

CONDENSED TANNINS INTERACTION WITH AND
MODIFICATION OF WHEAT GLUTEN PROTEINS

A Dissertation

by

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ABSTRACT

Proanthocyanidins (PA) strongly complex proteins, which could be exploited in food systems to modify protein polymer structures and their functionality to benefit nutrition and health. Wheat is widely consumed, and its gluten proteins are structurally favorable for interaction with PA. In this work, we investigated effect of PA of different MW on gluten protein rheology and film properties, and assessed the mechanisms behind these interactions.

Polymeric PA from sorghum (mean degree of polymerization, mDP 19.5) dramatically strengthened wheat gluten; e.g., at 2.5 mg/g flour, PA doubled insoluble polymeric protein (IPP) in weak gluten flour, increased mix time by 75% and dough elasticity by 29%. Oligomeric PA from grape seed (mDP 8.3) was less effective (increased IPP by 75% and dough elasticity by 16%). Uniquely for a gluten strengthening agent, PA did not decrease dough extensibility. Polymeric PA increased gluten film strength (e.g., at 10 mg/g gluten, force to extend was 2.2X greater than control without reduced extensibility). Thus, PA may improve gluten film flexibility and structural integrity. Polymeric PA also significantly ($p < 0.05$) increased gluten film stability to protease enzyme degradation versus tannic acid and control, which could allow for targeted delivery of micronutrients in the gastrointestinal tract.

Polymeric and oligomeric PA had equilibrium dissociation constants of 0.6 and 2.1 mol PA/mol of glutenins, respectively, indicating polymeric PA interacted with glutenins more efficiently; a similar effect was seen in gliadins. Compared to the control,

polymeric PA at 30 mg/g protein decreased soluble glutenins (31%) and gliadins (20%). Within glutenins, polymeric PA decreased soluble high molecular weight glutenin subunits (HMW-GS) more so than low MW-GS (79 vs 6%) and further reduced solubility of the larger x-type HMW-GS more than y-type. Similarly, polymeric PA decreased solubility of the largest ω -gliadin fraction the most, suggesting increased complexing efficiency with higher MW gluten fractions. Surface hydrophobicity of glutenins, but not gliadins, was reduced by PA (69 – 75% vs control). Overall evidence indicates PA complexed glutenins by hydrophobic interaction and hydrogen bonding, but gliadins mainly by hydrogen bonding.

DEDICATION

To my family.

Most especially to my mom, Diane Mick, and my husband, Joey Girard.

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The methodology and part of the data presented in Chapters III (Wheat Flour Protein Profiling – SE-HPLC methodology and data) and IV (Reversed Phase HPLC and Electrophoresis – methodology and data) were provided by Dr. Scott Bean, Dr. Mike Tilley, and Dr. Sherry Adrianos of the USDA-ARS Center for Grain & Animal Health Research in Manhattan, KS.

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CHAPTER I

INTRODUCTION

Tannins are secondary metabolites found in various plants, including fruits and seeds such as cocoa, grape, and some sorghum varieties. These polymeric phenolic compounds are known to strongly complex with proteins. A well-known consequence of this is the astringent oral sensation as when drinking a dry wine; tannins bind to oral proteins and reduce tongue lubrication. These interactions have been used industrially (e.g., to tan leather) and in food processing (e.g., to clarify wine (Sarni-Manchado, Deleris, Avallone, Cheynier & Moutounet, 1999)). Tannins' propensity to complex proteins could be exploited in food systems to modify protein polymer structures to benefit nutrition and health.

Tannins are primarily divided into two groups: condensed tannins (proanthocyanidins, PA) consisting predominantly of polymerized flavan-3-ols, and hydrolysable tannins (e.g., tannic acid) composed of gallic acid esterified to glucose (Hagerman et al., 1998). Tannins have a range of conformations, degree of polymerization (DP), monomeric unit composition, and flexibility, which are all important factors in determining how they interact with proteins. Of the two tannin types, PA have been shown to have a greater affinity for proteins (Baxter, Lilley, Haslam & Williamson, 1997, Emmambux & Taylor, 2003, Hagerman, Rice & Ritchard, 1998). This is likely because of structural differences: PA are primarily linear, whereas

hydrolysable tannins have a more spherical arrangement with hydroxyl groups conforming together in the center of the structure (Hagerman, Rice & Ritchard, 1998).

Protein-PA interactions are noncovalent, mainly hydrogen bonding and hydrophobic interactions (Hagerman, Rice & Ritchard, 1998, Oh, Hoff, Armstrong & Haff, 1980). The phenolic hydroxyl groups and hydrophobic regions of PA complex with the carbonyl groups and hydrophobic amino acids, respectively, of proteins. Higher molecular weight (MW) PA cross-link protein more efficiently (Frazier, Papadopoulou, Mueller-Harvey, Kisson & Green, 2003, Harbertson, Kilmister, Kelm & Downey, 2014, Zeller et al., 2015) as they offer greater number of hydroxyl groups and more hydrophobic regions in close proximity for interaction with proteins. Protein structure also affects PA binding; large proteins with open conformation and high proportion of proline have a high affinity for PA (Hagerman & Butler, 1981). Thus, high MW PA will likely have strong interactions with conformationally open, high MW proteins that could alter the protein polymer structure and its functionality.

Wheat is widely consumed; it provides about 20% of calories worldwide (FAO Statistics Division, 2013). Wheat gluten, which comprises ~15% of wheat flour, is perhaps the most uniquely functional plant protein as it confers an uncommon property onto doughs: viscoelasticity. Two main protein types comprise gluten: gliadins and glutenins. Glutenins are divided into high MW glutenin subunits (HMW-GS, 60-90+ kDa) and low MW glutenin subunits (LMW-GS, 30-60 kDa), which are similar in MW to gliadins (32-74 kDa) (Fido, Bekes, Gras & Tatham, 1997, Lew, Kuzmicky & Kasarda, 1992, Shewry, Tatham, Forde, Kreis & Mifflin, 1986, Shewry, Halford & Tatham, 1992).

Gliadins are generally globular whereas glutenins have a more elongated structure (Fido, Bekes, Gras & Tatham, 1997, Lew, Kuzmicky & Kasarda, 1992, Shewry, Tatham, Forde, Kreis & Miflin, 1986, Shewry, Halford & Tatham, 1992). While gluten proteins contain only a small proportion of cysteine (~2 mol%) (Ewart, 1967, Rombouts, Lamberts, Celus, Lagrain, Brijs & Delcour, 2009), intermolecular disulfide bonds are the major drivers of gluten polymerization. Glutenins are largely responsible for gluten strength as glutenins form more intermolecular disulfide bonds than gliadins, thus creating a larger, denser protein matrix (MacRitchie & Gupta, 1993); gliadins impart elasticity to dough structure (Uthayakumaran, Newberry, Keentok, Stoddard & Bekes, 2000). Gluten proteins are rich in the amino acids glutamine (~32 mol%) and proline (~14 mol%) (Ewart, 1967, Rombouts, Lamberts, Celus, Lagrain, Brijs & Delcour, 2009), which are particularly amenable to hydrogen bonding and hydrophobic interactions, respectively, thus favorable for PA interaction.

When PA complex with protein, they alter protein rheology. Tannins (condensed and hydrolysable) improved strength of plant protein films, as in sorghum kafirin (Emmambux, Stading & Taylor, 2004), corn zein (de Freitas et al., 2017), and wheat gluten (Hager, Vallons & Arendt, 2012) films. Tannic acid increased wheat flour dough stability and bread loaf volume (Wang et al., 2015, Zhang, Cheng, Jiang, Wang, Yang & He, 2010). In a wheat flour dough, tannic acid increased insoluble protein, decreased surface hydrophobicity, and altered gluten secondary structure (Wang et al., 2015), suggesting tannic acid cross-linked gluten through noncovalent interactions. Generally larger proteins with open conformations are more likely to interact with PA than smaller,

globular proteins (Hagerman & Butler, 1980a, Hagerman & Butler, 1980b), which suggests PA are likely to preferentially interact with glutenins over gliadins. PA-glutenin interactions could greatly increase the macropolymer size and gluten network density, thus strength, without losing the extensibility conferred by gliadins.

PA can also reduce caloric impact of foods by binding macronutrients or inhibiting digestive enzymes. PA complex with protein and reduce protein digestibility (Dunn, Yang, Girard, Bean & Awika, 2015, Feeny, 1968). In sorghum porridge (Lemlioglu-Austin, Turner, McDonough & Rooney, 2012) and wheat flour tortillas (Dunn, Yang, Girard, Bean & Awika, 2015), PA also slowed starch digestion *in vitro*. This modulation of digestion can increase satiety and decrease total calorie consumption (Holt & Miller, 1995, Holt, Brand, Soveny & Hansky, 1992, Willis, Eldridge, Beiseigel, Thomas & Slavin, 2009). Recent work showed PA encapsulated in sorghum kafirin microparticles slowed carbohydrate digestion in mice, reducing the glycemic response (Links, Taylor, Kruger, Naidoo & Taylor, 2016). By complexing with starch, especially amylose, PA can alter starch digestibility (Amoako & Awika, 2016b, Barros, Awika & Rooney, 2012, Barros, Awika & Rooney, 2014). PA can also inhibit digestive enzymes including amylases (Gonçalves, Mateus & De Freitas, 2011), proteases (Gonçalves, Soares, Mateus & De Freitas, 2007), and lipases (Moreno, Ilic, Poulev, Brasaemle, Fried & Raskin, 2003). Thus, PA could be used as a weight management tool, which would be greatly beneficial with the ever-increasing rise in global obesity and associated secondary diseases.

With the known consequences of PA-protein interactions, PA could likely expand gluten functionality as a natural ingredient. Beyond naturally improving gluten functionality, gluten-PA interactions could also have novel applications to benefit food quality and health. It is likely that differences in gluten subunit composition (e.g.: gliadins vs glutenins) and PA MW will influence the nature of gluten interaction with PA.

The objectives of this study are:

- I. Determine effect of proanthocyanidin interactions with wheat proteins on gluten rheology
- II. Establish mechanisms of proanthocyanidin-gluten protein interactions
- III. Investigate effect of proanthocyanidin-gluten cross-linking on gluten film formation and stabilization

CHAPTER II

LITERATURE REVIEW

Defining Tannins

Tannins are astringent phenolic compounds that occur in a wide range of plant species and are well-known for binding proteins. All plants have phenolic compounds. These secondary metabolites are not essential for the plant's life but serve important roles from attracting pollinators to defending from predation to antimicrobial and wound healing properties (Bi & Felton, 1995, Coley, Bryant & Chapin III, 1985, Kessler & Baldwin, 2007). Phenolic compounds consist of at least a benzene ring with a hydroxyl group attached. There are wide variety of phenolic compounds, but the classes most important to the human diet are phenolic acids and flavonoids. These are generally found in highest concentrations in the outer layers of plant tissues; e.g.: in cereal pericarp and fruit peels.

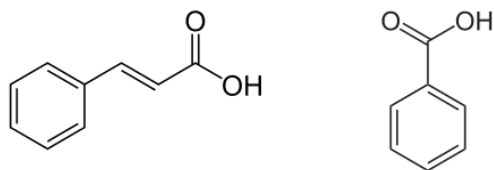


Figure 1 - Cinnamic (left) and benzoic (right) acids.

Phenolic acids consist of a C₆-C₁ or C₆-C₃ structure, with numerous variations. Phenolic acids are ubiquitous: most plants contain variants of cinnamic and/or benzoic acids (Fig 1). These commonly include hydroxycinnamic acids, like caffeic and ferulic

acid, and hydroxybenzoic acids, like gallic (a monomeric unit of hydrolysable tannin) and vanillic acid (Fig 2).

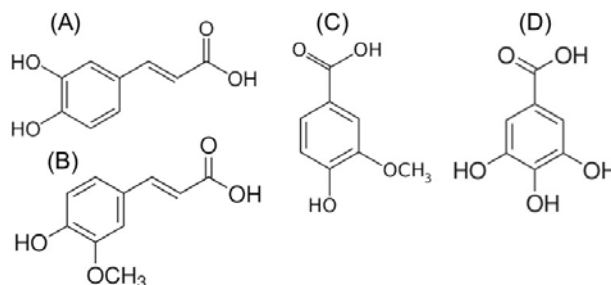


Figure 2 - (A) Caffeic acid, (B) ferulic acid, (C) vanillic acid, and (D) gallic acid.

Flavonoids have a base structure of C₆-C₃-C₆ and include many different classes. The most important flavonoid classes for edible plants are flavan-3-ols, anthocyanidins, flavonols, flavanones, flavones, and isoflavones (Fig 3). Flavan-3-ols include catechins, condensed tannins (proanthocyanidins), and theaflavins and are found especially in teas, grapes, and cocoa. Flavan-3-ols are the most commonly consumed flavonoid (Gu et al., 2004) and are known for their bitterness and astringency.

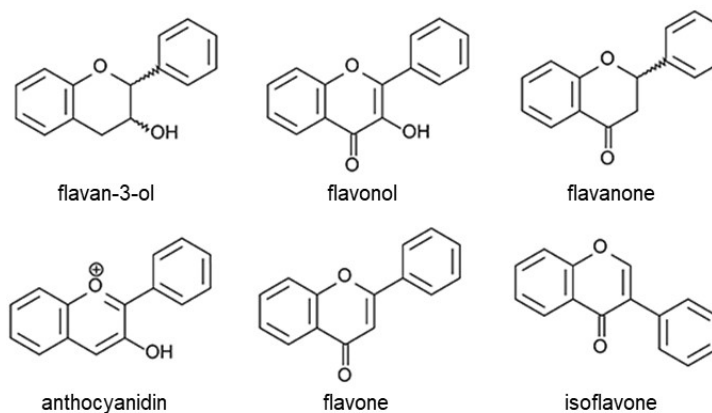


Figure 3 - Basic flavonoid structures.

Tannins can be divided into three groups: condensed tannins (proanthocyanidins, PA) consisting primarily of polymerized flavan-3-ols (Fig 4A), hydrolysable tannins (e.g., gallotannins and ellagitannins) composed of gallic acid esterified to glucose (Fig 4B), and phlorotannins composed of phloroglucinol subunits (found only in marine brown algae, not pictured) (Hagerman et al., 1998). PA are found in many fruits and beverages like teas, grapes, cocoa, and wine as well as in some grains like sorghum, finger millet, common beans, and cowpea (Gu et al., 2004, Hagerman & Butler, 1980a, Ojwang et al., 2013, Prior & Gu, 2005). Hydrolysable tannins occur in fruits and nuts, especially berries (Clifford & Scalbert, 2000).

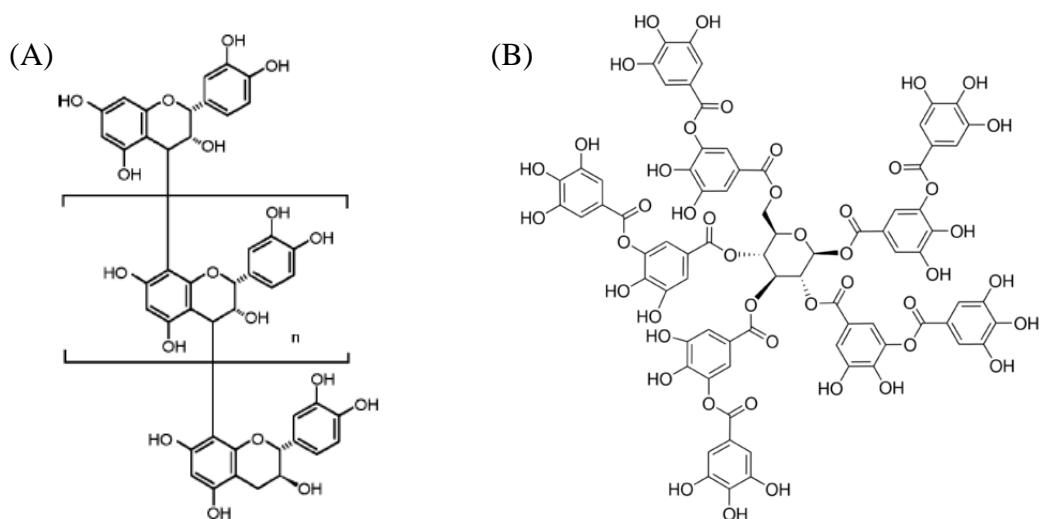


Figure 4 – (A) Condensed tannin and (B) hydrolysable tannin (gallotannin).

Proanthocyanidin Structure

Flavan-3-ols are the primary base units of PA that are polymerized into two forms: A-type, which have C2 → C7 ether bonds with C4 → C8 interflavan bonds, and

B-type, which have C4 → C8 or C4 → C6 interflavan bonds (Fig 5) (Geissman & Yoshimura, 1966, Jacques, Haslam, Bedford & Greatbanks, 1974). Of the two, B-type are far more common in the human diet (Prior & Gu, 2005). All else equal, intermonomeric linkages (as in A-type PA) decrease protein binding (Kolodziej, Haberland, Woerdenbag & Konings, 1995), probably because the intramolecular double bond limits PA flexibility.

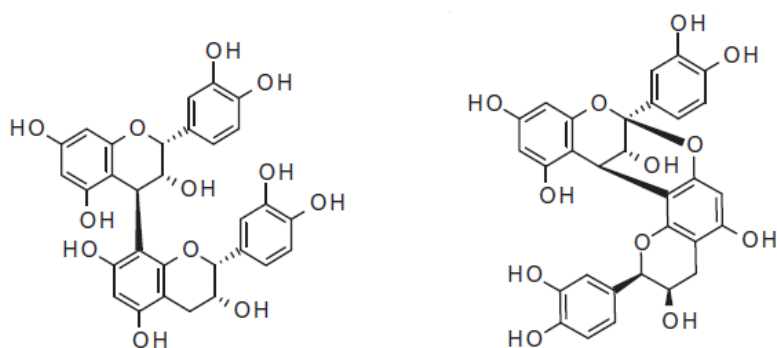


Figure 5 - B-type with C4 → C8 interflavan bond (left) and A-type (right) proanthocyanidins.

PA are formed in the flavonoid biosynthetic pathway and generally polymerize late in the plant maturation cycle as carbocations of quinone methides and leucoanthocyanidin derivatives condense with catechin or epicatechin (Dixon, Xie & Sharma, 2005, Winkel-Shirley, 2001, Zhao, Pang & Dixon, 2010). Based on their primary hydroxylation, PA can be further subdivided into procyanidin (most common in foods), prodelfinidin, propelargonidin, etc. (Fig 6).

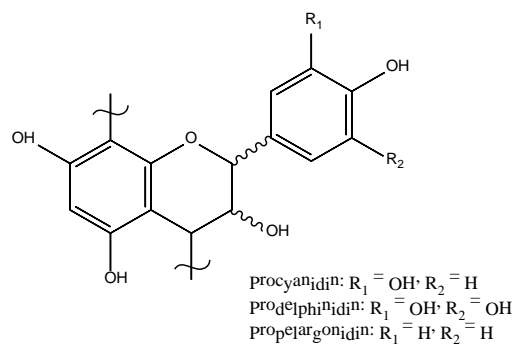


Figure 6 - Structure of proanthocyanidins with differences based on B-ring hydroxylation.

PA have a range of conformations, degree of polymerization (DP), monomeric units, and structural flexibility (Table 1) which are all important factors in determining how they will interact with macronutrients. However, these factors are difficult to study directly as PA are structurally complex thus hard to isolate and characterize.

Table 1 - Proanthocyanidins (PA) in select foods.

Source	Primary PA type	Mean DP
Hi-Tannin Sorghum	Procyanidin	16.0
Grape Seed	Procyanidin	8.1
Cocoa	Procyanidin	8.0
Cranberry	A-type, Procyanidin	8.3
Cinnamon	A-type, Propelargonidin, Procyanidin	6.5

***Data sourced from: Gu et al., 2004, Hagerman, Rice & Ritchard, 1998, Labarbe, Cheynier, Brossaud, Souquet & Moutounet, 1999, Prior & Gu, 2005, Urpi-Sarda et al., 2009.**

Potential Health Benefits of Phenolic Compounds

A wide range of phenolic compounds found in fruits and vegetables are associated with beneficial health outcomes, including lower incidence of cardiovascular disease (Block, Patterson & Subar, 1992, Hertog, Feskens, Kromhout, Hollman & Katan, 1993, Hertog et al., 1995), anticarcinogenic effects (Hertog et al., 1995, Knekt et

al., 1997, Owen, Giacosa, Hull, Haubner, Spiegelhalder & Bartsch, 2000), and prevention of Type II diabetes (Kar, Laight, Rooprai, Shaw & Cummings, 2009, Kim & Park, 2012, Kim, Hyun & Choung, 2006). It was previously thought that the mechanism of health benefit of these phenolics hinged on their antioxidant properties, due to the strong antioxidant capacity of phenolics *in vitro* (Hagerman et al., 1998, Rice-Evans, Miller & Paganga, 1997, Sánchez-Moreno, Jiménez-Escrig & Saura-Calixto, 2000). This is likely one mechanism by which phenols are bioactive. However, based on the abundance of phenolic metabolites found *in vivo* (Gu, House, Rooney & Prior, 2007, Margalef, Pons, Bravo, Muguerza & Arola-Arnal, 2015, Rechner, Spencer, Kuhnle, Hahn & Rice-Evans, 2001, Rondini, Peyrat-Maillard, Marsset-Baglieri & Berset, 2002), it is likely that there are many beneficial mechanisms based upon modulatory actions by phenolic metabolites.

In general, the body recognizes phenolics as xenobiotics. Phenolics that are absorbed in the small intestine undergo modification to form glucuronidated, methylated, or sulfated metabolites, which either efflux via ATP transporters or enter into the bloodstream and undergo phase II metabolism in the liver (Crozier, Jaganath & Clifford, 2009). Tannins largely survive digestion to the colon (Rios, Bennett, Lazarus, Remesy, Scalbert & Williamson, 2002), where they can be metabolized into a multitude of metabolites by colonic bacteria (Gu, House, Rooney & Prior, 2007, Margalef, Pons, Bravo, Muguerza & Arola-Arnal, 2015). Tannins have also been shown *in vitro* to bind proteins like angiotensin I converting enzyme (ACE), which may prevent vasoconstriction (Ottaviani, Actis-Goretta, Villordo & Fraga, 2006), or low-density

lipoproteins, which can prevent their oxidation and subsequent atherosclerosis plaque formation (Porter, Krueger, Wiebe, Cunningham & Reed, 2001).

