Wheat bran valorization for proteins and bioactive peptides

by

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Abstract

Over the past several decades, significant efforts have been made towards the valorization and value-addition of agricultural waste and byproducts. One area being explored is the valorization of wheat bran: a major byproduct of the wheat milling industry. Wheat bran has excellent nutritional value, containing approximately 10 to 15% protein by weight. However, its direct incorporation in human diets has been limited due to unappealing sensory characteristics, which has resulted in wheat bran being used primarily for animal feeds. To address this challenge, we propose that the separation of proteins in wheat bran from undesirable components could enhance its suitability as a food ingredient for human consumption. In addition to the use of wheat bran protein as a food ingredient, there has also been a surge in the study of bioactive peptides for use in nutraceuticals, or 'functional foods". We have also proposed that wheat bran proteins could be used as a readily available feedstock for the production of bioactive peptides.

The objectives of this study were specifically focused on the following goals: 1) Optimization of protein extraction efficiency and purification methods, 2) Production and characterization of peptides from purified wheat bran proteins, and 3) Screening of wheat bran proteins and peptides for antioxidant and anti-aging activities using in-vitro bioactivity assays. The experiments conducted in this study focused on optimization of protein extraction methods based on Osborne fractionation, alkaline extraction, and deep-eutectic solvent (DES) extraction methods. Each of these protocols were performed and evaluated via LECO analysis of the bran residues after extraction and by performing Bradford assays on protein extracts. A chromatographic purification method was then adapted for use with wheat bran protein extracts using inexpensive, 500Å underivatized silica gel with a two-phase capture and elution phase step gradient. The effectiveness of this method was further confirmed using size exclusion high

performance liquid chromatography (SEC-HPLC) analysis. The purified proteins were then used to produce peptides via enzymatic hydrolysis catalyzed by pepsin and trypsin. Each Osborne protein fraction, and each corresponding tryptic- and peptic-peptide were then screened for potential biological activity via the DPPH assay, as well as the percent inhibition of enzymes associated with skin cellular aging. The results of these assays were evaluated using one-way ANOVA statistical analysis, and the activity of each protein or peptide sample was compared to the standard, ascorbic acid.

The results of this study showed that the modified Osborne fractionation extraction protocol was the most efficient, achieving a percent recovery of 74.75% of the total protein in the bran. The alkaline extraction and the DES extraction achieved percent recoveries of only 24.77% and 24.84% of the total protein in the bran, respectively. The purity of each Osborne fraction extracted from wheat bran was then tested using SDS-PAGE and SEC-HPLC. Significant impurities were observed in every sample as visible in the SEC-HPLC chromatograms. These impurity peaks disappeared from the chromatograms of proteins purified by the 500Å silica gel column and matched the retention times of the species isolated during the capture phase. This confirmed that the adapted 500Å silica gel column method successfully and reliably removed lower molecular weight impurities from each protein fraction. Analysis of the bioactivity assay results using the one-way ANOVA identified seven different peptide and protein samples that were statistically comparable to ascorbic acid. UPLC-MS analysis of these samples confirmed the presence of peptides through observation of characteristic peptide clusters in the mass spectra.

This study details the optimization of a protein extraction method, the adaptation of an inexpensive, efficient, and effective method for purifying wheat bran proteins, and the screening

of wheat bran proteins and protein hydrolysates for in-vitro biological activity. The results of these studies suggest that wheat bran has excellent potential for valorization from a low-value industrial byproduct into more valuable nutraceutical products for the consumer market.

Table of Contents

List of Figures

List of Tables

Acknowledgements

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Chapter 1 - Proteins and Peptides of Wheat Bran: Extraction, Characterization, and Bioactivity Screening.

1.1 Abstract:

Advancements in agriculture made over the last century have revolutionized human society, resulting in significant improvements to virtually every aspect of life around the globe. These advancements have exponentially augmented humanity's ability to cultivate immense quantities of food. This abundance has ultimately led to the growth of the largest human population to exist on Earth at any other time in history, and it grows larger every year. Although the agricultural industry is now able to produce enormous quantities of food crops, one consequence of these advancements is the generation of comparable amounts of waste and byproducts of substantially lower economic value. These byproducts and waste streams consist of chemically diverse organic biomass, but due to their complex nature, these raw materials have remained largely under-utilized. Significant efforts are currently underway towards the valorization of these materials by developing processing methodologies to extract as much value out of them as possible. Coupled with their diverse chemical profiles, the abundance and inexpensive costs associated with byproduct and waste streams make them prime candidates as renewable natural resources and industrial feedstocks. An excellent example is wheat bran, a major byproduct of the wheat industry. Wheat bran itself boasts a well-balanced nutritional profile, as well as several phytochemical constituents with positive health effects. Despite these factors, wheat bran is almost entirely used as a component for animal feeds due to its unappealing sensory qualities when incorporated into human diets. This review examines the potential for wheat bran to serve as a source of high-value products relevant to meeting the

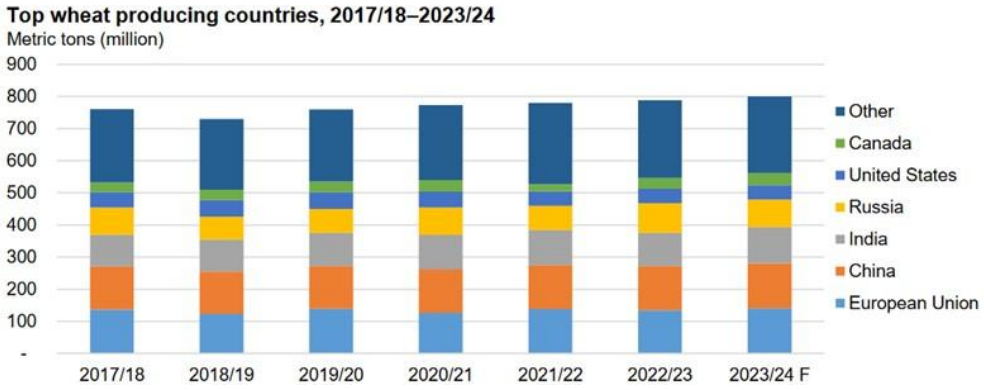
nutritional requirements of the ever-growing global population, as well as a source of medicinally relevant bioactive peptides. Multiple perspectives of wheat bran are considered, such as production of wheat bran by the agricultural and milling industries, sophisticated chemical processing strategies, and applications in biotechnology and medicine.

1.2: Introduction

1.2.1 Wheat Bran: A Byproduct of the Milling Industry:

Wheat is a vital crop because its flour is well suited for the production of baked goods, such as bread, pasta, and pastries (Shewry, 2009). The annual global production of wheat exceeds 600 million tons, and it can be cultivated throughout the world in many different latitudes and elevations (Shewry, 2009).

Figure 1.1. Global wheat production from 2017 to 2024, USDA Economic Research Service, USDA Foreign Agricultural Service, Production Supply, and Distribution database, (Sowell & Swearingen, 2023).



Wheat kernels are composed of three main sections; the outer layers are classified as bran, and the inner sections are classified as the endosperm and germ. Bran itself is composed of several sublayers classified into distinct sections based on biological function (Jerkovic et al., 2010). The outer layers consist of the epidermis and hypodermis, functioning to protect the seed from environmental degradation(Jerkovic et al., 2010). The intermediate layers consist of various

cross and tube cells, nuclear tissue, and the testa layer(Jerkovic et al., 2010). The innermost layer of wheat bran is the aleurone, which is involved primarily with metabolic and germination processes (Jerkovic et al., 2010). Bran is produced during the milling process where it is separated from the endosperm and germ, usually by roller milling (Onipe et al., 2015). Total annual production of wheat bran through this process is estimated at 150 million tons, but the vast majority of this material is used in animal feeds(Prückler et al., 2014),(Katileviciute et al., 2019). This is primarily due to the negative sensory effects imparted by wheat bran to food products(Onipe et al., 2015). Despite these drawbacks to its use in human diets, wheat bran has an excellent nutritional profile. It is high in dietary fiber at roughly 53% by weight in the form of cellulose, lignan, and xylan constituents (Onipe et al., 2015). It also contains significant quantities of protein, approximately 15% by weight (Mérillon & Ramawat, n.d.) with a more balanced amino acid composition than wheat flour(De Brier et al., 2015), as well as a variety of vitamins, minerals, and bioactive phytochemicals (Katileviciute et al., 2019). The main focus of this review is wheat bran proteins and their comprising peptides, but the other nutritional constituents of wheat bran have been extensively studied and reported in the literature (Onipe et al., 2015)(Prückler et al., 2014)(Katileviciute et al., 2019). Due to the significant concentration of protein in wheat bran, it has been considered for use as a source of supplemental plant-based protein to help meet the nutritional requirements in human diets (De Brier et al., 2015). In addition to serving as an alternative source of dietary protein, multiple studies have shown that proteins derived from wheat bran contain peptide sequences with beneficial bioactivity. The bioactive peptides encrypted within the overall protein sequence do not exhibit bioactivity until liberated through various hydrolytic processes, but once released they have been shown to possess antioxidant, blood pressure lowering, and ACE-inhibitory effects (Mahato, 2003),(Zou et

al., 2020). Although wheat bran has historically been considered a low value byproduct, further investigations have consistently demonstrated its potential as a versatile feedstock for multiple value-added products.

1.3 Wheat Bran Protein Extraction:

1.3.1 Plant Protein Extraction Methods:

The extraction of plant proteins for use as food ingredients and as sources of bioactive peptides has been achieved in numerous studies carried out over the past several decades. A number of these studies have been compiled in table 1.1. Seminal work by Osborne classifies proteins based on their solubility in aqueous media (Osborne, 1907). These fractions include albumins, globulins, prolamins, and glutelins. Albumins are characteristically soluble in water, while globulins are soluble in dilute salt solutions (Tamayo Tenorio et al., 2018), (Osborne, 1907). Prolamins are soluble in aqueous ethanol typically 60-70% by volume. Glutelins are soluble in acidic or basic solutions.

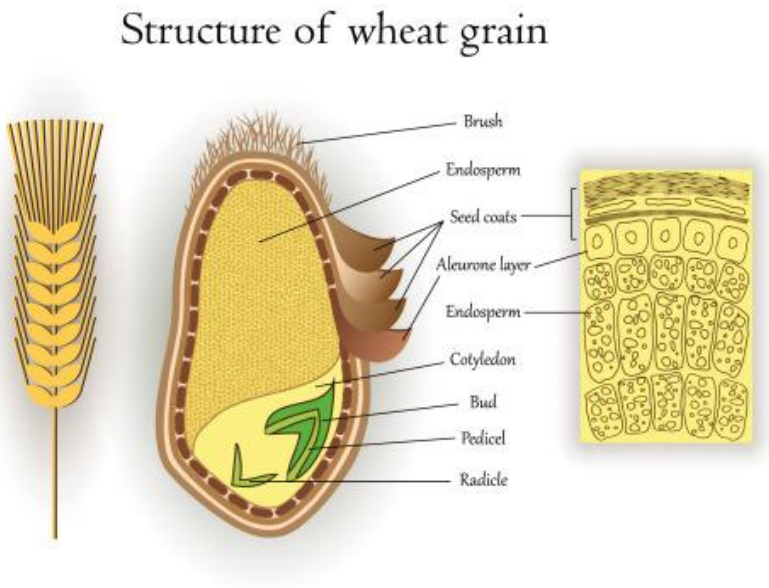
Table 1.1: Compilation of protein extraction methods.

Reference	Objective	Pretreatment	Key Findings
Osborne, 1907	Sequential liquid extractions	Milling	Proteins extracted, crudely separated via solubility
Roberts et. al., 1985	Alkaline extraction	Cellulase	Alkaline extraction 12-16 hrs, high %-recovery
Neudoerffer, 1989	Enzyme pretreatment, protein solubilization	Fungal cellulase + commercial protease	Pretreatments increased protein solubility to 54%
Beaugrand et. al., 2004	Structural investigation	Xylanase pretreatment	Analysis of sub-cellular heterogeneity of wheat bran
Jerkovic et al., 2010	Protein distribution	Dissection, aqueous extraction	Proteomic analysis of protein distribution in wheat bran layers
De Brier et al., 2015	Osborne fractionation	Xylanase, cellulase, ball-milling	Proteins extracted, characterized via SEC-HPLC and SDS-PAGE
Sardari et al., 2019	Osborne fractionation	Multiple, milling, defatting, de-starching	62% of whole proteins extracted, up to 91.6% in degraded form (insoluble proteins extract)

Initial studies on wheat bran protein extraction involved alkaline extractions with varying degrees of heating without separation of proteins based on their Osborne solubility. Several of these studies have also evaluated the use of enzymes to degrade cell walls, such as in the case of Roberts et. Al., 1985 (Roberts et al., 1985). This study found that addition of cellulase did not significantly improve the extraction of proteins and solids from wheat bran (Roberts et al., 1985). Several other studies have been performed on the use of enzymatic treatments on the degradation of cell walls for the purpose of increasing protein recovery with varying results (Neudoerffer, 1969)·(Waszczynskyj, 1980). The study conducted by Neudeorffer evaluated multiple wheat bran pretreatments. Each pretreatment consisted of crude enzyme preparations derived from different species of fungi combined with commercial proteases. Each treatment was evaluated based on the % reducing sugar accumulation, loss of holo- and alpha-cellulose, and the change in protein solubilization. The nutritional profile of enzyme-modified wheat bran in rats was also explored by measuring improvements in protein digestibility. Two top-performing pretreatment fomulas were identified that included a crude enzyme preparation from the fungi T. viride and M. verrucaria. These crude enzyme preparations were combined with Rhozyme PF and Rhozyme 41, as well as purified bacterial type VII, Subtilisin protease from Sigma Chemical Company. The results of these pretreatments consisted of significant decreases of cellulose and holocellulose, accumulation of reducing sugar, and 50% to 54% solubilization of protein. Waszczynskyj's study evaluated increases in protein extracted from full-fat wheat bran using simple alkaline extraction methods when subjected to carbohydrase pretreatments. This study resulted in an increase from 30% of the total nitrogen to 38.5% when the enzymatic pretreatment was used. Another study with xylanase reported preferential accumulation of xylanase in the aleurone layers, providing evidence of subcellular heterogeneity of the cell walls in different

layers of the bran (Beaugrand et al., 2004). In one study, 2-dimensional-gel electrophoresis was used to identify 832 unique protein spots from wheat bran extracts, of which 742 were identified using MASCOT and 403 via GPM (Jerkovic et al., 2010). This study was able to identify many of the proteins that comprise the proteome of wheat bran and elucidate its function (Jerkovic et al., 2010). Each of the three sections of wheat bran have been shown to contain distinct types of proteins, from defense enzymes in the outer layers to storage and metabolism proteins in the aleurone layer (Jerkovic et al., 2010). Defense enzymes cooperate within the outer layers to provide multiple lines of defense against microbial organisms. Enzymes in the outer layer prevent the degradation of cell walls, as well as to deactivate digestive enzymes secreted by pathogenic fungi (Jerkovic et al., 2010). Enzymes of the intermediate layer act as a secondary line of defense against invading microorganisms by deactivating their secreted pathogenic enzymes once the first line of defense has been breached (Jerkovic et al., 2010). Enzymes in the aleurone layer are primarily responsible for metabolic functions, as well as storage proteins that are used during germination (Jerkovic et al., 2010). For the purposes of total protein extraction for use in food and derivatization, the distinction between the different types of protein is trivial as these layers are not precisely separated. It is noteworthy though as it highlights the difficulty in protein extraction as the proteins are essentially laminated within multiple sublayers of the bran.

Figure 1.2: Illustration of the multiple sublayers comprising wheat bran (Bold, 2022).

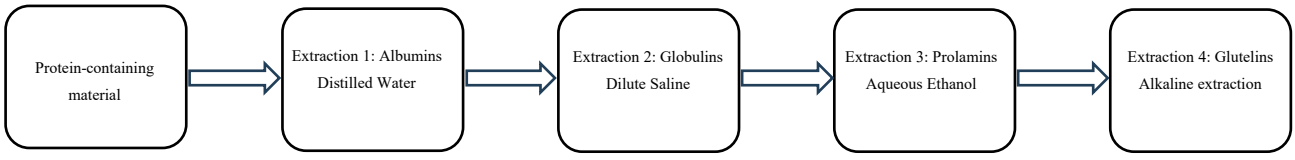


One simple strategy that has been used with consistent success is particle size reduction through milling. In 2015 De Brier *et al*., successfully increased protein recovery from 60 to 77% through ball milling of coarse bran (De Brier et al., 2015). Another study evaluated coarse vs fine bran particle sizes and showed consistently higher protein recovery with particle size reduction (Sardari et al., 2019).

1.3.2 Osborne Fractionations:

Osborne Fractionations have become a popular alternative to bulk extraction by aqueous alkaline extraction solutions. In Osborne fractionations, each Osborne classification of protein is extracted sequentially.

Figure 1.3: General flowchart of Osborne fractionation extractions of protein from biological samples.



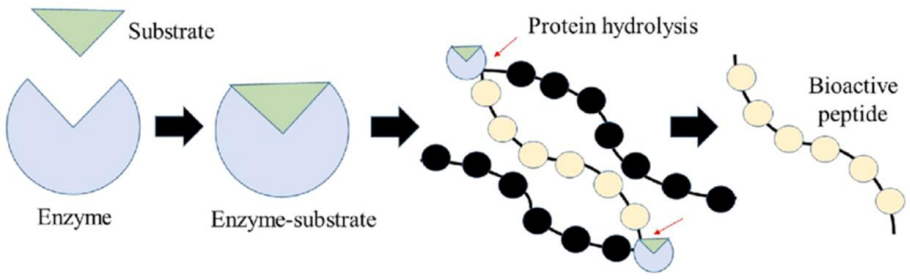
Once centrifuged and separated from solids, the supernatants are decanted and collected as the crude Osborne fraction. This process has continued to be refined and optimized for use in many cereals, as well as for wheat bran (Sardari et al., 2019). These fractions can then be further purified using a variety of protein purification methods. These can include dialysis, ultrafiltration and diafiltration, evaporative methods of concentration; as well as various methods of chromatography such as size exclusion, ion exchange, and affinity chromatography, depending on the desired application. Once sufficiently purified from the organic biomass, proteins can then be further derived into peptides.

1.4 Production of Bioactive Peptides from Wheat Bran Proteins:

1.4.1 Enzymatic Protein Hydrolysis:

Proteins are polymers composed of multiple polypeptide chains. As such, these polypeptide chains can be hydrolyzed into peptide fragments through numerous different strategies. In its most basic form, hydrolysis of a protein's peptide bonds is facilitated either by acidic or alkaline conditions (Tsugita & Scheffler, 1982). Alternatively, enzymes such as proteases are widely used to catalyze the hydrolysis of protein isolates. Enzymes hydrolyze proteins at specific amino acid sequences called recognition sites, with varying degrees of specificity depending on the particular enzyme (Tsugita & Scheffler, 1982).

Figure 1.4: Illustration of enzymatic hydrolysis of protein polypeptide chains, (Cruz-Casas et al., 2021)**.**



Because of this specificity, the peptides and protein hydrolysates produced using enzymatic hydrolysis exhibit a high degree of reproducibility (Sewczyk et al., 2018). Peptides generated via hydrolysis are often characterized using liquid chromatography and mass spectrometry of the entire mixture to generate a peptide fingerprint, or proteolysome for a given enzyme (Sewczyk et al., 2018). In addition to enzymatic production strategies, several fermentation techniques have also been explored to improve the functionality of wheat bran, as well as to produce bioactive peptides from wheat bran proteins, such as the use of solid-state fermentation (Table 1.2) (Manfredini et al., 2021), (X. Jiang et al, 2021).

Table 1.2: Microbial fermentation methods used to improve functionality and nutritional utility of wheat bran.

Microorganism	Method	Substrate	Reference	Key Findings
B. subtilis	Solid-state or	Wheat bran	(Lim et al., 2017)	Increased levan, γ-PGA, and peptides.
L. brevis, K. exigua	24 hr fermentation	Wheat bran	(Coda et al., 2014)	Increased phytase activity, peptide and total free amino acid content, protein digestibility, antioxidant capacity, and nutritional index.
L. rhamnosus	Solid-state fermentation	Wheat bran-whey permeate	(Bertsch et al., 2020)	Increased antioxidant activity, free and bound phenolic acid content, bioaccessibility of free total phenolic acids.
L. rhamnosus	48-hr fermentation	Wheat bran	(Spaggiari et al., 2020)	Phytic acid degradation, arabinoxylan solubilization, increased antioxidant activity.
B. licheniformis, others (comparison)	48-72hr fermentation	Wheat bran	(Liu et al., 2023)	Beneficial effect on growth performance in growing pigs, increased nutrient digestibility, antioxidant capacity, and gut microbiota.
Yeast, L. bulgaricus, S. thermophiles	Solid-state fermentation	Wheat bran	(Zhao et al., 2017)	Increased water extractability of arabinoxylans, total dietary and soluble fiber, improved hydration properties

The simplest version of enzymatic hydrolysis involves protein substrates and homogenous proteolytic enzymes stirring in reactors at temperatures and pH's corresponding to the optimal activity conditions for a specific enzyme (Sewczyk et al., 2018), (Lin et al., 2013). A compilation

of common protease enzymes has been compiled in table 1.3, the majority of which are available commercially from common chemical vendors.

Table 1.3: Examples of Common Protease Enzymes Used for Production of Bioactive Peptides.

Enzyme	Source Protein	Sequences	Bioactivity	Reference
Pepsin	Plant, animal, egg, dairy	NA	ACE-inhibitory, anti-cancer, antioxidant, metal-binding, antidiabetic, anti-inflammatory, antimicrobial	(Ashaolu et al., 2024)
Trypsin	Whey	ALC(carbamidomethyl)SEK, ELKDLK, ALPMHIR	Antioxidant, cytoprotective	(Ballatore et al., 2020)
Alcalase	Fish, soy bean	SeMDPGQQ, TSeMMM, YLDNY, SPPYWPY, YLDNY	Metal chelating, antioxidant, anti-hyperuricemic	(Tacias-Pascacio et al., 2020)
Papain, Bromelain	Dairy, animal, plant proteins	PGWNQWFL, VEVLPPAEL	Anti-cancer, antioxidant, antithrombotic, anti-diabetic, anti-hypertensive, cytoprotective	(Mazorra-Manzano et al., 2018)
Pancreatin	Quinoa	Not identified	Antioxidant	(Daliri et al., 2021)
Ficin	Whey	Not identified	Antioxidant	(Kheroufi et al., 2022)
Pronase	Soy	Not identified	ACE-inhibition, anti-thrombotic, antioxidant	(Gibbs et al., 2004)
Thermolysin	Dairy	LVRTPEVDDEALEK, SAPLRVY, VAGTWYS, LIVTQTMKG, others	ACE-inhibition,	(Hernández-Ledesma et al., 2006)

Once sufficient time has passed to facilitate the desired hydrolysis of a protein, the reaction is stopped by inactivating the enzyme, typically by pH- or heat-denaturation (Sewczyk et al., 2018). Although enzyme-catalyzed hydrolysis results in a more consistent hydrolysate product, the requirement of large quantities of enzymes to produce a single batch of hydrolysates at scale is a major disadvantage. This is especially true for less common enzymes, as they are significantly more expensive, if commercially available. If an enzyme is not commercially available, more often than not, it will be prohibitively expensive/complicated to produce in-house, assuming a laboratory is even equipped to do so. In some cases, it may be worthwhile to

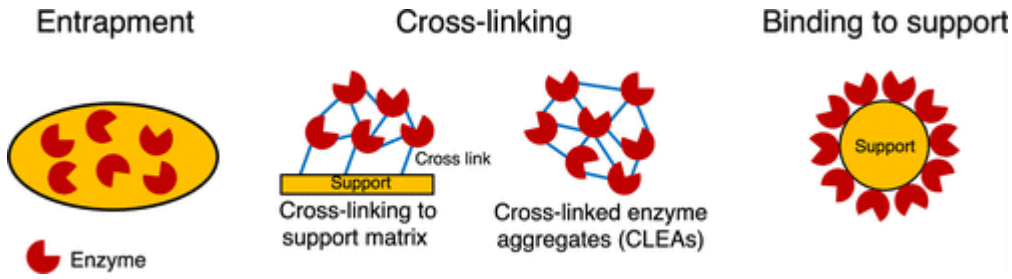
explore less-common enzymes to evaluate whether the effort to produce them in larger quantities is a worthwhile endeavor. Several strategies have been devised over the years to maximize the efficiency of such enzymes or even allow them to be recycled for multiple batch reactions. These strategies often involve immobilization of enzymes on solid support frameworks and are discussed in further detail in section 1.4.2.

1.4.2 Immobilized Enzymatic Hydrolysis:

Immobilization strategies that secure enzymes onto solid supports have been developed to address a number of challenges associated with enzymatic reactions. Immobilization of an enzyme onto a solid support essentially results in the formation of heterogeneous enzymatic catalyst: one that is not dissolved within the reaction solution. A key advantage of a heterogeneous catalyst is that it can be eliminated from a reaction more quickly than homogenous catalysts via filtration. This allows heterogeneous catalysts to be easily and efficiently recovered for reuse in multiple batches, or in continuous flow systems. This helps reduce the cost of production of consistent protein hydrolysates, as the most expensive component of the reaction can be recycled (Sewczyk et al., 2018)·(Lin et al., 2013). There are numerous methods for immobilizing enzymes on solid media, such as deposition on solids, adsorption on porous media, immobilization on polyketones via hydrogen bonding, covalent immobilization, physical entrapment, affinity tag immobilization, encapsulation in liposomes, and immobilization on polymer substrates (Homaei et al., 2013). Each of these strategies has advantages and disadvantages in varying conditions that need to be considered when designing reactions. Generally speaking, the immobilization of enzymes on solid supports stabilizes the

enzyme's structure and helps to maintain enzymatic activity in a more diverse range of reaction conditions (Homaei et al., 2013).

Figure 1.5: Illustration of enzyme immobilization strategies, (Tang et al., 2024)**.**



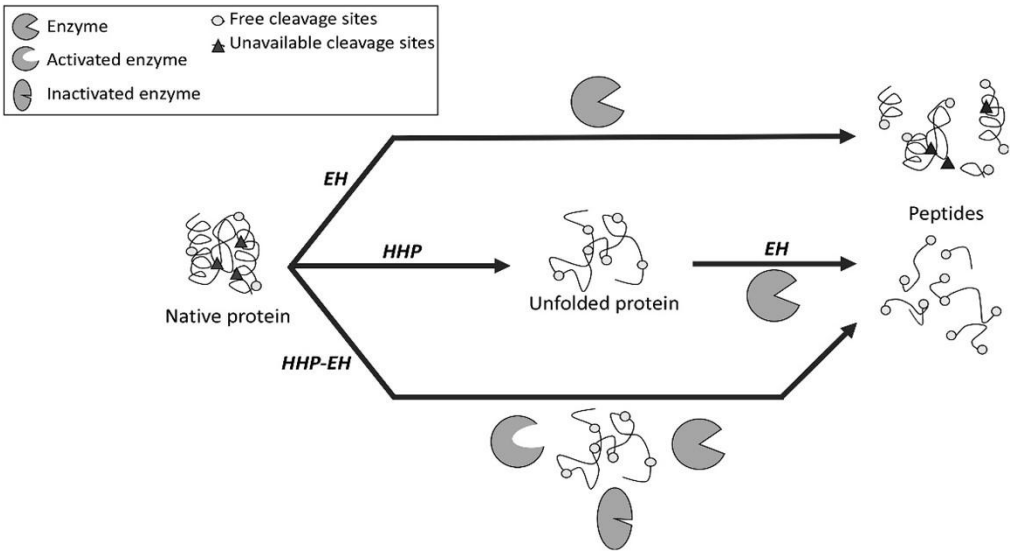
The additional stability imparted by the support media does come with a tradeoff in the form of decreased activity for the majority of cases though. One important example is higher Michaelis constants due to reduced accessibility of the enzyme for the substrate that have been observed (Homaei et al., 2013). Despite these drawbacks however, the use of immobilized enzymes in industrial applications offers significant advantages over homogenous enzymatic methods, and as this technology is further developed, it is likely that these issues will be resolved.

1.4.3: High Hydrostatic Pressure (HHP) Enzymatic Protein Hydrolysis:

Another method for the improvement of enzymatic hydrolysis of proteins is the use of high hydrostatic pressure treatments, or HHP. This method of processing involves denaturation of native proteins using high pressures, often 100-1000 MPa, before, during, or after enzymatic treatments. Numerous studies have shown that exposure of native proteins to HHP treatments successfully increases the degree of hydrolysis by protease enzymes (Landim et al., 2023). A primary driving force for the development of HHP-treatment strategies has been focused on the removal of allergenic sequences within proteins, some of which are resistant to traditional hydrolytic methods (Landim et al., 2023). HHP increases the efficiency of enzymatic hydrolysis by causing proteins to undergo reversible structural changes due to the destabilization of intramolecular interactions that maintain the 3D-structure of the protein under normal conditions

(Landim et al., 2023). Figure 1.6 depicts a generalized illustration of the effects of HHP on enzymatic hydrolysis.

Figure 1.6: Illustration of the effect of HHP on enzymatic hydrolysis of proteins to produce peptides,(Marciniak et al., 2018).



Disruption of these forces results in the unfolding of the protein, which increases the availability of more peptide bonds for enzymatic hydrolysis. Although this can be beneficial for increasing the degree of hydrolysis, the unfolding of proteins exposes the hydrophobic residues that are normally shielded by hydrophilic residues. This can result in accumulation of denatured proteins into insoluble aggregates that can block cleavage sites or cause precipitation of proteins out of the reaction solution, both of which negatively impact yields (Landim et al., 2023). Because of this, lab-scale optimization of the HHP conditions is necessary to determine the most effective pressures for specific enzymes, prior to scaling up for commercial production (Landim et al., 2023). Several studies conducted on HHP with various enzymes have been compiled in table 1.4.

Table 1.4: Examples of HHP-assisted Enzymatic Digestion Methods for Food Protein Isolates.

Protein Isolate	Hyperbaric Conditions	Digestive Enzyme	Results	Peptides Identified	Reference
Soy	500-600MPa, 20 min, 20°C	Pepsin, Pancreatin	Improved digestibility	Not identified	(Dan et al., 2010)
Whey	550 MPa, 3-4min, 20°C	Multi-stage: Pepsin (first) trypsin, chymotrypsin, peptidase (second)	Production of antioxidant peptides, improved digestibility	Not identified	(Iskandar et al., 2015)
Milk (β-lactoglobulin)	600 MPa, 10 min, 30-44°C	Chymotrypsin	Reduced immune response	Not identified	(Bonomi et al., 2003)
Buckwheat	600 MPa, 60°C, 30 min	Trypsin	Improved digestibility, reduced allergenicity	Not identified	(Deng et al., 2015)
Wheat Bran	600 MPa, 40-70°C, 5 min	UltraFlo XL	Breakdown of cellulose, increase in digestibility	Not identified	(Jiménez-Pulido et al., 2024)
Peanut	40-80 MPa, 1hr	Alcalase	Production of antioxidant peptides	Molecular weight distribution assessed, structures not identified	(Dong et al., 2011)
Chickpea	100-600 MPa, 50°C 2-40min	Alcalase	Production of antioxidant peptides	Molecular weight distributions assessed, structures not identified	(T. Zhang et al., 2012)

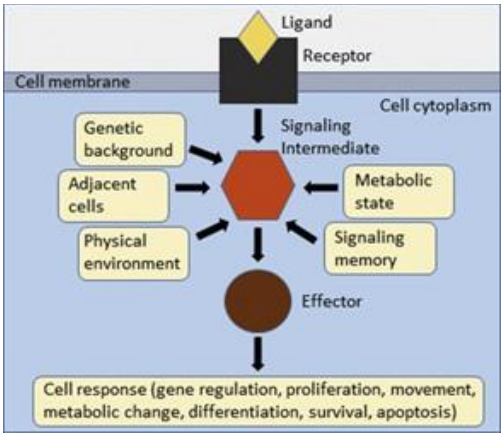
1.5 Bioactive Peptides: Physiological Importance:

1.5.1 Peptide Signaling Molecules:

Peptides play important roles in many physiological systems and biochemical processes. There are several representative classes of endogenous bioactive peptides, such as peptide hormones including vasopressin, lutein(Martini & Tagliazucchi, 2023) (Martini & Tagliazucchi, 2023); neuropeptides, including oxytocin and enkephalin(Martini & Tagliazucchi, 2023); and growth factors, such as epidermal growth factor(Berlanga-Acosta et al., 2009), human platelet-derived growth factor (PDGF) (Steed, 2006) , and human vascular endothelial growth factor (VEGF165) (Hanft et al., 2008). One of the most noteworthy examples of the biological function

of endogenous peptides is their role as biochemical messengers in cell signaling pathways. In these systems, a particular peptide binds to a receptor site on a protein or a G-coupled receptor protein (GPCR) on a cell's surface to trigger a cascade of subsequent reactions within a cell (Forbes & Krishnamurthy, 2023). This process of signal transduction results in the transmission of biochemical information throughout an organism. These ligand-receptor signaling mechanisms direct virtually all cellular processes within an organism (Forbes & Krishnamurthy, 2023). A brief illustration of signal transduction is depicted in figure 1.7.

