BLUEBERRY CELL WALL FRACTIONATION AND INTERMOLECULAR BINDING BETWEEN PECTIN RICH FRACTIONS AND ANTHOCYANINS

by

ZHUANGSHENG LIN

ABSTRACT

Blueberry cell wall fractions extracted with increasingly strong solvents were subjected to GC-MS for neutral sugar profile and glycome profiling. Water soluble (WSF), chelator soluble (CSF) and sodium carbonate soluble (NSF) fractions were pectin rich fractions with 50-60% uronic acid content. Pectin rich fractions had xyloglucan, HG backbone and arabinogalactans epitopes, and arabinose and galactose were the two major neutral sugars. WSF and CSF had high Mw of 450 kDa in the major eluting peak, while NSF had smaller Mw of 170 kDa. Pectin rich fractions were incubated with anthocyanin solutions under pH 2-4.5, and bound and free anthocyanin were separated by centrifugal ultrafiltration. WSF, CSF and NSF all reduced free anthocyanin pigment concentration in the filtrates, and the binding was more significant at pH 2 and pH 3.6. Ionic interaction between pectic free carboxyl groups and anthocyanin flavylium cation and anthocyanin aromatic stacking were concluded as the major binding mechanisms.

INDEX WORDS: Blueberry, Pectin, Anthocyanin, Intermolecular binding, Glycome profiling, Centrifugal ultrafiltration, HPSEC-MALS-RI

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B.S. Iowa State University, 2012

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirement for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2014

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August 2014

DEDICATION

THANK YOU MOM AND DAD

GO JASON

KEEP PASSIONATE AND CARRY ON

ACKNOWLEDGEMENTS

I would like to thank Dr. Louise Wicker for her sincere guidance and her trust throughout my pursuit of the degree. She is a great advisor. I appreciate her support intellectually, spiritually, and financially to let me test my ideals in the lab, to help me learn from where I fail, and most importantly, to show me not to be frustrated when 'life pulls the vacuum'. I would like to thank Dr. Mark Harrison and Mrs. Karen Simmons for recruiting me to the department and selecting me as a recipient of a departmental assistantship. It has been a great adventure at the UGA. I would like to thank Dr. Jeffrey Urbauer for serving in my committee and all the help he and Mrs. Urbauer generously provided. I would like to thank Dr. Joan Fischer for serving in my committee and her advice on anthocyanins. I would like to thank Dr. Rakesh Singh for his leadership and his effort on establishing such a good image for the program. I would like to thank Dr. Sivakumar Pattathil and Michael Hahn for the glycome profiling assay. I would like to thank the my labmates, Brittnee Thirkield, Jiyoung Jung, Jeewon Koh and Jiyeon Lee for all the thick and thin, as well as the 'hot and cool' we've been through together, and they all become good memories.

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CHAPTER 1

INTRODUCTION AND INTERATURE REVIEW

Introduction

Pectin is a hetero polysaccharides extracted from plant cell wall. Anthocyanins are a class of phenolic compounds from vascular plant. Pectin is one of the most complex plant food polysaccharides, and anthocyanins are one of most important visible pigments in plants. Studies on both pectin and anthocyanin are still ongoing processes, and recent research interests focus on revealing the complex structure of pectin (Maxwell, Belshaw, Waldron, & Morris, 2012; Yapo, 2011), biomedical application of pectin (Munarin, Tanzi, & Petrini, 2012; Wicker et al., 2014), biotransformation of anthocyanins *in vivo* (Fleschhut, Kratzer, Rechkemmer, & Kulling, 2006; Keppler & Humpf, 2005), *in vivo* health promoting benefits of anthocyanin (Wallace, 2011; Wang & Stoner, 2008), others. Dozens of studies showed clear evidence on interactions between pectin and anthocyanin. Researchers who studied anthocyanins in flowers as color pigments found that pectin formed large non-dialyzable molecules with anthocyanin-copigment complexes (Asen, Stewart, Norris, & Massie, 1970; Bayer, Egeter, Fink, Nether, & Wegmann, 1966). Recent studies discovered that pectin solubilized anthocyanin-metal chelate complexes and prevented complex precipitation in an aqueous environment, making anthocyanin a potential natural blue colorant in beverage application (Buchweitz, Carle, & Kammerer, 2012; Buchweitz,

Nagel, Carle, & Kammerer, 2012). Pectin enhanced the anthocyanin pigment stability and extended pigment degradation half-life in model fruit jam systems (Buchweitz, Speth, Kammerer, & Carle, 2013a; Holzwarth, Korhummel, Siekmann, Carle, & Kammerer, 2013; Kopjar et al., 2009). Cell wall composites containing pectin tightly bind anthocyanin, and the binding model containing both dietary fiber and anthocyanin may have synergistic effect on promoting colon health (Padayachee et al., 2013; Padayachee et al., 2012). However, the mechanism for pectin and anthocyanin interaction is not well understood. In this literature review, current knowledge on pectin and anthocyanin are discussed, and studies that involve pectin and anthocyanin interaction are summarized.

Pectin

Pectin is a hetero plant polysaccharide that is present in the middle lamella, primary and secondary plant cell walls (Voragen, Coenen, Verhoef, & Schols, 2009). The most common and conventional food application of pectin relies on its gelling and thickening properties. Pectin is an important food additive in jams, jellies, and in the confectionary industry, as well as a stabilizer in acid milk products (Stephen & Phillips, 2010). Conventional pectin structure is visualized as linear polygalacturonic acids of 15-30 units with partial methylesterification (Morell, Baur, & Link, 1934), and this ideal pectin model has been used in the various handbooks since the nineties of the last century (Voragen et al., 2009). Commercial pectin is extracted mainly from citrus peels and apple pomaces using hot acid hydrolysis (60-90^{o}C) for extended hours followed by alcohol precipitation (Voragen et al., 2009). The most important chemical feature of pectin structure that contributes to different gelling properties that has been

described in almost all food chemistry handbooks is the degree of methylesterification (DE) of the carboxyl groups on the pectin polygalacturonic acid backbone (Stephen & Phillips, 2010). High methoxyl (HM) pectin forms a gel in low acid (pH 2.8-3.5) and high sugar conditions. High sugar content lowers water activity and therefore favors chain-chain hydrogen and hydrophobic interaction. Low pH lessens the negative charges on carboxyl groups and diminishes electrostatic chain repulsion. Low methoxyl (LM) forms a gel in the presence of divalent cations, such as calcium ion, which forms calcium bridges with carboxyl groups on two pectin chains.

Studies on pectin structures and functional properties over the last decades have drastically changed our understanding of pectin. It has become clear that pectin is a versatile polysaccharide (Voragen et al., 2009), both structurally and functionally.

Pectin Structure

Pectin is composed of at least 17 different monosaccharides, ferulic acid and protein moieties (Scheller, Jensen, Sørensen, Harholt, & Geshi, 2007; Voragen et al., 2009; Yapo, 2011). The pectin backbone has a chain structure of α-1-4 linked D-galacturonic acid units interrupted by α-1-2- L-rhamnose insertion at adjacent or alternate positions. Other sugar constituents are attached as the side chains. D-galactose, L-arabinose are the most common (Stephen & Phillips, 2010). D-xylose, D-glucose, D-mannose, L-fucose and D-glucuronic acid are less common. More rare sugars such as 2-O-methylfucose, 2-O-methylxylose, D-apiose, 3-C-carboxy-5-deoxy-L-xylose and 2-keto-3-deoxy-D-mano-octulosonic acid have also been reported (Scheller et al., 2007). Most of the sugars occur in short side chains of one to three units, except for D-galactose and L-arabinose, which are present in more complex side chains of arabinans and

arabinogalactans. Arabinans have an α-1-5-linked L-arabinose backbone branched with L-arabinose residues. Arabinogalactans occur in the two structurally different forms, arabinogalactan-I and arabinogalactan-II. Arabinogalactan-I has a 1-4-linked linear backbone of D-galactose residues with 20-40% α-1-5-L-arabinose residues in short side chains. Arabinogalactan-II is a highly branched polysaccharide with rhamified chains of D-galactose residues joined by 1-3 and 1-6 linkages. Arabinogalactan-II chains are generally terminated by L-arabinose residues (Stephen & Phillips, 2010). Pectin also has non-sugar substituents, essentially methoxyl groups, acetyl groups, phenolic acids, proteins and amide groups in commercial samples. The percentage of galacturonic acid backbone carboxyl groups esterified with methyl groups defines the degree of methylesterification (DE). If more than 50% carboxyl groups are methylesterified, the pectin is called high methoxyl (HM) pectin; if less than 50%, it is called low methoxyl (LM) pectin. Acetic acid is acetylated at C-2 and C-3 of the galacturonic acid backbone. Acetylation impairs the gelling properties of pectin (Stephen & Phillips, 2010), for example, sugar beet pectin with higher degree of acetylation is not used for gelling purposes (Funami et al., 2011; Williams et al., 2005). Industrially, LM pectin is treated with ammonia to amidate carboxyl groups on galacturonic acid to make amidated pectin. Amidated pectin has unique gelling features (Stephen & Phillips, 2010). Commercial pectin is mainly extracted from citrus peels and apple pomances, and is sometimes extracted from sugar beet (Wicker et al., 2014). The major application for sugar beet pectin is as an emulsifying or stabilizing agent rather than gelling agent (Funami et al., 2011). Structurally, sugar beet pectin is rich in pectic hairy region and non-sugar moieties such as protein and ferulic acid, and the backbone is often acetylated (Williams et al., 2005). Commercial pectin has to contain no less than 65%

galacturonic acid to meet the specifications of the Food and Agriculture Organization (Maxwell et al., 2012).

Based on the backbone structures and neutral sugar side chain constituents, pectin is divided into four different regions, namely homogalacturonan (HG), rhamnogalacturonan-I (RG-I), rhamnogalacturonan-II (RG-II), and xylogalacturonan (XG) (Scheller et al., 2007; Yapo, 2011). HG is also called the 'smooth region', due to the fact that it is composed of the simplest α-1-4-galacturonic acid backbone with partial methylesterification and acetylation, but with no neutral sugar side chains attached. RG-I is the only pectin region that has the rhamnified polygalacturonic acid backbone. The rhamnose residues might be substituted with neutral sugar side chains of arabinans, arabinogalactan-I, or arabinogalactan-II. Sometimes the arabinose and galactose residues can be substituted with ferulic acid. RG-II has the same backbone as HG, but the structure is not very well understood because the complex side chains constituents, including those rare neutral sugars. RG-II is the most complex pectin region consisting of at least 12 different side chains and more than 20 different linkages. XG is similar to HG except that it is has branches of single xylose or short β-1-4-linked xylose chain. The four pectin regions are covalently linked to each other, but it is not clear how the different pectin regions are connected (Voragen et al., 2009; Yapo, 2011).

Pectin Functional Properties

Besides the application in food system as gelling, thickening and stabilizing food additives, recent research focuses on revealing the health promoting effects of pectin as a bioactive polysaccharides (Wicker et al., 2014). As dietary fiber, pectin, has the potential to

lower blood cholesterol levels and affect glucose metabolism by lowering the glycemic response curve. Numerous studies suggest that dietary fiber has beneficial effects in the prevention of diverticulosis, colon cancer, coronary heart disease, hypercholesterolemia and diabetes (Voragen et al., 2009). Modified pectin, which has small molecular weight that, is absorbable by the human body can bind to pro-metastatic protein galectin-3 (GAL3) and inhibit the ability of GAL3 to promote cell adhesion and migration (Maxwell et al., 2012). This indicates the potential application of pectin as non-toxic approach to prevent or reduce carcinogenesis. Pectin is an excellent candidate as a colon targeted drug delivery vehicle due to the selective digestion by colon microflora with minimal degradation in gastriointestinal tract (Jung, Arnold, & Wicker, 2013; Wong, Colombo, & Sonvico, 2011). Unlike cellulose, pectin as soluble dietary fiber undergoes nearly complete fermentation by the colon microflora and leads to generation of short-chain fatty acids, which lower the colonic pH and may reduce colon tumor development (Moore, Park, & Tsuda, 1998).

Pectin Extraction

Pectin is found in the middle lamella or within the plant primary and secondary cell walls. Inside the plant cell, pectin forms a network that binds to the interacting cellulose and hemicellulose. Thus, it contributes to the structural integrity of cell walls and resistance to pathogen attack (Scheller et al., 2007). In dicotyledonous plants, pectin accounts for 35% of fiber, and cellulose and hemicellulose account for 30%. Grasses and wood tissues contain 2-10% and 5% pectin respectively (Voragen et al., 2009). Pectin is believed to bind to hemicellulose and the crystalline structure of cellulose of plant cell walls or bind to the crystalline structure of

cellulose with hemicellulose inside cell walls (Holzwarth, Korhummel, Carle, & Kammerer, 2012). It is suggested that in cell walls that contain low amount of xyloglucan, pectic arabinans and galactans side chains coat cellulose microfibrils (Zykwinska, Ralet, Garnier, & Thibault, 2005). Physical, chemical, and enzymatic treatments are used to extract the pectin from cell wall matrix. Industrially, pectin is extracted from citrus peel, apple pomaces, or sugar beet pulp, byproducts from fruit or sugar processing. Industrial extraction conditions are optimized to emphasize extraction yield and desirable DM, therefore physical maceration and hot acid hydrolysis of cell wall material for several hours are applied (Voragen et al., 2009). Acid hydrolysis not only breaks down chemical bonds in the cell wall matrix and enhances pectin yields, but also removes neutral sugar side chains, breaks down ester links and pectic backbone (Stephen & Phillips, 2010). To better understand the structure of pectin, on a laboratory scale, plant cell wall materials are precipitated in alcohol to prepare alcohol insoluble solids (AIS). Most of the free sugars, phenolic compounds, proteins and lipids are removed from AIS and the residue is composed of pectin, hemicellulose, cellulose and lignin (Eaks & Sinclair, 1980; Fügel, Schieber, & Carle, 2006). Chemical fractionation is conducted to extract pectin rich fractions from AIS. AIS are usually sequentially extracted with water, chelator buffer solution, weak acids and finally, sodium hydroxide solution or sodium carbonate. The extracts are dialyzed to remove buffer salts and then precipitated in alcohol or freeze dried to concentrate pectic substances. The water and chelator extraction are non-degradative; therefore, more intact pectin structures are preserved (Stephen & Phillips, 2010). Pectin extracted with chelator buffer in general originates from the middle lamella, where it is present in the form of calcium pectate, while protopectin tightly anchored in the cell walls is extracted by weak acid and weak alkali, which are

degradative extractants (Stephen & Phillips, 2010). Enzyme assisted pectin extraction is often used to cleave pectin structures into smaller extractable fractions to facilitate structural understanding of this polysaccharide. Some example enzymes used in pectin studies are endo-polygalacturonase, rhamnogalacturonase, endo-arabinanase, endo-galactanase (Stephen & Phillips, 2010).

Blueberry Pectin

Blueberry pectin has not been well characterized for structural or functional properties. The pectin in blueberry has been studied to monitor pectin transition during fruit development and during storage. AIS from blueberries of different maturity were prepared and used to study pectin transitions during fruit development between 30-57 days after full bloom (Proctor & Peng, 1989). In the report, the amount of uronic acid was assumed to be the amount of pectin. The reported total pectin content ranged from 280 to 400 mg uronic acid per gram AIS. The total pectin content decreased two-fold in the fruits during fruit development, and the corresponding water soluble pectin increased from about 20% to 60% of total pectin content in the AIS. Total pectin content in chelator soluble pectin increased slightly, while in alkali soluble pectin it decreased from about 65% to 20%. AIS from cull lowbush blueberry was prepared using citric acid acidified ethanol, and pectin content during storage was reported (Chen & Camire, 1997). The amount of uronic acid was assumed to be the amount of pectin. The reported pectin content ranged from 270-320 mg uronic acid per gram blueberry. Total pectin content increased during storage from 3 to 7 months, and the degree of methylesterification ranged from 66 to 77%. Both studies suggested the high pectin content in blueberry cell wall. Since Proctor and Peng (1989)

used laboratory scale AIS preparation and fractionation without concentrating each pectin fractions, and Chen and Camire (1997) only prepared AIS, neither study had literally extracted pectin from blueberry. In recent research, AIS from blueberry of different maturity and sequentially extracted water soluble fraction (WSF), chelator soluble fraction (CSF), sodium carbonate soluble fraction (NSF), and two potassium hydroxide soluble fractions (Vicente et al., 2007) were studied. AIS contained between 210 to 330 mg uronic acid per gram AIS. Total uronic acid content in the AIS did not change at different fruit development stages, and it was concluded pectin depolymerization was not associated with blueberry fruit softening during ripening. Arabinose and xylose were the major neutral sugar presented in AIS, with arabinose the major neutral sugar in the pectin rich fractions WSF, CSF, and NSF. The uronic acid content of pectin rich fractions was measured on AIS weight basis, therefore, no conclusion can be drawn on pectin content of extracted blueberry pectin based on results from this study.

Anthocyanins

Anthocyanins are pigments in vascular plants responsible for the bright orange, pink, red, violet and blue colors in flowers and fruits, for example, blueberry. The name anthocyanin is derived from Greek words *anthos*, meaning flower and *kianos* meaning blue (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009). The study of anthocyanins raises a lot of interest in recent years due to the potential application as natural food colorants and health promoting features.

Anthocyanin Structure

Anthocyanins are phenolic compounds (Castañeda-Ovando et al., 2009), and the basic structures of anthocyanins are anthocyanidins (Figure 1.1), which consist of an aromatic A ring bound to an heterocyclic oxygen containing C ring and a third aromatic B ring. When anthocyanidins are present in their glycosidic form, with attached sugar moiety, they are known as anthocyanins. Glycosidic substitution can take place at 3, 5, and 7 positions on anthocyanidins, and the sugar moieties can be acylated with organic acids (Wrolstad, Durst, & Lee, 2005). Up to date, there are 23 anthocyanidins reported (Castañeda-Ovando et al., 2009) and more than 540 anthocyanin pigments have been identified in nature (Wrolstad et al., 2005). The six most common anthocyanidins found in nature are pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin (Durst & Wrolstad, 2001). The main differences between different anthocyanidins are the number and combination of hydroxyl and methoxyl groups, the nature and number of sugar moieties, and the organic acids acylation on the sugar moieties (Kong, Chia, Goh, Chia, & Brouillard, 2003).

The structure of anthocyanins, especially the C ring structure, is pH dependent. In fact, anthocyanins can be found in four different chemical forms, namely, flavylium cation, quinoidal base, carbinol pseudo base, and chalcone form, at different pH conditions (Giusti & Wrolstad, 2001). At pH 1, the flavylium cation is the predominate form. At very acidic pH media (pH 0.5), only the flavylium cation form of anthocyanins can be found. Increasing pH reduces the flavylium cation concentration, as it is can be hydrated by nucleophilic attack of water to the carbinol form, or by proton loss to form the quinoidal base form. As pH goes even higher, for

example, to neutral pH 7, the chalcone structure can be formed due to the ring opening of carbinol. At slightly acidic environments of pH 4-6, all four structural forms can coexist (Castañeda-Ovando et al., 2009). The equilibrium between quinoidal base and carbinol pseudo base or chalcone is controlled via flavylium cation (Figure 1.2). The flavylium cation can reversibly transform to quinoidal base form or carbinol pseudo base/chalcone form. When pH increases, the equilibrium shifts towards increasing the amount of quinoidal base or carbinol pseudo base/chalcone form, and when pH decreases, the equilibrium shifts towards increasing the amount of flavylium cation.

Anthocyanin Stability

The monomeric anthocyanins are highly unstable and are susceptible to degradation. The stability of anthocyanins depends on several factors such as pH, monomeric anthocyanin chemical structure, temperature, pigment concentration, light, oxygen, solvents, enzymes, other phenolic compounds, proteins, and metal ions (Castañeda-Ovando et al., 2009). Since anthocyanin structure changes under different pH conditions, anthocyanin stability is very sensitive to pH. Anthocyanins are more stable in acidic media and in low pH value. At pH value higher than 7, anthocyanins undergo a degradation process depending on their glycosidic substitution (Castañeda-Ovando et al., 2009).

For anthocyanin aglycones, the chalcone form with open ring structure can transform to diketone, which is susceptible to degradation into phenolic acid and aldehyde (Keppler & Humpf, 2005) (Figure 1.2). The glycosidic substitution of anthocyanidins and the number and placement of hydroxyl and methoxyl groups on the aglycone have a significant effect on

anthocyanin stability. In general, the presence of additional hydroxyl or methoxyl groups on the B ring decreases the stability of anthocyanidins (Castañeda-Ovando et al., 2009; Fleschhut et al., 2006); therefore, pelargonidin with no hydroxyl or methoxyl substitution is reported to be the most stable anthocyanidins (Fleschhut et al., 2006). The increased hydroxylation of the aglycone stabilizes anthocyanidins, for example, delphinidin with three hydroxyl groups on B ring is more stable than cyanidin with two B ring hydroxyl groups in acidic methanol (Dao, Takeoka, Edwards, & Berrios, 1998). The anthocyanins with glycosidic substitution generally have higher stability (Wrolstad et al., 2005). In contrast to anthocyanin aglycones, anthocyanins with one glycosidic substitution or two glycosidic substitutions are sequentially more stable under neutral pH conditions (Fleschhut et al., 2006). The sugar substitution at the 3 position on anthocyanidin C ring prevents diketone formation, thus blocking the pathway to degradation into phenolic acid and aldehyde. Sugar moieties can also stabilize the vertical stacking models between anthocyanins and between anthocyanin and phenolic copigments by superimposing them with hydrogen bonds, contributing to increased stability (Goto & Kondo, 1991). Acylation of phenolic compounds also enhances anthocyanin stability. When two or more acyl aromatic moieties present in anthocyanins, a sandwich type of intramolecular self-association can take place. The acyl aromatic moieties can vertically stack on aromatic nuclei of aglycone. The superimposed hydrophilic sugar moieties further stabilize the vertical stacking. The hydrophobic stacking could be further stabilized by hydrogen bonding on the hydrophilic moieties (Goto & Kondo, 1991).

Anthocyanin as a Natural Colorant

Anthocyanin pigments are responsible for the red to purple color in many fruits and vegetables. As synthetic colorants can contribute to attention deficit hyperactivity disorder (ADHD) (McCann et al., 2007), the blue color hue naturally produced by anthocyanin pigments is of particular interest in food industry. Only few natural blue colorants are commercially available for coloring food and beverages (Buchweitz, Carle, et al., 2012). The color of anthocyanin pigments can be affected by pH, copigments, and concentration (Mazza & Brouillard, 1990), all of which limit the application of anthocyanin as a natural colorant.

Anthocyanins have different structures at different pH conditions as explained above, and different anthocyanin structures in aqueous solutions exhibit different color hue (Castañeda-Ovando et al., 2009). At low pH, flavylium cation predominates and anthocyanins solutions are red. At intermediate pH, solutions tend to be colorless due to the colorless carbinol pseudo base form. At higher pH, equilibrium shifts towards quinoidal base form, giving anthocyanin solutions blue color hue. The color intensity and spectral properties of anthocyanins can be affected by copigmentation effect, with the addition of copigments. Copigmentation is a phenomenon that the pigments form molecular or complex associations with other organic compounds, or metal ions and result in a change or increment of color intensity (Boulton, 2001). Copigmentation effect produces a hyperchromic effect and a bathochromic shift in the anthocyanin typical absorption region. The copigments can be flavonoids, alkaloids, amino acids, organic acids, nucleotides, polysaccharides, metals or anthocyanins (Castañeda-Ovando et al., 2009). Copigments can stabilize flavylium cations and shift the equilibrium towards forming

anthocyanin flavylium cations; the shift in spectral absorption range enhances red pigment intensity. Therefore, most copigmentation studies on anthocyanin are conducted under weak acid conditions (pH 3-6), where colorless carbinol pseudo base form exists. The copigmentation effect is generally more apparent at higher pH (Lewis, Walker, & Lancaster, 1995).

Anthocyanins and anthocyanidins with ortho-dihydroxyl groups in the B ring (cyanidin, delphinidin, and petunidin) are able to form metal-anthocyanin chelate complexes (Boulton, 2001), and result in significant hyperchromic effect and bathochromic shift (Buchweitz, Nagel, et al., 2012). The chelate complexes also stabilize anthocyanin color and weak acid pH condition (pH 5-6) is favored for cyanidin and delphinidin aluminum and iron chelate formation (Bayer et al., 1966). At pH lower than 3, the phenolic oxygen atom is protonated, while at pH higher than 3, small and multiply charged metal ions like aluminum and irons displace the phenolic proton on flavylium B ring and form deeply colored anthocyanin-metal chelate complexes (Buchweitz, Carle, et al., 2012).