Potential Health Benefits of Proanthocyanidins

PA have myriad potential health benefits including: anti-inflammatory effects, chemoprevention, anti-bacterial and anti-viral properties, and protection against cardiovascular disease (Awika, Yang, Browning & Faraj, 2009, Bors, Michel & Stettmaier, 2000, Hagerman et al., 1998, Howell, Reed, Krueger, Winterbottom, Cunningham & Leahy, 2005, Kaur, Singh, Gu, Agarwal & Agarwal, 2006, Riedl & Hagerman, 2001, Sato, Maulik, Ray, Bagchi & Das, 1999, Yousef, Saad & El-Shennawy, 2009). Some of these health benefits stem from strong PA antioxidant properties (Bagchi, Garg, Krohn, Bagchi, Tran & Stohs, 1997). Even when complexed with macronutrients, PA maintain most of their antioxidant capacity and could thus act as a radical sink *in vivo* (Riedl & Hagerman, 2001). Flavan-3-ols impact health in three main ways: direct bioactive properties, interactions with microflora, and interactions with macronutrients.

Flavan-3-ol monomers, and to a lesser extent dimers and trimers, are absorbed *in vivo*, evidenced by the flavan-3-ols and their glucuronidated, methylated, or sulfonated forms in the urine or plasma of rats (Deprez, Mila, Huneau, Tome & Scalbert, 2001, Gonthier, Donovan, Texier, Felgines, Remesy & Scalbert, 2003, Margalef, Pons, Bravo, Muguerza & Arola-Arnal, 2015). PA larger than DP = 3 survive digestion to the colon (Rios, Bennett, Lazarus, Remesy, Scalbert & Williamson, 2002) where they are

fermented by various bacteria into low MW phenolic acids including hydroxylated phenylacetic, phenylpropionic, and phenylvaleric acids (Deprez et al., 2000). These molecules can then be absorbed and undergo phase II metabolism. Benzoic, phenylacetic, and phenylpropionic acid metabolites were found in the serum and urine of rats fed sorghum PA (Gu, House, Rooney & Prior, 2007). Similarly, benzoic, phenylacetic, and phenylpropionic acids and valerolactone derivatives were found in the plasma of rats fed grape seed PA (Margalef, Pons, Bravo, Muguera & Arola-Arnal, 2015).

PA and their low MW phenolic acid metabolites can also exert a modulating effect on the microbiota in the colon. Monomeric flavan-3-ols incubated with fecal bacteria generated valerolactones and phenylpropionic acid, and led to an increase in the *Clostridium coccooides-Eubacterium rectale* group, *Bifidobacterium* spp. and *Escherichia coli* while decreasing *Clostridium histolyticum* (Tzounis et al., 2008). When PA (DP 2-3) were added to cultured fecal bacteria, *Lactobaccillus* spp. and *Enterococcus* spp. increased and *Clostridium histolyticum* decreased (Cueva et al., 2013). Human consumption of high MW cocoa PA for 4 weeks increased *Bifidobacterium* spp., *Lactobaccillus* spp., and *Enterococcus* spp. but decreased *Clostridium* spp. in human fecal samples (Tzounis, Rodriguez-Mateos, Vulevic, Gibson, Kwik-Urbe & Spencer, 2011). Broadly, *Bifidobacterium* spp. and *Lactobaccillus* spp. have health promoting functions while *Clostridium* spp. have deleterious effects (Gibson & Roberfroid, 1995), so PA have been found to exert a positive effect on colonic bacteria.

Beyond that, PA alter digestibility of foods by blocking digestive enzymes and reducing or slowing macronutrient digestion (Amoako & Awika, 2016b, Barros, Awika & Rooney, 2012, Dunn, Yang, Girard, Bean & Awika, 2015, Gonçalves, Mateus & De Freitas, 2011). It has been shown *in vitro* that PA can interact with and inhibit digestive enzymes including amylases (Gonçalves, Mateus & De Freitas, 2011), proteases (Gonçalves, Soares, Mateus & De Freitas, 2007), and lipases (Moreno, Ilic, Poulev, Brasaemle, Fried & Raskin, 2003). PA are also known to complex with protein and reduce protein digestibility (Cousins, Tanksley, Knabe & Zebrowska, 1981, Dunn, Yang, Girard, Bean & Awika, 2015, Feeny, 1968).

Other *in vitro* studies have shown that PA can interact with starch, especially amylose, and decrease rapidly digestible starch, increase slowly digestible starch, and create resistant starch (Amoako & Awika, 2016b, Barros, Awika & Rooney, 2012, Barros, Awika & Rooney, 2014). In a sorghum porridge (Lemlioglu-Austin, Turner, McDonough & Rooney, 2012) and wheat flour tortilla (Dunn, Yang, Girard, Bean & Awika, 2015), PA also slowed starch digestion *in vitro*. This modulation of digestion can increase satiety and decrease total calorie consumption (Holt & Miller, 1995, Holt, Brand, Soveny & Hansky, 1992, Willis, Eldridge, Beiseigel, Thomas & Slavin, 2009). A human trial (N=10) showed consumption of a sorghum-based drink with PA prior to glucose solution decreased glycemic response (Anunção et al., 2016), likely because the PA interacted with digestive enzymes or macronutrients. By slowing or inhibiting digestion and increasing satiety, PA could be a valuable weight management tool.

Wheat Gluten

Wheat is a globally important grain. Of all grain crops worldwide, wheat is cumulatively planted on the greatest amount of acreage (Statista, 2017), ahead of corn and rice. Wheat provides about 20% of calories worldwide (FAO Statistics Division, 2013) and is widely consumed in a variety of products. These include the bread forms of many cultures, various bakery goods, pasta and noodle applications, etc. Refined wheat flour is composed of starch (80-85%, dry basis), proteins (11-17%, dry basis), non-starch polysaccharides (2-3%, dry basis), lipids (2-3%, dry basis), and ash (< 1%, dry basis).

Though wheat gluten proteins comprise less than 1/5th of wheat flour, they are largely responsible for wheat flour's ability to form leavened bread. Gluten confers a unique property onto doughs: viscoelasticity. This property allows for a dough which can be stretched (extensibility) and will spring back together (elasticity): it allows for doughs to be sheeted, manipulated, formed into a variety of products, and to leaven. No other grain proteins have equivalent properties.

Chemistry of Gluten's Components: Glutenins and Gliadins

Two protein types comprise gluten: alcohol-soluble gliadins and alcohol-insoluble glutenins. Gliadins are generally globular with MW 32-74 kDa (Fido, Bekes, Gras & Tatham, 1997, Shewry, Tatham, Forde, Kreis & Mifflin, 1986). Glutenins have a more elongated structure with bimodal distribution: smaller, low molecular weight glutenin subunits (LMW-GS, 30-60 kDa) and larger, high MW-GS (HMW-GS 65-90+kDa) (Lew, Kuzmicky & Kasarda, 1992, Shewry, Halford & Tatham, 1992).

Glutenins and gliadins can each be further subdivided by protein types based on their electrophoretic mobility (Fig 7). Gliadins consist of alpha (~32 kDa), gamma (~38-42 kDa), omega (44-74 kDa) fractions (Fido, Bekes, Gras & Tatham, 1997, Shewry, Tatham, Forde, Kreis & Miflin, 1986). HMW-GS consist of x-type (larger) and y-type (smaller) subunits and are named based on which loci (1A, 1B, or 1D) they are encoded upon (Payne & Lawrence, 1983, Payne, Law & Mudd, 1980). LMW-GS are characterized by the first amino acid of their sequence: isoleucine, methionine, or serine (LMW-GS-*i*, -*m*, or -*s*) (D'Ovidio & Masci, 2004).

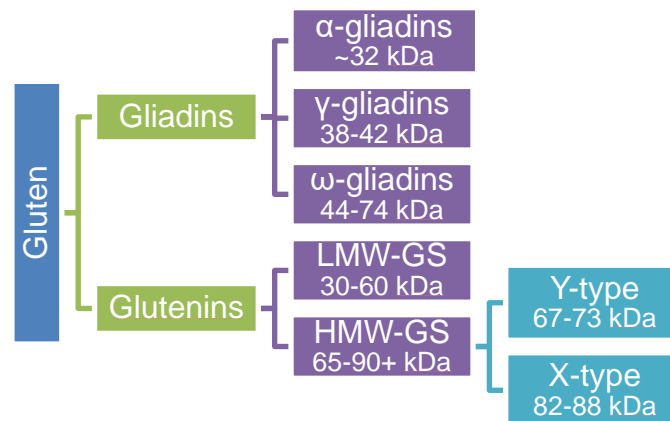


Figure 7 - Gluten protein subfractions.

As a whole, gluten is rich in the amino acids glutamine (~32 mol%) and proline (~14 mol%) with only a small proportion of cysteine (~2 mol%) (Ewart, 1967, Rombouts, Lamberts, Celus, Lagrain, Brijs & Delcour, 2009). Glutenins form macropolymers through intermolecular disulfide bonds; these macropolymers impart strength to the gluten structure through the aforementioned disulfide bonds and through

hydrogen bonds which further stabilize the structure. Gliadins typically form intra-, but not inter-, molecular disulfide bonds, thus are monomeric. They primarily interact with the macropolymer through noncovalent bonds (hydrogen bonds and hydrophobic interaction) (Fig 8).

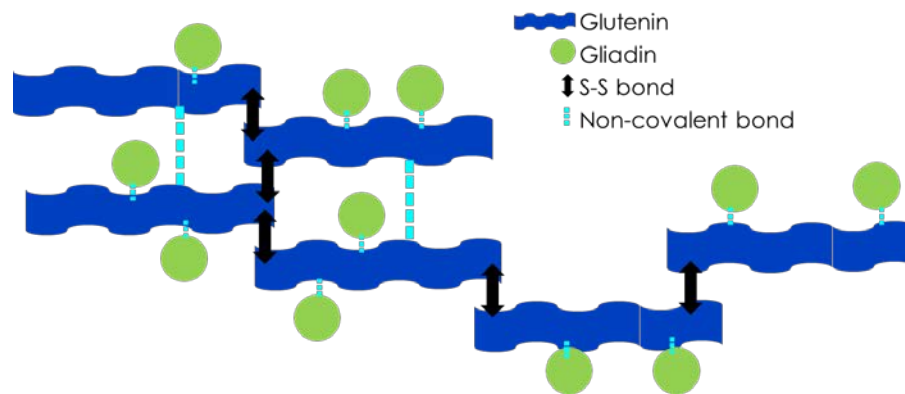


Figure 8 - Stylized gluten network structure.

The unique viscoelasticity of gluten allows wheat flour dough to capture gasses released from yeast during fermentation, expand and leaven, and maintain structure as the product is set and baked (Hoseney & Rogers, 1990). In general, gliadins impart extensibility to gluten whereas glutenins confer elasticity (Uthayakumaran, Newberry, Keentok, Stoddard & Bekes, 2000). The image Dimler published in 1963 of hydrated gluten, gliadins, and glutenins (Fig 9) illustrates these characteristics and is widely used to demonstrate the differences between gluten components.

Within wheat there is a large range of gluten quality: simply, the protein's impact on viscoelasticity. Across wheat gluten quality literature, the subjective terms of "strong" and "weak" are used to refer to gluten strength (Jazaeri, Bock, Bagagli, Iametti,

Bonomi & Seetharaman, 2015, Josephides, Joppa & Youngs, 1987, MacRitchie, 1973, Miller & Hosney, 1999). This designation has been used since at least 1900, when Helen W. Atwater wrote in a USDA bulletin that flours are classified as strong or weak based on the their quantity of gluten and thus their bread-making ability (Atwater, 1900).

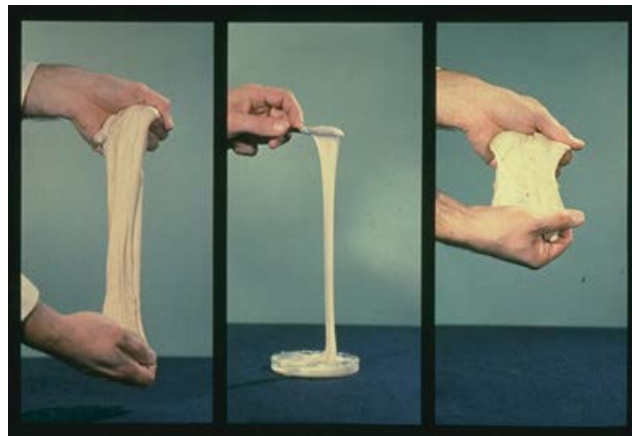


Figure 9 – Hydrated wheat gluten (left), gliadins (middle), glutenins (right) (reprinted from Dimler, 1963).

Protein quantity is of broad importance: more proteins create a larger, more cohesive gluten network resulting in a bread with greater volume (Finney & Barmore, 1948). But, quality plays an even larger role. Gluten quality is determined by the HMW-GS present, MW distribution of polymeric proteins (HMW-GS:LMW-GS), and ratio of glutenins:gliadins. Specific HMW-GS correlate to better bread quality (e.g.: *Glu-D1* 5+10 are superior to *Glu-D1* 2+12) (Payne, Corfield, Holt & Blackman, 1981, Payne, Corfield & Blackman, 1979). A higher HMW-GS:LMW-GS indicates a superior quality, as the HMW-GS are larger and create a denser gluten matrix (MacRitchie & Gupta,

1993). The ratio of glutenins to gliadins is also important, with balance required to get a gluten that is elastic and extensible enough to expand during fermentation without being too tight to allow expansion (Doekes & Wennekes, 1982, MacRitchie, 1987, Uthayakumaran, Newberry, Keentok, Stoddard & Bekes, 2000).

Gluten quality has a great impact on baked product quality. The product that has been most extensively studied is pan bread, and with good reason, as this is the most common method of wheat consumption in the US and throughout Europe. Strong gluten refers to that which will perform well in bread as it is extensively cross-linked when mixed and offers sufficient extensibility and elasticity; weak gluten refers to gluten that is less elastic. Soft wheats have weaker gluten: products made from soft wheat (cookies, cakes, pies, etc.) do not benefit from strong gluten and are actually superior with undeveloped gluten that will not toughen the product. By contrast, hard wheats are used for products like breads and noodles and require strong gluten for the desired organoleptic properties in finished goods. Of course, genetic traits can create variation within each subtype (hard vs soft), but environmental conditions also play a significant role in gluten quality (Peterson, Graybosch, Baenziger & Grombacher, 1992), with fluctuations seen in the product from year to year based on weather patterns.

General Uses of Gluten

Wheat gluten's unique properties allow for its use in an array of products (Table 2). Gluten is predominantly used as endogenous proteins in wheat flour for structure and function of baked food products (e.g.: bread, tortillas, cake, etc.). After hydration and

with mechanical energy applied, glutenins and gliadins interact through disulfide bonding and noncovalent interactions to create a continuous gluten matrix. This gluten matrix entraps air cells created during mixing and captures gas released due to leavening of baked goods. During the baking process, the gluten matrix allows for further expansion, and then sets the structure as the gluten proteins are denatured.

Millers and bakers often use redox reagents to manipulate wheat gluten quality. Oxidizing agents (e.g.: bromates, azodicarbonimide) strengthen gluten by oxidizing the thiol of cysteine residues and driving intermolecular disulfide bond formation. This increases the macropolymer size and creates a denser network. Reducing agents (e.g.: L-cysteine, antioxidants) break disulfide bonds thus decreasing the size of the macropolymers and weakening the gluten structure.

Table 2 - Wheat gluten functions and products.

Gluten Function	Product
Capture gas and allow for product expansion	Fermented baked goods (e.g.: bread, bagels, etc.)
Structure and texture	Most baked goods (e.g.: bread, tortillas, cake, muffin, cookies, etc.); pasta and noodles
Textural agent and protein source	Meat replacers, RTE cereal, fish food, pet food
Film forming ability	Bioplastic films
Thickening agent	Soups, gravies, sauces
Fermentation substrate	Soy sauce, beers
Bonding agent	Adhesives

Beyond its use in baked goods, gluten can function as a textural agent and protein source in meat replacers. For this application, gluten is generally extruded to restructure the polymer and mimic the texture of meat, as in textured vegetable protein. Gluten is plasticized (melted) and the proteins bonds are dissociated and reassociated during the

extrusion process. The protein associations are generally disulfide bonds initiated by hydrophobic interactions (Li & Lee, 1996a, Li & Lee, 1996b). Processing conditions greatly affect the protein extrudate structure, with lower mechanical energy and temperature leading to a less dense gluten network and smooth-surfaced extrudates (Redl, Morel, Bonicel, Vergnes & Guilbert, 1999).

Gluten has also been used for biofilm applications (Gontard, Guilbert & Cuq, 1992). Gluten films can be created by solution-casting or compression molding/extrusion. In either method, gluten functions to create a highly cross-linked polymer network. Biopolymers are ecofriendly alternatives to plastic because they rely on renewable resources for production, they are biodegradable, and there is a possibility to make them safe for biomedical or edible applications. For instance, wheat gluten films degraded in farmland soil within 50 days (Domenek, Feuilloley, Gratraud, Morel & Guilbert, 2004), and edible wheat gluten films have been created (Gontard, Guilbert & Cuq, 1992). However, biopolymers often have inferior properties to plastics (e.g.: weaker, less extensible, higher water vapor permeability (Hernandez-Izquierdo & Krochta, 2008)).

Gluten and Diet

Wheat gluten is present in many whole grain and all whole wheat food products, which provide wholesome calories, beneficial fiber, and phenolic compounds to the human diet. Gluten is a primary source of protein in many cultures throughout the world. Over the last 50 years, worldwide consumption of wheat increased by almost 25% from

416 calories/person/day in 1962 to 526 calories/person/day in 2011 (FAO Statistics Division, 2013).

There are reasons to avoid gluten consumption including celiac disease and gluten allergy. An estimated 1% of the world population has celiac disease (Rubio-Tapia, Ludvigsson, Brantner, Murray & Everhart, 2012), an immune response to the prolamin fraction of wheat (gliadins), rye (secalins), and barley (hordeins) proteins that, over time, damages the villi of the small intestine and inhibits nutrient absorption (Wieser & Koehler, 2008). Another reason for gluten avoidance is gluten allergy, which affects less than 1% of all populations (Tatham & Shewry, 2008).

Proanthocyanidin Interaction with Proteins

When consumed, tannins are usually found to be bitter or astringent; this mouth-drying feeling occurs because tannins precipitate salivary proteins thereby reducing tongue lubrication. The astringent effect may be a human evolutionary trait to prevent continued ingestion of these so-called “antinutritional” factors. Tannins have been considered antinutritional factors because they can interact with macromolecules and enzymes thus preventing digestion and subsequent nutrient absorption (Chang & Fuller, 1964, Feeny, 1968, Goldstein & Swain, 1965, Schaffert, Lechtenberg, Oswald, Axtell, Pickett & Rhykerd, 1974).

Between hydrolysable and condensed tannins, condensed (PA) have been shown to have a greater affinity for proteins (Baxter, Lilley, Haslam & Williamson, 1997, Emmambux & Taylor, 2003, Hagerman, Rice & Ritchard, 1998, Lu & Bennick, 1998).

This is because of their structural differences. PA are largely linear with many hydroxyl groups and hydrophobic regions in close proximity available for hydrogen bonding and hydrophobic interactions, respectively (Fletcher, Porter, Haslam & Gupta, 1977, Zanchi, Konarev, Tribet, Baron, Svergun & Guyot, 2009). Hydrolyzable tannins have a more spherical structure (Hagerman, Rice & Ritchard, 1998), thus some of their hydroxyl groups are not available for interaction with macronutrients.

Many factors affect PA-protein interactions, including, most importantly, protein and PA size and conformation. Generally larger proteins with open conformations and a higher concentration of proline (e.g.: glutenin proteins) are more likely to interact with PA (Hagerman & Butler, 1980a, Hagerman & Butler, 1980b). As for PA, those with larger DP (e.g.: sorghum PA), more flexibility, and greater number of hydroxyl groups interact more strongly with proteins (Hagerman, Rice & Ritchard, 1998, Harbertson, Kilmister, Kelm & Downey, 2014).

How Proanthocyanidins Interact with Protein

PA interact with proteins largely through hydrogen bonding and hydrophobic interaction (Hagerman & Butler, 1980a, Hagerman & Butler, 1980b, Hagerman & Butler, 1981, Haslam, 1974, Oh, Hoff, Armstrong & Haff, 1980). PA-protein bonds are irreversible, though not covalent (Hagerman, Rice & Ritchard, 1998). Extensive cross-links occur between PA and proteins due to PA's many hydroxyl groups and aromatic rings with hydrophobic regions. Most of the sophisticated modeling of these interactions have employed well-defined peptides or proteins such as polyamino acids, gelatin,

bovine serum albumin, or salivary or digestive enzymes (Deaville, Green, Mueller-Harvey, Willoughby & Frazier, 2007, Frazier, Papadopoulou, Mueller-Harvey, Kisson & Green, 2003, Kilmister, Faulkner, Downey, Darby & Falconer, 2016, Poncet-Legrand, Gautier, Cheynier & Imberty, 2007, Silva, García-Estévez, Brandão, Mateus, De Freitas & Soares, 2017). However, wheat proteins are complex and have structures that are not fully understood. This makes it difficult to predict their interaction with PA.

Recent research suggests that cereal proteins interact extensively with PA. Taylor et al. (2007) showed the sorghum prolamin, kafirin, can be cross-linked with sorghum PA, thereby decreasing both protein digestibility and biodegradation. PA encapsulated in kafirin microparticles had an antioxidant effect (Taylor, Taylor, Belton & Minnaar, 2009) and slowed carbohydrate digestion in mice, which can reduce the glycemic response (Links, Taylor, Kruger, Naidoo & Taylor, 2016), likely because PA were bound to proteins and inhibited digestive enzymes. Studies have also shown that tannins (condensed and hydrolysable) can alter strength of plant protein films in kafirin (Emmambux, Stading & Taylor, 2004), zein (de Freitas et al., 2017), and wheat gluten (Hager, Vallons & Arendt, 2012) films.

Work on PA interactions with wheat gluten proteins is sparse. Tannic acid formed haze in solution through bonds with gliadins (Siebert, Carrasco & Lynn, 1996). This tannin-protein effect was used to clarify and reduce astringency of wine by adding gluten to precipitate grape PA (Marchal, Marchal-Delahaut, Michels, Parmentier, Lallement & Jeandet, 2002, Maury, Sarni-Manchado, Lefebvre, Cheynier & Moutounet, 2003). Grape seed PA cross-linked with specific, partially digested gliadin fractions

(Dias, Perez-Gregorio, Mateus & De Freitas, 2016). Sorghum bran containing PA decreased protein solubility and reduced starch digestibility in wheat flour dough, showing PA interacted with gluten and slowed enzyme degradation of starch (Dunn, Yang, Girard, Bean & Awika, 2015). Because of their large size, relatively high concentration of glutamine and proline amino acids (which promote hydrogen bonding and hydrophobic interactions, respectively), and generally open structure, gluten proteins are likely to interact strongly with PA.

