Potential of Hordein as a Biodegradable Food Packaging Material

Thesis submitted for the degree of Doctor of Philosophy in Food and Nutrition Sciences

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Declaration

I confirm that is my own work and the use of all material from other sources has been properly and fully acknowledged.

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Abstract

Conventional plastic packaging is almost non-degradable, leading to significant environmental concerns. As a result, developing biodegradable packaging materials has attracted considerable attention from both industry and researchers. This study explores the potential of hordein, a protein extracted from spent barley, as a biodegradable packaging material. The high glutamine content and presence of hydrophobic amino acids, such as proline, leucine, and valine, endow hordein with strong aggregation properties and notable hydrophobicity.

However, hordein-based films tend to have poor mechanical properties. Therefore, addition of individual plasticizers, glycerol (GLY) and PEG400 and their combinations were investigated in this research in order to enhance its property. The composite plasticizer formed by 25% PEG and 75% GLY demonstrated a synergistic effect, and significantly improved tensile strength and elongation at break of hordein film. Furthermore, the optical property of hordein film was also optimized in this research. Heat treatment was able to unfold the protein structure, exposing thiol groups to promote cross-linking through disulfide bonds and strengthening the protein network. Similarly, transglutaminase (TGase) could induce covalent cross-linking by forming isopeptide bonds between glutamine and lysine residues. To further enhance the mechanical properties of the hordein films, heat treatment at 85^{o}C for 30 mins and TGase added at 0, 10, 20, 30 and 40 U/g were applied to assess their impacts on hordein film quality. Heat treatment produced a thinner film without compromising its mechanical property, while 20 U/g TGase achieved optimal mechanical properties. Although improvements were observed, the mechanical properties still fell short of currently used competing alternatives.

Given hordein's excellent hydrophobicity and UV-blocking properties, the possibility of using hordein as a supplementary ingredient to enhance the properties of starch-based films was explored in this work. Starch films tend to be inherently brittle and sensitive to moisture, despite their superior film-forming ability. Corn starch was identified as the optimal base material for film production, outperforming potato and wheat starch in comparative analyses. Hordein was incorporated into the corn starch matrix at varying mass ratios (10%, 20%, 30% and 40%) to assess its effect on the properties of starch film. The results showed that the addition of hordein significantly increased film thickness, tensile strength (TS), and UV-blocking performance of starch film.

Overall, this study provides valuable insights into the characteristics of hordein and its potential application in the development of biodegradable food packaging films, offering a promising alternative to conventional plastic materials.

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Chapter 1

Introduction to hordein as a potential biodegradable material for food packaging

Chapter 1: Introduction to hordein as a potential biodegradable material for food packaging

1.1 Background

1.1.1 The need for biodegradable packaging for food applications

Traditional food packaging materials, derived from fossil fuels, impose significant environmental pressure. On one hand, fossil fuel resources are unsustainable; on the other, plastic packaging is non-biodegradable. The increasing demand for packaging materials not only accelerates the depletion of natural resources but also worsens environmental degradation (Gan & Chow, 2018). Statistics show that approximately 4.8 to 12.7 million metric tons of plastic were discarded into the worldwide oceans in 2010, and over 30% of landfill waste in 2014 came from plastic packaging (UNEP, 2016). Moreover, global plastic production and consumption are estimated to grow at a rate of 4% annually, with the food packaging sector accounting for 7% of total plastic production (Statista, 2019).

In recent years, there has been a growing interest in developing biodegradable packaging materials. Biodegradability refers to the breakdown of materials through enzymatic reactions by microorganisms such as bacteria, yeast, and fungi, converting them into carbon dioxide, water, biomass, and hydrocarbons under aerobic conditions, or into methane and biomass under anaerobic conditions (Doi & Fukuda, 1994). Biopolymers, typically derived from natural resources such as proteins, polysaccharides, and lipids, are commonly used either alone or in combination for producing biodegradable packaging. These materials are characterized by good biodegradability, non-toxicity, and excellent film-forming properties. However, packaging materials made from natural components often suffer from poor mechanical properties and water resistance, significantly limiting their industrial applications. To address these shortcomings, substantial efforts have been made including optimizing production conditions, employing chemical or biosynthetic methods, using modification techniques (such as the addition of plasticizers, heat treatment, and enzyme treatment), and incorporating composite materials like nanomaterials and plant essential oils (Xu et al., 2012; Stuchell & Krochta, 1994; Tang et al., 2005; Arfat et al., 2015; Xu et al., 2019).

This thesis provides a comprehensive literature review, summarizing the research progress of biodegradable film from the perspective of biopolymer chemical structures. It explains the factors affecting the quality formation of biodegradable packaging and examines the interactions between biopolymers and nanomaterials/essential oils from the angel of molecular structure and chemical bonds (Chapter 2).

1.1.2 Choice of protein - Hordein

Brewers' spent grain (BSG) is one of the key byproducts of the brewing industry, representing approximately 85% of total waste and amounting to approximate 40 million tons annually worldwide (Stojceska, 2019; Mussatto, 2014). BSG contains 15–20% protein, predominantly barley hordein, an alcohol-soluble glycoprotein that serves as a storage protein in barley (Arendt & Zannini, 2013). The high glutamine content and the presence of hydrophobic amino acids, such as proline, leucine, and valine, endow hordein with strong aggregation properties and notable hydrophobicity, which makes hordein a promising candidate for developing biodegradable food packaging (Zhao et al., 2010).

Nevertheless, similar to other protein-based films, hordein films have limitations in mechanical performance, including low tensile strength and poor elongation at break, which restrict their applications in flexible packaging systems (Cho & Rhee, 2009).

1.1.3 Optimizing the properties of hordein-based films

To enhance the mechanical properties of protein-based films, several approaches have been commonly employed, including optimizing production conditions, utilizing chemical or biosynthetic methods, applying modification techniques (e.g., the addition of plasticizers, heat treatment, and enzyme treatment), and incorporating composite materials (e.g., nanomaterials and plant essential oils, etc.). In this study, the following methods were explored:

i) Plasticizer addition: Plasticizers can modify the protein film structure, improving flexibility and reducing brittleness (Cao et al., 2009; Gennadios et al., 1996). The effectiveness of plasticizers depends on their composition, molecular size, and shape (Sothornvit & Krochta, 2001). Common plasticizers include ethers (Gorrasi and Vertuccio, 2014), long-chain fatty acids (Erickson et al., 2014; Xu et al., 2012), and glycerol (GLY) (Parris and Coffin, 1997; Xu et al., 2012), each exhibiting different plasticizing effects on protein films. In recent years, composite plasticizers have gained attention for their synergistic effects, achieving a better balance between strength, flexibility, and stability. The interaction between plasticizers and polymers becomes more complex in composite systems, as the mechanical properties and barrier performance are influenced by the molecular weight, ratio, and compatibility of each component in the mixture (Masamba et al., 2016). This complexity makes composite plasticizers more versatile and effective in optimizing film properties. For instance, Xu et al. (2012) demonstrated that a combination of glycerol and oleic acid lowered the glass transition temperature (Tg) of zein films, with mechanical testing showing a slight improvement in elongation at break (EB). Similarly, Huo et al. (2018) reported that a 1:1 ratio of GLY and PEG improved the elongation at the break of zein films at a plasticizer concentration of 0.45 g plasticizer/g zein. In this project, the effectiveness of GLY, PEG400 and their combination on the quality of hordein film were investigated in Chapter 3.

ii) Heat Treatment: Heating unfolds protein structures, exposing thiol and hydrophobic groups, which subsequently form disulfide bonds and cross-linked network structures (Altschul, 1985; Gennadios and Weller, 1991). This process could enhance the mechanical properties of films based on gliadin, kafirin, and soy protein (Byaruhanga et al., 2007;

Stuchell and Krochta, 1994).

iii) Enzyme Treatment: Transglutaminase (TGase) is known to enhance protein cross-linking through three primary reactions: the formation of isopeptide bonds between glutamine and lysine residues, acyl transfer reactions, and deamidation (DeJong and Koppelman, 2002; Ohtsuka et al., 2001). TGase has been shown to improve the mechanical properties of various protein films, including those made from soy protein isolate, whey protein, zein hydrolysates, αs1-casein, sodium caseinate, quinoa protein isolate, chitosan, and gelatin (Tang et al., 2005; Oh et al., 2004; Motoki et al., 1987; Patzsch et al., 2010; Escamilla-García et al., 2019; Masamba et al., 2016; Lim et al., 1999). The effects of TGase and heating treatment on the quality of hordein film will be discussed in Chapter 4.

1.1.4 Optimizing the performance of starch-based films by adding hordein

Starch, a natural polysaccharide, is widely recognized for its abundance, renewability, biodegradability, low cost, and excellent film-forming properties (Ortega‐Toro et al., 2017). Consequently, it has emerged as a preferred material for developing biodegradable food packaging. However, films derived from native starch often suffer from inherent limitations, including poor mechanical properties, high water sensitivity, and significant brittleness (Shah et al., 2015). Hordein, while exhibiting relatively weak mechanical performance, is distinguished by its pronounced hydrophobicity due to high glutamine content and substantial levels of hydrophobic amino acids, such as proline, leucine, and valine (Zhao et al., 2010). Zein, an alcohol-soluble protein with comparable properties, has been found successfully enhanced the functional properties of starch-based film including mechanical strength and water barrier performance (Corradini et al., 2007; Habeych et al., 2008). So far there is limited information on the impact of hordein on starch film, we hypothesized that there is synergistic effect between starch and hordein due to their complementary characteristics of hydrophilicity and hydrophobicity.

Given these findings, the combination of starch with hordein holds considerable potential for improving the mechanical and barrier properties of biopolymer films, providing a promising pathway for the advancement of biodegradable packaging materials. The results will be discussed in Chapter 5.

1.2 Thesis hypothesis

H1: The addition of plasticizers can improve the performance of hordein protein films.

H2: The addition of composite plasticizers has a synergistic effect, which can more significantly improve the mechanical deficiencies of hordein protein films.

H3: Heat treatment can enhance the mechanical properties of hordein protein films.

H4: The addition of transglutaminase can enhance the mechanical properties of hordein protein films.

H5: A combination of heat and enzyme treatments can work synergistically to improve the mechanical properties of hordein protein films.

H6: Incorporating hordein protein as a supplementary material into starch films can improve the properties of starch-based films.

1.3 Aims and objectives of the project

Considering the research hypothesis, this project aims to:

i) Investigate the effects of various plasticizers on the quality of hordein films (Chapter 3, H1).

ii) Identify the optimal ratio of blended plasticizers for enhancing hordein film properties (Chapter 3, H2).

iii) Determine the impact of heat treatment (Chapter 4, H3) and transglutaminase (TGase) treatment (Chapter 4, H4) on the quality of hordein film.

iv) Assess whether there is a synergistic effect between heat treatment and TGase addition on the mechanical properties of hordein films, making them more suitable for industrial applications (Chapter 4, H5).

v) Identify a starch with optimal quality for film production among the popular potato starch, wheat starch and corn starch (Chapter 5, H6).

vi) Explore the interactions between the selected starch and hordein at different ratios to optimize the quality of the composite films (Chapter 5, H6).

1.4 Thesis outline

This thesis begins with critical evaluation of the research progress in the production of biodegradable films from the perspective of biopolymer molecular structures and chemical bonds formation, then highlighting hordein as a potential material to develop biodegradable food packaging (Chapter 2), Chapter 3 reports on the interactions between hordein extracted from beer production waste and various plasticizers, which shows significantly improvement in the hordein film properties. To further enhance the properties of hordein films and align them with industrial requirements, the effects of heat treatment and transglutaminase (TGase) addition on quality of hordein films were studied (Chapter 4). Given the time-consuming and costly extraction of hordein, as well as the poor mechanical performance of hordein films even after various treatments, hordein being a supplemental material for biodegradable packaging was further explored. After comparing films made from commercially popular starches, corn starch was identified as the best performing starch in film production among them. Incorporating different levels of hordein in corn starch film on the quality of starch films were analyzed in Chapter 5. Finally, the thesis concludes with a general discussion (Chapter 6), summarizing the research findings, identifying current limitations and areas for future work, and highlighting the contributions of this study to food science and packaging technology.

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Zhao, J., Tian, Z. and Chen, L. (2010) "Effects of Deamidation on Structure and Functional Properties of Barley Hordein," *Journal of agricultural and food chemistry*, 58(21), pp. 11448–11455.

Chapter 2

Development of biodegradable packaging for food applications

Chapter 2: Development of biodegradable packaging for food applications

Abstract:

Traditional plastic packaging is rarely degradable and has a negative impact on the environment. Therefore, developing biodegradable packaging has attracted lots of attention from both industry and researchers. Biopolymers are usually made from natural ingredients, such as proteins, polysaccharides and lipids. Most of these materials have good degradability, non-toxicity and good film-forming properties, but poor mechanical and barrier properties. Reinforcing these biopolymers by the addition of nanomaterials (forming composites) can improve their mechanical strength, oxidation resistance, antibacterial and gas/moisture barrier properties. Moreover, the inclusion of essential oils to the biopolymer films can synergistically improve the shelf life of food products due to the improvement of anti-oxidation and anti-bacterial properties of the composite film. This review offers critical evaluation of research progress in nanocomposite films and the beneficiary effect of inclusion plant essential oils from the perspective of molecular structure and chemical bonds formation.

Key words: Biopolymers; films; nanomaterials; essential oil; chemical structure

2.1 Introduction

The basic functions of food packaging are to protect food products from mechanical damage, environmental hazards and microbial contamination with aim to achieve quality stability and longer shelf life. Packaging has become one of the largest industries in the world, accounting for approximately 2% of GDP (Mihindukulasuriya & Lim, 2014). Traditional food packaging materials are based on fossil fuels, which create huge pressure on the environment. On one hand, the supply of fossil fuels is not sustainable; on the other hand, the plastic packages are not degradable. The increasing demand in packaging materials not only accelerates the consumption of natural resources, but also deteriorates the environment (Gan & Chow, 2018). According to the statistics, about 4.8 million to 12.7 million metric tons of plastic were thrown into the sea in 2010, while more than 30% of landfilled waste was from plastic packaging in 2014 (UNEP, 2016). Moreover, it has been estimated that the production and consumption of plastics all over the world are increasing at the rate of 4% every year, while food packaging industry contributes 7% of total plastic output (Statista, 2019). Therefore, it is essential for food industry to take the initiatives to reduce usage of plastic packaging and develop alternatives of novel packaging materials to make the food production more sustainable.

In the past few years, more and more research has been conducted to develop degradable packaging materials. "Biodegradable" is defined as a material that can be degraded under the enzymatic reaction of organisms such as bacteria, yeast, fungi and turned into carbon dioxide, water, biomass and hydrocarbons under aerobic conditions, or methane and biomass under anaerobic conditions (Doi & Fukuda, 1994). Biopolymers are usually natural ingredients, such as proteins, polysaccharides and lipids, alone or in combination are often used to produce biodegradable packaging. Most of them have good degradability, non-toxicity and good film-forming properties. For example, proteins can interact with polysaccharides carrying opposite charges to form self-assembled structure through ionic interaction and develop film (Derkach et al., 2020). Safety has been one of the biggest selling points for protein, carbohydrate and lipids-based packaging materials because there is no risk of toxic chemical migration from packaging materials to food products (da Silva et al.,2020; Hosseini et al.,2015). Some biodegradable materials, such as chitosan have antibacterial properties. Zhang et al. (2020) reported that chitosan could work with cellulose nanofiber (CNF) and trans-cinnamaldehyde (TC) to extend the shelf life of food products due to its strong antibacterial activity. However, packaging materials made from natural ingredients usually have poor mechanical properties and water resistance, which greatly limit their applications in industrial practice.

To address the quality defects of biodegradable packaging, significant efforts have been made over the past twenty years. These include adjusting production conditions, utilizing chemical or biological synthesis, employing modification techniques (e.g., the addition of plasticizers, heat treatment, enzymatic treatment), and incorporating composite materials. Xu, Chai, and Zhang (2012) found that the elongation at break (EB) of zein film increased with a combination of glycerol and oleic acid, while Stuchell and Krochta (1994) observed that heat treatment significantly enhanced the mechanical properties of soy protein isolate films. Additionally, transglutaminase (TGase) has been shown to improve the mechanical properties of various protein films, including those made from soy protein isolate, whey, zein hydrolysate, αs1-casein, sodium caseinate, quinoa protein isolate, chitosan, and gelatin (Tang et al., 2005; Oh et al., 2004; Motoki et al., 1987; Patzsch et al., 2010; Escamilla-García et al., 2019; Masamba et al., 2016; Lim et al., 1999). Furthermore, functional ingredients such as plant essential oils and nanomaterials have been explored to optimize biodegradable packaging by enhancing mechanical, barrier, antioxidative, and antibacterial properties. For example, Arfat et al. (2015) and Sahraee et al. (2017a) found that ZnO nanoparticles (ZnONP) improved the mechanical, water barrier, and antibacterial properties of fish protein isolate/fish peel gelatin-based composite films. Synergistic effects between nanofibers and oxidized metal nanoparticles have also been reported, such as chitosan nanofiber (CHNF) with ZnO nanoparticles (Amjadi et al., 2020a), TiO2 nanoparticles with betanin (Amjadi et al., 2020b), and ZnO nanoparticles with cellulose nanofibers (Ahmadi et al., 2020). Moreover, essential oils, known for their antioxidative and antibacterial properties, have shown potential to enhance packaging materials. Research has explored the use of essential oils such as thyme essential oil (TEO), rosemary essential oil (REO), Zataria multiflora Bioss. essential oil (ZME), and Trachyspermum ammi (Ajowan) essential oil (AJEO).

Many research results demonstrate that there are many superiorities of nanocomposites including strengthening mechanical, barrier properties and antibacterial effects, but there are no clear laws and regulations on the use of biodegradable packaging materials and nanomaterials in food. FDA regulations stipulate that all products that contain nanomaterials or use nanotechnology for hazardous products will not be processed (FDA, 2014). Due to the uniqueness of each nanomaterial, the risk assessment must be based on specific circumstances (EU, 2012a, EU 2012b). The European Commission Regulation (No. 10/2011) on plastics and other materials in contact with food was implemented in 2011 (EC, 2011) and states that the migration limit for substances from food contact packaging materials to food product is set to 10 mg/dm^2 excluding nanoparticles and emphasized the safety evaluation of nanomaterials should be based on case by case. Many other countries including Canada, Australia, New Zealand, Korea, China and Japan have no specific regulations on nanomaterial packaging.

In recent years, there have been many literature reviews on the progress of biodegradable packaging materials, however the relationship between chemical structures and properties of biodegradable packaging has not been fully explained. Therefore, this report will discuss the research progress of biodegradable film from the angle of chemical structure of biopolymer to explain the property formation of biodegradable packaging and discuss the interaction with nanomaterials and plant essential oils in the aspect of molecular structure and chemical bonds formation.

2.2 Development of food packaging using biopolymers

2.2.1 Packaging requirement for food products

The main function of food packaging is to protect the product from damage caused by external mechanical forces, and environmental hazards including insects, dust, oxygen, water vapor and microorganisms with aim to maintain its quality and extend the shelf life. Food can be damaged or contaminated at any stage of processing, storage, transportation, distribution and sale. Therefore, food packaging should have excellent mechanical properties to resist the damage, good barrier property to oxygen and moisture, non-toxic and inert to microorganisms in order to effectively prevent food contamination at various stages (Nasser et al., 2020).

Mechanical property is considered as one of the most basic requirements for food packaging materials (Liu et al., 2019). In addition, water vapor permeability (WVP) and oxygen permeability (OP) are also key attributes indicating the performance of packaging materials. For meat products, oxidation can cause discoloration, poor odour and shortened shelf life (Mathew, 2017). Therefore, good oxygen barrier properties can effectively extend the shelf life of meat products. For the packaging and preservation of fresh fruits and vegetables, limiting the moisture loss of vegetables and fruits is the key for maintaining their appearance, texture and flavour (Robertson, 2009).

Overall, mechanical and barrier properties are the two critical properties for food packaging. If antibacterial or/and antioxidative function can be added, it would be the surplus for food packaging, as the shelf life of food can be further extended. Fellows (2008) pointed out that poor mechanical property is one of the main limitations for biodegradable packaging to be widely used in industry compared with synthetic materials.

2.2.2 Types of biopolymers for developing packaging materials

The biopolymers can be categorized into three types, i.e. natural polymers, polymers derived from chemical synthesis, and polymers derived from biological synthesis according to their chemistry features. I) Natural polymers obtained directly from biomass include protein, lipid and polysaccharides. For protein, the popular ones are gluten, whey protein, soy protein etc, while cellulose, starch and chitosan are good examples of polysaccharides. II) Derived polymers from chemical synthetic reactions include alcohol polyvinyl (PVA), polylactic acid (PLA), ethylene–vinyl alcohol (EVOH) and polycaprolactones (PCL). III) Derived polymers from biological synthesises mainly include bacterial cellulose and polyhydroxyalcanoates (PHAs) from genetically modified or natural microorganisms (Fabra, López-Rubio and Lagaron, 2014).

The research advances associated with each type of biopolymers are summarized in Table 2.1. Plant/animal protein, polysaccharides and lipids are natural polymers and can be used directly to develop biodegradable packaging. Proteins from wheat, soy, corn were given extensive attention due to their availability and cheap price. In general, animal proteins are more expensive compared with plant protein, but whey protein stands in a unique position due to the valorisation of food waste from dairy industry. Along with whey protein, soy protein, zein protein and wheat gluten, sunflower protein, peanut proteins also attracted a good level of attention. Polysaccharides are popularly used in the production of degradable packaging. Most polysaccharides such as cellulose, chitosan, starch, fructose and alginates have good film-forming properties and gas/oil barrier properties, but poor water barrier properties. According to Table 2.1, chitosan is considered as one of the most popular polysaccharides for production of biodegradable packaging due to its good compatibility with other film forming composites, antibacterial activity and wide availability, although it has poor mechanical properties and water barrier properties. Unlike proteins and polysaccharides, lipids are often considered as supplement to improve the hydrophobicity properties of the film rather than work independently as the main film forming agents. The popular lipids including palm fruit oil, beeswax, lecithin and clove bud essential oil etc, possess strong hydrophobicity have been used to improve the water barrier of the composite film. In addition, many essential oils were also added to the matrix to improve the antibacterial and antioxidant properties of the film. Synthetic polymers have good water barrier and optical properties compared to natural polymers. From a cost perspective, synthetic polymers are typically cheaper to produce at scale, benefiting from established petrochemical processes, but natural and bio-derived polymers are more sustainable.

Table 2.1 Popular types and properties of bio polymers in film production

Polymer classification	Materials	Key examples	Strength	Weakness	Enhancement strategy	Remark	Reference
Natural polymers	Proteins	Whey proteins	Good gas barrier properties; better tensile strength; good use of dairy waste	Poor water vapor barrier properties; poor mechanical properties	Ultrasound; heat; enzyme can be used to improve the property; chitosan; glycerol	Abundant, low cost; ecologically sustainable; nutritional	Schmid & Müller, 2019; Ferreira et al., 2009
		Soybean	Abundant; inexpensive; biodegradable; nutritional; good oxygen and oil barrier when the humidity is low	Poor mechanical property; high sensitivity to moisture; beany flavour	Heat, pH; enzyme; gelatin; glycerol; poly (vinyl alcohol)		Cao, Fu, & He, 2007; Su et al., 2010
		Corn zein	Good Antioxidative property; good film forming property; biodegradable	Poor mechanical properties; poor water vapor barrier	High-Amylose Corn Starch; sorbitol; oleic acid; glycerol; urea and formamide; pH		Ryu et al., 2002; Trujillo-de Santiago et al., 2014
		Wheat gluten	Good mechanical properties; low cost; good oxygen barrier; film formation; renewability	Poor water vapor barrier properties	High processing temperature; glycerol		Mojumdar et al., 2011; Kayserilioglu et al., 2003

	Sunflower protein	Abundant; low cost; good film forming properties; biodegradable	Poor mechanical properties; poor water vapor barrier	PH; glycerol; heat		González-Pérez & Vereijken, 2007; Salgado & Petruccelli, 2011
Polysaccharides	Cellulose	Good mechanical properties; totally transparent; good thermal property	Highly sensible to water presence	Heat; Starch; glycerol; polyethylene glycol	Non-toxic and widely available materials in nature; good permeability to carbon dioxide and oxygen; effective in the protection of food with low moisture	El Halal et al., 2016; Chinnan & Park, 1995
	Chitosan	Great film-forming properties; antibacterial and antifungal activities; good resistance to fat and oil, oxygen	Highly sensitive to moisture; poor mechanical properties;	Heat; Glycerol; Tween 20; konjac glucomannan; starch; oleic acid		Skurtys et al., 2011; Campos et al., 2011 ; Tripathi, Mehrotra & Dutta, 2008; Vargas et al. 2008
	Pectin	Good mechanical properties; good barrier to oil and gas	Poor resistance to moisture, low elongations and is quite brittle	Crosslinked with polyvalent cations can exhibit fair mechanical properties; lactic acid; chitosan; glycerol		Liu, Fishman, & Hicks, 2007; Hoagland & Parris, 1996

		Starch	Translucent or transparent; low cost; biodegradable	Poor mechanical properties; poor water vapor barrier	Glycerol; potassium sorbate; poly (vinyl alcohol)		Flores, 2011; Lawton, 1996
		Alginates	Abundant; inexpensive; biodegradable; good film forming properties	Poor water vapor barriers	Can produce a strong insoluble, or gel, polymer when reacting with calcium cations or polyvalent metal; gelatin, soy protein isolate; whey protein		Erginkaya et al., 2014; Harper et al., 2013
	Lipids	Palm fruit oil	Favored water resistance, water vapor barrier, transparency, and elongation	Oil impaired transparency and overall tensile properties of the films	Ultrasonic; Tween 80 and Span 80; glucerol	Good water barrier properties; low cost; supplementary ingredient; lipid does not form film independently and usually act as supplement ingredients.	Rodrigues et al., 2016
		Beeswaxes	Reducing moisture permeability	Poor mechanical properties	Gum cordia; PEG400		Saucedo-Pompa et al., 2007; Haq et al., 2016
		Lecithin	Important emulsifiers; good water barrier properties	Poor mechanical properties	Glycerol; Yucca schidigera extract		Dias et al., 2013
		clove bud essential oils	Antibacterial activity	Tensile and water vapor properties, poor transparency	Allspice; cinnamon		Du et al., 2009
Derived polymers from chemical synthetic reactions	polycaprolactones (PCL)		Good transparency; good film forming properties	Poor mechanical properties	Heat; starch; stearic acid; citric acid	Good film forming properties; good water barrier properties; good transparency	Correa et al., 2017

	Poly-lactic acid (PLA)	Good mechanical properties; good thermal properties	Poor water vapor barriers	Zataria multiflora Bioss. essential oil; propolis ethanolic extract; cellulose nanofiber		Rezaeigolestani et al., 2017
	Polyvinyl alcohol (PVA)	Good water barrier properties; good film forming properties	Poor mechanical properties	Cellulose nanofibers; cellulose nanocrystals		Lee et al., 2020
Derived polymers from biological synthesises	Bacterial cellulose	Synthesized in a pure way; biodegradable; no toxic	High cost of production	Dried process; storage relative humidity conditions; chitin multi-nanofibers		Cheng, Catchmark, & Demirci, 2009; Yang et al., 2020

2.2.3 Relationship between quality of biodegradable packaging and their chemical structure.

The characteristics of the film are affected by the inherent characteristics of the material such as molecular size, surface charge, crystallinity, hydrophobicity/hydrophilicity and the three-dimensional shape, and the external processing factors such as processing temperature, pH, ionicity, salt, relative humidity, shear force and pressure during processing and storage (Dangaran et al., 2009). In this section, the relationship between the chemical structure of biodegradable packaging and the quality attributes will be illustrated.

2.2.3.1 Film forming properties

Biopolymers consist of long chains of repeating molecular units (monomers) which could intertwine and overlap, creating a continuous and stable network. Through drying process to remove solvents, this network can form a thin and cohesive layer, i.e. film. The intermolecular force including van der Waals forces, hydrogen bonding, and covalent binding can help the biopolymer chains adhere to each other, contributing to the film's integrity and stability (Bauer et al., 1998).

Proteins can be found naturally as fibrous proteins or globular proteins. There is a rotation of the spheres themselves and parallel connections between the fibrils. The mechanical properties of protein layers are better than polysaccharides due to their unique structure (Mohamed et al., 2020). Polysaccharides are composed of long chains of sugar units (monosaccharides) linked through glycosidic bonds. In polysaccharide-based films, the network structure primarily depends on hydrogen bonding between hydroxyl groups of sugar units, as well as van der Waals forces. These films generally form cohesive networks due to these relatively weaker bonds, making them flexible and transparent but with moderate mechanical strength and barrier properties. Polysaccharide-based films often struggle with water solubility and moisture sensitivity due to the hydrophilic nature of the sugar units (Han, 2005).

2.2.3.2 Mechanical properties

Good mechanical properties are considered as one of the basic requirements for food packaging, because film with good flexibility or strength can protect the food during production, transport, storage and sale (Sothornvit and Rodsamran 2008). Tensile strength (TS), elastic modulus (EM) and elongation at break (EB) are popularly used to evaluate the mechanical properties of packaging materials. EB indicates the ability to change shape before breaking, EM shows the stiffness of the film, and TS is used to indicate the ability to remain unbreakable under tensile stress (Yang et al., 2019). Huang et al. (2020) pointed out that the ideal mechanical properties of packaging materials should meet the requirements of breaking elongation greater than 10% and tensile strength greater than 10 MPa. The higher the TS and EB, the stronger the packaging material.

In principle, the mechanical property of biopolymer-based film is governed by the composition, structure and environmental conditions (Vargas et al., 2008). Proteins have extremely high intermolecular bonding potential which determines that protein-based films have better mechanical properties and gas barrier properties than polysaccharide-based films. Especially some hydrophobic proteins (for example hordein and zein) have better water barrier properties. Hordein, according to the Goldman-Engelman-Steitz (GES) scale, one-third of the amino acids in hordein are hydrophobic, with high levels of Leu, Val, Phe, and Tyr (Xia et al., 2011). In addition, a study reported that increasing zein concentration in chitosan-based edible films resulted in increased elasticity of the films with improved barrier properties (Escamilla-García et al., 2013). Protein structure is far more complicated including secondary, tertiary, and quaternary structures, and stabilized by various forces including peptide bond, ionic bond, hydrophobic interaction, and disulfide bond which ensure the stability and mechanical property of the film, while polysaccharide-based film H bonds are the primary interaction which is far less and diverse compared to protein-based film (Han, 2005). All proteins have complex polymeric structures; however, they are not structurally stable by nature. Using different strategies, the mechanical characteristics of protein-based films can be enhanced, such as by introducing a plasticizer into the protein matrix (Lamp, Kaltschmitt and Dethloff, 2022). Huo et al. (2018) reported using a 1:1 ratio of GLY and PEG, the plasticized zein films showed good elongation at break at 0.45 g plasticizer/g zein.

To improve mechanical properties, different strategies have been employed by modifying the intermolecular interactions within biopolymers. For protein-based film, treatments including thermal denaturation, chemical hydrolysis, enzymatic treatment, and chemical crosslinking are popularly used to enhance the interaction force for strength and stability of protein-based film (Han, 2005). Heating unfolds proteins, exposing sulfhydryl and hydrophobic groups, which subsequently form disulfide bonds and create a cross-linked network structure (Altschul, 1985; Gennadios and Weller, 1991). Similar findings were reported by Tang et al. (2009) that the elongation at break (EB) of vicilin-rich protein isolate films (from red bean, kidney bean, mung bean, and soy protein isolate) was unaffected by heating, though tensile strength (TS) increased. Tang et al. (2005) found that treating cast soy protein isolate (SPI) films with microbial transglutaminase (MTGase) reduced the elongation at break (EB) values by 34%, 22%, and 73% for films with glycerol, a glycerol-sorbitol mixture (1:1), and sorbitol, respectively. This suggests that MTGase treatment results in a more compact and less elastic film structure. Similarly, formaldehyde-treated pea protein films and aldehyde-crosslinked glutenin-rich films also show that increases in tensile strength (TS) are frequently accompanied by decreases in EB. (Guéguen et al., 1998; Hernández-Muñoz et al., 2004).

For polysaccharide-based films, strategies such as pH adjustment, crosslinking, and the incorporation of fillers play significant roles in enhancing mechanical properties. Teklehaimanot et al. (2020) demonstrated that alkaline treatment of pre-gelatinized maize starch improves surface activity, facilitating better interactions between starch and zein, which contributes to enhanced film structure. Crosslinking reactions are another common approach, as they strengthen the interactions between polysaccharide molecules, creating a firmer and more stable network, which leads to improved mechanical performance (Zhao et al., 2019). Additionally, studies have shown that incorporating organic acids like tartaric, oxalic, malic, and citric acid can enhance both mechanical and water vapor barrier properties of polysaccharide films. These acids act as plasticizers, participating in polymer-polymer interactions by forming hydrogen bonds (Cagri et al., 2001). Organic acids, such as sorbic acid esters, can increase elongation while reducing tensile strength, as observed in films made from tapioca starch, sweet potato starch, and whey protein (Flores et al., 2010; Shen et al., 2010).

