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Extraction and Functional Enhancement of Wheat Bran Proteins for Sustainable Food Applications

MSc research project; Chemical Engineering

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Abstract

The growing demand for sustainable protein sources calls for innovative approaches to valorize agricultural byproducts. This study focuses on the extraction and functional modification of wheat bran proteins—albumins, globulins, and glutelins—with the aim of developing sustainable food ingredients with enhanced techno-functional properties.

Proteins were sequentially extracted using a modified Osborne method. In the batch extraction, 60.8% of the total protein content was recovered across all fractions, while in the continuous extraction, the total recovery was 22.7%. Enzymatic crosslinking was performed using microbial transglutaminase (TGase), catalyzing covalent bonds between glutamine and lysine residues to enhance structural stability. Pretreatments such as heat treatment and the use of the reducing agent β-mercaptoethanol were applied to improve crosslinking efficiency. SDS-PAGE analysis confirmed the formation of high-molecular-weight aggregates, particularly for albumins and glutelins at a 1:2 enzyme-to-protein ratio.

Functional properties—solubility, emulsifying capacity, and foaming behavior—were evaluated across a pH range. While solubility decreased after crosslinking (by up to 50%), foaming capacity and stability improved significantly, particularly at pH 7 and 9, with foam stability increasing from 50% to 86% at pH 7. However, no significant enhancement in emulsifying properties was observed compared to the non-treated protein.

Additionally, process modeling in SuperPro Designer and kinetic analysis in MATLAB provided insights into the scalability and industrial relevance of the extraction process. Overall, this research shows that wheat bran proteins have potential as functional ingredients in sustainable food systems. However, optimization of crosslinking conditions is required to consistently enhance their functional performance for broader food applications.

Contents

1 Introduction

With a growing global population projected to exceed nine billion by 2050, the demand for sustainable, nutritious, and affordable protein sources is rapidly intensifying [1]. Conventional animal-based proteins, while nutritionally valuable, pose substantial environmental burdens, including high land use, greenhouse gas emissions, and water consumption. In contrast, plant-based proteins are increasingly recognized as a more sustainable and accessible alternative, due to their lower production costs, reduced ecological impact, and long history of agricultural cultivation. However, the broader use of plant proteins in food systems is challenged by their lower protein quality and less favorable functional properties, such as solubility, emulsification, foaming, and gelling, especially in comparison to animal-derived proteins [1].

To bridge this gap, researchers are increasingly focused on underutilized plant protein sources and strategies to improve their extraction and functional performance. One such promising source is wheat bran, a byproduct of the wheat milling industry. Although traditionally used as low-value animal feed, wheat bran is rich in dietary fiber, phenolic compounds, minerals, and proteins, containing approximately 12–20% protein depending on wheat variety and processing conditions [2, 3]. Presence of amino acids, particularly lysine, histidine, and arginine, makes it a nutritionally attractive candidate for valorization in the context of future food systems [3].

Despite its compositional potential, the utilization of wheat bran protein in food applications remains limited. The primary obstacle lies in the structural complexity of the bran tissue. Proteins are embedded within dense cell wall matrices composed largely of arabinoxylans and lignin, which restrict enzymatic and solvent access, reducing both extractability and digestibility [3, 4]. These barriers necessitate the development of tailored extraction protocols, combining physical and chemical pretreatments, to access specific protein fractions with minimal degradation of functional properties.

Although wheat bran proteins are successfully extracted, their techno-functional properties often remain inferior to those of more commonly used proteins. Properties such as solubility across a range of pH values, water and fat absorption, foam formation and stability, and gelation behavior are typically weak, thus limiting their incorporation into complex food matrices such as dairy analogues, meat alternatives, or bakery products [2, 4]. These limitations highlight the need for post-extraction protein modification techniques that can tailor the structural and interfacial behavior of wheat bran proteins.

Among various approaches, enzymatic crosslinking using transglutaminase (TGase) has emerged as a particularly effective strategy to improve the functional performance of plant proteins. TGase catalyzes the formation of covalent bonds between the ε-amino group of lysine and the γ-carboxamide group of glutamine, creating stable and extended protein networks. This enzymatic process significantly enhances gel strength, water-holding capacity, elasticity, and thermal stability, all of which are desirable properties in structured food products [5, 6].

Wheat bran proteins, being heterogeneous in nature and comprising a mixture of albumins, globulins, glutelins, and prolamins, may particularly benefit from targeted crosslinking interventions [2, 7, 8]. Several studies have demonstrated that the application of TGase, especially in combination with pretreatments such as heating or the use of reducing agents like β-mercaptoethanol (βME), can significantly improve protein functionality [9, 10]. These pretreatments help to unfold protein structures, increasing the exposure of reactive amino acid residues, and thereby enhancing crosslinking efficiency [9].

Valorizing wheat bran proteins through extraction and functional modification not only contributes to a more sustainable protein supply, but also supports circular economy principles by upgrading a widely available agricultural byproduct into a high-value food ingredient. Nonetheless, the effectiveness of such strategies depends on careful consideration of the extraction conditions, protein composition, and the compatibility of modification techniques with food-grade applications [1, 2].

In addition to lab-scale experimentation, it is essential to evaluate the feasibility of applying these

extraction and modification strategies at industrial scale. While laboratory protocols provide valuable insight into extraction yields and protein behavior, scale-up introduces new challenges related to mass transfer, solvent consumption, equipment design, and process economics. Therefore, this study also incorporates process modeling using SuperPro Designer to simulate and analyze the upscaling of protein extraction from wheat bran. By integrating experimental data with flow-sheet modeling, the project aims to evaluate key parameters such as throughput, solvent usage, and output yields, while providing an overview of the potential challenges in scaling up the industrial implementation of wheat bran protein valorization.

Therefore, the present study aims to extract, modify, and characterize wheat bran proteins using enzymatic crosslinking, with the objective of enhancing their functional properties for application in sustainable protein-enriched food formulations. In parallel, process modeling is employed to evaluate the scalability and industrial relevance of the developed extraction strategy, bridging the gap between laboratory innovation and practical application.

2 Literature review

2.1 Wheat bran

Wheat bran is the outer layer of the wheat kernel, obtained as a byproduct during the milling process, where the starchy endosperm is separated for flour production [11]. Though traditionally used as animal feed, wheat bran is increasingly recognized for its rich nutritional profile, including dietary fiber, minerals, antioxidants, and high-quality plant proteins [2].

Anatomically, the wheat kernel consists of three major parts: the bran, endosperm, and germ. The bran makes up roughly 15–20% of the whole grain and includes several sublayers: the outer pericarp (epicarp, mesocarp, and endocarp), the seed coat (testa), the nucellar layer, and the aleurone layer [3, 12]. The aleurone layer, in particular, is rich in proteins, dietary fiber, lipids, and minerals [7, 12]. Figure 1 illustrates the organization of these layers.

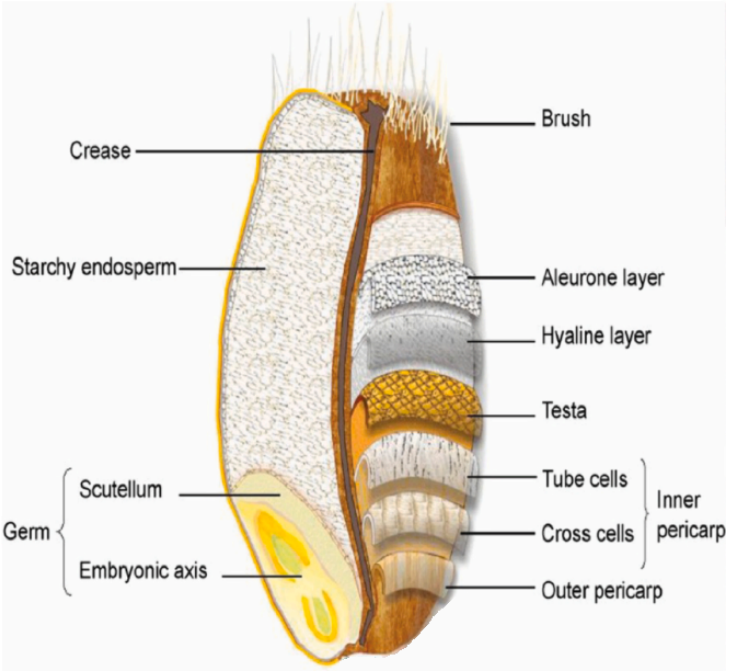

Figure 1: Anatomical cross-section of the wheat kernel showing outer layers (bran) and inner endosperm. Reproduced from Bilal et al. (2025) [13].

While rich in nutrients, the extractability of proteins from bran is limited by the presence of cell wall polysaccharides, which enclose the protein bodies—particularly in the aleurone cells. To efficiently access these proteins, physical (e.g., fine milling) and chemical (e.g., alkaline extraction) treatments are often employed [3].

Wheat bran contains approximately 12–20% protein (dry weight basis), with the exact content depending on the wheat variety and milling process [3, 8]. These proteins are generally classified based on solubility using the Osborne fractionation system into four main types: albumin, globulin, prolamin, and glutelin [8].

The distribution and functionality of these proteins are not only determined by solubility but also by their localization within the bran layers. For example, albumin and globulin are predominant in the outer bran layers, while glutelins are concentrated in the aleurone [3].

Taken together, this layered structure and the biochemical diversity of wheat bran highlight the importance of tailoring extraction techniques to target specific protein types, depending on the intended application.

2.1.1 Albumin

Albumins are water-soluble proteins that represent around 2.4–3.1% of the total content of wheat bran [7], which corresponds to roughly 23.5% of the total extractable protein from wheat bran [8]. They have a compact, globular structure that contributes to their high solubility in water and stability in aqueous systems. These proteins are predominantly found in the outer layers of the bran and are rich in essential amino acids such as lysine and methionine [3, 7]. Albumins exhibit excellent functional properties, including high foaming capacity and emulsifying activity, making them suitable for various food applications [7]. Their composition includes both hydrophilic and hydrophobic amino acid residues, allowing them to interact at the oil–water interface, an important feature for emulsifying properties [7, 8]. They typically range from 20–50 kDa in size [8].

2.1.2 Globulin

Globulins are soluble in salt solutions and account for approximately 1.9–2.0% of the whole bran mass [7], which corresponds to roughly 15.5% of the total extractable wheat bran protein [8]. Globulins are also globular in structure but less water-soluble than albumins. They are primarily located in the outer layers of the bran and are rich in arginine and lysine [3, 7]. While they do exhibit some emulsifying capability, their performance is generally lower compared to albumins. Globulins have been associated with bioactive properties, including angiotensin-converting enzyme (ACE) inhibitory activity, suggesting potential health benefits [7]. They are typically soluble in saline conditions and fall within a broader molecular weight range of 20–200 kDa [8].

2.1.3 Prolamin

Prolamins are alcohol-soluble proteins that make up about 2.5% of the total mass of wheat bran [8], which is 18.5% of the total extractable wheat bran protein [7]. Although less studied than albumins or glutelins, prolamins are notable for their high proline and glutamine content, which contributes to their hydrophobicity. This limits their solubility in water-based systems and narrows their functional role, though they may still contribute to structural properties when combined with more soluble protein fractions [11].
Given its low abundance, limited solubility, and marginal functional role, prolamin extraction is not pursued in this study.

2.1.4 Glutelin

Glutelins are soluble in dilute acid or alkali solutions and represent the largest fraction of wheat bran proteins, glutelins represent approximately 5.2% of the total mass of wheat bran [7], accounting for approximately 25.5% of total extractable wheat bran protein [8]. Glutelins have a more complex, aggregated structure due to strong intermolecular disulfide bonds, making them insoluble in water or salt solutions. Their disulfide bonding contributes to structural rigidity, beneficial for forming gels but limiting in flexibility [7]. They are predominantly found in the aleurone layer and are rich in hydrophobic amino acids [3]. Glutelins contribute to the structural properties of dough, such as elasticity and strength, and have

been associated with antioxidant activities [7]. With molecular sizes typically ranging from 50–100 kDa [8].

2.2 Extraction method

Efficient protein extraction from wheat bran is crucial to unlocking its potential as a functional food ingredient. The extraction method significantly influences not only yield and purity, but also the techno-functional properties of the recovered proteins [14]. A commonly applied approach is Osborne fractionation, which leverages differences in protein solubility across solvents—namely water, saline solutions, aqueous ethanol, and dilute alkali—to sequentially extract albumins, globulins, prolamins, and glutelins, respectively [8, 15].

However, extraction efficiency is often limited by the structural complexity of wheat bran, particularly the encapsulation of proteins within aleurone cells and cell wall matrices rich in arabinoxylans [3, 14]. Therefore, pretreatments such as fine or superfine milling are essential to disrupt these structures and improve protein accessibility. Bran with particle sizes below 50 µm has shown markedly increased extractability and solubility, likely due to a larger surface area and more extensive cell wall disruption [3].

Physicochemical parameters such as pH and ionic strength also play a key role in selectively solubilizing protein fractions. Salt concentrations (e.g., 1 M NaCl) facilitate the extraction of salt-soluble globulins, while alkaline conditions (pH 9–11) are effective in solubilizing glutelins, which are more tightly bound within the cell wall matrix [8]. These conditions affect not only extraction efficiency but also the functional quality of the protein isolates, such as solubility, emulsifying capacity, and foaming behavior [8, 14].

Beyond classical solvent systems, various enzymatic and mechanical pretreatments have been explored to further improve yields. Enzymes such as cellulase and xylanase can hydrolyze structural polysaccharides, thereby releasing embedded proteins, while ultrasonication enhances mass transfer and disrupts residual bran structure [7, 12]. A sequential strategy combining mild aqueous extraction, enzyme activation, and Osborne fractionation has achieved protein recoveries of up to 91.6%, including both native and denatured fractions [12].

From a process engineering perspective, both batch and continuous extraction modes have been applied. Batch systems offer greater flexibility and control over key parameters such as residence time and solvent ratio, making them suitable for lab-scale optimization. In contrast, continuous systems offer higher throughput and are more amenable to industrial implementation, particularly in the context of integrated biorefinery concepts [12].

The effectiveness of protein extraction protocols varies widely across studies, due to differences in wheat bran composition, particle size, pretreatment, and extraction conditions. Factors such as pH, solvent type, temperature, and the use of additives or enzymes all influence both the extractability and quality of protein fractions [15]. As a result, direct comparisons between studies are often limited, and optimal conditions must be tailored to the desired application and target functionality.

In this project, the Osborne fractionation method was selected due to its capacity to selectively extract protein classes while preserving their native structure [12]. Figure 2 illustrates this method. It is particularly well suited for complex substrates like wheat bran, where proteins are embedded in a dense matrix of dietary fibers and arabinoxylans [3].

The rationale behind the sequence lies in the distinct solubility profiles of the protein fractions. Water-soluble albumins are first extracted using just water, salt-soluble globulins are extracted next using a buffered saline solution, as these two fractions are the most accessible and loosely bound. Next, prolamins, characterized by their high content of non-polar amino acids, are extracted using aqueous ethanol,

which disrupts hydrophobic interactions. Finally, dilute alkali is used to extract glutelins, which are more tightly associated with the bran matrix and require alkaline pH for solubilization. [8]

The strength of this method lies in its ability to fractionate proteins based on solubility without relying on harsh denaturing conditions in the early steps. This enables the recovery of proteins with partially preserved native structure, which is essential for linking extraction protocols to functional behavior in food applications [12].

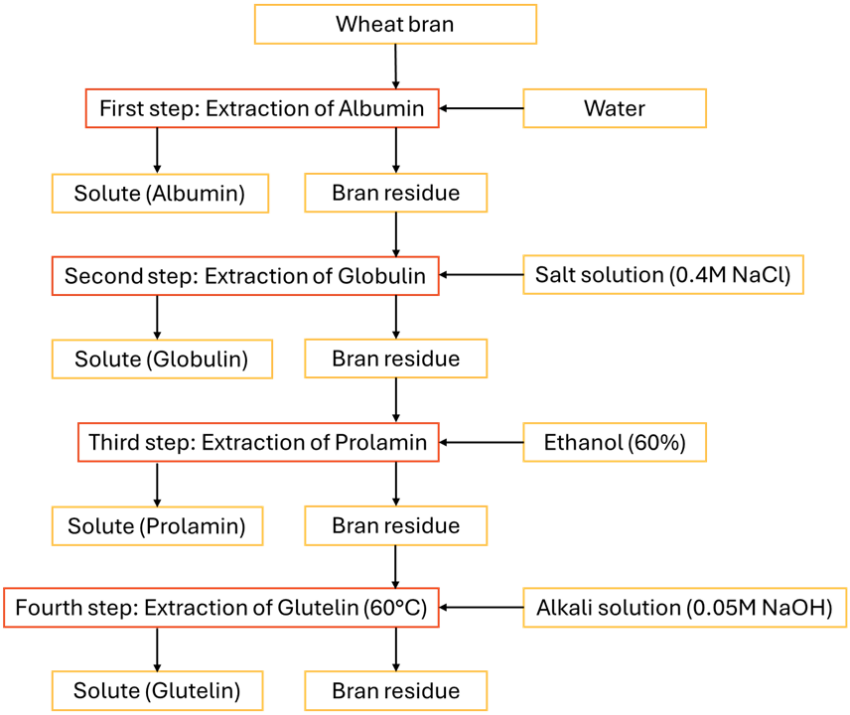

Figure 2: Flowchart of the Osborne fractionation method for wheat bran proteins.

2.3 Crosslinking

Since the extracted protein is intended for food applications, it would be beneficial to enhance the functional properties. Functional properties can be enhanced by crosslinking. Crosslinking is a molecular process in which covalent or non-covalent bonds are formed between protein chains, resulting in extended protein networks. This technique is widely used in food systems to enhance properties such as gel strength, water retention, elasticity, and thermal stability [5, 16]. In particular, plant-based proteins, which often exhibit inferior functional performance compared to their animal-derived counterparts, benefit significantly from crosslinking to improve emulsification, foaming, and gelling properties [1, 6, 10].

Crosslinking can be particularly beneficial in wheat bran protein applications. Native wheat bran proteins often have limited solubility and poor functional performance [3, 15]. By introducing crosslinks, the network-forming abilities of plant proteins can be significantly enhanced, making them more suitable for applications requiring gel strength, foam stability, or emulsifying capacity [6, 17].

Among the enzymatic crosslinkers, transglutaminase (TGase) is the most commonly used in the food industry [18]. TGase catalyzes an acyl-transfer reaction between the γ-carboxamide group of protein-bound glutamine and the ε-amino group of lysine, forming an ε-(γ-glutamyl)lysine bond, as shown in Figure 3. As a by-product, ammonia is released during the reaction [19]. This crosslinking can occur both

intra- and intermolecularly, stabilizing the protein structure without the addition of foreign chemicals, making it an appealing clean-label approach for food structuring [5, 20]. As a result, TGase is extensively applied to improve textural and functional properties of various plant and dairy proteins [6, 9, 18].