Potential Applications of Proanthocyanidin-Gluten Interactions

PA-gluten interactions could be used to strengthen the gluten matrix, to stabilize gluten film structure, or to slow or inhibit macronutrient digestion. As strong antioxidants, PA will break disulfide bonds as was observed for tannic acid (Wang et al., 2015, Zhang, Cheng, Jiang, Wang, Yang & He, 2010) and phenolic acids (Han & Koh, 2011). This generally decreases dough strength by breaking apart gluten macropolymers and increasing protein extractability (Han & Koh, 2011). However, because of PA propensity to cross-link proteins via hydrogen bonding and hydrophobic interaction, it may overcome the antioxidant weakening effect and actually strengthen the gluten matrix. Tannic acid, of unspecified chemistry, has been shown to increase wheat flour dough stability and bread loaf volume (Wang et al., 2015, Zhang, Cheng, Jiang, Wang, Yang & He, 2010). Tannic acid increased insoluble proteins in gluten, decreased surface hydrophobicity, and altered gluten secondary structure (Wang et al., 2015), suggesting tannic acid cross-linked gluten through noncovalent interactions.

Complexing PA with gluten could also strengthen and stabilize gluten film-forming properties. This has not yet been studied in wheat gluten. However, tannic acid showed a strengthening effect in wheat gluten films at a low usage level (5%) while greater usage (up to 30%) made films overly stiff and brittle (Hager, Vallons & Arendt, 2012). In a corn prolamin (zein) film, addition of PA in the form of Pinhão (*Araucaria angustifolia* (Bertol.) Kuntze) extract, increased film strength (de Freitas et al., 2017). PA was also shown to improve sorghum kafirin films, especially at low usage levels (5%) (Emmambux, Stading & Taylor, 2004). The ability of PA to cross-link protein could stabilize the film forming properties of gluten for use as a stand-alone film or in foams (e.g.: cake, bread).

As found with other cereal proteins, PA-gluten interactions would likely limit macronutrient digestibility (Links, Taylor, Kruger, Naidoo & Taylor, 2016, Taylor, Bean, Ioerger & Taylor, 2007, Taylor, Taylor, Belton & Minnaar, 2009). PA encapsulated by sorghum kafirin survived digestion to the small intestine and modulated blood glucose level (Links, Taylor, Kruger & Taylor, 2015, Links, Taylor, Kruger, Naidoo & Taylor, 2016). Similarly, sorghum bran containing PA reduced starch digestibility in wheat flour dough, showing PA interacted with gluten and slowed enzyme access to macronutrients (Dunn, Yang, Girard, Bean & Awika, 2015). Because starch granules are dispersed throughout the gluten matrix, increasing density of the protein matrix will likely hamper the ability of enzymes to access the starch for digestion as hypothesized by Dunn et al. (2015). Thus, gluten complexed with PA could be used to

reduce spikes in postprandial blood glucose and likely increase satiety by slowing digestion.

If not digested and absorbed in the upper gastrointestinal tract, the PA-complexed protein will survive to the colon where it will be fermented by various bacteria (Margalef, Pons, Bravo, Muguerza & Arola-Arnal, 2015, Wen, Wen, Zong, Linhardt & Wu, 2017, Zhang, Wang, Li, Ho, Li & Wan, 2016). While PA and undigested carbohydrates have beneficial colonic fermentation products, proteins typically do not. High protein, reduced carbohydrate diets were shown to increase proportions of undesirable colon substrates (branched-chain fatty acids, phenylacetic acid, and N-nitroso compounds) (Russell et al., 2011). However, this must be viewed in perspective: the presence of the PA, and plausibly some resistant starch, would likely be more beneficial.

CHAPTER III
EFFECT OF CONDENSED TANNIN PROFILE ON WHEAT FLOUR DOUGH
RHEOLOGY*

Introduction

Plant tannins have been utilized for millennia to form leather by cross-linking collagen in animal hides. Tannins can be divided into two groups: condensed tannins (proanthocyanidins, PA) consisting of polymerized flavanols, and hydrolysable tannins (e.g., tannic acid) composed of gallic acid esterified to glucose. Of the two tannin types, PA have been shown to have a greater affinity for proteins (Emmambux & Taylor, 2003, Hagerman, Rice & Ritchard, 1998). Higher molecular weight (MW) PA cross-link protein more efficiently, with at least three flavanol subunits required to bind protein. Binding also depends on protein structure, thus proteins with large, open conformation have a high affinity for PA (Hagerman & Butler, 1981). The mechanisms for cross-linking include hydrogen bonding and hydrophobic interaction (Butler, Riedl, Lebryk & Blytt, 1984).

Proanthocyanidin-protein interactions have a variety of consequences. When complexes form, they can aggregate and precipitate out of beverages such as juice, wine,

*Reprinted with permission from “Effect of Condensed Tannin Profile on Wheat Flour Dough Rheology” by Girard, A.L., Castell-Perez, M.E., Bean, S.R., Adrianos, S.L., Awika, J.M., 2016. *Journal of Agricultural and Food Chemistry*, 64, 7348-7356, Copyright 2016 by American Chemical Society.

and beer (Siebert, 2006) . PA also bind with salivary proteins and create the oral sensation of astringency (Maury, Sarni-Manchado, Lefebvre, Cheynier & Moutounet, 2003). Because they bind with macronutrients (starch and protein) or enzymes (such as α -amylase), PA can slow or inhibit macronutrient digestion (Amoako & Awika, 2016b, Amoako & Awika, 2016a, Gonçalves, Mateus & De Freitas, 2011), thereby decreasing caloric impact of foods. Recent work showed PA encapsulated in sorghum kafirin microparticles slowed carbohydrate digestion in mice, reducing the glycemic response (Links, Taylor, Kruger, Naidoo & Taylor, 2016). Because of PA's affinity for kafirin and kafirin's slow digestibility, PA remained active as an enzyme inhibitor in the small intestine (Links, Taylor, Kruger, Naidoo & Taylor, 2016).

Tannins also alter protein rheology; condensed and hydrolysable tannins increased the strength of plant protein films, based on sorghum kafirin (Emmambux, Stading & Taylor, 2004) and wheat gluten (Hager, Vallons & Arendt, 2012). Although tannins are antioxidants, which generally act as reducing agents and weaken dough, their ability to cross-link proteins may overcome this effect to strengthen dough. Work specifically with gluten-PA interactions is sparse. Tannic acid bound one of the gluten proteins, gliadin, resulting in protein aggregation and haze formation (Siebert, 2006), and grape seed PA cross-linked with specific, partially digested gliadin fractions, some containing celiac disease epitopes (Dias, Perez-Gregorio, Mateus & De Freitas, 2016). Gluten has been used to clarify and reduce astringency of wines through precipitation of grape PA (Maury, Sarni-Manchado, Lefebvre, Cheynier & Moutounet, 2003). Sorghum bran containing PA was shown to interact with wheat flour dough by decreasing protein

solubility and reducing starch digestibility (Dunn, Yang, Girard, Bean & Awika, 2015). However, none of the aforementioned studies looked at the effect of the gluten-PA interaction on gluten rheology.

Wheat gluten is, perhaps, the most uniquely functional plant protein. Its complexation with tannins presents interesting opportunities to alter and expand its functionality. Previous studies on wheat gluten have used commercial tannic acid of unspecified chemistry (Wang et al., 2015, Zhang, Cheng, Jiang, Wang, Yang & He, 2010). The authors reported a somewhat mixed effect of tannic acid on gluten strength, likely due to insufficient cross-linking of wheat protein to overcome the antioxidant effect of tannic acid on gluten. Emmambux and Taylor (2003) showed that PA bound kafirin more strongly than tannic acid, similar to findings by Hagerman et al. (1998) with bovine serum albumin. The PA have a higher MW and a much larger structure, which may explain their better binding efficiency to proteins (Emmambux & Taylor, 2003, Hagerman, Rice & Ritchard, 1998). Besides the work on gluten-PA precipitation in wines (Maury, Sarni-Manchado, Lefebvre, Cheynier & Moutounet, 2003), no studies have looked at the effect of gluten or PA MW profile on gluten-PA binding and possible impact on gluten rheology.

With the known consequences of PA on collagen and other proteins high in proline with open, non-globular structure, it is likely that the PA could be used as a natural ingredient to expand gluten functionality. Such functionalities could target improved dough strength, formation of edible films, and decreased caloric impact of

food macromolecules. This study aimed to determine the effect of PA MW profile on rheology of wheat flours with differing gluten composition.

Materials and Methods

Materials

Proanthocyanidins of different MW profiles were obtained from sorghum and grape seed. Sorghum PA were extracted from high tannin sorghum bran using 70% (v/v) aqueous acetone following procedure previously described (Barros, Awika & Rooney, 2012); it comprised mostly high MW (degree of polymerization, DP > 10) units. Grape seed PA were obtained from NuSci Institute & Corp (Walnut, CA) and contained a mixture of monomers, dimers, oligomers (DP 3-7), and some polymers (DP 8+). Catechin hydrate (94% pure catechin) obtained from Sigma-Aldrich (St. Louis, MO) was used as a positive control. All solvents were HPLC or analytical grade. Gallic acid, Folin-Ciocalteu reagent, ethanolamine, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and procyanidins B1 and C1 were purchased from Sigma-Aldrich (St. Louis, MO). Trolox from TCI America (Portland, OR).

Two wheat flours were selected for their similar protein content (14%) but differing protein composition: a commercial bread flour (Hummer Flour, Horizon Milling, Wayzata, MN) with strong gluten and a weak gluten flour from an experimental wheat line (Texas A&M University) modified via high molecular weight glutenin subunit (HMW-GS) deletions (*Glu-D1* 10y and *Glu-B1* 18y) to increase gluten extensibility (Jondiko, Alviola, Hays, Ibrahim, Tilley & Awika, 2012).

Proanthocyanidin Quantification

Proanthocyanidins were characterized with a normal phase HPLC method described by Langer et al. (2011) using a Develosil Diol column (250mm x 4.6 mm, 5 µm particle size, Phenomenex, UK). Fluorescence was measured at excitation wavelength of 230 nm and emission at 321 nm. The mobile phase was (A) acetic acid in acetonitrile (2:98) and (B) acetic acid in methanol and water (2:95:3) with a flow rate of 0.6 mL/min. The elution gradient (B) over the 83 min run time was: 0-3 min, 7% isocratic; 3-57 min, 7-37.6%; 57-60 min, 37.6-100%; 60-67 min, 100% isocratic; 67-73 min, 100-7%; 73-83 min, 7% isocratic. Molecular weight distribution of monomers through trimers was quantified based on available standards (catechin, procyanidin B1, and procyanidin C1). Quantity of PA with DP greater than four was extrapolated based on procyanidin C1 (trimer) peak response as described by Ojwang et al. (2013).

Phenolic Content and Antioxidant Estimation

Phenolic content was estimated with a modified Folin-Coicalteu method as recently described (Dunn, Yang, Girard, Bean & Awika, 2015). Antioxidant capacity was measured using a modified Trolox equivalent antioxidant capacity (TEAC) assay (Awika, Rooney, Wu, Prior & Cisneros-Zevallos, 2003). Catechin, grape seed PA, and sorghum PA were each dissolved in 1% HCl in MeOH. Trolox served as the standard and values were expressed as µM TEAC/g sample. Standards and samples (100 µL) were reacted with dilute ABTS^{•+} solution (2900 µL) for 30 min; then absorbance read at 734 nm.

Wheat Flour Protein Profiling

Flour protein and moisture content were analyzed with near-infrared spectroscopy following AACCI Method 39-11.01 (AACC International). To characterize the protein composition, dough samples comprising flour, water, salt (2% of flour), and treatments (catechin and grape seed and sorghum PA at 0-2.5 mg PA/g flour) were mixed to full dough development per mixograph data, freeze-dried, and ground to a particle size of < 0.1 mm. Each sample was separated into 50% (v/v) aqueous 1-propanol soluble and insoluble fractions based on method of Schober et al. (2006). The soluble fraction was analyzed with size exclusion HPLC (SE-HPLC) to quantify soluble polymeric protein (SPP) and monomeric proteins (Schober, Bean & Kuhn, 2006). The insoluble polymeric protein (IPP) fraction was sonicated at 10 W for 30 sec in 50% (v/v) aqueous 1-propanol to solubilize remaining protein, and then analyzed with SE-HPLC (Bean, Lyne, Tilley, Chung & Lookhart, 1998). Protein fractions were compared using HPLC area under curve. Previous studies (Bean, Lyne, Tilley, Chung & Lookhart, 1998, Schober, Bean & Kuhn, 2006) on separating gluten fractions with 50% 1-propanol indicate SPP includes soluble glutenin polymers with a lower average MW than the insoluble glutenin polymers of IPP. Thus, if the IPP increases, the proteins have increased in size and decreased in solubility.

Dough Mixing Properties

To determine effect of PA on dough mixing properties, doughs were analyzed with a mixograph (National Manufacturing, Lincoln, NE) following AACCI Method 54-

40.02 (AACC International). Treatments (catechin, grape PA, and sorghum PA) were used at 0.8, 1.6, and 2.5 mg PA equivalents/g flour (based on HPLC quantification). These levels were chosen based on preliminary tests with sorghum PA: 0.8 mg PA/g flour was the lowest concentration to show noticeable effect on mixograph parameters. Flour absorption was based on control (no extract) water absorption as determined by mixograph and held constant with all treatments to limit variability.

The standard mixograph run time of 8 min allowed most of the doughs to hydrate, fully develop the gluten matrix, and breakdown through overmixing; one exception was the high level sorghum PA treatment with the strong gluten flour where the mixing time had to be increased to 13 min to capture the full mixing profile. The following mixogram data points were used to describe dough behavior: time to midline peak, width at midline peak, width 2 min after peak, and peak angle. Taken together, these are potential indicators of dough strength: larger values of each indicator suggest stronger dough.

Large Deformation Rheometry

Dough extensibility indicates processability as large deformations occur during normal baking processes, and extensibility can predict dough behavior and finished product quality (Dobraszczyk & Salmanowicz, 2008). Thus, dough extensibility was measured using a Kieffer dough and gluten extensibility rig attached to a texture analyzer (TA.XT2i, Texture Technologies, Scarsdale, NY/Stable Micro Systems, Godalming, Surrey, UK) with a modified Smewing method (1995). Flour, water, salt

(2% of flour), and treatments (catechin and grape seed and sorghum PA at 0-2.5 mg PA/g flour) were mixed to optimal dough development time based on mixograph data. Dough balls were rested in proofing chamber (32 °C, 70% RH) for 10 min. Then the dough was placed in the grooved Teflon mold coated in mineral oil and rested for 45 min at ambient conditions inside a plastic bag to prevent drying. Dough strips were carefully removed from the mold and clamped into place on the platform of the extensibility rig. Parameters recorded were dough extensibility and resistance to extension. Five replications were run for each sample.

Small Deformation Rheometry

In contrast to mixing properties and dough extensibility, fundamental rheometry may detect relatively modest changes in dough material properties which can greatly impact finished product quality (Tronsmo, Magnus, Baardseth, Schofield, Aamodt & Færgestad, 2003, Tuncil, Jondiko, Castell-Perez, Puerta-Gomez & Awika, 2016). Stress and frequency sweeps and creep tests were performed on a HAAKE Rheostress 6000 rheometer (Thermo Scientific, Waltham, MA). A parallel plate geometry was used with the top smooth plate with a diameter of 20 mm and a gap between top and bottom plates of 1 mm. Doughs were made of flour, water, and treatments (catechin and grape seed and sorghum PA at 0-2.5 mg PA/g flour) mixed to optimum per mixogram data. After mixing, samples were divided into balls (~15 mm diameter), covered to prevent drying, and rested for 60 min to allow dough to relax. Samples were then placed between plates and analyzed immediately. All samples were run under temperature control at 21 °C (to

mimic dough behavior at room temperature) with the help of a TC-81 Peltier system (Thermo Fisher Scientific, Waltham, MA). Stress sweeps (1 Hz, 0.1-100 Pa) were performed to determine the linear viscoelastic region (LVR) of each sample to ensure the rheological properties were constant. Stress of 1 Pa was within the LVR of all samples, thus used for frequency sweeps (1 Pa, 0.1–10 Hz) to determine the storage (G') and loss (G'') moduli. The complex modulus (G^*) was calculated from G' and G'' as ($G^* = [(G')^2 + (G'')^2]^{1/2}$) (Steffe, 1996). G^* gives a good indication of dough strength, with stronger doughs exhibiting larger G^* , assuming $G' > G''$ (Tuncil, Jondiko, Castell-Perez, Puerta-Gomez & Awika, 2016). The tangent of the phase angle ($\tan \delta$) was calculated as G''/G' (Steffe, 1996). $\tan \delta$ can range from 0 (perfectly elastic material) to infinity (perfectly viscous material) (Steffe, 1996).

Creep and recovery tests (300 s at 250 Pa, 300 s at 0 Pa) were performed to characterize gluten elasticity. Constant stress was applied, followed by a period of no stress (creep and recovery test), and deformation during both cycles was recorded. Time and stress were applied based on literature (Schober, Clarke & Kuhn, 2002, Tronsmo, Magnus, Baardseth, Schofield, Aamodt & Færgestad, 2003). From the creep test data, deformation (or compliance) and recovery was calculated using a Burgers model which combines Kelvin-Voigt and Maxwell elements to quantify viscoelastic behaviors (Steffe, 1996). The four element Burgers model was used to quantify this data, written as a creep compliance function:

$$J = f(t) = J_0 + J_1 \left[1 - \exp\left(\frac{-t}{\lambda_{ret}}\right) \right] + \frac{t}{\mu_0} \quad (1)$$

with J_0 representing the instantaneous creep compliance, J_1 the retarded compliance, λ_{ret} the retardation time of the elastic component, and μ_0 the Newtonian viscosity (Steffe, 1996).

Relative recovery (Schober, Clarke & Kuhn, 2002) was also calculated from creep and recovery data:

$$Recovery = \frac{(J_{300s} - J_{600s})}{J_{300s}} \quad (2)$$

with J_{300s} representing maximum compliance during deformation and J_{600s} minimum compliance during recovery. Relative recovery indicates the percentage of sample which returned to its original form after deformation; i.e.: a perfectly elastic material would have 100% recovery.

Statistical Analysis

All tests and treatments were run in duplicate, unless otherwise stated. Means, standard deviations, and coefficients of variation were calculated using Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA). Data analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC). ANOVA used to detect treatment effects. A 5% significance level determined differences between treatments (Tukey's HSD). Dunnett's test was used to compare treatments to the control, with a 5% significance level.

Results and Discussion

Proanthocyanidin Quantification and Phenol and Antioxidant Estimation

Grape seed PA contained 583 mg PA/g extract with 45% of that being polymeric PA (DP 8+); sorghum PA contained 158 mg PA/g extract with a higher ratio (93%) of polymeric PA (Fig 10 and Table 3). This is in line with the ratio of PA with DP > 10 found in grape seeds (31%) and whole grain high tannin sorghum (86%) by Gu et al. (2004). Folin phenol content (mg GAE/g PA) of sorghum PA and grape seed PA was 1971 and 874, respectively (Table 3). Antioxidant capacity ($\mu\text{M TEAC/g PA}$) was 188 for catechin, 152 for grape seed PA, and 414 for sorghum PA.

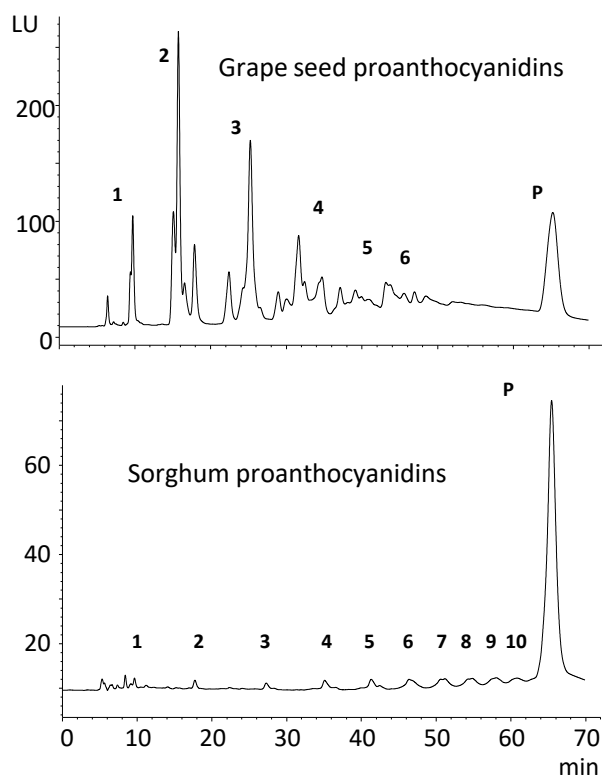


Figure 10 - HPLC chromatograms for grape seed and sorghum proanthocyanidins. Numbers above peaks indicate degree of polymerization with P > 10.

Table 3 - Quantitative profile of proanthocyanidins (PA) used in this study^a

	Proanthocyanidin Source		
	Catechin	Grape seed	Sorghum
PA content [mg PA/g extract]	942	577 ± 66	158 ± 18
<i>degree of polymerization (DP) 1-2 [%]</i>	100 c	5.9 ± 0.6b	0.3 ± 0.1 a
<i>DP 3-7 [%]</i>	0 a	49.3 ± 6.3 c	6.8 ± 1.3 b
<i>DP 8+ [%]</i>	0 a	45.8 ± 5.1 a	93 ± 1.4 c
Phenol content [mg GAE/g PA]	-	883 ± 107 a	1971 ± 432 b
AOX capacity [µM TEAC/g PA]	188 ± 39 b	153 ± 15 a	414 ± 12 c

^aDegree of polymerization data obtained using normal phase HPLC with fluorescence detection. Values with different letters within row are significantly different ($p < 0.05$).

Catechin served as the positive control because it is the most common monomeric unit of PA in both sorghum and grape seed. As catechin is a monomeric flavanol, it does not participate in protein-PA cross-linking (Emmambux & Taylor, 2003). Thus, catechin would only provide an antioxidant effect by breaking disulfide bonds and weakening gluten. Sorghum PA had the strongest antioxidant capacity per g PA (Table 3); however, it also had a higher proportion of polymeric PA than grape seed PA. Grape seed PA had the lowest antioxidant capacity and a blend of PA chain lengths with a lower average MW profile than sorghum PA. The higher antioxidant capacity of sorghum PA indicates it has a high average MW and a structure which exposes its many hydroxyl groups. Thus the treatments used in this study provided a good opportunity to determine the interactive effect of PA antioxidant properties and MW profile on gluten-PA cross-linking and gluten properties.

Effect of Proanthocyanidins on Flour Gluten Profile

The protein contents of strong gluten and weak gluten flours were similar (14.1 and 13.7%, respectively, db), but they showed vastly different protein compositions. SE-HPLC indicated strong gluten flour had almost twice as much IPP as weak gluten flour (84 vs 43 AU, respectively) (Fig 11), while the SPP contents were similar (86 vs 90 AU, respectively, data not shown). This agrees with Jondiko et al. (Jondiko, Alviola, Hays, Ibrahim, Tilley & Awika, 2012). As IPP contains the largest and least soluble polymeric proteins (Schober, Bean & Kuhn, 2006), higher IPP indicates stronger dough due to increased disulfide bonding and larger polymeric proteins (Bock & Seetharaman, 2012).