Anthocyanins can also play the role of copigment, therefore, the color intensity of anthocyanin aqueous solution does not necessarily increase linearly as anthocyanin concentration increases. It is reported that an increment of anthocyanin concentration from 10^{-4} to 10^{-2} M increased the pigment absorbance by 300-fold (Asen et al., 1970).

Anthocyanin Health Benefits

Anthocyanins have also shown an array of possible health promoting benefits, such as the potential to reduce risk for cardiovascular disease and some cancers, , preventing oxidative damage to DNA (Kong et al., 2003). Anthocyanins showed powerful systematic antioxidant,

anti-inflammatory, antimicrobial, and anticarcinogenic activities in *in vitro* studies (Mazza, 2007). Anthocyanins have the ability to reduce cancer cell proliferation and inhibit tumor formation (Lila, 2004). The anticarcinogenic activities of anthocyanins are related to radical scavenging activity, stimulation of phase II enzymes, reduction of cell proliferation, inflammation, angiogenesis and invasiveness, and induction of apoptosis and differentiation (Wang & Stoner, 2008). *In vivo* studies show that dietary anthocyanins inhibit some cancers of the gastriointestinal tract and topical application of anthocyanin inhibits skin cancer (Wang & Stoner, 2008). Increased anthocyanin consumption may reduce the risk of cardiovascular disease; anthocyanins may regulate a variety of signaling pathways associated with the development of cardiovascular diseases (Wallace, 2011). Large amounts of anthocyanins (up to 85% depending on the sugar moiety of the anthocyanin) *in vivo* can reach the colon, and have potential to influence colon health (Kahle et al., 2006). Anthocyanins can be fermented by human gut microflora and promote the growth of *Bifidobacterium spp.*, *Lactobacillus-Enterococcus spp.* (Leijdekkers et al., 2014), which are probiotic bacteria in human gut that promote human health (Hord, 2008). Blueberry extracts rich in anthocyanins have prebiotic effects on growth of pure cultures of *Lactobacillus rhamnosus* and *Bifidobacterium breve*, and increase the growth of *Lactobacilli spp.* and *Bifidobacterium spp.* in human fecal cultures (Molan, Lila, Mawson, & De, 2009).

Anthocyanin Measurement

Since anthocyanin pigments are rare natural colorants responsible for blue color in food and beverage application, and anthocyanin health benefits have raised increasing interests,

qualitative and quantitative measurement of anthocyanin pigment are crucial. The simplest way to quantitatively measure total anthocyanin pigment content is to use UV-Vis spectroscopy (Wrolstad et al., 2005). Since monomeric anthocyanin pigments exhibit reversible structural and color changes due to changes in pH, total monomeric anthocyanin content can be measured by UV-Vis spectroscopy under different pH condition. At pH 1, the colored flavylium cation predominates, and has a typical absorption band between 490 to 550 nm regions of the visible spectra. At pH 4.5, the colorless hemiketal form predominates. The typical absorption band region of anthocyanin pigments make it possible to measure anthocyanin content of crude anthocyanin extracts, as other phenolic compounds in the crude extract have λ-max in the UV range. The maximum absorbance and molar absorptivity of most monomeric anthocyanins have been well documented in literature; cyanidin-3-glucoside in 0.1N HCl has maximum absorbance at 520 nm, with molar absorptivity of 25740 (Giusti & Wrolstad, 2001). Total monomeric anthocyanin pigment content can be calculated with optical density difference measured at λ-max corrected against haze (measured at 700 nm), molecular weight of monomeric pigment and molar absorptivity. The pH difference method is a rapid and accurate colorimetric method for measuring total monomeric anthocyanin content, regardless of presence of degraded pigments or other interfering compounds (Giusti & Wrolstad, 2001). A major limitation of this method is that this assay is based on assumption that at pH 4.5, no flavylium cation form exists. In reality, minor flavylium cation form of anthocyanins exist in pH 4.5 condition (Castañeda-Ovando et al., 2009; Wrolstad et al., 2005).

The most widely used analytical methods for anthocyanin analysis are HPLC-DAD and HPLC-MS (Buchweitz, Carle, et al., 2012; Hidalgo et al., 2012; Padayachee et al., 2012; Yi, Akoh, Fischer, & Krewer, 2006). Chromatography technique separates different anthocyanins. HPLC-MS is used for identification of individual anthocyanins based on the molecular weight and fragmentation profile. HPLC-DAD is used for quantification purposes of individual anthocyanin peak, and external standard, e.g. cyanidin-3-glucoside is essential to determine the quantity of anthocyanins (Durst & Wrolstad, 2001). However, this method is limited due to the fact that different anthocyanins have different extinction coefficient from the external standard, therefore, the quantity of anthocyanins other than cyanidin-3-glucose determined by this method is not perfectly accurate (Wrolstad et al., 2005).

Additionally, NMR can also be used for structural analysis of anthocyanins. Mass spectrometry is commonly used for common anthocyanin identification, but for anthocyanins with complex sugar substitution and organic acid acylation, structural information cannot be obtained based on mass spectrometry (Goto & Kondo, 1991). For example, vitisin A, an anthocyanin derivative found in red wines, has 68 mass units greater than malvidin, and with NMR analysis the complex structure of vitisin A was interpreted (Bakker et al., 1997).

Pectin and Anthocyanin Interaction

Pectin and Anthocyanin Color Stability

Copigmentation of anthocyanins, for example with metals, phenolic acids and other anthocyanins, is the main mechanism of stabilizing color in plants (Davies & Mazza, 1993), while pectin has not been proved to be a copigment for anthocyanins, pectin interacts with

anthocyanins and enhances color intensity (Mazzaracchio, Pifferi, Kindt, Munyaneza, & Barbiroli, 2004). In one study, five different anthocyanin glucosides (pelargonidin, cyanidin, delphinidin, malvidin and petunidin) were investigated for copigmentation effect with some organic compounds at pH 3.0 and 4.3 (Mazzaracchio, Pifferi, Kindt, Munyaneza, & Barbiroli, 2004). Phenols and phenolic acids showed remarkable bathochromic and hyperchromic effect on interaction with anthocyanins, attributed by the vertical stacking of aromatic rings of copigments and anthocyanin aromatic nuclei. Aliphatic acids such as lactic acid and galacturonic acid showed weak copigmentation effect with anthocyanins with more than two hydroxyl groups on the B rings, possibly due to electrostatic interaction. Macromolecules such as cellulose, lignin and oat bran were not anthocyanin copigments, but they adsorbed anthocyanins and resulted in decreased spectral absorbility. High methoxyl apple pectin did not show significant copigmentation effect with anthocyanins, indicating that apple pectin is not a copigment for anthocyanins (Mazzaracchio, Pifferi, Kindt, Munyaneza, & Barbiroli, 2004). A similar conclusion was drawn from another study, in which anthocyanins were mixed with various polysaccharides including pectin at pH 2 and pH 4 to investigate shift in λmax and change in absorbility, and no significant copigmentation effect was observed between pectin and anthocyanins (Lewis et al., 1995).

The study of anthocyanin pigment color originated from the studies of red, purple, blue color of anthocyanin based pigments in flowers. Earlier studies suggested that anthocyanins containing at least two hydroxyl groups on the B rings can form anthocyanin metal chelate complexes with aluminum and iron and give rise to blue color hue (Bayer et al., 1966). Bivalent

metal ions such as calcium and magnesium may also form chelate complexes but have low complex stability, therefore, aluminum and iron are the only two metal ions responsible for forming blue anthocyanin metal chelate complex pigment in flowers (Bayer et al., 1966). Although anthocyanin metal chelate complex gives intense and stable blue color hue, the limitation of the metal chelate complex model lies in the poor solubility in aqueous solution, especially anthocyanin iron chelate complex (Bayer et al., 1966; Buchweitz, Nagel, et al., 2012). Polysaccharides, most likely pectin, are associated with the anthocyanin based pigments in flowers. Protocyanin is the first naturally occurring metal chelate complex which is carried by polysaccharide (Bayer et al., 1966). A stable, non-metallic, anhydrobase of delphinidin-3,5-glucoside was isolated from Prof. Blaauw Iris, and associated with a pectin, and complexed with a flavone copigment (Asen et al., 1970). Apple pectin added to delphinidin solution isolated from larkspur resulted in less than 2% delphinidin passing through dialysis membrane (Bayer et al., 1966). The collective evidence from earlier studies shows that polysaccharides, especially pectin cannot be ignored as they play a role in preventing anthocyanin metal chelate complexes from precipitation in aqueous solutions. As a result, in a recent study, different types of pectin (sugar beet pectin, sugar beet pectin fractions, citrus pectin, apple pectin, low methoxyl pectin, high methoxyl pectin and amidated pectin) were studied in terms of stabilizing anthocyanin metal chelate complexes in aqueous solution. In two consecutive studies conducted by the same group, sugar beet pectin (SBP) and pectic polysaccharide fraction (PPF) isolated from sugar beet pectin were studied to examine the copigmentation effect of pectin and anthocyanin rich solutions and with glucosides of 5 different anthocyanins (pelargonidin, cyanidin, delphinidin, malvidin and petunidin) at various acidic pHs (Buchweitz, Carle, et al., 2012; Buchweitz, Nagel, et al., 2012).

It was concluded that the copigmentation effect was contributed by anthocyanin metal chelate complex formation instead of anthocyanin pectin interaction. The magnitude of bathochromic shift and hyperchromic effect was correlated with aluminum and iron content in the pectin samples. As expected, the copigmentation effect was observed only between anthocyanin glucosides with more than 2 hydroxyl groups on the B rings and aluminum and iron pectin fraction. Control experiments carried out in aluminum and iron containing buffer solutions devoid of pectin showed similar copigmentation effect; however, anthocyanin iron chelate complex precipitated within 2 hours and after 24 hours the supernatant was colorless, while anthocyanin aluminum chelate complex precipitated after 24 hours (Buchweitz, Nagel, et al., 2012). PPF hindered anthocyanin metal complex precipitation and despite the fact that pigments had different stability, no precipitation was observed in model solutions consisting of pectic substances, metal and anthocyanins. In a follow up study conducted by the same group, amidated and high methoxylated citrus pectin were prepared with anthocyanin rich extracts from different fruits in the presence of ferric ion, to investigate the color and stability of anthocyanin iron chelate complexes in the model systems, and the model system containing anthocyanin iron chelate complex and added pectin had enhanced stability of chelate complex (Buchweitz, Brauch, Carle, & Kammerer, 2013).

Pectin and Anthocyanin Stability

Model food systems with pectin addition have enhanced anthocyanin pigment retention during storage. For example, model strawberry jams prepared with added pectin had enhanced anthocyanin pigment retention during storage (Kopjar et al., 2009), and model breads with

blackcurrant polyphenol extracts and pectin addition had enhanced polyphenol retention, including anthocyanin after baking (Sivam, Sun-Waterhouse, Perera, & Waterhouse, 2012). In one study, strawberry jams and spread were prepared with added pectins to evaluate anthocyanin pigment stability and degradation half-life during storage (Holzwarth et al., 2013). It was reported that model systems with lower water activity resulted in higher anthocyanin pigment stability, and LM pectin contributed to higher anthocyanin retention and longer pigment half-life comparing to HM pectin. In another study, amidated, low methoxyl and high methoxyl citrus and apple pectin and sugar beet pectin were added in black current extract (Buchweitz, Speth, et al., 2013a). After 6 weeks of storage, total anthocyanin content in black current extract with no pectin added was reduced to 37.2%, while total anthocyanin content in extracts with pectin added contained 65.9-75.5% of the initial anthocyanin pigments. Best stabilization was obtained with amidated pectin, followed by low methoxyl and high methoxyl pectin. Citrus pectin stabilized anthocyanin better than apple pectin with similar degree of methylesterification and amidation, and sugar beet pectin enhanced anthocyanin pigment stability moderately. After 18 weeks of storage, amidated, low methoxyl and high methoxyl pectin contributed to 24.2%, 18.1% and 14.2% as well as 30.0%, 22.3% and 17.8% pigment retention for apple and citrus pectin respectively, and sugar beet pectin contributed to about 16% pigment retention. In another study conducted by the same group of researchers, the same various types of pectin were added to strawberry anthocyanin extract (Buchweitz, Speth, Kammerer, & Carle, 2013b). Pigment retention differences were ascribed to pectin sources, with apple pectin and sugar beet pectin enhanced the anthocyanin stability moderately, while citrus pectin had the least anthocyanin stabilizing effect. After 18 weeks of storage, anthocyanin pigment in control strawberry

anthocyanin extract decreased to about 10% initial pigment content. Apple pectin and sugar beet pectin contributed to 16.3-20.2% pigment retention, while citrus pectin contributed to 10.3-14.6%. The variation in the degree of methylesterification and amidation had negligible effects on pigment stability in strawberry extract. It is suggested pectin enhances anthocyanin pigment stability by immobilizing water (Mazzaracchio et al., 2004). In accordance with the assumption, higher sugar concentration enhances anthocyanin stability (Hubbermann, Heins, Stöckmann, & Schwarz, 2006; Stintzing & Carle, 2004), and anthocyanin microencapsulates with higher water binding capacity resulted in higher anthocyanin retention after incubation in gastric fluids (Berg, Bretz, Hubbermann, & Schwarz, 2012).

Pectin and Anthocyanin in vivo Bioavailability

Anthocyanins have potential health promoting benefits, but the bioavailablity in vivo is low (Fleschhut et al., 2006). For example, the urinary anthocyanin excretion of elderberry extract and blueberry extracts consumed by elderly women was 0.077% and 0.004% respectively (Wu, Cao, & Prior, 2002). The structure and stability of anthocyanin are pH sensitive and anthocyanin is unstable under pH higher than 7 (Castañeda-Ovando et al., 2009), which is the intestinal pH. Therefore, some encapsulation systems have been investigated to protect anthocyanins. For example, black carrot extracts were sprayed dried with maltodextrin (Ersus & Yurdagel, 2007), black currant anthocyanins were encapsulated in glucan gel (Xiong, Melton, Easteal, & Siew, 2006), and blackberry anthocyanin extracts were encapsulated with curdlan, pectin and sodium alginate (Ferreira, Faria, Grosso, & Mercadante, 2009). A few studies evaluated the anthocyanin stability in simulated gastric and intestinal environments protected by gastric intestinal system.

Three encapsulation systems, amide pectin, whey protein isolate and shellac coated maltodextrin were evaluated for anthocyanin release in simulated gastric and intestinal fluids for blackberry extract (Oidtmann et al., 2011). All encapsulation systems retarded early anthocyanin release in gastric fluid and enhanced available anthocyanin pigment by more than 20% in intestinal fluid; however, the encapsulation systems investigated failed to fully prevent anthocyanin release in gastric fluid (Oidtmann et al., 2011). The ideal anthocyanin encapsulation system would fully prevent early release of anthocyanins in the stomach, so that early anthocyanin degradation before reaching small intestine can be prevented. A few of studies have investigated the application of pectin on improving anthocyanin *in vivo* bioavailability. A recent study evaluated anthocyanin encapsulation using shellac coated maltodextrin with 2% (w/w) pectin added as core material, and the encapsulates were subjected to incubation in gastric fluid (Berg et al., 2012). The study reported encapsulates with added sugar beet pectin had lowest gastric anthocyanin release, with more than 50% total anthocyanin retention. Encapsulates with added HM pectin had lower anthocyanin release than with LM pectin, and encapsulates with added citrus pectin had lower anthocyanin release than with apple pectin. In another study, plant cell wall composites composed of cellulose or cellulose/pectin, and plant cell wall prepared from black carrot were submerged in purple carrot juice to allow anthocyanin binding, and the cell wall models with bound anthocyanin were subjected to acidified methanol extraction, and incubation in gastric and in intestinal fluids (Padayachee et al., 2013). The authors reported tight anthocyanin binding with the cell wall models. About 20% total anthocyanin were extracted using acidified methanol, and very minimal anthocyanin release after gastric intestinal incubation (Padayachee et al., 2013). The tight binding between anthocyanin and cell wall models affected

anthocyanin bioaccessibility and therefore, bioavailability for small intestinal absorption, but may not affect the bioaccessibility once reaching the colon. In fact, dietary fibers are beneficial to colon health (Marlett, McBurney, & Slavin, 2002), and pectin has potential application for colon target drug and phytochemical delivery (Jung et al., 2013; Wicker et al., 2014). Both pectin and anthocyanin can be metabolized by gut microflora and have potential prebiotic effect on promoting gut health (Hidalgo et al., 2012; Molan et al., 2009; Wicker et al., 2014; Wong, Colombo, & Sonvico, 2011). Studies that evaluate pectin and anthocyanin are scarce, and no study has evaluated the binding between pectin and anthocyanin. For procyanidins , pectin has significantly higher affinity than xyloglucan, starch and cellulose (Le Bourvellec, Bouchet, & Renard, 2005).

Hypothetical Binding Mechanisms

The mechanism for pectin and anthocyanin binding is not well understood and no study has investigated the binding mechanism. It is hypothesized and is assumed in most studies that pectin binds with anthocyanin through ionic interaction between pectic free carboxyl groups and anthocyanin flavylium cations (Asen et al., 1970; Buchweitz, Carle, et al., 2012; Holzwarth et al., 2013; Padayachee et al., 2012). It is also suggested that hydrophobic interactions between methoxyl groups of the anthocyanin B-ring and pectin methoxyl groups may contribute to the binding (Mazzaracchio et al., 2004). Studies that evaluated both LM and HM pectins had discrepancies on the effect of LM and HM pectin addition on anthocyanin pigment stability (Buchweitz, Speth, et al., 2013a, 2013b; Holzwarth et al., 2013; Kopjar et al., 2009). Anthocyanin pigments can have intermolecular association by stacking of aromatic nucleis, and

the stacked structures are protected by hydrogen bonds formed by superimposed sugar moieties (Goto & Kondo, 1991). It is suggested recently that the pectin and anthocyanin binding has two phases, which start with initial binding between pectin and anthocyanin, followed by anthocyanin stacking (Padayachee et al., 2012). In the study conducted by this group, composites composed of cellulose or cellulose/pectin submerged in purple carrot juice contributed to 13-18% anthocyanin binding within the first few hours, and prolonged exposure to resulted in up to 80% anthocyanin binding (Padayachee et al., 2012). Confocal microscopy revealed the local high concentrations of anthocyanins within the cell wall composites along with regions with no bound anthocyanin (Padayachee et al., 2013).

Summary

Both pectin and anthocyanin have health promoting benefits, and it seems like pectin is able to bind with anthocyanin. Blueberry contains a significant amount of pectin and is a rich source of anthocyanins. Preliminary studies showed blue stained AIS prepared from blueberry and blue stained blueberry pectin. The blue colors were not removable after alcohol and acetone washes, nor by extensive dialysis against water. Pectin in blueberry may have unique anthocyanin binding properties, and the bound pectin-anthocyanin mixture may have synergistic effect on promoting human health, especially colon health. In the next chapters, blueberry cell wall was sequentially fractionated and pectin rich fractions were identified and characterized. Intermolecular binding between pectin rich fractions and anthocyanin was studied. The components and polymer characteristics of the pectin rich fractions, and hypothetical pectin anthocyanin binding mechanisms are discussed. For the first time, blueberry pectin is studied for

the functional properties, and pectin and anthocyanin binding is examined under well designed conditions.

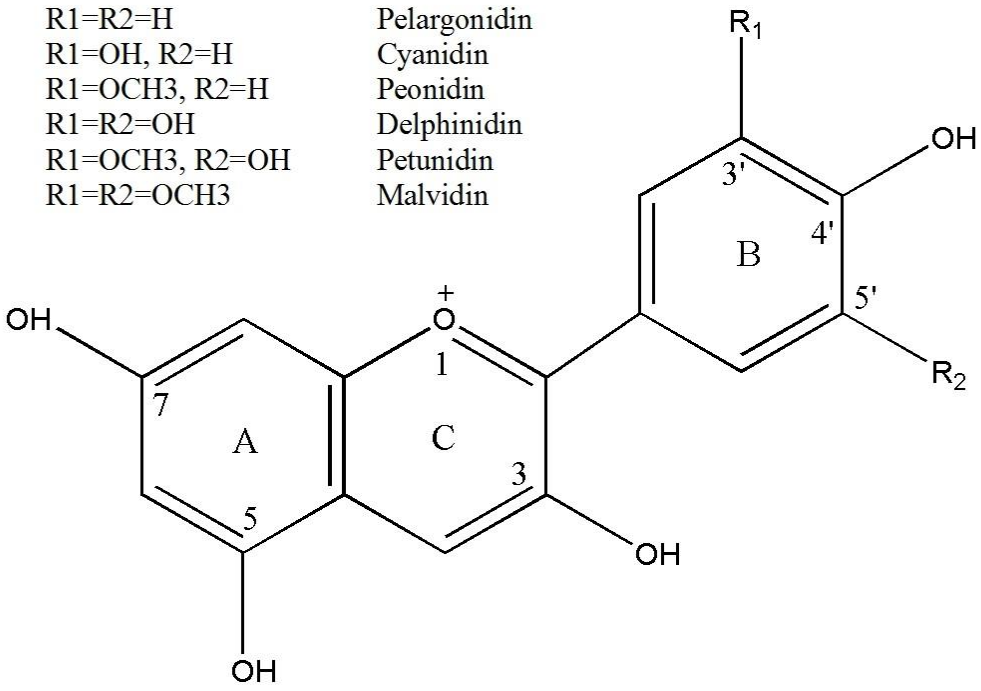

Figure 1.1. Anthocyanin aglycone structure.

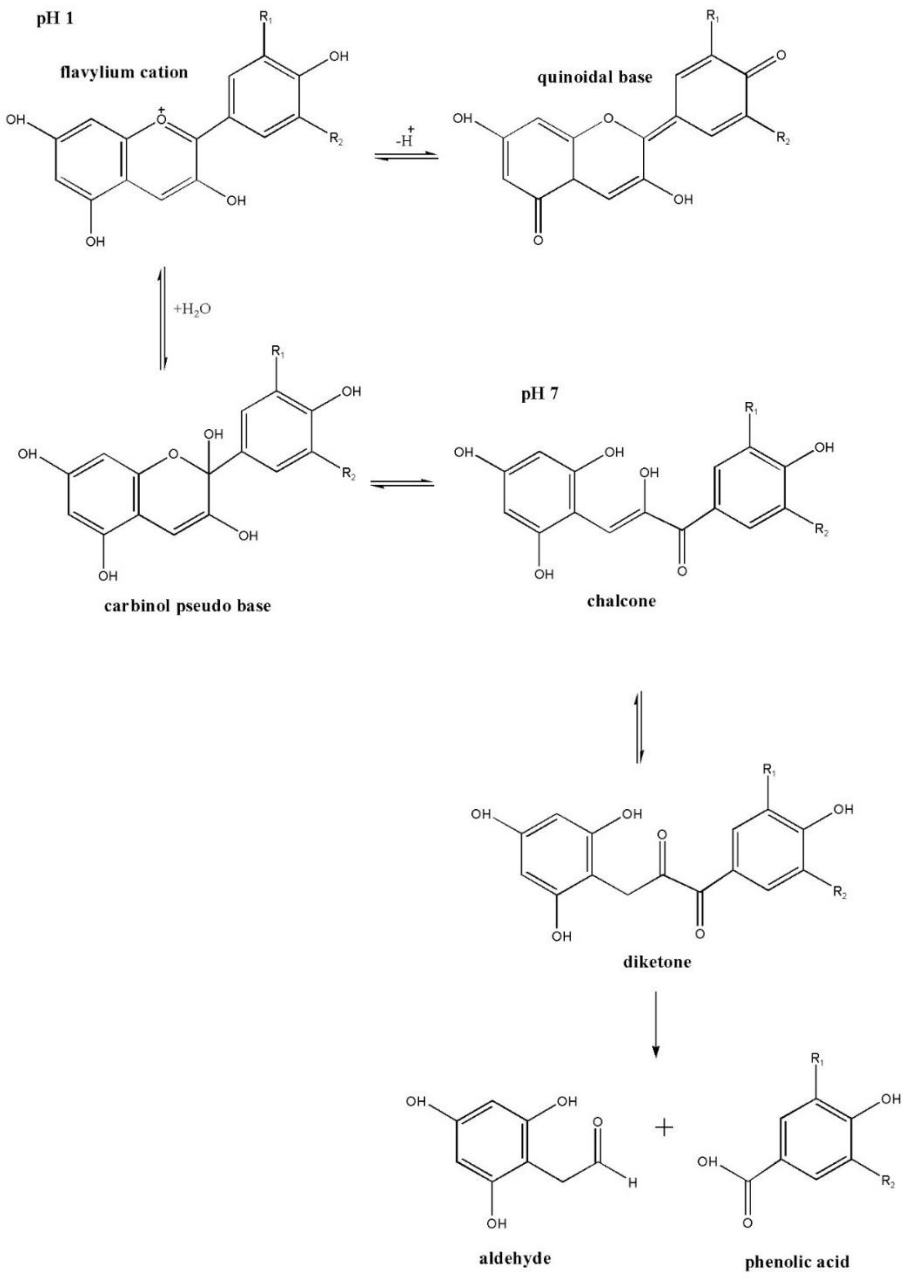

Figure 1.2. Anthocyanin transformation under different pH and chemical degradation.

