THESIS

PHYSICOCHEMICAL MODIFICATION OF GLIADIN BY BLACK TEA POLYPHENOLS: INSIGHT TOWARDS A POTENTIAL NUTRACEUTICAL THERAPY FOR CELIAC DISEASE

Submitted by

Paul Mathews

Department of Food Science and Human Nutrition

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Advisor: Charlene Van Buiten

Chris Gentile
Jean Chung

ABSTRACT

PHYSICOCHEMICAL MODIFICATION OF GLIADIN BY BLACK TEA POLYPHENOLS: INSIGHT TOWARDS A NUTRACEUTICAL THERAPY FOR CELIAC DISEASE

Celiac disease is an autoimmune disorder that affects approximately 1% of the global population. The pathogenesis of celiac disease is complex, involving the innate and adaptive immune responses. Exposure to gluten amongst genetically susceptible individuals initiates and propagates the disease process, with autoimmunity against endogenous tissue-transglutaminase enzymes manifesting intra- and extra-intestinal symptoms. Currently, the only mitigation strategy for celiac disease is an adherence to a gluten-free diet, which can be difficult to maintain.

Recent advances in synthetic and natural products chemistry may offer therapeutic alternatives to the total abstinence from gluten containing products. The overarching objective of our research is to develop a nutraceutical approach to treating celiac disease using dietary polyphenols from tea. Within this thesis, we used a multi-spectroscopic approach to show that black tea polyphenols, which are rich in theaflavins and other flavanols, interact with gluten proteins *in vitro* to form colloidal complexes that result in structural change to the protein. These changes have the potential to reduce the immunogenicity of gluten via interference with digestion, sequestration, and conformational changes which may reduce recognition of the protein by immune cells. The interactions investigated here offer promise as a nutraceutical, plant-based therapy to acute gluten exposure in susceptible individuals.

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CHAPTER I: LITERATURE REVIEW

1.1 Introduction

Gluten sensitivities have an estimated prevalence of 5% worldwide, with celiac disease accounting for estimated 1-2% of global population.[1] A range of sensitivities exist corresponding to different autoimmune and allergenic responses, with celiac disease (CD) developing the most severe chronic symptoms. The etiology of CD is a complex interplay of environmental triggers with innate and adaptive immune responses in genetically susceptible individuals. Despite the complexity of pathology, the immunostimulatory nature of gluten and especially the poorly digested subfraction of gluten, gliadin, is unequivocally associated with disease onset and progression.[2–6] The unique physicochemical properties of gluten proteins are implicated as the molecular basis of immunogenicity.

Gluten comprises proline-rich gliadin and glutenin subunits. In CD, partially digested gliadin peptides are implicated in disease onset and progression. Partially digested gliadin is a high-affinity ligand for tissue transglutaminase-2 (TG2), which further deamidates gliadin peptides.[2] Digested and deamidated peptides, as well as TG2-peptide haptens are strong ligands for MHC-II immune receptors expressed by individuals possessing celiac associated haplotypes (HLA-DQ8/2.5^{+}).[7] Digested and deamidated gliadin peptides have displayed increased immunogenicity over native gliadins,[89–11] therefore, approaches to disrupt digestion of gliadin or epitope recognition by MHC-II complexes may improve celiac disease outcomes.

To date, no known cure for celiac disease exists, however a range of treatments have emerged as potential intersectional and preventative therapies. Immune system modulation,[12]

exogenous protease supplementation,[13] synthetic peptide blocking[14] and natural products display potential to mitigate disease symptoms and progression.[7] Of these, natural products in the form of plant polyphenols offer a promising new avenue. Polyphenols, in addition to being safe to consume and associated with many positive health outcomes, interact non-covalently with digestive enzymes and proline-rich proteins. These physicochemical interactions may allow polyphenols to mask or sequester digested proline-rich gliadin peptides.

Previous work by our group with green tea polyphenols has shown promise as a novel nutraceutical therapy strategy.[3,15] In addition to increased popularity in the West, black tea contains a more complex oligomeric polyphenolic profile which may exhibit improved gliadin-binding potential. The objective of this literature review is to provide relevant background on the physiology and immunology of celiac disease, describe molecular basis of pathology and the implications for a nutraceutical therapy approach and review the established physicochemical properties of proteins and polyphenols within the context of celiac disease.

1.2 Gluten: A Wheat Storage Protein

Gluten is a class of prolamin storage proteins found in the endosperm of Triticacea family, namely wheat, rye and barley. These proteins are characterized as sulfur-rich, sulfur-poor, low molecular weight (LMW) and high molecular weight (HMW) prolamins and glutelins, with monomeric and polymeric forms. Gluten proteins contain highly conserved and repetitive motifs rich in glycine, glutamine and proline, which account for up to 70% of amino acid content.[16] Structurally similar but evolutionarily distinct analogues of these proline rich proteins are found in rice, maize and oats. Of note, wheat contains other water-soluble albumins and globulins which are not directly associated with CD but play other functional properties in food products.[16]

Gluten proteins can be further classified into their main subunits, glutenin and gliadin. These provide wheat doughs with desirable rheological properties such as extensibility and elasticity. Gluten can first be obtained by rinsing the starch away from wheat flour, followed by solubilization in dilute acid, with further ethanolic fractionation of gliadin and glutenin.[16,17] Glutenins are grouped by high and low molecular weight fractions (HMW, 70-90 kDa; LMW, 20-45 kDa).[16] These proteins form strong fibrillar networks, strengthened by disulfide bonds and give wheat-based doughs their strength and elasticity. Gliadins are globular proteins responsible for the plastic and extensible nature of doughs. Among gliadins, alpha, gamma and omega subfractions exist, with proline and glutamine concentrations of 16% and 36%, 15% and 35%, and 27% and 37%, respectively.[1] The presence of cysteine residues imparts strong disulfide bonding, and the hydrophobic nature of prolamins are both associated with the extensibility and strength of gluten.[16] The unique rheological properties make gluten a very useful protein in food technology, with applications as binders, fillers, encapsulations, foaming agents and emulsifiers.

The increasing prevalence of celiac disease and related wheat sensitivities has led some to suggest that modern wheat breeding has fundamentally changed the composition of gluten proteins, however data does not exist to support this claim. Wheat has been selected for these properties over millennia of agriculture, however, no major differences between consensus and repeat motifs have been identified between wild and cultivated varieties.[17] Further agronomic work has in fact shown a decrease in relative proportions of the most immunostimulatory gliadin proteins.[18,19] Conflicting data exist regarding the changes in micronutrient and protein content in modern cultivars.[20,21] The biggest

contributors to changes in modern wheat are attributed to fertilization and cultivar, though soil health and weather also play significant roles in nutrient composition of final grain products.[22,23]

When considering agronomic and genomic characteristics of wheat proteins as potential intervention points for celiac disease management, it is noteworthy that gluten proteins are coded for by over 100 genes.[24] Furthermore, given the hexaploidy nature of modern wheat, it is difficult to attribute immunogenicity to a single locus.[24] The heterogeneity of the protein, and existence of overlapping peptide epitopes must be considered when investigating molecular mechanisms for targeted treatment of gluten induced toxicity. Subsequent sections explain the physicochemical characteristics of gliadin peptides, molecular specificity of pathogenesis, and illuminate the potential of polyphenols to form non-specific interactions with gluten proteins.

1.2.1 Structural Properties of Gluten Proteins Lead to Immunogenicity

To better understand the mechanisms of gluten sensitivity, a closer look at the structure and behavior of gluten proteins is needed. Although different manifestations of wheat intolerance exist, and wheat contains many proteins, digested gliadin peptides are strongly associated with immunogenicity and have heretofore been the primary subject of investigation.[2,8,25,26]

The secondary structures of these gluten proteins, especially proline-rich gliadins, make them particularly resistant to digestion by the endogenous digestive proteases including pepsin, trypsin, and intestinal brush border enzymes[2,27]. The presence of proline and glutamine repeat motifs (Fig 1) form secondary structures containing rigid polyproline-II helices (PPII), as confirmed by multiple spectroscopic studies.[1,4,28,29] These structures are implicated in incomplete digestion and remain present following enzymatic digestion.[2,30]

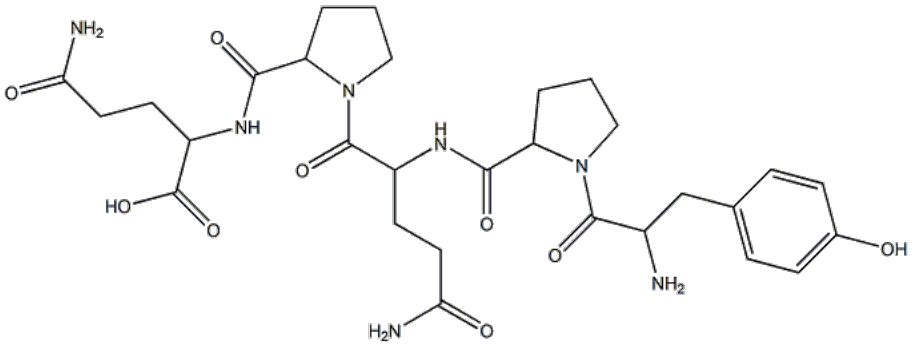


Figure 1. Characteristic gliadin structure. Example structure of a digested gliadin peptide containing repeat proline and glutamine amino acids. Due to the heterogenous nature of gliadin proteins and difference in digestion efficiency by endogenous enzymes, various structures are present in native and digested gliadin. Image courtesy of ChemicalBook CAS database. https://m.chemicalbook.com/ChemicalProductProperty_EN_CB3493924.htm accessed 3/24/22

Of the many digestive fragments, a 33-mer peptide (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF, residues 57-89, including many overlapping epitopes) (Fig. 2) and p31-43 peptide have been widely recognized as potent immunostimulatory fragments.[2,4,7,26]

Figure 2. Structure of canonical 33mer immunostimulatory gliadin.
This common digestion fragment is rich rich in proline, glutamine, tyrosine and phenylalanine, and has been established as high toxicity to epitope. Image generated in PepDraw (www.pepdraw.com)

In genetically predisposed individuals (HLA-DQ8/2.5^{+}) these PPII structures are strong ligands for MHCII presentation subsequent immune activation.[5,8] The immunogenicity of such digested fragments is increased by deamidation of glutamine residues to glutamine by TG2, an endogenous tissue repair enzyme.[31–33] Coupled with genetic factors for disease onset, specific physicochemical properties of gluten proteins are implicated in triggering of disease. The

interplay between host physiology and molecular features of gliadin are explained in the following section.

1.3 Celiac Disease

1.3.1 Symptoms

CD has a distinct genetic component; individuals possessing the HLA-DQ2.5^{+} and HLA-DQ8^{+} haplotypes (MHCII receptors on antigen presenting cells) may develop the disease.[32,34] Although intestinal damage has been associated with gluten in the absence of CD,[35] CD is the gluten sensitivity most associated with negative health effects, with symptoms ranging from abdominal distention and diarrhea to serious damage to intestinal mucosal architecture.[36] In severe cases, the oral ingestion of even small amounts of gluten trigger and propagate an inflammatory and autoimmune responses which target intestinal epithelial cells (IECs), culminating in severe villous atrophy and crypt hyperplasia.[37] This reduction of intestinal surface area can lead to nutrient malabsorption. The immune system also develops tolerance toward gliadin peptides and TG2 enzymes. The confirmation of intestinal lesions as well as serum titers of anti-gliadin/anti-TG2 antibodies are the gold standards in diagnosing the disease.[31]

In addition to intestinal damage, CD may manifest as extra-intestinal conditions such as dermatitis herpetiformis and gluten ataxia.[38,39] In the former, skin lesions appear.[39] In the latter, varying degrees of neurological pathologies have been observed, including mental fogginess and loss of coordination.[33,40] In both cases, research suggests that autoreactive antibodies are mounted to isozymes of TG2, resulting in attack of neurological or epithelial tissues. Additionally, some work has suggested a link between gluten sensitivity and other conditions like bone mineral deficiency[41] and thyroid dysfunction.[42]

Currently, there is no cure for CD other than a lifelong adherence to a gluten-free diet. Maintaining a gluten-free diet is difficult for many reasons. Gluten is widely used as an additive to foods, medicines and cosmetic products for its functions as a binder and filler. Gluten-free products may likewise be contaminated from shared equipment or ingredient cross-over. Although the market for gluten free and grain free products is growing, it is much costlier and difficult to consistently obtain gluten free products.[43] In addition to availability, persons with gluten sensitivities may face social difficulties in a food system structured around wheat products[43].

For most individuals, avoidance of gluten results in a reduction in symptoms and substantial recovery of intestinal atrophy, however, certain memory functions of the immune system have been shown to preserve T-cell tolerance to gluten, perpetuating intestinal pathologies.[8] Interestingly, the adaptive immune response associated with celiac disease does not generate memory B-cells, and antibody titers of auto immune antibodies generally drop when a gluten-free diet is adopted.[8]

1.3.2 Pathogenesis

The pathogenesis of CD results from a complex and not yet fully elucidated immune response involving both adaptive and innate pathways. Figure 3 displays the multifaceted nature of pathology.