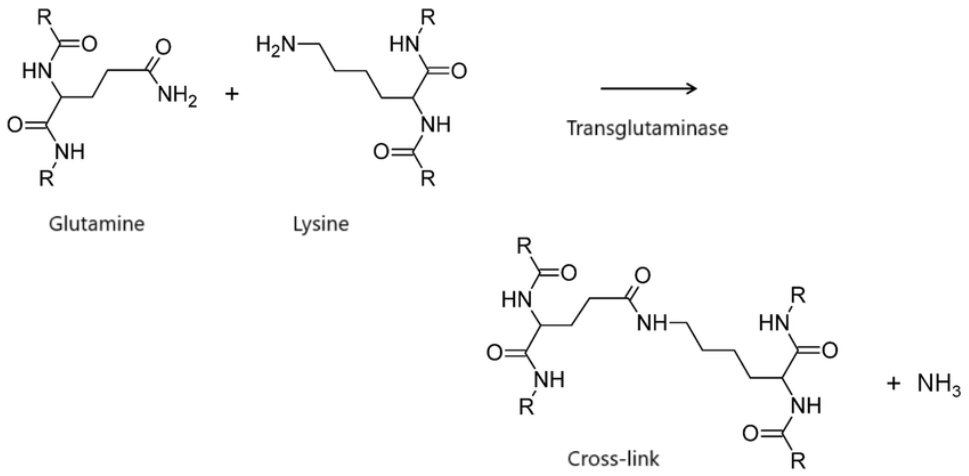

Figure 3: Enzymatic crosslinking catalyzed by transglutaminase. The enzyme forms a covalent isopeptide bond between the γ-carboxamide group of a glutamine residue and the ε-amino group of a lysine residue within peptide chains, resulting in protein crosslinking and the release of ammonia (NH_3). R represents the remainder of the peptide chains.

Microbial transglutaminase, typically derived from *Streptoverticillium* species, offers further advantages. It is calcium-independent and active in a broad range of pH and temperatures, making it versatile for many food processing conditions [5, 21]. Its action alters protein conformation and polymerization, leading to improved functional properties. For example, TGase-treated wheat, soy, or barley proteins showed increased water-holding capacity, fat adsorption, and emulsion stability [22, 23]. In dense plant protein systems, such as pea and mung bean isolates, TGase significantly improved mechanical strength and elasticity, as well as gel network integrity [24].

The efficiency of TGase crosslinking can be significantly enhanced through pretreatments such as heating or reducing agent application [16, 18]. Heat pretreatment partially unfolds protein structures, exposing more glutamin and lysine residues, which serve as TGase substrates [10]. Reducing agents break disulfide bonds, because of this, the protein structure can even open up further to increase crosslinking efficiency. Shand et al. (2008) [6] and Liu et al. (2021) [10] combined TGase treatment with heat pretreatment which greatly enhanced the gelation and water-holding properties of soy, wheat and pea proteins. Also, foaming stability was enhanced in rapeseed proteins using TGase-crosslinking combined with protein unfolding [25].

To stimulate crosslinking, β-mercaptoethanol (βME) can be used as reducing agent to partially unfold the protein structure prior to TGase addition. βME acts by reducing disulfide bonds between cysteine residues, thereby loosening the tertiary structure of proteins and exposing the buried lysine and glutamine residues. Previous studies have shown that reducing agents such as βME or DTT (dithiothreitol), especially when combined with heat pretreatment, can substantially enhance protein polymerization by TGase [9, 10, 18]. However, a major limitation of βME is its non-food-grade status. Due to its strong odor, potential toxicity, and lack of regulatory approval for food use, βME is not suitable for industrial food applications. Its use is restricted to analytical or experimental settings, such as laboratory-scale

screening of protein modification strategies. For food-grade applications, milder and safer alternatives, such as L-cysteine or physical pretreatments like mild heating or ultrasound, are typically preferred [21]. Nevertheless, βME was selected in this study to specifically evaluate the mechanistic impact of enhanced crosslinking on the functional properties of wheat bran proteins under controlled laboratory conditions.

In this study, a commercially available meat glue preparation was used as the source of transglutaminase [26]. The use of commercial TGase products offers the advantage of high availability and standardized enzymatic activity. However, the commercial TGase preparation used in this study contains additional components such as sodium caseinate and lecithin, which can serve as alternative substrates or influence protein interactions [5]. As a result, part of the enzymatic activity may be consumed by crosslinking these excipient proteins instead of the target wheat bran proteins, potentially reducing the crosslinking efficiency. Maltodextrin, although not a protein, may also influence the viscosity or dispersion behavior of the enzyme preparation. Therefore, while commercial TGase preparations are practical for experimental use, their composition must be carefully considered when interpreting the crosslinking results.

2.4 Functional Properties

The functional properties of wheat bran protein are central to its applicability in food systems, especially for developing plant-based alternatives. These properties—including solubility, emulsifying capacity, foaming ability, and gelation—are strongly dependent on the protein composition (albumins, globulins, glutelins), extraction method, processing history, and post-treatment modifications such as enzymatic crosslinking [7, 11]. Among modification tools, TGase has emerged as a valuable enzymatic agent for enhancing techno-functional traits through protein–protein crosslinking [5].

2.4.1 Solubility

Solubility is the most basic yet crucial functional property because it underpins the effectiveness of proteins in emulsions, foams, and gels. Protein solubility reflects the protein's ability to interact with water and remain in solution. Wheat bran proteins exhibit pH-dependent solubility, with markedly higher solubility in alkaline conditions compared to acidic ones. The solubility of wheat bran proteins is pH-dependent, with minimum solubility near the isoelectric point and enhanced solubility under alkaline conditions. The iso-electric point of wheat bran proteins are around pH 4-5, at this pH the proteins are precipitating [8, 27]. Albumins, being inherently water-soluble and structurally flexible, demonstrate the highest solubility, while globulins and glutelins require salt or alkaline conditions for solubilization [7, 8]

Idris et al. (2003) [8] reported that solubility increased significantly with the adjustment of pH and the addition of sodium chloride, facilitating enhanced foaming and emulsifying abilities. Similarly, Alzuwaid et al. (2020) [2] observed that wheat bran protein concentrate demonstrated excellent solubility across various pH levels, supporting its functional versatility in food systems. This trait is particularly advantageous in beverages and other aqueous-based food applications.

Solubility is not only influenced by pH and ionic strength but also by protein conformation and aggregation state. Bioprocessing techniques such as enzymatic hydrolysis or fermentation can increase solubility by partially degrading structural barriers or modifying surface charge [4, 7]. TGase, depending on the application conditions, is expected to reduce solubility due to aggregation. However, it can also stabilize partially unfolded proteins in aqueous media, preserving solubility for further functional performance [5, 18].

2.4.2 Emulsification

The emulsifying capacity of a protein is defined by its ability to stabilize oil–water interfaces, which is essential in the production of dressings, sauces, and beverages. Emulsification depends on a protein's amphiphilic nature, molecular flexibility, and ability to adsorb rapidly at oil–water interfaces. Wheat bran proteins offer relatively high emulsifying activity due to their balanced hydrophilic-hydrophobic profiles [4, 8, 15]. Emulsifying capacity is further enhanced by alkaline extraction in pea protein, which promotes unfolding and interface adsorption [28].

The surface-active nature of wheat bran proteins, particularly after alkaline extraction, supports their role as effective emulsifiers [8]. Guo and Mu (2011) [29], working on sweet potato proteins as an analog, emphasized the effect of protein concentration on emulsion droplet size and stability. Their findings highlight general principles applicable to plant proteins: optimal concentration improves emulsification by reducing droplet size and increasing the interfacial protein layer.

TGase improves emulsifying stability by reinforcing the interfacial protein network, preventing coalescence and creaming. Studies on other plant proteins, such as soy and rapeseed, showed that TGase-treated proteins form stronger and more elastic films around oil droplets, thereby increasing the emulsion's physical stability and viscosity [10, 25]. Such effects are expected to translate similarly to wheat bran proteins, particularly in systems where albumin and glutelin fractions dominate.

2.4.3 Foaming

Foaming ability and stability are particularly important for aerated food products. These depend largely on the protein's ability to adsorb at air–water interfaces and to form cohesive interfacial films. Wheat bran proteins foaming performance correlates strongly with solubility and surface activity [27]. Proteins with flexible structures, such as albumins, typically perform better as they can rapidly adsorb at air–water interfaces and form cohesive films [28, 30]. Foaming properties are enhanced by partial unfolding and increased surface activity [4, 8].

Adebiyi and Aluko (2011) [28] showed that water-soluble protein fractions from yellow pea exhibited superior foaming capacity and stability, highlighting the role of solubility and molecular flexibility. For wheat bran proteins, Idris et al. (2003) [8] demonstrated that both foaming capacity and stability improved with increased solubility at alkaline pH levels. Recent reviews (e.g., Prajapat et al (2023) [30]) have further emphasized that structural modifications, such as enzymatic hydrolysis or thermal treatments, can enhance the foaming properties of cereal proteins by increasing their molecular flexibility and surface activity. This opens avenues for optimizing wheat bran proteins in aerated food systems.

The improvement of foam stability via TGase crosslinking has been reported in multiple plant protein systems [16, 25]. TGase treatment enhances foam stability by forming covalent bonds at the air–water interface, producing elastic interfacial films that resist collapse. This enzymatic crosslinking reduces molecular mobility but strengthens structural integrity, resulting in higher foam stability [10, 18, 25]. However, excessive crosslinking can limit flexibility, reducing foamability, hence dosage and incubation time must be optimized [31].

2.4.4 Gelation

Gelation refers to the protein's ability to form a three-dimensional network, it is critical for developing desirable textures in products such as meat analogs, dairy alternatives, and desserts. Wheat bran glutelins are particularly relevant here due to their molecular weight and bonding capacity [7]. Gelling properties are influenced by protein concentration, heating conditions, pH, and ionic strength [28, 30].

Ahmedna et al. (1999) [27] reported that solubilized wheat proteins form gels at elevated concentrations, particularly when combined with calcium chloride, suggesting the potential for texture customization through salt interactions. Increased pH and salt concentrations are known to promote protein unfolding and network formation, which can improve gelling behavior in many plant protein systems [27, 30]. The presence of disulfide linkages and hydrophobic interactions further supports stable gel network formation. Moreover, Janssen et al. (2024) [14] confirmed that aqueous-extractable wheat bran proteins can support semi-solid food structures through their gelling capabilities, especially when subjected to specific pH and ionic conditions.

TGase significantly enhances gelation through the formation of ε-(γ-glutamyl)lysine crosslinks between glutamine and lysine residues, improving gel strength, elasticity, and water-holding capacity [6, 17, 24]. Comparative studies on pea, soy, and mung bean proteins show that TGase-treated gels demonstrate improved fracture stress, shear strength, and thermal stability, properties desirable in high-moisture plant-based food products [6, 24]. Furthermore, the degree of crosslinking achieved by TGase is influenced by factors such as enzyme concentration and incubation temperature, with higher concentrations leading to more robust protein networks [17].

2.4.5 Nutritional value

Beyond techno-functionality, wheat bran protein offers considerable nutritional value. With a typical protein content of 12–20% and a good distribution of essential amino acids, especially lysine and methionine, it serves as a promising plant-based alternative to animal proteins [4, 7]. Albumins and globulins contribute substantially to lysine content, which is often limited in cereals [11].

Importantly, mild extraction and enzymatic treatment (including TGase) can help retain amino acid integrity, thus preserving digestibility. While crosslinking could, in theory, hinder protease accessibility, most studies report no negative effect on bioavailability when TGase is applied under controlled conditions [5, 10, 18]

Together, wheat bran proteins exhibit a range of functional properties that make them promising ingredients for various food applications. Their solubility, emulsifying capacity, foaming ability and gelation behaviour are governed by intrinsic molecular characteristics and are highly sensitive to extraction condition and processing treatments. Among post-extraction modifications, enzymatic crosslinking via TGase has shown significant potential to enhance the techno-functional performance of these proteins. Through the formation of covalent bonds, TGase improves gel strength, foam and emulsion stability, and water-holding capacity, without compromising nutritional value when applied under controlled conditions. This positions TGase-modified wheat bran protein as a versatile, sustainable, and value-added ingredient for the development of plant-based, high-protein, and clean-label food products. This project will explore the optimization of TGase application in wheat bran systems to optimize functional properties for targeted food applications.

3 Material and Methods

3.1 Material

Wheat bran was provided by Koopmans. Milling was performed using a jaw crusher to achieve a particle size of approximately 700 µm.

3.2 CHNS Analysis

The nitrogen (N), and sulfur (S) content of wheat bran and the extracted protein fractions were determined using elemental analysis. This analysis was performed by a commercial laboratory using a standard CHNS protocol, which involves combustion of the sample and detection of the resulting gases using an elemental analyzer. The results obtained from this analysis were used to calculate the protein content based on nitrogen content, as proteins are primarily composed of nitrogen-containing amino acids. Proteins are known to contain approximately 16% nitrogen on average [32], and a conversion factor of 6.25 (100 / 16 = 6.25) is commonly applied to estimate protein content from nitrogen [33]. The calculation of protein content is as follows:

$$Protein\,content = Nitrogen\,content \times 6.25 \tag{1}$$

3.3 Extraction

The proteins can be extracted either in batch or continuously from the wheat bran. Proteins were sequentially extracted from wheat bran based on their differential solubility using a modified Osborne method [12]. Each protein fraction—albumins, globulins, and glutelins—was extracted sequentially from the same initial wheat bran sample using water, salt, and alkaline solutions, respectively.

3.3.1 Batch

Albumins were extracted using a bran-to-solvent ratio of 1:10 (w/v) at room temperature for 30 minutes. To extract albumins water was used as solvent. The slurry was filtered through cheesecloth to separate the liquid extract from the bran residue. This extraction step was repeated two more times using fresh water for each cycle. The three water extracts were combined.

Globulins were subsequently extracted from the water-extracted bran residue using a phosphate buffer (NaH_2PO_4/Na_2HPO_4, pH 7) containing 0.4 M NaCl. The extraction was carried out as mentioned above.

Glutelins were then extracted from the bran residue, immediately following the globulin extraction. The extraction was performed as mentioned above using 0.05 M NaOH at 60°C.

The prolamin fraction was not extracted in this study.

3.3.2 Continuous

A continuous extraction method was used to isolate wheat bran proteins sequentially based on their solubility, extracting albumins, globulins, and glutelins using distilled water, salt solution, and alkaline solution, respectively. Each subsequent solvent was added directly to the residual bran slurry, and solute concentrations were adjusted based on the remaining liquid volume in the system.

For albumin extraction, wheat bran was mixed with distilled water at a substrate-to-solvent ratio of 1:37 (w/v) in a stirred tank and continuously circulated past a 0.5 µm filtration unit. For each liter of extract collected, an equal volume of fresh distilled water was added to maintain a constant volume.

Protein concentration in the collected fractions was estimated using the Bradford assay. The extraction was discontinued once the protein concentration dropped below 0.05 g/L.

Globulin extraction was performed using the residual slurry and a sodium phosphate buffer (pH 7) containing 0.4 M NaCl. The buffer concentration was adjusted to account for the volume of residual liquid and added solvent. The extraction followed the same protocol as for albumins. Protein concentration was again estimated using the Bradford assay.

Glutelin was extracted from the same slurry using 0.05 M NaOH at 60°C. The extraction procedure followed the same approach as for the previous fractions. The extraction was discontinued when the protein concentration, as estimated by Bradford assay, was below 0.05 g/L.

The prolamin fraction was not extracted.

3.3.3 Concentrating

Protein extract (albumin, globulin, and glutelin) was concentrated using a 10 kDa molecular weight cut-off (MWCO) ultrafiltration unit. During this process, proteins were retained in the retentate, while smaller molecules passed through the membrane as filtrate. The protein-rich retentate was then frozen and subsequently freeze-dried for further analysis.

3.4 Bradford assay

The concentration of protein was determined by using Bradford assay kit from Bio-Rad. Bovine serum albumin (BSA) at a concentration of 1.23 mg/mL was used as the standard. The absorbance was measured at 595 nm using a UV–Vis spectrophotometer. A calibration curve was constructed by plotting the absorbance values against the known protein concentrations, which was subsequently used to calculate the protein concentrations of the unknown samples. The calibration curve can be found in Appendix 8.1.

3.5 Crosslinking

To crosslink the extracted protein fractions, a commercially available transglutaminase preparation (”meat glue”) was used as the enzymatic agent [26]. Proteins were first dissolved in 0.05 M phosphate buffer (pH 6.8), after which TGase was added to initiate the crosslinking reaction. The crosslinking reactions were performed in 10 mL Falcon-tubes under continuous mixing on a tube rotator.

The reaction conditions were systematically varied across different trials. Variables included:

- Enzyme-to-protein ratio
- Incubation time
- Reaction temperature

Additionally, the protein was subjected to pretreatments prior to addition of the enzyme including either:

- Heat treatment at 95°C for 5 minutes, or
- Addition of 0.83% (v/v) βME as reducing agent followed by heat treatment at 95°C for 5 minutes

The combinations of reaction conditions and pretreatments can be found in Table 1.

The crosslinking reaction was stopped by heating the mixture in a 95°C water bath for 10 minutes to inactivate the enzyme.

In every experiment, a control sample was included in which the enzyme was added but immediately heat-inactivated to account for non-enzymatic effects under the same reaction conditions.

Table 1: Overview of crosslinking conditions applied to extracted protein fractions. Different enzyme-to-protein ratios, incubation times, and temperatures were tested, along with optional heat pretreatment and/or reduction with βME (0.83% v/v). The table includes all combinations used in the experimental design for albumin, globulin, and glutelin fractions.

Protein sample	TGase:protein ratio (w/w)	time	temp	heat pretreatment (95°C for 5 min)	βME pretreatment (0.83% (v/v))
Albumin	1:2	3 h	40°C		
Albumin	1:2	3 h	40°C	x	
Albumin	1:2	3 h	40°C	x	x
Glutelin	1:2	3 h	40°C		
Glutelin	1:2	3 h	40°C	x	
Glutelin	1:2	3 h	40°C	x	x
Globulin	1:1	3 h	40°C		
Globulin	1:1	3 h	40°C	x	
Globulin	1:1	3 h	40°C	x	x
Albumin	1:5	3 h	40°C		
Albumin	1:5	3 h	40°C	x	
Albumin	1:5	3 h	40°C	x	x
Albumin	1:7	3 h	40°C		
Albumin	1:7	3 h	40°C	x	
Albumin	1:7	3 h	40°C	x	x
Glutelin	1:5	3 h	40°C		
Glutelin	1:5	3 h	40°C	x	
Glutelin	1:5	3 h	40°C	x	x
Glutelin	1:7	3 h	40°C		
Glutelin	1:7	3 h	40°C	x	
Glutelin	1:7	3 h	40°C	x	x
Albumin	1:2	24 h	4°C		
Albumin	1:20	24 h	4°C		
TGase	-	24 h	4°C		
Albumin	1:2	3 days	40°C	x	x
Albumin	1:5	3 days	40°C	x	x
Albumin	1:2	24 h	40°C	x	x
Albumin	1:5	24 h	40°C	x	x
Albumin	1:2	24 h	40°C		
Albumin	1:2	24 h	40°C	x	

3.6 Determination of Crosslinking

3.6.1 SDS-PAGE

SDS-PAGE was performed to analyze protein crosslinking and molecular weight distribution. Gels were prepared with a 4% stacking gel and a 12% resolving gel. Protein samples (100 µl) were mixed 1:1 with reducing sample buffer consisting of Tris-Glycine SDS with 10% (v/v) βME. The mixtures were heated in a 95°C water bath for 10 minutes and briefly centrifuged prior to loading.

Samples were loaded in equal volumes within each gel, alongside 2 µl of Biorad Precision Plus Protein Dual Color Standard as a marker in a separate lane. Electrophoresis was carried out at 180 V using 1× Tris-Glycine SDS running buffer. After the run, gels were stained for 30 minutes with Coomassie Brilliant Blue solution and destained overnight using a standard destaining solution.

3.6.2 Particle size analysis

Particle size measurements were carried out using dynamic light scattering (DLS) instrument to assess protein aggregation and crosslinking. Protein samples were prepared at a concentration of 0.1% (w/v) in distilled water without filtration. Measurements were performed in a standard disposable cuvette using the instrument's default settings at 25°C.