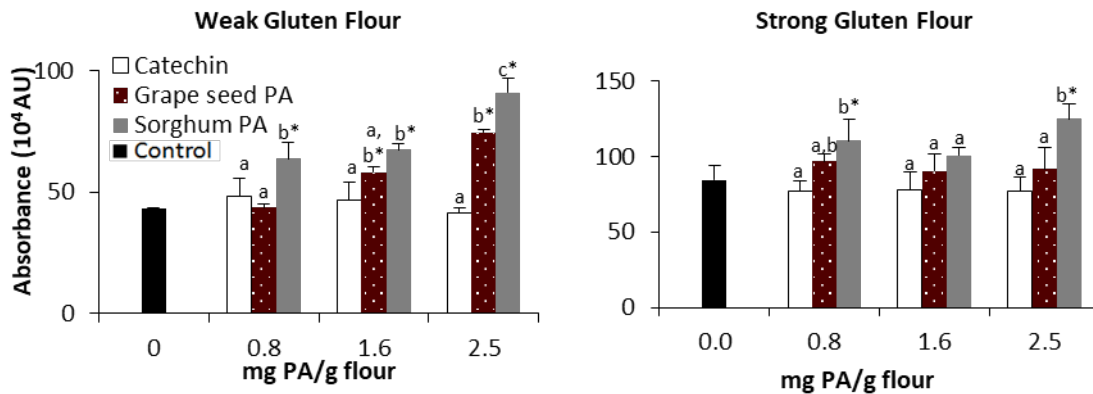


Figure 11 - Effect of catechin, grape seed, and sorghum proanthocyanidins (PA) on insoluble polymeric protein (IPP) for weak gluten, and strong gluten flour. Letters above bars indicate significant difference ($p < 0.05$, Tukey's HSD) between treatments at given level; stars indicate significant difference ($p < 0.05$, Dunnett's) from control (black bar).

SE-HPLC of treatments (Fig 11) showed that the PA treatments increased IPP; sorghum PA increased IPP more than grape seed PA, indicating high MW PA (sorghum PA) cross-links gluten more efficiently than lower MW PA (grape seed PA). On the

other hand, catechin slightly (though not significantly) reduced IPP in the strong gluten flour, likely by reducing disulfide linkages. Furthermore, the PA effect on IPP was greater with the weak gluten flour than the strong gluten flour. For example, at 2.5 mg PA/g flour, sorghum PA more than doubled the IPP in the weak gluten flour compared to an increase of 50% in the strong gluten flour. Also, the grape seed PA significantly increased the IPP in weak gluten flour but not the strong gluten flour (Fig 11). No significant differences in SPP were observed based on treatments; however, increase in IPP did correspond to decrease in the monomeric proteins of the flours, especially the weak gluten flour (data not reported). The different effects in weak versus strong gluten flour may be due to PA preferentially interacting with monomeric proteins over polymeric proteins. Similarly, Maury et al. (2003) found that gluten with lower MW precipitated high MW PA more selectively than high MW gluten. The data suggests that the PA is interacting with both monomeric and polymeric proteins; likely some of the monomeric proteins are becoming SPP and IPP, and some of the SPP is becoming IPP. Cross-linking gluten proteins with PA, especially sorghum PA, created larger, less soluble proteins. In fact, sorghum PA increased IPP in the weak gluten flour to a level equivalent to IPP content in the strong gluten flour (Fig 11), which suggests sorghum PA may increase the strength of weak gluten to behave like a strong gluten flour.

Because sorghum PA was highly polymerized, it had more hydroxyl groups and hydrophobic regions to cross-link with gluten (Hagerman, Rice & Ritchard, 1998) thus increased IPP more effectively than grape seed PA. Previous work showed sorghum PA irreversibly bound kafirin and created haze in solution (Emmambux & Taylor, 2003),

and increased protein size. High IPP has been shown to correlate with stronger gluten rheological characteristics such as mixing strength, extensibility, and elasticity (Schober, Bean & Kuhn, 2006, Zhang, Jondiko, Tilley & Awika, 2014). Thus, cross-linking, as with high MW PA, leads to increased IPP and stronger dough.

Effect of Proanthocyanidins on Dough Mixing Properties

As expected, strong gluten flour had higher mixogram parameters: longer time to peak (4.2 vs 2.0 min), greater band width at peak (36 vs 19%), greater band width 2 min after peak (18 vs 5%), and peak angle (112 vs 99°) compared to weak gluten flour (Fig 12; Table 4). The weak gluten data was similar to recent results (Tuncil, Jondiko, Tilley, Hays & Awika, 2016). The weak gluten flour has HMW-GS deletions because it was developed for flatbread applications: it is intended to have a high extensibility and low elasticity (Jondiko, Alviola, Hays, Ibrahim, Tilley & Awika, 2012).

Fig 12 and Table 4 show the effect of treatments on mixograms and key mixogram parameters. Catechin, as expected, had a weakening effect on both doughs. The PA treatments, on the other hand, increased dough strength, with the sorghum PA producing a much stronger effect than grape seed PA. For example, sorghum PA addition to the weak gluten flour resulted in a mixogram and mixogram parameters that were almost similar to the strong gluten control while the strong gluten flour treated with sorghum PA required a much longer mixing time (13 min) to show typical dough breakdown (versus typical mixing time of 8 min).

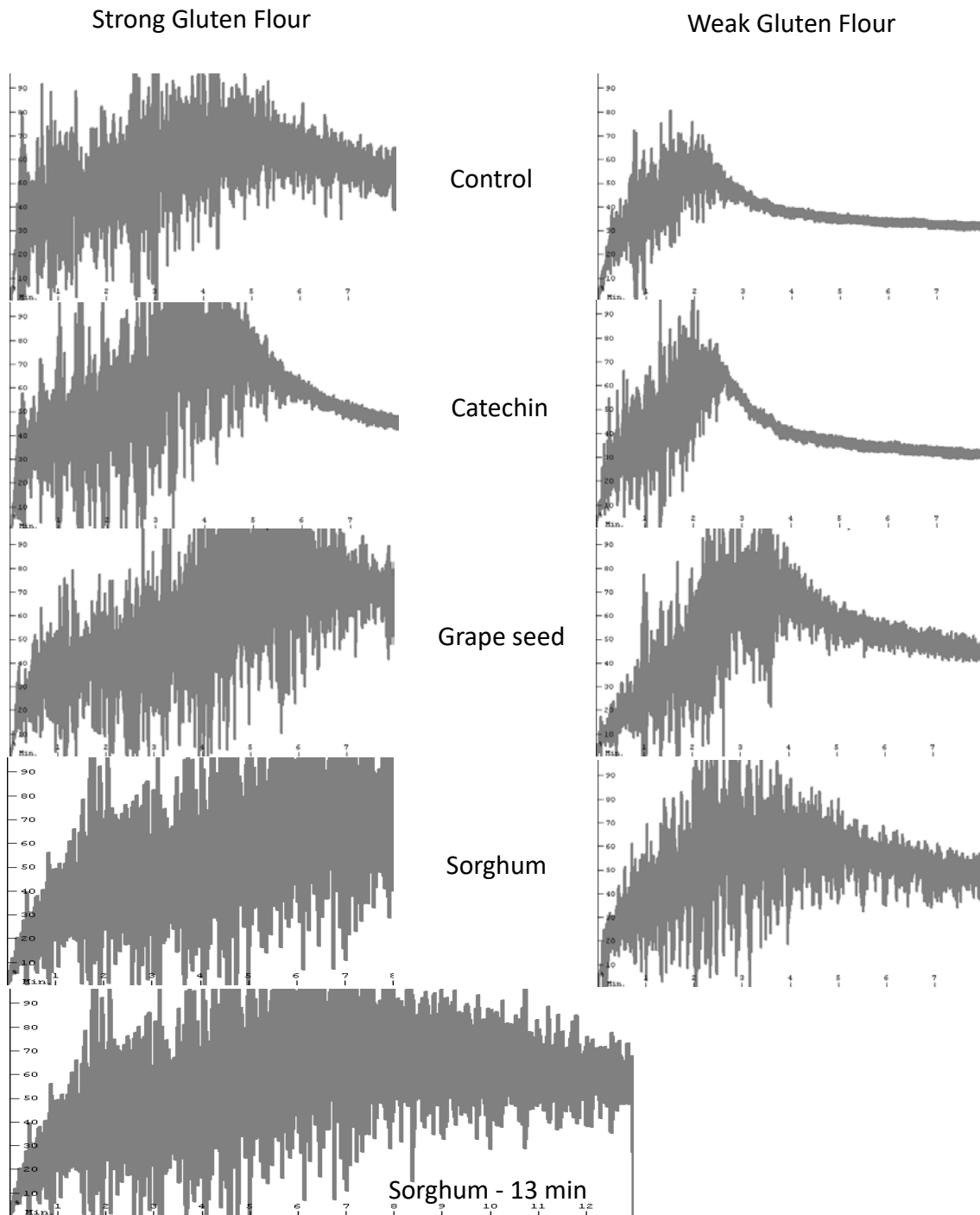


Figure 12 - Effect of catechin, grape seed, and sorghum proanthocyanidins (PA) on mixing properties of strong gluten flour (left) versus weak gluten flour (right) at a 2.5 mg PA/g flour addition level. Note: Standard mixing time was 8 min, except sorghum PA-strong gluten dough that required 13 min mixing.

Table 4 - Effect of catechin, grape seed, and sorghum proanthocyanidins on dough mixing properties^a

Flour	Treatment	Peak Time [min]	Peak Width [%]	Width + 2 [%]	Peak Angle [°]
	Control	4.2 c	36 c	18 b	112 c
Strong	Catechin	4.4 c	32 bc	6 a	89 a
Gluten	Grape Seed	5.9 d	39 c	17 b	106 b
	Sorghum	6.9 e	40 c	29 c	129 d
	Control	2.0 a	19 a	5 a	99 b
Weak	Catechin	2.1 a	22 ab	5 a	80 a
Gluten	Grape Seed	3.4 b	31 bc	8 a	84 a
	Sorghum	3.5 b	35 c	15 b	116 c
	<i>Average CV</i>	<i>4%</i>	<i>7%</i>	<i>13%</i>	<i>3%</i>

^aProanthocyanidins used at 2.5 mg/g flour. Values followed by the same letter in the same column are not significantly different ($p < 0.05$). Peak time = time to midline peak; peak width = average width at midline peak; width + 2 = average width at 2 min after midline peak.

The weakening effect of catechin was more prominent on the strong gluten flour. Catechin shifted the redox reaction from disulfide bonds to free –SH, thus broke the gluten cross-linkages and weakened the dough. This antioxidant effect is consistent with results of other studies (Han & Koh, 2011, Kerr, Hosney & Faubion, 1993).

Grape seed PA had only a modest effect on the strong gluten flour, with the most obvious effect being an increase in mixing time (Table 4). However, its effect on the weak gluten flour was more pronounced (Fig 12, Table 4), which corresponds to the effect seen on the gluten profile. Curiously, grape seed PA decreased the peak angle in both flours (Table 4). This suggests that the oligomeric PA in grape seed modestly strengthened dough during mixing but made it more susceptible to overmixing due to the antioxidant effect. So, grape seed PA appears to have a mixed effect on gluten by not strengthening all parameters, similar to reports for tannic acid (Wang et al., 2015, Zhang,

Cheng, Jiang, Wang, Yang & He, 2010). Zhang et al. (2010) and Wang et al. (2015) found that tannic acid increased mix time; however, Wang et al. (2015) found tannic acid did not increase mixogram peak width or width 2 min post peak. Grape seed PA, and tannic acid, may not cross-link protein as extensively as they break disulfide bonds, thus the sum of the effects result in increased mix time but decreased mix tolerance.

Sorghum PA significantly strengthened the doughs as evidenced by an increase in all key parameters in both flours (Table 4), likely due to intra- and inter-molecular cross-linking of soluble protein fractions with the glutenin to increase polymer size. The cross-linking effect of sorghum PA was at a high enough level that it transformed a very weak dough to a behavior similar to a strong gluten bread dough (Fig 12 and Table 4). Sorghum PA also increased the strength of the strong gluten flour such that the mixing time had to be extended from 8 to 13 min to capture the full mixing profile (Fig 12 and Table 4). This is supported by the gluten profile data showing the largest increase in IPP with sorghum PA addition (Fig 11). Though sorghum PA had the highest antioxidant capacity (Table 3), its affinity for gluten protein overcame the antioxidant dough weakening effect and strengthened the dough. This indicates the higher average DP in sorghum PA is much more favorable for gluten cross-linking and strengthening than the mainly oligomeric grape seed PA. Thus, polymeric PA may be useful as a natural dough strengthening agent.

Effect of Proanthocyanidins on Dough Extensibility

Dough resistance to extension, indicative of dough strength, differed significantly ($p < 0.05$) between weak gluten flour (0.24 N) and strong gluten flour (0.95 N) controls (Fig 13); which agrees with the mixogram data. The weak gluten flour dough was three times as extensible as the strong gluten flour dough (77 vs 23 mm, respectively).

Because the weak gluten wheat was developed for use in flatbreads, it lacks specific HMW-GS, which makes its dough highly extensible and less elastic, properties desirable for flatbread processing (Jondiko, Alviola, Hays, Ibrahim, Tilley & Awika, 2012).

Sorghum PA increased dough resistance to extension in both strong and weak gluten flours, while grape seed PA had insignificant effect in strong gluten flour but increased resistance in weak gluten flour. Catechin generally decreased resistance to extension in weak gluten flour. Compared to the control, sorghum PA at 2.5 mg PA/g flour nearly doubled resistance to extension for the strong gluten flour (0.95 to 1.70 N) and increased it for the weak gluten flour from 0.24 to 0.31 N (Fig 13). However, nearly all treatments increased dough extensibility.

Although the PA treatments significantly increased dough strength compared to the control, as evidenced by the mixogram (Table 4) and resistance to extension results (Fig 13), they did not decrease dough extensibility as would be expected. In fact, grape seed PA increased extensibility at all levels used. At 2.5 mg PA/g flour, grape seed PA increased strong gluten flour extensibility from control of 23 mm to 38 mm and weak gluten flour from 77 mm to 117 mm. This was likely due to dual action of PA: cross-linking gluten thus strengthening it (Zhang, Cheng, Jiang, Wang, Yang & He, 2010),

while breaking disulfide bonds through antioxidant activity (Han & Koh, 2011, Kerr, Hosney & Faubion, 1993) thus allowing dough to stretch more.

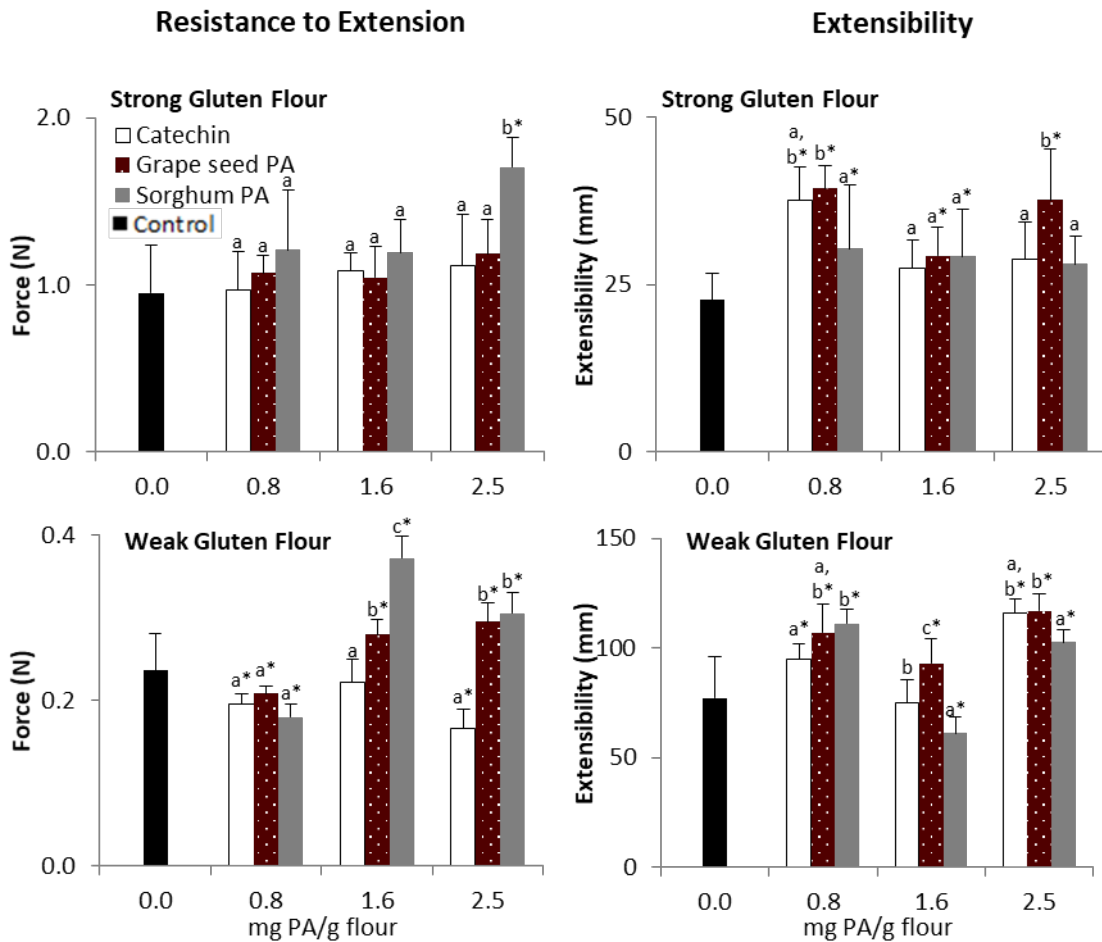


Figure 13 – Effect of catechin, grape seed, and sorghum proanthocyanidins (PA) at three levels on resistance to extension, and extensibility of strong and weak gluten flours as measured by a TA.XTplus texture analyzer. Letters above bars indicate significant difference ($p < 0.05$, Tukey's HSD) between treatments at given level; stars indicate significant difference ($p < 0.05$, Dunnett's) from control (black bar).

Interestingly, although sorghum PA at the levels used is a stronger antioxidant than grape seed PA (Table 3), it had a greater dough strengthening effect. This suggests

that the high DP PA complexed extensively with gluten to overcome the loss of disulfide linkages. Thus, gluten-PA interactions appear to be significant and depend upon the MW of PA. This effect on gluten strength may be a useful property to increase strength and flexibility of gluten-based films and coatings. Tannic acid was reported to make gluten films strong but brittle (Hager, Vallons & Arendt, 2012). Perhaps sorghum PA could improve strength and flexibility of such films based on the increased resistance to extension without subsequent negative effect on extensibility.

Effect of Proanthocyanidins on Dough Viscoelasticity

For all treatments and controls, G' (elastic modulus) was higher ($p < 0.05$) than G'' (viscous modulus) indicating a predominantly elastic character, a well-known property of wheat flour dough. As expected, complex modulus (G^*) of strong gluten dough was greater than weak gluten dough, while the opposite was true for $\tan \delta$ (Fig 14A and Table 5). Catechin treatments generally had the lowest G^* of all treatments, and lower than the controls, for both strong gluten and weak gluten flours, confirming its weakening effect on gluten.

Sorghum PA had the highest G^* in strong gluten and weak gluten flours (Figure 14A) indicating it created the most elastic dough of the treatments. The strong gluten flour with grape seed PA behaved very similarly to the control, whereas in weak gluten flour, grape seed PA increased G^* . The evidence confirms the large deformation data (Fig 13), suggesting PA, especially high MW, cross-linked with gluten and created a more elastic dough. Interestingly, Zhang et al. (2010) found that tannic acid decreased G'

and G'' , which indicates a decrease in G^* as compared to control dough. This implies that tannic acid decreased dough elasticity.

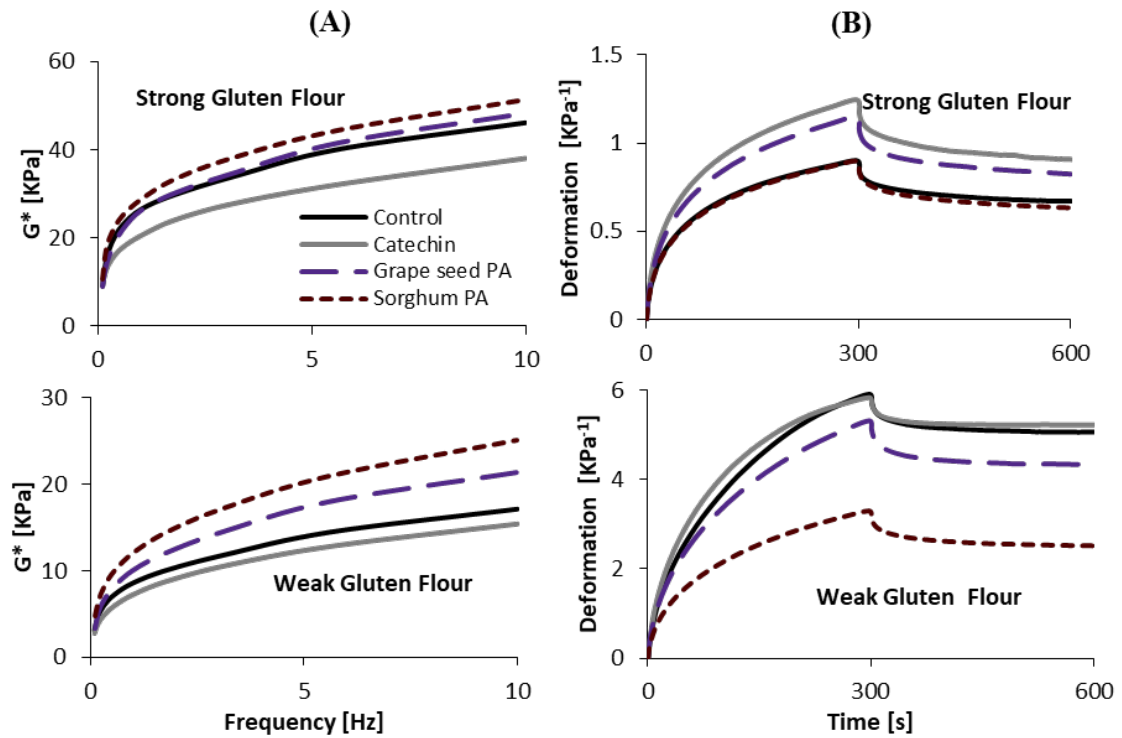


Figure 14 – Effect of catechin, grape seed, and sorghum proanthocyanidins (PA) at 2.5 mg PA/g flour on complex modulus (G^*) (A) and creep and recovery response (B) of strong and weak gluten flours.

