Wheat Proteins-based Bioplastics; Synthesis, Properties, and Applications.

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Wheat Proteins-based Bioplastics; Synthesis, Properties, and Applications.

A thesis submitted to the Department of Biotechnology, Quaid-I-Azam University Islamabad, in partial fulfillment of the requirements for the degree of Master of Philosophy in Biotechnology.

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In the name of Allah, The Most Gracious, The Most Merciful.

Declaration

I hereby declare that the work presented in this thesis is my own effort, except where otherwise acknowledged, and that the thesis is my own composition. No part of this thesis has been previously published or presented for any other degree or certificate.

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Certificate of Approval

This is to certify that the research work presented in this thesis, entitled "Wheat Proteins-based Bioplastics; Synthesis, Properties, and Applications" was conducted by Miss Ayesha Asif under the supervision of Dr. Faiza Rasheed.

No part of this thesis has been submitted anywhere else for any degree. This thesis is submitted to the Department of Biotechnology, Faculty of Biological Sciences, Quaid-I-Azam University, Islamabad, Pakistan in partial fulfillment of the requirements for the Degree of master's in philosophy in the field of Biotechnology from the Department of Biotechnology, Faculty of Biological Sciences, Quaid-I-Azam University, Islamabad, Pakistan.

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Dated: _____

وَمَا تَوْفِيقِي إِلَّا بِاللَّهِ

And verily my success is only by Allah (Surah Hud – 11:88)

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Ayesha Asif

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Abbreviations	Description
WG	Wheat gluten
3D	Three dimensional
IG	Industrial gluten
FC	Folin-Ciocalteu
MPa	Mega Pascals
Gly	Glycerol
BSA	Bovine serum albumin
rpm	Rotations per minute
MP	Monomeric proteins
PP	Polymeric proteins
DW	Distilled water
SE-HPLC	Size exclusion high-performance liquid chromatography

ABSTRACT

Proteins have numerous non-edible and edible uses. Wheat flour contains starch and wheat proteins. Gluten protein from wheat is widely used in the food and bioplastic industry but industrially available gluten is not 100% pure. Industrially separated gluten has different associated impurities e.g., lipids, starch, carbohydrates, etc. High-temperature exposure up to 160°C required for its separation from starch, resists the free flow of starch molecules, their entrapment causes the formation of hard particles and aggregated, a pre-crosslinked network of gluten formed. Lower solubility, protein content, and water absorption capacities of bioplastics prepared from untreated industrial gluten limit applications of industrial gluten. Different methods for wheat gluten purification including mild, mechanical, and enzymatic treatments through cellulase and amylase were developed for increasing the protein content and purity of gluten. Lowry assay was performed to determine the protein content of differently treated samples. Protein content increased with an increase in purity. The protein content of enzymatically treated mild and mechanically extracted gluten was higher as compared to untreated gluten samples. Films from untreated and treated samples were prepared and free swelling capacity was evaluated through the tea bag assay. Enzymatically treated gluten samples have shown higher absorption capacities in distilled water and saline solutions. The protein solubility of samples was analyzed through size-exclusion high-performance liquid chromatography (SE-HPLC). Industrial untreated gluten samples showed lower solubilities after repeated extractions in contrast to samples that were purified through different methods and showed high solubilities. Results from the different analyses have shown that mild, mechanical, and enzymatic treatment of gluten had less aggregated and loosely bound protein networks. Biobased gluten films with improved water absorption capacities have shown good potential to be used in hygiene products as a sustainable alternative to synthetic plastics.

1. INTRODUCTION AND REVIEW OF LITERATURE

Proteins are nanometer-scale molecular machines that perform different biological functions [1]. They serve as the foundation for every cell in our bodies as well as every other living organism across all kingdoms. Although DNA molecule encodes the information required for life to continue, proteins are responsible for the essential life processes including maintenance, replication, reproduction, and defense [2].

The value of proteins to living organisms cannot be overstated. The function of proteins is essential to almost all biological activities. They generally consist of different enzymes that are involved in metabolic processes, maintenance, and growth, function as hormones, and signaling molecules, regulate the immune system and physiological pH and serve as storage and transport proteins [3]. At about 50% of tissue's dry weight, they make up the majority of the organic compounds in vertebrates. Amino acids are the building blocks of proteins. In long chains, hundreds to thousands of twenty different amino acids are linked together to form proteins [4].

Twenty naturally occurring amino acids are more common than other unique ones with specific functions. These twenty amino acids can be arranged in many ways to form proteins, which are constrained by stereochemical characteristics and defined by the genetic code. Regarding their roles, these proteins have transient or constitutive expressions in cells [5, 6].

Protein production for industrial applications first began at the turn of the 20th century, the sector looked for new components to increase process efficiency and find alternatives to make it more economically feasible. Advancements in bioprocess technology offer a wide range of possibilities for producing proteins with improved properties for applications in different industrial operations [7]. Industrial applications of proteins are depicted in Figure 1.1.

1.1 Protein structure and shapes

Amino acids, which are building blocks of proteins, are made up of two different chemical groups, NH2 (amine), a basic group, and COOH (carboxyl), an acidic group, both are joined to organic acid's alpha carbon (carbon adjacent to the carboxyl group). A peptide bond is formed when the amine group of one amino acid and the carboxyl group of another amino acid are linked together [8].

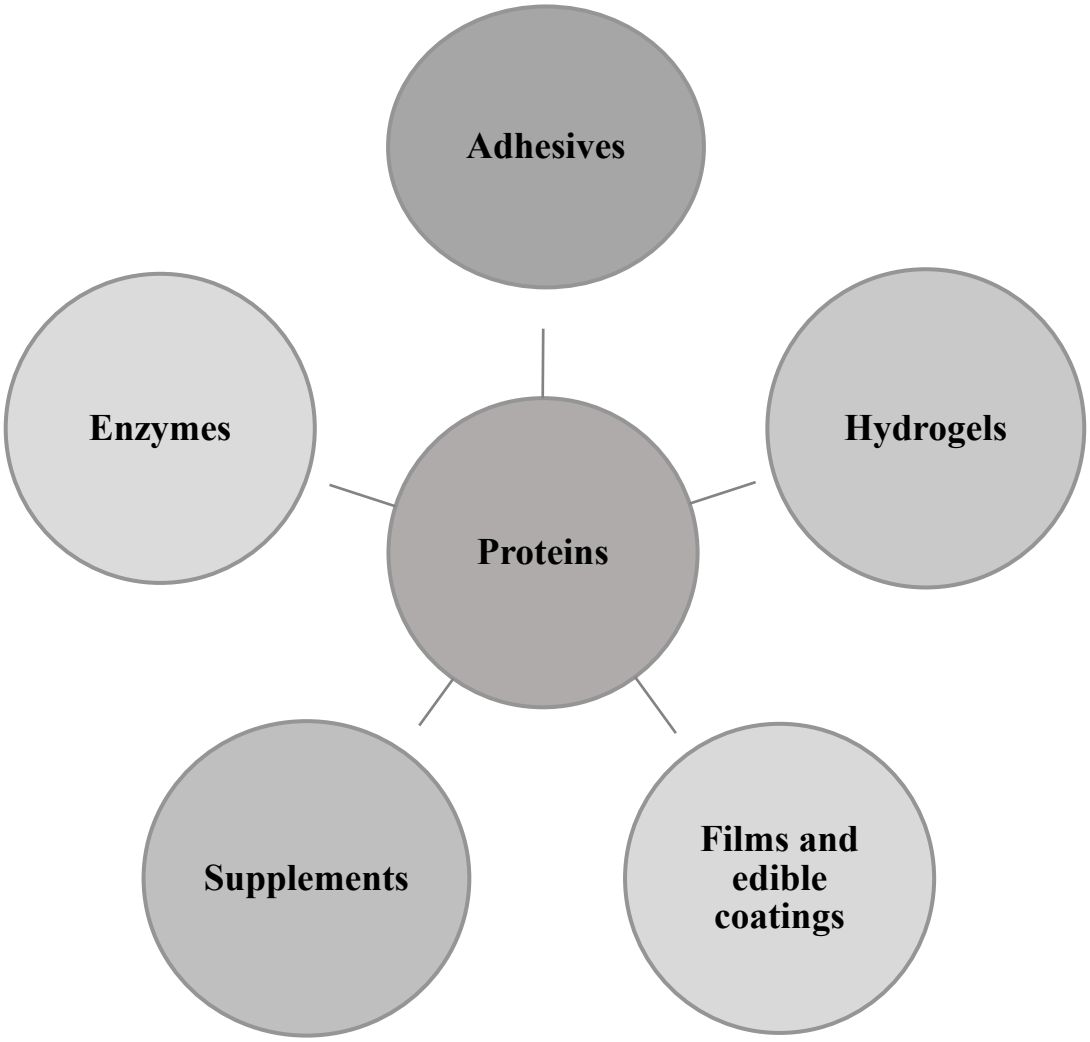

Figure 1.1: Industrial importance of proteins.
The inherent chemical properties of animal and plant-based proteins are exploited in various industries.

The primary structure of proteins is also referred to as polypeptide chain, that is formed when amino acids are arranged in a linear sequence. Regions of protein folding or coiling, e.g. pleated sheets and alpha helices, which are supported by hydrogen bonds are referred to as secondary structures [9].

The beta-sheet and alpha-helix forms predominate among the several secondary structure conformations that are available. Random coils are disorganized polypeptide chain segments that can also be found. Patterns of hydrogen bonding, amino acid composition, and sequence in the polypeptide chain determine all secondary structural forms in proteins [10]. The 3D structure of proteins is referred to as tertiary structure. The term quaternary structure describes protein molecules made up of several polypeptide chains or subunits [11].

Each protein has a distinctive molecular shape in its original state. Proteins can be divided into two categories: fibrous and globular. In globular proteins, molecules fold into an ovoid or compact spheroid with three similar-length axes. They include antibodies, hemoglobin, hormones, and enzymes, and they are more sensitive to pH and temperature than fibrous proteins [9]. In fibrous proteins, extended sheets or fibers are formed from parallel polypeptide chains, where the longitudinal axis prevails over the transversal one. They are found in structures like connective tissue fibers that require a lot of physical power [4].

Historically, the solubility of proteins was used to categorize them e.g. Prolamins (zein, gliadin, etc.) are soluble in 50-80% ethanol, and now a more precise classification system based on function and structure is used in which amino acid sequences are compared e.g. vicilin family of seed storage proteins are found in legumes, share similar physical and functional characteristics due to their considerable amino acid sequence similarity [10].

Examples of proteins with specific functions include collagen, which is widely present in tendons, bones, cartilage, and skin. Keratin proteins are present in skins, hair, horn, and feathers [9]. Hemoglobin and myoglobin are transport proteins, responsible for the transport of oxygen in blood and muscles respectively. Globulins, albumins, and fibrinogen are blood plasma proteins. Actin, Troponin, myosin, and myofibrils are skeletal muscle proteins [4].