Figure 1.7: Basic illustration of cellular signal transduction, (Luker, 2021).



Peptide ligands are the most abundant type of signaling ligands found in human biochemistry(Forbes & Krishnamurthy, 2023)·(Cooper, 2000), and in turn peptide-protein interactions are implicated in virtually all human diseases(Tsai et al., 2022). These facts illustrate the immense potential of peptides in the development of therapeutics for the treatment of disease, and as expected, the study of bioactive peptides as medically relevant therapeutics and health-promoting compounds has its roots in the study of these systems, beginning with the study of human peptide hormones(Wang et al., 2022a). The development of exogenous bioactive peptides for therapeutics and health-promoting products has had great success in recent years and has garnered significant attention in pharmaceutical research since the 1950s(Wang et al., 2022a).

1.5.2 Peptide Therapeutics:

Many natural and synthetically modified peptides have been developed and approved for a diverse array of applications, such as in oncology, pain management, metabolic diseases such as diabetes, various cardiovascular conditions(Wang et al., 2022a), as well as for their roles in wound healing(Berlanga-Acosta et al., 2009),(Steed, 2006),(Hanft et al., 2008). Numerous therapeutic bioactive peptides are currently in preclinical development for the treatment of other conditions and have had promising results in animal models for the treatment of conditions such as obesity(Kumar, 2019), tumor cell targeting immunotherapies(Zhang et al., 2022), antiviral and antimicrobials, as well as many other applications involving human health and disease(Martini & Tagliazucchi, 2023). Each individual facet of human disease that is currently being targeted through bioactive peptide treatment strategies could constitute its own review to address all of the bioactive peptides currently being studied, but a brief compilation of a number of representative publications and reviews has been included in table 1.5 to illustrate the current state of the art in this area of research. The vast body of work currently underway on the development of bioactive peptide therapeutics stands to illustrate the realization of their immense potential in future treatment options for numerous health issues.

Table 1.5: Examples of Food-Derived Bioactive Peptides Investigated as Potential Treatments for Human Disease.

Disease/Target	Peptide	Source	Mechanism of Action	Reference
Cancer	Lunasin	Wheat	Core histone acetylation inhibition	(Jeong et al., 2007)
Cancer	Tripeptides VVV, VWV	Wheat	Dv11 PDZ antagonism/inhibition	(Lee et al., n.d.)
Cancer	Pentapeptide EQRPR	Rice (bran)	Multi-action antiproliferative	(Kannan et al., 2010)
Cancer	SSDEEVREEKELDLSSNE	Wheat (germ Protein)	Core histone acetylation inhibition	(Karami et al., 2019)
Cancer	CPe-III-S	Chickpea (semi-synthetic)	P53-dependent apoptosis	(Xue et al., 2015)
Obesity	LGP, IGP, LRP, VY, IY, TF	Wheat Bran (autolysis)	ACE-inhibition	(Nogata et al., 2009a)
Non-alcoholic steatohepatitis	LRP, LQP	Wheat Bran (autolysis)	AMPK/ACC upregulation	(Kawaguchi et al., 2017)
Hypertension	IR	Pea (protein hydrolysate)	Renin inhibition	(H. Li et al., 2010)
Hypertension	IAP	Wheat	ACE-inhibition	(Motoi & Kodama, n.d.)
Microbes	Multiple	Wheat (gluten hydrolysate)	Antimicrobial	(Khosravi et al., 2022)

1.5.3 Bioactive Peptides Derived From Plant and Animal Proteins:

In addition to development as pharmaceutically relevant therapeutics, exogenous peptides derived from natural protein sources have also been explored for their health-promoting effects. This area of research colloquially referred to as nutraceuticals has been developed to include a wide range of protein sources, from animal proteins(Bhat et al., 2015), marine sponges (Fusetani' & Matsunaga, 1992), as well as plant-derived proteins(Fan et al., 2022). These are of significant interest in health-promoting products as once peptides are liberated from their parent proteins, they can exert their beneficial effects by acting on several physiological systems within the body. Several products are currently available that incorporate bioactive peptides which have been empirically demonstrated to exhibit beneficial health effects and high safety profiles(Bhat

et al., 2015). Although the mechanisms of action and structure-activity relationships of many bioactive peptides derived from food proteins have been characterized in the literature(Fan et al., 2022),(Y. Li & Yu, 2015),(Yang et al., 2021) the sheer number of peptides made available through hydrolytic and enzymatic strategies leaves much to be elucidated in this rapidly developing area of research. In the following section the properties of protein hydrolysates and peptides derived from food-protein sources is discussed as an elementary starting point for the discussion of these compounds.

1.6: Properties of Protein Hydrolysates and Peptides:

Protein hydrolysates and peptides offer significant advantages over whole proteins in several ways. An important example is that hydrolysates and peptides have improved functional properties, such as higher solubility than whole proteins. Large protein molecules often exhibit poor solubility which in turn decreases their absorption in the gastrointestinal tract(Adler-Nissen, n.d.). Several studies have shown that protein hydrolysates and peptides have greater solubility and absorption than whole or intact proteins, and in turn higher bioavailability(Adler-Nissen, n.d.),(Abeer et al., 2021),(Thomson & Buckley, 2011). The differences in absorption between proteins and protein hydrolysates appear to be specific to individual proteins and corresponding protein hydrolysates though, as well as manufacturing techniques and the hydrolysis conditions employed therein(Thomson & Buckley, 2011). As previously discussed, different enzymes have specific activities for the hydrolysis of specific amino acid sequences in the protein's primary sequence. For this reason, it is important to take the manufacturing process into consideration when comparing the absorption rates of protein hydrolysates from different suppliers(Thomson & Buckley, 2011). As research in this area continues, many examples will likely be reported, but the general rule of thumb that smaller protein fragments are more soluble and absorbed more

effectively than intact proteins has been demonstrated in the literature for many cases (Thomson & Buckley, 2011).

In addition to improved functional properties and bioavailability, hydrolysis of proteins is also associated with decreased or even eliminated allergenicity for a variety of plant and animal-based proteins(Guadix et al., 2006),(Ragno et al., 1993),(Calcinai et al., 2022). Different proteases have been shown to impart varying degrees of allergenicity suppression, but screening studies have been conducted to evaluate the efficiency of multiple enzymes for individual protein substrates(Calcinai et al., 2022). The reduction of allergenicity to food proteins has significant implications for individuals with gluten intolerances and allergies such as in the case of celiac patients, as it could potentially allow for the incorporation of wheat-gluten hydrolysates into their diet while avoiding the detrimental effects usually associated with gluten consumption.

One of the most noteworthy properties of protein hydrolysates and peptides is the therapeutic bioactivity of certain peptide sequences. Peptides with high bioactivity are typically smaller than 1kDa and consist of 2 to 20 amino acids (Zou et al., 2020). Peptides obtained from the hydrolysis of plant and animal proteins have been reported that possess antihypertensive, anti-inflammatory, antimicrobial, antioxidant, opioid, anti-obesity, blood-sugar regulation, and antiaging activities(Akbarian et al., 2022). A peptide derived from a marine sponge exhibiting anti-HIV activity has also been reported(Plaza et al., 2009). Several anti-cancer peptides are also known, exerting their therapeutic effects via cytotoxicity, inducing apoptosis, and several other mechanisms of action(Akbarian et al., 2022). Many bioactive peptides have been characterized and even developed as pharmaceutical active ingredients and have been approved for clinical use(Wang et al., 2022b).

1.7: Production and Modification of Bioactive Peptides, and Future Areas of Exploration:

As mentioned previously, bioactive peptides are typically produced through techniques such as enzymatic hydrolysis, microbial fermentation, recombinant production; but they are also produced and modified using synthetic methods. The most relevant methods for generating bioactive peptides from wheat bran proteins are catalyzed hydrolytic methods, but peptides with demonstrated bioactivities could be modified to improve their pharmaceutical chemical properties. One important property that can be modified relatively easily is a molecule's cellular permeation and cellular distribution. A common synthetic strategy used to increase cytosolic distribution and cellular permeation in peptide drugs is synthetic addition of a palmitoyl group or other fatty acid to the n-terminus of a peptide to increase hydrophobicity(Mahato, 2003). These strategies are already widely employed for the development of peptide drugs and could realistically be applied to bioactive peptides derived from natural sources to improve their pharmaceutical properties in later stages of development.

Bioactive peptides currently derived from wheat bran have been reported to exhibit antioxidant(Mahato, 2003),(Zou et al., 2020) and antihypertensive ACE-inhibiting activity(Mahato, 2003),(Nogata et al., 2009). In these studies, multiple protease enzymes have been screened and their resulting proteolysomes are separated based on peptide size for further analysis. These fractions are then tested in various in-vitro bioactivity assays, such as radical scavenging assays, antioxidant capacity assays, as well as in-vitro assays to study their effects on biological mechanisms to target diseases(Mahato, 2003),(Zou et al., 2020),(Nogata et al., 2009). Common radical scavenging assays used to determine the antioxidant potential of bioactive peptides include the superoxide anion radical scavenging activity assay, the DPPH assay, and the

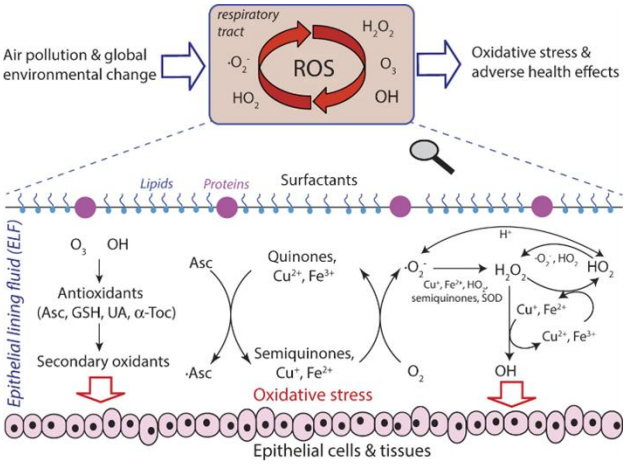
Trolox equivalent antioxidant capacity assay, where the potential antioxidant species is tested based on its ability to deactivate radical species in solution. This corresponds to potential radical scavenging activity in-vivo, which is important as the accumulation of radical species in the body can lead to the subsequent accumulation of reactive oxygen and nitrogen species that can damage tissues and cause disease(Akbarian et al., 2022). These tests have been used extensively in the testing of numerous bioactive peptides for antioxidant activity and are prevalent throughout the literature. In addition to these tests the fatty acid oxidation inhibition assay is also quite common, where the antioxidant species are compared to known antioxidants' ability to prevent the peroxidation of long chain fatty acids(Wang et al., 2022b). Several mechanisms of action have been proposed based on the results of these assays in combination with the structural information obtained through sequencing of the peptide fragments. It has been reported that peptides that exhibit the most significant bioactivity are usually composed of hydrophobic amino acids(Wang et al., 2022b). Tyrosine is an important amino acid for antioxidant bioactive peptides because of its phenolic moiety, which serves as a potent scavenger for free radicals(Wang et al., 2022b). It has also been reported that bioactive peptides with sulfur-containing residues in the presence of hydrophobic residues experienced improved solubility and possessed significant antioxidant activity(Wang et al., 2022b).

ACE-inhibition activity assays are another important factor for screening the bioactivity of new peptides and protein hydrolysates, as previous studies have shown that peptides produced from wheat bran protein isolates using alcalase had antihypertensive effects in rats when administered orally(Zou et al., 2020). The inhibition of angiotensin-converting enzyme results in the lowering of blood pressure by preventing the endogenous biochemical process that converts the hormone angiotensin I into angiotensin II(Brown & Vaughan, 1998). Angiotensin II causes

vasoconstriction and triggers the release of aldosterone which has further impacts on the kidneys' control of electrolytes and fluids in the body by limiting the extent to which they are removed from the bloodstream(Brown & Vaughan, 1998). The protein bradykinin is an endogenous protein that lowers blood pressure that is broken down by angiotensin-II(Brown & Vaughan, 1998), therefore the inhibition of the formation of angiotensin-II by ACE-inhibiting bioactive peptides should also result in the accumulation of blood pressure-lowering agents, and ultimately a therapeutic effect. The use of protein hydrolysates with ACE-inhibitor activity derived from wheat bran to achieve this goal is very attractive, as they could serve as natural alternatives to synthetic medications.

Another assay that is commonly used is a metal chelation test, where the ability of the peptides to bind to a sequester metal species is evaluated. The ability to chelate undesirable metal species is important as these metallic ions catalyze the formation of reactive or radical species in the body. Transition metals such as iron and copper are common in bioinorganic systems but can also generate destructive oxygen species such as the superoxide anion, hydrogen peroxide, and hydroxyl radicals(Csire et al., 2020). An illustration of this process is depicted in figure 1.8.

Figure 1.8: Illustration of the formation of reactive oxygen species from copper and iron in-vivo that ultimately lead to oxidative stress, (Lakey et al., 2016).



In these assays the chelating ability of a bioactive peptide is determined based on the decrease in UV absorption of a reactive metal species such as iron or copper salts (Csire et al., 2020)·(Wickramasinghe et al., 2022). Multiple studies have demonstrated that both synthetic and naturally sourced peptides can act as iron (III) chelators, as well as radical scavengers (Csire et al., 2020)·(Wickramasinghe et al., 2022), ultimately preventing the formation and accumulation of reactive oxygen species in in-vitro studies by 95-99% (Csire et al., 2020).

1.8: Future outlooks:

The use of wheat bran as an industrial feedstock and a protein resource has immense potential, with many studies and applications already being explored to make the most of this abundant resource. Its status as a low-value, but highly abundant raw material imparts attractive economic margins to its use in valorization processes. Aside from animal feeds, it is currently underutilized for industrial applications despite its high nutritional profile and diverse chemical composition. Current developments in biochemical processing techniques have made the industrial use of hydrolytic enzymes more economically feasible for the production of consistently reproducible batches of protein hydrolysates. Additionally, recent work has demonstrated that bioactive peptides have enormous potential as nutritional supplements, as well as therapeutic compounds. They have been demonstrated to be effective for not only the treatment of diseases, but as preventative agents for the development of disease due to oxidative stress. Several wheat bran peptides have been identified with antioxidant and antihypertensive activity, but it is likely that there are still peptide sequences with bioactivities that have yet to be discovered. Although these advances have contributed significantly to the development of wheat bran as a more valuable resource, further research is still needed to evaluate the full extent to which wheat bran can be utilized.

Chapter 2 - Extraction and Purification of Wheat Bran Proteins

2.1 Abstract

This chapter details the extraction and purification of wheat bran proteins from hard red winter wheat bran. Several previous publications were considered when planning the extraction optimization, and these studies were narrowed down to three representative extraction methods. The first was a rudimentary alkaline extraction, while the second method selected was a modified Osborne fractionation. The third method tested was extraction using a deep eutectic solvent, originally designed for extracting protein from fava beans. Each of these extraction methods was tested in triplicate at least twice. The percent recovery of each trial was calculated based off of the remaining protein in the extracted wheat bran, determined by LECO analysis via the Dumas method of combustion. The highest performing extraction method was found to be the modified Osborne fractionation, with an average percent recovery of approximately 80% by LECO analysis. Further investigation using the Bradford assay showed that the extracts themselves consisted of approximately 74% protein, but using either method of protein quantitation, the modified Osborne fractionation outperformed both of the other extraction methodologies tested.

Once extracted and lyophilized, crude protein extracts were purified using a 500Å underivatized silica gel column equipped in a dry-column vacuum chromatography configuration. The mobile phase consisted of a 2-step gradient, that was adapted from Ghose et al., 2004, with an initial capture phase of 0.025M phosphate buffer at a pH of 7. The elution phase consisted of 0.1 to 0.025M arginine, which gave comparable results. These impurities were analyzed via SEC-HPLC to confirm that they were in fact lower molecular weight impurities. Additionally, crude and purified proteins were analyzed via SEC-HPLC to illustrate

the increase in concentration of the protein, as well as the removal of impurities from the crude protein samples by the 500Å si-gel column. The crude and purified proteins were also analyzed via SDS-PAGE to provide an estimation of the molecular weights of the most prominent protein bands in each Osborne fraction.

2.2 Introduction:

2.2.1: Background Information

Many different protein extraction methods have been established and described in the literature spanning several decades of work. Each of these publications' methods exhibit different degrees of simplicity and complexity, with wide ranges of percent recoveries. In addition to these variables, many of the protein extraction methods in the literature have not been designed or even optimized for use with wheat bran proteins. As a result, the use of an extraction method for a specific organism such as a legume could have significantly different results when performing the exact same procedure on a cereal grain. Furthermore, because wheat bran is a part of a biological organism, there are cellular structures that can impair the amount of protein that can be extracted. To circumvent this, many strategies have been proposed in previously established methods that incorporate an array of different pre-treatments to maximize the amount of protein that can be extracted from the wheat bran. Some of these strategies include reducing the particle size of the wheat bran by milling, or degradation of the cellular structures of the bran using enzyme pre-treatments(Roberts et al., 1985),(Waszczynskyj, 1980), (Neudoerffer, 1969). In addition to pre-treatments, some strategies incorporate the use of different types of extraction solvents, such as the Osborne fractionation's sequential extractions using different aqueous buffers(Osborne, 1907),(Sardari et al., 2019). Further investigation has also been carried out using more exotic, deep eutectic solvent systems that incorporate different polar organic solvents

to help extract as much protein as possible using a more complex chemical strategy(Hewage et al., 2024). Taking all of these variables into consideration, the vast body of prior work that has been carried out on food-protein extractions provides many starting points for the investigation of the best conditions for extracting protein from a new feedstock. However, because of all of the different strategies that have been developed, this large body of work can also provide too many starting points. Many pre-treatment options with large differences in material costs can make it difficult to know where to begin, as well as numerous publications claiming to achieve the highest yields with a particular crop that fail to be reproduced in another, or even a different variety of the same crop can cause confusion regarding the true reproducibility of a method. It is because of these factors and potential discrepancies that the investigation of a new extraction methodology from a series of previously established procedures was investigated and optimized for protein extraction from hard red winter wheat bran.

2.2.2 Extraction Efficiency Tests:

The primary goal of this investigation was to ensure that the highest yields possible could be obtained for this particular cultivar of wheat bran, and that the most efficient and effective method was used and proven to be reproducible and reliable. The investigation began by evaluating the percent recoveries of three different extraction methods reported in the literature. The first method investigated was based off the work of Roberts et. al., in 1985(Roberts et al., 1985). This extraction method uses a simple alkaline extraction at a pH of 12 for 16 hours, followed by two washings with pH 12 distilled water. This is by far the simplest extraction method, and according to the publication, should have achieved respectable yields of 74% of the total protein (Roberts et al., 1985). The simplicity of this method, combined with the high

reported yields made this method very attractive, and they were the primary reasons it was selected for evaluation.

The second extraction method selected for investigation with wheat bran proteins was a deep-eutectic solvent system, adapted from the work of Hewage et. al., in 2024(Hewage et al., 2024). The deep-eutectic solvent system consisted of choline chloride and glycerol dissolved in distilled water. This method was originally designed for the extraction of protein from fava beans, and it provided an average percent recovery of 65.42 ±6.53 % of the total protein content of the fava beans. The advantages of this method were that it was moderately high yielding, while only requiring a single round of extractions using a single solvent system. The solvent system was more complex than the simple alkaline extraction, but it was also cited as being higher yielding, and an environmentally friendly solution to obtaining higher protein yields using mild extraction conditions (Hewage et al., 2024). The extraction conditions also eliminated exposure of protein to harsh conditions, such as alkaline conditions at a pH of 12, that could result in base-catalyzed hydrolysis of the proteins. These considerations were the deciding factors that led to the selection of this method for further investigation with hard red winter wheat bran.

The third extraction methodology selected for investigation with wheat bran proteins was a modified Osborne fractionation. In this extraction method, wheat bran proteins were extracted in five sequential extraction steps according to their Osborne classifications: the solubility of a group of proteins in different aqueous extraction media(Osborne, 1907). These classifications consisted of albumins, which are soluble in distilled water; globulins, which are soluble in aqueous saline solutions; prolamins, which are soluble in aqueous ethanol; glutelins, which are soluble in aqueous acidic or alkaline solutions; and the insoluble fraction, which must be

extracted using harsh alkaline conditions at high temperatures(Osborne, 1907). The Osborne fractionation is more tedious than single-step extractions, but this method has stood the test of time since its inception in the early 1900s. This is primarily because of the high specificity of each extraction media for a particular type of protein. This coupled with the incorporation of five different extractions, each performed in triplicate, make the Osborne fractionation a high-yielding, reliable protein extraction method for cereal grains. Previous publications have reported yields as high as 62.5% of the total protein content(Sardari et al., 2019). These results were comparable to the DES extraction method, and although the Osborne fractionation is a lengthier process, the option to separate the different classifications of proteins in sequential steps was attractive for further analysis of peptides produced from these proteins via enzymatic hydrolysis. Peptides with high biological activity in invitro assays could be more easily narrowed down if the proteins are separated prior to hydrolysis, rather than determining which peptide came from a particular parent protein when produced in a more complicated protein cocktail.

The extraction efficiencies of these methods were compared through two analytical methods. The first analytical method was LECO analysis, using the Dumas method of combustion to estimate the total protein content in the bran before and after extraction. The second analytical method was the Bradford assay, a spectroscopic method that estimates the amount of protein in a protein extract using a color change reaction with the Bradford reagent. The percent recoveries for each extraction method were calculated based on these results, and the method with the highest percent recovery was selected for further investigation. This involved scaling up the extraction method, purification of the protein extracts using a 500Å underivatized silica gel column, and finally analysis of extract purity before and after purification on the column via SDS-PAGE and SEC-HPLC analysis.

2.2.3 Purification of Crude Protein Extracts on 500Å underivatized silica gel:

Once the highest yielding method had been established, the lyophilized crude protein extracts obtained using the best method were purified using a 500Å underivatized silica gel column set up in a dry column vacuum chromatography configuration. The proteins were purified using a two-phase step gradient, consisting of a capture phase of 0.025M phosphate buffer that deprotonates the silanol groups of the stationary phase, giving it a high affinity for proteins. Under these conditions, the proteins adhere strongly to the underivatized silica gel, and impurities are washed off of the column. The elution of these species was monitored via UV-Vis analysis at 280nm, and once the chromatogram returned to baseline in the capture phase, the mobile phase was switched to the second phase, or the elution phase. This phase used a 0.025M to 0.1M solution of arginine to disrupt the interactions of the proteins with the underivatized silica gel stationary phase and resulted in the elution of the proteins from the column almost directly with the solvent front. Fractions were collected, and the elution was monitored at 280nm to confirm that the entire protein sample had been eluted from the column. Fractions of interest from the chromatograms were collected and lyophilized for further analysis. Fractions obtained from the elution phase contained significant quantities of arginine, but due to the significant differences in size of proteins and the single amino acid, the excess arginine was easily removed from the purified protein samples using an ultrafiltration stirred cell equipped with a 3kDa molecular weight cutoff membrane. Following the removal of the arginine, the purified and polished protein samples were collected from the stirred cell and lyophilized again for stability during storage.

2.2.4 Protein Analysis via SEC-HPLC and SDS-PAGE

Crude proteins, proteins purified using a 500Å underivatized silica gel column, and the impurity fractions from the capture phase of each column were isolated for analysis. Size Exclusion High Performance Liquid Chromatography (SEC-HPLC) analysis was performed for each of these samples to evaluate the removal of lower molecular weight impurities from the protein samples using the 500Å si-gel column. The 600 kDa reference, 69385 Protein Standard Mix 15 was obtained from Sigma Aldrich, and was used to determine the approximate retention times of different proteins on the SEC column. This standard was referred to as “Protein Standard d". A sample of pure arginine was used to determine if any arginine remained in the protein samples collected from the 500Å si-gel column and 3kDa ultrafiltration polishing step. The arginine sample was also used as a reference to determine approximate retention times for small molecules on the SEC column.

SDS-PAGE was performed to illustrate the increase in concentration and purity of the proteins before and after the column. The SDS-PAGE gels obtained from BioRad had a lower molecular weight cutoff of 10kDa. As a result, any lower molecular weight impurities present in either the crude protein samples or the purified protein samples could not be directly visualized on the gel. However, the differences in protein concentrations required to achieve acceptable SDS-PAGE results did help illustrate the increase in concentration and purity of the protein samples. Compared to the crude extracts, proteins purified on the 500Å si-gel column required significantly lower sample concentrations. The lanes of the purified proteins were also straighter, and the resolution of individual bands improved dramatically.

2.3 Materials and methods:

2.3.1 Hard Red Winter Wheat Bran: Particle Size Reduction:

Hard red winter wheat bran was obtained as a donation from the Hal Ross flour mill in Manhattan Kansas. Initial protein content was measured using a LECO 928 series using the Dumas method of combustion, with a protein coefficient of 6.31 for wheat bran, according to the Conversion factors obtained from USDA Circular No. 183. August 1931, revised Feb 1941. The initial protein content of the unprocessed wheat bran was 16.28% by weight. The particle size of the coarse wheat bran was reduced by pin milling, and the bran was milled over six passes. This resulted in a fine bran powder suitable for further processing and extraction. The resulting bran powder was stored in a refrigerator at 4°C.

2.3.2 Defatting Pin Milled Wheat Bran Powder

Pin milled wheat bran powder was defatted using hexanes using a 1:3 ratio of wheat bran to hexanes. For most batches of defatted wheat bran, 100g of pin-milled wheat bran was defatted using 300 mL of hexanes. The wheat bran-hexanes slurry was stirred for 30 minutes using a mechanical overhead stirrer in a beaker, with an aluminum foil cover to prevent splashing. The first hexane-fat solution was filtered off the wheat bran powder using a vacuum filtration apparatus and ceramic Buchner funnel equipped with a 200-micron mesh filtration bag. Following filtration, the wheat bran was transferred back to the original beaker for two subsequent lipid extractions. A total of three lipid extractions were performed on the pin milled wheat bran. On the final filtration, 300 mL of hexanes were used to wash the wheat bran of any residual lipids, and the removal of virtually all of the lipids from the bran was visually confirmed by washing with hexanes until the filtrate running off of the wheat bran in the filter was clear. Following defatting, residual hexanes were removed from the defatted wheat bran powder using

a rotary evaporator. Defatted wheat bran was then stored in the refrigerator at 4°C prior to further processing.

2.3.3 Extraction of Protein From Defatted Wheat Bran Powder:

Three types of extraction methodologies adapted from publications by (Roberts et al., 1985), (Hewage et al., 2024), (Sardari et al., 2019b) were performed and compared to obtain the highest percent recovery possible. These extractions consisted of a 16-hour alkaline extraction at a pH of 12 (Roberts et al., 1985), a deep-eutectic solvent extraction (Hewage et al., 2024), and a modified Osborne fractionation Sardari et al., 2019. Percent recoveries were determined by running LECO analysis on extracted wheat bran using the Dumas method of combustion and comparing the remaining protein content to the original protein content of defatted, pin milled wheat bran powder. Protein concentrations in the extraction solutions were also measured using the Bradford assay to determine the approximate protein concentration of the protein extracts. The highest performing extraction method was selected for further investigation, which was the modified Osborne fractionation adapted from Sardari et al., 2019. Crude protein extracts were lyophilized and stored in the freezer at -20°C prior to further analysis and purification.

2.3.4 Simple Alkaline Extraction of Protein from Defatted Wheat Bran Powder

The first method tested was adapted from the work of Roberts et al., 1985. This procedure employed a simple alkaline extraction procedure, where pin-milled and defatted hard red winter wheat bran was subjected to a 16-hour extraction. The extraction was carried out using 15g of pin milled, defatted hard red winter wheat bran powder per 150 mL of distilled water. The pH of the extraction solution was adjusted to 12 using NaOH. Following the adjustment of the pH, the extraction solution was incubated at 60°C in a shaker bath set to 200 rpm. After the 16-hour extraction, the extract solution was decanted from the extracted wheat

bran, neutralized with HCl, and saved for further processing. The extracted bran was then washed twice with distilled water with a pH of 12 (adjusted to 12 using sodium hydroxide), and these washings were decanted off, neutralized with HCl to a pH of 7, and combined with the original neutralized extract. The pooled neutralized extract solution was stored at 4°C prior to further processing/analysis. The extracted wheat bran residue was washed with distilled water to remove the residual NaOH from the extraction and washing solutions, and dried overnight in an oven at 60°C. The percent recovery was evaluated by measuring the remaining protein in the extracted wheat bran using a 928 series LECO combustion analyzer.

2.3.5 Deep Eutectic Solvent Extraction of Protein from Defatted Wheat Bran Powder

The DES extraction procedure was adapted from the work of Hewage et al., 2024. In this procedure, a deep-eutectic solvent was prepared by combining 84g of choline chloride with 168g glycerol. This mixture was heated at 80°C until the solution was clear, and then cooled to room temperature. Once cooled, 168mL of distilled water was added and stirred until homogenous. This resulted in a 60% DES solution, the highest yielding ratio reported by Hewage et al., 2024. The extractions were carried out in triplicate, using 1g of pin milled, defatted hard red winter wheat bran powder and 28g of 60% DES solution. The samples were vortexed prior to incubation at room temperature for 1 hour on a shaker set to 200 rpm. Following the 1-hour incubation, the samples were centrifuged at 10,000 rcf at 4°C for 5 minutes, and the supernatants were decanted and stored for further analysis. This process was repeated for a total of 3 iterations, and each of the three supernatants were pooled. The extracted wheat bran was washed with distilled water to remove residual DES solvent and dried in an oven at 60°C overnight prior

to LECO analysis. In addition to LECO analysis, a Bradford assay was also run on the extracts to quantify the amount of protein within the extracts.

2.3.6 Modified Osborne Fractionation of Protein from Defatted Wheat Bran Powder

The modified Osborne fractionation procedure was adapted from Sardari et al., 2019. In this procedure, a ratio of 10g pin milled, defatted wheat bran was extracted in 100 mL of extraction solvent. Each extraction was performed in triplicate. The albumin fraction was extracted first using distilled water, with a 30-minute shaking at 200 rpm at room temperature. The samples were then either centrifuged for 20 minutes at 6,000 rcf, at 4°C, or strained through a 200mesh filter bag for larger extractions. The supernatants or filtrates were collected and pooled. This process was repeated three times, and the supernatants were collected and pooled after each centrifugation.

This process was repeated for each Osborne classification, but with 0.4M NaCl in 0.05M phosphate buffer for the globulin fraction, 70% ethanol for the prolamin fraction, and 0.05M NaOH for the glutelin fraction. Finally, the insoluble fraction was extracted by refluxing the wheat bran in 0.5M NaOH for 1 hour at 100°C. Following the 1-hour reflux, the reaction vessel was allowed to cool to room temperature, and the wheat bran slurry was poured into a 1L centrifuge bottle lined with a 200-mesh filter bag. The extract solution was removed from the wheat bran by straining it through the bag, and the extracted wheat bran was washed with distilled water to remove the residual extract solution and sodium hydroxide. The extracted wheat bran was then dried in an oven at 60°C overnight and stored in the refrigerator at 4°C prior to LECO analysis.

The prolamin fraction was rotary evaporated to remove ethanol from the extract solution. The glutelin and insoluble fractions were neutralized with HCl to a pH of 7 prior to further processing to prevent the hydrolysis of the proteins in the extract solutions. Samples of these extracts were analyzed using the Bradford assay to quantify the amount of protein in the extract solutions.

2.3.7 Protein Purification via Underivatized 500Å Silica Gel Column Chromatography

Crude protein extracts were purified via column chromatography, using a method adapted from (Ghose et al., 2004). This method employed a stationary phase media of underivatized silica gel with a pore size of 500Å. A step-phase gradient was used according to the previously established method, with a capture phase of 0.025M phosphate buffer at a pH of 7, and an elution phase of 0.025-0.1M arginine. 0.025M and 0.1M arginine gave comparable results. Solid crude protein extracts were reconstituted in 0.025M phosphate buffer, and were loaded onto the column, using a bed of acid-washed sand to protect the top of the column bed. The chromatography apparatus consisted of a typical sintered column set up in a dry column vacuum chromatography apparatus configuration. Fractions were analyzed using a microplate reader at 280 nm. Protein positive fractions were ultrafiltered to remove arginine using a stirred cell equipped with a 3kDa molecular weight cutoff ultrafiltration membrane. Purified protein samples were then collected and lyophilized for storage, prior to further analyses. The column was regenerated for multiple uses by washing the column with 2-3 column volumes of arginine, then 2-3 column volumes of 10% v/v acetic acid, followed by 2-3 column volumes of 30% v/v ethanol (Ghose et al., 2004). Following these steps, the column was regenerated by re-equilibrating the silica gel with 2-3 column volumes of pH 7, 0.025M phosphate buffer. Once re-equilibrated, the column was ready for re-use, with no detectable breakdown in resolution or

change in elution behavior of either the impurities in the capture phase, nor the proteins in the elution phase.