Table 5 – Effect of catechin, grape seed, and sorghum proanthocyanidins on frequency sweep and creep-recovery of wheat dough^a

Flour	Treatment	Frequency Sweep ^b				Burgers Model				Relative Recovery		
		G'	G''	tan δ	G*	Instant Compliance, J ₀ [KPa ⁻¹]	Retarded Compliance, J ₁ [KPa ⁻¹]	Viscosity, μ_0 [KPa]	Retardation Time, λ_{ei} [s]	Max. Compliance, J _{30s} [KPa ⁻¹]	Min. Compliance, J _{60s} [KPa ⁻¹]	Relative Recovery [%]
	Control	43 d	16 c	0.38 a	46 d	0.07 a	0.63 a	1120 c	10.6 a	0.87 a	0.65 a	25 a
Strong	Catechin	39 c	16 c	0.41 ab	42 c	0.09 a	0.85 a	769 b	11.4 a	1.20 a	0.91 a	25 a
Gluten	Grape Seed	44 d	19 d	0.44 b	48 de	0.09 a	0.76 a	769 b	11.6 a	1.11 a	0.83 a	26 a
	Sorghum	48 e	19 d	0.41 ab	51 e	0.08 a	0.61 a	1000 c	11.2 a	0.87 a	0.63 a	27 a
	Control	15 a	8 a	0.53 cd	17 a	0.32 c	3.56 c	120 a	17.0 c	5.70 c	5.07 cd	11 b
Weak	Catechin	14 a	8 a	0.55 d	16 a	0.37 c	4.11 c	167 a	17.4 c	5.69 c	5.23 d	8 b
Gluten	Grape Seed	19 b	10 b	0.53 cd	21 ab	0.27 bc	3.00 bc	127 a	15.1 bc	5.08 c	4.34 bc	15 ab
	Sorghum	22 c	11 b	0.50 c	25 b	0.18 b	1.91 b	213 a	12.9 ab	3.15 b	2.51 b	20 ab
	<i>Average CV</i>	5%	4%	1%	5%	7%	7%	11%	5%	3%	6%	14%

^aProanthocyanidins used at 2.5 mg/g flour. Values followed by the same letter in the same column are not significantly different ($p < 0.05$).

^bFrequency sweep data at 10 Hz.

Effect of Proanthocyanidins on Dough Creep and Recovery Profile

All doughs showed nonlinear deformation during the loading period and partial recovery during the relaxation period—a typical viscoelastic behavior (Steffe, 1996). Values of retarded compliance (J_1), a measure of dough deformation using the Burgers model (Equation 1), for strong and weak gluten control doughs were 0.63 and 3.6 KPa^{-1} , respectively (Fig 14B and Table 5). As in Tronsmo et al. (2003), high compliance values were associated with weak flour. Relative recovery of strong gluten flour (25%) was much greater than weak gluten (11%), indicating that strong gluten flour had greater elasticity and strength.

None of the treatments significantly affected the strong gluten dough, though catechin and grape seed PA slightly increased the deformation implying a loss of elasticity (Fig 14B, Table 5). Sorghum and grape seed PA did significantly increase the weak gluten dough elasticity, while catechin showed a very slight (statistically insignificant) weakening effect. The greatest impact was seen with sorghum PA which decreased retarded compliance of weak gluten dough by almost 50% versus control (1.9 vs 3.6 KPa^{-1} , respectively). This was supported by the increase in relative recovery of the weak gluten flour with sorghum PA (from 11% in control to 20%) to a level similar to the strong gluten control (25%). The data confirms that sorghum PA increased elasticity and strength of the weak gluten dough to make it behave like the strong gluten dough; this agrees with the mixogram data (Fig 12, Table 5). The grape seed PA showed a small (not statistically significant) increase in weak gluten dough elasticity. Again, the mixed

oligomers of the grape seed PA did not bind the gluten proteins as efficiently as the high MW sorghum PA.

The effect of PA between flours with different gluten compositions is obvious: PA showed a greater strengthening effect on the weaker gluten flour. The lower MW gluten proteins, primarily gliadin, have shown a greater affinity for binding to and precipitating grape PA (Maury, Sarni-Manchado, Lefebvre, Cheynier & Moutounet, 2003). Due to its lower IPP content, the weak flour had proportionately more of the lower MW proteins available to cross-link with PA into polymeric proteins in dough. This then strengthened the dough as evidenced by stronger mixing profile, greater resistance to extension, and increased elasticity. Perhaps there are differences between the hydrophobicity of the proteins in the gluten sources; previous research indicated soft wheat had a high number of low affinity hydrophobic regions, while hard wheat had a low number of high affinity regions (Bock & Seetharaman, 2012, Huschka, Bonomi, Marengo, Miriani & Seetharaman, 2012). As hydrophobic interactions are one of the ways PA can cross-link proteins (Hagerman & Butler, 1981), a higher number of hydrophobic regions in the weak gluten flour would allow for increased interactions.

Polymeric PA are a promising natural ingredient to improve and expand gluten functionality. The MW profile of the PA is critical to such functionality, with high MW PA being more effective at strengthening gluten. The potential breaking of disulfide bonds, while yet still increasing elasticity and maintaining or increasing extensibility, is a novel dough effect. It suggests a potential of high MW PA to stabilize gluten films while maintaining their resilience, which may enhance volume of baked goods and allow

higher inclusion of beneficial nonwheat ingredients, improve the integrity of edible barriers and biofilms, among other benefits. The fact that the high MW PA effect was more pronounced in the weak gluten suggests such PA could function as a natural dough improver. Also, the effects were observed at relatively low PA concentrations (≤ 2.5 mg/g flour or 0.25%) which are not likely to negatively impact sensory properties in baking and related application.

CHAPTER IV

INTERACTION MECHANISMS OF CONDENSED TANNINS (PROANTHOCYANIDINS) WITH WHEAT GLUTEN PROTEINS*

Introduction

Proanthocyanidins (PA), commonly referred to as condensed tannins, strongly complex with proteins to alter their structure and functional properties. These interactions are usually noncovalent, primarily hydrogen bonding (Hagerman, Rice & Ritchard, 1998) and hydrophobic interactions (Oh, Hoff, Armstrong & Haff, 1980). The interactions have long been employed industrially (e.g., to tan leather) and in food processing (e.g., to clarify wine (Cerpa-Calderón & Kennedy, 2008, Sarni-Manchado, Deleris, Avallone, Cheynier & Moutounet, 1999)). PA have phenolic hydroxyl groups and hydrophobic regions that complex the carbonyl groups and hydrophobic amino acids, respectively, of proteins.

The PA-protein interactions could be exploited to modify polymer structures in food systems to benefit nutrition and health. Applications could include targeted delivery of micronutrients to specific regions of the digestive tract through micro/nanoencapsulation (Links, Taylor, Kruger, Naidoo & Taylor, 2016) or slowed

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glucose metabolism and reduced caloric impact of foods (Amoako & Awika, 2016b, Dunn, Yang, Girard, Bean & Awika, 2015). We recently showed that sorghum-derived PA dramatically increased wheat gluten strength without negatively impacting dough extensibility (Girard, Castell-Perez, Bean, Adrianos & Awika, 2016). This suggests that PA can alter the gluten polymer in a unique way and can serve as a natural mechanism to expand gluten functionality. Tannic acid was reported to increase wheat dough stability and bread loaf volume (Wang et al., 2015, Zhang, Cheng, Jiang, Wang, Yang & He, 2010). Gluten-PA interactions have also been investigated as a possible means to reduce consequences of celiac disease by binding specific gliadin fractions (Dias, Perez-Gregorio, Mateus & De Freitas, 2015).

Gluten proteins are rich in glutamine (~35 mol%) and proline (~14 mol%) (Delcour & Hoseneey, 2010), which are particularly amenable to complexation with PA via hydrogen bonds and hydrophobic interactions, respectively. Intermolecular disulfide bonds between cysteine residues are major contributors to gluten polymerization during dough mixing. However, as strong antioxidants, the PA inhibit disulfide cross-linkage formation, as was shown for tannic acid (Wang et al., 2015), which would weaken gluten as observed for the monomeric catechin (Girard, Castell-Perez, Bean, Adrianos & Awika, 2016). Thus, the gluten strengthening effect of tannins, despite their strong antioxidant effect, suggests extensive cross-linkage formation with gluten proteins.

Gluten comprises two main proteins: alcohol-soluble gliadins and alcohol-insoluble glutenins. Of the two primary gluten proteins, glutenins are largely responsible for gluten strength. Glutenins can be divided into high MW subunits (HMW-GS; 65-90+

kDa) and the smaller low MW subunits (LMW-GS; 30-60 kDa) (Delcour & Hosoney, 2010). Gliadins consist of three fractions: α - (MW ~31 kDa), γ - (~35 kDa), and ω -gliadins (44-80 kDa) (Barak, Mudgil & Khatkar, 2015). Glutenins, especially, HMW-GS, have a more elongated, rod-like structure than the spherical gliadins. Binding affinity of PA is impacted by protein size and shape: PA have a higher affinity for larger and less compact proteins (Hagerman & Butler, 1981). It is thus likely that PA preferentially interact with HMW-GS over other gluten proteins. Differences in gluten subunit composition may influence the nature of gluten interaction with PA.

Gluten-PA interactions present interesting opportunities, not only to naturally improve gluten functionality, but also to potentially expand its applications to benefit food quality and health. However, as yet, mechanisms for PA-gluten interactions are largely unknown. In this work, we investigate how glutenin and gliadin sub-fractions interact with PA of different MW profiles.

Materials and Methods

Materials

Gluten from two wheat (*Triticum aestivum* L.) flours with similar protein contents (14%) but different protein compositions were compared: a strong gluten commercial bread flour (Hummer Flour, Horizon Milling, Wayzata, MN) with HMW-GS *Glu-A1 2**, *Glu B1 7+9*, *Glu D1 5+10* and a weak gluten flour from an experimental wheat line developed by Texas A&M University with HMW-GS *Glu-A1 2**, *Glu B1 17x*, *Glu D1 5x*. This line has specific HMW-GS deletions (*Glu-D1 10y* and *Glu-B1*

18y) intended to increase gluten extensibility for flatbread applications (Jondiko, Yang, Hays, Ibrahim, Tilley & Awika, 2016, Tuncil, Jondiko, Tilley, Hays & Awika, 2016); its protein profile was previously reported (Zhang, Jondiko, Tilley & Awika, 2014). The allelic compositions were confirmed by lab-on-a-chip electrophoresis (described in Electrophoresis section). To obtain gluten, flour was defatted with hexane three times (once with 4 mL hexane/g flour, twice with 2 mL hexane/g flour); starch was then removed from gluten following the AACCI Method 38-10.01 (AACC International). Washed gluten was freeze-dried, ground, and stored at -20 °C until used. Nitrogen content was determined by combustion, and total protein was calculated using a 5.7 conversion factor.

Sorghum (*Sorghum bicolor*) and grape (*Vitis vinifera* L.) seed PA were compared because sorghum PA are mostly polymeric (degree of polymerization, DP>8) while grape seed PA have a lower DP profile (Girard, Castell-Perez, Bean, Adrianos & Awika, 2016). Sorghum PA were extracted from high tannin sorghum bran with aqueous acetone as previously described (Barros, Awika & Rooney, 2012). Freeze-dried sorghum PA were re-solubilized in 80% (v/v) ethanol and applied to a column of Sephadex LH-20. After removing monomeric phenolics and low MW PA with 80% (v/v) ethanol, the PA were recovered with 70% (v/v) acetone. The acetone was removed under vacuum, and the remaining sample freeze-dried and stored at -20 °C until used. Grape seed PA were from NuSci Institute & Corp (Walnut, CA). The PA monomer, (+)-catechin, was used as a positive control. Catechin hydrate (94% pure catechin) was from Sigma-Aldrich (St. Louis, MO).

All solvents were HPLC or analytical grade. Gallic acid, Folin-Ciocalteu reagent, ethanolamine, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), procyanidins B1 and C1, and bromophenol blue were from Sigma-Aldrich (St. Louis, MO). Trolox and phloroglucinol were from TCI America (Portland, OR). Dithiothreitol was from Fisher Scientific (Hampton, NH). Sephadex LH-20 was from GE Healthcare (Uppsala, Sweden).

Proanthocyanidin Characterization

Proanthocyanidins were quantified using normal phase HPLC with fluorescence detection, and phenolic content and antioxidant capacity estimated spectrophotometrically as described previously (Girard, Castell-Perez, Bean, Adrianos & Awika, 2016). Mean degree of polymerization (mDP) was estimated using HCl to depolymerize PA and the nucleophile phloroglucinol to form adducts with extensional units of polymeric PA (Cerpa-Calderón & Kennedy, 2008).

Wheat Gluten Sub-Fraction Isolation

Gluten fractions were isolated based on the method described by Sapirstein and Fu (1998) so that interaction of PA with specific subfractions could be analyzed. Aqueous 1-propanol (50% v/v) solubilized the gliadins and soluble polymeric glutenins. After centrifuging and extraction of the insoluble fraction (insoluble polymeric glutenins), the propanol concentration was increased to 70% (v/v) to precipitate the soluble polymeric glutenins. The insoluble polymeric glutenins were solubilized in 50%

(v/v) 1-propanol with 1% dithiothreitol (DTT) to break the disulfide cross-linkages.

After propanol was removed under vacuum, gliadins solubilized in 70% (v/v) 1-propanol and glutenins solubilized in 50% (v/v) 1-propanol with 1% DTT were freeze-dried, ground, and stored at -20 °C until used. The DTT remained with the glutenins to inhibit disulfide formation; DTT was not found to complex PA nor interfere with PA-gluten interactions (Hagerman, Rice & Ritchard, 1998).

Nephelometry

Kinetics of PA binding with gluten proteins was measured following the method described by Carvalho, Mateus, & de Freitas (2004) with minor modifications. Isolated gliadins or glutenins (10 mg) were dissolved in 50% (v/v) 1-propanol, 50% (v/v) 0.1 M acetate buffer (1 mL, pH 5). Catechin, grape seed or sorghum PA were added at 0-1500 mg PA/g protein. The mixture was shaken for 30 s, then absorbance was read at 630 nm at 0, 5, 10, 20, 40, 60, and 1440 min on a BioTek Synergy HT plate reader (Winooski, VT) using 24-well polystyrene plates. Equilibrium dissociation constant (K_d) was calculated from the PA-protein binding curve, based on best fit regression of absorbance vs mg PA/g protein (SigmaPlot 13). The molar ratio of PA to protein binding was estimated based on mDP of the PA and average M_w of 65 and 35 kDa for glutenins and gliadins, respectively (Delcour & Hosney, 2010). The amount of protein bound per unit PA was obtained from the regression plot of proteins precipitated by 0–500 mg PA/g protein (the range within which proteins in all treatments were fully precipitated from solution, as quantified by nitrogen combustion method).

Reversed Phase HPLC

Reversed phase HPLC was used to determine the interaction of various fractions of glutenins and gliadins with PA. Freeze-dried purified gliadin or glutenin (1 mg) fractions were resolubilized in 50% (v/v) aqueous 1-propanol (1 mL) for gliadins and 50% (v/v) aqueous 1-propanol + beta-mercaptoethanol for glutenins, and mixed with sorghum PA (0-30 mg PA/g protein) as described in Taylor, Bean, Ioerger, & Taylor (2007).

Soluble proteins (not precipitated by PA) were analyzed via RP-HPLC on an Agilent 1260 HPLC (Agilent Technologies, Palo Alto, CA) using a Jupiter C-18 column, 250 x 4.6 mm, 5 μ m diameter and 300Å pore size (Phenomenex, Torrance, CA). The glutenins were separated using the method of Marchylo et al. (1989) using the following parameters: column temperature 70 °C; injection volume 20 μ l; eluting system solvent A: Water +TFA (0.1% v/v); solvent B: ACN + TFA (0.05% v/v); 23% B to 60% B with run time 40 min and post time 10 min; flow rate: 1.0 ml/min. Detection will be done by UV absorbance at 210 nm. The PA-precipitated glutenins were analyzed by lab-on-a-chip electrophoresis (next section). Gliadins were separated as described by Waga et al. (2012) using the C-18 column described above. Elution solvents A and B were water containing 0.1% trifluoroacetic (v/v) and acetonitrile containing 0.05% trifluoroacetic (v/v), respectively. The elution linear gradient was 25% B to 50% B for 80 min, flow rate 1.0 ml/min, column temperature of 70 °C, detection at 210 nm. Gliadin peaks were classified into three groups with retention times ranging between: 1) 20 - 26 min: ω -

gliadins; 2) 26 - 40 min: α -gliadins; 3) 40 - 60 min: γ -gliadins. Manual integration of peaks was done using Agilent ChemStation software.

Electrophoresis

Glutenin proteins which were precipitated from solution by sorghum PA were analyzed with lab-on-a-chip electrophoresis to identify how the various HMW-GS interact with PA. The precipitates were dissolved in sodium dodecyl sulfate (SDS)-triethanolamine solution based on method by Hagerman and Butler (1980b). The SDS was used to release the proteins from PA-gluten interactions, denature the proteins, and uniformly charge them for electrophoresis. Triethanolamine was used to increase the solution pH and assist in disrupting PA-gluten interactions. The solutions were visually clear with no apparent undissolved residue. The proteins will be analyzed for HMW-GS using an Agilent 2100 bioanalyzer with a Protein 230 chip kit (Agilent Technologies, Palo Alto, CA) (Uthayakumaran, Batey & Wrigley, 2005).

Surface Hydrophobicity

Surface hydrophobicity was measured to determine possible extent to which PA-gluten interactions involve hydrophobic regions of gluten proteins. A modified bromophenol blue assay (Chelh, Gatellier & Santé-Lhoutellier, 2006) was used, as proteins do not need to be solubilized with this method. Washed gluten, or purified gliadins or glutenins, (to yield 5 mg protein/mL buffer) was mixed with 1 mL of 20 mM sodium phosphate buffer and 200 μ L of bromophenol blue indicator. PA treatment (0-

200 µg PA/g protein) was added to the buffer and briefly vortexed with the gluten before adding indicator. After vortexing (10 min) and centrifuging (15 min, 1800g, 20 °C), the supernatant (300 µL) was diluted with the buffer (2700 µL). This was briefly vortexed and absorbance read at 595 nm.

ATR-FTIR

Proanthocyanidin-gluten complexes were examined by attenuated total reflectance – Fourier transform infrared (ATR-FTIR) spectroscopy to determine effect of PA on protein secondary structure. However, due to overlap of PA bands and gluten's amide I, II, and III bands, this method did not prove effective.

Statistical Analysis

All tests and treatments were run in duplicate, unless otherwise stated. Data analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC). ANOVA was used to detect treatment effects. A 5% significance level determined differences between treatments (Fisher's LSD). Dunnett's test was used to compare treatments to the control at 5% significance level.

Results and discussion

Profiles of Sorghum and Grape Seed Proanthocyanidins

Sorghum PA contained 301 mg PA/g extract which was mostly polymeric (DP 8+, 87%) while grape seed PA had 584 mg PA/g extract with mix of 6%

monomers/dimers, 49% oligomers (DP 3-7), and 45% polymers (DP 8+) based on analysis by normal phase HPLC-FLD (Table 6). Sorghum PA had higher Folin phenol content than grape seed PA (1571 vs 874 mg GAE/g PA, respectively) and higher antioxidant capacity (271 vs 152 μ M TEAC/g PA, respectively).

Acid depolymerization and subsequent phloroglucinol adduct formation revealed sorghum PA had a higher mDP than grape seed PA; 7.8 vs 3.3, respectively. However, both PA types had low conversion (40%) of PA to monomers, which resulted in underestimating mDP. Correcting for the underestimation ($\times 2.5$), the sorghum PA can be assumed to have an estimated mDP of approx. 19.5, and the grape seed PA an estimated mDP of 8.3, which agree with data from other authors; mDP 6 – 10 for grape seed PA (Pappas, Kyraleou, Voskidi, Kotseridis, Taranilis & Kallithraka, 2015) and 17 for sorghum PA (Frazier et al., 2010). These results confirm the higher MW profile of sorghum PA than grape seed PA. In general, sorghum and grape seed PA are structurally similar, both primarily consist of C4-8 interflavan linkages, and are dominated by catechin and epicatechin units (Gu et al., 2003, Prieur, Rigaud, Cheynier & Moutounet, 1994, Qi, Zhang, Awika, Wang, Qian & Gu, 2016), even though grapes also contain some prodelfinidin (galocatechin) units (Gu et al., 2003, Prieur, Rigaud, Cheynier & Moutounet, 1994, Qi, Zhang, Awika, Wang, Qian & Gu, 2016). Acid catalyzed hydrolysis and oxidation of both sorghum and grape seed PA in our lab yielded predictable contents of predominantly cyanidin, further confirming overall structural similarity (data not shown).

Table 6 - Quantitative profile of sorghum and grape seed proanthocyanidins (PA)

	Catechin	Grape seed	Sorghum
Estimated mDP ^a	-	8.3 ± 0.5	19.5 ± 2.5
PA content [mg PA/g extract]	942	584 ± 73	301 ± 21
DP ^b 1-2 [%]	100 c	5.8 ± 0.7 b	1.1 ± 1.0 a
DP 3-7 [%]	-	48.8 ± 5.7 b	12.3 ± 2.1 a
DP 8+ [%]	-	45.4 ± 5.4 a	86.6 ± 1.3 b
Phenol content [mg GAE/g PA]	-	874 ± 105 a	1571 ± 214 b
AOX capacity [µM TEAC/g PA]	188 ± 39 b	152 ± 14 a	271 ± 17 c

^aMean degree of polymerization obtained from acid catalyzed hydrolysis; ^bDegree of polymerization obtained using normal phase HPLC with fluorescence detection. Values ± SD, different letters within row indicate significant difference ($p < 0.05$). AOX capacity; antioxidant capacity measured using the TEAC method.

Proanthocyanidin-Protein Binding Properties

The interaction of PA with solubilized glutenin and gliadin proteins was nearly instantaneous, as haze formation was nearly complete (80 – 95%) at time 0, complete at 10 min, and remained stable over a 60 min period. Thus, 10 min was used as equilibrium binding time for measuring the PA-protein interactions.

The PA-gluten protein binding curves generally demonstrated that sorghum PA had higher binding affinity for both glutenins and gliadins than grape seed PA (Fig 15). Calculated equilibrium dissociation constants (K_d) were lower for sorghum PA than grape seed PA, confirming the higher binding affinity of sorghum PA to the gluten protein fractions (Table 7). The sorghum PA K_d values were 67 – 71% lower than grape seed PA for glutenin fractions, and 64 – 85% lower for the gliadins (Table 7). For example, 0.6 mol sorghum PA was required to precipitate 1.0 mol glutenins isolated from the strong gluten wheat, whereas 2.1 mol grape seed PA was required to precipitate 1 mol glutenins from this wheat sample (Table 7). Also worth noting was the fact that

the gliadins required more PA (approx. 2–3-fold higher molar ratio) to precipitate than the glutenins, perhaps due to their smaller size and globular nature, which reduce cross-linking efficiency by PA.

