EFFECT OF ENZYME-ASSISTED EXTRACTION OF ANTHOCYANINS FROM PURPLE SWEET POTATO ON TOTAL SOLUBLE SOLIDS AND PIGMENT YIELD

An Undergraduate Research Scholars Thesis

by

MARITZA ASHTON SIRVEN

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ABSTRACT

Effect of Enzyme-Assisted Extraction of Anthocyanins from Purple Sweet Potato on Total Soluble Solids and Pigment Yield. (May 2015)

Maritza Sirven
Department of Nutrition and Food Science
Texas A&M University

Research Advisor: Dr. Stephen Talcott
Department of Nutrition and Food Science

An increased interest in health and wellness has led to consumer demand for natural alternatives to synthetic food dyes. The aim of this research was to increase the efficiency of extraction of anthocyanins from purple sweet potatoes (Ipomoea Batata) for large-scale production of natural food colorant. Protease, alpha-amylase, pectinase, and cellulase enzymes were assessed for their ability to increase pigment yield and decrease soluble solids content in the final concentrate of anthocyanins. Pectinase increased pigment yield by 50% and had 57% higher soluble solids content than the control when partitioned with Amberlite FPX-66, a non-ionic resin used to adsorb anthocyanins and remove water and polar components from extracts. Although there was a higher soluble solids content in the final concentrate, this was due to the increase in pigment yield. Therefore, pectinase is a viable processing aid that can deliver higher quality product and increase efficiency of extraction.

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CHAPTER I

INTRODUCTION

Natural colors in the food industry

Consumers have become more concerned about the physiological effects of synthetic ingredients in processed foods. This has put pressure on the food industry to be more transparent about the source of their ingredients and to find healthy, 'natural' alternatives. Food, Drug, and Cosmetic Certified (FD&C) colorants have been especially scrutinized for their use in food. Possible links between synthetic colorants and toxicological and negative psychological effects in children has led to consumer demand for natural alternatives.[1] American consumers perceive colors exempt form certification as having a higher functional quality in food, leading to an increase in their demand. As of now, natural food colorants have overtaken the market share of FD&C colors.

A number of food companies have already reformulated their products using natural pigments instead of FD&C colorants. A notable example is Pepperidge Farm's 'Goldfish Colors.' A well-established company, such as Pepperidge Farm, repositioning a product to showcase natural colors demonstrates the relevance of natural colors and sets a precedent for other companies.

One of the most common natural colorants already used in the food industry is anthocyanin extracts and juices.[2] However, replacing FD&C colors with these compounds poses a number of challenges. First, many anthocyanins lack heat stability. Pasteurization, one of the most moderate food processing heat treatments, results in 8-14% loss of pigment.[3] Second, the hue reflected by anthocyanins is most stable at very high acidity levels. . There are few, if any, foods that can exhibit their maximal color capacity with anthocyanins due to a low pH alone. Finally, anthocyanins are naturally synthesized and extracted from crop commodities. While the

concentration of synthetic colors can be stoichiometrically predicted and made any time of the year, concentration of anthocyanins varies per each individual crop and relies on harvest yield and season.

Despite the challenges of using anthocyanins in food products, many efforts persist in using this class of compounds for the replacement of FD&C Red #40 (Allura Red), one of the most widely used synthetic food colorants.[4] Anthocyanins reflect a hue comparable to that of Red #40, making them highly desirable over other red-colored compounds. Color matching is important in the food industry because color is highly influential on how quality and flavor of a food is perceived. Anthocyanins are also water-soluble, increasing their applicability in a variety of food matrices. Because anthocyanins reflect a variety of colors in the acidic pH range, colors other than those reflective of Red #40 can be produced, increasing their versatility. Overall, anthocyanins can meet consumer and industry needs as a natural alternative for synthetic colorants.