2.3.8 SDS-PAGE Analysis of Crude and Purified Protein Extracts

Purity analysis of crude protein extracts was performed using SDS-PAGE by reconstituting lyophilized crude protein in 4x Laemmli buffer to obtain a protein concentration of 0.1 to 0.4mg/µL. Sample tubes were heated at 100°C for 5 minutes. 10 µL of each fraction were loaded onto 16.5% Mini Tris-Tricine Gels (Bio-Rad, Hercules, CA, USA), resulting in 1 to 4 mg of protein per lane. Purified proteins were reconstituted using 5.0mg of lyophilized, purified protein in 100 µL of 4x Laemmli buffer. These samples were heated at 100°C for 5 minutes, and 2.5µL of SDS-PAGE sample was loaded onto each gel lane for a final protein concentration of 0.125mg of protein per lane. Electrophoresis was conducted under a constant voltage of 200V using a Bio-Rad Powerpack 1000 unit. 5µL of molecular weight standards from 10 to 250 kDa were used to determine the molecular weights of the protein bands using 5 µL of Precision Plus Protein Dual Color Standard (Bio-Rad, Hercules, CA, USA). Gels were stained for 1 hour with a staining solution composed of 40% acetic acid, 10% methanol, and Coomassie Brilliant blue in water. Once stained, the gels were carefully removed from the stain solution, rinsed with ultrapure r.o. water, and destained overnight using a destaining solution composed of 10% acetic acid, 40% methanol, and 50% distilled water. Following destaining, gels were rinsed with ultrapure r.o. water and placed on a glass plate with a white background for imaging.

2.3.9 Purity Analysis via Size Exclusion High Performance Liquid Chromatography (SEC-HPLC)

Because the SDS-PAGE gels had a minimum molecular weight threshold of 10kDa, lower molecular weight impurities could not be visualized using SDS-PAGE. An Agilent 1100-

seried HPLC equipped with a Phenomenex Yarra 3µm SEC-4000 column, (300x7.8mm) was used instead to show the removal of impurities by analyzing the protein samples before and after being purified on the 500Å si-gel column. The impurity peak from the capture phase was also collected and analyzed for each Osborne fraction via SEC-HPLC to confirm that the impurities removed in the capture phase were in fact lower molecular weight, non-protein impurities, as expected. Retention time mapping using SEC-HPLC allowed for the approximate estimation of molecular weights of species of interest obtained from the 500Å si-gel column because the SEC column separated constituents in a sample based on molecular size. In this process, larger molecules eluted first, and smaller molecules eluted later in the chromatogram. Using protein standard d (69385 Protein Standard Mix 15 from Sigma Aldrich) and Arginine as the lower molecular weight standard, relative molecular sizes of unknown species could be estimated based on their retention times relative to these standards. Arginine was used as a standard for mapping the retention times of lower molecular weight impurities, as it has a molecular weight of 174.2g/mol, and was not present in the capture phase. It was also completely removed from the protein samples via 3kDa mw cutoff ultrafiltration and was not detected in any of the protein samples purified on the 500Å column. These factors made arginine an ideal choice for use as a retention time mapping standard, in addition to being detectable at 214 and 220 nm. A protein standard was also used to determine the retention times of various proteins on the SEC-HPLC column. SEC-HPLC was performed on each of the samples using an isocratic run consisting of of 60% water with 0.1% trifluoroacetic acid, and 40% acetonitrile with 0.1% trifluoroacetic acid. The flow rate was set at 0.4 mL/min, with each analysis set for 60 minutes. Elution was monitored at 214, 220, and 280nm. Samples were prepared at approximately 1mg/mL, using pH 6.8 phosphate buffered saline to reconstitute each lyophilized sample. Impurity samples for the

albumin, globulin, and prolamin isolated impurities were slightly higher in concentration, due to the fact that the phosphate buffer from the capture phase could not be separated from the lower molecular weight impurities via ultrafiltration.

2.4 Results and Discussion:

2.4.1 Comparisons of Protein Extraction Methods

Percent recoveries for each extraction method were determined via LECO analysis using the Dumas method of combustion. The amount of protein remaining in the extracted wheat bran was compared to the initial protein content of unprocessed wheat bran. The results of the LECO analyses for the alkaline extractions, Osborne fractionations, and DES extractions are shown below in table 2.1, compared to the initial protein content of unprocessed wheat bran.

Table 2.1: Comparison of extraction methods evaluated in this study. Percent protein by weight in bran was determined by LECO analysis of unprocessed wheat bran. Percent protein extracted was determined via Bradford assay.

Sample ID	% Protein by weight in Bran	% Protein Remaining	% Protein Extracted
Unprocessed WB	16.28[a]	100.00	0.00
Alkaline Extract	12.25[b]	75.23	24.77
DES Extract	12.24[b]	75.16	24.84
Osborne Fractionation	4.11[b]	25.25	74.75

a: %protein by weight in bran was determined via LECO analysis of unprocessed wheat bran. b: % protein by weight in bran was determined by measuring the percentage of protein extracted from a particular bran sample, multiplied by its original bran content, determined via LECO analysis.

The results of this experiment showed that the Osborne fractionation procedure extracted an average of 74.75% of the total protein content. These results outperformed the alkaline extract reproduced from the work of Roberts et. al., in 1985,(Roberts et al., 1985), which achieved an average percent recovery of 24.77%. The modified Osborne extraction also outperformed the deep eutectic solvent method(Hewage et al., 2024), which achieved an average percent recovery

of only 24.84%. Based on these results, the Osborne fractionation was selected for further development. Several more Osborne fractionations were performed, resulting in a range of percent recoveries, listed in table 2.2. The highest yielding extraction achieved a yield of 85.47%, but this method achieved an average percent recovery of 80.10%.

Table 2.2:Further assessment of the modified Osborne fractionation method. Percent recoveries were determined via LECO analysis of extracted wheat bran.

Trial	% recovery	Wheat bran: solvent ratio
MS-WB-03	80.89	2.5g: 25mL
MS-WB-04	78.41	2.5g:25mL
MS-WB-05	85.47	2.5g:25mL
MS-WB-06	73.05	2.5g:25mL
MS-WB-28	82.69	5.0g:50mL
Average	80.10%	-

The percent recovery results of the Osborne fractionation procedure were satisfactory; although there were slight fluctuations in percent recoveries of protein, the average was around 80%, which outperformed not only the other two extraction methods, but also outperformed the expected results based on the results from the original publication. Once the effectiveness of this extraction method had been demonstrated and shown to be reproducible, the extraction efficiency was analyzed at each step in the Osborne fractionation. The percent recoveries of each step are tabulated in table 2.3.

Table 2.3:Sequential analysis of each step of the Osborne fractionation, experiment MS-WB-05, determined via LECO analysis of the remaining protein content of extracted wheat bran compared to unprocessed wheat bran.

Osborne Fraction	Protein extracted [mg] (calculated via Bradford Assay)	Individual Fraction % Recovery	Total % Recovery
Pin milled, defatted WB	804.63 mg (prior to extraction)	0.00	0
Albumin	92.48 mg	11.49	11.49
Globulin	137.31 mg	17.06	28.55
Prolamin	30.84 mg	3.83	32.38
Glutelin	145.50 mg	18.08	50.46
Insoluble Fraction	195.30 mg	24.28	74.74
Total	601.43 mg	NA	74.74

Additional analysis of each step in the Osborne fractionation was performed using the Bradford assay to quantify the amount of protein in each sequential extract. These values are tabulated in table 2.4.

Table 2.4: Sequential analysis of each step of the Osborne fractionation, experiment MS-WB-28, protein quantified via Bradford assay. Initial protein content was determined via LECO analysis.

Osborne Fraction	% Protein remaining	Total % Recovery	Individual Fraction %
Pin milled, defatted WB	16.28	0	-
Albumin + Globulin	11.25	30.91	30.91
Prolamin	10.71	34.20	3.29
Glutelin	6.72	58.73	24.53
Insoluble Fraction	2.37	85.47	26.74

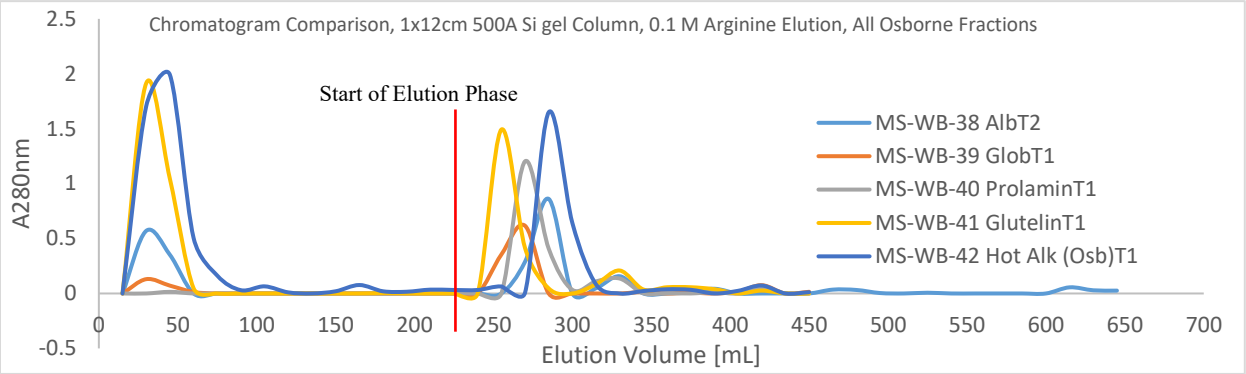
The results of the Bradford assay protein quantitation were slightly less than the LECO analysis data, but the total percent recovery was still significantly higher than either of the alternative

extraction methods. The modified Osborne fractionation continued to be the preferred extraction method for use with wheat bran protein extraction.

2.4.2 Purification of Crude Protein Extracts via 500Å Silica Gel Column Chromatography

Lyophilized crude Osborne protein extracts were reconstituted in pH = 7 phosphate buffer and applied to the 500Å si-gel DCVC column. The capture phase step gradient of pH 7 phosphate buffer was used to elute the non-protein impurities from the column, and the elution of these species was monitored at 280nm until a consistent return to baseline was achieved. The fractions that contained the impurity peak were collected for lyophilization and analysis via SEC-HPLC. Once the chromatogram returned to baseline, the capture phase buffer was depleted via elution from the column, and the mobile phase was switched to the elution phase step gradient, 0.1 to 0.025M arginine. These concentrations achieved similar, if not identical results. The elution of this step in the gradient was monitored at 280nm to confirm protein-positive fractions, and fractions were collected until a consistent return to baseline was achieved. Once the chromatogram returned to baseline and all of the protein was collected from the column, the protein-positive fractions were collected and lyophilized, prior to further analyses. Figure 2.1 shows the overlaid chromatograms for each of the five Osborne fractions, illustrating the consistency in the elution of both impurity fractions in the capture phase, and protein fractions in the elution phase of the step gradient. Although these chromatograms were generated with 0.1M arginine, identical elution profiles were observed for 0.025M arginine elution gradients.

Figure 2.1:Overlaid chromatograms generated for each Osborne fraction. Peaks eluting around 25-50 mL during the capture phase were isolated as impurity fractions. Peaks eluting between 250 and 350mL were protein fractions collected during the elution phase of the step gradient. Chromatogram generated using a 0.1M arginine elution gradient.



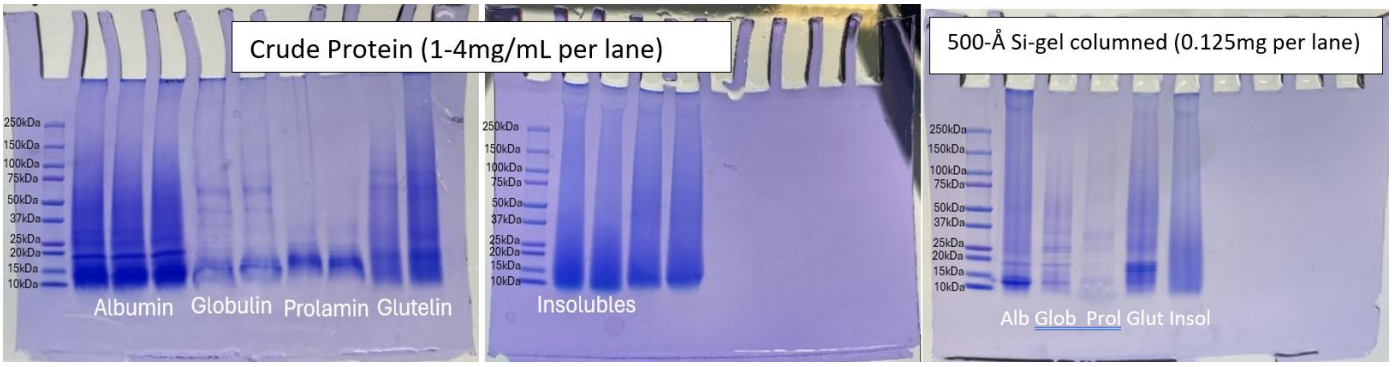
2.4.3 SDS-PAGE Analysis of Crude and Purified Proteins

SDS-PAGE was performed to further investigate the size of the proteins extracted from wheat bran, as well as the effect of the 500Å si-gel column on protein concentration and purity. Crude proteins required sample concentrations of 0.1-0.4mg/µL and sample volumes of 10µL per lane, equivalent to 1 to 4 mg protein per lane. Purified proteins required significantly less material, with samples prepared at a concentration of 0.05mg/µL. Initially,10µL of each purified sample were loaded and ran on the gels, but the results of these trials were overloaded lanes with poor band resolution. Subsequent trials were performed using reduced sample volumes at the same concentration of 0.05mg/mL until optimal bands were obtained. Additionally, the lanes of the purified proteins were much straighter than the crude samples, likely due to the removal of significant quantities of sodium chloride and other lower molecular weight constituents.

Proteins purified on the 500Å si-gel column achieved better resolution and more easily visualized protein bands. The majority of the protein bands observed in the SDS-PAGE gels ranged from 10 to 37 kDa. SDS-PAGE analysis of the proteins before and after purification on the 500Å si-gel column were unable to visualize the impurities in the crude proteins and could

not show their removal after being columned. This was due to the 10kDa molecular weight threshold of the SDS-PAGE gels. The results of the SDS-PAGE analyses did show an obvious improvement in the quality of the lanes of the gels. This was attributed to the removal of significant quantities of impurities from the protein samples, which was especially obvious in the case of the globulins. Crude globulin samples contained large quantities of salt that made preparation of the crude samples difficult. This resulted in streaky, crooked lanes, with poor resolution of protein bands after staining and destaining. Removal of the salt and lower molecular weight impurities using the 500Å Si-gel column made it much easier to measure out the correct amount of protein for each Osborne fraction at 5 mg of protein per 100µL of 4x Laemmli buffer from Bio-Rad. Initial SDS_PAGE trials were ran with sample volumes of 10µL, but the lanes of these gels were clearly overloaded and had poor band resolution. Subsequent trials were conducted using smaller sample volumes to improve sample loading and resolution on the gels. The best SDS-PAGE results were obtained using a sample volume of 2.5µL at a concentration of 5mg per 100µL, equivalent to 0.125mg of protein per lane. The lanes of this gel were far straighter and had more visual protein bands compared to the crude protein samples, illustrated by the comparison in figure 2.2. These SDS-PAGE results suggested that the 500Å si-gel column successfully increased protein concentrations and removed lower molecular weight impurities such as sodium chloride. Despite these results, further analysis using SEC-HPLC was required to characterize the lower molecular weight constituents in the impurity fractions obtained during the capture phase of the step gradient. SEC-HPLC analysis was also used to confirm the complete removal of these constituents from the protein fractions.

Figure 2.2: SDS-PAGE of crude protein extracts vs proteins purified using the 500Å silica gel column. Crude proteins required concentrations of 1-4mg per lane, whereas purified proteins were ran using only 0.125 mg per lane for optimal band visualization. The proteins purified on the 500Å si-gel column provided significantly better SDS-PAGE results, with straighter lanes and more well-defined protein bands. Samples of purified protein with equivalent concentrations to crude protein samples were also ran, but resulted in poor banding and overloaded lanes.



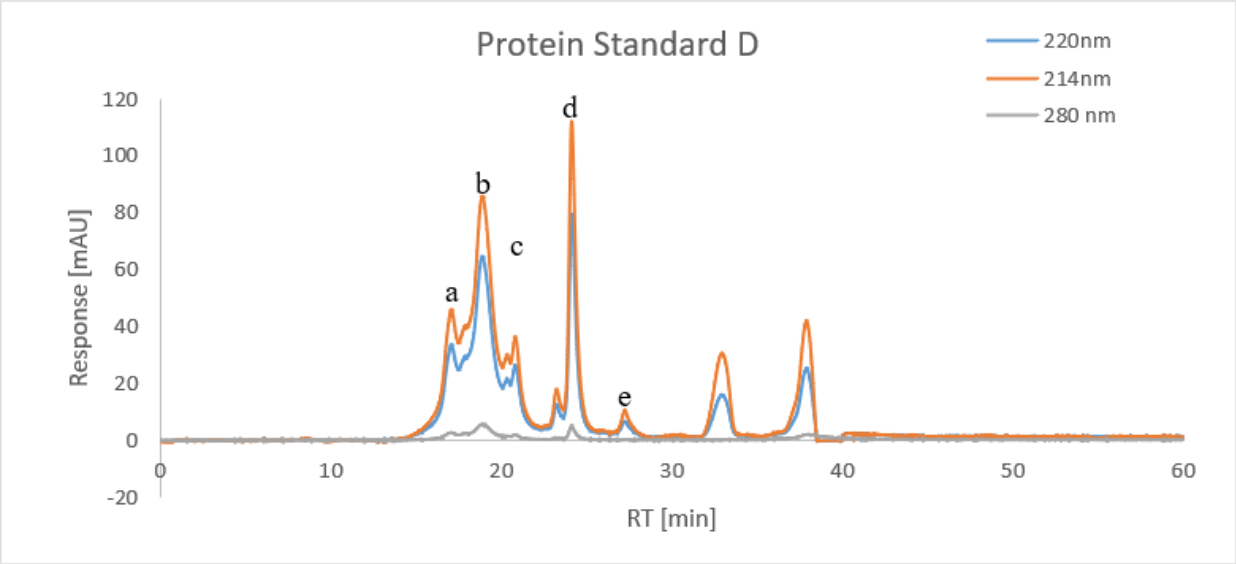
2.4.3 SEC-HPLC Analysis of Crude Protein Extracts

Purity analysis of crude protein extracts was performed using SEC-HPLC using an Agilent 1100-series HPLC equipped with a Phenominex Yarra 3µm SEC-4000 Size-exclusion chromatography column. An isocratic mobile phase was used, consisting of 60% water with 0.1% TFA and 40% acetonitrile with 0.1% TFA. A flow rate of 0.4mL/min over 60 minutes was used to generate chromatograms for each Osborne protein fraction before and after purification on the 500Å underivatized silica gel column. The impurity fractions collected during the capture phase step of the 500Å si-gel column were also isolated for each Osborne fraction for SEC-HPLC analysis. These samples were ran to demonstrate that no protein was lost during the capture phase of the step gradient ran on the 500Å si-gel column, and that the capture phase was successful in removing lower molecular weight impurities from the protein extracts. A protein standard was used as a reference for retention time mapping according to the sizes of different protein standards. Unfortunately, due to the age of this standard it was useful only for demonstrating the approximate retention times for proteins in general. Standard d's small

molecular weight standard, p-aminobenzoic acid was not useable for determination of retention times for lower molecular weight impurities. Instead, a sample of arginine was used to determine the retention times for lower molecular weight constituents. A standard sample of 200 proof ethanol was also used to identify the retention time of ethanol in the prolamin samples, as they had to be reconstituted in 70% ethanol in phosphate buffered saline for SEC-HPLC analysis. Samples of crude albumins, globulins, prolamins (70% ethanol, 30% pH 6 PBS), glutelins, and hot alkaline extracted insoluble fractions were reconstituted in pH 6 phosphate buffered saline at a concentration of 1mg solid per mL of reconstitution buffer. Impurity samples were reconstituted in 2 mL of pH 6 phosphate buffered saline.

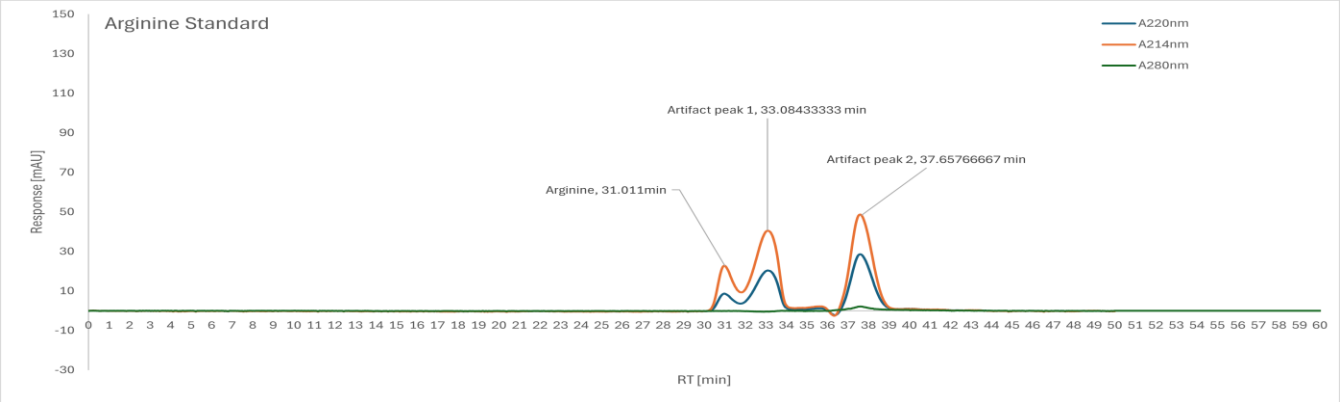
The results of the SEC-HPLC analysis showed clear increases in protein concentration after purification on the 500 Å si-gel column, as well as increases in protein purity. Protein standard “d” (protein standard 69385 protein standard mix 15 from Sigma Aldrich) was used to associate retention times with approximate molecular weights, illustrated in figure 1. These consisted of thyroglobulin (a), with a mw of 670kDa and RT of 17.19 min, gamma globulin (b), with mw of 150 kDa and RT of 18.99 min, ovalbumin (c), with a mw of 44.3 kDA, and RT of 20.92 min, and Ribonuclease A (d), with a mw of 13.7 kDA and RT of 24.16 min. The last and smallest standard in standard d was p-aminobenzaldehyde (e), with a mw of 137.14g/mol with a retention time of 27.33 min.

Figure 2.3: SEC-HPLC analysis of protein standard d.



Unfortunately, this standard had degraded to the point that it was unreliable for exact retention time mapping of individual proteins, as there were discrepancies between the retention times of p-aminobenzoic acid and the arginine sample, which should have had similar retention times due to their similar molecular weights. Arginine, with a molecular weight of 174.2g/mol, eluted consistently around 31 minutes. P-aminobenzoic acid should have eluted even later, as its molecular weight is 137.14g/mol, but this peak was not readily observed in standard d's chromatogram. This was likely due to previous, long-term storage at room temperature, inside the HPLC's autosampler. As a result, protein standard d was used as a rough benchmark for protein retention, while the SEC analysis of arginine was used to determine the retention of small molecular weight constituents and impurities. The standard sample of arginine was analyzed via SEC-HPLC and was also used to account for any residual arginine in the purified proteins, but no residual arginine was detected in any of the purified protein samples. The arginine sample's primary purpose was then to serve as a standard for a lower molecular weight constituents and is shown in figure 2.4.

Figure 2.4: SEC-HPLC analysis of pure arginine.



Overall, Crude protein samples had much smaller peaks, as well as peaks observed at longer retention times past 28 minutes, associated with lower molecular weights. Samples of protein fractions purified on the 500 Å silica-gel columns exhibited much sharper peaks with larger responses. Additionally, peaks at longer retention times were eliminated from the chromatograms. Analysis of the impurity fractions collected during the capture phase of the 500 Å silica-gel column purifications showed peaks that corresponded to lower molecular weight constituents only. None of the retention times corresponded to the proteins the impurity fractions were separated from, indicating that the capture phase successfully retained the protein on the 500Å silica-gel stationary phase. These results clearly illustrate that purification of crude protein extracts using a 500 Å, underivatized silica-gel column with a 0.025M phosphate buffer capture phase and 0.025M arginine elution phase step gradient successfully removed non protein, lower molecular weight impurities from the crude protein extracts.

SEC-HPLC analysis of crude albumin extracts in figure 2.5 showed small peaks with protein-retention times, but it was difficult to see peaks associated with lower molecular weight impurities. Post-column albumins showed much larger responses, with peaks having retention times ranging from 19 to 26 minutes, shown in figure 2.6. Isolated albumin impurities were also analyzed to show that the impurities removed from the crude albumin sample were non-protein,

lower molecular weight constituents with a retention time of 45.9 minutes, illustrated in figure 2.7.

Figure 2.5: SEC-HPLC analysis of crude albumin extract, pre 500Å si-gel column.

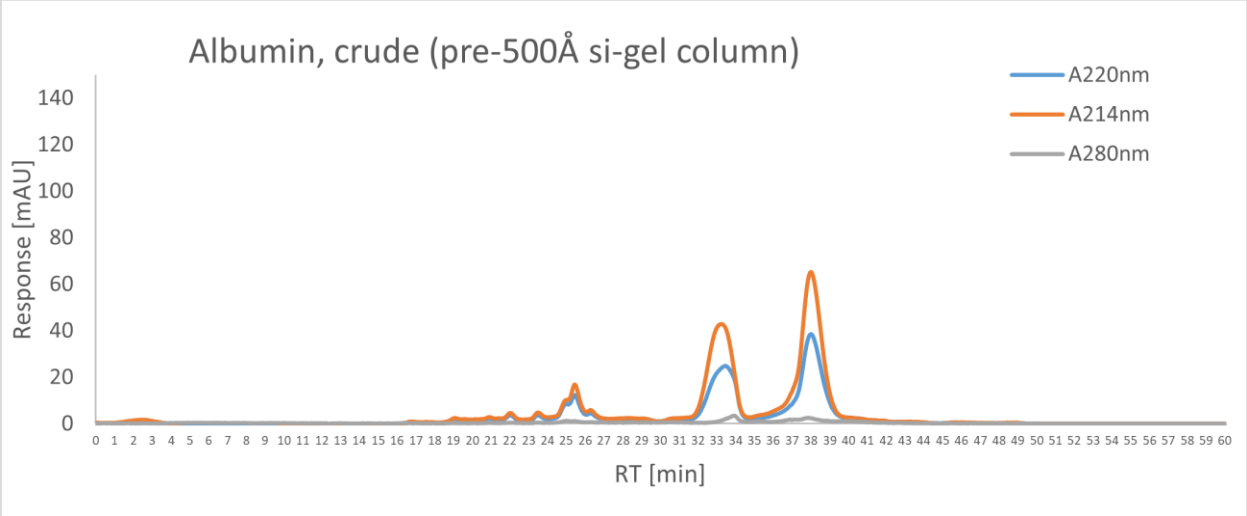


Figure 2.6: SEC-HPLC analysis of albumin extract purified on the 500Å si-gel column.

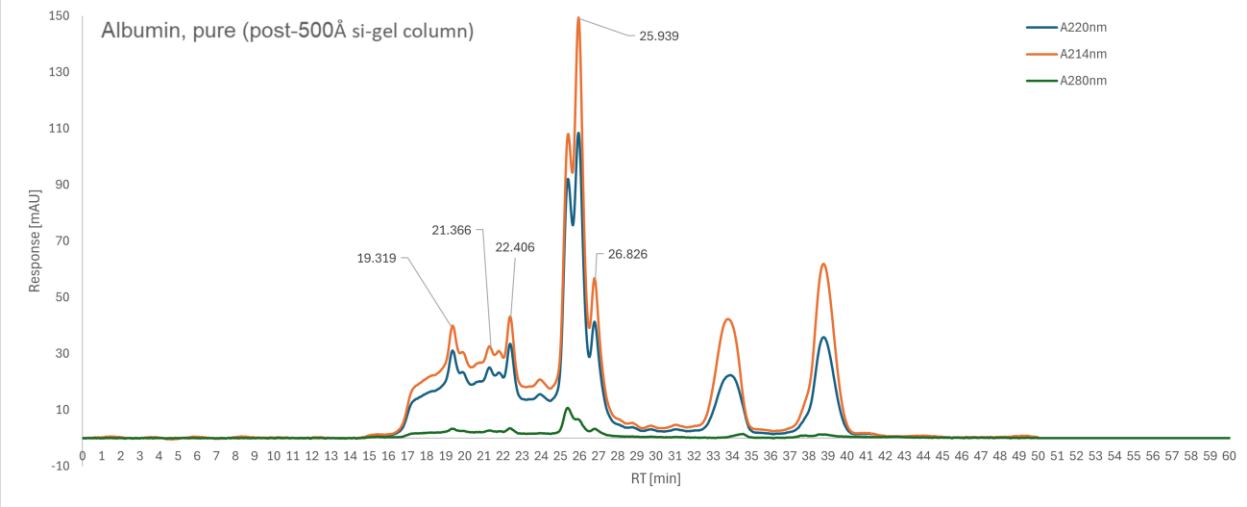
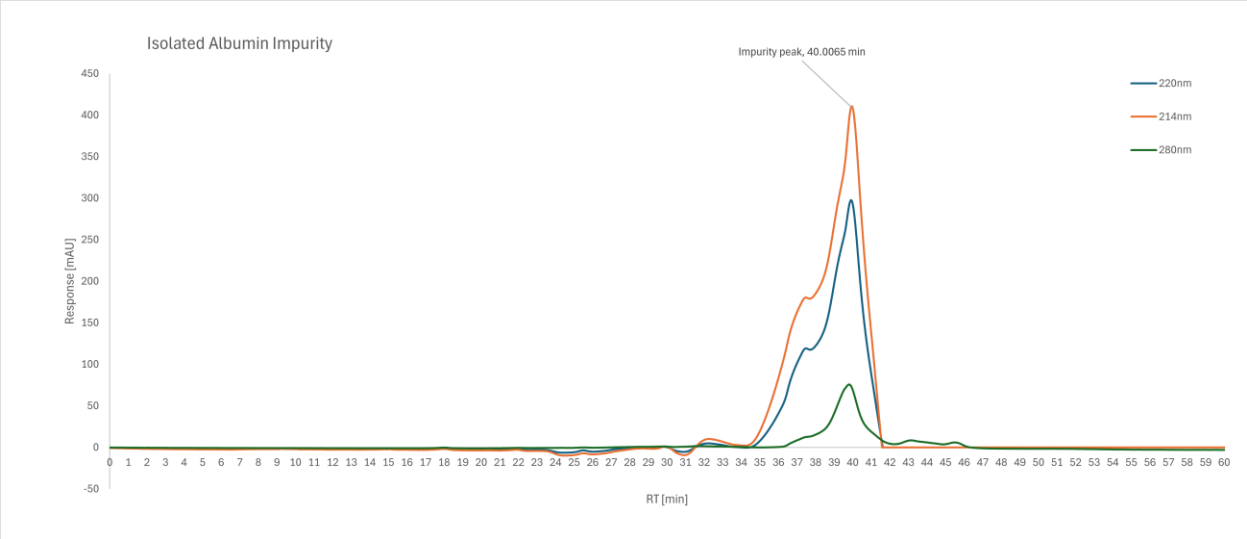
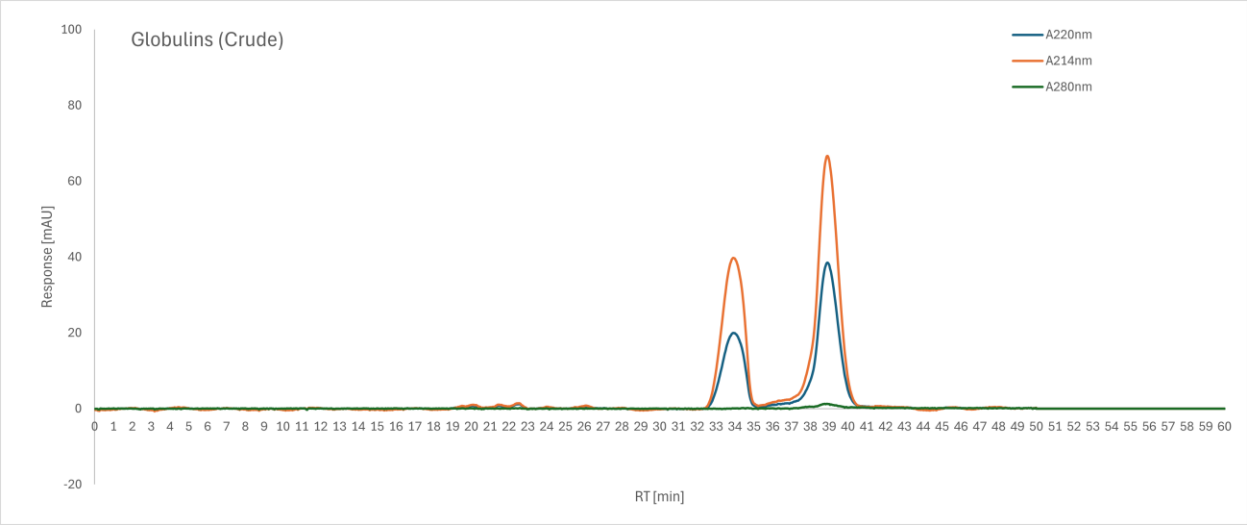


Figure 2.7: SEC-HPLC analysis of isolated albumin impurity fraction, obtained during the capture phase step of the 500Å si-gel column purification of crude albumin extract. Concentration was high enough to engulf both artifact peaks at ~35 and ~39 minutes.