References

Asen, S., Stewart, R. N., Norris, K. H., & Massie, D. R. (1970). A stable blue non-metallic co-pigment complex of delphanin and C-glycosylflavones in Prof. Blaauw Iris. *Phytochemistry*, *9*(3), 619-627. doi: http://dx.doi.org/10.1016/S0031-9422(00)85702-7

Bakker, J., Bridle, P., Honda, T., Kuwano, H., Saito, N., Terahara, N., & Timberlake, C. F. (1997). Identification of an anthocyanin occurring in some red wines. *Phytochemistry*, *44*(7), 1375-1382. doi: http://dx.doi.org/10.1016/S0031-9422(96)00707-8

Bayer, E., Egeter, H., Fink, A., Nether, K., & Wegmann, K. (1966). Complex Formation and Flower Colors. *Angewandte Chemie International Edition in English*, *5*(9), 791-798. doi: 10.1002/anie.196607911

Berg, S., Bretz, M., Hubbermann, E. M., & Schwarz, K. (2012). Influence of different pectins on powder characteristics of microencapsulated anthocyanins and their impact on drug retention of shellac coated granulate. *Journal of Food Engineering*, *108*(1), 158-165. doi: http://dx.doi.org/10.1016/j.jfoodeng.2011.06.035

Boulton, R. (2001). The Copigmentation of Anthocyanins and Its Role in the Color of Red Wine: A Critical Review. *American Journal of Enology and Viticulture*, *52*(2), 67-87.

Buchweitz, M., Brauch, J., Carle, R., & Kammerer, D. R. (2013). Application of ferric anthocyanin chelates as natural blue food colorants in polysaccharide and gelatin based gels. *Food Research International*, *51*(1), 274-282. doi: http://dx.doi.org/10.1016/j.foodres.2012.11.030

Buchweitz, M., Carle, R., & Kammerer, D. R. (2012). Bathochromic and stabilising effects of sugar beet pectin and an isolated pectic fraction on anthocyanins exhibiting pyrogallol and catechol moieties. *Food Chemistry, 135*(4), 3010-3019. doi: http://dx.doi.org/10.1016/j.foodchem.2012.06.101

Buchweitz, M., Nagel, A., Carle, R., & Kammerer, D. R. (2012). Characterisation of sugar beet pectin fractions providing enhanced stability of anthocyanin-based natural blue food colourants. *Food Chemistry, 132*(4), 1971-1979. doi: 10.1016/j.foodchem.2011.12.034

Buchweitz, M., Speth, M., Kammerer, D. R., & Carle, R. (2013a). Impact of pectin type on the storage stability of black currant (Ribes nigrum L.) anthocyanins in pectic model solutions. *Food Chemistry, 139*(1–4), 1168-1178. doi: http://dx.doi.org/10.1016/j.foodchem.2013.02.005

Buchweitz, M., Speth, M., Kammerer, D. R., & Carle, R. (2013b). Stabilisation of strawberry (Fragaria x ananassa Duch.) anthocyanins by different pectins. *Food Chemistry, 141*(3), 2998-3006. doi: http://dx.doi.org/10.1016/j.foodchem.2013.04.117

Castañeda-Ovando, A., Pacheco-Hernández, M. d. L., Páez-Hernández, M. E., Rodríguez, J. A., & Galán-Vidal, C. A. (2009). Chemical studies of anthocyanins: A review. *Food Chemistry, 113*(4), 859-871. doi: http://dx.doi.org/10.1016/j.foodchem.2008.09.001

Chen, H.-C., & Camire, M. E. (1997). RECOVERY OF ANTHOCYANINS, PECTIN, AND DIETARY FIBER FROM CULL LOWBUSH BLUEBERRIES. *Journal of Food Quality, 20*(3), 199-209. doi: 10.1111/j.1745-4557.1997.tb00464.x

Dao, L. T., Takeoka, G. R., Edwards, R. H., & Berrios, J. D. J. (1998). Improved Method for the Stabilization of Anthocyanidins. *Journal of Agricultural and Food Chemistry, 46*(9), 3564-3569. doi: 10.1021/jf980359v

Davies, A. J., & Mazza, G. (1993). Copigmentation of simple and acylated anthocyanins with colorless phenolic compounds. *Journal of Agricultural and Food Chemistry, 41*(5), 716-720. doi: 10.1021/jf00029a007

Durst, R. W., & Wrolstad, R. E. (2001). Separation and Characterization of Anthocyanins by HPLC *Current Protocols in Food Analytical Chemistry*: John Wiley & Sons, Inc.

Eaks, I. L., & Sinclair, W. B. (1980). CELLULOSE-HEMICELLULOSE FRACTIONS IN THE ALCOHOL-INSOLUBLE SOLIDS OF VALENCIA ORANGE PEEL. *Journal of Food Science, 45*(4), 985-988. doi: 10.1111/j.1365-2621.1980.tb07493.x

Ersus, S., & Yurdagel, U. (2007). Microencapsulation of anthocyanin pigments of black carrot (Daucus carota L.) by spray drier. *Journal of Food Engineering, 80*(3), 805-812.

Ferreira, D. S., Faria, A. F., Grosso, C. R. F., & Mercadante, A. Z. (2009). Encapsulation of blackberry anthocyanins by thermal gelation of curdlan. *Journal of the Brazilian Chemical Society, 20*(10), 1908-1915.

Fleschhut, J., Kratzer, F., Rechkemmer, G., & Kulling, S. (2006). Stability and biotransformation of various dietary anthocyanins in vitro. *European Journal of Nutrition, 45*(1), 7-18. doi: 10.1007/s00394-005-0557-8

Fügel, R., Schieber, A., & Carle, R. (2006). Determination of the fruit content of cherry fruit preparations by gravimetric quantification of hemicellulose. *Food Chemistry, 95*(1), 163-168. doi: http://dx.doi.org/10.1016/j.foodchem.2005.02.011

Funami, T., Nakauma, M., Ishihara, S., Tanaka, R., Inoue, T., & Phillips, G. O. (2011). Structural modifications of sugar beet pectin and the relationship of structure to functionality. *Food Hydrocolloids*, *25*(2), 221-229. doi: 10.1016/j.foodhyd.2009.11.017

Giusti, M. M., & Wrolstad, R. E. (2001). Characterization and Measurement of Anthocyanins by UV-Visible Spectroscopy *Current Protocols in Food Analytical Chemistry*: John Wiley & Sons, Inc.

Goto, T., & Kondo, T. (1991). Structure and Molecular Stacking of Anthocyanins—Flower Color Variation. *Angewandte Chemie International Edition in English*, *30*(1), 17-33. doi: 10.1002/anie.199100171

Hidalgo, M., Oruna-Concha, M. J., Kolida, S., Walton, G. E., Kallithraka, S., Spencer, J. P., & de Pascual-Teresa, S. (2012). Metabolism of anthocyanins by human gut microflora and their influence on gut bacterial growth. *J Agric Food Chem*, *60*(15), 3882-3890. doi: 10.1021/jf3002153

Holzwarth, M., Korhummel, S., Carle, R., & Kammerer, D. (2012). Impact of enzymatic mash maceration and storage on anthocyanin and color retention of pasteurized strawberry purées. *European Food Research & Technology*, *234*(2), 207-222. doi: 10.1007/s00217-011-1601-y

Holzwarth, M., Korhummel, S., Siekmann, T., Carle, R., & Kammerer, D. R. (2013). Influence of different pectins, process and storage conditions on anthocyanin and colour retention in strawberry jams and spreads. *LWT - Food Science and Technology*, *52*(2), 131-138. doi: http://dx.doi.org/10.1016/j.lwt.2012.05.020

Hord, N. G. (2008). Eukaryotic-microbiota crosstalk: potential mechanisms for health benefits of prebiotics and probiotics. *Annu Rev Nutr, 28*, 215-231. doi: 10.1146/annurev.nutr.28.061807.155402

Hubbermann, E., Heins, A., Stöckmann, H., & Schwarz, K. (2006). Influence of acids, salt, sugars and hydrocolloids on the colour stability of anthocyanin rich black currant and elderberry concentrates. *European Food Research and Technology*, *223*(1), 83-90. doi: 10.1007/s00217-005-0139-2

Jung, J., Arnold, R. D., & Wicker, L. (2013). Pectin and charge modified pectin hydrogel beads as a colon-targeted drug delivery carrier. *Colloids Surf B Biointerfaces, 104*, 116-121. doi: 10.1016/j.colsurfb.2012.11.042

Kahle, K., Kraus, M., Scheppach, W., Ackermann, M., Ridder, F., & Richling, E. (2006). Studies on apple and blueberry fruit constituents: do the polyphenols reach the colon after ingestion? *Mol Nutr Food Res, 50*(4-5), 418-423. doi: 10.1002/mnfr.200500211

Keppler, K., & Humpf, H.-U. (2005). Metabolism of anthocyanins and their phenolic degradation products by the intestinal microflora. *Bioorganic & Medicinal Chemistry, 13*(17), 5195-5205. doi: 10.1016/j.bmc.2005.05.003

Kong, J.-M., Chia, L.-S., Goh, N.-K., Chia, T.-F., & Brouillard, R. (2003). Analysis and biological activities of anthocyanins. *Phytochemistry, 64*(5), 923-933. doi: http://dx.doi.org/10.1016/S0031-9422(03)00438-2

Kopjar, M., Pilizota, V., Tiban, N. N., Subaric, D., Babic, J., Ackar, E., & Sajdl, M. (2009). Strawberry jams: influence of different pectins on colour and textural properties. *CZECH JOURNAL OF FOOD SCIENCES, 27*, 20-28.

Le Bourvellec, C., Bouchet, B., & Renard, C. M. G. C. (2005). Non-covalent interaction between procyanidins and apple cell wall material. Part III: Study on model polysaccharides. *Biochimica et Biophysica Acta (BBA) - General Subjects, 1725*(1), 10-18. doi: http://dx.doi.org/10.1016/j.bbagen.2005.06.004

Leijdekkers, A. G., Aguirre, M., Venema, K., Bosch, G., Gruppen, H., & Schols, H. A. (2014). In vitro fermentability of sugar beet pulp derived oligosaccharides using human and pig fecal inocula. *J Agric Food Chem, 62*(5), 1079-1087. doi: 10.1021/jf4049676

Lewis, C. E., Walker, J. R. L., & Lancaster, J. E. (1995). Effect of polysaccharides on the colour of anthocyanins. *Food Chemistry, 54*(3), 315-319. doi: 10.1016/0308-8146(95)00026-F

Lila, M. A. (2004). Anthocyanins and human health: an in vitro investigative approach. *Journal of Biomedicine & Biotechnology, 2004*(5), 306-313.

Marlett, J. A., McBurney, M. I., & Slavin, J. L. (2002). Position of the American Dietetic Association: Health Implications of Dietary Fiber. *Journal of the American Dietetic Association, 102*(7), 993-1000.

Maxwell, E. G., Belshaw, N. J., Waldron, K. W., & Morris, V. J. (2012). Pectin – An emerging new bioactive food polysaccharide. *Trends in Food Science & Technology, 24*(2), 64-73. doi: 10.1016/j.tifs.2011.11.002

Mazza, G. (2007). Bioactivity, absorption and metabolism of anthocyanins. *Acta Horticulturae*(744), 117-125.

Mazza, G., & Brouillard, R. (1990). The mechanism of co-pigmentation of anthocyanins in aqueous solutions. *Phytochemistry, 29*(4), 1097-1102. doi: 10.1016/0031-9422(90)85411-8

Mazzaracchio, P., Pifferi, P., Kindt, M., Munyaneza, A., & Barbiroli, G. (2004). Interactions between anthocyanins and organic food molecules in model systems. *International Journal of Food Science & Technology, 39*(1), 53-59. doi: 10.1111/j.1365-2621.2004.00747.x

McCann, D., Barrett, A., Cooper, A., Crumpler, D., Dalen, L., Grimshaw, K., . . . Stevenson, J. (2007). Food additives and hyperactive behaviour in 3-year-old and 8/9-year-old children in the community: a randomised, double-blinded, placebo-controlled trial. *The Lancet, 370*(9598), 1560-1567.

Molan, A. L., Lila, M. A., Mawson, J., & De, S. (2009). In vitro and in vivo evaluation of the prebiotic activity of water-soluble blueberry extracts. *World Journal of Microbiology and Biotechnology, 25*(7), 1243-1249. doi: 10.1007/s11274-009-0011-9

Moore, M. A., Park, C. B., & Tsuda, H. (1998). Soluble and insoluble fiber influences on cancer development. *Critical Reviews in Oncology/Hematology, 27*(3), 229-242. doi: http://dx.doi.org/10.1016/S1040-8428(98)00006-7

Morell, S., Baur, L., & Link, K. P. (1934). THE METHYLGLYCOSIDES OF THE NATURALLY OCCURRING HEXURONIC ACIDS: III. POLYGALACTURONIC ACID-METHYLGLYCOSIDES DERIVED FROM PECTIN. *Journal of Biological Chemistry, 105*(1), 1-13.

Munarin, F., Tanzi, M. C., & Petrini, P. (2012). Advances in biomedical applications of pectin gels. *International Journal of Biological Macromolecules, 51*(4), 681-689. doi: http://dx.doi.org/10.1016/j.ijbiomac.2012.07.002

Oidtmann, J., Schantz, M., Mäder, K., Baum, M., Berg, S., Betz, M., . . . Richling, E. (2011). Preparation and Comparative Release Characteristics of Three Anthocyanin Encapsulation Systems. *Journal of Agricultural and Food Chemistry, 60*(3), 844-851. doi: 10.1021/jf2047515

Padayachee, A., Netzel, G., Netzel, M., Day, L., Mikkelsen, D., & Gidley, M. (2013). Lack of release of bound anthocyanins and phenolic acids from carrot plant cell walls and model composites during simulated gastric and small intestinal digestion. *Food Funct, 4*(6), 906-916. doi: 10.1039/C3FO60091B

Padayachee, A., Netzel, G., Netzel, M., Day, L., Zabaras, D., Mikkelsen, D., & Gidley, M. J. (2012). Binding of polyphenols to plant cell wall analogues – Part 1: Anthocyanins. *Food Chemistry, 134*(1), 155-161. doi: http://dx.doi.org/10.1016/j.foodchem.2012.02.082

Proctor, A., & Peng, L. C. (1989). Pectin Transitions During Blueberry Fruit Development and Ripening. *Journal of Food Science, 54*(2), 385-387. doi: 10.1111/j.1365-2621.1989.tb03088.x

Scheller, H. V., Jensen, J. K., Sørensen, S. O., Harholt, J., & Geshi, N. (2007). Biosynthesis of pectin. *Physiologia Plantarum, 129*(2), 283-295. doi: 10.1111/j.1399-3054.2006.00834.x

Sivam, A. S., Sun-Waterhouse, D., Perera, C. O., & Waterhouse, G. I. N. (2012). *Exploring the interactions between blackcurrant polyphenols, pectin and wheat biopolymers in model breads; a FTIR and HPLC investigation*.

Stephen, A. M., & Phillips, G. O. (2010). *Food polysaccharides and their applications*: CRC Press.

Stintzing, F. C., & Carle, R. (2004). Functional properties of anthocyanins and betalains in plants, food, and in human nutrition. *Trends in Food Science & Technology, 15*(1), 19-38. doi: http://dx.doi.org/10.1016/j.tifs.2003.07.004

Vicente, A. R., Greve, L. C., Labavitch, J. M., Powell, A. L. T., Ortugno, C., & Rosli, H. (2007). Temporal Sequence of Cell Wall Disassembly Events in Developing Fruits. 2. Analysis of Blueberry (Vaccinium Species) [electronic resource]. *Journal of Agricultural and Food Chemistry, 55*(10), 4125-4130. doi: http://dx.doi.org/10.1021/jf063548j

Voragen, A. G., Coenen, G. J., Verhoef, R. P., & Schols, H. A. (2009). Pectin, a versatile polysaccharide present in plant cell walls. *Structural Chemistry, 20*(2), 263-275.

Wallace, T. C. (2011). Anthocyanins in Cardiovascular Disease. *Advances in Nutrition: An International Review Journal, 2*(1), 1-7. doi: 10.3945/an.110.000042

Wang, L. S., & Stoner, G. D. (2008). Anthocyanins and their role in cancer prevention. *Cancer Lett, 269*(2), 281-290. doi: 10.1016/j.canlet.2008.05.020

Wicker, L., Kim, Y., Kim, M.-J., Thirkield, B., Lin, Z., & Jung, J. (2014). Pectin as a bioactive polysaccharide – extracting tailored function from less. *Food Hydrocolloids*(0). doi: http://dx.doi.org/10.1016/j.foodhyd.2014.01.002

Williams, P. A., Sayers, C., Viebke, C., Senan, C., Mazoyer, J., & Boulenguer, P. (2005). Elucidation of the Emulsification Properties of Sugar Beet Pectin. *Journal of Agricultural and Food Chemistry, 53*(9), 3592-3597. doi: 10.1021/jf0404142

Wong, T. W., Colombo, G., & Sonvico, F. (2011). Pectin matrix as oral drug delivery vehicle for colon cancer treatment. *AAPS Pharmscitech, 12*(1), 201-214. doi: 10.1208/s12249-010-9564-z

Wong, T. W., Colombo, G., & Sonvico, F. (2011). Pectin matrix as oral drug delivery vehicle for colon cancer treatment. *AAPS PharmSciTech, 12*(1), 201-214. doi: 10.1208/s12249-010-9564-z

Wrolstad, R. E., Durst, R. W., & Lee, J. (2005). Tracking color and pigment changes in anthocyanin products. *Trends in Food Science & Technology, 16*(9), 423-428. doi: 10.1016/j.tifs.2005.03.019

Wu, X. L., Cao, G. H., & Prior, R. L. (2002). Absorption and metabolism of anthocyanins in elderly women after consumption of elderberry or blueberry. *Journal of nutrition, 132*(7), 1865-1871.

Xiong, S., Melton, L. D., Easteal, A. J., & Siew, D. (2006). Stability and Antioxidant Activity of Black Currant Anthocyanins in Solution and Encapsulated in Glucan Gel. *Journal of Agricultural and Food Chemistry, 54*(17), 6201-6208. doi: 10.1021/jf060889o

Yapo, B. M. (2011). Pectic substances: From simple pectic polysaccharides to complex pectins—A new hypothetical model. *Carbohydrate Polymers, 86*(2), 373-385. doi: 10.1016/j.carbpol.2011.05.065

Yi, W., Akoh, C. C., Fischer, J., & Krewer, G. (2006). Effects of phenolic compounds in blueberries and muscadine grapes on HepG2 cell viability and apoptosis. *Food Research International, 39*(5), 628-638. doi: http://dx.doi.org/10.1016/j.foodres.2006.01.001

Zykwinska, A. W., Ralet, M.-C. J., Garnier, C. D., & Thibault, J.-F. J. (2005). Evidence for In Vitro Binding of Pectin Side Chains to Cellulose. *Plant Physiology, 139*(1), 397-407. doi: 10.1104/pp.105.065912

CHAPTER 2

BLUEBERRY CELL WALL FRACTIONATION, CHARACTERIZATION AND GLYCOME PROFILING[1]

[1]Lin, Z., Pattathil, S., Hahn, M., Wicker, L., To be submitted to Carbohydrate Polymers.

Abstract

Blueberry powder was precipitated in alcohol and sequentially extracted using increasing strong solvents to generate five cell wall fractions. Water soluble fraction (WSF), chelator soluble fraction (CSF) and sodium carbonate soluble fraction (NSF) were pectin rich fractions with high uronic acid content above 22 μg/mg AIS, while 4% potassium hydroxide soluble fraction (4KSF) and 24% potassium hydroxide soluble fraction (24KSF) were hemicellulose rich fractions. Monoclonal antibody (mAbs) assay glycome profiling revealed that pectin rich fractions had pectin homogalacturonan (HG) backbone, arabinogalactan (AG), and xyloglucan (XG) epitopes, but had no xylan epitopes. 4KSF and 24KSF had rich profile of xyloglucan and xylan epitopes. Arabinose and galactose were the two major neutral sugars in the pectin rich fractions, and xylose was the predominant neutral sugar in hemicellulose rich fractions, more than 70 mol%. WSF*, CSF* and NSF* from the second extraction had uronic acid content of 500-600 μg/mg and the degree of methylesterification was between 26-39% analyzed by FTIR. HPSEC-MALS-RI chromatography revealed both WSF* and CSF* had polydispersed and high molecular weight of around 450 kDa in the major eluting peaks, while NSF* had lower molecular weight of 177 kDa.

Keywords: Blueberry, Pectin, Hemicellulose, Glycome Profiling, HPSEC-MALS-RI.

Introduction

Pectin presents in the middle lamella, primary and secondary cell walls (Voragen, Coenen, Verhoef, & Schols, 2009) and links to and surrounds the cellulose together with hemicellulose to constructure the structure of plant cell wall (Foster, 2011). Pectin is a versatile heterogeneous polysaccharide extracted from plant cell walls, and the application of pectin is not limited to making jams and jellies (Voragen et al., 2009). Pectin can be used to emulsify and stabilize beverages, forming hydrogels for colon targeted delivery, reduce low density lipoprotein cheloesterol as a one type of dietary fiber and influence gut microflora metabolism (Wicker et al., 2014). Low methoxyl and charge modified high methoxyl pectin form hydrogel beads as a colon targeted delivery vehicle (Jung, Arnold, & Wicker, 2013). Pectin has dual advantages for colon targeted delivery because it undergoes minimal degradation in the gastrointestinal tract and is fermentable by gut microflora (Wong, Colombo, & Sonvico, 2011). Sugar beet pectin derived oligosaccharides have potential prebiotic effect and modifies colonic microflora (Leijdekkers et al., 2014). Commercially, pectin is extracted from citrus peels, apple pomaces, or sugar beet pulps using acid hydrolysis followed by alcohol precipitation, and pectin can be extracted from various sources such as carrot, plum, potato, mango, sunflower, grape, peach and etc. using hot water, oxalate chelator, weak alkali, etc. (Stephen & Phillips, 2010). Blueberry contains a significant amount of pectin (Chen & Camire, 1997). Alcohol insoluble solids (AIS) prepared from blueberry contained between 30-40% galacturonic acid content (Proctor & Peng, 1989; Vicente et al., 2007). Previous studies on blueberry pectin focused on pectin transition during fruit development (Proctor & Peng, 1989) and during storage (Chen & Camire, 1997), and fruit

softening associated pectin depolymerization (Vicente et al., 2007). Blueberry pectin is not well characterized in terms of uronic acid content, molecular weight and pectin structure, nor has any functional property been studied.

Blueberry is a rich source of anthocyanins (Chen & Camire, 1997; Yi, Fischer, Krewer, & Akoh, 2005), a class of phenolic compounds that showed strong antioxidant, anti-inflammatory, antimicrobial, and anti-carcinogenic activities *in vitro* studies (Mazza, 2007). In human and animal models less than 1% of the intact consumed anthocyanidin glycosides is absorbed and excreted in urine (Keppler & Humpf, 2005), and large amounts anthocyanins *in vivo* reach the colon (Kahle et al., 2006). Anthocyanin has prebiotic effect on colonic microflora and promotes the growth of probiotic colon bacteria (Hidalgo et al., 2012). Our preliminary data showed that water soluble pectin extracted from blueberry had non-dialyzable purple color, which was most likely contributed by anthocyanin pigments. Recent studies suggested interactions between pectin and anthocyanins. Pectin stabilizes anthocyanin metal chelate complex and prevents complex precipitation in aqueous solution (Buchweitz, Brauch, Carle, & Kammerer, 2013; Buchweitz, Carle, & Kammerer, 2012; Buchweitz, Nagel, Carle, & Kammerer, 2012). Pectin containing cell wall composites bind anthocyanin pigments (Padayachee et al., 2012), and have low *in vitro* pigment release in gastric and intestinal fluids (Padayachee et al., 2013). Blueberry pectin may have potential anthocyanin binding properties, and blueberry pectin with bound anthocyanins may have dual prebiotic effect on promoting gut health.

