Intermolecular Binding of Blueberry Pectin-rich Fractions and Anthocyanin

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Abstract

Pectin was extracted from blueberry powder into three fractions of water soluble (WSF), chelator soluble (CSF) and sodium carbonate soluble (NSF). The fractions were incubated with cyanidin-3-glucoside (C3G), a mixture of five anthocyanidins (cyanidin, pelargonidin, malvidin, petunidin and delphinidin) or blueberry juice at pH 2.0-4.5. Free anthocyanins and bound anthocyanin-pectin mixtures were separated by ultrafiltration. WSF bound the least amount of anthocyanin at all pH values. CSF had stronger anthocyanin binding ability at pH 2.0-3.6, while NSF had stronger anthocyanin binding ability at pH 3.6-4.5. The pectin and anthocyanin binding was lowest at pH 4.5 and higher at pH 2.0-3.6. Nearly doubling C3G pigment content increased bound anthocyanin percentage by 16-23% at pH 3.6, which favored anthocyanin aromatic stacking, compared to 3-9% increase at pH 2.0. 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

1. Introduction

Anthocyanins are naturally found in some flowers, fruits and vegetables and are responsible for red, purple and blue colors (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009). They are probably the most important visible pigment in plants besides chlorophyll (Kong, Chia, Goh, Chia, & Brouillard, 2003). Anthocyanins 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 reduce cancer cell proliferation and inhibit tumor formation (Lila, 2004). The potential health promoting benefits *in vivo* may be limited by the low systematic bioavailability (Fleschhut, Kratzer, Rechkemmer, & Kulling, 2006).

Anthocyanin are phenolic compounds which consist of an aromatic A ring bound to an heterocyclic oxygen containing C ring and a third aromatic B ring (Castañeda-Ovando et al. 2009). The main differences between anthocyanidins are the number and combination of hydroxyl and methoxyl groups on anthocyanidins, the nature and number of sugar moieties, and the organic acids acylation on the sugar moieties (Kong, Chia, Goh, Chia, & Brouillard, 2003). 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). Anthocyanins are found in four chemical forms at different pH: flavylium cation, quinoidal base, carbinol pseudo base, and chalcone form (Giusti & Wrolstad, 2001). At acidic pH near 0.5, only the

flavylium cation form of anthocyanins is found. Increasing pH reduces flavylium cation concentration as it can be hydrated by nucleophilic attack of water to carbinol form, or a proton loss to quinoidal base form. At slightly acidic environment near pH 4-6, four structural forms coexist (Castañeda-Ovando et al., 2009). As pH increases further to neutral pH 7, the ring opening of carbinol and development of chalcone form occurs. The equilibrium between quinoidal base and carbinol pseudo base or chalcone is controlled via flavylium cation. 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.

Depending on the sugar moiety, up to 85% of anthocyanins, reaches the colon *in vivo* and potentially influences colonic health (Kahle et al., 2006). Anthocyanins are fermented by human gut microflora and promote the growth of probiotics like *Bifidobacterium spp.*, *Lactobacillus-Enterococcus spp.* (Leijdekkers et al., 2014). Blueberry extracts rich in anthocyanin 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).

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, pectins are bioactive polysaccharide that have health promoting benefits (Wicker et al., 2014). As a soluble dietary fiber, pectin has the potential to lower blood cholesterol levels and affect glucose metabolism (Stephen & Phillips, 2010), and many studies have suggested the potential effects on risk for diverticulosis, colon cancer, coronary heart disease, cholesterolemia and diabetes (Voragen et al., 2009). A recent

review described the binding of modified pectin to pro-metastatic protein galectin-3 (GAL3) and inhibition of cell adhesion and migration (Maxwell, Belshaw, Waldron, & Morris, 2012). Pectin is an excellent candidate as a colon targeted drug delivery vehicle due to the selective digestion by colon microflora with minimal degradation in gastro-intestinal tract (Jung, Arnold, & Wicker, 2013; Tin Wui Wong, Gaia Colombo, & Fabio Sonvico, 2011). Unlike cellulose, pectin as a soluble dietary fiber undergoes nearly complete fermentation by colonic microflora and leads to generation of short-chain fatty acids, which lowers the colonic pH and prevents colon cancer (Moore, Park, & Tsuda, 1998). Pectin is a diverse polysaccharide consisting of three main structural domains of homogalacturonan, rhamnogalacturon I and rhamnogalacturon II (Voragen et al., 2009). The galacturonic acid may be methyl esterified-uncharged, de-esterified-charged, or de-esterified-protonated-uncharged, with an apparent pKa near 3.5-4.5 (Voragen et al., 2009). In addition to total charge, distribution of charge influences the ability of pectin to bind cations (Sperber, Schols, Cohen Stuart, Norde, & Voragen, 2009).

