study biocompatibility and biodegradation properties in rodent models (J. Taylor et al., 2015).

1.7.3 Biodegradable films

Concerns about the limited availability of fossil fuel feedstocks for synthetic polymers and the persistent and non-degradable nature of synthetic polymer plastics have fueled a research boom, with attendant concerns about their disposal and environmental pollution (J. Taylor et al., 2013). Isolated sorghum protein as a bio-based material that has been shown to produce biodegradable films and has been used as an extender in plywood adhesives (Ramos et al., 1984) and other low-cost adhesives, wallboard, and packaging materials (Bean et al., 2006). This combined with the fact that sorghum is a relatively drought-resistant grain, suggests that sorghum has the potential to be a valuable renewable resource for bioindustrial products, especially in arid regions where other crops are not easily grown (Bean et al., 2006).

Adhesion of proteins to wood surfaces is due to the diffusion, wetting and penetration of the porous wood structure by the proteins during coagulation, achieving mechanical interlocking, physical attraction and chemical bonding between wood and proteins, followed by entanglement through physical attraction and chemical interactions and crosslinking. Protein-to-wood and protein-to-protein binding during heat setting (N. Li et al., 2011). As the protein concentration increases, more protein is available to bond with the wood and provide greater strength.

However, as protein concentration increases beyond optimal levels (>16%), protein-protein interactions may dominate protein-wood interactions, resulting in uneven protein distribution or insufficient exposure of hydrophobic groups on the wood surface. As a result, a limited contribution to the improvement in adhesion performance was observed when the protein concentration reached 16% (Y. Wang et al., 2007).

1.8 Conclusions

Due to its hypoallergenic nature and its ability to thrive in drought conditions, sorghum stands out as an excellent raw material and a reliable source of protein. While sorghum is primarily utilized as animal feed in the United States, it holds significant potential for diverse food applications, serving as a concentrated protein source for inclusion in gluten-free foods. However, there exists a notable disparity between the current state of sorghum and its proteins and the desired nutritional and functional properties, hindering their broader utilization in the food industry. There is considerable promise for leveraging sorghum proteins in applications such as food films for edibles and degradable biofilms for non-food purposes.

Consequently, future research avenues could concentrate on developing cost-effective, food-compatible, and safe methods for concentrating and/or extracting sorghum proteins, particularly those capable of being scaled up to commercial levels. Additionally, modifying the functional properties of sorghum proteins is essential to broaden their application range in both food and non-food sectors.

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Table 1-1 Functionalities of sorghum proteins

Source	Treatments	Protein content %	WHC (g/g)	OHC (g/g)	EC & ES	FC % & FS%	Solubility %	References
Decorticated white-regular sorghum	70% (v/v) aqueous ethanol containing 0.3% of sodium metabisulfite	83.6	2.31	2.31	EC = 1.263	\	at as-is pH, 0.55	(Espinosa-Ramírez & Serna-Saldívar, 2016)
Whole white-regular sorghum		72.71	2.48	2.34	EC = 1.557		at as-is pH, 1	
Decorticated white-waxy sorghum		81.54	1.99	2.18	EC = 1.64		at as-is pH, 0.48	
Whole white-waxy sorghum		73.44	2.01	2.11	EC = 1.734		at as-is pH, 0.95	
Decorticated red-regular sorghum		93.34	2.46	2.04	EC = 1.108		at as-is pH, 0.85	
Whole red-regular sorghum		88.69	2.65	2.4	EC = 1.13		at as-is pH, 0.51	
Decorticated high-tannin sorghum		92.34	2.65	2.76	EC = 1.081		at as-is pH, 0.59	
Whole high-tannin sorghum		74.58	2.96	2.75	EC = 1.596		at as-is pH, 0.96	
Commercial white regular sorghum (Non-tannin, decorticated)	Dry-milling + 70% aqueous ethanol and sodium metabisulfite at 65 ℃	85.56	1.85	1.57	EC < 2 m^2/g at pH 3 or 5; EC > 3 m^2/g at pH 9	\	\	(Espinosa-Ramírez et al., 2017)
	Dry-milling + 1.25% Alcalase (protease) + 70% aqueous ethanol and sodium metabisulfite at 65 ℃	83.78	1.85	1.69	EC < 2 m^2/g at pH 3 or 5; EC > 7 m^2/g at pH 9			
	Wet-milling + 70% aqueous ethanol and sodium metabisulfite at 65 ℃	94.87	2.82	2.39	EC < 2 m^2/g at all pH			

	Wet milling + 1.25% Alcalase (protease) + 70% aqueous ethanol and sodium metabisulfite at 65 ℃	92.78	2.78	2.56	EC < 2 m^2/g at all pH			
White sorghum	Wet milling + 70% (v/v) aqueous ethanol containing 0.1% (w/v) sodium metabisulfite and. 0.1% (w/ v) sodium hydroxide extraction	94.23	2.2	1.41	EC > 20 m^2/g; ES > 60%	ND	\	(Amoura et al., 2020)
	Dry milling + same method	90.07	1.82	1.34	EC > 20 m^2/g; ES > 60%	ND		
Red sorghum flour	Extract at pH 8	38.98			EC = 125.16; ES = 14.76	\	at pH= 7, 14.5	(Dias de Carvalho et al., 2024)
	Extract at pH 10	44.59			EC = 108.96; ES = 15.27		at pH= 7, 17.68	
	Extract at pH 12	43.02			EC = 102.49; ES = 20.81		at pH= 7, 64.77	

*ND: not detected

Chapter 2 - Comparative analysis of the physicochemical characteristics of kafirin proteins extracted from various grain sorghums and their distillers' grains

Abstract

Sorghum is a globally significant cereal crop that is a potential source of innovative plant proteins. Kafirin is the primary storage protein in grain sorghum. This study aims to conduct a comparative analysis of the physicochemical and functional properties of kafirin proteins extracted from different types of sorghums, as well as their distillers' grains (DGs), to understand the effect of sorghum type and fermentation process on protein properties. Kafirin protein was extracted from seven distinct types of grain sorghums and their DGs using a glacial acetic acid method. The protein content, SDS-PAGE profiles, molecular size distributions, secondary structure composition, surface hydrophobicity, *in vitro* protein digestibility, and other functionalities of the extracted kafirins were characterized.

The protein content of extracted kafirins ranged from 75% to 85%. The α-helix structure was predominant in kafirin proteins. SDS-PAGE results showed that the kafirins from different sorghum raw materials had similar band profiles, except that new bands in the range of 15-20 kDa and 25-37 kDa were observed for the kafirin samples from flours but not DGs. The surface hydrophobicity of the kafirins varied between 51.37-59.02 μg SDS/mg protein, and the fermentation process further altered the surface hydrophobicity of the extracted kafirin to some extent. The kafirin from black sorghum DGs had slightly lower *in vitro* protein digestibility (around 75.53%) compared to other sorghum DGs (75.34-79.33%), which may be due to the high tannin content in the black sorghum variety. The functional properties of extracted kafirins

were varied to some extent. This study provides fundamental knowledge of the protein properties associated with different sorghum types and their distillers' grains, which will aid in the future production and wider applications of sorghum-derived proteins.

Keywords: Sorghum protein, kafirins, fermentation, distiller's grains, physicochemical property, protein functionality

2.1 Introduction

In the contemporary food system, addressing challenges such as the depletion of freshwater sources, habitable land, arable areas, and greenhouse gas emissions is paramount (Pöri et al., 2023). Furthermore, the increasing awareness of obtaining high-quality proteins in the diet has spurred researchers to explore more eco-friendly and sustainable protein sources (Kumar et al., 2021). Plant-derived proteins have emerged as viable alternatives to animal proteins in the human diet, owing to their multifaceted benefits, including sustainable sourcing, provision of essential phytochemicals and fibers, and reduction of saturated fat and cholesterol intake (Akharume et al., 2021). Yet, while conventional protein isolates such as wheat gluten and soy protein play an important role in food, their consumption is constrained by dietary restrictions among individuals with conditions such as celiac disease and soy allergies (Dias de Carvalho et al., 2024). This situation has sparked a marked increase in consumer interest towards gluten-free resources.

Sorghum (*Sorghum bicolor L.*), a gluten-free cereal, ranks as the fifth-most produced cereal crop globally, following corn, wheat, rice, and barley (Perraulta Lavanya et al., 2021). It is a versatile, drought-tolerant crop predominantly grown in semi-arid regions of Africa, Asia, Australia, and North and South America (Hassan, 2023). Sorghum serves as a staple food in Asian and African countries, where it is consumed in various forms such as bread, porridges, and crackers. In contrast, in Western countries like the United States, Australia, and Brazil, sorghum is primarily cultivated for animal feed or bioethanol production (Abah et al., 2020; Hassan, 2023).

The commercial production of bioethanol from grains like sorghum for biofuel results in the significant generation of dried distillers' grains with solubles (DDGS), a by-product rich in

proteins (Lau et al., 2015). Nowadays, the DDGS are predominantly utilized as a supplement in animal feed. However, the residual portions are often discarded as waste, posing environmental risks (Böttger & Südekum, 2018). Hence, it is important to explore technologies to unlock the value of these under-utilized DDGS, thereby mitigating the environmental burden associated with their disposal. Sorghum distillers' grains, as a major co-product of bioethanol fermentation, present a valuable, abundant, and readily available feedstock with protein contents ranging from 30% to 40% (Wang et al., 2009).

Sorghum proteins represent a significant macronutrient source within sorghum grains, with an average protein content of approximately 11%. These proteins can be categorized into albumins, globulins, prolamins, and glutelins based on their solubility characteristics. Among these, kafirin, a prolamin, constitutes the majority of the total protein content, accounting for up to 70% in sorghum endosperm (J. R. N. Taylor et al., 2006). Kafirins are further classified into four subunits, including α-kafirin (23-25 kDa), β-kafirin (16-20 kDa), γ-kafirin (28-50 kDa), and δ-kafirin (13 kDa), with α-kafirin typically located in the core and surrounded by β- and γ-kafirin (Belton et al., 2006). The β- and γ-kafirin subunits at outer region are often cross-linked due to their high cysteine content, rendering kafirin insoluble and hydrophobic (Semwal & Ms, 2022). However, isolating kafirin from sorghum grains poses challenges due to its distribution within the endosperm, where it forms agglomerations with the glutelin matrix around starch granules (Castro-Jácome et al., 2020).

To enhance the value of sorghum DDGS, protein extraction processes can be applied to this material. Commonly employed methods for kafirin extraction include precipitation at the isoelectric point, solubilization in alcohol solvents, wet milling, sonication, and hydrolysis. Precipitation method capitalizes on the insolubility of proteins at their isoelectric point, involving

alkaline or acid extraction techniques (Dias de Carvalho et al., 2024; Wang et al., 2009). The use of alcohol extraction systems, employing nonpolar solvents such as ethanol or reducing agents, is another prevalent approach due to the high solubility of sorghum prolamins in nonpolar solvents (Espinosa-Ramírez & Serna-Saldívar, 2016; Ioerger et al., 2020). Wet milling, based on physical separation principles, is often combined with chemical reagents and enzymes to enhance the extractability of sorghum proteins (Espinosa-Ramírez et al., 2017; Amoura et al., 2020). Additionally, sonication and enzymatic methods employ physical equipment or enzymes to augment protein extractability (Semwal & Ms, 2022; Wulandari et al., 2023).

In our study, glacial acetic acid was utilized as the primary solvent to extract kafirins from sorghum samples. In contrast to methods employing aqueous ethanol, which often result in elevated temperatures during extraction and may consequently disrupt protein structures, the use of glacial acetic acid offers the advantage of a low dielectric constant, facilitating the dissolution of hydrophobic proteins at ambient temperature (J. Taylor et al., 2005). Subsequent pH adjustment to the isoelectric point of sorghum proteins further facilitated the protein precipitation and isolation. The objective of our work was to characterize the extracted kafirins from various sorghum types and to evaluate the impact of type and the fermentation process on the physicochemical characteristics and functionalities of kafirin proteins.

2.2 Materials and Methods

2.2.1 Materials

Red-normal (Red-NLM-20 and Red-NLM-SB), red-waxy (Red-Waxy), white-normal (White-4525, White-32020, and White-F1000), and black-normal sorghums (Black-NLM-16) were obtained from a commercial supplier (Nulife Market company, Scott City, KS, USA). Sorghum distillers' grains (DGs) were byproduct obtained from Chinese baijiu fermentation as

described in our previous study (Liu et al., 2023). Sodium metabisulfite, glacial acetic acid, NaOH, and hexane were purchased from Sigma-Aldrich.

2.2.2 Preparation of kafirin proteins

The acetic acid method was referenced by Wang et al. (2009) with some modifications. Both the original grains and DGs were ground into fine flours using a coffee grinder, ensuring that 70% of the samples passed through a 0.355 mm screen (sieve No. 45). Subsequently, 100 g of sorghum flours or 50 g of sorghum DGs were presoaked in 4 times their volume of 0.5% (w/w) sodium metabisulfite and stirred for 24 hours. The liquid part was removed by centrifugation (Avanti® J-E high-speed centrifuge, Beckman Coulter Inc., Brea, CA, USA) for 20 min (at 8000 ×g), followed by the addition of 5 volumes of acetic acid and stirring for 2 h at room temperature (RT). The mixture was centrifuged again to obtain the supernatant at 8000 ×g for 15 min. The pH of the supernatant was slowly adjusted to 5.0 using 50% (w/v) NaOH, with an ice bath employed during this process. The suspension was then kept at 4 ℃ overnight, followed by centrifugation (8000 ×g, 15 min) to collect the sediment. The sediment was washed with distilled water three times, followed by centrifuging (8000 ×g, 10 min) and subsequently freeze-dried (Freezone 4.5, Labconco Corporation, Kansas City, MO, USA). The dried protein samples were defatted using hexane three times, and the final protein powders were collected. All protein samples were stored in the refrigerator at 4 ℃ for later use. The protein content of the kafirin proteins was determined using a FP928 LECO nitrogen analyzer (LECO Corporation, Saint Joseph, MI, USA) according to AOAC method 990.03 with a conversion factor of 6.25. The equations below represented the calculations of yield and protein recovery rate:

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

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

2.2.3 Color analysis

The color of kafirin proteins extracted from both flours and DGs and commercial pea protein, soy protein, and wheat gluten were measured using a Minolta Chroma Meter CR-300 colorimeter (Minolta Camera Co., Osaka, Japan). L* (lightness), a*(redness to greenness), b* (yellowness to blueness) values were collected and averaged from duplicate samples.

2.2.4 FTIR spectroscopy

Fourier transform infrared (FTIR) spectra were acquired using a PerkinElmer Spectrum 400 FT-IR/FTNIR Spectrometer (PerkinElmer, Inc., Waltham, MA, USA) equipped with an attenuated total reflectance cell (ATR). Each protein sample underwent 64 scans within the range of 400-4000 cm^{-1} at a 4 cm^{-1} resolution (Chen et al., 2019). Analysis of the secondary structure of proteins was focused on the amide I region (1600 – 1700 cm^{-1}). Relative areas of the amide I region were quantified using OriginPro 2016 software (OriginLab, Inc., Northampton, MA, USA).

2.2.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein profiles were determined via SDS-PAGE, following the protocol by Hong et al. (2022) with minor adjustments. 150 mg of proteins were mixed with 10 mL of PBS buffer (pH= 6.8) containing 2% (w/v) SDS to form stable suspension (shaken for 2 h at 250 rpm). After centrifugation (8000g, 5 min), the supernatant was collected as sample solution. Protein samples were treated under non-reducing conditions. Briefly, sample solution was combined with 3 volumes of 4 x Laemmli buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and boiled for 5 min. After cooling, 10 μL of the mixture was loaded into the wells of a 4-20% Mini-Protean TGX gel (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Precision Plus Protein Dual Color

Standards (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were loaded parallelly as molecular weight (MW) markers. Electrophoresis was conducted at 200 V in 1X Tris/Glycine/SDS running buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for approximately 38 min. The gel was stained with diluted Brilliant Blue R Concentrate solution (Sigma, St. Louis, MO, USA) for 8 min, rinsed with distilled water overnight, and washed with destaining solution (10% v/v acetic acid and 30% v/v methanol) until the background was cleared.

2.2.6 Surface hydrophobicity

Surface hydrophobicity (H_0) was determined using sodium dodecyl sulfate (SDS) binding method, following the protocol outlined by Tang et al. (2021). The suspension was prepared by mixing 10 mg of each protein with 40 mL of 0.1 mmol/L SDS buffer, followed by shaking for 1 h to ensure thorough dispersal. The suspension was then transferred into a SnakeSkin^{TM} dialysis tubing (35 mm dry I.D.) with MW cut-off of 3.5 kDa (Thermo scientific, Rockford, IL, USA) and dialyzed for 48 h in distilled water, with the resulting solution volume recorded. Thereafter, an aliquot of the dialyzed solution (10 mL) was mixed with 25 mL of chloroform and 5 mL of 24 mg/L methylene blue. The mixture was shaken for 5 min before centrifugation at 2500 g for 15 min, and the lower chloroform layer solution was analyzed using a double beam spectrometer (VWR UV-6300PC, Radnor, PA, USA) at 655 nm. SDS solutions with various concentrations were prepared to establish a standard curve. Surface hydrophobicity was determined based on the amount of SDS bound to the protein.

2.2.7 *In vitro* protein digestibility

Protein digestibility was assessed following Hsu et al. (1977) with some modifications. A 30 mL solution with a protein concentration of 6.25 mg/mL was prepared in distilled water and pH was adjusted to 8.00 (± 0.02) using 1 N HCl and/or 1 M NaOH. A multienzyme solution was

prepared with 1.6 mg/mL trypsin, 3.1 mg/mL α-chymotrypsin, and 1.3 mg/mL protease in distilled water, maintaining pH at 8.00 (± 0.02). While mixing 3 mL of enzyme solution with the sample solution at 37°C for 10 min, pH changes were monitored using a pH meter. The digestibility was calculated using the equation from Tinus et al. (2012):

$$Protein\ digestibility\ \% = 65.66 + 18.10\ \times \Delta pH_{10\ min}$$

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

2.2.8 Total tannin content determination

The total tannin content was determined following a combined approach based on methods by Price et al. (1978) and Subbiah et al. (2021) with some modifications. To extract phenolic compounds, 100 mg protein sample was mixed with 800 µL methanol, followed by agitation in darkness for 20 min at 250 rpm. The supernatant was collected after centrifugation at 3000 g for 15 min. Thereafter, 25 µL of phenolic extracts, 25 µL of 32% v/v sulfuric acid (dissolved in methanol), and 150 µL of 4% w/v vanillin solution (dissolved in methanol) was added to a 96-well microplate sequentially and incubated for 30 min in darkness. The absorbance was measured at 500 nm using a microplate spectrophotometer (BioTek, Synergy H1 Hybrid, Highland Park, Winooski, VT). The total tannin content was quantified using a catechin calibration curve with concentrations ranging from 0 to 1 mg/mL, and was expressed in mg catechin equivalent per gram of protein weight.