The purpose of this study was to identify and characterize pectin rich fractions from blueberry AIS for future blueberry pectin functional property studies on anthocyanin binding.

Blueberry cell wall extracts were subjected to monoclonal antibody (mAbs) assay glycome profiling for identification of major pectic and hemicellulosic epitopes. Polymer characteristics including molecular weight and estimated polymer shape of pectin rich fractions were analyzed by HPSEC-MALS-RI and will be discussed.

Materials and Methods

Materials and Reagents

Freeze-dried blueberry powder (Tiflbue/Rubel 50/50 blend Lot #30711) was obtained from U.S. High Bush Blueberry Council (Folsom, CA). Sodium borohydride, sulfuric acid, meta-hydroxydiphenyl, trifluoroacetic acid (TFA), dimethyl sulphoxide (DMSO), 1-methylimidazole, dichloromethane, myo-inositol, D(+)-xylose, D-glucuronic acid, D(+)-mannose, D(+)-galactose, L-rhamnose, α-L(-)-fucose, and α-arabinose were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Sodium tetraborate decahydrate, sodium azide were purchased from Fisher Scientific (Fair Lawn, NJ). Sulfamic acid and trans-1,2-diaminocyclohexane- N,N,N',N'-tetraacetic acid monohydrate (CDTA) were purchased from Acros Organics (Waltham, MA). Sodium phosphate dibasic, sodium phosphate monobasic, sodium nitrate, sodium acetate, potassium hydroxide, sodium borohydride, glacial acetic acid, ammonium hydroxide, and phenol were purchased from J.T. Baker (Phillipsburg, NJ). Material for glycome profiling can be found on Pattathil et al. (2010).

Preparative Extraction for Glycome Profiling

AIS Precipitation and Sequential Fractionation

Freeze-dried blueberry powder was suspended in 10 volumes of 95% (v/v) ethanol, and the suspension was mixed in 70^{o}C water bath for 1 hour. The alcohol insoluble solids (AIS) was washed with 4 volumes of ethanol and 1 volume of acetone, and was dried overnight under fume hood.

An aliquot of 300 mg of AIS was suspended in 30 mL 50 mM sodium acetate (pH 5.2) with 0.02% sodium azide extraction buffer and mixed at room temperature for 24 hours. The suspension was centrifuged and filtered. The supernatant was designated as water soluble fraction (WSF) supernatant and was stored in 4^{o}C cold room. The pellets were washed with MiliQ water (Millipore, Billerica, MA) and suspended in 30 mL 50 mM CDTA, 50 mM sodium acetate (pH 5.2) with 0.02% sodium azide extraction buffer and mixed at room temperature for 24 hours. Suspension was centrifuged and filtered as above. The supernatant was designated as chelator soluble fraction (CSF) and stored in cold room. The pellets were washed as above and were suspended in 30 mL 50 mM sodium carbonate, 20 mM sodium borohydride extraction buffer and treated as above. The supernatant was designated as sodium carbonate soluble fraction (NSF) and stored as above. The washed pellets were suspended in 30 mL 4% (w/v) potassium hydroxide, 0.1% (w/v) sodium borohydride extraction buffer and treated as above. The supernatant was designated as 4% potassium hydroxide soluble fraction (4KSF) and stored as above. The washed pellets were suspended in 30 mL 24% (w/v) potassium hydroxide, 1% (w/v) sodium borohydride extraction buffer and treated as above. The supernatant was designated as

24% potassium hydroxide soluble fraction (24KSF). NSF, 4KSF, and 24KSF supernatants were adjusted to neutral pH with glacial acetic acid. WSF, CSF, NSF, 4KSF, and 24KSF were extensively dialyzed against MiliQ water. The dialysates were lyophilized and grinded into powders. The sequential fractionation procedure was performed in sextuplicate.

Glycome Profiling

The glycome profiling assay followed the protocol of Pattathil, Avci, Miller, and Hahn (2012). The two randomly selected extracts WSF, CSF, NSF, 4KSF, or 24KSF were subjected to glycome profiling.

Total Sugar Assay

Total sugar assay was done using phenol-sulfuric acid micro plate assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956a; Masuko et al., 2005). Two extracts of WSF, CSF, NSF, 4KSF or 24KSF were randomly selected and dissolved in deionized water, and an aliquot of each samples was mixed with 5% (w/v) phenol in test tube. An aliquot of concentrated sulfuric acid was added into each test tube, mixed, and incubated for 20 min at room temperature. After incubation, an aliquot of mixture was transferred into a Costar 3598 ELISA plate and absorbance readings were measured at 490 nm. Total sugar content was calculated relative to glucose standard. The assay was performed in duplicate.

Uronic Acid Assay

Uronic acid content analysis followed modified protocols of Melton and Smith (2001), with addition of sulfamic acid to reduce the interference from neutral sugars. The six extracts of

WSF, CSF, NSF, 4KSF and 24KSF were combined to give enough yield. The combined WSF, CSF, NSF, 4KSF, or 24KSF was hydrolyzed in chilled sulfuric acid with several drops of ethanol to prevent charring. The hydrolysates of WSF, CSF and NSF were diluted to proper concentration, and aliquots of diluted hydrolysates were mixed with 4M sulfamic acid/potassium sulfamate solution (pH 1.6). Aliquots of mixtures were added slowly into test tubes with chilled 75 mM sodium tetraborate in sulfuric acid. The test tubes were incubated in 100^{o}C water bath for 20 min and chilled in ice for another 10 min. An aliquot of 0.15% (w/v) meta-hydroxydiphenyl in 0.5% (w/v) sodium hydroxide was added into each tube, and waited for 4 min before absorbance readings were taken at 525 nm. Uronic acid content of each sample was calculated relative to D-galacturonic acid standard. The assay was performed in triplicate.

GC-MS/Neutral Sugar Analysis

Neutral sugar composition analysis using Gas Chromatography-Mass Spectrometry (GC-MS) followed modified protocol of Blakeney, Harris, Henry, and Stone (1983). Combined WSF, CSF, NSF, 4KSF or 24KSF was hydrolyzed 2N trifluoroacetic acid (TFA) under flushed nitrogen gas for 1 hour at 121^{o}C in heat block. Myo-inositol was added as an internal standard. The hydrolysates were filtered through 13mm 25μm polyethersulfone (PES) filter (Whatman, Maidstone, UK) to remove insoluble materials, and aliquots were evaporated to dryness at 90^{o}C under nitrogen gas in concentrator. The dried hydrolysates were re-dissolved in water, neutralized with 15M ammonia, and reduced in 2% (w/v) sodium borohydride in dimethyl sulphoxide (DMSO) at 40^{o}C for 90 min. After reduction, glacial acetic acid was added to decompose sodium borohydride, and 1-methylimidazole was added followed by 2 mL acetic

anhydride. After 10 min incubation at room temperature, MiliQ water was added to decompose excess acetic anhydride. The acetylated alditols were extracted with dichloromethane, and were stored in septum capped GC vial at -40^{o}C before GC analysis. Aliquots of alditol acetate derivatives were injected into HP model 6890 GC (Palo Alto, CA) with a 30 m x 0.25 mm i.d. x 0.25 µm BPX70 capillary column (SGE Inc. Austin, TX). The detector temperature was held at 250^{o}C, and a linear oven temperature gradient from 170^{o}C to 230^{o}C with 2^{o}C/min increment, and a 5 min hold at 230^{o}C. The different alditol acetates were identified by comparing with standards of xylose, glucuronic acid, mannose, galactose, rhamnose, fucose, and arabinose alditol acetate derivatives. Neutral sugar composition was calculated quantitatively relative to myo-inositol internal standard.

Second Extraction

AIS Precipitation and Sequential Fractionation

The protocol was similar to AIS precipitation and sequential fractionation described above with modifications. AIS* from second extraction was prepared as described above.

For sequential fractionation, 1 gram of AIS* was suspended in 100 mL 50 mM sodium acetate (pH 5.2) with 0.02% sodium azide extraction buffer and mixed at room temperature for 24 hours. Suspension was centrifuged and filtered to collect supernatant. The supernatant was designated as water soluble fraction (WSF*), and stored at 4^{o}C. The pellets were washed with MiliQ water, suspended in 100 mL 50 mM EDTA, and 50 mM ammonium oxalate in 50 mM sodium acetate (pH 5.2) with 0.02% sodium azide extraction buffer and treated as above. The supernatant was designated as chelator soluble fraction (CSF*) and stored at 4^{o}C. The washed

pellets were suspended in 100 mL 50 mM sodium carbonate, 20 mM sodium borohydride extraction buffer and treated as above. The supernatant was designated as sodium carbonate soluble fraction (NSF*) and stored at 4^{o}C. The washed pellets were suspended in 4% (w/v) potassium hydroxide, 0.1% (w/v) sodium borohydride extraction buffer and treated as above. The supernatant was designated as 4% potassium hydroxide soluble fraction (4KSF*) and stored at 4^{o}C. The washed pellets were suspended in 24% (w/v) potassium hydroxide, 1% (w/v) sodium borohydride extraction buffer and treated as above. The supernatant was designated as 24% potassium hydroxide soluble fraction (24KSF*). NSF*, 4KSF*, and 24KSF* supernatants were adjusted to neutral pH with glacial acetic acid. WSF*, CSF*, NSF*, 4KSF*, and 24KSF* supernatant were extensively dialyzed against deionized water. The dialysates were freeze dried and grinded into powders. The AIS precipitation and sequential fractionation procedure was performed in duplicate.

Uronic Acid Assay

Uronic acid assay followed the exact same protocol as described above. WSF*, CSF*, NSF*, 4KSF* and 24KSF* were subjected to uronic acid assay. The assay was performed in triplicate.

Total Sugar Assay

Total sugar content was determined by method of Dubois, Gilles, Hamilton, Rebers, and Smith (1956b). WSF*, CSF*, NSF*, 4KSF*, or 24KSF* was suspended in deionized water to form suspension of concentration suitable for analysis. An aliquot of each samples were mixed with 0.5 mL 5% (w/v) phenol. An aliquot of concentrated sulfuric acid was added directly to the

liquid surface in each test tube. Test tubes were incubated for 20 min at room temperature and absorbance were measured at 490 nm. Total sugar content was calculated relative to glucose standards. The assay was performed in triplicate.

Protein Content

WSF*, CSF*, NSF*, 4KSF* or 24KSF* was dissolved in deionized water to form suspensions with proper concentration. Aliquots of sample suspension were mixed with Bradford reagent (Thermo Scientific, Rockford, IL) in 96 well microplate (Fisher Scientific, Waltham, MA). Microplate was shaken for 30 seconds in Bio-Rad iMark^{TM} microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA) and incubate at room temperature for 10 min. Absorbance was measured at 595 nm. Protein content was calculated in relative to immunoglobulin G (IgG) standard. The assay was performed in triplicate.

Degree of Methylesterification

Degree of methyesterification was measured using FTIR followed protocol of Chatjigakis et al. (1998) with modifications. Samples were analyzed using Perkin Elmer Spectrum 400 (Waltham, MA). Peak heights at 1750 cm^{-1} and 1630 cm^{-1} were measured. The degree of methylesterification was calculated by height esterified carboxyl group (1750 cm^{-1}) divided by the sum of peak heights of esterified carboxyl group (1750 cm^{-1}) and free carboxyl group (1630 cm^{-1}).

HPSEC-MALS -RI

The high performance size exclusion chromatography (HPSEC) system consisted of an Infinity 1260 isocratic pump with a degasser (Agilent Technologies, Santa Clara, CA), a SpectraSYSTEM AS1000 auto sampler (Thermo Fisher Scientific Inc., Waltham, MA), a PL-Aquagel-OH mix column (300 x 7.5 mm) with PL-Aquagel-OH guard colum (50 x 7.5 mm) (Agilent Technologies, Santa Clara, CA), a Dawn Heleos-II multi-angle light scattering (MALS) detector (Wyatt Technology Corporation, Santa Barbara, CA), and an Optilab T-rEX differential refractive index (RI) detector (Wyatt Technology Corporation, Santa Barbara, CA). WSF*, CSF*, or NSF* was suspended in eluent buffer to make around 2 mg/mL sample dispersion, and was filtered through 13mm 25 µm PES filter (Whatman, Maidstone, UK) before injection. The flow rate was set at 0.5 mL/min, with 10 mM sodium phosphate and 100 mM sodium nitrate, pH 7 as an eluent buffer. The assay was performed in duplicate.

Statistical Analysis

Glycome profiling results of each sample were presented as a heat map using modified version of R-Console software. Absorbance values of ELISA response were converted into color gradients using a set of color keys.

All other statistical analysis was conducted with SAS® 9.3 (SAS Institute Inc., Cary, NC). Means of uronic acid content, protein content, total sugar content, Mw and Rw were analyzed using ANOVA and compared using Fischer's least significant difference at statistical significance of $P<0.05$.

Results and Discussion

Preparative Extraction for Glycome Profiling

The uronic acid assay was conducted on the cell wall fractions to evaluate the pectin content. WSF, CSF and NSF had uronic acid content of 25.0, 22.3 and 27.6 μg/mg AIS respectively, while 4KSF and 24KSF contained around 2 μg uronic acid/mg AIS (Figure 2.1a). WSF, CSF and NSF had significantly higher uronic acid content than 4KSF and 24KSF, therefore, the first three fractions were pectin rich fractions. On a laboratory scale, AIS prepared from cell wall materials are generally fractionated by sequential extraction with water, chelator buffer, weak acids and weak alkali solution (Stephen & Phillips, 2010), to pull out pectin fractions from the cell wall preparation. The residues are mainly hemicellulose, cellulose and lignin (Lawther, Sun, & Banks, 1995). To further extract hemicellulose fractions, strong alkali solutions are usually used. Arabinose and galactose were the two most abundant neutral sugars in WSF, CSF and NSF (Figure 2.2). WSF contained up to 60 mol% arabinose and about 20 mol% galatose, CSF contained about 50 mol% both arabinose and galactose, and NSF contained around 35 mol% and 55 mol% arabinose and galactose respectively. Arabinose was reported as the highest amount of neutral sugar found in pectin rich fractions prepared from ripe blueberry (Vicente et al., 2007). Arabinose forms arabinans and arabinogalactans in pectin, both of which may be associated with the pectic hairy region (Voragen et al., 2009; Yapo, 2011). The majority of galactose might be contributed by galacturonic acid forming the backbone structure of pectin. The neutral sugars were reduced to sugar alcohol derivatives by sodium borohydride; therefore, monosaccharides and monosaccharide uronic acid forms cannot be distinguished (Blakeney et al., 1983). A small

amount of galactose may be from pectic side chains of galactans and arabinogalactans. Since the neutral sugars are reduced to sugar alcohol derivatives, monosaccharides and monosaccharides in uronic acid forms cannot be distinguished (Blakeney et al., 1983). The hemicellulose rich fractions, 4KSF and 24KSF contained a majority, more than 70 mol% of xylose, and arabinose, mannose, galactose and glucose in minor amount. Xylose is the key monosaccharides forming xyloglucans and xylans (Scheller & Ulvskov, 2010).

The five cell wall fractions were subjected to glycome profiling analysis. Glycome profiling is an ELISA-based immunological approach to analyze and characterize plant cell walls polysaccharide (Pattathil et al., 2010). Plant cell wall materials are analyzed by a collection of about 200 monoclonal antibodies (mAbs), which are grouped into classes based on specific binding with glycan epitopes. A comprehensive picture of plant cell wall polysaccharide composition can be generated by the binding between each class of mAbs and glycan epitopes (Pattathil et al., 2012). A bar chart at the top indicates the total glycan content in the cell wall material, and provides a quantitative idea of the glycan epitopes content combined with the heat map. All pectin rich fractions WSF, CSF and NSF contained xyloglucan (XG), homogalacturonan (HG) backbone, and arabinogalactan (AG) epitopes, but no xylan epitopes (Figure 2.3). WSF had strong mAbs binding in the XG region, especially in the non-fuc XG-1 and non-fuc XG-2 groups. It indicates that a significant amount of XG was co-extracted with this pectin rich fraction. XG has a glucose backbone branched with xylose (Scheller & Ulvskov, 2010), and WSF contained 6 and 10 mol% of xylose and glucose, respectively (Figure 2.2). CSF had strong binding in both HG backbone 1 and HG backbone 2 groups, and in all the AG groups.

CSF had binding with mAb JIM 137 in the RG-1b group, and binding in the XG groups, but arabinose and galactose were the only two neutral sugars found in CSF. Rhamnose, xylose and glucose may exist in small amount in CSF, and was reported in chelator soluble fraction extracted from ripe blueberry (Vicente et al., 2007). CSF may consist predominantly pectic HG regions and RG-I region highly branched with arabinogalactans, and a small amount of xyloglucan. NSF has equal distribution of XG, pectic HG backbone, and AG epitopes. Considering the particularly high total soluble sugar content per gram AIS, NSF may have comparable or even richer content of xyloglucan, pectic HG backbone and arabinogalactan epitopes comparing to WSF and CSF. Rhamnose, xylose and glucose were reported in small amount in the sodium carbonate soluble fraction extracted from ripe blueberry (Vicente et al., 2007).

4KSF and 24KSF had binding in both XG and xylan regions (Figure 2.4). 4KSF had equal amount of binding intensity with both XG and xylan specific mAbs, 24KSF had strong binding in the XG epitope region, including fucosylated XG, and comparably weak binding in the xylan epitope region. 4KSF may contain more xylans and 24KSF. Although both 4KSF and 24KSF contained 2-5 mol% mannose, the hemicellulose rich fractions contained no galactomannan and acetylated mannan epitopes.

Second Extraction

On sample weight basis, CSF and NSF contained relatively low uronic acid content. CSF and NSF contained about 10% and 27% uronic acids, respectively (data not shown). Alditol acetate derivatives prepared from CSF during GC-MS neutral sugar assay had low signal

intensity compared with internal standard (chromatograph not shown). For commercial pectin, the Food and Agriculture Organization has a specification for pectin, including no less than 65% uronic acids (Maxwell, Belshaw, Waldron, & Morris, 2012). It was suspected that a certain amount of CDTA residue did not pass through dialysis tubing and remained in the CSF. CDTA is not easily removed by dialysis during pectin extraction (Mort, Maness, Pierce, & Moerschbacher, 1991). Some residual CDTA may also carried over to NSF, resulting in lower than true uronic acid content in NSF. EDTA is commonly used to as a chelating agent for pectin extraction (Stephen & Phillips, 2010). For the second extraction, CDTA was replace by EDTA and ammonium oxalate to make the chelator soluble extraction buffer. The cell wall fractions from second extraction were designated as WSF*, CSF*, NSF*, 4KSF* and 24KSF*.

On AIS weight basis, the pectin rich fractions from the second extraction, WSF*, CSF* and NSF* had 43.8, 30.1 and 36.1 μg uronic acid per mg AIS, between 10 to 20 times the uronic acid measured in hemicellulose rich fractions; 4KSF* and 24KSF* had 2.3 and 2.5 μg uronic acid per mg AIS, respectively (Figure 2.1b). On an AIS weight basis, WSF*, CSF* and NSF* had 30-75% more μg uronic acid/mg AIS than corresponding pectin rich fractions extracted in the preparative extraction (Figure 2.1a). The difference may be contributed by the slightly higher uronic acid content in the AIS* (242 μg/mg) from second extraction than previous AIS (227 μg/mg) (data not shown), and modification of the extraction condition. On a sample weight basis, pectin rich fractions contained 509, 567 and 572 μg uronic acid/mg, respectively, and the hemicellulose rich fractions contained 43 and 44 μg uronic acid/mg, respectively (Table 2.1). WSF*, CSF* and NSF* contained significantly higher uronic acid content than 4KSF* and

24KSF* on both AIS weight and sample weight basis. The cell wall fractions from second extraction had total soluble sugar between 300 and 450 μg/mg. The total sugar content in WSF*, CSF* and NSF* was mainly contributed by pectin polygalacturonic acid backbone and neutral sugars in hairy region. The total sugar in 4KSF* and 24KSF* was xylose. WSF*, CSF* and 24KSF* contained low protein content, less than 40 μg/mg, while NSF* and 4KSF* contained higher protein content of 250 and 187 μg/mg, respectively. The high protein content in NSF* may be associated with arabinogalactan to form arabinogalactan protein. The degree of methylesterification of the pectin rich fractions were between 26-39%, therefore, all the pectin rich fractions were low methoxyl pectins. The low DE maybe attributed to pectin methylesterase activity in blueberry. Some pectin methylesterase might be active during the freeze dried blueberry powder processing, and cleaved some pectin methyl esters during the grinding.

HPSEC-MALS-RI analysis on WSF*, CSF* and NSF* showed major light scattering (LS) and refractive index (RI) peaks eluting at 6.5-8.5 mL, and small LS peaks eluting after 9 mL (Figure 2.5). The lower RI signal relative to the LS signal at 6.5-8.5 mL indicates a small amount of large molecular weight compound; the higher RI signal relative to the LS signal after 9 mL indicates a large amount of low molecular weight materials. Weight average molecular weight (Mw), weight average root mean square radius (Rw), polydispersity, and RMS conformation plot slope were calculated for the major eluting peaks at 6.5-8.5 mL (Table 2.2). The major eluting peaks of WSF*, CSF* and NSF* had Mw of 456, 453 and 177 kDa, respectively. WSF* and CSF* had high molecular weight and relatively intact pectin structure due to the non-degradative extraction with water and chelating buffer, while NSF* was the

protopectin with lower Mw due to the destructive weak alkali condition (Stephen & Phillips, 2010). Commercial citrus pectin assayed in our lab had Mw between 100-200 kDa (Corredig, Kerr, & Wicker, 2000; Kim, Teng, & Wicker, 2005), and sugar beet pectin had Mw around 400 kDa (Jung & Wicker, 2012). All pectin rich fractions were polydisperse, with the ratio of Mw/Mn much higher than 1. The RMS conformation plot slope of WSF*, CSF* and NSF* were 0.40, 0.12, and 0.20, respectively. RMS conformation plot slope gives good estimation of polymer shape (Wyatt, 1993). Slope value around 0.33 indicates sphere, near 0.5-0.6 indicates random coil, and approximately 1 indicates rigid rod. WSF* had sphere/random coil shape, while CSF* and NSF* more compact polymer shape. WSF*, CSF* and NSF* had a decreasing trend of Rw of 82 nm, 47 nm and 36 nm, respectively. The high Rw of WSF* is another indication of the less compact shape of WSF*. The mass recovery of the major eluting peaks of WSF*, CSF* and NSF* was around 35% (data not shown), therefore only a part of the cell wall materials was investigated. Some insoluble material might be retained on filter.

Conclusions

Pectin rich fractions extracted from blueberry contained 50-60% uronic acid and were not rich source of pectin, but had unique proportions. Xyloglucans were co-extracted in the pectin rich fractions by sequential AIS fractionation with water, chelating agent and weak alkali. Pectin rich fractions contained arabinogalactans, which were most likely associated with pectin hairy region. The high protein content in NSF may indicate the presence of arabinogalactan proteins. The pectin fractions extracted with water and chelator buffer retained the intact pectin structure

with high molecular weight, and the fraction extracted with degradative extraction buffer had lower molecular weight.

Table 2.1. Chemical characterization of blueberry cell wall fractions from second extraction, water soluble fraction (WSF*), chelator soluble fraction (CSF*) and sodium carbonate soluble fraction (NSF*).

	UA (μg/mg)	TS (μg/mg)	Protein (μg/mg)
WSF*	$509^{b} \pm 7$	$303^{d} \pm 9$	$23^{d} \pm 1$
CSF*	$567^{a} \pm 9$	$317^{c} \pm 9$	$33^{c} \pm 1$
NSF*	$572^{a} \pm 24$	$445^{a} \pm 14$	$250^{a} \pm 8$
4KSF*	$43^{c} \pm 2$	$333^{b} \pm 7$	$187^{b} \pm 11$
24KSF*	$44^{c} \pm 1$	$435^{a} \pm 7$	$33^{c} \pm 1$

*indicates the fractions were from second extraction using EDTA and ammonium oxalate to make chelator soluble buffer. Means (± standard deviation) of uronic acid (UA), total sugar (TA) and protein are significantly different ($P<0.05$) if they share different superscripts in the same column.