Anthocyanin biosynthesis and chemistry

Anthocyanins are found in nature as the red, blue, and purple pigments in flowers, vegetables, roots, tubers, legumes, and fruits.[5] A physical function of anthocyanins in plants is their ability to attract pollinators and seed dispersers, facilitating their reproduction.[6] Furthermore, these compounds play a biological and chemical role in plant metabolism. Anthocyanins are synthesized in plants as a response to heat, light, weather, and disease stressors.[5] Their chemical structure allows them to act as a radical-scavenging antioxidant, donating hydrogen atoms to free radicals to prevent undesirable reactions.[5] Anthocyanins also aid in the protection of chloroplasts. Chloroplasts are a vital organelle in the regulation of photosynthesis, but when exposed to an

excess of light they can become damaged. Because anthocyanins have the ability to absorb light in the same range (200-700nm), they help relieve excess strain on chloroplasts.[5]

Anthocyanins are a class of flavonoids, or compounds characteristic for their $C_6H_{12}C_6$ molecular structure.[7] The algycon, anthocyanidin, is the backbone of all anthocyanin derivatives. Anthocyanidins contain two aromatic rings referred to as Ring A and B, and one heterocyclic ring referred to as Ring C.[2] There are six algycons (anthocyanidins) predominantly found in nature: cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin. These specific algycons are differentiated by methoxy and hydroxyl substituents on the B ring. Anthocyanindins are considered anthocyanins when glycosylated with a sugar moiety. Because hydroxyl groups are present at each individual ring of the main six algycons, glycosidic bonds can be found at the A, B, and/or C ring(s).[8] The sugar moieties of the glycoside can also be acylated with aromatic and/or aliphatic acids.[9] The variation and numerous combinations of functional groups has led to the existence of more than five hundred anthocyanin derivatives.[2] Variation in substituents and sugar and acid linkages also causes differentiation in stability between anthocyanins; some are more chemically stable than others. Generally, more hydroxyl groups decrease stability while more methoxy groups and acylation increases stability.[8]

Color reflected by anthocyanins interrelates with the chemical stability of the compound. Hue and saturation are dependent on pH, concentration, and copigmentation.[8] At pH 1 monomeric anthocyanins are stable as the *red* flavylium cation form. If the pH increases to pH 2-4, keeping concentration of a given solution of anthocyanins constant, the chemical compound's equilibrium shifts in favor of the *blue* quinoidal base form. At pH 5-6, four compounds coexist in equilibrium – flavylium cation, quinoidal base, *colorless* carbinol pseudobase, and *yellow*

chalcone. However, carbinol pseudobase predominates at this pH range. In neutral environments (pH >7) anthocyanins degrade into smaller organic products.[2]

Benefits of anthocyanins from purple sweet potato

Purple sweet potatoes (PSPs) are originally grown in Japan, New Zealand, and Korea.[9] However, the United States has begun domestically producing PSP to sell as produce or as raw material for the production of natural anthocyanin colorant. There are many advantages of using PSP as a source of anthocyanin extracts and juices. PSPs have a higher concentration of anthocyanins per kilogram of potato compared to other fruit and vegetable sources.[10] As previously mentioned, some anthocyanin derivatives are more stable than others depending on the substituents and sugar/acid linkages on the algycon. 98% of anthocyanins from PSP are acylated diglucosides of cyanidin and peonidin.[3] Acylated anthocyanins can exhibit intramolecular copigmentation. Copigmentation is a phenomenon where anthocyanins stack on top of each other due to hydrophobic attraction of the cyclic rings. Copigmentation results in a bathochromic and hyperchromic shift in the absorption maximum, causing the compound to reflect a more red and vibrant color, which is desirable for the natural food colorant industry.[8] Researches have proposed the "sandwich structure" where two acylated anthocyanins are stacked on top of each other in the middle, acyl linkages are stacked on the outside of the anthocyanins, and sugar moieties act as spacers in between the acyl group and algycon.[4] Stacking of anthocyanins not only allows more color to prevail at a larger pH range, but also makes the compounds less susceptible to hydration, or a nucleophilic attack by water on C-2.[4] Common sources of anthocyanins such as blueberry, grapes, and elderberries are mainly composed of mono, di-glucosides. These anthocyanin derivatives are more susceptible to hydration or a nucleophilic attack by water under low acid conditions, and therefore a larger concentration of pigment would