2.2.9 Functional properties

2.2.9.1 Water/ oil holding capacity and protein solubility

Water and oil holding capacities were assessed following the method described by Espinosa-Ramírez & Serna-Saldívar (2016) with some modifications. For water holding capacity, 0.25 g of the protein (W_0) was mixed with 7.5 mL of distilled water in a 15 mL centrifuge tube (W_2) and agitated at 300 rpm for 30 min. Following centrifugation at 4500 g for

15 min, the tube was inverted for 5 min to remove excess water, and the final weight (W_1) was recorded. Water holding capacity (WHC) was calculated using the formula:

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

The precipitate after WHC test was collected and freeze-dried, solubility was calculated by the difference in weight between the original protein sample and the precipitate divided by the original protein sample weight, as shown in the equation below:

$$Solubility\ \% = \frac{wt\ of\ original\ protein\ sample\ (g) - wt\ of\ precipitate(g)}{wt\ of\ original\ protein\ sample\ (g)} \times 100$$

For oil holding capacity, 0.25 g of protein (O_0) was blended with 7.5 mL of soybean oil in a 15 mL centrifuge tube (O_2). After shaking, centrifugation, and draining excess oil, the final weight of the precipitate was recorded (O_1). The calculation of oil holding capacity (OHC) followed the equation:

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

2.2.9.2 Emulsifying properties

Both emulsifying capacities (Emulsification Activity Index (EAI) and Emulsion Stability Index (ESI)) were determined following a turbidimetric method described by Espinosa-Ramírez & Serna-Saldívar (2016) with modifications. The procedure involved combining 0.25 g of proteins with 25 mL of 100 mM phosphate buffer (pH = 7) and stirring until a uniform solution was formed. Subsequently, 8.33 mL of soybean oil was dispersed into the solution and homogenized using a Polytron PT 2500 E homogenizer (Kinematica AG, Switzerland) at 14,000 rpm for 1 min at RT. After homogenization, 50 µL of the emulsion was mixed with 2.95 mL of 0.1% (w/v) SDS buffer and vortexed. The absorbance of the diluted emulsion was measured at

500 nm (A_0) and again after 10 min (A_{10}). The Emulsification Activity Index (EAI) and Emulsion Stability Index (ESI) were then calculated using the following formulas:

$$T = 2.303 \times \frac{A_0}{L} \times D$$

$$EAI\ (m^2/g) = \frac{2 \times T}{\varphi \times C \times 1000}$$

$$ESI\ (min) = \frac{A_0}{A_0 - A_{10}} \times t$$

where *T* is the turbidity of emulsion, *A* is the absorbance at 500 nm, *D* is dilution factor, *L* is the path length of light (m), φ is the oil volume fraction, *C* is the protein concentration in the dispersion (mg/ mL), *t* is 10 min.

2.2.10 Statistical analysis

The experiments were conducted at least duplicate, and results were expressed as mean values ± standard deviations (SD). Statistical analysis was performed using IBM SPSS Statistics software (version 27.0.1., Armonk, NY, USA). One-way analysis of variance (ANOVA) was conducted, followed by Tukey's test for multiple comparisons to assess mean differences. Statistical significance was set at $P < 0.05$.

2.3 Results and Discussions

2.3.1 Protein extraction and recovery

The results in Table 1 reveal significant variations in the protein content, yield, and protein recovery among different sorghum types of DGs and flour samples. The kafirins extracted from DGs and flours exhibited protein content ranging from 68.88% to 88.47%, which are higher than that of 49.76% to 88.47% reported by Musigakun & Thongngam (2007), Wang et al. (2009), and Pontieri et al. (2019). The choice of extraction method has significant impact on the purity of the extracted kafirin proteins. In general, acetic acid and NaOH-ethanol methods produced proteins

with high purity (Wang et al., 2009). Regardless of source (DGs or flours), kafirin protein content from red and black sorghum types tended to be higher than that from white sorghum types, being consistent with the findings of Espinosa-Ramírez & Serna-Saldívar (2016). Across different types, kafirin protein content extracted from DGs was significantly higher than that from flours ($P < 0.05$), except for Red-NLM-20, of which kafirins from both DG and flour had similar protein content (87.91% and 88.47%, respectively).

In terms of yield, the Red-Waxy (DGs) sample displayed the highest yield at 17.53%, while Red-Waxy (Flour) exhibited the lowest yield at 3.97%. Notably, the yield of kafirin extracted from DGs (average of 13.96%) was also significantly higher than that from flours (average of 5.70%) ($P < 0.05$). Furthermore, the yields of extracted kafirins were similar across different sorghum types(red, black, and white types), which was in agreement with previous study (Espinosa-Ramírez & Serna-Saldívar, 2016). Protein recovery rates of extracted kafirin proteins ranged from 24.90% (Red-NLM-SB (DGs)) to 57.16% (Black-NLM-16 (flour)). Kafirins extracted from both black sorghum DG and flour showed the highest protein recovery rates. However, protein recovery rates of kafirin extracted from either DGs or flours did not exhibit significant differences ($P < 0.05$). These findings suggest that both sorghum type and fermentation process can influence the protein extraction process.

2.3.2 Color properties

Color analysis provides crucial information regarding the visual appearance of samples, influencing consumer acceptance and product quality assessment. The L* value indicates lightness, with higher values representing lighter colors and lower values indicating darker colors. The a* value signifies the redness to greenness axis, while the b* value indicates the yellowness to blueness axis. The color parameters are summarized in Table 2.

Based on the L* values, kafirins extracted from both white sorghum DGs and flours exhibited the highest lightness, being around 83.94-85.77, while those extracted from black sorghum types (DGs or flour) had the lowest lightness, being approximately 71.74-75.46. These findings align with results reported by Espinosa-Ramírez et al. (2017), where kafirins from white sorghums showed higher lightness values ranging from 86.62 to 89.40. Moreover, there are no significant difference in L* values of kafirins among the DGs and flours samples. For commercial plant proteins, soy protein isolate (SPI)exhibited relatively higher lightness compared to wheat gluten and pea protein isolate (PPI), yet still lower than that of kafirin proteins derived from white sorghum types.

Kafirins extracted from black sorghum DGs or flours demonstrated the highest a* values (6.27 and 5.88, respectively) compared to those from red and white sorghum types, indicating a more reddish color. Additionally, a* values of kafirins extracted from sorghum DGs were higher than those from flours within the same sorghum types. PPI exhibited the highest a* value among commercial plant proteins, which was 4.91. Conversely, kafirins extracted from black sorghum types displayed the lowest b* values, with 10.59 for DG and 9.75 for flour. PPI exhibited the highest b* value, indicating a yellowish color, and all commercial plant proteins demonstrated higher b* values compared to sorghum kafirins. In the study by Espinosa-Ramírez & Serna-Saldívar (2016), kafirin extract exhibited lower a* values but relatively higher b* values compared to our study. This difference in color attributes could be attributed to variations in the pigment compounds and genetic makeup of different sorghum types, influencing the color of sorghum grains. Different sorghum varieties possess distinct genes and tannin content, leading to variations in pericarp color or the formation of pigmented testa (Rashwan et al., 2021).

2.3.3 Protein secondary structure

The Fourier transform infrared (FTIR) spectroscopy analysis provided insights into the structural characteristics of the proteins in the samples, which are pivotal for understanding their functional properties. The secondary structural elements, including α-helix, β-sheet, β-turn, and random coil structures, were derived from the FTIR spectra and are presented in Table 3.

The α-helix content represents the spiral-shaped secondary structure of proteins, with higher percentages indicating greater structural stability (Swanson & Sivaramakrishnan, 2014). Kafirins extracted from different types exhibited similar α-helix content, approximately 71.40% in DG-extracted kafirins and 59.27% in flour-extracted kafirins, suggesting minimal influence of sorghum type on protein structure. Red-NLM-20 (Flour) exhibited significantly lower α-helix content compared to other flour-extracted kafirins ($P < 0.05$), while kafirins from DGs generally exhibited higher α-helix content compared to flour-extracted kafirins. This phenomenon may be attributed to structural changes induced by the fermentation process, which involves heat treatment for starch hydrolysis and ethanol distillation. The observed increase in α-helix content after thermal treatment was also reported in the study by Semwal & Ms (2022). Remarkably, all kafirin proteins exhibited significantly higher α-helix content (66.76%) compared to commercial plant proteins (18.50%).

Beta-sheet content reflects the presence of extended sheet-like structures within proteins. Although the proportion of β-sheet structure did not significantly differ among sorghum types with different colors ($P < 0.05$), kafirins extracted from sorghum DGs generally exhibited higher β-sheet content compared to those from sorghum flours, except for the Red-NLM-20 (Flour) exhibited higher β-sheet content compared to DGs (51.68% versus 27.48%). PPI demonstrated

the highest β-sheet content (51.75%) among all samples, indicating the predominant presence of sheet-like structures.

The β-turn content represents the tight turns or bends within protein structures. White-4525 (Flour) exhibited the highest β-turn percentages among all protein samples, suggesting increased flexibility or bending within its protein structure. In sorghum DGs, kafirins extracted from white sorghum types (11.82%) had higher β-turn content compared to red and black sorghum types (5.85% and 5.16%, respectively), while in sorghum flours, kafirins extracted from black sorghum typeshad the highest β-turn content. Interestingly, kafirins extracted from sorghum DGs had lower β-turn content compared to those from sorghum flours, indicating the impact of the fermentation process on protein structure. Consequently, the fermentation process led to a deduction in α-helix and β-sheet structures while β-turn structure in extracted kafirins increased. Contrarily, the study by Shah et al. (2021) observed the opposite structural changes in kafirin extracts, in which the β-sheet structure increased as the α-helix and random coil structures in kafirin extracted from DDGS decreased. This is possibly due to the differences in extraction methods used, as the extraction method that used above was ethanol-NaOH method. Moreover, the β-turn content of commercial plant proteins (5.88%) was generally lower than kafirin proteins extracted from sorghum flours (18.49%).

Random coil content indicates the absence of defined structural elements within proteins. Notably, all sorghum samples exhibit negligible percentages of random coil content, suggesting well-defined structural elements within the proteins analyzed. In contrast, commercial plant proteins displayed a larger proportion of random coil structures, with an average of 42.81%.

2.3.4 SDS-PAGE

SDS-PAGE profile of kafirin proteins in non-reducing condition is shown in Figure 1. Kafirins are found in different forms including monomeric form, oligomeric form, or polymeric complexes (Belton et al., 2006). Similar band profiles were observed for kafirins extracted from various sorghum DGs and flours, with comparable band intensities, being consistent with findings by Shah et al. (2021). The presence of residues above 250 kDa, identified as high molecular polypeptides, suggests the formation of protein aggregates possibly due to the extreme extraction conditions, such as extremely low pH, as reported by Dias de Carvalho et al. (2024). The alkaline medium hazardously alters protein structures, leading to cross-linking between proteins and aggregation (Rawel et al., 2001).

The faint bands above 100 kDa may indicate trimers or oligomers, as observed in previous studies (Castro-Jácome et al., 2020). The bands around 75 kDa can be corresponding to residue glutelin fraction (Ioerger et al., 2020). A distinct dark and broad band around 50 kDa, identified as a dimer of γ-kafirin (Belton et al., 2006), was observed in both DG and flour extracts, being consistent with findings by Shah et al. (2021). The faint band at approximately 36 kDa, observed only in flour-extracted kafirins, may be related to the globulin fraction. Another band at around 28 kDa, exclusively present in kafirins from sorghum flours, likely represents the co-migration between an α-polypeptide and a γ-polypeptide (Shah et al., 2021). Notably, the band in the 20-25 kDa range appeared darker and broader in kafirins extracted from sorghum DGs compared to those from flours, suggesting a higher abundance of α_1- and α_2-kafirin in DG-extracted kafirins.

Additionally, the band at around 16 kDa was exclusively observed in flour-extracted kafirins, being possibly β-kafirin (Belton et al., 2006), which might be influenced by the fermentation process applied to sorghum DGs. The small residues observed in the 10-15 kDa

range exclusively in kafirin extracted from sorghum DGs, as reported previously, may be attributed to extreme conditions such as high acidity, or heat treatment during the fermentation process (Dias de Carvalho et al., 2024).

2.3.5 Surface hydrophobicity (H_0)

The surface hydrophobicity value (H_0) is a critical factor that can affect protein functional properties. This parameter also provides information about protein unfolding and stability and can relate to the concentration of exposed hydrophobic amino acid residues of proteins (Zhi et al., 2022). The results of H_0 are presented in Table 4.

In DG-extracted kafirins, H_0 ranged from 51.37 to 59.02 μg SDS/mg protein, with Red-Waxy (DGs) exhibiting the highest surface hydrophobicity. Notably, these values remained similar across red, black, or white sorghum types, suggesting that sorghum type had a minimal influence on the surface hydrophobicity of DG-extracted kafirins. Conversely, in flour-extracted kafirins, the highest H_0 value (average) was observed in white sorghum type (56.91 μg SDS/mg protein), followed by red and black sorghum types, with Red-NLM-20 (Flour) displaying the lowest surface hydrophobicity (52.02 μg SDS/mg protein). Interestingly, a comparison between DG- and flour-extracted kafirins revealed fluctuations in H_0 values, with an increase observed for Red-Waxy (Flour)and Black-NLM-16 (Flour) after fermentation, while Red-NLM-20 (Flour)and White-4525 (Flour) experienced a decrease. These variations in H_0 values may be attributed to structural alterations induced by heat treatment and the fermentation process, which can either inhibit or expose hydrophobic residues depending on the specific sample type. In the study by Semwal & Ms (2022), the thermal treatments led to a decrease in H_0 values of kafirin proteins, which was attributed to the unfolding of proteins, resulting in fewer exposed hydrophobic sites. Among the samples, SPI exhibited the highest surface hydrophobicity (68.00 μg SDS/mg

protein), followed by PPI (64.87 μg SDS/mg protein). However, it is noteworthy that previous studies have reported relatively lower surface hydrophobicity for PPI and SPI due to their high content of hydrophilic amino acids. Therefore, the observed high surface hydrophobicity in this study may attributed to the high solubility or protein content of SPI and PPI.

2.3.6 *In vitro* protein digestibility and total tannin content

The *in vitro* protein digestibility represents the percentage of protein that is digested under simulated physiological conditions. The results are presented in Table 4. For DG-extracted kafirins, protein digestibility ranged from 74.80% to 79.33%, with White-32020 (DGs) exhibiting the highest value (79.33%) and Black-NLM-16 (DGs) displaying the lowest (74.80%). The variability in protein digestibility among sorghum types can be influenced by several factors, including genotype variations, grain and protein body structure, protein cross-linking, and the presence of phenolic compounds (Khoddami et al., 2023). Conversely, kafirins extracted from sorghum flours generally showed higher digestibility (76.25% to 79.33%) than that extracted from DGs (74.80% to 79.33%). The observed lower digestibility in DGs-extracted kafirins may be attributed to the fermentation process, which inhibits enzyme action sites in the samples, rendering the DG-extracted kafirins less digestible (Day & Morawicki, 2018). Despite flours resulted in an overall higher digestibility, Black-NLM-16 (Flour) still demonstrated the lowest digestibility among the flour-extracted kafirins. To further elucidate this phenomenon, we investigated the total tannin content in protein samples.

As shown in Table 4, proteins extracted from black sorghum types (both DG and flour) had the highest total tannin content. The DG-extracted kafirin exhibited a total tannin content of 2.44 mg catechin equivalents/g protein, while the flour-extracted kafirin had a higher content of 5.70 mg catechin equivalents/g protein, being consistent with findings from the study by De

Morais Cardoso et al. (2017). This finding suggests a negative correlation between *in vitro* protein digestibility and total tannin content (r = -0.42). Tannins have the capability to bind with proteins and proteases and form complexes, which may render them resist to enzyme hydrolysis. Additionally, tannin-protein interactions can denature enzymes, leading to digestive enzyme inhibition (Girard & Awika, 2018). In addition, it is important to note that some proteins with relatively low total tannin content still exhibited low digestibility, potentially due to factors such as low solubility. Among the commercial plant-based proteins analyzed, SPI exhibited the highest *in vitro* protein digestibility (86.20%), followed by PPI (85.75%) and wheat gluten (83.31%), which were comparable with the results from Qin et al., 2022. The higher digestibility of SPI, PPI, and wheat gluten can be attributed to their protein profiles, high solubility, and low or no tannin content compared to sorghum kafirin proteins.

2.3.7 Water holding capacity and oil holding capacity

Water holding capacity (WHC) and oil holding capacity (OHC) are pivotal parameters that influence a protein's ability to retain water and bind oil, respectively, profoundly impacting food texture and flavor during application. Table 5 presents the WHC and OHC values obtained in our study. For DG-extracted kafirins, WHC ranged from 2.12 to 3.42 g water/g protein, with the highest values observed in red sorghum types and the lowest in black sorghum types. Conversely, flour-extracted kafirins displayed WHC values ranging from 2.08 to 2.61 g water/g protein, with a similar trend of higher values in red sorghum types and the lowest in white sorghum type. This discrepancy may arise from variations in protein composition among different sorghum color types. Notably, except for Black-NLM-16, kafirins extracted from sorghum DGs generally exhibited higher WHC values than those extracted from flours. Comparable results were obtained from the study by Espinosa-Ramírez & Serna-Saldívar (2016),

with WHC values ranging from 1.99 to 2.96 g water/g protein, and the kafirin extracted from red sorghum flour had higher WHC values as well. However, in the study by Amoura et al. (2020), the WHC of kafirins extracted from dry-milled or wet-milled methods were 1.82 g water/g protein and 2.20 g water/g protein, respectively, which were relatively lower than the WHC values in this study. These differences may be attributed to extraction methods used and pretreatments.

Among the protein samples analyzed, SPI exhibited the highest WHC (10.01 g water/ g protein), followed by PPI (6.04 g water/ g protein) and kafirin proteins. In contrast, wheat gluten showed the lowest WHC (1.47 g water/g protein) among the protein samples, reflecting differences in their protein structures and compositions. Lam et al. (2018) reported WHC values of 4.0 g water/g protein and 4.6 g water/g protein for commercial PPI and SPI, respectively, which were lower than the values here, indicating potential differences in extraction or drying methods and protein sources.