Table 2.2. Characterization of pectin rich fractions from second extraction, water soluble fraction (WSF*), chelator soluble fraction (CSF*) and sodium carbonate soluble fraction (NSF*).

	DE (%)	Mw (kDa)	Rw (nm)	Polydispersity	RMS conformation plot slope
WSF*	39	$456^{a} \pm 26$	$81.9^{a} \pm .9$	1.7 ± 0.1	0.40 ± 0.02
CSF*	30	$453^{a} \pm 31$	$46.7^{b} \pm 1.0$	2.0 ± 0.1	0.12 ± 0.01
NSF*	26	$177^{b} \pm 4$	$36.0^{c} \pm 0.8$	1.7 ± 0.0	0.20 ± 0.03

*indicates the fractions were from second extraction using EDTA and ammonium oxalate to make chelator soluble buffer. DE stands for degree of methylesterification; Means of weight average molecular weight (Mw) and weight average root mean square radius (Rw) are significantly different ($P<0.05$) if they share different superscripts in the same column. RMS stands for root mean square.

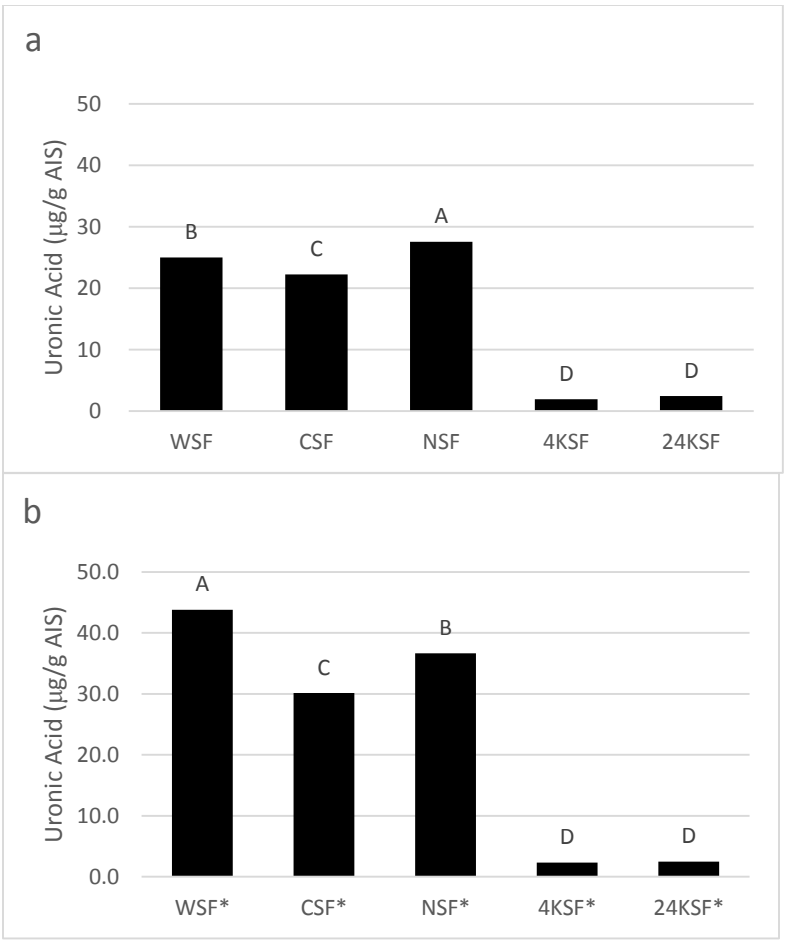

Figure 2.1. Uronic acid content of cell wall fractions, water soluble fraction (WSF), chelator soluble fraction (CSF), sodium carbonate soluble fraction (NSF), 4% potassium hydroxide soluble fraction (4KSF) and 24% potassium hydroxide soluble fraction (24KSF) on AIS weight basis: (a) preparative extraction for glycome profiling; (b) second extraction. *indicates the fractions were from second extraction using EDTA and ammonium oxalate to make chelator soluble buffer. Letters on the top of bars indicate statistical difference ($P<0.05$) between the mean uronic acid content in the same graph. AIS from preparative extraction for glycome profiling and AIS* from second extraction had statistically different ($P<0.05$) mean uronic acid content of 227 and 242 ug/mg AIS, respectively.

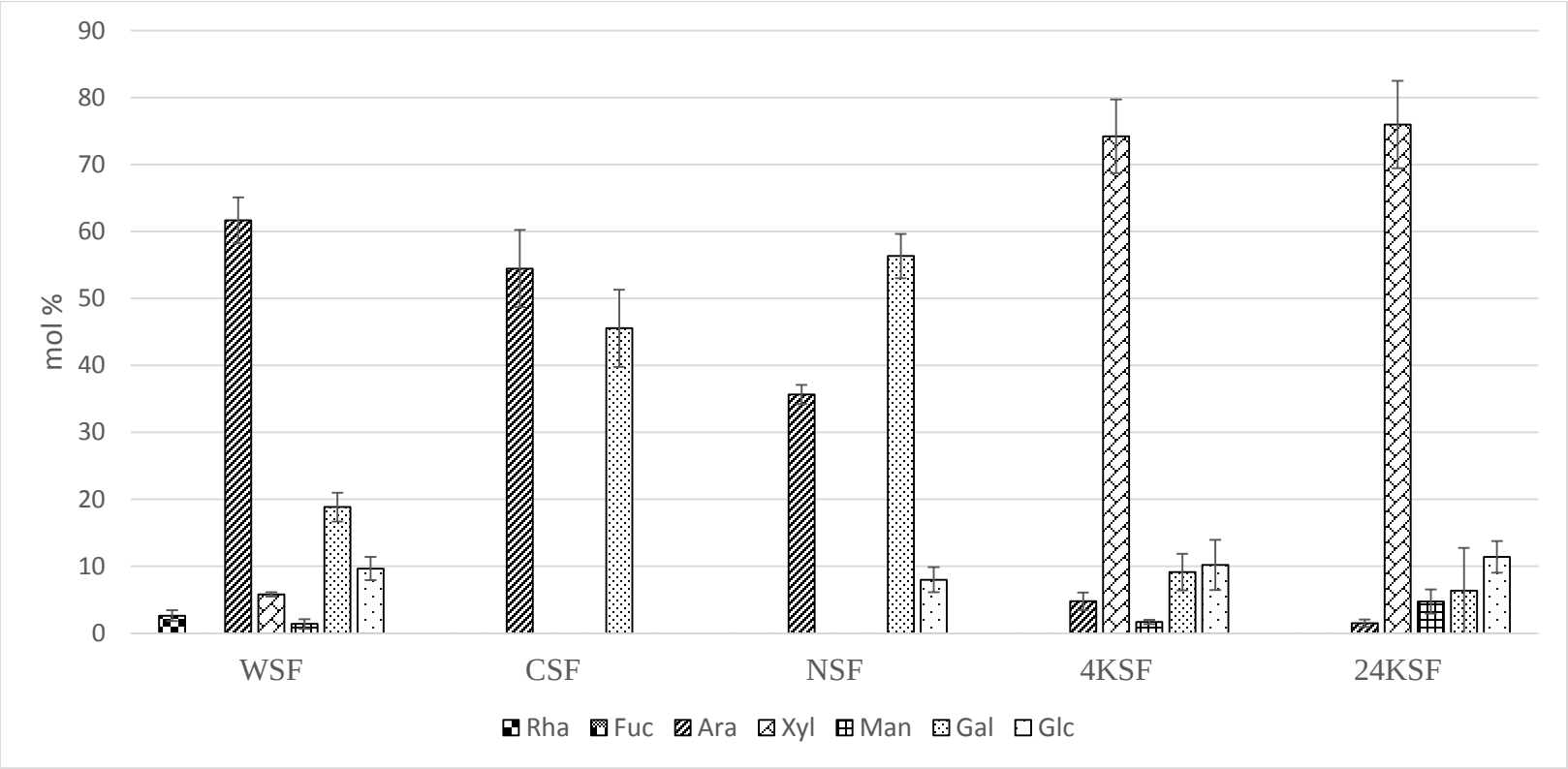

Figure 2.2. Neutral sugar profile of blueberry cell wall fractions, water soluble fraction (WSF), chelator soluble fraction (CSF) and sodium carbonate soluble fraction (NSF). Bars with different pattern fills indicate different neutral sugars, rhamnose (Rha), fucose (Fuc), arabinose (Ara), xylose (Xyl), mannose (Man), galactose (Gal), and glucose (Glc).

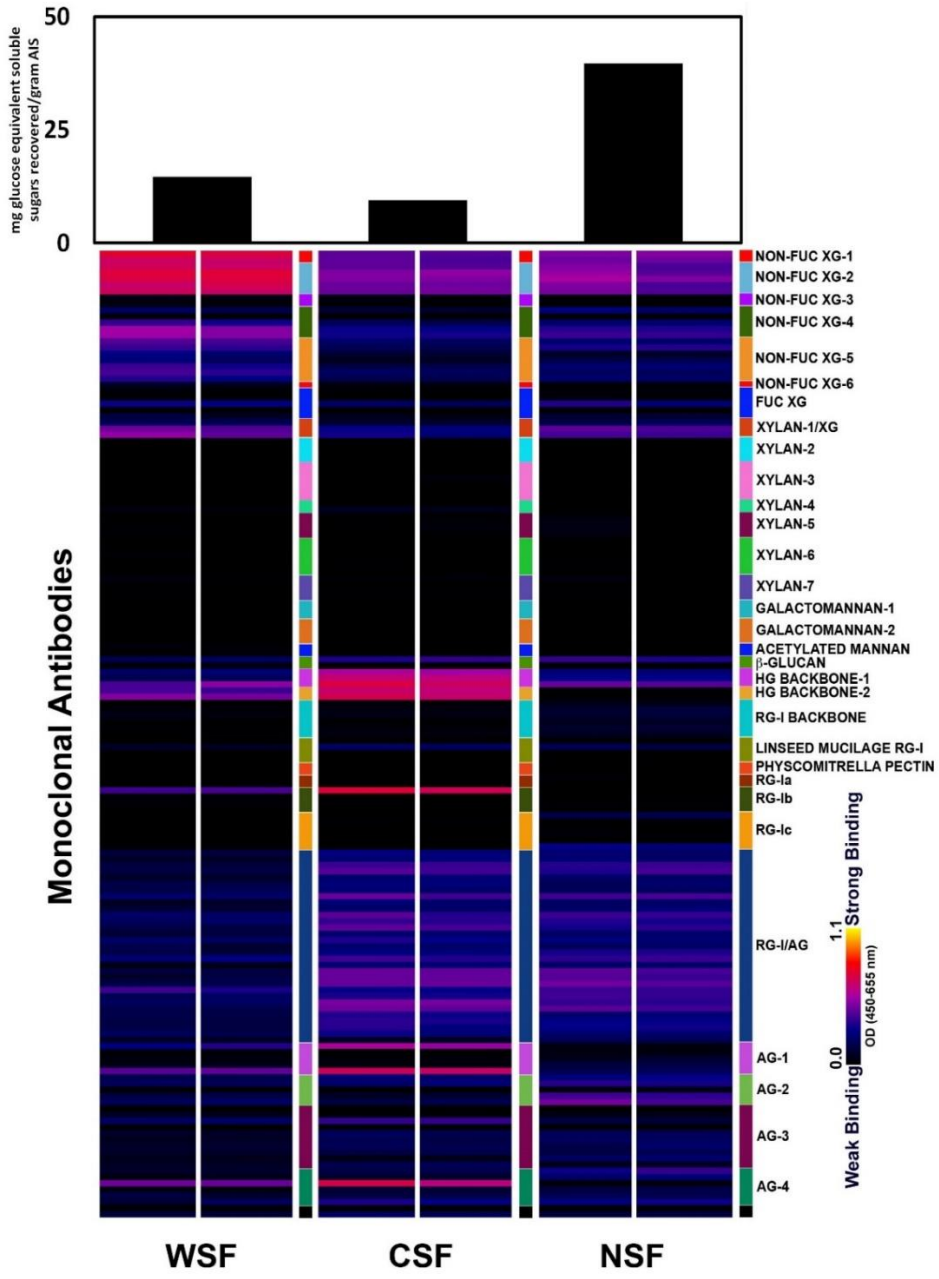

Figure 2.3. Glycome profiling of blueberry pectin rich fractions, water soluble fraction (WSF), chelator soluble fraction (CSF) and sodium carbonate soluble fraction (NSF). The vertical color-coded strips show the clades of cell wall glycan-binding antibodies. Each successive cell wall solvent is shown on the bottom. The bar graph shows the amount of glucose equivalent soluble sugar in the cell wall materials recovered.

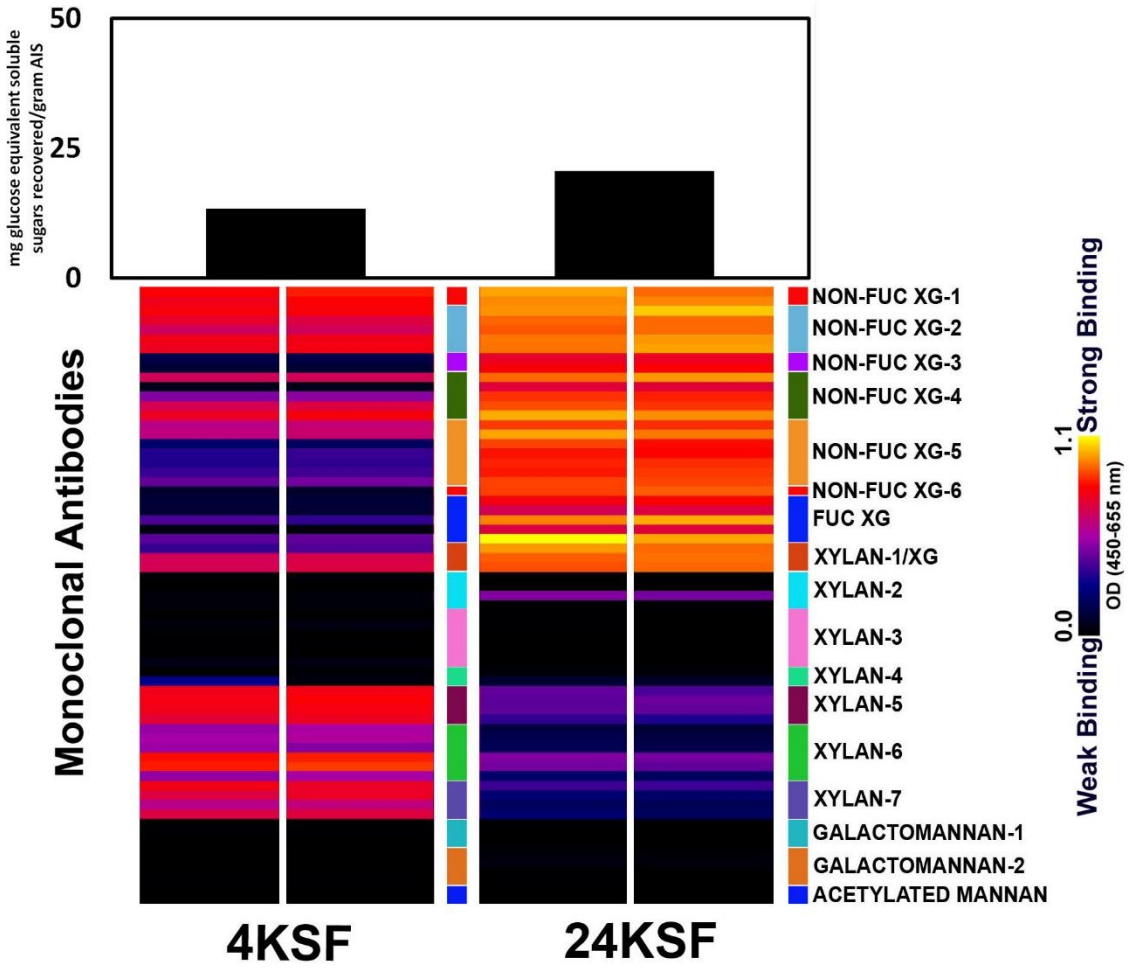

Figure 2.4. Glycome profiling of blueberry hemicellulose glycan epitopes region of 4% potassium hydroxide soluble fraction (4KSF) and 24% potassium hydroxide soluble fraction (24 KSF). The vertical color-coded strips show the clades of cell wall glycan-binding antibodies. Each successive cell wall solvent is shown on the bottom. The bar graph shows the amount of glucose equivalent soluble sugar in the AIS recovered.

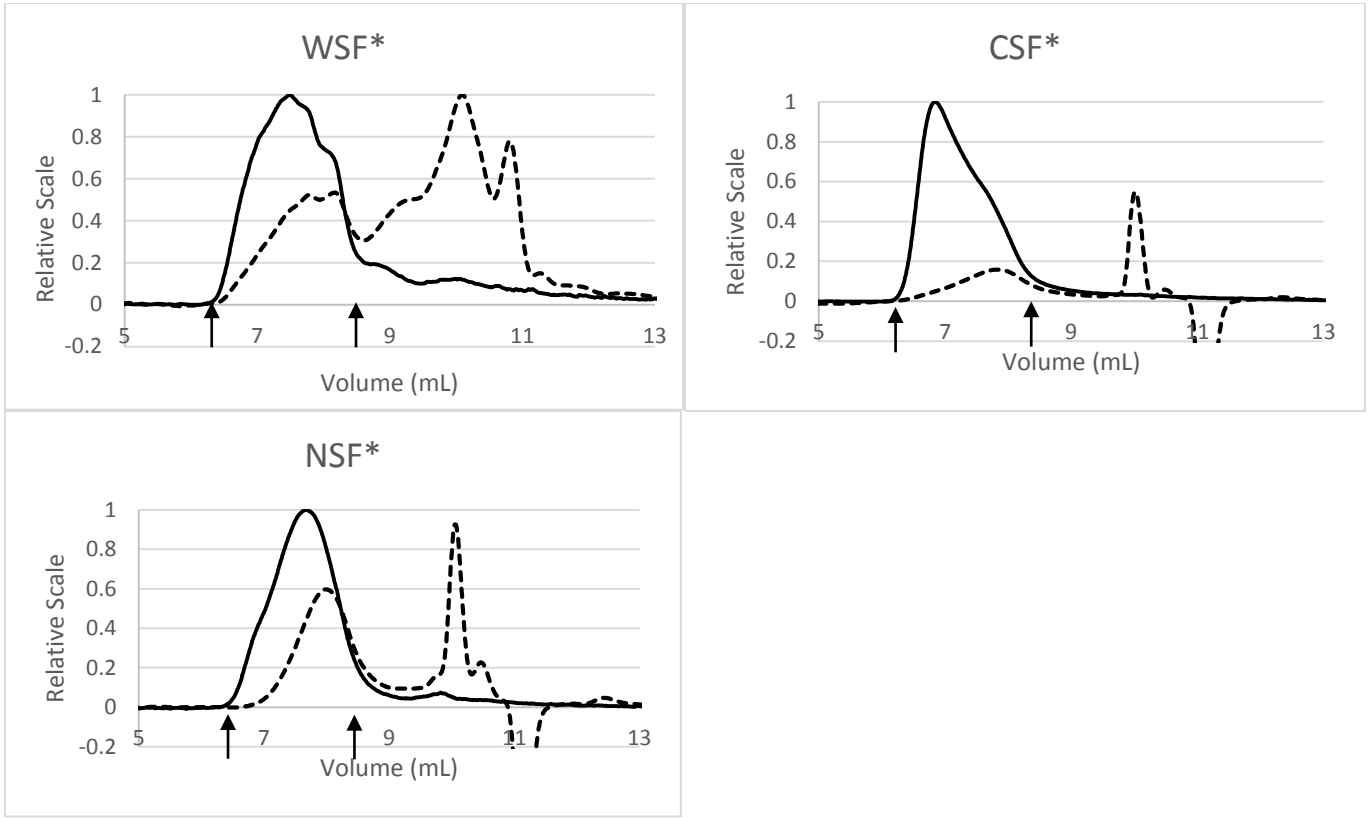

Figure 2.5. Representative elution profiles of pectin rich fractions from second extraction, water soluble fraction (WSF*), chelator soluble fraction (CSF*), and sodium carbonate soluble fraction (NSF*) monitored by multi angle light scattering (MALS) and differential refractive index (RI). *indicates the fractions were from second extraction using EDTA and ammonium oxalate to make chelator soluble buffer. Areas between two arrows are peak areas for weight average molecular weight (Mw), weight average root mean square radius (Rw), polydispersity, and RMS conformation plot slope calculation. Solid line indicates light scattering (LS) signal; dotted line indicates refractive index (RI) signal.

References

Blakeney, A. B., Harris, P. J., Henry, R. J., & Stone, B. A. (1983). A simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohydr Res, 113*(2), 291-299. doi: http://dx.doi.org/10.1016/0008-6215(83)88244-5

Buchweitz, M., Brauch, J., Carle, R., & Kammerer, D. R. (2013). Colour and stability assessment of blue ferric anthocyanin chelates in liquid pectin-stabilised model systems. *Food Chemistry, 138*(2–3), 2026-2035. doi: http://dx.doi.org/10.1016/j.foodchem.2012.10.090

Buchweitz, M., Carle, R., & Kammerer, D. R. (2012). Bathochromic and stabilising effects of sugar beet pectin and an isolated pectic fraction on anthocyanins exhibiting pyrogallol and catechol moieties. *Food Chemistry, 135*(4), 3010-3019. doi: http://dx.doi.org/10.1016/j.foodchem.2012.06.101

Buchweitz, M., Nagel, A., Carle, R., & Kammerer, D. R. (2012). Characterisation of sugar beet pectin fractions providing enhanced stability of anthocyanin-based natural blue food colourants. *Food Chemistry, 132*(4), 1971-1979. doi: 10.1016/j.foodchem.2011.12.034

Chatjigakis, A. K., Pappas, C., N.Proxenia, O.Kalantzi, P.Rodis, & Polissiou, M. (1998). FT-IR spectroscopic determination of the degree of esterification of cell wall pectins from stored peaches and correlation to textural changes. *Carbohydrate Polymers, 37*(4), 395-408. doi: http://dx.doi.org/10.1016/S0144-8617(98)00057-5

Chen, H.-C., & Camire, M. E. (1997). RECOVERY OF ANTHOCYANINS, PECTIN, AND DIETARY FIBER FROM CULL LOWBUSH BLUEBERRIES. *Journal of Food Quality, 20*(3), 199-209. doi: 10.1111/j.1745-4557.1997.tb00464.x

Corredig, M., Kerr, W., & Wicker, L. (2000). Molecular characterization of commercial pectins by separation with linear mix gel permeation columns in-line with multi-angle light scattering detection. *Food Hydrocolloids, 14*(1), 41-47. doi: 10.1016/S0268-005X(99)00044-2

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956a). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry, 28*(3), 350-356.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956b). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry, 28*, 350-356.