1.2 Industrial importance of proteins

When it comes to human health and nutrition, proteins are crucial macronutrients Their amino acid composition, digestibility, bioavailability, purity, and processing have a significant impact on their nutritional quality. Because of their numerous non-edible and edible uses, and

biodegradability, plant-derived proteins have recently acquired popularity when animal-derived proteins are unable to meet the demands of the world's population. The food and processing sectors are very interested in the physicochemical, functional, and structural characteristics and amino acid content of plant-based proteins [12].

1.2.1 Supplements

The human diet includes proteins as a crucial dietary supplement. Diet nutritional value is increased by dietary supplements, which supply extra amounts of nutrients. To maintain the health of people of all ages, proteins can be employed as a significant supplement in the human diet. The functions of proteins include maintaining weight [13], bone health, and fulfilling nutritional requirements [14]. Proteins are bioactive substances, that have been demonstrated for maintaining cardiovascular health and defending the body against various ailments [15].

Plant proteins satisfy the protein needs of athletes, are responsible for muscle growth improvement, and lower cholesterol [16-18]. Plant sources to be used as supplements include cereals (barley, maize, sorghum, rice, wheat) legumes (pea, soybean, chickpea), different dry fruits and seeds (flaxseeds, pumpkin, sunflower) [19].

A leguminous seed, lupin has a lower oil content of about 5.95 percent and protein content equivalent to soybeans i.e. about 32.2 percent [20]. For both animals and humans, they have been used as protein supplements as they are widely available globally and have desirable chemical properties [21].

Some infants and adults are allergic to protein supplements that are cow milk-based, so soy protein is used as an alternative protein supplement for them. Soy flour i.e., defatted can be used as a protein source in the human diet because of its lower content of sugars, and fats, high content of essential amino acids, and absence of cholesterol. Serum cholesterol levels can be lowered by the intake of plant proteins e.g. soybeans proteins [22].

Combining pulses or grains with other foods is a successful fortification technique to receive the required amounts of important amino acids. Legume cereal mixtures sufficiently enhance food products' nutritious qualities. The addition of faba bean flour and wheat mixture in bread and bakery products can increase amino acid levels. 35.7 percent protein content is present in thirty percent of faba bean flour so the protein content of wheat bread can be increased from 11.6 percent to 16.5 percent after the addition of this faba bean flour [23].

Due to the presence of antioxidants e.g. carotenoids and its high concentration of important amino acids, the use of the blue-green algae spirulina for humans as a protein supplement is now being researched [24].

1.2.2 Films and edible coatings

The development of plant-based edible food coatings is driven by the growing customer desire for healthy, stable, and safe meals as well as awareness of the damaging environmental consequences of nonbiodegradable substances from the packaging of food. For food packaging and coating, proteins serve as sustainable sources as they are natural polymers that are edible, biodegradable, and eco-friendly. Thin covering layers prepared from edible compounds, used for packaging foods, are described as edible films and coatings. These films can block oxygen and moisture entry, and solute movement, without changing the texture and taste of food products [25].

For the preparation of these edible films, globular proteins are mostly used which are denatured by acids, bases, solvents, and heat that shape them so can be used for film preparation. Interchain interactions, and strong bonding, result in maintained films with lower permeability of vapors, liquids, and gases [12].

At low humid conditions, these coatings serve as high O_2 blockers. Different plant and dairy proteins are attracting sources for edible food packaging development. The gluten from wheat, zein from corn, soy proteins, etc. are used for film preparation [26, 27].

Proteins from yellow peas are employed for film preparation with excellent mechanical and thermal properties [28]. Edible films with antibacterial and antioxidant activities are prepared when the peel powder of pomegranate is mixed with the protein of mung bean [29].

1.2.3 Adhesives

Scientists are encouraged to search for other applications of proteins other than the food industry. Characteristics of proteins make them an excellent candidate to be used as an alternative to dangerous chemical adhesives [12]. The reaction between crosslinkers and active groups of protein molecules improves their performance and properties when used as adhesive agents. Adhesives based on proteins show water-resistant properties as on protein unfolding, hydrophobic groups are exposed, so water interruption is avoided, and hydrophilic group exposure creates densely cross-linked structures [30].

Petroleum-based substances, e.g., resins based on formaldehyde, are carcinogenic and used for wood adhesive development. So, in recent years the trend shifted towards eco-friendly and high-quality adhesives. By the use of modifiers along with proteins, sustainable protein-based adhesives are prepared. These modifiers include phosphoric acid [31], lignin [32], etc.

In the wood industry cotton seed proteins are being used as environment-friendly adhesives [31, 33-35]. Proteins of cotton seeds show high strength and hot water-resistant properties [36]. After oil extraction from cameline seeds, the by-product is cameline protein. This protein has been used as an adhesive. Proteins with improved performance and quality for adhesives preparation help to improve the wood cost/benefit ratio in the plywood industry [37].

1.2.4 Hydrogels

Hydrogels are polymeric crosslinked 3D structures i.e., can absorb water or biological fluids to about a thousand times their weight. The 3D structure of hydrogels is formed by physical or chemical crosslinking of synthetic, hybrid, or natural polymers [38].

Protein-based hydrogels are considered favorable biomaterials Due to their ability to respond to environmental stimuli e.g. temperature, and pH, by changing their physicochemical features, such as swelling, they can be employed to deliver chemicals at a precise time and location [39].

Hydrogels are used for diagnosis, biosensors, tissue engineering, regenerative medicines, wound dressing, drug delivery, superabsorbent, food additives, pharmaceuticals, biomedical implants, cell encapsulation, contact lenses, etc. [40-43].

For the delivery of bioactive compounds and drugs, protein-based hydrogels are preferred over synthetic polymers. As protein structure is unique because of the presence of basic and acidic groups that can donate and accept protons. Controlled drug release occurs from hydrogel depending upon medium pH [44]. Both heat and cold-set hydrogels based on globular proteins can be prepared [45]. In heat-activated hydrogels, globular proteins are unfolded and disrupted due to heat energy influence, resulting in hydrophobic area exposure and the formation of disulfide bonds. Aggregation of protein moieties that are unfolded, occurs due to beta-sheet structure involvement, resulting in heat set gels [46].

Cold set hydrogels are prepared through the following steps: At 1st protein is heated at neutral pH, resulting in the unfolding and denaturation of peptide chains. And then different treatments, e.g. acidification or by using salts, are performed to lower repulsion to create cross-linkages between peptide chains [47]. After protein denaturation, its functional groups in a

surrounding medium are exposed that interact with other bioactive compounds by electrostatic, hydrophobic interactions, or hydrogen bonding. These interactions can be altered and tailored, providing control over gel texture and structure, and as a result desired compound can be carried and delivered [48].

Hydrogel based on soybean protein and corn fiber has been prepared for delivering riboflavin, a thermolabile bioactive compound [47].

Utilizing animal proteins may offer several benefits, including high absorbability and minimal toxicity of the breakdown products (as animal proteins are smaller) [49, 50]. Through hydrolysis of collagen, gelatin protein is obtained i.e., considered as GRAS (generally recognized as safe), by the FDA (Food and Drug Administration), and is used in pharmaceutical and food products. Due to the creation of triple helices in gelatin, which leads to chains being entangled and network forming, gelatin produces thermo-reversible hydrogels [49, 51].

For microencapsulation of EGCG (epigallocatechin gallate), an antioxidant, gelatin-based micro hydrogels are prepared, in which gelatin serves as wall material for its encapsulation [51]. These hydrogels exhibit delayed release and good bioaccessibility after gastrointestinal tract (GIT) digestion. Gelatin-based films with high mechanical strength and antimicrobial activities have been prepared that can be used as packaging material [52].

Oval albumin, an egg white protein, i.e. acidic and globular has been used to prepare different structures [53, 54]. In food products, it can be used as a foaming agent and emulsifier. In functional foods, it serves as a carrier of bioactive compounds e.g., curcumin-loaded nanogel based on ovalbumin and dextran with high storage and pH stability was prepared. Through heat treatment, nanoparticles of ovalbumin-linoleic acid were prepared. These nanoparticles can bind with linoleic acid due to hydrophobic surfaces. So, they serve as nanocarriers of unsaturated fatty acids which have applications in food industries [55].

Milk proteins are classified as casein and whey proteins where lactoferrin is minor and alpha and beta-lactoglobulin are major whey proteins. Because of their functional and structural varieties, their use as bio-active encapsulation agents has been studied [56, 57]. Through cold gelation, microparticles based on sodium alginate and whey protein have been prepared [58]. In food products, these particles serve as flavor carriers and texture modifiers. Soft gels based on proteins, with desired mechanical properties, easy swallowing, and mastication e.g., food products for elderlies, have been prepared.

Alternatively, non-animal protein sources are gaining popularity as consumers are looking to have a diet with more plant-based alternatives and fewer animal-based options [59]. Plant proteins offer low prices, and higher sustainability when compared with animal proteins [49, 50].

Vegetable proteins lowered the risk of type two diabetes [60, 61]. Leguminous seeds are the richest protein source with low prices, beneficial health effects, high nutritional value, and availability [62]. Bioactive agents that are lipophilic can be encapsulated by zein proteins present in a kernel of corn, i.e. alcohol soluble and water-insoluble [63]. Tangeretin, a flavonoid, was encapsulated in nanoparticles based on zein and beta-lactoglobulin. In food products, they can be added as functional components [64]. In addition, films based on zein serve as a carrier for active compound delivery e.g. catechin, lysozyme, and niacin [65].

Soybean proteins are biodegradable and readily available. Materials based on these proteins can be used in tissue engineering, film preparation, delivery of bioactive compounds, in food emulsions [49]. Nanoparticles based on soy proteins were synthesized for carrying vitamin B12 [66].

1.2.5 Enzymes

Enzymes are proteins in nature that act as catalysts in various chemical reactions. Enzymes are often referred to as industrial enzymes because they are frequently used to speed up the production of products and industrial processes. Enzymes have been used for cheesemaking, brewing, baking, and other processes since ancient times [67]. Enzymes can now be widely used in a variety of industrial processes and products e.g. paper and pulp, leather, textile industries, and detergents [68]. Through advancements in bioprocess and recombinant DNA technology, it became feasible to produce well-characterized, purified enzymes on large scales [69].

Due to advancements in protein engineering, enzymes are modified according to properties of interest, which lead to the growth of this industry both in terms of complexity and size [67, 70-72]. Most industrial enzymes currently in use are hydrolases, which include lipases and proteases. These enzymes are widely employed in the chemical, dairy, and detergent industries. The second largest group consists of carbohydrases, e.g., cellulases and amylases [67, 72, 73]. In addition to these, other enzymes for higher efficiency are also required in different applications in various industries.