The polymers obtained directly from biomass including protein, polysaccharides and lipids have the advantages of being non-toxic, degradable and easy to obtain. Most polysaccharides have good gas barrier properties but often have poor mechanical properties and water resistance (Campos, Gerschenson and Flores, 2011). For example, chitosan has a strong film-forming ability due to its cationic property and high molecular weight along with antibacterial and antifungal activities. but it is highly sensitive to moisture (Ferreira et al., 2009). Studies have demonstrated that protein-based edible films are the most attractive and provide mechanical stability compared to carbohydrates and lipids (Nandane & Jain, 2015). The composite biodegradable film is composed of mixtures of polysaccharides, proteins and/or lipids that can take advantage of the unique functional characteristics of each compound to improve mechanical properties (Bourtoom 2008). Cao, Fu and He (2007) examined the mechanical attributes of soy protein isolate (SPI) film and reported that the film had poor mechanical properties and was brittle and hard to handle. When SPI and gelatin composite films were prepared, the TS and EB significantly improved.

Nanomaterials such as nanofibers and inorganic nanoparticles have been added to the composite film to further improve the mechanical properties of biopolymer film. Carvalho et al. (2020) reported that adding chitin nanofibers and curcumin micro/nanoparticles to bacterial cellulose can improve mechanical properties. Ranjbaryan et al. (2020) reported that adding cellulose nanofiber into the sodium caseinate film can increase the mechanical properties significantly.

2.2.3.3 Barrier properties

The barrier properties of food packaging materials to water vapor, oxygen and light significantly affect the shelf life of the product (Lagarón, 2011). Azeredo et al., (2012b) reported that waterproof biopolymer films could effectively extend the shelf life of products.

2.2.3.3.1 Water barrier property

Different product has specific requirement for the water permeability of packaging materials. The water blocking performance of the film is usually evaluated by gravimetric analysis. The film acts as a semi-permeable barrier between a high relative humidity (RH) environment and another low RH environment, and its water diffusion is monitored by gravimetric analysis. The water vapor permeability (WVP) value has been the most studied and reported parameter so far.

WVP is affected by the mobility and free volume of macromolecules, as well as the integrity, hydrophilicity, hydrophobicity and crystalline amorphous ratio of the film. Protein contains hydrophilic parts which would interact with water due to the polar-polar interaction. Therefore, the water resistance of protein-based films is generally poor. Adding hydrophobic materials through chemical and enzymatic methods or physical methods would reduce the water permeability (Bourtoom, 2009). Lipids can be obtained from animals, insects and plants with the diverse structures including phospholipids, phospholipids, fatty alcohols, fatty acid monoglycerides, diglycerides, triglycerides and terpenes. Belitze et al. (2009) and Akoh and Min (2008) reported adding lipids in films can effectively improve water barrier properties due to the increase in hydrophobicity in the composite matrix. In order to improve the water resistance of polysaccharides, Vargas et al. (2009) added different concentrations of oleic acid to chitosan film. He found that WVP decreased with the increase of the oleic acid content. Ojagh et al. (2010) reported that cinnamon oil as composite of chitosan coatings could extend the shelf life of rainbow trout during cold storage due to the improvement of water barrier property and antioxidative property of the film. Alginate has the unique ability to irreversibly react with multivalent metal cations, especially calcium ions, to produce water-insoluble polymers, thereby it can be used to improve the water barrier properties (Olivas et al., 2008). Composite film by blending polysaccharides and proteins can also offer better water barrier property. Erdohan and Turhan (2005) observed that films made from a methylcellulose (MC)-whey protein blend exhibit lower water vapor permeability (WVP) than those made from a single biopolymer. This is because the addition of MC increases the solid content and forms a denser film matrix with smaller pore sizes (Gutsche & Yoshida, 1994). The linear structure of MC allows its polymer chains to pack tightly, reducing the film’s permeability to water vapor and enhancing its barrier properties (Chen, 1995). Similarly, the addition of pullulan to the whey protein film could also reduce the permeability of water vapor (Gounga, Xu and Wang, 2007). Nanocomposites have attracted lots of attention in the recent years. Lee et al. (2020) reported that the addition of cellulose nanocrystals, cellulose nanofibers and dialdehyde cellulose nanofibers to polyvinyl alcohol (PVA) can significantly improve the waterproof performance, and improvement in water barrier property.

2.2.3.3.2 Oxygen barrier properties

Oxygen permeability is also another important attribute for packaging materials, especially for modified atmosphere packaging. Limiting the contact of food with oxygen is essential, especially for food products containing high level of fat or compositions susceptive to oxygen, because it can lead to loss of sensory quality (odor, color, taste and texture) and nutrients (Sothornvit and Pitak 2007). Oxygen permeability is also another important attribute for packaging materials, especially for modified atmosphere packaging. Limiting the contact of food with oxygen is essential, especially for food products containing high levels of fat or compositions susceptive to oxygen, because it can lead to loss of sensory quality (odour, color, taste and texture) and nutrients (Sothornvit and Pitak 2007). In fruits and vegetables, respiration and transpiration affect shelf life. Oxygen is necessary for respiration, which reduces carbohydrates and weight, impacting quality (Oms-Oliu et al., 2008; Galus & Kadzińska, 2015). Reducing oxygen levels below 10% can control respiration, but excessively low levels cause anaerobic respiration, leading to flavor loss and bacterial growth. Low O2 and high CO2 concentrations can reduce C2H2 production (Shayanfar, 2014). Protein-based films are widely used for fruit and vegetable preservation. Fat deterioration in peanuts can be prevented with gluten and SPI coatings (Paul, 2019). Gelatin-based films extended the shelf life of carrots and strawberries (Wang et al., 2005; Fakhouri et al., 2014). In dairy products, high oxygen levels accelerate oxidation and microbial growth. Cheese, which consumes oxygen and releases CO2, requires packaging with low oxygen permeability to prevent fat oxidation and microbial growth. Edible films help extend the shelf life of various cheeses (Chen et al., 2019). For fresh meat, maintaining the red oxymyoglobin color requires high oxygen permeability packaging or MAP with high O2 levels. However, high oxygen concentrations promote the growth of Gram-negative bacteria, particularly on ground meats. Traditional MAP with 70–80% O2 and 20–30% CO2 can inhibit microbial growth, though lactic acid bacteria like Carnobacteria spp. may still grow (Petersen et al., 1999). High CO2 concentrations are effective for lighter colour meats, though levels above 25% may cause off-flavours and discolouration in poultry (Bartkowski, Dryden and Marchello, 1982).

Adding antioxidant active compounds to packaging is one of the main ways to extend the shelf life of food. The antioxidant active agent is composed of synthetic antioxidants and natural antioxidants. The main sources of natural antioxidants are plant extracts and essential oils, such as carvacrol, citral and lemongrass essential oils (Rojas-Graü et al.). Yu et al. (2014) reported that adding tea and rosemary extract to the composite film had better antioxidant effect than the composite film made with synthetic α-natural phenol. Carvalho et al. (2019) reported that adding thyme essential oil to whey protein-based film significantly improved the antioxidant properties.

2.2.3.4 Anti-bacterial properties

Antibacterial packaging can extend the shelf life of food by reducing the microbial load, and it can be classified into two groups, film with natural antibacterial property and film with added antibacterial agents. For polymer with natural bacterial property, chitosan is one of the popular ingredients which can form film naturally and it also has antibacterial property. Ziani et al., (2009) reported that the positive charge on the amino group of chitosan can react with the negative charge on the phospholipid bilayer in the bacterial cell wall to form pores which would increase the membrane permeability and lead to the cell death. Antibacterial agents such as metal/metal oxides, inorganic clays, natural biological materials (such as essential oils), synthetic antimicrobial agents and enzymes can also be incorporated into the packaging film (;Vásconez et al., 2009). These reagents can be incorporated into packaging materials or coated or fixed on packaging materials (Arvanitoyannis and Stratakos, 2012). The antibacterial efficiency of packaging films can be evaluated by different methods, including coverage test/direct contact with growth medium (laboratory medium or natural food) and gas phase method, while the latter is suitable for volatile compounds and their applications (Du et al., 2009a). Hosseini et al. (2009) reported that adding clove, thyme and cinnamon essential oils to chitosan-based films can inhibit Listeria monocytogenes, Salmonella enteritidis, Staphylococcus aureus and Pseudomonas aeruginosa. Devlieghere et al. (2004) reported that due to consumer demand for healthy food without chemical additives, it has become a trend to choose antimicrobial agents from natural sources. Therefore, antimicrobial agents including organic acids, polybasic chitosan, certain nisin fragments, lactoperoxidase system, plant extracts and essential oils could be the ideal candidates for developing biodegradable packaging with antibacterial property.

2.3 Nanomaterials used to improve the quality of biodegradable film

Although packaging films are composed of carbohydrates, proteins and lipids are biodegradable, non-toxic and environmentally sustainable, their mechanical properties, water resistance and antibacterial activity are generally poor, which have greatly limited their wide applications in food production (Youssef & El-Sayed, 2018). However, addition of nanomaterials could significantly improve their antibacterial activity, thermal, mechanical and barrier properties, while retain their biodegradable and non-toxic properties (Youssef et al., 2015; Youssef et al., 2017). In general, there are two types of nanomaterials which are added into biopolymer mass, i.e. nanofibers and nanoparticles as indicated in Table 2.2 Nanofibers are nanoscale materials with a fibrous morphology, while nanoparticles are discrete particles with all three dimensions in the nanoscale. The interaction between polymers and nanomaterials, whether nanofibers or nanoparticles, is primarily governed by intermolecular forces such as van der Waals forces, hydrogen bonding, and electrostatic interactions. The specific nature and strength of these interactions depend on the chemical composition and surface properties of both the polymer and the nanomaterial.

Table 2.2 Research progress of using nanomaterials in developing biodegradable packaging

Nanomaterials	Base Material	Mechanical property	Thermal property	Barrier property	Optical property	Antibacterial property	Reference
Chitosan nanofiber	Whey protein isolate/ emulsified cinnamon oil /nano-formulated CiEOSS	TS, Young's modulus (YM) increased and EB decreased		Increased water, oxygen and UV light barrier properties			Mohammadi et al., 2020
Bacterial cellulose nanofibers	Polyvinyl alcohol gel/ via ultrasonication treatment	TS, tensile modulus (TM) increased and EB decreased	Increased the thermal resistance	Increased water barrier property	Transparency decreases slightly with increased nanofiber		Abral et al., 2019
Cellulose nanofiber	Thyme essential oil/ whey protein isolate	TS, elastic modulus (EM) increased and EB decrease		Increased water and oxygen barrier properties.			Carvalho et al., 2018
Chitosan (CSNF) nanofiber/ ZnO nanoparticles (ZnONPs)	Gelatin	TS, YM increased and EB decreased		Increased the water barrier properties		The antimicrobial effect was measured on S. aureus E. coli P. aeruginosa	Amjadi et al., 2019
Cellulose nanofiber (CNF)/ TiO2	Rosemary essential oil/			CNF increased the water barrier properties			Alizadeh Sani et al., 2017

nanoparticles （TiO2 NPs）	whey protein isolate						
Cellulose nanofiber	Propolis ethanolic extract/ Zataria multiflora Bioss. essential oil/ poly-lactic acid	TS, EM increased and EB decrease					Rezaeigolestani et al., 2017
Cellulose nanofiber	Polydextrose incorporated whey protein isolate/ probiotic	TS increased and EB decreased		Increased the water barrier properties			Karimi et al., 2020
Chitin nanofiber/ bacterial cellulose nanofiber	Curcumin	TS and EB increased		Increased		The antimicrobial effect on S. aureus E. coli	Yang et al., 2020
Chitin nanofiber	Ajowan essential oil (AJEOSS)/ carboxymethyl cellulose/ Gelatin	TS increased and EB decreased		Increased		Supressing growth of S.aureus	Azarifar et al., 2019
TiO2 nanoparticles (TiO2-NPs)	Zein/ Sodium alginate/ Betanin		Increased			Supressing growth of S. aureus E. coli P. aeruginosa	Amjadi et al., 2019b

Rosin modified cellulose nanofiber (R-CNF)	polylactic acid/chitosan	TS, YM and EB increased		Inhibiting E. coli and B. subtilis.	Niu et al., 2017
ZnO nanoparticles (ZnO-NPs)	Polyvinyl alcohol (PVA) / Starch	TS, YM increased and EB decreased	Increased the water barrier properties	Inhibiting S. typhimurium	Jayakumar et al., 2019
Ag nanoparticles (Ag-NPs)	Polyvinyl alcohol (PVA)-Montmorillonite clayGinger extract		Increased water and light barrier properties	Inhibiting S. aureus, S. Typhimurium	Mathew et al., 2019
TiO2 nanoparticles (TiO2-NPs)	Polyvinyl alcohol (PVA)-chitosan	TS and EB increased	Increased water and gas barrier properties	Supressing E. coli, S. aureus, Salmonella, enterica, Listeria monocytogenes	Lian, Zhang, and Zhao, 2016

2.3.1 Enhancing mechanical property

As indicated in Table 2.2, nanomaterials have been added to natural polymers to enhance the mechanical property, including chitosan nanofiber, cellulose nanofiber, Chitin nanofiber, TiO2 nanoparticles, and Ag nanoparticles etc. Among them, cellulose nanofibers (CNF) are one of the most popular nanomaterials. CNF with flexibility, high stiffness and low density, can provide high surface area to interact with biopolymers to enhance their mechanical property (Deng, Jung, & Zhao, 2017). Therefore, CNF became a promising candidate material to act as reinforcing filler in biopolymer-based film (Niu et al., 2018). Shabanpour et al. (2018) reported that incorporating cellulose nanofibers into fish protein based edible film can significantly improve its tensile strength from 6 MPa to 8.94 MPa. Alizadeh Sani et al. (2017) found that the addition of 2.5% CNF could improve the mechanical property of edible film based on whey protein isolate (WPI) and reduce the surface roughness as well. The density increase of WPI matrix film due to the strong interaction between CNF could be the key reason for this improvement (Carvalho et al., 2018; Ribeiro-Santos et al., 2018). Rezaeigolestani et al. (2017) incorporated CNF into the PLA matrix film and found that the TS increased by 32% and EM increased by 19%, but the EB value did not change at all. Compared with vegetable sourced cellulose, bacterial cellulose nanofiber has gradually increased its popularity due to its purity. Abral et al. (2019) added bacterial cellulose nanofiber to the PVA-based film, and found that bacterial cellulose (BC) nanofiber has good dispersion property within PVA matric and it can increase in the number of hydrogen bonds between composite molecules, which leads to the increase in tensile strength (TS) and tensile modulus (TM). However, the compatibility between CNF with other composites has been one of the main obstacles hindering its application. For example, the hydrophilicity of CNF is not compatible with the hydrophobic PLA. So far, several chemical strategies have been put forward to adjust the compatibility between PLA and CNF, such as esterification (Espino-Perez, et al., 2014; Sato et al., 2016), salinization (Robles et al., 2015) and polymer grafting (Hatton et al., 2016; Navarro et al., 2015). Niu et al. (2017) showed adding rosin to the film based with CNF and PLA would form an ester group between the -OH group of CNFs and the -COOH group on the resin acid of the rosin. As a result, the compatibility of CNF and PLA and the mechanical properties of the composite film were greatly improved.

High volume ratio, biodegradability, easy production and high mechanical strength are the characteristics of chitosan nanofibers (CSNF) which determine its popularity in composite films. Mohammadi et al. (2020) shows that adding CSNF to the whey protein isolate (WPI) film can significantly increase the TS strength. They proposed that the cross-linking between WPI and CSNF, good miscibility and compatibility help reduce the mobility and elasticity of the base film, hence great improvement in mechanical properties are expected. Similar improvement in mechanical property was also observed in composite film containing gelatin (G), ZnONPs and CSNF. The interconnected porous structure studied by scanning electronic microscope provides useful evidence for the enhanced mechanical strength of the composite (Amjadi et al., 2019).

Metal based Nanoparticles including TiO_2, ZnO and Ag are also found useful in improving mechanical property of composite film. Sahraee et al. (2017b) reported that ZnONP could improve the mechanical property of gelatin-based nanocomposite films (GNCF). Similar improvement was also achieved by adding TiO_2 into Polyvinyl alcohol (PVA)-chitosan based film (Lian, Zhang & Zhao, 2016) and Zein/ Sodium alginate/ Betanin film (Amjadi et al., 2020), adding Ag in Polyvinyl alcohol-based film (Mathew et al., 2019), and adding ZnO in Polyvinyl alcohol (PVA) / Starch based film (Jayakumar et al., 2019). Amjadi et al., (2019) found that addition of CHNF and ZnONPs into gelatin film could develop a high dense and less permeable polymer network. The enhanced mechanical strength could be attributed to the increase in the intermolecular interactions between nanoparticle and other composites within the film matrix. A model used to disclose the interactions between nanoparticle and biopolymers is proposed by Abutalib and Rajeh (2020) that electrostatic charge and H bonding are the main contributors to stabilize the doped structure between nanoparticles and polymers and enhance the mechanical property of composite film. Lan et al (2021) reported that interfacial Ti-O coupling between apple pomaces extract (APE) and TiO2 nanoparticles enhanced the mechanical properties of the chitosan-TiO2-apple pomace film. In addition, nanoparticles have high mechanical strength, and easy dispersion within the polymer matrix, which may also contribute to the enhanced mechanical property. Abutalib and Rajeh (2021) proposed nanoparticles with high tensile characteristics are positioned themselves as the centre of the stress with extra stress shift from the main polymers, which consequently lead to the increase in Young's modulus, tensile strength, and stiffness of the Ag/TiO_2 / chitosan/ polyethylene oxide composite film. What’s more, there are some researche showing that mechanical properties are related to crystallinity. Amjadi et al. (2020) reported that adding TiO2NP to the zein/sodium alginate/betaine base film can significantly improve the mechanical properties. This may be due to the increase in crystallinity caused by the addition of TiO2NPs, and lead to the increase in TS. This is consistent with the results reported by El-Aassar et al. (2016) that introducing TiO2NPs into polyvinyl alcohol-based nanofibers increased the tensile strength.

However, the effects of nanomaterials on EB property of composite film are varied. Abral et al. (2019) showed that after adding bacterial cellulose (BC) nanofibers to the PVA-based film, the fiber dispersion of BC nanofibers led to an increase in the number of intermolecular bonds between BC nanofibers and PVA, thereby lead to improvement of TS and TM of composite materials. Although the TS increased, with the increase of BC fiber content, a reduction of elongation at break was observed by Abutalib and Rajeh (2021).

2.3.2 Improving the water barrier properties

The water vapor, oxygen and light barrier properties in packaging applications are very important, and good barrier properties can effectively extend the shelf life of products (Lagarón, 2011). Water vapor permeability (WVP) and water solubility (WS) are key properties to consider for food packaging because many chemical and physical deterioration reactions during food storage are directly related to water. Protein and polysaccharide based composite materials contain hydrophilic groups in its polymer chain, hence their inhibitory effect on water molecules is relatively poor. Therefore, incorporating functional groups and reducing hydrophilic sites within biopolymer films are useful to overcome this defect. Mohammadiet al. (2020) reported that by adding chitosan nanofiber

(CSNF) into whey protein isolate (WPI) based films, the WS value of the film was significantly reduced. This may be due to the close interaction between CSNF, WPI and glycerol leading to the blockage of reactive hydroxyl groups forming H bond with water, consequently, a densely structured composite film was formed. Similar result was also observed by Jafari et al (2016) that the incorporation of 2% chitin nanofibers in the chitosan film achieved the lowest WS index of the prepared composite material, the concentration of CHNF increased, and the solubility of the films also increased. The lower solubility at 2% CHNF content is likely due to strong intermolecular interactions between the CHNF nanoparticles and the polymer matrix, which limit water penetration and dissolution. FTIR spectroscopy has been popularly used to investigate interaction among the functional groups of the composites within biopolymer matrix, while a higher transmittance (T) value of O-H stretching in FTIR graph can indicate the increase in hydrophobicity of biopolymer films. Abral et al. (2019) pointed out that adding bacterial cellulose nanofiber into PVA matrix resulted in an increase of hydrophobicity due to the increase in the intermolecular hydrogen bonds between BC fiber and PVA matrix which was evidenced by an increased T value. Therefore, the diffusion of water molecular within the film was reduced.

Nanofiber may also work as filler to reduce the chance of water molecules to pass through the film, in another word, change the porosity. Azarifar et al. (2020) reported that increase in the concentration of chitin nanofiber (CHNF) could improve the film structure by reducing its porosity and increase the compactness of the film. As a result, the barrier properties to water and oxygen of the composite film can be improved. Hosseini et al. (2016) reported similar observation, and found chitosan nanoparticles could reinforce the water barrier property of fish gelatin films. But conflicting results were reported by Abdulkhani et al. (2014) that adding 1% cellulose nanofibers to the PLA matrix increased WVP by 2%. They claimed that the hydrophilicity of cellulose nanofibers may be responsible of the decrease in water barrier property. However, in the studies conducted by Rezaeigolestani et al., (2017) and Karimi et al., (2020), neutral effect of cellulose nanofiber on water vapor barrier were reported and they claimed that the negative effects are very small and could be neglected.

For metal ion-based nanoparticle, Amjadi et al. (2020) found that incorporated TiO_2NPs into Zein/sodium alginate film could improve the water barrier properties of the composite film which is consistent with the report by Sekar et al. (2019). The reason for this improvement is that inorganic nanoparticles reduce the interaction of biopolymers with water by forming the H-bonds. Hence, the surface hydrophobicity of composite film is increased.

2.3.3 Thermal properties

Enhancing the thermal properties of biofilms increases their durability, versatility, and applicability in environments requiring higher heat resistance, making them more suitable for both industrial and consumer use. Differential scanning calorimetry (DSC) is popularly used to measure the thermal property of composite film. Abral et al. (2019) reported that the addition of BC nanofibers to the PVA matrix film improved the thermal resistance due to the reduction of free OH groups in the bio-nanocomposite film and formation of new interfacial bonding between BC nanofiber and PVA matrix. Amjadi et al. (2020) drew similar conclusion when added TiO2NPs to zein based film. They claimed that adding TiO2NPs could encourage the formation of hydrogen bonds between zein and TiO2NPs and lead to the increase in the compactness of the polymer chain and the overall crystallinity. Therefore, many researchers believe the formation of cross-linked bonds between the various components of nanofibers would limit the mobility of polymer chains, thereby an improved thermal stability was expected (Nista et al., 2015; Torres-Giner et al., 2009).

2.3.4 Antibacterial properties

Generally, antimicrobial compounds can be divided into two main types, inorganic and organic materials. Enzymes, polymers and organic acids are the main organic materials, and metal oxides or metal nanoparticles are the main inorganic materials. The main difference between them is that inorganic materials have higher thermal stability than organic materials. Therefore, metal oxides and metal nanoparticles are more likely to withstand harsh processing conditions (Carbone et al., 2016; Gan and Chow, 2018).

Nanoparticles could form reactive oxygen species (ROS) which would affect the structure of biological molecule and damage enzymes involved in the cell metabolism and eventually lead to the death of bacteria, therefore, excellent antimicrobial activity is demonstrated (Abutalib and Rajeh, 2021). According to Table 2.2, zinc oxide nanoparticles (ZnONPs) are popular antibacterial composite which have been incorporated in variety of composite film. They have been approved by the U.S. Food and Drug Administration (FDA) and are generally considered safe (GRAS) (Noshirvani et al.2017). Amjadi et al. (2019) reported that chitosan nanofiber (CSNF) itself does not have any significant antibacterial activity, but can work synergistically with ZnONPs to inhibit the growth of microorganism. This result agrees with the findings of Almasi et al. (2018) that the positively charged amine groups in CSNF can interact with the anions of the microbial cell membrane. The addition of CSNF to ZnONP can enhance the penetration of Zn^{2+} ions and reactive oxygen species (ROS) produced by ZnONPinto the cell wall to react with the cytoplasm and kill the bacteria (Jahed et al. 2017). The antibacterial activity of ZnONP against gram-positive bacteria is higher than that of gram-negative bacteria because of the structural difference of the two types bacteria. For gram-positive bacteria, their cell walls are consisting of multiple layers of peptidoglycan (Shahmohammadi Jebel and Almasi 2016). However, there is a thin layer of peptidoglycan on the cell wall of gram-negative bacteria, and the outer membrane is mainly composed of lipopolysaccharide (Shankar et al. 2015). The outer membrane of gram-negative bacteria can limit the penetration of Zn^{2+} ions and reactive oxygen species (ROS) produced by ZnONP, thereby reducing the antibacterial ability of ZnONP (Espitia et al., 2012). Similar results are also reported in the composite film of bacterial cellulose (Shahmohammadi Jebel and Almasi 2016) and gelatin (Shankar et al. 2015) with ZnO nanoparticles incorporation.

Titanium dioxide nanoparticles (TiO_2-NPs) is another popular choice to be incorporated into composite films to improve their antibacterial activity (Karthikeyan et al., 2017; Montaser et al., 2019). Alizadeh Sani et al. (2017) reported that using nanocomposite films containing TiO2-NPs could reduce the total viable count (TVC) of food sample, because TiO_2 nanoparticles is primarily due to their ability to disrupt microbial cell membranes, altering their permeability and compromising the cell's function. This disruption allows the nanoparticles to penetrate the cell and damage essential biomolecules like DNA and proteins. Additionally, when exposed to light, TiO_2 nanoparticles produce reactive oxygen species (ROS), which further damage cellular components, ultimately leading to cell death (Ando et al., 2015; Jayaseelan et al., 2013). Lian, Zhang, and Zhao (2016) proved that adding TiO2NPs to Polyvinyl alcohol (PVA)/chitosan-based composite film could significantly improve inhibitory effects of composite film on *E. coli, S. aureus*, *Salmonella enterica* and *Listeria monocytogenes*. In addition, Mathew et al. (2019) reported that the silver nanoparticles have a significant inhibitory effect on *S. aureus* and *S. Typhimurium* when incorporated in the biodegradable film, but it has lower stability and volatility at elevated temperatures.

2.4 Essential oils used to enhance the quality of biodegradable film

Essential oils (EOs) are multi-component volatile oils, which can be extracted from various parts of plants, such as wood, bark, branches and roots, etc., while physical methods including pressing and distillation have been the main methods for its production (Silvestre et al., 2019; Vergis et al., 2013). EOS contain volatile terpenoids and/or non-terpenoids (Edris, 2007; Kumari et al., 2014) which are generally considered safe (GRAS) in the United States and can be used in food directly (Kim et al., 1995). Each EO is composed of many different compounds, containing 85–99% of volatile compounds and 1–15% of non-volatile compounds. The chemical composition of plant EOs depends on the type of plant species, its geographic location, maturity stage and the method of obtaining EOs (Nazzaro ei al.,2017). Aldehydes, terpenoids, esters, ketones, acids, alcohols and phenols are considered as the main compounds contained in EOs (Dini, 2016).

EOs has been widely adapted in the development of biodegradable packaging in recent years, and results show that EOs based antibacterial packaging can be used to extend the shelf life of meat, vegetable, fruits and dairy products (Han 2000; Surendhiran et al., 2020). Essential oils can destroy the structure of enzymes, phospholipids in cell membranes, and the genetic composition of microorganisms to achieve the antibacterial effect (Ruiz-Navajas et al., 2013). Essential oils also have excellent antioxidant properties, which are beneficial for storing oxygen sensitive food products, especially product with high fat level. Therefore, EOs have become more and more popular in recent years (Deng et al., 2020; Heredia-Guerrero et al., 2018). In general, application of essential oils in active packaging can be either in the form of films to cover the food or coating on the surface of food (Ribeiro-Santos et al., 2017). Table 2.3 summarizes the research progress about incorporating essential oils in the biopolymer film. As indicated in Table 2.3, effects of EOs on the physical and antibacterial properties of the biopolymer-based film vary differently.

Table 2.3 Research progress on the effect of essential oils on the quality of polymer film

Essential oil	Polymers	Function	Reference
Carvacrol	Polycaprolactone	Enhanced the antibacterial and antioxidant activities	Tampau et al. (2018)
Thyme EOs	Gelatin	Antibacterial activity against Campylobacter jejuni in chicken	Lin et al. (2018)
Cinnamaldehyde EOs	Zein	Antibacterial activity against the growth of bacteria on mushrooms	Shao et al., (2019)
Rosemary EOs	Zein	Antibacterial activity against S. aureus and E. coli	Amjadi et al., (2020)
Tea tree EOs	chitosan	Antibacterial activity against *Salmonella* spp. in chicken	Cui et al., (2018)

Oregano EOs	Starch	Enhanced the antimicrobial and antioxidant properties	Fonseca et al. (2019)
Cinnamon oil	Chitosan-gum	Enhanced the water barrier properties and antioxidant properties	Xu et al. (2019)
Oregano oil	Soy Protein	Enhance mechanical properties and water vapor barrier property; Antibacterial activity against E. coli and S. aureus	Dos Santos Paglione et al. (2019)
Laurel essential oils	Chitosan	Enhanced the antimicrobial properties; Extend the shelf life of pork	Wu et al. (2019)
Rosemary oil, mint oil	Chitosan pectin and starch	Reduced tensile strength and water barrier properties; Antibacterial activity against B. subtilis, E. coli and L. monocytogenes	Akhter et al., (2019)
Rosemary essential oil	Starch-carboxy methyl cellulose	Enhanced the water barrier properties; Antibacterial activity against S. aureus	Mohsenabadi et al. (2018)
Cinnamon essential oil	Polylactic acid	Antibacterial activity against E. coli and S. aureus; Extend the shelf life of pork	Wen et al. (2016)

2.4.1 Effect of EOs on the antibacterial properties of composite film

Fat solubility is the essential property of most EOs compounds which enable them to penetrate into the cell dermis and cell membranes easily. Thereby they exert a multi-channel antibacterial mechanism through the interaction with intracellular proteins, DNA, metabolism, enzymes, and energy production, etc. Phenolics are the most common antibacterial components in EOs, including thymol, cinnamaldehyde, carvacrol and eugenol, etc., and have significant antibacterial effects (Bhavaniramya et al., 2019). In addition, the antibacterial activity of EOs is related to the type of bacteria. According to research findings, the antibacterial effect of EOs on gram-positive bacteria is better than that of gram-negative bacteria because their cell walls are different. The cell wall of gram-positive bacteria is mainly made of peptidoglycan, but EOs compounds are hydrophobic molecules which allow them to easily penetrate into gram-positive bacteria (Trombetta et al., 2005). On the other hand, the peptidoglycan layer of gram-negative bacteria has an outer membrane composed of a double layer of phospholipids, which can prevent hydrophobic compounds from penetrating into the cell membrane, so gram-negative bacteria have stronger resistance to EOs (Nazzaro et al., 2013; Vaara, 1992). Sánchez-González et al. (2010) demonstrated that the composite films containing tea tree essential oil (TTO) were effective in delaying the growth of P. italicum. However, at lower concentrations of TTO, no significant antimicrobial activity was observed. A noticeable inhibition of fungal growth only occurred when the ratio of TTO to chitosan exceeded 1. In addition, Chen et al. (2020) found that the cellulose nanofiber packaging film infused with oregano essential oil (OEO) effectively prevented bacterial growth on its surface, achieving a 99.99% inhibition rate against *E. coli* and *L. monocytogenes*. This result highlights the critical role of OEO in significantly reducing the growth of these harmful bacteria, demonstrating its potent antimicrobial properties.

2.4.2 Effect of EOs on the antioxidant properties of composite film of EOs

Since EOs are considered as natural antioxidants, and most of them have non-toxic properties, using EOs instead of synthetic oxidants is becoming more and more popular (Mimica-Dukić et al., 2016). Zheng et al. (2019) reported that adding eugenol to the edible chitosan-based film significantly improved the antioxidant activity. The antioxidant activity of essential oils depends on their composition and antioxidant degradation ability. Antioxidant ability is usually related to the presence of compounds that may contain hydrogen atoms and hydroxyl (-OH) which can prevent the generation of new free radicals (Dima et al., 2014). Del

Ré and Jorge (2012) reported that the antioxidant activity in thyme essential oil is mainly due to the presence of the phenolic compounds including thymol and carvacrol, both of which contain an aromatic ring attached to the hydroxyl group.

