The Pennsylvania State University
The Graduate School
College of Agricultural Sciences

PHYSICOCHEMICAL MODIFICATION OF GLIADIN BY DIETARY
POLYPHENOLS AND THE POTENTIAL IMPLICATIONS
FOR CELIAC DISEASE

A Dissertation in
Food Science
by
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ABSTRACT

Celiac disease is an autoimmune enteropathy that affects approximately 1% of the world population. Characterized by an adverse reaction to gluten protein, celiac disease manifests in the small bowel and results in inflammation and increased permeability of the gut barrier. This is followed by an immune response mounted against not only gluten, but also tissue transglutaminase 2 (TG2), a gluten-reactive enzyme secreted by intestinal epithelial cells. Despite the growing number of individuals affected by this disease, the only reliable intervention strategy available is lifelong adherence to a gluten-free diet. Novel strategies for treating or preventing celiac disease include synthetic pharmaceuticals to modify the gut barrier, parabiotic infection to reduce inflammatory cytokine release and administration of a synthetic polymer that binds gluten proteins and prevents their digestion and absorption. Interestingly, polyphenols possess similar abilities as this polymer to interact with protein.

Polyphenols are a structurally diverse class of compounds that are ubiquitous to plants. The bioactivity of many of these compounds has been explored extensively both in vivo and in vitro, demonstrating antioxidant, anti-carcinogenic and anti-inflammatory properties. Polyphenols have also been studied for their anti-nutritional effects, with high polyphenol diets resulting in decreased absorption of macro- and micronutrients. The ability of polyphenols to interact with protein has been studied extensively with respect to interactions with proline rich salivary proteins due to the role those interactions play in the tactile oral sensation of astringency.

Interestingly, gluten proteins possess some structural similarities to salivary proteins including high frequencies of proline and secondary structure motifs that lend
themselves to interacting with polyphenols through noncovalent interactions with the protein main chain. Based on these similarities, I hypothesized that gluten proteins would interact with polyphenols through noncovalent interactions and result in the formation of protein-polyphenol complexes in vitro. Furthermore, I expected that these interactions would limit the damaging effects of gluten proteins on intestinal epithelial cells in a celiac disease model by modifying or sequestering the protein, preventing its digestion and the resultant production of immunostimulatory fragments or impeding its recognition by cellular binding sites. My findings suggest that dietary polyphenols are able to interact with gluten proteins, forming protein-polyphenol complexes that exert some benefit towards reducing the intestinal permeability and inflammation associated with celiac disease.

My initial investigation focused on the mechanisms of interaction between α2-gliadin (57-89), a hydrolytically stable and immunologically relevant fragment of gluten that is produced during digestion, and three polyphenols: gallic acid (GA), epigallocatechin gallate (EGCG) and theaflavin (TF). Though each of these compounds are found in tea and are therefore readily accessible in the human diet, they are also structurally distinct from one another. Saturation transfer difference nuclear magnetic resonance revealed that each of these compounds interacted with α2-gliadin (57-89) and while an overarching trend of phenolic groups playing an important role in interaction emerged, only EGCG demonstrated specific spatial orientation upon interaction with α2-gliadin (57-89). Additionally, EGCG and TF possessed similar affinities for interaction with α2-gliadin (57-89) despite TF having a greater molecular weight and increased frequency of potential binding sites. These findings suggested that the structural
conformation of phenolic ligands influence the propensity of those compounds to bind to α2-gliadin (57-89) rather than the interaction being dependent on frequency of potential hydrogen bond donors and acceptors alone.

Given the importance of the structure of α2-gliadin (57-89) in recognition by immune receptors, I next examined the physical impact of interaction with EGCG on the peptide. The reaction formed protein-polyphenol aggregates, which were first observed using cryo-transmission electron microscopy. Using a variety of analytical techniques, I outlined the biphasic reaction mechanism that occurs upon exposure of α2-gliadin (57-89) to an excess of EGCG and elucidate the structural changes that interaction with EGCG exerts on the peptide. Further, I used computational modeling software to illustrate these interactions in silico. Using 2D NMR and circular dichroism, I determined that the complexes formed modified the structure of α2-gliadin (57-89) as observed by chemical shift changes in main chain hydrogens and an increase in the overall relative helicity of the peptide. These interactions may help mitigate the immune response associated with celiac disease by obscuring recognition of α2-gliadin (57-89) by human leukocyte antigen-DQ2 (DQA1*05:01, DQB1*02:01).

The objective of my next study was to determine the extent to which polyphenol complexation may disrupt celiac disease pathogenesis in vitro using a more translatable system. Purified polyphenols were replaced with decaffeinated green tea extract while gliadin, both native and enzymatically digested, was used in place of α2-gliadin (57-89). A critical step in the primary phase of celiac disease pathogenesis is the enzymatic hydrolysis of gliadin during digestion. The immunogenic fragments produced are able to stimulate intestinal permeability and are recognized by TG2 and immune cells. While the
aforementioned studies suggest that polyphenols may disrupt the recognition of these fragments via sequestration and structural changes, I sought to also elucidate the impact of polyphenol supplementation on the formation of these digestive products. Upon addition of green tea extract to in vitro digestion of gliadin, immediate precipitation of protein was observed and quantified via SDS-PAGE and subsequent densitometric analysis of the proteins in the supernatant. Furthermore, green tea extract addition resulted in dose-dependent retention of proteins in the 30-45 kDa molecular weight range in comparison to the untreated control. Based on these findings, I hypothesized that (1) precipitation of gliadin by polyphenols is effective in sequestering gliadin proteins from digestive enzymes and (2) green tea polyphenols exert inhibitory effects directly on digestive enzymes, reducing their activity.

Co-precipitation of native and pepsin-trypsin digested (PT) gliadin with green tea polyphenols was measured as increases in turbidity. Analyses of the supernatant revealed that at a 1:1 ratio of PT-gliadin to green tea extract, 93.4% of protein and 34.8% of green tea polyphenols were precipitated upon combination. Precipitation was also observed upon interaction between green tea extract and native, undigested gliadin. These precipitation profiles suggest that green tea polyphenols effectively bind to gliadin in vitro while a portion of the polyphenols remain unbound. It was also determined that green tea polyphenols dose-dependently inhibit both pepsin and trypsin. Collectively, these findings demonstrate multiple mechanisms for protective effects of green tea polyphenols against the production and availability of gliadin fragments.

The effect of green tea polyphenol supplementation on gliadin-mediated permeability was tested next, as intestinal permeability stimulated by exposure to gliadin...
is a hallmark symptom of celiac disease. This was done using an *in vitro* model for the intestinal brush border wherein human colonic cells (Caco-2) were grown to form a polarized monolayer on a permeable transwell. Permeability was measured as changes in transepithelial electrical resistance (TEER). In both the presence and absence of gliadin, green tea extract increased TEER, demonstrating an improvement in barrier integrity. This suggests that in addition to preventing the damaging effects of gliadin on intestinal permeability, green tea polyphenols exert protective effects on the gut barrier. This protective effect was further demonstrated as GTE suppressed the secretion of proinflammatory cytokines IL-6 and IL-8, which play important roles in gut barrier maintenance and lymphocyte recruitment, respectively.

The overall findings of these studies suggest that the anti-nutritional characteristics of tea polyphenols can be used to physically sequester gliadin proteins, disrupting key steps in disease pathogenesis, and contribute to amelioration of celiac-related intestinal permeability. This is the first study to test a plant-derived dietary compound as a potential therapeutic agent for celiac disease. This potential treatment strategy features a variety of advantages including ease of incorporation into the diet, developed understanding of toxicity risks and a large body of work supporting supplementation of these compounds to treat other ailments. The interaction mechanisms outlined here in a simple *in vitro* system provide a foundation for future exploration into the potential for polyphenol supplementation to be used as a nutraceutical approach to treating celiac disease.
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<td>IEL</td>
<td>intestinal epithelial lymphocyte</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>STD-NMR</td>
<td>saturation transfer difference nuclear magnetic resonance</td>
</tr>
<tr>
<td>ITC</td>
<td>isothermal titration calorimetry</td>
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<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
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<tr>
<td>EGCG</td>
<td>epigallocatechin gallate</td>
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<tr>
<td>TF</td>
<td>theaflavin</td>
</tr>
<tr>
<td>GA</td>
<td>gallic acid</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility</td>
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<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
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<tr>
<td>TEER</td>
<td>transepithelial electrical resistance</td>
</tr>
<tr>
<td>PT</td>
<td>pepsin-trypsin digested</td>
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<tr>
<td>TJ</td>
<td>tight junction</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IEC</td>
<td>intestinal epithelial cell</td>
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<td>MMP</td>
<td>matrix metalloproteinase</td>
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“If the patient can be cured at all, it must be by means of the diet.”

– Dr. Samuel Jones Gee

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Chapter 1  Literature Review

1.1  Introduction

Celiac disease is an autoimmune enteropathy characterized by an adverse reaction in the small intestine to gluten, a protein found in wheat, barley and rye. Initial symptoms of celiac disease are gastrointestinal discomfort as well as permeability and inflammation of the small bowel, while long-term exposure to gluten can lead to extensive damage of the small intestine, leading to impaired nutrient absorption and chronic conditions related to malnutrition.\(^1\) Despite affecting nearly 1% of the population, there is no known cure for celiac disease, and the most reliable treatment currently available is lifelong adherence to a gluten-free diet.\(^2\)

Celiac disease, while not a classically defined food allergy, is one of many gastrointestinal food-based hypersensitivities. Food hypersensitivities affect millions of individuals in the United States alone, with food allergy diagnosis rates increasing markedly in the past decade. The incidence of food allergies in children increased by 50% between 1997 and 2011, and the rate of diagnosis for celiac disease, which more than doubled between 2004 and 2008, continues to climb as information about diagnostic symptoms becomes more widely accepted.\(^3,4\) In the United States alone, as many as 15 million people are living with food allergies and 3 million people are living with celiac disease.\(^5,6\) The economic burden of food hypersensitivities is profound, with healthcare costs of an estimated $30 billion dollars per year in the United States alone.\(^4,7\) Personal healthcare expenses for individuals with celiac disease average nearly $4,000 more per person per year than non-affected individuals, even after diagnosis.\(^8\) Further, the seeming ubiquity of gluten in the Western diet presents an additional cost to individuals with CD,
as gluten-free alternatives to commonly consumed foods like crackers, pasta and cakes cost an average of 240% more than their gluten-containing counterparts.  

The only reliable strategy for preventing celiac disease symptoms upon the consumption of gluten is eliminating gluten from the diet altogether, which can be both costly and inconvenient. A variety of alternative strategies have been explored, from gut barrier-modifying pharmaceuticals to intentional hookworm infection, which has been shown to reduce cytokine secretion in the gut. Another strategy employed has been the use of a synthetic polymer made of hydroxyethyl methacrylate and sodium-4-styrene sulfonate (poly(HEMA-co-SS); BL-7010), which was shown to bind gluten proteins in the gut, thereby preventing their breakdown by digestive enzymes and sequestering the protein from initiating gluten-mediated symptoms in the gut such as inflammation and the celiac-related immune response.

Sequestration of macro- or micronutrients is typically described as anti-nutritional and something to be avoided; however, this may be an effective strategy for mitigating or eliminating symptoms associated with celiac disease. Polyphenols are a structurally diverse class of secondary plant metabolites that have demonstrated anti-nutritional effects upon consumption by humans and animals alike due to their ability to inhibit digestive enzymes and interact with other dietary compounds, including proteins. Interactions between polyphenols and proteins have been studied extensively for their role in the tactile sensation of oral astringency, which results from the precipitation of proline-rich salivary proteins. Polyphenol-protein interactions have been explored as potential therapeutic routes for food allergies, showing that immunoglobulin (Ig) E
responses can be reduced and digestion of allergenic proteins can be blocked by protein-polyphenol interactions.\textsuperscript{16–19}

Gluten makes for a particularly interesting protein target for polyphenol interactions as it shares structural similarities with salivary proteins (i.e., they are rich in proline residues and possess a natively unfolded structure with polyproline II helical (PPII) motifs).\textsuperscript{20,21} In contrast to classical food allergens, the propagation of celiac disease and recognition of gluten by immune receptors is dependent on these structural characteristics. The coincidence that proline-rich PPII helices are preferential targets for the formation of MHC class II complexes in the celiac immune response\textsuperscript{22} and for the formation of protein-polyphenol complexes\textsuperscript{23} makes polyphenolic supplementation a potential approach for the treatment or prevention of celiac disease.

1.2 Gluten

Gluten is a storage protein found in wheat, barley and rye. It is heterogeneously composed of nearly 40 highly homologous proteins classified by two subunits- glutenins, a cysteine-rich class of fibrous proteins, and gliadins, which are classified as prolams due to their richness in proline and glutamine residues.\textsuperscript{24} Prolamins are found in a variety of cereal seeds, and make up approximately half of the total protein found in mature grains, with the exception of oats and rice which, notably, are gluten-free cereal grains.\textsuperscript{25} Gluten was the first prolamin to be isolated and studied in 1745, which was followed by a body of work wherein T. B. Osborne (considered by many to be the “father of plant protein chemistry”) developed a classification system for plant proteins based on solubility.\textsuperscript{26} These classifications included protective proteins, structural and metabolic
proteins and storage proteins. Prolamins, which are soluble in organic solvent, fall under the classification of storage proteins.\textsuperscript{26} As such, wheat prolams are the primary proteins found in the endosperm of grains.\textsuperscript{24} Despite their similar solubility patterns and primary sequences of amino acids containing proline and glutamine, prolams are structurally diverse, ranging from 10 to 100 kDa.\textsuperscript{24} Further classification of prolams separates proteins by their molecular weight (MW) and relative sulfur (S) content, creating the groupings high MW prolams (6-10\% total prolams), S-rich prolamins (70-80\%) and S-poor prolams (10-20\%).\textsuperscript{26}

The sulfurous component of prolams is cysteine. Cysteine frequency in the S-rich class of prolams is 2-3\% compared to 30-40\% glutamine and 15-20\% proline.\textsuperscript{26} However, cysteine’s ability to form disulfide bonds is of key importance with respect to the functionality of gluten during the production and processing of grain-based food products. The primary example of a grain-based food product that highlights gluten functionality is bread. Composed of glutenins and gliadins interacting with one another through disulfide bonds, gluten is responsible for the physicochemical properties of dough that allow stretching and the trapping of air during manipulation and other processing procedures such as proofing and baking. The viscoelasticity of dough is derived from the viscosity of gliadin and elasticity of glutenin as the two subunits come together through inter- and intramolecular disulfide bonding during the mixing of dough. During bread production, gluten formation occurs through three steps: (1) hydration, (2) oxidation and (3) kneading. During hydration, water is added to flour, which allows for the “unraveling” of glutenins and gliadins, partial solubilization of the proteins, and mobilization of functional groups like thiols. Enzymes and starches also become hydrated
during this step, which contribute to the overall flavor and some functional properties of grain-based products.\textsuperscript{27} Hydration also mobilizes oxidants, which affect cysteine residues within glutenin and gliadin, allowing them to interact with one another and to form disulfide bonds.\textsuperscript{27,28} Intramolecular disulfide bond formation is prevalent for gliadins, while glutenins also form intermolecular bonds. Intermolecularly bonded glutenins allow for the formation of linear and branched polymeric networks, while gliadins form intramolecular disulfide bonds within those networks resulting in a viscoelastic mass.\textsuperscript{27,29}

While thiols in flour can become oxidized during an aging or maturation period, modern processes typically include the addition of oxidizing agents such as bromates or ascorbic acid.\textsuperscript{28} Mixing, or kneading, allows distribution of this network throughout the dough.\textsuperscript{27}

The end result is the formation of thin strands of protein that are sufficiently extensible and elastic to trap the CO\textsubscript{2} produced by yeast during bread making (Figure 1.1). Beyond bread, gluten is also the protein responsible for the pliability of pasta dough. In baked goods where lighter, fluffier textures are desired, gluten formation is avoided or weakened by minimal mixing/kneading or the addition of reducing agents that prevent the formation of disulfide bonds.\textsuperscript{30}

\textbf{Figure 1.1} Gluten comprises two main subunits, gliadins (globular prolamins) and glutenins (fibrous glutelins). After hydration with water, and oxidation of cysteine residues within each subunit, glutenins and gliadins form a network stabilized by inter- and intramolecular disulfide bonds. This protein mass is distributed throughout the dough by kneading.

\textit{Adapted from Fasano 2011}\textsuperscript{31}
After kneading and leavening, the viscoelastic network of gluten traps air to give volume and porosity in baked goods. The white “strands” shown in this image are made up of gluten and the open pockets shown are able to trap CO$_2$ produced during yeast fermentation. *Photo by Kelsey Tenney © 2015*

Gluten has also been used as a functional additive in a variety of food and non-food products. It can be commonly found as a thickener or binder in products like salad dressings, sauces and even meat products.$^{32}$ Notably, isolated wheat gliadin has also been explored as a delivery system for environmentally sensitive bioactive compounds like resveratrol through the formation of nanoparticles.$^{33}$ However, despite its importance in the quality of baked goods and utility as a functional ingredient, sales of products that are conspicuously formulated and marketed as “gluten-free” grew 34% between 2009 and 2014, is expected to slow only slightly to 19.2% growth by 2019.$^{34}$ The motivation to exclude gluten from food products is driven by consumer demand. While 19% of gluten-free consumers cite weight loss goals, 37% of consumers claim “better overall health” as their main reason for avoiding gluten.$^{35}$ According to the 2009-2014 National Health and Nutrition Examination Survey (NHANES) study, these individuals account for 0.61% of
the population. However, for another 1.08% of people surveyed by NHANES, the reason for avoiding gluten is much more serious, as gluten is the environmental trigger for the autoimmune digestive disorder celiac disease.

1.3 Celiac Disease

Celiac disease is one of the most prevalent autoimmune diseases in the world, affecting approximately 1% of the population in both Europe and the United States. According to the 2009-2010 NHANES, overall American prevalence is 0.71%, or 1 in 141 people. There is a notable discrepancy in prevalence between racial groups, as celiac disease is more likely to affect non-Hispanic whites at a rate of 1%. These statistics include individuals who were undiagnosed prior to this study, which highlights the unique issue of under-diagnosis that is encountered with celiac disease. While most food allergies and hypersensitivities result in immediate and visually recognizable responses ranging from minor (e.g. skin rash, flushing, coughing) to severe (e.g. low blood pressure, vomiting, anaphylaxis), some individuals with celiac disease are asymptomatic, or symptoms such as gastrointestinal upset are simply overlooked and not thought of as a reason to seek medical assistance. The result of this is reflected in the 2009-2010 NHANES study, where 35 of the individuals surveyed were found to have celiac disease according to serologic testing, yet 29 of those individuals were not aware of their diagnosis. Understanding of the magnitude of this disorder has continued to grow over the last decade as prevalence has been revealed thanks to large epidemiological studies like the one previously described, as well as improved serologic tests employing the use of the most up-to-date methods of disease identification.
Despite the development of serologic tests in the 1980s and ‘90s, the only reliable way to definitively determine whether an individual has celiac disease is a small bowel endoscopy and biopsy.\(^{41}\) Coupled with the non-specific symptoms associated with celiac disease (e.g. gastrointestinal upset, bloating, diarrhea), diagnosis of celiac disease is often delayed; in 2001, the reported average time between initial recognition of symptoms and diagnosis of celiac disease was 11 years.\(^{5}\) Interest in celiac disease from a public health perspective has increased as prevalence rates have increased five-fold over the last 30 years.\(^{42}\) However, as of 2017 there is no known cure for celiac disease, and the only reliable treatment is lifelong adherence to a gluten-free diet.

1.3.1 Risk Factors

1.3.1.1 Genetic Susceptibility

Celiac disease is dependent upon both genetic and environmental factors, though as of 2017, the link between the two is still not completely understood. Investigation into a genetic risk factor for celiac disease was motivated by increased disease rates for siblings and children of individuals with celiac disease.\(^{43}\) Evidence for a genetic predisposition for celiac disease exists, with nearly 100% of individuals with celiac disease possess the genotype for class II human leukocyte antigen (HLA) DQ2 (DQA1*0501-DQB*0201; 90%) or DQ8 (DQA1*0301-DQB*0302; 8-10%).\(^{44,45}\) The significance of genetic predisposition is highlighted by a study of genetic markers for celiac disease in Italian children, where it was determined that the likelihood of individuals with DQ2 or DQ8 having celiac is 1:7, while likelihood of disease for someone without either predisposing factor is 1:2518.\(^{43}\)
Despite these findings, celiac disease cannot be entirely attributed to the HLA-DQ2/8 haplotypes, as HLA-DQ2 has a prevalence of approximately 30% in the Caucasian population compared to the 1% prevalence of the disease. This suggests that the HLA-DQ2/8 haplotype, while a prerequisite, is not independently sufficient for disease development. The frequency of other genetic variants in celiac populations have been explored with the goal of characterizing addition genetic linkages. Celiac disease risk is increased in populations with other autoimmune disorders. These associations have led to the exploration of variants in genes on the 2q33 (CELIAC3) chromosome-CD28, CLTA4 and ICOS. Related to the regulation of T-cell immune responses, the genes in this region of CELIAC3 have been implicated as risk factors for type-1 diabetes, multiple sclerosis, asthma, autoimmune hypothyroidism and Graves’ disease. Rather than differences in individual variations, strong associations between celiac disease and the CLTA4 haplotype have been documented, with multiple studies concluding that the over-reactive immune response to gluten seen in celiac disease may arise in part from underexpression of CLTA4.

1.3.1.2 Environmental Influence

Gluten ingestion is a known environmental trigger for celiac disease, but the prevalence of individuals who are genetically susceptible for development of celiac disease without having an adverse reaction to gluten suggests that other factors influence the disease. Prevalence of celiac disease over time has outpaced the increase in number of cases that could be attributed to more sensitive diagnostic tests, as well as increases that could be due solely to genetic changes in the population. Moreover, the increases in
celiac disease prevalence coincide with an increase in the prevalence of many other autoimmune disorders including inflammatory bowel disease, type-1 diabetes and multiple sclerosis.\textsuperscript{52–54}

Many hypotheses relating to the environmental influences of celiac disease development are related to human development, particularly childhood feeding habits and antigen exposure. For many years, it was thought that the time of introduction to gluten in the diet could influence whether a genetically susceptible child would develop celiac disease or not. A “window of tolerance” hypothesis was therefore developed, wherein it was thought that introducing children to gluten during breastfeeding between four and seven months of age would promote tolerance of gluten proteins. Initially investigated in Sweden, this hypothesis gained traction in the United States after a study, later argued to be statistically underpowered, noted that children at risk for type-1 diabetes were less likely to develop celiac disease after having gluten introduced into their diet during the “window of tolerance”.\textsuperscript{42} These findings were further supported by a similar study carried out in German children at-risk for type-1 diabetes.\textsuperscript{55} However, recent randomized and controlled trials do not support the “window of tolerance” hypothesis, and have instead demonstrated that time of introduction to gluten has no effect on development of celiac disease regardless of genetic susceptibility or age of the children involved.\textsuperscript{42,56,57} Within these studies it was also determined that the dose of gluten administered to children also has no effect on disease development.\textsuperscript{57} Further, despite its numerous other benefits to infant health, breastfeeding had no effect on a child’s susceptibility for developing celiac disease.\textsuperscript{56,57} Overall, recent studies do not support the idea of childhood dietary patterns directly influencing celiac disease development.
Another popular theory regarding the influence of the environment on celiac disease development is the “hygiene hypothesis”, wherein it is speculated that decreased antigenic load and frequency of infections during childhood may cause immune system imbalance and over-activity later in life. This theory was developed based on the inverse relationship between frequency of infectious diseases such as measles, tuberculosis and mumps and the frequency of autoimmune disorders such as type-1 diabetes, asthma, multiple sclerosis and celiac disease. In developed countries where the greatest incidence of celiac disease is observed, there is also more widespread use of antibiotics and decreased exposure to microorganisms.

However, data supporting the relationship between intestinal infection and celiac disease development contradicts the hygiene hypothesis, as both viral and bacterial infections have been shown to increase celiac disease development risk in infants and adults. In a study of Swedish children born between 1987 and 1997, neonatal infection was found to be one of the main risk factors for developing celiac disease. Specific viral infections that have been linked to celiac disease are adenovirus, hepatitis C, reovirus and rotavirus. Adenovirus type 12 has been implicated as a trigger for celiac disease, and it is most often found in the intestinal tract. One study showed that 89% of people with untreated celiac disease tested positive for adenovirus type 12 antibodies compared to 30-33% of people with treated celiac disease and 0-12.8% of control subjects. However, these results have not been consistently repeatable and a causative link between adenovirus infection and celiac disease development has not been proven. Patients with chronic hepatitis C have been shown to be twice as likely to have celiac
disease than the general population, making hepatitis C the most common liver disease associated with celiac disease development.62

Reoviridae are another infectious agent that has been shown to have a strong link to celiac disease development. Individuals with celiac disease tend to have higher anti-reovirus titres compared to control patients. Further, celiac disease patients following a gluten-free diet expressed higher levels of interferon regulatory factor 1 (IRF1) in the small intestine, which suggests irregular immune homeostasis. The persistence of IRF1 overexpression despite clearing reovirus infection and adhering to a gluten-free diet suggests that reovirus infection in combination with genetic risk factors for celiac disease may result in the development of the overactive immune response to gluten.59

Compelling evidence linking celiac disease development to rotavirus also exists. Rotavirus infection is one of the most common causes of childhood gastroenteritis worldwide, resulting in increased inflammation and permeability as well as structural changes to the epithelium. Rotavirus has also been implicated in the development of type-1 diabetes. In a longitudinal study of genetically predisposed children two years old and younger, it was found that two or more cases of rotavirus infection significantly increased the risk of developing celiac disease.60 The fact that rotavirus has been shown to alter the intestinal mucosa and is associated with celiac disease risk is similar to clinical findings in a case study where a two-year-old boy presented symptoms of celiac disease including high IgA and IgG anti-gliadin antibodies and diarrhea. Upon biopsy, he was shown to have increased intestinal epithelial lymphocytes (IELs) and partial atrophy of intestinal villi. Stool samples revealed that he was also suffering from Giardia lamblia infection. Interestingly, after clearing the giardiasis, the child’s celiac disease symptoms subsided,
even upon reintroduction of gluten to the diet. These findings support the previous notion that compromised mucosa from an infection may have an impact on the state of celiac disease, but in this case, the impact of *G. lamblia* infection did not seem to have long-term repercussions.