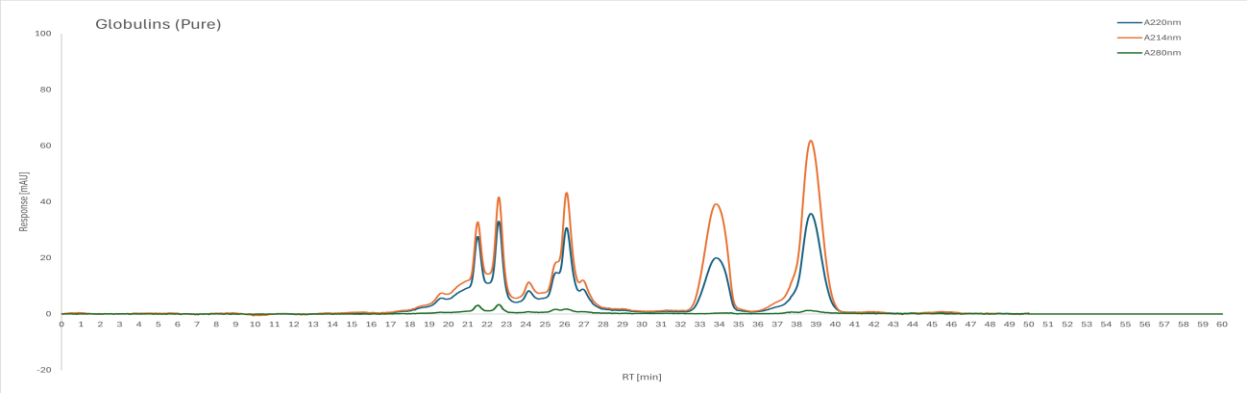
Crude globulin samples were heavily contaminated with NaCl, which resulted in very low responses for globulin proteins during SEC-HPLC analysis of crude globulin extract samples. These low responses with retention times ranging from approximately 20 to 25 minutes are illustrated in figure 2.8.

Figure 2.8: SEC-HPLC analysis of crude globulin extracts, prior to purification on 500Å si-gel column.



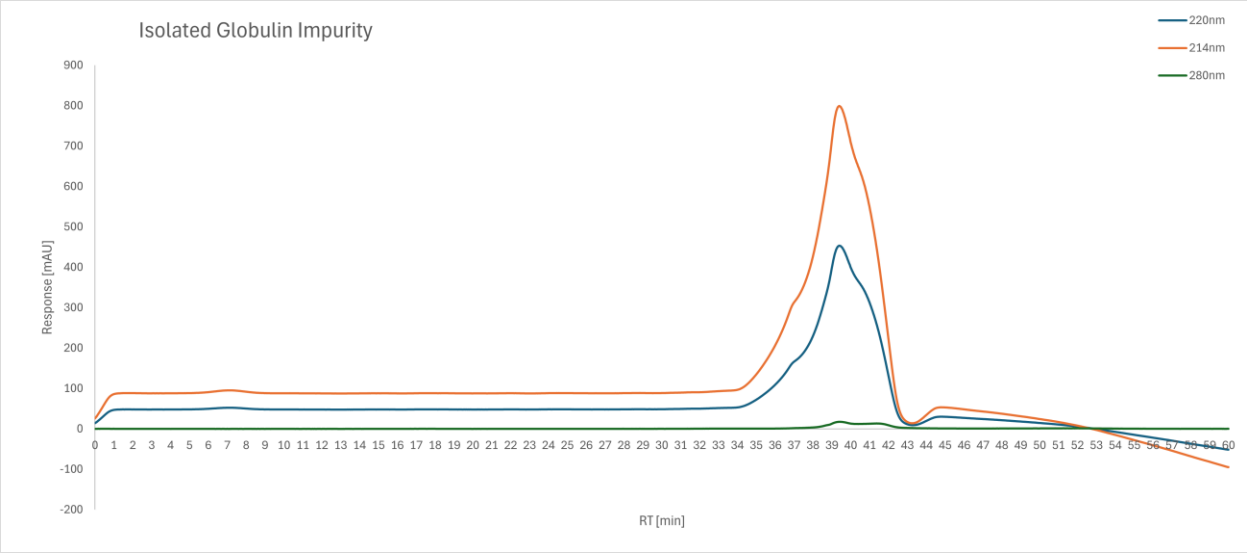
After purification on the 500Å si-gel column, globulin peaks increased dramatically in size, as shown in figure 2.9. Protein signals from approximately 17 minutes to 28 minutes increased significantly, and retention times associated with lower molecular weights remained at zero.

Figure 2.9: SEC-HPLC analysis of globulin extract after purification via 500A si-gel column.



SEC-HPLC analysis of the impurity fraction obtained during the purification of crude globulin samples showed retention times around 35-42 minutes, illustrated in figure 2.10. These retention times indicate the removal of lower molecular weight impurities from the crude globulin sample, aside from NaCl.

Figure 2.10: SEC-HPLC analysis of isolated globulin impurity from the 500A si-gel column. Sample was concentrated enough to engulf both artifact peaks at ~35 and ~39 minutes.



Prolamins purified on the 500Å si-gel column increased in concentration, and upon SEC analysis it was found that there was a low molecular weight constituent with a retention time of 44 minutes that also had a high affinity for the silica gel stationary phase, exhibiting very similar behavior to proteins in the capture-elution step gradient system. This could indicate the presence of denatured protein fragments that could be the result of damaged proteins that denatured during the rotary evaporation of ethanol from the extract. Further investigation needs to be carried out to determine the exact identity of the constituents in this peak, but based on previous literature publications these unknown constituents behave very similarly to proteins on this column system, only with reduced size.

Figure 2.11: SEC-HPLC analysis of crude prolamins, prior to purification on the 500Å si-gel column.

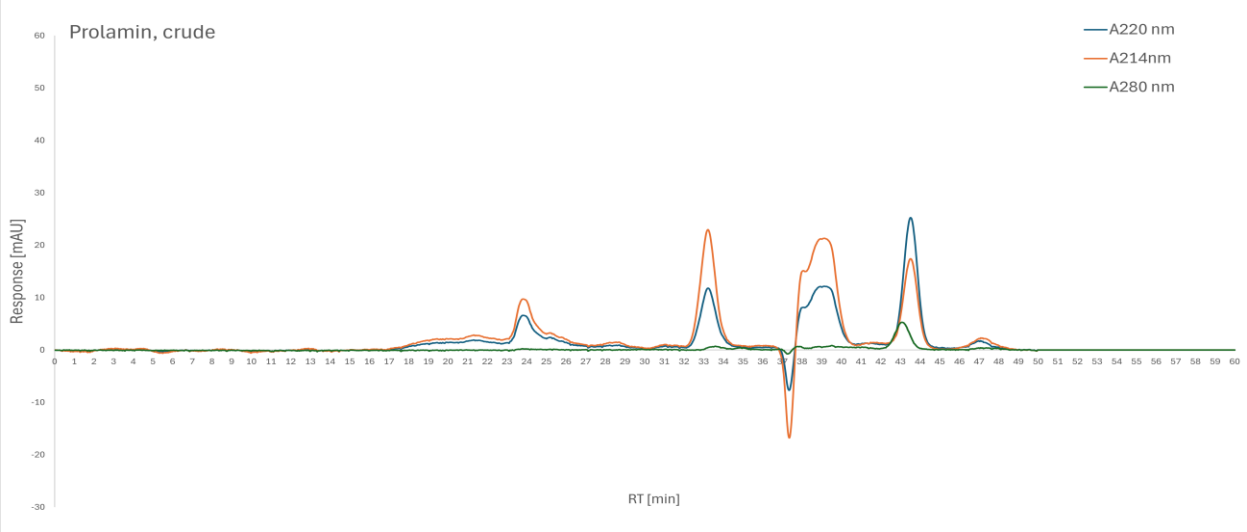


Figure 2.12: SEC-HPLC analysis of prolamins purified on the 500 A si-gel column. protein peaks increased, but the peak at 44 minutes also increased. Currently unsure of the identity of this constituent but based on its behavior and affinity for the stationary phase during the

capture step, it is possible that this constituent is derived from the protein, and may be a protein fragment resulting from denaturation.

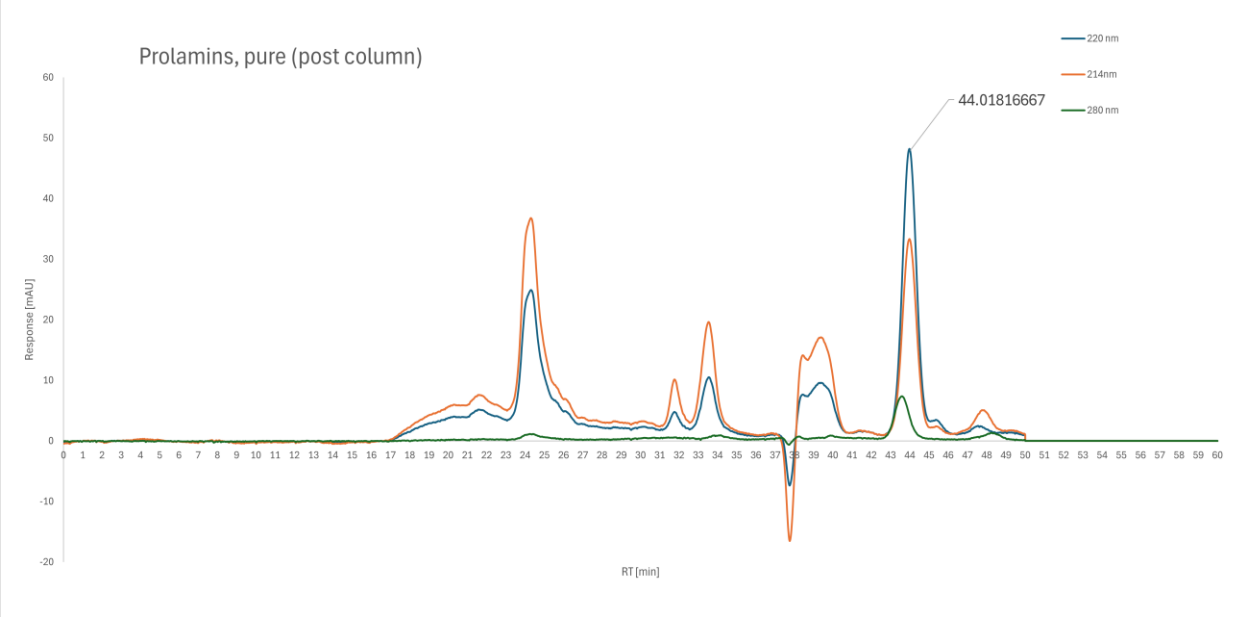
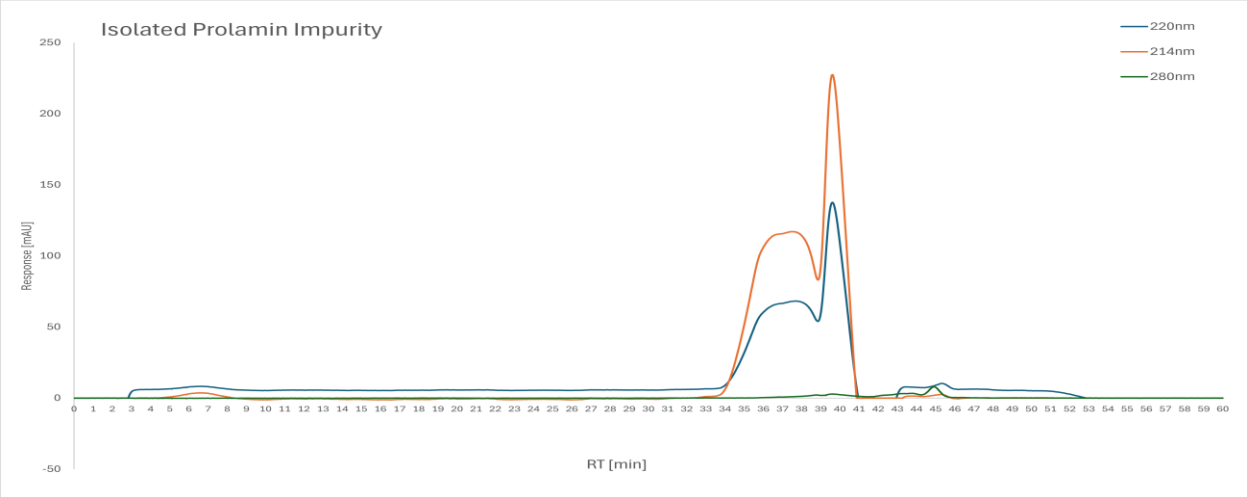


Figure 2.13: SEC-HPLC analysis of isolated prolamin impurity from the 500A si-gel column. Concentration was high enough to partially engulf both artifcat peaks around 35 and 39 minutes, with the primary impurity peak eluting around 37.8 minutes.



SEC analysis of crude glutelins in figure 2.14 showed significant levels of impurities, with peaks observed at 41.9 and 44 minutes. These peaks were not observed in figure 2.15, which depicts the chromatogram for the glutelin protein purified on the 500 Å si-gel column. The

disappearance of these peaks illustrated successful removal of lower molecular weight impurities from the sample using the capture-elution step gradient on the 500 Å si-gel column.

Figure 2.14: SEC-HPLC analysis of glutelins prior to purification on the 500A si-gel column. Low mw impurities observed at RT's 41.9 and 44 minutes.

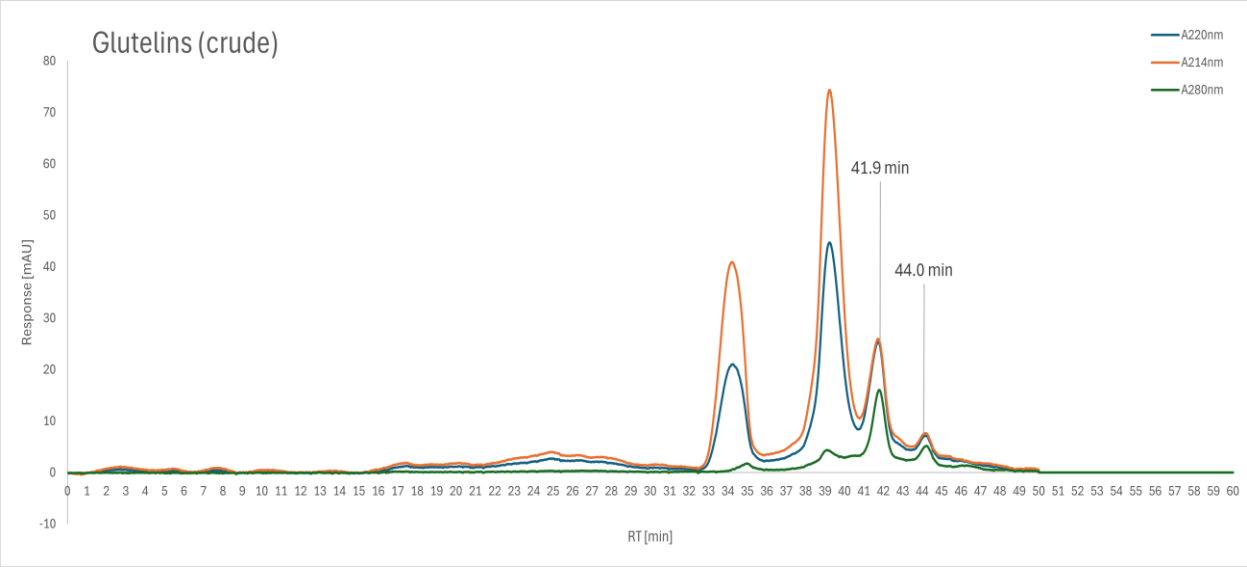
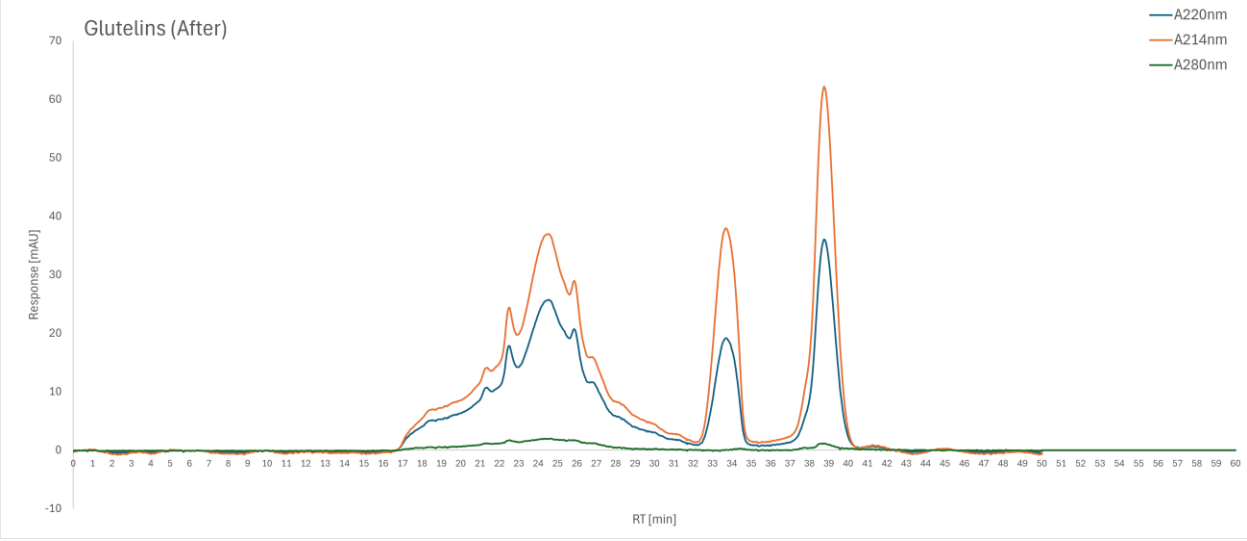
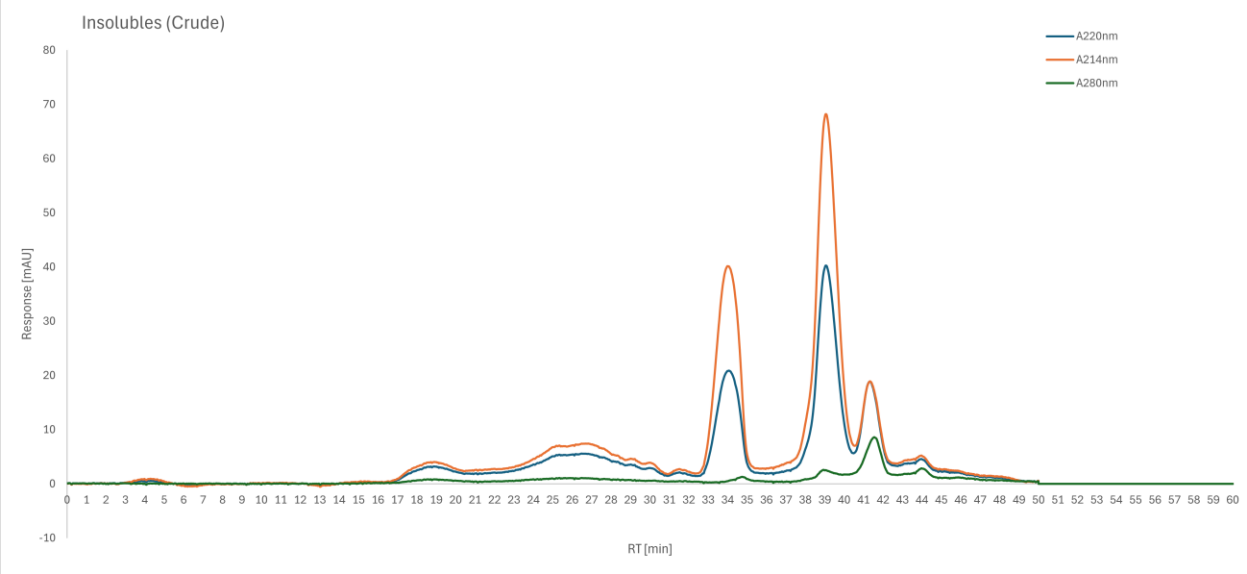


Figure 2.15: Glutelins after purification on the 500 A si-gel column. Low molecular weight impurity peaks at 41.9 and 44 minutes disappeared, and protein peaks from approximately 17 to 30 minutes increased significantly.



SEC analysis of the crude insoluble fraction in figure 2.16 showed 2 lower molecular weight impurity peaks with retention times of approximately 41 and 43 minutes, in addition to the protein signals from ~17 to 30 minutes.

Figure 2.16: SEC-HPLC analysis of crude insoluble fraction, protein signals observed, as well as lower molecular weight impurities.



Purification of the insoluble protein fraction using the 500Å si-gel column resulted in removal of the lower molecular weight impurity signals with retention times of ~ 41 and 43 minutes, indicated by the disappearance of these peaks in figure 2.17. Additionally, peaks associated with protein retention times on the SEC-HPLC increased significantly from 15 to 30 minutes.

Figure 2.17: SEC-HPLC analysis of insoluble fraction after purification on the 500Å si-gel column.

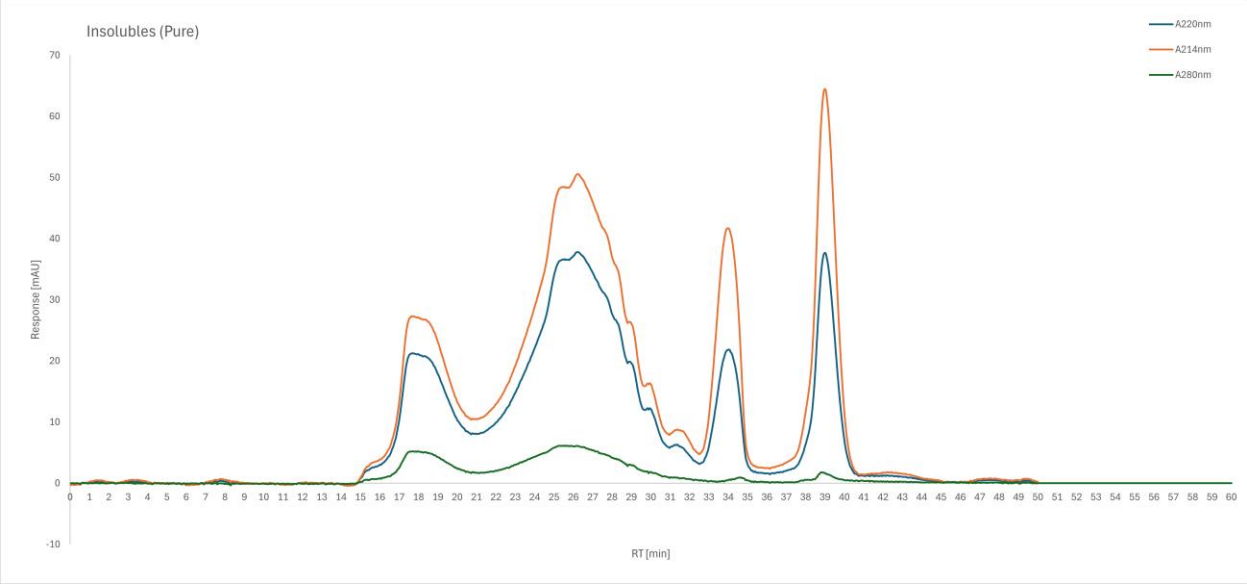
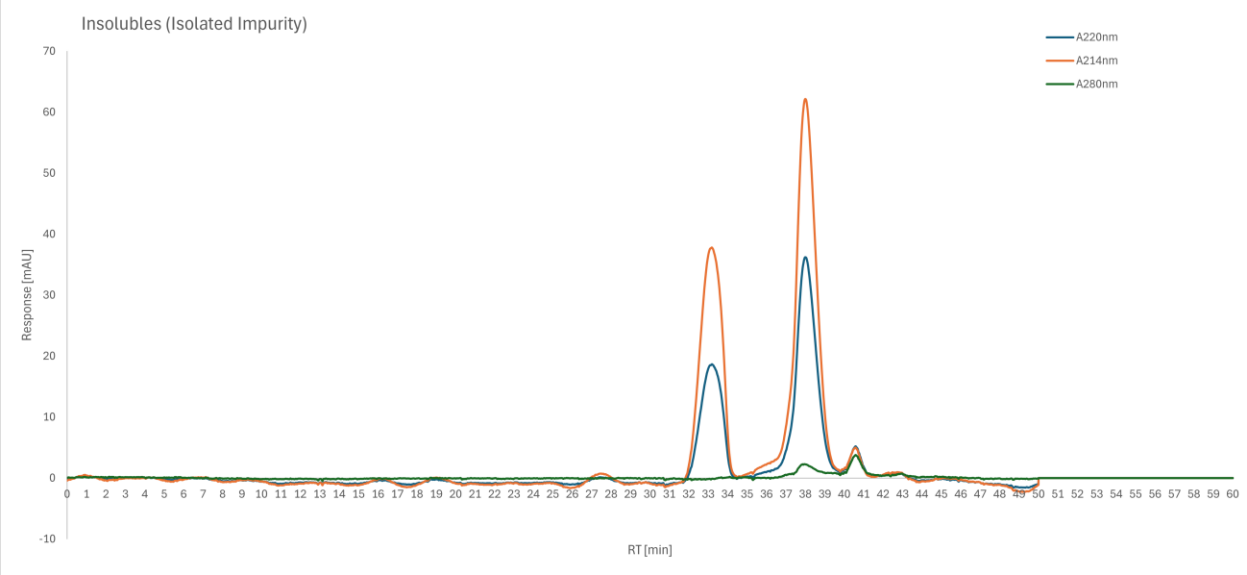


Figure 2.18 depicts the capture phase peak isolated during purification of the insoluble fraction on the 500Å si-gel column. The impurity peak had a retention time of 40.58 minutes, with no peaks associated with proteins observed in the chromatogram. These results confirmed the successful removal of lower molecular weight impurities from the insoluble fraction using the 500Å si-gel column using the capture phase step gradient.

Figure 2.18: SEC-HPLC analysis of the isolated impurity obtained during the purification of the insoluble protein fraction via the 500Å si-gel column.



2.5 Conclusions:

In conclusion, three different protein extraction methods were tested and compared based on their extraction efficiencies. Of the three methods tested, the modified Osborne fractionation extraction method adapted from Sardari et al., 2019, gave the highest percent recoveries. This method consistently yielded an average of 80.10% of the total protein content when analyzed via LECO analysis. When the protein extracts themselves were quantified, the total percent recovery dropped slightly to 74.74%, but this was still significantly better than either of the other two extraction methods. The deep eutectic solvent extraction adapted from Hewage et. al.,(Hewage et al., 2024) achieved an average percent recovery of 24.84%. This was similar to the reported

yields from the publication of 23.15±2.31, but these results were not acceptable compared to the results of the modified Osborne fractionation. The simple alkaline extraction adapted from Roberts et. al.,(Roberts et al., 1985) gave a percent yield of 24.77. This was on par with the DES extraction, but again, this procedure did not provide high enough yields for continued use with the hard red winter wheat bran study. These results were also significantly lower than the yields reported by Roberts et al., which should have been closer to 74%(Roberts et al., 1985). Despite these failures, the modified Osborne fractionation adapted from (Sardari et al., 2019b) gave satisfying results, with consistent yields averaging from 74 to 80%, depending on the method of analysis.

The crude Osborne fraction extracts were purified using the 500 Å si-gel column using a capture-elution step gradient. This protocol was adapted from Ghose et al., 2004, and was found to be quite useful in removing non-protein impurities from crude protein extracts quickly, economically, and when combined with dry column vacuum chromatography, there was great potential for scalability. The use of dry column vacuum chromatography is well suited for use with this mobile phase system as well, because both the capture phase and the elution phase are aqueous solutions. Application of vacuum to this system has virtually zero chance of issues with volatility that can occur when DCVC is used with low boiling point organic solvents. This method is also very fast and efficient, with protein samples purified in one chromatography step in as little as 30 minutes. Larger protein samples take longer, but with the use of an appropriately wide column with a bed height of 7cm, overall processing times can be increased. SDS-PAGE and SEC-HPLC analysis of protein extracts before and after purification on a 500Å silica-gel column using the pH 7 0.025M phosphate buffer capture phase and an arginine elution phase step gradient at concentrations of 0.1 to 0.025M showed that the chromatography system was

able to concentrate the crude wheat bran proteins, as well as remove most of the lower molecular weight impurities from each of the Osborne fractions. The only major exception was an unknown lower molecular weight impurity in the prolamin extracts that eluted from the SEC-HPLC column at 47.96 minutes. This unknown species had a very similar affinity for the 500Å silica-gel column as the prolamin protein during both the capture phase and elution phase in the step gradient, and its signal intensity increased along with the protein peak signal intensities after being concentrated after being ran on the 500Å silica-gel column. This behavior is suggestive of a protein-based derivative with a lower molecular weight, possibly protein sub-fragments or peptides generated through denaturation during previous processing steps of the prolamin fraction. This could be due to the denaturation that takes place once most of the ethanol has been rotary evaporated off of the initial prolamin extract. During this processing step a significant amount of protein was observed aggregating out of solution due to the decreased concentration of ethanol. Further investigation will be required to positively identify this species in future work. Despite this discrepancy, the other Osborne fractions responded well to purification on the 500Å silica-gel column, and SEC-HPLC analysis indicated successful removal of lower molecular weight impurities from the crude protein extracts.

SDS-PAGE analysis of the proteins before and after purification on the 500Å si-gel column were unable to visualize the impurities in the crude proteins and could not show their removal after being columned because the SDS-PAGE gels had molecular weight thresholds of 10kDa. The results of the SDS-PAGE analyses did show improvements in the quality of the lanes of the gels. SDS-PAGE gels of crude protein extracts required sample concentrations of 1 to 4 mg per lane, while proteins purified on the 500Å silica-gel column were ran at 0.125mg per lane. The lanes of this gel were far straighter and improved protein banding that was easier to

visualize and differentiate the molecular weights compared to the SDS-PAGE gels of the crude protein samples. With these data supporting the successful purification of the Osborne fraction proteins, the focus of the project turned to the production of protein hydrolysates, or peptides from the wheat bran proteins using the enzymes pepsin and trypsin. These enzymes were chosen to simulate endogenous digestive processes that could produce the same peptides in the human gastrointestinal tract upon ingestion of these wheat bran proteins. The details of this study are discussed in chapter 3, but they could not have been carried out accurately without the work performed in chapter 2. Testing of the proteins and peptides with lower molecular weight impurities such as polyphenols would likely have skewed the results of bioactivity assays, such as the DPPH antioxidant assay, because polyphenols themselves have been reported to have antioxidant bioactivity(Verma et al., 2008). The purification of these proteins using the methods detailed in chapter 2 was an imperative step for more accurately screening in-vitro biological activities of only the wheat bran proteins and peptides using the assays discussed in chapter 3.

Chapter 3 - Wheat Bran Peptides: Production, Characterization, and Bioactivity Screening

3.1 Abstract:

Once extracted and sufficiently purified, the wheat bran proteins underwent enzymatic hydrolysis to produce protein hydrolysates, or peptides. These reactions were optimized by performing them first with bovine casein obtained from Sigma Aldrich. These preliminary tests evaluated a range of enzyme-to-substrate ratios, as well as different reaction times to determine the best conditions for use with wheat bran proteins. Pepsin and trypsin were selected for the hydrolysis reactions as they are digestive enzymes found in the human gastrointestinal tract. These hydrolysis reactions were designed to simulate the digestion of wheat bran proteins in the human GI-tract to predict the hydrolysate products and peptides that would be produced upon consumption of wheat bran proteins. The hydrolysates/peptides produced from these reactions and the proteins they were derived from were then subjected to 4 different bioactivity screening assays to determine the antioxidant capacity and anti-aging enzyme-inhibition effects of these substances. The peptic and tryptic peptides were compared to one another and to their parent proteins, as well as to a standard inhibitor, ascorbic acid. This use of the ascorbic acid standard provided a less abstract comparison of activities by establishing a dose-response ratio of the unknown peptides/proteins that was compared to a comparable amount of vitamin C. As a generalized example, this method allows the comparison of 5mg of vitamin C at x% inhibition vs 5 mg of wheat bran peptide/protein, which achieved a percent inhibition of y%. The results of these assays suggested that the peptides and proteins of wheat bran have significant potential for further development as nutraceutical ingredients, with comparable antioxidant activity and enzyme-inhibition activity to the ascorbic acid standard. Further testing will be required before

they are ready for the consumer market, but these preliminary results were very promising for the development of health-promoting products.