2.4.3 Effect of EOs on the mechanical properties of composite film

Many studies showed that incorporating EOs in hydrophilic biofilms could reduce tensile strength (TS) and increase elongation at break (EB). The decrease in tensile strength is attributed to the formation of a discontinuous heterogeny EOs membrane structure (Benavides et al., 2012). Essential oils can break the strong interaction between polymer chains and result in weaker bonds, thereby mechanical resistance is reduced (Sanchez-Garcia et al., 2010; Shen and Kamdem, 2015). Yang et al. (2020) reported a reduced mechanical strength of composite film based on bacterial cellulose/chitin nanofiber when curcumin, a polyphenol compound, was added. However, research also showed that adding any EOs containing thymol or carvacrolcould could lead to a decrease in elastic modulus (EM) and TS but an increase in EB (reference missing). This may be because EOs help the movement of adjacent polymer chains and increase the flexibility of composite materials (Rhim et al., 2006). This phenomenon is called plasticization and it can make hard polymers (such as PLA) to be more flexible.

2.4.4 Effect of EOs on the Optical properties of composite film

From a market perspective, the optical properties of packaging films are very important and will affect consumers' choices of products (Abdollahi et al., 2012). The transparency of the film is affected by the internal structure of film formed during the drying process, the nature of the dispersion, the size of the dispersion, the amount and color of the added EOs and other factors (Fabra et al., 2009). Generally speaking, adding EOs will decrease the transparency of the film due to the difference in refractive index between film composites and the EOs (Sánchez-González et al., 2011; Wu et al., 2017), or the colorant compounds in the EOs (Tongnuanchan et al., 2013). Mohammadiet al. (2020) reported that adding cinnamon essential oil (4 wt% of WPI) to whey protein isolate/chitosan nanofiber composite film significantly reduced the transparency of the film. Chen et al. (2020) reported similar results that the transparency of cellulose nanofiber film was significantly reduced by adding the oregano essential oil (4 wt%, CNF based) film. However, conflict results were also reported. Sánchez-González et al., (2010) found that the incorporation of tea tree oil into the chitosan matrix increased the transparency of the film. They proposed that tea tree oil may destroy the original dense polymer network of the chitosan matrix and make the light easy to pass through, hence increase in optical property is expected.

2.4.5 Effect of EOs on the water barrier property of composite film

Since EOs are a hydrophobic compound that can reduce the water permeability, it is expected that the water barrier performance should be improved by adding EOs to biofilms (Sánchez-González, Cháfer et al., 2010). Silveira et al. (2020) reported that the WVP of the starch cellulose nanofiber-based films decreased with the level of tea tree essential oil increase in the film matrix. The decrease in WVP indicates the cohesion of the polymer chains formed, and the strong intermolecular interaction that creates obstacles for the diffusion of water vapor through the matrix. However, there are also many studies showings that EOs have no effect on WVP (Martucci et al., 2015). Resianingrum et al. (2016) reported that the introduction of lemongrass EOs has no significant effect on the WVP rate of the tapioca starch-based edible film. This can be explained by the high ratio of the hydrophilic component within the film matrix, while high water vapor transmission rate is the main feature of this film. Overall, the impact of EOs on the water barrier property not only depends on the hydrophobic-hydrophilic ratio of the film, but also on other factors, such as porosity and cracks, etc in the film structure. (Azadbakht et al., 2018).

2.4.6 Drawbacks and future trends of using EOs

When EOs are incorporated into food packaging, they can improve antioxidative and antimicrobial properties, but their use also poses some risks. Firstly, almost 90-95% of the essential oils are volatile components, and only 5-10% are non-volatile substances. The volatile components in EOs are easily affected by external conditions such as air, heat, light and radiation, which greatly restrict their application (Zhu et al., 2020). Secondly, EOs are oxidizable. For example, monoterpene is the main compound responsible for the antioxidation of EOs, but it is easily oxidized after contact with air (Sedaghat Doost et al., 2020). Moreover, most EOs have a strong aromatic odor. Therefore, using EOs as composite agents in a food packaging may mask or interfere with the original odor of the food and affect consumer acceptance.

In order to avoid the loss of EOs caused by high volatility, heat, light sensitivity and other poor characteristics and improve their stability, nano emulsification and encapsulation techniques have been used to overcome these problems. Lin et al. (2019) reported that using nano-encapsulation technology including chitosan nanoparticles and gelatin nanofibers can effectively embed Moringa oil in the composite film, and prevent the loss of active ingredients. Wen et al. (2017) incorporated cinnamon essential oil/β-cyclodextrin inclusion complex into polylactic acid nanofibers through electrospinning technology and found the negative impact of EOs on foods were greatly reduced.

2.5 Conclusions

The development of nanotechnology will have a huge impact on food packaging. This review discussed the research progress of food packaging containing natural polymers, nanomaterials and essential oils in details, and critically evaluated the relationship between film composites and their physical and chemical properties formation from the perspective of chemical structure. According to the mechanical properties, barrier properties, antibacterial properties and other properties of the biodegradable composite film, nanomaterials and essential oils could help overcome the weakness in mechanical strength and offer anti-bacterial / anti-oxidation benefits which would accelerate the further application of biodegradable packaging in food production. However, the laws and regulations about nanomaterials used in biodegradable packaging are different in each country, which would create barrier for its further application. Therefore, more toxicological studies associated with nanomaterials application in developing biodegradable packaging are needed. In addition, production of composite films is also involved with high economic costs. Extracting biopolymers from food waste and converting them into biodegradable packaging materials could be an effective strategy to reduce the production cost and make the food production system sustainable.

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Chapter 3

Improving the quality of hordein based biofilm using composite plasticizers.

Chapter 3: Improving the quality of hordein based biofilm using composite plasticizers.

Abstract

Hordein, a protein extracted from beer production by-products, was used to prepare hordein-based films via the casting method. The films were plasticized with individual plasticizers—polyethylene glycol (PEG) and glycerol (GLY)—as well as composite plasticizers with varying PEG and GLY ratios. The films were evaluated for their mechanical properties, transparency, and water barrier properties. Scanning electron microscopy (SEM) and Fourier-transform infrared spectroscopy (FTIR) were employed to analyze their morphology and chemical structure. The results showed that glycerol significantly improved the mechanical properties of the films, enhancing both tensile strength (TS) and elongation at break (EB) compared to films plasticized with PEG alone. On the other hand, PEG alone notably increased the water barrier properties of the films compared to glycerol. The composite plasticizers exhibited a synergistic effect, with the film containing 25% PEG and 75% GLY showing optimal performance, featuring a more compact structure, higher TS, and improved EB compared to films with single plasticizers.

Keywords: Hordein, glycerol, PEG400, composite plasticizers, film properties, packaging.

3.1 Introduction

Environmental pollution from oil-derived plastic materials is one of the most severe issues of this century. Consequently, replacing conventional petroleum-based polymers with biodegradable alternatives derived from renewable resources is a promising solution to solve this problem (Porta, 2019). There are three major types of biodegradable materials commonly used for food packaging: polysaccharides, lipids, and proteins (Paramawati et al., 2001). Different from polysaccharides and lipids, proteins have a large number of functional groups to be reacted with, which makes it possible to meet demand in various applications (Huo et al., 2018). However, a significant drawback for biopolymer-based packaging materials is that they don't often offer sufficient mechanical properties to meet the industrial application, and most protein-based films exhibit poor water vapor barrier capacity and high sensitivity to moisture, causing films to disintegrate upon contact with water due to their high hydrophilicity (Azeredo & Waldron, 2016; Zink et al., 2016). Therefore, enhancing the hydrophobic properties and mechanical strength of protein-based packaging materials is highly desired for food packaging (Reddy & Yang, 2010).

Extracting hordein from the by-products of beer production was considered as a valorization process to reduce food waste and improve the sustainability of food production. Similar to wheat protein, hordein possesses attractive properties, including good cohesion, elasticity, and film-forming abilities (Olivato et al., 2012). Additionally, according to the Goldman-Engelman-Steitz (GES) scale, one-third of the amino acids in hordein are hydrophobic, with high levels of Leu, Val, Phe, and Tyr (Xia et al., 2011). This hydrophobicity gives hordein good water barrier properties, making it a promising biodegradable material for food packaging applications.

However, like other protein-based films, hordein-based films have limited application in flexible packaging because of low tensile strength and elonagtion break (Cho & Rhee, 2009). To improve the flexibility and overcome the brittleness of films, plasticizers are typically added to soften the structure (Cao et al., 2009; Gennadios et al., 1996). The effectiveness is dependent on the composition, size, and shape of the plasticizer used (Sothornvit & Krochta, 2001). In addition, research about barley protein has been limited to the chemical composition, protein quality, and amino acid composition of barley, but only limited studies have been reported about the properties of hordein-based films. However, hordein is similar to zein protein, Ethers (Gorrasi and Vertuccio, 2016), long-chain fatty acids (Erickson et al., 2014; Xu et al., 2012), and glycerol (GLY) (Parris and Coffin, 1997, Xu et al., 2012) are the most common plasticizers and exhibit a diverse effect on zein film plasticizations. In recent years, efforts to enhance plasticizing efficiency have led to the use of combined plasticizers to explore potential synergistic effects. For instance, Xu et al. (2012) found that the glass transition temperature (Tg) of zein film was decreased by a combination of glycerol and oleic acid, while mechanical testing indicated that there was a slight increase in the EB. Huo et al. (2018) reported using a 1:1 ratio of GLY and PEG, the plasticized zein films showed good elongation at break at 0.45 g plasticizer/g zein.

Although hordein has great potential as a biodegradable material for both food and pharmaceutical applications, limited research has been reported on hordein. In this study, hordein films were prepared and treated separately with polyethylene glycol (PEG) and glycerol (GLY), as well as with blended plasticizers at different ratios of PEG and GLY, to investigate the effects of various plasticizers on the quality of hordein films. The composite films were evaluated in terms of mechanical properties, optical properties and water permeability properties. Fourier-transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM) were also employed to understand the chemical and microstructural changes. The primary aim of this work was to optimize the usage of plasticizers to produce high quality hordein film for food application.

3.2 Material and methods

3.2.1 Raw materials

Hordein was extracted from barley flour (Buy Wholefoods Online, Kent, UK) using a 75%

aqueous ethanol solution according to the methods described by Wang et all (2010).

Barley flour was mixed with hexane (≥95%) at room temperature, using a flour-to-hexane ratio of 1:4 (w/v) for 4 hours, followed by centrifugation at 7000xg at 24℃ for 30 minutes. The sample was then air-dried overnight. Defatted flour was washed with 0.1 M NaCl solution using a magnetic stirrer (FOUR E'S SCIENTIFIC) at 600 rpm for 30 minutes. After washing, the solution was centrifuged at 6000xg for 20 minutes to remove albumin, globulin, and gliadin. This process was repeated twice. The same steps were then followed using deionized water, washing and centrifuging twice to remove residual NaCl. The precipitate was added to 75% ethanol at room temperature for 1.5 hours, and centrifugated at 8000xg for 30 minutes. The supernatant containing hordein residue was filtered using filter paper (Whatman 4) to remove impurities. A vacuum rotary evaporator was used to partially evaporate the ethanol at 50°C for 2.5 hours. The resulting solution was stored at 4°C overnight for cold precipitation. Hordein protein was isolated through centrifugation at 8500xg for 15 minutes at 4°C. The precipitate was then freeze-dried using freeze-drier (CHRIST-Beta 1-8 LCS plus) for 4 days and stored for further use.

The yield of the extracted hordein was approximately 5.8%. The characterization of the hordein extract was conducted using the Kjeldahl method, and the protein content of the extract is 84.4%, which is consistent with the result reported by Qazanfarzadeh et al. (2021). Glycerol (≥99.0%) and PEG400 were both purchased from Sigma Chemical Co. (UK).

3.2.2 Film production

The extracted hordein powder 5g was dissolved in 20ml of 75% ethanol solution at room temperature, followed by the addition of a designated plasticizer and stirring for 15 minutes. Glycerol was used as a plasticizer at concentrations of 25%, 30%, and 35%. Similarly, PEG was used at the same concentrations 25%, 30%, and 35%. For the composite plasticizer, a total of 30% was used with varying ratios of 25%PEG75%GLY, 50%PEG50%GLY and 75%PEG25%GLY. All the levels were calculated based on the weight of hordein. 6ml of the mixture was then poured into glass Petri dishes and placed in a vacuum oven at 40℃ without vacuum for 24 hours before peeling off the film. The films were conditioned at 57%

RH and 20℃ for 48 hours before measurement.

Glycerol and PEG400 are both non-toxic, inexpensive, and effective plasticizers. Glycerol's simple structure and high water solubility enable it to degrade rapidly, especially in water and soil, where microorganisms accelerate the process, often completing it within a few days. In contrast, PEG400 is biodegradable but breaks down more slowly. Microorganisms produce enzymes that degrade PEG400 into smaller molecules, eventually forming water and carbon dioxide. The biodegradability of PEG varies with molecular weight, generally decreasing as molecular weight increases. Low molecular weight PEG, such as PEG400, exhibits better biodegradability (Otal et al., 1997). In Experiment 1, hordein films were treated with varying concentrations of the single plasticizer glycerol (GLY) and evaluated for their mechanical properties, optical properties, water permeability, FTIR, and SEM analysis. In Experiment 2, hordein films were treated with different concentrations of the single plasticizer PEG and similarly assessed in terms of mechanical properties, optical properties, water permeability, FTIR, and SEM. In Experiment 3, based on the optimal total plasticizer content determined from Experiments 1 and 2, hordein films were treated with different ratios of GLY and PEG, and evaluated for their mechanical properties, optical properties, water permeability, FTIR, and SEM analysis.

3.2.3 Thickness and moisture content

The thickness of the films was measured with an electronic digital micrometer with a sensitivity of 0.001 mm. At least 10 random spots were chosen for measurement. The obtained thickness was further used to calculate WVP and transparency.

Moisture content was determined according to the method described by Zahedi, Fathi-Achachlouei, and Yousefi (2018). The weight of film sample (2 cm × 2 cm) was recorded before (*Wi*) and after (*Wd*) drying at 105 °C in oven for 24 h and weight loss (%) was calculated as the moisture content according to the following equation:

$$\text{Moisture content (\%)} = [(Wi - Wd)/Wi] \times 100$$

Wi – Initial weight of film before drying

Wd- weight of film after drying

3.2.4 Optical properties:

3.2.4.1 Transparency

The opacity of the films was measured using a NIR-VIS-UV Cary7000 spectrophotometer (Agilent Technologies, Santa Clara, US) at wavelengths from 200 to 800 nm, and three readings were taken per specimen. Transparency value was calculated using the equation provided by Han & Floros (1997):

$$\text{Transparency value}(\text{mm}^{-1}) = -\log T_{600}/\text{d}$$

T_{600} is the fractional transmittance at a wavelength of 600 nm; d is the thickness(mm) of the film. The higher value of transparency value presents the lower film transparency.

3.2.4.2 Color measurement:

The color properties of the films were determined using a CR-400 Chroma Meter (Konica Minolta, Japan) based on the CIELab color space, evaluating lightness (L*), red/green (a*), and yellow/blue (b*) against a white background (L* = 94.1, a* = 0.49, b* = 4.13). Data were gathered from 5 measurements at different points on the film.

3.2.5 Mechanical properties:

Tensile strength (TS) and elongation at break (EB) were measured using a TA. XT2i Texture Analyzer (Stable Microsystems, Godalming, UK) according to the method described by Patzsch et al. (2010). Samples were conditioned at 57% relative humidity for 48 hours before the measurement. Each film was cut into strips (Length 0.80 cm × width 0.50 cm) and fixed with the clamps. The test was conducted using a 30 kg load cell at 50 mm/min primary grip breaking speed for tensile strength and elongation at break, data were gathered from 5 strips of each film.

3.2.6 Water vapor permeability (WVP):

Films were conditioned at 57% relative humidity for 72 hours before the measurement. A piece of film was sealed on top of individual glass vials (The bottle has a bottom diameter of 4 cm, a height of 7 cm, and an open mouth with a diameter of 1.58 cm) filled with silica gel, and the vials were placed in a desiccator containing a saturated solution of potassium sulfate (98% RH). The vials' weights were recorded every 24 hours for 7 days. WVTR was the slope of linear regression curve of weight gain and was used to derive WVP using the equation:

$$\text{WVTR } (g/m^2 \cdot day) = \text{slope/area}$$

$$\text{WVP}(g.mm/\boldsymbol{m^2}.d.kPa) = (\text{WVTR x L })/\Delta P$$

WVTR ($g/m^2 \cdot day$): Water Vapor Transmission Rate. Slope (g/day): The rate of weight gain or loss in the test. Area (m^2): The surface area of the film exposed to water vapor transmission. L is thickness(mm), ΔP is difference in water vapor partial pressure(kPa) between both sides of the film, respectively.

3.2.7 Fourier Transform Infrared Spectrometer (FTIR)

To study the chemical structures and interactions between components, the FTIR spectra of films were obtained using an FTIR spectrophotometer (Spectrum 100, PerkinElmer, US) with an Attenuated Total Reflectance (ATR) attachment and selecting a wavenumber resolution of 4 cm^{-1} for 16 scans from 4200 to 650 cm^{-1}.

3.2.8 Scanning electron microscope (SEM)

Small square sections were cut from the main film and were attached to an aluminium SEM stub using a double-sided sticky, conducting carbon tab and coated with gold for 2.5 mins at a setting of approximately 0.9kV at ~30mA using an Edwards S150B gold coater. Scanning electron microscopy (FEI SEM, Thermo scientific, US) was performed on a Quanta 600 equipped with a field emission gun. Samples were examined at accelerating voltages of [X] kV under [Y] vacuum conditions. The SEM image was captured with magnification at 2000x.

3.2.9 Statistical analysis

Overall, there were 3 factors including GLY, PEG and ratio of GLY and PEG involved in this experiment, each factor had 3 levels. Therefore overall, there were 9 treatments, and each treatment had 3 replicates. The data for each factor was analyzed individually using one-way ANOVA (IBM SPSS Statistics 28.0.0.0 software, UK). Duncan's multiple comparison test was used to determine significant differences between means at significance level of 0.05.

3.3 Results and discussion

3.3.1 Experiment 1-Effect of GLY on the quality of hordein film

3.3.1.1Thickness and moisture content

Thickness can affect the mechanical and barrier properties of films (Fundo et al., 2015). As shown in Table 3.1, the thickness of the hordein-glycerol films in this study varied with the concentration of glycerol. The film with 35% glycerol exhibited the highest thickness (69.33 ± 13.79 µm), which was significantly different from the films with 25% and 30% glycerol (64.44 ± 16.63 µm and 62.79 ± 13.23 µm, respectively). This finding contrasts with reports by Arfat et al. (2014) and Chang and Nickerson (2013), who found that increasing the glycerol concentration during film preparation did not significantly affect thickness ($p > 0.05$). This discrepancy might be attributed to differences in the film-forming materials or preparation methods. However, our results align with observations by Nuthong et al. (2009) who reported a positive correlation between glycerol concentration and film thickness in porcine plasma protein-based films. This thickness increase could be attributed to glycerol's ability to absorb moisture and disrupt the polymer chain network, creating expanded structures with water molecules (Lim et al., 2020).

According to Table 3.2, the moisture content of hordein film with GLY addition at 25, 30 and 35% ranges from 5.92% to 7.02% which are much lower than that of hordein/chitosan film (15.03%) (Cheng et al, 2022). The low moisture content could be attributed to the hydrophobicity of hordein protein and the engagement of binding site of -OH from glycerol, which leave much less binding site for water to attach to the hordein molecule. As a result, much lower moisture content would be expected. Xu et al (2024) reported the range of moisture content for zein composite films is from 10-17.5% vs 25-28% whey protein composite films (Kontogianni, et al, 2022), which further confirm that the protein with strong hydrophobicity would have much lower moisture content. In general, glycerol is a hydrophilic plasticizer, so the moisture content of films is expected to increase with higher glycerol content. However, as shown in Table 3.2, the moisture contents are not significantly different among all the films. This may be explained that limited hydrophilic binding sites are available for water to form H bonding, and varying glycerol content did not alter the moisture content of the composite films significantly.

Table 3.1 Thickness and color of the hordein-glycerol films.

	Thickness(μm)	a*	b*	L*
25%GLY	64.44 ± 16.63^a	-1.88 ± 0.24^a	28.27 ± 3.28^a	86.51 ± 1.38^a
30%GLY	62.79 ± 13.23^a	-1.67 ± 0.32^b	28.33 ± 2.79^a	86.05 ± 1.62^a
35%GLY	69.33 ± 13.79^b	-1.64 ± 0.20^b	26.56 ± 2.04^a	86.54 ± 0.74^a

Means with different letter in the same column are significantly different (P<0.05).

Table 3.2 Moisture content and WVP of hordein-glycerol films.

	Moisture content (%)	WVP(g.mm/m^2.d.kPa)
25%GLY	6.69 ± 0.87^a	12.55 ± 0.61^a
30%GLY	7.02 ± 0.94^a	12.47 ± 1.45^a
35%GLY	5.92 ± 0.62^a	18.66 ± 0.99^b

Means with different letter in the same column indicate significantly differences between groups (P<0.05).

Table 3.3 Light transmittance and transparency values of hordein-glycerol films.

	Transmittance (%) at wavelength (nm)								Transparency
	200	280	350	400	500	600	700	800	Value (mm^{-1})
25%GLY	0.33	0.77	22.77	52.68	78.07	87.22	89.72	90.72	0.92 ± 0.08^{a}
30%GLY	0.35	0.90	30.11	58.50	80.08	87.73	89.78	90.67	0.86 ± 0.19^{a}
35%GLY	0.39	1.29	33.93	61.04	80.93	87.87	89.75	90.50	0.82 ± 0.15^{a}

Means with different letter in the same column indicate significantly differences between groups (P<0.05).

3.3.1.2 Optical properties

3.3.1.2.1 Transparency

The light transmission (%) and transparency of the hordein-based films are shown in Table 3.3. The films restricted the transmission of UV light in the range of 280–200 nm, and the transmission of UV light was found to be negligible at 280-200 nm for all films, which indicates the films prepared from hordein showed a great barrier property for UV light transmission, could prevent light transmission in the UV range. According to the Goldman-Engelman-Steitz (GES) scale, approximately one-third of the amino acids in barley protein are hydrophobic, with high levels of leucine, valine, phenylalanine, and tyrosine (Xia et al., 2011). Notably, amino acids such as tyrosine, phenylalanine, and tryptophan are known for their strong UV absorption properties (Guerrero et al., 2011), which likely contributes to the excellent UV barrier properties observed in the films.

The results suggest that, within the tested concentration range (25-35%), glycerol does not appear to substantially influence the transparency of hordein films. This observation aligns with several studies on different protein-based films. For instance, Arfat et al. (2014) found no significant differences in transparency values between films with 30% and 50% glycerol. Similarly, Nemet, Šošo, and Lazić (2010) reported that glycerol content did not affect the transparency of chicken breast protein edible films. Furthermore, Vanin et al. (2005) observed that both plasticizer type and concentration had no effect on the transparency of pig skin gelatin films. However, it's worth noting that Gontard et al. (1994).

reported increased film transparency with increasing glycerol concentration, attributing this to glycerol's inherent transparency and its ability to enhance dispersion within the film matrix. This discrepancy might stem from differences in the film-forming materials, the range of glycerol concentrations tested, or specific film preparation methods.

3.3.1.2.2 Color

The color parameters of the films are represented by a* (red/green hue), b* (yellow/blue hue), and L* (lightness hue). As shown in Table 3.1, a significant difference in a* value was observed between 25% glycerol film and the films with 30% and 35% glycerol. Specifically, the a* values for 25% glycerol (-1.88 ± 0.24) was lower than these with 30% and 35% glycerol (-1.67 ± 0.32 and -1.64 ± 0.20, respectively). This indicates that films with higher glycerol content move towards red hue. However, there are no significant differences in b* (yellow/blue) and L* (lightness) among the films, suggesting that glycerol concentration has a minor effect on these color properties.

3.3.1.3 Mechanical properties

The mechanical properties test assesses the tolerance of films to different stresses during handling and application, which is crucial for evaluating packaging materials (Nurul Syahida et al., 2020). Tensile strength (TS) and elongation at break (EB) were measured to determine the mechanical properties of films with varying glycerol concentrations. Due to the three hydroxyl groups present in glycerol, it is expected to form extensive hydrogen bonds with protein molecules at amino, amide, carboxyl, and hydroxyl sites (Xia et al., 2011).

As depicted in Table 3.4, the films with 25% and 30% glycerol exhibited comparable TS values, indicating that up to 30% glycerol effectively maintains the film's strength. However, the film with 35% glycerol showed a significant decrease in TS. This decrease is likely due to the excess plasticizer disrupting the protein network and weakening the film structure. This observation is consistent with the findings of Valderrama Solano & Rojas de Gante (2014) who reported a similar trend of decreasing TS with increasing glycerol content (30-40%) in blue corn flour films. They attributed this decrease in TS to the plasticizing effect of water. Glycerol, being hygroscopic, increases the moisture content of the films. As water is a powerful plasticizer in starch- and protein-rich materials, the increased moisture content further plasticizes the films, leading to lower tensile strength. This explanation is supported by Dias et al. (2010) who also observed a decrease in tensile strength, in starch films with higher moisture content.

The elongation at break of the hordein-glycerol films is depicted in Table 3.4. The films with 30% and 35% glycerol exhibited significantly higher EB compared to the film with 25% glycerol, indicating improved flexibility and extensibility. This result aligns with the general understanding that plasticizers can enhance flexibility and extensibility by interacting with protein chains and reducing intermolecular forces. The molecular structure of glycerol, which possesses a small chain, allows it to enter the polymeric network more easily, further increasing its effectiveness as a plasticizer (Cuq et al., 1997). This, coupled with its ability to increase water content, contributes to the observed increase in EB. Valderrama Solano & Rojas de Gante (2014) also reported a similar trend of increasing EB with increasing glycerol content in blue corn flour films.

The film with 30% glycerol appears to achieve an optimal balance, providing enhanced flexibility without compromising tensile strength. This optimal balance at 30% glycerol might be related to the formation of favorable interactions with hordein chains at this specific concentration, as suggested by the smooth and homogenous surface morphology observed in SEM images (Figure 3.2).

Table 3.4 Mechanical properties of hordein-glycerol films. All films are derived from film-forming solutions containing 30% plasticizers in total.

	Tensile strength (KPa)	Elongation at break (%)

25%GLY	126.30 ± 20.30^{a}	103.75 ± 0.33^{a}
30%GLY	139.25 ± 9.81^{a}	105.50 ± 0.35^{b}
35%GLY	110.44 ± 12.88^{b}	105.35 ± 0.91^{b}

Means with different letter in the same column indicate significantly differences between groups (P<0.05).

3.3.1.4 Water vapor permeability (WVP)

WVP, a measure of water diffusion, is an important attribute to reflect the water controlling property of packaging materials between food and its surrounding, in general, low WVP is desired for packaging materials as it can help retain the quality of food products. As shown in Table 3.2 the water vapor permeability (WVP) of hordein films increased with the glycerol content increased from 30% to 35%. Similar results were previously reported by Xia et al. (2011), who also observed that higher glycerol content increased water vapor permeability of barley protein films This increase can be attributed to the hydrophilic nature of glycerol. An effective plasticizer, which interacts with hordein polymer chains and reduces the intermolecular force between them, making it more flexible and less dense the increased flexibility and porosity would increase the chance for water molecules to pass through the film, consequently a high WVP is expected. Of course, the hydrophilic property of glycerol would also increase the chance of interaction with water, facilitating the absorption and transport of water through the film. Kovtun et all (2024) reported that 36% glycerol addition could disrupt the crystalline structures and increase the amorphous region of biopolymer, but 22% glycerol did not penetrate the crystalline of biopolymer, and only fostered the reorganization of the amorphous regions. The interaction between glycerol at different levels and biopolymers provided useful support to the change of WVP at different level of glycerol.

3.3.1.5 Fourier transform infrared spectroscopy (FTIR)

FTIR was used in this study to investigate the interactions between the GLY and hordein in the formed hordein film. The FTIR spectra of hordein films with 25%, 30%, and 35% glycerol are shown in Figure 3.1. Distinct changes in the spectra were observed as the glycerol concentration increased. For the 25% GLY sample, key peaks were observed at 3272 cm^{-1} and 2934 cm^{-1}, attributed to O-H/N-H stretching and C-H stretching vibrations, respectively (Kohsari et al., 2016). The Amide I band at 1651 cm^{-1}, associated with C=O stretching, and the Amide II band at 1539 cm^{-1}, representing N-H bending and C-N stretching, reflect the characteristic secondary structure of the protein matrix (Li et al., 2021). The sharpness of these peaks suggests a well-ordered protein structure with limited plasticization. As the glycerol content increased to 30%, the intensity of the 3272 cm^{-1} peak increased slightly, indicating enhanced hydrogen bonding between glycerol and the protein molecules (Casariego et al., 2009). Similarly, the 2934 cm^{-1} peak increased in intensity, suggesting enhanced molecular flexibility due to increased alkyl chain interactions. The Amide I and II bands showed slight broadening at 1651 cm^{-1} and 1539 cm^{-1}, respectively, reflecting a disruption of the protein's ordered structure as glycerol induces plasticization, thereby reducing protein-protein hydrogen bonds and increasing the flexibility of the film. In the 35% GLY sample, these effects were further pronounced. The 3272 cm^{-1} peak showed a marked increase in intensity, signifying extensive hydrogen bonding between glycerol and the protein matrix. The 2934 cm^{-1} peak continued to rise in intensity, further indicating increased molecular mobility. The Amide I and II bands broadened significantly, suggesting that the protein structure becomes more disordered with higher glycerol content. This broadening of the Amide peaks points to a greater disruption of the hydrogen bonding network within the protein structure, leading to a more amorphous and flexible film.

Quantitative analysis showed a progressive increase in the intensity of the O-H/N-H and C-H stretching peaks, as well as significant broadening of the Amide I and II bands, as glycerol content increased from 25% to 35%. These changes indicate that glycerol acts as a plasticizer, weakening the protein-protein interactions and introducing greater flexibility into the film. The results suggest that higher glycerol concentrations promote more extensive hydrogen bonding between glycerol and protein molecules, leading to a more pliable film with less structural order. Thus, the FTIR data confirm that glycerol significantly affects the molecular structure and properties of hordein-based films, with increasing glycerol concentrations leading to greater plasticization and structural flexibility. The observed surface morphology of the 35% GLY film aligns with the FTIR analysis, which showed a further broadening of the Amide bands and a significant increase in hydrogen bonding between glycerol and the protein matrix. These findings suggest that higher glycerol content disrupts the protein structure, leading to a more amorphous film.

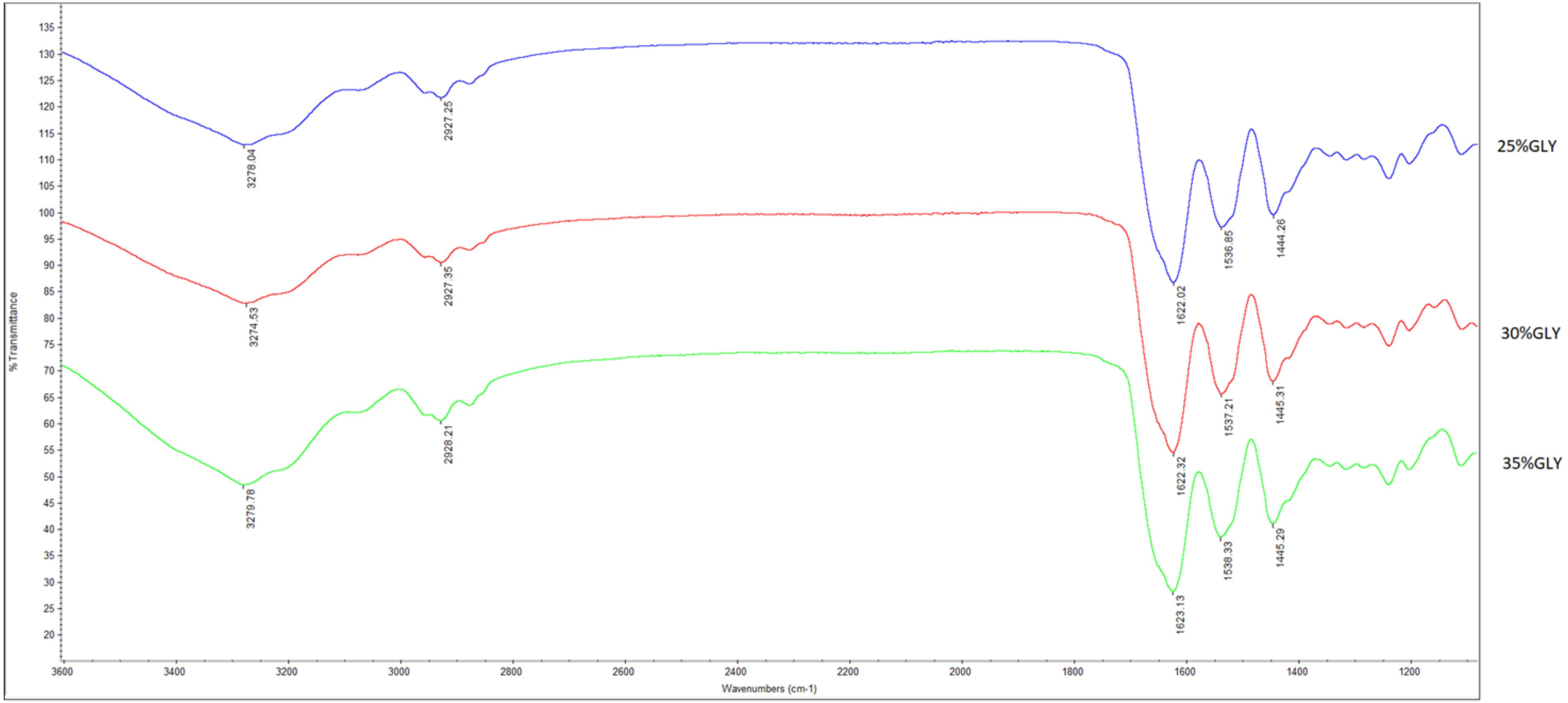

Fig. 3.1 Comparison of FTIR spectra of hordein with different concentrations of glycerol composite films.