Proteases are proteolytic enzymes also known as proteinases and serve as the largest enzymatic group that undergoes peptide bond hydrolysis in proteins. They are classified into endo peptidases and exopeptidases, used in food processing, detergents for dishwashing and laundry, leather processing, waste treatments, etc. Their main application is the removal of protein stains and deposits [74-76] so widely used in detergents either in liquid or powder forms for laundry or dishwashing. They serve as a tool for modifying food protein properties widely used in food processing for enhancing flavor, nutritional and functional properties of proteins [77]. Acidic proteases can coagulate casein (milk protein), i.e., required for curd formation for cheese preparation, so they can be used in place of rennet (calf enzymes). They can also serve as a debittering agent, as they can break peptides that are a reason for the bitter taste in cheese [78]. Alkaline proteases are used in leather industries from hide soaking till the end to get the final product, in place of dangerous chemicals used for batting, dehairing, and soaking [79, 80]. This treatment is favorable in terms of reducing pollution and saving energy [79].

Lipases belong to the hydrolases class that hydrolyze triglycerides to fatty acids and glycerol [81]. They are present in animals, plants, and fungi. In vegetable oils, the process of phospholipid removal through selective phospholipase has been recently introduced. Laundry and dishwashing detergents also contain lipases for lipid stain removal.

In the leather industry, the processing of raw materials, hides, and animal skin degreasing is a crucial step. Surfactants and organic solvents are used in conventional degreasing techniques, posing major environmental risks like emissions of volatile organic compounds. By hydrolyzing triglyceride through lipases, (the primary type of fat contained in animal skins) to fatty acids and glycerol, lipase can extract grease and lipids from hides and skins. Lipases that are acid-active and alkaline-stable can both be used to degrease hides. In the paper and pulp industry pitch, composed of sterols, fatty acids, and waxes causes problems. So, lipases are used for their removal. During paper production from pulp, they are used to control pitch accumulation during synthesis. Additionally, lipases are employed to remove ink from recycled paper, resulting in a product with greater brightness and less ink residue when compared with chemically treated pulps [69].

Amylase is a hydrolase that breaks starch into sugars and acts on alpha 1,4 glycosidic bonds [82]. This enzyme has applications in detergent, paper, textile, fermentation, and food industries [83]. In bread baking, alpha-amylase is added [84] to the dough catalyzes the breakdown of the starch in wheat into smaller destrins. Additionally, alpha-amylase produces

more sugar in the dough during bread baking, which enhances the bread's flavor, toasting capabilities, and crust color They have an antistaling effect during the baking of bread and prolong the time during which baked goods retain their softness, extending the shelf life of these goods. The manufacturing of starch syrups and fruit juices, as well as brewing, digestive aid preparation, and different food processing activities also employ amylases. The preparation of animal feed with amylases increases the fibber's digestibility.

Pectinases hydrolyze pectin, which is frequently present in plants [85, 86]. These enzymes have applications in the clarification and extraction of fruit juices [87]. Pectinase breaks down the soluble pectin in fruit pulp, making it easier to press the fruit, extract the juice, and separate the precipitate using sedimentation, centrifugation, and filtration. The kind and quantity of the enzymes used, fruit type, and reaction temperature, affect how long a pectinase treatment will take. To increase juice extraction, pectinases have also been used along with other enzymes e.g. cellulases and xylanases. Wastewater containing pectin is released by enterprises that process vegetables for food. To remove pectinaceous material the wastewater must undergo treatment with alkaline nature pectinase [88].

1.3 Wheat proteins

Wheat (Triticum aestivum L.), is a widely cultivated crop in the world and one of the main sources of staple food [89]. For human consumption, 90% of wheat grown is utilized and 10% is used for industrial reasons, such as the manufacturing of dextrose, malt, gluten, and starch, as well as seeds for the following crop [90].

It is a favorable crop because of its low price, high nutrition value, and ability to be grown under different climate conditions [91]. It contains fibers, lipids, proteins, and minerals e.g. iron, magnesium, manganese, sodium, zinc., niacin, thiamin, folate, and riboflavin [92]. For human life, proteins serve as a vital nutrient. Thousands of different proteins arise from different combinations of monomeric amino acids that are building blocks of heteropolymers, i.e. proteins [93]. Content, size distribution, and composition i.e., either polymeric or monomeric, of wheat proteins are factors that affect bread quality [94-96]. As a result of the abundance of polar and non-polar amino acids that they contain, proteins (animal or plant-derived) are the ideal raw material for the development of bioplastics [93]. Proteins can be used to create blends or composites of desirable qualities since they are simple to process and can bind to a variety of surfaces [97].

Among cereal crops, wheat after maize is 2nd largest crop and it offers a fantastic base for making a variety of different food products [98]. Because of the dough-forming quality of wheat flour i.e., required for producing a wide range of food products, it is considered a unique crop. Wheat flour contains starch and wheat proteins. These proteins are classified based on their ability to form a viscoelastic network. The proteins that can form this network are termed gluten proteins and the rest are non-gluten-forming proteins [99]. Gluten is proteinaceous, viscoelastic, and cohesive material that is obtained from wheat after starch isolation [100].

Based on solubilities, non-gluten proteins are classified as globulins which are insoluble in water but soluble in salt solutions and water-soluble albumins. Gluten proteins that are insoluble in salt solutions and water consist of glutenin (insoluble in ethanol) and gliadins (soluble in ethanol). Wheat flour contains different components [92, 101] as shown in Figure 1.2. The reason for water insolubility of gluten proteins includes high glycine and proline content which are non-polar amino acids and low content of ionizable side chain amino acids. Glutenin is a polymer with a molecular weight of eighty thousand to greater than twenty million. Monomeric gliadins have a molecular weight ranging from thirty thousand to sixty thousand. The viscoelastic characteristics of hydrated gluten proteins are distinctive [102].

The most crucial structural components of the gluten network are the disulphide bonds that are created by crosslinking of cysteine residues [103-105] (Figure 1.3). The most popular (and well-known) application of gluten proteins, such as the wheat flour component, includes the making of bread, cakes, pretzels, and cookies [99].

1.3.1 Gluten production

Commercialized wheat gluten is produced through the following methods:

In 1835, the so-called Martin or dough technique was created in Paris. A dough formed by kneading water and wheat flour [106]. The dough is then gently agitated under running water to separate the starch and gluten, which the water carries [100, 107, 108]. Due to the starch granules' larger size and high density than gluten, they can be separated from the wheat flour during this fractionation step using a variety of techniques, including centrifugation, use of decanters, hydro cyclones, or sieving (screening) [109]. After proper drying and further grinding, gluten is still able to rehydrate and form an elastic and cohesive dough [110].

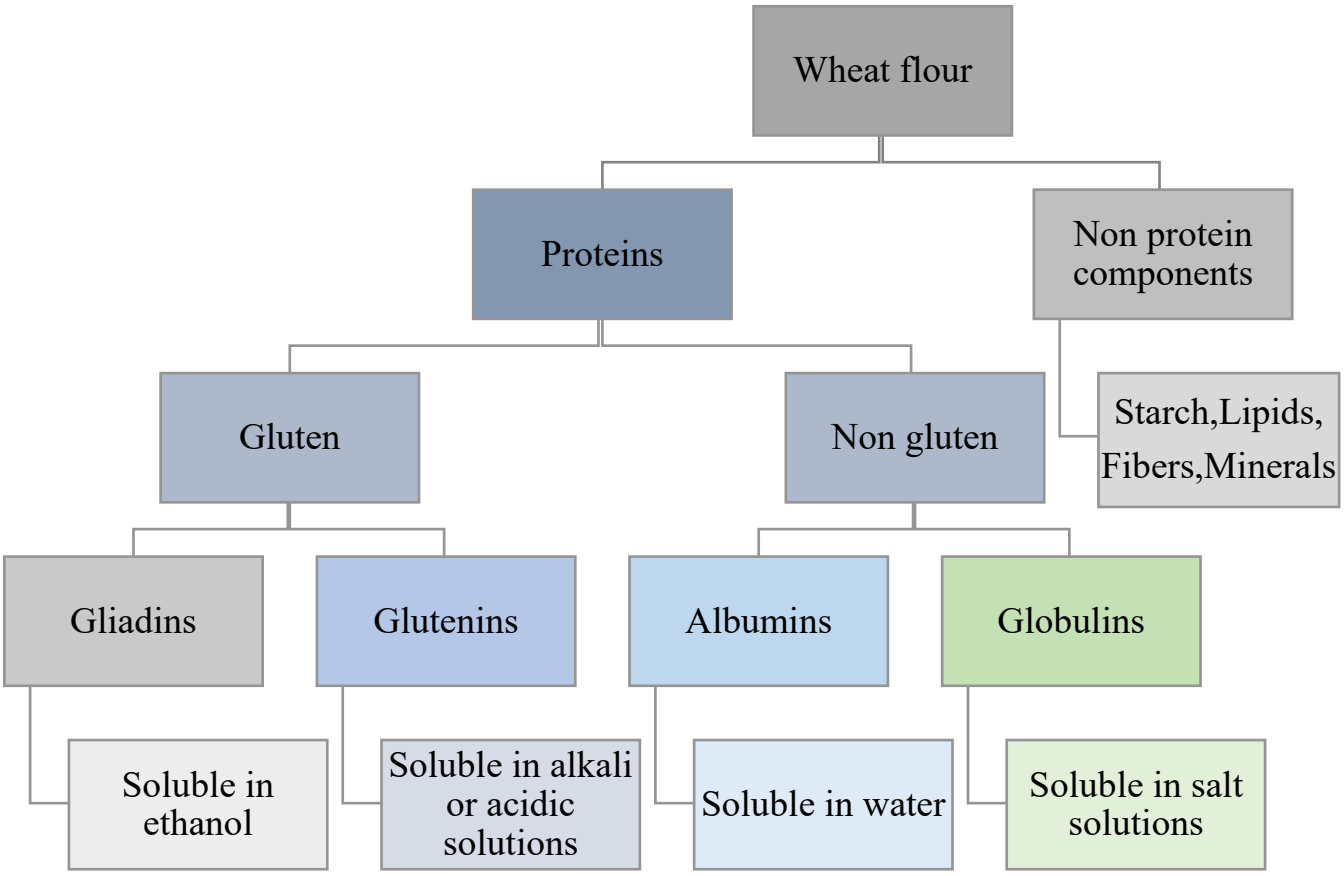

Figure 1.2: Schematic representation of components of wheat flour.
Wheat flour contains both protein and non-protein components and their individual components show solubility in chemically distinct solvents.

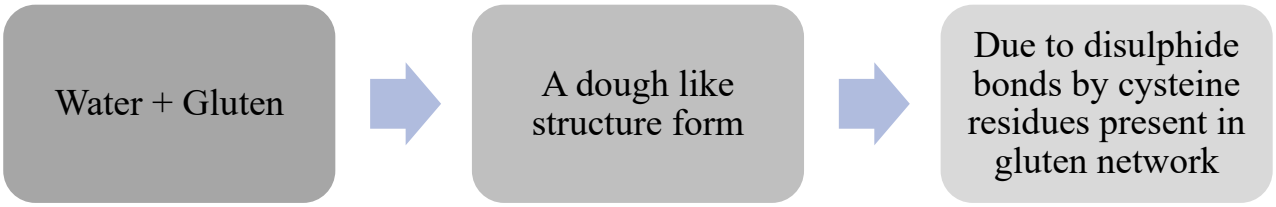

Figure 1.3: Hydration of gluten.
Disulphide bonds between cysteine residues are responsible for the formation of an elastic dough-like structure when gluten is mixed with water.