have to be used to achieve the same hue strength compared to anthocyanins from PSP.[8] Other sources of acylated anthocyanins do not match the hue FD&C Red #40 as well as PSP anthocyanins. For example, red cabbage acylated anthocyanins appear to be more purple than red.[4] There are also physical benefits of using PSP for the production of natural food colorant over other sources. PSP are less sensitive to damage compared to soft fruits such as grapes, cabbage, and berries. PSP also have a longer shelf life compared to grapes, cabbage, and berries, which only last up to a week.

Extraction methods of anthocyanins from purple sweet potato

Many works have been aimed at elucidating the most effective method for extracting anthocyanins from purple sweet potato and other anthocyanin sources. Amongst the most common are aqueous extraction, solvent extraction, and microwave-assisted extraction. Each of these methods poses their own set of challenges. While solvent extraction yields more total pigment than microwave-assisted extraction, solvents have toxicological and safety hazards.[11] Solvents also evaporate at considerably low temperatures, making companies liable for pollution. Aqueous extraction would be optimal comparing efficiency and safety to micro-assisted and solvent extraction.

Because anthocyanins are located in the vacuole, it has been hypothesized that breaking down the cell wall aids in pigment release.[12] Researchers typically grind PSPs to particulate sizes in order to optimize damage to cell walls and increase surface area for higher extraction yield. An issue with rupturing PSP cells is that intrinsic polyphenol oxidase is exposed to oxygen and oxidizes naturally present polyphenols. Although PPO does not affect anthocyanins directly they still follow a co-oxidation reaction. The *o*-quinones generated are very reactive and condense

with anthocyanins to form brown pigments. It has been found that a pre-heating step at 90°C for 10 minutes to deactivate PPO followed by 70°C aqueous extraction was efficient.[13]

Although typical extraction processes apply physical stress to the cell wall by heat and shear, some researchers hypothesize that cell wall material is still not completely ruptured or that anthocyanins become entangled in cell wall material, preventing their isolation.[12]

Enzymes are not a new processing aid utilized in the food industry. Enzymes are used in the production of a variety of products from cheeses to high fructose corn syrup.[14] The use of cell wall degrading enzymes has been used frequently in the processing of grape products such as wine and juice. Researchers have found that using pectinases in wine making increased anthocyanin content from 10-30%, enhanced color, decreased processing time, and increased filtration 10-fold.[14] In juice processing, pectinases increased bilberry juice yield by 116-118% and black current juice yield by 133-135%.[14]

It was first hypothesized that polysaccharides and proteins were adsorbing to Amberlite resin with anthocyanins, increasing soluble solids in the final concentrate. Therefore, proteases, alpha amylases, and cell wall degrading enzymes would increase the solubility of these macromolecules, decrease the amount of soluble solids in the final extract, and increase soluble solids in the unbound water-soluble partition. It was discovered that cell wall degradation has a large impact on pigment yield as previous studies have found with other anthocyanin containing product.

CHAPTER II

METHODS

Enzyme-assisted extraction of anthocyanins from purple sweet potatoes

Purple sweet potatoes were grown and harvested via Avoca, Inc., Merry Hill, North Carolina. PSPs were then shipped to Texas A&M University right after harvesting, ensuring freshness. Once received, the produce was stored in the dark at room temperature.