Regarding OHC, kafirin proteins extracted from sorghum DGs displayed values ranging from 2.16 to 3.24 g oil/g protein, with the highest values observed in red sorghum types (average of 3 g oil/g protein) and the lowest in white sorghum types (average of 2.49 g oil/ g protein), although not significantly different ($P < 0.05$). In contrast, kafirin proteins extracted from sorghum flours exhibited OHC values ranging from 1.97 to 2.41 g oil/g protein, with higher values in white sorghum types (average of 2.41 g oil/g protein) and the lowest in black sorghum type (1.97 g oil/g protein). A study by Espinosa-Ramírez & Serna-Saldívar (2016) also showed a similar range of OHC from 2.04 to 2.76 g oil/g proteinand kafirins extracted from white sorghum flours had higher OHC, which was consistent with the results in this study. Notably, DG-extracted kafirins demonstrated significantly higher OHC than flour-extracted kafirins ($P <$

0.05), indicating the unfolding of proteins from the fermentation process. Additionally, DG-extracted kafirins contained fewer carbohydrate components, which were hydrolyzed to produce ethanol, allowing the proteins to interact more with the oil fraction. The OHC of commercial plant proteins generally fell below that of kafirin proteins, as lipids and proteins interact through the binding of the aliphatic chains of lipids to the nonpolar side chains of amino acids, and proteins with higher hydrophobicity tend to have a greater propensity to hold oils (Lam et al., 2018).

2.3.8 Solubility

The solubility indicates the proportion of protein that is soluble in a given solvent, typically water. Higher solubility values imply greater protein solubility and dispersibility in water, which can be advantageous in various applications such as food processing and formulation.

In Table 5, among DG-extracted kafirins, white sorghum types exhibited the highest solubility, with a mean value of 15.80%, followed by black sorghum types at 13.05%, and red types at 9.31%. Conversely, solubility of flour-extracted kafirins ranged from 8.65% to 28.32%, with white sorghum type also demonstrating the highest solubility. The solubility trend observed in our study aligns with findings reported by Espinosa-Ramírez & Serna-Saldívar (2016), although the overall values of which were dramatically lower compared to our study due to different testing methods. Nevertheless, the consistent trend indicates that kafirins extracted from white sorghum varieties tend to be more soluble.

Interestingly, DG-extracted kafirins showed significantly lower solubility compared to flour-extracted ones, suggesting that sorghum proteins may undergo structural modifications during heat treatment and fermentation, resulting in decreased intermolecular electrostatic repulsions and colloidal stability against gravitational sedimentation, thereby reducing solubility

(Ryan et al., 2013). This observation contrasts with the study of Semwal & Ms (2022), which reported an increase in kafirin solubility after heat induction compared to the control, indicating the impact of thermal treatment on sorghum proteins. The difference may be attributed to variations in the conditions employed for thermal treatments relative to the fermentation process. Furthermore, among the commercial plant protein samples, SPI displayed the highest solubility at 24.71%.

2.3.9 Emulsifying properties

The emulsifying activity index (EAI) indicates the ability of proteins to form emulsions, with higher values suggesting better emulsifying properties, and the emulsifying stability index (ESI) represents the ability of emulsions to resist phase separation over time, with higher values indicating better stability. The emulsifying properties are presented in Table 5.

For DG-extracted kafirins, the EAI ranged from 5.20 to 8.95 m^2/g. The highest EAI was observed in the black sorghum type (8.95 m^2/g), followed by white sorghum types (mean value of 6.72 m^2/g) and red sorghum types (averaged to 6.12 m^2/g). In contrast, among flour-extracted kafirins, white sorghum type exhibited the highest EAI (8.58 m^2/g). The kafirins had a obviously lower EAI ranging from 1.08 to 1.73 m^2/g reported by Espinosa-Ramírez & Serna-Saldívar (2016), in which the kafirins extracted from white sorghum varieties had a higher EAI compared to that from red varieties aligned with the trend in this study. Conversely, Dias de Carvalho et al. (2024) reported significantly higher EAI values ranged from 102.49 to 125.16 m^2/g, suggesting potential differences in raw materials or extraction methods. Interestingly, fermentation led to a decrease in EAI for DG-extracted kafirins compared to those from sorghum flours, with the exception of Black-NLM-16, which showed an increase. Moreover, SPI and PPI demonstrated

higher EAI compared to extracted kafirins, while wheat gluten exhibited the lowest EAI among the protein samples (5.13 m^2/g).

Regarding ESI values, kafirins extracted from sorghum DGs exhibited significantly high values ranging from 28.75 to 85.56 min. Red sorghum types displayed the highest ESI (64.07 min) compared to black and white types. Conversely, ESI values in kafirins extracted from sorghum flours were significantly lower, ranging from 21.04 to 26.14 min, with red sorghum types still exhibiting the highest ESI (25.37 min) among flour-extracted kafirins. These differences in ESI values may reflect variations in the physicochemical characteristics of proteins, droplet size distribution, and repulsive forces between droplets. The results reported by Dias de Carvalho et al. (2024), had lower ESI ranging from 14.76 to 20.81 min compared to our study, also suggesting potential differences in experimental conditions or protein sources. SPI and PPI had ESI values comparable to those in kafirins extracted from sorghum flours, while wheat gluten had the lowest ESI value among all protein samples (18.32 min).

2.4. Conclusion

Kafirins extracted using the glacial acetic acid method consistently exhibited protein contents ranging from 68.88% to 88.47%. The observed variations in the color of the kafirins reflect the color differences in their original samples. The α-helix structure predominated in kafirin proteins, with ratios ranging from 39.85% to 75.93%. SDS-PAGE profiles showed similarities across different kafirins, although three bands were absent in the 25-37 kDa and 15-20 kDa ranges in DG-extracted kafirins. Kafirins extracted from black sorghum types demonstrated the lowest digestibility, which corresponded to the highest levels of total tannin content. This finding suggests a negative correlation between *in vitro* protein digestibility and

total tannin content, with a correlation coefficient of -0.42. The functionalities of kafirins extracted from DGs or flours exhibited some variations.

DG-extracted kafirins generally displayed higher protein content, surface hydrophobicity, and water and oil holding capacities but lower digestibility and solubility compared to those extracted from sorghum flours. These differences may be attributed to structural changes induced by the fermentation process and underscore the pivotal role of processing methods in shaping the functional properties of kafirin proteins. Comparisons with commercial plant proteins highlighted the distinct functional attributes of kafirin proteins, with SPI and PPI demonstrating higher water holding capacity, solubility, and emulsifying activity, while kafirin proteins excelled in oil holding capacities.

In summary, these findings offer valuable insights into the impact of sorghum types and fermentation processes on the characteristics and functional properties of kafirin proteins, thereby enhancing fundamental understanding of kafirin proteins. These results have the potential to further advance the utilization of sorghum proteins in both food and industrial applications.

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Figures and Tables

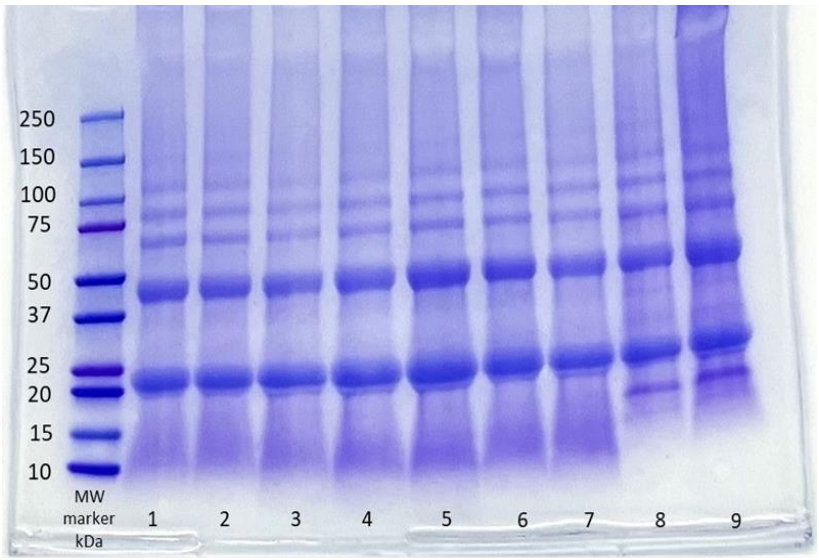

Figure 2.1 SDS-PAGE profile of extracted kafirin proteins (non-reducing condition).

Note: from left to right, the columns are standard, (1) Red-NLM-20 (DGs), (2) Red-Waxy (DGs), (3) White-32020 (DGs), (4) White-F1000 (DGs), (5) Black-NLM-16 (DGs), (6) Red-NLM-SB (DGs), (7) White- 4525 (DGs), (8) Red-NLM-20 (Flour), (9) White-4525 (Flour). (DGs) means the kafirins are extracted from DGs and (Flour) means the kafirins extracted from flours.

Table 2-1 Protein content, yield, and recovery rate of extracted kafirin proteins

Samples	Protein content (%, db)	Yield (%)	Protein recovery (%)
Red-NLM-20 (DGs)	87.91 ± 1.90^{a}	15.02 ± 0.14^{cd}	36.98 ± 1.15^{c}
Red-Waxy (DGs)	84.19 ± 4.95^{ab}	17.53 ± 1.46^{b}	41.87 ± 1.02^{b}
White-32020 (DGs)	82.69 ± 0.71^{abc}	13.20 ± 0.45^{e}	35.65 ± 1.53^{c}
White-F1000 (DGs)	79.94 ± 0.71^{bcd}	13.77 ± 0.49^{de}	36.10 ± 0.98^{c}
Black-NLM-16 (DGs)	83.91 ± 1.72^{ab}	15.82 ± 0.17^{cd}	43.13 ± 1.35^{b}
Red-NLM-SB (DGs)	83.78 ± 0.13^{ab}	8.30 ± 0.13^{a}	24.90 ± 4.97^{d}
White-4525 (DGs)	75.69 ± 4.33^{d}	14.06 ± 1.02^{de}	36.33 ± 0.55^{c}
Red-NLM-20 (Flour)	88.47 ± 3.05^{a}	4.63 ± 0.17^{h}	37.03 ± 2.63^{c}
Red-Waxy (Flour)	76.91 ± 4.02^{cd}	3.97 ± 0.34^{h}	26.98 ± 0.90^{d}
Black-NLM-16 (Flour)	76.69 ± 0.43^{cd}	8.14 ± 0.26^{f}	57.16 ± 0.68^{a}
White-4525 (Flour)	68.88 ± 0.59^{e}	6.04 ± 0.34^{g}	28.87 ± 0.73^{d}

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 based on as-is basis. (DGs) means the kafirins are extracted from DGs and (Flour) means the kafirins extracted from flours.

Table 2-2 Color properties of extracted kafirin proteins and commercial plant proteins

Samples	Color		
	L*	a*	b*
Red-NLM-20 (DGs)	79.93 ± 0.19^{fg}	4.50 ± 0.08^{d}	12.84 ± 0.18^{d}
Red-Waxy (DGs)	78.61 ± 0.09^{h}	4.89 ± 0.02^{c}	11.69 ± 0.04^{h}
White-32020 (DGs)	83.94 ± 0.33^{b}	1.81 ± 0.01^{j}	11.93 ± 0.08^{g}
White-F1000 (DGs)	85.73 ± 0.16^{a}	1.57 ± 0.03^{l}	12.81 ± 0.14^{d}
Black-NLM-16 (DGs)	75.46 ± 0.17^{j}	6.27 ± 0.03^{a}	10.59 ± 0.02^{j}
Red-NLM-SB (DGs)	79.98 ± 0.05^{fg}	3.96 ± 0.02^{e}	12.15 ± 0.07^{f}
White-4525 (DGs)	85.56 ± 0.04^{a}	1.67 ± 0.05^{k}	12.64 ± 0.09^{e}
Red-NLM-20 (Flour)	80.55 ± 0.06^{e}	3.71 ± 0.04^{f}	11.53 ± 0.07^{i}
Red-Waxy (Flour)	79.73 ± 0.05^{g}	3.55 ± 0.02^{g}	9.84 ± 0.06^{k}
Black-NLM-16 (Flour)	71.74 ± 0.07^{k}	5.88 ± 0.02^{b}	9.75 ± 0.04^{k}
White-4525 (Flour)	85.77 ± 0.12^{a}	1.29 ± 0.02^{m}	11.58 ± 0.02^{hi}
PPI	77.65 ± 0.02^{i}	4.91 ± 0.01^{c}	20.89 ± 0.05^{a}
SPI	82.85 ± 0.03^{c}	1.97 ± 0.01^{i}	17.59 ± 0.11^{b}
Gluten	81.87 ± 0.06^{d}	2.20 ± 0.02^{h}	16.27 ± 0.02^{c}

Note: Results are expressed as mean ± SD (n = 2). Different letters indicate significant differences in the same column ($P < 0.05$). (DGs) means the kafirins are extracted from DGs and

(Flour) means the kafirins extracted from flours. SPI means soy protein isolate and PPI means pea protein isolate.

Table 2-3 Secondary structure of proteins from FTIR

Samples	Protein secondary structure ratio %			
	α-Helix	**β-Sheet**	**β-Turn**	**Random coil**
Red-NLM-20 (DGs)	68.02 ± 9.05^{abc}	27.48 ± 8.77^{bc}	4.49 ± 0.27^{d}	0.00 ± 0.00^{c}
Red-Waxy (DGs)	71.55 ± 2.08^{ab}	20.31 ± 3.55^{bcd}	8.13 ± 1.47^{cd}	0.00 ± 0.00^{c}
White-32020 (DGs)	73.08 ± 0.37^{a}	19.88 ± 4.60^{bcd}	7.04 ± 4.97^{cd}	0.00 ± 0.00^{c}
White-F1000 (DGs)	60.31 ± 6.82^{bc}	14.83 ± 2.43^{de}	24.86 ± 4.40^{b}	0.00 ± 0.00^{c}
Black-NLM-16 (DGs)	75.93 ± 4.00^{a}	18.91 ± 1.42^{bcde}	5.16 ± 2.58^{d}	0.00 ± 0.00^{c}
Red-NLM-SB (DGs)	74.94 ± 0.57^{a}	20.14 ± 0.29^{bcd}	4.92 ± 0.28^{d}	0.00 ± 0.00^{c}
White-4525 (DGs)	73.47 ± 5.16^{a}	22.95 ± 5.78^{bcd}	3.57 ± 0.62^{d}	0.00 ± 0.00^{c}
Red-NLM-20 (Flour)	39.85 ± 0.16^{d}	51.68 ± 2.77^{a}	8.47 ± 2.61^{cd}	0.00 ± 0.00^{c}
Red-Waxy (Flour)	72.25 ± 5.38^{ab}	14.93 ± 1.15^{de}	12.81 ± 4.23^{c}	0.00 ± 0.00^{c}
Black-NLM-16 (Flour)	66.20 ± 3.02^{abc}	12.97 ± 0.65^{de}	20.83 ± 2.37^{b}	0.00 ± 0.00^{c}
White-4525 (Flour)	58.77 ± 0.16^{c}	9.38 ± 0.41^{e}	31.84 ± 0.57^{a}	0.00 ± 0.00^{c}
PPI	16.24 ± 7.93^{e}	51.75 ± 7.75^{a}	4.92 ± 1.09^{d}	27.09 ± 0.91^{b}
SPI	24.64 ± 2.94^{e}	17.92 ± 4.19^{cde}	7.70 ± 4.04^{cd}	49.73 ± 5.29^{a}
Gluten	14.63 ± 5.72^{e}	28.73 ± 3.41^{b}	5.03 ± 0.17^{d}	51.61 ± 2.13^{a}

Note: Results are expressed as mean ± SD (n = 2). Different letters indicate significant differences in the same column ($P < 0.05$). (DGs) means the kafirins are extracted from DGs and (Flour) means the kafirins extracted from flours. SPI means soy protein isolate and PPI means pea protein isolate.

Table 2-4 Surface hydrophobicity, digestibility, and total tannin content of proteins

Samples	Surface hydrophobicity (µg SDS/ mg protein)	*In vitro* protein digestibility %	Total tannin content (mg catechin equivalents/g protein)
Red-NLM-20 (DGs)	51.37 ± 0.31^{k}	77.24 ± 0.51^{de}	0.45 ± 0.02^{e}
Red-Waxy (DGs)	59.02 ± 0.64^{c}	79.05 ± 0.51^{c}	0.49 ± 0.01^{e}
White-32020 (DGs)	56.52 ± 0.55^{ef}	79.33 ± 0.13^{c}	0.02 ± 0.01^{h}
White-F1000 (DGs)	55.32 ± 0.39^{g}	77.97 ± 0.51^{d}	0.08 ± 0.02^{g}
Black-NLM-16 (DGs)	55.63 ± 0.06^{fg}	74.80 ± 0.13^{h}	2.44 ± 0.05^{b}
Red-NLM-SB (DGs)	57.53 ± 0.22^{d}	75.34 ± 0.13^{gh}	1.05 ± 0.33^{c}
White-4525 (DGs)	53.22 ± 0.70^{hi}	75.80 ± 0.03^{fg}	0.00 ± 0.00^{h}
Red-NLM-20 (Flour)	52.02 ± 0.11^{jk}	79.24 ± 0.06^{c}	0.46 ± 0.03^{e}
Red-Waxy (Flour)	56.99 ± 0.33^{de}	79.33 ± 0.38^{c}	0.69 ± 0.04^{d}
Black-NLM-16 (Flour)	52.89 ± 0.11^{ij}	76.25 ± 0.38^{f}	5.70 ± 0.04^{a}
White-4525 (Flour)	56.91 ± 0.22^{de}	77.06 ± 0.77^{e}	0.01 ± 0.01^{h}
PPI	64.87 ± 0.36^{b}	85.75 ± 0.06^{a}	0.15 ± 0.01^{f}
SPI	68.00 ± 0.53^{a}	86.20 ± 0.38^{a}	0.00 ± 0.00^{h}
Gluten	53.89 ± 0.61^{h}	83.31 ± 0.13^{b}	0.74 ± 0.03^{d}

Note: Results are expressed as mean ± SD (n = 2). Different letters indicate significant differences in the same column ($P < 0.05$). (DGs) means the kafirins are extracted from DGs and (Flour) means the kafirins extracted from flours. SPI means soy protein isolate and PPI means pea protein isolate.