The batter process is a variation of the dough process, which was created at the USDA's Northern Regional Research Laboratory during World War II. For making dough, flour, and water are combined. The batter obtained is vigorously stirred with extra water after resting for thirty minutes. Finally, it is separated from the starch liquor using sieve bends, gyratory screens, and rotary screens [106].

These products need to be dried before being marketed after the aqueous methods used to separate starch and gluten. Since gluten is heat-sensitive, the drying step is essential for maintaining the functional qualities of the product while also ensuring the product's microbiological stability. Heating gluten up to 70 °C has no negative effects on its vitality, but over 80 °C, denaturation occurs [111]. Spray drying and drum drying [112] are used for this purpose in addition to ring dryers. The product is ground into tiny particles and marketed as vital wheat gluten after drying [113].

1.3.2 Industrial importance of gluten

Gluten is widely used in food and bioplastic industries. In the baking industry wheat gluten is the main ingredient [114-116]. It is used for the production of special bread, e.g., panettone, for which the volume is crucial and the fermentation stage is lengthy. It is used for the fortification of flours with low gluten quality and quantity [117].

The practice of flour fortification with gluten is routinely employed by the milling and baking industries. The strength, dough handling, tolerance to mixing, shelf life, softness, and yield, are all properties improved with gluten addition [113]. In pasta gluten addition lower stickiness, provide firmness, and can improve breakage resistance, and heat tolerance, in retorted items in cans [118].

As thickening agent gluten is used for soy sauce and fruit puree production, in addition, it is also used for the manufacturing of monosodium glutamate because of its high content of glutamine [110]. Due to its viscoelasticity, gluten is utilized to substitute the milk protein in some cheese products, giving the cheese a distinctive texture and improving its eating quality. Gluten is used in dog biscuits to make them breakage resistant, particularly while shipment and packaging [118].

Plastics, the most used man-made materials today and ubiquitous in all facets of our lives, were created 110 years ago. They are synthetic polymers that are readily mouldable into a wide range of items and shapes for different applications [119]. From food packaging to water bottles, construction materials, and medical supplies, each person disposed of fifty-two kg of plastic waste per year [120].

Petroleum-based plastics cannot degrade, they pose a threat to the environment. It is now clear how dangerous it is for the world's seas, freshwater bodies, and land to become clogged with this very persistent plastic garbage [121, 122].

Studies on particle toxicity show that plastic particles can harm the lungs and intestines, and small particles in particular can cross the placenta, cell membranes, and blood-brain barrier, which can result in many health issues [123, 124].

Remains of petroleum-based plastics with high carbon profiles and the associated environmental issues are another worry that is being raised internationally. Because crude oil is a nonrenewable resource and its price has been steadily rising, experts are actively looking for suitable replacements for it [125].

Due to health and economic issues caused by traditional petroleum-based plastics, researchers have been motivated to create plastics, often known as bioplastics, which demonstrate to be environmentally acceptable alternatives to toxic petroleum-based plastics [126]. Natural materials like polysaccharides e.g. starch, lignin, chitin, proteins e.g. gelatin, gluten, casein, and lipids e.g., animal fats and plant oils, are used to synthesize bioplastics [127]. Because they are synthesized from organic resources that are renewable, bioplastics are now recognized as an alternative to traditional plastics and are anticipated to reduce our reliance on fossil fuels and waste generated from plastics [128].

Bioplastics from wheat gluten can be synthesized through compression molding, extrusion, and casting processes [129]. Transparent, water-resistant, and homogenous films have been obtained through casting techniques [130, 131]. The casting process is well suited for coated material production like paper coating, or to directly spread the solution of film forming on seeds. The casting of films based on wheat gluten has shown impressive gas barrier properties e.g. barrier against oxygen, so they don’t get moist [131]. Due to gas barrier properties, these

films based on gluten can serve as packaging material providing a suitable atmosphere around fruits and vegetables [132].

Different plasticizers are used during the synthesis of these bioplastics including sorbitol, fatty acids, glycerol, etc. to overcome the brittleness because of excessive interactions between protein chains [129, 133]. Hydrogen and disulphide bonds affect the properties and structure of these bioplastics based on gluten protein [134]. Due to the interaction between lignin present in natural fibers and gluten protein, mechanical and water resistance properties of gluten bioplastics are improved by the use of these natural fibers e.g. by using wood fibers, hemp fibers [129], coconut fibers [133], jute fibers [135].

Fish scales (FS), renewable and easily available, a waste product of the seafood industry, have been used along with gluten to synthesize bioplastics. These FS/gluten composites have shown higher tensile strength up to 6.5-7.5MPa as compared to bioplastics prepared without employing fish scales with a strength of 3.4 MP. The reason for increased tensile strength is the strong adhesion between gluten and fish scale powder [136].

Palm oil has been used in various products e.g., cosmetics, soaps, etc. [137-139]. Fibers from, OPEFB (oil palm empty fruit bunch), have been used to increase the flexural and tensile strength, elongation, and stiffness of different matrices [140, 141]. It has been used to prepare fiber/gluten composites that show higher tensile strength up to 13.75MPa and lower water absorption properties. The increase in strength is due to the strong adhesion between fibers and gluten [142].

Bioplastics based on gluten by using KCl (potassium chloride source in agriculture), modified by citric acid, have been prepared [143]. Issues related to applying fertilizers on crops like their controlled release and pollution caused by them when they reach water bodies can be lowered by encapsulating or entrapping fertilizers in polymeric matrices. By entrapment of fertilizers in polymers that are biodegradable, they can be released in a controlled manner [144-146]. Calcium carbonate can improve the mechanical properties and tensile strength of plastic materials [147, 148].

Shell waste of shrimp serves as a renewable and alternative source of CaCO3 [149-152]. Bioplastics based on gluten and shrimp shell waste have shown improved properties in terms of tensile strength and degradation properties. The tensile strength increased and the degradation process slow down, by employing shrimp shell waste along with gluten as compared to bioplastics synthesized without them [153].

SAMs (Superabsorbent materials) are another use of protein-based bioplastics. Superabsorbent are materials that can retain and absorb water 10 times its weight. These materials have a wide range of applications e.g., in health care, hygiene products, wastewater treatment, and in drug delivery [154-156].

Bioplastic from soy protein demonstrated superabsorbent properties because of high amino acid content e.g. glutamic and aspartic acid residues when functionalized with succinic anhydride, resulting in water uptake greater than 3,600 percent [154].

PPP (Plasma Porcine Protein) possesses a high content of charged amino acids and has the potential application to be used as source material of bioplastics that are superabsorbent attractive alternative bioresources to conventionally used SAMs. The application of bioplastic from PPP in the packaging industry has already been researched [157-159].

Completely natural superabsorbent materials can be synthesized from wheat gluten [160]. The gliadins and glutenin proteins of gluten interact in many ways through physical and covalent interactions and form stable networks, these cross-linked networks can play an important role in the formation of superabsorbent. The presence of a high concentration of charged amino acids in gluten makes it a promising choice to produce a chemically functionalized biobased superabsorbent. The chemical functionalization of wheat gluten proteins can have a significant impact on their performance [161]. Different chemical additives have been incorporated into gluten to increase its polymerization and boost its absorbency. For instance, sulfuric acid reacts with the hydroxyl groups of serine and tyrosine residues and forms sulfuric acid esters. Phosphorylated gluten is produced by the reaction of phosphoric acid with hydroxyl groups of amino acids in gluten. The capacity of these modified gluten samples increases up to a hundred to thousand times their weight in water [160]. Citric acid has also been used to modify gluten by forming citrates and crosslinking it further. By heating citric acid at high temperatures, the carboxylic acids on citric acid form the anhydride by losing a molecule of water. Citric acid-modified gluten proteins are used to synthesize the natural superabsorbent that can absorb up to 78 times its weight in water [162].

In another study, a nontoxic acylation agent Ethyleneditetraacetic dianhydride (EDTAD) was used to modify the wheat gluten protein. This EDTAD-treated wheat gluten was able to absorb 1000, 150, and 100%, water, saline, and blood respectively higher than those of wheat gluten powder [163]. The mechanically compressed particles will be most likely to retain more liquid than swelling soft foams, which is an important property for applications such as sanitary pads

and diapers [161]. Superabsorbent polymers are primarily used as an absorbent for water and aqueous solutions for diapers, adult incontinence products, personal care, feminine hygiene products, medical products, and in other similar applications. Different modification approaches have been adapted for the value addition of gluten.

Through acid or alkali treatment, deamidation of gluten occurs [164, 165]. Additionally, certain peptides may hydrolyze as a result of acidic deamidation, forming polypeptides with lower molecular weights that are more soluble. Peptide hydrolysis is typically not a result of alkali treatment; however, it may induce cross-links by rupturing the disulfide bonds of cystine [164]. Alkali or acid deamidation involves neutralization, which generates large amounts of salts that must often be eliminated, frequently via iso-electric precipitation [166]. Deamidated gluten can be used for foam stabilization or emulsification in food because it is readily dispersible. Products that have been treated with chlorosulfonic acid, phosphoric acid, and sulfuric acid, bind water far more strongly than unmodified glutens do; in certain situations, they can bind up to two hundred times their weight in water [167]. Other treatments include succinic anhydride-treated gluten, to increase its solubility at pH 7, which is close to the pH at which native gluten is least soluble [168].

Modification through physical means is performed through UV irradiation, high-pressure processing, and texturization by extrusion.

To give gluten a fibrous structure, simulating meat fibers, extrusion technology is frequently used. Microfibrils or thin filaments are created when wheat-protein molecules align during the extrusion process and they assemble to form a macroscopic fibrous structure. Texturized wheat gluten has a fleshy, laminated appearance due to fibrous strands hydration [169]. Pressure causes a change in gluten properties either to more solid-like at an increased pressure of about eight hundred megapascals, to more liquid-like at lower pressure of two hundred megapascals [170]. Under mild treatment conditions, non-covalent bonds appeared to be weakened but additional chemical cross-links appeared as the severity of the treatment increased. Crosslinking between the protein chains increases the tensile strength of gluten-based films [171].

For modifying baking performance and dough properties, enzymes used for modification include hemicellulose, protease, transglutaminase, lipoxygenase, and amylase [172].

Enzymatically solubilized gluten shows properties similar to deamidated gluten e.g. emulsion formation and foam stability. But enzymatic treatment is more beneficial e.g. mixing time of dough is reduced by the addition of one to two percent of enzymatically solubilized gluten, and the same level of increase occurs after the addition of chemicals e.g. ascorbic acid or cysteine for improving loaf volume [173].