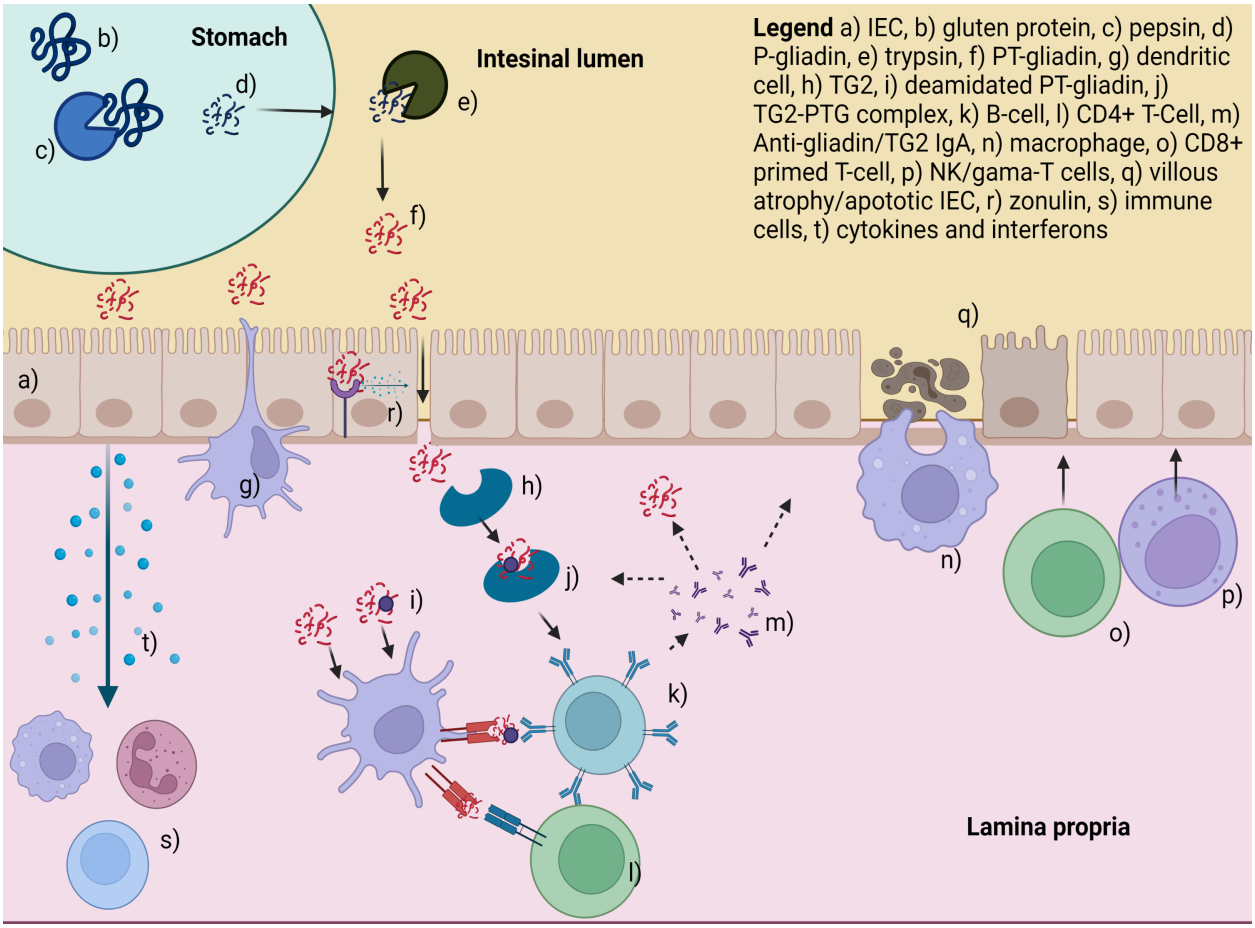


Figure 3. Diagram of celiac disease pathogenesis. Native gluten proteins are partially digested by pepsin (c) and trypsin (e) in the stomach and small intestine to PTG. PTG can induce innate inflammatory cascades (t), recruiting immune cells (s). From there they may pass through the epithelial layer to the lamina propria para- or trans-cellularly, which may be affected by zonunlin dependent signaling (r). PTG is recognized by HLA DQ2.5/8+ dendritic (g) or other APCs and present epitopes to B and T cells (k,l). TG2 (h) also binds to PTG, deamidating it (dPTG)(i). TG2-PTG and dPTG may also be recognized by B-cells. Recognition by B cells initiates an anti-gliadin/anti-TG2/anti-epithelial antibody response: this combined with innate inflammation leaves to recruitment autophagic IELs, such as NK (P), CD8+/y T cells and macrophages, to epithelial cells leading to progressive villous atrophy and crypt hyperplasia (q)

Gliadins which have undergone proteolysis by digestive enzymes including pepsin and trypsin (PT-gliadin) may interact with IECs to initiate an innate inflammatory response.[15,44] From the intestinal lumen, digested peptides can migrate to the lamina propria either via passive paracellular means or via active transcellular endocytosis. Once across the epithelial layer of the

intestine, gluten peptides become available for deamidation by TG2 and increased recognition by APCs. There is not consensus on the mechanism of transport, but it has been suggested CD71 mechanisms may protect gliadin from endosomal degradation, resulting in transcellular of gliadin peptides and IgA-linked gliadin complexes from the lumen to lamina propria.[45,46]. Increased intestinal permeability upon gluten challenge has also been demonstrated in vitro, via modulation of tight junction protein via CXCR3/MyD88/zonulin pathways,[44] suggesting paracellular transport of peptides. Additionally, native and digested gliadin have been shown to spontaneously form fibrils at low concentrations, further complicating physicochemical behavior, with potential effects on influence cellular adhesion and trafficking.[1]

PT-gliadin is also modified by tissue-transglutaminase-2 enzymes. The unique conformation of PPII structural motifs, as well as the primary structure arrangement of lysine and glutamine residues make gliadin an attractive substrate for TG2.[47] This molecular structure has displayed affinity for the digested gliadin peptides, such as the 33mer, than for TG2's natural substrate.[48] TG2 deamidates glutamine residues to glutamate acid by removal of the amine group.[14] The site of deamidation correlates to affinity increases for HLA-DQ2.5 and HLA-DQ8 haplotypes: in one study deamidation of glutamines at 4th, 6th and 7th positions along the peptide backbone were associated with increased affinity for HLA-DQ2.5^{+}, and deamidation of the 1st and 9th with HLA-DQ8^{+}.[31,32] The conversion of glutamine to glutamate creates a negatively charged side chain which increases binding into the HLA binding pocket, assisted by steric repulsion of proline residues.[32] In addition to increasing immunogenicity of gliadin peptides, TG2 becomes an autoimmune target in CD pathogenesis. Anti-TG2 IgA and IgG are hallmarks of CD, and illuminate the coordinated adaptive response of APCs, CD4^{+}/CD8^{+} T-cells, and B-cells.[8,32]

A key step in disease pathogenesis is recognition and presentation of the immunogenic peptides and haptens by antigen presenting dendritic cells (APCs) present in the lamina propria and lymph tissue centers (GALT). In celiac diseases patients, presentation of gluten peptides MHC-II initiates an adaptive immune response involving primed $CD4^+$ and $CD8^+$ T cells and anti-gliadin/TG2 IgA producing B cells, y-T-cells as NK subtypes.[34]

It has been suggested that initial loss of oral tolerance may be abetted by a type 1 interferon (IFN-alpha) response typically associated with viral infection, mediating a T_H1 response.[5] IL-15 has been widely implicated in initiating and propagating the immune response,[5,6,12] with multiple cell-type and tissue-specific responses contributing to histological damage.[6,35] The inflammatory mechanisms of IL-15 are varied and complex, but its importance in CD cannot be understated, as high concentrations produced by IECs are a reliable indicator of disease development.[6]

In summary, gluten peptides may increase intestinal permeability and upregulate innate inflammatory pathways.[1] In genetically predisposed individuals, an adaptive immune response is mounted against gluten peptides, tissue transglutaminase and IECs, resulting in histological damage and elevated serum antibodies. This summary of potential innate and adaptive mechanisms highlights the complexity of pathogenesis. Further detailed interpretation of immunology is beyond the scope of this research, but many reviews of the interplay between T- and B-cell immune tolerance are available.[5,8,34] Despite the complex physiological cascade, the structural properties of digested gluten, especially gliadin, are large determinants of innate and adaptive responses.

1.3.3 Potential Therapeutic Approaches

The pathogenesis of CD is a complex interplay of innate and adaptive immune response with many potential intervention points. One such approach is immune modulation and suppression. Anti-IL-15 and c-jun antisense siRNA showed ability to restore IL-15 homeostasis in biopsies of active celiac disease, potentially downregulating cytotoxicity of T-cells.[12] Antigen based vaccines have also been investigated. The NEXVAX2 epitope-specific vaccine has undergone Phase II trials with limited success in participant tolerance and dampening IFN-y and IL-2 associated responses.[49] However, further clinical trials have been discontinued due to insignificant improvements compared to placebo.[50] Vaccine-based therapies may be hindered by the complex interplay between innate cytokine responses and B-cell and T-cell responses unique to CD pathogenesis. Furthermore, the multitude of immunostimulatory peptides (themselves containing multiple overlapping epitopes) make generation of specific antigen vaccines difficult.[51]

Interfering with the recognition of gliadin peptides by enzymes and immune cells has also been explored. Co-administration of synthetic blocking peptides and gluten reduced TG2 deamidation activity by up to 35%.[14] Synthetic compounds have also been engineered to outcompete gluten proteins at HLA-DQ2 binding sites, with one group suggesting 100-200 fold increases in affinity for synthetic peptides. However, their *in silico* work did not translate to meaningful changes *in vivo*[52]. Simply locking of HLA/MHC-II is further limited by the many cell types participating in the CD cascade. Enzyme prolyl-protease supplementation to further degrade gluten proteins beyond the extent possible by endogenous digestive enzymes[13]. A comprehensive review on gluten-targeting therapies is available, which highlights modification of gluten digestion and interference of epitope presentation.[7] The approaches mentioned here highlight the molecular basis of CD and the potential points of intervention, namely; digestion,

antigen recognition, immune signaling. Although there is no "magic bullet" to prevent CD, emergent strategies may attack the disease on multiple fronts.

Our research is interested in exploiting the known mechanisms of polyphenol-protein interactions, especially the physical sequestration of proteins, in the context of CD.[30,53–55]Beyond the general health benefits of polyphenols, the physicochemical properties of polyphenols can be exploited to modify proteins and enzymes, potentially improving CD outcomes at multiple intervention points. Previous work has investigated the interaction between wheat proteins, enzymes and polyphenolic compounds, demonstrating the potential of various polyphenol sources to interact with proteins and digestive enzymes. Our group has previously investigated interactions between green tea polyphenols and gliadin proteins, showing promise of polyphenols to modulate CD pathogenesis via: modification and sequestration of proteins, improving intestinal permeability and dampening innate immune response (*in vitro*).[3,15] The following sections describe the nature of polyphenol chemistry as it relates to protein and enzyme interactions, adjacent health promoting effects, and their potential in the context of CD.[56–59]

1.4 Polyphenols

While most plants produce polyphenolic compounds as secondary metabolites, the focus of this research is on black tea polyphenols, with an emphasis on condensed polycyclic structures; catechins, theaflavins and procyanidins. Many excellent reviews on the chemical structure, pharmacognosy and plant physiology of polyphenols are available.[60–62] The following section highlights information most relevant to this research.

1.4.1 Biosynthesis

The biosynthesis of plant-based polyphenolics originates in the shikimate pathway. Starting with phenylalanine, the aromatic B ring and chromane ring are enzymatically generated via phenylalanine ammonia lyase. Ring A arises from sequential decarboxylation reactions of malonyl-CoA subunits. Final flavonoid synthesis involves generation of intermediates cinnamate via PAL, condensed naringenin chalcone via chalcone synthase and isomerization to flavanone precursors via chalcone flavone isomerase[62].

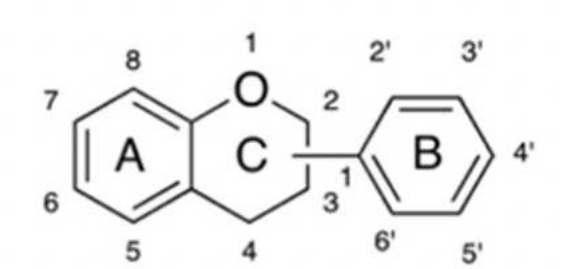


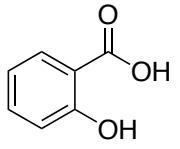
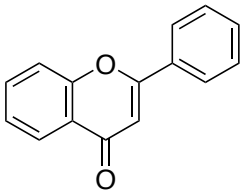
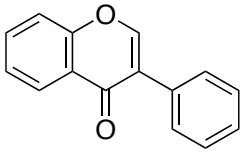
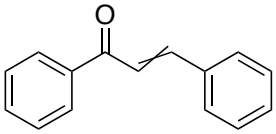
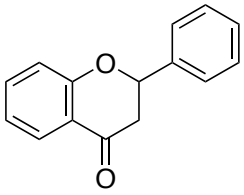
Figure 4. Flavonoid backbone. Further modification of this structure gives rise to thousands of flavonoid derivatives and related compounds. Image adapted from Van Buiten, 2018.