Table 2-5 Water/ oil holding capacity, solubility, and emulsifying properties of proteins

Samples	Water holding capacity (g water/ g protein)	Oil holding capacity (g oil/ g protein)	Solubility %	Emulsifying activity index (m^2/ g)	Emulsifying stability index (min)
Red-NLM-20 (DGs)	2.85 ± 0.02^{d}	2.97 ± 0.09^{ab}	8.99 ± 0.05^{i}	5.45 ± 0.05^{g}	58.06 ± 1.84^{c}
Red-Waxy (DGs)	2.84 ± 0.08^{d}	2.81 ± 0.22^{bc}	11.64 ± 0.40^{h}	5.34 ± 0.05^{g}	85.56 ± 1.91^{a}
White-32020 (DGs)	2.41 ± 0.02^{g}	2.58 ± 0.24^{cd}	12.30 ± 0.54^{h}	7.15 ± 0.04^{e}	41.55 ± 1.55^{f}
White-F1000 (DGs)	2.71 ± 0.06^{de}	2.74 ± 0.31^{bc}	15.34 ± 0.26^{f}	5.20 ± 0.04^{g}	79.87 ± 1.96^{b}
Black-NLM-16 (DGs)	2.12 ± 0.03^{i}	2.57 ± 0.02^{cd}	13.05 ± 0.08^{g}	8.95 ± 0.30^{b}	55.10 ± 0.86^{d}
Red-NLM-SB (DGs)	3.42 ± 0.04^{c}	3.24 ± 0.03^{a}	7.29 ± 0.20^{j}	7.58 ± 0.04^{d}	48.59 ± 0.66^{e}
White-4525 (DGs)	2.41 ± 0.04^{g}	2.16 ± 0.01^{efg}	19.78 ± 0.09^{c}	7.81 ± 0.07^{d}	28.75 ± 0.12^{g}
Red-NLM-20 (Flour)	2.61 ± 0.02^{ef}	2.04 ± 0.06^{fg}	8.65 ± 0.31^{i}	6.11 ± 0.02^{f}	24.60 ± 0.65^{hi}
Red-Waxy (Flour)	2.56 ± 0.01^{f}	2.28 ± 0.02^{ef}	15.99 ± 0.40^{f}	7.89 ± 0.13^{d}	26.14 ± 0.53^{h}
Black-NLM-16 (Flour)	2.25 ± 0.01^{h}	1.97 ± 0.05^{g}	17.49 ± 0.36^{e}	7.94 ± 0.26^{d}	21.04 ± 0.05^{j}
White-4525 (Flour)	2.08 ± 0.01^{i}	2.41 ± 0.09^{de}	28.32 ± 0.47^{a}	8.58 ± 0.18^{c}	23.42 ± 0.06^{ij}
PPI	6.04 ± 0.08^{b}	1.19 ± 0.02^{h}	18.57 ± 0.46^{d}	10.65 ± 0.07^{a}	21.53 ± 0.36^{j}
SPI	10.01 ± 0.19^{a}	1.46 ± 0.01^{h}	24.71 ± 0.10^{b}	9.00 ± 0.04^{b}	21.07 ± 0.53^{j}
Gluten	1.47 ± 0.03^{j}	1.17 ± 0.01^{h}	5.78 ± 0.02^{k}	5.13 ± 0.43^{g}	18.32 ± 1.03^{k}

Note: Results are expressed as mean ± SD (n = 2). Different letters indicate significant differences in the same column ($P < 0.05$). (DGs) means the kafirins are extracted from DGs and (Flour) means the kafirins extracted from flours. SPI means soy protein isolate and PPI means pea protein isolate.

Chapter 3 - Enzymatic production and physicochemical and functional properties of sorghum protein isolates

Abstract

Grain sorghum has emerged as a promising source for producing alternative proteins, yet current extraction methods often lack efficiency. In this study, we developed a novel enzymatic approach using α-amylases and cellulases on sorghum materials 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) using both white and red sorghums. 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 (protein content 75-76%) and DDG (protein content 45-50%). Physicochemical properties and functionalities of the isolated sorghum proteins were analyzed and compared with those of 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 commercial plant protein isolates. These characteristics remained consistent across the different sorghum protein samples. 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.

Keywords: Sorghum protein isolation, wet milling, enzyme processing, protein functionality

3.1 Introduction

With the increasing population and emphasis on personal nutrition (Fischer et al., 2023), it is estimated that protein production for human food may need to double by 2050 (Alrosan et al., 2022). Therefore, developing alternative proteins to those conventional animal proteins is crucial (Rajpurohit & Li, 2023). 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 grains, legumes, and oilseeds (Kumar et al., 2022).

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 (Tanwar et al., 2023). 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 (Dias de Carvalho et al., 2024).

The protein content of sorghum varies from 6% to 18%, with an average of 11% (Lasztity, 2017). 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 protein extraction, such as aqueous ethanol, glacial acetic acid, hydrochloric acid, or

sodium hydroxide with the addition of reducing agents (Pérez Sira & Lares Amaiz, 2004, Taylor et al., 2005, Wang et al., 2009, Bean et al., 2006, Espinosa-Ramírez & Serna-Saldívar, 2016). Despite these methods, protein yield has remained relatively low, ranging from only 32.0% to 59.3% (Li et al., 2018), as these methods can only extract kafirin but leave the glutelin protein in the residue.

Wet milling, a common industrial process that separates components like germ, endosperm, bran, fiber, starch, and protein in cereal grains (De Mesa-Stonestreet et al., 2010), results in a relatively moderate protein content in the gluten meal fraction, ranging from 44.3% to 58.2% (Buffo et al., 1998; Xie & Seib, 2000), 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. (2006) 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 (Souza & Magalhães, 2010). Cellulase, another widely used industrial enzyme, degrade cellulose by hydrolyzing β-1,4-glycosidic bonds (Ejaz et al., 2021). 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 extracted 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 enzymes on protein characteristics.

3.2 Materials and methods

3.2.1 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^{TM} DA-16) as described previously (M. Liu et al., 2023). Commercial soybean protein isolates (SPI), pea protein isolates (PPI), and wheat gluten were used for comparison. Cellulase (Cellic Ctec2, 1,000 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).

3.2.2 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 (Pulivarthi et al., 2021), 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 DGs (RDG) and white sorghum DGs (WDG).

Wet milling procedures were carried out following the method described by Wang & Chung (2002) with some modification. 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 ℃ for 36 hours. 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 15,000 rpm 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 (Bé =1.04) with distilled water before being pumped onto 5.08-cm × 2.44-m aluminum starch tables inclined at a slope of 1 inch 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 5,000 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 ℃ 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 procedures of Wang & Chung (2002).

3.2.3 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. (2006) 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 ℃ 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 ℃ then maintained for 2 h to hydrolyze the starch component. The slurry was then centrifuged (8000g, 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.

3.2.4 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, 08-01.01, 30-10.01, 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:

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

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

3.2.5 Scanning electron microscope (SEM)

The morphology of the protein powders was analyzed using SEM (S-3500N, Hitachi Scientific Instruments, Mountain view, CA, USA) at magnifications of 100× and 1000× with 5kV power.

3.2.6 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) (Chen et al., 2019). The relative areas of the amide I region were determined using OriginPro 2016 software (OriginLab, Inc., Northampton, MA, USA).

3.2.7 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The protein profiles were determined by SDS-PAGE based on the method of Hong et al. (2022) 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 x 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.

3.2.8 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).

3.2.9 Protein solubility

The solubility of proteins was assessed following a modified method described by K. Liu & Hsieh (2008) 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)**: 8M Urea and 50 mM dithiothreitol (DTT) and 2% (w/v) sodium dodecyl sulfate (SDS) + 2M 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 + 2M thiourea + 2% (w/v) CHAPS in phosphate buffer (D+S+T+C+P); (3) **IEF w/o DTT**: 8M Urea and 2% (w/v) SDS + 2M thiourea + 2% (w/v) CHAPS in phosphate buffer (U+S+T+C+P); (4) **IEF w/o Urea and DTT**: 2% (w/v) SDS + 2M thiourea + 2% (w/v) CHAPS in phosphate buffer (S+T+C+P); (5) **IEF w/o Thiourea, SDS, and CHAPS**: 8M Urea and 50 mM DTT in phosphate buffer (U+D+P); (6) **IEF w/o DTT, thiourea, SDS, and CHAPS**: 8M 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 8000g 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.

3.2.10 *In vitro* protein digestibility

The protein digestibility was determined as described by Hsu et al. (1977) 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 ℃

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 ℃ 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. (2012):

$$Protein\ digestibility = 65.66 + 18.10 \times \Delta pH_{10\ min}$$

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

3.2.11 Surface hydrophobicity

Surface hydrophobicity (H_0) was determined using sodium dodecyl sulfate (SDS) binding method according to Tang et al. (2021). 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.

3.2.12 Free and total sulfhydryl content

The free and total sulfhydryl contents were determined following the method of Beveridge et al. (1974). A 75 mg sample was dispersed in 10 mL of Tris-Gly-Urea buffer (containing 0.086mol/ 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 hour 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 ℃ 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:

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

$$Disulfide\ bonds\ content\ (\mu M/g) = (Total\ SH\ content - Free\ SH\ content)/2$$

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/ (1.36×10^4) [1.36×10^4 was the molar absorptivity of Ellman' reagent and10^6 was for conversions from the molar basis to the μM/mL basis and from mg sample to g sample].

3.2.13 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 (2016) 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:

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

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:

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

3.2.14 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 $P < 0.05$.

3.3 Results and discussion

3.3.1 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% (Espinosa-Ramírez et al., 2017; Buffo et al., 1998) . 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 extracted samples weight relative to the initial material weight, while the protein recovery percentages indicate the proportion of the extracted 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 increase 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. (2020) 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 extract from sorghum flours increased significantly (approximately 20%, $P<0.05$) after the addition of cellulase in their extraction 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 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) (Y. Wang et al., 2009); 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 concentrating. In most cases, the incorporation of

cellulase led to increased protein content, indicating its potential in enhancing protein extraction 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. (2020), 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.

3.3.2 Proximate analysis 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 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 (2016b) 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).

3.3.3 SEM analysis

The microstructure of both sorghum proteins and commercial proteins is shown in Figure 1. Under SEM, the isolated sorghum proteins 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. (2023), where amylase was used to degrade starch, leading to higher sorghum protein content. In contrast, Musigakun & Thongngam (2007) 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 (Hamaker & Bugusu, 2003). Comparison of Figures 1A, 1B with Figures 1C, 1D 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 (Neumann et al., 1984). Additionally, Figure 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. (2009), who used the ethanol method to extract kafirins, exhibited mainly spherical particles. Comparatively, the microstructures of SPI and PPI (Figure 1E and 1F) exhibited similar spherical shapes with relatively smooth surfaces. In contrast, the wheat gluten in Figure 1G displayed numerous aggregates, due to the intrinsic cohesivity and high processing temperature employed in industrial production.

3.3.4 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}) (Kong & Yu, 2007). 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}) (Vanga et al., 2016). 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 (Hamaker & Bugusu, 2003). 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. (2020), Elkhalifa et al. (2009), and, Emmambux & Taylor (2009), 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. (2017)' s report of a predominant β-sheet structure in extracted kafirin. However, in the study by Espinosa-Ramírez & Serna-Saldívar (2016b), 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.

3.3.5 SDS-PAGE

The SDS-PAGE profile provided clear molecular weight information for different proteins. Figures 2a and 2b 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 37-75 kDa likely corresponds to the dimer (45 kDa) in sorghum proteins (Byaruhanga et al., 2005) and the faint bands of high molecular weight above 75 kDa are probably kafirin trimers and tetramers. Ioerger et al. (2020) 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 (2009) and Wang et al. (2009) 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. (2017) 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 (2016b) 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 (2009).

The profiles of PPI and SPI were initially faint under non-reducing conditions, but became clearer upon the addition of the reducing 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 (Zhao et al., 2020). 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 (Adal et al., 2017). 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) (Gao et al., 2010).

3.3.6 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 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. (2011).

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 (Temba et al., 2016). 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) (Khan et al., 2017). 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. (2011). This characteristic can contribute to their functionalities of low solubility in water and low water holding capacity, as substantiated in subsequent tests.

3.3.7 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.

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, Figure 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 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 (2016b) 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.

3.3.8 *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) , Qin et al., 2022). 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, 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% (Espinosa-Ramírez & Serna-Saldívar, 2016b), 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 (Duodu et al., 2003). Additionally, the high-temperature extraction process employed for sorghum proteins may have contributed to decreased protein digestibility, as reported by Emmambux & Taylor (2009). Espinosa-Ramírez et al. (2017) 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 (Dias de Carvalho et al., 2024). Under optimal conditions, sorghum tannins can bind and precipitate proteases and proteins significantly. (Butler et al., 1984). 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.

3.3.9 Surface hydrophobicity

Surface hydrophobicity reflects the exposure of hydrophobic amino acid residues on the protein surface and can affect protein solubility and interfacial interactions (Tomaz, 2017). 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.

3.3.10 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 (Taylor & Richardson, 1980; Shimada & Cheftel, 1988; Van Der Plancken et al., 2005).

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 extraction process, some free sulfhydryl groups might have been lost.

3.3.11 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 (Zayas, 1997). 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. (2020), 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. (2017) 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 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 (Belton et al., 2006). 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.

3.4 Conclusions

The utilization of both wet milling and enzymatic treatments proves beneficial for enhancing sorghum protein concentration. While amylase and cellulase 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 also increases protein recovery to approximately 92% using

white sorghum gluten meal. While this enzymatic treatment improves protein isolation from sorghum flours, its impact on sorghum distillers' grains is less pronounced.

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. 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.

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Figures and tables

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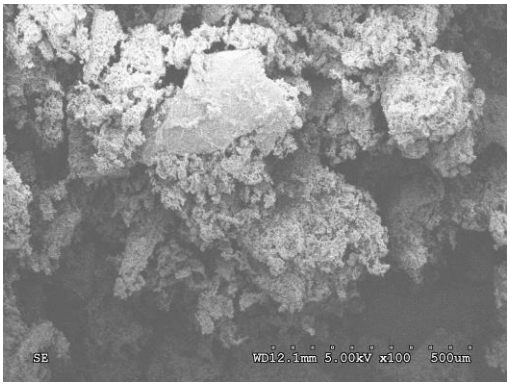

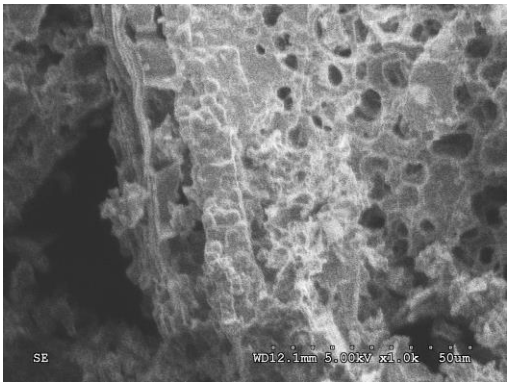

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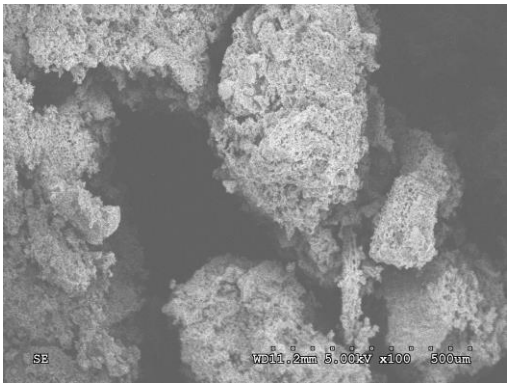

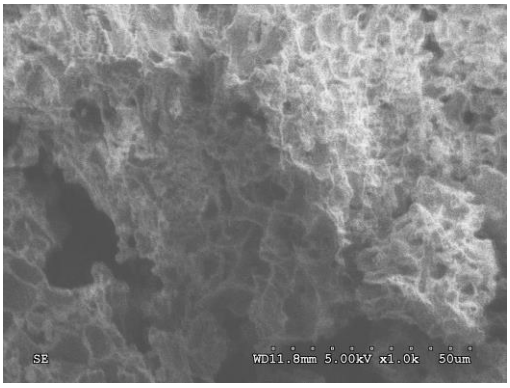

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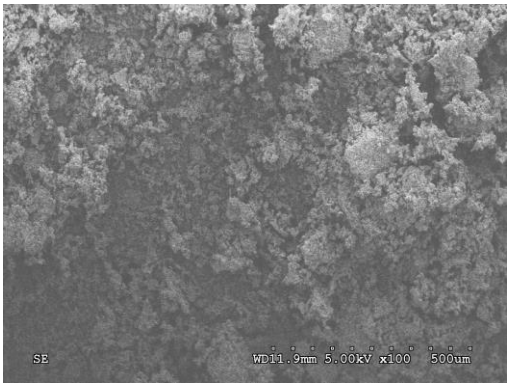