The addition of lipase to wheat flour increases gluten content from 8.7% -10.9%. The addition of cellulase increased gluten content to about 12.7%. Protein recovery in soluble and separated gluten is an important factor in wheat flour processing properties. Protein recovery is defined amount of recovered protein in gluten divided by the protein amount in flour and it must be high in gluten, while protein loss in soluble must be lower [174].

Carbohydrates bound with gluten can be removed by using starch-degrading enzymes, to get high water-soluble protein content. For this purpose, alpha-amylase is used to degrade starch. Wheat gluten is treated with alpha-amylase at a seventy-five-degree temperature, for two hours, in the presence of water, to degrade gluten's polysaccharide content and obtain solids in aqueous suspension which is then separated through centrifugation or filtration. 1st protein fraction is obtained by drying of solid suspension and 2nd protein fraction is liquid i.e., left after drying. After combining these fractions' total protein concentration, more than sixty percent is obtained [175].

The coagulation index is also a processing property indicator. It is a measure of the rate at which larger gluten particles form from smaller ones. It can be determined by the gluten amount on top of two sieves with size ranges from 250-400pm, divided by the total gluten amount on sieve size >125pm. Enzymes in addition to flour improve the coagulation index from 15-20 percent. Protease addition also enhances soluble protein content due to proteolysis. Amylase, protease, cellulase, and hemicellulose increased protein recovery, gluten yield, and coagulation index, without affecting the quality of gluten [174].

1.3.3 Limitations of industrial gluten

Despite the fact that industrial gluten is labeled as a protein concentrate, this product does not simply include proteins, it has approximately protein content (73-82%)[100], moisture content (6-8%), carbohydrates(3-20%, primarily starch), lipids (5- 8%) varying levels of minerals (0.5-1.5%), fibers (less than 1%), [113].

Gluten-based bioplastics with fish scale powder and shrimp shell waste were prepared with improved mechanical properties when compared with bioplastics prepared from neat gluten. Neat gluten-based bioplastics showed lower tensile strength that was 3.40 MPa when compared with bioplastic prepared after the addition of powder from shrimp shell and fish scale that showed a tensile strength of 6.53 MPa [153] and 7.51 MPa [136]respectively.

Tensile strength of 10.41 MPa was obtained in the case of bioplastics prepared from the addition of oil palm empty fruit bunch fiber **(**OPEFB) that was highest in comparison with a tensile strength of 9.25 MPa of gluten-based bioplastic [142].

1.4 Aims and Objectives

The overall objectives of this study were to develop and test the non-toxic and eco-friendly methods that include mild mechanical and enzymatic treatments with cellulase and amylase, to purify gluten and increase its protein content. Protein content was determined through the Lowry assay. Solubility of untreated and treated samples determined through SE-HPLC. Films were prepared and their absorption capacity was determined through tea bag assay.

Specific objectives of the thesis were:

- Purification of gluten protein through different nontoxic, ecofriendly methods including mild, mechanical, and enzymatic treatments.
- To determine the effect of different purification methods on gluten protein content through the Lowry assay.
- Difference in solubility of samples was evaluated through SE-HPLC
- Casting of gluten-based films
- Determining free swelling and absorbance capacities of films through the tea bag method.
- Evaluation of films to be used as superabsorbent for hygiene products.

2. MATERIALS AND METHODS

2.1 Materials

Lowry reagent**,** FC (Folin–Ciocalteau), BSA (Bovine serum albumin), Cellulase, and Amylase were sourced from Sigma-Aldrich. Wheat gluten with protein, moisture, starch, fat, and ash content is 77.7%, 6.9%, 5.8%, 1.2%, and 0.9% respectively with additional cellulose and fibers, of dry weight) was graciously provided by Lantmännen Reppe AB, Sweden. Acetonitrile and trifluoro acetic acid (TFA) were sourced from Merck, Germany. Glycerol, Urea, and Gellan gum powder were sourced from Merck Pharma.

2.2 Wheat gluten purification

For gluten purification and modifying protein content, 3 methods were tested and developed (Figure 2.1) that were easily scalable and involved minimal use of chemicals to obtain clean wheat gluten particles. Treated and untreated samples shown in Figure 2.2.

2.2.1 Mild treatment

For gluten extraction, 30g of wheat flour was wrapped in a muslin cloth and washed under running water, till we get a rubbery mass of wheat gluten. This mass was at first, dried in an incubator at 45°C for two days. and then milled through the grinder. For removal of maximum starch, obtained gluten was again resuspended in water and centrifuged for 15 min at 5000 rpm. This gluten is referred to as mild gluten as the high temperature, pressure, and energy required for drying gluten is avoided. This procedure is a modification of the standard dough-washing method.

2.2.2 Mechanical treatment

In this method, 100g of industrially available gluten was added through a sieve (to prevent agglomeration) to 1800ml water. Stirring of the resulting mixture was done for one hour, then shaking for four hours at 200rpm, and then centrifugation at room temperature for fifteen minutes at 9000rpm. After centrifugation, the supernatant was discarded, and the pellet was subjected to washing with distilled water, oven drying, and milling through a grinder to obtain powder form.

2.2.3 Enzymatic treatment

In this method, gluten was treated with alpha-amylase and cellulase for the digestion of starch and bran particles. In enzymatic treatment, both mechanically and mildly extracted gluten were treated with cellulase. For this treatment 25g of each sample was added to 100uL cellulase and acetic acid 0.5M, (pH 4.5 to 5). For maximum cellulose digestion, the resulting mixture was

heat treated at 40°C. The resulting samples were washed, oven-dried, and then milled to a fine powder-like form. This obtained powder was then treated with amylase 31.1 units per mg in acetic acid 0.5M with pH 6. In a water bath, samples were kept for a day for the maximum enzymatic reaction. These samples were again subjected to washing, oven drying, and milling to get the powdered form for further analysis.

2.3 Lowry method

Lowry et al proposed a method for protein quantification of samples, an alternative to other procedures.

From stock solutions, reagents for this assay were prepared as:

- FC (Folin–Ciocalteu) reagent prepared in ratios of 1:1.25 i.e., in distilled water of 6ml, 5ml of FC was added.
- BSA (Bovine serum albumin) reagent prepared by adding 1mg BSA in the distilled water of 10ml.
- Stock solution of Lowry reagent

Gluten samples with and without treatment were used for the Lowry assay (Figure 2.3). In 100ml distilled water, 1.25g of gluten samples and NaOH of 0.06g, were added. The mixture was stirred for 30min at 400rpm. Then the mixture was put in a water bath at a temperature of 60°C. When this temperature reached the water bath was turned off and samples were taken out after 30min. This step allows the hydrolysis of gluten samples by NaOH. Hydrolysates were then filtered. In a microplate of 96 wells, samples were poured along with negative and positive controls. In the first six wells of three rows, a gluten sample of 100µl along with an FC reagent of 100µl and Lowry solution of 100µl were added. In the first three wells of the fourth row, as a negative control, distilled water of 100µl along with 100µl of FC and Lowry reagent were added. In the next five wells of the same row, gluten samples of 250µl were added. In four wells of the fifth row, FC and Lowry reagents along with BSA serial dilutions were added as a positive control. Absorbance at 630 nm was recorded.

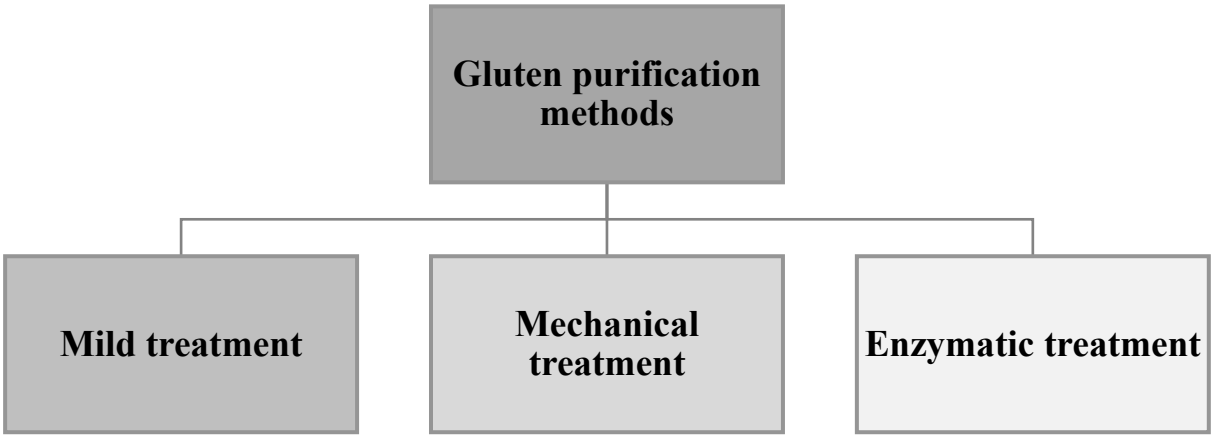

Figure 2.1: Gluten purification methods

Figure 2.2: Treated and untreated samples
(a) Untreated industrial gluten **(b)** After enzymatic treatment

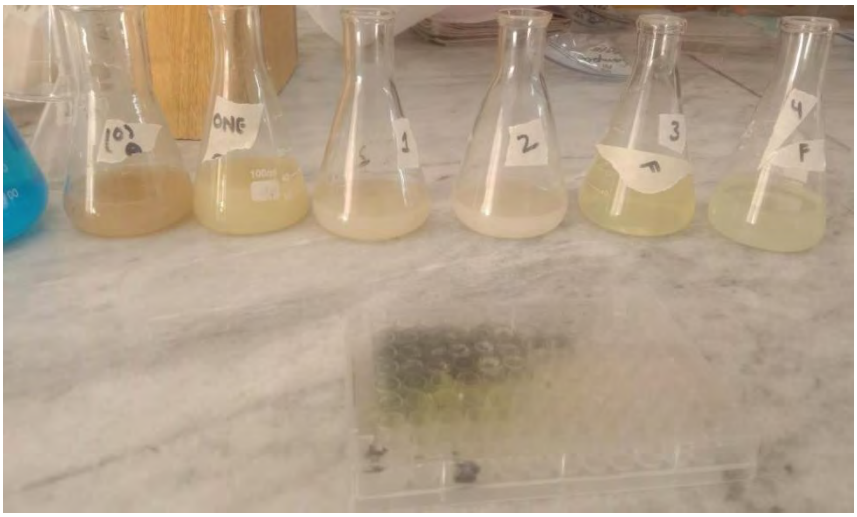

Figure 2.3: Sample preparation for Lowry assay.
Untreated and treated gluten samples were prepared and poured in 96 well microplate.

2.4 SE-HPLC (Size exclusion high-performance liquid chromatography)

To determine protein solubility and distribution, SE-HPLC, with 3 step extraction process was performed. In these steps, the buffer used for extraction contains 0.05M NaH_2PO_4 and 0.5% (w/v) SDS. A 16.5mg sample was used in each case. This procedure was performed according to [176] along with modifications.