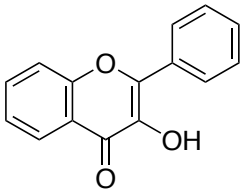
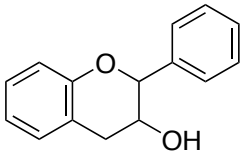
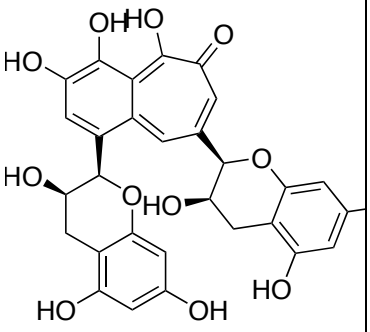
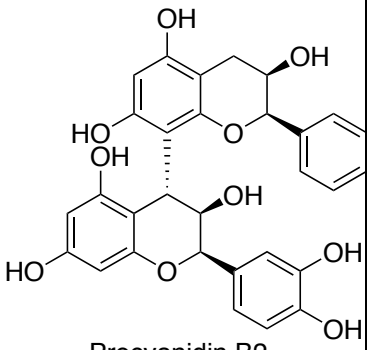
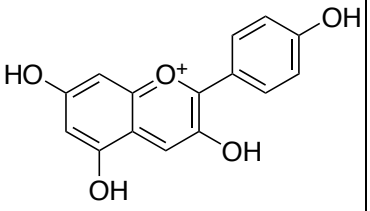
1.4.2 Sources and Structures

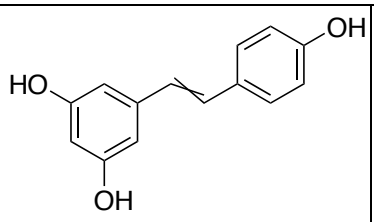
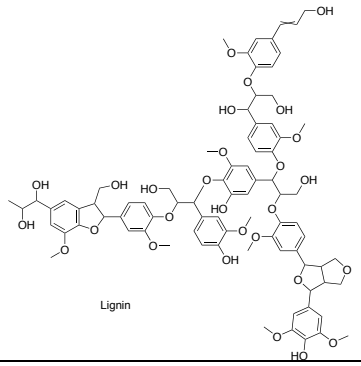
Monomeric, oligomeric and polymeric phenol-containing molecules are found throughout the plant kingdom in a variety of plant tissues. They are considered secondary metabolites, serving as molecular defense mechanisms against herbivory, bacterial and fungal infestation, as well as providing protection from UV radiation.[63] Common foods rich in phenolics include coffee, cacao, tea, berries and fruits, whole cereal grains (bran layer), and many culinary spices and herbs. As a part of foods, they exhibit many bioactive properties due to their interactions with proteins, gut flora, and cellular enzymes.[62] The base phenol unit contains an aromatic benzene ring with one or more

hydroxyl (-OH) moieties attached. From this building block a range of polymers and derivatives exist, with over 8000 known phenolic structures.[60] The classes of polyphenols found in tea include phenolic acids and flavonoids. Table 1 identifies common sources, classes and structures of phenolic compounds and their derivatives.

Table 1. Common sources, classes and structures of phenolic compounds and their derivatives. Structures generated in ChemDraw JS (PerkinElmer).

Phenolic Class	Example Structure	Compounds	Food Sources	Reference
Phenolic acids	 hydroxybenzoic acid	Derivatives of hydroxybenzoic and cinaminnic acid; gallic, vanilic, synringic acids, caffeic, ferulic, sinapic, chlorogenic acids	Cereals, oil seeds, legumes, fruits, vegetables	62,64
Flavones	 flavone	Luteolin, apigening glycosides, molymethoxylated tangeretin, nobilitn, sinensetin	Parsley, celery, citrus	63
Isoflavones	 isoflavone	Genestein, daidzein, glycitein; hydroxylated and methylated coumestans	Soy, Lucerne, clover	61,63
Chalcones	 chalcone	Isolequiritigenin, butein, isobavachalcone	Apples, hops, medicinal plants	65
Flavanones	 flavanone	Naringenin, hesperitin, eriodictyol, neohesperidose glycosides; prenylated, furanylated, pyranolated deirvatives	Grapefruit, kiwi, banana, cereal grains	63,66

Flavonols	 flavonol	Quercetin, kaempferol, myrectin; glycoside derivative	Onions, kale, leeks, broccoli, tea	61
Flavanols	 flavan-3-ol	Catechin, epigallocatechin, epigallocatechin gallate	Green tea	15
Oxidized flavonoids	 Theaflavin	Theabrownin, thearubigins, theaflavins and galloylated derivatives, theasinsens	Black/Oolong /Pu-ehr tea	67–69
Procyanidins (condensed tannins)	 Procyanidin B2	Procyanidin B1, B2, B3	Grapes, wine	70
Anthocyanins	 pelargonidin	Pelargonidin, cyanidin, delphinidin, peonidin, petuniden	Berries, eggplant, cabbage, cherries	63

Stilbenes	 resveratrol	reservatrol	Grapes	63
Lignins	 Lignin	Nitrogen and hydroxyl containing heteropolymers;	Woody plant growth	63

Of particular interest to this research are the oxidized flavanols and their derivatives. Black tea contains a subset of these flavanols, namely theaflavins, theabrownins and thearubigins, which are produced by oxidation and fermentation of tea leaves during processing. The convergence of B rings of two catechin/catechin-gallate molecules results in a benzotropolone molecule linking two flavonoid backbones (Rings A and C). Theaflavin can be substituted with 1 or 2 gallate moieties, resulting in 3-gallate, 3'-gallate, or 3,3'-digallate compounds.

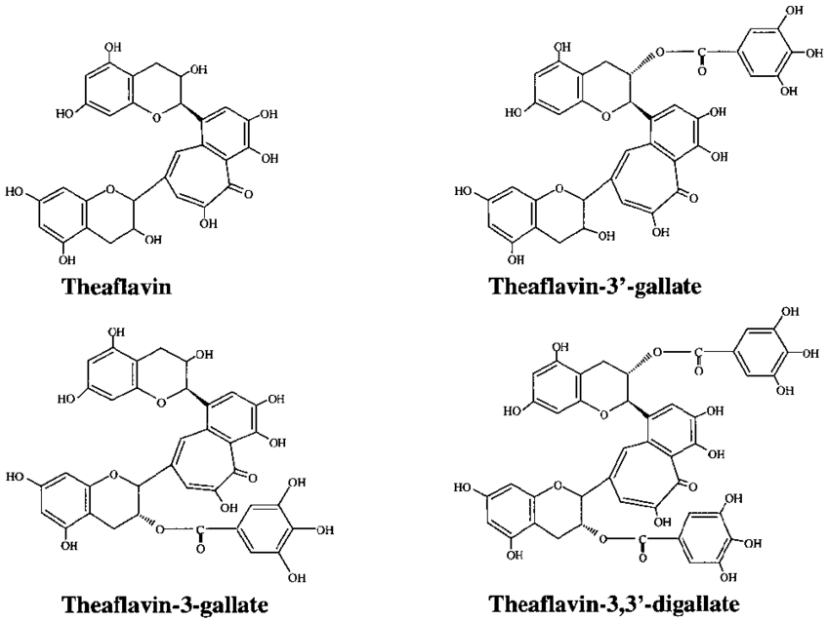


Figure 5. Theaflavin. Oxidation of catechins during black and oolong tea production produces benzotropolone structures with two flavonoid moieties. Theaflavin can be further modified by addition of gallate groups. (Wikipedia commons).

Thearubigins, theabrownins and other oxidation products are also formed in the enzymatic oxidation and condensation of green tea, as larger oligomeric structures based on the catechin building blocks.[67] The autooxidation of green tea catechins in digestion has also been shown to produce theaflavin-like molecules, the theasinensens, which may display similar bioactivity to theaflavins.[68] The effects of tea processing on the phenolic profile have been well elucidated in chromatographic studies.[71–73]

1.4.3 Bioavailability

The absolute bioavailability of polyphenols for target tissues via systemic circulation is still up for debate, but generally, polyphenols are known to have low systemic bioavailability. Their large molecular weight and polar side groups inhibit transcellular absorption in by

epithelial cells.[74,75] Furthermore, the relatively extreme pH changes and enzymes present during digestion can affect the structure and antioxidant potential of many polyphenols. [68]

1.4.3.1 Digestive stability

Although the flavonoid backbone is generally stable at acidic pH, acid-catalyzed hydrolysis of substituent groups (galloyls, esters and sugars) may remove some derivative moieties from flavonoid base structure. Catechin degradation has been observed during *in vitro* digestion, with galloyl groups especially susceptible to hydrolysis.[68,76] In one study, digestion of catechin-gallates resulted in losses of 60-100% of species, as well as formation of new theasinensin autooxidation products following alkaline phase.

A multitude of studies have investigated changes in antioxidant potential that may occur during digestion of phenolic compounds. Significant reduction in phenolic antioxidant potential through oral, gastric and duodenal phases of digestion, has been observed as measured by DPPH, FRAP and ABTS antioxidant potential assays.[77] In oral and duodenal environments, enzymes cleave glucosides to generate aglycone forms; colonic fermentation may result in ester cleavage and generation of smaller phenolic acid metabolic by-products. Antioxidant capacities of tea drinks and fruits showed decrease in total polyphenols and overall antioxidant capacity following *in vitro* digestion.[78,79] Correlation between total polyphenol content (TPC) and antioxidant potential appeared to decrease following digestion, indicating the change of biological activity despite retention (or increase) of Folin-Ciocalteu detectable phenolic groups.[78] A study on the stability of carob fruit polyphenols revealed digestion induced loss of antioxidant activity, with an overall increase in TPC. The study found phenolic acids to be more stable than flavanols and note the role of the food matrix in protecting against enzymatic and pH degradation.[76]

Of relevance to this research, oral digestive enzymes cleave glycosylated phenolic compounds to aglycone forms, but as catechins and procyanidins are not glycosylated, they pass through this step unaffected. In agreement with previous research, the authors also found that digestion conditions may also promote isomerization and racemization of chiral centers on polyphenols.[77]

Encouragingly, theaflavins have been shown to possess resilience to acidic digestion conditions, withstanding the expected cleavage of galloyl groups. Despite eventual degradation in simulated duodenal environments, theaflavins concentration remained relatively stable (80% intact) for up to 4 hours in alkaline conditions.[69] In summary, the chemical environment can degrade or alter flavonoid structure, but multiple studies support that flavonoids are at least partially resistant to digestive processes, allowing for their bioactivity to occur at intestinal sites and upon absorption.

1.4.3.2 Absorption

Phenolics are primarily metabolized in the small intestine and liver by endogenous phase II enzymes, including UDP-glucuronosyl transferase, sulphotransferase and catechol-o-methyltransferase.[63] Phenolics that pass through to the colon are further metabolized and modified by gut flora, where they can reenter enterohepatic circulation.[80] Gut flora metabolizes polyphenols into various metabolites, including cleavage products, further conjugates and fission products, which can be either reabsorbed or excreted.[81]

The generally low bioavailability of phenolic compounds may be attributed to their large steric bulk and hydrophilic hydroxyl groups. No specific receptors for catechins have been observed on intestinal cell membranes, with most absorption being via passive diffusion.[81] Once in cells, efflux systems including multidrug resistance-associated protein (MRP) pumps and P-

glycoprotein mediated mechanism pump polyphenols back into the luminal space,[81] limiting the amount that transverses the basolateral membrane and enters lamina propria. In one *in vitro* study, the bioavailability of back tea theaflavins ranged from an apparent permeability coefficient (P_{app}) of 0.44 x 10^{-7} to 3.64 x 10-07 cm/s (P_{app} values lower than 1 x 10^{-6} indicate very low bioavailability), with an efflux ratio > 1.[74] This study observed increased expression of p-glycoprotein and related ABC super family transporters upon the exposure of Caco-2 IECs to theaflavin, indicating an increased efflux response. Theaflavins that were transported basolateral membrane were degraded and de-galloylated into simpler forms. The authors suggest that theaflavin may interact with membrane bound receptors to increase transcriptional activation of efflux transporters. The authors also note that enzymes traditionally associated with phase II metabolism were not expressed in the Caco-2 model, due to the absence of glucuronidated, sulfated and methylated derivatives.[74]

These results suggest limited applicability of polyphenols to be used in the tradition of Western medicine, where maximum bioavailability and systemic circulation is the goal. However the low bioavailability of flavonoid-type molecules may result in nano- or micro-molar accumulation of polyphenols at the luminal membrane of intestinal epithelial cells[30,74]. This potentially results in high local concentration effects of polyphenols in the intestinal environment, of particular interest for the polyphenol-protein interactions discussed in subsequent sections.

1.4.4 Protein-Polyphenol Interactions

1.4.4.1 Self organization

Polyphenols have been shown to self-associate using an isodesmic model, wherein the aromatic rings form stacks, aided by hydrogen bonding, hydrophobic and Van der Waals

forces.[3,7,82,83] This self-association may change available binding sites for protein-polyphenol interactions, change the reaction kinetics/stoichiometry and alter phase dependent solubility processes.