Extraction method was based off that of Cipriano et al.[13] From a randomly selected potato, 50 g were sliced into 2 cm rounds and pre-heated for 10 minutes in 90C deionized water. PSP was then finely chopped in a domestic food processor for 2 minutes, scraping down sides every 30 seconds to ensure uniform particle size. PSP was then pureed with 300 ml (6:1 v/w) 0.5 M pH 3.8 citrate buffer in a standard kitchen blender for 2 minutes. The mixture was divided into equal parts by volume and poured into two capped 500 ml media bottles. Media bottles were placed in a shaking thermostatic water bath and brought to a constant temperature of 45C. One bottle was dosed with 0.02% v/v Crystalzyme 200XL, a polygalacturonase enzyme with specificity for hydrolysis of alpha-1-4-galacturonic acid linkages, 200,000 depectinizing units min -1, provided by DSM. The other bottle served as the control. Media bottles were left in thermostatic water bath 3 hours for extraction. After extraction, bottles were taken out of water bath and set aside to cool for 10 minutes at room temperature. Mixtures were divided into 50 ml falcon tubes and centrifuged 3500 x g using Eppendorf Centrifuge 5810R for 15 minutes. The supernatant was poured over a bed of 30 g acid-washed diatomaceous earth to clarify. Amberlite FPX-66, a non-ionic adsorbent resin, was prepared by first washing with 1 Column Volume (CV) of methanol followed by 2 CV's of acidified water (0.01% HCl). Clarified extracts were then loaded into the

column and water-soluble components of the extract were partitioned with 2 CV of acidified water (0.01% HCl). The eluent was collected for later analysis. 4 CVs of acidified methanol (0.01% HCl) were used to elute anthocyanins. Solvent was evaporated off at 45C using a Rotovap and isolate was brought up to final volume using 0.5 M citrate buffer pH 3.

HPLC Analysis

Anthocyanins in the final extracts were analyzed using a HPLC equipped with a photodiode array detector (PDA) at 520 nm. Individual anthocyanins were separated using a 250 4.6 mm Acclaim 120 C18 column with a C18 guard column. Mobile phases used were 10% acetic acid and 5% methanol in water (Phase A) and 1% acetic acid and 1% formic acid in methanol with a flow rate of 0.7 ml/min. A gradient solvent program was run with Phase A starting at 100%. Phase B increased from 0% to 20% in 5 minutes, 20% to 25% in 15 minutes, and from 25% to 30% in 2 minutes before being re-equilibrated back to 0% in 4 minutes and 30 seconds. Concentration of individual anthocyanins was quantified in cyanidin-3-glucoside equivalents.

Spin Solids

Spin Solids was used to compare effectiveness of enzymatic treatments on PSP citrate mixture by measuring decrease in solids over time. PSP was shredded into uniform particles with a domestic food processor and then homogenized in a standard kitchen blender with 0.5M pH 3.8 citrate buffer. Mixture was separated into 15 ml falcon tubes and dosed with 0.1% v/v enzymes. Enzymes used were Crystalzyme 200XL and Validase TRL. Falcon tubes were placed in a shaking thermostatic water bath at 45C for 2, 6, 12, and 24 hours. After a time interval elapsed, falcon tubes were centrifuged 3500 x g for 15 min and %solids was recorded.

Pectin Quantification

Galacturonic acid was quantified using the m-hydroxydiphenyl according to Kintner et al.[15] 6 ml 0.0125 M sodium tetraborate in concentrated sulfuric acid was added to 1 ml aliquot of water-soluble partition from extraction. The resulting solution was cooled on ice and then vortexed for 25 seconds. Samples were heated for 5 minutes in a 100C water bath and then cooled in an ice water bath. 100 µl 0.15% m-hydroxydiphenyl in 0.5% sodium hydroxide was added to each sample and vortexed for 25 seconds to fully develop color. Absorption was measured after bubbles in the solution dissipated at 520 nm. Concentration of galacturonic acid was quantified in mg/ml galacturonic acid.