For every step of extraction, a 1.4ml buffer was used. The sequence of extraction was as followed:

- 1st step extraction: extraction of proteins soluble in the phosphate-SDS buffer.
- 2nd step extraction: Along with buffer, samples were sonicated for thirty seconds.
- 3rd step extraction: Along with buffer, samples were sonicated three times for the 30s and 120 seconds.

The Soniprep 150 from Tamro, Mölndal, Sweden, an ultrasonic disintegrator at amplitude 5 equipped with a three mm exponential microtip, was employed. Weight and amount of extracted proteins, were determined by using HPLC (Waters, Milford, USA), outfitted with column (Phenomenex, Torrance, USA) and Biosep SEC-4000, (Phenomenex, Torrance, USA) column, detected at 210 nm. 2 solvents (water contains 0.1 percent TFA and acetonitrile containing 0.1 percent TFA), used in ratios of 1:1 for protein elusion. Monomeric and polymeric proteins from extraction steps were separately explained.

2.5 Preparation of gluten-based films

Films from wheat gluten samples that were untreated and treated, were prepared through the given procedure, at 1st, 4g gluten was dissolved in 100 ml of distilled water. For gluten hydrolysis, 0.15g of NaOH was added. The mixture was stirred for 2 hours and kept in a water bath for 40 min at 60°C. At room temperature, the hydrolysate was cooled and then filtered. In 35ml of gluten extract, 0.2g gellan gum, and 300µl of glycerol were added. The solution was further stirred for 30 minutes. In Petri plate solution was poured and put in an incubator at 52°C, for two days for the complete drying of films (Figure 2.4).

2.5.1 Tea-bag assay

According to the tea bag method, the water absorbance of gluten-based films was recorded. From the shop tea bags with mesh sizes 25-50µm, were purchased. Tea bags with dimensions of 40×60 mm, 3 sides heat sealed, were used. 4th side of the tea bag was sealed after filling it

with 0.2g of sample. To determine water absorbance, tea bags with rod hooked were dipped in beakers containing distilled water and saline solution (Figure 2.5). After certain time intervals, tea bags were dried by patting them with a towel for 8 seconds, to remove extra liquid (Figure 2.6). Free swelling capacity was calculated according to the following equations [163]:

Wblank = Wwb / Wdb

FSC = (Wi – (Wb. Wblank)) - (Wd) / Wd

Where Wb is: Weight of tea bag without sample

Wd: Dry sample weight

Wdb: weight of 3 empty dry tea bags

Wwb: wet weight of 3 empty dry bags.

Wi: wet tea bag weight after 1,5,10,20,60 minutes and after 24 hours.

The complete procedure for film preparation was as follows (Figure 2.7).

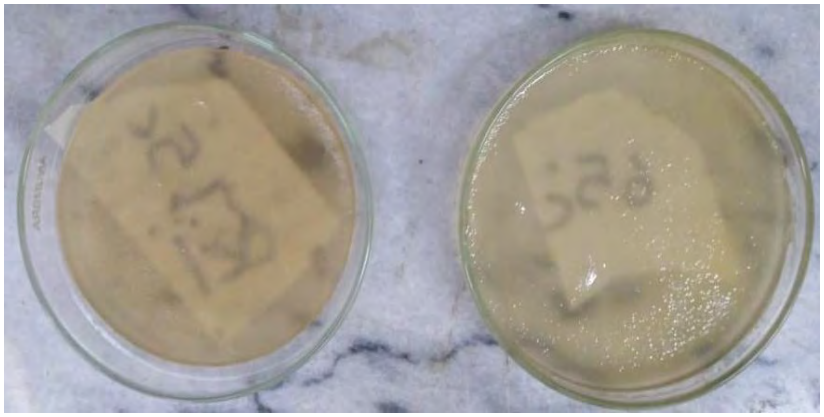

Figure 2.4: Incomplete drying of films

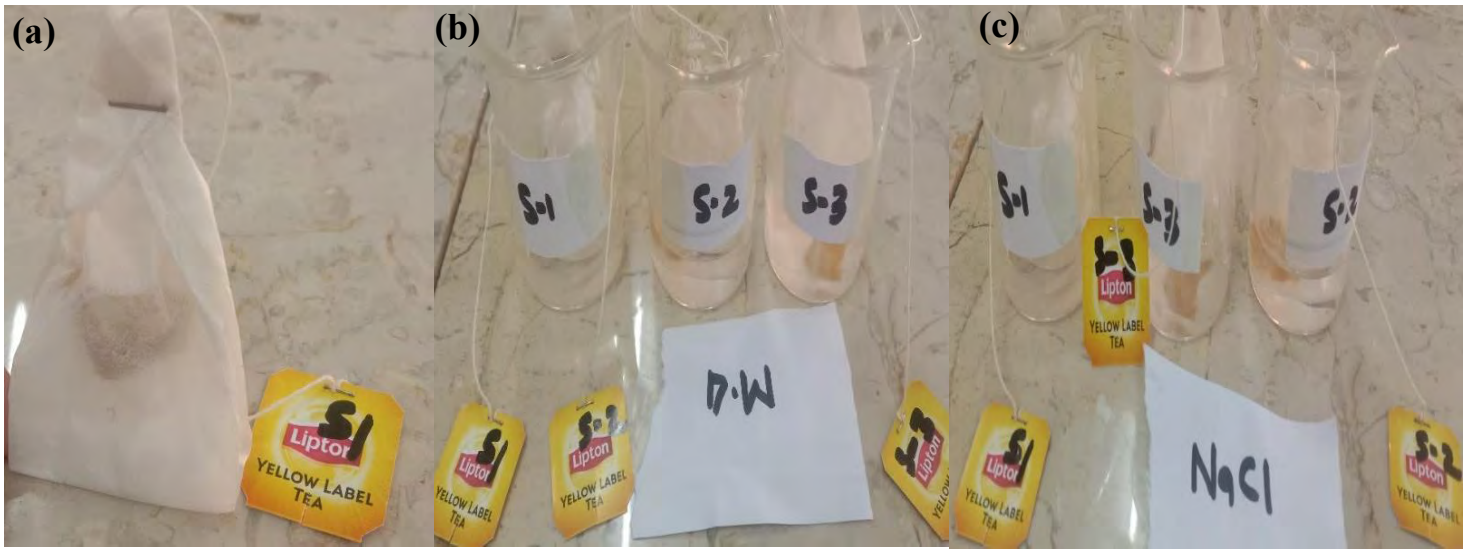

Figure 2.5: Tea bag assay
(a) Tea bag with film sample **(b)** Tea bags with samples in distilled water **(c)** Tea bags with sample in saline medium

Figure 2.6: Samples after 20 mins.
Tea bags were taken out from distilled water and saline solutions and were dried for eight seconds.

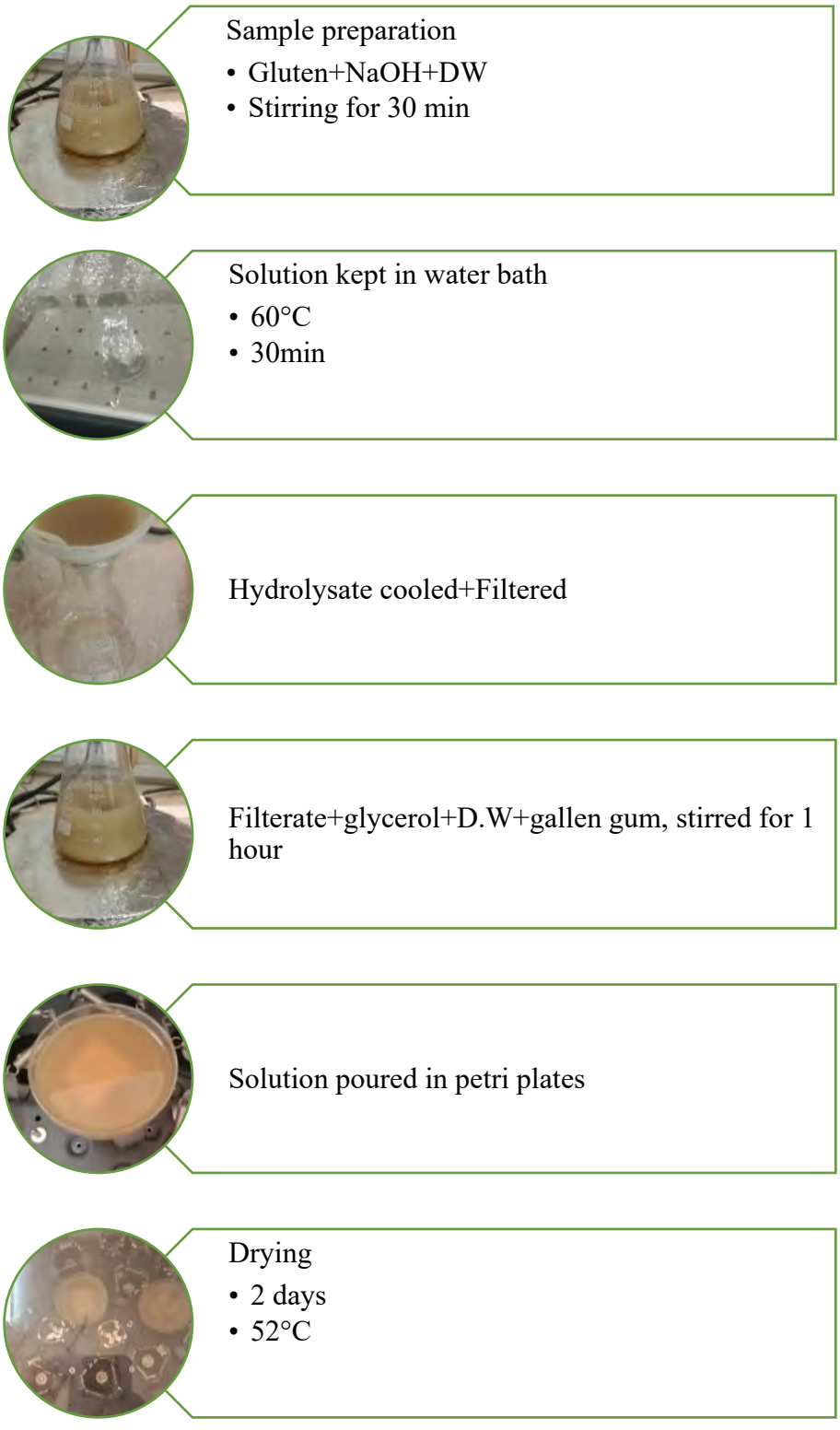

Figure 2.7: Schematic representation of film preparation

3. RESULTS

3.1 Protein quantification through Lowry assay

The protein content of all samples was quantified through the Lowry method (Table 3.1).

Untreated industrial gluten had lower protein content when compared with gluten samples treated differently. Harsh treatment was done for the separation of gluten from starch at the industrial level. Higher protein content was obtained from mildly extracted gluten samples than from industrial untreated gluten. In the mechanical treatment of gluten, more washing and mixing led to higher removal of starch so higher protein content was obtained as compared to untreated and mildly treated gluten.