1.4.4.2 Molecular Basis of Interaction

Polyphenolic compounds interact with proteins and enzymes under physiological conditions with various non-covalent bonding mechanisms. This effect has been exploited in food product processing, with applications such as emulsifiers,[84–86] dough rheology adjuvants (green tea wheat dough paper), precipitating/clarifying agents,[87] and protein delivery systems.[84] These interactions have been explored with multiple analytical techniques, including NMR (STD-,COSY-,PGSE-), circular dichroism spectroscopy, dynamic light scattering, analytical ultracentrifugation, transmission electron microscopy, Viscometry, UV-Vis and fluorescence spectroscopy and molecular docking studies.[2–4,54,84,88,89] The cross utilization of these methods has produced interesting data on protein-polyphenol interactions, such as: specificity of binding sites,[84,90,91] formation of different oligomeric species,[92]free-energy changes of association,[91] affinity and disassociation constants,[93] viscosity,[88] changes in net-protein charge.[87]

The binding between proteins and polyphenols involves different types of interactions, namely hydrogen bonding between phenolic hydroxyl groups and amides and carbonyls; Van der Waals interactions between ring systems; ionic bonding between oxidized phenolics and charged residues; and certain covalent interactions between nucleophilic sulfhydryl or amide groups and phenolic electrophiles.[54,94]

1.4.4.3 Haze, precipitation and astringency

The formation of haze in beverages containing proteins and polyphenols, such as beer, has been attributed to formation of colloidal polyphenol-protein complexes. The overall colloidal system has been shown to be affected by: pH, with lower pH reducing haze by affecting protein solubility and isoelectric point; % of alcohol or organic solvent which affects hydrophobic binding; degree of polymerization (DP) affects the size of cross-linked networks, with larger or multi-hydroxylated phenol enabling a protein-polyphenol scaffold, and smaller phenols simply binding to proteins.[70,87] These interactions are quite consistent and display simple binding kinetics, allowing researchers to quantitatively assess haze formation by the measured addition of proanthocyanins (tannins) to beer.[87]

This DP-dependent phenomenon has also been demonstrated between globular proteins and polyphenols.[70,92] Interaction strength increased with degree of polymerization, with monomeric catechins and dimeric procyanidins displaying weaker interactions than large tannins. Procyanidin DP above 5.5 significantly increased binding affinity.[92] However DP>12 reduced precipitating capacity likely due to steric hindrance.[95] Concentration-dependent effects suggest positive cooperativity between binding sites, with dimerization events altering the affinity for subsequent protein agglomeration. It was also observed that affinity of polyphenols increased near the isoelectric point of the protein.[92] These findings highlight a few key processes relevant to subsequent enquiry into gluten-polyphenol interactions, namely that proline rich proteins interact with polyphenols to form colloidal haze.

The formation of protein-polyphenol aggregates can induce phase change beyond the colloidal phase, resulting in precipitation of proteins. This interaction has been exploited in the wine industry, where the deliberate addition of gluten precipitates soluble tannins, which can then be removed.[96] This high affinity interaction between polyphenols and proline-rich proteins

also explains the phenomenon of oral astringency. Proline-rich salivary proteins interact with polyphenols in food, reducing oral saliva amounts and precipitating onto taste receptors, resulting in the sensations of astringency.[54,88,94]

The mechanism for polyphenol-protein interaction can be explained by spontaneous monomeric polyphenol and protein binding, followed by continual addition and dimerization/polymerization of multiple proteins and/or polyphenols.[54] The level of precipitation was shown to be related to binding, but pH induced changes to protein conformation and redox potential of polyphenols displayed greater impact on solubility of aggregates than degree of binding alone.[54] The effects of pH cannot be understated as the changes in ionic environment change the chemical potential of the solution. Once a phase boundary is reached and the miscibility of substances changes, the reduced activation energy can induce spontaneous spinodal decomposition and sudden precipitation.[54]

1.4.4.4 Proline-Polyphenol interactions

Proline-polyphenol binding occurs via established protein-polyphenol interactions but may be strengthened due to the physicochemical nature of proline-rich peptides. Like mechanisms to polyphenol self-association, the heterocyclic nature of proline residues creates ring stacking interactions, largely driven by hydrophobic forces.[3] The aromatic amino acid phenylalanine may also participate with phenolic structures, though it is often buried within peptides and less accessible; conversely, charged residues such as arginine may strengthen the association via hydrogen bonding with hydroxyl groups.[54] Effect degree of polymerization affects available peptide binding sites appears to extend to proline-rich peptides, with larger polyphenols having a smaller number, n, than smaller polyphenols. Size of protein has also been demonstrated to affect degree of binding. In one study, a proline-rich 19-mer peptide was shown

to bind with more than 50x the affinity than a 7-mer of similar composition. The authors posit that the cooperativity of binding sites induces longer peptides to "wrap around" polyphenols at multiple sites. Dynamic light scattering (DLS) by the same authors evidences the formation of two classes of particles, one smaller d=~80nm and one larger d=~500nm, dependent on concentration of polyphenol.[54]

Studies of flavan-3-ols (catechins) and polyphenols have shown that monomeric polyphenol-protein complexes is dependent on initial protein concentration and ratio of protein to polyphenol, consistent with previous studies suggesting self-aggregating events. The degree of galloylation of the flavan-3-ol also affects particle size and aggregation, with more galloyl groups and higher concentration leading to more cross-linking, larger particles and greater precipitation than less galloylated catechins.[97] As colloidal formation, aggregation and precipitation are not unimolecular processes, it is also important to consider the self-aggregating properties of the protein, which may change binding sites and solubility. Gluten proteins have demonstrated self-association behavior,[1] complicating the stoichiometry of polyphenol-gluten binding kinetics.

The relationship between globular and proline-rich proteins and polyphenols has also been investigated from a food processing perspective, with potential for application in digestion solution environments. Girard *et al.* have investigated the effects of DP on wheat gluten-polyphenol interactions. Overall the study found that smaller catechins reduce dough strength, which they attribute to a primarily antioxidant phenomenon, whereas larger condensed tannins strengthened dough by crosslinking proteins.[70] The antioxidant capacity of the polyphenol may affect gluten dough structure by reducing disulfide (S-S) linkages.[98]

In a progressive study on the effects of tea polyphenols on the rheology of wheat noodles, improved rheological properties on wheat noodle with addition of all tea polyphenols was demonstrated. Using FTIR, tea polyphenols were shown to modify secondary structure of gluten proteins, encouraging α-helix formation. Consistent with previous studies, tea polyphenols reduced disulfide bonds and promoted polymerization of gluten proteins, with increased protein-chain dimensions and overall increases in dough strength observed.[99] Although these studies do not specifically address physiological solution behavior of polyphenols and proteins, they further support the model of consistent gluten-polyphenol interactions resulting in structural change to monomeric and polymeric peptides.

1.4.5 Health Effects of Tea Polyphenols

Many *in vitro*, *in vivo* and epidemiological studies have established the bioactivity of polyphenols toward useful human health outcomes. Excellent reviews on polyphenol bioactivity are available.[61,62,75] Some relevant findings are summarized below.

1.4.5.1 Antioxidant Effects

The antioxidant capacity of polyphenols is largely a function of conjugated electron systems present in aromatic hydroxylated compounds. The ability of hydroxyl groups to donate protons or H-atoms arises from the delocalization of charge throughout the conjugated system. Phenolic groups can remain stable despite being oxidized, and thus act as donors or quenchers for reactive oxygen species (ROS).[71,77] ROS are free radicals produced during normal metabolism and cellular activities. Although the body contains many endogenous enzymes to maintain redox balance, excessive ROS can overwhelm the innate capacities of the system, leading to oxidative damage to tissue[77]. Generally, an imbalanced redox environment is

damaging to most cells, with downstream negative effects on various physiological processes. ROS induced inflammation has been associated with increased risk for metabolic syndrome,[100] hypertension,[101] atherosclerosis,[102] downstream cardiovascular stress,[103] and cancer and mutagenesis.[104]

The intrinsic antioxidant potential of polyphenols presents a direct mechanism of ROS reduction, however multiple studies indicate that polyphenols may upregulate antioxidant enzymes and/or suppress pro-oxidant processes via stimulation of membrane bound or intracellular transcription factors. For example, fruit and tea polyphenols have been shown to inhibit NF-e2, a key transcription factor in expression of pro-oxidant catalase and glutathione peroxidase.[80] The ability of polyphenols to inhibit lipid peroxidation via lipid peroxidase inhibition may be associated with decreased risks of cellular membrane damage.[80] This intervention may reduce the formation of cell-penetrating lipid oxidation products, which may go on damage cellular metabolites and DNA.

1.4.5.2 Anti-Inflammatory and Immune Modulation

Another demonstrated benefit of plant polyphenols arises from modulation of the inflammatory immune response. The catechin EGCG has shown efficacy in mitigating adhesion and migration of $CD8^+$ cytotoxic T-cells via blocking or downregulating CD11b receptors, a key ligand in ICAM-I binding.[57] EGCG was also shown to impair chemokine production in TNF-α-stimulated Caco-2 cells, lowering transcription of inflammation-associated chemokines IL-8, macrophage inflammatory protein MIP-3a, and prostaglandin E_2 (PGE_2). Tea polyphenols can act as an inhibitory ligand for PPAR-y, a potent transcription factor in inflammatory pathways, as well as inhibitors of inflammatory pathway molecules of MAPK, JNK and NF-kB inflammatory signaling.[80]

1.4.5.3 Digestive Enzyme Inhibition

Polyphenols, especially flavonoids and their derivatives, have been widely investigated for their role in digestive enzyme inhibition. Indeed, some of the purported weight loss, anti-hyperlipidemic and anti-hypercholesteremic effects may be due to the inhibition of key amylases and lipases. Modulation of starch digestion, lipase binding by EGCG,[91] tea polyphenols binding with amylase, pepsin, trypsin and lipase,[91] as well as various flavonoid-enzyme interactions have been demonstrated.[93,105]

1.4.5.4 Synergistic effects

Theaflavins have been shown to improve efficacy selective toxicity of numerous cancer treatments, perhaps by modulating accessory pathways, improving cellular response or preserving the activity of the drug and its health benefits; such as with cisplatin.[106]

In summary, the antioxidant activity, transcription factor interactions, inflammatory ligand binding and enzyme inhibition give polyphenols potential to disrupt several physiological pathways associate with cellular oxidation, inflammation, metabolic syndrome, and carcinogenesis. As a result of anti-inflammatory and anti-oxidant mechanisms, polyphenols also promise to mitigate damage to many systems of the body, including the kidneys, skin and neurological tissues.[75]

1.5 Celiac Disease and Polyphenols

In addition to the anti-inflammatory and antioxidant capacity of polyphenols, the established interactions between proteins and polyphenols position black tea polyphenols as potential therapeutic devices in the maintenance and prevention of celiac disease pathogenesis.

Previous work by our group with similar compounds has applied these physicochemical interactions in the context of celiac disease, with promising results. Exploiting protein-polyphenol chemistry, green tea polyphenols (GTP) were shown to mitigate the gliadin induced inflammation *in vitro*. [3] In this study, the interactions between green tea compounds and gliadin proteins resulted in complexation and precipitation of protein. Using a Caco-2 cell model, co-administration of GTP upon gliadin challenge reduced intestinal permeability and dampened innate cytokine profiles associated with CD.[3]

A later study employing NMR, circular dichroism and dynamic light scattering, observed that EGCG-gliadin interactions induced structural change in CD-associated peptides. Building on previous finding of precipitation and improved epithelial integrity, these methods explored the potential of epitope masking or immunogenicity reduction via structural change to the peptides.[3] Beyond tea polyphenols, mitigation of gliadin immunogenicity in the presence of polyphenols has been observed, with one study showing artichoke and cranberry extracts providing the strongest effect.[59] Procyanidins have also been investigated and show promise in reducing gliadin toxicity via similar mechanisms.[30] Non-covalent binding between wheat gliadin and the flavanol quercetin has also been reported.[90] Comprehensive reviews about this topic can be found.[7,107]

The physicochemical behavior of polyphenols and gluten proteins as a means of CD mediation is a novel and exciting avenue of research. Many plant-based compounds have been investigated for their health effects and influence on proteins, but to date no studies using theaflavin-rich black tea have been performed in the context of CD.

CHAPTER II: HYPOTHESIS & OBJECTIVES

In the context of celiac disease, the physicochemical properties polyphenol-protein/enzyme interactions, as well as intrinsic health promoting effects of polyphenols offer the following potential therapeutic avenues:

1. Tea polyphenols interfere with native gliadin or digestive enzymes, resulting in the incomplete degradation of gliadin proteins to their immunogenic peptides
2. Sequestration of digested gliadin by protein-polyphenol binding may help shield APCs from gliadin peptides
3. Changes to electrostatic and conformation environment of gliadins induced by polyphenol binding may reduce immunogenicity to HLA-haplotypes.
4. Intrinsic anti-inflammatory and anti-oxidant properties, which may improve epithelial cell resilience to gluten challenge and dampen exaggerated immune responses associated with CD pathogenesis.

The objective of the present study was to characterize the molecular interactions between black tea polyphenols and gliadin as a basis of physicochemical reduction of gliadin toxicity in celiac disease. Our hypotheses were (i) black tea polyphenols precipitate gliadin proteins potentially reducing the concentration of peptide available as substrate for pathological cascades, and (ii) interaction may result in modification of the protein structure, which is implicated in CD pathogenesis. We tested this hypothesis by a series of in vitro experiments to examine the solution properties and conformational attributes of gliadin in the presence of black tea polyphenols.

CHAPTER III: MATERIALS AND METHODS

3.1 Materials

Gliadin (Spectrum, Gardena, CA) and black tea polyphenol extract (Pure Bulk, Rosenburg, OR) were used as the starting material for all experiments. Pepsin (1:10000, VMR, Solon, OH) and Trypsin (1:250, MP, Solon, OH) were used for *in vitro* digestion of gliadin. In all experiments MilliQ Ultrapure 18.2 Mohm water (Millipore, Billerica, MA) was used. Buffers for sample dilution were prepared from di-sodium phosphate (Millipore, Billerica, MA) and sodium monobasic phosphate (VWR, Solon, OH). Sodium hydroxide (Santa Cruz Biotechnology, Dallas, TX) and sulfuric acid (VWR, Radnor, PA)) were used to adjust pH. Sonication of samples was performed in a Branson 1800 sonicator (Fisher Scientific, Hampton, NH).

3.2 *In vitro* digestion of gliadin

The *in vitro* pepsin-trypsin digestion procedure was adapted from Van Buiten and colleagues.[15] Three independent replications were performed to generate pepsin-trypsin digested gliadin (PTG) for subsequent experiments.