CHAPTER III

RESULTS AND DISSCUSSION

It was originally hypothesized that proteins contained in PSP were also adsorbing onto Amberlite resin with anthocyanins. Depending on the solubility of the proteins present, it was also hypothesized that proteins were either co-eluting with the anthocyanin concentrate or staying entrapped by the resin, ultimately clogging the column. When Validase AFP 1000L was used to assist extraction, the resulting concentrate had 36.3% less total pigment than the control (Figure 1). The enzyme either had a degrading effect on anthocyanins or the extraction conditions were not optimal for the enzyme. Alpha-amylase was also evaluated to determine if starch had an effect on pigment yield. The resulting extraction yielded 35.8% less pigment than the control (Figure 2). This could also be due to degrading effects of anthocyanins by the enzyme or lack of optimization of enzyme. However, Crystalzyme 200XL increased pigment yield by 25.57% and cellulose had approximately the same yield as the control. In future studies, Validase AFP 1000L should be trialed again at more acidic conditions

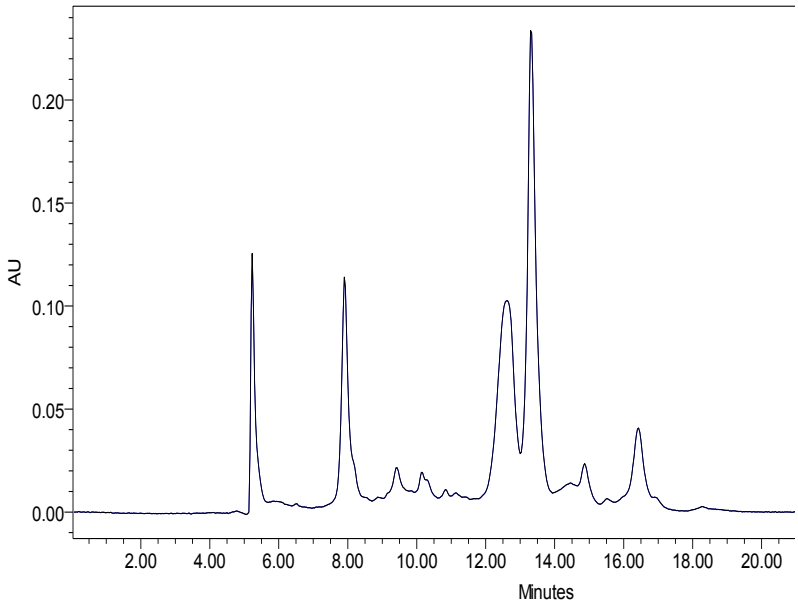

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Figure 1: HPLC chromatogram of protease-assisted extraction (0.1% v/v Validase AFP 100L) of anthocyanins from PSP.

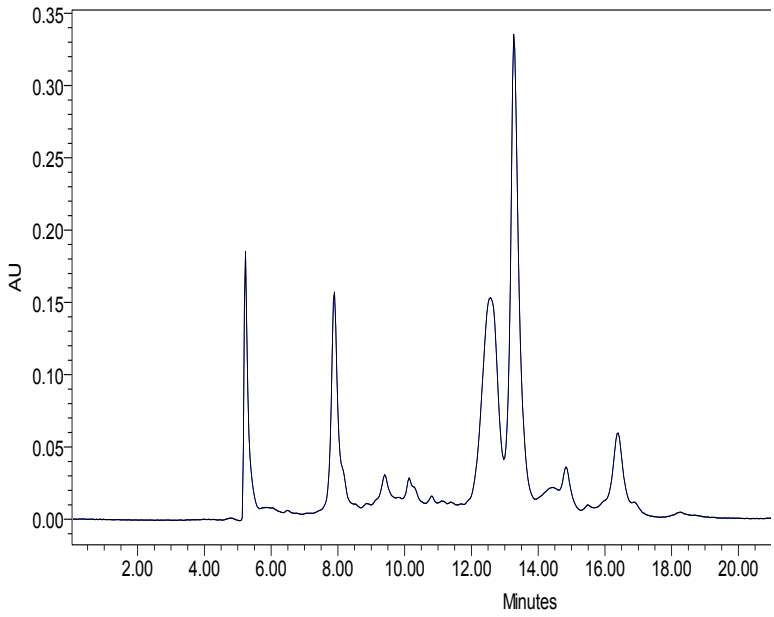

Figure 2: HPLC chromatogram of amylase-assisted extraction (0.1% v/v Validase BAA 100L) of anthocyanins from PSP.