For each sample, two size measurements were recorded with a zeta potential measurement performed in between, using the integrated DLS and electrophoretic light scattering (ELS) functionality. This approach was used to assess the stability of the protein suspension and to verify whether the application of the electric field during zeta potential analysis affected the particle size distribution. Particle size distributions were reported based on intensity.

3.6.3 Berthelot assay

To quantify the ammonia content in the crosslinked samples, a Berthelot assay was performed. Reagent 1 was prepared by dissolving 6.906 g of sodium salicylate and 0.225 g of sodium nitroprusside in 250 ml of 0.5 mol/l sodium hydroxide solution. Reagent 2 was prepared by adding 7.5 ml of sodium hypochlorite solution (10–15% available chlorine) to 250 ml of 1.0 mol/l sodium hydroxide solution. A standard curve was prepared using 0.01% NH_4Cl solution. The calibration curve can be found in Appendix 8.1. For each reaction, 100 µl of sample or standard was mixed with 450 µl of Reagent 1 and 450 µl of Reagent 2. The mixtures were incubated at room temperature for 30 minutes, after which absorbance was measured at 660 nm using a UV–Vis spectrophotometer.

3.7 Functional properties

3.7.1 Solubility

The solubility of the crosslinked wheat bran albumin fractions was assessed based on the method described by Thiansilakul et al. (2007) [34], with minor modifications. Protein solutions (1% w/v) were prepared in distilled water, and the pH was adjusted to 3, 5, 7 or 9 using 1 M HCl or 1 M NaOH. The mixtures were stirred at room temperature for 30 minutes and subsequently centrifuged at 10,000 ×g for 5 minutes. Protein concentration in the supernatant was determined using the Bradford assay. The protein solubility index (%) was calculated according to the following equation:

$$Protein\ solubility\ index(\%) = \frac{A}{B} \times 100\% \tag{2}$$

where A reperesents the protein content in the supernatant and B is the protein content in the original sample.

3.7.2 Emulsification

The emulsifying activity index (EAI) and emulsifying stability index (ESI) of the crosslinked wheat bran albumin fractions were assessed following the procedure of Guo and Mu (2011) [29], with slight modifications. Protein suspensions (1% w/v) were prepared and adjusted to pH values of 3, 5, 7, and 9 using 1 M HCl or 1 M NaOH. The protein solution was mixed with sunflower oil at a 3:1 (v/v) ratio and homogenized using a high-speed homogenizer at 24,000 ×g for 1 minute. Immediately after homogenization, and again after 10 minutes of standing, 50 µL aliquots were collected from the bottom of the test tube. Each aliquot was diluted 100-fold with a 0.1% SDS solution, vortexed, and the absorbance at 500 nm was measured using a microplate spectrophotometer. The EAI and ESI values were calculated using the following formulas:

$$EAI(m^2/g) = \frac{2 \times 2.303 \times A_0 \times dilution factor}{c \times 1 \times (l - \phi) \times 10,000} \tag{3}$$

$$ESI(min) = \frac{A_0 \times \Delta t}{A_0 \times A_{10}} \tag{4}$$

with A_0 and A_{10} as the absorbances of the emulsion at 0 and 10 minutes, respectively, c as the initial protein concentration in g/mL, l as the optical path (0.01 m), ϕ as the oil volume fraction and Δt as time in minutes.

3.7.3 Foaming

The foam capacity (FC) and foam stability (FS) of the crosslinked wheat bran albumin fractions were evaluated according to the method outlined by Vinayashree and Vasu (2021) [31], with slight modifications. A 0.5% (w/v) protein solution was prepared, and the pH was adjusted to 3, 5, 7, or 9 using 1 M HCl or 1 M NaOH. Ten milliliters of the solution were transferred to a 50 mL graduated cylinder and homogenized at 13,500 ×g for 1 minute at room temperature. Foam capacity was calculated based on the increase in volume immediately after homogenization, while foam stability was assessed by measuring the change in foam volume after 10 minutes of standing.

To assess the individual effects of TGase and albumin, two controls were included: a TGase-only control containing 1.67 mg/mL TGase, and an albumin-only control containing 3.33 mg/mL albumin. These concentrations reflect the 1:2 enzyme-to-protein ratio used in the 5 mg/mL crosslinked samples.

The following formulas were applied to calculate FC and FS:

$$Foam\ capacity(\%) = \frac{Volume\ after\ homogenization - Volume\ before\ homogenization}{Volume\ before\ homogenization} \times 100\% \tag{5}$$

$$Foam\ stability(\%) = \frac{Volume\ after\ 10\ minutes - Volume\ before\ homogenization}{Volume\ after\ homogenization - Volume\ before\ homogenization} \times 100\% \tag{6}$$

4 Results and Discussion

4.1 CHNS Analysis

The CHNS analysis was used to determine the nitrogen content of wheat bran, which serves as the basis for estimating its protein content. The results are shown in Table 2.

Table 2: Results of CHNS elemental analysis and estimated protein content of wheat bran

Sample	N (%)	S (%)	Protein content (%)
Wheat bran	2.45	0.48	15.31

The calculated protein content of 15.31% is close to the experimentally determined values reported in the literature, which typically range from 12–20% depending on wheat variety, cultivation region, and harvest season [4, 7, 14]. Minor deviations may also arise due to differences in the amino acid composition.

The measured sulfur content of 0.48% reflects the presence of sulfur-containing amino acids such as cysteine and methionine [7]. Although sulfur content is not used to directly estimate total protein, it may offer compositional insight by reflecting the presence of these amino acids, which contribute to both the nutritional quality and functional behavior of proteins [35].

4.2 Extraction

4.2.1 Batch Extraction

The protein content extracted from the batch extraction is shown in Table 3. Both the results of the amount of protein determined by Bradford assay after extraction and the amount of protein measured after concentration and freeze-drying are shown.

The total protein content of wheat bran, based on CHNS analysis, is 15.31%, so the maximum amount of protein that can be obtained from 50 g of wheat bran is 7.66 g. Therefore, the extraction yield of the batch extraction process is $\frac{1.388+0.345+2.925}{7.66} \times 100 = 60.8\%$, indicating that 60.8% of the total protein in the wheat bran was extracted. This is lower than the extraction yields reported by Idris et al. (2003) and Uttam et al. (2023), who reported maximum extractable protein fractions of 82.5% and 79.5%, respectively [8, 7].

To enable comparison, the extraction yield of each protein fraction in this study was calculated relative to the maximum extractable amounts reported by Idris et al. (2003) [8]. According to their findings,

Table 3: Protein extracted from batch extraction of wheat bran. Recovery represents the fraction relative to the total protein content in the wheat bran. Extraction yield is calculated as percentage of the theoretical extractable protein fraction according to Idris et al. (2003) [8].

Protein fraction	Albumin	Globulin	Glutelin
Bradford assay (mg)	1387.8	345.3	2925.1
Recovery (%)	18.1	4.5	38.2
Extraction yield (%)	77.0	29.0	149.8
Weight after freeze-drying (mg)	1764.7	1375.2	1547.8
Recovery (%)	23.0	18.0	20.2
Extraction yield (%)	97.9	116.1	79.2

up to 82.5% of the total protein in wheat bran can be extracted. The extractable protein fractions for albumin, globulin, and glutelin were reported as 23.5%, 15.5%, and 25.5% of the total protein content, respectively. These values were used as theoretical benchmarks to determine the extraction yield in this study.

Notably, the prolamin fraction—which accounts for up to 18.5% of the extractable protein according to Idris et al. (2003) [8] was not extracted in the present work. This omission limits the overall recovery and means that comparisons are based solely on the albumin, globulin, and glutelin fractions.

The theoretical protein extraction of these three fractions from 50 g of wheat bran with 15.31% protein content can be calculated as:

$$Wheat\ bran(g) \times protein\ content \times yield\ protein = 50 \times 15.31\% \times (23.5 + 15.5 + 25.5)\% = 4.94\,g$$

Thus, based on the reference extraction yields, the expected extractable protein from the selected fractions amounts to 4.94 g, which corresponds to 64.5% of the total protein in wheat bran. The observed extraction of 4.66 g (sum of Bradford values for albumin, globulin, and glutelin) is close to this theoretical value, though some individual deviations are noteworthy.

For instance, the glutelin fraction as measured by the Bradford assay shows an extraction yield exceeding 100%, which may be attributed to incomplete separation during sequential extraction, potentially resulting in the co-extraction of residual globulin and prolamin proteins. Conversely, the mass recovered after freeze-drying of the glutelin fraction was lower than expected, which may reflect losses during concentration or freeze-drying, or precipitation inefficiencies inherent to alkaline extraction procedures [2, 4]. As reported by Idris et al. (2003) [8], the solubility ranges of different protein fractions can overlap, making perfect separation challenging in stepwise extraction procedures.

In addition, for the albumin and globulin fractions, the recovered mass after freeze-drying was notably higher than the protein-specific values determined by Bradford assay. This suggests contamination with non-protein substances such as buffer salts, which were not removed during filtration and remained after freeze-drying [7, 25]. To accurately determine the protein purity of the freeze-dried samples, further analyses such as total nitrogen determination or amino acid quantification would be required in future research.

Another important factor contributing to the lower extraction yields compared to literature is the difference in quantification methods. Both Idris et al. (2003) [8] and Uttam et al. (2023) [7] used the Kjeldahl method to determine protein content in their extracts, which quantifies total nitrogen, including that from insoluble or denatured proteins and non-protein nitrogen sources. In contrast, this study used the Bradford assay, which selectively detects soluble proteins with certain amino acid profiles, and may underestimate total protein, particularly in alkaline or salt-rich fractions. This methodological difference inherently leads to lower apparent yields [28].

Finally, no mechanical or physical enhancement methods such as ultrasonication were employed, unlike in the study by Uttam et al. (2023) [7], where sonication increased albumin extraction yield by 26%.

4.2.2 Continuous Extraction

The protein content extracted from the continuous extraction is shown in Table 4. The total protein content of wheat bran, based on CHNS analysis, is 15.31%, so the maximum amount of protein that 100 g of wheat bran contains is 15.31 g. The extraction yields shown in Table 4 are calculated in the same manner as described in Section 4.2.1.

In the continuous extraction process, a total of 15 L of distilled water was used for albumin extraction, 9 L of salt solution for globulin, and 12 L of NaOH solution for glutelin. Notably, the extraction was

Table 4: Protein extracted from continuous extraction of wheat bran. Recovery represents the fraction relative to the total protein content in the wheat bran. Extraction yield is calculated as percentage of the theoretical extractable protein fraction according to Idris et al. (2003) [8].

Protein fraction	Albumin	Globulin	Glutelin
Bradford assay (mg)	1418.4	905.9	1146.1
Recovery (%)	9.3	5.9	7.5
Extraction yield (%)	39.6	38.1	29.4
Weight after freezedrying (mg)	2228.9	1334.4	1893.9
Recovery (%)	14.6	8.7	12.4
Extraction yield (%)	62.1	56.1	48.6

paused overnight after 8 and 14 L of albumin extract, and after 4 L of glutelin extract. These interruptions may have affected the stability or solubility of proteins in the slurry, potentially lowering the overall yield due to aggregation, microbial activity, or settling effects [4, 8, 14].

The theoretical protein extraction of these three fractions from wheat bran, which corresponds to the yield of 64.5% can be calculated as follows:

$$Wheat\ bran(g) \times protein\ content \times yield\ protein = 100 \times 15.31\% \times (23.5 + 15.5 + 25.5)\% = 9.87\,g$$

However, the experimental data obtained from Bradford assays after extraction showed a significantly lower total extraction of 3.47 g, corresponding to a 22.7% yield. This is substantially lower than the yields obtained in batch extraction (Section 4.2.1), which reached up to 60.8%. This substantial difference indicates that much of the protein was not successfully extracted, and several factors may have contributed to this discrepancy:

First, the extraction setup may have suffered from mass transfer limitations due to insufficient mixing within the vessel. Although each extraction step allowed time for solubilization until protein concentrations fell below 0.05 g/L, the lack of active agitation or circulation within the slurry may have led to stagnant zones. This could result in incomplete protein release, particularly from the interior of bran particles or compacted areas at the bottom of the vessel [12].

Second, unlike the batch process, in which each extraction step was followed by filtration to remove all soluble components before applying the next solvent, the continuous process lacked such intermediate separation. This means that residual salts, solubilized proteins, or pH-altering substances from one extraction step may have carried over into the next. Such carryover could have altered the solvent environment, reducing protein solubility or interfering with subsequent extractions, particularly for pH-sensitive fractions like globulin [8, 27].

Third, although the extraction was halted when protein concentrations fell below 0.05 g/L, this threshold may not guarantee complete recovery. Some proteins may remain in the bran if they are loosely bound or diffuse slowly into the solvent, especially when the mixture is not actively stirred or kept in motion. While the system did resume movement after overnight pauses, it is possible that partial settling or phase separation occurred during these interruptions. According to Sardari et al. (2019), such settling effects can reduce protein availability and impair recovery in subsequent extraction stages [12].

The protein obtained after freeze-drying totaled 5.46 g, which corresponds to a yield of 35.7%. This value reflects the protein recovered after concentration and freeze-drying. Losses of protein are expected to occur during these steps. The degradation or aggregation of proteins during filtration could contribute to these losses [2]. Incomplete filtration may cause some proteins to become trapped or degraded, reducing their solubility and thereby decreasing the amount of recoverable protein [4].

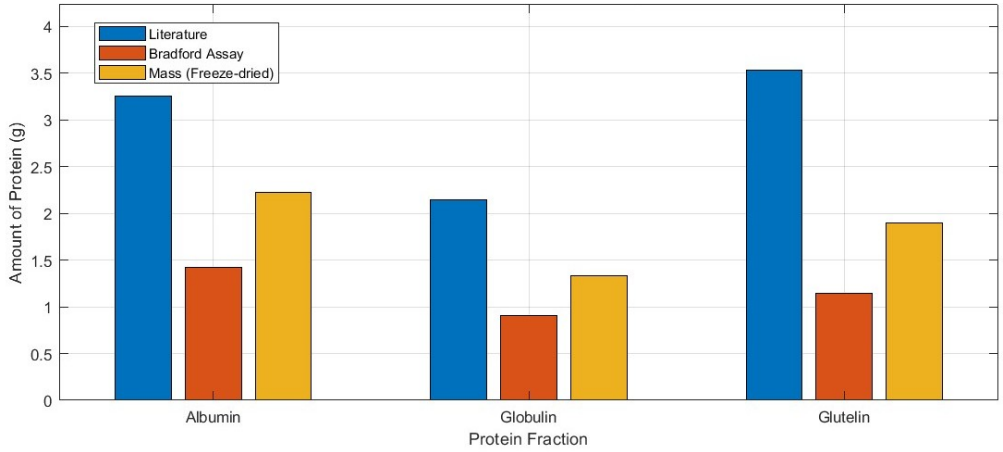

Figure 4: Extracted protein per fraction (albumin, globulin, glutelin) in the continuous extraction process. Shown are the theoretical extractable values based on Idris et al. (2003) (blue), measured protein via Bradford assay (red), and total freeze-dried mass (yellow). The figure illustrates deviations between expected and measured protein yields, and potential effects of impurities and underestimation by Bradford.

However, an interesting observation is that the protein mass after freeze-drying is higher than that obtained after the initial extraction. This suggests that there may have been inaccuracies in the Bradford assay measurement or the presence of impurities in the protein fraction, such as salts or residual carbohydrates, which could contribute to the observed increase in protein mass [25, 28]. To confirm the actual protein content and assess the purity of the freeze-dried samples, further analyses such as total nitrogen quantification (e.g., Kjeldahl or Dumas method) or amino acid profiling would be necessary in future research [7].

4.3 Crosslinking

4.3.1 SDS-PAGE

To determine enzymatic crosslinking, various analytical methods can be employed. One commonly used technique is sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), which enables the comparison of protein molecular weight distributions before and after enzymatic treatment. Upon successful crosslinking, proteins form larger aggregates, which appear as bands with reduced electrophoretic mobility, typically appearing higher in the gel. As a result, the intensity of the original monomer bands typically decreases, since crosslinked proteins are no longer present as discrete, low-molecular-weight proteins [16, 23].

The SDS-PAGE results of 1:2 enzyme-to-substrate crosslinked albumin are shown in Figure 5. After 3 hours of TGase incubation (lane 3), a slight accumulation of high-molecular-weight protein bands is observed, although there is no pronounced decrease in the monomer band intensity. This suggests that some crosslinking occurred, but the reaction may have been limited by the native conformation of the albumin proteins, which restricts access to reactive glutamine and lysine residues. Previous studies have similarly observed limited crosslinking efficiency in untreated plant proteins, likely due to steric hindrance or compact protein folding [16, 23].

Thermal pretreatment (lanes 4–5) and combined heat with βME (lanes 6–7) reported stronger crosslinking effects compared to untreated samples. Heat likely caused partial unfolding of the protein structure,

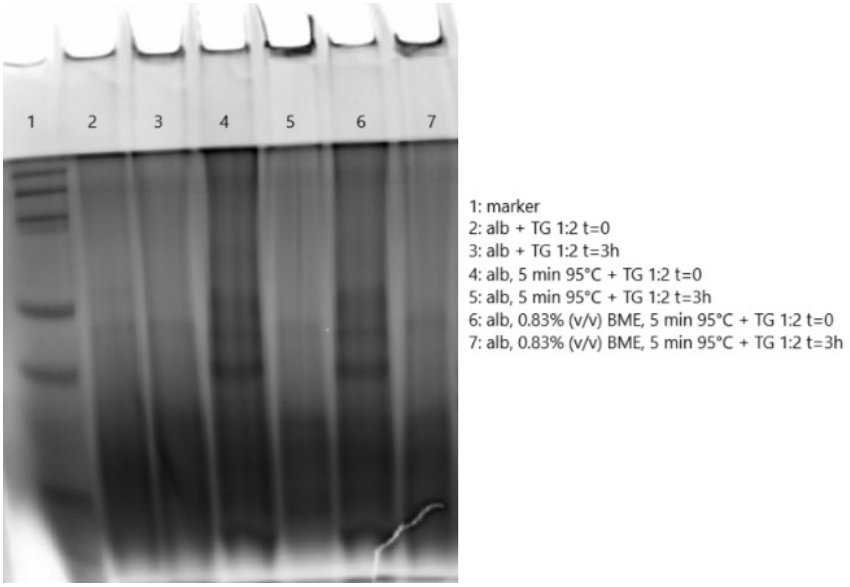

Figure 5: SDS-PAGE analysis of wheat bran-derived albumin incubated with TGase (3h) under different pretreatment conditions. The enzyme-to-substrate ratio was 1:2 (w/w)

exposing reactive sites to TGase. This effect was amplified by βME, which reduces disulfide bonds, thereby increasing molecular flexibility. These pretreatment strategies are known to enhance TGase-mediated crosslinking in plant proteins by improving the accessibility and mobility of reactive residues [16, 24].

Notably, lane 5 displays an intense band at the top of the stacking gel and a clear reduction in monomer intensity in the resolving region of the gel. These changes indicate protein aggregation into larger molecular structures, consistent with TGase-induced crosslinking. Similar observations were reported by Basman et al. (2002) [23], who showed that TGase treatment of wheat and barley proteins led to the disappearance of monomer bands and the formation of insoluble aggregates. These findings confirm that pretreatment significantly enhances crosslinking and affects the resulting molecular weight distribution of the proteins.