In 50 mL centrifuge tubes, 600 mg +/- 1mg of gliadin was combined with 30 mL of 10 mM phosphate buffer (gastric conditions; pH 2) for final gliadin concentration of 20 mg mL^{-1}. Concentrated pepsin stock was added to the reach a final pepsin concentration of 0.3mg mL^{-1}. Samples were immediately vortexed and scraped down to push all aggregated gliadin into solution.

The samples were placed in preheated (37°C) shaking water bath (Precision Scientific, Winchester, VA). The samples were incubated with shaking for a total of 2 h at 37°C, and vortexed after 1 h. Samples were then removed from the shaking bath and the pH was rapidly

increased to 7.4 with NaOH to neutralize pepsin activity. Trypsin was then added to a reach a final concentration of 0.3 mg mL^{-1}. The samples were vortexed and scraped down again, and then returned to the shaking water bath for 4 h, with a vortex step at 2 h. After 4 h, all samples were removed from the water bath. Samples were incubated in boiling water for 20 min to deactivate enzymes and stop digestion.

Following digestion, samples were stored in 50 mL centrifuge tubes and frozen at -20°C. Frozen PTG solutions were lyophilized for 36 hours (HarvestRight, North Salt Lake, UT) until a dry powder was produced. The resulting powder was homogenized with a mortar and pestle and stored at -20°C until future experiments.

3.3 Inhibition of digestion by black tea polyphenols

The method for digestion of gliadin was modified by the addition of black tea polyphenol (BTP) extract to the starting digestion. 600 mg +/- 1mg of gliadin was combined with 0, 125, 300, or 600 mg of black tea extract. Samples were dissolved in 10 mM phosphate buffer (pH 2.0) for final gliadin concentration of 20 mg mL^{-1} and final ratios of 0:1, 0.25:1, 0.5:1, and 1:1 (BTP:PTG). BTP-modified digestion was conducted under the same times and conditions as previously described for gliadin alone.

After digestion and enzyme deactivation, all samples were separated into suspension, supernatant, and pellet aliquots. Samples were vortexed to homogenize, and 15 mL of sample was immediately aliquoted to new 50 mL tubes. The remaining sample was centrifuged at 3500 rpm for 5 min at 5°C and the supernatant and pellet were separated. All sample aliquots were frozen lyophilized for 36 hours. Samples were ground to fine powder using a mortar and pestle. Lyophilized samples were stored at -20°C for further analyses.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to assess effects of BTP on digestion of gliadin. In each gel, native gliadin was compared to PTG and black tea modified digestion products. Table 2 shows the well layout.

Table 2. Well layout of SDS-PAGE experiment.
Gel loading scheme: Sn=suspension aliquot, St=supernatant aliquot, Pt=pellet aliquot. To obtain 1mg mL-1 protein across all samples, the presence of BTP solutes was adjusted for by proportionally increasing weights of PTG samples containing BTP.

Well	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Sample (BTP:PTG)	MW Marker	Native Gliadin	PTG	0.25:1			0.5:1			1:1		
Subsample	n/a	n/a	n/a	Sn	St	Pt	Sn	St	Pt	Sn	St	Pt
Protein concentration (mg mL^{-1})	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Freeze-dried samples were resuspended in ultrapure water and combined with a loading buffer of 2x-Tris-glycine SDS buffer (Novex, Life technologies, Carlsbad, CA) and b-mercaptoethanol (95:5 v/v) to achieve final PTG concentrations of 1 mg ml^{-1}. Samples and MW standard (Novex, Invitrogen, Thermo Fisher, Carlsbad, CA) were loaded onto 4-20% tris-glycine gels (Novex WedgeWell, Invitrogen, Thermo Fisher, Carlsbad, CA), placed in a double-sided vertical mini-cell electrophoresis system (Invitrogen, Thermo Fisher, Carlsbad, CA) and covered with Tris-Glycine SDS 10x running buffer (Invitrogen, Thermo Fisher, Carlsbad, CA). Electrophoresis was performed with an initial voltage of 80V for 15min, followed by 125V for 80min until MW bands reached the bottom of the gel.

After electrophoresis, gels were fixed in a solution of 50% methanol, 40% acetic acid and 10% water for 1 h on a shaking plate at 150 rpm. After rinsing with ultrapure water, gels were stained with Coomassie Blue R250 (RPI, Mt. Prospect, IL) staining solution of 1% Coomassie

Blue, 50% methanol and 49% water for 2 h, followed by rinsing with a destain solution of 50% methanol, 10% acetic acid and 40% water for 4 h. Gels were rinsed and destained a final time with 10% methanol 10% acetic acid 80% water overnight. Destained gels were stored in dilute acetic acid for imaging. Gels were imaged EpiChemi^3 Darkroom (Analytik Jena GmbH, Upland, CA), and processed in VisionWorksLS (Analytik Jena GmbH, Upland, CA) and Image Studio Ver. 5.2 (LI-COR, Lincoln, NE). Coomassie Blue filter settings were chosen for image acquisition.

3.4 Co-precipitation of gliadins by BTP

BTP and PTG were co-incubated, and precipitation and haze formation were measured spectrophotometrically. Stocks were prepared from lyophilized PTG and BTP in 10mM phosphate buffer at pH of 6.8 at a range of concentrations (0.1-4.0mg mL^{-1}). This pH approximated the slight acidity of the duodenal environment. In a clear 96-well microplate, (VWR, Radnor, PA) 100 μL of each PTG and BTP sample was aliquoted to create an array of increasing concentrations. 100 μL of each sample was combined with 100 μL of buffer for later blank subtraction. The 96-well plate was shaken for 5 minutes and then transferred to a Biotek Synergy3 UV-VIS plate reader (Agilent, Santa Clara, CA). Data were acquired using Gen5.11 software (Agilent). Absorbance was measured at 400 nm, to assess turbidity and haze formation, based on a previous method.[15]

3.5 Polyphenol precipitation by PTG

Co-precipitation experiments were performed to measure the relative amounts of gliadin and black tea polyphenols remaining unbound in solution after the observed formation of haze and precipitation. The Folin-Ciocalteu assay for total polyphenols was used to determine the

concentration of polyphenols remaining in solution after co-precipitation with gliadin. A gallic acid solution was prepared at 2.0 mg mL^{-1} and diluted to create a 6-point calibration curve. BTP and PTG stocks were prepared in 10 mM phosphate buffer at pH 6.8. All samples contained 0.25 mg mL^{-1} BTP. For BTP and PTG samples, PTG was combined at concentrations of 0.25, 0.50, 0.75 and 1.0 mg mL^{-1} (1:1, 0.5:1, 0.33:1, 0.25:1 BTP:PTG ratio). Corresponding concentrations of PTG were created and subtracted from spectra during analysis. Samples were vortexed and allowed to co-incubate for 5 minutes at room temperature. Samples were then centrifuged at 3500 rpm for 5 min at room temperature. Supernatant was collected and used for analyses. 10 μL of supernatant was combined with 790 μL buffer and 50 μL Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO). This was protected from light and incubated for 5 min with shaking at 200rpm. 150 μL of sodium bicarbonate was added to the sample before incubation at 37°C for 30 minutes. Samples were plated in triplicate in a clear, non-treated 96-well plate (VWR, Radnor, PA) and absorbance was measured at 765 nm (Synergy3, Agilent). Data were acquired using Gen5.11 software (Agilent). Changes in gallic acid equivalents (GAE) corresponding to changes in solution polyphenols were calculated from the calibration curve.

3.6 Protein precipitation by black tea polyphenols

Reduction in gliadin concentration after co-precipitation with BTP was measured using the EZQ Protein Quantitation Assay (Invitrogen, Thermo Fisher, Eugene, OR). BTP and PTG stocks were prepared in 10mM phosphate buffer at pH 6.8. PTG was prepared at 2.0 mg mL^{-1} and diluted to create a 6-point calibration curve. All samples contained PTG 1mg mL^{-1}. For PTG and BTP samples, BTP was combined at concentrations of 0.25, 0.33, 0.5 and 1.0 mg mL^{-1} (0.25:1, 0.33:1, 0.5:1 and 1:1 BTP:PTG ratio). Corresponding concentrations of BTP were created and subtracted from spectra during analysis. Samples were vortexed and allowed to co-

incubate for 5 minutes at room temperature. Samples were centrifuged at 3500 rpm for 5min at room temperature. Supernatant was collected and used for analyses. 1 μL of sample was spotted onto EZQ assay paper, affixed to the 96-well cassette, and developed according to kit instructions. Assay paper was read on an infinite m200 UV-Vis/Fluorescence reader (Tecan, Switzerland) using Tecan i-Control software (Tecan, Switzerland. Acquisition parameters were excitation at 485 nm, emission at 590 nm, 10 reads per well, with 9 nm/20 nm excitation/emission bandwidth. Changes in PTG were calculated from calibration curve data.

3.7 Protein Structure Analysis

Circular dichroism spectroscopy provides a spectral fingerprint of proteins and peptides. The differential absorption of circularly polarized light, reported in mdeg, is dependent on protein secondary structures, and give insight into the relative proportion of helical, sheet, turn and disordered protein structures within a sample.[108] A circular dichroism spectropolarimeter (Jasco-J1100, Easton, MD) was used to collect spectral data on PTG or pepsin with and without BTP complexation. PTG, pepsin and BTP solutions were prepared in 10mM phosphate buffer at pH 6.8. These stocks were diluted to achieve final concentrations of PTG or pepsin of 0.1 mg mL^{-1} for optimal instrument sensitivity. PTG or pepsin were combined with BTP concentrations of 0.025, 0.05 and 0.1 mg ml^{-1} (0.25:1, 0:5:1, 1:1 BTP:PTG/PEPSIN). Corresponding concentrations of BTP were created and subtracted from spectra during analysis. Samples were vortexed and allowed to co-incubate for 5 minutes before being analyzed in a 1 mM quartz cuvette. 3 spectra were acquired and automatically accumulated in Jasco Spectral Acquisition software. For all analyses, the following parameters were used: wavelength: 260-190nm; scanning speed: 50nm min^{-1}; CD scale: 2000 mdeg/1.0 dOD; D.I.T.: 0.5 sec; bandwidth: 1.00 nm; data pitch: 1.0 nm; holder temp: 37 ^{o}C. Data were processed in JASCO Spectral Analysis

software. Blanks and control absorbances were subtracted and spectra were smoothed using the Savitsky-Golay algorithm with a 13-step deconvolution. Relative helicity (RH) was obtained by calculating the ratio of mdeg at 222 nm and 208 nm. For pepsin spectra, Jasco Secondary Structure Evaluation (SSE) software was used to assign relative proportions secondary structures.

3.8 Protein-Polyphenol Binding Affinity

Fluorescence spectroscopy gives insight into the binding behavior of fluorescent molecules. In this experiment, the intrinsic fluorescence of tyrosine (Tyr) and phenylalanine (Phe) amino acids present in gliadin was probed (Fig. 2). The ability of black tea polyphenols to quench intrinsic fluorescence when complexed to PTG gives insight into the interaction. Intrinsic fluorescence of PTG in the absence and presence of BTP was measured using an infinite m200 fluorescent plate reader (Tecan, Switzerland). PTG was prepared at 1 mg mL^{-1} in acetate-filtered 10mM phosphate buffer, with 68mM sodium chloride (Santa Cruz Biotechnology, Dallas, TX) at pH 6.8. Sodium chloride was added to improve spectral resolution and signal to noise (s/n). PTG sample was prepared at 1 mg mL^{-1} PTG, and PTG with BTP samples containing increasing concentrations of BTP (0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1.0 mg mL^{-1}). Blank samples containing only BTP were prepared for spectra correction. Samples and co-incubated at room temperature for 5 minutes and vortexed before analysis. 200 µL of each standard or treatment was plated in triplicate in a black 96-well plate. To optimize spectra, a range of excitation wavelengths was tested. 275 nm was selected for the highest resolution (s/n of PTG versus buffer) spectra. Emission data was collected from 300-500nm. Sample fluorescence intensity was correct by subtracting buffer or BTP controls.

3.9 Statistical analysis

All data are presented as the mean +/- SEM and completed in triplicate unless otherwise indicated. Normality of residuals and homogeneity variance of data was assessed using Shapiro-Wilkes and/or Levene's tests in R studio software. Non-parametric analysis of variance testing was performed with Kruskal-Wallis and Dunn's multiple comparison tests in GraphPad Prism v. 9.0 (GraphPad, San Diego, CA). Values of $p < 0.05$ were considered significant.

CHAPTER IV: RESULTS AND DISCUSSION

4.1 SDS-PAGE of digestion products suggests inhibition of gliadin digestion by black tea polyphenols

Treatment of proteins and peptides with denaturing agents disrupts quaternary and tertiary protein structures, which can then be analyzed using SDS-PAGE. The forces that determine protein conformation devolve from denaturation, resulting in linear chains with charged residues which can be separated on a gel with electric current. Using a molecular weight (MW) standard (Novex, Invitrogen, Thermo Fisher) with known masses, the relative abundance and distribution and MW of proteins or peptides in a heterogenous mixture can be estimated. This is done by examining density and migration distance of proteins/peptides, with denser bands representing higher concentrations, and distance of migration inversely related to size of protein/peptide. The denaturation step of this technique proved especially useful when analyzing poorly soluble and self-aggregating gliadin protein products. Moreover, change in electrostatic environment from denaturing agents disturb the non-covalent interactions between PTG and BTP, allowing for characterization of proteins in digestion solutions.

By examining gels, the degree of digestion of gliadin in the presence or absence of BTP was qualitatively determined. The relative abundance of high or low molecular weight peptides informed the extent of proteolysis of PTG compared to native, undigested gliadin. Among all three digestion trials, gels displayed comparable patterns in terms of distribution and concentration of protein bands.