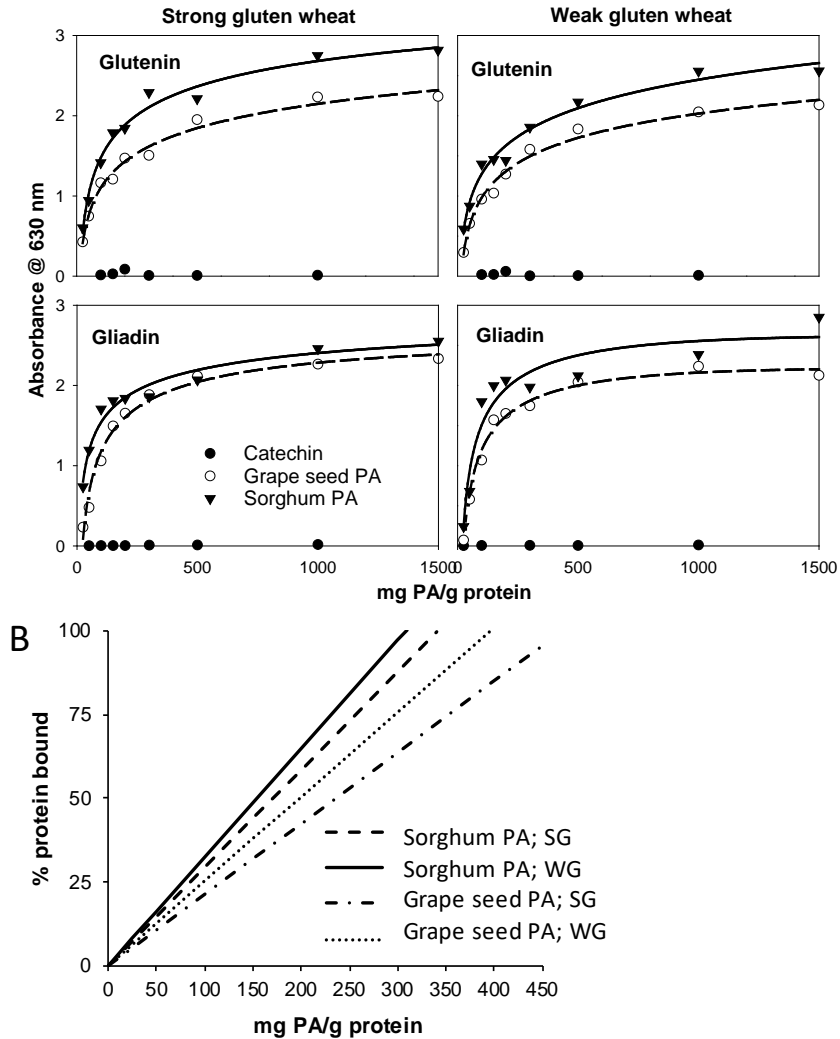


Figure 15 - Binding properties of grape seed and sorghum proanthocyanidins (PA) with gluten proteins isolated from strong and weak gluten wheat samples measured using nephelometry. Binding affinity with best fit regression (SigmaPlot) curves used to estimate binding constants (A), and regression curve for gluten precipitated per unit amount of PA within the linear range (B). Absorbance at 630 nm was measured after 10 min reaction. Protein content of precipitates was measured using nitrogen combustion. SG – strong gluten, WG – weak gluten.

Table 7 - Binding constants of proanthocyanidins (PA) with gluten fractions

	Glutenins		Gliadins	
	K_d^a	$\frac{[PA]:}{[Protein]^b}$	K_d	$\frac{[PA]:}{[Protein]}$
<i>Strong gluten</i>				
Grape seed PA	2.1 b	12.7 b	1.3 c	24.6 c
Sorghum PA	0.6 a	3.9 a	0.2 a	7.7 a
<i>Weak Gluten</i>				
Grape seed PA	2.4 c	10.7 b	1.1 a	26.1 c
Sorghum PA	0.8 a	3.6 a	0.4 b	10.5 b

^aEquilibrium dissociation constant (mol PA/mol protein). ^bMinimum molar concentration of PA required to precipitate 1 mol of protein. Values based on average MW 65 and 35 kDa for glutenins and gliadins, respectively (Delcour & Hoseney, 2010). Different letters within column not significantly different ($P < 0.05$, LSD).

The higher mDP sorghum PA has a greater number of hydroxyl groups in proximity and hydrophobic aromatic rings available for interaction with gluten, it was therefore able to complex more effectively with the gluten protein fractions than the lower mDP grape seed PA. This agrees with other work showing that higher MW PA cross-linked cereal prolamins more effectively (Dias, Perez-Gregorio, Mateus & De Freitas, 2015, Emmambux & Taylor, 2003). Zeller et al. (2015) also recently reported that higher mDP (~18) PA precipitated bovine serum albumin and alfalfa proteins more effectively than lower mDP (~9) PA. The present data helps explain our recent finding that showed the sorghum PA more effectively strengthened gluten than the grape seed PA (Girard, Castell-Perez, Bean, Adrianos & Awika, 2016). Similar higher binding of carbohydrates has also been reported for higher mDP PA compared to lower mDP PA (Amoako & Awika, 2016b, Amoako & Awika, 2016a, Barros, Awika & Rooney, 2014).

The monomeric catechin did not affect turbidity, indicating it did not precipitate the gluten fractions (Fig 15). Similarly, Emmambux and Taylor (2003) only saw a small increase in haze of the sorghum prolamins, kafirin, with high concentrations of catechin while sorghum PA greatly increased kafirin haze due to PA-protein complexation. Our previous work showed that catechin significantly reduced gluten strength and mixing stability due to its ability to inhibit disulfide linkage formation (antioxidant effect) (Girard, Castell-Perez, Bean, Adrianos & Awika, 2016). The fact that catechin did not precipitate the gluten proteins suggests it does not contribute to any new gluten polymer formation like the polymeric PA. Thus, catechin's weakening effect on gluten previously reported vis a vis the strengthening effect of the PA (Girard, Castell-Perez, Bean, Adrianos & Awika, 2016) provides indirect insight on the extensive magnitude of gluten protein cross-linking by PA that overcomes the strong PA antioxidant effect (Table 6).

Interaction of Proanthocyanidins with Glutenin and Gliadin Subfractions

To determine if PA preferentially bound specific glutenin and gliadin subfractions, different concentrations of sorghum PA were added to solubilized glutenins and gliadins, and the non-precipitated proteins (not complexed with PA) were analyzed by RP-HPLC. In both strong and weak gluten samples, the HMW-GS reduced most dramatically with increasing PA concentration (Fig 16), indicating the sorghum PA preferentially bound HMW-GS over LMW-GS. For instance, in the strong-gluten HMW-GS content decreased 76%, from 42 AU in the control to 9 AU, in the 30 mg sorghum PA/g protein treatment, while the LMW-GS content only decreased 6%, from

84 to 79 AU, respectively (Table 8). The effect of sorghum PA on weak-gluten glutenins was similar in magnitude (Fig 16) with HMW-GS decreasing 74% and LMW-GS decreasing 12%.

The HMW-GS range in size from 65 to 90+ kDa, while LMW-GS range from 30 to 60 kDa (Delcour & Hoseney, 2010). The HMW-GS have an elongated rod-like structure, as their composition is repeating units of primarily glutamine, proline, and glycine which form loose beta-spirals in between the spherical N- and C-termini (Shewry, Halford, Tatham, Popineau, Lafiandra & Belton, 2003). This elongated structure allowed for better access to amino acid side groups for more extensive hydrogen bonding and hydrophobic interactions than the more compact LMW-GS fractions. Because the HMW-GS are most strongly associated with dough strength (Shewry, Halford & Tatham, 1992), their preferential complexation with the PA, which presumably further increases their size, may explain the dramatic effect of the sorghum PA on dough strength we previously observed (Girard, Castell-Perez, Bean, Adrianos & Awika, 2016). In this previous work, we found that sorghum PA restored the 'bread wheat-like' mixing behavior to a weak gluten wheat with deletions of specific HMW-GS essential for bread quality.

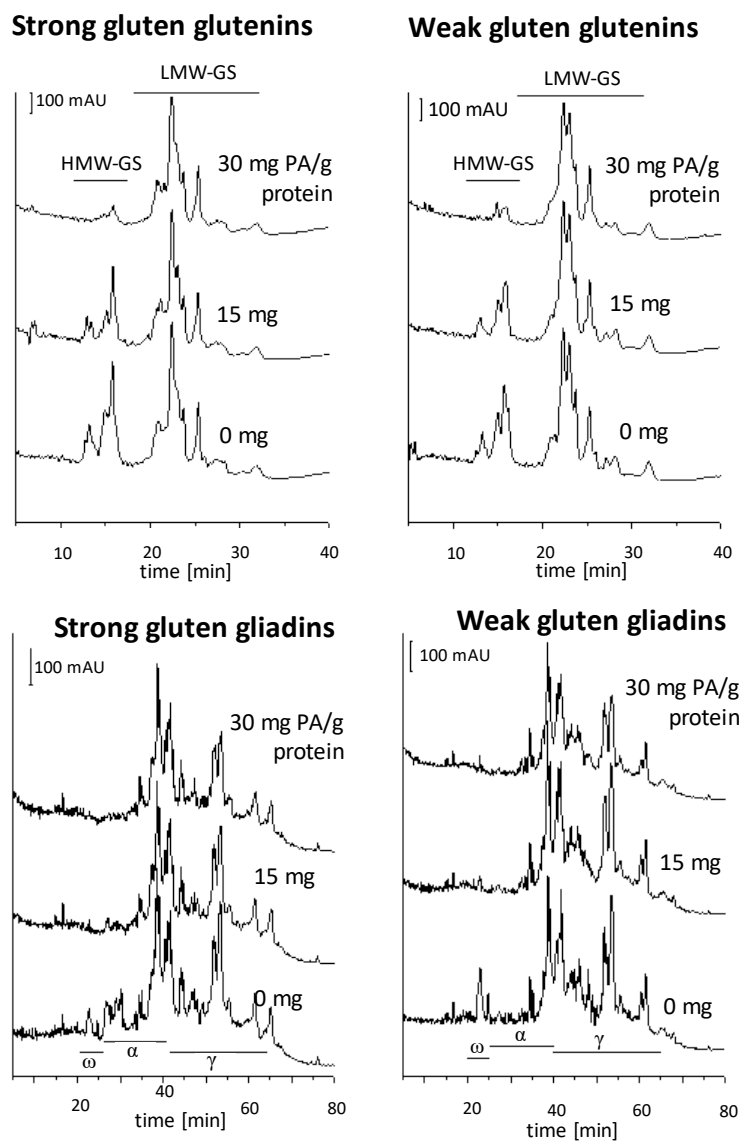


Figure 16 - Effect of sorghum PA on solubility of wheat glutenin and gliadin fractions. Chromatograms represent protein fractions that were not precipitated by sorghum proanthocyanidins (PA) at different levels of addition. Y-axis is absorbance at 214 nm. HMW-GS, high molecular weight glutenin subunits; LMW-GS, low molecular weight glutenin subunits.

Table 8 – Effect of sorghum proanthocyanidins (PA) on solubility of gluten fractions and sub-fractions

mg sorghum PA/g gluten	Strong gluten				Weak gluten			
	0	7.5	15	30	0	7.5	15	30
<i>Non-precipitated gluten fractions^a</i>								
total gliadin	325 ± 16	294 ± 7	274 ± 1	260 ± 1	309 ± 12	315 ± 10	321 ± 20	272 ± 10
ω-gliadin	15 ± 0.1	9 ± 0.1	8 ± 0.1	7 ± 0.3	21 ± 2	14 ± 1	12 ± 1	10 ± 0.2
α-gliadin	113 ± 5	94 ± 3	85 ± 0.1	82 ± 0.1	78 ± 4	83 ± 3	85 ± 7	76 ± 3
γ-gliadin	197 ± 11	191 ± 5	181 ± 0	170 ± 2	209 ± 6	218 ± 6	224 ± 12	186 ± 7
total glutenin	127 ± 3	114 ± 6	92 ± 4	88 ± 3	148 ± 2	141 ± 15	121 ± 6	102 ± 1
LMW-GS	84 ± 1	79 ± 1	71 ± 2	79 ± 3	101 ± 2	102 ± 7	93 ± 2	89 ± 1
HMW-GS	42 ± 2	36 ± 4	22 ± 3	9 ± 0.2	47 ± 0.1	39 ± 7	28 ± 4	12 ± 0.1
<i>Precipitated HMW-GS subfractions^b</i>								
<i>1Ax2*</i>	2	9 ± 1.9	25 ± 0.6	43 ± 2.9	nd	nd	nd	18 ± 0.9
<i>1Bx7</i>	3	7 ± 0.3	21 ± 0.1	35 ± 1.3	nd	nd	nd	nd
<i>1Bx17</i>	nd	nd	nd	nd	nd	nd	nd	6 ± 0.3
<i>1By9</i>	nd	1 ± 0.1	4 ± 0.8	11 ± 0.9	nd	nd	nd	nd
<i>1Dx5</i>	nd	58 ± 6.3	90 ± 5.2	44 ± 6.1	nd	nd	nd	13 ± 0.1
<i>1Dy10</i>	2	1 ± 0.5	4 ± 1.1	nd	nd	nd	nd	nd

^aData obtained using RP-HPLC on protein fractions which remained in solution after PA precipitation. ^bThe proteins precipitated by PA from glutenin fraction were re-solubilized with SDS-triethanolamine, and the resolubilized HMW-GS subfractions analyzed by lab-on-a-chip electrophoresis; nd – not detected. All values reported as $\times 10^3$ absorbance units \pm SD.

Generally, sorghum PA also decreased total soluble gliadins indicating PA interacted with and precipitated gliadins, but to a lesser extent than the glutenins. For example, in strong-gluten and weak-gluten gliadin fractions at 30 mg PA/g protein, total gliadins (sum of soluble α -, γ -, ω -gliadins) decreased by 20% and 12%, respectively; these values were 31% for total glutenins (sum of soluble HMW-GS and LMW-GS) from both strong-gluten and weak-gluten samples (Table 8).

As with the glutenins, specific subfractions of gliadins also preferentially interacted with the PA. The PA bound the majority of the ω -gliadins at 30 mg PA/g protein (Fig 16, Table 8). Omega-gliadins are the largest (MW 44-80 kDa), least globular, least hydrophobic, and have the highest glutamine content of all the gliadin fractions (Barak, Mudgil & Khatkar, 2015). Thus, like the HMW-GS, the ω -gliadins have the size and structural features that make them more amenable to complex with PA than the other gliadin subfractions (Hagerman, Rice & Ritchard, 1998). The abundance of glutamine units may further promote hydrogen bonding. The α - and γ - gliadins have a more compact structure and are smaller (~31 and 35 kDa, respectively) than ω -gliadins (Barak, Mudgil & Khatkar, 2015), which may explain why they interacted less with the PA. For example, in the strong-gluten gliadins at 30 mg PA/g protein, the α - and γ - gliadins content decreased 27% and 14%, respectively, versus 53% decrease in the ω -gliadins content (Table 8).

Proanthocyanidins complexed the glutenins to a greater extent than gliadins, evidenced by the larger decrease in soluble glutenins. As a whole, glutenins are larger than gliadins and have a more open structure which increase PA affinity for them. As

gliadins are primarily responsible for gluten extensibility (Delcour & Hoseney, 2010), their lower binding affinity (relative to glutenins) by the PA may help explain the previously reported increased gluten strength by PA without significant reduction in gluten extensibility (Girard, Castell-Perez, Bean, Adrianos & Awika, 2016).

Interaction of Proanthocyanidins with High Molecular Weight Glutenin Subunits

The HMW-GS are considered the most important determinants of gluten strength and bread-making quality of wheat (Payne, Nightingale, Krattiger & Holt, 1987), thus insight on their specific interaction with PA is useful to help identify dough-strengthening mechanisms of the PA. Lab-on-a-chip electrophoresis was used to determine if specific HMW-GS were preferentially bound by the PA. In this test, the proteins precipitated by the PA were redissolved in SDS-triethanolamine and analyzed. In strong gluten, which had HMW-GS 1Ax2*, 1Bx7, 1By9, 1Dx5, and 1Dy10, the x-type subunits were bound to a greater extent than the y-type (Table 8). The x-type HMW-GS have a greater MW (82-88 kDa) than y-type (67-73 kDa) (Shewry, Halford & Lafiandra, 2003). It is likely that this size difference impacted binding affinity with PA which confirms affinity of PA for larger MW gluten subtypes.

Specific subunit pairs affect gluten strength differently, with subunits 1Dx5 + 1Dy10, 1Ax1 or 1Ax2*, and 1Bx17 + 1By18 generally performing better in breads than other alleles encoded on their respective chromosomes (1D, 1A, 1B) (Shewry, Halford, Tatham, Popineau, Lafiandra & Belton, 2003). The strong gluten wheat sample had most of the bread-type HMW-GS alleles, and its complexation with PA further increased the

glutenin size, as evidenced by their increased precipitation from solution (Table 8). This likely contributed to the rather extreme dough strength previously reported (Girard, Castell-Perez, Bean, Adrianos & Awika, 2016). By binding preferentially to glutenin subunits most critical to gluten strength, the PA may thus not only improve the strength of weak gluten, but also enhance strong gluten's resilience to mechanical abuse (i.e. mixing tolerance), or ability to carry non-wheat ingredients, like high dietary fiber formulations.

In the weak gluten wheat sample with some HMW-GS deletions, the x-type HMW-GS 1Ax2*, 1Bx17, and 1Dx5 present were all complexed by the PA (Table 8), thus likely increasing glutenin polymer size as in the strong-gluten HMW-GS. However, the sensitivity of the methods enabled us to only reliably quantify the precipitates in the 30 mg PA/g protein treatment. Nevertheless, the evidence points to similar selective binding of PA to specific HMW-GS subunits most critical to the gluten strength, as observed in the strong gluten wheat sample.

Effect of Proanthocyanidins on Gluten Surface Hydrophobicity

Surface hydrophobicity was analyzed to determine contribution of hydrophobic interactions to PA-gluten binding. As expected PA, but not catechin, reduced surface hydrophobicity of both strong and weak gluten (Fig 17). Clear dose responses were observed for decreased surface hydrophobicity as PA concentration increased. Furthermore, sorghum PA were more effective at lowering surface hydrophobicity. For example, at 25 mg PA/g protein, sorghum PA decreased surface hydrophobicity in

strong gluten from 116 to 67 μg bromophenol blue (BPB) bound, whereas grape seed PA had no effect. This trend was the same in weak gluten. Because it has a larger mDP, and therefore more aromatic rings in close proximity available for hydrophobic interactions, the sorghum PA likely participated in more hydrophobic interactions with gluten. This helps explain why the high MW sorghum PA increased gluten strength at a lower usage rate than relatively lower MW grape seed PA (Girard, Castell-Perez, Bean, Adrianos & Awika, 2016).

Isolated glutenins surface hydrophobicity exhibited a dose response decrease similar to gluten with increased PA addition (Fig 17). This indicates that hydrophobic interactions likely play a key role in PA-glutenin complex formation. On the other hand, PA had little to no effect on gliadins surface hydrophobicity (data not shown). Gliadins have similar amino acid profile to glutenins, but unlike glutenins, gliadins do not have the central domain with extensive repeating units (Shewry, Halford & Lafiandra, 2003). Therefore, they have more compact structures, which offer few exposed hydrophobic amino acids side chains for hydrophobic interaction. This suggests that hydrophobic interactions play a non-significant role in PA-gliadins complexation.

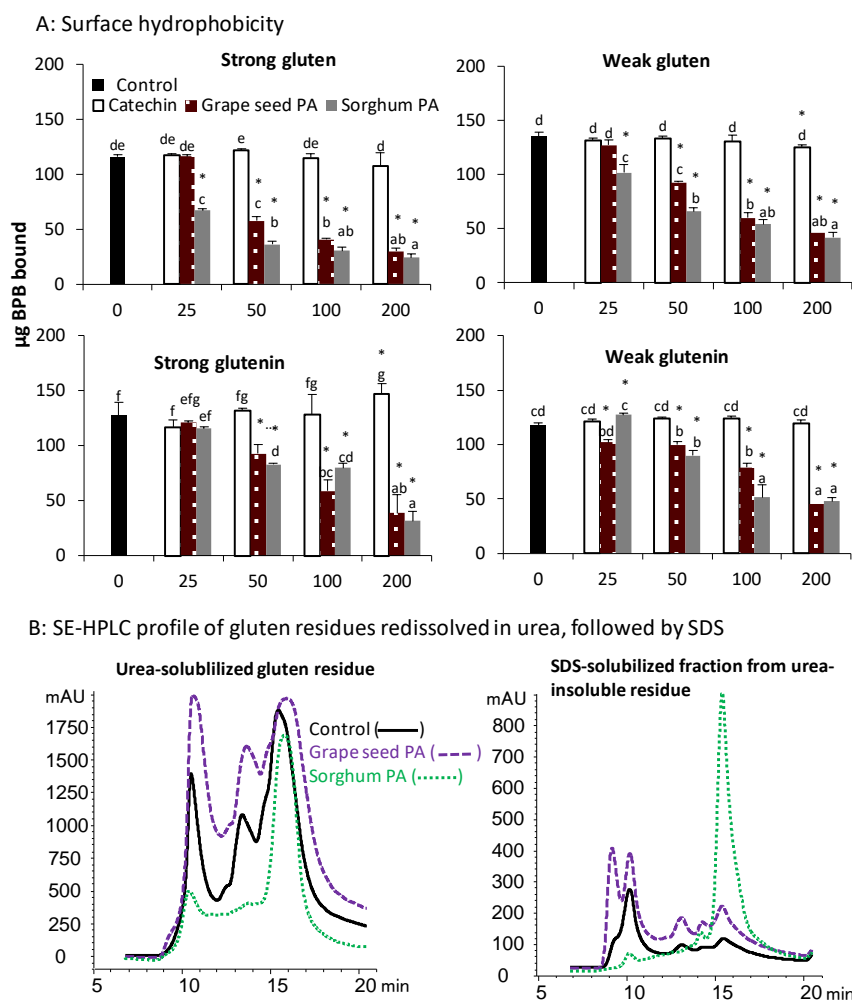


Figure 17 - Contribution of hydrophobic interactions and hydrogen bonding to gluten-PA binding.
A) Effect of catechin, grape seed and sorghum proanthocyanidins (PA) on surface hydrophobicity of strong and weak gluten samples and their respective glutenin fractions. BPB, bromophenol blue. Error bars illustrate \pm SD, letters indicate significant difference among treatments ($p < 0.05$, Fisher's LSD), and stars indicate difference ($p < 0.05$, Dunnett's test) from control. Gliadin treatments yielded no significant differences. B) Size exclusion chromatograms of insoluble (in 50% 1-propanol with 1% DTT) strong gluten protein residue resolubilized in 8 M urea (to break hydrogen bonds, left), followed by 4% SDS on urea insoluble fraction (to disrupt hydrophobic interactions, right). Sorghum and grape seed PA treatments at 25 mg/g.