SDS-PAGE analysis (Figure 6) was used to evaluate TGase-mediated crosslinking of wheat bran-derived glutelin under various pretreatment conditions. Compared to the control sample, the untreated glutelin incubated with TGase for 3 hours (lane 3) shows a visible reduction in monomer band intensity and the appearance of bands in the high-molecular-weight region near the top of the gel, indicating protein crosslinking. Such shifts are consistent with TGase-catalyzed ε-(γ-glutamyl)lysine bond formation as similarly observed in legume protein isolates by Ali et al. (2010) [16].

Thermal pretreatment (lanes 4–5) and combined heat with βME (lanes 6–7) led to a slight enhancement of higher molecular weight aggregates, although the overall differences in band intensity between conditions were moderate. This suggests that, while denaturation and disulfide bond reduction can facilitate TGase access to reactive sites, their effect on crosslinking efficiency in glutelin was limited under the tested conditions. Similar trends were reported in studies by Basman et al. (2002) [23], where the extent of polymerization varied depending on the degree of protein unfolding and the accessibility of reactive glutamine and lysine residues. In line with the current observations, glutelin showed limited crosslinking compared to other protein fraction, possibly due to its more compact or aggregated native structure.

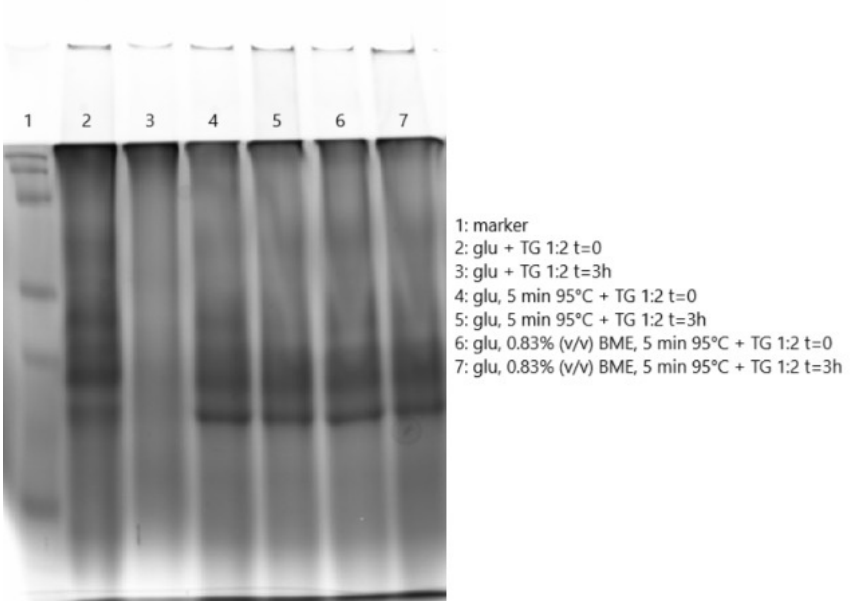

Figure 6: SDS-PAGE analysis of wheat bran-derived glutelin incubated with TGase (3h) under different pretreatment conditions. The enzyme-to-substrate ratio was 1:2 (w/w)

SDS-PAGE analysis (Figure 7) was used to assess TGase-mediated crosslinking of wheat bran-derived globulin after various pretreatment conditions. The untreated globulin control (lane 2) showed multiple distinct bands corresponding to different subunits, consistent with the heterogeneous structure of globulins of different protein sources reported in the study of Basman et al. (2002) [23].

After 3 hours of TGase incubation without pretreatment (lane 4), only a modest decrease in band intensity was observed, suggesting limited crosslinking activity under native conditions. In contrast, samples subjected to heat pretreatment (lane 6) or a combination of heat and βME (lane 8) showed a clear reduction in monomeric bands and increased presence of high-molecular-weight aggregates near the top of the gel. These results suggest enhanced TGase activity when the protein is unfolded, consistent with literature on other plant protein sources indicating that pretreatment can increase the accessibility of reactive glutamine and lysine residues required for ε-(γ-glutamyl)lysine bond formation [16, 23].

Nevertheless, compared to albumin and glutelin, the globulin fraction appeared overall less responsive to crosslinking. Notably, the distinct globulin band from the control (lane 2) remained clearly visible after TGase treatment, implying that effective crosslinking of globulin was limited under the tested conditions. This limited reactivity may be attributed to a lower availability of reactive amino acid residues or a more compact quaternary structure that hinders enzyme access, consistent with prior findings that compact native structures may hinder crosslinking in dense protein blends [24]. Similar observations were reported for globulin-rich fractions from soy and barley proteins, where extensive crosslinking was achieved only under highly optimized conditions [23].

It is also important to note that, given the relatively high enzyme-to-substrate ratio used, some of the high-molecular-weight bands observed, particularly in the pretreated samples (lanes 6 and 8), may result from self-crosslinking of TGase or other proteinaceous components present in the commercial enzyme preparation, as previously noted by Basman et al. (2002) [23] as a possible artefact. This should be considered when interpreting the SDS-PAGE patterns.

Overall, since globulin constitutes only a minor fraction of the total wheat bran protein, exhibits lim-

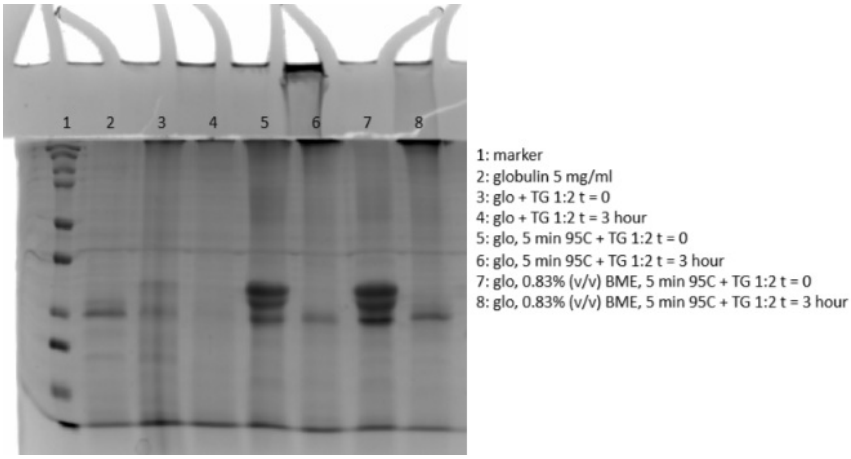

Figure 7: SDS-PAGE analysis of wheat bran-derived globulin incubated with TGase (3h) under different pretreatment conditions. The enzyme-to-substrate ratio was 1:1 (w/w)

ited functional properties [8], and showed no clear enhancement in crosslinking behavior, this protein fraction will not be further discussed in the remainder of this thesis.

To evaluate the effect of enzyme dosage on crosslinking efficiency, wheat bran-derived albumin was incubated with TGase at a reduced enzyme-to-substrate ratio of 1:5 (w/w). As shown in Figure 8, a mild reduction in the monomeric albumin band was observed after 3 hours of incubation, particularly in the thermally pretreated sample (lane 5) and in the sample pretreated with both heat and β-mercaptoethanol (βME) (lane 7). This decrease suggests that some degree of protein crosslinking occurred, even at the lower enzyme concentration.

The formation of high-molecular-weight aggregates was limited or diffuse, indicating reduced crosslinking efficiency compared to previous trials using a 1:2 ratio. Nonetheless, the visible shift in banding patterns confirms that TGase remains catalytically active at reduced dosages. This aligns with findings by Schlangen et al. (2023) [24], who observed that TGase can still induce crosslinking at low concentrations in soy and wheat proteins, although typically less efficiently and often requiring extended incubation or protein unfolding. Pretreatments that disrupt tertiary structure enhance residue accessibility for ε-(γ-glutamyl)lysine bond formation [16, 23].

In practical terms, these results highlight a trade-off between enzyme cost and functional modification: reduced TGase dosages can still promote crosslinking, but achieving meaningful effects requires optimization of pretreatment and processing conditions.

At enzyme-to-protein ratios of 1:5 and 1:7 (w/w), the crosslinking efficiency of TGase on wheat bran-derived albumin was markedly reduced (Figure 9). Only limited changes were observed after 3 hours of incubation, even in pretreated samples. A slight accumulation of high-molecular-weight aggregates was seen in thermally and chemically pretreated albumin (lane 7), though monomeric bands remained largely intact. These results suggest that under such low enzyme dosages, crosslinking activity becomes strongly dependent on protein accessibility rather than enzyme concentration alone, a pattern also noted by Schlangen et al. (2023) [24] for soy and wheat blends, where low TGase levels required extensive protein denaturation to induce meaningful crosslinking.

Interestingly, one pretreatment condition (lane 6) displayed a distinct intermediate band not seen elsewhere, which may reflect partial intra- or intermolecular bond formation without full aggregation. This

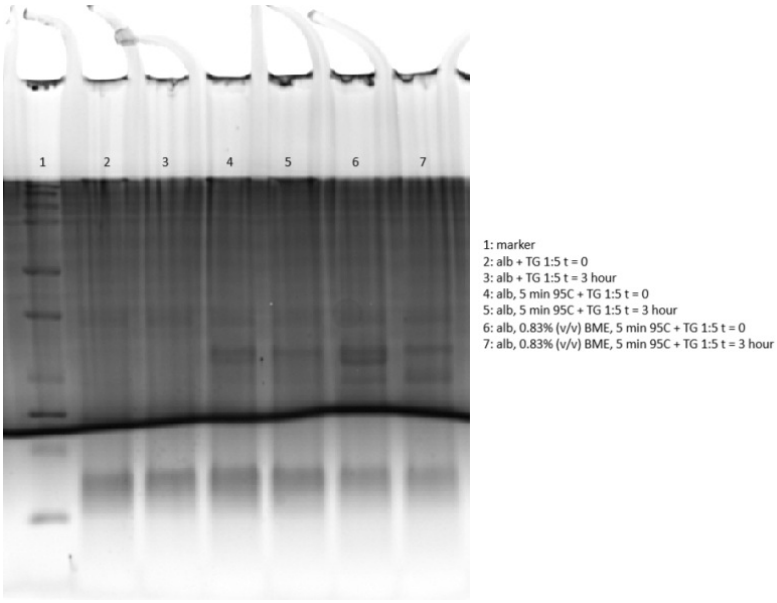

Figure 8: SDS-PAGE analysis of wheat bran-derived albumin incubated with TGase (3h) under different pretreatment conditions. The enzyme-to-substrate ratio was 1:5 (w/w)

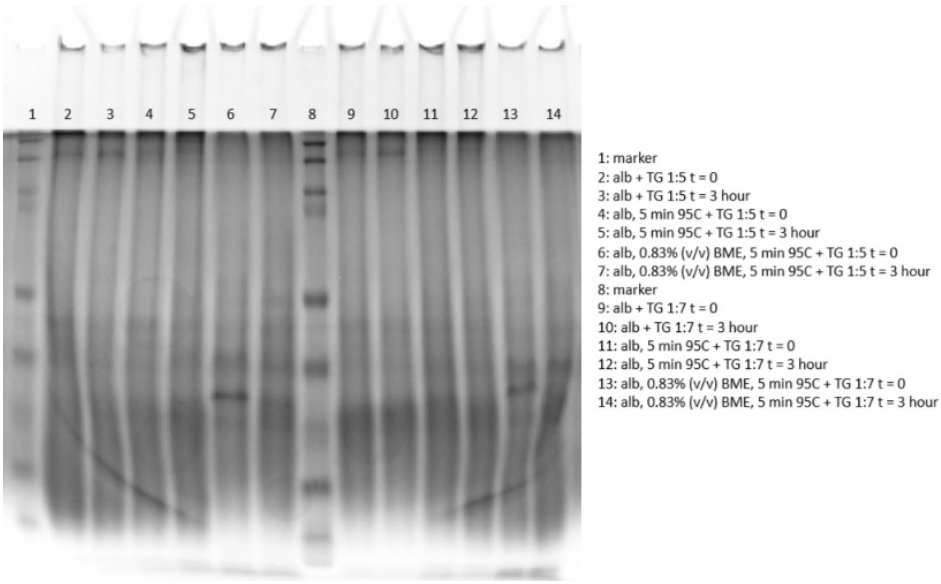

Figure 9: SDS-PAGE analysis of wheat bran-derived albumin incubated with TGase (3h) under different pretreatment conditions. The enzyme-to-substrate ratio was 1:5 (w/w) and 1:7 (w/w)

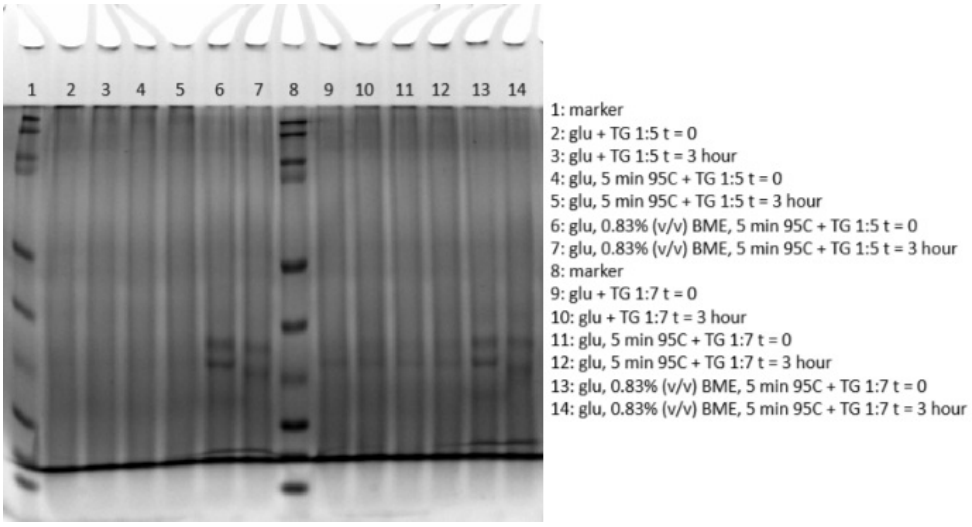

Figure 10: SDS-PAGE analysis of wheat bran-derived glutelin incubated with TGase (3h) under different pretreatment conditions. The enzyme-to-substrate ratio was 1:5 (w/w) and 1:7 (w/w)

aligns with findings by Ali et al. (2010) [16], who observed that pretreatment with reducing agents can expose buried glutamine and lysine residues but does not always lead to extensive polymerization unless paired with sufficient TGase dosage.

A similar trend was observed for wheat bran-derived glutelin (Figure 10). Despite slight decreases in monomer intensity in βME-treated samples, no visible aggregation was observed, suggesting that this fraction is poorly susceptible to crosslinking under reduced enzyme levels. This outcome may be attributed to the more compact or aggregated nature of glutelin, which reduces enzyme accessibility, an effect similarly reported for plant protein isolates by Basman et al. (2002) [23] and Alzuwaid et al. (2020) [2].

Taken together, these results underscore the importance of both enzyme dosage and structural pretreatment in determining crosslinking efficiency. While TGase can remain active at reduced concentrations, its functional impact appears limited unless supported by conditions that increase substrate mobility and residue exposure. This trade-off is crucial in industrial applications, where enzyme costs must be balanced against desired modifications in texture, solubility, or stability [21].

To evaluate the crosslinking potential of wheat bran-derived albumin under recommended conditions, samples were incubated with TGase at 4°C for 24 hours, mimicking commercial cold storage protocols.

Despite the extended incubation, no visible differences in monomeric band intensity were observed between the 0 and 24 h timepoints for either the 1:2 or 1:20 enzyme-to-substrate ratio (Figure 11, lanes 4 and 6). This absence of high-molecular-weight aggregates suggests that TGase activity is severely limited under refrigeration, consistent with reports that microbial TGase exhibits optimal catalytic efficiency around 50°C [5].

In contrast, control samples containing only the commercial TGase blend (lanes 7–8) showed clear changes in banding pattern after cold incubation, indicating some degree of self-polymerization or interaction among the carrier proteins present in the preparation. This observation aligns with earlier concerns that protein additives in TGase formulations (e.g., casein) may be crosslinked even in the absence of substrate, potentially confounding SDS-PAGE interpretations [23].

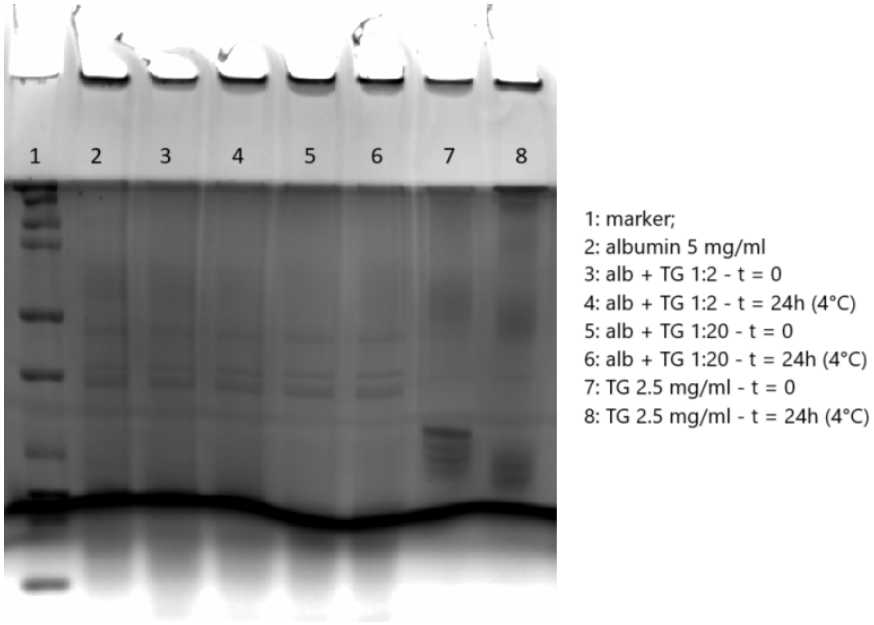

Figure 11: SDS-PAGE analysis of wheat bran-derived glutelin crosslinked with TGase under cold storage conditions (4°C, 24h). The enzyme-to-substrate ratio was 1:2 (w/w) and 1:20 (w/w)

Together, these findings underscore the importance of both temperature and formulation composition when evaluating TGase performance under realistic storage or application conditions.

Following the earlier observation that βME pretreatment enhanced TGase-mediated crosslinking of albumin, a prolonged incubation period was applied to evaluate whether this effect could be amplified. After 3 days, both enzyme concentrations (1:2 and 1:5) resulted in visible shifts in the SDS-PAGE patterns, indicating successful crosslinking. As expected, the 1:2 ratio led to a stronger depletion of monomeric bands and accumulation of high-molecular-weight aggregates. In contrast, the 1:5 condition demonstrated a clear shift in banding patterns indicative of crosslinking, although without a distinctly prominent high-molecular-weight band at the gel top.

These outcomes are consistent with studies demonstrating that longer incubation times can partly offset reduced enzyme dosages, particularly when protein unfolding is enhanced through heat and reducing agents [16, 24]. This likely reflects the gradual formation of ε-(γ-glutamyl)lysine bonds as reactive sites become more accessible [5]. Still, the comparatively faint aggregates at 1:5 highlight that low enzyme levels limit reaction kinetics, even under favorable structural conditions.

Overall, these findings confirm that prolonged reaction time in combination with structural pretreatment enables functional crosslinking at reduced TGase dosages. Yet, consistent with earlier studies, higher enzyme loads still yield more extensive polymer networks, which may be essential in applications requiring strong gelation or network formation [5]. However, from an industrial perspective, extended incubation times are generally undesirable due to increased energy consumption, process inefficiency, and the elevated risk of microbial contamination during prolonged protein handling at elevated temperatures [5].