There is some evidence of pectin and anthocyanin interaction. Researchers who studied anthocyanins in flowers as color pigments found that pectin formed large non-dialyzable, co-pigment complexes with anthocyanin (Asen, Stewart, Norris, & Massie, 1970; Bayer, Egeter, Fink, Nether, & Wegmann, 1966). More recent studies report that pectin solubilized anthocyanin-metal chelate complexes and prevented pigment precipitation in aqueous environments, making anthocyanin 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 half-life in a model fruit jam system (Buchweitz, Speth, Kammerer, & Carle, 2013a; Holzwarth, Korhummel, Siekmann, Carle, & Kammerer, 2013; Kopjar et al., 2009). Cell wall composites containing pectin 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 likely (Asen et al., 1970; Buchweitz, Nagel, et al., 2012; Holzwarth et al., 2013; Padayachee et al., 2012).

In human and animal models, less than 1% of consumed anthocyanins are 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); it is possible that anthocyanins exhibit a prebiotic effect, promoting colonic health. If pectin binds anthocyanins, it suggests that blueberry pectin-rich fractions may have dual advantages of protecting anthocyanins in the human body and promoting colonic health. To date, no research has been conducted involving a direct system on pectin and anthocyanin binding, and no study has been conducted on the physico-chemical 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 solutions, and free and bound anthocyanin-pectin mixtures were separated by centrifugal ultrafiltration. The objective was to directly measure the binding between pectin and anthocyanins.

2. Material and Methods

2.1. Materials and Reagents

Freeze-dried blueberry powder (Tifblue/Rubel 50/50 blend) was provided by U.S. Highbush 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).

2.2. Pectin-rich Fraction Extraction and Characterization

2.2.1. Extraction of Pectin-rich Fractions

Approximately 250 grams of freeze-dried blueberry powder (U.S. Highbush Blueberry Council) were mixed in 10 volumes of 95% ethanol at 70°C for 2 hours. The mixture was centrifuged at 6,000g at 4°C for 20 min and was filtered through Miracloth (Millipore, Billerica, MA) under vacuum to obtain the pellet. Pellets were washed in 4 volumes of ethanol, centrifuged and filtered 4 times; a final wash with 4 volumes of acetone, centrifugation and filtration yielded 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°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°C. The pellets were mixed with 500 mL 50 mM 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 residue 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 freeze dried and stored at

116 4°C until analysis.

117 **2.2.2. Uronic Acid Assay**

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

127 **2.2.3. Total Sugar Content**

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

135 **2.2.4. Protein Content**

136 WSF, CSF or NSF was dissolved in deionized water and an aliquot of suspension was
137 mixed with Bradford reagent according to manufacturer's guide (Thermo Scientific, Rockford,

IL) in 96 well microplate (Fisher Scientific, Waltham, MA) to estimate protein content by dye binding assay. The microplate was shaken for 30 seconds in Bio-Rad iMark™ microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA) and incubated at room temperature for 10 min. Absorbance was measured in microplate reader at 595 nm. Protein content of each sample was calculated relative to immunoglobulin G (IgG) standard. Each pectin-rich fraction was sampled in duplicate and measurements were performed in triplicate.

2.2.5. Degree of Methyl Esterification

Degree of methyl esterification (DE) was analyzed using Fourier Transform Infrared (FTIR) spectroscopy (Perkin Elmer Spectrum 400, Waltham, MA), following a modified protocol of Chatjigakis et al. (1998). Peak heights were measured at 1750 cm^{-1} and 1630 cm^{-1} , representing esterified and free carboxyl groups, respectively. The degree of methyl esterification was calculated by the height of the peak attributed to the esterified carboxyl group divided by the sum of peak heights of esterified and free carboxyl group.