The Relative Role of Hydrophobic Interactions in Gluten Polymerization by Proanthocyanidins

To provide preliminary evidence on direct contribution of hydrophobic interactions to gluten cross-linking by PA, size exclusion chromatography was used to assess the gluten profile of insoluble (in 50% v/v 1-propanol with 1% DTT) strong gluten protein residues treated with sorghum and grape seed PA at 25 mg/g (where the differential effect of sorghum vs grape seed PA on surface hydrophobicity was most apparent, Fig 17A), relative to control. Because of the dominant role of hydrogen bonding in non-covalent gluten polymerization, the insoluble gluten residues were first resolubilized in 8 M urea to break hydrogen bonds. The subsequent urea-insoluble residue was then dissolved in 4% SDS to disrupt hydrophobic interactions.

Profile of urea-soluble gluten residue was remarkably different for sorghum PA treatment compared to grape seed PA or control (Fig 17B). Clearly, urea was able to solubilize more of the grape seed PA-treated and control gluten residue than sorghum PA treated residue, especially the higher MW peaks at 10.5 and 13.5 min that were largely absent in the sorghum PA treatment (Fig 17B). This suggests that the sorghum PA-treated residue likely formed more urea-insoluble polymers; likely via hydrophobic interactions. Subsequent solubilization of the urea-insoluble residues in SDS appeared to confirm this; sorghum PA-treatment produced a distinctly different profile and much higher peak than the control and grape seed PA treatment (Fig 17B). This seems to confirm that hydrophobic interactions are indeed important to sorghum PA-gluten polymerization. Thus the cooperative binding model (involving both hydrogen bonding

and hydrophobic interactions) proposed by Kilmister, Faulkner, Downey, Darby, & Falconer (2016) for BSA-tannin interactions may be valid for PA-gluten interactions as well.

Conclusion

Proanthocyanidins precipitated glutenins to a greater extent than gliadins, suggesting more extensive cross-linkages between the glutenins were facilitated by the PA. Our data also generally suggests that PA-gliadin interactions are largely hydrogen-bond driven, whereas PA-glutenin complexation involves cooperative hydrogen bonding and hydrophobic interactions. The overall evidence indicates that PA interaction with gluten proteins is not entirely random, and could thus be manipulated to produce desired rheological behavior. Higher MW increased binding affinity of PA for gluten proteins, perhaps due to enhanced opportunity for cross-linkages by the presence of more hydroxyl groups and aromatic rings in close proximity. Both the strong and weak gluten proteins used in this study surprisingly had similar binding affinities to PA, likely because they both contained the large x-type HMW-GS, which were preferentially bound by PA. In all, the PA-gluten protein interactions depend on the protein MW profile and structure, as well as the PA degree of polymerization. This suggests that selection of raw materials (wheat type and tannin profile) and processing conditions could be optimized to alter gluten functionality for desired applications. Such applications may include, not only natural dough strengthening, but also bioactive/edible

films for targeted delivery of bioactive compounds and nutrients, food matrix modification to slow macronutrient digestibility profile, among others.

CHAPTER V
EFFECT OF PROANTHOCYANIDIN-GLUTEN CROSS-LINKING ON GLUTEN
FILM FORMATION AND STABILIZATION

Introduction

Tannins are known to strongly complex with proteins. For instance, condensed tannins (proanthocyanidins, PA) have been shown to interact with cereal proteins through non-covalent bonds (hydrogen bonding and hydrophobic interaction), which produce larger protein macropolymers thereby decreasing chain mobility and altering protein rheology (Emmambux, Stading & Taylor, 2004, Hagerman, Rice & Ritchard, 1998). Tannins can increase wheat flour dough strength, as reported by Wang et al. (2015) and Zhang et al. (2010). Besides affecting physical properties, cereal protein-PA interactions have been shown to slow or decrease macronutrient digestibility (Dunn, Yang, Girard, Bean & Awika, 2015, Taylor, Bean, Ioerger & Taylor, 2007) through interacting with digestive enzymes directly or complexing macronutrients thereby hindering enzyme access.

A potential application of gluten-PA interaction is the production of films for edible and/or biodegradable packaging or biomedical applications. Biopolymer films have limitations compared to plastic films; generally, biopolymer films are weaker and less extensible, with higher water vapor permeability (Hernandez-Izquierdo & Krochta, 2008). However, gluten has beneficial properties including lower water vapor

permeability compared to other proteins (Gennadios, Brandenburg, Weller & Testin, 1993).

PA may increase strength and decrease permeability of gluten films, especially at low usage levels. Tannic acid was shown to strengthen gluten film; however, it was used at high levels (5-30% of wheat gluten) which led to overly brittle films with undesirable properties (Hager, Vallons & Arendt, 2012). Both tannic acid and PA increased sorghum kafirin film tensile stress, decreased water absorption and oxygen permeability, and increased glass transition temperature (Emmambux, Stading & Taylor, 2004). But these were also used at high levels (up to 20% of kafirin) which had deleterious effects resulting in stiffer, less plastic films that quickly disintegrated in water (Emmambux, Stading & Taylor, 2004). Because PA cross-link proteins and can greatly increase gluten strength without necessarily reducing extensibility (as discussed in Chapter III), it is likely that PA will enhance gluten film properties at low levels of use.

Gluten-PA films could be used to decrease the amount of plastic packaging. Also, the antioxidant properties of PA may function in active food packaging (Li, Miao, Wu, Chen & Zhang, 2014). Because PA decreases the rate of protein digestion (Taylor, Bean, Ioerger & Taylor, 2007, Taylor, Taylor, Belton & Minnaar, 2009), these films may also be a good way to encapsulate micronutrients for controlled release in the lower GI tract. Other possible biomedical applications include implants, sutures, and scaffolds.

In addition, gluten film formation and stabilization is an important function in food processing, not only in doughs, but also in batter-based systems. Due to their gluten cross-linking, the PA could also increase batter viscosity which could enhance stability

of inclusions in batter-based products like cake, and increase pickup of batter in coatings for fried products (Bettge & Morris, 2007).

Gluten-PA interactions may improve gluten functionality through strengthening and stabilizing gluten films and foams. However, this has not been studied in depth. The current study aimed to determine the effect of gluten-PA complexation on wheat gluten film properties and wheat flour batter stability.

Materials and Methods

Materials

Wheat gluten (79.4% protein) was from Sigma-Aldrich (St. Louis, MO). A commercial pastry wheat flour (White Spray, Ardent Mills, Denver, CO, 8.5% protein) was used for batter systems. Tannic acid (ACS grade) and catechin hydrate (94% pure catechin) were from Sigma-Aldrich (St. Louis, MO). Pepsin (P7000) from Sigma-Aldrich (St. Louis, MO) was used for protein digestion. All solvents were ACS grade. PA was extracted from high tannin sorghum bran as described in Chapter III.

Gluten Film Formation

Films were cast based on the methods of Emmambux et al. (2004) and Hager et al. (2012). Gluten (2.2 g) was solubilized in 14 mL EtOH with 900 mg glycerol; the pH was adjusted to 4.0 with acetic acid. Then, 15 mL water, shortening (0 or 5%), and 1 mL EtOH, containing treatment (catechin, tannic acid, and PA at 0-10 mg/g gluten), was added. This was heated at 70 °C for 10 min while stirring to allow for complexation of

gluten and treatments. Aliquots of 13 g were poured into polyethylene petri dishes (8.5 cm D) and dried overnight at 40 °C. After completely drying, samples were equilibrated for 24+ h in a desiccator at ~50% RH. Average film thickness was 0.25 mm.

Bound Tannin Content of Films

To determine the amount of tannin that complexed with gluten, and thus became unextractable, the amount of catechins and tannins solubilized from film in 70% (v/v) aqueous acetone for 20 min was analyzed. The catechin and tannin added (0-10 mg/g gluten) minus the amount solubilized was assumed to equal bound catechin and tannin. Catechin and tannin content of the solutions was quantified with normal phase HPLC, using a Supelcosil LC-Diol column (250 mm x 4.6 mm, 5 µm particle size, Supelco, Bellefonte, PA) as described in Chapter III.

Gluten Film Physical Properties

Images of the films were taken using field emission scanning electron microscopy (FE-SEM). Each dry film was placed on double-sided adhesive carbon tape and mounted onto a stainless-steel stub. A sputter coater, 208 HR (Cressington, USA), coated the samples with a platinum:palladium mixture (80:20%) to a thickness of ~5 nm. The samples were observed in a JSM-7500F FE-SEM (JEOL, Japan) at an accelerating voltage of 5 kV, working distance of 18 mm. Film surface observations were made using a secondary electron (SE) detector at magnification range (500 – 5,000 X) and

resolution of 1 – 10 μm . Representative gluten film micro images were taken using an automatic image capture software (PC-SEM).

Thickness of each film was measured with a digital calipers. $L^*a^*b^*$ color was measured with a Minolta CR-310 Chroma Meter (Konica Minolta, Ramsey, NJ), with the translucent films on a white glass background.

Extensibility was assessed with a texture analyzer (TA.XT2i, Texture Technologies, Scarsdale, NY/Stable Micro Systems, Godalming, Surrey, UK) using a modified method from Emmambux et al. (2004). Strips of film (60 x 6 mm) were cut and placed between tensile grips (40 mm apart), with cardboard on each of the grips to minimize slippage of strips and tearing from the grips. The films were extended to rupture from the top with a pretest and test speed of 0.5 mm/s, with a trigger force of 5 g. Extension was assessed on 10 film strips per treatment.

Aqueous Stability and Water Holding Capacity of Gluten Films

The stability and water holding capacity (WHC) of gluten films at different pH conditions were determined. Oven-dried (40 °C for 24 h) film strips (1 x 1 cm) were weighed and placed in aqueous phosphate buffer solutions of various pH (2 and 7) and temperature (25 and 37 °C). At set time points (1 h, 2 h, 24 h), films were removed, carefully blotted dry, and weighed. These samples were further dried at 40 °C for 24 h and weighed. The stability was reported as the percent of film insoluble in buffer solution; and WHC as the amount of water the film absorbed while in solution.

Gluten Film Digestibility

A modified *in vitro* protein digestion method was used to assess digestive stability of film (Mertz et al., 1984). Film pieces (1 cm x 1 cm) were placed in a 0.2 M phosphate buffer (pH 2) containing pepsin (0.06 mg pepsin/ml). This was gently shaken at 37 °C for 5, 10, or 20 min. After digestion, 2 M NaOH was added to increase pH to 7 and inactivate pepsin. Films were carefully removed from the solution, dried, and weighed.

Flour Batter Viscosity

Effect of PA on wheat flour batter viscosity was measured. Batters of 40% pastry flour and 60% water were mixed with treatments of catechin, tannic acid, and PA at 0-25 mg/g flour, and the sample placed in a 100 mL beaker up to the 70 mL line. Samples rested for 30 min before analyzing. Viscosity was measured on a Brookfield DV-III Rheometer (Brookfield Engineering Laboratories, Inc., Stoughton, MA) with the #6 spindle at 25, 50, and 100 rpm each for 1 min. Samples were maintained at 22 °C.

Flour Batter Properties During Cooking (RVA)

Rapid Visco Analyzer (RVA, Perten Instruments, Springfield, IL) was used to determine effect of PA, tannic acid, and catechin on starch pasting properties during a cook-cool cycle. Samples of 3 g pastry flour and 25 g water plus treatments (catechin, tannic acid, and PA at 0-25 mg/g flour) were briefly pre-blended, then analyzed by RVA. The test was run with a temperature gradient of: 0.0-1.0 min at 50 °C, 1.0-8.5 up

to 95 °C, 8.5-13.5 hold at 95 °C, 13.5-21.0 down to 50 °C, and 21.0-23.0 hold at 50 °C.

The paddle was controlled to 960 rpm for 10 sec, followed by 160 rpm for the remainder of the test period.

Batter Stability

Influence of PA on wheat flour batter stability was measured. Batters of varying pastry flour to water ratio (20:80, 30:70%) were mixed (treatments of catechin, tannic acid, and PA at 0-25 mg/g flour) in 15 mL tubes. The sample was rested, and the volume of liquid separating from the batter was recorded at 15 min intervals up to 1 h, then every 1 h thereafter until equilibrium.

Statistical Analysis

All tests and treatments were run in duplicate, unless otherwise stated. Data analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC). ANOVA was used to detect treatment effects. A 5% significance level determined differences between treatments (Fisher's LSD). Dunnett's test was used to compare treatments to the control at 5% significance level.

Results and Discussion

Physical Properties of Gluten Films

All of the films cast were translucent, but unfortunately had pinholes and/or sub-surface bubbles in their structure visible to the naked eye. Thus, assessing properties like

film water vapor permeability was not possible. Film color was not greatly affected by addition of catechin and tannic acid (Table 9), whereas PA gave the films a reddish-brown tint. This seemed to be inherent color from the powder extracts added to the film forming solution: PA had an intense brown color while catechin and tannic acid were both light tan. Similarly, Emmambux et al. (2004) found that PA tinted sorghum kafirin films, but tannic acid did not.

Analysis of film phenolic extract showed negligible amounts of tannic acid and PA were extractable (Table 9): this indicates tannic acid and PA were irreversibly bound within the film. As expected, catechin showed high extractability from the film (6.1 mg/g gluten extractable versus 10 mg/g gluten in formulation). The data confirms that tannic acid and PA form insoluble complexes with gluten, whereas catechin does not, as discussed in Chapter IV, and previously reported by Wang et al. (2015).

Table 9 - Effect of 10 mg/g catechin, tannic acid, or proanthocyanidins on gluten film color and extractable film tannin content.

Film	L*	Color		Extractable Tannin ^a (mg/g gluten)
		a*	b*	
Control	95.5	-0.7	10.2	nd
Catechin	89.5	0.3	19.8	6.1
Tannic Acid	90.0	1.5	10.5	nd
Proanthocyanidins	66.2	16.8	33.7	nd

^aExtracted in 70% aqueous acetone and quantified by normal phase HPLC. nd: none detected.

FE-SEM images show the control, tannic acid, and PA films had cracks on their surfaces whereas the catechin film had a fairly smooth surface (Fig 18). The control film had much more pronounced cracks in its structure than the other films. In contrast, the

catechin film surface was smooth, suggesting catechin acted as a plasticizer. This is likely because catechin reduced gluten polymer size by breaking disulfide bonds, thus allowed for higher protein mobility and a more flexible structure as the proteins realigned. However, this may not necessarily imply a stronger film because catechin was previously shown, in Chapter III, to weaken dough.

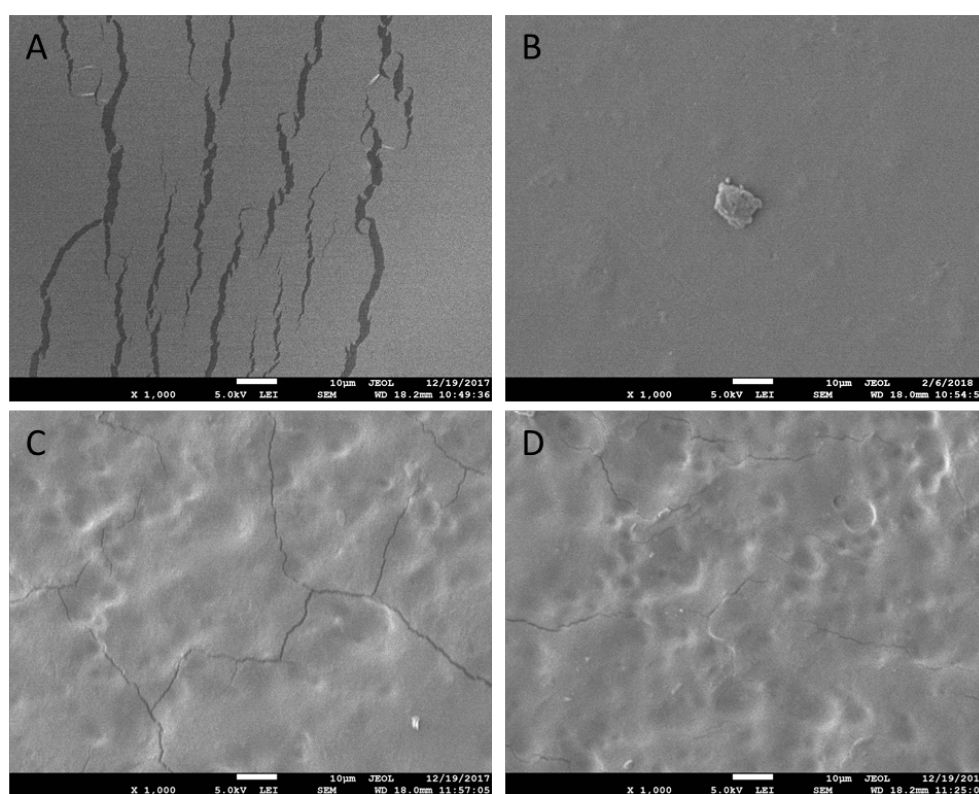


Figure 18 – Field emission scanning electron microscopy showing effect of tannins on film properties. A. control, B. 10 mg/g catechin, C. 10 mg/g tannic acid, and D. 10 mg/g proanthocyanidins. All images at 1000X magnification. White bar represents 10 µm.

Tannic acid and PA films had fissures, but much smaller than the control.

Further, the PA film had smaller fissures than the tannic acid film (Fig 18). The PA and tannic acid films also had surface indentations which appear to be regions of bubble

collapse during film dehydration. This suggests that the tannins were able to alter the protein structure and film integrity by their ability to cross-link gluten. Between the two, PA appeared to provide greater structural integrity.

Effect of Tannins on Gluten Film Strength and Extensibility

Film strength and extensibility are important factors in determining ability of protein films to withstand applied forces such as foam expansion during leavening or package handling. The extensibility characteristics of gluten films (average thickness 0.25 mm) were assessed with a texture analyzer. The average force to extend the control gluten film was 1.1 N (Fig 19). PA treatment significantly ($p < 0.05$) increased force to extend gluten film (2.4 N), while catechin and tannic acid slightly, though not significantly, increased force (1.8 N). None of the treatments significantly affected the distance film extended before rupture (Fig 19). Strong, dense protein structures offer high resistance to extension, while highly extensible films have good flexibility.

The strengthening effect of PA on gluten was greater than that of tannic acid (2.2X vs 1.6X, respectively), suggesting PA created a denser protein matrix through more extensive cross-linking. While PA-gluten interactions created stronger films, evidenced by the increased force to extend, these interactions did not significantly change extensibility (Fig 19). Previous studies showed that tannic acid and PA increased the tensile strength (i.e., force to extend) of kafirin film, but decreased elongation (i.e., extensibility, Emmambux, Stading & Taylor, 2004), and that tannic acid in a gluten film had similar effect (Hager, Vallons & Arendt, 2012). Thus, PA-gluten interactions are unique among cereal protein-tannin complexes and allow for stronger films without

necessarily decreasing its extensibility at a low usage level (10 mg PA/g gluten). This may be useful in applications like foams where extensibility is critical to leavening ability, or biopolymer films where strength and flexibility are crucial.

Catechin also slightly, though not significantly, increased the force to extend compared to the control (1.6X) but did not alter extensibility (Fig 19). Because catechin is a strong antioxidant that breaks disulfide bonds but does not cross-link proteins, it likely increased gluten polymer mobility during film formation thus allowing for closer protein associations. Addition of sodium sulfite, a reducing agent, to gluten film was shown to increase the film tensile strength and decrease its extensibility (Gennadios, Weller & Testin, 1993). The authors posited that because the reducing agent broke disulfide bonds, the protein chains were more mobile and able to form more interactions during film formation.

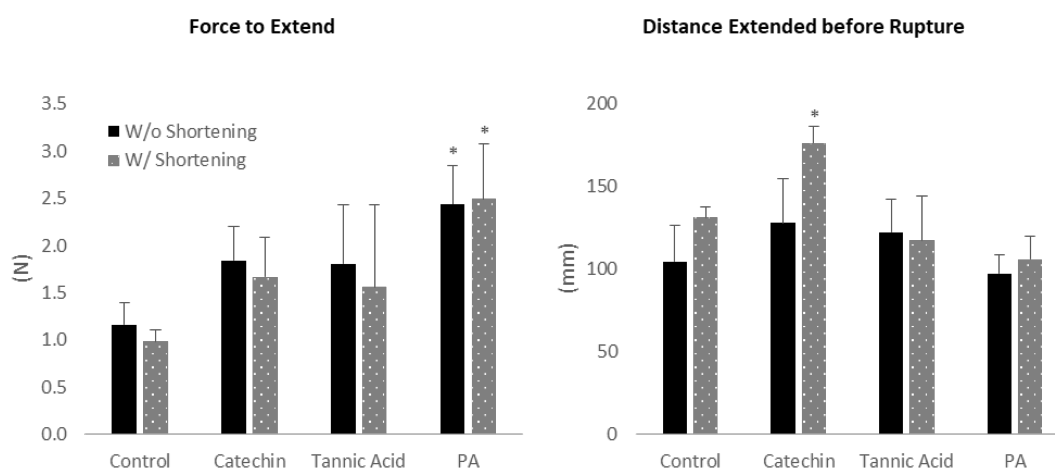


Figure 19 - Effect of 10 mg/g catechin, tannic acid, and proanthocyanidins (PA) with or without 5% shortening on force to extend film (left) and distance film extended before rupture (right). Stars indicate significant difference ($p < 0.05$) from respective control.

Effect of Shortening on Gluten Film Strength and Extensibility

Shortening is a common ingredient in formulations that rely on the film forming ability of gluten because shortening associates with gluten and increases film flexibility. Lipids are also common plasticizers in biofilms. Shortening was tested in the films to determine if its hydrophobic nature would affect tannin-gluten interactions. Addition of shortening at 5% to the control, catechin, and tannic acid films resulted in slightly, but not significantly, decreased force to extend (18, 11, and 14%, respectively, Fig 19). Shortening can disrupt the continuity of the gluten matrix by preventing gluten intermolecular interactions, thus decreasing its integrity. Conversely, the slight decrease in force to extend was not observed in the PA-gluten film (Fig 19).

The largest effect of shortening was seen in the catechin treatment, where shortening significantly increased the film extensibility (176 mm) versus catechin without shortening (125 mm) and the control film with shortening (131 mm). As mentioned previously, reducing agents, such as antioxidants like catechin, break disulfide bonds resulting in smaller proteins with better mobility. The addition of shortening may have further increased extensibility by allowing shortening to interact efficiently with hydrophobic areas of the gluten exposed by breaking disulfide bonds.