To reduce the risk of microbial contamination associated with prolonged incubation, a 24-hour TGase treatment was evaluated as a more practical alternative. As shown in Figure 13, the samples incubated

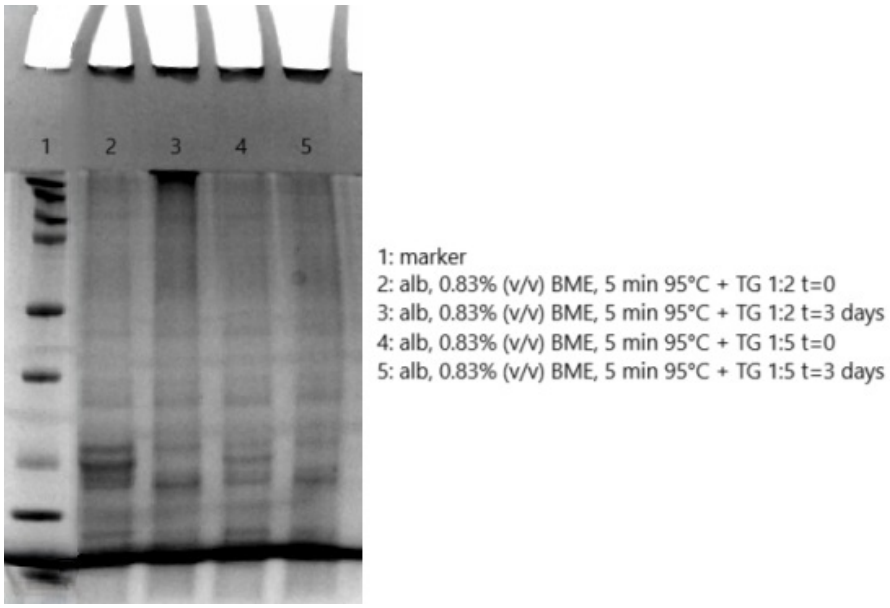

Figure 12: SDS-PAGE analysis of wheat bran-derived albumin incubated with TGase (3 days) under βME pretreatment conditions. The enzyme-to-substrate ratio was 1:2 (w/w) and 1:5 (w/w)

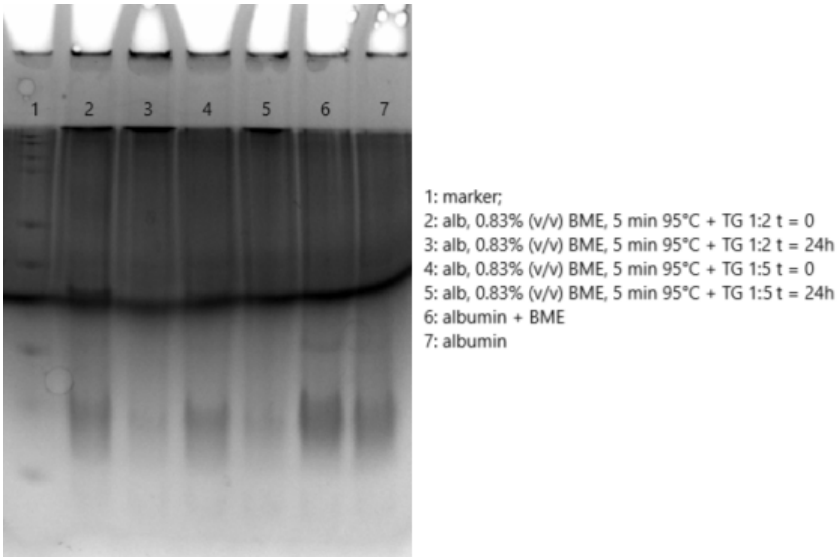

Figure 13: SDS-PAGE analysis of wheat bran-derived albumin incubated with TGase (24h) under βME pretreatment conditions. The enzyme-to-substrate ratio was 1:2 (w/w) and 1:5 (w/w)

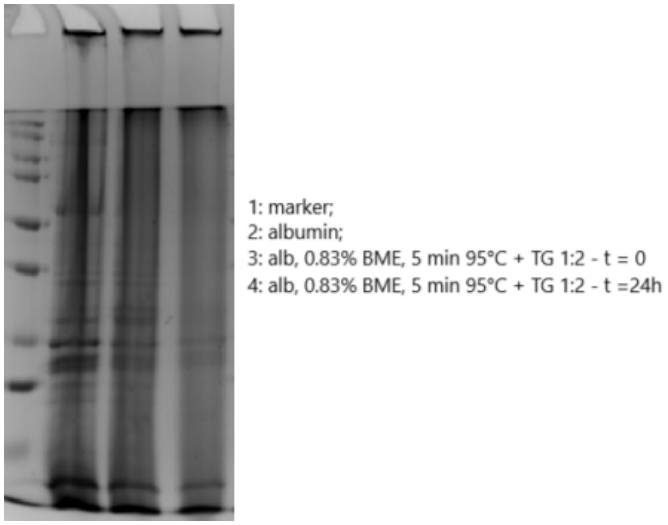

Figure 14: SDS-PAGE analysis of wheat bran-derived albumin immediately after ultrafiltration without intermediate drying steps incubated with TGase (24h) under βME pretreatment conditions. The enzyme-to-substrate ratio was 1:2 (w/w)

for 24 hours (lanes 3 and 5) clearly show a reduction in monomeric albumin bands compared to their respective time-zero samples (lanes 2 and 4). In lane 3, additional intense bands appear near the top of the resolving and stacking gel, suggesting the formation of high-molecular-weight aggregates. Although lane 5 also shows crosslinking, the effect is largely limited. This confirms that even at shorter incubation times, effective polymerization can occur, particularly at higher enzyme dosages, when reactive sites are made accessible through unfolding [16].

Control samples without TGase showed no changes in banding, confirming that βME alone does not alter protein migration. These findings align with earlier observations that reducing agents improve substrate reactivity for TGase and that crosslinking efficiency remains enzyme-dose dependent [16, 24]. From an application perspective, limiting reaction time to 24 hours is more compatible with food industry standards, as extended incubation increases energy demands and microbial risks [5].

To evaluate whether albumin can be effectively crosslinked immediately after membrane concentration, without prior freeze-drying, TGase treatment was applied to freshly concentrated protein (3 mg/mL), based on a Bradford assay.

As shown in Figures 14 and 15, effective crosslinking occurred in all samples, as evidenced by a marked reduction in monomeric band intensity after 24 hours. In particular, the TGase-treated sample in lane 4 (Figure 14) displayed a marked depletion of the albumin monomer band compared to the untreated and time-zero controls (lanes 2 and 3), confirming that freeze-drying is not essential for effective crosslinking. This aligns with previous findings that structural pretreatment combined with moderate enzyme dosages can enable efficient crosslinking within 24 hours [16, 24].

Although the total protein load in Figure 15 was lower than in Figure 14, a clear reduction in monomer intensity was still observed after 24 hours, both with and without heat pretreatment. Notably, the heat-pretreated sample (lane 6) exhibited a prominent high-molecular-weight band near the top of the stacking gel, indicative of extensive protein aggregation due to enzymatic crosslinking [16, 23].

Performing the crosslinking step directly after concentration offers additional advantages: it eliminates the need for freeze-drying, which is time- and energy-intensive, and may lead to protein losses

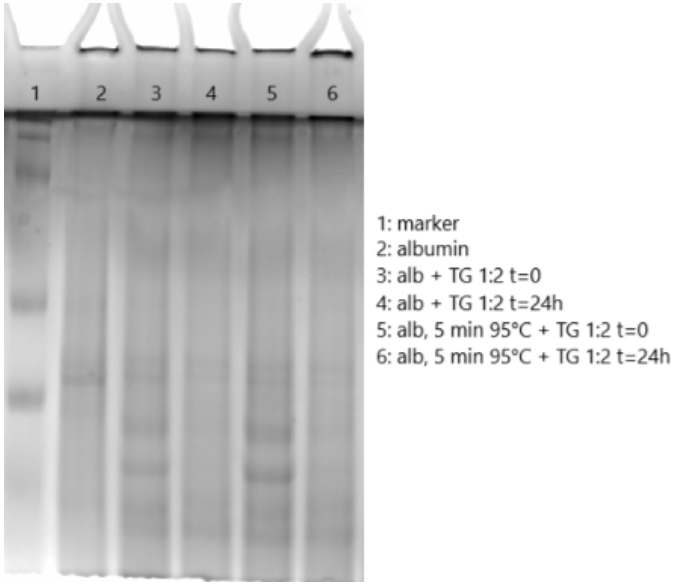

Figure 15: SDS-PAGE analysis of wheat bran-derived albumin immediately after ultrafiltration without intermediate drying steps incubated with TGase (24h) under different pretreatment conditions. The enzyme-to-substrate ratio was 1:2 (w/w)

caused by rehydration inefficiencies or adsorption to container surfaces [21]. Therefore, applying TGase immediately after concentration improves overall process efficiency and protein recovery, making this approach more attractive for scale-up and application in protein ingredient production [5].

It is important to note that SDS-PAGE provides qualitative information on the presence of crosslinked protein but does not exactly quantify the extent of crosslinking or the size distribution of aggregates in solution. Therefore, SDS-PAGE results should be interpreted in combination with complementary techniques for a comprehensive understanding of the aggregation behavior.

4.3.2 Particle size analysis

To complement the SDS-PAGE analysis and gain further insight into the aggregation behavior of TGase-treated albumin samples, dynamic light scattering was performed. The tables below summarize the Z-average hydrodynamic diameters, polydispersity indices, and peak distributions of the four TGase-treated samples. These measurements provide information on the average particle sizes and heterogeneity within the protein dispersions. While larger particle sizes generally suggest the formation of bigger aggregates consistent with crosslinking activity, particle size data alone cannot definitively confirm crosslinking and should be considered together with other analytical results [36].

The Z-average (nm) gives the intensity-weighted hydrodynamic diameter, calculated from the cumulants analysis. This value is highly sensitive to large aggregates, as larger particles scatter light much more strongly [37]. Because light scattering is intensity-weighted, even a small number of large aggregates can disproportionately increase the Z-average. The polydisperisty index (PDI) is a measure of the width of the size distribution. Values below 0.3 indicate a homogeneous (monodisperse) sample, while values above 0.5 suggest high variability in particle sizes and possible sample instability or aggregation. A PDI of 1 often indicates extreme heterogeneity or measurement interference due to turbidity [37, 36]. The peak mean (nm) is the mean diameter of the main particle population, based on the peak(s) identified

in the intensity distribution. This reflects the most abundant particle size class. The peak area is the relative contribution of each peak to the total intensity. This reports the proportion of the sample contributing to the intensity at that peak. If two peaks are reported, the sample contains at least two dominant particle size populations [36].

Table 5: Dynamic light scattering results for wheat bran-derived albumin treated with TGase (0h and 24h) under ME pretreatment. Samples were freeze-dried before analysis. Enzyme-to-substrate ratios of 1:2 and 1:5 (w/w) were used.

Albumin samples	**Z-average (nm)**	**Polydispersity**	**Peak mean (nm)**	**Peak area**
Alb, 0.83% (v/v) βME, 5 min 95°C + TG 1:2 t=0	5445	0.4168	1: 1699 2: 173.4	77.59% 22.41%
Alb, 0.83% (v/v) βME, 5 min 95°C + TG 1:2 t=24h	7276	1	1: 1104	100%
Alb, 0.83% (v/v) βME, 5 min 95°C + TG 1:5 t=0	8336	0.456	1: 188.6	100%
Alb, 0.83% (v/v) βME, 5 min 95°C + TG 1:5 t=24h	4305	0.4855	1: 2140	100%

Following the SDS-PAGE results (Figure 13), the particle size analysis in Table 5 further confirmed the aggregation behavior induced by TGase treatment. The sample incubated for 24 hours with a 1:2 TGase-to-protein ratio exhibited a monomodal peak at 1104 nm and an exceptionally high Z-average of 7276 nm with a PDI of 1, suggesting extensive aggregation and extreme heterogeneity. Although such high Z-average values exceed those typically reported for TGase-treated plant proteins, usually below 1000 nm [9, 19], the deviation may reflect the complex matrix composition of the wheat bran extract. Notably, aggregation appears to have already started at time zero, as the same sample showed a bimodal distribution with peaks at 173 nm and 1699 nm (with the latter accounting for 77.6% of total signal), indicating rapid onset of clustering after enzyme addition. Comparable early aggregation behavior, though without enzyme involvement, was observed by Wang et al. (2014) [38] following heat treatment of soybean protein isolate.

In comparison, the 1:5 TGase samples displayed different behavior. At time zero, the sample showed a single dominant peak at 189 nm (PDI 0.456), suggesting limited aggregation. After 24 hours, however, a new peak at 2140 nm emerged, pointing to progressive crosslinking. This behavior is consistent with previous reports indicating that TGase-induced crosslinking can progress over time, with initial particle growth followed by possible restructuring or stabilization of aggregates [9, 38]. Although the Z-averages were high at both time points, it decreased during crosslinking from 8336 nm to 4305 nm.

These unusually high Z-average values, some exceeding 8000 nm, are significantly larger than typically observed for TGase-treated plant proteins such as soy, rapeseed, or whey protein isolates, which generally remain below 1000 nm even after crosslinking [19, 9]. One likely explanation for this discrepancy is the presence of residual starch in the albumin extracts. Starch granules or partially gelatinized starch can absorb water, swell during heat pretreatment, and scatter light strongly, even at low concentrations [39]. This can inflate the Z-average and PDI due to the intensity bias of DLS toward large structures [19]. High turbidity was observed in the samples, making it difficult to determine whether the measured particle sizes correspond solely to protein aggregates or were influenced by the presence of starch particles.

The particle size analysis of albumin samples before freeze-drying of Figure 14 is summarized in Table 6. At time zero, the sample treated with TGase at a 1:2 enzyme-to-protein ratio exhibited a Z-average size

Table 6: Dynamic light scattering results for wheat bran-derived albumin treated with TGase (0h and 24h) under ME pretreatment. Measurements were performed directly after ultrafiltration. Enzyme-to-substrate ratio was 1:2 (w/w).

Albumin samples before freezedrying	Z-average (nm)	Polydispersity	Peak mean (nm)	Peak area
Alb, 0.83% (v/v) βME, 5 min 95°C + TG 1:2 t=0	2723	1	546.1	100%
Alb, 0.83% (v/v) βME, 5 min 95°C + TG 1:2 t=24h	3087	0.4147	1423	100%

of 2723 nm with a very high polydispersity index (PDI = 1), indicating a highly heterogeneous population dominated by larger aggregates [10]. The peak mean of 546.1 nm represents the main particle population detected at this time point. According to Stetefeld et al. (2016) [36], such broad distributions often reflect multiple scattering species, particularly when early aggregation stages and incomplete crosslinking are present.

After 24 hours of incubation with TGase, the Z-average increased slightly to 3087 nm, while the PDI decreased to 0.415, suggesting that the size distribution became more homogeneous over time. The shift in peak mean to 1423 nm further supports the hypothesis that protein aggregates grow and reorganize into more compact and stable forms over time [19]. This trend is consistent with prior observations in TGase-treated soy protein isolate and whey protein isolate, where prolonged incubation times led to increased particle size and decreased PDI due to gradual formation of high molecular weight polymers [10].

Compared to the previous measurements on freeze-dried or concentrated samples, these values confirm that significant protein aggregation occurs early upon TGase addition and evolves during incubation. Nonetheless, a decreasing PDI suggests that despite ongoing aggregation, the resulting structures become more uniform—a behavior also described in TGase-treated ovalbumin systems, where crosslinking followed a progression toward homogenization with time [9].

The moderate Z-average values and reduced PDI after 24 hours may reflect partial stabilization of aggregates prior to freeze-drying. According to Wang et al. (2014) [38], such stabilization often accompanies a shift in secondary structure and surface hydrophobicity during heat-induced aggregation, which can be further enhanced by enzymatic crosslinking.

However, notable turbidity was observed in the solution, which may have been influenced by residual starch and/or βME. Both are known to increase solution turbidity and promote non-specific aggregation. In particular, starch granules can interfere with light scattering measurements by contributing to background noise and polydispersity, as noted in wheat flour systems treated with TGase [17]. Moreover, βME has been reported to affect the structural integrity of proteins and enhance aggregation under certain conditions, thus potentially skewing particle size measurements [9].

Table 7: Dynamic light scattering results for wheat bran-derived albumin treated with TGase (0h and 24h) under heat pretreatment. Measurements were performed directly after ultrafiltration. Enzyme-to-substrate ratio was 1:2 (w/w).

Albumin samples	Z-average (nm)	Polydispersity	Peak mean (nm)	Peak area
Alb, 5 min 95°C + TG 1:2 t=0	182.5	0.3816	1: 263.8 2: 39.1	93.4% 4.777%
Alb, 5 min 95°C + TG 1:2 t=24h	305.7	0.3798	1: 394.1 2: 4339	92.6% 7.405%

βME can influence the DLS measurement because the solvent selected in the Zetasizer is water, in

which the proteins are dissolved. However, βME is also present in the solvent, although it was not explicitly selected as the solvent in the Zetasizer. This may affect the accuracy of the measurement, as βME can alter the refractive index of the solvent and interact with proteins, potentially promoting non-specific aggregation or unfolding [36]. To determine if crosslinking occurred in samples without βME, albumin samples were prepared and crosslinked with TGase in the absence of βME.

For the next measurements, possible starch was removed by centrifuging the protein extract at 10,000 × g for 5 minutes prior to TGase treatment. This step was included to reduce background turbidity and eliminate insoluble components such as residual starch, which are known to interfere with light scattering measurements [17]. Particle size analysis was then performed on these samples (Table 7).

The samples without βME showed substantially smaller Z-average sizes at t=0 (182.5 nm) and more moderate increases after 24 hours of incubation with TGase (305.7 nm). Polydispersity indices remained relatively low (0.38), indicating more homogeneous particle populations compared to the βME-treated samples. This improved homogeneity may reflect the absence of interfering agents and a cleaner protein matrix, allowing TGase to act more selectively on accessible glutamine and lysine residues [19]. A plausible explanation for this improved homogeneity is that centrifugation prior to TGase treatment effectively removed impurities and residual starch, reducing sample turbidity and non-specific aggregation. This interpretation aligns with earlier findings in enzymatically crosslinked proteins, where reducing non-protein particulates improved DLS signal quality and aggregate definition [9].

The bimodal peak distributions observed at 24 hours, with a major peak around 394 nm and a minor population near 4339 nm suggest the formation of initial protein aggregates consistent with enzyme-mediated crosslinking. Such dual-peak distributions are characteristic of systems undergoing stepwise aggregation, where early oligomers coexist with fewer large polymers [10].

These results are more in line with previously reported particle sizes for TGase-treated plant proteins, which generally remain within the submicron to low micron range [9, 19].

4.3.3 Berthelot assay

To further quantify the extent of TGase-induced crosslinking in the albumin samples, the Berthelot assay was performed to measure the amount of ammonia released during the enzymatic reaction. TGase catalyzes the formation of isopeptide bonds between specific glutamine and lysine residues, releasing ammonia as a byproduct [19] (see Figure 3). Therefore, higher ammonia concentrations directly correlate with increased crosslinking activity, making ammonia measurement a valuable quantitative indicator of the degree of enzymatic crosslinking.

Albumin samples treated with TGase, both with and without pretreatment, were measured at 0 and 24 hours of incubation. A calibration curve was created using NH_4Cl.