Enzymatic treatment of mechanically and mildly extracted gluten affects the network formation of protein, as extra impurities including starch, fibers, etc. were removed to a higher extent. Functional groups were more accessible for new interactions and bonding, resulting in a less aggregated and less crosslinked protein network that ultimately causes more protein content recovery.

3.2 Protein solubility through SE-HPLC analysis

The protein solubility of differently treated and untreated samples was determined through SE-HPLC (Figure 3.1). SE-HPLC analysis indicates the solubility of proteins in terms of monomeric proteins (MP) and polymeric proteins (PP).

With and without enzymatic treatment, mechanically and mildly extracted gluten indicated high solubilities as compared to industrial gluten powder that was untreated. With and without enzymatic treatment, mechanically extracted gluten, indicate less solubility in 1^{st} and 2^{nd} extractions With and without enzymatic treatment, mildly extracted gluten, indicates high solubility in the first and second extractions.

Table:3.1 Protein content of untreated and treated samples

Samples (W.G)	Protein content (%)
Industrial	73
Mild	80
Mechanical	86
Enzymatic (Mild+ Mechanical)	90

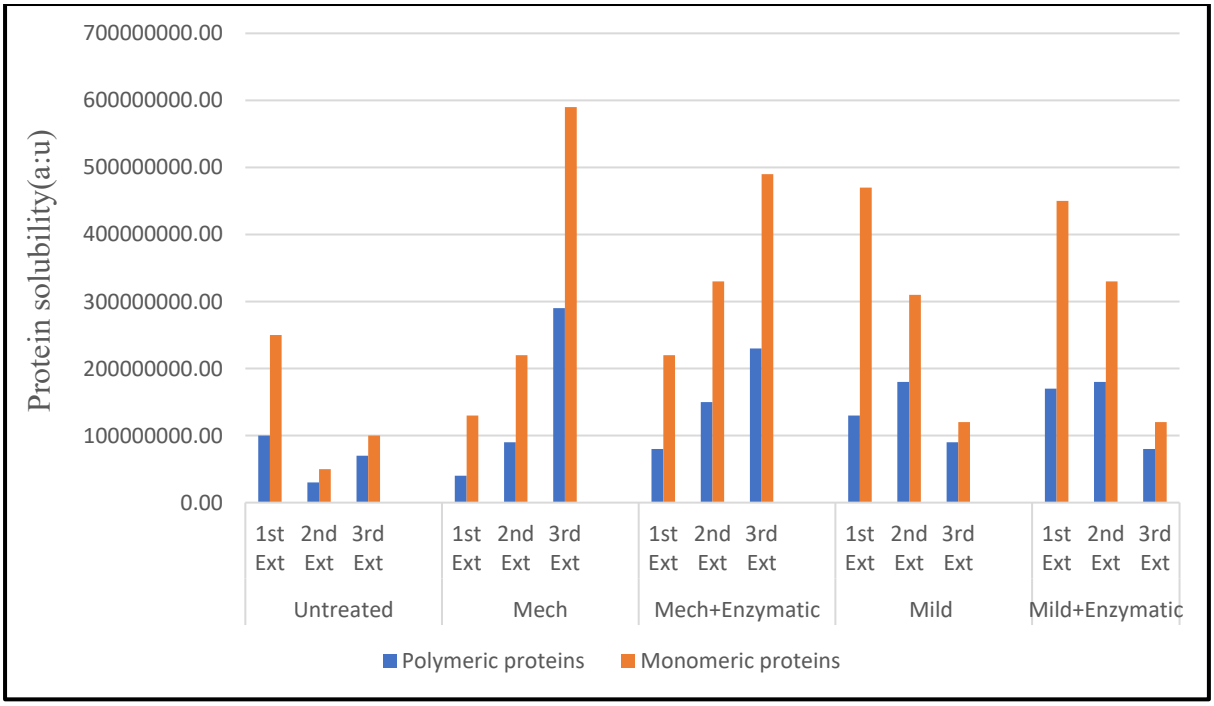

Figure 3.1: SE-HPLC pattern for protein solubility of samples
1st Ext-3rd Ext= 1st-3rd extractions. Protein solubility and size are determined from samples of untreated, mech(mechanical), mech+ enzymatic (mechanical and enzymatic), mild, mild+ enzymatic (mild and enzymatic) treated gluten samples. 1st Ext (Buffers), 2nd Ext (Thirty seconds sonication+ buffer), 3rd Ext (Thirty and 120-seconds sonication+ buffer).

3.3 Prepared films from untreated and treated gluten samples

Films from untreated industrial gluten, gluten that was purified through mild treatment, and gluten sample that were purified after enzymatic treatment with cellulase and amylase were prepared (Figure 3.2). Free swelling and water absorption capacities of films from treated and untreated gluten samples in distilled water and saline solutions were determined through the tea bag method. The swollen samples of different films shown in Figure 3.3.

3.3.1 Absorption capacity of films in distilled water

The absorbance capacities of films in distilled water were determined (Figure 3.4). Water absorbance capacity calculated through the tea bag method after certain time intervals indicated that enzymatically treated samples have higher water absorbing and free swelling capacities after different time intervals. Industrial untreated gluten indicated lower water absorption capacities in both distilled water and saline solutions when compared with mild and enzymatic treated. Water absorption of industrially untreated samples increased from 15g/g at 1min to 20g/g after 24 hours. Enzymatically treated gluten samples showed higher water absorption capacities from 15g/g at 1min to 25g/g after 24 hours than mild and untreated. In mildly treated samples absorption increases from 16g/g at 1 minute to 23g/g after the day. However, water absorption increases in gluten samples treated and untreated over a period of time. After 24 hours the highest water absorption was recorded.

3.3.2 Absorption capacity of films in saline solution

The absorption capacity of films from untreated and treated samples in saline solution was determined through the tea bag method (Figure 3.5). In saline solution, industrially untreated samples uptake 16g/g at 1 min to 20g/g after 24 hours. Mildly treated samples show absorption from 20g/g at 1 min to 27g/g after 24 hours, and enzymatically treated samples indicated the highest uptake from 22g/g at 1 minute to 33g/g after 24 hours.

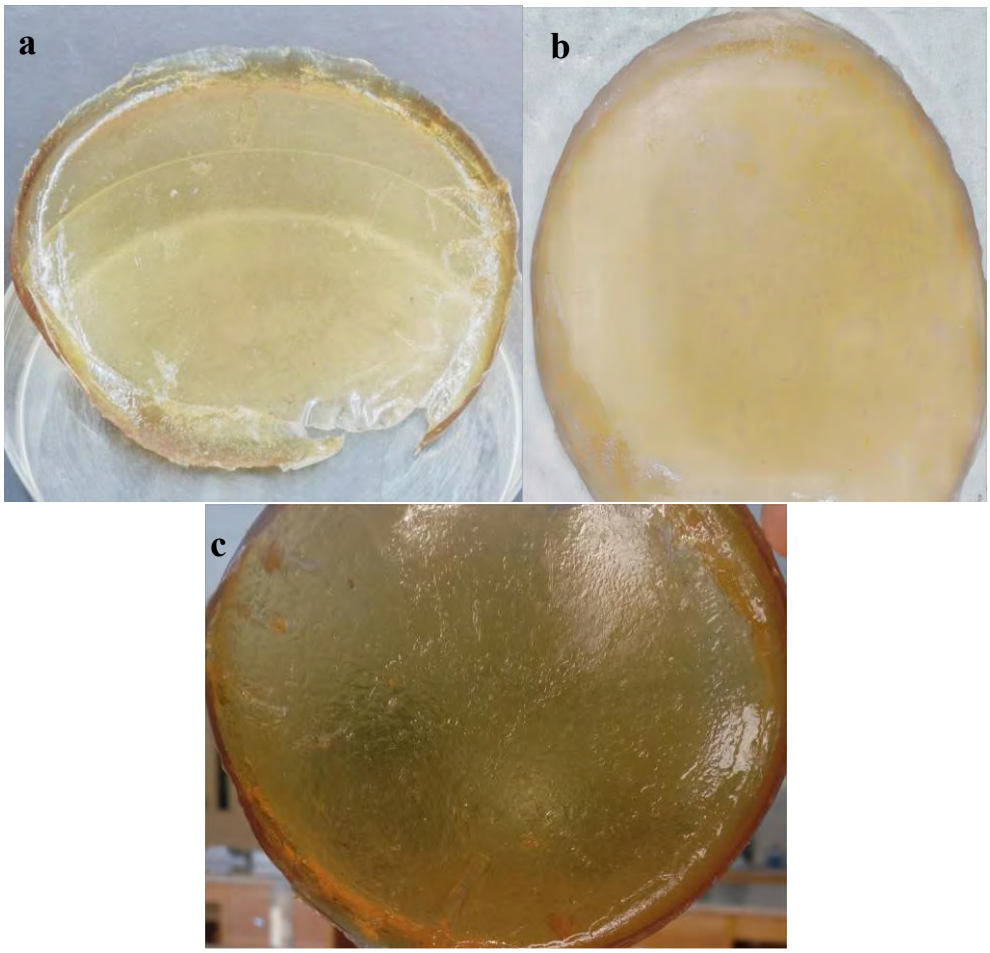

Figure 3.2: Films from untreated and treated samples
a: untreated, **b:** mildly treated, **c:** enzymatically treated gluten samples were prepared

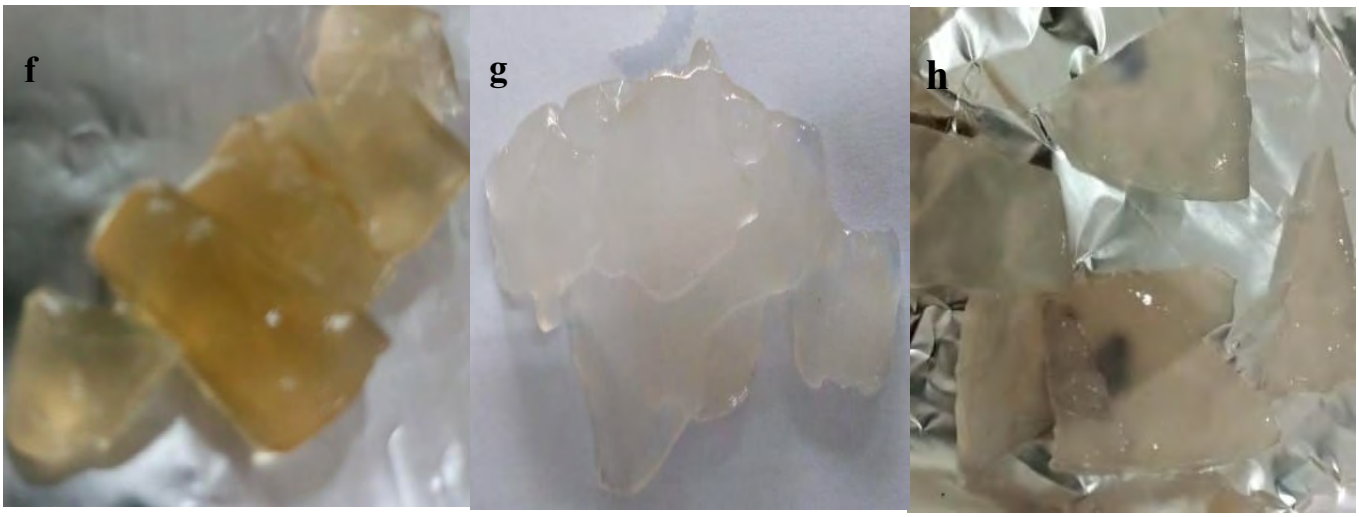

Figure 3.3: Swollen sample of films
f: Swollen sample from the enzymatically treated film, **g:** from mildly treated **h:** from untreated film sample