While a majority of studies relating intestinal infection to celiac disease development have been carried out with children as subjects, infection with other pathogens have been linked to the loss of immune tolerance to gluten in adults. In one notable case study, a 29-year old woman experienced continuous gastrointestinal discomfort for over five years after infection with *Campylobacter jejuni*. Though initially diagnosed with post-infective irritable bowel syndrome, the patient was eventually screened for celiac disease where she tested positive for several celiac disease serological markers and her biopsy showed elevated IELs and partial villous atrophy. She was re-tested one year later after following a gluten-free diet and showed recovery from all symptoms.

Pathogens are not the only microorganisms that may play a role in the development of celiac disease. A growing body of evidence suggests that gut microbiota influences immune homeostasis. As gut-associated lymphoid tissue (GALT) makes up 70% of the immune system, changes in microbial populations have the potential for downstream extraintestinal manifestations including decreased protection against toxins or pathogens, malnutrition due to impaired nutrient absorption and hypersensitivity to dietary antigens. It has been shown that individuals with celiac disease have altered gut microbiota composition and function compared to healthy individuals, regardless of their state of diagnosis. Similar to celiac disease itself, the development of this
dysbiosis is thought to be influenced by both genetic and environmental factors. Early gut microbiota composition of infants with the HLA-DQ2 haplotype have been shown to differ from that of non-susceptible infants, with at-risk individuals showing higher levels of Firmicutes (Clostridium spp.) and Proteobacteria (Enterobacteriaceae), but lower levels of Actinobacteria (Bifidobacterium spp.). More clinical studies are needed to determine whether differences in gut microbiota cause celiac disease development or arise as a result of the disease.

As is the case with many other chronic and autoimmune diseases, there does not seem to be a singular cause for celiac disease development. Celiac disease etiology is influenced by complex interplay between a variety of genetic risk-factors and environmental conditions. Because of this, many of the strategies for treating celiac disease that have been explored to date deal with pathogenesis and symptom management rather than preventing disease development. The multi-step pathway of celiac disease pathogenesis provides many potential therapeutic targets.

1.3.2 Pathophysiology

Protein digestion takes place in the small intestine as luminal and brush border enzymes are secreted, hydrolyzing enzymes into free amino acids and di- and tripeptides by targeting specific cleavage sites dependent on the enzyme. Gluten, particularly gliadins, is exceptionally resistant to enzymatic hydrolysis during digestion in all individuals, regardless of whether they suffer from celiac disease or not. Digestive enzymes produced by the stomach, pancreas and brush border lack the ability to fully hydrolyze the proteins with high frequencies of proline residues, a characteristic of gluten
proteins. The incomplete enzymatic hydrolysis of gluten results in larger polypeptide digestive products, which have been shown to stimulate a host of deleterious effects on the small intestine including cytotoxicity, immunomodulation and gut permeation.\textsuperscript{31} One notable digestive product of $\alpha$-gliadin is a 33-amino acids peptide that has been heavily studied for its dominant role in stimulating the celiac disease immune response. With the sequence LQLQPFQPQLPYPQPQLYPQPQLYPQPQPF, this peptide features six overlapping epitopes for enzymatic binding and immune recognition, which will be subsequently discussed in section \textbf{1.3.2.2 The Adaptive Immune Response}.\textsuperscript{21,70}

For individuals without celiac disease, these larger digestive fragments of gluten protein enter the mucosa via enterocyte endocytosis. As their transport occurs through lysosomes, these fragments undergo further digestion and are released to the lamina propria as free amino acids and small non-immunogenic peptides. In cases where these peptides stimulate IgA release before crossing the brush border, the gliadin-IgA complexes are likely trapped in mucus and excreted rather than absorbed and digested.\textsuperscript{71}
Figure 1.3 Schematic representation of celiac disease. Numbers (i-v) correspond to 1.3.2.1 Luminal and Early Mucosal Events. Gliadins are first (i) enzymatically hydrolyzed into smaller digestive peptides. These peptides (ii) interact with the CXCR3 receptor on the surface of intestinal epithelial cells to stimulate intestinal permeability through the production (iii) zonulin via the MyD88 pathway. Zonulin (iv) disrupts tight junction proteins, stimulating intestinal permeability. This also (v) stimulates damage to the epithelial cells, which secrete proinflammatory cytokines and become infiltrated with intraepithelial lymphocytes. Numbers (vi-x) correspond to 1.3.2.2 The Adaptive Immune Response. Cellular damage stimulates the secretion of (vi) enzyme tissue transglutaminase 2, which deamidates glutamine residues on gliadin in the lamina propria. After deamidation, (vii) antigen presenting cells recognize gliadins and (vii) present the antigens to helper T cells. The helper T cells (ix) recruit and activate killer T cells to attack epithelial cells producing stress signals, which B cells product antibodies against gliadin and tissue transglutaminase 2.
1.3.2.1 Luminal and Early Mucosal Events - The Innate Immune Response

For individuals who do have celiac disease, undigested fragments of gliadin cross the brush border via transcellular and paracellular mechanisms, but do not get further processed by the gut into free amino acids or non-immunogenic peptides.\textsuperscript{71–73} One mechanism of gliadin transport is protected transcytosis due to abnormal expression of CD71 in the enterocytes of individuals with celiac disease. CD71 has been identified as a recycling receptor, protecting transferrin from lysosomal degradation during endocytosis. Studies have shown that CD71 elicits a similar effect on gliadin during transcytosis upon complexation with IgA, blocking the further degradation of immunogenic peptide fragments and allowing them to enter the lamina propria intact.\textsuperscript{74}

Paracellular leakage of gliadin peptides into the lamina propria has also been explored.\textsuperscript{31,73,75,76} Studies have shown that gliadin digestive products are able to bind to the luminal chemokine receptor CXCR3, the expression of which is elevated in active celiac disease.\textsuperscript{73} Binding to this receptor stimulates the recruitment of myeloid differentiation factor 88 (MyD88). MyD88 induces the release of pro-inflammatory cytokines, particularly interleukin (IL)-15, which signals for lymphocytes to infiltrate the submucosa.\textsuperscript{77}

IL-15 is a pro-inflammatory cytokine that is influenced by innate immune signaling in the gut.\textsuperscript{78} Though dysregulated expression of IL-15 has been implicated in many inflammatory autoimmune diseases,\textsuperscript{79} it is considered to be the “hallmark of celiac disease” due to chronic upregulation in the intestinal epithelium and lamina propria.\textsuperscript{78} Overexpression of IL-15 interferes with normal immune homeostasis by preventing TGF-\(\beta\) from suppressing T cell activation.\textsuperscript{80} As a result, the intestinal mucosa of individuals
with active celiac disease features abnormally high levels of intraepithelial lymphocytes (IELs). These IELs contribute to villous atrophy and overall mucosal damage observed in active celiac disease by killing epithelial cells producing stress signals, including IL-15. Though IL-15 can also be produced by dendritic cells in the lamina propria, IL-15 production by intestinal epithelial cells has been shown to be a prerequisite for the villous atrophy observed upon gluten challenge in a HLA-DQ8 mouse model. As such, preventing gliadin-mediated inflammation and IL-15 production may be an effective strategy for mitigating intestinal damage.

During celiac disease pathogenesis, MyD88 also signals the production of zonulin by intestinal epithelial cells (IECs). An endogenous mediator of intestinal barrier function, zonulin can trigger the disassembly and downregulation of intercellular junction proteins between IECs by directly activating the epidermal growth factor receptor or indirectly via proteinase-activated receptor 2. Zonulin acts rapidly and reversibly, explaining both the rapid onset time of symptoms after gluten challenge and the remission of mucosa damage and gastrointestinal discomfort when a gluten-free diet is maintained.

Tight junctions (TJ) and adherent junctions (AJ) are two classes of intercellular structures found in polarized epithelial cells such as those in the intestinal mucosa. They serve as molecular gates to the body, regulating the transport of nutrients and protecting the body from antigens, toxins and macromolecules (Figure 1.4). TJs are closest to the apical membrane of the cell and include claudins and occludins. These transmembrane proteins are linked to cytoskeletal actin via TJ-associated proteins, zonula occludens. TJs also feature junctional adhesion molecules (JAM). AJs, which include cadherins, are
necessary for the formation of TJs and are located beneath TJ complexes. TJs and AJs are
dynamic structures within the mucosa, able to be modified by a variety of stimuli
including nutrients, cytokines, toxins and pathogenic bacteria.82

Disassembly of TJs and AJs occurs via vesicular transport of proteins from the
cell membrane to alternative locations within the cell, or by rearrangement of the actin
cytoskeleton. Gliadin-stimulated zonulin release has been shown to alter junctional
properties of intestinal epithelial cell lines IEC-6 and Caco-2, altering the localization of
TJ proteins claudin-3 and -4 and causing the polymerization of actin filament.83,84
Exposure of gliadin also downregulates the expression of some TJ and AJ proteins,
including E-cadherin, occludin and zonula occludins-1 (ZO-1).83 These alterations to
junctional proteins decrease the functionality of the intestinal barrier, increasing
permeability and allowing leakage of small molecules into the lamina propria. This
increase in paracellular flux provides an alternative route for gliadin to cross the brush
border.31 Intestinal permeability is often referred to colloquially as “leaky gut”, and has
been implicated in a variety of autoimmune disorders wherein non-self-antigens other
than gliadins are able to enter the lamina propria and elicit an immune response.85
Figure 1.4 Intercellular junction proteins and junction-associated proteins. TJs (claudin, occludin, ZO-1/2/3) and AJs regulate the paracellular transport of nutrients past the brush border of the intestines. Also featured are JAM family proteins, an additional class of TJ proteins. Adapted from Visser et al.55
1.3.2.2 The Adaptive Immune Response

Upon crossing the brush border and entering the lamina propria, gliadin peptides encounter an endogenous enzyme called tissue transglutaminase 2 (TG2), which is released from cells during inflammation.\textsuperscript{86} TG2 is ubiquitous in the human body and has been implicated in a variety of biological processes including extracellular matrix formation, cell differentiation and signal transduction, though its most common role in the intestinal mucosa is tissue repair.\textsuperscript{87,88} Typically, TG2 catalyzes covalent crosslinking between proteins via isopeptidyl bonds between glutamine and lysine.\textsuperscript{86} Notably, it has shown specific affinity for glutamine residues located one amino acid away from a proline residue- a pattern that is heavily conserved within the proline and glutamine rich digestive products of gliadin.\textsuperscript{70} Early studies implicating TG2 in celiac disease pathogenesis showed that jejunal tissue samples taken from individuals with treated and untreated celiac disease demonstrated increased TG2 activity compared to healthy controls.\textsuperscript{89} TG2 plays two important roles in pathogenesis, the first being related to the presentation of gliadin as an antigen. TG2-catalyzed deamidation of glutamine to glutamic acid within gliadin peptides results in a change in charge of the peptide from neutral to negative. This negative charge increases the affinity for gluten peptides to form major histocompatibility complex (MHC) class II complexes with HLA-DQ2 and HLA-DQ8 molecules on antigen presenting cells (APCs).\textsuperscript{70} The second role of TG2 in celiac disease pathogenesis is critical in the classification of celiac disease as an autoimmune disorder when, up to this point, the immune response has been centered on dietary antigens. When TG2 binds to gliadin for deamidation, it forms a transient TG2-gliadin complex. This complex can be recognized as an antigen by HLA-DQ2 and HLA-DQ8.
MHC class II molecules in a similar fashion to the recognition of the peptide alone. However, in this case, TG2 is also recognized and presented as an antigen despite its endogenous production by epithelial cells.

The adaptive immune response associated with celiac disease is initiated by MHC class II molecules on the surface of APCs in the lamina propria. Tissues taken from individuals with active celiac disease have increased numbers of APCs compared to healthy controls, but can return to normal levels with a gluten-free diet. MHC class II molecules found on the surface of these APCs are able to bind to both deamidated gliadin as well as gliadin-TG2 complexes, presenting each as antigens to naïve T cells. MHC class II molecules have been found to preferentially bind to the main chain of peptide ligands through hydrogen bonding, as opposed to MHC class I molecules which bind to N and C termini of peptides.\textsuperscript{90,91} Interestingly, MHC class II molecules demonstrate affinity for polyproline II helical peptides and the P6 binding pocket of DQ2 preferentially and tightly binds proline residues.\textsuperscript{92} These structural preferences of HLA-DQ2 make the proline and glutamine-rich fragments of gliadin that transcend the brush border, which also tend to form polyproline II helices, attractive targets for binding and forming MHC class II complexes.\textsuperscript{93} Recalling the aforementioned highly immunostimulatory 33-amino acid peptide produced by enzymatic hydrolysis in the lumen, there are several amino acid sequences that are recognized as epitopes in MHC class II complex formation (\textbf{Figure 1.5}).
Figure 1.5 Gliadin 33-mer featuring 3 glutamine residues capable of being deamidated by TG2 and 6 overlapping epitopes recognizable by MHC class II molecules. Adapted from Qiao et al.70

Presentation of gliadin and gliadin-TG2 by MHC class II molecules activates T helper (Th) cells in the lamina propria.94 Upon recognition of gliadin and TG2, gliadin-reactive CD4+ cells follow the Th1/Th0 pathways and release pro-inflammatory cytokine interferon (IFN)-γ, which activates signal transducer and activator of transcription 1 (STAT1) and IRF1.95 IL-10, an anti-inflammatory cytokine, is also upregulated in active celiac disease, though the ratio between IL-10 and IFN-γ is lower in active celiac disease than treated or healthy controls, suggesting that IFN-γ is of greater importance to the disease state.96 IFN-γ is responsible in part for mucosal damage—degradation of the extracellular matrix and basement membrane of epithelial cells—by stimulating the release and activation of matrix metalloproteinases (MMP).77,97 IL-1β and TNF-α have been shown to contribute to extracellular matrix degradation by activating MMP-1 and -3.98,99 Mucosal damage is also carried out by CD8+ cytotoxic T cells, which are stimulated by the release of cytokines from the CD4+ T cells. These T cells cause the formation of mucosal lesions in the small intestine by stimulating apoptosis in the enterocyte through production of cytotoxic compounds Fas ligand and granzyme.77 CD4+ T cells further propagate the immune response by activating effector B cells, or plasma cells. These plasma cells produce anti-gliadin and anti-TG2 IgA and IgG antibodies. Dissimilar to most other instances of T cell dependent activation of B cells, B cell activation in celiac disease does not result in the production of memory B cells. As a
result, gliadin and TG2 antibodies disappear from circulation after approximately one month of following a gluten-free diet.\textsuperscript{100}

1.3.3 Clinical Features

1.3.3.1 Gastrointestinal Manifestation

Presentation of symptoms of celiac disease function on a gradient of exposure. For some individuals with celiac disease, as little as 50 mg of gluten per day is enough to elicit a physiological response\textsuperscript{101} in the blanket form of “gastrointestinal discomfort”, including diarrhea, abdominal pain, bloating, and constipation.\textsuperscript{44} Persistent diarrhea is the primary identifying symptom of celiac disease among adults, with 50\% of cases reporting the symptom.\textsuperscript{44} As of 2017, the absence of cure for celiac disease means that even when an individual has adhered to a gluten-free diet for a significant amount of time, inadvertent ingestion of gluten will result in the aforementioned physiological response. Other physical symptoms result from long-term gluten exposure.

1.3.3.2 Nutritional Deficiency and Growth Problems

One notable feature of active celiac disease is impaired absorption of macro- and micronutrients. Steatorrhea, a direct result of fat malabsorption, has been noted in up to 20\% of newly diagnosed cases of celiac disease.\textsuperscript{102} Additionally, according to a 2013 study of 80 Dutch adults, up to 87\% of untreated patients with celiac disease were deficient in at least one vitamin or mineral. Folate and vitamins A, B\textsubscript{6}, and B\textsubscript{12} were among the most common vitamins that celiac disease patients were deficient in, along with zinc and iron as minerals of common deficiency. Further, 32\% of the study
participants were anemic. Interestingly, these deficiencies did not correlate with degree of histological abnormalities, suggesting that absorption of nutrients can be impaired regardless of the severity of the disease state.\textsuperscript{103} Other nutritional issues associated with celiac disease are reduced bone density, arthritis and dental problems due to enamel defects.\textsuperscript{104}

Often, celiac-related nutrient deficiencies can lead to undesired weight loss in adults, though the impact that celiac disease can have on growth is most often apparent in children and adolescents. In fact, some of the earliest recorded cases of celiac disease in the 1800s describe children who, in addition to experiencing abdominal distention, constipation, diarrhea and vomiting, were also anemic and small for their ages. The common symptom of “failure to thrive” inspired the early name for the disease- “celiac infantilism” or “intestinal infantilism”.\textsuperscript{105} In modern times, children with celiac disease still display decreased growth velocity in terms of both height and weight; in 2004, 8.3% of children evaluated for short stature were diagnosed with celiac disease. Though not associated with growth and stature, delayed puberty has also been noted in many adolescents with celiac disease.\textsuperscript{44,104} Delayed puberty in individuals with celiac disease is well-documented, but the mechanism is not fully understood. Hypothesized explanations include targeting of hormones or endocrine organs by autoimmune factors or malabsorption of micronutrients important to sex hormones.\textsuperscript{104}

\textbf{1.3.3.3 Extraintestinal Manifestations and Associated Conditions}

Abnormal endocrine function is one of several conditions that has been associated with celiac disease.\textsuperscript{104} It has been noted in an increased rate of thyroiditis in adults with
celiac disease and increased rates of insulin-dependent diabetes in children with celiac
disease.\textsuperscript{106} Often, these conditions are monoglandular- for instance, adrenal insufficiency,
or pituitary disease- but the number of polyglandular syndromes have increased over the
last several years.\textsuperscript{106}

Celiac disease symptoms have been shown to manifest in organ systems other
than the obvious gastrointestinal tract and the aforementioned endocrine system. One
target organ of note is the skin. Dermatitis herpetiformis is a skin condition that was
noted in some of the earliest recorded cases of celiac disease.\textsuperscript{105} Characterized by
symmetrical vesicular rashes on the trunk and extremities, diagnosis can be confirmed via
biopsy, which reveals deposits of granular IgA. This occurs as a result of TG2 antibodies
binding to a skin transglutaminase, tissue transglutaminase 3.\textsuperscript{104,107,108} In addition to
dermatitis herpetiformis, eczema and psoriasis often present as associated conditions with
celiac disease.\textsuperscript{104,107}

An extraintestinal manifestation of celiac disease that makes up 50\% of the
reported neurological conditions associated with the disease is gluten ataxia, or
discoordination of limbs and movement as a result of an overactive immune system and
deposition of antibodies in the brain targeting Purkinje cells.\textsuperscript{104,109} Contrary to typical
celiac disease and related symptoms, gluten ataxia is often not reversible, especially after
advanced progression of the disease. Other associated neurological conditions include
autism, schizophrenia and anxiety, each of which require further controlled studies to
elucidate the links between those conditions and celiac disease.\textsuperscript{104}

Links have been made between celiac disease and a variety of other organs
including the spleen, liver, kidneys and pancreas. As an important factor in the immune
system, splenic function is often disrupted in active celiac disease, with 30% of undiagnosed patients demonstrating hyposplenism or asplenism. As previously mentioned, there is a strong link between hepatitis C diagnosis and celiac disease, associating celiac disease with liver dysfunction. Many celiac disease patients display elevated liver enzyme levels, which is suggestive of inflammation or injury of the liver. This coincides with celiac disease patients’ increased risk for liver failure (2-6 fold) and death due to cirrhosis (8-fold increase). However, the underlying mechanism for these problems is currently unclear. Kidney problems related to celiac disease include increased risk for glomerulonephritis and renal disease, and pancreatitis and exocrine insufficiency have been shown to commonly affect individuals with celiac disease as well. Interestingly, both of these conditions are often also related to type-1 diabetes, which is a condition commonly associated with celiac disease.

Celiac disease has many associated conditions or comorbidities in addition to type-1 diabetes, many of which are also related to impaired immune function or genetic mutation in similar areas of the chromosome. Some of these include autoimmune thyroid disease, Turner syndrome, Down syndrome and IgA deficiencies. These additional comorbidities as well as asymptomatic celiac disease cause inconsistent symptom presentation among many individuals, which greatly complicates diagnosis. In fact, in many cases, individuals with celiac disease do not know that they have been suffering from the disease until participation in studies that involve apparently healthy individuals, wherein histological screening reveals mucosal alterations associated with celiac disease. This asymptomatic manifestation is often referred to as “silent” celiac disease, though post-hoc interviews about general well-being often reveal that individuals with silent
celiac disease were suffering from celiac-related illnesses such as iron deficiency, fatigue, reduced bone mineral density and behavioral disturbances such as depression or irritability.\textsuperscript{111}

1.3.4 Diagnosis

The myriad non-specific symptoms associated with celiac disease has made diagnosis based on symptom presentation alone extremely difficult, which results in many individuals living with undiagnosed celiac disease. However, diagnosis rates have improved with improved understanding of the disease itself as well as development of more specific diagnostic tests. The general process flow of celiac disease diagnosis is outlined in Figure 1.6.

![Celiac disease diagnosis algorithm](Adapted from Barker and Liu.\textsuperscript{44})
1.3.4.1 Serologic Testing

Patients demonstrating classic symptoms of celiac disease will often first undergo one or more serologic tests, wherein the patient’s serum is tested for circulating antibodies that have been implicated in celiac disease, each of varying sensitivity and specificity.\(^{44}\) The preferred and most sensitive of tests available is for IgA antibodies for TG2. An alternative test can be used to detect anti-TG2 IgG if the patient is IgA deficient, which is commonly seen in celiac disease.\(^{44,112}\) Each of these tests have demonstrated 95-98% sensitivity with 94-98% specificity.\(^{44}\) In addition, less specific tests include those for anti-endomysial antibodies, which are produced in the small intestine, as well as anti-gliadin antibodies. However, each of these options are less sensitive than those against TG2.\(^{44}\)

1.3.4.2 Abnormal Histology

Despite the role of serologic testing in improving the diagnosis rate of celiac disease, observation of the mucosal architecture of the small intestine via endoscopy and biopsy is considered to be the “gold standard” for celiac disease diagnosis, as it allows for determination of the severity of the disease with near 100% specificity for some mucosal alterations. Notable changes in histology are surface damage to enterocytes, infiltration of IELs and the blunting of villous structure- crypt hyperplasia and villous atrophy- which results in the loss of small intestinal surface area.\(^{113}\)
Endoscopies can provide valuable information to clinicians by allowing visualization of the small intestinal mucosa and subsequent identification of features that are characteristic of celiac disease (Figure 1.7). Common markers of damage are a reduction of Kerckring’s folds, scalloping of duodenal folds and the development of a mosaic pattern or micronodules on the mucosal surface. Endoscopic markers are less sensitive as primary diagnostic tools than serologic tests at 47-100% accuracy, as less severe intestinal damage can be difficult to visually detect. Interestingly, the sensitivity of each endoscopic marker is inversely related to its specificity with loss of folds being the most sensitive, present in 97% of celiac disease patients, but also the least specific at 47%. This is compared to the mosaic and nodularity, present in 6 and 12% of patients, respectively, but each showing specificity of up to 100%. With all factors considered, despite not being as sensitive as serologic testing, observable signs of celiac disease upon endoscopy is highly specific for diagnosis (92-100%).

Figure 1.6 Small bowel endoscopies of (a) healthy and (b) celiac disease patients. Distinct histological differences are visible in the form of scalloping in (b). From Presutti et al. 2007.
Endoscopy is often paired with a duodenal biopsy, or removal of tissue from the duodenum in four different areas and examined under a microscope by a histopathologist. The clinical criteria for scoring the severity of intestinal damage has been broken down into two systems: (1) Marsh-Oberhuber,\textsuperscript{118,119} the traditional system, and (2) the simplified Corazza-Villanacci system.\textsuperscript{120} The Marsh-Oberhuber system, or Modified Marsh Classification, utilizes four criteria- two quantitative and two qualitative markers relating to IEL frequency and villi/crypt architecture, respectively, to make up six separate histology scores.\textsuperscript{119} While robust and still widely-accepted as a histological scoring method, its repeatability has been questioned, leading to the development of a simplified system made up of only three classifications (Figure 1.8).\textsuperscript{119,120}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{histological_scoring.png}
\caption{Comparison of histological scoring criteria in the Marsh-Oberhuber system and the Corazza-Villanacci system. While robust and quantitative, the Marsh-Oberhuber system has shown low reproducibility and is more labor intensive than the simplified version proposed by Corazza and Villanacci.}
\end{figure}
As celiac disease is characterized by a heightened immune response, celiac disease patients often demonstrate a greater number of intraepithelial lymphocytes in the mucosa compared to healthy controls.\textsuperscript{121} One of the main criticisms of the Marsh-Oberhuber scoring system is the labor-intensive counting IELs per 100 enterocytes in histology samples, though criticism also exists regarding the enumeration of IELs at all, as even after over two decades of using Marsh scoring, pathologists have not reached consensus on what a “normal” IEL count is for the small intestine, with values between 12 and 40 IELs per 100 enterocytes having been considered.\textsuperscript{121} These inconsistencies contribute to the low reproducibility of the Marsh-Oberhuber system.\textsuperscript{120} The Corazza-Villanacci system, on the other hand, utilizes the qualitative aspect of the Marsh-Oberhuber system for a more simplified approach to diagnosis (\textbf{Figure 1.9}).