3.2 Introduction:

3.2.1 Optimization of Enzymatic Hydrolysis Reactions:

Following extraction and purification, wheat bran proteins were subjected to enzymatic hydrolysis to produce protein hydrolysates, or peptides. Two versions of these hydrolysates were produced using either the enzyme pepsin, or the enzyme trypsin. These reactions were performed individually, using either pepsin or trypsin, and were not combined to form hybrid hydrolysates or peptides at any point. The enzymatic hydrolysis reactions were optimized using bovine casein obtained from Sigma Aldrich. Different enzyme to substrate ratios were tested, as well as different reaction times. All other variables were held constant, such as temperature and pH, which were selected to simulate biological conditions in the gastrointestinal tract. Optimal conditions were evaluated by testing the peptide products via the detergent DPPH assay, to determine which set of conditions gave the highest percent inhibition of DPPH radical, based on the amount of remaining DPPH radical. The conditions that performed the best in the DPPH assay with the lowest percent-remaining DPPH radical were then selected for use with the wheat bran proteins.

3.2.2 In-Vitro Biological Activity Assays:

The peptides produced from the optimized reaction conditions with wheat bran proteins were then evaluated for potential bioactivity using four different chemical in-vitro bioassays. These consisted of the protein-compatible detergent DPPH antioxidant assay, the collagenase inhibition assay, the elastase inhibition assay, and the hyaluronidase inhibition assay. The DPPH assay measured the antioxidant capacity of a substance based off its ability to quench radical

DPPH• molecules in a detergent-based solution that was capable of solvating both the radical species, as well as a protein or peptide species. This version of the DPPH assay allowed for the assessment of proteins in particular, as traditional DPPH assays are typically carried out in methanol. The decrease in absorbance over time determined the unknown substance of interest's antioxidant capacity, based off of the percent DPPH remaining at 30 minutes.

In addition to the DPPH antioxidant capacity assay, the enzyme inhibition assays were designed to determine the ability of an unknown substance to inhibit the breakdown of a particular tissue structural component substrate, such as collagen, elastin, or hyaluronic acid. These biomolecules are responsible for the youthful appearance of skin and are broken down by cellular aging processes through a variety of mechanisms(Nicklisch & Waite, 2014),(Van Wart & Steinbrink, 1981), (Liyanaarachchi et al., 2018). Reactive oxygen species, free radicals, as well as their respective hydrolytic enzymes that are also present in the body can all have a negative impact on these molecules. In each of these four types of assays, samples of unknown peptic and tryptic peptides generated from each Osborne fraction protein, as well as samples of unhydrolyzed protein from each Osborne fraction were compared to a known inhibitory standard, ascorbic acid. The samples tested in each of these bioactivity assays were evaluated in triplicate. The results of these bioactivity assays showed promising preliminary results for the development of these substances as antioxidant and anti-aging nutraceuticals, with comparable activities to similar concentrations of ascorbic acid. However, these substances require further testing with in-vitro cellular studies, and in-vivo studies to confirm their efficacy in actual biological systems, in addition to purely chemical/biochemical assays. Nevertheless, the results obtained in this study strongly suggest that the peptide hydrolysates and proteins of wheat bran are worth further exploration in these areas.

3.3. Materials and Methods

3.3.1. Optimization of Enzymatic Hydrolysis Reactions

The hydrolysis reaction conditions for pepsin were adapted from Vanvi & Tsopmo, 2016, while the conditions for trypsin hydrolysis were adapted from Jin et al., 2020. Protein hydrolysis with pepsin or trypsin obtained from Sigma Aldrich was optimized using bovine casein obtained from Sigma Aldrich, but any protein could theoretically be used to optimize the hydrolysis reaction. Four enzyme-to-substrate ratios were tested, and each E/S ratio was assessed at four different reaction times. These consisted of E/S ratios of 1:20, 1:30, 1:40, and 1:50, at 30 minutes, 60 minutes, 90 minutes, and 120 minutes. The temperature was held constant at 37°C to simulate biological conditions. The pH of the hydrolysis reactions tested were selected based on the optimal pH's for trypsin and pepsin. These digestive enzymes are found in specific locations within the GI-tract, with pepsin localized in the stomach and trypsin in the small intestine. As a result, the optimal pH for pepsin was approximately 2, and the optimal pH of trypsin was closer to 9. To simulate these physiological conditions and to achieve the most optimal pH for the function of each enzyme, the pH of the trypsin hydrolysis reactions was held at 9.1 using an ammonium carbonate buffer. Similarly, the pH of the pepsin hydrolysis reactions was held at a pH of 2.4 using 1% formic acid. These samples were incubated at 37°C for each reaction time being tested. Once the reaction time had commenced, the enzymes were deactivated via heat denaturation by heating the reaction tube at 100°C in boiling water for 15 minutes. The deactivated reaction tube was then centrifuged at 10,000 rcf for 15 minutes at 4°C to remove residual protein/denatured enzyme. The supernatants containing the peptides/ protein hydrolysates were frozen and lyophilized. Once fully lyophilized, yields were determined by

measuring the lyophilized hydrolysates/peptides. The freeze-dried hydrolysates/peptides were stored in the freezer prior to further analyses. Table 3.1 depicts the different E/S ratios and reaction times tested during the optimization of the hydrolysis reactions using casein as the substrate.

Table 3.1:Different E/S ratios tested at different reaction times. Each reaction was held at 37°C, with a pH of 2 for pepsin-catalyzed hydrolysis reactions, and a pH of 9.1 for trypsin-catalyzed hydrolysis reactions.

E/S ratio	Time 1	Time 2	Time 3	Time 4
1:20	30 min	60 min	90min	120 min
1:30				
1:40				
1:50				

The detergent DPPH assay was performed to determine the most optimal hydrolysis conditions. This procedure was adapted from Nicklisch & Waite, 2014, with volumes adjusted to 250 µL for use in a Biotek Synergy H1 microplate reader. The antioxidant capacity of each peptide and protein sample consisting of 4.15mg peptide/protein was compared to the antioxidant capacity of 5.5mg ascorbic acid. Each sample was tested in triplicate, and the antioxidant capacity was calculated using equation 1:

$$1) \quad \%DPPH\ remaining = \left(\frac{mols\ DPPH\ sample}{mols\ DPPH\ pos.\ control}\right) * 100$$

Optimized reaction conditions were then applied to wheat bran proteins purified on the 500Å underivatized silica gel column. Hydrolysis reactions were run on each individual Osborne fraction, with one set hydrolyzed by pepsin and the other set hydrolyzed by trypsin. E/S ratios of 1:20 and 120-minute reaction times were selected based off of the optimized reaction conditions obtained for casein. In the casein trials the most optimal conditions were 1:20 at 90 minutes, and these results were extrapolated to 120 minutes to ensure enough time was allowed for full

hydrolysis. A one-way ANOVA statistical analysis was performed on these datasets using SAS studio to identify any statistical variance between the different reaction conditions tested.

3.3.2 Protein-Compatible Detergent DPPH Antioxidant Capacity Assay

The detergent DPPH assay was performed on each of the wheat bran protein Osborne fractions, as well as the tryptic wheat bran peptides and peptic wheat bran peptides. The assay was carried out according to the procedure adapted from (Nicklisch & Waite, 2014), with volumes adjusted for use in an Agilent Synergy H1 microplate reader using clear 96 well plates with 300µL wells. The antioxidant capacity was compared to ascorbic acid, with a relative comparison of 7.89mg of each wheat bran protein/peptide sample to 10.46mg of ascorbic acid. The antioxidant capacity was calculated using equation 1, with antioxidant capacity measured as the %DPPH remaining after 30 minutes. It is important to note that higher percentages of DPPH radicals remaining in solution indicate a weaker antioxidant capacity, while lower percentages of DPPH remaining indicate a stronger antioxidant. The antioxidant capacities for each substance, denoted as % DPPH remaining, were then plotted in a bar graph for comparison. A one-way ANOVA statistical analysis was performed using SAS studio to identify any statistical differences between the different peptides and protein samples tested using the DPPH assay.

1) $$\%\text{DPPH remaining} = \left(\frac{\text{mols DPPH sample}}{\text{mols DPPH pos. control}}\right) * 100$$

The 0.1M citrate-phosphate assay buffer containing 0.3% Triton X-100 detergent used in this assay was prepared per the procedure in Nicklisch & Waite, 2014. A 0.1M solution of citric acid was prepared, followed by a 500mL stock solution of 0.2M Na_2HPO_4. 37.440 mL of the 0.1M citric acid solution was added to 482.56mL of the 0.2M Na_2HPO_4 stock solution, resulting in 520 mL of 0.1M citrate-phosphate buffer. To this solution, 1.560mL of Triton X-100 detergent was

added to the solution using a 1000µL micropipette, with care being taken during the measurement of the detergent due to its high viscosity.

A 0.1M stock solution of ascorbic acid was prepared from 0.176g ascorbic acid dissolved in 10.0mL of assay buffer. This stock solution was used to prepare a 0.1mM stock solution of ascorbic acid, by adding 50µL of the 0.1M ascorbic acid solution to 49,950µL of assay buffer for a total volume of 50mL.

Ascorbic acid standards were prepared using the 0.1mM stock solution over the range detailed in table 3.2.

Table 3.2: Preparation of ascorbic acid standards. A range of standards was prepared initially to determine the concentration of ascorbic acid to find a concentration of ascorbic acid that was comparable to the antioxidant capacity of the unknown wheat bran samples.

Ascorbic acid [µM]	Volume 0.1mM Ascorbic acid stock solution [µL]	Volume assay buffer [µL]
2.5	125	4875
5.0	250	4750
10.0	500	4500
15.0	750	4250
20.0	1000	4000
Negative control	0	5000
Positive control (no antioxidant)	0 (DPPH solution only)	5000

Wheat bran protein and peptide samples were prepared by reconstituting the lyophilized protein/peptide hydrolysate at a concentration of 33.22mg solid per mL of assay buffer. This resulted in 7.89mg of sample per well. The lyophilized samples were reconstituted in assay buffer, vortexed thoroughly until fully dissolved.

237.5µL of each ascorbic acid sample or wheat bran protein/peptide sample were pipetted into a 300µL 96 well plate in triplicate samples. Pathlength correction samples of distilled water were also used in triplicate samples. 237.5µL of each wheat bran protein and peptide samples were then transferred to the 96 well plate in triplicate samples. Once the 96 well plate was set up,

the DPPH standard curve wells were prepared per the ratios in table 3.3. The 2mM DPPH solution was prepared after every other component of the assay was prepared and transferred to the 96 well plate, due to the unstable nature of the DPPH radical. Because of this, only the assay buffer volumes were pipetted into the wells for the DPPH standard curve.

Table 3.3: Preparation of the DPPH standard curve.

Sample [uM]	Volume DPPH (2mM) [µL]	Volume Assay Buffer [µL]
Blank	0.00	250.0
25	3.125	246.9
50	6.25	243.7
100	12.5	237.5
150	18.7	231.25
200	25.0	225.0

Once all of the wells were prepared, the 2mM stock solution of DPPH was prepared using 10mg of DPPH solid dissolved in 12.68mL of HPLC grade methanol. 10mg of DPPH solid was weighed into a tared glass vial. Once the appropriate amount of DPPH was measured out, the vial was wrapped in aluminum foil to block out as much light as possible. 12.68mL of methanol was added, and the cap of the vial was shut. A small aluminum foil lid was added to the vial to ensure virtually all light was kept out of the DPPH solution. The vial was vortexed thoroughly, and the foil was removed briefly to ensure the DPPH was fully dissolved. The solution was allowed to sit on the benchtop for 5 minutes, after which time the vial was vortexed again. This solution was transferred into a multi-channel pipette reservoir, and the specified volumes of 2mM DPPH solution were added to the DPPH standard curve wells. Each of the standards were also prepared in triplicate. Once the standard curves were prepared, 12.5µL of 2mM DPPH solution were added to each of the ascorbic acid and wheat bran protein/peptide samples. After the addition of DPPH to each well, the contents of the wells were mixed via

pipetting using ½ the volume of the well for a total of 3 pumps. This step was imperative for obtaining accurate data, and if skipped, the absorbances of the sample wells would increase, rather than decrease, resulting in unusable results. The importance of this mixing step cannot be stressed enough. Once the samples in the 96 well plate were fully prepared, the plate was placed in the microplate reader, and data points were taken at 515nm at 60 second intervals for each sample over the course of 2 hours. The absorbance of the DPPH was measured and converted to the concentration of DPPH using the standard curve. The concentration of DPPH was then used to calculate the number of moles of DPPH remaining at time, t. The percent remaining DPPH was calculated from the moles of DPPH remaining at each time, t. The percent DPPH remaining was then plotted for each ascorbic acid sample, as well as each wheat bran protein/peptide sample in a bar graph. This graph illustrates the relative antioxidant capacities of each sample, in terms of percent DPPH remaining at 30 minutes. The 250mM ascorbic acid sample was selected as the primary standard for comparison, as it gave a satisfactory comparison of the antioxidant capacity of a known antioxidant, ascorbic acid, and at this concentration, the antioxidant capacity was well within the range of the unknown wheat bran protein and peptide samples. This concentration of ascorbic acid at the volumes used in the assay equated to 10.46mg ascorbic acid, which was close to the 7.89mg of wheat bran protein/peptide in each of the unknown samples.

3.3.3. Collagenase Inhibition Assay

The collagenase inhibition assay was adapted from Van Wart & Steinbrink, 1981. In short, this assay measured the hydrolysis of the synthetic peptide substrate FALGPA (N-[3-(2-

furyl)acryloyl]-Leu-Gly-Pro-Ala). The hydrolysis of FALGPA by collagenase results in a decrease in absorbance over time, which is used to determine the % inhibition using equation 2:

$$2) \quad \%\ \text{inhib.} = \left(1 - \left(\frac{\text{neg control abs.}}{\text{sample abs.}}\right) * 100\right)$$

Each sample was prepared in triplicate, and a one-way ANOVA was performed in SAS studio to identify any variance in the percent-inhibitions of each sample.

0.1M FALGPA substrate and 0.5-5.0 units (5.0mg collagenase, 0.5mL tricine buffer) of collagenase from Clostridium histolyticum (Sigma Aldrich, USA) were prepared in 50mM tricine buffer (50mM tricine, 10mM CaCl2, 400mM NaCl, with a pH of 7.5, adjusted with HCl and NaOH). Wheat bran peptide and protein samples were reconstituted in tricine buffer such that each sample contained 0.030mg of peptide/protein. The standard inhibitor ascorbic acid was reconstituted in tricine buffer (1mg/mL) such that each ascorbic acid standard contained 0.020 mg, and the negative control was distilled water. 60µL of tricine buffer, 10µL collagenase solution, and 30µL of inhibitor solution were added to a 96 well plate and incubated for 15 minutes at room temperature before proceeding. 20µL of FALGPA substrate was added, with the negative control receiving 20µL of r.o. water, and the positive control receiving 20µL of 1mg/mL ascorbic acid. Immediately following addition, the wells were mixed by pipetting ½ of the volume of the well three times. Once the samples in the 96 well plate were fully prepared, the plate was inserted into the Synergy H1 microplate reader for analysis at 345nm, with data points taken every 60 seconds over a period of 30 minutes. The percent inhibition for each sample was calculated using equation 2, using the absorbances measured at 20 minutes. These values were plotted as a bar graph for direct comparison of percent inhibitions for each sample. Unknown wheat bran protein and peptide samples were compared to the 250mM ascorbic acid sample. At

the concentrations and volumes used in the assay, this equated to 0.020mg ascorbic acid vs 0.030mg wheat bran protein/peptide.

3.3.4. Elastase Inhibition Assay

This assay was adapted from Ritthibut et al., 2021, modified for use with the Synergy H1 microplate reader. All samples were prepared in triplicate, and a one-way ANOVA was performed in SAS studio to identify variance in the dataset.

The assay buffer consisted of a 2mM Tris-HCl buffer with a pH of 8.0. This buffer was prepared using 24.23mg Tris-HCl, 9µL of 37% HCl, initially dissolved in ~50mL, then diluted to a final volume of 100mL. The substrate succ-Ala-Ala-Ala-p-nitroanilide was prepared in a 3mg/mL stock solution by dissolving 10.5mg of this substrate in 3.5mL of Tris buffer. 10µL of 1.1U/mL porcine pancreas elastase in 1mL of Tris buffer was prepared by diluting 26.570µL of the 6.0U/mL, 6.9mg/mL concentrate (obtained from Sigma Aldrich) with 973.43µL of tris buffer. Ascorbic acid inhibitor standards were prepared for comparison to the unknown wheat bran protein and peptide samples. A 500mM stock solution of ascorbic acid was prepared using 4.403g ascorbic acid and 50 mL of assay buffer. A series of standard dilutions was prepared to determine the concentration of ascorbic acid that had a percent inhibition similar to the unknown wheat bran proteins and peptide samples. These standard dilutions were prepared according to the ratios in table 3.4.

Table 3.4: Ratios for preparation of ascorbic acid standards.

Ascorbic Acid [mM]	Volume 500mM Ascorbic Acid Stock Solution [mL]	Volume Assay Buffer [mL]
Blank	0.0	10.0
25	0.5	9.5
50	1.0	9.0
100	2.0	8.0
250	5.0	5.0

Unknown wheat bran protein and peptide samples were prepared by reconstituting 10mg of solid lyophilized sample in 333µL assay buffer, and vortexing the sample until fully dissolved. 30µL of each inhibitor solution (ascorbic acid and wheat bran protein/peptide samples) were transferred to the 96 well plate, with samples prepared in triplicate. The positive control received 30µL of the known inhibitor ascorbic acid, and the negative control received 30µL of assay buffer, rather than an inhibitor sample. 10µL of 1.1U/mL elastase stock solution was then added to each sample well, followed by 100µL of tris buffer. This mixture was incubated at 25°C for 10 minutes. Following incubation, 40µL of 3mg/mL substrate stock solution was added to each sample well using a multi-channel pipette and a multi-channel pipette disposable reservoir. The wells were thoroughly mixed by pipetting the well three times using the multi-channel pipette set at one half of the total well volume. Wells labeled as water received 180µL of distilled water for pathlength correction. The plate was then shaken for 20 minutes at 25°C. After this incubation, the absorbances of the wells were measured at 410nm. The percent inhibition of each sample was determined using equation 3.

3) $$Inhib.activity\ (\%) = \left[\frac{(Blank\ with\ enzyme - blank\ no\ enzyme) - (Inhibitor\ with\ enzyme - Negative\ control)}{(Blank\ with\ enzyme - blank\ no\ enzyme)}\right] * 100$$

The calculated percent inhibitions for each sample and for the 250mM ascorbic acid sample were plotted in a bar graph for comparison of the activity of 0.9mg of each wheat bran protein/peptide sample per well, versus 1.32mg of ascorbic acid per well. Finally, a one-way ANOVA statistical analysis was performed in SAS studio to determine the statistical variance of the percent inhibitions of each sample.

3.3.5 Hyaluronidase Inhibition Assay

This assay was adapted from a previously published protocol by Liyanaarachchi et al., 2018. 0.1M sodium acetate buffer was prepared by dissolving 7.721g sodium acetate and

352.5mg acetic acid in 1L of distilled water. The pH of this buffer was adjusted to 3.5 using HCl. A stock solution of 4200U/mL hyaluronidase was prepared by reconstituting 10mg of hyaluronidase obtained from Sigma Aldrich in 2.381mL of acetate buffer. A 5% DMSO solution was prepared from 2.5mL DMSO and 47.5mL distilled water, for reconstitution of inhibitor samples. A 12.5mM stock solution of $CaCl_2$ was prepared using 0.0694g $CaCl_2$ dissolved in 50mL of distilled water. The substrate solution was prepared at a concentration of 12mg/mL using 60mg sodium hyaluronate in 6mL of acetate buffer. A 0.9M solution of NaOH was prepared using 3.5997g NaOH dissolved in 100mL distilled water, and a 0.2M sodium borate solution was prepared from 7.627g sodium borate dissolved in 100mL distilled water. The p-dimethylaminobenzaldehyde reagent was prepared by dissolving 0.25g p-dimethylaminobenzaldehyde, 21.88mL glacial acetic acid, and 3.12mL 10N HCl. Three positive controls were tested due to issues encountered during initial tests of the assay with ascorbic acid. These issues were resolved in subsequent tests, but a sample of ascorbic acid, EDTA-dihydrate, and 1,10-phenanthroline were used for comparison to the wheat bran protein and peptide unknowns. Each of the positive controls was prepared by dissolving 5mg of solid inhibitor in 5mL of 5% DMSO for a final concentration of 1mg/mL. The wheat bran peptide and protein samples were prepared by reconstituting 5mg of lyophilized extract in 5mL of 5% DMSO. After obtaining successful trials with ascorbic acid, the samples of EDTA-dihydrate and 1,10-phenanthroline were abandoned in favor of ascorbic acid, as it is representative of an actual dietary supplement.

The assay procedure involved a sequential addition of reagents, each followed by an incubation step, for a total of 5 steps. Each sample was prepared in triplicate, using a clear 96 well plate with a maximum volume capacity of 300µL. In the first step, 10µL hyaluronidase

solution was added to each well, followed by 50µL of inhibitor. The negative control received 50µL of 5% DMSO solution, and the positive controls received 50µL of either ascorbic acid, EDTA-dihydrate, or 1,10-phenanthroline. This mixture was incubated at 37°C for 20 minutes. 20µL of 12.5mM $CaCl_2$ was then added to each well, and the mixture was incubated at 37°C for 10 minutes. 50µL of sodium hyaluronate substrate was added to each well carefully, due to the solution's high viscosity, and the plate was incubated at 37°C for 40 minutes. 10µL of 0.9m NaOH and 20µL of sodium borate were added to each well, and the plate was incubated in a hot air oven at 100°C for 3 minutes, with a piece of glass covering the wells to prevent evaporation. After this incubation the plate was removed from the oven and allowed to cool to room temperature. Once cooled, 50µL of p-dimethylaminobenzaldehyde was added to each well, and the plate was incubated at 37°C for 10 minutes. After the final incubation period, the absorbance of each well was measured at 585nm. The percent inhibition was calculated using equation 4.

$$4) \quad \%\, inhib. = \left(1 - \left(\frac{neg.\, control\ abs.}{sample\ abs.}\right) * 100\right)$$

The average percent inhibition was calculated for each wheat bran protein or peptide sample, as well as each standard inhibitor. The ascorbic acid standard was selected for comparison to the unknown samples, as it had an average percent inhibition of 91.18%. The volumes used of each inhibitor resulted in a comparison of 0.05mg ascorbic acid vs 0.05mg wheat bran protein/peptide sample per well. Finally, a one-way ANOVA was performed in SAS studio on the percent inhibition data to identify any statistical variance in the dataset.

3.3.6 UPLC-QToF Analysis of Wheat Bran Peptides

Samples of the wheat bran peptides produced using trypsin were analyzed using a Waters Acquity I class UPLC inlet equipped with a Waters Acquity 2.1x50mm, 1.6µm reverse phase column, and a Waters Xevo-G2-XS-QTof mass spectrometer. Each sample was prepared at

concentrations of 0.4mg/mL in UPLC-grade water with 0.1% formic acid. The UPLC conditions are listed in table 3.5.

Table 3.5: Gradient used to obtain the UPLC-MS data for tryptic peptide samples.

Time [min]	Flow Rate [mL/min]	% H2O (1% FA)	%MeCN (1% FA)	Curve
Initial	0.200	100.0	0.0	Initial
1.00	0.200	94.0	6.0	6
52.00	0.200	75.0	25.0	6
53.00	0.200	0.0	100.0	6
58.00	0.200	50.0	50.0	6
63.00	0.200	100.0	0.0	6
68.00	0.200	100.0	0.0	6

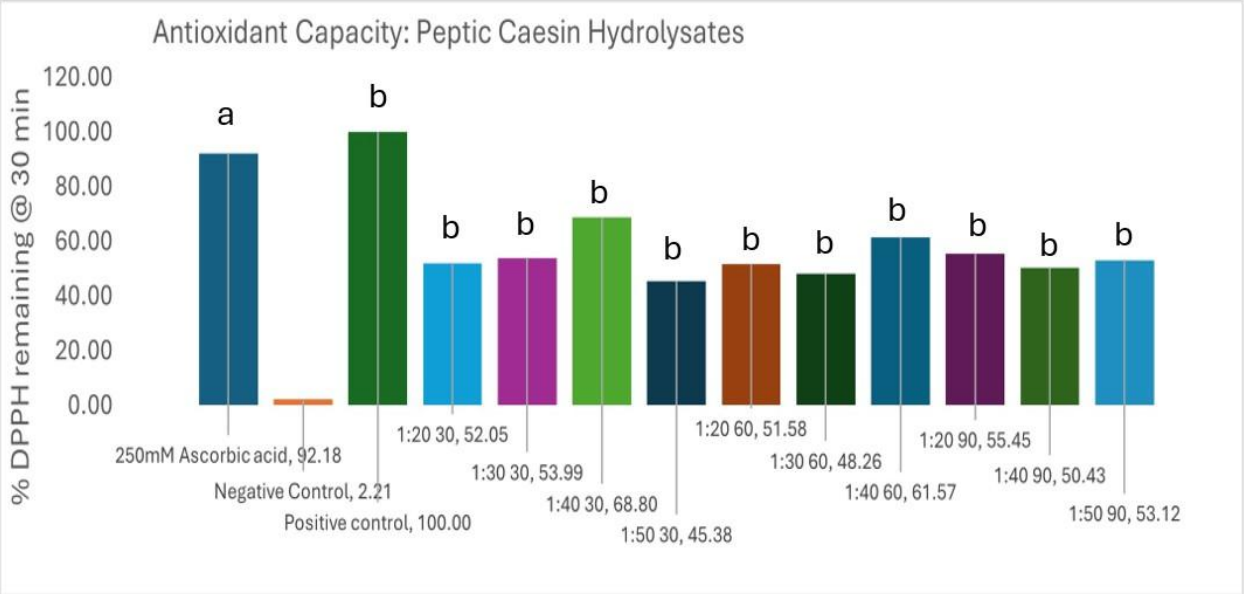
The mass spectrometer was configured for positive ionization in MSe mode. Due to the lack of processing software for peptide structural characterization, the sequences of the peptides produced for each Osborne fraction were unable to be identified. Despite this setback, the spectra obtained for each sample were able to confirm the presence of multiple peptides in each sample, indicating the successful formation of peptides from each of the wheat bran proteins using trypsin. In future work, the software ProteinLynx Global Server will be used to perform large database searches using this experimental data to identify the structures of each peptide analyzed in this study.

3.4. Results and Discussion

3.4.1 Optimization of Enzymatic Hydrolysis Reactions

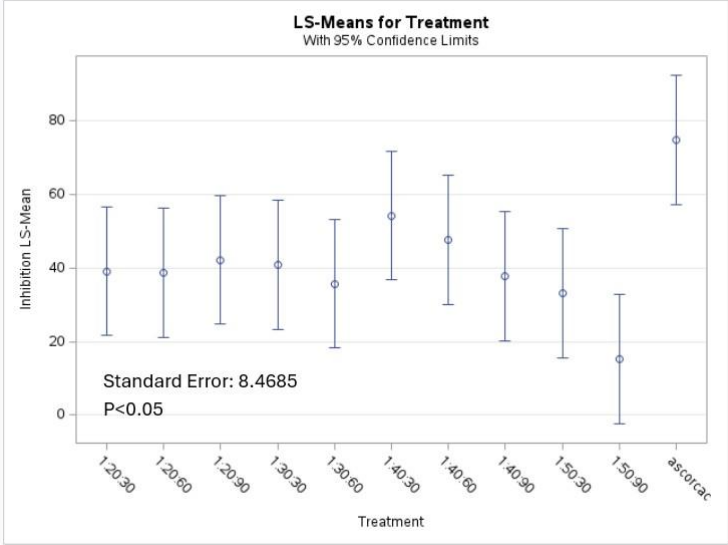
Each E/S ratio at each reaction time tested was analyzed using the detergent DPPH assay, adapted for use in the Synergy H1 microplate reader. The results of this assay are presented in figure 3.1, and they show the antioxidant capacities calculated for each sample represented as the percent DPPH remaining at 30 minutes.

Figure 3.1: DPPH assay for optimization of protein hydrolysis reactions catalyzed by pepsin. Some samples were lost due to leaking reaction tubes during heat denaturation, but the dataset provided a general picture of the antioxidant capacities for a wide range of E/S ratios and reaction times. Different letters above bars indicate statistical significance, (P<0.05), determined via one-way ANOVA.



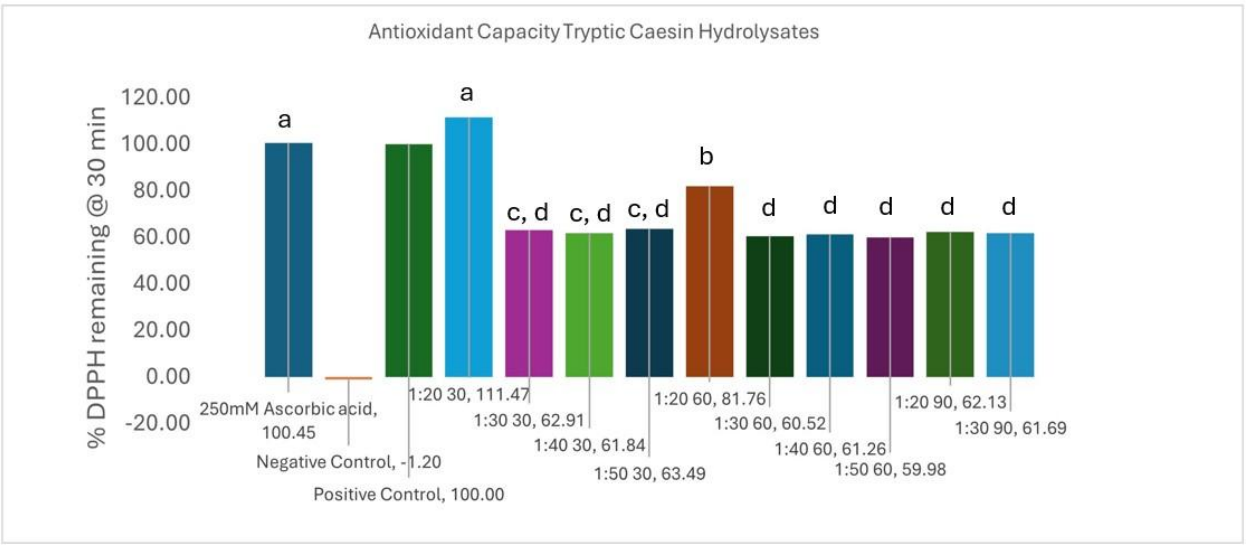
The one-way ANOVA statistical analysis of the results of the pepsin hydrolysis of casein showed no statistically significant differences ($P < 0.05$) between the different hydrolysis reactions, indicating that the antioxidant capacities of each reaction were close enough for the different E/S ratios and reactions times to make little difference. For this reason, the maximum E/S ratio of 1:20 and the maximum reaction time of 120 minutes was selected to ensure the most theoretically optimal reaction conditions were achieved for the hydrolysis of proteins by pepsin. The results of this statistical analysis are provided in figure 3.2.

Figure 3.2: One way ANOVA statistical analysis performed on the antioxidant capacities of the peptic hydrolysates of caesin. Note: 1:20:30 is E/S ratio 1:20, for 30 minutes, and so on. P<0.05.



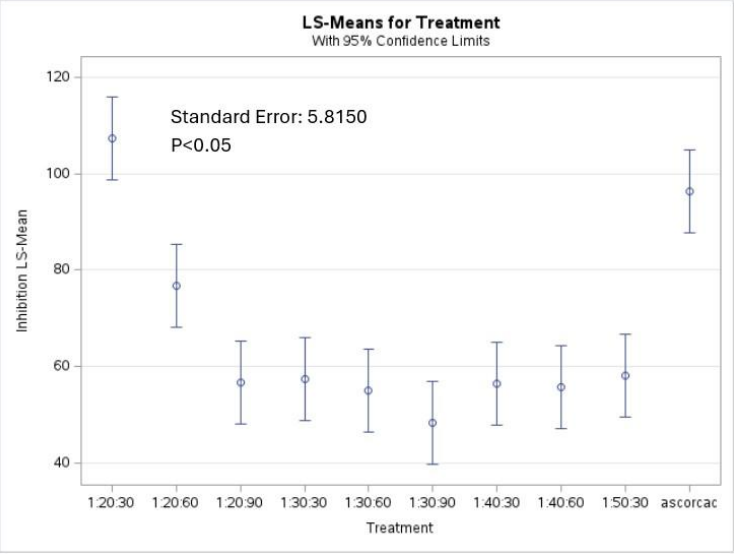
The DPPH assay was also performed on the tryptic hydrolysates of casein, with a one-way ANOVA statistical analysis of variance performed on the dataset to identify any statistically significant differences between the E/S ratios and reaction times tested. Figure 3.3 shows the antioxidant capacities for each of the sample reactions.

Figure 3.3: DPPH assay ran on tryptic casein hydrolysates to determine the most optimal reaction conditions. Different letters above bars indicate statistical significance, (P<0.05), determined via one-way ANOVA.