Crystallzyme 200XL and Validase TRL were accessed by Spin Solids method their ability to break down soluble solids. Cystallzyme 200XL decreased %solids on average faster than Validase TRL (Figure 3 and 4). However, from Figure 3 it can be deduced that Validase TRL could possibly break down a greater %solids given a longer period of time, making it more effective than Crystallzyme 200XL. In future studies, Validase TRL can be dosed at a higher activity to match that of Crystallzyme 200XL for comparison. It would make sense that a cellulase would be more effective at reducing soluble solids based off the compositional analysis of PSP.

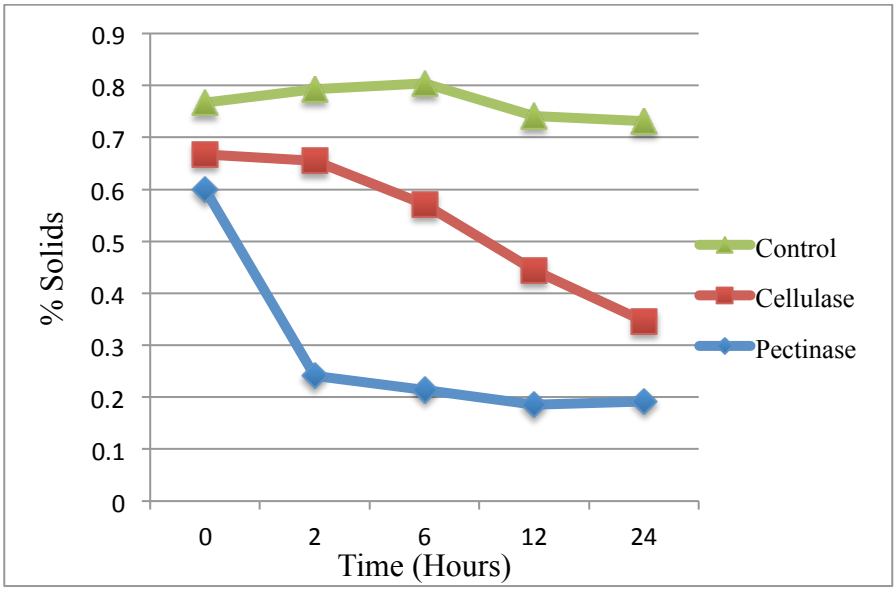

Figure 3: %Solids over time of 0.1% v/v enzyme dosed PSP mixture compared to control. Pectinase used was Crystallzyme 200 XL and cellulose used was Validase TRL.