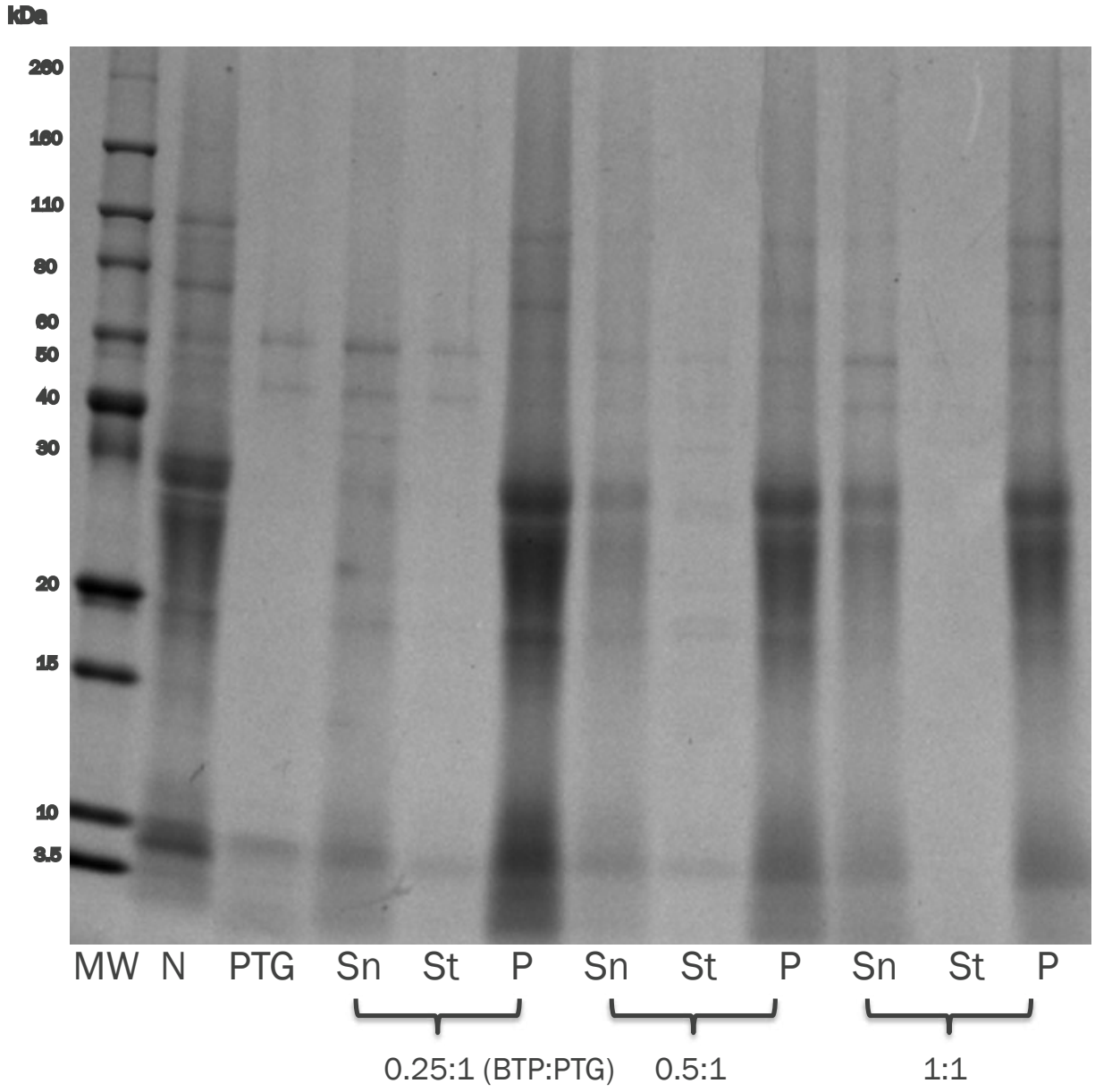


Figure 6. Gliadin digestion products.
Representative SDS-PAGE of gliadin digest (PTG) with and without BTP compared to native gliadin (N) (n=3). In the absence of polyphenols native gliadin is fully digested to small (<3.5kDa peptides), which can diffuse out of the gel. Centrifuged pellet samples, containing most of the protein, suggest digestion inhibition. The similarity to native gliadin and presence of higher MW peptides suggests that BTP supplementation, even at low relative concentrations reduces proteolysis of native gliadin. Lane labels correspond to sample aliquot: Sn=suspension; St=supernatant; P=pellet.

Native gliadin displays a high concentration of proteins between 20 and 30 kDa, as well as a dense band from 5 to 10 kDa. Compared with native gliadin, PTG in the absence of BTP shows a near complete loss of higher-MW (>20kDa) peptides. The changes in protein/peptide distribution on SDS-PAGE gels (Fig 6) suggest that co-administration of black tea polyphenols inhibit digestion, as evidenced by increased retention of higher MW proteins (20-30 kDa) with BTP present. At all BTP doses, the wells with resuspended pellet (Pt) contain protein profiles resembling that of native gliadin, which suggests that gliadin is precipitated by BTP prior to digestion.

Across all gels, PTG appeared as faint protein bands. In wells containing suspension samples of PTG with BTP, the protein bands appear much fainter than native gliadin, despite having the same approximate loading concentration. This might be attributed to run off or diffusion of more completely digested small-MW peptide.[1]

Across all wells, two faint bands are visible at ~45 and 60 kDa. These values are beyond the expected MW of pepsin or trypsin (34.5 kDa and 23.3k Da respectively). Monomeric gliadins have been previous identified to have a MW range from 29 kDa-59 kDa.[109] potentially corresponding the faint bands observed in the gels. Analytical ultracentrifugation has also shown a MW for gliadins of 25.7 kDa for monomeric gliadin proteins, consistent with bands of native gliadin and BTP inhibited pellet samples.[110] Gliadin can polymerize via disulfide bonding (S-S) and self-associate in the solution,[1] however S-S and non-covalent aggregates would be disrupted by denaturing agents. A possible explanation for these peaks might be covalent linkage between polyphenols and protein, which has been suggested by previous studies.[90,111,112] The retention of these bands in all samples suggest the presence of intrinsically non-digestible gliadins.

Inhibition of digestion implies a reduction in the formation of highly immunogenic peptide fragments, potentially serving as an intervention point upon acute gluten exposure. This experiment however does not clarify whether BTP directly inhibits pepsin and trypsin or sequesters PTG substrate. As elucidated in the following sections, these results may be attributed to structural modification of digestive enzymes and/or precipitation of polyphenol-protein complexes.

4.2 PTG and BTP precipitate out of solution upon complexation

Polyphenols and proteins interact in solutions via primarily non-covalent mechanisms.[54] Previous studies have found differences in binding behaviors based on polymer properties of both proteins and polyphenols, with size and electrochemical microenvironments affecting the outcomes of complexation. It has been suggested that degree of polyphenol polymerization affects the interactions with polyphenols, with smaller monomeric and dimeric polyphenols interacting via antioxidant/redox mechanisms, and oligomeric polyphenols forming cross-linked networks of protein.[54,59,70] The length of peptide has also been shown to affect the nature of the interaction, with longer peptides displaying higher affinity by "wrapping around" polyphenols.[54] The following set of experiments was concerned with cross-linking type events that result in the formation of polyphenol-protein colloids. These complexes are of biological relevance as aggregation results in phase-change and precipitation from solution,[88] potentially reducing the concentration of gliadin peptides present for further deamidation and immune cell recognition.

4.2.1 PTG forms colloidal aggregates with BTP

Turbidity is a measure of haze and light scattering within a solution and is indicative of colloidal interactions.[87] Proteins and polyphenols have demonstrated interactions that result in colloid formation which can be detected using UV-Vis spectroscopy.[15,87] Sufficient aggregation

between the two chemical classes can induce a phase-change, resulting in precipitation of complexes out of solution.[54] This assay spectrophotometrically measured turbidity by absorbance of samples at 400nm (Fig 7a). This method was adapted from previous work.[15] Visible protein-polyphenol precipitates exceed detection limits but were nonetheless visually observable and confirmed precipitation of large aggregates (Fig 7b).

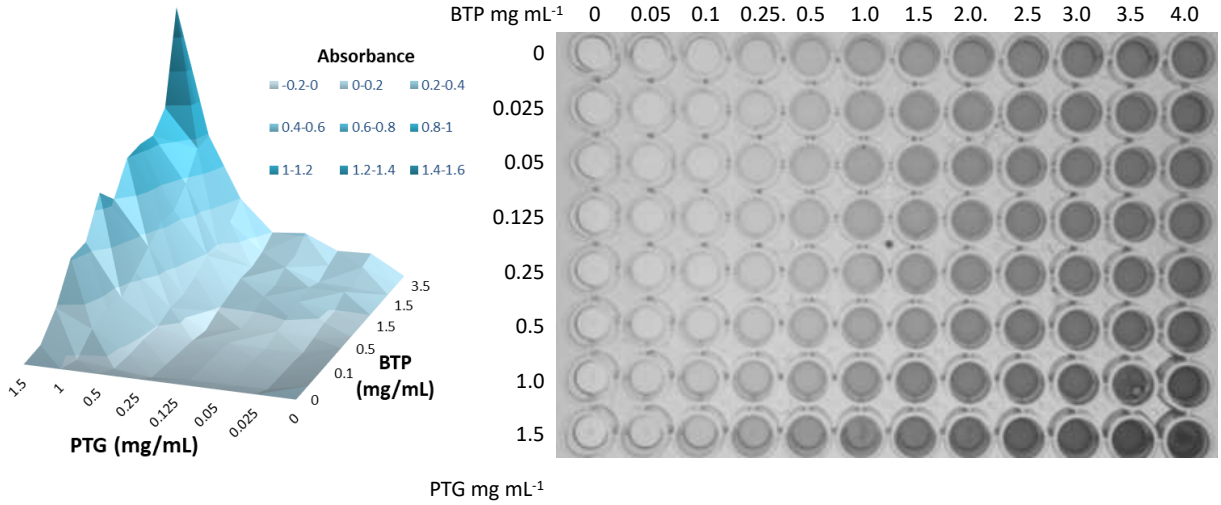


Figure 7. Turbidity resulting from PTG and BTP complexation.
Mean absorbance readings at 400nm of PTG co-incubated with BTP (n=3). PTG increases along the x-axis, BTP increases along the y-axis, and blank adjusted absorbance readings are reported on the z-axis. B) Image of co-precipitation experiment plate. Visible precipitate observable in lower-right corner of image with increasing concentration ratios of BTP:PTG.

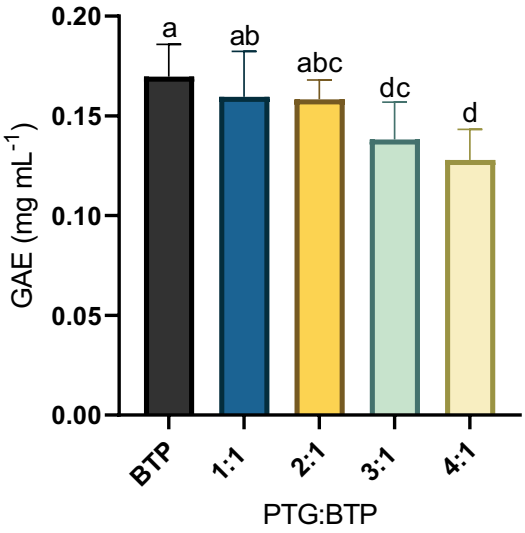
As Figure 7a illustrates, neither PTG nor BTP alone display significant increase in absorption at 400 nm as concentrations increase. However, absorbance increases sharply with of increasing ratios of BTP to PTG, with increases in turbidity observed at ratios as low as 0.1:1 BTP:PTG. Visible precipitate appears at 1:1 ratio. This suggests the formation of colloidal complexes.

Although this experiment was conducted with PTG and not native gliadin, the precipitation is consistent with that observed in the SDS-PAGE experiment, in which majority of protein appeared in the wells containing precipitated pellet aliquots. Coprecipitation of native and PT-gliadin with polyphenols suggests that polyphenols can be applied in the food system to sequester gliadin proteins before and after digestion, serving as a potential roadblock of celiac disease development. To quantify the effects of PTG and BTP complexation, the following two experiments measure the respective reduction of both species.

4.2.2 Complexation of protein to BTP reduces polyphenol concentrations

The Folin-Ciocalteu assay is a method for determining the total phenolic content (TPC) of a solution based on a calibration to a standard curve of gallic acid, with results are reported in gallic acid equivalents (GAE). Polyphenols reduce Folin-Ciocalteu's reagent via a redox reaction, which results in a change in solution color which can be quantitatively measured by UV-Vis absorbance at 765 nm. Following complexation of polyphenols and PTG, the samples were centrifuged, and the supernatant reserved. The TPC of the supernatant revealed, by difference, the quantity of polyphenols involved in the precipitation reaction.

As shown in Figure 8a, a reduction in polyphenols is seen at all PTG concentration ratios.



Ratio	Mean reduction in BTP (%)	SEM
BTP	-	-
1:1 (BTP:PTG)	5.25	1.24
0.5:1	8.88	2.09
0.33:1	18.52	4.36
0.25:1	24.14	5.69

Figure 8. Precipitation of BTP by PTG complexation.
A) Gallic acid equivalents (GAE) as measured by Folin-Ciocalteu assay of 0.25 mg mL^{-1} BTP control and BTP with increasing concentrations of PTG (n=6). Significant differences determined by Kruskal-Wallis and Dunn's multiple comparison test. B) Mean % reduction in polyphenols +/- SEM, as quantified by GAE (n=6). Increasing concentrations of PTG reduce TPC of supernatant solution in a dose-dependent manner.