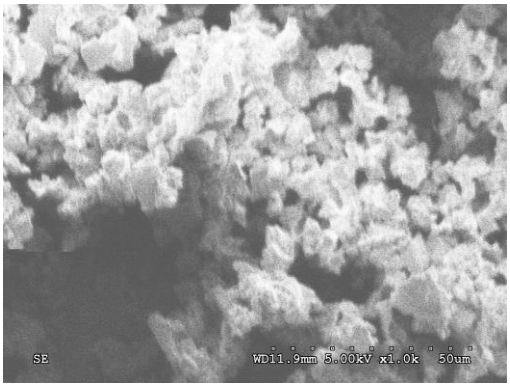

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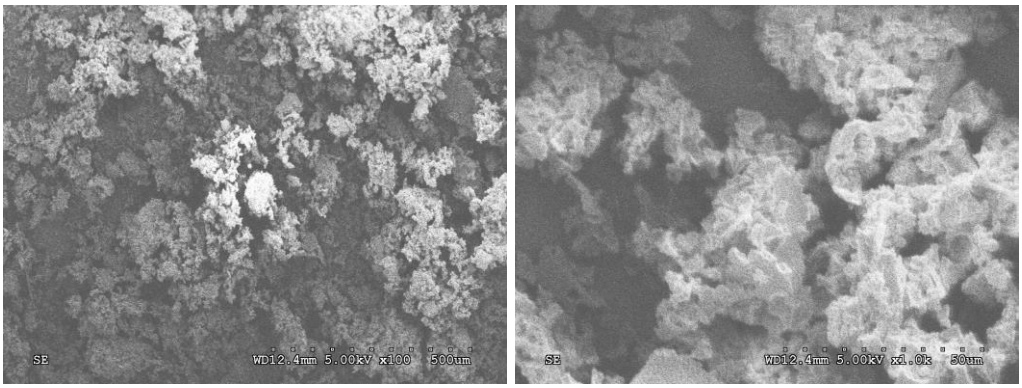
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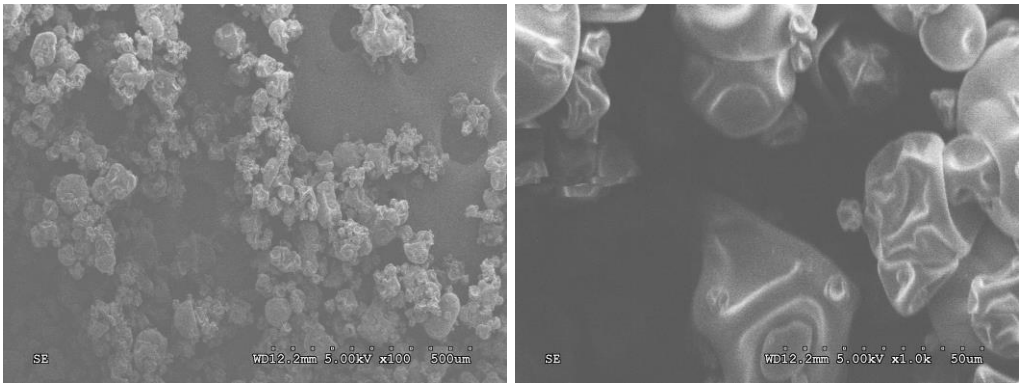
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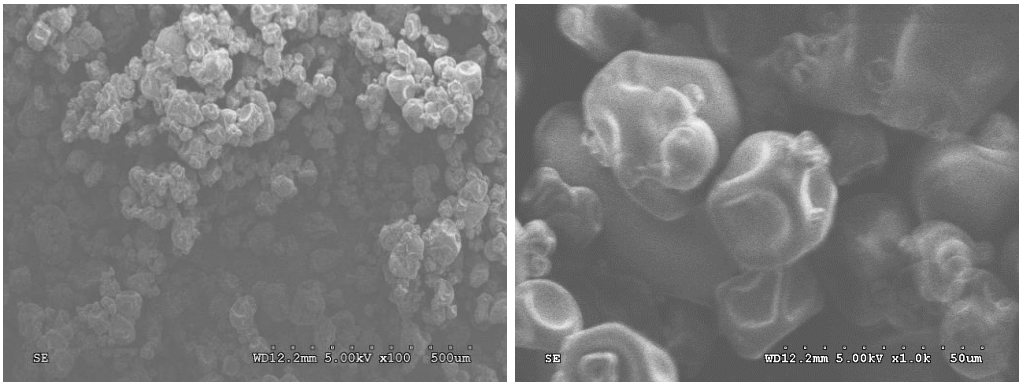
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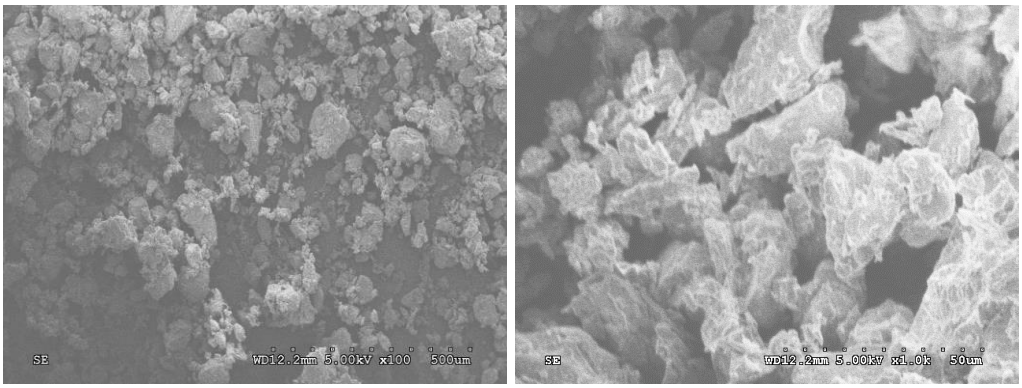

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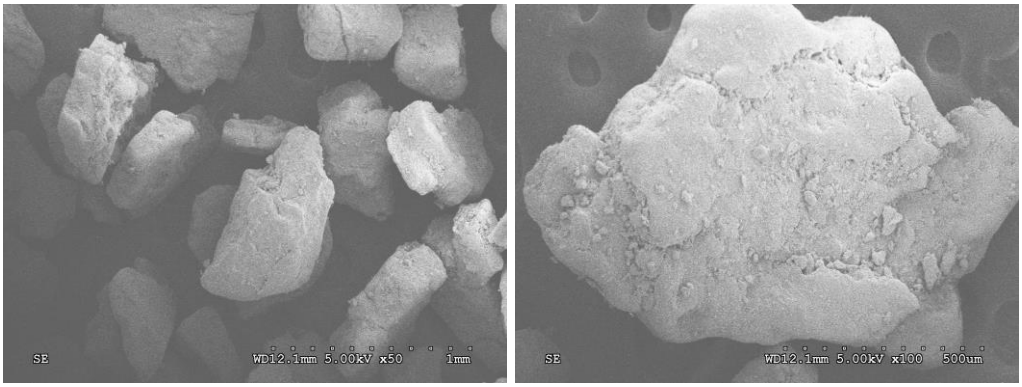

Figure 3.1 SEM analysis of proteins

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.

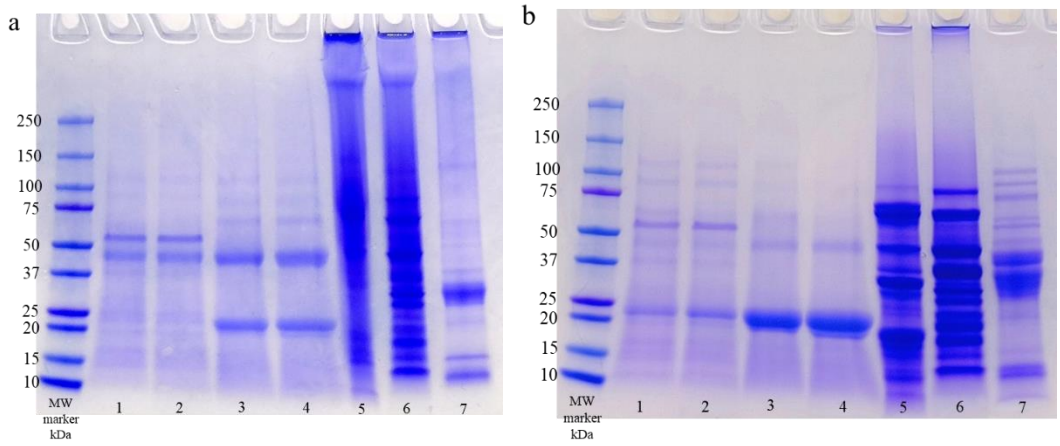

Figure 3.2 SDS-PAGE profile of proteins: a. non-reducing condition; b. reducing condition.

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.

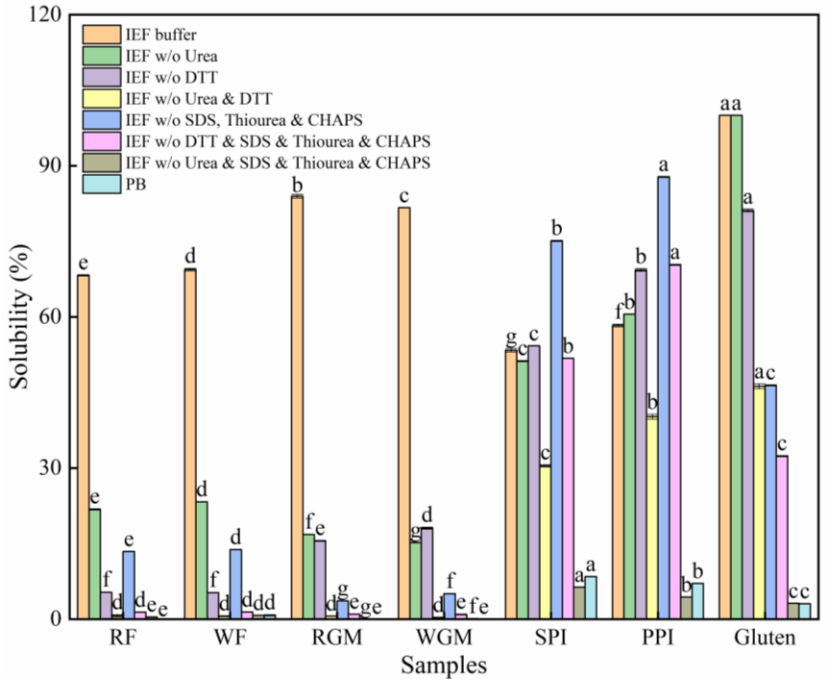

Figure 3.3 Solubility results of proteins in different extraction solvents

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.

Table 3-1 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 DGs	28.42	RDG (A)	RDG (A + C)
White sorghum DGs	26.66	WDG (A)	WDG (A + C)

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

Table 3-2 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.

Table 3-3 Composition of isolated sorghum proteins

Samples	Protein content %	Crude fat content %	Moisture content %	Ash content %
RF (A)	51.98 ± 0.54^{j}	8.38 ± 0.12e^{fg}	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.

Table 3-4 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.

Table 3-5 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.

Table 3-6 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	√					

Table 3-7 Water and oil holding capacity and *in vitro* protein 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.

Table 3-8 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.

Chapter 4 - Physicochemical and functional properties of plant proteins before and after extrusion texturization

Abstract

With the growing demand for protein intake, animal meat production has experienced rapid expansion. However, the utilization of animal meat poses numerous environmental, animal welfare, biodiversity, and human health concerns. Plant-based meat alternatives offer significant potential to partially replace animal-based meat. Texturized vegetable proteins (TVPs) represent critical meat substitutes produced through extrusion processing. Therefore, in our study the low-moisture extrusion process was performed using a twin-screw extruder to texturize raw materials with varying proportions of cold-swelling proteins (CS) ranging from 0 to 90%. The ingredients, raw-material blends, and resulting texturized proteins were systematically characterized to understand the impact of CS ratios and extrusion processing on the physicochemical and functional properties of the proteins. Fourier transform infrared spectroscopy (FTIR) revealed a decrease in α-helix structure accompanied by an increase in β-sheet content after extrusion, indicating significant conformational changes in protein structures. Solubility tests conducted with different extracting buffers highlighted disulfide bonds as the primary force maintaining fibrous structure integrity. The results demonstrated a significant decrease ($P < 0.05$) in free amino and sulfhydryl group content in the proteins, as well as surface hydrophobicity after extrusion. Protein digestibility, water holding capacity, and oil holding capacity exhibited fluctuating trends with varying CS ratios. Both emulsifying and foaming properties were diminished post-extrusion. Moreover, there was a general increase in least gelation concentration in texturized proteins, indicating reduced gel-forming properties. In summary, the extrusion

process induced protein-protein interactions within samples and led to diverse changes in various properties, enabling the development of improved fibrous structures suitable as meat alternatives with excellent texture, thereby enhancing their utility in various industries.

Keywords: Plant-based proteins, extrusion process, texturized proteins, protein-protein interactions

4.1 Introduction

Animal-based proteins are favored in the food markets due to their desirable taste, complete amino acid composition, and good functionalities for high-quality food production (Ozturk & Hamaker, 2023). However, meat products have significant impacts on the anthropogenic environment. Their production processes require substantial water and land resources and contribute to elevated greenhouse gas emissions, including methane, carbon dioxide, and ammonia (Fresán et al., 2019; Aiking & de Boer, 2020). Additionally, the transportation of meat products presents challenges due to their perishable nature, necessitating cold environments. During this supply chain, they are susceptible to being disrupted or affected by diseases like swine flu and avian flu (Ozturk & Hamaker, 2023). Besides, some studies have shown that several human diseases such as obesity, type 2 diabetes, cardiovascular disease, stroke, and colorectal cancer have a relationship with the red meat diet (Willett et al., 2019) and the high fat content of meat is also associated with a high-risk factor for human health (X. Zhang et al., 2022). Consequently, the attention of consumers has transferred to find more environmentally friendly and healthy protein sources. Meat alternatives, including plant-based, cell-based (cultured meat), fermentation-based (mycoprotein) (Souza Filho et al., 2019), algae-based, and insect-based sources, have emerged as viable options.

Plant-based sources are commonly utilized due to their lower costs but replicating the texture of meat-based products to achieve similar mouthfeel, texture, and sensory characteristics presents a critical challenge. Animal proteins possess a muscle structure absent in plant-based proteins, necessitating the formation of fibrous structures using plant-based sources, which requires specific processing techniques and ingredients. Various processing techniques, such as extrusion, shear-structuring (shear cell), 3D printing, electrospinning, and freeze-structuring, can

be employed to create fibrous anisotropic structures (Ozturk & Hamaker, 2023). Among these, extrusion technology is the most mature technique for producing meat analogues, offering advantages such as low processing costs, continuous production, improved energy utilization, and high output (T. Zhang et al., 2023). According to the differences in moisture content, the extrusion method can be divided into two types: low-moisture extrusion and high-moisture extrusion. Low-moisture extrusion, conducted at moisture levels of 30 to 40%, produces texturized vegetable proteins (TVP), which require rehydration or further processing before use, but have a longer shelf life (Y. Zhang & Ryu, 2023; Webb et al., 2023). High-moisture extrusion, performed at moisture levels of about 60-65%, yields high moisture meat analogs (HMMA) with a meat-like texture that can be used without further hydration needs but have a shorter shelf-life due to their high moisture content (Akdogan, 1999; Webb et al., 2023).

In addition to processing techniques, the selection of appropriate raw materials is crucial for extrusion. Soybean proteins have long been favored due to their balanced amino acid composition and widespread consumer acceptance (Sui et al., 2021). Pea proteins are gaining attention for their cost-effectiveness, lack of allergens, non-genetically modified nature, and cholesterol-free properties (McCarthy et al., 2016; Y. Liu et al., 2020). Wheat gluten, a by-product of wheat flour isolation, offers favorable viscoelastic and thermal coagulation characteristics (X. Zhang et al., 2022). These proteins possess unique functionalities, categorized based on their water absorption capacity, viscosity peak time, and least gelation concentration into cold-swelling proteins (CS) and heat-swelling proteins (HS) (Flory et al., 2023). Proteins exhibiting cold-swelling properties showed a greater propensity for crosslinking, resulting in a porous internal structure with reduced layering. Conversely, proteins with heat-swelling characteristics led to a denser, more stratified extrudate structure (Flory et al., 2023).

In this study, raw materials with varying CS ratios were used, and twin-screw extrusion under low moisture conditions was conducted. Analyses of both raw materials and resulting extruded products were performed to elucidate the underlying mechanisms involved in extrusion texturization. The analyses included assessments of protein secondary structure, solubility, free amino content, free sulfhydryl content, surface hydrophobicity, in vitro protein digestibility (IVPD), water holding capacity (WHC), oil holding capacity (OHC), emulsifying activity index (EAI), emulsifying stability index (ESI), foaming capacity (FC), and least gelation concentration (LGC). Our objective is to comprehend changes in physicochemical properties and functionalities of samples during extrusion and provide fundamental insights into the effects of CS ratio and extrusion processes on protein structures.

4.2 Materials and methods

4.2.1 Materials

The samples used in this study remained the same with those detailed in the research conducted by Flory et al. (2023), comprising six treatments with varying cold-swelling protein ratios: 0% CS, 30% CS, 40% CS, 50% CS, 60% CS, and 90% CS. These samples consisted of blends of soybean protein isolate (SPI), two variants of soybean protein concentrate (Arcon F and Arcon S SPC), soybean flour, pea protein isolate (PPI), and wheat gluten (Gluten). According to the classification provided in the aforementioned study, the protein sources can be categorized into two groups based on their water absorption index (WAI) and the time of viscosity formation. Proteins exhibiting a high WAI (> 4.0 g/g) and rapid viscosity peak formation (< 4 min) were classified as cold-swelling proteins, while those demonstrating different characteristics were referred to as heat-swelling proteins. Based on the report from Flory et al. (2023), the soybean protein isolate (SPI), soybean protein concentrate (SPC Arcon S

type), and pea protein isolate (PPI) were identified as CS proteins, while soybean protein concentrate (SPC Arcon F type) and wheat gluten were classified as HS proteins. Although samples with varying cold swelling ratios utilized different protein ingredients, the overall protein content across all samples remained relatively consistent. Both individual ingredients and the resulting raw-material blends, as well as the texturized proteins from the treatments, were utilized in the subsequent experiments. The protein samples mentioned were all ground into fine powder using a coffee grinder and subsequently stored in a refrigerator at 4°C for later use.

4.2.2 FTIR Spectroscopy

FTIR spectroscopy was conducted using a PerkinElmer Spectrum 400 FT-IR/FTNIR Spectrometer (PerkinElmer, Inc., Waltham, MA, USA) equipped with an attenuated total reflectance cell (ATR) accessory. The samples were kept on diamond crystal and were secured tightly with a clamp for the analysis. Each sample underwent 64 scans within the range of 400–4000 cm^{-1}, with a 4 cm^{-1} interval. The relative areas of the amide I region (1600 cm^{-1} to 1700 cm^{-1}) were determined using OriginPro 2016 software (OriginLab, Inc., Northampton, MA, USA).

4.2.3 Solubility

The determination of protein solubility were conducted in different extracting buffers to analyze the protein-protein interactions indirectly, following the method from Liu & Hsieh (2008) with modifications. 200 mg sample was mixed with 10 ml of each extractant and shaken for 2 h (300 rpm) at room temperature to get even suspension. The supernatant was obtained by centrifugation at 8000 g for 15 min. The protein content in the supernatant was quantified using the Bradford assay. The extractants include: (1) **Isoelectric focus buffer (IEF)**: 8M urea and 50 mM dithiothreitol (DTT) and 2% (w/v) sodium dodecyl sulfate (SDS) + 2M 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 + 2M thiourea + 2% (w/v) CHAPS in phosphate buffer (D+S+T+C+P); (3) **IEF w/o DTT**: 8M urea and 2% (w/v) SDS + 2M thiourea + 2% (w/v) CHAPS in phosphate buffer (U+S+T+C+P); (4) **IEF w/o Urea and DTT**: 2% (w/v) SDS + 2M thiourea + 2% (w/v) CHAPS in phosphate buffer (S+T+C+P); (5) **IEF w/o Thiourea, SDS, and CHAPS**: 8M urea and 50 mM DTT in phosphate buffer (U+D+P); (6) **IEF w/o DTT, thiourea, SDS, and CHAPS**: 8M 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). Protein solubility was assessed by comparing the protein content in the supernatant to that of the samples, presenting it as a ratio.