Table 8: Ammonium concentrations measured by the Berthelot assay in albumin samples following TGase treatment under various pretreatment conditions.

Sample	NH_4 (µmol)
Alb + TG 1:2 t=0 h	0.053
Alb + TG 1:2 t=24 h	0.203
Alb, 5 min 95°C + TG 1:2 t=0 h	0.095
Alb, 5 min 95°C + TG 1:2 t=24 h	0.162
Alb, 0.83% βME, 5 min 95°C + TG 1:2 t=0 h	0.259
Alb, 0.83% βME, 5 min 95°C + TG 1:2 t=24 h	0.244

The increase in ammonia concentration over the 24-hour incubation period supports the progressive

nature of the enzymatic crosslinking reaction, observed in both the non-pretreated and heat-pretreated samples. However, in samples pretreated with βME, a reduction in ammonia release was seen, which is likely due to βME interference with the Berthelot assay. This is in line with earlier findings showing that reducing agents like βME can interfere with colorimetric assays by reacting with intermediate compounds or altering pH sensitivity [40]. This does not necessarily indicate lower crosslinking activity, but rather limits the reliability of the Berthelot assay in these samples.

Interestingly, the non-pretreated samples showed a higher ammonia release after 24 hours than the heat-pretreated samples. This contrasts with prior studies showing that thermal denaturation typically increases the accessibility of reactive glutamine and lysine residues, thereby enhancing TGase activity [19].

A possible explanation is that although heat treatment unfolds proteins and exposes reactive groups, it may also promote rapid aggregation or misfolding, especially when not tightly controlled. These aggregates can shield reactive sites or form intramolecular bonds that compete with TGase-mediated crosslinks, ultimately reducing crosslinking efficiency [9, 16]. Furthermore, compact misfolded structures may physically hinder the enzyme's access to its target residues [25].

Thus, the higher ammonia release in the non-pretreated samples might reflect more efficient crosslinking due to better substrate accessibility in the absence of thermally induced aggregation.

Taken together, the SDS-PAGE profiles, particle size data, and ammonia measurements provide converging evidence for TGase-induced crosslinking in the wheat bran albumin fraction. SDS-PAGE confirmed the disappearance of monomeric bands and the formation of high molecular weight aggregates, while dynamic light scattering (DLS) measurements revealed increased particle sizes and polydispersity, particularly after prolonged incubation. Although the Berthelot assay was affected by interfering compounds such as βME, ammonia production in non-pretreated and heat-pretreated samples supported enzymatic crosslinking activity. These results demonstrate that crosslinking was successfully induced, though further studies using purified protein fractions and interference-free conditions are recommended to verify the extent and uniformity of the reaction.

Based on the combined results from SDS-PAGE, particle size analysis, and the Berthelot assay, the condition involving heat-pretreated albumin incubated with TGase at a 1:2 enzyme-to-protein ratio for 24 hours was selected for subsequent functional property assessment. This treatment exhibited clear signs of protein aggregation, measurable ammonia release, and a relatively homogeneous particle size distribution, without the assay interferences observed in reducing-agent-treated samples.

4.4 Functional Properties

To evaluate the effects of enzymatic crosslinking on wheat bran albumin, a series of functional properties tests were analysed on the crosslinked albumin samples. Initially, the functional properties of the non-treated albumin were characterized and used as a positive control. Subsequently, the control (t=0) and crosslinked (t=24h) samples were analyzed.

The sample crosslinked for 24 hours (enzyme-to-protein ratio 1:2, heat-pretreated) was selected for functional analysis (namely emulsification, foaming and solubility) due to comparatively higher crosslinking.

These analyses provide a foundation to understand how crosslinking influences the solubility, emulsification, and foaming behaviors of wheat bran albumin, which are critical for its potential applications in food formulations.

4.4.1 Solubility

Figure 16 shows the solubility of albumin extracted from wheat bran at different pH values[1]. The solubility follows the typical U-shaped curve, with a minimum observed around the isoelectric point (pI) of albumin, approximately around pH 4. This decrease in solubility near the pI is attributed to the net zero charge on the protein, leading to reduced electrostatic repulsion and increased aggregation [8, 28].

At pH values away from the pI, solubility increases sharply, reaching values above 90% under alkaline conditions. This is consistent with the fact that albumin was originally extracted based on its solubility in water, which has a neutral pH around 7; therefore, high solubility near this pH is expected. However, solubility values below 100% are common because freeze-drying can cause protein oxidation or structural changes that reduce solubility [41]. These findings align with previous studies on wheat bran albumin and other plant proteins, confirming its high water solubility and structural flexibility [7, 27].

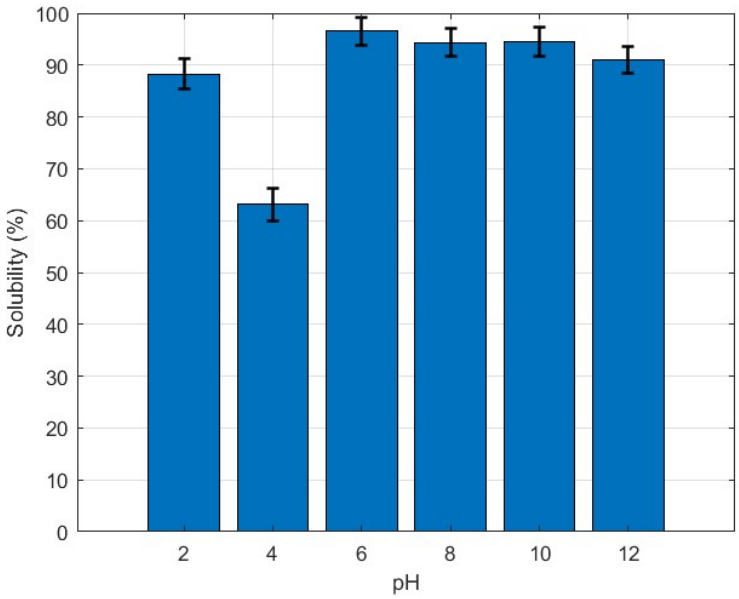

Figure 16: Solubility of wheat bran albumin at various pH (mean ± SD, n=3), determined as described in Equation 2. See Appendix 8.3 for detailed data.[1]

To evaluate the impact of TGase crosslinking on albumin solubility, samples of albumin were prepared with crosslinking times of 0 hours (control) and 24 hours. Due to limited sample availability, solubility was assessed at four selected pH values (3, 5, 7, and 9), with single measurements per condition.

Figure 17 summarizes the results. A clear decrease in solubility is observed after 24 hours of crosslinking compared to the control (0 hours) across all tested pH values. The reduction in solubility after 24 hours of crosslinking is significant and consistent across all tested pH values, indicating that enzymatic crosslinking broadly decreases albumin solubility by promoting protein aggregation and structural changes that limit protein-water interactions [34]. These results are in agreement with previous studies on TGase-treated wheat and soy proteins [19, 38]. This finding aligns with previous reports showing that TGase-mediated crosslinking can induce protein aggregation and reduce solubility due to altered surface hydrophobicity and steric hindrance [34].

However, it is important to note that TGase itself is present in relatively high concentrations in the

[1]These data were kindly provided by PhD Varshini Krishnamoorthy, who performed the measurements.

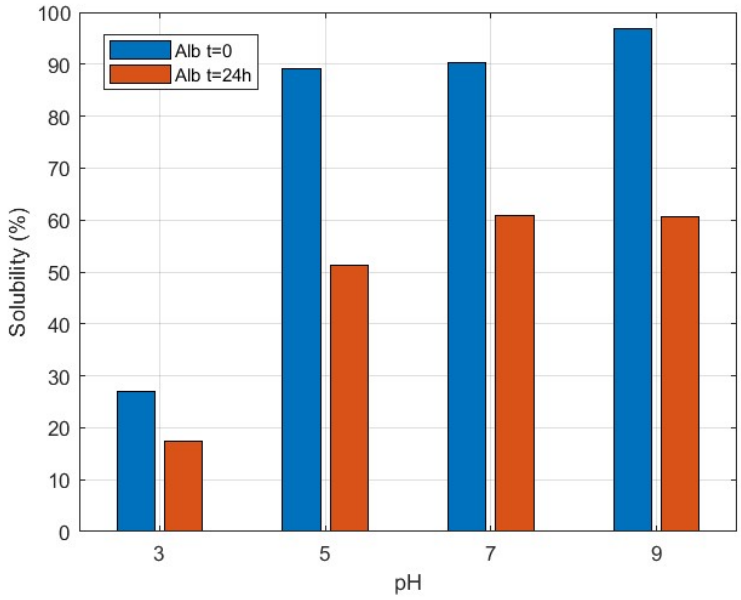

Figure 17: Albumin solubility before and after crosslinking (heat pretreatment 5 min 95°C, enzyme-to-protein 1:2 w/w, 40°C, t=0 and t=24h) at various pH. See Appendix 8.3 for detailed data.

crosslinked samples and can influence solubility independently of its crosslinking function. Some studies have reported that TGase can bind to protein substrates or form insoluble complexes, which may contribute to the decrease in solubility [5, 17]. Nevertheless, since a t=0 control sample containing TGase was included in the experimental design, the pronounced reduction in solubility after 24 hours can largely be attributed to albumin aggregation resulting from the crosslinking process.

4.4.2 Emulsification

The EAI and ESI of egg-yolk[1], non-treated albumin[1] and crosslinked albumin samples (t=0 and t=24h) were assessed at different pH values (3, 5, 7, and 9), with results presented in Figures 18 and 19.

The EAI reflects the protein's ability to stabilize the oil-water interface. Non-treated albumin, as shown in Figure 18, exhibits relatively high EAI values, though it is consistently lower than egg yolk, which is considered a gold standard emulsifier in food systems due to its amphiphilic structure and phospholipid content [29, 10].

At pH 3, egg yolk reached an EAI of 11.19 m²/g, while albumin displayed a lower value of 8.26 m²/g. Similar differences were observed at pH 5 (egg yolk: 12.94 m²/g; albumin: 3.71 m²/g) and pH 9 (egg yolk: 20.7 m²/g; albumin: 8.21 m²/g), indicating that egg yolk performs better as an emulsifier at these pH values as well. This trend highlights the stability of egg yolk as an emulsifier across a broad pH range, while albumin's EAI remains consistently lower across all pH values. However, at pH 7, albumin reached 8.87 m²/g, closely approximating egg yolk at 9.79 m²/g. Similar results were found in other studies on wheat bran and on field pea isolates, which exhibit relatively high EAI under neutral conditions [7, 28].

Upon addition of TGase (t=0), EAI values dropped significantly at all pH levels compared to the non-treated albumin, possibly due to immediate aggregation or conformational changes reducing interfacial activity. This observation is consistent with reports on soy and hyacinth bean proteins, where partial unfolding or initial aggregation upon TGase exposure reduced emulsifying capacity before structural

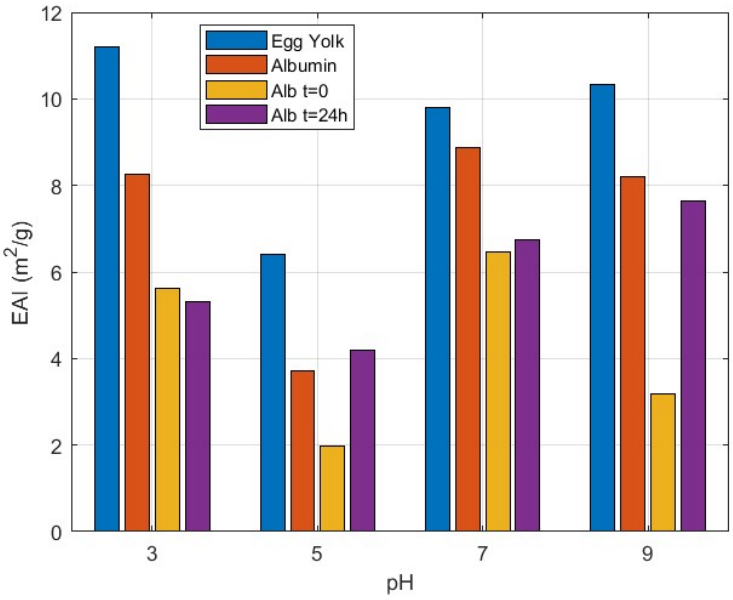

Figure 18: Emulsifying Activity Index (EAI) of egg yolk, non-treated albumin and albumin after crosslinking (heat pretreatment 5 min 95°C, enzyme-to-protein 1:2 w/w, 40°C, t=0 and t=24h) at various pH. See Appendix 8.3 for detailed data.

reorganization occurs [16, 18].

After 24 hours of crosslinking, a modest recovery of EAI is observed at some pH values. At pH 3, the EAI remains nearly unchanged (5.62 to 5.32 m²/g), while at pH 7, a slight increase is noted (6.47 to 6.73 m²/g). More substantial increases are seen at pH 5 (from 1.97 to 4.18 m²/g) and pH 9 (from 3.19 to 7.63 m²/g). These results align with previous findings that TGase crosslinking can improve EAI under certain conditions for plant proteins such as pea and soy [10, 18]. Ali et al. (2010) [16], for example, reported significantly improved EAI in crosslinked pea protein isolates at pH 4, a pH typically associated with low emulsifying performance. A similar trend is observed here at pH 5. This is particularly relevant, as many food emulsions (e.g. dressings, sauces, and beverages) are formulated within the acidic range of pH 3–5 [**?**], highlighting potential applicability for crosslinked albumin in such systems.

Nonetheless, it is important to note that crosslinking does not significantly improve EAI over the non-treated albumin in this study. At pH 3, 7, and 9 the value of the crosslinked albumin (t=24h) does not outperform the non-treated albumin, and only shows moderate improvement at pH 5. Moreover, the addition of TGase appears to suppress emulsifying activity initially (t=0), possibly due to aggregation or loss of flexibility. Overall, these results suggest that under the conditions used in this study, TGase crosslinking does not confer consistent functional advantages in emulsifying activity, and may even hinder EAI in some cases.

The ESI measures the ability of a protein to resist coalescence and separation over time, indicating the stability of the emulsion system [10]. The ESI results for egg yolk, non-treated albumin, and crosslinked albumin samples (t=0 and t=24h) were assessed at different pH values (3, 5, 7, and 9), as shown in Figure 19.

At pH 3, egg yolk shows the highest ESI of 40.21 min, significantly higher than non-treated albumin (17.91 min). This suggests that egg yolk has a stronger ability to stabilize the emulsion at acidic

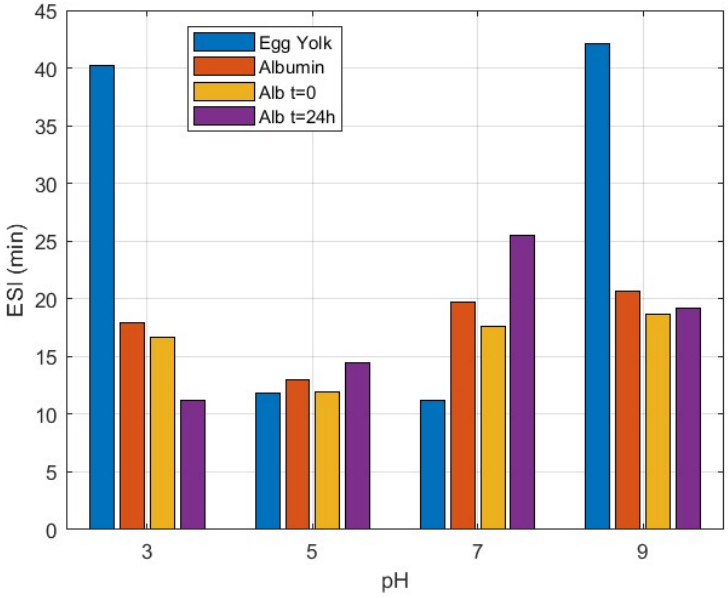

Figure 19: Emulsifying Stability Index (ESI) of egg yolk, non-treated albumin and albumin after crosslinking (heat pretreatment 5 min 95°C, enzyme-to-protein 1:2 (w/w), 40°C, t=0 and t=24h) at various pH. See Appendix 8.3 for detailed data.

pH. At pH 5, egg yolk and non-treated albumin exhibit similar ESI values (11.84 and 12.94 minutes, respectively), indicating comparable emulsion stability. At pH 7, non-treated albumin exhibits a higher ESI value (19.68 minutes) compared to egg yolk (11.16 minutes), indicating superior ESI at neutral pH. Similar neutral pH behavior has been reported for other plant proteins, including field pea and sweet potato isolates [28, 29]. This, combined with a comparable EAI, suggests that albumin could serve as a good alternative to egg yolk for emulsification purposes under neutral conditions. In contrast, at pH 9, egg yolk demonstrates a much higher ESI (42.16 min) than non-treated albumin (20.7 min), indicating that egg yolk outperforms albumin in ESI at alkaline pH.

Upon TGase crosslinking (t=24h), the ESI results exhibit variability across pH values. At pH 3, the ESI for crosslinked albumin (11.23 min) is notably lower than that of the control albumin (16.69 min), suggesting that crosslinking reduces ESI under acidic conditions. However, at pH 5, the ESI of crosslinked albumin increases to 14.41 min, compared to 11.89 min for the control, indicating a minor improvement in stability at slightly acidic pH. At pH 7, the ESI for crosslinked albumin is 25.52 min, significantly higher than the control value of 17.58 min, suggesting that crosslinking notably enhances ESI near neutral pH. At pH 9, crosslinked albumin (19.18 min) shows an increase over control albumin (18.65 min), though the difference is less in alkaline conditions. Similar findings have been reported in studies on other protein sources [10, 16]. Ali et al. (2010) [16] demonstrated that TGase treatment significantly enhanced the ESI of hyacinth bean protein at pH values of 2, 4, 6, and 8. The results found in Figure 19 similarly indicate that crosslinking generally increases ESI under neutral and alkaline conditions, while it may decrease stability in acidic environments.

Overall, a moderate increase in the ESI of TGase-crosslinked albumin was observed at pH 5, accompanied by a slight improvement in EAI compared to the non-treated sample. Since this pH falls within the typical range for many food emulsions, such as sauces and dressings [**?**], the use of crosslinked albu-

min under these crosslinking conditions may offer some functional benefits in these applications at pH 5. However, the observed improvements are relatively limited, and given the additional processing steps and enzyme costs involved, the use of TGase-crosslinked albumin may not be practically or economically justified for emulsification purposes.

4.4.3 Foaming

The results for FC and FS of non-treated albumin, TGase in water, and TGase crosslinked albumin (t=0 and t=24h) at different pH values (3, 5, 7, and 9) are presented in Figures 20 and 21. The results provide insight into TGase treatment, including crosslinking, influences the foaming properties of wheat bran albumin under various pH conditions. This is particularly relevant for applications in aerated food products such as mousses, whipped toppings, and bakery items [10].