\textbf{Figure 1.9} Representative biopsies of histological scoring using each of the currently accepted scoring systems- Marsh-Oberhuber and Corazza-Villanacci. Each set demonstrates crypt hyperplasia and various degrees of villous atrophy used to make diagnostic decisions. \textit{Marsh-Oberhuber images from Polanco Allué 2011}\textsuperscript{122} and Corazza-Villanacci images from Corazza et al. 2007.\textsuperscript{120}
The severity of intestinal damage is measured by changes in the mucosal architecture of the small intestine—namely, crypt hyperplasia and villous atrophy. In a healthy small intestine, the surface area of the organ is increased by numerous folds. The surface is further increased by the formation of villi, vascularized projections on the surface of the folds that are lined by epithelial cells, creating the brush border of the small intestine. The epithelial cells themselves are physiologically distinct, with microvilli forming on the apical membrane. Increased surface area of the small intestine allows for more efficient nutrient absorption throughout the course of digestion. Intestinal crypts are indentations within the small intestine that comprise stem cells and are responsible for the renewal of brush border epithelial cells during normal epithelial shedding. In celiac disease, crypts can become hyperplastic due to increased proliferation of the cells they contain. In this case, the crypts become shallow and eventually disappear completely, leading to a flattening of the small intestinal surface. Similarly, the villi become atrophic as a result of the onslaught of inflammation and cytotoxicity stimulated by gluten ingestion, shortening at first and eventually wearing down to the point of the intestinal surface becoming completely flat.

1.3.5 Treatments and Therapies

As of 2017, the only reliable method for avoiding the symptoms and intestinal damage associated with celiac disease is adherence to a gluten-free diet. Despite the inconvenience and high associated costs of a gluten-free diet, it has been proven in case-controlled studies to be an effective strategy for the elimination of gastrointestinal symptoms. The earliest documented cases of celiac disease being mitigated by a gluten-
free diet were not intentionally gluten-free. In 1887, Dr. Samuel Jones Gee noted remission of gastrointestinal symptoms in child patients when fed a diet exclusively of oysters, followed by relapse upon introduction of a typical diet. In 1924, Dr. Sidney Haas reported similar findings after feeding eight children a banana-based diet which excluded grains. Gluten was identified as the dietary component of interest in celiac disease in 1953, a realization which was closely followed by dietary intervention studies confirming the findings. In an American study of 215 patients with celiac disease, 76% of participants reported the subsiding of abdominal pain and 41% reported decreased frequency of diarrhea after 6 months of a gluten-free diet. In a majority of these cases, symptoms subsided in less than one month on the gluten-free diet. Further, a gluten-free diet can reverse mucosal damage. In a prospective study of 65 Italian adults, 66% of the participants achieved full histological recovery with a Marsh-Oberhuber score of 0 after one year of adhering to the gluten-free diet and another 32% achieved partial recovery.

The downsides of the gluten-free diet include cost and nutritional implications. As discussed previously in this review, gluten-free versions of commonly consumed foods like cakes, breads and crackers can cost over double the price of their gluten-containing counterparts. Additionally, the nutritional implications of consuming a gluten-free diet have been called into question. One study tracking the diets of adolescents following a gluten-free diet showed macronutrient imbalance by excessive protein and fat consumption and low amounts of carbohydrates. Patients were also lacking in intake of fiber and interestingly, calcium and iron. Based on this finding, it appears that
following a gluten-free diet may not help resolve some of the nutritional deficiencies that patients present at the time of diagnosis.

Many strategies have been explored to help individuals with celiac disease maintain normal diets without the restrictive nature of the gluten-free diet. These strategies have come in the form of both synthetic and naturally-derived options, and target multiple stages of celiac disease pathogenesis by interacting directly with either the body or with gluten proteins (Figure 1.9, Table 1.1).

**Figure 1.10** Therapeutic targets for celiac disease. Protein-targeted therapies modify gluten from chemical and structural standpoints, whereas pathology-targeted therapies modify the immune system or the processing of gluten proteins by the body.
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<th>Identifier/Phase</th>
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**Table 1.1** Recent and ongoing clinical trials for novel therapeutics as of May 2017 (clinicaltrials.gov).
1.3.5.1 Pathology-Focused Therapies

1.3.5.1.1 Immunomodulation

As previously mentioned, the sensitization of the digestive system to gluten remains a largely misunderstood factor in the development and progression of celiac disease. Celiac disease is characterized by the upregulation of many facets of immune signaling, making immunosuppression a potential therapeutic strategy. Many believe that the microbial environment of the gut may play a role in the development of celiac disease, making it an interesting target for reversing the associated symptoms. This can be achieved through the diet by administering probiotics. A probiotic is defined by the FAO/WHO as a “live microorganism, which when administered in adequate amounts confers a health benefit on the host”\textsuperscript{130}. Some bacterial cultures have been shown to possess immunomodulatory activity within the context of celiac disease. \textit{Bifidobacterium bifidum} IATA-ES2 and \textit{B. longum} ATCC 15707, for example, have been demonstrated to suppress IFN-\(\gamma\) secretion by peripheral blood mononuclear cells (PBMCs) in the presence of gliadin. \textit{B. bifidum} IATA-ES2 was also shown to stimulate lower levels of IEC permeability than \textit{Escherichia coli} CBL2 and \textit{Shigella} sp. CBD8, which trigger TNF-\(\alpha\) and IFN-\(\gamma\) secretion\textsuperscript{131,132}. Data from clinical trials supporting the use of probiotics to treat celiac disease in humans have shown varied results, especially concerning the impact on histological outcomes. However, a clinical trial using \textit{B. infantis} showed reduced serologic markers after three weeks of supplementation alongside a gluten-containing diet compared to the controls, and patients noted an improvement of gastrointestinal symptoms\textsuperscript{133}. 
Bacteria are not the only microorganisms that are able to confer immunosuppressive effects that may be beneficial for individuals with celiac disease. With respect to the “hygiene hypothesis”, infection with helminths as parabiotic agents has been investigated in individuals with celiac disease. Particular attention has been paid to *Necator americanus*, a gastrointestinal nematode (hookworm) that has been shown to be well-tolerated by a majority of individuals with no deleterious effects to the intestinal mucosa or overall health of individuals in developed countries. In one *in vivo* study, hookworm infection reduced gluten-mediated IFN-γ in celiac disease patients and gluten challenge did not induce mucosal damage in the presence of infection. In 8 out of 12 participants, Marsh scores were either sustained or improved, suggesting that the immunosuppressive effects of *N. americanus* have the potential to be used to promote immune homeostasis in celiac disease patients.

Immunosuppression can also be achieved by non-biological or dietary factors, as demonstrated by clinical trials testing the efficacy of IL-15-mediating antibodies and T cell antagonists. Given the critical role of IL-15 in recruitment of IELs to the small intestinal mucosa and overarching impact on immune dysregulation, disruption of IL-15 signaling has been explored as an option for managing gluten-mediated mucosal damage. When mice overexpressing IL-15 were injected with TM-β1 antibody, the antibody blocked the IL-15 receptors on CD122 CD8⁺ T cells, resulting in a reversal of characteristically high levels of CD8⁺ T cells to levels similar to the non-transgenic control mice. TM-β1 treatment also improved villous architecture in treated mice, and resulted in macroscopic pathological changes including a reduced number of mucosal lesions in the small intestine and a reduction in splenic size. Functioning in a similar
fashion is vedolizumab, an α4β7 integrin antibody which has been studied in phase 2 clinical trials for its use in treating celiac disease, ulcerative colitis and Crohn’s disease with success by disrupting the trafficking of IELs in the gut without affecting other organ systems.137,138

A similar approach to reducing the immune response of celiac disease is employed by vercirron (previously CCX282-B), an orally administered chemokine (CCR) 9 receptor antagonist. CCR9 is a receptor expressed on CD4+ and CD8+ T cells that is involved with the recruitment and homing of those cells to the small intestine, which ultimately results in the destruction of the small intestinal mucosa during celiac disease.139 Vercirron has also been studied as a treatment for other autoimmune enteropathies including Crohn’s disease and inflammatory bowel disease (IBD) with success.140 Clinical remission in Crohn’s disease patients was achieved in a majority of patients in a phase II trial.140 In a murine model of TNF-ARE, vercirron protected against both inflammation and histological damage in 70% of mice.139,141 This treatment is unique in its striking specificity for CCR9 even in the presence of CCX-CKR, a receptor of the same chemokine ligand 25.142

Immunomodulation via tolerance development is an avenue of treatment that has been explored with NexVax2, a vaccine comprised of peptides recognized by CD4+ T cells in individuals with the HLA-DQ2 haplotype that is administered subcutaneously on a weekly basis.143 While Phase 1b clinical trials did not show complete desensitization, improvement of the celiac disease state was observed as decreased T cell mobilization.144
1.3.5.1.2 Antigen Presentation

The onset of the celiac disease-related adaptive immune response is reliant on the presentation of gliadin peptides as antigens. Inhibiting or otherwise impairing antigen presentation has been shown to be an effective strategy through several approaches. One approach focuses on impairing the processing of gliadin peptides required for antigen presentation. As previously discussed, deamidation of glutamine residues by TG2 greatly enhances the affinity of the peptide for the MHC class II binding pocket. One study found that incubation of gliadins with synthetic “blocking peptides” reduced TG2 activity in vitro.\textsuperscript{145} Synthesis of gluten epitopes with azidoproline in the place of two proline residues demonstrated the efficacy of a similar “blocking” approach at the antigen presentation level, as the modified epitopes were able to bind to HLA-DQ2 binding pockets and prevent T cell proliferation, though the binding was not competitive enough with unmodified gliadin to pursue in vivo testing.\textsuperscript{146} Binding optimization experiments have led to the development of synthetic peptides that are able to bind to HLA-DQ2 with 100-200 fold greater affinity than gliadin epitopes and modified versions of the immunodominant gliadin 33-mer, though they have demonstrated varying success in their ability to prevent T cell activation.\textsuperscript{147,148}

Direct inhibition of TG2 has been explored ex vivo with active-site TG2 inhibitor R283. Pre-treatment of tissue with R283 prevented gliadin-mediated T cell activation in duodenal biopsies, but the effect was diminished when gliadin had been deamidated prior to addition to the culture, suggesting that the prevention of deamidation was the critical factor at play.\textsuperscript{149} A similar approach has been taken with the pharmaceutical therapy zedira, or ZED1227, which has completed phase 1 clinical testing.\textsuperscript{150}
Beyond deamidation, the processing of antigens by MHC class II molecules is dependent on another enzyme called cathepsin S. A cathepsin S inhibitor, RG7625, has been approved for investigation in clinical trials to measure its efficacy for prevention of antigen presentation in celiac disease (clinicaltrials.gov).

1.3.5.1.3 Gut Barrier Function

Gut barrier function plays a key role in celiac disease pathogenesis, as paracellular transport is one means of partially digested gliadins passing the brush border and entering the lamina propria.\textsuperscript{31,73,75,76} For this reason, methods to combat celiac disease by mitigating gliadin-mediated intestinal permeability have been explored.

In addition to conferring immunomodulatory benefits, probiotic supplementation has also been shown to improve barrier function both \textit{in vitro} and \textit{in vivo}. Both \textit{B. longum} CECT 7347 and \textit{L. casei} ATCC 9595 have been shown to improve gut barrier function, reduce inflammation and repair gliadin-mediated intestinal damage in gluten-sensitized mice.\textsuperscript{151,152} Another lactic acid bacteria \textit{B. lactis} has demonstrated protective effects against gliadin-mediated IEC permeability and TJ disruption \textit{in vitro}. In addition to preserving transepithelial electrical resistance (TEER) of Caco-2 cell monolayers, \textit{B. lactis} appeared to prevent the disruption of TJ protein ZO-1 as observed by immunofluorescent microscopy. It has been hypothesized that this preservation of barrier integrity is due to modification of cyclooxygenase (COX) in epithelial cells. \textit{B. lactis} has been shown to upregulate expression of COX-1 while concurrently downregulating COX-2, a balance that favors the mucosal membrane maintenance while preventing inflammation.\textsuperscript{153}
Larazotide acetate (AT-1001) is a synthetic 8-amino acid peptide that preserves gut barrier function by acting as a zonulin antagonist and preventing the disruption of tight junction proteins stimulated by gliadin. In vitro, larazotide acetate has been shown to prevent gliadin-mediated permeability in Caco-2 cells by preserving tight junction proteins, an effect which also prevented the paracellular transport of FITC-labeled gliadin.\(^{154}\) Based on the idea that the paracellular transport of gliadin is a rate-limiting factor in pathogenesis, larazotide acetate has been shown to effectively prevent the destruction of IECs and associated immune response that occurs when gliadin is able to transcend the intestinal barrier. This has been demonstrated in both transgenic HLA-HCD2/DQ8 mice\(^{155}\) and in a number of clinical trials. While the primary outcome of the initial clinical trial (i.e., to increase lactulose to mannitol ratios in the urine signifying a change in barrier permeability) was not achieved, participants reported decreased severity of gastrointestinal symptoms upon gluten challenge and serologic testing showed that patients in the larazotide acetate group did not generate anti-TG2 antibodies. The absence of change in blood titres as well as sustained levels of IFN-\(\gamma\) suggest that larazotide acetate does prevent the passage of gliadin to the lamina propria,\(^{156}\) and complements findings from animal studies demonstrating its efficacy in preventing both TJ disruption and IEL infiltration.\(^{157}\)

1.3.5.2 Protein-Focused Therapies

One problematic aspect of most pathogenesis-focused therapies is the necessity for some steps of the adverse reaction to gluten to take place in order for the therapeutic target to become available. As previously described, the passage of immunostimulatory
peptides into the lamina propria stimulates inflammation and the infiltration of IELs to the mucosa as primary adverse effects in pathogenesis. While many of the pathogenesis-focused therapies target events after passage of gliadin to the lamina propria, a variety of protein-focused therapies exist, which focus on modifying the immunological potential of gluten prior to consumption and absorption.

1.3.5.2.1 Non-Toxic Wheat Cultivars

Simple removal of gluten from wheat, barley and rye would appear to be an obvious method for preventing adverse effects of gluten during digestion by individuals with celiac disease, but as discussed previously, gluten proteins play an important functional role in food quality. Alternative species of the *Triticum* genus other than *Triticum aestivum* (common wheat) have been explored for differential or reduced toxicity for celiac disease patients, which may arise from the absence of celiac-specific epitopes within gluten amino acid sequences. Attempts to find or generate a celiac-safe wheat have been met with variable results. Several ancient wheat strains have been shown to contain greater amounts of immunostimulatory proteins with increased immunogenicity over those which have been characterized in *T. aestivum*, but *T. monococcum* has been identified as a species of particular interest after genetic sequencing of different wheat cultivars to find naturally-occurring amino acid substitutions encoded for α-gliadins that decrease the immunogenicity of the resultant gliadin peptides. While early *in vitro* studies suggested α-gliadin substitutions of serine for proline within immunogenic residues reduced the stimulation of T cells, *ex vivo* and *in vivo* studies provided conflicting findings where both *T. monococcum* lines Monlis and
ID331 activated gluten-reactive T cells and the Monlis line induced IL-15 expression in biopsies of villi, crypts and the lamina propria.\textsuperscript{160} In vivo testing of the safety of T. monococcum for celiac disease patients revealed that, despite the relative successes of earlier trials, ingestion stimulated the generation of anti-TG2 antibodies and the formation of mucosal lesions over the course of 60 days.\textsuperscript{161}

1.3.5.2.2 Enzymatic Hydrolysis

The incomplete digestion of gliadin in the lumen has also been targeted as a therapeutic route with varying levels of success. Despite the proteolytic efficacy of Lactobacillus during sourdough bread fermentation to degrade gluten peptides further than typical processing procedures,\textsuperscript{162} no therapeutic outcome was detected when $\alpha$-gliadins processed through sourdough fermentation were exposed to TG2.\textsuperscript{163} However, orally administered enzyme supplements such as Aspergillus niger prolyl endopeptidase (AN-PEP) and two proteases from Sphingomonas capsulate (ALV003) have demonstrated prevention of histopathological changes upon gluten challenge in clinical trials.\textsuperscript{164,165}

1.3.5.2.3 Sequestration

In contrast to the method of assisting complete breakdown of gliadin, inhibition of luminal processing has also been shown to be an effective method for reducing immunogenicity, as demonstrated by synthetic polymer of hydroxyethyl methacrylate-styrene sulfonate, known as poly(HEMA)-co-SS or commercially as BL-7010.\textsuperscript{166} BL-7010 interacts with $\alpha$-gliadin at both gastric and intestinal pH levels, disrupting the
enzymatic hydrolysis of gliadins and preventing the formation of immunogenic and cytotoxic peptides.\textsuperscript{12,167} Upon interaction, BL-7010 also elicits a structural change on α-gliadin peptides.\textsuperscript{168} The biological implications of these interactions have been demonstrated \textit{in vitro} with two cell culture lines, \textit{in vivo} with transgenic HLA-HCD4/DQ8 gluten-sensitized mice and \textit{ex vivo} with duodenal biopsies from celiac disease patients. The protective effects of BL-7010 against gliadin-mediated intestinal damage were demonstrated by maintenance of ZO-1 localization along the lateral membrane of Caco-2/15 cells.\textsuperscript{11} These findings were further supported by the decrease in gliadin-mediated horseradish peroxidase-flux in gluten-sensitized mice in the presence of BL-7010, which preceded the attenuation of the gliadin-mediated immune response, as measured by the decreased IEL counts and the absence of lesions in the small intestinal mucosa.\textsuperscript{11} A similar study measured the production of anti-gliadin IgA, showing that BL-7010 also improved celiac-related serology, and \textit{ex vivo} testing of biopsy specimens showed decreased secretion of TNF-α and IL-10 in the presences of the polymer as well.\textsuperscript{12}

Despite many promising possibilities for celiac disease treatment being studied in pre-clinical and clinical trials, the gluten-free diet is still the only reliable option for preventing the symptoms and immune response associated with celiac disease. An area that remains understudied is the potential impact of dietary supplements and naturally-derived compounds on gluten digestion and processing. Of interest are polyphenols, which have been shown to have anti-nutritional properties with respect to proteins. Protein-polyphenol interactions have been studied extensively for their impact on protein structure and function, as well as the downstream biological implications of interactions...
including prevention of absorption and digestion and modification of bioavailability and accessibility. Given the preliminary success of BL-7010 as a synthetic sequestrant, polyphenols may exhibit similarly protective effects against celiac disease via interaction with gluten proteins.

1.4 Polyphenols

Polyphenols are a structurally diverse class of secondary metabolites produced by plants to aid with non-growth processes such as defense against external stressors, hormone release and signaling within the plant during ripening. Ubiquitous in plants, polyphenols have been widely studied with regard to their potential impact on human health. While the consumption of polyphenols is often associated with health benefits, their ability to obstruct the absorption of nutritive compounds has also been explored as a negative characteristic. After a brief introduction to polyphenols from a chemical standpoint, this section will focus on the anti-nutritional mechanisms and biological impact of polyphenols within the context of human health, as well as applications of this concept that have already been explored with respect to food hypersensitivities.

Polyphenols are organic compounds composed of multiple phenol groups (Figure 1.1a). Some can be loosely classified as biopolymers, especially in the case of lignins and tannins, which tend to have MWs between 3,000 and 20,000 g/mol. Other classes of polyphenols include phenolic acids (e.g., benzoic and cinnamic acids), polyphenolic amides (e.g., capsaicinoids), flavonoids and unclassified non-flavonoids (e.g., ellagic acid, curcumin). Flavonoids are a broad group of polyphenols that are distinguished by their structure- a C6-C3-C6 backbone with two phenolic rings (Figure 1.1b). While
most polyphenols are highly conjugated and feature multiple phenolic hydroxyl groups, variations in hydroxylation and structural conformation dictate differences in the classification and functional characteristics. For this reason, flavonoids can be further broken down into sub-classifications. One such class of flavonoids is flavonols, also known as catechins (Figure 1.11d). All catechins feature two phenolic rings (A and B) linked by a dihydropyran heterocycle, and can be differentiated from one another by isomeric configuration, hydroxylation and the substitution of gallyl groups within the structure. Catechin monomers can also condense to form larger molecules like the polymeric procyidanins (PCs) or dimeric theaflavins (TF) (Figure 1.11e, 1.11f).
Figure 1.11 Structure of basic flavonoids and polymerization products. *Structures from Balentine et al.*\textsuperscript{174} and *Higdon et al.*\textsuperscript{175}
1.4.1 Dietary Sources of Flavonoids and their Impact on Health

Flavonoids can be found in many edible plants including berries, citrus fruits, soy, cocoa, leafy vegetables, root vegetables and legumes, making incorporation into the diet relatively easy. According to the 2007-2010 NHANES study, the average flavonoid intake for American adults was 200.1 ± 8.9 mg/d. Common contributors of flavonoids to the diet are citrus juices, berries, and wine, but the major source of flavonoids in the American diet by a significant margin is tea.\(^{172}\) As the second most commonly consumed beverage in the world, tea has been studied extensively in terms of flavonoid composition, as well as the impact that tea consumption and tea flavonoids have on human health.\(^{173}\)

1.4.1.1 Tea Flavonoids

Tea is made from the plant *Camellia sinensis*, which can be processed in different ways to yield products of differing chemical and sensorial properties (Figure 1.1).\(^{174}\) Prior to processing, tea leaves contain a variety of monomeric catechins including (\(-\))-epicatechin (EC), (\(-\))-epigallocatechin (EGC), (\(-\))-epicatechin gallate (ECG) and (\(-\))-epigallocatechin gallate (EGCG) (Figure 1.11d). Green tea, which makes up 20% of worldwide tea consumption, is processed by steaming or frying leaves immediately after the withering process in order to halt enzyme activity and to preserve monomeric catechins. This is in contrast to black tea processing, wherein leaf maceration after withering allows for activation of oxidative enzyme and the formation of dimeric and polymeric oxidation products of catechins (e.g., thearubigins and theaflavins) that are not found in green tea (Figure 1.11f, 1.11g). Black tea is the most popularly consumed type
of tea, at 78% of total worldwide tea consumption. The remaining 2% of tea consumed worldwide is oolong, which is only partially oxidized.\textsuperscript{175}

It is critical to note that the biological activity of tea can be heavily impacted by the processing of the product, as it affects the overall flavonoid composition. The bioactive catechin EGCG can be used as an example to highlight this point. Physiologically-relevant doses of EGCG as an isolated, purified compound have been shown to have a potential role in reducing obesity, preventing metabolic syndrome and hepatic steatosis in mice fed a high-fat diet for 16 weeks.\textsuperscript{176} When translating these findings to dietary recommendations, the EGCG content of the tea would be critical to know in order to achieve the doses discussed in the study, but as discussed, processing affects the flavonoid content. For this reason, a recommendation to consume black tea, which typically contains 0 to 70 mg EGCG per cup, would provide a markedly different dose of the bioactive component in comparison to oolong or green tea, which contain between 30 and 130 mg EGCG per cup.\textsuperscript{175} Similarly, theaflavins and thearubigins are found only in teas which have undergone the oxidative process of leaf maceration. As a result, green tea would not be a suitable dietary source of those compounds.
Figure 1.1 Process flow of green and black tea production. Despite both beginning as *Camellia sinensis* leaves, the difference between immediate steaming and enzyme deactivation versus leaf maceration and enzyme activation results in distinct differences in total flavonoid composition of the final products. Adapted from Balentine et al.
Epidemiological evidence suggests that tea flavonoids can beneficially impact human health, demonstrating anti-inflammatory, anti-carcinogenic and anti-obesity properties \textit{in vitro} and \textit{in vivo}. Often contributing to each of these is the antioxidative capacity of flavonoids. As a class of compounds, polyphenols have the ability to both scavenge free radicals and prevent radical formation, although antioxidative potential can vary between compounds and applications.\textsuperscript{177} EGCG has been widely studied for its antioxidative properties, which have been shown to be matrix-dependent \textit{in vitro}.