2.2.6. 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 column (50 x 7.5 mm) (Agilent Technologies, Santa Clara, CA), Dawn Heleos-II multi-angle laser light scattering (MALS) detector (Wyatt Technology Corporation, Santa Barbara, CA), and Optilab T-rEX differential refractive index (RI) 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 μ m polyethersulfone (PES) filter (Whatman, Maidstone, UK) just prior to injection. 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).

2.3. Intermolecular Binding between Pectin-rich Fractions and Anthocyanins

2.3.1. Pectin Dispersions

Blueberry pectin-rich fractions, WSF, CSF and NSF were dissolved in buffer solutions: 25 mM potassium chloride (pH 2.0), 25 mM sodium acetate (pH 3.6), or 25 mM sodium acetate (pH 4.5) 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°C before further analysis.

2.3.2. Anthocyanin solutions

Cyanidin-3-glucoside (C3G) chloride was dissolved in MilliQ water (Millipore, Billerica, MA) to make stock solution. Aliquots of stock solution was diluted to one of two concentrations, one at a lower concentration (~0.3 mg/mL) and one at a higher concentrations (~0.6 mg/mL) with pH 2.0, pH 3.6 and pH 4.5 buffer solutions described above. Anthocyanin aglycones, cyanidin, pelargonidin, malvidin, peonidin, and delphinidin chlorides, were dissolved in pH 2.0 buffer and were filtered through a 0.2 μ m PES syringe filter (Whatman, Maidstone, UK) to remove insoluble anthocyanin aglycones. Aliquots of anthocyanin aglycone solutions were adjusted to selected concentration in pH 2.0 buffer. Blueberry juice was made from freeze-dried blueberry powder by mixing blueberry powder in MilliQ water with 0.02% sodium azide. The

mixture was centrifuged at 6,000g at 4°C for 20 min and the supernatant was filtered through a 0.45 µm PES syringe filter (Whatman, Maidstone, UK) to remove insoluble plant cell wall materials. The filtered juice was diluted to desired concentrations in pH 2.0, pH 3.6 or pH 4.5 buffers. The pH of all anthocyanin solutions was adjusted to target pH using either 1N hydrochloric acid or 1N sodium hydroxide if necessary. The C3G equivalent anthocyanin pigment content of each anthocyanin solution was measured before the binding study to monitor pigment concentration changes during incubation.

2.3.3. Pectin Anthocyanin Binding

Equal volumes of each pectin dispersion and anthocyanin solution were mixed in an Amicon 0.5 mL centrifugal ultrafilter tube with 30kDa Millipore Ultracell low-binding regenerated cellulose membrane (Millipore, Billerica, MA). Control groups were obtained by mixing aliquots of each pectin solution or anthocyanin solution with an equal volume of buffer solution with corresponding pH. Samples 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°C for 20 min to separate free anthocyanin, 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.

2.3.4. Anthocyanin Pigment Test

Anthocyanin pigment concentration in the filtrate was analyzed using a modified protocol of Giusti and Wrolstad (2001), with 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) = $[(A_{520} - A_{750})_{pH1} - (A_{520} - A_{750})_{pH4.5}] \times MW \times DF / (\epsilon \times L)$, in which MW is the molecular weight, DF is the dilution factor, ϵ is extinction coefficient and L is the path length of microplate. Molecular weight of C3G (449.2 Da) was used for MW, DF was 10, 25740 cm²/mol was used for extinction coefficient and 0.645 cm was used as the path length of microplate. Measurements were conducted in duplicate.

2.4. 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.

3.0 Results and Discussion

3.1. 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 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 methyl esterification of all three pectin-rich fractions was lower than 50% measured by FTIR, indicating the pectin was low methyl esterified. The total soluble sugar content of WSF, CSF and NSF were 328, 279 and 496 $\mu\text{g}/\text{mg}$ 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 methyl esterification, total soluble sugar and protein content of the pectin-rich fractions were similar to the pectin-rich fractions extracted in a previous study on a smaller scale (data not shown).