The one-way ANOVA was performed on the data set obtained for the tryptic hydrolysates of casein. The results of this analysis are shown in figure 3.4. No statistical differences ($P<0.05$) were observed for the majority of the dataset, but reactions with larger enzyme-to-substrate ratios and shorter reaction times exhibited statistically lower antioxidant capacities. This was illustrated by the higher % DPPH remaining for reaction E/S ratios of 1:20 with reaction times of 30 and 60 minutes. These reactions were still on par with the ascorbic acid standard, but reactions that had E/S ratios of 1:20 to 1:50 at any of the other reaction times tested resulted in better antioxidant capacities that did not differ significantly from one another.

Figure 3.4: One-way ANOVA statistical analysis for the optimization reactions hydrolyzing casein with trypsin. Note: 1:20:30 is E/S ratio 1:20, for 30 minutes, and so on. P<0.05.

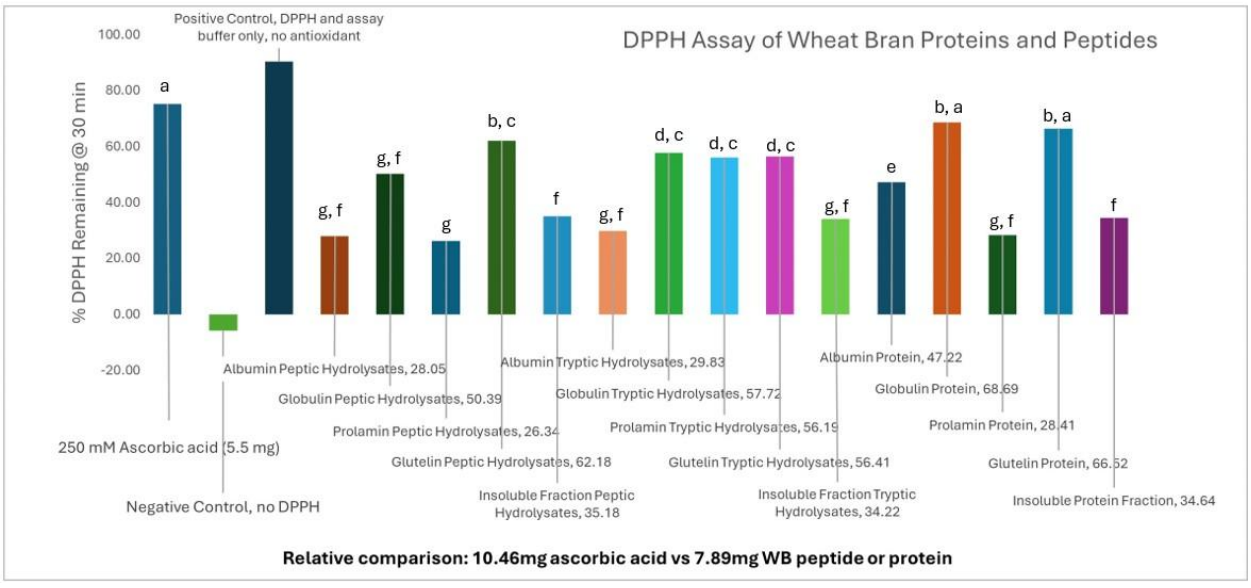


Based on these results, the highest enzyme to substrate ratio of 1:20 was selected, with a reaction time of 120 minutes to ensure that the most optimal reaction conditions were provided for the hydrolysis of wheat bran proteins using trypsin. With the optimized reaction conditions established, the hydrolysis reactions using pepsin and trypsin were performed on the wheat bran proteins of each individual Osborne fraction per the adapted procedures established in section 3.3.1.

3.4.2 Detergent DPPH Antioxidant Capacity Assay of Wheat Bran Proteins and Peptides

After hydrolysis of each wheat bran protein obtained through Osborne fractionation and purification on the 500Å silica gel column, the resulting tryptic and peptic hydrolysates were assayed via the detergent DPPH assay. In addition to the peptic and tryptic hydrolysates/peptides, each of the wheat bran protein Osborne fractions purified on the 500Å silica gel column were also assayed. This was done to determine if the antioxidant capacity increased or decreased following the hydrolysis reactions. The results of these assays are provided in figure 3.5. A one-way ANOVA test was also performed on the dataset using SAS studio.

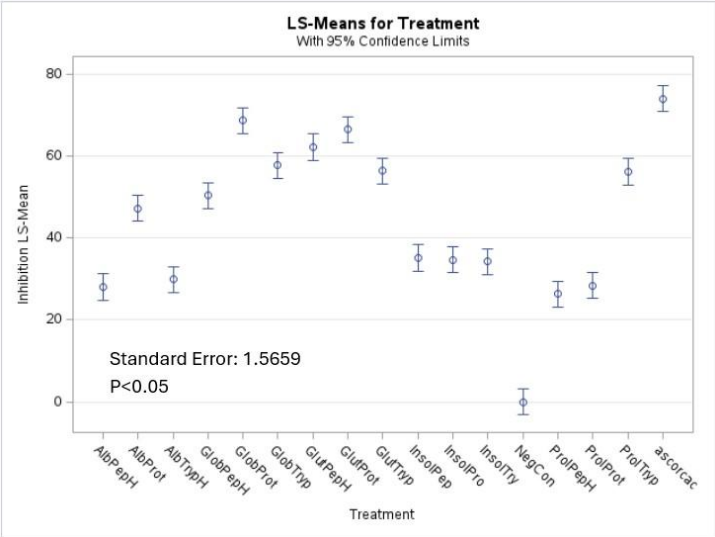
Figure 3.5: Antioxidant capacities for each wheat bran peptic and tryptic hydrolysate sample, as well as each wheat bran protein Osborne fraction. Antioxidant capacity determined via the detergent DPPH assay, with antioxidant capacity represented as the % DPPH remaining at 30 minutes. Different letters above bars indicate statistical significance, (P<0.05), determined via one-way ANOVA.



The results of the one-way ANOVA statistical analysis on this dataset are provided in figure 3.6. The results of this analysis illustrated the significant differences between the peptic and tryptic hydrolysates, as well as the whole proteins with the highest antioxidant capacities.

The samples with the lowest antioxidant capacities (highest % DPPH remaining) were the globulin proteins (GlobProt), glutelin protein (GlutProt), peptic glutelin hydrolysates (GlutPepH), tryptic glutelin hydrolysates (GlutTrypH), and tryptic globulin hydrolysates (GlobTryp). The samples with the highest antioxidant capacities according to the results of this analysis were the peptic albumin hydrolysates (AlbPepH), the tryptic albumin hydrolysates (AlbTrypH), peptic prolamin hydrolysates (ProlPepH), prolamin protein (ProlProt). The rest of the samples fell somewhere in between. The relative comparison of the ascorbic acid standard inhibitior and the wheat bran peptide and protein unknowns were 10.46mg ascorbic acid vs 7.89mg wheat bran protein or peptide. The results of this comparison showed that a similar, but slightly higher dose of ascorbic acid by weight had a lower antioxidant capacity at 75.25% DPPH remaining than the wheat bran peptides and proteins. The wheat bran proteins also exhibited lower percentages of remaining DPPH radical when compared to ascorbic acid, though the Globulin and glutelin proteins were not far off at 68.69% and 66.52%, respectively; despite these being the lowest performing samples of the entire DPPH assay dataset, these samples still outperformed the ascorbic acid standard.

Figure 3.6: One way ANOVA statistical analysis of wheat bran proteins and peptides vs the known dietary antioxidant ascorbic acid, or vitamin C. The relative comparison was 10.46mg ascorbic acid vs 7.89mg wheat bran protein/peptide sample. Note: abbreviations are ProtPepH: Protein classification, tryptic or peptic hydrolysis, H for hydrolysates. AlbPepH = albumin peptic hydrolysates. AlbTrypH = albumin tryptic hydrolysates. AlbProt = albumin protein. Ascorcac = ascorbic acid standard. NegCon = negative control. P<0.05.



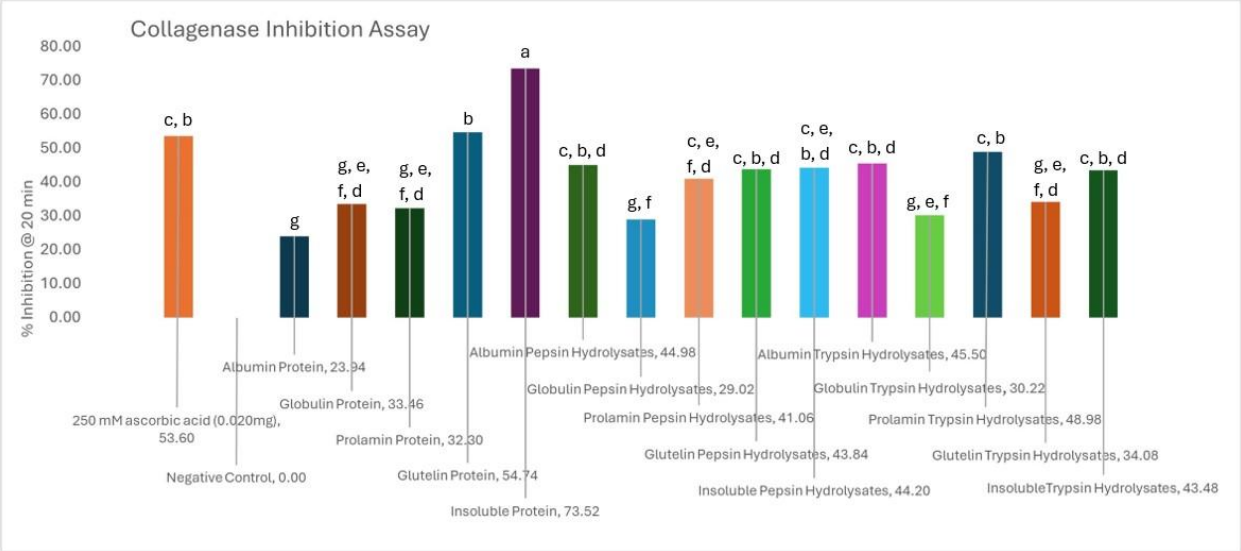
Following the analysis of antioxidant capacity, the anti-aging assays focused on the inhibition of enzymes that degrade tissues responsible for the youthful appearance of skin were assayed.

3.4.3 Collagenase Inhibition Assay

The results of the collagenase inhibition assay for each of the wheat bran peptide and protein samples are provided in figure 3.7. The results in figure 3.7 illustrate a comparison of 0.020mg ascorbic acid vs 0.030mg of wheat bran peptide/protein sample per well. The highest percent inhibition was obtained by the insoluble protein fraction at 73.52%. This was likely due to the base-catalyzed hydrolysis of the insoluble proteins into peptide fragments during the alkaline conditions at 100°C required for the extraction of the insoluble protein fraction. These results were likely due to higher concentrations of peptides/protein hydrolysates that were formed from the insoluble parent proteins. The unselective nature of a base-catalyzed hydrolysis that would be promoted under the extraction conditions of the insoluble fraction would also

produce a more diverse series of peptides than a highly selective enzymatic hydrolysis reaction. It would be expected then that all of the different peptides in the insoluble protein fraction would be able to inhibit the collagenase enzyme to a greater extent than a more limited series of peptides produced from a more selective hydrolytic reaction. The SDS-PAGE results from chapter 2 lend support to this hypothesis, as there were no specific bands observed in the insoluble fraction samples. Instead, a large streak that became more concentrated at lower molecular weights around 10kDa were observed. This evidence supports the idea that the harsh extraction conditions resulted in a diverse series of lower molecular weight protein sub-units, which in turn supports the hypothesis that these lower mw protein derivatives would be able to inhibit the collagenase enzymes more so than other samples.

Figure 3.7: Results of the collagenase inhibition assay ran on wheat bran proteins and wheat bran peptides produced from pepsin and trypsin hydrolysis. E/S ratio of 1:20, 120 minute reaction time at 37°C. Different letters above bars indicate statistical significance, (P<0.05), determined via one-way ANOVA

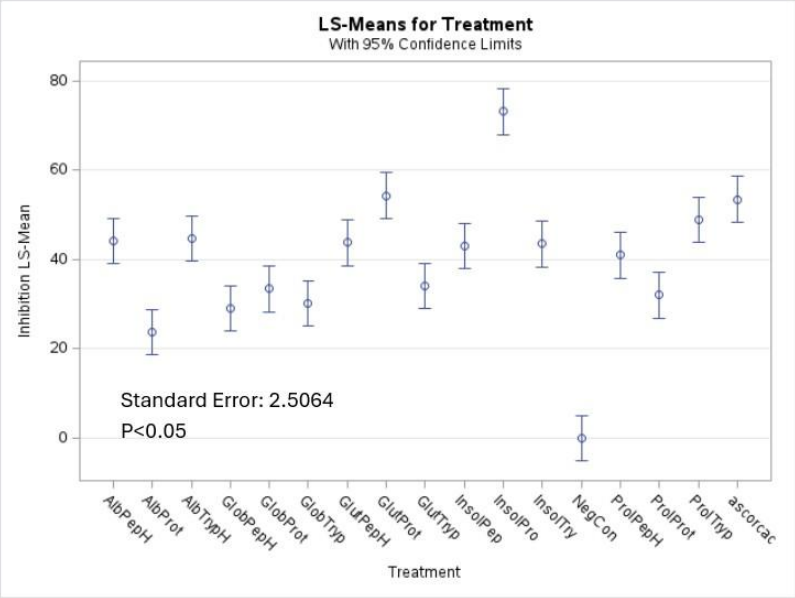


The lowest percent inhibition of collagenase was seen in the albumin protein sample. This was likely due to the fact that the albumin proteins were extracted under the most mild conditions, using distilled water at room temperature. The mechanism of inhibition of the albumin proteins

is not known, but this sample provided the lowest percent inhibition out of all of the other samples.

A one-way ANOVA statistical analysis was carried out in SAS studio on this dataset to determine the statistical variance between the samples. The results of this analysis are provided in figure 3.8. Some statistically significant differences were observed for this dataset, but many of the wheat bran proteins and peptides exhibited statistically similar percent inhibitions. This analysis also illustrated that the insoluble protein fraction's percent inhibition of collagenase was significantly higher than all other wheat bran protein and peptide samples, as well as being significantly higher than the ascorbic acid standard (denoted ascorcac in the one-way ANOVA). The lowest percent inhibition observed in the dataset was again the albumin proteins, but it was statistically similar to the peptic globulin hydrolysates (GlobPepH), the globulin protein fraction (GlobProt), the tryptic globulin hydrolysates (GlobTryp), tryptic glutelin hydrolysates (GlutTryp), and the prolamin proteins (ProlProt). Further analysis of the one-way ANOVA data showed that although the albumin protein fraction did obtain the lowest percent inhibition, its performance was very similar to several of the other lower-performing samples.

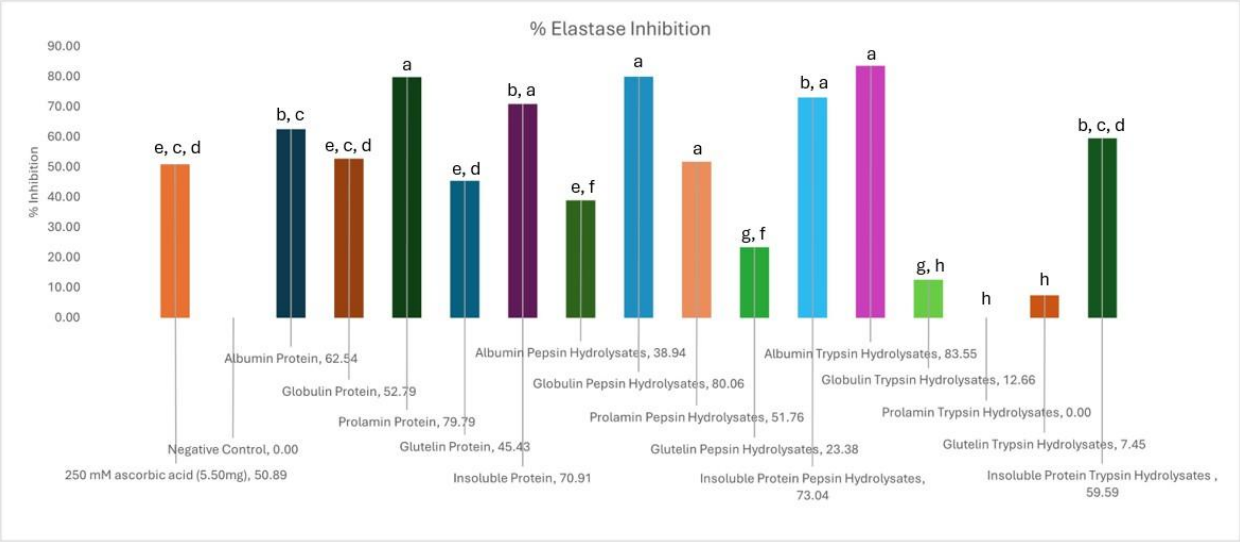
Figure 3.8: One-way ANOVA statistical analysis of the results from the collagenase inhibition assay ran with wheat bran proteins and peptides. Note: abbreviations are ProtPepH: Protein classification, tryptic or peptic hydrolysis, H for hydrolysates. AlbPepH = albumin peptic hydrolysates. AlbTrypH = albumin tryptic hydrolysates. AlbProt = albumin protein. Ascorcac = ascorbic acid standard. NegCon = negative control. P<0.05.



3.4.4 Elastase Inhibition Assay

Following the collagenase assay, the elastase inhibition assay was performed on the wheat bran proteins and peptide samples. This assay gave a decent in-vitro representation of the ability to prevent the breakdown of elastin, another tissue responsible for the youthful appearance of skin, similar to collagen. The results of the elastase inhibition assay are provided in figure 3.9.

Figure 3.9: Results of the elastase inhibition assay performed with wheat bran proteins and peptides. Comparison of unknown samples derived from wheat bran were compared to ascorbic acid, with a relative comparison of 1.32mg ascorbic acid to 0.90mg wheat bran protein/peptide. Different letters above bars indicate statistical significance, (P<0.05), determined via one-way ANOVA



The majority of the wheat bran protein and peptide samples possessed at least some degree of inhibition activity for elastase, with the exception of the tryptic prolamin hydrolysates/peptides. This sample failed to inhibit elastase to any degree, with no concrete explanation as to why. It was particularly interesting that this sample possessed no inhibition Staactivity for elastase while the prolamin protein fraction resulted one of the highest percent inhibitions out of the entire dataset. Furthermore, the pepsin hydrolysates of prolamin resulted in a relatively high percent inhibition of elastase, at 51.76%. In general, the hydrolytic enzyme pepsin cleaves proteins with lower specificity than trypsin. Based on these observations, a hypothesis could be argued that the peptides produced by the more specific enzyme trypsin eliminated a key functionality that was retained in the pepsin hydrolysates. The identity of this functionality has yet to be identified though and would require further investigation to characterize it's structure. This would be an important area of continued exploration, as the

differences in activity based on the different structures produced by the two enzymes is suggestive of a distinct structure-activity relationship.

A similar phenomenon was observed for the globulin proteins and peptides. In this sample set, the globulin proteins (GlobProt) possessed a percent inhibition of 52.79%, while the peptic hydrolysates of the globulin fraction (GlobPepH) increased to 80.06%. The tryptic hydrolysates of the globulin protein (GlobTrypH) fell to 12.66%. This trend was consistent with the results of the prolamin samples. Interestingly, the reverse of this trend was observed for the albumin protein and peptide samples. In this dataset, the albumin protein sample (AlbProt) possessed a percent inhibition of 62.54%, while the peptic albumin hydrolysates (AlbPepH) only achieved a percent inhibition of 38.94%. The tryptic hydrolysates of the albumin fraction (AlbTrypH) on the other hand achieved a percent inhibition of 83.55%. The trend for the albumin samples suggests that the more specific hydrolysis by trypsin produced a more potent set of peptides than the less-specific hydrolysis reaction catalyzed by pepsin. Determination of the structures of these peptides would be equally useful for determination of a more comprehensive structure-activity profile for the peptide-inhibition of elastase.

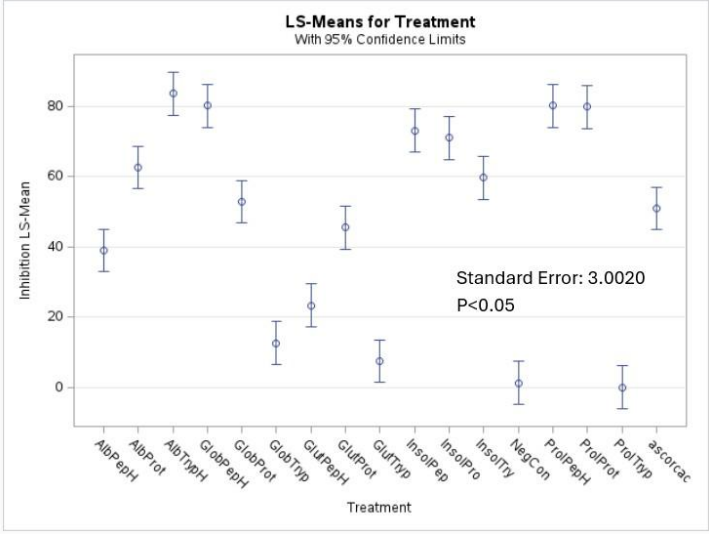
The glutelin protein and peptide samples produced a unique trend, in that the glutelin protein (GlutProt) had the highest percent inhibition of the three sample categories at 45.43%. The peptic glutelin hydrolysates (GlutPepH) decreased to a percent inhibition of 23.38%, and the tryptic glutelin hydrolysates (GlutTrypH) only achieved 7.45%. The opposite of this trend was observed for the insoluble protein and peptide samples, which each had consistently high percent inhibitions. The insoluble protein fraction (InsolProt) had a percent inhibition of elastase at 70.91%, while the peptic hydrolysates of the insoluble fraction (InsolPepH) was measured at

73.04%. The tryptic hydrolysates of the insoluble fraction (InsolTrypH) were slightly lower at 59.59%, but this was one of the higher percent inhibitions of the entire dataset.

The results of the one-way ANOVA are provided in figure 3.10 and show the statistical variances in the elastase inhibition dataset. The results of this analysis illustrate the differences in percent inhibition from protein to tryptic or peptic hydrolysate samples. The differences in activity between a particular protein and its peptic or tryptic hydrolysates were statistically significant for every sample but the insoluble fraction and the prolamin fraction. In the case of the insoluble fraction, the differences in percent inhibition of the protein (InsolProt) were not significantly different from the insoluble peptic hydrolysates (insolPep), nor from the insoluble tryptic hydrolysates (InsolTryp). However, there was a slightly significant difference in the percent inhibitions of the peptic insoluble hydrolysates and the tryptic insoluble hydrolysates. Such similar results of the insoluble protein fraction and its peptide derivatives could again be explained by the harsh extraction conditions this sample was subjected to. The alkaline extraction solution being heated to 100°C could easily result in base-catalyzed hydrolysis of the protein through non-specific hydrolysis reactions, resulting in a diverse mixture of peptides or protein sub-fragments. The similar results of the protein and the peptic hydrolysates support this hypothesis to a degree, given that hydrolysis facilitated by pepsin is less-specific than trypsin and would be closer to a base-catalyzed hydrolysis reaction. This could result in similar hydrolysate products as those that were already present in the insoluble protein fraction from the extraction, but the extra two hours of hydrolysis reaction time could increase the concentration of hydrolysates relative to the original insoluble protein sample. This hypothesis is supported by the results of the elastase inhibition assay, as although the difference was insignificant, the percent inhibition of the peptic hydrolysates of the insoluble protein fraction did increase slightly from

70.91% to 73.04%. The decrease in percent inhibition by the tryptic hydrolysates of the insoluble protein fraction may further support this hypothesis, as the more specific hydrolysis reaction could have destroyed some of the functionality of the insoluble fraction's peptides. The mechanism of this inhibition has yet to be elucidated, but a possible explanation could be that the hydrolysis at a critical lysine or arginine residue(Manea et al., 2007) may have removed or altered a functionality that was integral to the inhibition of the elastase enzyme. Further investigation is needed in this area, but this would be an ideal starting point for the establishment of a structure-activity relationship for these compounds as well.

Figure 3.10: One way ANOVA analysis of the elastase inhibition dataset. Note: abbreviations are ProtPepH: Protein classification, tryptic or peptic hydrolysis, H for hydrolysates. AlbPepH = albumin peptic hydrolysates. AlbTrypH = albumin tryptic hydrolysates. AlbProt = albumin protein. Ascorcac = ascorbic acid standard. NegCon = negative control. P<0.05.

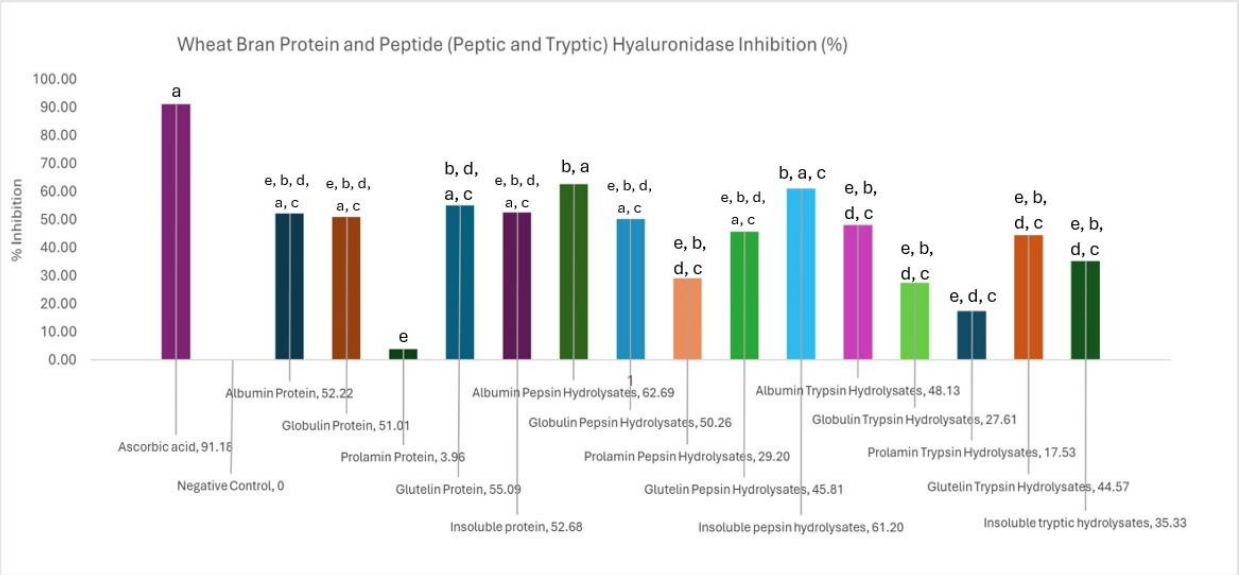


3.4.5 Hyaluronidase Inhibition Assay

The results of the hyaluronidase inhibition assay are provided in figure 3.11 and illustrate interesting activities for the wheat bran protein and peptide samples. The unknown samples derived from the wheat bran protein fractions were compared to ascorbic acid, at a relative comparison of 0.05mg ascorbic acid vs 0.05mg wheat bran protein/peptide. At first glance, all of

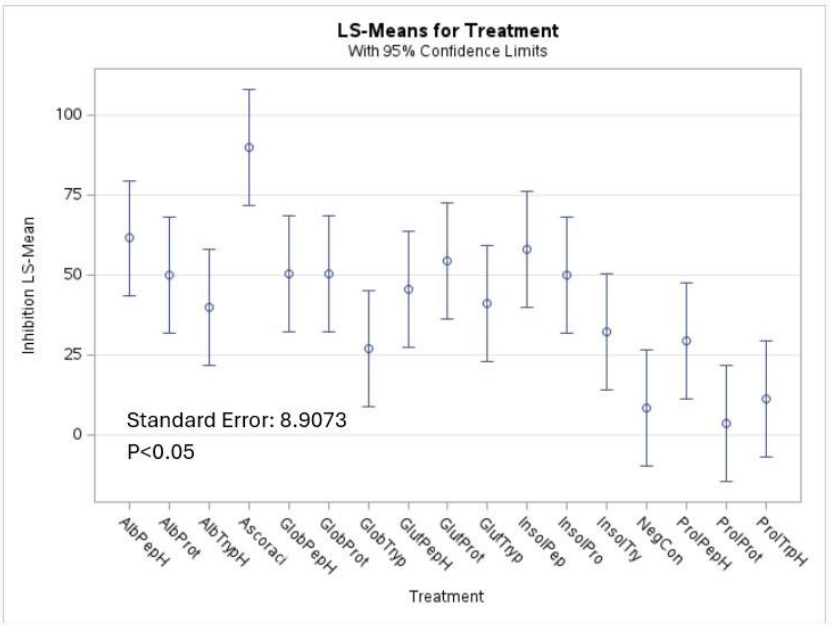
the wheat bran protein and peptide samples had a lower percent inhibition than the ascorbic acid sample, which achieved a percent inhibition of 91.18%.

Figure 3.11: Results of the hyaluronidase inhibition assay ran on wheat bran proteins and peptides produced by pepsin and trypsin. Wheat bran unknowns were compared to ascorbic acid with a relative comparison of 0.05mg ascorbic acid vs 0.05mg wheat bran protein/peptide. Different letters above bars indicate statistical significance, (P<0.05), determined via one-way ANOVA



Further analysis of this dataset using a one-way ANOVA statistical analysis performed in SAS studio showed a series of interesting trends in the overall dataset. The results of this statistical analysis are provided in figure 3.12.

Figure 3.12: One-way ANOVA statistical analysis of variance for the hyaluronidase inhibition assay ran with wheat bran proteins and peptides. Note: abbreviations are ProtPepH: Protein classification, tryptic or peptic hydrolysis, H for hydrolysates. AlbPepH = albumin peptic hydrolysates. AlbTrypH = albumin tryptic hydrolysates. AlbProt = albumin protein. Ascorcac = ascorbic acid standard. NegCon = negative control. P<0.05.



The results of the one-way ANOVA confirmed that the percent inhibition of the ascorbic acid standard was significantly higher than the majority of the wheat bran protein and peptide unknown samples. A closer look at the trends within each of the different Osborne classifications shows that the percent inhibitions for an individual protein sample did not differ significantly from either of the peptides produced using trypsin or pepsin hydrolysis for the entire dataset. Albumin proteins and peptides performed the best relative to the other Osborne fractions, followed by the insoluble fraction, globulin fraction, and glutelin fraction samples. The prolamin samples resulted in the lowest percent inhibition of the entire dataset.

The trends observed within each Osborne fraction showed interesting activity. The average albumin protein sample (AlbProt) had a reasonably high percent inhibition for hyaluronidase at 52.22%, but the peptic hydrolysates of albumin (AlbPepH) exhibited the highest

percent inhibition of the entire dataset at 62.69%. The tryptic hydrolysates of albumin (AlbTrypH) had a slightly lower percent inhibition, at 48.13%, indicating a loss of activity relative to the parent albumin proteins. Despite these differences in activity, the percent inhibitions were not found to be significantly different for any of the albumin samples. The trend was identical for the insoluble fraction, with the insoluble peptic hydrolysates (InsolPep) achieving a percent inhibition of 61.20%, the insoluble protein (InsolProt) achieving a percent inhibition of 52.68%, and the insoluble tryptic hydrolysates achieving a percent inhibition of 35.33%. Further comparison showed that the percent inhibitions of the albumin protein samples were more similar to the results of the albumin tryptic hydrolysates. The trends of the insoluble fraction samples were identical to the trends found within the albumin samples. None of the insoluble tryptic hydrolysates were found to be significantly different according to the one-way ANOVA statistical analysis, although the tryptic hydrolysates and the insoluble protein samples were more similar than the insoluble proteins and the peptic hydrolysates. The Insoluble protein (InsolProt) sample had a percent inhibition of 57.89%, while the peptic hydrolysates (InsolPep) was 50.05%, and the tryptic hydrolysates (InsolTryp) achieved a percent inhibition of 32.24%.

The percent inhibitions for the prolamin samples initially followed a different trend, with the prolamin protein possessing an average percent inhibition of 3.96%, the prolamin peptic hydrolysates an average of 29.20%, and the prolamin tryptic hydrolysates an average of 17.53%. When analyzed via the one-way ANOVA in SAS studio, the average percent inhibition for the prolamin protein was calculated to be 11.34%. This discrepancy was due to the calculation of the average from the average of three percent inhibitions, rather than the three absorbances measured for the prolamin protein samples. The more accurate percent inhibition calculation is likely the 3.96% inhibition, as this gave a better representation of the percent inhibition based on the actual

physical measurements of the triplicate samples. Adjusting these calculations in the statistical analysis did not change the results much, as the prolamin tryptic hydrolysates were also calculated to have an average of 3.39%. The tryptic hydrolysates of the prolamin fraction were found to be statistically insignificantly different from the peptic hydrolysates, and in turn the prolamin proteins would not be statistically significantly different either.