Foster, T. J. (2011). Natural structuring with cell wall materials. *Food Hydrocolloids, 25*(8), 1828-1832. doi: http://dx.doi.org/10.1016/j.foodhyd.2011.05.016

Hidalgo, M., Oruna-Concha, M. J., Kolida, S., Walton, G. E., Kallithraka, S., Spencer, J. P., & de Pascual-Teresa, S. (2012). Metabolism of anthocyanins by human gut microflora and their influence on gut bacterial growth. *J Agric Food Chem, 60*(15), 3882-3890. doi: 10.1021/jf3002153

Jung, J., Arnold, R. D., & Wicker, L. (2013). Pectin and charge modified pectin hydrogel beads as a colon-targeted drug delivery carrier. *Colloids Surf B Biointerfaces, 104*, 116-121. doi: 10.1016/j.colsurfb.2012.11.042

Jung, J., & Wicker, L. (2012). Laccase mediated conjugation of sugar beet pectin and the effect on emulsion stability. *Food Hydrocolloids, 28*(1), 168-173. doi: 10.1016/j.foodhyd.2011.12.021

Kahle, K., Kraus, M., Scheppach, W., Ackermann, M., Ridder, F., & Richling, E. (2006). Studies on apple and blueberry fruit constituents: do the polyphenols reach the colon after ingestion? *Mol Nutr Food Res, 50*(4-5), 418-423. doi: 10.1002/mnfr.200500211

Keppler, K., & Humpf, H.-U. (2005). Metabolism of anthocyanins and their phenolic degradation products by the intestinal microflora. *Bioorganic & Medicinal Chemistry, 13*(17), 5195-5205. doi: 10.1016/j.bmc.2005.05.003

Kim, Y., Teng, Q., & Wicker, L. (2005). Action pattern of Valencia orange PME de-esterification of high methoxyl pectin and characterization of modified pectins. *Carbohydr Res, 340*(17), 2620-2629. doi: 10.1016/j.carres.2005.09.013

Lawther, J. M., Sun, R., & Banks, W. B. (1995). Extraction, fractionation, and characterization of structural polysaccharides from wheat straw. *Journal of Agricultural and Food Chemistry, 43*(3), 667-675. doi: 10.1021/jf00051a021

Leijdekkers, A. G., Aguirre, M., Venema, K., Bosch, G., Gruppen, H., & Schols, H. A. (2014). In vitro fermentability of sugar beet pulp derived oligosaccharides using human and pig fecal inocula. *J Agric Food Chem, 62*(5), 1079-1087. doi: 10.1021/jf4049676

Masuko, T., Minami, A., Iwasaki, N., Majima, T., Nishimura, S., & Lee, Y. C. (2005). Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. *Anal Biochem, 339*(1), 69-72. doi: 10.1016/j.ab.2004.12.001

Maxwell, E. G., Belshaw, N. J., Waldron, K. W., & Morris, V. J. (2012). Pectin – An emerging new bioactive food polysaccharide. *Trends in Food Science & Technology, 24*(2), 64-73. doi: 10.1016/j.tifs.2011.11.002

Mazza, G. (2007). Bioactivity, absorption and metabolism of anthocyanins. *Acta Horticulturae*(744), 117-125.

Melton, L. D., & Smith, B. G. (2001). Determination of the Uronic Acid Content of Plant Cell Walls Using a Colorimetric Assay *Current Protocols in Food Analytical Chemistry*: John Wiley & Sons, Inc.

Mort, A. J., Maness, N. O., Pierce, M. L., & Moerschbacher, B. M. (1991). Problems encountered during the extraction, purification, and chromatography of pectic fragments, and some solutions to them. *Carbohydr Res, 215*(1), 219-227.

Padayachee, A., Netzel, G., Netzel, M., Day, L., Mikkelsen, D., & Gidley, M. (2013). Lack of release of bound anthocyanins and phenolic acids from carrot plant cell walls and model composites during simulated gastric and small intestinal digestion. *Food Funct, 4*(6), 906-916. doi: 10.1039/C3FO60091B

Padayachee, A., Netzel, G., Netzel, M., Day, L., Zabaras, D., Mikkelsen, D., & Gidley, M. J. (2012). Binding of polyphenols to plant cell wall analogues – Part 1: Anthocyanins. *Food Chemistry, 134*(1), 155-161. doi: http://dx.doi.org/10.1016/j.foodchem.2012.02.082

Pattathil, S., Avci, U., Baldwin, D., Swennes, A. G., McGill, J. A., Popper, Z., . . . Hahn, M. G. (2010). A Comprehensive Toolkit of Plant Cell Wall Glycan-Directed Monoclonal Antibodies. *Plant Physiology*(2), 514. doi: 10.2307/25680872

Pattathil, S., Avci, U., Miller, J. S., & Hahn, M. G. (2012). Immunological approaches to plant cell wall and biomass characterization: Glycome Profiling. *Methods In Molecular Biology (Clifton, N.J.), 908*, 61-72. doi: 10.1007/978-1-61779-956-3_6

Proctor, A., & Peng, L. C. (1989). Pectin Transitions During Blueberry Fruit Development and Ripening. *Journal of Food Science, 54*(2), 385-387. doi: 10.1111/j.1365-2621.1989.tb03088.x

Scheller, H. V., & Ulvskov, P. (2010). Hemicelluloses. *Plant Biology, 61*(1), 263.

Stephen, A. M., & Phillips, G. O. (2010). *Food polysaccharides and their applications*: CRC Press.

Vicente, A. R., Greve, L. C., Labavitch, J. M., Powell, A. L. T., Ortugno, C., & Rosli, H. (2007). Temporal Sequence of Cell Wall Disassembly Events in Developing Fruits. 2. Analysis of Blueberry (Vaccinium Species) [electronic resource]. *Journal of Agricultural and Food Chemistry, 55*(10), 4125-4130. doi: http://dx.doi.org/10.1021/jf063548j

Voragen, A. G., Coenen, G. J., Verhoef, R. P., & Schols, H. A. (2009). Pectin, a versatile polysaccharide present in plant cell walls. *Structural Chemistry, 20*(2), 263-275.

Wicker, L., Kim, Y., Kim, M.-J., Thirkield, B., Lin, Z., & Jung, J. (2014). Pectin as a bioactive polysaccharide – extracting tailored function from less. *Food Hydrocolloids*(0). doi: http://dx.doi.org/10.1016/j.foodhyd.2014.01.002

Wong, T. W., Colombo, G., & Sonvico, F. (2011). Pectin matrix as oral drug delivery vehicle for colon cancer treatment. *AAPS PharmSciTech, 12*(1), 201-214. doi: 10.1208/s12249-010-9564-z

Wyatt, P. J. (1993). Light scattering and the absolute characterization of macromolecules. *Analytica Chimica Acta, 272*(1), 1-40. doi: http://dx.doi.org/10.1016/0003-2670(93)80373-S

Yapo, B. M. (2011). Pectic substances: From simple pectic polysaccharides to complex pectins—A new hypothetical model. *Carbohydrate Polymers, 86*(2), 373-385. doi: 10.1016/j.carbpol.2011.05.065

Yi, W., Fischer, J., Krewer, G., & Akoh, C. C. (2005). Phenolic compounds from blueberries can inhibit colon cancer cell proliferation and induce apoptosis. *Journal of Agricultural and Food Chemistry, 53*(18), 7320-7329.

CHAPTER 3

INTERMOLECULAR BINDING BETWEEN PECTIN RICH FRACTIONS AND ANTHOCYANIN[2]

[2]Lin, Z., Fischer, J., Wicker, L., To be submitted to Food Chemistry.

Abstract

Pectin was extracted from blueberry powder into three fractions of water soluble (WSF), chelator soluble (CSF) and sodium carbonate soluble (NSF), and were incubated with anthocyanin rich solutions, cyanidin-3-glucoside (C3G) solutions, five anthocyanidin solutions and blueberry juice at pH 2-4.5. The mixtures were filtered through 30 kDa centrifugal ultrafilter to separate free anthocyanins and bound anthocyanin-pectin mixtures. Pectin rich fractions bound anthocyanins and resulted in reduced free anthocyanin content in anthocyanin rich solutions. WSF bound the lowest amount of anthocyanin pigments at all pH values. CSF had stronger anthocyanin binding ability at pH 2-3.6, while NSF had stronger anthocyanin binding ability at pH 3.6-4.5. The pectin and anthocyanin binding was pH dependent, with the lowest amount binding at pH 4.5 comparing to pH 2-3.6. Nearly doubling C3G pigment content increased bound anthocyanin percentage by 16-23% at pH 3.6, which favored anthocyanin aromatic stacking, comparing to 3-9% increase percentage wise at pH 2. Ionic interaction between anthocyanin flavylium cations and free pectic carboxyl groups, and anthocyanin stacking may be two major mechanisms for pectin and anthocyanin binding.

Keywords:

Blueberry, pectin, Anthocyanin, Cyanidin-3-glucoside, Intermolecular Binding, Centrifugal Ultrafilter

Introduction

Anthocyanins are phenolic compounds naturally found in flowers and some fruits and are responsible for red, purple and blue colors (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009), and they are probably the most important visible pigment in plants besides chlorophyll (Kong, Chia, Goh, Chia, & Brouillard, 2003). Anthocyanins and have shown an array of potential health benefits, such as modulating cardiovascular disease biomarkers, reducing tumor development in rodents, and preventing oxidative damage to DNA (Kong et al., 2003). Increased anthocyanin consumption may reduce the risk of cardiovascular disease; anthocyanins may regulate a variety of signaling pathways associated with the development of cardiovascular diseases (Wallace, 2011). Anthocyanins have the ability to reduce cancer cell proliferation and inhibit tumor formation (Lila, 2004). The powerful health promoting benefits *in vivo* are limited by the low systematic bioavailability (Fleschhut, Kratzer, Rechkemmer, & Kulling, 2006). The monomeric anthocyanins are highly unstable and are susceptible to degradation. The stability of anthocyanins depends on several factors such as pH, monomeric anthocyanin chemical structure, temperature, pigment concentration, light, oxygen, solvents, enzymes, other phenolic compounds, proteins, and metal ions (Castañeda-Ovando et al., 2009).

Pectin is a versatile polysaccharides found in the middle lamella, primary and secondary cell wall of plants (Voragen, Coenen, Verhoef, & Schols, 2009). Besides the application in food system as gelling, thickening and stabilizing agents, pectin is a bioactive polysaccharide that has health promoting benefits (Wicker et al., 2014). As a soluble dietary fiber, pectin in general has potential to lower blood cholesterol levels and affect glucose metabolism (Stephen & Phillips,

2010), and many studies suggested the potential effects on preventing diverticulosis, colon cancer, coronary heart disease, cholesterolemia and diabetes (Voragen et al., 2009). For example, modified pectin with small molecular weight can bind to pro-metastatic protein galectin-3 (GAL3) and inhibit the ability of GAL3 to promote cell adhesion and migration (Maxwell, Belshaw, Waldron, & Morris, 2012). Pectin can be used for colon targeted drug delivery due to the selective digestion by colon microflora with minimal degradation in gastriointestinal tract, and is fermentable by colonic microflora, which leads to short-chain fatty acid production (Jung, Arnold, & Wicker, 2013; Wong, Colombo, & Sonvico, 2011).

In many studies, there is evidence of pectin and anthocyanin interaction. Researchers who studied anthocyanins in flowers as color pigments found that pectin formed large non-dialyzable molecule with anthocyanin-copigment complexes (Asen, Stewart, Norris, & Massie, 1970; Bayer, Egeter, Fink, Nether, & Wegmann, 1966). More recent studies reported that pectin solubilized anthocyanin-metal chelate complexes and prevented pigment precipitation in an aqueous environment, making anthocyanins a potential natural blue colorant for beverage applications (Buchweitz, Carle, & Kammerer, 2012; Buchweitz, Nagel, Carle, & Kammerer, 2012). Pectin also enhances the anthocyanin pigment stability and extends pigment degradation half-life in a model fruit jam system (Buchweitz, Speth, Kammerer, & Carle, 2013; Holzwarth, Korhummel, Siekmann, Carle, & Kammerer, 2013; Kopjar et al., 2009). Cell wall composites containing pectin can bind anthocyanins and have low pigment release in simulated gastric and intestinal fluids (Padayachee et al., 2013; Padayachee et al., 2012). The mechanism for anthocyanin and pectin interaction is not known, but non-covalent chemical interaction, most likely ionic interaction, are proposed (Asen et al., 1970; Buchweitz, Nagel, et al., 2012;

Holzwarth et al., 2013; Padayachee et al., 2012). To date, no research was conducted involving a direct system on pectin and anthocyanin binding, and no study was conducted on functional properties of blueberry pectin.

In this study, blueberry pectin rich fractions were extracted from blueberry alcohol insoluble solids (AIS) using water, chelator buffer and weak alkali buffer. Pectin rich fractions were incubated with anthocyanin rich solutions, and free anthocyanins and bound anthocyanin pectin mixtures were separated by centrifugal ultrafiltration. The objective was to directly measure the binding between pectin and anthocyanins. It is reported that in human and animal models less than 1% of intact anthocyanins consumed were absorbed or excreted in urine (Fleschhut et al., 2006; Keppler & Humpf, 2005). A large amount of anthocyanins *in vivo* reached the colon (Kahle et al., 2006), and exhibited prebiotic effect on promoting colonic health. If pectin binds anthocyanins, it may suggest that blueberry pectin rich fractions may have dual advantages of protecting anthocyanins in the human body and promoting colonic health.

Material and Methods:

Materials and Reagents

Freeze-dried blueberry powder (Tiflbue/Rubel 50/50 blend) was provided by U.S. High Bush Blueberry Council (Folsom, CA). Cyanidin-3-glucoside chloride, cyanidin chloride, malvidin chloride, delphinidin chloride, peonidin chloride and pelargonidin chloride were purchased from Chromadex (Irvine, CA). Other chemicals were purchased from Sigma-Aldrich Inc. (St. Louis, MO).

Pectin Rich Fraction Extraction and Characterization

Extraction of Pectin Rich Fractions

Approximately 250 grams of freeze-dried U.S. highbush blueberry powder was mixed in 10 volumes of 95% ethanol at 70^{o}C for 2 hours. The mixture was centrifuged at 6000g at 4^{o}C for 20 min and was filtered through miracloth (Millipore, Billerica, MA) under vacuum to obtain the pellets. Pellets were washed in 4 volumes of ethanol, centrifuged and filtered 4 times, and finally washed with 4 volumes of acetone, centrifuge and filtered to obtain alcohol insoluble solids (AIS). The AIS were dried under the fume hood overnight. AIS were fractionated according to a modified protocol of Vicente et al. (2007). Briefly, an aliquot of 50 grams of AIS was suspended in 500 mL 50 mM sodium acetate buffer (pH 5.2) with 0.02% sodium azide for 24 hours at room temperature. The mixture was centrifuged and filtered to separate the pellets and supernatant. The supernatant was designated as water soluble fraction (WSF) and stored at 4^{o}C. The pellets were washed with deionized water and were mixed in 500 mL 50 mM EDTA, 50 mM ammonium oxalate, 50 mM sodium acetate (pH 5.2) with 0.02% sodium azide for 24 hours at room temperature. The mixture was treated as described above, and the supernatant was designated as chelator soluble fraction (CSF). CSF supernatant was stored at 4^{o}C. The pellets were then mixed in 500 mL 50mM sodium carbonate, 20 mM sodium borohydride buffer for 24 hours at room temperature. The mixture was treated as above and the supernatant was designated as sodium carbonate soluble fraction (NSF) supernatant. The pH of the NSF supernatant was adjusted to neutral pH with glacial acetic acid, and WSF, CSF and NSF supernatants were dialyzed extensively against deionized water to remove salt residues from the extraction buffer solution. The dialysis tubing (Spectrum Laboratories, Inc. Rancho Dominguez, CA) had a

molecular cutoff of 6-8 kDa. Dialyzed supernatants were then freeze dried and stored at 4^{o}C until analysis.

Uronic Acid Assay

Uronic acid content analysis followed protocols of Melton and Smith (2001) with minor modifications. WSF, CSF or NSF was hydrolyzed in concentrated sulfuric acid and was diluted to a measurable volume with deionized water. Aliquots of hydrolysates were mixed with 4M sulfamic acid/potassium sulfamate (pH 1.6) and were added slowly into the chilled 75 mM sodium tetraborate in sulfuric acid. The mixture was incubated in water bath at 100^{o}C for 20 min. Aliquots of 0.15% (w/v) meta-hydroxydiphenyl in 0.5% (w/v) sodium hydroxide were added into chilled mixture and stood for 4 min to allow color development. Absorbance was measured at 525nm. Uronic acid content was caulculated relative to D-galacturonic acid standard. Each pectin rich fraction was sampled in duplicate and measurements were performed in triplicate.

Total Sugar Content

Total sugar content analysis followed the method of Dubois, Gilles, Hamilton, Rebers, and Smith (1956). WSF, CSF or NSF was dissolved in deionized water and an aliquot of each sample was mixed with 5% (w/v) phenol. Concentrated sulfuric acid was added directly towards liquid surface into each test tube. Test tubes were incubated for 20 min at room temperature and absorbance was measured at 490 nm. Total sugar content was calculated relative to glucose standard. Each pectin rich fraction was sampled in duplicate and measurements were performed in triplicate.

Protein Content

WSF, CSF or NSF was dissolved in deionized water and an aliquot of suspension was mixed with Bradford reagent (Thermo Scientific, Rockford, IL) in 96 well microplate (Fisher Scientific, Waltham, MA). The microplate was shaken for 30 seconds in Bio-Rad iMark^{TM} microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA) and incubated at room temperature for 10 min. Absorbance was measured in microplate reader using 595 nm microplate filter. Protein content of each sample was calculated in relative to immunoglobulin G (IgG) standard. Each pectin rich fraction was sampled in duplicate and measurements were performed in triplicate.

Degree of Methylesterification

Degree of methyesterification (DE) was analyzed using FTIR following a modified protocol of Chatjigakis et al. (1998). Samples were analyzed using Perkin Elmer Spectrum 400 (Waltham, MA). Peak heights at 1750 cm^{-1} and 1630 cm^{-1}, which represent esterified carboxyl group and free carboxyl group were measured. The degree of methylesterification was calculated by height esterified carboxyl group divided by the sum of peak heights of esterified carboxyl group and free carboxyl group.

HPSEC-MALS-RI

The high performance size exclusion (HPSEC) system consisted of an Infinity 1260 isocratic pump with an inline degasser (Agilent Technologies, Santa Clara, CA), SpectraSYSTEM AS1000 auto sampler (Thermo Fisher Scientific Inc., Waltham, MA) with 50 µL injection loop, PL-Aquagel-OH mix column (300 x 7.5 mm) with PL-Aquagel-OH guard

colum (50 x 7.5 mm) (Agilent Technologies, Santa Clara, CA), Dawn Heleos-II multi-angle laser light scattering detector (Wyatt Technology Corporation, Santa Barbara, CA), and Optilab T-rEX differential refractive index detector (Wyatt Technology Corporation, Santa Barbara, CA). Eluent buffer was 100 mM sodium nitrate in 10 mM pH 7 sodium phosphate buffer. During the analysis, flow rate was set at 0.5 mL/min. WSF, CSF and NSF were dissolved in eluent buffer to make 2 mg/mL solution, and were filtered through 13 mm 0.45 μm polyethersulfone (PES) filter (Whatman, Maidstone, UK) to remove insoluble materials. Each pectin rich fraction was sampled in duplicate and injection was conducted in duplicate. Eluting chromatographs were analyzed with ASTRA software Version 6.0.5.3 (Wyatt Technology Corporation, Santa Barbara, CA).

Intermolecular Binding between Pectin Rich Fractions and Anthocyanins

Pectin Dispersions

Blueberry pectin rich fractions, WSF, CSF and NSF were dissolved in pH 2 (25 mM potassium chloride), pH 3.6 (25 mM sodium acetate) or pH 4.5 (25 mM sodium acetate) buffer solutions to make 10 mg/mL pectin dispersions. All buffer solutions contained 0.02% sodium azide as antimicrobial. The pH values were adjusted to target pH with either 1N hydrochloric acid or 1N sodium hydroxide if necessary. Each pectin dispersion was made in duplicate and stored at 4^{o}C before further analysis.

Anthocyanin rich solutions

Cyanidin-3-glucoside (C3G) chloride was dissolved in MiliQ water (Millipore, Billerica, MA) to make stock solution. Aliquots of stock solution was diluted to proper concentrations, one

at lower concentration (~0.3 mg/mL) and one at higher concentrations (~0.6 mg/mL) with pH 2, pH 3.6 and pH 4.5 buffer solutions described above. Anthocyanin aglycones, cyanidin, pelargonidin, malvidin, peonidin, and delphinidin chlorides, were dissolved in pH 2 buffer solution and were filtered through 0.2 μm PES syringe filter (Whatman, Maidstone, UK) to remove insoluble anthocyanin aglycone salts. Aliquots of anthocyanin aglycone solutions were adjusted to proper concentration in pH 2 buffer. Blueberry juice was made from freeze-dried blueberry powder by mixing blueberry powder in MiliQ water with 0.02% sodium azide. Mixture was centrifuged at 6000g at 4°C for 20 min and supernatant was filtered through 0.45 μm PES syringe filter (Whatman, Maidstone, UK) to remove insoluble plant cell wall materials. The filtered juice was diluted to desired concentration in pH 2, pH 3.6 or pH 4.5 buffers. The pH of all anthocyanin rich solutions was adjusted to target pH using either 1N hydrochloric acid or 1N sodium hydroxide if necessary. C3G equivalent anthocyanin pigment content of each anthocyanin rich solution was measured before binding study to monitor pigment concentration changes during incubation.

Pectin Anthocyanin Binding

Experimental groups were set up by mixing equal volume aliquots of each pectin dispersion and anthocyanin rich solution in Amicon 0.5 mL centrifugal ultrafilter tube with 30kDa Millipore Ultracell low-binding regenerated cellulose membrane (Millipore, Billerica, MA). Control groups were set up by mixing aliquots of each pectin solution or anthocyanin rich solution with equal volume buffer solution with corresponding pH in centrifugal ultrafilter. Mixtures were mixed with a vortex and incubated at 4°C under dark condition by wrapping in aluminum foil to minimize pigment degradation during incubation. After 18 hours of incubation,

centrifugal ultrafilters were centrifuged in Eppendorf 5415R centrifuge (Eppendorf, Hamburg, Germany) at 14,000g 4^{o}C for 20 min to separate free anthocyanin, and pectin and bound anthocyanin mixture. Anthocyanin pigment content of the filtrates was measured using microplate reader and expressed as C3G equivalent (Giusti & Wrolstad, 2001). Free anthocyanin content of experimental groups were calculated by subtracting anthocyanin content in the filtrate of pectin control from anthocyanin content in the filtrate of experimental groups of corresponding pectin and pH. Bound anthocyanin pigment content was calculated by difference between free anthocyanin content of anthocyanin pigment control and corresponding free anthocyanin content of experimental group.

Anthocyanin Pigment Test

Anthocyanin pigment concentration in the filtrate was analyzed using a modified protocol of Giusti and Wrolstad (2001), by utilizing a microplate reader. An aliquot of filtrate was pipetted into a microplate well pre-pipetted with 25 mM potassium chloride (pH 1) or 400 mM sodium acetate (pH 4.5) buffer solution. Absorbance readings were taken at 520 nm and 750 nm using a Bio-Rad iMark microplate reader (Bio-Rad Laboratories, Hercules, CA). Anthocyanin pigment content was calculated as C3G equivalent. The equation: pigment content (mg/mL) = $[(A520\text{-}A750)_{pH1}\text{-}(A520\text{-}A750)_{pH4.5}]$xMWxDF/(ƐxL), in which MW is the molecular molecular weight, DF is the dilution factor, Ɛ is extinction coefficient and L is the pathlength of microplate. Molecular weight of C3G (449.2 Da) was used for MW, DF was 10, 25740 cm^2/mol was used for extinction coefficient and 0.645 cm was used as the pathlength of microplate. Measurements were conducted in duplicate.

Statistical Analysis

Statistical analysis was conducted with SAS® 9.3 (SAS Institute Inc., Cary, NC). Means of all experiment results were analyzed using ANOVA and compared using Fischer's least significant difference at statistical significance of $P<0.05$.

Results and Discussion

Pectin Characterization

The uronic acid contents of the pectin rich fractions WSF, CSF and NSF were 516, 627 and 580 μg/mg sample respectively (Table 3.1). All the pectin rich fractions contained more than 50% uronic acid, but less than 65%, which is the minimum uronic acid content of commercial pectin required by the Food and Agriculture Organization (Maxwell et al., 2012). The laboratory scale extraction is generally milder than commercial extraction method with hot acid hydrolysis, therefore, intact pectin structures with neutral sugar as well as other non-sugar moieties can be retained (Wicker et al., 2014). The pectin rich fractions extracted from our previous study also contained non-uronic acid part, xyloglucans and arabinogalactans. The degree of methylesterification of all three pectin rich fractions was lower than 50% measured by FTIR, indicating the pectin was low methylesterified. The total soluble sugar content of WSF, CSF and NSF were 328, 279 and 496 μg/mg respectively. WSF and CSF contained a low protein content of less than 3%, while NSF had around 24% protein. The uronic acid content, degree of methylesterification, total soluble sugar and protein content of the pectin rich fractions were similar to the pectin rich fractions extracted in previous study on smaller scale.