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 1, see Supplement). The lower RI signal relative to the light scattering signal in peak eluting at 6.5-8.5 mL indicates that a small amount of large molecular weight components; the higher RI signal relative to the lower scattering signal in peaks eluting after 9 mL indicates a larger amount of lower molecular weight materials. Weight average molecular weight (M_w), weight average root mean square radius (R_w) and polydispersity were calculated for the major eluting peak (Table 1). Both WSF and CSF had high weight average molecular weight (M_w) in the 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 M_w values that ranged from 100-200 kDa (Corredig, Kerr, & Wicker, 2000; Kim, Teng, & Wicker, 2005) and around 400 kDa (Jiyoung Jung &

Wicker, 2012). It is likely that 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 the highest 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 conformation plot slopes below 0.33. WSF had a conformation plot slope of 0.25, higher than that of CSF and NSF, therefore WSF was less compact than CSF and NSF. All three pectin-rich fractions were polydisperse with multiple peaks in the chromatograph (Figure 1; Supplement). Polydispersity was estimated as the ratio of the weight average to number average molecular weight; major eluting peaks were polydisperse, with polydispersity values above 1.5 (Table 1).

In this study, the free anthocyanin content in pectin controls prepared at 5 mg/mL concentration were measured (data not shown). WSF control had a high amount of 12.5 µg/mL free anthocyanin content, and CSF and NSF controls contained trace amount of free anthocyanins. Both dialyzed WSF supernatant and freeze dried WSF had a purple color. Freeze dried CSF was white, but was pink when dissolved in aqueous solution. Both dialyzed NSF supernatant and freeze dried NSF had a brown color. Since anthocyanin is the major pigment in the blueberry fruits, the color of the pectin-rich fractions was most likely contributed by anthocyanins. AIS prepared from freeze dried blueberry powder was washed with ethanol and acetone to remove free pigments and sugars. All pectin-rich fractions were extensively dialyzed against deionized water in dialysis tubing 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). It was expected that any residual free

anthocyanin would be removed during dialysis. This suggests 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).

3.2. Binding with C3G

The initial anthocyanin content of C3G solutions, and free anthocyanin content of the control and experimental groups of the study conducted on pectin-rich fractions and C3G were summarized in Table 2. The initial anthocyanin concentration was adjusted to approximately 60 µg/mL; 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 co-pigmentation. Co-pigmentation is a phenomenon in which 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 co-pigmentation 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 2.0-4.5, free anthocyanin concentration was reduced significantly ($P < 0.05$) after incubation, from 63-66 µg/mL to 15-46 µg/mL (Table 2). All pectin-rich fractions bound anthocyanin pigments, giving less free anthocyanin in the filtrates. At pH 2.0 and pH 3.6, CSF showed the lowest free pigment content of 14.5 µg/mL and 19.0 µg/mL respectively, compared

to WSF and NSF. At pH 4.5, WSF and CSF had similar amounts of free anthocyanin pigment of around 46 $\mu\text{g/mL}$, while NSF had lower anthocyanin concentration of 40.3 $\mu\text{g/mL}$. Across all pH values, WSF bound the least amount of anthocyanin, compared to CSF and NSF. The low C3G binding by WSF might be due to the high amount of endogenous anthocyanins contained in WSF. CSF bound more C3G pigment than WSF and NSF at pH 2.0 and pH 3.6, while NSF bound more C3G pigment at 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 2). At pH 4.5, all three pectin-rich fractions bound 27-37% anthocyanins, while at pH 2.0-3.6, 33-78% anthocyanins were bound (Figure 2). Pectin-rich fractions bound less anthocyanins at pH 4.5 than at pH 2.0-3.6. WSF and NSF bound about 20% and 10% more C3G pigment respectively at pH 3.6 than at pH 2.0. CSF bound about 10% more C3G pigments at pH 2.0 than at pH 3.6. The binding between pectin and anthocyanins is pH dependent. At a lower pH, anthocyanin flavylum cations with positive charge are more prevalent than the charge less quinoidal base, carbinol pseudo base or chalcone forms, while at a weak acidic pH, charge less anthocyanin forms are more prevalent (Castañeda-Ovando et al., 2009). Below the apparent pectin pKa of approximately 3.5-4, free carboxyl groups are mostly protonated at pH 2.0. All blueberry pectin-rich fractions characterized in this study were low methoxyl pectin with DE value below 50%. The ionic interaction between negatively charged carboxyl groups and positively charged anthocyanin flavylum cations may be the main mechanism, for pectin and anthocyanin binding. Ionic interaction has been suggested in most studies involving pectin anthocyanin interactions (Buchweitz, Nagel, et al., 2012; Mazzaracchio, Pifferi, Kindt, Munyaneza, & Barbiroli, 2004; Padayachee et al., 2012). The variation of

anthocyanin binding percentage at pH 2.0 and pH 3.6 of all pectin-rich fractions may be explained by the amount of pectic free carboxyl groups.