Structurally, EGCG differs from other catechins as it features an \textit{ortho}-trihydroxyl group in the B ring and a galloyl moiety.\textsuperscript{178} The trend for di- vs. trihydroxy substitutions to affect redox potential has been reported for other catechins, showing that structurally similar compounds such as catechin, EC and EGC possess similar redox potentials.\textsuperscript{174} The bioactivity of tea flavonoids is not limited to the neutralization or prevention of free radicals, and/or protection against oxidative damage. The role that flavonoids can play in cell signaling and anti-inflammatory compounds has been investigated \textit{in vitro} and \textit{in vivo} through their ability to induce or inhibit enzymes that play important roles in cell maintenance.\textsuperscript{175} Flavonoids have been shown to modify the activity of enzymes associated with oxidative stress, such as inducible nitric oxide synthase (iNOS), lipoxygenases, and COX. Endogenous enzyme iNOS is responsible for the production of nitric oxide ($\textbullet\text{NO}$), which is a highly reactive compound capable of oxidizing cellular proteins and DNA,\textsuperscript{175,179} and whose activity has been shown to be decreased by tea flavonoids.\textsuperscript{175,180,181} Similarly, the ability of flavonoids to compromise the activity of lipoxygenases and cyclooxygenases has been used to explain a potential mechanism for the anti-cancer properties of flavonoids. Enzymes 15-lipoxygenase and COX-2 have both
been found to be upregulated in colon cancer cells compared to normal epithelial cells, but this increase in activity can be reversed in vitro in human mucosal tissue, and in vivo in mice and rats given tea flavonoids in their diet in the form of green tea extract added to their drinking water.\textsuperscript{175,182}

The anti-inflammatory capabilities of flavonoids have been investigated with respect to a variety of disease states. Obesity is characterized by a chronic, low-grade inflammation, which plays a role in the development of other diseases such as type-2 diabetes and cardiovascular disease. EGCG has the ability to decrease tumor necrosis factor (TNF)-\(\alpha\) signaling in vitro and in vivo. In the development of atherosclerosis, TNF-\(\alpha\) induces endothelial cell secretion of monocyte chemoattractant protein (MCP)-1, which is associated with the development of atherosclerotic plaques. Pre-treatment of porcine endothelial cells with EGCG prevented TNF-\(\alpha\)-induced MCP-1 upregulation.\textsuperscript{183} In liver tissue of obese mice, EGCG prevented steatohepatitis and improved insulin resistance by similarly decreasing TNF-\(\alpha\), linked to an overall suppression of NF-\(\kappa\)B signaling.\textsuperscript{184} The suppression of NF-\(\kappa\)B signaling by EGCG also influences the immune system in obese individuals, reducing the impact of the obesity-induced inflammatory state by promoting the proliferation of regulatory T cells and secretion of anti-inflammatory cytokine IL-10.\textsuperscript{185}

**1.4.1.2 Flavonoids as an Alternative Therapy for Inflammatory Bowel Disease**

In general, flavonoids have been implicated as potentially beneficial in the mediation of inflammatory bowel disease (IBD), which includes ulcerative colitis (UC) and Crohn’s disease. Both of these diseases are characterized by chronic inflammation.
within the gastrointestinal tract. While UC primarily affects the colon, Crohn’s disease can manifest along the entirety of the gastrointestinal tract, most commonly appearing in the terminal ileum.\textsuperscript{186} A key difference between celiac disease and IBD is IBD’s lack of a specific environmental/dietary trigger. However, IBD shares a number of similarities with celiac disease not only pertaining to what is known about it, but also to what remains unknown. UC and Crohn’s disease are both thought to develop as a result of both genetic and environmental factors. Although those environmental factors are presently undefined, hypotheses include drug exposure, the microbiome and stress. Furthermore, the pathogenesis of each of these conditions feature both innate and adaptive immune responses in the intestinal mucosa and present similar symptoms and physiological effects such as gastrointestinal discomfort, inflammation of the gastrointestinal tract and intestinal barrier permeability.\textsuperscript{186,187} Similar to celiac disease, IBD treatment is focused on symptom management and disruption of the immune response via administration of anti-inflammatories and immunosuppressants, although alternative and dietary therapies are currently being explored. Among them are pre- and probiotics, as well as flavonoid supplementation.\textsuperscript{188}

Studies on the potential for flavonoids as a complementary therapy for IBD have shown that the anti-inflammatory and immunomodulatory effects of tea flavonoids observed in liver tissue and endothelial cells - inhibition of COX-2, decreased NO production, NF-κB suppression - are also observed in the intestinal mucosa, resulting in overall amelioration of the inflammatory state of IBD.\textsuperscript{181,187,189} Histological damage such as crypt hyperplasia and IEL infiltration can also be prevented by flavonoid treatment.\textsuperscript{187,190} Decreased IEL infiltration as a result of flavonoid treatment is
demonstrative of the therapeutic effect that flavonoids can have on the immune
dysregulation that is associated with IBD. By suppressing NF-κB, EGCG is able to
reduce TNF-α expression by peritoneal macrophages,\textsuperscript{189,191} and intestinal epithelial cell
lines Caco-2 and IEC-6 (rat small intestine).\textsuperscript{187,190,191} EGCG has also been shown to
modify cytokine secretions in Caco-2 cells, reducing proinflammatory cytokines IL-6 and
IL-8,\textsuperscript{192} which play a role in intestinal barrier permeability and IEL homing,
respectively.\textsuperscript{193,194}

1.4.2 Anti-Nutritional Effects

Despite their numerous health benefits, polyphenols are sometimes referred to as
“anti-nutrients”, as polyphenol-rich diets have been associated with reduced absorption
and digestibility of micro- and macronutrients. Underlying mechanisms driving the anti-
nutritional effects of polyphenols in the diet include metal chelation, digestive enzyme
inhibition and protein sequestration, which will be discussed in the proceeding sections.

1.4.2.1 Metal Chelation

Polyphenols are able to chelate metals such as iron and copper at physiological
pH levels, as phenols can become deprotonated between pH 5.0 – 8.0 in the presence of
cations. The affinity of polyphenols for cations increases when the polyphenols are
structured in a way that allows bidentate chelation due to proximal positioning of
hydroxyl groups within a phenolic ring structure. Iron (III), iron (II), zinc (II) and
aluminum (III) have all been shown to interact with polyphenols.\textsuperscript{195} Grapeseed extract,
which contains a variety of polyphenols, as well as purified EGCG, have both been
shown to dose-dependently chelate iron in physiological conditions, inhibiting the uptake and transport of iron in Caco-2 cells. Animal and clinical trials using polyphenol-containing whole foods corroborate these findings.

1.4.2.2 Enzyme Inhibition

The inhibition of digestive enzymes by extracts of plant products including grape seed, berries and tea has been demonstrated in vitro. This effect does not only apply to endogenous enzymes; bacterial enzymes can also be affected. Enzyme inhibition has been shown to be a therapeutic target for obesity and metabolic syndrome, as decreased absorption of fat and carbohydrates can result in improved health outcomes. For this reason, lipase and amylase inhibition by polyphenols and polyphenol-rich diets have been examined extensively with obesity-related outcomes.

Inhibition of digestive proteases has also been studied, with polyphenols showing inhibitory effects on trypsin activity, while conflicting data exists with regard to pepsin. Studies on the impact of polyphenols on protein digestion and absorption demonstrate the efficacy of polyphenols in inhibiting digestive proteases. In a clinical trial of Egyptian boys consuming diets of fava beans and wheat, Hussein and Abbas determined that the procyanidins in the husks of the fava beans decreased net protein utilization. Upon removal of the husks, these effects were reversed. In vitro, green tea extract and its constituent compounds have been shown to inhibit trypsin activity. Pepsin, on the other hand, has been shown to be inhibited, unaffected and even activated in the presence of polyphenols in three different studies.
Based on both empirical data and *in silico* modeling studies, the inhibition of digestive enzymes by polyphenols appears to be due to direct interactions between the polyphenols and the enzymes, as polyphenols have a natural propensity to interact with proteins.\(^{210}\) However, when the target substrates of an enzymatic reaction are also proteins, the possibility that polyphenols sequester the substrate from enzymatic catalysis cannot be ruled out out.

### 1.4.3 Protein-Polyphenol Interactions

Polyphenolic interaction with proteins has been well-characterized, particularly with respect to the role that these interactions play in food quality. The ability of flavonoids to contribute to important qualities like color, flavor and mouthfeel require an understanding of their physicochemical interactions with other compounds in the food system being studied and with the consumer. Tea and wine provide simple examples for how this phenomenon can affect a product. When milk is added to tea, the polyphenols in solution can become bound by milk proteins, which alters polyphenol bioavailability and, thus, the nutritional value of both proteins and polyphenols to consumption.\(^{211}\) In drinking a glass of wine, a consumer effectively mixes the tannins from the beverage with their own salivary proteins, resulting in protein-polyphenol interactions and precipitation. This is detected by the consumer as the tactile oral sensation of astringency.\(^{212}\)

Protein-polyphenol interactions can occur via covalent or non-covalent mechanisms. Covalent interactions typically occur between functional groups on the proteins and phenolic oxidation products (i.e., quinones), which can occur naturally or
during processing. Non-covalent interactions between proteins and polyphenols often involve flavonoids, although the structural diversity of flavonoids as a class of compounds introduces complexity to the understanding and characterization of interactions. For example, affinity for interaction can be influenced by galloylation - a factor that has been shown to come into play in comparison of protein binding capacity of EC versus EGC and EGCG.

Protein structure can be used to predict the characteristics of interaction between proteins and polyphenols. Structurally-defined proteins will often display specific binding pockets whereas non-specific interactions are observed with proteins that have only secondary structural motifs. In either case, non-covalent interactions are the driving force of interaction including van der Waals interactions, π-stacking and hydrogen bonding. These interactions have been studied and modeled extensively in relation to the aforementioned salivary proteins, which are rich in proline. Proline-rich proteins have high affinities for polyphenols, facilitated by the structural conformations that occur with high frequencies of proline. The first is pyrrolidine ring formation of the proline functional group, which allows for π-stacking with polyphenolic rings. Proline also induces an extended/disordered structure, often featuring PPII helices. This type of secondary structure allows increased accessibility to the protein backbone for interaction with polyphenols. These interactions can induce structural changes upon the protein, which in turn can affect protein function. The interactions that occur between proline-rich proteins and polyphenols result in the formation of aggregates, which can precipitate from solution (Figure 1.13).
1.4.4 Gluten-Polyphenol Interactions

Gluten proteins are an interesting candidate for interaction with polyphenols due to the structural characteristics that a majority of gluten proteins possess. As previously mentioned, gluten is rich in proline and is not found to possess a defined structure beyond PPII repeat motifs. Interactions between gluten and polyphenols have been demonstrated in a food processing setting, with gluten added to wine as a fining agent. Fining is defined as the removal of an undesirable compound from a wine by the addition of a precipitating agent, followed by removal of the precipitate formed.\textsuperscript{218} Gluten has been added to both red and white wines in attempts to improve mouthfeel, clarify and generally confer chemical stability to the wines.\textsuperscript{219,220}
The interactions between gluten and a variety of anthocyanins and procyanidins have also been investigated, demonstrating the formation of gluten-polyphenol complexes.\textsuperscript{221–224} While these studies provide basic information about whether interactions between phenolics and gluten occur, they lack in both mechanistic underpinnings and biological outcomes of the interactions.

### 1.4.5 Influence of Polyphenols on Food Allergies

Polyphenols have been shown to influence the immune system within the context of food allergies both directly, as immunomodulators, and indirectly as sequestrants of allergenic proteins.

#### 1.4.5.1 Immunomodulation

Polyphenols have been shown to possess immunomodulatory activity as demonstrated by their ability to disrupt cell signaling pathways, modify cytokine production and concomitantly affect T cell proliferation and migration. While previously discussed within the context of inflammatory disorders, polyphenols exhibit similar effects in allergic disorders (\textbf{Figure 1.14}).\textsuperscript{225}
Figure 1.13 Mechanisms of action for polyphenols in food allergies. Polyphenols have been shown to modify the allergic response to allergenic food proteins by (i) binding to the antigens and preventing recognition by antigen presenting cells. T cell function is impaired (ii) through the induction of apoptosis and (iii) inhibition of cytokine production, including IL-4, -5 and -13. Polyphenols can also (iv) prevent the production of immunoglobulin E antibodies by B cells and (v) prevent the degranulation of mast cells, which release histamine and propagate the inflammatory response associated with allergies. Adapted from Singh et al. 2011.

Apple polyphenols have been found to dose-dependently suppress the expression of MHC class II molecules when tested in ovalbumin-stimulated dendritic cells via upregulation of MARCH1, which downregulates surface molecules. However, TNF-α was upregulated and IL-10 was downregulated,\textsuperscript{226} demonstrating that these particular compounds are not protective against inflammation. In a similar study, apple polyphenols have shown the prevention of IgE-mediated allergic responses to ovalbumin in mice, including anaphylaxis. Immune signaling in the gut was affected by consumption of apple extract, as IL-5, IL-13 and CCL11 were all downregulated. The allergenicity of ovalbumin was greatly reduced in the presence of apple polyphenols according to an IgG
ELISA, suggesting that the modification to the immune signaling pathway may be due to sequestration of the allergenic protein.\textsuperscript{16}

\subsection*{1.4.5.2 Binding and Sequestration of Allergenic Proteins}

The use of polyphenols as protective agents against food allergens has been explored most notably with the creation of hypoallergenic peanut butter.\textsuperscript{17,19,227,228} In that study, Chun and Champagne demonstrated that complexation of peanut allergens Ara h 1 and Ara h 2 with caffeic, chlorogenic or ferulic acid all resulted in significantly reduced IgE binding.\textsuperscript{19} A follow-up study used tannic acid as a precipitation agent, removing Ara h 1 and Ara h 2 from solution and similarly preventing recognition by IgE.\textsuperscript{227} Further investigation of this topic has led to the development of an edible peanut butter matrix with reduced allergenicity containing polyphenols from a variety of plant sources including cranberries, cinnamon and green tea.\textsuperscript{17,228} The polyphenol-fortified matrices demonstrated reduced allergenicity by way of reduced IgE binding, basophil activation and mast cell degranulation, but the stability of the complexes and maintenance of hypoallergenicity was dependent upon the type of polyphenol used.\textsuperscript{228}

The mechanistic explanation for decreased binding and recognition of allergenic proteins has been explored using an array of analytical techniques and purified forms of the allergenic proteins. Studies characterizing the interactions between EGCG and peanut allergens Ara h 2 and Ara h 6 demonstrate binding specificity upon formation of protein-polyphenol complexes. Additionally, binding elicits a structural change on the allergens, modifying the frequencies of $\alpha$-helices and $\beta$-sheets within each. \textit{In silico} analyses revealed potential binding sites on each Ara protein, which is useful in predicting the
impact of binding on epitope recognition by immune cells. The mechanistic findings of this study could potentially help explain the effects of reduced allergenicity in the previous peanut allergen study; however, the absence of mechanistic data in the first and biological application in the second prevent the drawing of clear conclusions.

1.5 Conclusions

Celiac disease is a complex autoimmune disorder and, despite the many significant discoveries and hypotheses relating to its etiology, many questions still remain regarding the reasons for disease development in some genetically susceptible individuals and not others. As a result, the goal for clinicians dealing with celiac disease has not been to prevent development, but to treat existing cases and minimize damage by prescribing a lifelong gluten-free diet. However, the ubiquity of wheat-based food products and the usefulness of gluten as a food processing aid makes complete avoidance of gluten very difficult for some individuals.

Fortunately, celiac disease pathogenesis has become more clear over the last two decades, revealing number of potential targets for new therapies or adjuvants to the gluten-free diet. Some notable contributions are pharmaceuticals designed to reduce T cell proliferation or prevent intestinal permeability. Other proposed therapies target gluten in the lumen before it reaches the brush border by either hydrolyzing the protein further into non-immunostimulatory fragments, or conversely, sequestering the protein from digestion completely.

The potential role for dietary polyphenols in intestinal inflammatory diseases has been explored with respect to ulcerative colitis and to a lesser extent, Crohn’s disease. In
these studies, flavonoids have been shown to demonstrate protective effects against inflammation, gut barrier permeability and immune dysregulation,\textsuperscript{187,189,192} which are all similarly associated with celiac disease pathogenesis. This suggests that flavonoids may confer health benefits to individuals with celiac disease.

Beyond the capacity for flavonoids to directly modify the intestinal inflammatory state, they also may be able to take on a role similar to the synthetic sequestrants previously discussed. By binding to gluten, polyphenols may protect the protein from digestion and absorption and thus, protect celiac disease patients from the adverse effects associated with gluten ingestion. Similar approaches have been applied to food allergens with success \textit{in vitro}. Despite some researchers having shown the potential for polyphenols to interact with gluten proteins, the underlying mechanisms driving these interactions and the biological implications of these mechanisms are unknown, making predictions for the value of these interactions for treating celiac disease disease difficult.

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2.1 Hypothesis & Specific Aims

Current strategies for treating celiac disease can be broken into two categories: (1) pathogenesis-focused strategies; and (2) protein-focused strategies. The former targets post-absorptive modification and immunological events, while the latter targets the digestion and absorption of gluten proteins by modification of the proteins in the lumen. One protein-focused therapy that has been explored for safety and efficacy is the oral administration of BL-7010, a synthetic polymer comprised of hydroxyethyl methacrylate and sodium-4-styrene sulfonate (poly(HEMA-co-SS) or BL-7010), which binds to gluten to form insoluble gluten-polymer complexes. This interaction has been shown to prevent the digestion and absorption of gluten, allowing it to be excreted without causing the inflammatory and autoimmune responses that are typically stimulated by gluten in an individual with celiac disease.1–5

Polyphenols, a class of secondary metabolites found ubiquitously in plants, have been studied extensively over the past decade for their anti-oxidative and anti-inflammatory properties, often relating to their role as anti-carcinogenic and anti-obesity compounds. Recently, studies have proposed beneficial roles for flavonoids in the diet to combat inflammatory bowel disease.6–10 Polyphenols also possess the potential to be anti-nutrients, blocking the absorption of micro- and macronutrients via chelation, precipitation and enzyme inhibition. Reduced digestion and absorption of proteins has been explored as both a health precaution, in terms of high-tannin animal feed,11 and as a health benefit through the production of hypoallergenic peanut products.12–14 It has been shown that by complexing allergenic food proteins to polyphenol-rich extracts of plant...
products such as cranberries and blueberries, the allergic response typically elicited by that allergen can be reduced.\textsuperscript{12,15}

Polyphenolic complexation of gluten has been studied, but without any characterization of interaction mechanisms or biological implications.\textsuperscript{16–20} Gluten is an attractive target for phenolic complexation, as it bears striking structural similarities to proline-rich salivary proteins, which are implicated in the protein-polyphenol precipitation reactions that drive the sensation of astringency. These interactions are principally driven by non-covalent interactions, and can occur between proteins and large tannins, but also smaller flavonoids such as those found in tea, which is the most common source of flavonoids in the American diet.

Based this information, I hypothesized that \textit{tea flavonoids would interact with gluten proteins to form protein-polyphenol complexes via non-covalent interactions and that the resulting complexes would demonstrate reduced ability to stimulate celiac-related symptoms \textit{in vitro}.}

The following aims were used to test this hypothesis:

1. Assess the influence of the molecular structure of tea phenolics gallic acid, epigallocatechin-3-gallate (EGCG) and theaflavin on their ability to interact with immunodominant gluten fragment $\alpha_2$-gliadin (57-89) using saturation transfer difference NMR. (\textit{Chapter 3})

2. Elucidate the mechanism and structural impact of EGCG complexation with $\alpha_2$-gliadin (57-89). (\textit{Chapter 4})

3. Investigate the effect of green tea extract supplementation on the digestion of gliadin \textit{in vitro}. (\textit{Chapter 5})
4. Investigate the impact of green tea extract on gliadin-mediated intestinal permeability and inflammation in vitro. (Chapter 5)

2.2 Significance

Celiac disease is an autoimmune enteropathy that affects an estimated 1% of the world population. While the etiology of the celiac disease is still not fully understood, the focus of current research is symptom-preventative therapies rather than treatments that prevent disease development. Despite the diagnosis rates of celiac disease continuing to climb, the only reliable method currently prescribed for avoiding celiac-related intestinal damage is complete avoidance of gluten in the diet. This has been shown to be expensive as well as difficult. Inadvertent ingestion of gluten while adhering to a gluten-free diet can occur as a result of cross contamination or from foods that fall within the legal limits to be labeled gluten-free, but still contain trace amounts of the protein that can trigger a reaction in some individuals.\(^{21}\) The Code of Federal Regulations Title 21, Section 101.91 states that a food that has been treated to remove gluten can be labeled “gluten-free” even if gluten remains in that product at a concentration of up to 20 ppm.\(^{22}\) This is despite safety analyses by the United States Food and Drug Administration, which determined that the level of gluten that would protect the most sensitive individuals from adverse reactions is less than 1 ppm of gluten per food product.\(^{23}\) This disconnect between regulations and clinical recommendation may contribute to the persistence of villous atrophy despite following a gluten-free diet, which was recorded in one study as affecting 39-43% of celiac disease patients.\(^{24}\)
Adjuvant therapies such as BL-7010, larazotide acetate, vercirnon and AMG 714 that target a variety of different steps in pathogenesis are still undergoing clinical testing. The research described herein provides foundation for the development of a nutraceutical approach to treating celiac disease symptoms using dietary polyphenols as a therapeutic agent. While the studies described here focus on the mechanism of interaction and primary biological impact on celiac disease in vitro, the safety of polyphenol supplementation is supported by many years of research focusing on the impact of polyphenol-rich diets and polyphenol supplementation on a variety of human health outcomes. Based on the findings described herein, it may be possible to use dietary polyphenols as a nutraceutical adjuvant to a gluten-free diet in the form of a supplement or by dietary recommendations.

2.3 Innovation

This work takes an innovative approach to food safety, nutrition and human health by exploring ways to reduce or eliminate the adverse effects of gluten in a celiac disease model by exploiting the natural phenomenon of protein-polyphenol interaction. To date, there have been no other studies that have explored the feasibility of using a common dietary component in treating celiac disease; rather, the majority of studies have focused on synthetic pharmaceutical compounds that have required extensive testing for safety and tolerance. While previous studies have highlighted the ability of gluten proteins and polyphenols to form complexes, my studies are the first to outline interaction mechanisms between gluten proteins and polyphenols, as well as investigate the biological impact that polyphenolic complexation of gluten has in vitro. This research
helps to predict the efficacy of dietary polyphenols in treating celiac disease, and provides a foundation for future in vivo and pre-clinical studies.

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Chapter 3 Influence of Molecular Structure on Interactions Between Tea Polyphenols and an Immunodominant Gluten Peptide

3.1 Abstract

Phenolics have been shown to exert anti-nutritional properties in humans through their interaction with other dietary components, resulting in decreased absorption of those compounds. However, this phenomenon may be beneficial; the ability of high molecular weight procyanidins to modulate the immunostimulatory effect of food proteins has been explored in recent years. The present study aims to identify differences in the mechanisms and affinities of low molecular weight tea polyphenols to interact with an immunostimulatory peptide from gluten, based on structural differences between the polyphenols. Gallic acid, (−)-epigallocatechin-3-gallate (EGCG) and theaflavin were investigated based on their prevalence as tea phenols and their structural diversity.

Saturation transfer difference nuclear magnetic resonance (STD-NMR) was used to map binding epitopes on each ligand and assess interaction strength. Ligand mapping revealed the importance of hydroxyl functional groups on all three phenols for interaction with the peptide. Despite structural differences, the dissociation constants of EGCG and theaflavin were not different from one another. Comparison of signal localization revealed differences in saturation signal distribution where EGCG’s gallate and galloyl groups were in greater proximity to the peptide than theaflavin’s benzotropolone rings. This suggests that the strength of interactions observed are influenced by the structural conformation of polyphenols working in concert with the hydrogen bonding array and molecular weight of the ligand.
3.2 Introduction

Tea (from *Camellia sinensis*) is one of the most widely consumed beverages in the world, second only to water. Rich in polyphenolic compounds, tea is considered to be a functional food with many established beneficial health effects.\(^1\) A 100 mL serving of green tea typically contains 250-350 mg of tea solids (dry weight), of which catechins make up 30-42%.\(^2\) Epigallocatechin-3-gallate (EGCG) is the predominant catechin found in green tea. It can also be found in black, white and oolong teas, along with gallic acid (GA), a low molecular weight (MW) phenolic compound.\(^2\) Theaflavin (TF), a polymeric product of enzymatic catechin oxidation, comprises up to 2-6% of the total solids in black tea extract.\(^2\) These compounds, as well as the foods from which they are derived, have been widely studied for their chemopreventative, cardioprotective, anti-inflammatory, anti-oxidative and anti-obesity effects both *in vitro* and *in vivo*.\(^3\)–\(^7\)

Conversely, flavonoids such as EGCG and TF may also exhibit anti-nutritional effects upon consumption due to their propensity to interact with digestive enzymes as well as other dietary compounds. Such interactions include inhibition of trypsin and pancreatic phospholipase A\(_2\), chelation of metals and precipitation of proteins.\(^8\)–\(^14\) Complexation of proteins with dietary polyphenols has been shown to affect the digestibility and bioaccessibility of each class of compounds.\(^12,13\) However, there exist cases in which the anti-nutritional effects that these compounds exert may actually be advantageous to the individual consuming them (e.g., in the case of food allergies). Dietary polyphenols have been shown to interact with a variety of proteins responsible for major food allergies including ovalbumin from eggs and 2S albumins from peanuts. As a result, the allergenicity of these proteins is reduced, either through structural
modification or decreased bioaccessibility. The polyphenols that have been explored for their efficacy in reducing immunogenicity are often high MW procyanidins derived from the skin and seeds of fruits like grape, cranberry and pomegranate. The prevalence of lower MW tea polyphenols already existing in the human diet without supplementation makes them an interesting and relevant target for similar immunostimulatory protein interaction studies with respect to future exploration for their use as nutraceutical therapeutic agents.

Protein-polyphenol interactions have been studied extensively as they relate to the tactile sensation of oral astringency, which appears to result in large part from the precipitation of proline-rich salivary proteins. In previous studies, it was discovered that polyphenols bind preferentially to proline residues in proteins. Hydrogen bonding, van der Waals interactions and π-π stacking all contribute to the formation of proline-rich protein-polyphenol complexes. Interestingly, proline is one of the most abundant residues in the amino acid sequence of gluten, a storage protein from wheat, barley and rye that is responsible for stimulation of the autoimmune response associated with celiac disease. Peptide α2-gliadin (57-89) (LQLQPF(PQPQLPY)3PQPQPF) has been identified as a physiologically stable immunostimulatory peptide in celiac disease and is produced upon enzymatic digestion of gluten both in vitro and in vivo. Similar to proline-rich salivary proteins, the high frequency of proline residues in α2-gliadin (57-89) causes the peptide to exhibit an extended, natively unfolded structure due to polyproline II helices. This structure has been shown to favor interaction with polyphenols due to the increased accessibility of polyphenols to potential binding sites within the protein. Gluten and gliadin-rich fractions of gluten have previously been shown to interact with
polyphenols (e.g., anthocyanins, coumarin, resveratrol) and procyanidins, though a mechanistic understanding of the interactions driving complex formation have, to my knowledge, yet to be reported.\textsuperscript{27–30}

The objective of this study was to assess the influence of the molecular structure of tea phenolics GA, EGCG and TF on their ability to interact with $\alpha_2$-gliadin (57-89) using saturation transfer difference nuclear magnetic resonance (STD-NMR). It was expected that $\alpha_2$-gliadin (57-89) would interact with dietary phenolics to form gliadin-phenolic complexes, similar to previously demonstrated protein-polyphenol interactions between proline-rich salivary proteins and dietary polyphenols.\textsuperscript{21,31–34} By exploring the interaction potential of three different tea-derived compounds, we were able to establish trends in the interactions based on structural characteristics of each.

### 3.3 Results

#### 3.3.1 Impact of Solvent System Selection

DMSO-$d_6$ was chosen as the solvent for these experiments based on the hypothesis that the phenolic hydroxyl groups of GA, EGCG and TF would play an important role in interacting with $\alpha_2$-gliadin (57-89). Aqueous solvents allow rapid exchange between phenolic hydrogens and water, inducing extensive signal line-broadening and preventing the detection of these groups.\textsuperscript{35} Furthermore, DMSO-$d_6$ assisted with the solubility of all samples, which precipitated in aqueous solvent. The impact of the chosen solvent on binding was investigated by running additional STD experiments with 15% DMSO-$d_6$/85% H$_2$O as the solvent, the least concentration of DMSO-$d_6$ at which the sample did not precipitate. The interactions observed with 100%
DMSO-d$_6$ were retained under these conditions for each polyphenol at a 50:1 ligand-to-protein ratio, with the exception of those signals related to the phenolic hydrogens for reasons previously discussed (Appendix, Figure 1).