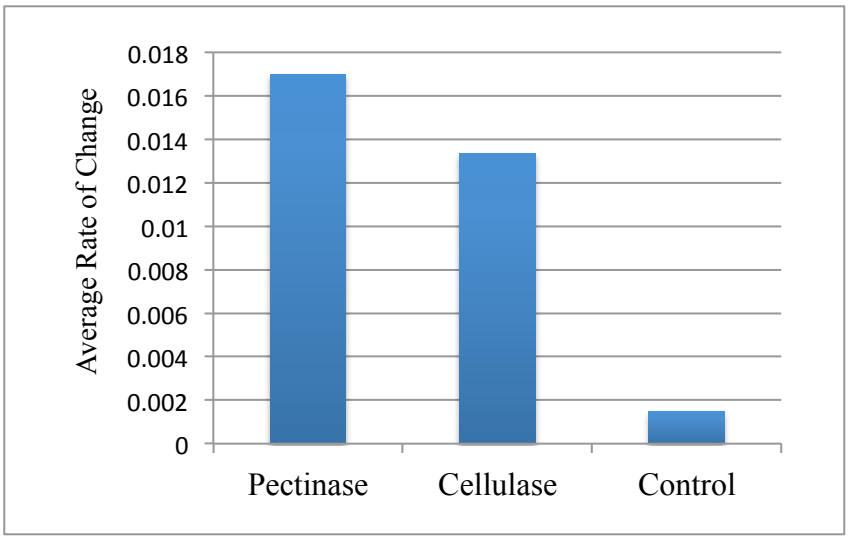

Figure 4: Average rate of change of % solids over 24-hour period.

Cystallyzme 200XL increased pigment yield by 50% compared to the control (Figure 5). The only difference between the control and enzyme chromatogram was the area of the peaks, showing that polygalcturonase had no detrimental effect on the individual compounds but was successful at extracting more pigment. M-hydroxydiphenyl method was used to measure galacturonic acid in unbound partition. There was 156 mg/ml galacturonic acid compared to the control with 68.4 mg/ml galacturonic acid (Figure 6). This shows that pectin from PSP was made more soluble by hydrolyzing polymers into individual galacutonic acid units.

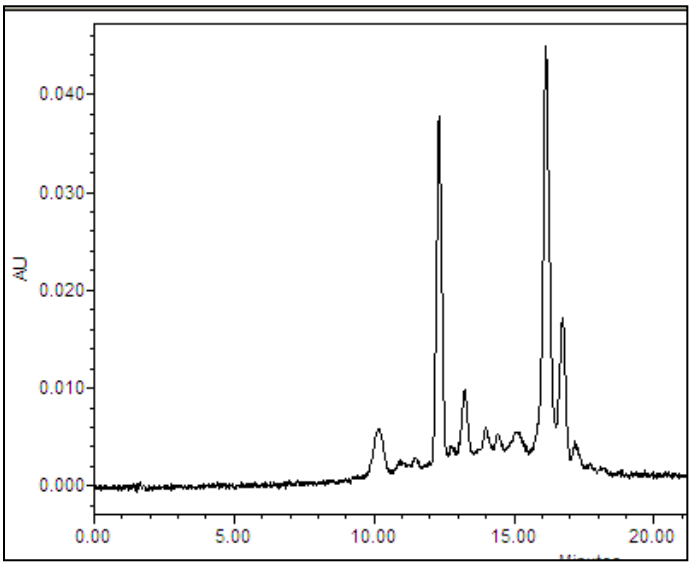

Figure 5: HPLC chromatogram of polygalacturonase-assisted extraction (0.02% v/v Crystallzyme 200XL) of anthocyanins from PSP.

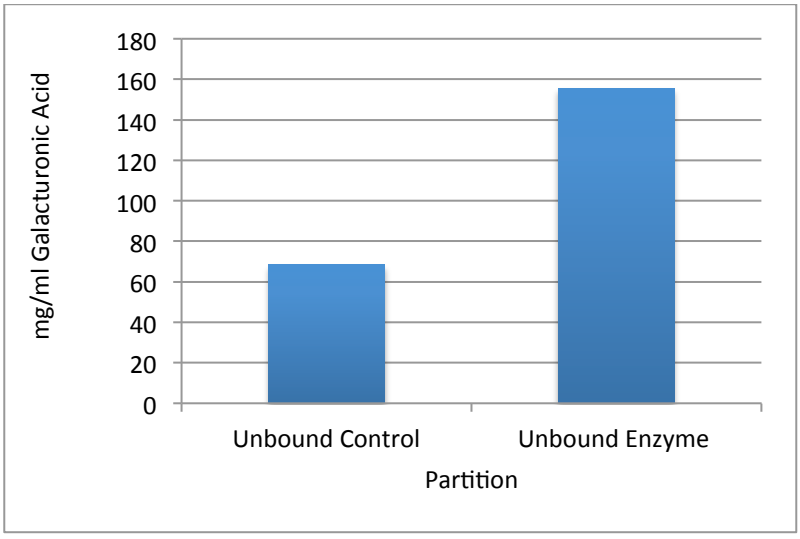

Figure 6: mg/ml galacturonic acid quantified in the unbound water-soluble partition when concentrating anthocyanins with Amberlite.