Initial C3G concentrations was increased to 105 µg/mL and binding between pectin-rich fractions and C3G was evaluated (Table 2). 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 co-pigmentation 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 2). At pH 2.0 and pH 3.6, CSF had the lowest free anthocyanin content of 16.5 µg/mL and 23.0 µg/mL respectively compared to WSF and NSF. At pH 4.5, 76-79 µg/mL free anthocyanins was measured when mixed with WSF and CSF; the free anthocyanin content was 69.1 µg/mL with NSF. Similarly to the study conducted at lower C3G concentration, at all pH values, WSF bound the least amount of anthocyanin, compared to CSF and NSF, and CSF bound more anthocyanins at pH 2.0-3.6, 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). At pH 4.5, all three pectin-rich fractions bound 29-37% anthocyanins, while at pH 2.0-3.6, pectin bound 39-87% anthocyanins. Similarly to the binding study conducted at lower C3G concentration, at pH 4.5, less anthocaynins were bound than at lower pH values. Notably, the percent anthocyanins bound by each pectin-rich fraction and at pH 2.0 or pH 4.5, was about the same, regardless of C3G concentration. The percentage bound anthocyanin at pH 2.0 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 near 60 µg/mL. Padayachee et al. (2012) evaluated anthocyanin binding of cell wall analogues consisting of pectin and cellulose and concluded that anthocyanin binding to plant cell walls was complex. They proposed a two phase binding model with initial anthocyanin binding onto actives sites followed by anthocyanin stacking, which accounted for the major amount of binding. At low pH, the prevalent anthocyanin flavylum 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. The 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 increased binding (16-28%) at pH 3.6 may be contributed by the stacking of quinoidal base forms.

3.3. Binding with Anthocyanin Aglycones

The binding between pectin-rich fractions and anthocyanin aglycones was evaluated. The initial pigment concentration, free anthocyanin in the anthocyanin control and experimental groups of study conducted on pectin-rich fractions and for five anthocyanin aglycone solutions are summarized in Table 3. The study was conducted only at pH 2.0, due to the poor solubility of aglycones at higher pH values. In nature, anthocyanins 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, the pigment content of the control groups was reduced from 58-75 µg/mL initially to 32-48 µg/mL (Table 3). Pelargonidin solution had the highest reduction of 58%, while delphinidin had the lowest pigment reduction of 22%. Pelargonidin has one hydroxyl group on the aromatic B ring, and delphinidin has three hydroxyl groups. Increased hydroxylation of the B ring of anthocyanin aglycone stabilizes anthocyanidins (Dao, Takeoka, Edwards, & Berrios, 1998). Anthocyanin

alycones are highly unstable and 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 di-ketone, a substrate that is further degraded into phenolic acid and aldehyde (Fleschhut et al., 2006). In the experimental groups, all anthocyanidin aglycone solutions had dramatic free anthocyanidin reduction, compared to the control groups (Table 3). CSF had nearly no free pigment in cyanidin, malvidin, and peonidin ($<1.2 \mu\text{g/mL}$) and minimal pigment in pelargonidin and delphinidin ($<3.2 \mu\text{g/mL}$) solutions. WSF and NSF had 9-16 $\mu\text{g/mL}$ free pigment in all anthocyanin aglycone solutions. WSF had slightly higher free pigment for cyanidin and malvidin solutions than NSF, and both WSF and NSF had the same free pigment amount content for the other three aglycone solutions.

Among all five anthocyanin aglycone solutions, all pectin-rich fractions showed very similar binding patterns. CSF bound close to 100% of the pigments and WSF and NSF bound 58-78% of the pigments (Figure 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). For WSF and the five anthocyanin aglycones, there was a strong positive correlation between binding percentage and the number of free hydroxyl groups in aglycone B ring ($r=0.7198$), but a strong negative correlation with the number of methoxyl groups ($r=-0.8745$). NSF and aglycone binding percentage showed a positive correlation between number of hydroxyl groups ($r=0.4585$), and a negative correlation between number of methoxyl groups ($r=-0.6850$). The number of hydroxyl substitution may enhance the binding between WSF or 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 4). The substitution pattern of the aromatic B ring does not greatly account for the majority of the bound anthocyanidins.