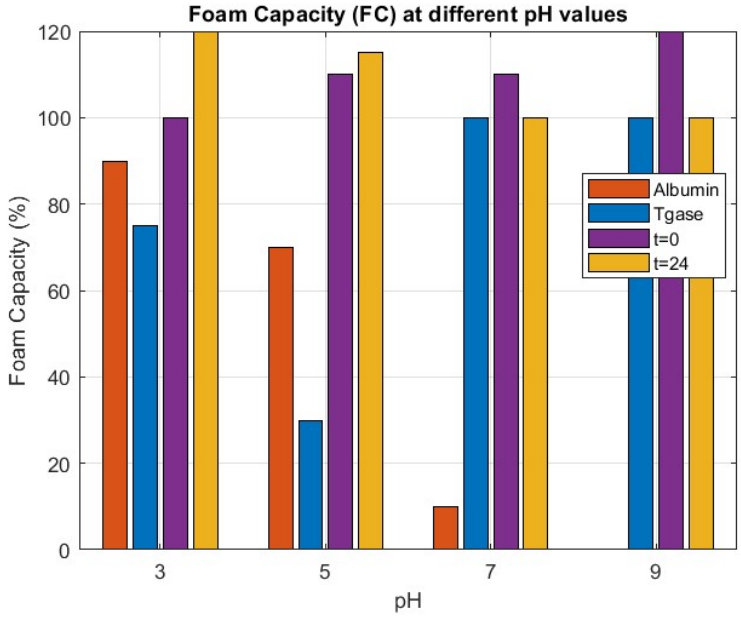

Figure 20: Foaming Capacity (FC) of non-treated albumin, TGase and albumin after crosslinking (heat pretreatment 5 min 95°C, enzyme-to-protein 1:2 (w/w), 40°C, t=0 and t=24h) at various pH. See Appendix 8.3 for detailed data.

The FC reflects the protein's ability to form foam, with the percentage representing the amount of extra volume the foam has formed compared to the original volume before air was introduced [16]. This explains why the FC can exceed 100%.

At pH 3, albumin at t=0 shows a FC of 100%, while the FC of crosslinked albumin (t=24h) increases to 120%. This result contrasts with expectations, as the isoelectric point of albumin is around pH 3-4, which typically leads to protein precipitation at this pH [7]. This is confirmed by the solubility results, where albumin solubility decreases at acidic pH values, promoting protein aggregation and precipitation. However, during homogenization, visible precipitates were largely removed, suggesting that sufficient surface-active protein remained in solution to enable foam formation. This phenomenon was also noted in other protein systems where partial precipitation does not prevent foaming [10]. The observed increase in FC suggests that TGase crosslinking enhances the ability of the protein to rapidly adsorb and unfold at the air–water interface, facilitating foam formation under acidic conditions [16].

At pH 5, the FC of albumin at t=0 increases from 110% to 115% at t=24h, indicating a slight improvement due to crosslinking. The FC of the TGase-only sample at pH 5 is relatively low (30%), likely due to TGase precipitation at this pH. This precipitation was visible in the samples and did not change after homogenization, confirming that TGase alone does not contribute significantly to foaming at pH 5. The combination of TGase and albumin enhances FC compared to the individual components. Notably, no precipitation was observed in this sample at pH 5, which is consistent with the solubility results. Therefore, TGase remains solubilized when combined with albumin, and this may explain why the FC is improved for both t=0 and t=24h samples.

While previous studies have reported peak foaming capacities for TGase-crosslinked plant proteins at mildly acidic pH values (around pH 4–6), such as in hyacinth bean and rice bran proteins [16, 42], the current results show the highest FC at pH 3. This suggests that wheat bran albumin may behave differently from other plant proteins, potentially due to structural differences or higher surface activity under more acidic conditions, as protein flexibility, hydrophobicity, and interfacial behavior are known to vary significantly between sources and influence foaming properties [42].

At pH 7, the FC for TGase-only is high (100%), while the non-crosslinked albumin shows only 10% FC. The high foam capacities observed at both t=0 (110%) and t=24h (100%) at this pH are likely due to the significant amount of TGase in the samples. This explanation also holds for the results at pH 9, where the FC for both t=0 and t=24h is similarly influenced by the presence of TGase. Interestingly, the FC decreases after crosslinking, which contrasts with findings from earlier studies on other protein sources, such as Liu et al. (2021) [10] and Ali et al. (2010) [16], who reported increased FC following TGase treatment. A possible explanation is that wheat bran albumin may lack the molecular flexibility necessary for optimal foaming at neutral and alkaline pH, reducing the effectiveness of TGase-induced structural modifications [42]. Additionally, excessive crosslinking could reduce FC, as reported by Wu et al. (2023), who found that extensive crosslinking at neutral pH decreases FC [42].

These findings suggest that TGase crosslinking appears to enhance FC under acidic conditions (notably at pH 3, slightly at pH 5). However, at neutral and alkaline pH (7 and 9), foaming capacity is primarily attributed to the TGase itself, not the albumin. Since non-crosslinked albumin showed negligible FC in these conditions, and crosslinking did not improve it, the application of crosslinked albumin for foaming purposes under neutral or alkaline pH appears limited. Similar conclusions have been drawn in earlier work, where excessive crosslinking reduced the interfacial activity and foaming ability of plant proteins [42].

FS compares the volume of foam immediately after air is introduced and after 10 minutes, reflecting the ability of the foam to resist collapse [8].

A general decreasing trend in FS is observed when comparing t=0 with t=24h across all pH values. This decrease is particularly significant at acidic pH. At pH 3, FS drops from 70% for t=0 to 12.5% for t=24h, and at pH 5, it decreases from 81.8% for t=0 to 26.1% for t=24h. The relatively high FS for t=0 at pH 5, compared to the TGase-only control, could explain why FC for t=0 is higher than for TGase alone, as no visible precipitant was observed in this sample. However, this improvement is not retained in the crosslinked albumin sample (t=24h), where FS is lower than all other samples.

At pH 7, the combination of albumin and TGase shows a positive effect on FS, indicating that the combination of these two components enhances stability compared to the controls. However, crosslinking the albumin does not contribute additional stability. This suggests that while TGase helps in forming stable foam, the crosslinking of albumin does not further enhance its ability to maintain FS at pH 7.

While there is no FC for albumin alone at pH 9, the albumin-containing sample shows greater FS than TGase alone (22.2%), suggesting that albumin contributes to foam stability even without initially

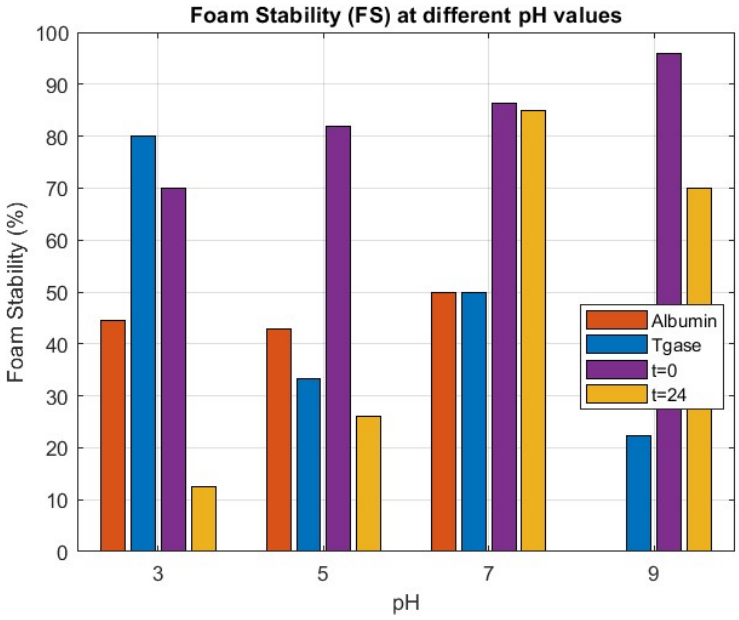

Figure 21: Foaming Stability (FS) of non-treated albumin, TGase and albumin after crosslinking (heat pretreatment 5 min 95°C, enzyme-to-protein 1:2 (w/w), 40°C, t=0 and t=24h) at various pH. See Appendix 8.3 for detailed data.

producing foam. This is possibly due to its interaction with TGase, reinforcing the interfacial network and preventing rapid foam collapse.

In contrast to the observed results, previous studies have generally reported an increase FS upon TGase-induced crosslinking of plant proteins across a range of pH values, particarly under acidic conditions [8, 16, 42]. Wu et al. (2023) [42] observed that rice bran protein isolate treated with TGase showed significantly improved foam stability at pH 5, attributed to enhanced interfacial film formation and reduced bubble coalescence.

However, in the case of wheat bran albumin, crosslinking appears to have the opposite effect, decreasing FS across all pH values. This unexpected outcome may be explained by the fact that crosslinking altered the balance between solubility and interfacial activity. While moderate crosslinking can improve protein functionality, even partial aggregation may reduce protein mobility at the air–water interface, thereby limiting the ability to form and stabilize foam. Similar observations have been reported in studies on other plant proteins, where the formation of crosslinked networks restricted interfacial rearrangement and ultimately decreased foam stability [20].

Due to time constraints and limited sample availability, gelation behavior and nutritional properties could not be experimentally assessed, despite their recognized importance as highlighted in the literature review.

5 Scaling Up the Extraction

To assess the practical feasibility of scaling up the protein extraction process from wheat bran, it is essential to translate the results obtained in the laboratory to a larger industrial context. Upscaling allows for the evaluation of how the laboratory process can be implemented in a cost-effective and technically viable manner. While lab-scale experiments provide valuable data on yields and process dynamics, they cannot fully account for scale-dependent factors such as material handling, equipment design, and flow behavior. Therefore, upscaling becomes a critical step in bridging the gap between laboratory-scale success and industrial-scale application.

The goal of this chapter is to explore the step towards scaling up by integrating insights from laboratory experiments with process modeling and simulation tools. Specifically, this chapter combines data analysis in MATLAB with process simulation in SuperPro Designer to evaluate the feasibility of scaling up the protein extraction process from wheat bran. While aspects such as costs and energy consumption were not considered, the simulation in SuperPro Designer, based on MATLAB calculations, provides insights into the required input quantities and the resulting output.

The basis for the upscaling study is the semi-continuous extraction process developed and tested at the laboratory scale. In this setup, a fixed amount of wheat bran was used for each extraction batch, while water was added in fractions and continuously drained. Specifically, 100 gram of wheat bran was combined with approximately 15 L of water, yielding 1.42 g of albumin. After this, the next fraction was extracted with in total 9 L of 0.4 M NaCl which amounted to 0.91 g of globulin. The last fraction was extracted with 12 L of 0.05 M NaOH at 60°C yielding to 1.15 g of glutelin. This semi-continuous configuration, where the solid phase remains constant while the liquid phase is replenished and extracted, serves as the conceptual model for the upscaling process described below.

5.1 MATLAB Analysis: Kinetic Modelling

To quantify the performance of the extraction process, a comprehensive data analysis was conducted in MATLAB, resulting in kinetic curve fitting. This provides valuable insights into the behavior of the extraction process and aids in the upscaling to industrial applications in SuperPro Designer. See Appendix 8.4 for the exact calculations.

A first-order extraction model was fitted to cumulative extraction data from fractionated samples using the equation:

$$E(V) = E_{max} \times (1 - e^{-kV}) \tag{7}$$

This model provides an estimate of both the maximum extractable protein (E_{max}) and the extraction constant (k) for each protein fraction [43]. V in this equation accounts for the volume of the solvent.

The plot in Figure 22 displays the cumulative protein extraction as a function of extraction volume. Each set of experimental data is plotted with circular markers, while the solid lines represent the fitted curves using Equation 7. Each protein fraction - albumin, globulin, and glutelin - is shown as a separate curve with the exponential model fit. The staggered x-axis represents the sequential addition of extraction liquids. The fitted curves highlight how each protein shows a characteristic saturation behavior.

The kinetic modeling results provide important insights into the dynamics of the extraction process. The parameter E_{max} represents the maximum extractable protein (g) from each fraction, while k is the extraction constant (L^{-1}) that describes how efficiently the protein is extracted as a function of solvent volume. The values for E_{max} and k were calculated for each protein fraction and are summarized in Table 9.

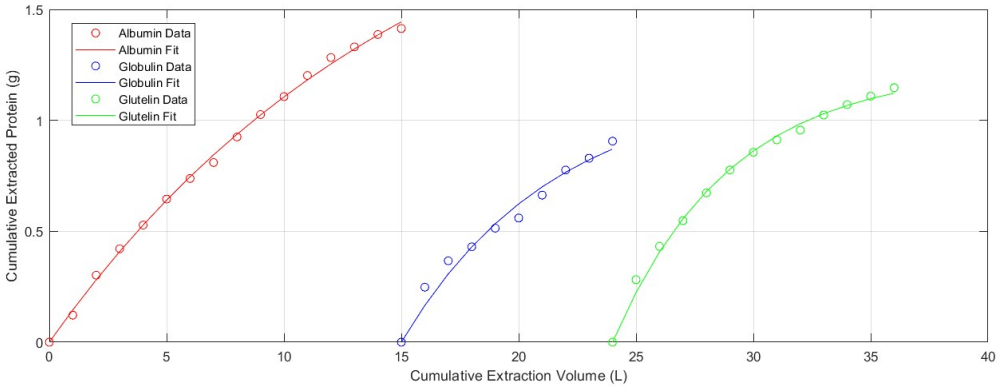

Figure 22: Cumulative protein extraction as a function of extraction volume for albumin (red), globulin (blue), and glutelin (green). The fitted curves show the characteristic saturation behavior for each protein, with albumin reaching saturation slowly, while glutelin exhibits rapid extraction dynamics.

Table 9: Maximum extractable protein (E_{max}) and extraction constants (k) for albumin, globulin, and glutelin from 100 g wheat bran derived from the kinetic model.

	Albumin	Globulin	Glutelin
E_{max} (g)	2.345	1.162	1.235
k (L^{-1})	0.064	0.154	0.200

This representation allows direct comparison of the extraction dynamics:

- Albumin (red): The extraction of albumin is relatively slow, as indicated by the less steep initial slope of the curve and the low k-value (0.064). This indicates that a larger solvent volume is required to achieve comparable yields. Nevertheless, albumin reaches a substantial maximum extractable amount of 2.345 g (E_{max}), showing that significant recovery is possible, although at higher solvent consumption.

- Globulin (blue): The extraction of globulin is faster than that of albumin, as indicated by the steeper initial curve and the higher k-value (0.154). This suggests that globulin requires less extraction volume to reach its saturation point. However, globulin can only reach an E_{max} of 1.162 g, which is the smallest fraction compared to albumin and glutelin, as reported in the literature [7]. This aligns with the lower extracted amount of globulin observed in the extraction process.

- Glutelin (green): Glutelin follows a similar pattern to globulin, but with a higher E_{max} (1.235 g), indicating a larger extractable quantity. Despite this, the extracted amount of glutelin remains considerably lower than that of albumin, even though literature reports comparable or slightly higher yields for glutelin relative to albumin [7]. The higher k-value (0.200) indicates that glutelin is extracted more rapidly and with less volume, reflecting its relatively high solubility and faster extraction dynamics. This could be due to the fact that glutelin is extracted at 60°C, a temperature that may enhance its solubility and extraction rate compared to the other proteins.

5.2 SuperPro Designer

SuperPro Designer allows for the creation of simplified process flows and material balances, providing both visual and quantitative insights into input and output streams. By simulating the process, potential challenges related to material throughput and solvent usage can be identified early in the upscaling process, facilitating more informed decisions on process optimization.

A preliminary model was constructed in SuperPro Designer to simulate the protein extraction process at a scale of 1 kg of wheat bran per batch. The objective of this simulation is to evaluate the feasibility of scaling the process from laboratory to industrial scale. Within SuperPro Designer, the extraction process is modeled as a mixer-settler extraction step followed by an ultrafiltration unit, where protein is extracted from wheat bran, and the residue is subsequently transferred to the next extraction step for the following protein fraction.

The extraction process is modeled based on the extrapolated efficiencies obtained from the MATLAB analysis. These efficiencies provide the maximum extractable protein for albumin, globulin, and glutelin, as well as the extraction constants (k) for each protein fraction. The model incorporates these values to predict the amounts of extracted protein, remaining solids, and water usage required for a semi-continuous process involving 1 kg of wheat bran. The inflow stream of wheat bran was set to 1 kg and mass compositions were calculated using the E_{max} calculated in Section 5.1. So, the mass compositions of albumin, globulin, and glutelin in the wheat bran were set to 2.345%, 1.162%, and 1.235%, respectively, based on their relative abundances. The goal is to achieve a minimum extraction yield of 80% for each protein fraction.

The simulation flowchart consists of the following key unit operations:

- Mixer-Settler Extraction: The extraction step simulates the contact between the wheat bran and the extraction solvent. The amount of solvent required for extraction is based on the laboratory experiments. During this stage, part of the protein is dissolved into the solvent.
- Ultrafiltration: After extraction, the protein solution is passed through a filtration unit to concentrate the extracted protein. A membrane filtration of 10 kDa is used. This step is essential for concentrating the protein solution and removing excess small solid material. For each filtration unit a concentration factor (feed/retentate) of 10 is used. If further concentration is required in the future, additional ultrafiltration units can be added. However, achieving higher concentrations in a single filtration unit is challenging.
- Solvent Recycling: After concentrating the extracted proteins, the solvent is recycled back into the extraction step. For each recycling stream a purge of 10% is taken out of the stream to prevent the build-up of contaminants and ensure the quality of the solvent.

5.2.1 Albumin extraction in SuperPro Designer

At laboratory scale, 15 L of albumin extract were obtained from a total of 18 L of solution, with 3 L of water required to maintain the wheat bran slurry flow. The albumin extraction yield is calculated as

$$\frac{E_{alb}(g)}{E_{max}(g)} = \frac{1.42}{2.345} = 60.6\%$$

indicating that 60.6% of the albumin relative to the maximum extractable amount (E_{max}) was recovered from 15 L of solvent per 100 g of wheat bran. To achieve a target yield of at least 80%, the required

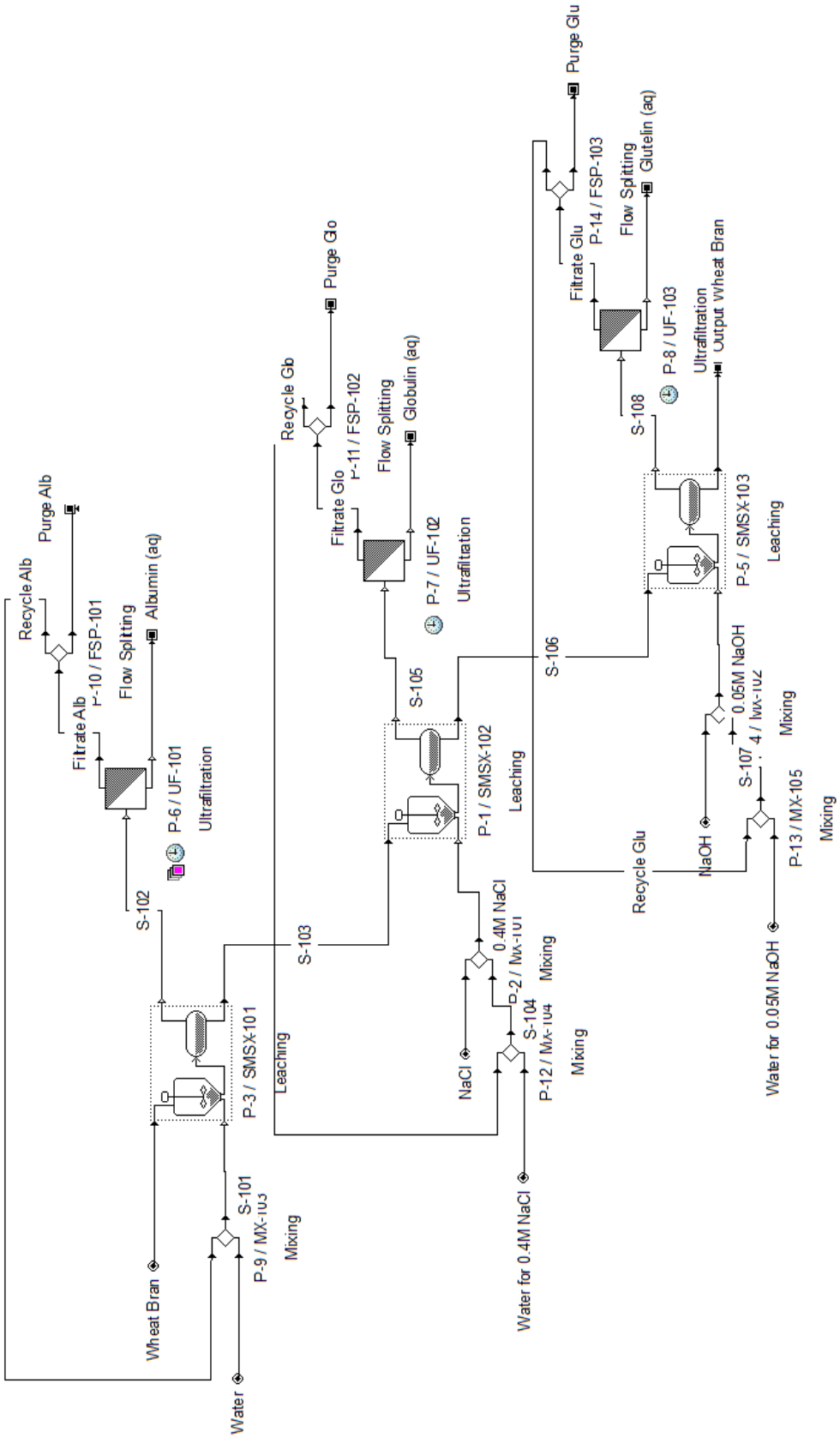

Figure 23: SuperPro Designer model simulation of the protein extraction from wheat bran.

solvent volume was calculated using:

$$\frac{E_{alb}(g)}{E_{max}(g)} = (1 - e^{-kV}) = 80\%$$

based on the parameters in Table 9, resulting in a required volume of 25 L. Including the additional 3 L that flows with the wheat bran to the subsequent extraction, the initial volume should be set at 28 L for the initial 100 g of wheat bran. Scaling this by a factor of 10 yields a volume of 280 L, with an anticipated solvent loss of 30 L.