4.2.4 *In vitro* protein digestibility

Protein digestibility was determined according to the method from Hsu et al. (1977) with some modifications. A 30 mL sample solution containing 6.25 mg of protein per milliliter was prepared, followed by adjustment of the pH to 8.00 (± 0.02) using 1 N HCl and/or NaOH. Simultaneously, a multienzyme solution was prepared consisting of 1.6 mg trypsin, 3.1 mg α-chymotrypsin, and 1.3 mg protease per milliliter of distilled water. The pH of the enzyme solution was similarly adjusted and maintained at 8.00 (± 0.02). After mixing 3 mL of the enzyme solution with the sample at 37°C for 10 min, the pH drop was recorded using a pH meter. The equation was based on the literature of Tinus et al. (2012):

$$Protein\ digestibility = 65.66 + 18.10 \times \Delta pH_{10\ min}$$

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

4.2.5 Determination of surface hydrophobicity and free sulfhydryl groups

Surface hydrophobicity (H_0) was determined using SDS binding method, in accordance with the approach detailed by Tang et al. (2021). The determination of free sulfhydryl content followed the method described by Beveridge et al. (1974).

4.2.6 Determination of free amino groups

The method outlined by Adler-Nissen (1979) was used to determine free amino content with some modifications. 0.5 mL of a sample solution, with a concentration of 4 mg/mL and dissolved in a 1% (w/v) SDS solution, was combined with 4 mL of 0.2125 M phosphate buffer (pH 8.2, the mixture of 4.5 mL 0.2125 M NaH_2PO_4 and 100 mL 0.2125 M Na_2HPO_4) and 4 mL of 0.1% (v/v) 2,4,6-trinitrobenzene sulfonic acid (TNBS). This suspension was then incubated in a 50°C water bath at 200 rpm for 1 h in darkness. Following the hour-long reaction, 8 mL of 0.1 N HCl was introduced to the solution, which was then stored at room temperature for 30 min. The resulting supernatant was obtained by centrifugation at 8000 g for 10 min, and its absorbance was measured using a double beam spectrometer (VWR UV-6300PC, Radnor, PA, USA) at 340 nm. Additionally, L-leucine solutions were prepared following the same procedure, but with varying concentrations (ranging from 0 to 2.4 mM), to establish a standard curve.

4.2.7 Functional properties

The water and oil holding capacity were quantified following the approach detailed by Espinosa-Ramírez & Serna-Saldívar (2016) with modifications. For water holding capacity determination, 0.25 g of the sample (W_0) was combined with 7.5 mL of distilled water in a 15 mL centrifuge tube (W_2) and shaken for 30 min at 300 rpm. After centrifugation at 4500 g for 15 min, the tube was inverted for 5 minutes to drain water residues, and the final weight was recorded (W_1). Water holding capacity was calculated using the formula:

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

Similarly, for oil holding capacity determination, a 0.25 g sample (O_0) was mixed with 7.5 mL of soybean oil in a 15 mL centrifuge tube (O_2). After shaking, centrifugation, and inversion to drain the oil, the final precipitate weight was recorded (O_1). The oil holding capacity was calculated as same:

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

The determination of both emulsifying capacity was conducted in accordance with a turbidimetric method detailed by Espinosa-Ramírez & Serna-Saldívar (2016) with some modifications. The procedure involved adding 0.25 g of samples to 25 mL of 100 mM phosphate buffer (pH = 7) and stirring until a uniform solution was achieved. Next, 25% (v/v) soybean oil was dispersed into the solution, followed by homogenization using homogenizer (Polytron PT 2500 E, Kinematica AG, Switzerland) at 14,000 rpm for 1 min at room temperature. After homogenization, 50 µL of the solution was mixed with 2.95 mL of 0.1% (w/v) SDS buffer and vortexed. The absorbance of the diluted emulsion was then measured at 500 nm (A_0) and again after 10 min (A_{10}). The Emulsification Activity Index (EAI) and Emulsion Stability Index (ESI) were subsequently calculated using the following formulas:

$$T = 2.303 \times \frac{A_0}{L} \times D$$

$$EAI\ (m^2/g) = \frac{2 \times T}{\varphi \times C \times 1000}$$

$$ESI\ (min) = \frac{A_0}{A_0 - A_{10}} \times t$$

where T is the turbidity of emulsion, A is the absorbance at 500 nm, D is dilution factor, L is the path length of light (m), φ is the oil volume fraction, C is the protein concentration in the dispersion (mg/ mL), t is 10 min.

The foaming capacity and stability of samples were assessed using the protocol described by Shen et al. (2021). The method described by Shen & Li (2021) was also employed to determine the least gelation concentration of samples.

4.2.8 Statistical analysis

The experiments were performed in at least duplicate, and the results were presented as mean values ± standard deviations (SD). Statistical analysis was carried out using IBM SPSS Statistics software (version 27.0.1., Armonk, NY, USA). One-way analysis of variance (ANOVA) was conducted, followed by Tukey's test for multiple comparisons to compare means. Statistical significance was determined at $P < 0.05$. Principal Component Analysis (PCA) was conducted using OriginPro 2016 software (OriginLab, Inc., Northampton, MA, USA) to analyze the relationships among different properties and samples (raw ingredients, raw material blends, and TVPs).

4.3 Results and discussions

4.3.1 Protein secondary structure

In Fourier Transform Infrared (FTIR) spectroscopy, various functional groups present within analyzed samples exhibit unique absorption patterns at distinct wavelengths, resulting in characteristic peaks in the spectrum. The amide I band, spanning from 1600 to 1700 cm^{-1}, is particularly informative as it reflects the structural arrangement of protein components, primarily associated with the C=O stretch vibration, and is highly sensitive to changes in secondary structure (Gao et al., 2023). Hence, our study focused solely on analyzing this specific band.

Quantification of changes in secondary structure was performed by assessing the peak areas of each fitted sub-peak within the amide I band. Table 1 presents the distribution of α-helix, β-sheet, β-turn, and random coil structures in the protein secondary structure, with the distribution ratio quantified using the method described by Kong & Yu (2007). Distinct absorption peak wavelengths are associated with each of these structural elements: α-helix (1654-1658 cm^{-1}, 1660-1666 cm^{-1}), β-sheet (1623-1629 cm^{-1}, 1631-1643 cm^{-1}, 1689-1698 cm^{-1}), β-turn (1666-1668 cm^{-1}, 1674-1687 cm^{-1}), and random coil structure (1646-1650 cm^{-1}). These specific wavelengths facilitate the identification and quantification of different protein secondary structures, providing valuable insights into structural alterations induced by sample formulation and extrusion texturization.

In Table 1, a varied trend in α-helix content was observed with increasing CS levels in raw-material blends. Samples with 0% CS (R) and 40% CS (R) exhibited notably higher α-helix content (33.20% and 39.86%, respectively), surpassing other raw samples significantly ($P < 0.05$). This phenomenon can be attributed to the presence of wheat gluten in these two samples compared to others. By combining the findings from Table S1 in the supplementary material, it becomes apparent that wheat gluten displayed the highest α-helix content among the proteins (46.82%). The α-helix structure, owing to its extensive hydrogen-bonding network, offers an advantage in non-polar solvents, which correlates with the low solubility of wheat gluten in most research (Gao et al., 2023). Furthermore, the predominant protein secondary structure in the raw material blends was the β-sheet structure. Analysis from Table S1 reveals that, apart from wheat gluten, nearly all raw ingredients exhibited a substantial proportion of β-sheet structure. In the research conducted by Zhang et al. (2022), native PPI demonstrated a high content of β-sheet structure at 48.33%. Similarly, Li et al. (2023) illustrated that various types of SPI predominantly

possessed a β-sheet structure (ranging from 37.80% to 42.56%). This finding was also corroborated by Meng et al. (2022). Additionally, the content of β-sheet and β-turn structures remained relatively stable across different levels of CS. Notably, 60% CS (R) sample exhibited a significant increase ($P < 0.05$) in random coil content compared to other CS levels, possibly due to its variation in ingredient composition.

After extrusion texturization, there was an increase in the content of β-sheet and β-turn structures in samples compared to the raw material blends, while the content of α-helix and random coil structures fluctuated. Initially, α-helix content decreased in samples with 0% to 40% CS (T), but then exhibited a non-significant increase in samples with 50% to 90% CS (T). The most significant reduction was observed in 40% CS (T) (decreased by 28.46%, $P < 0.05$). This decline could be attributed to the intense shear and thermal stresses experienced during extrusion, leading to protein unfolding and reorientation. However, the increase in α-helix content in samples containing higher levels of cold-swelling proteins suggests that there may be less alteration in protein structure after extrusion texturization. This observation is consistent with most previous studies, where the α-helix structure either decreased or did not undergo significant changes after extrusion, even when samples were composed of different protein sources (B. Zhang et al., 2022; Meng et al., 2022; X. Zhang et al., 2022; Li et al., 2023; Gao et al., 2023).

The majority of protein secondary structure in the texturized protein samples were also comprised β-sheet structures, accounting for around 55.18% to 71.86%. This prevalence of β-sheet structures may be attributed to their stability in aggregates (B. Zhang et al., 2022). After the extrusion process, there was a significant increase in β-sheet content, although some samples exhibited slight decreases (e.g., 50% CS (T)) or remained relatively stable (e.g., 40% CS (T)).

Under the extreme conditions of extrusion texturization, irreversible protein denaturation occurred, leading to the transition from α-helix to β-sheet structure (Gao et al., 2023). X. Zhang et al. (2022) demonstrated that the α-helix structure decreased while the β-sheet structure underwent a significant increase after extrusion, suggesting the stretching of polypeptide chains to promote the formation of an ordered fibrous structure. However, the relationship between the content of α-helix or β-sheet structure and the quality of fibrous end products remains unclear. Notably, the 60% CS (T) displayed a significant increase in β-turn content after extrusion (7.19%), indicating the formation of more flexible protein structures. Additionally, the 40% CS (T) exhibited a significant increase in random coil structures post-extrusion, suggesting a transformation from ordered to disordered structures induced by the extrusion process.

4.3.2 Solubility

The solubility of samples is a critical indicator of their potential applications. However, most texturized samples often exhibit extremely low solubility in water, necessitating an investigation into the bonds or interactions that predominantly influence solubility. To address this, we employed multiple solvents containing various functional reagents to disrupt non-covalent or covalent bonds and enhance the solubility of the samples. This approach allowed us to identify the bonds formed during the extrusion texturization process that contribute most to the observed low solubility. Several solvents were utilized to dissolve the six texturized samples: phosphate buffer to extract proteins in their native state, can possibly disrupt the electrostatic interaction, urea to disrupt hydrogen bonding, and SDS, CHAPS, and thiourea for breaking down hydrophobic interactions (K. Liu & Hsieh, 2008). Additionally, DTT was employed to cleave disulfide bonds. The solubility results of the extrudates with increasing CS ratio are shown in Figure 1.

According to Flory et al. (2023), the protein solubility of the raw material blends with different CS ratios ranged from 20.04% to 46.20% in water. But after extrusion texturization, the solubility of the texturized proteins in phosphate buffer (native state) notably decreased, with values ranging only from 0.64% to 1.74%. However, upon the addition of various chemical buffers, their solubility dramatically increased, changing from average 1.05% (in PB) to 80.93% (in IEF buffer). This phenomenon indicates that many chemical bonds or interactions were formed in proteins during extrusion texturization, resulting in aggregate formation. The IEF buffer, containing all the reactive agents, achieved the highest solubility for these samples, ranging from 70.72% to 88.95%. Remarkably, the 0% CS (T) exhibited the highest solubility (88.95%), which gradually decreased with increasing CS ratios. This trend suggests that higher cold swelling ratios may contribute to reduced solubility, possibly due to differences in protein structure alterations during extrusion.

When urea was excluded from the solvent, solubility decreased across all CS levels compared to the solubility of samples in IEF buffer. This decline underscores the importance of hydrogen bonding in maintaining protein aggregates. With the increase of CS ratios, reductions fluctuated, with the extrudates with 0% CS and 40% CS presenting relatively lower reductions (13.96% and 12.22%) compared to other extrudates. However, the extrudate with 50% CS experienced the highest decrease (31.92%). These differences were not only caused by the extrusion process but also by the variation in formulations of samples with different cold swelling ratios.

Excluding dithiothreitol (DTT), the reducing agent, from the solvent, resulted in a more significant decrease on solubility ($P < 0.05$). On average, solubility decreased by approximately 60.08%, indicating a strong response to disulfide bond disruption. Notably, the solubility of the

texturized samples with 0% and 40% CS experienced drops of 72.24% and 72.88%, respectively, while the decrease in other samples was around 50%. This phenomenon may also be attributed to differences in their formulation, as only these two samples contained wheat gluten, which is rich in disulfide bonds (Table S3), as confirmed by the test of free -SH group determination (Table 2). Conversely, PPI and SPI (or SPC) may have more non-covalent bond sites and a greater tendency to form hydrogen bonding.

The combination of excluding both urea and DTT (IEF w/o Urea & DTT) further reduced solubility across all CS levels, suggesting synergistic effects of disrupting both hydrogen and disulfide bonds. The extrudate with 0% CS experienced the most decrease, with an 81.29% reduction, followed by the extrudate with 40% CS experienced 72.88% reduction. This phenomenon confirms the reason explained above.

Excluding SDS, thiourea, and CHAPS (IEF w/o SDS, thiourea & CHAPS) led to a moderate reduction in solubility across CS levels, decreasing by 33.52% compared to the solubility in IEF buffer alone, indicating the role of hydrophobic interactions in protein solubility. Intereatingly, the reduction in solubility observed in IEF w/o SDS, thiourea & CHAPS buffer was higher than that in IEF w/o Urea buffer, suggesting that hydrophobic interactions may contribute more to protein aggregation during extrusion texturization compared to hydrogen bonding. But both hydrophobic interactions and hydrogen bonding have the function to remain constant during extrusion (K. Liu & Hsieh, 2008). From the results above, we can infer a sequence of driving forces for different covalent or non-covalent bonds during extrusion texturization: disulfide bond > hydrophobic interaction > hydrogen bonding. The most important chemical bond which may maintain the integrity and fibrous of the extrudates was disulfide bond, affecting solubility the most. However, other situations exist, Y. Zhang and Ryu (2023)

found out that the most critical force during aggregation was hydrophobic interaction in PPI texturized samples. X. Zhang et al. (2022) and Osen et al. (2015) found that hydrogen bonding played a more important role during extrusion process. The differences in these studied may be attributed to the sample materials or the conditions of extrusion processing (different temperature or moisture).

4.3.3 Determination of free amino groups

The free amino content of samples serves as a crucial indicator of their nutritional value and functionality in food products, while also revealing Maillard reaction and the formation of peptide bonds and other chemical linkages involving -NH_2 during extrusion. The results are presented in Table 2.

Within raw material blends, there was a clear increasing trend in free amino content with increased cold swelling (CS) ratios, ranging from 3.47 to 6.90 mmol/g protein. This trend suggests that cold swelling proteins facilitate the exposure of free amino groups from protein matrices, possibly due to their more native stage and higher initial free amino content in the original ingredients (Table S2). Specifically, the free amino groups in the 50% CS (R) and 90% CS (R) samples were significantly ($P < 0.05$) higher than in other samples. This difference may be attributed to variations in sample formulation, notably, these two samples were the only ones that included PPI in their formulations, which had the highest free amino content among all the raw ingredients.

After extrusion texturization, there was a significant decrease in free amino content compared to raw material blends ($P < 0.05$). This reduction could be attributed to thermal degradation, Maillard reactions (which occur between amino groups and reducing sugars), or other processing-induced transformations leading to the loss of free amino groups and their

conversion into other compounds. During extrusion processing, concomitant processes such as protein denaturation, starch dextrinization, lipid oxidation, cellulose degradation, the Maillard reaction, and water redistribution collectively influence the final product's attributes, encompassing its color, flavor (J. Zhang et al., 2019).

Notably, the extrudates with 0% CS and 40% CS exhibited the lowest free amino content compared to other texturized samples. This difference may also be attributed to the distinct formulation of these two samples, as they were the only ones with the addition of wheat gluten, which contained the lowest free amino content (3.07 mmol/g protein) among the ingredients. Conversely, the free amino content showed no significant difference between native PPI and extrudates in the study conducted by Osen et al. (2015), suggesting that the reactions involving free amino group was not evident.

4.3.4 Determination of free sulfhydryl groups

The free sulfhydryl content of proteins is a crucial parameter that reflects the degree of disulfide bond formation and protein structural changes, which can imply the denaturation degree of protein structure. In this study, the free sulfhydryl content of samples was examined and presented in Table 2.

The free sulfhydryl content exhibited a moderate increase trend with varying CS levels in raw material blends, indicating increased accessibility of sulfhydryl groups when samples contained a larger proportion of cold-swelling proteins. As shown in Table S3, the cold-swelling proteins (SPI, SPC (Arcon S), and PPI) had a higher free sulfhydryl content, which also corroborates the results above. Notably, 90% CS (R) exhibited the highest free sulfhydryl content (3.24 μmol/g protein) followed by 50% CS (R) (3.12 μmol/g protein), which may result from the high SPI content in these two samples.

After extrusion, the values of the sulfhydryl content were all decreased significantly from the average 2.78 μmol/g protein to 1.90 μmol/g protein ($P < 0.05$). Particularly, the extrudate with 90% cold swelling ratio experienced the most significant decrease, dropping to 1.32 μmol/g protein after extrusion. The mechanical shear forces during extrusion can cause physical disruption to proteins, exposing sulfhydryl groups. Subsequently, the high temperatures involved in extrusion can lead to the oxidation of sulfhydryl groups, resulting in their conversion to disulfide bonds, which aligns with the inference drawn from the solubility test (Mosibo et al., 2022). This oxidative process can decrease the concentration of free sulfhydryl groups in the sample. Comparative results can also be observed in previous studies that used wheat gluten or a combination of PPI, SPI, gluten, and corn starch as raw ingredients (Gao et al., 2023; Y. Zhang & Ryu, 2023). Conversely, samples comprised of SPI, PPI, or even oat protein concentrate only, after the extrusion process, showed an increase in free sulfhydryl content (B. Zhang et al., 2022; Pöri et al., 2022; Li et al., 2023). These differences may be attributed to the variation in raw materials and different extrusion conditions. Since under certain conditions, the extrusion process can break down disulfide bonds and unfold the protein structure in the samples. In addition, extrudates with the increasing CS levels demonstrated a decrease in free sulfhydryl content, indicating that different cold swelling ratios in samples can be affected differently by the thermal process.

4.3.5 Surface hydrophobicity

Surface hydrophobicity is a key parameter that reflects the changes on protein conformations and functional properties. The hydrophobic power has the important effect on maintaining the protein tertiary and quaternary structure (Gao et al., 2023). The surface hydrophobicity results are exhibited in Table 2.

The surface hydrophobicity of raw material blends exhibited a mixed pattern with varying CS levels with an average of 61.55 μg SDS/mg protein. Notably, 60% CS (R) demonstrated the highest surface hydrophobicity of 69.05 μg SDS/ mg protein, indicating its higher exposure of hydrophobic regions on the protein surface. The ingredients for 60% CS (R) also had a relatively high surface hydrophobicity ranged from 53.28 to 77.65 μg SDS/ mg protein, as shown in Table S2. However, the surface hydrophobicity of the samples with 50% and 90% CS only had an average value of 56.05 μg SDS/mg protein. This phenomenon may result from the inclusion of pea protein isolate in their formulation, which had the lowest surface hydrophobicity of 50.69 μg SDS/mg protein among the raw ingredients.