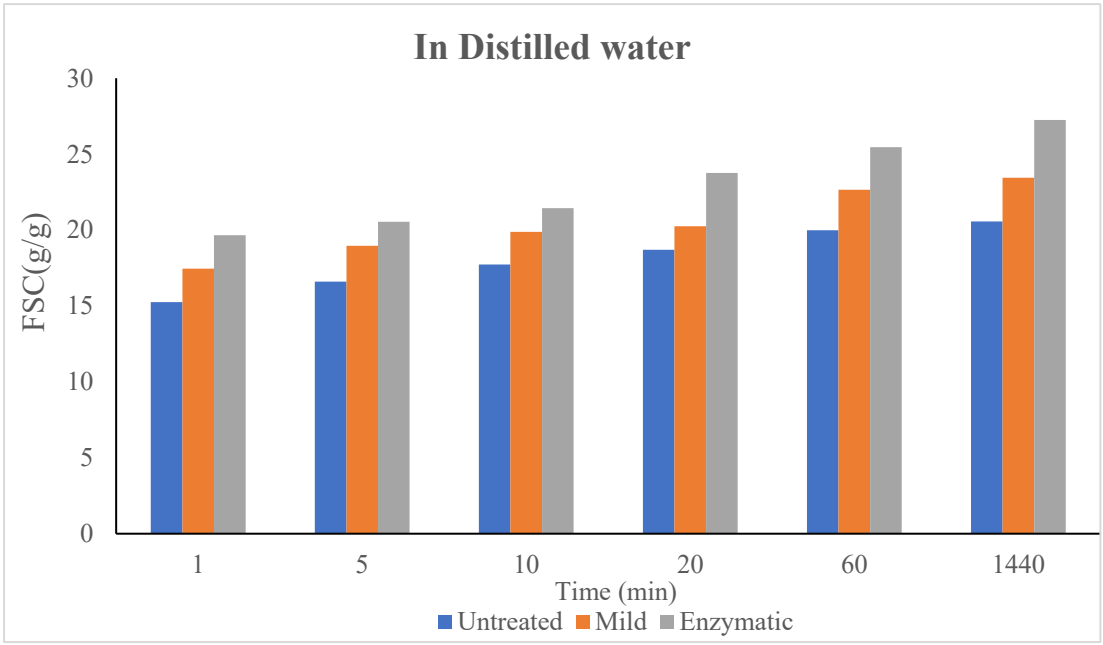

Figure 3.4: FSC of films in distilled water
From tea bag assay FSC (free swelling capacity) of film samples after different time intervals was determined. Enzymatically treated gluten samples showed high absorption capacity in distilled water than mild and untreated samples

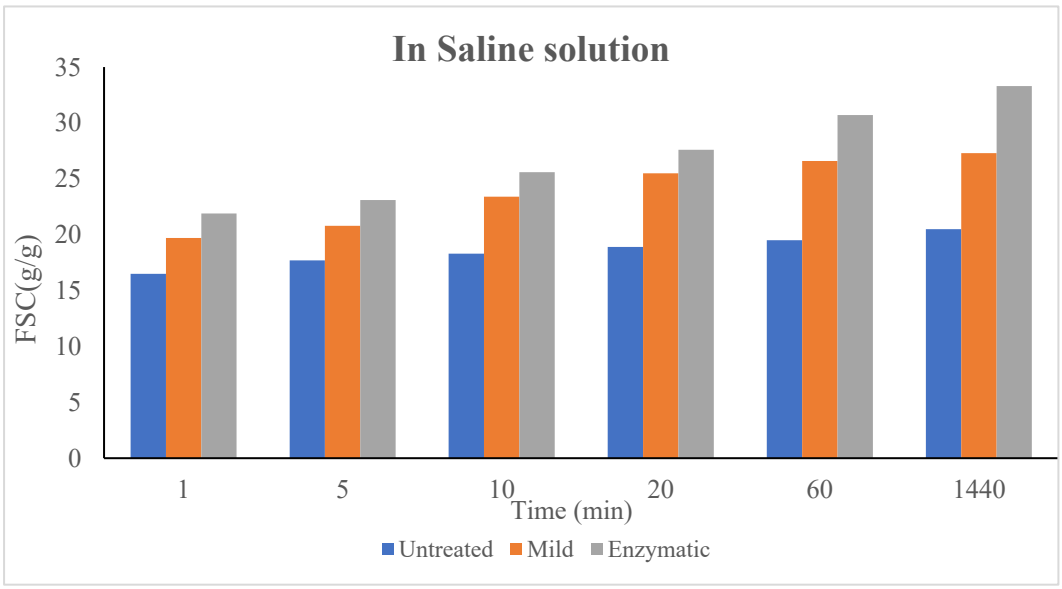

Figure 3.5: FSC of films in saline solution
From tea bag assay FSC (free swelling capacity) of film samples after different time intervals was determined. Enzymatically treated gluten samples showed high absorption capacity in saline medium than mild and untreated samples.

4. DISCUSSION

Wheat gluten protein is widely used in food and bioplastic industries. The most popular (and well-known) application of gluten proteins, such as the wheat flour component, includes the making of bread, cakes, pretzels, and cookies [99]. Bioplastics from wheat gluten can be synthesized through compression molding, extrusion, and casting processes [129]. Transparent, water-resistant, and homogenous films have been obtained through casting techniques [130, 131]. Despite industrial applications of the gluten protein, industrially available gluten has some limitations associated with it, i.e., this product does not simply include proteins, different impurities e.g. starch, minerals, carbohydrates, and lipids with varying percentages are present [100, 113]. Therefore, bioplastics prepared from industrial gluten without chemical modification have shown low tensile strength and other desirable properties as compared to bioplastics prepared from modified gluten protein [136, 142, 153].

In this study, three methods for gluten purification were developed and tested to increase the purity of gluten samples and to modify protein content. They were mild, mechanical, and enzymatic treatments. The Lowry method was performed to determine the protein content of samples. Untreated industrial gluten had lower protein content when compared with gluten samples treated differently. Harsh treatment was done for the separation of gluten from starch at the industrial level. As for industrial separation of gluten, grains were milled into flour, then high-speed mixing of water and flour, gluten was separated from starch through centrifugation and then dried through a ring dryer with an outlet temperature of 60°C and inlet temperature of 100°C. Protein functionality is negatively affected by harsh treatment due to polymerization and lower interaction of protein. So, the, possibility of new bond formation and exchanges also gets limited [177].

During the mixing process, mildly extracted gluten entanglements were disrupted [178]. Therefore, higher protein content was obtained than industrial untreated gluten. In the mechanical treatment of gluten, more washing and mixing led to higher removal of starch so higher protein content was obtained as compared to untreated and mildly treated gluten.

Enzymatic treatment of mechanically and mildly extracted gluten affects the network formation of protein, as extra impurities including starch, fibers, etc. were removed to a higher extent. Functional groups were more accessible for new interactions and bonding, resulting in a less

aggregated and less crosslinked protein network that ultimately causes more protein content recovery.

The SE-HPLC results have shown that with and without enzymatic treatment, mechanically and mildly extracted gluten indicated high solubilities as compared to industrial gluten powder that was untreated. Compact aggregated protein structure with entangled non-protein and protein molecules causes lower solubility. Starch molecules become entrapped and their free flow is resisted in an entangled protein network [179]. For gluten preparation at the industrial level, a higher temperature about 160°C, is provided for the separation of gluten from starch, which causes hard particle formation and agglomeration of starch molecules left behind [177]. With and without enzymatic treatment, mechanically extracted gluten, indicate less solubility in 1st and 2nd extractions, due to disulphide bonds while high solubility in the third extraction where samples were sonicated three times [180, 181]. With and without enzymatic treatment, mildly extracted gluten, indicate high solubility in first and second extractions, suggesting a protein network that is open, loosely bound, and without the disulphide bonds.

Free swelling capacity and water absorption capacity are directly related to each other. High water absorbance, swelling, cohesive properties, large and strong networks, and a large number of charged amino acids are properties of gluten to be used as SAP (super absorbent polymer), in daily care, and disposable products [182, 183].

Pre-crosslinking and protein aggregation occur during the harsh industrial separation of gluten from starch. So, for new interactions low protein content is available. During film formation, this aggregated protein network prevents further interactions due to the inaccessibility of functional groups [177]. Therefore, lower water absorption was recorded in untreated industrial gluten samples. Mildly treated samples showed higher absorption than untreated and lower absorption than enzymatically treated. During mild treatment for gluten separation, the protein network is non-aggregated, reactive functional groups e.g., SH, NH_2, and COOH, etc. are highly accessible so there are more chances for new interactions e.g. isopeptide or disulphide interactions occur during film formation [184-186].

During enzymatic treatment, extra impurities e.g., fiber, and starch content is removed to increase protein content and to get a purified form of gluten. This enzymatically treated gluten indicates higher water absorbance and free swelling capacities in distilled water and saline solution when compared with untreated and mildly extracted gluten. Cellulase undergoes hydrolysis of glycosidic bonds of cellulose [187]. Glycosidic linkages of the starch chain are

hydrolyzed by alpha-amylase. The unfolding of secondary and tertiary conformation of gluten is inhibited by entrapped starch molecules that interact with gliadins and glutenin through non-covalent and covalent interactions [188]. A less aggregated protein network allows higher chances for new interactions to develop.

The protein content determined through the Lowry assay of mild, mechanical, and enzymatically treated samples with cellulase and amylase results in increased protein content from 80%,86%, and 90% respectively when compared with harshly separated untreated gluten samples with a protein content of 73%. In SE-HPLC, samples that were given different treatments had shown higher solubilities due to increased content of monomeric proteins, and open less aggregated protein network, while lower solubility in the case of untreated gluten sample due to press crosslinked protein network. Water absorption capacities of bio-based films prepared from untreated and treated samples determined through tea bag assay had shown that untreated gluten samples had lower water absorption capacity due to tightly bound protein network and treated gluten samples had greater water absorption and free swelling capacities due to avoidance of pre-crosslinking and aggregated protein structure that happened during the harsh separation of gluten. Biobased films with increased water absorption capacities can be used in hygiene products. Bioplastics from gluten samples that were separated through the non-toxic and eco-friendly method can further be investigated to be used in different applications as a replacement for synthetic plastics derived from petroleum-based products.

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