Polymer information was obtained with size exclusion chromatography and multi-angle light scattering (MALS) and refractive index (RI) analysis. Eluting profiles of all three pectin rich fractions had major peak eluting between 6.5 and 8.5 mL and smaller peaks after 9 mL (Figure 3.1). The lower RI signal relative to the LS signal in peak eluting at 6.5-8.5 mL indicates that a small amount of a large molecular weight compound; the higher RI signal relative to the LS signal in peaks eluting after 9 mL indicates a larger amount of lower molecular weight materials. Weight average molecular weight (Mw), weight average root mean square radius (Rw) and polydispersity were calculated for the major eluting peak (Table 3.1). Both WSF and CSF had high weight average molecular weight (Mw) in their first eluting peaks of 602 kDa and 485 kDa respectively. The molecular weight for blueberry pectin extracted by non-degradative extraction solvent are higher than reported for commercial pectins. Citrus and sugar beet pectins had Mw values that ranged from 100-200 kDa (Corredig, Kerr, & Wicker, 2000; Kim, Teng, & Wicker, 2005) and around 400 kDa (Jung & Wicker, 2012). Most likely, the mild laboratory extraction conditions maintained the integrity of the carbohydrate polymers. NSF had a lower Mw of 169 kDa, compared to WSF or CSF. The weak alkali condition was degradative and broke cell wall materials to extract some tightly bound pectic substances in the AIS (Stephen & Phillips, 2010).WSF had highest the weight average root mean square radius (Rw) radius of 75 nm, while the NSF had the lowest Rw of 35 nm. The polymer shapes of all three fractions were spherical, with RMS conformation plot slope below 0.33. WSF had RMS conformation plot slope of 0.25, higher than that of CSF and NSF, therefore WSF was a less compact than CSF and NSF. All three pectin rich fractions were polydispersed with multiple peaks in the chromatograph (Figure 3.1), had polydispersed major eluting peaks, with all the polydispersity values above 1.

In this study, the free anthocyanin content in pectin controls prepared at 5 mg/mL concentration were measured (data not shown).WSF control contained a high amount of 12.5 μg/mL free anthocyanin content, and CSF and NSF control contained trace amount of free anthocyanins. Both dialyzed WSF supernatant and freeze dried WSF had purple color; freeze dried CSF was white, but when dissolved in aqueous solution, it had pinkish color. Both dialyzed NSF supernatant and freeze dried NSF had a brown color. Since anthocyanin is the major pigment in the blueberry fruits, the stained color on the pectin rich fractions was most likely contributed by anthocyanins. AIS prepared from freeze dried blueberry powder were washed with ethanol and acetone to remove all free pigments and sugars. All the pectin rich fractions were extensively dialyzed against deionized water in dialysis tubings with 6-8 kDa molecular cutoff. Most anthocyanin pigments were removed during AIS preparation, since organic solvents are used for anthocyanin extraction (Bakker et al., 1997; Chen & Camire, 1997). Any residual free anthocyanins would have been removed during dialysis. There may be tightly bound anthocyanin pigments on blueberry pectin rich fractions. The natural anthocyanin may bind tightly on the surface of pectin rich fractions through hydrogen bond and/or hydrophobic interactions, similar to staining mechanisms between natural colorants and cotton (Holme, 2002; Padayachee et al., 2013).

Binding with C3G

The initial anthocyanin content of C3G solutions, and free anthocyanin content the control and experimental groups of the study conducted on pectin rich fractions and C3G were summarized in Table 3.2. The initial anthocyanin concentration was adjusted to around 60 μg/mL, and after incubation, the control anthocyanin concentration increased, from 59-61 μg/mL

to 63-66 μg/mL. The pigment increase was mainly contributed by anthocyanin intermolecular copigmentation. Copigmentation is a phenomenon that the pigments form molecular or complex associations with other organic compounds, or metal ions and result in a change or increment of color intensity (Boulton, 2001). Anthocyanin copigmentation is the main mechanism of stabilizing color pigment in plants (Davies & Mazza, 1993). Compounds that have aromatic rings can vertically stack on anthocyanin aromatic nuclei through hydrophobic interaction or aromatic interaction and sugar moieties can stabilize the vertically stacked anthocyanin aglycones by superimposing them with hydrogen bonds (Goto & Kondo, 1991). In the experimental groups, in which anthocyanin solutions were incubated with pectin rich fractions at pH 2-4.5, free anthocyanin concentration reduced significantly after incubation, from 63-66 μg/mL to 15-46 μg/mL (Table 3.2). All pectin rich fractions bound anthocyanin pigments, allowing less free anthocyanin in the filtrates. At pH 2 and pH 3.6, CSF allowed the lowest free pigment content of 14.5 μg/mL and 19.0 μg/mL respectively, comparing to WSF and NSF. At pH 4.5, WSF and CSF allowed the same amount of free anthocyanin pigment of around 46 μg/mL, while NSF allowed lower anthocyanin concentration of 40.3 μg/mL. Across all pH values, WSF bound the least amount of anthocyanin, comparing to CSF and NSF, and it might be due to the high amount of endogenous anthocyanins WSF contained. CSF bound more C3G pigment than WSF and NSF at pH 2 and pH 3.6, while NSF bound more C3G pigment pH 4.5. CSF had better anthocyanin binding ability at low pH values, while NSF bound anthocyanin better at higher pH.

The bound anthocyanin percentage was calculated for each pectin rich fraction and each pH value to compare the effect of pH (Figure 3.2). At pH 4.5, all three pectin rich fractions

bound 27-37% anthocyanins, while at pH 2-3.6, 33-78% anthocyanins were bound (Figure 3.2). Pectin rich fractions bound less anthocyanins at pH 4.5 than at pH 2-3.6. WSF and NSF bound about 20% and 10% more C3G pigment respectively at pH 3.6 than at pH 2. CSF bound about 10% more C3G pigments at pH 2 than at pH 3.6. The binding between pectin and anthocyanins are pH dependent. At lower pH, anthocyanin flavylium cations with positive charge are more prevalent than chargeless quinoidal base, carbinol pseudo base or chalcone forms, while at weak acidic pH, chargeless anthocyanin forms are more prevalent (Castañeda-Ovando et al., 2009). The apparent pKa of pectin is near 3.5-4.5 (Sperber, Schols, Cohen Stuart, Norde, & Voragen, 2009), therefore, at pH 2, free carboxyl groups are partially protonated. All blueberry pectin rich fractions characterized in this study were low methoxyl pectin with DE value below 50%. Ionic interaction between negatively charged carboxyl groups and anthocyanin positively charge flavylium cations may be the main mechanism, if not the only mechanism, for pectin and anthocyanin binding. Ionic interaction has been suggested in most studies involving pectin anthocyanin interaction (Buchweitz, Nagel, et al., 2012; Mazzaracchio, Pifferi, Kindt, Munyaneza, & Barbiroli, 2004; Padayachee et al., 2012). The variation of anthocyanin binding percentage at pH 2 and pH 3.6 of all pectin rich fractions could be explained by the amount of pectic free carboxyl groups.

Initial C3G concentrations were nearly doubled to around 105 μg/mL and binding between pectin rich fractions and C3G were evaluated (Table 3.3). The control pigment concentration increased from 103-108 μg/mL initially to 109-126 μg/mL after incubation. The pigment increase was due to anthocyanin intermolecular copigmentation explained above. All experimental groups had reduced free C3G pigment content, from 109-126 μg/mL for the control

groups to 17-79 μg/mL for the experimental groups (Table 3.3). At pH 2 and pH 3.6, CSF allowed the lowest free anthocyanins of 16.5 μg/mL and 23.0 μg/mL respectively comparing to WSF and NSF. At pH 4.5, WSF and CSF allowed 76-79 μg/mL free anthocyanins, while NSF allowed lower the free anthocyanins of 69.1 μg/mL. Similar to the study conducted at lower C3G concentration, across all pH values, WSF bound the least amount of anthocyanin, comparing to CSF and NSF, and CSF bound more anthocyanins at pH 2-3.6, while NSF bound more anthocyanins at pH 4.5.

The bound anthocyanin percentage was calculated for each pectin rich fraction and each pH value to compare the effect of pH (Figure 3.3). At pH 4.5, all three pectin rich fractions bound 29-37% anthocyanins, while at pH 2-3.6, 39-87% anthocyanins were bound. Similar to the binding study conducted at lower C3G concentration, at pH 4.5, less anthocaynins were bound than at lower pH values. Percentage wise, the bound anthocyanins for each pectin rich fractions and each pH combination did not decrease when the C3G concentration was nearly doubled. The bound anthocyanin percentage at pH 2 increased 3-9%, the bound anthocyanin percentage at pH 3.6 increased 16-28%, and the bound anthocyanin percentage at pH 4.5 increased 1-3%. It is clear that the binding sites of all pectin rich fractions were not saturated when C3G pigment concentration was at around 60 μg/mL. Padayachee et al. (2012) evaluated anthocyanin binding of their cell wall analogue composites consisted of pectin and cellulose and concluded the anthocyanin binding to plant cell walls was not limited to the bindings sites. They proposed a two phase binding model with initial anthocyanin binding onto actives sites followed by anthocyanin stacking, which accounted for the major amount. At low pH, the prevalent anthocyanin flavylium cation has the aromatic A ring and heterocyclic C ring next to it on the

same plane, while having the aromatic B ring on another plane. Anthocyanin quinoidal base form, which populates as pH increases, has all three rings on the same plane (Goto & Kondo, 1991), and facilitates anthocyanin stacking. Anthocyanin stacking might be associated with pectin and anthocyanin binding. The binding 16-28% binding percentage increase at pH 3.6 may be contributed by the stacking of quinoidal base forms.

Binding with Anthocyanin Aglycones

The binding between pectin rich fractions and anthocyanin aglycones was evaluated and the initial pigment concentration, free anthocyanin in the anthocyanin control and experimental groups of study conducted on pectin rich fractions and five anthocyanin aglycone solutions were summarized in Table 3.4. The study was conducted only at pH 2, due to the extremely poor solubility of aglycones at higher pH values. Anthocyanins in nature are usually in glycosidic forms and are water soluble (Castañeda-Ovando et al., 2009; Wrolstad, Durst, & Lee, 2005). Without sugar moieties, anthocyanin aglycones have poor solubility in water. After incubation, pigment content of the control groups reduced from 58-75 μg/mL initially to 32-48 μg/mL (Table 3.4). Pelargonidin solution had the highest reduction of 58%, while delphinidin had the lowest pigment reduction of 22%. Pelargonidin has one hydroxyl group on aromatic B ring, and while delphinidin had three hydroxyl groups. Increased hydroxylation of B ring of anthocyanin aglycone can stabilize anthocyanidins (Dao, Takeoka, Edwards, & Berrios, 1998). Monomeric anthocyanins are highly instable and are susceptible to degradation (Castañeda-Ovando et al., 2009). Glycosidic substitution increases anthocyanin stability (Wrolstad et al., 2005), while anthocyanin aglycones without sugar substitution are susceptible to formation of diketone, a substrate to be further degraded into phenolic acid and aldehyde (Fleschhut et al., 2006). In the

experimental groups, all anthocyanidin aglycone solutions had dramatic free anthocyanin reduction, comparing to the control groups (Table 3.4). CSF allowed close to zero free pigment in pelargonidin and malvidin solutions and very minimal pigment in cyanidin, peonidin and delphinidin solutions. WSF and NSF allowed 9-16 μg/mL free pigment in all anthocyanin aglycone solutions. WSF allowed slightly higher free pigment for cyanidin and malvidin solutions than NSF, and both WSF and NSF allowed the same free pigment amount content for the rest three aglycone solutions.

Among all five anthocyanin aglycone solutions, all pectin rich fractions showed very similar binding patterns, with CSF binding close to 100% pigments and WSF and NSF binding 58-78% pigments (Figure 3.4). Pearson correlation analysis was performed for pigment binding percentage of WSF and NSF, between the number of hydroxyl groups and methoxyl groups on anthocyanin B ring (data not shown). WSF and five anthocyanin aglycone binding percentage had strong positive correlation between the number of hydroxyl groups in aglycone B ring ($r=0.7198$), and strong negative correlation between the number of methoxyl groups ($r=-0.8745$). NSF and aglycone binding percentage showed moderate positive correlation between number of hydroxyl groups ($r=0.4585$), and strong negative correlation between number of methoxyl groups ($r=-0.6850$). The number of hydroxyl substitution may enhance the binding between WSF and NSF, and anthocyanin aglycone forms; the number of methoxyl groups negatively impacted the binding. Regardless of the slight impact of structural differences on binding percentage, WSF bound 58-74% pigment in all aglycone solutions and NSF bound 66-78% (Figure 3.4). The substitution pattern of the aromatic B ring does not greatly account for the majority of the bound anthocyanins.

Binding with Blueberry Juice Anthocyanins

Blueberry juice prepared from freeze dried blueberry powder was incubated with pectin rich fractions at pH 2-4.5 to see if pectin rich fractions bind with blueberry juice anthocyanins. The initial anthocyanin concentration, and free anthocyanin in the control and experimental groups of experiments conducted on pectin rich fraction and blueberry juice were summarized in Table 3.5. After incubation, the anthocyanin concentration in the control groups increased from 41-42 μg/mL to 44-48 μg/mL. The control pigment increase after incubation was due to anthocyanin intermolecular copigmentation and copigmentation with organic acids naturally presented in blueberry. The blueberry contained petunidin-3-glucoside, peonidin-3-glucoside, delphinidin-3-glucoside and cyanidin-3-galactoside in major amount, and cyanidin-3-glucoside, peonidin-3-galactoside and malvidin-3-glucoside in minor amount (Yi, Fischer, Krewer, & Akoh, 2005). Gallic acid, caffeic acid, *p*-coumaric acid, ferulic acid, catechin, quercetin and kaempferol are some phenolic acids found in blueberry (Yi, Akoh, Fischer, & Krewer, 2006). All the experimental groups had reduced free anthocyanin content of 17-33 μg/mL (Table 3.5). At pH 2, CSF allowed lowest free anthocyanins of 17.4 μg/mL comparing to other two pectin rich fractions. At pH 3.6, CSF and NSF allowed the lowest free pigment content of 18-18.5 μg/mL. At pH 4.5, NSF allowed the lowest free pigment content of 26.0 μg/mL. The binding between each pectin rich fraction and blueberry juice anthocyanins was similar to that of C3G solutions. Across all pH values, WSF allowed the highest amount of free anthocyanin, comparing to CSF and NSF. WSF bound the least amount of blueberry juice anthocyanins at all pH values. At lower pH values of 2 and 3.6, CSF allowed low free anthocyanin comparing to WSF and NSF, whole at pH values of 3.6 and 4.5, NSF allowed the low amount of free anthocyanins. CSF

tended to bind more blueberry juice anthocyanin at the lower pH values, and NSF tended to bind more at the higher pH values.

The bound blueberry juice anthocyanin percentage wise was calculated for each pectin rich fraction and each pH value so that the effect of pH can be compared (Figure 3.5). In general, at pH 4.5, all pectin rich fractions bound the least amount of anthocyanins, comparing to binding at pH 2-3.6. Except for WSF at pH 2, all pectin rich fractions bound more anthocyanins at pH 2 and pH 3.6 than at pH 4.5. The blueberry juice anthocyanin binding pattern across difference pH values was similar to that in C3G studies. Ionic interaction between anthocyanin flavylium cations and petic carboxyl groups is the main mechanism for pectin and anthocyanin binding. Note that anthocyanin content to pectin content ratio used in this study was about 1:10 on weight basis, which was similar to the ratio of anthocyanin to pectin content in whole blueberries (Chen & Camire, 1997). When blueberry is consumed as a whole berry, a large amount of blueberry anthocyanins may bind to blueberry pectin in stomach (pH 2) and reduce the bioaccesssibility of anthocyanin for absorption in the small intestine. According to a recent study conducted on cell wall composites consisted of pectin and cellulose, bound anthocyanin on cell wall composites had minimal release in gastric and intestinal fluids (Padayachee et al., 2013). The bound pectin and anthocyanin mixture may be utilized by colonic microflora once reaching the colon.

Conclusion

In this study, direct binding between blueberry pectin rich fractions and anthocyanins were demonstrated. All blueberry pectin rich fractions reduced the free anthocyanin pigment content in C3G solutions, anthocyanin aglycone solutions and blueberry juice. The binding is pH dependent, with the lowest amount anthocyanin binding at pH 4.5. Nearly doubling C3G

concentration increased binding percentage by 16-23% at pH 3.6. Ionic interaction between positively charge anthocyanin flavylium cations and free pectic carboxyl groups and anthocyanin aromatic stacking on bound anthocyanins might be the two major mechanisms for anthocyanin and pectin binding. WSF had endogenous anthocyanins and had relatively poor anthocyanin binding ability. CSF had relatively strong anthocyanin binding ability at pH 2-3.6, while NSF had relatively strong anthocyanin binding ability at pH 3.6-4.5. The pectin bound anthocyanins may have enhanced bioavailability for colonic microflora fermentation and promoting colonic health.

Table 3.1. Characterization of blueberry pectin rich fractions, water soluble fraction (WSF), chelator soluble fraction (CSF) and sodium carbonate soluble fraction (NSF).

	UA (µg/mg)	TS (µg/mg)	Protein (µg/mg)	DE (%)	Mw (kDa)	Rw (nm)	Polydispersity	RMS conformation plot slope
WSF	$516^{c} \pm 7$	$328^{b} \pm 4$	$26^{b} \pm 1$	36	$602^{a} \pm 29$	$75.2^{a} \pm 2.2$	1.6 ± 0.2	0.25 ± 0.04
CSF	$627^{a} \pm 19$	$279^{c} \pm 5$	$28^{b} \pm 1$	28	$485^{b} \pm 28$	$53.8^{b} \pm 0.2$	1.9 ± 0.1	0.14 ± 0.03
NSF	$580^{b} \pm 19$	$496^{a} \pm 9$	$238^{a} \pm 9$	26	$169^{c} \pm 4$	$35.4^{c} \pm 0.8$	1.6 ± 0.0	0.14 ± 0.01

Means of uronic acid (UA), total sugar (TS), protein, Weight average molecular weight (Mw) and weight average root mean square radius (Rw) are significantly different ($P<0.05$) if they do not share the same superscript in the same column. DE stands for degree of methylesterification.

Table 3.2. Cyanidin-3-glucoside (C3G) equivalent anthocyanin pigment content (μg/mL) of initial C3G solutions, and free anthocyanin in the C3G control solutions and experimental groups of the study conducted on C3G solutions and blueberry pectin rich fractions, water soluble fraction (WSF), chelator soluble fraction (CSF) and sodium carbonate soluble fraction (NSF) at pH 2-4.5.

	pH 2	pH 3.6	pH 4.5
C3G Initial	$61^{b} \pm 1.5$	$59.8^{b} \pm 0.3$	$58.5^{b} \pm 0.5$
C3G Control	$65.9^{a} \pm 1.1$	$63.2^{a} \pm 1.3$	$63.2^{a} \pm 0.3$
WSF+C3G	$44.4^{c} \pm 0.9$	$29.9^{c} \pm 0.5$	$46.0^{c} \pm 4.2$
CSF+C3G	$14.5^{e} \pm 2.9$	$19.0^{e} \pm 0.1$	$45.5^{c} \pm 1.5$
NSF+C3G	$31.4^{d} \pm 0.5$	$25.6^{d} \pm 1.2$	$40.3^{d} \pm 2.3$

Means of pigment content are significantly different ($P<0.05$) if they share different superscripts in the same column.

Table 3.3. Cyanidin-3-glucoside (C3G) equivalent anthocyanin pigment content (μg/mL) of initial C3G* solutions, and free anthocyanin in the C3G* control solutions and experimental groups of the study conducted on C3G* solutions and blueberry pectin rich fractions, water soluble fraction (WSF), chelator soluble fraction (CSF) and sodium carbonate soluble fraction (NSF) at pH 2-4.5.

	pH2	pH3.6	pH4.5
C3G* Initial	$108.3^{b} \pm 0.8$	$104.3^{b} \pm 1.2$	$103.0^{b} \pm 0.9$
C3G* Control	$125.9^{a} \pm 2.9$	$109.0^{a} \pm 3.9$	$110.5^{a} \pm 1.7$
WSF+C3G*	$77.5^{c} \pm 3.0$	$44.9^{c} \pm 1.2$	$78.9^{c} \pm 1.4$
CSF+C3G*	$16.5^{e} \pm 1.6$	$23.0^{e} \pm 0.8$	$75.9^{d} \pm 0.7$
NSF+C3G*	$55.7^{d} \pm 3.4$	$30.2^{d} \pm 0.1$	$69.1^{e} \pm 0.4$

* indicates the experiments were conducted with elevated C3G pigment concentration. Means of pigment content are significantly different ($P<0.05$) if they share different superscripts in the same column.

Table 3.4. Cyanidin-3-glucoside (C3G) equivalent anthocyanin pigment content (μg/mL) of initial anthocyanin aglycone solutions, cyanidin (CYD), pelargonidin (PG), malvidin (MV), peonidin (PN) and delphinidin (DP), and free anthocyanin in the control anthocyanin aglycone solutions and experimental groups of the study conducted on five anthocyanin aglycone solutions and blueberry pectin rich fractions, water soluble fraction (WSF), chelator soluble fraction (CSF) and sodium carbonate soluble fraction (NSF) at pH 2.

	CYD	PG	MV	PN	DP
Initial	$74^{a} \pm 0.0$	$74.7^{a} \pm 2.5$	$72.2^{a} \pm 0.6$	$57.5^{a} \pm 3.3$	$61.8^{a} \pm 4.0$
Control	$45.4^{b} \pm 1.8$	$31.7^{b} \pm 1.2$	$38.4^{b} \pm 1.0$	$35.4^{b} \pm 0.7$	$48.2^{b} \pm 3.1$
WSF	$12.7^{c} \pm 0.2$	$9.9^{c} \pm 1.5$	$16.2^{c} \pm 1.2$	$13.3^{c} \pm 1.0$	$12.6^{c} \pm 0.6$
CSF	$0.6^{e} \pm 0.1$	$1.2^{d} \pm 1.4$	$-0.2^{e} \pm 0.3$	$0.5^{d} \pm 0.3$	$3.2^{d} \pm 0.2$
NSF	$9.9^{d} \pm 0.2$	$8.8^{c} \pm 1.5$	$12.9^{d} \pm 0.7$	$12.2^{c} \pm 0.6$	$13.4^{c} \pm 1.8$

Means of pigment content are significantly different ($P<0.05$) if they share different superscripts in the same column.

Table 3.5. Cyanidin-3-glucoside (C3G) equivalent anthocyanin pigment content (μg/mL) of initial blueberry juice (BJ), and free anthocyanin in the BJ control and experimental groups of study conducted on BJ and blueberry pectin rich fractions, water soluble fraction (WSF), chelator soluble fraction (CSF) and sodium carbonate soluble fraction (NSF) at pH 2-4.5.

	pH2	pH3.6	pH4.5
BJ Initial	$41.0^{b} \pm 0.8$	$40.2^{b} \pm 0.8$	$41.9^{b} \pm 1.5$
BJ Control	$44.7^{a} \pm 2.2$	$44.1^{a} \pm 0.9$	$47.8^{a} \pm 1.4$
WSF+BJ	$30.9^{c} \pm 0.6$	$25.3^{c} \pm 1.1$	$32.6^{c} \pm 0.6$
CSF+BJ	$17.4^{e} \pm 0.2$	$18.0^{d} \pm 0.8$	$33.1^{c} \pm 0.6$
NSF+BJ	$20.3^{d} \pm 0.9$	$18.5^{d} \pm 0.2$	$26.0^{d} \pm 0.9$

Means of pigment content are significantly different ($P<0.05$) if they share different superscripts in the same column.