3.3.1.6 Scanning electron microscope (SEM)

Figure 3.2 presents the SEM images of hordein-glycerol films with varying glycerol content. At 25% GLY, the film surface appears slightly rough. As glycerol content increases to 30%, the surface becomes smoother. This observation correlates with the FTIR spectra analysis (Figure 3.1), which revealed a broadening of the Amide I and II bands and increased intensity of the O-H/N-H and C-H stretching peaks, indicating stronger glycerol-protein interactions. However, at 35% GLY, the surface exhibits pronounced roughness and visible aggregate. This phenomenon might be attributed to the significant phase separation induced by the polarity difference between hordein aggregates and the solvent during film drying (Gao et al., 2022). Additionally, glycerol's ability to disrupt hydrogen bonds between protein molecular chains, as observed in zein films (Gao et al., 2022), could further contribute to the increased roughness and aggregate formation in the 35% GLY hordein films. This observation is different from the findings of Boyacı et al. (2019), who reported porous cross-sections in zein films plasticized solely with glycerol. However, in our SEM analysis, no such porosity was observed. This difference may be attributed to the fact that, although both hordein and zein are prolamins, hordein appears to be more compatible with glycerol, leading to a more uniform, less porous structure (Mohamed et al., 2022).

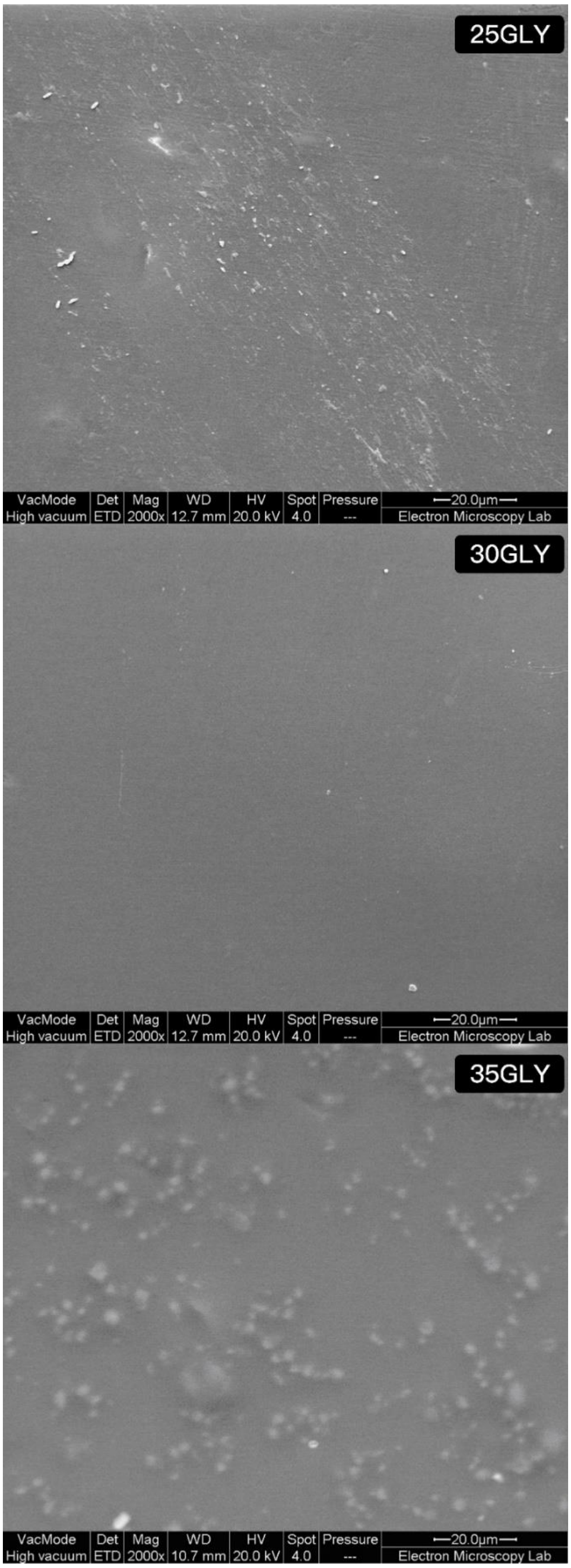

Fig. 3.2 SEM micrographs of hordein film surface with 25%, 30% and 35% GLY.

3.3.2 Experiment 2- Effect of PEG on the quality of hordein film

3.3.2.1 Thickness and moisture content

The thickness of hordein-PEG films exhibited a positive correlation with PEG concentration. As shown in Table 3.5, while films with 25% PEG (45.44 ± 13.83 μm) and 30% PEG (45.58 ± 14.36 μm) displayed similar thicknesses, a statistically significant increase was observed at 35% PEG (57.00 ± 16.92 μm). This indicates a threshold concentration beyond which PEG has a more pronounced impact on the film structure. Similar findings were reported by Pongjanyakul & Puttipipatkhachorn (2007), who observed a comparable effect of plasticizers on film properties. As a plasticizer, PEG disrupts protein-protein interactions, increasing chain mobility expanding the protein network, and increasing molecular volume (Ili Balqis et al., 2017). Additionally, specific interactions between PEG and hordein, such as hydrogen bonding, may modify protein packing, resulting in a greater film volume (Qussi and Suess. 2005).

Table 3.6 shows that the moisture content of films with 25% and 30% PEG did not exhibit significant differences, but the film with 35% PEG had a higher moisture content. This is likely because PEG is a hydrophilic plasticizer, and a higher concentration of PEG would increase the moisture content of composite films by attracting and retaining more water molecules.

Table 3.5 Thickness and color of the hordein-PEG films.

	Thickness(μm)	a*	b*	L*
25%PEG	45.44 ± 13.83^{a}	-1.17 ± 0.19^{a}	20.07 ± 4.10^{a}	88.25 ± 1.53^{a}
30%PEG	45.58 ± 14.36^{a}	-1.20 ± 0.13^{a}	21.74 ± 2.40^{a}	88.14 ± 2.19^{a}
35%PEG	57.00 ± 16.92^{b}	-1.13 ± 0.20^{a}	19.89 ± 3.39^{a}	88.13 ± 1.34^{a}

Means with different letter in the same column indicate significantly differences between groups (P<0.05).

Table 3.6 Moisture content and WVP of hordein-PEG films.

	Moisture content (%)	WVP(g.mm/m^2.d.kPa)
25%PEG	5.88 ± 0.64^{a}	5.96 ± 0.88^{a}
30%PEG	5.93 ± 0.42^{a}	6.27 ± 1.29^{a}
35%PEG	7.61 ± 1.34^{b}	9.66 ± 0.95^{b}

Means with different letter in the same column indicate significantly differences between groups (P<0.05).

Table 3.7 Light transmittance and transparency values of hordein-PEG films.

	Transmittance (%) at wavelength (nm)								Transparency Value (mm^{-1})
	200	280	350	400	500	600	700	800	
25%PEG	0.27	0.74	17.75	47.94	76.09	86.45	89.57	90.89	1.40 ± 0.13^{a}
30%PEG	0.33	0.93	24.57	54.57	79.09	87.55	90.03	91.07	1.23 ± 0.06^{ab}
35%PEG	0.45	1.38	38.17	64.16	82.49	87.86	90.39	91.14	0.99 ± 0.22^{b}

Means with different letter in the same column indicate significantly differences between groups (P<0.05).

3.3.2.2 Optical properties

3.3.2.2.1 Transparency

Based on the light transmission (%) and transparency data presented in Table 3.7, the films effectively blocked UV light in the 280–200 nm range, with negligible transmission observed across all films. This indicates that hordein-based films with PEG provide an excellent barrier against UV light transmission.

Table 3.7 also shows that the transparency of hordein-PEG films decreases as PEG concentration increases, meaning that the films become more transparent with higher PEG content. The transparency values for films with 25% and 30% PEG are relatively similar, with no significant difference between them. However, a noticeable increase in transparency is observed at 35% PEG. As noted in the SEM images (Figure 3.4), PEG shows lower compatibility with the protein matrix, and as the PEG concentration rises, more pores form in the film. At lower concentrations, PEG enhances chain mobility and creates a more uniform protein network. However, at higher concentrations, PEG may reach its solubility limit within the barley protein matrix, leading to increased voids and pores in the structure. These structural changes may allow more light to pass through, contributing to the observed increase in transparency (Cao et al., 2009).

3.3.2.2.2 Color

According to Table 3.5, the PEG concentration does not significantly affect the color properties of the hordein-PEG films.

3.3.2.3 Mechanical properties

In general, plasticizers reduce the associations between polymer chains within a film matrix, often resulting in a decrease in tensile strength (TS) values (Paramawati et al., 2001). However, as shown in Table 3.8, the TS values significantly increased with higher PEG concentrations ($p<0.05$). According to the FTIR results (Figure 3.3), this increase in TS can be attributed to the higher concentration of PEG, which promotes more hydrogen bonding within the protein chains, leading to a tighter and more structured network that enhances mechanical strength. Additionally, the hordein film with 35% PEG is thicker, which further contributes to its increased TS by providing greater resistance to mechanical stress. Similar findings were reported by Honary & Orafai (2002), who observed that higher PEG concentrations led to increased tensile strength at the breaking point. This can be explained by gel theory, which suggests that in solution, active center forces attract polymer molecules to each other, creating bonds that are in dynamic equilibrium (constantly forming and breaking). Water molecules and plasticizers compete for these bonding sites, reducing the number of active centers and polymer-polymer contacts.

Table 3.8 shows that films with higher PEG concentrations exhibit greater elongation at break (EB), with the 35% PEG film achieving the highest EB. This finding is consistent with the study by Qussi and Suess (2005), who observed that increasing PEG concentrations in shellac films enhanced both elongation and flexibility. The increase in PEG concentration is associated with a larger molecular volume and a higher number of hydroxyl groups, which allows for stronger interactions with the polymer chains. Additionally, PEG is known to lower the glass transition temperature (Tg) of hordein, causing the material to behave more like a rubbery, elastic substance at room temperature, thereby increasing its EB. This behavior is similar to what has been observed in physical-mechanical studies of shellac polymers, where the addition of plasticizers reduced Young's modulus, indicating a decrease in rigidity, while increasing elongation at break. The reduction in Young's modulus and the increase in EB are both attributed to the plasticizing effect of PEG, which lowers Tg and modifies the interactions between the polymer molecules (Honary & Orafai, 2002).

Table 3.8 Mechanical properties of hordein-PEG films. All films are derived from film-forming solutions containing 30% plasticizers in total.

	Tensile strength (KPa)	Elongation at break (%)
25%PEG	8.20 ± 3.77^{a}	102.18 ± 0.37^{a}
30%PEG	64.30 ± 11.81^{b}	102.78 ± 0.44^{b}
35%PEG	125.56 ± 13.83^{c}	104.76 ± 0.38^{c}

Means with different letter in the same column indicate significantly differences between groups (P<0.05).

3.3.2.4 Water vapor permeability (WVP)

Results on the water vapor permeability for hordein films with different concentrations of PEG (25%, 30%, and 35%) are presented in Table 3.6. The WVP of hordein-based films exhibited a clear trend of increasing permeability with higher PEG content. At 25% PEG, the WVP is no significant difference between this value and that of the 30% PEG film, suggesting that increasing the PEG concentration from 25% to 30% has a minimal effect on the film's permeability to water vapor. However, at 35% PEG, the WVP significantly increased. According to Guilbert and Biquet (1996), the addition of a plasticizer modifies the properties of the film by reducing the intermolecular bonds between the polymer chains, thus increasing the WVP of the film. The similar results were observed with the plasticized coating films of zein (Li et al., 2010). Additionally, SEM (Figure 3.4) results indicate that the compatibility between PEG and hordein is suboptimal, with visible pores and noticeable phase separation. In films with high PEG concentrations, a significant number of pores are present, potentially forming pathways that allow water vapor to penetrate more easily, leading to increased WVP values.

3.3.2.5 Fourier transform infrared spectroscopy (FTIR)

The effects of different PEG content on the secondary structure of hordein in the films were investigated using FTIR, as depicted in Figure 3.3**.** The spectra illustrate the characteristic peaks of hordein films with 25%, 30%, and 35% PEG. At 25% PEG, the O-H/N-H stretching peak at 3271 cm^{-1} suggests moderate hydrogen bonding between PEG and the protein matrix while the C-H stretching peak at 2933 cm^{-1} indicates balanced alkyl group interactions (Casariego et al., 2009; Xu et al., 2024). The sharp Amide I band at 1642 cm^{-1} (C=O stretching) and the defined Amide II band at 1539 cm^{-1} suggest a well-ordered protein structure (Li et al., 2022; Li et al., 2021). As PEG content increases to 30%, the O-H/N-H stretching peak at 3271 cm^{-1} intensifies, indicating stronger hydrogen bonding. Similarly, the C-H stretching peak at 2933 cm^{-1} shows a slight intensification, suggesting enhanced molecular mobility. However, the Amide I and II bands at 1642 cm^{-1} and 1539 cm^{-1}, respectively, begin to broaden, indicating that increased PEG content disrupts the ordered protein structure, leading to a more flexible conformation. At 35% PEG, the O-H/N-H stretching peak reaches maximum intensity, suggesting strong hydrogen bonding and a more plasticized film structure. The C-H stretching peak at 2933 cm^{-1} further intensifies, indicating a greater presence of flexible polymer chains. The Amide I and II bands at 1642 cm^{-1} and 1539 cm^{-1} show significant broadening, reflecting a shift towards a more amorphous and flexible film with reduced protein structural order.

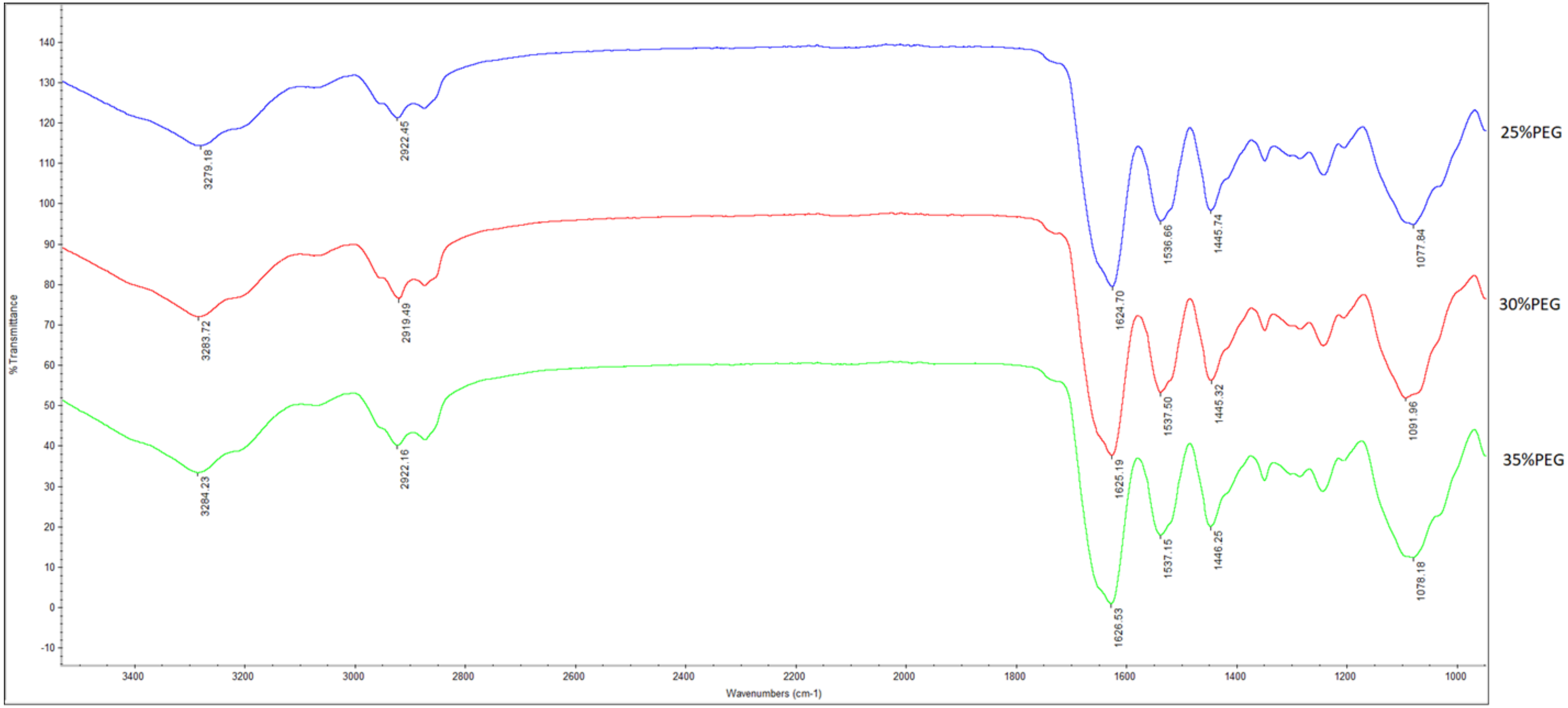

Fig. 3.3 Comparison of FTIR spectra of hordein with different concentrations of PEG composite films.

3.3.2.6 Scanning electron microscope (SEM)

According to Figure 3.4, all the SEM images of hordein-PEG films show some pores on the surface. This may be due to as the solvent evaporates, this phase separation leads to the formation of large pores. This observation is consistent with previous studies reporting porous cross-sections in zein films (Y. Li et al., 2022; Qiao et al., 2023; L. Zhang, Li, et al., 2022). As the concentration of PEG increased, more pores formed in the film. At a PEG concentration of 35%, the film exhibited not only increased pore formation but also noticeable surface aggregation, which could be attributed to the PEG molecules being large and occupying significant space in the solution. With low concentration, PEG can fit in the space between hordein proteins to reduce the interaction and improve the flexibility of the film. However, with the concentration increase, PEG can effectively reduce the amount of free water available for hordein solvation in ethanol due to its strong hydrophilic property (Sim, et al.,2012), which would provide more chance for hordein molecules to interact more with each other rather than hinder the interactions, consequently the aggregation and precipitation could be expected.

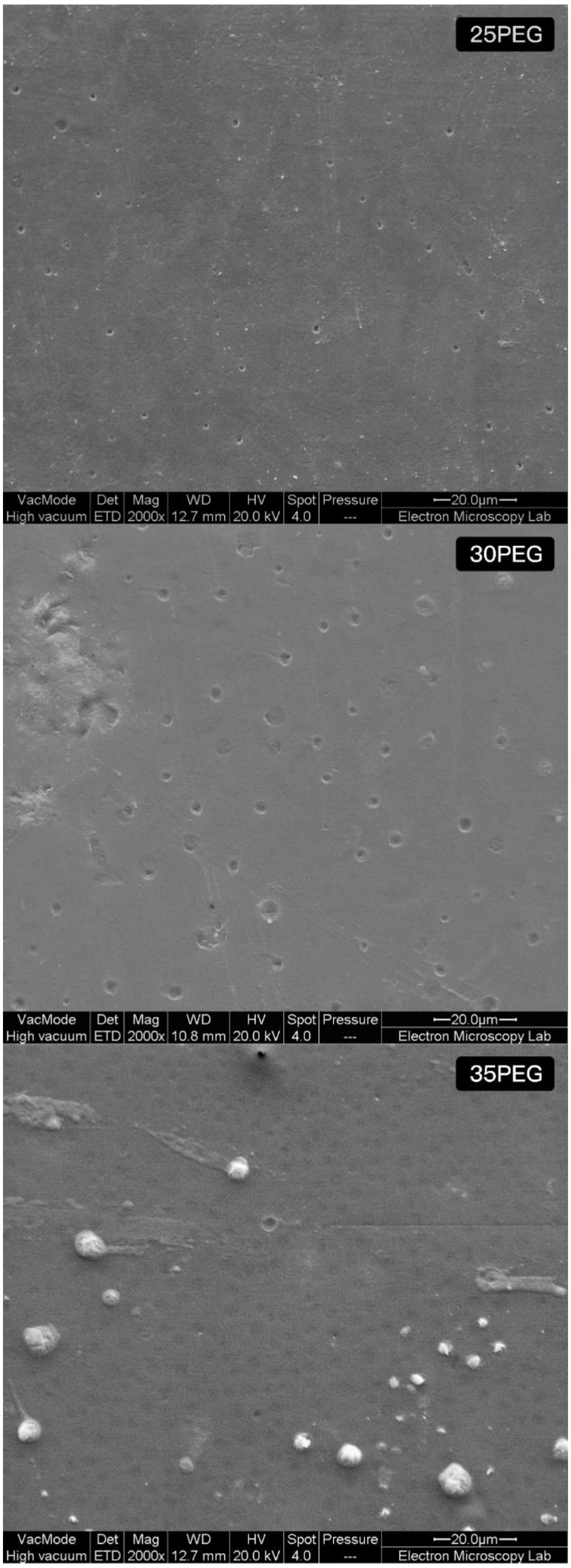

Fig. 3.4 SEM micrographs of hordein film surface with 25%, 30% and 35% PEG.

3.3.3 Experiment 3-Effect of composite plasticizers including GLY and PEG on the quality of hordein film

3.3.3.1 Thickness and moisture content

As shown in Table 3.9, the thickness of the hordein films was significantly influenced by the plasticizer composition. The film plasticized with 100% glycerol exhibited the greatest thickness (62.79 ± 13.34 µm), which was significantly higher than all other formulations ($p < 0.05$). This result is consistent with the properties of glycerol, whose small molecular size and three hydroxyl groups enable it to effectively penetrate and disrupt the hydrogen bonding network within the protein matrix. This disruption increases chain mobility and creates additional free volume, allowing the film to take up and retain more water, thus contributing to the observed increase in thickness (Lim et al., 2020). Similarly, Müller et al. (2008) reported that glycerol-plasticized films tend to be thicker due to glycerol's high moisture absorption, which causes the films to swell more than those containing other plasticizers. In contrast, the film plasticized with 100% PEG had the lowest thickness (45.58 ± 14.36 µm). This can be attributed to PEG's larger molecular size and fewer hydroxyl groups compared to glycerol, resulting in less disruption of the hydrogen bonding network within the protein matrix. Additionally, PEG has lower hydrophilicity, which leads to reduced water absorption and swelling, ultimately contributing to the thinner film (Ili Balqis et al., 2017).

Interestingly, films plasticized with a combination of PEG and glycerol exhibited intermediate thickness values, falling between the extremes of the 100% glycerol and 100% PEG films. This trend aligns with the FTIR results (Figure 3.5), which showed that higher glycerol concentrations result in more hydrogen bond disruption and a more disordered protein structure, while higher PEG concentrations lead to less plasticization and a more preserved protein matrix. The combination of glycerol and PEG allows these plasticizers to complement each other, balancing flexibility and structural integrity.

As shown in Table 3.10, the moisture content of all the films is not significantly different from each other ($p>0.05$). This may be because both PEG and glycerol are hydrophilic plasticizers, and all the films were derived from film-forming solutions containing a total of

30% plasticizers, so the moisture content did not deviate significantly.

Table 3.9 Thickness and color of the hordein-composite plasticizer films.

	Thickness(μm)	a*	b*	L*
100%GLY	62.79 ± 13.34^{a}	-1.67 ± 0.33^{a}	28.33 ± 2.79^{a}	86.05 ± 1.62^{a}
25%PEG75%GLY	56.88 ± 12.57^{b}	-1.67 ± 0.09^{a}	25.88 ± 2.29^{b}	86.74 ± 1.09^{a}
50%PEG50%GLY	56.25 ± 11.04^{b}	-1.75 ± 0.11^{ab}	26.14 ± 2.29^{b}	86.68 ± 0.84^{a}
75%PEG25%GLY	57.29 ± 14.84^{b}	-1.86 ± 0.10^{b}	25.20 ± 1.89^{b}	87.14 ± 0.68^{ab}
100%PEG	45.58 ± 14.36^{c}	-1.20 ± 0.13^{c}	21.74 ± 2.40^{c}	88.14 ± 2.19^{b}

Means with different letter in the same column indicate significantly differences between groups (P<0.05).

Table 3.10 Moisture content and WVP of hordein-composite plasticizer films.

	Moisture content (%)	WVP(g.mm/$\boldsymbol{m^2}$.d.kPa)
100%GLY	7.02 ± 0.94^{a}	12.47 ± 1.45^{a}
75%GLY25%PEG	6.55 ± 0.2^{a}	8.74 ± 2.13^{b}
50%GLY50%PEG	6.1 ± 1.06^{a}	8.54 ± 0.11^{b}
25%GLY75%PEG	6.24 ± 1.09^{a}	8.41 ± 1.08^{b}
100%PEG	5.93 ± 0.42^{a}	6.27 ± 1.29^{b}

Means with different letter in the same column indicate significantly differences between groups (P<0.05).

Table 3.11 Light transmittance and transparency values of hordein-composite plasticizer films.

	Transmittance (%) at wavelength (nm)								Transparency Value (mm^{-1})
	200	280	350	400	500	600	700	800	
100%GLY	0.35	0.90	30.11	58.50	80.08	87.73	89.78	90.67	0.86 ± 0.19^{a}
25%PEG75%GLY	0.39	0.91	23.97	55.20	78.83	87.30	89.73	90.69	1.06 ± 0.04^{b}
50%PEG50%GLY	0.32	0.75	15.28	46.71	75.63	86.40	89.50	90.73	1.13 ± 0.06^{b}
75%PEG25%GLY	0.32	0.79	18.56	47.87	76.10	86.87	89.65	90.81	1.07 ± 0.08^{b}
100%PEG	0.33	0.93	24.57	54.57	79.09	87.55	90.03	91.07	1.23 ± 0.06^{b}

Means with different letter in the same column indicate significantly differences between groups (P<0.05).

3.3.3.2 Optical properties

3.3.3.2.1 Transparency

As shown in Table 3.11, all the films demonstrated excellent UV barrier properties in the 200–280 nm range, which were not influenced by the type or concentration of plasticizers. This strong UV protection is attributed to the amino acids present in hordein (Li et al., 2004).

Additionally, the 100% GLY films exhibited the highest transparency. This is likely due to the smaller molecular size of glycerol compared to PEG, allowing it to disperse more homogeneously within the film matrix. In contrast, PEG, being larger, was more heterogeneously dispersed, causing increased light scattering. The reduction in network homogeneity caused by PEG incorporation led to decreased transparency (Chang & Nickerson, 2013). Furthermore, several studies have reported a blooming or blushing phenomenon occurring on the surface of films plasticized with PEG. This is due to PEG's lower compatibility with the protein matrix, resulting in phase separation or physical exclusion, which significantly increases film opacity (Orliac et al., 2003; Cao et al., 2009).

3.3.3.2.2 Color

The color of the hordein films was significantly influenced by the type and concentration of plasticizer used (Table 3.9). All films exhibited a greenish tint, as indicated by the negative a* values. The intensity of the green hue increased with higher PEG content, with the 75% PEG 25% GLY film showing the most pronounced green color (a* = -1.86 ± 0.10). The b* values revealed that films with higher glycerol content appeared more yellow. This can be attributed to the fact that the 100% GLY film had the greatest thickness, while the addition of PEG led to a decrease in thickness, with the 100% PEG film being the thinnest. Since hordein has a natural yellow-brown color, thicker films appear more yellow due to the increased material density, which enhances light absorption and scattering, making the yellow hue more prominent. Kaewprachu et al. (2018) reported that plasticizers are generally colorless and, therefore, do not directly affect film color. However, Kaewprachu et al. (2015) noted that the source and concentration of proteins have a greater impact on film color than the type of plasticizer used. Similarly, Ramos et al. (2013) observed that WPC (whey protein concentrate) films exhibited higher b* values than WPI (whey protein isolate) films, likely due to the slightly yellowish color of WPC powders, which may be attributed to the presence of contaminants like fat and phospholipids.

The lightness (L* value) of the films was also influenced by the plasticizer composition. The 100% PEG film exhibited the highest lightness (L* = 88.14 ± 2.19), likely because PEG increases the transparency of the film, allowing lighter to pass through with minimal scattering or absorption. This results in a lighter appearance, as more light is reflected and transmitted through the film.

3.3.3.3 Mechanical properties

The composition of glycerol and PEG plasticizers significantly influenced the mechanical properties of hordein films. As shown in Table 3.12, films with 100% GLY and 75% GLY25% PEG exhibited significantly higher tensile strength compared to other formulations ($P<0.05$), with TS gradually decreasing as the PEG concentration increased. This may be because glycerol, being a smaller molecule with high content, can more effectively fill gaps and form a denser network, resulting in optimal interaction with the hordein polypeptide chains (Xia et al., 2011). This is supported by SEM (Figure 3.6) analysis revealing the smoothest surfaces for 100% GLY and 25%PEG75%GLY films and FTIR (Figure 3.5) analysis demonstrating increased hydrogen bonding with higher GLY concentrations.

Regarding film flexibility, the elongation at break (EB) values peaked at 25% and 50% PEG in composite plasticizers but decreased with further increases in PEG concentration. This trend is similar to the results of Huo et al. (2018), suggesting that an optimal PEG concentration enhances flexibility, while excessive PEG disrupts the network, leading to reduced flexibility. Glycerol primarily reduces protein-protein interactions by creating a hydrated environment that keeps proteins solvated and separated. In contrast, PEG, being a larger molecule, exerts a steric exclusion effect, preventing proteins from aggregating and allowing for the formation of a flexible protein network. However, when the PEG ratio exceeds 50%, the steric exclusion becomes excessive, leading to a discontinuous hordein network. FTIR data revealed that higher PEG content resulted in narrower Amide I and Amide II bands, indicating a more ordered, rigid protein matrix. Furthermore, SEM images (Figure 3.6) showed that increasing PEG content beyond 50% resulted in a more textured surface with pores, making the films more brittle and reducing their elongation at break.

Table 3.12 Mechanical properties of hordein-composite plasticizer films. All films are derived from film-forming solutions containing 30% plasticizers in total.

	Tensile strength (KPa)	Elongation at break (%)
100%GLY	139.25 ± 9.81^{ab}	105.50 ± 0.35^{a}
75%GLY25%PEG	144.53 ± 13.23^{a}	107.58 ± 2.82^{b}
50%GLY50%PEG	124.47 ± 26.75^{bc}	108.16 ± 3.40^{b}
25%GLY75%PEG	117.92 ± 16.75^{c}	105.44 ± 0.94^{a}
100%PEG	64.30 ± 11.81^{d}	102.78 ± 0.44^{c}

Means with different letter in the same column indicate significantly differences between groups (P<0.05).

3.3.3.4 Water vapor permeability (WVP)

As shown in Table 3.10, the WVP of the 100% glycerol film is significantly higher than that of all other films, and the WVP of the films decreases with the addition of PEG. This difference could be attributed to the variation in their hydrophilic properties. While both PEG and glycerol are hydrophilic, glycerol possesses greater hydrophilicity due to its three hydroxyl groups compared to the two terminal hydroxyl groups of PEGS (Xia et al., 2011). This is consistent with Li et al. (2010) reported that the WVP of zein films containing PEG 400 at various concentrations was clearly lower than that of the respective films containing glycerol as a plasticizer, which might be because glycerol has high capacity to interact with water, which facilitates its solubilization and permeation through the film (Galdeano et al., 2009). Additionally, in composite plasticizer films, the concentration of glycerol was decreased compared to the 100%GLY film, combined with the smaller molecular size of glycerol, allowing it to fill the gaps between polypeptide chains and the larger PEG molecules, forming a denser network that reduces water vapor permeability. This result is consistent with the findings reported by Huo et al. (2018) regarding the synergistic plasticization effect in zein films.

3.3.3.5 Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of hordein films with different concentrations of PEG and glycerol are presented in Figure 3.6. The 100% glycerol film shows a high degree of plasticization, with an intense O-H/N-H stretching peak at 3275.93 cm^{-1} and C-H stretching at 2928.98 cm^{-1}, indicating extensive hydrogen bonding and molecular mobility. The broadening of the Amide I (1623.27 cm^{-1}) and Amide II (1535.86 cm^{-1}) bands suggests that the protein structure is highly disrupted, making the film highly flexible but potentially less structurally stable.