The albumin extract is then concentrated by ultrafiltration. The rejection coefficient of the membrane is set at 0.9, reflecting the typical molecular size of albumin (20–50 kDa). This ensures substantial retention of albumin, while water and smaller molecules permeate. This resulted in 28.0 L of albumin solution at a concentration of 0.70 g/L.

To minimize water usage, the filtrate is recycled back into the extraction process. Consequently, the fresh water inflow required is reduced to 53.2 L.

5.2.2 Globulin Extraction in SuperPro Designer

Following albumin extraction, the residual slurry is transferred to the next extraction step, where 0.4 M NaCl solution is added to facilitate globulin extraction. The presence of NaCl improves globulin solubility in the aqueous phase, enhancing extraction efficiency. In laboratory experiments, 9 L of 0.4 M NaCl were used; combined with 3 L of residual water from the albumin extraction, the total solvent volume was adjusted to achieve the target molarity. Solvent loss was again observed at 3 L.

This step yielded 0.91 g of globulin, corresponding to a recovery yield of:

$$\frac{E_{glo}(g)}{E_{max}(g)} = \frac{0.91}{1.162} = 78.3\%$$

. The volume required to reach an 80% extraction yield, calculated with the kinetic model of Equation 7, is 10.5 L. Considering solvent loss and scaling by a factor of 10, the solvent volume for this step should be 135 L. Since 30 L of water remain from the previous extraction, an additional 105 L of NaCl solution is needed. To achieve a 0.4 M NaCl concentration, 3.16 kg of NaCl must be added.

Ultrafiltration concentration of globulin employs a rejection coefficient of 0.95, reflecting its larger molecular size compared to albumin. This results in an output volume of 10.5 L with a globulin concentration of 0.85 g/L.

The ultrafiltration filtrate is recycled into the extraction process, allowing the required fresh water addition to be reduced to 20 L, with only 0.61 kg of fresh NaCl needed.

5.2.3 Glutelin Extraction in SuperPro Designer

The remaining slurry proceeds to the final extraction, where a temperature of 60°C and 0.05 M NaOH are applied to promote glutelin solubilization. In laboratory-scale experiments, 14 L of solvent were used, including 3 L retained with the wheat bran. The glutelin recovery yield was notably high:

$$\frac{E_{glu}(g)}{E_{max}(g)} = \frac{1.15}{1.235} = 93.1\%$$

This high extraction efficiency indicates that the majority of the extractable glutelin was recovered, leaving little room for improvement by adding more solvent. Consequently, when scaling by a factor of 10,

the originally used 140 L of NaOH solution, combined with the 30 L retained with the wheat bran, totaling 170 L of solvent, is deemed sufficient for effective glutelin extraction without the need for additional solvent input. The corresponding amount of NaOH required to achieve the 0.05 M concentration is 0.34 kg. However, with an additional 30 L of solvent coming from the globulin extraction, only 140 L of NaOH solution is needed.

For ultrafiltration, a rejection coefficient of 0.85 is assumed, considering the higher extraction temperature and possible molecular size changes. This results in 14.0 L of glutelin solution at 0.71 g/L.

Due to the recycling process, the input of fresh water is reduced to 26.6 L, with 68 g of NaOH required as fresh inputs.

Overall, in this modeled extraction, 28 L of 0.70 g/L albumin, 10.5 L of 0.85 g/L globulin, and 14.0 L of 0.71 g/L glutelin were extracted from 1 kg wheat bran. The waste streams generated in the process consist of three purge streams and the excess wheat bran residue, which combine to form a total of 47.3 L of solvent and 961.6 g of residue wheat bran.

The combined modeling and simulation approach offers a foundation for optimizing and scaling the protein extraction process towards industrial implementation.

6 Conclusion

This study demonstrated that wheat bran, an underutilized byproduct of the milling industry, holds considerable potential as a sustainable source of functional plant proteins. Through sequential extraction using a modified Osborne method, key protein fractions—albumins, globulins, and glutelins—were successfully isolated. Albumins and glutelins emerged as the most promising fractions in terms of yield and techno-functional performance, whereas globulins showed limited extractability under the applied conditions.

Enzymatic crosslinking with microbial transglutaminase was investigated to enhance the functional properties of the extracted proteins. Pretreatments such as heat treatment and the addition of βME were applied to partially unfold the protein structure, thereby improving the accessibility of reactive amino acids for crosslinking. SDS-PAGE analysis confirmed the formation of high-molecular-weight protein aggregates, particularly for albumins and glutelins, when a 1:2 enzyme-to-protein ratio was used. Additional enzyme ratios were tested to optimize the balance between crosslinking efficiency and process cost, as excessive crosslinking or high enzyme concentrations may adversely affect protein functionality.

Functional properties, which are essential for the incorporation of plant proteins into complex food systems, were evaluated across different pH values. The tested properties included solubility, emulsifying capacity, and foaming behavior. A 24-hour crosslinked albumin sample with heat pretreatment was selected for these analyses, and was compared to non-treated albumin and a $t = 0$ crosslinked control sample. The results showed that extensive crosslinking reduced protein solubility, consistent with aggregate formation. The emulsifying capacity of the crosslinked albumin was decreased compared to the non-treated protein, while emulsifying stability was improved at certain pH values. However, overall, emulsification was not enhanced. In contrast, foaming capacity and stability were significantly improved in the crosslinked samples compared to the non-treated albumin, particularly at pH 7 and 9, though these results may have been influenced by the high TGase concentration. Overall, crosslinking under the tested conditions did not consistently improve the functional properties, suggesting that milder crosslinking through reduced enzyme dosage and/or shorter reaction times could yield better results.

Additionally, process modeling using SuperPro Designer and kinetic analysis in MATLAB provided valuable insights into the scalability of the extraction process. The simulations highlighted key parameters for optimizing throughput and solvent usage, demonstrating the feasibility of industrial-scale implementation. In the simulated process, 28 L of 0.70 g/L albumin, 10.5 L of 0.85 g/L globulin, and 14.0 L of 0.71 g/L glutelin were extracted. Through solvent recycling, the system was further optimized, as the need for fresh solvent was reduced.

In conclusion, the valorization of wheat bran proteins through targeted extraction presents a viable pathway toward more sustainable and circular food systems. While enzymatic crosslinking with TGase, as applied in this study, did not consistently enhance protein functionality, further research into food-grade pretreatments and crosslinking optimization will be critical to fully unlock wheat bran's potential as a valuable functional ingredient for future-proof food applications. The following recommendations outline key avenues for future research:

- **Assessment of Protein Purity and Composition:** Future studies should include complementary methods such as Kjeldahl analysis, CHNS, or amino acid profiling to more accurately determine the protein content and purity of extracted fractions. This is essential for validating functional improvements and ensuring consistent quality across batches.

- **Optimization of Crosslinking Conditions:** The present work highlighted that extensive crosslinking can negatively impact solubility and emulsifying properties. Future studies should focus on

optimizing TGase dosage and reaction time to achieve a balance between improved foaming stability and retention of solubility and emulsification capacity. Investigating alternative, food-grade pretreatments, such as mild heating or enzymatic unfolding, could enhance crosslinking efficiency without compromising functional properties.

- **Broader Functional Characterization:** Expanding the analysis to include gelation behavior would provide a more comprehensive understanding of the functional improvements achieved through crosslinking, particularly for applications in meat analogues and dairy substitutes.
- **Nutritional and Sensory Evaluation:** While this study focused on techno-functional aspects, the nutritional quality and sensory attributes of crosslinked wheat bran proteins require evaluation to confirm their suitability for consumer food products.
- **Industrial-Scale Validation:** Further process development, including pilot-scale trials and techno-economic assessments, is necessary to validate the scalability and commercial viability of wheat bran protein extraction and modification strategies proposed in this work.

By addressing these research directions, a clearer pathway can be established toward the integration of wheat bran proteins as functional, sustainable ingredients in next-generation food products.

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8 Appendix

8.1 Calibration curves

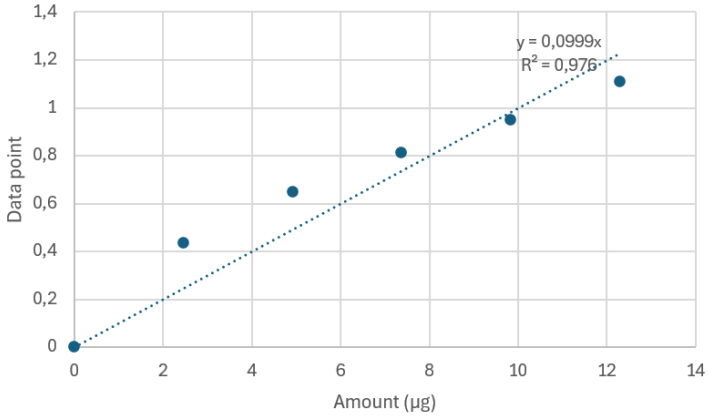

Figure 24: Calibration curve for the Bradford protein assay using bovine serum albumin (BSA) as standard. The data points represent the blank-corrected absorbance at 595 nm, plotted against the BSA amount (µg). This curve was used to determine the protein content of unknown samples.

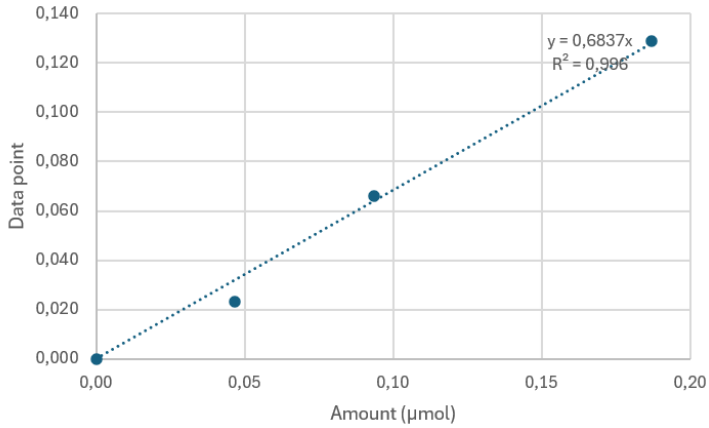

Figure 25: Calibration curve for the Berthelot assay using ammonium chloride (NH_4Cl) as standard. The data points represent the blank-corrected absorbance at 660 nm, plotted against the amount of ammonium (µmol). This curve was used to quantify ammonia released from crosslinked protein samples.

8.2 CHNS

Table 10: Results Elemental Analysis

Name	N (%)	S (%)
Wheat Bran	2.44	0.55
Wheat Bran	2.46	0.41
Protein extracted wheat bran	0.90	0.17
Protein extracted wheat bran	1.10	0.15

8.3 Functional Properties Data

Table 11: Solubility of wheat bran albumin protein [1] (mean ± SD, %).

pH	2	4	6	8	10	12
Solubility (%)	88.3 ± 3.0	63.1 ± 3.2	96.5 ± 2.7	94.3 ± 2.7	94.5 ± 2.7	91.0 ± 2.6

Table 12: Albumin solubility before and after crosslinking (t=0 and t=24h, %).

pH	3	5	7	9
Alb t=0 (control) (%)	27.0	89.1	90.4	96.9
Alb t=24h (crosslinked) (%)	17.3	51.3	60.9	60.6

Table 13: Emulsifying Activity Index (EAI) for different samples at various pH values

pH	**Egg Yolk**[1]	**Albumin**[1]	**Alb t=0**	**Alb t=24h**
3	11.19	8.26	5.62	5.32
5	6.41	3.71	1.97	4.18
7	9.79	8.87	6.47	6.73
9	10.33	8.21	3.19	7.63

Table 14: Emulsifying Stability Index (ESI) for different samples at various pH values

pH	**Egg Yolk**[1]	**Albumin**[1]	**Alb t=0**	**Alb t=24h**
3	40.21	17.91	16.69	11.23
5	11.84	12.94	11.89	14.41
7	11.16	19.68	17.58	25.52
9	42.16	20.7	18.65	19.18

Table 15: Foam Capacity (FC) for different samples at various pH values

pH	**Albumin (t=0)**	**Albumin (t=24h)**	**Albumin**	**TGase Only**
3	100%	120%	90%	75%
5	110%	115%	70%	30%
7	110%	100%	10%	100%
9	120%	100%	0%	100%

[1]These data were kindly provided by PhD Varshini Krishnamoorthy, who performed the measurements.

Table 16: Foam Stability (FS) for different samples at various pH values

pH	Albumin (t=0)	Albumin (t=24h)	Albumin	TGase Only
3	70.0%	12.5%	44.4%	80.0%
5	81.8%	26.1%	42.9%	33.3%
7	86.4%	85.0%	50.0%	50.0%
9	95.8%	70.0%	x	22.2%

8.4 Calculations cumulative model

```
% Initial protein content (literature vs experimental)
initial_protein_content_lit = 16.8; % from literature (g per 100g of
    dry bran)
initial_protein_content_exp = 15.31; % from CHNS analysis (g per 100g
    of dry bran)

extractable_protein = 82.5; % according to literature
bran_mass = 100; % in g
total_protein_lit = (initial_protein_content_lit *
    extractable_protein / 10000) * bran_mass;
total_protein_exp = (initial_protein_content * extractable_protein /
    10000) * bran_mass;

% Experimental data
V_values_alb = [0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14,
    15]; % Volumes in L
V_values_glo = [0, 1, 2, 3, 4, 5, 6, 7, 8, 9]; % Volumes in L
V_values_glu = [0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12]; % Volumes
    in L]
C_values_alb = [0, 0.122, 0.180, 0.119, 0.107, 0.117, 0.093, 0.072,
    0.115, 0.101, 0.081, 0.095, 0.081, 0.048, 0.056, 0.027]; % Albumin
    (g/L)
C_values_glo = [0, 0.248, 0.119, 0.063, 0.084, 0.046, 0.103, 0.113,
    0.053, 0.077]; % Globulin (g/L)
C_values_glu = [0, 0.282, 0.150, 0.116, 0.125, 0.103, 0.080, 0.056,
    0.044, 0.068, 0.047, 0.038, 0.038]; % Glutelin (g/L)

% Calculate cumulative proteinextraction (g)
E_values_alb = cumsum(C_values_alb); % every fraction = 1 L
    proteinmass = concentration
E_values_glo = cumsum(C_values_glo);
E_values_glu = cumsum(C_values_glu);

% Model for cumulative extraction
% E(V) = E_max * (1 - exp(-k * V))
cumulative_model = @(params, V) params(1) * (1 - exp(-params(2) * V))
    ; % params = [E_max, k]
```

```
% Initial estimation
init_guess = [2, 0.5]; % [E_max (g), k (1/L)]

% Curve fit
params_alb = lsqcurvefit(cumulative_model, init_guess, V_values_alb,
    E_values_alb);
params_glo = lsqcurvefit(cumulative_model, init_guess, V_values_glo,
    E_values_glo);
params_glu = lsqcurvefit(cumulative_model, init_guess, V_values_glu,
    E_values_glu);

% Maximum extracted protein & k-values
E_max_alb = params_alb(1);
E_max_glo = params_glo(1);
E_max_glu = params_glu(1);
k_albumin = params_alb(2);
k_globulin = params_glo(2);
k_glutelin = params_glu(2);

E_max_ex = total_protein_exp - (E_max_alb + E_max_glo + E_max_glu);

% Fitvalues
E_fit_alb = cumulative_model(params_alb, V_values_alb);
E_fit_glo = cumulative_model(params_glo, V_values_glo);
E_fit_glu = cumulative_model(params_glu, V_values_glu);

% Verschuif V-waarden om doorgang te simuleren
V_values_glo_shifted = V_values_glo + 15; % start na Albumin
V_values_glu_shifted = V_values_glu + 24; % start na Globulin

% Fitwaarden met verschoven V's
E_fit_glo_shifted = cumulative_model(params_glo, V_values_glo_shifted
    - 15); % let op: fit gebruikt originele V
E_fit_glu_shifted = cumulative_model(params_glu, V_values_glu_shifted
    - 24); % idem

% Plot
figure;
plot(V_values_alb, E_values_alb, 'ro', 'DisplayName', 'Albumin');
    hold on;
plot(V_values_alb, E_fit_alb, 'r-', 'DisplayName', 'Albumin model');

plot(V_values_glo_shifted, E_values_glo, 'bo', 'DisplayName', '
    Globulin');
plot(V_values_glo_shifted, E_fit_glo_shifted, 'b-', 'DisplayName', '
```

```
    Globulin model');

plot(V_values_glu_shifted, E_values_glu, 'go', 'DisplayName', '
    Glutelin');
plot(V_values_glu_shifted, E_fit_glu_shifted, 'g-', 'DisplayName', '
    Glutelin model');

xlabel('Cumulative Extraction Volume (L)');
ylabel('Cumulative Extracted Protein (g)');
title('Cumulative Protein Extraction per Protein Fraction');
legend('Albumin Data', 'Albumin Fit', 'Globulin Data', 'Globulin Fit
    ', 'Glutelin Data', 'Glutelin Fit');
grid on;

% Show results
fprintf('\n--- Albumin extraction ---\n');
fprintf('E_max: %.3f g | k: %.3f per L\n', params_alb(1), params_alb
    (2));

fprintf('\n--- Globulin extraction ---\n');
fprintf('E_max: %.3f g | k: %.3f per L\n', params_glo(1), params_glo
    (2));

fprintf('\n--- Glutelin extraction ---\n');
fprintf('E_max: %.3f g | k: %.3f per L\n', params_glu(1), params_glu
    (2));
```