### 3.3.2 Epitope Mapping Reveals Localization of Interaction Sites on Phenolic Ligands

The interaction of each polyphenol with $\alpha_2$-gliadin (57-89) was confirmed by qualitative observation of the ligand signal in STD-NMR difference spectra at all ligand concentrations, suggesting that interaction occurs between GA, EGCG and TF at concentrations as low as 5 times excess to $\alpha_2$-gliadin (57-89). Resonance assignments were made according to previously reported chemical shift data for each compound. The importance of individual ligand moieties to each interaction can be described by relative degree of saturation, a measurement of which protons are nearest to the protein. As binding to the protein causes saturation of ligand protons, the protons nearest to the protein are the most saturated and thus produce a more intense signal.$^{36}$

Interactions between GA and $\alpha_2$-gliadin (57-89) appear to be primarily driven by interaction with the phenolic hydroxyl groups (Figure 3.1). The absence of signal originating from aromatic hydrogens in the difference spectrum at 6.9 ppm in Figure 3.1c suggests that the formation of gliadin-phenolic complexes in solution relies on hydrogen bond formation. Notably, interactions are also observed with GA’s carboxyl group. However, the relative degree of saturation of the carboxyl hydrogen is significantly lower than those of the phenolic hydrogens. This suggests that GA’s aromatic ring is likely to be oriented within the binding pockets of $\alpha_2$-gliadin (57-89),
with the carboxyl group facing outwards from the peptide, further from interaction sites than its hydroxyl group counterparts (Figure 3.1d).

**Figure 3.1** (a) $^1$H NMR spectrum of GA (25 mM). (b) Reference spectrum of GA and $\alpha_2$-gliadin (57-89) in a 100:1 molar ratio and (c) corresponding difference spectrum, shown at a 1:4 ratio. (d) Relative degree of saturation of GA hydrogens upon interaction with $\alpha_2$-gliadin (57-89) normalized to that of H-3,5. STD signal from H-2,6 could not be measured due to overlap with $\alpha_2$-gliadin (57-89) signal, though there did not appear to be an increase in intensity that could have potentially been attributed to the ligand.
Figure 3.2 (a) $^1$H NMR spectrum of EGCG (25 mM). (b) Reference spectrum of EGCG and α$_{2}$-gliadin (57-89) in a 100:1 molar ratio and (c) corresponding difference spectrum, shown at a 1:4 ratio. (d) Relative degree of saturation of EGCG hydrogens upon interaction with α$_{2}$-gliadin (57-89) normalized to that of H-11’. The following hydrogens that produced detectable STD signals that are not listed are H-2, 6.7%; H-3, 6.0%. Saturation signals for H-10,12; H-9’,13’ and H-9,13 are quantified as averages due to overlapping signals. STD signal from H-5 could not be measured due to overlap with α$_{2}$-gliadin (57-89) signal. (e) Average relative degrees of saturation per EGCG ring constituent. Different letters denote significant differences in relative degree of saturation between ring constituents ($p \leq 0.05$).
Interaction between α2-gliadin (57-89) and EGCG was also observed by STD-NMR (Figure 3.2). Localization of more intense STD signals within the structure of EGCG suggest the possibility of preferential binding or interaction specificity (Figure 3.2d). According to the relative degrees of saturation, the hydroxyl hydrogens of the B-ring (H-10, H-11, H-12) are in closest proximity to α2-gliadin (57-89), more so than H-9 and H-13 (i.e., the aromatic hydrogens of the same ring). H-9 and H-13 share a similar relative degree of saturation with all hydrogens on the D-ring with the exception of H-11’, the most saturated hydrogen in EGCG. In a comparison of the overall saturation of each ring constituent of EGCG, the B- and D-rings showed the greatest degree of saturation with no significant difference between one another (Figure 2e). All hydrogens on the A- and C-rings show a lesser degree of saturation than those of the B- and D-rings, suggesting further distance from the peptide and a lesser role in the overall interaction. These trends suggest the importance of the EGCG’s gallate and galloyl moieties with respect to interaction with α2-gliadin (57-89). As relative degree of saturation is a measurement of relative spatial proximity between ligand and peptide, one can conclude that the flexibility of the galloyl moieties allow insertion into binding pockets, whereas A- and C-ring hydrogens are more likely to be affected by steric hindrance and play a less meaningful role with respect to their interaction with α2-gliadin (57-89).
Figure 3.3 (a) $^1$H NMR spectrum of TF (25 mM). (b) Reference spectrum of TF and $\alpha_2$-gliadin (57-89) in a 100:1 molar ratio and (c) corresponding difference spectrum, shown at a 1:8 ratio. (d) Relative degree of saturation of TF hydrogens upon interaction with $\alpha_2$-gliadin (57-89) normalized to that of H-7,7'. The following hydrogens which produced detectable STD signals that are not listed are: H-8', 17.9%; H-4', 28.3%; H-9, 46.1%. The following hydrogens did not produce any STD signals above the limit of detection: H-2, H-3, H-2', H-6', H-3', H-2'', H-4'', H-7''. Saturation signals for H-8'', H-9'' and H-10'' were quantified as an average due to their overlapping signals, as were those for H-7',7 and H-9',9. (e) Average relative degrees of saturation per TF ring constituent. No significant differences were observed between groups.
The relative degrees of saturation of TF hydrogens show similar patterns of localization not based on the constituent rings, but rather the structure of the polyphenol as a whole (Figure 3.3). No significant differences between the relative degree of saturation of the interaction sites were observed with the exception H-8’ and H-6’ and the hydrogens which did not produce a STD signal above the limit of detection (H-2, H-3, H-2’, H-3’, H-6’, H-2’’, H-4’’, H-7’’). Noticeably, all TF hydroxyl hydrogens did produce a STD signal, suggesting their importance in the molecule’s ability to interact with α2-gliadin (57-89) (Figure 3.3d). Furthermore, the hydrogens that did not produce a detectable STD signal are all located directly on aromatic or dihydropyran heterocyclic rings. The localization of ligand interactions sites to the outer edges of TF suggest decreased ability of the compound to interact with α2-gliadin (57-89) despite the greater number of potential proton donors and acceptors in its molecular structure.

3.3.3 Affinity of Phenolic Compounds to α2-Gliadin (57-89) is Affected by Phenolic Structural Conformation

The strength of interactions between α2-gliadin (57-89) and each phenolic compound were quantified by calculation of dissociation constants (K_D), the concentration of ligand required to saturate 50% of the available binding sites on the peptide. Figure 3.4 shows experimental data points (symbols) and lines of best fit for K_D calculation according to Equation 3.2. No differences in K_D of individual hydrogens within each compound were observed, therefore these individual values were pooled into averages for each ligand. The K_D for GA was calculated to be 19.25 ± 4.92 mM, nearly tenfold greater than that calculated for EGCG (2.51 ± 0.79 mM) and TF (2.33 ± 1.35
Based on these findings, the affinities of each compound for \( \alpha_2 \)-gliadin (57-89) can be ranked as TF \( \equiv \) EGCG > GA.

**Figure 3.4.** STD amplification factor as a function of ligand concentration for each phenolic compound. Symbols represent experimental averages for each ligand hydrogen signal while lines represent the line of best fit according to Equation 2. Dissociation constants represent the values corresponding to each compound as a whole.

### 3.4 Discussion

Based on the reported ligand mapping experiments, one can conclude that phenolic structure directly affects the propensity of phenolic compounds GA, EGCG and TF to interact with \( \alpha_2 \)-gliadin (57-89). As previously stated, TF features a greater number of potential proton donors or acceptors for hydrogen bond formation; as such, a greater affinity between TF and \( \alpha_2 \)-gliadin (57-89) would be expected. However, structural conformation and location of phenolic rings within the structure plays an important role in binding affinity. This was demonstrated by Cala et. al. (2010) with procyanidins of similar MWs interacting with proline-rich salivary protein IB-7\( _{14} \). It was found that procyanidins B1 and B3, which adopted a compact conformation based on \( \pi \)-\( \pi \) stacking, had higher \( K_D \) values and thus lower affinity to the peptide than procyanidins B2 and B4, which adopted more extended conformations. These extended conformations resulted in
greater affinity for interaction with the peptide, as defined by a lower $K_D$.\textsuperscript{31} Our findings suggest that the potential of EGCG to adopt an extended conformation compared to TF compensates for the comparatively lower number of potential bindings sites and lower MW, resulting in similar affinities for interaction with $\alpha_2$-gliadin (57-89) (Table 3.1).

To our knowledge, this is the first study to characterize intermolecular interactions between $\alpha_2$-gliadin (57-89) and dietary phenolic compounds. However, our findings are corroborated by a large body of work exploring the interaction between polyphenols and proteins that possess similar characteristics to $\alpha_2$-gliadin (57-89) in terms of both proline content and structure. With a proline content of 39.4\% and a high frequency of polyproline II helix repeat motifs, $\alpha_2$-gliadin (57-89) bears strikingly similar characteristics to proline-rich salivary proteins IB-5, MP-5,\textsuperscript{19,31,32} and IB-9,\textsuperscript{37} which are made up of 41.4, 42.1 and 43.2\% proline, respectively.\textsuperscript{31,32,37,38} Notably, IB9 (3.531 kDa) is also similar in MW to $\alpha_2$-gliadin (3.911 kDa), which is another driving force behind the propensity of proteins and polyphenols to interact.\textsuperscript{35} Each of these peptides have been shown to interact with polyphenols of various MWs and structures with dissociation constants ranging from 0.4 to 8.0 mM.\textsuperscript{31,32}
Table 3.1 Comparison of MW and number of potential hydrogen bonding sites of tea phenolics GA, EGCG and TF with calculated dissociation constants. MW and number of potential bonding sites have been implicated in the ability of phenolic compounds to interact with proteins. Our findings suggest that the orientation of interaction sites on EGCG’s structure compensates for the lower MW and overall less interaction sites. Different letters denote significant differences between $K_D$ values ($p < 0.05$).

<table>
<thead>
<tr>
<th>Compound</th>
<th>MW</th>
<th>Potential Hydrogen Bonding Sites</th>
<th>Calculated $K_D$ Value (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA</td>
<td>170.12</td>
<td>6 (4 –OH, 2 CH)</td>
<td>$19.25 \pm 4.92^b$</td>
</tr>
<tr>
<td>EGCG</td>
<td>458.37</td>
<td>17 (8 –OH, 8 CH, 1 CH$_2$)</td>
<td>$2.51 \pm 0.79^a$</td>
</tr>
<tr>
<td>TF</td>
<td>564.49</td>
<td>22 (9 –OH, 11 CH, 2 CH$_2$)</td>
<td>$2.33 \pm 1.35^a$</td>
</tr>
</tbody>
</table>

The present work demonstrates the ability of the dietary phenolics GA, EGCG and TF to interact with $\alpha_2$-gliadin (57-89), an immunodominant peptide in celiac disease. Epitope mapping of each ligand reveals the importance of molecular structure in the ability of these phenolics to interact with proteins, as interactions of $\alpha_2$-gliadin (57-89) with TF and EGCG demonstrated similar $K_D$ values despite the greater number of potential interaction sites in the structure of TF. Despite limitations regarding non-physiological solvent systems and concentrations, these results support an interest for further investigation of these interactions to elucidate protein-based binding sites and potential impact on the structure and immunostimulatory properties of $\alpha_2$-gliadin (57-89). Alternative therapies for the treatment and prevention of symptoms associated with celiac disease have been explored extensively, though their availability for general use is limited. The potential development of a supportive treatment of celiac disease with naturally derived, dietary compounds (e.g., phenolics) would provide a safe and accessible alternative for the prevention or alleviation of symptoms of the associated autoimmune response.
3.5 Materials and Methods

3.5.1 Materials and Sample Preparation

The $\alpha_2$-gliadin (57-89) peptide LQLQPF(PQPQLPY)$_3$PQPQPF was synthesized with 95-97% purity by 21st Century Biochemicals (Marlboro, MA). Gallic acid (97.5% purity) was purchased from Sigma-Aldrich (St. Louis, MO) while EGCG and theaflavin were purchased as standards from Quality Phytochemicals (East Brunswick, NJ) at 98% purity. NMR samples were prepared immediately prior to analysis by dissolving $\alpha_2$-gliadin (57-89) and each ligand in dimethylsulfoxide-d$_6$ (DMSO-d$_6$, 99.8%, deuterium; EMD Millipore, Billerica, MA) to achieve a final concentration of 0.25 mM in 500 µL. Ligand mapping and buildup experiments were performed with 25 mM ligand per sample, or 100-fold excess. For $K_D$ determination experiments, EGCG and theaflavin were added to samples in amounts corresponding to 5-100 fold excess of the protein while GA was added in amounts corresponding to 5-500 fold excess.

3.5.2 NMR Spectroscopy

All NMR experiments were performed on a Bruker Avance-III-HD 500-MHz instrument operating at a $^1$H frequency of 500.20 MHz using a 5-mm Prodigy BBO BB-1H/19F/D Z-GRD probe at temperature of 298 K. Prior to STD-NMR experiments, structural conformation of each phenolic compound was confirmed using a 2D NMR experiments $^1$H-$^{13}$C heteronuclear multiple quantum correlation (HMQC) and $^1$H-$^{13}$C heteronuclear multiple bond correlation (HMBC) spectra.

STD-NMR is a pseudo-2D experiment in which a receptor (e.g., a protein) is selectively irradiated to transfer saturation signals to areas of the ligand that are in contact.
with the receptor, resulting in a decreased signal intensity from the free ligand. This also provides information regarding which parts of the ligand are in contact with the receptor.\textsuperscript{36} This method allows for semi-quantitative characterization of binding epitopes on ligands through calculation of the relative degree of saturation of each proton involved in the interaction as well as determination of the dissociation constant (K\textsubscript{D}) of each ligand. In all STD-NMR experiments, on-resonance irradiation of peptide \(\alpha_2\)-gliadin was performed at 1.84 ppm while off-resonance irradiation was set to 40 ppm. These conditions were selected based on preliminary experimentation with the peptide and each ligand alone, ensuring that the on-resonance irradiation frequency would not overlap with ligand resonances, but would fully saturate the peptide (Appendix, Figures 2, 3). The water peak originating from wet DMSO-d\textsubscript{6} was suppressed in all experiments using excitation sculpting in order to preserve exchangeable protons. Spectra were acquired using 100 50-ms E-Burp pulses for selective saturation of the peptide with a total saturation time of 5.0s, a 12.0s relaxation delay and an acquisition time of 1.7s for an overall recycle delay of 14s.\textsuperscript{39} Other parameters were 90° pulse of 10.13 \(\mu\)s @ 20 W, spectral width of 19.2 ppm and 32 scans per irradiation frequency (in blocks of 8 scans at each on/off irradiation) and receiver gain of 203.\textsuperscript{40}

Ligand mapping and K\textsubscript{D} determination experiments were performed using the described parameters. Data acquisition and processing were performed with TopSpin 3.2 (Bruker, Billerica, MA) and MestReNova 10.0.1 (Mestrelab Research, Santiago de Compostela, Spain), respectively.
3.5.3 Ligand Mapping

Binding epitopes on each ligand were identified by the presence of ligand signal in difference spectra. The importance of individual protons in each interaction was evaluated by comparing their relative degrees of saturation. These values were calculated by setting the most intense ligand signal to 100% and normalizing all other signals accordingly, given similar relaxation rates of each hydrogen in the ligand molecules (Appendix, Figure 4, Table 1).\(^\text{40}\)

3.5.4 Determination of Dissociation Constants

In order to acquire a 1D STD-NMR difference spectrum, on-resonance spectra were subtracted from the off-resonance spectrum. Ligand signals were identified in all STD-NMR difference spectra and integration regions were saved for identical processing of each replicate within a treatment group. Integral values of each proton displaying interaction with \(\alpha_2\)-gliadin (57-89) were used to calculate STD amplification factors \(A_{\text{STD}}\) with Equation 3.1,\(^\text{40}\)

\[
A_{\text{STD}} = \frac{I_0 - I_{\text{SAT}}}{I_0} \times \left(\frac{[L]}{[P]}\right) = \frac{I_{\text{STD}}}{I_0} \times \left(\frac{[L]}{[P]}\right),
\]

where \(I_{\text{SAT}}\) corresponds to signal intensity of the spectrum saturated on-resonance, and \(I_0\) is the signal intensity of the spectrum saturated off-resonance.

\(\frac{I_0 - I_{\text{SAT}}}{I_0}\) was calculated for each signal using the integral region calibration method described by Viegas et al. (2011) where one integral was set to 100 in each reference spectrum and used to calibrate the corresponding difference spectrum, taking ligand and protein concentration ([L] and [P], respectively) into account. \(K_D\) values for each proton were calculated with GraphPad Prism 6.0 (La Jolla, CA) by fitting \(A_{\text{STD}}\)
values to a nonlinear least-squares-fitting curve using Equation 3.2, where $\alpha_{\text{STD}}$ is the maximum amplification factor.\textsuperscript{40}

$$A_{\text{STD}} = (\alpha_{\text{STD}}[L])/(K_D+[L])$$  \hspace{1cm} (3.2)

### 3.5.5 Statistical Analysis

All analyses were repeated in triplicate ($n = 3$). Relative degree of saturation values are presented as mean ± standard deviation. One-way ANOVA analysis was paired with Tukey’s test for determining differences between the relative degrees of saturation of each proton identified in binding. Differences of $p < 0.05$ were considered significant. Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad, La Jolla, CA).

### 3.6 Acknowledgements

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### 3.7 References


(20) De Freitas, V.; Mateus, N. Protein/Polyphenol Interactions: Past and Present


Chapter 4  Structural Modification of $\alpha_2$-Gliadin (57-89) Upon Interaction with (-)-Epigallocatechin-3-Gallate

4.1 Abstract

(-)-Epigallocatechin-3-gallate (EGCG), a major phenolic constituent of tea, has has been shown to have biological activity against major inflammatory pathways involved with food allergies and intolerances. One proposed mechanism for this effect is sequestration and structural modification of immunostimulatory proteins as a result of direct interactions with EGCG. The present study employs a variety of biophysical techniques with the aim of elucidating the mechanism(s) by which EGCG interacts with $\alpha_2$-gliadin (57-89), a immunodominant peptide in celiac disease pathogenesis. We have demonstrated that EGCG interacts with $\alpha_2$-gliadin (57-89) in a multi-phase reaction driven by non-specific binding, forming polydisperse particles that increase in size as a function of EGCG concentration. Structural changes in the peptide were also observed upon interaction with EGCG. Based on previous reports of binding specificity of enzymes and antigen presenting cells in celiac disease pathogenesis, our data provides foundational support for the use of EGCG to immunostimulatory epitopes of $\alpha_2$-gliadin (57-89).

4.2 Introduction

According to NHANES 2007 – 2010, the average adult in the United States consumes approximately 200 mg of flavonoids per day, the majority of which are consumed in the context of green or black tea.\(^1\) The health benefits associated with tea and its constituent flavonoids have been extensively explored, both \textit{in vitro} and \textit{in vivo}.\(^2\)\(^-\)\(^5\)
In recent years, attention has turned to the potential for flavonoids to aid in the treatment of inflammatory disorders including inflammatory bowel disease (IBD) and the alleviation of symptoms associated with food allergy.\textsuperscript{6–10} These studies have demonstrated that flavonoids can be multi-functional with respect to prevention and reversal of the disease states investigated. One flavonoid of particular interest is EGCG, the major catechin found in green tea.

EGCG can mitigate the excessive generation of radical oxygen species (ROS) by both RAW264.7 macrophages and Caco-2 cells, suggesting a protective role against IBD.\textsuperscript{11,12} Additionally, EGCG has been shown to block the nuclear factor (NF)-\kappaB pathway in intestinal cells and \textit{in vitro} models of IBD, attenuating the secretion of pro-inflammatory cytokines interleukin (IL)-6 and IL-8 and defining the ability of EGCG to directly affect cell signaling.\textsuperscript{9,13–15} This ability is realized also in models of food allergy, as EGCG is able to mediate the degranulation of mast cells via inhibition of histidine decarboxylase as well as reduce the expression of key proteins involved in immune cell recruitment.\textsuperscript{16–18} As food allergies are stimulated by contact with an external antigen, “epitope masking”, or physically blocking recognition of immunostimulatory proteins from recognition, has also been explored as a function for polyphenols such as EGCG in mitigating the symptoms of food allergy. This has been demonstrated most notably with peanut proteins and procyanidins from cranberries and blueberries, which were demonstrated to result in decreased binding by IgE and attenuation of histamine and $\beta$-hexoaminidase release.\textsuperscript{19} In other studies, peanut proteins have been shown to undergo conformational changes upon binding to EGCG, suggesting that structural modification that may contribute to the decreased IgE recognition described by Plundrich et al.
EGCG has also been shown to structurally modify ovalbumin, a major allergen found in eggs, preventing uptake of the allergen by monocytes and thus attenuating the allergic response.  

Celiac disease is an autoimmune enteropathy that shares characteristics with both IBD and food allergies. In celiac disease, gluten proteins from wheat, barley and rye stimulate a host of symptoms that primarily manifest in the small intestine, inducing inflammation and damage to the enterocytes. Gluten proteins are rich in proline residues and feature repeat motifs of polyproline II (PPII) helices and random coils, structures that are important for their recognition by key receptors in pathogenesis. Tissue transglutaminase (TG2) is an endogenous enzyme secreted by enterocytes in response to gluten proteins passing the brush border that cross-links with gluten, preferentially deamidating glutamine residues one amino acid away from proline residues. Human leukocyte antigen (HLA)-DQ2 (DQA1*05:01, DQB1*02:01) recognizes gluten epitopes and presents them to T cells, activating the adaptive immune response which leads to destruction of small intestinal architecture and secretion of antibodies against gluten and TG2.

The present work explores the use of a dietary polyphenol (EGCG) as a gliadin protein binding agent which, if successful, could represent a novel therapeutic concept for alleviating celiac disease symptoms by masking binding sites and epitopes capable of being recognized by TG2 or HLA-DQ2. Characterization of the etiology of the CD autoimmune response has led to the discovery of physiologically stable, 33-amino acid fragment of α2-gliadin that is produced upon enzymatic digestion of gluten both in vitro and in vivo. This immunodominant 33-mer, α2-gliadin (57-89)
(LQLQPF(PQPQLPY)_3PQPQPF), contains six overlapping epitopes (1 α-I, PFPQPQLPY; 3 α-II, PQPQLPYPQ; 2 α-III, PYPQPQLPY) that are able to be deamidated by TG2 and be recognized by HLA-DQ2 (Figure 4.1).

![Figure 4.1](image)

**Figure 4.1** Recognition of immunostimulatory gliadin peptide LQPFPQPELPY by HLA-DQ2, driven by structural specificity for an unfolded peptide capable of forming hydrogen bonds within the binding pocket.²³

The tendency for these proteins to form polyproline II helices allows for increased accessibility of polyphenols to potential binding sites within the proteins, favoring noncovalent interactions such as hydrogen bonding, van der Waals interactions and π-π stacking.²⁴ This phenomenon has been explored with respect to proline-rich salivary proteins and wine tannins, as precipitation interactions between the two are thought to drive the oral sensation of astringency. Interactions between EGCG and proline-rich salivary proteins have been shown to modify the structure of salivary proteins and result in precipitation of both compounds.²⁵ Structural characterization of the α₂-gliadin (57-89)
33-mer has revealed that the peptide transitions between extended PPII helices and type
II β-turns depend on solvent conditions and temperature,\textsuperscript{26} suggesting that structural
modification as a result of binding with EGCG may also be possible.

The α\textsubscript{2}-gliadin 33-mer of interest exhibits physicochemical similarities to proline-rich salivary proteins in terms of molecular weight, proline content and secondary
structure motifs.\textsuperscript{27–30} Moreover, previous studies have characterized interactions between
the 33-mer and EGCG from the perspective of the ligand, revealing the importance of the
gallate and galloyl groups as interaction sites (\textbf{Chapter 3}). In the present work, we
further explored the potential for EGCG to bind to the 33-mer, this time from the
perspective of the peptide rather than the ligand, in order to elucidate the structural
implications of these interactions. From a functional perspective, interaction with EGCG
that results in modification of the 33-mer structure may provide the groundwork for
developing a nutraceutical approach to preventing the autoimmune response associated
with CD by preventing the recognition of the 33-mer by TG2 and/or HLA-DQ2. This
work provides mechanistic insight to this potential therapy as a precursor to \textit{in vitro}
studies focusing on biological implications of the interaction.

\subsection*{4.3 Materials and Methods}

EGCG (> 98% purity) was purchased from Quality Phytochemicals (East
Brunswick, NJ) and α\textsubscript{2}-gliadin (57-89) (LQLQPF(PQPQLPY)\textsubscript{3}PQPQPF; MW = 3911.4
kDa; 95-97% purity) was synthesized by 21st Century Biochemicals (Marlboro, MA).
4.3.1 Cryo-Transmission Electron Microscopy

Sample solutions (3 µL) were deposited onto glow-discharged 200-mesh carbon-coated copper grids (Ted Pella Inc., Redding, CA), blotted to thin film and immediately vitrified in liquid ethane using a Vitrobot cryo-station (FEI, Hillsboro, OR). Specimens were examined at 120 kV and up to 360,000x magnifications in a Tecnai G2 Spirit BioTWIN transmission electron microscope (FEI, Hillsboro, OR) in low dose conditions.