The 25P75G film exhibits the most significant plasticization among the tested formulations. The strong O-H/N-H stretching band at 3277.98 cm^{-1} suggests extensive hydrogen bonding interactions within the film matrix, likely attributed to the high glycerol content. This observation aligns with Fan et al. (2018) reporting that a lower wavenumber and lower amplitude of the Amide A peak indicate the increase of hydrogen bonding for the molecular interaction, which suggests that a higher concentration of glycerol in the film results in more hydrogen bonds. Additionally, the prominent C-H stretching at 2926.94 cm^{-1} indicates increased alkyl chain mobility, further supporting the enhanced flexibility of the 25P75G film. The broadening of the Amide I (1623.78 cm^{-1}) and Amide II (1537.94 cm^{-1}) bands, representing C=O stretching and N-H bending vibrations in the protein backbone, respectively, suggests a significant disruption of the protein secondary structure (Grdadolnik & Marechal, 2000). This disruption is likely due to the plasticizers' interference with protein-protein interactions, leading to a more flexible but less ordered film structure.

In contrast, the 50P50G film demonstrates a more balanced profile. The O-H/N-H stretching peak at 3279.62 cm^{-1} and C-H stretching at 2925.12 cm^{-1} indicate moderate plasticization, suggesting a more balanced interaction between the plasticizers and the hordein matrix. The Amide I (1624.46 cm^{-1}) and Amide II (1538.65 cm^{-1}) bands are slightly narrower compared to 25P75G, indicating that the protein structure retains more order. The 75P25G film shows further reduced plasticization, as evidenced by the O-H/N-H stretching peak at 3280.29 cm^{-1} and C-H stretching at 2922.47 cm^{-1}. The narrower Amide I (1624.22 cm^{-1}) and Amide II (1537.60 cm^{-1}) bands reflect a more ordered protein matrix, suggesting a more rigid, less flexible film. This trend can be attributed to the higher PEG content in the 75P25G film.

For the 100% PEG film, the O-H/N-H stretching peak at 3284.79 cm^{-1} and the C-H stretching at 2922.87 cm^{-1} indicate minimal plasticization. The sharp Amide I (1625.50 cm^{-1}) and Amide II (1536.80 cm^{-1}) bands demonstrate that the protein structure is largely preserved, resulting in a more structurally intact film. Finally, the pure hordein exhibits minimal plasticization, with an O-H/N-H stretching peak at 3278.71 cm^{-1} and weaker C-H stretching at 2924.31 cm^{-1}. The sharp Amide I (1624.31 cm^{-1}) and Amide II (1517.98 cm^{-1}) bands indicate a well-ordered protein structure, characteristic of an unplasticized.

In conclusion, films plasticized with a combination of PEG and glycerol outperform those with a single plasticizer. Glycerol enhances the plasticizing effect, while PEG helps preserve the protein structure, creating a synergistic effect when used together. Based on the results, both the 25P75G and 50P50G formulations demonstrate strong potential for selection due to their balanced plasticization and structural order.

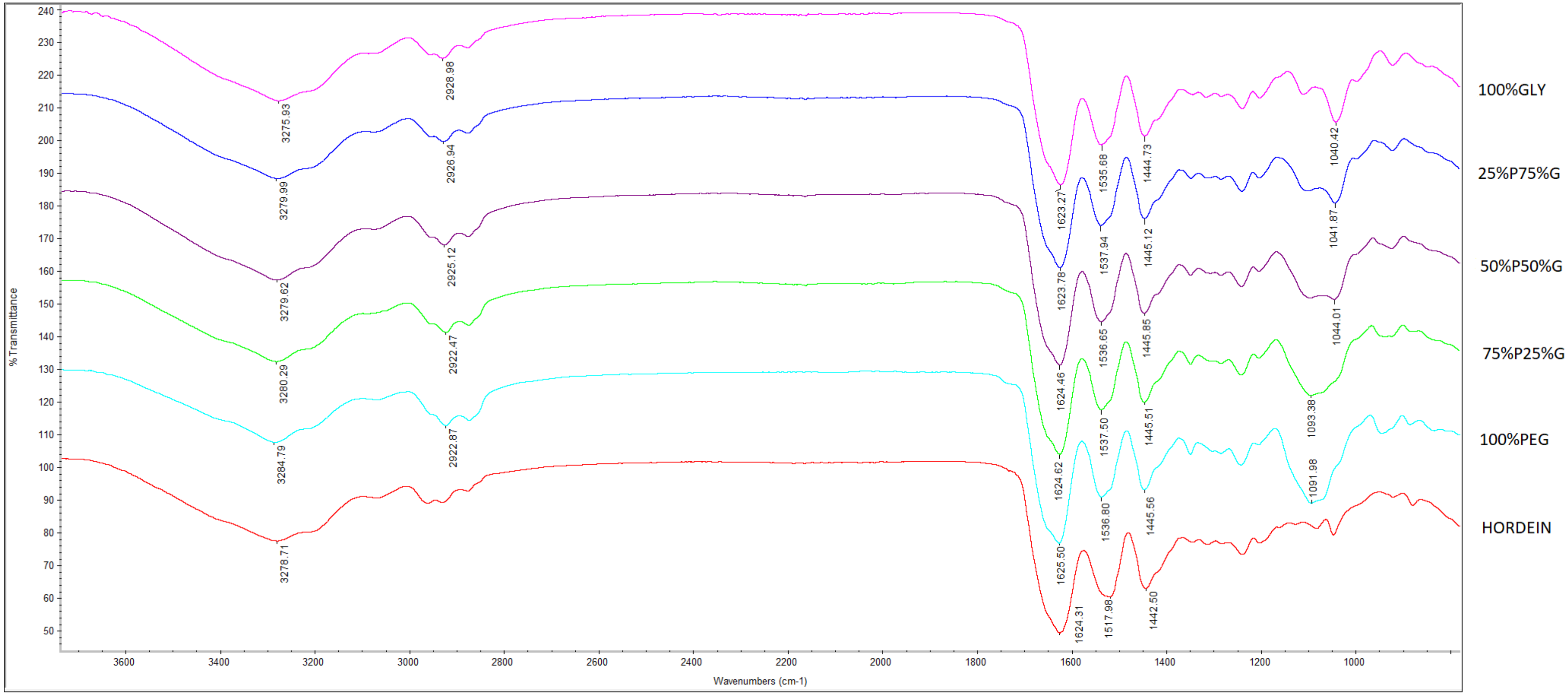

Fig. 3.5 Comparison of FTIR spectra of hordein and hordein with different ratios of glycerol and PEG composite films.

3.3.3.6 Scanning electron microscope (SEM)

As shown in Figure 3.6, the 100% GLY film exhibited a smooth and homogeneous surface, indicative of significant plasticization. This is attributed to glycerol's small molecular weight and hydrophilic nature, allowing it to diffuse and interact efficiently with the protein matrix (Faradilla et al., 2019). Although PEG was added to the 25%PEG75%GLY film, glycerol remained the dominant plasticizer, resulting in a smooth surface. The FTIR results indicate that the addition of 25%PEG contributed some rigidity to the film structure, but glycerol's plasticizing effect was still the primary influence. Increasing the PEG content to 50% resulted in a more textured surface and few pores, which could be attributed to the formation of microdomains with varying PEG concentrations, creating localized regions of stiffness within a more flexible glycerol-rich matrix. At higher PEG concentrations (75%PEG25%GLY), the SEM images revealed a rough and uneven surface with visible microcracks. This suggests that PEG having higher molecular weight could not migrate to hydroxyl groups easily, leading to phase separation, so the higher PEG content led to a high degree of phase separation detected in films, which indicated less compatibility and deficiency in polymers' interactions and hindered glycerol's plasticizing effect (Laohakunjit & Noomhorm, 2004; Faradilla et al., 2019). Finally, the 100% PEG film exhibited the roughest surface with pronounced cracks, indicative of significant rigidity and brittleness.

Films with 100% GLY and 25%PEG75%GLY had the smoothest surfaces, likely due to glycerol’s ability to enhance connectivity between protein chains. The combination of high glycerol concentrations with PEG helps fill the gaps between hordein chains, forming a smooth surface and dense network. Glycerol and PEG, due to their differing molecular sizes, complement each other by filling gaps of varying sizes between hordein polymer chains, contributing to the observed surface smoothness.

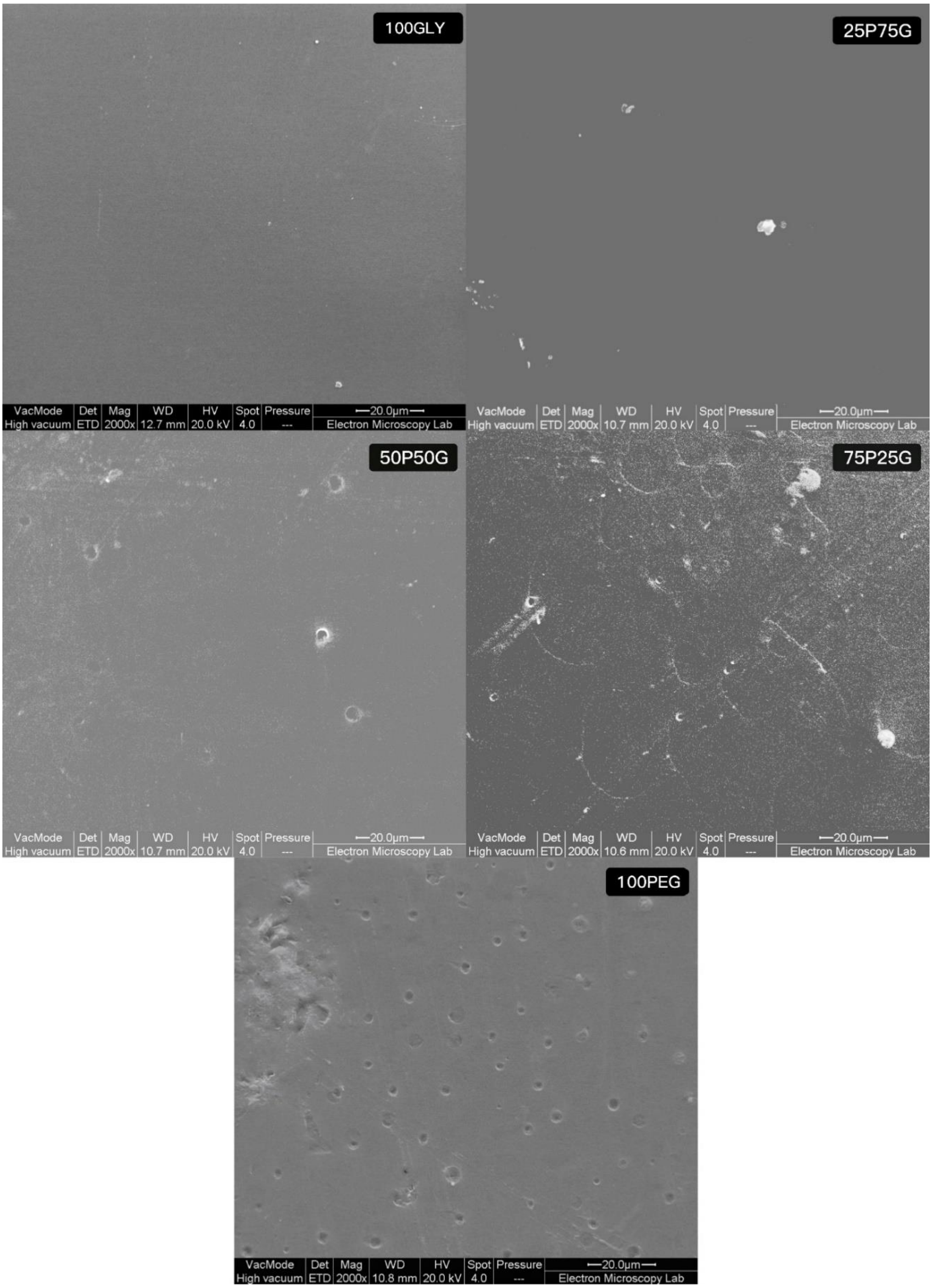

Fig. 3.6 SEM micrographs of films surface for 100%GLY, 75%GLY+25%PEG, 50%GLY+50%PEG, 25%GLY+75%PEG, 100%PEG with hordein. All films are derived from film-forming solutions containing 30% plasticizers in total.

3.4 Conclusion

In this study, the effects of two individual plasticizers (GLY and PEG) at different concentrations and various ratios in composite plasticizers on hordein-based films were investigated. The results revealed that the type of plasticizer significantly affects both the mechanical and water barrier properties of the hordein-based films. Films with GLY alone exhibited better mechanical properties compared to those with PEG alone. However, hordein-PEG films demonstrated superior water barrier properties compared to hordein-glycerol films. The blend of different ratios of PEG and GLY showed a synergistic effect, with the 75% GLY + 25% PEG hordein film presenting excellent properties, including a more compact structure, higher tensile strength, and lower water vapor permeability compared to films with individual plasticizers.

In conclusion, hordein films with composite plasticizers show promise as biodegradable protective food packaging. Composite plasticizers can optimize both mechanical and water barrier property of hordein films; however, their breaking strength still falls short of industrial requirements for product applications. Future research should focus on improving the mechanical properties of hordein films through methods such as enzymatic treatment, heat treatment, or the incorporation of nanomaterials.

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CHAPTER 4

Property of hordein-based film affected by Transglutaminase and heat treatment

Chapter 4: Property of hordein-based film affected by Transglutaminase and heat treatment

Abstract

Hordein, a protein derived from the by-product of beer production, shows promise for developing biodegradable packaging. However, its mechanical properties are currently inadequate for practical use in the food industry. Heat treatment can unfold proteins to expose sulfhydryl groups, facilitating crosslinking through disulfide bonds and enhancing the protein network. Similarly, Transglutaminase (TGase) can induce covalent crosslinking by forming isopeptide bonds between glutamine and lysine residues. We hypothesized that combining TGase and heat treatment could improve the mechanical properties of hordein-based films. In this study, hordein-based films with varying TGase concentrations (10, 20, 30 and 40U/g based on the hordein weight), with and without heat treatment, were evaluated from the aspects of mechanical properties, optical properties, and water barrier property. Atomic Force Microscope (AFM), Fourier-transform infrared spectroscopy (FTIR), and thermal analysis were used to evaluate structural integrity. Results indicated that heat treatment reduced the thickness and transparency of hordein film, but did not affect the tensile strength and elongation. Higher TGase concentrations (above 30 u/g) increased film thickness, while elongation at break decreased with the increase of TGase concentrations. Tensile strength peaked at 20U/g TGase concentration.

Keywords: Hordein, Transglutaminase, heat treatment, film properties, packaging

4.1 Introduction

Environmental pollution from petroleum-based plastics has emerged as one of the most critical issues of this century, primarily due to the substantial greenhouse gas emissions and the pollution of marine and terrestrial habitats throughout their lifecycle (Oktavilia et al., 2020). Until now, food packaging materials are mainly produced from petrochemical polymers, due to their good mechanical properties, lightness and attractive price. However, the low degradation rate of these materials has given rise to serious environmental problems (De-la-Torre, 2020). Biodegradable films, prepared from naturally occurring resources such as carbohydrates, lipids, and proteins, are a promising alternative to conventional plastics. Among these, proteins have garnered attention due to their abundance and superior film-forming capabilities, offering better mechanical and barrier properties compared to polysaccharide and lipid films (Kaewprachu et al., 2016). Proteins, composed of a diverse range of amino acid monomers, possess complex secondary, tertiary, and quaternary structures that facilitate crosslinking and intermolecular bonding, enhancing film properties (Gennadios, 2002). Furthermore, protein films can be modified through various treatments, such as enzymes, heat treatment, pressure, irradiation, lipid interface, acids, alkalis, and metal ions to optimize film preparation (Zhang and Mittal, 2010).

Brewers spent grain (BSG), a primary by-product of the brewing industry, accounts for 85% of its total waste, with approximately 40 million tonnes generated annually worldwide (Stojceska, 2019; Mussatto, 2014). BSG contains 15% to 20% protein, predominantly hordein, a prolamine glycoprotein found in barley that functions as a storage unit (Arendt and Zannini, 2013). Hordein's high glutamine content and hydrophobic amino acids, such as proline, leucine, and valine, provide strong protein aggregation and high hydrophobicity, making it a potential candidate for biodegradable film production (Zhao et al., 2010). However, hordein films exhibit brittle mechanical characteristics, as well as weak water holding capacity (WHC) and oil holding capacity (OHC), limiting their application (Wang et al., 2010; Cho and Rhee, 2009). Thus, there is a need for further research to enhance hordein film properties.

Previous studies have demonstrated that heat treatment can induce cross-linking in proteins, thereby enhancing the mechanical properties of protein films, including those made from prolamine kafirin and soy protein (Byaruhanga et al., 2007; Stuchell and Krochta, 1994). Heating unfolds proteins, exposing sulfhydryl and hydrophobic groups, which subsequently form disulfide bonds and create a cross-linked network structure (Altschul, 1985; Gennadios and Weller, 1991). Similar findings were reported by Hoque et al. (2010) for films prepared from cuttlefish (Sepia pharaonis) skin gelatin, where heating increased both tensile strength (TS) and elongation at break (EB). Likewise, Stuchell and Krochta (1994) found that heat treatment significantly improved the mechanical properties of soy protein isolate films.

Transglutaminase (TGase, EC 2.3.2.13) is an enzyme known to enhance protein cross-linking through three reactions: forming isopeptide bonds between glutamine and lysine residues, acyl-transfer reactions, and deamidation (DeJong and Koppelman, 2002; Ohtsuka et al., 2001). As shown in Figure 4.1A, transamidation occurs when the ε-amino group of a protein's lysine residue acts as an acyl acceptor. This process leads to the transfer of an acyl group to the lysine residue, resulting in the formation of ε-(γ-glutamyl) lysine (Gln-Lys) inter- or intramolecular covalent crosslinks (CL). Additionally, the protein involved is rich in essential amino acids, as depicted in Figure 4.1B. Furthermore, in the absence of free amine groups, TGase can catalyze the deamidation or esterification of the γ-carboxamides of glutamine residues. In this scenario (Figure 4.1C), a water molecule acts as an acyl acceptor via its hydroxyl groups (Zhang et al., 2023; Akbari et al., 2021). TGase has shown to improve the mechanical properties of various protein films, including those made from soy protein isolate, whey, zein hydrolysate, αs1-casein, sodium caseinate, quinoa protein isolate, chitosan, and gelatin (Tang et al., 2005; Oh et al., 2004; Motoki et al., 1987; Patzsch et al., 2010; Escamilla-García et al., 2019; Masamba et al., 2016; Lim et al., 1999).

There is limited information about using enzymatic method and/or heat treatment in developing hordein film. In this study, hordein-based films were prepared with varying TGase concentrations, with and without heat treatment. The quality of these films was assessed in terms of mechanical characteristics, color, thickness, and water vapor permeability. Fourier-transform infrared spectroscopy (FTIR) and Atomic Force Microscope (AFM) were also employed to analyze the chemical and microstructural changes. The aim was to determine whether the combination of heat treatment and TGase could improve the mechanical properties of hordein-based films, making them more viable for industrial applications.

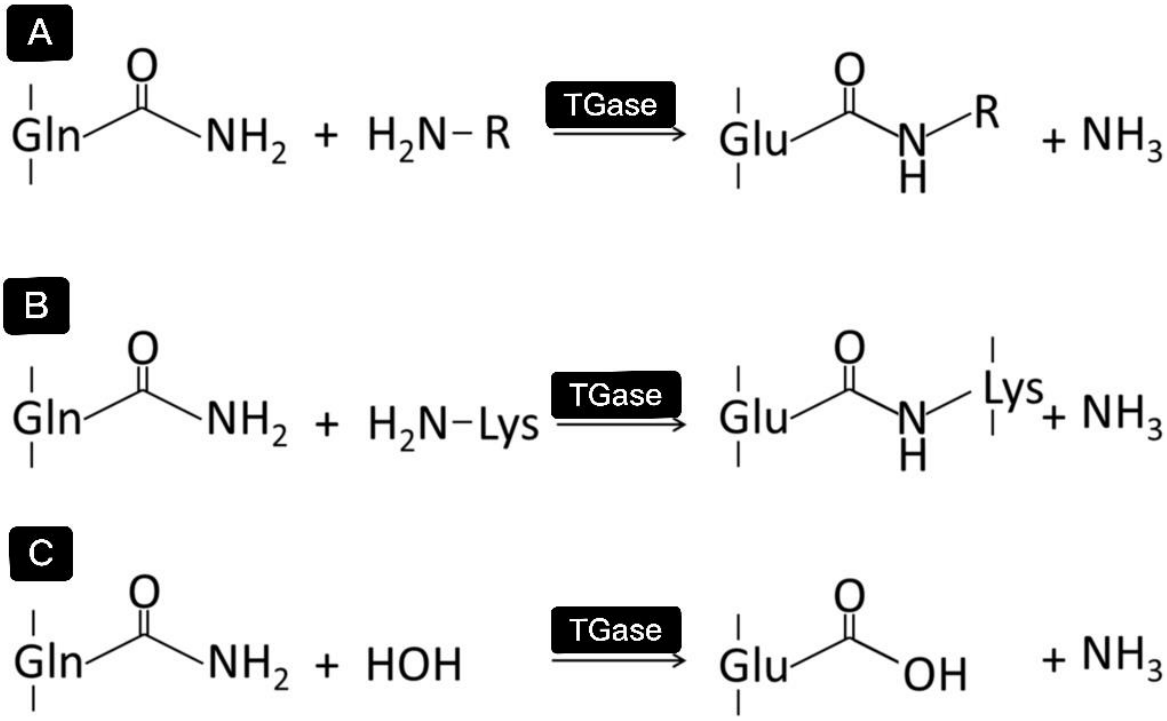

Fig. 4.1 The reactions catalyzed by transglutaminase: (A) Acyl transfer reaction; (B) Cross-linking reaction between Gln and Lys residues of proteins; (C) Deamidation with water (Zhang et al., 2023; Akbari et al., 2021)

4.2. Materials and methods

4.2.1 Materials

Hordein was extracted from barley flour (Buy Whole Foods Online Ltd., UK) using a 75%

aqueous ethanol solution according to the method described by Wang et al. (2010) Detailed procedure can be found in the section 2,1 Materials and method in Chapter 2. The yield of hordein extract is 84.4%. The characterization of the hordein extract by the Kjeldahl method was consistent with results reported by Qazanfarzadeh et al. (2021). Glycerol (≥99.0%) and PEG400 were both purchased from Sigma Chemical Co. (UK). Microbial Transglutaminase (TGA; TG Activa WM, 100 U/g enzyme activity, enzyme content 1% in maltodextrin) was obtained from Ajinomoto (Paris, France).

4.2.2 Film preparation

The specific extraction procedure is the same as outlined in Chapter 3. 8% hordein solution was prepared by dissolving hordein powder in a 75% ethanol solution at room temperature by continuously stirring at 600 rpm at room temperature on a magnetic stirrer (FOUR E'S SCIENTIFIC). For samples without heat treatment, it was mixed with a 30% composite plasticizer containing GLY and PEG with a ratio of 3:1 by stirring for a further 15 minutes. For samples with heat treatment, the mixture was heat treated for 30 minutes at 85°C after glycerol was added, and the forming solution was cooled to room temperature. TGase at 0, 10, 20, 30, and 40U/g were added to both heat or without heat the hordein solution based on hordein weight (grams), respectively, and mixed with a magnetic stir at room temperature for 1 hour to facilitate the TGA reaction. A factorial design was used including 2 factors, enzyme (5 levels) and heat treatment (2 levels) and overall, 10 treatments were carried out. All the films were formed at 40℃ for 24hrs in a vacuum oven (Townson and Mercer Limited), then conditioned at 20℃ and relative humidity (RH) 52% for 48hrs before tests.

4.2.3 Thickness

The thickness of the films was measured with an electronic digital micrometer with a sensitivity of 0.001 mm. At least 10 random spots were chosen for analysis. The average of the 10 repeated reading was calculated to present the thickness of the film. The thickness data were used for further analysis of WVP and mechanical properties.

4.2.4 Optimal properties:

4.2.4.1 Transparency

The opacity of the films was measured using a NIR-VIS-UV Cary7000 spectrophotometer (Agilent Technologies, Santa Clara, US) at wavelengths from 200 to 800 nm, with three repeated measurements for each sample. A film sample was cut to a rectangular shape (40 × 40 mm). Transparency values were calculated using the equation by Han & Floros (1997):

$$\text{Transparency value} = -\log T_{600}/\text{d}$$

T_{600} is the fractional transmittance at a wavelength of 600 nm; d is the thickness(mm) of the film. The higher value of transparency value presents the lower film transparency.

4.2.4.2 Color measurement

The color properties of the films were determined using a CR-400 Chroma Meter (Konica Minolta, Japan) based on the CIELab color space with values including lightness (L*), red/green (a*), and yellow/blue (b*). The sample was measured against a white background with L* = 94.1, a* = 0.49, b* = 4.13. Five repeated measurements were conducted on each sample and average was used to present the color feature of the film.

4.2.5 Mechanical properties:

Tensile strength (TS) and elongation at break (EB) were measured using a TA-XT2i Texture Analyzer, (Stable Microsystems, Godalming, UK), as described by Patzsch et al. (2010). Samples were conditioned for 48 hours at 57% relative humidity and room temperature. Each film was cut into strips (0.8 cm × 0.5 cm) and tested using a 30 kg load cell and a 50 mm/min primary grip breaking speed. Fives strips were measured for each film sample. TS values were calculated by dividing maximum loads by specimen cross-sectional areas. The percentage of elongation at break (EB) was determined by dividing the extension at break of the strip by its original length and multiplying by 100 (Díaz et al., 2016).

4.2.6 Water behavior

4.2.6.1 Moisture content of hordein film

Moisture content was determined according to the method described by Zahedi, Fathi-Achachlouei, and Yousefi (2018). A square film sample (2 cm × 2 cm) was dried at 105 °C in oven for 24 h, and weight change before (Wi) and after (Wd) drying were recorded and moisture content was calculated as the weight loss in percentage according to the following equation:

$$\text{Moisture content (\%)} = [(W\text{i} - W\text{d})/W\text{i}] \times 100$$

Wi – Initial weight of film before drying
Wd- weight of film after drying

4.2.6.2 Water vapor permeability (WVP):

Films were conditioned at 57% relative humidity for 72 hours. A piece of film was sealed on top of glass vial (The bottle has a bottom diameter of 4 cm, a height of 7 cm, and an open mouth with a diameter of 1.58 cm) filled with silica gel, and the vial was placed in a desiccator containing a saturated solution of potassium sulfate with relative humidity at 98%. The vials' weights were recorded every 24 hours for 7 days. WVTR was calculated from the slope of linear weight gain and used to derive WVP using the equation:

$$\text{WVTR (g/m}^2\text{·day)} = \text{slope/area}$$

$$\text{WVP(g.mm/}\boldsymbol{m^2}\text{.d.kPa)=(WVTR x L)/}\Delta\text{P}$$

WVTR (g/m²·day): Water Vapor Transmission Rate. Slope (g/day): The rate of weight gain or loss in the test. Area (m²): The surface area of the film exposed to water vapor transmission. L is thickness(mm), ΔP is difference in water vapor partial pressure(kPa) between both sides of the film, respectively.

4.2.7 Fourier Transform Infrared Spectrometer (FTIR)

To study the chemical structures and interactions between components, the FTIR spectra of films were obtained using an FTIR spectrophotometer (Spectrum 100, PerkinElmer, US) with an Attenuated Total Reflectance (ATR) attachment and selecting a wavenumber resolution of 4 cm−1 for 16 scans from 4200 to 650 cm−1.

4.2.8 Atomic Force Microscope (AFM)

Film surface morphology was studied using an Atomic Force Microscope (Oxford Instruments Asylum Research, UK). Samples were examined on an Oxford Instruments Asylum Cypher ES. Films were sectioned and attached to a steel puck using a double-sided sticky carbon tab. AFM tips AC160TS were used for examining the samples in alternating contact mode (AC) mode. AC160TS tips had a drive frequency range of (201 - 341.8) kHz and a spring constant in the range of (8.53 - 37.30) N/m, with tip radius 7nm. Scanning frequencies of (0.4 - 1) Hz were used. Images obtained were examined in the Gwyddion software package.

4.2.9 Statistical analysis

All treatments were repeated three times in consideration of batch effect. All data were analyzed using IBM SPSS Statistics 28.0.0.0 software (IBM Corp, New York, USA) and expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA for differences among all the treatments and two-way ANOVA for assessing the factors effect and their interaction. Duncan's test was used for multiple comparison, a significance level 0.05 was used throughout the analysis.

4.3 Results and discussion

4.3.1 Physical properties of hordein-based films

4.3.1.1 Thickness

As shown in Table 4.1, heat treatment significantly reduced the thickness of the hordein-based films compared to those without heat treatment ($P<0.05$). This aligns with the findings of Stuchell and Krochta (1994), who reported that soy protein films cast from unheated solutions were thicker than those cast from heated solutions. They suggested that heating denatures the protein, promoting increased interactions, tighter packing, and reduced chain mobility of the polymer chains, which leads to thinner films. Additionally, TGase concentration significantly affects film thickness ($P<0.05$). Higher concentrations of TGase (≥30U/g) significantly increased the thickness of the films. This increase can be attributed to the enhanced cross-linking induced by TGase, which boosts the dry matter content; as shown in Table 4.1, higher concentrations of TGase (30 and 40 U/g) resulted in lower moisture content, which leads to higher dry matter content. This finding is consistent with the results reported by Mirpoor et al. (2022), who observed that TGase addition significantly increased the thickness of Hemp (Cannabis sativa) seed oilcake films. Similarly, Tang et al. (2005) found that adding TGase to soy protein films led to increased thickness. According to Table 4.1, there is no significant interaction between TGase concentration and heat treatment ($P=0.259$). This suggests that the effects of TGase and heat treatment on film thickness are independent rather than synergistic.

4.3.1.2 Optimal properties

4.3.1.2.1 Transparency

Based on the light transmission (%) and transparency data presented in Table 4.3, the hordein-based films effectively blocked UV light in the 280–200 nm range, with negligible transmission observed across all films. This indicates that all the hordein-based films, regardless of heat treatment or TGase addition, provide an excellent barrier against UV light transmission.

As shown in Tables 4.1 and 4.3, the transparency of the hordein-based films remained relatively consistent, with no significant differences between films, whether treated with TGase or subjected to heat treatment. Choi and Han (2002) similarly reported that the transparency of pea protein isolate films was unaffected by heat treatment. Hoque et al. (2010) also found no significant differences in transparency between gelatin films prepared from film-forming solutions with or without heat treatment. Ahammed et al. (2021) observed comparable results, reporting that TGase did not impact the transparency of gelatin films. However, when zein was added to gelatin-based films, it reduced transparency. In contrast, Tang et al. (2005) noted that TGase treatment reduced the transparency of soy protein isolate films, attributing the decrease to cross-linking or aggregation induced by the enzyme, which increased the turbidity of the film-forming dispersion.

4.3.1.2.2 Color

Color is fundamental properties of films that significantly influence consumer willingness to purchase. The color is featured with L* for lightness, a* for greenness, and b* for yellowness. The results in Table 4.1 indicate that heat treatment significantly affects the L* and b* values of the films ($P<0.05$), making the films lighter (higher L* values) and less yellow (lower b* values), but it does not significantly impact the a* value ($P>0.05$). This similar with findings by Tarhan and Şen (2022), who reported that heat-treated whey protein isolate films exhibited higher L* values, while a* and b* values showed no significant changes. The differences in color attributes between hordein-based and whey protein films may be due to the natural color differences in proteins.

TGase concentration significantly affected the film colour ($p<0.05$). Higher TGase concentrations resulted in films with stronger green hue (lower values). Notably, the addition of 10 U/g TGase resulted in the lightest and least yellow films. However, as the TGase concentration increased beyond this point, the lightness value decreased, and the yellowness increased. This trend differs from the findings of Ahammed et al. (2021), who reported that TGase cross-linking did not affect the color of gelatin-zein films. This discrepancy could be attributed to the difference between proteins. For gelatine, it is naturally colourless protein, hence changing extent of enzymatic reaction did not affect the colour. However, hordein protein naturally has light yellow colour, the TGase catalysed cross-linking changed the molecule arrangement and made the film denser and more compact, consequently, the light reflection changed. Uresti, Ramirez, López-Arias, and Vázquez (2003) also reported that the covalent crosslinking induced by transglutaminase decreased the lightness of fish film. Contrary to that observed in hordein-based films, the color of gelatin films became lighter as the transglutaminase reaction occurred. However, overall, the colour change caused by TGase is not in a great extent. Further sensory test should be conducted to verify if this change can affect the consumer's acceptability.