3.4. Binding with Blueberry Juice Anthocyanins

Blueberry juice prepared from freeze dried blueberry powder was incubated with pectin-rich fractions at pH 2.0-4.5 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 are summarized in Table 4, (See Supplement). After incubation, the anthocyanin concentration in the control groups increased from 41-42 $\mu\text{g/mL}$ to 44-48 $\mu\text{g/mL}$. The control pigment increase after incubation was due to anthocyanin intermolecular co-pigmentation and co-pigmentation with organic acids naturally presented in blueberry. Blueberry contains petunidin-3-glucoside, peonidin-3-glucoside, delphinidin-3-glucoside and cyanidin-3-galactoside in major amounts, and contains cyanidin-3-glucoside, peonidin-3-galactoside and malvidin-3-glucoside in minor amounts (Yi, Fischer, Krewer, & Akoh, 2005). Gallic acid, caffeic acid, *p*-coumaric acid, ferulic acid, catechin, quercetin and kaempferol are phenolic acids found in blueberry (Yi, Akoh, Fischer, & Krewer, 2006). All experimental groups had reduced free anthocyanin contents ranging between 17-33 $\mu\text{g/mL}$ (Table 4, Supplement). At pH 2.0, CSF had the lowest free anthocyanin of 17.4 $\mu\text{g/mL}$ compared to 30.9 $\mu\text{g/mL}$ in WSF or 20.3 $\mu\text{g/mL}$ in NSF. At pH 3.6, CSF and NSF had the lowest free pigment content of 18-18.5 $\mu\text{g/mL}$, compared to 25.3 $\mu\text{g/mL}$ in WSF. At pH 4.5, the lowest free pigment content of 26.0 $\mu\text{g/mL}$ was observed in NSF, compared to about 33 $\mu\text{g/mL}$ in WSF or CSF. The binding pattern between each pectin-rich fraction and blueberry juice anthocyanins was similar to that of C3G solutions. WSF bound the least amount of blueberry juice anthocyanins at all pH values. At lower pH values of 2.0 and 3.6, CSF had lowest

free anthocyanin compared to WSF and NSF; at pH values of 3.6 and 4.5, NSF had the lowest 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 percentage of bound blueberry juice anthocyanin was calculated for each pectin-rich fraction and each pH value so that the effect of pH could be compared (Figure 5; see Supplement). In general, at pH 4.5, all pectin-rich fractions bound the least amount of anthocyanins, compared to pH 2.0-3.6. Except for WSF at pH 2.0, pectin-rich fractions bound more anthocyanins at pH 2.0 and pH 3.6 than at pH 4.5. The blueberry juice anthocyanin binding pattern across difference pH values was similar to that observed in C3G studies. Ionic interaction between anthocyanin flavylum cations and pectic 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: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 under stomach conditions (pH 2.0) and reduce the bio-accessibility 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). Further pectins sourced from apple or sugar beet, with different structural features, stabilize anthocyanins more effectively than citrus (Buchweitz, Speth, Kammerer, & Carle, 2013b).

Depending on the form of anthocyanin or the pH, the binding affinity to citrus pectin with 14% DE, a binding affinity, K_d of $\sim 2 \mu\text{M} \sim 180$ to $250 \mu\text{M}$, was measured indirectly using saturation transfer difference NMR spectroscopy. At any pH range, the blueberry pectin used in

this study, at DE values between 26 and 36%, has less total charge than citrus pectin at 14% DE. Pectin affinity for cations and functionality will vary depending on total charge but also charge distribution (Sperber et al. 2009), block limit of DE (Lee, Rivner, Urbauer, Garti, & Wicker, 2008), which in turn influences the functionality of pectin as a delivery vehicle (Wicker et al. 2014). In addition, other pectin structural features, such as MW, homogalacturonan or rhamnogalacturonan domains, uronic acid to neutral sugar ratio influence health benefits attributed to pectin (Wicker & Kim, 2015). Presumptively, the greater the binding affinity, the greater the protection against pre-mature gastric release or external degradative conditions (Jung et al. 2013).