Compared to a control of BTP at 0.25 mg mL^{-1}, increasing concentration ratios of PTG significantly reduced supernatant concentrations of black tea polyphenols (in GAE). Compared to the control, a significant reduction in BTP was observed when PTG was in 3-fold or 4-fold excess (p=0.0002 and p<0.0001, respectively). Significant decreases were observed between ratio of 1:1 and 0.33:1 and 0.25:1 (BTP:PTG) (p=0.0402 and 0.0002, respectively), as well as between 0.5:1 and 0.25:1 (BTP:PTG) (p =0.0004), suggesting a dose dependent mechanism. Mean % reduction of polyphenols (GAE) by PTG ratio are given in Figure 8b. At a 1:1 ratio, a modest reduction of 5.25% TPC is observed. With increasing concentrations of PTG, more polyphenols are precipitated from solution, reaching 24.5% at 4-fold PTG excess. The known self-aggregating behavior of gliadins[1] may skew the estimated stoichiometry of the reaction, as gliadin peptides may form larger complexes that limit the number of polyphenol crosslinked per

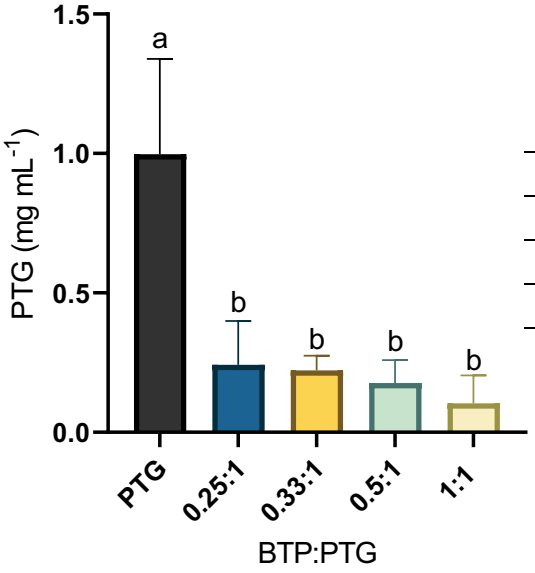
peptide. The binding affinity and specifics of interaction addresses this question in sections 4.3 and 4.4. The poor solubility of gliadins in aqueous solution[16,113] might also lead to exaggerated precipitation of protein relative to polyphenols. The following experiment addressed this question from the analytical perspective of PTG concentrations, giving a clearer image of the effects BTP induces on PTG solution behavior.

4.2.3 Black tea polyphenols induce precipitation of PTG

To complete the picture of co-precipitation, this experiment directly quantified the main compound of interest, PTG. The fluorescence technique used in this assay, as well as in section 4.4, are ideal for limiting background signal from polyphenols due to differences in emission and excitation of the different fluorophores.

The Invitrogen EZQ protein quantitation kit determines protein concentration based on the binding and detection of a proprietary fluorescent dye. This assay was chosen for its superior detection of PTG compared to commonly used Bradford assay, which did not detect the digested peptides reliably. The bicinchoninic acid (BCA) assay was likewise avoided due to BTP interference with assay reagents. In this experiment a calibration curve was generated from known concentrations of PTG. In the EZQ assay, the dye fluoresces with an emission wavelength 280 nm of excitation wavelengths of 450/618 nm. Upon complexation of protein the dye becomes excited at 485 nm with a peak emission at 590 nm. Following centrifugation of samples, protein-polyphenol complexes are precipitated, allowing for detection of remaining PTG in solution. Compared to a control of PTG without BTP, the relative changes in PTG concentration were calculate from fluorescence intensity data. Figures 9a and 9b show PTG concentration by black tea polyphenol ratio and mean % reduction in PTG, respectively. Compared to control PTG at 1.0mg mL^{-1}, all samples containing BTP displayed significant (p-

>0.05) reduction in protein concentration. No significant differences were observed between ratios of BTP.



Ratio	Mean % reduction in PTG	SEM
PTG	-	-
0.25:1 (BTP:PTG)	69.77	6.24
0.33:1	77.30	3.02
0.5:1	80.97	2.52
1:1	90.75	2.48

Figure 9. Precipitation of PTG by BTP complexation.
A) PTG control and PTG with BTP treatment concentration (mg mL^{-1}) as measured by EZQ protein quantitation assay kit (n=4). Significant differences determined by Kruskal-Wallis and Dunn's multiple comparison test. B) Mean % reduction in PTG concentration +/- SEM, at different BTP ratios as quantified by EZQ fluorescence assay (n=4).

These results are consistent with the co-precipitation and Folin assays, suggesting that polyphenols precipitate proteins at intestinal pH. The reductions in protein concentration compare with our previous work with green tea polyphenols, where 1:1 ratios precipitated 93.42% +/- 9.2 of the protein[15]. The degree of cross-linking or oxidation activity of polyphenols has been correlated with DP, with larger compounds participating in aggregation events. As BTP contains larger condense phenolics, we expected to see strong crosslinking and precipitation black tea polyphenols.[70,92]

Dramatic precipitation of PTG was observed with relatively low BTP addition, with 69% of PTG falling out of solution at a 0.25:1 BTP:PTG ratio. In the context of a meal or acute gluten exposure, a black tea polyphenol intervention would necessarily be administered at much lower molar concentrations than the protein. This experiment suggests that protein precipitation, and therefor sequestration of PTG from TG2 or APCs, may be achieved at manageable doses of black tea polyphenols. As a reference point, a cup (236mL) of black tea contains 200-250 mg of BTP (approximately 1mg mL^{-1}). Considering the average adult stomach size of ~0.9L, and the potential of theaflavins to accumulate in the intestinal lumen[62,74], concentrations used in this ratio may be deliverable and relevant within physiological context .

In addition to sequestration of peptides via precipitation, binding of peptides by polyphenols may also change to protein structure by altering the electrostatic environment. The following section explores the conformation changes induced to protein by black tea polyphenols via circular dichroism analysis.

4.3 Circular Dichroism

Circular dichroism is a spectrophotometric technique used to evaluate the conformation of proteins or polymeric structures. Circular dichroism spectroscopy provides a spectral fingerprint of molecules. The differential absorption of circularly polarized light, reported in mdeg, is dependent on protein secondary structures, and give insight into the relative proportion of helical, sheet, turn and disordered protein structures within a sample.[108]

In the case of proteins, when circularly polarized light is passed through a dilute sample, the torsion angles present on amide bonds differentially absorb and rotate the light as it passes through the sample to the detector.[108] The amide bonds of secondary structures of proteins, such as alpha helices, beta sheets and polyproline II helices, generate characteristic spectra when

exposed to circularly polarized light. Corroborating CD spectral information with established structural data from NMR and X-ray crystallography allows for accurate and consistent estimation of protein secondary structure.[114] The binding kinetics and affinity between enzymes or proteins and their ligands are sensitive to the electronic and steric environment; small conformational changes in secondary structure may produce large differences bioactivity. CD is thus an excellent tool for investigating pharmacokinetics, generating information about ligand binding. In the case of black tea polyphenols and gliadin proteins, CD spectra gives information about the conformational changes induced by polyphenol-protein binding.

The effect of ligand binding can be inferred from changes to spectra broadly and at specific intervals. Broad spectral changes induced by ligand, such as blue or ref shifts in minima/maxima, changes to shoulder regions and amplitude shifts indicate modifications to proteins.[2,108,114] Secondary structure evaluation algorithms are used to provide insight into changes to helical, sheet, turn and disordered protein structure. Another useful metric is relative helicity (RH), which can be calculated by taking the ratio molar ellipticities (mdeg) of 222 nm and 208nm. RH gives insight into the helical structure of the peptide, with an increase in RH corresponding to an increase in order.[3,115]

4.3.1 BTP induces conformation change and increases relative helicity of PTG

Figure 10a shows processed spectra of PTG and PTG with BTP complexation. In agreement with previous CD work with gliadin proteins, the spectra display characteristic features of PPII and alpha helices, namely the negative maxima from 202-208nm and a shoulder region around 220-226 nm.[2,4,59] In all treatments, slight red-shifting occurs at the

negative maxima, with the most exaggerated shift of ~4nm occurring with 0.5:1 BTP:PTG ratio.

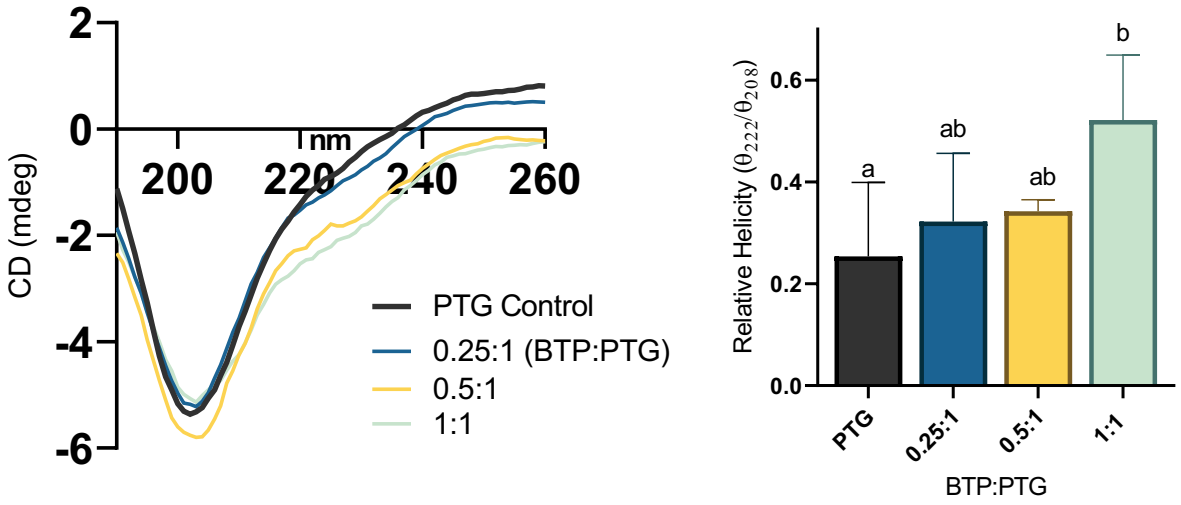


Figure 10. Structural modification of PTG.
A) Mean spectra of PTG at 0.1mg mL^{-1}, and PTG with increasing concentrations of BTP (n=4). Spectra were smoothed and adjusted by subtracting corresponding buffer or BTP blank containing subtracted. B) Relative helicity of PTG at 0.1 mg mL^{-1} and PTG with increasing concentrations of BTP (n=4). Significant differences determined by Kruskal-Wallis and Dunn's multiple comparison test.

RH appears to trend toward increase with increasing concentrations of BTP (Figure 10b). Compared to PTG control, a significant increase in RH was observed at a 1:1 (p=0.0174). Greater intensity of the peak in the 200-208 nm range (absolute value of theta) suggests increased helical signal,[112] in agreement with an increase in RH. Previous spectroscopic methods have found that polyphenol binding to proteins is primarily non-covalent, spontaneous, and exothermic, resulting in a decrease in entropy as complexation induces compaction of the protein.[87,88] Increasing concentrations of BTP may likewise alter the total solution properties and Increase in RH may be reflective of this compaction upon complexation. This change suggests that black tea polyphenols affect secondary structure conformation. Previous work has

demonstrated proteins "wrap around" polyphenols.[54] This interaction may explain the changes in secondary structure torsion angles observed.

Compared to previous work by our group with green tea EGCG and synthetic α_2-gliadin synthetic fragments, BTP appears to have a stronger and significant effect on helical conformation of digested gliadin peptides.[3] Notably, the conformational changes observed in this work occurred at lower polyphenol concentration compared to our previous work with EGCG. This difference in protein structure modification may be attributed to the difference between purified EGCG and the components in our bulk black tea extract. The oxidative processes occurring during black tea fermentation result in the generation of multiple condensed polyphenolic species, with theaflavin and its derivatives the most widely distributed compound.[69,72,73,74] Black tea extract, while containing some EGCG-like catechins, comprises larger MW compounds. Compared to the EGCG (MW=459.372g mol^{-1}), theaflavin (564.49 g mol^{-1}) and thearubigen (902 g mol^{-1}) are significantly bulkier. Coupled with the established cross-linking activity of large MW phenolics, BTP's enhanced modification to protein conformation may be related to MW, steric interactions, and solution behavior of its components. Further molecular modeling and spectroscopic studies may elucidate the mechanics of binding.

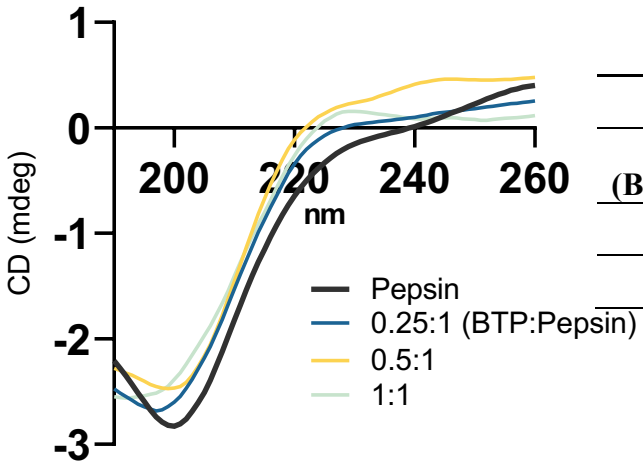
It has been suggested that recognition binding of PTG to TG2 for deamidation and recognition of PTG by MHC-II receptors is highly stereospecific, with the order and relative exposure of prolines factoring into ligand affinity.[8,32] Changes to the helical nature of the PPII structure could result in altered features to the stereochemical and electronic environment, potentially reducing the substrate affinity for TG2, resulting in lowered production of the highly immunogenic deamidated peptide. The electrostatic and steric

forces present in PTG have been shown to correlate with ligand affinity toward TG2 and HLA-DQ2.5/8^{+} binding sites.[8,11,32] and therefor essential in disease onset and progression. Given the specific molecular basis of antigen recognition, polyphenol induced conformation change may act as an epitope masking effect, in which complexed gliadins have reduced binding kinetics and affinity toward antigen presenting cells and TG2.