According to Table 4.2, there is significant interaction between heat treatment and TGase concentration on the colour parameters, i.e. L*, a* and b*. ($P < 0.05$), which indicates that the effect of heat treatment is dependent on the concentration of TGase. Heat treatment leads to a lighter coloured film with high L* and low b*, while TGase concentration affects greenness and lightness, with an optimal concentration of 10 U/g resulting in the lightest and least yellow films.

4.3.1.3 Mechanical properties

Heat treatment has been reported to unfold sections of the three-dimensional structure of proteins, leading to intermolecular polymerization through -SH, S-S exchange reactions, and hydrophobic bonds, thereby creating an intermolecular network that could increase tensile strength (Guo et al., 2012). However, as shown in Table 4.1, heat treatment did not affect the tensile strength or elongation at break of the films. Hoque et al. (2010) observed similar results in films prepared from cuttlefish (Sepia pharaonis) skin gelatin, where heating at 80°C resulted in mechanical properties comparable to control films without heat treatment. This may be due to degradation occurring at higher temperatures (>70°C), where shorter gelatin chains are unable to form strong film networks capable of resisting mechanical forces. Tang et al. (2009) also reported that the elongation at break (EB) of vicilin-rich protein isolate films (from red bean, kidney bean, mung bean, and soy protein isolate) was unaffected by heating, though tensile strength (TS) increased. This is consistent with the findings of Stuchell and Krochta (1994), where heating (at 85°C) of soy protein isolate (SPI) films with 20% glycerol did not affect EB. Similarly, Liu et al. (2004) reported that heating peanut protein films at 60°C to 90°C did not impact EB, but temperatures above 70°C increased TS. The varying impact of heat treatment on film properties may depend on the protein source, differences in film-forming mechanisms, and the types of bonds involved.

TGase concentration had significant impact on tensile strength ($p<0.05$). The highest tensile strength is observed at 20 U/g TGase, while higher concentrations (30 U/g TGase and 40 U/g TGase) result in lower tensile strength. As shown in Figure 4.3, the decrease in tensile strength (TS) may be attributed to the poor binding of excessively high concentrations of TGase with barley hordein, leading to the formation of aggregates. These results agree with Jiang et al. (2007) that 4-10U/g TGase resulted in a higher tensile strength of soy protein isolate films, but a reduced tensile strength of protein film was observed at TGase over 20 u/g. The varying effect of enzyme concentration on tensile strength of the film was also reported in other proteins like soy protein isolate (Tang et al., 2005) and deaminated gluten (Larré et al., 2000). The increase in tensile strength at low TGase concentrations is due to the increased cross-linking between peptide chain which leads to an ordered structure for better mechanical strength. However, with the increase of TGase concentration, excessive cross-linking might reduce the distance of polypeptide chain and lead to the protein coagulation, consequently the formed pores reduce the mechanical strength of protein network, which can also be evidenced in the microstructure of the film (Figure 4.3). In terms of the flexibility of the film, elongation at break decreases with the increase of TGase concentration which is in line with the result of Jiang et al. (2007) who reported that elongation of soy protein isolate films decreased with the increased TGase concentration.

The decrease in elongation might be due to the excessive cross-linking catalysed by TGase at higher concentration which limits the mobility of molecules and results in a reduction in flexibility. Moreover, there is dissolving issue that a higher concentration of TGase might not be fully solubilized in the hordein solution; hence beneficial effects of TGase reaction are not observed at higher concentration.

Table 4.2 indicates that the control sample without TGase and heat (T0 Noheat) sample exhibits excellent flexibility but has the lowest tensile strength. In contrast, the 20T Heat sample demonstrates the best overall mechanical performance, combining the highest tensile strength with a good elongation break. This suggests that a moderate concentration of TGase (20 U/g) provides an optimal balance of strength and flexibility for hordein-based films.

4.3.1.4 Water behavior

4.3.1.4.1 Moisture content of hordein film

Table 4.1 reveals that heat treatment did not significantly affect the moisture content of hordein-based films (P>0.05), while the concentration of transglutaminase (TGase) had a more noticeable impact. Films without TGase or with lower TGase concentrations (10 and 20 U/g) exhibited the highest moisture content. As TGase concentrations increased to 30 and 40 U/g, the moisture content significantly decreased to 5.19% and 5.59%, respectively. These findings are consistent with those reported by Kaewprachu et al. (2017), where a gradual reduction in moisture content was observed as MTGase levels increased in fish myofibrillar protein (FMP) films. The decrease in moisture content is likely due to TGase-induced crosslinking, which reduces the protein's ability to absorb moisture by increasing the proportion of hydrophobic groups (Tang et al., 2005). Similarly, Jiang and Tang (2013) found a reduction in moisture content in gelatin films when 4 U/g of MTGase was incorporated. Tang et al. (2005) also reported that in soy protein isolate films, TGase reduced moisture content by crosslinking protein amino groups, particularly lysine residues, which are normally available for binding water through hydrogen bonding. This crosslinking makes fewer amino groups available for moisture uptake, further explaining the observed reduction in moisture content.

4.3.1.4.2 Water vapor permeability (WVP)

Table 4.1 shows that heat treatment does not significantly affect the WVP (water vapor permeability) of hordein-based films (P>0.05), and no synergistic effect was observed between heat and TGase treatment (P>0.05). While some studies suggest that heat treatment reduces WVP by increasing protein cross-linking, thereby restricting polypeptide chain mobility and reducing water diffusion (Hernández-Muñoz et al., 2004; Angellier-Coussy et al., 2011; Aguirre et al., 2011), our findings show no significant change in WVP due to heat treatment. This is consistent with Schmid et al. (2017), who found that although heat-treated whey protein films formed covalent bonds through disulfide linkages, as confirmed by protein solubility studies, there was no significant difference in WVP between native and heat-denatured whey protein films. Additionally, Hoque et al. (2010) reported varying WVP in cuttlefish skin gelatin films heated at different temperatures, with increased WVP observed at 40–70°C, but no difference at 80°C compared to the control. This could be attributed to the exposure of hydrophobic domains of gelatin chains at higher temperatures. Similarly, the lack of significant WVP change in our study may be due to the specific behavior of hordein proteins under heat treatment.

Table 4.1 demonstrates that TGase concentration had a statistically significant impact on WVP ($p < 0.05$), although the effect size was small. The WVP value decreased from 6.30 to 5.08 g.mm/m^2.d. KPa. While no significant changes were observed at lower TGase concentrations, a substantial reduction occurred at the highest concentration (40 U/g). This result is consistent with the findings of Wang et al. (2015), who reported that lower TGase concentrations did not significantly alter WVP in gelatin-calcium carbonate composite films, while higher concentrations yielded a significant reduction. Similarly, De Carvalho & Grosso (2004) reported a significant decrease in WVP in TGase-modified films compared to both native and chemically modified films. They attributed this to the enzymatic introduction of ε(γ-glutaminyl)-lysyl isopeptide bonds, enhancing the film's water barrier properties. Weng et al. (2010) also showed a decrease in WVP of fish scale gelatin films (with and without SPI) with increasing TGase concentration. These findings are consistent with other studies on TGase-induced lizardfish scale gelatin films, suggesting that TGase-induced crosslinking reduces the free volume within the gelatin matrix, thereby decreasing film permeability. This reduced free volume is likely a result of the increased crosslinking within the gelatin structure (Wangtueai et al., 2010; DeCarvalho and Grosso 2004).

4.3.2 Characterization of hordein-glycerol films

4.3.2.1Fourier transform infrared spectroscopy (FTIR)

Based on Figure 4.2, in the Amide I band (1600–1700 cm^{-1}), which is associated with C=O stretching and reflects the secondary structure of proteins, broadening was observed with increasing TG concentration, particularly in heat-treated films. This broadening indicates enhanced crosslinking between protein chains. According to peak assignment ranges, β-sheet structures (1607–1640 cm^{-1} and 1682–1696 cm^{-1}) likely become more prominent with increasing TGase concentrations, while α-helices (1651–1664 cm^{-1}) and β-turns (1664–1681 cm^{-1}) diminish. This shift towards a more β-sheet-rich structure indicates a more ordered, rigid protein matrix, which can be attributed to TGase's formation of covalent isopeptide bonds between glutamine and lysine residues (Chen et al., 2024). The presence of these crosslinks increases the structural stability of the films, especially in heat-treated samples, where the structural changes are more pronounced due to the enhanced efficiency of the TGase reaction under heat.

The Amide II band (1500–1590 cm^{-1}), which corresponds to N-H bending and C-N stretching, also exhibits a shift and broadening as TG concentration increases, more significantly in heat-treated films. This change reflects the disruption of hydrogen bonds and modifications to the peptide backbone due to TG crosslinking (Lin et al., 1991). The decrease in the amplitude of the Amide II band, especially in heat-treated films, points to stronger protein-protein interactions and reduced N-H bond flexibility, which deamination chemical modifications resulting from TG treatment could explain the decreased intensity in this region. The Amide III band (1200–1400 cm^{-1}), though less prominent, provides insights into C-N stretching and N-H bending vibrations, further illustrating the increase in β-sheets and random coils with higher TG concentrations and heat treatment. The broadening of this band suggests additional conformational changes, confirming the formation of a more organized and stable protein network. The O-H/N-H stretching region (3270–3300 cm^{-1}), related to hydrogen bonding, shows a shift to lower wavenumbers in heat-treated films, indicating stronger hydrogen bonding due to TG crosslinking. This enhanced hydrogen bonding contributes to the film's increased structural integrity (Chen et al., 2025). In contrast, the non-heat-treated films show less of a shift, reflecting weaker hydrogen bonding and lower crosslinking efficiency.

Similarly, the C-H stretching region (2900–2930 cm^{-1}), which reflects alkyl chain mobility, decreases in intensity as TG concentration increases, particularly in heat-treated films. This suggests a reduction in chain mobility as the protein network becomes tighter and more rigid due to cross-linking. The non-heat-treated films exhibit smaller changes, reinforcing the role of heat in enhancing TG-mediated crosslinking.

4.3.2.2 Atomic Force Microscope (AFM)

Figure 4.3 illustrates the AFM images of hordein films subjected to heating or treated with varying concentrations of TGase. The images reveal that images reveal that in the film without heat and TGase (NoheatT0), the surface is rough and uneven with small irregularities, indicating a poorly structured or crosslinked film. The films with no heat and 10U/g of TGase (NoheatT10) showed slight improvement in surface smoothness, although small aggregates and imperfections remain, suggesting that low concentrations of TGase induce minimal crosslinking, but the effect is not substantial. NoheatT20 films demonstrate a smoother surface compared to NoheatT10, but still not as smooth as films treated with both TGase and heat. However, the films treated with TGase concentrations above 30 U/g, whether heated or unheated, exhibit aggregates that contribute to increased surface roughness, which might be because excessive TGase may cause phase separation or uneven crosslinking (Masamba et al., 2016).

In contrast, the T0Heat film exhibits numerous pores, likely due to the thermal denaturation of hordein. Without TGase to stabilize the structure, the protein network becomes less organized and loses integrity under heat. This is supported by the FTIR results (Figure 4.2), where the broadening of the Amide I band, in the absence of crosslinking agents like TGase, is associated with protein denaturation and structural disorder. As observed in NoheatT0, the broad Amide I band reflects a disordered protein structure, indicating weaker molecular packing and increased susceptibility to pore formation.

Table 4.1 Effects of heat treatment/TGase on thickness, color, transparency, moisture content, WVP, tensile strength and elongation at break of hordein-based films (mean ± SD).

Treatment	**Thickness (μm)**	**L***	**a***	**b***	**Transparency Value (mm^{-1})**	**Moisture content (%)**	**WVP (g.mm/m^2.d.KPa)**	**Tensile strength (KPa)**	**Elongation at break (%)**
No heat	66.31 ± 11.87^{a}	78.62 ± 1.67^{a}	-0.90 ± 0.48^{a}	27.66 ± 2.03^{a}	1.22 ± 0.16^{a}	6.39 ± 3.02^{a}	5.83 ± 0.78^{a}	96.40 ± 12.94^{a}	107.14 ± 4.53^{a}
Heat	62.30 ± 12.89^{b}	79.39 ± 1.81^{b}	-0.77 ± 0.45^{a}	26.64 ± 2.00^{b}	1.30 ± 0.16^{a}	6.70 ± 1.72^{a}	5.61 ± 0.93^{a}	96.14 ± 17.67^{a}	106.73 ± 4.39^{a}
0TGase	62.71 ± 12.84^{a}	77.97 ± 1.77^{a}	-0.58 ± 0.46^{a}	28.16 ± 1.91^{a}	1.32 ± 0.14^{a}	8.92 ± 3.20^{a}	6.30 ± 0.59^{a}	92.78 ± 7.85^{a}	113.31 ± 3.66^{a}
10 TGase	62.50 ± 10.68^{a}	80.15 ± 1.83^{b}	-1.10 ± 0.41^{b}	25.97 ± 2.26^{b}	1.24 ± 0.19^{a}	6.54 ± 1.75^{ab}	5.91 ± 1.10^{ab}	98.34 ± 8.68^{a}	108.40 ± 1.91^{b}
20 TGase	60.51 ± 12.79^{a}	79.30 ± 1.42^{c}	-0.92 ± 0.33^{b}	26.90 ± 1.70^{c}	1.32 ± 0.20^{a}	6.49 ± 1.64^{ab}	5.28 ± 0.68^{b}	112.69 ± 8.69^{b}	105.58 ± 1.75^{c}
30 TGase	67.83 ± 12.23^{b}	79.01 ± 1.45^{c}	-0.87 ± 0.39^{bc}	27.43 ± 1.96^{ac}	1.18 ± 0.14^{a}	5.19 ± 0.98^{b}	6.03 ± 0.66^{ab}	96.70 ± 10.55^{a}	104.08 ± 1.90^{d}
40 TGase	67.97 ± 12.56^{b}	78.60 ± 1.70^{ac}	-0.69 ± 0.57^{ac}	27.28 ± 2.01^{ac}	1.25 ± 0.11^{a}	5.59 ± 2.65^{b}	5.08 ± 0.65^{b}	78.81 ± 17.73^{c}	103.13 ± 3.31^{d}
Heat*TGase P value	0.259	<0.001	0.021	0.002	0.751	0.207	0.575	0.003	0.209

Means with different letter in the same column indicate significantly differences between groups (P<0.05).
N.B. TGase stands for “transglutaminase”

Table 4.2 Effect of different concentrations of TGase under and without heat treatment on the thickness, color, moisture content, WVP, tensile strength and elongation at break of hordein-based films (mean ± SD).

Treatment	Thickness (μm)	L*	a*	b*	Moisture content (%)	WVP (g.mm/m2.d.KPa)	Tensile strength (KPa)	Elongation at break (%)
0T No heat	63 ± 12.36^{abc}	77.10 ± 0.89^{a}	-0.51 ± 0.31^{d}	29.34 ± 0.86^{a}	10.39 ± 4.19^{a}	6.34 ± 0.73^{a}	87.57 ± 5.29^{ab}	114.12 ± 3.93^{a}
10T No heat	65 ± 10.09^{bcd}	79.56 ± 2.15^{cd}	-1.09 ± 0.52^{a}	$26.49 \pm 2.65^{\mathrm{def}}$	5.45 ± 1.96^{b}	5.62 ± 1.16^{ab}	98.79 ± 6.72^{c}	107.87 ± 1.37^{b}
20T No heat	64 ± 12.76^{bc}	78.69 ± 1.12^{bcd}	-0.93 ± 0.35^{abc}	27.67 ± 1.12^{bcd}	5.40 ± 1.69^{b}	5.52 ± 0.67^{ab}	109.93 ± 6.79^{d}	105.42 ± 1.83^{cd}
30T No heat	71.67 ± 11.47^{d}	78.49 ± 0.64^{bc}	-1.01 ± 0.21^{ab}	28.22 ± 0.75^{ab}	5.62 ± 0.87^{b}	6.12 ± 0.84^{ab}	98.38 ± 9.44^{c}	104.77 ± 2.15^{cde}
40T No heat	67.93 ± 11.14^{cd}	79.29 ± 1.93^{cd}	-0.95 ± 0.68^{abc}	26.58 ± 2.50^{cdef}	5.07 ± 2.95^{b}	5.53 ± 0.57^{ab}	84.83 ± 16.46^{a}	102.83 ± 1.28^{e}
0T Heat	62.41 ± 13.54^{abc}	78.84 ± 2.01^{bcd}	-0.66 ± 0.57^{cd}	26.98 ± 1.95^{bcde}	7.45 ± 1.23^{ab}	6.28 ± 0.59^{a}	98.38 ± 6.13^{c}	112.43 ± 3.27^{a}
10T Heat	60 ± 10.83^{ab}	80.75 ± 1.23^{e}	-1.10 ± 0.26^{a}	25.45 ± 1.73^{f}	7.63 ± 0.49^{ab}	6.20 ± 1.19^{a}	97.93 ± 10.41^{c}	108.90 ± 2.24^{b}
20T Heat	56.90 ± 11.98^{a}	79.92 ± 1.44^{de}	-0.91 ± 0.33^{abc}	26.14 ± 1.86^{ef}	7.58 ± 0.56^{ab}	5.03 ± 0.73^{ab}	115.64 ± 9.70^{d}	105.75 ± 1.72^{c}
30T Heat	64 ± 11.92^{bc}	79.53 ± 1.83^{cd}	-0.73 ± 0.47^{bcd}	26.65 ± 2.47^{cdef}	4.76 ± 1.03^{b}	4.62 ± 0.34^{ab}	95.14 ± 11.62^{bc}	103.45 ± 1.42^{de}
40T Heat	68 ± 14.00^{cd}	77.91 ± 1.13^{ab}	-0.43 ± 0.25^{d}	27.99 ± 1.02^{abc}	6.10 ± 2.83^{b}	5.72 ± 0.85^{b}	73.64 ± 17.69^{e}	103.38 ± 4.42^{de}

Means with different letter in the same column indicate significantly differences between groups (P<0.05).
N.B. T stands for 'TGase'.

Table 4.3 Effect of different concentrations of TGase under and without heat treatment on the transparency of hordein-based films (mean ± SD).

Treatment	**Transmittance (%) at wavelength (nm)**								**Transparency**
	200	280	350	400	500	600	700	800	**Value (mm^{-1})**
0T No heat	0.28	0.54	7.05	33.00	67.00	82.39	86.58	88.32	1.26 ± 0.12^{a}
10T No heat	0.30	0.61	11.97	39.36	70.61	84.20	87.95	89.61	1.15 ± 0.10^{a}
20T No heat	0.24	0.52	7.93	33.58	67.47	82.91	87.32	89.32	1.28 ± 0.28^{a}
30T No heat	0.24	0.54	8.53	34.37	68.00	83.12	87.18	88.93	1.12 ± 0.08^{a}
40T No heat	0.28	0.77	9.97	36.27	68.06	81.96	85.89	87.61	1.28 ± 0.15^{a}
0T Heat	0.26	0.52	6.91	31.85	65.71	82.00	86.43	88.20	1.38 ± 0.17^{a}
10T Heat	0.25	0.49	7.57	31.85	66.15	83.20	87.84	89.86	1.33 ± 0.24^{a}
20T Heat	0.24	0.52	9.15	34.88	68.01	83.69	87.99	89.91	1.36 ± 0.11^{a}
30T Heat	0.26	0.64	11.34	36.99	68.51	83.48	87.50	89.26	1.23 ± 0.18^{a}
40T Heat	0.28	0.80	10.40	35.47	67.51	82.64	86.89	88.80	1.22 ± 0.06^{a}

Means with different letter in the same column indicate significantly differences between groups (P<0.05).
N.B. T stands for ‘TGase’.

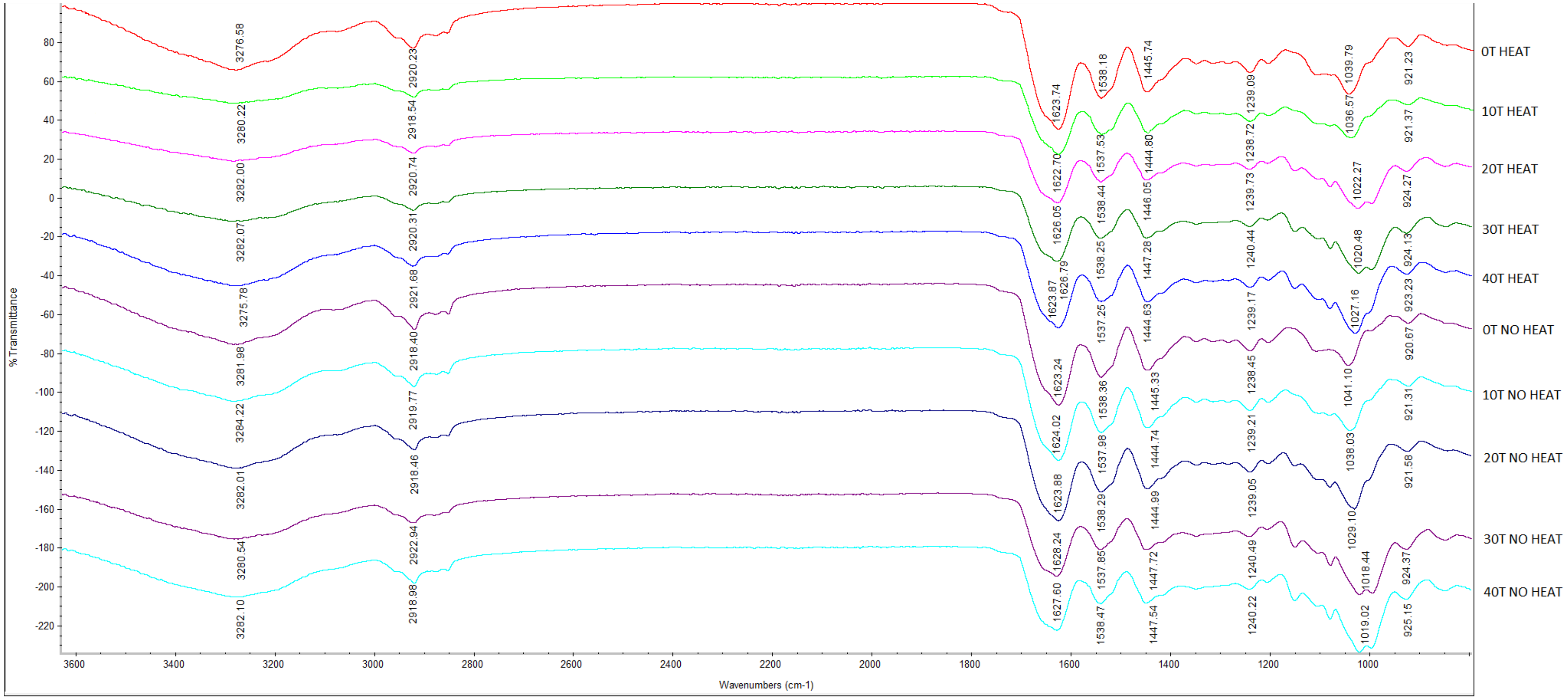

Fig. 4.2 ATR-FTIR spectra of the different concentrations of TGase under and without heat treatment of hordein films. 0T, 10T, 20T, 30T, and 40T stand for adding 0U/g, 10U/g, 20U/g, 30U/g and 40U/g transglutaminase, respectively.

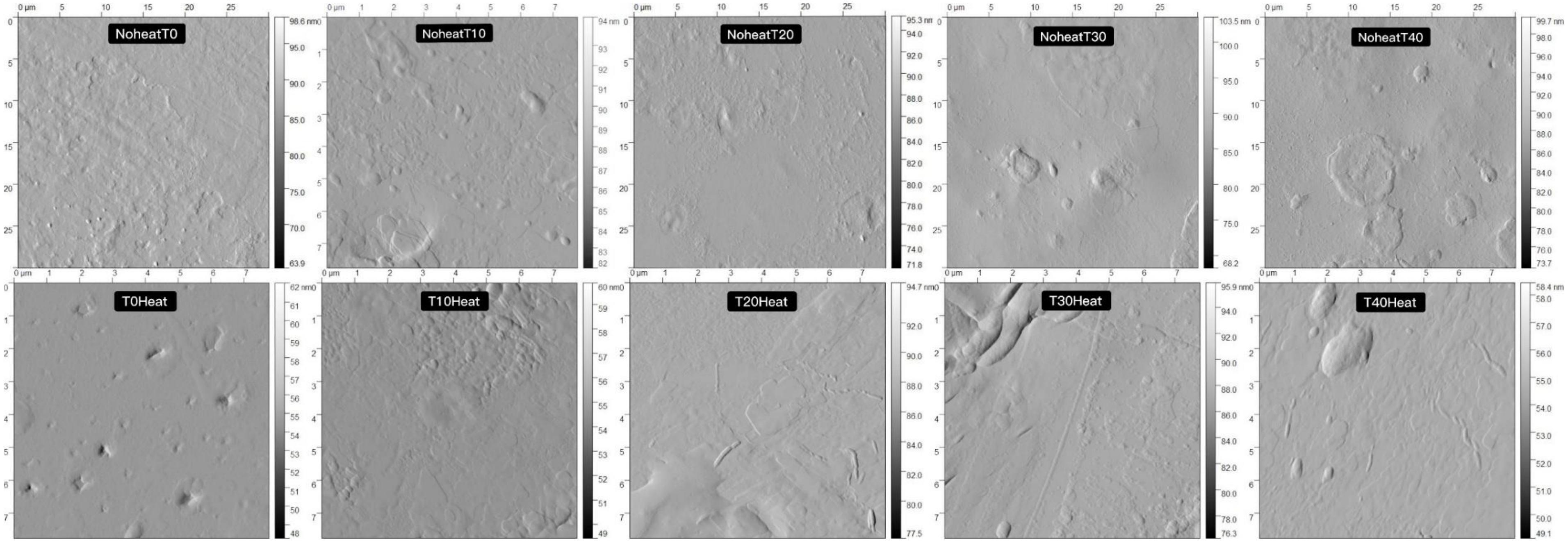

Fig. 4.3 AFM images of hordein films treated with heat or without heat at various concentrations of TGase .

4.4 Conclusion

Compared to untreated hordein-based films, heat-treated barley hordein films exhibited reduced thickness, while water vapor permeability, moisture content, tensile strength, and elongation at break remained largely unaffected. Treatment with high concentrations of TGase (above 30 u/g) increased the thickness and reduced the moisture content of the films. The tensile strength initially increased with TGase concentration but then declined. However, the elongation at break decreased with increasing TGase concentrations. Regardless of heat treatment, films with 20 u/g TGase demonstrated the highest tensile strength.

Therefore, heat treatment is not an effective method for improving the mechanical properties of barley hordein films. Although TGase addition enhances mechanical properties, it still falls short of meeting market requirements. As a preliminary study on barley hordein films, it is noteworthy that despite the suboptimal mechanical properties, the films exhibit good water vapor and UV barrier properties, making them a potential candidate for food packaging, especially for high-moisture foods. Further research is warranted.

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CHAPTER 5

Improving the Quality of Starch Film through Hordein Integration

Chapter 5: Improving the Quality of Starch Film through Hordein Integration

Abstract

The persistence of petroleum-based plastics in the environment necessitates the development of biodegradable alternatives. Starch films have good film-forming properties but are often brittle and moisture-sensitive. Hordein protein with good hydrophobic property may have the potential to improve the quality of starch film. To leverage the strengths of each component, a two-stage experimental design was employed. Corn starch was identified as the optimal base material for film production, outperforming potato and wheat starch in comparative analysis. Subsequently, hordein was incorporated into the corn starch matrix at varying mass ratios (10%, 20%, 30% and 40% based on the weight of corn starch) using the casting method. The resulting composite films were characterized from the aspect of mechanical, barrier, optical, and microstructural properties. The incorporation of hordein significantly enhanced the tensile strength of the corn starch films compared to starch without hordein, while 40% incorporation achieved the highest tensile strength. However, high hordein content (≥30%) led to a decrease in elongation at break, highlighting a classic trade-off between strength and flexibility. The addition of hordein also increased film thickness, reduced transparency and significantly increased UV blocking. However, there were no significant changes in moisture content and water vapor permeability (WVP), suggesting limited modification in hydrophilic property which could be explained by the poor compatibility at high concentration. In conclusion, corn starch offers good choice for developing biodegradable packaging, while 10% hordein incorporation to corn starch offered the best balance of optimal quality including mechanical properties, transparency and WVP, demonstrating its potential for developing sustainable packaging materials with enhanced performance.

Keywords: Starch, Hordein, film properties, packaging.

5.1 Introduction

Petroleum-based plastics are non-biodegradable and contribute to environmental pollution, prompting researchers to seek biodegradable alternatives (Tyński, et al., 2019). Biodegradable films derived from natural resources like carbohydrates, lipids, and proteins offer promising substitutes for traditional plastics (Dirpan, Ainani, and Djalal, 2023). Moreover, composite films incorporating two or more of the aforementioned components are extensively researched, with the aim of optimizing the physicochemical properties of the films (Achari, Heggannavar, & Kariduraganavar, 2020).

Starch, a natural polysaccharide material, exhibits advantages such as widespread availability, good renewability, biodegradability, low cost, and excellent film-forming capabilities (Ortega-Toro, et al., 2017). Consequently, starch has emerged as a preferred material for developing biodegradable food packaging. Nevertheless, films made from natural starch often exhibit drawbacks, including poor mechanical properties, high water sensitivity, and notable brittleness. (Shah, U. et al., 2015). Hence it is useful to find effective strategies to reduce the hydrophilic property of starch films and enable them for a wide range of food products.

Hordein, a protein extracted from Brewers Spent Grain (BSG)—a major by-product of the brewing industry presents a promising opportunity for value-added applications (Stojceska, 2019; Mussatto, 2014). Hordein is characterized by its high glutamine content and a significant presence of hydrophobic amino acids such as proline, leucine, and valine. These biochemical properties contribute to strong protein aggregation and high hydrophobicity, which are desirable traits for the development of biodegradable films (Zhao et al., 2010). However, hordein films exhibit brittle mechanical characteristics, limiting their application (Wang et al., 2010; Cho and Rhee, 2009). While there are no reports on blending hordein with starch, zein—another prolamin protein with properties similar to hordein—has been combined with starch to improve the material properties of both biopolymers (Corradini et al., 2007; Habeych et al., 2008). The complementary characteristics of hydrophilic starch and hydrophobic zein have been shown to enhance the overall film properties. Therefore, blending starch with hordein has the potential to improve the mechanical and barrier properties of the films, offering a promising solution for biodegradable packaging applications.

In this study, a two-stage experimental design was employed. The first stage focused on identifying a suitable type of starch with optimum film quality. While the interactions between the selected starch and hordein at different ratios were investigated to optimize the quality of composite film in the second stage. Once the optimal starch type was selected, the second stage involved the incorporation of hordein at varying levels to optimize the properties of the starch film. Starch films of different types, as well as corn starch/hordein composite films, were prepared using the drop-casting method. The films were evaluated from the aspects of mechanical properties, color, thickness, and water vapor permeability. Fourier-transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM) were also utilized to investigate the chemical and microstructural changes of the films.

5.2 Materials and methods

5.2.1 Materials

Barley flour, potato starch containing 20% amylose and corn starch containing 28% amylose were purchased from Buy Whole Foods Online Ltd., UK. Wheat starch containing 26% amylose was purchased from Foo Lung Ching Kee Ltd. Glycerol (≥99.0%) and PEG400 were both purchased from Sigma Chemical Co. (UK). Hordein was extracted from barley flour using a 75% aqueous ethanol solution, yielding 84.4% protein based on the previously described extraction method (Wang et al., 2010). The characterization of the hordein extract by the Kjeldahl method was consistent with results reported by Qazanfarzadeh et al. (2021). The specific extraction procedure is the same as outlined in Chapter 3.

5.2.2 Experiment handling

5.2.2.1 Stage 1: identification of starch type with optimal film quality

Potato starch, wheat starch and corn starch were chosen to compare their film-forming quality. The films were fabricated by employing the casting method according to previous research with slight adjustments (Shah et al., 2016, Liang & Wang, 2018). 5g of starch was placed in a beaker containing 100 ml deionized water, and 30% composite plasticizer (comprising 75%glycerol and 25%PEG) was added based on the weight of starch. The mixture was stirred at 400rmp on a hotplate (Four E's Scientific, China) at 90°C for 20 minutes to form starch film-forming dispersion. The resulting dispersion was further heated at 90°C in a water bath (Grant Instruments, UK) or 30 minutes to achieve complete gelatinization of the starch. The dispersion was then cooled to 40°C at room temperature before casting. 15 ml of the dispersion was poured into a Petri dish and then put into an oven (Sanyo, Japan) at 35°C for 20 hours for drying at 8% relative humidity (RH). Then, the formed films were conditioned in a desiccator at 20°C with 52% RH for 7 days before further testing. The quality of starch films was assessed from mechanical properties, color and water vapor permeability which served as judgement points to identify the optimal starch to work with hordein for film production.