The trend in the one-way ANOVA analysis of the glutelin samples showed that the glutelin protein samples (GlutProt) had the highest percent inhibitions of hyaluronidase at 55.09%, followed by the glutelin peptic hydrolysates (GlutPepH) at 45.81%, followed by the glutelin tryptic hydrolysates (GlutTrypH) at 44.57%. This is indicative of a slight loss in inhibition activity after hydrolysis into peptides by both the pepsin and trypsin enzymes, although pepsin resulted in a slightly better percent inhibition. The results of the one-way ANOVA also confirmed that these percent inhibitions were not significantly different for any of the three glutelin samples, regardless of being composed of protein or protein hydrolysates. The reasons for the lack of significant difference between the protein and peptide samples is not known, but further investigation into the structural differences between the protein and peptide samples may be able to explain the lack of an appreciable difference. Structural analysis of the peptides may be able to elucidate similar structure-activity relationships that each of these samples' constituents share and could confirm similar functionalities that contribute to the inhibition of hyaluronidase.

3.4.6 Top Performing Samples

From each of the peptides and proteins screened in this study, 11 samples exhibited antioxidant or enzyme inhibitions statistically similar to ascorbic acid. These samples were

tabulated in table 3.6, which shows the sample and the corresponding assay or assays that it performed well in.

Table 3.6: Tabulated results of top-performing wheat bran protein or peptide samples. Samples were selected based on statistical similarity to the assay's ascorbic acid standard sample, or the top-performing sample in the case of the DPPH assay.

Peptide/Protein Sample Statistically Similar to Ascorbic Acid Standard	Corresponding Assay(s)
Albumin Peptic Hydrolysates	DPPH, Collagenase, Elastase, Hyaluronidase
Globulin Peptic Hydrolysates	Elastase
Prolamin Peptic Hydrolysates	DPPH, Elastase
Glutelin Peptic Hydrolysates	Collagenase, Elastase
Insoluble Peptic Hydrolysates	DPPH, Elastase, Hyaluronidase
Albumin Tryptic Hydrolysates	DPPH, Collagenase, Elastase
Globulin Tryptic Hydrolysates	Elastase
Prolamin Tryptic Hydrolysates	Collagenase
Glutelin Tryptic Hydrolysates	NA
Insoluble Tryptic Hydrolysates	DPPH, Collagenase, Elastase
Albumin Protein	Elastase
Globulin Protein	NA
Prolamin Protein	DPPH, Elastase
Glutelin Protein	Collagenase, Hyaluronidase
Insoluble Protein	DPPH, Elastase, Collagenase

The results in table 3.6 represent the highest performing samples in each of their respective bioactivity assays. Some of the samples were found to be statistically similar to the ascorbic acid standards in multiple assays, while other samples excelled in only one or two assays. A few hypotheses have been proposed in sections 3.4.2 to 3.4.5 that attempted to explain the trends observed in each of the bioactivity assay data sets. Overall, a general trend was observed that suggested the highest-performing samples were either exposed to more denaturing and/or hydrolytic conditions during the Osborne fractionation extractions, or enzymatic hydrolysis conditions that could facilitate less-specific cleavages and therefore a larger number of smaller peptides. This observation was consistent with the general rule-of-thumb for bioactive peptides that smaller peptides correlate to higher degrees of biological activity(Zou et al., 2020).

This hypothesis was further supported by the results of the bioactivity assays that showed hydrolysates produced by pepsin had higher bioactivities across a larger number of assays than hydrolysates produced by trypsin. The peptic hydrolysates also out-performed the majority of the protein samples that were not subjected to either form of enzymatic hydrolysis. However, proteins extracted through harsher alkaline conditions capable of non-specific base-catalyzed hydrolysis reactions also resulted in better performance across multiple assays, a fact that is illustrated by the insoluble protein and insoluble protein hydrolysates. These insoluble protein and insoluble protein hydrolysates were found to be consistently top-performing samples in the DPPH, Collagenase, and Elastase assays. Compared to the results of the peptic hydrolysates and the insoluble fraction samples, hydrolysates produced by trypsin exhibited slightly worse performance. This was attributed to trypsin's higher selectivity for lysine and arginine hydrolysis sites, which would likely result in the formation of fewer, but larger peptides with reduced bioactivity. Despite these hypotheses and the supporting results of the bioactivity assays, further work devoted to structural characterization of these peptides and structure-activity relationships will be required to better-determine the operative mechanisms that led to these trends.

3.4.7 UPLC-QToF Analysis of Wheat Bran Peptides

A common theme observed in each of the bioactivity assays was the lack of significant differences in the percent inhibitions of a specific protein relative to its peptic and tryptic hydrolysate products. The common hypothesis that has been proposed to explain these trends is that the proteins and peptides may share similar functionalities to one another that are responsible for the inhibition of the enzyme in the assay. Another trend that was observed was reduced activity or improved activity of a particular type of hydrolysate. In some examples, the less specific peptic hydrolysate product resulted in higher percent inhibition than the more

specific tryptic hydrolysate product. The proposed explanation for this trend was that the less specific hydrolysis reaction produced a larger variety of peptides, with a larger number of peptides containing a functionality that was able to inhibit the enzyme in the assay. In addition to this possibility, it was also hypothesized that the specific hydrolysis reaction catalyzed by trypsin could be producing fewer peptides than the less specific reaction catalyzed by pepsin and could even be unintentionally destroying key functionalities based on lysine and arginine cleavages at the c-terminus of the junction (Manea et al., 2007). The operative mechanism of inhibition by these samples has yet to be elucidated, but further work to characterize the structures of these peptides and proteins would be instrumental in determining the functional groups responsible for inhibition of the enzymes in each assay. By determining the structures, the structure-activity relationships can be analyzed for each peptide and protein in a sample set.

To determine the structures of these peptides, each sample was prepared at a concentration of 0.4mg/mL in HPLC-grade water with 0.1% formic acid. These samples were then analyzed using the Waters Acquity I-class UPLC, and the Waters Xevo-G2-XS-QTof mass spectrometer. The results of these analyses are provided in figures 3.13-3.16. Unfortunately, due to software limitations, the spectra obtained for each of the samples were unable to be matched to any spectra provided in any peptide fragmentation spectral libraries. In future work, the software ProteinLynx Global Server will be used to determine the structures of each of these species. Upon acquisition of this information, the structures of the peptides with the highest in-vitro biological activities found in this study will be identified and synthesized. Performing the same bioactivity assays on the synthetic peptides would provide a high-confidence assessment of the results obtained from the extracted peptide mixtures. Results found to be inconsistent with the bioactivity results in this study would be able to be contested with a greater degree of

certainty, as a single peptide species would be evaluated rather than a more complex mixture of peptides.

Figure 3.13: Chromatogram of the albumin tryptic peptide sample. Two primary peaks, with a total of four peaks of interest were observed upon closer evaluation of the peak at 55.44 minutes.

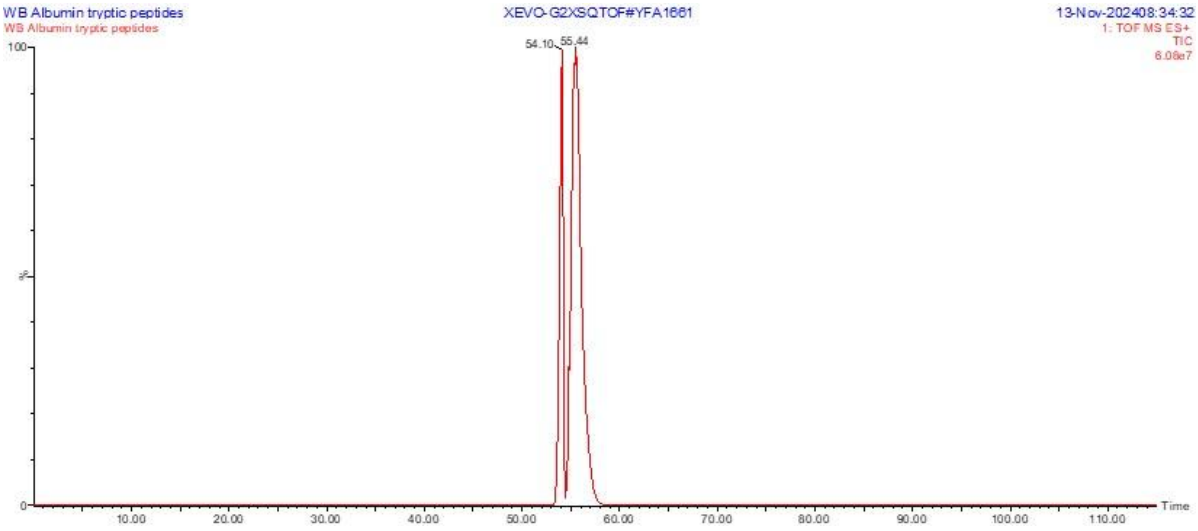


Figure 3.14: Mass spectrum of the peak at 54.10 minutes. Peaks consistent with peptide samples were observed at m/z's at 1367, 697, 421, and 328.

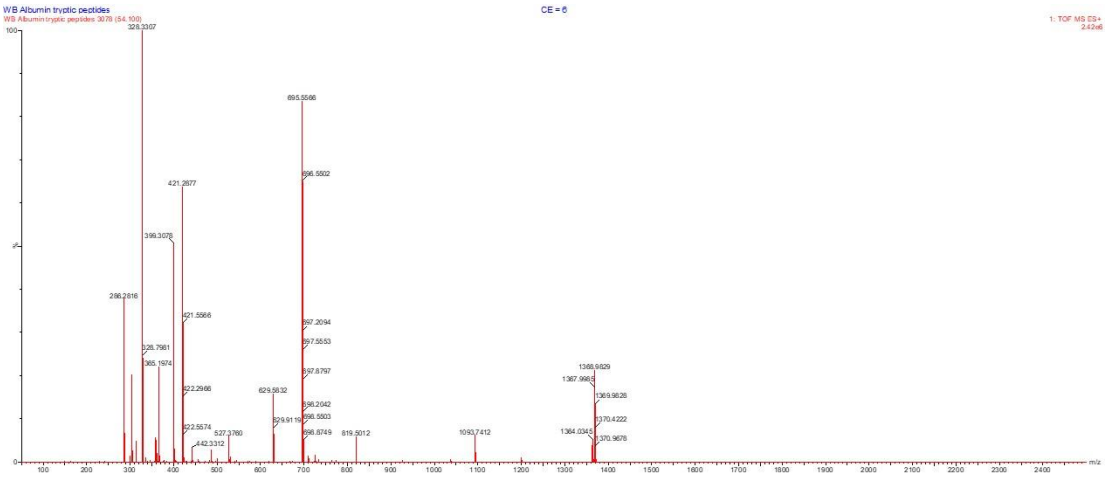


Figure 3.15: Mass spectrum of the peak of interest in the tryptic albumin hydrolysate sample at 55.439 minutes. Characteristic peptide peaks were observed at m/z's of 1364-1370, 695-699, and 328-330.

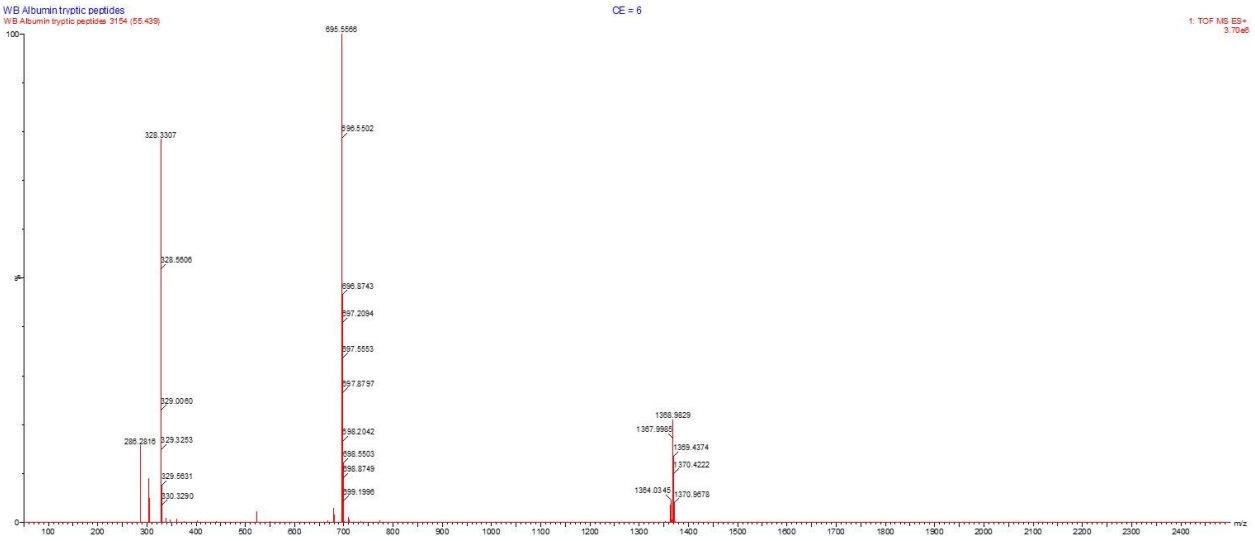
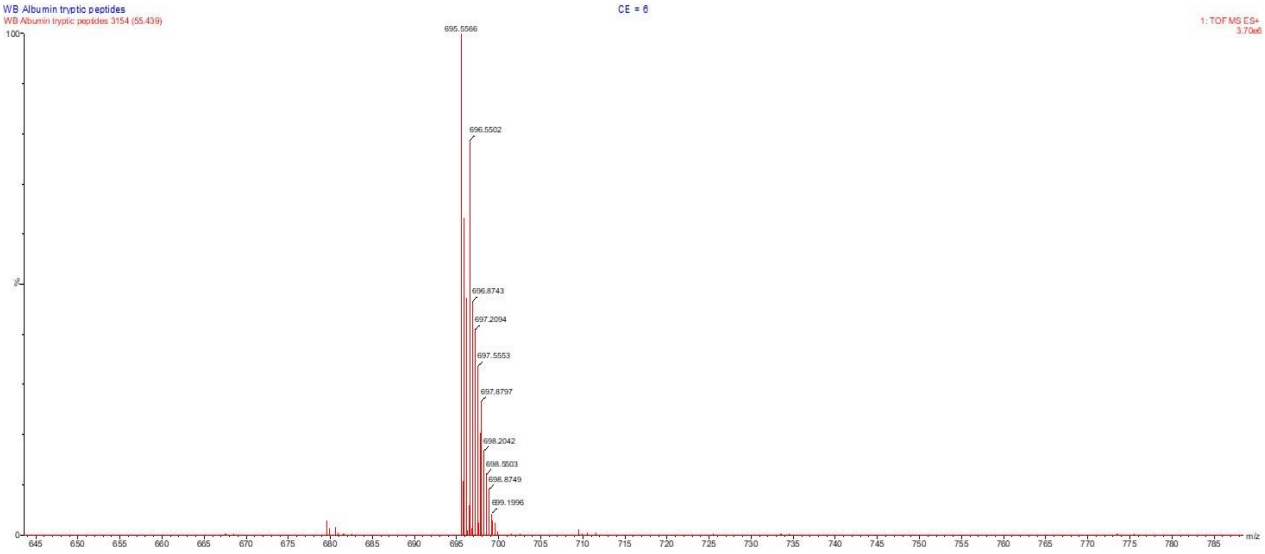


Figure 3.16: Mass spectrum of the peak of interest in the tryptic albumin hydrolysate sample at 55.439 minutes. Zoomed in to emphasize the characteristic peptide spectral pattern observed from the m/z of 695 to 699.



Although the structures of these peptide samples were unable to be identified, the mass spectra of the peaks of interest were clearly representative of peptide mass spectra, identified by the presence of peptide clusters (G. Zhang et al., 2010). These data confirmed that the hydrolysate samples produced by trypsin did in fact contain a mixture of peptides with potential

biological activity. Similar results were obtained for each of the other peptide samples derived from the globulin, prolamin, glutelin, and insoluble Osborne fractions, and their spectra have been included in Appendix A.

3.5 Conclusions

In summary, the hydrolysis reactions of protein samples were successfully optimized for trypsin and pepsin using casein as a test protein. The results of these optimization reactions were assessed using the detergent DPPH assay, and statistical analysis was carried out to determine if any of the reaction conditions tested had statistically significant results. Because the majority of the antioxidant capacities measured for each set of casein hydrolysis conditions were statistically identical, the results of this DPPH assay were used to eliminate under-performing conditions. The results of these tests were extrapolated to the highest enzyme to substrate ratio, with a reaction time of 120 minutes to ensure the largest amount of peptides possible were produced. These conditions were run with each Osborne protein fraction individually, and the peptides/protein hydrolysates from each reaction were collected via centrifugation and lyophilized. The lyophilized peptide samples were then screened for biological activity using a series of in-vitro assays. The antioxidant capacity of each sample was assessed, followed by a collagenase inhibition assay to determine each sample's ability to prevent the breakdown of collagen. The elastase inhibition assay was performed after the collagenase inhibition assay to determine the extent of each sample's ability to prevent the breakdown of elastin. Finally, the hyaluronidase inhibition assay was performed to assess each sample's ability to inhibit the breakdown of hyaluronic acid, another key component of healthy and youthful skin. The preliminary results of these assays were quite promising and showed comparable results to ascorbic acid standards. Several samples were identified that exhibited statistically similar results

to the ascorbic acid standard used in each assay, and these samples were provided in table 3.6.

In future studies, the structures of each of the peptide and protein samples need to be characterized via mass spectrometry. This is imperative, as the structure of each peptide is vital to its function in any chemical or biochemical environment. Each peptide identified in these samples should then be synthesized via solid-state peptide synthesis to obtain a completely pure sample, and the assays should be ran again to confirm the results of the extracted compounds. This strategy would effectively narrow down the highest-performing peptides even further and provide in-vitro bioactivity screening results with even higher confidence; performing the assays on pure, synthetic peptides would allow for the assessment of individual peptides, rather than more complex mixtures. Trends in similar functional groups on the peptides with similar bioactivities would be able to be evaluated, and structure-activity relationships could be established. This information would provide significantly better insights into the operative mechanisms of antioxidant activities, as well as the mechanisms of enzyme inhibition for each sample.

In addition to characterizing the properties of the native peptide/ proteins, the pharmaceutical properties of these compounds could be evaluated, such as the partition coefficients, and cellular permeability of an individual peptide. Compounds that would not perform well in in-vivo, for example, due to poor membrane permeability, could be altered synthetically to provide for better cellular penetration. Such a strategy could be carried out using common synthetic techniques already employed by the pharmaceutical industry, such as the addition of lipophilic palmitoyl groups (Mahato, 2003).

The results obtained by this study suggest that further research and development of the peptides and proteins extracted from hard red winter wheat bran has considerable potential and

would be a promising strategy for the valorization of wheat bran through its use as an industrial feedstock. The use of wheat bran as an inexpensive and abundant resource to produce value-added products, especially for the development of nutraceutical and cosmeceutical products, is strongly supported by the preliminary data presented in this study. The in-vitro bioactivity assays performed in this study successfully identified 13 samples with bioactivities that were found to be statistically similar to or better than ascorbic acid in four different biological activity assays. This is especially noteworthy considering that ascorbic acid is a well-known antioxidant that has been studied extensively for the health-promoting effects that stem from its powerful antioxidant properties(Brauchla et al., 2021). The data presented in this study also suggests that future work focused on characterizing the individual constituents in these samples would quickly identify a series of peptides and proteins that could serve as lead compounds for the development of more effective health-promoting ingredients. Although more work is required to achieve this goal, these potentially health-promoting constituents extracted from wheat bran could eventually become available on the consumer market as health-promoting and anti-aging nutraceutical and cosmeceutical products.

Chapter 4 - Overall Summary and Future Research

Wheat bran has excellent potential for use as an industrial feedstock as it is an inexpensive, abundant, and renewable resource. Wheat bran is composed of 15 to 16% protein on average, making it particularly useful as a source of protein. Unfortunately, the negative sensory qualities of wheat bran have contributed significantly to the underutilization of wheat bran in human diets. These qualities have effectively prevented the potential of wheat bran from being realized, both from a nutritional perspective as well as from an economic perspective. The fact that humans do not want to consume wheat bran because it has an undesirable taste and a gritty texture when incorporated in breadmaking has kept the value of wheat bran low since the conception of industrial wheat production. From a certain point of view this is regrettable, as wheat bran's desirable nutritional profile would make it an excellent addition to human diets. However, from the perspective of valorization, the low price of wheat bran combined with the abundance of wheat bran as an industrial byproduct has set the stage for wheat bran to become an ideal industrial feedstock to produce value-added products.

This study has demonstrated that wheat bran protein can be successfully extracted and purified using simple extraction and purification techniques such as Osborne fractionations and column chromatography methods. In addition to more conventional chromatography methods, it was also demonstrated that the incorporation of dry column vacuum chromatography techniques and solid-phase extraction column techniques were able to concentrate wheat bran proteins and remove lower molecular weight impurities from protein samples reliably and consistently. The purification methods explored in this study were also carried out using inexpensive and re-usable stationary phases such as underivatized silica gel, rather than more expensive and complex chromatography media such as size exclusion or affinity chromatography media. The

purification of proteins on underivatized silica gel was also achieved using environmentally friendly mobile phases, using a 0.025M phosphate buffer for the capture step and 0.025 to 0.1M solutions of arginine for the elution of the proteins. Finally, the arginine in the elution step was easily removed from the protein sample using a 3kDa molecular weight cutoff ultrafiltration step. The use of this purification method eliminated the need for long dialysis steps and sped up the purification process by eliminating the need for ultrafiltration of large volume extracts. Rather than multiple-day long purification steps, this column protocol was able to isolate proteins in 30 minutes to 3 hours, depending on the scale of the samples being purified.

This study also demonstrated that wheat bran proteins could be further derivatized into peptides using the digestive enzymes pepsin and trypsin. The preliminary results of the in-vitro biological activity assays performed on the proteins and peptides of wheat bran identified 13 different samples derived from wheat bran proteins that exhibited promising bioactivities. The results of each bioactivity assay underwent statistical analysis using a one-way ANOVA test and were found to be statistically similar to comparable doses of ascorbic acid, a well-established dietary antioxidant. In addition to these higher-performing samples, nearly all of the other protein and peptide samples exhibited at least some degree of antioxidant or enzyme-inhibiting activity, with only a few examples of samples that exhibited zero activity. In each instance, if a sample performed poorly in one assay, there was a counter example where its performance was significantly better than the assay in which it failed to achieve satisfactory performance.

Each of the tryptic peptides of the Osborne protein fractions was analyzed via UPLC-MS using a Waters Acquity I class UPLC inlet coupled to a Waters Xevo-G2-XS QTof mass spectrometer. The results of these analysis positively identified peptide spectra for each sample, with a minimum of four individual peptide species in each sample. Unfortunately, due to

software limitations, further structural analysis of the constituents of these samples is still needed. Characteristic peptide mass spectra were observed for these samples, but the sequences of these peptides still need to be identified through large searches of peptide fragmentation spectral databases. Despite these setbacks, the spectra obtained from each of these samples should be sufficient to identify their structures.

Once the structures of these peptides have been identified, it will be possible to establish structure-activity relationships between the functional groups specific to the peptides in a particular sample and the performance of that peptide in a particular bioactivity assay. If a specific class of functional group such as an aromatic or basic functional group is found across each of the highest performing peptide samples in a given assay, it would support the hypothesis that that particular class of functionality is implicated in the antioxidant or enzyme inhibition mechanism. To take it a step further, by assessing the chemistry of said functionality, conclusions could be drawn to more specific mechanisms that could be operative in these reactions. The structural information obtained for a specific peptide would be vital supporting evidence for proposing the mechanism of such a reaction or molecular interaction. Some hypotheses have been proposed to explain the differences in biological activities between the different samples tested in this study, such as higher activities associated with samples that were exposed to conditions that could give rise to less-specific hydrolysis conditions. Although the chemical theory supports these conclusions, without elucidating the structures of each of the active constituents in these samples, these hypotheses are largely conjectural. There remains a significant amount of work before these products will be ready for sale as nutraceuticals or cosmeceuticals, but the results of this study suggest that further exploration of the proteins and peptides derived from wheat bran for this purpose would be a worthwhile endeavor.

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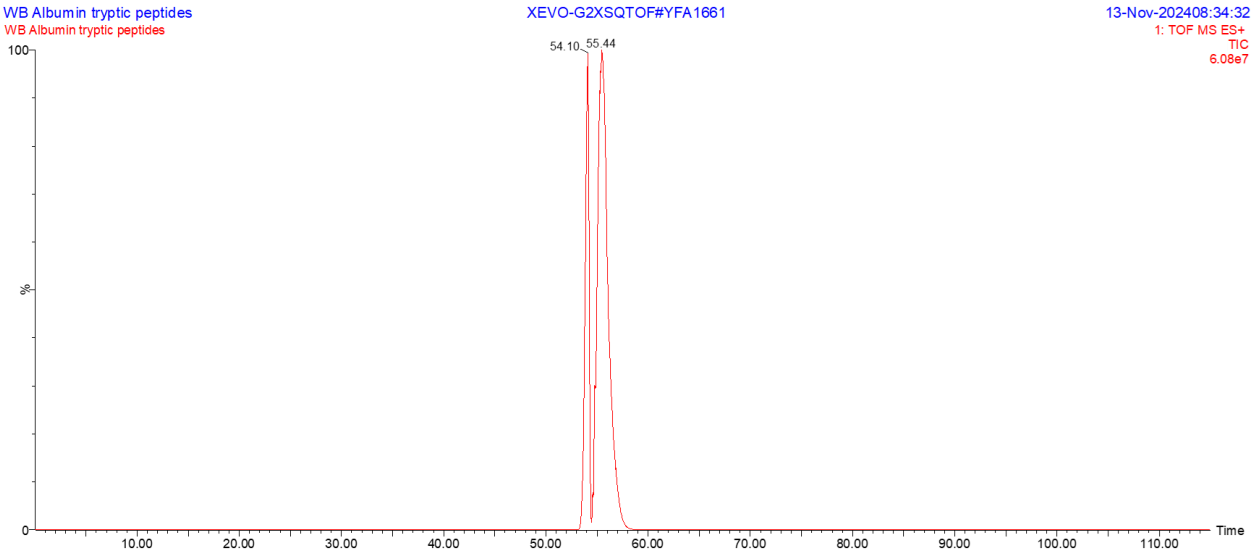
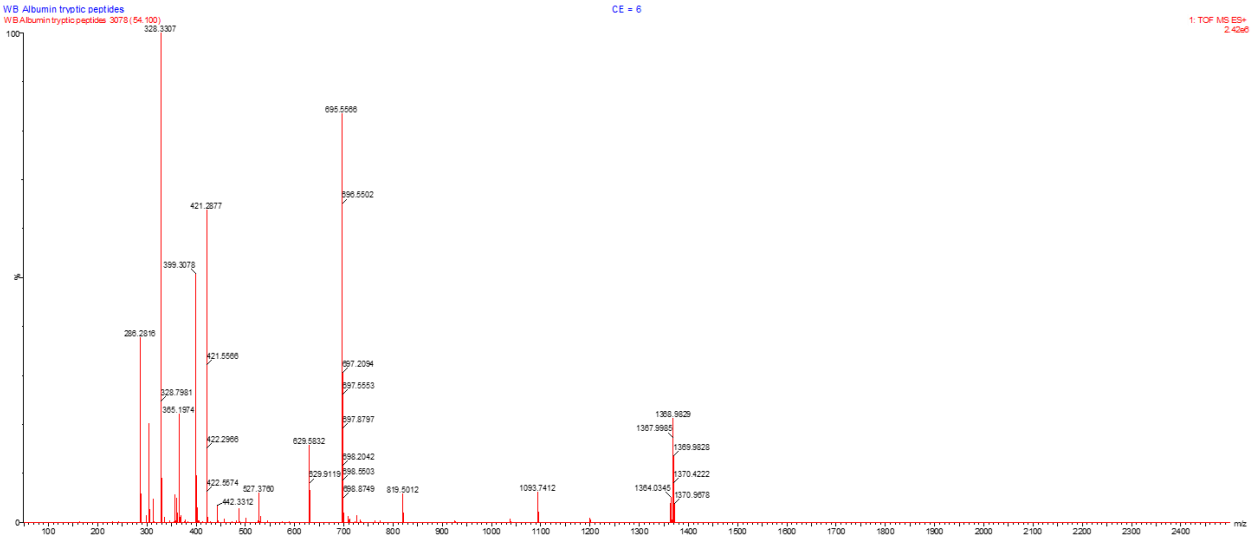
Appendix A - Mass Spectra of Tryptic Wheat Bran Peptides

UPLC chromatograms for tryptic wheat bran peptides discussed in chapter 3 have been included in Appendix A.

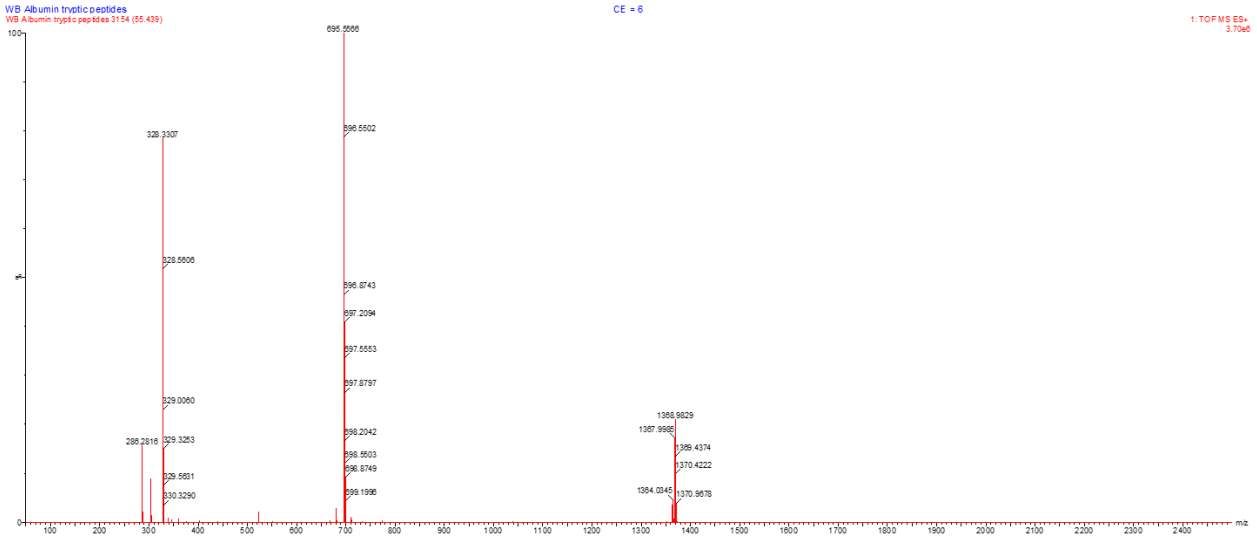
Appendix Figure A.1. UPLC Chromatogram for Tryptic Wheat Bran Albumin Peptides.



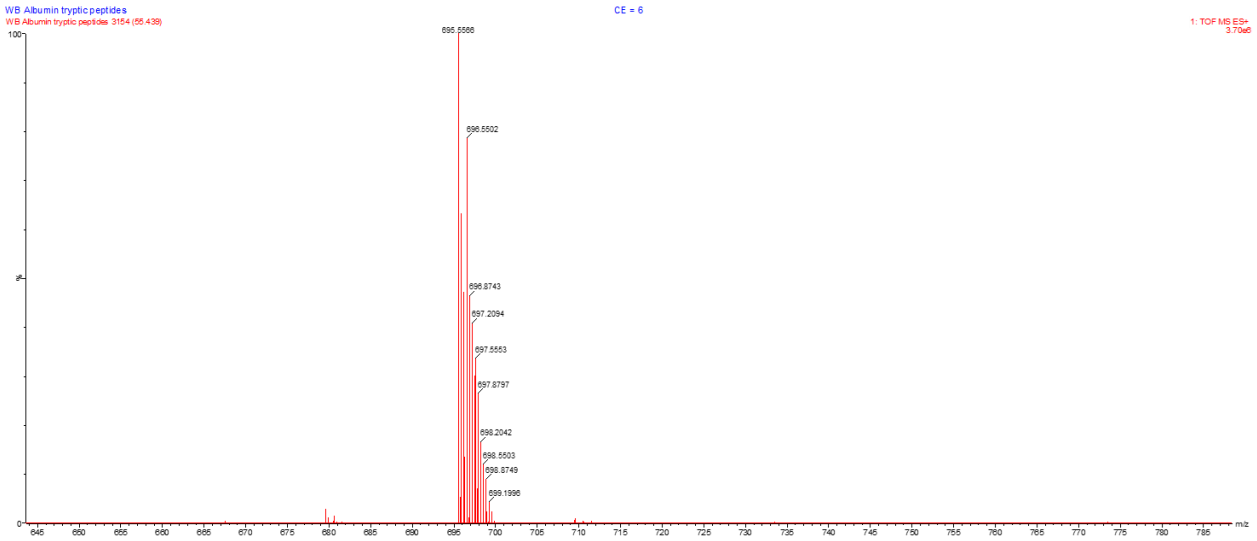
Appendix Figure A.2: Mass spectrum for the peak of interest located at RT 54.10 min, (wheat bran albumin tryptic peptides).