4.3.2 Isothermal Titration Calorimetry

An isothermal titration calorimeter (MicroCal Auto-iTC200, Malvern Instruments, Westborough, MA) was used to measure the enthalpies of mixing for α2-gliadin (57-89) and EGCG. Experiments were conducted at 37°C in 10 mM sodium phosphate buffer, pH 6.8. EGCG (3.2 mM) was titrated into a cell containing 400 µL of 12.8 mM α2-gliadin (57-89) as 38 injections of 1 µL each. EGCG was injected into buffer alone as a control, and the results were subtracted from the EGCG/α2-gliadin (57-8) results. Each injection lasted 2 s and an interval of 300 s was maintained between injections. The cell was stirred at 750 rpm throughout the experiment.

4.3.3 NMR Spectroscopy

All NMR experiments were performed on a Bruker Avance-III-HD 500-MHz instrument operating at a 1H frequency of 500.20 MHz using a 5-mm Prodigy BBO BB-1H/19F/D Z-GRD probe at temperature of 298 K. Solvent suppression was achieved using the Sinc1.100 excitation sculpting sequence. Data acquisition and processing for
all experiments were performed with Topspin 3.2 (Bruker, Billerica, MA) and MestReNova 10.0.1 (Mestrelab Research, Santiago de Compostela, Spain).

2D NMR samples were prepared with 0.25 mM 33-mer in 10 mM phosphate buffer, pH 6.8 with 15% DMSO-d₆ to aid with solubility. EGCG was added to protein-ligand samples 1 h prior to analysis to achieve a final concentration of 12.5 mM EGCG, or 50-fold ligand excess. All experiments were run at a temperature of 297 K with a spectral width of 20 ppm (¹H) and 170 ppm (¹³C). TOCSY experiments were performed using 256 increments and 24 scans for a total time of 63 hours and 15 minutes. Mixing times of 20, 45, 65, 75 and 110 ms were used during acquisition of the 33-mer alone and later merged for assignment of the spectra. A mixing time of 110 ms only was used for acquisition of the 33-mer/EGCG complex. ¹H-¹³C HSQC experiments were acquired using 256 increments and 136 scans for a total time of 16 hours and 18 minutes.

Partial assignment of the 33-mer was achieved based on previously published literature regarding chemical shift values for amino acids leucine, glutamine, proline, phenylalanine and tyrosine.

4.3.4 Dynamic Light Scattering

Dynamic light scattering (DLS) experiments were carried out using a Viscotek 802DLS with OmniSIZE software (Malvern Instruments, Malvern, UK). Initial samples of varying 33-mer and EGCG concentrations were screened for detectable colloids and to ensure experimental concentrations remained below the turbidity threshold.

EGCG was added to 12.8 µM α2-gludin (57-89) at molar ratios of 5-50 times excess EGCG in 10 mM sodium phosphate buffer, pH 6.8. Samples were prepared 1 h
prior to analysis, which was carried out at 37°C. Hydrodynamic radii \( (R_h) \) and estimated molecular weights were measured based on 10 separate measurements of 10 s each. The diffusion coefficients of the EGCG/33-mer aggregates formed were calculated using the Stokes-Einstein Equation (Equation 4.1) where \( k \) corresponds to the Boltzmann constant, \( T \) corresponds to temperature and \( \eta_0 \) corresponds to solvent viscosity.

\[
D = \frac{kT}{6\pi\eta_0 R_h}
\]  

\( \text{(4.1)} \)

Further experiments characterizing pH and concentration effects were carried out at pH 2.0, 6.8 and 7.5 with concentrations of 0.25 µM \( \alpha_2 \)-gliadin (57-89) and 0.4-50 times excess EGCG in 10 mM sodium phosphate buffer.

4.3.5 Circular Dichroism

Circular dichroism (CD) spectra were recorded in the far-UV region on a Jasco J-1500 CD spectrometer in a 0.1 mm path length cuvette. EGCG spectra were recorded at each experimental concentration as controls to subtract from the corresponding EGCG/33-mer spectra. All spectra were averaged from 3 scans. Changes in secondary structure were calculated as changes in relative helicity using Equation 4.2.

\[
\frac{[\theta]_{222}}{[\theta]_{208}} = \text{relative helicity}
\]  

\( \text{(4.2)} \)

Further experiments characterizing pH and concentration effects were carried out at pH 2.0, 6.8 and 7.5 with concentrations of 0.25 µM \( \alpha_2 \)-gliadin (57-89) and 0.4-10 times excess EGCG in 10 mM sodium phosphate buffer using a 1 mm cuvette.
4.3.6  *In silico* Modeling

The structure of α2-gliadin (57-89) was predicted by I-TASSER.\textsuperscript{33-35} Atomic coordinates for EGCG were gathered from previously published literature.\textsuperscript{36} EGCG was manually moved and rotated to amino acid residues suggested to be involved with interactions based on NMR data using *Coot*.\textsuperscript{37} 33-mer/EGCG complex model were submitted to YASARA for energy minimization\textsuperscript{38} and the resulting model was analyzed using PyMol (The PyMOL Molecular Graphics System, Version 1.3).

4.3.7  Statistical Analysis

All analyses were repeated in triplicate (n = 3) and analyzed with one-way ANOVA and two-way ANOVA analysis for samples with varied pH experiments, then paired with Tukey’s test for honestly significant differences. Differences of $p < 0.05$ were considered significant. Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad, La Jolla, CA).

4.4 Results and Discussion

4.4.1  EGCG Interacts with α2-gliadin (57-89) to Form Insoluble Complexes

The formation of insoluble aggregates upon mixing the 33-mer and EGCG in sodium phosphate buffer (pH 6.8) are summarized in Figure 4.1. Lower concentrations of protein allowed the formation of colloidal particles; however, increasing the concentration of protein resulted in the formation of haze and eventual phase separation as determined by the turbidity exceeding the upper detection limits of the Viscotek instrument used for DLS. At a concentration of 25 µg/ml 33-mer, no particle aggregation
was observed even upon the addition of excess (100 x) EGCG relative to the 33-mer. This is in contrast to greater concentrations of the 33-mer (0.25 mg/mL), where as little as 5 times excess concentration of EGCG caused phase separation. At a 33-mer concentration of 0.05 mg/mL, the turbidity threshold was above 75 times excess EGCG, whereas doubling the protein concentration decreased the turbidity threshold by more than half, with phase separation occurring at 25 times excess EGCG.

**Figure 4.2** Phase diagram of EGCG/33-mer mixtures indicating turbidity thresholds at varying protein concentrations.

Cryo-TEM was used to visualize the size and shape of aggregates formed between the 33-mer and EGCG at 25 times concentration excess at pH 6.8. The micrograph (Figure 4.3) shows (a) supramolecular assembly of 33-mer nanospheres when alone in solution,²⁶ (b) EGCG forming “fluffy” aggregates characteristic of polyphenols in aqueous solution³⁹ and (c) multiple organizations of EGCG/33-mer co-precipitates. The particles in Figure 4.3c are uniquely heterogeneous in size and shape in comparison to
those seen in Figures 4.3a and 4.3b. The major conformations seen here are (i) round, dense aggregates that have been associated with EGCG/proline-rich salivary proteins and (ii) fibrillar, ribbon-like structures that suggest the formation of a gel-like structure as a result of protein-polyphenol crosslinking. These conformations have been noted previously upon interactions between poly(L-proline) and EGCG.

Figure 4.3 Cryo-TEM images of (a) the 33-mer, (b) EGCG and (c) EGCG/33-mer complexes.
4.4.2 Cooperative, Non-Specific Binding Interactions Induce Subtle Changes in Peptide Secondary Structure

4.4.2.1 Reaction Thermodynamics

The EGCG/33-mer interactions observed by DLS (Figure 4.2) and cryo-TEM (Figure 4.3c) were investigated further to elucidate the reaction thermodynamics and structural fate of the 33-mer during the formation of the observed insoluble complexes. Observation of the reaction thermodynamics by ITC showed that the 33-mer contains multiple binding sites, resulting in what appears to be a cooperative, biphasic reaction (Figure 4.4). The relatively small exchange of heat observed suggests that these interactions are non-covalent. Demonstrative of a multivalent interaction, these findings corroborate previous identification of localized binding sites on the B and D rings of EGCG. The A ring in EGCG’s structure has also been shown to interact with the 33-mer, though with less intensity (Chapter 3). The 33-mer also features 13 proline residues and 5 aromatic amino acids which represent potential interaction sites for polyphenols.\(^{40}\)

The primary endothermic phase observed suggests initial reactions are driven by hydrophobicity of the peptide and EGCG. Hydrophobic interactions between proline-rich proteins and polyphenols have been well-documented as occurring between ring structures within the peptide, such as aromatic rings and pyrrolidine rings which, in this case, can be found in the structures of phenylalanine, tyrosine and proline. The co-desolvation of the peptide and polyphenol in these reactions displaces water, creating an endothermic response.\(^{41}\) The weak exothermic reaction is likely derived from the formation of hydrogen bonds, which are able to form between peptide bond carbonyls and phenolic hydroxyl groups.\(^{42}\) The continuation of endothermic reactions to saturation
suggest further hydrophobic interaction, though rather than direct interactions between the peptide and EGCG, the increasing enthalpy may be due to EGCG/33-mer complexes aggregating.\textsuperscript{24}

Figure 4.4 (a) Raw ITC data demonstrating a biphasic interaction as EGCG is titrated into $\alpha_2$-gliadin (57-89). Sections (I-IV) correspond to areas of structural changes described by DLS and CD. (b) The raw data demonstrated a complex isotherm of both endothermic and exothermic reactions upon each injection. (c) EGCG titration into buffer yielded weak endothermic responses, which were subtracted from the data.
The stoichiometry of the endothermic reaction phases can be estimated to be \(~5:1\) (EGCG:33-mer) for the initial phase of the reaction based on the inflection point in (I). The formation of insoluble complexes between proline-rich proteins and polyphenols is most favorable at (polyphenol binding site):(protein binding site) ratios of approximately 1:1.\(^{24,42}\) Taking the 3 ligand binding sites and 18 potential peptide binding sites (proline and aromatic residues) into account, this figures into a (ligand binding site):(protein binding site) ratio of 0.83:1, a favorable condition for complex formation.\(^{24}\)

### 4.4.2.2 Insoluble Complexes Increase in Hydrodynamic Radius as a Function of EGCG Concentration

With understanding that the conditions of the ITC experiment resulted in the formation of colloidal complexes (Figure 4.2), we sought to characterize the evolution of complexes formed during ITC titration in terms of \(R_h\) and diffusion coefficient in a separate DLS experiment run at the same conditions as the ITC experiment (Figure 4.5).

The findings from ITC show that an endothermic reaction occurs as the molar ratio of EGCG:33-mer increases from 0-10, at which point the binding isotherm suggests that a point of saturation has been reached.\(^{30}\) The saturation of binding sites within a peptide by polyphenols has been described previously as the formation of a “polyphenolic coating”.\(^{43}\) Our data show only modest increases in \(R_h\) and MW are noted throughout this titration period. The stacking of phenolic rings onto proline and aromatic residues up to a point of binding site saturation has been documented with other proline-rich proteins with varying implications for particle size that are dependent on both the protein and the polyphenol. Where our data shows that particle size does not change upon
initial interactions with EGCG, other studies have shown decreases in particle size as measured by DLS at this stage. The rationale for the decrease is that complexation with polyphenols may cause proteins to become more compact.\textsuperscript{24,39}

**Figure 4.5** Dynamic light scattering reveals increasing (a) hydrodynamic radius and diffusion coefficient of EGCG/33-mer complexes as a function of increasing EGCG concentration. (b) This data was also used to estimate molecular weight of complexes. Measurements were taken in 10 mM sodium phosphate buffer, pH 6.8.
Differences in the formation trends of protein-polyphenol aggregates can be explained by differences in the experimental systems being studied. From the polyphenol perspective, compounds with more hydroxyl groups and greater degrees of galloylation and polymerization typically have greater affinity to interact with proteins, however, as demonstrated in Chapter 3, the structural orientation of these groups play a role in binding, as greater flexibility of molecules lends itself to cooperative binding, allowing polyphenols to act as multidentate ligands.\textsuperscript{44–46} The capacity of peptides to interact with polyphenols is dictated not only by the availability of binding sites, but also by primary sequence, which plays a role in steric hindrance as well as flexibility of the peptide chain. It has been shown that sequences high in proline and glycine favor polyphenol interaction,\textsuperscript{47} as do peptides that feature tandem repeats or repeating prolines in the primary sequence.\textsuperscript{30,48} Hydrophobicity of the peptide alone also influences interactions with polyphenols.\textsuperscript{49} Because EGCG has been used in many of the experiments that have shown peptides compacting upon interaction, we can deduce that the difference is driven by the peptide being studied. While the 33-mer does feature tandem repeats and a high concentration of proline (39\% of total residues), these prolines are not mutually adjacent as they typically are in salivary proteins. Moreover, the 33-mer lacks glycine altogether, causing it to likely be less flexible than those proteins usually studied by these methods and thus less capable of forming a measurably more compact structure upon initial interaction with EGCG.

Increases in $R_h$ and MW were recorded as EGCG concentrations increased beyond 10 times molar excess of the 33-mer. These increases corresponded to the weakly exothermic reaction measured by ITC between 10 and 20 times molar excess of EGCG.
One explanation for the observed exothermic response is the formation of hydrogen bonds within complexes, which serve to stabilize the complexes. However, the increase in $R_h$ suggests that crosslinking between EGCG/33-mer complexes via noncovalent interactions are beginning to occur as well. As binding site saturation is achieved within the peptide structure, EGCG molecules oriented towards the surface of the complex, or the “polyphenol coating” are able to bind to other EGCG/33-mer complexes causing the formation of intermolecular bridges, or cross-links.\textsuperscript{24} This phenomenon explains the sharp increase in $R_h$ and MW measured, which cannot be explained simply by an additive effect of EGCG alone continuing to bind to singular complexes.

In the second endothermic phase taking place between 20 and 40 times molar excess of EGCG, continuous growth of particle size is again observed, culminating in a stabilized maxima of 52.9 nm ± 3.5 from 35 to 50 times molar excess EGCG that corresponds to the stabilized $\Delta H_{obs}$ of part IV in Figure 4.4a.

4.4.2.3 Elucidation of Peptide Interaction Sites and Evidence for Structural Change

Cooperative binding mechanisms driven both by entropy and enthalpy similar to what was observed in the present study have been reported in the case of interactions between polyphenols and proline-rich salivary proteins.\textsuperscript{30,40,41,48,49} Polyphenols like EGCG have been shown to interact with multiple areas on a single peptide, causing the peptide to “wrap around” the polyphenols. This results in a modification of the structure of the protein, which was hypothesized based on the findings of the ITC experiment. 2D NMR experiments were performed in order to observe modifications to the spectral signals which can arise as a result of either direct binding with EGCG or conformational
changes induced by binding elsewhere on the peptide (Figures 4.6-4.12). Crosspeaks were assigned to amino acids based on previously determined chemical shift values and correlation patterns with adjustment for solvent effects and neighboring amino acid residues.31,51

The ¹H-¹³C HSQC experiment allowed for elucidation of structural changes as observed by changes in ¹³C chemical shifts (Figures 4.6-4.8). Superimposition of the EGCG/33-mer spectrum over the spectrum of the 33-mer alone shows slight changes in each nucleus, though more pronounced in the proton spectrum as the chemical shift spread is smaller and more sensitive to changes. The changes in the HSQC spectra as a result of EGCG addition are primarily 15-30 Hz upfield on f₂.
Figure 4.6 Full 2D $^1$H-$^{13}$C HSQC NMR spectra of $\alpha_2$-gliadin (57-89) before (gray) and after (color) addition of EGCG in 10 mM sodium phosphate buffer, pH 6.8 with 15% v/v DMSO-d$_6$. 
Figure 4.7 $^1$H-$^{13}$C HSQC comparison of aliphatic sidechain crosspeak region of $\alpha_2$-gliadin (57-89) with (color) and without (grey) EGCG in 10 mM sodium phosphate buffer, pH 6.8 with 15% v/v DMSO-d$_6$. 
Figure 4.8 $^1$H-$^{13}$C comparison of aromatic crosspeak region of $\alpha_2$-gliadin (57-89) with (color) and without (grey) EGCG in 10 mM sodium phosphate buffer, pH 6.8 with 15% v/v DMSO-$d_6$. 
As TOCSY measures proton chemical shifts along f1 and f2, it is more sensitive to subtle changes in chemical shift than the HSQC experiments (Figures 4.9-4.12). These data demonstrate primarily upfield shifts as a result of interaction with EGCG. These shifts can be attributed to binding rather than bulk or solvent effects, as the DMSO-d₆ signal remains unchanged between the two spectra. Our findings from ITC demonstrated that the interaction mechanism could not be fit to a one-site binding model, suggesting multiple binding sites. This is corroborated by these findings where changes in chemical shift are not isolated to specific residues. This is unsurprising due to the size and simplicity of the peptide in question, as well as the high frequency of residues that have been implicated as potential binding sites for polyphenolic interaction. Of the 33-mer’s primary sequence, 13 amino acids are proline and 5 possess aromatic R-groups; thus, 54% of the 33-mer’s amino acids are potential binding sites for EGCG.

Upfield shifts observed in the NMR spectra have been shown to result from the binding of aromatic rings to the peptide of interest, as has been shown previously.⁵⁰–⁵² The stacking of aromatic rings between two molecules causes the electron density of that area to increase, inducing the observed upfield shifts of the proton nuclei, which can be observed most clearly with the tyrosine signals shown in Figure 4.10.⁵¹ The greatest shifts noted appeared in the HN-Hα crosspeak regions shown in Figure 4.11, which suggest the importance of the peptide backbone take place as a result of (i) interaction with EGCG both as a potential area for hydrogen bond formation or (ii) overarching changes to the chemical environment cause by interaction, such as structural modification.⁵¹ In silico modeling was used to depict examples of the types of interactions observed with NMR (Figure 4.13).
Figure 4.9 Full 2D $^1$H-$^1$H TOCSY NMR spectra of $\alpha_2$-gliadin (57-89) before (gray) and after (color) addition of EGCG in 10 mM sodium phosphate buffer, pH 6.8 with 15% v/v DMSO-d$_6$. 
Figure 4.10 $^1$H-$^1$H TOCSY comparison of aromatic crosspeak region of $\alpha_2$-gliadin (57-89) with (color) and without (grey) EGCG in 10 mM sodium phosphate buffer, pH 6.8 with 15% v/v DMSO-d$_6$. 
Figure 4.11 $^1$H-$^1$H TOCSY comparison of sidechain HN and HN-Hα crosspeak regions of α2-gliadin (57-89) with (color) and without (grey) EGCG in 10 mM sodium phosphate buffer, pH 6.8 with 15% v/v DMSO-d$_6$. 
Figure 4.12 H-1 H TOCSY comparison of Hα/Hβ crosspeak regions of α2-gliadin (57-89) with (color) and without (grey) EGCG in 10 mM sodium phosphate buffer, pH 6.8 with 15% v/v DMSO-d6.
Figure 4.13 Computational modeling of EGCG binding sites on the 33-mer. (a) Peptide structure was predicted using I-TASSER. EGCG interacts with (b) proline residues and (c) aromatic residues via $\pi - \pi$ stacking, a hydrophobic interaction. (d) Amide-side chains on glutamine residues are available for hydrogen bond formation with the phenolic hydroxyl groups on EGCG’s ring constituents. (e) The loose, flexible structure of the random coil peptide may allow for EGCG to form hydrogen bonds with the peptide backbone’s amide and carbonyl groups.
Structural modification of the 33-mer upon interaction with EGCG was confirmed with CD (Figure 4.14). By calculating the relative helicity of the peptide over the course of titration with EGCG, we determined that the 33-mer undergoes a disorder-to-order transition during the final stage of interaction. Interestingly, at this point, increases in $R_h$ and MW have subsided (Figure 4.5) but heat is still absorbed by the system per ITC (Figure 4.4). This may be due to the desolvation of functional groups, as the peptide undergoes rearrangement within the EGCG/33-mer complex system.

The ability of EGCG to induce a conformational change in a protein upon binding has been shown previously, notably causing a similar disorder-to-order transition in proline-rich salivary protein IB-5, which shares structural similarities to the 33-mer in terms of molecular weight, primary amino acid sequence and natively unfolded structure. However, it should be noted that the conformational change that was observed in IB5 occurred much earlier on in the titration period (EGCG:protein = 6:1) and the experimental conditions differed in terms of pH and protein concentration.

Figure 4.14 Impact of EGCG interaction on secondary structure of 33-mer. $\theta_{222}/\theta_{208}$ was calculated for treatments as a measurement of changes in peptide helicity.
4.4.3 Complexes Form at Various Gastrointestinal pH Conditions

Among the differences in test conditions between these experiments and those previously characterizing proline-rich protein interactions with polyphenols is pH. As a primary interest in these interactions is their contribution to astringency in wine, these systems are often tested at acidic pH (~3.5). Further, variations in concentration have been explored that may affect the course of the reaction. An understanding of the effect that pH plays in terms of protein-phenolic interactions is essential in developing a potential therapy for any disease state involving the digestive tract. Each experiment to this point has been carried out at pH 6.8, characteristic of the duodenojejunal junction, where the symptoms of celiac disease tend to manifest.

In order to investigate the impact of pH throughout the digestive tract on the complexes formed, DLS and CD were run at pH 2.0, pH 6.8 and pH 7.5. The concentration of the 33-mer was increased tenfold for these experiments. Overall, the trends observed in terms of particle size and diffusion coefficient were not affected by pH, showing that increasing EGCG concentration result in the formation of insoluble complexes (Figure 4.15). The notable differences in particle size occur at lower concentrations ratios. Where increases in \( R_h \) was not observed in the initial experiments until after 10 times EGCG concentration was achieved, the increases in particle size observed here are immediate and dependent on pH. At pH 2.0, which represents the gastric environment, larger particles were observed to form upon the first EGCG addition, whereas the increase in particle size is more graduate for higher pH levels. This may be attributed to the solubility of the peptide as well as the stability of EGCG at each pH level. Nevertheless, as the titration proceeded in each case, the development of
similarly sized particles occurred at the same rate. The plateau of $R_h$ as EGCG:33-mer approached 50 suggests that the reaction proceeds in a similar fashion was what was discussed for lower concentrations.

![Graph](image)

**Figure 4.15** EGCG/33-mer aggregate particle sizes (a) increase as a function of EGCG concentration at pH 2.0, 6.8 and 7.5 and (b) decrease in diffusivity. Diffusion coefficients are influenced by EGCG concentration ($p < 0.0001$) whereas particle size is affected by both EGCG concentration and pH ($p < 0.001$) and the interaction between the two parameters ($p = 0.004$).
A non-significant change in peptide structure from disordered to ordered was observed at these increased concentrations (p = 0.08, Figure 4.16); however, the change was observed at only 10 times molar excess of EGCG, which is lower than the observed minimum concentration excess in previous experiments (Figure 4.14). This is may be due to a phenomenon known as “macromolecular crowding”, wherein the reduction in free water caused by increased concentration of macromolecules can induce protein folding and affect conformational stability. The potential for crowding to induce a conformational change in the peptide with lower concentrations of EGCG has important implications for this interaction, as an in vivo system would be much more complex than what has been tested in vitro with these experiments. Though a more complex environment would introduce competition for binding between EGCG and the 33-mer, these findings suggest that the presence of potential competitors and other molecules may initiate conformational changes with a lesser amount of available EGCG.

**Figure 4.16** Changes in relative helicity of α₂-gliadin 57-89 in the presence of 250 μM EGCG (EGCG:33-mer = 10).
4.5 Conclusions

Our findings demonstrate that interactions between EGCG and α2-gliadin (57-89) occur through four distinct energetic phases which correspond to the formation of insoluble complexes and result in structural modification of the peptide (Figure 4.17). The initial endothermic phase (I) of the reaction corresponded to hydrophobic interactions as EGCG stacks onto the 33-mer. Dynamic light scattering revealed increases in hydrodynamic radii of particles through the following weak exothermic phase (II), driven by polar interactions or hydrogen bonding, and further endothermic reactions (III) culminating in the reaction reaching a saturation point (IV). Structural changes to the peptide backbone were characterized by both 1H-1H TOCSY NMR and CD. Changes in chemical shifts within the HN-Hα crosspeak region of the spectrum suggested modification to the chemical environment through interactions with or refolding of the peptide backbone. Examination of the relative helicity of the peptide within the EGCG/33-mer complex by CD revealed that a structural change did indeed take place, increasing helicity as a result of a disorder-to-order transition.