After extrusion texturization, the surface hydrophobicity of samples showed a significant decrease compared to pre-extrusion samples (from 61.55 to 40.78 μg SDS/ mg protein, $P < 0.05$). This decrease may be due to the temperature and shearing forces inside the extrusion process that led to the denaturation and aggregation of proteins. As proteins aggregate, the hydrophobic components may become buried deeper within the protein structure driven by hydrophobic interactions, resulting in lower hydrophobicity. Additionally, saccharides can combine with the protein samples after the extrusion process, resulting in an increase in hydrophilic groups, which may also affect the surface hydrophobicity of extrudates (Cheng et al., 2022). These results are consistent with those obtained in the study by Gao et al. (2023), where the surface hydrophobicity of wheat gluten samples all experienced a dramatic decrease after extrusion, even under different extrusion conditions. Similar results were observed in the study by Meng et al. (2022). Additionally, extrudates with higher CS levels demonstrated a slight increase in surface hydrophobicity, ranging from 35.80 to 49.80 μg SDS/mg protein, suggesting that variations in protein formulations may affect protein aggregation or structural rearrangement

during extrusion. The 0% CS (T) may experience a more extensive protein modification and aggregation under the extrusion condition.

4.3.6 *In vitro* protein digestibility

In vitro protein digestibility (IVPD) is a critical parameter for assessing the nutritional quality and bioavailability of protein-rich foods. In Table 2, raw material blends with higher cold swelling ratios demonstrated higher IVPD, ranging from 85.21% to 91.18%, indicating enhanced protein digestibility with increasing cold swelling ratio. This trend suggests that the cold-swelling proteins have better native structures and higher accessibility of proteolytic enzymes to peptide bonds, thereby promoting protein digestion.

After extrusion texturization, the IVPD of samples showed moderate changes compared to raw material blends. The majority of the samples became more digestible after extrusion process, except for 40% CS (T) and 90% (T) samples, which decreased to 86.20% and 89.82%, respectively. This phenomenon may be attributed to protein denaturation induced by the extrusion process, which can either expose or inhibit reactive sites to the digestive proteases, thus affecting protein digestibility. Additionally, the moisture and thermal conditions in the extruder may affect starch granules, leading to starch gelatinization. This gelatinization allows the release of proteins from the protein-starch matrix, potentially enhancing protein digestibility. Moreover, the extrusion process may degrade anti-nutrients that affect protein digestibility, facilitating better enzyme access to samples (Omosebi et al., 2018). Studies by Gao et al. (2023) and Omosebi et al. (2018) also support the notion that the extrusion process increases the digestibility of samples. Conversely, the extrudate with 50% CS exhibited the highest IVPD post-extrusion (90.37%), indicating the potential synergistic effects of sample formulation and extrusion on protein digestibility.

4.3.7 Water and oil holding capacity

The behavior of extruded products in aqueous environments is elucidated by their hydration properties, with the water holding capacity (WHC) being of utmost significance. The porosity and cell size of samples can be inferred from the value of WHC (Lin et al., 2002). The oil holding capacity (OHC) takes into account the amount of fat that can be absorbed per gram of protein. Both WHC and OHC are key parameters that influence the texture, stability, and sensory attributes of food products. The results were collected in Table 3.

The WHC of raw materials demonstrated an increasing trend from 1.62 g water/g protein to 4.69 g water/g protein, except for 50% CS (R) containing only 2.32 g water/g protein. This variation may be attributed to the significantly lower WHC of its ingredients like SPC (F) and PPI (Table S4). These WHC results of raw ingredients align with findings from Webb et al. (2023) and indicate that the protein structure or subunits of ingredients can result in different functionalities, thereby affecting WHC values. After extrusion, samples with a cold-swelling protein proportion above 30% showed a decrease in WHC. This suggests that samples with a lower proportion of cold-swelling proteins may experience an increase in air cells and porosity after extrusion. The extrusion process induces the unfolding proteins, leading to the exposure of additional hydrophilic groups, thereby facilitating potential interactions with water molecules (Mosibo et al., 2022). However, samples with higher levels of cold-swelling proteins may not benefit as much from the extrusion process and may even cause the destruction of the sponge-like structure already present in their raw materials. Different protein sources may undergo varied changes in structure when subjected to the extrusion process. Conversely, some samples showed higher WHC compared to raw materials in previous research (Samard & Ryu, 2019). The extrudate with 90% (R) showed the most significant decrease in WHC after extrusion,

suggesting a more severe modification. This result aligns with findings by Samard & Ryu (2019) and Y. Zhang & Ryu, (2023), indicating that extrudates with higher PPI content may lead to lower WHC values due to a less sponge-like structure after extrusion, hindering water absorption.

All samples exhibited relatively lower oil holding capacity compared to the values of water holding capacity. The OHC of raw materials showed a slight increasing tendency from 0.82 g oil/g protein to 0.96 g oil/g protein with increasing CS%, except for 50% CS (R) with only 0.59 g oil/g protein. This observation may be attributed to the formulation of this sample, where PPI also had the lowest OHC value, suggesting a reduced presence of non-polar amino acids (Table S4). After extrusion texturization, the OHC values of majority of extrudates significantly increased, while 40% CS (T) and 60% CS (T) showed a slight decrease compared to the raw material blends ($P < 0.05$). These results may arise from the dissociation of proteins, which expose non-polar amino acid side chains to a greater extent, facilitating interactions with oil molecules (Mosibo et al., 2022). In addition, the porous microstructures of extrudates may better trap oil droplets, increasing OHC.

4.3.8 Emulsifying properties

Emulsifying properties encompass both the ability to form droplets during homogenization and, subsequent to emulsion formation, their capacity to maintain stability and resist changes over time (Chen et al., 2011). Emulsifying activity index (EAI) and emulsifying stability index (ESI) are crucial parameters that determine the emulsifying properties of proteins, influencing their applicability in various food and industrial applications.

In Table 3, the emulsifying activity index of raw materials had a fluctuating trend ranging from 8.44 m^2/ g to 11.41 m^2/ g. Notably, the sample with 50% CS had the highest EAI (11.41

m^2/ g) followed by the sample with 30% CS (10.82 m^2/ g). However, the EAI values decreased after the extrusion process, ranging from 1.59 m^2/ g to 6.50 m^2/ g, indicating the poor emulsion ability of extrudates. The sample with 30% CS had the highest EAI among the texturized samples (6.50 m^2/ g). The formation of protein aggregates during the extrusion process led to the low solubility of the extrudates, resulting in their poor emulsion ability. The proteins may not dissolve well in water, making it difficult for them to act as effective emulsifiers. This result contrasts with previous studies where some samples exhibited better emulsification ability after extrusion (B. Zhang et al., 2022; Gao et al., 2023). The differences may stem from variations in sample formulation or different extrusion conditions used.

The emulsifying stability index of raw materials had a slight increase trend based on the gradient of cold swelling ratio, ranging from 15.99 min to 22.84 min, while the ESI of extrudates exhibited a decreasing trend from 58.81 min to 32.92 min except for 30% CS (T) and 60% CS (T), which had the lowest values of 14.78 min and 21.19 min, respectively. Interestingly, after extrusion, the ESI increased in most samples except for 30% CS (T) and 60% CS (T), which were the only two containing only soybean protein and flour in their formulation. This result aligns with the study by Gao et al. (2023).

4.3.9 Foaming properties

The foaming capacity (FC) of both raw materials and extrudates was evaluated to assess their suitability for various food applications. FC was measured at the 0-minute mark when the foam was initially made. The FC of raw materials ranged from relatively high, ranging from 81.67% to 100.50%, with 40% CS (R) showing the highest FC value. After a 90-minute period, only 0% CS (R) and 40% CS (R) experienced the most significant decrease of around 20%,

while 50% CS (R) and 90% CS (R) maintained the highest FC at 75%. This result underscores the good foaming capacity of PPI.

After extrusion process, the FC of extrudates dropped dramatically to the average of 10.86%, with 30% CS (T) and 60% CS (T) showing relatively higher values (20.00% and 26.67%, respectively). However, after only a 30 min period, the FC of 30% CS and 60% CS decreased to 0%, while other samples still retained some foam. After another 15 minutes, only 0% CS (T) and 30% CS (T) had a FC of 1.67%, but by the 60-minute mark, all extrudates had lost their foam. Good solubility is a decisive condition for good foaming capacity. The irreversible thermal denaturation of proteins during the extrusion process resulted in a dramatic decrease in sample solubility, leading to the low FC of the extrudates. This finding aligns with the study by Gao et al. (2023), which also observed similar changes in FC after extrusion.

4.3.10 Least gelation concentration

The least gelation concentration (LGC) of raw materials was from 17% to 20%, with 30% CS (R) and 40% CS (R) exhibiting the highest values, indicating their poor ability to form a gel. Samples with a higher proportion of cold-swelling proteins demonstrated better gelation ability. Among the raw ingredients, cold-swelling proteins also displayed low LGC values (Table S4), consistent with findings from studies by Webb et al. (2023) and Flory et al. (2023). After the extrusion process, the texturized samples either had larger LGC values or maintained similar values compared to the raw materials, ranging from 19% to above 20%. This phenomenon may be attributed to the extrusion process leading to the formation of a large proportion of protein aggregates, resulting in folded protein structures. These folded structures were less conducive to absorbing water and trapping it to form a gel-like product. Notably, both 50% CS (T) and 60% CS (T) exhibited the largest LGC values, both exceeding 20%.

4.3.11 Principal component analysis

Principal component analysis was conducted to elucidate the relationships between diverse physicochemical and functional properties and the samples. The analysis revealed that the first (PC1) and second (PC2) principal components collectively explained 55.71% of the total variance in the dataset. In the PCA plot, samples forming clusters denote similar characteristics, while those positioned distantly indicate distinct features. Notably, most TVPs and raw material blends were separated primarily along PC1, whereas differentiation based on lower (0% to 40% CS) and higher (50% to 90% CS) cold-swelling protein ratios occurred along PC2, illustrating sample disparities. 0% CS (R), 30% CS (R), 40% CS (R), PPI, and wheat gluten demonstrated positive associations with foaming capacity, *in vitro* protein digestibility, and β-turn structure. However, wheat gluten exhibited weaker associations with the analyzed properties compared to other samples. Conversely, raw material blends with higher cold-swelling protein ratios (50% to 90%), SPI, SPC (Arcon S), and soy flour had a positive correlation with random coil structure, water holding capacity, free sulfhydryl content, free amino content, emulsifying activity index, and surface hydrophobicity. SPC (Arcon F) and most TVP samples were positively associated with β-sheet structure, emulsifying stability index, and least gelation concentration, whereas weaker connections were observed for 0% CS (T) and 40% CS (T). This phenomenon demonstrates that the extrusion process induced changes in the protein structure of TVP samples, resulting in different properties

4.4 Conclusions

In conclusion, this study investigated various physicochemical properties of texturized protein samples with different cold swelling (CS) ratios before and after extrusion processing. Increasing cold-swelling protein ratios in raw material blends correlated with elevated free amino

content, free sulfhydryl content, *in vitro* protein digestibility, water holding capacity, and emulsifying stability index, indicating the higher water affinity of cold-swelling proteins.

After the extrusion process, significant alterations in the physicochemical properties of the protein samples and most samples exhibited distinct property trends across CS levels compared to raw materials. α-Helix decreased with the increase of β-sheet structure. The solubility of extrudates in their native state decreased notably by 79.88% compared to solubility in IEF buffer, with disulfide bonds identified as the primary driving force behind the formation of stable fibrous structures.

Both free amino content and free sulfhydryl content decreased after extrusion, indicative of structural cross-linking and the formation of Maillard reactions. Surface hydrophobicity and IVPD exhibited fluctuating trends dependent on the CS ratio. WHC and OHC were influenced in varying degrees across samples, likely owing to differences in protein sources among the raw materials. Furthermore, emulsifying properties were compromised after extrusion, as evidenced by decreased EAI and ESI values. Foaming capacity was drastically reduced, attributed to irreversible thermal denaturation of proteins and decreased sample solubility. Moreover, the LGC values indicated a limited ability of texturized samples to form gels post-extrusion.

After extrusion texturization, the structure and properties of texturized proteins have undergone significant changes due to the protein aggregation under high-temperature and high-shear condition. This study enhances our comprehension of protein-protein interactions and alterations in protein properties during the texturization process, offering valuable insights for future production and broader applications of texturized vegetable proteins.

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Figures and tables

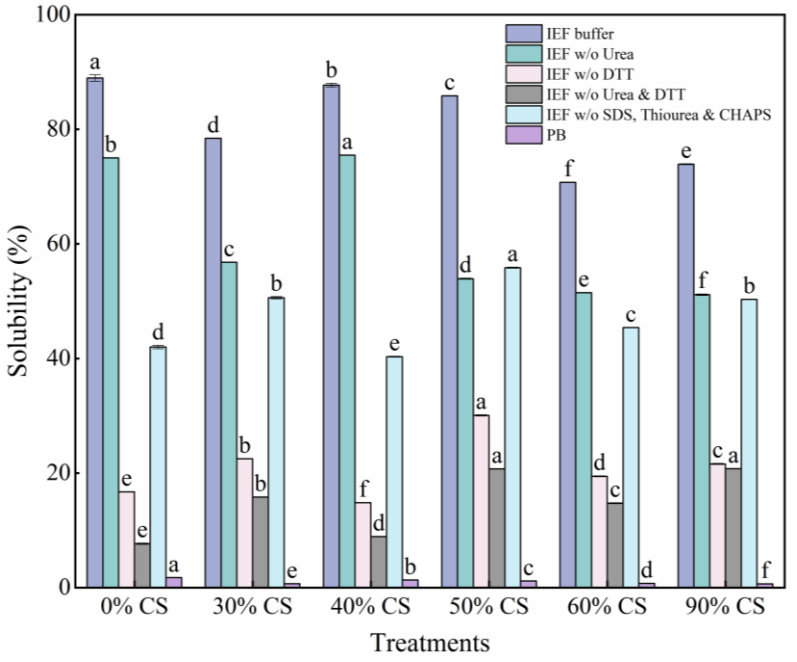


Figure 4.1 Protein solubility in different tailored buffers

Note: Different letters indicate significant differences in the same column ($P < 0.05$). CS means cold-swelling protein and (T) means the texturized proteins or extrudates. DTT: dithiothreitol; SDS: sodium dodecyl sulfate; CHAPS: 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate.

A B

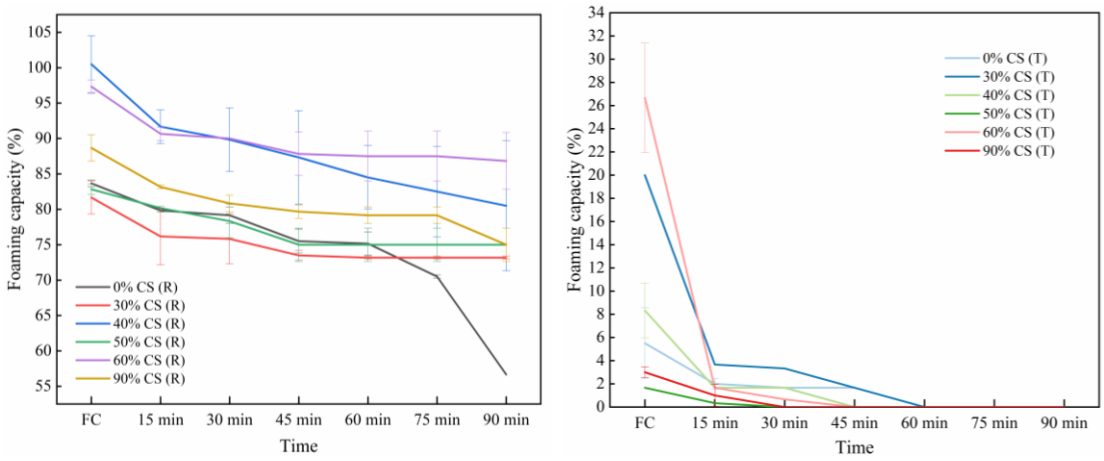


Figure 4.2 Foaming properties of raw materials and texturized proteins

Note: A: (R) means the raw-material blends, B: (T) means the texturized proteins or extrudates. CS means cold-swelling protein.

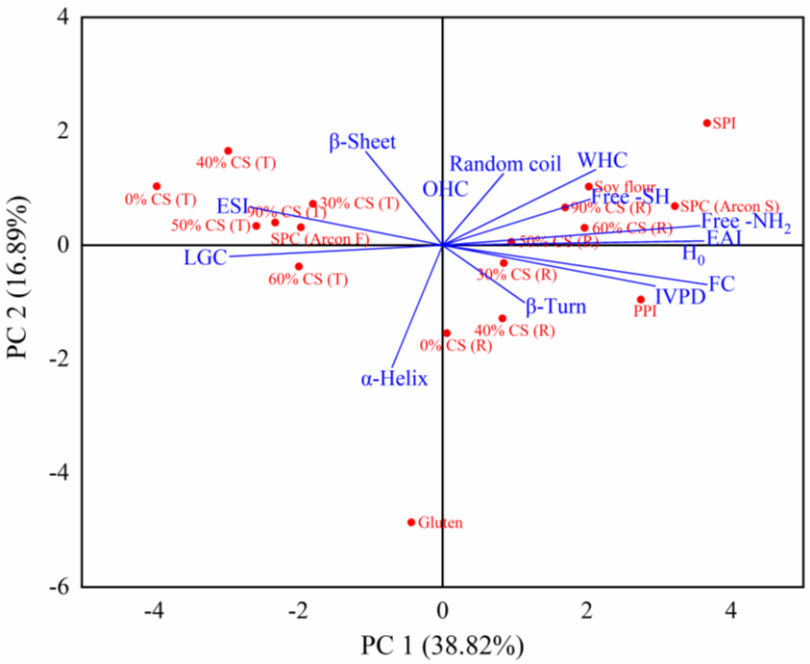


Figure 4.3 Principal component analysis (PCA) biplot

Note: This figure describes the relationship among different properties and different samples. SPI means soy protein isolate, SPC (Arcon F or Arcon S) means soy protein concentrate (Arcon F or Arcon S), PPI means pea protein isolate, Gluten means wheat gluten. CS means cold-swelling protein, (R) means the raw-material blends, and (T) means the texturized proteins or extrudates. Protein secondary structures: α-helix, β-sheet, β-turn, random coil. Free -NH2: free amino content; Free -SH: free sulfhydryl content; H0: surface hydrophobicity; IVPD: in vitro protein digestibility; WHC: water holding capacity; OHC: oil holding capacity; EAI: emulsifying activity index; ESI: emulsifying stability index; FC: foaming capacity; LGC: least gelation concentration.