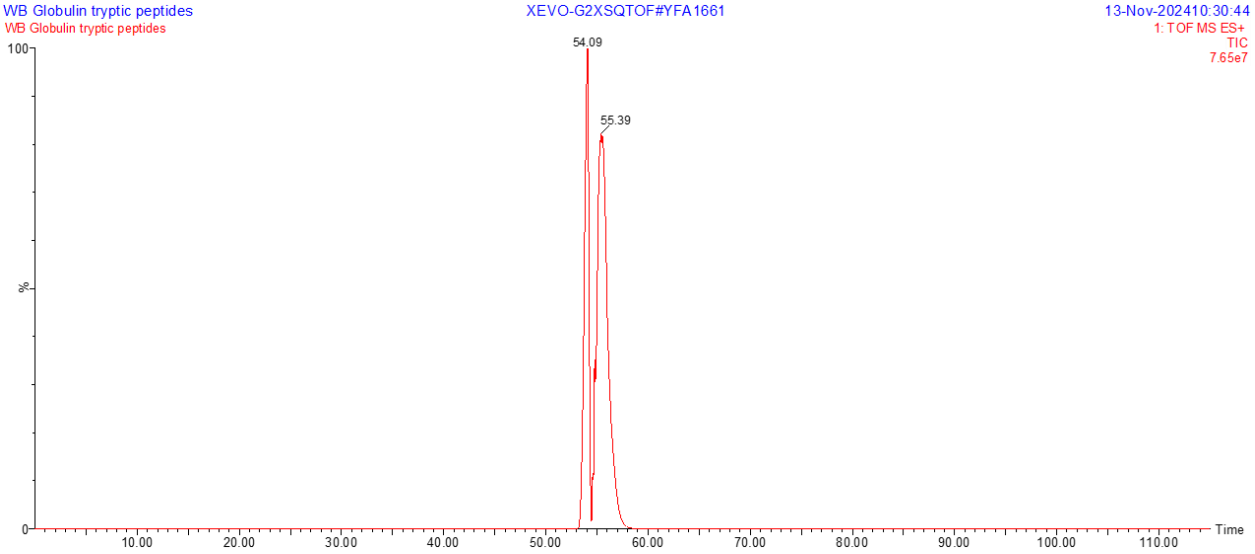
Appendix Figure A.3: Mass spectrum for the peak of interest located at RT 54.439 min, (wheat bran albumin tryptic peptides).



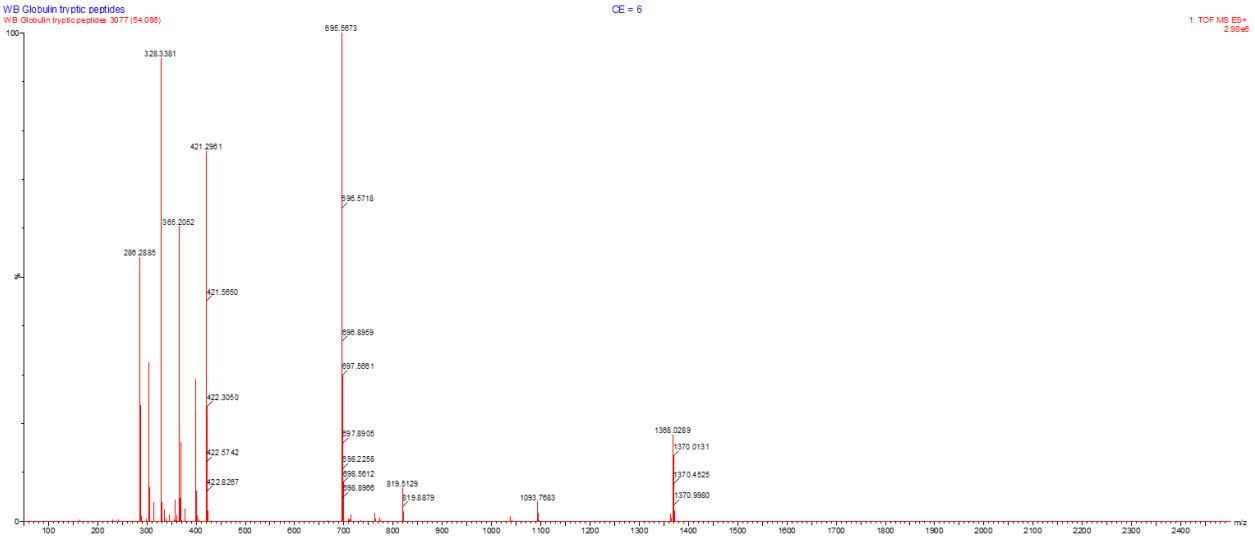
Appendix Figure A.4: Mass spectrum for the peak of interest located at RT 439 min, zoomed in to illustrate typical peptide spectral cluster behavior, (wheat bran albumin tryptic peptides).



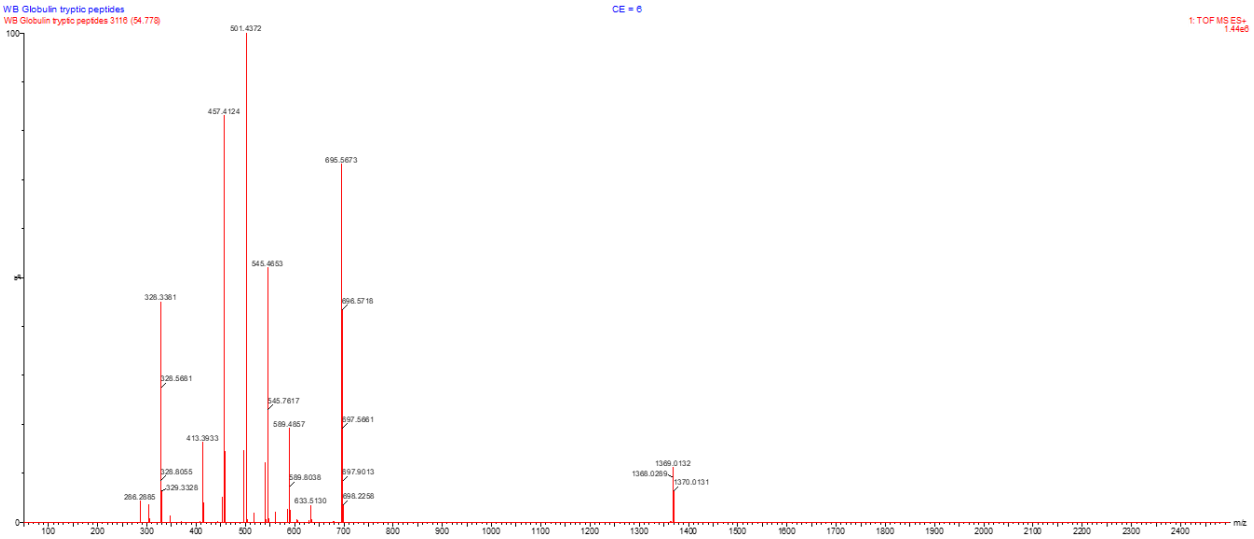
Appendix Figure A.5: UPLC Chromatogram for Tryptic Wheat Bran Globulin Peptides.



Appendix Figure A.6: Mass spectrum for the peak of interest located at RT 54.439 min, (wheat bran globulin tryptic peptides).

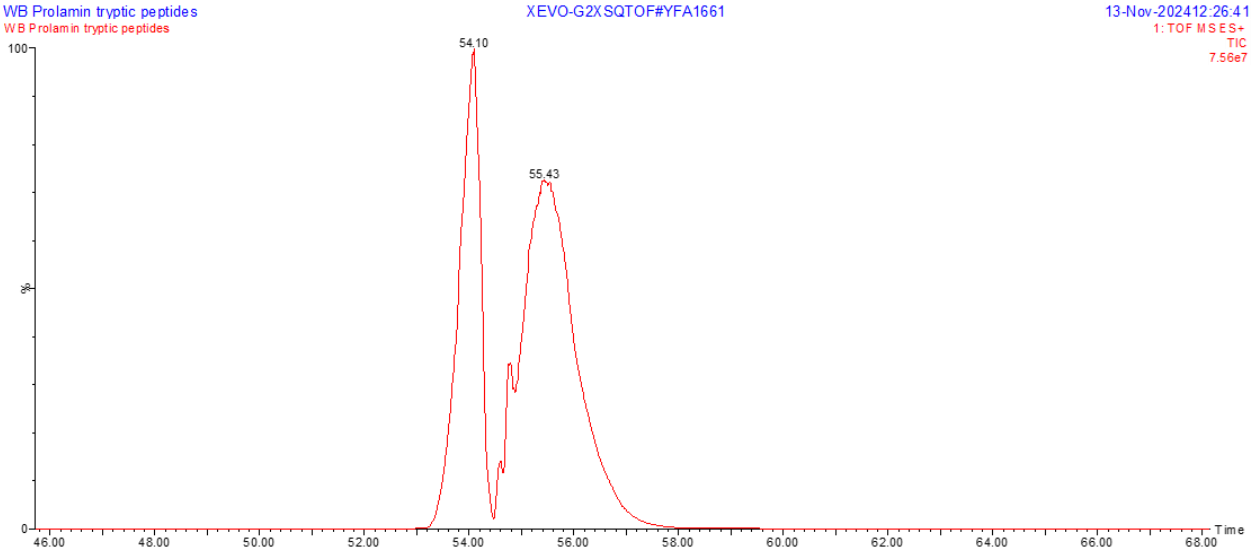


Appendix Figure A.7: Mass spectrum for the peak of interest located at RT 54.778 min, (wheat bran globulin tryptic peptides).

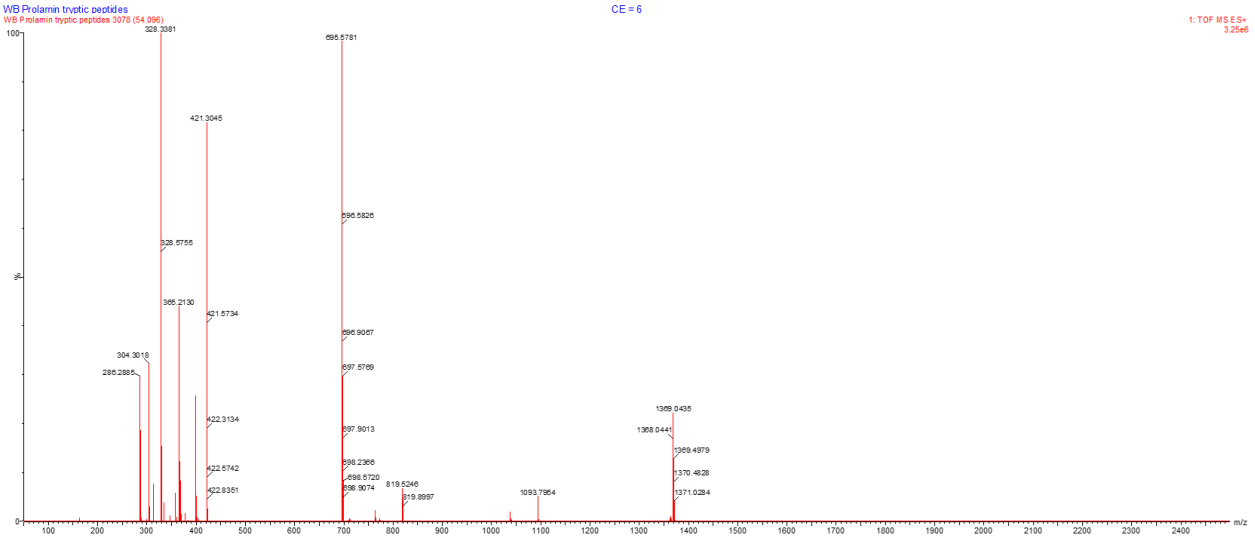


Appendix Figure A.8: Mass spectrum for the peak of interest located at RT 55.390 min, (wheat bran globulin tryptic peptides).

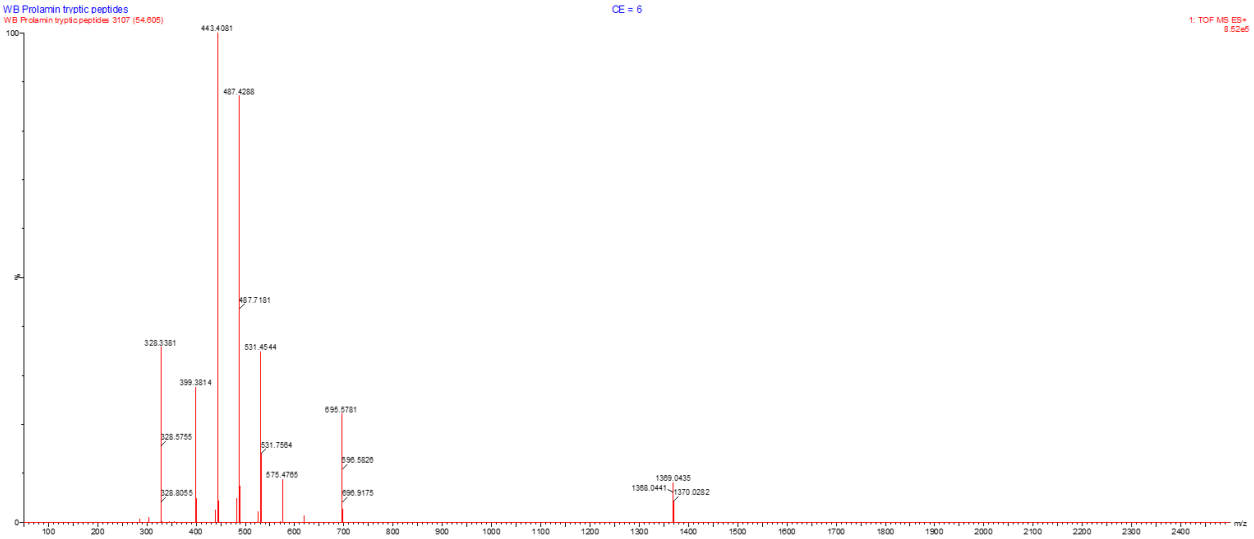
Appendix Figure A.9: UPLC Chromatogram for Tryptic Wheat Bran Prolamin Peptides.



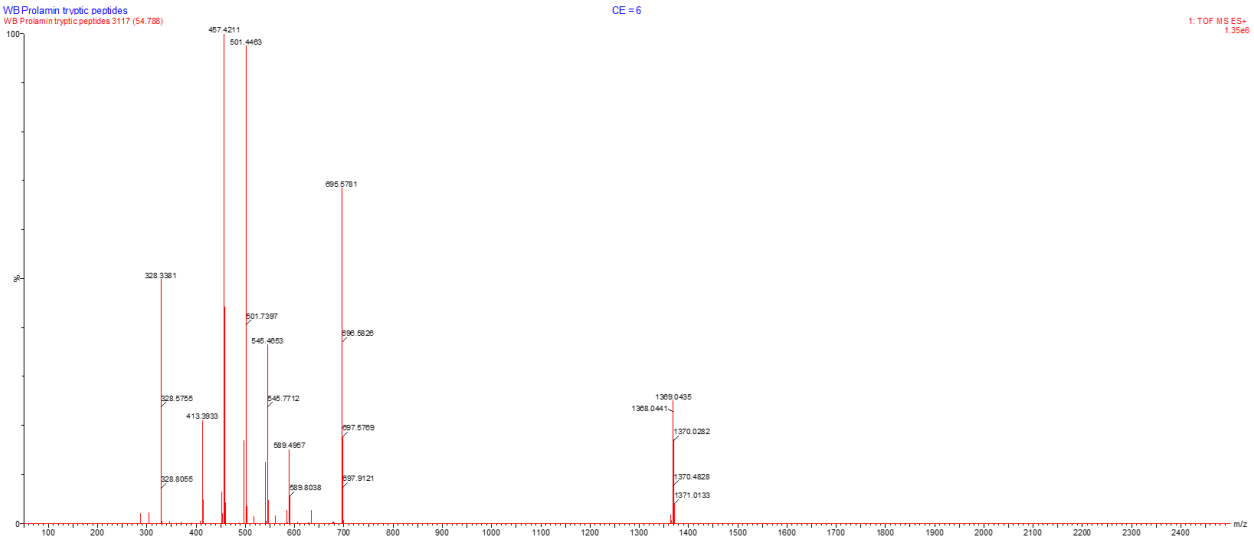
Appendix Figure A.10: Mass spectrum for the peak of interest located at RT 54.096 min, (wheat bran prolamin tryptic peptides).



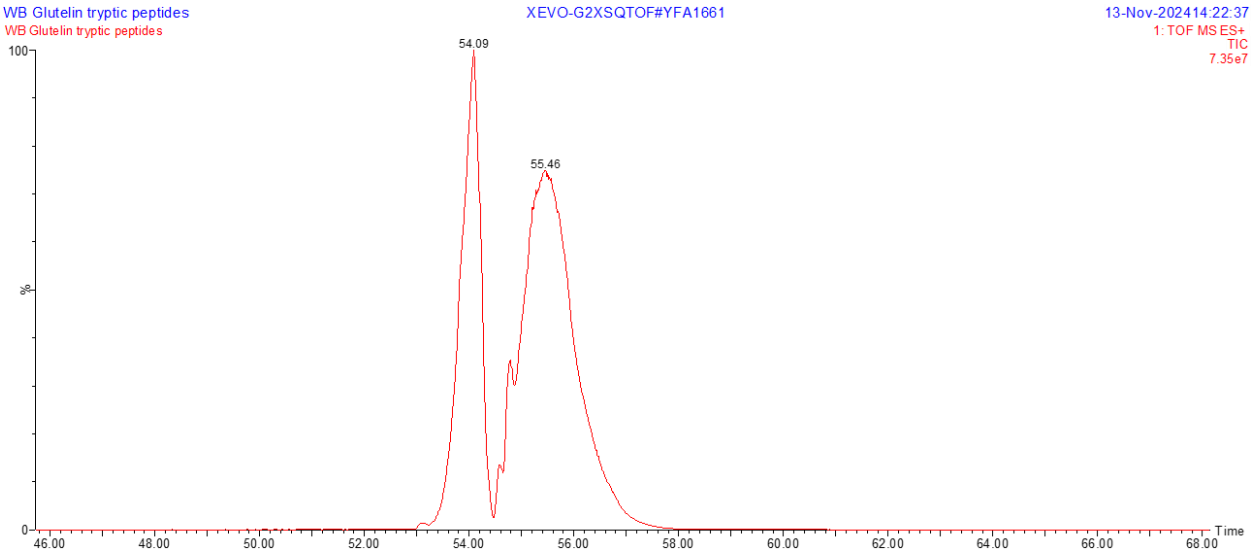
Appendix Figure A.11: Mass spectrum for the peak of interest located at RT 54.605 min, (wheat bran prolamin tryptic peptides).



Appendix Figure A.12: Mass spectrum for the peak of interest located at RT 54.788 min, (wheat bran prolamin tryptic peptides).



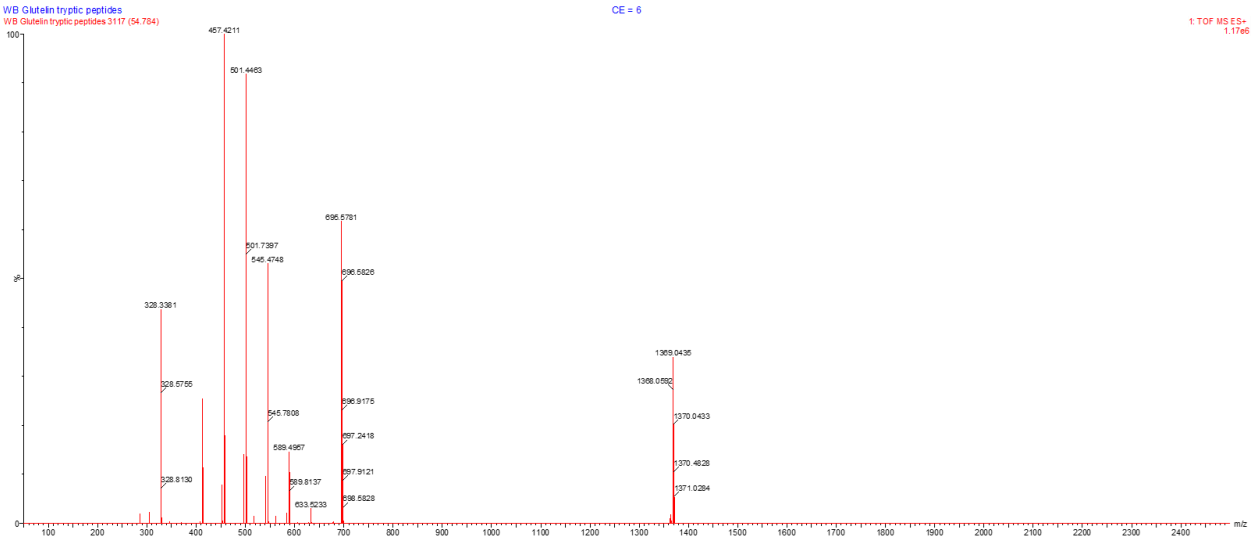
Appendix Figure A.13: UPLC Chromatogram for Tryptic Wheat Bran Glutelin Peptides.



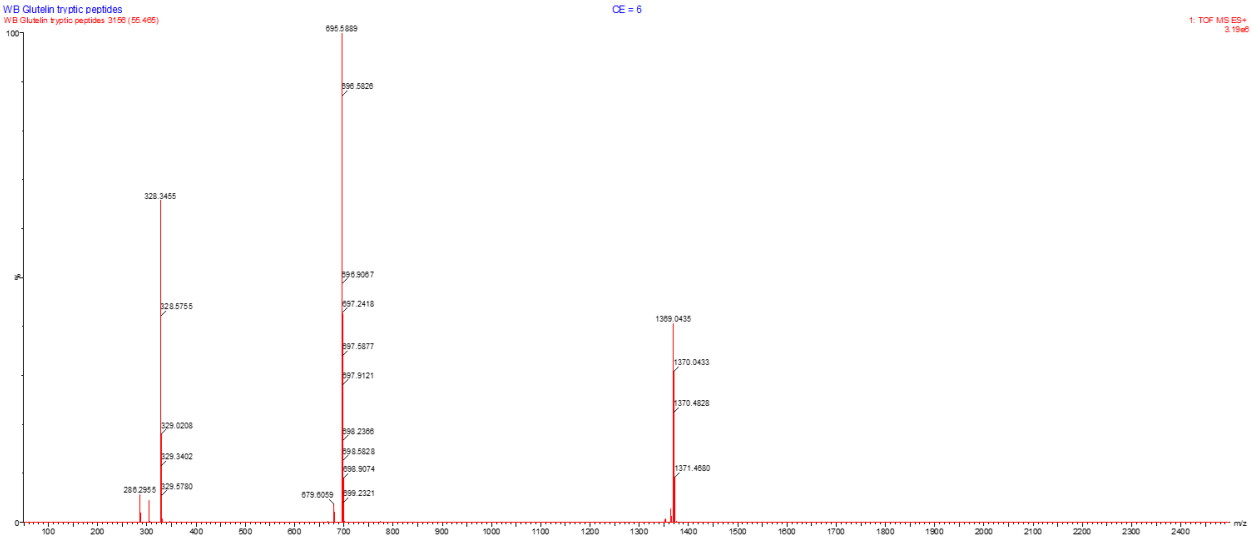
Appendix Figure A.14: Mass spectrum for the peak of interest located at RT 54.092 min, (wheat bran glutelin tryptic peptides).

Appendix Figure A.15: Mass spectrum for the peak of interest located at RT 54.584 min, (wheat bran glutelin tryptic peptides).

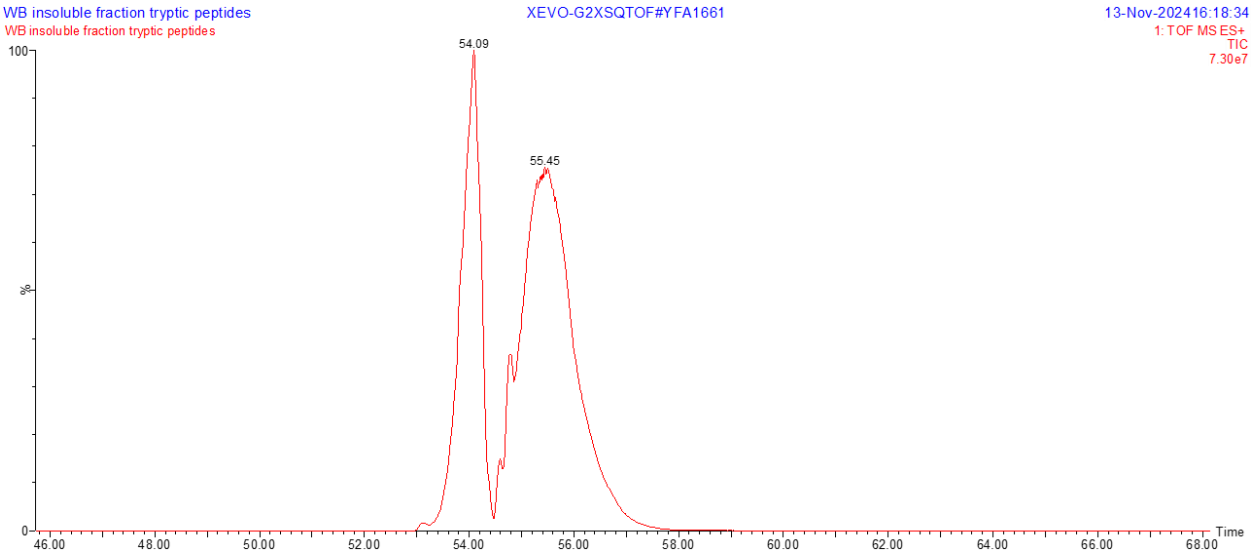
Appendix Figure A.16: Mass spectrum for the peak of interest located at RT 54.784 min, (wheat bran glutelin tryptic peptides).



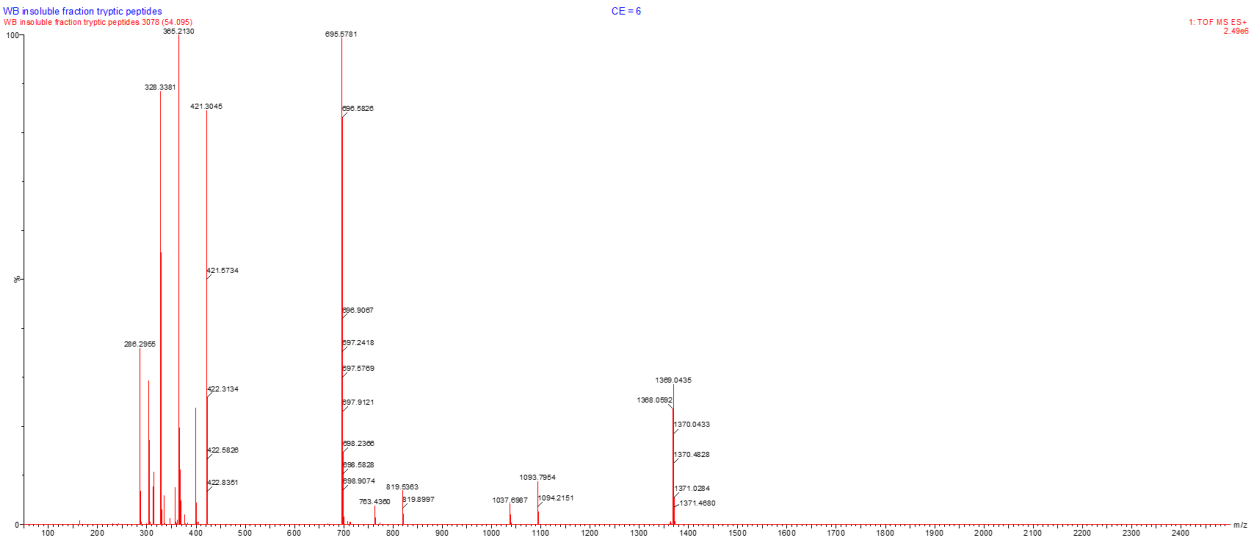
Appendix Figure A.17: Mass spectrum for the peak of interest located at RT 55.465 min, (wheat bran glutelin tryptic peptides).



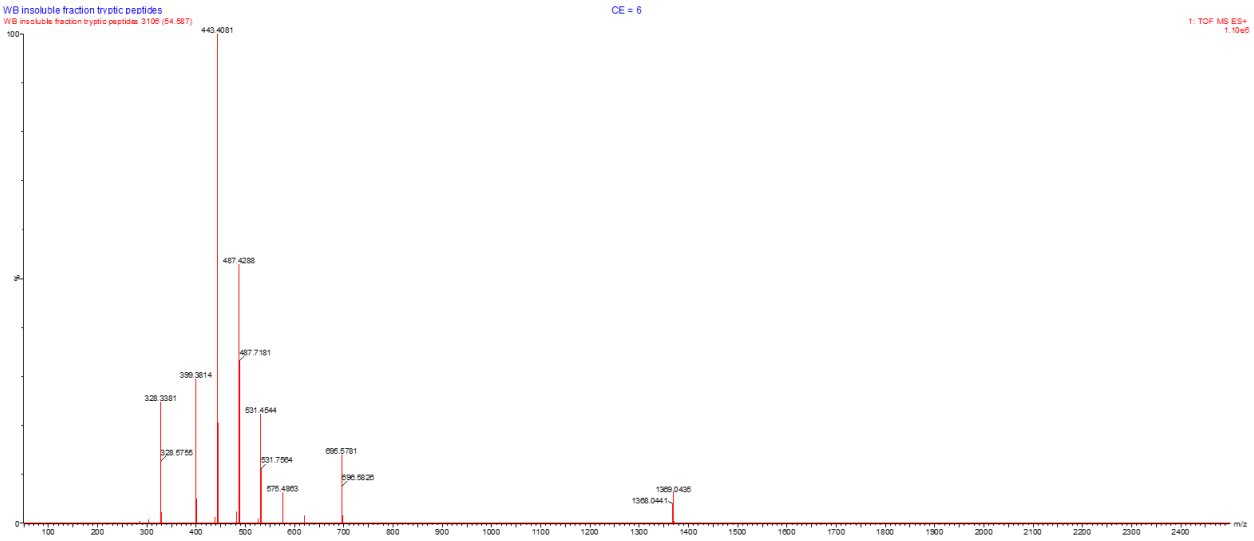
Appendix Figure A.18: UPLC Chromatogram for Tryptic Wheat Bran Insoluble Protein Fraction Peptides.



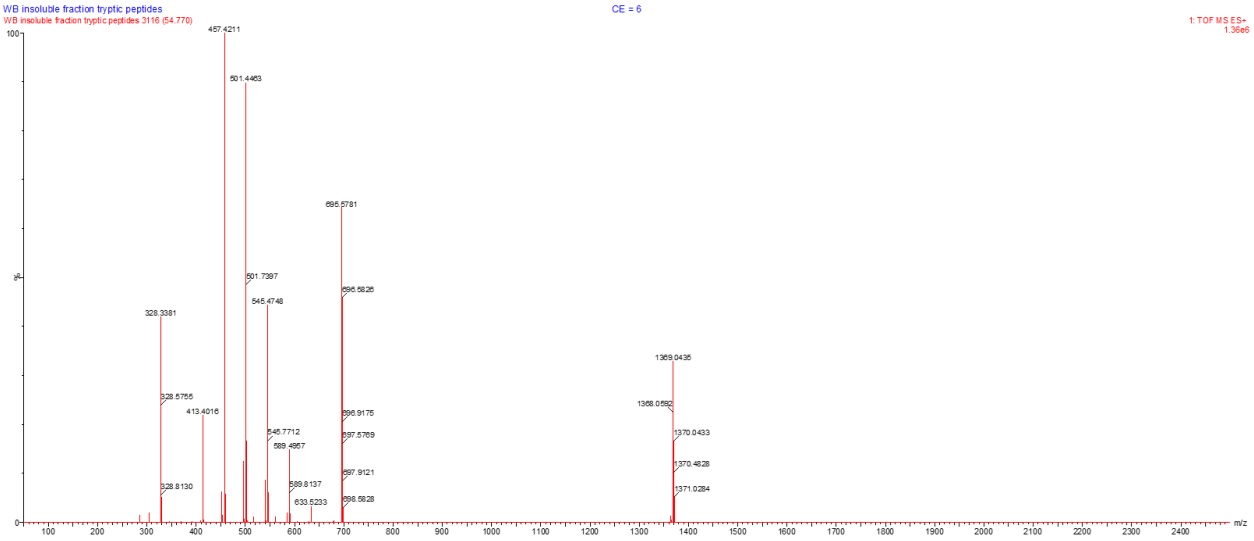
Appendix Figure A.19: Mass spectrum for the peak of interest located at RT 55.465 min, (wheat bran insoluble protein fraction tryptic peptides).



Appendix Figure A.20: Mass spectrum for the peak of interest located at RT 54.587 min, (wheat bran insoluble protein fraction tryptic peptides).



Appendix Figure A.21: Mass spectrum for the peak of interest located at RT 54.770 min, (wheat bran insoluble protein fraction tryptic peptides).



Appendix Figure A.22: Mass spectrum for the peak of interest located at RT 55.450 min, (wheat bran insoluble protein fraction tryptic peptides).