The ability of EGCG to interact with α2-gliadin (57-89) and to elicit a conformational change on the peptide highlights the potential for polyphenols, such as EGCG, to be used as a nutraceutical approach to mitigating the symptoms and immune response associated with celiac disease. Celiac disease pathogenesis is based heavily upon the deamidation of glutamine within gluten peptide fragments and upon the recognition of epitopes containing proline and glutamine residues by immune cells. Each of these potential receptors, TG2 and HLA-DQ2, respectively, feature binding pockets specific to the structural characteristics of gliadin in terms of both amino acid sequence as
well as extended structuration. Minor changes to amino acids within highly immunogenic gliadin fragments have been shown to greatly decrease recognition by HLA-DQ2 and thus, have the potential to prevent an immune response. Although direct changes to the amino acid sequence (i.e. substitution) in vivo would be impossible, these findings support the potential for development of a post-digestion mechanism for blocking gliadin peptide recognition through sequestration of binding epitopes and structural modification of the immunostimulatory peptide. Further studies are needed to test the efficacy of these interactions against immune recognition both in vitro and in vivo.
Figure 4.17 Schematic representation of observed interaction and physical implications of EGCG/33-mer complex formation based on ITC, DLS, NMR and CD. Initial samples of α2-gliadin (57-89) exhibit $R_h$ (represented by dotted lines) that do not change significantly over the course of the endothermic reactions taking place during phase I of the titration. As EGCG stacks onto the 33-mer, evidence of weak exothermic reactions suggests the formation of hydrogen bonds and crosslinking between protein-polyphenol complexes, supported by increasing $R_h$. Further endothermic reactions coincide with continued increasing $R_h$ up to a point where particle size no longer increases, but NMR and CD suggest structural change to the peptide backbone.
4.6 Acknowledgements

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4.7 References


Bordenave, N.; Hamaker, B. R.; Ferruzzi, M. G. Nature and Consequences of Non-


Chapter 5  Green Tea Polyphenols Interact with Gliadin Peptides to Mitigate Primary Symptoms of Celiac Disease In Vitro

5.1 Abstract

(-)-Epigallocatechin-3-gallate (EGCG), the major phenolic constituent of green tea, has been reported to mitigate a number of inflammatory and hypersensitivity disorders in laboratory models, and has been shown to moderate pathways related to food allergies in vitro. Here, we sought to determine the impact of decaffeinated green tea extract (GTE) on the digestion of gliadin protein and production of smaller immunostimulatory peptides in vitro. Complexation of GTE and gliadin was confirmed by monitoring increase in turbidity as a result of co-precipitation upon titration of GTE into a gliadin solution. This phenomenon was observed again during in vitro digestion when gliadin was also exposed to digestive proteases pepsin and trypsin. SDS-PAGE revealed that GTE not only precipitated gliadin from solution, but also inhibited the hydrolytic activity of pepsin and trypsin, resulting in decreased production of low molecular weight gliadin digestion products. Enzyme inhibition assays revealed that GTE dose-dependently inhibits pepsin (IC$_{50}$ = 29 µg/mL) and trypsin (IC$_{50}$ = 88 µg/mL) in mixed and uncompetitive manners, respectively, with respect to substrate concentration. Using a Caco-2 cell model of the human small intestine, we have shown that complexation of gliadin with GTE reduces gliadin-stimulated intestinal permeability. Furthermore, gliadin-stimulated release of proinflammatory cytokines IL-6 and IL-8 was mitigated by the addition of GTE at a 1:1 ratio (gliadin:GTE). Our findings provide further support for the potential beneficial effects of GTE as an adjuvant therapy for celiac disease.
5.2 Introduction

Celiac disease is an autoimmune enteropathy that is triggered by ingestion of gluten, a glutamine- and proline-rich protein found in wheat, barley and rye.\textsuperscript{1} Pathogenesis begins with incomplete digestion of gluten proteins in the small intestine, which trigger an increase in intestinal permeability via the release of zonulin, a tight junction inhibitor.\textsuperscript{2} This increases paracellular transport of gliadin which, in addition to transcellular transport, allows an influx of undigested gliadins to the lamina propria.\textsuperscript{3–7}

The influx of gliadin to the lamina propria stimulates the release of proinflammatory cytokine IL-15, a proinflammatory cytokine that is clinically associated with celiac disease. IL-15 then signals the infiltration of lymphocytes to the submucosa, which kill the epithelial cells producing stress signals. Damage to the intestinal epithelial cells results in macroscopic changes to the small intestine including the formation of mucosal lesions, hyperplasia of intestinal crypts and atrophy of the villi.\textsuperscript{8} The loss of villous structure leads to a decrease in the surface area of the small intestine and impaired nutrient uptake, causing celiac disease to present a variety of extraintestinal manifestations relating to malnutrition including anemia and reduced bone density.\textsuperscript{9} In response to cellular damage, intestinal epithelial cells secrete a protein called tissue transglutaminase (TG2), which is normally associated with wound healing.\textsuperscript{10} TG2 deamidates glutamine residues on the gliadin fragments in the lamina propria, increasing their affinity to bind with human leukocyte antigen (HLA)-DQ2/8 major histocompatibility complex class II molecules on antigen presenting cells.\textsuperscript{11} Antigen presentation of gliadin and gliadin bound to TG2 results in the activation of CD4\textsuperscript{+} T cells.\textsuperscript{12} In addition to mediating the release of proinflammatory cytokines IFN-\gamma, IL-1\beta
and TNF-α, which contribute to degradation of the extracellular matrix of intestinal epithelial cells, CD4+ T cells stimulate the production of anti-gliadin and anti-TG2 antibodies by plasma cells. These antibodies allow the immune response to continue to propagate.

Despite affecting approximately 1% of the world population, there is no known cure for celiac disease and the only reliable treatment currently available to patients is lifelong adherence to a gluten-free diet. However, the gluten-free diet has been shown to be expensive as well as difficult to adhere to, with many individuals unintentionally ingesting gluten, in some cases unbeknownst to them, resulting in continued development of mucosal lesions in the small intestine. For this reason, the development of adjuvant therapies for celiac disease has been of interest. Among the proposed therapies are enzyme supplements that can be taken orally to further digest immunostimulatory epitopes, antagonists of specific celiac disease symptoms such as intestinal permeability and a synthetic polymer that binds to gluten in the lumen, preventing digestion of the protein and stimulation of disease symptoms and the autoimmune response.

The use of the synthetic binder, a polymer of hydroxyethyl methacrylate and sodium-4-styrene sulfonate (poly(HEMA-co-SS); BL-7010), has been shown to be a potentially effective strategy for mitigating the symptoms and immune response associated with celiac disease. Poly(HEMA-co-SS) functions by binding to gluten proteins in the lumen, making them resistant to digestion and absorption, which prevents the initiation of the celiac disease symptoms and the previously described immune response. The biological implications of poly(HEMA-co-SS) have been studied primarily
with gliadin, the more immunostimulatory subunit of gluten. Poly(HEMA-co-SS) has been shown to form insoluble complexes with gliadin in solution and cause a change in the secondary structure of the protein, both of which may contribute to the protective effects of poly(HEMA-co-SS).\textsuperscript{28,29,31} Further, \textit{in vitro} digestion studies showed that poly(HEMA-co-SS) supplementation hindered enzymatic hydrolysis of gliadin.\textsuperscript{28} Gliadin-mediated intestinal permeability can be reduced by poly(HEMA-co-SS) \textit{in vitro}, as evidenced by the preservation of cytoskeletal structures and tight junction (TJ) proteins when the polymer was added to Caco-2 cell cultures at a 1:1 ratio with \(\alpha\)-gliadin.\textsuperscript{28} When tested in gliadin-sensitive transgenic mice, gavage with poly(HEMA-co-SS) after gluten challenge protected against intestinal permeability and mucosal alterations including infiltration of intraepithelial lymphocytes and villous atrophy.\textsuperscript{28} These findings support the potential of gliadin sequestration as a possible adjuvant therapy for celiac disease.

While poly(HEMA-co-SS) is a synthetic polymer, compounds with protein-binding capabilities have been found to exist in nature and more importantly, in commonly consumed food products. A notable class of compounds that are able to bind proteins are polyphenols, a secondary metabolite found in plants that have been widely studied for their benefits to human health. Flavonoids are a class of polyphenols that are found in tea, which is not only the main source of flavonoids in the adult American diet, but is also the second most commonly consumed beverage worldwide.\textsuperscript{32,33} Protein-polyphenol interactions have been studied in terms of their role in the tactile oral sensation of astringency, wherein proline and aromatic residues within salivary proteins act as binding sites for hydrophobic interactions with phenolic rings.\textsuperscript{34} Protein structure has been shown to be critical to these interactions. High frequencies of proline allow
proteins to adopt extended structures such as a polyproline II helices, which expose the protein backbone. This reduces steric hindrance by larger R groups and allows greater potential for hydrogen bond formation between polyphenols and backbone carbonyl and amide groups. 34,35 Protein-polyphenol interactions can affect digestibility and bioavailability of proteins directly by sequestering proteins from digestive proteases as well as indirectly by inhibiting digestive protease activity. 36–38

Flavonoids have been studied for their potential in preventing or treating a variety of chronic conditions including obesity, cardiovascular disease and a variety of cancers. They have also been studied in recent years as a treatment for inflammatory bowel disease, a chronic condition characterized by inflammation of the gastrointestinal tract. 39,40 Flavonoids have been shown to ameliorate symptoms of inflammatory bowel disease (IBD), preventing damage to the small intestinal mucosa40,41 and suppressing the immune response associated with IBD via downregulation of necrosis factor(NF)-κB signaling in endothelial cells and TNF-α by peritoneal macrophages. 42,43 Secretion of proinflammatory cytokines IL-6 and IL-8 by intestinal epithelial cells has been shown to be reduced by epigallocatechin-3-gallate (EGCG), a major constituent of green tea. 44,45 These cytokines are important markers for intestinal inflammatory damage and are associated with intestinal permeability and intraepithelial lymphocyte migration, respectively. 46,47

IBD shares a number similar symptoms with celiac disease including intestinal permeability and elevated levels if intraepithelial lymphocytes, both indicative of mucosal damage and dysregulation of the immune system. 39,45 Moreover, the etiologies of each also feature genetic predispositions that explain part, but not all, of disease
development.\textsuperscript{39,46} However, celiac disease is unique in its requirement for exposure to gluten antigens, the specific environmental trigger for the disease.\textsuperscript{1} The beneficial effects of dietary flavonoids in the treatment of IBD may also apply to celiac disease, as the diseases share several symptoms including chronic intestinal inflammation and permeability of the gut barrier. Moreover, the ability of flavonoids to interact with proteins like gluten, disrupting digestion and absorption in a similarly protective manner as sequestrants like poly(HEMA-co-SS), suggests that the benefits of flavonoids may be twofold for individuals with celiac disease.

Previous studies from our group characterized the interactions between EGCG and $\alpha_2$-gliadin (57-89), a proteolytically stable digestive fragment of gliadin that features six overlapping immunostimulatory epitopes recognizable by antigen presenting cells with HLA-DQ2 MHC class II molecules. We have demonstrated that interaction with EGCG \textit{in vitro} results in the formation of insoluble aggregates via hydrophobic interaction, and that these interactions modify the structure of the peptides (\textit{Chapter 3, Chapter 4}). The present study describes the potential for green tea extract to be used as an alternative treatment for celiac disease by interacting with gliadin. We hypothesized that green tea polyphenols may block gluten toxicity by inhibiting the digestion of gliadin, thereby reducing its ability to initiate permeability and inflammation of the small intestine. Our objectives were to characterize the formation of gliadin-green tea polyphenol complexes \textit{in vitro}, elucidate the impact of green tea polyphenols on gliadin digestion and digestive protease function and determine the efficacy of green tea extract on gliadin-mediated intestinal permeability and inflammation. To our knowledge, this is
the first study to demonstrate the potential protective effects of green tea polyphenols against gliadin-mediated intestinal damage within the context of celiac disease.

5.3 Materials and Methods

Gliadin, pepsin, trypsin and Folin-Ciocalteu reagent were obtained from Sigma Aldrich (St. Louis, MO). Decaffeinated GTE was donated by Nature’s Sunshine Products, Inc. (Spanish Fork, UT). Per HPLC analysis performed by the manufacturer, the GTE used in this study contained 806 mg/g total polyphenols, 653 mg/g of which were catechins. EGCG was found at a concentration of 413 mg/g.

Gliadin was digested in vitro to form PT-gliadin as described previously. Gliadin was suspended in 0.2 N HCl at a concentration of 0.1 g/mL and stirred continuously at 37°C. Pepsin (2.0 g/L) was added to the suspension after 10 min, and the solution was left to stir for 2 h. After 2 h, the pH was raised to 7.4 with 2.0 N NaOH and 2.0 g/L of trypsin was added to the solution before an additional 4 h of continuous stirring at 37 °C. Enzymes were inactivated by boiling the solution for 30 min after completion of the mixing period. The solution was subsequently frozen at -80°C and lyophilized for use in the following experiments.

5.3.1 In vitro Digestion

The impact of GTE on the digestion process was tested by dissolving gliadin in 0.2 N HCl at 20 mg/mL with varying concentrations of green tea extract (0, 5, 10, 20 mg/mL) and with continuous stirring at 37 °C. Pepsin was added at 0.3 mg/mL and digestion proceeded for 2 h before raising the pH to 7.4 with 2 N NaOH and adding 0.3
mg/mL trypsin. The solution was then mixed at 37 °C for an additional 4 h. The reaction was quenched by boiling the solution for 30 min. Samples were then aliquoted into two—one saved as a suspension and the other centrifuged to separate insoluble particles from solution. These samples were further analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

5.3.1.1 SDS-PAGE

Suspension and supernatant aliquots from each digestion experiment were mixed with Laemmli sample buffer (Bio-Rad, Hercules, CA) and electrophoresed on Mini-PROTEAN TGX precast gels (Bio-Rad, Hercules, CA) containing 4-20% polyacrylamide. Gels were subjected to a constant voltage of 80 V for 15 minutes followed by 45 minutes of 100 V until the dye front reached the bottom of the gel. Gels were stained with Coomassie R-250 dye (Sigma-Aldrich, St. Louis, MO) and imaged using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

5.3.2 Co-Precipitation Profiling

Samples in the following experiments were prepared in 10 mM phosphate buffered saline (PBS) at pH 6.8. All experiments were completed in triplicate. PBS, PT-gliadin or native gliadin and GTE were all used as controls.

To analyze the precipitation of gliadins by GTE, gliadin (PT and native gliadin, final concentrations of 0 – 4 mg/mL) was added to GTE (0 – 1.5 mg/mL). Samples were prepared in 96-well plates and shaken at 300 rpm for 2 h prior to analysis.
Turbidity was measured at 400 nm using a Multiskan Go UV/Vis Plate Reader (Thermo Scientific, Ashville, NC). Controls were subtracted from each value. After turbidity readings, samples were removed from wells and centrifuged (10 min at 1300g). The supernatant was used for further analysis.

Percent precipitation of total polyphenols was measured using the Folin-Ciocalteu method. Soluble protein remaining in the supernatant was measured using the EZQ protein quantitation kit (Life Technologies, Grand Island, NY). Percent protein precipitated was calculated by comparing the protein content of the controls to the protein content of each supernatant.

5.3.3 Enzyme Activity

Inhibition of pepsin was measured using a commercially-available fluorimetric method (Molecular Probes, Eugene, OR). Pepsin (50 µL) was prepared in 10 mM HCl, pH 2.0 for a final assay concentration of 2.7 µg/mL and added to GTE (50 µL, 0 – 500 µg/mL) in a 96-well plate. A fluorogenic substrate (BODIPY FL® casein, 10 µg/mL, 100 µL) added to each well to start the reaction. The plate incubated at ambient temperature protected from light for 1 h prior to measuring fluorescence at $\lambda_{\text{ex}} = 485$ and $\lambda_{\text{em}} = 530$ using a Fluoroskan Ascent FL (Thermo Scientific, Ashville, NC).

The inhibitory activity of GTE against trypsin was measured using a modified version of the method developed by Liu and Markakis, where trypsin activity is monitored calorimetrically by the release of p-nitroanilide upon enzymatic cleavage of N-$\alpha$-benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA). A 40 mg/mL stock solution of BAPNA in dimethyl sulfoxide was prepared, then diluted to 400 µg/mL using
pre-warmed assay buffer (50 mM Tris buffer, pH 8.2, 10 mM CaCl$_2$) prior to analysis. A 0.2 mg/mL trypsin stock solution was prepared in 1 mM HCl (pH 2.5) with 2.5 mM CaCl$_2$, and was diluted to 16 µg/mL with 1 mM HCl prior to analysis. GTE (0 – 500 µg/mL) was added in 1 mL volumes to 2 mL of the BAPNA solution, and the reaction was started by adding trypsin to a final concentration of 2.7 µg/mL. The reaction was incubated for 10 min at 37 °C before being stopped by the addition of 0.5 mL 30% acetic acid. Absorbance was measured at 385 nm using a Multiskan Go UV/Vis plate reader (Thermo Scientific, Ashville, NC).

The mechanisms of pepsin and trypsin inhibition by GTE were measured by performing analogous experiments to those previously described, but with modifications. GTE was held at concentrations of 0, 50 or 100 µg/mL while substrate concentrations were varied from 0 – 2 µM BODIPY FL® casein for pepsin and BAPNA for trypsin. All experiments were repeated in triplicate.

5.3.4 Caco-2 cells

Caco-2 cells were donated by Lankenau Institute for Medical Research (Wynnewood, PA). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with sodium pyruvate supplemented with 10% fetal bovine serum, 1% non-essential amino acids, and 50 U/mL penicillin and 50 µg/mL streptomycin (Corning Life Sciences, Corning, NY). Cells were maintained at 37 °C in a 5% CO$_2$, 95% air humidified atmosphere. Cells were passaged twice weekly at 70% confluence.
5.3.4.1 Permeability

A transwell monolayer model was used to measure paracellular permeability as a function of transepithelial electrical resistance. Cells were grown on polycarbonate transwells (0.33 cm², 0.4 µM pore size; Corning Life Sciences, Corning, NY) at a density of 2 x 10⁵ cells/mL. Media was refreshed every 2-3 days for 21 days until cells reached confluence. On day 21, cells were treated with 1 mg/mL PT-gliadin, 1 mg/mL GTE or 1 mg/mL 1:1 GTE:PT-gliadin in DMEM. DMEM was used as a negative control. Transepithelial electrical resistance (TEER) was measured as Ω·cm² using a Millicell ERS-2 Epithelial Volt-Ohm Meter (Millipore Corp, Billerica, MA) prior to and immediately after treatment as well as 1, 3, 4 and 24h after treatment. This experiment was repeated in duplicate with three replicates of each treatment within each experiment. Results are expressed as percent of the baseline TEER value.

5.3.4.2 Inflammation and Proliferation

Cells were plated in 24-well plates at 2 x 10⁵ cells/mL and treated 48 h after plating with 250 µL of 1 mg/mL PT-gliadin, 1 mg/mL GTE or 1 mg/mL 1:1 GTE:PT-gliadin in DMEM. DMEM was used as a negative control. Media was aliquoted at 30 min, 1 h, 4 h and 24 h, then stored at -80°C until analysis. Levels of IL-6 and IL-8 were assayed in duplicate by electrochemiluminescence using a multiplex V-Plex kit from Meso Scale Discovery (MSD; Meso Scale Discovery, Rockville, MD). Sample concentrations were determined based on a standard curve generated by MSD Workbench software.
After 24 h of the aforementioned treatments, cells were trypsinized and counted using a hemocytometer to determine the impact of each treatment on cell proliferation over the 24 h period. Proliferation experiments were repeated in triplicate.

5.4 Results and Discussion

Luminal sequestration of gliadin proteins by synthetic polymer poly(HEMA-co-SS) has been shown to be effective as a potential therapy for celiac disease by preventing digestion and absorption of the protein, resulting in an absence of gliadin-mediated toxicity. The present study explored the potential of green tea extract as a sequestrant of gliadins, investigating the impact of polyphenol complexation on digestion as well as the stimulation of gliadin-mediated damage to the small intestine in vitro.

5.4.1 Gliadin Digestion is Inhibited by Green Tea Extract

Pathogenesis of celiac disease begins when partially digested gliadin peptide cross the brush border intact as a result of protected transcytosis or paracellular transport, which is increased as a result of zonulin-mediated intestinal permeability. Gliadin molecular weights fall between 28-55 kDa, but hydrolysis by pepsin and trypsin produces lower molecular weight digestive products (Figure 5.1).

In vitro digestion of gliadin in the presence and absence of GTE revealed that GTE inhibits digestion in a dose-dependent fashion and that the addition of GTE to the digestive process allows greater removal of gliadin proteins from solution by centrifugation (Figure 5.2). For each treatment (0, 0.25x, 0.5x, 1.0x GTE), electrophoretic separation was run on the sample before and after centrifugation.
Figure 5.1. Molecular weight profiles of native and pepsin-trypsin digested (PT) gliadin.

Figure 5.2 Dose-dependent precipitation and inhibition of gliadin digestion. Control gliadin shows complete digestion of the protein pre- and post-centrifugation. Supplementation of GTE caused decreased formation of low MW digestion products and complete removal of protein in the supernatant due to gliadin involvement in gliadin/GTE complexes.
In the control sample (Figure 5.2), complete digestion of the protein can be observed along with the majority of the sample remaining in the supernatant post centrifugation. The samples treated with GTE show incomplete digestion of the protein as noted by the presence of bands within the 31.0-66.0 kDa MW range. Further, centrifugation of the samples treated with GTE resulted in the absence of protein signal, suggesting that the majority of the protein in the sample was precipitated by GTE throughout the course of digestion. These findings suggest two separate mechanisms of digestion inhibition- substrate sequestration, wherein gliadins bound by GTE are protected from enzymatic hydrolysis, and direct inhibition of digestive proteases.

The absence of protein signal in the supernatant of only the centrifuged digestion samples that contained GTE suggests that interactions with GTE causes the formation of insoluble precipitates, making gliadin resistant to digestion and able to be removed from solution. To test this, we combined gliadin with GTE at different concentration ratios and measured turbidity and percent precipitation of polyphenols. Both native and PT-gliadins were tested to determine whether hydrolysis of the protein had an effect (i.e., if GTE is capable of precipitating gliadin that has already begun the digestive process). As the treatment in the cell experiments was a 1:1 ratio of GTE to gliadin, we measured the percent protein precipitation in the 1:1 sample of PT-gliadin.

It was determined that both native and PT-gliadin are susceptible to interaction with GTE, resulting in the formation of insoluble aggregates as measured by increased in turbidity (Figure 5.3). Infrared imaging of the GTE/gliadin complexes showed conformational differences between GTE/native gliadin and GTE/PT-gliadin. While GTE/native gliadin formed large, dense aggregates (Figure 5.3a), interaction with PT-
gliadin resulted in the formation of haze (Figure 5.3d). These conformational differences contribute to the differences in measured turbidities of the solutions, which are markedly different. While GTE/native gliadin demonstrates trends towards increased turbidity as a function of GTE concentration regardless of gliadin concentration (Figure 5.3b), GTE/PT-gliadin haze formation appears to be dependent on the concentrations of each, also resulting in an overall greater turbidity (Figure 5.3e). This is likely due to the differences in the molecular weight and particle size of the protein in solution prior to the addition of GTE, as turbidity measurements are affected by concentration and geometry of particles. Although the overall mass protein concentration is the same between samples, PT-gliadin features a greater number of individual particles per mg as a result of enzymatic hydrolysis, which explains the differences between the two samples.52

Analysis of the supernatant of both samples revealed losses in overall concentrations of polyphenols in each sample which, in combination with the loss of protein signal in SDS-PAGE, corroborates the idea that the precipitates formed are comprise both gliadin and GTE. In the GTE/native gliadin samples, maximum polyphenolic precipitation was achieved at a 1:1.5 ratio of GTE:gliadin (w/w), with 33.35% of total polyphenols being removed from solution (Figure 5.3c). This is in contrast to GTE/PT-gliadin samples, where phenolic precipitation was greatest at the lowest GTE concentration, with 72.53% of total polyphenols precipitated when the sample contained a ratio of 0.05:1 GTE:PT-gliadin (w/w) (Figure 5.3f). Similar to the results shown by SDS-PAGE, protein analysis of the supernatant showed that GTE is an effective sequestrant of PT-gliadin, precipitating 93.4% ± 9.2 of protein at an addition ratio of 1:1. (Figure 5.4).
Figure 5.3 GTE interacts with gliadins both before and after enzymatic hydrolysis. Precipitation profiles of (a-c) native gliadin and (d-f) PT-gliadin with GTE. Native gliadin was precipitated by GTE to form larger dense particles (a) compared to the haze formed by PT-gliadin (d), resulting in lower overall turbidity (b vs. e).
Inhibition of gliadin digestion and absorption via physical sequestration has been shown to be an effective strategy for mitigating celiac disease symptoms. This has been demonstrated by the administration of a polymeric binding agent, which can hinder enzymatic hydrolysis of the protein.\textsuperscript{28} In addition to demonstrating a capacity to bind to gliadin proteins \textit{in vitro} and prevent enzymatic hydrolysis in that way, GTE can also interact directly with digestive proteases pepsin and trypsin to inhibit their function \textit{in vitro}.

The inhibition of digestive proteases by dietary polyphenols has been studied with respect to the impact on digestibility and accessibility of proteins in the human diet. Inhibition has been shown to occur primarily through non-covalent interactions.\textsuperscript{53} The structure of the polyphenols studied has been shown to play an important role with respect to inhibitory activity, with increasing hydroxyl groups within a polyphenol structure being associated with greater affinity for interaction with trypsin.\textsuperscript{54} As a result, food sources of polyphenols are likely to exhibit varying degrees of inhibitory activity due to variation in polyphenolic profile. In general GTE is associated with trypsin

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\textbf{Figure 5.4}. GTE interaction with PT-gliadin results in co-precipitation of both the protein and polyphenols.
inhibitory activity,\textsuperscript{37,38} though conflicting data exists regarding whether GTE inhibits or activates pepsin activity.\textsuperscript{38,55}

The present study shows that GTE inhibits both pepsin and trypsin in a dose-dependent manner (Figure 5.5). The IC\textsubscript{50} of GTE for pepsin was 29 µg/mL, while the IC\textsubscript{50} for trypsin was 88 µg/mL, suggesting that pepsin is more sensitive to inhibition by GTE. The mode of inhibition for each enzyme was determined by Michaelis-Menten kinetic analysis of analogous experiments with varied substrate concentration and constant GTE concentrations. Kinetic analysis revealed the GTE exerts mixed inhibition on pepsin and uncompetitive inhibition on trypsin, causing decreased maximum velocity for both enzymes with increasing concentrations of GTE (Figure 5.6, Table 5.1). The increase in K\textsubscript{m} observed upon addition of GTE to pepsin suggests that GTE has a greater affinity for pepsin when it has not yet bound to gliadin. This can be characterized as mixed inhibition. Conversely, the decreasing K\textsubscript{m} observed for trypsin suggests that GTE is an uncompetitive trypsin inhibitor, more likely to interact with trypsin once trypsin has already bound to gliadin (Figure 5.7).\textsuperscript{56}

![Figure 5.5](image_url)

**Figure 5.5.** GTE dose-dependently inhibits (a) pepsin and (b) trypsin *in vitro*. Values are expressed as mean ± SEM from three independent experiments.
Figure 5.6. Kinetic analysis of GTE on (a) pepsin and (b) trypsin. Values are expressed as mean ± SEM from three independent experiments. BODIPY-casein and N-α-benzoyl-DL-arginine 4-nitroanilide were used as substrates for pepsin and trypsin, respectively.