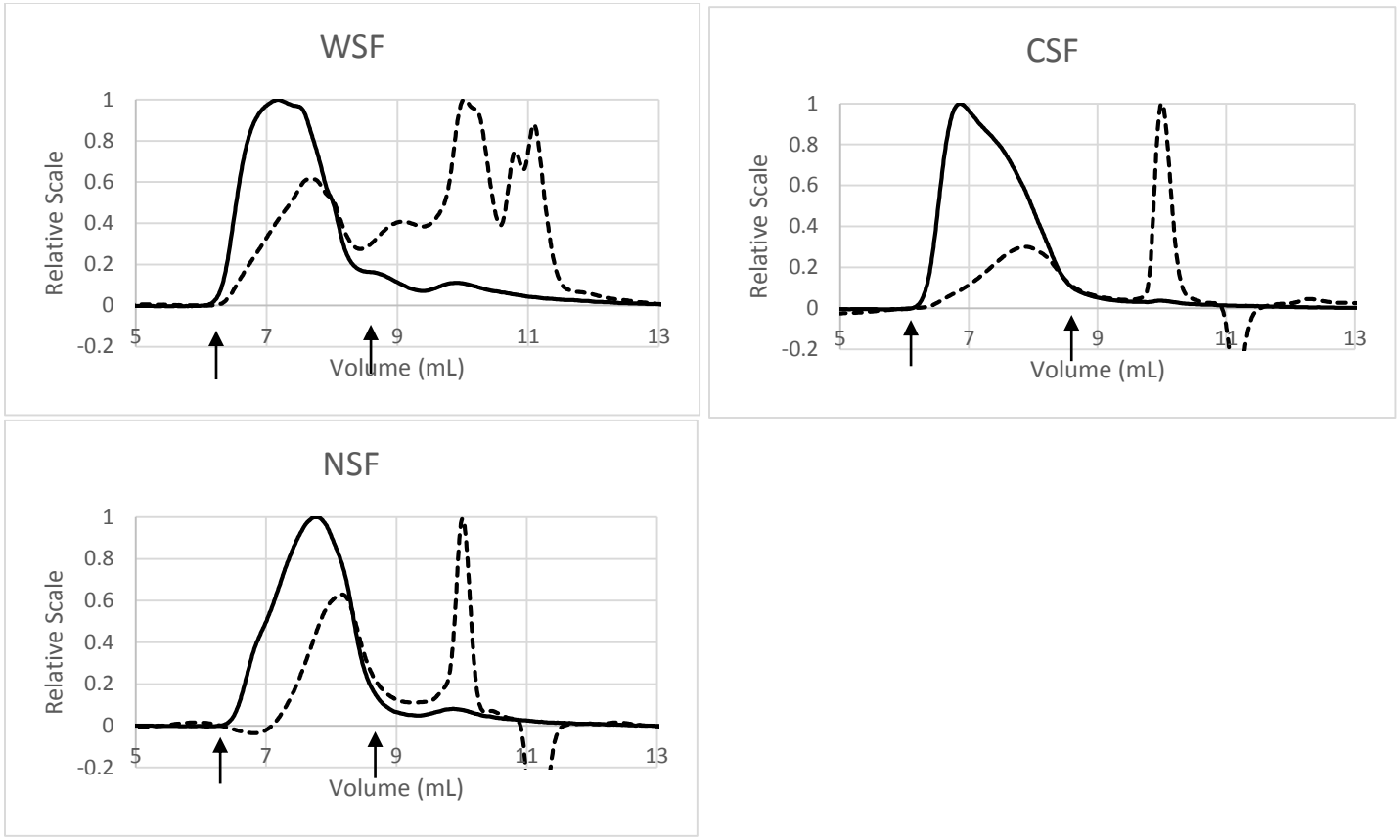

Figure 3.1. Representative eluting profiling of water soluble fraction (WSF), chelator soluble fraction (CSF) and sodium carbonate soluble fraction (NSF). Solid line indicates light scattering (LS) signal and dotted line indicates refractive index (RI) signal. All the scales were adjusted to relative scale. Areas between two arrows are peak areas for weight average molecular weight (Mw), weight average root mean square radius (Rw), and polydispersity calculation.

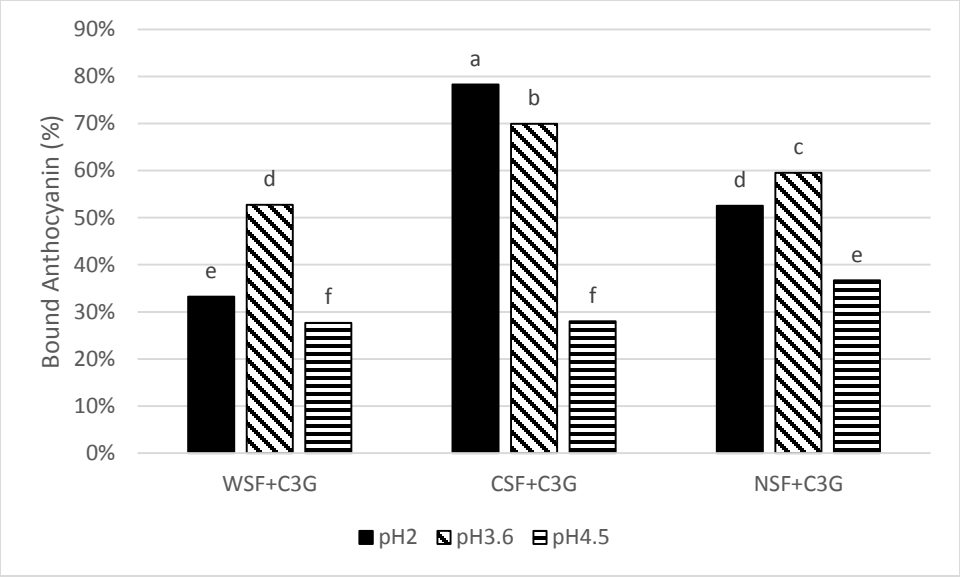

Figure 3.2. Bound anthocyanin pigment percentage for cyanidin-3-glucoside (C3G) with initial anthocyanin pigment concentration of around 60 μg/mL and three blueberry pectin rich fractions, water soluble fraction (WSF), chelator soluble fraction (CSF) and sodium carbonate soluble fraction (NSF) at pH 2-4.5. Bars with different pattern fills indicate different pH values. Bars with different letters are significantly different ($P<0.05$) in the same graph.

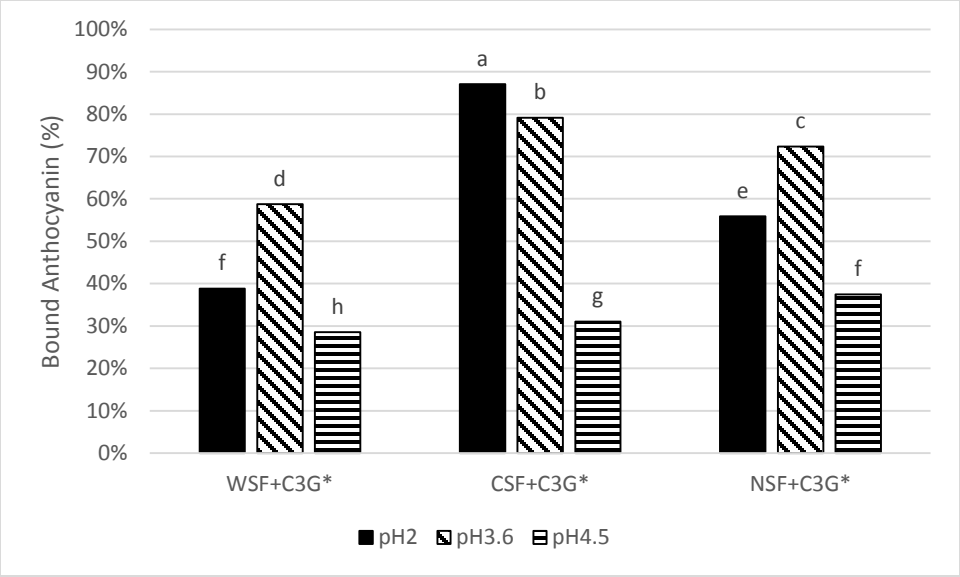

Figure 3.3. Bound anthocyanin pigment percentage for cyanidin-3-glucoside (C3G*) with initial anthocyanin pigment concentration of around 105 μg/mL and three blueberry pectin rich fractions, water soluble fraction (WSF), chelator soluble fraction (CSF) and sodium carbonate soluble fraction (NSF) at pH 2-4.5. * indicates the experiments were conducted with elevated C3G pigment concentration. Bars with different pattern fills indicate different pH values. Bars with different letters are significantly different ($P<0.05$) in the same graph.

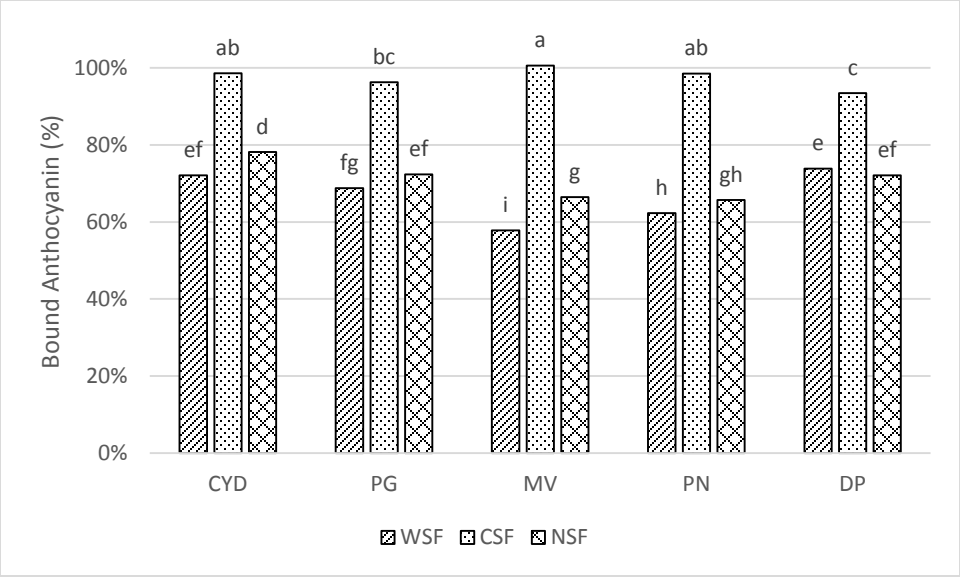

Figure 3.4. Bound anthocyanin pigment percentage of cyanidin (CYD), pelargonidin (PG), malvidin (MV), peonidin (PN) and delphinidin (DP) and three blueberry pectin rich fractions, water soluble fraction (WSF), chelator soluble fraction (CSF) and sodium carbonate soluble fraction (NSF) at pH 2. Bars with different pattern fills indicates different pectin rich fractions. Bars with different letters are significantly different ($P<0.05$) in the same graph.

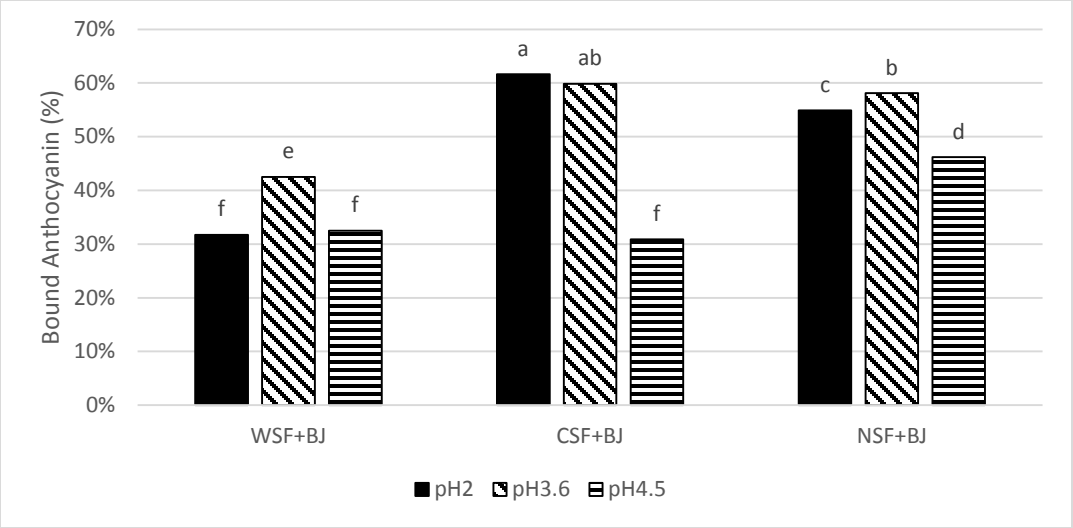

Figure 3.5. Bound anthocyanin pigment percentage for blueberry juice (BJ) and three blueberry pectin rich fractions, water soluble fraction (WSF), chelator soluble fraction (CSF) and sodium carbonate soluble fraction (NSF) at pH 2-4.5. Bars with different pattern fills indicate different pH values. Bars with letters are significantly different ($P<0.05$) in the same graph.

References

Asen, S., Stewart, R. N., Norris, K. H., & Massie, D. R. (1970). A stable blue non-metallic co-pigment complex of delphanin and C-glycosylflavones in Prof. Blaauw Iris. *Phytochemistry, 9*(3), 619-627. doi: http://dx.doi.org/10.1016/S0031-9422(00)85702-7

Bakker, J., Bridle, P., Honda, T., Kuwano, H., Saito, N., Terahara, N., & Timberlake, C. F. (1997). Identification of an anthocyanin occurring in some red wines. *Phytochemistry, 44*(7), 1375-1382. doi: http://dx.doi.org/10.1016/S0031-9422(96)00707-8

Bayer, E., Egeter, H., Fink, A., Nether, K., & Wegmann, K. (1966). Complex Formation and Flower Colors. *Angewandte Chemie International Edition in English, 5*(9), 791-798. doi: 10.1002/anie.196607911

Boulton, R. (2001). The Copigmentation of Anthocyanins and Its Role in the Color of Red Wine: A Critical Review. *American Journal of Enology and Viticulture, 52*(2), 67-87.

Buchweitz, M., Carle, R., & Kammerer, D. R. (2012). Bathochromic and stabilising effects of sugar beet pectin and an isolated pectic fraction on anthocyanins exhibiting pyrogallol and catechol moieties. *Food Chemistry, 135*(4), 3010-3019. doi: http://dx.doi.org/10.1016/j.foodchem.2012.06.101

Buchweitz, M., Nagel, A., Carle, R., & Kammerer, D. R. (2012). Characterisation of sugar beet pectin fractions providing enhanced stability of anthocyanin-based natural blue food colourants. *Food Chemistry, 132*(4), 1971-1979. doi: 10.1016/j.foodchem.2011.12.034

Buchweitz, M., Speth, M., Kammerer, D. R., & Carle, R. (2013). Impact of pectin type on the storage stability of black currant (Ribes nigrum L.) anthocyanins in pectic model

solutions. *Food Chemistry, 139*(1–4), 1168-1178. doi: http://dx.doi.org/10.1016/j.foodchem.2013.02.005

Castañeda-Ovando, A., Pacheco-Hernández, M. d. L., Páez-Hernández, M. E., Rodríguez, J. A., & Galán-Vidal, C. A. (2009). Chemical studies of anthocyanins: A review. *Food Chemistry, 113*(4), 859-871. doi: http://dx.doi.org/10.1016/j.foodchem.2008.09.001

Chatjigakis, A. K., Pappas, C., N.Proxenia, O.Kalantzi, P.Rodis, & Polissiou, M. (1998). FT-IR spectroscopic determination of the degree of esterification of cell wall pectins from stored peaches and correlation to textural changes. *Carbohydrate Polymers, 37*(4), 395-408. doi: http://dx.doi.org/10.1016/S0144-8617(98)00057-5

Chen, H.-C., & Camire, M. E. (1997). RECOVERY OF ANTHOCYANINS, PECTIN, AND DIETARY FIBER FROM CULL LOWBUSH BLUEBERRIES. *Journal of Food Quality, 20*(3), 199-209. doi: 10.1111/j.1745-4557.1997.tb00464.x

Corredig, M., Kerr, W., & Wicker, L. (2000). Molecular characterization of commercial pectins by separation with linear mix gel permeation columns in-line with multi-angle light scattering detection. *Food Hydrocolloids, 14*(1), 41-47. doi: 10.1016/S0268-005X(99)00044-2

Dao, L. T., Takeoka, G. R., Edwards, R. H., & Berrios, J. D. J. (1998). Improved Method for the Stabilization of Anthocyanidins. *Journal of Agricultural and Food Chemistry, 46*(9), 3564-3569. doi: 10.1021/jf980359v

Davies, A. J., & Mazza, G. (1993). Copigmentation of simple and acylated anthocyanins with colorless phenolic compounds. *Journal of Agricultural and Food Chemistry, 41*(5), 716-720. doi: 10.1021/jf00029a007

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry, 28*, 350-356.

Fleschhut, J., Kratzer, F., Rechkemmer, G., & Kulling, S. (2006). Stability and biotransformation of various dietary anthocyanins in vitro. *European Journal of Nutrition, 45*(1), 7-18. doi: 10.1007/s00394-005-0557-8

Giusti, M. M., & Wrolstad, R. E. (2001). Characterization and Measurement of Anthocyanins by UV-Visible Spectroscopy *Current Protocols in Food Analytical Chemistry*: John Wiley & Sons, Inc.

Goto, T., & Kondo, T. (1991). Structure and Molecular Stacking of Anthocyanins—Flower Color Variation. *Angewandte Chemie International Edition in English, 30*(1), 17-33. doi: 10.1002/anie.199100171

Holme, I. (2002). Recent developments in colorants for textile applications. *Surface Coatings International Part B: Coatings Transactions, 85*(4), 243-264. doi: 10.1007/BF02699548

Holzwarth, M., Korhummel, S., Siekmann, T., Carle, R., & Kammerer, D. R. (2013). Influence of different pectins, process and storage conditions on anthocyanin and colour retention in strawberry jams and spreads. *LWT - Food Science and Technology, 52*(2), 131-138. doi: http://dx.doi.org/10.1016/j.lwt.2012.05.020

Jung, J., Arnold, R. D., & Wicker, L. (2013). Pectin and charge modified pectin hydrogel beads as a colon-targeted drug delivery carrier. *Colloids Surf B Biointerfaces, 104*, 116-121. doi: 10.1016/j.colsurfb.2012.11.042

Jung, J., & Wicker, L. (2012). Laccase mediated conjugation of sugar beet pectin and the effect on emulsion stability. *Food Hydrocolloids, 28*(1), 168-173. doi: 10.1016/j.foodhyd.2011.12.021

Kahle, K., Kraus, M., Scheppach, W., Ackermann, M., Ridder, F., & Richling, E. (2006). Studies on apple and blueberry fruit constituents: do the polyphenols reach the colon after ingestion? *Mol Nutr Food Res, 50*(4-5), 418-423. doi: 10.1002/mnfr.200500211

Keppler, K., & Humpf, H.-U. (2005). Metabolism of anthocyanins and their phenolic degradation products by the intestinal microflora. *Bioorganic & Medicinal Chemistry, 13*(17), 5195-5205. doi: 10.1016/j.bmc.2005.05.003

Kim, Y., Teng, Q., & Wicker, L. (2005). Action pattern of Valencia orange PME de-esterification of high methoxyl pectin and characterization of modified pectins. *Carbohydr Res, 340*(17), 2620-2629. doi: 10.1016/j.carres.2005.09.013

Kong, J.-M., Chia, L.-S., Goh, N.-K., Chia, T.-F., & Brouillard, R. (2003). Analysis and biological activities of anthocyanins. *Phytochemistry, 64*(5), 923-933. doi: http://dx.doi.org/10.1016/S0031-9422(03)00438-2

Kopjar, M., Pilizota, V., Tiban, N. N., Subaric, D., Babic, J., Ackar, E., & Sajdl, M. (2009). Strawberry jams: influence of different pectins on colour and textural properties. *CZECH JOURNAL OF FOOD SCIENCES, 27*, 20-28.

Lila, M. A. (2004). Anthocyanins and human health: an in vitro investigative approach. *Journal of Biomedicine & Biotechnology, 2004*(5), 306-313.

Maxwell, E. G., Belshaw, N. J., Waldron, K. W., & Morris, V. J. (2012). Pectin – An emerging new bioactive food polysaccharide. *Trends in Food Science & Technology, 24*(2), 64-73. doi: 10.1016/j.tifs.2011.11.002

Mazzaracchio, P., Pifferi, P., Kindt, M., Munyaneza, A., & Barbiroli, G. (2004). Interactions between anthocyanins and organic food molecules in model systems. *International Journal of Food Science & Technology, 39*(1), 53-59. doi: 10.1111/j.1365-2621.2004.00747.x

Melton, L. D., & Smith, B. G. (2001). Determination of the Uronic Acid Content of Plant Cell Walls Using a Colorimetric Assay *Current Protocols in Food Analytical Chemistry*: John Wiley & Sons, Inc.

Padayachee, A., Netzel, G., Netzel, M., Day, L., Mikkelsen, D., & Gidley, M. (2013). Lack of release of bound anthocyanins and phenolic acids from carrot plant cell walls and model composites during simulated gastric and small intestinal digestion. *Food Funct, 4*(6), 906-916. doi: 10.1039/C3FO60091B

Padayachee, A., Netzel, G., Netzel, M., Day, L., Zabaras, D., Mikkelsen, D., & Gidley, M. J. (2012). Binding of polyphenols to plant cell wall analogues – Part 1: Anthocyanins. *Food Chemistry, 134*(1), 155-161. doi: http://dx.doi.org/10.1016/j.foodchem.2012.02.082

Sperber, B. L. H. M., Schols, H. A., Cohen Stuart, M. A., Norde, W., & Voragen, A. G. J. (2009). Influence of the overall charge and local charge density of pectin on the complex formation between pectin and β-lactoglobulin. *Food Hydrocolloids, 23*(3), 765-772. doi: http://dx.doi.org/10.1016/j.foodhyd.2008.04.008

Stephen, A. M., & Phillips, G. O. (2010). *Food polysaccharides and their applications*: CRC Press.

Vicente, A. R., Greve, L. C., Labavitch, J. M., Powell, A. L. T., Ortugno, C., & Rosli, H. (2007). Temporal Sequence of Cell Wall Disassembly Events in Developing Fruits. 2. Analysis

of Blueberry (Vaccinium Species) [electronic resource]. *Journal of Agricultural and Food Chemistry, 55*(10), 4125-4130. doi: http://dx.doi.org/10.1021/jf063548j

Voragen, A. G., Coenen, G. J., Verhoef, R. P., & Schols, H. A. (2009). Pectin, a versatile polysaccharide present in plant cell walls. *Structural Chemistry, 20*(2), 263-275.

Wallace, T. C. (2011). Anthocyanins in Cardiovascular Disease. *Advances in Nutrition: An International Review Journal, 2*(1), 1-7. doi: 10.3945/an.110.000042

Wicker, L., Kim, Y., Kim, M.-J., Thirkield, B., Lin, Z., & Jung, J. (2014). Pectin as a bioactive polysaccharide – extracting tailored function from less. *Food Hydrocolloids*(0). doi: http://dx.doi.org/10.1016/j.foodhyd.2014.01.002

Wong, T. W., Colombo, G., & Sonvico, F. (2011). Pectin matrix as oral drug delivery vehicle for colon cancer treatment. *AAPS PharmSciTech, 12*(1), 201-214. doi: 10.1208/s12249-010-9564-z

Wrolstad, R. E., Durst, R. W., & Lee, J. (2005). Tracking color and pigment changes in anthocyanin products. *Trends in Food Science & Technology, 16*(9), 423-428. doi: 10.1016/j.tifs.2005.03.019

Yi, W., Akoh, C. C., Fischer, J., & Krewer, G. (2006). Effects of phenolic compounds in blueberries and muscadine grapes on HepG2 cell viability and apoptosis. *Food Research International, 39*(5), 628-638. doi: http://dx.doi.org/10.1016/j.foodres.2006.01.001

Yi, W., Fischer, J., Krewer, G., & Akoh, C. C. (2005). Phenolic compounds from blueberries can inhibit colon cancer cell proliferation and induce apoptosis. *Journal of Agricultural and Food Chemistry, 53*(18), 7320-7329.

CHAPTER 4

CONCLUSIONS

Blueberry pectin rich fractions extracted from blueberry contained 50-60% uronic acids and were not rich source of pectin. Pectin rich fractions contained co-extracted xyloglucans and high amount of arabinogalactans, which may be associated with pectic hairy region. NSF contained high amount of proteins, which may indicate the presence of arabinogalactan proteins. The pectin rich fractions extracted with water and chelator buffer retained the intact pectin structure with high molecular weight, and the fraction extracted degradative weak alkali had low molecular weight. All blueberry pectin rich fractions bound anthocyanins and reduced free anthocyanin content when incubated with anthocyanin rich solutions. The binding was pH independent, with higher binding at low pH 2-3.6, suggesting ionic interaction between pectic free carboxyl groups and anthocyanin flavylium cations may be the main binding mechanism. Nearly doubling C3G concentration increased binding percentage by 16-23%, suggesting anthocyanin stacking may be involved. WSF had endogenous anthocyanins and had relatively poor anthocyanin binding ability. CSF had relatively strong anthocyanin binding at pH 2-3.6, while NSF had relatively strong anthocyanin binding ability at pH 3.6-4.5.