To further assess the increase in efficiency promoted by the enzyme, Brix of soluble solids were measured in the clarified extraction, unbound water-soluble partition, and bound final anthocyanins concentrate. Polygalacturonase assisted extraction resulted in 57% increase soluble solids in the unbound partition, correlating with the greater amount of galacruonic acid found in the enzyme treated fraction (Figure 6). Although the goal of this study was to decrease soluble solids in the final anthocyanin concentrate, a higher soluble solids content was found in the bound enzyme extracted fraction (Figure 7). However, this correlates with the higher concentration of pigment as determined HPLC analysis (Figure 5).

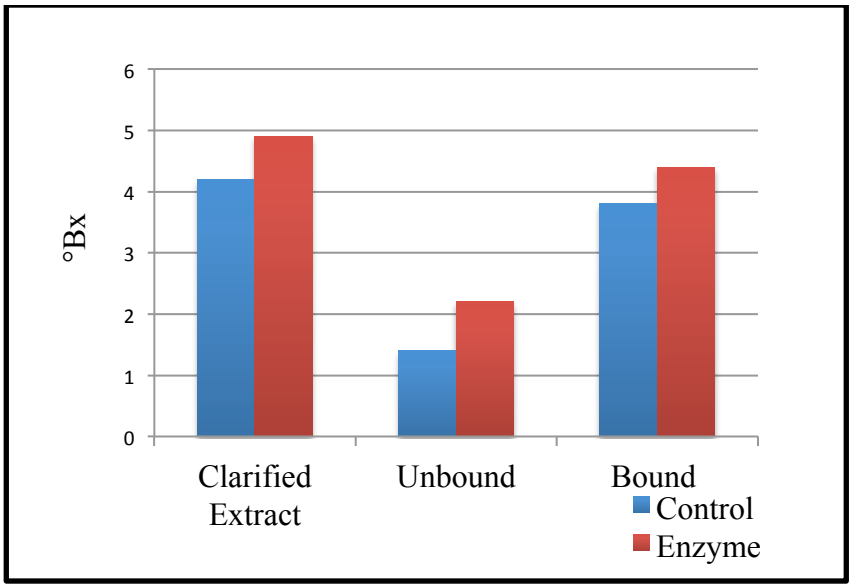

Figure 8: Comparison of Brix after subsequent partitions.

CHAPTER IV

SUMMARY AND CONCLUSION

The results as of now are inconclusive. Although protease showed a decrease in pigment yield, this may be due to heat degradation of anthocyanins and lack of optimization of Validase-AFP. However, analysis of soluble protein using Biuret Method revealed that there was 0% concentration soluble protein in the final concentrate. Therefore, protease was ruled out as potential processing aid for extraction of anthocyanins from PSP. Alpha-amylase also showed a decrease in pigment yield compared to the control. This is most likely due to lack of optimization of enzyme. There is evidence that cell wall degrading enzymes are the most effective at increasing pigment yield and decreasing soluble solids, especially pectinase. This is consistent with the findings of Padayachee et al who proposed that pectin interferes with pigment release from the vacuole by ionic and hydrophobic interactions.[12]

More research needs to be done on the effectiveness of enzymes on pigment yield and reduction of soluble solids. Future studies will include a comparison of individual cell wall degrading enzymes and enzyme cocktails. Efforts will be made to show the ability of enzymes to enhance processing efficiency by decreasing turbidity and increasing ease of filtration.

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