5.2.2.2 Stage 2: interaction between the chosen starch and hordein at different ratios

After comparing the film-forming properties, corn starch was selected to investigate the interaction between starch and hordein at different ratios in order to improve the quality of the composite film. A 5% (m/v) corn starch solution was prepared by dissolving 2.5 g of corn starch in 50 ml of deionized water, along with a 30% composite plasticizer composed of 75%GLY and 25%PEG. The mixture was heated at 90°C for 20 minutes and stirred at 400 rpm to ensure proper dispersion and dissolution. After cooling the starch solution to room temperature, it was mixed with the hordein solution. To prepare a 33.3% (g/v) hordein solution, 2.96 g of hordein extract (84.4% purity) was dissolved in 75 ml of 75% ethanol with continuous stirring to accelerate the dissolution process. To achieve hordein levels of 10%, 20%, 30%, and 40% based on starch weight, varying volumes of hordein solution (7.5, 15, 22.5, and 30 ml, respectively) were mixed with 50 ml of corn starch solution. The mixtures were stirred for 15 minutes to ensure uniformity. Since higher hordein solution concentrations increased the solvent volume, the volume poured into Petri dishes was adjusted to maintain a consistent dry weight of starch and hordein (0.75 g) per dish. The films were dried at 35°C for 20 hours in an oven at 8% relative humidity (RH), then stored in a desiccator at 20°C and 52% RH for 7 days before further testing.

5.2.3 Fourier Transform Infrared Spectrometer (FTIR)

To study the chemical structures and interactions between components, the FTIR spectra of films were obtained using an FTIR spectrophotometer (Spectrum 100, PerkinElmer, US) with an Attenuated Total Reflectance (ATR) attachment and selecting a wavenumber resolution of 4 cm−1 for 16 scans from 4200 to 650 cm−1.

5.2.4 Scanning electron microscope (SEM)

Small square sections were cut from the main film and were attached to an aluminium SEM stub using a double-sided sticky, conducting carbon tab and coated with gold for 2.5 mins at a setting of approximately 0.9kV at ~30mA using an Edwards S150B gold coater. Scanning electron microscopy (Cambridge Instruments SEM, UK) was performed on a Cambridge Instruments Stereoscan 360. Images were obtained using an operating voltage of [X] kV. The backscatter detector was used for imaging.

5.2.5 Viscosity

Viscosity of different concentrations of corn starch/hordein mixed solution was conducted using a Physica MCR 101 Rheometer (Anton Paar, Ostfildern, Germany) using a bob and cup. Shear rate was increased from 0.1 to 100/s and reduced back from 100 to 0.1/s. The measurements were taken at 25 °C.

5.2.6 Thickness

The thickness of the films was measured with an electronic digital micrometer (Fisher Scientific, United Kingdom) with a sensitivity of 0.001 mm. At least 10 random spots were chosen for analysis. The obtained data were used for further analysis of WVP and mechanical properties.

5.2.7 Mechanical properties:

Tensile strength (TS) and elongation at break (EB) were measured using a TA-XTS Texture Analyzer, Stable Microsystems (Godalming, UK), as described by Patzsch et al. (2010). Samples were conditioned for 7 days at 57% relative humidity. Each film was cut into strips (0.8 cm × 0.5 cm) and tested using a 30 kg load cell and a 50 mm/min primary grip breaking speed for tensile strength and elongation tests.

5.2.8 Optimal properties

5.2.8.1 Transparency

The opacity of the films was measured using a NIR-VIS-UV Cary7000 spectrophotometer (Agilent Technologies, Santa Clara, US) at wavelengths from 200 to 800 nm, with measurements repeated three times. Transparency values were calculated using the equation by Han & Floros (1997):

$$\text{Transparency value} = -\log T_{600}/\text{d}$$

T_{600} is the fractional transmittance at a wavelength of 600 nm; d is the thickness(mm) of the film. The higher value of transparency value presents the lower film transparency.

5.2.8.2 Color measurement

The color properties of the films were determined using a CR-400 Chroma Meter (Konica Minolta, Japan) based on the CIELab color space, evaluating lightness (L*), red/green (a*), and yellow/blue (b*) against a white background. Five repeated measurements were conducted on each sample and average was used to present the color feature of the film.

5.2.9 Water behavior

5.2.9.1 Film moisture content:

Moisture content was determined according to the method described by Zahedi, Fathi-Achachlouei and Yousefi (2018). The weights of the film sample (2 cm × 2 cm) before (Wi) and after (Wd) drying at 105 °C in oven for 24 h were recorded and moisture content was calculated as the percentage of weight loss by using the equation,

$$\text{Moisture content (\%)} = [(W\text{i} - W\text{d})/W\text{i}] \times 100$$

Wi – Initial weight of film before drying
Wd- weight of film after drying

5.2.9.2 Water vapor permeability (WVP):

Films were firstly conditioned at 57% relative humidity in a desiccator for 7 days. A piece of film was placed on top of individual glass vials (The bottle has a bottom diameter of 4 cm, a height of 7 cm, and an open mouth with a diameter of 1.58 cm) filled with silica gel, and the edges were then sealed using parafilm to ensure all moisture absorption was attributed to the area covered by the film, then vials were placed in a desiccator containing a saturated solution of potassium sulfate (98% RH). The vials' weights were recorded every 24 hours for 7 days. Water vapor transfer rate (WVTR) and WVP were by using the equation:

$$\text{WVTR } (\text{g/m}^2\cdot\text{day}) = \text{slope/area}$$

$$\text{WVP}(\text{g.mm}/\boldsymbol{m^2}\text{.d.kPa})=(\text{WVTR x L })/\Delta\text{P}$$

WVTR ($\text{g/m}^2\cdot\text{day}$): Water Vapor Transmission Rate. Slope (g/day): The rate of weight gain or loss in the test. Area (m^2): The surface area of the film exposed to water vapor transmission. L is thickness(mm), ΔP is difference in water vapor partial pressure(kPa) between both sides of the film, respectively.

5.2.10 Statistical analysis

All treatments were repeated three times in consideration of the batch effect. All data were analyzed individually using one-way ANOVA (IBM SPSS Statistics 28.0.0.0 software, UK). Duncan's multiple comparison test was used to determine significant differences between means at a significance level of 0.05.

5.3 Results and discussions

5.3.1 Stage 1: identification of starch type with optimal film quality

5.3.1.1 Thickness

As shown in Table 5.1, the potato starch film is the thickest and the wheat starch film has the lowest thickness ($p<0.05$). That could be explained by the amylose level in the starch products. Potato starch contains 20% amylose, while wheat starch has 26% and corn starch has 28% amylose, respectively. According to the morphological property of starch molecule, amylose has a lined structure which allows it to develop compact structure. In contrast, amylopectin is more branched and not easy to compact together. Consequently, an increased presence of amylopectin within the molecular structure is associated with an augmented thickness. Potato starch has a higher prevalence of amylopectin (Basiak et al., 2017), so higher thickness is expected. According to Singh et al (2003), potato has high swelling powder compared to corn starch and wheat starch due to its high level of phosphate groups. The phosphate group would create repulsion force between adjacent amylopectin chains to increase hydration which resulted in a thicker film.

5.3.1.2 Color

Color is a basic property of films that determines consumers' willingness to purchase, and it is affected by the type of starch, size and shape of starch granules plus the thickness of the film. The color parameter L* is the lightness, a* is the color change from greenness to redness, and b* is the blueness to yellowness. In the current investigation, wheat and corn starch have higher L* values than potato starch, while the difference could be explained by the amylose level. As mentioned earlier, amylose has a more compact structure, facilitating greater reflection of light, thereby resulting in higher L* values. Although there are significant differences in a* and b* among the three starch products, the small variation of the a* and b* values possibly would not interfere with consumers' perception about food (Basiak et al., 2017). Regarding the morphology aspect, the supportive side (referring to the surface in contact with the Petri dish during the film-drying process) of all starch films exhibited a lustrous appearance. The side of exposing air during drying appeared matte, aligning with the observations made by Bonilla et al. (2013).

Table 5.1 Thickness, color, moisture content, and WVP of wheat, corn and potato starch-based films (mean ± SD).

	Thickness (μm)	**a***	**b***	**L***	**Moisture content (%)**	**WVP (g.mm/m2.d.KPa)**
Wheat starch	40.33 ± 1.83^{a}	-0.53 ± 0.03^{a}	4.25 ± 0.07^{a}	93.71 ± 0.34^{a}	18.66 ± 0.61^{a}	9.85 ± 0.6^{a}
Corn starch	44.67 ± 6.29^{b}	-0.85 ± 0.06^{b}	$5.11 \pm 0.12^{\mathrm{b}}$	93.87 ± 0.18^{a}	18.02 ± 1.15^{a}	10.08 ± 1.46^{a}
Potato starch	58 ± 8.87^{c}	-0.49 ± 0.03^{c}	4.36 ± 0.08^{c}	92.99 ± 0.57^{b}	17.45 ± 0.52^{a}	12.81 ± 0.86^{b}

Means with different letter in the same column indicate significantly differences between groups (P<0.05).

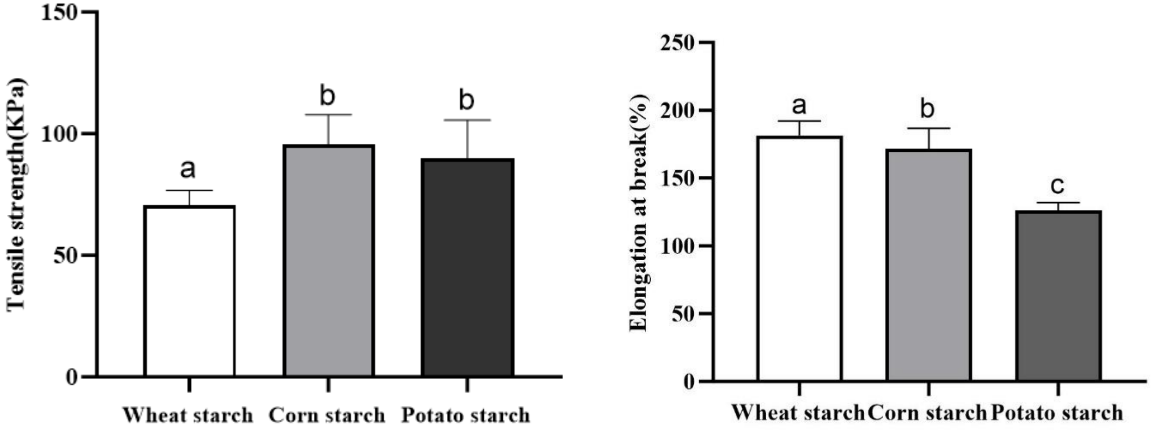

Fig. 5.1 Tensile strength and elongation at break of wheat, corn and potato starch-based films. Different letters in each figure indicate significant differences between groups (P<0.05).

5.3.1.3 Moisture content and WVP

Table 5.1 indicate that there were no significant differences in the moisture content among the three types of starch films. However, the water vapor permeability (WVP) of the potato starch film was significantly higher than that of wheat and corn starch films ($p<0.05$). This finding contrasts with the results of Dai et al. (2019), who reported no significant difference in WVP between corn, wheat, and potato starch films. Alves et al. (2007) highlighted that WVP in edible films depends on various factors, including film integrity, thickness, glycerol content, and the hydrophilic-to-hydrophobic ratio. Rindlav-Westling et al. (1998) noted that amylopectin-based films generally exhibit higher WVP than amylose-based films, possibly because amylose's linear structure promotes tighter molecular packing, making it harder for water vapor to pass through. Given that potato starch has the lowest amylose content, this could explain its higher WVP. Additionally, Rindlav-Westling et al. (1998) found that differences in crystallinity among amylopectin films did not significantly affect WVP. Therefore, the discrepancy in results between studies may be attributed to differences in the starch film preparation processes.

5.3.1.4 Mechanical properties

As presented in Figure 5.1. There is no difference in tensile strength for films of corn starch and potato starch, but both films have significantly higher tensile strength than wheat starch film ($p<0.05$). The amylose/amylopectin ratio and the thickness of the films could explain the differences in mechanical strength. Domene-Lopez et al. (2019) reported that amylose was one of the most important factors affecting the tensile behavior, and high amylose resulted in high tensile strength. Corn starch contains the highest amylose, which can form more compact structure, therefore, the highest tensile strength is expected for corn starch-based film. Tensile strength is positively correlated with thickness, thicker film will result in a higher tensile strength. According to Table 5.1, films made from potato starch is much thicker than these from corn/wheat starch, hence higher tensile strength was expected, although the amylose level is not the highest one.

In terms of elongation, Figure 5.1 shows that wheat starch films are more deformable than corn and potato starch films, while the potato starch film has the lowest elongation at break ($p<0.05$). Basiak et al. (2017) reported that mechanical resistance of starch film is mainly related to the thickness. According to Table 5.1, potato film had the highest thickness and resulted the stronger resistance for deformation, hence the elongation rate is lower. For the difference between corn starch film and wheat starch film, the amylose content in the starch may explain the difference. Palviainen et al (2001) indicated that starch with high amylose content offered more rigid film due to the increased crystallinity. Corn starch has 28% vs 26% amylose in wheat starch, therefore, high rigidity in corn starch was expected compared to wheat starch, i.e. low elongation rate for corn starch. These results are also consistent with the inverse relationship between tensile strength and elongation observed by Żołek-Tryznowska and Kałuża (2021).

The tensile strength value achieved in this work (potato, corn and wheat starch) ranges from (70.57~95.64 KPa) which are lower than the starch film (3.29~6.56MPa) reported by Basiak et al. (2017) and 1.49~3.05 MPa reported by Żołek-Tryznowska and Kałuża (2021). The deviation in the tensile strength could be explained by thickness content. Jansson and Thuvander (2004) reported that the starch film strength is strongly dependent on the thickness with higher strength for thicker film. In this work, films had thickness 0.04~0.06mm, which are much thinner than 0.05~0.11mm in the work of Basiak et al. (2017) and 0.27~0.33mm in the work of Żołek-Tryznowska and Kałuża (2021). For the film flexibility, the moisture content may explain the deviation in elongation rate compared to other published work. According to Basiak et al. (2017) and Żołek-Tryznowska and Kałuża (2021), moisture contents of starch film ranged from 20%~30%, while around 18% of moisture content was obtained in the starch film. As water is effective plasticizer for starch, it could increase the flexibility with high moisture content. In addition, the level of plasticizer used in the film formation could also greatly affect the elongation rates. 50% of plasticizer addition in other research's experiments would have stronger plasticizing result compared to 30% addition in this work.

Corn, potato and wheat are primary agricultural products, and corn starch contributed to 84% of starch market (Basiak et al., 2017). From the cost point of view, corn starch is cheapest one among them. In this work, corn starch film achieved the best balance of tensile strength and elongation at break compared to other potato starch and wheat starch with comparable results in moisture content and color quality, hence corn starch was selected for further investigation.

5.3.2 Stage2: Investigation of corn starch/hordein composite films

5.3.2.1 Fourier transform infrared spectroscopy (FTIR)

Based on Figure 5.2, the FTIR spectra provide insights into the chemical structure of corn starch films incorporated with varying concentrations of hordein at 10% (CS10H), 20% (CS20H), 30% (CS30H), and 40% (CS40H). In the O-H/N-H stretching region (3200–3400 cm^{-1}), all films exhibit broad peaks, indicating hydrogen bonding. As the concentration of hordein increases, this peak broadens slightly, suggesting stronger hydrogen bonding interactions between starch and hordein due to the introduction of amino acid side chains in hordein. In the C-H stretching region (2900–3000 cm^{-1}), the bands remain relatively consistent across all samples, indicating that alkyl chain mobility is not significantly impacted by the addition of hordein. A key observation is the significant absorption peak around 1652 cm^{-1} present in all hordein-incorporated films (CS10H, CS20H, CS30H, and CS40H). This peak, attributed to C=O stretching vibrations, is characteristic of protein structures and is absent or less pronounced in the pure corn starch film. The increasing intensity of this peak with higher hordein content suggests a proportional increase in protein interactions within the film matrix, reflecting the successful integration of hordein. On the other hand, the increased intensity could also imply the secondary structure changes, FTIR spectroscopy reveals that the incorporation of hordein into corn starch films leads to significant alterations in the protein secondary structure. Specifically, there is a noticeable shift towards β-sheet dominance, suggesting enhanced crosslinking and structural rigidity. This is evident from the increase in β-sheet peaks within the Amide I region (1607–1640 cm^{-1} and 1682–1696 cm^{-1}), an effect that becomes more pronounced with increasing hordein concentrations. Furthermore, the addition of hordein, particularly at higher concentrations (20%-40%), corresponds with a decrease in α-helix (1651–1664 cm^{-1}) and β-turn (1664–1681 cm^{-1}) regions, indicating a potential reduction in flexibility (Chen et al., 2024). In summary, while the native corn starch matrix already possesses a relatively ordered structure, the introduction of hordein, even at low concentrations (10%), further promotes the formation of a highly organized and rigid film characterized by a predominance of β-sheets.

Further analysis of the spectra reveals absorption peaks in the 1366-1367 cm^{-1} range across all samples, linked to C-H bending vibrations (Yang, L. et al., 2014). This consistent feature indicates that the basic starch structure remains intact despite the incorporation of hordein. Additionally, in the region around 1142-1149 cm^{-1}, another set of absorption peaks is observed, likely corresponding to C-O-C stretching vibrations typical of starch structures. The presence of these peaks in all samples, including those with hordein, suggests that the fundamental polysaccharide backbone of the starch is preserved (Wongphan & Harnkarnsujarit, 2020). The peak at 1017 cm^{-1} is found in all films with hordein addition but not observed in pure starch film, and this peak is associated with C–O stretching vibrations, representing the amino acid residuals containing carboxyl groups or amide groups such as aspartic acid, glutamic acid, asparagine and glutamine on the side chain of polypeptides.

Overall, the FTIR analysis demonstrates that the addition of hordein to corn starch films leads to noticeable changes in the chemical structure, particularly with the introduction of protein-specific absorption peaks at 1652 cm^{-1}. As the concentration of hordein increases, these peaks become more prominent, indicating an increased presence of protein interactions within the film. These structural changes are likely to influence the physical and functional properties of the films, correlating with the variations in thickness, color, and transparency as hordein concentration increases.

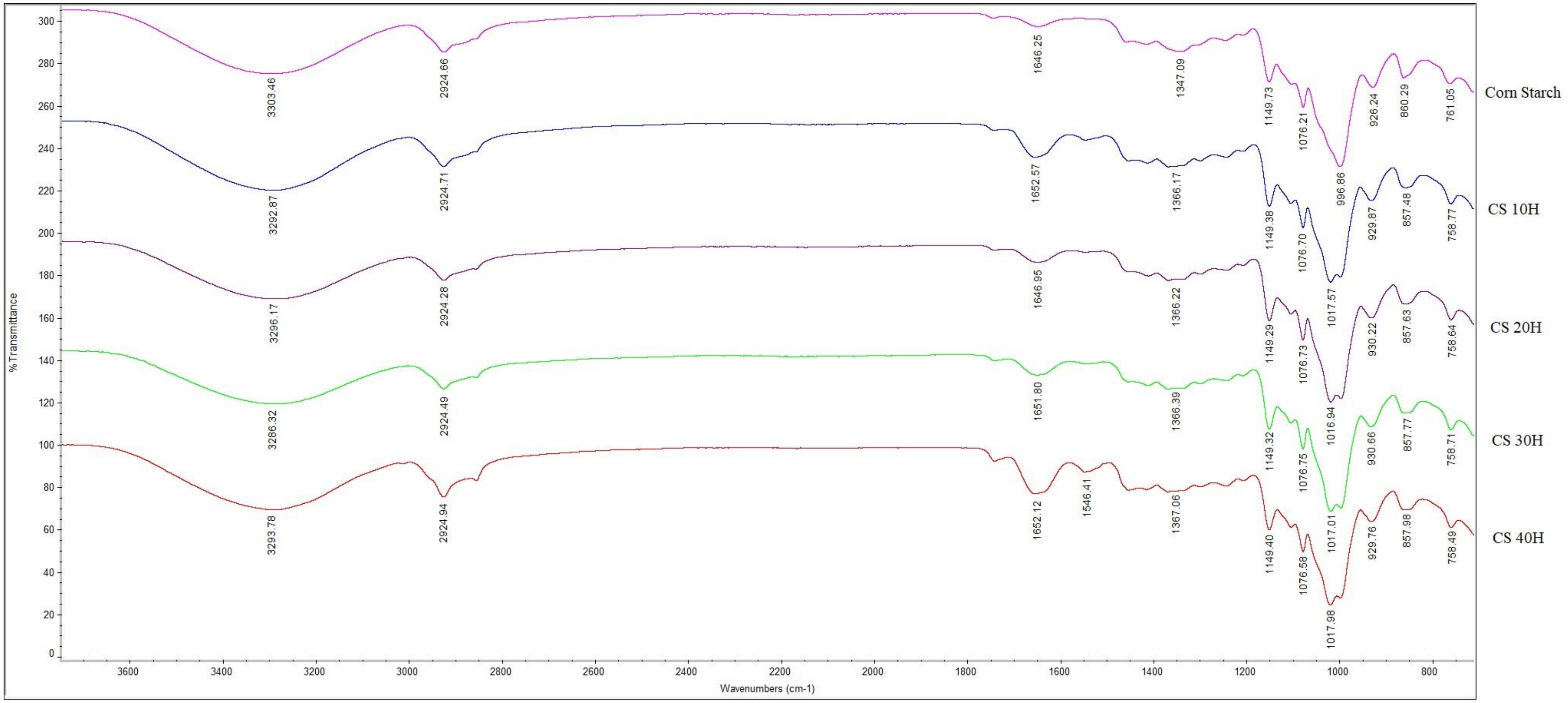

Fig. 5.2 Comparison of FTIR spectra of corn starch/hordein composite films.

5.3.2.2 Scanning electron microscope (SEM)

According to Figure 5.2, FTIR spectra reveal key chemical changes in the film's structure, particularly the increasing intensity of the amide I band at 1652 cm^{-1}, which is characteristic of protein secondary structures. This peak's growth with higher hordein content indicates a successful integration of hordein into the starch matrix, reflecting an increase in protein interactions within the film. These interactions likely contribute to the observed changes in the films' physical properties. The Figure 5.3 SEM images depict the microstructure changes of the film with varying concentration of hordein addition. The pure corn starch film, as seen in the SEM image, is characterized by a uniform and dense structure, which aligns with the FTIR results indicating a consistent polysaccharide network.

With the increase of hordein concentration, roughness on the surface increased along with sign of protein aggregation, which could be attributed to the interactions between starch and hordein molecules, as suggested by the increased intensity of the amide I band in the FTIR spectra (Habeych et al., 2006, and Leroy et al. 2012). At higher hordein concentrations (CS20H and CS30H), more pronounced surface irregularities and a less compact structure were disclosed. This microstructural disruption is also reflected in the FTIR spectra, where the intensity of the amide I band continues to increase, indicating that more protein integration would interfere with the cohesiveness of starch network, leading to less homogeneity. Finally, in the CS40H sample, significant protein aggregation and a highly irregular surface is observed, reflecting a stronger interference with starch network due to the incompatibility which leads to more gaps and holes found in the film.

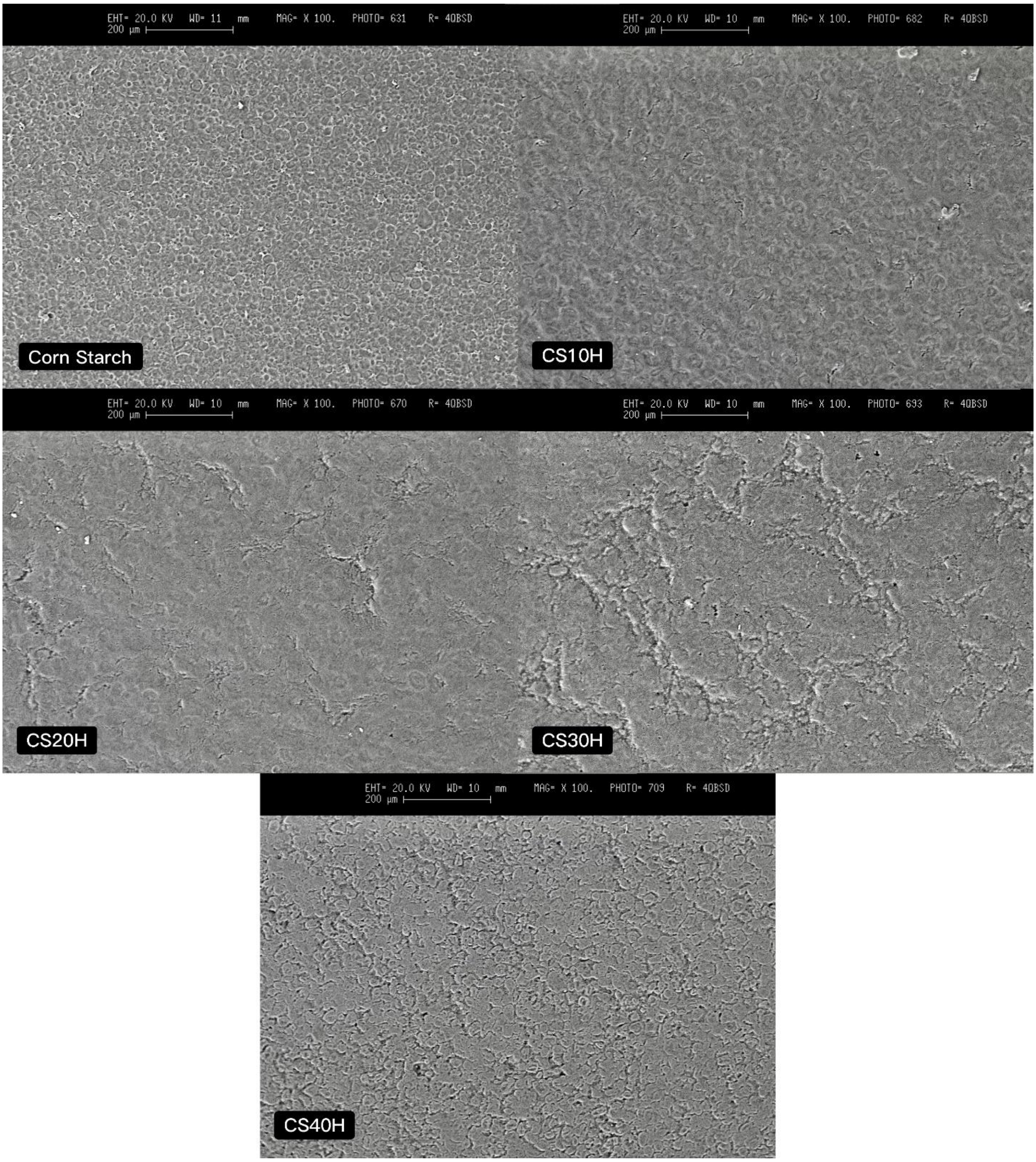

Fig. 5.3 SEM micrographs of corn starch/hordein composite films

5.3.2.3 Viscosity

The rheological behavior of the corn starch/hordein composite films (as shown in Figure 5.4) reveals that viscosity decreases as hordein content increases. This trend, evident in the viscosity versus shear rate graph, is supported by both Figure 5.3 SEM and Figure 5.2 FTIR analyses, which highlight the structural changes induced by hordein incorporation.

This finding is consistent with previous studies on starch-protein composite films. For instance, Corradini et al (2005) reported a viscosity reduction of corn starch solution when zein was incorporated. Similarly, Welday et al (2015). reported that blending zein with pre-gelatinized maize starch reduced the apparent viscosity compared to the pre-gelatinized maize starch alone. Qiu et al (2015) also showed that the viscosity of waxy corn starch-zein composite solution decreased with increasing zein content, attributing to the substitution of starch with zein.

Corn starch films exhibit the highest viscosity across all shear rates, indicating a strong, cohesive network within the starch matrix. This observation aligns with the SEM images, a dense, uniform microstructure. However, the addition of hordein disrupts this starch network. Even at a low concentration (CS10H), a noticeable viscosity reduction is observed. This reduction coincides with the emergence of phase separation and surface roughening in SEM images and the appearance of a protein-specific peak (1652 cm^{-1}) in FTIR spectra, indicating hordein integration within the film. This observation aligns with the findings of Liu et al. (2019), who reported that the addition of zein to starch solution led to a decrease in peak viscosity, trough viscosity, final viscosity, and breakdown. They attributed this to the hydrophobic nature of zein, which can reduce water binding and amylose leaching, thereby improving the thermal stability of the composites. Additionally, they suggested that zein could inhibit the recrystallization of starch chains during cooling, further contributing to the observed viscosity reduction.

This trend of decreasing viscosity continues as hordein concentration increases (CS20H, CS30H, and CS40H). SEM analysis reveals increasingly pronounced phase separation and a less compact microstructure, particularly at higher hordein concentrations. This morphological disruption is mirrored in the FTIR spectra, where the protein-specific peak intensifies, signifying stronger protein interactions within the film matrix. These interactions further weaken the starch network, facilitating flow under shear and resulting in lower viscosities.

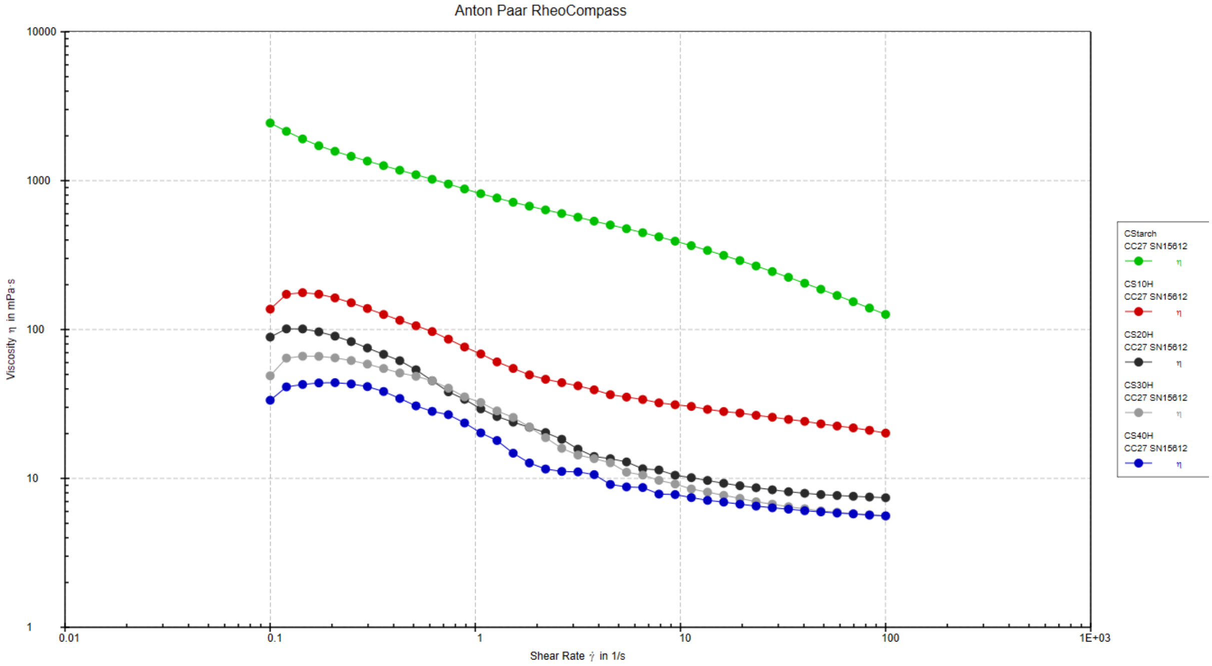

Fig. 5.4 Viscosity corn starch/hordein composite paste

5.3.2.4 Thickness

According to Table 5.2, the thickness of the films was significantly affected by the addition of hordein at different concentrations. The inclusion of hordein led to an increase in the thickness of starch films, but further increases in hordein concentration did not result in additional increases in the thickness of the starch/hordein composite films. The initial increase in thickness compared to pure starch films can be attributed to the filler effect of hordein when incorporated into the starch network. However, the lack of further increase in thickness from 10% to 40% hordein suggests limited incorporation. This is consistent with findings by Basiak et al. (2014), who reported that films made from pure starch or protein isolates (e.g., pure wheat starch or whey protein films) were the thinnest, while films made from starch and protein isolate mixtures were thicker than those made from a single component.

5.3.2.5 Optical properties

5.3.2.5.1 Color

In terms of color, the a* values, indicating red-green hue, became more negative with increasing hordein content, suggesting a shift towards greener color. Similarly, the b* values, indicating yellow-blue hue, increased, reflecting a transition towards more yellowish tones. The lightness of the films, measured by the L* value, decreased from 92.43 ± 0.15 for the pure corn starch film to 82.29 ± 0.56 for the CS40H film, demonstrating a reduction in brightness as hordein content increased. These color changes can be attributed to the intrinsic yellowish-brownish color of the hordein protein, and the color impact became more dominant as its concentration increased in the composite films (Pratap ‐ Singh, A. et al., 2020).