4.3.2 BTP induces minor change to overall secondary structure of pepsin

This experiment explored the potential of black tea polyphenols to noncovalently alter pepsin structure. Enzyme activity (and therefor generation of immunogenic peptides) can be affected by pH, solvent conditions, as well as the presence of inhibitors. Although this assay does not provide specificity of inhibition, observed alteration to enzyme secondary structure may partially explain the previously demonstrated inhibition of gliadin digestion in the presence of black tea polyphenols.

Spectra and SSE evaluations are presented in Figure 11, with changes to overall spectra observed with BTP addition.



	Helix	Sheet	Turn	Other
Pepsin	2.04	38.86	13.42	45.68
0.25:1 (BTP:Pepsin)	3.82	38.66	13.02	44.5
0.5:1	0.62	40.34	12.14	46.9
1:1	2.58	39.7	13.32	44.4

Figure 11. Structural analysis of pepsin.
A) Spectra of pepsin 0.1 mg mL^{-1}, and of pepsin with increasing concentrations of BTP, prepared in 10mM phosphate buffer at pH 2.0. B) Mean SSE (n=5) designations, determined in Jasco SSE.

Compared to the control pepsin, BTP addition induced red shifting to all points of the spectra, as well as decrease in intensity (absolute value of theta) of negative bands. This shift may indicate reduction of overall helical character and increase of disordered structure.[116] RH calculations did not show significant difference with BTP addition. This is not surprising, given the, the increased molecular complexity (presence of multiple ordered secondary structures, and size of pepsin (molecular weight of 35 kDa, 362 amino acid residues) compared to gliadin peptides. SSE was instead used to quantify changes to the enzyme.

Jasco SSE software was used to determine distributions of secondary structures in control pepsin compared to pepsin in the presence of black tea polyphenols. The software applies a partial least squares analysis based on similarities the processed spectra to a library of proteins. The library matches structural features of circular dichroism spectra to

NMR and X-ray crystallography assignments, producing an estimation of relative proportions of protein secondary structures.

SSE assignments indicate fluctuations to the alpha-helical content of pepsin in the presence of BTP, however no correlation was observed with increasing ratios of polyphenols to protein. At the lowest ratio (0.25:1 BTP:Pepsin) helicity increases, following by a rapid decrease at 0.5:1. At 1:1 a slight increase was observed. From these results, it is unclear whether black tea polyphenols alter helical conformation with any dose dependency. This effect might be explained by a dose dependent interconversion of secondary structures at different concentrations of BTP. Additionally, the lower pH of this experiment (pH 2.0 compared to 6.8 for PTG experiments) may alter the solution properties of black tea polyphenols.

However, minor transformations to helical composition of the enzyme suggests that BTP induces non-specific conformational change. As such, secondary structure modification upon interaction suggests, change to the electronic environment of the enzyme, this may partly explain the effects on digestion observed. Future studies involving spectra in the near-UV (260-320) may provide more information on the extent of enzyme structural modification induced by BTP.

4.4 Fluorescence quenching of PTG by BTP supports molecular binding

Gliadin proteins and peptides contain intrinsic tyrosine, tryptophan and phenylalanine fluorophores which can be detected using fluorescence spectroscopy.[2,90,117] The intensity of a fluorescence signal is affected by the electrochemical environment surrounding the fluorophore; this technique can provide information about binding of polyphenols and provide insight into the binding-induced changes to the peptide conformation. Using an excitation wavelength of 275 nm and a maximum absorbance from 340-370 nm, the intrinsic fluorophores on PTG were probed

for intensity with increasing concentration ratios of black tea polyphenols. The effect of BTP on fluorescence intensity of PTG at room temperature (22°C) is shown in Figure 12.

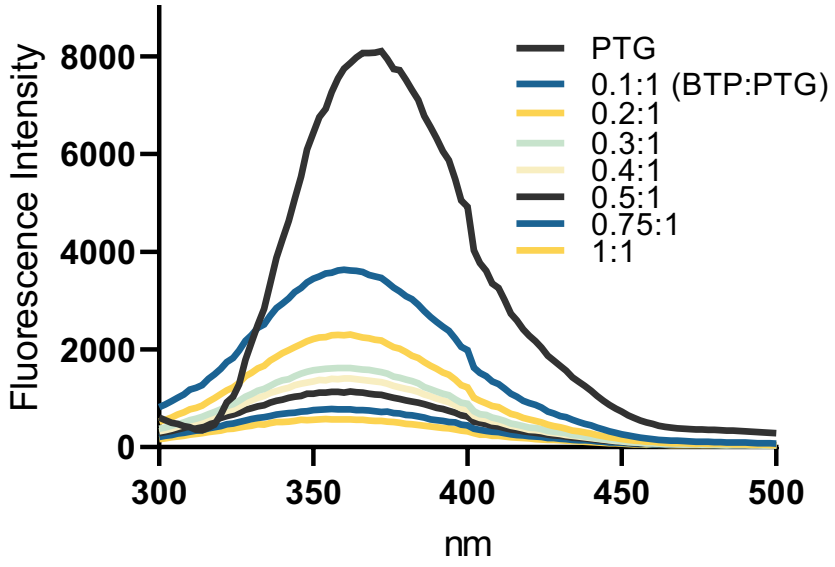


Figure 12. Fluorescence spectra of PTG-BTP interactions.
Fluorescence emission spectra at λem=275nm of PTG prepared at 1.0mg mL^{-1} in 10mM phosphate buffer with 68mM NaCl, pH 6.8 at room temperature and with increasing concentration of BTP. Each spectrum represents the mean fluorescence (n=4) after correction of BTP fluorescence.

In addition to changes in signal intensity, shifts of absorbance maxima indicate changes to the steric environment surrounding fluorophores.[1,90] Conformational changes induced by quencher can drive hydrophobic residues to the interior of the protein and reduce the fluorescent signal.[1,93] The blue-shifting of 10-12nm observed with BTP addition may be explained by increases to hydrogen bonding and compaction of the protein structure.[30,90,93] This is consistent with previous research on polyphenol-protein interactions,[3,54] and agrees with the increase in RH observed in our circular dichroism studies.

Information about the binding kinetics (K_a/K_{sv}) and number of sites (n, Hill Coefficient) can be determined by analyzing fluorescence data with the Stern-Volmer equation:

$$\frac{F_0}{F} = 1 + k_q\tau_0[Q] = 1 + K_{sv}[Q]$$

Where F_0 and F are the fluorescent intensities of PTG and PTG with BTP, respectively, the bimolecular quenching constant is (k_q), τ_0 is the fluorescence lifetime of PTG without BTP, [Q] is the concentration of quencher (BTP), and K_{sv} is the Stern-Volmer constant. Stern-Volmer plots are presented in Figure 13 (A-C). Modification of the Stern-Volmer plot gives information about binding affinity (K_a)

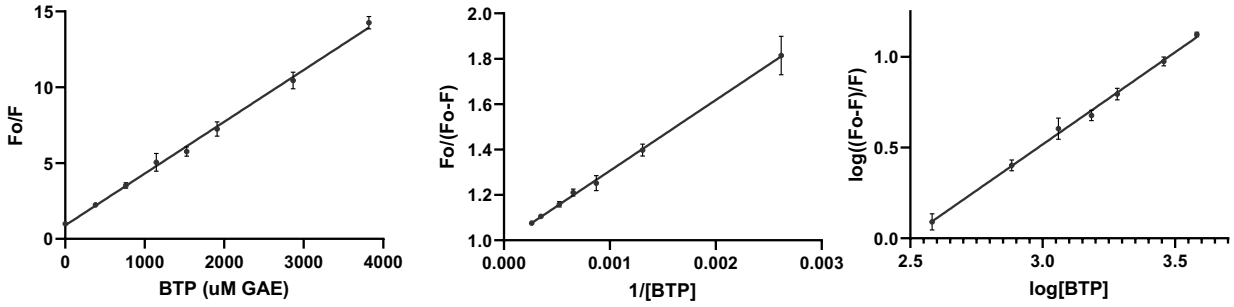


Figure 13. Stern-Volmer plots of BTP and PTG binding affinities
Plots of A) Stern-Volmer (y = 0.0034x + 1, R^2 = 0.9972), B) modified Stern-Volmer (y = 311.98x + 0.9945, R^2 = 0.9988), and C) double log transformed Stern-Volmer (y = 1.0155x - 2.5294, R^2 = 0.9976) showing quenching of intrinsic gliadin fluorophores Trp, Tyr and Phe with increasing concentration of BTP. BTP was converted from w/v to molar values based on mean GAE calculated in previous experiments. Maximum fluorescence intensities of control PTG (F0) at 370nm and 358-362nm for PTG with BTP (F) were used.

Table 3. Stern-Volmer calculated binding affinities for PTG and BTP
K_{sv}, K_a and Hill Coefficient (n) values obtained from Stern-Volmer calculations (Fig 13)

K_{sv} (μmol•L^{-1})	K_a (μmol•L^{-1})	n
0.0034 +/- 5.3x10^{-5}	313.71 +/- 7.46	1.02 +/- 0.02

K_{sv} relates to the interaction between quencher and fluorophore and was determined to be 0.0034 +/- 5.3 x 10^{-5} umol L^{-1}. A good linear fit of the Stern-Volmer plot (A) suggests static or dynamic quenching.[93]To determine whether the quenching mechanism is dynamic or static the bimolecular quenching constant, (k_q) is calculated by dividing K_{sv} values (slope of Stern-Volmer plot A) by the fluorescence lifetime of PTG in the absence of BTP (τ_0). Lacking experimental fluorescence lifetime data in these experiments, a static mechanism was chosen based on previous findings conducted at comparable conditions flavonoids and globular proteins,[91] procyanidins and gluten proteins,[30] and between wheat glutens and quercetin.[90]

With a static model, K_a can be calculated from modified Stern-Volmer plots A and B, respectively. K_a was calculated to be 313.71 +/- 7.46 umol L^{-1}. A high K_a suggests high affinity interaction between peptide and polyphenol that can overcome the electrostatic repulsive forces.[90]

The Hill coefficient for number of binding sites (n) can be calculated using the double log-transform of the Stern Volmer plot (Figure 13, plot C).[90] A coefficient of n=1.0155 indicates a single binding site for black tea polyphenols and PTG, which agrees with previous work on polyphenol-protein interaction.[30,91] In agreement with previous work between protein-polyphenol binding kinetics, a peptide may interact with one phenolic by "wrapping" around.[54] Subsequent

oligomerization events have also been suggested, where in multiple polyphenols and gliadins are crosslinked, giving a molecular dynamics explanation for aggregation and precipitation.

These *in vitro* results suggest the efficacy of polyphenol-gliadin binding at duodenal pH, which may be applied toward sequestering or epitope masking gliadin proteins from immune recognition and subsequent pathogenesis.

CHAPTER V: CONCLUSIONS & FUTURE DIRECTIONS

This work has expanded upon previously established interaction mechanisms between polyphenols and proteins within the celiac disease model. The *in vitro* experiments conducted confirm black tea polyphenol's ability to modify gliadin protein conformation and solution behavior at various stages of digestion, as well as insight into potential inhibition of digestive enzymes. The multi-spectroscopic experimental design used in this thesis thoroughly explore the potential molecular mechanisms of interaction.

The digestion and precipitation experiments suggest that black tea polyphenols cause precipitation of gliadin proteins and digested peptides at physiological pH. The formation of these complexes in the intestinal lumen may potential reduce innate inflammatory and intestinal-permeability effects of gliadin proteins due to the increased size and polarity of complexes, as well as dampening recognition by zonulin-linked and cytokine producing pathways. Further research with these compounds in the context of innate intestinal signaling should explore this potential effect.

Further work is needed to investigate the potential inhibition of TG2 activity with black tea polyphenols, either by direct enzyme inhibition or sequestration/alteration of substrate. In

addition to the promise of digestive inhibition, reducing deamidation of peptides via enzyme interference may provide another therapeutic strategy.

Due to the heterogenous nature of the PTG and BTP starting material, the effect size may be dampened by averaging bulk solution effects. Future work with isolated/synthetic peptides and black tea polyphenols such as theaflavin will give more information on specific molecule interactions. However, as food is administered in a complex and heterogenous matrix this data is encouraging for its application to commonly consumed products.

Future experimentation with these compounds should employ fluorescence-based techniques to compare binding mechanisms at different pH and solution conditions. Previous work has demonstrated changes in the binding affinity and kinetics based on pH for other flavonoid species.[30,93] which has important implications in considering the intestinal versus gastric effects of black tea polyphenol activity.

In addition to the general prophylactic benefits attributed to polyphenols, this work on black tea polyphenols and gliadin interactions suggests potential for intercepting different points of CD pathogenesis. The administration of black tea polyphenols to improve gluten toxicity outcomes, whether as an adjuvant supplement or acute exposure should be further investigated as a nutraceutical therapy. Future work in biological systems should investigate the effects of black tea polyphenols to acute gluten exposure as well as in pathology prevention, maintenance, and remission scenarios of CD patients.

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