Table 5.1 Kinetic analysis of GTE inhibition of digestive proteases.$^*$

<table>
<thead>
<tr>
<th></th>
<th>Pepsin</th>
<th>Trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$</td>
<td>$V_{max}$</td>
</tr>
<tr>
<td>GTE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
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<td>6.48±0.33$^a$</td>
</tr>
<tr>
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</tr>
<tr>
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<td>3.15±0.27$^c$</td>
</tr>
</tbody>
</table>

Mixed: $K_m^{GTE} > K_m$

Uncompetitive: $K_m^{GTE} < K_m$

$V_{max}^{GTE} < V_{max}$

$V_{max}^{GTE} < V_{max}$

$^*$Concentration of GTE is expressed as µg/ml. The units of $K_m$ are expressed as mmol/L Nα-benzoyl-DL-arginine for trypsin and µmol/L BODIPY FL casein for pepsin. The units of $V_{max}$ are expressed as µmol p-nitroaniline per min per mg protein for trypsin and pmol BODIPY FL per min per µg protein for pepsin. Values are expressed as mean ± the standard deviation of the mean of three independent experiments. Values in the same column not sharing common letters are significantly different (p < 0.05).
Figure 5.7. Schematic representation of inhibition of digestive proteases by GTE. Pepsin is inhibited through mixed inhibition, where the inhibitor (I) has a greater affinity for the enzyme alone (E) than then enzyme-substrate complex (ES). Trypsin inhibition is uncompetitive, with the inhibitor having greater affinity for ES than E.

Luminal sequestration of gliadin has been shown to be an effective strategy for mitigating celiac disease symptoms. Physical interactions between gliadin and sequestrants causes the gliadin to become resistant to digestion, preventing the formation of the lower MW digestive fragments that have been implicated in stimulating celiac disease-related immunogenicity and cytotoxicity.\textsuperscript{28,30} Overall, our findings confirm that the inhibition of gliadin digestion by GTE occurs as a function of two separate mechanisms- protein sequestration and enzyme inhibition (Figure 5.8).
5.4.2 Green Tea Extract Exerts a Protective Effect on Intestinal Epithelial Cells

The biological implications of complexation with GTE on gliadin-mediated permeability and inflammation of the small intestine were tested using pre-digested PT-gliadin mixed with GTE at a 1:1 (w/w) ratio and the Caco-2 cell line. Previous studies have shown that inhibiting the formation of immunostimulatory and cytotoxic digestive fragments of gliadin may be an effective strategy for preventing celiac disease symptoms;\textsuperscript{28,30} therefore, we chose to investigate the system from the perspective of toxic gliadin fragments having already been produced by digestive enzymes and GTE administered as a therapy post-ingestion.

During celiac disease pathogenesis, gliadin causes the disruption of tight junction proteins and polymerization of cytoskeletal actin, resulting in permeability of the brush border.\textsuperscript{2} This occurs when gliadin binds to CXCR3 receptors on the surface of the
intestinal epithelium, which signal for the release of zonulin, an endogenous mediator of
gut barrier function that triggers disassembly and downregulation of junction proteins.49
This phenomenon can be replicated using Caco-2 cells plated on transwells. Caco-2 cells
are derived from human adenocarcinoma cells, but serve as an effective in vitro model
for the small intestine and celiac disease. After 14-21 days of growth, these cells are able
to form a polarized monolayer expressing tight junction proteins and brush border
enzymes. Further, they are able to produce cytokines IL-6, -8, -15 and TNF-α, all of
which have been implicated in celiac disease pathogenesis and intestinal permeability.57
Changes in permeability were monitored by measuring the TEER of each monolayer in
comparison to the baseline value before the beginning of the experiment. A reduction in
TEER is associated with an increased in permeability; conversely, an increase in TEER
suggests improved barrier function.

Our findings show that GTE exerts a protective effect against gliadin-mediated
intestinal permeability (Figure 5.9). These protective effects can be observed as early as
1 h into the treatment, with both the GTE and 1:1 (PT:GTE) treatment demonstrating
similar increases in TEER, approximately 12% greater than baseline. Gliadin-mediated
permeability appears at the 1h time point and is maintained through 24 h, but the
protective effects of GTE are sustained. As gliadin stimulates intestinal permeability by
binding to the CXCR3 receptor on the surface of intestinal epithelial cells, a potential
mechanism for the protective effect of GTE against gliadin-mediated permeability is
sequestration of gliadin and prevention of recognition by the CXCR3 receptor.
Figure 5.9 Changes in TEER as a result of gliadin and GTE addition. Gliadin-mediated permeability is demonstrated by decreases in TEER. GTE supplementation increases TEER in the presence and absence of gliadin, suggesting an improvement in barrier integrity. Values in the same time point not sharing common letters are significantly different (p < 0.05).

In addition to intestinal permeability, gliadin stimulates mucosal damage in individuals with celiac disease. This damage can be characterized by the secretion of cytokines. IL-15 is a proinflammatory cytokine that is considered to be the hallmark of celiac disease, playing a critical role in signaling for the infiltration of intraepithelial lymphocytes to the intestinal mucosa. IL-10 is an anti-inflammatory cytokine that plays an important role in immune regulation, and is overexpressed in active celiac disease.

Changes in secretion of these cytokines were tested for, but the concentrations fell below the limit of detection of our assays. However, increases in IL-6 and IL-8 secretion were mitigated by GTE (Figure 5.10, Figure 5.11).

IL-6 is a proinflammatory cytokine associated with the aforementioned intestinal permeability observed upon gliadin challenge in celiac disease, and it has been found to be expressed in both acute and chronic inflammatory processes.
gliadin stimulated a significant increase in IL-6 production, which has been mediated by the addition of GTE at a 1:1 ratio. IL-6 production by cells treated with PT:GTE was less than even that of the control treatment, and the cells treated with GTE only did not show any detectable secretion of IL-6 within the first 4 h of the experiment, representative of the residence time of dietary components in the gut during digestion. The protective effects of GTE against IL-6 production are sustained over 24 h, even as IL-6 production by the control cells increases to similar levels as those treated with PT-gliadin. These findings reflect those of the intestinal permeability experiment, as IL-6 induces the expression of claudin-2, a tight junction protein that increases intestinal permeability and paracellular transport.\textsuperscript{43,60}

![Figure 5.10](image)

**Figure 5.10** Gliadin-mediated IL-6 production is downregulated by GTE. Experiments completed in duplicate, expressed as mean ± standard deviation. Values in the same time point not sharing common letters are significantly different (p < 0.05).
In addition to reducing IL-6 production, GTE also reduced the production of IL-8. The production of IL-8 in celiac disease pathogenesis is stimulated by gliadin binding to CXCR3, the same receptor responsible for signaling the production of zonulin. \(^{61}\) IL-8 is responsible for the recruitment and activation of intraepithelial lymphocytes in the small intestinal mucosa. Elevated intraepithelial lymphocytes in the mucosa is a histological marker used in celiac disease diagnosis. \(^{62}\) Figure 5.11 shows the protective effect of GTE against IL-8 production, which is elevated by PT-gliadin within the first 0.5 h of treatment. The suppressive effect of GTE addition to PT-gliadin treated cells is demonstrated throughout the course of the experiment, with PT:GTE-treated cells producing significantly less IL-8 than both the PT-gliadin and control treatments. The protective effect of GTE alone is apparent as well, based on similarly suppressed IL-8 production throughout the 24 h experiment.
With respect to the elevated levels of IL-6 and IL-8 in the control samples, it is important to note that each of the applied treatments affects cell proliferation and, thus, the apparent concentrations of inflammatory cytokines shown are likely affected by the number of cells. Cell proliferation experiments demonstrated that cell growth rate was modified by the presence of the treatment groups; however, the cell counts at the time of the experiment were not different from one another, validating the comparisons between experimental groups.

**Figure 5.12** PT-gliadin and GTE modify cell growth rates over the course of 24 h in comparison to the untreated control. Cells were initially plated with a seeding density of 2 x 10^5 cells/mL.

Mediation of proinflammatory cytokines such as IL-6 and IL-8 has been investigated as a treatment for inflammatory bowel disease, a gastrointestinal condition with mucosal symptoms similar to those observed in celiac disease. Green tea polyphenols and purified EGCG have been shown to mediate TNF-α-stimulated IL-8 release by IEC-6 cells as well as prevent DSS-induced IL-6 and TNF-α secretion in a murine model of ulcerative colitis. This protective effect translated to amelioration of DSS-induced colonic lesions.
5.5 Conclusions

Flavonoids such as those found in green tea have been studied for their efficacy in treating a variety of inflammatory disorders, including those localized in the gastrointestinal tract.\textsuperscript{42,63–65} Our findings support the potential for green tea extract supplementation to be used as an adjuvant therapy for controlling celiac disease. The protective effects conferred to intestinal epithelial cells \textit{in vitro} suggest that GTE can ameliorate gliadin-induced permeability and inflammation. As demonstrated here, this can occur through direct interaction with digested gliadin fragments, which results in the formation of insoluble GTE/gliadin precipitates. These interactions are also observed with undigested gliadin, which, in concert with the ability of GTE to inhibit digestive enzymes, results in decreased formation of the gliadin peptides implicated in initiating damage to the intestinal epithelium. Future studies should focus on applying this treatment method to a more complex system \textit{in vitro} or \textit{ex vivo} to determine the impact of GTE/gliadin interactions on the celiac disease response to gliadin as a whole, including the activation of gluten-sensitive immune cells.

5.6 Acknowledgements

Caco-2 cells were donated by Dr. James Mullin from the Lankenau Institute for Medical Research (Wynnewood, PA). This project was supported by a National Institute of Food and Agriculture Predoctoral Fellowship awarded to C. V. under Grant no. 2016-67011-24702, USDA Agriculture and Food Research Initiative.
5.7 References


6.1 Conclusions

Results from my studies suggest that dietary polyphenols exert protective effects against celiac disease through physical interaction with gliadin proteins. These interactions occur primarily between polyphenol galloyl moieties and a variety of protein binding sites including proline residues, aromatic residues and the peptide backbone (Chapter 3, Chapter 4). Interactions occur between polyphenols from green tea and both undigested and digested gliadins to form insoluble complexes with reduced ability to initiate celiac-related symptoms. However, complexation of gliadin with polyphenols prior to digestion results in decreased formation of gliadin digestive products. This is due to both digestive resistance of polyphenol/gliadin complexes as well as the inhibitory activity of polyphenols against digestive proteases. Complexation of gliadin by polyphenols results in protection of the intestinal epithelium through suppression of proinflammatory cytokines and prevention of gliadin-mediated intestinal permeability (Chapter 5). These effects are summarized in Figure 6.1.
Figure 6.1 Summary of research findings, comparing (a) celiac disease pathogenesis to (b) protective mechanisms of GTE. In celiac disease pathogenesis, (i) gliadin in the lumen is (ii) enzymatically hydrolyzed by digestive proteases. Hydrolyzed gliadins (iii) stimulate intestinal permeability and upregulate paracellular transport. This also (iv) stimulates the production of proinflammatory cytokines. Gliadin (v) becomes deamidated by tissue transglutaminase 2 in the lamina propria, allowing for (vi) antigen presentation and (vii) recruitment and activation of intraepithelial lymphocytes. The protective mechanisms of GTE demonstrated by this research begin with (i) gliadin and green tea extract in the lumen in vitro. (ii) Interaction between GTE and native gliadins prevents gliadin digestion through substrate sequestration and enzyme inhibition. (iii) Gliadins already digested by proteases can also interact with GTE. Included in these digestive products is (iv) immunostimulatory α2-gliadin (57-89), which interacts with EGCG, a main constituent of GTE to form insoluble complexes with modified secondary structure. These interactions occur along the peptide backbone, among other binding sites in the peptide including proline and aromatic R groups. (v) Interactions between GTE and enzymatically hydrolyzed gliadins prevents gliadin-mediated intestinal permeability and reduces cellular damage as measured by the secretion of proinflammatory cytokines IL-6 and IL-8.
My overarching hypothesis for these studies was that tea flavonoids interact with gluten proteins to form protein-polyphenol complexes via non-covalent interactions, and that the resulting complexes would demonstrate reduced ability to stimulate celiac-related symptoms in vitro. Initial investigations characterizing the interactions between dietary polyphenols and α₂-gliadin (57-89), an immunostimulatory digestive fragment of gliadin, showed that the molecular structure of the dietary polyphenols applied to the system would play an important role in the formation of protein-polyphenol complexes (Chapter 3). While all three of the phenolic compounds studied were able to interact with α₂-gliadin (57-89), interactions with epigallocatechin-3-gallate (EGCG) were found to be localized on the gallyl moieties of the compound, in contrast to an absence of localization and apparent steric hindrances that were observed with interactions between α₂-gliadin (57-89) and theaflavin. With both polyphenols demonstrating similar binding affinities for α₂-gliadin (57-89), EGCG was chosen for further study based on its observed interaction trends and prevalence in polyphenol-rich foods and beverages.

Further exploration of the interactions between EGCG and α₂-gliadin (57-89) (33-mer) revealed that the formation of EGCG/33-mer complexes occurs as a result of non-covalent interactions, namely hydrophobic interactions between polyphenolic rings and proline and aromatic residues as well as hydrogen bond formation between phenolic hydroxyl groups and amide and carbonyl groups from the peptide backbone (Chapter 4). The formation of insoluble EGCG/33-mer aggregates in vitro may play a role in preventing celiac disease pathogenesis by blocking immunostimulatory epitopes from recognition.
As EGCG is the main phenolic constituent of green tea, green tea extract (GTE) was chosen to measure the protective effects of dietary polyphenols against celiac disease \textit{in vitro}. I determined that GTE can prevent the digestion of gliadins into immunostimulatory and cytotoxic digestive products through direct interaction with gliadins, causing the formation of insoluble precipitates, as well as inhibition of digestive proteases. Enzymatically hydrolyzed gliadins showed reduced capacity to stimulate intestinal permeability and the production of proinflammatory cytokines IL-6 and IL-8 by Caco-2 cells when co-incubated with GTE, suggesting that the complexation of gliadin proteins by GTE may prevent gliadin from exerting celiac disease-related intestinal damage (Chapter 5).

The efficacy of luminal gliadin sequestration as an adjuvant therapy to the gluten-free diet in celiac disease has been demonstrated using a synthetic polymer that prevents digestion of the protein and subsequent intestinal damage.\cite{1,2} Overall, the results from my dissertation research suggest that dietary polyphenols may be applied in a similar manner, as they are also capable of interacting with and preventing digestion of gliadins \textit{in vitro}, mediating celiac-related symptoms and improving gut barrier function even in the absence of gliadin.
6.2 Future Work

These studies are the first to examine the impact of gliadin-polyphenol complexation on gliadin-mediated damage in a celiac disease model. As a result, there are many opportunities for further exploration of this topic, some of which are outlined in this section.

6.2.1 Elucidate Impact of EGCG Complexation on Immunostimulatory Capacity of α₂-gliadin (57-89) Ex Vivo and In Silico

Chapter 3 describes the formation of insoluble complexes between EGCG and α₂-gliadin (57-89), an immunodominant 33-mer peptide with six overlapping epitopes recognizable by class II human leukocyte antigens (HLA) DQ2 (DQA1*0501-DQB*0201) and DQ8 (DQA1*0301-DQB*0302). The results suggest that the immunostimulatory epitopes of α₂-gliadin (57-89) would be blocked from recognition by immune cells through interaction with EGCG. However, a clearer understanding of the biological impact of these interactions requires further study.

As I explained in Chapter 1, the deamidation of gliadin peptides is an important step in celiac disease pathogenesis. Deamidation occurs through interaction with endogenous enzyme tissue transglutaminase 2 (TG2) as it crosslinks with glutamine residues located one amino acid away from a proline residue. The deamidation of glutamine enhances recognition of gliadin by HLA-DQ2/8 MHC class II molecules significantly.³⁴ Measurement of the impact of EGCG complexation on the deamidation of the 33-mer by TG2 could be achieved by incubating the complexes with TG2 and using MALDI-TOF to characterize the reaction products.³⁵
A greater understanding of the impact of EGCG complexation of α2-gliadin (57-89) on the celiac disease immune response could be achieved by using gastrointestinal explant cultures. While individual cell lines and co-cultures provide important foundational information for disease research, the use of gastrointestinal explants provides superior understanding of inflammatory diseases. Treatment of celiac disease gastrointestinal explants with EGCG/33-mer complexes would allow us to determine whether EGCG complexation disrupts recognition of the 33-mer by HLA-DQ2/8 and if complexation affects the uptake of gliadin antigens and subsequent activation of gliadin-reactive T cells. A simpler method for testing this would be using a co-culture model, wherein intestinal epithelial cells such as Caco-2 cells are cultured with macrophages, though the specificity for gluten-reactivity would be impaired in comparison to the explant model. Biological markers of interest upon EGCG/33-mer challenge include IL-6, IL-8, IL-10, IL-15 and TNF-α.

Flavonoids have been shown to modify immune responses through a variety of mechanisms including disruption of redox homeostasis and inhibition of histamine release. From the perspective of physical interactions preventing immune responses, EGCG has demonstrated the ability to prevent uptake of ovalbumin antigens by antigen-presenting cells though direct interaction with ovalbumin proteins. In silico modeling of interactions between EGCG and α2-gliadin (57-89) could be used to determine whether the potential decrease in recognition of α2-gliadin (57-89) by HLA-DQ2/8 in the aforementioned gastrointestinal explant culture occurs as a result of the peptide’s involvement in EGCG/33-mer complex formation. In silico modeling of the complexes
can be used to evaluate how complexation with EGCG affects the binding affinity to form a HLA-DQ2/8 MHC class II complex.

6.2.2 Investigate Impact of EGCG Interaction on Other $\alpha_2$-gliadin Fragments

Digestion of $\alpha_2$-gliadin results in the production of other peptides apart from (57-89) that have been implicated in the initiation of specific biological responses in celiac disease (Figure 6.2). Using the methods described to characterize interactions between EGCG and $\alpha_2$-gliadin (57-89), it may be possible to elucidate further protective effects of EGCG upon binding with these peptides. Findings from these experiments may help define specific mechanisms behind the findings described in Chapter 5, where interactions between pepsin-trypsin digested gliadin and GTE was shown to protect against gut barrier permeability and IL-8 release.

\[
\begin{align*}
\text{FLGQQQQSFPQ} & \quad \text{apoptosis} \\
\text{QQQQQQQQQQQQQQQILQQILQQ} & \quad \text{permeability} \\
\text{PPYCTIVFGITNYR} & \quad \text{IL-8 release}
\end{align*}
\]

Figure 6.2 Additional specific peptides implicated in celiac disease.

6.2.3 Investigate Efficacy of Green Tea Polyphenols on Celiac Disease In Vivo

To further proceed towards clinical relevance, an additional objective would be to investigate the potential protective effects of GTE against celiac disease in vitro. Investigation of the potential protective effect of GTE against celiac disease in vitro would be a necessary objective for proceeding towards clinical relevance. A starting
point for *in vitro* testing would be a mouse model. HLA-DQ8/HCD4 transgenic mice are sensitive to gluten and demonstrate physiological responses such as intestinal permeability, the production of anti-gliadin antibodies and development of histological indicators for celiac disease (elevated intraepithelial lymphocytes, villous atrophy).⁹

Overall efficacy of gliadin sequestration by GTE against celiac disease symptoms could be tested by daily administration of gliadin that has been pre-treated with GTE to gluten-sensitized mice for three weeks. Pre-treatment of gliadin with GTE would mitigate interference of other dietary compounds interacting with GTE and allow the study to focus on the proof of principle. Control groups would include a gluten-free/GTE group and a gluten group. The mice would need to be sacrificed and their organs harvested 24 h after the last oral challenge. Net protein utilization would be assessed by measuring protein in the feces. Decreased protein utilization would indicate that gliadin is protected from digestion. The biological outcomes of interest in these experiments would be (1) prevention of intestinal permeability, (2) prevention of morphological abnormalities (3) decreased generation of anti-gliadin IgA. Permeability of the harvested small intestines could be measured using an Ussing chamber and a fluorimetric or radiolabeled substrate and recording the flux of said substrate over time. Histological changes could be measured by staining the harvested tissue and evaluating villous-to-crypt ratios under a light microscope. Anti-gliadin antibodies can be measured using an enzyme-linked immunosorbent assay.

Depending on the success of the initial mouse experiment, the application of GTE could be further explored from the perspective of dietary incorporation, where gluten-sensitized mice could be fed GTE via their water source and introduced to small amounts
of gluten in their diets. The efficacy of GTE as a treatment post-exposure could also be explored, where mice are exposed to gluten in the diet followed by a supplemental dose of GTE to determine whether luminal interactions between GTE and gluten can reduce celiac disease-related symptoms.

6.2.4 Observational Cohort Study on Dietary Polyphenol Intake and Mucosal Health of Individuals with Celiac Disease

The persistence of mucosal lesions and villous atrophy in individuals with celiac disease despite adherence to a gluten-free diet outlines the need for alternative treatments or adjuvant therapies against gluten ingestion. If GTE is shown to prevent celiac disease symptoms in the experiments described previously, it would be interesting to assess the potential protective effects of polyphenols for individuals with celiac disease that may already exist in the diet. This could be done through an observational cohort study wherein HLA-DQ2/8-positive participants with celiac disease are asked to keep track of their meals in a food diary, noting details such as where the meal was consumed and who prepared it (e.g., home, restaurant, acquaintance). After one month of the food diary, participants would then fill out a disease-related questionnaire asking about symptoms typically related to gluten consumption (gastrointestinal distress, bloating, diarrhea, headache) that they have experienced over the course of the last month. They would then be tested for biological markers of celiac disease including elevated anti-gliadin and anti-TG2 IgA and a duodenal biopsy would be collected for histological evaluation.

The food diary would be used to estimate the typical polyphenol intake of each participant, allowing for determination of any associations between polyphenol intake
and the clinical presentation of celiac disease symptoms despite attempted adherence to a gluten-free diet. The food diary would allow for determination of an individual participant’s behaviors as far as putting themselves at risk for inadvertently consuming gluten by eating at restaurants where cross contamination risk is greater, or by consuming foods that contain wheat, barley or rye but qualify as “gluten-free” per FDA regulations.\(^\text{10}\) Associations between a high polyphenol diet and decreased prevalence of mucosal lesions despite partaking in activities that put an individual at risk for gluten consumption may suggest a protective effect of a polyphenol rich diet for individuals with celiac disease.

### 6.3 Closing Words

Despite affecting millions of people, the only reliable therapy for celiac disease is adherence to a gluten-free diet, which still often results in unintentional ingestion of gluten and damage to the small intestine. My research suggests that individuals with celiac disease may potentially be able to turn to commonly consumed dietary compounds as a source of relief from gliadin-mediated symptoms.

### 6.4 References


APPENDIX

Methodological Support for STD-NMR Experiments

Figure 1. STD Experiment with EGCG and α2-gliadin (57-89) in 15% DMSO-d6/85% H2O. Reference (a) and difference (b) spectra of EGCG and α2-gliadin (57-89) at a 50:1 molar ratio in 15% DMSO-d6/85% H2O. The observed ligand signals in the difference spectrum appear as a result of interaction between α2-gliadin (59-89) and EGCG.

Figure 2. Difference Spectra of Phenolic Ligands. STD experiments on GA (a), EGCG (b) and TF (c) at 25 mM acquired in DMSO-d6 in the absence of peptide receptor α2-gliadin (57-89) result in very small ligand signal in the difference spectra. These negative controls demonstrate the presence of ligand signal in the experiments run in the presence of α2-gliadin (57-89) appear as a result of peptide-ligand interaction and saturation transfer rather than direct saturation of the ligands. In all experiments, the control difference spectra acquired with the ligand alone were subtracted from the experimental difference spectra.
Figure 3. STD-NMR of $\alpha_2$-gliadin (57-89) in the Absence of Ligands. Off-resonance (reference) (a), on-resonance (b) and difference (c) spectra of $\alpha_2$-gliadin (57-89) at a concentration of 0.25 mM in DMSO-$d_6$. Spectra are presented at 1:1:1 ratio. The presence of peptide signal in (c) results from the attenuation at the saturation point (1.8 ppm) and spin diffusion for the remaining signals.

Figure 4. Calculation of $T_1$ Proton Relaxation Time for Ligand EGCG. Inversion-Recovery was used to measure the relaxation time of EGCG signal in a sample of EGCG and $\alpha_2$-gliadin (57-89) at a 100:1 molar ratio in DMSO-$d_6$. 
<table>
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<tr>
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<tr>
<td>H-10,12</td>
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<tr>
<td>H-10',12'</td>
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<td>H-11</td>
<td>1.88</td>
</tr>
<tr>
<td>H-11'</td>
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</table>

Table 1. $T_1$ Proton Relaxation Times for Ligand EGCG. Values were calculated from Inversion-Recovery experiments (Figure S4). The 0.5-3 second relaxation rates of the EGCG protons ensure full relaxation of all of the protons within the 14-second recycle delay of the experiment.
VITA

Charlene B. Van Buiten

Education
Ph.D. Food Science, The Pennsylvania State University, University Park, PA 2017
B.S. Nutritional Sciences, University of Connecticut, Storrs, CT 2012

Selected Presentations & Publications

Grants
“Physicochemical Modification of an Immunodominant Gliadin Peptide with Dietary Polyphenols and the Potential Implications for Celiac Disease” (Lead Author and PI)
USDA National Institute of Food and Agriculture Predoctoral Fellowship
Total Award: $70,874 01/2016 – 01/2018

“Investigation of a Novel Polymer-Base Fining System for the Removal of Foxy/Native Aroma in Vitis vinifera Wines” (Lead Author and Co-PI)
Pennsylvania Wine Marketing & Research Board – Research, Marketing and Education Grant
Total Award: $12,960

“Physicochemical Modification and Sequestration of an Immunodominant Gliadin Peptide by Dietary Polyphenols” (Lead Author and PI)
The Pennsylvania State University College of Agricultural Sciences Graduate Student Competitive Grant
Total Award: $2,000

Selected Awards
2016 Harold F. Martin Graduate Assistant Outstanding Teaching Award Pennsylvania State University
2013, 2015 Robert D. and Jeanne L. McCarthy Graduate Teaching Award Pennsylvania State University