5.3.2.5.2 Transparency

Based on the light transmission (%) and transparency data presented in Table 5.3, hordein-modified corn starch films show significantly improved UV-blocking properties, particularly in the lower wavelength range (200–280 nm). Pure corn starch films exhibit relatively high transmittance at 200 nm (3.77%) and 280 nm (74.87%), indicating they allow a substantial amount of UV light to pass through. However, as the hordein concentration increases from CS10H to CS40, the transmittance at 200 nm sharply decreases, reaching as low as 0.24% for CS40. This substantial reduction suggests that incorporating hordein enhances the film's UV-blocking capacity, which is attributed to the amino acids present in hordein the presence of amino acids in hordein (Li et al., 2004). Additionally, the transparency of the films decreased as the hordein content increased, with transparency values increasing from 0.06 ± 0.07 for the pure corn starch film to 0.84 ± 0.04 for CS40H. This reduction in transparency can be attributed to the intrinsic brownish color of the hordein protein, as well as the increased thickness of the films resulting from the incorporation of hordein.

Table 5.2 Thickness, color, moisture content, and WVP of corn starch/hordein composite films. (mean ± SD).

	Thickness (μm)	**a***	**b***	**L***	**Moisture content (%)**	**WVP (g.mm/m2.d. KPa)**
Corn starch	69.67 ± 5.56^{a}	-0.95 ± 0.16^{a}	$5.10 \pm 0.13^{\mathrm{a}}$	92.43 ± 0.15^{a}	17.35 ± 1.72^{a}	8.02 ± 1.08^{a}
CS10H	82.33 ± 6.79^{b}	-1.39 ± 0.14^{b}	$11.16 \pm 0.35^{\mathrm{b}}$	89.0 ± 0.21^{b}	17.47 ± 1.68^{a}	7.74 ± 0.51^{a}
CS20H	81.67 ± 9.13^{b}	-1.44 ± 0.09^{b}	$15.71 \pm 1.24^{\mathrm{c}}$	85.90 ± 0.46^{c}	17.42 ± 2.28^{a}	7.42 ± 0.88^{a}
CS30H	84.00 ± 11.63^{b}	-1.09 ± 0.10^{c}	$18.96 \pm 0.61^{\mathrm{d}}$	83.99 ± 0.52^{d}	17.24 ± 1.74^{a}	7.12 ± 1.01^{a}
CS40	85.33 ± 11.06^{b}	-0.90 ± 0.17^{a}	21.01 ± 0.49^{e}	82.29 ± 0.56^{e}	15.39 ± 2.98^{a}	6.36 ± 0.55^{a}

Means with different letter in the same column indicate significantly differences between groups (P<0.05).

Table 5.3 Effect of different concentrations of TGase under and without heat treatment on the transparency of hordein-based films.

Treatment	**Transmittance (%) at wavelength (nm)**								**Transparency Value (mm^{-1})**
	200	280	350	400	500	600	700	800	
Corn starch	3.77	74.87	87.52	90.71	90.78	90.78	90.88	90.81	0.60 ± 0.07^{a}
CS10H	0.66	9.75	63.22	78.93	86.43	88.79	89.48	89.58	0.63 ± 0.02^{ab}
CS20H	0.42	1.83	45.19	68.81	82.66	87.68	89.29	89.74	0.70 ± 0.05^{bc}
CS30H	0.32	0.98	34.77	61.23	79.36	86.60	88.99	89.79	0.74 ± 0.06^{c}
CS40	0.24	0.62	23.15	51.22	74.68	84.72	88.15	89.35	0.84 ± 0.04^{d}

Means with different letter in the same column indicate significantly differences between groups (P<0.05).
N.B. T stands for 'TGase'.

5.3.2.6 Mechanical properties

The mechanical properties of the corn starch/hordein composite films were significantly influenced by the addition of hordein. As shown in Table 5.4, the tensile strength of the films increased with increasing hordein concentrations, while elongation at break (EB), a measure of flexibility, showed a decreasing trend. This finding aligns with previous studies that have reported similar trends in pre-gelatinized maize starch-zein blend films (Teklehaimanot et al., 2020).

The pure corn starch film exhibited a TS of 77.67 ± 5.96 KPa. Incorporating hordein led to a marked improvement in TS, with the highest TS reaching 104.47 ± 12.14 MPa for CS40H. This progressive enhancement suggests that hordein acts as a reinforcing agent within the film matrix, enhancing its resistance to tensile forces. This observation aligns with Chiemela et al. (2011), who attributed the increased TS in cassava starch films with soy protein concentrate to the formation of a dense matrix due to strong starch-protein interactions. Additionally, Gounga et al. (2007) found that higher protein content in the film-forming solution led to increased protein aggregation and improved film strength. Specifically, hordein molecules are known to form strong hydrogen bonds with each other, creating a more rigid network within the film matrix and contributing to the observed increase in tensile strength.

This reinforcement effect, however, comes at the expense of flexibility. The pure corn starch film displayed an EB of 159.97 ± 9.42%, indicating good flexibility. However, the addition of hordein progressively reduced the EB to 136.32 ± 8.69%, 123.96 ± 5.65%, 115.37 ± 5.07%, and 111.28 ± 3.43% when hordein incorporation increased from 10% to 40%, respectively. This decline suggests that the increased rigidity from hordein incorporation limits the film's ability to stretch, which could be explained by the disruption of amorphous starch network. Zeng et al (2011) found that zein with relatively compact structure would damage the stress of starch matrix when it was inculpated into the starch network. This result disagrees with the findings of Chiemela et al. (2011), who reported an increase in EB with increasing soy protein concentrate addition in cassava starch films. They attributed this increase to a decrease in starch crystallinity, which enhanced the flexibility of the films (Rindlav et al., 1997). The addition of soy protein concentrates reduced amylose content, thereby decreasing starch crystallinity and increasing the elongation. Similarly, researchers also reported that the increased peak viscosity values in composite blends have been associated with higher gel strength and elasticity (Chinma et al., 2011; Adebowale et al., 2005). In the present study, addition of hordein led to a decrease in viscosity, which could explain the observed decrease in EB. Moreover, according to Figure 5.2 and Figure 5.3, both FTIR and SEM results suggests that the integration of hordein introduced structural heterogeneities that could negatively impact the film's overall performance, particularly its flexibility and barrier properties.

The mechanical performance of the corn starch/hordein composite films demonstrated a classic trade-off between strength and flexibility. While hordein effectively enhanced the tensile strength of the films, it also reduced their elongation at break. This highlights the importance of optimizing hordein concentration to achieve a balance between these properties, depending on the specific application requirements. Further research could explore methods to mitigate the loss of flexibility, such as incorporating plasticizers or modifying the processing conditions, without compromising the strength enhancement provided by hordein. Current commercial biodegradable plastics usually have tensible strength ranging from 12.4 to 55.2MPa, and elongation at break up to 400% (La Mantia et al., 2017), the starch based biodegradable packaging still have big room for further improvement.

Table 5.4 Tensile strength and Elongation at break of the corn starch/hordein composite films.

	Tensile strength (KPa)	**Elongation at break (%)**
Corn Starch	77.67 ± 5.96^{a}	159.97 ± 9.42^{a}
CS10H	92.20 ± 10.64^{b}	$136.32 \pm 8.69^{\mathrm{b}}$
CS20H	94.07 ± 8.69^{b}	123.96 ± 5.65^{c}

CS30H	96.43 ± 9.80^{b}	115.37 ± 5.07^{d}
CS40H	104.47 ± 12.14^{c}	111.28 ± 3.43^{d}

5.3.2.7 Moisture content and WVP of corn starch/hordein composite films

Hordein is known for its high content of hydrophobic amino acids, which suggests that an increase in its concentration within the film would enhance the overall hydrophobicity of the film (Gilhotra & Mishra, 2008; Li, Li, Huang, Luo, & Mei, 2021). This increase in hydrophobicity would theoretically inhibit the interaction between the film and water molecules, thereby reducing the film's ability to absorb and retain moisture, leading to a decrease in both moisture content and water vapor permeability. However, as indicated in Table 5.2, the moisture content and WVP of the films did not exhibit significant changes across samples with different hordein concentrations, although a decreasing trend in WVP was observed with the increase of hordein level. This unexpected outcome suggests that the added hordein content may not be high enough to alter the hydrophobicity of the film. Another plausible explanation could be related to the compatibility between hordein and corn starch. If the fusion or compatibility between these two components is not optimal, it could result in uneven distribution or aggregation of hordein molecules, limiting their ability to influence the film's overall hydrophobicity, so the effectiveness of hordein in modifying the film's properties could be reduced (Teklehaimanot et al., 2020). As observed in Figure 5.3, SEM results disclosed that the addition of hordein, particularly at higher concentrations, led to the formation of more gaps within the composite film, which could promote moisture transmission. As a result, the reduced water vapor permeability due to the increase of hydrophobicity was cancelled out. Similar observations have been reported in studies where the addition of zein protein to starch films resulted in altered morphology and increased porosity (Habeych et al., 2008; Leroy et al., 2012), and these gaps/cracks could counteract any potential benefits of increased hydrophobicity by providing more pathways for water vapor to permeate the film.

5.4 Conclusion

This study identified corn starch as the optimal base material for developing bio-composite films compared to wheat and potato starch. Incorporating hordein into the corn starch matrix significantly enhanced tensile strength, though this improvement came at the cost of flexibility, as elongation at break decreased with increasing hordein content. No significant changes in moisture content or water vapor permeability were observed, indicating poor compatibility between hordein and corn starch at the tested concentrations. Despite this limitation, the addition of hordein significantly improved the UV-blocking capacity of the corn starch films, making them particularly suitable for packaging light-sensitive food products. Among the formulations, the film with 10% hordein concentration demonstrated the best balance of properties, showing promise for sustainable packaging applications.

While the mechanical properties of the corn starch/hordein composite films were enhanced, further research is needed to meet market requirements. This study serves as a preliminary investigation into the potential of these bio-based materials. Future research should focus on improving the compatibility between corn starch and hordein to fully realize their synergistic effects on mechanical and barrier properties. With further development, corn starch/hordein composite films hold significant promise as sustainable and high-performance materials for food packaging applications.

5.5 Reference

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Chapter 6

General discussion and future work

Chapter 6: General Discussion and Recommendation for Future Work

6.1 Thesis hypothesis

H1: The addition of plasticizers can improve the performance of hordein protein films.

H2: The addition of composite plasticizers has a synergistic effect, which can more significantly improve the mechanical deficiencies of hordein protein films.

H3: Heat treatment can enhance the mechanical properties of hordein protein films.

H4: The addition of transglutaminase can enhance the mechanical properties of hordein protein films.

H5: A combination of heat and enzyme treatments can work synergistically to improve the mechanical properties of hordein protein films.

H6: Incorporating hordein protein as a supplementary material into starch films can improve the water vapor barrier properties, mechanical strength, and UV-blocking properties of starch-based films.

6.2 Key findings of the thesis

In this study, hordein protein was extracted from barley flour with protein concentration at 84.4%, In Chapter 3, the effects of two plasticizers, glycerol (GLY) and polyethylene glycol (PEG), on the quality of hordein films were investigated. The hordein films containing only GLY exhibited better mechanical properties compared to those with PEG only. However, compared to hordein-GLY films, the hordein-PEG films showed better water resistance. Additionally, the effects of different ratios of composite plasticizers (GLY and PEG) on hordein protein films were evaluated. The mixture of PEG and GLY in varying proportions had different effect, with the 75% GLY + 25% PEG hordein protein film displaying superior properties, including a more compact structure, higher tensile strength, and lower water vapor permeability compared to films containing only one plasticizer. However, the mechanical property still could not meet the requirements for industrial applications.

To further improve the mechanical properties of the hordein protein film, heat treatment was explored, as previous studies have shown that heat-induced crosslinking can enhance the mechanical properties of protein-based films, such as those made from zein, kafirin, and soy protein (Byaruhanga et al., 2007; Stuchell and Krochta, 1994). Heating causes the protein to unfold, exposing thiol and hydrophobic groups, which subsequently form disulfide bonds and create a crosslinked network structure (Altschul, 1985; Gennadios and Weller, 1991). We applied heat treatment to the hordein protein films, but compared to untreated films, the heat-treated hordein films did not achieve a stronger network for better mechanical property and water barrier, which could be attributed to loss of hydrogen bonding after heating treatment and a reduced film thickness.

Transglutaminase (TGase) is an enzyme known to enhance protein crosslinking through three types of reactions: the formation of isopeptide bonds between glutamine and lysine residues, acyl transfer reactions, and deamidation (DeJong and Koppelman, 2002; Ohtsuka et al., 2001). Many studies have shown that TGase can improve the mechanical properties of various protein-based films, including those made from soy protein isolate, whey protein, hydrolyzed zein, αs1-casein, sodium caseinate, quinoa protein isolate, chitosan, and gelatin (Tang et al., 2005; Oh et al., 2004; Motoki et al., 1987; Patzsch et al., 2010; Escamilla-García et al., 2019; Masamba et al., 2016; Lim et al., 1999). Thus, TGase was incorporated into the hordein protein films at different concentrations with combination of heat and non-heat treatments. The results showed that treatment with high concentrations of TGase (above 30 U/g) increased the film's thickness and reduced its moisture content. The tensile strength initially increased with the increase of TGase concentrations but peaked at 20U/g of TGase, while the elongation at break consistently decreased with the increase of TGase concentration. Regardless of heat treatment, films containing 20 U/g of TGase exhibited the highest tensile strength. Thus, heat treatment was not found to be an effective method for improving the mechanical properties of the hordein protein film. Although the addition of TGase improved the mechanical properties, the performance still fell short of market requirements. Notably, despite the suboptimal mechanical properties, the films exhibited good barrier properties in water vapor and UV light, making them potential candidates for food packaging, particularly for high-moisture and photosensitive foods.

Furthermore, given that the mechanical properties of the hordein protein films still did not meet market demands after several attempts, and the lengthy extraction process of hordein potentially resulted in high costs, we decided to leverage the strong hydrophobicity and UV-blocking capabilities of hordein as supplementary material to improve the properties of cost-effective starch films. Corn, potato, and wheat are major agricultural products, with corn starch accounting for 84% of the starch market (Basiak et al., 2017). Compared to films made from wheat, potato, and corn starch, corn starch film had the best balance between tensile strength and elongation at break, with comparable results in moisture content and color quality. Thus, corn starch was selected for further study. hordein was incorporated into the corn starch matrix at different concentrations and results indicated that it significantly increased tensile strength, although elongation at break decreased with increasing hordein content. Unfortunately, although water vapor barrier properties showed a promising trend with increasing hordein concentrations, no significant changes in water vapor permeability were observed. According to morphology study (SEM images), the gaps or cracks formed in film may explain the discrepancy between theoretical result and practical data, suggesting poor compatibility between hordein and corn starch at the tested concentrations. Despite this limitation, the addition of hordein significantly improved the UV-blocking ability of corn starch films, making them particularly suitable for packaging light-sensitive foods. Among all formulations, the film with 10% hordein concentration exhibited the best overall performance balance, showing promise for sustainable packaging applications.

Although the mechanical properties of the corn starch/hordein composite film were enhanced, the full potential of hordein protein was not fully realized. Future research could focus on improving the compatibility between corn starch and hordein to maximize their synergistic effects on mechanical and barrier properties.

6.3 Limitations and suggested future work

This thesis provides valuable insights into the development of biodegradable packaging using the combination of hordein protein with plasticizers, heat and enzyme treatments, as well as the integration of hordein with starch, highlighting the potential applications of hordein protein in developing degradable packaging materials. However, there are still several areas that require further exploration, which were not covered in this thesis due to time constraints or being beyond its scope. Future work could focus on addressing two key issues raised in this thesis:

How to enhance the compatibility between hordein protein and starch?

- Chemical Crosslinkers: Introducing chemical crosslinkers, such as glutaraldehyde or citric acid, can form covalent bonds between the hydroxyl groups in starch and amino groups in hordein. This chemical crosslinking creates a more unified and stable matrix, reducing phase separation and improving mechanical properties (Wei et al., 2024; Priya et al., 2014).

- Emulsifiers: Adding emulsifiers like lecithin or Tween 80 can reduce surface tension between the hydrophobic hordein and the hydrophilic starch, improving dispersion and creating a more uniform film matrix. Emulsifiers stabilize the system by allowing better mixing of the two phases, preventing phase separation and enhancing film quality (Yang et al., 2024).

How to better tailor the films for food packaging003F

- Barrier Properties: Improving the water vapor permeability (WVP) and oxygen barrier properties is crucial for food packaging. This can be achieved by adding nanoclay, wax, or essential oils as hydrophobic additives, which reduce the film's water permeability and improve its barrier against oxygen, extending the shelf life of packaged food.

- Mechanical Strength: Enhancing the tensile strength and elongation at break of the films is essential for handling and durability in packaging applications. This can be done by optimizing plasticizer content, introducing crosslinking agents, or adding nanofillers like silica nanoparticles or graphene oxide to improve mechanical properties.

- Antimicrobial and Antioxidant Properties: Adding natural extract like essential oils, plant polyphenols, or chitosan can provide antimicrobial and antioxidant properties, which are beneficial for preserving food freshness. These bioactive agents can inhibit the growth of pathogens and delay food oxidation.

In addition to these topics, future research should also explore methods for scaling up the industrial production of hordein protein to increase yields and reduce extraction costs.

6.4 Contribution

This study provides an in-depth understanding of the properties of hordein protein and its advantages as a packaging material, offering comprehensive scientific evidence for future research on hordein protein. From an innovative perspective, there are limited research on hordein protein in developing biodegradable packaging in terms of interaction with plasticizer, response to heat and enzyme treatment, compatibility with starch, thus making a significant contribution to the existing body of knowledge in the biodegradable packaging materials industry. Furthermore, this research contributes to the United Nations Sustainable Development Goals (SDGs), such as “Sustainable use and conservation of ocean resources” (SDG 14) and “Taking urgent action to combat climate change” (SDG 13) (United Nations Sustainable Development Goals, 2023). Therefore, this study indirectly lays the groundwork for the development of sustainable and ethical biodegradable materials. In essence, this PhD thesis significantly advances the application of hordein protein in the packaging industry and leaves room for future research in this field.

6.5 Conclusion

This study comprehensively explored the color, mechanical properties, barrier properties, as well as chemical and microstructural changes of hordein protein as a packaging material. Its distinctive barrier properties in water vapor and UV light suggest its potential as an additive in the food packaging industry. The research highlights that, like other protein-based films, hordein protein films have the drawback of insufficient mechanical properties, and the extraction process results in low yields and high costs. However, hordein shows great potential as an additive material.

Overall, this study makes a significant contribution to the understanding of the properties of hordein protein and its application in developing biodegradable packaging for food application.

6.6 References

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Appendix

The differential scanning calorimetry (DSC) method used in Chapter 2 followed the steps below:

1. Data storage was turned on at the start of the procedure.

2. The instrument was zeroed for heat flow at -85.0°C.

3. The end of cycle 1 was marked.

4. A ramp heating rate of 20.00°C/min was applied, increasing the temperature to 130.00°C.

5. The end of cycle 2 was marked.

6. A ramp cooling rate of 20.00°C/min was applied, decreasing the temperature back to -85.00°C.

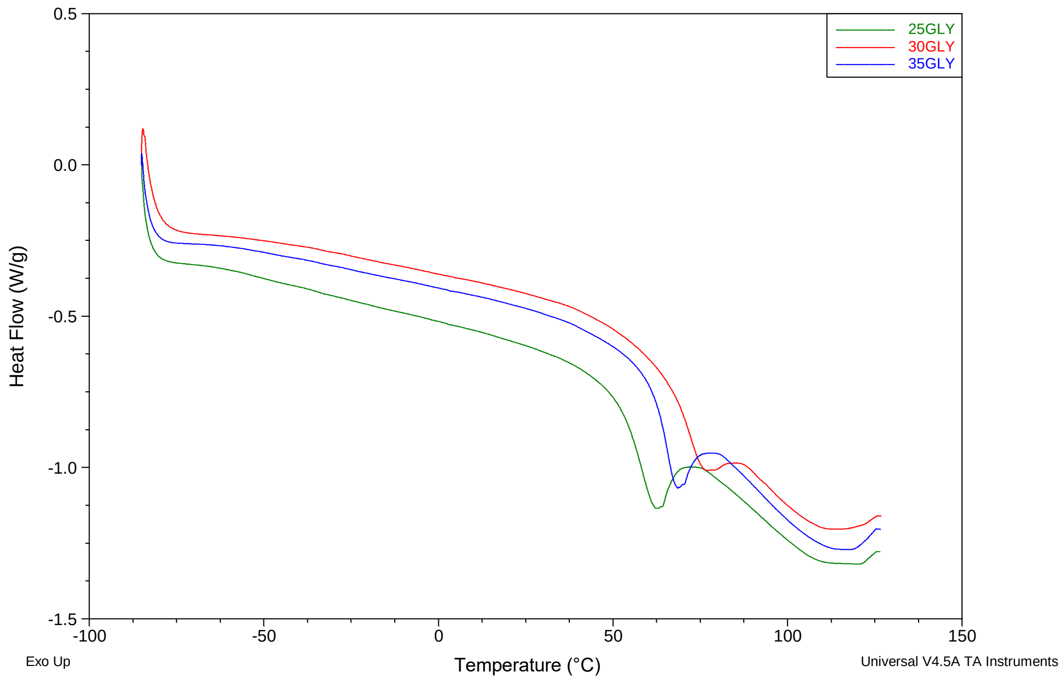
7. The end of cycle 3 was marked.

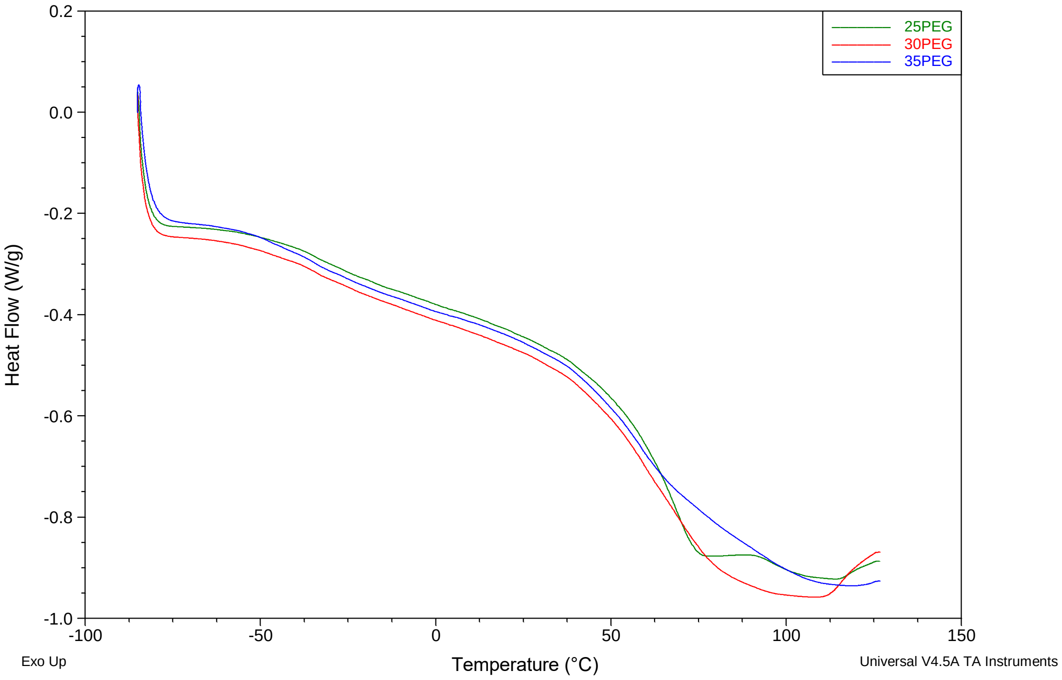
8. Data storage was turned off.

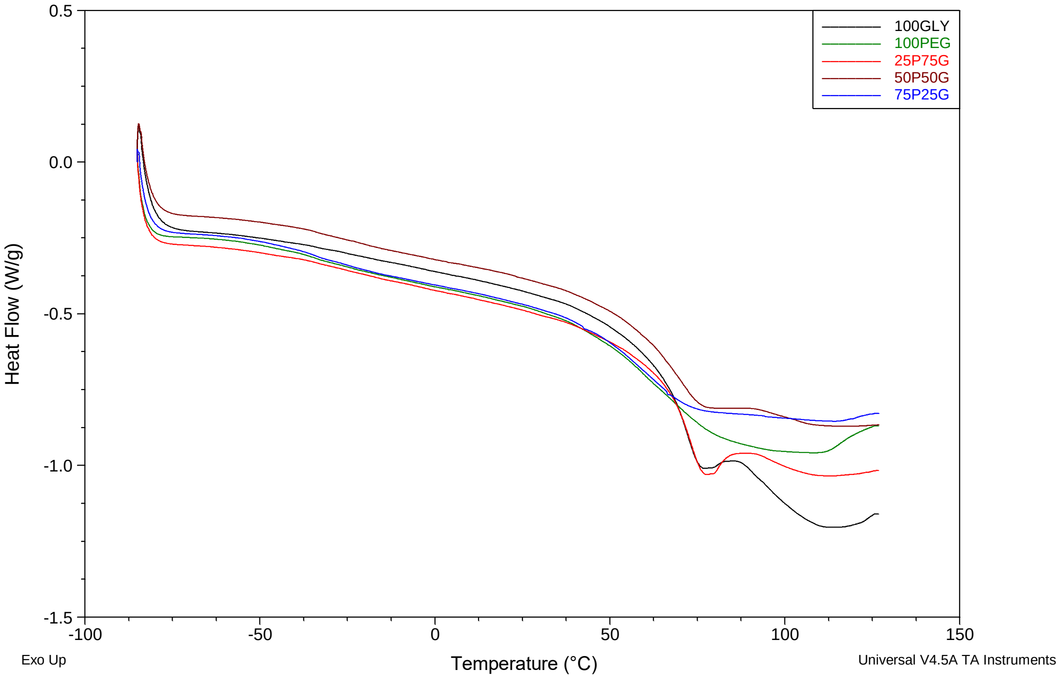
9. The system equilibrated at 40.00°C.

10. External events were turned off.

11. The method concluded at this point.




25PEG
30PEG
35PEG
Heat Flow (W/g)
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-100
-50
0
50
100
150
Temperature (°C)
Exo Up
Universal V4.5A TA Instruments


100GLY
100PEG
25P75G
50P50G
75P25G
Heat Flow (W/g)
0.5
0.0
-0.5
-1.0
-1.5
-100
-50
0
50
100
150
Temperature (°C)
Exo Up
Universal V4.5A TA Instruments

The differential scanning calorimetry (DSC) method used in Chapter 3 followed the steps below:

1. Data storage was turned on at the start of the procedure.

2. The instrument was zeroed for heat flow at -85.0°C.

3. The end of cycle 1 was marked.

4. A ramp heating rate of 20.00°C/min was applied, increasing the temperature to 130.00°C.

5. The end of cycle 2 was marked.

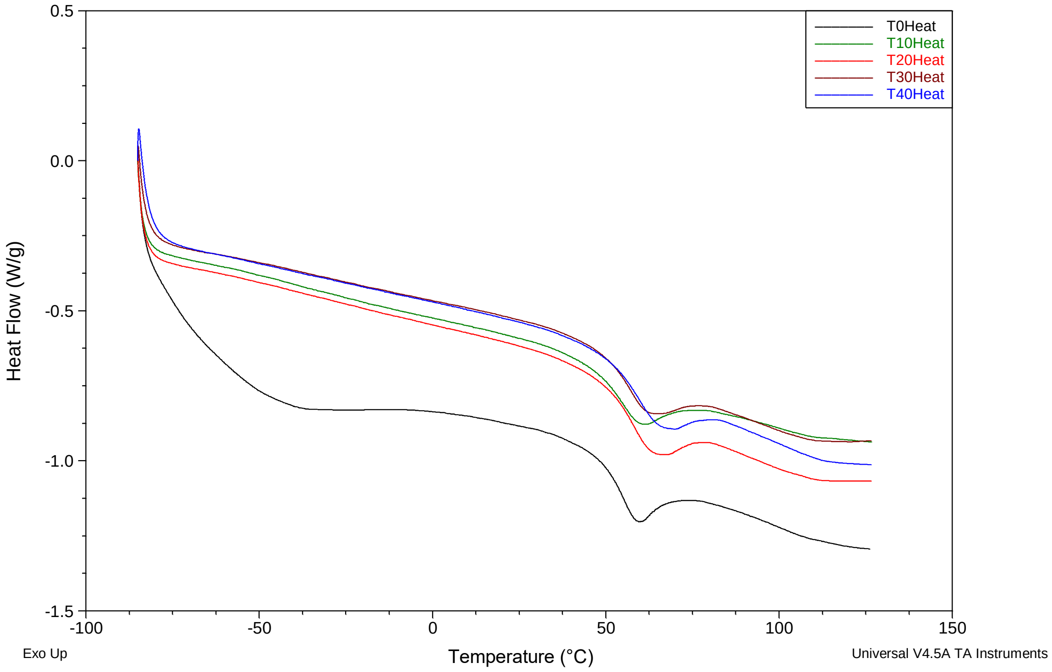
6. A ramp cooling rate of 20.00°C/min was applied, decreasing the temperature back to -85.00°C.

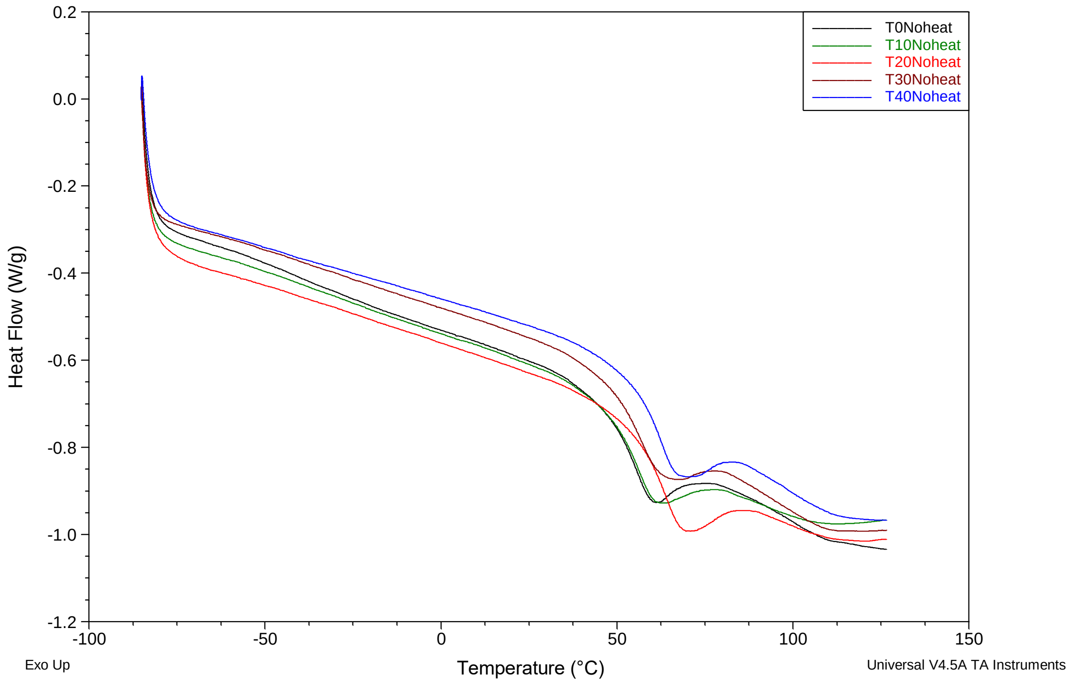
7. The end of cycle 3 was marked.

8. Data storage was turned off.

9. External events were turned off.

10. The method concluded at this point.




0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
Heat Flow (W/g)
-100
-50
0
50
100
150
Temperature (°C)
Exo Up
Universal V4.5A TA Instruments
T0Noheat
T10Noheat
T20Noheat
T30Noheat
T40Noheat

The differential scanning calorimetry (DSC) method used in Chapter 4 followed the steps below:

1. Data storage was turned on at the start of the procedure.

2. The system equilibrated at 150.00°C.

3. The sample was held isothermal for 60.00 minutes.

4. The end of cycle 1 was marked.

5. The system then equilibrated at -70.00°C.

6. The sample was held isothermal for 3.00 minutes.

7. The end of cycle 2 was marked.

8. A ramp heating rate of 20.00°C/min was applied, increasing the temperature to 220.00°C.

9. The end of cycle 3 was marked.

10. Data storage was turned off.

11. External events were turned off.

12. The method concluded at this point.