Five percent lipid addition also slightly, but not significantly, decreased extensibility of the tannic acid film (Fig 19), further suggesting disruption of the gluten matrix. Gontard et al. (1994) found that 20% (w/w) beeswax in gluten decreased film puncture strength and extensibility. Similarly, lipid addition was shown to decrease strength and extensibility with sodium caseinate and whey films (Khwaldia, Banon,

Desobry & Hardy, 2004, Pérez-Gago & Krochta, 2001). The lack of lipid effect on PA-gluten film strength may be a factor of PA interacting with shortening. Tea catechins (including (epi)catechins and (epigallo)catechin gallates) were shown to coalesce olive oil out of emulsion, likely through direct interaction with the lipid or incorporation of the catechins into the lipid layer (Shishikura, Khokhar & Murray, 2006).

As an observation, the control and catechin samples tore in unpredictable manners when extended whereas tannin, particularly PA, films extended until they snapped in a straight line, perpendicular to the force of extension. This suggests a difference in film network structure, likely based on the difference in protein association. The control and catechin films were formed by gluten-gluten interactions which allowed the film to develop irregular tears during extension. However, the tannic acid and PA films had gluten-gluten and tannin-gluten interactions which seemed to keep the film intact until the final structure failure. Thus, tannin-gluten complexation may be useful to improve film structural integrity.

Effect of Tannins on Film Solubility

To investigate if films could potentially withstand pH conditions associated with digestion, film solubility in aqueous solution at pH 2 and 7 (stomach and intestinal pH, respectively) and at 25 and 37 °C (room and body temperature, respectively) was determined. Preliminary data testing various time points showed that solubility plateaued at 2 h, so that time was used for further testing. At pH 2, the PA film was significantly less soluble than the control, catechin, and tannic acid films at both 25 and 37 °C (Table

10). This suggests that PA-gluten interactions increased film stability in solution and likely protein network density, supported by increased film strength with PA treatment (Fig 19). The solubility of PA film increased at 37 °C compared to 25 °C (45% insoluble vs 51%, respectively), suggesting H-bonds, which become unstable with increasing temperature, had a role in stabilizing the PA-gluten interactions. At both 25 and 37 °C, PA films were more insoluble with shortening addition than without (Table 10) suggesting hydrophobic interaction were also important. PA participates in both hydrogen bonds and hydrophobic interactions with gluten (Chapter IV); lipid addition may strengthen these hydrophobic interactions.

Surprisingly, tannic acid showed no difference in solubility from the control or catechin at either pH or temperature (Table 10), suggesting that at 10 mg/g gluten, tannic acid had little effect on protein film stability in solution. At pH 2 and 37 °C, PA decreased water-holding capacity (WHC) as compared to the control (8.2X vs 9.5X, respectively). This suggests that the PA-gluten cross-links were dense and prevented water absorption, even at elevated temperatures. However, catechin and tannic acid films did not significantly alter WHC. This supports the effect seen in the solubility data, where catechin and tannic acid had negligible effect on the gluten film in solution. In general, 5% shortening addition decreased film WHC at pH 2 and 37 °C with the same trends as without shortening: PA treatment had the lowest WHC (Table 10). This suggests that PA may limit gluten film swelling and increase its stability in an aqueous environment.

Table 10 - Effect of 10 mg/g catechin, tannic acid, and proanthocyanidins with or without shortening on solubility and water holding capacity of gluten films.

	pH 2				pH 7			
	% film insoluble		water holding capacity		% film insoluble		water holding capacity	
	25 °C	37 °C	25 °C	37 °C	25 °C	37 °C	25 °C	37 °C
<i>w/o shortening</i>								
Control	41 a	38 a	8.1 a	9.5 de	71 a	69 a	2.7 a	2.8 a
Catechin	39 a	38 a	7.9 a	9.9 e	72 a	74 a	2.0 a	3.2 a
Tannic Acid	40 a	38 a	7.8 a	8.9 cd	71 a	69 a	2.5 a	3.1 a
Proanthocyanidins	51 b	45 b	7.3 a	8.2 ab	72 a	70 a	2.3 a	3.4 a
<i>w/ 5% shortening</i>								
Control	42 a	38 a	7.4 a	8.3 bc	72 a	69 a	2.2 a	2.6 a
Catechin	42 a	41 a	7.6 a	8.5 bc	73 a	76 a	1.7 a	3.3 a
Tannic Acid	42 a	38 ab	7.7 a	8.5 bc	72 a	69 a	2.3 a	2.5 a
Proanthocyanidins	56 b	48 bc	6.8 a	7.5 a	72 a	70 a	1.9 a	2.4 a

Different letters within row indicate significant difference ($p < 0.05$, LSD).

All films were more insoluble at pH 7 (average 71%, Table 10) than pH 2. There were no differences among treatments, with or without shortening. As the film formulation included 29% (w/w dry matter) glycerol, it appears the glycerol component was solubilized, but not the gluten proteins at pH 7. This was not surprising as the pI of gluten is ~7.6 (Gennadios, Brandenburg, Weller & Testin, 1993); because a protein's pI is the pH at which it has no net charge in solution, it is insoluble. So, while gluten films were stable in replicated intestinal pH (7), acidic environments like the stomach will solubilize gluten. Thus, any effort to improve gluten film stability for controlled release of nutrients must protect the film from disintegrating in strong acid (low pH).

The WHC of the films (control and all treatments) were also similar at pH 7 / 25 and 37 °C: average WHC 2.6X weight of film, but considerably lower than at pH 2 (Table 10). The gluten films did not absorb as much water at pH 7 (near their pI) because the proteins had low net charge and were closely associated.

Effect of Tannins on Film Digestibility

Films were assessed for protein digestibility by measuring amount of film left intact after incubating at 37 °C with pepsin in a pH 2 buffer for 5, 10, or 20 min. Films with low protein digestibility will survive stomach digestion better and be able to protect and distribute micronutrients to the lower gastrointestinal tract. In treatments without shortening, 45% of the PA film was intact after 20 min of *in vitro* digestion vs 34, 31, and 37% for the control, catechin, and tannic acid films, respectively (Fig 20). This suggests that tannins, but especially PA, reduced protein digestion in gluten films. This is not surprising as tannins are known reduce digestibility by interacting directly with protein (Cousins, Tanksley, Knabe & Zebrowska, 1981, Feeny, 1968) and inhibiting pepsin (Gonçalves, Soares, Mateus & De Freitas, 2007). The fact that PA-gluten film was less digested than tannic acid-gluten film suggests that PA interactions led to a stronger protein matrix, less accessible to enzymatic digestion than tannic acid. This is in concurrence with the extensibility (Fig 19) and solubility (Table 10) data suggesting PA had a greater cross-linking effect on gluten than tannic acid. Among the gluten films, only the catechin treatment increased digestibility. This was likely because catechin breaks disulfide bonds, but does not complex proteins, thus it made gluten more accessible to pepsin for digestion.

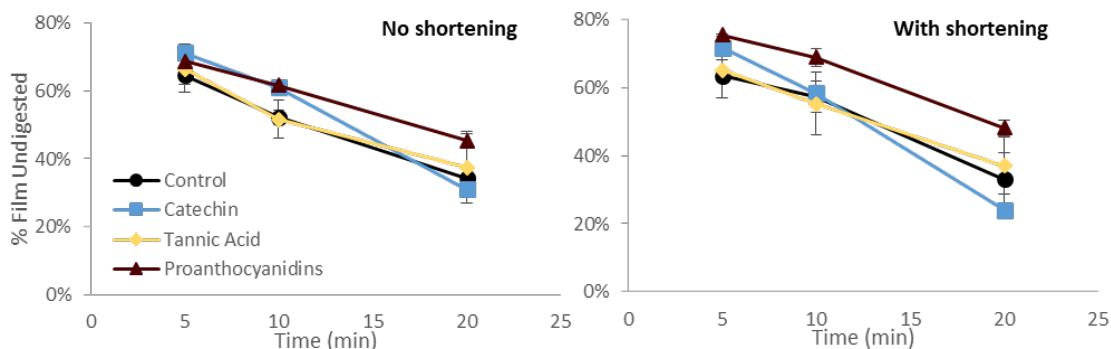


Figure 20 - Effect of 10 mg/g catechin, tannic acid, and proanthocyanidins with or without 5% shortening on protein digestibility of gluten films.

Adding shortening (5%) to the films did not significantly affect the protein digestibility of the control and tannic acid films at 20 min (33% and 37% intact, respectively, Fig 20). However, shortening did further decrease digestion of PA-gluten (48% vs 45% without shortening). The decrease in PA-gluten film digestion in presence of shortening is supported by the evidence that PA and shortening may be enhancing hydrophobic interaction between PA and gluten (Fig 19 and Table 10): these interactions could slow enzymatic degradation, likely through altered protein matrix density. Shortening did not affect digestion of tannic acid-gluten film indicating hydrophobic interactions are less relevant to the tannic acid-gluten complex.

Adding 5% shortening to catechin film further increased its protein digestibility, (31% insoluble without shortening versus 24% with) after 20 min *in vitro* digestion (Fig 20). As mentioned in earlier sections, catechin breaks disulfide bonds thus allowing lipids to better interact with gluten. This likely interrupted the gluten structure and increased enzyme access to protein polymers.

Effect of Tannins on Wheat Flour Batter Viscosity and Stability

To determine effect of tannins on wheat flour batter viscosity, flour + water batters were mixed and viscosity analyzed on a Brookfield viscometer. At consistent speed and temperature (25 rpm, 22 °C) the catechin batter viscosity was similar to the control (1640 vs 1660 cP, respectively, Fig 21). Tannic acid increased batter viscosity to 2200 cP, while PA further increased viscosity to 3280 cP. The data suggests that both tannins increased protein polymer size, thus contributing to increased batter viscosity. The much greater effect of PA vs tannic acid further confirms the ability of PA to create larger and stronger gluten complexes than tannic acid.

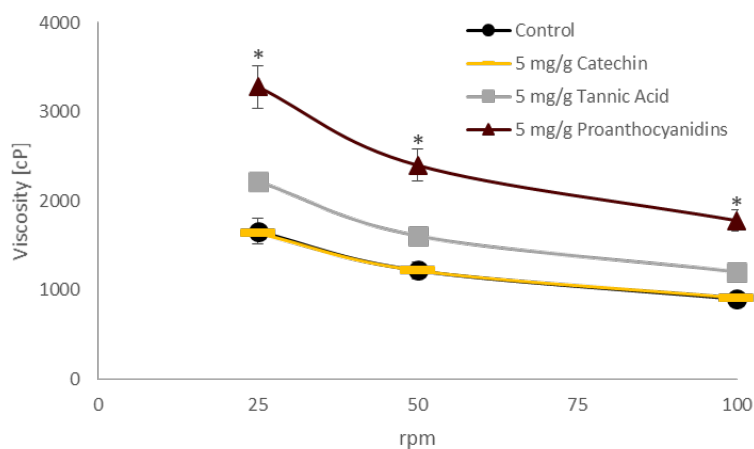


Figure 21 - Effect of catechin, tannic acid, and proanthocyanidins on viscosity of pastry flour batters at 22 °C. Batter was prepared by mixing 40% pastry flour (pre-blended with catechin, tannic acid, or PA) and 60% water (w/w) with an electric whisk for 2 min. Error bars illustrate \pm SD. Stars indicate significant difference ($p < 0.05$) from control.

The stability of pastry flour batters were tested by volumetrically measuring liquid that separated from the batter over a 4 h period. In general, 20% pastry flour in

80% water (w/w) formed an unstable batter, with visible separation occurring within 3 min after mixing. The control and 25 mg/g catechin batter behaved similarly over time (Fig 22), with an average liquid separation of 17% at 15 min up to 53% at 4 h. This suggests that while the disulfide reducing ability of catechin altered film structure (Fig 19 and 20), it did not greatly affect batter viscosity (Fig 21) or stability (Fig 22).

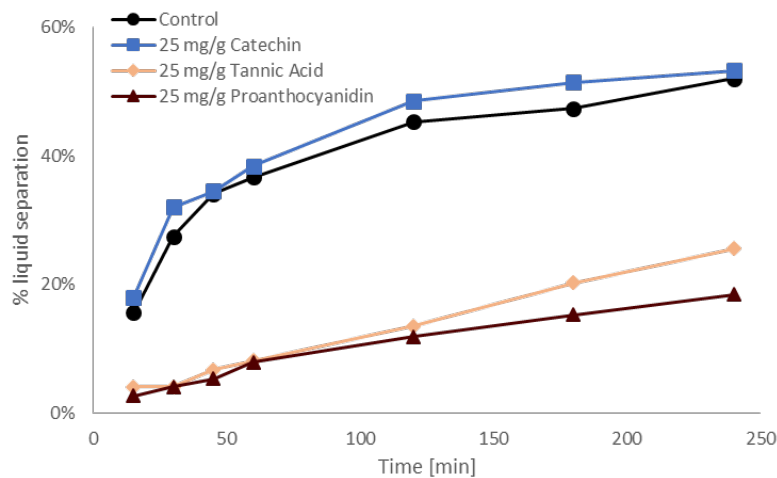


Figure 22 - Effect of catechin, tannic acid, and proanthocyanidins on stability of pastry flour batters. Batter was prepared by adding 20%:80% (w/w) pastry flour:water with treatments of catechin, tannic acid, and PA to a 15 mL tubes and vortexing 1 min. Samples were rested and liquid separation read volumetrically.

Tannic acid and PA at 25 mg/g flour significantly decreased the separation of liquid from the batter (Fig 22), likely through cross-linking gluten and enhancing its network structure and viscosity (Fig 21). For instance after 15 min rest, the tannic acid and PA treated batters only had 4% and 3% liquid separation, respectively, versus 16% for the control. Up to 1 h, the tannin treatments behaved similarly with only 8% liquid separation versus 38% for the control. However, from 1 to 4 h, tannic acid treated batter

had more liquid separation than PA (Fig 22). In fact at 4 h, PA batter had only 18% liquid separation, which was similar to the control at 15 min (16%), while tannic acid had 26% liquid separation. This indicates that PA-gluten interactions are highly stable in batters. As batter-type products are typically chemically leavened and do not contain highly developed gluten matrices, having stable, viscous rheology is important to maintain air cell inclusion and capture gas expansion, without allowing coalescing of gas cells. Addition of PA to batters may help improve stability and could mitigate detrimental effect of inclusions (e.g.: bran, fruit pieces, chocolate chips) on volume.

Effect of Tannins on Batter Viscosity during Cook-Cool Cycle

To assess the effect of tannin-gluten interactions on starch viscosity profile during the cooking process, batters were tested using the RVA. While the starch pasting properties of time to peak and pasting temperature were largely unaffected by the treatments, the peak viscosity increased significantly with the tannic acid and PA treatments (Table 11, Fig 23). PA at 25 mg/g flour usage level significantly increased peak viscosity (53% compared to control); tannic acid increased peak viscosity by 16%. Even at 5 mg/g flour usage level, the PA increased peak viscosity by 19% compared to the control while tannic acid increased peak viscosity by 9% (Table 11). Again, PA exhibited a greater effect on viscosity than tannic acid implying PA created stronger complexes with gluten proteins, and almost certainly starch components (Amoako & Awika, 2016b, Barros, Awika & Rooney, 2012). These are significant increases in viscosity even compared to hydrocolloids commonly used in the food industry. For

example, xanthan and alginate at 10 mg/g wheat flour increased maximum viscosity by 5% and 13%, respectively (Rojas, Rosell & De Barber, 1999). A higher viscosity throughout baking (i.e.: peak viscosity) improves retention of gas cells and prevents coalescing.

Table 11 - Effect of catechin, tannic acid, and proanthocyanidins on pasting properties of pastry flour measured by RVA.

	Peak Time [min]	Pasting Temp [°C]	Peak Viscosity [cP]	Final Viscosity [cP]	Breakdown [cP]
Control (Pastry Flour)	9.0 a	87.5 a	1571 a	2106 a	558 a
<i>Catechin</i>					
5 mg/g flour	9.0 a	87.7 a	1486 a	1960 a	541 a
25 mg/g flour	8.9 a	87.2 a	1561 a	2025 a	579 ab
<i>Tannic Acid</i>					
5 mg/g flour	9.0 a	87.1 a	1716 a	2282 a	646 ab
25 mg/g flour	9.2 a	88.1 a	1824 a	2222 a	649 ab
<i>Proanthocyanidins</i>					
5 mg/g flour	9.0 a	87.3 a	1863 a	2235 a	778 bc
25 mg/g flour	8.9 a	86.3 a	2400 b	2838 b	953 c

Different letters within row indicate significant difference ($p < 0.05$, LSD).

The tannin-induced increase in starch viscosity was also seen in the final viscosity, primarily in the 25 mg PA/g flour treatment, which had a final viscosity of 2838 cP vs the control of 2106 cP (Table 11). PA alters starch pasting properties and increases viscosity, mostly through interactions with amylose (Amoako & Awika, 2016b, Barros, Awika & Rooney, 2012). Catechin had negligible effect on peak and final viscosity; suggesting no meaningful interaction with starch, as was previously reported (Amoako & Awika, 2016b).

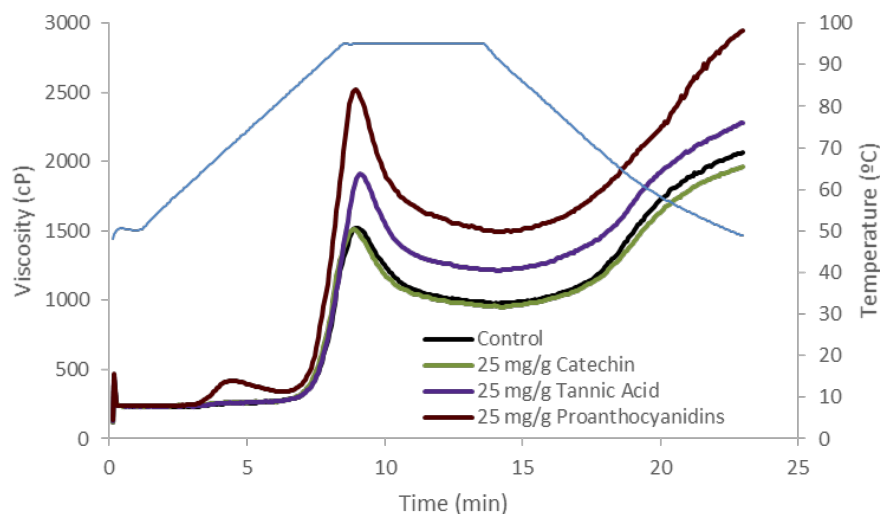


Figure 23 - Effect of catechin, tannic acid, and proanthocyanidins on RVA profile of pastry flour (8.5% protein).

RVA profiles are mainly a function of starch, and it is thus likely that some of the tannins interacted with starch, increasing both peak and final viscosity. PA interaction with starch is known to increase starch peak viscosity (Amoako & Awika, 2016b, Barros, Awika & Rooney, 2012). Protein interactions play a role in viscosity, albeit typically minor compared to starch. Potassium bromate, an oxidizing agent used to increase gluten strength by increasing disulfide bonds, was not shown to affect pasting characteristics of wheat flour, whereas a reducing agent, l-cysteine, was shown to decrease peak viscosity (Ravi, Manohar & Rao, 1999). High peak viscosity is a desirable attribute to prevent collapse of batter-based goods (e.g.: cake, gluten-free bread) through stabilizing the gaseous phase of batters.

Interestingly, PA treatment produced a small peak at about 3 min, 60 °C (Fig 23). This may be a result of the gluten denaturing during the cooking process, thus unravelling and exposing hydrophobic amino acid residues. These residues may have

cross-linked with PA thereby increasing viscosity. Legrain et al. (2008) showed that extractable gluten proteins, especially glutenins, decreased under a cooking treatment beginning ~60 °C, as the glutenins were cross-linked via disulfide bonds triggered by protein denaturation. The fact that tannic acid did not have similar effect suggests that hydrophobic reactions between PA and gluten were largely responsible. This is likely because of the difference in tannin structure: PA are primarily linear and somewhat flexible to be able to interact with gluten as it changes conformation whereas tannic acid has a more spherical arrangement (Hagerman, Rice & Ritchard, 1998) and may have been sterically hindered from interacting with gluten.

Proanthocyanidins, and to a lesser extent tannic acid, increased gluten film strength and wheat flour batter viscosity and stability. PA also decreased film solubility and digestibility, and addition of lipids to gluten-PA film enhanced this effect. This effect could be used to create better biofilms or to target delivery of micronutrients in the digestive tract. The film strengthening and stabilizing effect of PA on foams could be useful as a natural viscosity modifier in food processing.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Summary

This study demonstrated that PA complex with gluten proteins via hydrophobic interactions and hydrogen bonding, thus greatly increasing gluten dough strength and stability even at modest usage level (≤ 2.5 mg/g flour or 0.25%). The MW profile of the PA plays a large role in this interaction, with high MW PA being more effective at strengthening gluten. Among the gluten proteins, PA precipitated glutenins to a greater extent than gliadins, and the larger x-type glutenins to a greater extent than the smaller y-type glutenins, suggesting that PA more extensively cross-linked larger gluten proteins. As PA and gluten MW distribution are important factors in their interaction, selection of raw materials (wheat protein composition or subfractions, and PA profile) and processing conditions could be optimized to alter gluten functionality for desired applications. This study also suggests that PA-gliadin interactions are largely hydrogen-bond driven, whereas PA-glutenin complexation involves cooperative hydrogen bonding and hydrophobic interactions. Because PA interaction with gluten proteins is not entirely random, it can be manipulated to produce desired rheological behavior.

The ability of a strong antioxidant, such as PA, to break disulfide bonds, while also increasing elasticity and maintaining or increasing extensibility, is a novel effect in a gluten matrix. This functionality of high MW PA was shown to stabilize gluten films while maintaining their resilience, evidenced by the increase in film strength and

decrease in film solubility and digestibility. In a batter system, high MW PA increased wheat flour batter viscosity and stability. High MW PA ability to stabilize films and foams through gluten interaction may have applications such as:

- enhanced volume of baked goods, enabling higher inclusion of beneficial nonwheat ingredients,
- improved integrity of edible barriers and biofilms,
- food matrix modifications to slow macronutrient digestibility profile, thus allowing targeted delivery of bioactive compounds and nutrients in the digestive tract.

This study did not elucidate the effect of PA interactions on gluten secondary structure. The precise locations of PA-gluten interactions were not identified (e.g.: specific amino acid side chains). Effect of PA on individual glutenin and gliadin rheology was not studied.

Recommendations for Further Research

To employ PA-gluten interactions for novel functionalities, further studies should assess:

1. Stability of PA-gluten complex over a range of processing and storage conditions.
2. Interaction of PA with specific glutenin and gliadin subunits, and their effect on protein behavior and functionality.
3. Effect of PA-gluten interactions in food matrices on macronutrient digestion and on colonic microbiota and their metabolites.

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