4. Conclusions

In this study, direct binding between blueberry pectin-rich fractions and anthocyanins was 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 flavylum 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 2.0-3.6, 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 promotion of colonic health.

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Figure 2. Percentage of bound anthocyanins to blueberry pectin-rich fractions: water soluble fraction (WSF), chelator soluble fraction (CSF) and sodium carbonate soluble fraction (NSF) at pH 2.0-4.5, calculated as cyanidin-3-glucoside (C3G) equivalent. Initial anthocyanin pigment concentration was approximately 60 $\mu\text{g/mL}$. 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. Percentage of bound anthocyanins to blueberry pectin-rich fractions: water soluble fraction (WSF), chelator soluble fraction (CSF) and sodium carbonate soluble fraction (NSF) at pH 2.0-4.5, calculated as cyanidin-3-glucoside (C3G) equivalent. Initial anthocyanin pigment concentration was approximately 105 $\mu\text{g/mL}$. * 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 4. Percentage of bound cyanidin (CY), pelargonidin (PG), malvidin (MV), peonidin (PN) or delphinidin (DP) and three blueberry pectin-rich fractions: water soluble fraction (WSF), chelator soluble fraction (CSF) and sodium carbonate soluble fraction (NSF) at pH 2.0. Bars with different pattern fills indicates different pectin-rich fractions. Bars with different letters are significantly different ($P < 0.05$) in the same graph.

1 Table 1. Characterization of blueberry pectin-rich fractions, water soluble fraction (WSF), chelator soluble fraction (CSF) and sodium
2 carbonate soluble fraction (NSF).

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

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

Table 2. Anthocyanin content ($\mu\text{g/mL}$) of initial, control and blueberry pectin experimental groups at pH 2.0-4.5, calculated as cyanidin-3-glucoside (C3G) equivalent

	60 $\mu\text{g/mL}$			105 $\mu\text{g/mL}$		
	pH 2.0	pH 3.6	pH 4.5	pH 2.0	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	108.3 ^b $\pm$ 0.8	104.3 ^b $\pm$ 1.2	103.0 ^b $\pm$ 0.9
C3G Control	65.9 ^a $\pm$ 1.1	63.2 ^a $\pm$ 1.3	63.2 ^a $\pm$ 0.3	125.9 ^a $\pm$ 2.9	109.0 ^a $\pm$ 3.9	110.5 ^a $\pm$ 1.7
WSF+C3G	44.4 ^c $\pm$ 0.9	29.9 ^c $\pm$ 0.5	46.0 ^c $\pm$ 4.2	77.5 ^c $\pm$ 3.0	44.9 ^c $\pm$ 1.2	78.9 ^c $\pm$ 1.4
CSF+C3G	14.5 ^e $\pm$ 2.9	19.0 ^e $\pm$ 0.1	45.5 ^c $\pm$ 1.5	16.5 ^e $\pm$ 1.6	23.0 ^e $\pm$ 0.8	75.9 ^d $\pm$ 0.7
NSF+C3G	31.4 ^d $\pm$ 0.5	25.6 ^d $\pm$ 1.2	40.3 ^d $\pm$ 2.3	55.7 ^d $\pm$ 3.4	30.2 ^d $\pm$ 0.1	69.1 ^e $\pm$ 0.4

Blueberry pectin-rich fractions, water soluble fraction (WSF), chelator soluble fraction (CSF) and sodium carbonate soluble fraction (NSF)

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

13 Table 3. Anthocyanidin content ($\mu\text{g/mL}$) of initial, control, and blueberry pectin experimental
 14 groups at pH 2.0 calculated as cyanidin-3-glucoside (C3G) equivalent

	CY	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

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

17 Initial anthocyanin aglycone solutions, cyanidin (CY), pelargonidin (PG), malvidin (MV),
 18 peonidin (PN) and delphinidin (DP), and free anthocyanin in the control anthocyanin aglycone
 19 solutions

20 Blueberry pectin-rich fractions, water soluble fraction (WSF), chelator soluble fraction (CSF)
 21 and sodium carbonate soluble fraction (NSF) at pH 2.0