Table 4-1 Protein secondary structure from FTIR

Samples	Protein secondary structure ratio %			
	α-Helix	**β-Sheet**	**β-Turn**	**Random coil**
0% CS（R）	33.20 ± 3.09^{b}	60.67 ± 3.32^{cd}	6.13 ± 0.23^{bcd}	0.00 ± 0.00^{e}
30% CS（R）	27.65 ± 0.00^{cd}	57.51 ± 0.00^{de}	3.24 ± 0.00^{d}	11.60 ± 0.00^{d}
40% CS（R）	39.86 ± 2.56^{a}	57.13 ± 1.38^{de}	3.01 ± 1.19^{d}	0.00 ± 0.00^{e}
50% CS（R）	22.45 ± 0.49^{de}	63.58 ± 0.50^{bc}	3.26 ± 0.23^{d}	10.72 ± 0.21^{d}
60% CS（R）	23.05 ± 0.92^{de}	51.23 ± 0.05^{f}	6.79 ± 0.31^{bc}	18.93 ± 1.18^{b}
90% CS（R）	25.98 ± 4.82^{cde}	57.74 ± 0.90^{de}	5.86 ± 3.72^{bcd}	10.42 ± 0.20^{d}
0% CS（T）	22.95 ± 1.03^{de}	71.84 ± 0.11^{a}	5.21 ± 0.91^{bcd}	0.00 ± 0.00^{e}
30% CS（T）	20.52 ± 1.27^{e}	71.86 ± 1.15^{a}	7.62 ± 0.11^{b}	0.00 ± 0.00^{e}
40% CS（T）	11.40 ± 0.20^{f}	57.91 ± 0.90^{de}	5.43 ± 0.24^{bcd}	25.26 ± 0.94^{a}
50% CS（T）	25.81 ± 3.20^{cde}	55.18 ± 0.39^{e}	3.64 ± 0.09^{d}	15.37 ± 2.90^{c}
60% CS（T）	23.87 ± 1.72^{de}	62.14 ± 3.06^{bc}	13.98 ± 1.33^{a}	0.00 ± 0.00^{e}
90% CS（T）	30.26 ± 3.34^{bc}	65.56 ± 2.21^{b}	4.19 ± 1.12^{cd}	0.00 ± 0.00^{e}

Note: Results are expressed as mean ± SD (n = 2). Different letters indicate significant differences in the same column ($P < 0.05$). CS means cold-swelling protein, (R) means the raw-material blends, (T) means the texturized proteins or extrudates.

Table 4-2 Protein free amino content, free sulfhydryl content, surface hydrophobicity and *in vitro* protein digestibility (IVPD)

Samples	Free amino content (mmol/ g protein)	Free sulfhydryl content (μmol/ g protein)	Surface hydrophobicity (μg SDS/ mg protein)	IVPD (%)
0% CS（R）	3.47 ± 0.05^{g}	2.21 ± 0.10^{de}	59.73 ± 1.96^{c}	85.21 ± 0.26^{g}
30% CS（R）	4.68 ± 0.02^{e}	2.66 ± 0.12^{bcd}	64.14 ± 1.20^{b}	87.38 ± 0.26^{e}
40% CS（R）	4.88 ± 0.02^{d}	2.61 ± 0.02^{cd}	64.28 ± 0.35^{b}	87.47 ± 0.13^{e}
50% CS（R）	5.53 ± 0.03^{b}	3.12 ± 0.24^{ab}	54.83 ± 0.97^{d}	89.37 ± 0.26^{c}
60% CS（R）	5.29 ± 0.05^{c}	2.83 ± 0.03^{abc}	69.05 ± 1.67^{a}	88.83 ± 0.26^{d}
90% CS（R）	6.90 ± 0.05^{a}	3.24 ± 0.04^{a}	57.27 ± 0.55^{cd}	91.18 ± 0.51^{a}
0% CS（T）	1.75 ± 0.02^{i}	2.03 ± 0.02^{e}	35.80 ± 1.72^{g}	86.11 ± 0.00^{f}
30% CS（T）	3.11 ± 0.05^{h}	1.99 ± 0.03^{e}	38.41 ± 1.35^{g}	89.64 ± 0.13^{c}
40% CS（T）	1.67 ± 0.03^{i}	2.06 ± 0.06^{e}	37.14 ± 1.44^{g}	86.20 ± 0.13^{f}
50% CS（T）	3.90 ± 0.05^{f}	2.03 ± 0.14^{e}	42.22 ± 0.83^{f}	90.37 ± 0.13^{b}
60% CS（T）	3.19 ± 0.03^{h}	1.96 ± 0.03^{e}	42.00 ± 0.14^{f}	89.46 ± 0.13^{c}
90% CS（T）	3.85 ± 0.06^{f}	1.32 ± 0.04^{f}	49.10 ± 1.29^{e}	89.82 ± 0.13^{c}

Note: Results are expressed as mean ± SD (n = 2). Different letters indicate significant differences in the same column ($P < 0.05$). CS means cold-swelling protein, (R) means the raw-material blends, (T) means the texturized proteins or extrudates.

Table 4-3 Water holding capacity (WHC), oil holding capacity (OHC), emulsifying properties, and leas gelation concentration (LGC)

Samples	WHC (g water/ g protein)	OHC (g oil/ g protein)	EAI (m^2/ g)	ESI (min)	LGC (%)
0% CS（R）	1.62 ± 0.01^{i}	0.82 ± 0.00^{f}	8.44 ± 0.13^{d}	15.99 ± 0.43^{g}	18^{d}
30% CS（R）	2.77 ± 0.03^{e}	0.85 ± 0.00^{ef}	10.82 ± 0.26^{b}	19.10 ± 0.94^{f}	20^{b}
40% CS（R）	3.23 ± 0.12^{c}	0.94 ± 0.01^{c}	10.05 ± 0.26^{c}	20.47 ± 0.18^{d}	20^{b}
50% CS（R）	2.32 ± 0.03^{g}	0.59 ± 0.02^{g}	11.41 ± 0.05^{a}	21.77 ± 0.88^{ef}	17^{e}
60% CS（R）	3.65 ± 0.03^{b}	0.96 ± 0.01^{c}	9.72 ± 0.02^{c}	22.84 ± 0.80^{e}	17^{e}
90% CS（R）	4.69 ± 0.02^{a}	0.93 ± 0.05^{cd}	10.01 ± 0.47^{c}	21.34 ± 0.64^{ef}	18^{d}
0% CS（T）	2.55 ± 0.03^{f}	0.89 ± 0.04^{de}	1.59 ± 0.02^{i}	58.81 ± 2.27^{a}	19^{c}
30% CS（T）	3.01 ± 0.01^{d}	1.04 ± 0.00^{b}	6.50 ± 0.10^{e}	14.78 ± 0.58^{g}	20^{b}
40% CS（T）	2.76 ± 0.01^{e}	0.84 ± 0.00^{f}	1.84 ± 0.20^{i}	40.60 ± 0.11^{b}	20^{b}
50% CS（T）	1.98 ± 0.02^{h}	1.03 ± 0.00^{b}	3.73 ± 0.18^{g}	32.92 ± 0.91^{d}	$> 20^{a}$
60% CS（T）	2.98 ± 0.02^{d}	0.89 ± 0.00^{d}	4.54 ± 0.45^{f}	21.19 ± 2.00^{ef}	$> 20^{a}$
90% CS（T）	2.97 ± 0.02^{d}	1.19 ± 0.00^{a}	2.75 ± 0.10^{h}	36.18 ± 2.07^{c}	19^{c}

Note: Results are expressed as mean ± SD (n = 2). Different letters indicate significant differences in the same column ($P < 0.05$). CS means cold-swelling protein, (R) means the raw-material blends, and (T) means the texturized proteins or extrudates, EAI means emulsifying activity index and ESI means emulsifying stability index.

Chapter 5 - Conclusions and perspectives

5.1 Conclusions

A growing proportion of the population is increasingly interested in substituting animal-based proteins with plant-derived alternatives, motivated by concerns regarding health, environmental sustainability, and food security. Sorghum proteins are emerging as a viable option within this context. This study seeks to explore the impact of sorghum genotype and fermentation processes on the functional and physicochemical properties of kafirin proteins; discover an optimized method for extracting sorghum proteins, investigate the effects of wet milling processes and enzymatic treatments on sorghum protein characteristics, and evaluate the influence of extrusion processes on protein-protein interactions in plant-based proteins.

In Chapter 1, a comprehensive review of sorghum proteins was conducted, covering their structural characteristics, extraction methodologies, functional attributes, and diverse applications. Chapter 2 delved into the comparative analysis of kafirin proteins extracted from distillers' grains (DGs) and sorghum flours. DG-extracted kafirins exhibited superior protein content, surface hydrophobicity, and water and oil holding capacities compared to their flour-extracted counterparts, possibly influenced by fermentation processes. Notably, kafirins derived from black sorghum varieties exhibited reduced digestibility, attributed to elevated tannin levels inherent to black sorghum grains.

Chapter 3 introduced a novel extraction method for sorghum proteins, encompassing kafirin and glutelin isolation. The approach entailed a combination of wet milling processes and enzyme treatments, yielding proteins with heightened purity and recovery rates. However, structural alterations were observed in proteins extracted via this method, resulting in diminished

total sulfhydryl content, water holding capacity (WHC), oil holding capacity (OHC), and protein digestibility.

In Chapter 4, a comparative analysis was conducted between raw material blends and samples post-extrusion. Solubility testing underscored the pivotal role of disulfide bonds in protein aggregation formation. Despite variations in cold swelling ratios across samples, no significant differences were noted. Extruded samples exhibited decreased levels of free amino and sulfhydryl content, as well as diminished emulsifying and foaming properties. These changes were primarily attributed to the high temperature and pressure conditions inherent to the extrusion process, which facilitated extensive protein aggregation.

5.2 Future perspectives

In addition to the research covered in this thesis, several avenues warrant further investigation: 1) Application studies: Conduct experiments to explore the diverse applications of sorghum proteins or kafirins in various food and non-food products. Investigate their potential as functional ingredients, texturizers, emulsifiers, or nutritional supplements. 2) Tannin removal methods: Develop and optimize methods for the removal of tannins from sorghum proteins to enhance their digestibility. Investigate strategies such as enzymatic treatments, adsorption processes, or chemical modifications to mitigate the negative effects of tannins on protein digestibility. 3) Extraction method optimization: Further optimize sorghum protein extraction methods by exploring different enzyme types, pH conditions, and combinations with physical treatments. Investigate how variations in extraction parameters impact protein yield, purity, functionality, and structural characteristics. 4) Extrusion process comparison: Compare extruded samples using different extrusion types, such as high moisture and low moisture extrusion, to assess potential differences in protein structure, functional properties, and applications.

Investigate how extrusion conditions influence protein aggregation, solubility, digestibility, and textural attributes of the final products.

Appendix

Chapter 4- Table S1: Secondary structure from FTIR for ingredients

Samples	Protein secondary structure ratio %			
	α-Helix	β-Sheet	β-Turn	Random coil
SPI	12.02 ± 0.70^{d}	58.61 ± 0.62^{c}	2.56 ± 0.28^{cd}	26.81 ± 0.20^{a}
SPC (Arcon F)	24.96 ± 0.80^{b}	75.04 ± 0.80^{a}	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}
SPC (Arcon S)	11.13 ± 0.44^{d}	60.32 ± 0.51^{c}	28.54 ± 0.07^{a}	0.00 ± 0.00^{c}
PPI	23.77 ± 0.56^{bc}	57.17 ± 1.22^{c}	19.06 ± 1.78^{b}	0.00 ± 0.00^{c}
Gluten	46.82 ± 5.54^{a}	32.59 ± 3.95^{d}	20.59 ± 1.60^{b}	0.00 ± 0.00^{c}
Soy flour	18.41 ± 0.94^{c}	69.35 ± 2.13^{b}	2.96 ± 0.88^{c}	9.28 ± 2.19^{b}

Note: Results are expressed as mean ± SD (n = 2). Different letters indicate significant differences in the same column ($P < 0.05$). SPI means soy protein isolate, SPC (Arcon F or Arcon S) means soy protein concentrate (Arcon F or Arcon S), PPI means pea protein isolate, Gluten means wheat gluten.

Chapter 4- Table S2: Free amino content, surface hydrophobicity, and *in vitro* protein digestibility (IVPD) for ingredients

Samples	Free amino content (mmol/ g protein)	Surface hydrophobicity (μg/ mg protein)	IVPD (%)
SPI	7.82 ± 0.06^{b}	77.65 ± 0.90^{a}	90.37 ± 1.15^{ab}
SPC (Arcon F)	3.28 ± 0.03^{e}	53.28 ± 1.22^{c}	86.02 ± 0.13^{c}
SPC (Arcon S)	5.48 ± 0.06^{d}	64.57 ± 1.35^{b}	89.46 ± 0.13^{b}
PPI	8.12 ± 0.03^{a}	50.69 ± 1.41^{c}	90.82 ± 0.26^{a}
Gluten	3.07 ± 0.02^{f}	51.45 ± 0.85^{c}	90.91 ± 0.13^{a}
Soy flour	5.85 ± 0.00^{c}	67.18 ± 1.18^{b}	79.51 ± 0.38^{d}

Note: Results are expressed as mean ± SD (n = 2). Different letters indicate significant differences in the same column ($P < 0.05$). SPI means soy protein isolate, SPC (Arcon F or Arcon S) means soy protein concentrate (Arcon F or Arcon S), PPI means pea protein isolate, Gluten means wheat gluten.

Chapter 4- Table S3: Free and total sulfhydryl content and disulfide bond content for ingredients

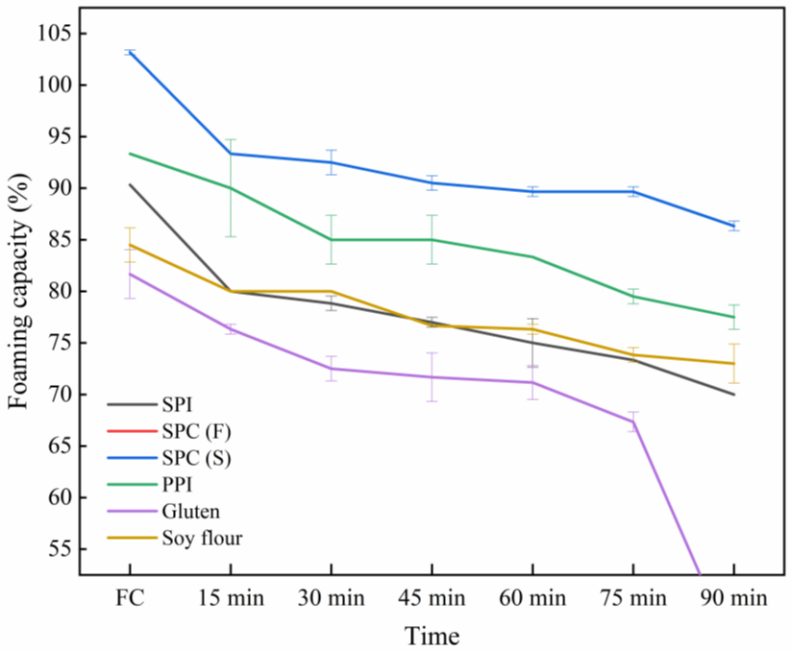
Samples	Free sulfhydryl content (µmol/ g protein)	Total sulfhydryl content (µmol/ g protein)	Disulfide bond content (µmol/ g protein)
SPI	1.46 ± 0.00^{d}	50.68 ± 0.10^{e}	24.61 ± 0.05^{d}
SPC (Arcon F)	0.45 ± 0.00^{e}	62.60 ± 0.14^{c}	31.08 ± 0.07^{c}
SPC (Arcon S)	2.92 ± 0.03^{c}	52.88 ± 0.26^{d}	24.98 ± 0.11^{d}
PPI	3.65 ± 0.06^{b}	46.49 ± 1.02^{f}	21.42 ± 0.48^{e}
Gluten	0.05 ± 0.15^{f}	77.63 ± 0.19^{b}	38.79 ± 0.17^{a}
Soy flour	4.40 ± 0.16^{a}	80.51 ± 0.22^{a}	38.06 ± 0.03^{b}

Note: Results are expressed as mean ± SD (n = 2). Different letters indicate significant differences in the same column ($P < 0.05$). SPI means soy protein isolate, SPC (Arcon F or Arcon S) means soy protein concentrate (Arcon F or Arcon S), PPI means pea protein isolate, Gluten means wheat gluten.

Chapter 4- Table S4: Water holding capacity (WHC), oil holding capacity (OHC), emulsifying properties, and leas gelation concentration (LGC) for ingredients

Samples	WHC (g water/ g protein)	OHC (g oil/ g protein)	EAI (m^2/ g)	ESI (min)	LGC (%)
SPI	5.84 ± 0.06^{a}	0.98 ± 0.02^{b}	10.45 ± 0.22^{c}	20.69 ± 1.45^{c}	14^{c}
SPC (Arcon F)	3.02 ± 0.01^{c}	0.79 ± 0.00^{d}	2.07 ± 0.03^{e}	39.79 ± 0.60^{a}	17^{a}
SPC (Arcon S)	5.20 ± 0.01^{b}	1.17 ± 0.00^{a}	9.96 ± 0.03^{c}	23.93 ± 0.19^{b}	13^{d}
PPI	3.07 ± 0.03^{c}	0.75 ± 0.02^{e}	12.35 ± 0.07^{a}	20.01 ± 0.66^{c}	16^{b}
Gluten	1.31 ± 0.01^{e}	0.82 ± 0.01^{c}	3.55 ± 0.40^{d}	24.41 ± 1.14^{b}	17^{a}
Soy flour	1.92 ± 0.02^{d}	0.98 ± 0.00^{b}	11.09 ± 0.42^{b}	21.34 ± 0.75^{c}	16^{b}

Note: Results are expressed as mean ± SD (n = 2). Different letters indicate significant differences in the same column ($P < 0.05$). SPI means soy protein isolate, SPC (Arcon F or Arcon S) means soy protein concentrate (Arcon F or Arcon S), PPI means pea protein isolate, Gluten means wheat gluten. EAI means emulsifying activity index and ESI means emulsifying stability index.



Chapter 4- Figure S1: Foaming property for ingredients

Note: SPI means soy protein isolate, SPC (F or S) means soy protein concentrate (Arcon F or Arcon S), PPI means pea protein isolate, Gluten means wheat gluten.